

# Modifications to the foot-and-mouth disease virus 2A peptide; influence on polyprotein processing and virus replication

**Kjær, Jonas; Belsham, Graham**

*Published in:*
Journal of Virology

*Link to article, DOI:*
10.1128/JVI.02218-17

*Publication date:*
2018

*Document Version*
Peer reviewed version

# Modifications to the foot-and-mouth disease virus 2A peptide; influence on polyprotein processing and virus replication

Jonas Kjær[1] and Graham J. Belsham[1*].

1: DTU National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771, Kalvehave, Denmark

*Corresponding author: Graham J. Belsham, email: grbe@vet.dtu.dk

Telephone: +45 3588 7985

Keywords: picornavirus; replicon; FMDV; ribosomal skipping; protease; StopGo

Short title: Role of the FMDV 2A peptide in virus replication

Figures: 7

Tables: 2

Words: 7546

## Abstract

Foot-and-mouth disease virus (FMDV) has a positive-sense ssRNA genome that includes a single, large, open reading frame encoding a polyprotein. The co-translational "cleavage" of this polyprotein at the 2A/2B junction is mediated by the 2A peptide (18 residues in length) using a non-proteolytic mechanism termed "ribosome skipping" or "StopGo". Multiple variants of the 2A polypeptide with this property among the picornaviruses share a conserved C-terminal motif (D(V/I)E(S/T)NPG↓P). The impact of 2A modifications within this motif on FMDV protein synthesis, polyprotein processing and virus viability were investigated. Amino acid substitutions are tolerated at residues $E^{14}$, $S^{15}$ and $N^{16}$ within the 2A sequence of infectious FMDVs despite their reported "cleavage" efficiencies at the 2A/2B junction of only ca. 30-50% compared to wt. In contrast, no viruses were rescued containing substitutions at residues $P^{17}$, $G^{18}$ or $P^{19}$ that displayed little or no "cleavage" activity *in vitro*, but wt revertants were obtained. The 2A substitutions impaired the replication of a FMDV replicon. Using transient expression assays, it was shown that certain amino acid substitutions at residues $E^{14}$, $S^{15}$, $N^{16}$ and $P^{19}$ resulted in partial "cleavage" of a protease-free polyprotein indicating that these specific residues are not essential for co-translational "cleavage". Immunofluorescence studies, using full-length FMDV RNA transcripts encoding mutant 2A peptides, indicated that the 2A peptide remained attached to adjacent proteins, presumably 2B. These results show that efficient "cleavage" at the 2A/2B junction is required for optimal virus replication. However, maximal StopGo activity does not appear to be essential for the viability of FMDV.

## Importance

Foot-and-mouth disease virus (FMDV) causes one of the most economically important diseases of farm animals. Co-translational "cleavage" of the FMDV polyprotein precursor at the 2A/2B junction, termed StopGo, is mediated by the short 2A peptide through a non-proteolytic mechanism which leads to release of the nascent protein and continued translation of the downstream sequence. Improved understanding of this process will not only give a better insight into how this peptide influences the FMDV replication cycle but may also assist the application of this sequence in biotechnology for the production of multiple proteins from a single mRNA. Our data show that single amino acid substitutions in the 2A peptide can have a major influence on viral protein synthesis, virus viability and polyprotein processing. It also indicates that efficient "cleavage" at the 2A/2B junction is required for optimal virus replication. However, maximal StopGo activity is not essential for the viability of FMDV.

## Introduction

Foot-and-mouth disease virus (FMDV) is the causative agent of foot-and-mouth disease, a highly contagious disease of domestic and wild cloven-hooved animal species. The virus has been successfully eradicated from Europe but is still endemic in many regions of the world (in Asia, Africa and the Middle East) and can potentially cause major outbreaks in domestic livestock elsewhere with severe economic losses (reviewed in [1]). FMDV is the prototypic member of the *Aphthovirus* genus within the family *Picornaviridae*. These viruses are small (ca. 25-30nm) and have a positive-sense ssRNA genome [2]. The FMDV genome is ~8.5 kb in length and includes a single, large, open reading frame (ORF) encoding a long polyprotein of over 2300 residues [3]. However, this polyprotein is never observed within infected cells due to rapid co- and post-translational processing to produce, initially, the mature Leader protein ($L^{pro}$) and the precursor proteins P1-2A, P2, and P3. The $L^{pro}$ is a papain-like protease and cleaves the polyprotein at its own C-terminus; that is the junction between $L^{pro}$ and the capsid precursor P1-2A [4, 5]. The 3C protease ($3C^{pro}$) is responsible for proteolytic cleavage of P1-2A to produce the structural proteins VP0, VP3 and VP1 plus the 2A peptide. The P2 and P3 precursors are also processed by $3C^{pro}$ to generate the non-structural proteins, required for the replication of the viral genome. The processing of VP0 to VP4 and VP2 occurs during encapsidation of the viral RNA although it can also occur on assembly of empty capsid particles [6, 7].

The separation of the P1-2A precursor from 2B (within P2) is achieved by yet another mechanism. There is considerable heterogeneity among the picornaviruses with respect to the 2A peptide/protein that is located on the C-terminal side of the capsid protein precursor. Both the size and the function of the different 2A species differ between different picornavirus genera [8]. The entero- and rhinovirus 2A proteins, termed $2A^{pro}$, are thiol proteinases of ~150 amino acids which catalyse the proteolytic cleavage of the junction between the P1 and

P2 precursors at their own N-termini, i.e. at the P1/2A junction [9, 10]. In contrast, in the aphthoviruses and cardioviruses (e.g. encephalomyocarditis virus (EMCV) and Theiler's murine encephalitis virus (TMEV)), the separation of the capsid precursor (P1-2A) from P2 (2BC) occurs at the 2A/2B junction, i.e. at the C-terminus of 2A. In FMDV, the 2A peptide is only 18 amino acids long and lacks any characteristic protease motifs [11–13]. Earlier studies have demonstrated that the "cleavage" at the 2A/2B junction is not dependent on the FMDV proteases $L^{pro}$ or $3C^{pro}$ nor on host cell proteases [12, 14, 15]. The FMDV 2A peptide contains a highly conserved amino acid sequence at its C-terminus, $D^{12}(V/I)E(S/T)NPG_{2A}{}^{\downarrow}P^{19}{}_{2B}$. This sequence induces a co-translational "cleavage" event that is referred to as "ribosomal skipping" [16] or, alternatively, "stop-carry on" or "StopGo" [17, 18]. The first residue of 2B (pro or P) is referred to as $P^{19}$ as it is a key part of the "cleavage" site. This motif, together with upstream amino acids that form an α-helix over most of its length, are believed to interact with the exit tunnel of the translating ribosome and prevent the formation of a peptide bond between the C-terminal amino acid, glycine (gly, or G), of 2A and the first residue, proline (P), of 2B [16, 19]. This produces a break in the growing amino acid chain, but the process of protein synthesis continues, without the requirement for a new translation initiation event. The same conserved motif is also present at the C-terminus of the cardiovirus 2A proteins that are substantially larger; the FMDV 2A sequence appears to be the minimal functional entity to break the growing polypeptide chain (this process will simply be described as cleavage subsequently, for convenience) and other functions have been assigned to the cardiovirus 2A protein as well [20].

The majority of studies on the function of the FMDV 2A peptide have been conducted using *in vitro* experiments with mRNAs encoding artificial polyproteins comprising two reporter proteins linked via the 2A peptide, thus generating two separate translation products (e.g. [16, 21, 22]). These previous studies have shown that specific amino acid substitutions within the

101 FMDV 2A sequence, and especially within the highly conserved $D^{12}(V/I)E(S/T)NPG_{2A}{}^{\downarrow}P^{19}{}_{2B}$
102 motif, drastically reduce the apparent cleavage efficiency and can even block it entirely.
103 These results indicate that these amino acid residues are critical for optimal ribosomal
104 skipping [16, 22]. Furthermore, in the context of a synthetic reporter polyprotein, assayed
105 within CHO cells, four different synonymous codons for residue $G^{18}$ of the 2A peptide were
106 shown to function with very similar apparent cleavage efficiencies at the 2A/2B junction but
107 the cleavage efficiency was not optimal, only 88-89% complete, in this system [23]. These
108 results were interpreted as showing that it is this amino acid residue rather than the nucleotide
109 sequence which is critical for achieving cleavage [23]. The 2A peptide has been shown to
110 mediate cleavage in all eukaryotic translation systems tested, whereas a number of artificial
111 polyproteins containing this sequence have been examined in prokaryotic systems and no
112 detectable cleavage products were observed [22].
113 The less conserved part of the 2A sequence, located upstream of the $D(V/I)E(S/T)NPG_{2A}{}^{\downarrow}P_{2B}$
114 motif, has also been shown to be important for optimal 2A function. Chimeric FMDV,
115 TMEV and EMCV 2A peptides were generated by replacing the N- or C-terminal portions
116 with another 2A variant and then assayed within artificial polyprotein systems where they
117 showed little or no activity [24]. In addition, when the FMDV 2A, in an artificial polyprotein
118 system, was elongated by the addition of up to 30 amino acids, from the upstream VP1, then
119 its apparent cleavage activity was enhanced [16, 22, 25]. Thus, the context of the 2A
120 sequence is important. The 2A peptides from other picornaviruses exhibited similar increases
121 in activity when elongated with 30 amino acids from their respective polyprotein precursors
122 [8]. Moreover, an extensive alanine (A), glycine (G) and proline (P) scanning mutagenesis of
123 the entire FMDV 2A peptide showed a decrease in apparent cleavage activity for all mutants
124 [24]. This supports the view that the specific identity of the amino acid at nearly all positions

within the 2A peptide is important for activity and that 2A peptides are fine-tuned to function as a single unit within their natural polyprotein.

In the studies of Loughran et al. [26], a number of mutations in the 2A coding sequences within the full-length TMEV and FMDV genomes were tested for their effects on virus viability and polyprotein processing. Modification of the SNPG$^{\downarrow}$P$_{2B}$ sequence to SNPL$^{\downarrow}$V$_{2B}$ at the 2A/2B junction blocked polyprotein cleavage. However, this modification had no significant effect on the growth of Theiler's murine encephalomyelitis virus (TMEV) whereas it was detrimental for the replication of mengovirus (another cardiovirus) and apparently lethal for FMDV. Thus, it was concluded that the 2A/P2 cleavage event is not essential for virus viability for certain cardioviruses but is critical for FMDV.

In this study, we have re-investigated the effect of 2A modifications in the context of the native FMDV polyprotein and its effect on virus protein synthesis and replication, virus viability and on polyprotein processing. In contrast to earlier studies, mutant infectious FMDVs having certain amino substitutions within the 2A peptide have been obtained but such changes do adversely affect virus replication and polyprotein processing to some degree.

## Results

### Effect of single amino acid substitutions in 2A on FMDV viability

Several studies using artificial polyprotein systems have demonstrated that nearly all positions of the 2A peptide are important for the "StopGo" activity and modifications can severely impair cleavage [22, 24]. To establish whether the StopGo activity plays a crucial role in FMDV viability, this study set out to investigate the constraints on the 2A sequence within the context of the full-length FMDV genome (see Fig. 1a).

To determine the viability of FMD viruses with single amino acid substitutions within the 2A peptide, selected modifications that were previously found to impair, to different extents, the

StopGo activity in artificial polyproteins systems [22, 24] were introduced into the plasmid, pT7S3, that contains the full length FMDV cDNA [27], using site-directed mutagenesis (see Methods). The resultant plasmids were linearized and RNA transcripts, prepared *in vitro*, were introduced into BHK cells by electroporation. Unexpectedly, all of the FMDV 2A mutants (Table 1) produced viable progeny viruses, with full CPE detectable after the second passage. The rescued viruses were then sequenced to identify possible adaptations or reversions (Table 1). After three passages of the 2A mutant viruses in cells, the viruses rescued from the transcripts encoding the N16H, E14Q, S15F and S15I modifications, had each retained the plasmid-derived amino acid substitutions. In contrast, the rescued viruses derived from the N16A, G18, P17A, P19G and P19A mutant transcripts all matched the wt sequence (i.e., the rescued viruses were not mutant) even when 2 nt changes were required to achieve this (e.g., see Fig. 2 for P19A mutant). To determine whether this reflected reversion to the wt sequence or some form of contamination/carryover of the wt sequence, three synonymous substitutions were inserted ca. 20 nucleotides downstream of the 2A/2B junction in the wt and the N16A, P17A, G18A and P19A mutant plasmids (see Fig. 2) after which the "marked" RNA transcripts were introduced into BHK cells. After three passages, the rescued mutant viruses had lost the 2A modification in each case but had, like the "marked" wt virus, each retained the three synonymous substitutions in the 2B coding region (see Fig. 2); this provides strong evidence that the presence of the wt sequence in the rescued viruses reflects reversion.

The growth characteristics of the wt and the viable 2A mutant viruses in BHK cells were examined in more detail by determining growth curves using a multiplicity of infection (m.o.i.) of 0.1. Surprisingly, both the wt and the viable 2A mutants grew with similar kinetics (Fig. 3a). Analysis of the FMDV capsid proteins within cells infected with the wt and the viable 2A mutant viruses, as determined by immunoblotting using anti-FMDV, is shown in

175 Fig. 3b. As expected, the production of the capsid proteins was similar for the wt and the 2A
176 mutants in each of the infected cell extracts (Fig. 3b, lanes 1-5).

177

178 **Requirements for efficient 2A/2B "cleavage" in its native context**

179 To examine the effects of the 2A mutants on the StopGo cleavage at the 2A/2B junction in its
180 natural context and in cells, a plasmid encoding a truncated FMDV polyprotein termed the
181 P1-2A-2BC-FLAG protein with a FLAG epitope at its C-terminus was generated (see Fig.
182 1d). The transient expression of this truncated viral polyprotein (without any proteases) was
183 designed to permit the simultaneous assessment of the production of the uncleaved P1-2A-
184 2BC-FLAG (ca. 135 kDa) and of the "cleavage" product 2BC-FLAG (ca. 54 kDa). The
185 coding sequences for the wt or mutant P1-2A-2BC-FLAG products were under the control of
186 the T7 promoter. The plasmids, were transfected into BHK cells that had been infected with
187 the recombinant vaccinia virus vTF7-3 [28] which expresses the T7 RNA polymerase. The
188 expression and processing of the proteins generated from these plasmids was visualized in
189 immunoblots using anti-FLAG antibodies. Expression of the wt cassette led to apparently
190 complete cleavage of the P1-2A-2BC-FLAG polyprotein as expected (Fig. 4a, lane 1) and
191 thus only the 2BC-FLAG product was observed. In contrast, the E14Q mutant generated a
192 mixture of both uncleaved and cleaved products (Fig. 4a, lane 5). Unexpectedly (c.f. [22,
193 24]), in the system used here, the S15A, S15F and S15I mutant proteins were each efficiently
194 cleaved (Fig. 4a, lanes 6-8). The mutants N16C, N16H, P19A, P19G, P19V and P19S all
195 produced a mixture of cleaved and uncleaved products (Fig. 4b, lanes 2, 3 and Fig. 4c, lanes
196 1-4). However, the D12A, V13A, E12A, N16A, N16V, N16W, P17A and G18A substitutions
197 resulted in the production of only the uncleaved product and hence these mutant 2A peptides
198 were all inactive in this system (Fig. 4a, lanes 2, 3, 4 and Fig. 4b, lanes 1, 4, 5, 6, 7). Overall,
199 there is partial agreement between the results described here, using assays of the 2A in its

near native context within cells, and those described previously [22, 24]. The main discrepancies concern the S15A, S15F and S15I mutants which resulted in essentially complete cleavage (≥90%) here but gave rather sub-optimal cleavage (42 and 39% of wt, respectively) *in vitro* [22] while the P19A, P19G, P19V and P19S mutants resulted in detectable, but low level, cleavage (8-20%) here but completely abrogated cleavage *in vitro* [22]. The same cell lysates were also analysed using an anti-FMDV capsid protein antibody to detect the intact polyprotein and the P1-2A product (data not shown). The pattern of results was fully consistent with those obtained using the anti-FLAG to detect the intact polyprotein and the 2BC-FLAG product. Thus it seems that the efficiency of cleavage detected in this assay system is higher than that observed using cell-free translation systems *in vitro*.

**Influence of the amino acid substitutions in FMDV 2A on FMDV RNA replication efficiency assessed using a replicon that expresses the *Gaussia* luciferase**

To evaluate the impact of the 2A mutants on the replication of viral RNA, nine different substitutions within the 2A coding sequence were introduced into a FMDV replicon (see Fig. 1b). In this replicon, the coding sequences for the FMDV structural proteins (VP1-VP3) have been replaced by the sequence encoding the *Gaussia* luciferase (Gluc) reporter protein, thus allowing replication to be readily monitored via measurement of Gluc expression. RNA transcripts were produced *in vitro* from the linearized plasmids and introduced into BHK cells using electroporation. As a negative control, a derivative of the wt-Gluc replicon was produced which lacks a portion of the coding sequence for 3Dpol (the RNA dependent RNA polymerase) and is termed wt-GlucΔ3D (see Fig. 1c). Lysates were prepared from cells at various times after electroporation with the different transcripts and assayed for Gluc activity (see Fig. 5). The wt-Gluc-Δ3D transcript produced Gluc initially, that was already detectable at 1 h post-electroporation, but no further increase in luciferase activity was observed after 2

h. This expression presumably represents the translation of the input RNA. In contrast, the replication-competent wt-Gluc, while generating an initially similar level of Gluc activity at 2 h, showed a sustained increase in expression at later time points. Interestingly, all of the 2A mutants expressed low levels of Gluc activity initially, almost 10-fold less than the wt-GlucΔ3D at 2 h. However, the expression increased to some degree at later time points; the level of Gluc expression first surpassed the polymerase knockout mutant after 6 h. It is noteworthy that the mutant transcripts with the E14Q, S15F, S15I and N16H changes, which were retained in the rescued viruses, did not have better RNA replication efficiencies than the other 2A mutants. This may reflect, to some degree, sub-optimal cleavage at the 2A/2B junction due to the absence of the upstream VP1 coding sequences in these replicons (see [25]).

**Influence of the StopGo function on the correct processing of the FMDV P1-2A precursor**

Hahn & Palmenberg [29] demonstrated that amino acid substitutions within the conserved D(V/I)E(S/T)NPG$_{2A}$$^{\downarrow}$P$_{2B}$ motif at the C-terminus of the 2A protein of EMCV not only severely reduced or abrogated the StopGo function but also impaired the subsequent cleavage of L-P1-2A by 3C$^{pro}$. The effects of substitutions in 2A on the FMDV P1-2A processing in cells has now been assayed using the truncated FMDV polyprotein termed P1-2A-2BC-FLAG (as above, see Fig. 1d) which was co-expressed with the FMDV 3C$^{pro}$. The wt and mutant P1-2A-2BC-FLAG plasmids encoding the N16A, P17A, G18A and P19A substitutions (shown in Fig. 4 to abrogate or impair (P19A) cleavage) were transfected, alone or together with a plasmid that expresses the 3C$^{pro}$ (as in [7]), into vTF7-3-infected BHK cells. Analysis of the FMDV P1-2A processing, was determined by immunoblotting using anti-VP2 antibodies and is shown in Fig. 6a. Expression of the wt plasmid alone led to

250 complete cleavage at the 2A/2B junction of the P1-2A-2BC-FLAG polyprotein, to yield P1-
251 2A, as expected (Fig. 6a, lane 1). Furthermore, co-expression of the wt product with the $3C^{pro}$
252 produced VP0 (from the P1-2A) also as expected (Fig. 6a, lane 2). When the mutant
253 plasmids, with defective cleavage at the 2A/2B junction, were expressed alone then the
254 larger, intact, P1-2A-2BC-FLAG product was detected (Fig. 6a, lanes 3, 5, 7, 9), as above
255 (see Fig. 3). In the presence of the $3C^{pro}$, the production of VP0, derived from P1-2A (both
256 detected with an anti-VP2 monoclonal antibody), was still readily apparent in each case (Fig.
257 6a, lanes 4, 6, 8, 10). These results were confirmed by immunoblotting using anti-FMDV
258 antibodies (Fig. 6b). Co-expression of the wt and mutant plasmids with the $3C^{pro}$ produced a
259 very similar pattern of detectable capsid proteins in each case (Fig. 6b, lanes 2, 4, 6, 8, 10).
260 Thus, abrogating cleavage at the 2A/2B junction did not block the processing of the capsid
261 precursor by $3C^{pro}$ in this system. It should be noted that this is in contrast to some earlier
262 studies [14], which showed that a truncated version of FMDV P1-2A (lacking the C-terminus
263 of VP1) could not be processed at all by $3C^{pro}$.
264
265 **Detection of a novel FMDV 2A-2B fusion protein using immunofluorescence**
266 The FMDV capsid protein precursor, P1-2A, is normally processed by the $3C^{pro}$ to VP0, VP3,
267 VP1 and 2A. In previous studies, it has been shown that when the cleavage of the VP1/2A
268 junction is impaired, then the presence of FMDV 2A (still attached to VP1, as VP1-2A) can
269 be detected in BHK cells by immunofluorescence using anti-2A antibodies [7, 30]. When the
270 2A is released from the VP1 then the 2A is no longer detectable (presumably it is either
271 degraded or not fixed in the procedure). Thus, it seemed possible that substitutions within the
272 2A peptide that impair the 2A/2B cleavage activity (and prevent formation of viable, mutant,
273 viruses), would result in the formation of detectable 2A-2B fusion proteins. Full-length
274 FMDV RNA transcripts, with or without modifications in 2A, were introduced into BHK

cells by transfection and after 8 hrs, the cells were stained with either anti-2A antibodies or anti-FMDV capsid protein antibodies. The FMDV VP1 K210E mutant, as previously described [7], which produces an uncleaved VP1-2A protein, was included as a positive control for the detection of 2A attached to an adjacent protein. FMDV capsid proteins could be detected in cells transfected with each of the RNA transcripts, as expected (see Fig. 7b-g). In contrast, no signal for the 2A peptide was observed in cells transfected with the wt O1K RNA (Fig. 7b) or in untransfected cells (Fig. 7a). However, the presence of FMDV 2A (still attached to VP1) was detected in cells transfected with the VP1 K210E mutant RNA (Fig. 7c), consistent with previous results [7, 30]. Furthermore, using the transcripts with the mutant 2A/2B junctions, the presence of FMDV 2A, presumably attached to 2B (and maybe VP1), could be detected in the transfected cells (Fig. 7d-g). It should be noted that it is not possible to detect the free 2A peptide by immunoblotting due to its small size (ca. 2 kDa) and attempts to identify the presence of the 2A fused to other proteins in extracts from these RNA transfected cells were unsuccessful (c.f. detection of VP1-2A within cells infected with the VP1 K210E mutant virus [7, 30]), presumably because the 2A could be attached to a number of different proteins, e.g. within 2A-2B, 2A-2BC, VP1-2A-2B and VP1-2A-2BC and not all cells take up and replicate the RNA transcripts.

## Discussion

The 2A peptide plays a significant role in the FMDV life cycle as it is required for the co-translational cleavage of the growing polyprotein into two separate entities at the junction between 2A and 2B. Related 2A peptide sequences are found in a variety of other members of the picornavirus family; this suggests that they contribute significantly to the correct production and function of the viral proteins.

299 Using artificial polyprotein systems, it has been well documented [18, 22, 24, 26] that point
300 mutations in the highly conserved $D^{12}$(V/I)E(S/T)NP$G_{2A}{}^{\downarrow}P^{19}{}_{2B}$ motif, located at the C-
301 terminus of FMDV 2A, can either severely reduce or completely abrogate cleavage activity.
302 In this study, we have extended these observations and investigated the effects of single
303 amino acid substitutions in 2A on FMDV RNA replication, on virus viability and on
304 polyprotein processing in its natural context within cells. The results presented here clearly
305 demonstrate that certain 2A mutants previously found to greatly impair the StopGo activity in
306 artificial polyproteins systems [22, 24] were still able to produce infectious viruses and thus
307 the wt sequence and maximal cleavage activity is not essential for virus viability. It was
308 anticipated that some mutations might have resulted in lethal phenotypes since earlier
309 mutagenesis studies using FMDV and EMCV did not produce any viable progeny when the
310 C-terminal 2A sequence was changed from SNPG$^{\downarrow}P_{2B}$ to SNPL$^{\downarrow}V_{2B}$ even after several
311 passages [26]. Interestingly, we were able to rescue viruses from all of the RNA transcripts.
312 When the apparent cleavage activity of the mutant 2A was low (<31% of wt activity) then it
313 was found that reversions to the wild type sequence had occurred. This indicates that some
314 RNA replication must have occurred (to allow the formation of wt revertants) despite the
315 presence of a defective 2A peptide. In contrast, mutants with a higher level of cleavage
316 activity (≥31% of wt) retained, in each case, the introduced amino acid substitutions in the
317 rescued viruses. These results clearly indicate that efficient 2A mediated cleavage activity is
318 advantageous for the virus but that optimal efficiency is not essential. This raises the question
319 of why the separation of the capsid proteins from the non-structural proteins is so
320 advantageous for some picornaviruses? It seems necessary for these viruses to have a 3C-
321 independent mechanism to break the polyprotein. Some members of the picornavirus family
322 (e.g. enteroviruses) possess a 2A protease to achieve the separation of the capsid protein
323 precursor from the rest of the polyprotein and the StopGo mechanism that occurs at the

2A/2B junction is clearly a distinct mechanism but one that is used by many members (e.g. aphthoviruses, cardioviruses, sapeloviruses, teschoviruses) of this virus family [8].

It has been speculated [13] that 2A can act as a translational regulator to modify the amount of the different parts of the polyprotein that are produced. In FMDV, the 2A peptide is located at the boundary between the upstream capsid proteins and the non-structural proteins involved in RNA replication. There could be two distinct functions for the 2A peptide. One primary function of 2A could be to achieve the cleavage of the polyprotein but it may also down-regulate downstream translation. Potentially, this could prove beneficial to the virus as the assembly of the FMDV capsid requires up to sixty copies of each of the four structural proteins whereas fewer copies of the proteins involved in the replication process are required. On the other hand, it could be considered that in the early stages of the virus infection, then it would seem advantageous to produce more of the proteins required for replication and processing than the capsid proteins. It is also noteworthy that most members of the picornavirus family that use a different mechanism for separation of the capsid proteins from the non-structural proteins do not apparently have any mechanism for modifying the ratio of proteins produced, thus the need for such a mechanism within the picornaviruses, in general, is not established. However, recently, Napthine et al., [31] have demonstrated that in EMCV a programmed -1 ribosomal frameshift occurs within the 2B coding region, just downstream of the 2A coding region. This frameshift results in the production of a distinct protein, termed 2B$^{*}$, and then termination of translation. The level of ribosomal frame shifting increases dramatically late in infection and thus the production of the non-structural proteins involved in virus replication is reduced at this time. The process requires the interaction of the EMCV 2A protein (ca. 16 kDa) with a stem-loop structure some 14 nt downstream of a “slip site” (GGUUUUU) within the 2B coding region. Although a U-rich motif (UUCUUUUUCU) is present just downstream of the coding region for the 2A/2B junction in the FMDV genome,

certain other elements of this process appear to be absent. As indicated above, the FMDV 2A is only 18 residues long and it lacks the cluster of basic residues (R95-R97) that appear to be important for the interaction of the EMCV 2A protein to the stem-loop structure that is critical for the high frameshift efficiency. Thus, currently, there is no evidence for such a process within FMDV.

Assessment of the RNA replication efficiency, using a replicon system, demonstrated that alterations in the 2A peptide have a clear, negative, effect on either the replication of the viral RNA or on the translation of the polyprotein. Clearly, the processes of translation and replication are linked since when translation of the polyprotein is reduced, then the levels of protein available to replicate the RNA are also reduced resulting in a lower level of RNA replication. As indicated above, it may be that the detrimental effect of the changes in 2A were accentuated by the absence of the VP1 coding sequence in the replicons. In the context of the full-length viral polyprotein, it was shown (see Figure 7) that blocking the cleavage at the FMDV 2A/2B junction produced fusion proteins containing 2A (presumably as 2A-2B or possibly VP1-2A-2B, before or after the cleavage of the VP1/2A junction by $3C^{pro}$). However, the addition of just 18 amino acids to the N-terminus of the 2B protein may be considered to be unlikely to cause this decrease in replication efficiency (indeed it has been shown that leaving the 2A peptide fused to the C-terminus of VP1 has no apparent effect on virus viability [7, 30]. It should be remembered, however, that the VP1/2A cleavage is the slowest of the 3C-mediated processing events within P1-2A [7, 14, 30]. Previously it has been found that cleavage at the VP1/2A junction in poliovirus appears to a have a role in processing of the capsid precursors since amino acid substitutions that prevented cleavage resulted in a P1 capsid precursor which was resistant to $3C^{pro}$ processing [10]. Furthermore, Hahn & Palmenberg demonstrated, using *in vitro* translation assays, that a mutation in the EMCV 2A impaired the processing of the L-P1-2A precursor by $3C^{pro}$ [29]. This may suggest

374 a critical role for the 2A cleavage to allow proper folding of the (L)-P1-(2A) precursor to
375 permit efficient cleavage by $3C^{pro}$. However, in our studies, blocking the cleavage at the
376 2A/2B junction did not block the processing of P1-2A by $3C^{pro}$ (see Fig. 6). It was also
377 observed with TMEV that normal capsid protein processing occurred in mutant viruses in
378 which the 2A/2B processing was blocked [26].
379 The *Gluc* replicon, as used here, lacks the coding sequences for the structural proteins except
380 for VP4, however, the replication / translation is still impaired in the 2A mutants compared to
381 the wild type (Fig. 5). It is, therefore, conceivable that the possible cleavage restrictions that
382 could govern the processing of the structural proteins also apply to the non-structural
383 proteins. This may mean that correct processing of these proteins, which are required for
384 RNA replication, is impaired, thereby resulting in lower RNA synthesis. Although the
385 processing of the FMDV P1-2A by $3C^{pro}$ appears to be unaffected when the 2A peptide is
386 mutated (Fig. 6), this does not rule out the possibility of a negative effect on the 2B-2C (or
387 P3) processing. Surprisingly, there was relatively little difference in the growth
388 characteristics between the viable 2A mutant viruses (E14Q, S15F, S15I and N16H) and the
389 wt (Fig. 3), which contrasts with the decrease in replication efficiency observed in the context
390 of a FMDV replicon. This could suggest that the changes in the 2A peptide influence the
391 initial rate of viral RNA replication but not the final virus yield.
392 Investigation of the effect of 2A mutations on the StopGo mechanism revealed that certain
393 amino acid substitutions are severely detrimental for the proper function of the 2A whereas
394 others only moderately impair the cleavage resulting in a mixture of products (some cleaved
395 and others not, see Fig. 4). Previous studies have suggested that the 2A geometry is the
396 determining factor for its function [19, 22]. The current hypothesis is that the N-terminal
397 portion of 2A (in an α-helical conformation) interacts with the ribosomal exit tunnel to confer
398 specific constraints required for the turn motif (ESNPG) to be in a position to influence

events within the peptidyl transferase centre of the ribosome. Some amino acid substitutions could severely change the conformation of the 2A, thereby preventing the disruption of the peptide bond formation between the G and P residues, and hence result in an uncleaved polyprotein. The substitutions N16C and N16H were found to result in cleavage although with decreased efficiency (both cleaved and uncleaved products were observed, see Fig 4). The function of residue N16 within 2A has not yet been determined, however it has been suggested that the N16 forms a hydrogen bond with E14 to stabilise the right turn [22]. The substitutions S15A, S15I and S15F were found to result in essentially complete cleavage in contrast to earlier studies [22] that reported a reduction in the cleavage activity. Comparison of the 2A sequence from different picornavirus species has shown that a variety of amino acids are allowed at this position within the C-terminus of 2A suggesting that this particular amino acid is of low importance for the StopGo function. However, Sharma et al. [24] demonstrated that substitution of S15 by glycine (G) (in the FMDV sequence), which influenced the peptide secondary structure, impaired function more significantly than Ala or Pro substitutions, suggesting that increased backbone flexibility imposed by the Gly residue at this position was especially detrimental [24].

Interestingly, the substitutions P19A, P19G, P19V and P19S greatly reduced the level of cleavage but did not abolish it (see Fig. 4). This is in contrast to previous studies [22] which have reported that these amino acid substitutions resulted in no apparent cleavage activity in an artificial polyprotein system. A model for the mechanism of 2A mediated cleavage developed by Donnelly et al. [16] suggests that the P19 residue (at the N-terminus of 2B) is an absolute requirement for cleavage as a poor nucleophilic character in this position is an integral part of the proposed mechanism. However, our data clearly shows that ala (A), ser (S) and val (V) residues are also functional at this position albeit with reduced activity. Rychlík et al. [32] demonstrated that A, S, and V are, in fact, also poor nucleophiles in the

context of ribosomal peptidyl transferase activity, however not to the same extent as P and G. This could explain why these amino acids are able to support the cleavage activity to some degree although not at a level compatible with virus viability. Although this does not account for the reduced cleavage activity observed for the P19G mutants, suggesting that another, not yet identified, characteristic of residue 19 must apply.

The study by Gao et al. [23] found that the codon usage for the NPGP motif is conserved among the seven FMDV serotypes. Through the use of mRNAs encoding artificial polyproteins comprising two reporter proteins, assayed within CHO cells, the study investigated the role of synonymous codons for the $G^{18}$. It was concluded that the different synonymous codon usage for $G^{18}$ did not influence the cleavage efficiency in that system. However, in separate studies, we have provided evidence that a clear codon bias operates to encode the NPG/P motif at the 2A/2B junction within FMDV-infected cells [33]. This raises the interesting possibility that the RNA sequence itself contributes to the cleavage event at the 2A/2B junction.

## Materials and methods

### Construction of plasmids containing full-length mutant FMDV cDNAs

The plasmid pT7S3 [27] contains the full-length cDNA for the O1Kaufbeuren B64 strain of FMDV. Modification of the coding sequence around the 2A/2B junction was achieved by a 2-step site-directed mutagenesis procedure, a variation of the QuickChange protocol (Stratagene), using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). The first round of PCR, using forward mutagenic 2A PCR primers (Table 2) with a single reverse primer 10PPN10 (Table 2) and the plasmid pT7S3 as template, generated an amplicon (ca. 450 bp) specifying particular amino acid substitutions within 2A. The primary PCR products were gel purified (GeneJet gel extraction kit, Thermo Fisher Scientific) and used as primers

449 for a second round of PCR with plasmid pT7S3 as template. The *Dpn-I* resistant full-length
450 products were selected in chemically competent *Escherichia coli* (*E.coli*) TOP10 cells
451 (Thermo Fisher Scientific), amplified, then the plasmid DNA was purified (Midiprep kit;
452 QIAGEN) and verified by sequencing of the 2A coding region with a BigDye Terminator v.
453 3.1 Cycle Sequencing kit and a 3500 Genetic Analyzer (Applied Biosystems).
454 The generation of plasmids with three synonymous mutations ca. 20 bp downstream of the
455 modified 2A/2B junction was achieved essentially as described above. The first round of
456 PCRs, used the forward mutagenic 2A_Synonymous_Fwd primer (Table 2) with a single
457 reverse primer 10PPN10 (Table 2) and plasmid pT7S3 as template. The primary PCR
458 products were gel purified (GeneJet gel extraction kit, Thermo Fisher Scientific) and used as
459 primers for a second round of PCR with modified versions of the pT7S3, with the codons for
460 $N^{16}$, $P^{17}$, $G^{18}$ or $P^{19}$ changed to encode an alanine (A) residue in each case, as templates.
461
462 **Construction of plasmids containing a FMDV replicon containing *Gaussia* Luciferase**
463 The *Gaussia* luciferase (Gluc) FMDV replicon was constructed by replacement of the coding
464 region for VP2, VP3, VP1 and 2A from pT7S3-NheI [34] with the coding region for Gluc
465 fused to FMDV 2A (as used in [35]). The Gluc-2A sequence was amplified by PCR using
466 primers 13APN1 and 13APN4 (see Table 1) using the rPad2GL BAC (see [35]) as template.
467 The amplicon was inserted into the vector pCR-XL-TOPO (Invitrogen), the *NheI-ApaI*
468 fragment was excised and inserted between the same sites within the ca. 5kb *XbaI*-fragment
469 from pT7S3-*NheI* (essentially as described previously [34]). The modified *XbaI* fragment
470 (now containing the Gluc-2A sequence) was reconstructed into the backbone of the O1K
471 FMDV cDNA within the *XbaI*-digested pT7S3 [27] and the orientation established by
472 restriction digestion (using *EcoRI* and *HpaI*). The Gluc FMDV replicon was termed wt-Gluc.

473 The replication-defective Gluc FMDV replicon termed wt-GlucΔ3D was prepared by
474 digesting the wt-Gluc plasmid with *BamHI* and *HpaI* to liberate a fragment of ca. 770 bp
475 corresponding to the 3'-terminus of the FMDV genome (including part of the 3Dpol coding
476 region, see Fig. 1). The large residual fragment was gel purified, blunt ended, self-ligated and
477 transformed into *E. coli*. The wt-GlucΔ3D plasmid DNA was purified (Midiprep kit;
478 QIAGEN) and verified by sequencng of the 3Dpol coding region, as above.
479
480 **Construction of plasmids containing FMDV P1-2A-2BC-FLAG cDNA cassettes**
481 The FMDV cDNA cassette, in the plasmid pP1-2A-2BC-FLAG, was prepared by PCR using
482 Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Briefly, the coding
483 region for P1-2A-2BC from O1K FMDV cDNA (as in pT7S3, [27]) was amplified with a
484 forward primer ATG_P1_fwd which incorporates an initiation codon and the reverse primer
485 2C_FLAG_Stop_rev that includes the sequence for a FLAG epitope tag followed by a
486 termination codon (see Table 2). The blunt-end amplicon (ca. 3670 bp) was ligated into the
487 pJET1.2 vector (Thermo Fisher Scientific) according to the manufacturer's instructions.
488 Sequencing revealed an unwanted initiation codon between the T7 promoter and the insert,
489 which was then removed. A 2-step site-directed mutagenesis PCR using Phusion High-
490 Fidelity DNA Polymerase as previously described with mutagenic PCR 2A primers (Table 2)
491 and 10PPN10 was used to produce the following plasmids encoding the indicated single
492 amino acid substitutions within 2A: pP1-2C-FLAG D12A, V13A, E14A, E14Q, S15A, S15F,
493 S15I, N16A, N16C, N16W, P17A, G18A, P19A, P19G, P19V and P19S. All plasmids were
494 propagated in *E. coli* TOP10 cells (Thermo Fisher Scientific), purified (Midiprep kit;
495 QIAGEN), and verified by sequencing.
496
497 ***In vitro* transcription**

498 Briefly, 5 µg of replicon plasmid or full-length FMDV plasmid were linearized by digestion
499 with *HpaI* (Thermo Fisher Scientific), purified (GeneJET PCR Purification Kit, Thermo
500 Fisher Scientific) and eluted in RNase-free water. Both replicon and full-length FMDV RNA
501 transcripts were prepared using the Megascript T7 kit (Ambion). Reaction mixtures were
502 incubated at 37°C for 4 h and treated with 2 units of TURBO DNase for 30 min after which
503 the RNA was purified using the MEGAclear Transcription Clean-Up Kit according to the
504 manufacturer's instructions. RNA integrity was assessed by electrophoresis using an
505 ethidium bromide-stained agarose gel (1%), in TBE buffer, and quantified by
506 spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific).
507
508

509 **Rescue of virus from full-length cDNA plasmids**

510 For rescue and passage of infectious FMDV, 5 µg full-length FMDV RNA was introduced
511 into BHK cells by electroporation (as described previously [36]). The cells were then
512 transferred to one well of a 6-well plate and incubated for 1-3 days at 37°C after which the
513 viruses were harvested by freezing. The rescued viruses were then amplified using additional
514 passages (P2 and P3) using fresh BHK cells. After the third passage (P3), viral RNA was
515 extracted (RNeasy Mini Kit, Qiagen) and converted to cDNA using ready-to-go you-prime
516 first-strand beads (GE Healthcare Life Sciences) with random hexamer primers. Amplicons
517 (ca. 660 bp), including the 2A coding region, were amplified by PCR (AmpliTaq Gold DNA
518 polymerase, Thermo Fischer Scientific) using the primers 8APN206 and 8APN203 (see
519 Table 2). Control reactions, without RT, were used to ensure that the analysed products were
520 derived from RNA and not from the presence of carryover plasmid DNA template. The
521 amplicons were visualized in 1% agarose gels, purified (GeneJET gel extraction kit, Thermo
522 Fischer Scientific) and sequenced as above. Sequences were analysed using Geneious 7.2
523 (Biomatters, Auckland, New Zealand).

524

525 ***Gaussia* luciferase assay**

526 BHK cells suspended in cold PBS were transferred to a 4 mm cuvette after which 2 µg
527 replicon RNA was added, briefly mixed, and the cells were electroporated (25 ms and 240 V;
528 one pulse) on a Gene Pulser X-Cell (Bio-Rad). Following incubation for 10 min at room
529 temperature, the cells were transferred to 5 wells of a 24-well plate (140 µl per well with 500
530 µl DMEM containing 5% FCS). Following incubation, at 37°C for the required time, the
531 medium was removed and the BHK cells were lysed by adding 100 µl of *Renilla* luciferase
532 assay lysis buffer (Promega) to the cells in each well (24-plate well) and incubated at room
533 temperature for 15 min. The luciferase activity was quantified in a Luminometer (Titertek-

534 Berthold) by addition of this lysate (20 µl) to Renilla Luciferase Assay reagent (100 µl)
535 according to the manufacturer's instructions.
536
537 **Virus growth kinetics**
538 Virus titres for the wt and the 2A mutant viruses: E14Q, S15I, S15F and N16H were
539 determined in BHK cells as $TCID_{50}$/ml, as described previously [37]. Monolayers of BHK
540 cells, grown in 96-well plates were infected with either wt or mutant FMDV at an m.o.i of 0.1
541 at 37°C. At 0, 2, 5, 10 and 24 hours post infection the infected cells were harvested by
542 freezing (at -80°C) to determine the virus yield as $TCID_{50}$/ml.
543
544 **Transient expression assays**
545 BHK cells (in 35mm wells) were grown to 90 % confluency and infected with vTF7-3, a
546 recombinant vaccinia virus that expresses the T7 RNA polymerase [28], as described
547 previously [38]. Briefly, following the infection, plasmid DNA (pP1-2A-2BC-FLAG and its
548 derivatives, 2µg) was transfected alone or, when indicated, with pSKRH3C (50 ng) [39] that
549 expresses FMDV $3C^{pro}$, using FuGene6 (Roche), into the infected BHK cells and incubated
550 overnight at 37°C.
551
552 **Western blotting**
553 Cell lysates for immunoblotting were prepared by addition of cold Buffer C (0.125 M NaCl,
554 20 mM Tris/HCl (pH 8.0), 0.5% NP-40) to the cells. After incubation (on ice, for at least 5
555 mins), the cell extracts were clarified by centrifugation (20000 g for 10 min) and Laemmli
556 sample buffer (with 25 mM DTT) was added (as described previously [40]) . Following
557 heating to 98°C for 5 min, samples were resolved by SDS-PAGE (4-15% polyacrylamide)
558 and transferred to a PVDF membrane (Bio-Rad) and blocked for 1 h in 5% PBS-Tween

(PBS, 0.1% Tween) with 5% non-fat milk. The membranes were incubated overnight at 4°C with either goat anti-FLAG antibodies (Abcam), guinea pig anti-FMDV O1 Manisa serum (to detect FMDV capsid proteins) or mouse anti-FMDV VP2 (4B2) monoclonal antibody [41], as used previously [7]. The membranes were washed 3x with PBS-Tween and incubated for 3 hours at room temperature with either HRP-conjugated anti-goat IgG (Dako), HRP-conjugated anti- guinea pig IgG (Dako) or HRP-conjugated anti-mouse IgG (Dako), respectively. The membranes were then washed 3x with PBS-Tween and bound proteins were detected using a chemiluminescence detection kit (ECL Prime, Amersham) with a Chemi-Doc XRS system (Bio-Rad). The intensities of the signals for the FLAG-tagged polyproteins were, when necessary, quantitated using ImageJ software (v1.50).

**Immunofluorescence assay**

Monolayers of BHK cells were grown on glass coverslips in 6 well plates and immediately prior to transfection, cells were washed briefly in PBS and the medium replaced with DMEM without serum. FMDV RNA transcripts were introduced into BHK cells using Lipofectin transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. After 8 h, the cells were fixed, stained and mounted as described previously [7, 30] using rabbit anti-FMDV O serum or rabbit anti-2A (ABS31, Merck) followed by a donkey Alexa Fluor 568-labelled anti-rabbit IgG (A10042, Life Technologies). The slides were washed in PBS after which they were mounted with Vectashield (Vector Laboratories) containing DAPI and images were captured using an epifluorescence microscope.

582 **Acknowledgements**

583 We wish to thank Li Yu (Chinese Academy of Agricultural Sciences, China) for providing us
584 with the anti-FMDV VP2 antibody. We would also like to acknowledge the excellent
585 technical assistance of Preben Normann and helpful advice from Thea Kristensen.

586 **References**


587 1. **Jamal SM, Belsham GJ**. 2013. Foot-and-mouth disease: past, present and future. Vet
588 Res 44:116–129.

589 2. **Martinez-Salas E, Belsham GJ**. 2017. Genome Organisation, Translation and
590 Replication of Foot-and-mouth Disease Virus RNA, p. 13–42. *In* Sobrino, F,
591 Domingo, E (eds.), Foot-and-Mouth Disease: Current Research and Emerging Trends.
592 Caister Academic Press.

593 3. **Belsham GJ**. 2005. Translation and replication of FMDV RNA. Curr Top Microbiol
594 Immunol 288:43–70.

595 4. **Medina M, Domingo E, Brangwyn JK, Belsham GJ**. 1993. The two species of the
596 foot-and-mouth disease virus leader protein, expressed individually, exhibit the same
597 activities. Virology 194:355–359.

598 5. **Strebel K, Beck E**. 1986. A second protease of foot-and-mouth disease virus. J Virol
599 58:893–899.

600 6. **Curry S, Fry E, Blakemore W, Abu-Ghazaleh R, Jackson T, King A, Lea S,**
601 **Newman J, Stuart D**. 1997. Dissecting the roles of VP0 cleavage and RNA packaging
602 in picornavirus capsid stabilization: the structure of empty capsids of foot-and-mouth
603 disease virus. J Virol 71:9743–9752.

604 7. **Gullberg M, Polacek C, Bøtner A, Belsham GJ**. 2013. Processing of the VP1/2A
605 junction is not necessary for production of foot-and-mouth disease virus empty capsids
606 and infectious viruses: characterization of "self-tagged" particles. J Virol 87:11591–
607 11603.

608 8. **Luke GA, de Felipe P, Lukashev A, Kallioinen SE, Bruno EA, Ryan MD**. 2008.

609 Occurrence, function and evolutionary origins of "2A-like" sequences in virus
610 genomes. J Gen Virol 89:1036–1042.

611 9. **Sommergruber W, Zorn M, Blaas D, Fessl F, Volkmann P, Maurer-Fogy I, Pallai**
612 **P, Merluzzi V, Matteo M, Skern T, Kuechler E**. 1989. Polypeptide 2A of human
613 rhinovirus type 2: Identification as a protease and characterization by mutational
614 analysis. Virology 169:68–77.

615 10. **Toyoda H, Nicklin MJH, Murray MG, Anderson CW, Dunn JJ, Studier FW,**
616 **Wimmer E**. 1986. A second virus-encoded proteinase involved in proteolytic
617 processing of poliovirus polyprotein. Cell 45:761–770.

618 11. **Belsham GJ**. 1993. Distinctive features of foot-and-mouth disease virus, a member of
619 the picornavirus family; aspects of virus protein synthesis, protein processing and
620 structure. Prog Biophys Mol Biol 60:241–260.

621 12. **Palmenberg AC, Parks GD, Hall D, Ingraham RH, Seng TW, Pallal P V.** 1992.
622 Proteolytic processing of the cardioviral cleavage in clone-derived P2 Region : primary
623 2A / 2B precursors. Virology 190:754–762.

624 13. **Tulloch F, Luke GA, Ryan MD**. 2017. Foot-and-mouth disease virus proteinases and
625 polyprotein processing, p. 43–59. *In* Sobrino, F, Domingo, E (eds.), Foot-and-Mouth
626 Disease: Current Research and Emerging Trends. Caister Academic Press.

627 14. **Ryan MD, Belsham GJ, King AMQ**. 1989. Specificity of enzyme-substrate
628 interactions in foot-and-mouth disease virus polyprotein processing. Virology 173:35–
629 45.

630 15. **Ryan MD, King AMQ, Thomas GP**. 1991. Cleavage of foot-and-mouth disease virus
631 polyprotein is mediated by residues located within a 19 amino acid sequence. J Gen

Virol 72:2727–2732.

16. **Donnelly M, Luke G, Mehrotra A, Li X, Hughes LE, Gani D, Ryan MD**. 2001. Analysis of the aphthovirus 2A/2B polyprotein "cleavage" mechanism indicates not a proteolytic reaction, but a novel translational effect: A putative ribosomal "skip." J Gen Virol 82:1013–1025.

17. **Atkins JF, Wills NM, Loughran G, Wu C-Y, Parsawar K, Ryan MD, Wang C-H, Nelson CC**. 2007. A case for "StopGo": reprogramming translation to augment codon meaning of GGN by promoting unconventional termination (Stop) after addition of glycine and then allowing continued translation (Go). RNA 13:803–810.

18. **Doronina VA, Wu C, de Felipe P, Sachs MS, Ryan MD, Brown JD**. 2008. Site-specific release of nascent chains from ribosomes at a sense codon. Mol Cell Biol 28:4227–4239.

19. **Ryan MD, Donnelly M, Lewis A, Mehrotra AP, Wilkie J, Gani D**. 1999. A model for nonstoichiometric, cotranslational protein scission in eukaryotic ribosomes. Bioorg Chem 27:55–79.

20. **Groppo R, Palmenberg AC**. 2007. Cardiovirus 2A protein associates with 40S but not 80S ribosome subunits during infection. J Virol 81:13067–13074.

21. **Donnelly M, Gani D, Flint M, Monaghan S, Ryan MD**. 1997. The cleavage activities of aphthovirus and cardiovirus 2A proteins. J Gen Virol 78:13–21.

22. **Donnelly MLL, Luke GA, Hughes LE, Luke G, Mendoza H, Dam E, Gani D, Ryan MD**. 2001. The "cleavage" activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring "2A-like" sequences. J Gen Virol 82:1027–1041.

655 23. **Gao Z, Zhou J, Zhang J, Ding Y, Liu Y**. 2014. The silent point mutations at the
656 cleavage site of 2A/2B have no effect on the self-cleavage activity of 2A of foot-and-
657 mouth disease virus. Infect Genet Evol 28:101–106.

658 24. **Sharma P, Yan F, Doronina VA, Escuin-Ordinas H, Ryan MD, Brown JD**. 2012.
659 2A peptides provide distinct solutions to driving stop-carry on translational recoding.
660 Nucleic Acids Res 40:3143–3151.

661 25. **Minskaia E, Nicholson J, Ryan MD**. 2013. Optimisation of the foot-and-mouth
662 disease virus 2A co-expression system for biomedical applications. BMC Biotechnol
663 13:67.

664 26. **Loughran G, Libbey JE, Uddowla S, Scallan MF, Ryan MD, Fujinami RS, Rieder**
665 **E, Atkins JF**. 2013. Theiler's murine encephalomyelitis virus contrasts with
666 encephalomyocarditis and foot-and-mouth disease viruses in its functional utilization
667 of the StopGo non-standard translation mechanism. J Gen Virol 94:348–353.

668 27. **Ellard FM, Drew J, Blakemore WE, Stuart DI, King AMQ**. 1999. Evidence for the
669 role of His 142 of protein 1C in the acid induced disassembly of foot and mouth
670 disease virus capsids. J Gen Virol 80:1911–1918.

671 28. **Fuerst TR, Niles EG, Studier FW, Moss B**. 1986. Eukaryotic transient-expression
672 system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA
673 polymerase. Proc Natl Acad Sci U S A 83:8122–8126.

674 29. **Hahn H, Palmenberg AC**. 1996. Mutational analysis of the encephalomyocarditis
675 virus primary cleavage. J Virol 70:6870–6875.

676 30. **Kristensen T, Normann P, Gullberg M, Fahnøe U, Polacek C, Rasmussen TB,**
677 **Belsham GJ**. 2017. Determinants of the VP1/2A junction cleavage by the 3C protease

in foot-and-mouth disease virus-infected cells. J Gen Virol 98:385–395.

31. **Napthine S, Ling R, Finch LK, Jones JD, Bell S, Brierley I, Firth AE**. 2017. Protein-directed ribosomal frameshifting temporally regulates gene expression. Nat Commun 8:15582.

32. **Rychlík I, Černá J, Chládek S, Pulkrábek P, Žemlička J**. 1970. Substrate specificity of ribosomal peptidyl transferase. The effect of the nature of the amino acid side chain. Eur J Biochem 16:136–142.

33. **Kjær J, Belsham GJ**. 2017. Selection of functional 2A sequences within foot-and-mouth disease virus; requirements for the NPGP motif with a distinct codon bias. RNA (in press). doi: e-pub October 2017 doi:10.1261/rna.063339.117

34. **Bøtner A, Kakker NK, Barbezange C, Berryman S, Jackson T, Belsham GJ**. 2011. Capsid proteins from field strains of foot-and- mouth disease virus confer a pathogenic phenotype in cattle on an attenuated, cell-culture-adapted virus. J Gen Virol 92:1141–1151.

35. **Risager PC, Fahnøe U, Gullberg M, Rasmussen TB, Belsham GJ**. 2013. Analysis of classical swine fever virus RNA replication determinants using replicons. J Gen Virol 94:1739–1748.

36. **Nayak A, Goodfellow IG, Woolaway KE, Birtley J, Curry S, Belsham GJ**. 2006. Role of RNA structure and RNA binding activity of foot-and-mouth disease virus 3C protein in VPg uridylylation and virus replication. J Virol 80:9865–9875.

37. **Reed LJ, Muench H**. 1938. A simple method of estimating fifty percent endpoints. Am J Hyg 27:493–497.

38. **Belsham GJ, Nielsen I, Normann P, Royall E, Roberts LO**. 2008. Monocistronic

mRNAs containing defective hepatitis C virus-like picornavirus internal ribosome entry site elements in their 5' untranslated regions are efficiently translated in cells by a cap-dependent mechanism. RNA 14:1671–1680.

39. **Belsham GJ, McInerney GM, Ross-Smith N**. 2000. Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. J Virol 74:272–280.

40. **Polacek C, Gullberg M, Li J, Belsham GJ**. 2013. Low levels of foot-and-mouth disease virus 3C protease expression are required to achieve optimal capsid protein expression and processing in mammalian cells. J Gen Virol 94:1249–58.

41. **Yu Y, Wang H, Zhao L, Zhang C, Jiang Z, Yu L**. 2011. Fine mapping of a foot-and-mouth disease virus epitope recognized by serotype independent monoclonal antibody 4B2. J Microbiol 49:94–101.

**Table 1: Amino acid sequences in the encoded 2A peptide within rescued viruses following three passages in BHK cells.** Sequence differences from wt are shown whereas identical amino acids are indicated by a (**.**). The wt and mutant 2A cleavage activities previously determined by Donnelly et al. [22] and Sharma et al.[24], *in vitro*, are indicated.

| Plasmid | "Cleavage" *in vitro* (%)[1] | Residue encoded in rescued viruses | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2A | | | | | 2B |
| **wt** | **90** | **E14** | **S15** | **N16** | **P17** | **G18** | **P19** |
| **E14Q** | **56** | **Q** | **.** | **.** | **.** | **.** | **.** |
| **S15F** | **39** | **.** | **F** | **.** | **.** | **.** | **.** |
| **S15I** | **42** | **.** | **I** | **.** | **.** | **.** | **.** |
| **N16A** | **0** | **.** | **.** | **.** | **.** | **.** | **.** |
| **N16H** | **31** | **.** | **.** | **H** | **.** | **.** | **.** |
| **P17A** | **0** | **.** | **.** | **.** | **.** | **.** | **.** |
| **G18A** | **0** | **.** | **.** | **.** | **.** | **.** | **.** |
| **P19A** | **0** | **.** | **.** | **.** | **.** | **.** | **.** |
| **P19G** | **11** | **.** | **.** | **.** | **.** | **.** | **.** |

**1:** Data from Donnelly et al. and Sharma et al. [22, 24].

720 **Table 2:** PCR primers used to create and sequence mutant FMDV cDNAs. Mutagenic
721 nucleotides are shown underlined.

| Primer name | Sequence (5′-3′) |
|---|---|
| Fwd_2A_D12A | AAGTTGGCGGGAGCCGTCGAGTCCAACCCTGG |
| Fwd_2A_V13A | AAGTTGGCGGGAGACGCCGAGTCCAACCCTGG |
| Fwd_2A_E14A | AAGTTGGCGGGAGACGTCGCGTCCAACCCTG |
| Fwd_2A_E14Q | GATGTCCAGTCCAACCCTGG |
| Fwd_2A_S15A | TTGGCGGGAGACGTCGAGGCCAACCCTG |
| Fwd_2A_S15F | GATGTCGAGTTTAACCCTGC |
| Fwd_2A_S15I | GATGTCGAGATTAACCCTGG |
| Fwd_2A_N16A | GTC CGC CCC TGG GCC CTT C |
| Fwd_2A_N16C | CGAGTCCTGCCCTGGGCCCTTCTTTTTCTCCGA |
| Fwd_2A_N16H | CGAGTCCCACCCTGGGCCCTTCTTTTTCTCCGA |
| Fwd_2A_N16V | CGAGTCCGTCCCTGGGCCCTTCTTTTTCTCCGA |
| Fwd_2A_N16W | CGAGTCCTGGCCTGGGCCCTTCTTTTTCTCCGA |
| Fwd_2A_P17A | GTCCAACGCTGGGCCCTTC |
| Fwd_2A_G18A | GTCCAACCCTGCGCCCTT C |
| Fwd_2A_P19A | CAACCCTGGGGCTTTCTT |
| Fwd_2A_P19G | CAACCCTGGGGGCTTCTT |
| Fwd_2A_P19S | CGAGTCCAACCCTGGGTCCTTCTTTTTCTCCGA |
| Fwd_2A_P19V | CGAGTCCAACCCTGGGGTCTTCTTTTTCTCCGA |
| 8APN203 | CTCCTTCAACTACGGTGCC |
| 8APN206 | CACCCGAAGACCTTGAGAG |
| 10PPN10 | CTTTGACCAACCCGGCCA |
| 13APN1 | CCGGGCCCAGGGTTGGACTCGAC |
| 13APN4 | CCGGATCCGCTAGCCATGGGAGTCAAAGTTCTGTTTGC |
| ATG_P1_fwd | ATGAATACTGGCAGCATAATAAACAACTAC |
| 2C_FLAG_Stop_rev | CTATTACTTGTCGTCATCGTCTTTGTAGTCCTGCTTGAAGATCG GGTGACTCGACAC |
| 2A_Synonomous_Fwd | TCTCCGACGTAAGATCAAACTTCTCCA |

722

**Fig. 1: Structure of the plasmids used in this study.** These include: (a) full-length FMDV O1K cDNA, (b) *Gluc* replicon cDNA and (c) RNA polymerase defective *Gluc* replicon cDNA. The plasmids were linearized using *HpaI* or *BlpI* prior to *in vitro* transcription. Panel (d): Schematic representation of the P1-2A-2BC-FLAG cDNA cassette expressed in transient expression assays (as described in Material and Methods).

**Fig. 2: FMDVs rescued from mutants N16A, P17A, G18A and P19A had reverted to the wt sequence.** Three synonymous mutations downstream of the 2A/2B junction were introduced into the wt and mutant N16A, P17A, G18A and P19A plasmids. The resultant RNA transcripts were introduced into BHK cells. The rescued viruses were analysed after 3 passages in BHK cells. The region of the FMDV genome including that encoding the 2A peptide was amplified by RT-PCR and the PCR products were sequenced. The chromatograms are shown, note the retained synonymous mutations ca. 20 nt downstream of the 2A/2B junction, that had been introduced as a marker.

**Fig. 3: Growth curves and assessment of the production of FMDV capsid proteins in BHK cells infected with wt and viable 2A mutant viruses. (a)** BHK cells were infected with wt and the indicated 2A mutants at an m.o.i. of 0.1 and virus was harvested by freezing at 0, 2, 5, 10 and 24 hours post-infection. Virus yields were determined as $TCID_{50}$ by titration in BHK cells. **(b)** Uninfected or FMDV-infected BHK (m.o.i. 0.1) cell lysates were analysed by SDS-PAGE and immunoblotting with antibodies specific for FMDV capsid proteins (anti-FMDV sera). Uninfected cells were used as a negative control. Molecular mass markers (kDa) are indicated on the left.

748 **Fig. 4: Transient expression assays to determine 2A/2B "cleavage" induced by the wt**
749 **and mutant FMDV cDNAs**. The indicated plasmids were transfected into vTF7-3 infected
750 BHK cells as described in Materials and Methods. After 24 hours, cell extracts were prepared
751 and analysed by SDS-PAGE and immunoblotting using an anti-FLAG antibody. The
752 uncleaved P1-2A-2BC-FLAG and the cleavage product (2BC-FLAG) are marked. Molecular
753 mass markers (kDa) are indicated on the left. The cleavage activities (percentage of cleaved
754 product) of the wt and each 2A mutant were determined by quantifying the intensity of the
755 signal for the FMDV capsid proteins using ImageJ (v1.50) and are indicated above each lane.

756

757 **Fig. 5: Expression of the luciferase reporter protein, Gluc, by a FMDV replicon**. BHK
758 cells were electroporated with wt or mutant RNA transcripts derived from the indicated
759 cDNAs and, at the indicated times, cell lysates were prepared and assayed for Gluc activity.
760 RLUs = Relative light units. Data are presented as mean ± standard deviation (SD) RLUs
761 from samples (n=3) harvested at the indicated times.

762

763 **Fig. 6: Transient expression assays to determine the influence of 2A substitutions on the**
764 **processing of the FMDV capsid precursor P1-2A**. The wt and mutant P1-2A-2BC-FLAG
765 plasmids were transfected alone or with pSKRH3C [40] (which expresses FMDV $3C^{pro}$), as
766 indicated, into vTF7-3 infected BHK cells as described in Methods. After 24 h, cell extracts
767 were prepared and analysed by SDS-PAGE and immunoblotting using anti-FMDV VP2
768 (panel a) and anti-FMDV antisera (to detect all FMDV capsid proteins) (panel b) as
769 indicated. Molecular mass markers (kDa) are indicated on the left.

770

771 **Fig. 7: Detection of FMDV 2A fusion proteins by IF staining within cells.** BHK cells were
772 untreated or transfected with wt or mutant FMDV RNA transcripts. At 8 h post-transfection,

773 the cells were fixed. FMDV capsid proteins or the FMDV 2A peptide were detected using
774 anti-FMDV O1K polyclonal antibodies (upper panels) or anti-2A antibodies (lower panels),
775 respectively, plus a secondary antibody labelled with Alexa Fluor 568 (red). The 2A
776 substitutions are indicated. Untransfected cells were used as a negative control whereas the
777 O1K VP1 K210E mutant, described previously [7], in which the 2A remains joined to VP1,
778 served as a positive control. The cellular nuclei were visualized with DAPI (blue). Bar, 50
779 µm.





Journal of Virology