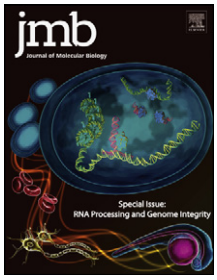

The NS1 Protein from Influenza Virus Stimulates Translation Initiation by Enhancing Ribosome Recruitment to mRNAs

Baptiste Panthu [1,2,3,4,5], Olivier Terrier [1,2,3,4,5,6], Coralie Carron [1,2,3,4,5,6], Aurélien Traversier [1,2,3,4,5,6], Antoine Corbin [1,2,3,4,5], Laurent Balvay [1,2,3,4,5], Bruno Lina [1,2,3,4,5,6], Manuel Rosa-Calatrava [1,2,3,4,5,6] and Théophile Ohlmann [1,2,3,4,5]

*1 - **CIRI,** International Center for Infectiology Research, Université de Lyon, 69364 Lyon, France*
*2 - **Inserm,** U1111, 69364 Lyon, France*
*3 - **Ecole Normale Supérieure de Lyon,** 69364 Lyon, France*
*4 - **Université Lyon 1,** Centre International de Recherche en Infectiologie, 69364 Lyon, France*
*5 - **CNRS,** UMR5308, 69364 Lyon, France*
*6 - **Laboratoire de Virologie et Pathologie Humaine VirPath,** Université Claude Bernard Lyon 1, Hospices Civils de Lyon, Faculté de médecine RTH Laennec, rue Guillaume Paradin, F-69008 Lyon, France*

***Correspondence to Théophile Ohlmann:** CIRI, International Center for Infectiology Research, Université de Lyon, 69364 Lyon, France. tohlmann@ens-lyon.fr*
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Abstract

The non-structural protein NS1 of influenza A viruses exerts pleiotropic functions during infection. Among these functions, NS1 was shown to be involved in the control of both viral and cellular translation; however, the mechanism by which this occurs remains to be determined. Thus, we have revisited the role of NS1 in translation by using a combination of influenza infection, mRNA reporter transfection, and *in vitro* functional and biochemical assays. Our data show that the NS1 protein is able to enhance the translation of virtually all tested mRNAs with the exception of constructs bearing the Dicistroviruses Internal ribosome entry segment (IRESes) (DCV and CrPV), suggesting a role at the level of translation initiation. The domain of NS1 required for translation stimulation was mapped to the RNA binding amino-terminal motif of the protein with residues R38 and K41 being critical for activity. Although we show that NS1 can bind directly to mRNAs, it does not correlate with its ability to stimulate translation. This activity rather relies on the property of NS1 to associate with ribosomes and to recruit them to target mRNAs.

Introduction

Influenza A viruses are major human pathogens with pandemic outbreaks such as in 1918 that had caused over 20 million deaths worldwide [1,2]. Influenza is an enveloped virus that belongs to the Orthomyxoviridae family and possesses 8 negative single-stranded RNA genomes that encode up to 17 different proteins [3]. The viral life cycle commences by virus entry into the cell where incoming vRNPs are transported toward the nucleus to undergo transcription and replication; these are performed by the viral RNA-dependent RNA polymerase with the support of the host RNA polymerase II and transcriptosome machinery [3]. The viral mRNAs harbor a 5′ methylated cap that is stolen from cellular transcripts in a process called “cap-snatching” [3]. This occurs by a subtle subversion of the cellular transcriptional machinery whereby the RNA-dependent RNA polymerase binds to the m^7GTP cap of cellular transcripts to cleave it about 9 to 17 nt downstream to their 5′ end. This cellular fragment of RNA is then used as a primer for viral transcription [4–6], resulting in the synthesis of properly capped mature viral mRNAs and their CRM1-dependent export to the cytoplasm, where they are translated by the host cell machinery into proteins [7]. Such hijacking is accompanied by the degradation of the cleaved cellular transcripts together with the impairment of nucleocytoplasmic export and contributes to the shut-off of cellular gene expression [8,9].

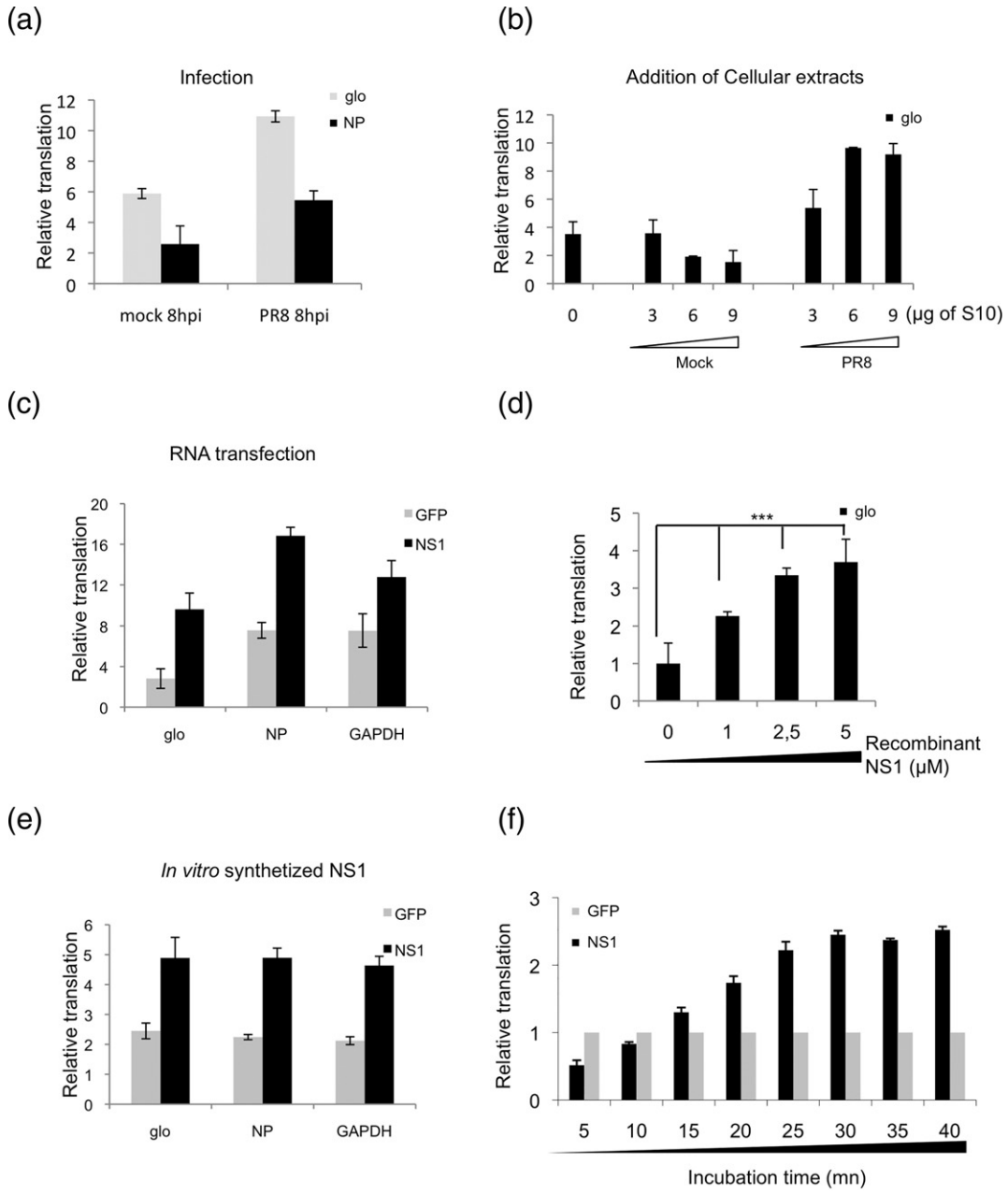


Fig. 1. Viral and cellular translation is enhanced in influenza-infected cells. (A) Capped and polyadenylated luciferase reporter mRNAs driven by the 5′UTRs derived from the β-globin (glo) or the NP (NP) segment of PR8 influenza strain were electroporated in A549 cells that were infected, or not (mock), with the influenza PR8 strain at a multiplicity of infection of 6. Then, 8 h post-infection, luciferase activity was determined. (B) Capped and polyadenylated luciferase reporter mRNAs driven by the β-globin 5′UTR were added to the RRL supplemented with increasing concentrations of cytoplasmic extracts (from 3 µg to 9 µg) prepared from infected or mock-infected A549 cells with the PR8 influenza strain. Luciferase activity was determined after 30 min of incubation at 30 °C. (C) Capped and polyadenylated luciferase reporter mRNAs driven by the 5′UTRs derived from β-globin (glo), GAPDH, or the NP genes were electroporated in A549 cells that have been previously transfected with RNAs coding for the GFP or the NS1 protein as indicated. Luciferase activity was determined for each construct 1 h post-electroporation. (D) Capped and polyadenylated luciferase reporter mRNAs driven by the 5′UTRs derived from β-globin was translated in the RRL in the presence of increasing amounts of recombinant NS1 protein as indicated. After 30 min of incubation, luciferase activity was determined. (E) Capped and polyadenylated luciferase reporter mRNAs driven by the 5′ UTRs derived from β-globin (glo), GAPDH, or the NP segment were translated in the RRL in the presence of *in vitro* synthesized GFP or NS1 proteins as indicated. (F) Capped and polyadenylated luciferase reporter mRNAs driven by the 5′ UTRs derived from β-globin were translated in the RRL in the presence of *in vitro* synthesized GFP or NS1 proteins. Luciferase activity was determined at different times as indicated on the figure (from 5 to 40 min of incubation). All results are expressed as mean +/- SD of three independent experiments.

In higher eukaryotes, translation begins by the attachment of the 40S ribosomal subunit to the 5′ capped end of the transcript followed by linear scanning until it reaches an AUG codon [10]. This process is mediated by a dozen of initiation factors among which is found the eIF4F complex that is composed of eIF4E, eIF4A, and eIF4G and serves to promote both ribosomal attachment to the mRNA molecule and scanning toward the AUG codon [11]. All known viruses are dependent on the host cell translational apparatus to express their own proteins. This creates a competitive situation between viral and cellular messengers, which are often bypassed by viral strategies to ensure the preferential use of ribosomes for viral mRNAs [12].

Although little is known about influenza translational control, previous reports have shown that both the structure of the viral 5′UTR and the interplay with the multifunctional RNA-binding NS1 protein are critical for viral protein expression. The viral 5′UTR is typically short (about 50 nts) and not particularly structured in terms of secondary and tertiary RNA interactions [13,14]. With the exception of their cap-snatched 5′ end, the composition of viral UTRs is highly conserved among the different Influenza A strains, and they contain motifs that are essential for replication and viability [13,14]. About this last point, initial studies reported that influenza 5′UTR could confer translational specificity and selective advantage over the cellular mRNAs, leading to the hypothesis that viral protein production was so efficient during infection [15,16]. However, this point of view has been challenged by Cassetti and colleagues, and further work is needed to understand how viral production can proceed in infected cells [17]. Other reports have suggested the involvement of NS1 protein in this mechanism [18,19]. The NS1 protein is a 230-aa protein divided into a 3-alpha-helix RNA-binding domain (RBD; 1–73) and an effector domain (aa 80–230) that are connected by a flexible linker region [20]. NS1 performs multiple accessory functions during viral infection such as mRNA processing and nuclear export and the control of the host cellular interferon-mediated antiviral response [21–23]. In addition to these pleiotropic functions, NS1 is also a key player in both viral and cellular translation through: (i) the enhancement of translation of viral transcripts by promoting a complex with, at least, eIF4GI, PABP1, and hStaufen [24–26]; and (ii) the stimulation of viral translation by binding to the AGCAAAAG motif present in the 5′UTR of influenza mRNAs [27–29]. Very recently, Anastasina and colleagues reported that the addition of NS1 to the reticulocyte lysate system resulted in the stimulation of non-viral reporter mRNAs, then suggesting a more global effect for the viral protein in cellular translation [30].

In this study, we have examined the molecular mechanism by which NS1 could promote protein synthesis. Using a combination of viral infection, *ex-vivo* and *in vitro* translation systems, and biochemical assays, we could demonstrate that NS1 strongly stimulates translation driven by virtually all cellular and viral 5′UTRs with the sole exception of the CrPV and DCV IRESes. Biochemical dissection of the different domains of NS1 showed that this mechanism requires the amino-terminal part of the protein and more particularly involves the positively charged residues implicated in RNA binding. However, translation stimulation is not due to the association of NS1 with transcripts but is rather caused by the association of NS1 with the ribosomal cellular fraction to promote an increased recruitment of ribosomes to the mRNA.

Results

Viral and cellular translation is enhanced in influenza-infected cells

It is now well established that the infection of cells with influenza viruses results in the overall decrease of cellular gene expression [31], which is merely due to the host transcriptional shut-off [18]. However, as this shut-off of gene expression is initiated in the nucleus and mainly caused by the misregulation of transcriptional and co-transcriptional events, the direct impact of influenza infection on translation in the cytoplasm is difficult to assess. To circumvent this, we have transfected *in vitro* synthesized reporter mRNAs into the cytoplasm of infected human epithelial lung cell line (A549) in order to bypass any nuclear steps of virus-induced shut-off. Reporter mRNAs used in this study are composed of the renilla open reading frame preceded by variable 5′UTRs that have been cloned from viral and cellular genes. The resulting mRNAs are expressed by T7 polymerase transcription and can bear, or not, a cap and a poly(A) tail at their 5′ and 3′ extremities. We transfected A549 cells that were mock-infected or infected with the PR8 influenza strain with reporters containing the 5′UTR of β-globin (glo) or the influenza NP gene (NP; Fig. 1a). Luciferase activity was measured 1 h post-transfection. This showed that protein synthesis was enhanced by about twofold in infected cells for both reporter genes (Fig. 1a, glo and NP). In parallel, we have used a rabbit reticulocyte lysate (RRL) supplemented with S10 extracts prepared from mock or A549 infected cells (see Materials and Methods). *In vitro* transcribed mRNAs containing the β-globin 5′UTR were translated for 30 min in supplemented RRL, and protein synthesis was measured and plotted in Fig. 1b. Data show that β-globin-driven translation was significantly enhanced by the addition of S10 made from infected cells. Thus, these data show that influenza viral infection can stimulate translation in the context of both infected cells and *in vitro* expression system. This suggests that one or several viral proteins may play a role on translation

either directly or indirectly. As the viral NS1 protein was previously described to enhance viral protein synthesis by several distinct mechanisms [12,19], we have expressed it from RNA transfection in A549 cells (Fig. 1c). *In vitro* synthesized mRNAs driven by a 5′ UTR derived from cellular (β-globin and GAPDH) or viral (NP) genes were also introduced in A549 cells expressing NS1 (Fig. 1c). Translation of all three reporter mRNAs was stimulated by the co-expression of NS1 compared to Green fluorescent protein (GFP), which was used as a control. Next, we have monitored the effects of adding purified recombinant NS1 protein that was expressed and purified from *Escherichia coli*. As shown in Fig. 1d, this also resulted in the

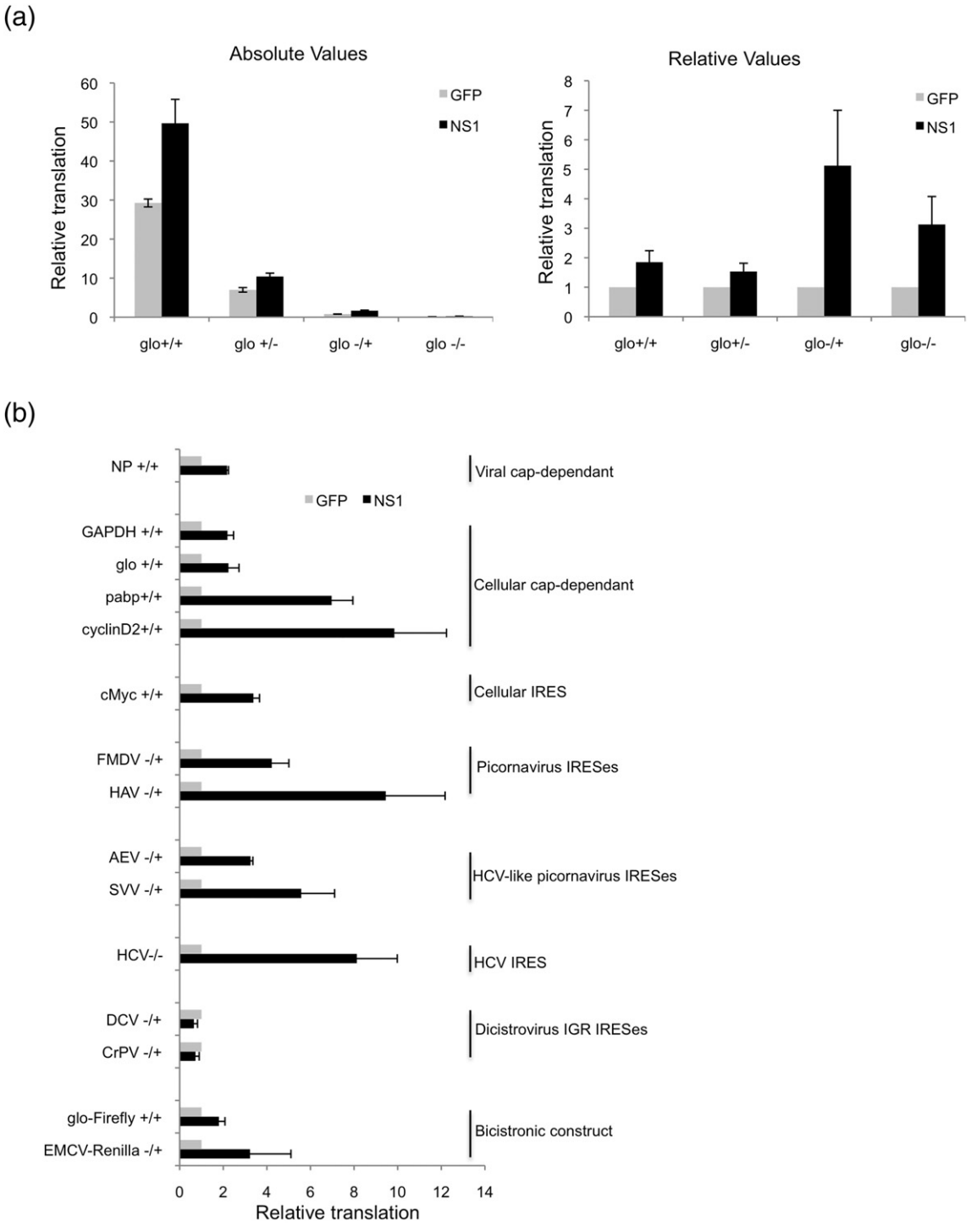


Fig. 2 (*legend on next page*)

enhancement of the globin 5′UTR-driven reporter gene in RRL. We also looked at the translation of glo/NP/GAPDH reporter mRNAs upon the addition of NS1 protein that had been previously synthesized *in vitro* in the RRL (see Materials and Methods). This also resulted in a significant enhancement of activity (Fig. 1e) and provided us with a very useful tool to produce the protein (and mutated versions of it) with no need for bacterial expression and purification. Finally, the effects of NS1 addition on translational stimulation of the globin 5′UTR-driven gene was also investigated over a time course running from 5 to 40 min of incubation (Fig. 1f). This shows linear stimulation of protein synthesis over the first 30 min of incubation; this set out the incubation time used for the remainder of this study.

Taken together, these results suggest that influenza infection stimulates cellular and viral translation and that this effect could be attributed to the NS1 protein.

NS1 stimulates protein synthesis at the initiation step

To further characterize the role of NS1 on translation, we then used a β-globin 5′UTR-driven mRNA, which was synthesized by *in vitro* transcription with or without a cap and/or a poly(A) tail (see Fig. 2a and Supplementary Fig. 1). Equal concentration of transcripts was added to the untreated RRL (uRRL) for 30 min in the presence of *in vitro* produced NS1 or control GFP proteins. Results in Fig. 2a are expressed as absolute (left panel) or relative (right panel) values that were normalized to the GFP control (set to 100%) in order to compare the effect in order to appreciate the effect of NS1 on each mRNA. Although translation was enhanced in all cases by the addition of NS1 (see black bars), this occurred to a variable extent with uncapped mRNAs being relatively more sensitive and stimulated by three- to fourfold. As the presence of the cap and poly(A) is known to be key determinants in translation initiation, this suggests that NS1 protein can be involved in this step of protein synthesis. To further investigate this hypothesis, we have next used a set of luciferase reporter mRNAs harboring different 5′UTRs as depicted in Fig. 2b and Supplementary Fig. 1; the status of their 5′ and 3′ ends regarding the presence of a cap and poly(A) tail is indicated by using the same annotation as above. Among them, we found 5′UTRs derived from (i) cellular transcripts that are translated via a canonical cap-dependent (GAPDH, β-globin, PABP, Cyclin D2) or an IRES (c-myc) mechanism and (ii) viral transcripts such as influenza NP or those containing IRESes from picornaviruses (FMDV, HAV, SVV, and AEV), hepacivirus (HCV), and dicistroviruses (CrPV and DCV). Each corresponding mRNA was produced by *in vitro* transcription, as it is found in its natural context regarding the presence of a cap and/or a poly(A) tail: for example, capped/polyadenylated for cellular genes, uncapped/polyadenylated for picornaviruses and dicistroviruses, and uncapped/non polyadenylated for HCV (see Supplementary Fig. 1). Luciferase activity was measured for all constructs in the presence of NS1 or GFP, and results are expressed relative to values obtained upon the addition of the GFP control protein (Fig. 2b). Translation of all reporter mRNAs was stimulated by the addition of NS1 with a magnitude varying from two- to about eightfold depending on the nature of their 5′UTR. We have also assessed the effect of NS1 on a dual firefly/renilla luciferase bicistronic mRNA containing the globin 5′UTR driving the Firefly gene and the EMCV IRES to express the Renilla gene. In this context, the addition of NS1 resulted in the enhancement of both cistrons (Firefly and Renilla) with a similar order of magnitude (Fig. 2b). Interestingly, only mRNAs that belong to the intergenic *Dicistroviridae* IRES family (DCV and CrPV) were not sensitive to NS1. This class of IRESes does not require any initiation factor to promote ribosomal attachment and initiation [32]. Taken together, these data indicate that the addition of the viral NS1 protein has different effects from no (CrPV and DCV) to medium (NP, GAPDH, glo, EMCV) and high (PABP, Cyclin D2, c-myc, FMDV, HAV, AEV, SVV, and HCV) levels of stimulation of protein synthesis from mRNAs that only differ by the nature and structure of their respective 5′UTR. Moreover, it can be suggested that NS1 would stimulate protein synthesis at the level of initiation.

Fig. 2. NS1 stimulates translation initiation. (A) Capped/polyadenylated (+/+), uncapped/polyadenylated (-/+), capped/non-polyadenylated (+/-), and uncapped/non-polyadenylated (-/-) luciferase mRNAs driven by the β-globin 5′UTR were translated in the RRL in the presence of *in vitro* synthesized GFP (gray bar) or NS1 (dark bar) proteins. Luciferase production was determined after 30 min, and results are presented as absolute renilla luciferase activity (left panel) or normalized to the values obtained with the GFP control that was set arbitrarily to 1 (right panel). (B) A spectra of luciferase-coding mRNAs driven by 5′UTRs (see Supplementary Fig. 1) isolated from cellular (GAPDH, β-globin, Pabp, Cyclin D2, c-myc) or viral (NP, FMDV, SVV, HAV, AEV, HCV, DCV, CrPV) genes were translated in the RRL in the presence of *in vitro* synthesized GFP (gray bar) or NS1 (dark bar) proteins. For each construct, the presence of a cap at the 5′ end and a poly(A) tail at the 3′ end is annotated by the symbol (+/+) and corresponds to the native context of the mRNA from which it is derived. Translation was carried out for 30 min at 30 °C, and results are expressed as % of stimulation relative to the GFP control that was set arbitrarily to 1. A bicistronic mRNA construct containing the firefly (first gene) and the renilla (second gene) luciferases with the EMCV IRES in the intercistronic spacer was also translated in a capped and polyadenylated (+/+) form under the same experimental conditions. All results are expressed as mean +/- SD of three independent experiments.

RDB domain of NS1 is required for *in vitro* translation stimulation

The NS1 protein has been previously described to exert a stimulatory role on translation by, at least, two distinct mechanisms: (i) it can bind directly to components of the translational apparatus (eIF4GI, PABP, Staufen) and (ii) it can interact directly with

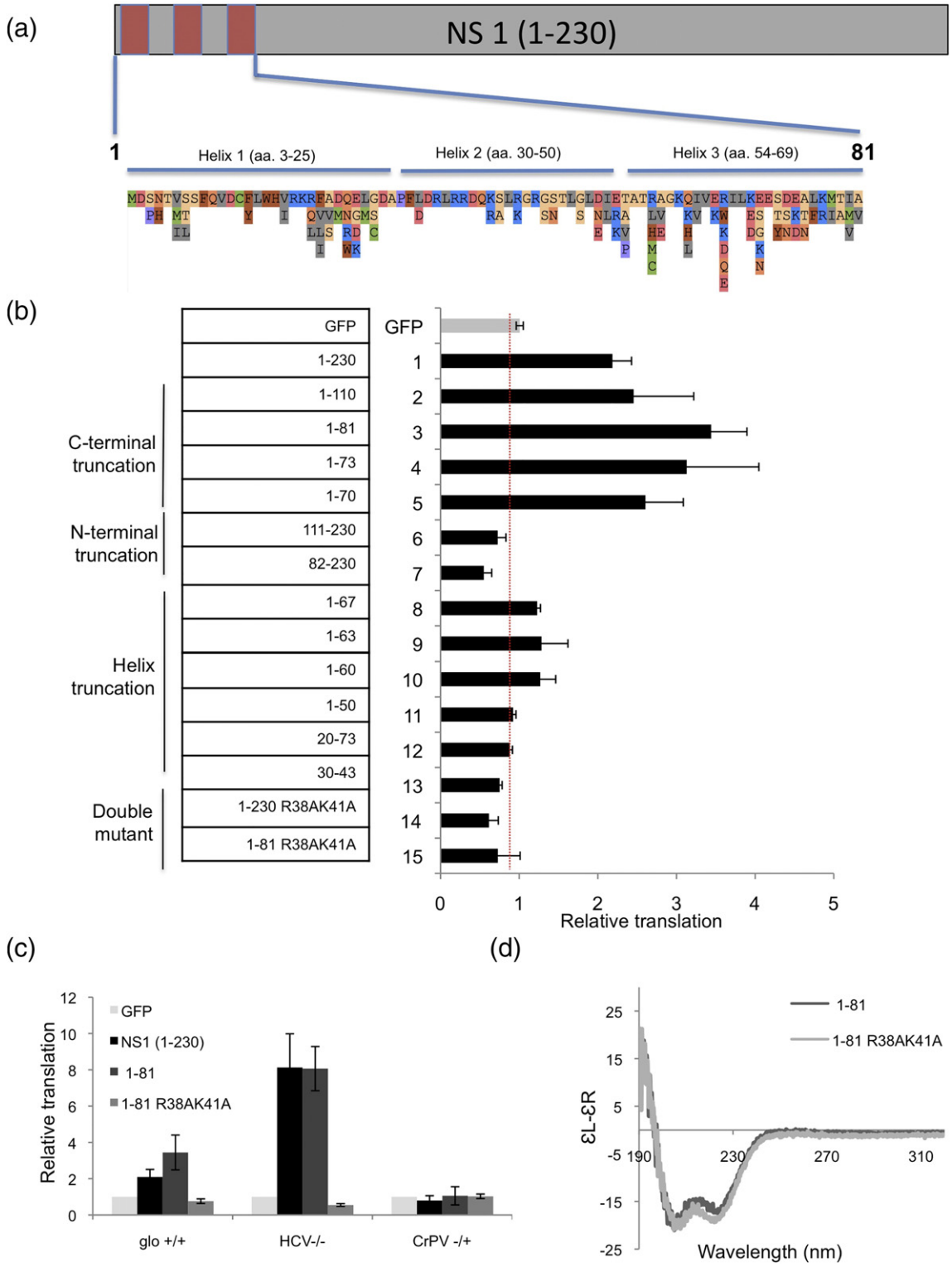


Fig. 3 (*legend on next page*)

viral mRNAs via its RBD domain [33]. As our data show that NS1 can stimulate translation *in vitro*, we have introduced deletions and mutations in the viral protein and tested their effects *in vitro*. Figure 3a shows a schematic representation of NS1 with the three alpha helixes that constitute the RBD (aa 1–81). A sequence alignment of the three helixes found in different viral strains was performed to determine the corresponding consensus sequence together with all alternative amino acids present at a frequency above 1% in the alignment of all known Influenza A NS1 sequences (Fig. 3a, lower panel). A series of deletions and point mutations was introduced and summarized in Fig. 3b. All proteins were produced as a single gene product of the predicted molecular weight by *in vitro* translation in the presence of [^{35}S]-Met (data not shown). Each mutant was added to an *in vitro* translation assay with β-globin-renilla luciferase reporter mRNA, and data were expressed relative to values obtained with the GFP control (Fig. 3b). Baseline of non-enhanced translation corresponding to the addition of GFP is marked by a dotted line. NS1 mutants that only harbored the RBD domain (Fig. 3b, lane 2: aa 1–110; lane 3: aa 1–81; lane 4: aa 1–73, and lane 5: aa 1–70) were all capable to enhance translation of the globin-renilla luciferase construct to the same level or even more than the full-length NS1 polypeptide (Fig. 3b, lane 1: aa 1–230). In sharp contrast, expression of the effector domain alone (Fig. 3b, lane 6 and lane 7) failed to increase luciferase production. Interestingly, removal of only 3 aa between position 67 and 70 was sufficient to abolish translation stimulation (Fig. 3b, lane 8: aa 1–67). Similarly, expression of individual or combination of segments of NS1 that contain helixes 1 and 2 (Fig. 3b, lanes 9–11), helixes 2 and 3 (Fig. 3b, lane 12), or helix 2 (Fig. 3b, lane 13) was not able to recover stimulatory effect, confirming the need for all the three helixes. As it has been previously described that the two positively charged residues R38 and K41 were critical for the activity of the RBD [33], we decided to change them to alanine (named mutant R38AK41A) and found that this double mutant had completely lost its ability to stimulate β-globin-renilla translation both within the context of the full-length protein (Fig. 3b, lane 14) and within the truncated amino-terminal part (Fig. 3b, lane 15). Consistent with above results, loss of activity with the R38AK41A mutant was also observed with a reporter mRNA containing the HCV IRES, whereas mutations did not affect translation driven by the CrPV IRES (Fig. 3c). It is noteworthy that the level of expression of both the NS1 and the R38AK41A mutant was verified and found to be similar for the two proteins (see Supplementary Fig. 2).

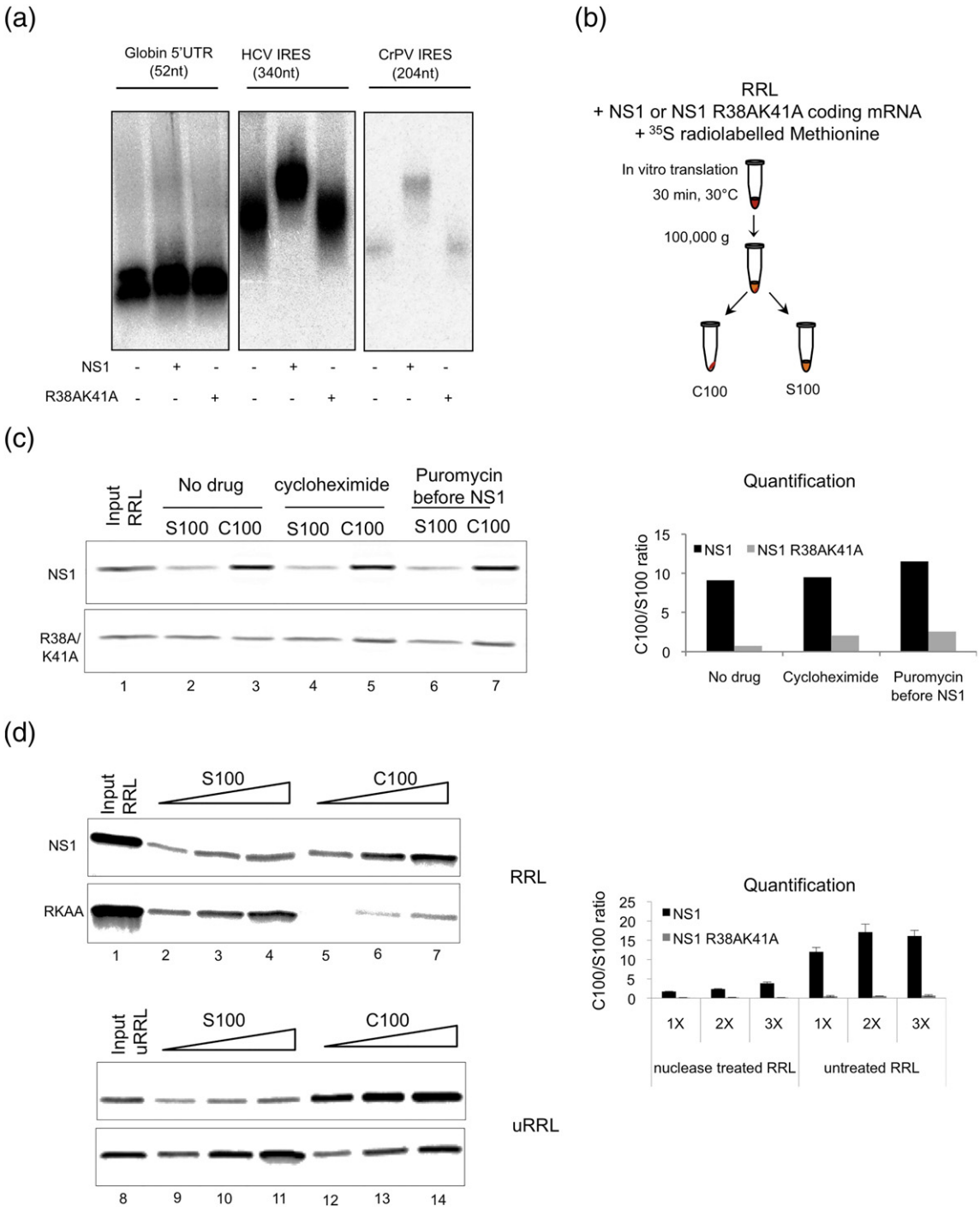
In order to investigate whether the R38AK41A mutation could perturb the overall structural shape of the polypeptide, we have used CD (Fig. 3d). Spectrum obtained with the WT and mutant protein showed a very similar profile and exhibited the typical pattern of α-helical folding, with a maximum at 190 nm and two minima, at 208 and 222 nm; the various CD deconvolution methods used indicated a predominant α-helix content (~65%) and no variation between the NS1 1-81 R38AK41A peptide and the NS1 1-81, indicating that these two mutations did not cause any change in the overall folding of the protein.

NS1 is associated with the ribosomal fraction

The NS1 protein was shown to interact with many different types of viral and cellular RNAs including viral genomic RNAs [34], viral mRNAs [33], U6 small nuclear RNAs [20,35], and double-stranded RNAs [35–37]. Recently, RNA motifs that bind with high affinity to NS1 have been identified by a systematic evolution of ligands by experimental enrichment method and must be displayed within a proper RNA structure to be recognized by NS1 [28]. To further investigate the basis of NS1 stimulation, we have looked for putative interactions with some of the 5′ UTRs used in this work. For this, electrophoretic migration shift assays (EMSAs) were performed using ^{32}P-labeled β-globin (52 nt) 5′UTR, HCV (340 nt), and CrPV (204 nt) IRESes incubated with recombinant NS1 or R38AK41A mutant proteins (see Supplementary Fig. 2). The ribonucleoprotein complex was run on a non-denaturing gel, and the position of the RNA was visualized by

Fig. 3. Domains of NS1 involved in translation stimulation. (A) Schematic cartoon of the NS1 protein with the position of the three helixes. The bottom part represents the amino acids consensus sequence for the first 81 aa (first line), and all amino acid variations are present above a 1% frequency in all known influenza A NS1 sequences and ordered by decreasing frequency. (B) Capped and polyadenylated luciferase reporter mRNA driven by the β-globin 5′UTR was translated in the RRL supplemented with *in vitro* translated: GFP, NS1 (1-230), or segments of NS1 proteins (as indicated, numbers refer to the positions of amino acids). Translation was monitored after 30-min incubation at 30 °C and is expressed as % of stimulation relative to the GFP control that was set arbitrarily to 1. Results are expressed as mean +/- SD of three independent experiments. (C) Luciferase mRNAs driven by β-globin 5′UTR (glo), HCV, or CrPV IRESes were translated in the RRL supplemented with *in vitro* translated NS1 (black), the amino terminus (1–81; dark grey), and the carboxy terminus (82–230, light grey) of NS1 or the GFP proteins (white). Translation was monitored after 30-min incubation at 30 °C and is expressed as % of stimulation relative to the GFP control that was set arbitrarily to 1. Results are expressed as mean +/- SD of three independent experiments. (D) NS1 (aa 1–81; dark gray) and R38AK41A mutant (light grey) secondary structure analyses by CD and recorded in the 190- to 320-nm regions at 20 °C using a spectropolarimeter from 25-μM samples in phosphate buffer. CD spectra are the reported variation of ellipticity (ΔL-ΔR), in units of M^{-1} cm^{-1}.

autoradiography (Fig. 4a). Addition of the NS1 protein induced a mobility shift of both HCV and CrPV RNAs (Fig. 4a, middle and right panels) but not with the β-globin 5′UTR (left panel). The addition of the R38AK41A mutant failed to promote complex formation on all RNAs tested (Fig. 4a, see β-globin, HCV, CrPV). The lack of any shift with the β-globin 5′ UTR in this assay (Fig. 4a, left panel) was surprising considering that the translation driven by β-globin was stimulated by NS1 (see Fig. 1 and 2). On the other hand, binding of NS1 to the CrPV IRES was also unexpected as the latter failed to be stimulated by the addition of the viral protein (see Fig 2b and 3c). Taken together, these data indicate a lack of correlation between mRNA/NS1 association and its ability to stimulate translation. This prompted us to



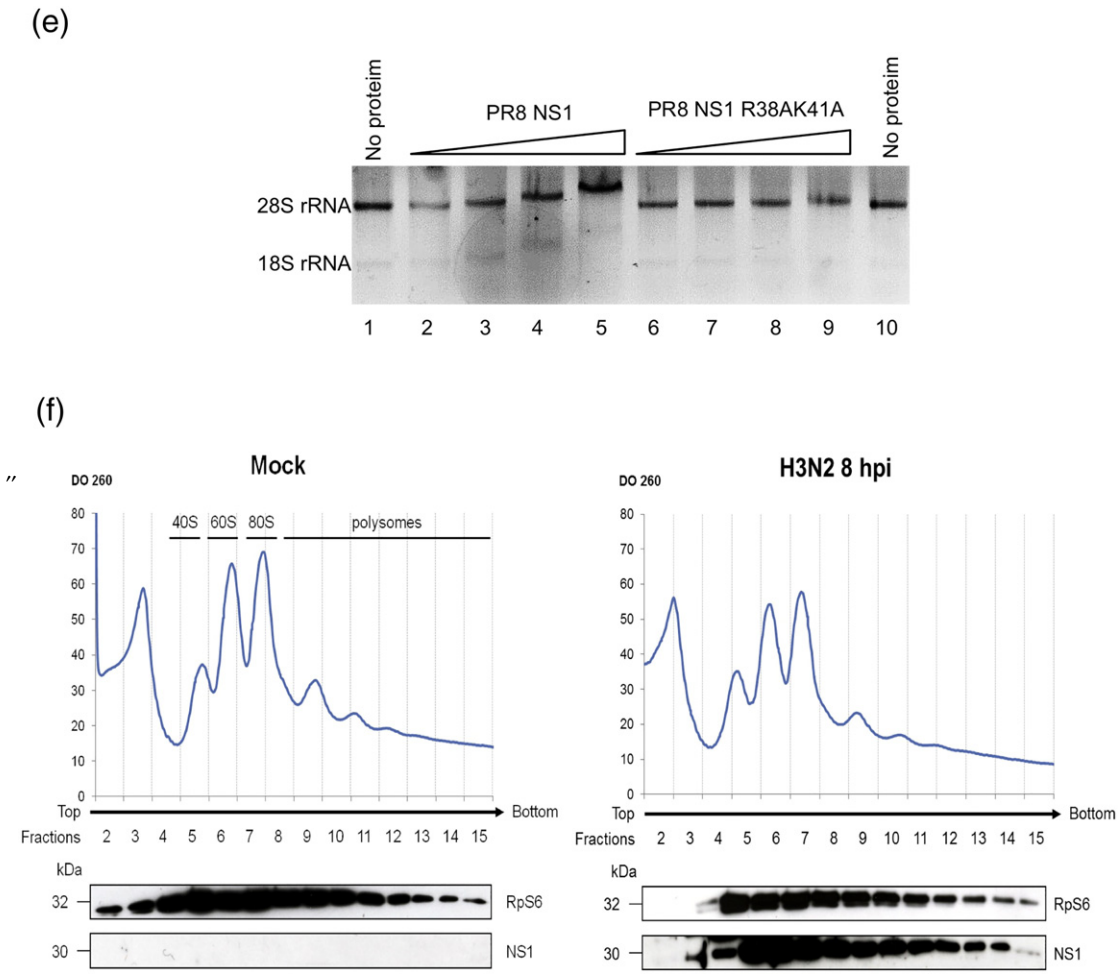


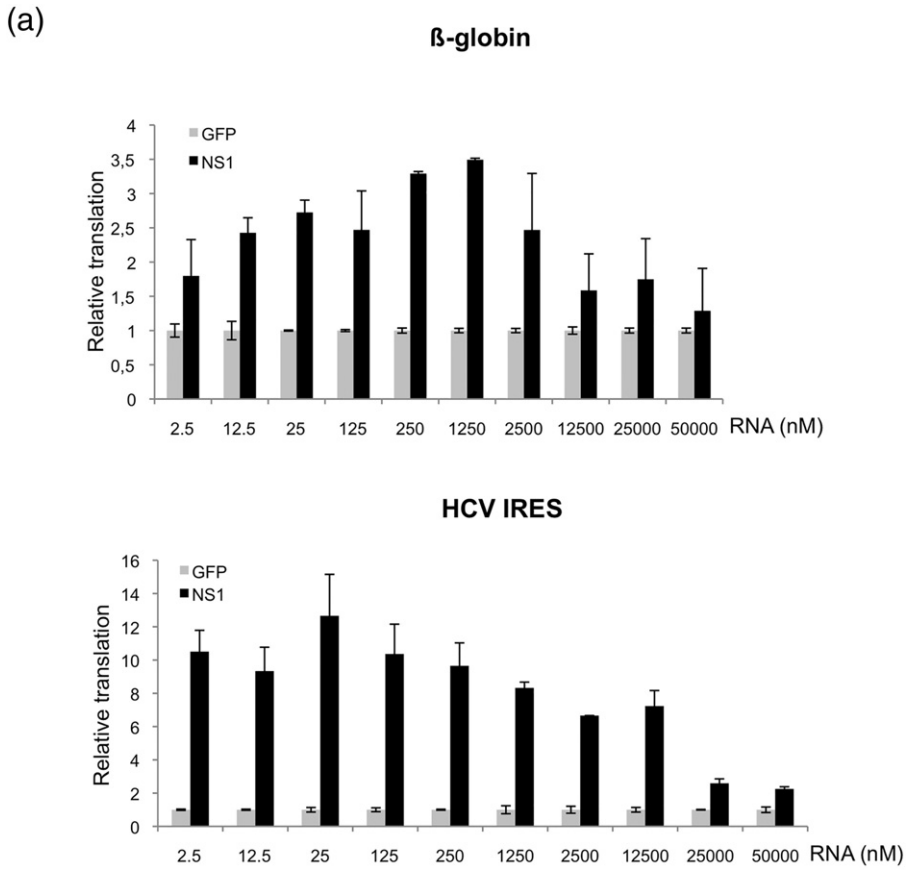
Fig. 4. NS1 associates with the ribosomal fraction.(a) EMSA with ^{32}P-labeled 5′UTRs derived from β-globin (52 nt), HCV (340 nt), and CrPV (204 nt) that were incubated for 15 min at 37 °C with 0, 1, 2.5, and 5 μM recombinant NS1 or R38AK41A mutant proteins in binding buffer (see Materials and Methods). The resulting ribonucleoprotein complexes were then resolved on a 1.5% non-denaturing gel and submitted to autoradiography. (b) Schematic cartoon showing the method used to fractionate the RRL into S100 and C100 fractions. (c) Luciferase mRNA driven by the HCV 5′UTR was translated in an RRL that was incubated with either *in vitro* synthesized and radiolabeled NS1 (upper panel) or the R38AK41A mutant (lower panel) proteins. In lanes 4 and 5, cycloheximide (100 ng/mL) was added after 10 min of incubation, whereas in lanes 6 and 7, puromycin (20 ng/mL) was added before the addition of the mRNA and the start of the incubation. After 10 min, the RRL was fractionated, and samples from the S100 and C100 fractions (as depicted on the cartoon) were resolved on a 12% SDS-PAGE gel and submitted to autoradiography and quantification in a PhosphorImager. The lane "Input RRL" corresponds to the signal obtained from the RRL before fractionation. Results obtained by quantification are expressed as the ratio of C100/S100 for each experimental condition (right panel). Please note that the C100 fraction is three times more concentrated than the S100 fraction. (d) A nuclease-treated (RRL, lanes 1 to 7) or untreated (uRRL, lanes 8 to 14) RRL was incubated with either *in vitro* synthesized NS1 (upper panel) or the R38AK41A mutant (lower panel) proteins and fractionated as described above. Increasing concentrations from both the S100 and C100 (1X (lanes 2, 5, 9, and 12), 2X (lanes 3, 6, 10, and 13), and 3X (lanes 4, 7, 11, and 14) were resolved on a 12% SDS-PAGE and submitted to autoradiography. The lane "Input" corresponds to the signal obtained from the RRL, or the uRRL, before fractionation. Results obtained by quantification are expressed as the ratio of C100/S100 for each experimental condition (right panel). (e) EMSA with rRNAs 18S and 28S extracted from epithelial lung cells that were incubated with increasing amounts of recombinant NS1 or R38AK41A mutant proteins. The resulting ribonucleoprotein complexes were then resolved on a 1.5% non-denaturing agarose gel containing BET and submitted to UV acquisition. (f) A cytoplasmic fraction of A549 cells (Mock; left panel) or A549 cells infected for 8 h with the H3N2-MO influenza strain (right panel) were treated with cycloheximide (100 μg/mL) before being layered on a 10–50% linear sucrose gradient and centrifuged at 36,000 rpm for 105 min in a SW41 rotor. Fractions were collected at OD254 nm with a Foxy Jr. gradient collector. Sedimentation and the position of 40S, 60S, 80S, and polysomes are indicated on the figure. For each fraction, western blot analysis was performed using polyclonal rabbit antibodies against the rpS6 ribosomal protein and the NS1 viral proteins as indicated on the figure. Positions of the molecular weight markers are indicated on the left side.

search for other potential RNA targets, and we focused on a possible interaction between NS1 and the ribosome.

Our recent study showed that RRL can be easily fractionated by centrifugation into a soluble and a ribosomal fraction [38]. Thus, we have exploited this property by using a RRL programmed with the HCV-renilla mRNA (as it responds to stimulation) and supplemented it with [^{35}S]-Met-labeled NS1 or the R38AK41A mutant (see Materials and Methods). Upon fractionation (see Fig. 4b), different amounts of the supernatant and ribosomal fractions were run on a SDS-PAGE gel and submitted to autoradiography. Data presented in Fig. 4c (left panel) show a very different pattern of distribution for the two proteins with NS1 located mostly in the C100 ribosomal fraction (Fig. 4c, upper panel, compare lanes 2 and 3), whereas the R38AK41A mutant remained in the S100 fraction (lower panel). It is important to note that the ribosomal pellet has been concentrated to 3X to highlight differences between NS1 and R38AK41A (see Materials and Methods). This suggests that NS1 has the ability to co-fractionate with ribosomes, and this requires the presence of RNA-interacting basic residues in the amino-terminal part of the polypeptide.

We further investigated whether NS1 associates with ribosome fraction during active translation of the mRNA. For this, we added the inhibitors cycloheximide (Fig. 4c, lanes 4 and 5) after 5 min of translation or puromycin (lanes 6 and 7) prior to the addition of the reporter mRNA. In both cases, the blockage of protein synthesis, either before or after the addition of the reporter gene, did not change the ribosomal distribution of NS1 or the mutant proteins, indicating that it does not require the translation of the mRNA *per se* (Fig. 4c, compare lanes 2 and 3 with lanes 4, 5, 6, and 7). A quantification of the intensity of the bands is given on the right panel for each condition.

Data above suggest that the localization of NS1 to the ribosomes could take place in the absence of any mRNA in the translational assay. To investigate this directly, we have compared the distribution of NS1 in a nuclease treated and in a uRRL, which contains endogenous β-globin and lipoxygenase mRNAs. The fractionation of these lysates was performed as described in Fig. 4b, and amounts of both the S100 and C100 were visualized by western blot (Fig. 4d, upper panel for RRL and lower panel for uRRL). In both cases, it confirms that the R38AK41A mutant



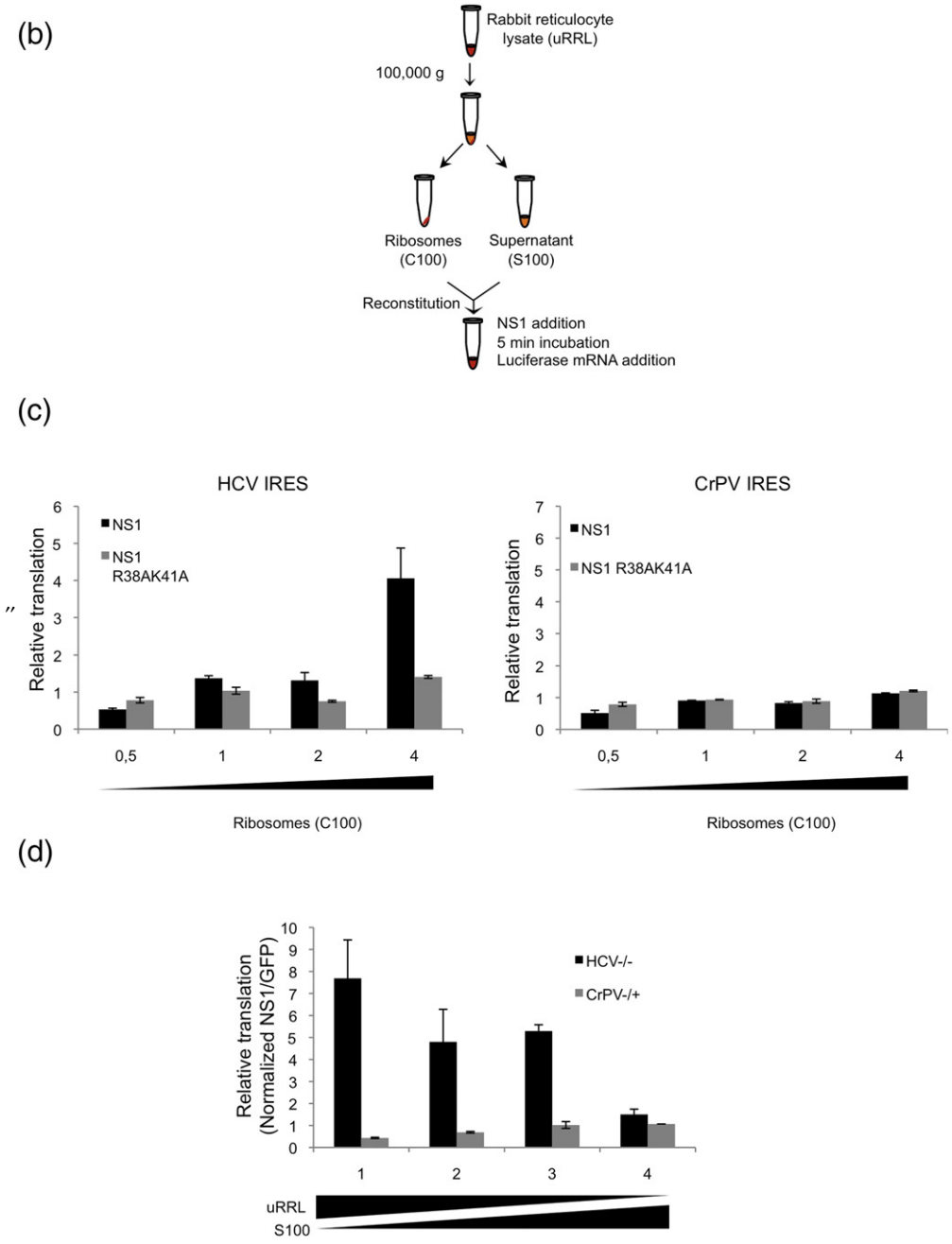


Fig. 5. NS1 can increase ribosomal uptake.(a) Increasing concentrations (from 2.5 to 50,000 nM) of renilla luciferase mRNAs driven by the 5′UTR of β-globin (upper panel) or HCV (lower panel) were translated in the RRL supplemented with either *in vitro* synthesized GFP (gray bar) or NS1 (dark bar) proteins. β-globin mRNAs harbor a cap and a poly(A) tail (+/+), whereas HCV mRNAs lacked both (-/-) as they are found in their natural context. Luciferase production was measured after 30-min incubation at 30 °C. (b) Schematic cartoon of the method used to fractionate and reconstitute the RRL. (c) An RRL has been reconstituted as depicted in (b) with increasing concentrations of ribosomes (from 0.5 to 4 µg per 10 µl of reaction mix). Renilla luciferase mRNAs driven by the HCV IRES (uncapped and non-polyadenylated; left panel) or the CrPV IRES (uncapped and polyadenylated; right panel) were translated in the presence of *in vitro* synthesized GFP, NS1 (dark bar), or the R38AK41A mutant (gray bar) proteins. Luciferase production was measured after 30 min, and the results are expressed relative to the value obtained with GFP for each construct. (d) Renilla luciferase construct driven by the HCV (dark bar) or CrPV (gray bar) IRESes was translated in an RRL that was supplemented with increasing amounts of the S100 supernatant [lane 1: 0% (vol/vol); lane 2: 10% (vol/vol), lane 3: 20% (vol/vol), lane 4: 30% (vol/vol)] issued from a fractionated lysate as depicted in (b). *In vitro* synthesized GFP or NS1 proteins were added to the translational mixture, and luciferase production was analyzed after 30 min of incubation. The results are expressed relative to the value obtained with the GFP protein for each construct. Results are expressed as mean +/- SD of three independent experiments.

protein remains in the S100 fraction, whereas NS1 preferentially associates to the ribosomes. Interestingly, association of NS1 to the ribosomes was even stronger in the case of the uRRL, which suggests that in this case, NS1 is bound both to the ribosomes and the mRNAs that are associated with the ribosome pellet. A quantification of the intensity of the bands is given on the right panel for each condition. This suggests that the distribution of NS1 to the ribosomes can take place even in the absence of any endogenous mRNA.

As this suggests that NS1 has affinity for the ribosome, we decided to investigate this further by looking at the direct interaction between the recombinant NS1 protein and ribosomal RNA (rRNA). Thus, we have used EMSA with both the WT and the R38AK41A mutated forms of the NS1 recombinant protein and purified rRNAs extracted from A549 cells (Fig. 4e). It can be observed that the addition of the WT recombinant protein induced a shift of mobility toward high molecular weight for both 28S rRNA and 18S rRNA (Fig. 4e, lanes 2–5), whereas this was not the case with the R38A/K41A mutant (lanes 6–9). This suggests that NS1 has the ability to directly bind both the 18S and 28S rRNAs, and this requires the presence of the Arg38 and Lys41 residues within the RNA-binding domain. It is also noteworthy that the apparent mobility of both rRNAs increases with the amount of recombinant NS1 protein added, which suggests cooperative and multiple interactions of the latter with the rRNAs (Fig. 4e).

Finally, we decided to investigate whether NS1 could be associated with the ribosomal fraction in the context of viral infection. For this, we have performed a polysome profile on sucrose gradient in mock (Fig. 4f, left panel) or in cells that were infected with the H3N2-MO influenza strain for 8 h (Fig. 4f, right panel). Polysome profiles were obtained by reading the absorbance at 254 nm, and 14 ribosomal fractions including 40S, 60S, 80S, and polysome fractions were collected from each gradient to be blotted using anti-rpS6 and anti-NS1 antibodies. In the two assays presented here, rpS6 was detected in all ribosomal fractions, as expected. Interestingly, we also noticed that the NS1 protein was constitutively distributed in all fractions derived from infected cells, suggesting a possible interaction between NS1 and the ribosome that might play a role in the control of translation in infected cells. This prompted us to investigate further the molecular basis of this mechanism.

NS1 enhances ribosome recruitment in a functional manner

We next looked for a functional link between NS1 association to the ribosomes and its ability to stimulate translation. In order to test this hypothesis, we have performed an mRNA dose curve in the presence of a fixed concentration of NS1 or the GFP control protein (Fig. 5a). As the concentration of endogenous ribosomes remains constant in this translational assay, the addition of increasing amounts of *in vitro* synthesized mRNAs (from 2.5 nM to 50,000 nM) will drastically change the ratio of RNA/ribosome. Reporter mRNAs with the 5′UTR of β-globin (upper panel) or the HCV IRES (lower panel) were translated from a very low (2.5 nM) to high concentration (50,000 nM) of transcripts, and activity was normalized to values obtained with the GFP control protein. Although for both constructs, NS1 stimulated translation, the magnitude of this stimulation was considerably higher when the RNA concentration was low (for HCV) to medium (for Globin), and then a sharp decrease at high concentration of RNA added to the translational assay. This indicates that NS1 stimulation is more efficient when endogenous ribosomes are in excess over the amount of RNA added to the translational mixture.

To investigate this further, we have used a fractionated/reconstituted assay; the detailed protocol used for this was recently described [38,39] and depicted in Fig. 5b. Briefly, a functional reconstituted lysate can then be assembled by adding back the two fractions (S100 and C100), and it retains about 90% of the translational efficiency of the parental lysate [38]. Application of this novel method provided us with a useful tool to manipulate the concentration of ribosomes that are added back in the reconstituted system by adjusting the ratio of S100/C100. This experimental setting was used to translate reporter constructs driven by the HCV (Fig. 5c, left panel) and CrPV (Fig. 5c, right panel) IRESes. Different concentrations of ribosomes (from 0.5 X to 4 X; see Materials and Methods) were added together with a fixed amount of the NS1 or R38AK41A mutant proteins. Translation driven by both the CrPV and HCV IRESes was not affected by the presence of an excess of ribosomes in the presence of the R38AK41A mutant (see gray bars). This is in agreement with our previous data showing that the excess of ribosomes does not stimulate translation [38]. This sharply contrasts with data obtained with the NS1 protein, which resulted in the increase of activity of translation driven by the HCV IRES when the concentration of ribosomes is increased (Fig. 5c, left panel, lane 4); however, this was not observed when the translation of the reporter construct was driven by the CrPV IRES (Fig. 5c, right panel, lane 4), in agreement with the data presented in Fig. 2.

In parallel, we have taken a complementary approach that consists of decreasing the pool of ribosomes available in the RRL by adding an excess of the S100 fraction (Fig. 5d). For this, the translation reaction is performed in a final volume of 50% (vol/vol) of reticulocyte lysate that can be adjusted as follows: 50% RRL/0% S100 (lane 1), 40% RRL/10% S100 (lane 2), 30% RRL/20% S100 (lane 3), and 20% RRL/30% S100 (lane 4). As the S100 is devoid of any

ribosome, this results in the dilution of the overall concentration of ribosomes available for translation. The HCV and CrPV reporter constructs are then added together with a fixed concentration of the GFP or NS1 proteins, and the results are normalized to the data obtained in the presence of the GFP protein used as a control. As expected, dilution of the pool of ribosomes by the S100 supernatant diminished the stimulatory effect of NS1 on the HCV construct (Fig. 5d, black bars, compare lane 1 with lane 4), but translation driven by the CrPV IRES remained unchanged (gray bars).

Discussion

Influenza A virus infection induces a shut-off of host gene expression by reducing cellular transcription, blocking cellular mRNA export, and causing mRNA degradation by cap-snatching [40]. All these nuclear events render difficult to assess the impact of viral replication on protein synthesis in the cytoplasm. In this study, we have used reporter mRNA transfection in influenza-infected cells together with the addition of infected extracts to the RRL (Fig.1). Both methods show that one or several viral proteins exerted a stimulatory effect on translation. A likely candidate was the non-structural protein NS1, which is required at multiple levels during viral replication both in the nucleus and the cytoplasm [19]. As far as translation is concerned, NS1 was previously shown to stimulate viral protein synthesis by an interplay with factors involved in the interferon pathway, by interacting with many different actors of the translational apparatus including eIF4G, PAPB, PKR, and hStaufen [24–26], and by binding directly to defined motifs located in the 5′UTR of influenza mRNAs [28]. In addition to its role on influenza mRNAs, a recent report shows that it can also activate the translation of other mRNAs such as those driven by the β-globin 5′UTR and the EMCV and HCV IRESes, but the mechanism by which this occurs remains to be solved [30]. By using a wide spectrum of cellular mRNAs and IRES-driven constructs, we have investigated the molecular basis of NS1 stimulation on protein synthesis to demonstrate its role on all mRNAs tested with the exception of the IRESes from dicistroviruses, namely DCV and CrPV IRESes (Fig. 2). More importantly, we could observe differences in the magnitude of stimulation depending on the presence of the cap and/or the poly(A) tail and also on the nature of the 5′UTR (Fig. 2). Such an observation pointed to a role for NS1 in the early steps of protein synthesis, at the level of translation initiation.

The lack of stimulation by the addition of NS1 to translation assays programmed with DCV and CrPV 5′UTRs was very informative about a possible role of NS1 on protein synthesis. Indeed, these intergenic dicistronic IRESes exhibit very peculiar features as they lack requirement for any components of the entire translational machinery, including initiator tRNA [41,42]. Interestingly, a recent study by Ramakrishnan and colleagues demonstrated that CrPV IRES has the ability to mimic a pretranslocation rather than an initiation state of the ribosome [43]; in other words, initiation does not occur on the CrPV IRES, and protein synthesis starts immediately with the elongation phase. The implication of the NS1 protein as an enhancer for translation initiation was initially proposed by Ortin and colleagues [27] more than 20 years ago. Our data are in complete agreement with this hypothesis, and we then set to investigate how NS1 was utilized to promote translation initiation.

Thus, we have first investigated which of the structural domains of NS1 could be involved in translation stimulation. By using deletion and mutation analysis, we demonstrated that: (i) the first 70 aa at the N-terminal part were required for translation stimulation and (ii) double mutation of RNA-interacting R38 and K41 amino acids (mutant R38AK41A) abolished this stimulation (Fig. 3). This R38AK41A mutant has been previously described and was shown to impair the RNA-binding activity of NS1 [44–47] and associated functions in viral replication [48]. It is noteworthy that the stimulation of translation with this mutant has also been documented if the latter was originated from the H5N1 and H5N2 influenza strains but not from the H1N1 one, which is the one used in this study [30,49]. This leads the authors to conclude a strain-specific activity of NS1 proteins on translation. In our hands, EMSA-binding experiments showed that the R38AK41A mutant protein was unable to complex with any of the mRNAs tested (β-globin, HCV, or CrPV), whereas the WT protein was found specifically associated to both HCV and CrPV IRESes but not with the β-globin 5′UTR. This is in agreement with reports showing that NS1 interacts preferentially with double-stranded (such as HCV and CrPV IRESes) rather than single-stranded RNAs (such as β-globin 5′ UTR) [35,36]. However, it also indicates that the direct association of the NS1 to the 5′UTR of a given transcript is not correlated with its capacity to stimulate translation from this given transcript; in other words, the fact that NS1 associates to the 5′UTR of a mRNA is not indicative of any translational enhancement (Fig. 4). A priori, this could be contradictory with the fact that the RBD domain is needed for stimulation (Fig. 3) unless NS1 can bind to RNAs other than messenger RNAs. A likely candidate to interact with NS1 would be the ribosome through the rRNAs. By using the fractionation of the reticulocyte lysate (Fig. 4), we observed the preferential association of NS1, but not the R38AK41A mutant, to the ribosomal fraction in a translation-independent manner (Fig. 4). Moreover, the association of NS1 to the ribosomal pellet was also evidenced in the nuclease-treated RRL, suggesting that NS1 can bind to the ribosome independently from the presence of the mRNA. However, a comparison of ribosomal fractions derived

from nuclease-treated RRL and uRRL revealed that the binding of NS1 was stronger when ribosomes were pelleted from the uRRL, hence in the presence of endogenous mRNAs (Fig. 4).

Association with the ribosomes was also evidenced *in vivo* in the context of influenza infection of A549 cells by performing polysome profile analysis on sucrose gradients in both mock and influenza-infected cells; this showed that the NS1 protein was associated to the 40S, 60S, and polysome fractions in infected cells (Fig. 4). Finally, EMSA between recombinant NS1 protein and isolated rRNAs confirmed a direct interaction in the context of the wild-type protein but not with the R38AK41A mutant form (Fig. 4). Interestingly, Marc and colleagues performed an *in vitro* evolution approach (systematic evolution of ligands by exponential enrichment) and suggested that NS1 could bind with high affinity RNAs bearing the "GUAAC" motif and its reverse complement [28]. Along this line, we found several copies of this motif in both 28S and 18S rRNAs (Supplementary Fig. 3) that systematically reside within conserved (single stranded) loops or bulges in the canonical rRNA structures (Supplementary Fig. 3), thus suggesting that NS1 could directly interact with such rRNA motifs.

Therefore, this prompted us to search for a functional relationship between the presence of NS1 on the ribosomal fraction and its ability to stimulate translation. As a first approach, we showed that maximal activity of NS1 was monitored when the ribosomal population was in excess over the concentration of mRNAs in the assay (Fig. 5a). This suggests that the role of NS1 would somehow reside in its ability to augment the ability of a given mRNA to recruit ribosomes. This was confirmed by using a fractionated/reconstituted lysate in which the final ribosome concentration was added in large excess, and it indicated that the NS1 protein, but not the R38AK41A mutant, could recruit these additional ribosomes in a translational competent manner (Fig. 5c). Conversely, lowering the concentration of ribosomes available in the RRL abolished the stimulatory effect of NS1 (Fig. 5d). Taken together, our data suggest a novel role for the NS1 protein, which can stimulate translation initiation on any mRNAs by increasing their ability to recruit ribosomes in a translational competent manner. However, it remains to be determined whether this is exerted at the level of initial ribosome binding or if it could increase the speed of ribosomal scanning. Nevertheless, as NS1-mediated stimulation is observed with HCV and HCV-like IRESes (Fig. 2, HCV, AEV, and SVV), this already points toward the former hypothesis, as these types of IRESes do not use ribosomal scanning to initiate translation [32]; however, further work is needed to validate this assumption.

In the context of the influenza viral life cycle, a global stimulation of both viral and cellular translation would most probably benefit the virus. Indeed, the rapid shut-off of host gene expression caused by the subversion of the transcriptional and export machinery [8,9] and degradation of cellular mRNAs by cap-snatching leads to a rapid and drastic diminution of the intracellular concentration of mRNAs in the infected cell. Such a situation should create adequate conditions for NS1 to recruit ribosomes from the large cellular pool available, and this would ultimately result in an increase of viral production.

Materials and Methods

DNA constructs

The β-globin, GAPDH, PABP, cyclinD2 cellular 5′ UTR, FMDV, HAV, AEV, DCV, HCV, and CrPV IRESes p0-renilla were previously described [50–52] (for signification of the acronyms, please refer to Supplementary Fig. 1). The NP 5′UTR of A/Puerto Rico/8/1934(H1N1) was constructed by inserting an oligo duplex corresponding to the sequence of the viral 5′UTR into PvuII/BamH1 sites and the p0-renilla or the HpaI/BamH1 sites of the pcDNA3.1-renilla. PMRF kindly provided by Prof. A.E. Willis [53] was used for the PCR amplification of Myc 5′UTR using specific primers with the same restriction site previously described to be cloned into p0-renilla vector. The pcDNA3.1 vector was modified after the CMV promoter in order to minimize the addition of unspecific nucleotides incorporated upstream of the 5′UTRs of interest. Position of the +1 transcription site was mapped by rapid amplification of cDNA extremity (Ambion kit). The p0-bicistronic construct was constructed by inserting the EMCV 5′UTR into the p0-β-globin-firefly vector (at the AflII restriction site). Truncated and mutated NS sequences were obtained by PCR amplification of the NS1 segment of the PR8 strain (H1N1) and inserted into the XhoI and EcoRI restriction sites of the pcDNA3.1 (hygro-) vector, p0-renilla vector, and pET-21. The pcDNA vector was used for cDNA transfection into cells, the p0 vector for *in vitro* transcription, and pET-21 for bacterial production of recombinant proteins.

In vitro transcription

RNAs were transcribed using the T7 RNA polymerase from templates linearized either at the AflII (for polyadenylated mRNAs) or at the EcoRV sites (for non-polyadenylated mRNAs). Uncapped mRNAs were obtained by using 1 μg of linear DNA template, 20 U of T7 RNA polymerase (Promega), 40 U of RNAsin (promega), 1.6 mM of each ribonucleotide triphosphate, and 3 mM DTT in transcription buffer [40mM Tris–HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl]. For capped mRNAs, the rGTP concentration was reduced to 0.32 mM, and 1.28 mM of m^7GpppG cap analog (New England

Biolabs) was added. The transcription reaction was carried out at 37 °C for 2 h, and the mRNAs were precipitated with ammonium acetate at 2.5-M final concentration. The RNA pellet was resuspended in 30-µL RNAse-free water, and RNA concentration was determined by reading the absorbance using Nanodrop technology. RNAs integrity was checked by electrophoresis on non-denaturing agarose gel.

Cell culture and mRNA transfection

Cell culture

A549 and MDCK cells were obtained originally from American Tissue Type Culture Collection and were typically grown in DMEM containing 10% fetal calf serum supplemented with 50 U/ml of penicillin and 50 µg/ml of streptomycin (PS) in a humidified atmosphere containing 5% CO_2 at 37 °C. MDCK were grown in serum-free Ultra-MDCK medium (Lonza) supplemented with 2 mM L-glutamine (Sigma Aldrich), 225 U/ml of penicillin, and 225 µg/ml of streptomycin (PS) in a humidified atmosphere containing 5% CO_2 at 37 °C.

RNA transfection

A549 cells were electroporated with 100 ng of *in vitro* synthesized mRNAs (see above) for 10^5 cells with the Neon™ system (life technology) or with Amaxa Nucleofector Technology as specified by the manufacturers. Cells were lysed 1 h post-transfection of the mRNA, and luciferase activity was determined.

Virus amplification and infection

Influenza A viral strain A/Puerto Rico/8/1934 (H1N1) (PR8) was obtained from the French national influenza monitoring network GROG (Groupes Régionaux d'Observation de la Grippe, Lyon, France) and was amplified by two passages in MDCK cells.

For infection, virus was inoculated at a multiplicity of infection of 6 in DMEM supplemented with 200 units/ml penicillin, 200 µg/ml streptomycin, 2 mM L-glutamine, and 0.5 µg/ml TPCK-trypsin (Roche diagnostics). After 2 h at 37 °C, the inoculum was removed and replaced by fresh medium. Cells were then incubated for 8 h before being collected. The efficiency of infection was monitored by FACS analysis using NP antibody coupled with red fluorophore.

Cells extract

All following steps were performed at 4 °C: 5.10^7 A549 cells were collected by centrifugation at 1000*g* for 5 min, rinsed 3 times with PBS, resuspended in an isovolume of hypotonic buffer [10 mM Hepes, 10 mM CH_3CO_2K, 1 mM $(CH_3CO_2)_2Mg$, and DTT 1 mM], and lysed with a potter dounce homogeneizer. The lysate was then centrifuged at 16,000*g* for 10 min, and the supernatant was collected to yield the S10 supernatant extract. S10 protein total concentration was determined by the Bradford method.

Purification of the NS1 recombinant protein

E. coli BL21 bacteria expressing the pET-21(+)-NS1 and pET-21(+)-NS1R38AK41A vectors were grown to an OD of 0.6–0.8 (at A_{600}) before induction with 0.2 mM IPTG for 2 h at 37 °C. Bacteria were centrifuged at 6,000 rpm for 10 min, and pellets were resuspended in 20 mM Tris–HCl (pH 7.5), 300 mM KCl, and 10% glycerol and sonicated on ice. The supernatant was collected by centrifugation at 10,000 rpm for 25 min and added to a 800-µl Bio-Rad Polyprep chromatography column (50% Ni-NTA, Quiagen). The protocol has been previously described by de Breyne *et al.* [54] and briefly consists of washing the column successively with 20 mL of buffer A-300 [20 mM Tris (pH 7.5), 300 mM KCl, and 10% glycerol], 10 ml buffer A-800 [20 mM Tris (pH 7.5), 800 mM KCl, and 10% glycerol], buffer A-100 [20 mM Tris (pH 7.5), 100 mM KCl, and 10% glycerol], and 30ml buffer A-100/I20 (A-100 plus Imidazole 20 mM) before elution with 6 mL buffer A-100/I300 (A-100 plus imidazole 300 mM) and finally dialyzed overnight into 25 mM Tris (pH 7.5) at 4 °C.

EMSA

[^{32}P]-UTP radiolabeled RNAs were synthesized by *in vitro* transcription as described above but using 0.5 mM rNTPs (without rUTP), 12.5 µM rUTP, and [$^{\alpha\text{-}32}$P]-rUTP (40 mCi/reaction). RNAs were treated with 2 U of RQ1 RNase-free DNase (Promega), precipitated with three volumes of 100% ethanol, and resuspended in water before being purified using Ilustra Microspin G25 column (GE Healthcare). For all reactions, 0.044 pmol (4.4 nM) of [^{32}P]-RNA was incubated for 15 min at 37 °C with recombinant NS1 or NS1R38AK41A [or elution buffer (H_2O) as control] in binding buffer [30 mM Tris–HCl (pH 7.0), 0.5 mM MgCl2, 0.1 mM ZnCl2, and 50 mM NaCl] in the presence of 40 U/reaction of RNAsin (Promega). After incubation, samples were analyzed on 1.5% non-denaturing Agarose TBE gels. Gels were dried, and images were acquired by using a PhosphorImager FLA 5100 (Fuji). rRNA extraction was performed on fractions using Phenol:chloroform liquid extraction followed by isopropanol/ethanol precipitation steps.

Analysis of ribosomes in infected cells by sucrose density gradient centrifugation

Eight hours after infection with the H3N2-MO strain, A549 cells were treated with 100 µg/ml cycloheximide (Sigma) for 10 min. The cytoplasmic fractions were prepared on ice as described above, except that cycloheximide was added to all samples. The

equivalent of 1 mg of total proteins was loaded on a 10–50% (wt/wt) sucrose gradient prealably poured with a Gradient Master former (BioComp Instruments, Fredericton, NB, Canada). Samples were centrifuged at 36,000 rpm for 105 min in a SW41 rotor at 4 °C (Optima L100XP ultracentrifuge; Beckman Coulter, Villepinte, France). Fractions were collected at OD254 nm using a Foxy Jr. gradient collector (Teledyne Isco, Lincoln, NE, USA).

Expression and purification of the NS1 recombinant protein

E. coli BL21 cells expressing the pET-21(+)-NS1 and pET-21(+)-NS1R38AK41A vector were grown until A_{600} reached 0.6–0.8 and then induced with 0.2 mM IPTG for 2 h at 37 °C. After centrifugation of bacterial cultures, pellets were resuspended in 20 mM Tris–HCl (pH 7.5), 300 mM KCl, and 10% glycerol and then sonicated on ice. Supernatants derived from the bacterial lysate by centrifugation were added to a Ni-NTA column that has been prepared by adding 800 ml of a 50 % Ni-NTA suspension (Qiagen) to a Bio-Rad Polyprep chromatography column. Washes and 2imidazole elutions were done according to the manufacturer. Samples obtained during every step were analyzed by 12% SDS-PAGE and Coomassie blue staining.

In vitro expression of GFP and NS1 proteins

In vitro transcribed mRNAs coding for GFP, NS1, and NS1 mutants were translated at 27 nM (final concentration) in a final volume of 10 µl [50% (vol/vol) RRL, Promega] supplemented with 75 mM KCl, 0.75 mM $MgCl_2$, and 20 µM aa mix. The translation mixture was incubated for 30 min at 30 °C before being frozen, aliquoted, and kept at – 80 °C.

Preparation of uRRL and *in vitro* translation assays

uRRL-The method used has been described previously [51] and can be briefly summarized as follows: 1 ml of uRRL was supplemented with 25 µM hemin (Fluka), 25 µg creatine phosphokinase (Sigma Aldrich), 5 mg creatine phosphate (Fluka), 50 µg of bovine liver tRNAs (Sigma Aldrich), and 3 mM of D-glucose (Sigma Aldrich).

In vitro translational assay. *In vitro* transcribed mRNAs were added at 2.7 nM to a final volume of 10 µl [50% (vol/vol) RRL (or uRRL), Promega] supplemented with 75 mM KCl, 0.75 mM $MgCl_2$, and 20 µM aa mix. The translation reaction was incubated for 30 min at 30 °C before the reaction is stopped by the addition of renilla lysis buffer (Promega). For radiolabeled proteins, 5 µCi of [^{35}S]-methionine [specific activity: >1000 Ci (37.0TBq)/mMole] was added in the presence of 20 µM of amino acids mix lacking methionine (Perkin Elmer).

Western blot and autoradiography

Samples were resolved on a 12% SDS-PAGE, transferred to PVDF membrane, and blotted using anti NS1 (Santa Cruz), anti NP (Santa Cruz), and anti RPS6 (Abcam) or dried and subjected to autoradiography for 12 h by the use of Kodak Biomax films (Fisher Scientific), and the signal was quantified by using a Molecular Dynamics PhosphoImager FLA 5100 (Fuji).

Renilla activity

Renilla activity was measured using the renilla luciferase Assay System (Promega Co, Madison, WI, USA) in a Mithras (Berthold technologies) with 50-µl substrate injection and 10 s of signal integration program.

CD Measurements

Far-UV CD spectra were recorded with a Chirascan spectrometer (Applied Photophysics, United Kingdom) calibrated with 1*S*-(+)-10-camphorsulfonic acid. Measurements were carried out at 25 °C in a 0.1-cm-path-length quartz cuvette (Hellma), with a typical peptide concentration of 25 µM. Spectra were measured at 190- to 320-nm wavelength range with an increment of 0.2 nm, band pass of 0.5 nm, and integration time of 1 s. Spectra were processed, baseline corrected, smoothed, and converted with Chirascan software. Spectral units were expressed as the mean molar ellipticity per residue by using the peptide concentration determined with UV light absorbance directly measured with a CD cell at 280 nm (e = 3,900 M^{-1} cm^{-1}) of the peptide solubilized into phosphate buffer. Estimation of the secondary structure content was carried out using the DICHROWEB server facilities† [55]. CD spectra are reported as variation of ellipticity (ΔL-ΔR), in units of M^{-1} cm^{-1}.

RRL or uRRL fractionation and reconstitution

uRRL or nuclease-treated RRL (Promega) preparation was performed as described previously [51]. All steps are performed at 4 °C. After centrifugation of 300 µl of uRRL for 2 h and 15 min at 75,000 rpm with a TLA 100.3 rotor from Beckman (R_{max} = 48.3 mm) to reach 240,000*g*, 250 µl of post-ribosomal supernatant (S100) was collected, frozen, and stored at – 80 °C. The extent of ribosome depletion from reticulocyte lysate was checked by translating 27 nM of *in vitro* transcribed capped and polyadenylated globin-renilla mRNA in the S100 and was validated when no luciferase activity could be detected. The ribosomal pellet (C100) was then rinsed three times in buffer R2 (20 mM Hepes, 10 mM NaCl, 25 mM KCl, 1.1 mM $MgCl_2$, and 7 mM β-mercaptoethanol) and resuspended in 100 µL of R2 buffer to get a 3X final concentration. The reconstituted

lysate is then assembled by mixing 5 μl of S100 supernatant with a scale from 0.5 to 4 μg of C100 as indicated on Fig. 5c. Typically, the standard reaction contains 5 μl of S100 with 1 μg of C100 (annotated 1X) in a final volume of 10 μl. The concentration of S100 to C100 can be adjusted as indicated in the figure. Upon reconstitution, the translation mixture is supplemented with 75 mM KCl, 0.75 mM $MgCl_2$, and 20 μM aa mix in a final volume of 10 μl.

Bioinformatics analysis

All NS1 nucleotide sequences present in the "nr" and "pat" section of Genebank were recovered (on February 6, 2013) using Blast searches on the NCBI Website and using PR8 sequence as a bait, with a 70% similarity cutoff, and filtered for Influenza A sequences with a complete NS1 open reading frame. The NS1 amino acid sequences were determined for the 19,310 sequences recovered; the 1-81 section was extracted for each of them, and identical sequences were eliminated. The resulting set of 180 non-redundant 1-81 sequences was aligned using the Muscle multiple alignment software [56] using default settings. The frequency of each amino acid was determined at each position and displayed by decreasing frequency order. However, amino acids present at a frequency of <0.01 were not displayed, in order to eliminate possible sequencing errors with no functional meaning. All steps (except multiple alignments) were performed using custom Perl scripts.

In silico analysis of NS1 RNA-binding sites on ribosomes

Exact matches to the four specific NS1 RNA-binding sites (as described by Marc *et al.* [28]): "GUAAC" and its reverse-complement "GUUAC", "AGCAAAAG", and "UGAUUGAAG") were searched in the four human rRNA Genebank RefSeq sequences (i.e., 28S: NR_003287, 18S: NR_003286, 5.8S: NR_003285, and 5S: AJ508600) using Perl scripts. We only found "GUAAC" and "GUUAC" sites in 28S and 18S rRNAs. The sites were then positioned on the human ribosome secondary structures published by Anger *et al.* [57], based on NR_003287 (28S) and X03205 (18S). The current version of NR_003287 differs from that in Anger *et al.* by a 4-nt offset.

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Abbreviations used:
RBD, RNA-binding domain; RRL, rabbit reticulocyte lysate; uRRL, untreated RRL; EMSA, electrophoretic migration shift assay; rRNA, ribosomal RNA.

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