A segmented form of foot-and-mouth disease virus interferes with standard virus: A link between interference and competitive fitness

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Abstract

Serial passage of foot-and-mouth disease virus (FMDV) in BHK-21 cells at high multiplicity of infection resulted in dominance of particles containing defective RNAs that were infectious by complementation in the absence of standard viral RNA. In the present study, we show that the defective FMDV particles interfere with replication of the cognate standard virus. Coinfections of defective FMDV with standard FMDV mutants that differ up to 151-fold in relative fitness have documented that the degree of interference is higher for low fitness than for high fitness standard virus. These comparisons suggest a likely overlap between those mechanisms of intracellular competition that underlie viral interference and those expressed as fitness differences between two viruses when they coinfect the same cells. Interference may contribute to the selective pressures that help maintain dominance of segmented defective RNAs over the standard FMDV genome.
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Introduction

High multiplicity of infection (MOI) passage of foot-and-mouth disease virus (FMDV) in BHK-21 cells led to dominance of encapsidated defective RNAs that were infectious by complementation in the absence of standard virus (García-Arriaza et al., 2004). The defective genomes that were identified at passage 260 included in frame deletions of 417, 999, and 1017 nucleotides that affected the L protease- and capsid-coding regions. The multipartite FMDV population was termed FMDV C-S8p260 and the corresponding RNAs were termed Δ417, Δ999, and Δ1017 (Fig. 1). The evidence that ΔRNAs are infectious by complementation and cause cytopathology in the absence of standard virus includes "two-hit" kinetics for cell killing, presence of defective genomes with undetectable standard RNA in individual viral plaques, and cell killing achieved after co-transfection of BHK-21 cells with transcripts produced in vitro from plasmids encoding Δ417 and Δ999 RNAs (again in the absence of standard RNA), but not by each individual defective RNA alone (García-Arriaza et al., 2004). Low MOI passage of FMDV C-S8p260 rescued FMDV with RNA of standard size, termed FMDV C-S8p260p3d, most likely through a homologous recombination event involving two defective RNAs (García-Arriaza et al., 2004; García-Arriaza et al., in preparation).

The acquisition of infectivity by complementation of two individually defective RNAs with internal deletions can be regarded as the first step of an evolutionary transition toward RNA genome segmentation to produce a bipartite viral system (two defective genomes encapsidated into separate particles). This poses, among others, the question of the driving forces that led to dominance of ΔRNAs over standard RNA. Examination of the evolutionary history of biological clone FMDV C-S8c1 in BHK-21 cells indicates that prior to dominance of ΔRNAs, two RNA species with deletions of 417 (the same deletion as in Δ417 of passage 260) and 402 nucleotides that affected the Lb-coding region, coexisted with standard RNA at passage 143 (Charpentier et al.,

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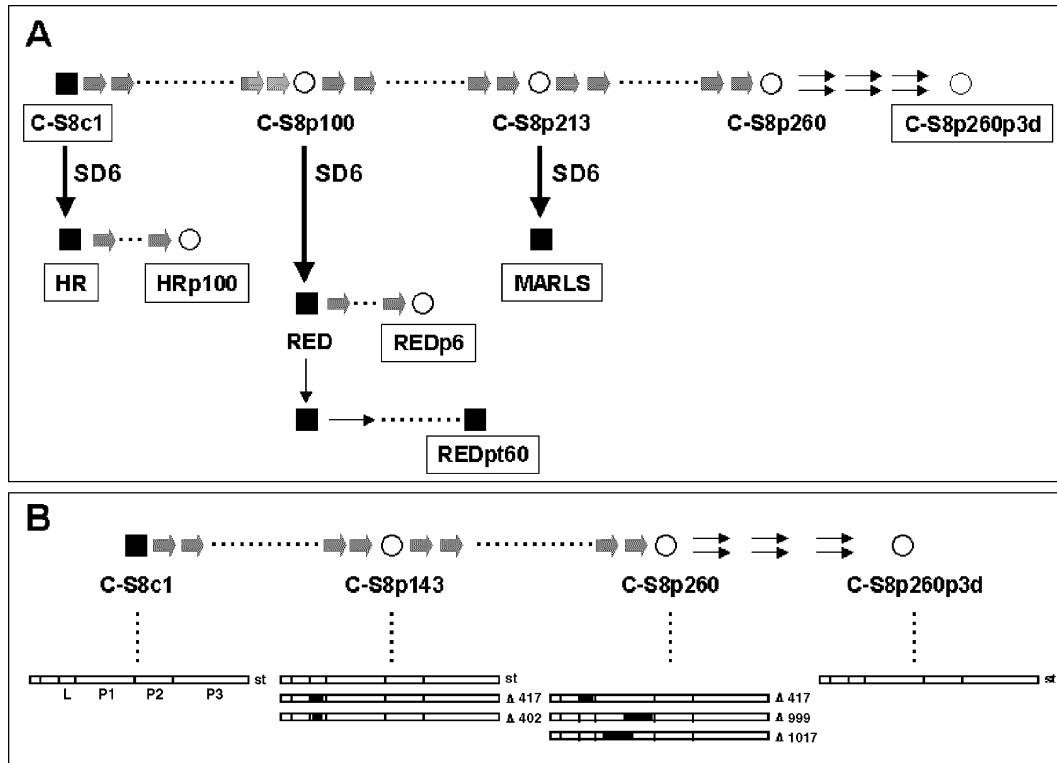 *E-mail address:* edomingo@cbm.uam.es (E. Domingo).

Fig. 1. Passage history of FDMV C-S8p260 and C-S8p260p3d, and origin of the standard FMDVs used in the present study. (A) Biological clones are depicted as filled squares and uncloned populations as empty circles. Large grey arrows indicate high MOI passages (1 to 5 PFU/cell); thick arrows indicate selection of mutants resistant to mAb SD6 (Mateu et al., 1990); thin arrows describe the isolation of biological clones (virus from individual plaques) after dilution of virus and plating on BHK-21 cell monolayers. Two parallel thin arrows indicate low MOI passages carried out to derive the unsegmented C-S8p260p3d from the defective RNA-containing C-S8p260 (García-Arriaza et al., 2004). The standard viruses used in the present study are boxed. Their origin is further detailed in Materials and methods; p followed by a number indicates passage number; pt refers to plaque-to-plaque transfer, carried out as described in Escarmís et al. (1996). (B) Genomes identified in FMDV populations C-S8p260 and C-S8p260p3d. The scheme of the FMDV genome is based on studies reviewed in Rowlands (2003) and in Sobrino and Domingo (2004); st, standard FMDV RNA; the position of deletions is indicated by filled areas and the length of the deletion in nucleotides is given following Δ. The genomic compositions are based on García-Arriaza et al. (2004).

1996) (Fig. 1B). These deleted RNAs maintained the correct open reading frame for polyprotein synthesis, contributed at least capsid protein VP1 for particle formation, required helper standard virus to produce virions, and did not interfere with the replication of standard virus (Charpentier et al., 1996). Additional evidence that Δ417 RNA does not interfere with standard virus replication was provided by similar progeny production following electroporation of BHK-21 cells with standard C-S8c1 RNA in the presence or absence of Δ417 RNA (González-López et al., 2004).

In contrast with the generation of deleted non-interfering RNAs when FMDV was passaged 143 times in BHK-21 cells (Charpentier et al., 1996), many RNA viruses of different families produce defective-interfering (DI) RNAs and particles upon few serial passages at high MOI in cell culture (reviews in Holland, 1990; Huang, 1973; Perrault, 1981; Roux et al., 1991). DI RNAs are deletion mutants of infectious virus that interfere with replication of the homologous virus and are dependent on standard virus for replication. DIs of the rhabdovirus vesicular stomatitis virus (VSV) contributed decisively to defining the dynamics of mutation, competition, and selection that occur

continuously in RNA virus populations (Holland et al., 1982) in what is now known as the quasispecies dynamics of RNA viruses (Domingo et al., 2005; Eigen and Biebricher, 1988). For the Picornaviruses poliovirus, Mengo virus, and encephalomyocarditis virus, DI RNAs with internal deletions affecting 3.7–16% of the 5′ portion of genomic RNA were characterized (Cole, 1975; Lundquist et al., 1979; McClure et al., 1980; Nomoto et al., 1979; Radloff and Young, 1983; Tershak, 1982). Genomic RNAs with deletions have also been identified in the progeny of various isolates of hepatitis A virus (Nüesch et al., 1988) and in the course of persistent infections of FMDV in cell culture (de la Torre et al., 1985), although their possible interfering activity was not established.

To gain insight into the mechanisms that conferred the multipartite FMDV C-S8p260 a selective replicative advantage over its unsegmented counterpart in BHK-21 cells, we have carried out several coinfection experiments of BHK-21 cells and examined relative viral yields. Here we report that FMDV C-S8p260 interferes with the replication of its cognate standard FMDV C-S8p260p3d. By comparing the degree of interference undergone by other standard FMDVs displaying a 151-fold range of relative fitness values, we

show that interference is fitness-dependent in that the lower the relative fitness of standard FMDV, the higher is the interference mediated by C-S8p260. Fitness is an important parameter, increasingly used in viral population dynamics, that quantifies the relative replicative capacity of a virus under a set of environmental conditions. Procedures to compare viral fitness were first developed by Holland et al. (1991) with VSV and applied to FMDV in cell culture (Martínez et al., 1991), in vivo (Carrillo et al., 1998), and to many other viruses (reviews in Domingo and Holland, 1997; Quiñones-Mateu and Arts, 2002). Therefore, the results reported here link two important parameters in virus population dynamics: classical interference and viral fitness. The results suggest that intracellular competition confers an advantage to the multipartite C-S8p260 version of FMDV, unless the opponent standard virus is endowed with high replicative fitness, as determined using standard fitness assays (Domingo and Holland, 1997; Holland et al., 1991; Quiñones-Mateu and Arts, 2002). To account for the experimental findings, we suggest as a working hypothesis that there is an overlap between intracellular mechanisms responsible for interference (as measured classically with two different viruses or a DI particle and the corresponding standard virus) and those that underlie differences in relative fitness between two mutants of the same virus.

Results

Interference by C-S8p260

Productive infection by population FMDV C-S8p260 requires coinfection by two classes of particles, one harboring Δ417 RNA and the other harboring either Δ999 or Δ1017 RNA (García-Arriaza et al., 2004) (Fig. 1B). Since dilution of virus is usually needed for titration, there is an ambiguity in the definition of plaque-forming-units (PFU) for C-S8p260 as compared with standard FMDV. This problem can be circumvented by correcting the number of observed PFU for the effect of dilution (Manrubia et al., submitted for publication) or by using the amount of genomic RNA, quantified by real time PCR, as the means to compare the number of viral particles included in the coinfection experiments. The latter procedure has been adopted throughout the present study.

The possible interference exerted by FMDV C-S8p260 on its standard C-S8p260p3d counterpart was tested using a functional assay consisting of measurement of the progeny of standard virus upon coinfection of BHK-21 cells with different proportions of FMDV C-S8p260 and FMDV C-S8p260p3d (Table 1). Progeny production by C-S8p260p3d was as low as 0.4% of the control infection when C-S8p260 was added in a 100-fold excess over C-S8p260p3d. In coinfections with equal amounts of the two viruses, a production of 31.5% relative to the control was observed (Table 1). Thus, the FMDV C-S8p260 population that consists of defective RNAs can interfere with the corresponding cognate standard FMDV.

The degree of interference depends on the fitness of the standard virus

To investigate whether interference by FMDV C-S8p260 was specific for its cognate standard FMDV C-S8p260p3d or it was also exerted on other standard FMDVs, BHK-21 cells were coinfected with FMDV C-S8p260 and a number of genetically and phenotypically marked standard FMDVs (MARLS, REDp6, REDpt60, HR, and HRp100; their origin is depicted in Fig. 1A and in Materials and methods). Each standard FMDV included an amino acid substitution at the G-H loop of capsid protein VP1 that renders the virus resistant to neutralization by monoclonal antibody (mAb) SD6 (Mateu et al., 1990). In addition, each standard FMDV displayed a different relative fitness in BHK-21 cells (Table 2). The mAb SD6 resistance marker allowed the specific quantification of the progeny of the standard virus. The results (Table 3 and Fig. 2) document interfering activity of C-S8p260 on all FMDVs tested, albeit to greatly different

Table 1
Progeny virus produced in coinfections of BHK-21 cells with FMDV C-S8p260p3d in the presence or absence of FMDV C-S8p260

C-S8p260p3d[a] (particles/cell)	C-S8p260[a] (particles/cell)	Input ratio (C-S8p260/C-S8p260p3d)	Progeny[b] (PFU/ml)	FMDV C-S8p260p3d[b] (%)
1.7×10^4	0	–	1.3×10^8	100
1.7×10^4	1.4×10^4	1	4.1×10^7	31.5
1.7×10^3	0	–	1.4×10^8	100
1.7×10^3	1.4×10^4	10	8.3×10^6	5.7
1.7×10^2	0	–	1.8×10^8	100
1.7×10^2	1.4×10^4	100	8.0×10^5	0.4
0	1.4×10^4	–	9.2×10^4	–

[a] The origin of C-S8p260p3d and C-S8p260 is described in the Introduction, in Fig. 1, and in Materials and methods. For each assay, 1×10^6 BHK-21 cells were infected with the indicated number of physical particles, as determined from the amount of genomic RNA quantitated by real-time PCR, as previously described (García-Arriaza et al., 2004). DMEM was used for cell culture and as the infection medium (Materials and methods) and was added instead of C-S8p260 in control infections and instead of C-S8p260p3d in the control for progeny production by C-S8p260 alone (indicated as 0 in the first two columns).
[b] Under the infection conditions used, progeny was contributed mainly by FMDV C-S8p260p3d, as established by control experiments (last row) and the large plaque morphology of the progeny virus (García-Arriaza et al., 2004).

Table 2
Relevant features of the FMDVs used in the interference assays

FMDV[a]	Amino acid substitution in the G-H loop of VP1[b]	Frequency of mAb SD6-resistant mutants[c]	Relative fitness[d]
C-S8c1	–	3.8×10^{-3}	1
C-S8p260	–	1.5×10^{-2}	35[e]
C-S8p260p3d	–	1.9×10^{-2}	70
MARLS	L-144 → S	1.5	118
REDp6	G-142 → E	1.3	15
REDpt60	G-142 → E	4.0	2
HR	H-146 → R	1.5	0.8
HRp100	H-146 → R	1.4	8

[a] The origin of the FMDVs used is depicted in Fig. 1 and detailed in Materials and methods.

[b] The amino acid sequence of the G-H loop of capsid protein VP1 of C-S8c1, C-S8p260, and C-S8p260p3d (positions 133–156) is TTTYT-ASARGDLAHLTTTHARHLP; the epitope defined by mAb SD6 is underlined (positions 138–147) (Mateu, 1995; Verdaguer et al., 1995).

[c] MAb SD6 has been previously described (Mateu et al., 1990) and the frequency of mAb SD6-resistant mutants was determined as detailed in Materials and methods. The higher frequency of mAb SD6-resistant mutants of C-S8p260 and C-S8p260p3d (which do not differ in amino acid sequence of the G-H loop of VP1) could be due to replacement H-197 → R found in antigenic site C of C-S8p260 and C-S8p260p3d but not of C-S8c1, in view of the evidence of interactions among antigenic sites of mutants of FMDV C-S8c1 (Baranowski et al., 2001); again, this possibility has not been investigated.

[d] Fitness values in BHK-21 are relative to C-S8c1 which is assigned a relative fitness of 1. Fitness values for REDp6, REDpt60, and HR were determined previously, and fitness values for MARLS, HRp100, and C-S8p260p3d were calculated for the present study. Procedures for fitness determinations and relevant references for known fitness values of FMDV mutants are detailed in Materials and methods.

[e] Probably an underestimate, as described in Materials and methods.

extents. Interference was not a consequence of some unspecific exclusion between two coinfecting viruses since interference exerted by C-S8c1 on MARLS was very low (29.1% progeny MARLS production in coinfections with a 100-fold excess of C-S8c1) (Table 3, footnote a). Interference by C-S8p260 was dependent on its infectivity since no interference on several standard viruses was observed using C-S8p260 preparations that were photoinactivated by treatment with neutral red (3-amino-7-dimethylamino-2-metyl-phenazine hydrochloride) and visible light (Acharya et al., 1989; Díez et al., 1990) (data not shown).

Differences in the extent of interference were observed between closely related FMDVs belonging to the same evolutionary lineage, and the differences were more pronounced when the interfering and standard viruses were used at a 1:1 particle ratio in the coinfections (compare Figs. 1 and 2 and Table 3). This suggested that relative fitness was a parameter likely to influence the degree of interference. Two different plots (Figs. 2A and B) correlated low fitness with susceptibility to interference by C-S8p260. The relationship between the proportion of standard virus found in the progeny and viral fitness was not linear, with a sharp increase in the degree of interference when the relative fitness of the standard virus was lower than 15 (Fig. 2B). Further evidence that

fitness rather than differences in the sequence of the G-H loop of VP1 that distinguishes the different variants tested (Table 2) was a determinant of the extent of interference was provided by the comparison of FMDV REDp6 and FMDV REDpt60. These two populations originated from the same biological clone FMDV RED (Fig. 1A) and both include substitution G-142 → E in VP1 (Table 2). They differ in 7.6-fold in relative fitness (Ruiz-Jarabo et al., 2002) because REDp6 resulted from subjecting RED to six large population passages [a process that results in fitness gain (Escarmís et al., 1999; Novella et al., 1995)] and REDpt60 was obtained after sixty plaque-to-plaque transfers of RED [a process that results in fitness decrease (Chao, 1990; Escarmís et al., 1996)] (the evolutionary history of RED and its derivatives is included in Fig. 1A). The 7.6-fold difference in relative fitness resulted in almost 10-fold higher interference on REDpt60 than on REDp6, in coinfections with the same proportion of C-S8p260 particles (Table 3 and Fig. 2). For MARLS and REDpt60, interference by C-S8p260 was confirmed by RT-PCR amplification of genomic RNA (Fig. 3).

Thus, FMDV C-S8p260, which is infectious by complementation of two defective genomes, interferes with its cognate standard FMDV and also with other standard FMDVs. When the number of interfering and interfered particles that enter the cell was comparable, low relative fitness of the standard virus resulted in a higher degree of interference.

Discussion

A connection between interference and fitness

The known evolutionary potential of RNA viruses (Domingo and Holland, 1997; Holland et al., 1982) has had an additional salient manifestation with the demonstration that high MOI passage of FMDV in cell culture resulted in dominance of particles that encapsidate defective genomes that are infectious by complementation (García-Arriaza et al., 2004) (Fig. 1). In the present report, we have shown that the complementing ΔRNAs of FMDV can interfere with replication of standard FMDV that in our experimental design included a mAb-resistance marker to distinguish the interfered FMDV from the interfering C-S8p260 by plating in the presence and absence of mAb SD6 (Table 3; Fig. 2). To our knowledge, this is the first report of DI particles in FMDV.

The results have raised the question of the relationship between interference and viral fitness. The term interference in virology refers to a number of distinct phenomena—mediated by different mechanisms—whose common outcome is inhibition of viral replication. Initially, it described inhibition of replication of one virus by another virus [reviews in (Condit, 2001; Youngner and Whitaker-

Table 3
Progeny virus produced in infections of BHK-21 cells with FMDV MARLS, REDp6, and REDpt60 in the presence or absence of FMDV C-S8p260[a]

FMDV 1[b] (particles/cell)	FMDV 2[b] (particles/cell)	Input ratio (virus2/virus 1)	Progeny[c] (PFU/ml)		SD6-resistant mutant (%)	Frequency of SD6-resistant mutants
			+mAbSD6	−mAbSD6		
MARLS (3×10^4)	None	–	2.2×10^8	1.8×10^8	100	1.2
MARLS (3×10^4)	C-S8p260 (1.4×10^4)	1	4.0×10^7	4.7×10^7	18.2	0.8
MARLS (3×10^3)	None	–	1.6×10^8	7.4×10^7	100	2.1
MARLS (3×10^3)	C-S8p260 (1.4×10^4)	10	5.7×10^6	7.5×10^6	3.6	0.8
MARLS (3×10^2)	None	–	7.2×10^7	2.9×10^7	100	2.5
MARLS (3×10^2)	C-S8p260 (1.4×10^4)	100	5.3×10^5	3.9×10^5	0.7	1.3
REDp6 (ND)	None	–	6×10^6	4.6×10^6	100	1.3
REDp6 (ND)	C-S8p260 (1.4×10^4)	1	8.8×10^5	2×10^6	14.7	0.4
REDp6 (ND)	None	–	1.5×10^7	9×10^6	100	1.7
REDp6 (ND)	C-S8p260 (1.4×10^4)	10	5.7×10^4	1.3×10^5	0.4	0.4
REDp6 (ND)	None	–	$>1.5 \times 10^7$	1.1×10^7	100	1.4
REDp6 (ND)	C-S8p260 (1.4×10^4)	100	1.1×10^4	7.6×10^4	>0.1	0.1
REDpt60 (1.9×10^4)	None	–	1.8×10^6	1×10^6	100	1.8
REDpt60 (1.9×10^4)	C-S8p260 (1.4×10^4)	1	2.8×10^4	1.5×10^5	1.6	0.2
REDpt60 (1.9×10^3)	None	–	2.8×10^6	4.5×10^5	100	6.2
REDpt60 (1.9×10^3)	C-S8p260 (1.4×10^4)	10	5.3×10^3	7×10^4	0.2	7.5×10^{-2}
REDpt60 (1.9×10^2)	None	–	2×10^6	7.7×10^5	100	2.6
REDpt60 (1.9×10^2)	C-S8p260 (1.4×10^4)	100	2.3×10^3	5.3×10^4	0.1	4.3×10^{-2}
C-S8p260 (1.4×10^4)	None	–	6.5×10^2	9.2×10^4	–	7.1×10^{-3}

[a] Data on the interference of C-S8p260 on HR and HRp100, and the controls that show the absence of interference of C-S8c1 on MARLS will be provided upon request. Values of %MAR virus are given in Fig. 2.

[b] The origin of the FMDVs used is given in the Introduction, Fig. 1, Table 2, and Materials and methods. Conditions of infection and determination of viral particles are described in footnote a of Table 1 and in Materials and methods. ND, not determined.

[c] The origin of mAb SD6 and procedures for determination of the frequency of mAb SD6-resistant mutants are described in footnote c of Table 2 and in Materials and methods.

Dowling, 1999)], including homologous interference by DI RNAs (Holland, 1990; Roux et al., 1991). Recently, a more general phenomenon of RNA interference mediated by small interfering RNAs has been characterized (Agrawal et al., 2003; Gitlin et al., 2002; Lecellier and Voinnet, 2004; Lopez and Arias, 2004). There may be a complex interplay between DI RNA- and small RNA-mediated interference, as in some plant virus systems in which reduction of viral load and attenuation of symptoms involve DI RNA-associated enhancement of posttranscrip-
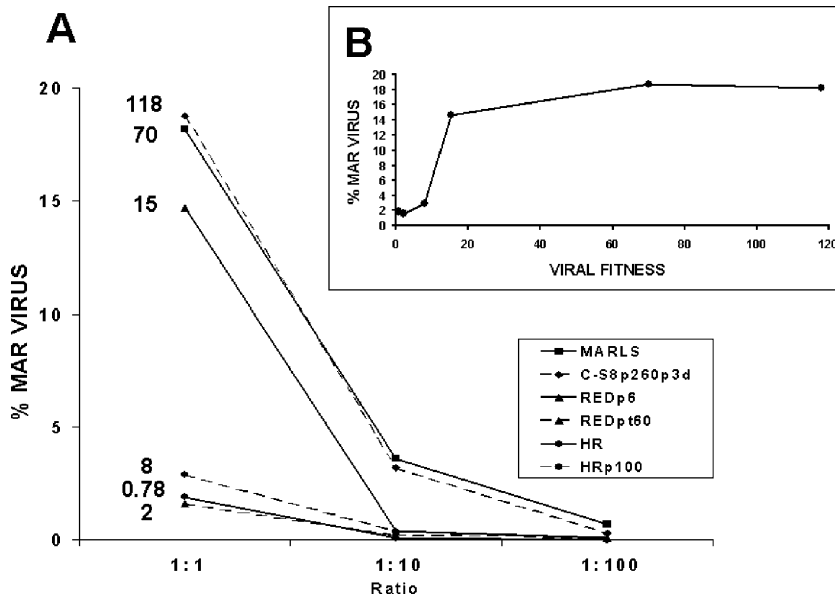

Fig. 2. Interference of C-S8p260 on different standard FDMVs. (A) The progeny production of standard virus (MAR, mAb-resistant) in coinfections with C-S8p260 at increasing ratios of standard to C-S8p260 is plotted, with an indication of the relative fitness of the standard FMDVs (number at the tip of each line). Data are from Tables 1 and 3, and the determination of fitness values is described in Materials and methods. (B) Representation of production of standard (MAR) FMDV as a function of relative fitness of the standard FMDV for the coinfections carried out with an initial 1:1 particle ratio of standard FMDV to FMDV C-S8p260. Data are the same as in A.
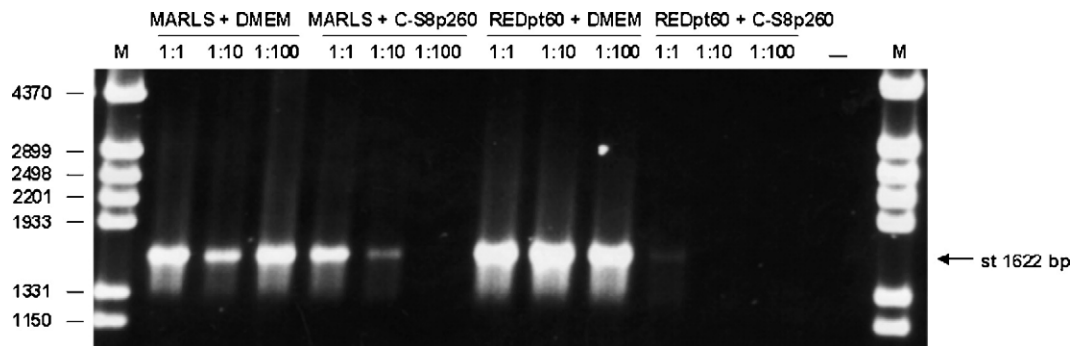
Fig. 3. Electropherogram showing the decrease in the amount of standard FMDV RNA in the progeny of coinfections of BHK-21 cells with C-S8p260 and either FMDV MARLS or REDpt60. Ratios (1:1, 1:10, 1:100) of the initial number of standard (MARLS or REDpt60) to C-S8p260 particles are indicated. DMEM denotes control infections carried out in parallel but in the absence of C-S8p260. The RT-PCR amplification used detected only standard FMDV RNA (García-Arriaza et al., 2004 and Materials and methods). The DNA product corresponding to standard (st) size RNA is indicated by an arrow on the right. Lane M: molecular size markers (HindIII-digested φ29 DNA; the corresponding sizes (bp) are indicated on the left); lane −, negative control, amplification without RNA. Use of ΔRNA-specific primers documented dominance of deleted RNAs (data not shown).

tional gene silencing (Simon et al., 2004). Still another meaning of interference relates to suppression of replication of individual viral variants by the mutant spectrum of viral quasispecies (Borrego et al., 1993; Chumakov et al., 1991; de la Torre and Holland, 1990; Teng et al., 1996), including suppression by the mutant spectrum of preextinction RNA populations (González-López et al., 2004). In this case, it has been proposed that interference is mediated by normal or aberrant expression from mutated RNAs of trans-acting non-functional proteins that jeopardize replication of infectious RNA.

Several molecular studies have indicated that DI RNAs replicate more rapidly than the corresponding standard virus and compete with standard virus for viral and cellular proteins needed to complete the virus infectious cycle (Holland, 1990). The lower interference by C-S8p260 exerted on high fitness virus (Figs. 2 and 3) suggests that intracellular competition may also underlie the interfering activity of C-S8p260, since high fitness may enhance the advantage of standard RNA in capturing viral and cellular resources in competition with ΔRNAs of C-S8p260. Additional interfering interactions, discussed in more detail elsewhere (Domingo et al., 2005), may be those mediated by dominant-negative mutants, as documented in classical viral genetics (reviews in Condit, 2001; Youngner and Whitaker-Dowling, 1999). Multiple mutants within the mutant spectra, showing different degrees of dominance, may collectively contribute a concerted suppressor activity on replication-competent genomes, favored by the multifunctional nature of most picornaviral proteins (Flint et al., 2004; Racaniello, 2001).

Most competition assays started at low MOI will include one or more rounds of infection at high MOI at late times post-infection following release of virus from the cells initially infected. Restricting the infection to short times poses technical difficulties in that rapid mutants may nevertheless reinfect cells and that cell to cell spread of virus on cell monolayers is difficult to control. The MOI-dependent competition events during

fitness assays have been discussed by Novella and colleagues with regard to complementation (Novella et al., 2004; Wilke et al., 2004).

Probing possible advantages of RNA genome segmentation

Our current investigations are directed to explore the reasons why particles containing ΔRNAs became dominant in competition with standard RNA at high MOI passage of biological clone C-S8c1. One of our working hypotheses is that a selective advantage could be provided by speed of replication. This mechanism was classically documented in pioneering experiments that revealed the progressive dominance of deleted RNAs in serial passages of Qβ RNA replicated in vitro by Qβ replicase, because the deleted forms completed rounds of template copying in a shorter time than full-length Qβ RNA (Biebricher, 1999; Mills et al., 1967; Sabo et al., 1977). Experiments are now in progress to try to compare the RNA synthesis rate of C-S8p260 and its standard counterpart. However, genome segmentation may provide a genetic system with other advantages, independent of (or together with) speed of replication. Indeed, segmentation has been regarded as a primitive form of sex, additional to homologous recombination which occurs with a high frequency during replication of FMDV in cell culture (King, 1988; King et al., 1981). Moreover, the different defective genomic segments in FMDV C-S8p260 contain redundant information (regulatory regions and most of the non-structural protein-coding region; see Fig. 1B) that enriches the information content of the mutant spectrum. By virtue of segmentation, part of the genetic information of FMDV C-S8p260 can be exchanged as a result of reassortment events. The several possibilities to account for an advantage of genome segmentation are now amenable to experimental investigation with the FMDV C-S8p260 system and its unsegmented cognate to test hypotheses that have been presented basically on theoretical grounds (Chao, 1991; Nee, 1987; Szathmary, 1992; Szathmary and Maynard

Smith, 1997). One interesting possibility is that when an RNA virus evolves towards producing DI RNAs–as in many negative-strand RNA viruses, perhaps due to facile *trans*-complementation–then the system is stuck in a dynamics of cyclic production of variants of the standard virus resistant to successive waves of DIs (Holland, 1984; Holland et al., 1979; Kirkwood and Bangham, 1994; Palma and Huang, 1974). In contrast, when the initial defective genome–such as Δ417 in the FMDV system–does not interfere with replication of the standard virus (Charpentier et al., 1996), there is a coexistence of deleted and standard RNA that allows for further evolutionary events such as generation of other defective RNAs that can complement the initial ones. It is only when complementation takes place that the defective genomes are sufficiently fit to successfully compete with the standard RNA. The interfering activity of defective particles, exerted on the cognate standard RNA (Table 1), may contribute to maintaining the dominance of complementing defective genomes. Yet according to the results reported here (Table 3), the interference should not be significant when complementing defective RNAs are first generated since, initially, the standard RNA must be in vast excess. Experiments are now in progress to try to clarify these issues.

Materials and methods

Cells, viruses, and infections

The origin of BHK-21 cells and procedures for cell growth, infection of cell monolayers with FMDV in Dulbecco's modification of Eagle's medium (DMEM), and for plaque assays in semisolid agar medium have been previously described (Domingo et al., 1980; Sobrino et al., 1983). All FMDV titrations carried out in this study were done in triplicate and standard deviations (not shown) never exceeded 15% of the mean. In all infection experiments, mock-infected BHK-21 cell monolayers were incubated in parallel to ascertain the absence of contamination; no evidence of contamination was seen at any time. FMDV C-S8c1 is a plaque-purified virus of the European serotype C, natural isolate C_1 Santa-Pau Spain 70 (Sobrino et al., 1983). FMDV C-S8p260 is a viral population obtained after 260 serial cytolytic passages of C-S8c1 at high MOI in BHK-21 cells (2×10^6 BHK-21 cells infected with the virus contained in 200 μl of the supernatant from the previous infection, estimated to contain 1×10^7 to 5×10^7 corrected PFUs) (corrected refers to the application of a correction factor to account for the effect of dilution on the titration of the multipartite FMDV; see Results, García-Arriaza et al., 2004, and Manrubia et al., in preparation). FMDV C-S8p260p3d is a viral population obtained after three serial cytolytic passages of C-S8p260 at low MOI in BHK-21 cells (2×10^6 BHK-21 cells infected with the virus contained in 200 μl of a 10^{-3} dilution of the supernatant from the previous

infection, or about 1×10^4 to 5×10^4 corrected PFUs) (García-Arriaza et al., 2004). FMDV MARLS is a mAb SD6-escape mutant obtained from FMDV C-S8c1 passaged 213 times in BHK-21 cells (Charpentier et al., 1996). MARLS includes the substitution L-144 → S at the G-H loop of capsid protein VP1 (Mateu, 1995). The fitness of MARLS in BHK-21 cells is about 130-fold higher than that of C-S8c1 (Sierra, 2001). REDp6 is a viral population obtained after 6 passages at high MOI of an FMDV RED, which is a mAb SD6 escape mutant obtained from a C-S8c1 passaged 100 times in BHK-21 cells (C-S8c1p100) (Ruiz-Jarabo et al., 2000, 2002). FMDV RED includes the substitution G-142 → E at the G-H loop of VP1 (Martínez et al., 1997). REDpt60 is a virus obtained after 60 plaque-to-plaque transfers of FMDV RED. REDp6 and REDpt60 maintain the resistance to mAb SD6 (Ruiz-Jarabo et al., 2002). REDpt60 has a relative fitness about 2-fold higher than that of C-S8c1 (Pariente, 2003; Pariente et al., 2003). REDpt60 has a relative fitness about 7.6-fold lower than that of REDp6 (Ruiz-Jarabo et al., 2002). FMDV HR is a mAb SD6 escape mutant of FMDV C-S8c1 (Mateu et al., 1990). Its capsid differs from that of C-S8c1 in only one amino acid (H-146 → R in VP1). The replicative fitness of HR in BHK-21 cells is about 0.8-fold that of C-S8c1 (Martínez et al., 1991). FMDV HRp100 is a viral population obtained after 100 serial cytolytic passages of HR at high MOI in BHK-21 cells; HRp100 maintains resistance to mAb SD6 (Holguín et al., 1997). FMDV C_{22}^9p50 and C_{22}^9p150 were obtained after 50 and 150 large population passages of clone C_{22}^9 in BHK-21 cells (MOI of 0.1–10 PFU/cell) (Escarmís et al., 1999). These two viruses were used as reference viruses for determinations of relative fitness, as described in a later section of Materials and methods.

MAb SD6-resistant mutants

The frequency of mutants resistant to neutralization by mAb SD6 was determined as previously described (Martínez et al., 1997; Ruiz-Jarabo et al., 2000, 2002). Briefly, about 10^5 to 10^6 PFU of virus were incubated for 60 min at room temperature either with 1:1 dilution of mAb SD6 (supernatant of hybridoma culture) or with DMEM, and then plated with or without a 1:20 dilution of the same mAb, respectively. Experiments with C-S8c1 (sensitive to SD6) and MARLS (resistant to SD6) were carried out to determine the dilution of SD6 required for SD6-resistant virus to yield the same number of plaques in the presence and absence of SD6, and the sensitive virus not to produce plaques in the presence of SD6. In the set of determinations summarized in Table 2, the frequency of mAb SD6-resistant mutants for C-S8c1 was about 10^2-fold higher than in previous determinations (Ruiz-Jarabo et al., 2003); the reason for this discrepancy has not been investigated, and it does not affect the results described here.

Interference competition assays

The interfering capacity of defective genomes present in C-S8p260 was studied by coinfecting 1×10^6 BHK-21 cells with mixtures of C-S8p260 and different non-defective standard FMDVs resistant to mAb SD6 to permit quantification of progeny C-S8p260 and standard virus. The number of viral particles used is indicated in the corresponding tables. The initial ratio of C-S8p260 to standard virus particles in the coinfections was 1, 10, or 100. After cytopathology (at 6 h to 22 h post-infection, depending on the standard FMDV tested), the viral progeny of each coinfection was analyzed by titration in presence and absence of mAb SD6 with a plaque assay in semisolid agar (Domingo et al., 1980). Several control assays indicated that the degree of interference was not significantly affected by the time post-infection (in the range of 6 h to 22 h) at which virus was analyzed. The infectivity of C-S8p260 virions was abolished by photoinactivation by neutral red and visible light, carried out as previously described (Díez et al., 1990).

Determination of relative fitness of HRp100 and C-S8p260p3d

The relative fitness of FMDV HRp100 and C-S8p260p3d was determined by growth competition in BHK-21 cells, as previously described (Duarte et al., 1992; Escarmís et al., 1996, 1999; Holland et al., 1991). The viral population to be assayed was mixed with appropriate proportions of either FMDV C_{22}^9p50 or C_{22}^9p150, which were used as reference viruses. For fitness determination of FMDV HRp100, the reference virus was C_{22}^9p50, since this virus has a relative fitness similar to that of C-S8c1 in BHK-21 cells (Escarmís et al., 1999). For fitness determination of FMDV C-S8p260p3d, C_{22}^9p150 was used as reference virus, since this virus has a fitness 10-fold higher than that of C-S8c1 in BHK-21 cells (Escarmís et al., 1999). For each determination, four serial infections were carried out at MOI of 0.1 PFU/cell. The proportion of the two competing genomes at different passages was determined by real-time RT-PCR, employing primers able to discriminate FMDVs C_{22}^9p50 or C_{22}^9 p150 RNA from HRp100 or C-S8p260p3d RNA. The nucleotide sequences of the primers will be provided upon request. The fitness vectors obtained for HRp100 and C-S8p260p3d corresponded to the equations $y = 0.5599e^{0.9201x}$; $R^2 = 0.953$; and $y = 0.8177e^{0.8467x}$, $R^2 = 0.921$, respectively. The antilogarithm of the vector slope is the fitness of the assayed virus relative to the reference virus (Holland et al., 1991). Since the procedure used to quantify the two competing viruses differs from the procedure used in previous fitness determinations (Pariente et al., 2001, 2003; Sierra, 2001; Sierra et al., 2000), we redetermined the relative fitness of MARLS using C_{22}^9 p150 as the reference virus. The value obtained was 118 ($y = 0.4879e^{1.0709x}$; $R^2 = 0.9573$), which is in good agreement with the value of 130 obtained previously (Sierra, 2001); the value 118 was used for comparative purposes in the present study (Table 2 and Fig. 2). The relative fitness values of all FMDVs tested in the present study have been included in Table 2 and Fig. 2. The determination of a relative fitness for C-S8p260 with current procedures is inaccurate due to the multipartite nature and dilution-dependent infectivity of this virus. A fitness determination using C_{22}^9 p150 as competitor yielded a relative fitness of 35 ($y = 0.6593e^{0.5467x}$; $R^2 = 0.9277$) for C-S8p260, but this is probably an underestimate of its true relative fitness value in BHK-21 cells.

RNA extraction, RNA quantification, cDNA synthesis, and PCR amplification

Viral RNA was extracted from the medium of infected cultures using Trizol (Invitrogene) as previously described (Sierra et al., 2000). RNA was quantified with Light Cycler instrument (Roche) using the Light Cycler RNA Master or RNA amplification SYBR Green I kits (Roche), according to the manufacturer's instructions, with purified FMDV C-S8c1 RNA as standard (García-Arriaza et al., 2004). Reverse transcription was performed with AMV reverse transcriptase (Promega) and PCR amplification was carried out using AmpliTaq polymerase (Perkin-Elmer), as specified by the manufacturers. To detect only standard FMDV RNA (and not ΔRNAs), the primers used were CACAGGGTTGGAGTTGCGCGAG-GGTG (sense orientation; positions 1305–1330) and GCACGTACGCCACCATGTACCGAG (antisense orientation; positions 2903–2926) (García-Arriaza et al., 2004). Amplification products were analyzed by agarose gel electrophoresis using HindIII-digested ϕ29 DNA as molar mass standards. Negative controls (amplifications in the absence of RNA) were included in parallel to ascertain absence of contamination by template nucleic acids. Buffers and templates were handled in separate locations, and no evidence of contamination was seen in the experiments reported here.

References

Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D., Brown, F., 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. Nature 337 (6209), 709–716.

Agrawal, N., Dasaradhi, P.V., Mohmmed, A., Malhotra, P., Bhatnagar, R.K., Mukherjee, S.K., 2003. RNA interference: biology, mechanism, and applications. Microbiol. Mol. Biol. Rev. 67 (4), 657–685.

Baranowski, E., Ruíz-Jarabo, C.M., Lim, P., Domingo, E., 2001. Foot-and-

mouth disease virus lacking the VP1 G-H loop: the mutant spectrum uncovers interactions among antigenic sites for fitness gain. Virology 288, 192–202.

Biebricher, C.K., 1999. Mutation, competition and selection as measured with small RNA molecules. In: Domingo, E., Webster, R.G., Holland, J.J. (Eds.), Origin and Evolution of Viruses. Academic Press, San Diego, pp. 65–85.

Borrego, B., Novella, I.S., Giralt, E., Andreu, D., Domingo, E., 1993. Distinct repertoire of antigenic variants of foot-and-mouth disease virus in the presence or absence of immune selection. J. Virol. 67 (10), 6071–6079.

Carrillo, C., Borca, M., Moore, D.M., Morgan, D.O., Sobrino, F., 1998. In vivo analysis of the stability and fitness of variants recovered from foot-and-mouth disease virus quasispecies. J. Gen. Virol. 79 (Pt 7), 1699–1706.

Chao, L., 1990. Fitness of RNA virus decreased by Muller's ratchet. Nature 348 (6300), 454–455.

Chao, L., 1991. Levels of selection, evolution of sex in RNA viruses, and the origin of life. J. Theor. Biol. 153 (2), 229–246.

Charpentier, N., Dávila, M., Domingo, E., Escarmís, C., 1996. Long-term, large-population passage of aphthovirus can generate and amplify defective noninterfering particles deleted in the leader protease gene. Virology 223 (1), 10–18.

Chumakov, K.M., Powers, L.B., Noonan, K.E., Roninson, I.B., Levenbook, I.S., 1991. Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. Proc. Natl. Acad. Sci. U.S.A. 88 (1), 199–203.

Cole, C.N., 1975. Defective interfering (di) particles of poliovirus. Prog. Med. Virol. 20, 180–207.

Condit, R.C., 2001. Principles of virology. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, vol. 1. Lippincott Williams and Wilkins, Philadelphia, pp. 19–51.

de la Torre, J.C., Holland, J.J., 1990. RNA virus quasispecies populations can suppress vastly superior mutant progeny. J. Virol. 64 (12), 6278–6281.

de la Torre, J.C., Dávila, M., Sobrino, F., Ortín, J., Domingo, E., 1985. Establishment of cell lines persistently infected with foot-and-mouth disease virus. Virology 145 (1), 24–35.

Díez, J., Dávila, M., Escarmís, C., Mateu, M.G., Dominguez, J., Pérez, J.J., Giralt, E., Melero, J.A., Domingo, E., 1990. Unique amino acid substitutions in the capsid proteins of foot-and-mouth disease virus from a persistent infection in cell culture. J. Virol. 64 (11), 5519–5528.

Domingo, E., Holland, J.J., 1997. RNA virus mutations and fitness for survival. Annu. Rev. Microbiol. 51, 151–178.

Domingo, E., Dávila, M., Ortín, J., 1980. Nucleotide sequence hetero-geneity of the RNA from a natural population of foot-and-mouth-disease virus. Gene 11 (3–4), 333–346.

Domingo, E., Martín, V., Perales, C., García-Arriaza, J., Arias, A., Escarmís, C., 2005. Viruses as quasispecies: biological implications. Curr. Top. Microbiol. Immunol., volume in preparation.

Duarte, E., Clarke, D., Moya, A., Domingo, E., Holland, J., 1992. Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. Proc. Natl. Acad. Sci. U.S.A. 89 (13), 6015–6019.

Eigen, M., Biebricher, C.K., 1988. Sequence space and quasispecies distribution. In: Domingo, E., Ahlquist, P., Holland, J.J. (Eds.), RNA Genetics, vol. 3. CRC Press, Boca Raton, FL, pp. 211–245.

Escarmís, C., Dávila, M., Charpentier, N., Bracho, A., Moya, A., Domingo, E., 1996. Genetic lesions associated with Muller's ratchet in an RNA virus. J. Mol. Biol. 264, 255–267.

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

Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2004. Principles of virology. Molecular Biology, Pathogenesis, and Control of Animal Viruses. 2nd ed. ASM Press, Washington, DC.

García-Arriaza, J., Manrubia, S.C., Toja, M., Domingo, E., Escarmís, C.,

2004. Evolutionary transition toward defective RNAs that are infectious by complementation. J. Virol. 78 (21), 11678–11685.

Gitlin, L., Karelsky, S., Andino, R., 2002. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 418 (6896), 430–434.

González-López, C., Arias, A., Pariente, N., Gómez-Mariano, G., Domingo, E., 2004. Preextinction viral RNA can interfere with infectivity. J. Virol. 78 (7), 3319–3324.

Holguín, A., Hernández, J., Martínez, M.A., Mateu, M.G., Domingo, E., 1997. Differential restrictions on antigenic variation among antigenic sites of foot-and-mouth disease virus in the absence of antibody selection. J. Gen. Virol. 78, 601–609.

Holland, J.J., 1984. Continuum of change in RNA virus genomes. In: Notkins, A.L., Oldstone, M.B.A. (Eds.), Concepts in Viral Pathogenesis, vol. 137–143. Springer-Verlag, New York.

Holland, J.J., 1990. Defective viral genomes. In: Fields, B.M., Knipe, D.M. (Eds.), Virology. Raven Press, New York, pp. 151–165.

Holland, J.J., Grabau, E.A., Jones, C.L., Semler, B.L., 1979. Evolution of multiple genome mutations during long-term persistent infection by vesicular stomatitis virus. Cell 16 (3), 495–504.

Holland, J.J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S., VandePol, S., 1982. Rapid evolution of RNA genomes. Science 215 (4540), 1577–1585.

Holland, J.J., de la Torre, J.C., Clarke, D.K., Duarte, E., 1991. Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. J. Virol. 65 (6), 2960–2967.

Huang, A.S., 1973. Defective interfering viruses. Annu. Rev. Microbiol. 27, 101–117.

King, A.M.Q., 1988. Genetic recombination in positive strand RNA viruses. In: Domingo, E., Holland, J.J., Ahlquist, P. (Eds.), RNA Genetics, vol. II. CRC Press Inc., Boca Raton Fl.

King, A.M., Underwood, B.O., McCahon, D., Newman, J.W., Brown, F., 1981. Biochemical identification of viruses causing the 1981 outbreaks of foot and mouth disease in the UK. Nature 293 (5832), 479–480.

Kirkwood, T.B., Bangham, C.R., 1994. Cycles, chaos, and evolution in virus cultures: a model of defective interfering particles. Proc. Natl. Acad. Sci. U.S.A. 91 (18), 8685–8689.

Lecellier, C.H., Voinnet, O., 2004. RNA silencing: no mercy for viruses? Immunol. Rev. 198, 285–303.

Lopez, S., Arias, C.F., 2004. Preface. Virus gene silencing by RNA interference. Virus Res. 102 (1), 1–2.

Lundquist, R.E., Sullivan, M., Maizel Jr., J.V., 1979. Characterization of a new isolate of poliovirus defective interfering particles. Cell 18 (3), 759–769.

Martínez, M.A., Carrillo, C., González-Candelas, F., Moya, A., Domingo, E., Sobrino, F., 1991. Fitness alteration of foot-and-mouth disease virus mutants: measurement of adaptability of viral quasispecies. J. Virol. 65 (7), 3954–3957.

Martínez, M.A., Verdaguer, N., Mateu, M.G., Domingo, E., 1997. Evolution subverting essentiality: dispensability of the cell attachment Arg–Gly–Asp motif in multiply passaged foot-and-mouth disease virus. Proc. Natl. Acad. Sci. U.S.A. 94 (13), 6798–6802.

Mateu, M.G., 1995. Antibody recognition of picornaviruses and escape from neutralization: a structural view. Virus Res. 38 (1), 1–24.

Mateu, M.G., Martínez, M.A., Capucci, L., Andreu, D., Giralt, E., Sobrino, F., Brocchi, E., Domingo, E., 1990. A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. J. Gen. Virol. 71, 629–637.

McClure, M.A., Holland, J.J., Perrault, J., 1980. Generation of defective interfering particles in picornaviruses. Virology 100 (2), 408–418.

Mills, D.R., Peterson, R.L., Spiegelman, S., 1967. An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule. Proc. Natl. Acad. Sci. U.S.A. 58, 217–224.

Nee, S., 1987. The evolution of multicompartmental genomes in viruses. J. Mol. Evol. 25 (4), 277–281.

Nomoto, A., Jacobson, A., Lee, Y.F., Dunn, J., Wimmer, E., 1979. Defective interfering particles of poliovirus: mapping of the deletion and evidence that the deletions in the genomes of DI(1), (2) and (3) are located in the same region. J. Mol. Biol. 128 (2), 179–196.

Novella, I.S., Duarte, E.A., Elena, S.F., Moya, A., Domingo, E., Holland, J.J., 1995. Exponential increases of RNA virus fitness during large population transmissions. Proc. Natl. Acad. Sci. U.S.A. 92 (13), 5841–5844.

Novella, I.S., Reissig, D.D., Wilke, C.O., 2004. Density-dependent selection in vesicular stomatitis virus. J. Virol. 78 (11), 5799–5804.

Nüesch, J., Krech, S., Siegl, G., 1988. Detection and characterization of subgenomic RNAs in hepatitis A virus particles. Virology 165 (2), 419–427.

Palma, E.L., Huang, A.S., 1974. Cyclic production of vesicular stomatitis virus caused by defective interfering particles. J. Infect. Dis. 126, 402–410.

Pariente, N., 2003. Base molecular y dinámica de la extinción del virus de la fiebre aftosa por combinación de un mutágeno e inhibidores, PhD Thesis. Universidad Autónoma de Madrid, Madrid, Spain.

Pariente, N., Sierra, S., Lowenstein, P.R., Domingo, E., 2001. Efficient virus extinction by combinations of a mutagen and antiviral inhibitors. J. Virol. 75 (20), 9723–9730.

Pariente, N., Airaksinen, A., Domingo, E., 2003. Mutagenesis versus inhibition in the efficiency of extinction of foot-and-mouth disease virus. J. Virol. 77 (12), 7131–7138.

Perrault, J., 1981. Origin and replication of defective interfering particles. Curr. Top. Microbiol. Immunol. 93, 151–207.

Quiñones-Mateu, M.E., Arts, E.J., 2002. HIV-1 fitness: implications for drug resistance, disease progression, and global epidemic evolution. In: Kuiken, C., Foley, B., Hahn, B.H., Marx, P., McCutchan, F.E., Mellors, J., Mullins, J.I., Sodroski, J., Wolinsky, S., Korber, B.T. (Eds.), HIV-1 Sequence Compendium. Theoretical Biology and Biophysics Group. Los Alamos National Laboratory, Los Alamos, NM, pp. 134–170.

Racaniello, V.R., 2001. Picornaviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology. Lippincott Williams and Wilkins, Philadelphia, pp. 685–722.

Radloff, R.J., Young, S.A., 1983. Defective interfering particles of encephalomyocarditis virus. J. Gen. Virol. 64, 1637–1641.

Roux, L., Simon, A.E., Holland, J.J., 1991. Effects of defective interfering viruses on virus replication and pathogenesis in vitro and in vivo. Adv. Virus Res. 40, 181–211.

Rowlands, D.J. (Ed.), Foot-and-mouth disease, Virus Res., vol. 91, pp. 1–161.

Ruiz-Jarabo, C.M., Arias, A., Baranowski, E., Escarmís, C., Domingo, E., 2000. Memory in viral quasispecies. J. Virol. 74, 3543–3547.

Ruiz-Jarabo, C.M., Arias, A., Molina-París, C., Briones, C., Baranowski, E., Escarmís, C., Domingo, E., 2002. Duration and fitness dependence of quasispecies memory. J. Mol. Biol. 315 (3), 285–296.

Ruiz-Jarabo, C.M., Miller, E., Gómez-Mariano, G., Domingo, E., 2003. Synchronous loss of quasispecies memory in parallel viral lineages: a deterministic feature of viral quasispecies. J. Mol. Biol. 333, 553–563.

Sabo, D.L., Domingo, E., Bandle, E.F., Flavell, R.A., Weissmann, C., 1977. A guanosine to adenosine transition in the 3′ terminal extracistronic region of bacteriophage Qb RNA leading to loss of infectivity. J. Mol. Biol. 112 (2), 235–252.

Sierra, S., 2001. Caracterización de la respuesta del virus de la fiebre aftosa a mutagénesis química. PhD Thesis. Universidad Autónoma de Madrid, Madrid, Spain.

Sierra, S., Dávila, M., Lowenstein, P.R., Domingo, E., 2000. Response of foot-and-mouth disease virus to increased mutagenesis. Influence of viral load and fitness in loss of infectivity. J. Virol. 74, 8316–8323.

Simon, A.E., Roossinck, M.J., Havelda, Z., 2004. Plant virus satellite and defective interfering RNAs: new paradigms for a new century. Annu. Rev. Phytopathol. 42, 415–437.

Sobrino, F., Domingo, E. (Eds.), 2004. Foot-and-Mouth Disease: Current Perspectives. Horizon Bioscience, Wymondham, England.

Sobrino, F., Dávila, M., Ortín, J., Domingo, E., 1983. Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell culture. Virology 128, 310–318.

Szathmary, E., 1992. Natural selection and dynamical coexistence of defective and complementing virus segments. J. Theor. Biol. 157 (3), 383–406.

Szathmary, E., Maynard Smith, J., 1997. From replicators to reproducers: the first major transitions leading to life. J. Theor. Biol. 187 (4), 555–571.

Teng, M.N., Oldstone, M.B., de la Torre, J.C., 1996. Suppression of lymphocytic choriomeningitis virus-induced growth hormone deficiency syndrome by disease-negative virus variants. Virology 223 (1), 113–119.

Tershak, D.R., 1982. Guanidine-resistant defective interfering particles of poliovirus. J. Virol. 41 (2), 615–625.

Verdaguer, N., Mateu, M.G., Andreu, D., Giralt, E., Domingo, E., Fita, I., 1995. Structure of the major antigenic loop of foot-and-mouth disease virus complexed with a neutralizing antibody: direct involvement of the Arg–Gly–Asp motif in the interaction. EMBO J. 14 (8), 1690–1696.

Wilke, C.O., Reissig, D.D., Novella, I.S., 2004. Replication at periodically changing multiplicity of infection promotes stable coexistence of competing viral populations. Evol. Int. J. Org. Evol. 58 (4), 900–905.

Youngner, J.S., Whitaker-Dowling, P., 1999. Interference. In: Granoff, A., Webster, R.G. (Eds.), Encyclopedia of Virology, vol. 2. Academic Press, San Diego, CA, pp. 850–854.