Supporting Information

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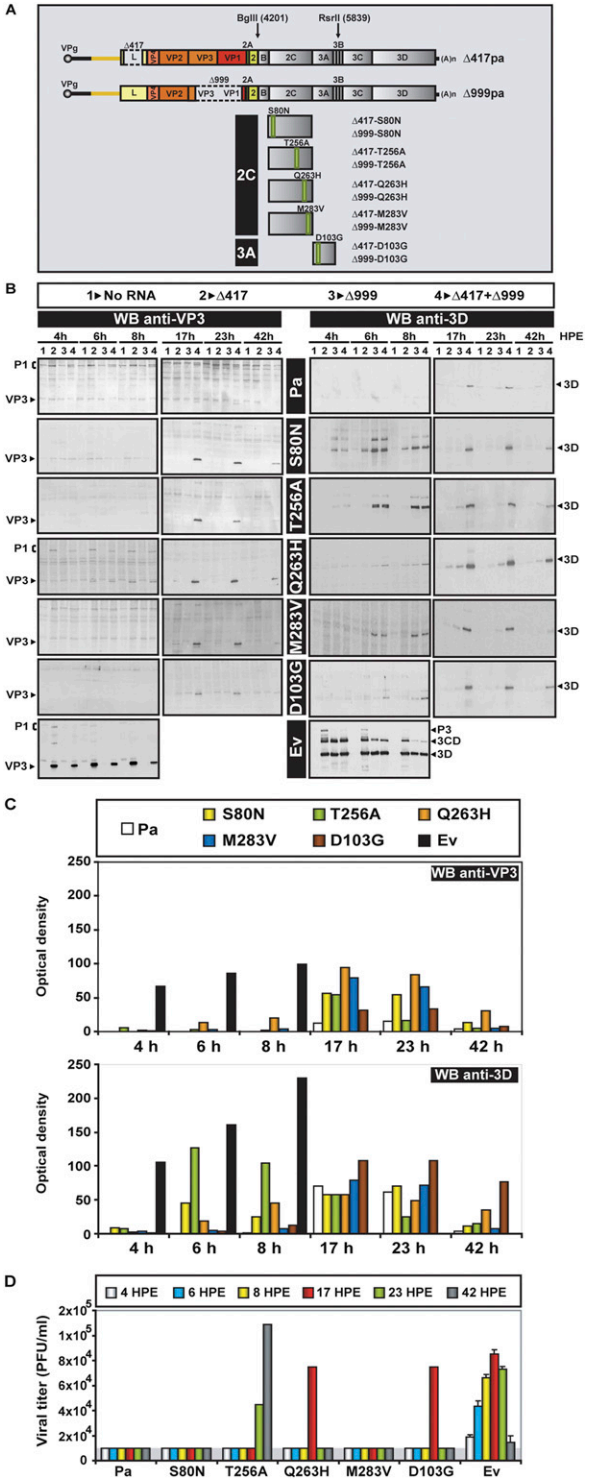

Fig. S1. Effect of amino acid substitutions in the expression of viral proteins from foot-and-mouth disease virus (FMDV) RNAs with internal deletions. (*A*) Scheme or Δ417pa and Δ999pa RNAs in which the deletions are present in the sequence context of non–structural-coding regions of the parental FMDV C-S8c1 (compare with Figs. 1 and 2). The position of the amino acid substitution in 2C and 3A and name of the mutants are indicated below the genomes. These

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mutations were chosen because they were the only nonsynonymous mutations present in the two ΔRNAs. (*B*) Western blot analysis of cellular extracts prepared at 4–42 h after electroporation (HPE) with either no RNA (mock-electroporated cells), electroporation with Δ417 RNA or Δ999 RNA, or coelectroporation with Δ417 RNA + Δ999 RNA (identified as 1, 2, 3, and 4, respectively, in the upper box, and at the top of each lane). Because the expression of viral proteins from parental ΔRNAs was not detected at early times (Fig. 2), the analyses were performed at 4–42 HPE. The horizontal filled boxes denote that the antibody used was either against VP3 (*Left*) or against 3D (*Right*). The amount of each extract was normalized relative to actin, detected with a specific monoclonal antibody (Fig. S2). The filled vertical boxes in the middle indicate that the electroporated RNA had the sequence of either Δ417pa or Δ999pa (Pa) or the sequence of Δ417pa or Δ999pa encoding the indicated amino acid substitution in 2C or 3A, or Δ417ev, Δ999ev (Ev) (compare with *A* and Fig. 2). The position of precursors P1, P3, and 3CD and processed proteins VP3 and 3D is indicated. The corresponding electropherograms for the same samples, and the measurement of shut-off of host cell protein synthesis, are given in Fig. S2. Electroporation by Δ417ev, Δ999ev, and Δ417ev + Δ999ev produced cytopathic effect at late HPE and no cell extracts could be prepared for analysis (two missing panels at the bottom). The origin of antibodies and procedures for electrophoresis and Western blot analysis are described in *Materials and Methods*. (*C*) Densitometric tracings of the bands corresponding to the Western blots for proteins VP3 and 3D of the coelectroporations of Δ417 RNA + Δ999 RNA shown in *B*. Sample identification in the abscissa is as in *B*. Values are given after background subtraction (average of several values around the band of interest). (*D*) Virus titers [corrected for two-hit kinetics (1, 2)] determined at the indicated HPE (top box) in the cell culture supernatants following coelectroporation with Δ417 RNA + Δ999 RNAs. Sample identification is as in *B*. Results are the average of three determinations, and SDs are given. The gray shaded area indicates the limit of detection of infectivity. Procedures are described in *Materials and Methods*.

1. Manrubia SC, Garcia-Arriaza J, Domingo E, Escarmís C (2006) Long-range transport and universality classes in *in vitro* viral infection spread. *Europhys Lett* 74(3):547–553.
2. Ojosnegros S, et al. (2011) Viral genome segmentation can result from a trade-off between genetic content and particle stability. *PLoS Genet* 7(3):e1001344.

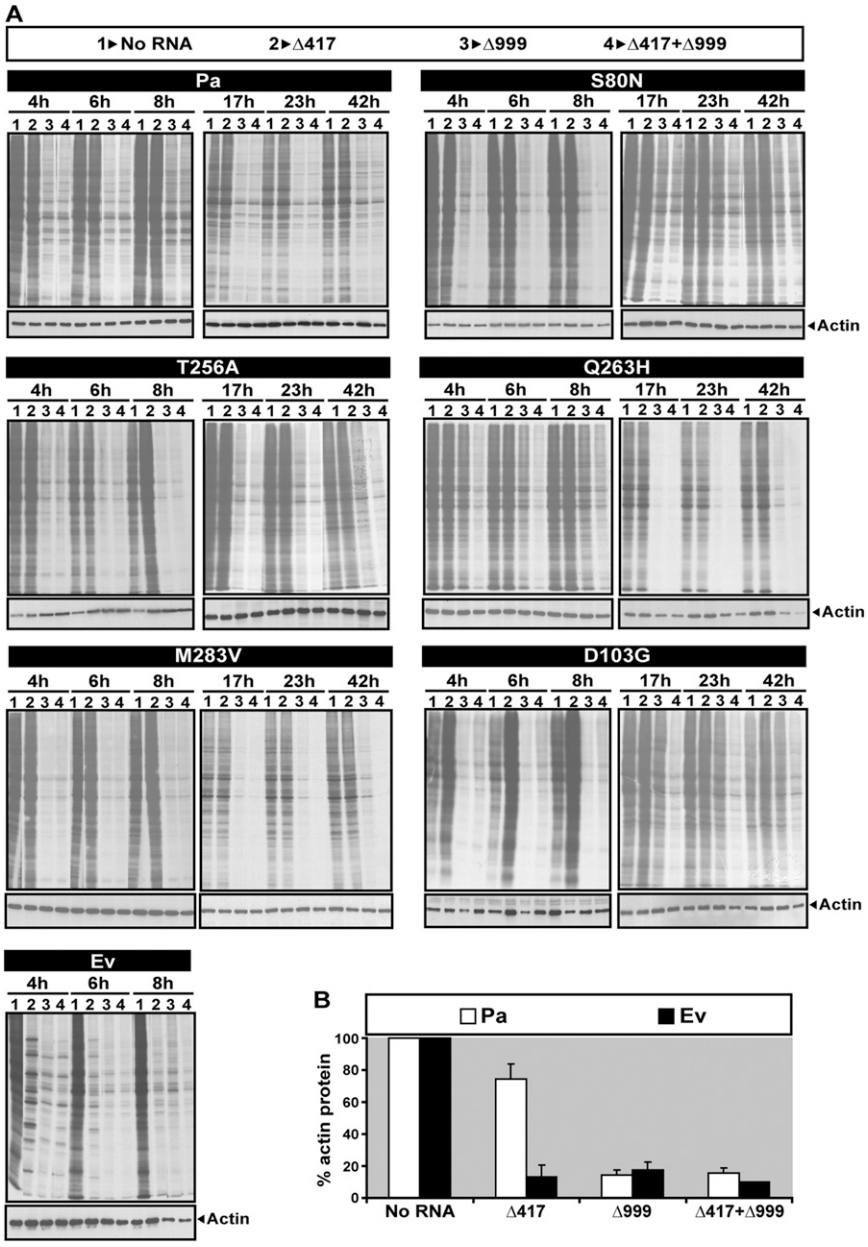

Fig. S2. Protein expression following electroporation or coelectroporation of baby hamster kidney-21 (BHK-21) cells with ΔRNAs. (*A*) Electropherograms corresponding to the same experiment whose Western blot analyses are depicted in Fig. S1. BHK-21 cells were mock-electroporated (No RNA), electroporated with either Δ417 RNA or Δ999 RNA, or coelectroporated with Δ417 RNA + Δ999 RNA (identified as 1, 2, 3, and 4, respectively, in the upper box and at the top of each lane). At 3, 5, 7, 16, 22, or 41 HPE, the cells were labeled with [^{35}S] Met-Cys for 1 h (indicated as 4, 6, 8, 17, 23, or 42 h at the top of each panel), and the cell extracts were analyzed by SDS/PAGE, followed by fluorography and autoradiography. The amount of extract in each lane was normalized relative to actin (bottom bands). (*B*) Estimate of the intensity of shut-off of host cell protein synthesis, obtained by densitometric tracings of the protein samples at 4, 6, and 8 HPE, following mock-electroporation (No RNA) or electroporation or coelectroporation with the ΔRNAs indicated in the upper box and in the abscissa. Values are expressed as percentage of the band corresponding to actin after background subtraction. Procedures are detailed in *Materials and Methods*.

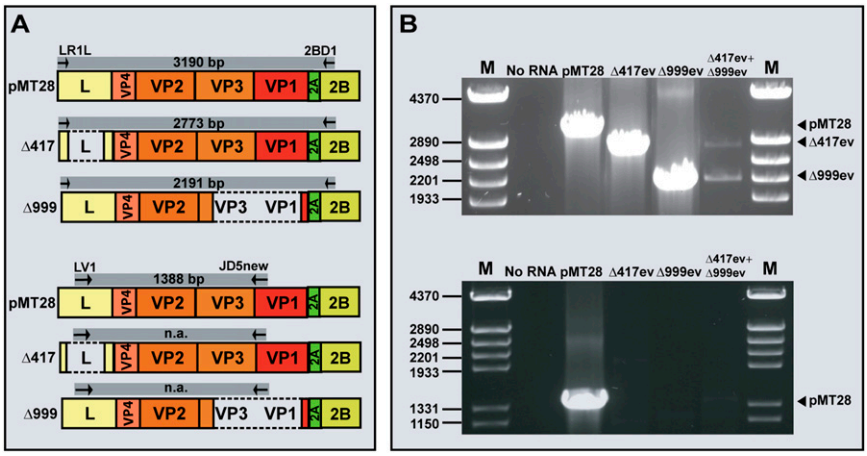

Fig. S3. Genome analysis of BHK-21 cell culture supernatants following electroporation with FMDV RNAs. (*A*) Viral RNAs were analyzed by RT-PCR using specific primers to distinguish standard FMDV RNA from ΔRNAs (small arrows linked by a shaded bar at the top of each genome type, with indication of the size in base pairs of the amplified DNA; n.a., no amplification expected; the nucleotide sequence and exact position of each primer are listed in Table S3). (*B*) RT-PCR amplification bands using RNA extracted from supernatant of BHK-21 cells at 4 h following either mock-electroporation or coelectroporation with Δ417ev + Δ999ev RNA. As controls, RT-PCR amplifications were performed in parallel using pMT28, Δ417ev, and Δ999ev RNAs obtained by in vitro transcription of the corresponding RNAs. A cDNA was first synthesized using primer 2BD1 or JD5new. Then, PCR amplification was performed using the primers corresponding to each genome type, as indicated in *A*. DNAs were analyzed by agarose gel electrophoresis. The size of the DNA expected from each genomic type is indicated on the right. Lane M contains the molecular mass DNA markers (Φ29 DNA digested with HindIII), and their size is indicated on the left. Procedures are described in *Materials and Methods*.

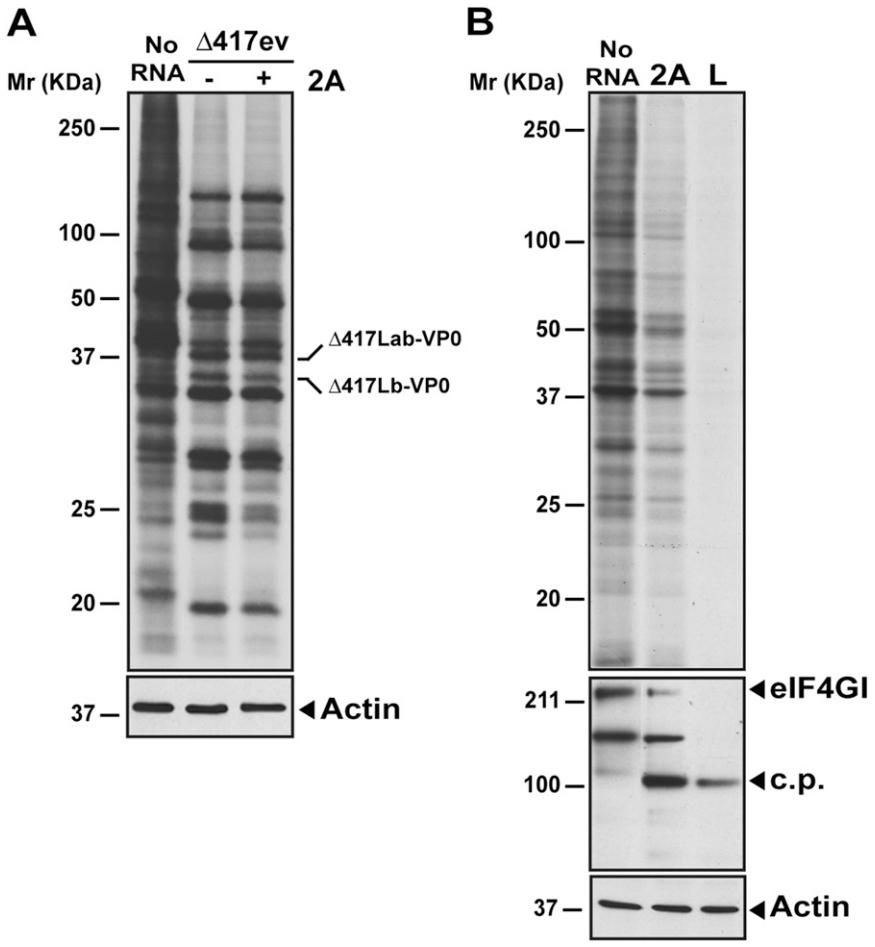

Fig. S4. Effect of poliovirus protein 2A on cellular and viral protein expression. (*A*) BHK-21 cells were either mock electroporated (No RNA) or electroporated with Δ417ev RNA in the absence or presence of poliovirus protein 2A expressed *in trans* (1). At 3 HPE, cells were labeled for 1 h with [^{35}S] Met-Cys, and the protein extracts were analyzed; the amount of extract was normalized using actin (bottom box). The position of Δ417Lab-VP0 and Δ417Lb-VP0 is indicated on the right. Molecular mass markers for proteins are indicated on the left. (*B*) (*Top*) BHK-21 cells were either mock electroporated (No RNA) or electroporated with RNAs expressing poliovirus protein 2A or FMDV Lab. At 3 HPE, cells were labeled for 1 h with [^{35}S] Met-Cys, and the protein extracts were analyzed. (*Middle*) Western blot analysis of eIF4GI and its cleavage product (c.p.). (*Bottom*) The amount of extract was normalized using actin. Molecular mass markers for proteins are indicated on the left. The constructions to express poliovirus 2A and FMDV L, as well as procedures for electroporation, electrophoresis, and Western blot analysis are described in *Materials and Methods*.

1. Ventoso I, Carrasco L (1995) A poliovirus 2A(pro) mutant unable to cleave 3CD shows inefficient viral protein synthesis and transactivation defects. *J Virol* 69(10):6280–6288.

Table S1. Mutations and corresponding amino acid substitutions in the FMDV populations passaged at high multiplicity of infection in BHK-21 cells

Region analyzed*	FMDV population†							Amino acid‡
					C-S8p260			
	C-S8p50	C-S8p100	C-S8p143	C-S8p200	Δ417	Δ999	p3d	
5′-UTR (1–1,038)		C247U	C247U	C247U	C247U	C247U	C247U	
		C338U	C338U	C338U	C338U	C338U	C338U	
						U467C/U (75%)		
	G476A	G476A	G476A	G476A	G476A	G476A	G476A	
					C511U/C (75%)			
							U518C	
	U856C	U856C	U856C	U856C	U856C	U856C	U856C	
							U1008C	
L (1,039–1,641)		A1043G	—					**N2S**
				G1066G/U (25%)		G1066U	G1066U	**V10L**
			C1105C/U (25%)					**P23S**
				A1154A/U (25%)	Δ417			**K39M**
				C1158C/A (25%)	Δ417			Syn
				C1180C/U (25%)	Δ417	C1180U	C1180U	**H48Y**
						A1628U	A1628U	**Q197L**
VP4 (1,642–1,896)					A1810G/A (50%)		A1810G	**T57A**
VP2 (1,897–2,550)					C2202U		C2202U	Syn
						C2250U		Syn
						G2285A		**G130D**
VP3 (2,551–3,207)						U2622C		Syn
		C2624U	C2624U	C2624U	C2624U	C2624U	C2624U	**A25V**
						G2763U		**R71S**
	C2897U/C (50%)	—				Δ999		**A116V**
				C3064G/C (75%)	C3064G	Δ999	C3064G	**H172D**
	G3067A	G3067A	G3067A	G3067A	G3067A	Δ999	G3067A	**E173K**
	C3202A	C3202A	C3202A	C3202A	C3202A	Δ999	C3202A	**Q218K**
VP1 (3,208–3,834)		A3328G	A3328G	A3328G	A3328G	Δ999	A3328G	**K41E**
			A3344G/A (50%)	A3344G/A (75%)	A3344G	Δ999	A3344G	**D46G**
		U3345A	—			Δ999		**D46E**
				U3387C/U (75%)	U3387C	Δ999	U3387C	Syn
				U3433C	U3433C	Δ999	U3433C	Syn
	A3668G/A (75%)	—				Δ999		**H154R**
					U3753C/U (75%)	Δ999	U3753C	Syn
	A3797A/G (25%)	A3797G	A3797G	A3797G	A3797G	A3797G	A3797G	**H197R**
2B (3,883–4,344)				A4036G/A (50%)	A4036G	A4036G	A4036G	**T52A**
2C (4,345–5,298)			G4583A	G4583A	G4583A	G4583A	G4583A	**S80N**
		G4650A	—					Syn
	C4748U/C (75%)	C4748U/C (75%)	C4748C/U (25%)	—				**T135I**
					C4824U/C (50%)	C4824U/C (50%)	C4824U	Syn
			A5110G	A5110G	A5110G	A5110G	A5110G	**T256A**
			G5133C	G5133C	G5133C	G5133C	G5133C	**Q263H**
				A5191G/A (50%)	A5191G	A5191G	A5191G	**M283V**
							C5214U	Syn
		G5295A	—					Syn
3A (5,299–5,757)			U5454C/U (50%)	U5454C/U (50%)	U5454U/C (25%)		U5454C	Syn
		A5524G	—					**I76V**
				A5606G/A (50%)	A5606G/A (75%)	A5606G		**D103G**
		A5713G	—		A5713G/A (50%)	A5713G/A (50%)	A5713G	**N139D**
3C (5,971–6,609)		U6372C	—					Syn

Table S1. Cont.

Region analyzed*	FMDV population†							Amino acid‡
					C-S8p260			
	C-S8p50	C-S8p100	C-S8p143	C-S8p200	Δ417	Δ999	p3d	
3D (6,610–8,009)					U6789U/C (25%)	U6789U/C (25%)	U6789C	Syn
							C6840U	Syn
		U6903C	—					Syn
	G7554U	G7554U	G7554U	—				Syn

*FMDV genomic region analyzed (residue numbering is according to ref. 1).
†The sequence of the entire viral genome was determined for C-S8p50, C-S8p100, C-S8p143, C-S8p200, and C-S8p260 (2). Mutations are relative to the sequence of the parental clone C-S8c1. Two residues separated by a bar indicate a mixture of two nucleotides in the population, according to the sequence peak pattern. The percentage indicates the proportion of the mutant. —, reversion of a mutation to the WT sequence.
‡Deduced amino acid substitutions relative to the sequence of the parental clone C-S8c1. Amino acid residues (single-letter code) are numbered individually for each protein from the N to the C terminus. Syn means synonymous mutation. Boldface type indicates a change in the amino acid residue. Procedures for nucleotide sequencing and identification of FMDV genomic regions are described in *Materials and Methods* of the main text.

1. Escarmís C, Dávila M, Domingo E (1999) Multiple molecular pathways for fitness recovery of an RNA virus debilitated by operation of Muller's ratchet. *J Mol Biol* 285(2):495–505.
2. García-Arriaza J, Ojosnegros S, Dávila M, Domingo E, Escarmís C (2006) Dynamics of mutation and recombination in a replicating population of complementing, defective viral genomes. *J Mol Biol* 360(3):558–572.

Table S2. Mutations and corresponding amino acid substitutions in Δ417pa, Δ999pa, Δ417ev, and Δ999ev FMDV RNAs

	FMDV genome[†]				
Genomic region*	Δ417pa	Δ999pa	Δ417ev	Δ999ev	Amino acid[‡]
5′-UTR (1–1,038)		U467C		U467C	
	G476A	G476A	G476A	G476A	
	C511U		C511U		
	U856C	U856C	U856C	U856C	
	C1025U		C1025U		
L (1,039–1,641)		G1066U		G1066U	**V10L**
		C1180U		C1180U	**H48Y**
		A1628U		A1628U	**Q197L**
VP2 (1,897–2,550)	C2202U		C2202U		Syn
		C2250U		C2250U	Syn
		G2285A		G2285A	**G130D**
		A2545G		A2545G	**K217E**
VP3 (2,551–3,207)		U2622C		U2622C	Syn
	C2624U	C2624U	C2624U	C2624U	**A25V**
		G2763U		G2763U	**R71S**
	C3064G		C3064G		**H172D**
	G3067A		G3067A		**E173K**
	C3202A		C3202A		**Q218K**
VP1 (3,208–3,834)	A3328G		A3328G		**K41E**
	A3344G		A3344G		**D46G**
	U3387C		U3387C		Syn
	U3433C		U3433C		Syn
	C3539U		C3539U		**P111L**
	U3753C		U3753C		Syn
	A3797G	A3797G	A3797G	A3797G	**H197R**
2B (3,883–4,344)	A4036G	A4036G	A4036G	A4036G	**T52A**
2C (4,345–5,298)	—	—	G4583A	G4583A	**S80N**
	—	—	C4824U	C4824U	Syn
	—	—	—	C4959U	Syn
	—	—	—	A4988G	**K215R**
	—	—	—	C5040U	Syn
	—	—	A5110G	A5110G	**T256A**
	—	—	G5133C	G5133C	**Q263H**
	—	—	A5191G	A5191G	**M283V**
	—	—	C5214U	—	Syn
3A (5,299–5,757)	—	—	A5606G	A5606G	**D103G**
3D (6,610–8,019)	—	—	—	C7074U	Syn

*FMDV genomic region; residue numbering is according to ref. 1.

[†]The sequence of the entire viral genome was determined for the indicated ΔRNAs. Mutations are relative to the sequence of the parental clone C-S8c1. The genomes are depicted in Fig. 2.

[‡]Deduced amino acid substitutions relative to the sequence of the parental clone C-S8c1. Amino acid residues (single-letter code) are numbered individually for each protein from the N to the C terminus. Syn means synonymous mutation. Boldface type indicates a change in the amino acid residue. Procedures for nucleotide sequencing and identification of FMDV genomic regions are described in *Materials and Methods*.

1. Escarmís C, Dávila M, Domingo E (1999) Multiple molecular pathways for fitness recovery of an RNA virus debilitated by operation of Muller's ratchet. *J Mol Biol* 285(2):495–505.

Table S3. Oligonucleotides used in the present study

Genomic region	Primer name	Sequence (5′-3′)*	Orientation†	Position‡
2B	2BR1	TTGGTGTCTGCTTTTGAGGAAC	F	3,988
3C	3CD1	CATGACCATCTTTTGCAGGTCAG	R	6,009
3A	3AR3	GATGACGTGAACTCTGAGCCCGC	F	5,704
3D	AV2new	TGTGGAAGTGTCTTTTGAGGAAAG	R	7,783
2C	mutSNu	TTGAAGAACGGGAACGTCCATATTGC	F	4,576
2C	mutSNd	CGTTCCCGTTCTTCAAACACACTTGG	R	4,591
2C	mutTAu	GAAGACACCCACGCCAATCCAGTGGC	F	5,098
2C	mutTAd	ACTGGATTGGCGTGGGTGTCTTCAAGTGC	R	5,120
2C	mutQHu	GGCAATGTTTCACTACGACTGTGCCC	F	5,121
2C	mutQHd	GGCACAGTCGTAGTGAAACATTGCC	R	5,145
2C	mutMVu	GAGATTGCAACAGGATGTGTTCAAGCCTCAACCACCCCTCCA	F	5,175
2C	mutMVd	GGGTGGTTGAGGCTTGAACACATCCTGTTGCAATCTCTTCATTTC	R	5,211
3A	mutDGu	CATCACCACCGATGGCCAGACACTTGACGAGGCGGAAAAG	F	5,592
3A	mutDGd	CCGCCTCGTCAAGTGTCTGGCCATCGGTGGTGATGTTTGC	R	5,626
L	5′NcoI-L	GGGGCCCCATGGGCAATACAACTGACTGTTTTATC	F	1,039
L	3′BamHI-L	CCCGGGGGATCCCTATCACTTGAGCTTTCGCTGAACGCT	R	1,641

*Underlined letters are nucleotides that have been modified with respect to the genomic sequence of the FMDV C-S8c1 to introduce the desired mutations.
†Genomic orientation of primer: forward (F) or reverse (R).
‡Position of the 5′-nucleotide of the primer; numbering of FMDV genomic residues is that described in ref. 1.

1. Escarmís C, Dávila M, Domingo E (1999) Multiple molecular pathways for fitness recovery of an RNA virus debilitated by operation of Muller's ratchet. *J Mol Biol* 285(2):495–505.

Table S4. Comparison of the predicted size of proteins expressed from pMT28 and ΔRNAs

Virus	Proteins*	Amino acid†	M_r (kDa)‡
pMT28	P1-2A	747	81.5
	VP0	303	33.0
	VP3	219	23.9
	VP1	209	22.8
Δ417	**Δ417-Lab-P1-2A**	**809**	**88.6**
	Δ417-Lb-P1-2A	**781**	**85.5**
	Δ417-M-P1-2A	**771**	**84.3**
	Δ417-Lab-VP0	**365**	**40.1**
	Δ417-Lb-VP0	**337**	**37.0**
	Δ417-M-VP0	**327**	**35.8**
	VP3	219	23.9
	VP1	209	22.8
Δ999	**Δ999-P1-2A**	**414**	**45.1**
	VP0	303	33.0
	Δ999-VP3	**81**	**8.8**
	Δ999-VP1	**14**	**1.6**

*The genomes and the predicted major precursors and mature proteins expressed from pMT28, Δ417, and Δ999 are depicted in Fig. 4 *A–C*. New proteins expected from the deletions in ΔRNAs are highlighted in bold.
†Deduced number of amino acids present in the expressed protein.
‡The expected molecular mass of major precursors and mature proteins was calculated based on the genomic sequences, excluding the deleted regions in the case of proteins expressed from ΔRNAs.