JOURNAL OF VIROLOGY, Aug. 2010, p. 8287–8299 Vol. 84, No. 16
0022-538X/10/$12.00 doi:10.1128/JVI.00058-10

Receptor Specificity of Influenza A H3N2 Viruses Isolated in Mammalian Cells and Embryonated Chicken Eggs▽†

James Stevens,[1]‡ Li-Mei Chen,[1]‡ Paul J. Carney,[1] Rebecca Garten,[1] Angie Foust,[1] Jianhua Le,[2] Barbara A. Pokorny,[2] Ramanunninair Manojkumar,[2] Jeanmarie Silverman,[2] Rene Devis,[2] Karen Rhea,[1] Xiyan Xu,[1] Doris J. Bucher,[2] James Paulson,[3] Nancy J. Cox,[1] Alexander Klimov,[1] and Ruben O. Donis[1]*

Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia[1]; Department of Microbiology and Immunology, New York Medical College, Valhalla, New York[2]; and Departments of Chemical Physiology and Molecular Biology, The Scripps Research Institute, La Jolla, California[3]

Received 12 January 2010/Accepted 18 May 2010

Isolation of human subtype H3N2 influenza viruses in embryonated chicken eggs yields viruses with amino acid substitutions in the hemagglutinin (HA) that often affect binding to sialic acid receptors. We used a glycan array approach to analyze the repertoire of sialylated glycans recognized by viruses from the same clinical specimen isolated in eggs or cell cultures. The binding profiles of whole virions to 85 sialoglycans on the microarray allowed the categorization of cell isolates into two groups. Group 1 cell isolates displayed binding to a restricted set of α2-6 and α2-3 sialoglycans, whereas group 2 cell isolates revealed receptor specificity broader than that of their egg counterparts. Egg isolates from group 1 showed binding specificities similar to those of cell isolates, whereas group 2 egg isolates showed a significantly reduced binding to α2-6- and α2-3-type receptors but retained substantial binding to specific O- and N-linked α2-3 glycans, including α2-3GalNAc and fucosylated α2-3 glycans (including sialyl Lewis x), both of which may be important receptors for H3N2 virus replication in eggs. These results revealed an unexpected diversity in receptor binding specificities among recent H3N2 viruses, with distinct patterns of amino acid substitution in the HA occurring upon isolation and/or propagation in eggs. These findings also suggest that clinical specimens containing viruses with group 1-like receptor binding profiles would be less prone to undergoing receptor binding or antigenic changes upon isolation in eggs. Screening cell isolates for appropriate receptor binding properties might help focus efforts to isolate the most suitable viruses in eggs for production of antigenically well-matched influenza vaccines.

Influenza A viruses are generally isolated and propagated in embryonated chicken eggs or in cultures of cells of mammalian origin. Human influenza viruses were previously noted to acquire mutations in the hemagglutinin (HA) gene upon isolation and culture in the allantoic sac of embryonated chicken eggs (herein simply referred to as "eggs") compared to the sequences of those isolated in mammalian cell substrates (herein referred to as "cells") (29, 30, 44, 53, 58). These mutations resulted in amino acid substitutions that were found to mediate receptor specificity changes and improved viral replication efficiency in eggs (37). In general, cell-grown viruses are assumed to be more similar than their egg-grown counterparts to the viruses present in respiratory secretions (30, 56). Since their emergence in 1968, influenza A (H3N2) viruses have evolved and adapted to the human host while losing their ability to be efficiently isolated and replicate in eggs, particularly after 1992 (37, 42, 48). The rate of isolation of H3N2 clinical specimens after inoculation into eggs can be up to ~30 times lower than that in mammalian cell cultures, highlighting the strong selective pressure for the emergence of sequence variants (77).

Virtually all influenza vaccines for human use were licensed decades ago by national regulatory authorities, which used a product manufactured from influenza viruses isolated and propagated exclusively in eggs; therefore, cell culture isolates have been unacceptable for this purpose (41, 71). The antigen composition of influenza vaccines requires frequent updates (every 2 years, on average) to closely match their antigenic properties to the most prevalent circulating antigenic drift variant viruses (51). The limited availability of H3N2 viruses isolated in eggs has on one or more occasions delayed vaccine composition updates and may have reduced the efficacy of vaccination against new antigenically drifted viruses (3, 34, 37).

Entry of influenza viruses into host cells is mediated by HA, which binds to sialic acid containing glycoconjugates on the surface of epithelial cells in the upper respiratory tract (2, 13). The nature of the linkage between sialic acid and the vicinal sugar (usually galactose) varies in different host species and tissues and may therefore determine whether an influenza virus binds to and infects avian or human cells (40, 46, 59, 62, 72–75). Human influenza viruses preferentially bind to α2-6-linked sialic acids, and avian viruses predominantly bind to α2-3-linked sialic acids (59). Previous studies with chicken embryo chorioallantoic membranes revealed differential lectin binding, suggesting that α2-3-linked but not α2-6-linked sialo-

* Corresponding author. Mailing address: Molecular Virology and Vaccines Branch, Influenza Division, NCIRD, CCID, Centers for Disease Control and Prevention, 1600 Clifton Road, Mail Stop G-16, Atlanta, GA 30333. Phone: (404) 639-4968. Fax: (404) 639-2350. E-mail: rvd6@cdc.gov.

† Supplemental material for this article may be found at http://jvi.asm.org/.

‡ These authors contributed equally to this study.

▽ Published ahead of print on 2 June 2010.

TABLE 1. Influenza H3N2 viruses analyzed in this study

Virus name	Abbreviation	Passage[a]	Accession no.[b]	Passage[a]	Accession no.[b]
Wyoming/03/2003[c]	WY03	C2	EU502435	E2	EF473604
Florida/2/2006	FL06	M2/C3	CY054272	Spfck2E4	CY058071
Honduras/3112/2006	HO06	X1/C3	CY054276	Spfck2E3	EPI155384
New Hampshire/3/2006	NH06	C4	CY054271	Spfck2E4	CY058070
New York/3/2006	NY306	M2/C3	CY054274	Spfck2E3	EF473473
New York/2/2006	NY206	M3/C3	CY054275	Spfck2E3	CY058073
New Jersey/2/2006	NJ06	C4	CY054273	Spfck2E4	CY058072
Pennsylvania/4/2007	PA07	C4	CY054277	Spfck2E5	CY058074
Wisconsin/3/2007	WI07	C4	EU199273	Spfck2E5	EU516105
Brisbane/10/2007	BR07	Cx/C7	EU161828	E2/E3	CY058075
Brisbane/10/2007- X-171[d]	X-171	NA[e]		E11/E3	EPI162188
Brisbane/10/2007-X-171A[d]	X-171A	NA		E11/E3	EPI162184
Brisbane/10/2007-X-171B[d]	X-171B	NA		E12/E3	EPI162186

[a] Spfck, primary chicken kidney cells; M, primary monkey kidney cells; C, MDCK cells; E, embryonated eggs; X, host unknown.
[b] GenBank or Global Initiative on Sharing Avian Influenza Data (www.gisaid.org) database accession number.
[c] Generated by reverse genetics.
[d] Reassortant containing internal protein coding genes from A/PR/8/34 virus.
[e] NA, not applicable.

sides are present on the epithelial cells (28). Human H3N2 viruses isolated in cell culture were reported to bind with a high affinity to α2-6-linked sialosides, while viruses isolated in eggs often had increased specificity for α2-3-linked sialosides (19, 20, 28). The functional classification of avian and mammalian influenza virus receptors is further complicated since *in vitro* and tissue-binding assays have led to new working hypotheses involving glycan chain length, topology, and the composition of the inner fragments of the carbohydrate chain as additional receptor specificity determinants (9, 17, 65, 66, 82). However, the significance of these *in vitro* properties remains unknown, since the structures of the natural sialosides on host cells that are used for infectious virus entry are undefined.

The techniques most widely used to study the interactions of the influenza virus with host cell receptors employ animal cells in various assay formats (36, 57, 59, 64, 69). To overcome the problems of cell-based techniques, new assays that rely on labeled sialyl-glycoproteins or polymeric sialoglycans have been developed (18). However, these assays are limited by having only a few glycans available in polymeric form and offer low throughput. In contrast, glycan microarrays can assess virus binding to multiple well-defined glycans simultaneously. Previous work with influenza live or β-propiolactone (BPL)-inactivated virions as well as recombinantly produced HAs revealed a good correlation with receptor specificity compared to that achieved by other methods of analysis (4, 11, 57, 58, 65–68).

Here we have compared paired isolates derived in eggs or cell cultures from the single clinical specimen to better understand their receptor binding specificity and its implications for vaccine production. We examined the differences in the sequences of the HAs between egg- and cell-grown isolates and analyzed their receptor binding profiles using glycan microarrays. Sequence analysis of the HA and glycan binding results revealed two distinct groups of viruses, with many egg isolates showing unexpectedly reduced levels of binding to α2-3 and α2-6 sialosides compared to the levels for the viruses isolated in mammalian cells. Furthermore, these studies highlighted that specific glycans may be important for H3N2 virus growth in eggs.

MATERIALS AND METHODS

Viruses and cells. Clinical specimens (respiratory secretions) and virus isolates were obtained from the WHO Global Influenza Surveillance Network. Virus isolation in cell culture was performed by inoculation of clinical materials into primary monkey kidney cells or Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection), cultured in Dulbecco's modification of Eagle's medium with trypsin supplementation as described previously (76) (Table 1). Isolation in eggs was performed as described previously (30, 76). Approximately 10 median (50%) egg infectious doses (EID_{50}s) of virus harvested from eggs were used for subsequent inoculation; the total number of passages was limited to the minimum required to obtain the necessary amounts of virus for analysis. The viruses and their passage histories are listed in Table 1, along with abbreviations. Both cell- and egg-adapted WY03 viruses were generated by reverse genetics (10, 16, 27, 45) on the basis of HA sequences of the cell and egg isolates and a complete wild-type genome (GenBank accession number EU502435). The HA genes of all virus stocks used in this study were sequenced to detect the emergence of sequence variants (and quasispecies) during growth and amplification. Detection of variant sequence populations was based on the presence of discrete overlapping peaks in the capillary sequencer chromatogram. Subdominant viral populations with a relative abundance of ≥20% were detected with confidence by this method. High-growth reassortant viruses with the HA and neuraminidase genes of BR07, the M gene from A/Puerto Rico/8/34, and the remaining genes from either parent were derived as described previously (32).

Structure modeling. The BR07 HA cell isolate and X-171B models were generated by homology modeling (61) using the structure of A/Aichi/2/1968 (Protein Data Bank accession number 2HMG) as the template.

Glycan microarray analyses. Viruses were grown in either MDCK cells or embryonated chicken eggs, clarified by low-speed centrifugation, and inactivated by treatment with BPL (0.05%) for 3 days at 4°C under conditions that did not significantly alter the hemagglutinating titers (5). Inactivated virus-containing supernatant liquids were concentrated as described previously (52). Virus stocks were aliquoted and stored at −80°C. Virus concentrations were determined from the amount of HA using 0.5% (vol/vol) turkey red blood cells, since they contain both α2-3 and α2-6 sialoglycans (70). A pilot study was performed to determine the impact of BPL inactivation on the profile of glycans bound by A/Brisbane/10/2007 (H3N2) reassortant X-171B; live and inactivated viruses were analyzed in the same experiment, as described previously (11).

Glycan microarray slides were produced specifically for influenza research at the CDC using the Consortium for Functional Glycomics (CFG) glycan library (see Table S1 in the supplemental material for the glycans used for analyses in these experiments), as described previously (4). Virus preparations were thawed and diluted in phosphate-buffered saline (PBS) with 3% (wt/vol) bovine serum albumin (BSA) to an HA titer of 128, established to be optimal for glycan array analyses in this study, as described below. Virus suspensions were applied to the slides, and the slides were incubated in a closed container (at 4°C) subjected to gentle agitation for 1 h. Unbound virus was washed off with brief sequential rinses in PBS with 0.05% Tween 20 (PBS-T) and PBS. The slides were then

TABLE 2. Amino acid differences for HA1 sequences of H3N2 cell and egg isolates

Group and virus	Host	Amino acid at position[a]:																			
		25	50	79	83	86[b]	138	140	142[b]	156	168	173	183	186	193	194	195[b]	219	223[b]	225[b]	226
Consensus sequence[c]		I	E	F	K	L	A	K	R	H	M	K	H	G	F	L	Y	S	V	N	I
Group 1																					
HO06	Cell			L		**I**							L								
HO06	Egg			L									L								
FL06	Cell										**M/V**									**N/D**	
FL06	Egg												**H/L**			**L/M**	**Y/F**				
NH06	Cell				E							E				**L/P**					
NH06	Egg				E				**G**[d]			E				**P**					
Group 2																					
NY306	Cell																		**I**		
NY306	Egg															**P**					
NJ06	Cell	V	G																**I**		
NJ06	Egg	V	G				**S**			**Q**				**G/V**							
NY206	Cell	V	G																	**D**	
NY206	Egg	V	G											**V**	**Y**			**Y**			
PA07	Cell							I												**N/D**	
PA07	Egg							I		**R**				**V**							
WI07	Cell							I				E									
WI07	Egg							I				E		**V**				**F**			
WY03-rg[e]	Cell		G												S					D	**V**
WY03-rg	Egg		G											**V**	S					D	

[a] Amino acid differences between egg and cell isolates are highlighted in boldface, whereas shared differences from the consensus sequence are shown as regular text.
[b] Positions not previously observed in published studies of egg-derived H3N2 viruses.
[c] This row shows the consensus amino acids of the H3 HA of Wisconsin/67/05-like viruses.
[d] The mutation appeared in egg passage 9.
[e] WY03-rg virus has additional amino acid differences compared to the sequences of the other H3N2 viruses listed in Table 1: T128A, N145K, F159Y, N189S, and P227S.

immediately incubated with sheep serum hyperimmune to H3 HA (30 min); a biotinylated anti-sheep IgG antibody (30 min), and a streptavidin-Alexa Fluor 488 conjugate (30 min) (Invitrogen, CA), with brief PBS-T/PBS washes being performed after the incubations. After the final PBS-T/PBS washes, the slides were briefly washed in deionized water, dried by a gentle steam of air, and immediately subjected to imaging. Fluorescence intensities were detected using a ProScanArray HT apparatus (Perkin-Elmer). Image analyses were carried out using ImaGene 8 image analysis software (BioDiscovery, El Segundo, CA). For a simplified graphical representation, data were processed in the Microsoft Excel program to group similar sialoglycans together. Glycans that bound to the antibody-only control slide (see Fig. S1 in the supplemental material) were removed from the results. A/Brisbane/10/2007 (H3N2) was analyzed in the glycan microarray at 2-fold incremental HA titers between 16 and 128 to determine the range of virus concentrations that would yield optimal fluorescence signals (see Fig. S3 in the supplemental material).

RESULTS

Amino acid substitutions in HAs of viruses isolated and propagated in embryonated eggs. We analyzed paired viruses isolated from nine clinical samples collected during 2006 and 2007 and propagated in either mammalian cells or embryonated eggs. One additional virus pair from a clinical specimen collected in 2003, WY03, was generated by reverse genetics, based on the available sequence information (see Table 1 for all virus abbreviations) (16, 27, 45). Sequence analysis of the HAs from the paired isolates shows that the majority of amino acid changes in the egg-grown virus relative to the sequence of the cell-grown virus occur at 8 of the 16 positions that have previously been reported to be egg isolation-associated sites in older viruses, i.e., positions 138, 156, 168, 183, 186, 193, 194, and 219 (Table 2) (19, 24, 26, 30, 31, 38, 43, 44, 78, 83, 84). This study also revealed changes at an additional seven new positions (positions 86, 142, 190, 195, 198, 223 and 225) that have not previously been associated with H3N2 virus growth in eggs, although two of these amino acid substitutions (at positions 190 and 195) were found only as mixed populations (Fig. 1; Tables 2 and 3). Notably, amino acid substitutions at two of these positions (190 and 225) were previously reported in egg-grown H1N1 viruses (19, 54) (Fig. 1).

Comparison of cell and egg isolates revealed no consensus amino acid substitutions that could be used to predict how future viruses will be affected by isolation and growth in eggs. Indeed, the results revealed from 1 to 4 amino acid substitutions in HA1 of each egg-grown virus. Interestingly, the Gly186Val substitution was common to 6 of the 10 viruses studied (NY206, NJ06, PA07, WI07, WY03, and BR07), while the Leu194Pro/Met replacement was common to four viruses (FL06, NH06, NY306, and BR07) (Tables 2 and 3). Of these two sites, variation at position 186 has been associated with egg propagation (19, 24, 26, 31, 38, 44, 78, 83) more frequently than

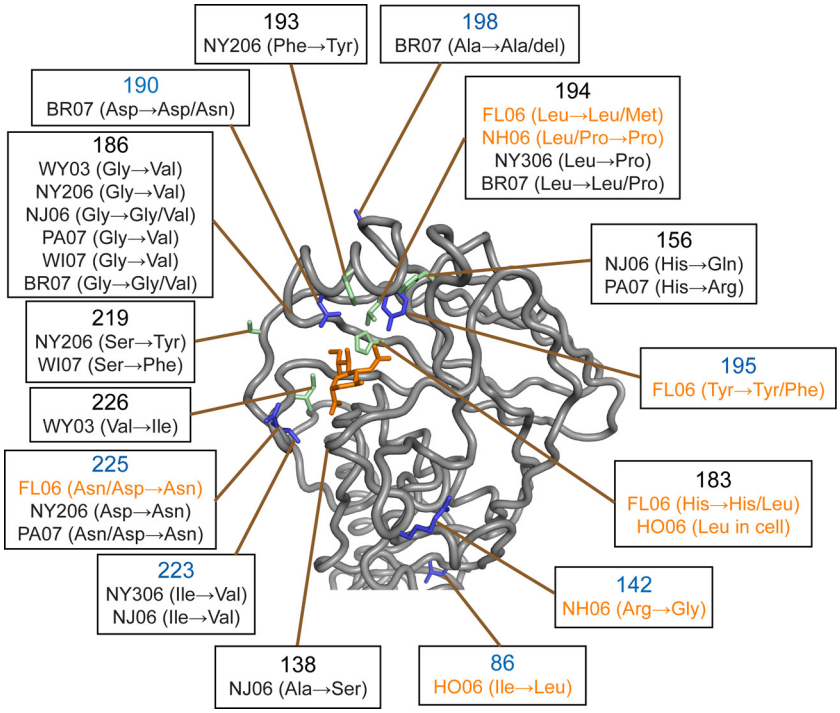


FIG. 1. Structural model of variable amino acid positions among H3N2 influenza viruses isolated in eggs. The structural model of the HA1 of BR07 HA highlights the differences noted among egg isolates from this study. Eight positions with substitutions that were previously reported in viruses isolated in eggs have their side chains colored pale green (except for Gly186, which has no side chain). Newly identified substitutions at seven sites have residue numbers in blue. The receptor binding site is occupied by sialic acid (orange). The viruses classified within group 1 are listed in orange fonts and those in group 2 in black.

variation at position 194 (26, 43). The HA1 sequences of the nine isolates from 2006 and 2007 differed from each other by 1 to 3 amino acids at six other positions not previously reported to be associated with egg isolation and propagation: positions 25, 50, 79, 83, 140, and 173 (Table 2).

Shared receptor binding properties of viruses isolated in mammalian cells or chicken eggs. To determine the receptor specificity of recent H3N2 viruses isolated in mammalian cells or in eggs, 10 paired egg and cell isolates were subjected to glycan microarray analysis. For egg-isolated viruses, only low-egg-passage-number viruses (i.e., passaged two to five times at a low multiplicity) were analyzed by the glycan microarray because very few further changes were observed at higher passage levels. To compare the viruses recovered in 2006 and 2007 to earlier isolates analyzed in studies described in the published literature (35), we included WY03 cell- and egg-like viruses generated from the available sequences by reverse genetics (Table 1). Pilot studies were conducted to evaluate the impact of inactivation with BPL and the virus concentration on glycan binding profiles, as described previously (11) (see Fig. S2 and S3 in the supplemental material). These studies showed that inactivation with BPL did not affect virus binding at the concentration established to be optimal (HA titer of 128) (see Fig. S2 and S3 in the supplemental material).

TABLE 3. Sequence variation in and around the HA receptor binding site of BR07 viruses

Virus	Host	Amino acid at position:					
		186	190[a]	194	198[a]	219	226
H3 consensus		G	D	L	A	S	I
BR07 cell isolate[b]	Cell						
BR07 egg isolate[b]	Egg	G/V	D/N	L/P	A/del		
X-171[c]	Egg	V			del		S
X-171A	Egg			P			
X-171B	Egg	V			del	Y	

[a] Positions not previously observed in published studies of H3 viruses.
[b] HA sequence differences for the cell- and egg-derived viruses determined in this study.
[c] The sequences of the PR8 reassortants are deposited in the Global Initiative on Sharing Avian Influenza Data database (www.gisaid.org). The accession numbers are shown in Table 1.

The glycan microarray results from this study revealed that certain glycans were ligands for most egg- and cell-derived H3N2 viruses analyzed. Glycan specificities shared by egg and cell-derived viruses were most likely present in the viruses in the original clinical specimen, since these two different host systems would not be expected to impose identical and parallel selective pressures. All viruses bound to a α2-6 sialylated tri-*N*-acetyllactosamine glycan in which the two proximal (reducing end) lactosamines are 1-3 fucosylated (Fig. 2 to 4; glycan 57 in the figures; Table 4). This polylactosamine structure was reported to be a terminal sequence in the N-linked glycans of some mammalian cells (9). In addition, ~75% of the isolates bound to a structurally related long linear α2-6 sialylated di-*N*-acetyllactosamine (Fig. 2 to 4, glycan 56), although egg isolates of NH06, NY306, PA07, WI07, and BR07 did not bind. This glycan was also detected as a terminal sequence in N-glycans of cultured human bronchial epithelial cells (9). Sev-

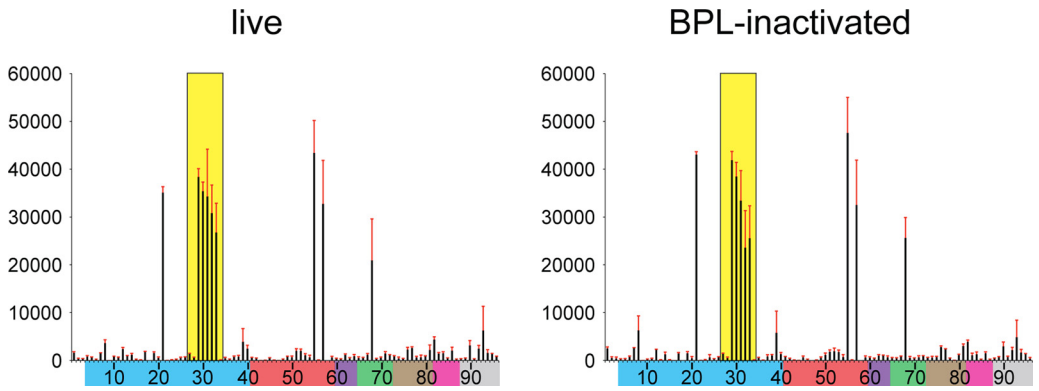


FIG. 2. Receptor specificity of group 1 human H3N2 influenza viruses. Sialoglycan binding of whole-virion preparations from viruses isolated in egg or cell culture host systems was analyzed by glycan microarray analysis. Different categories of glycans on the array (*x* axis) are highlighted in colors, the identity of each numbered glycan is provided in Table S1 in the supplemental material. The bars denote the fluorescent binding signal intensity, and error bars indicate the standard errors. Yellow boxes highlight α1-3 fucosylated sialoglycans. The group 1 virus cluster appears to have minimal changes in receptor specificity compared with those of both the egg- and cell-derived isolates.

eral other structurally diverse glycans were also broadly recognized receptors: (i) most viruses bound α2-6 sialylated *N*,*N*′-diacetyllactosediamine (LacDiNAc) (glycan 55); the exceptions were cell and egg isolates of WI07 and egg isolates of NH06 and NY306; (ii) most viruses bound the α2-6 sialylated-sulfated *N*-acetyllactosamine structure (glycan 41); the exceptions were egg isolates of NY306, PA07, WI07, and BR07; and (iii) most viruses bound a sialyl Lewis x motif (glycans 29 and 30); the exceptions were cell and egg isolates of HO06 and the FL06 cell isolate. Glycans 41 and 55 have been detected in normal or inflamed human tissues (14, 15), whereas sialyl Lewis x glycans are present in the human respiratory tract (1, 23). As with some H3N2 viruses analyzed previously, the isolates in this study (except cell-derived WY03) bound poorly or not at all to α2-6 sialylated biantennary glycans, typically found on membrane glycoproteins (glycans 46 to 49) (35, 66). Collectively, these findings suggest that some H3N2 viruses in original clinical specimens may bind a more diverse set of sialoglycans than was previously reported.

Categorization of viral isolates based on receptor binding profile and isolation substrate. The isolate pairs analyzed separated into distinct groups on the basis of their glycan binding profiles. The results for group 1 viruses, comprising FL06, HO06, and NH06, were characterized by the similar binding profiles for both their cell- and egg-derived isolates (Fig. 2). Viruses had restricted binding to a core subset of linear glycans terminated with α2-6 sialyl *N*-acetyllactosamine (glycans 53 to 57), although GalNAc was tolerated in place of Gal (α2-6 sialylated LacDiNAc [glycan 55]). For the three viruses, limited α2-3 specificities were apparent, with good binding only to the N-linked α2-3 sialylated LacDiNAc (glycan 21) occurring (60). Comparing HA sequences between the cell and egg isolate pairs, HO06 revealed only one amino acid difference, an isoleucine at position 86 of the cell isolate and a leucine in the egg counterpart (Ile86Leu). NH06 had only a mixed sequence population at position 194 (Pro/Leu in cell-derived virus and Pro in egg-derived virus), while the two FL06 sequences revealed mixed populations at five residues in their HA1 sequences, which may explain the minor differences in their binding profiles.

Comparison by glycan microarray of the cell- and egg-grown viruses for the second group (group 2), comprising NY306, NJ06 NY206, PA07, WI07, WY03, and BR07 viruses, revealed a much more significant change in receptor specificity (Fig. 3 and 4). Cell-grown viruses revealed a much broader specificity for binding to many α2-3 and α2-6 glycans, as well as the α2-3/α2-6 disialoside and biantennary N-linked glycans (60-64) relative to that of group 1 cell isolates (Fig. 2 and 3). Notably, the binding of cell-derived NY206 to α2-3 glycans was relatively weaker than that by the other viruses in group 2. In addition, egg-isolated viruses generally revealed a significant reduction in α2-6 binding, although binding to one or more of the α2-6 sialyl *N*-acetyllactosamine motifs (glycans 53 to 57) was maintained, as seen in the group 1 viruses. The most dramatic reduction was seen for the 2007 Wisconsin egg isolate (WI07), which bound weakly to all α2-6 sialylglycans. Binding to α2-3 avian-type receptors was also reduced for all of these group 2 egg-derived isolates. Only fucosylated α2-3 glycans (glycans 28 to 33) were maintained or enhanced upon egg isolation/propagation (Fig. 3). Despite these dramatic differences in binding profiles, comparison of the HA1 sequences revealed that at the protein level, the egg- and cell-derived isolate pairs differed by only 2 amino acid substitutions (WY03, NY306, WI07), 3 substitutions (PA07), or 4 substitutions (NJ06, NY206, BR07) (Table 2 and 3). In five out of the six group 2 viruses shown in Table 2, a Gly186Val substitution was introduced during egg isolation and propagation, together with a second substitution in or near the loop at position 220 (220 loop; NJ06, NY206, PA07, WI07, WY03). The only egg-derived virus not to conform to this observation was NY306, in which a Leu194Pro substitution was introduced within the alpha helix that sits atop the receptor binding site (RBS), and an Ile223Val substitution was introduced in the 220 loop. Interestingly, in three of the previous five viruses (NY206, NJ06, and WI07), the 220-loop substitutions introduced bulkier and/or hydrophobic residues when they were adapted to eggs. The remaining two viruses, WY03 and PA07, had changes at positions 226 and 225, respectively, positions previously identified in modifying receptor specificity in H3 and H1 viruses (39, 48, 66, 83). The structural basis for group 1 or 2 binding

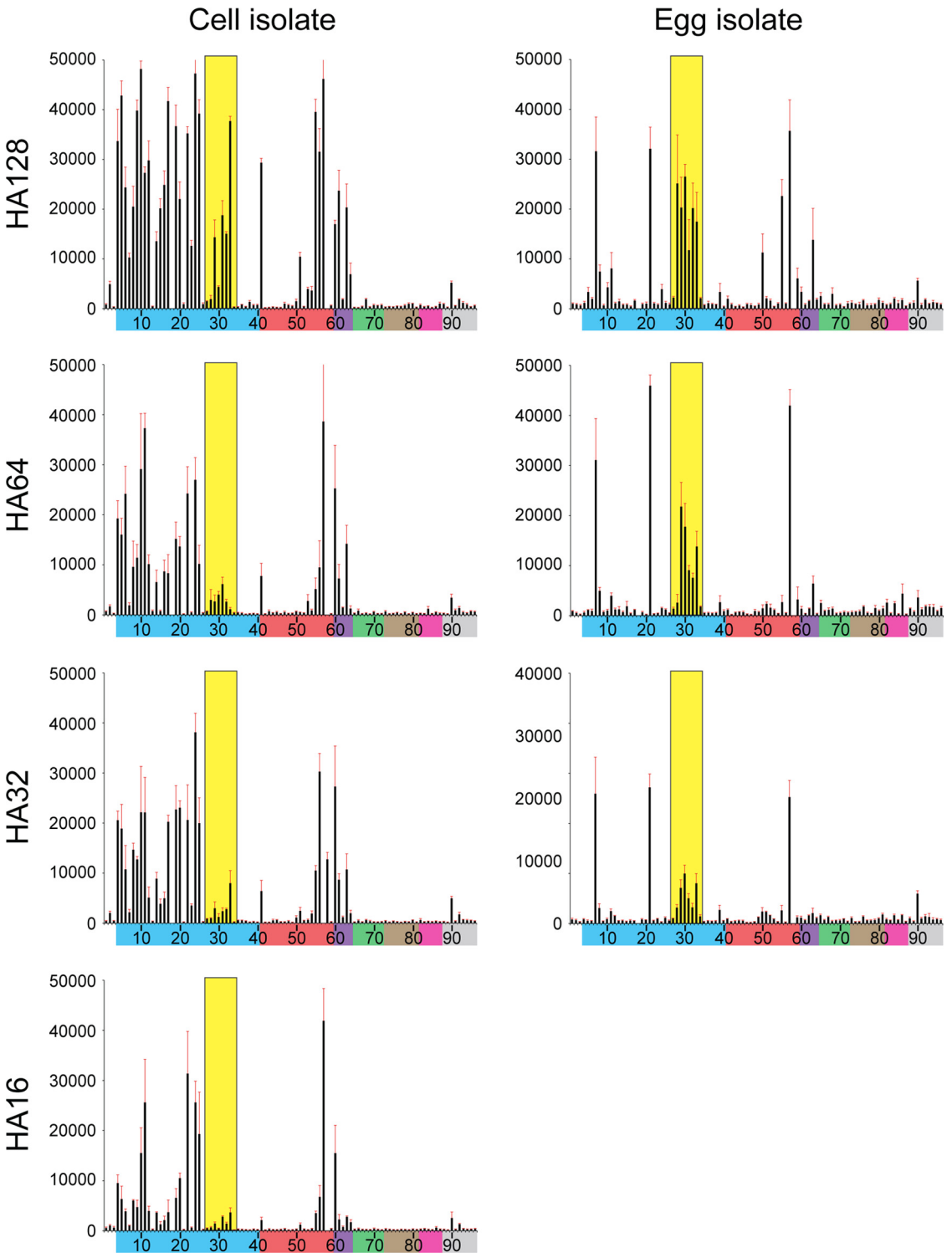


FIG. 3. Receptor specificity of group 2 human H3N2 influenza viruses. Glycan microarray analysis reveals that viruses designated to be in group 2 have more dramatic changes in receptor specificity when both egg- and cell-derived isolates were compared. The results revealed that cell-derived isolates possess a broad specificity for binding to many α2-3 and α2-6 sialylated glycans, while isolation in eggs resulted in a significant reduction in α2-3 and α2-6 binding. Data are presented as described in the legend to Fig. 2.

profiles remains to be determined; these data do not allow a direct linkage between HA sequence and receptor function, precluding attempts to predict receptor binding profiles on the basis of amino acid sequences.

The WY03 virus (also in group 2) was of particular interest since the sialoside binding pattern of the egg variant-derived vaccine reassortant has already been published (35). The egg-derived isolate showed binding comparable to that published

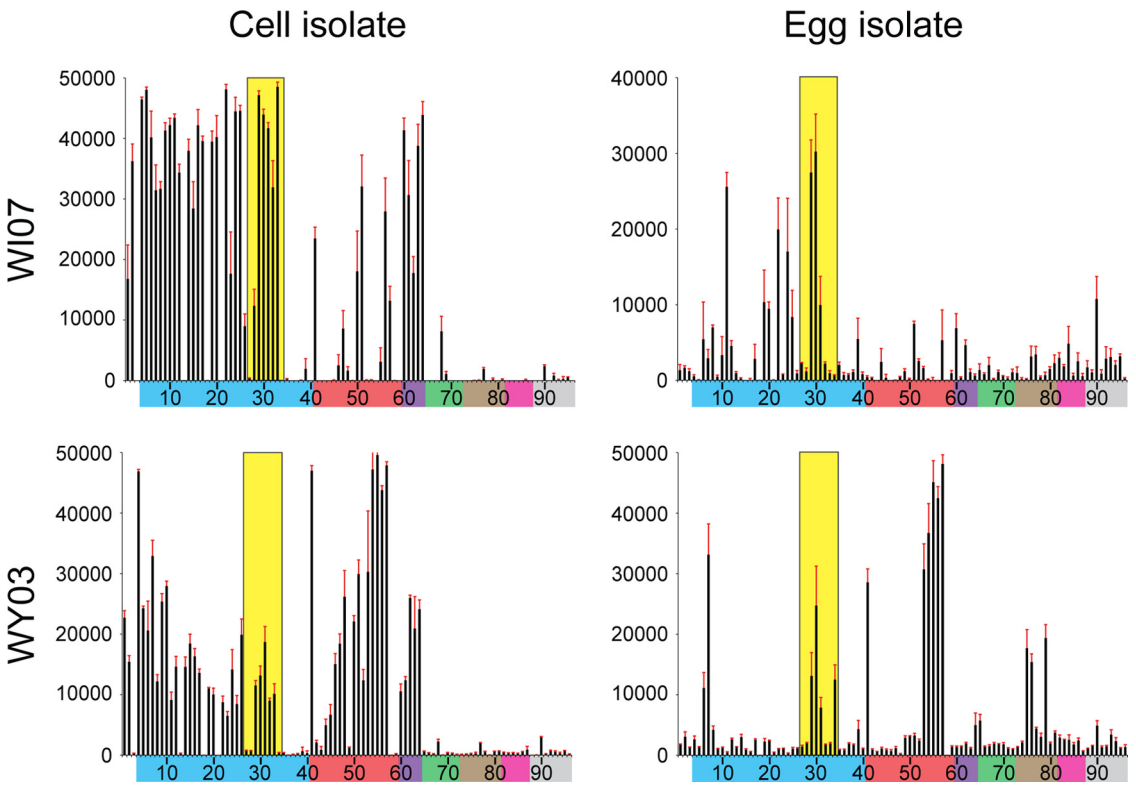


FIG. 3—*Continued*.

previously. However, the paired MDCK cell culture isolate from the same patient specimen exhibited the broad specificity characteristic of other group 2 viruses isolated in MDCK cell culture (Fig. 3). In addition to α2-3 and α2-6 binding, WY03 egg-derived virus also bound weakly to α2-8 glycans (glycans 75, 76, and 79; see Fig. 3 and reference 35), in contrast to the virus from MDCK cell cultures. This specificity was also previously reported for an egg-adapted NWS-Memphis/31/1998 (H3N2) reassortant virus (81), raising the possibility that 8′-polysialic acid receptor binding by influenza A (H3N2) viruses may result from isolation in eggs.

Receptor binding properties of BR07 egg isolate after reassortment for vaccine production. We also compared the BR07 cell isolate to the egg counterpart and to three clonal populations derived from the latter by limiting-dilution passage in eggs to generate high-growth reassortants for vaccine production (32) (Table 1). Compared to the cell isolate, biologically cloned egg-derived viruses had one to three changes in the HA. X-171 had three substitutions (Gly186Val, Ala198 deletion [del], and Ile226Ser), X-171A had a single change (Leu194Pro), and X-171B also had three substitutions (Gly186Val, Ala198 deletion, and Ser219Tyr) in their HA1 (Table 3).

Sequence analysis of the uncloned egg-isolated BR07 virus after five egg passages revealed the persistence of a heterogeneous virus population with variable residues at positions 186 (Gly/Val), 190 (Asp/Asn), 194 (Leu/Pro), and 198 (Ala/deletion) in the HA (Table 3). In agreement with these observed substitutions, glycan microarray analysis of the egg- and cell-grown BR07 viruses revealed characteristic group 2 profiles (Fig. 4). Since the heterogeneous populations in the BR07 egg-grown virus that was analyzed included substitutions identified in the X-171, X-171A, and X-171B reassortants, it was not surprising to see the binding profiles for these viruses yielding results similar to those for the uncloned original egg isolate (Fig. 4). The most unexpected result, though, appeared with the high-growth X-171B reassortant. Glycan microarray analysis of X-171B revealed a highly restricted pattern of binding to only eight glycans on the microarray: N-linked α2-3 sialylated LacDiNAc (glycan 21), sialyl Lewis x motifs (glycans 29 to 33), α2-6 sialylated LacDiNAc (glycan 55), and α2-6 sialylated tri-*N*-acetyllactosamine (glycan 57) (Fig. 4; Table 4). This short list of candidate sialoglycans may help identify the receptor(s) essential for efficient virus propagation in chicken embryos.

DISCUSSION

Successful replication of influenza viruses in eggs was first reported in 1940 (6). The allantoic sacs of embryonated eggs became an ideal substrate for influenza vaccines because they supported high antigen yields. The vast majority of current influenza vaccines are also produced in eggs from viruses propagated exclusively in eggs because vaccines produced in cell culture systems have not yet been licensed in most countries (22, 33, 50). Although the egg allantoic sac has been used successfully to produce influenza vaccines for 60 years, recent human H3N2 viruses have largely lost the ability to replicate in this host unless they acquire certain mutations in the HA (48, 80). This would not become a major problem if a vaccine virus seed could be used for many years after its derivation in eggs.

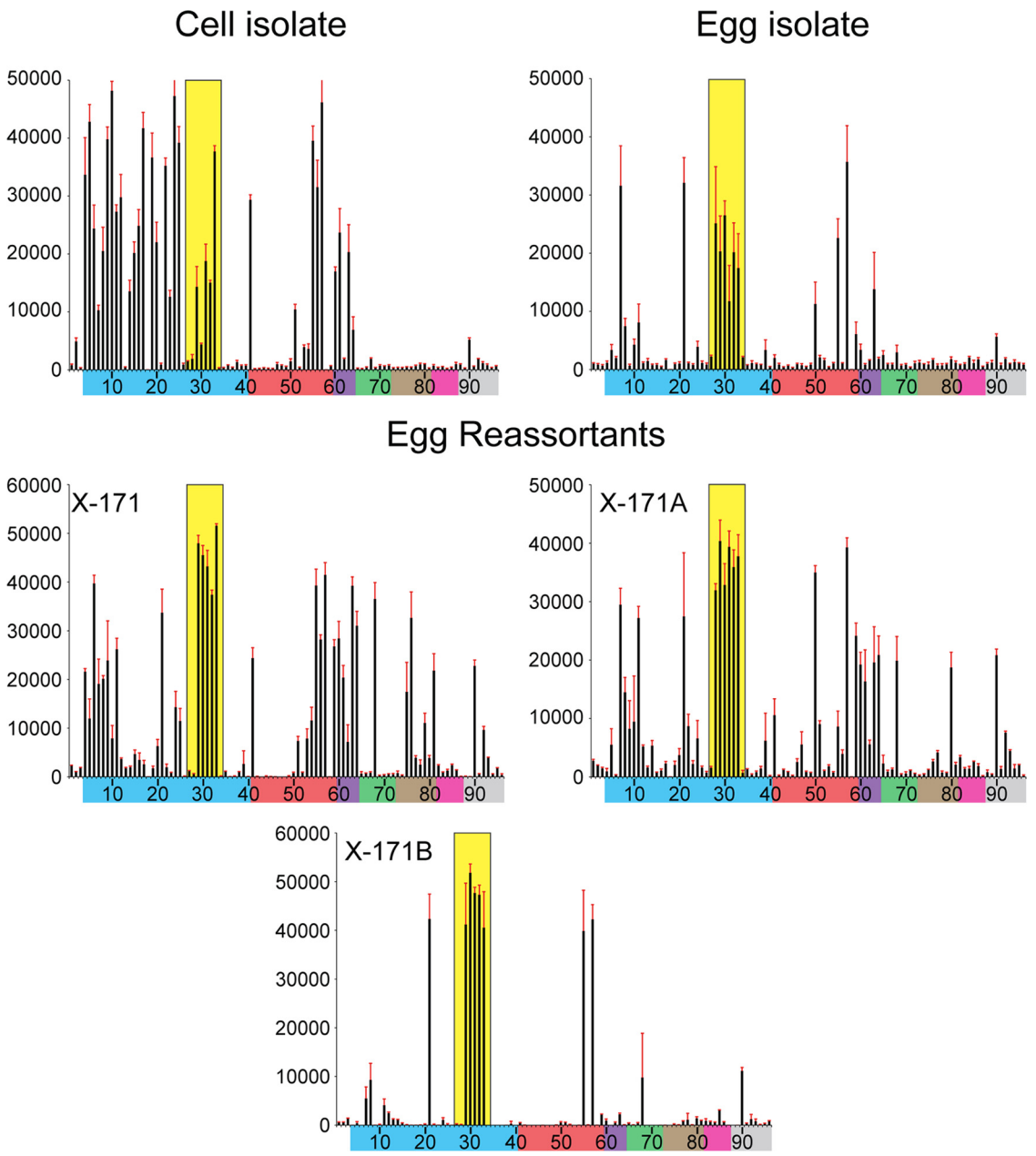


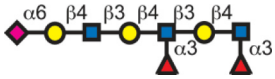
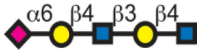
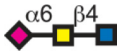
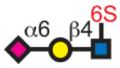
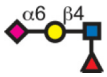
FIG. 4. Glycan microarray analysis of BR07 influenza viruses and reassortants. BR07 isolated in cells or eggs as well as high-growth reassortant virus clones were analyzed. Note that the binding profile is similar to that for the viruses designated as being in group 2 (Fig. 3). Of the egg-adapted reassortants, the high-growth X-171B virus reveals a restricted binding profile, indicating the types of sialoglycans that may be present in eggs to support a growth rate higher than that for the parent virus.

However, rapid antigenic drift mandates frequent updates in influenza vaccine composition, which requires derivation of new virus seeds in eggs within a short period of time (21).

Host selection of influenza A virus mutants has been known for over 65 years (7, 8, 12, 55), but we have not yet fully characterized the role of egg propagation on amino acid changes and virus receptor specificity. Cell-grown viruses have been demonstrated to accumulate fewer mutations than viruses present in the respiratory tract (29, 30, 53, 58) and thus are more representative of the wild-type virus circulating in the human population.

Previous studies have identified 16 positions at which substitutions emerged in the HA of H3N2 viruses isolated in eggs. However, the majority of these studies reported using viruses isolated between 1986 and 1990 (19, 24, 26, 30, 31, 44, 56, 83), while one studied a single 1997 virus (43) and two studies looked at a A/Fujian/2002-like virus (38, 78). Our studies with 9 pairs of viruses isolated in 2006 and 2007 identified substitutions at 8 of these 16 previously reported positions, and all were close to the RBS (Fig. 1). Formal demonstration of their role in viral replication in the egg host system would require detailed analysis of isogenic mutant viruses. However, their

TABLE 4. Sialoglycan ligands shared by the majority of H3N2 viruses in this study

Glycan no.	Common name[a]	Structure	Exceptions[b]
57	Fucosylated α2-6 sialyl tri-*N*-acetyllactosamine		None
56	α2-6 sialyl di-*N*-acetyllactosamine		NY306-E, PA07-E,[c] WI07-E, NH06-E, BR07-E[c]
55	α2-6 sialyl *N*,*N*′-diacetyllactosediamine		NY306-E, WI07-C, WI07-E, NH06-E
41	Sulfated α2-6 sialyl *N*-acetyllactosamine		NY306-E, PA07-E, WI07-E, BR07-E
29, 30	Sialyl Lewis x		HO06-C, HO06-E, FL06-C

[a] See Table S1 in the supplemental material for complete glycan names.
[b] Virus isolated in the indicated host did not show significant binding to the indicated sialoside in the glycan array.
[c] The suffix denotes the host in which virus was isolated: E, chicken embryo; C, mammalian cells.

potential significance for replication in eggs can be inferred from the nonrandom distribution of the 16 changes in the HA (Table 2) with regard to spatial and population diversity considerations. First, the majority of the variation occurred at only eight amino acid positions, all of which are vicinal to the receptor binding site in the HA1. Second, most changes involved the 16 amino acids previously reported to vary in relation to isolation in eggs (19, 24, 26, 30, 31, 38, 43, 44, 56, 78, 83).

On the basis of their binding profiles to glycan microarrays, the virus pairs studied could be divided into two groups. Group 1 HAs had only minimal differences in glycan microarray binding, and this was perhaps reflected in the somewhat reduced number of substitutions observed between the cell- and egg-derived HAs. Glycan array analysis of these cell isolates revealed that they already possessed a restricted sialoside binding profile. This property was associated with specific amino acids at critical positions; e.g., cell-derived NH06 had a mixed population at position 194 (Leu/Pro), while the HO06 cell-derived isolate already had a Leu at position 183, previously reported to be egg-selected (38). Group 2 virus isolates from eggs, however, had more dramatic changes with respect to their glycan binding profiles; receptor recognition was restricted to a smaller subset of glycans compared to the size of the subset in their cell grown counterparts. These changes suggested that egg isolation was frequently associated with a Gly186Val substitution in combination with a substitution in the 220 loop of HA. The NY306 egg isolate was the exception, having a Leu194Pro substitution besides the 220-loop modification.

Substitutions were also observed at seven additional sites that had not been previously reported for H3N2 viruses isolated in eggs (colored blue in Fig. 1). Five of the seven sites (190, 195, 198, 223, and 225) are within or proximal to the RBS. Position 190 substitutions have been associated with the replication of H1N1 viruses in eggs (39, 66), while position 198 was deleted in one of the BR07 clones (discussed below). Residues 223 and 225 are in the 220 loop, which was previously reported to be subject to changes during growth in eggs (44). Collectively, changes at these five positions merit further studies to determine their role in efficient viral replication in the egg host system.

Our results from the BR07 egg-isolated high-growth reassortants can shed light on virus-host interactions in this system. The X-171A clone had a single change (Leu194Pro), while the X-171B clone had two amino acid substitutions and one deletion (Leu186Pro, Ala198del, and Ser219Tyr) (Table 3). The uncloned BR07 isolated in eggs revealed heterogeneous populations at three of the four positions (Gly/Val at position 186, Leu/Pro at position 194, Ala/del at position 198), confirming the presence of most of these changes prior to selection of clonal reassortant viruses. Asp/Asn at 190 was the only change not seen in the cloned reassortants. Interestingly, two of the three changes observed in the high-growth reassortant (X-171B) were present in the egg passage 5 virus and predominated in the passage 9 virus (data not shown), suggesting that the Gly186Val/Ala198del combination is beneficial for growth in eggs. These two changes, in combination with an additional Ser219Tyr substitution (near the 220 loop), resulted in a dramatic switch in receptor specificity with a highly restricted binding profile (Fig. 4). Furthermore, a high-growth virus with a deletion in the HA (Ala198del) was obtained in eggs, despite the scarcity of trinucleotide deletions relative to single base substitutions in the original virus population, suggesting that the Ala198del/Gly186Val combination imparted a replication advantage in eggs. Antigenic analyses of the cell and egg isolates by hemagglutination inhibition tests using a panel of ferret antisera to A/Brisbane/10/2007 wild-type virus and each of its reassortants failed to reveal a ≥8-fold and reciprocal titer differences, although a partial (nonreciprocal) ≤8-fold difference was noted in these studies (data not shown).

Disruption of the helix at position 190 resulting from the introduction of a Leu194Pro substitution in BR07 (as well as in NH06 and NY306) appears to be correlated with a glycan binding profile similar to that of the X-171B high-yield reassortant (Fig. 3 and 4). The same appears to happen for the deletion at position 198, although its true effect cannot be determined due to the additional substitutions at positions 186, 219, and 226, which may also play a role (as seen with the other

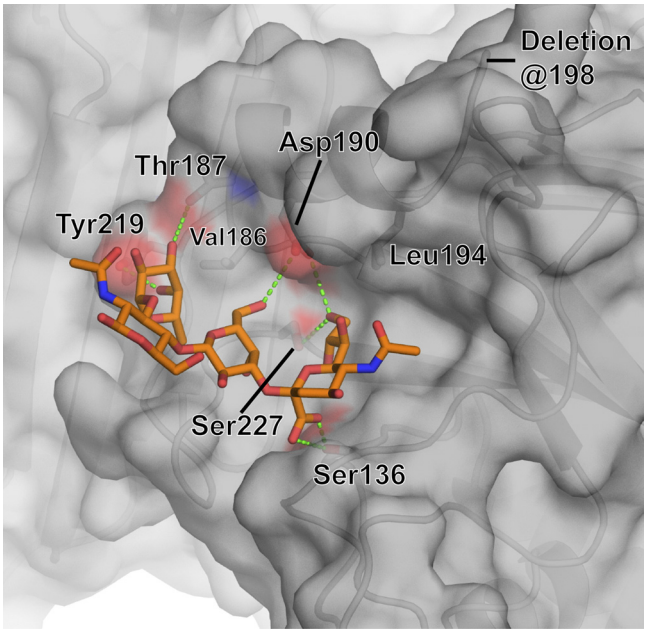


FIG. 5. Structural model of BR07 X-171B HA receptor binding site with a bound fucosylated sialoglycan. The avian receptor analog Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ (sialyl Lewis x, 29 in the microarray; see Table S1 in the supplemental material) was modeled into the RBS of the BR07 HA model. Putative hydrogen bond interactions between the glycan and the HA RBS are shown as green broken lines.

group 2 viruses). Position 186 and the 220-loop substitutions all introduce bulkier and/or hydrophobic residues to the left side of the RBS (Fig. 1). Comparing glycan microarray binding of X-171 and X-171B, which differ only in the position of the substitution on the 220 loop (Ile226Ser in X-171 and Ser219Tyr in X-171B), suggests that position 219 is the critical residue responsible for a more restrictive binding profile. Modeling a sialyl Lewis x glycan (Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ) into a hypothetical BR07 binding pocket indicates that the fucose could directly interact with the hydroxyl of the phenolic side chain of the Tyr and might therefore selectively stabilize these glycans (Fig. 5). In contrast, an Ile226Ser substitution introduces a potential hydrogen bond to the O-4 of galactose, which is less selective.

The narrow receptor specificity of the high-growth X-171B reassortant raises the possibility that these glycans might be present and used by the virus during egg growth: α2-3 sialylated LacDiNAc (glycan 21), sialyl Lewis x (glycans 29 to 33), α2-6 sialyl LacDiNac (glycan 55), α2-6 sialyl fucosyl tri-lactosamine (glycan 57), and sialylated *N*-glycolyl Lewis x (glycan 68). However, to date, these structures have not been identified as terminal sequences on N-linked glycans of either embryonated chicken egg chorioallantoic or amniotic cells (63), though neutral nonsialylated glycans containing LacDiNAc as well as fucosylated *N*-acetyllactosamine have been described in the N-glycan structures of a chicken eggshell protein, ovocleidin-116 (47), indicating that chickens have the biosynthetic capability to make the corresponding sialylated structures.

Kumari et al. recently analyzed MDCK cell-grown viruses isolated between 2003 and 2005 and found restricted specificity to only α2-6 glycans (35). Except for WY03, all the virus pairs presented in this study are isolated from 2006 onwards and revealed a much broader specificity. A possible reason for this difference may be due to a Ser193Phe substitution that emerged in 2005 and that is currently the dominant residue in circulating strains. Analysis of the HA sequences from the study of Kumari et al. reveals a serine at this position. Position 193 is in the alpha helix that sits atop the RBS, and the bulky aromatic side chain is predicted to extend over the receptor pocket. This, in conjunction with substitutions (Ser189Asn and Ser227Pro) on the left side of the RBS in regions of importance for egg adaptation, appeared in viruses from 2004 onwards and may contribute to the increased α2-3 binding observed in this study. Interestingly, Gulati et al. reported that cell-derived Brisbane/10/07-like viruses bound to very few sialoglycans on the array, primarily α2-6 sialyl-polylactosamines (25). The reason why their findings are so different from those for the viruses analyzed here is unknown. An analysis of the virus dilution of both cell- and egg-grown A/Brisbane/10/2007 (H3N2) virus at 2-fold incremental HA titers between 32 and 512 yielded an overall reduction in overall glycan signals rather than enrichment of the single long polylactosamine reported previously (see Fig. S3 in the supplemental material), suggesting that a drastic difference in the HA titers used between these different laboratories is not the reason. The rapid evolution of the receptor binding preferences of H3N2 antigenic variants that emerged between 2003 and 2007 (Fujian/410-like, Wisconsin/67-like, California/7-like, and Brisbane/10-like viruses) highlights the need to reevaluate functional predictions with any future variant viruses of interest (25, 35).

Compared to viruses isolated in MDCK cell culture, egg isolates from 2006 and 2007 can have both modestly and dramatically different glycan microarray profiles. In addition, substitutions at different positions, such as 194 (X-171A) and 186/198 (X-171B), appear to produce similar binding profiles, thus illustrating the complexity of the system that we are attempting to understand. Williams and Robertson (79) reported that substitutions in the vicinity of the RBS appear to be responsible not only for modulating attachment to receptors but also for triggering virus penetration into the target cells. The mutations acquired by group 1 egg-derived isolates (and some of those of group 2-derived egg isolates) may modulate postbinding events mediating endocytosis and membrane fusion.

Although early studies showed that the sequences of HA genes of H3N2 viruses isolated in cell culture are generally identical to those sequenced directly from nasal secretions of patients, recently circulating viruses may also undergo host selection during isolation in mammalian cells (30, 49). Isolation of H3N2 viruses in MDCK cells has been progressively more difficult in recent years (49). This obstacle has been overcome by utilizing a transfectant MDCK cell line expressing higher levels of α2-6 sialosides, suggesting that the paucity of these receptors in regular MDCK cells may consequently exert selective pressure for selection of viruses with enhanced binding of α2-3 sialylated glycans. Most group 2 viruses isolated in cells show moderate to high levels of α2-3 sialoside specificity. Viruses in this group with strong α2-3 binding specificity also bind to α2-3 linked N-glycans as well as mixed 2-3/2-6 biantennary glycans (glycans 60 and 61, respectively). In contrast, this is not seen in many of the egg isolates. This raises the possi-

bility that the α2-3 specificity seen in the MDCK cell-grown virus could reflect selection of viruses with increased specificity for α2-3 N-linked glycans to compensate for the low level of α2-6 N-linked glycans. Thus, the differences in glycan specificity seen between the egg- and cell-grown viruses could result from selective pressures exerted by both laboratory hosts.

These results also provide a snapshot of the receptor specificity of human H3N2 influenza viruses circulating in 2006 and 2007. Two long α2-6 sialylated glycans (glycans 56 and 57) were the ligands preferred by most viruses, a finding consistent with that from a previous report of a study that used recombinant HA (66). Three trisaccharides less frequently considered influenza virus receptors were the ligands widely used by these viruses. Two were α2-6 sialylated LacDiNAc and sulfated *N*-acetyllactosamine (glycans 55 and 41, respectively). Interestingly, the sialyl Lewis x glycan, whose presence in airway epithelial cells has been well documented (1, 23), was also an excellent ligand for many of the viruses in this panel, suggesting that α2-3-linked sialic acids should not be excluded as potential receptors for human influenza viruses. The consensus glycans for the 2006 and 2007 viruses includes the three previously reported ligands for the recombinant HA of A/Moscow/10/1999 (H3N2) virus (66): sulfated α2-6 sialyl *N*-acetyllactosamine (glycan 41), α2-6 sialyl di-*N*-acetyllactosamine (glycan 56), and α2-6 sialyl fucosylated tri-*N*-acetyllactosamine (glycan 57), indicating substantial conservation of receptor specificity (Table 4). The use of glycan arrays to analyze the receptor specificity of influenza viruses promises to advance our understanding of a very complex and dynamic virus-host interface. However, the body of information on influenza virus receptor specificity derived from glycan array analyses is still insufficient to interpret the general significance of the data.

Eggs will remain the most economical and practical vaccine production substrate until a cell-based alternative is fully established. Understanding how receptor binding is evolving in human H3N2 viruses circulating globally may explain the basis of poor replication in eggs and the significance of more radical structural changes, such as the Ala198 deletion or Leu194Pro substitution by BR07. Such changes may affect secondary structures in the HA and thus could impact the antigenicity of site B. Structural analysis of these changes will help to clarify their effects in both regards. Further studies will establish the feasibility of analyzing the receptor binding profiles of cell isolates to identify those that match a previously determined glycan binding profile correlated with permissive growth in eggs. As shown with group 1 viruses, the original sequences in certain clinical specimens may be less prone to the type of changes acquired by the HA1 of BR07 (group 2) to generate the high-growth reassortant in eggs. Such viruses would reduce the risk of potential antigenic changes in seed viruses selected for vaccine production.

ACKNOWLEDGMENTS

We thank the WHO Global Influenza Surveillance Network for providing influenza virus isolates and Ian Barr for the viruses isolated in eggs for reassortant production. The glycan microarray data presented here will be made available online through the Consortium for Functional Glycomics website upon publication (www.functionalglycomics.org).

The glycan microarray was produced for the Centers for Disease Control and Prevention by using a glycan library generously provided by the Consortium for Functional Glycomics, funded by National Institute of General Medical Sciences grant GM62116.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry.

REFERENCES

1. **Allahverdian, S., K. R. Wojcik, and D. R. Dorscheid.** 2006. Airway epithelial wound repair: role of carbohydrate sialyl Lewisx. Am. J. Physiol. Lung Cell. Mol. Physiol. **291:**L828–L836.
2. **Baum, L. G., and J. C. Paulson.** 1990. Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem. Suppl. **40:**35–38.
3. **Belongia, E. A., B. A. Kieke, J. G. Donahue, R. T. Greenlee, A. Balish, A. Foust, S. Lindstrom, and D. K. Shay.** 2009. Effectiveness of inactivated influenza vaccines varied substantially with antigenic match from the 2004-2005 season to the 2006-2007 season. J. Infect. Dis. **199:**159–167.
4. **Blixt, O., S. Head, T. Mondala, C. Scanlan, M. E. Huflejt, R. Alvarez, M. C. Bryan, F. Fazio, D. Calarese, J. Stevens, N. Razi, D. J. Stevens, J. J. Skehel, I. van Die, D. R. Burton, I. A. Wilson, R. Cummings, N. Bovin, C. H. Wong, and J. C. Paulson.** 2004. Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. Proc. Natl. Acad. Sci. U. S. A. **101:**17033–17038.
5. **Budowsky, E. I., E. A. Friedman, N. V. Zheleznova, and F. S. Noskov.** 1991. Principles of selective inactivation of viral genome. VI. Inactivation of the infectivity of the influenza virus by the action of beta-propiolactone. Vaccine **9:**398–402.
6. **Burnet, F. M.** 1940. Influenza virus infections of the chick embryo lung. Br. J. Exp. Pathol. **21:**147–153.
7. **Burnet, F. M., and D. H. Bull.** 1943. Changes in Influenza virus associated with adaptation to passage in chick embryos. Aust. J. Exp. Biol. Med. **21:**55–69.
8. **Burnet, F. M., J. D. Stone, et al.** 1949. The genetic character of O-D change in influenza A. Br. J. Exp. Pathol. **30:**419–425.
9. **Chandrasekaran, A., A. Srinivasan, R. Raman, K. Viswanathan, S. Raguram, T. M. Tumpey, V. Sasisekharan, and R. Sasisekharan.** 2008. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nat. Biotechnol. **26:**107–113.
10. **Chen, L. M., C. T. Davis, H. Zhou, N. J. Cox, and R. O. Donis.** 2008. Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. PLoS Pathog. **4:**e1000072.
11. **Childs, R. A., A. S. Palma, S. Wharton, T. Matrosovich, Y. Liu, W. Chai, M. A. Campanero-Rhodes, Y. Zhang, M. Eickmann, M. Kiso, A. Hay, M. Matrosovich, and T. Feizi.** 2009. Receptor-binding specificity of pandemic influenza A (H1N1) 2009 virus determined by carbohydrate microarray. Nat. Biotechnol. **27:**797–799.
12. **Choppin, P. W., and I. Tamm.** 1959. Two kinds of particles with contrasting properties in influenza A virus strains from the 1957 pandemic. Virology **8:**539–542.
13. **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.
14. **Crottet, P., Y. J. Kim, and A. Varki.** 1996. Subsets of sialylated, sulfated mucins of diverse origins are recognized by L-selectin. Lack of evidence for unique oligosaccharide sequences mediating binding. Glycobiology **6:**191–208.
15. **Degroote, S., E. Maes, P. Humbert, P. Delmotte, G. Lamblin, and P. Roussel.** 2003. Sulfated oligosaccharides isolated from the respiratory mucins of a secretor patient suffering from chronic bronchitis. Biochimie **85:**369–379.
16. **Fodor, E., L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, and A. Garcia-Sastre.** 1999. Rescue of influenza A virus from recombinant DNA. J. Virol. **73:**9679–9682.
17. **Gambaryan, A., S. Yamnikova, D. Lvov, A. Tuzikov, A. Chinarev, G. Pazynina, R. Webster, M. Matrosovich, and N. Bovin.** 2005. Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. Virology **334:**276–283.
18. **Gambaryan, A. S., and M. N. Matrosovich.** 1992. A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. J. Virol. Methods **39:**111–123.
19. **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.
20. **Gambaryan, A. S., A. B. Tuzikov, V. E. Piskarev, S. S. Yamnikova, D. K. Lvov, J. S. Robertson, N. V. Bovin, and M. N. Matrosovich.** 1997. Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6′-sialyl(N-acetyllactosamine). Virology **232:**345–350.

21. **Gerdil, C.** 2003. The annual production cycle for influenza vaccine. Vaccine **21:**1776–1779.
22. **Green, I. J.** 1962. Serial propagation of influenza B (Lee) virus in a transmissible line of canine kidney cells. Science **138:**42–43.
23. **Groux-Degroote, S., M. A. Krzewinski-Recchi, A. Cazet, A. Vincent, S. Lehoux, J. J. Lafitte, I. Van Seuningen, and P. Delannoy.** 2008. IL-6 and IL-8 increase the expression of glycosyltransferases and sulfotransferases involved in the biosynthesis of sialylated and/or sulfated Lewis x epitopes in the human bronchial mucosa. Biochem. J. **410:**213–223.
24. **Gubareva, L. V., J. M. Wood, W. J. Meyer, J. M. Katz, J. S. Robertson, D. Major, and R. G. Webster.** 1994. Codominant mixtures of viruses in reference strains of influenza virus due to host cell variation. Virology **199:**89–97.
25. **Gulati, S., D. F. Smith, and G. M. Air.** 2009. Deletions of neuraminidase and resistance to oseltamivir may be a consequence of restricted receptor specificity in recent H3N2 influenza viruses. Virol. J. **6:**22.
26. **Hardy, C. T., S. A. Young, R. G. Webster, C. W. Naeve, and R. J. Owens.** 1995. Egg fluids and cells of the chorioallantoic membrane of embryonated chicken eggs can select different variants of influenza A (H3N2) viruses. Virology **211:**302–306.
27. **Hoffmann, E., and R. G. Webster.** 2000. Unidirectional RNA polymerase I-polymerase II transcription system for the generation of influenza A virus from eight plasmids. J. Gen. Virol. **81:**2843–2847.
28. **Ito, T., Y. Suzuki, A. Takada, A. Kawamoto, K. Otsuki, H. Masuda, M. Yamada, T. Suzuki, H. Kida, and Y. Kawaoka.** 1997. Differences in sialic acid-galactose linkages in the chicken egg amnion and allantois influence human influenza virus receptor specificity and variant selection. J. Virol. **71:**3357–3362.
29. **Katz, J. M., M. Wang, and R. G. Webster.** 1990. Direct sequencing of the HA gene of influenza (H3N2) virus in original clinical samples reveals sequence identity with mammalian cell-grown virus. J. Virol. **64:**1808–1811.
30. **Katz, J. M., and R. G. Webster.** 1992. Amino acid sequence identity between the HA1 of influenza A (H3N2) viruses grown in mammalian and primary chick kidney cells. J. Gen. Virol. **73**(Pt 5)**:**1159–1165.
31. **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**(Pt 7)**:**1311–1316.
32. **Kilbourne, E. D., J. L. Schulman, G. C. Schild, G. Schloer, J. Swanson, and D. Bucher.** 1971. Related studies of a recombinant influenza-virus vaccine. I. Derivation and characterization of virus and vaccine. J. Infect. Dis. **124:**449–462.
33. **Kistner, O., P. N. Barrett, W. Mundt, M. Reiter, S. Schober-Bendixen, and F. Dorner.** 1998. Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. Vaccine **16:**960–968.
34. **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.
35. **Kumari, K., S. Gulati, D. F. Smith, U. Gulati, R. D. Cummings, and G. M. Air.** 2007. Receptor binding specificity of recent human H3N2 influenza viruses. Virol. J. **4:**42.
36. **Levinson, B., D. Pepper, and G. Belyavin.** 1969. Substituted sialic acid prosthetic groups as determinants of viral hemagglutination. J. Virol. **3:**477–483.
37. **Lu, B., H. Zhou, W. Chan, G. Kemble, and H. Jin.** 2006. Single amino acid substitutions in the hemagglutinin of influenza A/Singapore/21/04 (H3N2) increase virus growth in embryonated chicken eggs. Vaccine **24:**6691–6693.
38. **Lu, B., H. Zhou, D. Ye, G. Kemble, and H. Jin.** 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. J. Virol. **79:**6763–6771.
39. **Matrosovich, M., A. Tuzikov, N. Bovin, A. Gambaryan, A. Klimov, M. R. Castrucci, I. Donatelli, and Y. Kawaoka.** 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. **74:**8502–8512.
40. **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. U. S. A. **101:**4620–4624.
41. **Matthews, J. T.** 2006. Egg-based production of influenza vaccine: 30 years of commercial experience. The Bridge **36:**17–24.
42. **Medeiros, R., N. Escriou, N. Naffakh, J.-C. Manuguerra, and S. van der Werf.** 2001. Hemagglutinin residues of recent human A(H3N2) viruses that affect agglutination of chicken erythrocytes. Int. Congr. Ser. **1219:**369–374.
43. **Medeiros, R., N. Naffakh, J. C. Manuguerra, and S. van der Werf.** 2004. Binding of the hemagglutinin from human or equine influenza H3 viruses to the receptor is altered by substitutions at residue 193. Arch. Virol. **149:**1663–1671.
44. **Meyer, W. J., J. M. Wood, D. Major, J. S. Robertson, R. G. Webster, and J. M. Katz.** 1993. Influence of host cell-mediated variation on the international surveillance of influenza A (H3N2) viruses. Virology **196:**130–137.
45. **Neumann, G., and Y. Kawaoka.** 1999. Genetic engineering of influenza and other negative-strand RNA viruses containing segmented genomes. Adv. Virus Res. **53:**265–300.
46. **Nicholls, J. M., M. C. Chan, W. Y. Chan, H. K. Wong, C. Y. Cheung, D. L. Kwong, M. P. Wong, W. H. Chui, L. L. Poon, S. W. Tsao, Y. Guan, and J. S. Peiris.** 2007. Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat. Med. **13:**147–149.
47. **Nimtz, M., H. S. Conradt, and K. Mann.** 2004. LacdiNAc (GalNAcbeta1-4GlcNAc) is a major motif in N-glycan structures of the chicken eggshell protein ovocleidin-116. Biochim. Biophys. Acta **1675:**71–80.
48. **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.
49. **Oh, D. Y., I. G. Barr, J. A. Mosse, and K. L. Laurie.** 2008. MDCK-SIAT1 cells show improved isolation rates for recent human influenza viruses compared to conventional MDCK cells. J. Clin. Microbiol. **46:**2189–2194.
50. **Palache, A. M., H. S. Scheepers, V. de Regt, P. van Ewijk, M. Baljet, R. Brands, and G. J. van Scharrenburg.** 1999. Safety, reactogenicity and immunogenicity of Madin Darby canine kidney cell-derived inactivated influenza subunit vaccine. A meta-analysis of clinical studies. Dev. Biol. Stand. **98:**115–125.
51. **Payne, A.** 1953. The influenza programme of WHO. Bull. World Health Organ. **8:**755–774.
52. **Polson, A.** 1993. Virus separation and purification methods. Marcel Dekker Inc., New York, NY.
53. **Robertson, J. S., J. S. Bootman, C. Nicolson, D. Major, E. W. Robertson, and J. M. Wood.** 1990. The hemagglutinin of influenza B virus present in clinical material is a single species identical to that of mammalian cell-grown virus. Virology **179:**35–40.
54. **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.
55. **Robertson, J. S., C. Nicolson, D. Major, E. W. Robertson, and J. M. Wood.** 1993. The role of amniotic passage in the egg-adaptation of human influenza virus is revealed by haemagglutinin sequence analyses. J. Gen. Virol. **74**(Pt 10)**:**2047–2051.
56. **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**(Pt 11)**:**2513–2518.
57. **Rogers, G. N., and B. L. D'Souza.** 1989. Receptor binding properties of human and animal H1 influenza virus isolates. Virology **173:**317–322.
58. **Rogers, G. N., R. S. Daniels, J. J. Skehel, D. C. Wiley, X. F. Wang, H. H. Higa, and J. C. Paulson.** 1985. Host-mediated selection of influenza virus receptor variants. Sialic acid-α2,6Gal-specific clones of A/duck/Ukraine/1/63 revert to sialic acid-α2,3Gal-specific wild type in ovo. J. Biol. Chem. **260:**7362–7367.
59. **Rogers, G. N., and J. C. Paulson.** 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology **127:**361–373.
60. **Saarinen, J., H. G. Welgus, C. A. Flizar, N. Kalkkinen, and J. Helin.** 1999. N-glycan structures of matrix metalloproteinase-1 derived from human fibroblasts and from HT-1080 fibrosarcoma cells. Eur. J. Biochem. **259:**829–840.
61. **Schwede, T., J. Kopp, N. Guex, and M. C. Peitsch.** 2003. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res. **31:**3381–3385.
62. **Shinya, K., M. Ebina, S. Yamada, M. Ono, N. Kasai, and Y. Kawaoka.** 2006. Avian flu: influenza virus receptors in the human airway. Nature **440:**435–436.
63. **Sriwilaijaroen, N., S. Kondo, H. Yagi, P. Wilairat, H. Hiramatsu, M. Ito, Y. Ito, K. Kato, and Y. Suzuki.** 2009. Analysis of N-glycans in embryonated chicken egg chorioallantoic and amniotic cells responsible for binding and adaptation of human and avian influenza viruses. Glycoconj. J. **26:**433–443.
64. **Stephenson, I., R. G. Das, J. M. Wood, and J. M. Katz.** 2007. Comparison of neutralising antibody assays for detection of antibody to influenza A/H3N2 viruses: an international collaborative study. Vaccine **25:**4056–4063.
65. **Stevens, J., O. Blixt, L. M. Chen, R. O. Donis, J. C. Paulson, and I. A. Wilson.** 2008. Recent avian H5N1 viruses exhibit increased propensity for acquiring human receptor specificity. J. Mol. Biol. **381:**1382–1394.
66. **Stevens, J., O. Blixt, L. Glaser, J. K. Taubenberger, P. Palese, J. C. Paulson, and I. A. Wilson.** 2006. Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J. Mol. Biol. **355:**1143–1155.
67. **Stevens, J., O. Blixt, J. C. Paulson, and I. A. Wilson.** 2006. Glycan microarray technologies: tools to survey host specificity of influenza viruses. Nat. Rev. Microbiol. **4:**857–864.
68. **Stevens, J., O. Blixt, T. M. Tumpey, J. K. Taubenberger, J. C. Paulson, and I. A. Wilson.** 2006. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science **312:**404–410.
69. **Suzuki, Y., Y. Nagao, H. Kato, T. Suzuki, M. Matsumoto, and J. Murayama.**

1987. The hemagglutinins of the human influenza viruses A and B recognize different receptor microdomains. Biochim. Biophys. Acta **903:**417–424.
70. **Thompson, C. I., W. S. Barclay, and M. C. Zambon.** 2004. Changes in in vitro susceptibility of influenza A H3N2 viruses to a neuraminidase inhibitor drug during evolution in the human host. J. Antimicrob. Chemother. **53:**759–765.
71. **Ulmer, J. B., U. Valley, and R. Rappuoli.** 2006. Vaccine manufacturing: challenges and solutions. Nat. Biotechnol. **24:**1377–1383.
72. **van Riel, D., V. J. Munster, E. de Wit, G. F. Rimmelzwaan, R. A. Fouchier, A. D. Osterhaus, and T. Kuiken.** 2006. H5N1 virus attachment to lower respiratory tract. Science **312:**399.
73. **van Riel, D., V. J. Munster, E. de Wit, G. F. Rimmelzwaan, R. A. Fouchier, A. D. Osterhaus, and T. Kuiken.** 2007. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am. J. Pathol. **171:**1215–1223.
74. **Varki, A.** 2007. Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature **446:**1023–1029.
75. **Varki, N. M., and A. Varki.** 2007. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest. **87:**851–857.
76. **WHO.** 2002, posting date. WHO manual on animal influenza diagnosis and surveillance. WHO, Geneva, Switzerland. http://www.who.int/NR/rdonlyres/EFD2B9A7-2265-4AD0-BC98-97937B4FA83C/0/manualonanimalaidiagnosisandsurveillance.pdf.
77. **WHO-GISN.** 2008. Process of influenza vaccine virus selection and development. WHO-GISN, Geneva, Switzerland.
78. **Widjaja, L., N. Ilyushina, R. G. Webster, and R. J. Webby.** 2006. Molecular changes associated with adaptation of human influenza A virus in embryonated chicken eggs. Virology **350:**137–145.
79. **Williams, S. P., and J. S. Robertson.** 1993. Analysis of the restriction to the growth of nonegg-adapted human influenza virus in eggs. Virology **196:**660–665.
80. **World Health Organization Global Influenza Program.** 2005. WHO manual on animal influenza diagnosis and surveillance. Department of Communicable Disease Surveillance and Response, World Health Organization, Geneva, Switzerland.
81. **Wu, W., and G. M. Air.** 2004. Binding of influenza viruses to sialic acids: reassortant viruses with A/NWS/33 hemagglutinin bind to α2,8-linked sialic acid. Virology **325:**340–350.
82. **Xu, D., E. I. Newhouse, R. E. Amaro, H. C. Pao, L. S. Cheng, P. R. Markwick, J. A. McCammon, W. W. Li, and P. W. Arzberger.** 2009. Distinct glycan topology for avian and human sialopentasaccharide receptor analogues upon binding different hemagglutinins: a molecular dynamics perspective. J. Mol. Biol. **387:**465–491.
83. **Xu, X., E. D. Kilbourne, H. E. Hall, and N. J. Cox.** 1994. Nonimmunoselected intrastrain genetic variation detected in pairs of high-yielding influenza A (H3N2) vaccine and parental viruses. J. Infect. Dis. **170:**1432–1438.
84. **Yang, Z.** 2000. Maximum likelihood estimation on large phylogenies and analysis of adaptive evolution in human influenza virus A. J. Mol. Evol. **51:**423–432.