A protein that replaces the entire cellular elf4F complex

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The elf4F cap-binding complex mediates the initiation of cellular mRNA translation. elf4F is composed of elf4E, which binds to the mRNA cap, elf4G, which indirectly links the mRNA cap with the 43S pre-initiation complex, and elf4A, which is a helicase necessary for initiation. Viral nucleocapsid proteins (N) function in both genome replication and RNA encapsidation. Surprisingly, we find that hantavirus N has multiple intrinsic activities that mimic and substitute for each of the three peptides of the cap-binding complex thereby enhancing the translation of viral mRNA. N binds with high affinity to the mRNA cap replacing elf4E. N binds directly to the 43S pre-initiation complex facilitating loading of ribosomes onto capped mRNA functionally replacing elf4G. Finally, N obviates the requirement for the helicase, elf4A. The expression of a multifaceted viral protein that functionally supplants the cellular cap-binding complex is a unique strategy for viral mRNA translation initiation. The ability of N to directly mediate translation initiation would ensure the efficient translation of viral mRNA.

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Introduction

Members of the hantavirus genus of the family Bunyaviridae are enveloped viruses harbouring three negative-sense, single-stranded genomic RNA molecules (Schmaljohn, 1996). The nucleocapsid peptide (N) has a vital function in hantavirus replication. Multiple studies show that N recognizes viral RNA (vRNA) with specificity indicative of its function during encapsidation (Gott et al., 1993; Severson et al., 1999, 2001; Osborne and Elliott, 2000; Jonsson et al., 2001; Jonsson and Schmaljohn, 2001; Mir and Panganiban, 2006). Each of the three genome segments form pseudocircular structures through a short imperfect ‘panhandle’ composed of hydro-

gen-bonded nucleotides from the 5′ and 3′ termini (Pettersson and von Bonsdorff, 1975; Obijeski et al., 1976; Raju and Kolakofsky, 1989). The terminal panhandle is both necessary and sufficient for high-affinity binding by N (Mir and Panganiban, 2004b, 2005). N also functions in viral genome replication, as complementary in vitro and in vivo studies indicate that N, from diverse negative-sense RNA viruses, serves in vRNA replication working in coordinated manner with the viral polymerase or through interaction with template RNA (Bridge and Elliott, 1996; Blakqori et al., 2003; Pinschewer et al., 2003; Kohl et al., 2004; Ikegami et al., 2005). Although hantavirus replication is exclusively cytoplasmic, generation of viral mRNA uses an orthomyxovirus-like cap-snatching mechanism yielding mRNAs with 5′ m7G caps derived from cellular mRNAs (Dunn et al., 1995; Garcin et al., 1995; Hutchinson et al., 1996).

The vast majority of eukaryotic mRNA translation is m7G cap dependent. Translation initiation involves the recognition of capped mRNA by a set of initiation factors (components of elf4F cap-binding complex) (Dever, 1999; Gingras et al., 1999; Richter and Sonenberg, 2005). This heterotrimeric complex includes elf4E, which directly binds to the mRNA cap (von der Haar et al., 2004; Richter and Sonenberg, 2005), and elf4A, which is a DEAD box RNA helicase (Rogers et al., 2002; Hernandez and Vazquez-Pianzola, 2005). The third component of the elf4F complex is elf4G, a peptide that interacts with both elf4E and elf4A (Mader et al., 1995; Hentze, 1997; Dever, 1999). In addition, elf4F interacts with elf3 (Hinnebusch, 2006) to bridge the mRNA–elf4F cap-binding complex and the 43S ‘pre-initiation complex.’ The 43S complex is composed of the 40S small ribosomal subunit, initiator methionine transfer RNA, elf2 and GTP (Hershey and Merrick, 2000). Scanning by this large set of proteins then proceeds from the capped 5′ end of the mRNA in a process that may require the helicase activity of elf4A (Rogers et al., 2002; Hernandez and Vazquez-Pianzola, 2005). When an AUG start codon in optimal context is encountered the 60S large ribosomal subunit and additional factors are recruited and translation begins (Kozak, 1991, 1992).

Typically, viruses use this cellular machinery for translation of their mRNAs, and most have capped mRNAs. However, the picornaviruses, some flaviviruses, a few additional viruses contain a cis-acting internal ribosomal entry sites (IRESs) to enable cap-independent ribosomal entry at a site in the mRNA immediately proximal to the start codon (Hellen and Sarnow, 2001; Jang, 2006). Along the same lines, poxviruses contain a cis-acting poly A sequence in their 5′ leader that facilitates association of the pre-initiation complex with viral mRNA (Shirokikh and Spirin, 2008). In the course of experiments to examine hantavirus nucleocapsid (N) protein function, we noted that the expression of N in cells appeared to surprisingly result in increased expression of heterologous indicator mRNAs. Here, we describe this phenomenon in detail. N can replace the activities of elf4F to mediate mRNA translation. In particular, N binds with high
affinity to the capped 5’ end of viral mRNAs, an activity that mimics that of eIF4E. N substitutes for the standard requirement for the bridging peptide, eIF4G, by directly recruiting the 43S pre-initiation complex to the 5’ mRNA cap. Finally, N replaces the helicase, eIF4A, in the cap-binding complex. Thus, this viral strategy is the functional complement to that of an IRES. N supplants the eIF4F complex in trans, whereas an IRES replaces cap-dependent translation in cis.

Results

N facilitates translation of capped mRNA

Co-expression of N with various reporter mRNAs yielded unexpected evidence, consistent with the idea that the steady-state expression of reporter proteins was augmented by N (data not shown). To examine this apparent N-dependent increase in protein expression, we co-transfected HeLa cells with increasing amounts of a plasmid that expresses Sin nombre hantavirus (SNV) N (or an empty expression vector) and a constant amount of a reporter plasmid expressing either green fluorescent protein (GFP) or luciferase (luc) mRNA. At 36 h after transfection, cells were harvested, and GFP expression was quantified by flow cytometry and luc was measured using a quantitative enzymatic assay. We observed a concomitant increase of about five-fold in both GFP expression and luc expression with increasing amounts of N expression plasmid (Figure 1A and B). Quantitative RT–PCR (real-time PCR) with primers corresponding to a segment in the centre of each reporter RNA. In both (A, B), the results of this latter analysis are depicted with light bars. 

When increasing amounts of bacterially expressed purified N, were added translation reaction with reporter RNA containing or lacking a 5’ m7G cap. When increasing amounts of capped but not uncapped penta- and hexanucleotide RNA at a K_d of 120–130 nM (Figure 3B). However, there was no detectible binding with either capped or uncapped tri- and tetranucleotide RNA (Supplementary Figure S1). We also examined interaction between N and free cap using fluorescence spectroscopic analysis. The interaction of N with free cap is at least three orders of magnitude weaker than that observed for N with capped penta- or hexanucleotide RNA (Supplementary Figure S2). These data suggest that translation enhanced by N is superior for capped mRNAs owing simply to the ability of N to bind to capped 5’ ends.

Hantaviruses do not abrogate general cellular mRNA translation. Nonetheless, if N enhances translation, viral mRNA might be preferentially translated relative to non-viral mRNA. The previously described ‘reporter RNA’ encoding N (Figure 2C) contained the N gene but lacked this viral 5’ non-coding region, and N-mediated translation of this RNA was similar to that of the GFP and luc reporter mRNAs. As with all bunyavirus, the 5’ ends of hantavirus mRNAs contain approximately 10 non-viral nucleotides that arise from cap-snatching. The 5’ non-coding region from hantavirus segment mRNA is 44 nt in length, not including non-viral nucleotides. We carried out a competitive assay to examine the translation of an mRNA containing the viral 5’ non-coding sequences relative to a second reporter (GFP) containing a non-viral leader of equal length. Equimolar amounts of these two capped RNAs were added together to reticulocyte extracts with increasing amounts of purified N. N-mediated

Figure 1 N increases the expression of reporter proteins. HeLa cells were transfected with a constant amount of reporter plasmid and increasing amounts of a plasmid expressing hantavirus N. Evaluation of N expression on western blots with anti-N antibody indicated that N expression increased along with increasing amounts of plasmid, as expected (not shown). At 36 h after transfection, cells were harvested and GFP or luc expression was quantified, by flow cytometry or enzymatically, respectively (dark bars). (A) Expression of GFP is shown. (B) luc as a function of increasing N is shown. Steady-state GFP mRNA and luc mRNA were quantified using ‘real-time’ RT–PCR with primers specific for a segment in the centre of each reporter RNA. In both (A, B), the results of this latter analysis are depicted with light bars.

N binds to 5’ caps and mediates preferential translation of viral mRNA

As N preferentially enhances the translation of capped mRNAs, we next asked whether N interacts with the 5’ end of capped RNAs. We synthesized radioactively labelled capped and uncapped RNAs, 3–6 nt in length (Figure 3A) and carried out filter binding studies with each of these short RNAs to assess binding by N. This indicated that N binds to capped but not uncapped penta- and hexanucleotide RNA at a K_d of 120–130 nM (Figure 3B). However, there was no detectible binding with either capped or uncapped tri- and tetranucleotide RNA (Supplementary Figure S1). We also examined interaction between N and free cap using fluorescence spectroscopic analysis. The interaction of N with free cap is at least three orders of magnitude weaker than that observed for N with capped penta- or hexanucleotide RNA (Supplementary Figure S2). These data suggest that translation enhanced by N is superior for capped mRNAs owing simply to the ability of N to bind to capped 5’ ends.

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Figure 2. N augments the translational expression of capped mRNA. We examined the effect of increasing N on the translational expression of three reporter mRNAs containing or lacking a 5' cap using rabbit reticulocyte lysates. Capped and uncapped mRNA encoding GFP, luc or N were translated in vitro in the presence of [35S]-methionine and increasing amounts of N in (A–C), respectively. Translation products were then electrophoresed on SDS polyacrylamide gels, and the amount of translation product was quantified by phosphorimage analysis. Translation of capped and uncapped RNA is depicted with filled and open squares, respectively. The amount of labelled protein synthesized with capped RNA in reactions lacking N was normalized to 1 (this cannot be indicated on the log scale). In the absence of N, expression of the indicator proteins was slightly higher with uncapped than capped RNA. This is consistent with earlier observations (Svitkin et al., 1996). Thus, the amount of expression at the lower concentrations of N is equivalent to background levels of expression from capped and uncapped RNA for each of the indicator RNAs.

Enhancement of translation was superior for the viral mRNA when compared with the GFP RNA, yielding an increase in viral mRNA expression of about seven-fold (Figure 4A). We generated two additional mRNAs in which the 44 nt leader regions were interchanged between the two reporter genes. Increasing amounts of N resulted in preferential expression of GFP from the chimaeric mRNA containing the capped viral 5' leader (Figure 4B). Thus, the translation of non-viral mRNA can be facilitated by N (Figure 2), but in a competitive reaction containing both viral and non-viral mRNA translation of mRNA containing the 5' non-coding sequences from the virus is robust when compared with mRNA harbouring a non-viral leader.

RNA-binding assays of N for each of these capped RNAs indicated that RNA containing the 5' leader region from viral mRNA interacted with N at significantly higher affinity than RNA with a non-viral leader (Figure 4C). Further, the viral leader region was sufficient for higher affinity binding by N. Preferential translation of viral mRNA, and high-affinity binding by N, is not diminished by the presence of capped non-viral nucleotides at the 5' end, as would be present on bona fide viral mRNA (Figure 4C) (Mir and Panganiban, submitted).

N stably binds to the 43S pre-initiation complex and replaces eIF4G

eIF4F binds to mRNA by way of eIF4E, and to the eIF3 and the 43S pre-initiation complex by way of eIF4G. To determine whether N interacts with the 43S pre-initiation complex, we added his6-tagged N to rabbit reticulocyte lysates, recovered N using Ni-NTA beads and used a quantitative assay for 18S rRNA to quantify 40S ribosomal subunits associated with N. These assays indicated that 18S RNA was associated with N (Figure 5A), suggesting that N interacts with 40S eukaryotic ribosomal subunit. We carried out a similar experiment with lysates derived from 293 cells expressing his6-tagged N following transfection with an N expression construct. Again we observed that 18S rRNA was recovered with N on Ni-NTA beads, indicating that N interacts directly or indirectly with the 43S pre-initiation complex in vivo (Figure 5A).

In the unphosphorylated form, eIF2α is a functional component of the 43S pre-initiation complex, whereas phosphorylated eIF2α is not associated with the pre-initiation complex. We determined whether eIF2α co-puriﬁed with his6-tagged N from lysates of transfected cells on Ni-NTA columns using western blot analysis with antibody speciﬁc for the unphosphorylated and phosphorylated forms of eIF2α. This indicated that unphosphorylated eIF2α but not phosphorylated eIF2α was associated with N (Figure 5B). Moreover, western blot analysis indicated that S6 ribosomal protein also co-puriﬁed with N (Figure 5B). These data again suggest that N interacts with the 43S pre-initiation complex. In contrast, components of the eIF4 cap-binding complex, eIF4E and eIF4G, did not co-puriﬁe with N in parallel western blots (Figure 5B). Thus, association of the 43S pre-initiation complex with N does not require the eIF4F cap-binding complex.

To verify that interaction between N and the 43S pre-initiation complex was mediated by way of direct interaction rather than indirectly through an mRNA bridge, we dissociated ribosomes into large and small subunits by incubation with puromycin, purified 40S small ribosomal subunits by sucrose gradient centrifugation, and asked whether N could interact directly with purified 40S subunits. The purified 40S subunits were resedimented, detected by monitoring optical density and yielded a sedimentation profile indicative of a homogeneous 40S preparation (Figure 6A). We synthesized 35S-labelled N in reticulocyte extracts, and puriﬁed the labelled protein by denaturation, recovery on Ni-NTA columns, and renaturation. Sedimentation analysis of this puriﬁed 35S-labelled N protein indicated that N migrated to a distinct position high in the gradient (Figure 6B). Signiﬁcantly, incubation of N with puriﬁed 40S subunits resulted in co-migration of N with small subunits indicative of interaction between N and the 40S subunit (Figure 6C). A radioactively labelled control protein (GFP) did not interact with 40S ribosomal subunits and remained near the top of the gradient (data not shown). These data indicate that N binds directly to a component of the small ribosomal subunit and that association is not through an mRNA bridge. However,
the data do not unequivocally distinguish between whether N interacts directly with the 40S subunit or with residual eIF3 bound to the 40S subunit.

We next asked whether N can facilitate loading of small ribosomal subunits onto the 5′ end of mRNA. A 415-nt-long mRNA containing a 15-nt-long poly A tail and 200-nt-long non-coding 5′ leader sequence was incubated in rabbit reticulocyte lysates indicative of enhanced ribosome recruitment of the 43S pre-initiation complex onto the isolated mRNA. We observed a significant increase in both 18S and 28S rRNA when both N and mRNA were present in rabbit reticulocyte lysates indicative of enhanced ribosomal loading onto the isolated mRNA (Figure 8). As the recruitment of the 43S pre-initiation complex to the 5′ end of mRNAs is conventionally considered to be the rate-limiting step for translation, these data suggest that N increases the rate of recruitment of the 43S pre-initiation complex onto mRNAs.

Cellular mRNA is circularized through association between eIF4G in the eIF4F cap-binding complex at the 5′ end and poly A-binding protein (PABP) at the 3′ end leading to more efficient translation (Tarun and Sachs, 1996; Gray et al., 2000). As eIF4G appeared to be dispensable for N-mediated translation initiation, we asked whether N may interact with PABP to effect circularization. We carried out co-precipitation experiments in which his6-tagged N from lysates of transfected cells was recovered on Ni-NTA beads and the recovered material was analysed by western blot analysis with anti-PABP antibody. On the basis of this approach, there was not detectible stable association between N and PABP (Supplementary Figure S3).

**N replaces eIF4A**

We next wanted to see whether N replaces the activity of the third constituent of the eIF4F complex, eIF4A. eIF4A is a DEAD box RNA helicase required for eIF4F function during cap binding and has been postulated to function in the scanning of the pre-initiation complex to the AUG start codon (Rogers et al., 2002; Hernandez and Vazquez-Pianzola, 2005). In comparison, N has an intrinsic ATP-independent activity that facilitates transient RNA duplex dissociation (Mir and Panganiban, 2006). eIF4A migrates on and off the eIF4F complex, where it functions in concert with eIF4E and eIF4G, and this interconversion between the complexed and free forms of eIF4A appears to be necessary for eIF4A function. We used a dominant-negative mutant of eIF4A defective in cycling through eIF4F complex, which dramatically inhibits translation in rabbit reticulocyte lysates, to see whether N mediates translation initiation in an eIF4F-independent manner (Pause et al., 1994). Wild-type and dominant-negative eIF4A were expressed in bacteria and purified (Figure 9A). Consistent with published characterization of this dominant-negative eIF4A protein (Pause et al., 1994), 2 μg of the dominant-negative mutant protein inhibi-
Figure 4 N preferentially augments the translation of viral mRNA. (A) Equimolar amounts of capped mRNA containing the 5’ untranslated region from S segment mRNA and encoding N (v-N), and an mRNA containing a non-viral leader region and encoding GFP (GFP) were added together to reticulocyte extracts containing increasing concentrations of N as indicated. The concentration of each mRNA was approximately 45 nM. Labelled N and GFP were separated by PAGE and quantified by phosphorimage analysis (shown below the graph). Similar results were obtained in three separate experiments. In (B), the leader regions from the mRNAs of (A) were interchanged. Thus, one mRNA contained the 5’ viral UTR preceding the GFP gene (v-GFP), and a second mRNA contained the non-viral leader preceding the N gene (N). Translation and quantitation were as in (A). (C) Radioactively labelled capped RNAs were used in binding reactions with purified N and the binding affinity ($K_d$) was determined for each. Viral sequences are shown schematically in grey, whereas non-viral sequences are in white. n-v-GFP contains a 9 nt non-viral cap simulating cellular RNA derived from cap-snatching (shown in black). Note: The leader regions are not shown to scale relative to the N and GFP genes. The untranslated leaders, GFP gene, and N gene are 43, 798, and 1287 nt in length, respectively. (D) Comparison of the leader sequences from GFP, v-GFP, and n-v-GFP mRNA and minus strand S segment viral RNA. Nucleotides required for high-affinity binding to the vRNA panhandle are depicted with shading and include nucleotides from both the 5’ and 3’ termini (Mir and Panganiban, 2005). The 5’ terminal nucleotides of + strand mRNA required for binding by N is also indicated by shading. As the termini of the viral genome segments consist of imperfect inverted repeats, the 5’ sequences of both plus and minus strand viral RNA are similar. Nucleotide differences in the 5’ sequence of mRNA relative to the 5’ sequence of minus strand vRNA are indicated with bold lettering. Leader sequences of v-GFP and n-v-GFP. The 9-nt-long non-viral leader of n-v-GFP, and the start codon of the mRNAs are underlined.
N replaces eIF4F

Discussion

**N as a translation initiation factor**

N-mediated translation initiation is a viral strategy that is the complement to the use of an IRES. The latter tactic is employed by the picornaviruses and some flaviviruses.

Although an IRES is a *cis*-acting element that functionally supplants the requirement for cap-dependent translation, N is a *trans*-acting element that replaces eIF4F.

It is likely that N mediates initiation through a simple mechanism. There is no overt similarity between N and the eIF4F components. Nonetheless, the three-dimensional structure of cellular cap-binding peptides human eIF4E includes two W residues (W52 and W102) that hold the guanine residue of a cap-analogue through stacking interactions. An acidic residue (E103) further stabilizes this association (Marcotrigiano et al., 1997; Matsuo et al., 1997). There may be weak alignment of this region with a segment of N of identical length (a.a. 119–166) containing appropriately spaced W119 and Y165E166 residues. Binding of eIF4G with the eIF3 and the 43S pre-initiation complex is mediated by a portion of the central region of eIF4G (Korneeva et al., 2000; Schutz et al., 2008). ClustalW comparison of N with this region of eIF4G indicates weak alignment with the amino-

Figure 5 N interacts with the pre-initiation complex. (A) N was incubated with rabbit reticulocyte lysates and recovered with Ni-NTA beads. The bound material was eluted from the Ni-NTA, RNA was purified, and 18S rRNA was quantified using real-time RT-PCR. The leftward graph depicts the relative amount of 18S rRNA associated with Ni-NTA beads in the absence and presence of N. The rightward graph depicts an analogous experiment carried out with 293 cells that were transfected with either an N-expressing plasmid or its parental vector, as a negative control. N was recovered from the lysates of transfected cells using Ni-NTA and 18S rRNA that co-purified with N quantified by real-time RT-PCR. (B) A set of western blots to examine the association of peptide constituents of the 43S pre-initiation complex, and the eIF4F cap-binding complex, that co-purify with N. N was expressed by transfection, isolated from the lysates of these cells using Ni-NTA columns, bound material was recovered and subjected to western blot analysis with primary antibodies as indicated. Peptides that co-purify with N (bound), or that flow through the column are indicated.

Figure 6 N binds directly to the small ribosomal subunit. 40S small ribosomal subunits were prepared by incubation of ribosomes in the presence of puromycin and purified from large ribosomal subunits and mRNA. (A) Purified 40S subunits were then resedimented on a sucrose gradient. (B) N protein was expressed by *in vitro* translation in the presence of 35S-methionine, purified from the translation mixture by denaturation with urea, recovery on Ni-NTA beads, renaturation by dialysis and sedimented in parallel with 40S subunits. (C) N was incubated with excess purified 40S subunits prior to sedimentation. Leftward fractions correspond to those from the bottom of the gradient.

Figure 7 N replaces eIF4F

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The experiments of (A, B), the total amount of DNA used in the transfections was held constant by the addition of parental vector.

GFP expression (dark bars) and steady-state GFP mRNA (light bars) were quantified as in (A). In the constant amount of 2A expression plasmid (0.05 μg) sufficient for significantly reducing translation of the reporter gene, and increasing amounts of an N expression plasmid. GFP expression (dark bars) and steady-state GFP mRNA (light bars) were quantified as in (A). In the experiments of (A, B), the total amount of DNA used in the transfections was held constant by the addition of parental vector.

preferentially recognized by N is situated in the viral 5' cap region. Nonetheless, the motif in viral mRNA is recognized at higher affinity than viral mRNA. During replication, viral mRNA synthesis precedes and overlaps with genome replication. It will be of interest to see common RNA-binding domains of N function in both processes.

It is likely that the general strategy of encoding a trans-acting factor to ensure efficient viral translation is not restricted to hantaviruses. It is probable that the N peptides of members of the other genera of the bunyavirus family, and perhaps the members of diverse families of segmented and non-segmented minus strand RNA viruses, also supplant the elf4F complex. Several observations hint that RNA viruses may use this general scheme. Subgenomic Sindbis virus mRNA is translated when elf4F is inactivated (Castello et al., 2006). Vesicular stomatitis virus impairs elf4F function through dephosphorylation but sustain translation of its own mRNAs (Connor and Lyles, 2002). Similarly, influenza mRNA translation can occur when elf4E is impaired (Burgui et al., 2007). Expression of Sendai virus N is required for the expression of a reporter gene from a viral vector (Wiegand et al., 2007). This could be due to an effect on transcription, as suggested by the authors, but their data are also consistent with a positive role of SeV N in translation.

**Implications for viral transcription**

The bunyaviruses are unique among the negative-stranded RNA viruses in that transcription requires concomitant translation of the nascent viral mRNA (Belloq and Kolakofsky, 1987; Barr, 2007). Coupling of transcription with translation appears to be necessary for successful RNA elongation by the RdRp through spurious premature transcription termination signals. Mechanistically, this may occur as ribosomes trailing the RdRp block the formation of higher order structures in the nascent RNA that function as inappropriate termination sites. N may promote more efficient loading of ribosomes onto nascent viral mRNA leading to higher ribosome density and ensuring mRNA elongation.

**Figure 7** N replaces elf4F. (A) HeLa cells were co-transfected with a plasmid expressing reporter GFP, along with increasing amounts of pF/HRV-16 2A, which expresses the 2A protease of HRV-16. GFP expression was quantified using flow cytometry as in Figure 1 (dark bars) and GFP mRNA was quantified using real-time PCR (light bars). (B) Cells were transfected with a constant amount of GFP expression plasmid, a constant amount of 2A expression plasmid (0.05 μg) sufficient for significantly reducing translation of the reporter gene, and increasing amounts of an N expression plasmid. GFP expression (dark bars) and steady-state GFP mRNA (light bars) were quantified as in (A). In the experiments of (A, B), the total amount of DNA used in the transfections was held constant by the addition of parental vector.

**Figure 8** N promotes ribosome loading. A synthetic mRNA containing 3' poly A was incubated in reticulocyte lysates to allow translation. The synthetic polyadenylated RNA was recovered from the translation mixture using oligo dT beads. Ribosomes associated with the polyadenylated RNA were quantified by real-time RT–PCR with primer sets specific for 18 and 28S rRNA.

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Apparent lack of circularization of hantavirus mRNA

Circularization of cellular mRNA mediated by the interaction between eIF4G and PABP enhances translation efficiency (Tarun and Sachs, 1996; Gray et al., 2000). However, circularization is probably not required for efficient N-mediated translation of viral mRNA, or else occurs through unidentified factors. Initiation can take place independently of eIF4F and we were unable to detect association between N and PABP. Moreover, of the three hantavirus mRNAs only that encoding the viral envelope protein is polyadenylated, whereas mRNA encoding N and the RdRp are not (Hutchinson et al., 1996).

It is worthwhile to contrast N-mediated translation initiation with viral peptides that associate with eIF4F. The potyviruses, a set of plant viruses related to the picornaviruses, encode a VPg that is attached to the 5’ end of the genome and that also operationally associates with eIF4E, potentially enabling functional circularization of the genome (Kang et al., 2005). Rotavirus NSP3A and Alfalfa mosaic virus (AMV) coat protein associate with both the 3' end of their respective genomes and with eIF4G (Piron et al., 1998; Bol, 2005). This association with the eIF4F complex also enables genome circularization as the 3' termini of these viral genomes are not polyadenylated. Thus, NSP3A and AMV coat function as surrogates for PABP. For these viruses, interaction with the eIF4F complex is required for efficient replication.

There are a remarkable number of activities and functions associated with hantavirus N. These include its structural role as the capsid protein, its role as the principle player mediating the encapsidation of vRNA, its role as an RNA chaperone capable of reconfiguring the higher order structure of RNA, its role in genome replication in coordination with the viral polymerase, and now an unexpected role as a translational initiation factor with multiple complementary activities dedicated to that function. At the same time, N-mediated translation of viral mRNA can probably be considered to be a narrowly focused function ensuring efficient production of the viral peptides. In this regard, translation initiation by N would not be accompanied by the elegant and subtle regulatory capacity of the multicomponent cellular translational complex.

Materials and methods

Plasmids

pSNV N TriEx 1.1 expresses N containing a C-terminal histidine tag both in vitro and in vitro (Mir et al., 2006). pEGF-P (Promega) and pGL3 plasmid (Clone Tech) were used for the expression of GFP and luciferase (luc), respectively. pF/HRV-16 2A, which expresses human rhinovirus 16 2A protease from the EMCV IRES at high level, was kindly provided by Yury Bochkov, Alex Aminev, and Ann Palmenberg (Bochkov and Palmenberg, 2006).

Flow cytometry

All transfections were carried out in triplicate in six-well plates. Flow cytometry was carried out using a FACScan (BD Biosciences), obtaining 10000 gated events for each sample. The fluorescence of the gated cells was quantified and a histogram was generated to display the distribution of fluorescence intensity in the cell population. The mean fluorescence value of positive and negative events was calculated.

Ribosome-loading assay

RNA molecules generated were 415 nt long, containing a 200 nt non-coding sequence at the 5’ end, followed by an AUG and 200 additional nucleotides followed by a 15-nt-long poly A tail. Here, 5 μg of this mRNA was added to 20 μl in vitro translation reactions with or without N and incubated at 30°C for 15 min. RNA was recovered with 20 μl of oligotex (poly-dT beads) and reverse transcribed using random primers as described in the ‘Real-time PCR’ section. Then, 2 μl of the resulting cDNA was used for the quantitation of 18S and 28S rRNA using appropriate primers with the standard curve method as in the Real-time PCR section.
Preparation of mRNA substrates for in vitro T7 transcription reactions

PCR products containing T7 promoters were gel purified and used as a template in T7 transcription reactions with Ribomax T7 (Promega). Following synthesis, template DNA was degraded with DNase I, the RNA was purified by RNAeasy (Qiagen), and stored in 10 µl aliquots at −70 °C. RNA molecules with a terminal 5’ m7G cap were synthesized by the incorporation of m7G cap analogue in the transcription reactions. Short RNA molecules, 3–6 nt long, were synthesized from a 100-nt-long DNA template containing a terminal T7 promoter. 5’ caps were incorporated in the short transcripts by adding m7G cap analogue to the T7 reaction mixtures. Transcription reactions were incubated at room temperature for 30–45 min and filtered through nitrocellulose filters. Reaction mixtures were fractionated on denaturing 18% polyacrylamide gels containing urea. This resulted in a ladder composed of RNAs 3, 4, 5 and 6 nt in length. RNA was recovered from gel slices and stored in 10 µl aliquots at −70 °C.

In vitro translation

Nucleic acid–treated rabbit reticulocyte lysates were used for the translation of mRNA in the presence and absence of supplemented, bacterially expressed nucleocapsid protein. Translation reactions were performed in triplicate. For each reaction, an appropriate amount of mRNA was added to a final volume of 100 µl of reticulocyte lysate, 1 µl amino-acid mixture minus methionine (1 mM), 1 µl 35S-methionine (1175 Ci/mmol), 2 µl RNase inhibitor (40 µg/µl), 4 µl mRNA in water (250 ng/µl), and 7 µl of RNase-free water. The final RNA concentration in the reactions was approximately 90 nM. Reaction mixtures were incubated at 30 °C for 30 min. Samples were electrophoresed on 10% SDS gels and quantified with a phosphorimager.

Real-time PCR

RNA was isolated from cells, or from rabbit reticulocyte lysates, using ‘RNAeasy’ (Qiagen) and treated with RNase-free DNase I. RNA was reverse transcribed using Mo-MLV reverse transcriptase (Applied Biosystems) using a 150-nt-long sequence of the GFP, or luciferase, gene. Primer validation was carried out following the manufacturer’s protocol (Applied Biosystems). Real-time PCR reactions were carried out in 20 µl, including 10 µl ribogar mastermix (Applied Biosystems), 2 µl of template, 3.6 µl of each forward and reverse primer, and 0.8 µl water. Each reaction was carried out in triplicate.

RNA filter binding

All filter binding reactions were carried out in RNA-binding buffer (Mir and Panganiban, 2004) at a constant concentration of RNA (1 µM) with increasing concentration of N protein. Reaction mixtures were incubated at room temperature for 30–45 min and filtered through nitrocellulose membranes under vacuum. The amount of RNA retained on the filter by virtue of N was measured using a scintillation counter. Data points were fit to a hyperbolic equation using the program Origin 6 (Microcal). The apparent dissociation constant (Kd) corresponded to the concentration of N protein required to obtain the half saturation in the binding profile.

Pull-down experiments with Ni-NTA

His-tagged N from reticulocyte lysates was recovered on, and eluted from, Ni-NTA columns (Qiagen) in 50 µl of elution buffer (50 mM NaH2PO4, 300 mM NaCl, and 500 mM imidazole). The eluted sample (25 µl) was used for the purification of total RNA. RNA was reverse transcribed and analysed by real-time PCR to measure 18S or 28S rRNA. In controls, recovery of protein from sonicated cells, and ammonium sulphate fractionation, the peptides were fractionated on a DEAE sephacel column using a 0.1–0.5 M KCl gradient. 10% of the eluted and 10% of the flowthrough material, respectively, were used for the detection of ribosomal proteins or other initiation factors involved in translation by western blot analysis.

40S ribosomal subunit preparations

40S ribosomal subunits were purified using a standard protocol (Pestova et al., 1996). Briefly, rabbit reticulocyte lysates were diluted 10-fold in the presence of 1 mM dithiothreitol (DTT), and centrifuged for 4 h at 100 000 × g using a fixed angle NVT 90 rotor. The resulting pellet was resuspended in 5 ml of buffer A (0.25 M sucrose, 0.05 M Tris–HCl (pH 7.5), 1 mM DTT, 6 mM MgCl2, and 0.1 mM EDTA), followed by the addition of 0.5 M KCl with continuous stirring on ice for 30 min. The mixture was centrifuged for 2 h at 180 000 × g using a NVT 90 rotor. The pellet was dissolved in a small volume of buffer A, layered over a sucrose cushion (1.0 M sucrose, 0.5 M KCl, 0.02 M Tris–HCl (pH 7.5), 2 mM MgCl2, and 0.1 mM EDTA) and centrifuged at 275 000 × g for 3 h. The pellet, containing 80S ribosomes, was resuspended in a small volume of buffer A and further diluted 10-fold in buffer B (0.5 M KCl, 0.05 M Hepes (pH 7.5), 2 mM MgCl2, and 1 mM puromycin). The mixture was incubated for 10 min on ice followed by further incubation for 10 min at 37 °C and 5 min on ice. The solution was layered onto a 5–20% sucrose gradient prepared in buffer C (0.5 M KCl, 0.05 M Hepes (pH 7.5), 5 mM MgCl2, 1 mM DTT, and 0.1 mM EDTA) and centrifuged at 50 000 r.p.m. for 3 h. Here, 0.25–m fractions were collected and monitored by checking their absorbance at 280 nm. Two peaks corresponding to 40S and 60S subunits were detected. The fractions containing 40S subunit were pooled and concentrated by further centrifugation at 70 000 r.p.m. for 10 h. The purified 40S subunit pellet was resuspended in subunit storage buffer (0.05 M Tris–HCl (pH 7.5), 0.25 M sucrose, 1 mM DTT, 0.1 mM EDTA, 10 mM KCl, and 1 mM MgCl2) and stored at −80 °C.

Interaction of 40S subunits with N

mRNA encoding N was translated in rabbit reticulocyte lysates and labelled with 35S-met. Labelled N was purified under denaturing conditions using Ni-NTA affinity column. Purified N was renatured, concentrated, and sedimented on a 10–30% sucrose gradient prepared in buffer C at about 0.2 M KCl. Fractions containing eIF4A were identified by western blot analysis with anti-eIF4A antibody (provided by the Sonenberg lab). Pooled fractions were diluted with buffer A (1:1 dilution) and fractionated on Hi-Trap blue columns (Pharmacia) using a 0.1–2 M KCl gradient. Fractions containing eIF4A were dialysed and fractionated on a mono Q sepharose column using sequential gradients of 120–160 mM KCl and 160–200 mM KCl. eIF4A elutes from the column between 180 and 190 mM KCl. Fractions containing eIF4A were pooled, concentrated, and used in translation experiments.

Expression and purification of wild-type and mutant eIF4AI

Plasmids expressing wild-type eIF4A and R362Q eIF4A were generously provided by Nahum Sonenberg and Colin Lister (Pause et al., 1994). Following expression in Escherichia coli BL21 cells, recovery of protein from sonicated cells, and ammonium sulphate fractionation, the peptides were fractionated on a DEAE sephacel column using a 0.1–0.5 M KCl gradient. eIF4A elutes from the column at about 0.2 M KCl. Fractions containing eIF4A were identified by western blot analysis with anti-eIF4A antibodies (provided by the Sonenberg lab). Pooled fractions were diluted with buffer A (1:1 dilution) and fractionated on Hi-Trap blue columns (Pharmacia) using a 0.1–2 M KCl gradient. Fractions containing eIF4A were dialysed and fractionated on a mono Q 5/5 column using sequential gradients of 120–160 mM KCl and 160–200 mM KCl. eIF4A elutes from the column between 180 and 190 mM KCl. Fractions containing eIF4A were pooled, concentrated, and used in translation experiments.

Supplementary data

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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N replaces eIF4F

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