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Contingent Neutrality in Competing Viral Populations

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The replicative fitness of a genetically marked (MARM-C) population of vesicular stomatitis virus was examined in competition assays in BHK-21 cells. In standard fitness assays involving up to eight competition passages of the mixed populations, MARM-C competes equally with the wild type (wt), but very prolonged competitions always led to the wt gaining dominance over MARM-C in a very slowed, nonlinear manner (J. Quer et al., J. Mol. Biol. 264:465–471, 1996). In the present study we show that a number of quite unrelated environmental perturbations, which decreased virus replication during competitions, all led to an accelerated dominance of the wt over MARM-C. These perturbations were (i) the presence of added (or endogenously generated) defective interfering particles, (ii) the presence of the chemical mutagen 5-fluorouracil (5-FU), or (iii) an increase in temperature to 40.5°C. Thus, the "neutral fitness" of the MARM-C population is contingent. We have determined the entire genomic consensus sequence of MARM-C and have identified only six mutations. Clearly, some or all of these mutations allowed the MARM-C quasispecies population to compete equally with wt in a defined constant host environment, but the period of neutrality was shortened when the environment was perturbed during competitions. Interestingly, when four passages of each population were carried out independently in the presence of 5-FU (but in the absence of competition), no significant differences were detected in the fitness changes of wt and MARM-C, nor was there a difference in their subsequent abilities to compete with each other in a standard fitness assay. We propose a model for this contingent neutrality. The conditions employed to generate the MARM-C quasispecies population selected a small number of mutations in the consensus sequence. It appears that the MARM-C quasispecies population has moved into a segment of sequence space in which the average fitness value is neutral but, under environmental stress, beneficial mutations cannot be generated rapidly enough to compete with those being generated concurrently by competing wt virus quasispecies populations.

Many concepts and principles of population genetics apply to RNA viruses (reviewed in references 7 and 8). Among them, the competitive exclusion principle, which states that in the absence of niche differentiation and with two species competing for limited resources, one of the species will eventually outcompete the other and become dominant in the population (12). In agreement with this principle, although competing vesicular stomatitis virus (VSV) quasispecies populations initially neutral coexisted for many generations, highly advantageous mutations occurred stochastically in the genome of one of the two competing quasispecies, resulting in the eventual displacement of the other (6). The same experiments provided support to another concept of population genetics, the Red Queen Hypothesis (33). The words of the Red Queen in Lewis Carroll's *Through the Looking Glass* are "it takes all the running you can do to keep in the same place." Analysis of competing populations showed that both the winners and the losers increased in fitness, but the winners did so at a higher level than the losers (6, 29).

In another series of independent competitions between a wild-type (wt) VSV and a neutral monoclonal antibody (MAb)-resistant mutant, termed MARM-C VSV, it was observed that the relative ratio between competitors remained constant for 8 to 10 passages, but after that the wt always displaced MARM-C, and the critical displacement transition occurred anywhere between passages 30 and 100 (29). Therefore, in contrast to other mutants of VSV employed in a previous study (MARM-D, -G, and -H) that exhibited stochastic displacements of either the wt or the MARM in the course of passages (6), MARM-C was consistently displaced in a nonlinear manner by wt parental virus (29). However, isolation of MARM-C and wt prior to extinction of the latter showed that, as in previous experiments, both populations gained fitness.

At least two possible mechanisms could be considered to explain the observation that MARM-C was prone to exclusion by the wt. One possible explanation is that, by chance, MARM-C lost fitness relative to the wt in up to 18 independent competitions, as predicted by the competitive exclusion principle of evolution (12). Another possible interpretation is that there may exist some type of genetic predetermination for MARM-C to be the loser in competition with wt, despite the apparent neutrality shown during short-term competitions. For instance,

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the quasispecies composition of MARM-C is unable to generate favorable mutations at a rate sufficient to compete successfully with wt quasispecies. In other words, MARM-C has a lower beneficial mutation rate than wt. The prediction is then that MARM-C will gain fitness at a lower rate than wt under other environmental conditions. Moreover, if the selective pressure is increased so there is a more imperative need for beneficial mutations, the extinction of MARM-C in competition with wt will be accelerated. In order to test this hypothesis, we introduced several environmental perturbations during the competitions, leading to increased selective pressure, including the presence of the mutagenic base analogue 5-fluorouracil (5-FU), defective interfering particles (DIPs), or high incubation temperature. All of them led to an accentuation of the selective disadvantage of MARM-C relative to wt. The entire genomic consensus sequences of each of these two viruses were obtained, which indicated that a few critical substitutions present in noncoding regions and in the polymerase open reading frame (ORF) are sufficient to promote this behavior. We suggest the concept of contingent neutrality to describe the situation in which a compromised constellation of mutations leading to temporary neutrality may limit the capability of a population to generate enough advantageous mutations quickly enough to outcompete related genomes.

MATERIALS AND METHODS

Cells and viruses. BHK-21 cells were grown as cell monolayers in Eagle minimum essential medium (MEM) containing heat-inactivated (60°C, 30 min) bovine calf serum. A wt population of VSV Mudd-Summer strain, Indiana serotype, and a genetically marked MAb-resistant mutant (MARM-C [29]) were used. The competing wt quasispecies population was previously replicated exclusively on BHK-21 cells, and it was stored frozen at −85°C. This wt stock was assigned a fitness value (*W*) of 1.0, to be used as a fitness internal reference point (16). MARM-C is a subclone of this wt stock, which was debilitated in fitness during 20 consecutive bottleneck (plaque-to-plaque) passages of a clone selected from wt as a MAb-resistant mutant (resistant to I1 MAb). The fitness of this MAb-resistant clone was then restored to neutrality by carrying out two large population passages in BHK-21 cells (transfer of 2×10^5 PFU at a multiplicity of infection of 0.1). MARM-C has an Asp→Ala substitution at amino acid 259 in the surface glycoprotein. This clonal MARM-C population exhibits a fitness value of 1.0 (neutral fitness) with respect to the competing parental wt population in short-term competitions (eight competition passages or fewer).

Virus passages and competitions in the absence of DI particles. Transfer of large virus populations and competition assays were carried out as previously described (10, 16). Briefly, samples of wt and MARM-C populations were mixed at a starting proportion of 1:1. From the same initial mixture, 12 independent competition series (labeled A through L) were performed. In each competition series, a monolayer of 2×10^6 BHK-21 cells was infected with approximately 2×10^5 PFU from the original mixture and incubated until the cytopathic effect was complete (about 24 h postinfection). Therefore, the average multiplicity of infection at each competition passage was about 0.1 PFU/cell. A higher multiplicity of infection may lead to accumulation of DI particles and possible interference with viral replication (15). MARM-C/wt ratios were calculated daily by triplicate plaque assay in the presence or absence of I1 MAb as described elsewhere (10, 16). Samples of each competition passage were frozen at −85°C for additional experiments.

Isolation of DIPs. DIPs were obtained after three consecutive undiluted passages of a mixture of MARM-C and wt at a very high multiplicity of infection (10^3 to 10^4 PFU/cell) as described by Doyle and Holland (9). DIPs from passage 3 were amplified using a wt clonal pool as a helper virus. A T-short (extremely deleted genome) DI particle was isolated by ultracentrifugation (35 min at 35,000 × *g* in a Beckman L5-50 rotor SW41-3128) in a continuous sucrose gradient (5 to 37% in 10 mM Tris-HCl–1 mM EDTA–0.1 M NaCl). The DIPs were recovered from the upper third of the gradient, resuspended in MEM, and frozen in small aliquots at −85°C. Samples were used for interference experiments as described elsewhere (17). The level of interference of the DIPs with MARM-C and wt clonal populations was measured as the titer decrease in the presence of different amounts of DI particles compared to parallel infections in the absence of DIPs. Similar interference values were obtained for both populations. A dilution of DI particles that gave 98% inhibition of VSV yields was used in the experiments described below.

Isolation of MARM-C and wt VSV from mixtures of viruses after competition passages. wt VSV was isolated (in the absence of MAb) after suitable dilutions of competition mixtures were plated on BHK-21 cells. The dilutions were made to ensure the presence of at least 50 PFU of wt to avoid bottleneck effects. Analysis of the virus preparations in the presence and absence of I1 MAb indicated the presence of a $<10^{-2}$ proportion of MARM-C in the population. MARM-C was isolated from competition mixtures after neutralization of all wt virus present by incubation with excess of I1 MAb, followed by attachment to BHK-21 monolayers, thorough washing to remove unattached virus, and incubation for 24 h at 37°C. Analysis of this virus population in the presence or absence of I1 MAb indicated no detectable wt virus.

Competition passages with environmental perturbations. Three types of environmental perturbations were introduced in the course of competition passages between wt and MARM-C: (i) DIPs, either endogenously produced or exogenously introduced; (ii) the presence of the mutagenic base analogue 5-FU; or (iii) an increase in temperature from the physiological 37°C to 40.5°C.

Serial undiluted passages ensure the cyclic presence of large amounts of DIPs in the populations (15). To generate endogenous DIPs, 2×10^6 BHK-21 cells were infected with about 10^8 to 10^9 PFU to reach a multiplicity of infection of 10^2 to 10^3 PFU/cell. The accumulation of DIPs induced a decrease in the production of infectious particles in agreement with previous observations (18). To add exogenous DIPs in the competitions, a proportion of isolated T short fraction DIPs that induced a 98% inhibition in the production of MARM-C and wt yield was introduced into the competition mixtures at the beginning of each passage. After each passage, the viral progeny was diluted 100-fold, and exogenous DIPs were added again to maintain a constant 98% inhibition. A total of three serial infections were carried out under the same conditions.

For the perturbation by 5-FU, this base analogue was added to cell monolayers at a concentration of 25 μg/ml for 6.5 h prior to infection, as previously described (14). The monolayers were then infected with either wt, MARM-C, or mixtures of the two viruses, including 25 μg of 5-FU per ml, which remained present throughout the infections. Four serial passages were carried out. The fitness of the resulting populations was determined as previously indicated. When required, wt and MARM-C were isolated from the competition mixtures, as described above.

The third perturbation consisted of an increase in temperature to 40.5 ± 0.5°C during competition passages. Viral production at 40.5°C was about 100-fold lower than at 37°C. The proportion of wt and MARM-C was monitored during three serial passages at 40.5°C.

Nucleotide sequence determinations. Viral RNA from 25 μl of virus stock was isolated with Tri-Reagent according to the manufacturer's recommendations. Purified RNA was dissolved in 10 μl of diethyl pyrocarbonate-treated water, and 1 μl was employed as template for reverse transcription. Reactions were done with Superscript II (Gibco-BRL) and random hexamers in a final volume of 20 μl. After 1 h of incubation at 37°C, reactions were frozen at −86°C for later use or employed (0.5 μl) for PCR amplification of overlapping fragments as described. Amplified fragments were used as templates to obtain the complete consensus sequence of wt and MARM-C in an automatic ABI Prism 3700 sequencer. Reactions were done using ABI Prism BigDye mixtures. The primers employed for PCR and sequencing are available from L. L. Rodriguez (U.S. Department of Agriculture, Plum Island, N.Y.) upon request.

Statistical analysis of data. Multiple time series of the proportion of MARM-C and wt VSV were analyzed using standard statistical tools (23).

RESULTS

The neutral behavior of MARM-C is lost upon serial undiluted passages which generate DIPs. To evaluate whether the spontaneous accumulation of DIPs could perturb the previously observed coexistence of wt and MARM-C (29), 12 independent competition series between the two viruses were allowed to proceed without any virus dilution, starting with a multiplicity of infection of about 0.1 PFU/cell. In subsequent passages, the accumulation of DIPs was inferred by the reduction in virus titer, in agreement with early work (18). The results showed the coexistence in about similar proportions of

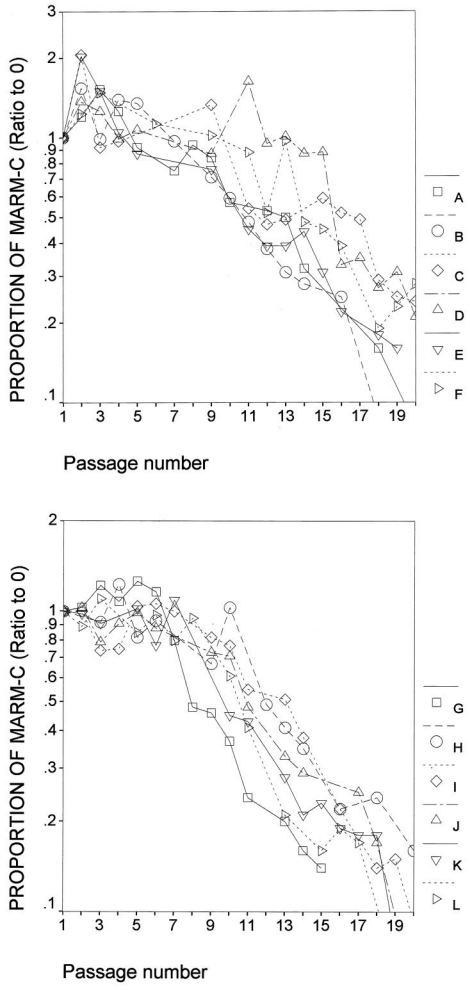

FIG. 1. Competition between wt and MARM-C VSV in serial undiluted passages. Results of 12 parallel competition experiments are shown in two panels for clarity. Procedures for infection of BHK-21 cells and determination of the proportion of wt and MARM-C genomes are detailed in Materials and Methods.

wt and MARM-C for about five to seven passages, and a decrease in the proportion of MARM-C for later passages (Fig. 1). In each of the 12 undiluted passage series, MARM-C decreased in proportion as previously observed in serial diluted competition passages (29), except that MARM-C/wt ratios started declining slightly earlier during undiluted passages (Fig. 1). These results make it unlikely that such a decrease was the result of stochastic effects which could lead to the dominance of either wt or MARM-C ($P < 0.001$, χ^2 test). It is important to note that DIP interference was observed before the proportion of MARM C to wt started to decrease, indicating that originally MARM C is not less fit under these experimental conditions.

Environmental perturbations promote the selective advantage of wt VSV. To test whether the addition of exogenous DIPs at each passage would also increase the dominance of wt over MARM-C, five competition series were carried out with (or without) the regular addition of purified DIPs, as detailed in Materials and Methods. The results showed that DIPs promoted a very rapid decrease in the proportion of MARM-C

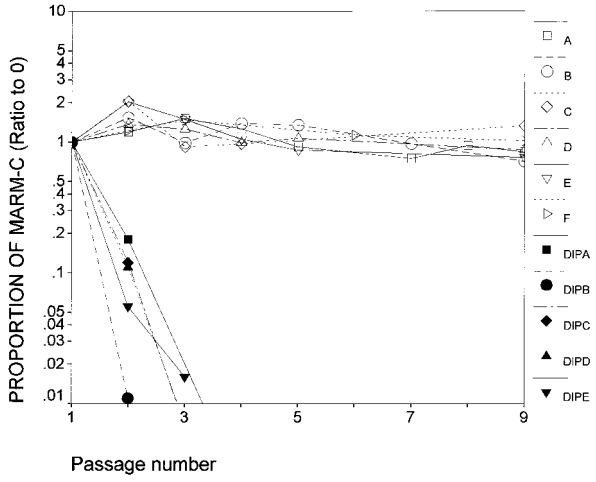

FIG. 2. Competition between wt and MARM-C VSVs in the absence (open symbols) or presence (solid symbols) of exogenous DIPs. DIPs were prepared as indicated in Materials and Methods. At each passage an amount of purified DIPs, giving a reduction of 98% in VSV production in a standard assay with VSV-Indiana, was added. The procedures for infection are detailed in Materials and Methods.

relative to wt compared to parallel competitions without addition of external DIPs (Fig. 2).

To test whether other types of environmental perturbations could have a similar effect, four diluted competition series were carried out in the presence of 5-FU and compared to six diluted competition passages without 5-FU. The results showed that 5-FU induced a rapid decrease in the proportion of MARM-C relative to wt, as seen when DIPs were exogenously added (Fig. 3).

Finally, modification of temperature was chosen as a perturbation that did not require the addition of any exogenous reagent (DIPs or 5-FU) during infection. A mixture of wt and MARM-C was divided in four aliquots and subjected to serial passages at 40.5°C. The results showed that infection at a high

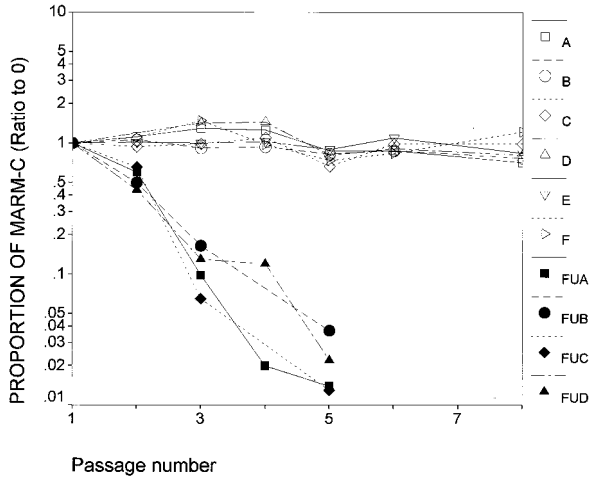

FIG. 3. Competition between wt and MARM-C VSVs in the absence (open symbols) or the presence (solid symbols) of 5-FU. The conditions of treatment with 5-FU and the procedures are detailed in Materials and Methods.

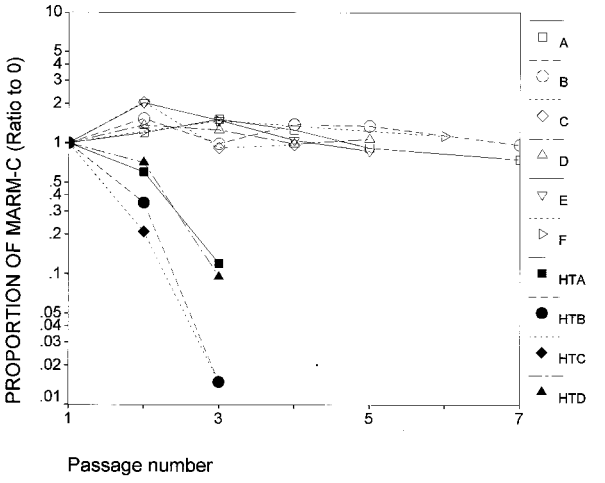

FIG. 4. Competition passages between wt and MARM-C VSVs at 37°C (open symbols) or at 40.5°C (solid symbols). The conditions for culture at high temperature are detailed in Materials and Methods.

temperature also promoted the dominance of wt over MARM-C (Fig. 4). Therefore, a number of environmental perturbations, all increasing the selective pressure exerted by the environment, led systematically to a faster dominance of wt VSV over MARM-C than that observed in unperturbed passages.

Fitness variations of viral subpopulations: similar behavior of wt and MARM-C. One possibility to explain the systematic decrease in the proportion of MARM-C in competition with wt is that environmental perturbations decreased the fitness of MARM-C more than that of wt, independently of the competition between the two viruses in mixed infections. To address this point, we examined the fitness of a number of VSV wt and MARM-C populations passaged in the presence of 5-FU. When either wt or MARM-C alone was subjected to four passages in the presence of 5-FU, both populations experienced a slight decrease in fitness (Table 1) as expected from previous work (22). No significant differences in the fitness decrease of wt and MARM-C were observed. In a second test, wt and MARM-C were isolated from each of the three competition series between the two viruses in the presence of 5-FU. The results indicated that in this case both viruses showed no detectable variation in fitness (neutral value), a result perhaps due to the viral amplification inherent to the procedure to isolate the wt and MARM-C subpopulations. Again, no differential effects on the two viruses were observed. These results suggest that MARM-C behaved as a neutral variant of wt VSV both with regard to the maintenance of its frequency in unperturbed population passages and to fitness variations. Yet, the presence of a wt competitor unveiled a selective impairment of MARM-C, and environmental stress accelerated its decrease in frequency during competition with parental wt.

Candidate mutations for the molecular basis of conditional neutrality. To quantitate the genetic distance between wt and MARM-C, the complete genomic sequences were determined by reverse transcription-PCR amplification and automatic sequencing. The protocol described in Materials and Methods allows the determination of the consensus or average sequence in the quasispecies populations. It is important to note that no cloning steps were involved and that the method employed a large amount of template. Therefore, the mutations found cannot be an artifact of the reverse transcription-PCR amplification, nor can they be due to random selection of minority components.

TABLE 1. Relative fitness of wt and MARM-C populations

VSV strain	Set	Mean fitness (W) ± SD
Initial populations[a]		
wt	1	0.6 ± 0.2
	2	0.49 ± 0.09
	3	0.51 ± 0.05
	4	0.43 ± 0.01
MARM-C	1	0.49 ± 0.01
	2	0.47 ± 0.07
	3	0.42 ± 0.08
	4	0.3 ± 0.2
Competitions[b]		
wt	1	1.16 ± 0.07
	2	1.04 ± 0.08
	3	1.1 ± 0.2
MARM-C	1	0.90 ± 0.09
	2	0.9 ± 0.1
	3	1.14 ± 0.04

[a] Initial populations of wt and MARM-C VSV strains were passaged four times in the presence of 5-FU. After that, fitness was measured in the absence of 5-FU as described in Materials and Methods.

[b] Three sets of competitions between wt and MARM-C at an input proportion of 1:1 were carried out in the presence of 5-FU. After four competition passages in the presence of 25 μg of 5-FU per ml, wt and MARM-C were isolated from the mixtures as described in the text and then competed against each other to determine the relative fitness of the isolated populations in the absence of 5-FU.

Seven mutations were observed distinguishing wt from MARM-C (Table 2). The U→G transversion at nucleotide 3853 (leading to an Asp→Ala substitution at amino acid 259 of the G protein) is the genetic marker that confers resistance to I1 MAb, and this was initially selected as a neutral genetic marker. Results from previous work showed that other populations carrying this mutation often can overcome wt in long-term competitions (6), suggesting that this mutation by itself is not responsible for the contingent neutrality of MARM-C. Two interesting features were observed among the six remaining mutations: first, the presence of two mutations in noncoding regions and, second, a 50:50 split between transversions and transitions. Mutation 2210 maps at the stop/polyadenylation signal in the P-M intergenic region, while mutation 3036 maps at the start signal in the M-G intergenic region. Mutations in both positions may have a significant effect on the

TABLE 2. Mutations in MARM-C compared to wt[a]

Nucleotide no.	Change	Position	Amino acid change
2210	U→G	Intergenic P-M	
3036	A→C	Intergenic M-G	
3853	U→G	G ORF	Asp→Ala[b]
7571	C→U	L ORF	Val→Leu
8422	C→U	L ORF	Silent
9701	G→A	L ORF	Thr→Ile
10420	A→C	L ORF	Phe→Leu

[a] Sequencing of the entire genomes of wt and MARM-C was carried out as described in Materials and Methods.

[b] Mutation that causes resistance to I1 MAb.

transcription patterns of VSV (2, 31, 32). Nevertheless, it is clear that some or all of these six mutations, which have not been previously reported in any VSV strain (GenBank database), can decrease the fitness of MARM-C in a contingent manner when this virus competes with its parental wt population.

DISCUSSION

Neutrality is a relative concept in that a neutral mutation may become selective under a different set of environmental conditions. Clear examples in virus populations are mutations leading to antigenic variation or antiviral resistance, which may be neutral or deleterious in the absence of antibodies or antiviral drugs and advantageous in the presence of antibodies or antiviral drugs (1, 3, 4, 24, 26). Our studies provide evidence of a different type of behavior of a mutant relative to its parental wt counterpart. The key feature is that while the mutant (MARM-C) acted as neutral in short-term competition, the neutral behavior faded upon further passage, as originally described (29). In this report we have shown that extinction of MARM-C was clearly accelerated not only under a specific type of perturbation but also under the influence of three quite disparate environmental perturbations, all involving increased selective pressure. The present results strongly suggest that the behavior of MARM-C is due to a lower beneficial mutation rate than that of the wt.

It should be noted that MARM-C has a history of genetic bottleneck, during which a significant fitness loss was observed due to the accumulation of deleterious mutations. Recovery to neutral fitness values was observed after only two large population passages. Sequence analysis revealed six nucleotide differences (three transitions and three transversions; Table 2) between wt and MARM-C (in addition to the I1 MAb resistance marker). During the evolution of both DNA and RNA genomes, transitions are much more frequent than transversions (19, 20, 25, 30, 34). Reversion of the three transversions would require many additional transversions that were not observed here. Recovery of apparent neutrality was likely due to compensatory mutation(s), as has been described for other viral systems (5, 11). The presence of mutations both in transcription signals and the L polymerase (and only in these) suggests epistatic interactions between the former and one or more of the latter. The availability of infectious clones for VSV (21, 35) will allow testing of this hypothesis.

The behavior of MARM-C can be interpreted in terms of fitness landscapes. The present results suggest that MARM-C has moved through an area in sequence space where the average fitness value was still maintained as neutral but, because the virus is located at a different fitness peak, it is more difficult for this population to move toward higher average fitness values than for its competitor wt. This type of fitness landscape, with multiple fitness peaks of different heights and shapes, was proposed by Wright (36) and is supported by our data. Even though there may be several alternative biological solutions for a selective constraint posed to a virus, not all genomes have the same probability to find one. The fact that MARM-C accumulated mutations that have not been found in other VSV strains supports this hypothesis.

We propose the term "contingent neutrality" to describe situations such as that reported here, wherein the impairment in the capability of a population to adapt is only manifested in the presence of a competitor. MARM-C replicating by itself gains fitness in an exponential manner (27), as wt does (28). MARM-C replicating in the presence of wt also gains fitness (29). However, the gains in fitness experienced by MARM-C populations during competition are consistently lower than those of wt, and thus MARM-C loses. This difference is increased under stressful environmental conditions, such as the presence of mutagen 5-FU, a high temperature, and DIPs. The results could be explained by MARM-C having an overall lower fitness than wt in stressful environments. However, two facts argue against this possibility. The first is the results shown in Table 1, which show that the relative fitness of wt and MARM-C populations were indistinguishable in the presence of 5-FU. Second, the competitions during undiluted passages (Fig. 1) showed no changes in the ratios at times when interference by DIPs was already observed. Overall, our data suggest that MARM-C, despite its initial neutrality in a constant host environment, has a lower beneficial mutation rate than the competing wt population, and this apparent impairment may be greatly magnified under stronger selective pressures.

These results are in agreement with the theory of quasispecies in that the unit of selection is not the individual genome but the whole quasispecies population. Virus clones of the same initial fitness levels may generate mutant distributions with different average fitness levels, and these distributions may be positively or negatively selected. Group or kin selection, in which a whole population is the unit of selection, is considered to be favored under conditions such that lack of further adaptation does not compromise survival (i.e., soft selection). However, traditional models of evolutionary biology require heterogeneous environments if group selection is to operate (13). The present report shows group selection operating in a constant and homogeneous environment and demonstrates that the level of selective pressure determines the extent of differences in the ability of MARM-C to generate a quasispecies population that can successfully compete with wt. MARM-C can and does gain fitness during replication. If individual selection was operating during competition and only the fitness of the individual mutants was relevant, we would observe some instances of MARM-C stochastically generating a superior mutant and dominating over wt, and this was never observed.

Contingency may contribute to limiting random drift during natural infections. Variants with an initial lower fitness will be outcompeted by wt. A second step of purifying selection may take place in a longer term. This step will target variation that, while initially neutral, places the quasispecies at a disadvantage for further adaptation.

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