

**DIFFERENTIAL EFFECTS OF NS1 PROTEINS OF HUMAN PANDEMIC H1N1/2009,
AVIAN HIGHLY PATHOGENIC H5N1 AND LOW PATHOGENIC H5N2 INFLUENZA A
VIRUSES ON CELLULAR PRE-MRNA POLYADENYLATION AND MRNA
TRANSLATION.**

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The non-structural protein NS1 of influenza A virus blocks the development of host antiviral responses by inhibiting polyadenylation of cellular pre-mRNA. NS1 also promotes the synthesis of viral proteins by stimulating mRNA translation. Here, we show that recombinant NS1 proteins of human pandemic H1N1/2009, avian highly pathogenic H5N1 and low pathogenic H5N2 influenza strains differentially affected these two cellular processes: NS1 of the two avian strains, in contrast to NS1 of H1N1/2009, stimulated translation of reporter mRNA in cell-free translation system; NS1 of H5N1 was an effective inhibitor of cellular pre-mRNA polyadenylation in A549 cells, unlike NS1 of H5N2 and H1N1/2009. We identified key amino acids in NS1 that contribute to its activity in these two basic cellular processes. Thus, we identified strain-specific differences between influenza virus NS1 proteins in pre-mRNA polyadenylation and mRNA translation.

Influenza A viruses of the *Orthomyxoviridae* family endemically infect both birds and mammalian species. In human, influenza A viruses replicate in the respiratory tract, whereas in avian species the primary site of virus replication is the intestinal tract. The site of viral entry is partly defined by the distribution of sialic acid molecules on cell surfaces. These surface molecules are recognized by viral hemagglutinin (HA) and neuraminidase (NA). There are sixteen HA (H1–H16) and nine NA (N1–N9) subtypes. At least 103 out of the possible 144 combinations have been found in wild birds, a primary host of influenza A viruses. Depending on the

composition of amino acids in the HA cleavage site avian influenza strains can be classified further as high (HPAI) or low pathogenic (LPAI) strains.

Only four influenza A subtypes have been detected in humans. The three pandemics of the 20th century were caused by H1N1 (1918), H2N2 (1957) and H3N2 (1968). The first pandemic in this century was caused by an H1N1/2009 strain of swine-origin. Highly pathogenic avian influenza H5N1 has also a pandemic potential (1). Pandemics can occur when novel influenza strains cross the species barrier (2). Pandemic strains can be generated by either reassortment of genomic segments, which take place when more than one strain infect the same cell, or by accumulation of specific mutations (virulence/pathogenicity markers) in the viral genome as a result of the error-prone viral RNA-dependent RNA polymerase activity (3). These two mechanisms are also important for the generation of the influenza A virus diversity.

Influenza A virus replicates for almost 2 days after infection before detection by the immune system (4). This evasion of immune surveillance involves NS1 interference with the expression of key proinflammatory cytokines via interactions with specific cellular factors (5). NS1 interacts with cleavage and polyadenylation specificity factor 4 (CPSF4) to inhibit polyadenylation of cellular pre-mRNA and, thereby, to block host transcription (6). The interaction of NS1 with the cellular translation apparatus stimulates the synthesis of viral proteins (7). The N-terminal 74 amino

acids of NS1 form a functional RNA-binding domain (RBD), whilst the C-terminal 150 amino acids form an effector domain (ED) which predominantly mediates interactions with cellular proteins (8).

Here we analyzed recombinant NS1 proteins of human pandemic H1N1/2009 and avian highly pathogenic (HP) H5N1 and low pathogenic (LP) H5N2 strains (Fig. 1A) for their ability to inhibit pre-mRNA polyadenylation and stimulate mRNA translation and showed that these proteins have strain-specific effects. Our analysis revealed key amino acids that are essential for the differential effects of NS1 on these two cellular processes.

EXPERIMENTAL PROCEDURES

Cloning and mutagenesis – NS1 genes were derived from pandemic H1N1/2009, HPAI H5N1 and LPAI H5N2. The H1N1/2009 was isolated from patients with influenza-like symptoms in Luxembourg in 2009 (GenBank: CAZ66453.1; Gerloff et al., unpublished data). The HPAI H5N1 strains were obtained from farm chicken (GenBank: CAQ58520.1; (9)) and the H5N2 strains were from healthy wild waterfowls (Snoeck et al., unpublished data).

The nucleotide sequence encoding NS1 protein of A/Luxembourg/43/2009 (H1N1/2009) strain was amplified from cDNA by PCR and cloned into the pET151/D-Topo vector (Invitrogen). The resulting plasmid pET151-NS1-H1N1/2009 encodes a polypeptide with an N-terminal His-tag, V5-epitope and TEV-protease cleavage site (MHHHHHHGKRIPNLLGLDSTENLYPQ/GIDPFT). The nucleotide sequences encoding NS1 proteins of A/chicken/Nigeria/OG10/2007 (HPAI H5N1) and A/Spor-winged goose/Nigeria/210/2008 (LPAI H5N2) viruses were amplified from cDNA by PCR and cloned into the pNic28-Bsa4 vector. The resulting plasmids, pNic-NS1-HPAI H5N1 and pNic-NS1-H5N2, encoded polypeptides with an N-terminal His-tag and TEV-protease cleavage site (MHHHHHHSSGVDLGSTENLYFQ/S). The R38A/K41A mutations (RK/AA) in H1N1/2009, H5N1 and H5N2 NS1, the deletion of C-terminal 11 amino acids in H5N2

NS1, the deletion of ED in H5N1 NS1 were introduced by site-directed mutagenesis using 5'-phosphorylated oligonucleotides and Phusion DNA-polymerase (Finnzymes). The Q25N/E26G, S48N, R67W or F103Y mutations were introduced into plasmids encoding RK/AA NS1 of H5N1. Mutations N25Q/G26E, N48S or W67R were introduced into plasmids encoding RK/AA NS1 of H1N1/2009.

The coding regions of wild type (wt) NS1 proteins with the tags and the protease-cleavage site were sub-cloned into the pIRES2-AcGFP vector (to generate pIRES-NS1-H1N1/2009, pIRES-NS1-HPAI H5N1 and pIRES-NS1-H5N2). This vector includes SV40 polyadenylation signals downstream of the NS1-AcGFP genes to direct proper processing of the 3' end of the bicistronic mRNA. The *G. gallus* CPSF4 fragment was inserted into the pNic28-Bsa4 vector to generate pNic-chCPSF4^{F1-3} plasmid expressing zinc-finger domains F1, F2 and F3 of CPSF4 (amino acids 1-114, CPSF4^{F1-3}). All constructs were verified by sequencing.

Protein expression and purification – NS1 proteins and their mutants, and CPSF4^{F1-3} were expressed in *E. coli* and purified. Briefly, bacterial cells were grown at 37°C in 0.5L of StabySwitch auto-inducible media (Eurogentec) until the absorbance at 600nm reached 1. Flasks were transferred to 17°C and cells were grown for 10h. Cells were collected by centrifugation and resuspended in 20ml TN buffer (Tris-HCl 20mM, pH 8.6, 100mM NaCl) containing 10mM imidazole. The cell suspensions were sonicated and centrifuged at 120,000g for 1h. Supernatants were loaded onto HisTrap columns (GE Healthcare) and proteins eluted with a linear 10–500mM gradient of imidazole. The N-terminal tags were cleaved from proteins by an overnight treatment with AcTev protease (Invitrogen). Proteins were diluted with TN buffer and loaded onto pre-assembled HisTrap and Q-HP columns (GE Healthcare). Columns were disassembled and proteins were eluted from Q-HP column with a linear 0.1–1.0M NaCl gradient. All proteins were concentrated and purified by gel filtration on Superdex-200 columns (GE Healthcare) pre-equilibrated with TN buffer.

To purify NS1:CPSF4^{F1-3} complex, the purified His-tagged CPSF4^{F1-3} was incubated with untagged NS1 for 30 min at 4°C in TN buffer containing 20mM imidazole. 250µl of Ni-NTA agarose beads (Qiagen) in TN buffer were added to the proteins and the mixture was incubated for another 30 min at 4°C. Beads were washed with TN buffer and remaining proteins were eluted with high imidazole and resolved by SDS-PAGE.

NS1-interacting partners were also purified from carcinomic human alveolar basal epithelial A549 cells expressing different His-tagged NS1 proteins. Briefly, 5x10⁷ A549 cells were transfected with 44 µg of pIRES-NS1-H5N1, pIRES-NS1-H5N2, or pIRES2-AcGFP vector using 132µl of Lipofectamine LTX (Invitrogen) and 44µl of Plus reagent (Invitrogen). Twenty four hours post transfection cells were trypsinized, collected by centrifugation, resuspended in 2ml TN buffer containing 10mM imidazole and sonicated. Lysates were treated or not with RNase A (Sigma-Aldrich; 0,1mg/ml) for 15 min at 4°C and centrifuged at 20000x g for 20min. 250µl of Ni-NTA agarose beads in TN buffer were added to the proteins and the mixture was incubated for another 30 min at +4°C. Beads were washed with TN buffer containing 10mM imidazole to remove unbound polypeptides. Remaining proteins were eluted with imidazole gradient and resolved by SDS-PAGE. Proteins were transferred to the Hybond-LFP membrane (GE Healthcare) using Novex Semi-Dry Blotter (Invitrogen). Membranes were incubated with primary rabbit polyclonal anti-CPSF4 (1:1000, AV40675, Sigma-Aldrich) and then with secondary Cy5-conjugated goat anti-rabbit IgG (1:5000, PA45011V, GE-Healthcare). His-tagged NS1 proteins were stained with Penta-His Alexa Fluor 647 conjugated antibody (1:1000; 35370, Qiagen). Blots were scanned using a Typhoon 9400 or Odyssey imager. The images were analyzed with ImageQuant (GE-Healthcare).

Analytical gel-filtration – The oligomeric states of NS1 proteins and its mutants were determined by analytical gel filtration at 20°C on a Superdex-200 10/30 column (GE Healthcare) in TN buffer at a flow rate of 0.5 ml min⁻¹. The column was calibrated using

molecular weight markers for gel filtration chromatography (Sigma-Aldrich).

Thermal shift assay – Thermal shift assay was performed with 3µg of purified NS1 proteins, NS1 mutants, CPSF4^{F1-3} or its complex with NS1 in TN buffer containing 900x dilution of SYPRO Orange dye (Invitrogen). The assay was carried out in the DNA engine Opticon 2 continuous fluorescence detection system (BioRad). The dye was excited at 490nm and the emission light was recorded at 575nm while the temperature was increased by increments of 0.5°C per minute from 20 to 90°C. Control assays were carried out to verify that no fluorescence signal was recorded in the absence of protein. The T_m were calculated from the maximum value of the negative first derivative of fluorescence intensity versus temperature; this is approximately the midpoint of the unfolding transition. A shift in T_m (i.e. in the presence of mutation), indicated a change in protein stability. An increase in T_m indicated a stabilization of the protein by an increase in structural order and a reduction in conformational flexibility, while a decrease in T_m indicates a destabilization. Each reaction was carried out in triplicate.

Gel shift assay – Purified NS1 or its mutants were incubated for 15 min on ice with yeast tRNA^{Asp} (10, 11) in 8µl reaction volumes. Subsequently, 2µl of the TN buffer containing 10% glycerol was added, and the samples were run in a 1% agarose gel containing 0.1µg/ml ethidium bromide in Tris-Borate EDTA buffer (5 V/cm, 90min).

In vitro translation assay – Luciferase T7 control DNA (1µg, Promega) was linearized with *XmnI* restriction enzyme (NEB). Unlabeled or [³²P] α-CTP (Perkin Elmer) -labelled capped polyadenylated luciferase mRNA were produced with mMESSAGE mMACHINE kit (Ambion). Luciferase was produced from resulting RNA using nuclease-treated rabbit reticulocyte lysate system (Promega) in the presence or absence of purified NS1 proteins. Translation reactions were carried out in 50µl containing 35µl of rabbit reticulocyte lysate, 40µM amino acid mixture, 80U RNaseOut (Invitrogen), 1µg mRNA. Reaction mixtures were incubated at

30°C. Reactions were stopped by transferring the tubes to 4°C, luciferase activity was assayed using a luciferase reporter gene assay kit (Roche) and luminescence was measured using Tecan or Paradigm plate reader (Beckman Coulter). Alternatively, luciferase expression was detected by Western blotting. Primary antibody was goat IgG polyclonal anti-luciferase (G745A, Promega) and the secondary antibodies was Cy5-conjugated donkey anti-goat IgG (ab6566-100, Abcam). The ³²P-labeled reporter mRNA was analyzed by electrophoresis in 1% agarose gel. The gel was dried and analyzed by phosphorimaging (Fuji BAS 1500). The RNA was quantified by ImageJ software.

Reverse transcription and real-time quantitative PCR

– One million of A459 cells were transfected with pIRES-NS1-H1N1/2009, pIRES-NS1-H5N1, pIRES-NS1-H5N1 (F103Y), pIRES-NS1-H5N2, or pIRES2-AcGFP as described above. The GFP-expressing cells were counted using Cellometer vision instrument (Nexcelom Bioscience). Total RNA was extracted from the cells with RNeasy Mini kit (Qiagen). cDNA synthesis was performed with random primers (Invitrogen) or dT20 primer (Eurogentec) and SuperScript III reverse transcriptase (Invitrogen). Polyadenylated and total RNA encoding β-actin and peptidylprolyl isomerase A (PPIA) were quantified. Briefly, real-time PCRs were done on DNA engine Opticon 2 continuous fluorescence detection system (BioRad) using Platinum Taq DNA polymerase (Invitrogen), SYBR Green, (Cambrex) and a set of specific primers: 5'-GGCCACGGCTGCTTC-3' and 5'-GTTGGCGTACAGGTCTTTGC-3' for β-actin, and 5'-TCCTGGCATCTTGTCCATG-3' and 5'-CCATCCAACCACTCAGTCTTG-3' for PPIA. The copy numbers of total and polyadenylated β-actin and PPIA RNAs were calculated using standard curve analysis as in (12). Each reaction was carried out in triplicates. The ratios of the averaged copy numbers of dT20 and random primer reverse-transcribed RNA of control transfected cells $\left(\bar{x}_{dT_{20neg}} / \bar{x}_{Rneg} \right)$ were divided by the dT20/random ratio for NS1 expressing cells $\left(\bar{x}_{dT_{20}} / \bar{x}_R \right)$. The change (F) in RNA

polyadenylation was expressed using the

$$\text{following equation, } F = \frac{\left(\bar{x}_{dT_{20neg}} / \bar{x}_{Rneg} \right)}{\left(\bar{x}_{dT_{20}} / \bar{x}_R \right)}.$$

RESULTS

NS1 protein of H1N1/2009 possesses higher thermal stability than NS1 proteins of avian H5N1 and H5N2 – In contrast to RBD and ED, which can be purified individually, full-length NS1 proteins are known to readily aggregate and precipitate at the concentrations required for biochemical studies. Surprisingly, we found that the full-length NS1 proteins of HPAI A/chicken/Nigeria/OG10/2007 (H5N1) and LPAI A/Spur-winged-goose/Nigeria/210/2008 (H5N2) strains could be expressed in *E. coli* and purified to homogeneity using a combination of affinity, anion-exchange and size-exclusion chromatography (Fig. 1B and E). In contrast, NS1 of pandemic H1N1/2009 strains precipitated after the last chromatographic step.

It has been shown that R38A and K41A mutations increase NS1 solubility (8). Therefore, RK/AA mutations were introduced into NS1 of pandemic H1N1/2009 and soluble protein was obtained after last purification step (Fig. 1C and F). These substitutions abrogated nucleic acid binding by NS1 as demonstrated by gel shift assays (Fig. 1H-J). The NS1 proteins of the three analyzed strains, including their RK/AA mutants, were purified as dimers, with an apparent molecular mass of about 60kDa (the predicted mass for an NS1 dimer is 52kDa).

The stability of the dimeric NS1 was probed by thermal denaturation assay. The NS1 of the pandemic H1N1/2009 had a significantly higher melting temperature than the H5N1 and H5N2 NS1 (T_m =65 vs. 51.0 and 51.5 °C; Fig. 1K and L). The increased thermal stability of H1N1/2009 NS1 was not caused by the RK/AA mutations or C-terminal deletion, which is naturally present in H1N1/2009 NS1, since the same mutations introduced into the H5N2 NS1, did not affect temperature-dependent unfolding of the protein (Fig. 1D, G and M). Altogether, these results show that

purified NS1 proteins of human pandemic H1N1/2009, and avian HP H5N1 and LP H5N2 strains have similar oligomeric status, but differ in their thermal stability.

H5N1 and H5N2, but not H1N1/2009 NS1, facilitate translation of reporter mRNA in vitro – NS1 proteins orchestrate multiple cellular processes. In particular, NS1 enhances the translation of reporter mRNA *in vivo* (7,21). In order to separate the effect of NS1 on translation from its functions in other cellular processes, purified recombinant NS1 proteins were tested in an *in vitro* translation reaction based on rabbit reticulocyte lysate containing capped polyadenylated mRNA encoding firefly luciferase. Luminescence was used as readout.

The wild-type NS1 protein of H5N1 stimulated expression of reporter mRNA compared to the control reaction, resulting in a 6-fold increase in luciferase activity within 2 hours of incubation (Fig. 2A). We also tested whether the RK/AA mutation may affect NS1-stimulated translation. The RK/AA mutant of H5N1 NS1 stimulated the synthesis of luciferase to similar level as wild type protein (Fig. 2A). Thus, the RNA-binding residues R38 and K41 are not involved in the stimulation of translation.

This observation allowed us to directly compare effects of the NS1 RK/AA mutants of the three viruses on their efficiency to stimulate translation. While RK/AA NS1 of both avian strains stimulated translation of reporter mRNA, NS1 of the human pandemic H1N1/2009 strain was almost inactive in this assay (Fig. 2B). To exclude the possibility that NS1 proteins act post-translationally, the production of reporter protein was monitored by Western blot (Fig. 2C). This confirmed that only NS1 proteins of avian but not of human pandemic strain efficiently stimulated translation of a reporter mRNA.

We next examined the effect of the NS1 on the stability of the reporter RNA during translation. For this, the optimal concentration of reporter mRNA to be added to the translation reaction was determined (Fig. 2D). The ³²P-labeled reporter mRNA at final concentration of 0.04 mg/ml was added to the

translation reactions containing no or RK/AA NS1 of H5N2. The reactions were stopped after different time intervals and reporter mRNA in each reaction was analyzed by electrophoresis in agarose gel and quantified (Fig. 2E and F). Comparison of luciferase mRNA in the translation reactions with and without NS1 showed that degradation of reporter mRNA during translation was independent on NS1. Thus, NS1 had no effect on the relative stability of reporter mRNA in cell-free translation system.

To map the region on NS1 which is essential for stimulation of translation, two deletion mutants were tested in the reporter assay. Deletion of 11 C-terminal amino acids in H5N2 NS1 (residues 219-230, ΔC), which are also naturally present in the wild-type H1N1/2009 NS1 and deletion of the entire NS1 ED in H5N1 NS1 (residues 75-225) did not affect translational stimulation (Fig. 2H and G). These results demonstrate that the RBD (residues 1-74) of NS1 is sufficient for the translation stimulation in rabbit reticulocyte lysates.

Since NS1 of H1N1/2009 was almost inactive in the translation reaction, we hypothesized that amino acids that differ between the human pandemic H1N1/2009 and avian strains within the RBD are likely to play a particular role. Sequence comparison of the three NS1 proteins revealed that NS1 of H1N1/2009 possesses four unique amino acids in its RBD that are different from H5N1 and H5N2 NS1 (25/26, 48 and 67; Fig. 1A). The side chains of these residues are solvent-exposed according to available structures of NS1 RBD, indicating that they are most probably implicated in interactions with cellular factors (Fig. 4B). Therefore, we introduced Q25N/E26G, S48N or R67W mutations corresponding to H1N1 NS1 into an H5N1 NS1 RK/AA background (loss-of-function) and N25Q/G26E, N48S, and W67R mutations corresponding to H5N1 NS1 into an H1N1/2009 RK/AA background (gain-of-function). Proteins were purified and tested in the *in vitro* translation assay. We found that while mutations corresponding to H1N1 diminished the ability of the H5N1 protein to stimulate translation, substitutions corresponding to H5N1 in H1N1/2009

background increased the ability to stimulate translation (Fig. 2I). Therefore, amino acids in positions 25/26, 48 and 67 of NS1 are essential for the stimulation of translation.

NS1 of H5N1 but not H5N2 and H1N1/2009 is an efficient inhibitor of the polyadenylation of cellular pre-mRNA – NS1 proteins of different influenza A virus isolates have been shown to interact with cellular CPSF4 protein and inhibit polyadenylation of cellular pre-mRNA, thus blocking host RNA transcription (22,23,24). However, NS1 proteins of pandemic H1N1/2009 strain and several other strains are unable to block general host gene expression, despite interacting with CPSF4 (Fig. 3A; (22,23,42)). We hypothesized that the stability of NS1:CPSF4 complex may play a particular role. Indeed, crystallographic studies identified a critical role for amino acids 103, 106, 108, 125 and 189 of NS1 for NS1:CPSF4 interaction (24). NS1 proteins of avian LP H5N2 have a unique Tyr in position 103. H1N1/2009 and H5N1 NS1 have a Phe at this position (Fig. 1A). A model of CPSF4:NS1 H5N2 based on the complex structure of NS1 ED and zinc-finger domains F2 and F3 of CPSF4 (amino acids 63-114, CPSF4^{F2F3}; PDB entry: 2RHK) suggested that the hydroxyl group of Y103 may not fit correctly into the hydrophobic pocket formed by side chains of P111, F112, L72 and Y88 of CPSF4 (Fig. 4C) and, therefore, could destabilize CPSF4:NS1 H5N2 interaction.

To test this hypothesis, we reconstituted NS1:CPSF4 complexes from purified recombinant CPSF4^{F1-3} and NS1 of H5N1 or H5N2 (Fig. 3B). These complexes were resistant to RNase treatment, indicating that NS1 proteins interact directly with CPSF4 *in vitro* (Fig. 3C). Thermal denaturation analysis showed that while the T_m of NS1 proteins were identical, the T_m of their complexes with CPSF4^{F1-3} differed by 3 degrees (Fig. 3D). NS1:CPSF4^{F1-3} complex containing H5N2 NS1 had lower thermal stability than the complex containing H5N1 NS1. This was in line with our observation that the substitution F103Y destabilized the CPSF4^{F1-3}:NS1 H5N1 complex. These results suggest that Y103 of H5N2 NS1 destabilizes its complex with CPSF4 by preventing

hydrophobic bonding between NS1 ED and CPSF4^{F2F3}.

To test whether H5N2 NS1 interacts with CPSF4 in human cells, A549 cells expressing His-tagged H5N2 NS1 were disrupted and proteins were purified on Ni-NTA agarose resin. In the control experiments, proteins were purified from the cells transfected with the control vector or plasmid expressing H5N1 NS1. Western blot analysis showed that CPSF4 together with H5N2 and H5N1 NS1 could be eluted from the resin with high imidazole (Fig. 3E), indicating that both NS1 proteins interact with CPSF4 *in vivo*. Moreover, these complexes were resistant to RNase treatment, indicating that NS1 proteins interact directly with CPSF4 in A549 cells (Fig. 3F). Altogether, these experiments showed that the NS1 proteins analyzed here bind CPSF4 *in vitro* and in cell culture, but the resulting NS1:CPSF4 complexes differ in stability.

To study the effects of Y103 of H5N2 NS1 protein on polyadenylation of cellular pre-mRNA, A549 cells were transfected with plasmids encoding wild type His-tagged H5N2 NS1. In the control experiments, cells were transfected with the control vector or plasmids expressing NS1 of H5N1, its F103Y mutant or NS1 of H1N1/2009. These plasmids also expressed GFP from a bicistronic mRNA. GFP fluorescence was used to reach the transfection efficiency of 70%. Twenty four hours post transfection, RNA was purified from the cells. Four pools of cDNA obtained by RT with the dT20 primer were used to estimate the amount of the polyadenylated RNA, another four pools of cDNA were synthesized with random primers and served as a reference for total RNA. Levels of the β -actin and PPIA cDNAs were calculated by qPCR. The ratios between polyadenylated and total RNA were calculated in cells transfected with NS1-expressing constructs and then normalized against the ratio obtained with cells transfected with the control vector. The effect of the different NS1 proteins on polyadenylation of β -actin and PPIA pre-mRNA is shown in Fig. 3G. A reproducible increase in inhibition of polyadenylation of two cellular pre-mRNAs was observed upon H5N1 NS1 transfection in A549 cells, while H5N2 NS1 and F103Y

mutant of H5N1 as well as H1N1/2009 NS1 were almost unable to inhibit polyadenylation of the pre-mRNAs (Fig. 3G). Thus, F103Y mutation present in the H5N2 NS1 attenuates polyadenylation inhibition.

Altogether, these experiments show that recombinant NS1 proteins of the analyzed influenza strains differ in their ability to inhibit pre-mRNA polyadenylation of two cellular pre-mRNAs. This difference may partly correlate with amino acid at position 103 that contributes to the structural stability of the NS1:CPSF4 complex. We conclude that NS1:CPSF4 complex stability is important for inhibition of cellular pre-mRNA processing.

DISCUSSION

NS1 protein ensures efficient influenza A virus replication for almost 2 days without detection by the host immune system (4), taking advantage of its multiple interactions with host factors (Table 1). It has been proposed that NS1 proteins of different influenza A virus strains differentially affect basic cellular processes (26). Using highly purified NS1 proteins we showed here that the NS1 of human pandemic H1N1/2009, avian HP H5N1 and LP H5N2 strains differentially affect cellular pre-mRNA polyadenylation and mRNA translation. This finding allowed us to identify key amino acids within NS1 that contribute to its structural stability and interactions with host CPSF4 and translation factors during pre-mRNA polyadenylation and mRNA translation, respectively.

Different structural stability of NS1 proteins - Purified NS1 proteins of human pandemic H1N1/2009, and avian HP H5N1 and LP H5N2 strains have similar oligomeric status, but differ in their thermal stability. The NS1 of the pandemic H1N1/2009 had a significantly higher melting temperature than the H5N1 and H5N2 NS1. H1N1/2009 NS1 in contrast to NS1 of avian strains has hydrophobic residues at positions 67, 84, 112, 119 and 171 (Fig. 1A). According to the available structures of NS1 (PDB ID: 3EE8, 3EE9, 3F5T, 3D6R, 2GX9), amino acids 67, 112 and 171 reside in the hydrophobic core of RBD or ED (Fig. 4A). H1N1/2009 NS1 residues W67, I112 and Y171, but not R/D67,

T112 and T/N171 of H5N1/H5N2 NS1 could be involved in the stabilization of NS1 hydrophobic core via binding to conserved hydrophobic residues 27, 68, 116, 141 and 202 (Fig. 4A and B). However, the R67W mutation introduced into H5N1 NS1 did not increase NS1 stability ($T_m^{R67W}=49.0^{\circ}\text{C}$ and $T_m^{WT}=50.5^{\circ}\text{C}$, unpublished data). Therefore, I112 and Y171 could increase structural stability of H1N1/2009 NS1. Several residues on the surface of NS1 monomer could also contribute to structural stability of the NS1 dimer and, perhaps, multimer, since it was shown that NS1 is able to form long filaments along dsRNA molecules (8). The enhanced structural stability of H1N1/2009 NS1 oligomers in comparison with H5N1 and H5N2 NS1 may result in the depletion of “active” NS1 molecules from reactions, i.e. cellular pre-mRNA polyadenylation and viral mRNA translation. Altogether, our results showed that NS1 proteins of different influenza strains differ in their structural stability.

Differential effects of NS1 proteins in translation - It has been shown that NS1 enhances the translation of mRNA in infected cells (7,21). Here we used an *in vitro* translation system to compare activities of purified recombinant NS1 proteins and to provide molecular insight into this process. We demonstrated that NS1 of avian HP H5N1 and LP H5N2, but not human pandemic H1N1/2009, efficiently stimulated translation of reporter mRNA. Surprisingly, the RNA-binding residues R38 and K41 of NS1 were not essential for the stimulation of translation.

It has been shown that the 1-114 region of NS1 is required for the stimulation of viral mRNA translation in transfected COS-1 cells (28). We demonstrate that an even shorter region of NS1 (amino acids 1-74) is required for the stimulation of mRNA translation in rabbit reticulocyte lysates. Moreover, we found that residues at positions 25/26, 48 and 67 are involved in translational stimulation (Fig. 4A). Because these residues are solvent exposed in NS1 structures, we hypothesised that the stimulation of translation by NS1 is mediated by interaction of NS1 with translation factors, PABC being a likely candidate because it binds the 1-81 region of NS1 (29). Altogether,

these results demonstrate that stimulation of translation by NS1 is strain-specific, and that amino acids at positions 25/26, 48 and 67 contribute to stimulation efficiency via interaction with host translation factors.

Differential effects of NS1 proteins in pre-mRNA processing – It has been shown that NS1 inhibits post-transcriptional 3'-end processing of cellular pre-mRNA by binding evolutionary conserved CPSF4 (Table 1), thereby blocking host gene expression (6,22). However, NS1 protein of pandemic H1N1/2009 strain was unable to block general host gene expression, despite its binding to CPSF4 (42). We found that NS1 of H5N2 and H5N1 also bind CPSF4 *in vitro* and *in vivo*, however NS1 of H5N2, like H1N1/2009 NS1, was unable to inhibit the polyadenylation of two host pre-mRNAs. We hypothesized that this effect may be associated with Tyr at position 103 in H5N2 NS1, which hydroxyl group may affect the interaction between NS1 ED and the CPSF4^{F2F3} (Fig. 4C). We showed that Y103, in contrast to Phe, affected the NS1:CPSF4 complex stability and weakened the inhibition of polyadenylation of two cellular pre-mRNAs. This observation is in line with previous study, in which Leu at position 103 also destabilized the NS1:CPSF4 interaction and weakened the inhibition of

cellular pre-mRNA polyadenylation (23,25). Thus, amino acid at position 103 in NS1 is essential for NS1:CPSF4 complex stability and inhibition of cellular pre-mRNA polyadenylation.

The NS1 protein of influenza A virus influences multiple cellular events that are important for virus replication. NS1 inhibits polyadenylation of host pre-mRNAs in the nucleus of infected cells (6). This ultimately leads to an impaired synthesis of cellular proteins in the cytoplasm, and thereby to an attenuation of the host immune response. We showed here that NS1 of avian HP H5N1, in contrast to human pandemic H1N1/2009 and avian LP H5N2, tightly controls cellular pre-mRNA polyadenylation. NS1 of avian HP H5N1 and LP H5N2, but not human pandemic H1N1/2009 strains stimulate the translation of mRNA. Thus, NS1 of HP H5N1, in contrast to NS1 of human pandemic H1N1/2009 and avian LP H5N2 strains, may contribute to increased virus proliferation. In conclusion, our results suggest strain-specific ways in which different NS1 proteins influence cellular pre-mRNA processing and mRNA translation to ensure preferential and efficient virus replication and identify critical amino acids of NS1 proteins that contribute to replication efficiency of influenza A virus strains.

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FOOTNOTES

Chicken cDNA was a kind gift from Lukasz Kowalik. Yeast tRNA^{Asp} was a kind gift from Anne-Catherine Dock-Bregeon. pNic28-Bsa4 vector was a gift from Opher Gileadi. We thank Aurélie Sausy, Emilie Charpentier, Sébastien De Landtsheer and Maxim Bepalov for technical support, and Andrey Golubtsov for critical reading of the manuscript. This work was supported by a generous Centre de Recherche Public (CRP)-Santé grant, European Cooperation in Science and Technology (COST)-B28 funding, Erkko funding, National Research Fund (FNR, Luxembourg) and CIMO PhD fellowships.

FIGURE LEGENDS

FIGURE 1. Purified recombinant NS1 proteins of three influenza A viruses possesses different thermal stability. (A) Sequence alignment of analysed NS1 proteins, generated using ESPript 2.2 program. Key residues and two deletions are indicated. (B-D) Elution profiles of NS1 wild-type and mutants from analytical gel-filtration (Superdex-200, 0.5 ml min⁻¹, absorbance at 280 nm). The elution peak of BSA, one of the six calibration standards, is shown. (E-G) SDS-PAGE analysis of NS1-containing fractions eluted from gel-filtration column. (H-J) Gel-shift assay monitoring binding of wild-type and RK/AA NS1 proteins to tRNA^{ASP}. Positions of free (F) and NS1-bound (S) RNA are marked. NS1 concentrations in the mixtures are indicated. (K-M) Thermal stability of purified NS1 proteins. Two thermal melting curves for each NS1 proteins are shown. Arrows indicate the T_m, which corresponds to the midpoint of the unfolding transition.

FIGURE 2. Differential effects of NS1 proteins on translation. (A) Wild-type H5N1 NS1 and its RK/AA mutant (4 μM) stimulate translation of capped luciferase mRNA in rabbit reticulocyte lysates to a similar extent. (B) H5N1 and H5N2 NS1 stimulate translation, while H1N1/2009 NS1 does not. Translation reactions were stopped at indicated time points and luciferase activity was measured by enzymatic assays. Control reactions were performed without NS1. All the reactions were performed in triplicates. (C) Western blot showing expression of reporter protein in (B). (D) Effect of mRNA concentration on basal and NS1-stimulated translation. Insert shows the fold increase in translation by RK/AA NS1 of H5N2 (4 μM). (E) Autoradiograph showing degradation of ³²P-labeled reporter mRNA (final concentration of 0.04 mg/ml) and agarose gel showing ribosomal RNA in translation reactions with and without RK/AA NS1 of H5N2 (4 μM). (F) Quantification of reporter mRNA in translation reactions. (G) Deletion of the 11 C-terminal amino acids in H5N2 NS1 does not abolish translation stimulation of capped luciferase mRNA. (H) RBD of H5N1 NS1 is sufficient to stimulate translation of capped reporter mRNA. Luciferase activity was measured after 1 h of incubation. (I) Effect of mutations introduced into RK/AA mutant of H5N1 NS1 or H1N1/2009 (4 μM) on translation stimulation. Luciferase activity was measured after 1h of incubation. F.l.– full-length. All the reactions were performed in triplicate (G-I).

FIGURE 3. Differential effects of NS1 proteins in pre-mRNA processing. (A) NS1 of H1N1/2009 interacts with CPSF4^{F1-3}. His-tagged CPSF4^{F1-F3} and untagged NS1 were incubated with Ni-NTA agarose beads. Unbound polypeptides were removed with buffer containing 20mM imidazole/1M NaCl (lane 1) and 50mM imidazole/50mM NaCl (lane 2). Remaining proteins were eluted with TN buffers containing 100 (lane 3), 150 (lane 4), 200 (lane 5) and 250mM imidazole (lane 6) and analysed on SDS-PAGE. (B) Purified His-tagged CPSF4^{F1-F3} and untagged NS1 of H5N1 and H5N2 co-elute from Ni-NTA agarose beads. Unbound polypeptides were removed with buffer containing 20mM imidazole/1M NaCl (lanes 1 and 4) and 50mM imidazole/50mM NaCl (lanes 2 and 5). Remaining proteins were eluted with TN buffers containing 250mM imidazole (lanes 3 and 6). (C) RNase treatment of purified His-tagged CPSF4^{F1-3} and H5N2 NS1 does not affect their subsequent co-purification on Ni-NTA resins. Lanes 1 and 2 - flow-through from the beads, lane 3 – wash, lane 4 –

elution with 50mM imidazole, lane 5 – elution with 100mM imidazole, lane 6 – elution with 250mM imidazole. (D) Difference in thermal stability (ΔT_m) between NS1:CPSF4^{F1-F3} complexes and NS1 alone. Complexes of CPSF4^{F1-F3} with wild-type or mutant F103Y NS1 of H5N1, or wild-type H5N2 NS1 were eluted from Ni-NTA agarose resin and their T_m were measured. T_m of NS1 proteins alone were measured and subtracted from T_m of their complexes with CPSF4^{F1-F3}. (E) H5N1 and H5N2 NS1 proteins co-elute with CPSF4 from an A549 cell lysate. Western blot of affinity purified proteins from A549 cells expressing His-tagged NS1 of HP H5N1, LP H5N2 or GFP. Cells were disrupted 24 h post transfection. Lysates were incubated with Ni-NTA agarose beads. Unbound proteins were eluted from beads with TN buffers containing 20mM imidazole (lanes 1, 5 and 9). Remaining proteins were eluted with TN buffers containing 50 (lanes 2, 6 and 10), 100 (lane 3, 7 and 11) and 250 mM imidazole (lane 4, 8 and 12). The fractions were resolved by SDS-PAGE followed by Western blotting using antibodies against His-tag and CPSF4. (F) Western blot showing co-purification of NS1 of H5N2 and CPSF4 from the lysate of A549 cells treated with RNase A. (G) Recombinant NS1 proteins differentially affect polyadenylation of β -actin and PPIA pre-mRNA in A549 cells. A549 cells were transfected with plasmids encoding wild type H5N2 NS1, H1N1/2009, H5N1 or F103Y mutant of H5N1 NS1. Twenty four hours post transfection, RNA was purified from the cells. Four pools of cDNA obtained by RT with the dT20 primer were used to estimate the amount of the polyadenylated RNA, another four pools of cDNA were synthesized with random primers and served as a reference for total RNA. Levels of the β -actin and PPIA cDNAs were calculated by qPCR. The ratios between polyadenylated and total RNA were calculated in cells transfected with NS1-expressing constructs and then normalized against the ratio obtained with cells transfected with the control vector. A value higher than 1 indicates that NS1 inhibited the polyadenylation of pre-mRNA. The results show means of three independent experiments.

FIGURE 4. Key amino acids of influenza A virus NS1 protein implicated in protein stability, cellular pre-mRNA polyadenylation and mRNA translation. (A) Ribbon representation of aligned ED monomers of NS1 carrying H1N1/2009 or H5N1-type of residues at positions 112 and 171 (PDB ID: 3M5R and 3F5T). (B) Ribbon representation of aligned RBD dimers of NS1 carrying H1N1/2009 or H5N1-type of residues at positions 25, 26, 48 and 67 (PDB ID: 3M8A and 2ZKO). (C) Ribbon representation of NS1 ED:CPSF4^{F2F3} complex focusing on a residue at position 103 which plays a key role in NS1:CPSF4 complex stability (PDB ID: 2RHK). The aromatic ring of F103 interacts with hydrophobic L72, Y88, F112 and, possibly, with P111 of CPSF4 (top). When the F103 is substituted with Tyr, its hydroxyl group may destroy the accurate network of hydrophobic interactions destabilizing the NS1:CPSF4 assembly (bottom). Side chains of these residues as well as conserved residues at positions 27, 68, 116, 141 and 202 are shown. (D) NS1 residues implicated in protein stability, nucleic acid binding, stimulation of translation and inhibition of polyadenylation analyzed in this study.

Table 1. Sequence identity and similarity between human and chicken NS1-interacting partners.

NS1 cellular partner	Identity / similarity, %	Reference
CPSF4	96/98	(6)
PABC	98/98	(29)
eIF4GI	45/55	(31)
PABPN1	45/54	(32)
Staufen1	62/66	(33)
CrkL	92/96	(34)
ISG15	31/50	(35)
NS1-BP	91/96	(36)
Nucleolin	63/76	(37)
NXF1	12/13	(38)
NXT1/NTF2-related export protein 2	80/93	(38)
Rae1	55/58	(38)
E1B-AP5	38/49	(38)
PI3K (p85 β)	71/81	(39)
PKR	37/52	(40)
TRIM25	48/65	(41)

Figure 1.

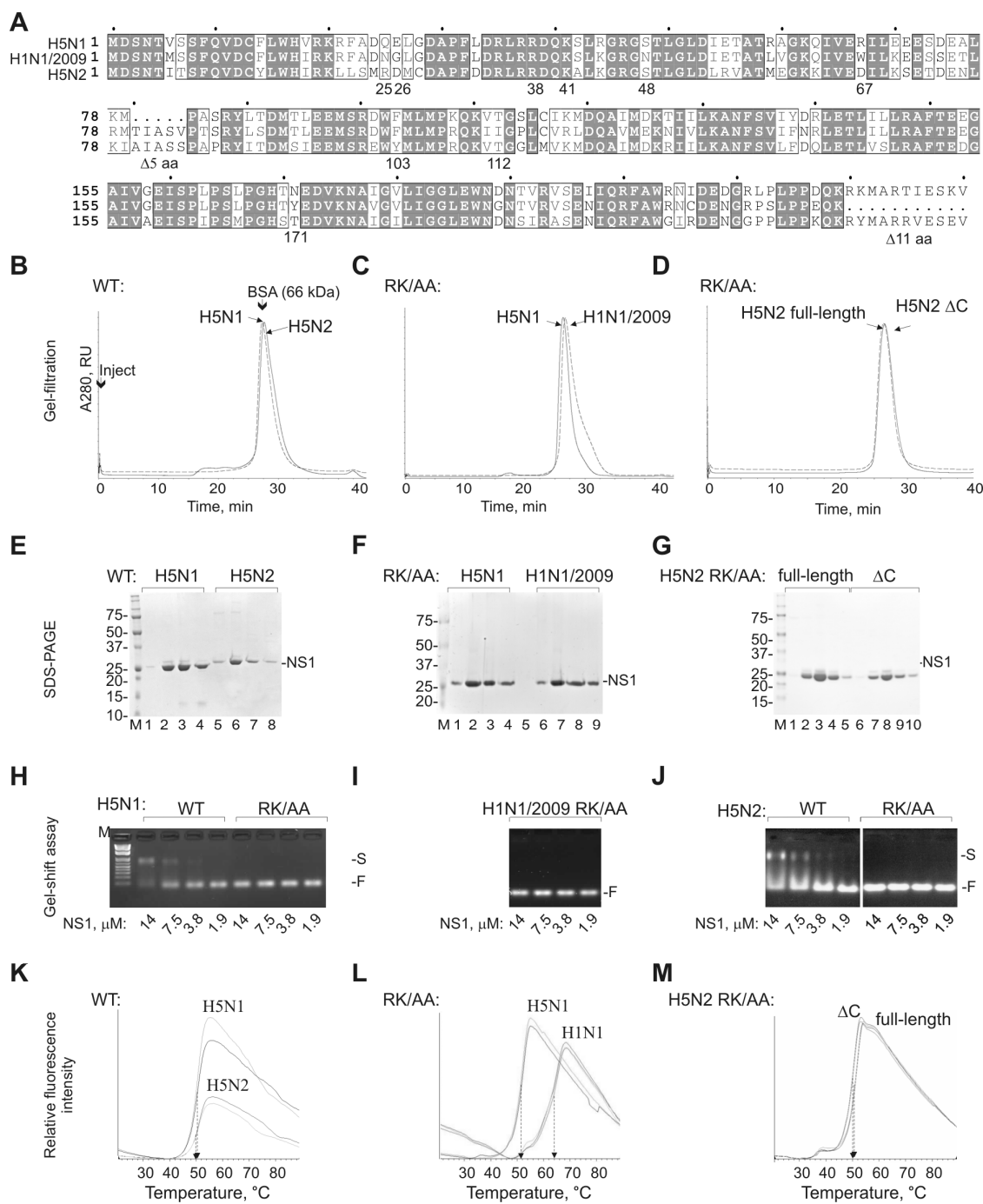


Figure 2.

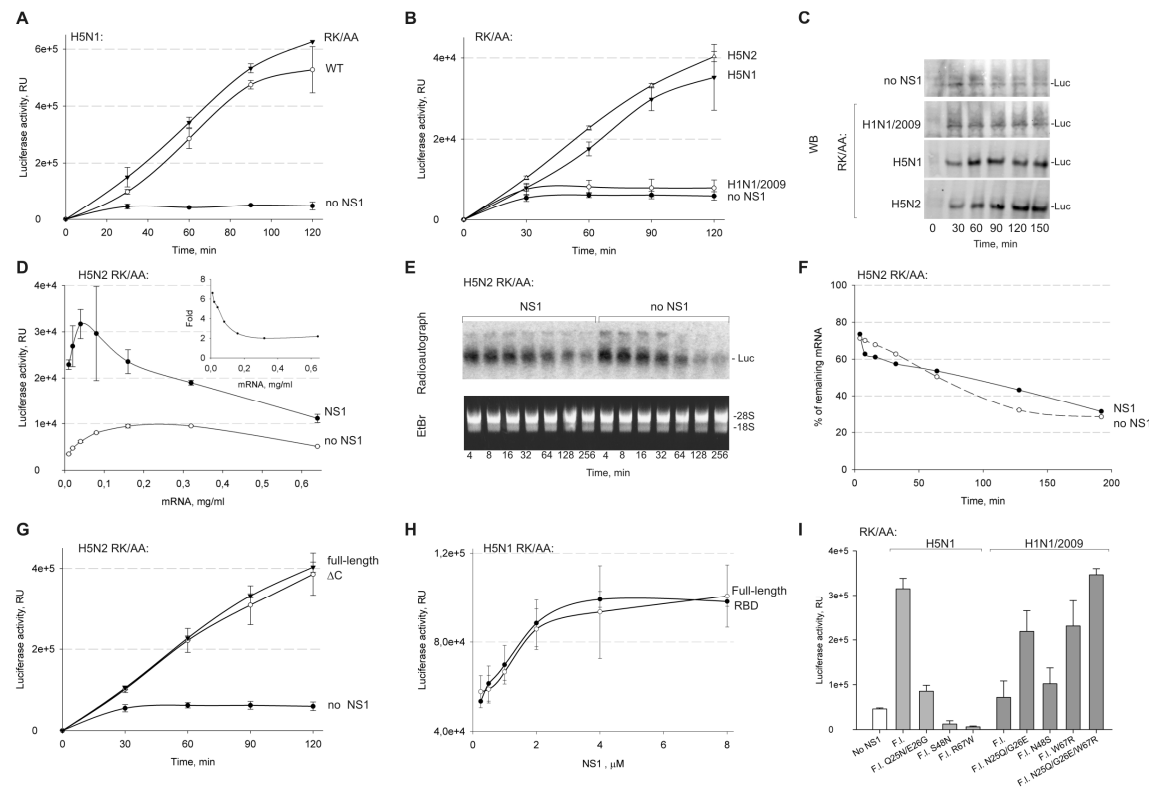


Figure 3.

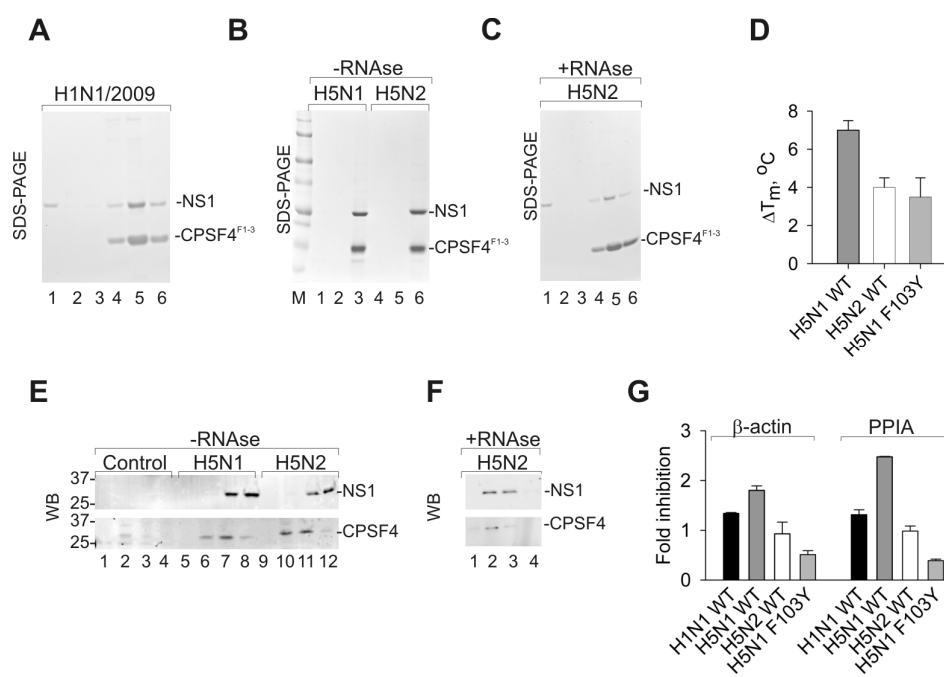
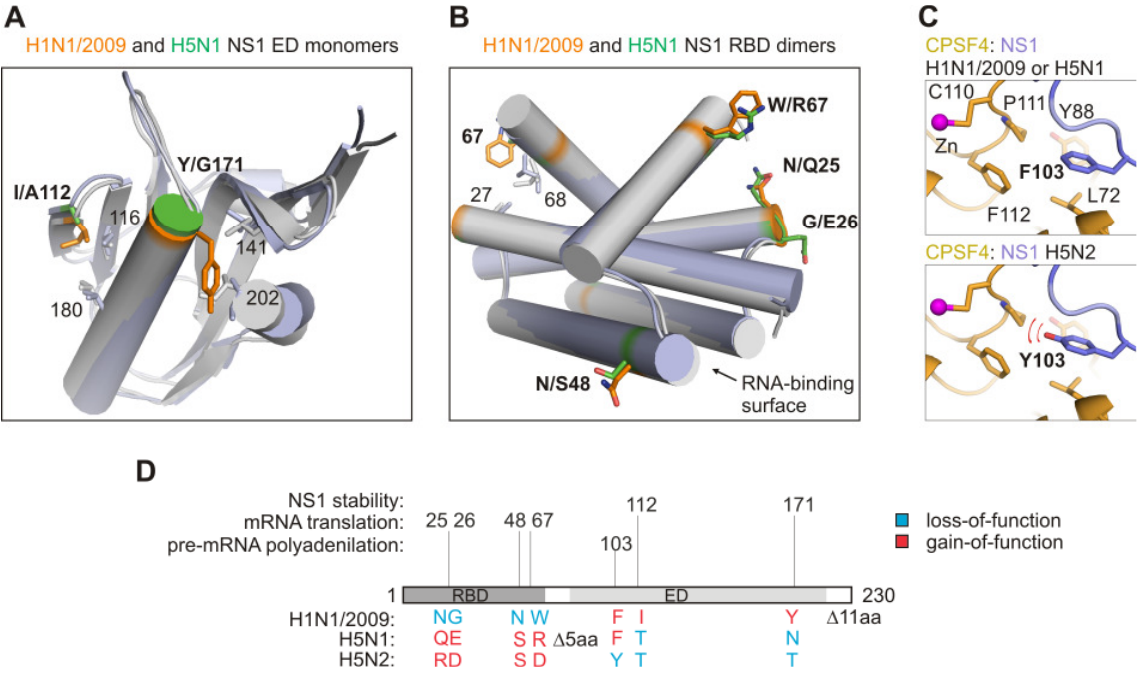


Figure 4.



Differential effects of NS1 proteins of human pandemic H1N1/2009, avian highly pathogenic H5N1 and low pathogenic H5N2 influenza A viruses on cellular pre-mRNA polyadenylation and mRNA translation

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