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GENOME REPLICATION AND REGULATION OF VIRAL GENE EXPRESSION

Fine Regulation of Influenza Virus RNA Transcription and Replication by Stoichiometric Changes in Viral NS1 and NS2 Proteins

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ABSTRACT In the influenza virus life cycle, viral RNA (vRNA) transcription (vRNA→mRNA) and replication (vRNA→cRNA→vRNA), catalyzed by the viral RNA-dependent RNA polymerase in the host cell nucleus, are delicately controlled, and the levels of the three viral RNA species display very distinct synthesis dynamics. However, the underlying mechanisms remain elusive. Here, we demonstrate that in the context of virus infection with cycloheximide treatment, the expression of viral nonstructural protein 1 (NS1) can stimulate primary transcription, while the expression of viral NS2 inhibits primary transcription. It is known that the NS1 and NS2 proteins are expressed with different timings from unspliced and spliced mRNAs of the viral NS segment. We then simulated the synthesis dynamics of NS1 and NS2 proteins during infection by dose-dependent transfection experiments in ribonucleoprotein (RNP) reconstitution systems. We found that the early-expressed NS1 protein can stimulate viral mRNA synthesis, while the late-expressed NS2 protein can inhibit mRNA synthesis but can promote vRNA synthesis in a manner highly consistent with the dynamic changes in mRNA/vRNA in the virus life cycle. Furthermore, we observed that the coexistence of sufficient NS1 and NS2, close to the status of the NS1 and NS2 levels in the late stage of infection, could boost vRNA synthesis to the highest efficiency. We also identified key functional amino acids of NS1 and NS2 involved in these regulations. Together, we propose that the stoichiometric changes in the viral NS1 and NS2 proteins during infection are responsible for the fine regulation of viral RNA transcription and replication.

IMPORTANCE In order to ensure efficient multiplication, influenza virus transcribes and replicates its segmented, negative-sense viral RNA genome in highly ordered dynamics across the virus life cycle. How the virus achieves such regulation remains poorly understood. Here, we demonstrate that the stoichiometric changes in the viral NS1 and NS2 proteins during infection could be responsible for the fine regulation of the distinct dynamics of viral RNA transcription and replication. We thus propose a fundamental mechanism exploited by influenza virus to dynamically regulate the synthesis of its viral RNA through the delicate control of viral NS1 and NS2 protein expression.

KEYWORDS influenza virus, viral RNA transcription and replication, NS1, NS2, stoichiometric change

Influenza viruses belong to the family *Orthomyxoviridae* and are divided into four types: influenza A, B, C, and D viruses (1, 2). Among them, influenza A virus (IAV) and influenza B virus (IBV) are the main pathogens leading to annual seasonal epidemics. Occasionally, IAVs also cause pandemics. IAVs are further divided into 18 hemagglutinin (HA) subtypes and 11 neuraminidase (NA) subtypes (3), and IBVs are classified into two lineages, Victoria and Yamagata. In recent years, annual seasonal epidemics have been caused mainly by

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H1N1 and H3N2 subtypes of IAVs and IBVs. The culprit of the recent 2009 pandemic was an IAV of the H1N1 subtype.

The genomes of IAV and IBV consist of eight single-stranded, negative-sense RNA segments encoding at least 10 major proteins (PB2, PB1, PA, HA, nucleoprotein [NP], NA, M1, M2, nonstructural protein 1 [NS1], and NS2) (4, 5). Each viral RNA (vRNA) segment exists in the form of a viral ribonucleoprotein (vRNP) complex in which the RNA is encapsidated by one copy of viral RNA-dependent RNA polymerase (RdRp), a heterotrimeric complex formed from PB2, PB1, and PA, and multiple copies of viral NP. The vRNP complex is the minimal functional unit for viral RNA transcription (vRNA→mRNA) and replication (vRNA↔cRNA) to occur in the nuclei of infected cells. The viral RdRp exploits completely different strategies for carrying out transcription and replication. Transcription relies on cap snatching a capped-RNA primer from host pre-mRNAs for initiation and reiteratively copying a stretch of U close to the 5′ end of vRNA for termination (6). Replication is a full-length copy between vRNA and its cRNA (7). The initiation of both vRNA and cRNA synthesis is primer independent but requires newly synthesized *trans*-activating RdRp to facilitate it (8). Along with the syntheses of vRNAs and cRNAs in the nucleus, they are encapsidated by the RdRp and NP proteins into vRNPs and cRNPs (2).

In the influenza virus life cycle, the production of both viral proteins and the three RNA species is delicately controlled. It is known that PB2, PB1, PA, NP, and NS1 are expressed early, while HA, NA, M1, and NS2 appear relatively late (9). Meanwhile, the three viral RNA species show very distinct synthesis dynamics across the life cycle. mRNA accumulation appears the earliest at the preliminary stage by primary transcription from the incoming virion vRNPs and then increases quickly to a maximum level at the middle stage by secondary transcription with the newly assembled vRNPs, followed by a sharp decrease to extremely low levels at the later stage. In the meantime, viral RNA replication is activated by the newly synthesized *trans*-activating RdRp. The accumulation of vRNAs appears to continue to increase until the end of the cycle, while the accumulation of its replicative intermediate cRNA remains at a low level throughout the life cycle (10–14). These distinct dynamic variations are certainly conducive to virus replication efficiencies. However, how the virus achieves such fine regulation remains poorly understood.

Several potential mechanisms were previously proposed for the dynamic regulation of viral RNA synthesis. It has been suggested that the amount of RdRp, particularly NP, could act as a switching factor for the transition from transcription to replication as large amounts of RdRp and NP are required for encapsidating vRNA into vRNP (15). Besides, it has been speculated that the sharp decrease in transcription at later stages might result from the degradation of polymerase II (Pol II) induced by viral infection because it could lead to the reduction of capped pre-mRNAs required for cap snatching to initiate mRNA synthesis (16). Moreover, viral NS2 has been shown to be able to differentially regulate viral RNA transcription and replication (11). It was also reported previously that the production of virus-derived small viral RNAs (svRNAs), facilitated by the presence of NS2, in addition to RdRp and NP, could regulate the switch from transcription to replication (17).

The NS1 and NS2 proteins of influenza virus are expressed from the unspliced and spliced mRNA transcripts of the NS segment, respectively. NS1 is an early protein with multiple functions (18, 19). It is able to antagonize innate immune responses and regulate the splicing, nuclear export, and translation of host cellular and viral mRNAs (19–22). The NS1 protein consists of an RNA-binding domain (RBD), an effector domain (ED), and a disordered C-terminal tail. Each domain contributes to its multifunctional traits by interacting with distinct cellular factors (19). The RBD allows NS1 binding to a variety of RNAs, including host small nuclear U6 RNAs, polyadenylated mRNAs, double-stranded RNAs (dsRNAs), and viral RNAs. The ED enables its interaction with the 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF30), by which it inhibits CPSF30 activities, leading to the inhibition of host mRNA translation (22–24). The C-terminal domain (CTD) is implicated in affecting virus fitness in human cells and virulence

in mice (18). In contrast, NS2 is a late-expressed protein, and its steady-state level of mRNA accounts for 10 to 15% of the unspliced NS mRNA level (9, 25). NS2 is considered to primarily mediate vRNP nuclear export and thus is also named nuclear export protein (NEP) (26). In addition, it has been reported that NS2 is capable of differentially manipulating transcription and replication. It was reported previously that the effects of NS2 on replication could be mediated by virus-derived svRNAs (17). However, the exact effects on the regulation of transcription and/or replication reported by different research groups are inconsistent (11, 12, 17, 27–31).

In this study, we found that NS1 and NS2 expressed from the viral NS segment in influenza virus RNP reconstitution systems are able to delicately regulate viral RNA transcription and replication in a manner highly consistent with the dynamics of the synthesis of the three viral RNA species shown in the virus life cycle. Our in-depth functional study further identified the key sites of NS1 and NS2 involved in mediating these effects. Based on these results, we propose that the stoichiometric changes in NS1 and NS2 during infection participate in the dynamic regulation of viral RNA transcription and replication.

RESULTS

Primary transcription can be differentially regulated by the early NS1 and late NS2 proteins during influenza virus infection. So far, the synthesis kinetics for the three influenza virus RNA species (mRNA, vRNA, and cRNA) in the influenza virus life cycle have not been systematically defined; for example, the reported peak time of viral mRNA synthesis varies from 2.5 to 6 h postinfection (hpi) under different infection conditions (10–14). Here, we first systematically examined the virus RNA synthesis kinetics of influenza A/WSN/1933 (WSN) virus at multiplicities of infection (MOIs) of 0.1, 1, and 10 in HEK 293T cells. The steady-state levels of the three viral RNA species of the NA segment at 0, 2, 4, 6, and 8 hpi were determined by primer extension analysis. It can be seen that the overall dynamic patterns of mRNA and vRNA accumulation are consistent under different MOI conditions in which mRNA appears first and then increases rapidly, followed by a sharp decrease; meanwhile, vRNA continues to increase until the end of the infection (Fig. 1A). However, the timing of the RNA accumulation kinetics varies significantly among different MOIs. With increasing MOIs of 0.1, 1, and 10, the time of the peak mRNA level shifted about 2 h forward, and the speed of vRNA accumulation also increased correspondingly. These results demonstrate that the kinetics of viral RNA synthesis is tightly associated with the viral multiplicity of infection and that its dynamics could be determined by the virus itself.

Previous studies have reported that the viral NS2 protein could inhibit viral RNA transcription and promote replication (11), but the role of the NS1 protein in the regulation of viral RNA synthesis remains unclear (12, 32). In addition, it has been reported that NS1, together with the three subunits of viral RNA polymerase (PB2, PB1, and PA), is considered an early-expressed protein during infection, and NS2 mRNA accounts for only 10 to 15% of the unspliced mRNA (9, 25). We speculated that the NS1 and NS2 proteins may participate in the dynamic regulation of the synthesis of viral RNAs. In order to directly visualize the timing of NS1 and NS2 expression across the virus life cycle, we generated a recombinant WSN virus with a Flag tag inserted at the N terminus of the NS open reading frame (ORF), referred to as the WSN/Flag-NS virus, leading to both the NS1 and NS2 proteins being expressed with the same Flag tag fused at their N termini (Fig. 1B). The growth kinetics of the WSN/Flag-NS virus were then examined, and it showed a peak titer of 8.3×10^7 PFU mL^{-1}, slightly lower than that of the wild-type WSN virus, with a peak titer of 3.4×10^8 PFU mL^{-1} (Fig. 1C). The expression levels of NS1 and NS2 were then checked at the indicated time points under infection at an MOI of 1 by Western blotting (Fig. 1D). It could then be directly visualized that the NS1 protein appeared at around 3 hpi and reached a plateau at around 6 hpi. In comparison with NS1, the NS2 protein appeared at around 6 hpi, and its expression then gradually increased.

In order to examine the effects of NS1 and NS2 on viral RNA synthesis without interference by other viral proteins in the context of virus infection, we examined the effects of

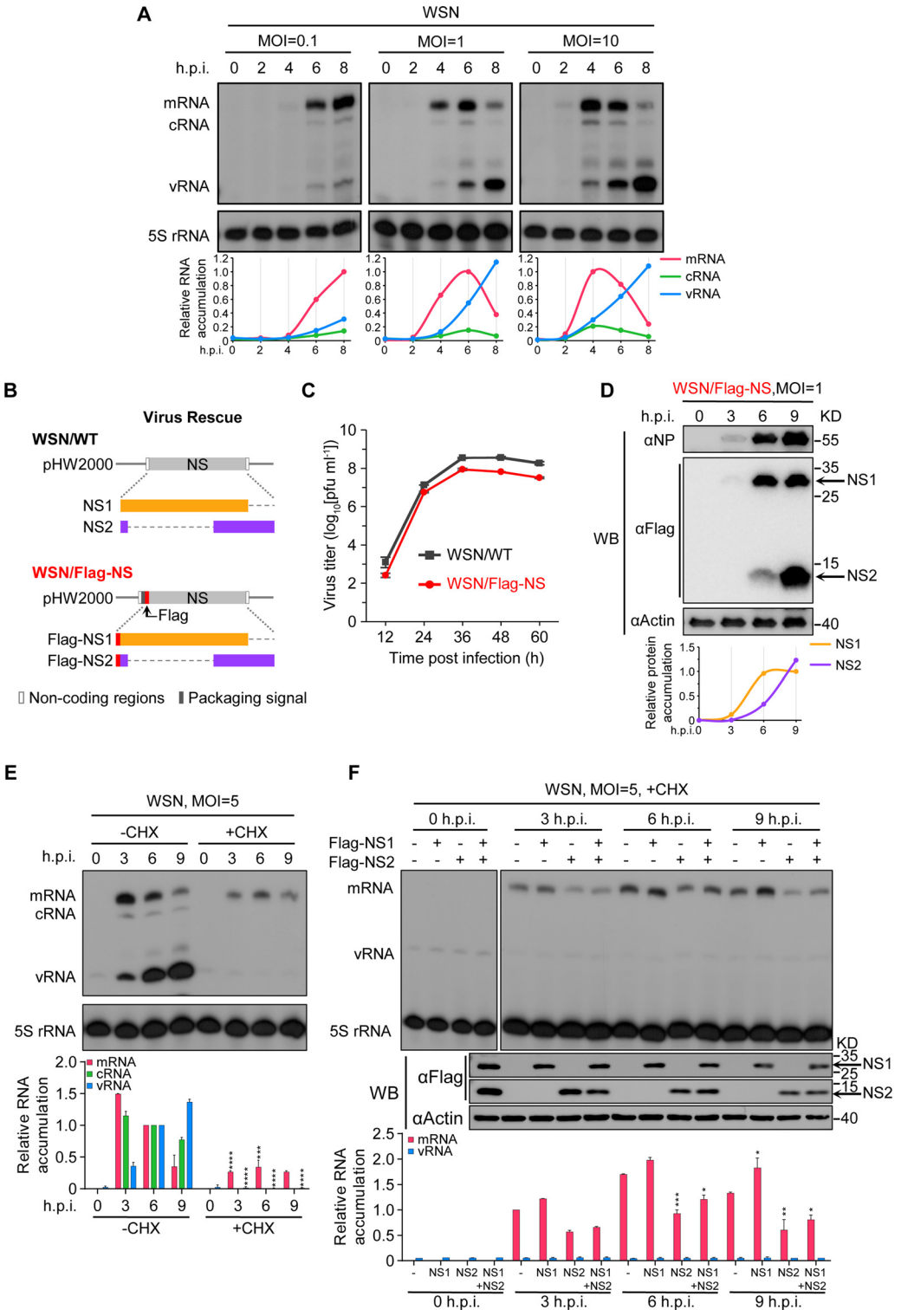

FIG 1 Primary transcription can be differentially regulated by NS1 and NS2 proteins during influenza virus infection. (A) HEK 293T cells were infected with WSN virus at MOIs of 0.1, 1, and 10. The steady-state levels of viral RNAs were determined by primer extension analysis using primers specific for the NA segment at the indicated hours postinfection. The levels of 5S rRNA were used as internal controls. Data representative of results from three independent experiments are shown. (B) Schematic representation of the NS segments of WSN WT virus and Flag-NS virus. The Flag tag (red bars), NS1 (orange bars), and NS2 (purple bars) are indicated. The first 30 nucleotides of the NS ORF (the start codon ATG was mutated to CTC to avoid translation initiation) were added upstream of the Flag tag to maintain the packaging signal (dark-gray bar). (C) Growth curves of WSN WT virus and Flag-NS virus in MDCK cells infected at an MOI of 0.001. At the indicated time points, the supernatants were harvested, and the virus titers were determined by a plaque assay. The data represent the means ±

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exogenously expressed NS1 and NS2 on viral primary transcription in infected cells with treatment with a protein translation inhibitor, cycloheximide (CHX). HEK 293T cells were transfected with pCAGGS Flag-NS1 and/or Flag-NS2 protein expression plasmids, and at 24 h posttransfection (hpt), the cells were infected with WSN virus (MOI = 5) in the presence of CHX (100 μg mL^{-1}) (15) (Fig. 1E and F). It can be seen that the expression of the NS1 protein significantly increased the mRNA levels; in contrast, the expression of the NS2 protein significantly reduced the mRNA levels, and the presence of the two proteins showed an intermediate effect. Together, these data suggest that viral RNA transcription during influenza virus infection can be differentially regulated by the early NS1 protein and the late NS2 protein.

NS1 and NS2 proteins differentially regulate viral RNA transcription and replication in the RNP reconstitution system. The NS1 and NS2 proteins of influenza virus are expressed from the unspliced and spliced mRNA transcripts of the viral NS segment, respectively. In order to further elucidate the regulatory roles of NS1 and NS2 in viral RNA transcription and replication during infection, we first studied the effects of NS1 and NS2, expressed from a single-templated cytomegalovirus (CMV) promoter-driven pCAGGS expression plasmid, on the RNP/NA reconstitution system of WSN virus. The pCAGGS protein expression plasmids expressing NS1 and/or spliced NS2 were first constructed by inserting the full coding sequence of the NS segment with a Flag tag coding sequence fused at its N terminus, followed by the introduction of stop codons upstream of the Flag-NS1 ORF and/or the Flag-NS2 ORF, respectively. Thus, the resulting four pCAGGS protein expression plasmids express Flag-NS1 and Flag-NS2 (Flag-NS), Flag-NS1, Flag-NS2, and Flag-ΔNS (as a control).

Subsequently, the RNP/NA reconstitution system of WSN virus, including PB1, PB2, PA, and NP protein expression plasmids and an RNA expression plasmid (pPOLI-NA) expressing the NA segment of the WSN virus, was transfected into HEK 293T cells, together with the above-described individual pCAGGS plasmids. The steady-state levels of the three viral RNA species (mRNA, cRNA, and vRNA) were examined at 24 and 48 hpt by primer extension analysis (Fig. 2A). It can be seen that, in comparison with the control, the expression of Flag-NS1 significantly increased the levels of accumulation of viral mRNA at 24 and 48 hpt. In contrast, the expression of Flag-NS2 resulted in reduced mRNA levels but increased vRNA and cRNA levels. Interestingly, when Flag-NS1 and Flag-NS2 were coexpressed by natural splicing, viral RNA replication was greatly enhanced. We further confirmed the effects of NS1 and NS2 by expressing untagged NS1 and/or NS2 proteins in the same RNP/NA reconstitution system (Fig. 2B) and in an RNP/M (M segment) reconstitution system (Fig. 2C). Furthermore, the effects of NS1 and NS2 on transcription were also confirmed at the protein translation level under the same experimental settings but with the replacement of the pPOLI-NA plasmid with either pPOLI-NA/NCR-eGFP (Fig. 2D) or pPOLI-PB2/NCR-eGFP (Fig. 2E) plasmids.

It has been reported that NS1 is able to interact with NP (33). In addition, NS2, together with M1, is able to interact with NP to form the nuclear export complex of vRNPs (34). To further examine whether NP is required for NS1 and/or NS2 to exert its regulatory effects on viral RNA transcription and replication, we examined the effects of NS1 and/or NS2 using a previously reported NP-free short-templated minireplicon system in which we transfected HEK 293T cells with PB1, PB2, and PA protein expression plasmids and an RNA expression plasmid (pPOLI-NP47) expressing a 47-nucleotide (nt)-long internally truncated NP segment

FIG 1 Legend (Continued)
standard deviations (SD) from three biological replicates. (D) HEK 293T cells were infected with WSN/Flag-NS virus at an MOI of 1. The levels of accumulation of the NS1 and NS2 proteins were analyzed by Western blotting at the indicated hours postinfection. β-Actin was detected as a loading control. Data representative of results from three independent experiments are shown. (E) Accumulation of viral RNAs in HEK 293T cells infected with WSN virus (MOI = 5) in the absence and presence of 100 μg mL^{-1} cycloheximide. (F) HEK 293T cells were transfected with plasmids expressing Flag-NS1 and/or Flag-NS2. At 24 hpt, cells were infected with WSN virus (MOI = 5) in the presence of 100 μg mL^{-1} cycloheximide. The steady-state levels of RNAs (E and F) and proteins (F) were determined by primer extension analysis (top) and Western blotting (WB) (bottom). The graphs in panels E and F show the relative mean intensities of viral RNAs normalized to 5S rRNA. The data represent the means ± SD from three biological replicates. Asterisks represent a significant difference from the control group (by one-way analysis of variance [ANOVA] with Dunnett's *post hoc* test) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

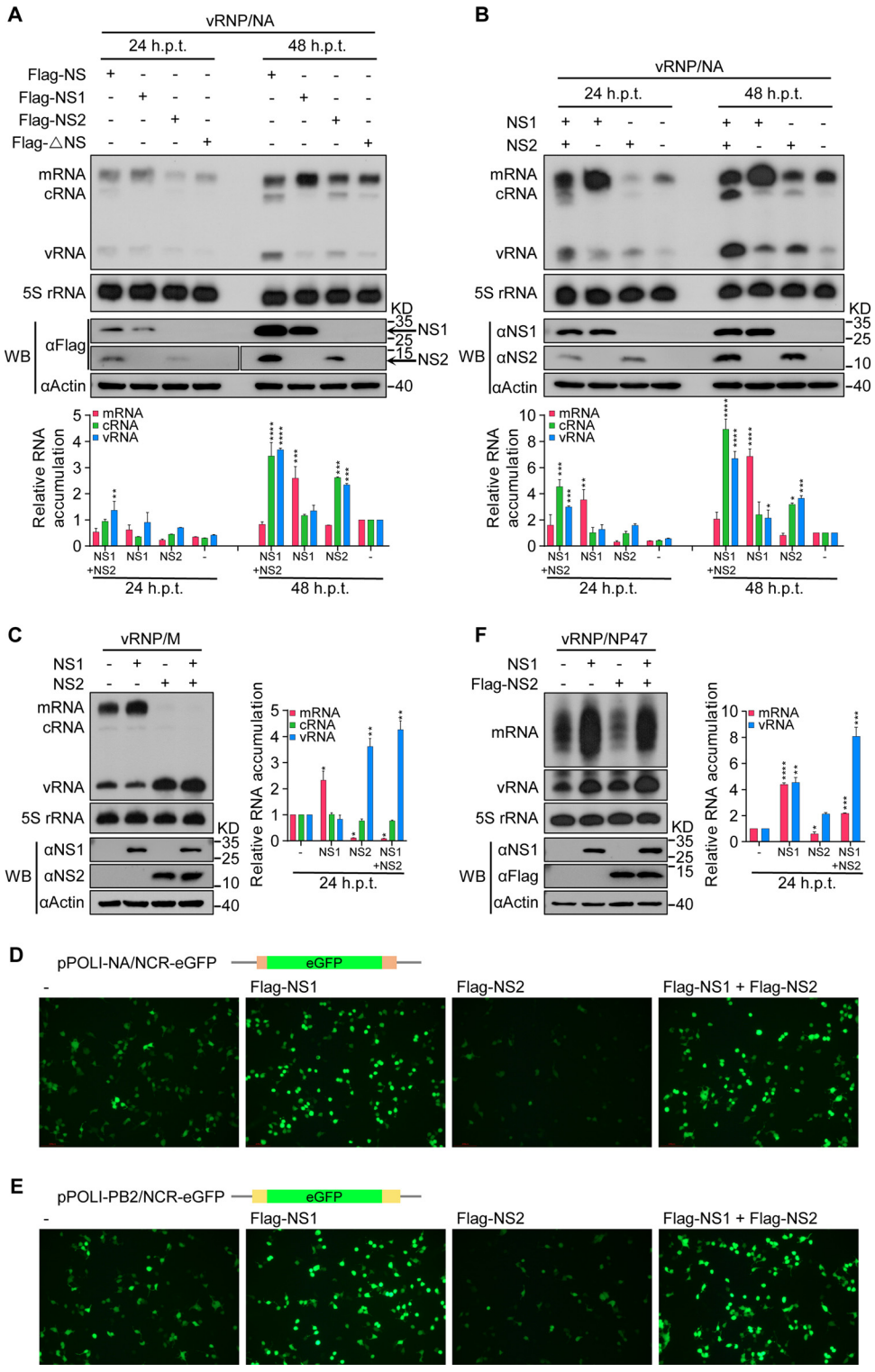

FIG 2 The viral NS1 and NS2 proteins differentially regulate viral RNA transcription and replication in the RNP reconstitution system. (A) Effects of Flag-NS1 and/or Flag-NS2 on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 and 48 hpt, respectively. (B) Effects of untagged NS1 and/or NS2 on the accumulation of viral RNAs detected as described above for panel A. (C) Effects of untagged NS1 and/or NS2 on the accumulation of viral RNAs in the RNP/M reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. (D and E) Regulatory effects of Flag-NS1 and/or Flag-NS2 on protein expression levels in RNP reconstitution systems. HEK 293T cells were transfected with plasmids expressing the PB2, PB1, PA, and NP proteins

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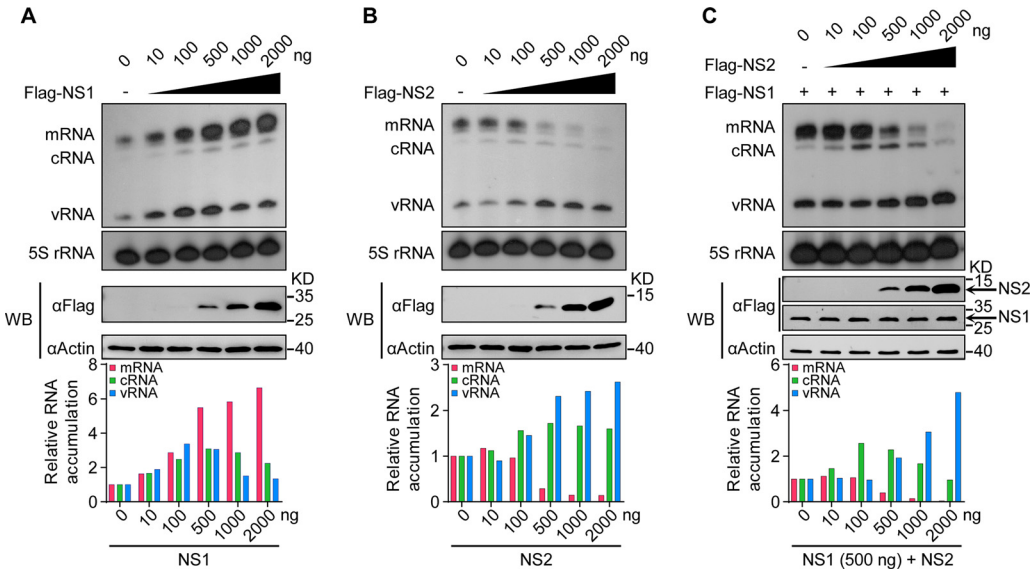

FIG 3 The effects of NS1 and NS2 on viral RNA transcription and replication are dose sensitive to NS1 and NS2 levels in the RNP reconstitution systems. (A and B) Dose-dependent effects of Flag-NS1 (A) or Flag-NS2 (B) on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. (C) Dose-dependent effects of Flag-NS2 in the presence of 500 ng of Flag-NS1 on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graphs show the relative intensities of viral RNA normalized to 5S rRNA.

(35, 36). We observed similar effects of NS1 and/or NS2 on the transcription and replication of the 47-nt-long template (Fig. 2F). These results indicate that the regulatory effects of NS1 and NS2 on viral RNA transcription and replication are independent of NP.

The effects of NS1 and NS2 on viral RNA transcription and replication are dose sensitive to NS1 and NS2 levels in the RNP reconstitution systems. Considering that NS1 and NS2 are expressed with different kinetics in the context of infection, we further characterized the dose effects of Flag-NS1 and/or Flag-NS2 on the regulation of viral RNA transcription and replication by varying the protein expression levels. We first simulated the early stage of infection in which HEK 293T cells were transfected with the RNP/NA reconstitution system together with gradually increasing levels of the pCAGGS Flag-NS1 expression plasmid. It can be observed that with increasing NS1 expression levels, the steady-state levels of viral mRNA were significantly increased in an NS1 dose-dependent manner, and the levels of accumulation vRNAs showed nonlinear variations but reproducibly appeared as a slight increase followed by a slight decrease (Fig. 3A). We concluded that NS1 is primarily capable of stimulating viral RNA transcription in a dose-dependent manner, which probably occurs in the early stage of infection. Besides, the results also suggested that NS1 is also implicated in differentially influencing the level of vRNA in a dose-sensitive manner. Next, we performed the same dose-dependent experiment by replacing the pCAGGS Flag-NS1 expression plasmid with the pCAGGS Flag-NS2 expression plasmid. We observed that with increasing NS2 expression levels, the viral mRNA levels were gradually reduced, whereas the levels of vRNA were gradually increased, both in NS2 dose-dependent manners (Fig. 3B).

Under the circumstances of influenza virus infection, as shown in Fig. 1D, as the late protein NS2 was produced at increasing levels in the presence of sufficient levels of the

FIG 2 Legend (Continued)
of WSN virus and the pPOLI-NA/NCR-eGFP (D) or pPOLI-PB2/NCR-eGFP (E) plasmid. The protein expression plasmids expressing Flag-NS1 and/or Flag-NS2 were cotransfected. The expression levels of GFP were examined by fluorescence micrographs at 24 hpt. (F) Effects of NS1 or/and NS2 on the accumulation of viral RNAs in the RNP/NP47 reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graphs in panels A to C and F show the relative mean intensities of viral RNA normalized to 5S rRNA. The data represent the means ± SD from three biological replicates. Asterisks represent a significant difference from the control group (by one-way ANOVA with Dunnett's *post hoc* test) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

early NS1 protein, we thus conducted a simulation experiment for a relatively later infection stage. HEK 293T cells were transfected with a fixed dose of the NS1 expression plasmid together with increasing amounts of an NS2 expression plasmid in the RNP/NA reconstitution system. The results showed that in the presence of a fixed level of NS1, increasing levels of NS2 gradually inhibited transcription and gradually promoted replication to a greater extent than the same level of NS2 present alone (Fig. 3C). Taken together, these results clearly demonstrate that the early-expressed NS1 protein is responsible for stimulating viral RNA transcription and that the late-expressed NS2 protein is responsible for switching viral RNA synthesis from a transcription-dominant to a replication-dominant status; moreover, the copresence of sufficient levels of NS1 and NS2 promotes replication to the highest efficiency, which is fully consistent with the viral RNA synthesis dynamics in the influenza virus life cycle shown in Fig. 1A.

NS1 and NS2 expressed from the NS segment dynamically regulate viral RNA transcription and replication in the RNP/NS reconstitution system. The above-described experimental results were obtained with the expression of NS1 and NS2 from a CMV promoter-driven protein expression plasmid, and dose variations were achieved by the transfection of different amounts of plasmids. To further confirm the regulatory effects of NS1 and NS2 in a way closer to a real virus infection as well as to examine the generality of these effects with a different viral RNA segment, we carried out experiments in the RNP/NS reconstitution system. The pPOLI-NS plasmid was modified by inserting a negative-sense Flag tag coding sequence at the N-terminal coding sequence of NS for the convenience of protein detection, followed by the introduction of various mutations to induce unspliced NS1 and spliced NS2 mRNA transcripts to express Flag-NS1 and Flag-NS2, Flag-NS1, Flag-NS2, or Flag-ΔNS (as a control) (Fig. 4A and B). The cotransfection of the four individual pPOLI-NS plasmids with PB1, PB2, PA, and NP of WSN virus in HEK 293T cells allowed us to examine the effects of the NS1 and/or NS2 protein from the real viral NS segment on the regulation of the transcription and replication of its own template. The NS1 and NS2 expression levels and the steady-state levels of the three NS viral RNA species can be determined by Western blotting and primer extension analyses simultaneously.

We performed a time course experiment, and cells were harvested at 12, 24, 36, and 48 hpt. As expected, the expression of the Flag-NS1 protein promoted the accumulation of mRNA to a greater extent than in the control samples, and the levels of vRNA accumulation were similar to those of the controls. In the Flag-NS2 samples, although an extremely low level of NS2 was detected, which was probably due to the transcription inhibition of NS2 on its own NS template, we observed a slight inhibition of mRNA accumulation and a slight promotion of vRNA accumulation, and the levels correlated well with the relative levels of NS2 detected. In contrast, in the Flag-NS samples with both NS1 and NS2 expressed by natural splicing, the levels of accumulation of vRNA were significantly enhanced, which was consistent with the effects of coexpressed NS1 and NS2, as shown in Fig. 2A and B. Meanwhile, the levels of mRNAs were significantly lower than those in the Flag-NS2 sample, which was due to the high levels of NS2 expressed in the system (Fig. 4C and D). These results are generally consistent with what we observed in the RNP/NA and RNP/M reconstitution systems but are closer to a real infection situation.

The regulatory effects of NS1 and NS2 on viral RNA transcription and replication are conserved among influenza A and B viruses. The genomic composition and organization of influenza B virus (IBV) are very similar to those of IAV, and its NS segment also encodes NS1 and NS2, which are expressed by the same splicing mechanism. To explore the generality of both NS1 and NS2 in the regulation of viral RNA synthesis as described above, we examined these effects using three other representative virus RNP/NA reconstitution systems (A/California/04/2009 [H1N1/CA04], A/Hong Kong/01/1968 [H3N2/HK68], and B/Yamagata/16/1988 [IBV/Yam]) (Fig. 5A and B). Consistently, all NS1 proteins could promote transcription and all NS2 proteins could inhibit transcription and promote replication in their corresponding RNP/NA reconstitution systems. Furthermore, when NS1 and NS2 were coexpressed, the viral mRNAs accumulated to an intermediate level, while the vRNA levels were greatly enhanced. We noted that a further increase in the vRNA level

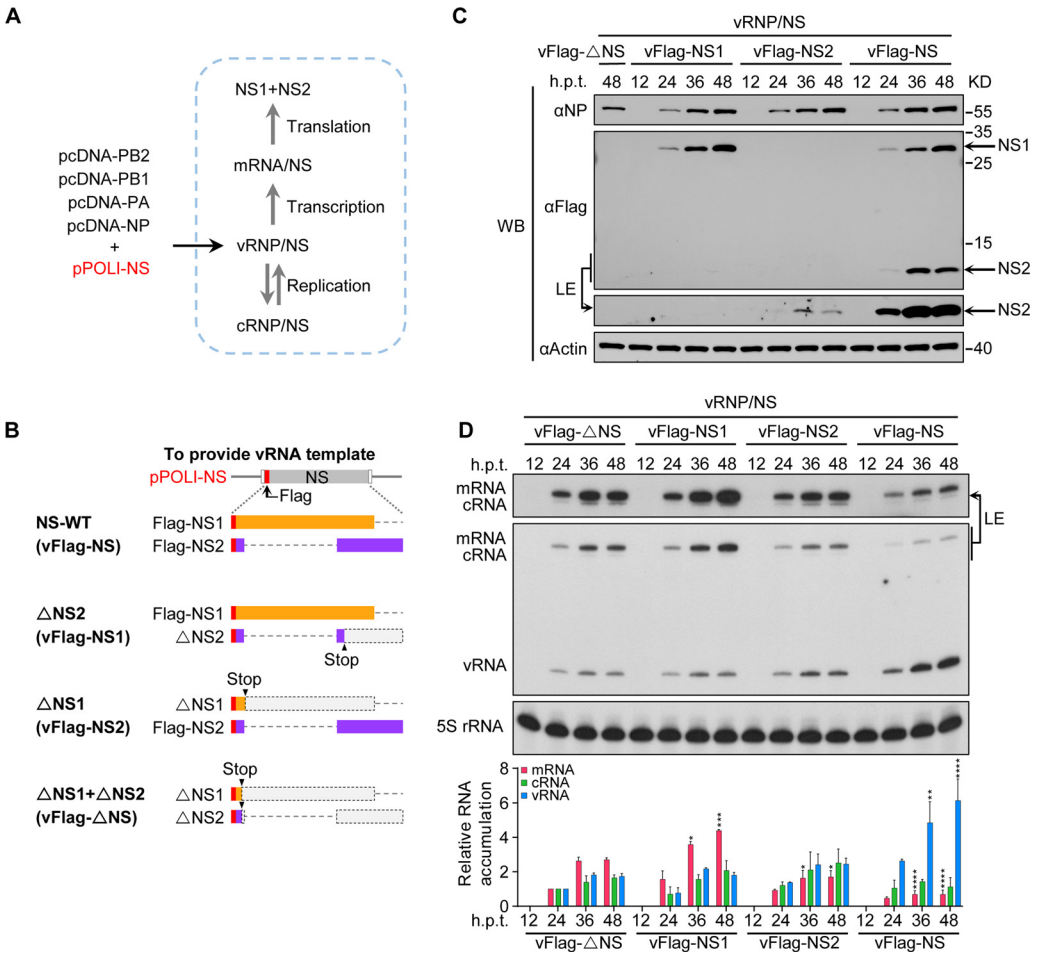

FIG 4 NS1 and NS2 proteins expressed from the viral NS segment differentially regulate viral RNA transcription and replication in the RNP/NS reconstitution system. (A) Schematic representation of the RNP/NS reconstitution system. The NS1 and NS2 proteins were expressed from mRNAs transcribed by viral RNA polymerase from the viral NS template provided by the pPOLI-NS plasmid. (B) Schematic representation of pPOLI-NS plasmids with various modifications to express different NS vRNA templates used for RNP/NS reconstitution systems. The Flag tag (red bars), the stop codon (black arrowheads), NS1 (orange bars), and NS2 (purple bars) are indicated. (C) The levels of viral proteins (NS1, NS2, and NP) in the RNP/NS reconstitution system with different pPOLI-NS templates in HEK 293T cells were analyzed by Western blotting at the indicated hours posttransfection. β-Actin was detected as a loading control. (D) The steady-state levels of NS RNAs in the RNP/NS reconstitution system with different pPOLI-NS templates in HEK 293T cells were determined by primer extension analysis using primers specific for the NS segment at the indicated hours posttransfection. LE, long exposure. The graph shows the relative mean intensity of viral RNA normalized to 5S rRNA. The data represent the means ± SD from three biological replicates. Asterisks represent a significant difference from the control group (by one-way ANOVA with Dunnett's *post hoc* test) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

in the presence of both NS1 and NS2 in the H3N2/HK68 RNP/NA reconstitution system was not apparent, which might be due to NS1/NS2 stoichiometric variation caused by the relatively low level of HK68/NS2 expressed in this system. In general, these results indicate that the capacities of the NS1 and NS2 proteins to regulate viral RNA transcription and replication are functionally conserved in IAV and IBV.

Amino acids R_{38} and K_{41} of NS1 are the key functional sites mediating transcription upregulation. NS1, a protein with a length of 230 amino acids (aa), contains an RNA-binding domain (RBD), an effector domain (ED), and a disordered C-terminal tail (18, 37). It has been shown that each domain could fulfill multiple functions, and function-correlated key amino acids have also been identified (19). For the purpose of identifying the key sites of NS1 responsible for transcription upregulation, we used a series of WSN NS1 deletion mutants and some key functional point mutants, and their effects on viral RNA synthesis were examined in the RNP reconstitution system of WSN virus (Fig. 6A) (33). The results showed that the expression of $NS1_{1-207}$ is capable of enhancing transcription to an extent similar to that of wild-type NS1 ($NS1_{WT}$), suggesting that

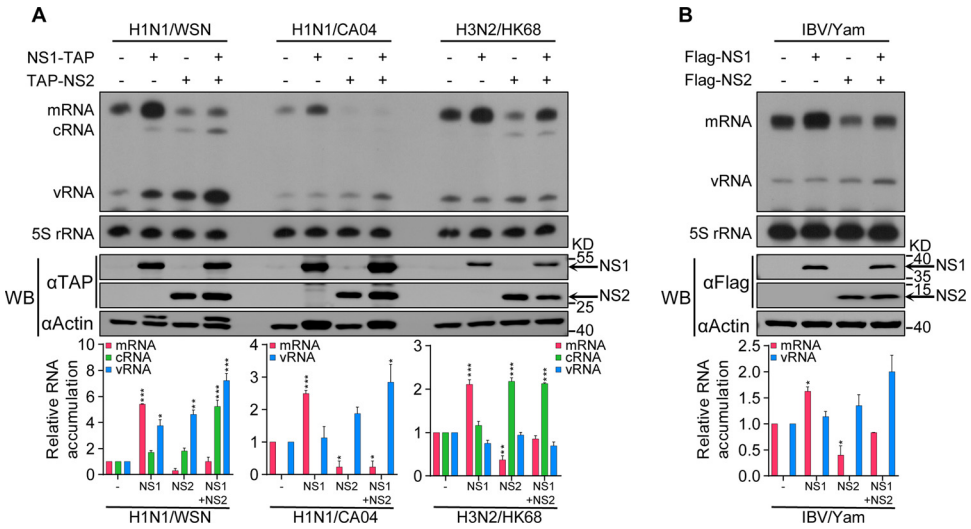

FIG 5 The regulatory effects of NS1 and NS2 on viral RNA transcription and replication are conserved in influenza A and B viruses. The effects of NS1 and/or NS2 on viral RNA transcription and replication in RNP/NA reconstitution systems derived from H1N1 (A/WSN/1933 [H1N1/WSN] and A/California/04/2009 [H1N1/CA04]) and H3N2 (A/Hong Kong/01/1968 [H3N2/HK68]) subtypes of influenza A virus (A) and B/Yamagata/16/1988 (IBV/Yam) of influenza B virus (B) in HEK 293T cells were determined. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graphs show the relative mean intensities of viral RNA normalized to 5S rRNA. The data represent the means ± SD from three biological replicates. Asterisks represent a significant difference from the control group (by one-way ANOVA with Dunnett's *post hoc* test) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

the disordered C-terminal tail does not participate in transcription upregulation (Fig. 6B). In comparison with $NS1_{1–207}$, the expression of $NS1_{1–162}$ and $NS1_{1–73}$ showed a very mild enhancing effect on transcription. The well-studied RBD-inactive mutant $NS1_{R38A/K41A}$, which can abolish the RNA-binding ability of NS1, showed significantly reduced effects on transcription upregulation compared to those of $NS1_{WT}$ or $NS1_{1–207}$. In contrast, another well-known ED-inactive mutant, $NS1_{F103S/M106I}$, which could abolish the capacity of NS1 to bind to host CPSF30 and, thus, cause NS1 to lose its inhibitory effect on cellular pre-mRNA processing, showed an enhancing effect on transcription similar to that of NS_{WT}. Interestingly, the combination of the two above-described mutants, a four-point mutant, $NS1_{38/41/103/106}$, could completely abolish the effects of NS1 on transcription upregulation. Meanwhile, we also observed that the levels of vRNA were upregulated with the expression of $NS1_{1–162}$, $NS1_{1–73}$, $NS1_{F103S/M106I}$, and $NS1_{38/41/103/106}$. The reason for this is unknown and awaits further investigation. Taken together, we concluded that both the RBD and ED of the NS1 protein are involved in regulating viral RNA transcription directly or indirectly. Moreover, amino acids R_{38} and K_{41} of NS1 are key functional sites that mediate this specific function. Based on this conclusion, we also conducted an $NS1_{R38A/K41A}$ dose-dependent experiment, which further confirmed our results (Fig. 6C).

Given that NS1 is the major viral interferon (IFN) antagonist, we further checked the effects of NS1 on IFN-deficient systems using either a STAT1 phosphorylation inhibitor, Ruxolitinib S enantiomer (S-ruxolitinib) (a highly selective and potent inhibitor of the JAK-STAT pathway [38]), or HEK 293T RIG-I knockout (293T RIG-I KO) cells. As shown in Fig. 6D, mRNA accumulation was promoted by the expression of $NS1_{WT}$ but not $NS1_{R38A/K41A}$. These results indicate that the regulatory effect of NS1 on viral RNA transcription is independent of its role as an IFN antagonist.

N-terminal amino acids 1 to 20 of NS2 mediate transcription inhibition, and the last C-terminal amino acid, I_{121}, mediates replication promotion independently. The NS2 protein consists of 121 amino acids, which are divided into an N-terminal domain (NTD) and a C-terminal domain (CTD) (39). The NTD of NS2 contains two nuclear export sequences (NESs) located at amino acids 12 to 21 and 31 to 40 (26, 40). In terms of the effects of NS2 on viral RNA synthesis, Robb et al. previously reported that NS2 is able to inhibit transcription and promote replication and that the deletion of amino

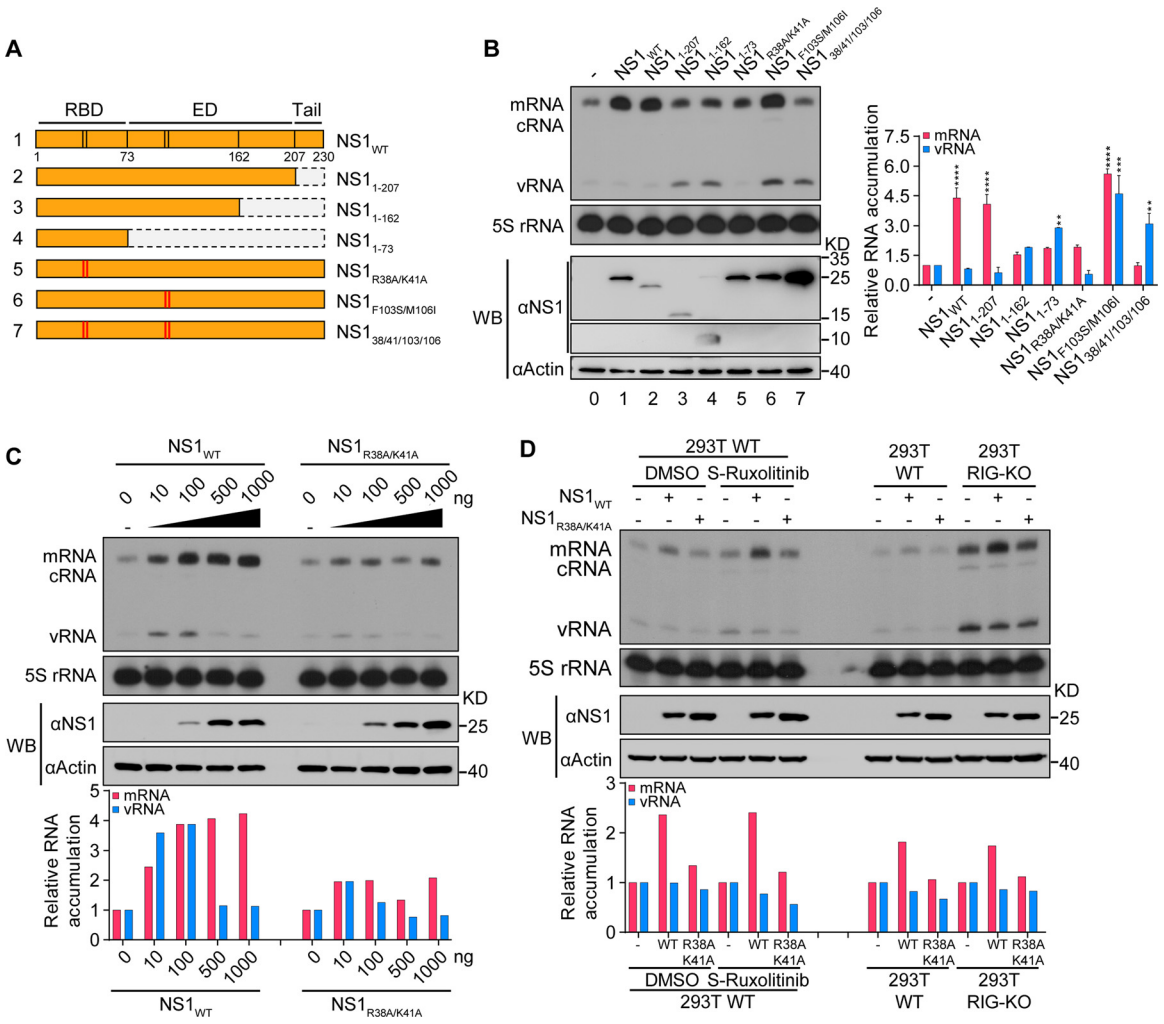

FIG 6 Amino acids R_{38} and K_{41} of NS1 are the key functional sites mediating transcription promotion. (A) Schematic representation of NS1 C-terminal deletion mutants and NS1 point mutants. (B) Effects of NS1 mutants on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graph shows the relative mean intensity of viral RNA normalized to 5S rRNA. The data represent the means ± SD from three biological replicates. Asterisks represent a significant difference from the control group (by one-way ANOVA with Dunnett's *post hoc* test) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (C) Dose-dependent effects of $NS1_{WT}$ (left) and $NS1_{R38A/K41A}$ (right) on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graph shows the relative intensity of viral RNA normalized to 5S rRNA. (D) Effects of NS1 on the accumulation of viral RNAs in the RNP/NA reconstitution system in IFN-deficient systems. (Left) HEK 293T cells were pretreated with S-ruxolitinib (4 μM) or dimethyl sulfoxide (DMSO) (as a control) for 1 h and then transfected with the plasmids of the RNP/NA reconstitution system of WSN virus, together with a plasmid expressing either the $NS1_{WT}$ or $NS1_{R38A/K41A}$ protein. (Right) HEK 293T cells (293T WT) and HEK 293T RIG-I knockout (293T RIG-I KO) cells were transfected with the same plasmids as the ones described above. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graph shows the relative intensity of viral RNA normalized to 5S rRNA.

acids 1 to 20 of NS2 does not affect these capacities (11). However, other reports demonstrated that the full-length NS2 protein could generally enhance polymerase activities in a concentration-dependent manner and that the removal of the last 3 amino acids completely abrogated this function (30, 31).

To determine the key sites of NS2 involved in inhibiting transcription but promoting replication, we constructed a number of N-terminal deletion mutants and C-terminal point mutants of WSN NS2 (Fig. 7A). The effects of these NS2 mutants were tested. Figure 7B shows that, in comparison with $NS2_{WT}$, the $NS2_{3A}$ triple mutant, with the last 3 amino acids mutated to alanine, is still capable of inhibiting transcription but loses the capacity to promote replication. We further generated three alanine point mutants ($NS2_{Q119A}$, $NS2_{L120A}$, and $NS2_{I121A}$) at the last 3 amino acids individually. In addition to

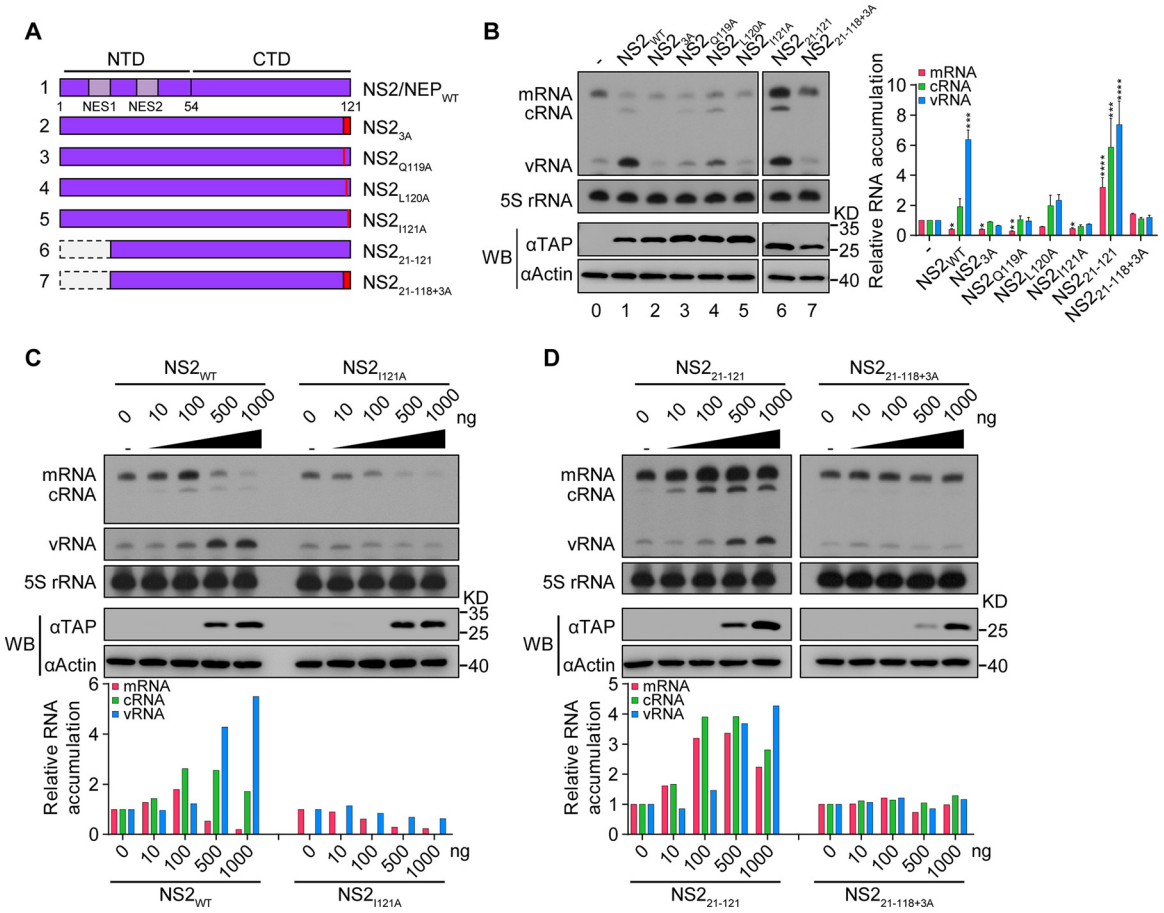

FIG 7 N-terminal amino acids 1 to 20 of NS2 mediate transcription inhibition, and the last C-terminal amino acid, I_{121}, of NS2 mediates replication promotion independently. (A) Schematic representation of NS2 N-terminal deletion mutants and C-terminal point mutants. (B) Effects of NS2 mutants on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graph shows the relative mean intensity of viral RNA normalized to 5S rRNA. The data represent the means ± SD from three biological replicates. Asterisks represent a significant difference from the control group (by one-way ANOVA with Dunnett's *post hoc* test) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (C) Dose-dependent effects of $NS2_{WT}$ (left) and $NS2_{I121A}$ (right) on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. (D) Dose-dependent effects of $NS2_{21-121}$ (left) and $NS2_{21-118+3A}$ (right) on the accumulation of viral RNAs in the RNP/NA reconstitution system in HEK 293T cells. The steady-state levels of RNAs and proteins (C and D) were determined by primer extension analysis (top) and Western blotting (bottom) at 24 hpt. The graphs in panels C and D show the relative intensities of viral RNA normalized to 5S rRNA.

their abilities to inhibit transcription, $NS2_{Q119A}$ and $NS2_{L120A}$ showed a slight promotion of replication, whereas $NS2_{I121A}$ completely abolished the ability of NS2 to promote viral RNA replication, behaving in the same manner as the $NS2_{3A}$ triple mutant. The effect of $NS2_{I121A}$ was further confirmed in a dose-dependent experiment. It can be seen that with increasing expression levels of $NS2_{I121A}$, the mRNA levels were gradually reduced, as expected, but the vRNA levels were not increased (Fig. 7C). These results not only confirm the essentiality of the last amino acid for regulating replication promotion but also clearly indicate that the two regulatory roles of NS2 in transcription and replication are uncoupled.

In terms of the transcription inhibition effects of NS2, Fig. 7B shows that the $NS2_{21-121}$ truncation mutant was able to enhance replication but lost the capacity to inhibit transcription. We further confirmed our results by combining the $NS2_{21-121}$ truncation with a mutation of the last 3 alanines. The results showed that the $NS2_{21-118+3A}$ mutant lost the functions in both transcription inhibition and replication promotion in the dose variation experiment (Fig. 7D). Overall, we conclude that N-terminal amino acids 1 to 20 of NS2 are important for NS2 to inhibit viral RNA transcription, while the last amino acid of the C terminus of NS2 is critical for stimulating replication.

DISCUSSION

It is well known that the production of influenza virus proteins and RNAs is tightly controlled to ensure efficient virus multiplication. Especially, the three viral RNA species showed very distinct synthesis dynamics in the virus life cycle. However, the mechanisms by which the virus achieves such fine regulation remain poorly understood. Here, in the context of infection, we show that the presence of NS1 is able to upregulate primary transcription, while the presence of NS2 does the opposite. Furthermore, using the RNP reconstitution system of WSN virus, by simulating the timing and the levels of NS1 and NS2 expression during infection, we show that different levels of NS1 and NS2 and their stoichiometric changes are capable of manipulating viral RNA transcription and replication in a way that is highly consistent with the dynamics of the synthesis of the three virus RNAs in the virus life cycle. Our in-depth functional study further identified that amino acids R_{38} and K_{41} of NS1 mediate transcription upregulation, while $NS2_{1-20}$ conveys transcription downregulation, and the last amino acid, I_{121}, of NS2 mediates replication upregulation. We also provide evidence that the regulatory roles of NS1 and NS2 in viral RNA transcription and replication are functionally conserved among different influenza A and B viruses. Based on these results, we thus propose, for the first time, that the expression of the early protein NS1 stimulates viral RNA transcription in the early stage of infection; the emergence of the late protein NS2 then gradually switches viral RNA transcription to replication; and, toward the end of infection, the copresence of sufficient NS1 and NS2 boosts vRNA synthesis to the highest efficiency (Fig. 8). This study suggests a fundamental mechanism exploited by influenza viruses to dynamically regulate viral RNA synthesis through the delicate control of the timing of the viral regulatory proteins.

NS1 has been reported to have multiple functions and acts as a regulatory hub to facilitate efficient virus replication. The most important function of NS1 is considered to be the suppression of host innate immunity (18). In addition, NS1 has been reported to be able to shut off host gene transcription and translation, delay virus-induced apoptosis, and manipulate host factors to support viral RNA splicing, nuclear export, and viral mRNA translation, etc. (19–22). Moreover, NS1 was vaguely implicated in the regulation of viral RNA synthesis but without explicit clarification (12, 32). In this report, we clarified that NS1 is able to promote viral RNA transcription in a dose-dependent manner (Fig. 3A and Fig. 6C), and we further demonstrated that the effect of NS1 is independent of the presence of NP (Fig. 2F) and independent of its role as a main IFN antagonist (Fig. 6D). Lin et al. previously reported that NS1 can interact with host RNA helicase A (RHA) in an RNA-dependent manner and enhances virus multiplication and viral RNA transcription (41). On the other hand, NS1 was also found to be able to bind a variety of RNAs, including host and viral mRNAs (19). Especially, Marc et al. reported previously that the RBD of NS1 could bind to virus-specific sequence patterns, the 8-nt-long motif at the 5′ ends of all positive-sense RNAs, in *in vitro* binding-selection experiments (24). Here, we identified that amino acids R_{38} and K_{41}, the key amino acids for RNA binding in the RBD of NS1, are involved in mediating the upregulation of viral mRNA synthesis. Together, we speculate that NS1 likely promotes viral mRNA synthesis by binding to the conserved noncoding regions (NCRs) of mRNAs. The underlying mechanism awaits further investigation.

NS2, also termed NEP, is expressed from a spliced transcript from the NS segment. In addition to its well-known nuclear export function, NS2 has been found to be involved in the regulation of viral RNA transcription and replication but with contradictory effects according to various reports (11, 12, 17, 27, 28, 30). Bullido et al. first reported that NS2 could inhibit the accumulation of all viral RNAs in the vaccinia helper virus infection system (27). However, Robb et al. reported that the expression of NS2 in the RNP reconstitution system reduced the accumulation of mRNAs but increased the accumulation of vRNAs (11). Perez et al. further reported that NS2 facilitates the production of virus-derived svRNAs that could specifically promote viral RNA replication (17, 29). Besides, Reuther et al. reported that the concentration of NS2 could either promote (25 ng) or inhibit (250 ng) the general viral RNA polymerase activities and that the deletion of the last 3 amino acids completely abolished this effect of NS2 (30). In

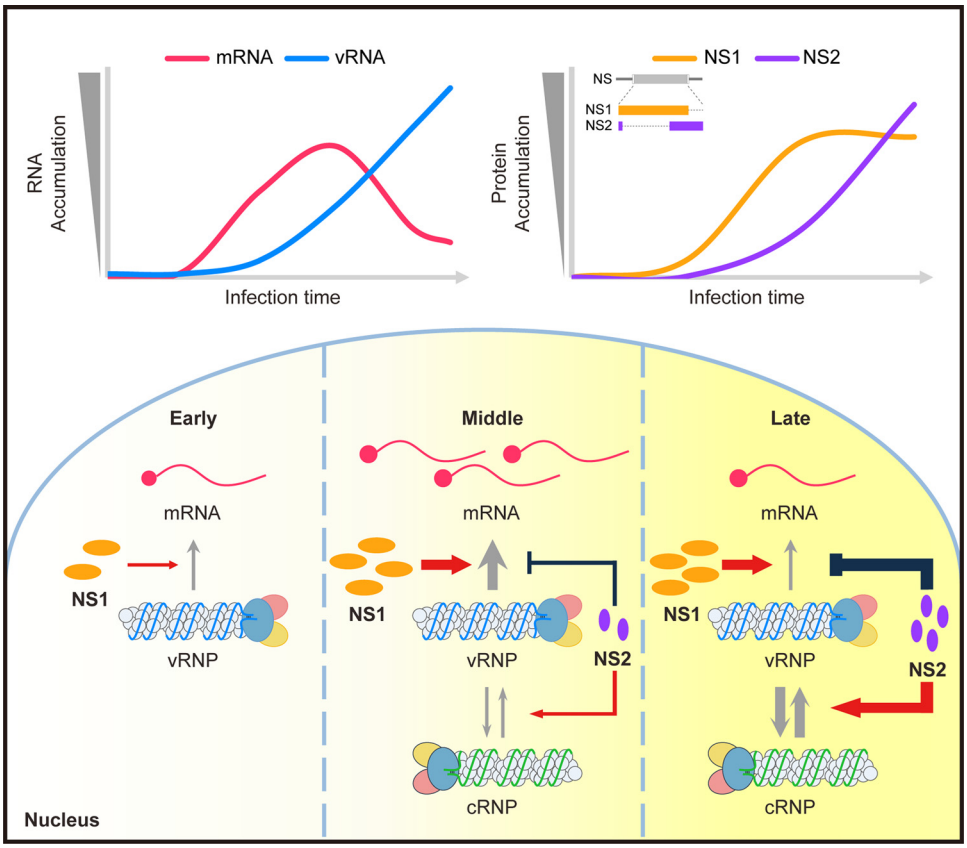

FIG 8 Proposed model for the regulatory effects of the early NS1 and late NS2 proteins on viral RNA transcription and replication during influenza virus infection. In the early stage of infection, viral RNA transcription is stimulated by an increase in the early protein NS1; as the infection proceeds, the emergence of the late protein NS2 manipulates a gradual switch from mRNA to vRNA synthesis; and later on, when both NS1 and NS2 accumulate to relatively high levels, vRNA production reaches its maximum efficiency.

this report, our results are largely consistent with those reported previously by Robb et al. Of note, in agreement with what Reuther et al. observed (30), we also observed a slight increase in polymerase activities when NS2 was expressed at low concentrations (10 to 100 ng) (Fig. 3B and C and Fig. 7C). Indeed, we found that the effect of NS2 on the manipulation of viral RdRp activities is highly sensitive to the relative protein concentrations of NS2, RdRp, and NP, which would probably explain the contradictory results reported previously by different groups.

By using a series of NS2 point and truncation mutants, we identified that the last amino acid, I_{121}, of NS2 was crucial for the function of NS2 in promoting replication, while the deletion of N-terminal amino acids 1 to 20 resulted in the loss of function of NS2 in transcription inhibition. Moreover, given evidence that only the C-terminal amino acids 59 to 116 of NS2 were structurally solved by crystallographic studies and that the N terminus and C terminus of NS2 were predicted to be intrinsically disordered (39, 42), the regulatory roles of NS2 are probably manipulated by its highly flexible N terminus and C terminus. On the other hand, it has been reported that NS2 can interact directly with PB1 and PB2 of the viral polymerase (28). We speculate that NS2, by binding to RdRp with its flexible N terminus and C terminus, could facilitate a conformational change of RdRp from a transcription-active conformation to a replication-active conformation. Further studies of the atomic structure of the viral RdRp-NS2 complex may provide comprehensive details.

More interestingly, here, we report for the first time that the simultaneous expression of NS1 and NS2 in the RNP reconstitution systems boosts the accumulation of vRNA to the highest level. This experimental condition well represents the status at later stages of infection in which NS1 and NS2 are copresent at relatively high levels and the vRNAs are

synthesized at maximum efficiency. This observation further highlights the delicacy of the virus in manipulating its RNA synthesis with its self-encoded proteins. Indeed, it was previously reported that experimental variations in the stoichiometric balance of NS1 and NS2 resulted in significantly reduced virus titers (43, 44). Together, given our observations that the early expression of NS1 stimulates transcription, the late expression of NS2 inhibits transcription and promotes replication, and the expression of both NS1 and NS2 further boosts vRNA synthesis to the highest levels, we thus conclude that stoichiometric changes in NS1 and NS2 play a critical role in controlling viral RNA synthesis across the virus life cycle.

In fact, the differential regulation of viral RNA transcription and replication by virus-encoded small proteins has been reported previously for vesicular stomatitis virus (45), Ebola virus (46), arenavirus (47), and respiratory syncytial virus (48, 49). A representative example is that the M2-1 protein encoded by the M2 mRNA of respiratory syncytial virus exerts the function of promoting transcription (48), while another protein encoded by this fragment, M2-2, plays a role in inhibiting transcription and promoting replication (49). Therefore, the self-regulation of virus RNA transcription and replication might be a general mechanism of negative-sense RNA viruses (NSVs).

In summary, based on our data, we propose for the first time that in the life cycle of influenza virus, the strategy for the successive expression of NS1 and NS2 through a splicing mechanism plays an important role in the dynamic regulation of viral RNA transcription and replication. Our findings may open up an avenue for further investigations of the regulation of viral RNA transcription and replication of influenza virus. We believe that structural elucidation of the mechanisms of how stoichiometric changes in NS1 and NS2 dynamically and differentially regulate viral RNA transcription and replication is of great significance.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby canine kidney (MDCK) (ATCC, CCL-34) and human embryonic kidney HEK 293T (ATCC CRL-3216) cells were purchased from the American Type Culture Collection (ATCC) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. HEK 293T RIG-I knockout (293T RIG-I KO) cells were kindly provided by Jianwei Wang and Xiaobo Lei (Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) and were cultured under the same conditions as the ones described above for HEK 293T cells. Cells were cultured in humidified incubators at 37°C with 5% CO_2. Recombinant influenza A/WSN/1933 (H1N1/WSN) virus was generated using the pHW2000 eight-plasmid system (50). The influenza WSN/Flag-NS virus was generated in the same way as the WSN virus except using the pHW2000-Flag-NS plasmid.

Antibodies. The antibodies used were as follows: mouse monoclonal anti-β-actin (catalog number A5441; Sigma-Aldrich), mouse monoclonal anti-Flag M2 (catalog number F3165; Sigma-Aldrich), rabbit anti-tandem affinity purification (TAP) tag (catalog number sc-25768; Santa Cruz), mouse monoclonal anti-influenza virus NP (catalog number MAB8251; Sigma-Aldrich), rabbit polyclonal anti-influenza virus NS1 (catalog number GTX125990; GeneTex), and rabbit polyclonal anti-influenza virus NS2 (catalog number GTX125953; GeneTex).

Plasmids. The pHW2000 eight-plasmid rescue system of influenza virus A/WSN/1933 (H1N1/WSN) was described previously (50). To rescue influenza WSN/Flag-NS virus, a Flag tag sequence was fused to the N terminus of the NS ORF of the pHW-2000-WSN/NS plasmid, and the first 30-nucleotide packaging signal of the NS ORF (the start codon ATG was mutated to CTC to avoid translation initiation) was added upstream of the Flag tag. The protein expression (PB2, PB1, PA, and NP) plasmids of the RNP reconstitution systems of A/WSN/1933 (H1N1/WSN) (51), A/California/04/2009 (H1N1/CA04) (52), A/Hong Kong/01/1968 (H3N2/HK68) (53), and B/Yamagata/16/1988 (IBV/Yam) viruses (54) were described previously. Plasmid pPOLI-WSN/NA expressing WSN NA vRNA was used in the RNP/NA reconstitution systems of the H1N1/WSN, H1N1/CA04, and H3N2/HK68 viruses. The B/Yam NA vRNA was expressed from a pDZ-muta-B/Yam/NA plasmid modified from a pDZ-B/Yam/NA plasmid (kindly provided by Adolfo García-Sastre, Icahn School of Medicine at Mount Sinai, New York, NY) by removing the CMV enhancer and the chicken β-actin promoter. pPOLI-NA/NCR-eGFP and pPOLI-PB2/NCR-eGFP were derived from the pPOLI-WSN/NA or pPOLI-WSN/PB2 plasmid by subcloning the enhanced green fluorescent protein (eGFP) gene in place of the original NA or PB2 ORF. The tagged or untagged CMV promoter-driven NS1 and NS2 expression plasmids were constructed in either pcDNA or pCAGGS plasmids with the tags fused at the indicated termini. The pCAGGS plasmids expressing NS1 and/or NS2 by natural splicing were generated by fusing a Flag tag at the N termini of the NS segments, and stop codons were introduced correspondingly by site-directed mutagenesis (ΔNS2 [stop codons at aa 24 and 25 of the NS2 ORF], ΔNS1 [stop codon at aa 15 of the NS1 ORF], or ΔNS1 and ΔNS2 [stop codons at aa 8 of the NS1 and NS2 ORFs]). The $NS1_{1–207}$, $NS1_{1–162}$, $NS1_{1–73}$, and $NS1_{R38A/K41A}$ mutants were gifts from Ervin Fodor (Sir William Dunn School of Pathology, University of Oxford, Oxford, UK). The other NS1 and NS2 mutants were constructed in our laboratory. In

the WSN RNP/NS reconstitution system, pPOLI-WSN/NS was modified to express vRNA templates of the NS segment with various modifications to allow the expression of NS1 and/or NS2 as expected.

Virus infections. HEK 293T cells were infected with WSN virus in DMEM supplemented with 0.5% FBS at an MOI of 0.1, 1, 5, or 10 based on the concentration of infectious viral particles (PFU per milliliter). Where required, cells were treated with 100 μg mL^{-1} cycloheximide as previously described (15). Cells were harvested at the indicated time points.

Growth curve analysis. MDCK cells were infected with WSN wild-type or Flag-NS virus at an MOI of 0.001 in DMEM supplemented with 0.5% FBS. At 12, 24, 36, 48, and 60 hpi, the supernatants were collected. The concentration of infectious viral particles (PFU per milliliter) was determined by a plaque assay in MDCK cells. The medium for the plaque assay was DMEM supplemented with 0.15% bovine serum albumin (BSA), 1 μg mL^{-1} tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin, and 1% agarose. The growth curves shown are the average results from three independent experiments.

RNP reconstitution and primer extension analyses. Approximately 10^6 HEK 293T cells or 293T RIG-I KO cells were transfected with 500 ng each of the PB2, PB1, and PA protein expression plasmids; 1,000 ng of the NP protein expression plasmids; and 500 ng of the vRNA expression plasmids (pPOLI-WSN/NA, pDZ-mut-B/Yam/NA, pPOLI-NA/NCR-eGFP, pPOLI-PB2/NCR-eGFP, or pPOLI-Flag-WSN/NS-related plasmids) using Lipofectamine 2000 and Opti-MEM according to the manufacturer's instructions. When using plasmids encoding short truncated vRNA templates (pPOLI-NP47), transfections were performed in the absence of pcDNA-NP (36). To inhibit the JAK-STAT pathway, HEK 293T cells were pretreated with S-ruxolitinib (final concentration of 4 μM) for 1 h and then transfected with the indicated plasmids (38). For all RNP reconstitution assays, a certain concentration of the empty vector was cotransfected to maintain equal concentrations of the total plasmid transfected. For primer extension analysis, total RNA was extracted using TRIzol reagent (Invitrogen) at the indicated time points. The steady-state levels of mRNA, cRNA, and vRNA of the WSN NA, M, NS, and NP47 templates were analyzed by primer extension analysis with ^{32}P-labled specific primers for negative- or positive-sense RNA of NA (11), M (11), NS (25), and NP47 (36). The primer used for the detection of the internal control 5S rRNA was described previously (11). The primers used for the detection of IBV/Yam NA RNAs (IBV/NA-vRNA, 5′-ACACTGTCACAGGTGTTGA-3′; IBV/NA-m/cRNA, 5′-CCGAATACAGTAAGTATGACAG-3′) were designed in this study. Primer extension products were analyzed on a 6% or 12% PAGE gel containing 7 M urea and detected by autoradiography.

Western blot analysis. Cells were lysed by using CytoBuster protein extraction reagent (Novagen), and the lysates were separated by SDS-PAGE and immunoblotted with the indicated primary antibodies, with anti-β-actin antibody as an internal control. Protein expression levels were visualized using an enhanced chemiluminescence detection kit (NCM Biotech) according to the manufacturer's instructions.

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