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# Active influenza virus neuraminidase is expressed in monkey cells from cDNA cloned in simian virus 40 vectors

(sialidase/glycoproteins/signal hypothesis/membrane protein)

ALAN R. DAVIS*, TIMOTHY J. BOS, AND DEBI P. NAYAK†

Jonsson Comprehensive Cancer Center and Department of Microbiology and Immunology, University of California, Los Angeles, School of Medicine, Los Angeles, California 90024

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**ABSTRACT** **We have replaced the late genes of simian virus 40 (SV40) with a cloned cDNA copy of the neuraminidase (NA; EC 3.2.1.18) gene of the WSN (H1N1) strain of human influenza virus. When the SV40–NA recombinant virus was complemented in a lytic infection of monkey cells with a helper virus containing an early region deletion mutant, influenza NA was expressed and readily detected by immunofluorescence as well as by immunoprecipitation of *in vivo* labeled proteins with monoclonal antibodies against NA. In addition, the expressed NA exhibited enzymatic activity by cleaving the sialic acid residue from α-2,3-sialyllactitol. The expressed protein was glycosylated and transported to the cell surface, and it possessed the same molecular weight as the NA of WSN virus grown in monkey cells. Because the structure of NA is quite different from that of other integral membrane proteins and includes an anchoring region at the $NH_2$ terminus consisting of hydrophobic amino acids, we also constructed deletion mutants of NA in this region. Replacement of DNA coding for the first 10 $NH_2$-terminal amino acids with SV40 and linker sequences had no apparent effect on NA expression, glycosylation, transport to the cell surface, or enzymatic activity. However, further deletion of NA DNA encoding the first 26 amino acids abolished NA expression. These data suggest that the hydrophobic $NH_2$-terminal region is multifunctional and is important in biosynthesis and translocation of NA across the membrane as well as in anchoring the protein.**

Influenza virus, a well-known human and animal pathogen, has been widely used for studying the biosynthesis, sorting, distribution, and orientation of membrane proteins (1–3). It is a negative-strand enveloped RNA virus possessing three membrane-associated proteins. Hemagglutinin (HA) and neuraminidase (NA, also known as sialidase, EC 3.2.1.18) are present on the outer envelope and are integral membrane proteins, whereas membrane (M) protein is associated with the inner lining of the lipid bilayer of the viral membrane.

Of the two surface proteins, HA has been studied extensively. Its three-dimensional structure, antigenic epitopes, receptor binding site for the sialic acid residue, cleavage site for activation, glycosylation points, and organization into trimeric structures have been defined (see refs. 4 and 5). The deduced amino acid sequence (see ref. 6) shows that the HA possesses the structural features of the integral membrane proteins—i.e., hydrophobic sequences are present in the proximity of both $NH_2$ terminus and COOH terminus and serve as signal sequence and stop transfer sequence (anchor sequence), respectively. Thus, the structural features that are required for translocation into the membrane according to the signal hypothesis (7) are present in viral HA.

However, similar information on the structure–function relationships of NA, the other important surface protein of the influenza virus, is not available. NA, like HA, undergoes antigenic shift and drift. Together with HA, NA plays an important role in viral epidemiology (6), viral pathogenesis (8), and host specificity (9) as well as maturation and release of virus particles (10, 11). The deduced amino acid sequence of NA (12–15) shows that it belongs to a different class of membrane proteins, possessing only one large hydrophobic region at the $NH_2$ terminus. Unlike the signal sequence of HA, this $NH_2$-terminal hydrophobic sequence is not cleaved but serves as the anchoring region of NA by associating with the lipid bilayer of the membrane (16). A number of integral membrane proteins such as isolmaltase (17) and aminopeptidase (18) possess structural features similar to those of influenza NA and remain attached to the membrane via the hydrophobic sequence of the $NH_2$ terminus. Obviously, the $NH_2$-terminal hydrophobic sequence is expected to play a crucial role in the processes involved in translocation, sorting, and topological orientation of these proteins on the membrane. As a first step towards studying the structure–function relationship of different domains of the NA in the biology of virus replication as well as in defining the steps involved in sorting, distribution, insertion, and topological orientation of NA into the membrane, we have cloned the NA gene of WSN (H1N1) virus, using the simian virus 40 (SV40) late promoter, and used it to transfect monkey cells, where it is expressed as functionally active protein.

## MATERIALS AND METHODS

**Virus Strains and Cells.** CV1P [obtained from P. Berg (19)] and CV1 monkey cells were grown in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% fetal bovine serum. Virus stocks of the A/WSN/33 strain of influenza virus were prepared in Madin–Darby bovine kidney (MDBK) cells as described (20).

**Plasmid Vectors.** We arranged to have NA cDNA expressed by employing the late SV40 promoter as previously described for HA (21). Briefly, NA cDNA was placed downstream from the SV40 late promoter in a vector (pA11SVL3) that contains the entire SV40 early region, the SV40 origin of replication, and the initiation and termination signals for late gene transcription but lacks most of the acceptor and donor sites for late gene splicing (22). Plasmid pA11SVL2 (22) was used to construct plasmid pA11SVL3 by placing an *Eco*RI site adjacent to the *Hpa* II site located 12 base pairs downstream from the agnogene ATG codon (23). SV40 DNA sequences of pA11SVL2

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Abbreviations: HA, hemagglutinin; NA, neuraminidase; SV40, simian virus 40; DME medium, Dulbecco's modified Eagle's medium; T antigen, tumor antigen.

* Permanent address: Wyeth Laboratories, Inc., Radnor, PA 19108.
† To whom reprint requests should be addressed.

and pA11SVL3 are inserted in pBR322 via a *Taq* I site (0.566 map unit) converted to an *Xba* I site in the early region. SV40 sequences were excised from pBR322 by *Xba* I cleavage. Religation prior to transfection restores early region function.

To produce a lytic infection SVSal·32 DNA was used as helper. SVSal·32 provided late gene function but lacked the early gene function, which was eliminated by inserting a *Sal* I linker into SV40 DNA at 0.32 map unit. SVSal·32 DNA was then inserted at the *Sal* I site of pBR322 to construct pSVSal·32.

**Transfection and Preparation of Virus Stocks.** SV40–NA recombinant DNA was excised from the vector with *Xba* I and isolated by agarose gel electrophoresis. Approximately 100 ng of this fragment and the SV40 insert of pSVSal·32 were recircularized separately at a DNA concentration of 4 μg/ml, combined, and used for transfecting (24) CV1P cells. Dishes were overlayed with agar for plaque production (19). After 12–14 days at 37°C confluent lysis was observed on the dishes and a lysate was prepared. Subsequently, the lysate was passaged once to prepare the virus stock.

**Antibodies and Immunofluorescent Staining.** Anti-N1 NA monoclonal antibodies prepared against PR8 virus (H1N1) were obtained from J. Schulman (py203) and W. Gerhard (H17, H18, NA66, NA112). All of these crossreacted with WSN virus NA. Anti-H1 HA monoclonal antibody (H15A13-18) against PR8 virus was obtained from W. Gerhard. Approximately 48 hr after infection cells were used for immunofluorescent staining with anti-NA monoclonal antibody followed by incubation with fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA).

**NA Assay.** Cells from a 60-mm dish were lysed in 200 μl of 50 mM Tris·HCl, pH 7.5/1% Triton X-100 and assayed for NA activity by determining extent of cleavage of the sialic acid residue from α-2,3-[$^3$H]sialyllactitol (25).

**Radiolabeling of Infected Cells and Analysis of Polypeptides.** At 45 hr after infection, cells were labeled 5 hr by using cysteine-free DME medium supplemented with 2% dialyzed fetal bovine serum and L-[$^{35}$S]cysteine at 50 μCi/ml (1 Ci = 3.7 × $10^{10}$ Bq). For tunicamycin treatment, cells were first treated for 1 hr at 37°C with tunicamycin (3 μg/ml) in DME medium containing 2% fetal bovine serum and then labeled with L-[$^{35}$S]-cysteine in DME medium containing tunicamycin (3 μg/ml). Subsequently, cells were lysed in RIPA buffer (26, 27) for 10 min at 0°C. Nuclei were removed by pelleting twice for 10 min in an Eppendorf centrifuge and the supernatant was incubated with 2 μl of anti-NA monoclonal antibody for 1 hr at 4°C. Antibody–antigen complexes were isolated by using staphylococcal protein A-Sepharose, washed four times, and subjected to electrophoresis on sodium dodecyl sulfate/10% polyacrylamide gels as described (26, 27).

## RESULTS

**Construction of SV40–NA Recombinant DNAs.** The cloning of the NA gene of the WSN virus (H1N1) and its complete sequence determination have been reported (13, 28). The cDNA clone used here contains the entire coding sequence (1,359 nucleotides coding for 453 amino acids) of NA, including the codons for translation initiation and termination. Major features of the sequence indicated a stretch of hydrophobic amino acids at positions 7–35 as well as three potential glycosylation sites.

We constructed SV40–NA recombinant DNAs that will express either the complete NA or variant forms lacking codons in the anchor region (Fig. 1). The NA cDNA was excised from pNA with *Sal* I and digested with nuclease *Bal* 31 in order to remove nucleotides at the 5′ and 3′ ends of NA cDNA. After addition of *Eco*RI linkers, the resected DNA was ligated into the *Eco*RI site of pBR322 and cloned in *Escherichia coli*. DNA

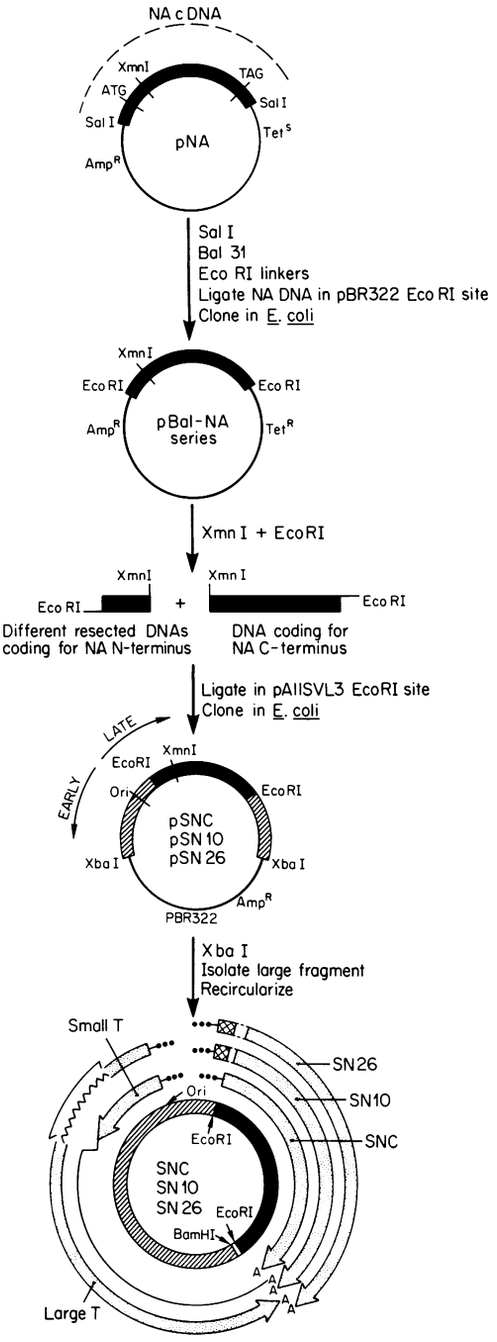


FIG. 1. Construction of SV40–NA recombinant DNAs that express influenza virus NA. Solid bar, the cloned NA DNA segments; hatched bar, SV40 DNA sequences; solid line, pBR322 DNA sequences. The mRNAs presumably transcribed from SNC, SN10, and SN26 and their protein products are shown outside the bottom circle. Cross-hatched areas indicate agnoprotein leader. Wavy lines indicate regions that are excised from the mRNAs. Tet$^S$, tetracycline sensitivity; Tet$^R$, tetracycline resistance; Amp$^R$, ampicillin resistance; Ori, origin of replication; T, tumor antigen.

sequence analysis showed that one clone contained only nine extra nucleotides past the G of the TAG termination codon of NA. This clone was selected for obtaining the DNA from the NA *Xmn* I site (position 291; ref. 13) through the terminal nucleotides and was used in construction of all three SV40–NA recombinant DNAs (labeled COOH terminus; Fig. 1). The DNA encoding the $NH_2$-terminal NA amino acids was obtained from three independent clones. The DNA used in constructing SNC contains 3 nucleotides from G·C linkers and 19 untranslated nucleotides at the 5′ end, followed by the ATG initiation codon.

SNC $NH_2$-MET-ASN-PRO-ASN-GLN-LYS-ILE-ILE-THR-ILE-GLY-SER-ILE-CYS-MET-VAL-VAL-GLY-ILE-ILE-SER-LEU-ILE-LEU-GLN-ILE-GLY-ASN-ILE-ILE-

SN10 $NH_2$-MET-VAL-LEU-ARG-PRO-asp-pro-asn-ser-GLY-SER-ILE-CYS-MET-VAL-VAL-GLY-ILE-ILE-SER-LEU-ILE-LEU-GLN-ILE-GLY-ASN-ILE-ILE-

SN26 $NH_2$-MET-VAL-LEU-ARG-PRO-asp-pro-asn-ser-GLY-ASN-ILE-ILE-

FIG. 2. Amino acid sequence encoded by SNC, SN10, and SN26. SNC consists of the complete amino acid sequence of WSN NA. SN10 and SN26 are fusion proteins. The boxed amino acids are derived from the SV40 agnogene (capital letters) and linker sequences (lower-case letters). All three are identical from amino acid 27 (Gly) of NA to the COOH terminus.

The DNA for SN10 construction lacks the sequence encoding the first 10 amino acids. Finally, DNA used to construct SN26 lacks the coding sequence for the first 26 amino acids. The DNA coding for the NA $NH_2$-terminal region of each of these clones was isolated after cleavage with *Eco*RI and *Xmn* I (labeled $NH_2$ terminus; Fig. 1).

NA genes were reassembled at the *Eco*RI site of the SV40 vector pA11SVL3, using each of the DNAs encoding the $NH_2$ terminus and the DNA encoding the COOH terminus. The plasmids constructed were termed pSNC, pSN10, and pSN26. SNC would be expected to produce the complete NA protein, beginning with its methionine codon. SN10 and SN26 were constructed such that the codons of the first five amino acids of the agnoprotein (29), including ATG, as well as four amino acids coded for by linker sequences, will be in phase with nucleotides coding for amino acids 11 and 27, respectively, of NA. As a result, SN10 and SN26 should produce fusion proteins consisting of a short agnogene leader peptide and linker peptide followed by NA proteins with deletions (Fig. 2). In SNC, however, only the unaltered NA and not the fusion protein is expected to be produced because of the presence of a termination codon (TAA) preceding ATG in the WSN NA gene (13). The predicted $NH_2$-terminal amino acid sequences of these proteins are shown in Fig. 2. The actual nucleotide sequences at the junction sites were confirmed by analysis through the *Eco*RI linker. In addition, the WSN HA gene previously expressed from SV40 vectors (21) was also inserted at the *Eco*RI site of pA11SVL3 for expression. The resulting DNA was termed pSHA.

**Expression of NA in CV1 Cells.** CV1P cells were transfected with SNC, SN10, or SN26 DNA, each along with SVSal-32 DNA, and virus stocks were prepared. Monolayers of CV1 cells were infected with each of the SV40–NA recombinant viruses and subsequently analyzed by indirect immunofluorescence. Fig. 3*A* shows that NA in SNC-infected cells is expressed and becomes concentrated in perinuclear regions. In addition, the NA is clearly present on the cell surface (Fig. 3*B*). SV40 T antigen, on the other hand, was present only in the cell nucleus (Fig. 3*C*) but not on the cell surface (Fig. 3*D*). The NA in SN10-infected cells showed a similar distribution, both within the cell and on the cell surface (data not shown). In each instance approximately 20% of the cells were positive. SN26-infected cells were negative by immunofluorescence with five different anti-NA monoclonal antibodies (pY203, H17, H18, NA66, and NA112) as well as anti-WSN antiserum prepared in rabbits (data not shown).

To examine the sizes of the NA proteins expressed, we infected CV1 cells with each of the SV40–NA recombinant viruses and labeled them with L-[$^{35}$S]cysteine at 45–50 hr after infection. Cells were lysed; labeled proteins were immunoprecipitated with anti-NA monoclonal antibody and analyzed by electrophoresis on a sodium dodecyl sulfate/polyacrylamide gel. Fig. 4 shows that SNC- (track 1) and SN10- (track 2) infected cells produce a protein ($M_r$, 56,000) that was indistinguishable from NA produced in WSN influenza virus-infected CV1 cells (track 6). The $M_r$ of the immunoprecipitated proteins agrees with previous estimates of NA that ranged between 48,000 and 63,000 (31–33) with a carbohydrate content of approximately 20% (31). The diffuse nature of the bands is also indicative of glycosylation. In SHA-infected cells, uncleaved HA ($M_r$, 70,000) was immunoprecipitated with an anti-HA monoclonal antibody (track 4). No proteins were immunoprecipitated in cells infected with SN26 virus (track 3) or mock-infected cells (track 5). SNC and SN10 proteins could not be immunoprecipitated by anti-HA monoclonal antibody (data not shown).

**Enzymatic Activity of Cells Infected with SV40–NA Recombinant Viruses.** NA activity in CV1 cells infected for 48 hr with each of the SV40–NA recombinant viruses was assayed by the

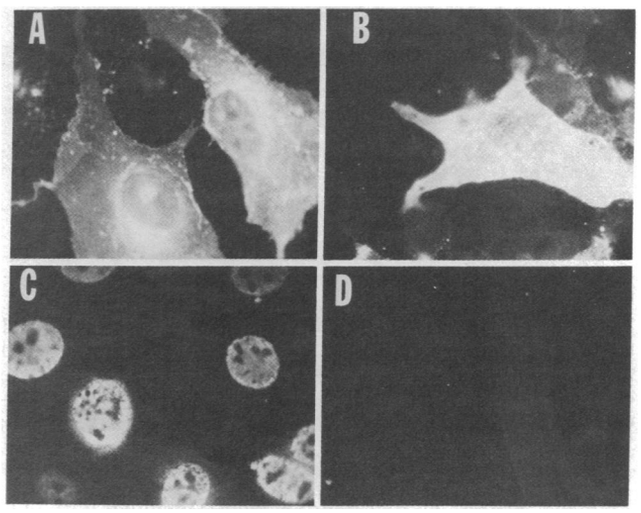


FIG. 3. Immunofluorescent antibody staining of CV1 cells infected with SNC. CV1 cells were infected with SNC virus and at 48 hr after infection were used for immunofluorescent staining. (*A*) For immunofluorescence within the cells acetone/methanol, 1:1 (vol/vol), was used for fixation. Cells were stained with anti-NA monoclonal antibody py203. (*B*) For surface immunofluorescence 3% (wt/vol) paraformaldehyde was used for fixation as described (30). Cells were stained with anti-NA monoclonal py203. (*C*) Acetone/methanol-fixed cells were stained with antiserum to SV40 T antigen. (*D*) Paraformaldehyde-fixed cells were stained with antiserum to SV40 T antigen.

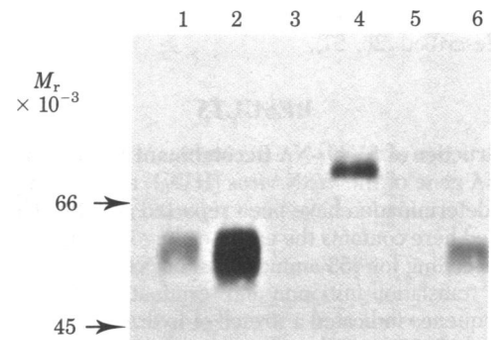


FIG. 4. Gel electrophoresis of NA produced in CV1 cells infected with SV40–NA recombinant virus or with influenza virus. L-[$^{35}$S]Cysteine-labeled proteins were immunoprecipitated with either anti-NA monoclonal antibody py203 (tracks 1–3 and 5 and 6) or anti-HA monoclonal antibody (track 4) and analyzed by gel electrophoresis. Autoradiography was for 16 hr. Tracks: 1, SNC virus; 2, SN10 virus; 3, SN26 virus; 4, SHA virus; 5, mock-infected; 6, CV1 cells were infected 8 hr at 2 plaque-forming units per cell with WSN influenza virus.

Table 1. NA activity produced in cells infected with SV40–NA recombinants or influenza virus*

| Virus | NA activity in lysate† |
|---|---|
| WSN | 1.00 |
| SNC | 0.25 |
| SN10 | 0.75 |
| SN26 | 0.00 |

* Lysates of CV1 cells infected with SV40–NA recombinant viruses were prepared 48 hr after infection and assayed for NA activity. Lysates of CV1 cells infected with WSN virus were prepared 8 hr after infection.

† NA activity relative to that of WSN-infected cells. Values corrected for difference in proportion of cells infected. SV40–NA recombinants infect approximately 20% of the cells, whereas WSN infects nearly 100% of cells.

hydrolysis of $\alpha$-2,3-sialyllactitol (25). Table 1 shows that the expressed NA is enzymatically active and that the amount of active NA produced per cell in SN10-infected cells is approximately equivalent to the amount produced per cell in influenza virus-infected cells. SNC-infected cells produce approximately one-third as much active enzyme per cell as did SN10- or influenza virus-infected cells. The amount of immunoprecipitated protein in SN10-infected cells was also approximately 3-fold greater than that in SNC-infected cells and was essentially equivalent to that of HA expressed from the same vector (Fig. 4). Again, no enzymatic activity was detected in cell lysates (Table 1) or culture supernatants (not shown) of cells infected with SN26 virus.

Kinetics of appearance of NA activity in cells infected with SN10 virus were also determined (Fig. 5). The maximal rate of synthesis of NA occurs between 30 and 40 hr after infection. This is similar to the time of maximal rate of synthesis of SV40 late proteins (34).

**Glycosylation of NA Produced with SV40–NA Recombinant Viruses.** Because the expressed NA migrates with NA produced in WSN-infected CV1 cells (Fig. 4), it appears that both SNC and SN10 are glycosylated. To confirm glycosylation of NA, cells infected with SNC, SN10, and SN26 viruses were treated with tunicamycin, an antibiotic known to block primary glycosylation of the nascent polypeptide (35). In the presence of tunicamycin the $M_r$ of immunoprecipitated proteins was reduced from 56,000 to approximately 48,000 in cells infected with SNC and SN10 viruses (Fig. 6). Similarly, the $M_r$ of HA was reduced from 70,000 to 62,000. This result is in agreement with

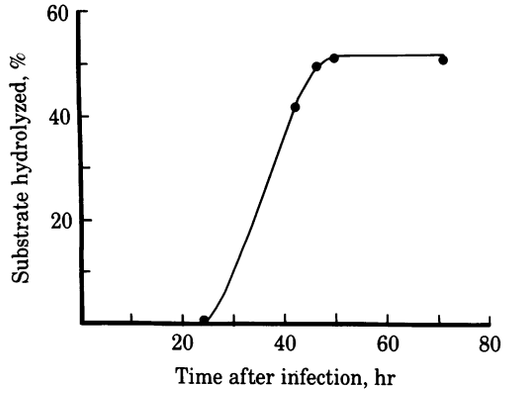


FIG. 5. Kinetics of appearance of enzymatic activity in CV1 cells infected with SV40–NA recombinant virus. Lysates of CV1 cells infected with SN10 were assayed for NA activity at different times. Substrate hydrolyzed is presented as cpm of [$^3$H]lactitol released/total cpm in a 2-hr assay with 40 $\mu$g of protein (25). Plateau level represents 21,439 cpm released.

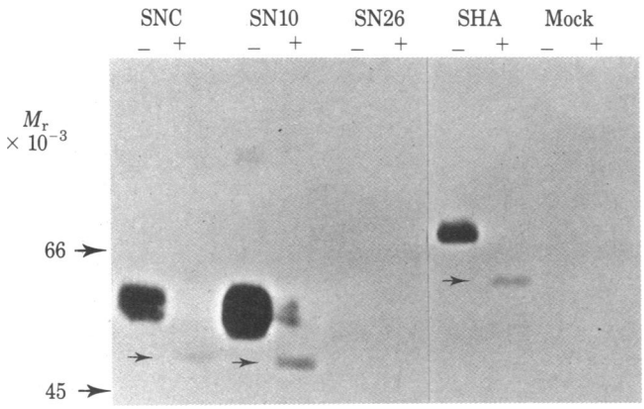


FIG. 6. Sodium dodecyl sulfate/polyacrylamide gel analysis of NA produced from cells infected with SV40–NA recombinant virus in the presence of tunicamycin. CV1 cells were infected for 45 hr with SNC, SN10, SN26, or SHA. Labeling was performed in the presence (+) or absence (–) of tunicamycin. L-[$^{35}$S]Cysteine-labeled proteins were immunoprecipitated with either anti-NA monoclonal antibody py203 (SNC, SN10, SN26, Mock) or anti-HA monoclonal antibody (SHA). Arrows indicate positions of unglycosylated proteins made in the presence of tunicamycin.

the $M_r$ of the NA polypeptide chain ($M_r$, approximately 50,000) predicted from the DNA sequence (13). Thus, the NA expressed in SNC- and SN10-infected cells is glycosylated.

**Properties of Deletion Mutant SN26.** The construction of SN26 depicted in Figs. 1 and 2 indicates that 20 of the 29 codons for hydrophobic amino acids in the NA $NH_2$-terminal sequence have been deleted. The DNA sequence of SN26 was rechecked by analysis through the *Eco*RI site approximately 100 base pairs in each direction. Additionally, the DNA of another clone of SN26 was used for transfection, a virus stock was prepared, and protein analysis was performed. Again, no NA protein was detected with this new virus stock (data not shown).

In addition, levels of NA-specific DNA and RNA were analyzed in SNC-, SN10-, and SN26-infected cells at 48 hr after infection by dot hybridization using nick-translated NA probe (28). NA-specific DNA levels obtained with each virus were approximately the same and NA-specific RNA levels did not vary more than 2-fold (data not shown). Thus, NA-specific DNA and RNA synthesis in SN26-infected cells is comparable to that in SN10- and SNC-infected cells, although no SN26 protein could be detected.

## DISCUSSION

We report here that the NA expressed from cloned cDNA appears indistinguishable from that produced during influenza virus infection in terms of molecular weight, glycosylation, and enzymatic activity. The expressed NA is also transported to the cell surface. These experiments therefore indicate that the NA protein itself contains the necessary signals for biosynthesis, glycosylation, translocation across the membrane, and transport to the cell surface.

It has been recently shown that the first 12 amino acids of N1 and N2 strains are strictly conserved in all type A influenza viruses, whereas the next 60 amino acids vary drastically between N1 and N2 strains (36). The function of the conserved amino acid sequence remains undefined. In this report we show that replacement of the first 10 of the 12 conserved amino acids has no apparent effect on the synthesis, glycosylation, or enzymatic properties of NA. Indeed, we have consistently observed that greater amounts of NA are produced in SN10-infected cells compared to the amount synthesized in SNC-infected cells (Fig. 4, Table 1). NA expressed in SN10-infected cells is

also transported to the cell surface. It appears, therefore, that the strict conservation of these amino acids is not required for the biosynthesis, translocation across the membrane, transport to the cell surface, or enzymatic function of NA. However, this highly conserved sequence may be involved in other functions. (*i*) This terminal sequence may be involved in sorting and directional transport. Whether the sorting and transport of NA is the same in SNC-, SN10-, or influenza virus-infected cells remains to be seen. We have recently shown that HA expressed from cDNA shows the same polarized expression on the cell surface of monkey kidney cells as the HA in influenza virus-infected cells (2). (*ii*) Sequence conservation may be required for interaction with other cellular and viral proteins such as M protein during incorporation of NA into the viral membrane. (*iii*) Alternatively, although unlikely, the amino acid conservation may be a reflection of nucleotide sequence conservation that may be required for a replicase recognition site or packaging of ribonucleoprotein.

SN26, which lacks DNA coding for all but nine hydrophobic amino acids from $NH_2$-terminal region, does not express detectable amounts of NA. Several explanations may account for the inability to detect NA protein in cells infected with this virus. There may be rapid degradation of this protein, if this region confers stability to the polypeptide. Yet even in pulse labels as short as 20 min no protein was observed, although SNC and SN10 proteins could be readily detected (data not shown). Alternatively, this region may indeed function as an extended signal sequence as previously suggested for NA (12). Indeed, when DNA coding for the signal sequence of HA was removed via recombinant DNA techniques (37), HA synthesis was drastically reduced (to approximately 1/100th). The small amount of HA synthesized was unglycosylated and remained cytoplasmic. When not bound to membranes, integral membrane proteins may be intrinsically unstable. It is also possible that polypeptide chain elongation may be blocked (38). We suggest that at least part of a critical signal sequence lies between amino acids 10 and 26 of NA. Furthermore, our studies suggest that the other small hydrophobic domain (amino acids 420–435) at the NA COOH terminus, if at all involved in the signal function, cannot be the only signal sequence. Because our data support that the hydrophobic $NH_2$ terminus functions both as a signal sequence and as an anchor sequence, translocation of NA is compatible with a modified signal hypothesis (39). In this model signals for cotranslational insertion need not always be transient and therefore may remain in the mature protein. Furthermore, the anchor need not always function as a stop transfer sequence because translocation across the membrane may take place via loop formation (39, 40).

The expression of NA from DNA reported here will enable the use of site-specific mutagenesis for further studies correlating specific parts of the NA polypeptide with specific functions. The nature and function of the hydrophobic $NH_2$ terminus and the detailed steps involved in translocation across the membrane can be further explored. In addition, the nature of the active site of the enzyme, thought to lie at the center of molecule (15), can be determined.

Finally, it has been found that several human genetic disorders termed sialidosis are associated with severe or total deficiency of NA activity. These include mucolipidosis and cherry-red spot myoclonus syndrome (see ref. 41). Gene transfer of functionally active NA would enable us to study the molecular mechanism of this disease process and eventually may aid in therapy.

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