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Relevance of the N-terminal and major hydrophobic domains of non-structural protein 3A in the replicative process of a DNA-launched foot-and-mouth disease virus replicon

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Abstract

A foot-and-mouth disease virus (FMDV) DNA-launched reporter replicon containing a luciferase gene was used to assess the impact of non-structural (NS) protein 3A on viral replication. Independent deletions within the N-terminal region (amino acid [aa] residues 6 to 24) and the central hydrophobic region (HR, aa 59 to 76) of FMDV NS protein 3A were engineered, and luciferase activity in lysates of control and mutated replicon-transfected cells was measured. Triple alanine replacements of the N-terminal triplet Arg 18- His 19 -Glu 20 and a single alanine substitution of the highly charged Glu 20 residue both resulted in a 70-80% reduction in luciferase activity when compared with wild-type controls. Alanine substitution of the 17 aa present in the central HR, on the other hand, resulted in complete inhibition of luciferase activity and in the accumulation of the mutated 3A within the cell nucleus according to immunofluorescence analysis. Our results suggest that both the aa sequence around the putatively exposed hydrophilic E20 residue at the N-terminus of the protein and the hydrophobic tract located between aa 59 and 76 are of major relevance for maintaining the functionality of the 3A protein and preventing its mislocalization into the cell nucleus.

Introduction

Foot-and-mouth disease (FMD) is a vesicular disease that is highly contagious, disseminates fast, and causes severe economic losses in endemic areas. Its reintroduction poses a threat to disease free-countries. The etiological agent is foot-and-mouth disease virus (FMDV), which belongs to the genus *Aphthovirus* of the family *Picornaviridae*. FMDV is a small non-enveloped virus with an icosahedral capsid. The FMDV particle encloses a genome of single-stranded positive-sense RNA of about 8500 nucleotides, which includes an open reading frame coding for four structural proteins (VP1-VP4) and several non-structural (NS) proteins (two forms of L [Lab and Lb], 2A, 2B, 2C, 3A, and three different forms of 3B, 3C and 3D) flanked by non-coding regions (UTR) at the 3´ and 5´ ends. The UTRs are directly involved in replication of the genomic viral RNA [1–3].

The NS protein 3A plays important roles in virus replication, virulence and host range [4, 5]. In a previous study, a single amino acid (aa) replacement (Q44R) in this protein conferred on FMDV the ability to cause vesicular lesions in guinea pigs [6]. Deletions and substitutions in the C-terminal region are associated with attenuation of the virus in cattle [4, 7] and a decrease in its replication rate [8]. This protein contains a hydrophobic region (HR) of 18 aa (positions 59-76) that is predicted to be a transmembrane region [2, 9, 10]. A previous study showed that, in cells transiently expressing FMDV 3A, about 50% of the cellular pool of the protein was recovered from the membrane fraction, suggesting an association of 3A with cellular membranes [9]. Additionally, it has been shown that FMDV 3A, unlike the corresponding protein of poliovirus (PV), is not responsible for blocking the endoplasmic reticulum (ER)-to-Golgi transport of proteins, and this function is instead carried out by the 2B and 2BC proteins [9]. The FMDV 3A protein is partially co-localized with ER and Golgi markers

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[11, 12] and recent evidence points to the involvement of the ER in virus replication [13]. Recently, Gonzalez-Magaldi et al. suggested that, topologically, FMDV 3A would correspond to a non-integral membrane protein [14]. However, further studies are necessary to assess the cellular location of this protein and its contribution to viral replication and host range.

Concerning to the role of 3A in viral replication, Rosas et al. [15] reported an increase in viral replication, plaque formation and viral titers in FMDV-infected BHK cells constitutively expressing the 3A, 3AB or 3ABC protein.

FMDV 3A is the viral protein with the lowest number of conserved residues in its aa sequence (37%), and its variability is comparable to that of the capsid proteins VP2, VP3 and VP4, suggesting that it is subjected to high selection pressure [16]. However, both the hydrophobic and hydrophilic domains, located at the N-terminal end of the 3A protein, are highly conserved in most FMDV strains [17, 18], and the majority of substitutions and deletions map to the C-terminal region in all variants (reviewed in reference 19).

The availability in our lab of a DNA-launched reporter replicon that does not generate virions and has a firefly luciferase (Luc) gene incorporated into the genomic background of FMDV allows the evaluation of changes in the coding or non-coding regions in a context similar to a viral infection, without the risks associated with live FMDV production [20]. RNA-based replicons driven by the T7 and SP6 prokaryotic promoters have been developed for members of the family *Picornaviridae*. These systems require *in vitro* transcription and subsequent transfection of cells with the synthesized RNAs [21]. We used a DNA-launched replicon to avoid the need for *in vitro* transcription and purification of RNA, which is usually needed with long RNA fragments to prevent initial non-replication-dependent reporter translation [21]. This system consists of an FMDV cDNA clone (pRep) derived from the genomic RNA of FMDV O1Campos strain in which the sequences coding for the structural proteins were replaced by the Luc reporter gene. Thus, the effect of site-directed mutagenesis on viral RNA replication can be assessed by measuring the levels of Luc activity found after transfection with each replicon.

Following this experimental approach, we have identified two specific 3A protein sequences, one mapping around a glutamic acid residue at position 20 at the N-terminus and the other within the central hydrophobic region, that have a major impact on the transcription and RNA replication performance of the FMDV replicon.

Materials and methods

Plasmids

A replicon plasmid (pK-VFA/O1C) derived from the FMDV strain O1/Campos was generated from viral RNA provided by the National Animal Health Service, SENASA, and used as a reverse genetic system. It was constructed on the background of plasmid pKF4 [22], adding a T7 promoter preceding a full-length cDNA copy of the viral genome (8267 nucleotides), followed by a unique site for the restriction enzyme *Eco*RV, which was used for linearization of the plasmid. The complete region coding for the structural proteins (VP1-4) was subsequently replaced in pK-VFA/O1C by the firefly luciferase coding gene (Luc) by overlap extension PCR (OE-PCR) [23], through two steps of overlapping with three segments: Lpro (amplified with the primers AflII.Fw and VP0.Luc.Rev), Luc (Luc.Fw and Luc.Rev), and 2ABC3A (2A.Fw and 2C3A.Rev) (Fig. 1C). The resulting plasmid, designed (pRep), has a complete L_{pro} and 6 aa from VP0 including the L_{pro} cleavage site, followed by the Luc gene, the intact cleavage site for $3C_{pro}$ between VP1 and 2A (last 6 aa of VP1), the rest of the genomic copy, a poly(A) tract of 20 nucleotides, and the *Eco*RV restriction site (Fig. 1, panel A). At the 5' end, the UTR possesses a poly(C) sequence composed of 20 nucleotides.

A defective pRep (pRepD) was obtained by introducing a deletion of the G at position 4120 of pRep, shifting the reading frame at the beginning of the 2B the coding region. The resulting replicon clone was dysfunctional and was used as a control for RNA transcription without RNA replication (Fig. 1, panel D).

FMDV 3A protein mutants

Three pRep mutants with serial deletions in the N-terminal region of 3A (pRep3AΔ6-11, pRep3AΔ6-17 and pRep3AΔ6-24) and the point mutants pRep3AQ18A/H19A/E20A and pRep3AE20A were constructed by amplification with primers carrying the correspondent mutation (Table 1) and further replacement of the amplified fragment in pRep. A further description is presented in Figure 1 (panel C).

In the mutant pRep3AΔHR, the hydrophobic region (aa 59–76) was replaced by eight alanines, using the overlapping PCR technique [23], with the primers 3A.Fw and ΔHR.Rev for the first segment and ΔHR.Fw and 3D.Rev for the second fragment (Table 1). The resulting overlapping PCR fragment was cloned in pRep between the *Eco*RI and *Mlu*I restriction sites (Figure 1C).
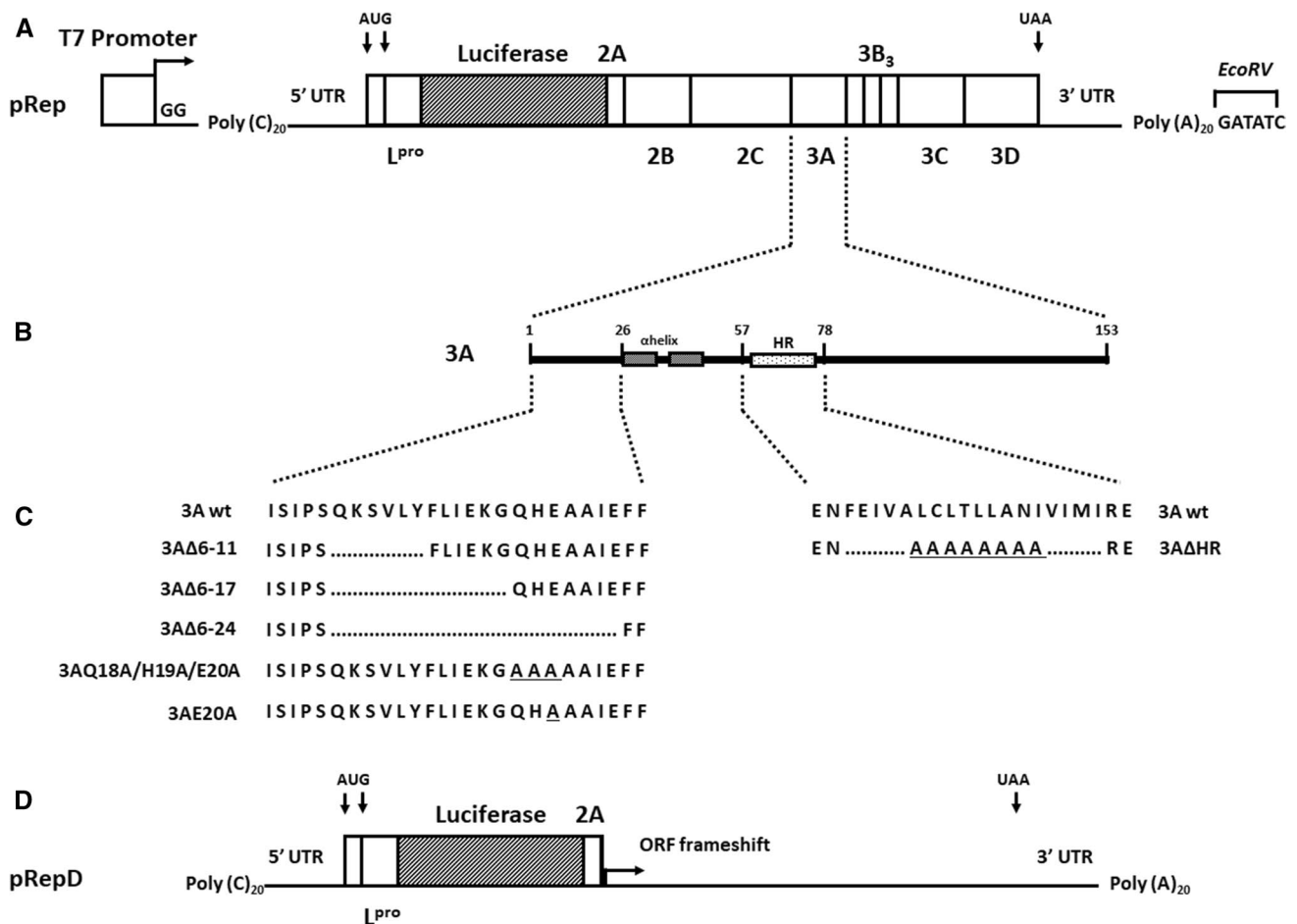
Fig. 1 Mutations introduced in a replicon plasmid carrying sequences of foot-and-mouth disease virus. (A) Partial schematic representation of the replicon plasmid pRep. Plasmid pRep contains a full-length cDNA copy of the O1 Campos strain of FMDV, where the capsid proteins have been replaced by a firefly luciferase reporter gene. The nonstructural viral proteins, T7 polymerase promoter, and 5' and 3' untranslated regions (UTRs) are indicated. (B) Schematic representation of FMDV non-structural protein 3A. The two alpha-helix structures (α-helix) and the hydrophobic region (HR) are indicated. (C) Engineered mutations. 3AΔ6-11, 3AΔ6-17 and 3AΔ6-24 are serial deletions of the N-terminus of 3A. 3AQ18A/H19A/E20A and 3AE20A are substitution mutants in which amino acids have been changed to alanine (underlined). 3AΔHR indicates the deletion of the HR, where amino acids 59 to 76 were replaced by eight alanines. (D) Partial schematic representation of the RNA derived from the replicon plasmid pRepD

FLAG-tagged plasmids

For immunofluorescence staining, plasmids containing the sequence of the 3A wt protein or the 3A mutants Δ6-24 and 3AΔHR were fused to the FLAG tag using PCR with specific primers (3A.Flag.Fw and 3A.Rev) and then cloned into the pEGFP-N1 plasmid backbone (Clontech Laboratories), deleting the EGFP gene sequence. The plasmids were designed as p3AF, p3AΔ6-24F and p3AΔHRF, respectively.

All plasmids used in this work were constructed using standard methods and confirmed by nucleotide sequencing (Macrogen, Korea). Restriction enzymes were purchased from Promega (USA), New England Biolabs (Hitchin, UK) and Thermo Fisher Scientific Inc. (USA). Oligonucleotides primers were obtained from IDT Technologies (USA), and

PCR amplification was performed with Pfx50 Taq polymerase (Thermo Fisher Scientific Inc., USA)

Luciferase reporter activity assay

Baby hamster kidney cells (BHK-21; ATCC CCL-10) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 7% fetal bovine serum (New Zealand Sourced, HyClone), 1 mM glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), 10 mM HEPES and 0.1 mM non-essential amino acids at 37 °C, 5% CO_2. Briefly, 70–80% confluent cell monolayers in 24-well plates were co-transfected using Lipofectamine-2000 (Thermo Fisher Scientific Inc., USA), following the manufacturer's instructions. The amount of DNA transfected was as follows: 0.5 µg of pRep

or each pRep mutant, 0.0025 µg of pTM1-Renilla and 0.05 µg of pCA-T7 [24], which provides the T7 polymerase.

Cell lysates were collected after 16 h, and luciferase activity was measured using a "Dual-Luciferase® Reporter Assay System" kit (Promega, USA), following the manufacturer's protocol, and read in a BioTek FLX800 luminometer. To normalize for differences in transfection efficiency, all experiments were performed using the plasmid pTM1-Renilla [25], which expresses *Renilla* luciferase transcribed from a T7 polymerase promoter. The normalized luciferase activity of pRep was considered 100%.

Multiplex RT-PCR

BHK-21 cells transfected as described above were pelleted at 3000 rpm, and total RNA was extracted using an SV Total RNA Isolation System® kit (Promega, USA) following the manufacturer's protocol. Purified DNAse-treated RNA was quantified in a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). Reverse transcription was performed using 1 µg of total purified RNA and 0.5 µg of random primers with the GoScript™ Reverse Transcription System (Promega, USA) under standard conditions. Two sets of primers were designed for multiplex PCR: for the detection of FMDV positive-stranded RNA, primers p1 (5' TGGCAATGTTTCAATACGACTGT 3' and p2: 5' CTCGACCCGATCAATCACCT 3'), which amplify a 110-bp fragment; for the detection of FMDV negative-stranded RNA, primers n1 (5' GAAGATCTGCCACCA TGATCTCAATTCCTTCCCA 3' and n2 5'GACGGATCC TCAGCTTGTGGTGTCTCCTCAACAG 3', amplifying a 491-bp fragment. The multiplex PCR was performed using a GoTaq® DNA Polymerase kit (Promega, USA), with a standard 30-cycle protocol and an annealing temperature of 60 °C. The amplified fragments were resolved by 2% agarose gel electrophoresis and stained with ethidium bromide. The amplified bands were observed and documented in a BioDoc-It™ UV System (UVP, USA).

Analysis of the degradation of eIF4G by Western blotting

Lysates from BHK-21 cells transfected as described above were resolved by two-step SDS-PAGE (8% and 12%), transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare), and blocked for 16 h at 4 °C in 5% PBS-T0.1 (PBS and 0.1% Tween 20, supplemented with 5% non-fat milk). The membrane was probed with rabbit anti-eIF4G (Cell Signaling, 1:1000) or rabbit anti-actin antibody (1:700. Sigma Chemical Company) in PBS-T 0.1 supplemented with 3% non-fat milk for 1 h at room temperature (RT). The membrane was then washed three times in PBS-T 0.1 and probed with peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, 1:5000) in 3% PBS-T0.1 for 1 h at RT. Membranes were washed three times in PBS-T0.1, and the antibody binding was detected by chemiluminescence assay using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific Inc.) in a G:Box equipment Chemi XRQ gel documentation system (Syngene).

For the evaluation of relative degradation of eIF4G, the values from the areas calculated from the band corresponding to cleaved eIF4G were normalized with respect to the amount of actin in each reaction, using the ImageJ program. The percentage of degradation eIF4G by pRep was taken to be 100%.

Immunofluorescence staining

BHK-21 cells grown on glass coverslips were transfected with plasmids p3AF, p3AΔ6-24F and p3AΔHRF, which contain a FLAG tag. At 16 h post-transfection, the cells were fixed in 3.7% formaldehyde for 15 min at RT, treated with 1 M glycine in PBS for 5 min, and permeabilized with PBS containing 0.2% Triton X-100 (PBS-TX) for 15 min, and blocked with 3% BSA in PBS-TX (PBS-TX-BSA) for 15 min. Coverslips were incubated with the monoclonal antibody Anti-FLAG®M2 (Sigma-Aldrich) diluted in PBS-TX-BSA for 1 h at RT, washed three times with PBS-T-BSA, and then incubated with anti-mouse Alexa Fluor® 546 (Thermo Fisher Scientific Inc.) for 1 h. Cell nuclei were stained with DAPI (4',6-diamino-2-fenilindol, 1 µg/ml) (Thermo Fisher Scientific Inc.) for 15 min. After the final washes with PBS-TX, coverslips were mounted in FluorSave™ Reagent (Merck Life Science), and the cells were observed under a fluorescence microscope (Eclipse E600, Nikon).

Statistical analysis

Statistical processing of data was performed using Prism 5® software (v5.03. Graph Pad Software, Inc.).

Results and discussion

We set out to study the impact of specific deletions and single amino acid substitutions within the 3A NS protein sequence on the replicative capacity of the FMDV genome. Our work was focused on the mutation of the R18H19E20 sequence at the N-terminal region and of a central hydrophobic stretch of 17 aa residues that had been proposed to be a transmembrane domain of the 3A protein.

The luciferase activity of the FMDV pRep replicon in transfected cells is dependent on the RNA replication carried out by the viral 3D polymerase encoded within the construct (Fig. 1A). The luciferase activity after transfection with pRep was 15-fold higher than that obtained by transfection

with the replication-defective pRepD, which has a frameshift downstream from the Luc gene and is only able to provide T7 polymerase-derived transcripts (Fig. 2A). These results indicate that the increase of Luc activity correlates with the presence of *de novo*-synthesized FMDV RNA generated by polymerase-3D-dependent replication and is not the consequence of early T7-polymerase-dependent transcription of the Luc gene in replicon-pRep-transfected cells.

Impact of the mutations in the 3A N-terminal and hydrophobic domain regions in the replicon system

BHK-21 cells transfected with replicons coding for the deletion mutants pRep3AΔ6-11 and pRep3AΔ6-17 showed Luc activity similar to that of pRep ($p = 0.77$ and $p = 0.33$; 101.91% and 93.65% activity, respectively, Fig. 2 B). This suggests that the region from aa 6 to 17 of 3A is not essential for replicon functionality. In contrast, pRep3AΔ6-24 showed a significant decrease in Luc activity ($p < 0.0001$; 30.69% activity), indicating that aa 18 to 23 in 3A may be critical for RNA replication (Fig. 2A and B).

Based on these results and in order to identify specific amino acids within the 3A sequence that are important for replicon activity, two independent mutants were generated by alanine (A) replacement of hydrophilic amino acids between positions 18 and 23 of the primary sequence. In the first mutant, pRep3AQ18A/H19A/E20A, alanine replaced the triplet QHE at positions 18, 19 and 20, respectively, while in the second mutant, pRep3AE20A, alanine replaced the highly charged glutamic acid at position 20 (E20) (Fig. 1). BHK-21 cells transfected with pRep3AQ18A/H19A/E20A or pRep3AE20A mutants showed a significant reduction in Luc activity (30.08% and 26.69%, respectively, with respect to the wt pRep, which represents the 100% control for Luc activity). This decrease in reporter activity was similar to that observed with mutant pRep3AΔ6-24, in which the N-terminal sequence (aa 6 to 24) has been deleted (Fig. 2A and B). This suggests that the conserved amino acids at positions 18, 19 and 20 [17] play a critical role in the replication of the analogous FMDV RNA transcripts, and possibly in FMDV replication. It is also worth noting that the single alanine replacement of residue E20 led to a drop in Luc activity that was similar to the one caused by the triple alanine replacement (Fig. 2). This suggests a major role for residue E20 within this highly hydrophilic domain at the N-terminal end of 3A, which is likely to be exposed in the native protein structure.

Regarding the impact of the central HR on replicon activity, we found that the mutant pRep3AΔHR (where positions 59 to 76 were substituted with eight alanines to disrupt the HR structure, Fig. 1C) exhibited strongly reduced Luc activity (5.18% of the control, $p < 0.0001$) when used to transfect BHK cells under the same conditions, suggesting a major role of the 3A HR sequence in replication.
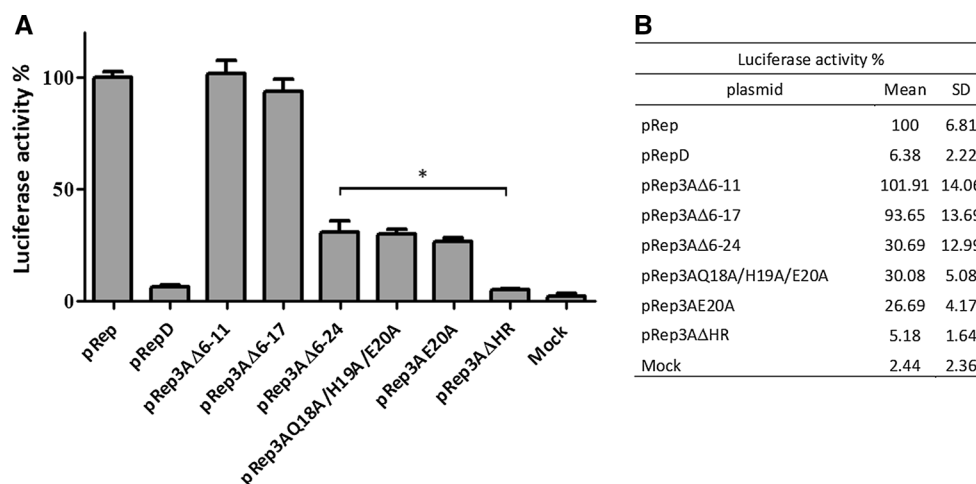

Luciferase activity %		
plasmid	Mean	SD
pRep	100	6.81
pRepD	6.38	2.22
pRep3AΔ6-11	101.91	14.06
pRep3AΔ6-17	93.65	13.69
pRep3AΔ6-24	30.69	12.99
pRep3AQ18A/H19A/E20A	30.08	5.08
pRep3AE20A	26.69	4.17
pRep3AΔHR	5.18	1.64
Mock	2.44	2.36

Fig. 2 Luciferase (Luc) activity reporter assay. (A) Analysis of the percent difference in Luc enzymatic activity after transfection of BHK-21 cells with the FMDV replicons carrying wt 3A and its mutants, using a "Dual-Luciferase® Reporter Assay System" kit (Promega). The plasmid pTM1-Renilla was included in each transfection for normalization. pRep, wt replicon; pRepD, dysfunctional replicon. The mutants are indicated on the *x*-axis. Values are obtained from three independent experiments, in which each condition was tested in duplicate. Values on the *y*-axis represent the mean and SD, expressed as % Luc activity. The asterisk indicates a significant difference with respect to the wt replicon pRep, analyzed by the Student *t*-test, at a 99% confidence level. *, $p < 0.0001$. (B) Luc activity values obtained for the constructions shown in panel A

Detection of positive- and negative-stranded replicon RNA in transfected cells

The presence of positive- and negative-stranded viral RNAs was analyzed using multiplex RT-PCR. Purified RNA from BHK-21 cells transfected with either pRep or its mutants was amplified as described in Materials and methods with specific primers to detect positive- and negative-stranded viral RNA based on the generation of 110- and 491-bp amplicons, respectively. Negative-stranded viral RNA was detected in pRep-transfected cells (491-bp band, Fig. 3, lane 2,) indicating that wt viral NS proteins were able to carry out replication of viral RNA encoded in the replicon. The presence of a 110-bp band was also detected (Fig. 3, lane 2). The absence of these bands in the mock control (Fig. 3, lane 10) confirms the specificity of this assay for detecting pRep-derived RNAs. Cells transfected with the defective replicon pRepD (Fig. 3, lane 3) did not yield the 491-bp band that would indicate the presence of the negative-stranded viral RNA, demonstrating the inability of this mutant to replicate viral RNA due to the frameshift mutation. Moreover, the 110-bp band obtained from pRepD transfection was much fainter than the one obtained from pRep-transfected cells (Fig. 3, lanes 2 and 3). In this case, the faint 110-bp band was derived from the early positive-stranded RNA transcribed by the T7 polymerase present in the replicon system. These results, although not quantitative, are consistent with the background level of Luc activity shown by pRepD when compared with that of pRep (Fig. 2B).

Deletion mutants pRep3AΔ6-11 and pRep3AΔ6-17 showed the same pattern as pRep (Fig. 3, lanes 4 and 5), indicating the completion of a full replication cycle, which correlates with the relative Luc activity found after
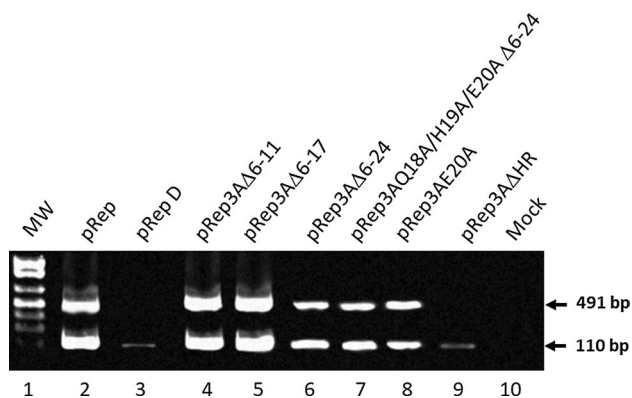
transfection (Fig. 2B). In mutants pRep3AΔ6-24, pRep3AQ18A/H19A/E20A, and pRep3AE20A (Fig. 3, lanes 6 to 8), both RNA species (positive and negative) were detected at lower intensity, indicating their diminished replication capability, which also correlates with the relative Luc activity (Fig. 2B). The HR mutant pRep3AΔHR (Fig. 3, lane 9) showed only the presence of positive-stranded RNA (110-bp band). The absence of negative-stranded RNA indicated that there was no replication activity, which correlated with the significantly lower relative Luc activity (Fig. 2B).

This experiment showed the existence of a correlation between full-cycle replication of replicons and Luc activity.

Analysis of the relative amount of degradation of the host cell eukaryotic initiation factor 4G

It is known that the FMDV L_{pro} protein specifically cleaves the eukaryotic initiation factor 4G (eIF4G) [26], and this event leads to the inability of the host cell to translate capped cellular mRNAs (host protein synthesis shutoff). The uncapped picornaviral RNA template meanwhile remains functional for protein synthesis because it is translated in an eIF4G-independent manner from an internal ribosomal entry site (IRES) located in the 5' UTR [27–31]. Cleavage of host factor eIF4G is therefore directly linked to the presence of the viral protein L_{pro} and thus constitutes an indirect parameter of *de novo* viral RNA transcription-translation activity in our system. To further study the impact of different mutations on pRep replication, lysates of BHK-21 cells transfected with either pRep or its mutants were analyzed by SDS-PAGE followed by immunoblotting with an anti eIF4G antibody (Fig. 4). The results show that this antibody recognizes a heterogeneous band of 120 kDa (eIF4Gcl) corresponding to cleaved forms of the eIF4G factor. This cleavage was readily detected in lysates from cells transfected with wt pRep (Fig. 4, lane 1), but not from either pRepD- or mock-transfected cells (lanes 2 and 9, respectively). Values of 100% and 0% were assigned to the density of eIF4Gcl bands present in pRep and pRepD lysates (Fig. 4, lanes 1 and 2, respectively, Table B) in order to estimate the relative proteolysis activity induced by the different mutant constructs. The percentages shown in the Table in Figure 4B represent the relative amounts of eIF4Gcl detected in the corresponding lysates. Values were normalized in all cases based on densitometry scanning of actin bands in the corresponding lanes.

The analysis shows that transfection of BHK cells with replicons coding for protein deletion mutant 3AΔ6-24, the triple mutant 3AQ18A/H19A/E20A, or the mutant pRep3AE20A resulted in a significant decrease in IF4G processing (6, 15 and 22%) when compared to cells transfected with pRep control expressing 3A wt (Fig. 4, lanes 5 to 7). Deletion of the putative hydrophobic transmembrane domain
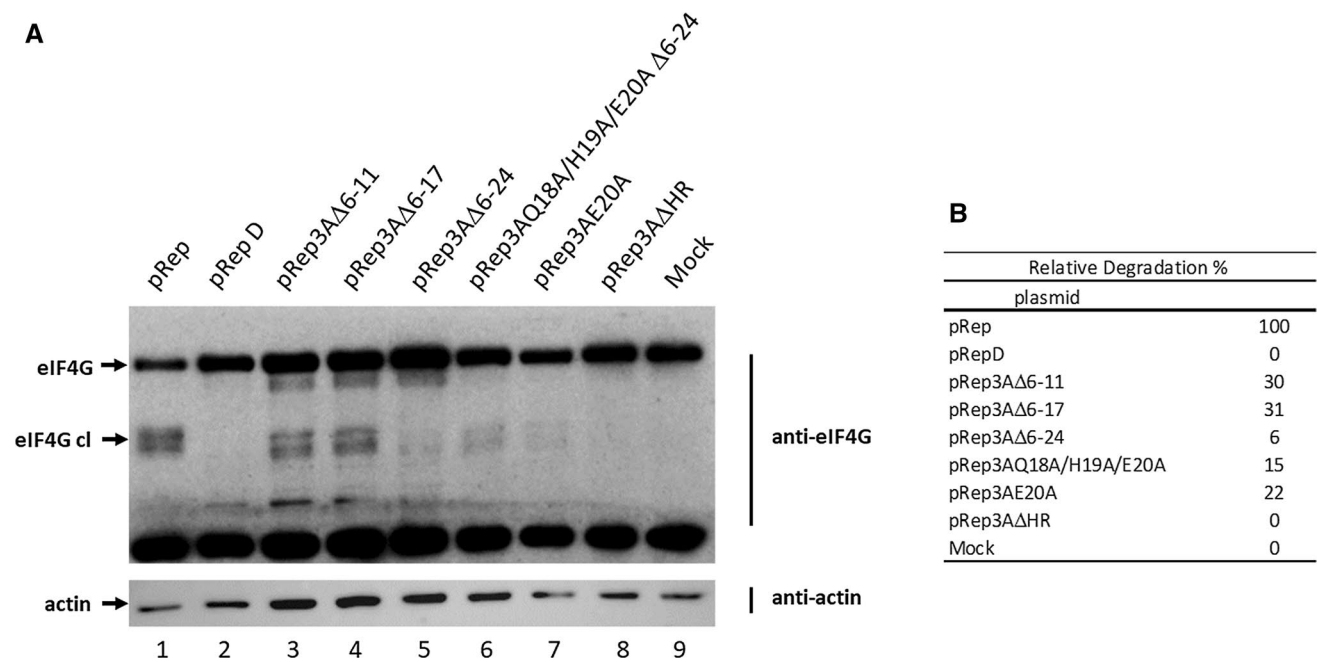

Fig. 3 Detection of positive- and negative-stranded replicon RNA. Ethidium-bromide-stained 2% agarose gel electrophoresis of multiplex RT-PCR of purified RNA from BHK-21 cells transfected with the different replicons carrying wt and mutated 3A. MW: molecular weight marker, 100 bp DNA Ladder® (Thermo Fisher Scientific Inc., USA). Arrows show the positions of the 491- and 110-bp bands

A

B

Relative Degradation %	
plasmid	
pRep	100
pRepD	0
pRep3AΔ6-11	30
pRep3AΔ6-17	31
pRep3AΔ6-24	6
pRep3AQ18A/H19A/E20A	15
pRep3AE20A	22
pRep3AΔHR	0
Mock	0

Fig. 4 Impact of the 3A mutations on the cleavage of the host cell factor eIF4G. (A) Extracts of BHK-21 cells transfected with the different replicons carrying wt and mutated 3A proteins were separated by 8%-12% SDS-PAGE and analyzed by Western blotting using anti-eIF4G and anti-actin antibodies. (B) Normalized relative degradation (%)

in the 3A sequence (HR) on the other hand, led to a complete inability to process the intracellular eIF4G (Fig. 4, lane 8). Deletion of aa 6 to 11 and 6 to 23 in the N-terminal portion of 3A seems to have a significant but relatively mild impact in L_{pro}-induced cleavage activity (reduction of 30% with respect to the control).

These results show an observable correlation with those obtained in the Luc activity assay (Fig. 2), indicating that both tests are reliable indicators of the transcription/translation activity and are sensitive enough to detect changes associated with 3A modifications. We conclude that both the aa triplet 18-20 (with emphasis on E20) and the HR domain of 3A have major relevance for the transcription/translation capabilities of the replicon.

Subcellular distribution of 3A proteins carrying mutations in the N-terminal or hydrophobic domain

The results described above suggest that the integrity of wild-type 3A is essential for a functional pRep replicon, and most likely for FMDV viral activity. Previous reports have linked the function of 3A to its capacity to associate with cellular structures, either membranous or non-membranous components, present in the perinuclear region of FMDV-infected cells [11, 12, 32]. In order to further explore on the role of 3A during virus infection, we studied the relevance of N-terminal and HR domain modifications on the subcellular location of this protein. Protein 3A wt and the mutants

3AΔ6-24 and 3AΔHR fused to a FLAG tag were independently and transiently expressed in BHK-21 cells, and the immunofluorescence profile was analyzed as described in Materials and methods.

As shown in Figure 5, a spotted pattern was observed in cells expressing 3A wt, which is compatible with the formation of small cytoplasmic vesicles reported previously in either FMDV type O infections [2, 9] and in cultured cells transiently expressing 3A [11, 12]. In contrast, cells expressing the 3AΔ6-24 mutant had the label dispersed throughout the cellular cytoplasm (Fig. 5).

Recently published studies have shown that 3A interacts with the DCTN3 protein (dynactin 3), a subunit of the dynactin complex that acts as a cofactor for dynein and is the motor base of microtubules, as well as with KTN1-AD (kinectin 1), a receptor for kinesin that is involved in kinesin-driven vesicle motility [33]. The interaction of 3A with both DCNT3 and KTN1 seems to be restricted to aa 15-21 and 64-77 [7]. It can thus be speculated that the loss of the particulate pattern and consequent cytoplasm dispersion shown by 3A lacking the N-terminal aa 6-24 sequence could be related to its inability to interact with the microtubule network of the cell.

Interestingly, transient expression of the protein lacking the HR domain resulted in most of the fluorescent signal being concentrated within the nucleus (Fig. 5). This suggests that the HR domain could be of major importance for anchoring 3A to either membranous or non-membranous

Fig. 5 Intracellular localization of nonstructural protein 3A wt and mutants. BHK-21 cells transiently transfected with FLAG-tagged plasmids expressing 3AF wt, 3AΔ6-24F, 3AΔHRF or mock transfected were fixed and stained with the monoclonal antibody Anti-FLAG®M2 (Sigma-Aldrich) and anti-mouse Alexa Fluor® 546 (Thermo Fisher Scientific Inc., USA) and observed under a fluorescence microscope (Eclipse E600, Nikon). While cells transfected with the wt replicon show a typical punctate pattern, the mutant with a deletion of aa 6-24 of 3A shows diffuse staining throughout the cytoplasm. In the absence of the HR region, the specific label for the 3A protein is concentrated in the cell nucleus. Cell nuclei were stained with DAPI
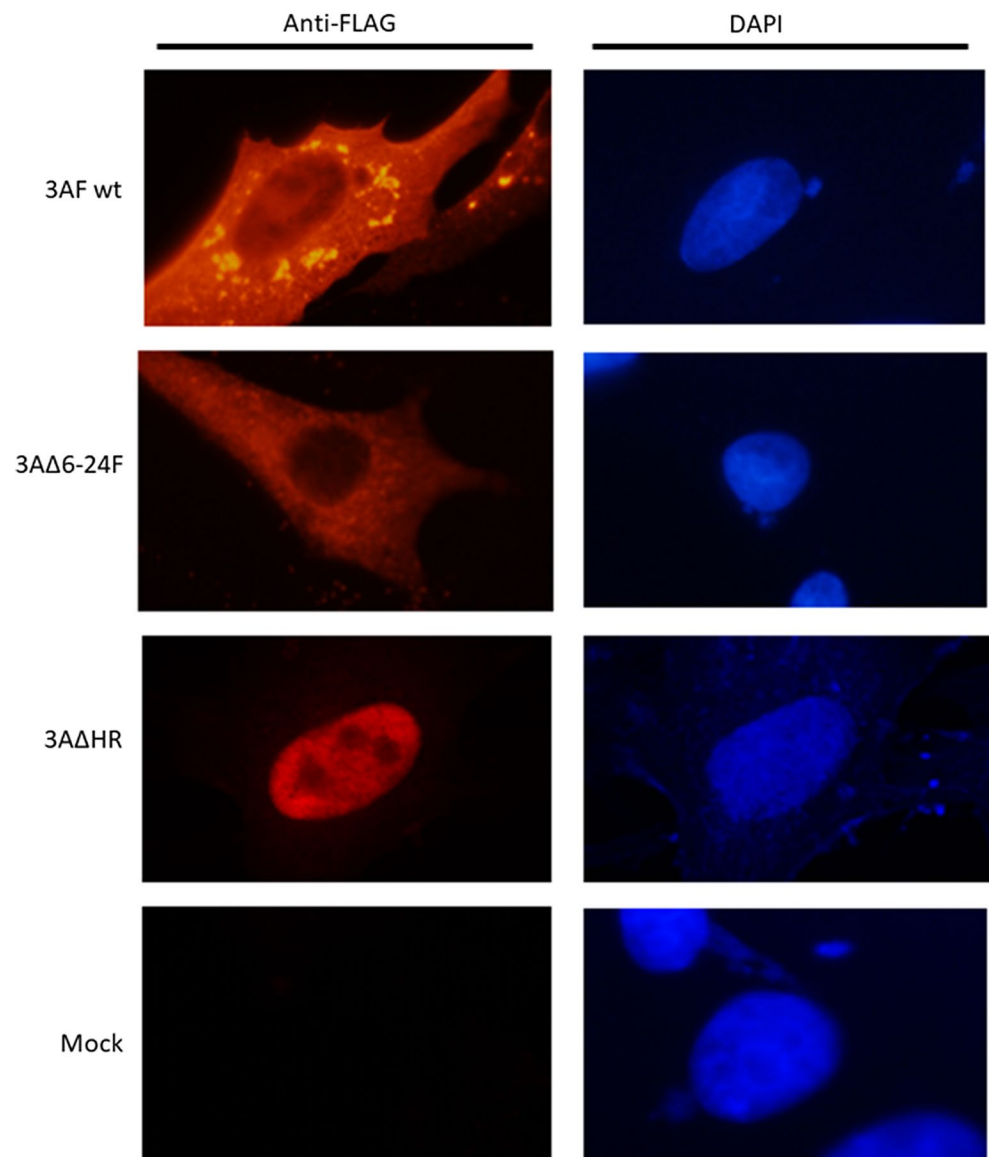
cytoplasmic structures. It also suggests that in the absence of the HR domain, putative nuclear localization sequences (NLS) present in 3A could become relevant for driving the protein into the cell nucleus.

The classical NLS is generally composed of a short stretch of basic amino acids (generally 5 aa) or by two short stretches (less than 5 aa) separated by a spacer region of approximately 10 aa (bipartite NLS). This signal must be exposed in the correct conformation to be accessible to the cell transport machinery [34, 35]. We were able to identify two sequences similar to that described by Do et al. [35] in the FMDV 3A protein. These motifs comprise the regions between aa 49 and 56 (**KR**AF**KR**L**K**) and between aa 77 and 84 (**R**ET**RKR**Q**K**), which contain five basic residues each (highlighted in bold), which are brought closer together when the HR domain is replaced by a stretch of eight alanine

residues. The results suggest that accessibility to these NLS sequences becomes possible due to the engineered changes in the putative transmembrane region of 3A, and it is possible that the HR deletion removes conformational constrains that prevent NLS recognition and accumulation of wt 3A in the cell nucleus.

Recently, an evaluation of a series of nine deletions in the dimerization and transmembrane domains of 3A in an infectious clone of the FMDV strain Asia1IND491/1997 was reported [36]. One of the nine deletions comprised almost the entire 3A sequence (aa 5 to 149), four were limited to the C-terminal half downstream of the putative transmembrane domain (aa 86-110, 101-149, 81-149 and 82-153), and the remaining four were designed to individually or jointly affect the transmembrane and dimerization domains at the N-terminus (aa 5-54, 21-50, 21-80 and 55-80). None of the

N-terminal deletions were viable, whereas the four C-terminal deletions yielded infectious viruses in cell cultures, confirming the relevance of the N-terminal region and showing the dispensability of some regions of the carboxy-terminal end of 3A [36].

Our results suggest that the NS protein 3A plays a major role in the replicative process of FMDV and that two well-defined regions, one mapped to and around the E20 residue and the other located within the hydrophobic stretch between residues 59 and 76 (HR), are essential for the function of this protein, while the HR region may be involved in the subcellular localization of the protein.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.
This article does not contain any studies with human participants or animals performed by any of the authors.

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