JOURNAL OF VIROLOGY, Feb. 2010, p. 1527–1535 Vol. 84, No. 3
0022-538X/10/$12.00 doi:10.1128/JVI.02069-09

The pH of Activation of the Hemagglutinin Protein Regulates H5N1 Influenza Virus Pathogenicity and Transmissibility in Ducks▽

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Received 30 September 2009/Accepted 7 November 2009

While the molecular mechanism of membrane fusion by the influenza virus hemagglutinin (HA) protein has been studied extensively *in vitro*, the role of acid-dependent HA protein activation in virus replication, pathogenesis, and transmission *in vivo* has not been characterized. To investigate the biological significance of the pH of activation of the HA protein, we compared the properties of four recombinant viruses with altered HA protein acid stability to those of wild-type influenza virus A/chicken/Vietnam/C58/04 (H5N1) *in vitro* and in mallards. Membrane fusion by wild-type virus was activated at pH 5.9. Wild-type virus had a calculated environmental persistence of 62 days and caused extensive morbidity, mortality, shedding, and transmission in mallards. An N114K mutation that increased the pH of HA activation by 0.5 unit resulted in decreased replication, genetic stability, and environmental stability. Changes of +0.4 and −0.5 unit in the pH of activation by Y23H and K58I mutations, respectively, reduced weight loss, mortality, shedding, and transmission in mallards. An H24Q mutation that decreased the pH of activation by 0.3 unit resulted in weight loss, mortality, clinical symptoms, and shedding similar to those of the wild type. However, the HA-H24$_1$Q virus was shed more extensively into drinking water and persisted longer in the environment. The pH of activation of the H5 HA protein plays a key role in the propagation of H5N1 influenza viruses in ducks and may be a novel molecular factor in the ecology of influenza viruses. The data also demonstrate that H5N1 neuraminidase activity increases the pH of activation of the HA protein *in vitro*.

Highly pathogenic H5N1 influenza viruses were transmitted to humans in 1997 in Southeast Asia (7) and have subsequently spread across Asia, Europe, and Africa (53). Millions of poultry have been culled to control outbreaks (19), and more than 250 human lives have been lost (http://www.who.int/csr/disease/avian_influenza/en/). These viruses appear currently to lack the molecular properties required for sustained transmission among humans. There is an urgent need to understand the molecular properties that contribute to the transmission and host range of these viruses for their effective surveillance and containment.

The transmissibility and pathogenicity of influenza A viruses, including the H5N1 subtype, in avian and mammalian species are determined by both viral and host factors (8, 39). One key factor is the multifunctional hemagglutinin (HA) protein. During viral entry, the HA protein binds to sialic acid-containing receptors on host cells; the virus then undergoes endocytosis, and its HA protein is activated at a low pH to cause the fusion of the viral and endosomal membranes (11, 41). The host range of influenza A viruses depends in large part on the receptor specificity of the HA protein. Avian influenza viruses generally bind to α(2,3) sialosides with greater affinity, while human influenza viruses usually bind to α(2,6) sialosides with greater affinity (4, 37). The receptor binding affinities and specificities of HA proteins also depend on internal linkages and modifications of inner oligosaccharides, and glycan microarray profiling has revealed differences in receptor binding between seasonal human influenza viruses and H5N1 viruses (23, 45, 46). Thus, the natural distribution of various sialosides in different tissues of different species helps to determine both tissue tropism and species specificity (31, 40, 50, 58). The posttranslational cleavability of the HA0 precursor protein into the fusion-capable HA1–HA2 complex is a critical determinant of the virulence of influenza viruses (16, 22, 55). The presence of a polybasic cleavage site in H5 and H7 influenza viruses allows HA protein cleavage in the *trans*-Golgi network by ubiquitous furin-like enzymes and is a marker of high pathogenicity (12, 38, 55).

During entry into host cells, influenza viruses are exposed to increasingly lower pHs until a threshold is reached at which HA protein trimers undergo irreversible conformational changes that promote membrane fusion (11, 41). Threshold pH values differ among influenza viruses, and a change in the pH of fusion of the HA protein can help influenza viruses to adapt to

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▽ Published ahead of print on 18 November 2009.

different cell lines (5, 25) and host species (13) or to the higher endosomal pH induced by high concentrations of the antiviral drug amantadine (6, 9, 42–44). In general, a high pH of HA protein activation could result in influenza virus inactivation in the environment or during transport to the cell surface for intracellularly cleaved HA proteins (2, 44). On the other hand, a low pH of HA protein activation could result in degradation in the lysosome as the pH of the endocytic pathway decreases from early endosomes to late endosomes to lysosomes (61). Therefore, for efficient propagation within a biological host and ecological niche, an influenza virus may have an optimal range of pHs of activation for the HA protein. Moreover, the optimal activation pH may change upon introduction of an influenza virus into a new host species or environment.

Aquatic birds are a natural reservoir of influenza viruses, but surprisingly little is known about the molecular basis of influenza virus propagation in these species. To test the hypothesis that the pH of activation of the HA protein contributes to the pathogenicity and transmissibility of H5N1 influenza viruses in the mallard, a prototypic aquatic bird, we previously generated four recombinant H5N1 viruses containing mutations that altered the acid stability of the HA protein without changing its level of expression, cleavage, receptor binding, or membrane fusion efficiency (36). Two of the mutations increased the pH of membrane fusion of the H5N1 HA protein ($Y23_1H$ and $N114_2K$), and the other two mutations reduced the pH of fusion ($H24_1Q$ and $K58_2I$). HA1 mutations $Y23_1H$ and $H24_1Q$ (H5 numbering with subscripts denoting HA1 and HA2 subunits) are located in the fusion peptide pocket and were originally chosen because of their presence in H1 and H9 subtypes, respectively. The $K58_2I$ mutation in the A-helix of HA2 was chosen because it decreases the pH of membrane fusion of the H3 HA protein by 0.7 unit (44). The $N114_2K$ mutation in the fusion peptide pocket was chosen because it increases the pH of membrane fusion by approximately 0.5 unit in H3 and H7 subtypes (6). Here we measured the effects of the H5 HA protein mutations on virus replication *in vitro*, on genetic stability after repeated passage in eggs, and on environmental stability. Mallards were inoculated with the recombinant viruses and were housed with contact ducks in order to determine the effects of the mutations on virus shedding, pathogenesis, and transmissibility. An $H24_1Q$ mutation in the HA protein was found to decrease the pH of activation by 0.3 pH unit, to increase the titers of infectious virus recovered from ducks' water dishes, and to prolong the persistence of infectious virus in the environment. In general, changes in the acid stability of the HA protein were found to alter H5N1 influenza virus replication, pathogenicity, and transmissibility.

MATERIALS AND METHODS

Viruses, plasmids, and cell culture. Recombinant viruses and plasmids containing HA protein mutations $Y23_1H$, $H24_1Q$, $K58_2I$, and $N114_2K$ were generated previously (36). All experiments using H5N1 influenza viruses were performed in a USDA-approved biosafety level 3+ containment facility. Monolayer cultures of Vero cells (ATCC CCL-81) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin. Monolayers of Madin-Darby canine kidney (MDCK) cells (ATCC CCL-34) were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin.

Virus growth kinetics. Single-step growth curves in MDCK cells were determined for each of the recombinant viruses. Confluent monolayers were infected at a multiplicity of infection (MOI) of ~3 PFU per cell. After 1 h of incubation, cells were first washed with a 0.9% aqueous NaCl solution (pH 2.2) to remove any free infectious virus particles and were then washed twice with phosphate-buffered saline (PBS) to adjust the pH. Cells were incubated at 37°C in MEM (containing 4% bovine serum albumin and 1% glutamine). Supernatants were collected 2, 4, 6, 8, and 10 h postinfection and were stored at −70°C. To determine multiple-step growth kinetics, MDCK cells were infected at an MOI of ~0.01 PFU/cell. After 1 h of incubation, cells were washed twice with PBS and were incubated at 37°C in MEM (containing 4% bovine serum albumin and 1% glutamine). Supernatants were collected 12, 24, 36, 48, 60, and 72 h postinfection and were stored at −70°C. The virus was titrated as described previously (60). Briefly, confluent MDCK cells were incubated for 1 h at 37°C with 10-fold serial dilutions of virus in 1 ml infection medium. The cells were then washed and overlaid with freshly prepared MEM containing 0.3% bovine serum albumin and 0.9% Bacto agar. After incubation at 37°C for 3 days, plaques were visualized by using a 0.1% crystal violet solution containing 10% formaldehyde.

Genetic stability. The H5N1 influenza viruses were serially passaged in 10-day-old embryonated chicken eggs to assess the genetic stability of the introduced mutations. Eggs were infected with 1 HA unit of sequence-confirmed virus. Allantoic fluid was collected, and the HA titer was measured to determine the dilution for subsequent passage of the virus. RNA was extracted and sequenced as described above.

Environmental stability. Stocks of recombinant viruses were diluted 1:50 in distilled water (pH 7.4) containing 2 mM HEPES buffer. Aliquots were incubated at 28°C (the approximate environmental temperature in Louisiana during the summer, allowing comparison with data from similar studies) (2). Aliquots were removed daily for 8 days, and their titers measured by plaque assay were compared to the initial virus titer. The sequential data were $\log_{10}$ transformed and analyzed by linear regression using GraphPad Prism software (GraphPad Software, La Jolla, CA). The gradient from this model was then used to calculate the estimated persistence of 1×10^6 PFU/ml of recombinant virus and the time required to reduce the infectivity of the initial inoculum by 90% (1 $\log_{10}$). Differences in the linear regression models were measured by using GraphPad Prism software.

Inoculation and transmission studies of mallards. Groups of three 4-week-old mallards (*Anas platyrhynchos*) were inoculated via intranasal, intraocular, and intratracheal instillation of $\sim 10^6$ 50% egg infective doses (EID_{50}) of virus in a 1-ml volume, as described previously (21). Two uninoculated contact ducks were placed in the cage with the inoculated ducks 24 h postinoculation (p.i.), and shared a common food and water source. Birds were weighed and observed daily for signs of morbidity or mortality over a period of 14 days. Birds that did not eat or drink on their own due to severe disease signs were euthanized, and their deaths were recorded on the following day of observation. Tracheal and cloacal swabs were collected from all ducks on days 3, 5, 7, and 10 p.i., and 0.5 ml of drinking water was sampled on days 1, 3, 5, 7, and 10 p.i. Influenza virus was detected by virus isolation in 10-day-old embryonated chicken eggs as previously described (14, 47). The virus was titrated in positive samples by calculating the EID_{50}, using the method of Reed and Muench (35); the lower limit of quantification was 0.75 $\log_{10}$ EID_{50}/ml. Swab samples with detectable influenza virus but titers below the limit of quantification were reported as having a titer of $<10^1$ EID_{50}/ml. All data shown were derived from two separate experiments. All animal experiments were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital (Memphis, TN) and were performed in compliance with relevant institutional policies, the Association for the Accreditation of Laboratory Animal Care guidelines, the National Institutes of Health regulations, and local, state, and federal laws.

Transient expression of HA and NA proteins. Monolayers of Vero cells in 6-well dishes (85 to 95% confluence) were transiently transfected with 1 μg of pCAGGS A/chicken/Vietnam/C58/04 HA DNA by using the Lipofectamine Plus expression system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transfected Vero cells were incubated for 4 h at 37°C. DMEM (containing 10% fetal bovine serum and 1% glutamine) was then added to cells, and cells were incubated for 16 h at 37°C. Cells were then treated as indicated for each experiment. Neuraminidase (NA) protein was expressed by using 0.1 to 1.0 μg of the pCAGGS A/chicken/Vietnam/C58/04 NA plasmid.

Syncytium assay. Monolayers of Vero cells grown in 6-well plates were transfected with 1.0 μg pCAGGS HA as described above or were infected with recombinant virus at an MOI of ~3 PFU per cell. At 16 h posttransfection or 6 h postinfection, cell monolayers were overlaid for 5 min with phosphate-buffered saline with magnesium and calcium (PBS+) that was adjusted to the reported pH with a 0.1 pH unit resolution using 0.1 M citric acid. Cells were neutralized by using DMEM (containing 10% fetal bovine serum and 1% glutamine) and were incubated at 37°C for 2 h. Samples were fixed and stained with a Hema 3 stat pack staining kit (Fisher) according to the manufacturer's instructions. Repre-

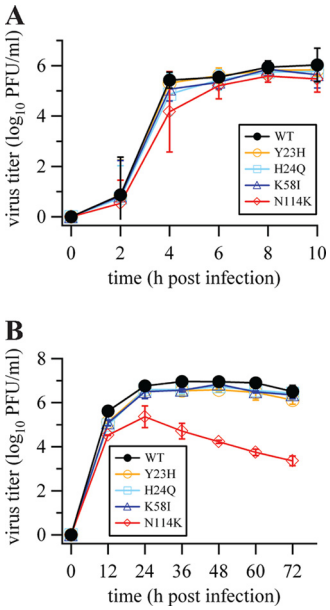

FIG. 1. Replication kinetics of recombinant A/chicken/Vietnam/C58/04 (H5N1) influenza viruses in MDCK cells. (A) For single-step growth curves, cells were infected at an MOI of 3 PFU/cell with wild-type virus or viruses containing HA protein mutation $Y23_1H$, $H24_1Q$, $K58_2I$, or $N114_2K$. (B) For multiple-step growth curves, cells were infected with the recombinant viruses at an MOI of 0.01 PFU/cell. The supernatant was collected at the indicated times, and the virus was quantified by a plaque assay. Each point represents the mean ± standard deviation from three experiments.

sentative microscopic fields were captured with a Nikon D70 digital camera attached to a Nikon Eclipse TS100 inverted microscope (26).

NA activity assay and NA inhibition. A modified fluorometric assay was used to determine the enzymatic activity of the NA protein present in transfected cell lysates with the fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Sigma, St. Louis, MO) (15, 34, 59). The fluorescence of the released 4-methylumbelliferone was measured in a Fluoroskan II spectrophotometer (Labsystems, Helsinki, Finland) using excitation and emission wavelengths of 355 and 460 nm, respectively. The enzymatic activity of NA protein was standardized to 0.1 mg total protein by using a bicinchoninic acid assay (Sigma, St. Louis, MO) and was expressed as the quantity of substrate (in picomoles) converted during a 30-min incubation at 37°C. The NA inhibitor oseltamivir carboxylate ([3*R*,4*R*,5*S*]-4-acetamido-5-amino-3-[1-ethylpropoxy]-1-cyclohexene-1-carboxylic acid) was provided by Hoffmann-La Roche, Ltd. (Basel, Switzerland). The compound was dissolved in distilled water, and aliquots were stored at −20°C until use. NA activity was inhibited by using a 4 μM concentration of oseltamivir carboxylate added immediately posttransfection or 1 h before the assay. The effect of an NA protein inhibitor on the pH of fusion was determined by performing a syncytium formation assay in parallel.

RESULTS

The HA protein mutations have little effect on the *in vitro* replication kinetics of recombinant H5N1 influenza viruses. In a previous study, we identified four mutant H5 HA proteins whose pH values of membrane fusion differed from that of wild-type HA protein when expressed from transiently transfected plasmid DNA (36). Here we determined whether the HA protein mutations affected the *in vitro* replication kinetics of recombinant H5N1 influenza viruses by generating single-step and multiple-step growth curves. Single-step growth curves showed that mutant and wild-type viruses grew at similar rates over the 10-h time course (Fig. 1A). In multiple-step growth curves, viruses containing the HA protein mutations $Y23_1H$, $H24_1Q$, and $K58_2I$ had replication rates similar to that of wild-type virus (Fig. 1B). Titers of the virus containing an $N114_2K$ mutation in the HA protein were similar to those of wild-type virus at the 12-h time point but were later reduced by 1 to 3 $\log_{10}$ units.

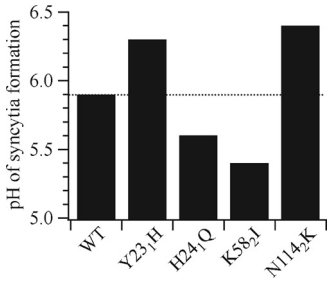

FIG. 2. The pH of HA-mediated membrane fusion by wild-type and mutant H5N1 influenza viruses in Vero cells was measured at 0.1 pH increments and is expressed as the highest pH value at which syncytium formation was observed.

The HA protein mutations alter the pH of membrane fusion *in vitro*. The cell surface expression, cleavage, receptor binding affinities, and membrane fusion efficiencies of the mutant HA proteins in Vero cells were previously found to be similar to those of wild-type virus (36). Moreover, infection of DF-1 primary chicken embryonic fibroblasts with the viruses resulted in HA protein properties similar to those found when Vero cells were infected with the viruses. To determine the effects of the mutations on the pH of membrane fusion, monolayers of MDCK cells were first infected with recombinant H5N1 influenza viruses at an MOI of ~3 PFU/cell and then exposed to PBS solutions of varying pHs, at a resolution of 0.1 unit. The highest pH at which cell-cell membrane fusion was induced in cells infected with wild-type virus was 5.9 (Fig. 2). The $H24_1Q$ and $K58_2I$ mutations reduced the pH of membrane fusion to 5.6 and 5.4, respectively, while the $Y23_1H$ and $N114_2K$ mutations increased the pH of membrane fusion to 6.3 and 6.4, respectively. The $N114_2K$ mutation, which increased the pH of HA activation by 0.5 pH unit, resulted in decreased virus fitness over several cycles of replication *in vitro*, while the other mutations did not alter *in vitro* replication kinetics.

The $N114_2K$ mutation is genetically unstable over multiple passages in eggs. To test the genetic stability of the HA protein mutations, wild-type and mutant viruses were passaged 10 times in 10-day-old embryonated chicken eggs. The sequence identity of each of the passage 1 (P1) recombinant viruses had been confirmed previously (36). Purified viral RNA sampled from allantoic fluid at P5 and P10 was sequenced. In parallel, syncytium formation assays were performed using Vero cells infected with P10 viruses to determine whether repeated passage in eggs resulted in any mutations that might alter the acid stability of the viral HA proteins (Table 1). Wild-type virus and viruses containing the HA protein mutation $H24_1Q$ or $K58_2I$ showed no additional mutations over the course of 10 passages and no change in the pH of membrane fusion. The virus containing the $Y23_1H$ mutation maintained the mutation for at least 5 passages in eggs and acquired an additional HA protein mutation, $R228_1I$, between P5 and P10. Residue R228 (H5 numbering) is located in the receptor-binding pocket of the HA1 subunit with its side chain facing away from the pocket

TABLE 1. Genetic stability of recombinant H5N1 influenza viruses containing HA protein mutations after serial passages in embryonated chicken eggs

P1 virus	ΔpH[a] at P1	Mutation[b] at:		ΔpH at P10
		P5	P10	
Wild type		—	—	
$Y23_1H$	+0.4	—	$R228_1I$	+0.4
$H24_1Q$	−0.3	—	—	−0.3
$K58_2I$	−0.5	—	—	−0.5
$N114_2K$	+0.5	$K114_2N$	$K114_2N$	+0.2

[a] ΔpH, change in the pH of membrane fusion from that of the wild-type virus as measured by a syncytium formation assay.
[b] —, no change in the HA protein sequence from that of the P1 virus. All of the mutations reported are in the HA gene (H5 numbering), and there were no amino acid sequence changes in the other genes.

(46, 57) such that the $R228_1I$ mutation may enhance receptor binding in eggs (56). Despite the extra $R228_1I$ mutation, the P10 virus caused membrane fusion at a pH of 6.3, as did P1 $Y23_1H$ virus without the additional $R228_1I$ mutation. The virus containing the $N114_2K$ mutation in the HA protein was the only recombinant virus whose pH of membrane fusion was altered at P10 from that for the P1 stock virus, a decrease from pH 6.4 to 6.1 (Table 1). Both P5 and P10 $K114_2N$ viruses showed reversion mutations, demonstrating that the $N114_2K$ mutation was not genetically stable and was selected against within 5 passages.

Changes in the pH of activation of the HA protein can alter the environmental stability of H5N1 influenza viruses. The environmental stability of highly pathogenic H5N1 isolates has been found to be lower than that of lower-pathogenicity viruses (1, 2). To determine whether changes in the pH of fusion of the HA protein alter the environmental stability of H5N1 viruses, we incubated the viruses in the present study at 28°C for 8 days and measured the virus titer as a function of time by a plaque assay. Data from each series were plotted, and the gradient of virus degradation was calculated by linear regression analysis (Table 2). The wild-type virus and the virus containing a $Y23_1H$ mutation in the HA protein showed similar rates of titer reduction (1 $\log_{10}$ unit every 10 days), a rate of degradation that matches those of other highly pathogenic H5N1 isolates (2). This result suggests that changes in the pH of fusion as great as +0.4 pH unit can be tolerated without a loss in environmental stability. Viruses containing the $H24_1Q$ or $K58_2I$ mutation, both of which promoted membrane fusion at lower pH values than wild-type virus,

TABLE 2. Environmental stability of H5N1 influenza viruses in water at 28°C

Virus	LRM[a]	R^2	Estimated persistence (days)[b]
Wild type	$6.8742 - 0.0974x$	0.7625	62 (10)
$Y23_1H$	$6.1717 - 0.0991x$	0.7599	61 (10)
$H24_1Q$	$6.9496 - 0.0775x$	0.8364	77 (13)
$K58_2I$	$6.6408 - 0.0761x$	0.7151	79 (13)
$N114_2K$	$5.6297 - 0.4234x$	0.9680	14 (2)

[a] LRM, linear regression model, where y is the virus titer ($\log_{10}$ PFU/ml) and x is persistence (in days).
[b] With a starting virus titer of 1×10^6 PFU/ml. Numbers in parentheses are days required to reduce the initial virus titer by 1 $\log_{10}$ unit.

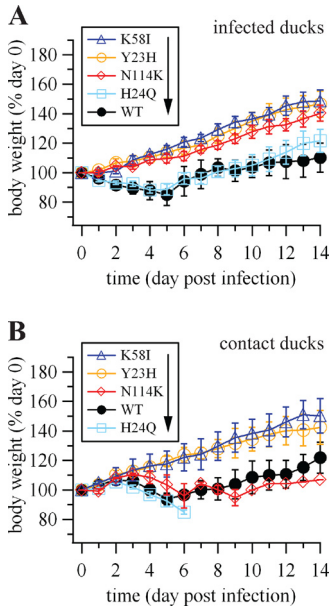

FIG. 3. Weight change in mallards infected with mutant and wild-type H5N1 influenza viruses. (A) Groups of ducks were inoculated with 10^6 EID_{50} of recombinant virus. (B) Contact ducks were introduced into each group's cage 1 day p.i. Ducks were weighed daily for 14 days. Data points (and error bars) represent the mean (± standard deviation) weight changes. Viruses listed in the figure keys are ordered by increasing weight loss.

were calculated to lose 1 $\log_{10}$ unit in their titers every 13 days. Thus, the two mutant viruses with lower pH values of activation retained infectivity longer than the wild-type virus. The virus containing an $N114_2K$ mutation rapidly lost infectivity in the environmental stability experiment, losing 1 $\log_{10}$ unit in its titer approximately every 2 days (Table 2). Therefore, an increase in the pH of HA activation to 6.4 due to the $N114_2K$ mutation resulted in greatly reduced environmental stability, and a decrease in the pH of activation of the HA protein to 5.6 or 5.4 due to the $H24_1Q$ or $K58_2I$ mutation, respectively, moderately increased environmental stability.

The pH of activation of the HA protein contributes to the pathogenicity and transmissibility of H5N1 viruses in mallards. To measure the biological properties of the mutant viruses in mallards, we inoculated duplicate groups of three animals and introduced two contact animals into the cage of each group after 1 day. The wild-type and $H24_1Q$ viruses induced considerable weight loss in both inoculated and contact animals (Fig. 3) and caused death in 60% and 70% of animals, respectively (Table 3). In contrast, the $Y23_1H$ and $K58_2I$ viruses did not induce weight loss or death in either inoculated or contact animals. Moreover, the $Y23_1H$ virus caused only cloudy eyes for 50% of the inoculated ducks, while the $K58_2I$ virus caused cloudy eyes only for one contact duck. While the virus containing an $N114_2K$ mutation in the HA protein did not induce weight loss or death in inoculated ducks, contact animals in this group unexpectedly showed weight loss after 4 days, and three of the four contact animals died. Neurological signs were observed in these contact animals, whereas none were observed in the inoculated group. Because of these unexpected findings, we sequenced viral RNA isolated from pos-

TABLE 3. Morbidity and mortality caused by the recombinant H5N1 influenza viruses in mallards[a]

Virus and infection route	No./total:		
	Dead	With cloudy eyes	With neurological signs[b]
Wild type			
Inoculation	4/6	5/6	3/6
Contact	2/4	4/4	1/4
$Y23_1H$			
Inoculation	0/6	3/6	0/6
Contact	0/4	0/4	0/4
$H24_1Q$			
Inoculation	3/6	4/6	3/6
Contact	4/4	2/4	2/4
$K58_2I$			
Inoculation	0/6	0/6	0/6
Contact	0/4	1/4	0/4
$N114_2K$			
Inoculation	0/6	2/6	0/6
Contact	3/4	2/4	2/4

[a] Data are from two separate experiments. In each, 3 ducks were inoculated with 10^6 EID_{50} of virus and 2 naïve contact birds were introduced into the cage 24 h p.i. Birds were observed daily.

[b] Twitching head, ataxia, violent tremors, severe torticollis, and/or loss of balance.

itive swabs from surviving inoculated and contact birds on days 7 and 10. In all cases, the $N114_2K$ mutation had reverted to the wild-type $N114_2$, as it had after serial passage in eggs (Table 1). This reversion offers the most plausible explanation for the increased transmissibility and pathogenicity in contact birds in the $N114_2K$ group. The reversion mutation also explains the greater weight loss, morbidity, and mortality in the contact birds than in the infected birds in this group. No other reversion mutations were sequenced from swabs of contact ducks infected with the other viruses, including the $H24_1Q$ virus.

To assess replication and transmission potential, titers of virus shed from the trachea and cloaca were measured (Table 4). Inoculated birds in all groups shed virus on day 3; therefore, it is clear that all of the viruses were capable of initial infection and replication. However, even at this early time point, the viruses containing HA protein mutation $Y23_1H$ or $K58_2I$ productively infected fewer ducks than wild-type and $H24_1Q$ viruses. Wild-type and $H24_1Q$ viruses were shed at similar levels on days 3 and 5. On day 7, wild-type virus was not shed, whereas the $H24_1Q$ virus continued to be shed. All contact birds in the wild-type and $HA24_1Q$ groups were shedding virus by day 3 p.i. (100% transmission). All contact birds in the $H24_1Q$ group succumbed to infection, whereas only half of the contact birds in the wild-type group died. The $Y23_1H$ virus was not detected in any contact birds throughout the experiment, showing that the mutation results in attenuated transmission compared to that of the wild-type virus. Five days p.i., the $K58_2I$ virus was detected in one inoculated bird and one contact bird, showing that its fitness and transmissibility were lower than that of the wild-type virus. The $N114_2K$ virus showed inconsistent shedding in inoculated birds. Inoculated

TABLE 4. Tracheal and cloacal shedding of H5N1 influenza viruses by inoculated and contact ducks

Virus	No. of inoculated or contact ducks shedding virus/total no. of ducks (mean titer of shed virus in positive swabs [$\log_{10}$ EID_{50}/ml][a])															
	Day 3 p.i.				Day 5 p.i.				Day 7 p.i.				Day 10 p.i.			
	Inoculated		Contact		Inoculated		Contact		Inoculated		Contact		Inoculated		Contact	
	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca	Trachea	Cloaca
Wild type	6/6 (2.5)	4/6 (1.4)	4/4 (3.1)	4/4 (1.6)	1/4 (<1)	2/4 (2.4)	3/3 (2.2)	2/3 (2.6)	0/2	0/2	1/2 (2.0)	0/2	0/2	0/2	0/2	0/2
$Y23_1H$	4/6 (1.6)	1/6 (1.3)	0/4	0/4	0/6	0/6	0/4	0/4	0/6	0/6	0/4	0/4	0/6	0/6	0/4	0/4
$H24_1Q$	6/6 (3.1)	5/6 (1.9)	4/4 (2.4)	4/4 (2.4)	3/5 (<1)	2/5 (1.6)	4/4 (2.3)	2/4 (1.4)	1/4 (1.5)	2/4 (2.0)	—[b]	—	0/2	0/2	—	—
$K58_2I$	4/6 (2.4)	3/6 (1.4)	1/4 (1.3)	0/4	0/6	1/6 (<1)	1/4 (1.3)	0/4	0/6	0/6	0/4	0/4	0/6	0/6	0/4	0/4
$N114_2K$	5/6 (3.2)	2/6 (3.1)	1/4 (1.8)	1/4 (1.5)	0/6	0/6	3/4 (1.3)	2/4 (2.0)	1/6 (2.5)	3/6 (1.1)	2/2 (2.1)	1/2 (1.8)	0/6	1/6 (1.8)	0/1	0/1

[a] Where swabs were positive but below the threshold of accurate measurement, a value of <1 $\log_{10}$ EID_{50}/ml was recorded.

[b] —, no animals survived.

TABLE 5. Titers of H5N1 influenza viruses in the water dishes of mallards[a]

Virus	Titer (log_{10} EID_{50}) on:				
	Day 1	Day 3	Day 5	Day 7	Day 10
Wild type	0.63	3.25	1.50	0	0
$Y23_1H$	0	0	0	0	0
$H24_1Q$	1.89	3.38	4.25	4.13	0
$K58_2I$	1.38	2.29	0	0	0
$N114_2K$	0	1	0.75	0	0

[a] Data are means from two separate experiments in which 3 ducks per group were inoculated with 10^6 EID_{50} of virus and 2 naïve contact birds were introduced into the cage 24 h p.i. Samples of drinking water (0.5 ml) were collected on days 1, 3, 5, 7, and 10 p.i. for virus titration in eggs.

birds shed virus on day 3 but not on day 5, yet some birds again shed virus on days 7 and 10. This result suggests that transmission to contact birds was mediated by the reverted $K114_2N$ virus, which was then transmitted back to inoculated ducks before being detected on days 7 and 10. On day 3 p.i., tracheal shedding was generally observed more often and at higher titers than cloacal shedding, consistent with previous work (48). The $H24_1Q$ and $K58_2I$ mutations did not appear to increase cloacal virus shedding; thus, small decreases in the pH of activation (and inactivation) of the HA protein may be insufficient to enhance virus replication in the low-pH environment of the duck digestive tract (49).

We also investigated shedding of the recombinant viruses by titrating virus in the ducks' water dishes. Wild-type virus was detected on days 1, 3, and 5 p.i. and had a peak titer of 3.25 log_{10} EID_{50} on day 3 (Table 5). No $Y23_1H$ virus was detected on any day, consistent with low shedding of this virus on day 3 and none on days 5, 7, and 10 p.i. The $K58_2I$ virus titer in the water dishes was comparable to that of the wild-type virus on days 1 and 3 but was subsequently undetectable, consistent with the pattern of virus shedding from the tracheae and cloacae of ducks (Table 4). The presence of the $N114_2K$ virus in water dishes on days 3 and 5 but not on day 1 is consistent with low-level shedding until after reversion. Higher titers of the $H24_1Q$ virus than of wild-type virus were detected in the water dishes on days 1 through 7, consistent with this mutant's greater environmental stability and lethality in contact ducks. Overall, our results show that the reduction of the pH of membrane fusion for the virus containing an $H24_1Q$ HA mutation enhances two properties that could promote H5N1 virus transmission in aquatic birds: shedding of virus into water and persistence of virus infectivity in water.

The properties of H5N1 influenza viruses reported in Fig. 1 to 3 and Tables 1 to 5 are summarized in Table 6.

NA activity promotes pH-mediated membrane fusion induced by the HA protein. The highest pH at which wild-type virus caused membrane fusion was 5.9 (Fig. 2); however, we previously found that wild-type HA protein expressed from transiently transfected plasmid DNA caused membrane fusion only when the pH was decreased to 5.5 (36). The occurrence of this change in the context of virus infection (with expression of all viral proteins) in the present study suggested that one or more of the other viral proteins promote acid-induced activation of the H5N1 HA protein. Previous studies have shown that the NA protein facilitates the entry of H3N2 influenza viruses (28, 32). To determine whether NA protein expression increases the pH of membrane fusion by the HA protein, we transfected Vero cells with the pCAGGS HA wild-type plasmid in the presence and absence of cotransfection with the pCAGGS NA wild-type plasmid. Titration showed that 0.1 μg of plasmid DNA produced neuraminidase activity similar to that in 10 μl of allantoic fluid containing virus (data not shown); therefore, a 1:0.1-μg ratio of HA to NA was used in all follow-up experiments. Transfected cells expressing the wild-

TABLE 6. Summary of properties of H5N1 influenza viruses[a] *in vitro* and in ducks

Mutation by H5 numbering[b]	Mutation by H3 numbering[c]	pH of membrane fusion[d]	*In vitro* growth rate[e]	Genetic stability[f]	Estimated environmental persistence[g]	Weight loss in directly infected ducks[h]	Mortality (%)[i]	Rank order of virus shedding[j]	Days on which virus was detected in ducks' water dishes[k]
$N114_2K$	$N114_2K$	6.4	+	No	14	−			
$Y23_1H$	$Y17_1H$	6.3	+++	Yes	61	−	0	4	None
Wild type		5.9	+++	Yes	62	+	60	2	1, 3, 5
$H24_1Q$	$H18_1Q$	5.6	+++	Yes	77	+	70	1	1, 3, 5, 7
$K58_2I$	$K58_2I$	5.4	+++	Yes	79	−	0	3	1, 3

[a] Recombinant influenza viruses in the background of A/chicken/Vietnam/C58/04 (H5N1).
[b] According to the number in the amino acid sequence of the H5 HA protein. The subscript "1" refers to numbering in HA1, and the subscript "2" refers to numbering in HA2, after cleavage.
[c] The number of the mutation in H5 has been converted to the conventional H3 numbering scheme.
[d] The highest pH at which syncytium formation was observed in Vero cells *in vitro*.
[e] Multiple-step growth rate in MDCK calls after infection with an MOI of 0.01 PFU/cell. Symbols represent a peak titer of ~5 log_{10} PFU/ml (+) or ~7 log_{10} PFU/ml (+++). Detailed data are reported in Fig. 1B.
[f] "No" means that the sequence reverted within 5 serial passages in the allantoic cavities of embryonated chicken eggs. "Yes" means that there were no mutations after 5 passages in eggs and no changes in the pH of membrane fusion after 10 passages in eggs.
[g] Expressed as the calculated number of days of virus persistence at 28°C (starting virus titer, 1×10^6 PFU/ml).
[h] −, continuous weight gain during the 14-day experiment; +, loss of 15 to 20% of the starting weight over the course of the first 5 days of infection.
[i] Calculated for a total of 6 directly infected ducks and 4 contact ducks. Data for the $N114_2K$ virus are excluded because this virus reverted to the wild-type sequence during the experiment.
[j] From tracheal and cloacal swabs taken from both directly infected and contact ducks. Data for the $N114_2K$ virus are excluded because this virus reverted to the wild-type sequence during the experiment. Detailed data are given in Table 4. Numbers are in descending rank order; i.e., 1 represents the highest level of shedding, and 4 represents the lowest.
[k] None, no detectable virus on days 1, 3, 5, 7, and 10. Data for the $N114_2K$ virus are excluded because this virus reverted to the wild-type sequence during the experiment. Detailed data are given in Table 5.

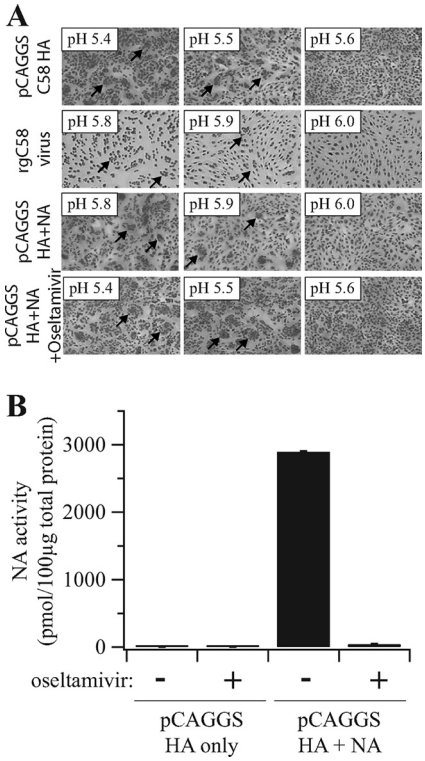

FIG. 4. Contribution of NA enzymatic activity to the pH of membrane fusion mediated by the HA protein. (A) Representative photomicrographs of syncytia showing the contribution of the NA protein to HA-mediated membrane fusion. The pH values are given in the top left corner of each micrograph. The arrows point to examples of syncytia. rgC58, reverse-genetics wild-type C58 strain of H5N1 influenza virus. (B) Mean neuraminidase activity as measured by a fluorescence-based assay using MUNANA as the substrate. Error bars represent the standard deviations from three independent determinations. Oseltamivir carboxylate (4 μM) was used to inhibit the enzymatic activity of the NA protein.

type H5N1 HA and NA surface proteins showed syncytium formation at pH 5.9, the same pH as that for wild-type virus (Fig. 4A). Having established that expression of the NA protein accounted for the observed increase in the pH of HA-mediated membrane fusion, we next examined whether NA enzymatic activity was responsible for the increase. NA enzymatic activity was eliminated in Vero cells cotransfected with pCAGGS HA and NA plasmids by treatment with oseltamivir carboxylate (3, 51) (Fig. 4B). The syncytium formation assay was repeated using cells coexpressing the HA and NA proteins in the presence of oseltamivir carboxylate. When NA enzymatic activity was inhibited by the drug, the pH of HA-mediated membrane fusion decreased to pH 5.5, the same value observed in cells expressing HA protein alone (Fig. 4A). These results are consistent with the promotion of H5 HA-mediated membrane fusion by N1 neuraminidase activity.

DISCUSSION

To investigate how the pH of activation of the HA protein influences the *in vitro* and *in vivo* properties of influenza viruses, we compared four recombinant viruses with altered pH-dependent HA protein stability to wild-type A/chicken/Vietnam/C58/04 (H5N1) virus. An $N114_2K$ mutation in the HA2 fusion peptide pocket region increased the activation pH of the HA protein from 5.9 to 6.4, allowing activation under mildly acidic conditions. This mutation dramatically reduced the fitness of the virus in three ways: (i) multiple-step replication *in vitro* was reduced by a factor greater than 10; (ii) infectivity in the environment decreased four times as rapidly as that of wild-type virus; and (iii) the virus reverted to the wild-type sequence within 5 passages in chicken eggs and after inoculation in mallards. The $N114_2K$ mutation may increase the pH of activation of the HA protein above the threshold pH at which a significant portion of intracellularly cleaved HA trimers become prematurely triggered, and inactivated, during transport to the cell surface (44). The HA protein mutations $Y23_1H$ and $K58_2I$ changed the activation pH of the HA protein to 6.3 and 5.4, respectively. While these two mutations had opposite effects on the activation pH, the recombinant viruses bearing the mutations had similar phenotypes. Despite *in vitro* replication rates similar to that of wild-type virus, the viruses bearing a $Y23_1H$ or $K58_2I$ mutation did not induce weight loss, neurological signs, or mortality in mallards, were not efficiently transmitted, and were shed significantly less. Overall, the data show that efficient and sustainable infection of mallards by H5N1 influenza virus is not supported by HA protein activation pH values less than 5.5 or greater than 6.2.

The results of experiments with the virus bearing an HA-$H24_1Q$ mutation suggest that robust infection in mallards is supported by activation pH values between 5.6 and 5.9. The data also raise the possibility that natural mutations that slightly reduce the pH of activation of the HA protein could increase the transmission of H5N1 influenza viruses among mallards. The wild-type virus and the HA-$H24_1Q$ virus had similar replication kinetics *in vitro* and induced similar weight loss, mortality, clinical signs, and shedding in mallards, but higher titers of the $H24_1Q$ virus were found in the ducks' water dishes, and the $H24_1Q$ virus retained infectivity ~20% longer than wild-type virus in an environmental stability experiment. The fact that all of the contact ducks succumbed to infection with transmitted $H24_1Q$ virus while only half died from transmitted wild-type virus also suggests that contact ducks were exposed to a larger inoculum of the $H24_1Q$ virus.

Our results demonstrate that the pH of activation of the HA protein plays a key role in the pathogenicity and transmissibility of H5N1 influenza viruses in mallards. Natural H5N1 virus isolates are highly pathogenic in many, but not all, duck species (21, 47, 48), and their transmission among wild ducks and from wild ducks to domestic poultry and mammals, including humans, has been a key element in their natural ecology (10, 33, 54). Moreover, wild ducks are thought to be a main reservoir of low-pathogenicity avian influenza viruses (33). The intraspecies and interspecies transmission of influenza viruses depends on at least four factors: (i) the amount of virus shed by the donor, (ii) the stability of the virus in the environment over time, (iii) the time between donor shedding and acceptor exposure, and (iv) the infectivity of the virus in the acceptor animal. Since the pH of activation of the HA protein was found here to determine both the amount of shedding from ducks and the stability of virus in the environment, this molecular property may have an essential role in the propagation of

H5N1 viruses in aquatic birds. Furthermore, HA mutations that maximize virus shedding and environmental stability via altered HA acid stability may be expected to promote both intraspecies and interspecies transmission. A broad survey of the environmental stability of 12 low-pathogenicity avian influenza viruses of various subtypes revealed that they were generally most stable at a slightly basic pH (7.4 to 8.2), a low temperature, and fresh to brackish salinity (1). The viruses lost infectivity much more rapidly after incubation under acidic conditions (pH <6.6), warmer temperatures, and higher salinity. Among the HA mutations characterized in the present study, the $N114_2K$ mutation increased the pH of activation to 6.4 while significantly reducing environmental stability, and the $H24_1Q$ and $K58_2I$ mutations reduced the pH of activation to 5.6 and 5.4, respectively, and moderately increased environmental stability. Thus, the pH of activation of the HA protein contributes to the duration of H5N1 influenza virus infectivity in the environment.

In the present study, an optimal range in the pH of activation of the HA protein supported the propagation of H5N1 influenza viruses in ducks. The adaptation of other subtypes of influenza viruses to different host tissues and species has been found to involve the selection of viruses with altered pH values for membrane fusion. A few passages of egg-grown recombinant X-31 influenza virus (H3N2 with the internal genes of A/PR/8/34 [H1N1]) in mammalian MDCK and Madin-Darby bovine kidney (MDBK) cells consistently resulted in HA protein mutations that increased the pH of HA-mediated membrane fusion from 5.2 to 5.6 to 5.8, and similar results were found after the passage of egg-grown A/Japan/305/57 (H2N2) virus in MDCK cells (25). The natural adaptation of H7N3 influenza viruses from wild ducks to turkeys coincided with two amino acid mutations in and near the HA2 stalk and a decrease in the pH of activation of the HA protein without a change in receptor binding (13). However, it is not known whether these mutations exclusively caused the reduction in the pH of membrane fusion, because the adaptation also resulted in a 23-amino-acid deletion in the NA stalk that reduced neuraminidase activity. We showed here that the absence of neuraminidase activity results in a lower pH of membrane fusion by the HA protein. Moreover, decreased neuraminidase activity in H3N2 influenza viruses has been shown to reduce virus entry (28, 32). In general, there may be a cooperative interaction between the neuraminidase activity of the NA protein and the fusogenicity of the HA protein. A functional balance between neuraminidase activity and the receptor binding activity of the HA protein is well known in many influenza virus subtypes (17, 20, 29, 30, 52).

In influenza viruses of the H3N2, H7N1, and H7N7 subtypes, an increase in the pH of activation of the HA protein results in resistance to high concentrations of amantadine (>0.1 mM), which raise the endosomal pH (6, 9, 18, 44). In a recombinant virus bearing the envelope glycoproteins of A/Netherlands/219/03 (H7N7), an $HA\text{-}G23_2C$ mutation in the fusion peptide that reduced the pH of membrane fusion from 5.4 to 4.4 reduced *in vitro* replication by more than 2 $\log_{10}$ units and increased the 50% mouse lethal dose by more than 3 $\log_{10}$ units (18). Thus, in mammalian species there may also be an optimal range of HA protein activation pHs that supports efficient virus replication, infection, and pathogenicity. Since high- and low-pathogenicity influenza viruses differ in their tissue tropism in avian and mammalian species (24, 27), the optimum pH values at which their HA proteins are activated to support successful infection and transmission may differ according to the influenza virus and the host species. Future investigation of the biological properties of the recombinant viruses from the present study with mouse and ferret models may reveal whether changes in the pH of activation of the HA protein support the adaptation and transmission of H5N1 influenza viruses in mammalian species, which are significant factors in the pandemic potential of these viruses.

ACKNOWLEDGMENTS

We thank Scott Krauss, Heather Forrest, David Carey, and Sharon Lokey for technical support in the BSL3+ facility. We thank Robert Lamb for the pCAGGS plasmid. We thank Rebecca DuBois and Stephen White for helpful discussions on protein structure and Sharon Naron for editing the manuscript. We thank the Hartwell Center for Bioinformatics & Biotechnology at St. Jude Children's Research Hospital for DNA sequencing.

This project was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract HHSN266200700005C, and by the American Lebanese Syrian Associated Charities (ALSAC).

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