The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04

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H5N1 influenza viruses transmitted from poultry to humans in Asia cause high mortality and pose a pandemic threat. Viral genes important for cell tropism and replication efficiency must be identified to elucidate and target virulence factors. We applied reverse genetics to generate H5N1 reassortants combining genes of lethal A/Vietnam/1203/04 (VN1203), a fatal human case isolate, and nonlethal A/chicken/Vietnam/C58/04 (CH58) and tested their pathogenicity in ferrets and mice. The viruses' hemagglutinins have six amino acids differences, identical cleavage sites, and avian-like α-(2,3)–linked receptor specificity. Surprisingly, exchanging hemagglutinin and neuraminidase genes did not alter pathogenicity, but substituting CH58 polymerase genes completely attenuated VN1203 virulence and reduced viral polymerase activity. CH58's NS gene partially attenuated VN1203 in ferrets but not in mice. Our findings suggest that for high virulence in mammalian species an avian H5N1 virus with a cleavable hemagglutinin requires adaptive changes in polymerase genes to overcome the species barrier. Thus, novel antivirals targeting polymerase proteins should be developed.

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Abbreviations used: a.i., after inoculation; EID, egg infectious dose; HA, hemagglutinin; MDCK, Madin-Darby canine kidney; NA, neuraminidase; RG, reverse genetics; SA, α-(2,3)–linked sialic acid.

In poultry, highly pathogenic avian influenza viruses of the H5 subtype spread systemically, causing death within a few days (1). In 2004, H5N1 avian influenza was reported in mammalian species during severe outbreaks in Asia (2–6). Fatal human infections were identified in Vietnam, Thailand, Cambodia, and Indonesia. These events increased concern about a potential influenza pandemic.

Human disease caused by H5N1 viruses has been characterized by viral pneumonia with acute respiratory distress syndrome, diarrhea, liver dysfunction, and neurological symptoms (7–11). Factors responsible for this high virulence in humans are not well characterized. In chickens, hemagglutinin (HA) with multibasic amino acids is important in virus dissemination and systemic spread (12). Human A/Vietnam/1203/04 (VN1203) and avian A/Chicken/Vietnam/C58/04 (CH58) H5N1 isolates, which are both of the Z genotype, have the multibasic amino acid motif PQRERRRKKR↓G in HA and are lethal to chickens (13). However, it is not known whether other adaptive changes in HA, such as those that determine receptor specificity, are crucial for interspecies transmission and virulence. Typical avian strains are characterized by HAs that bind α-(2,3)–linked sialic acid (SA) receptors, while those of human strains bind α-(2,6)–linked SA (14). Less well understood is the role of internal gene segments. Polymerase subunit PB2 and nonstructural NS1 protein were reported to be important for high virulence of 1997 H5N1 viruses in mouse and pig models (15, 16). Virulence factors of newly emerging H5N1 Z genotype viruses have not been mapped. Mapping of viral virulence factors in different mammalian hosts would provide more information about their contribution to pathogenicity.

Ferrets are considered the best small animal model of human influenza. Mice are often used in influenza studies, but, human influenza viruses typically do not replicate in mice without adaptation, whereas they cause an acute, respiratory illness in both ferrets and humans. We and

others previously demonstrated that highly pathogenic H5N1 viruses that were isolated from birds and humans in 2004 form groups according to their pathogenicity in ferrets (13, 17). VN1203, which was isolated from a fatal human case, is highly lethal to ferrets and mice (13, 18). Sequence analysis of this virus's complete genome and that of nonlethal CH58 revealed amino acid differences encoded by the genes (13). Specifically, there are three amino acid differences in PB1 gene, four in PB2, four in PA, five in HA, one in NP, five in NA, one in M, and eight in NS. Here, we demonstrate the use of a model system in which recombinant viruses are generated by reverse genetics (RG) to study pathogenicity in ferrets and mice. This approach allowed us to distinguish whether high virulence of H5N1 VN1203 as compared with CH58 is caused by its surface glycoproteins HA and neuraminidase (NA), its NS gene, or its polymerase genes.

RESULTS

Model system for evaluation of pathogenicity in ferrets and mice

Human isolate VN1203 and chicken isolate CH58 are both lethal to chickens; however, only the human isolate is lethal to ferrets (13). To determine what gene segments contribute to VN1203 high virulence and systemic spread in ferrets and mice as compared with CH58, we used the eight-plasmid system (19). We constructed two eight-plasmid sets encoding individual genes of VN1203 and CH58, to generate RG VN1203 and CH58 recombinant viruses by DNA transfection. Sequence analysis showed that the RG viruses were identical to their respective parental viruses. To test the pathogenicity of the RG viruses, we intranasally inoculated ferrets with 10^6 50% egg infectious dose (EID_{50}) of virus. Infection with VN1203 virus was lethal to all three ferrets as early as 5 d after infection, but CH58 was not (Fig. 1 A). Ferret body temperatures were ~2°C higher after inoculation with VN1203 than CH58. Ferrets inoculated with VN1203 lost >20% of their body weight (Fig. 1 B), whereas those in the CH58 group maintained or increased their weight. These findings were similar to those observed after inoculation (a.i.) with the respective parental virus strains (13).

To determine the lethal dosage of VN1203 and CH58 in mice, we inoculated C57BL6 mice with different dilutions of the recombinant viruses. Mice inoculated with 10^3 EID_{50} of VN1203 showed considerable weight loss, and all died (Fig. 1, C and D). In contrast, mice inoculated with the same dose of CH58 virus showed no disease signs, even those infected with 10^6 EID_{50} of CH58 survived. Therefore, VN1203 was highly lethal and CH58 nonlethal in both mammalian models under these infection conditions.

Infection of Madin-Darby canine kidney (MDCK) cells with VN1203 resulted in large plaque formation (Fig. 1 E), and infection with CH58 resulted in smaller plaques. The VN1203 plaques had a mean size of 2.72 ± 0.17 mm. In contrast, plaques created by CH58 were on average 0.67 ± 0.17 mm which were significantly smaller than plaques of the human virus (Student's *t* test; $P < 0.001$).

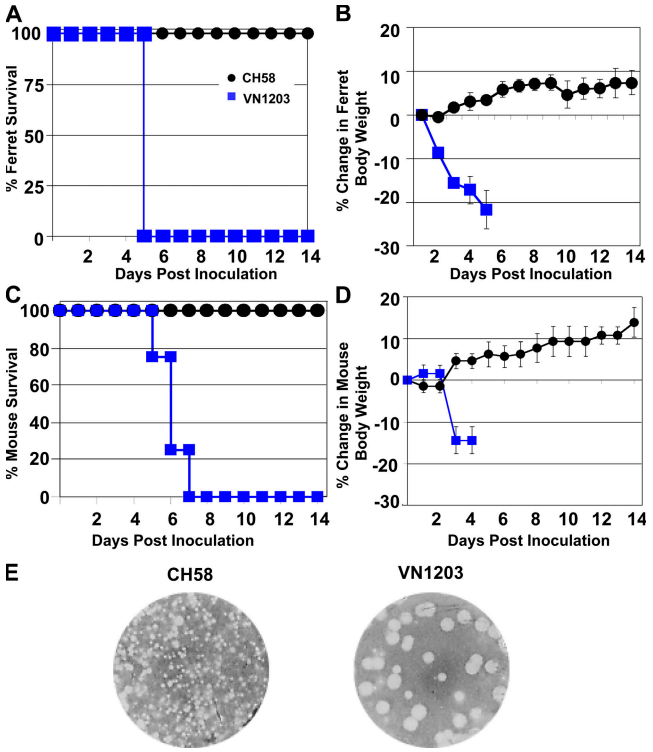

Figure 1. Effect of inoculation of ferrets and mice with VN1203 and CH58. (A) Survival rate of ferrets after intranasal inoculation with 10^6 EID_{50} of RG VN1203 ($n = 3$) or CH58 ($n = 3$) virus. (B) Mean ± SE percent weight change of groups of three ferrets after inoculation. (C) Survival rate after intranasal inoculation of groups of six mice with 10^3 EID_{50} of VN1203 or CH58. (D) Mean ± SE percent weight change of mice after inoculation. (E) Plaques on MDCK cells after titration of CH58 and VN1203 virus.

To monitor viral growth and spread, we titrated virus of ferret nasal wash specimens and organ homogenates. On days 3 and 5 a.i., virus titers were between 1 and 4 EID_{50}/ml logs higher in nasal washes from ferrets inoculated with VN1203 than in those inoculated with CH58 (Fig. 2 A). High virus titers were detected 4 d a.i. in brain, spleen, liver, and lung tissue homogenates from a ferret inoculated with VN1203 (Fig. 2 B).

Days 4 and 5 a.i. of ferrets with VN1203, histologic alterations were observed in the lungs, liver, and brain (Fig. 2 C). Lung peribronchiolar pneumonia was characterized by bronchiolar necrosis, hyperplasia, and intraluminal necrotic and inflammatory cells. Alveoli had hyperplasia and inflammatory cells. In the liver, there was hepatocellular vacuolization, biliary necrosis, and inflammatory cells. In the brain's neuropil, there was neuronal degeneration associated with inflammatory cells. Monocyte infiltrates were also in meninges and perivascular spaces. In contrast, the lungs, livers, and brains of ferrets inoculated with CH58 showed no apparent changes. In sum, the RG 1203 and CH58 viruses were identical to their respective parental viruses in genotype and phenotype.

BSL3+ isolators' space constraints allow testing of few animals per experiment. Therefore, we focused on reassortants

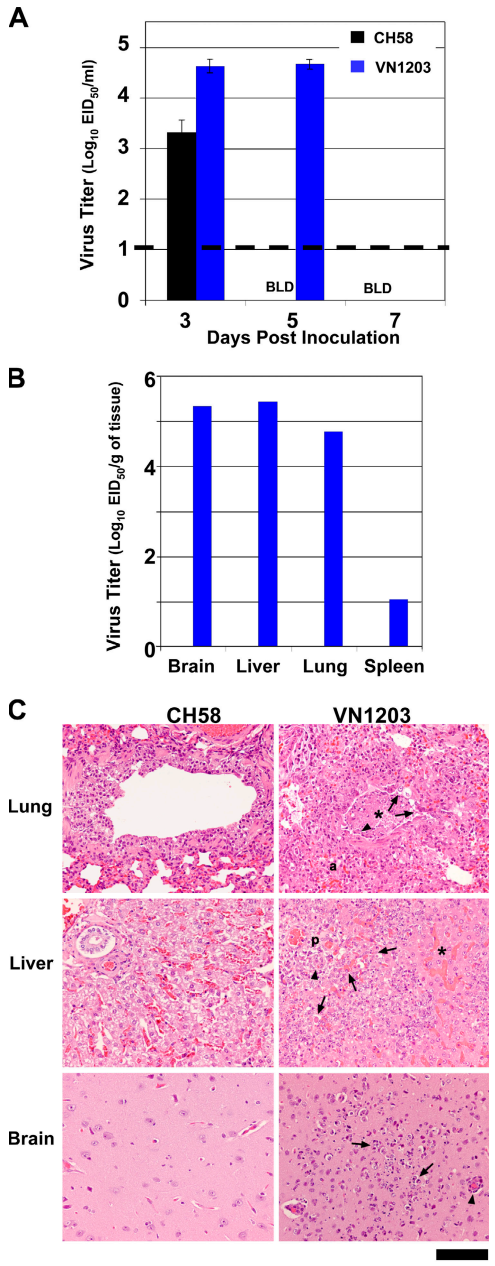

Figure 2. Virus titers and tissue pathology of RG VN1203 and CH58 H5N1 viruses in ferrets. (A) Mean virus titers ± in ferret nasal washes after VN1203 or CH58 inoculation. Dashed line indicates detection limit of 10^1 EID_{50}/ml. BLD, below level of detection. Error bars indicate that all animals deceased at this time point. (B) Viral titers in ferret organ homogenates 4 d a.i. (C) Hematoxylin and eosin–stained sections (20X) of ferret lung, liver, and brain 4–5 d a.i. All tissues from CH58 inoculation were normal. VN1203 ferret lung shows bronchiole (*) with epithelium loss (▲) and epithelial regeneration (↑), intraluminal necrotic and inflammatory cells in bronchioles and alveoli (A). Liver shows inflammatory cell infiltrate in parenchyma and portal tract (p) with blood vessels and biliary duct. Biliary epithelium necrosis (▲) and liver cells (↑). Normal liver cells (*) surround necrotic foci with inflammatory cells. Inflammatory cell infiltrate associated with degenerating and necrotic neurons (↑) and perivascular inflammatory cells (▲). Bar, 50 μm.

selected according to their virus life cycle function: surface glycoproteins, polymerase complex genes, and NS gene (Fig. 3). A previous systematic sequence comparison of all gene segments indicated that VN1203 and CH58 viruses differed most greatly in these genes (13).

Surface glycoprotein–reassortants do not alter pathogenicity

Systemic spread and high lethality of VN1203 in ferrets and mice could be caused by altered tissue tropism resulting from changes in HA and NA. VN1203 and CH58 differ by six amino acids in each surface glycoprotein (13). We therefore generated an RG VN1203-CH58(HA,NA) virus that had VN1203 six internal genes and CH58 HA and NA genes (Fig. 3). All ferrets inoculated with this recombinant virus died between days 6 and 11 a.i. (Fig. 4 A). All mice inoculated with VN1203-CH58(HA,NA) virus died 8 d a.i. (Fig. 4 B). Survival rate, weight loss, viral titers, and disease severity caused by VN1203-CH58(HA,NA) was similar to that of VN1203 in both models (Fig. 4).

10 days a.i. of ferrets with VN1203-CH58 (HA,NA), similar pathology as with VN1203 were observed in lungs, livers, and brains (Fig. 4 F). The lungs had bronchiolar necrosis, hyperplasia, and intraluminal necrotic and inflammatory cells (Fig. 4 F, top left). Within liver parenchyma, there was hemorrhage associated with necrotic and vacuolated hepatocytes (Fig. 4 F, top right). Brain alterations were consistent with a later infection stage. In addition, there was neuronal degeneration and gliosis (Fig. 4 F, bottom left). Moreover, the brain also had liquefactive necrosis (malacia) surrounded by foamy macrophages (gitter cells) and reactive astrocytes (Fig. 4 F, bottom right).

After titration on MDCK cells, VN1203-CH58(HA,NA) grew large plaques (2.87 ± 0.19 mm), as had VN1203 (Fig. 4 G). RG virus with surface glycoproteins of the human isolate and its remaining genes from the chicken isolate (CH58-VN1203(HA,NA)) grew small plaques (0.48 ± 0.17 mm). Therefore, plaque size in MDCK cells, lethality, and pathogenicity of these viruses in mice and ferrets were not affected by these sequence changes in HA and NA.

To determine whether VN1203 and CH58 viruses differed in their HA receptor specificity, we measured the viruses' affinity toward synthetic SA substrates p3′SL or p6′SL. In this assay, the lower the dissociation constant (K_d [μM of sialic acid]), the more strongly virus binds to the receptor. VN1203 had a K_d of 1.50 ± 0.21 to 3′SL but a $K_d > 100$ to 6′SL. Therefore, VN1203 has a greater affinity for α-(2,3)–linked than α-(2,6)–linked SA receptors. Similarly, CH58 had a K_d of 1.10 ± 0.10 toward 3′SL and a $K_d > 100$ for 6′SL. Thus, both VN1203 and CH58 have preferential binding affinity for α-(2,3)–linked SA receptors, typical of avian viruses.

Contribution of NS gene to lethality of VN1203 in ferrets but not mice

Many studies have established that NS1 regulates innate immunity by modulating the host type I interferon response, which has both antiviral and immunoregulatory functions.

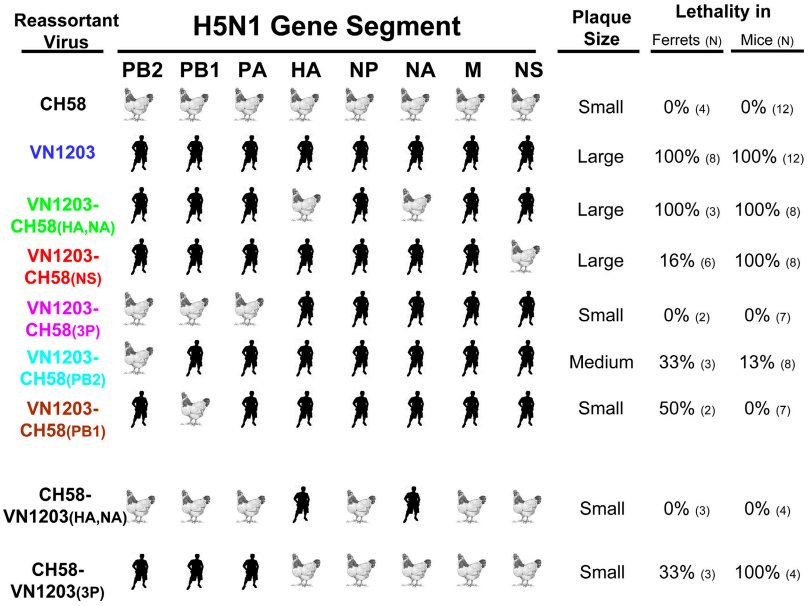

Figure 3. Generation of reassortant viruses with summary of plaque morphology and lethality. Human and chicken symbols denote source of gene segment (human VN1203 isolate or chicken CH58 isolate). The colors of the names of the RG reassortant viruses correspond to the colors in all data graphs of animals infected with those viruses. Mean plaque size was categorized as small (<1 mm), medium (1–2 mm), or large (>2 mm). The percent lethality caused by each RG virus and total number (N) of ferrets or mice used is indicated.

We therefore generated single-gene reassortants that combined CH58 NS with the remaining seven VN1203 genes. Five out of the six ferrets inoculated with VN1203-CH58(NS) virus survived (Fig. 5 A). They demonstrated 20% less weight loss than did ferrets inoculated with VN1203 (Fig. 5 B). All mice inoculated with single-reassortant VN1203-CH58(NS) lost weight and died by day 8 a.i. (Fig. 5, C and D). Thus, NS–reassortants show differences in lethality between ferrets and inbred mice.

Polymerase complex contribute to lethality of VN1203 in ferrets and mice

11 amino acid differences in polymerase genes (4 in PB2, 3 in PB1, and 4 in PA) distinguish VN1203 and CH58. Inoculation with reassortant virus VN1203-CH58(3P)—consisting of CH58 PB2, PB1, and PA genes and the remaining genes from VN1203—was not lethal to ferrets and mice (Fig. 6, A and C). Animals inoculated with VN1203-CH58(3P) showed no weight loss (Fig. 6, B and D). As with CH58, VN1203-CH58(3P) virus generated small plaques (0.88 ± 0.50 mm), on MDCK cells (Fig. 6 E). Thus, attenuation and reduction in plaque size, compared with VN1203 plaques, is mediated by polymerase complex genes.

Ferrets inoculated with single-gene reassortant VN1203-CH58(PB2) experienced modest weight loss, and two out of three ferrets survived (Fig. 6, A and B). Ferret nasal viral titers were 2 and 1 log lower on days 3 and 5 a.i., respectively, in those inoculated with chicken PB2 reassortants. Mice inoculated with VN1203-CH58(PB2) showed no weight loss, and one of eight mice died 10 d a.i. (Fig. 6, C and D). The generation of a RG virus of VN1203 with a point mutation in PB2 changing lysine (K) at position 627 to glutamic acid (E) was not lethal when administered to mice (unpublished data). This demonstrates that K^{627} of VN1203 PB2 contributes to lethality.

Ferret inoculation with single-gene reassortant VN1203-CH58(PB1) resulted in 50% survival (Fig. 6 A). Infection of mice with the same reassortant resulted in 15% weight loss 7 d a.i. However, all mice gained weight after day 9 and survived (Fig. 6, C and D). Interestingly, this single-gene CH58(PB1) reassortant also grew significantly smaller plaques (0.29 ± 0.04 mm), than VN1203 on MDCK cells (Fig. 6 E) (Student's *t* test, $P < 0.001$). These results underscore the importance of genetic changes in PB2 and PB1 to VN1203 replication efficiency. Collectively, these results demonstrate that the polymerase genes contribute to pathogenicity in both mice and ferrets.

To determine whether the three VN1203 P-genes are sufficient for high lethality, we generated a reassortant virus comprising the human isolate's polymerase genes and the remaining five segments from the chicken isolate. Inoculation of mice with CH58-VN1203(3P) resulted in severe loss of body weight and 100% lethality between days 9 and 11 a.i. Two of three ferrets inoculated with this CH58-VN1203(3P) virus survived and weight loss was similar to those infected with VN1203-CH58(NS) (Fig. 5). Thus, the three human P-genes are sufficient to convert a nonlethal virus into a lethal virus in mice but not in ferrets.

VN1203 polymerase complex possesses significantly higher transcription/replication activity

To assay polymerase activity of VN1203 and CH58, we transfected 293T cells with plasmids containing their PB2,

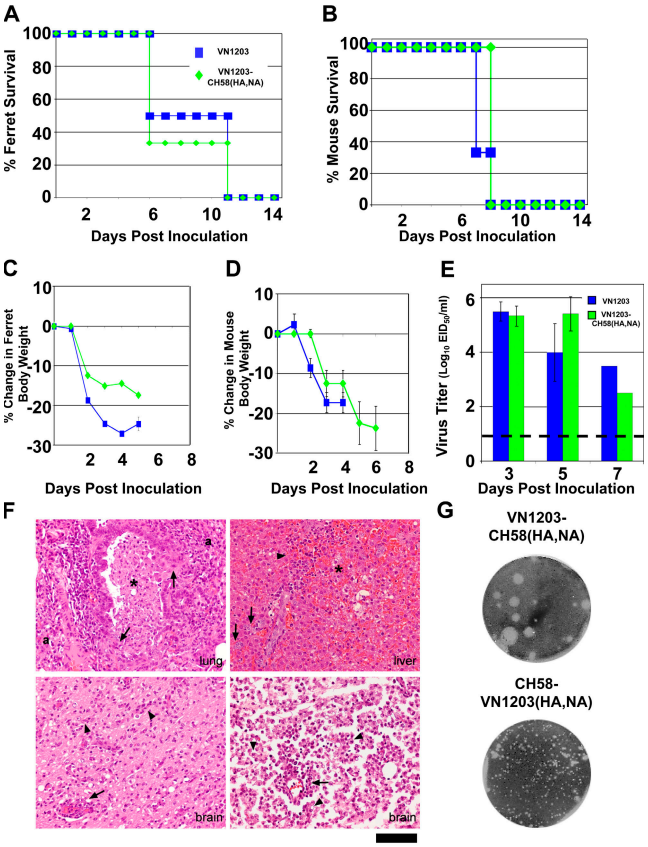

Figure 4. Reassortment of HA and NA does not affect lethality or pathogenicity in vivo or in cell culture. Survival (A) of ferrets and (B) mice a.i. with VN1203 ($n = 2$ ferrets, 8 mice) or VN1203-CH58(HA,NA) ($n = 3$ ferrets, 8 mice). Mean weight change (C) of ferrets and (D) mice ± SE. (E) Mean viral titers from ferret nasal washes ± SE. Dashed line indicates detection limit of 10^1 EID_{50}/ml. (F) Hematoxylin and eosin–stained sections (20X) of ferret lung, liver and brain 10 d a.i. with VN1203-CH58(HA,NA). Lung (top left) shows bronchiole (*) with epithelial hyperplasia (↑), necrotic debris in lumen and inflammatory cells in alveoli (A). Liver (top right) shows disrupted parenchyma with vacuolated hepatocytes associated with hemorrhage (*). Monocytes (▲) surround parenchyma and hyperplastic biliary cells (↑) extend into parenchyma. Brain (bottom left) shows perivascular inflammatory cells (↑), hypercellular neuropil with capillary endothelial hyperplasia (▲), gliosis and monocytes. Brain region (bottom right) with liquefactive necrosis and foamy macrophages (gitter cells, ▲) and perivascular monocytes (↑). Bar, 40 μm. (G) Plaque formation after virus titration on MDCK cells.

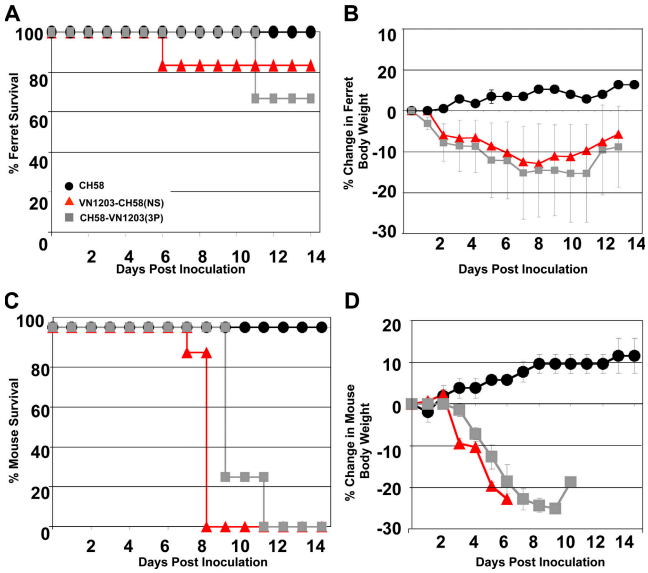

Figure 5. NS genes contribute to lethality of VN1203 in ferrets but not in mice. Survival rate (A), and mean weight change ± SE (B) of ferrets inoculated with CH58 ($n = 3$), VN1203-CH58(NS) ($n = 6$), or CH58-VN1203(3P) ($n = 3$) reassortant virus. Survival rate (C) and mean weight change ± SE (D) of mice ($n = 8$) inoculated with CH58 ($n = 8$), VN1203-CH58(NS) ($n = 8$), or CH58-VN1203(3P) ($n = 4$).

PB1, PA, and NP genes and a luciferase reporter plasmid. Because cDNA encoding luciferase is controlled by the influenza A virus M segment's noncoding region, luciferase levels reflected overall transcription and replication activity of the polymerase complex (20). Luciferase activity in human embryonic kidney 293T cells cotransfected with plasmids containing VN1203 polymerase complex and NP had luciferase activity (relative light units; RLUs) 3.5-fold that of cells cotransfected with CH58 polymerase complex and NP (Student's t test; $P < 0.01$) (Fig. 6 F). Therefore, the VN1203 polymerase complex possessed significantly higher transcription/replication activity than the CH58 polymerase complex.

To compare polymerase activity of VN1203 PB2 and PB1 segments, we replaced the individual PB2 and PB1 segments of VN1203 with those of CH58 (Fig. 6 F). When VN1203-PB2 was replaced with CH58-PB2, luciferase activity was only 22% of VN1203. When VN1203-PB1 was replaced with CH58-PB1, luciferase activity was 58% of VN1203 (Fig. 6 F). The effect of CH58 NP was assayed by cotransfecting VN1203 PB2, PB1, and PA plasmids with the CH58 NP plasmid. CH58 NP did not decrease the high transcription/replication activity of the VN1203 polymerase as there was no notable difference in luciferase activity as compared with the transfection of VN1203 PB2, PB1, PA and NP plasmids (unpublished data). Therefore, both CH58 PB2 and PB1 reduced the VN1203 transcription/replication activity.

DISCUSSION

Here, we have demonstrated the use of a model system that uses RG to map some factors that contribute to high virulence of a 2004 H5N1 influenza virus isolate. We showed that inoculation of ferrets with recombinant human influenza virus isolate VN1203, unlike inoculation with CH58, is followed by rapid systemic spread to the brain and liver, resulting in high lethality. Survival of all mice and ferrets inoculated with a RG VN1203 virus that had its polymerase genes derived from the chicken isolate showed that these genes are important for virulence. In the reverse experiment using the chicken-isolate backbone with the VN1203 polymerase genes, all inoculated mice died. Therefore, polymerase subunits

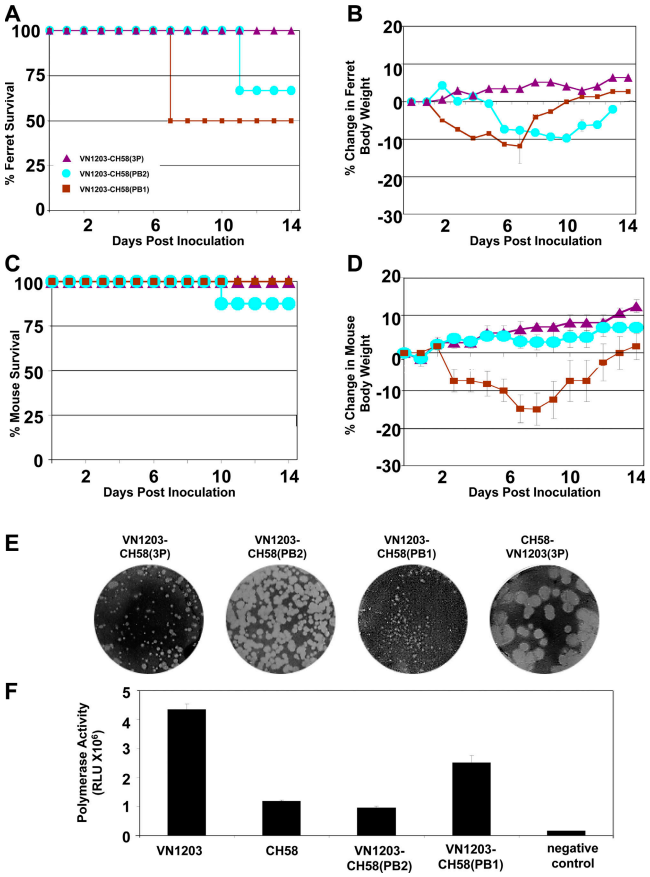

Figure 6. Polymerase complex genes contribute to lethality of VN1203. Survival rate (A) and mean weight change ± SE (B) of ferrets inoculated with polymerase gene–reassortant viruses. Survival rate (C) and mean weight change ± SE (D) of mice inoculated with polymerase gene–reassortant viruses. (E) Plaque formation in MDCK cells after virus titration. (F) Polymerase activity assayed by viral UTR-driven luciferase reporter gene. 293T cells transfected with plasmids containing VN1203 or CH58 PB2, PB1, PA, and NP genes plus a luciferase reporter plasmid, or with only VN1203 NP and the reporter plasmid (negative control). After 24 h, luciferase activity was assayed in cell extracts. Results are mean ± SE of triplicate transfections.

PB2, PB1, and PA are central to influenza's replication cycle and required for viral RNA replication and transcription (21). The NH_2-terminal region of PB1 interacts with the COOH-terminal region of PA, whereas the COOH-terminal region of PB1 interacts with the NH_2 terminus of PB2 (22). K^{627} of PB2 was previously demonstrated to be required for 1997 H5N1 viruses' high virulence in mice (16, 23), which was consistent with our finding that the human virus isolate whose PB2 contained K^{627} was more lethal than that with E^{627}.

Despite clear evidence of K^{627} role in the virulence and host range restriction (24), some lethal 1997 and 2004 H5N1 isolates, such as A/quail/Vietnam/36/04 (H5N1) viruses, have avian-like residue E^{627}, indicating the importance of other genes or of the other three amino acids in PB2. Indeed, the attenuation of PB1-reassortants indicated that PB1 contributes significantly to this effect. The same three amino acids of VN1203 changes in PB1 are found in the isolate A/quail/Vietnam/36/04 (H5N1), which is lethal to ferrets (13). Viral-like reporter replication was more efficient when PB1 is derived from an avian virus (25).

It was difficult to conclusively determine the contributions of single P genes to pathogenicity because of the small number of ferrets used. However, different growth properties observed in three different mammalian species (a canine cell line, mice, and ferrets) and the mini-genome assay in human cells suggest a general, not species-specific, enhancement of replication. Collectively, these results suggest that before and/or after transmission from chickens to humans, adaptive changes appear in PB2 and PB1 that result in better interaction of polymerase subunits and/or between polymerase subunits and host factors, leading to more rapid generation of infectious virus particles. It is possible that rapid growth is prerequisite for systemic spread after the initial infection in small animal models. The lack of evidence for systemic spread in humans indicates that efficient growth in yet to be identified cell types may contribute to severe disease and death in humans.

HA's role in virus cell binding and entry implies it is important for efficient virus spread after infection (26). The presence of HA's multibasic cleavage site is well correlated with influenza A viruses' high pathogenicity (12). This sequence results in the cleavage of HA by intracellular furin-like proteases (27). In this study, both the chicken and human isolate have polybasic amino acids in the connecting HA peptide which is sufficient for rapid systemic viral spread, resulting in death of chickens (28) and is essential for the human 1997 H5N1 isolates' virulence in mice (16). However, contributions of HA receptor-binding properties to cell tropism and virus transmission are not known. Our finding that recombinant human virus containing the chicken HA retained its lethality demonstrates that HA residues outside the cleavage site do not have a critical impact on tropism. Thus, VN1203 (H5N1) influenza's systemic spread and neurotropism are not the result of changes in receptor binding. H5N1 viruses whose HA is selective for α-(2,3)–linked SA can spread efficiently after experimental infection. It is noteworthy that CH58 and VN1203 and most recent H5N1 viruses have S^{223} in their HA and that this residue located in the 220-loop of the HA is critical for receptor specificity to α-(2,3)–SA (29). Substitution of S^{223} to N^{223} has been shown to alter receptor specificity to both avian-like α-(2,3)–linkage and humanlike α-(2,6)–linkage (29). Inefficient binding and cell entry of an avian H5N1 virus in a mammalian host can be compensated by intracellular cleavage of the HA and by efficient replication/transcription of the viral genome by PB1 or PB2. Thus, multibasic amino acids in the cleavage site of the HA are necessary but not sufficient for high lethality in mammalian species.

Viral pneumonia with acute respiratory distress syndrome is the main cause of rapid death in human cases of H5N1 influenza infection (7). H5N1/97 viruses induce much higher

transcription of proinflammatory cytokines in human primary monocyte-derived macrophages in vitro, particularly TNF-α and interferon-β, than do H3N2 human influenza viruses (30). The nonstructural (NS) gene-segment of H5N1/97 viruses contributed to the increase in TNF-α induced by the virus. Although PB1 and PB2 roles appear to be independent of the host and cell type, different effects of genetic changes in the CH58-NS segment in ferrets and mice suggest differences in the host factors regulated by NS1. NS1 is known to have the potential to inhibit host type I interferon responses to virus (31, 32). It is possible that ferrets and feral mice express functional interferon-inducible antiviral Mx protein, whereas many inbred strains, such as the C57BL6 mice in these studies, do not (33, 34). Mx has been shown to specifically inhibit influenza A (35, 36). Mice expressing Mx could be used to determine whether observed differences in the role of NS1 reflect an experimental limitation, such as dependence of lethality on virus dose, or indeed reflect species-specific interaction with the innate immune system. There is one amino acid difference between the NS2/NEP protein of VN1203 and CH58, which is unlikely to play a role in the differences in pathogenesis. However, as to whether the 10–amino acid deletion at the COOH terminus of VN1203 and/or specific amino acids in the NS1 protein are responsible for differences in pathogenesis should be further investigated.

Our results suggest that antivirals directed against the polymerase proteins could be developed. The mini-genome assay we established in this study for highly pathogenic H5N1 viruses can perhaps be used in high-throughput screening. Inhibitors that reduce polymerase activity about two- to threefold in cell culture–based assays are candidates for therapeutic testing in the mouse or ferret model. These new types of antivirals could complement the use of NA inhibitors and the M2-ion channel blocker amantadine.

Although H5N1 viruses such as VN1203 may be too lethal to cause a pandemic, our results demonstrate that changes in only the polymerase genes of an H5N1 influenza virus are sufficient to dramatically alter its pathogenic potential. Even changes in single segments PB2 and/or PB1 attenuated the human virus isolate's pathogenicity. 1957 and 1968 influenza pandemics were caused by replacement of the PB1 gene with avian PB1 (37). All eight virus segments causing the 1918 pandemic originated from an avian host (38). Thus, three pandemic viruses had avian-like PB1 genes that may be important to high replication efficiency, as we have demonstrated here with VN1203. Isolation of PB2 K^{627} in migrating birds (39, 40) is cause for concern because it appears to be a critical step for adaptation to a mammalian host. Those viruses may acquire additional changes in PB2, PB1 and HA resulting in the ability to spread from human to human.

MATERIALS AND METHODS

Generation of recombinant viruses by RG. A/Vietnam/1203/04 (H5N1) and A/chicken/Vietnam/C58/04 (H5N1) influenza viruses were obtained from the World Health Organization collaborating laboratories and propagated in allantoic cavities of 10-d-old embryonated chicken eggs. Experiments were conducted in biosafety level 3+ (BSL3+) conditions approved for work with these viruses. RT-PCR was used to amplify all eight viral genes, and viral cDNAs were inserted into dual-promoter plasmid pHW2000 (19). All plasmids were sequenced, and QuikChange Site-Directed Mutagenesis kits (Stratagene) were used to generate coding sequences in plasmids identical to PCR fragment sequences. Recombinant viruses were generated by DNA transfection of MDCK/293 T cells. Transfection supernatant was injected into 10-d-old embryonated chicken eggs, and virus stock prepared, sequenced and titrated.

Sequence analysis. Viral RNA isolated directly from virus-containing allantoic fluid using RNA isolation kit (RNeasy; QIAGEN). The universal primer set for influenza A virus was used for RT-PCR (41). Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital determined DNA template sequence using Big Dye Terminator (v.3) chemistry and synthetic oligonucleotides. Samples were analyzed on 3700 DNA analyzers (Applied Biosystems).

Inoculation of ferrets. Male ferrets, (Marshall's Farms) aged 3–5 mo and seronegative by HA inhibition test for exposure to circulating influenza B and H1N1, H3N2, and H5N1 influenza A viruses, were used. Ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 EID_{50} of infectious virus. Clinical signs of infection, weight, and temperature were recorded daily. Body temperature was measured using subcutaneous implantable temperature transponders. All animals impaired in their ability to eat or drink were killed. All animal studies were conducted under applicable laws and guidelines and after approval by St. Jude Children's Research Hospital animal care and use committee.

Titration of virus in ferret upper respiratory tract. On days 3, 5, and 7 after inoculation, ferrets were anesthetized with intramuscularly injected ketamine (25 mg/kg), and 0.5 ml of sterile PBS was introduced into each nostril and collected in containers. Virus was titrated in 10-d-old embryonated chicken eggs and expressed as $\log_{10}EID_{50}$/ml. Limit of detection was 1 $\log_{10}EID_{50}$/ml.

Virus titration and histopathologic analysis of ferret organs. Lung, brain, spleen, and liver were collected 4 d a.i. or at time of death. Samples were weighed and homogenized in PBS. Homogenates were titrated in 10-d-old embryonated chicken eggs to determine $\log_{10}EID_{50}$ per tissue gram. Samples were fixed in 10% buffered formalin, paraffin embedded, cut into 5-μm sections, mounted on slides, stained with hematoxylin and eosin, and examined by light microscopy.

Inoculation of mice. 7-wk-old C57BL6 female mice (The Jackson Laboratory) were lightly anesthetized with isoflurane and inoculated intranasally with 10^3 EID_{50} of infectious virus in 50 μl of PBS. Weight, clinical signs of infection, and survival were recorded daily.

Plaque assay. Confluent monolayers of MDCK cells were inoculated with 10-fold dilutions of influenza virus and incubated at 37°C for 1 h. The inoculum was removed, and cells were washed and overlaid with MEM containing 1% agarose and 0.2% serum albumin. After 3 d at 37°C, cells were stained with 0.1% crystal violet in 37% formaldehyde solution and plaque morphology evaluated. Plaque size was measured using fine scale magnifying comparator (6X). The probability of a significant difference in plaque size between viruses was computed using a Student's paired *t* test, with a two-tailed distribution.

Assay of virus binding to sialic acid–containing substrates. Virus binding to fetuin was measured by direct solid-phase assay with immobilized virus and horseradish peroxidase-conjugated fetuin (42, 43). Affinity to 3′- and 6′-sialylglycopolymer obtained by conjugation of 1-*N*-glycyl derivative of 3′-sialyllactose (p3′SL) or 6′-sialyllactose (p6′SL) was measured in competitive assay based on inhibition of peroxidase-labeled fetuin binding (44). Sialoglycopolymers were gifts from N. Bovin (Shemyakin Institute of

Bioorganic Chemistry, Moscow, Russia). The dissociation constant (K_d) of virus–receptor analogue complex was calculated for each virus as mean of four experiments.

Luciferase assays. 293T cells subconfluent monolayers (7.5×10^5 cells in 35-mm dishes) were transfected (Mirus Bio) with 2 μg of luciferase reporter plasmid (EGFP open reading frame in pHW72-EGFP substituted with luciferase gene [reference 20]) and mix of PB2, PB1, PA, and NP plasmids of VN1203 and CH58 viruses in quantities of 1, 1, 1, and 2 μg, respectively. After 24 h, cell extracts that were prepared in 500 μl of lysis buffer and luciferase levels were assayed with Luciferase Assay System (Promega) and BD Monolight 3010 luminometer (BD Biosciences). Experiments were performed in triplicate.

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