Cellular Microbiology (2015) **17**(4), 520–541
doi:10.1111/cmi.12381
First published online 19 November 2014

Inhibition of host protein synthesis by Sindbis virus: correlation with viral RNA replication and release of nuclear proteins to the cytoplasm

Miguel A. Sanz,* Manuel García-Moreno and Luis Carrasco
Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, C/Nicolás Cabrera 1, 28049 Madrid, Spain

Summary

Infection of mammalian cells by Sindbis virus (SINV) profoundly blocks cellular mRNA translation. Experimental evidence points to viral nonstructural proteins (nsPs), in particular nsP2, as the mediator of this inhibition. However, individual expression of nsP1, nsP2, nsP3 or nsP1-4 does not block cellular protein synthesis in BHK cells. Trans-complementation of a defective SINV replicon lacking most of the coding region for nsPs by the co-expression of nsP1-4 propitiates viral RNA replication at low levels, and inhibition of cellular translation is not observed. Exit of nuclear proteins including T-cell intracellular antigen and polypyrimidine tract-binding protein is clearly detected in SINV-infected cells, but not upon the expression of nsPs, even when the defective replicon was complemented. Analysis of a SINV variant with a point mutation in nsP2, exhibiting defects in the shut-off of host protein synthesis, indicates that both viral RNA replication and the release of nuclear proteins to the cytoplasm are greatly inhibited. Furthermore, nucleoside analogues that inhibit cellular and viral RNA synthesis impede the blockade of host mRNA translation, in addition to the release of nuclear proteins. Prevention of the shut-off of host mRNA translation by nucleoside analogues is not due to the inhibition of eIF2α phosphorylation, as this prevention is also observed in PKR$^{-/-}$ mouse embryonic fibroblasts that do not phosphorylate eIF2α after SINV infection. Collectively, our observations are consistent with the concept that for the inhibition of cellular protein synthesis to occur, viral RNA replication must take place at control levels, leading to the release of nuclear proteins to the cytoplasm.

Received 20 May, 2014; revised 30 September, 2014; accepted 10 October, 2014. *For correspondence. E-mail masanz@cbm.csic.es; Tel. +34 911964517; Fax +34 914974799.

Introduction

The genome of alphaviruses is a positive RNA molecule (gmRNA) of about 9–11 kb, which directs viral protein synthesis upon infection (Griffin, 2007). The genome contains two cistrons: the first is located in the 5′ two-thirds of the genome and encodes nonstructural proteins (nsPs) that form the viral replication machinery (Jose *et al.*, 2009). The second open reading frame encodes for the structural proteins that form part of the virus particles, and are synthesized from a subgenomic mRNA (sgmRNA; Strauss and Strauss, 1994). Sindbis virus (SINV) is one of the most studied members of alphaviruses and represents a good model to analyse the regulation of protein synthesis in virus-infected cells. After virus entry, the genome is delivered to the cytoplasm and is translated to produce nsP1-4, which participates in genome replication and transcription (Strauss and Strauss, 1994). These nsPs are synthesized from a single AUG initiation codon rendering two precursor polyproteins, which are proteolytically processed by nsP2 (de Groot *et al.*, 1990). After initiation at the first AUG initiation codon present in gmRNA, the majority of ribosomes (90–95%) translates this template until a stop codon (UGA) is encountered, producing the precursor P123. However, in a small proportion of cases, there is readthrough of this stop codon, generating the precursor P1234. The four nsPs are generated after proteolytic cleavage of the polyprotein precursors. The exact function of the individual proteins has been the subject of intensive research in several laboratories. Thus, nsP1 is an abundant component of the replicative complex and exhibits its activity either as a mature protein or in the form of precursors P123 or P1234. nsP1 is a palmitoylated protein that participates directly in initiation

and elongation of the minus-strand RNA synthesis (Hahn *et al*., 1989; Wang *et al*., 1991). During this process, nsP1 interacts with nsP4 (Shirako *et al*., 2000; Fata *et al*., 2002). Different moieties have been recognized in nsP1. The N-terminal region exhibits methyltransferase and guanylyltransferase activities involved in capping the viral positive-strand RNAs (Mi *et al*., 1989; Peranen *et al*., 1995; Ahola *et al*., 1997). Association with cellular membranes is promoted by an amphipatic helix located in the middle region of nsP1 (Peranen *et al*., 1995) that serves to anchor viral replicative complexes to membranes (Salonen *et al*., 2003). Several domains have also been distinguished in nsP2: an amino-terminal RNA helicase domain, a central protease domain that catalyses all cleavage reactions between the nonstructural proteins and an inactive RNA methyltransferase-like domain (Gomez de Cedron *et al*., 1999; Russo *et al*., 2006). Apart from its function in viral RNA synthesis, nsP2 is also involved in the interference with cellular transcription and translation. Accordingly, nsP2 can antagonize cellular antiviral responses triggered by alphavirus infection (Garmashova *et al*., 2006; Breakwell *et al*., 2007). Indeed, a proportion of nsP2 localizes to the nucleus and blocks cellular RNA export to the cytoplasm (Rikkonen *et al*., 1994; Breakwell *et al*., 2007). The precise role of nsP3 in virus replication remains poorly understood. nsP3 can be organized into three domains: an amino-terminal macro or X domain (Koonin *et al*., 1992), a central alphavirus specific region and a carboxyl region with an hypervariable sequence. Residues located just after the macro domain of nsP3 play a role in positioning of the P23 cleavage site (Lulla *et al*., 2012). Additionally, the carboxyl terminal region of nsP3 contains several phosphorylation sites (Vihinen *et al*., 2001). nsP3 blocks the formation of host cellular stress granules (SGs) through the interaction of its carboxy terminal domain with Ras-GTPase activating protein-binding protein (G3BP), leading to the disruption of these granules that are involved in innate antiviral mechanisms (Fros *et al*., 2012; Panas *et al*., 2012). Finally, nsP4 is an RNA-dependent RNA polymerase that is involved in the synthesis of the different viral RNAs, i.e. gmRNA, sgmRNA and minus-stranded RNA complementary to the genome (Rubach *et al*., 2009). Both P123 + nsP4 and nsP1 + P23 + nsP4 preferentially synthesize the negative strand of viral RNA (Lemm *et al*., 1994; Shirako and Strauss, 1994). The final cleavage event of P23 produces fully mature nsPs and switches the RNA template for synthesis of positive sense genomic and subgenomic RNAs. The correlation between P23 cleavage and the change from negative to positive sense RNA production is poorly defined. The P23 cleavage site is located at the base of a narrow cleft and is not readily accessible, suggesting a highly regulated cleavage. Mutations in nsP2 or nsP3 near this cleavage site may alter viral replication and pathogenicity (Shin *et al*., 2012).

Most cytolytic animal viruses induce a profound decrease of cellular protein synthesis in infected cells, particularly during the late phase of infection (Bushell and Sarnow, 2002). This is the case for SINV, which ablates cellular translation approximately 3 h after virus entry, while the protein-synthesizing machinery is preferentially dedicated to translate the viral sgmRNA (Strauss and Strauss, 1994; Griffin, 2007). Distinct mechanisms are responsible for the interference of cellular macromolecular synthesis by the different animal viruses. Alphaviruses induce the phosphorylation of eIF2α in vertebrate cells, leading to an inhibition of host mRNA translation (McInerney *et al*., 2005). However, infection of cells deficient in PKR (protein kinase activated by dsRNA), such as $PKR^{-/-}$ mouse embryonic fibroblasts (MEFs) by SINV also produces this blockade, despite the fact that no increased eIF2α phosphorylation is observed (Gorchakov *et al*., 2004; Ventoso *et al*., 2006). These observations suggest that SINV employs additional mechanisms, separate to eIF2α phosphorylation, to interfere with cellular protein synthesis. For example, the imbalance of ionic concentrations in the cytoplasm of infected cells, or the release of cellular proteins from the nucleus to the cytoplasm have been implicated as responsible for this inhibition (Garry *et al*., 1979; Garry, 1994; McInerney *et al*., 2005). Analysis of a number of alphavirus nsP variants suggest that nsP2 is chiefly responsible for the inhibition of cellular macromolecular synthesis after viral infection (Gorchakov *et al*., 2004; Garmashova *et al*., 2006; Frolov *et al*., 2009). Accordingly, SINV with a single nsP2 point mutation at proline 726 exhibits defects in host translational shut-off (Frolov *et al*., 1999). The possibility that the translational efficiency and the quantity of subgenomic mRNA play a role in the inhibition of host protein synthesis has also been proposed (Patel *et al*., 2013). Regardless, the cellular shut-off is efficiently induced by replicons lacking the coding region for the viral structural proteins (Frolov and Schlesinger, 1994; Sanz *et al*., 2007). These observations are consistent with the concept that nsPs participate and are necessary to trigger shut-off of host protein synthesis. In the present work, we have analysed the expression of nsPs and their potential relationship with the inhibition of cellular mRNA translation. In addition, viral RNA replication and the redistribution of nuclear proteins to the cytoplasm have been examined under restrictive conditions. Our findings suggest an association among these three phenomena: the blockade of host translation, the level of viral RNA replication and the redistribution of nuclear proteins to the cytoplasm.

Results

Individual expression of SINV nsPs in BHK-T7 cells

Previous work demonstrated that replicons expressing only SINV nsPs have the ability to interfere with host translation (Frolov and Schlesinger, 1994; Sanz *et al*., 2007). Moreover, SINV bearing mutations in nsP2 are defective in blocking cellular protein synthesis (Frolov *et al*., 1999). Thus, we tested the activity of the individual expression of nsP1, nsP2 and nsP3, and expression of nsP1-4, on the endogenous translation of cellular mRNAs. In order to achieve high-level synthesis of nsPs, we used BHK-T7 cells that constitutively express the T7 RNA polymerase. This system has been employed successfully to express the precursors of Semliki Forest virus (SFV) nsPs (Spuul *et al*., 2011). BHK-T7 cells were transfected with pTM1 plasmids containing the coding region for SINV nsPs downstream of the IRES element of encephalomyocarditis virus EMCV(IRES). As shown in Fig. 1A, cells transfected with pTM1-nsP1, pTM1-nsP2 or pTM1-nsP3 synthesized a new product that may correspond to the individual viral proteins. Both nsP1 and nsP3 were clearly detectable at 8 and 18 hpt, whereas nsP2 was synthesized at lower levels. However, the synthesis of new proteins was not evident by radioactive labelling after transfection of BHK-T7 cells with pTM1-nsP1-4. We employed poliovirus 2A protease (PV $2A^{pro}$) as a positive control as it is a viral protein that profoundly inhibits cellular translation, while the expression of PV 2C was employed as a negative control as it is a viral protein that does not directly affect cellular protein synthesis (Redondo *et al*., 2011). Densitometric analysis of cellular actin revealed that PV $2A^{pro}$ inhibited its synthesis by more than 90% at 8 hpt, suggesting that transfection of BHK-T7 cells was very efficient (Fig. 1A and B). In contrast, inhibition of cellular actin synthesis was 39% by expression of PV 2C. This partial blockade of cellular translation by PV 2C at 8 hpt may be due to the efficient transcription of this plasmid followed by translation of the corresponding mRNA. Additionally, BHK cells expressing nsP2, nsP3 or nsP1-4 exhibited an inhibition of actin synthesis similar to that observed with PV 2C (34, 48 and 42%, respectively) at 8 hpt (Fig. 1A and B). Expression of nsP1, which is very efficient at this time, provoked a 72% decrease in actin synthesis. Strikingly, when the synthesis of cellular actin was measured at 18 hpt, PV $2A^{pro}$ still blocked its production by 95%, whereas PV 2C showed no inhibition (3%) at this time. Reduced blockade of cellular protein synthesis was also observed with the SINV nsPs, including protein nsP1, which caused an inhibition of 24% at this time. Collectively, these results show that individual expression of SINV nsPs does not abrogate cellular translation to the extent shown by a genuine inducer of the shut-off of host translation, PV $2A^{pro}$. The partial inhibition detected at 8 hpt by SINV nsPs might be due to the high efficiency of transcription and translation of this system, which decreases after prolonged incubation (18 hpt), rather than to the activity of the SINV proteins on the translational machinery. If one individual nsP was responsible for the inhibition of cellular protein synthesis, we would expect that after prolonged synthesis (18 hpt), this inhibition would be cumulative, but rather, we observed that the blockade of cellular translation declines with time.

To further show that the protein species observed in Fig. 1A corresponded to the translation of mRNAs bearing the EMCV IRES, and also to assess whether nsP1-4 is expressed from the corresponding plasmid, we took advantage of the ability of PV $2A^{pro}$ to enhance picornavirus IRES-driven translation (Castello *et al*., 2011). In addition, expression of PV $2A^{pro}$ decreases endogenous cellular protein synthesis resulting in a more prominent and clear synthesis of proteins from pTM1 in this system, thus facilitating their detection. Strikingly, transfection of pTM1-nsP1-4 in cells previously transfected with pTM1-2A resulted in the appearance of protein species that corresponded to precursor and mature nsPs (Supporting Information Fig. S1A). Moreover, the synthesis of nsP1, nsP2 and nsP3 was more clearly distinguished when PV $2A^{pro}$ was present. Consistent with these results obtained in cultured cells, *in vitro* transcription/translation of the corresponding plasmids in rabbit reticulocyte lysates rendered proteins with the expected molecular mass (Supporting Information Fig. S1B). In the case of nsP1-4 mRNA, different products were produced because of the proteolytic processing of the polyprotein, rendering a variety of proteolytic precursors and mature proteins.

To further assess if these proteins corresponded to genuine SINV nsPs, we analysed their expression by immunoblotting using polyclonal antibodies to nsP1, nsP2 and nsP4. Additionally, we compared the levels of production of these proteins in BHK-T7 cells with those produced in SINV-infected cells. As these proteins were recognized by the specific antibodies and migrated similarly as in infected cells (Fig. 1C), we can conclude that genuine nsP1, nsP2 and nsP4 are being synthesized using the T7-expressing system. Moreover, nsP1 or nsP2 were produced at higher levels at 8 hpt when compared with that observed during virus infection at 8 hpi. Further, as shown in the labelling experiments (Fig. 1A), the level of nsP3 synthesis should be much greater in this system relative to infected cells, where nsPs cannot be directly detected by radioactive labelling.

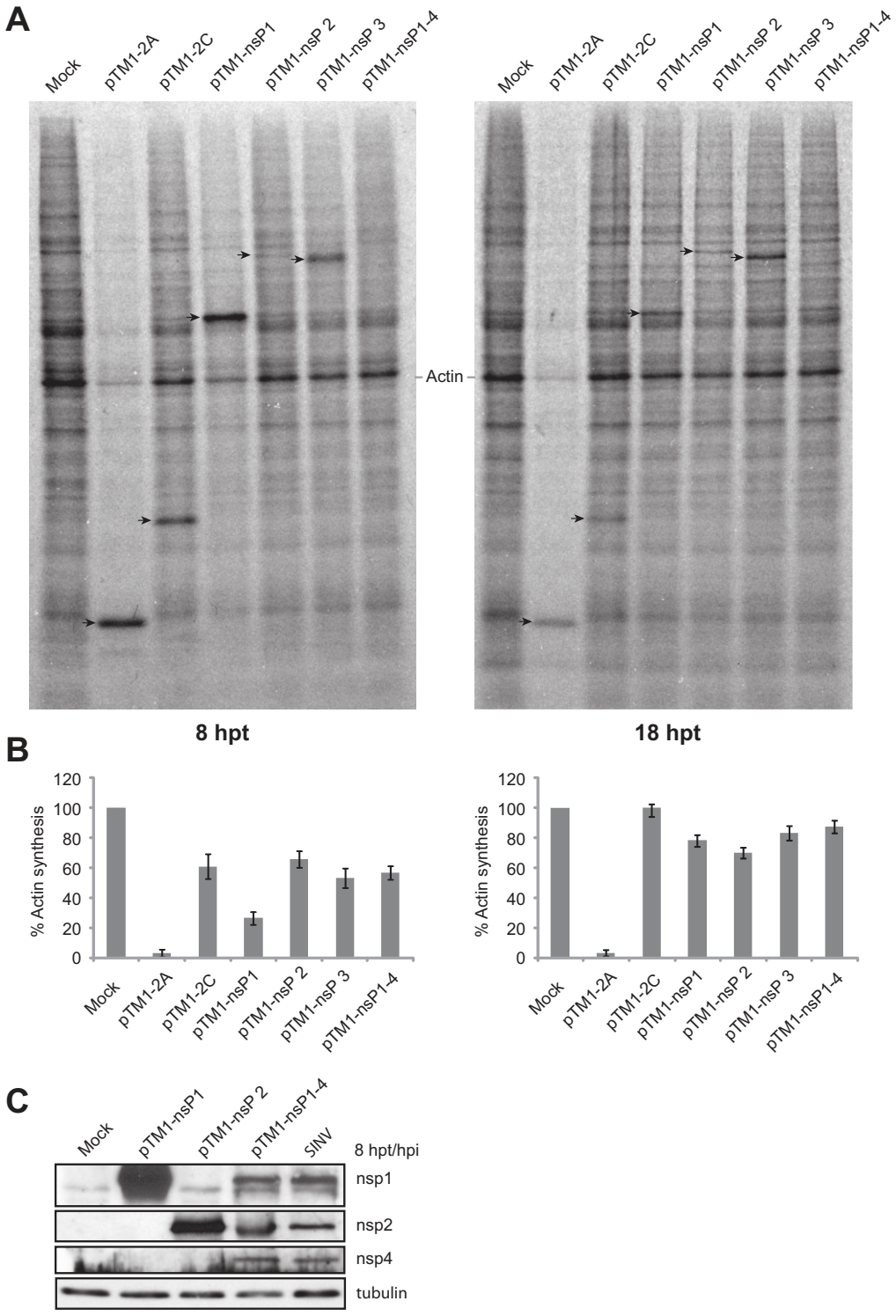


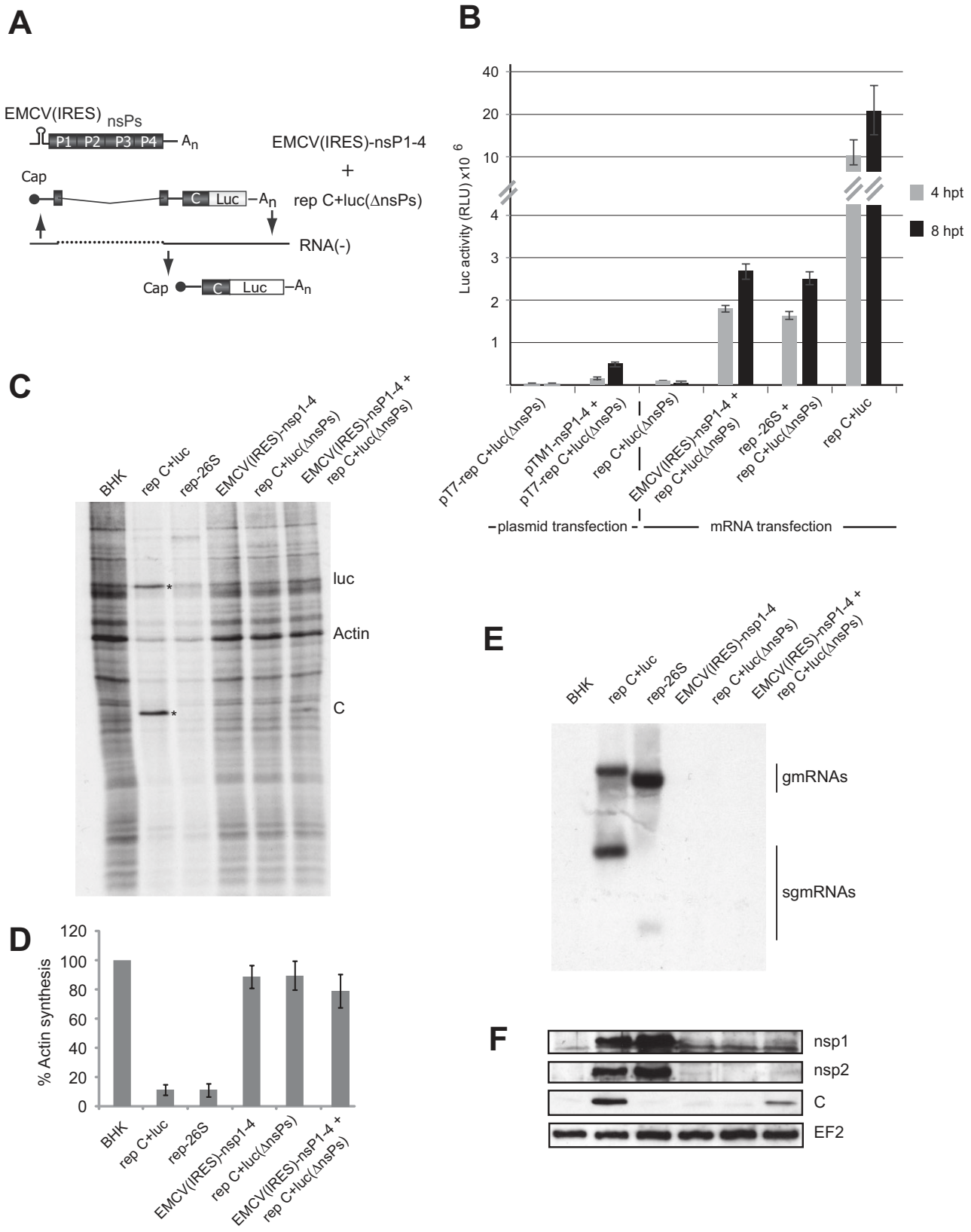
Fig. 1. Individual expression of SINV nsPs in BHK-T7 cells.
A. BHK-T7 cells seeded on 24-well plates were transfected with the different pTM1 plasmids (0.8 μg per well) and protein synthesis was analysed at 8 or 18 hpt by incorporation of [^{35}S]Met/Cys for 30 min followed by SDS-PAGE, fluorography and autoradiography. Arrows indicate the migration of the recombinant proteins.
B. The percentage of actin synthesis in transfected cells related to mock-transfected cells was calculated from values obtained by densitometric scanning of the corresponding bands and are plotted as means ± SD of three representative experiments.
C. Samples obtained from cells transfected with the indicated plasmids (8 hpt) and from SINV-infected cells (10 pfu cell^{-1}, 8 hpi) were subjected to immunoblotting using specific anti-nsP1, anti-nsP2, anti-nsP4 and anti-tubulin antibodies. Tubulin was used as a loading control.

Functionality of nsP1-4 determined by complementation of a defective SINV replicon

In order to assess whether the nsPs synthesized after plasmid transfection of pTM1-nsP1-4 were active and functional in viral RNA replication, we performed a complementation assay with a SINV replicon that lacks the majority of the nsP-coding region. This plasmid contains luciferase (luc) as a reporter placed after the coding region of the capsid protein (C) (see scheme of rep C+luc(ΔnsPs) in Fig. 2A). Two different complementation approaches were used: in the first approach, two plasmids (pT7-rep C+luc(ΔnsPs) and pTM1-nsP1-4) were co-transfected; in the second approach, we co-transfected two RNAs obtained by *in vitro* transcription of the plasmids, rep C+luc(ΔnsPs) and EMCV(IRES)-nsP1-4. Viral RNA replication can be complemented in *trans* in BHK-T7 cells transfected with plasmids encoding SFV nsPs (Spuul *et al*., 2011); however, in that study, transfections of DNA plasmids or RNA replicons were not compared. Transfection of DNA plasmid pT7-rep C+luc(ΔnsPs) alone produced virtually no luc activity, whereas co-transfection with pTM1-nsP1-4 induced significant luc synthesis (Fig. 2B). Presumably, in these cells, the T7 RNA polymerase synthesizes mRNAs EMCV(IRES)-nsP1-4 and rep C+luc(ΔnsPs) and the complementation occurs (as indicated in Fig. 2A). Notably, co-transfection of these mRNAs previously obtained by *in vitro* transcription led to a much greater production of luc (Fig. 2B). This result is of interest as it indicates that the mRNA EMCV(IRES)-nsP1-4 synthesized produces precursor and mature proteins that are active in SINV RNA replication and the subsequent transcription of the sgmRNA. Further, it indicates that complementation of the defective replicon rep C+luc(ΔnsPs) is approximately 10–15-fold more efficient when transcribed RNAs are transfected relative to plasmid transfection. Of note, a low level of luc synthesis occurred when capped RNA rep C+luc(ΔnsPs) was transfected alone (Fig. 2B). This activity may correspond to the inefficient translation of the second cistron (C+luc) present in this RNA, perhaps after ribosome reinitiation on this cistron. In addition, we measured luc activity from cells co-transfected with rep C+luc(ΔnsPs) and a non-defective replicon, rep-26S, which gives rise to all functional nsPs. In this way, the effectiveness of complementation of EMCV(IRES)-nsP1-4 RNA was compared with that obtained with the replicon rep-26S. Complementation *in trans* by nsPs derived from EMCV(IRES)-nsP1-4 is similar to that obtained with rep-26S (Fig. 2B). This suggests that the proteins synthesized from EMCV(IRES)-nsP1-4 mRNA are as functional as those derived from the replicon rep-26S. Both types of complementation *in trans* yield up to 90% less luc activity than rep C+luc (Fig. 2B).

As the expression of nsPs had little effect on cellular translation (Fig. 1), we wondered whether the expression of these proteins together with the replication of a viral RNA upon complementation could induce an inhibition of cellular protein synthesis. To this end, we examined protein synthesis in BHK cells with nsPs complementing rep C+luc(ΔnsPs) (Fig. 2C). BHK cells were transfected with EMCV(IRES)-nsP1-4 mRNA, with rep C+luc(ΔnsPs) or with both combined. As controls for this assay, we used rep C+luc and rep-26S, which renders a minimal sgmRNA with the 5′UTR sequence but without the sequence for structural proteins. At 7 hpt, cells were labelled with [^{35}S]Met/Cys for 30 min. As expected, a strong inhibition of cellular translation was observed in cells transfected with rep C+luc or rep-26S, whereas cellular protein synthesis persisted in cells transfected with EMCV(IRES)-nsP1-4 or with rep C+luc(ΔnsPs) (Fig. 2C and D). Interestingly, protein synthesis also continued

Fig. 2. Complementation of a defective replicon by expression of SINV nsPs.
A. Schematic representation of the EMCV(IRES)-nsp1-4 mRNA and defective replicon rep C+luc(ΔnsPs), and the processes of replication and transcription of sgmRNA C+luc.
B. BHK-T7 cells previously seeded on 24-well plates were transfected with XhoI-linearized pT7-rep C+luc(ΔnsPs) plasmid only (0.4 μg) or co-transfected with pTM1-nsP1-4 (0.4 μg). In parallel, cells were transfected with the *in vitro* obtained mRNA rep C+luc(ΔnsPs) (2.5 μg) or co-transfected with this mRNA (2.5 μg) and EMCV(IRES)-nsP1-4 (2.5 μg). Cells were also co-transfected with rep C+luc(ΔnsPs) (2.5 μg) and rep-26S (2.5 μg) or transfected only with rep C+luc (2.5 μg). At 4 and 8 hpt, cells were harvested and luc activity was determined. Luc activity results are displayed as means ± SD of three independent experiments performed in triplicate.
C. BHK cells previously seeded on 24-well plates were transfected with the different mRNAs (5 μg per well) obtained by *in vitro* transcription, and at 7 hpt protein synthesis was analysed by incorporation of [^{35}S]Met/Cys for 30 min followed by SDS-PAGE, fluorography and autoradiography. Asterisks indicate C and luc proteins.
D. The percentage values of actin protein synthesis in transfected cells compared with mock-transfected cells were calculated by densitometric scanning and are represented as means ± SD of three independent experiments.
E. BHK cells previously seeded on 24-well plates were transfected with 2.5 μg of each mRNA or co-tranfected with 2.5 μg EMCV(IRES)-nsP1-4 and 2.5 μg rep C+luc(ΔnsPs) and treated with [^{3}H]Uridine in the presence of actinomycin D from 3 to 7 hpt. Then, total RNA was extracted and analysed by electrophoresis in denaturing gels, fluorography and autoradiography as indicated in *Materials and methods*.
F. Samples obtained in panel C were used in immunoblot analyses with anti-nsp1, anti-nsp2 or anti-C antibodies. The sample corresponding to rep C+luc was diluted 10-fold in the analysis of C. Anti-EF2 was used as a loading control.


A
EMCV(IRES)
nsPs
P1 P2 P3 P4
An
EMCV(IRES)-nsP1-4
+
Cap
C Luc
An
rep C+luc(ΔnsPs)
RNA(-)
Cap
C Luc
An
B
Luc activity (RLU) x10 6
40
20
10
4
3
2
1
4 hpt
8 hpt
pT7-rep C+luc(ΔnsPs)
pTM1-nsP1-4 + pT7-rep C+luc(ΔnsPs)
rep C+luc(ΔnsPs)
EMCV(IRES)-nsP1-4 + rep C+luc(ΔnsPs)
rep -26S + rep C+luc(ΔnsPs)
rep C+luc
plasmid transfection
mRNA transfection
C
BHK
rep C+luc
rep-26S
EMCV(IRES)-nsp1-4
rep C+luc(ΔnsPs)
EMCV(IRES)-nsP1-4 + rep C+luc(ΔnsPs)
luc
Actin
C
D
% Actin synthesis
120
100
80
60
40
20
0
BHK
rep C+luc
rep-26S
EMCV(IRES)-nsp1-4
rep C+luc(ΔnsPs)
EMCV(IRES)-nsP1-4 + rep C+luc(ΔnsPs)
E
BHK
rep C+luc
rep-26S
EMCV(IRES)-nsp1-4
rep C+luc(ΔnsPs)
EMCV(IRES)-nsP1-4 + rep C+luc(ΔnsPs)
gmRNAs
sgmRNAs
F
nsp1
nsp2
C
EF2

unabated in cells co-transfected with EMCV(IRES)-nsP1-4 and rep C+luc(ΔnsPs). This result suggests that the replication of this defective SINV replicon after complementation with nsPs is not sufficient to abrogate cellular translation.

In a parallel experiment, the synthesis of viral RNA was studied in cells transfected with the different RNAs described above. To do this, transfected cells were incubated with [^{3}H]Uridine from 3 to 7 hpt in the presence of 2.5 μg ml^{-1} actinomycin D. Results showed that cells transfected with rep C+luc or rep-26S synthesized genomic or subgenomic RNAs (Fig. 2E). In contrast, no viral RNAs were detected in cells co-transfected with EMCV(IRES)-nsP1-4 and rep C+luc(ΔnsPs), suggesting that although complementation can be demonstrated through measurement of luc activity, the production of RNA is below the threshold of detection. Nevertheless, the synthesis of C protein can be observed by immunoblotting in co-transfected cells (Fig. 2F), although the level of this protein is approximately 100-fold lower relative to cells transfected with rep C+luc. In conclusion, this low RNA replication rate has no consequences for the blockade of host translation.

Release of nuclear proteins in SINV-replicating cells

Infection of mammalian cells by a variety of animal viruses induces the formation of SGs containing some nuclear proteins that are released to the cytoplasm (Valiente-Echeverria *et al*., 2012). Alphavirus infection elicits the release of T-cell intracellular antigen (TIA-1) from the nucleus to the cytoplasm, giving rise to the transient accumulation of SGs (Panas *et al*., 2012; Garcia-Moreno *et al*., 2013). Interestingly, the release of some nuclear proteins by SFV infection, such as TIA-1, has been involved in the shut-off of host translation (McInerney *et al*., 2005). In addition to TIA-1, other nuclear proteins appear in the cytoplasm after SINV infection, including hnRNP K, hnRNP A1 and HuR proteins (Burnham *et al*., 2007; Lin *et al*., 2009; Gui *et al*., 2010; Dickson *et al*., 2012). Thus, initially, we decided to examine if two nuclear proteins, TIA-1 and polypyrimidine tract-binding protein (PTB), changed their cellular distribution in SINV-infected BHK cells and in cells transfected with the SINV replicon. Polypyrimidine tract-binding protein is a ubiquitous RNA-binding protein that is involved in a number of diverse cellular functions including splicing regulation, polyadenylation and viral mRNA translation (Sawicka *et al*., 2008). This protein is released from the nucleus to the cytoplasm in picornavirus and coronavirus-infected cells (Back *et al*., 2002; Borghese and Michiels, 2011; Sola *et al*., 2011); to our knowledge, redistribution of PTB has not been examined in alphavirus-infected cells. To this end, BHK cells were infected with SINV or transfected with replicon rep C+luc and at 5 or 7 hpt, respectively, the subcellular distribution of TIA-1 and PTB was analysed by confocal microscopy (Fig. 3). Clearly, these proteins have a preferential nuclear localization in uninfected BHK cells, whereas they were released from nucleus to cytoplasm both in infected and in transfected cells. Densitometric analysis of the TIA-1 signal indicated that in uninfected cells, approximately 55–60% of the total amount of TIA-1was present in the nucleus. However, both in infected cells and in cells transfected with rep C+luc TIA-1 accumulated over 82–90% in the cytoplasm (Fig. 3A). Similar results were obtained whit PTB that exhibited a nuclear level of 75–80% in mock-infected BHK cells, whereas this amount was reduced to 11–15% in infected cells and 11–18% in rep C+luc-transfected cells (Fig. 3B). Therefore, there is a correlation between SINV replication and the release of proteins from nucleus to cytoplasm. Next, we wished to examine the release of nuclear proteins in cells co-transfected with rep C+luc (ΔnsPs) and EMCV(IRES)-nsP1-4 compared with cells transfected with rep C+luc (Figs 4A and B). As shown above, although inefficiently, viral replication can be induced by trans-complementation. The presence of luc tested with specific antibodies indicates SINV RNA replication, transcription of the sgmRNA C+luc and subsequent translation to produce luc in transfected cells. In cells transfected with rep C+luc, a clear re-localization of TIA-1 to the cytoplasm was observed in cells synthesizing luc whereas it largely remained nuclear in cells co-transfected with rep C+luc (ΔnsPs) and EMCV(IRES)-nsP1-4, even in cells with conspicuous luc production (Fig. 4A). Thus, in rep C+luc-transfected cells, the nuclear signal for TIA-1 related to mock-transfected cells drops by 74–80% and in cells co-transfected with rep C+luc (ΔnsPs) and EMCV(IRES)-nsP1-4 diminishes only by 2–6%. Similar results were obtained with PTB (Fig. 4B). The immunofluorescence signal associated to PTB into the nucleus of cells transfected with rep C+luc drops by 70–75% as compared to mock-transfected cells, but in cells co-transfected with rep C+luc (ΔnsPs) plus EMCV(IRES)-nsP1-4 decreases only by 1–4%. These observations are consistent with the idea that a correlation exists between the level of viral RNA replication, the redistribution of nucleocytoplasmic shuttling proteins as TIA-1 or PTB to the cytoplasm and the inhibition of cellular protein synthesis.

To analyse the contribution of nsPs to the potential release of PTB to the cytoplasm, we also tested BHK-T7 cells transfected with plasmids pTM1-nsP1-4 or pTM1-nsP2. The pTM1-BHK-T7 system allows the expression of higher levels of nsPs, compared with transfection of EMCV(IRES)-nsP1-4 mRNA. Thus, the location of PTB

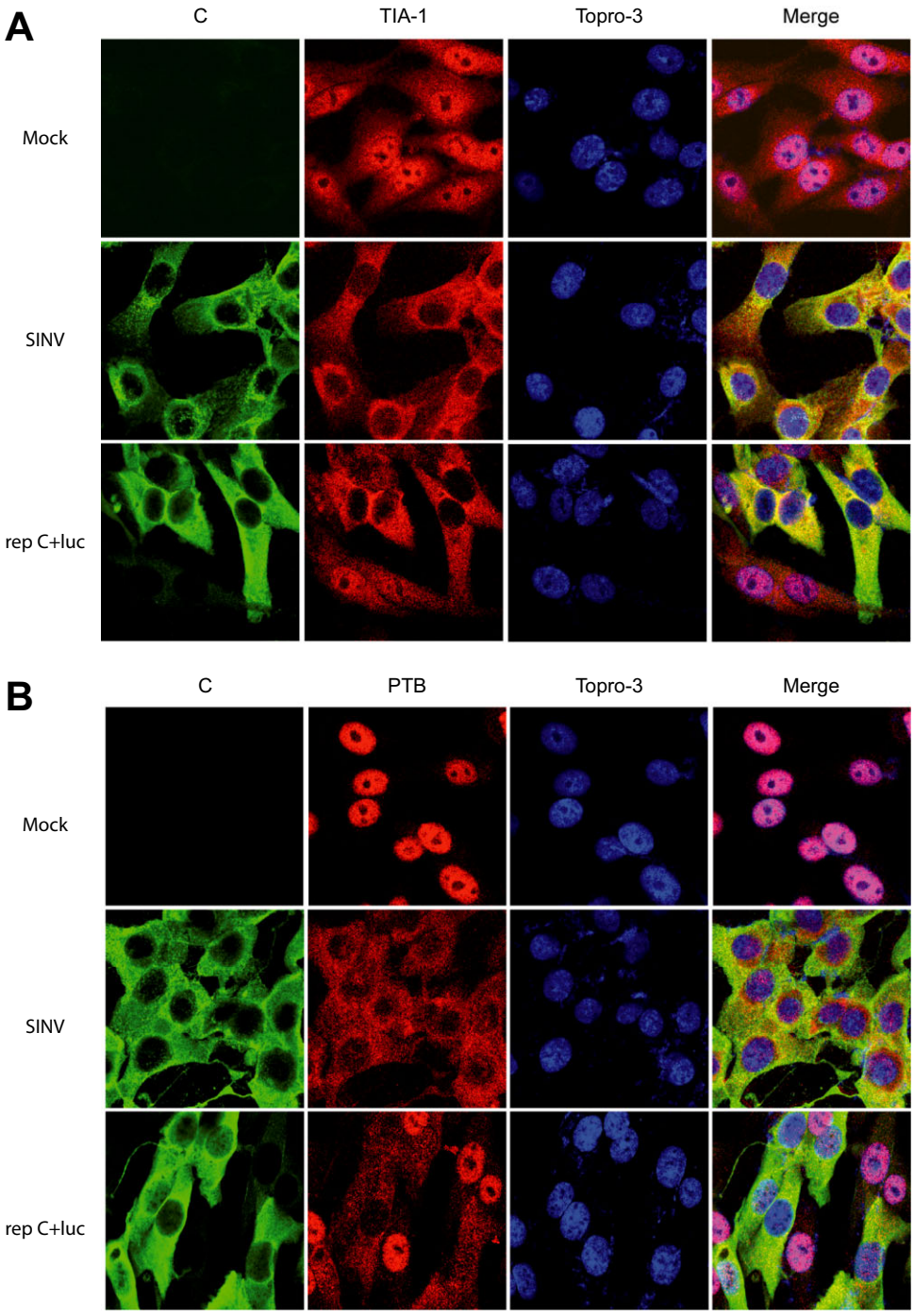


Fig. 3. Subcellular localization of TIA-1 and PTB in SINV-replicating cells. BHK cells seeded on coverslips in 24-well plates were mock-infected, infected (10 pfu cell^{-1}) or transfected with rep C+luc (1 μg) and at 5 hpi or 7 hpt, respectively, cells were processed for immunofluorescence.
A. SINV-replicating cells were detected with rabbit anti-C antibodies (green) and TIA-1 with goat anti-TIA-1 (red).
B. Alternatively, PTB was detected with mouse anti-PTB antibodies (red). Topro-3 was used to stain the nuclei (blue).

at 18 hpt was assessed in BHK-T7 cells transfected with pTM1-nsP1-4 or pTM1-nsP2, using anti-nsP1 or anti-nsP2 antibodies to detect the corresponding proteins in transfected cells. Notably, PTB exhibited a normal nuclear location in cells expressing nsP2 or nsP1-4 (Supporting Information Fig. S2), indicating that expression of SINV nsPs does not affect the redistribution of PTB between nucleus and cytoplasm. These results are

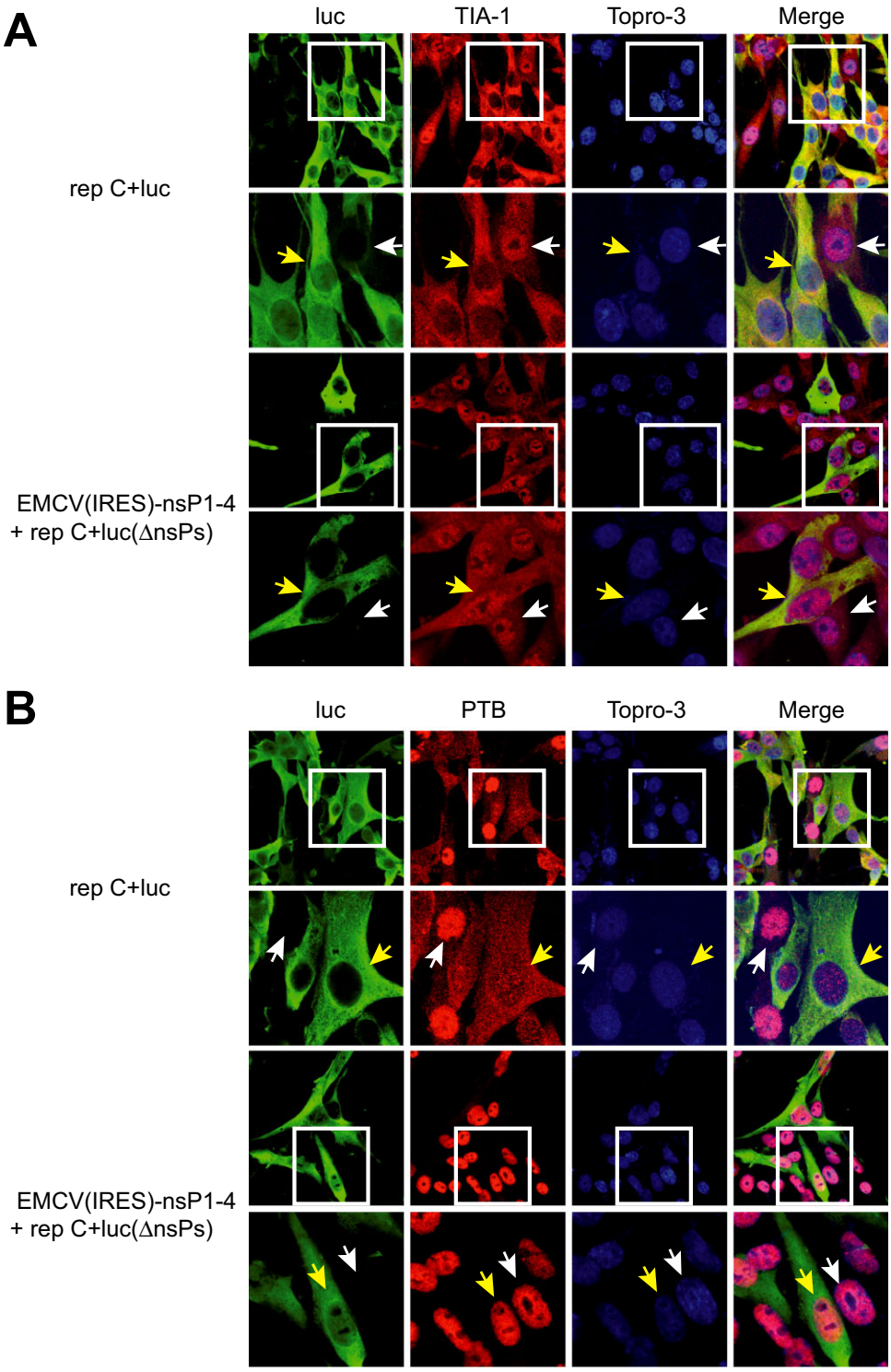


Fig. 4. Release of TIA-1 and PTB in SINV-replicating cells when the replication occurs in 'cis' or by trans-complementation.
A. BHK cells seeded on coverslips in 24-well plates were transfected with rep C+luc (1 μg) or co-transfected with rep C+luc(ΔnsPs) (1 μg) and EMCV(IRES)-nsP1-4 (1 μg); at 7 hpt, cells were fixed and processed for immunofluorescence. Replicating cells were detected with rabbit anti-luc antibodies (green) and goat anti-TIA-1 detected the subcellular location of this protein (red). Yellow and white arrows indicate the nuclei of transfected and untransfected cells respectively.
B. BHK cells were processed as in A and a mouse monoclonal anti-PTB antibody was used to detect PTB (red). Topro-3 was used to stain the nuclei (blue). Yellow and white arrows indicate the nuclei of transfected and untransfected cells respectively.

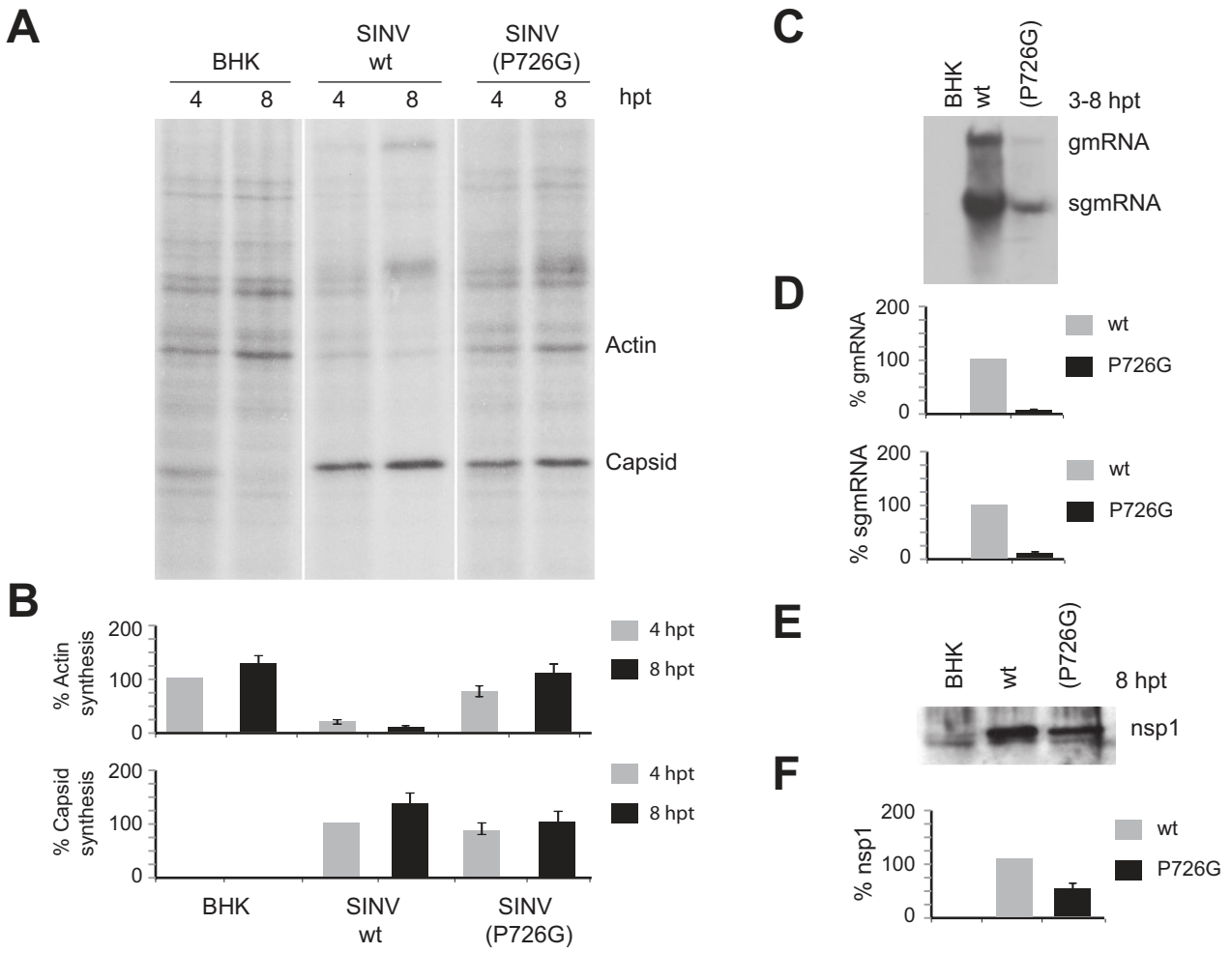


Fig. 5. Protein synthesis in BHK cells transfected with the SINV variant P726G.
A. BHK cells seeded on 24-well plates were transfected with *in vitro* synthesized mRNA (5 μg per well) from pT7SVwt or pT7SV nsP2(P726G). At 4 and 8 hpt, protein synthesis was analysed by incorporation of [^{35}S]Met/Cys for 30 min followed by SDS-PAGE, fluorography and autoradiography.
B. The percentages of actin synthesized related to mock-transfected cells at 4 hpt were estimated from values obtained by densitometric analysis (upper panel). The percentage values of C synthesis related to SINV wt at 4 hpt are also shown (lower panel). The values displayed are the mean ± SD of three independent experiments.
C. BHK cells were transfected as in A and treated with [^{3}H]Uridine in the presence of actinomycin D from 3 to 8 hpt. Then, total RNA was extracted and analysed by electrophoresis in denaturing gels followed by fluorography and autoradiography.
D. The percentage values of gmRNA and sgmRNA production from mutant P726G related to SINV wt are displayed as means ± SD of three representative experiments.
E. BHK cells were transfected as in A, and at 8 hpt, cells were harvested and analysed by immunoblotting with anti-nsP1 antibodies.
F. The ratio of nsP1 accumulation from the variant P726G versus SINV wt is indicated as the mean ± SD of three different experiments.

consistent with the findings reported in Fig. 1, demonstrating that these proteins do not block cellular protein synthesis at 18 hpt.

Protein synthesis and release of nuclear proteins in cells transfected with the defective variant SINV nsP2 (P726G)

As the SINV variant with a point mutation in nsP2 (P726G) is defective in the shut-off of host protein synthesis (Frolov *et al.*, 1999; Sanz *et al.*, 2007), we used this to test the potential parallelism between the ablation of host translation and the release of nuclear proteins. Initially, BHK cells were transfected with *in vitro* synthesized gmRNAs SINV wild type (wt) or SINV nsP2 (P726G), and protein synthesis was analysed by radioactive labelling. Whereas transfection of cells with SINV wt gmRNA profoundly arrested cellular protein synthesis at 8 hpt, transfection of the variant P726G resulted in a partial inhibition of protein synthesis (Fig. 5A), estimated at 33% by densitometric analysis (Fig. 5B). Thus, this SINV variant was not entirely defective in its ability to interfere with cellular protein

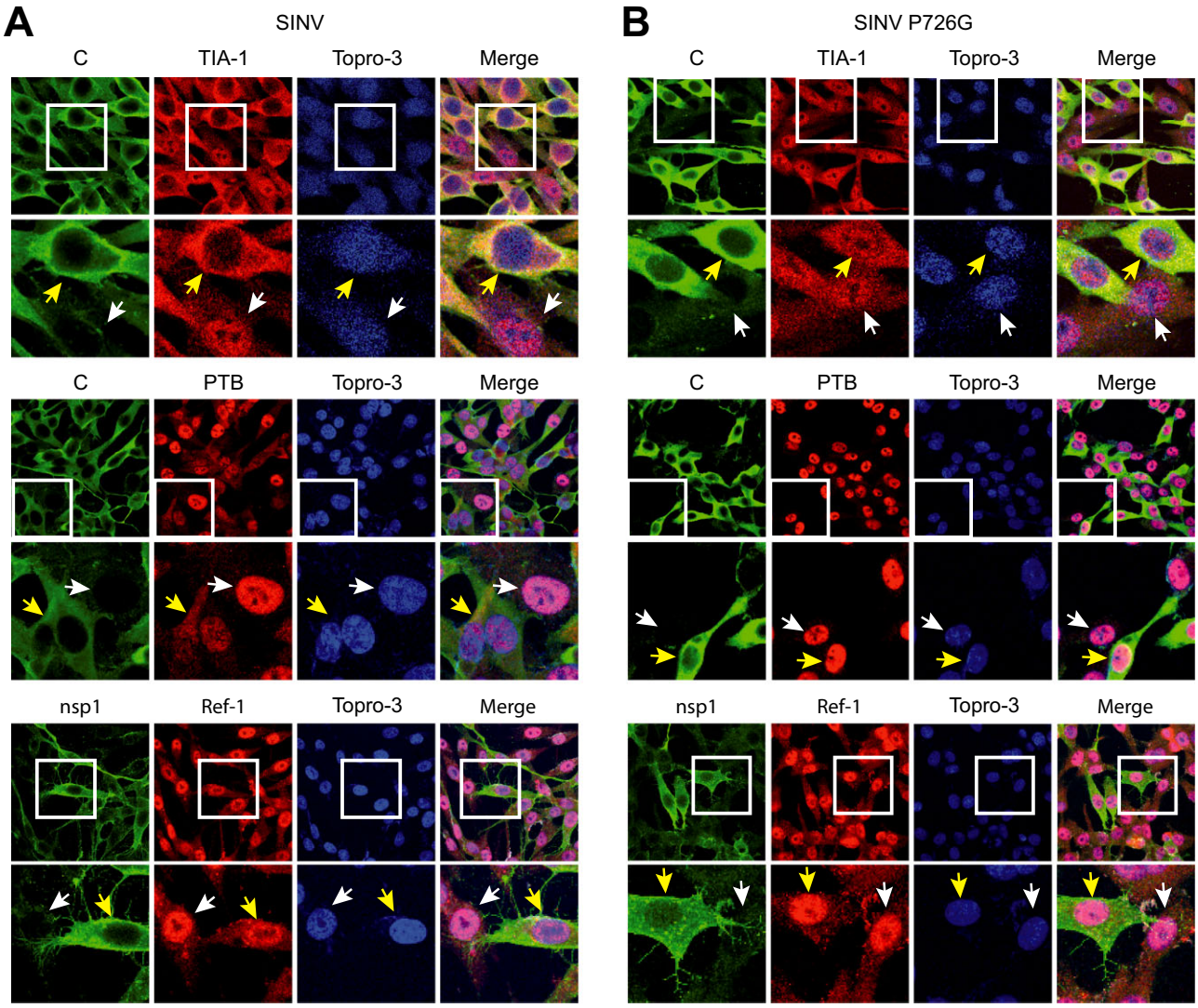


Fig. 6. Release of nuclear proteins in cells transfected with the SINV variant P726G. BHK cells were seeded on coverslips in 24-well plates and transfected with *in vitro* synthesized mRNA (1 μg per well) from pT7SVwt (A) or pT7SV(P726G) (B). At 7 hpt, cells were fixed and processed for immunofluorescence. Rabbit anti-C antibodies were used to detect SINV-replicating cells (green) and goat anti-TIA-1 or mouse monoclonal anti-PTB antibodies to detect the subcellular location of these proteins (red). Alternatively, rat anti-nsp1 antibodies were used to detect SINV-replicating cells (green) and rabbit anti-Ref-1 antibodies to detect the subcellular location of Ref-1 (red). Topro-3 was used to stain nuclei (blue). Yellow and white arrows indicate the nuclei of transfected and untransfected cells respectively.

synthesis. Next, the synthesis of viral RNAs was assayed by [^{3}H]Uridine incorporation in the presence of 2.5 μg ml^{-1} actinomycin D from 3 to 8 hpt. Compared with SINV wt, there was a significant reduction in the synthesis of viral RNAs, such that the amount of gmRNA SINV (P726G) was 4% of control, while the amount of sgmRNA was approximately 10% compared with control (Fig. 5C and D). Curiously, production of nsP1 measured by immunoblot was 47% compared with control cells transfected with SINV wt gmRNA (Fig. 5E and F), whereas the synthesis of C at 4 and 8 hpt was 83% and 75% of control respectively (Fig. 5A and B). Therefore, it appears that there is no correlation between the synthesis of viral mRNAs and the quantity of viral proteins synthesized. In conclusion, the major defect exhibited by this SINV variant is in the synthesis of viral RNAs, whereas viral proteins are still synthesized, although to a lower extent. Our next aim was to analyse the release of nuclear proteins to the cytoplasm in cells transfected with gmRNA from SINV wt or SINV nsP2 (P726G) (Fig. 6A and B). Sindbis virus-replicating cells were detected by immunofluorescence of proteins C and nsP1. The redistribution of TIA-1, PTB and additionally redox factor-1 (Ref-1) was examined. Of note, SINV wt induced the release of both TIA-1 and PTB to the cytoplasm at 7 hpt to a similar extent as with the replicon rep C+luc described above, although

with more variability between individual cells. This may be due to different viral replication levels in the different cells as a consequence of secondary infections propitiated by viruses produced in the transfected cells (Fig. 6A). In contrast, Ref-1 preserved its nuclear localization by about 90–96% in cells that produced nsP1 as related to uninfected cells, indicating that clearly not all nuclear proteins are redistributed to the cytoplasm in SINV-replicating cells. Interestingly, the SINV nsP2 variant (P726G) had little effect on the redistribution of nuclear proteins TIA-1, PTB and Ref-1 (Fig. 6B). Thus, the signal of TIA-1 at the nucleus of these cells reaches 88–93% of that of uninfected cells and similar values were obtained for PTB (86–93%) and Ref-1 (91–96%). Transfected cells were also evaluated at 24 hpt (Supporting Information Fig. S3). At this time, cells transfected with gmRNA from SINV wt showed clear signs of an infection-associated cytopathic response. This was not apparent in SINV nsP2 (P726G) transfected cells, which did not show changes in the pattern of distribution of TIA-1 or PTB. Therefore, in SINV nsP2 (P726G)-replicating BHK cells, a correlation exists between the inhibition of viral RNA replication and the release of nuclear proteins, in addition to the failure to completely block cellular mRNA translation.

Action of inhibitors of RNA synthesis

As the above observations suggested a correlation between the shut-off of host protein translation, the level of viral RNA replication and the release of some nuclear proteins to the cytoplasm, we next examined the effect of inhibiting viral RNA replication. To this end, two nucleoside analogues, 6-aza-uridine and ribavirin, which interfere with the metabolism of nucleosides and nucleotides and have detrimental effects on both cellular and viral RNA synthesis, were employed to inhibit viral replication (Carrasco and Vazquez, 1984; Vignuzzi *et al*., 2005). BHK cells were infected with SINV for 1 h to allow virus entry, and then the medium was exchanged with addition of inhibitors. This protocol avoids the potential inhibitory effects of nucleoside analogues on virus attachment and entry. Protein synthesis was estimated at 5 hpi. As observed in Fig. 7A, increasing concentrations of 6-aza-uridine and ribavirin led to a partial decline of protein synthesis in uninfected BHK cells. Densitometric measurements indicated that cellular actin represented 60% of control at 0.5 mM 6-aza-uridine and 74% at 0.2 mM ribavirin (Fig. 7B). Under these conditions, SINV infection does not intensify the inhibition of actin synthesis. In SINV-infected BHK cells, actin synthesis was blocked by 97% in the absence of inhibitors. Conversely, the decrease of actin synthesis was 8% in SINV-infected cells, relative to uninfected BHK cells, in the presence of 0.5 mM 6-aza-uridine, whereas the blockade of actin synthesis was 18% in SINV-infected cells incubated with 0.2 mM ribavirin (Fig. 7A and B). As a control, we also tested the inhibitory effect of actinomycin D that potently blocks cellular RNA synthesis but has almost no effect on the synthesis of SINV RNAs. As expected, actinomycin D treatment from 0 to 5 hpi had little effect on protein synthesis both in infected and uninfected cells (Fig. 7A).

To measure the effects of the inhibitors on cellular and viral RNA synthesis, [^{3}H]Uridine incorporation was performed from 3 to 5 hpi, and RNA was extracted and separated in agarose gels. Both nucleoside analogues potently inhibited cellular and viral RNA synthesis (Fig. 7C); transcription of sgmRNA was inhibited by approximately 90% with 0.5 mM 6-aza-uridine and 98% with 0.2 mM ribavirin, although these data are approximations because cellular mRNA (as 28S rRNA), overlap with the viral mRNAs in gels. Predictably, actinomycin D inhibited cellular RNA synthesis only (Fig. 7C). Additionally, the production of nsPs and sPs were measured in treated and untreated infected cells by immunoblotting. As noted previously, and also in several reports, there was no correlation between the level of mRNA and the production of viral proteins (Fig. 7C and D). Accordingly, the production of nsP2 in ribavirin-treated cells decreased proportionately less than the production of gmRNA. Similarly, in ribavirin-treated cells, synthesis of C and E1 glycoproteins decreased less than synthesis of sgmRNA (Fig. 7C and D). Comparable results were obtained in 6-aza-uridine-treated cells.

Finally, the release of TIA-1 and PTB was analysed in SINV-infected BHK cells treated with nucleoside analogues. Whereas treatment of control-uninfected BHK cells with these compounds had no effect in the redistribution of PTB or TIA-1, both inhibitors blocked the release of PTB to the cytoplasm in SINV-infected cells (Fig. 8). The nuclear signal of PTB in infected cells treated with 6-aza-uridine varies between 63% and 72% of total signal, while it ranged between 11% and 16% in infected untreated cells. Similarly, ribavirin treatment led to PTB retention in the nucleus (65–72% of total signal). Moreover, treatment with nucleoside analogues also reduced the accumulation of TIA-1 in the cytoplasm of infected cells (Supporting Information Fig. S4). Thus, in agreement with previous results, these findings indicate that the shut-off of host translation after SINV infection is restricted when viral RNA synthesis is blocked and nuclear proteins are not released to the cytoplasm.

Inhibition of cellular protein synthesis in PKR$^{-/-}$ MEFs

An important contributing factor to the arrest of host mRNA translation is the activation of PKR after SINV infection of mammalian cells (Ventoso *et al*., 2006; Garcia-Moreno *et al*., 2013). This activation leads to the phosphorylation of eIF2α, blocking cellular but not viral

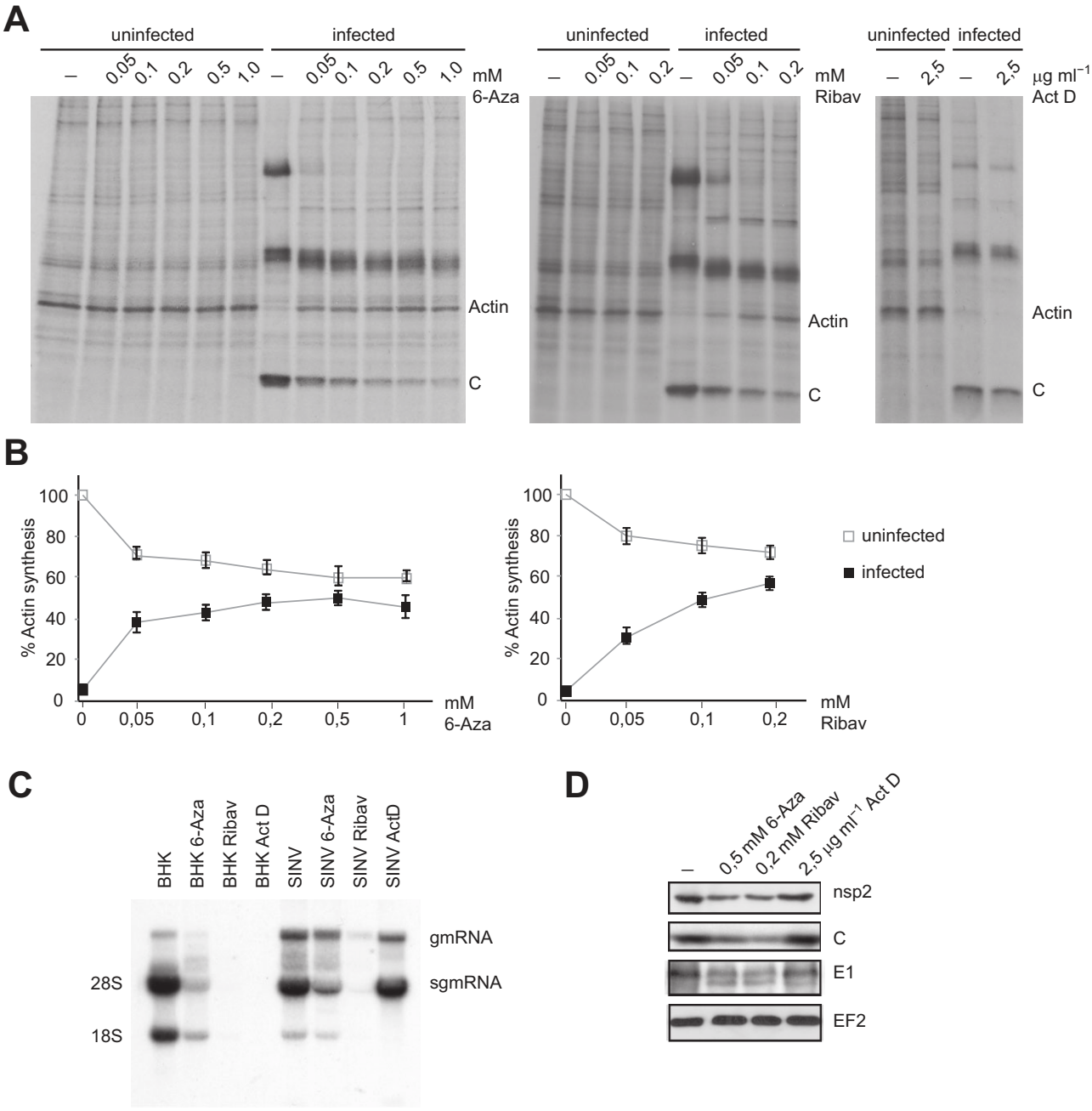


Fig. 7. Protein and RNA synthesis in SINV-infected BHK cells treated with 6-aza-uridine, ribavirin or actinomycin D.
A. BHK cells were infected (10 pfu cell^{-1}) for 1 h, followed by replacement of the infective medium with fresh medium supplemented with different concentrations of 6-aza-uridine and ribavirin or 2.5 µg ml^{-1} actinomycin D. At 5 hpi, protein synthesis was analysed by incorporation of [^{35}S]Met/Cys followed by SDS-PAGE, fluorography and autoradiography.
B. Graphs represent the percentages of actin synthesis in comparison with uninfected and untreated cells, and are displayed as means ± SD of three independent experiments.
C. Mock and SINV-infected BHK cells were treated with 0.5 mM 6-aza-uridine, 0.2 mM ribavirin, 2.5 µg ml^{-1} actinomycin D or maintained untreated; at 3hpi, [^{3}H]Uridine was added and at 5.5 hpi total RNA was extracted and analysed by electrophoresis in denaturing gels followed by fluorography and autoradiography.
D. BHK cells were infected and left untreated or treated with the indicated concentrations of the different compounds once the infective medium was removed. At 5 hpi, cells were harvested and analysed by immunoblot with anti-nsP2, anti-C or anti-E1 antibodies. An anti-EF2 antibody was used as a loading control.

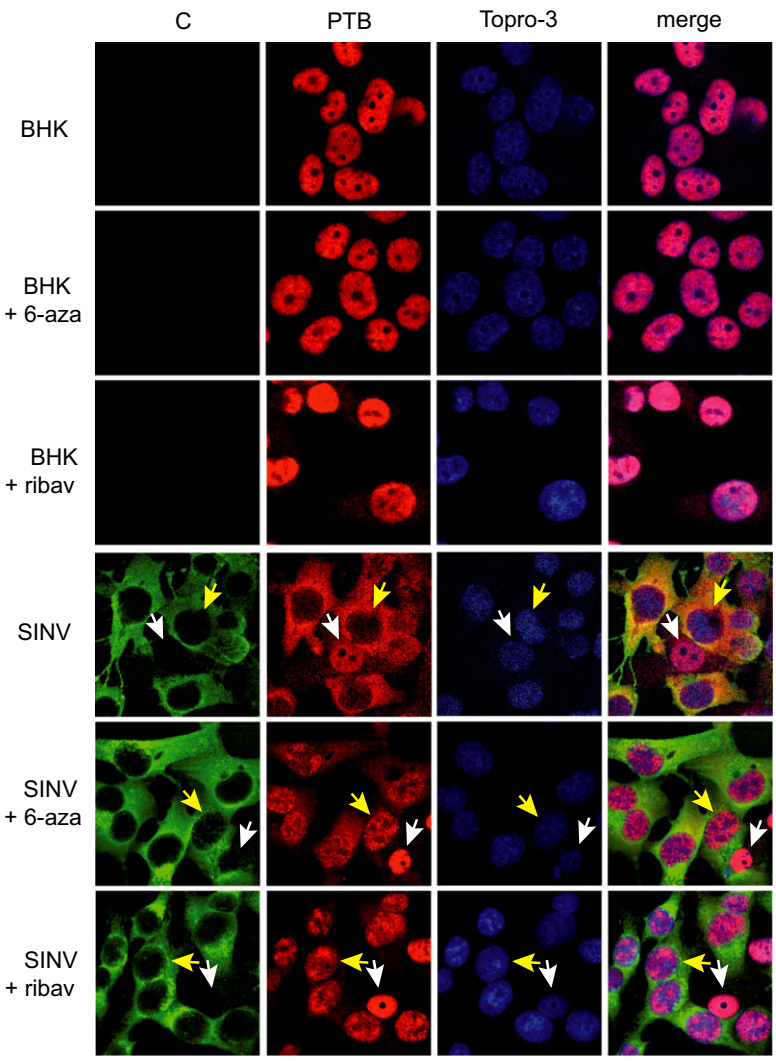


Fig. 8. Release of PTB in SINV-infected BHK cells treated with 6-aza-uridine or ribavirin. Mock and SINV-infected BHK cells (1 pfu cell^{-1}) were treated with 0.5 mM 6-aza-uridine, 0.2 mM ribavirin or left untreated; at 5 hpi, cells were fixed and processed for immunofluorescence. Rabbit anti-C antibodies were used to detect SINV-infected cells (green) and mouse monoclonal anti-PTB antibodies to detect the subcellular location of this protein (red). Topro-3 was used to stain the nuclei (blue). Yellow and white arrows indicate the nuclei of infected and uninfected cells respectively.

mRNA translation. Thus, it was important to analyse whether the nucleoside analogues prevented eIF2α phosphorylation, and whether this was the principal reason for the lack of shut-off of host translation observed in BHK cells. To this end, phosphorylation of eIF2α and the inhibition of cellular protein synthesis were examined in BHK cells treated with 0.5 mM 6-aza-uridine or 0.2 mM ribavirin (Fig. 9A and B). As anticipated, SINV infection of BHK cells induced eIF2α phosphorylation (Fig. 9A, lower panel). Interestingly, ribavirin largely prevented eIF2α phosphorylation, whereas this prevention was only partial in 6-aza-uridine-treated cells (Fig. 9A, lower panel). We reasoned that the prevention of the shut-off of host translation by the nucleoside analogues in BHK cells may be due to their interference with eIF2α phosphorylation. To test this, we used PKR$^{-/-}$ MEFs that do not undergo eIF2 inactivation following SINV infection (Ventoso *et al.*, 2006). Indeed, no phosphorylation of the eIF2 α subunit was evident in SINV-infected PKR$^{-/-}$ MEFs (Fig. 9C, lower panel). Similar to previous results, inhibition of protein synthesis by 0.5 mM 6-aza-uridine or 0.2 mM ribavirin was 33% and 46%, respectively, in uninfected cells (Fig. 9C and D). Notably, treatment with the nucleoside analogues prevented the inhibition of cellular protein synthesis by SINV infection even when no phosphorylation of eIF2α took place; in the presence of 0.5 mM 6-aza-uridine, SINV infection inhibited actin synthesis by 3%, related to 6-aza-uridine-treated uninfected cells, whereas this inhibition was 10% with ribavirin. In contrast, the decrease in actin synthesis was 95% in untreated PKR$^{-/-}$ MEFs after SINV infection related to untreated and uninfected cells (Fig. 9C and D).

Next, we assessed the location of PTB in PKR$^{-/-}$ MEFs infected with SINV. Consistent with the results obtained in BHK cells, a potent release of PTB occurred in PKR$^{-/-}$ MEFs infected with SINV containing less than 15% of PTB

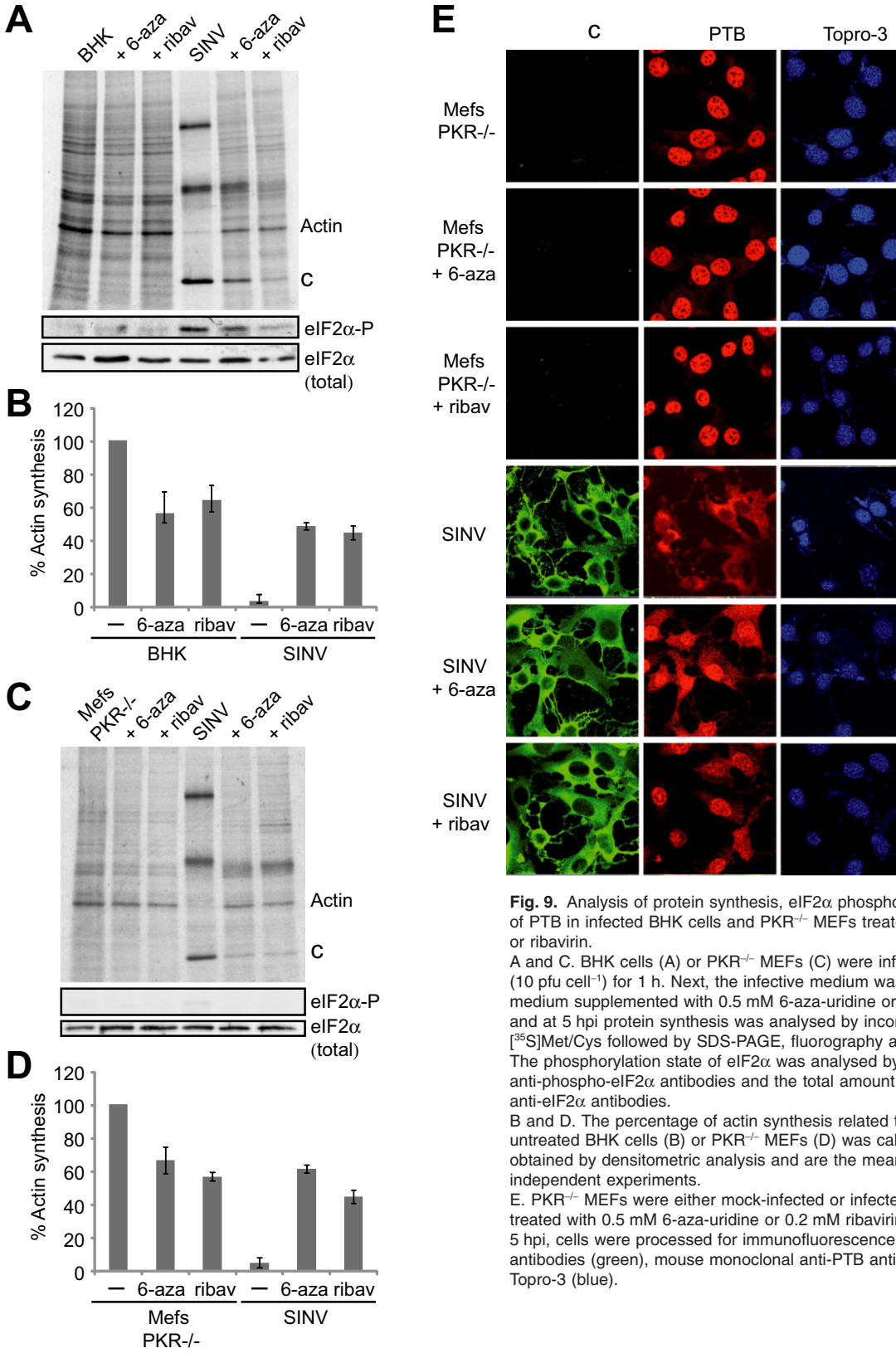


Fig. 9. Analysis of protein synthesis, eIF2α phosphorylation and release of PTB in infected BHK cells and PKR$^{-/-}$ MEFs treated with 6-aza-uridine or ribavirin.

A and C. BHK cells (A) or PKR$^{-/-}$ MEFs (C) were infected with SINV (10 pfu cell^{-1}) for 1 h. Next, the infective medium was replaced with fresh medium supplemented with 0.5 mM 6-aza-uridine or 0.2 mM ribavirin, and at 5 hpi protein synthesis was analysed by incorporation of [^{35}S]Met/Cys followed by SDS-PAGE, fluorography and autoradiography. The phosphorylation state of eIF2α was analysed by immunoblot with anti-phospho-eIF2α antibodies and the total amount of eIF2α with anti-eIF2α antibodies.

B and D. The percentage of actin synthesis related to uninfected and untreated BHK cells (B) or PKR$^{-/-}$ MEFs (D) was calculated from values obtained by densitometric analysis and are the mean ± SD of three independent experiments.

E. PKR$^{-/-}$ MEFs were either mock-infected or infected with SINV and treated with 0.5 mM 6-aza-uridine or 0.2 mM ribavirin, or left untreated. At 5 hpi, cells were processed for immunofluorescence with rabbit anti-C antibodies (green), mouse monoclonal anti-PTB antibodies (red) and Topro-3 (blue).

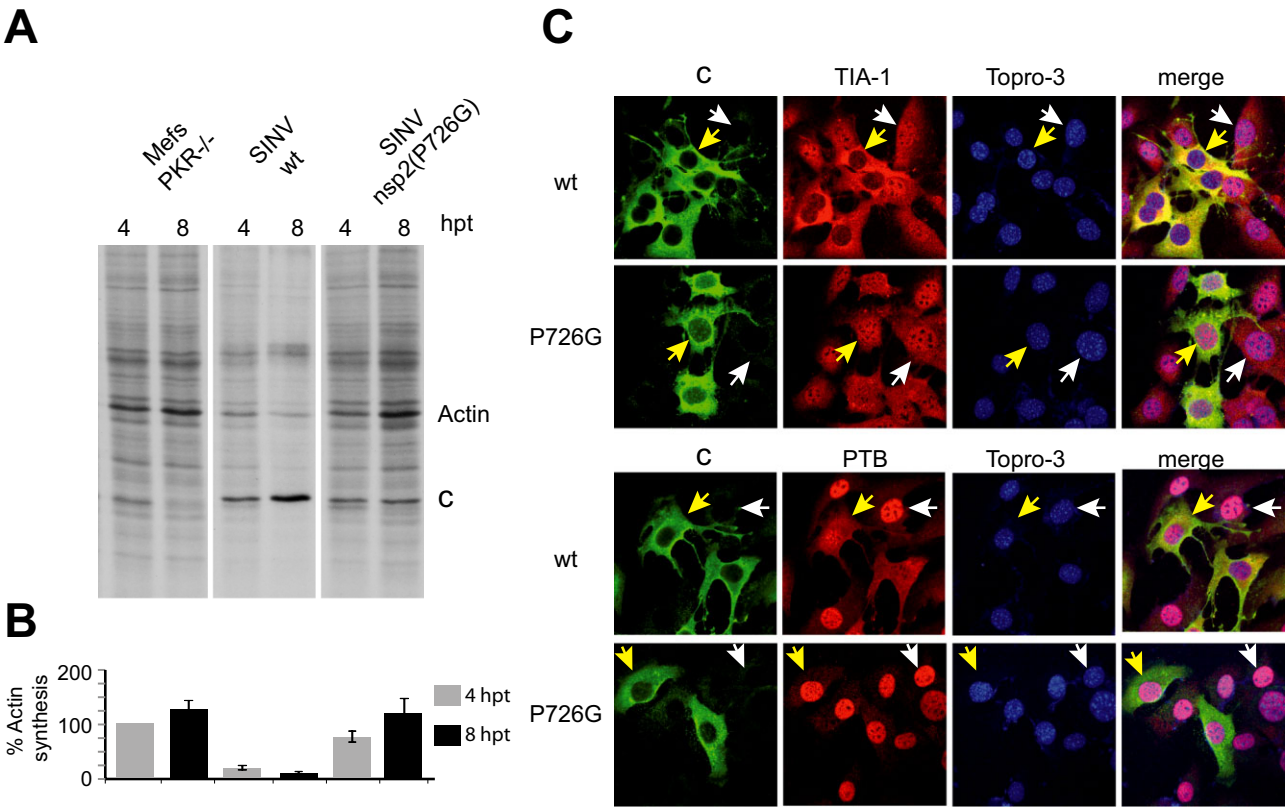


Fig. 10. Protein synthesis and release of TIA-1 and PTB in PKR$^{-/-}$ MEFs transfected with SINV wt or SINV nsP2 (P726G) gmRNAs.
A. PKR$^{-/-}$ MEFs were transfected with *in vitro* generated gmRNAs of SINV wt or the variant SINV nsP2 (P726G). At 4 or 8 hpt, protein synthesis was analysed by incorporation of [^{35}S]Met/Cys followed by SDS-PAGE, fluorography and autoradiography.
B. The percentages of actin synthesized in cells transfected with SINV wt or the variant P726G related to mock-transfected cells at 4 hpt are displayed as means ± SD of three representative experiments.
C. PKR$^{-/-}$ MEFs were transfected as in A, and processed for immunofluorescence at 8 hpt with rabbit anti-C antibodies (green), goat anti-TIA-1 or mouse monoclonal anti-PTB antibodies (red) and Topro-3 (blue). Yellow and white arrows indicate the nuclei of transfected and untransfected cells respectively.

in the nucleus of infected cells. However, in infected cells treated with nucleoside analogues around 68–72% of PTB remained into the nucleus (Fig. 9E). Thus, the inability of SINV to induce shut-off in the presence of 6-azauridine or ribavirin is not related to the prevention of eIF2α phosphorylation.

Finally, we tested shut-off of host translation in PKR$^{-/-}$ MEFs infected with the SINV nsP2 variant (P726G). Cells were transfected with *in vitro* synthesized gmRNAs from SINV wt or SINV nsP2 (P726G) and protein synthesis was estimated at 4 and 8 hpt. Compared with SINV wt, virtually no inhibition of cellular translation was observed in PKR$^{-/-}$ MEFs after infection with the SINV variant (Fig. 10A and B), suggesting that the partial inhibition observed in BHK cells (Fig. 5A) may be due to the presence of PKR in these cells. Further, in agreement with the results obtained in BHK cells, TIA-1 and PTB accumulated in the cytoplasm of SINV wt gmRNA-transfected cells, and their nuclear location was unchanged after transfection of SINV nsP2 (P726G) (Fig. 10C). Taken together, these results support an association between the shut-off of host translation and the exit of nuclear proteins to the cytoplasm. Additionally, the prevention of SINV-induced cellular protein synthesis blockade by nucleoside analogues or the SINV variant nsP2 (P726G), is more clearly observed in PKR-defective cells.

Discussion

Infection of mammalian cells with a number of animal viruses leads to a profound inhibition of cellular macromolecular synthesis, blocking innate antiviral responses. Alphavirus replicons encoding solely nsPs interfere with cellular translation to a degree similar to that observed with non-defective viruses (Frolov and Schlesinger, 1994; Sanz *et al.*, 2007). Several observations point to nsP2 as the mediator of these inhibitory effects (Frolov *et al.*, 1999; Gorchakov *et al.*, 2004; 2005; Garmashova *et al.*,

2006). However, a mutant in the 5′UTR sequence of sgmRNA of SINV that expresses more nsPs than wt virus is defective in the shut-off of host mRNA translation (Patel *et al*., 2013). We have analysed the inhibition of cellular mRNA translation mediated by individual nsPs and nsP1-4. We found that individual expression of nsP1, nsP2 and nsP3 or nsP1-4 has little effect on cellular protein synthesis. In the case of nsP1-4, not only mature nsPs are synthesized, but also their precursors, which is more akin to the situation observed in SINV-infected cells. As a control, we tested the expression of PV $2A^{pro}$ that induces a profound arrest in cellular mRNA translation upon cleavage of eIF4G (Castello *et al*., 2011), in clear contrast to SINV nsPs. Additionally, PV $2A^{pro}$ substantially modifies the shuttling of proteins between nucleus and cytoplasm, after cleavage of several nuPs (Park *et al*., 2008; 2010; Castello *et al*., 2009; Alvarez *et al*., 2011; 2013). In contrast, SINV nsPs did not elicit the release of nuclear proteins TIA-1 and PTB.

Our results are consistent with the idea that inhibition of host translation in alphavirus-infected mammalian cells requires a high level of viral RNA replication. Indeed, in cells co-transfected with rep C+luc(ΔnsPs) and EMCV(IRES)-nsP1-4 mRNA, there is low-level RNA replication, no release of nuclear proteins and the synthesis of cellular proteins is not blocked. A similar situation arises for the variant SINV nsP2 (P726G), which has little effect on cellular mRNA translation, particularly in $PKR^{-/-}$ MEFs, concomitant with a low level of viral RNA replication. Analogous to SINV nsP2 (P726G), two variants of SFV with point mutations in nsP2 that severely reduce the levels of viral RNA accumulation, were also unable to induce shut-off (Tamm *et al*., 2008). Notably, this inhibition of host protein synthesis is prevented with nucleoside analogues that diminish viral RNA synthesis. These nucleoside analogues inhibit cellular transcription to a similar extent as actinomycin D, but additionally block viral transcription. Actinomycin D treatment does not impede the process of cellular shut-off in SINV-infected cells, whereas 6-aza-uridine or ribavirin prevents this. Further support for this notion stems from the observation that SINV infection of mosquito cells, which results in low levels of viral RNA replication, does not abolish protein synthesis (Kim *et al*., 2004). In conclusion, for the shut-off of host mRNA translation to take place, synthesis of SINV nsPs is not sufficient and viral RNA replication is necessary. This replication should have to take place at normal levels, because no inhibition of host translation occurs when viral RNA replication is compromised. Notably, shut-off of cellular protein synthesis coincides with release of nuclear proteins to the cytoplasm and efficient viral RNA replication. This viral replication might trigger a cellular response that obstructs the shuttling of proteins between nucleus and cytoplasm. Therefore, it could be possible that one or more nuclear proteins are inhibitory for cellular translation when they are present in the cytoplasm. Of note, a number of nuclear proteins are released to the cytoplasm in various animal viruses studied (Li and Nagy, 2011). In the case of SINV, hnRNP K protein can be found enriched in membranous fractions containing SINV replication complexes, interacting with sgmRNA (Burnham *et al*., 2007). Also, hnRNP A1 can bind to the 5′-UTR of SINV genomic RNA and facilitates translation (Lin *et al*., 2009; Gui *et al*., 2010). HuR protein also relocalizes to the cytoplasm after alphavirus infection (Dickson *et al*., 2012) and interacts with a U-rich element in the 3′-UTR of SINV transcripts, mediating viral RNA stabilization (Garneau *et al*., 2008; Sokoloski *et al*., 2010). Recently, it was proposed that the 3′-UTR of SINV serves as a sponge for HuR contributing to disrupt cellular posttranscriptional control (Barnhart *et al*., 2013). In addition, PTB interacts with coronavirus RNAs and negatively affects the production of viral RNA (Sola *et al*., 2011). Also, several hnRNPs and other nuclear proteins have been located in the antiviral granules formed as an innate host response to combat poxvirus infection (Rozelle *et al*., 2014). When MEFs defective in TIA-1 are infected with SFV, the shut-off of host translation was significantly delayed as compared with control MEFs (McInerney *et al*., 2005). Undoubtedly, there are a large number of shuttling proteins that could be involved in the inhibition of cellular translation that remain to be studied.

The replication of viral genomes in the cytoplasm of infected cells is associated with profound rearrangements of cellular membranes. There are several possible explanations that might account for the connection between inhibition of host translation, viral RNA replication and the redistribution of nuclear proteins to the cytoplasm. One such possibility is that the appearance of large quantities of viral sequences in the cytoplasm induces a redistribution of nuclear proteins (Barnhart *et al*., 2013). Another possibility is that viral RNA replication itself induces this release, as this phenomenon is coupled to the rearrangement of intracellular membranes (Kujala *et al*., 2001; Miller and Krijnse-Locker, 2008). This rearrangement can distort the shuttling of proteins between the nucleus and the cytoplasm. In the case of alphaviruses, the replication complexes are associated with newly formed membranous structures named spherules (Kujala *et al*., 2001; Spuul *et al*., 2011). These spherules are connected to the cytoplasm by a narrow neck, through which the synthesized viral RNAs can be delivered. Expression of SFV nsPs in the absence of RNA replication does not induce formation of these structures. Indeed, the size of these spherules is dependent on the length of the replicative viral RNA (Kallio *et al*., 2013). If the shut-off of cellular protein synthesis is due to the increase of nuclear proteins in the cytoplasm, a

number of new questions arise: (i) which nuclear proteins are able to block cellular mRNA translation?; (ii) why is this inhibition selective for cellular versus viral mRNA?; (iii) what is the molecular mechanism that triggers the release of nuclear proteins?; and (iv) is the presence of a specific viral RNA sequence responsible for this inhibition? Although the inhibition of host mRNA translation can take place by redundant mechanisms, such as redistribution of nuclear proteins, modification of eIFs, ionic imbalance or mRNA competition, it is possible that one of the most important factors to explain this event is the re-localization of nuclear proteins. Future work along this line will provide insight into the molecular mechanisms employed by alphaviruses and other animal viruses to interfere with cellular functions and with innate antiviral responses.

Experimental procedures

Cell lines and viruses

Baby hamster kidney (BHK-21, and clone BSR-T7/5 designated as BHK-T7) cells (Buchholz *et al*., 1999) or MEFs derived from PKR$^{-/-}$ mice (Yang *et al*., 1995) were used to perform the experiments. Sindbis virus stock was prepared from a pT7 SVwt infective cDNA clone (where wt is wild type; Sanz and Carrasco, 2001). Viral infections were carried out in Dulbecco's modified Eagle's medium (DMEM) without serum for 60 min to permit virus attachment. This medium was then removed and infection continued in DMEM with 10% fetal calf serum.

Plasmids and recombinant DNA procedures

pT7 SVwt was used as the parental plasmid for all constructs. pT7-rep C+Luc, pT7-rep C+luc(ΔnsPs) and pT7-rep-26S (Sanz *et al*., 2007), pTM1-2A (Ventoso *et al*., 1998) and pTM1-2C (Aldabe *et al*., 1996) have been described. Plasmids pTM1-nsP1, pTM1-nsP2 and pTM1-nsP3 were constructed by inserting the corresponding polymerase chain reaction (PCR)-generated nsP sequences into the pTM1 plasmid. Thus, the nsP1 PCR product was digested with Nco I and Bam HI, the nsP2 PCR product was partially digested with Nco I and completely with Bam HI, and the nsP3 PCR product was partially digested with Nco I and completely with Xba I. Digested products were cloned into the corresponding sites of pTM1. pTM1-nsP1-4 was constructed by inserting the Eco 47III/Xho I fragment obtained from pT7-rep-26S into the corresponding sites of pTM1-nsP1.

Oligonucleotides

Oligonucleotides for PCR amplification were 5′Nco-nsp1 cgc gcgccatggagaagccagtagtaaac; 3′Bam-nsp1 gctgagggatcctatgct ccgatgtccgcctg; 5′Nco-nsp2 gcgcgcccatggcattagttgaaaccccg; 3′Bam-nsp2 ctgcagggatccttaggctccaactccatctcttg; 5′Nco-nsp3 cgcgcgccatggcgccgtcataccgcacc; 3′Xba-nsp3 ctgcagtcttagatt atcagtattcagtcctcctgc.

In vitro *transcription and transfection*

Plasmids digested with Xho I were used as templates for *in vitro* RNA transcription with T7 RNA polymerase (Promega). The transcription mixture always contained an m^{7}G(5′)ppp(5′)G cap analogue (New England Biolabs) except where translation driven by EMCV IRES was assayed. The mRNAs used for transfection were transcribed *in vitro*, treated with DNAse A and purified using an RNA cleanup kit (Qiagen). For DNA transfection, plasmids were directly employed. To prepare the transfection mixture, 2 μl of Lipofectamine 2000 reagent (Invitrogen) was added to 50 μl of Opti-MEM I Reduced Serum Medium (Opti-MEM I; Invitrogen) and then incubated for 5 min at room temperature. Simultaneously, 5 μg mRNA or 0.8 μg DNA were mixed in 50 μl of Opti-MEM I for each L-24 well and then incubated at room temperature for 5 min. Finally, these solutions were mixed and incubated for 30 min at room temperature. For transfection of BHK cells, growth medium was removed and 100 μl of Opti-MEM I plus 100 μl of the transfection mixture were added to each well. Cells were then incubated at 37°C for 2 h, followed by replacement with fresh medium.

Antibodies

The primary antibodies used in this work included: rabbit polyclonal antibodies raised against purified C protein, rat polyclonal antibodies raised against bacterially produced nsP1 and rabbit polyclonal antibodies anti-glycoprotein E1, all produced in our laboratory. Rabbit polyclonal antibodies anti-nsP2 and anti-nsP4 were a kind gift from Dr. Richard W Hardy (Indiana University, USA). A mouse anti-PTB monoclonal antibody was purchased from Invitrogen. Polyclonal antibodies anti-TIA-1 (C-20): sc-1751, rabbit polyclonal antibodies anti-luciferase, goat polyclonal antibodies anti-EF2 (p19): sc-13003 and rabbit polyclonal anti-eIF2α were purchased from Santa Cruz Biotechnology, mouse monoclonal anti-α-tubulin was purchased from Sigma-Aldrich, rabbit polyclonal antibodies raised against Ref-1/Aly were a generous gift from Dr. Elisa Izaurralde, Max Planck Institute for Developmental Biology, Tübingen, Germany, and rabbit polyclonal antibodies raised against phospho-eIF2α (serine 51) was purchased from Cell Signaling Technology.

Analysis of protein synthesis by radioactive labelling

Protein synthesis was measured by incubating cells with 0.2 ml DMEM without methionine-cystine supplemented with 2 μl EasyTagTM EXPRESS ^{35}S Protein Labeling mix, [^{35}S]Met/Cys (11 mCi ml^{-1}, 37.0 Tbq mmol^{-1}; Perkin Elmer) per well of a 24-well plate for 30 min. To assess the effect of ribavirin, 6-azauridine or actinomycin D, these compounds were added to the labelling medium at the indicated concentrations. Cells were then collected in loading buffer [62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol, 17% glycerol and 0.024% bromophenol blue] and autoradiographic analysis was performed following SDS-polyacrylamide gel electrophoresis.

Analysis of viral and cellular RNA synthesis by radioactive labelling

[^{3}H]Uridine (740 GBq mmol^{-1}, 30 μCi ml^{-1}, final concentration) was added to the culture medium and its incorporation was

employed to detect viral or cellular RNA synthesis by electrophoresis in denaturing gels, followed by fluorography and autoradiography. To this end, total RNA from approximately 2.5×10^6 cells was extracted using the RNeasy Mini Kit (Qiagen) and resuspended in 40 μl water. Samples (20 μl) were denatured by standard methods with glyoxal and dimethyl sulfoxide, and separated by electrophoresis in 0.8% agarose gels containing 10 mM phosphate buffer. Gels were treated with the autoradiography enhancer EN^{3}HANCE (PerkinElmer), dried, and exposed to X-ray film at −70°C.

Measurement of luciferase activity

Cells were lysed in a buffer containing 0.5% Triton X-100, 25 mM glycylglycine (pH 7.8) and 1 mM dithiothreitol. Luciferase activity was detected using the Luciferase assay system (Promega) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

Immunofluorescence microscopy

BHK cells seeded on coverslips were transfected with the indicated RNAs or infected with SINV. At the times indicated, cells were fixed in 4% paraformaldehyde (PFA) for 15 min, washed twice with phosphate buffered saline (PBS), and then permeabilized for 10 min with 0.2% Triton X-100 in PBS. All antibody incubations were carried out for 1 h in PBS containing 5% fetal calf serum and 0.1% Triton X-100. Coverslips were washed three times with PBS between primary and secondary antibody incubations, mounted using ProLong Gold anti-fade reagent (Invitrogen) and observed on a Radiance 2000 confocal laser scanning microscope (Bio-Rad/Zeiss). Specific antibodies conjugated to Alexa 555 or Alexa 488 were used as secondary antibodies.Topro-3 was used to stain the nuclei. ImageJ 1.47v software was used to determine the cellular distribution of TIA-1 and PTB by densitometric analysis of their immunofluorescence signals.

Acknowledgements

This study was supported by a DGICYT (Dirección General de Investigación Científica y Técnica. Ministerio de Economía y Competitividad, Spain) grant (BFU2012-31861). MGM is holder of a FPI (Formación de Personal Investigador) fellowship. The Institutional Grant awarded to the Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM) by the Fundación Ramón Areces is acknowledged.

References

Ahola, T., Laakkonen, P., Vihinen, H., and Kaariainen, L. (1997) Critical residues of Semliki Forest virus RNA capping enzyme involved in methyltransferase and guanylyltransferase-like activities. *J Virol* **71:** 392–397.

Aldabe, R., Barco, A., and Carrasco, L. (1996) Membrane permeabilization by poliovirus proteins 2B and 2BC. *J Biol Chem* **271:** 23134–23137.

Alvarez, E., Castello, A., Carrasco, L., and Izquierdo, J.M. (2011) Alternative splicing, a new target to block cellular gene expression by poliovirus 2A protease. *Biochem Biophys Res Commun* **414:** 142–147.

Alvarez, E., Castello, A., Carrasco, L., and Izquierdo, J.M. (2013) Poliovirus 2A protease triggers a selective nucleocytoplasmic redistribution of splicing factors to regulate alternative pre-mRNA splicing. *PLoS ONE* **8:** e73723.

Back, S.H., Kim, Y.K., Kim, W.J., Cho, S., Oh, H.R., Kim, J.E., and Jang, S.K. (2002) Translation of polioviral mRNA is inhibited by cleavage of polypyrimidine tract-binding proteins executed by polioviral 3C(pro). *J Virol* **76:** 2529–2542.

Barnhart, M.D., Moon, S.L., Emch, A.W., Wilusz, C.J., and Wilusz, J. (2013) Changes in cellular mRNA stability, splicing, and polyadenylation through HuR protein sequestration by a cytoplasmic RNA virus. *Cell Rep* **5:** 909–917.

Borghese, F., and Michiels, T. (2011) The leader protein of cardioviruses inhibits stress granule assembly. *J Virol* **85:** 9614–9622.

Breakwell, L., Dosenovic, P., Karlsson Hedestam, G.B., D.'Amato, M., Liljestrom, P., Fazakerley, J., and McInerney, G.M. (2007) Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. *J Virol* **81:** 8677–8684.

Buchholz, U.J., Finke, S., and Conzelmann, K.K. (1999) Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J Virol* **73:** 251–259.

Burnham, A.J., Gong, L., and Hardy, R.W. (2007) Heterogeneous nuclear ribonuclear protein K interacts with Sindbis virus nonstructural proteins and viral subgenomic mRNA. *Virology* **367:** 212–221.

Bushell, M., and Sarnow, P. (2002) Hijacking the translation apparatus by RNA viruses. *J Cell Biol* **158:** 395–399.

Carrasco, L., and Vazquez, D. (1984) Molecular bases for the action and selectivity of nucleoside antibiotics. *Med Res Rev* **4:** 471–512.

Castello, A., Izquierdo, J.M., Welnowska, E., and Carrasco, L. (2009) RNA nuclear export is blocked by poliovirus 2A protease and is concomitant with nucleoporin cleavage. *J Cell Sci* **122:** 3799–3809.

Castello, A., Alvarez, E., and Carrasco, L. (2011) The multifaceted poliovirus 2A protease: regulation of gene expression by picornavirus proteases. *J Biomed Biotechnol* **2011:** 369648.

Dickson, A.M., Anderson, J.R., Barnhart, M.D., Sokoloski, K.J., Oko, L., Opyrchal, M., *et al.* (2012) Dephosphorylation of HuR protein during alphavirus infection is associated with HuR relocalization to the cytoplasm. *J Biol Chem* **287:** 36229–36238.

Fata, C.L., Sawicki, S.G., and Sawicki, D.L. (2002) Modification of Asn374 of nsP1 suppresses a Sindbis virus nsP4 minus-strand polymerase mutant. *J Virol* **76:** 8641–8649.

Frolov, I., and Schlesinger, S. (1994) Comparison of the effects of Sindbis virus and Sindbis virus replicons on host cell protein synthesis and cytopathogenicity in BHK cells. *J Virol* **68:** 1721–1727.

Frolov, I., Agapov, E., Hoffman, T.A., Jr, Pragai, B.M., Lippa, M., Schlesinger, S., and Rice, C.M. (1999) Selection of RNA replicons capable of persistent noncytopathic replication in mammalian cells. *J Virol* **73:** 3854–3865.

Frolov, I., Garmashova, N., Atasheva, S., and Frolova, E.I. (2009) Random insertion mutagenesis of Sindbis virus nonstructural protein 2 and selection of variants incapable

of downregulating cellular transcription. *J Virol* **83:** 9031–9044.

Fros, J.J., Domeradzka, N.E., Baggen, J., Geertsema, C., Flipse, J., Vlak, J.M., and Pijlman, G.P. (2012) Chikungunya virus nsP3 blocks stress granule assembly by recruitment of G3BP into cytoplasmic foci. *J Virol* **86:** 10873–10879.

Garcia-Moreno, M., Sanz, M.A., Pelletier, J., and Carrasco, L. (2013) Requirements for eIF4A and eIF2 during translation of Sindbis virus subgenomic mRNA in vertebrate and invertebrate host cells. *Cell Microbiol* **15:** 823–840.

Garmashova, N., Gorchakov, R., Frolova, E., and Frolov, I. (2006) Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J Virol* **80:** 5686–5696.

Garneau, N.L., Sokoloski, K.J., Opyrchal, M., Neff, C.P., Wilusz, C.J., and Wilusz, J. (2008) The 3′ untranslated region of Sindbis virus represses deadenylation of viral transcripts in mosquito and Mammalian cells. *J Virol* **82:** 880–892.

Garry, R.F. (1994) Sindbis virus-induced inhibition of protein synthesis is partially reversed by medium containing an elevated potassium concentration. *J Gen Virol* **75** (Part 2)**:** 411–415.

Garry, R.F., Bishop, J.M., Parker, S., Westbrook, K., Lewis, G., and Waite, M.R. (1979) Na+ and K+ concentrations and the regulation of protein synthesis in Sindbis virus-infected chick cells. *Virology* **96:** 108–120.

Gomez de Cedron, M., Ehsani, N., Mikkola, M.L., Garcia, J.A., and Kaariainen, L. (1999) RNA helicase activity of Semliki Forest virus replicase protein NSP2. *FEBS Lett* **448:** 19–22.

Gorchakov, R., Frolova, E., Williams, B.R., Rice, C.M., and Frolov, I. (2004) PKR-dependent and -independent mechanisms are involved in translational shutoff during Sindbis virus infection. *J Virol* **78:** 8455–8467.

Gorchakov, R., Frolova, E., and Frolov, I. (2005) Inhibition of transcription and translation in Sindbis virus-infected cells. *J Virol* **79:** 9397–9409.

Griffin, D.E. (2007) Alphaviruses. In *Fields Virology*, 5th edn. Knipe, D. (ed.). Philadelphia: Lippincott Williams & Wilkins, pp. 1023–1067.

de Groot, R.J., Hardy, W.R., Shirako, Y., and Strauss, J.H. (1990) Cleavage-site preferences of Sindbis virus polyproteins containing the non-structural proteinase. Evidence for temporal regulation of polyprotein processing *in vivo*. *EMBO J* **9:** 2631–2638.

Gui, H., Lu, C.W., Adams, S., Stollar, V., and Li, M.L. (2010) hnRNP A1 interacts with the genomic and subgenomic RNA promoters of Sindbis virus and is required for the synthesis of G and SG RNA. *J Biomed Sci* **17:** 59.

Hahn, Y.S., Strauss, E.G., and Strauss, J.H. (1989) Mapping of RNA- temperature-sensitive mutants of Sindbis virus: assignment of complementation groups A, B, and G to nonstructural proteins. *J Virol* **63:** 3142–3150.

Jose, J., Snyder, J.E., and Kuhn, R.J. (2009) A structural and functional perspective of alphavirus replication and assembly. *Future Microbiol* **4:** 837–856.

Kallio, K., Hellstrom, K., Balistreri, G., Spuul, P., Jokitalo, E., and Ahola, T. (2013) Template RNA length determines the size of replication complex spherules for Semliki Forest virus. *J Virol* **87:** 9125–9134.

Kim, K.H., Rumenapf, T., Strauss, E.G., and Strauss, J.H. (2004) Regulation of Semliki Forest virus RNA replication: a model for the control of alphavirus pathogenesis in invertebrate hosts. *Virology* **323:** 153–163.

Koonin, E.V., Gorbalenya, A.E., Purdy, M.A., Rozanov, M.N., Reyes, G.R., and Bradley, D.W. (1992) Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. *Proc Natl Acad Sci USA* **89:** 8259–8263.

Kujala, P., Ikaheimonen, A., Ehsani, N., Vihinen, H., Auvinen, P., and Kaariainen, L. (2001) Biogenesis of the Semliki Forest virus RNA replication complex. *J Virol* **75:** 3873–3884.

Lemm, J.A., Rumenapf, T., Strauss, E.G., Strauss, J.H., and Rice, C.M. (1994) Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. *EMBO J* **13:** 2925–2934.

Li, Z., and Nagy, P.D. (2011) Diverse roles of host RNA binding proteins in RNA virus replication. *RNA Biol* **8:** 305–315.

Lin, J.Y., Shih, S.R., Pan, M., Li, C., Lue, C.F., Stollar, V., and Li, M.L. (2009) hnRNP A1 interacts with the 5′ untranslated regions of enterovirus 71 and Sindbis virus RNA and is required for viral replication. *J Virol* **83:** 6106–6114.

Lulla, A., Lulla, V., and Merits, A. (2012) Macromolecular assembly-driven processing of the 2/3 cleavage site in the alphavirus replicase polyprotein. *J Virol* **86:** 553–565.

McInerney, G.M., Kedersha, N.L., Kaufman, R.J., Anderson, P., and Liljestrom, P. (2005) Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation. *Mol Biol Cell* **16:** 3753–3763.

Mi, S., Durbin, R., Huang, H.V., Rice, C.M., and Stollar, V. (1989) Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. *Virology* **170:** 385–391.

Miller, S., and Krijnse-Locker, J. (2008) Modification of intracellular membrane structures for virus replication. *Nat Rev Microbiol* **6:** 363–374.

Panas, M.D., Varjak, M., Lulla, A., Eng, K.E., Merits, A., Karlsson Hedestam, G.B., and McInerney, G.M. (2012) Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection. *Mol Biol Cell* **23:** 4701–4712.

Park, N., Katikaneni, P., Skern, T., and Gustin, K.E. (2008) Differential targeting of nuclear pore complex proteins in poliovirus-infected cells. *J Virol* **82:** 1647–1655.

Park, N., Skern, T., and Gustin, K.E. (2010) Specific cleavage of the nuclear pore complex protein Nup62 by a viral protease. *J Biol Chem* **285:** 28796–28805.

Patel, R.K., Burnham, A.J., Gebhart, N.N., Sokoloski, K.J., and Hardy, R.W. (2013) Role for subgenomic mRNA in host translation inhibition during Sindbis virus infection of mammalian cells. *Virology* **441:** 171–181.

Peranen, J., Laakkonen, P., Hyvonen, M., and Kaariainen, L. (1995) The alphavirus replicase protein nsP1 is membrane-associated and has affinity to endocytic organelles. *Virology* **208:** 610–620.

Redondo, N., Sanz, M.A., Welnowska, E., and Carrasco, L. (2011) Translation without eIF2 promoted by poliovirus 2A protease. *PLoS ONE* **6:** e25699.

Rikkonen, M., Peranen, J., and Kaariainen, L. (1994) Nuclear targeting of Semliki Forest virus nsP2. *Arch Virol Suppl* **9:** 369–377.

Rozelle, D.K., Filone, C.M., Kedersha, N., and Connor, J.H. (2014) Activation of stress response pathways promote formation of antiviral granules and restrict virus replication. *Mol Cell Biol* **34:** 2003–2016.

Rubach, J.K., Wasik, B.R., Rupp, J.C., Kuhn, R.J., Hardy, R.W., and Smith, J.L. (2009) Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro. *Virology* **384:** 201–208.

Russo, A.T., White, M.A., and Watowich, S.J. (2006) The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. *Structure* **14:** 1449–1458.

Salonen, A., Vasiljeva, L., Merits, A., Magden, J., Jokitalo, E., and Kaariainen, L. (2003) Properly folded nonstructural polyprotein directs the Semliki Forest virus replication complex to the endosomal compartment. *J Virol* **77:** 1691–1702.

Sanz, M.A., and Carrasco, L. (2001) Sindbis virus variant with a deletion in the 6K gene shows defects in glycoprotein processing and trafficking: lack of complementation by a wild-type 6K gene in trans. *J Virol* **75:** 7778–7784.

Sanz, M.A., Castello, A., and Carrasco, L. (2007) Viral translation is coupled to transcription in Sindbis virus-infected cells. *J Virol* **81:** 7061–7068.

Sawicka, K., Bushell, M., Spriggs, K.A., and Willis, A.E. (2008) Polypyrimidine-tract-binding protein: a multifunctional RNA-binding protein. *Biochem Soc Trans* **36:** 641–647.

Shin, G., Yost, S.A., Miller, M.T., Elrod, E.J., Grakoui, A., and Marcotrigiano, J. (2012) Structural and functional insights into alphavirus polyprotein processing and pathogenesis. *Proc Natl Acad Sci USA* **109:** 16534–16539.

Shirako, Y., and Strauss, J.H. (1994) Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *J Virol* **68:** 1874–1885.

Shirako, Y., Strauss, E.G., and Strauss, J.H. (2000) Suppressor mutations that allow sindbis virus RNA polymerase to function with nonaromatic amino acids at the N-terminus: evidence for interaction between nsP1 and nsP4 in minus-strand RNA synthesis. *Virology* **276:** 148–160.

Sokoloski, K.J., Dickson, A.M., Chaskey, E.L., Garneau, N.L., Wilusz, C.J., and Wilusz, J. (2010) Sindbis virus usurps the cellular HuR protein to stabilize its transcripts and promote productive infections in mammalian and mosquito cells. *Cell Host Microbe* **8:** 196–207.

Sola, I., Galan, C., Mateos-Gomez, P.A., Palacio, L., Zuniga, S., Cruz, J.L., *et al*. (2011) The polypyrimidine tract-binding protein affects coronavirus RNA accumulation levels and relocalizes viral RNAs to novel cytoplasmic domains different from replication-transcription sites. *J Virol* **85:** 5136–5149.

Spuul, P., Balistreri, G., Hellstrom, K., Golubtsov, A.V., Jokitalo, E., and Ahola, T. (2011) Assembly of alphavirus replication complexes from RNA and protein components in a novel trans-replication system in mammalian cells. *J Virol* **85:** 4739–4751.

Strauss, J.H., and Strauss, E.G. (1994) The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58:** 491–562.

Tamm, K., Merits, A., and Sarand, I. (2008) Mutations in the nuclear localization signal of nsP2 influencing RNA synthesis, protein expression and cytotoxicity of Semliki Forest virus. *J Gen Virol* **89:** 676–686.

Valiente-Echeverria, F., Melnychuk, L., and Mouland, A.J. (2012) Viral modulation of stress granules. *Virus Res* **169:** 430–437.

Ventoso, I., Barco, A., and Carrasco, L. (1998) Mutational analysis of poliovirus 2Apro. Distinct inhibitory functions of 2apro on translation and transcription. *J Biol Chem* **273:** 27960–27967.

Ventoso, I., Sanz, M.A., Molina, S., Berlanga, J.J., Carrasco, L., and Esteban, M. (2006) Translational resistance of late alphavirus mRNA to eIF2alpha phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. *Genes Dev* **20:** 87–100.

Vignuzzi, M., Stone, J.K., and Andino, R. (2005) Ribavirin and lethal mutagenesis of poliovirus: molecular mechanisms, resistance and biological implications. *Virus Res* **107:** 173–181.

Vihinen, H., Ahola, T., Tuittila, M., Merits, A., and Kaariainen, L. (2001) Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J Biol Chem* **276:** 5745–5752.

Wang, Y.F., Sawicki, S.G., and Sawicki, D.L. (1991) Sindbis virus nsP1 functions in negative-strand RNA synthesis. *J Virol* **65:** 985–988.

Yang, Y.L., Reis, L.F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., *et al*. (1995) Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J* **14:** 6095–6106.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Protein synthesis in BHK-T7 cells transfected with different plasmids. Effect of PV 2Apro. (A) BHK-T7 cells were transfected first with pTM1-2A (0.8 μg per well) and 1 h later with 0.8 μg of the different pTM1 plasmids. Protein synthesis was analysed 8 h after the first transfection by incorporation of [^{35}S]Met/Cys followed by SDS-PAGE, fluorography and autoradiography. (B) Nuclease-treated RRL was used to translate 100 ng of mRNAs transcribed *in vitro* from the different pTM1 plasmids. Protein synthesis was analysed by radioactive labelling, SDS-PAGE, fluorography and autoradiography.

Fig. S2. Distribution of PTB in cells expressing SINV nsPs. BHK-T7 cells seeded on coverslips in 24-well plates were transfected with 0.4 μg pTM1-nsP1-4 or 0.4 μg pTM1-nsP2, and were processed for immunofluorescence at 18 hpt. Rat polyclonal anti-nsP1 or rabbit polyclonal anti-nsP2 antibodies

were used to detect the transfected cells (green), and mouse monoclonal anti- PTB detected the subcellular location of this protein (red).

Fig. S3. Distribution of TIA-1 and PTB in cells transfected with SINV or SINV nsP2 (P726G) gmRNAs. BHK cells were transfected with 5 μg per well of genomic mRNAs synthesized by *in vitro* transcription from pT7SVwt or pT7SV(P726G) and processed for immunofluorescence at 24 hpt. After fixation, cell monolayers were incubated with rabbit anti-C (green) and goat anti-TIA-1 (red) (A) or mouse anti-PTB (red) antibodies (B). Topro-3 was used to stain the nuclei (blue).

Fig. S4. Release of TIA-1 in SINV-infected BHK cells treated with ribavirin or 6-aza-uridine. Mock and SINV-infected BHK cells were fixed at 5 hpi and processed for immunofluorescence. Rabbit anti-C antibodies were used to detect SINV-infected cells (green) and goat anti-TIA-1 antibodies to detect the subcellular location of this protein (red). Topro-3 was used to stain the nuclei (blue).