**Preextinction Viral RNA Can Interfere with Infectivity**

Claudia González-López, Armando Arias, Nonia Pariente, Gema Gómez-Mariano, and Esteban Domingo*

*Centro de Biología Molecular “Severo Ochoa,” Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*

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When the error rate during the copying of genetic material exceeds a threshold value, the genetic information cannot be maintained. This concept is the basis of a new antiviral strategy termed lethal mutagenesis or virus entry into error catastrophe. Critical for its success is preventing survival of residual infectious virus or virus mutants that escape the transition into error catastrophe. Here we document that mutagenic foot-and-mouth disease virus (FMDV) RNA can interfere with and delay viral production up to 30 h when cotransfected in BHK-21 cells with standard RNA. Interference depended on the physical integrity of preextinction RNA and was not observed with unrelated RNAs or with nonmutated, defective FMDV RNA. These results suggest that this type of interference requires large size, preextinction FMDV RNA and is mediated neither by small interfering RNAs nor by RNAs that can compete with infectious RNA for host cell factors. A model based on the aberrant expression of mutated RNA as it is expected to occur in the initial stages of the transition into error catastrophe is proposed. Interference mediated by preextinction RNA indicates an advantage of mutagenesis versus inhibition in preventing the survival of virus escape mutants during antiviral treatments.

High mutation rates and quasispecies dynamics (17, 21, 23) confer great adaptability to RNA viruses and represent a major obstacle for the prevention and control of RNA viral diseases (17, 25). However, theoretical studies have provided evidence that for any replication system there is a maximum error rate compatible with maintenance of the information encoded in the replicating genome (2, 22, 23, 37, 46). The larger the complexity of the genome, the higher the copying fidelity needed to maintain the encoded information. The irreversible transition into loss of genetic information is termed entry into error catastrophe, and the critical average error rate at the transition point is the error threshold (2, 22, 23, 37, 46). This concept has encouraged research in a new antiviral strategy termed lethal mutagenesis (12, 22, 29, 32). In the case of viruses, crossing the error threshold should result in a transition from a productive to an abortive infection. Virus extinction associated with enhanced mutagenesis has been documented with a variety of virus-host systems (1, 12, 13, 27, 29, 31–34, 39, 43, 44; for reviews, see references 16, 22, and 26), including prevention of the establishment of a persistent lymphocytic choriomeningitis virus (LCMV) infection in vivo (42).

Studies with the animal picornavirus foot-and-mouth disease virus (FMDV) in cell culture have established that virus extinction through enhanced mutagenesis is favored by low viral load and low viral fitness (44). As a consequence, combination treatments involving a mutagenic agent and antiviral inhibitors were more effective than a mutagenic agent alone in driving FMDV to extinction (39). When the mutagenic activity was insufficient for a given fitness level of FMDV, inhibitor-resistant, extinction-escape mutants of FMDV were selected (38). Selection of inhibitor-escape mutants of viruses is a major cause of failure of antiviral treatments based on the administration of inhibitors (15, 20, 35, 41). Quasispecies dynamics suggest an experimentally testable advantage of an error catastrophe-based antiviral approach, consisting of the adverse effect of a highly-mutated-mutant spectrum on any residual viral infectivity remaining in the infected cell. De la Torre and Holland (14) showed that a standard mutant spectrum of vesicular stomatitis virus (VSV) could suppress the infectivity of VSV mutants of superior fitness. We hypothesized that this effect should also occur with a mutant spectrum on its way to extinction and that such a spectrum should hinder progression of infection by residual nonmutated RNA. Here we report RNA cotransfection experiments that show that preextinction FMDV RNA interferes with infectious FMDV RNA. Interference was not observed with unrelated RNAs, with an unmutated defective FMDV RNA with a deletion of 417 nucleotides, or with the same preextinction RNA reduced in size by mild alkaline treatment. The results identify an advantage of mutagenesis as an antiviral strategy through suppression of residual infectivity by complex mutant spectra produced near the transition into error catastrophe.

**MATERIALS AND METHODS**

**Cells, viruses, and infections.** The origin and growth conditions of BHK-21 cells have been previously described (18, 45). FMDV C-S8c1 is a biological clone of natural isolate C-Sta Pau-Spain 70, obtained as previously described (45). Infections in liquid culture medium and plaque assays in semisolid agar medium were done as previously described (18, 45). Mock-infected cells and serial infections with supernatants of mock-infected cells were maintained in parallel during FMDV infections to control for the absence of viral contamination.

**Infectious center plaque assay.** Different amounts of C-S8c1 RNA (10^7 to 10^11 molecules) and preextinction RNA genomes (10^7 to 10^10 molecules) were elec-
troptorated into 1.2 × 10^7 BHK-21 cells. Electroporated cells were serially diluted in PBS, and each diluted cell suspension was added to 35-mm plates containing BHK-21 cells at 80% confluence and incubated at 37°C for 2 h in 7% CO₂. Then semisolid agar medium was added, and the cells were incubated for another 48 h before staining for plaque counting (5, 18, 45). Control experiments confirmed that, as expected, cellular RNA did not induce plaque formation.

Mutagenic and antiviral treatments. 5-fluorouracil (FU) was used as the mutagenic base analog, and guanidine hydrochloride (G) was used as the inhibitor of FMDV replication, as previously described (38, 39, 44). FU medium contained FU (200 μg/ml) and FUG medium contained FU (200 μg/ml) and G (4 mM). Solutions were sterilized by filtration and stored a maximum of 15 days. All media were supplemented with 2% fetal calf serum. The effect of medium with FU and FUG on cell viability and control experiments to ascertain that virus extinction was not due to the toxicity of FUG were described previously (39, 44). Infections in the presence of FUG were performed as previously described (37, 38). The passage number is indicated by p (i.e., C-S8c1 FUGp1 is passage 1 of FMDV C-S8c1 in FUG medium). To test for virus extinction, serial infections and blind passages in the absence of FU and G were carried out as previously described (39). The number of cells involved in infection and the multiplicity of infection for each experiment are indicated in the corresponding figure legends.

Viral and cellular RNAs. Viral RNA was extracted from the medium of infected or electroporated cultures as previously described (44) and quantitated with Light Cycler instruments (Roche) by using the Light Cycler RNA Master SYBR Green 1 kit (Roche) according to the manufacturer’s instructions with purified FMDV C-S8c1 RNA as a standard. Three FMDV genomic regions were subjected to RNA quantification: residues 3175 to 3518 (within the VP1-coding region), residues 4175 to 4473 (within the 3C-coding region), and residues 6610 to 6816 (within the 2C-coding region). Low-molar-mass preextinction viral RNA was obtained by incubation of preextinction RNA with 200 mM sodium bicarbonate (pH 9.0) for 30 min at 90°C and neutralized by addition of the same volume of 42 nM HEPES (pH 7.0). Radiolabeled pMTA417 RNA (described below) was treated in parallel to calibrate size reduction. The size of the RNA was in the range of 20 to 100 nucleotides, as measured by 6% acrylamide-urea gel electrophoresis. pMTA417 RNA is a transcript from a full-length defective FMDV RNA that has a deletion spanning residues 1153 to 1571 (9). A cDNA copy of FMDV Δ417 RNA was cloned into plasmid pGEM-1 and transcribed with SP6 RNA polymerase, as previously described (50; J. F. Garcia-Arriaza, E. Domingo, and C. Escarms, unpublished data). Human immunodeficiency virus (HIV) pol RNA is a 141-residue-long RNA transcribed by using T3 RNA polymerase (8) from plBlastscript SK (+) containing a 66-bp fragment spanning nucleotides 1629 to 1692 from the start of the HIV pol open reading frame (51). A mixture of 23S and 16S Escherichia coli rRNA (Roche) was used as a nonviral RNA control in coelectroporation experiments.

Cell electroporation. BHK-21 cell monolayers were detached, washed with electroporation buffer (21 mM HEPES [pH 7.0], 137 mM NaCl, 5 mM KCl, 0.7 mM NaHPO₄, 6 mM glucose), and introduced in a 0.4-cm cuvette (Bio-Rad). RNA (0.05 to 5.5 ng) was electroporated into 1.2 × 10^7 cells by applying a pulse of 280 V, 250 μF, and 400 Ω with a Bio-Rad gene pulser. Under these conditions, the proportion of BHK-21 cells expressing 3D antigen (detected with a monoclonal antibody 3H11 specific for FMDV 3D) at 18-h postelectroporation with 100 C-S8c1 RNA molecules per cell was 30%. Electroporated cells were incubated in 25-cm flasks in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum at 37°C in 7% CO₂ for 4 h. The medium was then removed and replaced by DMEM with 2% calf serum. At different times postelectroporation, aliquots of culture supernatant were assayed for the presence of FMDV or interferon.

Interferon assay. Intracellular and extracellular interferon (IFN) levels produced by BHK-21 cells transfected with preextinction RNA were quantitated by a cytopathic-effect inhibition assay using VSV infection of BHK-21 cells and murine α interferon (IFN) and L929 cells as the positive controls (11).

Molecular cloning and nucleotide sequencing. Nucleotide sequencing of reverse transcription (RT)-PCR products, molecular cloning into pGEM-4Z, and nucleotide sequencing of individual clones were carried out as previously described (3, 24, 38, 44). The regions sequenced corresponded to residues 3193 to 3869 (VP1-coding region) and 6609 to 8035 (3D-coding region). To ensure that sequences of individual clones represented different genomes from the mutant spectrum, an excess template for the RT-PCR amplification was ascertainment by amplification of dilutions of the template preparations as detailed by Airaksinen et al (1). Mutation frequencies were calculated as previously described (3, 24, 38, 44).

RESULTS

Kineton FMDV production upon electroporation with viral RNA. Preextinction FMDV C-S8c1 RNA was obtained by passage of the virus in the presence of FUG, as detailed in Materials and Methods (Fig. 1). The mutation frequency of its mutant spectrum was 3.9 × 10⁻⁴ and 7.1 × 10⁻⁵ substitutions per nucleotide for the VP1- and 3D-coding regions, respectively. These values were 2.6 and 3.3 times higher than those for the control populations passaged in parallel in the absence of mutagens. No differences between the consensus nucleotide sequences of preextinction RNA and those of the parental C-S8c1 RNA were observed. Mutation frequencies and the invariance of consensus sequences are in agreement with previous characterizations of preextinction FMDV RNA (39, 44). To compare the capacities of FMDV C-S8c1 RNA and of increasingly mutagenized C-S8c1 RNA to produce infectious progeny, equivalent amounts of RNA were electroporated into BHK-21 cells and the FMDV titters in the supernatant of electroporated cell cultures were determined at different times up to 45-h postelectroporation (Fig. 2). Based on an average of 50 preextinction RNA genome molecules per cell, no detectable FMDV production occurred up to 44-h postelectroporation (Fig. 2). Based on an average of 10² preextinction RNA genome molecules per cell, virus production was detected at 30-h postelectroporation, and the virus titer at 30- to 40-h postelectroporation was 10³-fold lower than the titer produced by an equal amount of C-S8c1 RNA (Fig. 2). This observed viral production is possible because of the absence of mutagens in the medium employed in these assays. At 50-h postelectroporation and at later times in which at least a second round of

FIG. 1. Preparation of preextinction FMDV RNA. BHK-21 cells (1 × 10⁷) were infected with 5 × 10⁶ PFU of FMDV C-S8c1 in either DMEM or FUG medium. Subsequent infections were carried out, infecting 1 × 10⁷ BHK-21 cells with 0.5 ml of the culture supernatant of the previous infection. Virus production was determined at each passage. The multiplicity of infection can be calculated from the virus titer shown in ordinate. The preextinction population (that preceding the population from which no infectivity or RT-PCR-amplifiable FMDV genomic material could be rescued after three blind passages in DMEM) (38, 39, 44) is indicated by an arrow. Titration was carried out in triplicate, and standard deviations (not shown) never exceeded 25%. The procedures are detailed in Materials and Methods.
infection of BHK-21 cells had taken place (45), differences in titers decreased substantially (data not shown). Progeny produced upon electroporation of C-S8c1FUGp1 RNA and C-S8c1FUGp2 RNA was intermediate between the production by unmutated, parental C-S8c1 RNA and preextinction RNA (Fig. 2). Thus, viral production upon electroporation of BHK-21 cells with FMDV RNA decreased as the viral population from which the RNA was obtained approached extinction.

Electroporation of $1.2 \times 10^6$ BHK-21 cells with $10^{10}$ molecules of C-S8c1 RNA produced a total of $10^9$ infectious centers, whereas no infectious centers could be detected after electroporation with the same amount of preextinction RNA. Thus, independent assays indicate a very low specific infectivity (PFU per genomic RNA molecule) of preextinction FMDV RNA, which in an infectious center assay was at least 10-fold lower than that of FMDV C-S8c1 RNA.

**Interference of preextinction RNA with the infectivity of C-S8c1 RNA.** To test whether a preextinction RNA population could exert a suppressive effect on the replication of standard FMDV C-S8c1 RNA, BHK-21 cells were electroporated with either C-S8c1 RNA or preextinction RNA or with mixtures of C-S8c1 RNA and either preextinction RNA or other related and unrelated RNAs in different proportions (Fig. 3). In three independent experiments, $1.2 \times 10^6$ C-S8c1 RNA molecules were electroporated into $1.2 \times 10^6$ BHK-21 cells, either alone or with $1.2 \times 10^7$ (Fig. 3A), $1.2 \times 10^8$ (Fig. 3B), or $1.2 \times 10^9$ (Fig. 3C) molecules of preextinction RNA or other related and unrelated RNAs. In all cases, the presence of preextinction RNA delayed by 30- to 40-h postelectroporation the production of viral progeny. At early times postelectroporation, viral production by the cells cotransfected with C-S8c1 RNA and preextinction RNA was similar or lower than viral production by preextinction RNA alone (Fig. 2 and 3). To gain insight into the possible mechanisms involved in interference by preextinction RNA, cotransfection with the same amounts of unrelated RNAs, namely E. coli rRNA and an HIV-1 transcript (described in Materials and Methods) was carried out in parallel. No interference was detected (Fig. 3), indicating that interference was not due to some nonspecific effect associated with perturbations derived from the penetration of foreign RNAs into BHK-21 cells. To determine whether competition for cellular factors could be the major determinant of interference, cotransfection of C-S8c1 RNA with a variant FMDV C-S8c1 RNA that included a 417-nucleotide deletion ($\Delta 417$, spanning FMDV C-S8c1 genomic residues 1153 to 1571 within the L-coding region) (9) was carried out. This defective RNA maintains the open reading frame to produce the FMDV polyprotein; its replication can be complemented by standard C-S8c1, is packaged into capsids, and in mixed infections can contribute VP1 protein for the particles of the complementing FMDV (9). No interference was observed with $\Delta 417$ RNA, suggesting that competition between viral RNAs for host cell factors is not a major determinant of the suppressive activity of preextinction RNA. To determine whether large preextinction RNA was necessary for the interfering activity, preextinction RNA was treated with a mild alkaline solution to produce a product heterogeneous in size, in the range of 20 to 100 nucleotides (details given in Materials and Methods). No interference was observed with equivalent proportions of small preextinction RNA compared with that for full-size preextinction RNA (Fig. 3). This result suggests that short RNA segments that could either provide small interfering RNAs, trigger unspecific cellular responses, or supply some inhibitor into the cells did not play a major role in interference by preextinction RNA. To ascertain that preextinction RNA did not elicit some unspecific antiviral state such as IFN production, the amount of intracellular and extracellular IFN in BHK-21 cells transfected with preextinction RNA was measured with a cytopathic-effect inhibition assay using VSV. The amount of IFN measured both intracellularly and extracellularly was $<1.5$ unit equivalents of murine IFN/ml, suggesting that neither IFN nor any other unspecific interfering activity was a significant component of the interference produced by preextinction FMDV RNA. We conclude that preextinction viral RNA can interfere with infectivity of viral RNA and that the interference is not triggered by unspecific responses of the cell to foreign RNA.
DISCUSSION

Genetic variation of RNA viruses through mutation, homologous and nonhomologous recombination, and genome segment reassortment constitutes a major difficulty for disease prevention and control (16, 17, 19). Difficulties arise from adaptability, which has its origins in the continuous production of mutant genomes, which favors a process of competition and selection of those mutant distributions best adapted to a given environment (17, 21). Quasispecies dynamics has several biological manifestations. Failure of an antiviral immune response to clear virus may be associated with the presence of antibody—or cytotoxic T cell—escape mutants of a virus (30, 47, 48, 52). Mutant spectra of viral quasispecies may also include mutants with altered cell tropism and host range, with obvious implications for viral disease emergence (for a review, see reference 4). Of direct relevance to therapy using antiviral inhibitors is the frequent (at times systematic) selection of inhibitor-resistant mutants (for a review, see references 15, 20, 35, and 41), which is a major cause of treatment failure, a justification for combination therapy (17, 28), and an incentive to investigate virus entry into error catastrophe as a new antiviral strategy (16, 22).

In virus population dynamics, there is a fundamental difference between antiviral approaches based on the inhibition of viral replication and those based on enhanced mutagenesis. In the former, virus mutant distributions showing decreased sensitivity to one or several inhibitors are endowed with a selective advantage in the presence of the inhibitors, such that, notwithstanding possible suppressive effects of mutant spectra (14, 49), they can build new, dominant mutant distributions manifesting some degree of resistance to the inhibitor(s). Despite possible fitness costs associated with the resistance mutations, further replication generates either additional mutations that strengthen the resistance phenotype or compensatory mutations that, while maintaining the same level of resistance to inhibitors, contribute increases in viral fitness (7, 36). In contrast, in the transition towards error catastrophe, either residual infectivity or, possibly, viruses showing resistance to the mutagens or inhibitors used (40, 53) encounter a highly complex mutant spectrum that may interfere with replication of viable RNA. In the preextinction populations obtained upon serial infections with FUG, the proportion of infectious RNA molecules was $2.5 \times 10^{-7}$ of the total, as determined by light-cycler RT-PCR (38).
Suppressive effects of mutant spectra were documented experimentally with VSV in cell culture (14), with variants of LCMV in vivo (49), and with live poliovirus vaccines (10). The interference of preextinction RNA on the replication of infectious RNA underlines an advantage of enhanced mutagenesis versus inhibition as an antiviral strategy. The observed interference was specifically associated with full-length, mutated, preextinction RNA, suggesting that it may be the expression (normal or aberrant) of altered proteins that jeopardizes the replication capacity of residual infectious RNA. The expression of altered proteins may occur through trans-acting functions such as those of modified capsid proteins that are not properly assembled with standard nonmutated proteins or modified proteases (L or 3C) that may compete with their wild-type counterparts for the processing of cellular factors or viral proteases (L or 3C) that may compete with their wild-type counterparts for the processing of cellular factors or modiﬁed single codon sites in vesicular stomatitis virus polymerases that may compete with standard nonmutated proteins acting functions such as those of modiﬁed single codon sites in vesicular stomatitis virus polymerases that may compete with standard nonmutated proteins.

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