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Molecular Determinants within the Surface Proteins Involved in the Pathogenicity of H5N1 Influenza Viruses in Chickens

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Although it is established that the cleavage site and glycosylation patterns in the hemagglutinin (HA) play important roles in determining the pathogenicity of H5 avian influenza viruses, some viruses exist that are not highly pathogenic despite possessing the known characteristics of high pathogenicity (i.e., their HA contains multiple basic amino acids at the cleavage site and has glycosylation patterns similar to that of the highly pathogenic H5 viruses). Currently little is known about the H5N1 viruses that fall into this intermediate category of pathogenicity. We have identified strains of H5N1 avian influenza viruses that have markers typical of high pathogenicity but distinctly differ in their ability to cause disease and death in chickens. By analyzing viruses constructed by reverse-genetic methods and containing recombinant HAs, we established that amino acids 97, 108, 126, 138, 212, and 217 of HA, in addition to those within the cleavage site, affect pathogenicity. Further investigation revealed that an additional glycosylation site within the neuraminidase (NA) protein globular head contributed to the high virulence of the H5N1 virus. Our findings are in agreement with previous observations that suggest that the activities of the HA and NA proteins are functionally linked.

Highly pathogenic (HP) avian influenza (HPAI) viruses, which are restricted to H5 and H7 subtypes, are capable of causing severe respiratory disease and mortality as high as 100% in infected chicken flocks. Although HPAI viruses are primarily of major concern to the poultry industry, because they cause severe economic losses, they have also become a human health concern because of their ability to transmit directly to humans.

The pathogenicity of avian influenza viruses is polygenic. Numerous studies evaluating reassortant viruses indicate that pathogenicity depends on the functional integrity of each gene and on a gene constellation that is optimal for infection (6, 22, 28). Studies of pathogenicity showed that the optimal combination of hemagglutinin (HA) and neuraminidase (NA) enabling the cleavage of HA was important. Webster et al. (37) showed, in the avian system, that viral reassortants that were pathogenic for chickens suggested a correlation between pathogenicity and the surface composition of avian influenza viruses.

HA is the surface glycoprotein through which virus particles bind to cell surface receptors containing sialic acid (40). The HA is synthesized as a polyprotein precursor (HA0) that is posttranslationally cleaved into two subunits. This cleavage step is necessary for virus infectivity (13, 15). Major factors affecting tissue tropism, systemic spread, and pathogenicity of avian influenza viruses are the amino acids at the cleavage site of HA0 and the distribution of proteases in the host (reviewed in reference 32). The acquisition of virulence in the field correlates with changes in glycosylation patterns of HA (3) and with addition of polybasic amino acids at the HA cleavage site (11, 24, 31). The presence of polybasic amino acids at the cleavage site of HA is characteristic of HP influenza A viruses of the H5 and H7 subtypes. This polybasic amino acid region is the target of not only trypsin-like proteases but also intracellular proteases, such as furin, which enable systemic spread of the virus and thereby increase its virulence.

Several studies indicate that the NA plays some role in pathogenicity (21, 27, 28, 38). The NA protein facilitates the mobility of virions by removing sialic acid residues from the viral HA during entry and release from cells (4, 14, 23). Virus particles with low NA activity cannot be efficiently released from infected cells (17, 19, 23). A balance in HA and NA activities is crucial: there must be enough HA activity to facilitate virus binding and enough NA activity to allow release of virus progeny (20). Studies showing a link between pathogenicity of influenza viruses and NA have been done mostly on the A/WSN/33 (H1N1) virus. Goto et al. (7) have shown that plasminogen-binding activity of the WSN NA is an important determinant of the pathogenicity of this virus in mice. They showed that greater NA activity results in higher HA cleavage in multiple organs, thereby enhancing virulence, specifically neurovirulence in mice (16).

The severity of disease in chickens infected with avian influenza virus generally ranges from asymptomatic infection to fatal systemic infection. Some viruses cause disease of intermediate severity with systemic infection but relatively low mortality. Currently, little is known about these viruses. Although it is established, as mentioned above, that the nature of the HA cleavage site plays an important role in determining pathogenicity, there are viruses with multiple basic amino acids at the HA cleavage site that are not always HP. Therefore, other determinants of pathogenicity are likely to exist in viruses of intermediate pathogenicity. For example, both the virulent and

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avirulent H5N2 viruses isolated during the Pennsylvanian outbreak in 1983 had identical amino acid sequences at the HA cleavage site and two other amino acids within HA were associated with the acquisition of virulence (39).

Although different strains of HPAI virus are known to have substantially different pathotypes, the effects of specific amino acid changes on the pathogenicity of the HPAI H5N1 viruses in chickens remains largely unexplored. We therefore exploited a powerful new technique for rescuing infectious influenza A virus from cloned cDNA (using an eight-plasmid DNA transfection system) (10) to identify the molecular determinants of pathogenicity of two HPAI viruses, A/chicken/Hong Kong/YU562/01 (H5N1) and A/goose/Hong Kong/437-10/99 (H5N1). These viruses have different pathotypes despite both possessing an HA glycoprotein with polybasic amino acids at the cleavage site and identical glycosylation patterns. Analysis of these HPAI H5 viruses established that amino acid differences in the viral surface proteins play a significant role in pathogenicity. Whereas the HA protein had the greater effect on pathogenicity, the NA protein also contributes to modulate pathogenicity. Using rescued viruses bearing recombinant HA and NA proteins we showed that amino acids in the globular head of HA, in addition to those within the cleavage site, and a glycosylation site in NA play a major role in pathogenicity.

MATERIALS AND METHODS

Viruses. The H5N1 influenza A viruses used in this study were A/chicken/Hong Kong/YU562/01 and A/goose/Hong Kong/437-10/99. Viruses were kindly provided by the surveillance team of the University of Hong Kong and stored in the repository of St. Jude Children's Research Hospital. The H5N1 viruses were propagated in 10-day-old embryonated chickens' eggs and handled at St. Jude Children's Research Hospital in biosafety level 3+ facilities approved by the U.S. Department of Agriculture and Centers for Disease Control and Prevention. The complete genome sequence data for both of the viruses used in these studies are available on the Influenza Sequence database at www.flu.lanl.gov.

Cloning of viral segments and recombinant HA and NA genes. Influenza virus RNA was isolated by using the RNeasy kit (QIAGEN) and transcribed to cDNA by using the Uni12 primer (AGCAAAAGCAGG). The cDNA was amplified by using segment-specific primers (9). The eight gene segments of the A/chicken/Hong Kong/YU562/01 (H5N1) virus and the HA and NA genes of the A/goose/Hong Kong/437-10/99 (H5N1) virus were cloned by digesting the PCR products with BsmBI or BsaI and ligating them into the cloning vector pDP2002. The eight plasmids containing the full-length cDNA of the A/chicken/Hong Kong/YU562/01 (H5N1) virus were designated pDPPB2YU562, pDPPB1YU562, pDPPAYU562, pDPHAYU562, pDPNPYU562, pDPNAYU562, pDPMYU562, and pDPNSYU562. The HA and NA plasmids of the A/goose/Hong Kong/437-10/99 (H5N1) virus were designated pDPHA437-10 and pDPNA437-10. To ensure that the cloned genes were identical to the template RNA, the inserted viral cDNA and template cDNA were sequenced by the Center for Biotechnology at St. Jude Children's Research Hospital by using rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems, Inc., Foster City, Calif.) and synthetic oligonucleotides. The amino acid sequences of the HA proteins of each virus were aligned with that of A/Duck/Singapore/3/97 (H5N1) and numbered on the basis of the X-ray crystallographic structure (8).

Plasmids encoding recombinant HA molecules from the H5 viruses were created by using various restriction enzymes and by religating fragments from plasmids pDPHAYU562 and pDPHA437-10. The plasmids encoding the recombinant rHA1 and rHA2 molecules were generated by digestion with EcoRI and BglII. These recombinant HA molecules created five amino acid changes. Plasmids rHA3 and rHA4 were created by digestion with BglII and BsmI, which generated two amino acid changes. The recombinant plasmids rHA5 and rHA6 were generated by digestion with BsmI and PstI, which created one amino acid mutation at the polybasic cleavage site of HA.

Various fragments obtained by restriction enzyme digestion of plasmids pDPNAYU562 and pDPNA437-10 were combined and religated to create amino acid changes within the NA molecules of A/chicken/Hong Kong/YU562/01 and A/goose/Hong Kong/437-10/99. Plasmids rNA1 and rNA2 were created by digestion with SalI and MunI; rNA4 was generated by digestion with SwaI and MscI, which resulted in five amino acid changes in the NA of A/goose/HK/437-10/99, including the addition of a potential glycosylation site; and rNA5 and rNA6 were created by using the Quick-Change site-directed mutagenesis kit (Stratagene) to remove (rNA5) or create (rNA6) a potential glycosylation site in the NA. The mutations were achieved by using the following specific sets of primers: for rNA5, forward primer 5′GAACGGACAGTAGTTTTTCGGTGA AGCAAGATATC3′ and reverse primer 5′GATATCTTGCTTCACCGAAAA ACTACTGTCCGTTC3′; for rNA6, forward primer 5′GAACGGACAGTAAC TTCTCGCTGAAGCAAGATATC3′ and reverse primer 5′GATATCTTGCTT CAGCGAGAAGTTACTGTCCGTTC3′

Generation of reverse-genetic reassortant viruses. Reassortant viruses were generated by DNA transfection as described previously (10). Briefly, 293T and MDCK cells were cocultured at a concentration of 0.2×10^6 to 1×10^6 cells of each cell line. The cocultured cells were transfected with 1 μg of each of the eight plasmids and 18 μl of transit LTI (Panvera, Madison, Wis.) in a total volume of 1 ml of OPTIMEM-I (Gibco, Grand Island, N.Y.). The DNA-lipid complexes were removed after 6 h, and fresh medium was added to the cell cultures. The cells were incubated for an additional 24 h, and then 0.5-μg/ml TPCK-treated trypsin (Worthington) was added. After a total of 72 h of incubation, the supernatant was removed and 100 μl was injected into the allantoic cavity of 10-day-old embryonated chicken eggs. After 48 h, the allantoic fluid was harvested, RNA was extracted and analyzed by reverse transcription-PCR, and each viral segment was partially sequenced to confirm the identity of the reassortant virus.

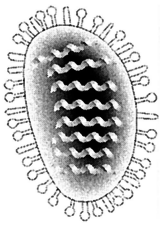
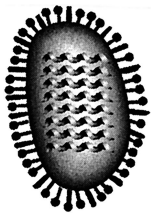
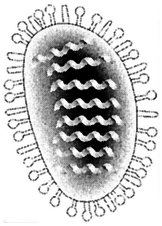
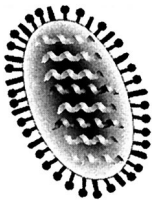
IVPI. The intravenous virus pathogenicity index (IVPI) was performed in duplicate and determined by the method described by Capua and Mutinelli (2). Infective allantoic fluid was diluted 1:10 in sterile phosphate-buffered saline. The diluted virus (0.1 ml) was injected intravenously into each of 10 six-week-old specific-pathogen-free chickens. The chickens were examined for clinical signs of viral disease every 24 h over a 10-day period. The birds received a score of 0 if they appeared normal, 1 if they were sick, 2 if they were severely sick, and 3 if they were dead. Chickens were classified as being sick if they displayed at least one clinical sign, such as depression; reluctance to move; tremors of the head; paralysis of the wings; incoordination of leg movements; cyanosis of the comb and wattles; or petechial hemorrhages on the legs, comb, or wattles. Chickens were classified as severely sick if they displayed more than one clinical sign. The index was then calculated as the mean score per bird per observation. An index of 3.00 indicates that all the birds died within 24 h, and an index of 0.00 means that no bird showed any clinical sign during the 10-day observation period.

Characterization of reverse-genetic viruses. Reverse-genetic viruses were injected intravenously at a 50% egg infectious dose (EID_{50}) of $10^{0.5}$ into seven 6-week-old specific-pathogen-free chickens. Starting 1 day after infection, one randomly selected chicken from each group was euthanized and its organs (brain, thymus, liver, spleen, pancreas, kidney, lung, and bursa of Fabricius) were harvested for determination of viral titer. The tissue samples (1 g/ml) were homogenized in sterile phosphate-buffered saline. Several dilutions of tissue homogenates were injected into the allantoic cavities of 10-day-old embryonated chicken eggs, and the EID_{50} was determined by the method of Reed and Muench (26).

Assays for CLD_{50} and CID_{50}. Six-week-old specific-pathogen-free chickens were inoculated intravenously with 10-fold dilutions of each virus. Each dilution was administered to four chickens, and the dilutions ranged from 10^7 to 10^{-2} EID_{50}. The chickens were observed over a 10-day period, and the number of chickens that died each day was recorded. On days 3 and 5 after infection, tracheal and cloacal swabs were taken and placed in isolation medium. Then 0.1 ml of swab medium was injected into 10-day-old embryonated chicken eggs, and the eggs were incubated for 48 h. The chicken eggs were then tested for virus by hemagglutination assay. The 50% chicken infectious dose (CID_{50}) was determined from the number of chickens having a positive swab (tracheal or cloacal) and was calculated according to the method of Reed and Muench (26).

Statistical analysis of CLD_{50}. To compare the 50% chicken lethal dose (CLD_{50}) values between the reverse-genetic viruses containing the recombinant HA or NA genes to the parental virus, we compared the hazards ratio between the viruses. We then performed Cox regression analyses to compare the hazards ratio for the specific comparison pairs. Only the two viruses in comparison are included in each model. In the regression, we included two predictors: a 10-based log of dilution as a continuous predictor and virus as the other predictor. Because there are many ties in death date among the chickens, Breslow's method is no longer appropriate to handle the tied situation (5, 12). Instead, we used the Exact method (1).

TABLE 1. IVPI and number of deaths after infection of chickens with wild-type and reverse-genetic viruses

Group[a]	Structure	IVPI[b]	Mortality (no. dead/no. infected) at[c]:	
			2 dpi	10 dpi
wt HP		3.00	8/10	10/10
wt MP		1.50	0/10	5/10
rg HP		2.88	6/10	10/10
rg MP HA/MP NA × HP int.		1.66	0/10	6/10

[a] wt, wild-type virus; rg, reverse-genetic virus; int., internal proteins; HP, highly pathogenic A/chicken/HK/YU562/01 (H5N1); MP, moderately pathogenic A/goose/HK/437-10/99 (H5N1).

[b] Ten 6-week-old specific-pathogen-free chickens were intravenously inoculated with a 1:10 dilution of the indicated virus. Chickens were observed over a 10-day period, and each bird was given a daily score of 0 if it appeared normal, 1 if it was sick, 2 if it was severely sick, or 3 if it was dead. The score was then calculated as the mean score per bird per observation.

[c] dpi, days postinfection.

RESULTS

H5N1 viruses differ in pathogenicity. The two HPAI H5 viruses we examined had markedly different IVPIs of 3.00 (A/chicken/Hong Kong/YU562/01) and 1.50 (A/goose/Hong Kong/437-10) (Table 1). This result indicates that the chicken H5N1 virus is more pathogenic than the goose H5N1 virus in chickens. Therefore, we designated the A/chicken/Hong Kong/YU562/01 (H5N1) virus as HP and the A/goose/Hong Kong/437-10/99 (H5N1) virus as "moderately pathogenic" (MP). The complete viral genomes of the two viruses were sequenced and compared. The following amino acid differences were observed between the HP and MP viruses: PB2 12, PB1 14, PA 16, HA 7, NP 5, NA 23, M 9, and NS 15. We also determined IVPIs for reverse-genetic reassortants of the HP virus bearing both surface proteins of the MP virus and for the reverse-genetic wild-type HP virus (Table 1). Infection with the parental HP virus and the reverse-genetic HP virus resulted in similar IVPIs and mortality patterns (Table 1). The IVPI and mortality pattern arising from infection with the reverse-genetic reassortant virus were similar to those observed for the wild-type MP virus. While there are several amino acid differences between these two viruses within the internal proteins, this finding indicates that the surface proteins of these H5N1 viruses are the major modulators of pathogenicity in chickens.

The pathotype of H5 viruses is modulated by the action of HA and NA. The HA proteins of the MP and HP viruses differ by only seven amino acids. The NA proteins of the two viruses differ by 23 amino acids, including 2 that create a potential glycosylation site on the NA of the HP virus, which is absent from the NA of the MP virus. To evaluate the contribution to pathogenicity of these different amino acid changes, we generated single-gene reassortants of the HP virus bearing either the HA or the NA of the MP virus and the other seven genes from the HP virus. We determined the CLD_{50} and CID_{50} of these single-gene reassortant viruses and those of the two-gene (HA and NA) or double-reassortant virus described above. The results showed (Fig. 1) that changes in both HA and NA affect the pathogenicity of these H5N1 viruses. Results obtained from infection with the single-gene reassortant viruses suggest that HA is the main determinant of pathogenesis, and its activity can be modulated by NA. The complete HP virus had a CLD_{50} of $10^{0.1}$ EID_{50}, and the single-gene reassortant having the HA of the HP virus and the NA of the MP virus had a slightly higher CLD_{50} of $10^{0.67}$ EID_{50}. These two viruses were clearly more pathogenic than the single-gene reassortant bearing the HA of the MP virus ($CLD_{50} = 10^{1.77}$ EID_{50}) and the double-reassortant bearing the HA and NA of the MP virus ($CLD_{50} = 10^{3.64}$ EID_{50}). A similar effect was observed during the progression and outcome of disease (Fig. 2). Chickens infected with the reverse-genetic HP virus had the lowest rate of survival: the first deaths occurred on day 2 after infection, although some chickens lived until day 4 after infection. Chickens infected with the single-gene reassortant of the HP virus bearing the NA of the MP virus also died on days 2 through 4 after infection. In contrast, chickens infected with the single-gene reassortant bearing the HA of the MP virus had a high survival rate and lived until day 5 after infection. Chickens infected with the same dose of the double-reassortant virus had a 100% survival rate. These results show that the HA and NA proteins both play a role in survival after infection, in which the HA protein is the major pathogenic determinant and the NA protein modulates disease progression.

To determine whether differences in pathogenicity were due to differences in tissue tropism, we determined virus titers in various organs after infection with the reverse-genetic viruses (Table 2). Viral load in the brain, thymus, liver, spleen, pancreas, kidney, and bursa of Fabricius was high at day 1 after infection for all of the reassortant viruses, which suggests that different pathotypes are not explained by differences in tissue tropism or differences in the ability to spread. Therefore, it was not surprising that the CID_{50} values for the four viruses were almost identical (data not shown). Although viral titers in the lungs at day 1 after infection were highest, by a factor of 10, in

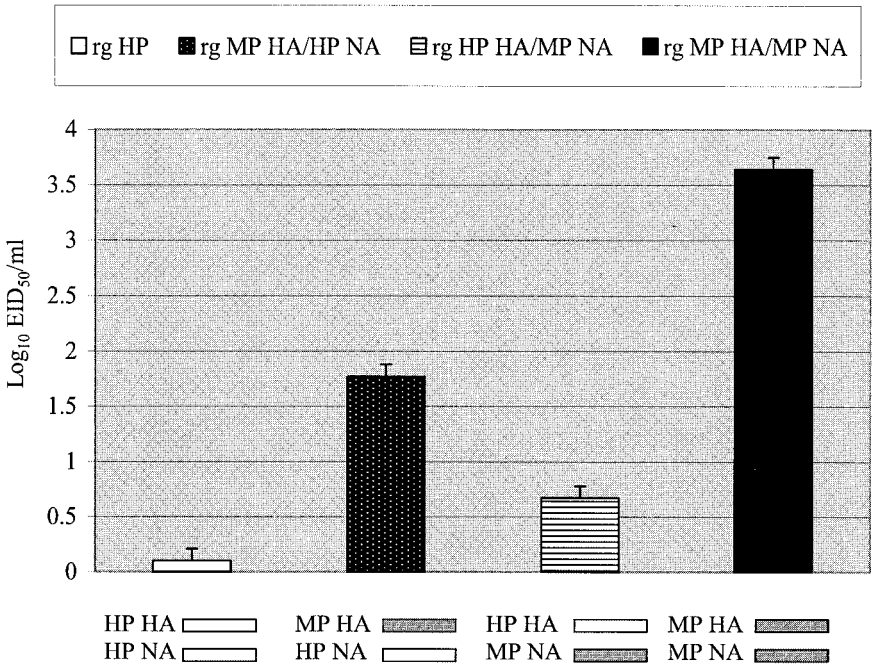


FIG. 1. Pathogenicity, as indicated by CLD_{50} (log_{10} EID_{50} per milliliter), of reverse-genetic (rg) reassortant viruses in chickens. The reverse-genetic viruses bear the surface proteins of the HP or MP viruses and contain the internal proteins of the HP virus.

chickens infected with viruses bearing the HA gene of the HP virus, by day 2 after infection, the lung titers of all four groups were similar. We also observed slightly higher viral titers in the thymus and bursa of Fabricius in chickens infected with viruses bearing the HA of the HP virus. High titers within these primary lymphoid organs may explain why chickens in these groups died sooner. In addition, chickens infected with the single-gene reassortant of the HP virus bearing the HA of the MP virus (and, therefore, the NA of the HP virus) continued to have virus in the lungs 6 days after infection, whereas the double reassortant (HA and NA of the MP virus) was cleared from the lungs of infected chickens by day 5 after infection. Therefore, it appears that the NA of the HP virus modulates pathogenicity by enabling the virus to avoid the host's clearance mechanisms, which, in turn, facilitates virus replication.

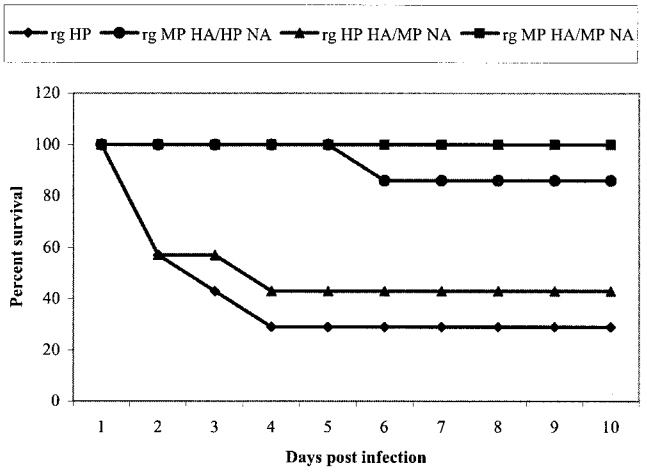


FIG. 2. Survival curve after intravenous injection of chickens with $10^{0.5}$ EID_{50}/ml of reverse-genetic (rg) viruses. The reverse-genetic viruses bear the surface proteins of the HP or MP viruses and contain the internal proteins of the HP virus.

Amino acid differences in the globular head and at the cleavage site of HA have a role in pathogenicity. To determine the overall effect of amino acid differences in the HA protein on differences in pathogenicity, we constructed recombinant HA proteins with different combinations of the HA sequences observed in the HP and MP viruses. Recombinant plasmids derived from the HA of the HP virus encoded four (rHA1), two (rHA3), and one (rHA5) of the amino acid substitutions found in the HA sequence of the MP virus. We generated reverse-genetic viruses bearing the recombinant HA proteins and having the NA and internal genes of the HP virus and compared their CLD_{50} values with those of the reverse-genetic HP virus (Fig. 3). Virus bearing HA encoded by rHA1 was less pathogenic ($CLD_{50} = 10^{0.67}$ EID_{50}) than the reverse-genetic wild-type HP virus. The pathogenicity of the virus bearing the HA encoded by rHA3 was similar to that of the reverse-genetic wild-type HP virus. The virus in which the HA contained the single-amino-acid substitution E338K (i.e., Glu338 to Lys; plasmid rHA5) was, surprisingly, less pathogenic than the reverse-genetic wild-type HP virus: it had a $CLD_{50} = 10^{0.5}$ EID_{50}. We had expected that a change from an acidic amino acid (glutamic acid) to a basic one (lysine) in the polybasic region of the HA cleavage site would result in higher rather than lower pathogenicity.

To determine whether amino acids substituted in the HA of the MP virus had the same effect as substitutions into the HA of the HP virus, we generated recombinant HA proteins (rHA2, rHA4, and rHA6) from the HA of the MP virus. We

TABLE 2. Virus titers of reverse genetic viruses in various organs of chickens

Day after infection	Virus[a]	Viral titers in organs harvested from infected chickens (log_{10} EID_{50}/ml)[b]							
		Brain	Thymus	Liver	Spleen	Pancreas	Kidney	Lung	Bursa of Fabricius
1	rg HP	3.75	4.25	2.50	4.75	3.75	3.25	6.25	4.50
	rg HA^{MP}, NA^{HP}	3.50	3.25	2.50	4.50	3.50	2.75	4.50	4.00
	rg HA^{HP}, NA^{MP}	3.75	4.25	2.50	4.75	3.50	3.00	6.00	4.50
	rg HA^{MP}, NA^{MP}	3.50	2.50	2.00	4.50	3.50	2.75	4.75	3.75
2	rg HP	5.00	4.25	2.00	2.25	1.00	3.25	6.25	4.50
	rg HA^{MP}, NA^{HP}	3.50	3.50	<	<	<	1.00	5.00	3.50
	rg HA^{HP}, NA^{MP}	4.75	3.50	<	<	1.00	4.50	5.25	4.75
	rg HA^{MP}, NA^{MP}	3.75	3.75	1.00	<	<	<	5.25	3.75
3[c]	rg HA^{MP}, NA^{HP}	2.00	1.50	1.00	2.50	1.00	2.25	4.75	3.50
	rg HA^{HP}, NA^{MP}	4.00	3.75	<	<	<	3.25	4.25	4.25
	rg HA^{MP}, NA^{MP}	1.50	1.00	<	<	<	<	3.50	2.00
4[d]	rg HA^{MP}, NA^{HP}	1.00	1.00	<	<	<	<	4.00	3.00
	rg HA^{MP}, NA^{MP}	1.00	<	<	1.00	<	1.00	2.50	2.25
5	rg HA^{MP}, NA^{HP}	<	<	<	<	<	<	4.75	<
	rg HA^{MP}, NA^{MP}	<	<	<	<	<	<	<	<
6[e]	rg HA^{MP}, NA^{HP}	<	<	<	<	<	<	2.25	<
	rg HA^{MP}, NA^{MP}	<	<	<	<	<	<	<	<

[a] Chickens were infected intravenously with $10^{0.5}$ EID_{50} of the virus listed. rg, reverse-genetic virus; HP, highly pathogenic A/chicken/Hong Kong/YU562/01 (H5N1); MP, moderately pathogenic A/goose/Hong Kong/437-10/99 (H5N1). All viruses contain the internal proteins (PB2, PB1, PA, NP, M, and NS) of the HP virus.

[b] Titers displayed are the log_{10} EID_{50} per milliliter of tissue homogenate. <, titer below level of detection.

[c] All of the chickens infected with the reverse-genetic HP virus were dead by day 3 after infection.

[d] All of the chickens infected with the reverse-genetic HA^{HP}, NA^{MP} virus were dead by day 4 after infection.

[e] One chicken infected with the reverse-genetic HA^{MP}, NA^{HP} virus died on day 5 after infection, leaving no chickens for sampling of organs on day 7 after infection. No virus was detected in chickens inoculated with the HA^{MP}, NA^{MP} virus on day 7 after infection.

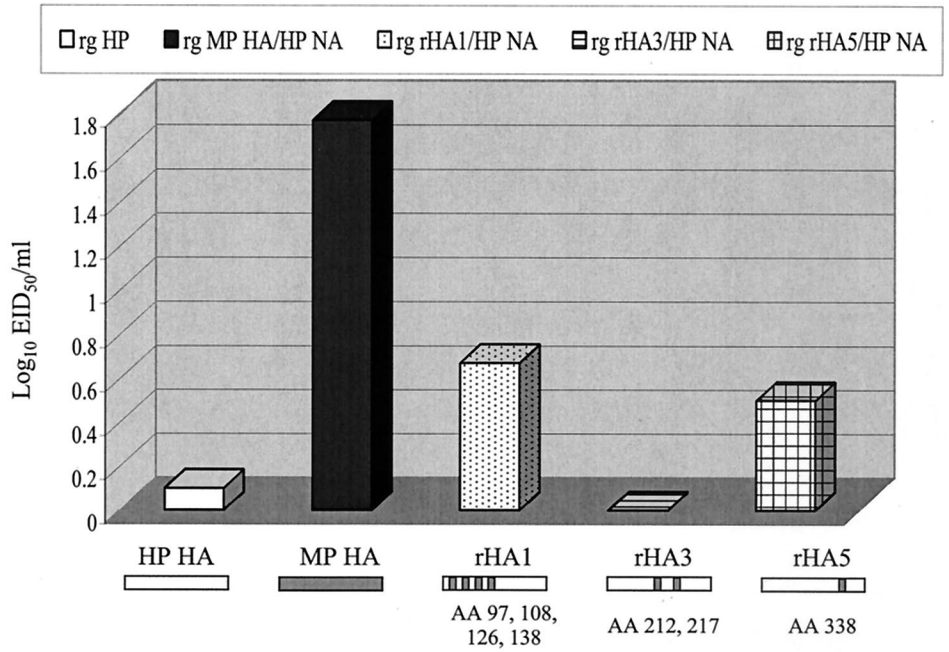


FIG. 3. Pathogenicity, as determined by CLD_{50}, of reverse-genetic (rg) reassortant viruses bearing recombinant HA, from the HP parental virus, in chickens. The reverse-genetic viruses have the NA and internal proteins of the HP virus and bear HA from the HP virus, HA from the MP virus, or recombinant HA encoded by plasmid rHA1, rHA3, or rHA5. AA, amino acids.

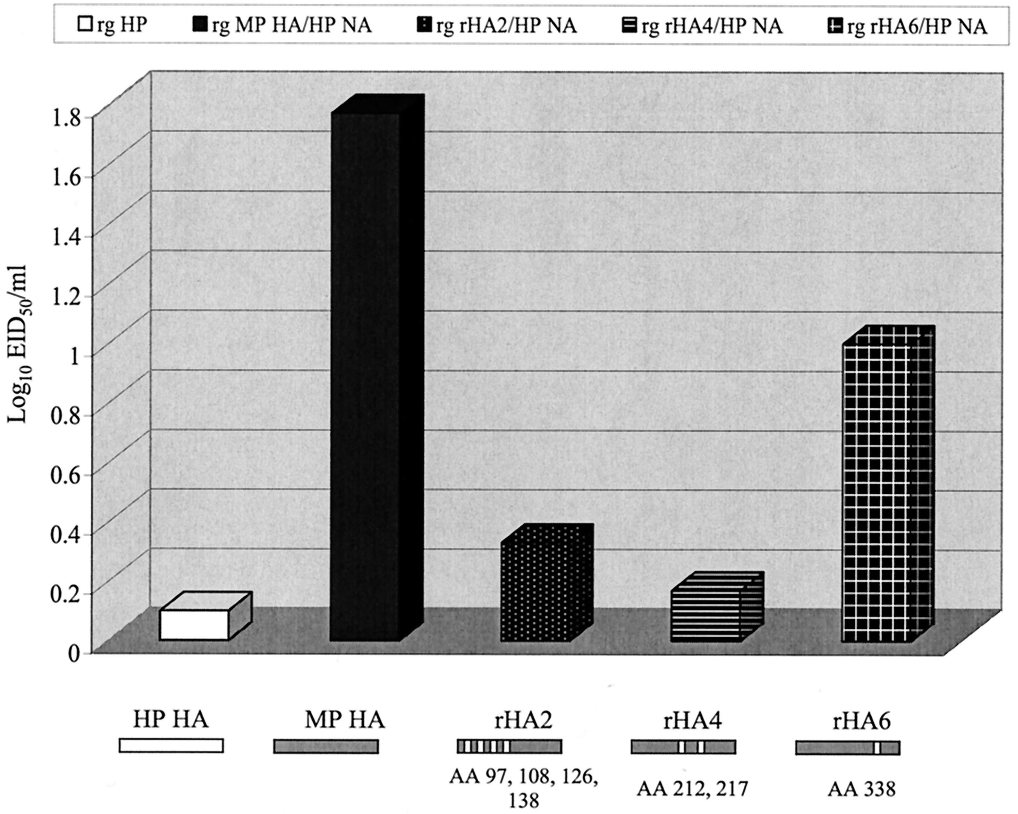


FIG. 4. Pathogenicity, as determined by CLD_{50}, of reverse-genetic (rg) reassortant viruses bearing recombinant HA, from the MP parental virus, in chickens. The reverse-genetic viruses have the NA and internal proteins of the HP virus and bear recombinant HA encoded by plasmid rHA2, rHA4, or rHA6. AA, amino acids.

then used reverse genetics to produce viruses bearing the recombinant HA proteins of the MP virus and having the NA and internal genes of the HP virus. We compared the CLD_{50} values determined for these viruses with that obtained for the single-gene reassortant bearing the HA of the MP virus and the NA of the HP virus (Fig. 4). The virus bearing HA encoded by rHA2 had a CLD_{50} ($10^{0.33}$ EID_{50}) that was lower than that of the single-gene reassortant bearing the HA of the MP virus and the NA of the HP virus ($CLD_{50} = 10^{1.77}$ EID_{50}). The virus bearing HA encoded by rHA4 had a markedly smaller CLD_{50} ($10^{0.17}$ EID_{50}) than that of the single-gene reassortant. Its pathogenicity was similar to that of the reverse-genetic wild-type HP virus. The substitutions encoded by rHA4, E212K, and P217S therefore resulted in increased pathogenicity, which suggests that these amino acid positions may be key determinants of pathogenicity. However, the effect was unidirectional: the virus bearing HA encoded by rHA3 (essentially the HP virus bearing HA with two amino acid substitutions, K212E and S217P) maintained the highly pathogenic phenotype.

Interestingly, amino acid replacement at the cleavage site (K338E) also reduced the CLD_{50} (10^{1} versus $10^{1.77}$ EID_{50}), making the rHA6-carrying virus more pathogenic than the single-gene reassortant virus bearing the HA of the MP virus. In summary, all substitutions within the HA of the MP virus resulted in increased pathogenicity.

A glycosylation site on the globular head of NA modulates pathogenicity. We generated several recombinant NA genes to determine which amino acids contributed to the pathogenicity of these H5N1 viruses. Because there are 23 amino acid differences between the two NAs, the first recombinant NAs we generated were made to determine which general region of the protein was important in pathogenicity. Plasmid rNA1 contained sequences encoding the hydrophobic stalk region of the NA of the HP virus and the globular head from the MP virus; plasmid rNA2, conversely, encoded the hydrophobic stalk region of the NA of the MP virus and the globular head from the HP virus. We generated reverse-genetic viruses bearing these recombinant NAs and having the HA and internal genes of the HP virus, and we compared their CLD_{50} values with those of the reverse-genetic wild-type HP virus and the single-gene reassortant bearing the HA of the HP virus and the NA of the MP virus (Fig. 5). The reassortant virus bearing NA encoded by rNA1 had a CLD_{50} ($10^{0.67}$ EID_{50}) equal to that of the single-gene reassortant bearing the NA of the MP virus ($10^{0.67}$ EID_{50}). The reassortant virus bearing the NA encoded by rNA2 had a CLD_{50} equal to that of the reverse-genetic wild-type HP virus ($10^{0.1}$ EID_{50}). These findings indicate that key amino acids within the globular head of NA are responsible for our observed differences in pathogenicity between the reverse-genetic wild-type HP virus and single-gene reassortant viruses bearing the NA of the MP virus.

Sequence analysis of the NA molecules of the two original wild-type viruses revealed the presence of an additional glycosylation site within the globular head of the NA of the HP

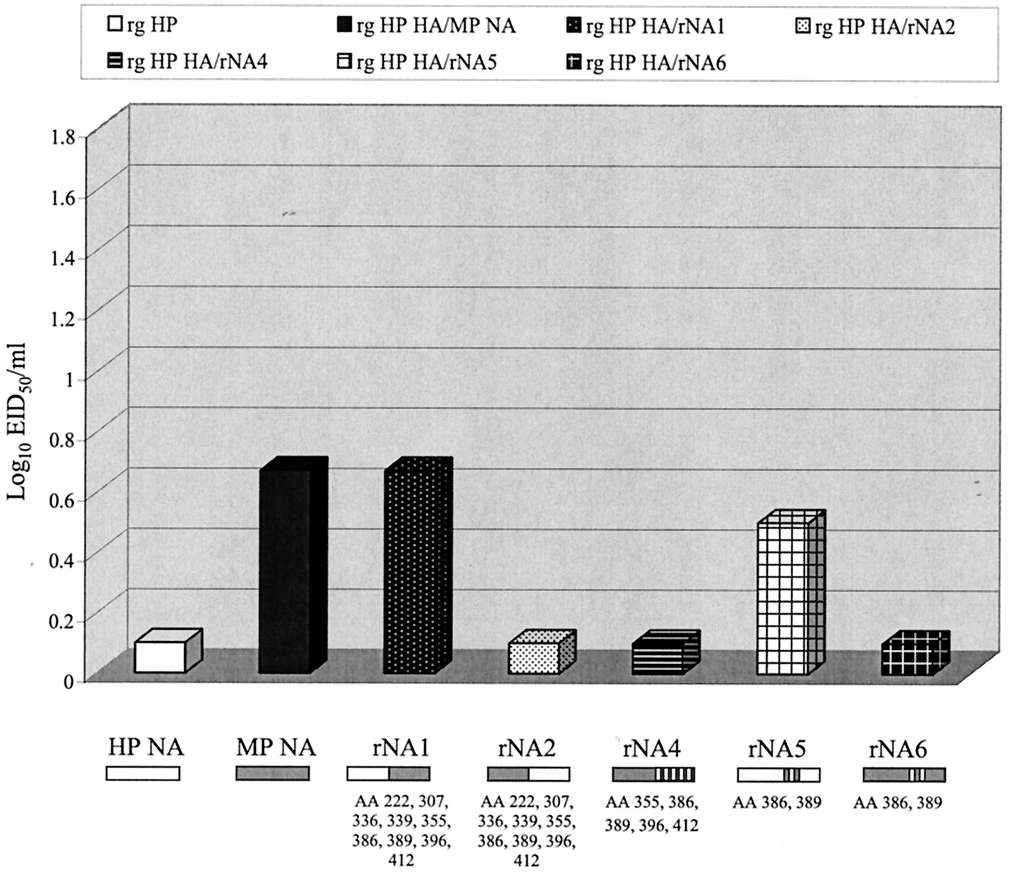


FIG. 5. Pathogenicity, as determined by CLD_{50}, of reverse-genetic (rg) reassortant viruses bearing recombinant NA, in chickens. The reverse-genetic viruses have the HA and internal proteins of the HP virus and bear recombinant NA encoded by plasmid rNA1, rNA2, rNA4, rNA5, or rNA6. AA, amino acids.

virus. To evaluate this site's effect on pathogenicity, we generated plasmid rNA4, which encodes a substitution that results in the addition of a glycosylation site to the NA of the MP virus. The CLD_{50} (Fig. 5) of the rNA4-containing reassortant virus was equal to that of the reverse-genetic wild-type HP virus ($10^{0.1}$ EID_{50}); it therefore had an increased pathogenicity. This finding suggested that the additional glycosylation site in NA is an important determinant of pathogenicity. We then generated two additional recombinant NAs to further define the role of the glycosylation site in pathogenesis. Plasmid rNA5 encodes a substitution that removes the glycosylation site from the NA of the HP virus. In contrast, rNA6 encodes a substitution that adds a glycosylation site to the NA of the MP virus. We generated reverse-genetic viruses bearing NA encoded by rNA5 or rNA6 and the HA of the HP virus and containing the internal proteins of the HP virus. The rNA5-containing virus had a higher CLD_{50} ($10^{0.5}$ EID_{50}) than did the reverse-genetic wild-type HP virus (CLD_{50} = $10^{0.1}$ EID_{50}): removal of the glycosylation site therefore reduced pathogenicity. The CLD_{50} of the rNA6-containing reassortant virus ($10^{0.1}$ EID_{50}) was lower than that of the single-gene HA reassortant virus ($10^{0.67}$ EID_{50}) and, interestingly, equal to that of the reverse-genetic HP virus. The addition of a glycosylation site to the NA of the MP virus increased the pathogenicity of this virus (rNA6-containing reassortant virus) and made it behave like the reverse-genetic HP virus.

We further analyzed the modulatory effect of NA on the pathotype of these H5 viruses by constructing reassortant HP viruses bearing the NA of the MP virus in combination with various engineered recombinant HA proteins. As expected, the NA of the MP virus was able to modulate the pathogenicity of the various reassortant viruses (Fig. 6) by making each virus less pathogenic than its counterpart carrying the NA of the HP virus (Fig. 3, 4, and 5). Taken together, our results suggest that the presence of an additional glycosylation site in the globular domain of NA plays an important role in determining virus pathogenicity and the ability of the virus to resist clearance from the lungs.

Statistical analysis of CLD_{50} values. Using the Cox regression model we compared the hazards of death for chickens infected with the viruses containing the recombinant HA or NA genes to those of their parental origin. The hazards ratios and their 95% confidence intervals were determined. Significant P values were <0.05. A hazards ratio of <1 means the virus's ability to kill chickens is inferior to the comparison parental virus. If the difference is significant, then in general, the 95% confidence interval for the hazards ratio does not cover 1. On the contrary, a hazards ratio of >1 indicates the

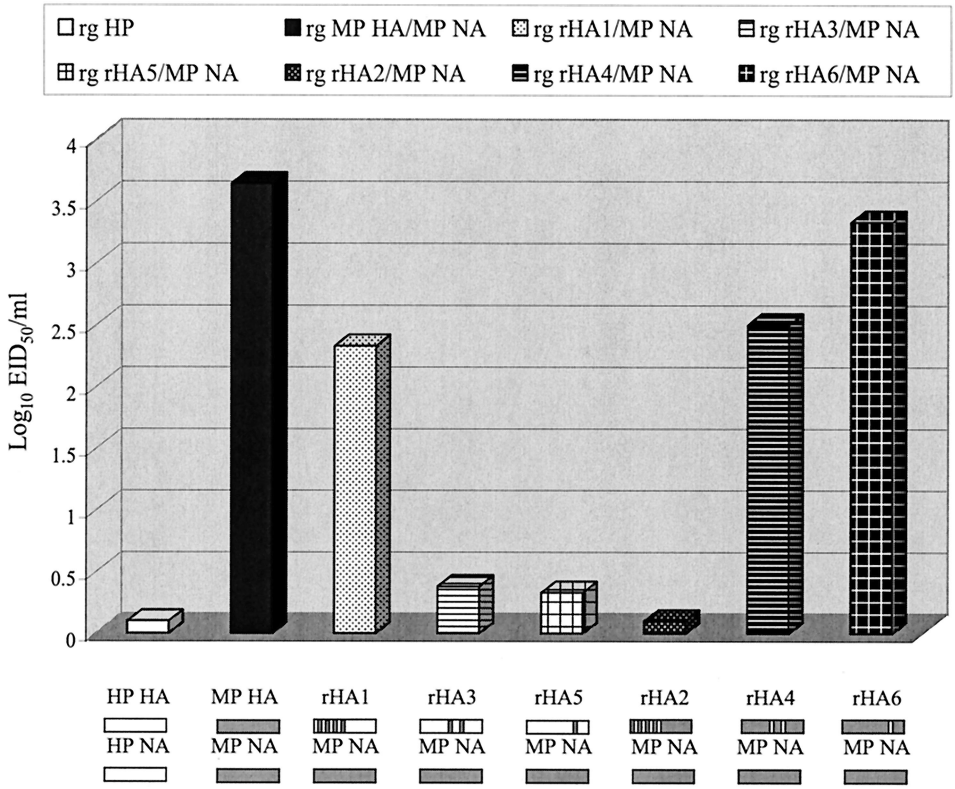


FIG. 6. Pathogenicity, as determined by CLD_{50}, of reverse-genetic (rg) reassortant viruses having recombinant HA, NA from the MP virus, and the internal proteins of the HP virus in chickens.

virus's ability to kill chickens is superior to its parental virus, to which it is compared. The single-gene reassortant viruses and the double-reassortant virus had hazards ratios of <1 and significant *P* values when compared to the HP virus, showing that the ability of these viruses to kill chickens is inferior to that of the HP virus. The viruses containing rHA1, rHA3, and rHA5 had hazards ratios and significant *P* values as expected when compared with the HP virus (rHA1 and rHA5, <1; rHA3, >1). The viruses containing rHA2 and rHA4 had hazards ratios that were >1 when compared to the single-gene reassortant virus containing the MP HA. The virus containing rHA6 was not statistically different from the single-gene reassortant virus. The viruses containing the recombinant NA genes all had hazard ratios as expected when compared to their parental NA donor; however, the *P* values for these viruses were not <0.05.

DISCUSSION

We have identified H5N1 viruses that contain markers typical of high pathogenicity but have different pathogenic phenotypes. Intravenous injection of the HP virus, A/chicken/Hong Kong/YU562/01, into chickens resulted in an IVPI of 3.00, which is the highest score possible. The MP virus, A/goose/Hong Kong/437-10/99, had a much lower IVPI of 1.50. Both viruses have HA1 domains with the same glycosylation patterns and polybasic amino acids at the HA cleavage site, but they show very different pathogenic phenotypes. Initial results (IVPIs and CLD_{50} values) showed that amino acids in the surface proteins play a major role in determining the pathogenic phenotype, as previously reported (21, 27, 28, 37, 38). However, we cannot exclude that the internal genes are also playing a role in the differences in the pathogenicity that were observed. Our present studies show that although pathogenicity is affected more by HA than by NA, variations in the NA also have a direct effect on the time course of the viral infection and disease outcome. Replacement of the HA and NA of the HP virus with the HA and NA of the MP virus resulted in a dramatic increase in the CLD_{50}, which indicated a decrease in pathogenicity of the HP virus. We conclude, therefore, that the activities of HA and NA are functionally linked and have a direct effect on pathogenicity.

Infection of chickens with the various reverse-genetic viruses resulted in different mortality patterns, including different timings of the onset of death. Early onset of death may be linked to high viral titers in the lungs, thymus, and bursa of Fabricius, which we found in chickens infected with the reverse-genetic HP virus or the single-gene reassortant of the HP virus having the HA of the HP virus and the NA of the MP virus. Previous studies have shown that an HP virus, A/turkey/Ont/7732/66 (H5N9), was associated with severe lymphopenia: high titers of virus were found in lymphoid tissues early in the infection process and were accompanied by severe damage to lymphocytes (34, 35). These studies and ours support the link between high virulence and high titers of virus in the lymphoid tissues.

Upon investigation of the specific amino acids within HA

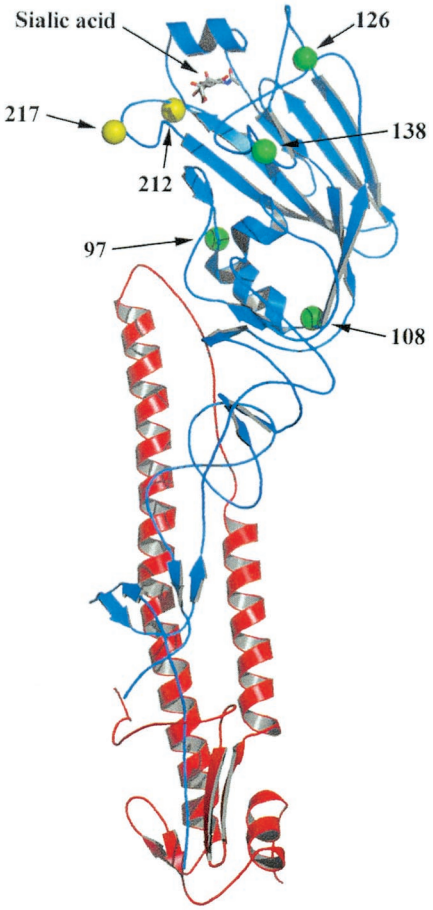


FIG. 7. Location of amino acid differences between the HP and MP viruses shown in the three-dimensional structure of A/Duck/Singapore/3/97 H5 HA (8). The blue portion of the molecule is HA1, and the red portion is HA2. The amino acids in green were changed in rHA1 and rHA2, and the amino acids in yellow were changed in rHA3 and rHA4.

that were responsible for pathogenicity, we found that changing HA amino acids 97, 108, 126, and 138 (rHA1 and rHA2) reduced the pathogenicity of the HP virus and increased the pathogenicity of the MP virus. Residues 97 and 108 are at either end of the 110 helix of HA1, which is packed against the tall turn of the helix-loop-helix motif in HA2 (Fig. 7). These interactions are broken upon transition to the fusion state of HA. Residue 97 points toward this tall turn in an adjacent monomer, and an aspartic acid at this position would introduce a negative charge in this region in the HP virus compared to the MP virus. Residue 108 packs into a hydrophobic core behind the helix away from HA2. An amino acid change from a threonine (in the HA of the MP virus) to an isoleucine (as in the HA of the HP virus) would create additional hydrophobic interactions in this region.

Residue 126 is located at the end of the 130 loop, which is directly involved in sialic acid binding, and is close to a neutralizing epitope (8, 25) (Fig. 7). Aspartic acid 126 in the MP virus HA interacts with serine 129 and seems to stabilize the conformation of this part of the loop. The longer side chain of glutamic acid in the HP virus HA would come too close to the hydroxyl of serine 129 to maintain this interaction. Therefore, the conformation of the 130 loop may differ subtly, depending on the residues occupying positions 126 and 129, and such subtle conformational variation may affect the precise receptor-binding properties of these viruses.

Residue 138 is located on a surface-exposed loop underneath the receptor binding site (8) (Fig. 7). It is therefore not involved directly with the receptor binding site or with a monomer-monomer interface. The HP virus has a hydrophobic leucine at this position compared to the polar histidine in the MP. A solvent-exposed hydrophobic residue in this region may cause some subtle alteration in the conformation of the 130 loop.

The results of the CLD_{50} assays showed that changing amino acids at HA positions 212 and 217 had a unidirectional effect on pathogenicity. Changing these amino acids increased the pathogenicity of the HA of the MP virus and of the HP virus. Residues 212 and 217 are at the edge of the receptor binding site behind the 220 loop, which is involved in receptor binding (Fig. 7). They are also at the ends of a loop that lies over three strands of a four-stranded beta-sheet in an adjacent monomer (in the trimer interface). Upon binding to the receptor in an avian host, this loop is the only region of the receptor-binding domain to undergo a conformational change. The crystal structure of H5 HA has the same amino acids as the MP virus in positions 212 (glutamic acid) and 217 (proline). The HP virus HA has a serine at position 217, which may introduce more flexibility in this region and allow a larger conformational change upon avian receptor binding than does the MP virus HA. In addition, the serine residue at position 217 in the HA of the HP virus introduces a polar hydroxyl group that could form a hydrogen bond with, perhaps, O7 of the *N*-acetylglucosamine attached to Asn165 of the adjacent monomer. This new hydrogen bond could potentially stabilize a specific conformation of the carbohydrate.

Although it is at the monomer-monomer interface, Glu212 in the HA of the MP virus (and in the crystal structure of H5 HA) appears not to interact directly with the adjacent monomer. The HA of the HP virus, however, has lysine at position 212, the long side chain of which could have the potential to form either a hydrogen bond across the monomer-monomer interface to a main chain atom between residues 206 and 208 or to form a salt bridge with Glu227 of the same monomer. Importantly, though, a change from glutamic acid (as found in the HA of the MP virus) to lysine (as found in the HA of the HP virus) in position 212 would reverse the point charge in this region from negative to positive.

Our experiments showed that by changing one amino acid in the cleavage site of HA we could modulate the virus's pathogenicity. The HA of the MP virus contained one more basic amino acid than did that of the HP virus. When lysine at position 338, in the cleavage site of the HA of the MP virus, was replaced with glutamic acid, as found in the HA of the HP virus, the MP virus became more pathogenic. The opposite was also true: when we replaced glutamic acid with lysine in the HP virus, pathogenicity was reduced. These results confirmed the importance of the composition of the cleavage site in pathogenicity, a finding similar to those described in previous reports (11, 24, 31) and also suggested that the presence of an acidic

residue within an otherwise polybasic region has great influence on the pathogenic phenotype of the virus.

Although further refinement of our findings could be accomplished by performing additional site-directed mutagenesis, our results clearly indicate that no group of amino acid differences were responsible for the virulence observed; rather the differences observed were dependent on the rest of the sequence of the HA. Therefore, the amino acids that we have identified play a role in virulence according to the rest of the HA amino acid sequence content.

HAs of other HP H5N1 viruses isolated in 1997 from chickens and humans have the same residues at positions 97 (aspartic acid), 108 (isoleucine), and 138 (leucine) (33) as found in the HP virus. Residues at positions 126 (aspartic acid), 212 (glutamic acid), 217 (proline), and 338 (lysine) in the HA of the H5N1 1997 viruses are the same as those in the MP virus. HAs of new, HP H5N1 viruses isolated in 2003 (G. Yi, personal communication) have the same amino acids as does the HP virus used in our experiments at positions 97, 108, 126, 212, and 217. Interestingly, the 2003 H5N1 isolates from humans and poultry all contained a basic amino acid at the cleavage site (Lys338), which would make these viruses less pathogenic than the HP virus used in this study.

Our results showed that NA is also important for pathogenicity in chickens and, specifically, that an additional glycosylation site in the NA of the HP virus is important for increased virulence. Although it has been proposed that glycosylation sites in HA enhance virulence and that these sites are closely associated with adaptation in chickens (3, 18), glycosylation sites in NA that affect pathogenicity in chickens have not previously been described. The mechanism by which an additional glycosylation site in NA may affect pathogenicity is unknown, but it is most likely to be linked to the cleavage of HA in cells (30). For example, the highly glycosylated NA of the HP virus may facilitate increased activation of the host proteases required to cleave the virus's HA, a mechanism suggested by Schulman (29). Although this additional glycosylation site was not present in the highly pathogenic viruses isolated from chickens and humans in 1997, it was present in the NA of the human influenza virus A/HongKong/213/03 (G. Yi, personal communication).

Our results also showed a strong cooperative effect between HA and NA in eliciting pathogenicity. This finding indicates that there is an important interplay between the two surface proteins and that a specific combination of their properties is required to achieve high pathogenicity. Indeed, highly balanced actions of HA and NA have been shown necessary to achieve a productive infection (20, 36). Our reverse-genetic single-gene reassortant viruses may have been less pathogenic than the parental HP virus because they had altered HA receptor-binding properties or NA receptor-destroying activity, and the cooperativity between the two proteins was lost. However, the double-reassortant virus containing both the surface proteins of the MP virus and the internal genes of the HP virus had what would be considered the optimal HA-NA combination (the one found in nature). However, pathogenicity of the virus was significantly different from that of the parental HP virus, indicating that the differences were not due to a lack of activity or infectivity but rather the HA and NA activities are functionally associated.

Identification of the amino acids in HA and NA that may be considered markers of high pathogenicity in chickens with the potential to cross to human or other mammalian species would help in identifying emerging strains of concern, particularly if the strains isolated were of low or moderate pathogenicity in chickens and had some or all of the amino acids residues identified here as having a direct effect on the pathogenicity of H5 viruses.

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