Persistence of foot-and-mouth disease virus in cell culture revisited: implications for contingency in evolution

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If we could rewind the tape of evolution and play it again, would it turn out to be similar to or different from what we know? Obviously, this key question can only be addressed by fragmentary experimental approaches. Twenty-two years ago, we described the establishment of BHK-21 cells persistently infected with foot-and-mouth disease virus (FMDV), a system that displayed as its major biological feature a coevolution of the cells and the resident virus in the course of persistence. Now we report the establishment of two persistently infected cell lines in parallel, starting with the same clones of FMDV and BHK-21 cells used 22 years ago. We have asked whether the evolution of the two newly established cell lines and of the earlier cell line would be similar or different. The main conclusions of the study are: (i) the basic behaviour characterized by virus–cell coevolution is similar in the three carrier cell lines, despite differences in some genetic alterations of FMDV; (ii) a strikingly parallel behaviour has been observed with the two newly established cell lines passed in parallel, unveiling a deterministic virus behaviour during persistence; and (iii) selective RT-PCR amplifications have detected imbalances in the proportion of positive- versus negative-strand viral RNA, mediated by both viral and cellular factors. The results confirm coevolution of cells and virus as a major and reproducible feature of FMDV persistence in cell culture, and suggest that rapidly evolving viruses may constitute adequate test systems to probe the influence of historical contingency on evolutionary events.

INTRODUCTION

A role for historical contingency in biological evolution is widely accepted. In the words of S. J. Gould, if we could rewind the tape of evolutionary history to the remote past and play it again, it would turn out entirely different (Gould, 1989). This fundamental issue has been approached with disparate replicative systems such as nucleic acid enzymes evolving in vitro (Joyce, 2004; Lehman, 2004) or adaptation of bacteria to thermostability (Couñago et al., 2006) but, for obvious reasons, only fragmentary experimental approaches are possible. Recurrence is the term used to describe the possibility of reaching the same end point multiple times when a biological system is allowed to evolve repeatedly starting from a given initial point (Lehman, 2004). Some rapidly evolving systems consisting of two interacting biological entities, can offer the possibility of probing the contribution of historical contingency versus predictable selection in a complex evolutionary process. Admittedly, present-day viruses represent highly evolved systems, and they constitute incomplete tools for investigating the reproducibility of evolutionary developments, at least as a general problem. Current genomics suggests that viruses have played an important role as agents of cellular evolution (Bushman, 2002; Villarreal, 2005; Forterre, 2006). The virus–cell interactions that are of evolutionary consequence may involve a number of mechanisms such as genome integration, gene transduction or differential survival of cell subpopulations, particularly in the course of persistent infections (Ahmed et al., 1981; Ahmed & Chen, 1999; Nathanson & Gonzalez-Scarano, 2007). Given the complexity of interactions involved, the independent initiation of a persistent infection from the same initial virus and cells may constitute a system for interrogating contingency versus recurrence of an evolutionary trajectory involving two interacting biological entities.

Foot-and-mouth disease virus (FMDV) is an important animal pathogen that usually produces an acute infection of cloven-hoofed animals, but it may also produce a persistent infection in ruminants (reviewed by Rowlands, 2003; Sobrino & Domingo, 2004; Mahy, 2005). BHK-21 cells persistently infected with FMDV were established and characterized in our laboratory (de la Torre et al., 1985,
The main feature of FMDV persistence in cell culture was a rapid coevolution of the cells and the resident virus, reflected in a gradual increase of resistance of the cells to infection by the parental FMDV, and a gradual increase of FMDV virulence towards the parental BHK-21 cells (de la Torre et al., 1988, 1989a; Martin Hernández et al., 1994; Sáiz & Domingo, 1996; Toja et al., 1999). These events conformed to the accepted definition of coevolution, meaning an interdependence of the evolution of two interacting biological entities (Futuyma & Slatkin, 1983; Woolhouse et al., 2002). The genetic instability of the BHK-21 cells and the infecting FMDV probably favoured their rapid coevolution. BHK-21 cells were isolated from a tumour of a Syrian hamster (Stoker & MacPherson, 1964), and manifested an increasing degree of cell transformation in the course of persistence (de la Torre et al., 1988, 1989a). FMDV displayed rapid genetic variation (de la Torre et al., 1985, 1988; Diez et al., 1990a), in agreement with the high mutability of RNA viruses (Batschelet et al., 1976; Drake & Holland, 1999).

Because of the genetic and phenotypic flexibility of the cells and the virus in the course of FMDV persistence in cell culture, we considered that it was uncertain whether, upon re-establishment of FMDV persistence using the same initial clonal populations of FMDV and BHK-21 cells, similar or different evolutionary events would be observed. Furthermore, other systems with which contingency in evolution has been addressed (Joyce, 2004; Lehman, 2004; Coun˜ago Holland, 1999).

Establishment of cell lines persistently infected with FMDV. To establish two new BHK-21 cell lines persistently infected with FMDV C-S8c1, the procedure described previously (de la Torre et al., 1985) was used: confluent BHK-21c1 cells were infected with FMDV C-S8c1 at a m.o.i. of 0.1 p.f.u. per cell. At 24 h post-infection (p.i.) cytopathology was extensive, but 10^{-3} to 10^{-4} of the initial number of cells remained attached to the dish. At 48 h p.i., the cells were washed extensively and allowed to grow in DMEM, 5 % FCS. The cells were subcultured every 2–3 days upon reaching confluence; each passage involved seeding of about 2 × 10^6 cells that were allowed to grow to about 6 × 10^6 cells. Two persistently infected BHK-21 cell lines were established in parallel; they are termed R-B and R-D. In this manuscript, the BHK-21 cells persistently infected with FMDV C-S8c1 that were established previously [termed c1-BHK-Rc1 in de la Torre et al. (1985)] are renamed R for simplicity.

Treatment of cells with ribavirin. A stock of ribavirin (1-b-d-ribofuranosyl 1,2,4-triazole-3-carboxamide) was prepared at 50 mM concentration in PBS and stored at −70 °C. It was diluted in DMEM, 5 % FCS as needed. To cure persistently infected cells of FMDV, cell monolayers (with about 6 × 10^6 cells) were treated with 500 μM ribavirin for 72 h (de la Torre et al., 1987; Airaksinen et al., 2003). They were then washed and passaged three times in the absence of ribavirin. The cured R-B and R-D cells are named C-B and C-D, respectively (for example, throughout the manuscript, including tables and figures, C-B15 and D-D15 denote R-B and R-D cells that were passaged 15 times and were then cured of FMDV by treatment with ribavirin). Curing was ascertained by absence of infectivity and of FMDV sequences amplifiable by RT-PCR (as described below).

Extraction of viral RNA. Extracellular viral RNA was extracted either from 150 μl supernatant of monolayers of lytically infected cells or from 500 μl supernatant of monolayers of persistently infected cells. The supernatant of monolayers of lytically infected cells or of FMDV C-S8c1 was kept at −70 °C. VR100 was derived from persistently infected BHK-21 cells at passage 100 (Diez et al., 1990a). MARLS is a monoclonal antibody-escape mutant isolated from population C-S8c1p213 (Charpentier et al., 1996). Clone H_{95} was obtained after 95 successive plaque-to-plaque transfers of H_{9} (a biological clone isolated from population C-S8c1p113) (Escarmis et al., 1996).

METHODS

Cells and viruses. BHK-21c1 is a clone of BHK-21 cells obtained as described previously (de la Torre et al., 1985). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 % fetal calf serum (FCS). The FMDV used was the biological clone C-S8c1, obtained by plating the natural isolate C-Sta Pau Sp/70 on BHK-21 cells (Sobrino et al., 1983). Both cells and virus were the same as used to establish the first FMDV carrier cell line (de la Torre et al., 1985). The stock of cells was kept in liquid nitrogen and FMDV C-S8c1 was kept at −70 °C. VR100 was derived from persistently infected BHK-21 cells at passage 100 (Diez et al., 1990a). MARLS is a monoclonal antibody-escape mutant isolated from population C-S8c1p213 (Charpentier et al., 1996). Clone H_{95} was obtained after 95 successive plaque-to-plaque transfers of H_{9} (a biological clone isolated from population C-S8c1p113) (Escarmis et al., 1996).

Infections. Procedures for infection of BHK-21 cell monolayers in liquid medium and plaque assays in semisolid agar medium were described previously (Sobrino et al., 1983; de la Torre et al., 1985).

Cell killing assay. The capacity of FMDV to kill BHK-21 cells was measured as described previously (Sevilla & Domingo, 1996; García-Arriazza et al., 2004; Herrera et al., 2007). The assay consists of determining the minimum number of p.f.u. required to kill 10^6 BHK-21 cells after variable times of infection. The assay was performed in 96 multiwell plates with monolayers of 10^4 BHK-21 cells per well, infected with serial dilutions of virus. Results are expressed as the logarithm of the number of p.f.u. needed for complete cell killing as a function of time post-infection (p.i.) (Sevilla & Domingo, 1996; García-Arriazza et al., 2004; Herrera et al., 2007).

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nucleotide corresponds to genomic residue 7493; numbering of genomic residues according to Escarmís et al., 1996) and oligo-A (5'-AGGAGATCATGGTTAAGTTGTC; antisense orientation; 5' nucleotide at genomic residue 7615) as primers. Quantification was relative to a standard curve obtained with known amounts of FMDV RNA, synthesized by in vitro transcription of DNA plasmid pMT28 (García-Arriaza et al., 2004). The specificity of the reaction was monitored by determining the denaturation curve of the amplified DNA and the size of the amplified DNA by agarose gel electrophoresis. Negative controls (without template RNA) were run in parallel with each amplification reaction.

**Quantification of positive- and negative-strand FMDV RNA.**
Total RNA was reverse-transcribed by employing the Transcriptor RT kit (Roche), using either oligonucleotide oligo-A or tag-oligo-U to amplify positive- or negative-strand FMDV RNA, respectively. ‘tag’ is the oligonucleotide 5'-AGTTTAAGAACCCCTCCGCC, corresponding to lymphocytic choriomeningitis virus (LCMV) RNA (segment L, positions 3662–3681), which does not hybridize with positive- or negative-strand FMDV RNA. Real-time quantitative PCR was carried out using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche), using either oligonucleotide oligo-A or tag-oligo-U to amplify positive- or negative-strand FMDV RNA. Real-time quantitative PCR was carried out using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche), using either oligonucleotide oligo-A or tag-oligo-U to amplify positive- or negative-strand FMDV RNA. Real-time quantitative PCR was carried out using the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche), using either oligonucleotide oligo-A or tag-oligo-U to amplify positive- or negative-strand FMDV RNA. Quantification of positive- and negative-strand FMDV RNA.

**RESULTS**

**Establishment of two BHK-21 cell lines persistently infected with FMDV**
To test whether newly established BHK-21 cells persistently infected with FMDV would behave similarly to a cell line characterized previously in our laboratory (de la Torre et al., 1985), we again established persistence in two parallel cultures, by growth of cells that survived a cytolytic infection, as detailed in Methods. The procedures and the starting cells and virus were the same as used when the first FMDV carrier cell line (termed R) was established (de la Torre et al., 1985). The two newly established cell lines, termed R-B and R-D, were passaged a total of 100 times. Cells were subcultured when just reaching confluence to prevent a cytopathology crisis involving cell detachment after confluence. The course of events, from the initial cytolytic infection to the full establishment of persistence, were very similar for R-B and R-D cells, and to the events recorded previously for R cells (de la Torre et al., 1985).

**Basic features of the cells are shared by the three persistently infected cell lines R, R-B and R-D**
In the course of persistence, R-B and R-D cells acquired a round morphology, increased their rate of duplication and became increasingly resistant to infection by FMDV C-S8c1, as documented previously for R cells (de la Torre et al., 1988) (results not shown). The cells were cured of FMDV by treatment with the nucleoside analogue ribavirin, and the cured cells maintained their resistance to FMDV C-S8c1, as measured by the extent of cytopathology and the number of viral progeny (Table 1).
positive- and negative-strand RNA. This observation followed a parallel variation in the proportion of positive-strand RNA. Again, strikingly, lineages R-B and R-D showed similar results. The amount of negative-strand FMDV RNA during persistence also produced a higher proportion of positive-strand RNA in the course of infection. The altered ratio of positive- versus negative-strand FMDV RNA is due to viral and cellular factors.

Deterministic events in two parallel lineages of persistently infected cells: infectivity and the ratio of positive- versus negative-strand FMDV RNA

The variation of extracellular and intracellular infectivity with cell passage number followed a very similar pattern in the R-B and R-D lineages (Fig. 1a). Strikingly, the virus titre fell below detectability at passage 30 and then increased transiently around passages 40–45, becoming undetectable again up to passage 100. The peak of infectivity at cell passage 45 and undetectable infectivity at cell passage 100 corresponded to the levels of intracellular structural and non-structural FMDV proteins, as quantified by Western blotting using specific monoclonal antibodies (Fig. 1b). As a control, extracts of C-B and C-D cells that had been cured of FMDV by treatment with ribavirin did not show a signal with the same monoclonal antibodies.

To investigate the relationship between infectivity and viral RNA levels, the numbers of extracellular and intracellular positive- and negative-strand FMDV RNA molecules were quantified at 16 different passages, spanning passages 2 to 100. The results (Fig. 2a) indicate a dominance of positive-strand RNA coincident with the passages at which virus infectivity was detectable (compare Figs 1a and 2a). When infectivity was not detected, the amount of negative-strand RNA was similar to or greater than the amount of positive-strand RNA. Again, strikingly, lineages R-B and R-D followed a parallel variation in the proportion of positive- and negative-strand RNA. This observation identifies a deterministic event at the level of intracellular viral RNA replication during viral persistence.

A crisis of cytopathology, when cells grow to over-confluence, is generally accompanied by an increase in FMDV titres (de la Torre et al., 1985). To investigate whether the peak of extracellular infectivity detected at passage 45 (Fig. 1a), which coincided with a maximum of positive-strand RNA (Fig. 2a), could be triggered by cells entering a phase of cytopathology, the numbers of extracellular and intracellular positive-strand RNA molecules were measured in R-B and R-D cells at passage 45 prior to confluence and after reaching confluence. The results (not shown) indicated that the numbers of extracellular and intracellular positive-strand RNA molecules at passage 45 were very similar prior to confluence and after reaching confluence, suggesting that the peak of extracellular infectivity detected was not due to cells entering a phase of cytopathology.

### Table 1. Resistance of R-B and R-D cells to infection by FMDV C-S8c1 and MARLS

<table>
<thead>
<tr>
<th>FMDV</th>
<th>Cells</th>
<th>BHK-21</th>
<th>C-B90</th>
<th>C-D90</th>
<th>C-B100</th>
<th>C-D100</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-S8c1</td>
<td></td>
<td>(4.1 ± 1.2) × 10^7</td>
<td>(7.2 ± 2.0) × 10^5</td>
<td>(2.6 ± 1.7) × 10^5</td>
<td>(4.4 ± 2.3) × 10^5</td>
<td>(3.3 ± 2.2) × 10^6</td>
</tr>
<tr>
<td>MARLS</td>
<td></td>
<td>(1.6 ± 0.8) × 10^7</td>
<td>(2.8 ± 1.9) × 10^5</td>
<td>(2.3 ± 2.4) × 10^5</td>
<td>(1.5 ± 1.4) × 10^6</td>
<td>(1.4 ± 0.9) × 10^8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[20 h p.i.]</td>
<td>[48 h p.i.]</td>
<td>[48 h p.i.]</td>
<td>[48 h p.i.]</td>
<td>[48 h p.i.]</td>
</tr>
</tbody>
</table>
replication. In the infections of late C-B and C-D cells with virus that produced a higher proportion of positive-strand RNA in the course of persistence, the dominance of the positive strand was diminished and, in some cases, the negative-strand RNA became dominant (Fig. 2b). When the same cells were infected with FMDV from a persistence passage in which negative strands dominated, the latter accentuated their dominance (Fig. 2b). Therefore, both cellular and viral factors contribute to the evolution of dominance of positive- versus negative-strand RNA in the course of viral replication.

The proportion of positive- and negative-strand FMDV RNA was also quantified at passages 45, 60 and 100 of R cells (those previously established in our laboratory; de la Torre et al., 1985, 1988). In all cases, in both intracellular and extracellular virus, levels of positive-strand RNA were 10²- to 10⁴-fold higher than negative-strand RNA. Although we cannot exclude the possibility that the dominance of the positive strand was lost at some other passages of R cells, the pattern of RNA polarity dominance in R cells was different from that in R-B and R-D cells. In turn, the results suggest that imbalances

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**Fig. 1.** Establishment of BHK-21 cell lines persistently infected with FMDV, and evolution of levels of infectivity and intracellular viral proteins. (a) Variation of extracellular (dashed lines) and intracellular (solid lines) infectivity with passage number in lineages R-B and R-D. The total number of p.f.u. in ~6 x 10⁶ cells was determined by plaque assay. Each value represents the mean ± SD (error bars) from triplicate assays. (b) Virus-specific proteins expressed by FMDV in R-B and R-D cells at cell passages 2, 45 and 100. R-B and R-D refer to R-B and R-D cells and C-B and C-D refer to the same cells cured of FMDV by treatment with ribavirin. Virus-specific proteins (VP3, 2C and 3D) were identified by reactivity with specific monoclonal antibodies in Western blot assays (Mateu et al., 1989; Perales et al., 2007). Procedures are detailed in Methods.
Fig. 2. Evolution of the number of positive- and negative-strand FMDV RNA molecules during persistence of FMDV in BHK-21 cells, and the influence of viral and cellular factors. (a) Quantification of extracellular (ec) or intracellular (ic) positive- (solid lines) and negative- (dashed lines) strand RNA as a function of passage number. At each passage analysed, total RNA was extracted either from the cell culture supernatant or from an extract of ~6×10^6 R-B or R-D cells. Each value represents the mean ± SD (error bars) from triplicate determinations. (b) Influence of the virus and of the host cells in the proportion of positive- (shaded bars) versus negative- (open bars) strand FMDV RNA. Parental BHK-21 cells, or R-B and R-D cells cured of FMDV by treatment with ribavirin (C-B and C-D), were infected either with FMDV from a passage in which positive-strand RNA was dominant (R-B5, R-B15, R-B25, R-D5, R-D15, R-D25) or with FMDV from a passage in which negative-strand RNA was dominant (R-B60, R-B70, R-B90, R-D60, R-D70, R-D90) [compare with (a)]. Each value represents the mean ± SD (error bars) of the number of RNA molecules (μl supernatant)^-1 from triplicate determinations. Procedures are detailed in Methods.
of positive- versus negative-strand RNA are not a necessary condition for maintaining FMDV persistence in BHK-21 cells, as also evidenced by the fact that, at passages 2–25 of R-B and R-D cells, persistence was maintained with continuous dominance of positive-strand RNA (Fig. 2a).

**Phenotypic and genetic changes of FMDV during persistence**

In the course of persistence in R cells, the resident FMDV became temperature sensitive (ts), acquired a small plaque morphology (0.5–1 mm versus 2–4 mm for C-S8c1) and increased its virulence for BHK-21 cells (de la Torre et al., 1985, 1988). These traits were also observed for FMDV rescued from R-B and R-D cells (Table 2). Virulence, quantified as the capacity to kill BHK-21 cells, increased with the time of residence of the virus in the carrier cells, reaching virulence levels comparable to those of FMDVs that have been passaged more than 100 times cytolytically (Herrera et al., 2007) (Fig. 3a and Table 3).

To compare the amounts of virus-specific proteins expressed by persistent virus when infecting BHK-21 cells cytolytically, either standard C-S8c1 or FMDV shed by R-B and R-D cells at cell passages 2 and 45 were used to infect BHK-21 cells and intracellular proteins were metabolically labelled and then analysed electrophoretically using actin as a control. The results (Fig. 3b) show that the persistent virus shed by R-B and R-D cells at passage 45 displays increased expression of viral proteins as well as increased shut-off of host-cell protein synthesis compared with the virus shed by R-B and R-D cells at passage 2, or with C-S8c1. Therefore, the increase in virulence of FMDV during persistence is also reflected in a more intense shut-off of host-cell protein expression.

To evaluate the extent of genetic variation undergone by FMDV during persistence in R-B and R-D cells, the entire genomic RNA of virus produced by R-B and R-D cells at passage 45 was sequenced and the sequences were compared with that of the parental FMDV C-S8c1. The results (Table 4) indicate that 62.5 % of the mutations found are common to the virus from R-B and R-D cells, and that only two amino acids (position 13 in VP3 and position 291 in 3D) differ between the R-B and R-D lineages. Thus, up to passage 45, the consensus nucleotide and deduced amino acid sequences showed minimal divergence between the two lineages.

It was not possible to amplify and determine the entire genomic nucleotide sequence of FMDV in R-B and R-D cells at passage 100, despite several attempts (probably due to the small amounts of viral RNA present). However, the consensus sequence of genomic residues 1897–3030 (residues encoding VP2 and part of VP3) and 6610–8019 (residues encoding 3D) could be determined, and they were compared with the consensus nucleotide sequence of VR100, the persistent virus shed by carrier cells at R cell passage 100 (Díez et al., 1990a; Toja et al., 1999). In the VP2- and VP3-coding regions examined, three mutations at genomic positions 2433, 2477 and 2517 that were not detected at passage 45 were dominant in the consensus sequence of R-D100 (Table 5). Interestingly, none of the mutations present in the 3D-coding region at passage 45 were maintained as dominant at passage 100 and, furthermore, four additional mutations, three of them leading to amino acid substitutions in either R-B and R-D, were present in the consensus sequences at passage 100 (compare Tables 4 and 5). The comparison with the corresponding regions of FMDV VR100 (Díez et al., 1990a; Toja et al., 1999) indicates that two mutations (D3009A and N3013H) were common to the three lineages (R, R-B and R-D cells). All the phenotypic traits analysed and part of the genetic changes undergone by persistent FMDV point to a remarkable reproducibility of the coevolutionary events during FMDV persistence in BHK-21 cells, despite an inherent genetic instability of both the cells and the resident virus.

### DISCUSSION

Coevolution of cells and virus has been described in the course of persistence of several animal viruses in cell culture (Takeo & Habel, 1959; Ahmed et al., 1981; Chiarini et al., 1983; Delli Bovi et al., 1984; Ron & Tal, 1985, 1986; Cummings & Rinaldo, 1989; Dermody et al., 1993; Calvez et al., 1995; Chen & Baric, 1996; Mrukowicz et al., 1998; Zhong et al., 2006). In other persistent infections in cell culture, no cell evolution was observed, and persistence was associated with defective interfering particles or with small-plaque and ts viral mutants.

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**Table 2. Phenotypic traits of FMDV C-S8c1 and its persistent derivatives**

R-B and R-D refer to the FMDV shed by R-B and R-D cells to the culture medium; the cell passage number (2, 20 or 45) is indicated. Viral yields from infections of BHK-21 with either the parental clone FMDV C-S8c1 or the persistent FMDVs shed by R-B and R-D cells at 37 and 42 °C are expressed as viral genomic RNA molecules per µl cell-culture supernatant. Quantification of RNA molecules was carried out by real-time RT-PCR. Each value represents the mean±SD from triplicate determinations. Procedures are detailed in Methods.

<table>
<thead>
<tr>
<th>FMDV</th>
<th>Yield at 37 °C</th>
<th>Yield at 42 °C</th>
<th>Plaque diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-S8c1</td>
<td>(4.4±1.2)·10⁶</td>
<td>(3.1±0.9)·10⁶</td>
<td>2–4</td>
</tr>
<tr>
<td>R-B2</td>
<td>(2.2±1.8)·10⁷</td>
<td>(1.0±2.5)·10⁶</td>
<td>2–4</td>
</tr>
<tr>
<td>R-B20</td>
<td>(2.6±2.4)·10⁷</td>
<td>(2.3±1.6)·10⁶</td>
<td>1–3</td>
</tr>
<tr>
<td>R-B45</td>
<td>(2.8±1.9)·10⁷</td>
<td>(2.4±3.1)·10⁷</td>
<td>0.5–1</td>
</tr>
<tr>
<td>R-D2</td>
<td>(1.8±2.2)·10⁷</td>
<td>(9.6±1.9)·10⁶</td>
<td>2–4</td>
</tr>
<tr>
<td>R-D20</td>
<td>(1.6±3.1)·10⁷</td>
<td>(3.2±2.7)·10⁶</td>
<td>1–3</td>
</tr>
<tr>
<td>R-D45</td>
<td>(5.6±2.8)·10⁷</td>
<td>(1.0±1.6)·10⁶</td>
<td>0.5–1</td>
</tr>
</tbody>
</table>
(Igarashi et al., 1977; Holland et al., 1980, 1982; Youngner & Preble, 1980). Our objectives in reanalysing FMDV persistence in BHK-21 cells were to evaluate whether the major biological events characterized previously would be repeated with the newly established cell lines and to examine features of FMDV replication with tools not available 20 years ago, notably quantitative PCR amplification.

The main biological features of FMDV persistence in BHK-21 cells, which is a coevolution of cells and virus, were repeated in the three cell lines. Mutations in the viral genome were not identical in the three lineages (Tables 4 and 5). However, amino acid substitutions D3009A and N3013H in VP3, which affect residues located around a pore at the capsid fivefold axes (Lea et al., 1994), became dominant in the three lineages. These two substitutions were previously identified in an antigenic variant of FMDV C-S8c1 (Holguin, 1996), and they are very rare in other FMDVs or picornaviruses (http://www.iah.bbsrc.ac.uk/virus/picornaviridae/SequenceDatabase/3Ddatabase/3D.htm). Substitutions C3007V and M3014L present in VR100 (Diez et al., 1990a; Toja et al., 1999) were not detectable in populations R-B and R-D (Table 5). In contrast, D3009A has invariably been found in FMDV from persistently infected BHK-21 cells, and this substitution was readily selected when FMDV C6Arg/85, an isolate of FMDV of a different subtype, associated with acute infections, was

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**Fig. 3.** Cell-killing capacity of FMDVs rescued from R-B and R-D cells. (a) Time needed by C-S8c1 and virus shed by R-B and R-D at cell passage 10, 20 and 45 to kill $10^6$ BHK-21 cells as a function of the initial number of infectious units (p.f.u.). Each value represents the mean ± SD (error bars) from triplicate assays. The data are summarized in Table 3. (b) Electrophoretic analysis of $^{35}$S-labelled FMDV and cellular proteins upon infection of BHK-21 cells with C-S8c1 or with virus shed by R-B and R-D at cell passage 2 and 45. Infections were carried out at an m.o.i. of 10 (p.f.u. per cell). Proteins were labelled with $^{35}$SMet–Cys from 1–2, 4–5 and 7–8 h p.i. using uninfected cultures treated in parallel (−virus) as a control (upper panel). Procedures are detailed in Methods.
Table 3. Cell-killing capacity of FMDVs rescued from R-B and R-D cells

Numbers of p.f.u. of C-S8c1 and of virus shed by R-B and R-D at cell passage 10, 20 and 45 needed to kill 10^6 cells in 24 h are based on the data shown in Fig. 3(a). The numbers in the first column indicate the cell passage number. Each virulence value represents the mean ± SD from triplicate assays; relative virulence values were normalized to the virulence of C-S8c1.

<table>
<thead>
<tr>
<th>FMDV</th>
<th>Virulence (p.f.u.)</th>
<th>Relative virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-S8c1</td>
<td>(1.9 ± 0.5) x 10^4</td>
<td>1</td>
</tr>
<tr>
<td>R-B10</td>
<td>(6.9 ± 1.0) x 10^3</td>
<td>2.7</td>
</tr>
<tr>
<td>R-B20</td>
<td>(1.6 ± 1.6) x 10^3</td>
<td>11.9</td>
</tr>
<tr>
<td>R-B45</td>
<td>(3.0 ± 1.1) x 10^2</td>
<td>63.3</td>
</tr>
<tr>
<td>R-D10</td>
<td>(1.2 ± 1.2) x 10^4</td>
<td>1.6</td>
</tr>
<tr>
<td>R-D20</td>
<td>(1.7 ± 1.1) x 10^4</td>
<td>11.2</td>
</tr>
<tr>
<td>R-D45</td>
<td>(2.8 ± 0.8) x 10^2</td>
<td>67.8</td>
</tr>
</tbody>
</table>

passaged in modified BHK-21 cells that had become partially resistant to FMDV in the course of persistence (Escarmis et al., 1998). Furthermore, replacement A3009T was observed in five out of six passage series in which VR100 was subjected to serial cytolytic infections in BHK-21 cells (Sevilla & Domingo, 1996). These observations suggest that fixation of D3009A was guided by some selective constraints that are encountered in carrier BHK-21 cells that have evolved to became partially resistant to the parental FMDV C-S8c1 (de la Torre et al., 1988; Diez et al., 1990a; Escarmis et al., 1998). Substitution D3009A caused a drastic reduction in plaque size and viability, and cytoplasmic replication of C-S8c1 with D3009A led to fixation of M3014L as a compensatory substitution. In turn, the introduction of the double substitution D3009A/M3014L facilitated the fixation of N3013H (Mateo & Mateu, 2007). In the FMDV rescued from R-B and R-D cells at passage 100, the only VP3 substitution that accompanies D3009A is N3013H (Table 5), and its possible compensatory role remains to be demonstrated. Also, the phenotypic implications of replacements around the FMDV pore at the fivefold axes, and their connections with persistence, require additional studies.

Repeated mutations in parallel evolutionary lineages have been reported in FMDV (Borrego et al., 1993; Martin-Hernández et al., 1994; Mateu et al., 1994; Ruiz-Jarabo et al., 2003) and in other picornaviruses (de la Torre et al., 1992; Borzakian et al., 1993; Chumakov et al., 1994; Couderc et al., 1994; Lu et al., 1996; reviewed by Domingo et al., 2001). The higher reproducibility of phenotypic than genotypic modifications during FMDV persistence relates to the general problem of mapping genotypes into phenotypes (Schuster, 1997; Schuster & Stadler, 1999; Table 3.)

Table 4. Nucleotide and deduced amino acid substitutions in the genomic RNA of FMDVs R-B45 and R-D45 compared with their parental virus C-S8c1

Residue numbering of the FMDV genome is given as described by Escarmis et al. (1996); amino acid residues (single-letter code) are numbered individually for each protein from the N terminus to the C terminus; genomic regions (5' fragment and pseudoknots at the 5' UTR; non-structural proteins L, 2B, 2C, 3A and 3D and capsid proteins VP3 and VP1) are based on Mahy (2005) and references therein. Silent (synonymous) mutations amounted to 41.7 and 35.7 % of the total number of mutations in the open reading frames of R-B and R-D, respectively. The 5' - and 3' -terminal 24 residues of the genome, the 22 residues at the 5' and 3' ends of the poly(C) and the homopolymeric regions [internal poly(C) and 3' poly(A)] were not sequenced.

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>Genomic region or encoded protein</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C-S8c1</td>
</tr>
<tr>
<td>365</td>
<td>S fragment</td>
<td>U</td>
</tr>
<tr>
<td>367</td>
<td>S fragment</td>
<td>A</td>
</tr>
<tr>
<td>452</td>
<td>Pseudoknots</td>
<td>U</td>
</tr>
<tr>
<td>1118 (27)</td>
<td>L</td>
<td>G (G)</td>
</tr>
<tr>
<td>1411 (125)</td>
<td>L</td>
<td>U (C)</td>
</tr>
<tr>
<td>2576 (9)</td>
<td>VP3</td>
<td>A (D)</td>
</tr>
<tr>
<td>2587 (13)</td>
<td>VP3</td>
<td>A (N)</td>
</tr>
<tr>
<td>2767 (73)</td>
<td>VP3</td>
<td>C (L)</td>
</tr>
<tr>
<td>2997 (149)</td>
<td>VP3</td>
<td>U (G)</td>
</tr>
<tr>
<td>3653 (149)</td>
<td>VP1</td>
<td>C (T)</td>
</tr>
<tr>
<td>4026 (48)</td>
<td>2B</td>
<td>A (K)</td>
</tr>
<tr>
<td>4539 (65)</td>
<td>2C</td>
<td>U (D)</td>
</tr>
<tr>
<td>5465 (56)</td>
<td>3A</td>
<td>A (K)</td>
</tr>
<tr>
<td>7071 (154)</td>
<td>3D</td>
<td>A (E)</td>
</tr>
<tr>
<td>7305 (232)</td>
<td>3D</td>
<td>A (Q)</td>
</tr>
<tr>
<td>7480 (291)</td>
<td>3D</td>
<td>A (T)</td>
</tr>
</tbody>
</table>
Table 5. Nucleotide and deduced amino acid substitutions of FMDVs VR100, R-B100 and R-D100 compared with their parental virus C-S8c1

Residues are numbered as described in Table 4. Mutations found in the consensus sequence of the indicated genomic residues (1897–3030, encoding VP2 and part of VP3, and 6610–8019, encoding 3D) are shown. Procedures for preparation of FMDV RNA and nucleotide sequence determination are described in Methods.

<table>
<thead>
<tr>
<th>Nucleotide position (amino acid residue)</th>
<th>Encoded protein</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C-S8c1</td>
</tr>
<tr>
<td><strong>Residues 1897–3030</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1944 (16)</td>
<td>VP2</td>
<td>U (T)</td>
</tr>
<tr>
<td>1956 (20)</td>
<td>VP2</td>
<td>G (G)</td>
</tr>
<tr>
<td>2316 (140)</td>
<td>VP2</td>
<td>A (L)</td>
</tr>
<tr>
<td>2325 (143)</td>
<td>VP2</td>
<td>C (Y)</td>
</tr>
<tr>
<td>2431–2433 (179)</td>
<td>VP2</td>
<td>CUC (L)</td>
</tr>
<tr>
<td>2470 (192)</td>
<td>VP2</td>
<td>G (A)</td>
</tr>
<tr>
<td>2473 (193)</td>
<td>VP2</td>
<td>G (G)</td>
</tr>
<tr>
<td>2477 (194)</td>
<td>VP2</td>
<td>C (A)</td>
</tr>
<tr>
<td>2517 (207)</td>
<td>VP2</td>
<td>C (N)</td>
</tr>
<tr>
<td>2569–2570 (7)</td>
<td>VP3</td>
<td>UG (C)</td>
</tr>
<tr>
<td>2576 (9)</td>
<td>VP3</td>
<td>A (D)</td>
</tr>
<tr>
<td>2587 (13)</td>
<td>VP3</td>
<td>A (N)</td>
</tr>
<tr>
<td>2590 (14)</td>
<td>VP3</td>
<td>A (M)</td>
</tr>
<tr>
<td><strong>Residues 6610–8019</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7595</td>
<td>3D</td>
<td>A (D)</td>
</tr>
<tr>
<td>7324</td>
<td>3D</td>
<td>G (V)</td>
</tr>
<tr>
<td>7358</td>
<td>3D</td>
<td>G (S)</td>
</tr>
<tr>
<td>7458</td>
<td>3D</td>
<td>C (H)</td>
</tr>
</tbody>
</table>

van Nimwegen *et al.*, 1999; Fontana, 2002; Fernández & Solé, 2007). In a simple but realistic scenario for theoretical studies, folding of RNA has been used as the phenotype, and evolution towards a different phenotype was preceded by drift in a vast neutral space of primary sequences (Schuster, 1997; Fontana & Schuster, 1998). What the results of FMDV persistence suggest is that, when the requirement of a genetic entity (in this case FMDV) is to reach a very complex set of phenotypic traits, needed to replicate and survive in a changing and increasingly hostile cellular environment, some specific genomic residues may be constrained. Thus, the degeneracy of sequence space when mapping a single phenotype such as RNA folding may be greatly decreased when mapping interconnected phenotypic traits.

At the phenotypic level, the need to cope with multiple constraints may be reflected in a number of deterministic features, notably variation in production of infectious virus and imbalances in the proportion of positive- versus negative-strand FMDV RNA, which occurred at virtually the same passage numbers of R-B and R-D cells (Figs 1a and 2a). Both cells and virus influenced the proportion of positive- versus negative-strand FMDV RNA (Fig. 2b), a feature which may have coevolved as a means to modulate the extent of viral RNA replication. A 310- to 820-fold excess of RNA of positive polarity over negative polarity has been quantified in the cell culture supernatant of cytolitic infections with FMDV C-S8c1, MARLS or H5 (results not shown), in agreement with determinations with other positive-strand RNA viruses (Cunningham *et al.*, 1990; Novak & Kirkegaard, 1991; Komurian-Pradel *et al.*, 2004). In several persistent infections, however, similar levels of positive- and negative-strand viral RNA have been observed (Cunningham *et al.*, 1990; Tam & Messner, 1999; Hohenadl *et al.*, 1991; Andreoletti *et al.*, 1997).

Deterministic events during RNA virus evolution, which were not imposed by obvious external selective pressures (drugs, antibodies) have been previously observed in competitions between a vesicular stomatitis virus wild-type clone and a neutral mutant derivative (Quer *et al.*, 1996, 2001). These studies defined the concept of contingent neutrality, meaning that, despite its neutrality, the mutant was more vulnerable to mutation than its parental clone. These results represented an experimental counterpart of the concept of ‘advantage of the flattest’ (advantage of a variant lying on a flat fitness surface) established with digital organisms (Wilke *et al.*, 2001). Another instance of determinism was the synchronous loss of memory genomes in parallel lineages of FMDV (Ruiz-Jarabo *et al.*, 2003). The initial formulation of quasispecies was deterministic, as are many initial theoretical treatments to place a problem in solvable mathematical terms, and determinism necessitated
an infinite population size of replicons (Eigen & Schuster, 1979). It could be inferred that a deterministic behaviour of a real viral quasispecies would be observed with higher probability when large viral population sizes are involved. However, during FMDV persistence, very low population sizes preceded the passages at which deterministic behaviour was manifested (passage 45 in Figs 1 and 2). In this case, determinism was probably imposed by selective demands of a biological environment acting on highly dynamic FMDV mutant repertoires since the early stages of persistence (Martín-Hernández et al., 1994).

Thus, if the tape of evolution were played again from the remote past, would it turn out similar to or different from what we know (Gould, 1989)? In comparing the unavoidably very fragmentary information that we have on major evolutionary transitions [the RNA world about 4 × 10^6 years ago (Eigen, 1992; Orgel, 2004) and the Cambrian explosion about 5 × 10^8 years ago (Gould, 1989; Conway Morris, 1998)] a major difference that could influence the weight of contingency is complexity of forms. Evolved, complex biological forms, as cells and viruses are, may be highly constrained to yield a specific biological solution when faced with a mutual interaction. The comparison of our results with previous tests of recurrence during RNA evolution in vitro suggests that, when a complex biological system has survival as its critical requirement, as is the case for cells and the resident virus during persistence, phenotypic alterations may be channelled towards a unique, reproducible outcome. In contrast, because of the larger degeneracy of the neutral sequence space (to reach alternative phenotypes) in primitive self-organized systems (such as in the RNA world; Eigen, 1992), the latter could have evolved towards alternative solutions in an unpredictable manner. If we are correct, the answer to S. J. Gould’s fundamental question would depend on how ‘remote’ the remote past is.

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