Genetically Engineered, Biarsenically Labeled Influenza Virus Allows Visualization of Viral NS1 Protein in Living Cells^{▽†}

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Real-time fluorescence imaging of viral proteins in living cells provides a valuable means to study virus-host interactions. The challenge of generating replication-competent fluorescent influenza A virus is that the segmented genome does not allow fusion of a fluorescent protein gene to any viral gene. Here, we introduced the tetracysteine (TC) biarsenical labeling system into influenza virus in order to fluorescently label viral protein in the virus life cycle. We generated infectious influenza A viruses bearing a small TC tag (CCPGCC) in the loop/linker regions of the NS1 proteins. In the background of A/Puerto Rico/8/34 (H1N1) (PR8) virus, the TC tag can be inserted into NS1 after amino acid 52 (AA52) (PR8-410), AA79 (PR8-412), or AA102 (PR8-413) or the TC tag can be inserted and replace amino acids 79 to 84 (AA79-84) (PR8-411). Although PR8-410, PR8-411, and PR8-412 viruses are attenuated than the wild-type (WT) virus to some extent in multiple-cycle infection, their growth potential is similar to that of the WT virus during a single cycle of infection, and their NS1 subcellular localization and viral protein synthesis rate are quite similar to those of the WT virus. Furthermore, labeling with membrane-permeable biarsenical dye resulted in fluorescent NS1 protein in the context of virus infection. We could exploit this strategy on NS1 protein of A/Texas/36/91 (H1N1) (Tx91) by successfully rescuing a TC-tagged virus, Tx91-445, which carries the TC tag replacement of AA79-84. The infectivity of Tx91-445 virus was similar to that of WT Tx91 during multiple cycles of replication and a single cycle of replication. The NS1 protein derived from Tx91-445 can be fluorescently labeled in living cells. Finally, with biarsenical labeling, the engineered replication-competent virus allowed us to visualize NS1 protein nuclear import in virus-infected cells in real time.

Real-time fluorescence imaging of single virus particles or viral proteins in live cells provides a valuable means to study virus-host interactions (4). Labeling influenza A virus particles by physically conjugating a lipophilic dye to the viral membrane has led to recent advances in dissecting the influenza A virus entry process (5, 21). However, studying postentry events, such as intracellular trafficking of the viral proteins, interactions between viral and cellular proteins, and assembly and budding of the virus, requires the use of genetically engineered, replication-competent fluorescent virus. Development of genetically engineered, replication-competent fluorescent influenza A virus has been hampered due to the nature of the viral genome. The influenza A virus genome consists of eight segmented RNA molecules of negative polarity (31). The capacity of each segment to accommodate foreign genes is limited, and fusion of fluorescent proteins, which are about 30 kDa, to influenza virus proteins does not produce viable viruses. Several replication-deficient fluorescent influenza A viruses have been developed (11, 16, 27). In these studies, the gene encoding the fluorescent protein was introduced into the

virus genome as a separate segment, which replaced one viral segment. The modified replication-deficient viruses and the strategy to generate such virus are useful in a genome-wide RNA interference (RNAi) screening for host factors required for influenza virus replication, vaccine development, or gene delivery, as well as in detecting neutralizing antibodies. None of these viruses were designed for live-cell imaging.

Recently, Adams et al. and Griffin et al. in the laboratory of R. Y. Tsien have developed a biarsenical tetracysteine (TC) technology (1, 13), which is an alternative to fluorescence protein labeling and has many advantages over fluorescent protein (26). This technology uses a small TC tag with the CCPGCC motif. Upon covalent binding of cysteine pairs to membrane-permeable biarsenical compounds, the TC-tagged proteins become fluorescent. Inserting such a small tag brings the risk of disrupting the overall structure of a targeted protein to a minimum. In addition, the biarsenical dye can bind to the TC tag right after the tagged protein is translated; therefore, the fusion protein becomes fluorescent more quickly than the fluorescent protein, which needs time to fold and autooxidize in order to form a functional chromophore after being synthesized (39). This technology has led to successful tracking of viral proteins, such as vesicular stomatitis virus M protein (8) and HIV Gag protein (12), viral component complexes, as with HIV (2), and *de novo* HIV production (41) in real time.

The objective of our study was to investigate whether biarsenical TC tagging technology could be used for influenza A virus. We tested this idea with NS1 (nonstructural protein 1) of

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influenza A virus. The RNA segment 8 of influenza A virus carries genes encoding two proteins: NS1 and NEP (nuclear export protein). NS1 is translated from unspliced mRNA (31) and can be divided into two functional domains, the RNA binding domain (RBD) near the N terminus and the effector domain (ED) in the C terminus (34). By interacting with RNA and cellular proteins, NS1 protein functions to regulate viral replication, viral protein synthesis, host innate and adaptive immune responses, and cellular signaling pathways (15). The RNA binding activity of the NS1 protein correlates with its abilities to inhibit cellular pre-mRNA splicing (24, 35) and to inhibit the 2'-5' oligo(A) synthetase/RNase L pathway, thus protecting the virus against the antiviral state induced by beta-interferon (IFN- β) (30). In addition, NS1 binds to numerous cellular factors to execute multiple functions during virus infection. For example, NS1 binds to cleavage and polyadenylation specificity factor (CPSF) and the poly(A)-binding protein II (PABII) to inhibit 3'-end processing of cellular pre-mRNAs (23); NS1 binds to the regulatory subunit p85 β of phosphatidylinositol 3-kinases (PI3Ks) and activates the PI3K/Akt pathway to inhibit apoptosis (9, 14, 36–38); NS1 binds to RIG-I (retinoic acid-inducible gene I product) and inhibits downstream activation of interferon regulatory factor 3 (IRF-3), thus preventing the transcriptional induction of IFN- β (29). Most recently, it was found that NS1 binds to the ubiquitin ligase TRIM25 (tripartite motif-containing protein 25) to evade recognition by the host viral RNA sensor RIG-I (10).

Given the multiple functions of NS1 during virus infection, fluorescent NS1 in the context of virus infection will be valuable for tracking the kinetics of NS1-host factor interactions during virus infection. In addition, NS1 is expressed abundantly in virus-infected cells, making it a good candidate for biarsenical labeling. Here we report a novel strategy to generate mutant influenza A viruses bearing a TC tag in NS1 protein. The TC-tagged influenza A viruses preserved the infectivity of the virus. With biarsenical labeling, the NS1 protein is fluorescent in infected live cells, allowing real-time visualization of NS1 nuclear import.

MATERIALS AND METHODS

Cells and viruses. A549 cells (human lung carcinoma cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen). Madin-Darby canine kidney (MDCK) cells were cultivated in minimal essential medium (MEM) (Invitrogen) supplemented with 10% FBS. Influenza A/Puerto Rico/8/34 (H1N1) (PR8) and A/Texas/36/91 (H1N1) (Tx91) were propagated in 11-day-old embryonated chicken eggs as described previously (36).

Plasmid construction and generation of mutant viruses. The TC tag-encoding sequence (TGCTGCCAGGATGCTGC) was introduced into the pHW198-NS (17) and pHW-Tx91-NS plasmids (Tx91 NS gene inserted into the pHW2000 vector in the laboratory of Y. Zhou) by standard overlapping PCR. Plasmid 410 carries a gene encoding PR8 NS1 with a TC tag inserted after amino acid 52 (AA52), plasmid 411 carries a gene encoding PR8 NS1 with amino acids 79 to 84 (AA79-84) replaced by a TC tag, plasmid 412 carries a gene encoding PR8 NS1 with a TC tag inserted after AA79, plasmid 413 carries a gene encoding NS1 with a TC tag inserted after AA102, plasmid 444 carries a gene encoding Tx91 NS1 bearing a TC tag inserted after AA52, and plasmid 445 carries a gene encoding Tx91 NS1 with AA79-84 replaced by a TC tag.

An eight-plasmid reverse genetics system (18) was utilized to rescue the viruses. To generate mutant PR8 viruses with NS1, plasmids pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW196-NA, and pHW197-M and one of the plasmids carrying a gene coding for the tag (plasmid 410, plasmid 411, plasmid 412, or plasmid 413) were used for transfection. To generate mutant Tx91 viruses, plasmids pDZ-Tx91-PB2, pDZ-Tx91-PB1, pDZ-

Tx91-PA, pDZ-Tx91-HA, pDZ-Tx91-NP, pDZ-Tx91-NA, pDZ-Tx91-M (40), and one of the plasmids carrying a gene coding for the tag (plasmid 444 or plasmid 445) were used for transfection. The rescued viruses were propagated in 10- to 11-day-old embryonated chicken eggs and characterized by sequencing of the reverse transcription-PCR (RT-PCR) product.

Virus growth curves and metabolic labeling. MDCK cells were infected either at a multiplicity of infection (MOI) of 0.001 (multiple-cycle growth curve) or at an MOI of 5 (single-cycle growth curve), the supernatant was harvested at the indicated time points, and virus titers were determined by plaque assays. For metabolic labeling, MDCK cells were mock infected or infected with virus at an MOI of 5. At predetermined times, cells were Met-Cys starved for 1 h with Met-free medium (Invitrogen) and were then labeled with [³⁵S]Met-Cys (Perkin-Elmer) for 1 h. Total extracts were processed by SDS-PAGE. The gel was visualized by phosphorimaging using a personal FX phosphorimager (Bio-Rad).

FLAsH labeling of virus-infected cells, immunofluorescence staining, and fluorescence microscopy. Biarsenical labeling of the infected cells with FLAsH (Invitrogen) was carried out per the manufacturer's instructions with modifications. A549 cells were infected with the virus at an MOI of 1. After virus adsorption, the cells were maintained in Opti-MEM (Invitrogen). One hour prior to observation, the cells were incubated with Opti-MEM containing 1 μ M FLAsH and 20 μ M bis-ethanedithiol (EDT) (Fluka) for 20 min. The cells were then washed two times with Opti-MEM containing 100 μ M EDT and 10 μ M β -mercaptoethanol (EMD).

To compare the patterns of NS1 subcellular localization detected by FLAsH labeling and antibody staining, the cells were stained with FLAsH, fixed at about 7 hours postinfection (h.p.i.) with phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 20 min, permeabilized with PBS containing 0.3% Triton X-100 at room temperature for 10 min, and incubated first with an NS1 antibody (38) for 1 h at room temperature and then with Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen). Images were obtained using a Carl Zeiss Axiovert 200M inverted fluorescence microscope.

For visualizing NS1 nuclear import, A549 cells seeded in glass-bottom 35-mm dishes (MatTek) were infected with PR8-411 (PR8 in which the TC tag was used to replace amino acids 79 to 84 [AA79-84]) at an MOI of 1. At 3 h.p.i., the medium was changed to Opti-MEM containing FLAsH (0.2 μ M), EDT (50 μ M), and β -mercaptoethanol (10 μ M). Between 4 and 7 h.p.i., live-cell fluorescence images of infected A549 cells were recorded every 3 min using a Leica SP5 microscope equipped with a 40 $\times$ oil objective with a numerical aperture (NA) of 1.25, an I3 filter cube, a PB450-490 excitation filter and an LP515 emission filter. All images were acquired with 500-ms exposures under the same illumination conditions. Images were analyzed using Leica LAS AF 1.8.2 software.

RESULTS

Generation of mutant viruses bearing TC-tagged NS1 protein. In this study, we attempted to generate an influenza A virus containing tetracysteine (TC)-tagged NS1 protein. Since NEP shares the first 10 amino acids with NS1 (Fig. 1C), direct fusion of a TC tag to the N terminus of NS1 will lead to tagging not only of NS1 but also of NEP. Meanwhile, fusion of the TC tag to the C terminus of NS1 will likely perturb the open reading frame (ORF) of NEP. We therefore attempted to integrate the TC tag into the ORF of NS1 with minimum disruption of NS1's function. Because previous studies suggested that short α -helices are the most suitable sites for insertion or substitution of a TC tag (25), we initially inserted a TC tag (CCPGCC) in several sites (after amino acid 20 [AA20], AA40, and AA63) in the α -helix regions of NS1 (Fig. 1A and C). However, no virus could be rescued, possibly due to severe disruption of NS1's structure and/or function by insertion of a TC tag. Given the flexible nature of the linker region in NS1 structure (3), we then tested whether the loop/linker regions in NS1 could be the viable sites for accommodating a TC tag. The following regions were tested in A/Puerto Rico/8/34 (PR8) NS1: a loop region connecting two α -helices at a RNA binding domain (RBD) (AA52), a highly variable linker region between the NS1 RBD and effector domain (ED)

FIG. 1. Strategies to introduce a tetracycline (TC) tag into influenza A virus NS1 protein. Ribbon representations of the NS1 structure (Protein Data Bank accession no. 3F5T [3]) are shown. Light blue arrows indicate the helix regions that cannot accommodate TC tags (A) and the loop/linker regions that can accommodate TC tags (B). (C) Schematic representation of the gene structure of influenza A virus segment 8 and of TC-tagged NS1 constructs.

(amino acids 79 to 84 [79-84]), and a linker region in the ED (AA102) (Fig. 1B). Four mutant viruses were generated using an eight-plasmid reverse genetics system, and the resulting viruses were designated PR8-410, PR8-411, PR8-412, and PR8-413. PR8-410, PR8-412, and PR8-413 viruses contain genes encoding NS1 with a TC tag inserted after AA52, AA79, and AA102, respectively; the PR8-411 virus contains genes encoding NS1 with a TC tag replacement of AA79-84 (Fig. 1C). The PR8-410, PR8-411, PR8-412, and PR8-413 viruses were propagated in 11-day-old embryonated eggs and had titers of 1.23×10^8 , 1.33×10^8 , 1.12×10^8 , and 3.3×10^7 PFU/ml, respectively.

Characterization of viruses with TC-tagged NS1. The effects of TC tagging of NS1 on virus infectivity were first assessed by monitoring the plaque size and multiple-cycle growth kinetics in MDCK cells. As shown in Fig. 2A, while the sizes of plaques formed by PR8-410, PR8-411, and PR8-412 viruses were similar to those formed by the WT virus, PR8-413 formed smaller

plaques, suggesting that PR8-413 is the most attenuated virus. MDCK cells were then infected at an MOI of 0.001, supernatant was harvested at 12-h intervals until 72 h.p.i., and virus titers were determined by plaque assay. Figure 2B showed that at a low MOI, the mutant viruses initially grew slower than the WT PR8 did; however, at 72 h.p.i., PR8-410, PR8-411, and PR8-412 viruses grew to titers approximately 1 log unit lower than that of the WT virus, indicating that insertion of the TC tag into the NS1 ORF caused some defects in NS1's function during multiple-cycle replication. Since the major function of NS1 is to antagonize interferon production during virus infection, thus limiting the spread of virus, the NS1 defects seen in multiple-cycle growth may not be apparent in single-cycle growth assay. We therefore determined virus titer after a single cycle of replication with the least attenuated virus, PR8-411. MDCK cells were infected by PR8-411 or WT PR8 at an MOI of 5. Virus titer in the supernatant was determined at 8 and 10 h.p.i. As seen in Fig. 2C, there is no significant difference in

FIG. 2. Characterization of recombinant A/Puerto Rico/8/34 (PR8) viruses bearing a TC tag in NS1. (A) Plaques formed by the WT PR8 virus and recombinant PR8-410, PR8-411, PR8-412, and PR8-413 viruses on MDCK cells. (B) Multiple-cycle growth curves of WT PR8, PR8-410, PR8-411, PR8-412, and PR8-413 on MDCK cells. Cells were infected in triplicate with each virus at an MOI of 0.001. Media were collected at 12-h intervals until 72 h.p.i., and the virus titers were determined by plaque assays on MDCK cells. Mean titer values at each time point were plotted. (C) Virus yield of PR8-411 and WT PR8 after a single cycle of infection. MDCK cells were infected at an MOI of 5, and the virus titers were determined at 8 and 10 h.p.i. by plaque assay. Mean titer values were plotted with error bars showing standard deviations. (D) Metabolic labeling of protein synthesis in MDCK cells. Cells were infected with the WT PR8, PR8-410, PR8-411, PR8-412, or PR8-413 virus at an MOI of 5. Cells were labeled with [35 S]Met for 1 h at the time points indicated. Cell lysates were analyzed by SDS-PAGE.

the amount of infectious virus production in WT PR8 and PR8-411, indicating that TC tagging in NS1 does not substantially compromise NS1's function during a single cycle of infection.

To examine whether insertion of a TC tag into NS1 would have any impact on viral protein synthesis rate, we infected MDCK cells with virus at an MOI of 5, and the kinetics of viral protein synthesis were monitored by metabolic labeling. As seen in Fig. 2D, during a single cycle of virus infection, no significant difference was detected in the time course and rate

of viral protein syntheses between WT PR8 virus, PR8-410, and PR8-411. NS1 derived from PR8-412 migrated slightly higher in the gel than NS1 from WT virus did, suggesting that insertion of a TC tag after AA79 altered NS1 mobility. PR8-413 synthesized less NS1 at 6 h.p.i.; however, the amount of NS1 protein detected at 8 h.p.i. was not dramatically decreased compared to that of WT virus.

To determine whether TC tagging on NS1 would alter the subcellular localization of NS1 in comparison with the WT NS1 protein, A549 cells were infected with virus at an MOI of 1,

FIG. 3. Intracellular localization of NS1 encoded by the WT PR8, PR8-410, PR8-411, PR8-412, or PR8-413 virus. A549 cells were infected with virus at an MOI of 1. At 6 h.p.i., cells were fixed, permeabilized, and stained with an NS1 antibody followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG.

and at 6 h.p.i., the cells were fixed, permeabilized, and stained with NS1 antibody (Fig. 3). NS1 proteins encoded by genes in PR8-410, PR8-411, and PR8-412 viruses, like the WT NS1 protein, were localized in the nuclei of infected cells. The majority of NS1 protein encoded by a gene in PR8-413 virus was localized in the cytoplasm. The mislocalization of PR8-413 NS1 may be attributed to the smaller plaque and the most retarded growth rate of the PR8-413 virus. Together, these data demonstrate that PR8-411 is the least attenuated virus and that replacing AA79-84 with a TC tag did not greatly perturb NS1 localization or function during a single cycle of infection.

FlAsH labeling of NS1 protein in virus-infected cells. To test whether the TC tag inserted in the NS1 protein could form a putative hairpin and specifically react with biarsenical reagent FlAsH, thus fluorescently labeling NS1 in live cells, we infected A549 cells with virus. At 6 h.p.i., FlAsH dye was added to the cell culture for 20 min followed by washing with wash buffer. The living cells were then observed by using a fluorescence microscope. While TC-tagged NS1 proteins derived from PR8-410, PR8-411, and PR8-412 viruses demonstrated specific FlAsH labeling and were primarily localized in the nuclei of the infected cells as reported previously (15), NS1 derived from virus PR8-413 did not exhibit distinctive fluorescence. No fluorescence could be detected in either mock-infected or untagged WT virus-infected cells (Fig. 4A). These data demonstrate that NS1 proteins encoded by genes in the PR8-410, PR8-411, and PR8-412 viruses could be labeled with a sufficient number of fluorophores for detection in the course of virus infection.

Taking into account the virus replication potential, NS1 subcellular localization, and the efficiency and specificity of FlAsH labeling, we concluded that while the PR8-411 virus retained the most infectivity during a single cycle of infection, its TC-tagged NS1 exhibited more distinctive fluorescence, with a

more favorable signal-to-noise ratio. Thereafter, PR8-411 was used for all further experiments.

In another set of experiments, we further observed the NS1 protein in living cells at 10 h.p.i. A549 cells were infected with PR8-411. At 9.5 h.p.i., FlAsH dye was added to the cells for 20 min. The living cells were then washed and observed by using a fluorescence microscope. Images were acquired with the same exposure time as for Fig. 4A. As seen in Fig. 4B, compared to the NS1 signal observed at 6 h.p.i., at 10 h.p.i., more NS1 was synthesized and NS1 was found not only in the nucleus but also in the nucleolus. No fluorescence could be detected in untagged WT virus-infected cells.

To further confirm that FlAsH-labeled protein reflects the authentic NS1 protein in virus-infected cells, we infected A549 cells with PR8-411 at an MOI of 1. At 6 h.p.i., cells were first labeled with FlAsH for 20 min, washed, fixed, and immunofluorescently stained with NS1-specific antibody. As shown in Fig. 4C, antibody-stained NS1 was primarily localized in the nucleus and was superimposable on that labeled by FlAsH, demonstrating that FlAsH labeling of the TC tag can truly detect NS1 protein in infected cells.

Rescue of TC-tagged NS1 in the background of Tx91 virus. We have identified that in PR8 NS1, a small linker region in the RBD (AA52) and a loop region that connects the RBD and ED (AA79-84) could accommodate a TC tag insertion or substitution and furthermore that TC tag inserted at these sites could be specifically labeled by the biarsenical dye FlAsH. We then tested whether this strategy can be extended to other influenza virus strains. Thus, we constructed two plasmids: plasmid 444 carries a gene encoding A/Texas/36/91 (Tx91) NS1 bearing a TC tag inserted after AA52, and plasmid 445 carries a gene encoding NS1 with AA79-84 replaced by a TC tag. Transfection of either plasmid (plasmid 444 or plasmid 445) together with seven other plasmids representing seven segments of Tx91 (40) resulted in one viable virus, Tx91-445,

FIG. 4. FIAsh labeling of NS1 in virus-infected A549 cells. (A) A549 cells were infected with WT PR8, PR8-410, PR8-411, PR8-412, or PR8-413 virus, the cells were labeled with FIAsh, and live cells were observed at 6 h.p.i. by using a fluorescence microscope. (B) A549 cells were infected with PR8-411 or WT PR8 virus and labeled with FIAsh. Fluorescent NS1 was observed and documented at 10 h.p.i. (C) FIAsh-labeled NS1 and antibody-stained NS1 are colocalized in the nuclei of infected cells. A549 cells were infected with PR8-411 at an MOI of 1. At 6 h.p.i., cells were stained with FIAsh for 20 min and then fixed, permeabilized, and stained first with an NS1 antibody and then with Alexa Fluor 594-conjugated goat anti-rabbit IgG. α -NS1, anti-NS1 antibody; DAPI, 4',6'-diamidino-2-phenylindole.

which has a TC tag replacement at AA79-84 in NS1. We could not rescue the Tx91 virus with NS1 with the TC tag inserted after AA52. As seen in Fig. 5A, Tx91-445 formed plaques that were similar in size to the plaques formed by the WT Tx91 virus. While the WT Tx91 virus grew to a titer of 3.2×10^7 PFU/ml on MDCK cells, the Tx91-445 virus grew to a titer of 2.15×10^7 PFU/ml. In contrast to PR8-411 virus, multiple-cycle growth kinetics and single-cycle virus yield of Tx91-445

FIG. 5. TC-tagged NS1 in the background of A/Texas/36/91 (Tx91). (A) Plaques formed by the WT Tx91 and Tx91-445 virus on MDCK cells. (B) Multiple-cycle growth curve of WT Tx91 and Tx91-445. MDCK cells were infected with the virus at an MOI of 0.001. (C) Single-cycle virus yield of Tx91 WT and Tx91-445. MDCK cells were infected with the virus at an MOI of 5. Virus titers were determined at 8 and 10 h.p.i. Mean titers were plotted with error bars showing the standard deviations. (D) FIAsh-labeled NS1 and antibody-stained NS1 are colocalized in the nuclei of Tx91-445-infected A549 cells. (E) Live-cell imaging of fluorescent NS1 observed at 10 h.p.i. Experiments were performed as described in the legend to Fig. 4B and C.

are quite similar to those of the WT Tx91 virus (Fig. 5B and C). These data indicated that TC tagging at AA79-84 did not affect Tx91 virus replication markedly. Furthermore, we infected A549 cells with Tx91-445 at an MOI of 1, and at 7 h.p.i., the

cells were first labeled with FIAsh and then immunofluorescently stained with NS1-specific antibody. As seen in Fig. 5D, two signals primarily localized in the nucleus overlapped perfectly, indicating that FIAsh-labeled NS1 represents the au-

FIG. 6. Real-time visualization of NS1 nuclear import. A549 cells were infected with PR8-411 at an MOI of 1. A low concentration of FLAsH was added to the cells and maintained throughout the course of the virus infection. Images were acquired every 3 min starting at 4 h.p.i. by using a fluorescence microscope. This figure shows snapshots of the cells from the time-lapse movie (Video S1 in the supplemental material) at specific times from 0 min to 171 min. The data show one representative cell of three independent experiments. The first frame of the time-lapse movie (Video S1) ($t = 0$ min) was modified to illustrate the nuclear and cell membranes.

thentic NS1 during virus infection. FLAsH-stained Tx91-445-infected living A549 cells were also observed. At 10 h.p.i., NS1 was localized in the nucleus, and the background noise was very low. WT Tx91-infected cells did not exhibit any fluorescence (Fig. 5E).

Live-cell imaging of NS1 nuclear import. Finally, to test whether the TC-tagged viruses are useful in real-time imaging, we utilized the PR8-411 virus to visualize NS1 protein trafficking in A549 cells. Given that the kinetics and mechanisms of NS1 nuclear import are unclear (15), we hoped that real-time visualization of NS1 would address two basic questions. (i) Is NS1 immediately imported into the nucleus once it is synthesized or is the newly synthesized NS1 initially sequestered in the cytoplasm and then later released and imported synchronously into the nucleus? (ii) How fast is the process of NS1 nuclear import? To answer these questions, PR8-411-infected cells were labeled with FLAsH first and time-lapsed images were acquired at 4–7 h.p.i. at 3-min intervals (Fig. 6) (see Video S1 in the supplemental material). By 4 h.p.i. ($t = 0$ min), NS1 had been synthesized and was found in the cytoplasm, with little, if any, in the nucleus. NS1 was synthesized continuously and accumulated in perinuclear regions (left side) by 48 min and 72 min. By 96 to 108 min, the nucleus-cytoplasm boundary was diffuse, possibly due to the binding of NS1-importin complexes to the fibrils of the nuclear pore complex (28), which is

responsible for the actual translocation. By 126 to 135 min, all the NS1 was imported into the nucleus. Note the slightly smaller size of the nucleus at 144 min, suggesting that it transiently contracted and then quickly recovered. This might explain the remodeling of the host nuclear architecture after influenza virus infection, which provides an optimized nuclear environment for viral replication (19). The time-lapse video showing the kinetics of NS1 cytoplasmic accumulation, nuclear translocation, and nuclear localization is provided as supplemental material (Video S1 in the supplemental material).

DISCUSSION

In this study, we developed a strategy to generate infectious influenza A viruses bearing a tetracysteine (TC) tag on the NS1 protein. The engineered viruses are replication competent, with marginal loss of infectivity during a single cycle of infection (PR8-411 and Tx91-445) and even during multiple cycles of infection (Tx91-445). Moreover, with biarsenical labeling, the TC-tagged NS1 protein is fluorescent, allowing real-time fluorescence imaging of NS1 nuclear import in the context of virus infection. This is the first report of successful application of biarsenical tetracysteine technology in influenza virus research.

To find a site in NS1 that can accommodate foreign tag

insertion, we initially tried several sites located in the helix region of the RNA binding domain (RBD) (Fig. 1A). All insertions are lethal to the virus, indicating that insertion of a short tag in the helix region of RBD disturbs the overall structure of NS1, thus impairing the functions of NS1 that correlate with RNA binding activity and NS1 dimerization. Inspired by the reports that (i) most recent H5N1 isolates have a five-residue deletion in the linker region between the RBD and effector domain (ED) of NS1 compared to H1N1 and H3N2 isolates (22); (ii) large-scale sequence analysis of NS1 revealed that Asp74-Leu77 and Lys79-Arg83 (conventional numbering, counting the five-residue deletion) are variable (6); and furthermore that (iii) the density corresponding to these five residues (75-79) in the linker region is not well defined in the X-ray structure (3), indicating the flexible feature of the linker, we thus postulated that it is very likely the linker region, especially the AA74-79 region, could accommodate insertion of a short tag without significant disruption of the function of the NS1 protein. We tested three such linker regions in NS1 for tag insertion: a linker region within RBD, a linker region within ED, and a linker between the RBD and ED (Fig. 1B). With PR8 NS1, insertion of a TC tag into three regions led to rescued viable viruses. Of these viruses, the PR8-410 (insertion within RBD), PR8-411 and PR8-412 (insertion/substitution between the RBD and ED) viruses exhibited plaques, NS1 subcellular localization, and protein synthesis rate that were similar to those of WT virus (Fig. 2 and 3). When we inserted the TC tag into these two sites in Tx91 NS1, we could rescue only the virus with the tag inserted at the linker region between the RBD and ED (Tx91-445 [Fig. 5]). The Tx91-445 virus grew as well as the WT Tx-91 virus during a single cycle and multiple cycles of infection, indicating that TC tagging has little effect on Tx91 NS1 function and hence on virus replication. Most noteworthy, this linker region could even accommodate insertion of 12 amino acids (expanded TC motif, FLNCCPGCC MEP) (data not shown). These results suggest that the linker region between the RBD and ED is tolerant of foreign sequence insertion. Identification of this insertion site is novel and valuable: it allows insertion of various small sequences, such as small tags, epitopes, and microRNA response elements (32) into NS1, which will lead to engineered influenza viruses of different applications. The discrepancy of TC tagging after AA52 between PR8 and Tx91 NS1 proteins may indicate that PR8 NS1 is structurally different from that of Tx91, especially at the RBD. Considering the results of previous studies that PR8 NS1 did not bind to CPSF30 (7) and did not activate transcription factor IRF-3 (7, 20), it is possible that PR8 NS1 employs a different mechanism to counteract interferon action. This may explain why we could insert a TC tag in PR8 NS1 in the loop region of RBD, whereas such insertion is lethal for Tx91.

To test whether the TC-tagged viruses are useful in real-time imaging to study influenza virus biology, we monitored NS1 intracellular trafficking. NS1 protein is primarily found in the nuclei of infected cells in the late phase of infection. However, the kinetics and mechanisms of NS1 protein nuclear import remain to be elucidated. It has been suggested that translocation of NS1 into the nucleus is rapid (33), and three mechanisms have been proposed concerning the regulation of NS1 nuclear transportation (15). Using TC-tagged virus PR8-411,

we could visualize the NS1 nuclear import in virus-infected cells in real time. Our results showed that NS1 was synthesized continuously and accumulated in the cytoplasm until about 4.5 h.p.i., when it began moving toward and congregate around the nucleus. The actual translocation of NS1 to the nucleus takes about 30 min (Fig. 6) (see Video S1 in the supplemental material). These results supported a model that newly synthesized NS1 is initially sequestered in the cytoplasm by a cellular or viral binding factor that masks the nuclear localization signal (NLS) (15), and at a certain stage of virus infection, the NLS is unmasked and the sequestered NS1 is released and imported synchronously to the nucleus. Although the detailed mechanism remains to be investigated, such as what these cellular or viral binding factors are, and what triggers the dissociation of the factors to unmask the NLS, our fluorescent NS1 virus provided a tool to study this process in the course of virus infection. From 1960 to 1980, several investigations using electron microscopy revealed striking ultrastructural changes within the nuclei and cytoplasm of influenza A virus-infected host cells (19). Our live-cell imaging showed that during NS1 nuclear import, the nucleus temporarily shrank (Fig. 6, 144 min) and then rapidly returned to its normal size (Fig. 6, 171 min). This observation may reflect the above-mentioned remodeling of the host nuclear ultrastructure.

In this study for the first time, we used the biarsenical tetracycline fluorescence system in influenza virus research by modifying the NS1 protein. The success of TC-tagged virus rescue and live-cell NS1 labeling in infected cells suggested that loop/linker regions might be viable alternatives for proteins whose N or C termini are not suitable for tagging. Moreover, the TC tag inserted in these regions has a high affinity for biarsenical dye, resulting in specific labeling of the fusion protein. The strategy presented here can be extended in principle to other segments, such as the M gene, which encodes M1 and M2 by splicing. Indeed, we have successfully rescued an influenza virus bearing TC-tagged M1 (Y. Li and Y. Zhou, unpublished data). If we are armed with a powerful microscope, these replication-competent fluorescent influenza viruses are extremely valuable: these viruses allow us to label nascent viral proteins, and therefore, are useful in studying dynamic events that occur in the influenza virus life cycle after entry into the cell, including protein trafficking, protein-protein interactions, virus assembly, and budding. Additionally, there are not yet any publications describing the use of biarsenical tetracycline technology in high-throughput screening (HTS). As the NS1 expression level represents the virus replication capability, influenza viruses bearing TC-tagged NS1 can be used in live-cell HTS for antivirals. This will greatly broaden and advance the application of biarsenical tetracycline technology in biomedical research and will significantly contribute to the combat against influenza epidemics and pandemics.

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