

# The Homologous Tripartite Viral RNA Polymerase of A/Swine/Korea/CT1204/2009(H1N2) Influenza Virus Synergistically Drives Efficient Replication and Promotes Respiratory Droplet Transmission in Ferrets

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We previously reported that influenza A/swine/Korea/1204/2009(H1N2) virus was virulent and transmissible in ferrets in which the respiratory-droplet-transmissible virus (CT-Sw/1204) had acquired simultaneous hemagglutinin (HA<sub>D225G</sub>) and neuraminidase (NA<sub>S315N</sub>) mutations. Incorporating these mutations into the nonpathogenic A/swine/Korea/1130/2009(H1N2, Sw/1130) virus consequently altered pathogenicity and growth in animal models but could not establish efficient transmission or noticeable disease. We therefore exploited various reassortants of these two viruses to better understand and identify other viral factors responsible for pathogenicity, transmissibility, or both. We found that possession of the CT-Sw/1204 tripartite viral polymerase enhanced replicative ability and pathogenicity in mice more significantly than did expression of individual polymerase subunit proteins. In ferrets, homologous expression of viral RNA polymerase complex genes in the context of the mutant Sw/1130 carrying the HA<sub>225G</sub> and NA<sub>315N</sub> modifications induced optimal replication in the upper nasal and lower respiratory tracts and also promoted efficient aerosol transmission to respiratory droplet contact ferrets. These data show that the synergistic function of the tripartite polymerase gene complex of CT-Sw/1204 is critically important for virulence and transmission independent of the surface glycoproteins. Sequence comparison results reveal putative differences that are likely to be responsible for variation in disease. Our findings may help elucidate previously undefined viral factors that could expand the host range and disease severity induced by triple-reassortant swine viruses, including the A(H1N1)pdm09 virus, and therefore further justify the ongoing development of novel antiviral drugs targeting the viral polymerase complex subunits.

Influenza A viruses are major viral respiratory pathogens that cause significant morbidity and mortality worldwide in the form of annually recurring seasonal epidemics. These viruses continue to be a threat to public health because of their unpredictable nature and the constant threat of new pandemic viruses. Influenza A viruses belong to the *Orthomyxoviridae* family of viruses, whose viral genome exists as eight single-stranded RNA segments with negative orientation (1). The segmented nature of the influenza virus genome is a key driver of the enigmatic viral evolution and ecology patterns observed through the years, because it allows reassortment in a suitable host (e.g., pigs) coinfecting with two or more viruses from different sources or lineages, which could lead to antigenic shift and the generation of pandemic viruses (2). Indeed, in early 2009, a novel reassortant influenza A H1N1 [A(H1N1)pdm09] virus, presumed to have come from pigs, caused the first human influenza pandemic of the 21st century (3, 4). The causative virus closely resembles the “triple-reassortant” swine H1 strains of the North American lineage that contain classical swine-like nucleoprotein (NP), nonstructural (NS) protein, human-like hemagglutinin (HA), basic polymerase 1 (PB1), and avian-like PB2 and acidic polymerase (PA) segments. However, A(H1N1)pdm09 had uniquely acquired the neuraminidase (NA) and matrix (M) segments of the Eurasian avian-like swine viruses (5, 6).

The emergence of the A(H1N1)pdm09 virus demonstrated that strains prevalent in swine populations can be the direct source of future influenza pandemics in humans. Some triple-reassortant swine viruses isolated in the United States before the 2009 pan-

demically induce severe disease in mice but are only moderately pathogenic in ferrets (7–9). However, their transmissibility by respiratory droplets (RD) in ferret models varies considerably depending on the HA and NA lineages (7). Recently, we have shown that the A/swine/Korea/1204/2009(H1N2) Korean swine virus, which is genetically related to North American strains and was similarly isolated before the 2009 pandemic, is virulent in ferrets and is efficiently transmitted to naive RD contacts (10). Viruses recovered from the RD contact ferrets (CT-Sw/1204) had acquired Asp-225-Gly (H3 numbering) and Ser-315-Asn (N2 numbering) mutations in their HA and NA proteins, respectively, contributing to the altered replicative ability, pathogenicity, and transmissibility. However, when HA<sub>225G</sub> and NA<sub>315N</sub> were incorporated into another H1N2 virus, A/swine/Korea/1130/2009 (Sw/1130), the same degree of pathogenesis and ease of RD transmission were not observed, indicating the genetic relevance of the internal gene constellation of CT-Sw/1204. In the present study, we attempted to further elucidate the viral factors that contribute to pathogenicity and transmissibility. To address these issues, we created a panel of reassortant CT-Sw/1204 and Sw/1130 viruses to

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**TABLE 1** Replication and virulence of recombinant parental and reassortant Sw/1130 viruses in mice

Recombinant virus <sup>a</sup>	Stock titer (TCID <sub>50</sub> /ml)	Lung viral titer (SD) <sup>b</sup>		
		3 days p.i.	5 days p.i.	MLD <sub>50</sub> <sup>c</sup>
<b>Sw/1130</b>	2.0 × 10 <sup>6</sup>	2.2 (0.2)	1.7 (0.1)	>6
CT-Sw/1204 <sub>HA</sub>	1.0 × 10 <sup>7</sup>	3.9 (0.3) <sup>d</sup>	3.5 (0.3) <sup>d</sup>	4.9
CT-Sw/1204 <sub>NA</sub>	2.0 × 10 <sup>6</sup>	2.4 (0.2)	2.1 (0.2)	>6
CT-Sw/1204 <sub>PB2</sub>	1.0 × 10 <sup>7</sup>	3.6 (0.2) <sup>d</sup>	3.4 (0.3) <sup>e</sup>	5.1
CT-Sw/1204 <sub>PB1</sub>	1.0 × 10 <sup>6</sup>	1.9 (0.1)	-	>6
CT-Sw/1204 <sub>PA</sub>	4.0 × 10 <sup>6</sup>	3.7 (0.3) <sup>e</sup>	4.2 (0.4) <sup>e</sup>	5.3
CT-Sw/1204 <sub>NP</sub>	2.0 × 10 <sup>6</sup>	2.7 (0.2) <sup>e</sup>	2.8 (0.2) <sup>e</sup>	5.5
CT-Sw/1204 <sub>M</sub>	2.0 × 10 <sup>6</sup>	2.2 (0.2)	2.1 (0.2)	>6
CT-Sw/1204 <sub>NS</sub>	6.3 × 10 <sup>6</sup>	2.4 (0.2)	3.0 (0.3)	5.5
CT-Sw/1204 <sub>HA-NA</sub>	4.0 × 10 <sup>7</sup>	3.7 (0.3) <sup>d</sup>	3.7 (0.3) <sup>d</sup>	4.5
CT-Sw/1204 <sub>3P</sub>	1.0 × 10 <sup>7</sup>	4.9 (0.4) <sup>d</sup>	3.7 (0.3) <sup>e</sup>	4.7
CT-Sw/1204 <sub>VRNP</sub>	2.0 × 10 <sup>7</sup>	4.9 (0.5) <sup>d</sup>	3.9 (0.3) <sup>e</sup>	4.7
<b>CT-Sw/1204</b>	3.2 × 10 <sup>8</sup>	5.9 (0.5) <sup>d</sup>	5.2 (0.4) <sup>e</sup>	3.5

<sup>a</sup> Reassortant virus names were assigned according to the origin of the exchanged genes (e.g., CT-Sw/1204<sub>HA</sub> denotes a reassortant bearing the HA of CT-Sw/1204 with the rest of its genes from Sw/1130). Parental strains are in boldface.

<sup>b</sup> Obtained from mice inoculated intranasally with 10<sup>5</sup> TCID<sub>50</sub> of the indicated virus; titers are expressed as log<sub>10</sub> TCID<sub>50</sub>/g. The dash denotes negative detection (<1.5 log<sub>10</sub> TCID<sub>50</sub>/g).

<sup>c</sup> Determined by inoculating mice with 10<sup>2</sup> to 10<sup>6</sup> TCID<sub>50</sub> of virus and expressed as the log<sub>10</sub> TCID<sub>50</sub> required to yield 1 LD<sub>50</sub>.

<sup>d</sup> *P* < 0.005 comparing virus titers relative to Sw/1130-inoculated mice (two-tailed unpaired *t* test).

<sup>e</sup> *P* < 0.05 comparing virus titers relative to Sw/1130-inoculated mice (two-tailed unpaired *t* test).

compare disease phenotypes in mice and ferrets and found that the pathogenicity and transmissibility of the CT-Sw/1204 virus may have been innate to the field virus isolate. We provide evidence that the viral RNA polymerases are critical factors for altered pathogenicity and virus dissemination.

## MATERIALS AND METHODS

**Cells and viruses.** Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium with Earle's salts (EMEM) (Lonza, Switzerland) containing 5% fetal bovine serum (FBS) (Gibco, NY), and 293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FBS. Primary normal human bronchial epithelial (NHBE) cells were purchased from ScienCell Research Laboratories (Carlsbad, CA) and grown in bronchial epithelial cell growth medium (BEpiCM; Gibco) as defined and recommended by ScienCell. All cells were incubated at 37°C in 5% CO<sub>2</sub>.

**Generation of recombinant and reassortant viruses.** The eight gene segments of the H1N2 swine viruses CT-Sw/1204 and Sw/1130 (10) were amplified and cloned into the pHW2000 plasmid vector to generate recombinant viruses through a plasmid-based reverse-genetics system as previously described (Tables 1 and 2) (11). All recombinant parental and reassortant viruses were rescued in cocultured 293T and MDCK cell mixtures transfected (TransIT-LT1; Mirus Bio) with the eight viral plasmids as indicated. The transfection medium was removed after 6 h and replaced with Opti-MEM I (Gibco). After 30 h, Opti-MEM I (500 μl) containing L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (0.2 μg/ml; Sigma-Aldrich) was added to the transfected cells. Supernatants were harvested 48 h posttransfection and used to inoculate MDCK cells for the production of stock viruses. The eight genes of each rescued virus were partially sequenced (Cosmo GeneTech, Seoul, Republic of Korea) to confirm the genotype and origin of the viral segments. The handling of viruses and virus rescue were performed in an enhanced biosafety level 3 (BSL3+) containment laboratory as approved by the Korean Centers for Disease Control and Prevention.

**TABLE 2** Replication and virulence of recombinant parental and reassortant CT-Sw/1204 viruses in mice

Recombinant virus <sup>a</sup>	Stock titer (TCID <sub>50</sub> /ml)	Lung viral titer (SD) <sup>b</sup>		
		3 days p.i.	5 days p.i.	MLD <sub>50</sub> <sup>c</sup>
<b>CT-Sw/1204</b>	3.2 × 10 <sup>8</sup>	6.5 (0.5)	6.2 (0.6)	3.5
Sw/1130 <sub>HA</sub>	5.0 × 10 <sup>6</sup>	4.8 (0.4) <sup>d</sup>	4.5 (0.4) <sup>d</sup>	4.8
Sw/1130 <sub>NA</sub>	8.0 × 10 <sup>7</sup>	6.3 (0.6)	6.0 (0.6)	3.7
Sw/1130 <sub>PB2</sub>	3.2 × 10 <sup>7</sup>	5.5 (0.5) <sup>d</sup>	5.1 (0.5) <sup>d</sup>	4.3
Sw/1130 <sub>PB1</sub>	8.0 × 10 <sup>7</sup>	6.5 (0.5)	6.1 (0.6)	3.5
Sw/1130 <sub>PA</sub>	1.3 × 10 <sup>7</sup>	6.0 (0.6) <sup>d</sup>	5.6 (0.5) <sup>d</sup>	4
Sw/1130 <sub>NP</sub>	8.0 × 10 <sup>7</sup>	6.1 (0.6)	5.9 (0.6)	3.7
Sw/1130 <sub>M</sub>	1.3 × 10 <sup>8</sup>	6.5 (0.6)	5.9 (0.5)	3.5
Sw/1130 <sub>NS</sub>	6.3 × 10 <sup>8</sup>	6.2 (0.6)	6.3 (0.6)	3.8
Sw/1130 <sub>HA-NA</sub>	1.3 × 10 <sup>7</sup>	5.1 (0.5) <sup>d</sup>	4.8 (0.4) <sup>d</sup>	5.2
Sw/1130 <sub>3P</sub>	6.3 × 10 <sup>6</sup>	4.5 (0.4) <sup>d</sup>	4.1 (0.3) <sup>d</sup>	4.7
Sw/1130 <sub>VRNP</sub>	6.3 × 10 <sup>6</sup>	4.6 (0.4) <sup>d</sup>	4.4 (0.4) <sup>d</sup>	5.1
<b>Sw/1130</b>	2.0 × 10 <sup>6</sup>	2.9 (0.30) <sup>d</sup>	2.5 (0.2) <sup>d</sup>	> 6

<sup>a</sup> Reassortant virus names were assigned according to the origin of the exchanged genes (e.g., Sw/1130<sub>HA</sub> denotes a reassortant bearing the HA of Sw/1130 with the rest of its genes from CT-Sw/1204). Parental strains are in boldface.

<sup>b</sup> Obtained from mice inoculated intranasally with 10<sup>5</sup> TCID<sub>50</sub> of the respective viruses; titers are expressed as log<sub>10</sub> TCID<sub>50</sub>/g.

<sup>c</sup> Determined by inoculating mice with 10<sup>2</sup> to 10<sup>6</sup> TCID<sub>50</sub> of virus and expressed as the log<sub>10</sub> TCID<sub>50</sub> required to yield 1 LD<sub>50</sub>.

<sup>d</sup> *P* < 0.05 comparing virus titers relative to CT-Sw/1204-inoculated mice (two-tailed unpaired *t* test).

**Viral growth kinetics *in vitro*.** Growth characteristics *in vitro* were compared by inoculating MDCK cells at a multiplicity of infection (MOI) of 0.001 and NHBE cells at an MOI of 0.1. Infectious cell culture supernatants were collected at 6, 12, 24, 48, and 72 h postinoculation (p.i.) for virus titration. All 24 recombinant parental and reassortant viruses were examined in six-well plates (in 2.5 ml trypsinized medium), and supernatants were collected at the designated time points (at 200 μl). The 50% tissue culture infective doses (TCID<sub>50</sub>) of viruses in stock cultures, nasal washes, tissue homogenates, and culture supernatants were endpoint titrated in MDCK cells and calculated by using the Reed and Muench method (12), with results expressed as log<sub>10</sub> TCID<sub>50</sub> per ml of supernatant or log<sub>10</sub> TCID<sub>50</sub> per gram of tissue, as appropriate. The limit of virus detection was 2.5 log<sub>10</sub> TCID<sub>50</sub> (*in vitro*) or 1.5 log<sub>10</sub> TCID<sub>50</sub> (*in vivo*) per unit sample tested, as indicated. Student's *t* test was used for statistical comparisons and calculated using GraphPad Prism software (version 6); the threshold of significance was set at a *P* value of <0.05.

**Experimental inoculation of mice and ferrets.** Groups of 16 6-week-old female BALB/c mice (weighing ≥ 18 g/mouse; Samtaco, Seoul, Republic of Korea) were anesthetized and inoculated intranasally with 10<sup>4</sup> or 10<sup>5</sup> TCID<sub>50</sub> of the designated virus (50-μl total volume). Three mice from each inoculation group were humanely killed 3 and 5 days p.i. for lung viral titrations; the remaining 10 mice of each group were monitored daily for up to 14 days for weight change, morbidity, and mortality. Mice that lost 30% or more of their starting body weight were humanely killed. The 50% lethal doses (LD<sub>50</sub>) of viruses that killed more than 50% of the mice were determined in additional groups of mice that were inoculated intranasally with 10-fold serial dilutions (10<sup>2</sup> to 10<sup>6</sup> TCID<sub>50</sub>/50 μl) of virus.

Groups of three 14- to 16-week-old female outbred ferrets (*Mustela putorius furo*; Wuxi Sangosho Pet Park Co., China) that were seronegative for influenza virus exposure/infection by hemagglutination inhibition (HI) and NP enzyme-linked immunosorbent assays (ELISAs) were inoculated intranasally (300 μl/nostril) and intratracheally (400 μl) with the indicated recombinant virus (10<sup>5.5</sup> TCID<sub>50</sub>/ml) as previously described (10). At 1 day p.i., each inoculated ferret was paired with a seronegative ferret (in a 1:1 setup) in barrier-separated transmission isolators (35 mm apart) that permitted only aerosol contact. At 5 days p.i., 1 of the 3 infected ferrets in each group was humanely killed for virus detection in various

tissues (trachea, lung, spleen, intestine, and brain) and for lung histopathologic examination. Signs of morbidity (e.g., weight change, fever, or sneezing) were monitored daily for 14 days. Each test group consisted of 2 infected and 2 RD contact ferrets. Viral growth in the upper respiratory tract was determined by collecting nasal washes from inoculated ferrets on days 1, 3, 5, 7, and 9 p.i. or daily up to 12 days p.i. from RD contact ferrets and then performing endpoint titration in MDCK cells.

Blood samples were collected from mice and ferrets before inoculation and at 18 to 21 days p.i. HI assays using 0.7% turkey red blood cells were used to analyze seroreactivity according to standard methods (13). Animal experiments were performed in a BSL3+ containment facility approved by the Korea Centers for Disease Control and Prevention, and general animal care guidelines required by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Republic of Korea, were followed.

**Mini-genome replication assays.** Luciferase mini-genome reporter assays were performed using influenza A virus M-driven luciferase reporter plasmid (pHW72-Luci\_M) (14). Briefly, 293T cells were prepared in 24-well plates 24 h before use and transfected with 0.1  $\mu$ g each of pHW72-Luci\_M, pHW2000-PB2, pHW2000-PB1, pHW2000-PA, pHW2000-NP, and pCMV- $\beta$ -galactosidase plasmids. After 6 h, the transfection medium was replaced with fresh DMEM containing 10% FBS. After 24 h, the cells were washed with PBS and lysed for 30 min with 100  $\mu$ l of lysis buffer (Promega). The cell lysates were then harvested, and luciferase activity was assayed in triplicate by using the Promega luciferase assay system. The results were normalized against the  $\beta$ -galactosidase activity level of the cells.

**Lung pathological examination.** Portions of tissue samples harvested from ferrets on day 5 postinoculation were fixed in 10% neutral buffered formalin. The tissues were then processed for paraffin embedding, sectioned (4  $\mu$ m thick), and examined in the pathology laboratory of Chungbuk National University Hospital by using standard hematoxylin and eosin staining and light microscopy (Olympus IX71;  $\times 40$  and  $\times 200$  magnifications).

## RESULTS

**Virus rescue and viability *in vitro*.** The inability of the Sw/1130 virus to induce severe clinical disease in mice and ferrets provided a framework with which to identify other viral factors of the CT-Sw/1204 virus that are required to cause noticeable morbidity. To determine which of the viral gene segments of CT-Sw/1204 other than HA<sub>225G</sub> and NA<sub>315N</sub> contributed to pathogenicity, we used Sw/1130 and CT-Sw/1204 to generate a series of reassortant viruses and compared their characteristics to those of the recombinant parental strains (Tables 1 and 2). Reassortant viruses were named according to the origin of their exchanged genes. For example, CT-Sw/1204<sub>HA</sub> denotes a reassortant bearing the HA of CT-Sw/1204 with the rest of its genes from Sw/1130. To examine independently the effects of the surface glycoproteins (HA and NA in combination), the tripartite viral RNA polymerase (3P) components (PA, PB1, and PB2), and the RNA polymerase components together with NP (i.e., viral ribonucleoprotein complex [vRNP]: 3P plus NP), we also generated CT-Sw/1204<sub>HA-NA</sub>, CT-Sw/1204<sub>3P</sub>, and CT-Sw/1204<sub>vRNP</sub> reassortant viruses.

The stock titer of the parental Sw/1130 recombinant virus was low ( $2.0 \times 10^6$  TCID<sub>50</sub>/ml) but was increased by the incorporation of the CT-Sw/1204 HA, PB2, HA-NA, 3P, and vRNP genes (i.e., stock titers reached at least  $10^7$  TCID<sub>50</sub>/ml) (Table 1). Among these recombinant viruses, the CT-Sw/1204<sub>PB1</sub> virus was particularly difficult to rescue. Although it was eventually rescued, it produced the lowest virus stock titers ( $8.0 \times 10^5$  TCID<sub>50</sub>/ml). However, most recombinant viruses in the background of CT-Sw/1204 produced higher stock titers (reaching up to  $10^8$  TCID<sub>50</sub>/ml) in

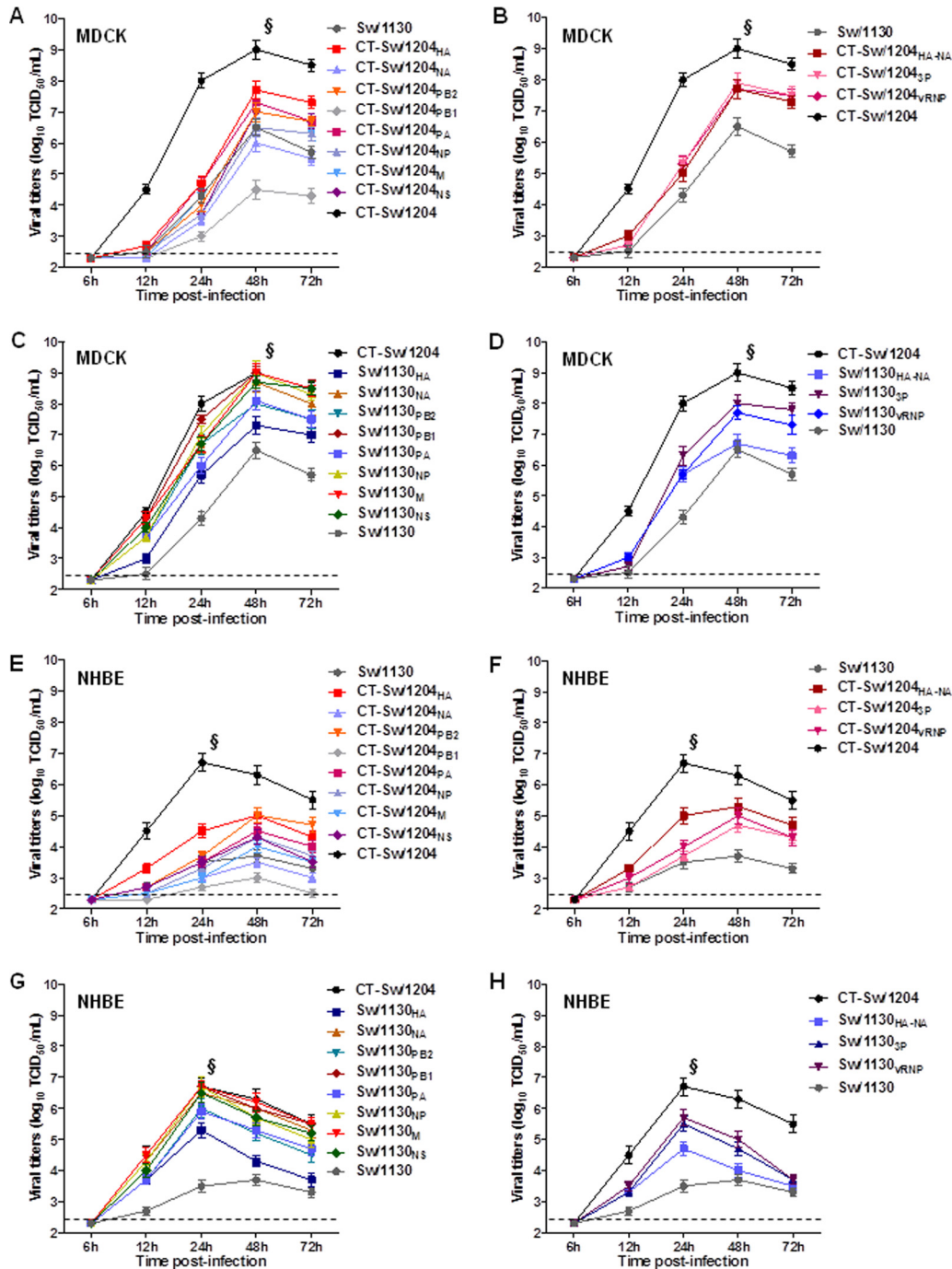
MDCK cells than did those in the Sw/1130 background. Replacing the HA, PB2, PA, HA-NA, 3P, and vRNP segments of CT-Sw/1204 with those from Sw/1130 reduced virus titers by at least  $1.0 \times 10^{1.5}$  TCID<sub>50</sub>/ml (Table 2).

**Comparison of the replicative abilities of recombinant viruses *in vitro*.** We first examined whether swapping the corresponding viral segments would affect viral growth kinetics in cell culture by comparing the amounts of infectious virus released in culture supernatants. In MDCK cells, the parental CT-Sw/1204 virus exhibited rapid growth, yielding mean peak titers that were at least 100-fold higher than those of the Sw/1130 virus ( $P < 0.001$ ) (Fig. 1A). Viruses possessing the HA of CT-Sw/1204 (i.e., CT-Sw/1204<sub>HA</sub> and CT-Sw/1204<sub>HA-NA</sub>) replicated more efficiently than did Sw/1130 (peak titers of 7.7 to 8.0 log<sub>10</sub> TCID<sub>50</sub>/ml versus 6.5 log<sub>10</sub> TCID<sub>50</sub>/ml) (Fig. 1A and B), whereas the Sw/1130<sub>HA</sub> and Sw/1130<sub>HA-NA</sub> viruses yielded lower mean peak titers than the parental CT-Sw/1204 virus (6.7 to 7.3 log<sub>10</sub> TCID<sub>50</sub>/ml versus 9.0 log<sub>10</sub> TCID<sub>50</sub>/ml) (Fig. 1C and D). The CT-Sw/1204<sub>PB1</sub> recombinant had the lowest replication kinetics (4.5 log<sub>10</sub> TCID<sub>50</sub>/ml mean peak titer), consistent with its low infectivity in these cells (Fig. 1A and Table 1).

Possession of the CT-Sw/1204 PB2, PA, 3P, or vRNP increased the replication efficiency of the Sw/1130 virus, reaching at least 7.0 log<sub>10</sub> TCID<sub>50</sub>/ml mean peak titers at 48 h p.i. (Fig. 1A and B), suggesting that these segments are essential for the optimal replication of CT-Sw/1204 in MDCK cells, apart from HA or HA-NA. In the background of the CT-Sw/1204 virus, Sw/1130<sub>PB1</sub>, Sw/1130<sub>NP</sub>, Sw/1130<sub>M</sub>, and Sw/1130<sub>NS</sub> displayed replicative abilities commensurate with those of CT-Sw/1204, indicating that replacing these segments does not significantly affect viral growth kinetics *in vitro* (Fig. 1C and D). However, Sw/1130<sub>PB2</sub>, Sw/1130<sub>PA</sub>, Sw/1130<sub>3P</sub>, and Sw/1130<sub>vRNP</sub> reassortants had slightly lower mean peak titers (7.7 to 8.0 log<sub>10</sub> TCID<sub>50</sub>/ml).

In primary NHBE cells, similar replication trends were observed. CT-Sw/1204 rapidly grew to high titers at 24 h p.i., whereas Sw/1130 produced lower viral titers that peaked at 48 h ( $P < 0.001$ ) (Fig. 1E). Among reassortant viruses, swapping the PB2 and PA segments marginally altered the replication of the corresponding parental strains; however, greater differences were observed with the homologous substitution of the viral RNA polymerases (i.e., CT-Sw/1204<sub>3P</sub> and CT-Sw/1204<sub>vRNP</sub> reassortants) (Fig. 1E to H). Regardless, more significantly different titers were produced when HA or HA-NA was replaced. Overall, growth of CT-Sw/1204 and Sw/1130 differed significantly *in vitro*, and substituting the viral surface genes, except for NA alone, subsequently affected *in vitro* viral growth.

**The heterotrimeric viral RNA polymerase of CT-Sw/1204, independent of HA and NA, contributes to efficient replication and pathogenicity in mice.** To determine whether the apparent variable growth kinetics in cultured cells also translate into distinct virus characteristics in animal models, we evaluated the replicative abilities and pathogenicities of the parental and reassortant viruses in groups of BALB/c mice. First, the highest possible standard dose for the recombinants possessing the CT-Sw/1204 viral segments in the background of Sw/1130 were used to inoculate groups of mice for determinations of morbidity and virus replication in the lungs (Table 1 and Fig. 2). The parental Sw/1130 recombinant virus produced significantly lower viral titers in harvested mouse lungs than did the recombinant CT-Sw/1204 virus ( $2.2 \log_{10}$  TCID<sub>50</sub>/g and  $5.9 \log_{10}$  TCID<sub>50</sub>/g, respec-



**FIG 1** Replicative abilities of recombinant viruses in cultured cells. MDCK (A to D) and primary NHBE (E to H) cells were infected with recombinant parental or reassortant virus. Virus titers in aliquots of harvested supernatants were determined at the indicated time points. The replicative abilities of parental viruses were compared with those of reassortants carrying segments from CT-Sw/1204 (A, B, E, and F) or Sw/1130 (C, D, G, and H). The values are means ( $\pm$  standard deviations [SD]) from three independently performed experiments at 37°C. The dashed lines indicate the detection limit ( $2.5 \log_{10}$  TCID<sub>50</sub>/ml). \$,  $P < 0.001$  relative to peak titers of recombinant parental Sw/1130 virus.

tively;  $P = 0.0021$ ) and did not induce appreciable signs of morbidity (Table 1).

Incorporation of PB1 from CT-Sw/1204 into the Sw/1130 background did not produce high viral titers in mouse lungs at 3 or 5 days p.i.; the reassortant virus (CT-Sw/1204<sub>PB1</sub>) could not be recovered from any mice at 5 days p.i., indicating attenuation,

which coincided with its low level of *in vitro* replication (Fig. 1A). However, expression of HA or HA-NA from CT-Sw/1204 increased replication of Sw/1130 by almost 100-fold ( $P = 0.002$  and  $0.004$ , respectively) (Table 1). Inoculated mice experienced at least 23% body weight reductions, and 40% of the mice died during the 14-day observation period after inoculation (Fig. 2A to D). Apart

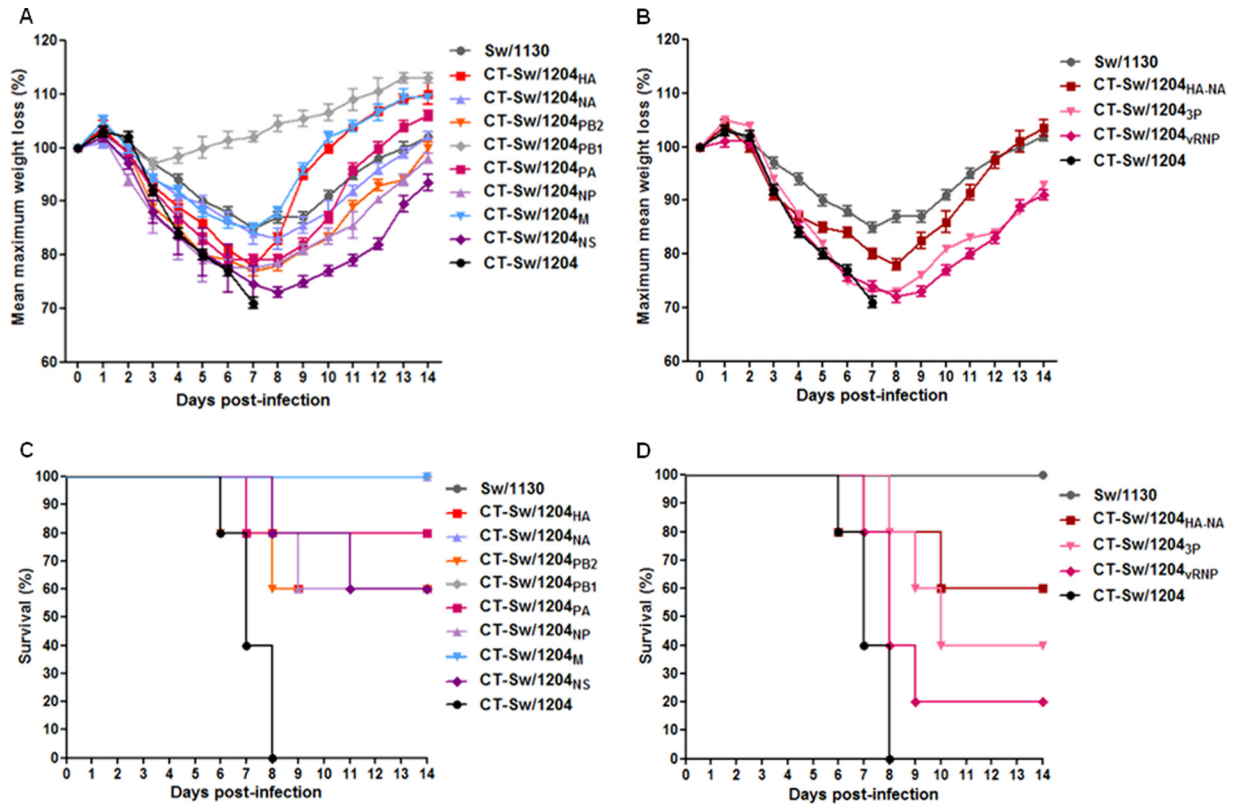


FIG 2 Pathogenicities of recombinant Sw/1130 reassortant viruses carrying CT-Sw/1204 segments in mice. Ten mice were inoculated intranasally with the indicated viruses. All groups were observed for morbidity (A and B) and mortality (C and D) for 14 days. The error bars indicate SD.

from those reassortants, possession of the CT-Sw/1204 PB2, PA, NP, NS, 3P, or vRNP complex significantly elevated virus replication (Table 1). All six of the reassortant viruses caused severe disease and lethality; however, the CT-Sw/1204<sub>3P</sub> and CT-Sw/1204<sub>vRNP</sub> reassortants appeared to be the most pathogenic, with 60% to 80% of mice experimentally inoculated with the viruses dying within 10 days (Fig. 2). The CT-Sw/1204<sub>NS</sub> reassortant also had a higher mean lung virus titer at 5 days p.i. than did the parental strain, but this difference was not statistically significant ( $P = 0.0853$ ), although 40% of the inoculated mice died.

We next assessed the growth kinetics of reassortant CT-Sw/1204 viruses bearing segments of the low-pathogenicity Sw/1130 virus in groups of mice to determine their virulence (Table 2 and Fig. 3). Yielding results similar to those discussed above, the parental CT-Sw/1204 virus replicated efficiently in mouse lungs, producing viral titers at 3 days p.i. that were at least 1,000-fold higher than those of the Sw/1130 virus ( $6.5 \log_{10}$  TCID<sub>50</sub>/g versus  $2.9 \log_{10}$  TCID<sub>50</sub>/g;  $P = 0.0075$ ) (Table 2). Replication of single-reassortant viruses containing the NA, PB1, NP, M, or NS of Sw/1130 did not significantly differ from that of the parental CT-Sw/1204 virus; however, the reassortants induced rapid reduction of mean body weight and started killing mice as early as 4 days p.i. (Fig. 3A and B). The death of mice infected with Sw/1130<sub>NP</sub> or Sw/1130<sub>NS</sub> was delayed by 1 or 2 days, but all mice eventually succumbed by 9 days p.i. In contrast to the lethal outcome induced by infection with these viruses, inoculation with Sw/1130<sub>HA</sub> induced significantly lower lung viral titers at both of the time points tested ( $P = 0.008$ ), which corresponded to <25% weight

loss and 20% survival rates (Fig. 3A and C). Although Sw/1130<sub>PB2</sub> and Sw/1130<sub>PA</sub> reassortant viruses also induced significantly lower titers in mouse lungs (Table 2), only the PB2 replacement could rescue 20% of the mice infected with the higher dose of virus. Sw/1130<sub>3P</sub> and Sw/1130<sub>vRNP</sub> reassortants had viral titers that were significantly lower than those of CT-Sw/1204 (Table 2). Hampered viral growth correlated with the reduced signs of clinical disease and weight loss that resulted in survival of 60% and 80% of inoculated mice, respectively (Fig. 3). These findings showed that the reciprocal constellation of the Sw/1130<sub>3P</sub> or Sw/1130<sub>vRNP</sub> segment is sufficient to alter the replication of CT-Sw/1204 despite the presence of HA and NA.

**Efficient viral growth mediates virulence of recombinant viruses.** We also determined the LD<sub>50</sub> in mice inoculated with 10-fold serial dilutions of the recombinant viruses (Tables 1 and 2). The fifty percent mouse lethal doses (MLD<sub>50</sub>s) of the recombinant parental CT-Sw/1204 and Sw/1130 viruses were  $3.5 \log_{10}$  TCID<sub>50</sub> and  $6 \log_{10}$  TCID<sub>50</sub>, respectively. As expected, the Sw/1130<sub>HA</sub> and Sw/1130<sub>HA-NA</sub> reassortant viruses had the greatest decrease in virulence (the MLD<sub>50</sub> increased from  $3.5 \log_{10}$  TCID<sub>50</sub> to  $5.2 \log_{10}$  TCID<sub>50</sub>), but these changes still could not completely attenuate CT-Sw/1204, indicating that the remaining viral gene segments contributed to virulence (Table 2). Recombinant CT-Sw/1204 viruses expressing the NA, PB1, NP, M, or NS of Sw/1130 retained the virulent phenotype (MLD<sub>50</sub>s ranged from  $3.5 \log_{10}$  TCID<sub>50</sub> to  $3.8 \log_{10}$  TCID<sub>50</sub>), whereas reassortant viruses carrying its PB2 or PA had moderately attenuated virulence ( $4.0 \log_{10}$  TCID<sub>50</sub> to  $4.3 \log_{10}$  TCID<sub>50</sub>). Incorporation of the vRNA polymerase subunit of

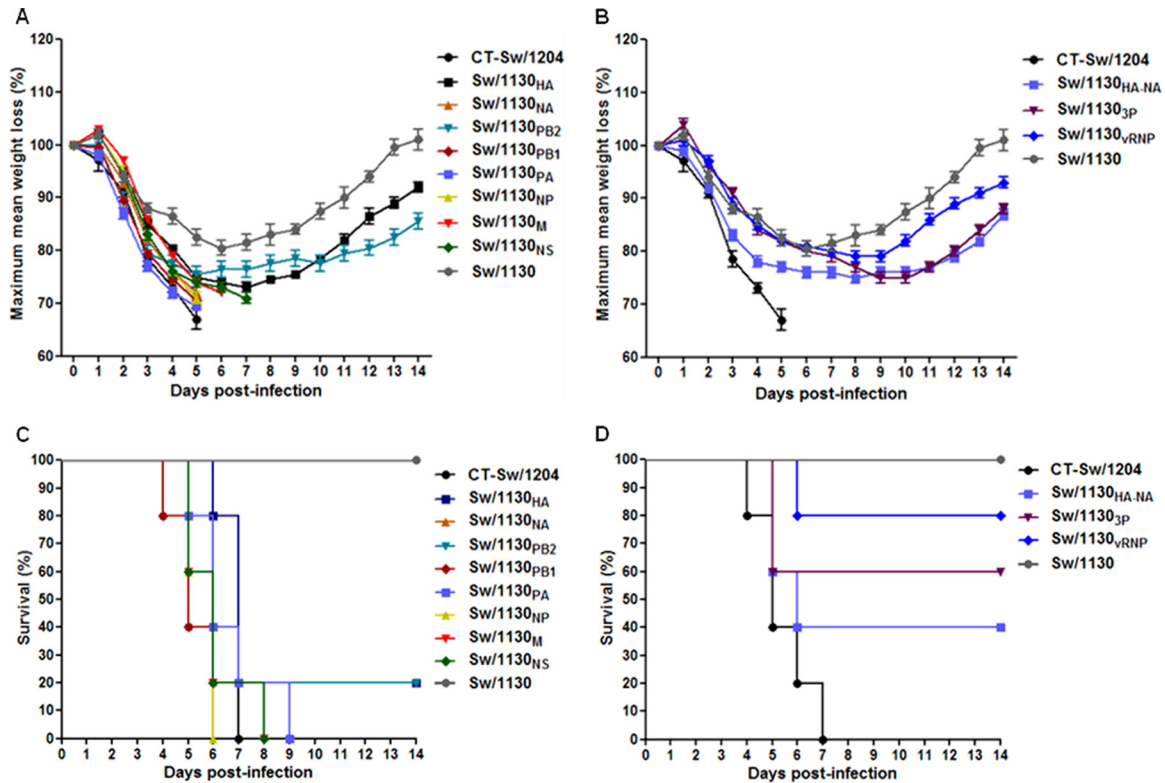
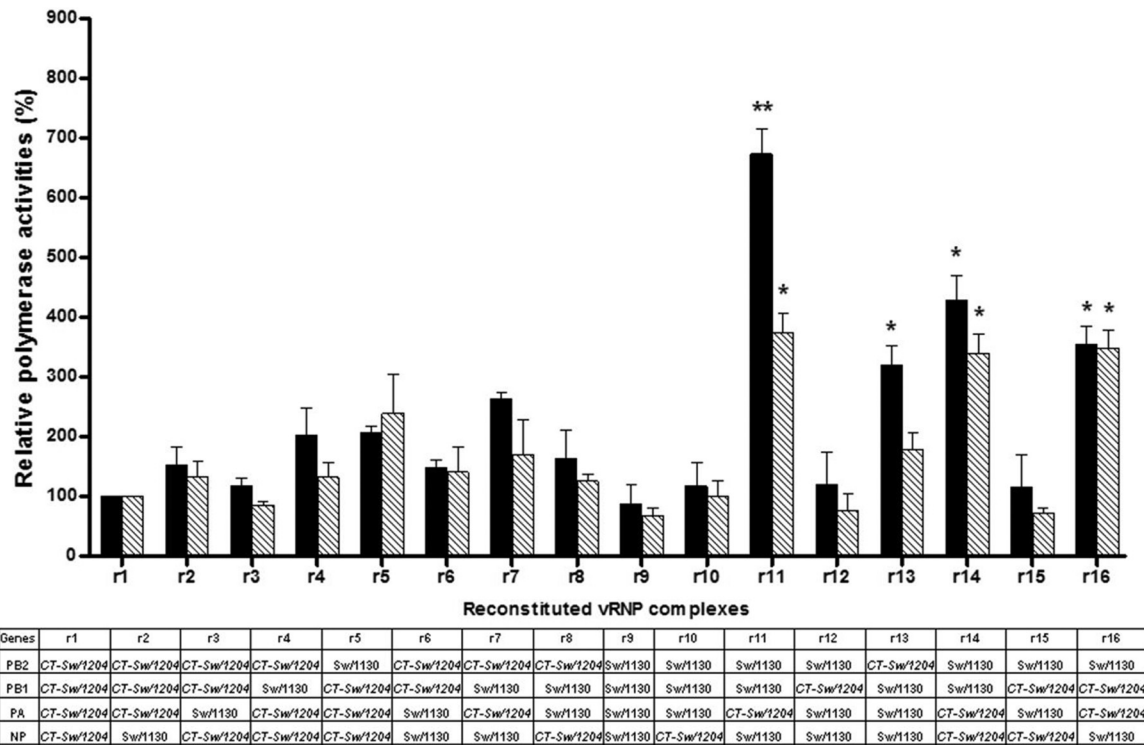


FIG 3 Pathogenicities of recombinant CT-Sw/1204 reassortant viruses carrying Sw/1130 segments in mice. Ten mice were inoculated intranasally with the indicated viruses. All groups were observed for morbidity (A and B) and mortality (C and D) for 14 days. The error bars indicate SD.

Sw/1130, irrespective of the source of NP, further dampened virulence (by 4.7 MLD<sub>50</sub> to 5.0 MLD<sub>50</sub>) relative to that observed with the individual Sw/1130<sub>PB2</sub> or Sw/1130<sub>PA</sub> replacement. Conversely, possession of the CT-Sw/1204 3P or vRNP complex increased virulence in mice, although the virulence was still considerably lower than that of the intact CT-Sw/1204 parental virus (4.7 MLD<sub>50</sub>s versus 3.5 MLD<sub>50</sub>s) (Table 1). These results are consistent with findings in the viral growth experiments in which CT-Sw/1204<sub>3P</sub> and CT-Sw/1204<sub>vRNP</sub> reassortants, apart from CT-Sw/1204<sub>HA</sub> and CT-Sw/1204<sub>HA-NA</sub>, replicated to higher titers in mouse lungs than did the parental Sw/1130 strain. Thus, the efficient viral growth induced by the viral polymerases contributed to the virulence of the CT-Sw/1204 virus.

As previously stated, the triple-reassortant internal gene cascades of CT-Sw/1204 and Sw/1130 share approximately 91% genetic identity, with no indications of further genetic reassortment with other viruses of different lineages. However, comparing the deduced amino acid sequences revealed molecular differences between corresponding viral segments of each virus, mostly in PB2, PA, and NS1, but these changes did not appear to be associated with markers that define pathogenicity or alter the host range (data not shown). Sequence variations possessed by CT-Sw/1204 were also different from those of an ancestral North American triple-reassortant swine H3N2 (A/swine/Texas/4199/1998) and a prototypical A(H1N1)pdm09 (A/California/04/2009) virus. However, some of these sites appear to have base residues similar to those of the 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) pandemic viruses, suggesting that acquisition of molecular adaptive mechanisms occurs during persistent circulation in swine hosts.

**Mini-genome replication assays for various combinations of reconstituted vRNP gene complexes.** The influenza virus vRNP complex, encoded by the PA, PB1, PB2, and NP genes, is responsible for the transcription and replication of the viral RNA genome. To obtain functional correlates that could help elucidate the mechanism underlying the observed differences in replication efficiency and pathogenic phenotype, we exploited a mini-genome replication reporter assay, which detects any potential contribution of the vRNP complex genes individually or synergistically. The polymerase activities of 16 possible combinations of vRNPs of CT-Sw/1204 and Sw/1130 origin were examined at 33°C or 37°C (Fig. 4), temperatures simulating those in the human upper and lower respiratory tracts (15). Under both thermal conditions, the intact CT-Sw/1204 vRNP complex (Fig. 4, r1) had higher polymerase transcription and replication efficiencies than did the reconstituted Sw/1130 (r9) (33% to 50% difference). Replacing the NP of CT-Sw/1204 with that of Sw/1130 (r2) increased polymerase activity by at least 30%, indicating that the viral origin of segment 5 in our study could not markedly affect the synergistic transcription and replication efficiencies of the 3 viral polymerase segments. Possession of PB1 (r4) or PB1-NP (r7) from Sw/1130 improved the polymerase activities of the CT-Sw/1204 virus by approximately 125% at 33°C and 30% to 70% at 37°C. Conversely, the vRNP complex carrying PA (r11) from CT-Sw/1204 consistently induced the highest activities among the various RNP combinations under any thermal conditions examined. Acquisition of CT-Sw/1204 PB2 (r13) resulted in an approximately 130% increase in polymerase activity over that of Sw/1130 at 33°C but only an approximately 70% increase at 37°C, whereas the CT-Sw/1204 NP (r10) or PB1 (r12) could not significantly elevate *in vitro* rep-



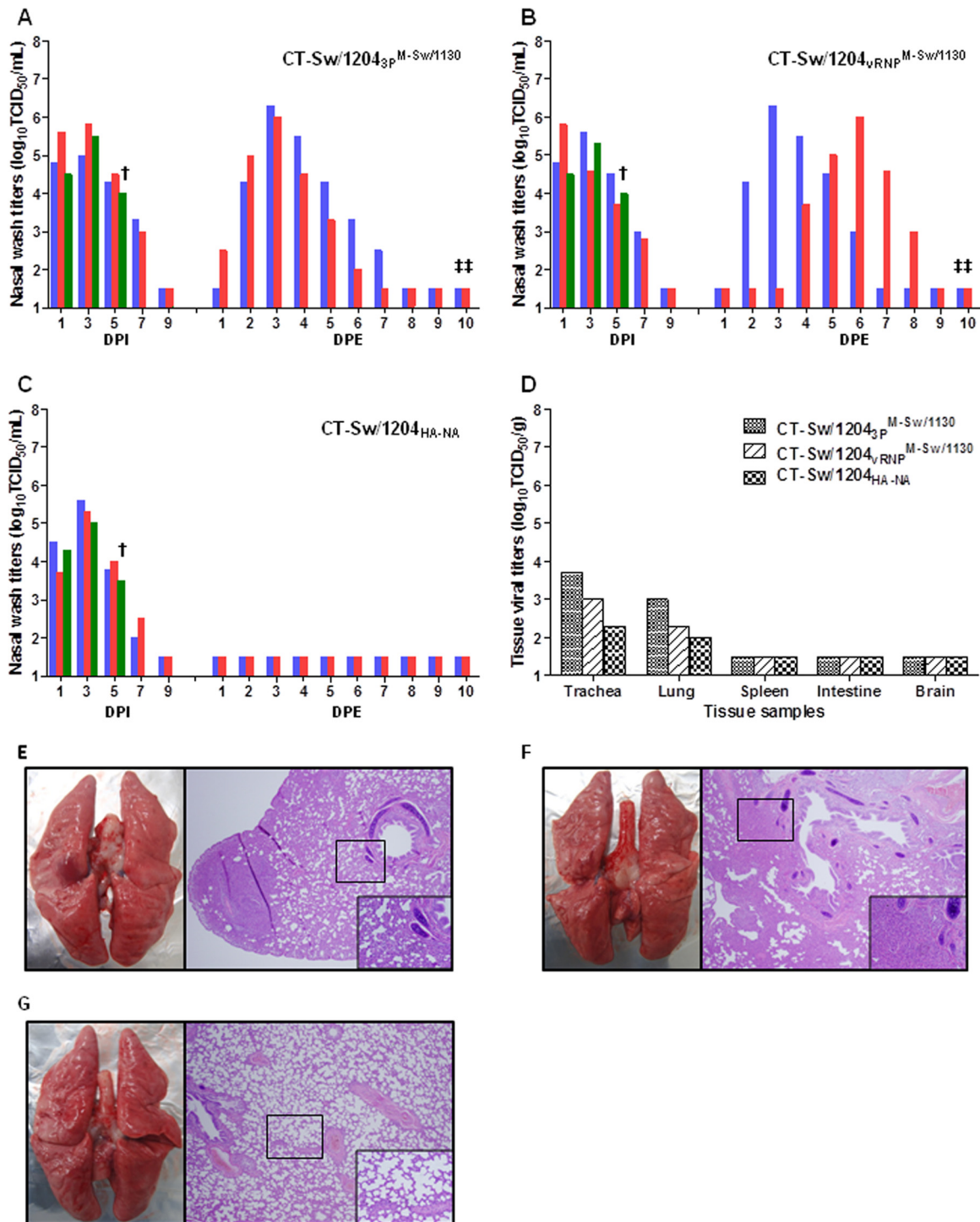
**FIG 4** *In vitro* polymerase activities of different viral RNP gene combinations. Four expression plasmids (PB2, PB1, PA, and NP) were used to yield the 16 RNP gene combinations of CT-Sw/1204 and Sw/1130, together with pHW72-Luci\_M, which carried the reporter luciferase gene. The transfected 293 cells were assayed for luciferase activity after 24 h of incubation at 33°C (upper respiratory tract temperature; solid bars) or 37°C (lower respiratory tract temperature; hatched bars). The values shown are means and standard errors of the mean of the results of 3 independent experiments performed in triplicate, which are standardized to the activity of the expression plasmids for the CT-Sw/1204 vRNP complex proteins (100%). Segments derived from CT-Sw/1204 are shown in italics. “r” denotes reconstituted. \*, *P* < 0.05, and \*\*, *P* < 0.01 compared to polymerase activities relative to the homologous CT-Sw/1204 vRNP (r1) complex (two-tailed unpaired *t* test).

lication and transcription at any given temperature (Fig. 4). Co-expression of CT-Sw/1204 PA with NP (r14) or PB1 (r16), but not with PB2 (as seen with r6 and r8), also significantly elevated the polymerase activity of Sw/1130, stressing the importance of the CT-Sw/1204 PA for the enhancement of transcription and replication activities.

**The viral polymerase of CT-Sw/1204 promotes RD transmission in ferrets.** Introduction of the HA<sub>225G</sub> and NA<sub>315N</sub> found in CT-Sw/1204 into the Sw/1130 virus increases pathogenicity in mice and replication in the upper respiratory tract of ferrets but does not cause efficient horizontal RD transmission (10), suggesting that these mutations are not sufficient to support virus dissemination through the air. To explore the potential role of the viral RNA polymerase subunit of CT-Sw/1204 in RD contact transmission, we generated 2 reassortant viruses in the genetic background of the Sw/1130 (M-Sw/1130) virus mutants that also have the HA<sub>225G</sub> and NA<sub>315N</sub> mutations: CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> and CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup>. For comparison, the double-reassortant CT-Sw/1204<sub>HA-NA</sub> virus in the context of the parental Sw/1130 was also tested.

Groups of 3 ferrets were inoculated intranasally and intratracheally (as before) with 10<sup>5.5</sup> TCID<sub>50</sub>/ml of the respective reassortant viruses. All strains replicated reasonably well in the upper respiratory tract for up to 7 days p.i., with maximum nasal wash titers of at least 4.6 log<sub>10</sub> TCID<sub>50</sub>/ml (Fig. 5). All ferrets inoculated with the CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> and CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> re-

assortants had moderate to severe signs of influenza marked by infrequent sneezing, a 10% to 15% mean body weight reduction, and temperature elevations of up to 2.3°C. In contrast, inoculation of the CT-Sw/1204<sub>HA-NA</sub> reassortant induced milder clinical disease, as demonstrated by 3.6% mean body weight losses and temperature elevations of only 1.6°C. Among the 3 recombinant viruses examined, only CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> and CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> were able to establish RD transmission to 2 seronegative RD contacts individually paired with inoculated ferrets (Fig. 5A to C). Exposure to CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> resulted in positive detection in nasal washes as early as 1 day postexposure (p.e.) (Fig. 5A), whereas CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> had delayed transmission kinetics, requiring 2 to 4 days p.e. for detection in both RD contacts (Fig. 5B). In tissues obtained at 5 days p.i. from 1 of the experimentally infected ferrets, CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> and CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> had higher growth capacity in the trachea and lungs than did CT-Sw/1204<sub>HA-NA</sub> (Fig. 5D). No infectious virions were recovered in any of the other tissue specimens tested (spleen, intestine, and brain). Furthermore, histopathologic examination of lung tissue sections indicated that CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> and CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> induced more visible macroscopic lesions, which were accompanied by prominent accumulation of immune cells (Fig. 5E to G). All experimentally infected ferrets and RD contacts except those paired with the CT-Sw/1204<sub>HA-NA</sub> inoculation group demonstrated seroconversion at 21 days p.i. (or 20 days p.e.).



**FIG 5** Replication and transmission of recombinant viruses in ferrets. (A to C) Groups of 3 ferrets were inoculated with the CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> (A), CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> (B), or CT-Sw/1204<sub>HA-NA</sub> (C) reassortant virus. One of three inoculated ferrets was sacrificed 5 days p.i. (†) for tissue viral titration (D) and histopathology (E). RD contacts were housed adjacent to the inoculated ferrets 1 day p.i. for viral exposure (1:1 setup). Each of the transmission test groups consisted of two directly infected animals and two RD contacts. Nasal wash titers are shown for individual ferrets (as indicated by the colors). The limit of virus detection was 1.5 log<sub>10</sub> TCID<sub>50</sub>/ml per g. ‡, seroconversion of RD contacts after exposure to inoculated ferrets as measured by hemagglutinin inhibition assays. (E to G) Hematoxylin and eosin staining of lung tissue from ferrets infected with CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> (E), CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> (F), and CT-Sw/1204<sub>HA-NA</sub> (G) are shown at low (×40) and high (×200; insets) magnifications.



## DISCUSSION

Here, we used reassortant viruses to determine which other viral factors contributed to the growth, pathogenicity, and transmissibility of the CT-Sw/1204 H1N2 virus. Substitution of the isogenic CT-Sw/1204 HA alone or in combination with NA into the closely related Sw/1130 H1N2 virus enhanced replication and pathogenicity in mice, confirming the role of the HA<sub>225G</sub> and NA<sub>315N</sub> mutations found in CT-Sw/1204 after a single passage in ferrets (10). However, the reciprocal expression of Sw/1130 HA or HA-NA in the background of CT-Sw/1204 could not render the recombinant reassortant virus completely attenuated, implying that the remaining viral segments are required for pathogenicity. Indeed, we show that possession of the individual CT-Sw/1204 internal gene segments conferred variable effects on replication and pathogenicity, implying that they play critical roles in these areas apart from the contribution of HA-NA.

We found that the *PB2*, *PA*, *NP*, and *NS* genes of CT-Sw/1204 could alter the growth and pathogenic phenotype of Sw/1130 to some extent in mice. Conversely, reassortant CT-Sw/1204 viruses containing PB2 or PA of Sw/1130, but not PB1, could attenuate clinical disease. However, beyond these results, we demonstrated that the combined expression of the viral RNA polymerase *PA*, *PB1*, and *PB2* genes of CT-Sw/1204 as a subunit, regardless of whether the NP was derived from CT-Sw/1204 or Sw/1130, had a prominent role in inducing efficient viral replication and lethality to mice. Therefore, our findings suggest that replication efficiency mediated by the epistatic gene interaction of the heterotrimeric RNA polymerase genes of CT-Sw/1204, rather than their individual expression, confers high pathogenicity independent of the balanced activity of the surface glycoproteins (HA<sub>225G</sub>-NA<sub>315N</sub>).

The influenza virus polymerase complex genes have been implicated in optimal growth and virulence of the 1918 pandemic virus (16–18) and of highly pathogenic avian influenza (HPAI) H5N1 viruses (19–22). As such, viral pathogenicity is generally attributed to a high level of replicative ability in a given host. Not surprisingly, expression of the homologous polymerase complex genes from CT-Sw/1204 in the context of the HA<sub>225G</sub> and NA<sub>315N</sub> mutations in Sw/1130 (i.e., CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> and CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup>) increased replication and invasive infection in the lungs of inoculated ferrets relative to that of the CT-Sw/1204<sub>HA-NA</sub> reassortant. However, neither CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> nor CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> was as virulent as the parental CT-Sw/1204 virus in this host (10), indicating the requirement for other viral factors in ferrets. Nonstructural protein 1 (NS1), encoded by the *NS* gene, confers efficient replication on human influenza A viruses and is a virulence determinant of HPAI H5N1 viruses (23–25). Noting that CT-Sw/1204 NS has the potential to increase viral growth and elevate pathogenicity, we speculate that segment 8 could also contribute to virulence in concert with the tripartite viral RNA polymerases. NS1 could interact with cognate PA and NP proteins to stabilize the CPSF30-NS1 protein complex (26) or with the vRNP complexes through NP (27), presumably to suppress antiviral responses or to regulate transcriptional activity, respectively. Thus, these interactions could be beneficial because the polymerase genes generally work in concert with the *NS* and *HA* genes to produce virulent viruses (28). Therefore, the overall polymerase functionality and its context within the other viral genes could be important in determining the ability of the CT-Sw/1204 virus to induce lethality in ferrets, and acquisition of the

HA<sub>225G</sub> and NA<sub>315N</sub> mutations exacerbates disease. Overall, these results show that the high pathogenicity of the CT-Sw/1204 virus involves interplay of multiple viral factors, further exemplifying the polygenic nature of virulence among influenza A viruses.

Respiratory droplet transmission has been mostly attributed to the receptor specificity of HA in H1 viruses (29, 30) or to balanced HA-NA activity (31, 32). However, the polymerase complex genes facilitate virus dissemination through the air (33, 34). We found that CT-Sw/1204<sub>3P</sub><sup>M-Sw/1130</sup> or CT-Sw/1204<sub>vRNP</sub><sup>M-Sw/1130</sup> promoted infection of RD contact ferrets, implying the indispensability of the tripartite viral RNA polymerases of CT-Sw/1204 for attaining RD transmission. Similarly, PB2 of the 1918 pandemic virus was required to support RD transmission of a recombinant avian virus carrying the 1918 HA (34). Possession of the CT-Sw/1204 HA-NA in the genetic background of Sw/1130 did not lead to efficient transmission, consistent with our previous results indicating that the HA and NA modifications alone are inadequate to mediate virus dissemination through the air. Hence, our findings confirm the notion that aerosol transmissibility may not always depend on receptor specificity or balanced activities of the surface glycoproteins in a given host (34).

The reconstituted vRNP complex of CT-Sw/1204 consistently had higher polymerase activity than did Sw/1130 at temperatures correlating with those in both regions of the human respiratory tract (15). Such selective advantage may have facilitated viral replication competence, allowing reassortant viruses carrying the CT-Sw/1204 polymerase segments to be readily available in the upper nasal cavity of infected ferrets for efficient transmission. It is apparent, therefore, that HA<sub>225G</sub> and NA<sub>315N</sub> in CT-Sw/1204 contributed to virulence and transmission by allowing virus dissemination in the upper and lower respiratory tract and that the heterotrimeric polymerase gene segments remained active in such tissue environments, facilitating robust replication.

The viral polymerase can mediate adaptation of influenza viruses in a new host (35). The internal gene segments of CT-Sw/1204 and Sw/1130 have notable amino acid differences, which are likely responsible for the altered growth, pathogenicity, and transmissibility of these strains. Of particular interest are positions 64 in PB2, 100 and 337 in PA, and 215 in NS1, all of which have been identified as persistent sites for adaptation and host shifting of influenza viruses, including in the 1918, 1957, and 1968 pandemic strains and H5N1 viruses (36–38). Although PB1 is a key component of the viral RNA polymerase complex functionally and structurally, replication impairment due to possession of CT-Sw/1204 PB1 alone suggests that the genetic modifications in segment 2 are deleterious in the context of the parental recombinant Sw/1130 virus. However, PB1 appears to function better with segments 1 and 3 from CT-Sw/1204, indicating its indispensability for the tripartite polymerase complex. Further studies will shed light on the specific role of these mutations in CT-Sw/1204 or other triple-reassortant swine viruses, including the genetically related A(H1N1)pdm09 virus.

Altogether, the data generated in this study confirm the importance of the HA<sub>225G</sub> and NA<sub>315N</sub> mutations to the CT-Sw/1204 virus. However, we also provide proof that the synergistic function of the homologous polymerase gene complex is critically important for the virulence and transmission of CT-Sw/1204. Because this report demonstrates that some viruses in the field may have made subtle, modest adaptations that could alter disease impact, continued monitoring is highly recommended to manage

risks associated with the emergence of strains that could become significant threats to public health. Along with the growing number of reports on the importance of the viral polymerase for replication, pathogenesis, and dissemination, our results justify the development of novel antiviral drugs aimed at curbing the functions of the viral polymerase complex subunits.

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