JOURNAL OF VIROLOGY, June 2005, p. 6763–6771 Vol. 79, No. 11
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.11.6763–6771.2005

Improvement of Influenza A/Fujian/411/02 (H3N2) Virus Growth in Embryonated Chicken Eggs by Balancing the Hemagglutinin and Neuraminidase Activities, Using Reverse Genetics

Bin Lu, Helen Zhou, Dan Ye, George Kemble, and Hong Jin*

MedImmune Vaccines, Inc., 297 N. Bernardo Ave., Mountain View, California 94043

Received 6 December 2004/Accepted 24 January 2005

The H3N2 influenza A/Fujian/411/02-like virus strains that circulated during the 2003-2004 influenza season caused influenza epidemics. Most of the A/Fujian/411/02 virus lineages did not replicate well in embryonated chicken eggs and had to be isolated originally by cell culture. The molecular basis for the poor replication of A/Fujian/411/02 virus was examined in this study by the reverse genetics technology. Two antigenically related strains that replicated well in embryonated chicken eggs, A/Sendai-H/F4962/02 and A/Wyoming/03/03, were compared with the prototype A/Fujian/411/02 virus. A/Sendai differed from A/Fujian by three amino acids in the neuraminidase (NA), whereas A/Wyoming differed from A/Fujian by five amino acids in the hemagglutinin (HA). The HA and NA segments of these three viruses were reassorted with cold-adapted A/Ann Arbor/6/60, the master donor virus for the live attenuated type A influenza vaccines (FluMist). The HA and NA residues differed between these three H3N2 viruses evaluated for their impact on virus replication in MDCK cells and in embryonated chicken eggs. It was determined that replication of A/Fujian/411/02 in eggs could be improved by either changing minimum of two HA residues (G186V and V226I) to increase the HA receptor-binding ability or by changing a minimum of two NA residues (E119Q and Q136K) to lower the NA enzymatic activity. Alternatively, recombinant A/Fujian/411/02 virus could be adapted to grow in eggs by two amino acid substitutions in the HA molecule (H183L and V226A), which also resulted in the increased HA receptor-binding activity. Thus, the balance between the HA and NA activities is critical for influenza virus replication in a different host system. The HA or NA changes that increased A/Fujian/411/02 virus replication in embryonated chicken eggs were found to have no significant impact on antigenicity of these recombinant viruses. This study demonstrated that the reverse genetics technology could be used to improve the manufacture of the influenza vaccines.

Influenza epidemics caused by different variants of the same influenza A virus subtypes or by influenza B virus are usually a result of changes to the antigenic glycoproteins of the virus, enabling escape from the host immunity. Significant antigenic drift is often associated with more severe influenza epidemics as the host immunity from the natural infection or vaccination becomes poorly protective against the drifted viruses. The emergence of A/Sydney/05/97-like strains in 1997 and the A/Fujian/411/02-like strains in 2003 resulted in influenza epidemics (3). In addition, replacement of the hemagglutinin (HA) with novel subtypes that have not been present in humans for long periods of time is defined as antigenic shift; this large antigenic change could cause an influenza pandemic. Vaccination plays a major role in the prevention of influenza and associated complications. However, the constant antigenic drift and periodic antigenic shift require that influenza virus vaccines be updated frequently to be effective against the circulating influenza strains. Currently, the licensed influenza virus vaccines in the United States are produced in embryonated chicken eggs. Occasionally, the prototype vaccine strains, such as A/Fujian/411/02, do not replicate well in eggs. This property makes them difficult to isolate in eggs, and it may be necessary to use cell culture to isolate these strains. The production of the vaccine may also be limited. Using reverse genetics to improve the ability of vaccine strains to replicate in eggs may be a critical step in delivering sufficient vaccines.

Replication of influenza virus in a host has been found to be associated with the receptor-binding activity of the HA and the neuraminidase (NA) activity of the NA molecule (28). HA and NA interact with sialic acid-containing receptor with conflicting activities. Influenza viruses bind to sialic acid residues present on cell surface glycoproteins or glycolipids through the receptor-binding site in the distal tip of the HA molecules followed by receptor-mediated endocytosis during viral entry (28, 50). The NA, on the other hand, cleaves the Neu5Ac moiety from the HA molecule to release the progeny virus from the cell membrane and to prevent aggregation of progeny virions (6, 27, 39). This NA enzymatic activity, however, also cleaves the receptor from the target cells. Therefore, the balance between the receptor-binding activity of the HA and the neuraminidase activity of the NA is critical for efficient virus replication in host cells (22, 23, 25, 35, 48). Although NA-deficient viruses have been made by passaging in the presence of exogenous bacterial neuraminidase and anti-NA antibodies, the released virions aggregated at the host cell surface (29). Adaptation to growth of NA-deficient virus in the absence of exogenous sialidase activity resulted in a concomitant decrease in the affinity of the HA protein for cellular receptors (16). When the NA activity was decreased due to anti-NA drug selection, concomitant HA mutations that lowered the HA

* Corresponding author. Mailing address: MedImmune Vaccines, Inc., 297 N. Bernardo Ave., Mountain View, CA 94043. Phone: (650) 603-2367. Fax: (650) 603-3367. E-mail: jinh@medimmune.com.

receptor-binding affinity were frequently isolated. These characteristics were necessary to facilitate progeny virus release and spread (1, 10, 11, 13, 35). It has also been shown that the deficiency in NA activity conferred by the shortened NA stalk can be compensated for by a decreased receptor-binding affinity of the HA in order to restore the balance of HA and NA (35). The converse has also been observed. Removal of the N-glycan attachment site near the HA receptor-binding pocket resulted in an increase in HA receptor-binding ability. This virus could replicate well only when matched with a NA of higher enzymatic activity (48). This balance of HA and NA activities is believed to be a limiting factor in the emergence of new HA and NA combinations. Concomitant changes in both HA and NA may be required for the adaptation of influenza viruses to new hosts in the natural environment.

Different receptor-binding specificity of human and avian viruses determines their preferential tropism to nonciliated cells and ciliated cells, which express 2,6-linked or 2,3-linked sialyl-galactosyl moieties, Siaα(2,6)Gal or Siaα(2,3)Gal, respectively (32). Since 1992, human influenza H3N2 viruses isolated from MDCK and Vero cell lines, which are rich in Siaα(2,6)Gal, were unable to agglutinate chicken red blood cells efficiently and grew poorly in embryonated chicken eggs (8, 34, 36, 38). Previous studies have indicated that amino acid changes in the HA receptor-binding region (8) or a different composition of the carbohydrates attached to the HA (36) could also change receptor-binding specificity.

To understand the molecular mechanisms of poor replication of the A/Fujian/411/02 H3N2 strain in embryonated chicken eggs, several related 6:2 reassortant H3N2 vaccine strains were produced by introducing the HA and NA segments of different H3N2 variants into the cold-adapted A/Ann Arbor/6/60 strain, the master donor virus (MDV-A) for FluMist (30). By selecting a common set of internal gene segments, any difference in the internal genes on replication would be minimized. Strains carrying the wild-type (wt) HA and NA in MDV-A maintain the cold-adapted, temperature-sensitive, and attenuated phenotypes (18, 19, 37). The amino acid residues in the HA and NA of influenza A/Fujian/411/02-like virus strains were evaluated for their effect on virus replication in MDCK cells and in embryonated chicken eggs. It was demonstrated that replication of 6:2 A/Fujian in eggs could be greatly improved by either increasing the HA receptor-binding activity or reducing the NA activity.

MATERIALS AND METHODS

Virus strains, cells, and antibodies. Wild-type influenza A virus strains, A/Fujian/411/02 (A/Fujian), A/Sendai-H/F4962/02 (A/Sendai), and A/Wyoming/03/03 (A/Wyoming) were obtained from the Centers for Disease Control and Prevention (Atlanta, GA) and amplified once in MDCK cells or in embryonated chicken eggs (eggs). The modified vaccinia virus Ankara strain expressing the bacteriophage T7 RNA polymerase (MVA-T7) (52) was grown in chicken embryonic kidney cells. HEp-2, COS-7, and MDCK cells (obtained from American Type Culture Collection, Manassas, VA) were maintained in minimal essential medium (MEM) containing 5% fetal bovine serum. Polyclonal antisera against A/Ann Arbor/6/60, A/Sendai-H/F4962/02, and A/Wyoming/03/03 were produced in chickens. Monoclonal antibodies against the NP protein of influenza A were obtained from BioDesign (Saco, MI).

Cloning of HA and NA expression plasmids. To make recombinant 6:2 reassortant viruses containing the HA and NA segments of H3N2 subtype and the six internal MDV-A RNA segments, the HA and NA cDNAs of wt A/Sendai-H/F4962/02 and A/Wyoming/03/03 were amplified by reverse transcriptase PCR (RT-PCR) using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and PFU DNA polymerase (Stratagene, La Jolla, CA), the extracted viral RNA as template and the H3 and N2 specific primers. HA-AarI5 (5′CACTTATTCACCTGCCTCAGGGAGCAAAAGCAGGGG3′) and HA-AarI3 (5′CCTAACATATCACCTGCCTCGTATTAGTAGAAACAAGGGTGTT3′) primers were used to amplify the HA segment. N2-AarI5 (5′CACTTATATTCACCTGCCTCAGGGAGCAAAAGCAGGAGT3′) and N2-AarI3 (5′CCTAACATATCACCTGCCTCGTATTAGTAGAAACAAGGAGTTT3′) primers were used to amplify the NA segment. Both the HA and the NA primer pairs contained the AarI restriction sites that were designed to be compatible to the BsmBI sites present in the pAD3000 polymerase I/polymerase II expression plasmid (15, 18). The HA and NA cDNA clones were sequenced and compared to the consensus HA and NA sequences that were obtained by direct sequencing of the HA and NA RT-PCR–amplified cDNA products. Any mutations introduced into the cDNA clones during the cloning process were corrected by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Measurement of the neuraminidase activity of the transiently expressed NA protein. To measure the neuraminidase activity of the NA proteins, wt NA and its modified derivatives were expressed from the plasmid-transfected cells. To obtain a high level of expression of the NA proteins, the NA RNA was transcribed from the T7 and cytomegalovirus promoters as the gene was inserted downstream of these dual promoters. HEp-2 cells in 10-cm dishes were infected with MVA-T7 at a multiplicity of infection (MOI) of 5.0 for 1 h followed by transfection of 5 μg of the NA plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The transfected cells were incubated at 35°C for 48 h. After washing with phosphate-buffered saline (PBS), the cells were scraped from the dishes and lysed in 100 μl of 0.125 M NaOAc (pH 5.0). The neuraminidase activity was determined by a fluorometric assay (40). After one freeze-and-thaw cycle, 50 μl of cell lysate was serially diluted twofold and incubated with 150 μl of 1.2 mM 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MU-NANA) substrate (Sigma, St. Louis, MO) at 37°C for 1 h and stopped by the addition of 75 μl of 1.0 M glycine (pH 10.4). The fluorescence level of the released chromophore 4-methylumbelliferone was determined at 362 nm with a SPECTROMAX plate reader. The level of each NA protein expressed in the transfected cells was monitored by Western blotting using chicken anti-A/Wyoming antisera. The neuraminidase activities of wt A/Sendai and A/Wyoming viruses containing 6.0 $\log_{10}$ PFU in 100 μl were also measured by the fluorometric assay.

Generation of recombinant 6:2 reassortants. Recombinant 6:2 reassortants that contained the HA and NA RNA segments of the H3N2 strains reassorted into MDV-A were generated according to the previously described procedures (15, 18). Briefly, a set of six plasmids containing the internal genes of MDV-A (18) together with the HA and NA expression plasmids were transfected into the cocultured COS-7/MDCK cells using TransIT LT1 reagents (Mirus, Madison, WI). The transfected cell culture supernatant was collected at 3 days posttransfection and used to infect fresh MDCK cells and 10-day-old embryonated chicken eggs. The infected MDCK cells were incubated at 33°C until 80 to 90% of the cells exhibited cytopathic effects. The infected embryonated chicken eggs were incubated at 33°C for 3 days, and the allantoic fluids were collected and stored at −80°C in the presence of the sucrose-phosphate-glutamic acid stabilizer (0.2 M sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, and 5.4 mM monosodium glutamate). The virus titer was determined by plaque assay in MDCK cells incubated under an overlay that consisted of 1× L15-MEM, 1% agarose, and 1 μg/ml TPCK (*L*-l-tosylamide-2-phenylethyl chloromethyl ketone)-trypsin at 33°C for 3 days. The plaques were enumerated by immunostaining using chicken anti-MDV-A polyclonal antibodies.

Receptor binding and replication of 6:2 recombinants in MDCK cells. HA receptor-binding and growth kinetics of recombinant 6:2 reassortants were determined by using MDCK cells. MDCK cells in six-well plates were infected with 6:2 A/Fujian, A/Sendai, A/Wyoming and two modified recombinant viruses at a MOI of 1.0. After 30 min of adsorption at either 33°C or 4°C, the infected cells were either washed three times with PBS or directly overlaid with 3 ml of Opti-MEM I containing 1 μg/ml TPCK-trypsin and incubated at 33°C. One set of the infected plates was fixed with 1% paraformaldehyde at 6 h postinfection for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 15 min followed by immunofluorescence analysis using anti-NP monoclonal antibodies. The cell images captured by an ORCA-100 digital camera were analyzed by Compix image capture and dynamic intensity analysis software, version 5.3 (Cranberry Township, PA), to calculate the percentage of infected cells. Another set of plates was incubated at 33°C. At various time intervals, 250 μl of culture supernatant was collected and stored at −80°C in the presence of sucrose-phosphate-glutamic acid prior to virus titration. After each aliquot was

TABLE 1. Comparison of wt and recombinant 6:2 A/Fujian/411/02-like strains in HA and NA sequences and their replication in MDCK cells and eggs

Virus (passage history)	Amino acid at position indicated for:								Virus titer (mean $\log_{10}$ PFU/ml ± SE) for:[a]			
	HA1				HA2	NA			MDCK		Egg	
	128	186	219	226	150	119	136	347	wt	6:2	wt	6:2
A/Fujian/411/02 (C1/C2)[b]	T	G	S	V	G[c]	E	Q	H	6.1 ± 0.3	6.2 ± 0.3	4.1 ± 0.6	<1.5
A/Sendai-H/F4962/02 (CxE8/E3)					E	Q	K	Y	8.1 ± 0.2	7.1 ± 0.1	9.0 ± 0.3	8.7 ± 0.2
A/Wyoming/03/03 (ck2E2/E9)	A	V	Y/F	I	E				6.7 ± 0.5	7.0 ± 0.4	8.9 ± 0.3	8.1 ± 0.1

[a] Virus titers were expressed as mean $\log_{10}$ PFU/ml ± standard errors (SE) from two or more experiments.
[b] wt A/Fujian had the H183L change after a one-time passage in MDCK cells and eggs.
[c] Recombinant 6:2 A/Fujian viruses with G150 or E150 replicated similarly in MDCK cells and eggs.

removed, an equal amount of fresh medium was added to the cells. The virus titer in these aliquots was determined by plaque assay in MDCK cells at 33°C.

Antigenicity of 6:2 recombinant viruses. The antigenicity of each virus was analyzed by hemagglutination inhibition (HAI) assay using ferret sera immunized with wt A/Sendai or wt A/Wyoming. Aliquots of 25 μl of twofold serially diluted ferret antisera were incubated with 25 μl of virus containing 4 HA units of 6:2 reassortant viruses at 37°C for 1 h followed by incubation with 50 μl of 0.5% turkey red blood cells (RBC) at 25°C for 45 min. The HAI titer was defined as the reciprocal of the highest serum dilution that inhibited hemagglutination.

RESULTS

Generation of 6:2 A/Fujian, A/Sendai, and A/Wyoming vaccine strains. Wild-type influenza A virus strains, A/Fujian/411/02, A/Sendai-H/F4962/02, and A/Wyoming/03/03 were amplified once in MDCK cells or in embryonated chicken eggs. As indicated in Table 1, A/Fujian was passaged for only 3 times in cell culture, whereas A/Sendai and A/Wyoming were passaged 11 times in eggs prior to sequence analysis. The HA and NA sequences of these three strains were determined by sequencing of the RT-PCR products using viral RNA extracted from these viruses. The differences in the HA and NA sequences of these three H3N2 strains are listed in Table 1. A/Sendai was identical to A/Fujian in the HA1 amino acid sequence but differed in the NA sequence at three amino acids, at positions 119, 146, and 347. A/Wyoming had a NA sequence identical to that of A/Fujian but differed from A/Fujian and A/Sendai in HA1 by four amino acids. In addition, both A/Sendai and A/Wyoming had Glu-150 instead of Gly-150 for A/Fujian in the HA2. After a one-time amplification in MDCK cells, the 183 residue in HA1 of wt A/Fujian mutated from His-183 to Leu-183 and it was difficult to isolate wt A/Fujian virus with His-183, indicating that the virus with His-183 had a growth disadvantage in MDCK cells.

These three wt viruses grew differently in MDCK cells, reaching tiers of 6.1, 8.1, and 6.7 $\log_{10}$ PFU/ml for wt A/Fujian, wt A/Sendai, and wt A/Wyoming, respectively. Of the three variants, wt A/Fujian replicated the most poorly in eggs, reaching a titer of 4.1 PFU/ml (Table 1). A/Fujian isolated from eggs also had the H183L change in the HA. In contrast, wt A/Sendai and wt A/Wyoming grew well in eggs, reaching titers of 9.0 and 8.9 $\log_{10}$ PFU/ml, respectively.

To confirm that the HA and NA segments of these H3N2 strains controlled virus replication in eggs and cell culture, the HA and NA gene segments were reassorted with the internal gene segments of the cold-adapted A/Ann Arbor/6/60 strain, the master donor virus for live attenuated influenza FluMist vaccines (MDV-A) to generate three 6:2 reassortant viruses. Replication of these three viruses was evaluated in MDCK cells and embryonated chicken eggs. The 6:2 A/Fujian showed a lower titer (6.2 $\log_{10}$ PFU/ml) than the 6:2 A/Sendai (7.1 $\log_{10}$ PFU/ml) and A/Wyoming (7.0 $\log_{10}$ PFU/ml) in MDCK cells. Similar to wt A/Fujian, 6:2 A/Fujian replicated poorly in embryonated chicken eggs and was not detectable by plaque assay (<1.5 $\log_{10}$ PFU/ml). The sequence difference at HA2 residue 150 had no impact on virus replication in MDCK cells and eggs, because recombinant 6:2 A/Fujian with HA2 G150 or E150 had identical replication properties in MDCK and eggs. The 6:2 A/Sendai and A/Wyoming replicated to higher titers, of 8.7 and 8.1 $\log_{10}$ PFU/ml, respectively. Thus, the transfer of the wt HA and NA gene segments into MDV-A did not change the capability of each virus to replicate in eggs.

Effect of amino acid changes in the NA on neuraminidase activities and virus replication. A/Fujian differed from A/Sendai by three amino acids in NA, E119Q, Q136K, and H347Y (Table 1), and these changes enabled A/Sendai to replicate in embryonated chicken eggs to a higher titer than A/Fujian. Substitutions of E119 by G, D, A, or V residues have been reported for several anti-neuraminidase drug-resistant strains that resulted in reduced neuraminidase activity (1, 2, 12, 45). To determine whether the E119Q, Q136K, or H347Y change in the NA had an effect on the NA activity of A/Fujian and on its ability to replicate in embryonated chicken eggs, single-and double-substitution mutations were introduced into A/Fujian NA expression plasmids, and the NA activity in the transfected HEp-2 cells was measured. In addition, recombinant viruses bearing these mutations in the A/Fujian NA were also recovered and their growth in MDCK cells and eggs was compared (Table 2). A/Fujian (E119/Q136/H147) had approximately 80% higher NA activity than A/Sendai (Q119/K136/Y147). The impact of single changes on the NA activity was determined. Q119 resulted in reduced NA activity of 66% of that of A/Fujian, and K136 exhibited 25% activity, whereas Y347 had a minimal effect on the NA activity. Double mutations K136/Y347, Q119/Y347, and Q119/K136 had NA activity at levels of 29%, 52%, and 25% of that of A/Fujian, respectively. These data indicated that these three NA residues affected the NA activity in the order of K136 > Q119 > Y347.

The correlation of the NA activity of the NA mutants with virus replication in embryonated chicken eggs was examined (Table 2). The six modified A/Fujian viruses with one or more of A/Sendai residues in the NA replicated well in MDCK cells reaching titers ranging from 6.2 to 6.9 $\log_{10}$ PFU/ml, but their replication in eggs was significantly different. FJ-Q119 and

TABLE 2. Effect of NA residues on virus replication in MDCK cells and eggs[a]

NA	NA residue			NA activity (mean ± SE)	Virus titer (Log_{10} PFU/ml)	
	119	136	347		MDCK	Egg
A/Fujian	E	Q	H	100	6.5	<1.5
FJ-Q119	Q			66 ± 3	6.7	<1.5
FJ-Y347			Y	99 ± 1	6.6	<1.5
FJ-K136		K		25 ± 1	6.6	4.8
FJ-K136/Y347		K	Y	29 ± 3	6.5	<1.5
FJ-Q119/Y347	Q		Y	52 ± 4	6.6	4.5
FJ-Q119/K136	Q	K		25 ± 1	6.2	6.2
FJ-Q119/K136/Y347 (A/Sendai)	Q	K	Y	21 ± 1	6.9	8.8

[a] The NA activities in the NA cDNA-transfected HEp-2 cells are expressed as percentages of those of A/Fujian (means ± SE) from four independent experiments. Recombinant 6:2 viruses were generated using A/Fujian HA and NA or A/Fujian NA with the mutations indicated.

FJ-Y347, which had 66% and 99% of the NA activity of A/Fujian, respectively, were unable to grow in eggs. FJ-K136, with 25% of the NA activity of A/Fujian, was able to grow to a titer of 4.8 log_{10} PFU/ml in eggs, but this was 4.0 log_{10} lower than that of A/Sendai (8.8 log_{10} PFU/ml). Unexpectedly, although K136/Y347 significantly decreased the NA activity in vitro, the recombinant virus carrying these two mutations (FJ-K136/Y347) was not able to replicate in embryonated chicken eggs. Q119/Y347, which had 52% of the NA activity of A/Fujian, replicated in eggs to a titer of 4.5 log_{10} PFU/ml. Q119/K136, which had NA activity slightly higher than that of A/Sendai, replicated to a titer of 6.2 log_{10} PFU/ml but was still 2.6 log_{10} lower than the titer for A/Sendai. These results indicated that each of the three NA residues differed between A/Fujian and A/Sendai and impacted virus replication differently. Although several NA mutations could reduce the NA activity to a level close to that of A/Sendai, only the Q119/K136 double mutation could result in significant improvement in virus replication in embryonated chicken eggs. Since this virus did not replicate as efficiently as A/Sendai virus in eggs, the Y347 residue was also required for efficient replication in eggs.

Effects of HA residues on virus replication. The changes of the four HA1 residues in A/Wyoming that differed from A/Fujian were investigated for their roles in virus replication. The single- and multiple-substitution mutations were introduced into A/Fujian HA cDNA, and the modified HA plasmids were introduced into MDV-A together with A/Fujian NA. All of the 6:2 reassortant virus mutants replicated well in MDCK cells but grew differently in embryonated chicken eggs (Table 3). The 6:2 reassortants with A/Fujian HA (T128/G186/S219/V226) were unable to replicate in eggs. A single T128A change did not improve virus growth in eggs. However, single G186V or V226I changes resulted in increased virus replication in eggs. Double G186V and V226I changes in HA enabled the virus to replicate efficiently in eggs. Additional substitutions at residues 128 and/or 219 did not significantly increase virus replication. Thus, a minimum of two changes, G186V and V226I, were sufficient to make 6:2 A/Fujian grow efficiently in eggs.

Adaptation of 6:2 A/Fujian/411/02. To determine whether the 6:2 A/Fujian strain could be adapted to grow in eggs, the virus was amplified in MDCK cells followed by passage in eggs (Table 4). When 3.0 log_{10} PFU of virus was inoculated into an egg, less than 1.5 log_{10} PFU/ml of virus was detected in the allantoic fluid. Infectious virus could not be recovered following passages of this material. During the second passage experiment, the amount of virus inoculated into embryonated chicken eggs was increased to 5.9 log_{10} PFU. A titer of 4.5 log_{10} PFU/ml was detected in the allantoic fluid (FJ-EP1), and an additional passage in eggs increased the virus titer to 6.2 log_{10} PFU/ml (FJ-EP2). A further passage in eggs (FJ-EP3) increased the virus titer to 8.2 log_{10} PFU/ml. Sequence analysis of the FJ-EP2 virus revealed an A-to-U mutation at nucleotide 625 in the HA RNA segment, which resulted in the H183L change in the HA protein. Further analysis showed this change could also occur during virus amplification in MDCK cells. The H183L mutation was also found in the wt A/Fujian HA during its replication in MDCK and eggs as described previously. An additional U-to-C mutation at nucleotide 754 of HA, resulting in a V226A substitution, was found in the FJ-EP3–amplified virus (Table 4). No changes in the NA segment were detected.

To confirm that H183L and V226A mutations in HA were indeed responsible for the increased replication of 6:2 A/Fujian in eggs, H183L and V226A were introduced into A/Fujian HA singly or in combination. Three recombinant viruses were obtained, and they grew to titers of 7.4 log_{10} PFU/ml for FJ-H183L, 7.9 log_{10} PFU/ml for FJ-V226A, and 8.4 log_{10} PFU/ml for FJ-H183L/V226A (Table 4). Recombinant 6:2 A/Fujian with a HA-183L change (FJ-183L) had a titer 1.2 log_{10} higher than the revertant with the same mutation (FJ-EP2). It was difficult to evaluate whether this 1.2 log_{10} difference was sig-

TABLE 3. Effect of HA residues on virus replication in eggs

Virus[a]	HA residue				Virus titer in egg (log_{10} PFU/ml)
	128	186	219	226	
A/Fujian	T	G	S	V	<1.5
FJ-A128	A				<1.5
FJ-V186		V			4.9
FJ-I226				I	5.2
FJ-V186/I226		V		I	7.6
FJ-V186/Y219/I226		V	Y	I	7.5
A/Wyoming	A	V	Y	I	7.3

[a] Virus recovered from the transfected cells contained A/Fujian NA and HA with the indicated amino acid changes.

TABLE 4. Effect of mutations in the HA of egg-adapted 6:2 A/Fujian on virus replication in eggs

Virus	Mutation at nucleotide (amino acid) indicated	Virus titer (log_{10} PFU/ml)
Egg-passaged		
FJ-EP1	Not determined	4.5[a]
FJ-EP2	A625U (H183L)	6.2
FJ-EP3	A625U (H183L), U745C (V226A)	8.2
Recombinant		
FJ-183L	A625U (H183L)	7.4
FJ-226A	T745C (V226A)	7.9
FJ-183L/226A	A625U (H183L), U745C (V226A)	8.4

[a] A dose of 5.9 log_{10} PFU of A/Fujian was inoculated into each egg in order to increase the possibility of reversion.

nificant, due to the rapid mutation of these viruses in eggs after another round of replication. Nevertheless, these results strongly indicated that H183L and V226A contributed independently to the improved replication of A/Fujian virus in embryonated chicken eggs.

Receptor-binding properties and replication of recombinant viruses. From the above studies, the three A/Sendai NA changes that reduced the NA activity of A/Fujian were shown to be sufficient for this virus to grow in eggs. On the other hand, the HA changes (G186V and V226I or H183L and V226A) might have increased receptor-binding affinity to compensate for the higher NA activity of A/Fujian. To determine whether the changes in the HA protein of A/Fujian increased its receptor-binding ability, binding of 6:2 A/Fujian carrying the HA-V186/I226 change and of egg-adapted 6:2 A/Fujian that contained HA-L183/A226 changes were compared to 6:2 A/Fujian, A/Sendai, and A/Wyoming. Each virus was adsorbed onto MDCK cells at an MOI of 1.0 for 30 min at 4°C or 33°C, the inoculum was removed, and the infected cells were either washed three times or not washed. After 6 h of incubation at 33°C, the percentage of infected cells was determined by immunofluorescence analysis using anti-NP antibody. As shown in Fig. 1, 6:2 A/Fujian and A/Sendai infected 26 to 27% of cells when adsorption was performed at 4°C, but the majority of viruses were readily removed by the washing step and only 3.1% and 4.1% of cells were infected after washing. At 33°C, washing greatly reduced the infection of the 6:2 A/Fujian virus (6.2% compared to 51.7%) but did not have a significant effect on the infection of 6:2 A/Sendai (42.8% compared to 37.8%). In contrast, 6:2 A/Wyoming or A/Fujian with HA-V186/I226 or HA-L183/A226 had similar infection rates no matter whether the cells were adsorbed at 4°C or 33°C and with or without a washing step. These data indicated that A/Fujian and A/Sendai HA had such a low binding affinity that the viruses attached to cells at 4°C could be readily washed off the cells. The binding and virus entry kinetics were faster at 33°C; thus, the washing step had a minimal impact on 6:2 A/Sendai virus infection. However, the majority of the bound 6:2 A/Fujian was washed off at the similar condition, which was likely due to the higher NA activity that prevented efficient virus binding at 33°C.

To determine whether the binding difference between these viruses affected virus growth kinetics in MDCK cells, the infected MDCK cells were incubated at 33°C and the culture supernatants were collected at various times for virus titration. When adsorbed at 33°C, 6:2 A/Fujian had slower growth kinetics and a lower titer (Fig. 2), and 6:2 A/Sendai and A/Fujian with HA-V186/I226 or HA-L183/A226 behaved similarly to 6:2 A/Wyoming. When adsorption was performed at 4°C, 6:2 A/Fujian as well as 6:2 A/Sendai had slower growth kinetics. The 6:2 A/Wyoming and the two A/Fujian variants grew similarly. These results were consistent with the virus-binding assay, whereas the washing step reduced efficient infection of A/Fujian at both temperatures.

Antigenicity of recombinant viruses. To examine whether viruses with the modified HA and NA residues affected virus antigenicity, HAI was performed using ferret anti-A/Wyoming and anti-A/Sendai sera (Table 5). The HAI antibody titer of wt A/Wyoming-immunized ferret serum was about 8- to 16-fold higher than ferret serum immunized with wt A/Sendai when measured with either 6:2 A/Fujian or A/Sendai virus. The two modified viruses (A/Fujian HA-V186/I226 and A/Fujian HA-L183/A226) had HAI titers similar to A/Wyoming and A/Sendai using either serum. These results indicated that the amino acid difference between A/Sendai and A/Wyoming and the modified HA viruses generated in this study did not alter virus antigenicity.

TABLE 5. Antigenicity of modified 6:2 A/Fujian viruses[a]

Virus	HA residue					NA residue			Antigenicity (log_2 HAI)	
	128	183	186	219	226	119	136	347	Anti-A/WY	Anti-A/SD
A/Fujian	T	H	G	S	V	E	Q	H	11	9
A/Sendai						Q	K	Y	12	8
A/Wyoming	A		V	Y	I				12	8
HA-V186I226			V		I			Y	11	9
HA-L183A226		L			A				11	9

[a] A/Fujian was grown in MDCK cells, and the rest of viruses were grown in eggs. Antigenicity was measured by HAI assay using wt A/Wyoming (anti-A/WY) or wt A/Sendai (anti-A/SD)-immunized ferret serum with the indicated virus antigens.

DISCUSSION

One of the challenging issues encountered during the vaccine strain selection process prior to the 2003-2004 influenza season was that the circulating A/Fujian/411/02 (H3N2) influenza virus lineages did not replicate well in embryonated chicken eggs, the substrate that is currently used for influenza vaccine production. Using the reverse genetics technology, we showed that the loss of balance of the HA and NA activities was responsible for poor replication of the prototype A/Fujian/411/02 strain in eggs. The A/Fujian virus could gain its efficient replication in eggs either by increasing its HA receptor binding affinity or by reducing its NA activity. This work also demonstrated the feasibility of improving influenza virus growth in embryonated chicken eggs by introducing specific changes in the HA or NA genes without affecting virus antigenicity.

It has been shown that the receptor-binding affinity of HA has to be balanced with the NA activity in order to achieve efficient virus replication in the host cells (16, 35, 47). Influenza virus can overcome host restriction and become adapted to a new host by making changes in HA or/and NA. The growth of influenza virus in chicken eggs often selects for subpopulations of the virus bearing HA that differ in one or more amino acids from the predominant sequence of the virus found replicating in humans (20, 41, 42). Egg adaptation of 6:2 A/Fujian resulted in H183L and V226A changes in the HA that enabled its efficient replication in eggs. Three of the A/Wyoming residues, G186V, S219Y (or S219F), and V226I, have been implicated to be acquired during virus amplification in eggs, as the original A/Wyoming/03/03 isolate did not have these changes (Michael Shaw [Centers for Disease Control and Prevention], personal communication). The G186V and V226I changes identified in the HA protein of A/Wyoming and the H183L and V226A in the HA of egg-adapted A/Fujian strain were shown to increase the ability of virus to bind to the cellular receptors as assayed in MDCK cells. The 183, 186, and 226 residues are located in the HA receptor-binding pocket (Fig. 3) (43, 49). The changes in these residues have been found previously to correlate with

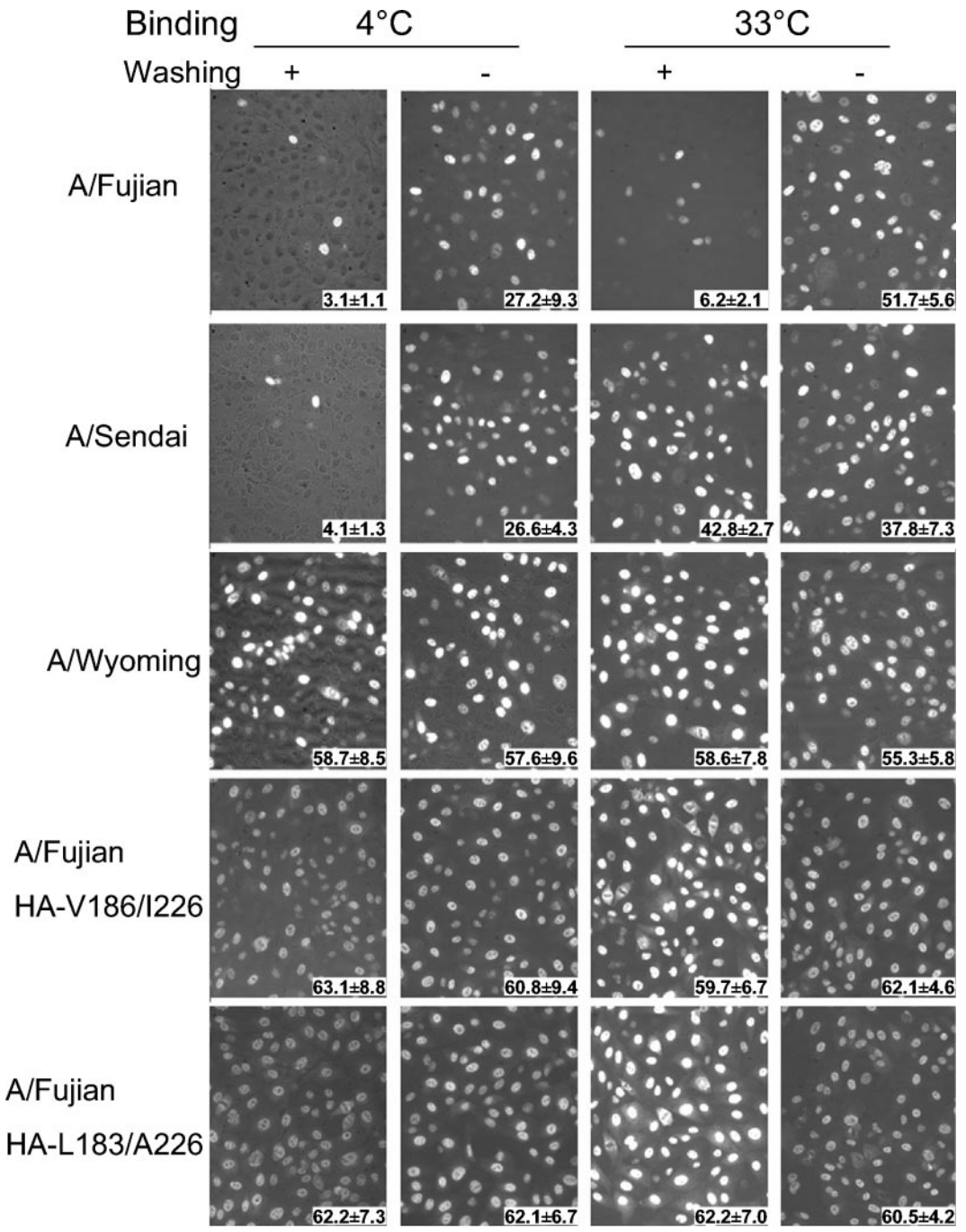

FIG. 1. HA receptor-binding affinity of recombinant viruses. The 6:2 A/Fujian, A/Sendai, A/Wyoming, and A/Fujian variants with V186 and I226 or L183 and A226 changes were adsorbed to MDCK cells at an MOI of 1.0 at 4°C or 33°C for 30 min, and the infected cells were washed three times (+) or left untreated (−). After 6 h of incubation at 33°C, the cells were processed for immunofluorescence staining. The percentage of infected cells (mean ± standard deviation) indicated in each image is an average for six images.

virus growth in embryonated chicken eggs (26, 42). Since replication of wt A/Fujian in MDCK cells also selected the H183L change, the increased HA-binding activity of the 6:2 A/Fujian HA variants also enabled their more efficient replication in MDCK cells.

The 226 residue of HA has been implicated in receptor-binding specificity, and the effects of variation at this position have been well documented (5, 31, 43). Mutations of the HA 226 residue from Q→L→I→V correlates with the progressive loss of HA binding to chicken erythrocytes (34). Although this 226 residue does not have direct contact with sialosides, the L226Q mutation has been shown to cause a narrowing of the receptor-binding pocket (49). Human influenza virus strains preferentially bind to Siaα(2,6)Gal, whereas avian strains pre-

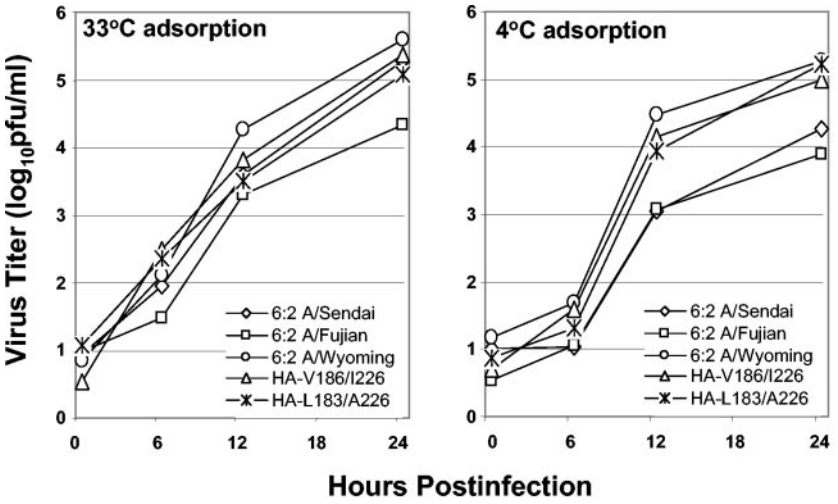

FIG. 2. Growth kinetics of recombinant viruses in MDCK cells. MDCK cells were infected at an MOI of 1.0 at either 33°C or 4°C for 30 min and washed three times with PBS prior to the addition of the medium containing 1.0 μg/ml of trypsin. The infected cells were incubated at 33°C and, at the indicated time intervals, the culture supernatants were collected and the virus amount was determined by plaque assay on MDCK cells.

fer Siaα(2,3)Gal, which are abundant in the chorioallantoic membranes of chicken eggs (4, 17, 32). A/Fujian-like strains agglutinate guinea pig and turkey RBC more efficiently than chicken RBC (data not shown), indicating that these recent H3N2 strains preferentially bind to Siaα(2,6)Gal (34, 36). The egg-adapted variants often change the receptor-binding specificity toward preferential recognition of Siaα(2,3)Gal (7). The V226I change may have made A/Fujian bind better to Siaα(2,3)Gal. However, the single 226 residue change may not be sufficient to specify receptor-binding specificity. The H3 subtype has evolved from the introduction of avian-derived H3 into the human populations from Q226 to L226, and L→Q→I→V changes at position 226 were found in the later isolates. Thus, other amino acid changes in the HA must have played a role in the growth of human H3 subtypes in humans and eggs. As demonstrated here, the V226I or V226A change was not sufficient for the A/Fujian virus to replicate in embryonated chicken eggs efficiently; an additional G186V or H183L change was needed. His-183 forms a hydrogen bond with the 9-hydroxyl group on sialic acid (44), and alteration in residue 226 usually causes the shift of His-183 (14). A previous study has shown that the S186I change enabled human isolates to grow in chicken eggs, which was accompanied by the increased binding to α2,3-linked analogs (7). The previously circulating A/Panama/2007/99 strain that replicated well in embryonated chicken eggs contained Leu-183. However, H183L alone was also not sufficient for A/Fujian to grow efficiently in eggs, and the concomitant V226A change may enable the efficient binding of HA to Siaα(2,3)Gal.

Viruses with reduced HA sialic acid-binding efficiency require less NA activity for egress from infected cells (9). The NA of A/Sendai differed from that of A/Fujian and A/Wyoming by three residues (E119Q, K136Q, and Y347H), and these three changes are all located in close proximity within the sialic acid-binding pocket (Fig. 3) (46), indicating their functional importance. These three A/Sendai NA residues resulted

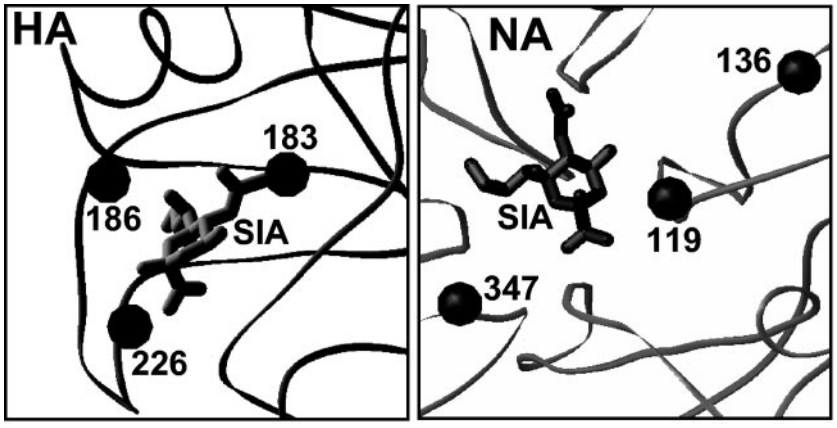

FIG. 3. Receptor-binding sites in HA and NA of influenza H3N2 subtypes. The residues that were shown to increase the HA receptor-binding affinity and to decrease the NA enzymatic activity in relation to sialic acid (SIA)-binding sites are indicated. The HA monomer was modeled using 5HMG (49), and the NA monomer was modeled based on 2BAT (46) using WebLab ViewerLite 3.10 (Accelrys, San Diego, CA).

in a fivefold reduction in its enzymatic activities, suggesting that the low NA activity contributed to the growth of A/Sendai in eggs. The NA Glu-119 residue is well conserved, and mutation at this residue has been found to correlate with virus resistance to drugs that inhibit neuraminidase activity. Several neuraminidase inhibitor-resistant variants selected in cell culture and in vivo have Glu-119 replaced by Gly, Ala, Asp or Val, which resulted in reduced NA activities (2, 33, 45). The replacement of Glu-119 by Gln has not been reported previously, and our studies showed that A/Sendai with a Gln-119 residue was also resistant to neuraminidase inhibitors zanamivir and oseltamivir (data not shown). Unexpectedly, the introduction of K136 and Y347 into A/Fujian NA did not enable the virus to replicate in eggs. Several attempts to adapt this virus to grow in eggs have been unsuccessful, indicating that three of the residues in A/Sendai NA may have been evolved in vivo instead of as a result of adaptation in eggs. Although H347Y did not appear to affect the NA activity as assayed in vitro, it resulted in reduced virus titer and smaller plaque size. Residue 347 is also located in close proximity to the sialic acid-binding site (Fig. 3), indicating its functional role in virus replication in vivo. Although 6:2 A/Fujian virus with reduced NA activity grew efficiently in eggs, it replicated poorly in ferrets and was poorly immunogenic (data not shown). A/Fujian HA variants with increased HA-binding activity were immunogenic, and the HAI antibody titers were similar to that of A/Wyoming, which should provide complete protection against wild-type challenge virus replication. Therefore, the virus with reduced NA activity is not recommended as an influenza vaccine candidate.

As demonstrated previously (7), egg adaptation of human viruses increases their affinity for Sia(α2,3)Gal-containing receptors and concomitantly impairs their ability to bind to Sia(α2,6)Gal-terminated receptors. These changes might also change virus antigenicity (21, 24, 26, 51). The data obtained in this study indicated that substitutions in the naturally occurring or egg-selected residues in the receptor-binding site of HA and NA of A/Fujian did not compromise virus antigenicity. We also demonstrated that the modified HA variants had immunogenicity similar to A/Fujian (data not shown). These results are consistent with the previous observation that an A/Memphis/7/90 Ile-186 substitution during egg passages did not significantly alter virus immunogenicity and protective efficacy (24, 26). Until an alternative system can be developed to manufacture influenza vaccines in substrates other than chicken eggs, embryonated chicken eggs remain to be the major means of producing both inactivated and live attenuated influenza vaccines. Results from this study indicated that the reverse genetics system could be used to improve influenza vaccine production by introducing residues to the HA and/or NA that would benefit virus replication in eggs without sacrificing virus antigenicity and immunogenicity. In addition, this system should also allow the rapid generation of 6:2 reassortant vaccines, which need to be updated frequently.

ACKNOWLEDGMENTS

We thank the staff of MedImmune Vaccine's tissue culture facility for providing tissue culture cells; the MVS group for supplying embryonated chicken eggs; Adam Seddiqui, Vicki Garcia, and the staff in the animal facility for the ferret studies; Chengjun Mo for helping with microscopy; Kutubuddin Mahmood for advice on HAI assays; Xing Cheng, Winnie Chan, and Mary Munoz for their excellent technical assistance; Chin-Fen Yang for the sequence information; and Min-Shi Lee for discussion and review of the manuscript.

REFERENCES

1. **Blick, T. J., A. Sahasrabudhe, M. McDonald, I. J. Owens, P. J. Morley, R. J. Fenton, and J. L. McKimm-Breschkin.** 1998. The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to 4-guanidino-Neu5Ac2en. Virology **246:**95–103.
2. **Blick, T. J., T. Tiong, A. Sahasrabudhe, J. N. Varghese, P. M. Colman, G. J. Hart, R. C. Bethell, and J. L. McKimm-Breschkin.** 1995. Generation and characterization of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en. Virology **214:**475–484.
3. **Centers for Disease Control and Prevention.** 2004. Update: influenza activity—United States, 2003–04 season. Morb. Mortal. Wkly. Rep. **53:**284–287.
4. **Couceiro, J. N., J. C. Paulson, and L. G. Baum.** 1993. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res. **29:**155–165.
5. **Daniels, P. S., S. Jeffries, P. Yates, G. C. Schild, G. N. Rogers, J. C. Paulson, S. A. Wharton, A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1987. The receptor-binding and membrane-fusion properties of influenza virus variants selected using anti-haemagglutinin monoclonal antibodies. EMBO J. **6:**1459–1465.
6. **Els, M. C., W. G. Laver, and G. M. Air.** 1989. Sialic acid is cleaved from glycoconjugates at the cell surface when influenza virus neuraminidases are expressed from recombinant vaccinia viruses. Virology **170:**346–351.
7. **Gambaryan, A. S., J. S. Robertson, and M. N. Matrosovich.** 1999. Effects of egg-adaptation on the receptor-binding properties of human influenza A and B viruses. Virology **258:**232–239.
8. **Grassauer, A., A. Y. Egorov, B. Ferko, I. Romanova, H. Katinger, and T. Muster.** 1998. A host restriction-based selection system for influenza haemagglutinin transfectant viruses. J. Gen. Virol. **79:**1405–1409.
9. **Gubareva, L. V.** 2004. Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res. **103:**199–203.
10. **Gubareva, L. V., R. Bethell, G. J. Hart, K. G. Murti, C. R. Penn, and R. G. Webster.** 1996. Characterization of mutants of influenza A virus selected with the neuraminidase inhibitor 4-guanidino-Neu5Ac2en. J. Virol. **70:**1818–1827.
11. **Gubareva, L. V., L. Kaiser, and F. G. Hayden.** 2000. Influenza virus neuraminidase inhibitors. Lancet **355:**827–835.
12. **Gubareva, L. V., L. Kaiser, M. N. Matrosovich, Y. Soo-Hoo, and F. G. Hayden.** 2001. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. J Infect. Dis. **183:**523–531.
13. **Gubareva, L. V., M. S. Nedyalkova, D. V. Novikov, K. G. Murti, E. Hoffmann, and F. G. Hayden.** 2002. A release-competent influenza A virus mutant lacking the coding capacity for the neuraminidase active site. J. Gen. Virol. **83:**2683–2692.
14. **Ha, Y., D. J. Stevens, J. J. Skehel, and D. C. Wiley.** 2001. X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc. Natl. Acad. Sci. USA **98:**11181–11186.
15. **Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. USA **97:**6108–6113.
16. **Hughes, M. T., M. Matrosovich, M. E. Rodgers, M. McGregor, and Y. Kawaoka.** 2000. Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. J. Virol. **74:**5206–52012.
17. **Ito, T., J. N. Couceiro, S. Kelm, L. G. Baum, S. Krauss, M. R. Castrucci, I. Donatelli, H. Kida, J. C. Paulson, R. G. Webster, and Y. Kawaoka.** 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J. Virol. **72:**7367–7373.
18. **Jin, H., B. Lu, H. Zhou, C. Ma, J. Zhao, C. F. Yang, G. Kemble, and H. Greenberg.** 2003. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology **306:**18–24.
19. **Jin, H., H. Zhou, B. Lu, and G. Kemble.** 2004. Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. J. Virol. **78:**995–998.
20. **Katz, J. M., M. L. Wang, and R. G. Webster.** 1990. Direct sequencing of the HA gene influenza (H3N2) virus in original clinical samples reveals sequence identity with mammalian cell-grown virus. J. Virol. **64:**1808–1811.
21. **Katz, J. M., and R. G. Webster.** 1989. Efficacy of inactivated influenza A virus (H3N2) vaccines grown in mammalian cells or embryonated eggs. J. Infect. Dis. **160:**191–198.
22. **Kaverin, N. V., A. S. Gambaryan, N. V. Bovin, I. A. Rudneva, A. A. Shilov, O. M. Khodova, N. L. Varich, B. V. Sinitsin, N. V. Makarova, and E. A. Kropotkina.** 1998. Postreassortment changes in influenza A virus hemagglutinin restoring HA-NA functional match. Virology **244:**315–321.
23. **Kaverin, N. V., M. N. Matrosovich, A. S. Gambaryan, I. A. Rudneva, A. A. Shilov, N. L. Varich, N. V. Makarova, E. A. Kropotkina, and B. V. Sinitsin.**

2000. Intergenic HA-NA interactions in influenza A virus: postreassortment substitutions of charged amino acid in the hemagglutinin of different subtypes. Virus Res. **66:**123–129.
24. **Kilbourne, E. D., B. E. Johansson, T. Moran, S. Wu, B. A. Pokorny, X. Xu, and N. Cox.** 1993. Influenza A virus haemagglutinin polymorphism: pleiotropic antigenic variants of A/Shanghai/11/87 (H3N2) virus selected as high yield reassortants. J. Gen. Virol. **74:**1311–1316.
25. **Klenk, H. D., R. Wagner, D. Heuer, and T. Wolff.** 2002. Importance of hemagglutinin glycosylation for the biological functions of influenza virus. Virus Res. **82:**73–75.
26. **Kodihalli, S., D. M. Justewicz, L. V. Gubareva, and R. G. Webster.** 1995. Selection of a single amino acid substitution in the hemagglutinin molecule by chicken eggs can render influenza A virus (H3) candidate vaccine ineffective. J. Virol. **69:**4888–4897.
27. **Lamb, R. A., and P. W. Choppin.** 1983. The gene structure and replication of influenza virus. Annu. Rev. Biochem. **52:**467–506.
28. **Lamb, R. A., and R. M. Krug.** 2001. *Orthomyxoviridae*: the viruses and their replication, p. 1487–1531. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields Virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.
29. **Liu, C., and G. M. Air.** 1993. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase genes. Virology **194:**403–407.
30. **Maassab, H. F.** 1967. Adaptation and growth characteristics of influenza virus at 25 degrees C. Nature **213:**612–614.
31. **Matrosovich, M. N., A. S. Gambaryan, A. B. Tuzikov, N. E. Byramova, L. V. Mochalova, A. A. Golbraikh, M. D. Shenderovich, J. Finne, and N. V. Bovin.** 1993. Probing of the receptor-binding sites of the H1 and H3 influenza A and influenza B virus hemagglutinins by synthetic and natural sialosides. Virology **196:**111–121.
32. **Matrosovich, M. N., T. Y. Matrosovich, T. Gray, N. A. Roberts, and H. D. Klenk.** 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc. Natl. Acad. Sci. USA **101:**4620–4624.
33. **McKimm-Breschkin, J. L., A. Sahasrabudhe, T. J. Blick, M. McDonald, P. M. Colman, G. J. Hart, R. C. Bethell, and J. N. Varghese.** 1998. Mutations in a conserved residue in the influenza virus neuraminidase active site decreases sensitivity to Neu5Ac2en-derived inhibitors. J. Virol. **72:**2456–2462.
34. **Medeiros, R., N. Escriou, N. Naffakh, J. C. Manuguerra, and S. van der Werf.** 2001. Hemagglutinin residues of recent human A(H3N2) influenza viruses that contribute to the inability to agglutinate chicken erythrocytes. Virology **289:**74–85.
35. **Mitnaul, L. J., M. N. Matrosovich, M. R. Castrucci, A. B. Tuzikov, N. V. Bovin, D. Kobasa, and Y. Kawaoka.** 2000. Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J. Virol. **74:**6015–6020.
36. **Mochalova, L., A. Gambaryan, J. Romanova, A. Tuzikov, A. Chinarev, D. Katinger, H. Katinger, A. Egorov, and N. Bovin.** 2003. Receptor-binding properties of modern human influenza viruses primarily isolated in Vero and MDCK cells and chicken embryonated eggs. Virology **313:**473–480.
37. **Murphy, B. R., and K. Coelingh.** 2002. Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. Viral Immunol. **15:**295–323.
38. **Nobusawa, E., H. Ishihara, T. Morishita, K. Sato, and K. Nakajima.** 2000. Change in receptor-binding specificity of recent human influenza A viruses (H3N2): a single amino acid change in hemagglutinin altered its recognition of sialyloligosaccharides. Virology **278:**587–596.
39. **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.
40. **Potier, M., L. Mameli, M. Belisle, L. Dallaire, and S. B. Melancon.** 1979. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-*N*-acetylneuraminate) substrate. Anal. Biochem. **94:**287–296.
41. **Robertson, J. S., C. Nicolson, J. S. Bootman, D. Major, E. W. Robertson, and J. M. Wood.** 1991. Sequence analysis of the haemagglutinin (HA) of influenza A (H1N1) viruses present in clinical material and comparison with the HA of laboratory-derived virus. J. Gen. Virol. **72:**2671–2677.
42. **Rocha, E. P., X. Xu, H. E. Hall, J. R. Allen, H. L. Regnery, and N. J. Cox.** 1993. Comparison of 10 influenza A (H1N1 and H3N2) haemagglutinin sequences obtained directly from clinical specimens to those of MDCK cell- and egg-grown viruses. J. Gen. Virol. **74:**2513–2518.
43. **Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley.** 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature **304:**76–78.
44. **Skehel, J. J., and D. C. Wiley.** 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutin. Annu. Rev. Biochem. **69:**531–569.
45. **Staschke, K. A., J. M. Colacino, A. J. Baxter, G. M. Air, A. Bansal, W. J. Hornback, J. E. Munroe, and W. G. Laver.** 1995. Molecular basis for the resistance of influenza viruses to 4-guanidino-Neu5Ac2en. Virology **214:**642–646.
46. **Varghese, J. N., J. L. McKimm-Breschkin, J. B. Caldwell, A. A. Kortt, and P. M. Colman.** 1992. The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. Proteins **14:**327–332.
47. **Wagner, R., M. Matrosovich, and H. D. Klenk.** 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev. Med. Virol. **12:**159–166.
48. **Wagner, R., T. Wolff, A. Herwig, S. Pleschka, and H. D. Klenk.** 2000. Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. J. Virol. **74:**6316–6323.
49. **Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley.** 1988. Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid. Nature **333:**426–431.
50. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56:**365–394.
51. **Wood, J. M., J. S. Oxford, U. Dunleavy, R. W. Newman, D. Major, and J. S. Robertson.** 1989. Influenza A (H1N1) vaccine efficacy in animal models is influenced by two amino acid substitutions in the hemagglutinin molecule. Virology **171:**214–221.
52. **Wyatt, L. S., S. T. Shors, B. R. Murphy, and B. Moss.** 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. Vaccine **14:**1451–1458.