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Foot-and-mouth disease virus 5'-terminal S fragment is required for replication and modulation of the innate immune response in host cells

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ABSTRACT

The S fragment of the FMDV 5' UTR is predicted to fold into a long stem-loop structure and it has been implicated in virus-host protein interactions. In this study, we report the minimal S fragment sequence required for virus viability and show a direct correlation between the extent of the S fragment deletion mutations and attenuated phenotypes. Furthermore, we provide novel insight into the role of the S fragment in modulating the host innate immune response. Importantly, in an FMDV mouse model system, all animals survive the inoculation with the live A_{24} FMDV-S_4 mutant, containing a 164 nucleotide deletion in the upper S fragment loop, at a dose 1000 higher than the one causing lethality by parental A_{24} FMDV, indicating that the A_{24} FMDV-S_4 virus is highly attenuated *in vivo*. Additionally, mice exposed to high doses of live A_{24} FMDV-S_4 virus are fully protected when challenged with parental A_{24} FMDV virus.

1. Background

Foot-and-mouth disease virus (FMDV) is a member of the genus Aphthovirus in the *Picornaviridae* family and it is a well-recognized pathogen in domestic and wild cloven-hoofed animals. Infected animals demonstrate fever and growth of vesicles, mainly in the mouth and on the feet, as well as reproductive losses. FMDV outbreaks have had severe socio-economic consequences, influencing both local and global economies.

The FMDV genome consists of a single-stranded, positive-sense RNA molecule of around 8400 nucleotides in length. The open reading frame (ORF), encoding the proteins of the virus, is flanked by 5' and 3' untranslated regions (5' UTR and 3'UTR, respectively). The ORF is first translated into a single polyprotein and then post-translationally cleaved into four structural proteins (VP1, VP2, VP3 and VP4) and ten non-structural proteins (L^{pro}, 2A, 2B, 2C, 3A, $3B_{1-3}$, $3C^{pro}$ and $3D^{pol}$) (Belsham, 2005). It has been demonstrated that the FMDV 5'UTR plays an important role in both the replication and cap-independent translation initiation of the viral polyprotein (Lopez de Quinto and Martinez-Salas, 1997). The 5'UTR of FMDV is unusually long compared to other members of the *Picornaviridae* family, and it consists of an S (short) fragment of 350–380 nucleotides, a poly(C) tract of 100–420 nucleotides, and a 700-nucleotide long L (long) fragment, which is composed of four pseudo knots (PKs), a *cis*-active replicative element (cre) and a type- II internal ribosome entry site (IRES) (Carrillo et al., 2005; Mason et al., 2003). A 93-nucleotide long 3'UTR is predicted to fold into two stem-loop structures (Serrano et al., 2006).

The S fragments folds into a long stem-loop (Clarke et al., 1987; Newton et al., 1985; Valdazo-Gonzalez et al., 2013) and it has been suggested to be important for FMDV replication, similar to the 5'terminal cloverleaf structure described for other members of the *Picornaviridae* family. FMDV isolates share close to 80% of S fragment nucleotide identity, suggesting a high degree of conservation (Carrillo et al., 2005). Early evidence indicates that host and viral proteins, including unknown 120 kDa and 30–34 kDa host proteins (Serrano et al., 2006), RNA helicase A (Lawrence and Rieder, 2009) and $3C^{pro}$ (Lawrence and Rieder, 2009), specifically bind to the FMDV S fragment. It has also been suggested that the S fragment may interact with the PCBP1 and PCBP2 proteins (Rodriguez-Pulido et al., 2011). Furthermore, Serrano et al. (2006) have provided evidence that the S fragment interacts with the 3'UTR of FMDV in the absence of cellular of viral proteins (Serrano et al., 2006). A similar stem-loop structure has been described for the S fragment of EMCV, although the cardiovirus S fragment is shorter, with only about 85 nucleotides in length (Duke et al., 1992). The highly base-paired cloverleaf structure of the poliovirus 5' UTR and the RNA elements of the 3'UTR (polyA tail) have been

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implicated in protein binding and in the initiation of negative strand synthesis (Herold and Andino, 2001). Many aspects of the S fragment RNA-protein interactions remain to be investigated.

Available biochemical and genetic evidence indicate that a viral infection triggers an innate immune response that is facilitated when an infected cell recognizes a specific molecular signature, known as a pathogen-associated molecular pattern (PAMP). The common PAMPs for RNA viruses include nucleic acid motifs and proteins (Jensen and Thomsen, 2012) and they are recognized by specific pathogen recognition receptor proteins (PRRs) (Jensen and Thomsen, 2012). Once PAMPs are sensed by the receptors, type I interferons (IFNs) and IFN-stimulated genes (ISGs) start being transcribed, which in turn activates the innate immune response. RNA elements encoded in the 5' and 3' UTRs of the FMDV genome, as well as the S fragment alone, could act as PAMPs and induce an antiviral state (Rodriguez-Pulido et al., 2011).

FMDV has been shown to evade the host innate immune response by cleaving the proteins necessary for host cell translation (Grigera and Tisminetzky, 1984) or by inhibiting the IFN response (de Los Santos et al., 2006). At early stages of infection the FMDV L^{pro} protease cleaves the host translation initiation factor eIF4G ablating the host cap-dependent mRNA translation (Devaney et al., 1988). Since FMDV is able to initiate its own translation through the IRES, it does not require eIF4G to translate the viral polyprotein. The L^{pro} also inhibits the induction of IFN-β mRNA and ISGs (de Los Santos et al., 2006). This inhibition is accomplished by degradation of NF-κB by L^{pro}, since the levels of NF-κB decrease dramatically as the viral L^{pro} enters the nucleus during a viral infection (de Los Santos et al., 2007; Grubman et al., 2008).

To gain a better understanding of the S fragment sequence-structural RNA element requirements in FMDV infectivity, we engineered internal deletions within the upper S fragment stem-loop and produced thirteen mutant full length cDNA clones with decreasing S stem-loop lengths. We then examined the effect of these deletions on: (i) FMDV viability, (ii) viral replication and translation, (iii) impact on cellular innate immune response and (iv) virulence in animals. We identified a correlation between the extent of the S fragment deletion in mutant viruses and their ability to trigger an increase in the IFN-β mRNA and several ISG mRNA levels in infected cells. Of note, transfection of primary host cells with mutant S4 fragment RNA (S_4 RNA) resulted in higher cytokine upregulation than the levels observed for the wild type A_{24} Cruzeiro S fragment RNA (intact S RNA). In a mouse model, both live A_{24} FMDV-S_4 or A_{24} FMDV-$S_{4\ 3B3D}$ mutant viruses exhibited a highly attenuated phenotype. Importantly, all inoculated animals survived and developed protective antibody responses against challenge with the wild type A_{24} FMDV. These findings support the relevance of the S fragment in FMDV replication and indicate that FMDV can tolerate deletions in upper S fragment stem-loop to some degree, without impairing virus viability. In addition, we demonstrated that the S fragment influences the induction of innate immune responses in the host cells and impacts virus pathogenesis in animals. Together, this evidence suggests that the engineered FMDV S fragment deletion mutants could aid in the development of safer vaccines candidates for disease control strategies.

2. Results

2.1. Generation of the S fragment deletion mutants

In this study, thirteen S fragment mutant FMDV plasmids (pA_{24} FMDV-S_1 to pA_{24} FMDV-S_{13}) were derived by site-directed mutagenesis using the FMDV pA_{24}Cru (Rieder et al., 2005) infectious cDNA clone backbone (Fig. 1). The S fragment deletions were designed considering the minimal free energy of Mfold predicted S fragment RNA secondary structures (MFold program, see Material and Methods) (Zuker, 2003). Importantly, the progressively shorter S fragments (drawn in Fig. 1B) did not appear to reconfigure the original S fragment predicted RNA secondary structure. The deletion mutants were named A_{24} FMDV-S_1-S_{13}, where A_{24} FMDV-S_1 carries the largest S fragment deletion (288 nt, see Fig. 1B), whereas A_{24} FMDV-S_{13} has the least number (17 nt) of nucleotides deleted. Each plasmid was verified by full-length genome sequencing. The parental and mutant plasmids were then linearized, *in-vitro* transcribed to RNA, electroporated into BHK-21 cells and evaluated for the development of cytopathic (CPE) effect in cell culture. Virus progeny was obtained for mutants A_{24} FMDV-S_{13} to A_{24} FMDV-S_4 RNAs in BHK-21 cells, although some of them required additional passages to produce full CPE (Supplementary Figure 1). In contrast, the S fragment deletions of more than 164 nucleotides from the upper S fragment stem-loop rendered the mutant RNAs (A_{24} FMDV-S_1 to A_{24} FMDV-S_3) non-viable. Two independently derived mutant clones were generated, fully sequenced and electroporated into BHK-21 cells, yet no viruses were derived after a total of eight blind passages (Fig. 1B). Sequencing analysis revealed that the S fragment deletions in the derived recombinant mutant viruses were maintained. Of note, mutant A_{24} FMDV-S_9 acquired 1 synonymous change in VP1 (L61) that did not alter the amino acid sequence (Supplementary Figure 1). All mutant viruses exhibited plaque morphologies (Fig. 1B) and titers in the range of 10^7 PFU/ml in BHK-21 cells similar to the parental wild type virus.

2.2. In vitro characterization of S fragment FMDV mutants

The growth properties of the recombinant viruses were examined in cell lines known to support FMDV replication, including BHK-21 (commonly used to propagate FMDV) and EBK (primary embryonic bovine kidney cells). Both the A_{24} FMDV parental virus and the derived A_{24} FMDV-S_4 mutant produced comparable titers in BHK-21 at multiplicity of infection of 1 (MOI of 1). The growth kinetics of parental A_{24} FMDV and A_{24} FMDV-S_{11}, A_{24} FMDV-S_8 and A_{24} FMDV-S_4 mutant viruses were next examined at MOI of 1 in one-step growth step curves, in the BHK-21 and EBK cell lines (Fig. 2A-B). At 2, 4, 6 and 24 h post infection (hpi), viral samples were taken and virus titers were determined in the BHK-21 cells. The titers of the three S fragment mutants were comparable to the parental A_{24} FMDV virus in BHK-21 cells lacking selective innate pressure at 24 hpi (Fig. 2A), although a slight delay in growth was observed at earlier time points (Fig. 2A). On the other hand, a significant growth attenuation was detected in the primary embryonic bovine kidney (EBK) cell cultures, showing two logs lower yields for the A_{24} FMDV-S_8 and A_{24} FMDV-S_4 mutant viruses, and a half log lower yield for the A_{24} FMDV-S_{11} mutant with respect to the parental virus (Fig. 2B). This data indicated that the growth properties of S fragment deletion mutants in host cells that possess selective innate pressures have changed, resembling those previously reported for attenuated FMDV strains (Uddowla et al., 2012).

2.3. In vitro characterization of A_{24} FMDV-S_4 variants

We were intrigued by the finding that while the A_{24} FMDV-S_4 mutant produces viable virus; removing additional nucleotides from the stem-loop resulted in a complete ablation of viral growth, as seen by the inability to recover viable viruses from A_{24} FMDV-S_3 - A_{24} FMDV-S_1 mutant RNAs. Specific deletions in regions adjacent to the existing deletion in the S_4 mutant were made by site directed mutagenesis to pinpoint the nucleotides whose loss could affect its viability (Fig. 3). First, we engineered GAGAAAU at nt 84–90 to replace the parental C-GCGGAG sequence. As described in Fig. 3A, the Mfold predicted loop structure and base pairing of the S_4 RNA changed as the result of the alteration. Nevertheless, these mutations rendered the genomic RNA non-viable. Fig. 3B displays additional nucleotide alterations at nts 87–91 (GGAGCU changed to CCUUAC) that created a large bulge (bulges contain one or more unpaired nucleotides) in the stem that also abolished viral growth. It is not possible to discriminate whether the lethal effect of multiple-nucleotide substitutions resulted from alterations of the secondary structure or primary sequence, or both. However,