Journal of General Virology (2013), **94**, 817–830 **DOI** 10.1099/vir.0.049171-0

Molecular dissection of a viral quasispecies under mutagenic treatment: positive correlation between fitness loss and mutational load

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Low fidelity replication and the absence of error-repair activities in RNA viruses result in complex and adaptable ensembles of related genomes in the viral population, termed quasispecies, with important implications for natural infections. Theoretical predictions suggested that elevated replication error rates in RNA viruses might be near to a maximum compatible with viral viability. This fact encouraged the use of mutagenic nucleosides as a new antiviral strategy to induce viral extinction through increased replication error rates. Despite extensive evidence of lethal mutagenesis of RNA viruses by different mutagenic compounds, a detailed picture of the infectivity of individual genomes and its relationship with the mutations accumulated is lacking. Here, we report a molecular analysis of a foot-and-mouth disease virus population previously subjected to heavy mutagenesis to determine whether a correlation between increased mutagenesis and decreased fitness existed. Plaque-purified viruses isolated from a ribavirin-treated quasispecies presented decreases of up to 200-fold in infectivity relative to clones in the reference population, associated with an overall eightfold increase in the mutation frequency. This observation suggests that individual infectious genomes of a quasispecies subjected to increased mutagenesis lose infectivity by their continuous mutagenic 'poisoning'. These results support the lethal defection model of virus extinction and the practical use of chemical mutagens as antiviral treatment. Even when extinction is not achieved, mutagenesis can decrease the infectivity of surviving virus, and facilitate their clearance by host immune responses or complementing antiviral approaches.

Received 24 October 2012
Accepted 10 December 2012

INTRODUCTION

Low fidelity polymerases and the absence of proofreading- and post-replicative-repair activities in RNA viruses are responsible for elevated mutation rates during replication, leading to highly heterogeneous viral populations termed quasispecies (Domingo, 2007; Domingo *et al.*, 2006, 2012; Lauring & Andino, 2010; Más *et al.*, 2010; Perales *et al.*, 2010). Intra-population genetic diversity permits rapid adaptability to complex environments, a trait which is critical for the establishment of infections in the host and successful virus propagation in nature (Bull *et al.*, 2010; Domingo, 2007; Pfeiffer & Kirkegaard, 2005; Vignuzzi *et al.*, 2006). The estimated error rates during RNA virus replication are in the range of 10^{-3} to 10^{-5} substitutions per nucleotide copied, values that are several orders of magnitude above those calculated for cellular DNA replication (Arias *et al.*, 2008; Arnold *et al.*, 2005; Batschelet *et al.*, 1976; Drake & Holland, 1999; Holland *et al.*, 1982). Theoretical predictions suggested that these error frequencies in RNA viruses are near to a maximum value compatible with maintaining genetic information and therefore, virus viability, namely the error threshold (Domingo, 2000; Eigen, 2002; Eigen & Biebricher, 1988; Eigen & Schuster, 1979; Swetina & Schuster, 1982). These

predictions encouraged a new antiviral strategy, termed lethal mutagenesis, based on the use of mutagenic compounds as antiviral agents (Holland *et al.*, 1990; Loeb *et al.*, 1999) with the aim of increasing replication error rates above the error threshold for virus viability (Eigen, 2002; Graci & Cameron, 2008; Loeb *et al.*, 1999). Experimental demonstrations of this hypothesis were obtained in cell culture with viruses from several different families, and considerable progress has been made on the molecular mechanisms that underlie decreases of infectivity and viral load associated with enhanced mutagenesis (Crotty *et al.*, 2000; Domingo *et al.*, 2012; Grande-Pérez *et al.*, 2002, 2005b; Holland *et al.*, 1990; Loeb *et al.*, 1999; Moreno *et al.*, 2011; Pariente *et al.*, 2001; Sierra *et al.*, 2000). In addition, recent studies have demonstrated that increased mutation frequencies in viral quasispecies, as a consequence of either *in vitro* chemical mutagenesis or the presence of a lower fidelity polymerase, typically result in decreased infectivity and pathogenesis *in vivo* (Gnädig *et al.*, 2012; Sanz-Ramos *et al.*, 2012).

A possible practical use of mutagens as antiviral agents is supported by several lines of evidence. Firstly, the finding that ribavirin (Rib) (1-β-D-ribofuranosyl-1-*H*-1,2,4-triazole-3-carboxamide), typically used as an antiviral compound against hepatitis C virus (HCV) infections, is mutagenic for several RNA viruses (Airaksinen *et al.*, 2003; Castro *et al.*, 2005; Crotty *et al.*, 2000; Moreno *et al.*, 2011) has encouraged the search of new mutagenic nucleoside analogues to target viral infections (Harris *et al.*, 2005; Moriyama *et al.*, 2008). Rib is an analogue of the standard nucleosides guanosine and adenosine, and its nucleoside-triphosphate derivative (RTP) has been shown to be incorporated by different viral polymerases in replicating RNA *in vitro* (Agudo *et al.*, 2010; Arias *et al.*, 2008; Castro *et al.*, 2005; Crotty *et al.*, 2000; Ferrer-Orta *et al.*, 2010; Maag *et al.*, 2001; Sierra *et al.*, 2007). However, whether Rib acts as a mutagen when clinically used to treat HCV infections is still an open question (Asahina *et al.*, 2005; Contreras *et al.*, 2002; Cuevas *et al.*, 2009; Chevaliez *et al.*, 2007; Dixit *et al.*, 2004; Kanda *et al.*, 2004; Lutchman *et al.*, 2007; Maag *et al.*, 2001; Perelson & Layden, 2007; Zhou *et al.*, 2003). A second piece of evidence supporting lethal mutagenesis *in vivo* came from a study with 5-fluorouracil, a mutagenic base analogue used in cancer chemotherapy (reviewed by Agudo *et al.*, 2009), which prevented the establishment of an infection by lymphocytic choriomeningitis virus (LCMV) in mice (Ruiz-Jarabo *et al.*, 2003). Currently, several new mutagenic nucleoside analogues are being synthesized and some of them assayed in clinical trials such as KP-1212/1416, tested for the treatment of human immunodeficiency virus-infected patients (Clay *et al.*, 2011; Harris *et al.*, 2005; Hicks *et al.*, 2012; Mullins *et al.*, 2011).

Previous studies with foot-and-mouth disease virus (FMVD) showed that the virus can be extinguished by mutagenic base or nucleoside analogues, and that mutagenized populations interfered with replication of the standard, infectious virus (González-López *et al.*, 2004, 2005; Pariente *et al.*, 2001; Perales *et al.*, 2009a, 2011; Sierra *et al.*, 2000). Parallel studies with LCMV also documented viral extinction and interference exerted by mutagenized quasispecies (Grande-Pérez *et al.*, 2005b; Martín *et al.*, 2010). These lines of evidence led to the proposal of a lethal defection model in which defectors (a class of defective, replication-competent viral genomes) interfere with the replication of infectious genomes (González-López *et al.*, 2004; Grande-Pérez *et al.*, 2005b; Martín *et al.*, 2010).

A pivotal aspect of lethal mutagenesis of RNA viruses, with important implications for the efficacy of virus extinction, which has not yet been addressed is the fitness cost associated with increased error rates among infectious genomes that replicate during lethal mutagenesis. Infectious genomes in mutagenized populations might be escaping mutagenesis by some unknown mechanism or, alternatively, they might be accumulating mutations that affect fitness to different extents. Despite an overall sixfold fitness decrease determined in pre-extinction FMDV populations (González-López *et al.*, 2005), such populations are likely to be formed by a complex composition of infectious, quasi-infectious, defectors, and non-infectious genomes, in which viral fitness might be sustained by a minority subpopulation of infectious genomes. Further, fitness and mutation frequency in a pre-extinction population might depend on different subsets of genomes. For these reasons we undertook a study of the infectious progeny recovered from a population subjected to mutagenesis, to contribute to an understanding of the molecular events ruling lethal mutagenesis. We have quantified the infectivity of biological clones isolated from Rib-mutagenized and non-mutagenized FMDV populations. The main findings of the present study are that: (i) a vast majority of the infectious clones isolated from mutagenized FMDV populations were less infectious than those from the parental untreated population, and those from passage 4 in the presence of Rib presented lower fitness values than those from passage 1, indicating a progressive loss of infectivity during successive rounds of replication in the presence of the mutagen. (ii) Fitness loss correlated with the number of mutations. We have extrapolated a total number of 22 mutations per genome as the average number of mutations to reach no infectivity, which can be regarded as an approximation to the extinction-threshold value for FMDV under Rib mutagenesis in BHK-21 cells. This value is less than 10-fold of the mutation frequency in populations replicating in the absence of mutagens, reinforcing the concept that error rates in RNA viruses are near the error threshold for maintenance of viability (Domingo, 2000; Eigen, 2002; Holland *et al.*, 1990). Comparison of the genetic divergence of these clones with the divergence that can be achieved by viral clones subjected to plaque-to-plaque transfers demonstrates the capacity of FMVD for fitness compensation when competition-selection is allowed. Little or no

opportunity exists for such compensation when the virus is immersed in an enhanced mutagenesis process.

RESULTS

Viral progeny production and cytopathology by FMDV passaged in the presence of Rib

To investigate the effect of increased mutagenesis on the virus infectivity we initially chose a high fitness FMDV population that might enable the isolation of biological clones within a wider range of fitness, thus facilitating this study. FMDV M6p0, a clonal population derived from high fitness FMDV C^9_{22}p150 (Arias *et al.*, 2004; Escarmís *et al.*, 1999), was subjected to four consecutive passages either in the absence or presence of 5 mM Rib (Fig. 1), a concentration that provides a strong antiviral activity (Agudo *et al.*, 2010). An initial drastic drop in viral titre was observed in the supernatant of BHK-21 cells collected after the first passage in the presence of Rib (Rp1) (Fig. 1). After the initial decrease, viral progeny production remained constant at passages 2 and 3, and decreased again at passage 4 (Rp4). The time needed for complete cytopathology of infected cells (time at which the cell culture supernatant was collected for analysis) was delayed at late passages (complete cytopathology observed at 24 h for passage 1 and >40 h for passages 2, 3 and 4).

Fitness of biological clones isolated from Rib-treated and untreated populations

To compare the relative replicative capacity (fitness) of clones in FMDV populations replicating in the absence and presence of Rib, individual biological clones were isolated from populations M6p0, M6p4, Rp1 and Rp4, and the virus titre per plaque was determined (Fig. 2). The viral titre per plaque had been previously proven a valid surrogate value for viral fitness in FMDV (Escarmís *et al.*, 2008; Lázaro *et al.*, 2003). Viral titres in biological clones isolated from both Rp1 and Rp4 populations were significantly lower than the corresponding values in clones from reference population M6p0 ($P=0.035$ and $P<0.0001$, respectively; two tailed *t*-test), suggesting that replication in the presence of Rib led to a loss of infectivity (Fig. 2). There was a trend to lower titres in passage 4 (Rp4) than in passage 1 (Rp1) ($P=0.085$; two tailed *t*-test). No significant difference was observed between viral clones from untreated populations M6p0 and M6p4 ($P=0.70$; two tailed *t*-test), which suggests that the decreased infectivity in clones from population Rp4 is a consequence of replication in the presence of Rib, and not of serial passage of FMDV in BHK-21 cells (Fig. 2 and Table 1). The mean viral titre calculated for clones derived from population M6p0 was 6.1×10^6 p.f.u. per plaque, and this mean titre was taken as representing fitness 1 (Fig. 2 and Table 1). The fitness value of each clone was then calculated as the viral progeny production per plaque at 20 h post-infection (p.i.), with respect to the average progeny production in plaques isolated from the reference population M6p0 (Table 1). Typically, largest fitness costs were associated with a small plaque size phenotype (Fig. 3). In Rp1, seven of eight clones exhibited relative fitness values below 1,

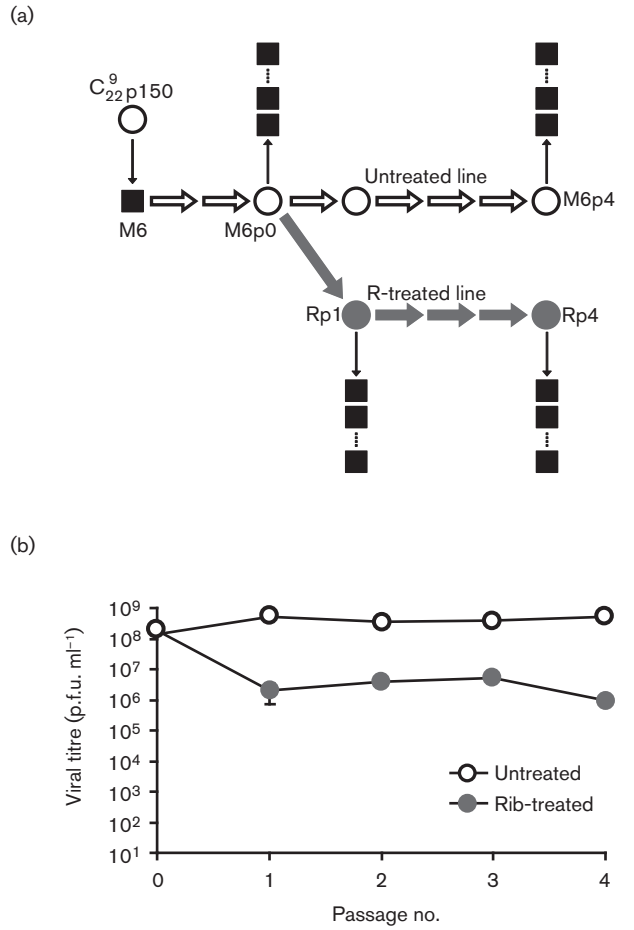

Fig. 1. Passage of FMDV in the absence and presence of Rib. (a) Schematic representation of passages and isolation of biological clones from FMDV populations passaged in the absence (untreated line, white wide arrows) or presence of 5 mM Rib (Rib-treated line, grey wide arrows). The origin of the FMDV population C^9_{22}p150 and its clonal derivative M6 is described in Methods. M6p0 (M6 passage 0; the reference FMDV population used in the present study) and M6p4 were obtained after 2 and 6 passages of clone M6, respectively. Rp1 and Rp4 were obtained after 1 and 4 passages of M6p0, respectively, in the presence of 5 mM Rib. The resulting FMDV populations are depicted as white circles (untreated) and grey circles (Rib-treated). Biological clones isolated from individual viral plaques are depicted as black squares. Black thin arrows represent biological cloning events to obtain virus from plaques. (b) Viral titre of FMDV populations passaged in the absence (white symbols) or presence of 5 mM Rib (grey symbols). Each value is the mean of at least three independent determinations. SD are given (in most cases hidden by the symbols). Procedures for FMDV infection and isolation of biological clones are detailed in Methods.

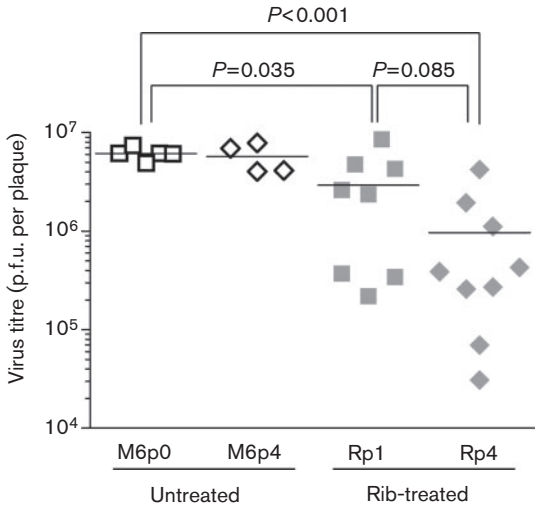

Fig. 2. Virus titre (p.f.u. per plaque) in clones isolated from untreated and Rib-treated FMDV populations, as indicated in the abscissa. Each value is the mean of at least three independent determinations. The origin of the viral populations analysed is depicted in Fig. 1. *P*-values of *t*-test analyses performed are given for comparisons between M6p0 and Rp1, M6p0 and Rp4, and Rp1 and Rp4; not shown in the figure is the *P*-value for the comparison between M6p0 and M6p4 ($P=0.70$). Procedures are described in Methods.

being three clones below 0.1, while in Rp4, all the nine clones presented fitness values below 1, and six of them had a value lower than 0.1. Relative fitness values were also calculated from the titre of plaques isolated at 24 h p.i. and they were similar to those obtained with plaques isolated at 20 h p.i. (Table 1). In summary, FMDV biological clones from populations subjected to Rib-treatment displayed lower fitness than biological clones from untreated populations, and fitness decreased upon FMDV passage in the presence of Rib, a trend that was not observed in clones from a population passaged in parallel in the absence of Rib.

Mutations in biological clones from Rib-treated populations

To quantify a possible connection between fitness loss and accumulation of mutations in the FMDV genome, full genome sequencing of eight biological clones isolated from the Rp4 population was performed. The largest numbers of mutations per genome were found in those clones with the lowest fitness values (29 and 26 mutations in clones with fitness values of 0.01 and 0.005, respectively). Conversely, the lowest number of mutations per genome in any of the clones from population Rp4 was 10, and corresponded to the highest fitness value of 0.7 found among the clones sampled from this population (Table 1). The difference between the number of mutations present in clones from Rib-treated and untreated populations was highly significant ($P<0.0001$, two tailed *t*-test). There was a positive correlation between mutations per genome and fitness loss, based on the values for clones from populations M6p4 and Rp4 ($P=0.0009$) (Fig. 4). Although with considerable scatter, the graph suggests that a total mean number of 22 mutations per genome (with the 95 % confidence interval spanning a range of 16.3–26.6 mutations) might lead to a theoretical relative fitness value of 0, suggesting a critical mutational load for virus extinction under Rib mutagenesis in the BHK-21 cell environment (see Discussion). Therefore, fitness loss correlated with the number of mutations accumulated per viable genome.

Distribution of mutations along the FMDV genome

A total of 158 mutations (19.8 mutations per genome) were identified in eight biological clones isolated from Rp4, relative to the sequence of parental population M6p0 (Fig. 5). By contrast, only 10 mutations were identified in the whole genome sequence analysis of four biological clones from the untreated population M6p4 (2.5 mutations per genome). In Rp4 clones, transitions G→A plus C→U amounted to 73 % of the total number of mutations, in remarkable contrast with untreated populations that showed only 8 % G→A plus C→U. The mutational bias associated with Rib mutagenesis is in agreement with the distribution of transitions observed previously for other FMDV populations subjected to Rib mutagenesis (Agudo *et al.*, 2010; Airaksinen *et al.*, 2003; Sierra *et al.*, 2007).

Strikingly, only two of the 158 mutations scored in the Rp4 clones were found more than once in the analysis: G931A (clones 7 and 9) and G941A (clones 1 and 3), both located within the internal ribosome entry site (IRES) element. This suggests that the mutational pressure elicited by Rib did not select for coincident changes that could be associated with decreased sensitivity to Rib (Fig. 5). No mutations resulted in amino acid substitutions that were previously related to Rib resistance in FMDV or other picornaviruses (Agudo *et al.*, 2010; Ferrer-Orta *et al.*, 2010; Levi *et al.*, 2010; Pfeiffer & Kirkegaard, 2003, 2005; Sierra *et al.*, 2007; Vignuzzi *et al.*, 2006, 2008) (Table 2). Mutations were distributed along the genome in a nearly random manner, with minor differences regarding the frequency of non-synonymous mutations depending on the coding region considered (Fig. 5). Non-structural proteins L and 3A presented a higher tolerance to non-synonymous mutations ($>4\times10^{-3}$ replacements per encoded amino acid) than mean, while proteins VP4 and 2B seemed less tolerant than mean ($<1\times10^{-3}$ replacements per encoded amino acid). Thus, the viable FMDVs replicating under Rib mutagenesis display fitness decreases that correlate with the number of mutations in their genomes.

Table 1. Relative fitness and fitness loss in clones isolated from populations passaged in the absence or presence of Rib

Viral population*	Clone no.†	Relative fitness‡	Fitness loss (20 h)§	Fitness loss (24 h)‖	No. mutations¶
M6p0	1	1.01	1.0	1.0	ND
	2	1.00	1.0	0.7	3
	3	0.80	1.3	0.9	0
	4	1.20	0.8	2.0	ND
	5	0.99	1.0	1.0	ND
M6p4	1	0.66	1.5	2.8	2
	2	1.13	0.9	1.2	4
	3	1.28	0.8	1.0	3
	4	0.67	1.5	1.9	1
Rp1	1	1.39	0.7	1.5	ND
	2	0.06	18.0	52.0	ND
	3	0.43	2.3	6.0	ND
	4	0.77	1.3	1.8	ND
	5	0.06	17.0	4.6	ND
	6	0.70	1.4	1.4	ND
	7	0.38	2.6	1.7	ND
	8	0.04	28.0	21.0	ND
Rp4	1	0.32	3.1	2.0	19
	2	0.005	200.0	45.0	26
	3	0.04	23.0	4.7	14
	4	0.01	88.0	94.0	29
	5	0.04	24.0	8.0	15
	6	0.18	5.0	4.9	ND
	7	0.06	16.0	24.0	21
	8	0.68	1.5	2.6	10
	9	0.07	14.0	23.0	24

*The origin of FMDV populations from which individual clones have been isolated is described in Methods and Fig. 1.
†Numbering of biological clones in each population is given, to facilitate the identification of the mutations present in each clone (compare with Table 2).
‡Relative fitness of plaques isolated at 20 h p.i. was calculated as the ratio between the number of p.f.u. per plaque and the mean number of p.f.u. per plaque in clones (1–5) isolated from reference population M6p0 (6.1×10^6 p.f.u. ml^{-1}) (see Methods).
§Relative fitness loss for plaques isolated at 20 h p.i., calculated as the reciprocal number of fitness values represented in the third column‡.
‖Relative fitness loss for plaques isolated at 24 h p.i., calculated as the reciprocal number of relative fitness obtained for plaques isolated at 24 h p.i. (not shown). Relative fitness values were calculated as the ratio between the number of p.f.u. in plaques isolated at 24 h p.i. for each clone relative to the mean number of p.f.u. per plaque in clones isolated in reference population M6p0 (4.3×10^6 p.f.u. ml^{-1}).
¶Total number of mutations per genome in clones isolated from untreated and Rib-treated populations, relative to the sequence of the reference M6p0 population. The complete genomic sequence of these clones was determined with the exception of the 5′ and 3′ genomic ends and the regions upstream and downstream the poly(C) tract, as described previously (Escarmís *et al.*, 1996). ND, Not determined. Mutations are listed in Table 2.

DISCUSSION

Lethal mutagenesis mediated by nucleoside analogues has emerged as an exciting approach to confront infectious diseases caused by RNA viruses (Anderson *et al.*, 2004; Domingo *et al.*, 2006, 2012; Eigen, 2002; Graci & Cameron, 2008; Grande-Pérez *et al.*, 2002; Holland *et al.*, 1990; Loeb *et al.*, 1999; Mullins *et al.*, 2011; Pariente *et al.*, 2001; Perales *et al.*, 2009b; Sierra *et al.*, 2000). Extinction of a viral population mediated by mutagenesis may involve at least: (i) unfaithful viral RNA replication that hampers the transmission of accurate genetic information; (ii) interference with the replication of infectious genomes exerted by aberrant gene products from defective viral genomes (defectors); and (iii) increased lethality that reduces the viral load. The main difference at the molecular level between antiviral strategies involving classic inhibitors and mutagens is that the latter leave their antiviral activity imprinted at several sites of the surviving viral genomes. Thus, decreased infectivity of virus populations subjected to mutagenesis is inherited by subsequent replicating progeny genomes, with little option of fitness recovery if enhanced mutagenesis continues.

In this work, we provide evidence of a positive correlation between the accumulation of mutations and fitness loss amidst viable genomes during mutagenesis. Increased Rib concentration correlated with higher levels of mutagenesis

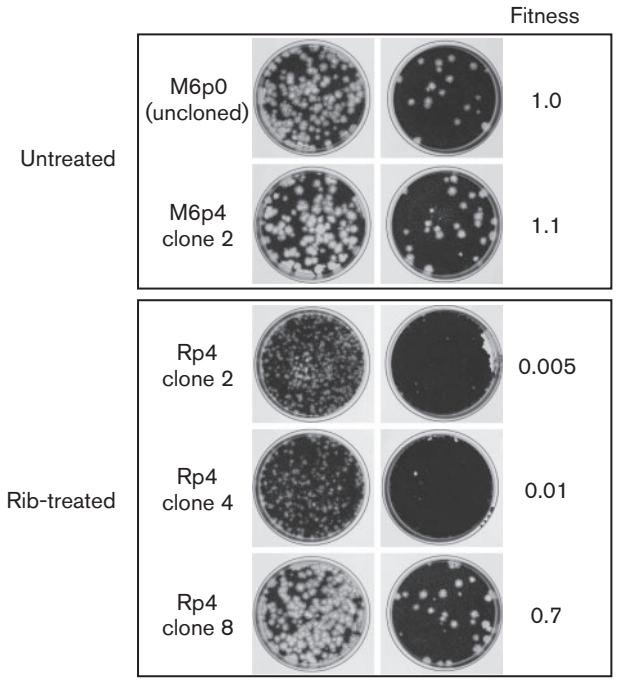

Fig. 3. Representative plaques of biological clones isolated from untreated populations M6p4 and Rib-treated population Rp4. Uncloned M6p0 and M6p4 clone 2 produced a large plaque phenotype, associated with high fitness values; the population M6p0 is assigned with a reference fitness value of 1.0. Fitness values for different biological clones isolated are relative to this fitness value of 1.0 in parental M6p0. Low fitness clones (Rp4 clones 2 and 4) presented smaller plaque size than the clone of highest fitness found in the analysis of Rp4 (clone 8). Appropriate (10^{-2} to 10^{-6}) dilutions of each virus (titres of clones are given in Fig. 2) were plated onto BHK-21 cell monolayers, and plaques allowed to develop for 20 h, as described in Methods.

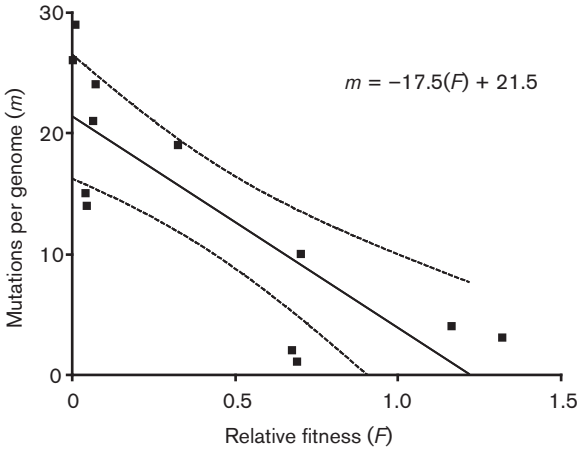

Fig. 4. Reciprocal correlation between relative fitness (F) and number of mutations per genome (m). Data correspond to values of fitness and mutations per genome of individual biological clones isolated from populations M6p4 and Rp4, listed in Table 1, and depicted in Fig. 1. The data have been fitted to a linear regression curve [equation $m=21.5-17.5(F)$; $R^2=0.69$], that establishes a reciprocal correlation between the two parameters ($P=0.0009$). The 95% confidence intervals of the regression curve are delimited by the dashed curves above and below the regression line.

of the FMDV genome (Agudo *et al.*, 2010; Sierra *et al.*, 2007). The extrapolated value of 22 mutations per genome as the error threshold value for FMDV genome replication (Fig. 4) has as its main limitation that this is an average figure, valid only under the conditions in which the measurements have been made. For example, three of the clones isolated from the population Rp4 included more than 22 mutations and, although highly affected, were still infectious (Table 2). Besides, some FMDV clones subjected to hundreds of plaque-to-plaque transfers (bottleneck passages) survived with even 122 mutations per genome (Escarmís *et al.*, 2008). This remarkable contrast illustrates a key feature of viral quasispecies dynamics. During plaque-to-plaque transfers, accumulation of mutations through operation of Muller's ratchet (Escarmís *et al.*, 2006) is accompanied by positive selection of those genomes that can form a visible plaque, no matter how infrequent they are. Myriads of unnoticed extinction events take place, but selection rescues the viable minorities. A poorly replicating virus may be outcompeted at early phases of plaque development by revertants or fitter genomes. Therefore, fitness values determined at 20 and 24 h of plaque development may overestimate the fitness value of the parental genome. The chances that compensatory or beneficial mutations can rescue high fitness genomes during replication under heavy mutagenesis in the absence of bottleneck events are extremely low. It was suggested that mutagenesis may select for robustness in RNA viruses and that this may impair extinction (Sanjuán *et al.*, 2007). However, experiments with LCMV failed to provide any evidence of selection for mutational robustness during lethal mutagenesis (Martín *et al.*, 2008), and the conclusion was supported by a theoretical model (O'Dea *et al.*, 2010). Significantly, FMDV can go to extinction with mutation frequencies in the range of 4×10^{-4} to 3×10^{-3} substitutions per nucleotide, while a clone can survive after hundreds of bottleneck transfers reaching 1.5×10^{-2} substitutions per nucleotide relative to its parental (untransferred) clone (Escarmís *et al.*, 2008).

According to the lethal defection model, one of the mechanisms by which defectors may exert their interfering activity is by expressing altered proteins that can either compete with their standard, fully functional counterparts or participate in protein complexes of lower stability (Grande-Pérez *et al.*, 2005a; Perales *et al.*, 2007). Interestingly, 18 of the 46 aa substitutions found in clones from the Rp4 population affect residues that are totally conserved among 103 natural FMDV isolates spanning all the seven serotypes described previously (Carrillo *et al.*, 2005), and seven of them affect the polymerase-coding

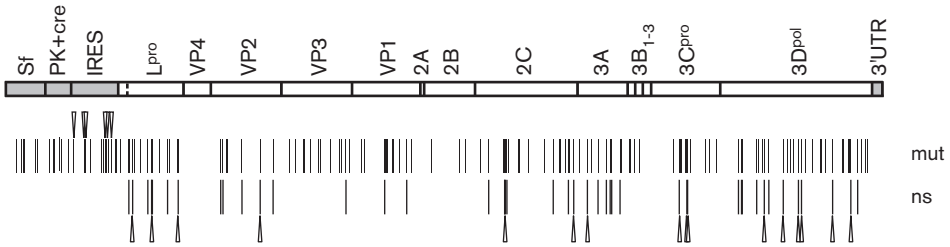

Fig. 5. Genomic distribution of mutations found in clones isolated from Rib-treated FMDV populations. A schematic representation of the FMDV genome is shown at the top (based in Mahy, 2005; Rowlands, 2003; Sobrino & Domingo, 2004). Grey boxes represent the 5′ and the 3′ UTR of the FMDV genome (Sf, S fragment; PK+cre, pseudoknots and *cis* replicating element; IRES, internal ribosome entry site). The central white box represents the polyprotein-coding region, with each individual protein indicated above its corresponding box. A mutation is represented as a vertical line below the affected genomic position (indicated as mut). Inverted triangles indicate the position of nucleotide residues strictly conserved in FMDV 5′ UTR (Carrillo *et al.*, 2005) (see text). Non-synonymous mutations (indicated as ns) are represented below their corresponding nucleotide substitutions. A triangle indicates the position of an amino acid residue strictly conserved in FMDV isolates (Carrillo *et al.*, 2005). The individual mutations are listed in Table 2.

region (3Dpol) (Table 2; Fig. 5). Thus, random mutations caused by lethal mutagenesis include deleterious mutations responsible for decreased infectivity. This is in agreement with estimates of a high proportion of deleterious and lethal mutations when mutations were engineered at random positions in viral genomes (Parera *et al.*, 2007; Sanjuán *et al.*, 2004). It is important to note that lethal mutations that might have occurred during mutagenic treatment, leading to non-infectious viruses, were not scored in the present analysis. The isolation of biological clones carried out here requires that all FMDV genomes retain a level of infectivity sufficient to develop plaques in cell culture. Some of the amino acid substitutions identified in 3Dpol are likely to alter polymerase activity and/or fidelity. In particular, residue A243 (mutated to Thr in 1 clone) in functional motif A interacts with the phosphate backbone of the incoming nucleotide, suggesting that its replacement may affect viral RNA synthesis (Ferrer-Orta *et al.*, 2007). Similarly, substitutions S58F or S67F may potentially affect viral polymerase fidelity since they are located in the same structural loop as residue G62 (loop α2-α3). G62 is equivalent to G64 in poliovirus (PV), a replacement that confers Rib-resistance and increases 3D(pol) template-copying fidelity in PV (Arias *et al.*, 2005; Pfeiffer & Kirkegaard, 2003; Vignuzzi *et al.*, 2006, 2008). Substitution G62S inflicted a strong selective disadvantage to FMDV, as a result of a defect in RNA replication, attributed to a distance effect from position 62 to the catalytic domain of the enzyme (Ferrer-Orta *et al.*, 2010). S58F and S67F were also identified in the mutant spectra for FMDV subjected to Rib mutagenesis, through selective amplification of A, U-rich genomes (Perales *et al.*, 2011). It is not known whether these replacements may affect RTP recognition and might be selected upon further passage of these populations in the presence of Rib.

Some other mutations identified in our study may also be responsible for decreased infectivity. E93 and P187 in leader protease L^{pro} (variable and conserved residues mutated to Lys and Ser, respectively; Table 2) participate in the proteolytic cleavage of eIF4G, required for shut-off of host cell protein synthesis, as well as for dimerization needed for self-cleavage from the rest of the polyprotein (Cencic *et al.*, 2007). K96 in VP2 (variable residue mutated to Arg; Table 2) establishes interpentameric interactions in the mature virion and its replacement for Ala has been shown to affect viral productivity (Mateo *et al.*, 2003). Likewise, eight of the 27 mutated UTR residues are strictly conserved among different isolates (Carrillo *et al.*, 2005) (Fig. 5). These changes are predicted to decrease the stability of 5′ UTR in six of eight biological clones, and C735U and G740A in Rp4 clone 7 are likely to alter IRES domain 3. Additionally, G398A (variable residue) in Rp4 clone 1 is expected to disrupt the conserved pseudoknot 1 structure in this clone. Obviously, direct interference assays are required to investigate whether the mutations listed in Table 2 can suppress or delay standard FMDV replication. Interestingly, VP4 and part of 3B- and 3C-coding regions (residues 1598–1989 and 5864–6167, respectively) are apparently refractory to the accumulation of nucleotide substitutions (Table 2, Fig. 5). MFOLD analyses showed that these regions are predicted to fold in relatively complex RNA secondary structures, although their possible functional relevance is not known. An attractive hypothesis is that alternative ORFs are expressed in these regions. *In silico* predictions suggest the existence of two small ORFs (encoding 59 and 88 aa products from genomic positions 1526 and 1784, respectively) overlapping with VP4. Analyses that predict alternative ORFs of 58 and 88 aa in FMDV C-S8c1, also predict ORFs of 108, 181 and 47 aa in the same region of FMDV C4, O and SAT-2 (GenBank accession nos: AJ133357, AY593808, GU384683 and NC_003992, respectively). These ORFs display different degrees of sequence identity, and their biological relevance remains unknown. Other examples are L* in Theiler's virus

Table 2. Nucleotide and amino acid substitutions found in clones isolated from the Rib-treated population Rp4

Genomic region*	Nucleotide substitution†	Amino acid replacement‡	Clone no.§
S fragment	U34C		1
	A96G		4
	G139A		2
	G161A		4
	A168G		5
	G276A		9
	C294U		7
	A296G		9
Pseudoknots	G398A		1
	U446C		5
	U452C		3
	C483U		2
	G512A		2
cre	U576C		4
IRES	G627A		4
	C735U		7
	G740A		7
	C786U		1
	C882U		5
	C908U		3
	A918G		4
	C921U		9
	G931A‖		7, 9‖
	G949A‖		1, 3‖
	G979A		2
	C1020U		4
	U1028C		2
L	U1061C¶	NC¶	1¶
	G1141A	D35N	9
	C1146U	—	4
	C1171U	P45S	3
	C1188U	—	8
	C1251U	—	3
	G1315A	E93K	2
	U1346C	V103A	4
	U1349C	I104T	2
	U1358C	I107T	1
	C1422U	—	4
	U1499C	V154A	2
	G1512A	—	4
	C1587U	—	2
	C1597U	P187S	3
VP4			
VP2	G1990A	V32I	1
	C2010U	—	9
	C2012U	A39V	5
	C2047U	—	4
	A2183G	K96R	5
	C2351U	T152I	3
	C2352U	—	4
	G2473A	G193S	7
VP3	C2624U	A25V	9
	A2676G	—	4
	C2679U	—	8
	C2754U	—	3
	C2764U	—	5

Table 2. cont.

Genomic region*	Nucleotide substitution†	Amino acid replacement‡	Clone no.§
	C2814U	—	2
	C2892U	—	3
	G2916A	—	4
	A3087G	—	4
	G3009A	—	7
	U3102C	—	7
	C3108U	—	9
	G3145A	V199I	7
	G3183U	—	4
VP1	U3285C	—	9
	G3321A	—	1
	A3508G	T101A	1
	C3513U	—	5
	C3525A	—	2
	C3528U	—	4
	C3537U	—	9
	C3576U	—	4
	G3582A	—	2
	C3646U	—	2
	A3712G	I169V	4
	U3753C	—	9
2A			
2B	C3939U	—	1
	G4200A	—	2
2C	C4260U	—	9
	C4392U	—	5
	C4469U	T42I	1
	C4611U	—	2
	G4618A#	V92M#	7#
	G4618U#	V92F#	2#
	C4624U	P94S	5
	A4636G	K98E	3
	C4653U	—	8
	C4740U	—	2
	C4753U	—	9
	G4836A	—	7
	G4989A	—	2
	U5066C	I241T	2
	A5124G	—	8
	C5139U	—	7
	U5179C	—	1
	C5206U	P288S	5
	C5214U	—	8
	G5254A	V304I	4
3A	C5301U	—	7
	G5380A	G28S	4
	C5382U	—	2
	G5415A	—	9
	C5421U	—	4
	G5482U	V62I	5
	G5557A	D87N	8
	U5594C	I99T	7
	G5605A	D103N	4
	C5681U	T128I	1
	G5700A	—	1
VPg1	G5772A	—	4

Table 2. cont.

Genomic region*	Nucleotide substitution†	Amino acid replacement‡	Clone no.§
	U5784C	—	9
	G5817A	—	1
VPg2	G5859A	—	2
	C5863U	—	8
VPg3			
3C	C6168U	—	3
	G6235A	V89M	3
	G6249A	G112S	4
	C6252U	—	2
	U6285C		9
	G6304A	—	4
	C6310U	—	7
	U6336C	P114S	9
	U6471C	—	8
	U6510C	—	7
	A6573G	—	7
3D	C6782U	S58F	1
	C6809U	S67F	9
	G6814A	E69K	1
	C6946U	P113S	2
	U6966C	—	4
	U7008C	—	7
	C7022U	T138M	8
	G7053A	—	3
	G7060A	E151K	3
	G7191A	M194I	1
	G7233A	—	7
	C7260U	—	2
	C7296U	—	9
	G7336A	A243T	2
	C7364U	A252V	5
	G7398A	—	4
	C7461U	—	7
	C7470U	—	1
	G7539A	—	9
	C7551U	—	4
	G7584A	—	9
	G7654A	D349N	9
	C7743U	—	7
	U7752C	—	5
	A7809G	—	9
	G7815A	—	7
	G7819A	A404T	9
	U7884G	I425M	5
	A7917G	—	7
	C7956U	—	5
	C7971U	—	2
3′ UTR		—	

*FMDV genomic region analysed. Viral RNA samples from biological clones isolated from the Rp4 population were obtained and full genomic sequences determined, as described in footnote ¶ of Table 1 and in Methods.

†Nucleotide substitutions found in the analysis. Numbering of genomic residues is according to Escarmís *et al.* (1996). Underlining in 5′ UTR substitutions indicates that the residue substituted is totally conserved in 103 different FMDV strains spanning all the seven FMDV serotypes, according to Carrillo *et al.* (2005).

‡Predicted amino acid substitution in the corresponding viral protein. The numbering of amino acid residues in each viral protein is that used in previous studies (Escarmís *et al.*, 1996). A dash represents a synonymous substitution. Substitutions in strictly conserved amino acid residues according to Carrillo *et al.* (2005) are underlined.

Table 2. cont.

§Each mutation is assigned to the corresponding biological clone number in which it was found (compare with Table 1).
‖Substitutions G931A and G949A were found each in two different clones.
¶NC, Non-coding region. Although position U1061 is located within two alternative AUG which both typically lead to the synthesis of a full viral polyprotein (with the sole difference of encoding a larger or shorter L protease isoform), viral polyprotein synthesis in FMDV M6 populations only proceeds from the second AUG located at position 1123. All FMDV populations derived from FMDV C^9_{22} lineage, such as M6 populations used in the present work, have residue U1056 deleted, leading to the truncated synthesis of viral polyprotein synthesis from the first AUG at position 1039 (Escarmís *et al.*, 1996, 1999).
#Position G4618 has been found replaced in two different clones, but was substituted by a different nucleotide (A in clone 7, U in clone 2).

and murine norovirus ORF4 product, involved in control of cellular antiviral responses (McFadden *et al.* 2011; Sorgeloos *et al.*, 2011). A recent study with unrelated murine noroviruses has revealed the existence of an alternative ORF with a key role in virulence overlapping with the expression of viral capsid (McFadden *et al.*, 2011), which might support the expression of overlapping ORFs in the FMDV capsid gene.

The current picture of the transition of viruses towards extinction includes the following steps: lethal defection, overt lethality and irreversible crossing of an error (or extinction) threshold (reviewed by Domingo *et al.*, 2012). The present study has clarified that even the most frequently encountered viable viruses rescued from a viral population subjected to enhanced mutagenesis have undergone fitness losses. Low fitness, RNA replication competent genomes, may either be actors or subjects of defection, in both cases contributing to the replicative collapse that precedes extinction.

METHODS

Cells, viral infections and mutagenic treatments. The origin of BHK-21 cells and procedures for their growth, infection with FMDV under liquid medium, and plaque assays under semisolid agar medium, were described previously (de la Torre *et al.*, 1988; Escarmís *et al.*, 1996; Sobrino *et al.*, 1983). FMDV M6 is a clone obtained after two consecutive plaque isolations from the population FMDV C^9_{22}p150. FMDV C^9_{22}p150 originated from a biological clone of FMDV C-S8c1 termed C^9_1 (described in Escarmís *et al.*, 1996) that was subjected to 22 serial plaque-to-plaque transfers, and then to 150 passages in BHK-21 cells at high m.o.i. (Escarmís *et al.*, 1999). FMDV C^9_{22}p150 reached a fitness of nine relative to its parental clone C-S8c1 (Escarmís *et al.*, 1999). Clone M6 has the same consensus nucleotide sequence as FMDV C^9_{22}p150 with the exception of six synonymous substitutions in its coding region. M6 population at passage 0 (M6p0) was obtained after two consecutive undiluted passages of clone M6 in BHK-21 cells (Fig. 1). M6p0 was then passaged either in the absence (untreated) or in the presence of 5 mM Rib (Rib-treated) (Fig. 1). For the passages in the presence of Rib, BHK-21 cells were pre-treated for 7 h with 5 mM Rib, and the same drug concentration was maintained in the culture medium during the infection, as described previously (Agudo *et al.*, 2010). A tenth of the volume from the supernatant of each infection was applied to a new BHK-21 cell monolayer (6×10^5 cells) and incubated at 37 °C for 1 h to allow particle adsorption. The inoculum was then removed, 1 ml of 5 mM Rib in Dulbecco's modified Eagle's medium added, and the infection allowed to proceed until complete cytopathology. Serial passages in the absence of Rib were carried out in parallel.

Biological cloning and determination of relative fitness. Biological clones were isolated from plaques developed after 30 h of infection on BHK-21 cell monolayers by standard procedures previously established in our laboratory (Sobrino *et al.*, 1983). At 30 h post-infection (p.i.), all viruses displaying a broad range of fitness values formed visible plaques of a size between 4 and 8 mm, favouring their identification and random (sized-independent) selection. To determine the infectivity of clones, a second consecutive biological cloning was carried out of plaques developed during 20 or 24 h p.i. At 20 h p.i., the viral progeny production per plaque was in the exponential phase in all the clones tested, while at 24 h p.i. a plateau was reached for certain clones. Fitness values in each clone were calculated as the ratio between the number of p.f.u. per plaque relative to the mean number of p.f.u. per plaque in five clones isolated from reference population M6p0. Mean viral titres of the same M6p0 clones isolated either at 20 or 24 h p.i. were 6.1×10^6 and 4.3×10^6 p.f.u. per plaque, respectively, with good agreement between values at the two time points (Table 1).

RNA extraction, RNA quantification, cDNA synthesis and PCR amplification. Viral RNA was extracted from the supernatant of infected cell cultures or from plaques recovered using Trizol (Invitrogen) as described previously (Escarmís *et al.*, 2008). RT-PCR amplification and nucleotide sequencing of the entire FMDV genome was performed as previously documented (Escarmís *et al.*, 2008). Reverse transcription and PCR amplification were carried out using Transcriptor (Roche) and Expand High Fidelity (Roche), respectively, as specified by the manufacturers.

Viral RNA and protein structure analysis. Structural prediction of protein alterations caused by amino acid substitutions identified in L^{pro}, capsid proteins (VP1, VP2, VP3 and VP4), 3C and 3D was performed using PYMOL program. Prediction of structural changes on the genomic 5′ UTR was carried out with MFOLD program (Zuker, 2003). The PKNOTSRG program (http://bibiserv.techfak.uni-bielefeld.de/pknotsrg/submission.html) was used to identify possible alterations in FMDV pseudoknots.

ACKNOWLEDGEMENTS

We thank E. García-Cueto for expert technical assistance. Work supported from Ministerio de Ciencia e Innovacion (MICINN) by grant BFU2008-02816/BMC, from Ministerio de Economía y Competitividad and Fundacion R. Areces by grant BFU2011-23604. CIBERehd (Centro de Investigacion Biomedica en Red de Enfermedades Hepaticas y Digestivas) is funded by Instituto de Salud Carlos III. A. A. was supported by an I3P contract from Consejo Superior de Investigaciones Cientificas (CSIC) and a Marie Curie IEF contract from European Commission.

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