Virology 380 (2008) 34–45

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Susceptibility to viral infection is enhanced by stable expression of 3A or 3AB proteins from foot-and-mouth disease virus ☆

María F. Rosas [a], Yuri A. Vieira [a], Raúl Postigo [a], Miguel A. Martín-Acebes [a], Rosario Armas-Portela [a,b,1], Encarnación Martínez-Salas [a], Francisco Sobrino [a,c,*]

[a] Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Madrid, Spain
[b] Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain
[c] Centro de Investigación en Sanidad Animal, INIA, Valdeolmos, Madrid, Spain

ARTICLE INFO

Article history:
Received 15 April 2008
Returned to author for revision 5 May 2008
Accepted 28 June 2008
Available online 9 August 2008

Keywords:
FMDV non-structural proteins
Stable and transient expression
Effect on cells
Virus growth modulation

ABSTRACT

The foot-and-mouth disease virus (FMDV) 3A protein is involved in virulence and host range. A distinguishing feature of FMDV 3B among picornaviruses is that three non-identical copies are encoded in the viral RNA and required for optimal replication in cell culture. Here, we have studied the involvement of the 3AB region on viral infection using constitutive and transient expression systems. BHK-21 stably transformed clones expressed low levels of FMDV 3A or 3A(B) proteins in the cell cytoplasm. Transformed cells stably expressing these proteins did not exhibit inner cellular rearrangements detectable by electron microscope analysis. Upon FMDV infection, clones expressing either 3A alone or 3A(B) proteins showed a significant increase in the percentage of infected cells, the number of plaque forming units and the virus yield. The 3A-enhancing effect was specific for FMDV as no increase in viral multiplication was observed in transformed clones infected with another picornavirus, encephalomyocarditis virus, or the negative-strand RNA virus vesicular stomatitis virus. A potential role of 3A protein in viral RNA translation was discarded by the lack of effect on FMDV IRES-dependent translation. Increased viral susceptibility was not caused by a released factor; neither the supernatant of transformed clones nor the addition of purified 3A protein to the infection medium was responsible for this effect. Unlike stable expression, high levels of 3A or 3A(B) protein transient expression led to unspecific inhibition of viral infection. Therefore, the effect observed on viral yield, which inversely correlated with the intracellular levels of 3A protein, suggests a transacting role operating on the FMDV multiplication cycle.

Introduction

Foot-and-mouth disease virus (FMDV) is the prototypic member of the aphthovirus genus within the family Picornaviridae (Bachrach, 1977; Domingo et al., 1990; Pereira, 1981; Sobrino et al., 2001) and the etiological agent of a devastating disease of livestock (Knowles et al., 2001). The viral particle is composed by a protein capsid that contains a positive-sense RNA molecule of about 8500 nucleotides that is infectious and encodes a single polyprotein, processed in infected cells to yield different polypeptide precursors and the mature viral proteins (Belsham, 2005; Sobrino et al., 2001). Polyprotein processing is carried out by *cis* and *trans*-acting viral proteases rendering the mature structural and nonstructural (NS) proteins (Ryan and Drew, 1994). The proteins encoded within the P1 region (VP1-4) form the capsid, while L protein and those encoded in the P2 (2B,2C) and P3 (3A, 3B, 3C and 3D) regions are NS proteins (Belsham and Martínez-Salas, 2004).

Picornavirus NS proteins, and some of their precursors, are involved in crucial aspects of viral cycle, such as RNA replication, rearrangements of intracellular membranes required for this process, pathogenesis and lysis of host cells (Andino et al., 1999; Bienz et al., 1983; Buenz and Howe, 2006; Cho et al., 1994; Choe et al. 2005; Whitton et al., 2005). Protein 3A is an example of this multifunctional role. In poliovirus (PV), the interaction between the RNA replication complex and intracellular membranes appears to be accomplished by proteins 3A and 2C, which have membrane-binding properties (Bienz et al., 1987; Semler et al., 1982). When expressed independently PV 3A co-fractionates with ER markers (Suhy et al., 2000) and its single transient expression can disrupt the secretory apparatus (Choe et al., 2005; Deitz et al., 2000; Doedens et al., 1997). On the other hand, 3AB presumably anchors 3B in the novo intracellular membranes originated during the initiation of RNA replication, where uridylylated 3B primes the synthesis of nascent viral RNAs (Andino et al., 1993; Lama et al., 1994; Lyle et al., 2002; Takegami et al., 1983; Towner et al., 1996). PV 3AB has a non-specific RNA-binding activity and associates

☆ Dedicated to the memory of Rosario Armas-Portela.

* Corresponding author. Mailing address: CBMSO, Cantoblanco 28049, Madrid, Spain. Fax: +34 91 1964420.
E-mail address: fsobrino@cbm.uam.es (F. Sobrino).

[1] Deceased.

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doi:10.1016/j.virol.2008.06.040

with the cloverleaf structure in the 5′ end of viral RNA and with the 3CD precursor to form a ribonucleoprotein complex required for PV RNA synthesis (Hope et al., 1997; Lama et al., 1994; Xiang et al., 1998; Xiang et al., 1995). Additionally, 3AB has been reported as a co-factor for 3Dpol activity (Lama et al., 1995).

The organization of the FMDV 3AB region is unique among the picornaviruses. Protein 3A extends its carboxy-terminus in at least 60 amino acids, a feature recently reported for bovine rhinovirus type 2 (Hollister et al., 2008). Also, three non-identical copies of 3B ($3AB_1B_2B_3$) are encoded and expressed in susceptible cells (Falk et al., 1992; Forss et al., 1984; Garcia-Briones et al., 2006; O'Donnell et al., 2001). Each of the 3B proteins can be *in vitro* uridylylated by 3Dpol (Ferrer-Orta et al., 2006; Nayak et al., 2005) and deletions of redundant copies led to a decrease in FMDV replication efficiency in cell culture (Falk et al., 1992) and attenuation in pigs (Pacheco et al., 2003). Engineered FMDV RNA with truncated 3A versions showed a decreased infectivity in bovine, but not in BHK-21 or porcine cells (Pacheco et al., 2003). Mutations in 3A, found in field isolates, are associated to FMDV hypervirulence in pigs and attenuation in cattle (Beard and Mason, 2000), and a single amino acid substitution in this protein can mediate adaptation of the virus to guinea-pigs (Nunez et al., 2001).

In comparison to other picornaviruses, differences in FMDV-induced membrane rearrangements in infected cells have been reported. FMDV infection is resistant to brefeldin A treatment (O'Donnell et al., 2001), a drug that disrupts the Golgi complex and inhibits replication of other picornaviruses, such as PV (Irurzun et al., 1992). In PV and coxackievirus, 3A, but not 3AB, mediates the inhibition of the anterograde traffic between the ER and the Golgi (Doedens et al., 1997), resulting in a reduced secretion of proteins such as MHC class I molecules (Choe et al., 2005) and TNF receptor (Neznanov et al., 2001). In FMDV, transient expression of 2BC, but not of 3A, has been reported to inhibit host protein secretion in Vero cells (Moffat et al., 2005).

FMDV 3A protein is predicted to contain a hydrophobic domain (Forss et al., 1984) and is found associated to crude membrane extracts from infected cells (Moffat et al., 2005). Fluorescence to 3A in infected cells partially co-localizes with the ER marker calreticulin and with the Golgi stacks protein p58 (Garcia-Briones et al., 2006; O'Donnell et al., 2001). Unlike what is observed in other picornaviruses, transient expression of 3AB proteins does not induce major rearrangements of intracellular membranes as inferred from immunofluorescence and electron microscopy (EM) studies (Garcia-Briones et al., 2006; O'Donnell et al., 2001). When transiently expressed, FMDV 3A preferentially localized at small vesicles (Moffat et al., 2005; O'Donnell et al., 2001). Interestingly, sequential addition of protein 3B copies resulted in a relocation of the 3AB products − hence 3A(B) − that became progressively compact and fibrous (Garcia-Briones et al., 2006). On the other hand, FMDV precursor $3AB_1B_2B_3C$ has been proposed to facilitate the transport of viral protease 3C to the nucleus in infected cells (Capozzo et al., 2002).

Cells transiently expressing PV membrane-binding proteins, including 3A, showed a markedly reduced ability to support PV replication as a consequence of the modification of ER membranes, suggesting that a functional replication complex is formed *in cis*, in a coupled process involving viral translation, membrane modification and viral RNA synthesis (Egger et al., 2000). To investigate the functional role of the FMDV proteins encoded in the 3AB region, we have generated BHK-21 transformed cells that stably express 3A protein, or the precursors $3AB_1$ and $3AB_1B_2B_3$. The level of expression of 3A found in these cells was low, and no detectable cell alterations were observed in the clones analyzed by electron microscope. Interestingly, 3A clones produced higher viral titers upon FMDV infection. This effect was also observed in $3AB_1$ and $3AB_1B_2B_3$ clones, suggesting that expression of proteins from the 3AB region can specifically enhance viral replication. This stimulation was specific for FMDV as it was not observed upon infection with encephalomyocarditis virus (EMCV) or vesicular stomatitis virus (VSV). In contrast, a decrease in FMDV, EMCV and VSV multiplication was observed in BHK-21 cells transiently expressing higher levels of these proteins. Thus, while low levels of expression of 3AB proteins resulted in a specific increase of FMDV multiplication, an unspecific effect was found in cells transiently expressing higher levels of these proteins, in which a decrease of FMDV, EMCV and VSV yield was observed.

Results

Selection of BHK-21 clones stably expressing 3A, $3AB_1$ or $3AB_1B_2B_3$ proteins

To investigate the effect of 3A or 3A(B) proteins on FMDV multiplication cycle, we derived BHK-21 cell clones stably expressing 3A alone or as fusion protein with one or three copies of FMDV 3B. To this end, plasmid pPur alone or in combination with pRSV derivatives expressing 3A, $3AB_1$ or $3AB_1B_2B_3$ from C-S8c1 FMDV, was transfected into BHK-21 cells and used for selection of stable transfectants, resistant to puromycin (Pur^R). The effect of transgene expression on cell survival was estimated from the number and size (diameter) of Pur^R clones that expressed the corresponding FMDV transcript (Table 1). Relative to the results obtained with cells transfected with pPur (Pur clones) alone or in combination with pRSV/L (Luc clones), a lower number of clones was recovered in two independent experiments in co-transfections with plasmids expressing the FMDV transcripts. The percentage of clones that could be expanded to obtain confluent monolayers was also reduced. Transcription of FMDV specific RNAs in the expanded clones was first monitored by RT-PCR amplification of cytoplasmic RNA, and later confirmed by nucleotide sequencing at passage 8th. The percentage of viable clones that showed detectable levels of the specific FMDV RNA ranged from 82% (3A) to 53% ($3AB_1$) (Table 1). The growth rate of 3A(B) clones was not noticeably different from that of the control Pur and Luc clones.

Expression of FMDV proteins, estimated by western blotting, was detected in the clones positive for FMDV RNA (representative examples are shown in Fig. 1A). The level of protein expression in the different clones was, in all cases, significantly lower than that observed in transfected cells transiently expressing these proteins from pRSV derivatives (see Materials and Methods), as confirmed by the quantification of the intensity of the 3A-specific bands (Fig. 1A), suggesting that high levels of expression of proteins from the 3AB region could be detrimental for cell viability. Additional faint bands of a size corresponding to various 3AB products were observed, as

Table 1
Selection of BHK-21 clones stably expressing 3A or 3A(B) FMDV proteins and its effect on cell growth

Plasmid	Pur^R [a]	Colony size [b]	Growth Capacity(%) [c]	Transcript detection (%) [d]
None (mock)	0/0	-	-	-
pPur	57/76	L	98	0 (8)
pRSV/L	51/75	L/M	92	0 (8)
pRSV/3A	33/64	M/S	62	82 (14)
pRSV/$3AB_1$	24/35	S/M	48	53 (10)
PRSV/$3AB_1B_2B_3$	27/29	M/L	58	67 (17)

[a] Number of cell clones visualized by crystal violet staining after 12 days of seeding at a 1:10 dilution of the initially co-transfected cells on a 100 mm Ø dish. The results of two independent experiments are presented.

[b] Average size of Pur^R clones: L, large (1.5-3 mm); M, medium (1-1.5 mm); S, small (0.5-1 mm). The first letter indicates the predominant colony size.

[c] Percentage of clones that were efficiently expanded to yield a monolayer of 4-6×10^6cells on a 60 mm Ø dish.

[d] Percentage of viable clones yielding positive RT-PCR amplification of the corresponding FMDV transcript. The number of clones positive for RT-PCR is indicated in brackets.

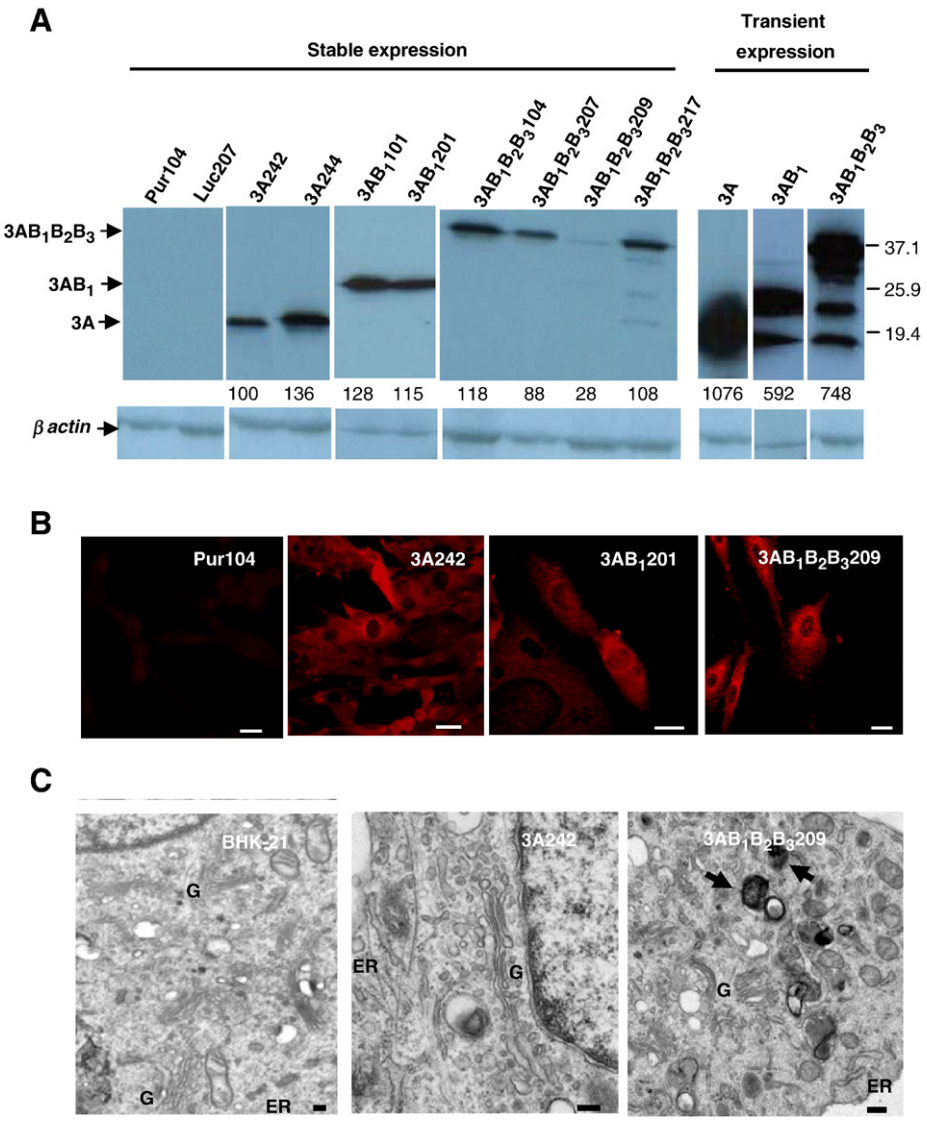


Fig. 1. Specific expression of FMDV proteins. (A) Western blot detection of 3A protein, using MAb 2C2, in transformed clones or in BHK-21 cells transiently expressing 3AB proteins. Pur104 and Luc207 correspond to two different negative controls, stably expressing only puromycin resistance or puromycin and luciferase, respectively. For transient expression, BHK-21 cells were processed at 24 h pt with the pRSV derivatives expressing the proteins indicated. In each case, the expression levels (intensity of the 3A/3A(B) protein band) given as percentage of that of clone 3A242, is indicated at the bottom of each line. Immunoblotting of β-actin was included as control of protein loading. (B) Representative microscope images of the distribution of 3A fluorescence in clones stably expressing 3A/3A(B) proteins. Cells were processed for immunofluorescence staining using MAb 2C2 (Bar: 20 μm). (C) Electron microscopy images of BHK-21 cells and clones stably expressing 3A242 and $3AB_1B_2B_3209$. Endo-membrane systems corresponding to endoplasmic reticulum (ER) and the Golgi apparatus (G) are indicated. Arrows point to residual vesicles accumulated in clone $3AB_1B_2B_3209$. Bar: 0.2 μm.

reported previously (Garcia-Briones et al., 2006). The identity of these bands, which could result from cleavage by cellular proteases, was confirmed in transfected cells transiently expressing the 3AB products, by immunobloting with MAb 1F8 targeted to 3B (data not shown). The amount of these proteins in the transformed cells was below the detection level of this antibody.

Stable expression of proteins from the 3AB region does not induce major cell rearrangements

FMDV 3A protein associates to membrane extracts from infected cells (Moffat et al., 2005), in which alterations of endomembranes, including the formation of vesicles of an unclear origin, have been reported (Garcia-Briones et al., 2006; Knox et al., 2005; Monaghan et al., 2004). To investigate whether expression of 3A or 3A(B) proteins induced alterations in cell membrane structures, we first performed immunofluorescence analysis. As shown in Fig. 1B, cells expressing proteins of the 3A region showed positive reaction in the cytoplasm with the specific anti-3A antibody, with a perinuclear distribution. Second, the EM images of negative stained cells from clones 3A242 and $3AB_1B_2B_3209$ revealed no major changes in the endomembrane system when compared to control BHK-21 cells, in which the integrity of the ER and the Golgi was maintained. The analysis of $3AB_1B_2B_3209$ cells showed an accumulation of lysosomes and residual vesicles that could suggest alterations in the degradative pathways (Fig. 1C).

Thus, no gross alterations of BHK-21 cell structures, including endomembranes as those observed during FMDV infection, were found in cells stably expressing 3A or 3A(B) proteins.

FMDV multiplication is specifically increased in cells expressing 3A, $3AB_1$ or $3AB_1B_2B_3$ proteins

To address the effect of the stable expression of proteins from the 3AB region on the FMDV multiplication cycle, we first analyzed virus plaque formation. To this end, monolayers of clones expressing these proteins were infected with FMDV isolate C-S8c1 (50 PFU) and the

virus plaques developed after 24 h were scored. In general, an increase was observed in the PFU recovered from monolayers expressing 3A or 3A(B) proteins. The percentages of PFU increase (PI) observed in each of the 36 clones expressing 3A, $3AB_1$ or $3AB_1B_2B_3$, relative to the PFU scored in the control Pur clones (average from 4 clones) – which were undistinguishable from those of a duplicate of BHK-21 cells infected in parallel (data not shown) – are summarized in Fig. 2A. Four additional control Luc clones gave PI ranging from 0 to 7%, supporting a lack of effect of the clonal selection on FMDV susceptibility. Except for five clones whose PI ranged from 10-50, the PFU values in the remaining

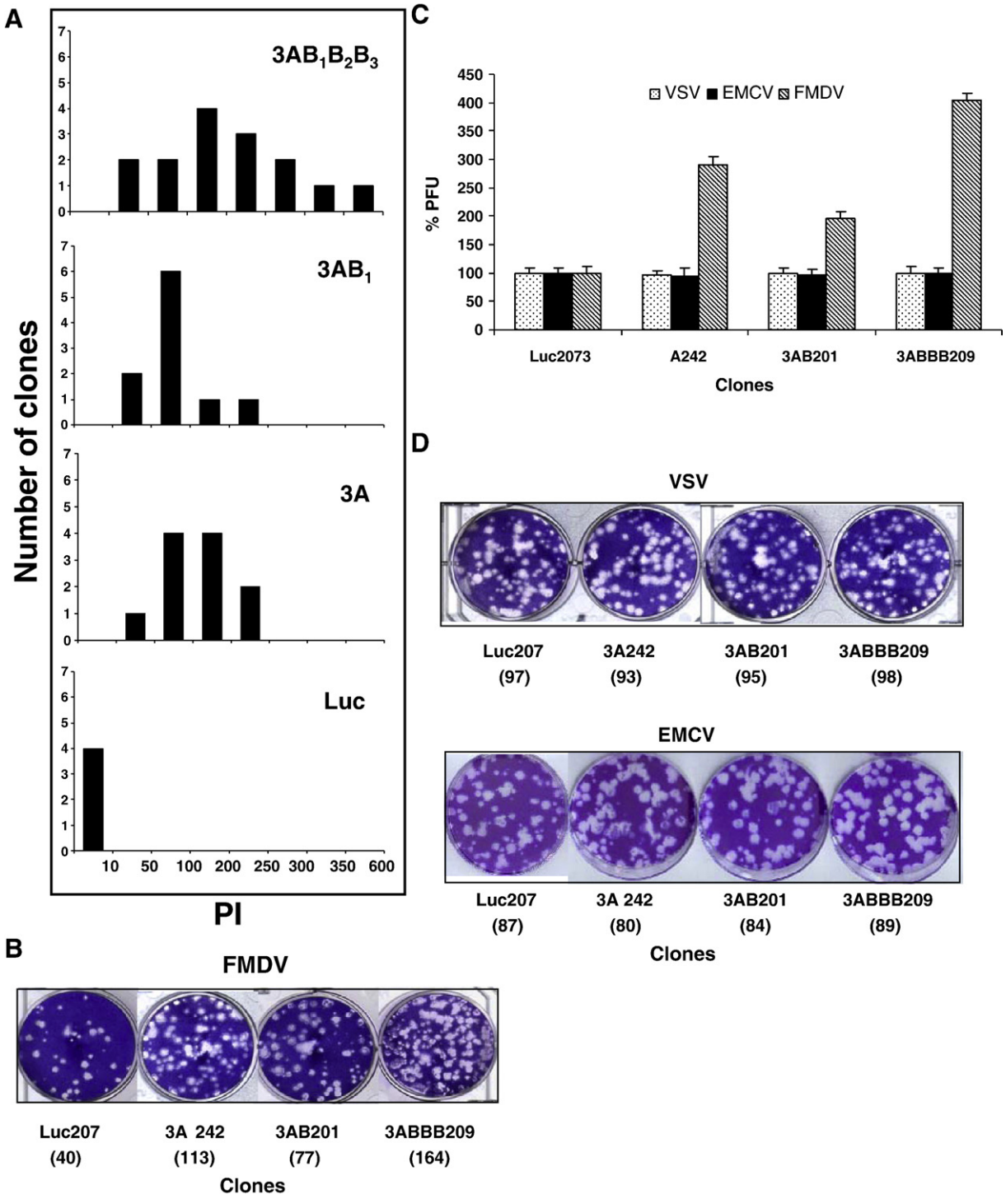


Fig. 2. Effect on virus plaque formation of the stable expression of FMDV 3AB proteins. (A) Frequency distribution of PFU increase (PI) upon infection of clones stably expressing 3A, $3AB_1$ or $3AB_1B_2B_3$. PI values are represented against the number of clones analyzed that survived puromycin selection in each group. Confluent monolayers were infected with about 50 PFU of FMDV and the virus plaques developed were scored at 24 h pi. PI values were calculated relative to the PFU recovered in the control Luc clones. SD was lower than 15% in all cases. The PI values for the clones analyzed in Fig. 1A were: 3A242 (207), 3A244 (120), $3AB_1$101 (90), $3AB_1$201 (102), $3AB_1B_2B_3$104 (27), $3AB_1B_2B_3$207 (234), $3AB_1B_2B_3$209 (300) and $3AB_1B_2B_3$217 (146). (B) Representative examples of FMDV PFU recovered in clones expressing 3A/3A(B) proteins (plaque counts are given in parenthesis). (C) The percentage of PFU recovered after infection with about 100 PFU / monolayer of VSV, EMCV or FMDV in clones expressing 3A/3A(B) proteins, relative to those recovered in the control Pur clones. Results with clone Luc207 are included. The means and SD from three independent experiments are shown. (D) Representative examples of VSV or EMCV plaques, recovered upon infection with about 100 PFU of each virus in clones expressing 3A/3A(B) proteins (plaque counts are given in parenthesis).

31 FMDV-specific clones (86%) were significantly higher (t-student for multiple comparisons, $p \leq 0.05$) than those of the control clones. The highest PI frequency was found for clones expressing $3AB_1B_2B_3$. An inverse correlation between the amount of FMDV protein detected in individual clones and the corresponding PI values was noticed (Fig. 1A and legend of Fig. 2A). Thus, clone $3AB_1B_2B_3104$, which showed a high level of $3AB_1B_2B_3$ expression, induced a low (27%) PI while clone $3AB_1B_2B_3209$, which showed a low expression level, exhibited one of the highest (300%) PI observed.

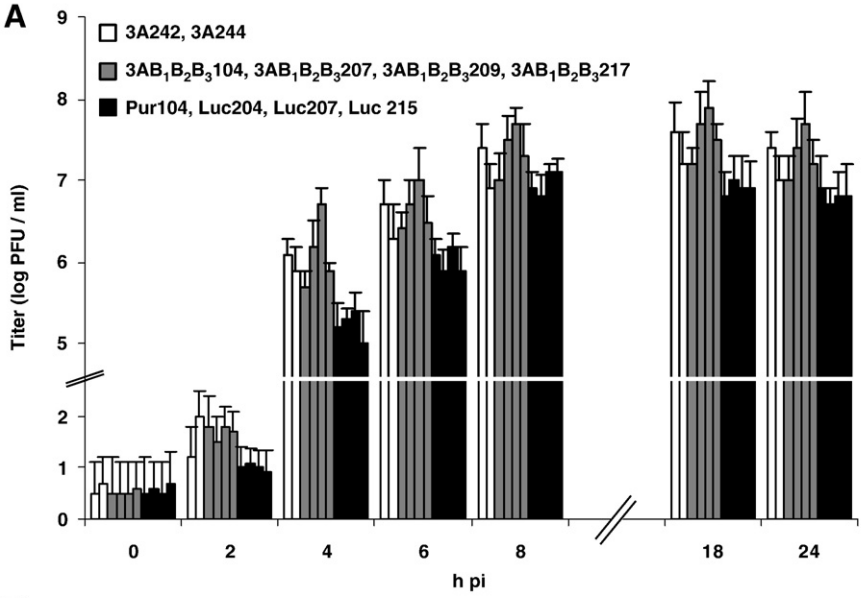
The specificity for FMDV of this increase in PFU was confirmed for clones 3A242, $3AB_1201$ and $3AB_1B_2B_3209$. When monolayers of these clones were infected with VSV, an unrelated rhabdovirus, or with the picornavirus EMCV, the number of plaques and their size was similar to those developed by the infected control clones, Luc207 (Fig. 2C and D) and Pur104 (data not shown). Thus, stable expression of the protein 3A specifically enhanced FMDV plaque formation in BHK-21 clones. This specific effect was also observed when the protein 3A was fused to 3B.

To further address the effect on the viral growth of the stable expression of proteins from the 3AB region, the virus titers recovered upon FMDV C-S8c1 infection of clones expressing 3A and $3AB_1B_2B_3$ were used to estimate the increase in viral yield, compared with the titers recovered from 4 independent Pur and Luc control clones. While no major morphological differences were observed in 3A(B)-infected clones relative to infected control clones, an advanced CPE (about 1 hour) was noticed by light microscopy. No significant differences were found in the virus titers recovered from control clones, which were undistinguishable from those of a duplicate of BHK-21 infected in parallel (data not shown). As shown in Fig. 3A, the highest titer increases were observed at 4 h pi, with differences up to ten-fold relative to the mean of control clones that, in all cases, were statistically significant, being the level of significance lower for clones expressing higher amounts of FMDV protein (i.e. clones 3A244 or $3AB_1B_2B_3104$) (Fig. 3B). This increase was also observed at later infection times to a lesser extent at 8 h pi, including 18 and 24 h pi, when cells had developed extensive cytopathic effect. Infected clones expressing $3AB_1B_2B_3$ showed, in average, higher titer increases (Fig. 3), being $3AB_1B_2B_3209$ the highest. These results were in agreement with those obtained in the PFU assay (see Fig. 2A). Again, the virus production exhibited by clones $3AB_1B_2B_3104$ and $3AB_1B_2B_3209$ correlated inversely with the expression level of the corresponding FMDV proteins (Fig. 1A).

To assess whether the increase in PFU recovery and virus yield correlated with the level of detection of FMDV capsid proteins, extracts from infected monolayers of 3A or $3AB_1B_2B_3$ clones were analyzed by western blot, using a MAb targeted to the capsid protein VP1. As shown in Fig. 4A, relative to the control Pur104, clones 3A242 and $3AB_1B_2B_3209$ showed higher intracellular levels of VP1 at the different times post infection analyzed. Likewise, an increase in the percentage of cells positive for VP1 detection by immunofluorescence was observed from 2 h pi. in monolayers infected with FMDV (Fig. 4B). This increase in the percentage of infected cells was specific for FMDV, as it was not detected in cells infected with VSV (data not shown).

The enhancement of proteins 3A or $3AB_1B_2B_3$ on viral yield is not due to a factor released to the growth medium

To address whether 3A and 3A(B) proteins were released from cells to the supernatant, facilitating subsequent infection events, we first analyzed the presence of FMDV 3A in the supernatant of transformed cell monolayers. To this end, supernatant aliquots (corresponding to about $2x10^7$ cells) were dialysed and then concentrated. To control protein stability in the concentration steps, immunoblotting of Pur104 supernatant supplemented with crude protein extracts from 3A or $3AB_1B_2B_3$ clones (containing about 40 μg) was included in parallel. The



B

Clones	3A242	3A244	$3AB_1B_2B_3$ 104	$3AB_1B_2B_3$ 207	$3AB_1B_2B_3$ 209	$3AB_1B_2B_3$ 217
p ≤	0.01	0.02	0.05	0.01	0.001	0.02

Fig. 3. FMDV yield in infected clones expressing 3AB proteins. (A) Time course of FMDV production in clones infected at a MOI of about 1. Four Luc and Pur control clones are included. The means and SD from three independent experiments are shown. (B) Significance (p values, obtained with the Student's t test for multiple comparisons) of the differences, at 4 h pi, among the mean of control clones and each of the 3A(B)-expressing clones analyzed.

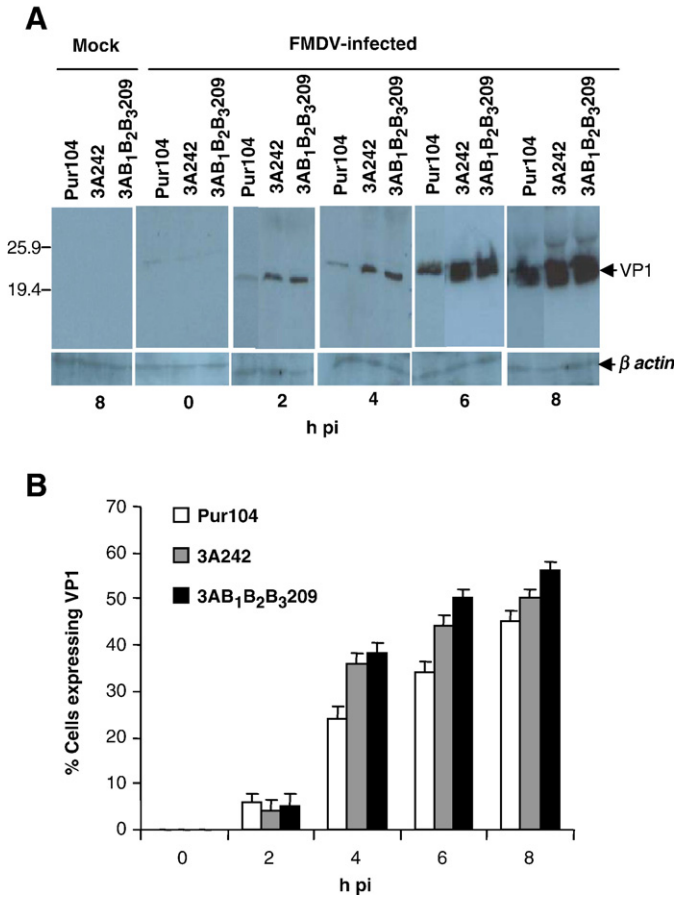


Fig. 4. Expression of VP1 capsid protein in FMDV infected 3A and 3A(B) clones. (A) Detection of VP1 protein in cell extracts collected at different h pi (MOI of about 1) by western blotting using MAb SD6. Immunoblotting of β-actin was included as control of protein loading. (B) Percentage of cells expressing VP1 at different h pi (MOI of about 10) detected by fluorescence. The means and SD from two independent experiments are presented.

results, shown in Fig. 5A, indicated that 3A was not secreted to the supernatant in amounts above 4 μg/2×10^7 cells, which was the lower limit of detection in this experimental approach. A similar analysis in BHK-21 cells transiently expressing high amounts of 3A or $3AB_1B_2B_3$ proteins was also negative (data not shown).

To further analyze the possibility that small amounts of 3A protein, below the detection level of western blotting, were exerting a functional role on subsequent virus infection, we determined the % of virus yield in BHK-21 cells growing in the presence of purified FMDV 3A protein. The results indicated that addition of a wide range of 3A (4 to 100 μg) to growing cell monolayers did not modify production of FMDV plaques (Fig. 5B). Likewise, addition of supernatant from cells transiently expressing 3A or $3AB_1B_2B_3$ proteins to BHK-21 monolayers did not modify virus yield (data not show).

Stable expression of 3A or $3AB_1B_2B_3$ proteins does not stimulate FMDV IRES-dependent translation

To determine whether the increase of FMDV yield described above resulted from an effect on IRES-driven translation, the earliest intracellular event during picornavirus gene expression (Belsham and Martínez-Salas, 2004), clones expressing 3A or $3AB_1B_2B_3$ proteins were used to assay FMDV IRES-dependent translation efficiency. As shown in Fig. 5C, no stimulation of internal initiation was observed in clones expressing 3A or $3AB_1B_2B_3$ proteins, relative to control clones. This result was in agreement with previous reports on the effect of PV 3A protein mutations that caused defective viral RNA synthesis, producing small plaques without modification of translation and polyprotein processing in cell-free systems.

Transient expression of proteins from the 3AB region induces a decrease of FMDV, EMCV and VSV multiplication

PV 3A transient expression has been shown to result in a markedly reduced ability of HeLa cells to support PV replication (Egger et al., 2000).

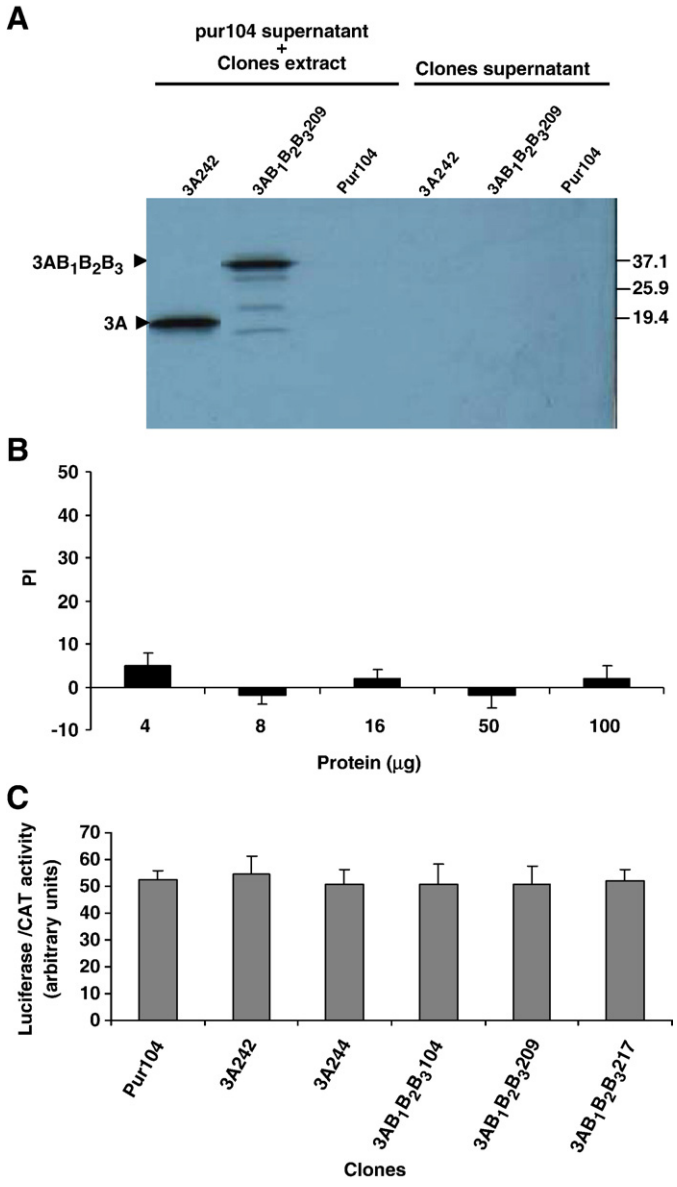


Fig. 5. Analysis of the release of 3AB proteins from FMDV clones. Effect on FMDV growth and IRES-dependent expression. (A) Analysis of FMDV 3A/3A(B) release to the cell supernatant. Western blot detection of 3A protein, using MAb 2C2, in concentrated supernatants of 2×10^7 cells stably expressing the indicated proteins. As a control of protein stability in the concentration steps, blotting of Pur104 supernatant supplemented with the indicated clone extracts containing FMDV 3A or $3AB_1B_2B_3$ protein (about 40 μg) was included. (B) Effect of purified FMDV 3A protein addition to the supernatant on virus yield. BHK-21 cells (about 2×10^6 cells, 35 mm Ø plate) grown in the presence or absence of the indicated amount of purified FMDV 3A protein were infected with FMDV (about 100 PFU/monolayer). The PFU developed were scored at 24 h pi and PI values were calculated relative to the PFU recovered in BHK-21 grown in absence of purified 3A protein. (C) Effect of FMDV 3A/3A(B) protein expression on FMDV IRES-dependent translation efficiency. IRES-dependent translation was estimated as luciferase activity normalized to CAT activity, measured in extracts from 3A or $3AB_1B_2B_3$ clones transfected with CMVpBIC bicistronic plasmid, relative to the value obtained in Pur104 transfected clones. The results were expressed in arbitrary units, error bars correspond to the SEM from two duplicated experiments.

To study whether the effect on FMDV multiplication of 3A or 3A(B) proteins could lead to different effects during transient expression, pRSV derivatives expressing 3A, $3AB_1$ or $3AB_1B_2B_3$, were transfected in BHK-21 cells. The levels of protein expression estimated by western blotting were higher than those observed in stable expression (Fig. 1A), being the highest levels detected at 18 to 24 h pt (data not shown). These high protein levels induced a detrimental effect in BHK-21 cells, as indicated by a 20 to 30 % reduction in cell viability at 24 h pt (data not shown).

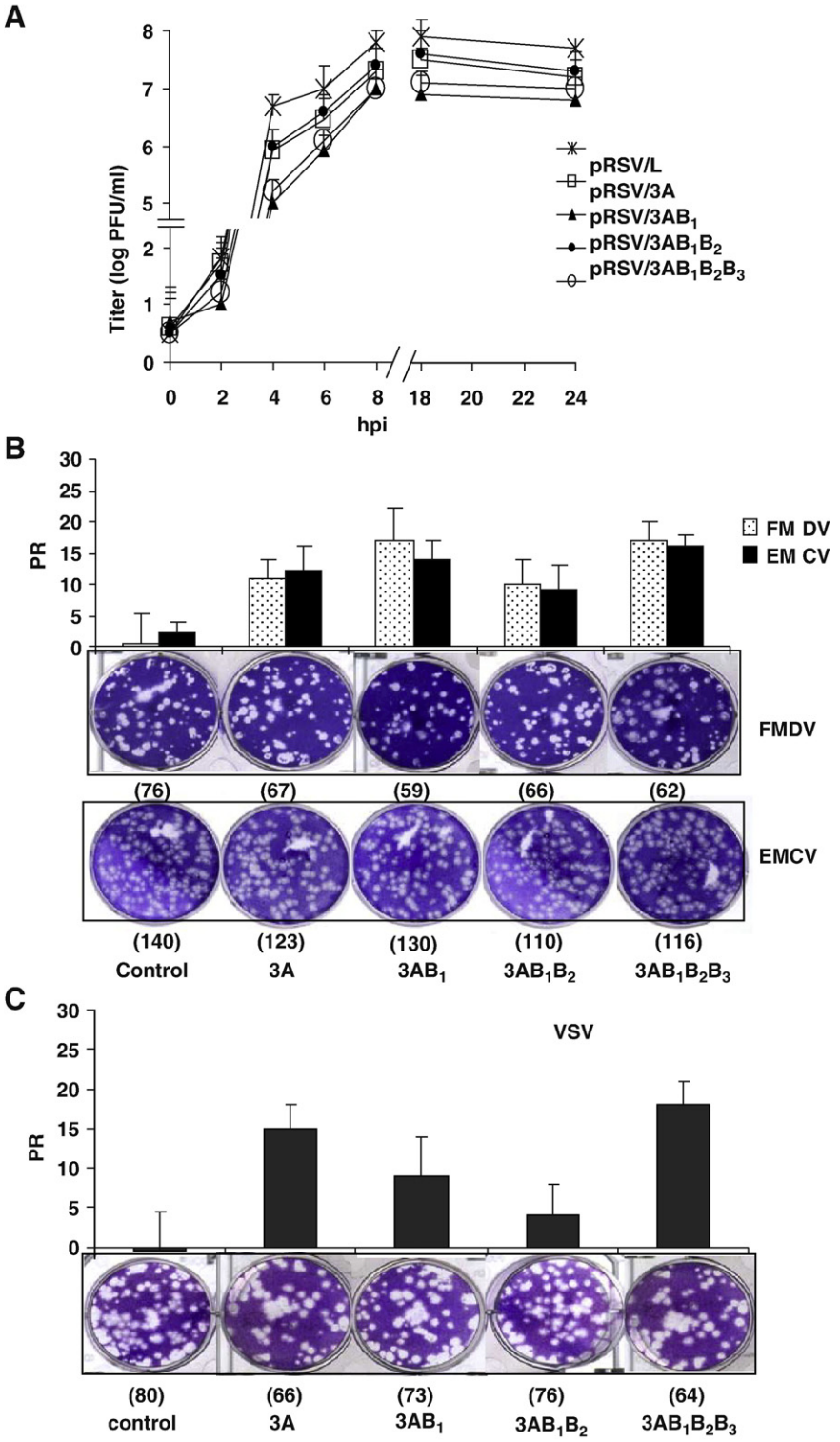


Fig. 6. Effect of transient expression of 3AB proteins on FMDV and VSV multiplication in BHK-21 cells. (A) Time course of FMDV titers recovered in the supernatants of transfected cells infected with FMDV (MOI of about 1). BHK-21 cells transfected with PRSV/L were included as control. The differences in virus titer observed at 4 h pi in cells expressing each of the four proteins from the 3AB region was significantly lower than that of control cells ($p \leq 0.001$). (B) FMDV and EMCV plaque formation in transfected cells. Monolayers, infected with about 100 PFU/monolayer of the indicated virus, were grown in the presence of agar. The PFU developed were scored at 24 h pi. PR values were calculated relative to the PFU recovered in cells transfected with PRSV/L. Non-transfected BHK-21 cells were included as control. Representative examples of the PFU recovered are shown (plaque counts are given in parenthesis). (C) VSV plaque formation. Cells were infected with VSV and processed as in (B). In all cases, the means and SD from three independent experiments are shown (plaque counts are given in parenthesis).

When BHK-21 cells transfected with pRSV/L were infected (at 16 h pt) with C-S8c1 FMDV, the viral titers in culture medium were undistinguishable from those found in mock-transfected cells, at any time-points analyzed (data not shown). Conversely, a reduction in the virus yield, relative to cells transfected with pRSV/L, was observed in cells transiently expressing FMDV 3A or 3A(B) proteins (Fig. 6A) at the different infection times. The virus titers were slightly lower in cells expressing $3AB_1$ and $3AB_1B_2B_3$ than in those expressing 3A and $3AB_1B_2$ (Fig. 6A). Similar reductions were observed in the capacity to produce FMDV plaques. In these assays, cells transiently expressing $3AB_1$ and $3AB_1B_2B_3$ exhibited the higher plaque reduction (PR) values, and no significant difference in the plaque size was observed (Fig. 6B). Similar results were obtained with 3A and 3A(B) transfected IBRS cells, a porcine line (data not shown). The magnitude of virus titer decrease was slightly lower than the virus titer increase observed in infected 3A/3A (B) clones.

The inhibition of the virus multiplication was not specific for FMDV, as indicated by the PR observed upon EMCV (Fig. 6B) and VSV (Fig. 6C) infection. In the VSV case, cells expressing $3AB_1B_2B_3$ showed the higher PR value, which was similar (17%) to that found for FMDV (Fig. 6C). Thus, in contrast to what observed in clones stably expressing proteins from the 3AB region, transient expression of 3A, $3AB_1$, $3AB_1B_2$ and $3AB_1B_2B_3$ results in a reduction of the FMDV yield and plaque formation in BHK-21 cells. This inhibitory effect extends also to the picornavirus EMCV and the RNA virus VSV, indicating that a non FMDV-specific mechanism/s is involved in this inhibitory effect.

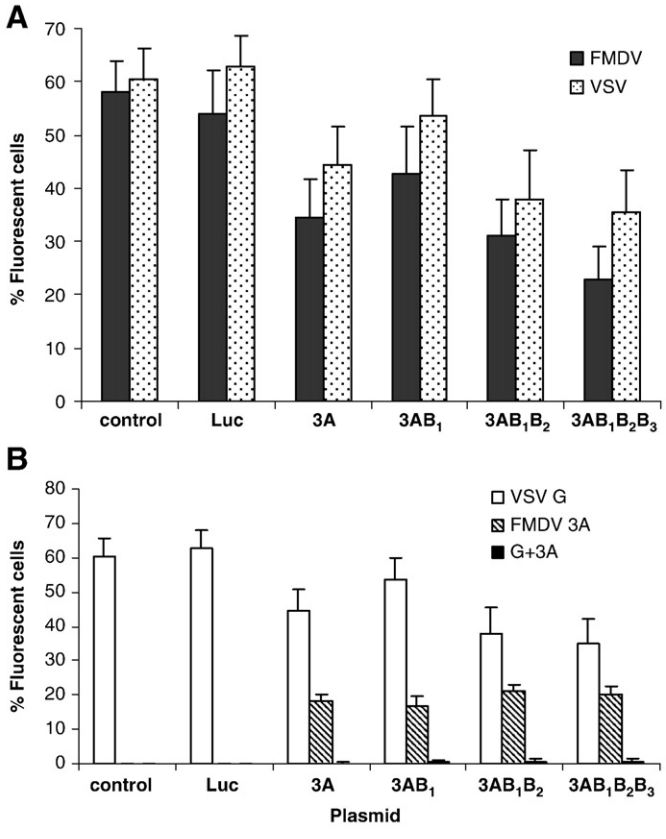


Fig. 7. Correlation between transient expression of 3A/3A(B) proteins and infection of single BHK-21 cells. Transfected monolayers, including those with pRSV/L (Luc), were infected (at 16 h pt) with FMDV or VSV (MOI of about 10). Non-transfected BHK-21 cells were included as control. After 4 h, the percentage of cells expressing 3A, VP1 (for FMDV infection) or G protein (for VSV infection) was monitored, using polyclonal serum 163 and MAb SD6 or I1, respectively. (A) Percentage of cells expressing VP1 (FMDV) or G (VSV) proteins. (B) Percentage of VSV infected cells expressing 3A, G or both proteins. In A and B, the mean and SD from two independent experiments (about 1000 cells were scored in each of them), are presented.

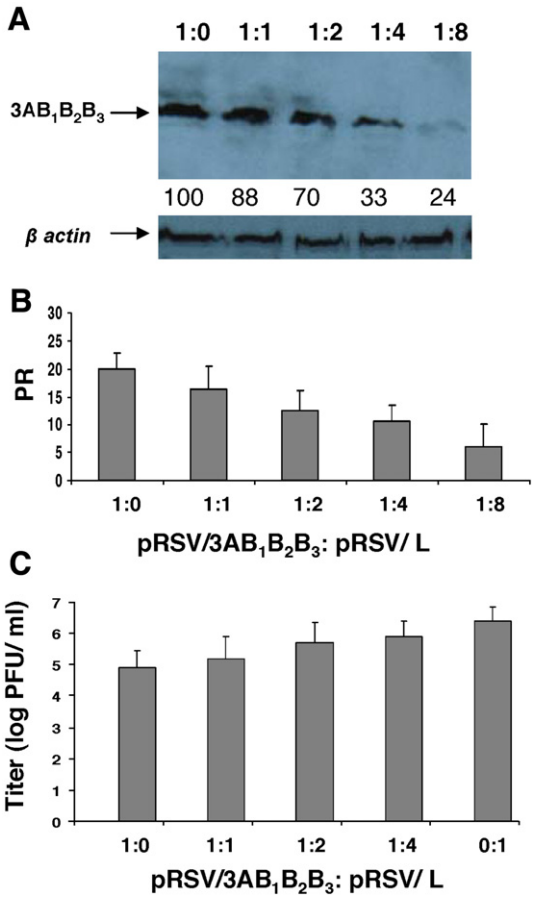


Fig. 8. Dose-effect inhibition of FMDV growth by transient expression of $3AB_1B_2B_3$. (A) Modulation of $3AB_1B_2B_3$ transient expression. Western blot detection of 3A protein, using MAb 2C2, in cell monolayers at 24 h post transfection with different ratios of pRSV/$3AB_1B_2B_3$: pRSV/L. The expression level (intensity of the $3AB_1B_2B_3$ protein band) given as percentage of that of cells transfected with pRSV/$3AB_1B_2B_3$ alone (1:0) is indicated at the bottom of each line. Immunoblotting of β-actin was included as control of protein loading. (B) FMDV plaque formation. Transfected cells, as in A, were infected with about 100 PFU/monolayer in the presence of agar. The PFU developed were scored at 24 h pi. PR values were calculated relative to the PFU recovered in cells transfected with PRSV/L (0:1). (C) Virus titers recovered from supernatants of transfected cells, as in A, at 4 h pi with FMDV (MOI of about 1).

Single cells transiently expressing 3AB proteins show a decreased level of FMDV infection

To determine whether transient expression of 3A, $3AB_1$, $3AB_1B_2$ or $3AB_1B_2B_3$ affected also the levels of FMDV protein synthesis in infected cells, the percentage of BHK-21 cells exhibiting VP1 fluorescence in infected monolayers was determined. Under the infection conditions used (MOI of 10), about 50% of the control cells transfected with pRSV/L were positive for VP1, while this percentage was reduced in cells expressing 3AB proteins (Fig. 7A) that ranged from 40% ($3AB_1$) to 25% ($3AB_1B_2B_3$). Similar reductions in cells positive for glycoprotein G fluorescence were observed upon infection of transfected cells with VSV (Fig. 7A). Double immunofluorescence assays indicated that a majority of cells showing intense green fluorescence to 3A did not exhibit red fluorescence to either FMDV VP1 or VSV G proteins. In VSV infected cells, a quantitative analysis was possible, as expression of 3A or 3A(B) products could only be due to plasmid transfection. The results obtained indicated that fluorescence to VSV G protein was detected in less than 1% of

cells expressing detectable levels of 3A or 3A(B) proteins (Fig. 7B). Thus, immunofluorescence detection of 3A/3A(B) proteins correlated with a lack of progress of VSV and FMDV infection.

Inverse correlation between transient expression level of proteins from the 3AB region and decrease of FMDV multiplication

To address whether the expression level of 3A(B) proteins in transfected cells influenced the extent of decrease of FMDV multiplication, BHK-21 cells were transfected with different ratios of pRSV/$3AB_1B_2B_3$ to pRSV/L. As shown in Fig. 8A, decreasing ratios (1:1 to 1:8) of pRSV/$3AB_1B_2B_3$ resulted in the expression of lower amounts of $3AB_1B_2B_3$ protein. The level of $3AB_1B_2B_3$ protein detection correlated with a decreasing trend in the PR observed upon FMDV infection of transfected cells (Fig. 8B), as well as with an increase in the virus yield recovered at 4 h pi (Fig. 8C). A similar trend was observed in the virus yield recovered at 6 and 8 h pi (data not shown). Thus, these results support a direct dose-effect of the inhibition exerted by the transient expression of $3AB_1B_2B_3$ protein.

Discussion

Here, we report the characterization of stable BHK-21 clones that express FMDV 3A, $3AB_1$ and $3AB_1B_2B_3$ proteins. To our knowledge, this is the first description among picornaviruses of selection of viable cells stably expressing proteins from this viral genomic region. The clones generated in this work provide a useful tool to study the role of proteins from the 3AB region in FMDV replication and its interaction with host cell components. The percentage of 3A or 3A(B) clones that were efficiently expanded to yield a monolayer (around 60%) was lower than that of control Pur and Luc clones, and appeared associated with low, but detectable, levels of protein expression. These results demonstrate that low levels of expression of these proteins are compatible with cell viability. No major cell rearrangements were found in clones stably expressing 3A, $3AB_1$ and $3AB_1B_2B_3$ (Fig. 1C), although the increase in lysosomes and residual vesicles observed in the EM images of cells from clone $3AB_1B_2B_3$209 (Fig. 1C), suggests that expression of these proteins could exert a certain level of toxicity. In addition, no evidence of alterations in the distribution of gp74 protein, a *cis*-Golgi marker whose pattern results relocated upon FMDV infection (Garcia-Briones et al., 2006), of the ER marker calreticulin and of the patterns of actin fibers and microtubules were observed in the 3A/3A(B) clones.

The low levels of 3A/3A(B) protein expression detected specifically enhanced viral yield, and plaque formation as well, upon FMDV infection. In addition, enhancement of both the amount of VP1 capsid protein and the percentage of cells expressing detectable levels of this protein was observed. The increase in virus yield was specific for FMDV, as it was not observed in clones infected with the related picornavirus EMCV or with VSV, an unrelated rhabdovirus, whose PFU were similar in number and size to those developed by control clones. These results suggest that blocking of unspecific anti-viral mechanisms, such us interferon (Chinsangaram et al., 1999; Thacore, 1978), do not play a relevant role in the increase of FMDV replication. On the other hand, the low dispersion of the FMDV titers among control clones (Luc and Pur) and its similarity to that of uncloned BHK-21 cells, points against: i) selection of pre-existing BHK-21 cells with a lower FMDV susceptibility, and ii) the decreased susceptibility being the result of an adaptation to the clonal selection itself.

A trend towards detection of lower amounts of 3A/3A(B) products in clones supporting higher levels of FMDV multiplication was noticed. Thus, one of the clones, $3AB_1B_2B_3$209, that showed a higher susceptibility to FMDV infection, expressed a low level of $3AB_1B_2B_3$ protein (see Figs. 1A, 2A and 3). Indeed, transfection of clone $3AB_1B_2B_3$209 with pRSV/$3AB_1B_2B_3$, resulted in a 14% reduction of the PI determined upon FMDV infection (data not shown), indicating that the enhancement of viral multiplication was, partially reversible and that this reversion was likely be due to a higher expression level of the 3A(B) protein. These results suggest that, in the absence of major cell rearrangements, low levels of 3AB proteins expressed *in trans* can specifically stimulate FMDV multiplication in BHK-21 cells. This effect could be mediated by alterations of cell components not detected in our analyses and/or by an enhancement of virus replication due to the presence in infected cells of pre-existing levels of 3A/3A(B) proteins. On the other hand, the effect of these proteins on viral yield only occurs when present intracellularly prior to virus infection (Figs. 5A, B).

In picornavirus infected cells translation of the viral message that depends on an internal ribosome entry site (IRES)-mediated mechanism (Martínez-Salas et al., 2008) is the first intracellular event, and therefore precedes viral RNA replication (Belsham and Martínez-Salas, 2004). A potential role of 3A protein in viral RNA translation was discarded by the lack of effect on FMDV-IRES dependent translation in any of the clones analyzed (Fig. 5C). Uridylylated 3B is required to initiate picornavirus RNA synthesis (Andino et al., 1993; Lyle et al., 2002; Towner et al., 1996) and for FMDV, the three 3B copies can be uridylylated *in vitro* (Nayak et al., 2005). Assuming that 3A(B) proteins expressed *in trans* can be uridylylated in the context of FMDV infection, the availability of 3B precursor in the $3AB_1$ and $3AB_1B_2B_3$ clones could contribute to the increase in FMDV replication. However, clones expressing 3A alone also showed similar levels of FMDV multiplication increase. In other picornaviruses the 3AB protein can be involved in both *cis* and *trans* acting functions. In PV, while P3 precursor, but not 3AB protein, can complement *in trans* RNA replication-defective mutants in 3A in a cell-free translation/replication assay (Towner et al., 1998), complementation of a 3B uridylylation-deficient mutant has been reported when 3AB, but not 3B or 3BC, was provided *in trans* using a dicistronic system in HeLa cells (Cao and Wimmer, 1995; Liu et al., 2007). Also, the growth of hepatitis A virus mutants with an impaired 3AB/3ABC processing can be enhanced by co-expressing 3AB or 3ABC (Kusov and Gauss-Muller, 1999). Likewise mutations in PV 3A protein producing decrease in virus yield do not affect translation, polyprotein processing, uridylylation of VPg or synthesis of negative-strand RNA, but markedly reduces positive-strand RNA synthesis (Teterina et al., 2003). Concerning FMDV 3A and 3A(B), further work is required to understand the mechanism underlying the increase in susceptibility promoted by the low levels of expression of these proteins to subsequent infection.

A different effect on virus multiplication was found in cells transiently expressing proteins from the 3AB region at levels that were, in average, seven-fold higher than those found in 3A/3A(B) clones, in spite of only about 20% of transfected cells showing detectable fluorescence to 3A. Under these high expression conditions, and as reported for PV replication (Egger et al., 2000), a non-specific inhibitory effect on FMDV yield was observed. Thus, the PFU and virus yield recovered upon FMDV infection were lower than in control transfected cells. The inhibition exerted by 3A/3A(B) transient expression affected, to a similar extent, the growth of EMCV and VSV. For both viruses, the number but not the size, of the PFU recovered upon infection was shown to be reduced, suggesting that the virus blockage induced by 3A/3A(B) mostly affects early steps of virus infection and that, once a cell becomes infected, the virus growth is similar to that of control cells. Consistently, quantitative analysis of infected cells expressing VP1 or G protein indicated that a majority of cells exhibiting high levels of 3A/3A(B) transient expression did not result productively infected by either FMDV or VSV, at times in which most of the control cells did. This inhibitory effect would become bypassed by the high amount of virus produced by non-transfected cells. A lack of type I IFN receptors and/or of functional effector pathways for these molecules has been reported for BHK-21 cells (Chinsangaram et al., 1999; Lam et al., 2005). Indeed, growth of BHK-21 cells in acid-treated culture

medium from BHK-21 cells infected with FMDV (Chinsangaram et al., 1999) did not affect PFU recovery upon subsequent infection with FMDV or VSV (data not shown). Thus, induction of type I interferon is not likely involved in the viral inhibition observed in transfected cells.

The stable clones generated in this work provide a tool to investigate the mechanism underlying the effect in susceptibility to FMDV infection induced *in trans* by different levels of expression of proteins from the 3AB region. Taken together, the results shown here indicate that 3A enhancing effect observed in transformed clones is independent of cell membranes rearrangements, it is unrelated to a factor release to the extracellular medium, and it appears to operate at a virus multiplication step posterior to the first rounds of viral RNA translation.

Materials and methods

Cells, viruses, antibodies and proteins

BHK-21 cells were grown as described (Sobrino et al., 1983). Viruses FMDV C-S8c1 (Sobrino et al., 1983), EMCV, kindly provided by L. Carrasco (CBMSO, Spain), and VSV, Indiana isolate (Novella et al., 1996), were grown and titrated as previously described (Rosas et al., 2003). The following antibodies to FMDV proteins were used: MAb SD6 to VP1 (Mateu et al., 1987), MAb 2C2 to 3A and 1F8 to 3B (De Diego et al., 1997) and polyclonal serum 163, produced by rabbit immunization with three consecutive injections of 3A recombinant protein (R. Armas, unpublished results). MAb I1 was used to detect VSV glycoprotein G (Lefrancois and Lyles, 1982); cell protein markers were detected using MAb AC-74 to β-actin (Sigma), MAb CC92 to the *cis*-Golgi network protein gp74 (Alcalde et al., 1994), rabbit polyclonal serum to the ER marker calreticulin (Abacam) and to βII-tubulin (Armas-Portela et al., 1999), and Alexa Fluor 488-labelled phalloidin (Molecular Probes). Horse radish peroxidase (HRP)-conjugated protein A (Sigma), HRP-conjugated rabbit anti-mouse IgG and horse anti-rabbit IgG (Amersham), and goat anti-mouse or anti-rabbit IgG coupled to Alexa Fluor 594 or 488 (Molecular Probes) were used as secondary antibodies. A C-S8c1 FMDV 3A protein expressed from the pRSET vector (Núñez et al, unpublished results) and purified trough a Ni-NTA Agarose (Qiagen) column (Hata et al., 2000) was used to asses its effect on the susceptibility of BHK-21 cells to FMDV infection.

Stable cell transformation and transient expression of FMDV proteins

cDNAs spanning FMDV 3A, $3AB_1$ or $3AB_1B_2B_3C$ coding region (Toja et al., 1999) were used to replace the luciferase gene in plasmid pRSV/L (de Wet et al., 1987) downstream of the Rous Sarcoma Virus (RSV) LTR promoter, as described (Garcia-Briones et al., 2006). For stable cell transformation, plasmids (5 μg) were co-transfected with pBSpacΔp (pPur) (de la Luna et al., 1988) at a molar ratio of 3:1 in BHK-21 cells (60 mm Ø dish), as previously described (Rosas et al., 2003). Plasmid pPur encodes puromycin acetyl-transferase, conferring resistance to puromycin. Cells resistant to puromycin (pur^R) (10 μg/ml) were selected, and independent pur^R clones, obtained by limiting dilution, were expanded in the presence of puromycin (2.5 μg/ml), to avoid loss of integrated DNAs (de la Luna and Ortin, 1992). Cell stocks were kept frozen in liquid N_2 for further analysis. All the Pur^R clones were stable, keeping the expression of the corresponding FMDV genes for, at least 10 serial passages, including more than 4 freezing and thawing cycles.

For transient expression assays, BHK-21 cells were transfected with pRSV derivatives expressing 3A, $3AB_1$, $3AB_1B_2$ or $3AB_1B_2B_3$ using lipofectamine plus (GIBCO, BRL), as described (Garcia-Briones et al., 2006).

Amplification and sequencing of FMDV RNA

Cell monolayers (about 4 x 10^6 cells), were lysed and the cytoplasmic RNA extracted, incubated with DNase RQ1 and RT-PCR amplified, as described (Rosas et al., 2003). Sequencing of 3A(B) RNA from positive clones was performed from amplified cDNAs, as reported (Baranowski et al., 2003) and confirmed the integrity of the corresponding FMDV transgene.

Virus yield assay

To assess the capacity of FMDV to grow in BHK-21 cells expressing FMDV 3A/3A(B) proteins, triplicates (about $5x10^6$ cells, 60 mm Ø plate) of monolayers at 16 h post-transfection (pt) or stably transformed clones cells were infected with FMDV C-S8c1 at a multiplicity of infection (MOI) — expressed as plaque forming units (PFU)/cell – of 1. After 1 h of adsorption, infection was allowed to proceed in DMEM supplemented with 4% FBS. Supernatant samples withdrawn at different times post-infection (pi) were used to estimate the viral titer (PFU/ml) on BHK-21 cells for transient expressions, or on Pur104 clone cells for stably transformed cells, as described (Bigeriego et al., 1999; Gutierrez et al., 1994). Results are the mean from three independent titer determinations.

Viral plaque assay

Determination of virus yield was performed as described (Gutierrez et al., 1993). Briefly, monolayers (about $5x10^6$ cells, 60 mm Ø plate) transiently transfected (16 h pt) or stably transformed cells were infected with FMDV (about 50 PFU), EMCV or VSV (about 100 PFU). At 24 h pi, cells were fixed and stained to determine the PFU developed. The percentage of virus yield reduction (PR) or virus yield increase (PI) was calculated as (PFU control – PFU) x 100 / PFU control, or as (PFU – PFU control) x 100 / PFU control, respectively, using the mean of three independent plaque assays. Cells transfected with pRSV/L for transient expression, or the average of four clones transformed with pPur alone for stably transformed cells were used as the control of PFU.

Protein detection

For western-blot, extracts were prepared from cell monolayers (35 mm Ø), proteins resolved on a 12% SDS-PAGE, transferred onto a nitrocellulose membrane and incubated with MAb 2C2 targeted to FMDV 3A (Garcia-Briones et al., 2006). Membranes were incubated with HRP-coupled anti-mouse antibody and proteins subsequently detected using an ECL kit (Amersham). A Typhoon equipment and software ImageQuant TL (Amersham) were used for quantification of the intensity of protein. For detection of intracellular virus by immunofluorescence, infected monolayers were processed as described (Garcia-Briones et al., 2006), using rabbit serum 163 to 3A, and MAbs SD6 and I1 to FMDV VP1 and VSV G proteins, respectively. Anti-rabbit or anti-mouse IgG coupled with Alexa Fluor 488 or 594 was used as secondary antibody.

Electron microscopy

Cells were grown on 100 mm Ø dishes, washed with PBS and fixed for 30 min in 1% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 at room temperature, for 1 h. Then, cells were scrapped in 1 ml of 0.1 M phosphate buffer, 0.5% bovine serum albumin, postfixed in 1% osmium tetroxide plus potassium ferrocianure for 1 h at 4 °C. After three washes with double distilled water, cells were treated with 0.15% tanic acid in phosphate buffer pH 7.4 for 1 min, washed again with buffer and twice with double distilled water prior the staining with 2% uranyl acetate for 1 h at room temperature. After three washes with double distilled water the samples were

dehydrated in ethanol following standard protocols and embedded in TAAB 812 resin (TAAB lab. Bershire, England). Cell samples were examined using a JEM-1010 electron microscope (Jeol, Japan) and images were acquired using a digital camera Bioscan 792 (Gatan, Inc 5933 Coronado Lane. Pleasanton, CA 94588).

Analysis of 3A protein release to the growth medium

Supernatant samples (10 ml) were withdrawn from monolayers (about $2x10^7$ cells, 100 mm Ø plate) of either BHK-21 cells transiently expressing FMDV 3A or $3AB_1B_2B_3$ protein (16 h pt), or from stably transformed clones. Samples were supplemented with 1 mM PMSF and protease inhibitor cocktail (Roche), dialyzed at 4 °C for 24 h against 20 mM Tris-HCl buffer, pH 7.4 (with changes every 8 h) and concentrated by freeze-drying until total dry up, for 24 h at -80°C and 0,07 mbar using a Virtis Sentry device. The samples were resuspended in Laemmli buffer and analyzed by western blot.

IRES-dependent translation activity

Bicistronic constructs (CMVpBIC) encompassing the FMDV IRES between the chloramphenicol acetyl transferase (CAT) and luciferase gene subcloned in pCDNA3 to generate transcripts from the CMV promoter, were transfected in stably transformed cells expressing 3A or $3AB_1B_2B_3$ proteins; as a control, Pur clones were transfected in parallel. Transfection of 80–90% confluent monolayers with CMVpBIC bicistronic plasmid was carried out using cationic liposomes as previously described (Lopez de Quinto et al., 2002). Extracts from 2 x 10^5 cells were prepared 20 h after transfection in 100 µl of 50 mM Tris–HCl, pH 7.8, 120 mM NaCl, 0.5% NP-40. Luciferase and CAT activities were measured as described (Martinez-Salas et al., 1993). Assays were performed at least two times.

Acknowledgments

We wish to thank J.I. Núñez, G. León and M. González-Magaldi for cloning and expression of FMDV 3A protein, and M. Saiz for discussions and critical reading of the manuscript. We also thank M. Dávila and E. Domingo for providing us with VSV Indiana isolate and with MAb I1, E. Brocci for MAb 2C2 and I. Sandoval for MAb CC92. This work was supported by Spanish grants from CICYT (BIO2005-07592-C02-01), MEC (CSD2006-07) (BFU-2005-00948), and by Fundación Severo Ochoa.

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