Editor-Communicated Paper

Note

(a) Extensive accumulation of influenza virus NS1 protein in the nuclei causes effective viral growth in VERO cells

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(e) Running head; NS1 LOCALIZATION IN VERO CELLS

(f) Key words: influenza virus, NS1, nuclear localization

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Abstract.

We previously showed that modified A/Puerto Rico/8/34 (PR8) influenza master strain had improved viral rescue and growth properties in African green monkey kidney (Vero) cell line by introducing NS gene of Vero-adapted A/England/1/53 (vaEng53). In the present study, it was found that the NS1 protein derived from vaEng53 was extensively accumulated in the nuclei than that of PR8. This accumulation was caused by 7 amino acid differences in C-terminal region of NS1 protein. These results suggest that specific accumulation of NS1 protein may contribute to efficient viral replication in Vero cells.

For the control of influenza, it is required the monitoring of virus infection in birds and mammals and the development of improved antiviral therapies and vaccines. The most widely used influenza vaccines are those made from subunits of inactivated viruses propagated in chicken embryonated eggs. Although routinely used to prepare influenza vaccines and diagnostic reagents, problems include the lack of reliable year-round supplies of high-quality eggs, the possible presence of pathogens, and the low growth of influenza virus in summer eggs (10). In order to resolve this problem, we demonstrated that well-characterized African green monkey kidney (Vero) cell line was available for cell-based vaccine production with reverse genetics as an alternative method. The nonstructural (NS) protein gene derived from Vero-adapted virus contributed effective viral growth in early stage of infection (11). In the present study, we investigated the relationship between localization of NS1 protein and growth kinetics of virus with mutant NS genes in Vero cells.

The plasmids carrying the eight gene segments of PR8 (H1N1) virus (pHW191 to pHW198) and plasmid carrying NS gene derived from Vero-adapted A/England/1/53 (H1N1) (vaEng53) (pHW/vaEng53-NS) (11) were used for the rescue of mutant viruses. Chimeric NS genes were constructed and modified by swapping first half and second half of *Nco*I or *Ssp*I digested fragments between PR8 and vaEng53 NS genes, respectively (Fig.1). Virus rescue and viral growth kinetics in Vero cells was performed by following the method previously described (11). Nuclear localization of each mutant NS1 protein was detected by immunofluorecent assay (6). Briefly, the viruses with different mutant NS gene were inoculated on confluent Vero cells with a multiplicity of infection (MOI) of 1 pfu/cell. Three, five, and nine hours post infection, cells were fixed with methanol and reacted with anti-NS1 monoclonal antibody (3). NS1 protein was detected with Alexa Fluor-488 goat anti-mouse IgG antibody (Invitrogen).

To confirm the effect of the replacement of the NS gene segment in PR8 on subsequent virus amplification in Vero cells, we tested triplicate samples of near-confluent Vero cells with PR8

and PR8/vaEng53-NS at a low MOI (=0.01) and monitored virus replication by assaying culture supernatants every 12 h. As shown in Fig. 2, vaEng53 NS gene was confirmed to contribute to the effective growth in Vero cells in early stage of virus replication. We, then, constructed carrying chimeric NS genes swapped the *Nco*I fragments between PR8 and vaEng53 NS genes in order to determine which region of the NS1 protein would be required for growth in early stage (Fig. 1). The mutant PR8 viruses with each chimeric NS gene were applied to assess the growth kinetics as above. The mutant PR8 including PE1-NS chimeric NS gene showed similar growth kinetics to that of PR8/vaEng53-NS. On the other hand, the growth of virus with EP1-NS chimeric NS gene was identical with that of PR8 (Fig. 2a). Moreover, to narrow the responsible region down for the phenomenon, two more chimeric NS genes were constructed by swapping *Ssp*I fragments (Fig. 1). Growth kinetics curves showed that PE2-NS gene gave a contribution for rapid replication of virus in Vero cells (Fig. 2b). These results indicate that near C-terminal region of NS1 protein is responsible for the rapid growth of the virus in Vero cells.

Amino acid alignment analysis revealed 15 amino acid differences on the NS1 protein between PR8 and vaEng53 (Fig. 1). Since 8 out of 15 amino acids did not affect to viral growth in Vero cells, the remaining 7 amino acid differences would be responsible to this phenomenon. We, then, focused on the nuclear localizing signal 2 (NLS2), PKQKRK motif (7) of the NS1 protein because the RNA binding domain, NLS1, and the effecter domain (described below and Fig. 1) were respectively common between both NS1 proteins. One amino acid difference was found in NLS2 between PR8 (PKQKR**E**) and vaEng53 (PKQKR**K**) NS1 proteins. To assess the localization of the NS1 protein in Vero cells, the mutant viruses were inoculated in Vero cells and NS1 protein was detected by immunofluorescence assay in different time points. As shown in Fig. 3, the NS1 protein derived from vaEng53 NS gene was detected in three hours post inoculation in Vero cells but not that of PR8. Five hours post inoculation, almost all of NS1 protein from vaEng53 NS gene was

accumulated in the nuclei of the cells with stronger signal than that of PR8. Four more hours later, the NS1 protein of PR8 was detected both in the nuclei and cytoplasm. Interestingly, NS1 protein from vaEng53 NS gene was still observed in the nuclei with strong signal at the same time point. In addition, PE1-NS1 and PE2-NS1 proteins were also detected similarly in the nuclei as that of vaEng53 NS gene (data not shown).

In the present study, we demonstrated that 7 amino acid substitutions in C-terminal region of NS1 protein from vaEng53 were responsible for the growth efficiency in Vero cells. Among these substitutions, one amino acid substitution detected in NLS2 motif may have a possibility to be related to the specific accumulation in the nuclei. The NS1 protein of influenza A virus regulates two post transcriptional events in the nucleus: the inhibition of the nuclear export of poly (A)-containing cellular and viral mRNAs, and the inhibition of pre-mRNA splicing (1, 5, 9, 12-14). These nuclear functions are caused by an RNA-binding domain near N-terminal, and an effecter domain in the carboxyl half of the molecule interacting with cellular nuclear proteins (12). Then, viral polymerase is able to generate capped primer effectively. Thus, specific accumulation of the NS1 protein in the nuclei may cause increased viral replication in Vero cells. In addition, NS1 protein represses the host cell antiviral response by multiple mechanisms. These mechanisms include the inhibition of the interferon-inducible double stranded RNA activated kinase PKR (protein kinase RNA-regulated) (2, 8, 17) and the blocking of interferon-β production by preventing NF-κB (18), interferon-regulatory factor (IRF) 3 (16), and IRF7 activation (15). It is unlikely, however, that antiviral NS1 functions are involved in efficiency of viral growth in Vero cells, because Vero cells are defective in the production of interferon (4).

For cell-based vaccine production, it is required to choose a suitable backbone considering the properties of each fragment like NS gene in this study. Although it is unknown that the influence of the remaining 6 amino acid substitutions in NS1 protein and nuclear exporting protein coded in

the same gene, the present study demonstrates that NS gene is a critical factor for controlling viral growth in Vero cells. It is important to understand the mechanisms how the NS gene derived from Vero-adapted strain affects on the viral replication in Vero cells.

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Figure legends.

Figure 1.

Schematic diagram for construction of chimeric NS1 proteins. Dot lines are shown as fragment swapping points by digestion of *Nco*I or *Ssp*I site, respectively. Arrows indicate the points of amino acid difference on NS1 protein. Three black bars indicate RNA binding domain and nuclear localizing signal 1 (NLS1), effecter domain, and NLS2, respectively.

Figure 2.

The growth characteristics of PR8 and PR8 with chmeric NS gene in Vero cells. Vero cells were inoculated with 0.01 MOI of each virus. Panel (a) shows the growth kinetics of PR8 (closed diamond) vs PR8 with vaEng53-NS (closed square), PE1-NS (open diamond), or EP1-NS (open circle) gene. Panel (b) shows the growth kinetics of PR8 (closed diamond) vs PR8 with vaEng53-NS (closed square), PE2-NS (open diamond), or EP2-NS (open circle) gene. Each plot is shown as average of 3 independent experiments.

Figure 3.

Nuclear localization of NS1 protein in Vero cells. Vero cells were inoculated with 1 MOI of PR8 (a-c) or PR8/vaEng53-NS (d-f). Cells were fixed at 3 hours (a, d), 5 hours (b, e), and 9 hours (c, f) post inoculation and NS1 protein was detected by indirect immunofluorescence using mouse anti-NS1 monoclonal antibody. Magnification is 1:400.

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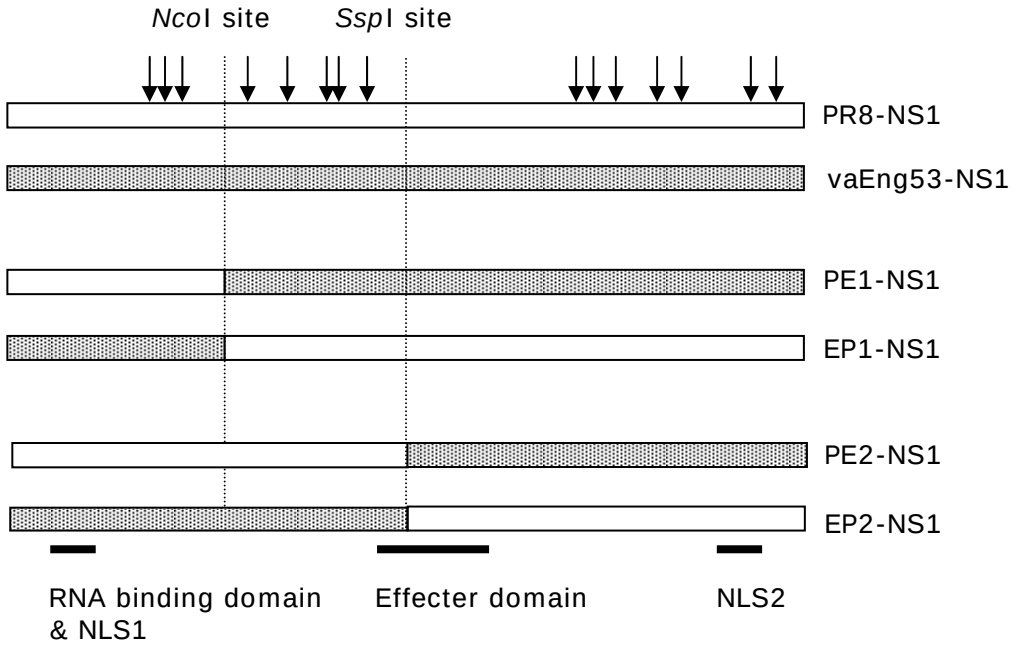
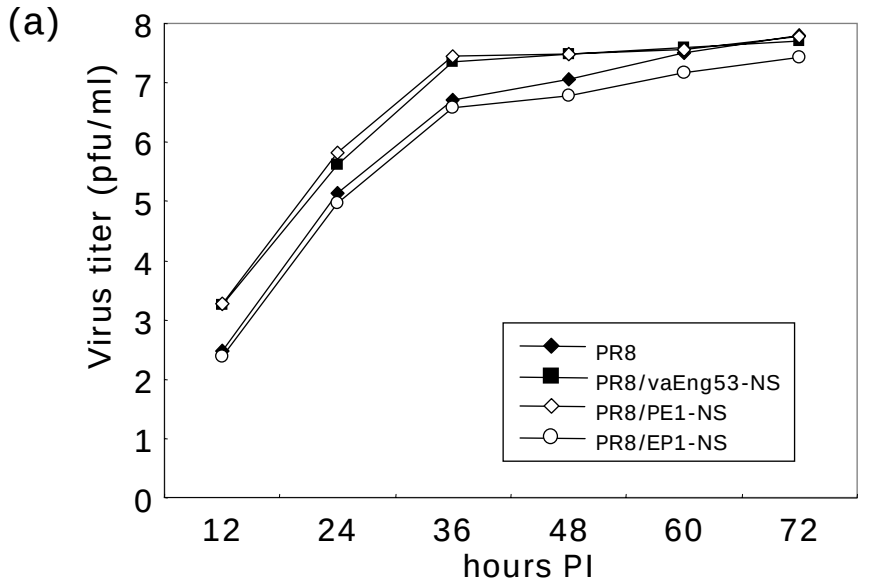
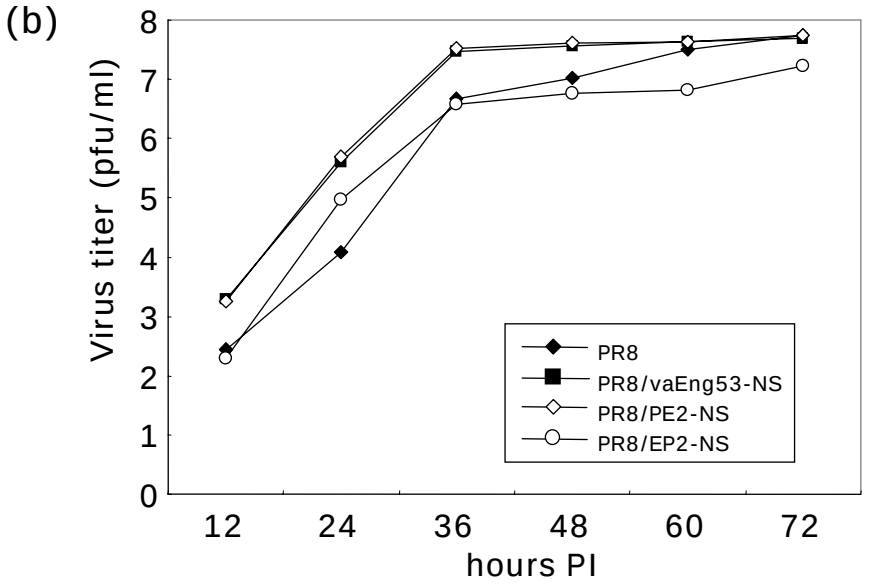
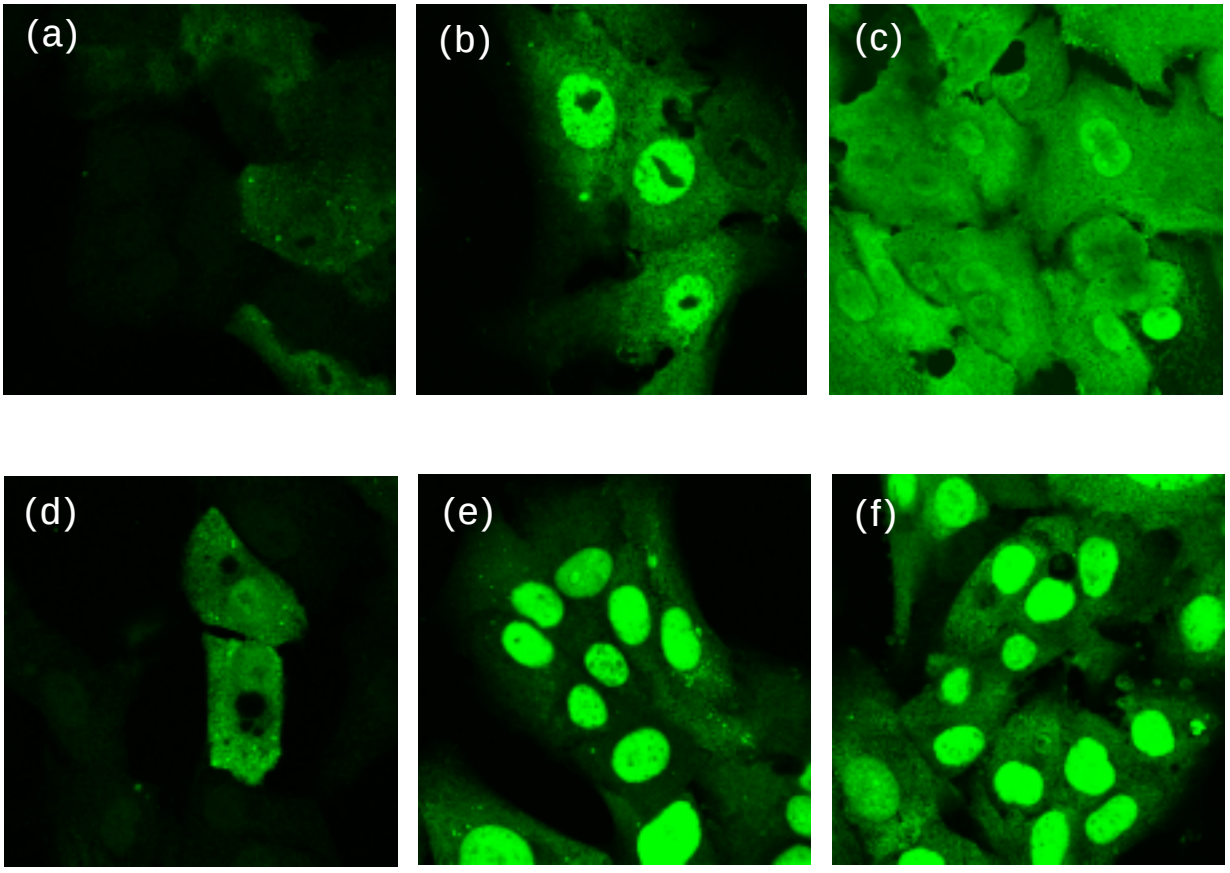


Ozaki and Kida Fig. 1





Ozaki and Kida Fig. 2



Ozaki and Kida Fig. 3