

# Rabies virus matrix protein interplay with eIF3, new insights into rabies virus pathogenesis

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## ABSTRACT

**Viral proteins are frequently multifunctional to accommodate the high density of information encoded in viral genomes. Matrix (M) protein of negative-stranded RNA viruses such as *Rhabdoviridae* is one such example. Its primary function is virus assembly/budding but it is also involved in the switch from viral transcription to replication and the concomitant down regulation of host gene expression. In this study we undertook a search for potential rabies virus (RV) M protein's cellular partners. In a yeast two-hybrid screen the eIF3h subunit was identified as an M-interacting cellular factor, and the interaction was validated by co-immunoprecipitation and surface plasmon resonance assays. Upon expression in mammalian cell cultures, RV M protein was localized in early small ribosomal subunit fractions. Further, M protein added *in trans* inhibited *in vitro* translation on mRNA encompassing classical (Kozak-like) 5'-UTRs. Interestingly, translation of hepatitis C virus IRES-containing mRNA, which recruits eIF3 via a different noncanonical mechanism, was unaffected. Together, the data suggest that, as a complement to its functions in virus assembly/budding and regulation of viral transcription, RV M protein plays a role in inhibiting translation in virus-infected cells through a protein-protein interaction with the cellular translation machinery.**

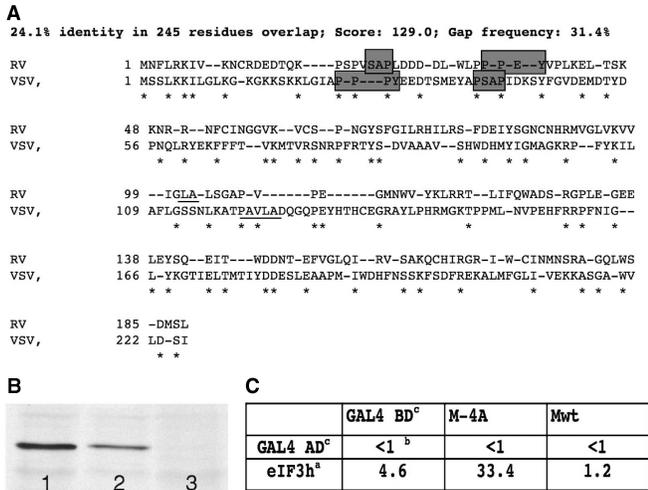
## INTRODUCTION

Rabies virus (RV) is the etiologic agent of a lethal encephalomyelitis. RV belongs to the *Rhabdoviridae* family and constitutes the prototype of the *Lyssavirus* genus. RV possesses a single negative-stranded RNA genome whose replication is exclusively cytoplasmic in infected cells. As for vesicular stomatitis virus (VSV), the most studied rhabdovirus, the RV particle contains five proteins, produced from five capped, methylated, polyadenylated viral mRNAs. Three of these viral proteins, the nucleoprotein (N), the phosphoprotein (P) and the RNA polymerase (L), form a helical ribonucleoprotein complex (RNP) in association with the genomic RNA. It is the N protein that directly encapsidates the viral genome, and the RNP is condensed into a coiled helical structure by the matrix protein (M). This structure is surrounded by an envelope derived from the cellular cytoplasmic membrane, via a budding event implicating an interaction between the viral M and glycoprotein (G). The intrinsic ability of the M protein to bud from the cell surface in the form of lipid-enveloped virus-like particles, even in the absence of any other viral components, provides strong evidence that M plays a major role in the late budding step of the virus life-cycle (1–3).

The M protein is the smallest and most abundant protein in the rhabdovirus virion. To date, the only rhabdovirus M protein submitted to crystallographic analysis is that of VSV. The two fragments resulting from trypsin cleavage (residues 48–121 and 122/124–229) fold in a single globular domain comprising five-stranded anti-parallel  $\beta$ -sheet packed against two  $\alpha$ -helices in the N-terminal part, connected via a 20-amino-acid linker to two-stranded  $\beta$ -sheet and an  $\alpha$ -helix in the C-terminus (4).

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**Figure 1.** (A) Alignment of VSV and RV matrix proteins. *In silico* comparison of VSV indiana strain matrix protein (genbankAC#NC\_001560) and RV matrix protein (AC#NC\_001542) was performed with SIM—Alignment Tool (<http://www.expasy.org/tools/sim-prot.html>) for protein sequences program. Conserved L-domains: PPxY and PSAP motifs are shaded in grey, hydrophobic AVLA sequence is underlined. (B) M-Gal4BD and M4A-Gal4BD expression levels in yeast AH109 cells. Total extracts from yeast expressing M4A-Gal4BD (lane 1), M-Gal4BD (lane 2) or untransformed control (lane 3) were subjected to SDS-PAGE analysis followed by immunoblotting with anti-Gal4-BD antibody (Clontech). (C) Quantification of the rabies wild-type and mutant matrix protein (M-4A, Mwt) interaction with eIF3h.  $\beta$ -Galactosidase activity. <sup>a</sup>eIF3h corresponds to the cDNA clone rescued from the human brain library screening. <sup>b</sup> $\beta$ -Galactosidase activity is expressed in standard units multiplied by 1000. *o*-Nitrophenyl- $\beta$ -D-galactopyranoside was used as the LacZ chromogenic substrate. <sup>c</sup>GAL4 BD and GAL4 AD are controls with empty vectors.

A comparison of the rhabdovirus M protein structure and domain organization with that of other negative-strand RNA virus M proteins and retroviral Gag proteins allows one to envisage several homologous sequence motifs, termed late domains, as playing an important role in viral budding (5). These are a proline-rich region, PPxY or PY (where x denotes any amino acid), and a P(T/S)AP sequence (Figure 1A). Further, in a recent study another important protein sequence (VSV's AVLA hydrophobic motif) has been shown to be important for VSV replication (6). An analogous motif is also present in RV M (see Figure 1A). Due to these common features, M proteins of rhabdoviridae should show functional parallels, though they share no global sequence homology. Indeed, the amino acid identity of RV and VSV matrix proteins is only 24.1%.

RV and VSV M proteins both possess the PY motif that has been implicated in a late step of virus budding, via an interaction with a WW domain of cellular partners (5,7). WW domains are 38–40 amino acids long, contain two highly conserved tryptophans, and are known to mediate protein–protein interactions (8). As mentioned above, the role of the rhabdovirus M protein in infected cells is

not limited to virus assembly and budding. Indeed, the RV M protein was shown to regulate the balance of viral RNA synthesis, by switching RNP activity from transcription to replication, and this function is dissociated from the role of M in virus budding (9,10). The M protein is now thought to be responsible for the cytopathic effect associated with VSV infection. The VSV M protein has been clearly implicated in the inhibition of cellular RNA and protein synthesis, hampering of nucleocytoplasmic transport and cell rounding due to the induction of apoptosis (5,7). While less pronounced than for VSV, RV infection also leads to a moderate inhibition of cellular gene expression (11,12), which would suggest that RV M protein could act via a different, as yet unknown, mechanism.

In line with the extreme competition between mRNAs within the eukaryotic cell with respect to components of the translational machinery, many viruses have developed mechanisms to modify certain eukaryotic translation initiation factors (eIFs), and thus inhibit host cell translation and hijack the translational machinery for their own profit. Numerous examples are available for positive-stranded RNA viruses (13). However, much less data are available for the negative-stranded RNA viruses. It has been shown that VSV infection leads to a shut-off of cellular and (later on) viral protein synthesis by two different mechanisms. The first one occurs early after infection and induces the dephosphorylation of the 7-methyl cap-binding protein eIF4E (a component of the multisubunit eIF4F complex) and eIF4E-binding protein 1. Hence, there is a reduction in the amount of eIF4E associated with eIF4G (another component of the multisubunit eIF4F complex), leading to the inhibition of host protein synthesis (14). The second mechanism used by VSV results in viral protein synthesis inhibition and implicates the phosphorylation of eIF2 (15). These data are in agreement with a recent report that shows the 7-fold more efficient translation for a VSV-derived messenger compared to a host-derived one in VSV-infected cells (16). This is intriguing because viral mRNAs possess 5'-UTR and 3'-UTR similar to host mRNAs. Hence, the question remains as to what are VSV mRNA cis-acting elements, and the viral and host proteins that determine/provide this selectivity.

For the moment, no direct evidence has been found concerning viral proteins that could be involved in the inhibition of host or viral protein synthesis. An early study has showed a positive effect of the VSV M protein on a reporter mRNA translation *ex vivo* (17), whereas the general effect of VSV M on host gene expression is negative (7). However, later works have showed that a point mutation in the VSV M protein leads to a defect in the inhibition of host and virus protein synthesis and mutation of the hydrophobic AVLA sequence leads to a reduced promotion of viral translation (6,14,15). Therefore the later data demonstrate the VSV M connection to translation process in viral-infected

cells and give indirect evidence of a matrix protein negative effect on host translation. To our knowledge no such information on RV M protein exists in the literature.

Here, we present the first identification of an RV M-interacting host partner unrelated to the budding process but involved in viral virulence. Based on an alanine-substituted PPxY motif form of M, we performed a yeast two-hybrid screening, which allowed a target with potential implications in the regulation of host translation to be identified, the h subunit of eIF3. This interaction was verified by co-immunoprecipitation and surface plasmon resonance assays. Fractionation by sucrose density gradient centrifugation of lysates of mammalian cells expressing the wt M protein revealed RV M protein within the early 40S fractions. Further, *in vitro* translation assays with M added *in trans* showed that matrix protein was a potent inhibitor of translation on mRNA encompassing classical (Kozak-like) 5'-UTRs. In contrast, M had no effect on translation driven by the hepatitis C virus (HCV) IRES that is competent for eIF3-independent recruitment of the 40S ribosomal subunit (18,19) and binds eIF3 through a different, sequence- or structure-specific mechanism ((20), for review see (21)). Thus, this work is the first direct demonstration that RV M can inhibit eukaryotic translation via a protein-protein interaction with eIF3 and it provides novel insights into the understanding of rabies infection pathogenesis.

## MATERIALS AND METHODS

**Plasmids**—Details of the plasmids used in this work are in Supplementary data.

### Yeast two-hybrid screening procedure

Two-hybrid bait plasmids containing the complete ORF of M or M4A fused to the Gal4p-DNA binding domain were made by inserting the respective PCR-amplified fragments into the pAS2 vector (Laboratoire du Métabolisme des ARN, Institut Pasteur) as described (22). The human brain cDNA library fused to Gal4p activation domain pretransformed in Y187 yeast strain was from Clontech (ref HY4004AH). This library was cloned into the pACTII vector using whole brain mRNAs. cDNAs obtained with both oligo-dT and random priming are expressed in frame with the Gal4 activation domain (Gal4-AD).

The baits were transformed into the AH109 yeast strain using LiAc. Two-hybrid screens were performed using a cell to cell mating protocol by testing successively HIS3 activation (SD/-Leu/-Trp/-His plates) and LacZ with an X-Gal overlay assay. Inserts of all positive clones were amplified by PCR and then sequenced.

For wild-type M and M4A expression in yeast see Supplementary data.

### *In vitro* transcription and translation

For *in vitro* transcription pOp24(A)<sub>50</sub> and pHCVp24(A)<sub>50</sub> plasmids were linearized by EcoRI; and pM(A)<sub>50</sub>, pM4A(A)<sub>50</sub> and pP(A)<sub>50</sub> plasmids by Asp718I. Details are in Supplementary data.

In reactions with purified Mflag and M4Aflag, the quantities of the proteins (indicated in Figure 5) were added to the *in vitro* translation performed in the presence of <sup>35</sup>S-methionine with a reaction mix of 50% of a RRL partially depleted of ribosomes (23), 20% by volume of H100 buffer. Translations were performed for 60 min at 30°C, and stopped by addition of an equal volume of 10 µg/ml RNaseA. The SDS-PAGE and further densitometric quantification of translation products was as described (23). Results of at least four independent experiments were averaged.

### Antibodies and co-immunoprecipitation

Goat anti-rabbit eIF3 antibody was prepared as described previously (24). Rabbit antibodies against rabies M and P were generously provided by Dr D. Blondel and Dr P. Perrin. Co-immunoprecipitation of eIF3 and *in vitro* translated rabies virus M or P proteins from RRL was performed as described (25) by immunoprecipitating the eIF3 complex, followed by detection of radio-labelled M or P protein in the immunoprecipitates by SDS-PAGE (23%).

### Polysome analysis of cellular extracts. Western blotting

BHK 21 cells were grown in 75 cm<sup>2</sup> flasks, and infected with vTF7-3 T7 recombinant vaccinia virus. One flask was transfected with a vector expressing flagged M protein under the control of the T7 promoter (20 µg). Two days post-transfection cells were harvested and polysome analysis was performed as described previously (26). Proteins were precipitated with trichloroacetic acid, subjected to SDS-PAGE and detected by western blotting. RNA purification from sucrose gradient fractions was performed using Trizol-LS reagent (Invitrogen).

### Expression and purification of flagged M and flagged M4A proteins

BHK 21 cells were transfected with vectors expressing flagged M and M4A proteins under the control of the T7 promoter (18 µg per 75 cm<sup>2</sup> flask) and with 2 µg of pEGFP-C1 as a transfection efficiency control. Two days post-transfection cells were lysed and flagged M and M4A proteins were purified using ANTI-FLAG M2 Affinity Gel (Sigma). The purified proteins were dialysed against 20 mM Tris-HCl pH7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 25% glycerol and stored at -80°C. Final concentrations of purified flagged M and flagged M4A proteins were estimated by western blot analysis using a flagged standard protein and represented 0.24 µM (400 µl) for M and 0.12 µM (600 µl) for M4A.

### Surface plasmon resonance assays

All assays were performed on a Biacore 2000 instrument equilibrated at 25°C in 20 mM HEPES pH 7.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM MgCl<sub>2</sub>. One hundred and forty RU of M or 475 RU of M4A, both dialysed beforehand against the running buffer, were covalently immobilized on the carboxymethylated surface of a CM5 sensorchip, using the Amine Coupling kit (Biacore AB).

Soluble recombinant human eIF3h protein (EIF3S3, Lab Vision corporation) was then injected for 6 min across the M and M4A surfaces, at 10 µl/min, at four different concentrations ranging from 5 to 50 nM. The raw profiles were double subtracted from those measured on a void reference surface and from those obtained through the injection of the sample buffer onto the M and M4A surfaces. The association and dissociation profiles were analysed with a non-linear least squares algorithm implemented in the BIAevaluation 3.2 software (Biacore), using single-exponential functions of time.

## RESULTS

### RV mutant M protein interacts with the h subunit of eIF3

In order to fulfil numerous functions within infected cells, viral proteins should recruit some cellular partners. We performed a differential two-hybrid screen in *Saccharomyces cerevisiae* (*S. cerevisiae*) using a full-length RV M protein as a bait against a commercial yeast pretransformed human brain cDNA library. A total of more than one million clones were screened. However, despite a high mating efficiency of 2.25% only three slowly growing colonies were obtained. These were later identified as false positive clones.

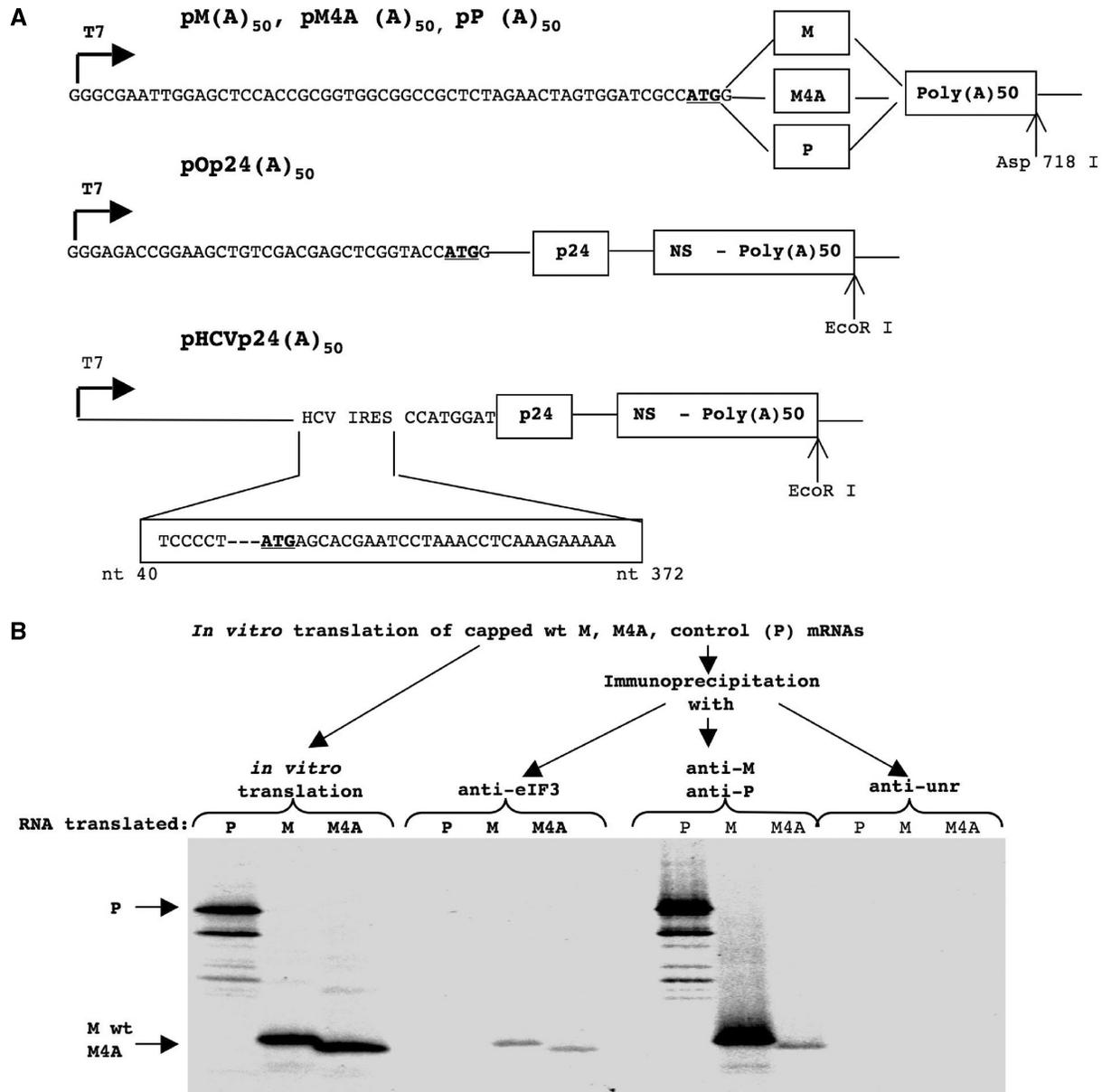
It is known that negative results in a two-hybrid screening could be an outcome of inefficient expression of a Gal4-fused protein or its low stability in yeast, which would result in an imbalance in the stoichiometry of the Gal4-binding and activation domains. Indeed, western blot analysis of fused proteins in yeast extracts, routinely used in our laboratory, almost failed to detect Gal4-M (Figure 1B). Previously, a two-hybrid screen, which used a 16-aa peptide containing the VSV PPxY motif as the bait revealed a strong interaction with the mouse Nedd4 protein WW domain (27). Further, mutations of Pro and Tyr to Ala within the PY motif have been shown to abrogate the protein interaction with Nedd4 (28). However, we did not find the equivalent interplay with a full-length RV M. The only yeast homologue of mouse Nedd4 is the Rsp5 protein, which, like Nedd4, contains several WW domains and a C-terminal E3 ubiquitin ligase domain (29). Rsp5 is an essential E3 ubiquitin ligase in *S. cerevisiae* and is known to be involved in the regulation of target gene expression via post-translational modifications as well as via

regulation of translation (30,31). One could speculate that Rsp5 interaction with the RV M PY motif might lower the quantity of RV M protein within a cell via one of these mechanisms and thus impede the two-hybrid screening.

We substituted the PPxY motif proline residues 34, 35, 36 and tyrosine 38 of the RV M protein by Ala to generate a plasmid carrying the M4A mutant in order to test our hypothesis and to overcome the yeast two-hybrid obstacle. Western blot analysis of yeast protein extracts for M and M4A-Gal4 fusion proteins demonstrated that the M4A-Gal4 concentration in a cell was at least 3-fold higher than that of M-Gal4 (Figure 1B). We applied M4A mutant protein as bait for the commercial yeast pretransformed human brain cDNA library used to screen wt M. We found that even with lower mating efficiency compared to that of wt M (1.3 and 2.25%, respectively), a major group of redundant sequences corresponding to 9 independent clones encoding the human translation initiation factor 3 subunit h (or p40; eIF3-P40 gene; Genbank Accession NM\_003756) was revealed. After plasmid rescue, a quantitative β-galactosidase assay confirmed the interaction between the Gal4-AD/eIF3h fusion protein and M4A, whereas wild-type M protein failed to display a detectable two-hybrid interaction with eIF3h (Figure 1C). Taken together, these data suggest that eIF3h could be a potential cellular partner of RV M.

### Anti-eIF3 antibodies specifically co-immunoprecipitate *in vitro* expressed RV M protein

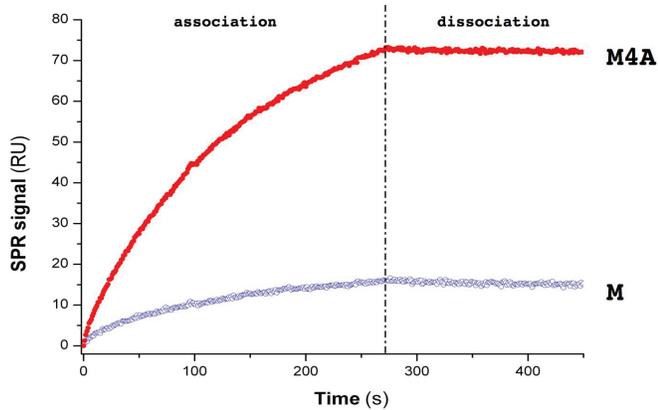
To test whether wild-type M protein can physically associate with eIF3, artificial cDNAs were constructed encoding M, M4A or control proteins under the control of a T7 promoter, and mRNA translation was assessed *in vitro* in nuclease-treated rabbit reticulocyte lysate (RRL). The constructions contained the wild-type M or M4A, or wild-type RV P protein coding regions, preceded by an identical 52 nt 5' UTR and an AUG codon in an identical, near-optimal Kozak consensus (see Figure 2A) (32). Capped mRNAs were synthesized *in vitro* from each of the cDNAs, which had first been linearized downstream of the protein coding sequence (Figure 2A), and translation reactions were immunoprecipitated with polyclonal antibodies raised against purified rabbit eIF3 (Figure 2B). Controls against non-specific pull-down of translation products included translations programmed with RV P mRNA, and immunoprecipitation using antibodies directed against an unrelated, non-canonical translation factor, unr (33). Co-immunoprecipitation analysis showed that wild-type and mutant M proteins do physically associate with components of the eIF3 complex in translation extracts, and failure to co-immunoprecipitate P protein showed the specificity of this association (2nd block of reactions, Figure 2B). Similarly, eIF3 was reproducibly co-immunoprecipitated by anti-M antibodies when reactions were programmed



**Figure 2.** (A) Schematic representation of reporter constructs used for *in vitro* translation. The end of the T7 promoter is arrowed, 5'-UTR sequences are given except for pHCVp24(A)<sub>50</sub> where a partial sequence of the IRES is presented in an open box. The translation initiation codon is shown in boldface and underlined. The various coding regions (M, M4A, P, p24) are shown as open boxes as are the 3'-UTRs followed by a 50-nt poly(A) sequence. NS signifies the 3'UTR of the influenza virus NS protein. The restriction endonuclease sites used for linearization prior to transcription are indicated. (B) Rabies virus M protein co-immunoprecipitates with eIF3 *in vitro*. Standard *in vitro* translation reactions (110  $\mu$ l reactions) were programmed with *in vitro* synthesized P (P lanes), M (M lanes) or M4A (M4A lanes) mRNAs. Reactions were incubated at 30 C for 90 min, and then analysed directly by SDS-PAGE (*in vitro* translation lanes), or subjected to immunoprecipitation using polyclonal antibodies against eIF3 (anti-eIF3 lanes), specific antibodies against the homologous M or P proteins (anti-M/P lanes) or unr (anti-unr lanes). Equal volumes of a translational reaction were used for each immunoprecipitation (35  $\mu$ l). Seven times more translational reaction output was loaded for immunoprecipitation lanes than for the control *in vitro* translation lanes. The positions of M, M4A and P proteins are indicated by arrows.

with M mRNAs, whereas antibodies raised against P protein failed to co-immunoprecipitate eIF3 from reactions programmed with P mRNAs (data not shown). It should be noted that the corollary experiment with mutated M4A mRNA was not feasible, because of

the weak reactivity of this protein with the anti-M antibodies used (see 3rd block of reactions, Figure 2B). Finally, no co-immunoprecipitation was observed using antibodies raised against unr (see 4th block of reactions, Figure 2B). Thus, wt and mutant M proteins,



**Figure 3.** Real-time surface plasmon resonance measurement of the interaction of eIF3 (50 nM) with immobilized M (blue empty circles) and M4A (red filled circles). RU: resonance units (1 RU corresponds to a variation of mass density of  $1 \text{ pg}\cdot\text{mm}^{-2}$ ).

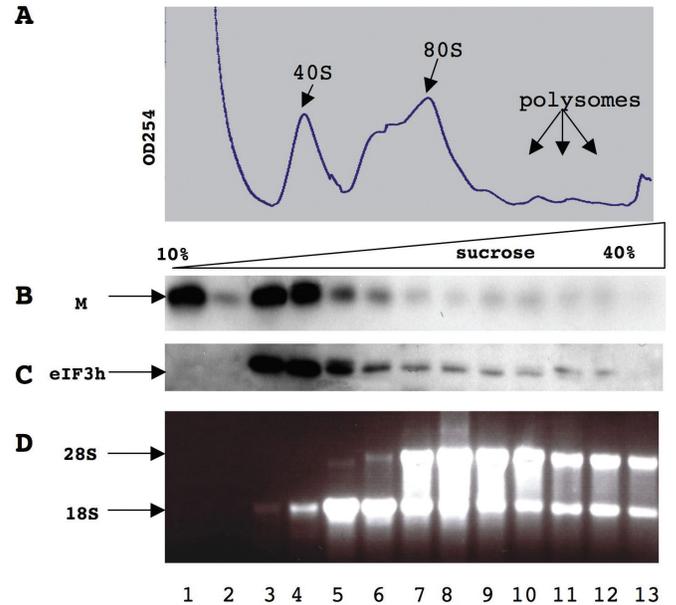
but not P protein, were immunoprecipitated from reactions by anti-eIF3 antibodies.

#### h subunit of eIF3 binds strongly to both M and M4A in surface plasmon resonance assays

We resorted to surface plasmon resonance assays to obtain further confirmation of a direct interaction between eIF3h and M, and to determine the characteristics of such an interaction. M and M4A, purified from eukaryotic mammalian cell cultures, were both covalently immobilized on individual flowcells of a sensorchip (140 RU of M or 475 RU of M4A), and eIF3h was brought in contact with them by a continuous flow. A specific signal could be detected in the two flowcells, suggesting that eIF3h is indeed capable of binding both wt M and the mutated M4A (Figure 3). The surface plasmon resonance signal was reproducibly proportional to the density of immobilized M (or M4A) protein, and dependent on the concentration of eIF3h injected. A full characterization of the kinetic parameters of both interactions was not possible, as the amounts of protein available were limiting. However, it clearly appears that, although the formation of the complex between eIF3h and M (or M4A) is rather slow ( $k_{\text{on}} < 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ), the stability of the complex once formed is very high ( $k_{\text{off}} < 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ ), with dissociation being barely detectable.

#### M protein is present in early small ribosomal subunits fractions

Since the h subunit is a part of the translation initiation factor eIF3, we wanted to examine whether the h-M interaction can be localized within the cellular translational machinery. Using centrifugation through a sucrose density gradient, we performed a ribosomal fraction analysis of lysates of cell cultures that expressed the wt M protein and examined the distribution of the matrix



**Figure 4.** Assessment of M protein association with ribosomes. Cytoplasmic lysates were prepared from BHK 21 cells expressing M protein. (A) An absorbance trace of the polysome profile (OD254), obtained by velocity sedimentation through a 10–40% sucrose gradient of lysates of cell cultures that expressed the wt M protein. The positions of the 40S, 60S, 80S and polysomal peaks are indicated. The top and the bottom of the gradient are shown as 10 and 40% of sucrose. (B) M was detected by western blotting with anti-flag antibodies. An arrow indicates the position of M protein. (C) eIF3h subunit was detected by western blotting with anti-eIF3h antibodies (anti-eIF3S3, Lab Vision corporation). An arrow indicates the position of eIF3h protein. (D), The position of the peak corresponding to the 40S ribosomal subunit has been confirmed by purification of RNA from each of the 13 sucrose gradient fractions, followed by electrophoresis through an agarose gel and staining with ethidium bromide. Arrows indicate positions of 18S and 28S ribosomal RNAs.

protein on it. A plasmid encoding wt flagged M protein under the control of the T7 promoter was transfected into BHK 21 cells co-infected with T7 recombinant vaccinia virus. This approach was used to overcome potential negative effects of the M protein on transcription and nucleocytoplasmic transport. Thus, as in the case of RV virus infection, M transcription and translation were both performed in the cytoplasm. Two days post-transfection, the cells were harvested and lysates were separated on a 10–40% sucrose density gradient. Polysome profiles were analysed by monitoring the absorbance at 254 nm (Figure 4A). Thirteen equal fractions were collected from the gradient, and they were subjected to western blot analysis to determine the distribution of M protein across the gradient (Figure 4B). wt M on the sucrose gradient showed a substantial amount associated with small ribosomal subunit. Furthermore, the western blot analysis of eIF3h subunit showed that it was localized in the same fractions of the gradient (Figure 4C).

Interestingly the patterns of distribution of the two proteins were almost identical. The assignment of OD<sub>254</sub> peaks corresponding to 40S and 60S subunits, intact ribosomes and polysomes was confirmed by gel electrophoresis of RNA extracted from each of the fraction, stained with ethidium bromide (Figure 4D). Thus, we found the RV M protein and eIF3h simultaneously present in the same fractions of the sucrose density gradient, corresponding to the beginning of 40S ribosomal subunit peak, suggesting that the matrix protein could be ribosome-associated in rabies-infected cells.

#### Wild-type M protein and M4A both inhibit translation

To study a possible role of M protein on translation we purified flagged wt M and M4A from eukaryotic mammalian cells. The purified proteins were tested for their effect on translation *in vitro* in an RRL that was partially depleted of ribosomes and associated factors (dRRL). This system was used rather than standard RRL as it has previously been shown to recapitulate more closely the requirements for efficient mRNA translation observed *in vivo* (23). Moreover, it allowed us to perform a direct study of M protein effect on translation without concomitant regulation of other cellular processes. We examined whether the presence of M or M4A proteins supplied *in trans* had an effect on the translation of M mRNA. A m<sup>7</sup>G-M-poly(A) mRNA (see Figure 2A) was translated in dRRL in the presence of increasing concentrations of flagged M or flagged M4A. Translation of M mRNA was inhibited in the presence of both proteins, with 50% inhibition of translation being observed at almost equimolar ratios of M protein to mRNA (Figure 5A). The translation inhibition could not be attributed to the protein buffer (0 (+) lanes, Figure 5A). To investigate whether the inhibition of translation by M protein could be seen for all capped and polyadenylated mRNAs, a capped reporter RNA, coding the human immunodeficiency virus p24 protein (see Figure 2A) was tested in the same *in vitro* system. Effectively, analogous results were observed (Figure 5B). Thus, M and M4A proteins both inhibit translation of capped polyadenylated mRNAs *in vitro* in the dRRL system. Fifty percent inhibition was observed with about 12 nM concentration of each protein (Figure 5A and B), corresponding to the molar ratios inhibitor:mRNA 1:1 in the case of M mRNA and 6:1 for the control mRNA. However, it should be noted that compared to M mRNA the concentration of m<sup>7</sup>G-p24-poly(A) mRNA was decreased, due to its highly efficient translation which would otherwise have impeded quantification of the results.

#### Effect of M protein on translation efficiency of mRNAs carrying the HCV IRES

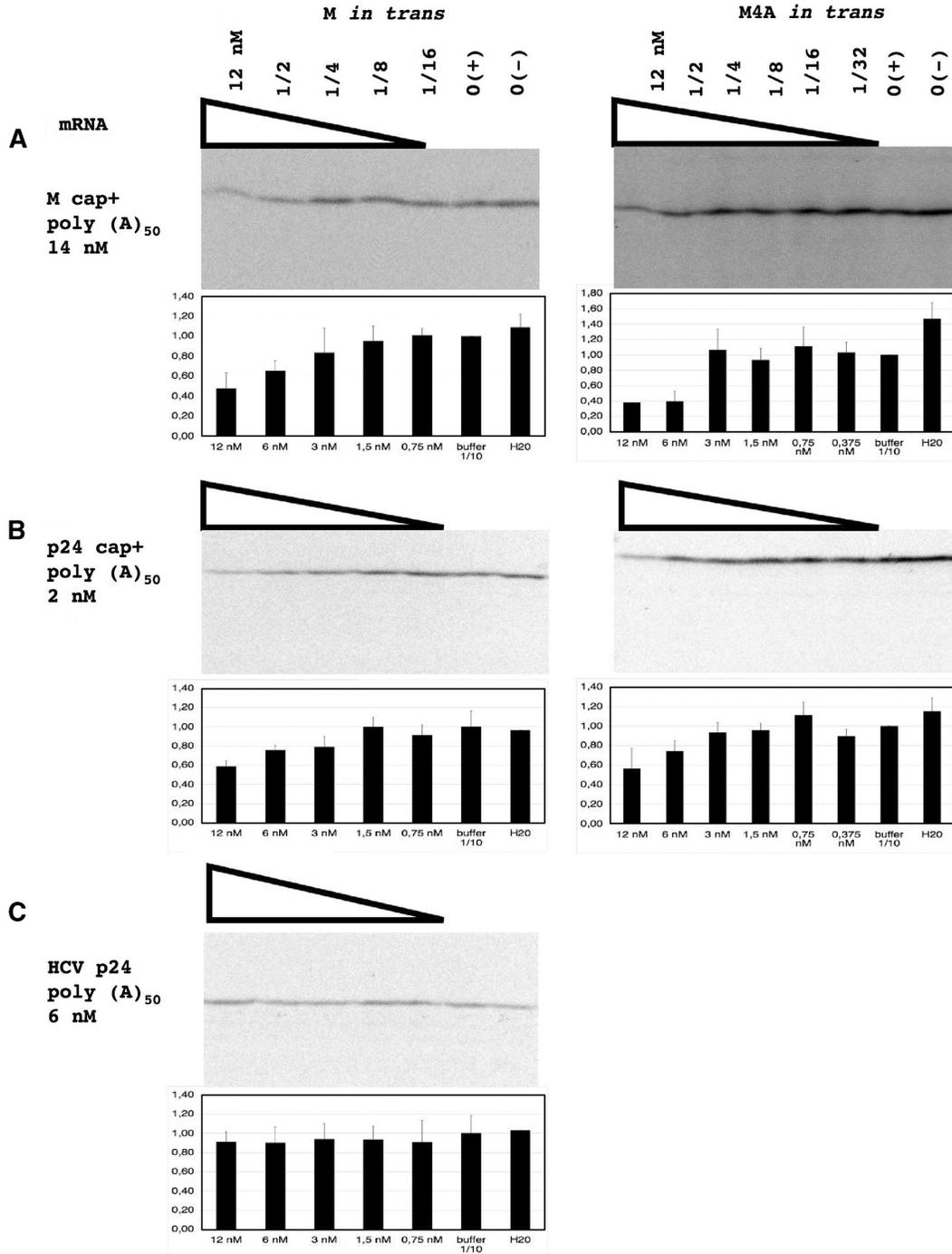
Having established that RV M protein (as well as its mutant form-M4A) seems to be a potent inhibitor of translation, the underlying mechanism of inhibition was

further investigated. The evidence presented above would suggest that M might act via the involvement of an interaction with h as part of eIF3. Thus, we decided to examine whether the translation of reporter mRNAs encompassing 5'UTRs capable of factor-independent binding of the mRNA to 40S ribosomal subunit was sensitive to the presence of M protein *in trans*. We employed the HCV IRES since it has been shown that the 40S ribosomal subunit is able to bind directly and specifically this element in the immediate vicinity of the P-site without the involvement of canonical initiation factors (18). A reporter mRNA carrying the HCV IRES in its 5'UTR and terminating with a poly(A) tail was used (see Figure 2A). As in the previous experiments, increasing doses of M protein were added in the dRRL translation system. However the addition of the M protein in the system had practically no effect on translational efficiency. In other words, the efficacy of mRNA translation in the presence or in the absence of M protein was virtually indistinguishable (Figure 5C). This suggests that RV M protein could function in an infected cell to inhibit the translation of those mRNAs that use a canonical mechanism of ribosome attachment to mRNA.

#### DISCUSSION

It is generally accepted that a single viral protein often implements several functions to ensure optimal replication. The M protein of negative-stranded RNA viruses such as Rhabdoviridae is one of the examples of multifunctional viral proteins. Indeed, previous studies on VSV M protein have demonstrated its implication in viral assembly and budding, and in the shut-off of cellular transcription and translation, which would provide adequate conditions for viral replication ((14,15), for a review see (5,7)). In the present work we undertook a search for potential RV M protein cellular partners and then explored processes in which the identified interaction could be involved. Indeed, since RV and VSV M proteins share only 24% amino acid identity (Figure 1A), functional and structural knowledge of VSV M protein cannot always be extrapolated to RV M and the latter protein needs to be examined separately.

First a yeast two-hybrid screening with the full-length RV M was performed to search for potential RV M-interactive cellular factors. However full-length wt M protein failed either to recapitulate the previously found interaction of VSV PY late domain with WW domain of Nedd4 (27) or to provide any promising M-cellular targets. Quantification of M-Gal4 protein concentration in yeast extracts allowed us to hypothesize that a PY interaction with an essential Rsp5 E3 ubiquitin ligase could direct RV M-Gal4 protein for degradation or regulate its efficacy of translation. In both cases that would result in a failure of the yeast two-hybrid screening. Thus, to abolish this interaction, for the following



**Figure 5.** Effects of M and M4A proteins on translation *in vitro*. Capped polyadenylated mRNA (12 nM) coding M protein (A), or a reporter mRNA (2 nM) coding the p24 protein (B), or uncapped pHCVp24-derived mRNAs (6 nM) (C) were translated in dRRL in the presence (+)/absence (-) of dialysis buffer or doubling dilutions of purified M or M4A proteins, as indicated above the panels. dRRL is RRL that has been partially depleted of ribosomes and associated factors by ultracentrifugation. Translation was analysed by SDS-PAGE and translation efficiencies derived from densitometric quantification of at least four independent experiments and related to respective 0(+) control experiments are plotted below each lane, with corresponding standard deviations.



experiment we used RV M protein that carried mutations within PY late domain (M4A) and deficient in the interaction with WW domains (28). The mutation should not change the globular fold of M, since Ala substitutions are situated within the protruding N-terminal part of the RV M protein. Moreover, a recent study of VSV M suggests that another protein sequence, located downstream from the PY motif, is responsible for interactions with host proteins (6). Therefore, the new two-hybrid approach, performed with the PPxY motif mutated M protein, allowed us to avoid Rsp5-PY two-hybrid screening bias, and resulted in the identification of an interaction with subunit h of eIF3.

The multisubunit eIF3 complex performs the principal role in ribosomal dissociation and anti-association (34–36). Recent studies have shown that the multisubunit eIF3 complex is likely to be an indirect target of attack by those viruses that provoke interferon-induced p56 synthesis. Indeed, human p56 can interact directly with the e subunit of eIF3, and mouse p56 with eIF3c. In both cases p56 binding leads to translation inhibition, interfering with eIF3/ternary complex interaction for human p56, and 40S ribosomal subunit-eIF3-ternary complex binding to eIF4F for mouse p56 (37,38). Furthermore, it has been found that a viral protein VPg of the positive-stranded RNA of caliciviruses directly binds to the eIF3d subunit. This protein–protein interaction is believed to inhibit cap-dependent initiation of translation and to recruit translation initiation complexes to calicivirus mRNAs, which are naturally uncapped (39). The role of the h subunit in eIF3 function is still not fully elucidated. Literature data suggest that while h might be dispensable to eIF3, it is used in mammalian cells to regulate eIF3 function. Interestingly, h subunit is overexpressed in breast and prostate cancer but its exact contribution in the pathogenesis of such cancers remains unknown (40). It also binds to DISC1, the product of the *Disrupted-In-Schizophrenia 1* gene, one of the candidate responsible for schizophrenia, and this binding could lead to accumulation of eIF3 in stress granules (41). Recently, a new h-protein interaction was found with human and *Xenopus* stem loop binding proteins (42). Finally, h subunit has been suggested to regulate translation of specific mRNAs in *Arabidopsis thaliana* via 5' mRNA leader sequences (43).

We used standard co-immunoprecipitation experiments to test whether the eIF3–M4A interaction could also be observed for the wt M protein. Hence, we could show that indeed RV M protein physically associates with components of the eIF3 complex in mammalian cell-free translation extracts (Figure 2B). Further evidence was provided by surface plasmon resonance assays. These *in vitro* assays equally confirmed that the h subunit of eIF3 was capable of interacting directly with both M and M4A, forming a very stable complex and that the profile of interaction of RV M or M4A–eIF3h proteins does not depend on the presence or absence of the PY motif (Figure 3).

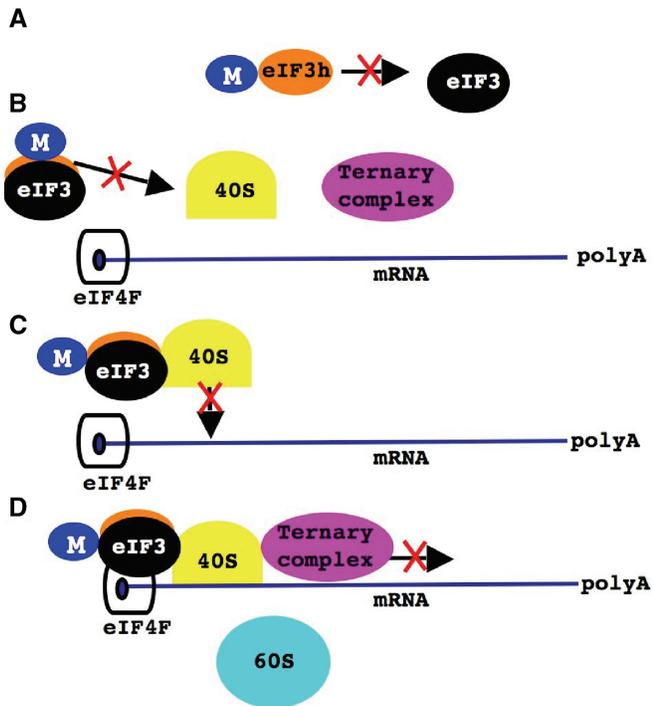
In RV-infected cells, the majority of the M protein is cytoplasmic, and to a lesser extent this protein is

associated with the inner leaflet of the plasma membrane, with traces also detected in the nucleus (44). We found the protein present in polyribosomal fractions and localized it to the early fractions of small ribosomal subunits (see Figure 4B). Moreover, this pattern of M protein distribution on polysomal fractions was analogous to that of eIF3h (see Figure 4C). Taking into account the results of plasmon resonance assay we believe that the presence of matrix protein in 40S subunit peak is due to the h subunit of eIF3. These results led us to explore the question of whether M protein presence on ribosomes could influence the translation in virus-infected cells.

We used *in vitro* translation assays to study the effect of M on translation. For this, the M and M4A proteins were purified from mammalian cell cultures to avoid the loss of potential post-translational modifications and the purified proteins were added to *in vitro* translation reactions. We observed an inhibition of translation of two capped and polyadenylated mRNAs with similar potencies for M and M4A proteins (see Figure 5A and B). Notably, the 50% of inhibition was obtained with molar ratios inhibitor:mRNA that could be considered as physiological, since during a viral infection, an infected cell produces preferentially viral proteins and M protein is one of the most abundant of these. This result allowed us to put forth the hypothesis that translation initiation on factor-dependent mRNAs could be inhibited by RV M protein, by a mechanism that involves an interaction between M and eIF3h. Such a mechanism of translational regulation has not been described to date.

If it is the interaction between M and eIF3h that inhibits canonical initiation of protein synthesis, this inhibition should be overcome when the role of eIF3 in translation initiation is different. To test this hypothesis, we employed the HCV IRES, which is known to be able to attach the 40S ribosomal subunit via factor-independent binding to mRNA at the translation initiation site (18). The presence of M protein *in trans* had no discernable effect on the translation of a reporter mRNA carrying the HCV IRES (Figure 5C). This is interesting, because in fact eIF3 does play certain roles in HCV IRES-driven translation. Although eIF3 is not required for accurate positioning of the 40S subunit on the start codon, it binds specifically to the HCV IRES (20). This sequence- or structure-specific binding is in contrast to canonical mRNAs. Moreover, eIF3 is required for the joining of a 60S subunit to the IRES-bound 48S complex (for a review see (21)). Thus, the absence of inhibition of translation from the HCV IRES by M protein shows that either the M–eIF3 interaction does not impede the specific binding of eIF3 to the HCV IRES or the different functions of eIF3 in HCV translation are not blocked by this interaction.

In summary, the results obtained in this work have allowed us to present several potential scenarios for M protein function as an inhibitor of canonical translation initiation. First, M could bind to free h subunits and titre them away from the translationally functional eIF3 complex (Figure 6A). Second, M could interact with an entire eIF3 complex but before the latter binds to the 40S subunit/mRNA, leading to the formation of



**Figure 6.** Proposed schematic models of the involvement of M/eIF3h interaction at different levels of translation initiation in an RV-infected cell. (A) M binds to a free eIF3h subunit and titeres it away from a translationally functional multisubunit eIF3 complex. (B) M binds to free eIF3, blocking its interaction with 40S. (C) M interaction with eIF3/40S leads to the formation of non-functional 40S complex. (D) M binds to the 48S complex and blocks some later stages of translation.

a non-functional pool of eIF3 (Figure 6B). Third, M could fix to the eIF3/40S complex without any other eIFs or mRNA (Figure 6C). Finally, binding could be directly to eIF3 as a part of the 48S translation initiation complex composed of 40S, eIFs and a ternary complex on mRNA, impeding efficient translation from occurring (Figure 6D). The enriched presence of M protein in the fractions corresponding to the 40S ribosomal subunit peak favours the last two scenarios of inhibition (Figure 6C and D). However, we cannot exclude that the M–eIF3h interaction leads to the formation of stress granules, which are aggregates of 40S, some translation initiation factors and numerous associated RNA-binding proteins. Further work will be required to validate these hypotheses and to draw a more detailed model of M protein function at the level of translation in a RV-infected cell.

Overall, the new insights that are beginning to emerge concerning translational control by *Lyssavirus* M protein are an important question in rabies pathogenesis. First, their study will help to clarify the mechanisms of regulation of translation employed by these viruses. Second, they provide an additional support that during a viral infection there is a modulation of gene expression at different levels: transcription, RNA transport, translation, degradation, etc. As such, this study should help to open up a plethora of new situations for analysis in the field of translational control of gene expression

and may provide new targets for the treatment and prevention of rabies disease.

#### Supplementary data

Supplementary data is available at NAR Online.

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