Virus Genes (2019) 55:440–447
https://doi.org/10.1007/s11262-019-01654-2

U13 → C13 mutation in the variable region of the NA gene 3′UTR of H9N2 influenza virus influences the replication and transcription of NA and enhances virus infectivity

Xi Li[1] · Yanci Chen[1] · Xin Wang[2] · Bo Peng[2] · Weihua Wu[2] · Hui Liu[2] · Ying Sun[2] · Xiujuan Tang[2] · Qing Zheng[1] · Shisong Fang[2]

Received: 30 January 2019 / Accepted: 5 March 2019 / Published online: 25 April 2019

Abstract

The untranslated regions within viral segments are the essential promoter elements required for the initiation of viral replication and transcription. The end of the UTR sequence and part of the ORF sequence constitute the packaging signal for progeny viruses. To explore the influence of single-point and multi-site joint mutations in the UTR of the NA gene on the viral expression, we select clones with upregulated expression of the reporter gene and analyze their sequence characteristics. Bioinformatics methods were used to analyze polymorphisms in the untranslated region (UTR) of the neuraminidase gene of the H9N2 influenza A virus. Using the RNA polymerase I reporting system with enhanced green fluorescence protein (EGFP) gene as the reporter gene, libraries containing random mutations at sites within the N2 UTR were constructed using random mutagenesis. The mutants were selected from the randomized mutagenesis libraries for the N2-UTR. The N2-UTR-RNA polymerase I fluorescence reporter system was identified by sequencing and transfected into infected MDCK cells. The expression of the reporter EGFP was observed using fluorescence microscopy, and the relative fluorescence intensity was measured using a multifunctional microplate reader to analyze the expression of the reporter gene (EGFP) qualitatively and quantitatively. Herein, an RNA polymerase reporter system was constructed to rescue the mutated viruses and measure their tissue culture infective dose (TCID50). The results showed that the U13 → C13 mutation in the 3′end of the NA gene promoted the expression of viral RNA and protein, and mutation of other sites within the UTR could differentially regulate viral genomic transcription and translation. These data showed that the U13 → C13 mutation within the variable region of the 3′UTR of the NA gene in the H9N2 influenza virus promotes viral genomic expression and infection.

Keywords H9N2 influenza A virus · The variable region · EGFP · N2-UTR · RNA polymerase I fluorescence reporter system

Statement

The paper expands upon functional research on base polymorphisms at nucleotide positions in the variable region of the UTR that have not been previously reported.

Introduction

From the exterior to the interior, the structure of the influenza A virus is composed of a capsule that contains surface glycoproteins, the matrix proteins, and the core regions [1]. The genetic material of the influenza A virus consists of eight single-stranded and negative-stranded viral RNA (vRNA) segments that are contained within an envelope.

Edited by Dr. Zhen F. Fu.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11262-019-01654-2) contains supplementary material, which is available to authorized users.

✉ Qing Zheng
tzhengq@jnu.edu.cn

✉ Shisong Fang
1229346705@qq.com

[1] College of Pharmacy, Jinan University, Guangzhou 510632, China

[2] Shenzhen Center for Disease Control and Prevention, Shenzhen 518055, China

The eight vRNA gene segments are approximately 13 kb in length and are known to encode the following influenza A viral proteins: PB1, PB2, PA, NP, M1, M2, HA, NA, NS1, and NS2 [2–8]. These proteins constitute the RNA polymerase, RNA binding protein, matrix protein, ion channel, surface glycoprotein, and nuclear transporter, all of which perform the basic functions of the virus. The viral RNA-dependent RNA polymerase (vRNPs) consists of vRNA, the RNA polymerase complex (RdRp), and the nuclear protein (NP), which are the smallest units of viral replication [9, 10]. The neuraminidase (NA) of influenza virus is a viral surface glycoprotein. As one of the most important antigens of influenza virus, NA can eliminate sialic acid residues on the surface of infected cells, release progeny virus from the host cells, and assist the progeny virus particles in moving into the respiratory mucus of the host to infect new cells [11, 12].

The full-length NA gene of the H9N2 influenza virus is 1467 nt in length; the first 1–13 nt of the 5′ UTR (untranslated region) and 1–12 nt of the 3′ UTR are highly conserved. The NA gene also contains an open reading frame (ORF) and an untranslated region (UTR) that is located on both sides of the ORF and can be divided into a variable region and a conserved region. The UTR is the essential promoter element required for initiating viral replication and transcription [13]; the end of the UTR sequence and part of the ORF sequence constitute the packaging signal for progeny viruses [14]. When using the reverse genetics system for virus rescue, it was found that the integrity and accuracy of the 3′ and 5′ sequences of the UTR directly affected the effectiveness of virus rescue and the infectivity of the progeny viruses. Zhao et al. [15] used the WSN (H1N1) reverse transcription gene system to study the HA-UTR gene (H1-H7 and H9) of the influenza A virus and found that (1) the 3′-UTR is more critical for progeny HA vRNA entry in new viruses than the 5′-UTR; (2) the specific UTR nucleic acid site and sequence length regulate the newly produced HA vRNA in progeny viruses. At present, studies have shown that the UTR at the end of the influenza A virus gene is mostly conserved. It is generally accepted that the nucleotides at positions 1–13 in the 5′-UTR and positions 1–3 and 5–12 in the 3′-UTR are highly conserved, apart from the U/C at position 4 in the 3′ end. Sun et al. [16] mutated C4 to U4 in the 3′-UTR of the PB1 gene of a highly pathogenic avian influenza virus (A/Vietnam/1194/2004 (H5N1)) and found that the recombinant virus promoted the transcriptional activity of RdRp. The LD50 of the H5N1 recombinant virus in a mouse animal model was lower than that of the PB1-3′-UTR-C4 virus, and the body weights in the experimental group were significantly reduced, suggesting that the PB1-3′-UTR-U4 mutant virus had greater pathogenicity. Similarly, it has been found that the HA/NA-3′-UTR-U4 can significantly increase the level of HA/NA-mRNA synthesis and stimulate the production of HA and NA proteins. It has been suggested that the presence of U4 in the 3′-UTR of the surface glycoprotein in influenza A virus can result in improved recognition and localization of RdRp and promote the transcription of viral proteins [17]. Scientists have been constantly researching and working to discover the functions of the bases in the UTRs of influenza viruses. However, systematic study of the functional research on base polymorphisms at various nucleotide positions in the variable regions of UTRs has not been previously reported.

To examine the effects of base mutations in UTRs on transcription and translation, we initially screened mutated UTR sequences for differential expression, which laid the foundation for further investigation of the effects of base polymorphisms in the UTRs of the H9N2 influenza virus on viral transcription and translation.

Methods

Construction of the mutation library

The NA genomic cDNA sequence information for the N2 subtype of the influenza A virus, which was obtained from the Influenza Virus Database at NCBI, was aligned using MEGA (Molecular Evolutionary Genetics Analysis) 7.0 software to screen out the 3′-UTR and 5′-UTR sequences and analyze the mutation sites within UTR sequences from the same evolutionary branch. The main sequence of the forward primer (F), which contained the EGFP KOZAK sequence and BsmbI restriction sites, was designed based on N2-5′-UTR cDNA. Similarly, the main sequence of the reverse primer (R), which contained the EGFP sequence and BsmbI restriction sites, was designed based on N2-3′-UTR cDNA. Based on the UTR sequence of the influenza virus N2 subtype, corresponding primer sequences containing random bases were designed (F/R: N2-5′-UTR/N2-3′-UTR-Majority; N2-5′UTR-Majority/N2-3′UTR; N2-5′-UTR/N2-3′-UTR). The N2-UTR mutation library was constructed using PCR that utilized the RNA polymerase I fluorescence reporter system plasmid as a template.

Construction of the RNA polymerase I fluorescence reporting system

Based on previous experiments [18], the humanized promoter (PIh) and murine terminator (t1) of RNA polymerase I were synthesized and the BsmbI restriction site (5′-GGA GACGGTACCGTCTCC-3′) was inserted between the promoter and the terminator to create the primary component of the RNA polymerase I fluorescence reporter system. This component was cloned into the reconstructive pMD18-T vector, from which non-specific BsmbI sites were removed using site-directed mutagenesis to construct the skeleton

of the RNA polymerase I fluorescence reporter system. The majority sequence, which was obtained via MegAlign software analysis of the N2-5′-UTR and N2-3′-UTR sequences, was spliced into the N2-5′-UTR-EGFP-3′-UTR target sequence. The target sequence was then ligated into the skeleton of the RNA polymerase I fluorescent reporter system to construct the pMD18-T-t1-N2-5′-UTR-EGFP-3′-UTR-PIh fluorescence reporter system.

Cell culture

Madin–Darby canine kidney (MDCK) cells were cultured in T25 cell culture flasks with Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in the presence of 5% CO_2.

Transfection of MDCK cells with recombinant plasmid

MDCK cells were infected for 2 h with H1N1 influenza virus [A/Shenzhen/42/2009(H1N1)] at an m.o.i. (multiplicity of infection) of 1, and then the medium was replaced with Opti-MEM (reduced serum medium). The cells were transfected with recombinant plasmid using Lipofectamine 2000 according to the manufacturer's protocol and maintained in an incubator at 35 °C in the presence of 5% CO_2. The expression of the fluorescent reporter gene was observed under a fluorescence microscope, and the relative fluorescence values were measured using a Victor multi-function microplate reader at 24 h, 36 h, 48 h, and 60 h post-transfection.

Real-time RT-PCR using SYBR PrimeScript

To precisely quantify the relative expression of mRNA, the one-step SYBR PrimeScript RT-PCR method was used. The sequence of the β-actin primer (Table 1) used for the MDCK cells was obtained from a previous report [19]. The total RNA in cells was harvested 24 h and 48 h after the positive clone plasmid was transfected into the infected cells. The experimental group consisted of N2-1, N2-2, N2-3, N2-5, N2-6, and N2-8, with the wild type as a control, and was named N2 [A/chicken/Beijing/0309/2013(H9N2)]. These RNA samples were subjected to one-step RT-PCR with specific primers, EGFP-F/R and β-actin-F/R, at 24 h and 48 h. The results were then analyzed using the $2^{-\Delta\Delta Ct}$ method with GraphPad Prism 5.0 mapping software to compare the relative differences in expression between the mutated and wild-type plasmids.

Table 1 The primer for RT-PCR

Primer	Sequence
β-actin-F	5′-CGTGCGTGACATCAAGGAAGAAG – 3′
β-actin-R	5′-GGAACCGCTCGTTGCCAATG – 3′
EGFP-F	5′- GCCACCATGGTGAGCAAGGGCGAG – 3′
EGFP-R	5′-TTACTTGTACAGCTCGTCCATG-3′

Rescue of viruses with U_{13} and C_{13} mutation

Reverse genetics system containing eight recombinant plasmids was provided by Shenzhen Center for Disease Control and Prevention. MDCK and 293T mixed cells were transfected with eight recombinant plasmids, PHW2000-PB1, PHW2000-PB2, PHW2000-PA, PHW2000-HA, PHW2000-NP, PHW2000-M, PHW2000-NS, and PHW2000-NA-U13 or PHW2000-NA-C13 with lipofectamine 2000 according to the manufacturer's protocol. After 6-h post-transfection, the medium was replaced with Opti-MEM (reduced serum medium) and maintained in an incubator at 37 °C in the presence of 5% CO_2. In order to detect the TCID50 of the recombinant viruses, the supernatant was collected at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h.

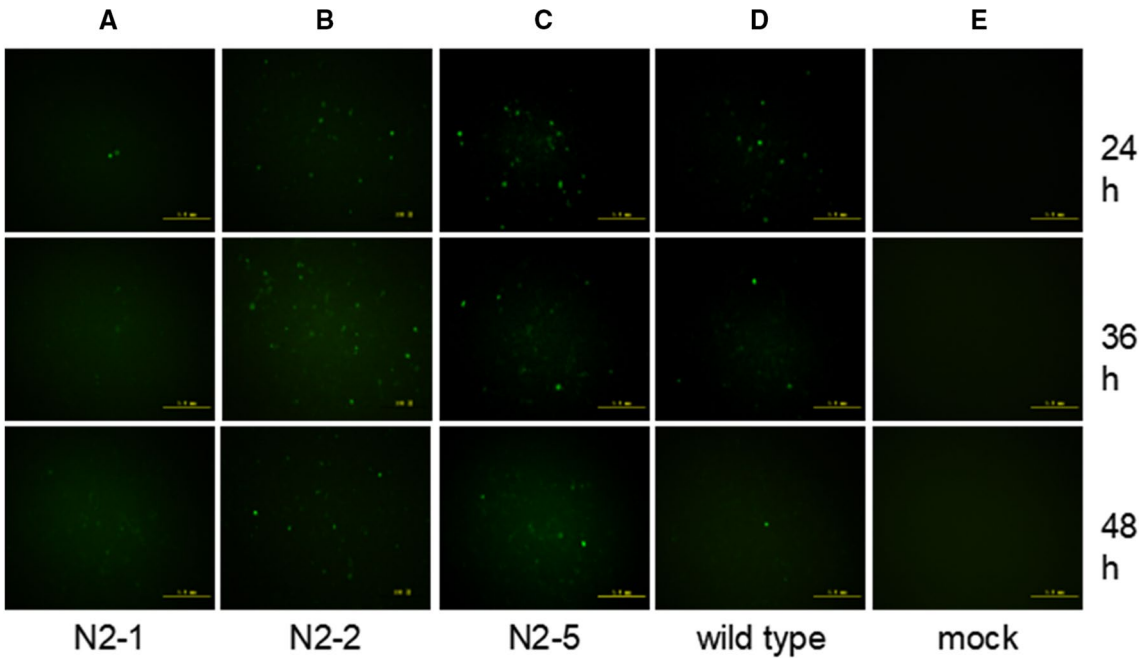
Results

Determination of the effects of the N2-UTR mutant strain on the EGFP expression level

To examine the influence of variations in the N2-UTR on EGFP expression, plasmids containing randomly mutated UTRs and the N2-UTR-RNA polymerase I fluorescence reporter system were transfected into MDCK cells that were infected with H1N1 influenza virus at an MOI of 1. The expression of EGFP was then observed using a fluorescence microscope at 24 h, 36 h, and 48 h. As shown in Fig. 1, the expression of the N2-1-3′/5′-UTR combined mutant strain was downregulated, while the expression of the N2-2/N2-5-3′/5′-UTR combined mutant strains was upregulated. Moreover, the results showed that the expression of the N2-1-3′-UTR mutation strains was decreased, while the expression of the N2-2/N2-5-3′-UTR mutation strains was increased (Fig. 2). However, there were no differences in the expression of the N2-1/N2-2/N2-5-5′-UTR mutation strains (Fig. 3).

Determination of relative fluorescence values

A multi-function microplate reader was used to analyze the relative fluorescence values (Rfu) of mutant strains in infected cells. MDCK cells were infected with H1N1 influenza virus at an m.o.i of 1. The plasmids containing randomly mutated N2-UTRs and the RNA polymerase I fluorescence reporter system were transfected into the infected



Notes:

Name of mutant strains	3’ UTR	5’ UTR
N2-1	U4→C4, C16→G16	C22→U22
N2-2	U13→C13	C22→U22
N2-5	U4→C4, C16→A16	C22→G22

Fig. 1 Effects of random N2-3′/5′UTR mutations on EGFP expression as observed using fluorescence microscopy (200×). **a** N2-1-3′/5′-UTR combined mutant strain. **b** N2-2-3′/5′-UTR combined mutant strains. **c** N2-5-3′/5′-UTR combined mutant strain. **d** Control: wild-type N2-UTR sequence [A/chicken/Beijing/0309/ 2013 (H9N2)]. **e** Negative control: mock

MDCK cells, and the Rfu values were measured at 24 h, 36 h, 48 h, and 60 h (excitation wavelength: 488 nm; absorption wavelength: 507 nm). The experiment was conducted independently three times. Using GraphPad Prism 5.0 mapping software, the normalized fluorescence intensity (the normal ratio of the Rfu value for the experimental group to that for MDCK cells infected with H1N1 influenza virus) was plotted on the ordinate, while time was plotted on the abscissa. A line chart of the intensity as a function of time was constructed. Line graphs showing the changes in fluorescence intensity over time were plotted using GraphPad Prism 5.0 mapping software; X represents the normalized fluorescence intensity as the ratio of the average Rfu of the experimental group to the Rfu measured in infected MDCK cells that did not contain mutant plasmid, while Y represents time. The experimental data were analyzed using a two-way ANOVA of multiple comparisons method to determine the significance of the differences. As shown in Fig. 4, the normalized fluorescence intensity for the experimental group strains N2-1-3′UTR (Fig. 4a) and N2-1-3′/5′UTR (Fig. 4c) was significantly downregulated compared with that of the standard strain. In contrast, the normalized fluorescence intensity for the mutant strains N2-2/N2-5-3′UTR (Fig. 4a) and N2-1/N2-5-3′/5′UTR (Fig. 4c) was upregulated. However, the normalized fluorescence intensity for the N2-1/N2-2/N2-5-5′-UTR mutant strains showed no significant differences (Fig. 4b).

The regulation of NA RNA by UTR mutations

To measure the effects of UTR mutations on viral RNA expression, MDCK cells were infected with H1N1 influenza

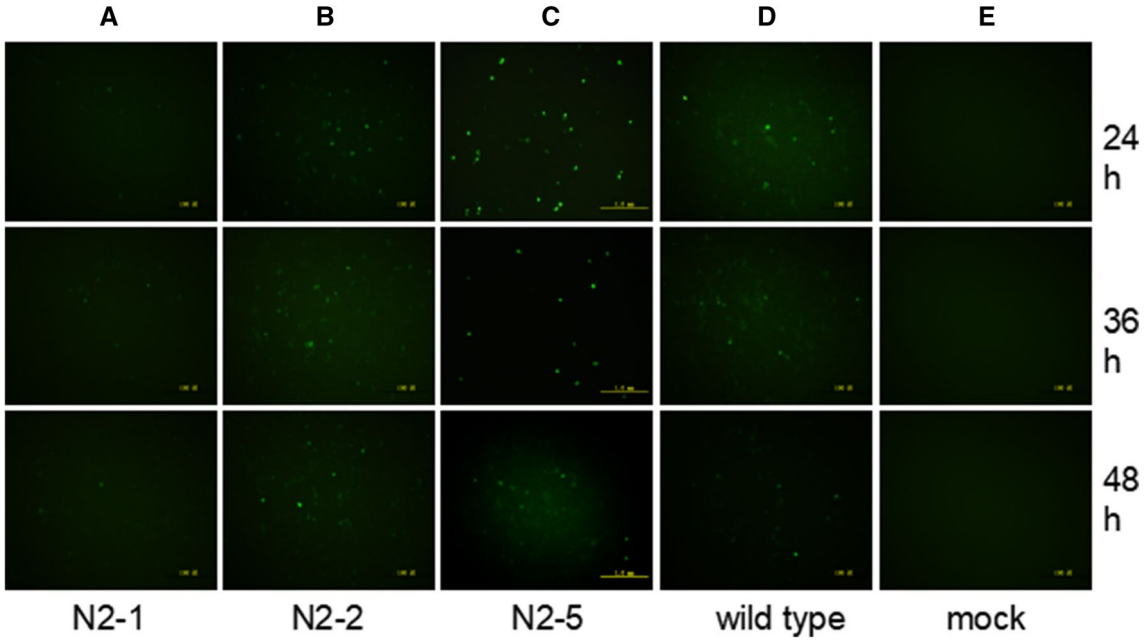


Fig. 2 Effects of random N2-3′UTR mutations on EGFP expression as observed using fluorescence microscopy (×200). **a** N2-1-3′-UTR mutant strain. **b** N2-2-3′-UTR mutant strains. **c** N2-5-3′-UTR mutant strain. **d** Control: wild-type N2-UTR sequence [A/chicken/Beijing/0309/ 2013 (H9N2)]. **e** Negative control: mock

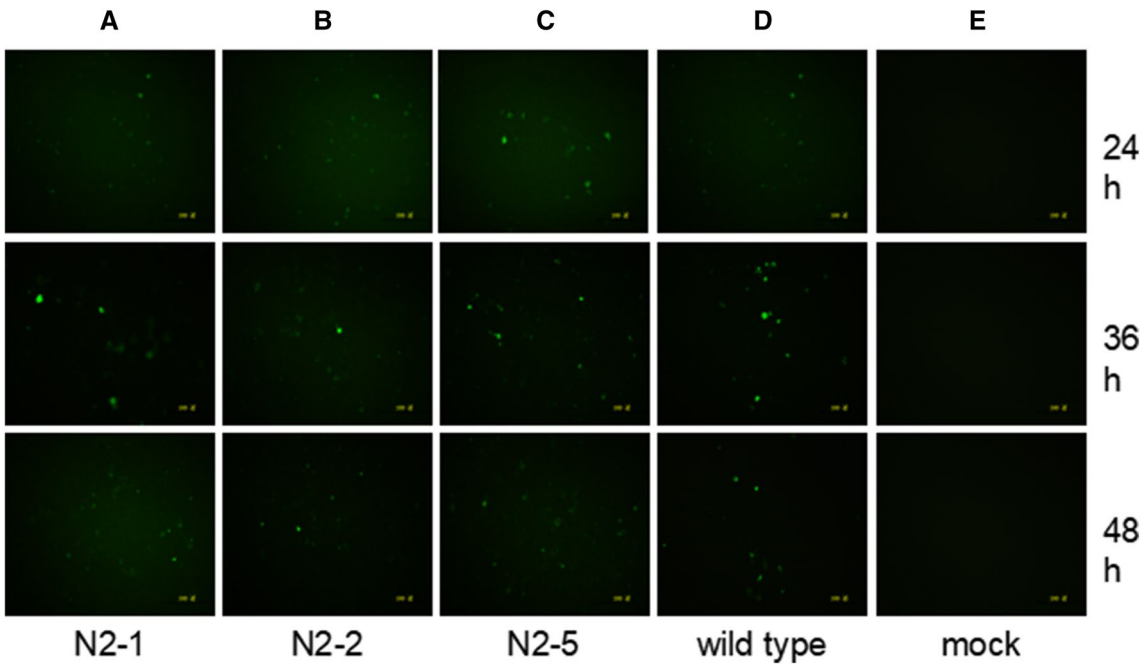


Fig. 3 Effects of random N2-5′UTR mutations on EGFP expression as observed using fluorescence microscopy (200×). **a** N2-1-5′-UTR mutant strain. **b** N2-2-5′-UTR mutant strains. **c** N2-5-5′-UTR mutant strain. **d** Control: wild-type N2-UTR sequence [A/chicken/Beijing/0309/ 2013 (H9N2)]. **e** Negative control: mock

virus at an MOI of 1, and then N2-UTR mutant plasmids were transfected into the cells. Twenty-four or 48-h post infection, the MDCK cells were harvested to extract total RNA. To quantify the vRNA expression of N2-UTR mutant strains, a real-time RT-PCR method that utilized SYBR Green I dye was employed, and the relative expression of

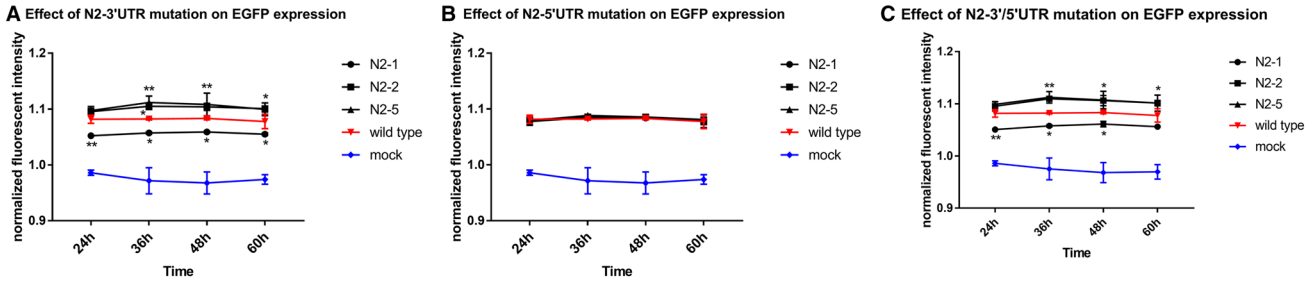


Fig. 4 Statistical analysis of the normalized fluorescence intensity of strains containing randomly mutated N2-UTR-EGFP sequences using a multilabel reader. **a** N2-3′-UTR mutant strain. **b** N2-5′-UTR mutant strains. **c** N2-3′/5′-UTR mutant strain. Positive control: the wild-type N2-UTR sequence [A/chicken/Beijing/0309/2013 (H9N2)]; negative control: mock of the RNA polymerase I fluorescence reporter system. The mean relative activity and standard deviation (error bars) are shown. The data were statistically analyzed using a two-way ANOVA method with GraphPad Prism Software. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

the RNA was calculated using the $2^{-\Delta\Delta Ct}$ method. GraphPad Prism 5.0 mapping software was used to construct a column chart in which X represents the $2^{-\Delta\Delta Ct}$ value and Y represents time (the dotted line Y = 1 represents the relative expression of the wild-type strain). Significant results were found; as shown in Fig. 5, relative expression in the N2-2/N2-5-3′UTR mutant strains and the N2-2/N2-5-3′/5′UTR combined mutant strains was upregulated, while the relative expression in the N2-1-3′UTR mutant strain and N2-1-3′/5′UTR combined mutant strain was clearly downregulated. However, variation in the N2-1/N2-2/N2-5-5′UTR strains made no significant contribution to the relative expression of RNA.

Growth characteristics of viruses by UTR mutations

The growth of the mutant viruses was characterized in MDCK cells. MDCK cells were infected with the two isogenic viruses at an MOI of 1, and maintained in serum-free DMEM. Supernatants were harvested at 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h. As shown in Fig. 6, peak yield of viruses was attained 24 h after infection. The TCID50 (Tissue culture infective dose) of the N2-2-3′UTR mutant virus and the N2-2-3′/5′UTR combined mutant virus were higher than wild type (Fig. 6a, c). Respectively, there is no difference between the TCID50 of the N2-2-5′UTR mutant virus and wild type (Fig. 6b).

Discussion

The highly conserved first 12 nucleotides of the 3′UTR and the first 13 nucleotides of the 5′UTR form the promoter for influenza virus vRNA [10]. The sequences of the 3′UTR and 5′UTR are complementary and form a looped structure that functions to bind and immobilize RdRp (RNA-dependent RNA polymerase) [20]. The influenza virus promoter induces transcription and replication of viral RNA via synergy with the virus RdRp. The conserved region is highly involved in the transcription, replication, and packaging processes of the virus. Synonymous mutations at the conserved site indicate that disruption of the looped structure in the conserved region will affect the production of infectious progeny virus [21]. Until now, few studies have shed light on the effects of mutations in the UTR of influenza A virus on gene transcription and translation. Jun Ma et al. [22] found

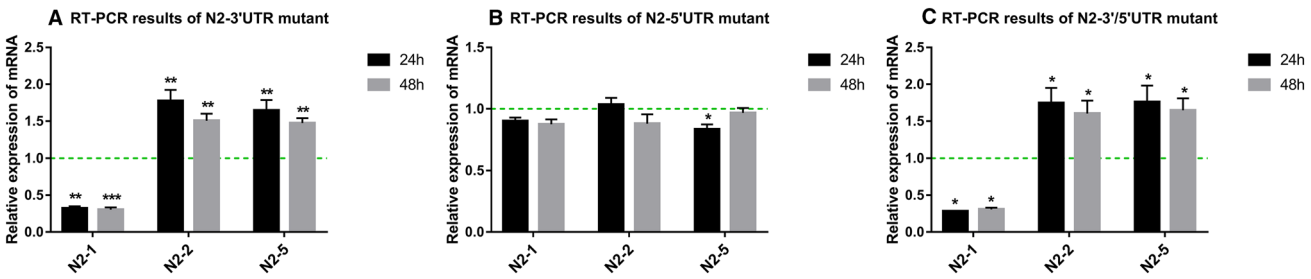


Fig. 5 Statistical analysis of the determination of the effects of random mutations in N2-UTRs on relative changes in gene expression using RT-PCR. **a** N2-3′-UTR mutant strain. **b** N2-5′-UTR mutant strains. **c** N2-3′/5′-UTR mutant strain. Y = 1 represents the positive control [wild-type N2-UTR sequence; A/chicken/Beijing/0309/2013 (H9N2)]. The mean relative activity values (±SEM) are shown. The *P* values representing significance for the experimental group were calculated using a one-way ANOVA method. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

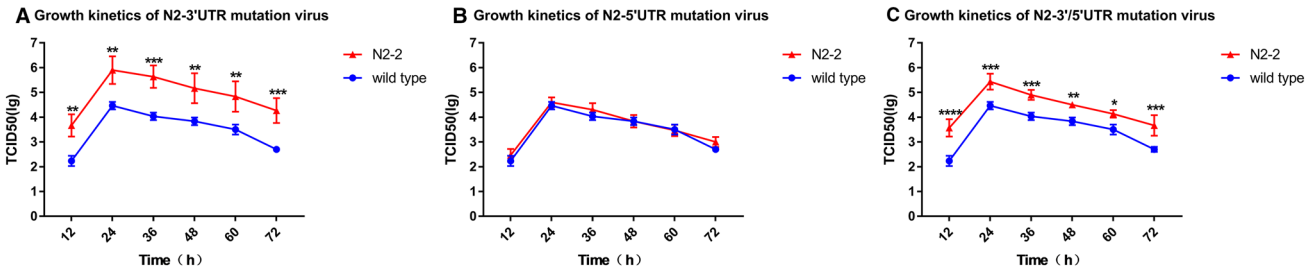


Fig. 6 Growth kinetics of the random mutations on N2-2-UTRs of H9N2 virus using TCID50. **a** N2-2-3′-UTR mutant virus. (U13→C13 mutation on 3′UTR) **b** N2-2-5′-UTR mutant virus. (C22→U22 mutation on 5′UTR) **c** N2-2-3′/5′-UTR mutant virus (U13→C13 mutation on 3′UTR; C22→U22 mutation on 5′UTR) wild type: A/chicken/Beijing/0309/2013 (H9N2). The *P* values representing significance for the experimental group were calculated using a two-way ANOVA method. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$

that natural mutation (U or C) of the nucleotide at position 4 in the 3′UTR can temporarily control the transcription of the virus. C→U at this position in the NA segment effects the level of mRNA and stimulates NA protein synthesis. Furthermore, Zhao et al. [15] suggested that, in addition to specific nucleotides, the length of the HA non-coding region is also involved in regulating the efficiency of vRNA integration. Mutation, deletion, and substitution within the variable region in the UTRs of influenza virus can significantly affect the transcription of vRNAs into mRNAs. The length, tertiary structure, and nucleotide conservation of the UTR in the HA and NA genes of influenza A virus have an impact on the transcription, replication, packaging, and expression of HA and NA viral vRNAs. Therefore, many studies have focused on the relationship between the conserved regions of the 3′/5′ UTRs and the transcription and replication of vRNA for each gene fragment [23–25]. Similarly, the variable regions in the 3′/5′UTRs have been observed to influence the packaging of progeny viruses [26, 27].

The western blot method was used to quantify the expression of eGFP protein. This was unsuccessful due to the low transfection efficiency, which resulted in low amounts of eGFP protein in the MDCK cells and no target band. Therefore, the examination of the N2-UTR mutant strains was performed using a fluorescence microscope (Figs. 1, 2, 3), a multi-microplate reader (Fig. 4), and RT-PCR quantitative analysis (Fig. 5). The results showed that the 3′/5′-UTR combined N2-2/N2-5 mutant strains and the 3′-UTR N2-2/N2-5 mutant strains were significantly upregulated. In addition, the N2-1-3′/5′-UTR combined mutant strains and the N2-1-3′-UTR mutant strain were downregulated. However, the N2-1/N2-2/N2-5-5′-UTR mutant strains showed no significant changes. Based on the comparison of the N2-UTR gene sequences in the mutant strains and the N2-UTR-Majority sequence obtained from phylogenetic tree and mutational site analysis, the following mutational sites were characterized: N2-1-3′-UTR (U4→C4, C16→G16), N2-2-3′-UTR (U13→C13), N2-5-3′-UTR (U4→C4, C16→A16), N2-1/N2-2-5′-UTR (C22→U22), N2-5-5′-UTR (C22→G22). Lee et al. [17] found that the C4 to U4 mutation in the 3′ UTR of the NA segment in the H1 subtype can significantly promote the synthesis of NA-mRNA and stimulate the synthesis of NA protein. Our results for the N2-2-3′UTR mutant strain showed that the U13→C13 variation at the 3′ end of the NA segment influences the expression of vRNA and viral protein, which could enhance the efficiency of transcription of cRNA into vmRNA and contribute to the ability of the viral infection. By aligning the N2-1/N2-5 UTR mutant gene sequences, it was shown that the downregulated N2 strain contained the U4→C4 and C16→G16 mutations at the 3′ end. Furthermore, the upregulated N2-5 strain contained the U4→C4 and C16→A16 mutations at the 3′ end, indicating that mutations at other positions within the UTRs can also affect NA protein expression. A possible explanation for the opposite trend is that the C16→A16 mutation antagonizes the downregulation of NA protein expression that is induced by the U4→C4 mutation; however, the specific mechanisms underlying this process require further study. In conclusion, the variation of NA in specific positions dramatically promotes the efficiency of viral replication and transcription, which is consistent with its infection: virus with higher transcription efficiency showed higher infection. The change in the relative effect on influenza virus infection may contribute to applying for vaccination with genetically engineered influenza viruses.

Funding This work was supported by the National Natural Science Foundation of China (81871631), the Shenzhen Special Fund Project for Strategic Emerging Industry Development (JCYJ20170306160244540), the Shenzhen Research Project for the Health and Family Planning System (201602002), and the Guangdong Provincial Medical Science and Technology Foundation (A2018269).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest to disclose.

Ethical approval This article does not contain any studies with human participants or animals that were performed by any of the authors.

References

1. Gao F, Shi Y (2014) Biological research of influenza virus structure in China for the last 40 years. Progr. Biochem. Biophys. 41(10):1029–1033
2. Gerber M, Isel C, Moules V et al (2014) Selective packaging of the influenza A genome and consequences for genetic reassortment. Trends Microbiol 22(8):446–455
3. Jagger BW, Wise HM, Kash JC et al (2012) An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science 337(6091):199–204
4. Muramoto Y, Noda T, Kawakami E et al (2013) Identification of novel influenza A virus proteins translated from PA mRNA. J Virol 87(5):2455–2462
5. Selman M et al (2012) Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. Emerg Microbes Infect 1(11):e42
6. Wise HM, Barbezange C, Jagger BW et al (2011) Overlapping signals for translational regulation and packaging of influenza A virus segment 2. Nucleic Acids Res 39(7):7775–7790
7. Wise HM, Foeglein A, Sun J et al (2009) A complicated message: identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J Virol 83(16):8021–8031
8. Wise HM, Hutchinson EC, Jagger BW et al (2012) Identification of a novel splice variant from of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. PLoS Pathog 8(11):e1002998
9. Vasin AV, Temkina OA, Egorov VV et al (2014) Molecular mechanisms enhancing the proteome of influenza A virus: an overview of recently discovered proteins. Virus Res 185:53–63
10. Fodor E (2013) The RNA polymerase of influenza a virus:mechanisms of viral transcription and replication. Acta Virol 57(2):113–122
11. Xie Y, Gong J, Li M et al. (2011) The medicinal potential of influenza virus surface proteins: hemagglutinin and neuraminidase. Curr Med Chem 18(7):1050–1066
12. Gong J, Xu W, Zhang J (2007) Structure and functions of influenza virus neuraminidase. Curr Med Chem 14(1):113–122
13. Barman S, Krylov PS, Turner JC et al (2017) Manipulation of neuraminidase packaging signals and hemagglutinin residues improves the growth of A/Anhui/1/2013 (H7N9) influenza vaccine virus yield in eggs. Vaccine 35(10):1424–1430
14. de Wit E, Spronken MI, Rimmelzwaan GF et al (2006) Evidence for specific packaging of the influenza A virus genome from conditionally defective virus particles lacking a polymerase gene. Vaccine 24(44–46):6647–6650
15. Zhao L, Peng Y, Zhou K et al (2014) New insights into the non-conserved noncoding region of the subtype-determiant hemagglutinin and neuraminidase segments of influenza A viruses. J Virol 88(19):11493–11503
16. Wei Sun J, Li P, Han et al (2014) U4 at the 3′ UTR of PB1 segment of H5N1 influenza virus promotes RNA polymerase activity and contributes to viral pathogenicity. Plos ONE 9(3):e93366
17. Lee KH, Seong BL (1998) The position 4 nucleotide at the 3′ end of the influenza virus neuraminidase vRNA is involved in temporal regulation of transcription and replication of neuraminidase RNAs and affects the repertoire of influenza virus surface antigens. J Gen Virol 79(8):1923–1934
18. Hoffmann E, Neumann G, Kawaoka Y et al (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci 97(11):6108–6113
19. Peng J, Zhang D, Ma Y et al (2014) Protective effect of fluvastain on influenza virus infection. Mol Med Rep 9(6):2221–2226
20. Noble E, Mathews DH, Chen JL et al (2011) Biophysical analysis of influenza A virus RNA promoter at physiological temperatures. J Biol Chem 286(26):22965–22970
21. Kobayashi Y, Dadonaite B, van Doremalen N et al (2016) Computational and molecular analysis of conserved influenza A virus RNA secondary structures involved in infectious virion production. RNA Biol 13(9):883–894
22. Ma J, Liu K, Xue C et al (2013) Impact of the segment-specific region of the 3′-untranslated region of the influenza A virus PB1 segment on protein expression. Virus Genes 47(3):429–438
23. Fodor E, Palese P, Brownlee GG et al (1998) Attenuation of influenza A virus mRNA levels by promoter mutations. J Virol 72(8):6283–6290
24. Lee MK, Bae SH, Park CJ et al (2003) A single-nucleotide natural variation (U4 to C4) in an influenza A virus promoter exhibits a large structural change: implications for differential viral RNA synthesis by RNA-dependent RNA polymerase. Nucleic Acids Res 31(4):1216–1223
25. Ng SS, Li OT, Cheung TK et al (2008) Heterologous influenza vRNA segments with identical non-coding sequences stimulate viral RNA replication in trans. Virol J 11:5:2
26. Crescenzo-Chaigne B, Barbezange C, van der Werf S (2008) Non coding extremities of the seven influenza virus type C vRNA segments: effect on transcription and replication by the type C and type A polymerase complexes. J Virol 5:132
27. Hutchinson EC, Von Kirchbach JC, Gog JR et al (2010) Genome packaging in influenza A virus. J Gen Virol 91(2):313–328