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Evolution of a Persistent Aphthovirus in Cytolytic Infections: Partial Reversion of Phenotypic Traits Accompanied by Genetic Diversification

NOEMI SEVILLA AND ESTEBAN DOMINGO*

Centro de Biología Molecular "Severo Ochoa," Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

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Foot-and-mouth disease virus (FMDV) shows a dual potential to be cytolytic or to establish persistent infections in cell culture. FMDV R100, a virus rescued after 100 passages of carrier BHK-21 cells persistently infected with FMDV clone C-S8c1, showed multiple genetic and phenotypic alterations relative to the parental clone C-S8c1. Several FMDV R100 populations have been subjected to 100 serial cytolytic infections in BHK-21 cells, and the reversion of phenotypic and genetic alterations has been analyzed. An extreme temperature sensitivity of R100 reverted totally or partially in some passage series but not in others. The small-plaque morphology reverted to normal size in all cases. The hypervirulence for BHK-21 cells did not revert, and even showed an increase, upon cytolytic passage. Most of the mutations that had been fixed in the R100 genome during persistence did not revert in the course of cytolytic passages, but the extended polyribocytidylate tract of R100 (about 460 residues, versus 290 in C-S8c1) decreased dramatically in length, to the range of 220 to 260 residues in all passage series examined. In passages involving very large viral populations, a variant with two amino acid substitutions (L-144→V and A-145→P) next to the highly conserved Arg-Gly-Asp (RGD motif; positions 141 to 143) within the G-H loop of capsid protein VP1 became dominant. A clonal analysis allowed isolation of a mutant with the single replacement A-145→P. Viral production and growth competition experiments showed the two variants to have a fitness very close to that of the parental virus. The results provide evidence that the repertoire of variants that could potentially become dominant in viral quasispecies may be influenced by the population size of the evolving virus. The net results of a series of persistent-infection passages followed by a series of cytolytic passages was progressive genomic diversification despite reversion or stasis of phenotypic traits. Implications for the evolution of RNA viruses are discussed.

Foot-and-mouth disease virus (FMDV) is an important animal pathogen which belongs to the aphthovirus genus of the *Picornaviridae* family (46). FMDV causes usually an acute, systemic infection of cloven-hooved animals, and it is cytolytic in cell culture. Occasionally, however, FMDV can establish persistent infections in ruminants (30, 36, 47, 49, 51) and in cell culture (9, 13, 23). Persistence of a clone of FMDV of serotype C (termed C-S8c1 [48]) in cloned BHK-21 cells (a population derived from a single cell) has been previously characterized (9, 10, 12, 13, 40). At the critical step of initiation of persistence, the determinant factor to ensure cell survival and sustained viral replication was the ability of the cells to rapidly become partially resistant specifically to FMDV (40). Once persistence was established, and upon serial passage of the carrier BHK-21 cells, the latter became progressively more resistant to FMDV C-S8c1 and the resident virus became gradually more virulent for BHK-21 cells (10, 12, 13, 40).

The virus rescued after 100 passages of the carrier cells, which is termed FMDV R100, showed a number of genetic and phenotypic alterations compared with the parental clone C-S8c1. The two viruses differed in about 1% of nucleotides in noncoding and coding regions (14, 27). The most dramatic genetic alteration seen in R100 was an extension of the polyribocytidylate [poly(C)] tract from about 270 residues in C-S8c1 to about 420 residues in R100, the longest homopolymeric tract described for a viral genome (27). A pyrimidine transition at the base of loop 3 of the internal ribosome entry site (IRES) led to a 1.5- to 5-fold increase in translation initiation activity of R100 relative to C-S8c1, suggesting that this mutation could contribute to the hypervirulence of R100 for BHK-21 cells (38, 39). Other phenotypic changes of R100 relative to C-S8c1 were attenuation for cattle and mice (15), small-plaque morphology (9), and at least in R55 (a virus ancestor of R100, shed by carrier cells at an earlier stage of persistence), considerable temperature sensitivity (9).

The ability of FMDV to produce both cytolytic and persistent infections in the same cells offered an opportunity to study the stability of the genetic and phenotypic alterations of the persistent R100 upon cytolytic passage. Six parallel passage series of R100 have been analyzed with regard to nucleotide sequences at noncoding and coding regions, the length of the poly(C) tract, and several phenotypic traits. We chose to analyze the virulence for BHK-21 cells, plaque morphology, and temperature sensitivity of the different populations. Here we report the results of such analyses and discuss their relevance to RNA virus evolution.

MATERIALS AND METHODS

Cells viruses and infections. The origins of the cloned BHK-21 cells and of plaque-purified FMDV C-S8c1, as well as procedures for growth of FMDV in liquid culture and plaque assays, have been previously described (18, 48). BHK-21 cell lines persistently infected with FMDV C-S8c1 were characterized by de la Torre et al. (9, 10, 12, 13). The persistent FMDV R100 was characterized by Díez et al. (14). Serial cytolytic infections with R100 were carried out initially in triplicate by infecting a monolayer of 2×10^6 to 4×10^6 BHK-21 cells with about 2×10^5 PFU of R100 (Fig. 1). Progeny virus was used to infect a fresh cell monolayer in the same way, and the process was repeated a total of 60 times.

* Corresponding author. Mailing address: Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Cantoblanco, 28049 Madrid, Spain. Phone: 34-1-397 84 85. Fax: 34-1-397 47 99. Electronic mail address: edomingo@mvax.cbm.uam.es.

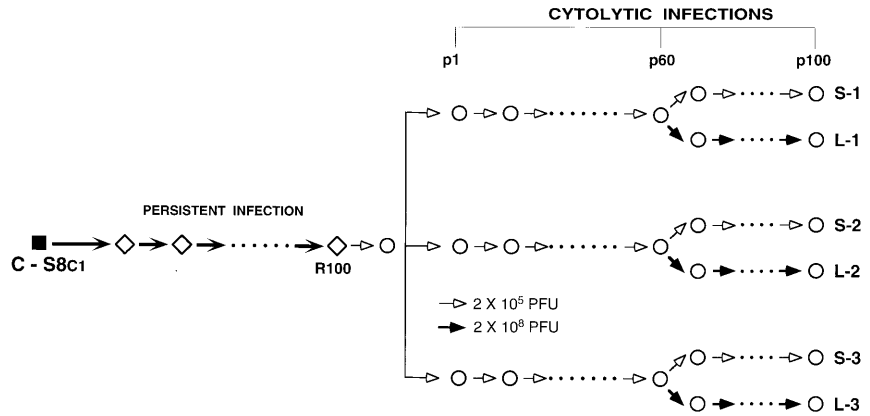

FIG. 1. Scheme of the passage history of FMDV populations analyzed in this study. A preparation of plaque-purified FMDV C-S8c1 (■) was used to establish a persistent infection of BHK-21 cells (◇) as described in reference 9. R100, the virus shed by Rp100 cells, was used to initiate three series of infectious involving 2×10^5 PFU per passage (—▷), and, from passage 61, a parallel series involving 2×10^8 PFU per passage (—▶). Procedures for infections of cell monolayers and plaque assays are described in Materials and Methods.

Then, each lineage was split in two. In one, serial infections were continued up to passage 100, using the same infection conditions. In the second lineage, however, each passage involved the infection of about 2×10^8 cells with about 2×10^8 PFU of virus. This type of infection, intended to minimize the bottleneck effect (limitation of viral population size) per passage, was continued up to passage 100 (Fig. 1). Mock-infected cells were maintained in parallel with no signs of cytopathology at any passage.

Phenotypic assays. Plaque morphology and temperature sensitivity were determined as previously described (9), and details are given in the relevant table footnotes. Virulence for BHK-21 cells was measured by determining the minimum number of PFU required to kill 10^4 BHK-21 cells.

Nucleotide sequencing and isolation of the poly(C) tract. For reverse transcriptase PCR (RT-PCR), the IRES, the L-protease-coding region, and the capsid-coding region of FMDV were copied into cDNA by using reverse transcriptase and then amplified by using *Taq* polymerase, using a number of oligonucleotide primers which have been previously described (2, 27). Some oligonucleotide primers were used for the first time in this work (Table 1). RT-PCR products were sequenced by the fmol method (Promega).

The genomic poly(C) tract of FMDV was isolated by digestion of purified virion RNA (10 ng) with 0.5 U of RNase T_1 (Calbiochem) in 24 μl of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA for 30 min at 37°C. Separation of the poly(C) tract by gel filtration, labeling with [γ-^{32}P]ATP and polynucleotide kinase, and determination of the molecular size of the poly(C) tract on urea-polyacrylamide gels were carried out as previously described (27).

RESULTS

Fixed and variable phenotypic traits upon passage of FMDV R100 in serial cytolytic infections. FMDV R100, the virus rescued from C-S8c1 carrier BHK-21 cells at passage 100 (13, 14), was passaged in cytolytic infections of BHK-21 cells as depicted in Fig. 1. This yielded viral populations R100 p100 S-1, R100 p100 S-2, R100 p100 S-3, R100 p100 L-1, R100 p100 L-2, and R100 p100 L-3. To explore whether phenotypic alterations that distinguished R100 from C-S8c1 had reverted in these viral populations, the temperature sensitivity, plaque morphology, and hypervirulence for BHK-21 cells were compared for C-S8c1, R100, and each of the six passaged viral populations.

The temperature sensitivity of R100 had not been previously analyzed, but its precursors FMDV R54 and R55 (the viruses shed by carrier BHK-21 cells at cell passages 54 and 55, respectively) showed a 200-fold decrease in viral yield at 42°C relative to FMDV C-S8c1 (9). Comparison of the yield of C-S8c1 R100 in infections of BHK-21 cells at 37 and 42°C revealed an extreme temperature sensitivity of R100, with viral yields of R100 about 10^5-fold lower than those of C-S8c1 at 42°C (Table 2). The temperature sensitivity of R100 did not revert to the same extent in the different series of infections. Partial reversion occurred in R100 p100 S-1 but not in R100 p100 S-2 or R100 p100 S-3 (Table 2). In contrast, partial reversion occurred in all of the L series, and population R100 p100 L-1 showed nearly normal yields at the restrictive temperature. The larger extent of reversion seen upon large-population passages than upon small-population passages of R100 (Table 2) suggests that the temperature sensitivity of R100 and its reversion may depend on two or more mutations.

TABLE 1. Oligonucleotide primers designed to amplify and sequence FMDV RNA

Genomic region	Oligonucleotide	Sequence (5′→3′)	Orientation	Position[a]
5′ untranslated	NR4	CACGATCTAAGCAGGTTTCC	Sense	246 (P, S)
L protease	LR1	GCTGTGGTAAACGCCATCA	Sense	742 (P)
	LR2	GGACAGGAACACGCTGTCT	Sense	1150 (P)
	LD1	GGGAACTCGAAAGCGA	Antisense	1322 (P, S)
P1	4R1	ACTGGCAGCATAATTAAC	Sense	52 (P, S)
	2R1	CTAGAGACGCGCGTTC	Sense	406 (P)
	2R2	GATGGGCGACATCAGT	Sense	638 (S)
	2R3	CAGGTGCCCAACAGATC	Sense	829 (S)
	3R1	ACACAGTACACCGGGAC	Sense	1201 (P, S)
	JD5	GCATGCGGTGGTACATGGC	Antisense	1263 (P, S)
	1R1	GCGCCACACCGTGTGTT	Sense	1927 (S)

[a] The 5′-terminal nucleotide of the primer. Each genomic region is numbered independently, as described in references 14 and 27. P, primer used for PCR amplification; S, primer used for nucleotide sequencing. Oligonucleotides were synthesized by Isogen Bioscience bv (Amsterdam, The Netherlands).

TABLE 2. Phenotypic traits of FMDV populations

FMDV	Plaque size[a] (diam, mm)	Temp sensitivity			Virulence for BHK-21 cells[b]
		Virus yield[c] (PFU/ml)		Ratio, 42°C/37°C	
		37°C	42°C		
C-S8c1	1.5–2	$(8 \pm 2) \times 10^6$	$(7 \pm 2) \times 10^5$	9×10^{-2}	5×10^5
R100	0.5–1	$(6 \pm 0) \times 10^6$	<5	$<10^{-6}$	5×10^2
R100 p100 S-1	2–4	$(5 \pm 1) \times 10^6$	$(9 \pm 7) \times 10^3$	2×10^{-3}	5
R100 p100 S-2	2–4	$(2 \pm 0) \times 10^7$	<5	$<2 \times 10^{-7}$	7
R100 p100 S-3	2–5	$(3 \pm 1) \times 10^7$	<5	$<2 \times 10^{-7}$	12
R100 p100 L-1	2–3	$(4 \pm 2) \times 10^6$	$(2 \pm 1) \times 10^5$	5×10^{-2}	5
R100 p100 L-2	2–3	$(1 \pm 0) \times 10^7$	$(4 \pm 2) \times 10^4$	4×10^{-3}	3
R100 p100 L-3	2–4	$(3 \pm 1) \times 10^7$	$(5 \pm 1) \times 10^4$	2×10^{-3}	6

[a] In all R100 p100 S and L populations, a small proportion (<20%) of the plaques were about 1 mm in diameter. This plaque size heterogeneity was not investigated further.

[b] Minimum number of PFU required for complete cell killing, as determined by infecting 10^4 BHK-21 cells with serial dilutions of the indicated virus. Two independent experiments yielded identical results. For C-S8c1 and R100, virulence was also measured as described in reference 40 (see also Materials and Methods).

[c] Yield upon infection of 2×10^6 to 3×10^6 BHK-21 cells with each virus at a multiplicity of infection of about 1 to 2 PFU per cell at the indicated temperature. Titrations were at 37°C in triplicate as described in Materials and Methods. Temperature sensitivity was tested for all samples in three independent experiments with identical results.

Plaque size reverted to the size of C-S8c1 or slightly larger in all cases (Table 2). Hypervirulence could not be compared by quantification of the number of cell survivors in a standard infection (40), since R100 and all R100 p100 populations did not yield any survivors, indicating hypervirulence for BHK-21 cells. A new virulence assay (described in Materials and Methods) revealed that the number of PFU required for complete killing of the cells in a standard BHK-21 cell monolayer was 10^3-fold smaller for R100 than for C-S8c1 and 40- to 100-fold smaller for any R100 p100 population than for R100 (Table 2). This result shows that in spite of being generated during cell-virus coevolution in carrier BHK-21 cells, the hypervirulence of R100 not only did not diminish upon cytolytic passage but increased considerably in all populations tested.

Partial phenotypic reversion was accompanied of genetic diversification. The partial phenotypic reversion seen in R100 subjected to cytolytic passage could be concomitant with reversion of mutations present in R100. Alternatively, further genetic diversification could occur in spite of phenotypic reversion. Nucleotide sequences of the 5′ untranslated region and of the L-protease- and capsid-coding regions were determined for all populations under study. The results (Fig. 2) show that most mutations which distinguished FMDV R100 from C-S8c1 were maintained in all R100 populations passaged in cytolytic infections. The pyrimidine transition (U-340→C) fixed at the base of domain 3 of the aphthovirus IRES of R100, which is responsible of enhanced translation activity (IRES position −376 [38, 39]), was maintained in all S and L populations. True reversions were observed in two cases: mutation A-1285→G, which corresponds to L-190→E in the L_b-coding region of R100 p100 L-2 and L-3; and mutation A-2147→G in P1, which resulted in substitution D-194→G of VP1 (amino acid 716 of P1). The latter mutation restored a G found at this position in many other FMDV type C isolates (42). This is the only capsid position at which one amino acid found uniquely in R100 (14) reverted upon passage of the virus in cytolytic infections. Except for substitution V-686→I in VP1, which was not exposed in the outer or inner capsid surface, all capsid substitutions affect solvent-accessible residues, according to the three-dimensional structure of C-S8c1 (34). In addition to the true reversions, 8 and 9 to 11 mutations were found in R100 p100 S and R100 p100 L populations, respectively, relative to R100 (Fig. 2). In consequence, the genetic distance between C-S8c1 and any of the R100 p100 populations was 1.2- to 1.3-fold larger than the distance between C-S8c1 and R100 (Table 3).

Unique double substitution at the FMDV loop upon large-population passage of variant R100. In each of the triplicate passage series in which 2×10^8 PFU was used per infection, a virus with two substitutions, L-144→V and A-145→P (variant LV/AP) in the G-H loop of VP1 (P1 amino acids 666 and 667, respectively), became dominant (Fig. 3). None of the three parallel passages involving infections with 2×10^5 PFU of R100 resulted in substitutions at this loop.

To determine the kinetics of the increase in proportion of the double substitution during serial passage of R100, RNA samples from population R100 L-1 at passages 60, 70, 80, and 90 were subjected to RT-PCR and the relevant P1 region was sequenced. In the sequencing gel, bands diagnostic of the two replacements were visible at passage 90 but not at passages 60, 70, and 80. A finer analysis of sequences at passages 80, 82, 83, 85, 87, 90, 92, 94, 97, and 99 revealed that the proportion of the two substitutions in the population increased in a nearly parallel fashion and gradually (Fig. 4). Since sequence analysis of RNA from FMDV R100 p85 L-1 revealed about 50% of the band diagnostic of each mutation, R100 p85 L-1 was subjected to a clonal analysis in an attempt to distinguish whether the two substitutions occurred simultaneously on the same genome or one substitution preceded the other. Analysis of 27 biological clones yielded three clones with A-145→P (variant AP) as the only substitution in the loop, three clones of the LV/AP group, and 21 clones indistinguishable from the parental FMDV R100. An additional 19 clones isolated from population R100 p90 L-1 yielded 13 LV/AP clones and six clones indistinguishable from R100. Finally, 10 clones from R100p80 L-1 had a loop sequence indistinguishable from that of R100, as expected from the average nucleotide sequence of R100 p80 L-1 (Fig. 4). Thus, the clonal analysis allowed the isolation of a variant FMDV with replacement A-145→P but not with L-144→V alone. This finding suggests either that the latter substitution in the absence of replacement A-145→P burdened the virus with a selective disadvantage or that the chance occurrence of mutations generated replacement A-145→P first, followed very rapidly by L-144→V. Substitution A-145→P did not require the short-term occurrence of L-144→V, since 20 serial infections of BHK-21 with a cloned variant AP did not result in any additional G-H loop substitution.

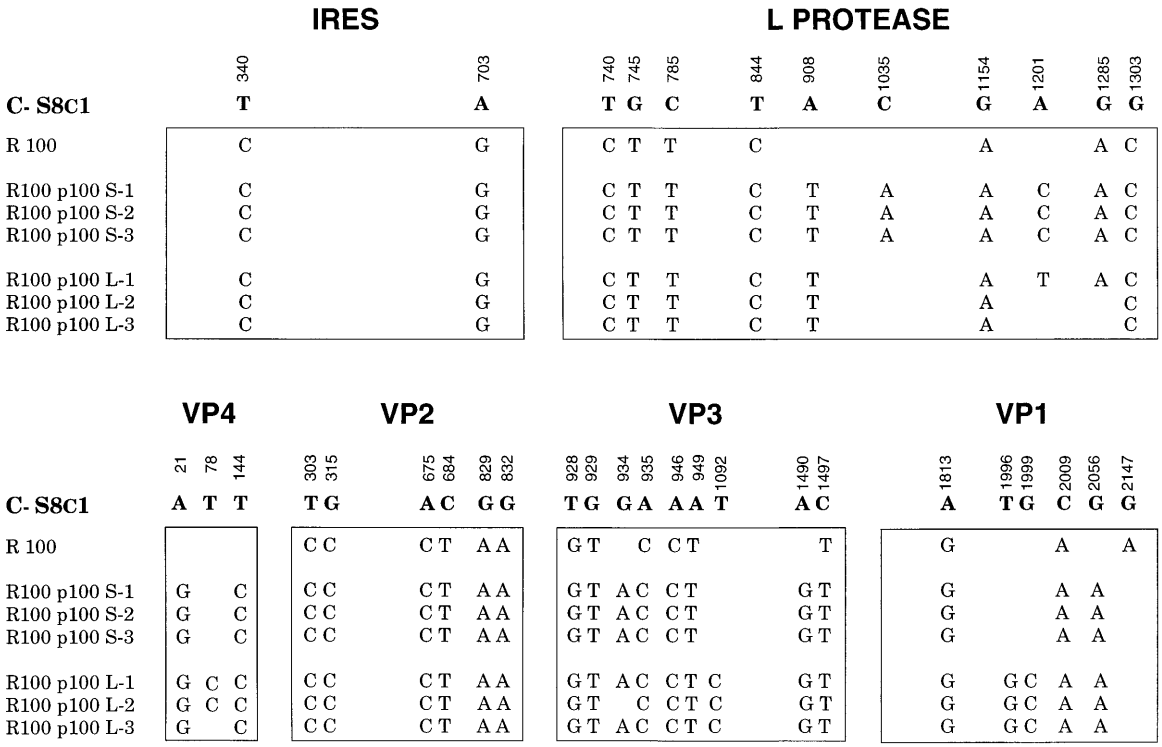

FIG. 2. Comparison of nucleotide sequences at three genomic regions of FMDV C-S8c1, R100, and R100 populations passaged in serial cytolytic infections. The origins of the viruses listed on the left are given in Materials and Methods and Fig. 1. The nucleotides of the IRES and of the L-protease-coding region are numbered consecutively as in reference 40, and those of the P1 region are numbered independently of L, and consecutively, as described in reference 14. The nonsynonymous mutations led to the following amino acid substitutions (given in parentheses, using the single-letter amino acid code) in L protease (amino acids numbered such that position 1 is the first methionine of the L_{ab} form of L): T-740→C (I-8→T), G-745→T (V-10→L), C-785→T (P-23→L), T-844→C (S-43→P), A-908→T (D-64→V), C-1035→A (N-106→K), G-1154→A (R-146→Q), A-1201→C (I-162→L), A-1201→T (I-162→F), G-1285→A (E-190→K), and G-1303→C (V-196→L). Mutations and amino acid substitutions in capsid proteins (amino acids numbered consecutively, with position 1 corresponding to the amino-terminal residue of VP4 as in reference 14) were G-829→A (A-277→T), G-832→A (G-278→S), T-928→G and G-929→T (C-310→V), G-934→A and A-935→C (D-312→A or D-312→T), A-946→C (N-316→H), A-949→T (M-317→L), A-1490→G (D-497→G), A-1813→G (T-605→A), T-1996→G (L-666→V), G-1999→C (A-667→P) (C-1999, and the corresponding amino acid P-667, was not completely dominant in R100 p100 L populations and coexisted with the residue of the parental R100 [14]), C-2009→A (T-670→K), G-2056→A (V-686→I), and G-2147→A (G-716→D).

Similar replication abilities of FMDV R100 variants harboring substitutions near the RGD motif. The dominance of variant LV/AP suggested that it may have a selective advantage over variant AP. The kinetics of extracellular production and intracellular accumulation of virus revealed no detectable differences between FMDV R100 and variants LV/AP or AP (data not shown). To test whether LV/AP manifested a selective advantage in direct competition with AP, each of the three LV/AP clones was mixed with an equal amount of one of the AP clones, chosen at random, to produce four competition mixtures. One of them was further divided in three series, giving a total of six competition series (Fig. 5). Sequence analysis at passages 10 and 20 revealed dominance of LV/AP in three cases and coexistence of the two variants in three cases. Since the triplicate series with the same starting clonal mixture produced dominance of LV/AP only in two series, the results suggest that the two variants are very similar with respect to fitness and that the random occurrence of mutations, or other chance events, in the course of the competition could modify the outcome of the competition. Since in no case did variant AP become dominant, the results do not exclude the possibility that mutants harboring the double substitution in the loop have a slight selective advantage over genomes harboring only the A-145→P replacement. This would not imply that the loop substitutions are directly responsible for the differences observed (see Discussion) but would provide an interpretation of

TABLE 3. Comparison of genomic RNAs of variant FMDVs

FMDV	Genetic distance (%)[a]		Length of the poly(C) tract (nt)[b]
	C-S8c1	R100	
C-S8c1		0.78	289–292
R100	0.78		463–474
R100 p100 S-1	1.00	0.28	227–241
R100 p100 S-2	1.00	0.28	236–242
R100 p100 S-3	1.00	0.28	241–246
R100 p100 L-1	1.03	0.31	234–245
R100 p100 L-2	0.97	0.31	240–251
R100 p100 L-3	0.97	0.31	225–230, 254–260[c]

[a] Percent nucleotide substitutions between C-S8c1 or R100 RNA and the RNA of the FMDVs indicated in the first column, applying the Kimura two-parameter model (33). Each value was derived by comparison of a total of 3,230 nucleotides corresponding to the 5′ noncoding and the L- and P1-coding regions. The mutations on which the calculations are based are depicted in Fig. 2.

[b] Determined by polyacrylamide-urea gel electrophoresis using a DNA sequence ladder as a size marker as previously described (27). Since the electrophoretic mobility of the poly(C) ladder is not identical to that of the DNA ladder, a correction factor of 1.11 was used to determine the length of the poly(C) tract (length given here equals the length seen on the gel as given by the DNA ladder × 1.11 [27]). The lengths of the poly(C) tracts determined for C-S8c1 and R100 by Escarmís et al. (27) were about 270 and 420 nucleotides (nt) instead of 289 to 292 and 463 to 474, respectively, found in this work. The reason for this difference has not been investigated but could be due to the use of different viral preparations in the two studies.

[c] This FMDV RNA was a mixture of genomes with poly(C) tracts of different mobilities (see Fig. 3).

	136				141		143	144	145				150
C - S8c1	Y	T	A	S	A	R	G	D	L	A	H	L	T	T	T
R100													K		
R100p100 S (-1, -2, -3)													K		
R100p100 L (-1, -2, -3)									V (P)				K		
R100p100 L-1, c1 - c10									V P				K		
R100p85 L-1									(V)(P)				K		
R100p85 L-1, c1 - c3									P				K		
R100p85 L-1, c4 - c6									V P				K		
R100p85 L-1, c7 - c27													K		

FIG. 3. Alignment of G-H loop sequences of VP1 of FMDV C-S8c1, its persistent derivative R100, and various populations resulting from cytolytic passage of R100. The origins of the different viral populations are described in Materials and Methods. Sequences from individual biological clones derived from populations R100 p100L-1 (c1-c10) and R100p85L-1 (clone 1 [c1] to clone 27) are boxed. An amino acid in parentheses indicates a mixed population which included also the corresponding amino acid of C-S8c1, as judged from the band pattern in sequencing gels (see Results and Fig. 4).

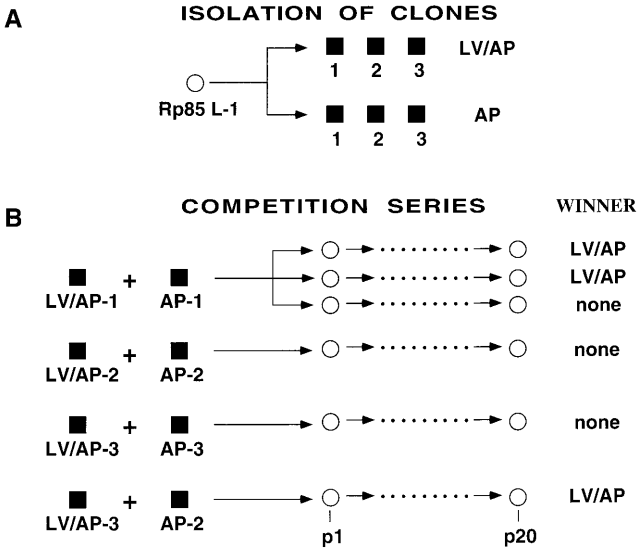

FIG. 5. Isolation of AP and LV/AP clones from FMDV population Rp85L-1 (A) and competition among them (B). Procedures for plaque isolations and for serial infections of BHK-21 cell monolayers are described in Materials and Methods. In these competitions among clones, each passage consisted in the infection of 5×10^6 BHK-21 cells with 5×10^5 PFU of virus. Sequence analysis was carried out by the fmol method after RT-PCR amplification of the relevant genomic region as described in Materials and Methods.

the dominance of LV/AP variants in the average population (Fig. 4).

The length of the poly(C) tract of R100 was greatly shortened upon cytolytic passage. A genetic difference that accompanied persistence of C-S8c1 was an increase in the length of the poly(C) tract from 275 residues in C-S8c1 to about 420 residues in R100 (27). To test whether cytolytic infections led to any change in the length of the poly(C) tract, the latter was measured for the parental R100 and each of the R100 p100 S and L populations. The results (Table 3 and Fig. 6) show a decrease in the length of the poly(C) tract from 463 to 474 nucleotides in R100 to about 220 to 260, depending on the population under consideration. This finding implies that virulence of FMDV for BHK-21 cells is independent of the length of the poly(C) tract, at least in the range of 220 to 470 residues. Thus, the transition from persistent to cytolytic infections of FMDV R100 was accompanied by reversion of several phenotypic traits, a dramatic shortening of the poly(C) tract, but no reversion of most other genetic changes in the 5′ noncoding and the L- and capsid protein-coding regions.

DISCUSSION

Fixed versus reversible phenotypic alterations. Some phenotypic traits that had been acquired by R100 in the course of its persistence in BHK-21 cells (small-plaque morphology and temperature sensitivity [9]) reverted completely or partially upon cytolytic passage (Table 2). This finding suggests that such traits in R100 were selected in the modified environment represented by the persistent infection. In contrast, the hypervirulence of R100 for BHK-21 cells was maintained, and further accentuated, upon cytolytic passage of R100 (Table 2). The most likely mechanism for sustained hypervirulence is its association with rapid completion of the infectious cycle and increased rate of cell death (13, 40). Any possible revertants

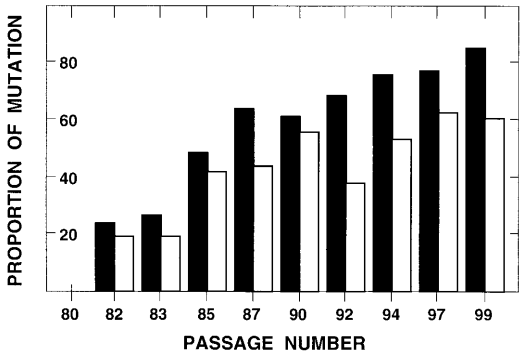

FIG. 4. Kinetics of the increase of proportion of mutations T-1996→G and G-1999→C of the P1-coding region in population R100 p100 L-1. The two transversions are diagnostic of amino acid substitutions L-144→V and A-145→P, respectively, in VP1. The proportion of each mutation was calculated from the densitometric tracings of the band pattern of a sequencing gel. Black bars, mutation T-1996→G; white bars, mutation G-1999→C.

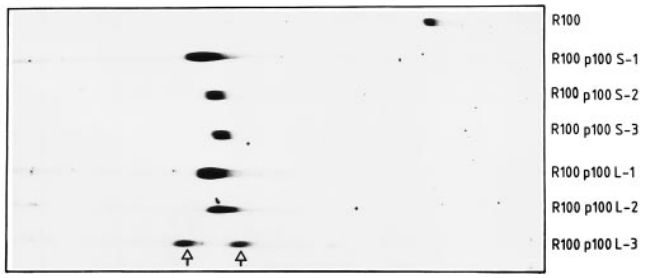

FIG. 6. Decrease in the size of the poly(C) tract upon cytolytic passage of R100. RNA from the indicated viruses was purified, digested with RNase T_1, and labeled with ^{32}P as described in Materials and Methods. Separation was by electrophoresis in a 6% polyacrylamide–8 M urea gel. A DNA sequencing ladder was run in the same gel (not shown) to determine the size of the poly(C) tracts (positions 463 to 474 for R100 and 225 to 260 for the R100 p100 S and L populations). Note the poly(C) tracts of different size in FMDV R100 p100 L-3; the arrows point to the tracts of 225 to 230 and 240 to 260 residues. The size of each poly(C) tract is listed in Table 3.

that were unable to complete an infectious cycle within the same time intervals as their hypervirulent parents would endure a very strong selective disadvantage and would be rapidly eliminated from the evolving population. Thus, although the hypervirulence of R100 was selected in response to an increased resistance of the carrier BHK-21 cells to FMDV C-S8c1 (10, 12), its nature confers a selective advantage to the virus beyond the particular environment in which the trait was first established. This hypervirulence constitutes an example of fixed phenotype, as opposed to reversible phenotypic traits which tend to be lost when the virus returns to its initial environment for replication.

Progressive genetic diversification at a limited number of genomic sites. Examination of genomic sequences in FMDV populations R100 p100 S and R100 p100L indicated that a large proportion of mutations occurred consistently in several of the parallel passage series (Fig. 2). We consider it highly unlikely that such sequence similarities were the result of cross-contamination among populations because several mutations occurred only in the L or S series, and some mutations were unique to one population. Also, the temperature sensitivity distinguished populations of the S series from those of the L series. Populations R100 p100 L-2 and L-3, which yielded similar amounts of virus at 42°C, differed in the length of the poly(C) tract: R100 p100 L-3 showed a unique doublet composed of two tracts different from that found in R100 p100 L-2 (Fig. 4). Furthermore, mutations consistently repeated in independent viral lineages have been previously found in FMDV (2, 32a, 40, 42) and in other picornaviruses. Examples are the poliovirus Sabin 1 vaccine strain passaged in vivo and in vitro (8, 35), wild-type Mahoney poliovirus type 1 during persistence in human neuroblastoma cell lines (6), and the precise reversion of four silent mutations introduced in independent clones of a temperature-sensitive mutant of poliovirus type 1 (11). The deterministic occurrence of the same or a limited repertoire of mutations in independent viral lineages replicating in the same environment is consistent with the restricted tolerance of the picornaviral capsid to accept amino acid substitutions as a result of functional and structural constraints (reviewed in references 21 and 50). In support of this suggestion is the high degree of acceptability of most amino acid replacements that distinguish R100 from R100 p100 S or L populations. Of a total of 10 different types of replacements, 8 had an acceptability of 4 or 5 according to the matrix of Feng et al. (28), which ranges from 0 (minimal acceptability) to 6 (replacement by the same amino acid). The proportion of nonsynonymous mutations in the L- and P1-coding regions was 70%, much greater than found in eukaryotic genes (44). This finding suggests either that R100 was subjected to positive selection during cytolytic passage or that restrictions to variation operated also at the RNA level (thus limiting the fixation of synonymous mutations) or both (20).

It has not been possible to distinguish whether phenotypic traits of FMDV reverted by true reversion of specific mutations or by additional, compensatory mutations. Whatever the mechanism involved, some phenotypes reverted while maintaining a limited but measurable genetic diversification. That is, even though R100 p100 S and L populations are genetically more distant from C-S8c1 than from R100, they are phenotypically closer to C-S8c1.

The length of the poly(C) tract is unrelated to the virulence of FMDV for BHK-21 cells. A possible association of the length of the poly(C) tract of FMDV with its virulence has been suggested (31). Our previous comparative analysis of the length of the poly(C) tract in R100 and C-S8c1 suggested that the increased length of this homopolymeric tract in R100 was compatible with hypervirulence for BHK-21 cells (27). The results reported in Table 3 clearly show that an extended poly(C) tract is not a requirement for such hypervirulence. The latter trait must be conferred by one or a combination of genomic mutations, the effects of which could be modulated, but not decisively determined, by the length of the poly(C) tract.

Population size and random mutational events in RNA virus evolution. The population size of the virus transmitted to susceptible cells has important effects on the fitness (or overall replication ability) of RNA viruses. Studies with bacteriophage $\phi 6$ (7) and with vesicular stomatitis virus (5, 25) showed that repeated plaque-to-plaque transfers (bottleneck events) led to stochastic, average fitness losses. In contrast, large-population passages of vesicular stomatitis virus resulted in exponential fitness gains (45). Likewise, nearly neutral or highly debilitated FMDV mutants rapidly gained fitness upon large-population infections in BHK-21 cells (2, 37). Plaque-to-plaque transfers isolate individual components of the quasispecies which tend to be suboptimal relative to the average (22, 26), whereas large-population passages favor competitive rating among variants and overall optimization of the quasispecies (26). The types of mutants that enter the competition, and thus the possible outcome of the competition, must be strongly influenced by the population size. With average mutant frequencies of about 10^{-4} mutations per nucleotide (4, 19, 24), infections involving 2×10^8 PFU per passage (as in the L series) incorporate a larger proportion of double and multiple mutants than infections involving 2×10^5 PFU (as in the S series). We suggest that FMDV with the double substitution in the loop was favored, in part for stochastic reasons, by the large population size of the virus involved in the L series of infections. Such double loop substitution was not found in the S-passage series or in any of 25 previously analyzed passage series which involved 10^4 to 10^5 PFU of C-S8c1 (2, 16, 32a). The occurrence of the double loop substitution cannot be attributed to a larger number of replication rounds undergone by FMDV in the L populations. In fact, because of the larger multiplicity of infection in the L series than in the S series, the number of replication rounds per passage was larger in the S series than in the L series. In the course of natural viral infections, the total viral load in an infected organism will increase whenever virus clearance is impaired, such as in cases of immunodeficiency and some types of nutritional deficiencies. The increase in population size will expand the repertoire of virus mutants in the quasispecies and thus the evolutionary potential of the virus.

The similarity in fitness of AP and LV/AP variants, indicated by the outcome of direct competitions among clones (Fig. 5), renders very difficult a statistical determination of a possible selective advantage of LV/AP over AP. A very large number of competitions would be required to try to substantiate that the two loop substitutions together, and no other genetic variations in the evolving genomes, were truly responsible for a selective advantage of LV/AP over AP variants. It cannot be excluded that under high-population passage conditions, LV/AP may manifest a stronger selective advantage over AP. What the results of growth competition between LV/AP and AP variants (Fig. 5) suggest is that loop replacements L-144→V and A-145→P, individually or together in the same VP1 molecule, must be well tolerated by these FMDV variants. This is remarkable in view of the evidence of the involvement of the RGD and neighboring residues in cell recognition (1, 29, 32, 41, 43) and the high conservation of positions 141 to 145 and 147 of the loop in type C viruses (19). In particular, recent results of inhibition of infectivity of FMDV C-S8c1 by synthetic peptides representing the VP1 G-H loop have shown that

replacements L-144→V and A-145→P diminished about 10- and 100-fold, respectively, the inhibitory activity of the peptide (43). In spite of such significant effects, it is clear that variants with replacement A-145→P could be isolated from population Rp85 L-1 (Fig. 3) and that they displayed an infectivity very similar to that displayed by R100 or variants LV/AP. Substitution L-144→V was not identified in the clonal analysis of R100 p85 and p90 L-1 populations (Fig. 5), nor it has been found among many monoclonal antibody-resistant or other C-S8c1-derived populations. However, a mutant including this amino acid substitution has recently been isolated from an FMDV population with a different passage history (36a). This result indicates that variants with L-144→V are also viable and supports the proposal that their absence in R100 p85 and R100 p90 L-1 populations was due to the chance occurrence of A-145→P prior to L-144→V.

Alternation of environments must be frequent in the replication cycle of viruses. In the case of animal infections with FMDV, the most frequent portal of entry is the upper respiratory tract, where localized virus replication occurs (3). This is followed by febrile viremia and colonization of the epithelium of vascular dermis, leading to vesicle formation (17, 52). This sequence of invasions of target cells is repeated after each transmission event, originating conflicting selective forces which, according to the results reported here, could result in limited phenotypic change with uninterrupted genetic diversification.

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