JOURNAL OF VIROLOGY, Nov. 1993, p. 6667–6673
0022-538X/93/116667-07$02.00/0

Vol. 67, No. 11

Glycosylation of Neuraminidase Determines the Neurovirulence of Influenza A/WSN/33 Virus

SHENGQIANG LI, JEROME SCHULMAN, SHIGEYUKI ITAMURA,† AND PETER PALESE*
Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029

Received 18 June 1993/Accepted 5 August 1993

The neuraminidase (NA) gene of influenza A/WSN/33 (WSN) virus has previously been shown to be associated with neurovirulence in mice and growth in Madin-Darby bovine kidney (MDBK) cells. Nucleotide sequence analysis has indicated that the NA of WSN virus lacks a conserved glycosylation site at position 130 (corresponding to position 146 in the N2 subtype). To investigate the role of this carbohydrate in viral pathogenicity, we used reverse genetics methods to generate a Glyc+ mutant virus, in which the glycosylation site Asn-130 was introduced into the WSN virus NA. Unlike the wild-type WSN virus, the Glyc+ mutant virus did not undergo multicycle replication in MDBK cells in the absence of trypsin, presumably because of lack of cleavage activation of infectivity. In contrast, revertant viruses derived from the Glyc+ mutant were able to replicate in MDBK cells without exogenous protease. Nucleotide sequence analysis revealed that the NAs of the revertant viruses had lost the introduced glycosylation site. In contrast to wild-type and revertant viruses, the Glyc+ mutant virus was not able to multiply in mouse brain. These results suggest that the absence of a glycosylation site at position 130 of the NA plays a key role in the neurovirulence of WSN virus in mice.

The neuraminidase (NA) of influenza virus is a surface glycoprotein consisting of a square boxlike head and a long narrow stalk which is anchored to the membrane (1, 6, 11, 16). The three-dimensional structure of the NA head of several influenza viruses has been determined (3, 7, 36). Sequencing of the A/Tokyo/3/67 virus NA protein suggested that four Asn residues within the head region are glycosylated (38). The Asn at position 146 (N2 numbering), a conserved residue among influenza A and B virus NA proteins, is associated with a complex sugar (38). This carbohydrate contains *N*-acetylgalactosamine, which is not found in other complex carbohydrate residues of the NA or the hemagglutinin (HA) of influenza viruses (4, 20, 37, 38). NA is able to catalyze the cleavage of the α-ketosidic linkage between the terminal sialic acid residue and the adjacent residue on the carbohydrate chain (17). The active site of the NA is located in the head region (12). Several functional roles of the NA during viral infection have been postulated. NA may cleave sialic acid in mucus, allowing penetration of virus to target respiratory epithelial cells (8, 9). It may also play an important role during the virus release process by cleaving sialic acid receptors from viral and cellular surfaces, preventing progeny virus from self-aggregating or adhering to cell membranes (30, 33, 39).

Influenza A/WSN/33 virus (WSN virus) is neurovirulent for mice. This character correlates with the growth properties of WSN virus in Madin-Darby bovine kidney (MDBK) cells. By analysis of reassortant viruses generated from WSN virus and the nonneurovirulent A/Hong Kong/68 virus, Schulman and Palese have shown that the NA gene of WSN virus confers its unique growth properties in MDBK cells by facilitating cleavage of HA (32). Sugiura and Ueda have used similar techniques to show that the WSN NA is required for its neurovirulence in mice (35). Although the mechanism by which the NA contributes to neurovirulence is not clear, presumably facilitation of HA cleavage in cells, in which cleavage activation of infection is not otherwise observed, is an important element (29). The nucleotide sequence of the NA gene of WSN virus has been determined by Hiti and Nayak (18). It has been observed that the WSN NA lacks a potential glycosylation site at position 130 (position 146 in N2 subtype NA) conserved in other influenza virus neuraminidases. It has been postulated that this change in structure could be correlated with neurovirulence (13, 22).

In this study, we used reverse genetics techniques and a new NA selection system to introduce the glycosylation site Asn-130 into the WSN virus NA. We report the in vitro and in vivo characterization of the resulting Glyc+ mutant virus as well as the isolation of revertant viruses. The fact that Glyc+ mutant virus fails to multiply in mouse brain suggests that the glycosylation of the NA can affect the neurovirulence of influenza WSN virus.

MATERIALS AND METHODS

Viruses and cells. Influenza A/WS/33 virus (WS virus), A/NWS/33 virus (NWS virus), and WSN/HK virus were grown in embryonated eggs. The WS virus was kindly provided by R. Lamb (Northwestern University, Evanston, Ill.). WSN/HK virus is a reassortant virus which derived its NA gene from influenza A/Hong Kong/68 virus (H3N2) and its remaining genes from A/WSN/33 virus (H1N1) (32). The wild-type (wt) WSN virus was obtained by transfection (see below) and was grown in MDBK cells. It should be noted that although for the purpose of comparison we refer to it as wt virus, WSN virus was derived by serial passage in mouse brain. Influenza virus Del 28N was grown in MDBK cells. Del 28N virus is a transfectant WSN virus which has a 28-amino-acid deletion in the stalk region of the NA (25). Both MDBK cells and Madin-Darby canine kidney (MDCK) cells were used for generation and characterization of transfectant viruses.

Plasmid constructs. Plasmid pT3NAwt was designed to facilitate the site-specific mutagenesis of NA. It contains three silent mutations at nucleotide positions 376 (T→A), 382 (C→T), and 412 (G→T), made to create unique *Dra*III and *Kpn*I restriction enzyme sites. pT3NAwt was constructed as

* Corresponding author.
† Permanent address: Department of Virology I, National Institute of Health, Toyama, Shinjuku-ku, Tokyo 162, Japan.

follows. Two polymerase chain reaction products were obtained by using pT3/WSN-NA (14) as the template and two primer pairs, (i) 5′-GTAAAACGACGGCCAGT-3′ and 5′-GTAAGGCACCTTGTGTCAGAAAAAAGG-3′ and (ii) 5′-GC G C G CCACAAGGTGCCTTACTGAATGACAAGCAT TCAAGGGGTACCTTTAAGG-3′ and 5′-CCCCACTGCAG ATGTATC-3′. The first primer is the universal M13 sequencing primer (U.S. Biochemical). The second and fourth primers are complementary to cRNA at positions 389 to 363 and 932 to 915, respectively. The third primer is complementary to viral RNA at positions 375 to 422. The two polymerase chain reaction products were digested with *Eco*RI and *Dra*III or *Dra*III and *Pst*I and were inserted in a trimolecular ligation reaction into *Eco*RI- and *Pst*I-digested pT3/WSN-NA. Plasmid pT3NAmt was constructed by replacing the *Dra*III-*Kpn*I fragment of pT3NAwt with a fragment which was made by annealing the oligonucleotides 5′-GTGCCTTACTGAATGA CAAGCATTCAAATGGTAC-3′ and 5′-CATTTGAATGCT TGTCATTCAGTAAGGCACCTT-3′.

Generation of transfectant virus. The ribonucleoprotein complex was prepared as described previously (23). Briefly, plasmid DNA was digested with *Ksp*632I and used as template for NA RNA synthesis in vitro in the presence of T3 RNA polymerase (Stratagene) and the purified influenza virus polymerase complex. The resulting NA ribonucleoprotein complex was transfected into MDBK cells, which were infected 1 h prior to transfection with WSN/HK or Del 28N virus. At 18 h posttransfection, the supernatant was collected and used for plaque assay. When WSN/HK virus was used as the helper virus, the plaque assay was done in MDBK cells in the absence of exogenous protease, as previously described (15). By using Del 28N as the helper virus, selection of transfectant virus was done in MDCK cells in the presence of trypsin at a final concentration of 2 μg/ml.

RNA sequencing. Direct sequencing of RNA derived from purified virions was done as previously described (23). The primer 5′-TGTCATAAGAGAGCC-3′ used for sequencing is complementary to the NA gene at positions 316 to 330.

Radioactive labeling and immunoprecipitation. MDBK cells were infected with virus at a multiplicity of infection (MOI) of 5 to 10. Eight hours postinfection, cells were labeled with L-[^{35}S]cysteine for 45 min. When indicated, tunicamycin was added to the medium 2 h prior to labeling at a final concentration of 2 μg/ml. The labeled cells were lysed in phosphate-buffered saline (PBS) containing 1.5% *n*-octylglucoside. For immunoprecipitation, the cell lysate was incubated overnight with a mixture of the anti-WSN virus NA monoclonal antibodies 3C8 and 10C9. The precipitated viral proteins were analyzed by electrophoresis on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel.

NA assay. The NA assay using fetuin as the substrate was done as previously described (2). Briefly, 50 μl of serial twofold dilutions of virus stock was incubated overnight with the same volume of fetuin (50 mg/ml) in the presence of 0.1 M phosphate buffer (pH 5.9) and 1.5 mM $CaCl_2$ at 37°C. After incubation, the *N*-acetylneuraminic acid released by the enzyme was chemically converted to a pink chromophore and extracted with 2.5 ml of *n*-butanol–HCl. The A_{549} was read.

For the NA assay using 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid as the substrate, virus was incubated with the substrate at final concentration of 1 mM at 37°C. The fluorescence was determined by using a Turner fluorometer model 111 at a sensitivity setting of 3× (28).

Infection of influenza virus in mice. Intracerebral inoculation of influenza virus was done as previously described (35). Briefly, 10^3 PFU of virus in 50 μl of PBS was inoculated intracerebrally into 6-week-old BALB/c mice. Five days postinfection, mouse brains were collected and homogenized in 1 ml of PBS containing 0.1% gelatin. The amount of infectious virus was determined by plaque assay in MDBK and MDCK cells in the presence of trypsin at a final concentration of 2 μg/ml.

To determine pneumovirulence, 6-week-old BALB/c mice were infected intranasally under anaesthesia with 10^4 PFU of virus in 50 μl of PBS. Three days postinfection, the lungs were collected and homogenized in 1.8 ml of PBS containing 0.1% gelatin. The virus titer was determined by plaque assay in MDBK cells in the presence of trypsin at a final concentration of 2 μg/ml.

RESULTS

Generation of the mutant virus containing a glycosylation site at position 130 in the NA. The glycosylation site at Asn-146 of influenza virus NAs (N2 numbering, corresponding to position 130 of WSN NA) has several outstanding characteristics. In the case of N2 viruses, the complex oligosaccharide attached to this position contains *N*-acetylgalactosamine, a sugar residue rarely found in *N*-glycosidically linked carbohydrates (37). In addition, this glycosylation site is located near the active site of the enzyme (12). Moreover, it is present in all influenza virus NAs except that of the neurovirulent WSN virus (13, 18). We also determined the nucleotide sequence of the NA of the nonneurovirulent WS virus, the parent strain of both neurovirulent WSN and NWS viruses (34). As shown in Table 1, the WS virus has a potential glycosylation site at position 130. Interestingly, the neurovirulent NWS virus has lost this glycosylation site, as has the WSN virus. The NWS contains a single nucleotide change resulting in Asn-130→Tyr, whereas the WSN virus contains a double base change resulting in Asn-130→Arg. These observations led us to investigate whether the absence of the glycosylation site was involved in the neurovirulence of WSN virus.

We took advantage of reverse genetics techniques and introduced a potential glycosylation site into the WSN NA. To facilitate the site-specific mutagenesis, unique *Dra*III and *Kpn*I restriction enzyme sites were introduced into the previously described plasmid pT3/WSN-NA (14) (Fig. 1). The resulting plasmid was designated pT3NAwt. Plasmid pT3NAmt contains two nucleotide changes which resulted in an Arg→Asn change restoring a potential glycosylation site at position 130. Using the previously described system (15), RNA derived from pT3NAmt was transfected into MDBK cells which were infected with WSN/HK helper virus. However, selection in MDBK cells in the absence of exogenous protease did not result in rescue of infectious virus (Fig. 1). These results raised the possibility that the glycosylation at position 130 interferes with the growth of WSN virus in MDBK cells.

We then developed another NA rescue system, in which Del 28N virus was used as helper virus (Fig. 1). Del 28N virus, which contains a deletion in the NA stalk, has previously been shown to replicate in MDBK cells to a level comparable to that of wt WSN virus. However, Del 28N virus was strongly attenuated in MDCK cells (25). Thus, this virus behaves like a host range mutant which can be used to select transfectant viruses growing in MDCK cells. When RNA derived from pT3NAwt was transfected into Del 28N virus-infected MDBK cells, up to 10^5 transfectant viruses were generated after selection in MDCK cells. In the same way, RNA derived from plasmid pT3NAmt was transfected into MDBK cells, and infectious virus was recovered after plaquing of the supernatant in MDCK cells in the presence of trypsin (2 μg/ml). The transfection efficiency and plaque morphology of the recovered

TABLE 1. Nucleotide and deduced amino acid sequences of the NA of influenza WS, NWS, and WSN viruses and of the genetically engineered Glyc+ mutant and its revertant viruses

Virus[a]	Nucleotide sequence at positions 407–415[b,c]	Amino acid sequence at positions 130–132[c]	Presence of glycosylation site
WS	AAT GGG ACC	Asn-Gly-Thr	+
NWS	TAT GGG ACC	Tyr-Gly-Thr	−
wt WSN	AGG GGG ACC	Arg-Gly-Thr	−
Glyc+ mutant	AA*T* GG*T* ACC	*Asn*-Gly-Thr	+
Revertant 1	AA*T* GG*T* G*C*C	*Asn*-Gly-Ala	−
Revertant 2	AA*A* GG*T* G*C*C	Lys-Gly-Ala	−

[a] The Glyc+ mutant virus was generated by using reverse genetics techniques. The revertant viruses, which were able to grow in MDBK cells, were isolated from Glyc+ virus.

[b] Determined by direct sequencing of RNA obtained from purified virus. The primer 5′-TGTCATAAGAGAGCC-3′ corresponds to positions 316 to 330 of the NA viral RNA (18).

[c] Italic letters indicate introduced mutations. Changes present in the revertant viruses are underlined.

virus were similar to those of wt virus derived from pT3NAwt. The introduced mutation at position 130(Arg→Asn) was confirmed by direct RNA sequencing (summarized in Table 1). This virus was designated the Glyc+ mutant virus.

Isolation of revertant viruses. We first examined the ability of the Glyc+ mutant to form plaques in MDBK and MDCK cells. As shown in Table 2, plaque formation by wt WSN virus in MDBK cells was not affected by trypsin, whereas the plaquing of the Glyc+ mutant virus was dependent on the concentration of trypsin. At 2 μg of trypsin per ml, the Glyc+ mutant virus plaqued as efficiently as wt virus did. When the trypsin concentration was only 1 μg/ml, the efficiency of plaquing was 10-fold lower. In the absence of trypsin, only a few tiny plaques were observed at very low dilutions of virus (Table 2). In MDCK cells, the Glyc+ mutant virus was able to form plaques in the absence of trypsin. However, trypsin at a concentration of 2 μg/ml improved the efficiency of plaquing by about 10-fold.

We isolated viruses from several plaques which were formed by the Glyc+ mutant virus in MDBK cells in the absence of trypsin. After replaquing, the revertant viruses retained the ability to grow in MDBK cells without trypsin. We then determined the nucleotide sequences of the revertant viruses in the NA region around the introduced glycosylation site Asn-130. As shown in Table 1, both revertant viruses possess point mutations in the nucleotides encoding the glycosylation motif. The most striking observation was that both revertant viruses contained a point mutation at nucleotide position 413 (A→G), which resulted in a change of the Thr-132 residue to Ala. Revertant 2 contained the additional change of Asn-130 to Lys, which is an amino acid similar to the Arg present in wt WSN virus NA. These results suggest that the introduction of the glycosylation site, and not the change of the amino acid (Arg→Asn), is responsible for the change of the growth properties of the Glyc+ mutant virus in MDBK cells.

In vitro characterization of the Glyc+ mutant virus. To confirm the effect of trypsin on infection of the Glyc+ mutant virus, a multicycle growth experiment was performed by infecting cells with virus at low MOI. As shown in Fig. 2A, the replication of wt and revertant viruses in MDBK cells did not increase in the presence of trypsin. It should be noted that the trypsin concentration of 1.5 μg/ml caused visible cell damage, which was reflected in slightly lower growth of the wt virus compared with that observed in the absence of trypsin. In the Glyc+ mutant virus-infected cells, no HA titer was detected when trypsin was absent. In the presence of trypsin, the Glyc+ mutant virus replicated to a level similar to that of wt WSN virus. These results demonstrate that the Glyc+ mutant virus lacks the ability to facilitate HA cleavage and thus requires trypsin for multicycle replication in MDBK cells. In MDCK cells, the Glyc+ mutant virus was able to replicate in the absence of trypsin. However, the level is lower than that of virus grown in the presence of trypsin (Fig. 2B). The revertant 1 virus replicated poorly in MDCK cells. It also formed very tiny plaques in this cell line (data not shown).

To investigate whether Asn-130 of the Glyc+ mutant virus

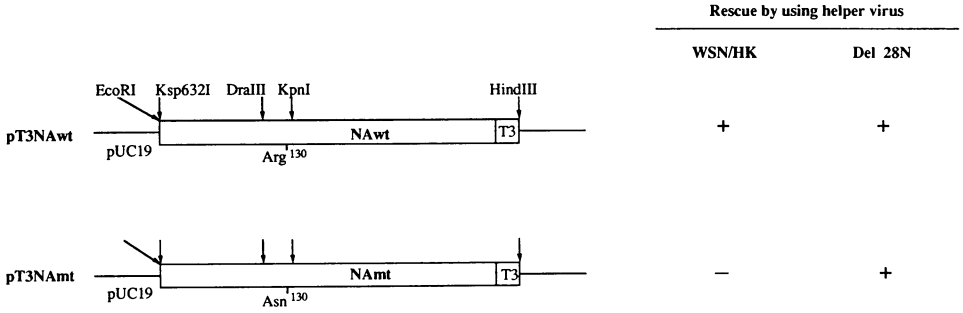

FIG. 1. Plasmid constructs and rescue of the mutant virus. Plasmid pT3NAwt was derived from the previously described pT3/WSN-NA (11). It contains the complete nucleotide sequence of the NA gene of WSN virus, the truncated bacteriophage T3 RNA polymerase promoter, and introduced unique *Dra*III and *Kpn*I restriction enzyme sites. For construction, see Materials and Methods. The arginine residue at position 130 is shown. In plasmid pT3NAmt, the Arg-130 was mutated to Asn to create a potential glycosylation site. Transfection of RNA derived from pT3NAwt or pT3NAmt into WSN/HK virus-infected MDBK cells was done as previously described (15). Transfection of synthetic RNA into Del 28N virus-infected MDBK cells and selection of transfectant virus in MDCK cells are described in Materials and Methods.

TABLE 2. Effect of trypsin on plaque formation by the Glyc+ mutant virus in MDBK and MDCK cells[a]

Virus	Virus titer (PFU/ml)			
	MDBK		MDCK	
	−Trypsin	+Trypsin	−Trypsin	+Trypsin
wt WSN	3.5×10^7	3.8×10^7 4.1×10^{7b}	7.0×10^7	6.8×10^7
Glyc+ mutant	2.0×10^2	1.1×10^8 1.1×10^{7b}	2.8×10^7	2.0×10^8

[a] For the plaque assay, cells were infected with serial 10-fold dilutions of virus. When indicated, trypsin was added at a final concentration of 2 μg/ml in the agar overlay.

[b] The final concentration of trypsin was 1 μg/ml.

NA is indeed glycosylated, we used radioactive labeling and immunoprecipitation to analyze the glycosylation pattern of the NA protein. As shown in Fig. 3, the glycosylated NAs of wt and revertant 1 virus migrated below the nucleoprotein (NP). It should be noted that in the absence of trypsin, the cleavage of WSN virus HA in MDBK cells is not visible after electrophoresis (32). However, the NA of the Glyc+ mutant virus migrated more slowly than the NA of wt virus and similar to the position of NP. After tunicamycin treatment, the NA proteins of all three viruses migrated to similar positions. The molecular weight difference between the NAs of Glyc+ mutant and wt viruses suggests that Asn-130 may be linked to a complex sugar such as the one found in the N2 subtype NA.

We then examined whether the glycosylation at position 130 alters the enzymatic activity of NA. Both a large substrate (fetuin) and a small substrate [2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid) were used for this study. As shown in Fig. 4, the enzymatic activities of wt and revertant 1 viruses were similar. This result indicated that mutations at positions 130 (Arg→Asn) and 132 (Thr→Ala) did not dramatically affect enzymatic activity. The activity of the Glyc+ mutant NA with use of the large substrate was significantly reduced (Fig. 4A). The enzymatic activity with the small substrate was also reduced (Fig. 4B). However, the level of the reduction (about 55%) was not as great as that observed with the large substrate.

Infectivity of the mutant virus in mice. To test for neurovirulence, we intracerebrally inoculated 1,000 PFU of virus into 6-week-old BALB/c mice. Five days postinfection, the brain tissue was collected and the presence of infectious virus was determined by plaque assay in MDBK and MDCK cells. As shown in Table 3, both wt WSN virus and revertant 1 virus were able to replicate in mouse brain. The titers of infectious virus recovered after revertant 1 and wt virus infection ranged from 10^4 to 10^5 PFU per brain. In contrast, no infectious virus was detected in brains from mice infected with the Glyc+ mutant virus. The detection limit of infectious virus was 100 PFU per brain. It should be noted that all mice infected with the wt WSN virus showed typical symptoms of neurological disease. Three of the five mice infected with the revertant 1 virus showed similar neurological symptoms, whereas all five mice infected with the Glyc+ mutant virus behaved normally.

To determine the infectivity of the Glyc+ mutant and revertant viruses in mouse lung, we intranasally inoculated under anaesthesia 10^4 PFU of virus into 6-week-old BALB/c mice. Three days postinfection, the lungs were collected and virus titers were determined by plaque assay in MDBK cells. As shown in Table 3, the virus titer recovered from mouse

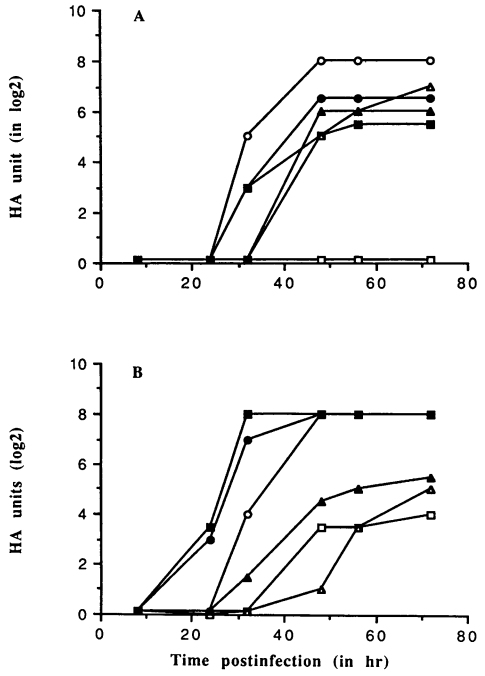

FIG. 2. Growth of wt, Glyc+ mutant, and revertant viruses in tissue culture. MDBK (A) and MDCK (B) cells were infected with wt virus (○, ●), Glyc+ mutant virus (□, ■), or revertant 1 virus (△, ▲) at an MOI of 0.001 in the absence (open symbols) or presence (closed symbols) of trypsin. The concentrations of trypsin used in MDBK and MDCK cells were 1.5 and 2 μg/ml, respectively.

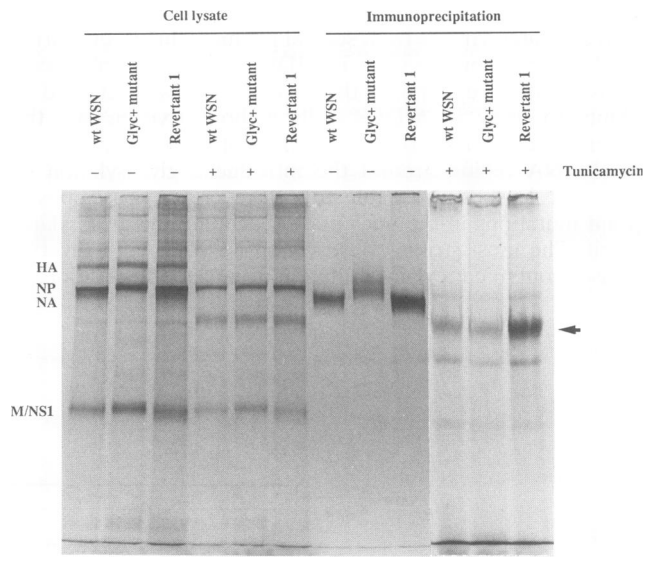

FIG. 3. Analysis of glycosylation pattern of neuraminidase. MDBK cells were infected with viruses at an MOI of 5 to 10. Eight hours postinfection, the cells were labeled with L-[^{35}S]cysteine. When indicated, cells were treated 2 h prior to labeling with tunicamycin at a final concentration of 2 μg/ml. The radioactively labeled cells were lysed in PBS containing 1.5% *n*-octylglucoside. For immunoprecipitation, the cell lysate was incubated with the anti-WSN virus NA monoclonal antibodies 3C8 and 10C9. Labeled and immunoprecipitated viral proteins were analyzed by electrophoresis on an SDS–12% polyacrylamide gel. The arrow indicates the position of the NAs in the presence of tunicamycin.

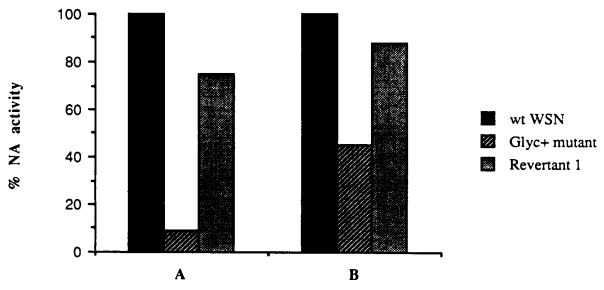

FIG. 4. NA activity of the wt, Glyc+ mutant, and revertant viruses. Enzymatic activities with fetuin (A) and with 2-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (B) were determined as described in Materials and Methods. NA activity is standardized with respect to HA units and is represented as activity relative to that of wt WSN virus (100%).

lungs infected with the Glyc+ mutant virus was similar to that observed after wt WSN virus infection. The revertant virus replicated about 10 times less efficiently in mouse lung compared with the wt and Glyc+ mutant viruses.

DISCUSSION

Neurovirulence in mice is a unique property of some influenza viruses, including WSN virus. Several genetic studies revealed that neurovirulence can be influenced by several genes (21, 26, 31). Sugiura and Ueda showed that the NA, M, and NS genes of WSN virus could contribute to neurovirulence (35). However, the NA gene is essential for neurovirulence, since reassortant viruses lacking the WSN NA were not able to multiply in mouse brain (35).

In this study, we have confined our attention to the role of NA and have identified a structural element within the NA which determines the neurovirulence of WSN virus. By using reverse genetics methods, a glycosylation site was introduced into the NA of the WSN virus. This was done by changing Arg in position 130 to Asn and thus creating an Asn-X-Thr motif. The resulting Glyc+ mutant virus required exogenous protease for its efficient replication in MDBK cells. Trypsin at appropriate concentrations increased the efficiency of plaque formation by more than 10^5-fold and allowed the multicycle replication of the Glyc+ mutant virus in this cell line. On the other hand, revertant viruses which contained mutations in the glycosylation triplets were able to multiply in MDBK cells in the absence of trypsin. Revertant 1 virus retained the Asn residue at position 130. Thus, it is most likely the carbohydrate, not the mutation of Arg to Asn, which is associated with the failure of the Glyc+ mutant virus to grow in MDBK cells. In MDCK cells, the Glyc+ mutant virus was able to replicate in the absence of trypsin. Revertant 1 virus was attenuated in tissue culture, especially in MDCK cells. Both revertant viruses contained mutations in positions which are conserved across different subtypes. Lentz et al. have previously shown that a change of Asn-146 to Ser in the Tokyo virus NA (N2 subtype) interferes with the transport and/or folding of the protein (22). Although the mutations present in the revertant are not lethal ones, they could affect the NA processing during viral infection. Further experiments would be needed to explore this possibility. It should be noted that we have not isolated a virus which reverts to the wt WSN NA sequence. Since a double base change would be needed to restore the Arg of the wt NA, this kind of reversion may occur at very low frequency.

TABLE 3. Infectivity of the Glyc+ mutant and revertant viruses in mice

Virus	Virus titer ($\log_{10}$ PFU)	
	Brain[a]	Lung[b]
wt WSN	4.86 ± 0.43	7.20 ± 0.24
Glyc+ mutant	<2.00[c]	7.02 ± 0.02
Revertant 1	3.48 ± 0.65	5.98 ± 0.32

[a] Mice were infected intracerebrally with 10^3 PFU of virus. Five days postinfection, the mouse brains were collected and the presence of infectious virus was determined by plaque assay in MDBK cells. The virus titer is expressed as the mean of titers obtained from five individual mice ± standard error.

[b] Mice were infected intranasally with 10^4 PFU of virus. Three days postinfection, the lungs were collected and the titer of infectious virus was determined by plaque assay in MDBK cells. The titer is expressed as the mean of titers obtained from five mice ± standard error.

[c] Detection limit was 10^2 PFU per brain.

The Glyc+ mutant virus was not able to multiply in mouse brain, suggesting that it had lost the properties of neurovirulence. No infectious virus was recovered after intracerebral inoculation of 1,000 PFU of the Glyc+ mutant virus, while 10^4 to 10^5 infectious virus particles were detected from brains of mice infected with either wt or revertant 1 virus. Furthermore, neurological signs were observed only in mice infected with the wt or revertant virus. It should be noted that the Glyc+ mutant virus stock contained revertant viruses at a frequency of about 10^{-5}. However, under the experimental conditions used in this study, the mutant virus inoculum (10^3 PFU) would not be likely to contain any revertant virus. We also did not observe the emergence of revertant viruses from mouse brain infected with the mutant virus. However, an increase of inoculum of the Glyc+ mutant virus might allow the isolation of revertant viruses in vivo. The Glyc+ mutant virus replicated in mouse lungs to a level similar to that of the wt WSN virus. These results suggest that glycosylation of the NA did not dramatically influence pneumovirulence.

The biological properties of the Glyc+ mutant virus revealed the importance of HA cleavage for neurovirulence of influenza virus. Analysis of reassortant viruses derived from the WSN and HK viruses previously showed that all reassortants which contained WSN NA were able to replicate in mouse brain and in a neuroblastoma cell line and had a cleaved HA in the latter system (29, 34). Studies by Scholtissek et al. also suggested that a cleavable HA derived from influenza virus A/FPV/Rostock/34 was essential for the neurovirulence of several reassortants (31). Thus, the cleavage of HA, which is required for initiation of infection, plays a crucial role in determining the neurovirulence of influenza virus. The NA of the WSN virus has been shown to facilitate the cleavage of HA (32). One likely possibility is that the WSN virus NA removes sialic acid from the HA surface and thus facilitates its cleavage by endogenous protease. How the glycosylation at Asn-130 could destroy the ability of the wt WSN NA to facilitate the cleavage of the HA remains unclear. However, analysis of the enzymatic activity revealed that the mutated NA had an altered activity with large substrates such as fetuin. We may speculate that the presence of a carbohydrate at Asn-130 interferes with the access to substrate, so that the mutated NA is not able to efficiently remove sialic acid from the surface of the HA. In this scenario, sialylated or partially sialylated HA would not be cleaved by the protease present in MDBK cells and mouse brain, and thus the Glyc+ mutant virus would not be neurovirulent.

It is known that the glycosylation of HA can play a role in the pathogenicity of some influenza viruses. As shown for the pathogenic A/chick/Penn/1370/83 virus, the loss of a carbohy-

drate at Asn-11 was responsible for the cleavability of the HA and an increase in the pathogenicity of the virus (19). Our results described above provide the first direct evidence that the glycosylation of NA can also be involved in viral pathogenicity. The reverse genetics approach and a new selection system to isolate influenza viruses with altered NA genes provided us with a tool with which to directly study the effect of a single mutation in the NA on the pathogenicity of a neurovirulent influenza virus.

The possibility of rescuing transfectant influenza virus containing a cDNA-derived NA gene has been extensively explored. On the basis of unique growth properties of WSN virus, Enami et al. have introduced the WSN NA gene into infectious virus particles (14, 15). This system was previously used to generate attenuated influenza virus (27) and to study the function of the stalk of the NA (10, 25). Recently, the possibility of using an NA-minus virus for rescue of the NA gene was described by Liu and Air (24). In the present study, we used a host range mutant to rescue transfectant virus. This system may have broad application, since the selection can be done in MDCK cells, which are suitable for the growth of a variety of influenza viruses. In addition, the transfection efficiency of 10^5 PFU/ml is similar to that obtained for other systems (15). Thus, the reverse genetics techniques described above may provide an excellent tool with which to study the structure-function relationship of the NA.

ACKNOWLEDGMENTS

We thank Robert Lamb for providing the WS virus and for suggesting that we analyze the NA of this nonneurovirulent isolate.

This work was supported by NIH grants (P.P.).

REFERENCES

1. **Air, G. M., and W. G. Laver.** 1989. The neuraminidase of influenza virus. Proteins Struct. Funct. Genet. **6**:341–356.
2. **Aminoff, D.** 1961. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. **81**:384–392.
3. **Baker, A. T., J. N. Varghese, W. G. Laver, G. M. Air, and P. M. Colman.** 1987. Three dimensional structure of the neuraminidase of subtype N9 from an avian influenza virus. Proteins Struct. Funct. Genet. **2**:111–117.
4. **Basak, S., D. G. Pritchard, A. S. Brown, and R. W. Compans.** 1981. Glycosylation sites of influenza viral glycoprotein: characterization of trypic glycopeptides from the A/USSR (H1N1) hemagglutinin glycoprotein. J. Virol. **37**:549–558.
5. **Basak, S., M. Tomana, and R. W. Compans.** 1985. Sialic acid is incorporated into influenza haemagglutinin glycoproteins in the absence of viral neuraminidase. Virus Res. **2**:61–68.
6. **Blok, J., G. M. Air, W. G. Laver, C. W. Ward, G. G. Lilly, E. F. Woods, C. M. Roxgurgh, and A. S. Inglis.** 1982. Studies on the size, chemical composition and partial sequence of the neuraminidase (NA) from type A influenza virus show that the N-terminal region of NA is not processed and serves to anchor the NA in the viral membrane. Virology **119**:109–121.
7. **Burmeister, W. P., R. W. Ruigrok, and S. Cusack.** 1992. The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. EMBO J. **11**:49–56.
8. **Burnet, F. M.** 1948. Mucins and mucoids in relation to influenza virus action. IV. Inhibition by purified mucoid of infection and haemagglutinin with the virus strain WSE. Aust. J. Exp. Biol. Med. Sci. **26**:381–387.
9. **Burnet, F. M., J. F. McCrea, and S. G. Anderson.** 1947. Mucin as a substrate of enzyme action by viruses of the mumps influenza group. Nature (London) **160**:404–405.
10. **Castrucci, M. R., and Y. Kawaoka.** 1993. Biologic importance of neuraminidase stalk length in influenza A virus. J. Virol. **67**:759–764.
11. **Colman, P. M.** 1989. Neuraminidase: enzyme and antigen, p. 175–210. *In* R. M. Krug (ed.), The influenza viruses. Plenum Press, New York.
12. **Colman, P. M., J. N. Varghese, and W. G. Laver.** 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature (London) **303**:41–44.
13. **Colman, P. M., and C. W. Ward.** 1985. Structure and diversity of influenza virus neuraminidase. Curr. Top. Microbiol. Immunol. **114**:177–255.
14. **Enami, M., W. Luytjes, M. Krystal, and P. Palese.** 1990. Introduction of site specific mutations into the genome of influenza virus. Proc. Natl. Acad. Sci. USA **87**:3802–3805.
15. **Enami, M., and P. Palese.** 1991. High efficiency formation of influenza virus transfectants. J. Virol. **65**:2711–2713.
16. **Fields, S., G. Winter, and G. G. Brownlee.** 1981. Structure of the neuraminidase in human influenza virus A/PR/8/34. Nature (London) **290**:213–217.
17. **Gottschalk, A.** 1957. Neuraminidase: specific enzyme of influenza virus and *Vibrio cholerae*. Biochim. Biophys. Acta **23**:645–646.
18. **Hiti, A. L., and D. P. Nayak.** 1982. Complete nucleotide sequence of the neuraminidase gene of human influenza virus A/WSN/33. J. Virol. **41**:730–734.
19. **Kawaoka, Y., C. W. Naeve, and R. G. Webster.** 1984. Is virulence of H5N2 influenza viruses in chickens associated with the loss of carbohydrate from the hemagglutinin? Virology **139**:303–316.
20. **Keil, W., R. Geyer, J. Dabrowski, U. Dabrowski, H. Nieman, S. Stirm, and H.-D. Klenk.** 1985. Carbohydrates of influenza virus. Structure elucidation of the individual glycans of the FPV hemagglutinin by two dimensional H n.m.r. and methylation analysis. EMBO J. **4**:2711–2720.
21. **Kilbourne, E. D.** 1963. Influenza virus genetics. Prog. Med. Virol. **5**:79–126.
22. **Lentz, M. R., R. G. Webster, and G. M. Air.** 1987. Site-directed mutation of the active site of influenza neuraminidase and implication for catalytic mechanism. Biochemistry **26**:5351–5358.
23. **Li, S., J. L. Schulman, T. Moran, C. Bona, and P. Palese.** 1992. Influenza virus transfectants with chimeric hemagglutinins containing epitopes from different subtypes. J. Virol. **66**:399–404.
24. **Liu, C., and G. M. Air.** 1993. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase gene. Virology **194**:403–407.
25. **Luo, G., J. Chung, and P. Palese.** 1993. Alteration of the stalk of the influenza virus neuraminidase: deletions and insertions. Virus Res. **29**:141–153.
26. **Mayer, V., J. L. Schulman, and E. D. Kilbourne.** 1973. Nonlinkage of neurovirulence exclusively to viral hemagglutinin or neuraminidase in genetic recombinants of A/NWS (H0N1) influenza virus. J. Virol. **11**:272–278.
27. **Muster, T., E. K. Subbarao, M. Enami, B. R. Murphy, and P. Palese.** 1991. An influenza A virus containing influenza B virus 5′ and 3′ noncoding regions on the neuraminidase gene is attenuated in mice. Proc. Natl. Acad. Sci. USA **88**:5177–5181.
28. **Myers, R. W., R. T. Lee, Y. C. Lee, G. H. Thomas, L. W. Reynolds, and Y. Uchida.** 1980. The synthesis of 4-methylumbelliferyl α-ketoside of N-acetylneuraminic acid and its use in a fluorometric assay for neuraminidase. Anal. Biochem. **101**:166–174.
29. **Nakajima, S., and A. Suguira.** 1979. Neurovirulence of influenza virus in mice. II. Mechanism of virulence as studied in a neuroblastoma cell line. Virology **101**:450–457.
30. **Palese, P., K. Tobita, M. Ueda, and R. W. Compans.** 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology **61**:397–410.
31. **Scholtissek, C., A. Vallbracht, B. Flehmig, and R. Rott.** 1979. Correlation of pathogenicity and gene constellation of influenza viruses. II. Highly neurovirulent recombinants derived from non-neurovirulent or weakly neurovirulent parent virus strains. Virology **95**:492–500.
32. **Schulman, J. L., and P. Palese.** 1977. Virulence factors of influenza A virus. WSN virus neuraminidase required for plaque production in MDBK cells. J. Virol. **24**:170–176.
33. **Seto, J. T., and R. Rott.** 1966. Functional significance of sialidase during influenza virus multiplication. Virology **30**:731–737.
34. **Sugiura, A.** 1975. Influenza virus genetics, p. 171–213. *In* E. D.

Kilbourne (ed.), The influenza virus and influenza. Academic Press, New York.
35. **Sugiura, A., and M. Ueda.** 1979. Neurovirulence of influenza virus in mice. I Neurovirulence of recombinants between virulent and avirulent strains. Virology **101:**440–449.
36. **Varghese, J. N., W. G. Laver, and P. M. Colman.** 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. Nature (London) **303:**35–40.
37. **Ward, C. W., and T. A. A. Dopheide.** 1981. Amino acid sequence and oligosaccharide distribution of the hemagglutinin from an early Hong Kong variant A/Aichi 2/68(X-31). Biochem. J. **207:**91–95.
38. **Ward, C. W., J. M. Murray, C. M. Roxburgh, and D. C. Jackson.** 1983. Chemical and antigenic characterisation of the carbohydrate side chains of an Asian (N2) influenza virus neuraminidase. Virology **126:**370–375.
39. **Webster, R. G., and W. G. Laver.** 1967. Preparation and properties of antibody directed against the neuraminidase of influenza virus. J. Immunol. **99:**49–55.