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# Influence of Mutagenesis and Viral Load on the Sustained Low-Level Replication of an RNA Virus

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Lethal mutagenesis is an antiviral strategy that aims to extinguish viruses as a consequence of enhanced mutation rates during virus replication. The molecular mechanisms that underlie virus extinction by mutagenic nucleoside analogues are not well understood. When mutagenic agents and antiviral inhibitors are administered sequentially or in combination, interconnected and often conflicting selective constraints can influence the fate of the virus either towards survival through selection of mutagen-escape or inhibitor-escape mutants or towards extinction. Here we report a study involving the mutagenesis of foot-and-mouth disease virus (FMDV) by the nucleoside analogue ribavirin (R) and the effect of R-mediated mutagenesis on the selection of FMDV mutants resistant to the inhibitor of RNA replication, guanidine hydrochloride (GU). The results show that under comparable (and low) viral load, an inhibitory activity by GU could not substitute for an equivalent inhibitory activity by R in driving FMDV to extinction. Both the prior history of R mutagenesis and the viral population size influenced the selection of GU-escape mutants. A sufficiently low viral load allowed continued viral replication without selection of inhibitor-escape mutants, irrespective of the history of mutagenesis. These observations imply that reductions of viral load as a result of a mutagenic treatment may provide an opportunity either for immune-mediated clearing of a virus or for an alternative antiviral intervention, even if extinction is not initially achieved.



## Introduction

Genetic variation of viruses is one of the major obstacles to controlling viral diseases using standard antiviral treatments. Viral population complexity and dynamics demand the development of new antiviral interventions. One of them is lethal mutagenesis of viruses, which consists in provoking decreases in viral load and eventually virus extinction by increasing the viral mutation rate during genome replication. Extinction is often achieved by replicating the virus in the presence of mutagenic

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Abbreviations used: FMDV, foot-and-mouth disease virus; R, ribavirin; GU, guanidine hydrochloride; HIV-1, human immunodeficiency virus type 1; PFU, plaque-forming units; 3D, three-dimensional; FU, 5-fluorouracil; MOI, multiplicity of infection; DMEM, Dulbecco's modified Eagle's medium.

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nucleoside analogues, one of the best-characterized families of mutagenic agents.[1–3] This antiviral strategy is still at the experimental stage, but it has been endorsed by several theoretical treatments (related to the quasispecies theory and others)[4–16] and by an increasing number of experiments with viruses in cell culture and *in vivo*[17–33] (reviewed by Anderson *et al.*,[34] Domingo *et al.*,[35] and Graci and Cameron[36]).

There is evidence that at least two mechanisms can mediate viral extinction. If the number of mutations introduced into the replicating viral genome by the mutagenic agent is low to moderate, a class of defective but replication-competent genomes, which have been termed "defectors," can be produced and can contribute to virus extinction.[25] Experimental and *in silico* evidence of the consequences of the presence of defectors during virus replication[25,37–39] led to the proposal of the lethal defection model of virus extinction. Defectors are RNA-replication-competent viruses that are produced by mutation of the standard virus and that, by themselves, do not necessarily complete an infectious cycle.[25,38] They differ from defective interfering RNAs in that the latter require the presence of the standard virus for replication.[40] In molecular terms, altered RNA structures required as biological signals, or altered gene products that perform suboptimally in processes needed to complete the virus life cycle, contribute to the replicative deterioration of the virus, leading eventually to its extinction. This process of stochastic extinction can only take place in finite populations, with a limited number of viral genomes infecting a single cell, since fluctuations in the number of standard virus *versus* the variable amount of defectors induce, in this case, the extinction of the population. Both the finding of genomes with lethal mutations in mutagenized foot-and-mouth disease virus (FMDV) populations[22,41] and the predictions of *in silico* simulations suggest that an accumulation of nonviable genomes and replicative competent defectors due to enhanced mutagenesis may be lethal for the virus and may cause extinction.[25]

Lethal defection might be expected to precede overt lethality in time during lethal mutagenesis treatments.[25] However, the mutation frequency in the mutant spectrum need not increase linearly during mutagenic treatments,[42] and virus elimination does not necessarily correlate with large increases in mutant spectrum complexity.[26] Thus, much needs to be learned about the molecular mechanisms that contribute to virus extinction in the process of lethal mutagenesis.

The effectiveness of classical nonmutagenic antiviral inhibitors is challenged by the problem on the selection of mutant viruses that are resistant (or have decreased sensitivity) to the inhibitors, a major problem in clinical practice.[43–53] More recently, selection of viral mutants with decreased sensitivity to the mutagenic purine analogue ribavirin (R) [1-(β-D-ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide] has been reported for a number of RNA viruses.[20,54–58] In the case of the picornavirus FMDV, replication in the presence of increasing concentrations of R resulted in the selection of a virus with three amino acid replacements (M296I, P44S, and P169S) sequentially added to the viral polymerase.[41,56,59,60] The biochemical and structural results with the single-mutant and triple-mutant polymerases have revealed a new mechanism of resistance to R that consists in biasing the incorporation of R to equilibrate the proportion of the four types of transition mutations in the mutant spectrum.[41]

In view of the incessant capacity of viruses to adapt to adverse environmental circumstances, including strong mutagenesis, it is critical to explore the key parameters that may help in designing lethal mutagenesis protocols that can best counteract the adaptive capacity of viruses. In our initial studies, following the well-established advantage of combination therapy to minimize the selection of inhibitor-escape variants,[43,53,61–69] the combination of a mutagenic agent and an antiviral inhibitor was shown to be more effective than a mutagenic agent alone in driving FMDV and human immunodeficiency virus type 1 (HIV-1) towards extinction.[33,70] Then, in line with the implication of defective but replication-competent genomes in viral extinction,[25,37,71] a sequential inhibitor–mutagen administration proved more effective than the corresponding combination treatment for the extinction of FMDV, at least within the range of mutagen and inhibitor concentrations tested.[72] In addition to the advantage of the sequential treatment being supported by a theoretical model,[72] experimental measurements of interference by specific FMDV mutants indicated that the interference was suppressed by the inhibitor of viral replication, but not by the mutagen.[72] Thus, defector genomes produced as a result of mutagenesis could exert their activity and contribute best to viral extinction when the mutagen and the inhibitor were not present simultaneously during viral replication.[72] *In silico* simulations also predict that the advantage of a sequential inhibitor–mutagen administration over the corresponding combination treatment is manifested only when a mutagenic agent is involved, and not when only nonmutagenic inhibitors are administered[72] (J. Iranzo *et al.*, manuscript in preparation).

The use of mutagenic agents and antiviral inhibitors, either in combination or sequentially, poses an evolutionary riddle consisting in mutagenic agents favoring the generation of inhibitor-escape viral mutants, but mutagenic agents may also decrease the viral load below a level necessary to select for inhibitor-escape mutants. Threshold levels of phenotypic variants present in viral populations are

critical to determining the biological behavior of the entire population.[73,74] In the present report, we investigate experimentally the interplay between two parameters that are important in determining viral survival *versus* extinction during lethal mutagenesis: the prior history of mutagenesis of a viral population and the viral load. The aim is to explore conditions that may lead to sustained low viral loads during replication that may improve the efficiency of lethal mutagenesis. We have used FMDV, R, and the inhibitor of viral replication, guanidine hydrochloride (GU),[21,31,70,72] to document that, as a result of subjecting FMDV to a sufficient level of mutagenesis by R, the population may attain a low viral load consisting of replication-competent viruses, and that during such low-level virus replication, the presence of GU did not result in the selection of GU-escape mutants. A lower number of passages (replication rounds) in the presence of R resulted, however, in the selection of GU-escape mutants. The results show that both the prior history of R mutagenesis and the viral load can contribute to determining the fate of a viral population either towards extinction or towards continued low-level replication. Implications for antiviral designs and possible modification of virus behavior derived from bottleneck events that may intervene during the intrahost evolution of a virus are discussed.

## Results

### Selection of GU-resistant mutants in FMDV populations subjected to R mutagenesis

R mutagenesis can drive FMDV to extinction through a decrease in specific infectivity [the ratio between the number of plaque-forming units (PFU) and the amount of viral RNA in a virus preparation][21,75] (Fig. 1). However, passage of the virus in the presence of increasing subinhibitory concentrations of R resulted in the sequential

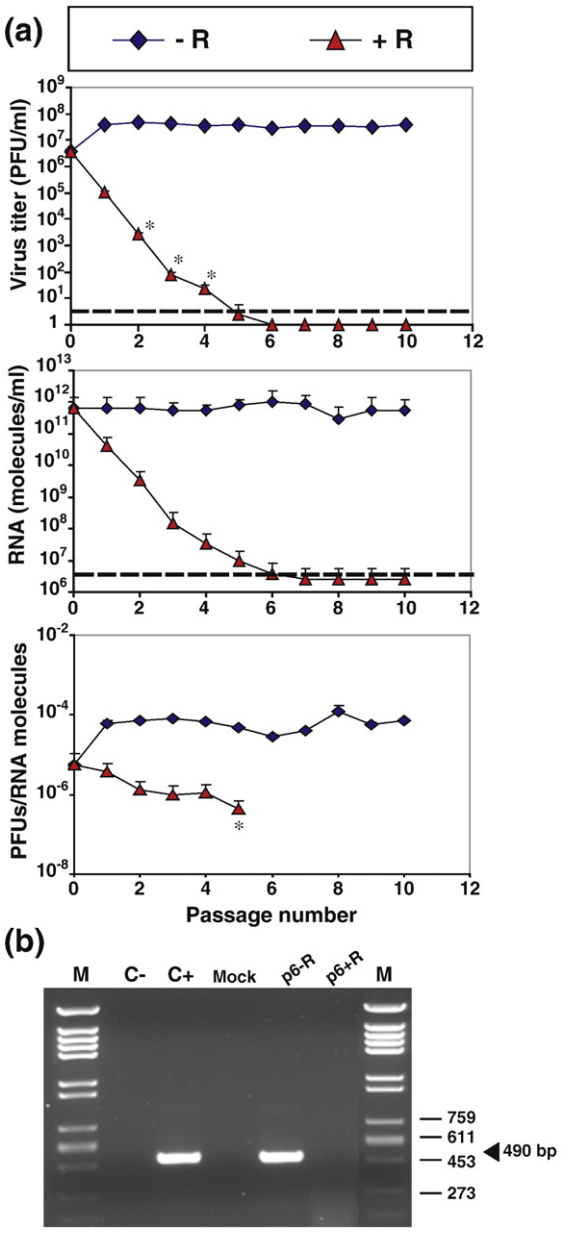


**Fig. 1.** Extinction of wild-type (progeny of pMT28) FMDV upon passage in the presence of R. BHK-21 cells ($2\times10^6$ cells) were initially infected with the wild-type virus at an MOI of about 0.3 PFU/cell. In successive passages, the same number of cells was infected with virus from the supernatant of the previous passage. The absence or the presence of R (5 mM) is indicated in the boxes at the top. (a) Infectivity, viral RNA levels, and specific infectivity in the supernatants of the infected cultures as a function of passage number. Viral RNA was extracted and quantified by real-time PCR. The virus titers and the amounts of FMDV RNA are expressed as the mean ± SD (error bars) of triplicate determinations. The discontinuous lines indicate the limit of detection of FMDV infectivity and viral RNA. Specific infectivity values (PFU/RNA molecules) were calculated from the infectivities and viral RNA levels given in the two previous panels of (a). The asterisks highlight the viral populations used for further experiments. (b) RT-PCR amplification used to detect viral RNA in the cell culture supernatants at passage 6 in the absence or in the presence of R (a). M, molecular size markers (HindIII-digested Φ29 DNA) [the corresponding sizes (base pairs) are indicated on the right]; C−, negative control (amplification without RNA); C+, PCR positive control; Mock, negative control corresponding to amplification of RNA extracted from the supernatant of mock-infected cells. The conditions for infection, mutagenic treatment, determination of FMDV infectivity by plaque assays, and quantification of FMDV RNA, as well as the positive and negative controls included in the assays, are described in Materials and Methods. Extinction of FMDV by R has been reproducibly observed in five different experiments. The upper panel in (a) had been previously published[75] and is shown here for completeness.

selection of replacements M296I, P44S, and P169S in the polymerase [three-dimensional (3D)], and these replacements mediated virus escape from extinction by R.[41,56,60,75] The R-driven decrease in the infectivity of wild-type FMDV prior to the selection of R-resistant mutants (Fig. 1) raised the possibility that a mutagenized FMDV population that was on its way towards extinction could be extinguished by the inhibitor GU[31,70,71] alone at a concentration that brings about the same decrease in infectivity as does the mutagenic agent (but without further mutagenesis). Alternatively, the history of enhanced mutagenesis by R might provide the viral population with a frequency of GU-resistant mutants sufficient to prevent extinction by the added inhibitor.

To investigate whether a concentration of GU that produces a decrease in viral load equivalent to that produced by 5 mM R (known to extinguish a clonal wild-type FMDV[75]) could achieve the extinction of R-treated FMDV, we first measured the inhibition of FMDV progeny production by different concentrations of GU. To this aim, a clonal population of FMDV (progeny from the transcript of pMT28)[76,77] was passaged two times in BHK-21 cells in the presence of 5 mM R and then once either in the absence of drugs, in the presence of 5 mM R, or in the presence of increasing concentrations of GU. [Whenever virus passages are carried out after replication in the presence (or in the absence) of a drug, the previous culture media and drugs (when present) are removed by washing the monolayer following virus attachment to the new cell monolayer.] The results documented that 6 mM GU exerted an inhibition equivalent to that produced by 5 mM R in one virus passage (Fig. 2). The inhibition exerted by GU is in agreement with previous measurements in independent experiments.[31,70,72] To test a possible influence of the history of R

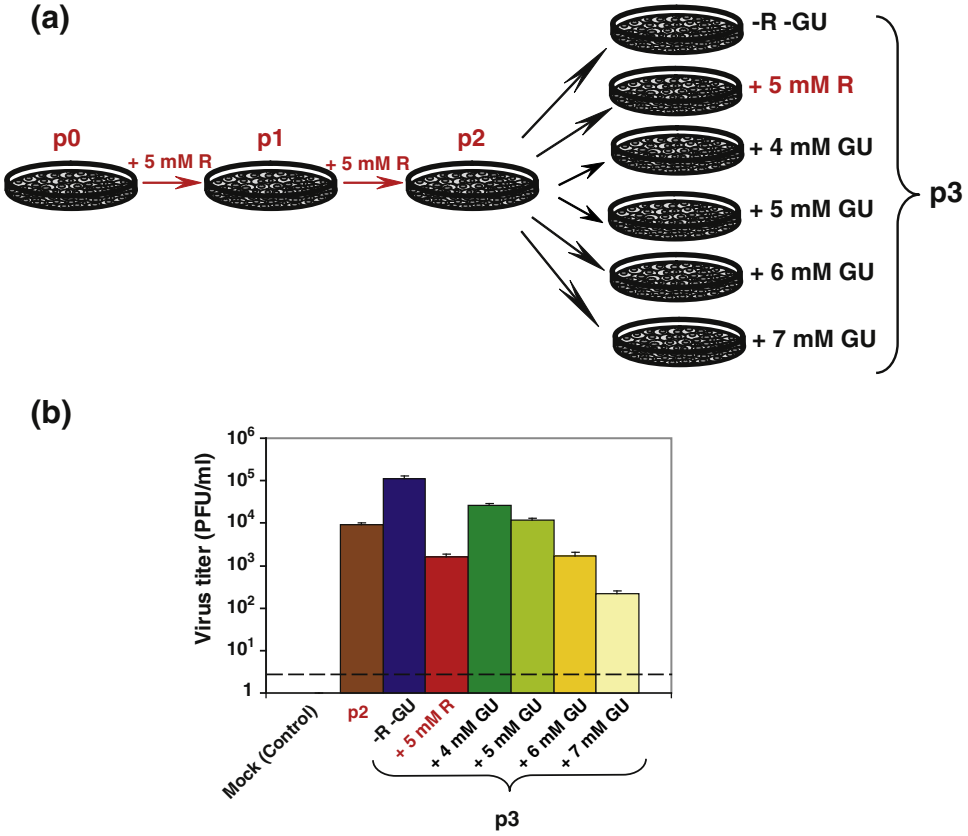


**Fig. 2.** Calibration of the inhibition produced in one virus passage by different concentrations of GU compared with 5 mM R. (a) Scheme of the protocol used. BHK-21 cells ($2\times10^6$ cells) were infected with FMDV (progeny of pMT28) at an MOI of 0.3 PFU/cell in the presence of 5 mM R. Then 0.2 ml of the infected cell culture supernatant (at 24 h postinfection) was used to carry out a second infection in the presence of 5 mM R to obtain FMDV p2 (as in the experiments shown in Fig. 1). BHK-21 cells ($2\times10^6$ cells) were infected with 0.2 ml of FMDV p2 either in the absence of any drug (−R and −GU; top), in the presence of 5 mM R, or in the presence of the indicated concentration of GU to yield six FMDV populations at passage 3 (p3). (b) Infectivities of the six viral populations obtained following the passage history shown in (a). Virus titers are expressed as the average of three determinations; mean ± SD (error bars) values are given. Mock (Control) (no bar value detectable) refers to the titer of a mock-infected culture maintained in parallel and is used to monitor the absence of contamination. Procedures are detailed in Materials and Methods.

mutagenesis on the response of FMDV to this treatment with GU, we further passaged viral populations obtained at passages 2, 3, and 4 in the presence of 5 mM R (abbreviated as Rp2, Rp3, and Rp4, respectively, and highlighted with an asterisk in Fig. 1a) either in the absence or in the presence of 6 mM GU, in both cases in the absence of R (Fig. 3). Virus titers, viral RNA molecules, and specific infectivities were determined in infected cell culture supernatants (Fig. 3). Recovery of infectivity upon interruption of a mutagenic treatment has been consistently observed in experiments with FMDV and lymphocytic choriomeningitis virus,[78,79] and it is the basis of the assay used to ascertain virus extinction (see Materials and Methods). The larger is the number of passages in the presence of R, the lower is the titer of the virus used to initiate the passages either in the absence or in the presence of GU (Fig. 3b). The kinetics of recovery of infectious progeny production (titer increase per passage) in the absence of GU was faster for Rp4 than for Rp2, although, in all cases, the populations regained similarly high levels of infectivity, viral RNA progeny production, and virus-specific RT-PCR-amplifiable material by passage 7 (Fig. 3b and d). Recovery of specific infectivity was observed, albeit with some fluctuations. Passage of the same Rp2, Rp3, and Rp4 populations in the presence of 6 mM GU led to a slower recovery of infectivity and viral RNA levels (Fig. 3c). In fact, population Rp4 yielded undetectable (or close to undetectable) infectivity and viral RNA levels. However, RT-PCR amplification of the cell culture supernatants of passage 10 in the presence of GU produced a DNA band diagnostic of viral genetic material (Fig. 3e). Thus, despite variations in the ability of the virus to regain the capacity to produce infectious progeny and viral RNA, in no case was extinction observed (as judged by the presence of viral nucleic acid amplified by RT-PCR; for requirements to consider FMDV extinct, see Materials and Methods). Considerable variations in specific infectivity (probably aggravated by very low infectivity and viral RNA values at some passages that render the calculation inaccurate) were observed in the passages in the presence of GU (Fig. 3c). Thus, a sequential treatment involving R first, followed by GU, did not result in FMDV extinction. Despite exerting a similar inhibitory activity to treatment with 5 mM R, we could not replace inhibition by 6 mM GU with inhibition by 5 mM R to drive FMDV towards extinction (compare Figs. 1 and 3).

The target of GU in picornaviruses is nonstructural protein 2C.[70,80–82] In previous studies on the extinction of FMDV by combination of the mutagenic pyrimidine analogue 5-fluorouracil (FU) and GU, escape from extinction was accompanied by selection of GU-resistant mutants that included replacements in 2C.[31,70,75] To investigate whether the failure of GU to drive R-treated FMDV to extinction was associated with selection of GU-escape mutants, we sequenced the 2C coding region of FMDV populations Rp2 and Rp3 that were passaged 10 times in the presence of 6 mM GU (Fig. 3c). The results (Table 1) indicate the presence of replacements K169R and M159L in populations Rp2 and Rp3, respectively, passaged 10 times in the presence of 6 mM GU. These substitutions were previously shown to confer FMDV resistance to GU.[70] Replacements K169R and M159L arise as a consequence of transition A4850G and of transversion A4819U, respectively, which are not favored by R mutagenesis. Replacements R55W and I248N in 2C were present in some populations passaged in the absence of GU. These substitutions have not been associated with resistance to GU, but replacements at the same positions have been found previously as compensatory substitutions selected upon the passage of some FMDV mutants that display defects in viral replication.[84–86] Interestingly, no amino acid substitutions became dominant in 2C of the FMDV Rp4 subjected to 10 passages in the presence of GU. This virus maintained low infectivity and low viral RNA levels, did not include any GU resistance mutation, and was not extinguished (Fig. 3c and e).

The results suggest that recovery of the replicative capacity of R-mutagenized FMDV populations in the presence of GU is favored by GU resistance mutations, as previously observed in extinction-escape mutants of FMDV subjected to lethal mutagenesis treatments that included GU.[70] Furthermore, a sufficiently R-mutagenized viral population maintained a low replicative level in the presence of GU without selecting inhibitor-escape mutants.

### FMDV infectivity of a mutagenized FMDV population cannot be recovered by expansion of infection

Population Rp4 passaged in the presence of 6 mM GU did not regain the capacity to yield infectious viruses under the standard conditions of infection [$2\times10^6$ BHK-21 cells infected at a multiplicity of infection (MOI) of $\leq 0.3$ PFU/cell, as detailed in Fig. 1], despite a transient infectivity above the detection level at passage 12 (Fig. 3c). Blind passages in the absence of drugs started with the cell culture supernatant of passage 14 did not result in recovery of infectivity. Since mutagenesis increases the mutation frequency of FMDV populations and since the mutagenized population may suppress the infectivity of standard FMDV,[37,71] we investigated the possibility that FMDV Rp4 could regain infectivity by expanding the number of target cells relative to the number of infectious particles, thus avoiding coinfection with defectors. To this aim, all

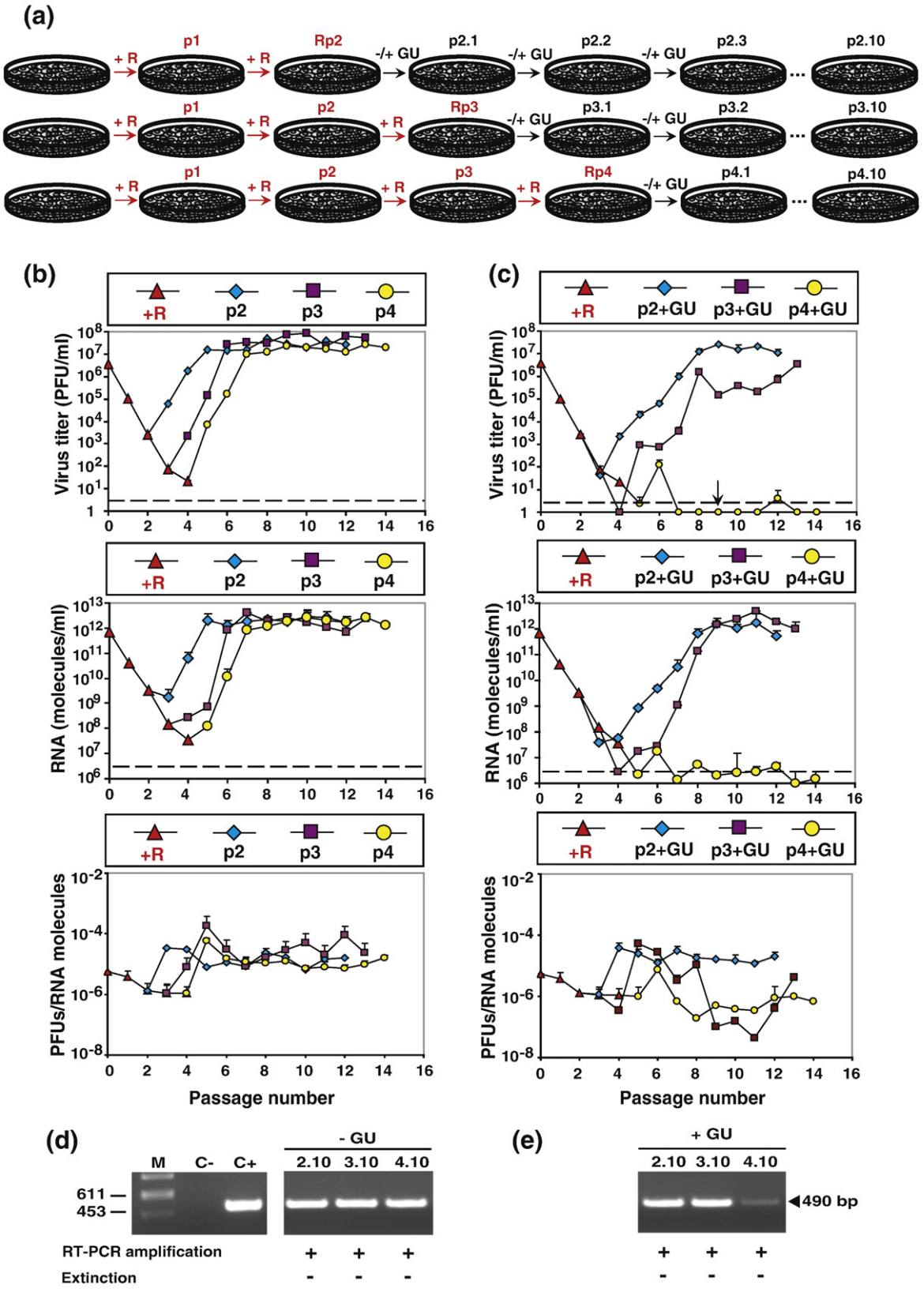


**Fig. 3** (*legend on next page*)

**Table 1.** Mutations in the 2C coding region (and deduced amino acid substitutions) identified in FMDV populations Rp2, Rp3, and Rp4 passaged 10 times in the absence or in the presence of GU

| Nucleotide position (amino acid residue)[a] | FMDV population[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | pMT28 | Rp2−GU | Rp2+GU | Rp3−GU | Rp3+GU | Rp4−GU | Rp4+GU |
| 4413 | C | C | C | C | C | U | C |
| 4507 (55) | C (R) | C | C | **U (W)** | C | C | C |
| 4819 (159) | A (M) | A | A | A | **U (L)*** | A | A |
| 4850 (169) | A (K) | A | **G (R)*** | A | A | A | A |
| 4860 | G | G | A | G | G | G | G |
| 5064 | A | A | A | A | G | A | A |
| 5087 (248) | U (I) | U | U | U | U | **A (N)** | U |

[a] Nucleotide positions and deduced amino acid positions (in parentheses) where mutations were located. The entire 2C coding region was sequenced. Residue numbering is in accordance with Escarmís *et al.*[83] Amino acid residues are numbered from the N-terminus to the C-terminus of 2C.

[b] The origin of the FMDV populations analyzed is described in Materials and Methods. The Rp2, Rp3, and Rp4 populations were passaged 10 times either in the absence of GU (−GU) or in the presence of 6 mM GU (+GU), and they are the populations described in Fig. 3. Mutations are relative to the corresponding sequence of pMT28 (second column). Letters in boldface highlight nonsynonymous mutations and corresponding amino acid substitutions (single-letter code). The asterisk denotes that the amino acid substitution was previously characterized as conferring resistance to GU.[70] The procedures for nucleotide sequencing are described in Materials and Methods.

the PFU produced by Rp4 at passage 5 in the presence of GU (arrow pointing to a total of nine passages in the first panel of Fig. 3c) were used to infect $18\times10^6$ BHK-21 cells (nine times the number of cells used in previous experiments), either in the absence or in the presence of 6 mM GU. The virus input involved 7 PFU and an MOI of about $4\times10^{-7}$ PFU/cell. No recovery of infectivity or virus-specific reverse-transcription-amplifiable material was observed even after five passages under such conditions (Fig. 4a–c). Using pMT28 as the infecting virus under the same conditions, we recovered infectivity and RT-PCR-amplifiable material upon passage in the absence of GU, but not in the presence of GU (Fig. 4d–f). Thus, an expansion of the number of cells exposed to the viral population derived from Rp4 did not result in recovery of infectivity either in the presence or in the absence of GU. In contrast, in a parallel experiment in which similar viral loads of pMT28 (without a history of R mutagenesis) were subjected to the same passage regime, pMT28 regained infectivity in the absence of GU, but not in its presence. The results suggest that a history of R mutagenesis precluded recovery of viral infectivity, and that a low viral load contributed to limiting FMDV replication in the presence of GU and in the absence of prior mutagenesis.

## Viral load influences the dominance of GU-escape FMDV mutants

The sustained low FMDV infectivity and RNA levels in FMDV passaged four times in the presence of R, and then further passaged in the presence of GU (Figs. 3 and 4), were favored by the prior history of R-mutagenesis (which led to a decrease in specific

**Fig. 3.** Selection of GU-escape mutants upon the passage of FMDV pMT28 in the presence of GU, starting at three different passages during treatment with R. (a) Scheme of the passage protocol used. The conditions of the infections of BHK-21 cells with FMDV C-S8c1 (progeny of pMT28) are described in Fig. 1. p, passage number; +R, presence of 5 mM R; −/+ GU, absence or presence of 6 mM GU. (b) FMDVs subjected to two, three, or four passages in the presence of 5 mM R (the same populations highlighted with an asterisk in Fig. 1, termed Rp2, Rp3, and Rp4) were further passaged in the absence of R or GU. For each passage, $2\times10^6$ BHK-21 cells were infected with the virus present in 0.2 ml of the infected cell culture supernatant from the previous passage, and the yield of virus infectivity and RNA molecules in the cell culture supernatants was determined. Each value represents the mean ± SD (error bars) of triplicate determinations. (c) Same as (b), except that FMDV subjected to two, three, or four passages in the presence of 5 mM R was further passaged in the absence of R, but in the presence of 6 mM GU. Infectivities and viral RNA levels were determined in triplicate, as described in (b). The discontinuous lines in (b) and (c) indicate the limit of detection of the number of viral plaques (for titer determination) and FMDV RNA (for real-time PCR quantification). The infectivity above the detection limit at passage 12 was scored in one of the triplicate titrations. (d and e) RT-PCR amplifications used to detect viral RNA in the cell culture supernatant of passage 10 of each of the series of the experiments in (b) and (c), respectively. In the lanes identified with −GU or +GU, 2, 3, and 4 indicate the number of virus passages in the presence of R, and 10 refers to the number of passages in the absence or in the presence of 6 mM GU. M, molecular size markers (HindIII-digested Φ29 DNA) [the corresponding sizes (base pairs) of the relevant bands are indicated]; C−, negative control (amplification without RNA); C+, PCR positive control. The conditions for infection, mutagenic treatment, determination of FMDV infectivity by plaque assays, and quantification of FMDV RNA are detailed in Materials and Methods.

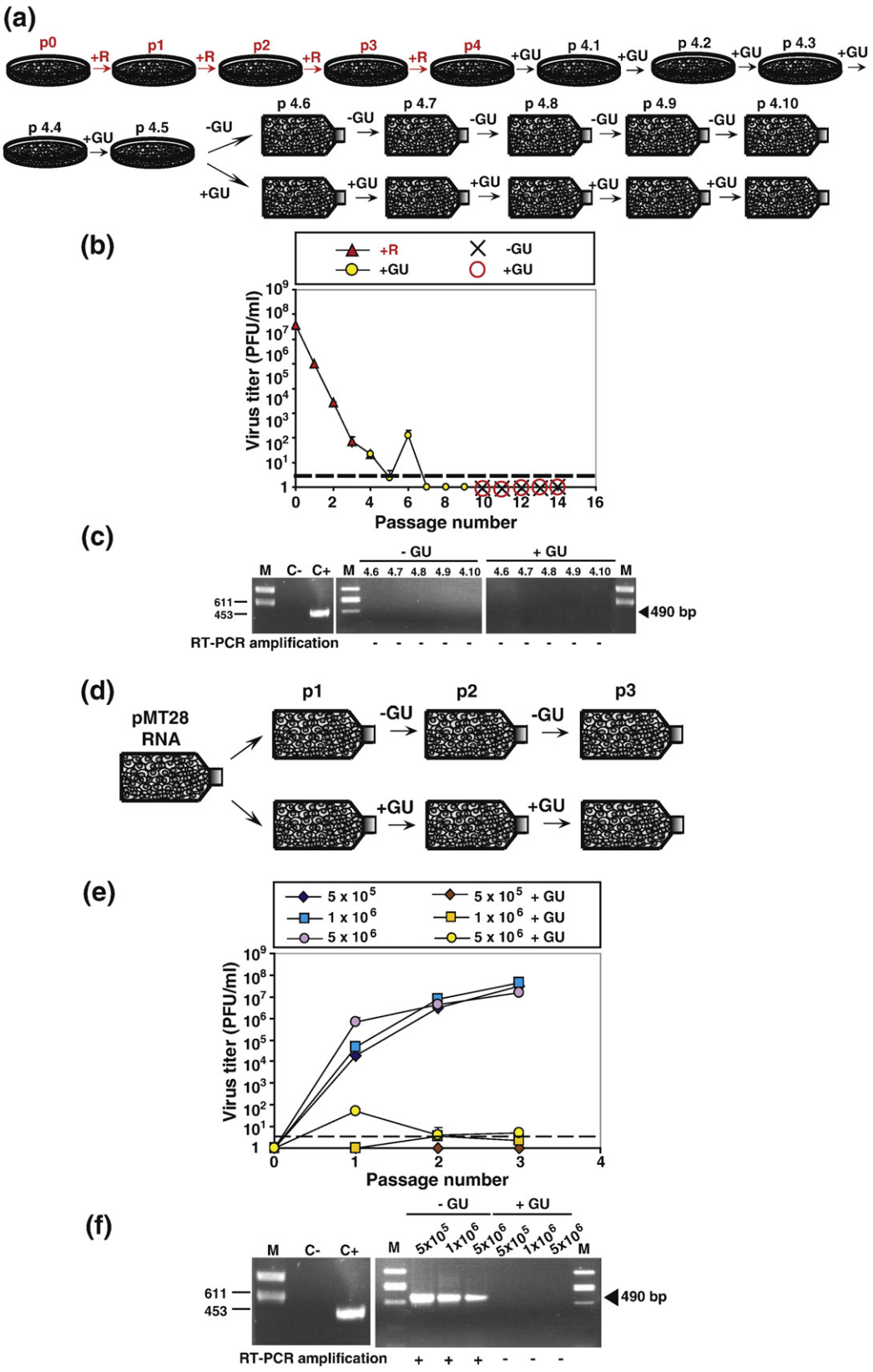


**Fig. 4.** (*legend on next page*)

infectivity) (Fig. 3) or by the decrease in viral load *per se*, irrespective of mutagenesis. A contribution of low viral load to preventing the recovery of infectious FMDV in the presence of GU was suggested by the inability of low levels of pMT28 to increase its progeny production in the presence of GU, but not in its absence (Fig. 4e and f). To investigate the effect of viral load on the selection of GU-escape mutants in the absence of mutagenesis, we passaged triplicate samples of FMDV containing either 20, 50, 75, or 2600 PFU [which approximates the range of infectious population sizes attained by populations Rp2, Rp3, and Rp4 passaged in the presence of R (compare these PFU values with those in Fig. 3b and c)] in the absence or in the presence of 6 mM GU. The results (Fig. 5) show that, in all cases, the infectivity was rapidly recovered when the virus was passaged in the absence of GU. In contrast, no recovery of viral infectivity occurred upon passage in the presence of GU when the initial total infectivity used for the first passage was 75 PFU or lower. This is in contrast to the selection of GU-resistant mutants and the recovery of infectivity of similar numbers of infectious particles but with a history of mutagenesis in population Rp3 (compare Figs. 3 and 5). However, when the initial infectivity was 2600 PFU, the triplicate samples passaged in the presence of GU (termed 2600.1+GU, 2600.2+GU, and 2600.3+GU) maintained detectable infectivity values, and in one of the triplicate samples (2600.2+GU), infectivity increased $5.2\times10^2$-fold from passage 1 to passage 5, reaching a level similar to that attained in the absence of GU (Fig. 5a). The increase in infectivity at passage 5 of replica 2 (2600.2+GU) was associated with an increase in the frequency of amino acid substitution K169R, which is known to confer resistance to GU[70] (Table 2). Passage 5 of replica 1 (2600.1+GU) included R134S in 2C, a substitution previously found in FMDV populations treated with a combination of GU and FU.[75] In replica 3 (2600.3+GU), substitution Y173 H in 2C became dominant (Table 2), but its possible involvement in GU resistance has not been determined. The amount of RNA at passage 5 of populations 20, 50, and 75 passaged in the presence of GU was not sufficient to yield a detectable RT-PCR product for sequencing.

The variation in viral load as a function of the initial infectivity suggests that 2600 PFU of FMDV might have been near a critical viral load that could lead either to sustained limited infectivity (in two of the replicas) or to a several-logarithm increase in infectivity (in one of the replicas) in the presence of GU (Fig. 5). Thus, viral load *per se*, independently of the contribution of enhanced mutagenesis, is an important determinant of the selection of GU-escape mutants. Of note, no FMDV extinction was observed upon treatment with GU even when the initial infectivity was as low as 20 PFU (Fig. 4b), emphasizing once more that, under equal decreases in viral load promoted either by a mutagenic agent or by an inhibitor, mutagenesis is the primary determinant of virus extinction[70] (see also Discussion).

## Mutagenesis can affect the selection of GU-resistant mutants of FMDV

Passages of FMDV in the presence of R altered the capacity of GU to select GU-resistant mutants, as evidenced by the selection of such mutants from populations Rp2 and Rp3, but not from Rp4 (Figs. 3 and 4; Table 1). To test whether FMDV populations Rp2 and Rp4 differed in mutant spectrum complexity, we determined the mutation frequencies and Shannon entropies for the 3D (polymerase) coding region for Rp2 and Rp4, and compared them with

**Fig. 4.** Expansion of the number of cells infected by mutagenized FMDV populations. (a) Scheme of the protocol used, with indication of the passage history of FMDV (progeny of pMT28) that involves the first four passages in the presence of 5 mM (R), in the absence of R, and in the presence of 6 mM GU; $2\times10^6$ BHK-21 cells (represented as a dish) were infected at each passage (further details are given in the legend to Fig. 3 and in Materials and Methods). Beginning with the supernatant of p4.5, the passages involved infection of $18\times10^6$ cells (indicated with a bottle) using 3 ml (out of a total of 15 ml) of the supernatant of the previous passage. Two sublines were initiated: one in the absence of 6 mM GU (−GU) and another in the presence of 6 mM GU (+GU). (b) Infectivity in the supernatants of BHK-21 cells of the experiment described schematically in (a), as indicated in the upper box. Values are expressed as the mean ± SD (error bars) of triplicate determinations. (c) RT-PCR amplification used to detect viral RNA in the cell culture supernatant of the last five passages of the experiment in (a). (d) Scheme of the passages of FMDV (progeny of pMT28). BHK-21 cells ($18\times10^6$ cells) were infected with PFU corresponding to either $5\times10^5$, $1\times10^6$, or $5\times10^6$ molecules of FMDV pMT28 determined by real-time RT-PCR. For passages 2 and 3, the same number of cells was infected with the supernatant from the previous passage (3 ml out of 15 ml). (e) Infectious progeny production in the infections of BHK-21 cells with pMT28 in the absence or in the presence of GU described in (d), as indicated in the upper box. Values are expressed as the mean ± SD (error bars) of triplicate determinations. (f) RT-PCR amplification used to detect viral RNA in the cell culture supernatant of the third passage of the experiment in (e). M, molecular size markers (HindIII-digested Φ29 DNA) [the corresponding sizes (base pairs) are indicated]; C−, negative control (amplification without RNA); C+, PCR positive control. In (b) and (e), the discontinuous line indicates the limit of detection of FMDV infectivity. The conditions for infection, mutagenic treatment, determination of FMDV infectivity by plaque assays, and quantification of FMDV RNA, as well as the positive and negative controls included in the assays, are described in Materials and Methods.

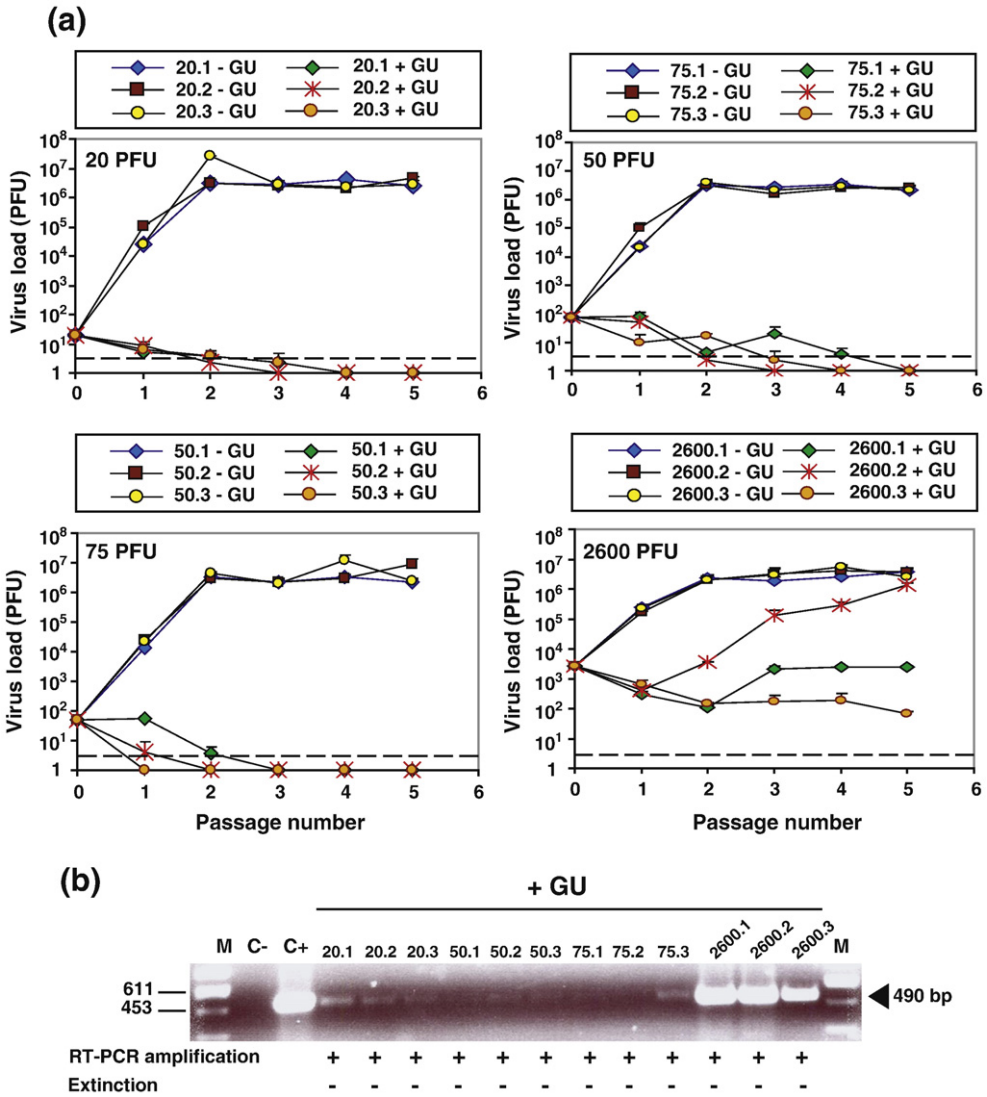


**Fig. 5.** Influence of viral load on the selection of GU-escape mutants of FMDV. (a) BHK-21 cells ($2\times10^6$ cells) were infected in parallel with either 20, 50, 75, or 2600 PFU of FMDV pMT28 in triplicate experiments. The numbers of PFU used are based on a titration of a virus stock whose value included a standard deviation that did not exceed 25% of the mean. Subsequent passages were carried out by infecting $2\times10^6$ BHK-21 cells with virus contained in 0.2 ml of supernatant from the previous passage, in the absence (−GU) or in the presence (+GU) of 6 mM GU, as indicated in the boxes above each panel (1, 2, 3, following the initial number of PFU, distinguish the triplicate assays). Titrations were carried out in triplicate, and standard deviations (error bars) are given. The conditions for infection, mutagenic treatment, and FMDV infectivity determination by plaque assay are detailed in Materials and Methods. (b) RT-PCR amplification of samples of the cell culture supernatant at passage 5 of the series treated with GU. In each lane, the origin of the sample is indicated at the top [same nomenclature as in the boxes in (a)]. M, molecular size markers (HindIII-digested Φ29 DNA) [the corresponding sizes (base pairs) are indicated on the right]; C−, negative control (amplification without RNA); C+, PCR positive control. RT-PCR was performed as described in Materials and Methods.

those of the parental FMDV population. The results (Table 3) indicate that the average mutation frequency among the components of the mutant spectrum of Rp2 and Rp4 was 3.7-fold and 4.5-fold higher, respectively, than that of the parental population (p0). Likewise, the Shannon entropy for the mutant spectrum of Rp2 and Rp4 was 1.7-fold and 1.8-fold higher, respectively, than that for p0. The increase in mutation frequency between either population Rp2 or population Rp4 and p0 is statistically significant ($p<0.01$ in both cases; chi-square test). In contrast, the corresponding increase between population Rp2 and population Rp4 is not statistically significant ($p=0.5$; chi-square test). Rp2 and Rp4 showed a higher frequency of G→A and C→U transitions, as expected from R mutagenesis

**Table 2.** Mutations in the 2C coding region (and deduced amino acid substitutions) identified in FMDV populations 2600.1, 2600.2, and 2600.3 passaged five times in the presence of GU

| Nucleotide position (amino acid residue)[a] | FMDV population[b] | | | |
|---|---|---|---|---|
| | pMT28 | 2600.1+GU | 2600.2+GU | 2600.3+GU |
| 4746 (134) | G (R) | **U (S)** | G | G |
| 4850 (169) | A (K) | A | **A/G (K/R)*** | A |
| 4860 (173) | U (Y) | U | U | **C (H)** |
| 5260 (306) | C (L) | C | **T (F)** | C |

[a] Nucleotide positions and deduced amino acid positions (in parentheses) where mutations were located. The entire 2C coding region was sequenced. Residue numbering is in accordance with Escarmís *et al.*[83] Amino acid residues are numbered from the N-terminus to the C-terminus of 2C.

[b] The populations are those started with 2600 PFU of FMDV pMT28 and passaged five times in the presence of GU, as described in Fig. 5a. Mutations are relative to the corresponding sequence of pMT28 (second column). Letters in boldface highlight nonsynonymous mutations and corresponding amino acid substitutions (single-letter code). Two residues separated by a bar indicate a mixture of two nucleotides present in about the same proportion, according to the sequence band pattern. The asterisk indicates that the amino acid substitution K169R was previously characterized as conferring resistance to GU.[70] The procedures for nucleotide sequencing are described in Materials and Methods.

(Table 3).[41,56] The frequency of G→A and C→U transitions increased in the evolution from population Rp2 to population Rp4, and two of the mutations in the mutant spectrum of Rp4 led to a termination codon, suggesting an increased frequency of defective genomes. Thus, despite not reaching statistical significance, the differences in mutant spectrum complexity and composition between Rp4 and Rp2 probably exerted some influence on the subsequent response of the viral population when confronted with replication in the presence of GU. The present study shows that both the history of mutagenesis (irrespective of differences in mutant spectrum complexity or composition reaching statistical significance) and low viral load are major determinants of a sustained viral replication without a selection of GU-resistant mutants.

## Discussion

The control of infections associated with highly variable viruses (including very important human pathogens such as HIV-1, hepatitis C virus, influenza virus, and several emerging and reemerging viruses) using inhibitors of viral replication remains a formidable task despite the continuous development of new antiviral agents.[87–90] The main problem stems from a general adaptive capacity of replicating viral populations that, in the case of drug treatment, is reflected in the selection of mutants resistant to one or multiple antiviral inhibitors. Selection is frequent probably because inhibitors exert a very focused selective pressure on targets that can be easily modified by modest numbers of amino acid substitutions while partly maintaining their functionality.[43–48,50–53] The frequent failure of classical antiviral therapy has been a major motivation to apply as antiviral therapy the concept of error catastrophe predicted by the quasispecies theory.[8,27,32,91] To this aim, mutagenic agents, together with antiviral inhibitors (administered either in combination or sequentially), are currently being tested to limit virus replication and to achieve virus extinction.

The understanding of the interplay between mutagenesis of the viral genome and inhibition of viral replication is still limited, but it is needed for the design of treatment protocols to eliminate the virus from infected cells. Assuming that a single-point mutation is sufficient to confer resistance to a drug, the probability of its generation is given by the product of the mutation rate and the viral population size. However, several factors may intervene to modify this simple equation. A history of mutagenesis may impede the fixation of drug resistance mutation through negative interactions among mutations in the same viral genome. As the population size increases, complementation among components of the mutant spectrum may enhance the replicative capacity of the ensemble,[57,71,92] but it may delay or prevent the fixation of beneficial mutations through clonal interference.[93,94] In the present study, we have approached the connection between mutagenesis and inhibition of virus replication by using as model system FMDV in cell culture, the mutagenic activity of R,[21,23,41,54–56,72,95–98] and the inhibition of RNA synthesis exerted by GU.[70,80–82] In this system, R mutagenesis may prevent the virus from increasing its capacity to produce progeny in the presence of GU (Fig. 3). The population that showed such an impairment had both a decreased viral load and an increased complexity of its mutant spectrum, both as a result of R treatment. Use of the same initial infectivity (50 PFU) either from population Rp3 or from virus pMT28 indicated that R mutagenesis favored selection of GU-resistant FMDV mutants (compare Figs. 3 and 5). In addition, a low viral load *per se* (not necessarily as a result of a prior history of enhanced mutagenesis) was sufficient to prevent a recovery of the capacity to increase the production of infectious progeny in the presence of GU (Figs. 4 and 5). Under the conditions of our experiments, a value of 2600 PFU for initiating the infection was identified as being near a critical threshold value that may drive the virus towards either increased progeny production or sustained low-level replication in the presence of GU. The

**Table 3.** Characterization of the mutant spectra of FMDV populations passaged in the presence of R

| | FMDV population[a] | | | | | |
|---|---|---|---|---|---|---|
| | p0 | | Rp2 | | Rp4 | |
| | Mutation[b] | Amino acid substitution[b] | Mutation[b] | Amino acid substitution[b] | Mutation[b] | Amino acid substitution[b] |
| | T6756C | — | C6891T | — | A6630G | — |
| | T7510C | S301P | G6897A | — | G6669A | — |
| | A7606G | M333V | G6899A | S97N | C6674T | A22V |
| | T7612G | S335A | T6932C | L108P | G6691A | G28S |
| | | | G6984A | — | T6717C | — |
| | | | T7004C | I132T | T6725A | STOP |
| | | | C7032T | — | A6733G | K42E |
| | | | C7096T | — | T6778C | F57L |
| | | | C7104T | — | C6834T | — |
| | | | C7115T | P169L | T6856C | S83R |
| | | | C7230T | — | C6865T | H86Y |
| | | | C7302T | — | G6871A | V88S |
| | | | G7368A | M253I | T6879C | — |
| | | | T7373C | I255T | G6889A | A94T |
| | | | G7406A | G266D | C6931T | L108S |
| | | | C7410T | — | C6954T | — |
| | | | G7428A | — | A7051G | K148E |
| | | | C7491T | — | A7068G | — |
| | | | T7509C | — | C7115T | P169L |
| | | | G7520A | S304N | C7171T | H188Y |
| | | | C7555T | L316F | A7195G | I196V |
| | | | T7569C | — | A7220G | H204R |
| | | | G7584A | — | G7208A | C200Y |
| | | | G7594A | D329N | G7244A | G212D |
| | | | A7597G | T330A | G7358A | S250R |
| | | | G7636A | A343T | T7408C | F267L |
| | | | T7744C | S379P | A7450T | T281S |
| | | | A7775G | H299R | G7494A | — |
| | | | G7798A | G397R | G7513A | A302T |
| | | | T7801C | F398L | A7522G | I305V |
| | | | A7807G | K400E | T7524C | — |
| | | | T7817C | M403I | T7547C | I314T |
| | | | G7834A | E409k | G7554A | — |
| | | | A7922G | E438G | G7632A | — |
| | | | C7943T | P445L | C7664T | A352V |
| | | | G7996A | V463M | C7693T | STOP |
| | | | C8013T | — | A7712G | D368G |
| | | | A8019T | — | A7716G | — |
| | | | | | T7737C | — |
| | | | | | C7755T | — |
| | | | | | T7757C | V383A |
| | | | | | A7781G | H391R |
| | | | | | C7821T | — |
| | | | | | C7845T | — |
| | | | | | T7851A | F414L |
| | | | | | A7871G | Q421R |
| | | | | | G7873A | E422K |
| | | | | | G7878A | — |
| | | | | | G7891A | A428T |
| | | | | | A7893G | — |
| | | | | | G7900A | — |
| | | | | | C7947T | — |
| | | | | | G7952A | G448D |
| | | | | | C7979T | S457L |
| Total mutations[c] | 4 | | 38 | | 54 | |
| Nucleotides sequenced | 10,000 | | 24,375 | | 29,473 | |
| Mutation frequency[d] | $4 \times 10^{-4}$ | | $15 \times 10^{-4}$ | | $18 \times 10^{-4}$ | |
| Shannon entropy (*S*)[e] | 0.53 | | 0.90 | | 0.96 | |
| G→A+C →U[f] | 0 | | 25 | | 30 | |
| Synonymous[g] | 1 | | 16 | | 20 | |
| Nonsynonymous[g] | 3 | | 22 | | 34 | |

value for this critical threshold is not unexpected if one assumes an average mutation frequency for FMDV of $10^{-4}$ per nucleotide and if one assumes that at least four point mutations in 2C have been assigned to the GU resistance phenotype.[31,70] Obviously, the critical infectivity values determined in our study apply to a specific virus–host system endowed with specific replicative properties and will require modification when applied to other virus–host systems.

Limitations of virus population size may underlie the absence or delay in the selection of drug-resistant viral mutants *in vivo*. Drug resistance mutations are infrequent in HIV-1-infected patients, in which viral load can be maintained at low levels.[99–101] However, virus evolution may continue as long as the virus replicates, even if replication involves a limited number of viral genomes and rounds of copying. What the low replicating load implies is that the probability of occurrence of one mutation (or a limited number of mutations) needed to confer resistance to a drug is low when the viral load is low. It must be also stressed that, in our experiments, the decrease in viral load that prevented extended replication in the presence of GU was the result of R mutagenesis, suggesting a critical involvement of mutagenesis in the events that could lead to sustained replication without selection of GU-resistant FMDV. An increased mutagenesis could impede fixation of beneficial mutations (in this case those that confer GU resistance) as a result of epistatic interference among mutations.[102,103] Previous studies with FMDV indicated also that mutagenesis—and not merely the inhibition of viral replication that accompanies mutagenesis—was required for FU to drive the virus towards extinction.[70]

Restriction of the selection of drug-escape mutants in viral populations imposed by a low viral load renders genetic bottlenecks (colonization of new cells, tissues, or organs by one infectious particle or by a limited number of infectious particles) as an important event in regard to response to antiviral treatment. Bottleneck events are probably frequent during viral infections *in vivo*.[104–115] Antiviral treatments with mutagens may give rise to transient decreases in population size, and the possible ensuing bottlenecks may relieve the system of the suppressive effects of mutagenized mutant spectra and may accentuate the stochastic (chance) nature of the selection of possible mutagen-escape or inhibitor-escape mutants.[92] The consequences of the interplay between mutagenesis, inhibition, viral load, and selection of inhibitor-escape mutants are relevant to a possible clinical application of lethal mutagenesis. Indeed, the results reinforce the concept that although extinction might not be achieved as a direct consequence of mutagenic-inhibitory treatment, decreases in viral fitness and viral load, even if transient, may provide the host immune system with an opportunity to clear the virus. Numerous studies have associated a high viral load during primoinfection (or as a result of nonresponsiveness to treatment) with disease progression in infections; this is deemed to be as relevant as the associations in human immunodeficiency virus or hepatitis C virus.[116–120] A sustained low viral load may also enhance the efficiency of alternative antiviral interventions that may also include a mutagenic agent.[75] Therefore, attaining a sustained low viral load, even though virus extinction is not achieved directly as a result of mutagenesis, can be a justified aim of a treatment based on lethal mutagenesis. The trajectory towards either virus survival or extinction is critically dependent on the enrichment of the population with inhibitor-escape mutants, which is favored by an increased mutational input but can be counterbalanced by the deterioration of the replicative capacity of the mutant ensemble as a result of mutagenesis.[42] In this respect, it will be important to compare simultaneous *versus* sequential treatment regimens[72] to achieve a low viral load in infected patients. The mechanisms that underlie lethal mutagenesis are not yet well understood, and their clarification is needed in the design of more effective protocols for antiviral therapies.

**Notes to Table 3:**

[a] The viral populations analyzed are those described in Figs. 2 and 3. p0 is the initial population, and Rp2 and Rp4 are the populations subjected to two and four passages, respectively, in the presence of 5 mM R.

[b] Mutations and deduced amino acid substitutions in the 3D (polymerase) coding region are relative to the sequence of the parental clone C-S8c1.[77] Amino acid residues (single-letter code) are numbered from the N-terminus to the C-terminus of 3D. The procedures for nucleotide sequencing and identification of FMDV genomic regions are described in Materials and Methods.

[c] Number of different mutations found comparing the sequence of each individual clone with the corresponding sequence of FMDV C-S8c1.[77]

[d] Mutation frequency is expressed in substitutions per nucleotide (total number of different mutations found divided by the total number of nucleotides sequenced).

[e] Shannon entropy gives the proportion of different sequences in the mutant spectrum. It is calculated with the formula $S = -[\sum(p_i \ln p_i)]/\ln N$, where $p_i$ is the proportion of each sequence of the mutant spectrum, and $N$ is the total number of sequences compared.

[f] Number of G→A+C→U substitutions found comparing the sequence of each individual clone with the corresponding sequence of FMDV C-S8c1.[77]

[g] The ratio of synonymous mutations to nonsynonymous mutations is 1.37 for Rp2 and 1.70 for Rp4. The difference is not statistically significant ($p = 0.78$; chi-square test).

## Materials and Methods

### Cells and viruses

The origin of BHK-21 cells and the procedures for cell growth in Dulbecco's modified Eagle's medium (DMEM) and for plaque assays in semisolid agar have been previously described.[121,122] The cell culture of infected and uninfected cells was carried out at 37 °C. The viruses used in the experiment are as follows: FMDV C-S8c1 is a plaque-purified derivative of serotype C isolate C1 Santa Pau-Sp70.[122] An infectious clone of FMDV C-S8c1, termed pMT28, was constructed by recombination into pGEM-1 plasmid subclones that represent the C-S8c1 genome, as described previously.[76,77] Thus, FMDV pMT28 used in the experiments is the progeny of infectious transcripts that express the standard FMDV C-S8c1. To control for the absence of contamination, we cultured mock-infected cells, and we titrated their supernatants in parallel with the infected cultures. No infectivity or sign of cytopathology in mock-infected cultures was detected in any of the experiments.

### Treatment with GU

A solution of GU (Sigma) in DMEM was prepared at a concentration of 50 mM, sterilized by filtration, and stored at 4 °C for a maximum of 14 days. Prior to use, the stock solution was diluted in DMEM to reach the desired concentration. For infections of BHK-21 cells with FMDV in the presence of GU, no pretreatment of the cell monolayer with GU was performed. After the addition and adsorption (60 min) of FMDV and washing of the cell monolayers, infections were allowed to continue in the presence of GU. For each passage, $2\times10^6$ BHK-21 cells were infected with virus from the cell culture supernatant from the previous infection (0.2 ml), and the new infection was allowed to proceed for about 24 h. The MOI for each passage can be calculated from the infectivity values given for each experiment described in Results. Cells infected in the absence of GU and mock-infected cells were maintained in parallel. No evidence of cell contamination with virus was observed at any time.

### Treatment with R

A solution of R (Sigma) in phosphate-buffered saline was prepared at a concentration of 100 mM, sterilized by filtration, and stored at −70 °C. Prior to use, the stock solution was diluted in DMEM to reach the desired R concentration. For infections in the presence of R, cell monolayers were pretreated for 7 h with 5 mM R prior to infection. FMDV C-S8c1 (progeny of pMT28) was passaged serially in the absence or in the presence of 5 mM R. Incubation of confluent BHK-21 cell monolayers (as used for the infections for FMDV) with 5 mM R for 31 h resulted in cell viabilities of 40–50%, as determined with trypan blue exclusion (range calculated in six different determinations). Therefore, the toxicity of R to the cells cannot account for FMDV extinction.[75] After adsorption of FMDV and washing of the cell monolayers, the infection was allowed to proceed in the presence of the same concentration of R. For each passage, $2\times10^6$ BHK-21 cells were infected with virus from the cell culture supernatant from the previous infection (0.2 ml), and the new infection continued for about 24 h. The MOI for each passage can be calculated from the infectivity values given for each experiment described in Results. Cells infected in the absence of R and mock-infected cells were maintained in parallel. No evidence of cell contamination with virus was observed at any time.

### Assessment of FMDV extinction

FMDV is considered extinct when no virus infectivity and no viral RNA that could be amplified by a highly sensitive RT-PCR protocol [a 3D coding region was amplified using, as primers, oligonucleotide A2SacI (5′-CACACATCGACCCTGAACCGCACCACGA; sense orientation; the 5′ nucleotide corresponds to genomic residue 6581) and oligonucleotide AV4 (5′-TTCTCTTTTCTCCATGAGCTT; antisense orientation; the 5′ nucleotide corresponds to genomic residue 7071)] can be demonstrated in the cell culture supernatant or after three blind passages of the supernatant in BHK-21 cells in the absence of any drug, as described in previous studies.[75] It should be noted that the loss of FMDV infectivity below the level of detection did not necessarily imply extinction. This was the case in the experiments on the treatment of FMDV with R and GU, described in Figs. 3 and 5. In the protocol in which low viral amounts were used to infect a large number of cells in an attempt to rescue the virus by expansion of the infections (Fig. 4), the absence of RT-PCR amplification fulfilled, in several cases, the requirement to consider the virus extinct (Fig. 4c and f). However, control experiments showed that the large dilution underwent by the minimal amount of virus present in the sample of Rp4 passaged 10 times in the presence of GU (Fig. 3c) was in itself sufficient to prevent a positive RT-PCR amplification using our standard protocols, despite the initial sample containing detectable RNA. A positive amplification might have been achieved by concentrating the culture medium or the extracted RNA, but concentration was not attempted because the occurrence of extinction was not the critical point under evaluation in the experiments of population expansion (Fig. 4).

### RNA extraction, RNA quantification, cDNA synthesis, PCR amplification, and nucleotide sequencing

Viral RNA was extracted from the medium of infected cells using Trizol (Invitrogen), as previously described.[83] Reverse transcription was performed with AMV reverse transcriptase (Promega), and PCR amplification was carried out using Expand High Fidelity (Roche), as specified by the manufacturers. The 3D coding region was amplified using, as primers, oligonucleotide PolC-KpnI (5′-GTTGGTACCCACTCTGCTGGAGGC; sense orientation; the 5′ nucleotide corresponds to genomic residue 6502) and oligonucleotide Rend (5′-GGATTAAGGAAGCGGGAAA; antisense orientation; the 5′ nucleotide corresponds to genomic residue 8115). The 2C coding region was amplified using, as primers, oligonucleotide 2BR2 (5′-GAAGACCTTGAGAGAGCAG; sense orientation; the 5′ nucleotide corresponds to genomic

residue 4318) and oligonucleotide 2CD4 (5′-ACACAGATTTTTGGGAAGGT; antisense orientation; the 5′ nucleotide corresponds to genomic residue 5326). Nucleotide sequences of genomic FMDV RNA were determined using the ABI 373 sequencer, as previously described.[41] To determine the complexity of the mutant spectra, we extracted FMDV RNA as described above and subjected it to RT-PCR to amplify the 3D coding region (residues 6610–8020), as described previously.[41] Genomic residues are numbered as described by Escarmís *et al.*[83] Amplification products were analyzed by agarose gel electrophoresis using HindIII-digested Φ29 DNA as molar mass standard. Negative controls (amplifications in the absence of RNA) were included in parallel to ascertain the absence of contamination by template nucleic acids. The basal mutation frequency among components of the mutant spectra of unpassaged FMDV populations (p0) was at least 2.3-fold higher than the frequency attributed to misincorporations during the RT-PCR amplification used.[123]

#### Quantification of FMDV RNA

Real-time quantitative RT-PCR was carried out using the Light Cycler RNA Master SYBR Green I kit (Roche), according to the instructions of the manufacturer and as described previously.[124] The 2C coding region of the FMDV genome was amplified using, as primers, oligonucleotide 2CR2 (5′-GGCAAACCCTTCAGCAGTAAG; sense orientation; the 5′ nucleotide corresponds to genomic residue 4924) and oligonucleotide 2CD3 (5′-CGCTCACGTCGATGTCAAAGTG; antisense orientation; the 5′ nucleotide corresponds to genomic residue 5047). Quantification was relative to a standard curve obtained with known amounts of FMDV RNA, synthesized by *in vitro* transcription of FMDV cDNA (plasmid pMT28). The specificity of the reaction was monitored by determining the denaturation curve of the amplified DNAs. Negative controls (without template RNA and RNA from mock-infected cells) were run in parallel with each amplification reaction to ascertain the absence of contamination with undesired templates.

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