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The N-terminal half of the influenza virus NS1 protein is sufficient for nuclear retention of mRNA and enhancement of viral mRNA translation

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ABSTRACT

A collection of C-terminal deletion mutants of the influenza A virus NS1 gene has been used to define the regions of the NS1 protein involved in its functionality. Immunofluorescence analyses showed that the NS1 protein sequences downstream from position 81 are not required for nuclear transport. The capacity of these mutants to bind RNA was studied by *in vitro* binding tests using a model vRNA probe. These experiments showed that the N-terminal 81 amino acids of NS1 protein are sufficient for RNA binding activity. The collection of mutants also served to map the NS1 sequences required for nuclear retention of mRNA and for stimulation of viral mRNA translation, using the NP gene as reporter. The results obtained indicated that the N-terminal 113 amino acids of NS1 protein are sufficient for nuclear retention of mRNA and stimulation of viral mRNA translation. The possibility that this region of the protein may be sufficient for virus viability is discussed in relation to the sequences of NS1 genes of field isolates and to the phenotype of known viral mutants affected in the NS1 gene.

INTRODUCTION

The influenza type A virus genome consists of eight single-stranded RNA molecules of negative polarity. They encode 10 different proteins, since the smallest segments encode two distinct gene products by differential splicing (for reviews, see 1,2). The first step in viral gene expression during productive infection is primary transcription from the incoming viral ribonucleoproteins (3). The expression of virus proteins, at least nucleoprotein (NP), leads to the shift to RNA replication by the synthesis of complete positive-polarity RNAs (cRNAs) (4,5), which serve as template for virion RNAs (vRNAs) synthesis. Transcription and replication of viral RNA takes place in the nucleus of the infected cell (6,7) and requires at least the activity of the three subunits of the polymerase (PB1, PB2 and PA) and the NP (8–12).

Early after the beginning of vRNA synthesis, transcription is coupled to replication. Some proteins, like NS1 or NP, are expressed earlier than others, like M1 or HA, because their vRNAs are replicated earlier (13). However, later in the process of vRNA synthesis, transcription is discontinued and viral protein synthesis relies on the use of previously synthesized mRNAs (13). In the course of infection, viral gene expression takes over the cell machinery. Such shut-off phenomena have been related to a series of alterations induced by the virus in the infected cell, such as nuclear retention and degradation of polymerase II transcripts in the nucleus (14), cytoplasmic degradation of pre-existing cellular mRNAs (15,16) and preferential utilization of the translation machinery by the viral-specific mRNAs (17). The virus supresses the activation of the PKR kinase by induction of a cellular inhibitor (18,19).

The NS1 protein is translated from the unspliced transcript of segment 8 (20,21) and is the only non-structural protein encoded by the virus. NS1 is a nuclear protein, both in the infected cell (22,23) and when expressed from cDNA (24–26). It accumulates in the nucleus early in the course of the infection, and in both the nucleus and the cytoplasm at later times (27). In addition, a portion of the NS1 protein has been found in association with polysomes (23,28). It has been shown that NS1 protein can bind various types of RNA, like vRNA, poly-A-containing RNAs, U6 snRNA and viral mRNA (29–33, and our unpublished results), and its expression from cDNA in cultured cells leads to a variety of alterations in cellular processes that involve RNA. These include nuclear retention of poly-A-containing mRNAs (30,34), alterations in the splicing of pre-mRNAs (30,34–36) and enhancement of the translation of viral, but not cellular, mRNAs (28,37). The spliced transcript of segment 8 encodes the NS2 protein (20,21), a small protein previously thought to be non-structural but recently found to be present in the virion in association with M1 protein (38,39).

Mutational analysis of the NS1 gene that involved the exchange of two or three consecutive amino acids at various positions along the NS1 protein led to the proposal that it contains two separate functional domains: an N-proximal domain (amino acids 19–38), that would be involved in RNA binding, and a C-proximal domain (amino acids 134–161), not required for RNA binding but presumably acting as effector for the functions of nuclear retention of poly-A-containing RNA and inhibition of splicing (35,40). In the present report, we used a series of C-terminal deletion mutants of NS1 protein to assay their activity in nuclear retention of poly-A-containing RNA and enhancement of viral

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mRNA translation, as well as their capacity to translocate to the nucleus and to bind RNA. The results obtained are consistent with the notion that the N-terminal half of the protein is sufficient for the biological activities tested. These results are discussed in the context of the genotype and phenotype of virus temperature-sensitive mutants and natural isolates.

MATERIALS AND METHODS

Biological materials

The COS-1 cell line (41) was obtained from Y.Gluzman and the HeLa cell line was purchased from the American Type Culture Collection. Cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal calf serum, as described (42). The Vaccinia recombinant virus expressing the T7 RNA polymerase (vTF7-3) (43) was kindly provided by B.Moss.

Plasmid pSVa963 contains the influenza virus nucleoprotein cDNA cloned under the SV40 control elements (44). Plasmid pGNP contains the NP gene under control of the T7 promoter (11). In plasmid pSVa232NS1 [a derivative of plasmid pSVa232N (45)], expression of influenza virus NS1 protein is driven by SV40 genome late control region (34). Hybrid plasmids expressing NP and NS1 genes (pSVaNPNS1) or NP and NS2 genes (pSVaNPNS2) have been reported previously (34). For the construction of pG4NS1 plasmid, the NS1 gene from plasmid pSVa232NS1 was isolated by digestion with *Asp*718 and *Eco*RI restriction enzymes and inserted between *Asp*718 and *Eco*RI sites of pGEM4 vector (Promega). Plasmid pvNSZ has been described previously (12). Plasmid pMS21, containing a TGE minigenome, was kindly provided by A.Izeta and L.Enjuanes.

For the preparation of antisera specific for NS1 protein, the open reading frame of NS1 gene was cloned into pRSET vector (Invitrogen). The resulting pRSHisNS1 plasmid was transferred to *Escherichia coli* BL21 DE3 pLys S cells and the protein was expressed by induction with IPTG. The protein was purified by affinity chromatography on agarose NTA–Ni^{2+} resin (Invitrogen) and used to immunize rabbits. Rabbit antiserum specific for the C-terminal half of the NP was a generous gift of A.Portela. Monoclonal antibody specific for the T7 tag present in the pRSET vectors was purchased from Novagen.

Mutant construction and characterization

Mutants pGNS1Δ82-238 and pGNS1Δ111-238 were constructed by digestion of plasmid pG4NS1 with *Nco*I or *Xcm*I, respectively, filling in with Klenow enzyme and religation. Mutants pGNS1Δ114-238 and pGNS1Δ212-238 were prepared by partial deletion of internal *Ava*II fragments, filling in with Klenow enzyme and religation. Mutants pGNS1Δ153-238 and pGNS1Δ172-238 were generated by site-directed mutagenesis using the Transformer kit of Clontech and oligonucleotides 5′-GCTTTCACCGAATAGGGAGCAATT-3′ and 5′-CATACT-ATATAGGATGTAAAAAAT-3′, respectively. To express the mutant NS1 proteins with an N-terminal His-tag, the corresponding mutant genes were transferred from the original pG4NS1 recombinants to plasmid pRSHisNS1 by swapping the *Eco*RI–*Sau*I restriction fragment. Likewise, the mutant genes were transferred to plasmids pSVa232NS1 and pSVaNPNS1 by swapping the corresponding *Asp*718–*Eco*RI DNA fragments.

DNA manipulation and cloning was carried out according to standard techniques (46), using *E.coli* DH-5 as host. The mutations in the NS1 gene were checked by dideoxy-termination sequencing with specific primers (47).

Cell transfection

For transfection of expression plasmids driven by the T7 promoter, COS-1 cell cultures were infected with Vaccinia vTF7-3 virus at a multiplicity of infection of 10 p.f.u./cell. At 1 h post-infection, the cells were transfected with a mixture of plasmid DNA and cationic liposomes (48). The cells were incubated overnight with the transfection mixture, washed with PBS and used for further analyses. Expression plasmids driven by polymerase II promoters were transfected similarly but excluding the Vaccinia virus infection.

RNA isolation and characterization

Nuclear and cytoplasmic fractions from cells transfected with polymerase II-expressing plasmids were prepared at 60 h post-transfection as described previously (34). Poly-A^+ RNA and poly-A^- RNA fractions were obtained by oligo-dT chromatography as described (49).

For preparation of labelled NP riboprobe, plasmid pGNP was digested with *Sca*I nuclease and transcribed with Sp6 RNA polymerase, in the presence of [^{32}P]GTP (400 mCi/pmol). Plasmid pMS21 was transcribed with T7 RNA polymerase. Labelled vNSZ probe was synthesized as described (12). Dot hybridization was carried out as described previously (28).

To assay the nucleocytoplasmic transport we followed the methods described previously (34), with slight modifications. In brief, cultures of transfected COS-1 cells were fractionated into nuclear and cytoplasmic fractions at 60 h post-transfection. A sample of pMS21-derived RNA was added to each fraction, total RNA was isolated and separated into poly-A^+ and poly-A^- RNAs by chromatography on oligo-dT cellulose (50). Portions of the poly-A^+ RNA were processed for dot-hybridization with an NP-specific probe to determine the NP mRNA content and with a pMS21-specific probe to normalize RNA recovery in the process. Hybridization was quantitated by microdensitometry of appropriate exposure of the films or in a phosphorimager.

Protein analyses

Unlabelled extracts from infected–transfected cells were prepared by solubilization in 100 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 7.5, 1% NP-40 (TNE-1% NP-40), and centrifugation for 10 min at 12 000 r.p.m. at 4°C. For protein labelling, cell cultures were infected–transfected as described above, but AraC (50 µg/ml) was included in every media used. At 5 h post-transfection the cells were washed with DMEM devoid of methionine and cysteine, depleted of these amino acids for 1 h in the same medium and incubated overnight with DMEM containing 1/10 the normal concentration of these amino acids and suplemented with [^{35}S]Met–Cys (20 µCi/ml). Finally, the cells were washed and total cell extracts were prepared in Laemmli sample buffer. Western blot and immunofluorescence analyses were carried out as described (28,36).

The assay for NS1-mediated enhancement of viral mRNA translation has been described previously (34). In brief, cultures of COS-1 cells were transfected as indicated above. At 60 h post-transfection, the cells were harvested and total cell extracts

were prepared from a portion of the culture. The content of NP was determined by western blot using an NP-specific polyclonal antibody and enhanced chemiluminiscence. The remaining portion of the culture was processed for the isolation of poly-A^+ RNA from the cytoplasmic fraction and the content of NP mRNA was determined as indicated above.

RNA binding

NS1-specific immunocomplexes were prepared by binding of NS1-specific rabbit serum to protein A–Sepharose for 60 min at room temperature and washing six times with TNE–1% NP-40 buffer. This preformed matrix was incubated for 60 min at room temperature with soluble extracts of cells infected with vTF7-3 virus and transfected with either pRSHisNS1 plasmid, pRSHisNS1 mutant plasmids or pRSET empty vector. After washing six times with TNE–1% NP-40–2 mg/ml heparin buffer, the immunocomplexes were incubated with labelled vNSZ probe for 30 min at 4°C in a buffer containing 50 mM Tris, 5 mM $MgCl_2$, 100 mM KCl. After washing three times with the same buffer, the bound material was analyzed for its RNA and protein contents. The RNA was isolated by treatment with proteinase K (50 μg/ml) for 30 min at 37°C and phenol extraction. Purified RNA was analyzed by electrophoresis in 4% polyacrylamide–urea gels. Total protein was extracted in Laemmli sample buffer and analyzed by western blot with anti-T7 tag antibody after electrophoresis in 7–15% polyacrylamide–SDS gradient gels.

RESULTS

Construction and characterization of NS1 protein mutants

In order to identify regions of the NS1 protein responsible for the multiple functions assigned to this small viral protein, a series of C-terminal deletion mutants was constructed, as detailed in Materials and Methods. The sizes of the NS1 protein derivatives encoded in such mutants ranged from 81 to 211 amino acids (Fig. 1A). Their expression was ascertained by means of the Vaccinia-T7 infection–transfection protocol. Cultures of COS-1 cells were infected with a Vaccinia recombinant virus expressing the T7 RNA polymerase [vTF7-3; (43)] and transfected with either pG4NS1 plasmid, each of its mutant derivatives or pGEM plasmid as a control. The infected–transfected cultures were continuously labelled with [^{35}S]Met–Cys and total cell extracts were prepared. The analysis of such extracts by polyacrylamide gel electrophoresis and autoradiography is shown in Figure 1B. The uneven distribution of methionine and cysteine residues in the NS1 protein led to a 3-fold underestimation of the expression level of mutant NS1Δ82-238 and a 2-fold underestimation of mutants NS1Δ111-238 and NS1Δ114-238. In spite of these corrections, the mutant protein of 110 amino acids was expressed to a reduced level as compared to wtNS1 protein or the rest of the mutants (see also Fig. 3 below for a further test of the expression of mutant proteins).

Nuclear translocation and RNA-binding properties of NS1 mutants

As a first step in the characterization of the NS1 mutants, we asked whether progressive deletion from the C-terminus would affect the translocation to the nucleus or alter the RNA-binding

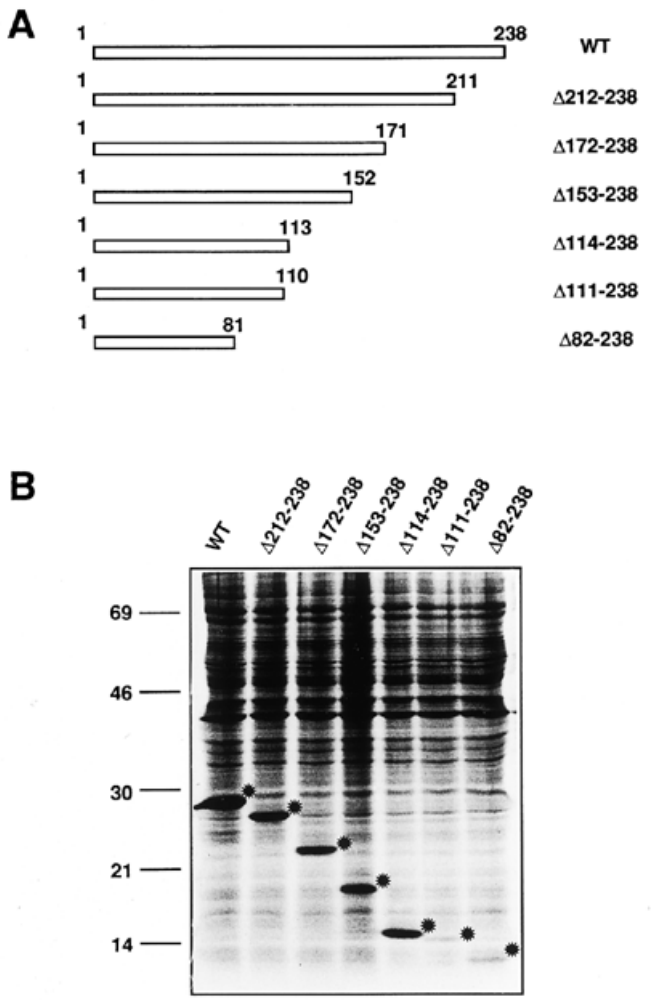

Figure 1. Structure and expression of NS1 protein mutants. (**A**) Diagram of the structure of NS1 mutant proteins used in this report. (**B**) Cultures of COS-1 cells were infected with vTF7-3 virus and transfected with pGEM plasmid, plasmid pG4NS1 or its mutant derivatives, as indicated on the top of the figure. The infected–transfected cultures were continuously labelled with [^{35}S]Met–Cys and total extracts were analyzed by electrophoresis in 7–15% polyacrylamide–SDS gels. The Met–Cys contents of mutants NS1Δ82-238 and NS1Δ111-238 are 3- and 2-fold lower, respectively, than the wtNS1 protein. The mobility of molecular weight markers is indicated to the left of the figure. The stars highlight the protein-specific bands.

properties of the mutant proteins. Previous work had indicated that NS1 protein contains two independent nuclear localization signals (NLSs), one located at residues 34–38 and the other close to the C-terminus (26). We studied the intracellular localization of wild-type or mutant NS1 proteins by indirect immunofluorescence of HeLa cells transfected with plasmids pSVa232NS1, 232Δ111-238, 232Δ114-238, 232Δ153-238, 232Δ172-238 and 232Δ212-238. The results presented in Figure 2 indicate that all mutants behave as wild-type NS1 with regard to nuclear transport. Mutant protein Δ82-238 could not be analyzed because the anti-NS1 serum used did not detect it. Thus, our results confirm those of Greenspan *et al.* (26) and verify that the NLS located close to the N-terminus of NS1 protein is sufficient for its translocation

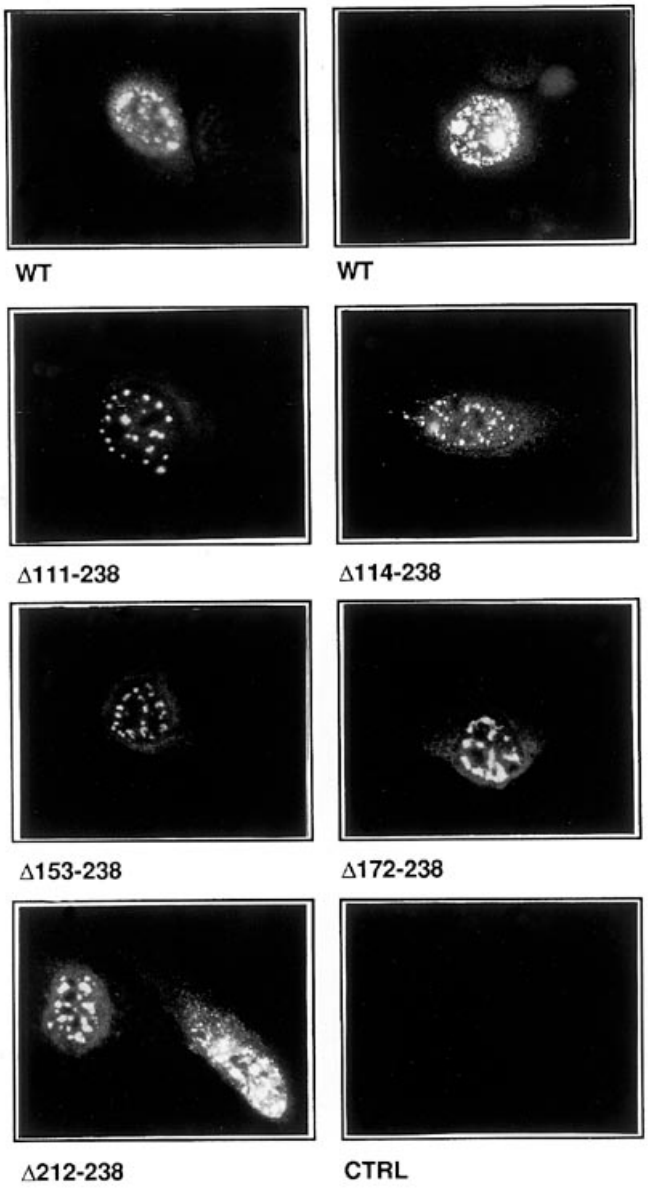

Figure 2. Intracellular localization of NS1 mutant proteins. Cultures of COS-1 cells were transfected with plasmid pSVa232NS1 (WT), its mutant derivatives or mock-transfected (CTRL). After 60 h of incubation, the cultures were fixed and processed for immunofluorescence using anti-NS1 rat serum and rhodamine-tagged goat anti-rat IgG antibodies.

into the nucleus. In addition, we could observe subtle differences in the intranuclear distribution of the mutant proteins: the smallest NS1 proteins showed a clearly distinct pattern of foci whereas wt NS1 protein and the largest mutants presented a more diffuse staining, also including foci (Fig. 2).

We next studied the RNA-binding properties of NS1 protein and its deletion mutants by *in vitro* retention of a model vRNA probe on NS1 protein immunocomplexes. COS-1 cell cultures were infected with Vaccinia vTF7-3 virus (43) and transfected with pRSHisNS1 plasmid or the corresponding deletion mutant plasmids. Either His-NS1 protein or His-NS1 mutant proteins thus expressed were bound to a preformed complex of anti-NS1

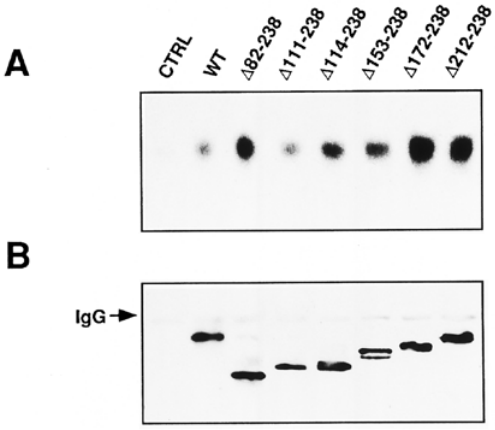

Figure 3. RNA-binding activity of NS1 mutant proteins. Soluble extracts from cells infected with vTF7-3 virus and transfected with pRSHisNS1 plasmid, its mutant derivatives or pRSET vector were incubated with a preformed complex of anti-NS1 IgG–protein A–Sepharose. After extensive washing, the immunocomplexes were incubated with vNSZ RNA and further washed. (**A**) The bound RNA was isolated and analyzed by electrophoresis in polyacrylamide–urea gels. (**B**) The bound proteins were analyzed by western blot using anti-T7 tag antibodies. IgG shows the position of the IgG of the immunoprecipitates, as detected in the western blots.

rabbit IgG–protein A–Sepharose. Previous experiments had shown that the rabbit anti-NS1 serum preferentially recognized the His-tag present in the His-NS1 protein used as immunogen (data not shown). The immobilized His-NS1 proteins were incubated with labelled vNSZ RNA (12), and the retained RNAs were isolated from a fraction of each sample and analyzed on sequencing gels. The binding was specific, since it could not be competed by a 1000-fold excess of unspecific, unlabelled RNA (data not shown). The results presented in Figure 3 indicate that all mutant proteins were able to bind RNA. The amount of wild-type or mutant protein present in the immunocomplexes was determined by western blot of other fractions of each sample with an anti-T7 tag monoclonal antibody (Fig. 3B). These results indicate that the 81 N-terminal residues of the NS1 sequence are sufficient for RNA binding.

Nuclear retention of mRNAs

Previous results from our laboratory had demonstrated that the expression of NS1 protein leads to the nuclear retention of the mRNAs of a coexpressed gene used as a reporter (34). To identify regions in the NS1 protein involved in such nuclear retention of mRNAs, we used the collection of deletion mutants described above in cell fractionation experiments. Cultures of COS-1 cells were transfected with pSVa963 plasmid (44), pSVaNPNS1 plasmid (34) or the corresponding recombinant plasmids in which the NS1 mutant genes substituted for the wild-type gene. The accumulation of NP mRNA in the poly-A^+ RNA isolated from either the nuclear or cytoplasmic fractions was determined by dot-blot hybridization with an NP-specific riboprobe. The results, presented in Figure 4 as the ratio of nuclear versus cytoplasmic accumulation of NP mRNA, are shown as the average and standard deviation of three to five independent experiments. The results obtained show that the NS1 sequences downstream of position 113 are dispensable for the nuclear retention phenotype. Thus, mutant NS1Δ114-238 behaves

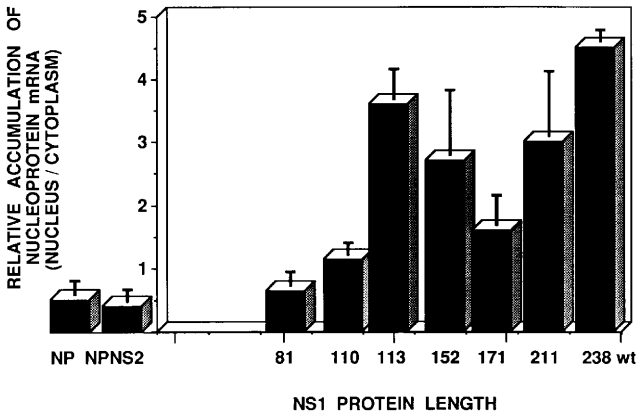

Figure 4. Nuclear retention of NP mRNA induced by NS1 mutant proteins. Cultures of COS-1 cells were transfected with plasmids pSVa963, pSVaNPNS1, its NS1 mutant derivatives as indicated in the figure or pSVaNPNS2 as a control. Nuclear and cytoplasmic fractions were obtained from the transfected cells and poly-A^+ RNA was isolated. The accumulation of NP mRNA was determined from either fraction by dot-blot using an NP-specific riboprobe. The results shown are the means and standard deviations of three to five independent experiments and are presented as the ratio of NP mRNA in the nuclear versus the cytoplasmic fraction.

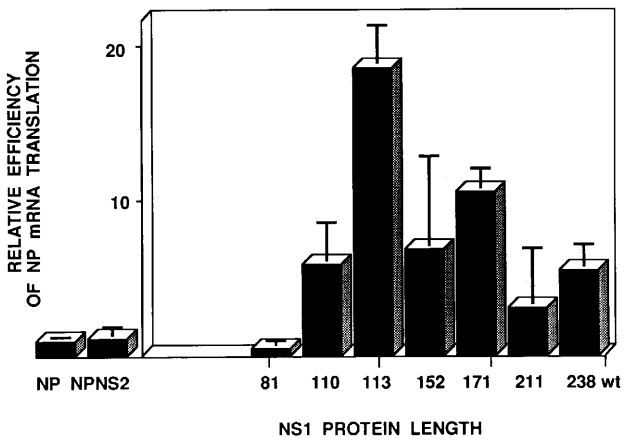

Figure 5. Stimulation of NP mRNA translation induced by NS1 mutant proteins. Cultures of COS-1 cells were transfected with plasmids pSVa963, pSVaNPNS1, its NS1 mutant derivatives as indicated in the figure or pSVaNPNS2 as a control. Using portions of the same transfected cultures, poly-A^+ RNA was isolated from the cytoplasmic fraction and total cell extracts were prepared. The accumulation of NP mRNA was determined by dot-blot using an NP-specific riboprobe and the content of NP in the total cell extracts was determined by western blot using an NP-specific serum. The efficiency of NP mRNA translation was calculated as the ratio of total NP protein versus NP mRNA present in the cytoplasm of the transfected cells. The results are presented as the ratio of the efficiencies of translation in cells co-expressing NP and either NS1 protein, NS1 mutant proteins or NS2 protein when compared to the values obtained for cells expressing only NP, and represent the means and standard deviations of three to four independent experiments.

essentially as wild-type NS1 and mutants NS1Δ153-238, NS1Δ172-238 and NS1Δ212-238 showed a clear nuclear retention of NP mRNA, although the dispersion of results was somewhat higher. On the other hand, the phenotype of mutant NS1Δ82-238 was indistinguishable from that obtained by the sole expression of NP or the co-expression of NP and NS2 protein, and mutant NS1Δ111-238 showed an intermediate phenotype. The amount of each mutant protein expressed in these transfections was difficult to determine because they were recognized with widely different efficiencies by the anti-NS1 serum available. Using the data obtained by continuous metabolic labelling (Fig. 1) and the accumulation of His-tagged protein (Fig. 3), we reasoned that the negative results obtained for mutant Δ82-238 cannot be interpreted as the consequence of a low level of protein expression. Thus, it is expressed to higher levels than mutant Δ111-238 and yet the latter shows an intermediae phenotype for nuclear mRNA retention (Fig. 4) and a positive phenotype for the stimulation of influenza mRNA translation (see below).

Enhancement of viral mRNA translation

We next examined the regions of NS1 protein required for the enhancement of translation of viral mRNAs (28). Cultures of COS-1 cells were transfected with plasmid pSVa963, expressing influenza virus NP (44), plasmid pSVaNPNS1 (34), expressing both NP and NS1 proteins or the corresponding recombinant plasmids with the NS1 deletion mutant genes. Transfection with plasmid pSVaNPNS2 (34), expressing NP and NS2 proteins was included as a negative control. The content of total NP and cytoplasmic NP mRNA was determined: total cell extracts from a portion of each transfected culture was assayed for NP by western blot. The other portion of each cell culture was separated into nuclear and cytoplasmic fractions, the poly-A^+ RNA from the cytoplasmic fraction was isolated and the content of NP mRNA was determined by dot-blot hybridization as indicated above. The ratio of total NP accumulated versus cytoplasmic NP mRNA was used as a measure of the translation efficiency of the NP mRNA in the transfected cells. The comparison of such a translation efficiency in cells expressing NP as a single transfected gene and in those co-expressing NP and NS1 verified that NS1 protein enhances the translation of NP mRNA, since we have previously shown that the stability of neither NP nor NP mRNA was altered by co-expression of NS1 protein (28). In Figure 5, the relative translation efficiencies obtained for the NS1 deletion mutants are presented in a comparison to that of wild-type NS1 protein. The results, averages and standard deviations for three to four independent experiments, indicated that the N-terminal 110 amino acids of NS1 protein are sufficient to observe the enhancement of NP mRNA translation. The results for mutants NS1Δ114-238 and NS1Δ172-238 indicated that they are even more active than wt NS1. The stimulation mediated by mutants NS1Δ111-238, NS1Δ153-238 and NS1Δ212-238 was more variable, but still clearly above the background of co-expression of NP and NS2 proteins. As observed in Figure 4 for nuclear retention of mRNA, mutant NS1Δ82-238 showed no detectable activity.

DISCUSSION

The results presented in this report support the notion that the N-terminal half of the NS1 protein is sufficient for many aspects of its biological activity. This has been tested by determination of the capacity of C-terminal mutants of the NS1 gene to retain poly-A^+ RNA in the nucleus of the cell (Fig. 4) and to enhance the translation of viral NP mRNA (Fig. 5). In addition, our results further confirm that the N-terminal NLS is enough to induce the translocation of NS1 protein into the nucleus (Fig. 2), as reported by Greenspan *et al.* (26), and that the N-terminal region of NS1 protein contains an RNA-binding domain (Fig. 3), as documented

earlier (51). The only function associated to NS1 protein that we have not mapped so far is its activity on splicing modulation (30,34–36).

The phenotypes associated to mutations in Influenza virus that map within the NS1 gene are diverse. Some mutants show a transcriptional block at non-permissive temperature (ts412, mutation R25K; ref. 52), while others are affected in viral gene expression at a post-transcriptional level (SPC45, mutation K62N; or ICR1629, mutation A132T; ref. 53). The first of such phenotypes can be interpreted in terms of the capacity of NS1 protein to bind vRNA (29,33) and to associate to transcription–replication complexes *in vivo* (54), while the second may be related to the inhibition of mRNA nucleo-cytoplasmic transport and the enhancement of viral mRNA translation (28,34,37). It has been reported recently that NS1 protein can inhibit the activation of PKR *in vitro* (55). These results are not surprising, since NS1 is able to bind double-stranded RNA, but their significance in the stimulation of viral mRNA translation is presently unclear. Thus, such an interpretation would not be compatible with the observed specificity of the stimulation (28,37) which is in line with the binding of NS1 to the 5′-UTR of NP mRNA (32). Furthermore, the phenotype of mutant NS1Δ82-238, fully capable of RNA binding (Fig. 3) and completely negative in the stimulation of NP mRNA translation (Fig. 5), rules out the possibility that simple RNA binding is sufficient to explain the stimulation of viral mRNA translation by NS1 protein.

The determination of the genetic alterations in the influenza virus mutants affected in the NS1 gene indicated that all mutations inducing a temperature-sensitive or host-range phenotype are located in the N-terminal portion of the protein: R25K (56), S83P (57), (K62N; A132T) (53) and Δ66-77 (58). This fact, together with the existence of a strong length polymorphism of NS1 protein among the different natural virus strains (59), indicates that our results might reflect a biologically relevant situation in nature. It is remarkable that the shortest NS1 protein found among influenza virus strains in nature contains only the 116 amino acids homologous to the N-terminus of the longer NS1 proteins described (60). Recently, a virus mutant was constructed by reverse genetics that expresses an NS1 protein containing only the N-terminal 110 amino acids. Such a virus showed a reduced yield but was able to stimulate late viral protein synthesis and was not temperature sensitive (M.Enami, personal communication). The phenotype of this virus mutant is in agreement with those of mutant plasmids pSVaNPNS1Δ111-238 and pSVaNPNS1Δ114-238 described here and confirm that the C-terminal half of the NS1 protein is not essential for virus viability.

It has been postulated that NS1 protein contains two functional domains: an RNA binding domain located between positions 19 and 38 in the sequence and an effector domain (positions 134–161) required for both alteration of splicing and nuclear retention of mRNAs (35,40,51). Our results are in agreement with the existence of the N-terminal RNA-binding domain but do not support the presence of any functional domain downstream of position 113 in the sequence, at least for nuclear retention of mRNAs and stimulation of viral mRNA translation. The data presented in this report (Figs 3–5), together with the previous results on the mapping of the minimal NS1 fragment capable of RNA binding (51), suggest that the sequence comprised between positions 73 and 113 might contain information required to retain poly-A^+ RNA in the nucleus and to stimulate the translation of Influenza virus mRNA. It is still possible that the predicted C-terminal effector domain (35,40,51) may play a role in splicing modulation, but our results, together with the properties of NS1 mutants and natural variants, strongly suggest that the essential biological activities of NS1 protein reside within its N-terminal half. In fact, the N-terminal RNA binding domain is conserved among Influenza A and B viruses, while the proposed effector domain close to the C-terminus is not conserved in type B viruses (61).

If only the N-terminal half of NS1 protein were essential for virus viability, an explanation should be provided for the presence of additional sequences in most of the NS1 proteins from natural isolates. Two alternative models for the evolution of the viral genetic information in segment 8 were put forward previously (62): (i) in an influenza virus ancestor, the NS1 gene completely occupied segment 8. The generation of a splice site by mutation allowed for the production of an alternative mRNA encoding NS2 protein and (ii) the NS1 and NS2 cistrons were colinear but not overlapping. Mutation of the ancestor NS1 terminator would extend the NS1 gene. Although it is difficult to eliminate any of these models, our results and the diversity of lengths in the NS1 protein from natural virus isolates (59,60) favor the second one. Thus, elimination of the ancestor terminator would incorporate additional protein sequeces to NS1. If these sequences did not interfere with NS1 function, they would not be selected against. Such an elongation of NS1 gene could have occurred stepwise and would explain the NS1 length polymorphism found in nature. It is also possible that these sequences incorporated onto an ancestor NS1 protein would have evolved to acquire new functions beneficial for virus replication or adaptation to the host, but mutations that would lead to premature termination, such as that described by Norton *et al*. (60) or artificial mutations as those described here would not have lethal effects.

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