Avian and mammalian reoviruses use different molecular mechanisms to synthesize their $\mu$NS isoforms

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Previous reports revealed that the M3 gene of both avian and mammalian reoviruses express two isoforms of the non-structural protein $\mu$NS in infected cells. The larger isoforms initiate translation at the AUG codon closest to the 5′ end of their respective m3 mRNAs, and were therefore designated $\mu$NS. In this study we have performed experiments to identify the molecular mechanisms by which the smaller $\mu$NS isoforms are generated. The results of this study confirmed the previous findings indicating that the smaller mammalian reovirus $\mu$NS isoform is a primary translation product, the translation of which is initiated at the internal AUG-41 codon of mammalian reovirus m3 mRNA. Our results further revealed that the smaller avian reovirus $\mu$NS isoform originates from a specific post-translational cleavage site near the amino terminus of $\mu$NS. This cleavage produces a 55 kDa carboxy-terminal protein, termed $\mu$NSC, and a 17 kDa amino-terminal polypeptide, designated $\mu$NSN. These results allowed us to extend the known avian reovirus protein-encoding capacity to 18 proteins, 12 of which are structural proteins and six of which are non-structural proteins. Our finding that avian and mammalian reoviruses use different mechanisms to express their $\mu$NSC isoforms suggests that these isoforms are important for reovirus replication.

INTRODUCTION

Mammalian reovirus (MRV) and avian reovirus (ARV) are species groups of the genus Orthoreovirus (Chappel et al., 2005), which differ in host range, degree of pathogenicity, genome coding capacity and in some biological properties. Mammalian reovirus is the prototype of the non-fusogenic orthoreoviruses and avian reovirus is the prototype of the fusogenic orthoreoviruses (Benavente & Martínez-Costas, 2007; Nibert & Schiff, 2001; Zhang et al., 2005). They are non-enveloped viruses that contain a genome formed by ten dsRNA segments enclosed within a double protein-capsid shell. The reoviral genes are transcribed by a virion-associated RNA polymerase to generate mRNAs that are identical to the positive-sense strand of the dsRNA segments encoding them. Viral transcripts, which are designated with lower-case letters, possess a type-1 cap at their 5′ ends and lack a polyadenylated 3′ tail (Banerjee & Shatkin, 1970; Furuichi et al., 1975; Martinez-Costas et al., 1995).

The replication and assembly of reoviruses takes place in distinctive cytoplasmic inclusions called viral factories (Fields et al., 1971), which contain structural and non-structural viral proteins but lack membranes and cellular organelles.

The observation that the M3-encoded non-structural $\mu$NS protein of either MRV or ARV is the only viral protein that forms factory-like inclusions when expressed in transfected cells suggests that $\mu$NS forms the framework of the viral factories in infected cells (Becker et al., 2003; Brandariz-Nuñez et al., 2010; Broering et al., 2002; Tourís-Otero et al., 2004b). Furthermore, it has been shown that viral core proteins and the non-structural protein $\sigma$NS associate with $\mu$NS inclusions in co-transfected cells, thus suggesting that $\mu$NS recruits these proteins to the viral factories of infected cells (Miller et al., 2003, 2010; Tourís-Otero et al., 2004a).

The M3 genes of MRV and ARV have been reported to express two protein isoforms in infected cells (Tourís-Otero et al., 2004b; Wiener et al., 1989). The smaller isoforms could originate from post-translational cleavage or by secondary initiation at in-frame, downstream AUG codons of their respective m3 mRNAs. Compelling experimental evidence suggests that the smaller MRV $\mu$NS isoform is a primary translation product that originates by initiation at an internal AUG codon of the MRV m3 mRNA: (i) pulse–chase analysis of MRV-infected cells failed to demonstrate a precursor–product relationship between the two MRV $\mu$NS isoforms.
(Wiener et al., 1989); (ii) two isoforms with electrophoretic mobilities similar to the ones produced in infected cells were synthesized by in vitro translation of the MRV m3 mRNA in reticulocyte lysates (Kobayashi et al., 2006; Wiener et al., 1989); (iii) the amino-termini of both MRV μNS and μNSC are blocked, thus suggesting that the two proteins are primary translation products (Wiener et al., 1989); and (iv) only μNSC is generated when the AUG codon closest to the 5′ end of the MRV m3 mRNA is mutated (Arnold et al., 2008; Kobayashi et al., 2006). If the smaller MRV μNS isoform originates from internally initiated translation, it would lack sequences from the amino terminus of μNS, and this has been experimentally demonstrated by subjecting the two MRV μNS isoforms to peptide mapping after N-chlorosuccinimide cleavage at tryptophan residues (Wiener et al., 1989). Hence, the smaller MRV μNS isoform was designated μNSC to indicate that it originates from the carboxy terminus of μNS.

The electrophoretic mobilities of the two MRV μNS isoforms suggests that they have a molecular mass difference of 5 kDa; therefore MRV μNSC could be produced by initiating translation at the in-frame downstream AUG codons specifying methionines 41 or 57, since initiation at the next in-frame downstream AUG (specifying methionine 111) would generate a protein ~12 kDa smaller than μNS (Fig. 1a). The observation that only MRV μNS, but not μNSC, is synthesized by translation of an MRV m3 mRNA version containing a mutated AUG-41 codon (Arnold et al., 2008; Kobayashi et al., 2006) suggests that μNSC begins at methionine 41 of μNS. However, the possibility of starting translation at methionine 57 has not yet been explored.

In contrast with the MRV situation, the mechanism by which the smaller ARV μNS isoform is produced has not yet been addressed. In this study we investigated the mechanisms used by these viruses to generate their smaller μNS isoforms.

**RESULTS**

**Two μNS isoforms are produced in ARV- and MRV-infected cells**

To identify the polypeptide isoforms expressed by the M3 genome segments of ARV and MRV, immunoprecipitation and immunoblot analysis of extracts from infected and uninfected cells were performed. The antiserum against MRV μNS immunoprecipitated several proteins from extracts of MRV-infected L929 cells (Fig. 2a, lane 4), but only the 80 and 75 kDa polypeptides were recognized by the antiserum in a Western blot assay (Fig. 2a, lane 6), indicating that they are MRV μNS isoforms. Similarly, the antiserum against ARV μNS immunoprecipitated three proteins of 70, 55 and 35 kDa from extracts of ARV-infected chicken embryo fibroblasts (CEF) (Fig. 2b, lane 4), but only the 70 and 55 kDa polypeptides were recognized by the antiserum in a Western blot assay (Fig. 2b, lane 6), indicating that they are ARV μNS isoforms. The 35 kDa protein is the viral non-structural protein σNS, which we have previously identified as a μNS-interacting protein (Touris-Otero et al., 2004b). Western blot analysis with anti-σNS antiserum confirmed the identity of this protein (result not shown).

It should be noted that whereas a molecular mass of 60 kDa has been described for the smaller ARV μNS isoform (Touris-Otero et al., 2004b), a closer examination of its electrophoretic mobility, by performing electrophoretic analysis with gradient and non-gradient gels containing different polyacrylamide concentrations and by using different MW markers, revealed that the smaller ARV μNS isoform migrates as a 55 kDa protein (data not shown). The expression of the two ARV μNS isoforms is not virus-strain or cell-type specific since the two isoforms were produced in: (i) CEF cells infected with the avian reoviruses S1133, 2408 and 173; and (ii) in avian CEF and DF-1 cells and mammalian Vero and HeLa cells infected with avian reovirus S1133 (data not shown).

The electrophoretic mobilities of the larger MRV and ARV μNS isoforms were those expected for the products where translation initiated at the AUG codons closest to the 5′ end of their respective m3 mRNAs (Fig. 1) and were coincident with the mobilities of the larger in vitro translation products of their respective m3 mRNAs (Fig. 3). Therefore, these proteins were designated μNS to indicate that they are the non-structural μ-class proteins encoded by the largest ORF of their respective M3 genes.

**Fig. 1.** Schematic representation of μNS-encoding transcripts. (a) MRV m3 mRNA. (b) ARV m3 mRNA. The AUG codons closest to the 5′ end and downstream in-frame AUG codons are underlined and numbered by codon underneath the mRNA sequence. The position of the first and last nucleotides, as well as the positions of the adenine nucleotides of the AUG codons are marked with an asterisk and numbered. AUG codons and their corresponding −3 and +4 positions are indicated by capital letters.
Once it was confirmed that ARV and MRV express two \( \mu \)NS isoforms, the main thrust of the present study was to identify whether the smaller \( \mu \)NS isoforms originate by initiating translation at an internal in-frame AUG codon or by post-translational cleavage of precursor \( \mu \)NS. We first examined the possibility of internal initiation.

Examination of the nucleotide sequences of ARV and MRV m3 mRNAs revealed that the AUG codons closest to their 5’ ends are in an optimal Kozak context (Kozak, 1991), since the two of them have purine nucleotides in positions -3 and +4 (Fig. 1). Consequently, these mRNAs should initiate translation exclusively at the AUG codons closest to their 5’ ends and therefore should be monocistronic.

However, compelling evidence (see Introduction) suggests that the smaller MRV \( \mu \)NS isoform is a primary translation product for which translation initiates at an internal AUG triplet of the MRV m3 mRNA. To assess whether the smaller ARV and MRV \( \mu \)NS isoforms are produced by secondary translation initiation, we followed two approaches. In the first approach, reticulocyte lysates were programmed with RNA isolated from both uninfected cells and reovirus-infected cells, were immunoprecipitated with the corresponding \( \mu \)NS antiserum, and the selected radioactive proteins were analysed by SDS-PAGE and autoradiography. The results revealed that while the two MRV \( \mu \)NS isoforms detected in infected cells (Fig. 3a, lane 6) were synthesized by \textit{in vitro} translation of the MRV m3 mRNA (Fig. 3a, lane 4), only the larger ARV \( \mu \)NS isoform, but not the 55 kDa isoform, was synthesized by \textit{in vitro} translation of the ARV m3 mRNA (Fig. 3b, compare lanes 4 and 6). These results suggest that the smaller MRV \( \mu \)NS isoform, but not its ARV counterpart, is a primary translation product of the m3 mRNA.

As stated in the Introduction, the molecular mass difference between the two MRV \( \mu \)NS isoforms (~5 kDa) suggests that the smaller MRV isoform could originate by initiating translation at the in-frame downstream AUG codons specifying methionines 41 or 57 (Fig. 2a). On the other hand, the predicted molecular mass difference between the two ARV isoforms is 15 kDa, implying that the smaller isoform might originate from translating translation at methionines 127, 140 or 208 of ARV \( \mu \)NS (Fig. 1b). The results shown in Fig. 3(c) revealed that the smaller MRV \( \mu \)NS isoform has an electrophoretic mobility similar to that of \textit{in vitro}-translated polypeptides that initiate translation at methionines 41 or 57 of MRV \( \mu \)NS, thus suggesting that this isoform could originate from initiating translation at one of these two AUG codons. It should be mentioned that the translated RNA in lane 1 of Fig. 3(c) is a version of a MRV m3 mRNA that contains 13 extra nucleotides at its 5’ end, and that synthesis of the 75 kDa isoform is barely detected following translation of this mRNA, suggesting that the AUG codon closest to the 5’ end of the MRV m3 mRNA is not leaky when preceded by a longer 5’ UTR. In contrast, the results shown in Fig. 3(d) indicate that the ARV \( \mu \)NS 55 kDa isoform migrates faster than the polypeptides for which translation initiates at methionines 127 and 140, but slower than the polypeptide for which translation initiates at methionine 208. These data indicate that the smaller MRV \( \mu \)NS isoform, but not its ARV counterpart, is a primary translation product of the m3 mRNA.

**The smaller MRV \( \mu \)NS isoform, but not its ARV counterpart, originates by initiation of translation at an internal AUG codon**

Once it was confirmed that ARV and MRV express two \( \mu \)NS isoforms, the main thrust of the present study was to identify whether the smaller \( \mu \)NS isoforms originate by initiating translation at an internal in-frame AUG codon or by post-translational cleavage of precursor \( \mu \)NS. We first examined the possibility of internal initiation.
Single mutations (AUG\[
\] were translated in reticulocyte lysates in the presence of specified by codons 1, 41 and 57 to leucines. These RNAs that only MRV-infected cells, were analysed by SDS-PAGE and as immunoprecipitated radiolabelled proteins from the C terminus of authentic 5\textsuperscript{\nu} transcribed MRV m3 mRNAs containing a type-1 cap and m57 of the translation product of the m3 mRNA, and was therefore designated MRV \(\mu\)NSC to indicate that it originates from the C terminus of \(\mu\)NS.

The results presented so far did not allow us to assess whether MRV \(\mu\)NSC starts translation at methionine 41 or 57 of \(\mu\)NS. To clarify this point, we generated \textit{in vitro}-transcribed MRV m3 mRNAs containing a type-1 cap and authentic 5\textsuperscript{\nu} and 3\textsuperscript{\nu} ends, either unmutated or containing single mutations (AUG→UUG) to change the methionines specified by codons 1, 41 and 57 to leucines. These RNAs were translated in reticulocyte lysates in the presence of \([35\text{S}]\)methionine, and the resulting radiolabelled proteins, as well as immunoprecipitated radiolabelled proteins from MRV-infected cells, were analysed by SDS-PAGE and autoradiography. The results shown in Fig. 3(e) revealed that only \(\mu\)NSC is synthesized when AUG codon 1 is mutated (lane 3), and only \(\mu\)NS is produced when AUG codon 41 is mutated (lane 4). In contrast, the two \(\mu\)NS isoforms detected in infected cells (lane 1), as well as by \textit{in vitro} translation of non-mutated m3 mRNA (lane 2), are similarly produced when AUG codon 57 is mutated (lane 5). Our finding that synthesis of \(\mu\)NSC is prevented by mutation of AUG codon 41, but not of AUG 57, indicates that \(\mu\)NSC originates from internal translation initiation at AUG codon 41 of MRV m3 mRNA.

**A single post-translational cleavage of ARV \(\mu\)NS yields 17 and 55 kDa polypeptides**

We next explored the possibility that the smaller \(\mu\)NS isoforms originate by post-translational cleavage. First of all, MRV- and ARV-infected cells were pulsed with \([35\text{S}]\)methionine and \([35\text{S}]\)cysteine for 10 min at 16 h post-infection (p.i.), and the cells were then lysed immediately or after chasing for different time periods in medium lacking radioactivity, but containing large excesses of non-radiolabelled methionine and cysteine. The resulting cell extracts were immunoprecipitated with the corresponding \(\mu\)NS-specific antisera and analysed by SDS-PAGE and autoradiography. The autoradiogram of the MRV samples revealed that the radioactive bands corresponding to the
two MRV μNS isoforms are already present in MRV-infected cells at the end of the radioactive pulse and that their relative intensities do not change significantly during the chase (Fig. 4a). In contrast, the radioactive band corresponding to the smaller ARV μNS isoform was barely detectable at the end of the labelling pulse, but its intensity increased progressively with longer chase periods (Fig. 4b).

These results demonstrate that the smaller ARV μNS isoform, but not its MRV counterpart, originates from post-translational cleavage.

If the 55 kDa ARV μNS isoform originates from a single μNS cleavage, a complementary 15 kDa polypeptide should be generated too. To detect the presence of this polypeptide in ARV-infected cells, cell extracts were immunoprecipitated with ARV-μNS-specific antiserum and the radioactive immunoprecipitated proteins were subsequently separated on an electrophoresis gel system (10% tricine–SDS-PAGE gel) specifically designed to resolve small peptides (Schägger & von Jagow, 1987). The autoradiogram, shown in Fig. 5(a), revealed the presence of a radioactive polypeptide band of ~17 kDa in immunoprecipitated extracts of ARV-infected cells (lane 2, marked with two asterisks) that did not show up in extracts from uninfected cells (lane 3). Furthermore, this polypeptide was recognized by ARV-μNS-specific antibodies in a Western blot assay of extracts from ARV-infected cells (Fig. 5b, lane 2), but not from uninfected cells (Fig. 5b, lane 1). These results indicate that the 17 kDa polypeptide is an ARV μNS isoform. To confirm this hypothesis, we performed a new pulse–chase analysis of ARV-infected cells, but the radioactive proteins were now resolved by the 10% tricine–SDS-PAGE system. The autoradiogram, shown in Fig. 5(c), revealed that, as with the 55 kDa μNS isoform, the 17 kDa polypeptide band was barely detectable at the end of the pulse period (lane 2), but its intensity gradually increased with longer chase periods (lanes 2–6). Furthermore, the increase in the intensity of both the 55 and 17 kDa bands was accompanied by a concomitant decrease in the intensity of the 70 kDa μNS protein band. Altogether, these results demonstrate that the 17 and 55 kDa polypeptides are ARV μNS isoforms that originate from a single post-translational cleavage of the 70 kDa ARV μNS precursor. It should be mentioned here that a faint 15 kDa band, which is recognized by anti-μNS antibodies, is also detected in infected cells (Fig. 5b, lane 2 and Fig. 5c, lane 6). We are now investigating whether this band corresponds to another ARV μNS isoform.

**ARV μNS is cleaved near its amino terminus**

To determine whether ARV μNS cleavage occurs near its amino- or carboxy-terminus, cytoplasmic extracts from both uninfected and ARV-infected CEF were incubated with μNS antiserum and the immunoprecipitated proteins were analysed by electrophoresis on a 10% tricine–SDS-polyacrylamide gel. Visualization of the protein bands by Coomassie blue staining revealed the presence of a 17 kDa polypeptide in immunoprecipitated extracts from infected cells (Fig. 6, lane 2, marked with two asterisks), which was not present in extracts from uninfected cells (Fig. 6, lane 1). The fact that this polypeptide is recognized by ARV μNS-specific antibodies in an immunoblot assay (Fig. 6, lane 5), indicates that it is an ARV μNS isoform. A slice containing the 17 kDa protein band was excised from the Coomassie-stained gel, digested with trypsin and subjected to mass
spectrometric analysis. The analysis revealed that this polypeptide contained two tryptic fragments whose amino acid sequences matched the regions containing residues 24–45 and 91–99 of the deduced ARV mNS amino acid sequence. These results demonstrate that the 17 kDa polypeptide is located at the amino terminus of mNS and hence it was designated mNSN. Conversely, the complementary 55 kDa isoform was termed mNSC.

Finally, we wanted to identify the ARV mNS cleavage site by subjecting gel-purified mNSC to Edman degradation analysis, but unfortunately mNSC could not be purified from the SDS-PAGE gel because it co-migrated in the gel with the immunoglobulin heavy chain (Fig. 6, lane 5).

DISCUSSION

Viruses use a variety of strategies to maximize protein expression from limited-size genomes; the most common ones are alternative translation initiation from polycistronic transcripts and proteolysis of primary translation products. In this report we provide evidence that two species groups of the genus Orthoreovirus use different strategies to generate their smaller mNS isoforms. Thus, MRV mNSC is produced by initiating translation at the internal AUG-41 codon of the MRV m3 mRNA, whereas ARV mNSC is generated by a single post-translational cleavage of the precursor mNS. In spite of being generated by completely different mechanisms, the two mNSC proteins lack sequences from the amino terminus of their respective precursors. These observations, and the facts that the initiator MRV mNS AUG codon is conserved
among all sequenced MRV M3 genes and that ARV \(\mu\)NSC is generated in cells infected with three different ARV isolates, suggest that \(\mu\)NSC may be important for reovirus replication. However, recent reports reveal that although MRV \(\mu\)NSC is still able to form inclusions, it is incapable of restoring MRV growth in cultured cells when \(\mu\)NS is absent (Arnold et al., 2008; Kobayashi et al., 2006, 2009). These observations suggest that both inclusion formation is an important, though not a sufficient, function of \(\mu\)NS during MRV reovirus replication, and that key functions for viral growth are localized within those amino-terminal sequences of MRV \(\mu\)NS that are missing in \(\mu\)NSC. These sequences have already been shown to be required for association with, and inclusion recruitment of, MRV proteins \(\mu\)2 and \(\sigma\)NS (Broering et al., 2002; Miller et al., 2003, 2010). We have similarly found that sequences contained within \(\mu\)NSN are necessary for association of ARV \(\mu\)NS with several structural and non-structural proteins (unpublished data). Although not strictly required for factory formation and viral growth on cultured cells, \(\mu\)NSC proteins might be important for reovirus replication and/or spread within host organisms, as suggested by Kobayashi et al. (2009).

The results reported by different laboratories strongly suggest that MRV \(\mu\)NSC originates by starting translation at an internal in-frame AUG codon of the MRV m3 mRNA. Our data confirmed this suggestion and further demonstrated that the starting codon for MRV \(\mu\)NSC synthesis is the one specifying Met-41 of MRV \(\mu\)NS, but not the one specifying Met-57. Ribosomes could initiate translation at codon 41 by either of two mechanisms: cap-independent internal entry and cap-dependent leaky scanning (Jackson, 2005; Sonenberg & Hinnebusch, 2009). Our finding that the synthesis of MRV \(\mu\)NSC is greatly increased upon mutation of the AUG closest to the 5′ end of the MRV m3 mRNA indicates that 40S ribosomal subunits start translation at codon 41 after scanning past the first AUG codon, and not by direct internal entry. At least two other reoviral proteins, MRV \(\sigma\)1S and ARV p17, have been reported to initiate translation from internal cistrons by a leaky scanning mechanism (Ernst & Shatkin, 1985; Racine et al., 2007). In both cases leaky scanning appears to be facilitated by the suboptimal context of the 5′-proximal AUG triplet of their respective mRNAs. However, the optimal context of the AUG codon closest to the 5′ end of the MRV m3 mRNA (Fig. 1a) should dramatically reduce or abolish translation initiation from downstream AUG codon 41 and hence MRV \(\mu\)NSC synthesis. There are some reported examples of leaky scanning despite the presence of a 5′-proximal AUG codon in a good Kozak-sequence context. This usually happens when the 5′ UTR is too short for the first AUG triplet to be efficiently recognized (Kozak, 1991; Ruan et al., 1994; Sedman et al., 1990; Spiropoulou & Nichol, 1993). Two observations suggest that the ability of small ribosomal subunits to scan past the optimal 5′-proximal AUG of the MRV m3 mRNA can be attributed to the small size of its 5′ UTR (18 nt; Fig. 1a); (i) synthesis of MRV \(\mu\)NSC is drastically reduced when reticulocytes are programmed with an m3 mRNA version that contains 13 extra nucleotides at its 5′ UTR (Fig. 3c, lane 1); and (ii) the optimal 5′-proximal AUG codon of the MRV m3 mRNA is leaky, whereas the one of its ARV counterpart, which contains a 6 nt-longer 5′ UTR, is not.

In contrast with the situation for MRV, the results of this study demonstrate that ARV \(\mu\)NSC originates from a single post-translational cleavage event, which occurs near the amino terminus of \(\mu\)NS. This cleavage, which occurs with ~30% efficiency, also generates a complementary N-terminal peptide of ~17 kDa. Thus, the ARV M3 gene expresses three protein isoforms, whereas only two are expressed by its MRV counterpart. Similarly to \(\mu\)NS, the ARV structural proteins \(\mu\)B and \(\sigma\)A have been shown to be partially cleaved near their amino termini to generate small amino-terminal peptides (\(\mu\)BN and \(\sigma\)AN, respectively) and larger carboxy-terminal proteins (\(\mu\)BC and \(\sigma\)AC, respectively) (Ji et al., 2010; Varela et al., 1996). Altogether, these results allow us to expand the protein repertoire known to be expressed by ARV to 18 polypeptides, 12 of which are primary translation products and six of which are non-structural proteins (Table 1).

Recent results from our laboratory indicate that ARV \(\mu\)NS cleavage is much less efficient in uninfected than in infected cells, suggesting that the protease involved is either a viral protein or a cellular protein whose activity is triggered by the viral infection. Experiments are in progress in our laboratory to identify the ARV \(\mu\)NS cleavage site and the mechanism involved in processing \(\mu\)NS.

**METHODS**

**Cells, viruses and antibodies.** Preparation of primary cultures of CEFs, and conditions for growing ARVs in CEFs and the MRV strain type 3 Dearing in monolayers of mouse L929 fibroblasts have been described previously (Grande & Benavente, 2000; Nibert & Fields, 2003, 2010). We have similarly found that sequences contained within \(\mu\)NSN are necessary for association of ARV \(\mu\)NS with several structural and non-structural proteins (unpublished data). Although not strictly required for factory formation and viral growth on cultured cells, \(\mu\)NSC proteins might be important for reovirus replication and/or spread within host organisms, as suggested by Kobayashi et al. (2009).

### Table 1. Avian reovirus genes and proteins

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1992). Rabbit polyclonal serum against ARV S1133 µNS protein was raised in our laboratory (Toursis-Otero, et al., 2004b), and rabbit polyclonal serum against MRV type 3 µNS protein was kindly provided by Dr Terence Dermody (Kobayashi, et al., 2009). Peroxidase-conjugated goat anti-rabbit antibody was purchased from Sigma.

**Viral infection, metabolic radiolabelling, cell fractionation, immunoassays and protein analysis.** Infection of cultured cells, metabolic radiolabelling, immunoprecipitation and immunoblotting were performed as previously described (Martinez-Costas et al., 1995; Toursis-Otero et al., 2004b; Varela et al., 1996). Proteins were analysed by SDS-PAGE (10% acrylamide) on discontinuous gels (Laemmli, 1970). The tricine–SDS-PAGE system described by Schägger & von Jagow (1987) was used when trying to resolve low-molecular-mass proteins. For visualization of the radioactive protein bands, the gels were dried and exposed to X-ray film (Agfa-Curix AFW).

For pulse–chase analysis, mock-infected and reovirus-infected cells were incubated for 2 h at 12 h p.i. in medium lacking methionine and cysteine, and then incubated for 10 min in the same medium supplemented with 500 µCi [35S]amino acids (methionine and cysteine) ml⁻¹. The cells were chased for the indicated times in non-radioactive medium supplemented with an excess of non-radio-labelled methionine and cysteine, and then lysed. The resulting cell extracts were immunoprecipitated with the corresponding anti-µNS antiserum and then analysed by SDS-PAGE (10% acrylamide) and autoradiography.

**Plasmid construction.** The construction of recombinant plasmids expressing full-length and amino-terminal truncations of ARV µNS have been described (Brandariz-Núñez et al., 2010). To generate a DNA insert encoding MRV µNS total RNA isolated from MRV-infected I929 cells was amplified by RT-PCR using the forward primer 5'-GCTTCCGATATGCGGCTAGTCAATCGC-3' and the reverse primer used was 5'-GCTAAAGGAAAATTGCGGCCGCTGATGAATGGGGGTCG-3'. The PCR product was digested with XhoI and NotI (Promega) and inserted into the XhoI and NotI sites of the pCINEo plasmid (Promega) to generate the recombinant plasmid pCINEo-MRVmuNS.

To express amino-terminally truncated versions of the MRV µNS protein, the insert contained within pCINEo-MRVmuNS was amplified by PCR. To generate Reo3muNS Δ40, the forward primer used was 5'-GCTTCCAGATATGCGGCTAGTCAATCGC-3' and the reverse primer used was 5'-GCTAAAGGAAAATTGCGGCCGCTGATGAATGGGGGTCG-3'. The PCR product was digested with XhoI and NotI (Promega) and inserted into the XhoI and NotI sites of the pCINEo vector. To generate amino-terminally truncated versions of the MRV µNS protein, the insert contained within pCINEo-MRVmuNS was amplified by PCR. To generate Reo3muNS Δ40, the forward primer used was 5'-GCTTCCAGATATGCGGCTAGTCAATCGC-3' and the reverse primer used was 5'-GCTAAAGGAAAATTGCGGCCGCTGATGAATGGGGGTCG-3'. The PCR product was digested with XhoI and NotI (Promega) and inserted into the XhoI and NotI sites of the pCINEo vector.

To generate templates that express MRV m3 mRNAs possessing the correct 5' and 3' termini, M3 sequences contained within the pCINEo-MRVmuNS plasmid were amplified by PCR using the forward primer 5'-TAAATACGACTCACTATAGGGATGAGCTGCTGTC-3' and the reverse primer 5'-GATGAACTGGGCTGCGGAA-3', in order to juxtapose the T7 promoter and the +1 nt of the MRV M3 gene. These primers were also used to amplify the mutated versions of the MRV M3 gene shown below. To mutate the MRV m3 AUG codons 1 (M1L), 41 (M41L) and 57 (M57L), we used a QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's specifications, by using pCINEo Reo3muNS-UTR as template. To mutate AUG codon 1 the forward primer was 5'-TGACCCGGTCTGCTCTGCCTTGCTCATGCGAAGGATTC-3' and the reverse primer was 5'-GAATCCGTTGAGTAGCGAACGACGTCAGACGTCAC-3'. To mutate AUG codon 41 the forward primer was 5'-ACTCCGTCTGATGGTCTGCAATCGGCGGTCG-3' and the reverse primer was 5'-CACCGGTATTGAGAAATCTCCACACAGACGGAGT-3'. To mutate AUG codon 57 the forward primer was 5'-AAATGAGAGGATCTCTGACTATCCATCA-3' and the reverse primer was 5'-GTGATAGTGATGAGACGACCCTTGTGCTAATT-3'. In all cases, a methionine-specifying AUG codon was replaced by a leucine-specifying UUG codon. The correctness of all recombinant plasmids was confirmed by nucleotide sequencing.

**In vitro transcription and translation.** The recombinant plasmids used as templates were linearized with NotI, purified by extraction with phenol/chloroform, precipitated with ethanol and resuspended in sterile water at a final concentration of 1 mg ml⁻¹. In vitro transcription from the T7 promoter was performed by using a RibomAX Large Scale RNA Production System (Promega), and 5'-capped RNA transcripts were synthesized by supplementing the transcription reaction mixtures with Ribo m7G Cap analogue (Promega) according to the manufacturer's specifications. The resulting RNAs were precipitated by the addition of one volume of 5 M ammonium acetate and were then washed in 70% ethanol, dried and resuspended in water. Transcription of the linearized pCINEo-MRVmuNS plasmid generated an MRV m3 mRNA version containing 13 extra nucleotides (from the pCINEo multiple cloning site) at the 5' end. In vitro translation was carried out by using a Rabbit Reticulocyte Lysate System (nuclease-treated; Promega) following the manufacturer's instructions, for 90 min at 30 °C in the presence of 50 µg RNA ml⁻¹ and 0.4 µCi [35S]methionine ml⁻¹ (Hartmann analytic KOSM-01).

**Isolation and mass spectrometric analysis of the 17 kDa ARV µNS isoform.** Approximately 10 x 10⁴ avian reovirus-infected CEF cells (10 p.f.u. per cell) were harvested at 18 h p.i. The cells were washed twice with PBS (pH 7.2) and then lysed by incubation for 30 min on ice with lysis buffer [10 mM Pipes, pH 6.8, 3 mM MgCl₂, 100 mM KCl, 300 mM sucrose, 1% Triton X-100 and protease inhibitors (Roche)]. The lysate was centrifuged at 16 500 g for 10 min at 4 °C, and the resulting supernatant was immunoprecipitated with ARV-µNS-specific antiserum. Immune complexes were resolved on a tricine–SDS-PAGE gel (10% acrylamide) (Schägger & von Jagow, 1987) and protein bands were visualized by staining with Coomassie brilliant blue G250. A slice containing the 17 kDa polypeptide band was excised from the gel, washed sequentially with 50 mM ammonium bicarbonate and acetonitrile, and then incubated for 8 h at 37 °C with 12.5 µg trypsin ml⁻¹ (Gibco) in 50 mM ammonium bicarbonate. Tryptic peptides were extracted by the addition of 70% acetonitrile containing 0.5% trifluoroacetic acid (TFA); the eluted peptides were dried by speed-vacuum centrifugation and finally resuspended in 4 µl of 30% acetonitrile and 0.1% TFA. A 0.5 µl aliquot of the mixture was added onto a matrix consisting of 0.5 mM 2,5-dihydroxybenzoic acid ml⁻¹ in water/acetonitrile (2:1) containing 0.1% TFA. Samples were measured on a 4800 MALDI-TOF-TOF Analyser (Applied Biosystems/MSD SCIEX) in positive ion reflector mode. Internal calibration of MALDI-TOF mass spectra was performed by using two trypsin autolysis ions with mass:charge ratios (m/z) of 842.510 and 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the m/z 800–3200 region. Proteins were identified by peptide mass fingerprinting and further MS/MS analysis for peptide sequencing. Following MALDI-TOF-TOF, the instrument was switched to MS/MS mode, and the five strongest peptides from the MS scan were isolated and fragmented by collision-induced dissociation with air. The ion acceleration voltage was 20 kV. The MALDI-MS data obtained were processed further by using the GPS Explorer software (Applied Biosystems) and using MASCOT to search against the Swiss-Prot database.
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