Journal of General Virology (2011), **92**, 2181–2190 DOI 10.1099/vir.0.030163-0

Differential effect of acute and persistent Junín virus infections on the nucleo-cytoplasmic trafficking and expression of heterogeneous nuclear ribonucleoproteins type A and B

Cynthia A. Maeto,†‡ María E. Knott,† Florencia N. Linero, Paula C. Ellenberg,§ Luis A. Scolaro and Viviana Castilla

Laboratorio de Virología, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

Correspondence
Viviana Castilla
viviana@qb.fcen.uba.ar

Received 28 December 2010
Accepted 31 May 2011

Heterogeneous nuclear ribonucleoproteins A and B (hnRNPs A/B), cellular RNA-binding proteins that participate in splicing, trafficking, translation and turnover of mRNAs, have been implicated in the life cycles of several cytoplasmic RNA viruses. Here, we demonstrate that silencing of hnRNPs A1 and A2 significantly reduces the replication of the arenavirus Junín virus (JUNV), the aetiological agent of Argentine haemorrhagic fever. While acute JUNV infection did not modify total levels of expression of hnRNPs A/B in comparison with uninfected cells, non-cytopathic persistent infection exhibited low levels of these cell proteins. Furthermore, acutely infected cells showed a cytoplasmic relocalization of overexpressed hnRNP A1, probably related to the involvement of this protein in virus replicative cycle. This cytoplasmic accumulation was also observed in cells expressing viral nucleoprotein (N), and co-immunoprecipitation studies revealed the interaction between hnRNP A1 and N protein. By contrast, a predominantly nuclear distribution of overexpressed hnRNP A1 was found during persistent infection, even in the presence of endogenous or overexpressed N protein, indicating a differential modulation of nucleo–cytoplasmic trafficking in acute and persistent JUNV infections.

INTRODUCTION

Junín virus (JUNV), a member of the family *Arenaviridae*, is the aetiological agent of Argentine haemorrhagic fever. These enveloped ssRNA viruses have a genome consisting of two RNA segments: the S segment encodes the structural nucleoprotein (N) and the glycoprotein precursor (GPC), which is secondarily cleaved into the envelope proteins G1 and G2; and the L segment encodes the viral polymerase (L) and a zinc-binding matrix protein (Z). Besides JUNV, other arenaviruses such as Lassa, Machupo, Guanarito and Sabiá viruses also cause severe haemorrhagic diseases in man. Specific rodents are the principal hosts of the arenaviruses and humans may become infected through direct contact with infected rodents or through inhalation of infectious rodent excreta and secreta (Charrel & de Lamballerie, 2010; Yun *et al.*, 2008).

JUNV establishes a persistent infection in its main reservoir in nature, the cricetid *Calomys musculinus*, or in cell cultures (Ellenberg *et al.*, 2002, 2004, 2007). Persistent infection in Vero cells is achieved after the acute phase of infection, which is characterized by the production of high titres of infectious virus and a marked cytopathic effect. During persistence cells remain unable to produce infectious virus, with a continuous synthesis of virus nucleoprotein (N) associated with blockage of the expression of the glycoprotein G1 and absence of cytopathic action (Ellenberg *et al.*, 2002, 2004). Little is known about the cell components involved in JUNV replication during acute infection or in the establishment and maintenance of persistent infection.

Several cytoplasmic RNA viruses alter the nucleo-cytoplasmic trafficking of cellular RNA and proteins and this may facilitate viral replication and help subvert the host antiviral response (Gustin 2003; Rodriguez *et al.*, 2003; Reid *et al.*, 2006; Frieman *et al.*, 2007). Heterogeneous nuclear ribonucleoproteins (hnRNPs) constitute a large group of RNA-binding proteins which participate in a variety of cellular functions, including mRNA splicing, trafficking, translation and turnover. The most abundant hnRNPs are those belonging to the A/B type, such as A1/A1b, A2/B1, B2 and A3, which have molecular masses of approximately 40 kDa.

†These authors contributed equally to this work.

‡Present address: Centro Nacional de Referencia para el SIDA, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 11, Buenos Aires, Argentina.

§Present address: Infectious Diseases Unit, Alfred Hospital, Level 2, Burnet Building, 85 Commercial Road, Melbourne, VIC 3004, Australia.

These proteins have a highly conserved amino-terminal domain, which contains two tandemly repeated RNA-binding domains that are directly involved in RNA binding, and a divergent glycine-rich domain at the carboxy terminus, which is implicated in protein–protein interactions. hnRNPs A1 and A2 are known to shuttle between the nucleus and the cytoplasm and this shuttling is mediated by a sequence (M9) located near the carboxy terminus of the proteins (Shi *et al.*, 2003).

hnRNP A/B family and hnRNP I (also known as poly-pyrimidine tract binding protein or PTB) have been shown to accumulate in the cytoplasm of cells infected with mouse hepatitis virus (MHV) in order to interact with and modulate MHV RNA synthesis (Shi *et al.*, 2000, 2003). In contrast, during poliovirus (PV) infection, hnRNP E is essential for the internal initiation of translation at internal ribosome entry sites (IRES) (Walter *et al.*, 1999), and the cytoplasmic relocalization of hnRNP C1 enables its association with viral proteins to promote replication (Brunner *et al.*, 2005). Furthermore, it has been demonstrated that alterations in the nucleo-cytoplasmic trafficking of several hnRNPs during vesicular stomatitis virus (VSV) infection might play a role in virus-induced apoptosis (Pettit Kneller *et al.*, 2009).

In an attempt to identify cellular factors involved in JUNV replication and to gain insight into virus–cell interactions that lead to the death or survival of the infected cell, in the present study we characterized the effect of expression of hnRNPs A/B during acute and persistent JUNV infections in Vero cells.

RESULTS

Knockdown of hnRNPs A/B by small interfering RNA (siRNA) reduces JUNV replication

To analyse the role of hnRNPs A1 and A2 in JUNV replication, we transfected Vero cells with siRNAs that targeted hnRNP A1 (siRNA A1) or A2 (siRNA A2) separately, or we used a mix of both of these siRNAs. A small non-interfering RNA was used as control. Cells were infected with JUNV (m.o.i. of 1) at 24 h post-transfection and cultures were further incubated for 24 h when extracellular virus production and the expression of viral N protein were evaluated (Fig. 1). Compared with control, single siRNA knockdown of either hnRNP A1 or A2 caused a 60 or 66 % reduction in virus titre, respectively (Fig. 1a). When both hnRNP A1 and A2 proteins were knocked down, the inhibition of virus production was 94 % (Fig. 1a) and a similar reduction in the expression of viral N protein, as assessed by indirect immunofluorescence, was achieved (Fig. 1b). The analysis by Western blot (Fig. 1c) showed that cells transfected with siRNA A1 displayed a marked reduction of the most intense band, revealed by an antibody that recognizes the hnRNPs belonging to the A/B group; this band corresponds to hnRNP A1, the major

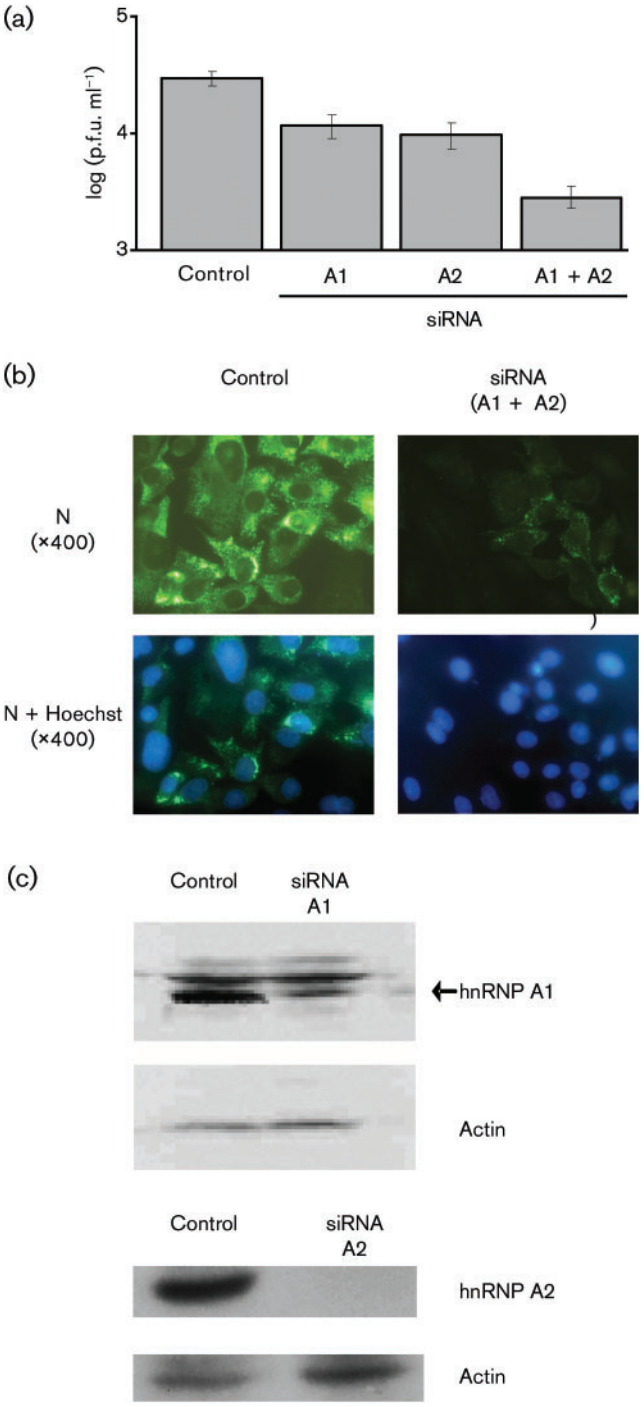


Fig. 1. Effect of hnRNP A1 and hnRNP A2 silencing on JUNV replication. Vero cells were transfected with siRNAs targeting hnRNP A1, A2, or A1 and A2 simultaneously, or with control RNA. After 24 h cells were infected with JUNV (m.o.i. of 1) and at 24 h post-infection (p.i.) virus yield was quantified by plaque assay. Viral titres are mean values from triplicates ± 95 % CI (a). Expression of viral N protein in cells treated with control RNA or siRNAs targeting hnRNP A1 and A2 was examined by immunofluorescence (b). Levels of hnRNP A1 and A2 in cells treated with siRNAs were analysed by Western blot by using H-200 antibody reactive against the hnRNP A/B group of proteins or anti-hnRNP A2 mAb (c).

protein among the hnRNP A/B group (Allemand *et al.* 2005). A high level of inhibition of hnRNP A2 expression by siRNA A2 was also achieved (Fig. 1c). These results indicate that the hnRNP A/B proteins are involved in acute JUNV infection.

JUNV-persistent infection alters hnRNP A/B expression

Next we examined whether the expression of hnRNPs A/B was modified by JUNV during either the acute or the persistent phase of infection. The characterization of acutely and persistently JUNV-infected cultures is shown in Fig. 2. Similarly to results obtained previously (Ellenberg *et al.* 2002, 2004), acute infection exhibited a progressive increase in extracellular virus yield during the first 72 h of infection (Fig. 2a), whereas persistently infected cells, called V3 cells, did not produce infectious virus. Furthermore, acutely infected cells expressed both N and G1 proteins, while N but not G1 protein could be detected in V3 cells (Fig. 2b). The analysis by Western blot showed that acute infection does not modify hnRNPs A/B expression levels with respect to non-infected cells; on the contrary, V3 cells exhibited a large reduction in the expression of these proteins (Fig. 2c). To confirm these results, we analysed the expression of hnRNPs A/B in several cell clones obtained from Vero (13 clones) or V3 cultures (10 clones). All clones obtained from V3 cells, which expressed N but not G1 protein and did not produce infectious virus (data not shown), displayed reduced levels of hnRNPs A/B compared with clones obtained from Vero cells (results obtained with some representative clones are shown in Fig. 2d).

JUNV infection induces changes in the intracellular localization of hnRNP A1

To investigate the effect of JUNV infection on the intracellular localization of hnRNP A1, we performed cell transfection with a plasmid that allows the expression of T7-tagged hnRNP A1. Fig. 3(a) shows the localization of hnRNP A1 in non-infected and infected cells; the percentage of cells with cytoplasmic hnRNP A1 is also stated. At 24 h p.i., approximately 60 % of infected cells exhibited a high cytoplasmic T7–hnRNP A1 fluorescence.

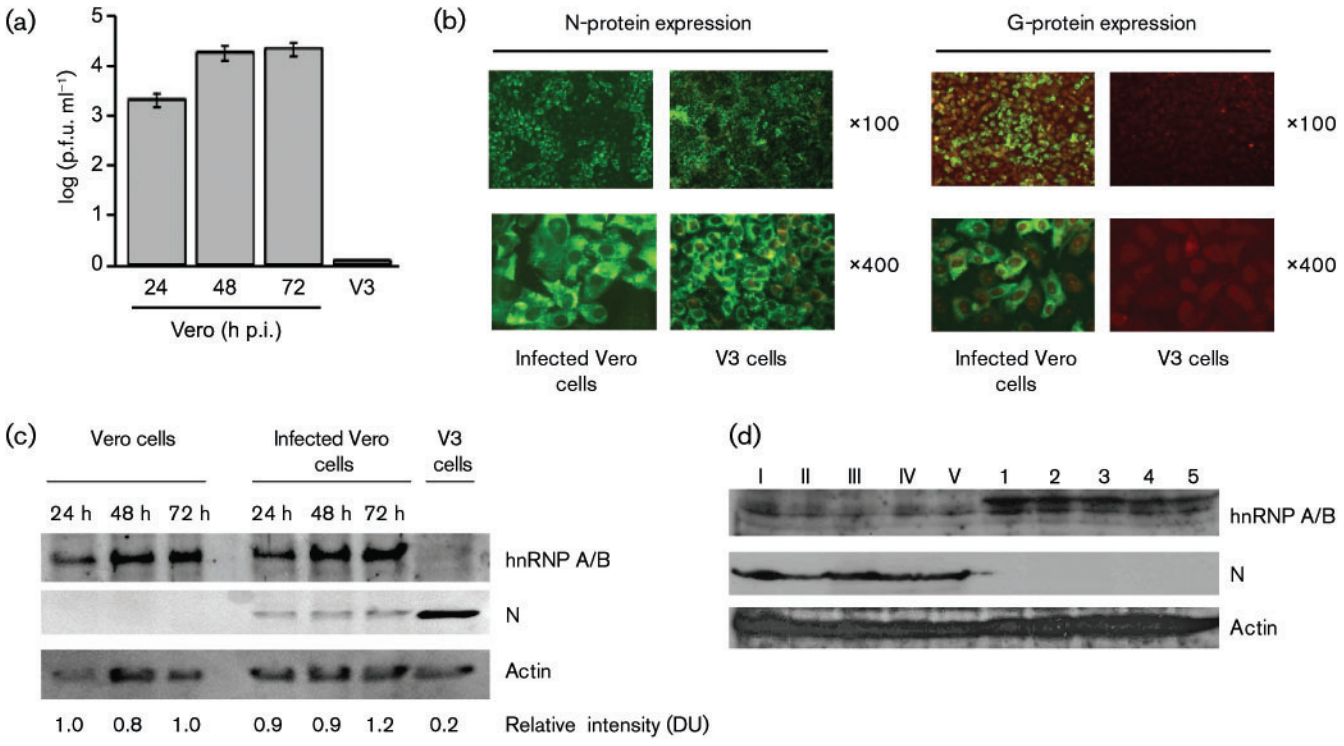
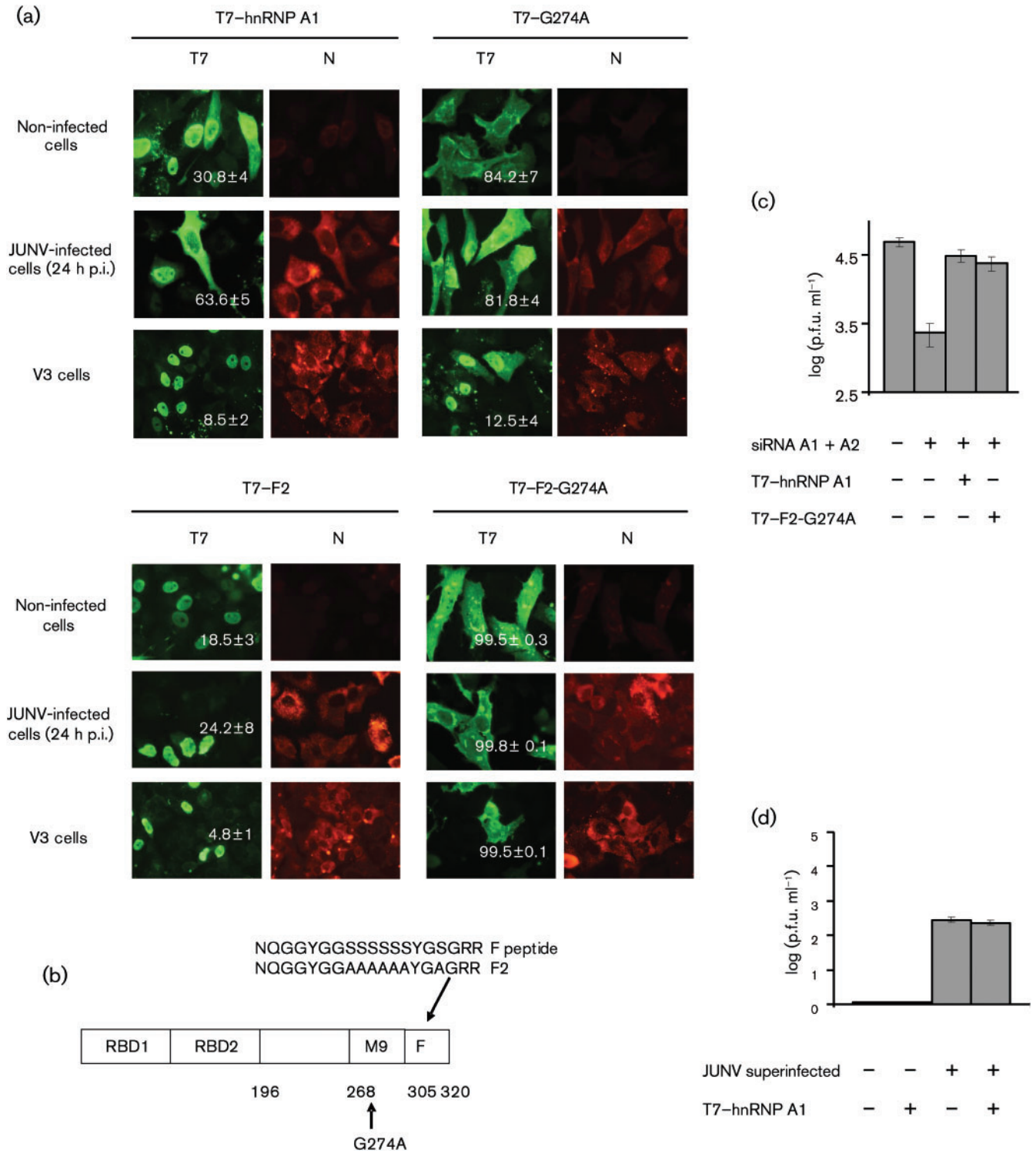


Fig. 2. Expression of hnRNPs A/B during acute and persistent JUNV infection. Supernatants from JUNV-infected Vero cells (m.o.i. of 1) after 24, 48 or 72 h of infection, or from V3 cultures, were titrated by plaque assay. Viral titres are mean values from triplicates ± 95 % CI (a). Expression of viral N and G1 proteins in JUNV-infected Vero cells (m.o.i. of 1) at 48 h p.i., or in V3 cells, was determined by immunofluorescence assay (b). Expression of hnRNPs A/B, actin and N proteins in non-infected or JUNV-infected Vero cells (after 24, 48 or 72 h), or in V3 cells, was analysed by Western blot. The relative intensity of a representative experiment was expressed as densitometry units (DU)=(intensity of hnRNP A/B band)/(intensity of band corresponding to actin) (c). Expression of hnRNPs A/B, actin and N protein in clones of Vero cells (1–5) or V3 cells (I–V) was analysed by Western blot (d).



In contrast, most of the non-infected cells and V3 persistently infected cells showed a nuclear pattern of distribution of T7–hnRNP A1 (Fig. 3a).

As mentioned above, the carboxy terminus of hnRNP A1 harbours a nuclear localization signal, the M9 motif, which is involved in the interaction with the import receptor transportin (Fig. 3b). A cytoplasmic form of hnRNP A1 has been generated by mutation of the M9 domain (G274A) (Guil *et al.*, 2006). The carboxy terminus of hnRNP A1 also presents the F peptide (Fig. 3b) that contains serine residues that are phosphorylated under stress conditions, resulting in cytoplasmic accumulation of hnRNP A1. Mutation of serine residues to alanine within this F peptide inhibits stress-induced cytoplasmic accumulation of hnRNP A1, yielding a mutant protein with nuclear

Fig. 3. Intracellular localization of hnRNP A1 (wt and mutants) in JUNV-infected Vero cells. (a) Intracellular localization of T7–hnRNP A1 in uninfected Vero cells, Vero cells infected with JUNV for 24 h or V3 cells. T7 tag was revealed by immunofluorescence using a rabbit polyclonal antibody and a FITC-conjugated secondary antibody. N protein was detected by using a mouse mAb as primary antibody and TRITC-conjugated anti-mouse immunoglobulins as secondary antibody. The quantification of the percentage of cells expressing hnRNP A1 predominantly in the cytoplasm was obtained by counting 250 co-transfected cells, and data included in the T7 panels represent the mean value of three separate experiments ± SD. (b) Diagram of the functional domains of hnRNP A1 and mutant proteins. (c) Vero cells were transfected with control RNA or siRNAs targeting hnRNP A1 and A2. At 24 h post-transfection cultures treated with siRNAs were transfected or not with plasmids encoding T7–hnRNP A1 or T7–F2-G274A and 24 h later the cultures were infected with JUNV (m.o.i. of 1). Culture supernatants were collected at 24 h p.i. and virus titres were determined. (d) V3 cells were transfected with T7–hnRNP A1 and after 24 h cultures were superinfected or not with JUNV (m.o.i. of 1). Culture supernatants were collected 24 h later and virus titres were determined. In (c) and (d), viral titres are mean values from triplicates ± 95 % CI.

localization called F2. Combining of these two mutations (within the M9 and F domains) results in the mutant F2-G274A (Fig. 3b), a cytoplasmic hnRNP A1 that cannot be phosphorylated within the F peptide (Guil *et al.*, 2006). Thus, in order to further characterize nucleo-cytoplasmic trafficking in acute and persistent JUNV-infected cultures, we examined the localization of these hnRNP A1 mutant proteins in both types of infection.

The mutant T7–G274A exhibited a cytoplasmic localization both in non-infected cells and in acutely infected ones; indeed, most of the cells in V3 cultures presented nuclear fluorescence, indicating that mutation of the M9 domain, which interferes with the interaction of hnRNP A1 with transportin, does not impede nuclear import of the protein into the nucleus of persistently infected cells (Fig. 3a). In contrast, T7–F2 mutant protein was exclusively nuclear in non-infected and infected cells, during both acute and persistent infections, corroborating that the export of hnRNP A1 to the cytoplasm requires phosphorylation of the F peptide. Finally, a similar cytoplasmic localization of the double mutant T7–F2-G274A was observed in all cell cultures tested, suggesting that the nuclear localization of the mutant T7–G274A in V3 cells might depend on the phosphorylation of serine residues of the carboxy terminus.

When we analysed whether overexpression of T7–hnRNP A1, wild-type (wt) or mutant proteins affected virus multiplication, we found similar virus yields were obtained in Vero cells transfected or not with each plasmid (data not shown). The lack of effect on virus production cannot be ascribed to a poor level of transfection since transfection efficiency, determined by immunofluorescence as the number of cells expressing the T7 tag divided by the total number of cells in several representative fields, was approximately 40 % for the four plasmids used. To better explore the effects of wt and mutant proteins on JUNV replication, Vero cells treated with the combined mix of siRNA A1 and siRNA A2 were then transfected with T7–hnRNP A1 or T7–F2-G274A and further infected with JUNV. When endogenous hnRNP A1 and hnRNP A2 were silenced, overexpression of hnRNP A1, wt or mutant, was able to partially suppress the inhibition of virus multiplication (Fig. 3c), indicating that the F2 and G274A mutations do not affect hnRNP A1 functionality. Since V3 cells exhibited very low levels of hnRNPs A/B, we explored the effect of T7–hnRNP A1 transfection on the production of infectious virus in persistent V3 cultures. Infectious particles in the supernatant of V3 cells were undetectable either in transfected or non-transfected cultures (Fig. 3d). It has been previously described how V3 cells are highly resistant to a new JUNV infection (superinfection) (Ellenberg *et al.*, 2002, 2004), thus we wanted to know whether overexpression of hnRNP A1 would allow the recovery of an infectious virus titre in superinfected V3 cells. V3 non-transfected cells superinfected with JUNV (m.o.i. of 1) displayed a reduction in virus yield of approximately 99 % with respect to the yield obtained in Vero cells infected at an identical m.o.i. (compare Fig. 3c with Fig. 3d). A similar low level of virus production was observed in T7–hnRNP A1-transfected, JUNV-superinfected V3 cells (Fig. 3d), indicating that overexpression of hnRNP A1 is not enough to restore the ability of these cultures to produce infectious virus at levels similar to those obtained in the acute infection. Furthermore, superinfection of V3 cells did not induce cytoplasmic accumulation of overexpressed T7–hnRNP A1 (data not shown).

We decided to analyse the effect of JUNV acute infection on the intracellular localization of PTB, another member of the hnRNP family. PTB contains a novel type of bipartite nuclear localization signal at the amino terminus of the protein named nuclear determinant localization type I, and importin α acts as cytoplasmic receptor for its nuclear import (Romanelli & Morandi, 2002). This protein is important for the life cycle of many viruses (Zúñiga *et al.*, 2009) and it has been demonstrated that viral infections might lead to its translocation to the cytoplasm (Agis-Juárez *et al.*, 2009). One role of cytoplasmic PTB that has been studied is the regulation of IRES-dependent translation of several cellular and viral mRNAs (Florez *et al.*, 2005; Sawicka *et al.*, 2008; Kafasla *et al.*, 2010). We performed an immunofluorescence assay to examine the expression of PTB in acutely JUNV-infected cells, and we determined that JUNV infection does not modify the nuclear pattern of PTB observed in non-infected cultures (Figs 4a, b). In contrast, PV infection caused the cytoplasmic relocalization of both PTB and T7–hnRNP

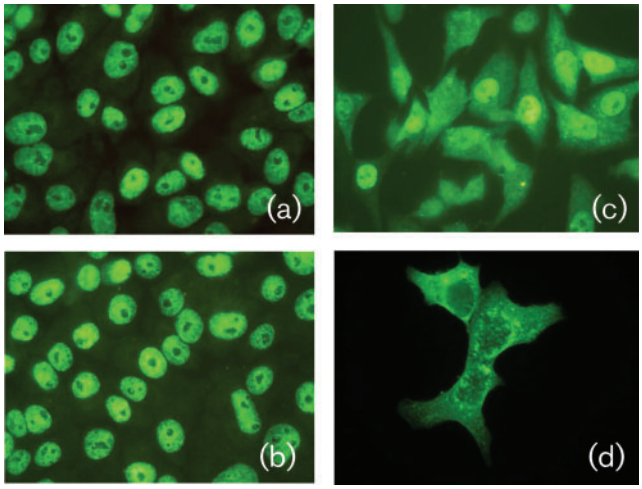


Fig. 4. Effect of JUNV infection on PTB intracellular localization. Expression of (a) PTB in uninfected Vero cells, (b) Vero cells infected with JUNV or (c) PV determined by indirect immunofluorescence assay. (d) Overexpression of T7–hnRNP A1, detected by indirect immunofluorescence assay, in PV-infected Vero cells.

A1 in Vero cells (Figs 4c, d) in accordance with previously reported results (Gustin &Sarnow, 2001; Gustin, 2003; Zúñiga *et al.*, 2009). Thus, our results indicate that acute JUNV infection does not cause a generalized alteration of nucleo-cytoplasmic shuttling of hnRNPs but specifically affects transportin-mediated traffic.

Viral N protein interacts with hnRNP A1

To establish whether a specific viral gene product was responsible for the cytoplasmic accumulation of hnRNP A1, we transfected Vero cells with T7–hnRNP A1 alone or in combination with plasmids expressing viral N protein or GFP, which was used as control. At 48 h post-transfection, an indirect immunofluorescence assay was performed and the number of co-transfected cells expressing hnRNP A1 in either the nucleus or the cytoplasm was counted in each sample. In accordance with the results mentioned above, a predominantly nuclear localization of hnRNP A1 was observed in Vero or V3 cells transfected with T7–hnRNP A1 alone (data not shown) or co-transfected with GFP-expressing plasmid (Fig. 5a). In contrast, Vero cells co-transfected with T7–hnRNP A1 and N-expressing plasmid exhibited a cytoplasmic pattern for hnRNP A1 in most cells analysed (Fig. 5b), suggesting a role of N protein in the accumulation of hnRNP A1 in the cytoplasm during acute JUNV infection. When co-transfection with T7–hnRNP A1 and N-expressing plasmids was performed in V3 cells, hnRNP A1 exhibited a predominantly nuclear distribution (Fig. 5b).

In order to investigate a possible interaction between hnRNP A1 and N protein we conducted a co-immunoprecipitation (IP) assay. Vero cells or Vero cells infected with JUNV at acute or persistent phase were transfected with the plasmid T7–hnRNP A1, and 24 h later cell lysates were obtained and immunoprecipitated with anti-N mAb. The analysis of the immunoprecipitates by Western blot, using anti T7 antibody, revealed that overexpressed hnRNP A1 co-immunoprecipitates with N protein from both acutely and persistently infected cultures (Fig. 5c). Interaction between N and T7–hnRNP A1 in V3 cells might occur with the small proportion of T7–hnRNP A1 that remained cytoplasmic. Therefore, our results indicate that N protein is able to interact with overexpressed hnRNP A1. However, in V3 cells this interaction might not be enough to retain hnRNP A1 within the cytoplasm (see the immunofluorescence assay shown in Fig. 3a).

In addition, co-immunoprecipitation of N protein from acute and persistent infections along with the over-expressed mutant protein T7–F2-G274A was also confirmed (data not shown), indicating that these mutations at the carboxy terminus do not interfere with the interaction of hnRNP A1 with the viral protein.

To confirm the interaction of N protein with endogenous hnRNP A1, we examined the ability of antibodies against hnRNPs A/B to precipitate N protein from acutely or persistently infected cells. Analysis of immunoprecipitated proteins by Western blot (using anti-N mAb) revealed the co-immunoprecipitation of hnRNP A/B and N protein only in acutely infected cells (Fig. 5d). N protein could not be detected in the immunoprecipitate obtained from V3 cells, probably owing to the low levels of endogenous hnRNPs A/B in these cultures (Fig. 2d).

DISCUSSION

The present study provides evidence that hnRNPs A/B play a role in JUNV multiplication. Knockdown of hnRNP A1 or A2 caused a modest reduction in the production of progeny virus, but when both cell proteins were silenced simultaneously a marked and greater inhibition of virus yield was achieved. The specificity of the siRNA inhibitory effect was confirmed when transient expression of hnRNP A1 was performed in siRNA-treated cultures, since over-expression of hnRNP A1 allowed the recovery of virus production. The participation of several members of the hnRNP A/B family has been reported not only in viral replication events (Shi *et al.*, 2003) but also in cellular RNA-splicing events (Caputi *et al.*, 1999; Mayeda *et al.*, 1994). Since all hnRNP A/B proteins are structurally similar and are particularly homologous in their RNA-binding domains it would not be surprising if they replaced each other in their functions.

Redistribution of hnRNPs to the cytoplasm during infection with cytoplasmic viruses would favour interaction between these factors and viral components, making possible or augmenting the efficiency of synthesis and/or translation of viral RNA (Shi *et al.*, 2000; Burnham *et al.*,

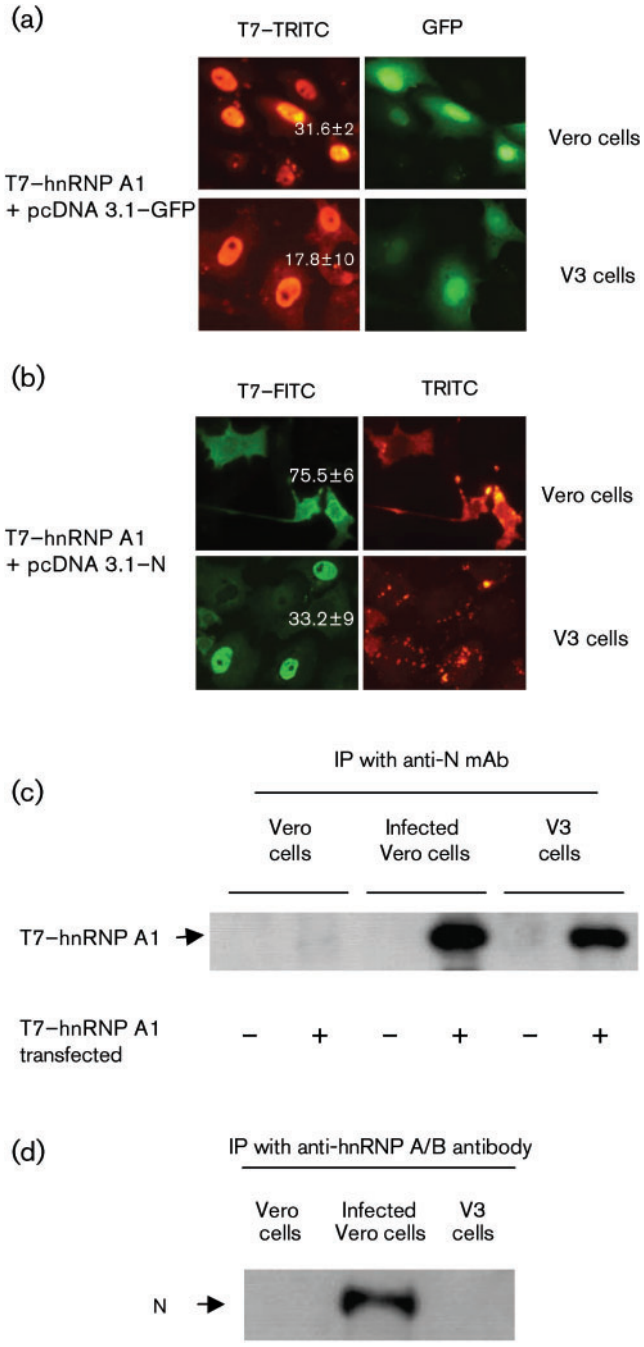


Fig. 5. Effect of N protein overexpression on intracellular localization of hnRNPA1. Intracellular localization of T7–hnRNP A1 in Vero or V3 cells co-transfected with plasmids T7–hnRNP A1 and pcDNA3.1–N or pcDNA3.1–GFP was analysed by using a double immunofluorescence assay. T7 tag was revealed with a rabbit polyclonal antibody using (a) a TRITC-conjugated or (b) a FITC-conjugated secondary antibody. N protein was detected using a mouse mAb as primary antibody and a TRITC-conjugated secondary antibody (b). Quantification of the percentage of cells expressing hnRNP A1 predominantly in the cytoplasm was obtained by counting 250 co-transfected cells; the figures shown within the T7 panels represent the mean values from three independent experiments ± SD (a and b). Lysates obtained from Vero cells, JUNV-infected Vero cells or V3 cells, transfected or not with T7–hnRNP A1 were immunoprecipitated with anti-N mAb. Co-immunoprecipitation of T7–hnRNP A1 was revealed by Western blot analysis (c). Lysates obtained from Vero cells, JUNV-infected Vero cells or V3 cells were immunoprecipitated with polyclonal antibody H-200 against hnRNPs A/B. Co-immunoprecipitation of the N protein was revealed by Western blot analysis (d).

2007; Cammas *et al.*, 2007; Kim *et al.*, 2007; Lin *et al.*, 2008, 2009; Gui *et al.*, 2010). A previous report showed that the arenavirus Pichinde virus causes a transient cytoplasmic translocation of hnRNP A1 (Bowick *et al.*, 2009). Here, we demonstrated that acute JUNV infection induces the cytoplasmic accumulation of hnRNP A1 in most infected cells but, in accordance with previous results obtained for hepatitis C virus-infected cells (Kim *et al.*, 2007), some infected cells exhibited a nuclear hnRNP A1 pattern of distribution. The m.o.i. of 1 employed in these experiments does not ensure a synchronic infection, so the heterogeneous pattern of hnRNP A1 localization observed after 24 h of infection might be attributed to the presence of cells at different stages of infection.

Another interesting finding was that cytoplasmic relocalization of hnRNP A1 in acute JUNV-infected cells might depend on serine phosphorylation at the F-peptide domain. It is well established that stress-mediated phosphorylation of hnRNP A1 abrogates the interaction with the import receptor transportin (Dreyfuss *et al.*, 2002), so a possible explanation for hnRNP A1 cytoplasmic accumulation in JUNV-infected cells would be virus-induced phosphorylation. As well as several transcription factors, some factors involved in mRNA processing, such as hnRNP K and hnRNP A1, are regulated by cell-signalling pathways. Stress-induced phosphorylation of hnRNP A1 is mediated by Mnk1/2 kinases and it is known that activation of mitogen-activated protein kinases (MAPKs) results in the activation of Mnk1 (Allemand *et al.*, 2005). Viruses rely upon cell signalling to regulate all processes within the cell that drive virus replication (Pleschka, 2008; Linero & Scolaro, 2009; Seo *et al.*, 2010), so we are actually investigating whether JUNV infection induces changes in hnRNP A1 phosphorylation through the activation of MAPK signal-transduction cascades.

We also demonstrated that overexpression of N protein induces cytoplasmic relocalization of hnRNP A1 in Vero cells. Furthermore, co-immunoprecipitation studies demonstrated the interaction between N protein and hnRNP A1. It was reported that cells infected with the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) exhibited cytoplasmic translocation of IRF-3, an interferon regulatory transcription factor, hence modulating the antiviral response of the host cell. Moreover, N protein from LCMV and other arenaviruses, including JUNV, induces cytoplasmic relocalization of IRF-3, suggesting that N protein would affect cell processes associated

with IRF-3 phosphorylation (Martínez-Sobrido *et al.*, 2006, 2007). Thus, additional studies will be needed to determine at what level JUNV N protein affects hnRNP A1 subcellular localization, and whether this effect is related to a modulation of hnRNP A1 phosphorylation.

While acute JUNV infection did not modify the total levels of hnRNP A/B expression with respect to uninfected cells, non-cytopathic persistent infection exhibited low levels of these cell proteins. Modulation of hnRNP expression by virus infections has been reported previously. hnRNP A1 is upregulated during differentiation of epithelial cells infected with human papillomavirus type 16, facilitating the appropriate alternative splicing necessary for virus late transcription (Cheunim *et al.*, 2008). In contrast, during the mid-to-late phase of PV infection, hnRNP E is cleaved by viral proteases 3C/3CD as a way to regulate virus translation and RNA replication (Perera *et al.*, 2007). The dynamic interplay between virus and host components is an important aspect of viral pathogenesis. Our results suggest that modulation of hnRNPs A/B might contribute to the regulation of virus replication and virus cytopathic effect, thus allowing the establishment or maintenance of persistent infection. Further studies will determine whether downregulation of the expression of hnRNPs A/B during persistent infection is the result of a blockage at the transcriptional or post-transcriptional level. We proved that transient expression of hnRNP A1 does not allow the recovery of virus production in V3 cells, indicating that other viral or cellular factors that have not yet been identified are responsible for the blockade of virus replication in this cell system. It is possible that during persistence the expression of many other cellular factors are altered. It is tempting to propose that a subpopulation of infected cells, carrying some features which favour the development of an equilibrium between virus replication and cell survival, might be selected at the end of the acute phase of infection, giving rise to a culture with relatively homogeneous characteristics, as can be seen in the case of the low levels of hnRNPs A/B displayed by different cellular clones obtained from V3 cells. Therefore, it is reasonable to consider that other cellular processes involved both in virus replication and in cell survival could be altered in these selected cell populations.

In contrast with the results obtained during acute infection, during persistent JUNV infection overexpressed hnRNP A1 is recruited almost exclusively within the nucleus, even when the nuclear localization signal M9 is mutated to abort the interaction with the import receptor transportin. These results indicate that persistently JUNV-infected cells may possess an alternative mechanism for nuclear import of this kind of shuttling protein. Since M9 has also been implicated in the export of hnRNP A1 to the cytoplasm, we cannot rule out deficient export of this protein in V3 cells (Guil *et al.*, 2006). Co-immunoprecipitation studies demonstrated that N protein can interact with hnRNP A1 in V3 cells; however, this interaction does not induce cytoplasmic accumulation of hnRNP A1. Thus, there may be additional factors contributing to the nuclear accumulation and/or retention of hnRNP A1 in the context of persistent infection. Subcellular localization of hnRNP A1 is an important determinant of apoptotic signalling, and it has been proven that cytoplasmic hnRNP A1 negatively regulates the translation of inhibitors of apoptosis such as XIAP and apaf-1 (Lewis *et al.*, 2007; Zhao *et al.*, 2009). We propose that, during acute infection, relocalization of hnRNP A1 to the cytoplasm may be involved in the induction of apoptotic cell death and the development of cytopathic action, as has been described for VSV infection (Pettit Kneller *et al.*, 2009). In contrast, non-cytopathic persistent JUNV infection may be related to low levels of hnRNPs A/B and modulation of nucleo-cytoplasmic trafficking, which results in the nuclear retention of overexpressed hnRNP A1.

In conclusion, we document here for the first time that acute and persistent JUNV infections differentially modulate hnRNP A/B expression. We propose that at the initial stage of productive infection these proteins might favour virus replication. It was demonstrated that arenavirus N and L proteins are the minimal *trans*-acting factors necessary to drive transcription and replication of minireplicons (Lee *et al.*, 2000; López *et al.*, 2001). However, the cellular proteins involved in viral RNA synthesis remain unknown. It is probable that, through its interaction with N protein, hnRNP A1 becomes part of the RNA replication complex and participates in viral genome transcription/replication. Furthermore, we do not rule out a possible role for hnRNPs A/B in virus translation, and we are now interested in the study of a possible interaction of these proteins with viral mRNAs and cellular translation factors. On the other hand, the achievement of a persistent infection might involve downregulation of cell proteins required for virus replication, such as hnRNPs A/B, as well as changes to nucleo-cytoplasmic trafficking that are implicated in cell survival processes.

METHODS

Virus and cells. Vero cells (ATCC CCL-81) were cultured in Eagle's minimum essential medium containing 5 % FBS and 50 μg ml^{-1} gentamicin (Sigma-Aldrich). A stock of JUNV, strain XJCl3, was obtained from BHK-21 cells (ATCC CCL-10) and a stock of PV type 3 (vaccine strain P3/Leon/12a, b) was obtained from Vero cells. Virus infectivity was quantified by plaque assay. Virus titres and 95 % confidence intervals (CI) were calculated according to the Poisson distribution. In each case three independent experiments were performed and only a representative experiment is shown.

Persistent cultures were obtained as described previously (Ellenberg *et al.*, 2002). Briefly, Vero cells were infected with JUNV (strain XJCl3) at an m.o.i. of 0.01. At the end of the acute phase of infection (30 days after infection) cells were subcultured, at a split ratio of 1 : 3, every two weeks for a 1 year period. Control non-infected Vero cells were subcultured in parallel. Vero and V3 cells were biologically cloned by limiting dilution as previously described (Ellenberg *et al.*, 2007).

siRNA and plasmid transfections. hnRNP A1 and hnRNP A2 silencing was accomplished by using commercial siRNAs (sc-35575

and sc-43841; Santa Cruz Biotechnology). Vero cells grown on coverslips in 24-well plates were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen). The sequence of the RNA used as control is 5′-GACCACAATTCTCGATATACAUU-3′. Cells were infected at 24 h post-transfection at an m.o.i. of 1. At 24 h p.i. extracellular medium was harvested to quantify virus production and cells were fixed with methanol for immunofluorescence assays.

Plasmids encoding hnRNP A1 wt (T7–hnRNP A1) and mutants (T7–G274A, T7–F2 and T7–F2-G274A) were kindly provided by Dr J. Cáceres (Guil *et al.*, 2006). Construction of N-protein-encoding plasmid (pcDNA3.1–N) and GFP-encoding plasmid (pcDNA3.1–GFP) was as previously described (Artuso *et al.*, 2009). Plasmids (1–2 μg ml^{-1}) were transfected into Vero cells grown in 24-well plates by using Lipofectamine 2000 (Invitrogen).

Immunofluorescence assay. For detection of viral proteins, cells fixed with methanol for 10 min at −20 °C were incubated with anti-N protein mAb (SA02 BG12) (1 : 300) or anti-G1 protein mAb (GB03BF11) (1 : 300) (Sánchez *et al.*, 1989) for 45 min at 37 °C followed by incubation with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) for 45 min at 37 °C.

Detection of T7-tagged hnRNP A1 (wt or mutant) was done in cultures fixed with 4 % paraformaldehyde for 15 min at room temperature (RT) followed by incubation with 0.2 % Triton X-100 for 10 min at RT. Cells were incubated at 37 °C for 45 min with rabbit polyclonal anti-T7-tag antibody (Abcam). Double staining of viral N protein and T7–hnRNP A1 was performed using TRITC-conjugated anti-mouse IgG and FITC-conjugated anti rabbit immunoglobulins (Sigma-Aldrich) as secondary antibodies.

Detection of PTB was conducted using a mouse mAb (Invitrogen) and FITC-conjugated anti-mouse IgG.

Nuclei were stained with Hoechst 33258 (1 μg ml^{-1}) for 15 min at RT. Coverslips were mounted on a 90 % glycerin solution in PBS (pH 7.2) containing 2.5 % 1, 4-diazabicyclo (2,2,2) octane (DABCO; Sigma-Aldrich) and visualized in a fluorescence microscope. The fluorescence intensities of 20 randomly selected fields (×100 magnification) was quantified by using IMAGE PRO EXPRESS, version 6.0 (Media Cybernetics).

Western blot assay. Cells were lysed and SDS-PAGE was performed on 10 % polyacrylamide gels. The resolved proteins were then transferred to a PVDF membrane (Hybond P; Amersham Pharmacia) in a dry system. NA05AG12 mAb (Sánchez *et al.*, 1989) and peroxidase anti-mouse IgG (Sigma-Aldrich) were used to reveal N protein. Detection of hnRNPs A/B and β-actin was performed by using the rabbit antibodies H-200 (Santa Cruz Biotechnology) (1 : 1000 dilution) and #4967 (Cell Signalling) (1 : 1000 dilution), respectively, with peroxidase anti-rabbit immunoglobulins as secondary antibody. Detection of hnRNP A2 was done by using the mouse mAb ab6102 (Abcam) and peroxidase anti-mouse immunoglobulins. The intensities of protein bands, visualized by chemiluminescence detection, were quantified by using SCION IMAGE for Windows. The relative intensity of each band was expressed as densitometry units (DU), which are defined as (intensity of the band)/(intensity of band corresponding to actin).

Co-immunoprecipitation assay. Uninfected or JUNV-infected Vero cells and V3 cells transfected or not with plasmids T7–hnRNP A1 or T7–F2-G274A were lysed into 75 μl of lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 % (w/v) SDS, 1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 0.4 mM PMSF and 1 μg aprotinin ml^{-1}). The lysates were subjected to centrifugation for 10 min at 10 000 ***g*** at 4 °C and the clarified supernatants were incubated with a 1 : 100 dilution of QB06 AEO5 anti-N mAb (Sánchez *et al.*, 1989) or polyclonal antibody H-200 against hnRNPs A/B for 30 min at 37 °C and 90 min at 4 °C on a rotator. Antibody–antigen complexes were precipitated with protein A–Sepharose beads (GE Healthcare) in lysis buffer for 30 min at 37 °C and 90 min at 4 °C on a rotator, followed by centrifugation for 10 min at 10 000 ***g***. The beads were washed four times with lysis buffer and then subjected to Western blot analysis using rabbit polyclonal antibody against the T7 tag or NA05AG12 anti-N mAb as primary antibodies.

ACKNOWLEDGEMENTS

We thank Dr Cáceres for his kind contribution of T7 plasmids and Dr Sánchez for providing JUNV mAbs. This work was supported by grants from Universidad de Buenos Aires (X206), Agencia Nacional de Promoción Cientiífica y Técnica (PICT 2007-00761) and CONICET (PIP 11220090100443) of Argentina. Dr L. A. Scolaro is a member of the Scientific Career and F. N. Linero and C. A. Maeto are fellows from CONICET.

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