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Optimizing Viral Protein Yield of Influenza Virus Strain A/Vietnam/1203/2004 by Modification of the Neuraminidase Gene▽

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The preparation of high-yield prepandemic influenza virus H5N1 strains has presented a challenge to both researchers and vaccine manufacturers. The reasons for the relatively low yield of the H5N1 strains are not fully understood, but it might be partially dependent on the interactions between the hemagglutinin (HA) or neuraminidase (NA) surface glycoprotein and other influenza virus proteins. In this study, we have constructed chimeras between the A/Puerto Rico/8/34 (PR8) NA gene and the A/Vietnam/1203/2004 (VN1203) NA gene that have resulted in an increase in the virus yield of the reassortant viruses without a significant loss of NA activity. By combining the amino terminus of NA from the PR8 strain with the carboxy terminus of NA from VN1203, the surface epitopes unique to the H5N1 VN1203 NA glycoprotein are maintained. This reassortant virus had a higher titer and total protein yield in eggs, grew to a higher titer, produced large plaques on MDCK cells, and retained NA activity. This work describes a novel recombinant technique designed to increase the yields of vaccine candidates for the production of pandemic influenza virus vaccines. The relationship between the infectivity and protein yield of the reassortants also is discussed.

One of the typical challenges that vaccine manufacturers face each influenza virus season is to rapidly grow enough of the selected strains to manufacture sufficient doses of vaccine to protect the entire at-risk population. The prevalent strains often do not grow well in chicken eggs, and higher-growth reassortants must be made in order to provide sufficient antigen for the >150 million doses needed for each fall season in the United States alone. To date, all of the vaccine strains licensed in the United States for the seasonal inactivated influenza virus vaccines have been made using classical egg reassortant techniques. More than one reassortant virus often must be prepared to select a strain adequate for vaccine manufacture. Studies of prepandemic strains, however, have shown that some do not grow very well using these traditional culture methods (24). Therefore, at the time of an outbreak, the challenge to produce millions of vaccine doses is even greater. An example of this is seen with the H5N1 strains, which were identified previously only in avian species, but in 1997 they were transmitted directly to humans from birds (23). After this species jump occurred, these highly pathogenic H5N1 viruses were responsible for more than 245 deaths in 15 countries (www.who.int).

Currently the only substrate that is used in the United States for influenza virus vaccine manufacture is embryonated chicken eggs. Vaccines against highly pathogenic avian viruses cannot be grown in this system, since these viruses would not only kill chicken embryos but also would pose significant risk to staff during the manufacturing process (22, 24). Reassortants were made that included the two glycoproteins from the prevailing avian virus strain while the internal genes were contributed by the WHO-recommended, classical donor vaccine strain, A/Puerto Rico/8/34 (PR8) (26). However, the H5N1 reassortant viruses did not grow as well in eggs as the PR8 strain (8). Experience with current H5N1 prepandemic vaccine strain reassortants has shown low antigen yield compared to that of seasonal influenza virus vaccine production. It is not fully understood why the H5N1 strain does not grow as robustly as other subtypes of influenza A viruses, but the observed restriction might have a basis in the interactions between the glycoproteins and the viral proteins encoded by the remaining six internal genes. Additionally, recent evidence shows that four times the usual dose of hemagglutinin (HA) is required for an H5N1 vaccine to achieve levels of protective antibody responses that are similar to those seen with the seasonal influenza virus vaccine (25), underscoring yet another reason to address the low growth of pandemic strains. While researchers have not focused significant effort on the contribution of the neuraminidase (NA) gene to the overall virus yield, it is known that its coordination with HA plays a significant role in viral growth (17). Neuraminidase function, which is critical to virus release (19, 20), also may act independently to determine the overall robustness of the virus and directly influence viral yield (16).

One strategy that may be used to evaluate the relative contribution of the NA protein of the PR8 strain, especially its N terminus domain, to overall yield is reverse genetics. This technique makes it possible to generate recombinant viruses that possess genes derived from a cloned cDNA and achieve the desired complement of internal genes and surface glycoproteins from various subtypes (6, 18). Here, we report the use of reverse genetics to generate several chimeric viruses that have an NA gene that incorporates portions of the PR8 NA into the A/Vietnam/1203/2004 (VN1203) NA sequences. These viruses

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are in the context of reassortants containing internal genes from PR8 and the HA glycoprotein contributed from either PR8 or the H5N1 isolate. The sequence of the original NA gene from the VN1203 H5N1 isolate contains a deletion and several amino acid differences from the PR8 (H1N1) NA gene. The length of the NA stalk, the region that holds the globular head domain away from the virion surface, can have a significant impact on virus growth (4). Therefore, the focused of this study was to determine if the PR8 stalk and tail could contribute to an increase in H5N1 yield while preserving NA activity and maintaining antigenicity.

MATERIALS AND METHODS

Plasmids, viruses, and cells. Plasmids containing the eight segments from A/Puerto Rico/8/34 (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW196-NA, pHW197-M, and pHW198-NS) and two segments from A/Vietnam1203/2004 (pHW-1203HA and pHW-1203NA) were obtained from Robert Webster at St. Jude Children's Research Hospital, Memphis, TN, where the VN1203 HA had been attenuated by the modification of the multibasic site from PQRERRRKKR ↓ G to PQIET. . . .R ↓ G. A series of influenza viruses containing the NA genes with various stalk deletions or modifications were generated using PCR. Primers 5′-CGGCGGCGGCGTCTCCGGGAGCAAAAGCAGG and 5′-TCATATGCATATAACCGGTGTGAATTGAATGACTGAC were used to make the amino half of the VN1203 NA gene by PCR, and primers 5′-CCGGTTATATGCATATGAATTCATCTCTTTGCCCCAT and 5′-CGGCGGCGGCGTCTCCTATTAGTAGAAACAAGG were used for the carboxyl half of the VN1203 NA gene. Using the two outside primers, a shortened NA gene was made by PCR that was missing the 33-amino-acid stalk region of VN1203 NA. This deletion in VN1203 NA maintained the correct reading frame, while the primers introduced EcoRI and AgeI restriction sites. The PR8 stalk was generated as an oligonucleotide and exchanged for the truncated VN1203 stalk using the EcoRI and AgeI sites. The amino terminus of PR8 NA was made by PCR using the primers 5′-GTACTGGTCGACCTCCGAAG and 5′-GTCTTTGCTGTATACAGCCCACCC. This was ligated into the vector containing the VN1203 NA gene using BsmBI enzyme and subcloned into the pHW-2000 plasmid. All constructs were sequenced to verify that the correct mutations had been introduced. Madin-Darby canine kidney (MDCK) cells and 293T cells were grown in Dulbecco's modified essential medium (DMEM) (Invitrogen, Carlsbad, CA) with 10 or 5% fetal bovine serum (HyClone, Logan, UT), respectively.

Production of reassortant influenza viruses carrying NA gene mutations. Reassortant influenza viruses carrying various HA and NA genes were generated by previously described reverse genetic techniques (6, 18), with modifications as described previously (14). Three days postinoculation, egg allantoic fluids were harvested and fluids with positive HA titers were stored at −80°C.

Titration of virus on MDCK cells. Plaque assays were performed on monolayers of MDCK cells in 12-well tissue culture plates. Serial dilutions were prepared from the viral stock of egg allantoic fluid and 200 μl of each dilution incubated on monolayers, in duplicate, for 1 h at 37°C and 5% CO_2 with occasional rocking. The inoculum was aspirated, and monolayers were overlaid with 1 ml of a 1:1 mix of 2× minimal essential medium (Invitrogen) and 1.5% agarose containing 1 μg/ml of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. Cultures were incubated for 3 days at 33°C, fixed with methanol, and stained with 1% crystal violet to reveal plaques.

Single-step growth curves. MDCK cell monolayers were infected at a high multiplicity of infection of 3 PFU/cell and then incubated for 1 h at 37°C before the inoculum was replaced with 1 ml of DMEM lacking serum but containing 1 μg/ml of TPCK-trypsin. Supernatant fluids (110 μl/sample) were collected at each time point (0, 6, 12, 24, and 36 h postinfection) and stored at −80°C until processed. The quantity of virus at each time point was determined by plaque assay.

EID_{50} and total protein yield assays. Embryonated 11-day-old chicken eggs were checked for viability and spray disinfected. Each virus was serially diluted from 10^{-1} to 10^{-9} in phosphate-buffered saline (PBS) containing 10 μg/ml gentamicin, and 0.2 ml was injected into the allantoic cavity of each of five eggs per dilution. Eggs were incubated at 33°C for 3 days before being chilled for 8 h at 4°C. Allantoic fluids were collected from each egg, and 50 μl was tested for hemagglutination with chicken erythrocytes (CBT Farms, Chestertown, MD). The Kärber formula was used to calculate the 50% egg infectious dose (EID_{50}) for each strain (11). For total protein yield, virus was pelleted according to a previously described method (13), with minor modifications. Approximately 60 ml of allantoic fluid was collected from HA-positive eggs and centrifuged at 2,000 × *g* for 25 min to remove egg debris. Concentrated virions were centrifuged through a discontinuous 15 to 30 to 60% sucrose gradient at 100,000 × *g* for 90 min. The band of viral particles was collected from the 30 to 60% interface, concentrated through a 25% sucrose cushion, and then resuspended in PBS before total protein was quantitated using a bicinchoninic acid assay (Pierce, Rockford, IL).

Neuraminidase assay. NA activity was determined as follows. A 25-μl volume of substrate (20 μM 4-methylumbelliferyl-*N*-acetylneuraminic acid [MU-NANA] [Sigma, St. Louis, MO] in PBS [pH 7.4]) was added to 25 μl of each sample containing 8 to 32 HA units. After 1 h at 37°C, reactions were stopped with 0.1 M glycine (pH 10.7) in 25% ethanol. Controls and standards were run in parallel, and the fluorescence was measured on a Victor V (Perkin Elmer, Waltham, MA) at an excitation of 355 nm and an emission of 460 nm for 0.1 s per well.

RESULTS

Construction of VN1203 NA and PR8 NA chimeric reassortant influenza viruses. Plasmids containing NA from PR8 (GenBank accession no. ABP64733) and the NA from VN1203 (GenBank accession no. EF541467) were used to generate chimeras between the two NA genes. Figure 1A contains the alignment of the amino-terminal region of NA from these two genes. The alignment of the stalk region of PR8 and VN1203 shows that the VN1203 NA has a six-amino-acid deletion and that 17 other amino acids of the stalk region differ from the sequence of PR8. The stalk domain is the least conserved region of the NA gene between these two strains; there is only 36% amino acid identity between PR8 and VN1203 NA in the stalk region, while there is 88% identity between the two in the remainder of the protein. To create NA chimeras, a portion of the VN1203 NA stalk was deleted by PCR, and then specific primers and PCR were used to incorporate segments of the PR8 NA gene. The schematic of the final NA constructs used in this study is shown in Fig. 1B. This group of chimeras provided us with several variations of the VN1203 NA gene that would allow us to discern whether the PR8 N terminus supplies an improved function needed for better growth and/or higher yield of the reassortant viruses. The swap or the deletion of the stalk would indicate if the length was of primary importance. Changing the entire N terminus would determine if these sequences of the NA protein were contributing to important interactions with HA or internal genes that were responsible for improved growth.

Generation of viruses by reverse genetics. Table 1 lists the 10 virus strains created and analyzed in this study and the respective HA and NA genes for each. All viruses were 6:2 combinations, using the six PR8 internal genes transfected along with HA from PR8 (to produce chimeric viruses JV15, JV16, JV17, JV18, and JV22) or the HA from VN1203 (to produce chimeric viruses JV19, JV20, JV21, JV23, and JV24) and various NA constructs as indicated in Table 1. Reassortant influenza viruses were rescued in cells and amplified in eggs, and then allantoic fluid was harvested, clarified, and frozen down as virus stock. Rescued viruses were examined by reverse transcription-PCR and sequencing to verify that they contained the intended HA and NA genes.

Growth of virus strains in eggs. The growth of rescued reassortant viruses was analyzed in eggs using an EID_{50} experiment performed with the primary harvest virus. Based on the hemagglutination results, the EID_{50} titer per milliliter was calculated. There was a range of approximately 2 $\log_{10}$ be-

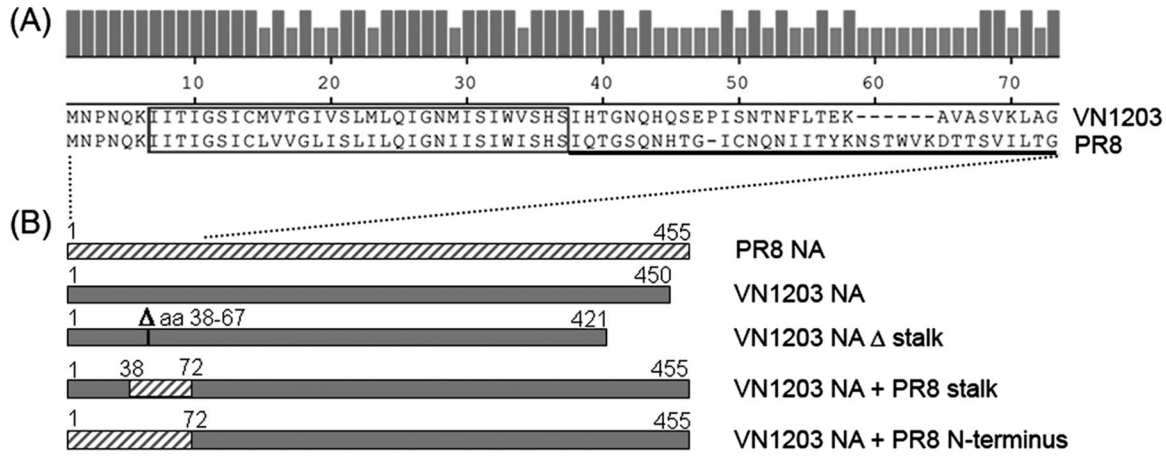

FIG. 1. Neuraminidase alignment and chimeras. (A) The alignment of the amino terminus of the NA gene segments from A/Vietnam/1203/2004 (top line) and A/Puerto Rico/8/34 (bottom line) is shown. The Clustal W method was used for this alignment, and identical amino acids are shown with taller bars, while changes are shown with shorter bars. The first six amino acids comprise the only cytoplasmic portion of the protein (1). The NA transmembrane region is boxed, and the stalk region is underlined. Deletions are indicated by a dash instead of an amino acid letter. (B) The first two constructs shown in this cartoon are PR8 (striped) and VN1203 (solid), respectively. Chimeras between the two NA genes are indicated by the insertion of the striped PR8 portion into the solid VN1203 NA gene, and the amino acid numbers indicate the length of the final constructs.

tween strains, but all viruses showed high infectivity rates, achieving at least 10^8 EID_{50} per ml. This result was interesting, as we had expected to see a significant decrease in the growth abilities of some strains. Specifically, JV17 and JV21, each of which contain an NA with a significantly shortened stalk region paired with PR8 HA and VN1203 HA, respectively, were expected to grow less efficiently than the other chimeric viruses, but only nominal decreases were seen. There also was a slight increase in growth efficiency between JV15 and JV16, in which the latter contains the NA gene from VN1203. Interestingly, in our system, the five strains that contained the VN1203 HA generally grew to infectious titers that were as high as those of strains containing the PR8 HA. The combination of the PR8 stalk or PR8 N terminus with the remainder of the VN1203 NA gene showed that this virus had an infectivity that was equivalent to that of the wild type.

Additionally, the morphology of the reassortant virions was analyzed by electron microscopy. The results indicated that JV24, which contains the PR8 N terminus on the VN1203 NA gene, had a normal spherical morphology similar to that of the PR8 wild type (data not shown). On the other hand, JV21 and JV17, both of which have the VN1203 NA gene with the deleted stalk, showed some filamentous particles and many segmented filaments of virus aggregates (data not shown).

TABLE 1. Virus strains generated by reverse genetics and growth efficiency as measured by EID_{50} and cell culture titers[a]

Virus	Gene designation		EID_{50}/ml	MDCK titer (PFU/ml)
	HA	NA		
JV15	PR8	PR8	$10^{9.2}$	$10^{7.5}$
JV16	PR8	VN1203	$10^{9.8}$	$10^{7.4}$
JV17	PR8	1203 with mutated stalk	10^{8}	0
JV18	PR8	VN1203 with PR8 stalk	$10^{8.8}$	$10^{7.0}$
JV22	PR8	VN1203 with PR8 N-term	$10^{9.2}$	$10^{7.5}$
JV19	VN1203	PR8	$10^{10.2}$	$10^{8.2}$
JV20	VN1203	VN1203	$10^{9.2}$	$10^{8.7}$
JV21	VN1203	VN1203 with mutated stalk	10^{9}	$10^{6.1}$
JV23	VN1203	VN1203 with PR8 stalk	$10^{9.2}$	$10^{8.6}$
JV24	VN1203	VN1203 with PR8 N-term	$10^{9.6}$	$10^{8.9}$

[a] All viruses have the six internal genes from PR8. N-term, N terminus.

Growth of the viruses in MDCK cells. The growth characteristics of reassortant viruses also were compared in cell culture. The titers of the 10 virus stocks are shown in Table 1 and reveal that when the VN1203 NA with the deleted stalk was paired with the PR8 HA (JV17), this virus showed no plaques on MDCK cells, even at the lowest dilution. To verify that it was not a limitation of our crystal violet staining method, the assay was repeated using anti-HA antibodies in a focus-forming unit assay, and no plaques were detected (data not shown). JV21, which contained the stalk-deleted VN1203 NA mutant glycoprotein paired with its cognate VN1203 HA, was restricted about 100-fold for replication in MDCK cells compared to that with the replication of the reassortant virus containing the parental NA protein.

Overall, it appears that reassortants containing HA from VN1203 grow as well on MDCK cells as virus containing HA from PR8. The data shown were representative of three experiments, and the standard deviations were within ± 1 $\log_{10}$.

It was further noted that the morphology of the plaques was not consistent between the various strains (Fig. 2). As noted above, JV17 did not form plaques on MDCK cells, though the virus grows in eggs. JV21, which is formed by pairing the stalk-deleted VN1203 NA with VN1203 HA, forms small pinpoint plaques on MDCK cell monolayers. In contrast, JV24, which contains VN1203 NA modified to express the PR8 N terminus, makes plaques that are larger than those of wild-type VN1203 HA and NA (JV20). This provides an interesting backdrop for further analysis to see if we can reliably modify the growth characteristics of influenza virus by pairing the extracellular domain of NA with the N-terminal 72 amino acids of PR8.

The replication of the modified viruses also was studied by

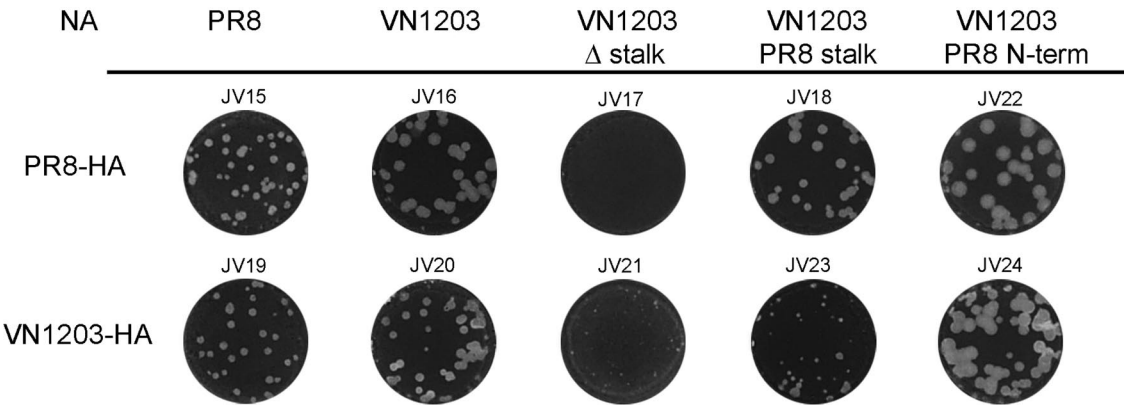

FIG. 2. Plaque morphology of the reassortant viruses on MDCK cell monolayers. Cell monolayers were fixed with methanol at 72 h postinfection and were stained with 1% crystal violet. The plates were dried, and then plaques were counted and photographed for an analysis of the various plaque sizes.

analyzing their growth kinetics in MDCK cells during a single-step time course (Fig. 3). Consistently with previous results, we were unable to see plaques from the JV17 virus at any time point on MDCK cells. JV21 showed an overall reduced growth phenotype, with a 2 to 4 $\log_{10}$ reduction throughout the time course. Interestingly, the replication of JV23, which contains the VN1203 NA with the PR8 stalk, was about 1 $\log_{10}$ lower than that of the other strains tested. When the entire N terminus of PR8 was combined with VN1203 NA (JV24) instead of just the stalk, replication returned to wild-type (JV15) levels. Overall, these results indicate that strains containing chimeric NA genes are able to grow quite well in MDCK cells, and in some cases they demonstrate kinetics that are close to those of the wild-type strains. In summary, the addition of the PR8 NA N terminus to the VN1203 NA resulted in a virus that grows very well on both MDCK cells and in eggs.

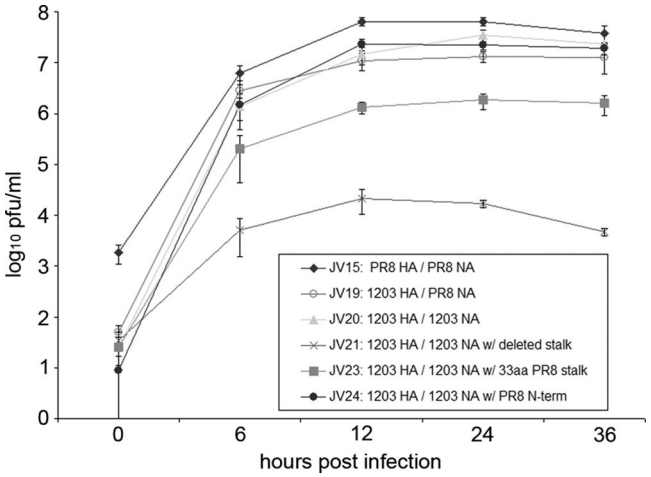

FIG. 3. Replication kinetics. Single-step growth curves of reverse genetics-generated viruses in MDCK cells. Cultures in 12-well dishes were infected at a multiplicity of infection of 3 PFU/cell, and samples were taken from the infected cell monolayer at the indicated time points. The titers of the samples on MDCK cells were determined. These growth curves are averages from three independent experiments, and standard errors are indicated. N-term, N terminus; aa, amino acid.

Total protein yield from reassortant viruses. Since each virus strain grew relatively well in eggs, we explored the relationship between virus growth and total viral protein yield in eggs. The allantoic fluid from infected eggs was clarified and concentrated, and viral particles were banded and pelleted before the protein concentrations were measured. Significant differences were observed in the amount of total protein and, presumably, the total number of viral particles that were produced by each of the strains (Fig. 4). The protein yield is presented as a percentage of the total protein obtained for the JV15 strain, which contains PR8 HA and PR8 NA. When PR8 HA was present, any version of the PR8 stalk or PR8 N terminus on the VN1203 NA demonstrated high levels of protein yield (JV15, JV18, and JV22). Only VN1203 NA (JV16) and the VN1203 NA with the stalk deleted (JV17) gave a reduced yield. In contrast, there was an overall decrease in the total protein yield when VN1203 contributed the HA gene to the new virus strains. The amount of HA also was tested and shown to remain proportional. For example, JV15 showed an inverse HA titer of 2,048, and that of JV19 was 1,024. The titer of JV21 was severalfold lower, at 64, while the JV24 reassortant showed an HA titer of 512. The viral proteins from the reassortant viruses also were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and showed the major viral proteins to be proportional.

The JV20 strain, with VN1203 HA and VN1203 NA only, provides ~37% of the yield of the wild-type PR8. When we incorporated the PR8 N terminus into the VN1203 NA (JV24), the yield was raised to 58%. These results confirmed that high infectivity in eggs or in MDCK cells (Table 1) may not accurately reflect the protein yield of the same strains grown in eggs. All of these results provide an interesting contrast to the MDCK plaque assay results, where the lower-yield viral strains (containing VN1203 HA) all had higher levels of growth in cell culture.

To verify that there were no artificial decreases in total protein from the loss of viral proteins due to the aggregation of viral particles or the adherence of viral particles to egg debris, we also employed a modification of the protocol by increasing the salt concentration to 1.5 M before the clarification spin (9).

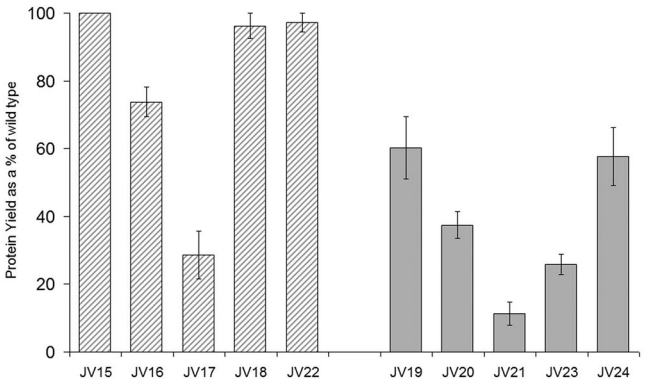

FIG. 4. Total protein. The viruses were propagated in the allantoic cavities of 9-day-old embryonated eggs by the inoculation of 0.2 ml of diluted virus stocks containing ~10^4 EID_{50} or 0.1 HA units/ml at 33°C for 2 days. Virus in allantoic fluid was concentrated and purified by banding in 15 to 60% sucrose gradients (14). Total protein yield was measured from virions purified from 60 ml of allantoic fluid. Viruses that contained the HA gene from PR8 are the striped bars on the left, while those with the VN1203 HA gene are represented by the solid bars on the right. The JV15 (wild-type) virus that contains HA and NA from PR8 was set at 100% for protein yield, and the remainder of the viruses are compared to this. This graph shows the averages from two independent experiments. JV15 (PR8 HA and PR8 NA), JV16 (PR8 HA and VN1203 NA), JV17 (PR8 HA and VN1203 NA with the stalk deleted), JV18 (PR8 HA and VN1203 NA with the PR8 stalk), JV22 (PR8 HA and VN1203 NA with the PR8 N terminus), JV19 (VN1203 HA and PR8 NA), JV20 (VN1203 HA and VN1203 NA), JV21 (VN1203 HA and VN1203 NA with the stalk deleted), JV23 (VN1203 HA and VN1203 NA with the PR8 stalk), and JV24 (VN1203 HA and VN1203 NA with the PR8 N terminus) were used.

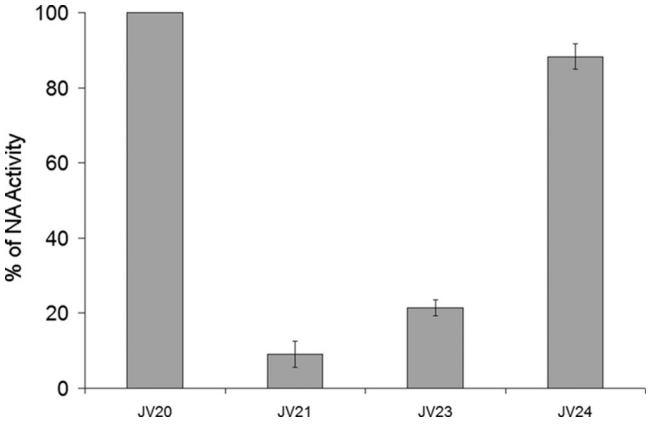

FIG. 5. Neuraminidase activity. The JV24 reassortant virus shows restored levels of NA activity. NA activity was measured at 12 h postinfection in MDCK cells and was normalized for HA in each strain at that time point. The mutant strains are compared to the JV20 virus, which contains both HA and NA from VN1203 and is set at 100% for NA activity. This graph shows the averages from three individual experiments.

It was found that this did not change the relative HA titer or the protein yields (data not shown).

NA activity. In order to determine if the NA activity was affected by the mutations made in the NA genes, the viral strains were inoculated onto MDCK cells, and samples were taken from each strain 12 h postinfection and NA activity measured (Fig. 5) after normalizing for HA units. When the NA activity of the JV20 strain (containing VN1203 HA and VN1203 NA) was set at 100%, there was a significant loss of NA activity for JV21, which carries the NA gene with the deleted stalk (JV21). Some NA activity was restored in JV23, when the PR8 stalk was swapped for the shorter VN1203 NA stalk. However, the addition of the entire PR8 amino terminus of the NA gene in JV24 results in NA activity that is approximately 94% of that seen with wild-type (JV15) strains. These results indicated that the reassortant virus, JV24, which contains the PR8 N terminus of NA on the VN1203 NA gene and has high growth in eggs and MDCK cells, also retains a significant portion of its NA activity.

DISCUSSION

An influenza virus pandemic is a looming danger for a naïve population. Since H5N1 influenza viruses continue to circulate asymptomatically in duck and geese populations, there needs to be a system in place to rapidly generate a vaccine to control a potential pandemic influenza virus outbreak. Novel avian virus strains have not been extensively manipulated in laboratory settings and often grow less efficiently in vaccine substrates than laboratory-adapted strains. By combining pandemic influenza virus genes with genes derived from a high-growth donor strain, such as PR8, reassortant viruses can be generated that will facilitate the rapid production of the pandemic vaccines in a timely manner. It is known that the contribution of the M gene from the PR8 vaccine donor strain is responsible for some of the high-growth characteristics of the PR8 strain (12). Recent evidence links the polymerase genes to the high level of virulence of H5N1 in mammalian species (21). It also has been shown that not all laboratory-adapted versions of PR8, which have been maintained in various ways in laboratory settings since its isolation in 1934, have the same growth characteristics (7). Although the incorporation of the internal genes from a laboratory-adapted strain, PR8, increases avian virus yield in eggs, the total protein yield of reassortant viruses may not be optimal. In this paper, we addressed these issues by delineating regions of the PR8 NA gene that can be used to modify the pandemic strain NA gene to increase total viral protein yield.

Since the NA genes of both VN1203 and PR8 are N1 subtypes, they have a high degree of identity at the amino acid level throughout a large portion of the protein. In contrast, the stalk regions, which hold the globular head domain off of the surface of the viral particle, have a much lower percentage of identical amino acids between these two strains. We postulated that the stalk region, either its length or sequence, is a critical component in generating a VN1203 vaccine strain with higher growth or increased protein yield. To accomplish this, we created a panel of NA constructs in which sections of the stalk were deleted or contained regions of the PR8 NA gene in place of the VN1203 NA gene. This was accomplished by either swapping out just the VN1203 NA stalk region for the PR8 stalk region or by substituting the entire amino terminus region, including the internal cytoplasmic tail, the transmembrane domain, and the stalk region. These NA constructs then were studied using reassortant viruses of two different backgrounds: first, a panel of 7:1 reassortant viruses were created that contained NA as the only gene contributed by the H5N1

strain, with the seven other genes being derived from PR8, or second, 6:2 reassortant viruses with HA from the H5N1 strain paired with the various NA constructs, and the remaining six genes coming from PR8.

Several properties of these viruses were analyzed in this study to characterize growth and antigen yield. While all 10 of the strains showed overall high levels of replication in eggs (with EID_{50} titers between 10^8 and $10^{10.2}$ [Table 1]), the overall total protein yields in eggs varied significantly between the various strains (Fig. 4). In fact, the five reassortant viruses that contained HA from VN1203 (JV19, JV20, JV21, JV23, and JV24) grew to EID_{50} titers comparable to those of viruses containing PR8 HA, but they had significantly lower protein yields. This goes counter to the thought that high infectivity in eggs directly correlates with high protein yield and an increase in the expected number of doses of vaccine per egg. This might indicate that in eggs, the system used for this study, the presence of the VN1203 HA did not hinder, and in fact might aid, the infectivity of the virus. Previous work has shown that deletions in the stalk region lead to the inability of the NA to properly function with the cognate HA and with a concomitant decrease in replication in some cell types or eggs (15). This phenomenon likely is strain dependent, though many short-stalk influenza virus strains replicate in tissue culture to levels equivalent to those of the parent virus (5).

Two of the strains that were tested contained a 33-amino-acid deletion of the NA stalk. Short-stalk mutants displayed moderate to severe growth defects. For example, JV17 did not form plaques on MDCK cells (Fig. 2). This inability to form plaques reinforces the idea that the balance of HA and NA is critical for efficient exit from and entry into the MDCK cells (17). Since the EID_{50} results for JV17, at 10^8 per ml, indicate a moderate level of replication, it is obvious that the deletion made in the stalk still is partially compatible with the H5 HA glycoprotein, but it is unable to infect neighboring cells to form plaques on MDCK monolayers. When the stalk-deleted NA was paired with the VN1203 HA, there was an increase in compatibility between the glycoproteins, and the JV21 strain formed pinpoint plaques on MDCK cells.

The JV24 virus, a 6:2 reassortant containing VN1203 HA, and a chimeric form of NA with the PR8 N terminus on the VN1203 NA, grew robustly in eggs and represents a candidate for use in vaccine production. The JV24 strain contained the carboxy terminus of the VN1203 NA, which includes the external epitopes that are present in the native VN1203 NA protein. The chimeric NA also provided the PR8 internal portion of the NA gene, which may allow for the more productive interactions with the other influenza virus proteins, including M1 protein from the PR8 strain. The percentage of NA and HA proteins in JV24 was compared to that in JV20 by Western blotting using antibody to VN1203 NA or PR8 HA antigen. The results indicated that JV24 contained slightly more NA protein (approximately 15% more) than JV20, but there were not differences in the percentages of HA proteins between JV24 and JV20 (data not shown), suggesting that a chimeric form of NA with the PR8 N terminus increases the incorporation of NA into the virus with the PR8 background and enhances the release of viral particles from cells. It has been suggested that the transmembrane domains and cytoplasmic tails of HA and NA play a role in association with M1 protein (2, 10, 27, 28), which also interacts with ribonucleoproteins (3, 13). The interaction of glycoproteins with M1 protein, therefore, may enhance the incorporation of ribonucleoprotein cores into viral particles and subsequently may increase the viral replication rate.

Both the PR8 and VN1203 NA genes are from the N1 subtype, although they demonstrate significant amino acid divergence in the stalk region. The molecular engineering of improved NA genes should not be limited to H5 strains only, and we plan to apply this to other clades of H5 and to H7N7 and H9N2 prepandemic strains as well. Preliminary alignments indicate that H7N7 (such as A/chicken/Netherlands/2586/2003) and H9N2 (A/chicken/Israel/1953/2004) isolates have significant divergence from PR8 NA and a longer stalk region than that of either the VN1203 or the PR8 NA protein (data not shown). Annual influenza virus strains that do not grow well in eggs also could have their yield significantly boosted by swapping in the PR8 NA N terminus for the original N terminus of the NA gene. This would leave the native NA antigenic region intact, but it could increase growth by efficiently complementing the six internal genes that come from PR8 when using reverse genetics to engineer reassortant viruses with the desired growth properties to rapidly initiate the manufacture of influenza virus vaccines.

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