

Effect of the fourth nucleotide at the 3' end of neuraminidase and matrix viral genomic RNA on the pathogenicity of influenza virus A/PR/8/34

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Twelve nucleotides located at the 3' end of viral genomic RNA (vRNA) are conserved among influenza A viruses (IAV) and have a promoter function. Hoffmann's 8-plasmid reverse genetics vector system introduced mutations at position 4, C nucleotide (C4) to U nucleotide (U4), of the 3' ends of neuraminidase (NA) and matrix (M) vRNAs of wild-type A/PR/8/34 (PR8). This resulted in a constellation of C4 and U4 vRNAs coding for low (polymerases) and relatively high (all others) copy number proteins, respectively. U4 has been reported to increase promoter activity in comparison to C4, but the constellation effect on the replication efficiency and pathogenicity of reverse genetics PR8 (rgPR8) has not been fully elucidated. In the present study, we generated 3 recombinant viruses with C4 in the NA and/or M vRNAs and rgPR8 by using reverse genetics and compared their pathobiological traits. The mutant viruses showed lower replication efficiency than rgPR8 due to the low transcription levels of NA and/or M genes. Furthermore, C4 in the NA and/or M vRNAs induced lower PR8 virus pathogenicity in BALB/c mice. The results suggest that the constellation of C4 and U4 among vRNAs may be one of the multigenic determinants of IAV pathogenicity.

Keywords: influenza A virus, pathogenicity, promoter, reverse genetics, viral replication

Introduction

The noncoding regions (NCRs) of the 3' and 5' ends of viral RNA (vRNA) of influenza A virus (IAV) form a 'corkscrew'-like structure and function as promoters for the transcription of messenger (mRNA), complementary RNA, and viral genomic RNA (vRNA) [2,5,16]. The promoter function is reported to be localized to 12 conserved nucleotides at the 3' end of vRNA, and nucleotides 9 to 11 were shown to be crucial for promoter activity [20]. Mutations at positions 11 and 12 of the 3' and 5' ends of neuraminidase (NA) vRNA of influenza A/WSN/33 reduced the NA mRNA and protein levels, as well as the virus titer, and resulted in attenuated phenotypes in mice [6,21]. Therefore, the NCRs of the 3' and 5' ends of vRNA may have an

important role in viral pathogenicity as well as viral gene expression.

The nucleotide at position 4 of the 3' end of NA vRNA has been shown to affect the transcription of vRNA, and a U nucleotide at position 4 (U4) increased the transcription level of NA vRNA above that from a C nucleotide (C4) [14]. Moreover, a U4 to C4 mutation affects viral transcription and replication activity by down-regulating polymerase recognition activity [11]. However, various mutant influenza A/PR/8/34 (PR8) viruses possessing different combinations of C4 and U4 did not produce different viral titers [4]. During adaptation in embryonated chicken eggs (ECEs), wild-type (wt) PR8 acquired multiple mutations in coding genes related to increased viral replication efficiency, and the genome segments of a high yield PR8 strain

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(reverse genetics PR8, rgPR8) was used for generation of recombinant vaccine strains by using reverse genetics. During analysis of the sequences of NCRs, we observed different combinations of U4 and C4 in NCRs of wtPR8 and rgPR8. The polymerases (PB1, PB2, and PA), NA, and matrix (M) vRNAs of wtPR8 possessed C4 rather than U4, but those of rgPR8 acquired U4 mutations in NCRs of the NA and M vRNAs [4,8,9]. Although PR8 virus have been widely used to inactivated vaccine backbone strain, the effect of U4 in NCRs of the NA and M vRNAs of rgPR8 have not been fully elucidated [8,17].

In the present study, we generated 4 recombinant viruses with C4 in the NA and/or M vRNAs and an rgPR8-like constellation of C4 and U4 by undertaking reverse genetics. We then compared their replication efficiency in ECEs, transcription levels of vRNA and mRNA of the NA and/or M genome segments in Madin-Darby canine kidney (MDCK) cells, and their pathogenicity in mice. Our results indicate that C4 in both the NA and M vRNAs decreased virus replication efficiency in ECEs and pathogenicity in mice. Thus, the position 4 nucleotide in the 3' end and the constellation of C4 and U4 among vRNAs may be multigenic determinants of pathogenicity. Studies to reveal the profiles of the promoters of IAVs may be valuable in the prediction of potential pathogenicity.

Materials and Methods

Viruses, eggs, cells, and plasmids

The rgPR8 virus was generated by using Hoffmann's 8-plasmid reverse genetics vector system [8] and was passaged

three times in 10-day-old specific pathogen-free (SPF) ECEs (VALO BioMedia, USA) before use. All of the influenza viruses were inoculated in 10-day-old ECEs via the allantoic cavity route, and the eggs were then incubated for 36 to 72 h. After chilling at 4°C overnight, the allantoic fluid was harvested and stored at -70°C until further use.

The 293T and MDCK cells were purchased from the American Type Culture Collection (USA) and maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, USA) supplemented with 5% fetal bovine serum (Invitrogen). The 293T cells were used to generate recombinant viruses through the reverse genetic process.

Site-directed mutagenesis

The bidirectional transcription vector, pHW2000, and 8 plasmid vectors with 8 genome segments of PR8 were obtained by following the process described by Hoffmann *et al.* [8]. To understand the effects of C4 in the M and NA vRNAs, we mutated nucleotide sequence T4 of the pHW197-M and pHW196-NA plasmids into C4 by applying site-directed mutagenesis (iNtRON Biotechnology, Korea). The mutated plasmids were named pHW197-M-C4 and pHW196-NA-C4. The mutagenesis primer sets are listed in Table 1, and the constellations of C4 and U4 among the viral genomes of each virus are presented in Table 2.

Recombinant virus generation by reverse genetics

The rgPR8 strain was generated by transfection using Hoffmann *et al.*'s 8 reverse genetics plasmids [8] as previously described, with some modifications. The recombinant viruses

Table 1. Primers used in this study

Primer name	Sequence (5'-3')	Usage
PR8-NAprom-mg-F	TCCGAAGTTGGGGGGAGCGAAAGCAGGAGTTTAAAATGA	Mutagenesis for NA vRNA-C4
PR8-NAprom-mg-R	TCGCTCCCCCAACTTCGGA	
PR8-Mprom-mg-F	TATTCGTCTCAGGAGCGAAAGCAGGTAGATATTGAAAGATGAG	Mutagenesis for M vRNA-C4
PR8-Mprom-mg-R	TCGCTCCCTGAGACGAATA	
vRNAtag	GGCCGTCATGGTGGCGAAT	Measurement of NA vRNA level
vRNAtag_PR8seg6_693F	GGCCGTCATGGTGGCGAAT ACTATAATGACTGATGGCCCCGAGT	
PR8seg6_843R	ACATCACTTTGCCGGTATCAGGGT	Measurement of NA mRNA level
mRNAtag	CCAGATCGTTCGACTCGT	
mRNAtag-PR8Seg6-dTR	CCAGATCGTTCGAGTCGT TTTTTTTTTTTTTT TGA ACA AAC TAC	Measurement of M vRNA level
PR8seg6-1318F	TGAATAGTGATACTGTAGATTGGTCT	
vRNAtag	GGCCGTCATGGTGGCGAAT	Measurement of M mRNA level
vRNAtag_PR8seg7_511F	GGCCGTCATGGTGGCGAAT TAGGCAAATGGTGACAACAACCAA	
PR8seg7_624R	GCTGCTTGCTCACTCGATCCAG	Measurement of M mRNA level
mRNAtag	CCAGATCGTTCGACTCGT	
mRNAtag_PR8seg7_dTR	CCAGATCGTTCGAGTCGT TTTTTTTTTTTTTT TAGTAGAAACAA	Measurement of M mRNA level
PR8seg7_936F	GAATATCGAAAGGAACAGCAGA	

NA, neuraminidase; vRNA, viral genomic RNA; C4, C nucleotide at position 4; M, matrix.

Table 2. Nucleotide sequence of the 3'-noncoding region (NCR) of wild-type (wt) A/PR8/34 (PR8), reverse genetics (rg) PR8, and the recombinant PR8 viruses generated in the present study

Coding protein	wtPR8	rgPR8	rPR8-NA-prom	rPR8-M-prom	rPR8-MN-prom
PB1	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu
PB2	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu
PA	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu	3'-ucgCuuu
HA	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu
NP	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu
NA	3'-ucgCuuu	3'-ucgUuuu	3'-ucgCuuu	3'-ucgUuuu	3'-ucgCuuu
M	3'-ucgCuuu	3'-ucgUuuu	3'-ucgUuuu	3'-ucgCuuu	3'-ucgCuuu
NS	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu	3'-ucgUuuu

PB1, PB2 and PA, three viral polymerase proteins; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural.

that possessed C4 in NA, M, and both vRNAs were generated by replacing pHW196-NA and/or pHW197-M with pHW196-NA-C4 and/or pHW197-M-C4 and were named rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8), respectively. Briefly, 293T cells were cultured (1×10^6 cells/well in 6-well plates) and transfected with 300 ng of each plasmid by using lipofectamine 2000 and plus reagents (Invitrogen) in a final volume of 1 mL of Opti-MEM (Invitrogen). After overnight incubation, 1 mL of fresh medium and 0.5 mg/mL of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, USA) were added. After 24 h, the culture medium was harvested and 200 μ L were injected into 10-day-old SPF ECEs via the allantoic cavity route. After incubating for 2 to 3 days, the allantoic fluid was harvested and tested with an hemagglutination test using 1% (v/v) chicken red blood cells according to the World Health Organization Manual on Animal Influenza Diagnosis and Surveillance. All experiments were performed after obtaining permission from the Seoul National University Institutional Biosafety Committee (SNUIBC) (approval No. SNUIBC-R150729-1).

Viral growth kinetics

Each recombinant virus (10 EID₅₀/200 μ L/ECE; EID₅₀, 50% egg infectious dose) was inoculated into eighteen 10-day-old SPF ECEs, and 3 ECEs were harvested at 8, 12, 16, 24, 32, and 48 h post-inoculation. To measure the virus titer at each time interval, the pooled sample was serially diluted 10-fold from 10^{-1} to 10^{-9} , and each dilution was inoculated into MDCK cells. The 50% tissue culture infection dose (TCID₅₀/mL) was calculated more than three times by using the Spearman-Kärber method [7].

vRNA and mRNA quantification

To determine the effects of the introduced mutations, the transcription levels of vRNA and mRNA of the M and NA genome segments were measured by performing two-step

real-time reverse transcription polymerase chain reaction (RT-PCR) as previously described [10,12], with some modifications. Briefly, the recombinant viruses were infected to confluent MDCK cells in a 12-well plate at 0.001 multiplicity of infection for 1 h and washed twice with phosphate buffer saline. Cells were cultured in maintenance medium (DMEM supplemented with 1% bovine serum albumin [fraction V] [Roche, Switzerland], 20 mM HEPES, antibiotic-antimycotic [Gibco, USA], and 1 μ g/mL of TPCK-treated trypsin [Sigma-Aldrich]) at 37°C under humidified 5% CO₂. Cells were harvested after incubation for 6 h, and total RNA was extracted by using the RNeasy Mini Kit (Qiagen, Germany). For the cDNA synthesis, an AmfiRivert cDNA Synthesis Platinum kit (GenDEPOT, USA) was used with the tagged primers (Table 1). Real-time PCR was conducted with SYBR GreenER qPCR SuperMix (Invitrogen) and the specific primer sets (Table 1) by using an ABI StepOne Real-time PCR machine (Applied Biosystems, USA). Transcription levels were normalized by using transcription levels of cellular GAPDH genes in the infected cells as an internal control. The relative transcription levels of vRNA and mRNA of each recombinant virus were represented by the ratio to those of rgPR8. Three independent experiments were performed.

Pathogenicity in BALB/c mice

Five-week-old female BALB/c mice were purchased from Narabiotech (Korea), and the mouse pathogenicity test was conducted by BioPOA (Korea). The mortality and weight loss of BALB/c mice were measured after intranasal inoculation of rPR8, rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8) as previously described [13], with some modifications. Briefly, each recombinant virus was diluted to 10^5 and 10^4 EID₅₀/50 μ L, and 5 mice were assigned to receive one dilution of the virus. Mice were anesthetized with Zoletil (15 mg/kg; Virbac, France) and mortality and weight loss were observed every day for 12 days. When the body weight of a mouse had decreased by more than 20%, the mouse was considered

moribund and was killed by CO₂ asphyxiation. All procedures performed in studies involving animals were approved by the Institute of Animal Care and Use Committee at BioPOA, Korea (approval No. BP-2017-001-2). The mouse experiments were carried out in accordance with the protocol of the National Institutes of Health's Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Statistical analyses

The significance of body weight and virus titer changes was evaluated by using one-way analysis-of-variance (IBM SPSS Statistics ver. 23; IBM, USA). The mortality differences observed in pathogenicity testing were assessed by using the Kaplan-Meier method (log-rank test, 95% confidence intervals). Statistical significance was defined as $p < 0.05$ and $p < 0.001$.

Results

Growth kinetics of recombinant viruses in ECEs

We generated four recombinant PR8 viruses: a rgPR8-like constellation of C4 and U4 (rgPR8), C4 in NA vRNA (rPR8-NA-prom), C4 in M vRNA (rPR8-M-prom), and a wtPR8-like constellation of C4 and U4 (rPR8-MN-prom) (Table 2), and compared their replication efficiency in ECEs. The rgPR8 virus could replicate in ECEs at 8 h post-inoculation, and its virus titers were significantly higher than those of recombinant viruses with C4 instead of U4 in the M and/or NA vRNAs at all of the time intervals that were compared ($p < 0.05$) (Fig. 1).

Comparison of relative vRNA and mRNA transcription levels of recombinant viruses

In order to verify the effects of the U4 to C4 substitutions on viral genome transcriptions, we compared relative vRNA and

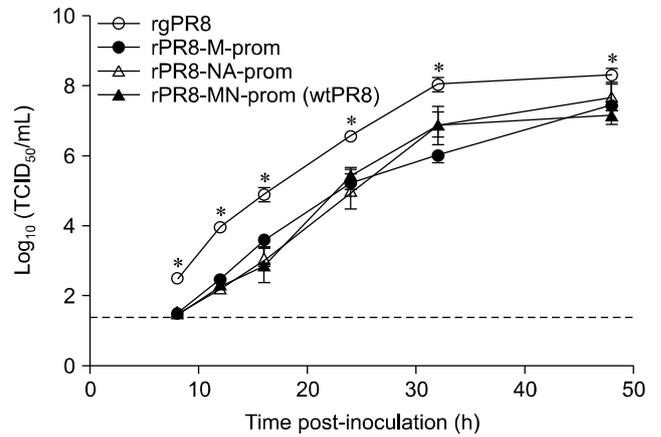


Fig. 1. Comparison of virus titers of recombinant viruses with different constellation of C nucleotide and U nucleotide at position 4 in the 3'-end of the noncoding region of the viral genome. Each recombinant virus (10 EID₅₀/200 μL/ECE; EID₅₀, 50% egg infectious dose; ECE, embryonated chicken egg) was inoculated into eighteen 10-day-old specific pathogen-free ECEs, and 3 ECEs were harvested at 8, 12, 16, 24, 32, and 48 h post-inoculation. The virus titers were measured by 50% tissue culture infection dose (TCID₅₀) assay in Madin-Darby canine kidney cells. *Asterisks represent a significant difference of virus titers between the reverse genetics PR8 (rgPR8) and the other recombinant viruses ($p < 0.05$).

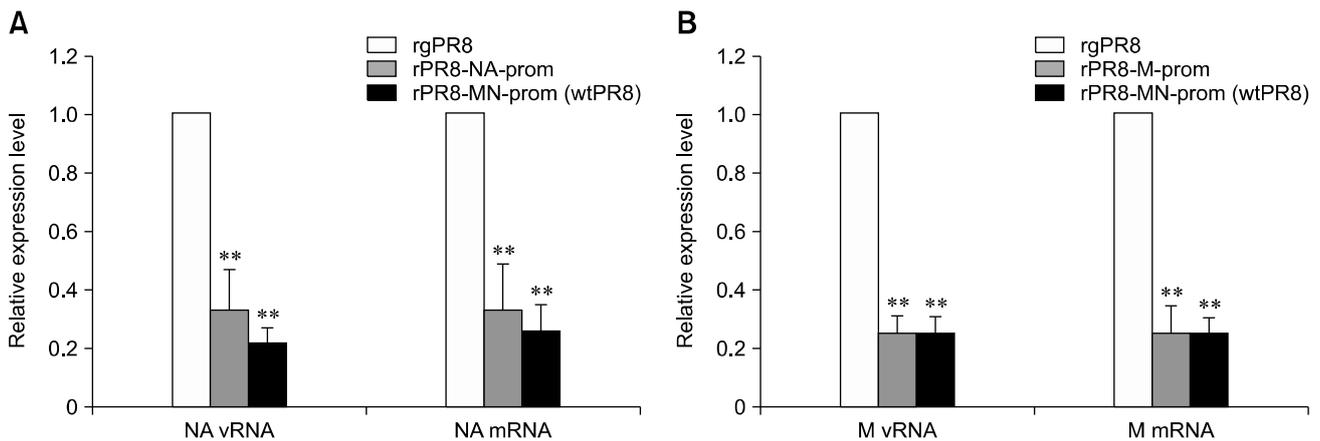


Fig. 2. Relative transcription levels of viral genomic RNA (vRNA) and messenger RNA (mRNA) of recombinant viruses. (A) Relative transcription levels of vRNA and mRNA of the neuraminidase (NA) genome segments. (B) Relative transcription levels of vRNA and mRNA of the matrix (M) genome segments. Madin-Darby canine kidney cells were infected by recombinant viruses at 0.001 multiplicity of infection at 37°C, and cell lysates were harvested at 6 h post-inoculation. The vRNA and mRNA transcription levels were normalized by the transcription levels of GAPDH gene of the infected cells. The relative transcription levels of vRNA and mRNA of each recombinant virus were represented by the ratio to those of rgPR8. The data presented are the average of three independent experiments. **Asterisks represent a significant difference between the rgPR8 and other recombinant viruses ($p < 0.001$).

mRNA transcription levels of the NA and M genome segments of recombinant viruses by using tagged-primer two-step real-time RT-PCR (Fig. 2). The U4 to C4 substitutions in NA vRNA decreased both vRNA and mRNA transcriptions of the NA genome segment by more than 50%. Similar to the NA results, C4 in M vRNA reduced the vRNA and mRNA transcriptions of the M genome segment. The wtPR8 (rPR8-MN-prom) showed downregulated transcription of both M and NA viral genome segments.

Comparison of pathogenicity of recombinant viruses in mice

Because the U4 to C4 substitutions affect the viral replication efficiency, we compared the pathogenicity of recombinant

viruses with that of rgPR8 in BALB/c mice. The 10^5 EID₅₀ inoculation of rPR8-NA-prom, rPR8-M-prom, and wtPR8 (rPR8-MN-prom) led to weight loss less than that of rgPR8, and caused less mortality and a greater mean time to death in mice (Fig. 3). Furthermore, the inoculation of 10^4 EID₅₀ recombinant viruses did not cause any death in contrast to that of 10^4 EID₅₀ rgPR8 (Fig. 3).

Discussion

The conserved, segment-specific NCRs of the 3' and 5' ends of the vRNAs of IAVs are related to viral RNA transcription and affect virus replication and pathogenicity [14,20-22]. The C4 to U4 substitution has been shown to increase the transcription of

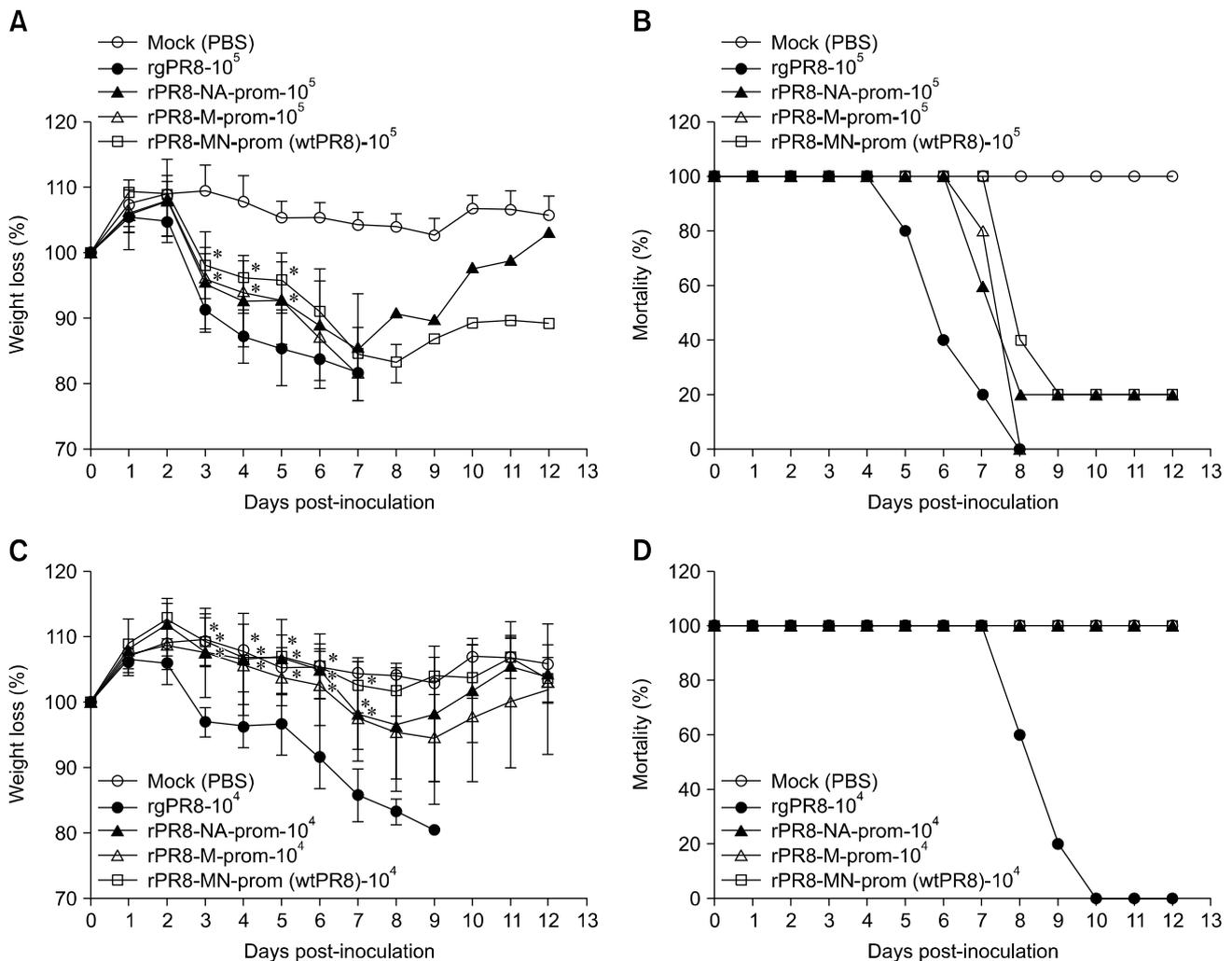


Fig. 3. Comparison of mouse pathogenicity of recombinant viruses. The 10^5 and 10^4 EID₅₀/50 μ L/mouse recombinant viruses were challenged to five 5-week-old BALB/c mice. Weight loss and mortality were monitored for 12 days. (A) Weight loss and (B) mortality of mice infected by 10^5 EID₅₀ of each virus. (C) Weight loss and (D) mortality of mice infected by 10^4 EID₅₀ of each virus. *Asterisks indicate weight loss of recombinant viruses is significantly different from that of rgPR8 ($p < 0.05$). EID₅₀, 50% egg infectious dose; PBS, phosphate-buffered saline; wtPR8, wild-type A/PR8/34.

NA vRNA of WSN/33 by 20-fold, but various combinations of C4 and U4 in vRNAs of PR8 did not affect the virus replication efficiency in 293T cells [14,22]. In the present study, we showed the virus titers of an rgPR8-like constellation of C4 and U4 (rgPR8) were significantly higher than those of C4 in NA vRNA (rPR8-NA-prom), M vRNA (rPR8-M-prom), and both vRNAs (rPR8-MN-prom) at all time intervals that were compared ($p < 0.05$) (Fig. 1). Considering that rgPR8 has constellations of U4 in 5 vRNA coding proteins (hemagglutinin [HA], nucleoprotein [NP], NA, M, and nonstructural [NS]) with relatively high copy numbers and C4 in 3 vRNA coding proteins (PB1, PB2, and PA) with relatively low copy numbers, an optimal constellation of C4 and U4 may result in a harmonized production of viral proteins to form virus particles. NA is important for virus budding, and matrix 1 protein is one of the major structural proteins that helps to export ribonucleoprotein complex to the cytoplasm [1,15]. Therefore, U4 to C4 substitutions in the NA and/or M vRNAs may cause a decrease in the replication efficiency of rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8). The virus titer of a recombinant PR8 virus generated with 8 plasmids with C4 was slightly higher than that of another PR8 virus generated with 8 plasmids with U4. However, the virus titers of 8 types of recombinant PR8 viruses with one C4 genome segment were not improved [4]. Thus, balanced production of low copy (PB1, PB2, and PA) and relatively high copy (HA, NP, NA, M, and NS) number viral protein transcripts by weak (C4) and strong (U4) promoters, respectively, may also be important for efficient virus replication.

Relative vRNA and mRNA transcription levels of the NA and M genome segments of recombinant viruses demonstrated that the U4 to C4 substitutions decreased both vRNA and mRNA transcription of NA and M genome segments (Fig. 2). The previously reported effects of C4 to U4 substitution on vRNA and mRNA transcription have been inconsistent. Lee and Seong [14] reported U4 downregulated vRNA but upregulated mRNA transcription levels. However, Jiang *et al.* [11] demonstrated C4 decreased both vRNA and mRNA transcription *in vitro*. Because strand-specific real-time RT-PCR with tagged primers could measure the amount of viral genomes more sensitively than conventional real-time RT-PCR or RNase protection assays, we concluded that U4 to C4 substitutions may reduce not only mRNA but also vRNA transcription of NA and/or M genome segments [12].

Reduced levels of NA due to decreased promoter activity by mutations at the 3' and 5' end NCRs of WSN/33 were reported to attenuate mutant viruses in mice [21]. The effect of the position 4 nucleotide on mice pathogenicity of WSN/33 mutants has been demonstrated, but the tested mutant viruses with all U4 or all C4 were considered too artificial [11]. The inoculation of 10^5 and 10^4 EID₅₀ dose of rgPR8 to each BALB/c mouse caused 100% mortality (Fig. 3). The inoculation of 10^5

EID₅₀ dose of rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8) caused 80%, 100%, and 80% mortality, respectively, along with greater mean death times. However, 10^4 EID₅₀ doses did not cause any mortality (Fig. 3). The 50% minimal lethal dose (MLD₅₀) of PR8 is slightly lower than the 10^4 EID₅₀ dose [19]. However, C4 in NA and/or M genes of PR8 may attenuate the viral pathogenicity to increase MLD₅₀ more than the 10^4 EID₅₀ dose. According to our results, rgPR8 was more pathogenic than rPR8-NA-prom, rPR8-M-prom, and rPR8-MN-prom (wtPR8) in BALB/c mice. Thus, U4 to C4 mutations in NA and/or M vRNA could attenuate viral pathogenicity of PR8 in BALB/c mice, as suggested previously [11].

To date, most full genome sequencing of IAVs has been conducted with primer sets designed on the basis of the conserved nucleotide sequences of the 3' and 5' ends of IAVs [9]. Thus, natural 3' and 5' end nucleotide sequences are rarely available among the reports of complete sequences of IAVs. Natural variability of the position 4 nucleotide in 8 vRNAs has been reported, and combinations of C4 and U4 in 8 vRNAs have been more variable than expected [2,18,22]. Therefore, sequencing the NCRs of IAVs may reveal important information regarding the variability of promoter profiles among gene segments and virus strains and may provide basic data to predict potential pathogenicity in mice [3,22].

In conclusion, the position 4 nucleotide of NA and M vRNA of IAVs is important for the effective replication of the PR8 virus in ECEs as well as having importance in virus pathogenicity in mice. Furthermore, an appropriate constellation of weak and strong promoters to low and high copy number protein-coding genome segments is important for virus replication and pathogenicity. Thus, sequence analysis of NCRs of a viral genome may be valuable in elucidating potential pathogenicities of IAVs in mice.

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Conflict of Interest

The authors declare no conflicts of interest.

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