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Importance of mRNA Secondary Structural Elements for the Expression of Influenza Virus Genes

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Abstract

Development of novel vaccines and therapeutics often requires efficient expression of recombinant viral proteins. Here we show that mutations in essential functional regions of conserved influenza proteins NP and NS1, lead to reduced expression of these genes *in vitro*. According to *in silico* analysis, these mRNA regions possess distinct secondary structures sensitive to mutations. We identified a novel structural feature within a region in NS1 mRNA that encodes amino acids essential for NS1 function. Mutations altering this mRNA element lead to significantly reduced protein expression. Conversely, expression was not affected by mutations resulting in amino acid substitutions, when they were designed to preserve this secondary RNA structural element. Furthermore, altering this structure significantly reduced RNA transcription without affecting mRNA stability. Therefore, distinct internal secondary structures of viral mRNA may be important for viral gene expression. If such elements encode amino acids essential for the protein function, then early selection against mutations in this region will be beneficial for the virus. This might point at yet another mechanism of viral evolution, especially for RNA viruses. Finally, introducing mutations into viral genes while preserving their secondary RNA structure, suggests a new method for the generation of efficiently expressed recombinants of viral proteins.

Introduction

Influenza NP and NS1 proteins are essential for viral replication. NP gene is highly conserved (Gorman et al., 1990; Ito et al., 1991). It possesses two sequences that can serve as nuclear localization signals (NLS), both of which are important for viral replication (Cros et al., 2005; Davey et al., 1985; Neumann et al., 1997; Ozawa et al., 2007). One NLS, residing in the center of the molecule (residues 198–216) is essential for its nuclear accumulation and viral RNA transcription (Ozawa et al., 2007). Another NLS, residing at the N-terminus (residues 3–13) is important for NP nuclear import (Cros et al., 2005; Wu et al., 2007). NP forms oligomers and its oligomerization is mediated by a tail loop within the residues 402–416 (Ye et al., 2006). It has been suggested that targeting essential NP regions (NLSs or oligomerization domains) may be a valuable strategy for the development of novel vaccines or therapeutics (Ohba et al., 2007; Ye et al., 2006).

NS1 is also conserved, and plays a major role in influenza pathogenesis (Lamb and Krug, 2001). Its deletion leads to virus attenuation (Donelan et al., 2003; Quinlivan et al., 2005; Solórzano et al., 2005; Wang et al., 2000, 2002). Vaccination with NS1 encoding vector might be beneficial (Falcón et al., 2005; Zhirnov et al., 2007). NS1 manifests many activities related to immune suppression and transcriptional regulation in infected cells (Chen et al., 1999; Dauber et al., 2006; de la Luna et al., 1995; Donelan et al., 2003; Enami et al., 1994; Fernandez-Sesma et al., 2006; Geiss et al., 2002; Kochs et al., 2007; Nemeroff et al., 1998; Salvatore et al., 2002; Wang et al., 2000; Zhirnov et al., 2002). Thus, development of an inactivated NS1 allele may be important for antiviral therapy (Ge et al., 2004) or vaccine development (Zhirnov et al., 2007).

NS1 possesses two main functional domains, an RNA-binding domain (RBD, amino acids 1–73) and an effector domain (amino acids 74–230), which are well conserved (Lamb and Krug, 2001; Qian et al., 1994). NS1 RBD is sufficient

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to prevent IFN induction (Wang et al., 1999, 2002). Results from several groups suggest that differences in NS1 sequence may at least partially account for increased pathogenicity of avian H5N1 influenza strains (Basler et al., 2001; Geiss et al., 2002) with mutations in NS1 gene being linked to H5N1 increased virulence and/or cytokine resistance (Seo et al., 2002; Zohari et al., 2008).

The influenza genome (eight vRNAs) is packed into the virion as an RNP containing 37–97 copies of NP and a single molecule of viral polymerase subunits PA, PB1, and PB2 (Lamb and Krug, 2001). Interaction of specific sequences located at the 5′- and 3′-termini of vRNA with viral polymerase complex is essential for influenza mRNA transcription (Lee et al., 2002, 2003). Therefore, studies of NP interaction with RNA have focused on various NP domains (Albo et al., 1995; Kobayashi et al., 1994; Mena et al., 1999) while utilizing synthetic mRNA target containing only the termini of vRNA (Elton et al., 1999).

Recent developments raised the possibility that internal features of influenza vRNA or mRNA may be important for viral pathogenesis. It was suggested that a unique single nucleotide substitution in NS1 associated with H5N1 strains had a significant effect on avian influenza virus replication by dramatically affecting the equilibrium between a hairpin and a pseudoknot conformation near the 3′ splice-site of the NS1 mRNA (Gultyaev et al., 2007).

Here we present evidence that elimination or disruption of several internal structural features of influenza NP- and NS1-encoding mRNAs results in a significant decrease of their expression *in vitro*. These regions correspond to amino acid sequences that are essential for NP and NS1 functions. Furthermore, we demonstrate that preservation of a newly identified loop/small hairpin element in the RBD-encoding part of NS1 mRNA is necessary and sufficient for efficient expression of NS1. Elimination or radical modification of this secondary RNA element leads to dramatic suppression of NS1 transcription and reduced mRNA levels, while mutations designed to preserve RNA conformation, carried no consequences for gene transcription and expression. In both cases, the RNA degradation rate was not affected. Viral RNA possessing distinct secondary structures in regions encoding for essential amino acid residues may have an additional evolutionary benefit for the virus because they will select against attenuating mutations at the early transcriptional stage.

Materials and Methods

Cells and plasmids

All experiments were performed using 293 human embryo kidney (HEK) cells. Deletion mutants of NS1 protein based on influenza A/WSN/33 (H1N1) sequence (GenBank sequence accession number J02150) have been described earlier (Ilyinskii et al., 2008b; Zhirnov et al., 2007) and are summarized in Figure 1. The following four novel site-specific mutants of NS1 were constructed: NS1mut3540, NS1 3541, NS1mut3841, and NS1mut3941 (see Results section for detailed description, summarized in Table 2). The full-length gene encoding influenza A/WSN/33 (H1N1) NP protein (GenBank sequence accession number V01084) was described earlier (Ilyinskii et al., 2008a). Deletion mutants NPΔ2–13, NPΔ2–13/198–216, NPΔ402–416, and NPΔ2–13/198–216/402–416 (see Fig. 3 for details) were generated by PCR-mutagenesis and cloned into modified pCAGGS plasmid as described (Ilyinskii et al., 2008a) and their sequence verified. An HA-tag (YPYDVPDYA)-encoding sequence or enhanced green fluorescent protein (EGFP) gene sequence were added at the 3′-terminus of all NP mutant genes to enable their efficient immunological detection. All novel mutated alleles of NS1 contained HA-tag at the 3′-terminus. Upon sequence confirmation, all mutant genes were cloned into the pCAGGS vector. NP–EGFP constructs were engineered as follows. The EGFP sequence was PCR-amplified using the following oligonucleotides: EGFP-start ata*GAATTCGTCGAC*CACCATG GTGAGCAAGGGCGAGG and EGFP–EcoRV tat*GATATC* GAGGTGGATCACAAAGTG (enzyme sites italicized, note Kozak sequence in front of starting ATG), cloned into pGEM-

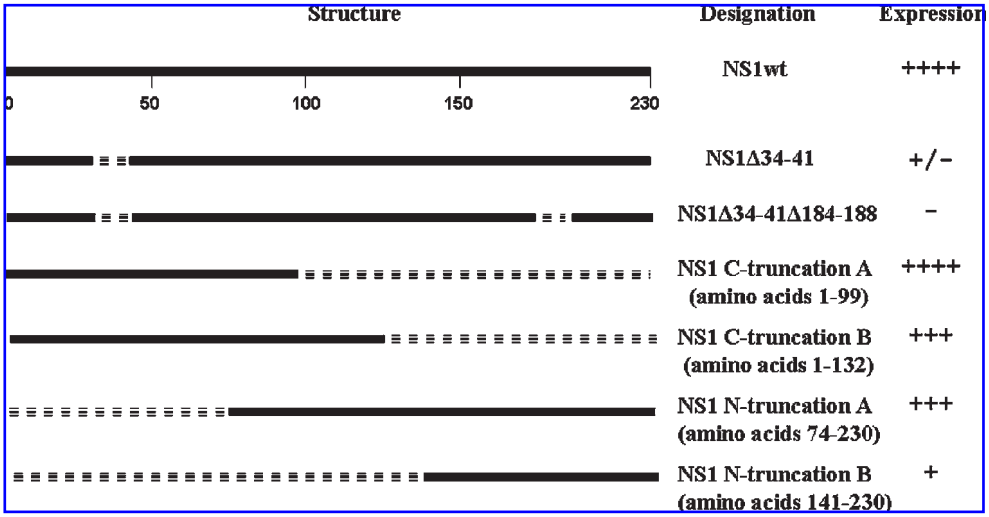

FIG. 1. Structure of earlier described influenza NS1 deletion mutants and their expression *in vitro*. Location of all deletions is shown. Expression of NS1Δ34–41 and NS1 N-truncation B was observed only if excessive amounts of plasmid DNA and/or long-term incubation with proteosomal inhibitor MG132 were used for transfection/protein detection (Ilyinskii et al., 2008a; Zhirnov et al., 2007).

Teasy, which was then sequenced with primers M13F and M13R. The plasmid EGFP–pGEM–Teasy was digested by partial hydrolysis with EcoRI and EcoRV and the EcoRI/EcoRV insertion was cloned between the EcoRI and EcoRV sites into pCAGGS carrying wild-type or mutant NP genes in front of the NP sequence.

Transfection and protein expression

293 HEK cells were transfected at 60–80% confluency in 35-mm plates with Lipofectamine 2000 (Invitrogen, Carsbad, CA) for 4 h or overnight (1.5 μg of total plasmid DNA per 5 μL LF2000 unless otherwise indicated). Control cells were transfected with the same amount of empty vector pCAGGS. Forty hours after the end of transfection, 10 μM emetine was added and samples were taken (2× wash in phosphate buffered saline (PBS), −80°C) at the indicated time points. All constructs were chased for various time periods falling within 0–7 h after emetine addition with or without proteasome inhibitor MG132 (10 μM). Cells were then homogenized on ice in lysis buffer (about 150 μL/35-mm dish) 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% Triton X-100 with protease inhibitors. Samples were adjusted for equal total protein and run on SDS-PAGE followed by immunoblotting with monoclonal anti-GFP antibody (Cell Signaling, Beverly, MA).

RT-PCR

mRNA synthesis was assessed 16 h after transfection with 0.5 μg of respective plasmids carrying wild-type or mutant NS1 alleles. Alternatively, actinomycin D (10 μg/mL) was added at 16 h after transfection and cell samples generated at 0, 4, and 8 h after its addition. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). RNA (1 μg) was subjected to reverse transcription from random hexaprimers using First-strand cDNA synthesis kit (Invitrogen). NS1sequences were amplified by specific primers using SYBR Green Mix (Applied Biosystems, Foster City, CA), and analyzed by Comparative Ct method using ribosomal protein L32 mRNA as a standard.

RNA structure modeling

The following software was used to assess the secondary structure of NS1 mRNA: mfold 3.2. (Zuker, 2003), RNAz and HotKnots (Ren et al., 2005). The Web version of mfold at http://mfold.bioinfo.rpi.edu/, with default parameters was used. Mfold predictions were verified by RNAfold of the Vienna RNA Structure tool set (Ren et al., 2005). Because neither RNAfold nor mFold can depict pseudoknots, JViz was used for their plotting.

Results

Deletion of NS1 amino acids 34–41 leads to suppression of NS1 expression in vitro

In our earlier studies we attempted to construct a series of NS1 mutants via targeted deletions or point-mutations (summarized in Fig. 1). NS1 mutants that are not capable of binding dsRNA were described in detail by several groups (Donelan et al., 2003; Wang et al., 1999). Reasoning that the deletion of this region will lead to NS1 inactivation, we designed and constructed two NS1 mutants, NS1Δ34–41 and NS1Δ34–41Δ184–188 in which amino acid region 34–41 was eliminated. The second of these mutants carried an additional small deletion in the effector domain, removing amino acids known to be essential for NS1 interaction with 30-kDa subunit of CPSF, a cellular factor required for the 3′-end processing of pre-mRNAs (Li et al., 2001; Noah et al., 2003). The expression of both was manifestly suppressed (Zhirnov et al., 2007). Additionally, a number of NS1 deletion mutants constructed at the same time demonstrated differing degrees of expression (Ilyinskii et al., 2008a). NS1 mutants were fused to peptide tags and antibodies to these tags were used for NS1 detection (Ilyinskii et al., 2008a). The patterns of expression and the schematic structure of NS1 mutants constructed prior to the current study are summarized in Figure 1 and Table 1. Collectively, three out of four NS1 mutants carrying a deletion of the 34–41 amino acid region had severe expression deficiencies, while two NS1 mutants that had the RBD region intact, were expressed as well as NS1wt (Ilyinskii et al., 2008a; Zhirnov et al., 2007). This raised the question of whether a particular feature of NS1 mRNA may be responsible for such a phenomenon.

mRNA structure of influenza NS1. The sequence of NS1 gene is 693 bp and the predicted secondary structure of its mRNA by mfold is presented in Figure 2. Upon its analysis, a particular structural feature was observed in the predicted NS1wt mRNA secondary structure (Fig. 2A and B), but is absent in NS1del34–41 mutant (Fig. 2C). This segment is at the top of a longer stem consisting of a loop and a small hairpin. In the mutant NS1del34–41 this "head" is replaced by a common loop.

The length of RNA is related to the accuracy of predictions of its secondary structure and evaluations by mfold showed

Table 1. NS1 Mutants and Their Expression *In Vitro*

Sequence	*Expression* in vitro
NS1wild-type	Expressed, results in stable protein
NS1Δ34–41 (amino acids 34–41 deleted)	Poorly expressed, 50–100 times <NS1wt
NS1Δ34–41Δ184–188 (deletion of amino acids 34–41 and 184–188)	Not expressed.
NS1-C-truncation A, amino acids 1–99	Expressed, results in stable protein.
NS1-C-truncation B, amino acids 1–132	Expressed, results in partially instable protein (slow degradation).
NS1-N-truncation A, amino acids 74–230	Expressed, results in stable protein.
NS1-N-truncation B, amino acids 141–230	Unstable protein, rapidly degraded.

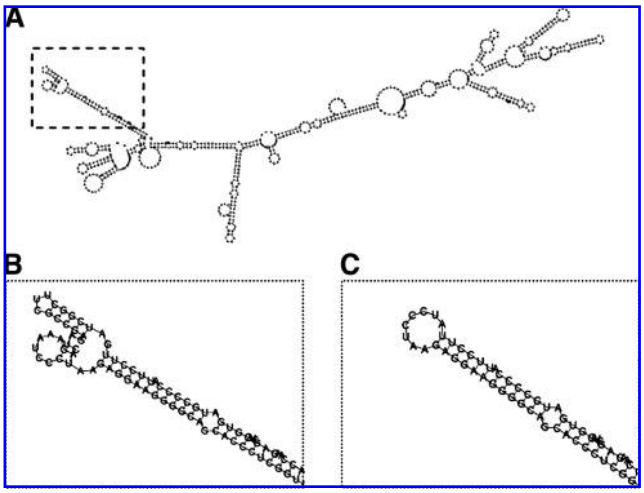

FIG. 2. Structure of NS1-encoding mRNA. Full (**A**) and partial (**B**, **C**; corresponding to the segment inside the dashed rectangle in **A**) NS1 mRNA structures (as predicted by mfold) are shown. Structures are for NS1wt (**A**, **B**) and NS1Δ34–41 (**C**). A specific hairpin/loop structural element present in NS1wt and absent in NS1Δ34–41 is shown by dotted rectangle in **B** and **C**, respectively.

that its prediction accuracy is sufficient for the shorter sequences (Wiese and Hendriks, 2006). We further verified our results by using a smaller part of NS1 nucleotide sequence, from its 5′-end until the end of a putative stem. Because mRNA starts to fold during transcription, it is likely that this shortened sequence will fold as predicted (Pan and Sosnick, 2006). The secondary structure prediction of such a shortened NS1 mRNA sequence while constraining the base pairing (ordering mfold to pair nucleotides 36–40 with 185–189, predicted to be paired in the whole structure) has given the same result identifying "loop/small hairpin" domain formed by nucleotides 99–131.

Expression of novel NP deletion mutants in vitro

Four mutants of influenza NP bearing deletions either of unconventional (NPΔ2–13) or of both NLS (NPΔ2–13Δ198–216), of oligomerization domain (NPΔ402–416) or in all of these three regions (NPΔ2–13Δ198–216Δ402–416) were constructed (Fig. 3A) and their *in vitro* expression was assessed as described (Ilyinskii 2008a, 2008b). Notably, all of these mutations were introduced into known functionally important regions of NP. The overall expression levels of all novel NP mutants were significantly lower compared to wild-type NP with NPΔ2–13 (30% from NPwt) and NPΔ402–416 (10% from NPwt) being less affected than other mutants that bore more profound sequence changes (Fig. 3A and B). While protein levels of most profoundly affected mutants NPΔ2–13Δ198–216 and NPΔ2–13Δ198–216Δ402–416 were somewhat restored by proteosome inhibitor, MG132, they remained far below the levels of NPwt (Fig. 3C). No increase of NPΔ2–13 or NPΔ402–416 levels was detected upon incubation with MG132, these remaining 3- and 10-fold less than NPwt. This demonstrates that the lower protein expression level is not due to increased proteosome-dependent degradation of the mutants.

mRNA structure of influenza NP

In the set of representative NP sequences (retrieved from the FLU database, http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) three structurally conserved and thermodynamically stable RNA secondary structures were found at the nucleotide positions 0–120 and 240–360 by RNAz. Two of these structures reside in the 5′-terminal 1–120 nucleotides and the third one in the region between 240–360 nucleotides. The first two regions were affected in NP NLS mutants NPΔ2–13, NPΔ2–13Δ198–216 and NPΔ2–13Δ198–216Δ402–416, while the third was affected in NPΔ402–416 and NPΔ2–13Δ198–216Δ402–416 (Fig. 4A, B, C, and E). While NPΔ2–13 had only the first conserved mRNA region affected, it also had a higher level of protein expression among NP mutants. Most interestingly, mRNA of NPΔ402–416 (and therefore, of NPΔ2–13Δ198–216Δ402–416) was predicted to miss "side arms" present in NPwt mRNA (Fig. 4D). This deletion partly overlapped the second conserved RNA motif in 240–360 bp. This short deletion per se resulted in a manifest decrease of protein expression (≥20-fold, see Fig. 3B and C). Similarly mRNA of NPΔ2–13Δ198–216 was missing another distinct structure present in NPwt and NPΔ2–13, and expression of corresponding protein was manifestly suppressed as well (∼20-fold). Similar to NS1, this lead us to analyze NP mRNA structure.

Design of site-specific NS1 mutants with and without changes in novel loop/hairpin structure

It seemed plausible from our data at this point that decreased *in vitro* expression levels of mutant proteins resulted from intervention into secondary structures of NS1 and NP mRNAs that were, in turn, encoding functionally important protein regions. To test this hypothesis we selected newly described secondary structure of NS1 mRNA (Fig. 5A). We reasoned that if this novel mRNA structural motif is important for NS1 expression, then mutations preserving this secondary RNA structure will not lead to NS1 expression reduction, while, conversely, its specific disruption will be detrimental for expression of NS1 mutants. Therefore, we designed four site-specific NS1 mutants that carry changes in this region (Table 2), that, according to our analysis, should result in no, little, or profound changes in the structure of this loop/hairpin structure in the 99–131-bp region (Fig. 5), while affecting or not affecting the amino acid sequence of the encoded region. Specifically, two mutants, NS1mut3541 and NS1mut3941, bore several amino acid changes while not affecting the putative hairpin/loop (Fig. 5B and C), another mutant NS1mut3540 had no amino acid changes and reoriented hairpin (no effect on the loop) and yet another mutant NS1mut3841 bore just two amino acid changes, but its secondary RNA structure was predicted to be completely perturbed resulting in pseudoknot formation (Fig. 5D).

Protein expression and transcription efficiency of novel NS1 mutants

Three of the four novel NS1 mutants were highly expressed *in vitro* similar or comparable to NS1wt, with NS1mut3541 being slightly less expressed than NS1mut3540 and NS1mut3941 (Fig. 6). At the same time, repeated attempts to detect any NS1mut3841 protein (the coding sequence of

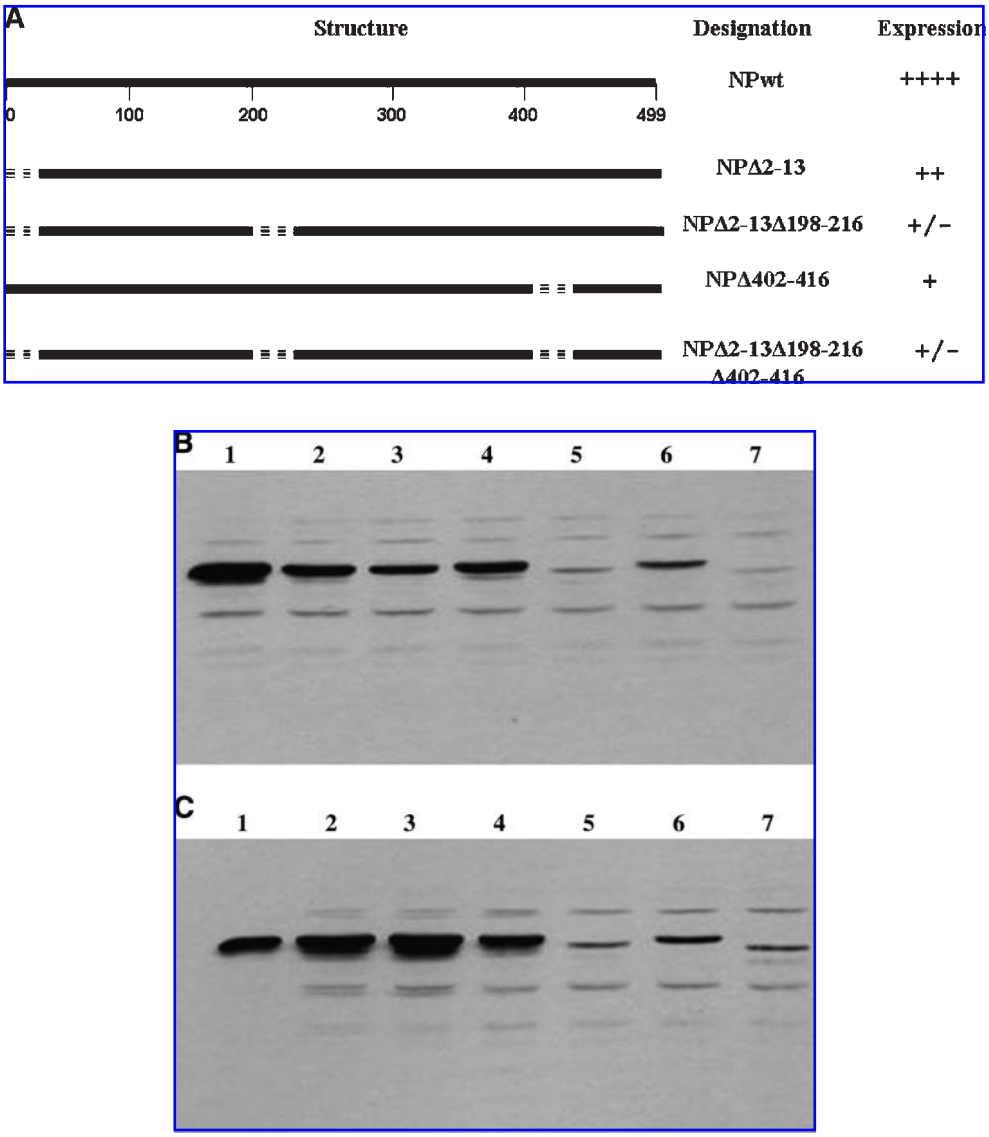

FIG. 3. Structure of novel NP deletion mutants and their expression *in vitro*. Location of all deletions is shown **A**. (**B**, **C**) Their expression level and metabolic stability. Lanes 1–3—NPwt, lane 4—NPΔ2–13, lane 5—NPΔ2–13Δ198–216, lane 6—NPΔ402–416, lane 7—NPΔ2–13Δ198–216Δ402–416. Amounts of DNA used for transfection of cells in lanes 2 and 3 were 1/3 and 1/9, correspondingly, from those in lanes 1 and 4–7 (**B**); amount of DNA in lane 1 was 1/3 from those in lanes 2–7 (**C**). Cells in lanes 3–7 were treated with 10 μM of MG132 for 6.5 h (**C**). Experiments in **B** and **C** were run in parallel. Expression of each protein was assessed in three independent experiments, which all gave the same result. Representative data is shown.

which has been rechecked and verified) failed, including utilization of excessive amounts of DNA for transfection (Fig. 6, lane 13) as well as Western blot overexposure (not shown).

When the levels of mRNA transcribed from recombinant plasmids encoding novel NS1 point mutants was tested by RT-PCR upon transient transfection, it was apparent that all mutant-expressing plasmids with the exception of NS1mut3841 produce similar levels of NS1-specific mRNA, which were not more than three-fold different than of NS1wt (Fig. 7). At the same time the level of NS1mut3841 mRNA at 16 h posttransfection was ~30-fold lower. Moreover, this reduction in RNA level was not due to enhanced mRNA degradation because treatment of transfected cells with actinomycin D, which significantly suppressed synthesis of control NS1wt mRNA and also of NS1mut3941 (Fig. 8), did not further reduce the level of NS1mut3841 mRNA.

Discussion

It is well known that RNA secondary structures of many RNA viruses are important for translation, replication, encapsidation, and binding *trans*-acting protein factors. RNA secondary structures may also become part of higher order tertiary RNA structures, which themselves may be functional (Davis et al., 2008; Simmonds et al., 2004). Furthermore, in some viral genera, notably influenza, hepatitis C and G viruses (HCV and HGV), there is both computational and experimental evidence for the existence of functional RNA

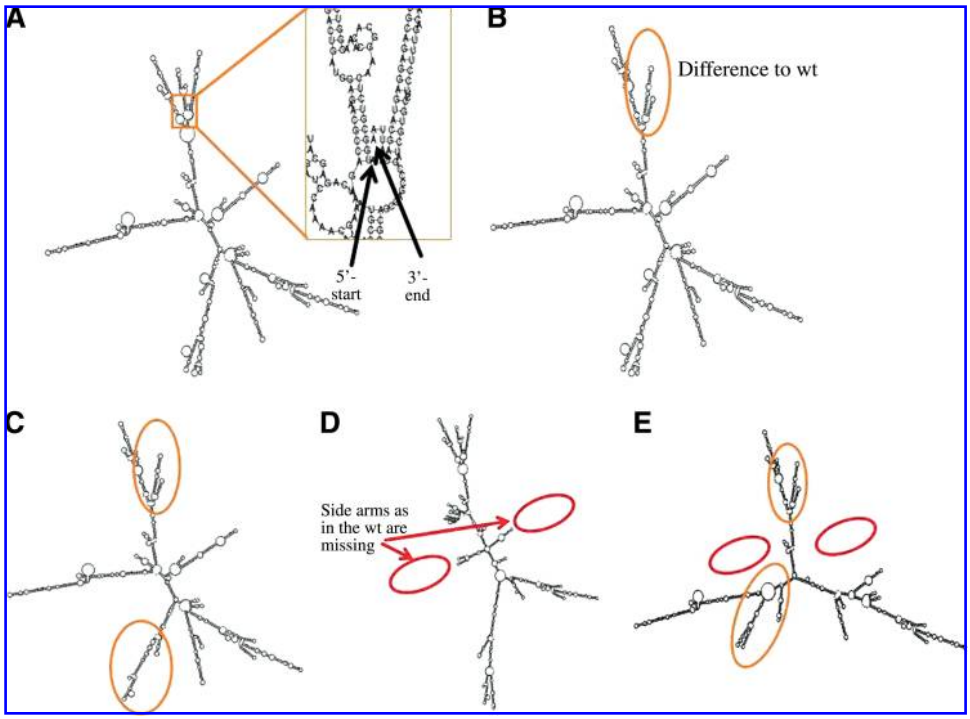

FIG. 4. Structure of NP-encoding mRNA as predicted by mfold. Results for NPwt (**A**), NPΔ2–13 (**B**), NPΔ2–13Δ198–216 (**C**), NPΔ402–416 (**D**) and NPΔ2–13Δ198–216Δ402–416 (**E**) are shown. The 5′- and 3′-termini are indicated by light brown rectangle (**A**) Changes as related to NPwt mRNA structure are shown by light brown ovals (**B**, **C**, **E**), missing structures are shown by red ovals (**D, E**).

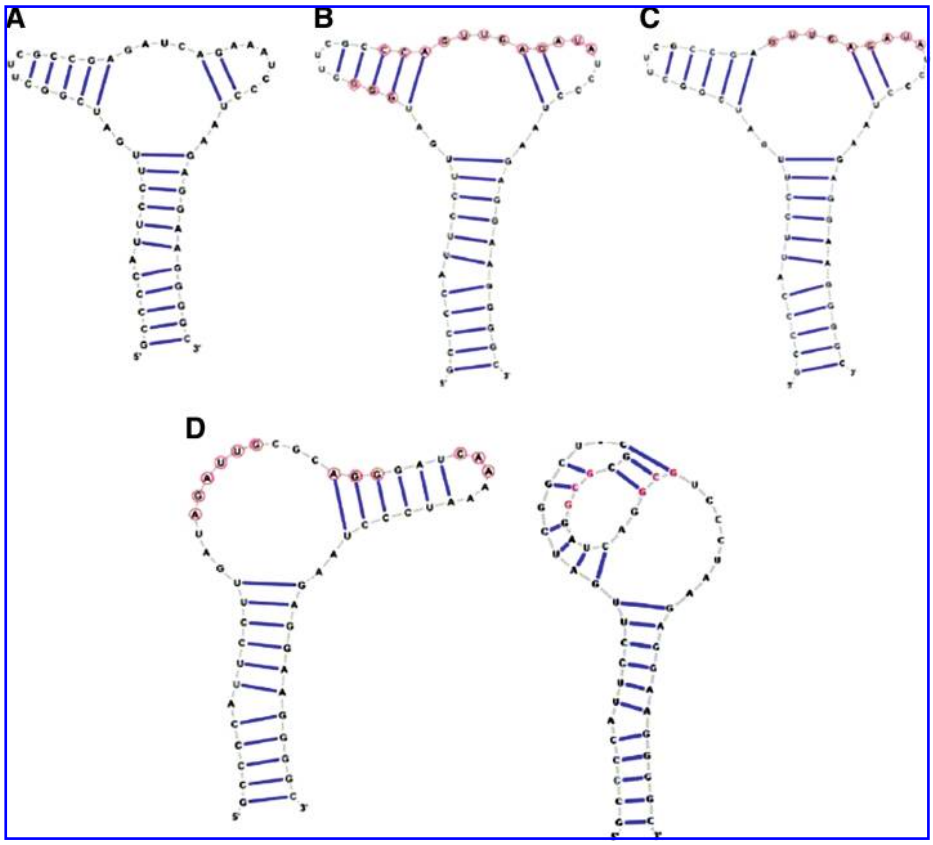

FIG. 5. Predicted structure of hairpin/loop region in mRNA of NS1wt (**A**), NS1mut3541 (**B**), NS1mut3941 (**C**), NS1mut3540 (**D**), and NS1mut3841 (**E**). Changed nucleotides are marked in red. Depicted are the nucleotides 88 to 141 (inclusive).

Table 2. New NS1 Mutants With Changes in Predicted Loop/Hairpin Structure

Mutant	*Sequence*												*Description*
	31	32	33	34	35	36	37	38	39	40	41	42	Wild-type Fig. 5A
NS1wt	CCA	TTC	CTT	GAT	CGG	CTT	CGC	CGA	GAT	CAG	AAA	TCC	
	P	F	L	D	R	L	R	R	D	Q	K	S	
	31	32	33	34	35	36	37	38	39	40	41	42	Loop preserved, hairpin
NS1mut3540	CCA	TTC	CTT	GAT	**AGA**	**TTG**	CGC	**AGG**	GAT	**CAA**	AAA	TCC	reversed amino acid
	P	F	L	D	R	L	R	R	D	Q	K	S	sequence preserved Fig. 5D
	31	32	33	34	**35**	36	37	**38**	**39**	**40**	**41**	42	Loop/hairpin preserved
NS1mut3541	CCA	TTC	CTT	GAT	**GGG**	CTT	CGC	**CCA**	**GTT**	**GAG**	**ATA**	TCC	amino acid sequence
	P	F	L	D	**G**	L	R	**P**	**V**	**E**	**I**	S	changed Fig. 5B
	31	32	33	34	35	36	37	**38**	39	40	**41**	42	Loop/hairpin and amino acid
NS1mut3841	CCA	TTC	CTT	GAT	CGG	CTT	CGC	**GCG**	GAT	CAG	**GCG**	TCC	sequence changed Fig. 5E
	P	F	L	D	R	L	R	**A**	D	Q	**A**	S	
	31	32	33	34	35	36	37	38	**39**	**40**	**41**	42	Loop/hairpin preserved,
NS1mut3941	CCA	TTC	CTT	GAT	CGG	CTT	CGC	CGA	**GTT**	**GAG**	**ATA**	TCC	amino acid sequence
	P	F	L	D	G	L	R	P	**V**	**E**	**I**	S	changed Fig. 5C

structures present within protein-coding regions (Davis et al., 2008; Gultyaev et al., 2007; McMullan et al., 2007; Simmonds et al., 2004; Tuplin et al., 2002, 2004; You et al., 2004), and it was suggested that the specific configurations of viral genomic RNAs may affect their detection by host cell defense mechanisms similar to structured RNA transcripts expressed by large DNA viruses (Davis et al., 2008). Our data demonstrates that distinct RNA secondary structures located in the regions encoding functionally essential amino acids may also be necessary for the efficient transcription and evolutionary conservation of viral genes.

For both NP and NS1 the deletion of known essential regions, such as NLSs and oligomerization domain of NP and RBD domain of NS1, results in a manifest expression defect in a recombinant system. Computational analysis indicates that mRNA regions encoding these essential protein domains possess secondary RNA structures that are disrupted by mutations leading to reduced protein expression. This observation may link two together.

Furthermore, we report here a novel loop/hairpin RNA structure in the RBD-encoding region of NS1 RNA. According to the results presented herein, the integrity of this novel loop/hairpin structure is imperative for the efficient expression of recombinant NS1. Those NS1 mutations that were designed to preserve its secondary structure conformation did not diminish NS1 expression, even if they induced amino acid changes in this region, which is essential for NS1 function *in vivo*. In contrast, changes that destroyed this mRNA structural feature were detrimental for NS1 expression. Such mutations resulted in reduced transcription levels of NS1 mRNA but did not affect its degradation rate.

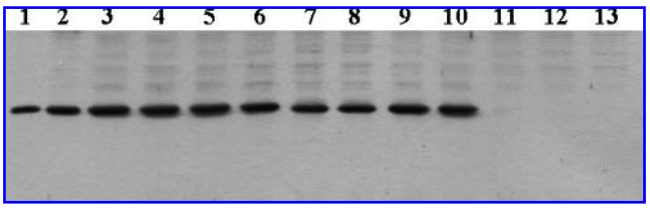

FIG. 6. Protein expression of NS1 mutants with changes in hairpin/loop structure. Lanes 1–4—NS1wt, lanes 5, 6—NS1mut3540, lanes 7, 8—NS1mut3541, lanes 9, 10—NS1mut3941, lanes 11–13—NS1mut3841. DNA amount used for transfection of cells in lane 13 is four-fold greater than in lanes 1–12, amounts of protein loaded in lanes 1 and 2 is 1/4 and 1/2, respectively, of amounts loaded in lanes 3–13. Experiments shown in lanes 3–13 were run in parallel. Expression of each protein was assessed in three independent experiments, which all gave the same result. Representative data is shown.

One question that may be asked is why the reduced expression has not been reported by those groups that have tested deletion mutants of NS1 and NP, undertaking detailed studies of NS1 capabilities for RNA binding or NP oligomerization (Li et al., 2001; Wang et al., 1999; Ye et al., 2006)? Noteworthy, when CPSF-binding site in NS1 was mutated leading to severe virus attenuation, the levels of mutant NS1 expression by the virus were not assessed (Noah et al., 2003). Also, it is likely that the employment of bacterially synthesized NS1 mutants coupled with GST tags used in those studies had avoided NS1 production deficiencies that became apparent in the eukaryotic system that we employed. Similarly, the effects of several known deletions on the expression of influenza NP have likely been underappreciated for the same reason (Ye et al., 2006).

Based on our data, we hypothesize that reduced transcription of those genes of RNA viruses that carry mutations in secondary RNA structures encoding essential protein amino acids is a yet another mechanism of viral evolution and conservation of essential protein domains via negative selection. We suggest that RNA of influenza and other RNA viruses contain essential secondary structural elements in the regions encoding functionally important elements of viral proteins. If such structures are disrupted due to mutations, gene transcription is significantly reduced. Thus, many mutations in these regions are effectively eliminated.

Such evolutionary mechanism should be beneficial for the virus. It will select against mutations in the essential regions of the protein early in viral replication cycle preventing the formation of the defective proteins. This will explain why mutagenic interventions into those regions of viral proteins that are essential for viral propagation significantly decrease the production and structural integrity of NP and NS1.

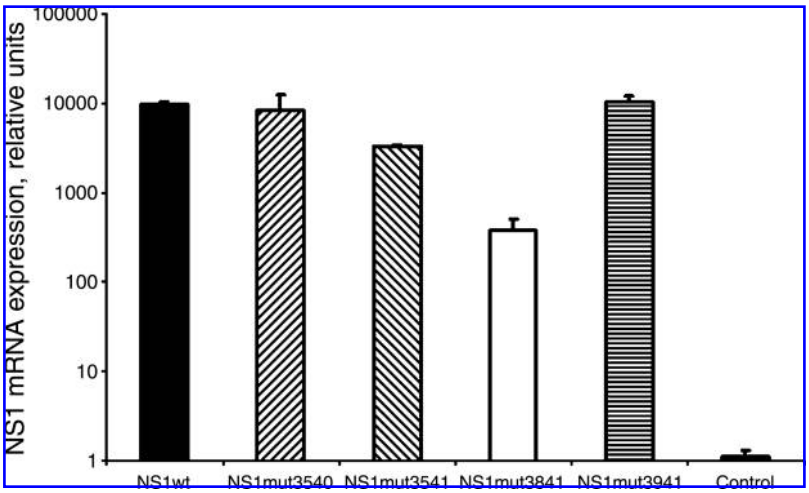

FIG. 7. Levels of NS1-specific mRNA as detected by RT-PCR upon transient transfection of HEK 293 cells. Experiment was done as described in Materials and Methods.

Additionally, the well-known evolutionary conservation of NP and NS1 sequences may be a consequence of necessity to maintain both protein and specific RNA structures. Interestingly, it was demonstrated that the nonpairing parts of RNA structures in the HCV (loop and bulge regions) evolve according to a selective process different from that of nonpairing nucleotides outside the structural regions (Pedersen et al., 2004a), thus supporting our hypothesis.

This hypothesis may be further tested in other viral models by comparing the relative presence and location of distinct secondary RNA structures within conserved and frequently mutating viral genes and their respective regions. For example, are secondary RNA structures equally frequent in conserved influenza genes (e.g. NP and NS1) as well as in the genes coding for hemagglutinin and neuraminidase? Furthermore, similar mechanism that immediately suppresses the synthesis of defective RNA molecules may also function during viral replication, thus selecting against those vRNA molecules that bear mutations in the essential regions of viral proteins. This possibility may be as well tested experimentally.

A specific mechanistic explanation of the observed phenomena remains to be deciphered. Secondary RNA structural features may bind to transcription factors. Alternatively, perturbations in secondary RNA structures may lead to reduced transcription levels due to the distorted thermodynamic stability of RNA. It also remains to be elucidated if newly synthesized RNAs interact with their templates differently depending on the presence or absence of the secondary structures within the transcripts, and if this contributes to a difference in transcription rates.

Based on our data, we propose a new method for the introduction of mutations into recombinant proteins and genes derived from RNA viruses. Apparently, it is necessary to generate and select those mutations that according to *in silico* analysis mimic the secondary structure of wild type mRNA. This method may be especially useful to design recombinant vaccines, where a viral gene has not only to be highly expressed, but also needs to be inactivated to alleviate safety concerns. Interestingly, several methods for identification of such RNA secondary structures within protein-coding regions have been proposed (Knudsen and Hein, 2003; Pedersen et al., 2004a, 2004b; Tuplin et al., 2002).

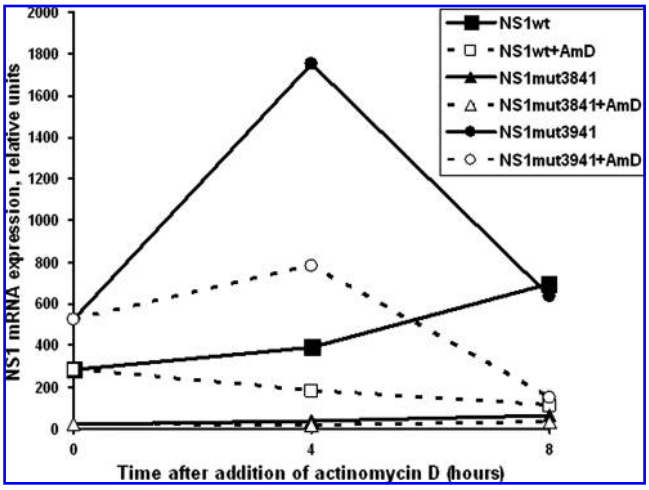

FIG. 8. Suppression of NS1mut3841 mRNA expression is not dependent on mRNA degradation, but results from inhibition of its synthesis *de novo*. Actinomycin D was added to the samples indicated at 16 h after transfection.

Author Disclosure Statement

While performing experiments described in this paper, D. Ilyinskii, G. Thoidis, and A. Shneider have been paid employees of Cure Lab, Inc.

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References

Albó, C., Valencia, A., and Portela, A. (1995). Identification of an RNA binding region within the N-terminal third of the influenza A virus nucleoprotein. J Virol 69, 3799–3806.

Basler, C.F., Reid, A.H., Dybing, J.K., Janczewski, T.A., Fanning, T.G., Zheng, H, et al. (2001). Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. Proc Natl Acad Sci USA 98, 2746–2751.

Chen, Z., Li, Y., and Krug, R.M. (1999). Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. EMBO J 18, 2273–2283.

Cros, J.F., Garcia-Sastre, A., and Palese, P. (2005). An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein. Traffic 6, 205–213.

Dauber, B., Schneider, J., and Wolff, T. (2006). Double-stranded RNA binding of influenza B virus nonstructural NS1 protein inhibits protein kinase R but is not essential to antagonize production of alpha/beta interferon. J Virol 80, 11667–11677.

Davey, J., Dimmock, N.J., and Colman, A. (1985). Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in Xenopus oocytes. Cell 40, 667–675.

Davis, M., Sagan, S.M., Pezacki, J.P., Evans, D.J., and Simmonds, P. (2008). Bioinformatic and physical characterizations of genome-scale ordered RNA structure in mammalian RNA viruses. J Virol 82, 11824–11836.

de la Luna, S., Fortes, P., Beloso, A., and Ortin, J. (1995). Influenza virus NS1 protein enhances the rate of translation initiation of viral mRNAs. J Virol 69, 2427–2433.

Donelan, N., Basler, C.F., and García-Sastre, A. (2003). A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. J Virol 77, 13257–13266.

Elton, D., Medcalf, L., Bishop, K., Harrison, D., and Digard P. (1999). Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding. J Virol 73, 7357–7367.

Enami, K., Sato, T.A., Nakada, S., and Enami, M. (1994). Influenza virus NS1 protein stimulates translation of the M1 protein. J Virol 68, 1432–1437.

Falcón, A.M., Fernandez-Sesma, A., Nakaya, Y, Moran, T.M., Ortín, J., and García-Sastre, A. (2005). Attenuation and immunogenicity in mice of temperature-sensitive influenza viruses expressing truncated NS1 proteins. J Gen Virol 86, 2817–2821.

Fernandez-Sesma, A., Marukian, S., Ebersole, B.J., Kaminski, D., Park, M.S., Yuen, T., et al. (2006). Influenza virus evades innate and adaptive immunity via the NS1 protein. J Virol 80, 6295–6304.

Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N., and Chen, J. (2004). Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc Natl Acad Sci USA 101, 8676–8681.

Geiss, G., Salvatore, M., Tumpey, T.M., Carter, V.S., Wang, X., Basler, C.F., et al. (2002). Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc Natl Acad Sci USA 99, 10736–10741.

Gorman, O., Bean, W.J., Kawaoka, Y., and Webster, R.G. (1990). Evolution of the nucleoprotein gene of influenza A virus. J Virol 64, 1487–1497.

Gultyaev, A.P., Heus H.A., and Olsthoorn, R.C. (2007). An RNA conformational shift in recent H5N1 influenza A viruses. Bioinformatics 23, 272–276.

Ilyinskii, P.O., Meriin, A.B., Gabai, V.L., Usachev, E.V., Prilipov, A.G., Thoidis, G., et al. (2008a). The proteosomal degradation of fusion proteins cannot be predicted from the proteosome susceptibility of their individual components. Protein Sci 17, 1077–1085.

Ilyinskii, P.O., Meriin, A.B., Gabai, V.L., Zhirnov, O.P., Thoidis, G., and Shneider, A.M (2008b). Prime-boost vaccination with a combination of proteosome-degradable and wild-type forms of two influenza proteins leads to augmented CTL response. Vaccine 26, 2177–2185.

Ito, T., Gorman, O.T., Kawaoka, Y., Bean, W.J., and Webster, R.G. (1991). Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J Virol 65, 5491–5498.

Knudsen, B., and Hein, J. (2003). Pfold: RNA secondary structure prediction using stochastic context-free grammars. Nucleic Acids Res 31, 3423–3428.

Kobayashi, M., Toyoda, T., Adyshev, D.M., Azuma, Y., and Ishihama, A. (1994). Molecular dissection of influenza virus nucleoprotein: deletion mapping of the RNA binding domain. J Virol 68, 8433–8436.

Kochs, G., Garcia-Sastre, A., and Martinez-Sobrido, L. (2007). Multiple anti-interferon actions of the influenza A virus NS1 protein. J Virol 81, 7011–7021.

Lamb, R., and Krug, RM. (2001). Orthomyxovirida: the viruses and their replication. In *Fields Virology*. 4th ed. P. Howley and D.M. Knipe, eds. (Lippincott, Williams & Wilkins, Philadelphia, PA), pp. 1487–1532.

Lee, M.T., Bishop, K., Medcalf, L., Elton, D., Digard, P., and Tiley, L. (2002). Definition of the minimal viral components required for the initiation of unprimed RNA synthesis by influenza virus RNA polymerase. Nucleic Acids Res 30, 1392–1397.

Lee, M.T., Klumpp, K., Digard, P., and Tiley, L. (2003). Activation of influenza virus RNA polymerase by the 5′ and 3′ terminal duplex of genomic RNA. Nucleic Acids Res 31, 1624–1632.

Li, Y., Chen, Z.Y., Wang, W., Baker, C.C., and Krug, R.M. (2001). The 3′-end-processing factor CPSF is required for the splicing of single-intron pre-mRNAs in vivo. RNA 7, 920–931.

McMullan, L.K., Grakoui, A., Evans M.J., Mihalik K., Puig M., Branch A.D., et al. (2007). Evidence for a functional RNA element in the hepatitis C virus core gene. Proc Natl Acad Sci USA 104, 2879–2884.

Mena, I., Jambrina, E., Albo, C., Perales, B., Ortin J., Arrese, M., et al. (1999). Mutational analysis of influenza A virus nucleoprotein: identification of mutations that affect RNA replication. J Virol 73, 1186–1194.

Nemeroff, M.E., Barabino, S.M., Li, Y., Keller, W., and Krug, R.M. (1998). Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3′end formation of cellular pre-mRNAs. Mol Cell 1, 991–1000.

Neumann, G., Castrucci, M.R., and Kawaoka, Y. (1997). Nuclear import and export of influenza virus nucleoprotein. J Virol 71, 9690–9700.

Noah, D.L., Twu, K.Y, and Krug, R.M. (2003). Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3′ end processing of cellular pre-mRNAS. Virology 307, 386–395.

Ohba, K., Yoshida, S., Zahidunnabi, D.M., Shimura, H., Sakamaki, N., Takeshita, F., et al. (2007). Mutant influenza A virus nucleoprotein is preferentially localized in the cytoplasm and its immunization in mice shows higher immunogenicity and cross-reactivity. Vaccine 25, 4291–4300.

Ozawa, M., Fujii, K., Muramoto, Y., Yamada, S., Yamayoshi, S., Takada, A., et al. (2007). Contributions of two nuclear localization signals of influenza A virus nucleoprotein to viral replication. J Virol 81, 30–41.

Pan, T. and Sosnick, T. (2006). RNA folding during transcription. Annu Rev Biophys Biomol Struct 35, 161–175.

Pedersen, J.S., Forsberg, R., Meyer, I.M., and Hein, J. (2004a). An evolutionary model for protein-coding regions with conserved RNA structure. Mol Biol Evol 21, 1913–1922.

Pedersen, J.S., Meyer, I.M., Forsberg, R., Simmonds, P., and Hein, J. (2004b). A comparative method for finding and folding RNA secondary structures within protein-coding regions. Nucleic Acids Res 32, 4925–4936.

Qian, X.Y., Alonso-Caplen, F., and Krug, R.M. (1994). Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. J Virol 68, 2433–2441.

Quinlivan, M., Zamarin, D., Garcia-Sastre, A., Cullinane, A., Chambers, T., and Palese, P. (2005). Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79, 8431–8439.

Ren, J., Rastegari, B., Condon, A., and Hoos, H.H. (2005). HotKnots: heuristic prediction of RNA secondary structures including pseudoknots. RNA 11, 1494–1504.

Salvatore, M., Basler, C.F., Parisien, J.P., Horvath, C.M., Bourmakina, S., Zheng, H., et al. (2002). Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis. J Virol 76, 1206–1212.

Seo, S.H., Hoffmann, E., and Webster, R.G. (2002). Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 8, 950–954.

Simmonds, P., Tuplin, A., and Evans, D.J. (2004). Detection of genome-scale ordered RNA structure (GORS) in genomes of positive-stranded RNA viruses: implications for virus evolution and host persistence. RNA 10, 1337–1351.

Solórzano, A., Webby, R.J., Lager, K.M., Janke, B.H., García-Sastre, A., and Richt, J.A. (2005). Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. J Virol 79, 7535–7543.

Tuplin, A., Wood, J., Evans, D.J., Patel, A.H., and Simmonds, P. (2002). Thermodynamic and phylogenetic prediction of RNA secondary structures in the coding region of hepatitis C virus. RNA 8, 824–841.

Tuplin, A., Evans, D.J., and Simmonds, P. (2004). Detailed mapping of RNA secondary structures in core and NS5B-encoding region sequences of hepatitis C virus by RNase cleavage and novel bioinformatic prediction methods. J Gen Virol 85, 3037–3047.

Wang, W., Riedel, K., Lynch, P., Chien, C.Y., Montelione, G.T., and Krug, R.M. (1999). RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA 5, 195–205.

Wang, X., Li, M., Zheng, H., Muster, T., Palese, P., Beg, A.A., et al. (2000). Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J Virol 74, 11566–11573.

Wang, X., Basler, C.F., Williams, B.R.G., Silverman, R.H., Palese, P., and García-Sastre, A. (2002). Functional replacement of the carboxy-terminal two-thirds of the influenza A virus NS1 protein with short heterologous dimerization domains. J Virol 76, 12951–12962.

Wiese, K.C., and Hendriks, A. (2006). Comparison of P-Rna-Predict and mfold—algorithms for RNA secondary structure prediction. Bioinformatics 22, 934–942.

Wu, W.W., Sun, Y.H., and Pante, N. (2007). Nuclear import of influenza A viral ribonucleoprotein complexes is mediated by two nuclear localization sequences on viral nucleoprotein. Virol J 4, 49.

Ye, Q., Krug, R.M., and Tao, Y.J. (2006). The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. Nature 444, 1078–1082.

You, S., Stump, D.D., Branch, A.D., and Rice, C.M. (2004). A *cis*-acting replication element in the sequence encoding the NS5B RNA-dependent RNA polymerase is required for hepatitis C virus RNA replication. J Virol 78, 1352–1366.

Zhirnov, O., Isaeva, E.I., Konakova, T.E., Thoidis, G., Piskareva, L.M., Akopova, I.I., et al. (2007). Protection against mouse and avian influenza A strains via vaccination with a combination of conserved proteins NP, M1 and NS1. Influenza Other Respir Viruses 1, 71–79.

Zhirnov, O.P., Konakova, T.E., Wolff., T., and Klenk, H.D. (2002). NS1 protein of influenza A virus down-regulates apoptosis. J Virol 76, 1617–1625.

Zohari, S., Gyarmati, P., Thorén, P., Czifra, G., Bröjer, C., Belák, S., et al. (2008). Genetic characterization of the NS gene indicates co-circulation of two sub-lineages of highly pathogenic avian influenza virus of H5N1 subtype in Northern Europe in 2006. Virus Genes 36, 117–125.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31, 3406–3415.

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