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Generation of highly productive and mammalian nonpathogenic recombinant H9N2 avian influenza viruses by optimization of 3'end promoter and NS genome

Se-Hee An[a], Chung-Young Lee[a], Jun-Gu Choi[e], Youn-Jeong Lee[e], Jae-Hong Kim[a,c], Hyuk-Joon Kwon[b,c,d,*]

[a] Laboratory of Avian Diseases, Republic of Korea
[b] Department of Farm Animal Medicine, Republic of Korea
[c] Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, 08826, Seoul, Republic of Korea
[d] Farm Animal Clinical Training and Research Center (FACTRC), GBST, Seoul National University, 25354, Pyeongchangdae-ro, Kangwon-do, Republic of Korea
[e] Avian Disease Division, Animal and Plant Quarantine Agency, 177, Hyeoksin 8-ro, Gyeongsangbuk-do, 39660, Republic of Korea

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ABSTRACT

We developed A/PR/8/34 (PR8) virus-based reverse genetics system in which six internal genes of PR8 and attenuated hemagglutinin and intact neuraminidase genes of field avian influenza viruses (AIVs) have been used for the generation of highly productive recombinant vaccine strains. The 6 + 2 recombinant vaccine strains can induce protective humoral immunity against intended field AIVs; however, the epitopes of B and T cells encoded by internal genes may be important for heterosubtypic protection. Therefore, it is advantageous to use homologous internal genes of field AIVs for recombinant vaccine strains. However, the rescue of recombinant viruses having whole internal genes of field AIVs by the PR8-based reverse genetics system was unsuccessful in some cases. Although partial replacement of an internal gene has been successful for generation of highly productive and mammalian nonpathogenic recombinant viruses, complete replacement of internal genes may be more favorable. In this study, we successfully generated complete recombinant H9N2 AIVs possessing 8 genomes of H9N2 AIVs by optimal combinations of 3' end promoter sequences of polymerase genomes, and a NS genome. All the generated recombinant viruses showed highly productive and mammalian nonpathogenic traits but some of them showed much higher virus titers in embryonated chicken eggs. Additionally, we found the same mutations of NS1 gene determined pathogenicity of AIVs in chicken embryos as well as mammals. Thus, the 3' end promoter optimization, and highly productive and mammalian nonpathogenic internal genes may be useful to develop vaccines against AIVs.

1. Introduction

Influenza A viruses (IAVs) have eight negative-sense, single-stranded RNA segments encoding polymerases (PB2, PB1, and PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M), and non-structural proteins (NS) (McCAULEY and Mahy, 1983). There are non-coding regions (NCRs) at the 5' and 3' ends of viral genominc RNA (vRNA), and the first 12 and 13 nucleotides at the each ends are highly conserved and play important roles in the viral replication (Neumann et al., 2004). The fourth nucleotide at the 3' end of vRNA is known as a promoter and an origin of replication, and genome segments with uridine at the fourth nucleotide of the promoter/origin (U4) showed higher replication and transcription levels than cytidine (C4) (Jiang et al., 2010; Lee et al., 2017b, 2003).

The Hoffmann's reverse genetics vector system is based on the high growth A/PR/8/34 (PR8) genome segments and possesses C4 in polymerase genome segments (PB2, PB1, and PA) and U4 in the others (HA, NP, NA, M, and NS) (Hoffmann et al., 2002, 2001). Therefore, the recombinant viruses generated through this system have the same promoter constellation, but the same constellation in different IAVs can cause different phenotypic results (Jiang et al., 2010; Lee et al., 2017b; Ping et al., 2015). Also, internal genes of PR8 were mostly used for recombinant vaccines because it is easily adaptable, its sequence is fully verified, and it facilitates high-growth in embryonated chicken eggs (ECEs) (Webby et al., 2004; Wood and Robertson, 2004). However, the use of internal genes homologous to those of field viruses is favorable to

* Corresponding author at: Laboratory of Poultry Production Medicine, College of Veterinary Medicine, Seoul National University, 08826 Seoul, Republic of Korea.
E-mail address: kwonhj01@snu.ac.kr (H.-J. Kwon).

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improve heterosubtypic protection efficacy of the vaccine strain because the internal genes are important for stimulating humoral and cellular immunity (Fujimoto et al., 2016; Townsend et al., 1984).

A/chicken/Korea/01310/2001(H9N2) (01310) was passaged 20 times (01310 CE20) through ECEs and 01310 CE20 has been used as a low-pathogenic avian influenza (LPAI) vaccine strain in Korea (Choi et al., 2008), but the rescue of complete recombinant virus possessing 8 genomic segments of 01310 CE20 could not be achieved using Hoffmann's vector system. The PB2 gene of 01310 CE20 is prototypic avian gene with decreased integrity of polymerase trimer and it may cause reduced viral replication and pathogenicity in mammalian hosts (Kim et al., 2014; Lee et al., 2017a). A human cell line, 293 T cell, has been used for generating recombinant viruses by reverse genetics, and host barrier may hinder replication of prototypic avian genomes resulting in unsuccessful virus rescue. Another Korea H9N2 strain, A/chicken/Korea/KBNP-0028/2000(H9N2) (0028), was also passaged 20 times in ECEs to increase productivity (Kwon et al., 2009). The PR8-derived recombinant viruses possessing PA or NS gene of 0028 did not cause a loss in body-weight but could replicate in the lungs of BALB/c mice (Kim et al., 2014, 2015). Therefore, investigating the effect of less competent PA and NS genes on the rescue of recombinant 0028 strains in 293 T cells may be valuable. Furthermore, 0028 did not cause mortality even after 72 h post inoculation via allantoic cavity route unlike 01310 CE20 and it may improve the productivity of vaccine strains by increasing virus titer and amount of harvested allantoic fluid (Kwon et al., 2009). WHO and World Organisation for Animal Health (OIE) also recommend low embryonic pathogenicity of vaccine strain. Therefore, investigation regarding molecular determinants affecting embryonic pathogenicity may also be valuable to improve vaccine productivity.

In this study, we hypothesized that optimal combinations of C4 and U4 in polymerase genomes might compensate for less competent avian polymerases and facilitate rescue and replication of complete recombinant H9N2 viruses possessing 6 internal genes of their own. We successfully rescued complete recombinant H9N2 recombinant viruses that are more replicative in ECEs and nonpathogenic for mammals. In addition, we demonstrated the common roles of NS1 gene and its mutations in embryonic as well as mammalian pathogenicity.

2. Materials and methods

2.1. Viruses, cells, eggs, and plasmids

The 01310 CE20 and 0028 (CE20) were propagated in 10-day-old SPF ECEs (Charles River Laboratories, North Franklin, USA). The 01310 CE22 and 0028 CE21 have same sequences with 01310 CE20 and 0028 CE20, and they are used in this study. 293 T, A549, and MDCK cells were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). 293 T and MDCK cells were maintained in DMEM (Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and A549 cell was maintained in DMEM/F12 supplemented with 10% FBS. To generate recombinant viruses, a Hoffmann vector system was used as described previously (Hoffmann et al., 2002, 2001). Recombinant viruses were generated and passaged two times in 10-day-old (10-d-o) SPF ECEs and then stored at −80 °C until experimental use.

2.2. Cloning and site directed mutagenesis of genes

Eight genome segments of 01310 CE22 and 0028 CE21 were amplified using Hoffmann's universal primer sets and cloned into the Hoffmann's bi-directional transcription vector, pHW2000, as reported previously (Hoffmann et al., 2002, 2001). The nucleotide sequences of the inserts were confirmed by sequencing with cmv-SF and bGH-SR primers listed in Table 1 (Macrogen Co, Seoul, Korea). The 4th nucleotide in 3'end of PB2, PB1, and PA genomes were mutated from C4 to U4 (C4U) by site-directed mutagenesis with the Muta-direct site directed mutagenesis kit (iNtRON Biotechnology, Seongnam-si, Korea). The mutagenesis primer sets used in this study are listed in Table 1. The mutated 0028 NS genome possessing single amino acid mutation of NS1 [G139D, G139 N, S151 T, or PL motif mutation (GSEV to EPEV)] were previously cloned into pHW2000 vector (Kim et al., 2015); we used the same plasmids after confirming the sequence of the inserts (Macrogen Co).

Table 1
Primer sets used for sequencing and mutagenesis of polymerase gene promoters.

primer	Sequence (5'-3')
cmv-SF	TAAGCAGAGCTCTCTGGCTA
bGH-SR	TGGTGGCGTTTTTGGGGACA
mPB2-F	AGTTGGGGGGGA *AGCAAAA* GCAGGTCAAATA
mPB2-R	TATTTGACCTGC *TTTTGCT* TCCCCCCCAACT
mPB1-F	AGTTGGGGGGG *AGCAAAA* GCAGGCAAACCA
mPB1-R	TGGTTTGCCTGC *TTTTGCT* CCCCCCCAACT
mPA-F	GAAGTTGGGGGGG *AGCAAAA* GCAGGTACTGATC
mPA-R	GATCAGTACCTGC *TTTTGCT* CCCCCCCAACTTC

2.3. Generation of recombinant viruses by reverse genetics

Eight plasmids possessing eight genomes were transfected into 293 T cells for the generation of recombinant viruses. One day before the transfection, 293 T cells were cultured in 6-well plate (5×10^5 cells/well) and 300 ng of each plasmids was transfected together into the cells using Lipofectamine and Plus reagent (Life Technologies) in 1 mL of Opti-MEM (Life Technologies). After overnight incubation, 1 mL of Opti-MEM and 0.5 μg/mL of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, USA) were added. After another overnight incubation, 0.2 mL of the harvested culture medium was inoculated into 10-d-o SPF ECEs using allantoic cavity route. The allantoic fluid was harvested from inoculated ECEs after 72h incubation at 37 °C and checked for the presence of recombinant viruses using plate hemagglutination test with 1% (v/v) chicken red blood cells according to the WHO Manual on Animal Influenza Diagnosis and Surveillance. Transfection was repeated three times to generate recombinant viruses independently.

2.4. Titration of viruses

The suspension containing recombinant viruses (E1) was diluted 100-fold and inoculated into three 10-d-o ECEs for virus propagation (E2). The harvested allantoic fluid was aliquoted and maintained at -80 °C until use. To measure the virus titer of the allantoic fluid, each sample was serially diluted by 10-fold and 0.1 ml of each dilution from 10^{-6} to 10^{-9} was inoculated into five 10-d-o ECEs. The 50% chicken embryo infectious dose (EID_{50}/mL) was calculated by the Spearman-Karber method (Hamilton et al., 1977).

2.5. Mini-genome assay

Promoter activity of recombinant viruses was determined by mini-genome assay as previously described (Lee et al., 2017a). Briefly, 293 T cells in 12-well plates were co-transfected with polymerase and NP genes of 01310 CE22, 0028 CE21, or PR8 together with pHW-NP-Luc and the Renilla luciferase plasmid pRL-TK (Promega, USA). After 24 h incubation at 37°C, the luminescence was measured using the Dual-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's instructions on a TECAN Infinite200 pro machine (Tecan Benelux, Giessen, Netherlands). The results are shown as the average and the standard deviation of three independent experiments.

2.6. Replication efficiency of recombinant viruses in MDCK cells

To compare the replication efficiency of recombinant viruses in MDCK cells, 10^7 EID_{50}/0.1 mL of each recombinant virus was 10-fold diluted and inoculated into confluent MDCK cells in 96-well plate (1×10^5 cells/well; 3 repeats for each diluted virus). After 72 h incubation in CO_2 incubator at 37 °C, the 50% tissue culture infectious dose ($TCID_{50}$/mL) was calculated as above.

2.7. Growth kinetics of recombinant 01310 CE22 viruses in A549 cells

Confluent A549 cells in 6 well plate were infected with 1 multiplicity of infection of recombinant 01310 viruses. After 1 h incubation, viruses were washed away with PBS and 1 mL of fresh DMEM and 0.2 μg/mL TPCK-trypsin were added. During 3 days incubation in CO_2 incubator at 37 °C, supernatant was harvested at 24, 48, and 72-hour post infection (hpi) and virus titers were measured as described above.

2.8. Effect of NS genome on the pathogenicity and productivity of AIVs in ECEs

Pathogenicity of recombinant virus was evaluated by measuring the mean death time (MDT) of chicken embryos (Hanson and Brandly, 1955). Each virus was diluted to 100 EID_{50}/0.1 mL and inoculated into ten 10-d-o ECEs via allantoic cavity route. During incubation at 37 °C, eggs were candled twice a day and death time of embryo was recorded. The MDT of each virus was calculated by average of death time of all infected ECEs. To compare the productivity of recombinant viruses, same experiment was performed as above except 3 days of incubation and chilling of dead ECEs at 4 °C. Then, the volume and hemagglutination unit (HAU) of harvested allantoic fluid were measured and multiplied to calculate total HAU of each virus.

2.9. Statistical analysis

The polymerase activity and virus titer were compared by one-way analysis-of-variance (IBM SPSS Statistics ver. 23; IBM, USA). Results were considered statistically significant if $p < 0.05$ and $p < 0.001$.

3. Results

3.1. Generation of recombinant 01310 CE22 and 0028 CE21 viruses by C4U mutation of promoter/origin in polymerase genomes

The PR8-derived 01310 CE22 virus (r310-PR8) possessing 6 internal genes of PR8 was generated in every transfection trials; however, we could not generate r310-C4 possessing 6 internal genes of 01310 CE22. The C4U mutations in PB2 and/or PB1 (r310-U4-PB21, r310-U4-PB2, and r310-U4-PB1), PB1/PA (r310-U4-PB1A), and PB2/PB1/PA (r310-U4) facilitated virus rescue. However, the U4 promoter mutation in only PA and in both PB2 and PA (r310-U4-PA and r310-U4-PB2A) did not facilitate virus rescue (Table 2). In contrast, recombinant 0028 CE21 viruses (r0028-C4, r0028-U4-PB2, r0028-U4-PB1, r0028-U4-PA, r0028-U4-PB21, r0028-U4-PB1A, r0028-U4-PB2A, and r0028-U4) were successfully generated in all kinds of promoter combination together with PR8-derived 0028 CE21 virus (r0028-PR8) (Table 3).

3.2. Replication efficiency of recombinant 01310 CE22 and 0028 CE21 viruses in ECEs

The titers of generated recombinant viruses were measured in ECEs. The titers (log_{10} EID_{50}/mL) of recombinant 01310 CE22 viruses ranged from 8.92 ± 0.14 to 9.58 ± 0.29, and the titers of r310-U4-PB1 and r310-U4-PB21 were higher than others, including r310-PR8 (Table 2). However, additional C4U mutation in PA of those two viruses (r310-U4-PB1A and r310-U4) decreased titer in ECEs (Table 2). The titers of recombinant 0028 CE21 viruses ranged from 8.67 ± 0.29 to 10.13 ± 0.15, and the titers of r0028-U4-PB21 and r0028-PR8 were higher than those of others (Table 3). Therefore, C4U mutations in both PB2 and PB1 increased titers of recombinant 01310 CE22 and 0028 CE21 viruses commonly in ECEs.

3.3. Comparison of polymerase activity between different combinations of C4U mutations in polymerase genomes

To investigate the effects of different combinations of C4U mutations of polymerase genomes on virus rescue, the polymerase activity was compared using mini-genome assay (Fig. 1). The polymerase activities of 01310 CE22 and 0028 CE21 mini-genomes were considerably weaker than that of PR8. It was difficult to differentiate between the activities of virus rescue-facilitating (r310-U4-PB2, r310-U4-PB1, r310-U4-PB1A, r310-U4-PB21, and r310-U4) and non-facilitating (r310-C4 and r310-U4-PB2A) C4U combinations of polymerases. Although the relative luciferase activities of both 01310 CE22 and 0028 CE21 mini-genomes were very low, all of the 0028 CE21 mini-genomes showed slightly higher activities than those of 01310 CE22 mini-genomes. Therefore, the polymerase activity of 0028 CE21 may be enough to produce recombinant 0028 CE21 viruses independent of the combinations of C4U mutations.

3.4. Comparison of replication efficiency of recombinant viruses in MDCK and A549 cells

As the recombinant 01310 CE22 and 0028 CE21 viruses were successfully generated from 293 T cells, we evaluated their replication efficiency in mammalian cell lines, MDCK and A549 cells. Although no significant difference in virus titer was observed between recombinant and parent viruses, recombinant 01310 CE22 viruses showed higher titers than 01310 CE22 virus in MDCK cells (Fig. 2). Furthermore, the titers of recombinant 01310 CE22 viruses were higher than that of recombinant PR8. However, recombinant 0028 CE21 viruses except for r0028-PR8 showed significantly lower virus titers than recombinant 01310 CE22 and PR8 viruses. For this reason, viral replication efficiency of recombinant 01310 CE22 viruses in A549 cells were further evaluated. All of the recombinant and parent 01310 CE22 viruses could not replicate in A549 cells during 72 hpi but recombinant PR8 virus showed the highest titer within 48 hpi (Fig. 3).

3.5. Effect of 0028 NS genome on the pathogenicity and productivity of recombinant 01310 CE22 viruses in ECEs

Previously, we demonstrated that the mutations of mammalian non-pathogenic 0028 NS1, GSEV to EPEV, S151 T and G139D, increased the mammalian pathogenicity of PR8-derived recombinant viruses possessing mutated 0028 NS1 genes, and the mammalian pathogenic mutations are shared by 01310 NS1 gene (Kim et al., 2015). To investigate the effect of NS genome on the embryonic pathogenicity of 01310 CE22, we generated recombinant 01310 viruses by replacing NS genome with parent (mammalian non-pathogenic) or mutated (mammalian pathogenic) 0028 NS genome. The recombinant viruses with parent (r310-NS28) and mutated 0028 NS genomes (r310-NS28-EPEV, r310-NS28-S151 T, r310-NS28-G139D, and r310-NS28-G139 N) were also generated by C4U mutations of PB2 and PB1 genomes. To compare the embryonic pathogenicity, the MDT of each recombinant virus was measured (Table 4). r310 was pathogenic and caused embryonic death within 48.8 ± 8 h, whereas r310-NS28 did not cause embryonic death even after 72 h incubation (94.4 ± 5). The recombinant viruses possessing EPEV (r310-NS28-EPEV), S151 T (r310-NS28-S151 T), G139D (r310-NS28-G139D), and G139 N (r310-NS28-G139 N) mutations showed a lower MDT than r310-NS28. Although the titers of r310-NS28-EPEV, r310-NS28-S151 T, and r310-NS28-G139D were significantly less than that of r310, r310-NS28 and r310-NS28-G139 N

Table 2
Genotype, rescue, and titer of recombinant 01310 CE22 virus.

Recombinant virus	3' end promoter[a]			Internal genes[b]	Rescue[c]	EID_{50}/mL (log_{10}) [d]
	PB2	PB1	PA			
r310-PR8	C4	C4	C4	PR8	+	8.83 ± 0.14
r310-C4	C4	C4	C4	01310 CE20	−	NT
r310-U4-PB2	U4	C4	C4	01310 CE20	+	9.00 ± 0.43
r310-U4-PB1	C4	U4	C4	01310 CE20	+	9.58 ± 0.29[e]
r310-U4-PA	C4	C4	U4	01310 CE20	−	NT
r310-U4-PB21	U4	U4	C4	01310 CE20	+	9.50 ± 0.50[e]
r310-U4-PB1A	C4	U4	U4	01310 CE20	+	9.00 ± 0.00
r310-U4-PB2A	U4	C4	U4	01310 CE20	−	NT
r310-U4	U4	U4	U4	01310 CE20	+	8.92 ± 0.14

[a] C4: the 4th nucleotide of the 3' end promoter is cytidine, weak promoter; U4: the 4th nucleotide of the 3' end promoter is uridine, strong promoter.
[b] The origin of six internal genes (PB2, PB1, PA, NP, M, and NS).
[c] The result of virus rescue after three times of transfection; +: successful (3/3), -: unsuccessful (0/3).
[d] EID_{50}/mL, mean 50% chicken embryo infectious dose/mL with standard deviation; NT, not tested.
[e] Significant difference ($p < 0.05$) from other recombinant viruses.

Table 3
Virus rescue and titer of recombinant 0028 CE21 virus.

Recombinant virus[a]	Rescue[b]	EID_{50}/mL (log_{10})[c]
r0028-PR8	+	9.95 ± 0.14[d]
r0028-C4	+	8.67 ± 0.29
r0028-U4-PB2	+	9.08 ± 0.18
r0028-U4-PB1	+	9.10 ± 0.30
r0028-U4-PA	+	8.69 ± 0.13
r0028-U4-PB21	+	10.13 ± 0.15[d]
r0028-U4-PB1A	+	8.93 ± 0.21
r0028-U4-PB2A	+	9.25 ± 0.50
r0028-U4	+	8.75 ± 0.25

[a] C4: the 4th nucleotide of the 3' end promoter is cytidine, weak promoter; U4: the 4th nucleotide of the 3' end promoter is uridine, strong promoter.
[b] The origin of six internal genes (PB2, PB1, PA, NP, M, and NS).
[c] EID_{50}/mL, mean 50% chicken embryo infectious dose/mL with standard deviation.
[d] Significant difference ($p < 0.05$) from other recombinant viruses.

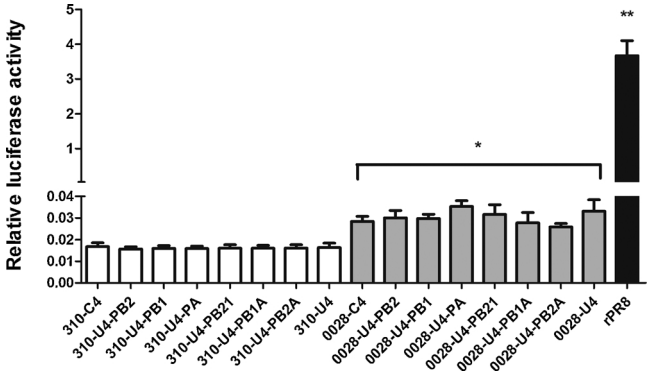

Fig. 1. Comparison of polymerase activity of 01310 CE22 and 0028 CE21 mini-genomes with different combinations of C4U mutations in polymerase genomes. pHW plasmids possessing polymerase genes (PB2, PB1, and PA) and NP were transfected into 293 T cells with pHW-NP-Luc and the Renilla luciferase plasmid pRL-TK, and the luminescence was measured. *, significant difference from 01310 CE22 mini-genome; **, significant difference from 01310 CE22 and 0028 CE21 mini-genomes ($p < 0.05$).

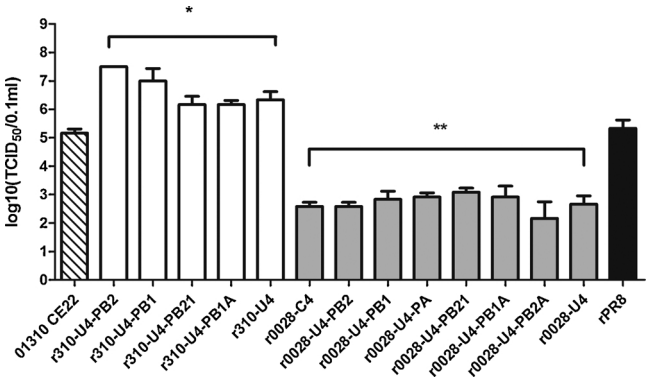

Fig. 2. Comparison of virus titers of recombinant 01310 CE22 and 0028 CE21 viruses in MDCK cells. 1×10^7 EID_{50}/0.1 mL of each virus was 10-fold diluted and inoculated into confluent MDCK cells. After incubation at 37 °C for 72 h, the virus titer ($TCID_{50}$/0.1 mL) was calculated. *, significant difference from parent 01310 CE22 and recombinant 0028 CE21 viruses; **, significant difference from other viruses ($p < 0.05$).

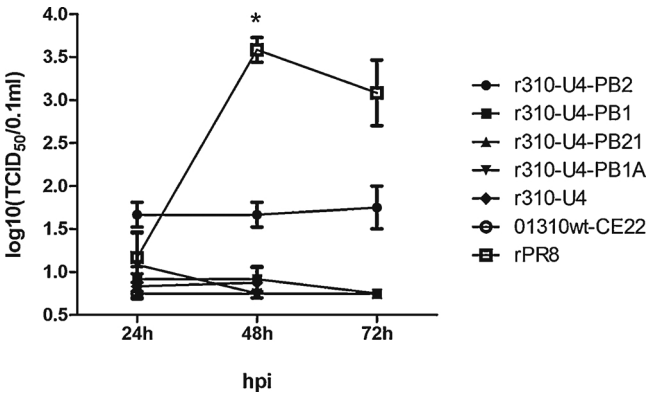

Fig. 3. Growth kinetics of recombinant 01310 CE20 viruses in A549 cells. One multiplicity of infection of each virus was inoculated into confluent A549 cells and supernatant was harvested at 24, 48, and 72 hpi. The $TCID_{50}$ in supernatant was determined in MDCK cells. *, significant differences from other viruses at 48 hpi ($p < 0.05$).

showed titers similar to r310 (Table 4). To compare the productivity of r310 and r310-NS28 in ECEs the total HAU was calculated by multiplying the HAU/mL and the volume of harvested allantoic fluid (Table 5). The total volume and HAU of r310 were less than those of r310-NS28. Therefore, r310-NS28 was more productive than r310 in ECEs.

4. Discussion

H9N2 AIVs became endemic in Asia, Middle East, and North Africa after severe outbreaks in poultry during 1990s (Alexander, 2007). They caused direct infection in humans and provided internal genes to

Table 4
Mean death time (MDT) and virus titer of 01310 CE22-derived recombinant virus.

Recombinant virus	MDT (Mean Death Time, h)[c]	Virus titer (log_{10} EID_{50}/mL)[d]
r310	48.8 ± 8	9.17 ± 0.29
r310-NS28	94.4 ± 5[a]	9.37 ± 0.12
r310-NS28-EPEV	61.6 ± 6[a]	8.70 ± 0.23[b]
r310-NS28-G139D	75.7 ± 1[a]	8.40 ± 0.12[b]
r310-NS28-G139N	82 ± 2.5[a]	9.33 ± 0.24
r310-NS28-S151T	67.6 ± 3[a]	8.53 ± 0.39[b]

[a,b]Significant difference from r310 ($p < 0.05$).

Table 5
Comparison of virus productivity of r310 and r310-NS28 in ECEs.

Harvested allantoic fluid	r310	r310-NS28
Volume (mL)[a]	11.9	13.3[d]
HAU (log_2)[b]	8.3	8.8
Total HAU[c]	3975.7	6412.8[d]

[a] Mean volume of harvested allantoic fluid from 10 ECEs.
[b] Mean hemagglutination unit (HAU)/25 μl of allantoic fluid from 10 ECEs.
[c] Median of total HAU of allantoic fluid from 10 ECEs.
[d] Significant differenc ($p < 0.05$) from r310.

human-lethal H5N1, H5N6, H7N9, and H10N8 AIVs (Gu et al., 2014; RahimiRad et al., 2016). The vaccines against H9N2 AIVs have been developed and used in the poultry farms (Choi et al., 2008; Sun et al., 2012). However, new vaccine strains have been recommended due to appearance of antigenic variants (Lee and Song, 2013).

PR8-based reverse genetics vector system has been successfully used for development of vaccines for humans and animals. As the 6 internal genes of PR8 improved efficient replication of 6 + 2 vaccine strains in ECEs, they were favorably used for vaccine development. However, internal genes of PR8 contained various mammalian pathogenicity related mutations including E627 K mutation in PB2 (Kim et al., 2014, 2015; Lee et al., 2017a, b). Therefore, the innate mammalian pathogenicity can be inherited to the vaccine strains. Although inactivated vaccine strains are produced under controls of strict biosecurity measures, mammalian non-pathogenic vaccine strains may be more favorable (Liu et al., 2012).

The single replacement of PB2 genomes of PR8 with 01310 CE20 increased replication efficiency of recombinant viruses in ECEs with decreased pathogenicity in BALB/c mice (Jang et al., 2017; Kim et al., 2014). The heterosubtypic protection efficacy of internal proteins may encourage efforts to replace all internal genes of PR8, but optimal 6 avian internal gene sets supporting generation of highly replicative and mammalian non-pathogenic recombinant viruses are not frequent (Liu et al., 2012; Shi et al., 2007).

In this study, we successfully generated complete recombinant 01310 virus by optimization of C4U mutations in polymerases. The C4U mutation of PB2 or PB1 genome may be minimum essential to compensate incompetent PB2 activity of 01310. However, C4U mutation only in PA genome was not enough for virus rescue and even inhibited virus rescue when paired with C4U mutation of PB2 genome. The low integrity of polymerase caused by 01310 PB2 may be overcome by increased expression of PB2 and PB1. PB2 is imported from the cytoplasm to the nucleus independently, but PB1 and PA form dimer in the cytoplasm to be imported efficiently into the nucleus. PB2 forms trimer with PB1/PA dimer in the nucleus (Huet et al., 2010). Therefore, increased levels of PB2 and PB1 molecules may increase active trimer formation. No effect of C4U mutation of PA genome supports the importance of PB1 in the nuclear translocation and trimer formation, but the negative effect of C4U mutation of PA together with PB2 was unexpected. Recently PA-X generated by ribosomal frameshift of PA was reported to contain only endonuclease activity (Jagger et al., 2012). PA-X distributes in the nucleus and cytoplasm, and degrades host mRNAs (Hayashi et al., 2016). Despite of contradiction, PA-X showed generally negative effects on viral replication and pathogenicity (Gao et al., 2015; Jagger et al., 2012). Increased PA expression may also increase PA-X expression, and the negative effects may also increase. Moreover, more efficient replication of r310-U4-PB21 and r0028-U4-PB21 than of r310-U4 and r0028-U4 in ECEs may also support a negative effect of increased PA and PA-X concentration (Ping et al., 2015). However, it is noteworthy that C4U mutations in all polymerases conferred positive and neutral effects on replication efficiency of WSN and PR8 in mammalian cells, respectively (Jiang et al., 2010; Ping et al., 2015). Therefore, the effects may be strain-specific.

Although C4U mutation of polymerases of 01310 PB2 facilitated virus rescue we could not find difference in the polymerase activity between the virus rescue-facilitating and non-facilitating mutations in 293 T cells (Fig. 1). However, the titers of recombinant viruses possessing virus rescue-facilitating mutations were higher in MDCK cells than 01310 CE22 (Fig. 2). Interestingly, the polymerase activities of 0028 CE21-derived mini-genomes were significantly higher than those of 01310 CE22-derived mini-genomes; however, the virus titers of 0028 CE21-derived recombinant viruses were significantly lower than those of 01310 CE22-derived recombinant viruses in MDCK cells. The relatively high polymerase activities of 0028 CE21 mini-genomes may be due to more competent PB2 than that of 01310 CE22 mini-genomes and the low replication efficiency of 0028 CE21-derived recombinant viruses can be explained by the low activities of PA and NS1 in mammalian hosts (Kim et al., 2014, 2015). Therefore, virus rescue and efficient replication of AIVs in different mammalian cells may be affected by multigenic traits.

MDCK cells are permissible to both avian and mammalian IAVs owing to expression of both avian and human receptors (Seitz et al., 2010). However, A549 cells expressed only human receptor and possessed active MxA, a potent antiviral protein (Holzinger et al., 2007). We verified mammalian pathogenicity of recombinant 01310 CE22 viruses using A549 cell model, which has been used for evaluation of mammalian pathogenicity of AIVs. In contrast to recombinant PR8, all recombinant 01310 CE22 and parent 01310 CE22 viruses did not replicate in A549 cells, and they may have lower mammalian pathogenicity than other viruses replicating in A549 cells.

The embryonic pathogenicity of highly pathogenic (HP) AIVs is higher than that of low pathogenic (LP) AIVs due to systemic replication of HP AIVs, which is supported by multi-basic amino acids in the cleavage site of HA (Scholtissek et al., 1988). However, the embryonic pathogenicity of LP AIVs containing only monobasic amino acid in the cleavage site had been unclear. In this study, we demonstrated the role of NS genome in embryonic pathogenicity of AIVs. The NS1 protein is a virulence factor that antagonizes host antiviral response mediated by type I interferons (IFN) and IFN-induced proteins (Hale et al., 2008). Several mammalian pathogenic mutations in NS1 gene were found in a previous study (Kim et al., 2015) and interestingly the mammalian pathogenicity related mutations of NS1, GSEV to EPEV, S151 T, and G139D also increased the embryonic pathogenicity. To our knowledge, the relationship between NS1 and embryonic pathogenicity, and the sharing of the same mutations for embryonic and mammalian pathogenicity were firstly demonstrated in this study.

In this study, we generated highly productive and mammalian non-pathogenic H9N2 vaccine strains by optimizing 3′end promoter and NS genome replacement. The optimization method and internal genes investigated in this study may be useful for the generation of more productive and safer vaccine strains against HPAIVs as well as antigenic variants of H9N2 AIVs.

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