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Manipulation of neuraminidase packaging signals and hemagglutinin residues improves the growth of A/Anhui/1/2013 (H7N9) influenza vaccine virus yield in eggs

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ABSTRACT

In 2013, a novel avian-origin H7N9 influenza A virus causing severe lower respiratory tract disease in humans emerged in China, with continued sporadic cases. An effective vaccine is needed for this virus in case it acquires transmissibility among humans; however, PR8-based A/Anhui/1/2013 (Anhui/1, H7N9), a WHO-recommended H7N9 candidate vaccine virus (CVV) for vaccine production, does not replicate well in chicken eggs, posing an obstacle to egg-based vaccine production. To address this issue, we explored the possibility that PR8's hemagglutinin (HA) and neuraminidase (NA) packaging signals mediate improvement of Anhui/1 CVV yield in eggs. We constructed chimeric HA and NA genes having the coding region of Anhui/1 HA and NA flanked by the 5′ and 3′ packaging signals of PR8's HA and NA, respectively. The growth of CVVs containing the chimeric HA was not affected, but that of those containing the chimeric NA gene grew in embryonated chicken eggs with a more than 2-fold higher titer than that of WT CVV. Upon 6 passages in eggs further yield increase was achieved although this was not associated with any changes in the chimeric NA gene. The HA of the passaged CVV, did, however, exhibit egg-adaptive mutations and one of them (HA-G218E) improved CVV growth in eggs without significantly changing antigenicity. The HA-G218E substitution and a chimeric NA, thus, combine to provide an Anhui/1 CVV with properties more favorable for vaccine manufacture.

1. Introduction

In 2013, a novel avian-origin H7N9 subtype influenza A virus emerged in China, causing severe lower respiratory tract disease in humans [1]. Zoonotic infections with this H7N9 virus are still being reported in China: as of June 22, 2016, a total of 795 laboratory-confirmed cases of human infection, including at least 308 deaths, had been reported [2,3].

The principal means of protection against influenza is vaccination. In September 2013, WHO recommended that an A/Anhui/1/2013 (Anhui/1, H7N9)–like virus be used to develop H7N9 vaccines for pandemic preparedness, and no changes have been recommended to date. Optimal vaccine production requires that candidate vaccine viruses (CVVs) grow at high yield in eggs without significant changes in antigenicity or immunogenicity. Initial Anhui/1 CVVs replicated poorly in eggs, although several egg-adaptive mutations in their HA's have been shown to improve their yield in eggs [4–6].

Influenza A viruses are enveloped, negative-strand, eight-segment RNA viruses of the *Orthomyxoviridae* family. The eight vRNAs (ranging from 890 to 2341 nt) consist of a long central coding region (open reading frame [ORF]) flanked by segment-specific non-coding regions (NCRs) of 19 to 58 nt. Within these NCRs, the extreme 5′ and 3′ termini (positive sense throughout the paper) nucleotides 12 and 13 (U12 and U13), respectively, are highly conserved. The termini are partially complementary, allowing the

Abbreviations: HA, hemagglutinin; NA, neuraminidase; WT, wild-type; CVV, candidate vaccine virus; ORF, open reading frame; NCR, non-coding region; nt, nucleotide; TIV, trivalent influenza vaccine; RT-PCR, reverse transcription–polymerase chain reaction; MDCK, Madin-Darby canine kidney cells; PFU, plaque-forming unit; HI, hemagglutination inhibition.

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vRNAs to fold back on themselves and form a so-called "panhandle" structure, and are the promoter elements necessary to initiate replication and transcription [7].

Two models have been proposed for the packaging of influenza A vRNAs into progeny virion: a random model and a segment-specific model, but growing evidence supports the latter [8]. Several studies point to the existence of segment-specific packaging signals in the 3′ and 5′ terminal regions, encompassing the NCRs and a part of the ORF extremes [9–25]. However, our knowledge of the subtype/lineage-specificity of packaging sequences is poor. The compatibility and interactions of lineage-specific packaging signals may play an important role in efficient packaging of the eight influenza genome segments into new virus particles, promoting efficient replication.

The influenza A components of inactivated trivalent influenza vaccine (TIV) are reassortant viruses containing gene segments from the master donor strain A/Puerto Rico/8/1934 (PR8, H1N1) and the gene segments encoding hemagglutinin (HA) and neuraminidase (NA) from circulating viruses. Although such reassortants are theoretically composed of only the HA and NA from the circulating viruses, in practice several high-yielding vaccine viruses generated by classical reassortment have retained other seasonal virus gene segments, most noticeably the PB1 segment, indicating this incorporation to be beneficial for virus production [26–28]. Being that reassortant viruses contain 6 gene segments from the donor virus and HA and NA from circulating viruses of various subtype/lineages, the compatibility of packaging signals in CVVs may pose a burden on optimal growth in eggs [29,30].

In recent years, several attempts have been made to exploit the terminal sequences of the PR8 donor virus' HA or NA to improve PR8-based CVV yield in eggs, and significantly improved growth of PR8-based A/Vietnam/1194/2004 (H5N1) or A/California/07/2009 (H1N1) CVV with PR8 HA or NA packaging signals has been demonstrated [31–33]. Here, we examine the role of PR8 HA and NA packaging signals on the growth of PR8-based Anhui/1 CVVs and show that NA but not HA packaging signals contribute to the higher growth of H7N9 vaccine viruses.

2. Materials and methods

2.1. Viruses

The wild-type (WT) Anhui/1 virus was kindly provided by the Chinese Center for Disease Control and Prevention, Beijing, China. Virus stocks were propagated in the allantoic cavities of 10- to 11-day-old, specific pathogen–free embryonated chicken eggs at 37 °C for 48 h. Viruses were titered by performing PFU assays in MDCK cells and hemagglutination assays using chicken red blood cells (CRBCs).

2.2. Determination of terminal sequence, cloning, and construction of chimeric HA and NA genes

Terminal sequences of Anhui/1 HA and NA were determined by using a method described elsewhere [34]. Briefly, vRNAs were circularized or concatemerized by using T4 RNA ligase (Promega Corporation, Madison, WI) per the manufacturer's instructions. Two sets of RT-PCR reactions (one reaction for each terminus) were performed for each gene segment (HA or NA). To determine the 5′ terminal sequence, an RT-PCR assay was performed by using the 3′ forward primer 5′-CCTTGTTCTACTAGC-3′ [34] and a segment-specific reverse primer. Similarly, to determine the 3′ terminal sequence, an RT-PCR reaction was performed by using a segment-specific forward primer and the 5′ reverse primer 5′-CC TGCTTTTGCTAGT-3′ [34], and PCR products were sequenced. The WT HA and NA gene segments from Anhui/1 virus were cloned into virus rescue plasmid pHW2000 [35]. The chimeric HA and NA genes were constructed by using mega-primer mutagenesis [36,37] to swap the terminal sequences of HA and NA with those of the PR8 HA and NA genes, respectively.

2.3. Generation of variant vaccine viruses by reverse genetics

Reassortant 6+2 vaccine viruses were generated by co-transfecting 293T cells with eight cDNA plasmids encoding the WT or chimeric HA and NA of Anhui/1 and the remaining 6 gene segments of PR8 as described previously [35]. Viruses rescued in 293T cells were propagated in the allantoic cavities of 10- to 11-day-old embryonated chicken eggs by inoculating cell culture supernatant (200 μL/egg) from transfected 293T cells at 37 °C for 48 h and titered by performing hemagglutination and PFU assays.

2.4. Serial passage in chicken eggs and cloning Anhui/1 HAs from egg-passaged CVVs

Anhui/1 CVVs were serially passaged in embryonated chicken eggs. HA and NA gene segments were amplified from viral RNA by one-step RT-PCR using segment-specific terminal primers and sequenced. HAs from egg-passaged CVVs were cloned into virus rescue plasmid pHW2000 [35]. All work was conducted prior to the US government's pause on gain-of-function research funding.

2.5. Virus purification

Virus purification was done as described before [38]. Briefly, 10- to 11-day-old embryonated chicken eggs were inoculated (allantoic cavity) with different viruses (egg passage 1 [E1] stock, 75 eggs for each CVV) and incubated at 37 °C for 48 h. Allantoic fluid (E2 virus) was harvested and clarified by centrifugation (Sorval SAL-1500 rotor) at low speed (10,000 rpm) for 15 min at 4 °C. Virus was inactivated by treatment with β-propiolactone (0.0005% v/v) at 4 °C for 3 days. Allantoic fluid containing the inactivated viruses was clarified at low speed (10,000 rpm) for 15 min at 4 °C and then pelleted by centrifugation (27,000 rpm in a Beckman SW32Ti rotor) for 90 min at 4 °C through a 25% sucrose cushion (33 mL allantoic fluid over 5 mL sucrose solution). Virus pellets were resuspended in PBS, and contents from all tubes were combined for each virus. The volume was adjusted to 18 mL with PBS, and the suspension was centrifuged at 24,000 rpm through a 20 mL, 25–70% continuous sucrose gradient for 2.5 h at 4 °C in a Beckman SW32Ti rotor. The virus band was harvested; the volume was then adjusted to 38 mL with PBS, and virus was pelleted by centrifugation (27,000 rpm) for 60 min at 4 °C in a Beckman SW32Ti rotor. The virus pellet was resuspended in PBS to a final volume that was 1/1000 the original volume of allantoic fluid, producing a 1000× virus concentrate.

2.6. Deglycosylation and SDS-PAGE analysis of viral proteins

Each virus concentrate was treated or mock-treated with 2 μL of PNGase F (New England Bio Labs, Ipswich, MA) in a 30-μL reaction volume (37 °C, 4 h), per the manufacturer's instructions, and mixed with 15 μL of SDS-protein sample buffer containing β-mercaptoethanol. The sample was heated in boiling water for 5 min before proteins were separated by SDS-PAGE in a 12% polyacrylamide gel (Mini-Protein TGX, Bio-Rad Laboratories Inc., Hercules, CA). Protein bands were stained with Coomassie blue dye, scanned, and analyzed by using ImageJ software (https://imagej.nih.gov/ij/).