Virus Mutation Frequencies Can Be Greatly Underestimated by Monoclonal Antibody Neutralization of Virions

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Monoclonal antibody-resistant mutants have been widely used to estimate virus mutation frequencies. We demonstrate that standard virion neutralization inevitably underestimates monoclonal antibody-resistant mutant genome frequencies of vesicular stomatitis virus, due to phenotypic masking-mixing when wild-type (wt) virions are present in thousandsfold greater numbers. We show that incorporation of antibody into the plaque overlay medium (after virus penetration at 37°C) can provide accurate estimates of genome frequencies of neutral monoclonal antibody-resistant mutant viruses in wt clones. By using this method, we have observed two adjacent G → A base transition frequencies in the I3 epitope to be of the order of 10⁻⁴ in a wt glycine codon. This appears to be slightly lower than the frequencies observed at other sites for total (viable and nonviable) virus genomes when using a direct sequence approach.

Generally high (but variable) mutation frequencies and rapid rates of evolution have been observed for many RNA viruses (for recent references and reviews, see references 1, 3-10, 17, 23, 24, 27, 29, 30, 32, 34-37, 40-42, 45-47). Resistance to monoclonal antibody (MAb) neutralization is a convenient and widely used method for estimating the frequency of monoclonal antibody-resistant mutants (MARMs), and a rather high but wide range of MARM frequencies have been observed for both RNA and DNA viruses (11, 12, 14, 24, 31, 33-35, 38, 39). It will require many years of careful study before mutation frequency averages (and high and low extremes) can be established for individual viruses and for all RNA (and DNA) viruses generally. This is an important goal for the understanding of virus evolutionary mechanisms. Different viruses can exhibit quite different rates and modes of evolution (3, 5, 6, 14, 17, 24, 27, 29, 34-37, 41, 42, 47). Even related human influenza viruses A, B, and C can evolve quite differently and at different rates, as shown by Palese and his colleagues (3, 36). At least under laboratory conditions (37, 41), the same vesicular stomatitis virus (VSV) clonal population can undergo either very rapid rates of evolution (during high-multiplicity passages or persistent infections) or extremely low rates of evolution (during dilute passages).

Batschelet et al. (1) were the first to determine the RNA base substitution frequency at a single defined site (in the Q β genome), and they estimated a frequency approximately 10⁻⁴ at that site. Domingo and colleagues have also reported high rates of evolution and high frequencies of mutation in the foot-and-mouth disease virus genome (4-6, 14). Similarly, Steinhauer et al. (40) have estimated base substitution frequencies between 10⁻³ and 10⁻⁴ at single selected sites in total (viable and nonviable) genomes of VSV. In order to compare mutation frequencies at selected sites in total genomes of VSV with those at selected sites in viable

genomes, we have measured MARM frequencies at the I3 epitope (44), in which only two adjacent G → A transitions in a single codon are able to generate detectable MARMs. Initially, we observed quite different MARM frequencies, depending upon the manner in which the virus pool was prepared prior to antibody selection. In particular, viruses picked directly from a plaque or from virus clonal pools subjected to one high-multiplicity passage exhibited MARM frequencies much lower than did clonal pools prepared by low-multiplicity passage of virus picked from a plaque. This strongly suggests phenotypic mixing and masking of MARM genomes by surface glycoproteins of the thousandsfold more numerous wild-type (wt) viruses. We demonstrate below that this is occurring.

Phenotypic mixing and masking occur during mixed infections for a variety of different virus types when surface proteins of one genotype enclose (or partially enclose) the DNA or RNA of another genome. This phenomenon was first observed and analyzed in bacteriophages (2, 28) and was later reported for many animal viruses (15, 16, 18-20, 26). Phenotypically masked or mixed animal virions are often referred to as pseudotypes (18).

MATERIALS AND METHODS

Cells and virus. Monolayer cultures of BHK₂₁ cells with Eagle minimal essential medium (MEM) containing 7% heat-inactivated (60°C, 30 min) calf serum were used for all virus propagation and plaque assays. The wt Mudd Summers strain of Indiana serotype VSV was used, following a number of plaque-to-plaque cloning rounds to prepare the clonal pools. In order to avoid any possible effects of temperature sensitivity of the mutants studied here, all plaque assays were carried out at 32°C and all clonal pools of VSV were prepared at 32°C. Clonal pools were high-titer virus stocks prepared by a single low-multiplicity passage of virus recovered directly from a well-isolated plaque. Mouse hybridoma cells were grown in MEM containing 20% heat-inactivated calf serum.

MAb. The VSV-neutralizing antibodies used here were produced and characterized by Lefrançois and Lyles (21, 22). The sequences of the epitopes to which the MAbs bound

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and the sequences of MARMs of VSV were determined by VandePol et al. (44). The mouse hybridomas producing these MABs were kindly provided by Leo Lefrancois of the Upjohn Co., Kalamazoo, Mich. We produced large quantities of each neutralizing antibody by replicating large numbers of each hybridoma in MEM in vitro at 37°C as nonadherent or loosely adherent cells. During the replication phase, the hybridoma cells grew rapidly, with frequent addition of fresh medium and frequent expansion of the numbers of culture flasks; expanded hybridoma cell populations were then allowed to reach maximal numbers in each flask and to secrete MAB during 48 to 72 h under conditions of high cell density and restricted cell replication. Several liters of each MAB were prepared, frozen in aliquots, characterized, and used for this study.

Virus neutralization (MARM phenotypic assays). Virus neutralization with MAB was carried out by mixing 0.1 ml of an appropriate dilution of infectious virus into 0.9 ml of MEM containing a maximally neutralizing concentration of each MAB contained in a sterile 5-ml Falcon polystyrene test tube. The test tube was flushed with a stream of 5% CO₂-95% air, firmly sealed to maintain a pH of approximately 7.2 to 7.4, and incubated at 37°C for 30 min; then aliquots were plated on BHK₂₁ cell monolayers for plaque quantitation of MARMs. For the quantitation carried out here, it was essential that all cell debris be removed from virus preparations (by brief microfuge centrifugation prior to neutralization), that MAB and virus be thoroughly mixed (by repeated inversion of the test tube) prior to incubation at 37°C, and that neutralizing levels of MAB be included in the agarose overlay medium to prevent wt breakout plaques.

MARM genotype assays under agarose overlays containing MAB. As described in Results, assays for MARM genotype must avoid neutralization of MARM genomes which are partially or completely coated with wt virus glycoproteins (phenotypic mixing and masking). To avoid this, we used a plaque assay method in which virus particles are allowed to attach and penetrate into cells of the assay monolayer at 37°C; then very high concentrations (completely neutralizing concentrations) of MAB are incorporated into the agarose overlay medium. After most attached viruses have penetrated beyond the influence of extracellular antibody, the antibody-containing gelled overlay prevents significant cell-to-cell spread of wt virus but does not arrest plaque development of MARM mutants. The antibody-containing overlay method for scoring genotypes in phenotypically mixed virions has been used previously for poliovirus RNA within Coxsackie virus capsids (16). To optimize this MARM genotype assay, the following precautions must be exercised. The period allowed for virus attachment and penetration must be long enough to allow penetration of most attached virus particles but not sufficiently long to allow production of any new virus progeny by the assay monolayer cells. VSV produces no new progeny particles during 1 h at 37°C; therefore, we routinely adsorbed 0.1 ml of each virus dilution to confluent monolayers of about 3×10^6 BHK₂₁ cells for 15 min at room temperature; this procedure was followed by 35 to 40 min of continued adsorption and penetration at 37°C. After this period, 7 ml of agarose overlay medium without antibody was added to control flasks (for total virus assays) and antibody-containing overlay medium was added to flasks for MARM genotype assays. The flasks were incubated at 32°C for about 40 h for plaque development, after which 0.2 ml of formaldehyde was added to each overlay flask for 20 to 30 min (to fix cells to the culture dish). The gelled overlay medium was carefully

removed, and the cell monolayers were stained with 1% crystal violet. It is important to note that wt virus is present at thousandsfold higher levels than are spontaneously arising MARM mutants (see below). Therefore the total number of viruses added (wt and MARM) must be considerably less than the number of cells in the monolayer or the monolayer will be destroyed. This usually limits the number of MARM plaques which can be counted in a typical wt virus population.

Finally, it should be emphasized that virus dilutions for the plaque assays must be very accurate. Any consistent dilution errors could produce major overestimates or underestimates of MARM frequency in experiments where control (no antibody) flasks register countable plaques after 10,000-fold greater dilution than do the MARM genotype (antibody-overlaid) flasks.

RESULTS

Standard neutralization tests can produce variable estimates of MARM frequency. When we initially attempted to obtain accurate estimates of MARM frequency, we often obtained quantitatively conflicting data. In particular, virus pools prepared by low-multiplicity infection of cells generally exhibited higher frequencies of MARM mutants than did virus pools prepared with the same starting virus inoculated at high multiplicities. This strongly suggested phenotypic masking of MARM genotypes, since high multiplicity ensures that most MARM-infected cells are also infected with a much greater number of wt virus particles. Furthermore, we observed very low frequencies of MARM phenotypes in virus pools picked directly from isolated plaques. Again, this suggested phenotypic masking, since the localization of total virus particles within the confines of a plaque prevents MARM mutants which arise in the plaque from escaping the phenotypic masking effect of the thousandsfold more numerous wt virus particles. The reconstruction experiments described below demonstrate strong phenotypic masking of MARM genotypes replicated together with larger numbers of wt genomes.

A simple direct plaque assay for MARM genotypes using MAB addition in the agarose overlay medium after virus penetration. As described in detail in Materials and Methods, neutralization of phenotypically masked MARM genotype PFU can be avoided if they are allowed to penetrate the plaque assay monolayer cells at 37°C prior to addition of MAB in the agarose overlay medium. We observed that when gradually increasing amounts of MAB were added with the overlay medium (after penetration at 37°C of about 100 to 200 PFU of wt virus), the size of wt plaques gradually decreased from 2 mm, average control size (with no antibody), to 1 mm, 0.5, 0.2 mm, etc., until no plaques were detectable. The concentration of MAB necessary to eliminate all plaques was approximately equal to the MAB concentration necessary for complete neutralization in a standard virion neutralization test at 37°C (see Materials and Methods). Therefore, in all MARM genotype assays described here, we have used agarose overlay medium containing MAB concentrations about 5- to 25-fold greater than the maximal virion neutralizing concentration. These very high MAB concentrations completely suppress wt plaque formation, with only a slight and negligible suppression of MARM plaque size and plaque numbers (0 to 20% reduction with the I3 MAB).

Reconstruction experiments to demonstrate phenotypic masking of MARM genotypes replicated together with greater

TABLE 1. Reconstruction experiments in which different input mixtures of wt and MARM viruses were used to quantitate phenotypic masking of output MARM genotypes after one passage in BHK₂₁ cells

Input MOI of wt	VSV epitope ^a	Input ratio (wt/MARM)	First passage yield ratios, wt/MARM ^b		
			wt/MARM phenotype (standard neutralization)	wt/MARM genotype (MAB in overlay only)	Genotype/phenotype ratios
100 (high)	I1	10/1	100,000/1	8.7/1	1,149/1
100 (high)	I2	20/1	5,000/1	18.5/1	270/1
100 (high)	I3	5/1	3,400/1	7/1	485/1
100 (high)	I3	5/1	4,000/1	7/1	571/1
10 ⁻³ (low)	I3	5/1	11/1	12/1	1/1
10 ⁻⁴ (low)	I3	100/1	1,600/1	220/1	7/1
10 ⁻⁴ (low)	I3	1,000/1	25,000/1	860/1	29/1
10 ⁻⁴ (low)	I3	3,000/1	59,000/1	3,500/1	16/1

^a Each VSV epitope corresponds to the MABs (and the defined MARM mutations in the VSV glycoprotein) which have been characterized previously. The I1 (or I8) MABs interact with the major antigenic epitope for which there are a number of different MARM mutations, whereas only one and two base substitutions are known to produce MARM at the I2 and I3 epitopes, respectively (21, 22, 44).

^b The mixture of wt and MARM viruses were used to infect 3×10^6 BHK₂₁ cells at multiplicities of wt virus indicated in the first column (wt/MARM ratios in this inoculum are indicated in column 3). Total virus yields (averaging about 10^{10} PFU/ml) were quantitated and compared with MARM neutralization phenotype PFU and MARM genotype PFU to calculate yield ratios. See Fig. 1 and Materials and Methods for description of the MARM (antibody overlay) genotype assay.

numbers of wt virus. To quantitate the effects of phenotypic masking, we prepared clonal pools of wt virus and MARM viruses, determined plaque titers of each, mixed them in known ratios for coinfection of BHK₂₁ cells, and then analyzed them for genotype and phenotype yields. Table 1 (top section) shows the results obtained when a single passage of mixed wt/MARM virus was carried out at high wt input multiplicity of infection (MOI) (100). The first passage progeny exhibited wt/MARM genotype ratios roughly equivalent to the input ratios used (about 20/1, 10/1, or 5/1). However, the wt/MARM phenotype ratios (determined by standard virion neutralization assays) were higher by 2 to 3 orders of magnitude, even though the wt/MARM ratios used for mixed inputs were quite modest (from 5/1 to 20/1).

Table 1 (lower section) also shows the results of similar experiments in which the wt/MARM input mixtures were added at a low MOI for a single passage (so that multiple rounds of virus replication occurred). Note that at an input MOI of 10^{-3} wt viruses per cell and a wt/MARM input ratio of only 5/1, there was little or no phenotypic masking observed after a single passage (i.e., the genotype/phenotype ratio was approximately 1). However, phenotypic masking was clearly detectable after a single low MOI passage at a wt/MARM input ratio of 100/1, and it regularly exceeded an order of magnitude at a ratio of 1,000/1 or more (the ratio which prevails in any clonal pool of wt virus). We show below that the usual ratio of wt virus to I3 MARM in a wt clonal pool is usually about 10,000/1 (the mutation frequency at the I3 epitope). The data in Table 1 show that mutation frequencies for MARM genomes can be underestimated by more than 1 order of magnitude if a virus pool was prepared by passage at a low MOI or by several orders of magnitude if it was prepared by passage at a high multiplicity. The difference between low and high MOI passages is due to the differing probabilities that cells replicating MARM genomes will also be infected with greater numbers of wt genomes. The data in Table 1 indicate that the MARMs are neutral mutants because the wt/MARM genotype output ratio approximates the input ratio ($\pm$ the expected variations in plaque assays).

Estimates of I3 MARM genome frequencies in VSV clonal pools by use of MAB neutralization tests require a correction factor (for phenotypic masking). The data in Table 1 clearly suggest that phenotypic masking can cause a high genotype/

phenotype ratio of MARM mutants present in VSV pools containing thousandsfold greater levels of wt virus (as will occur in wt clonal pools). This suggests that mutation frequencies for MARM genomes have generally been underestimated with MAB neutralization assays and that a correction factor should therefore be applied. For example, Portner et al. (31) observed similar MARM frequencies for VSV, Sendai virus, and influenza A viruses (frequencies about $10^{-4.5}$), but it is likely that the genotype frequency is higher than this neutralization phenotype frequency.

In Table 2, we show I3 MARM frequencies in five different clonal pools of VSV obtained by careful neutralization of virions from each pool. Frequencies approximating 10^{-5} were observed in approximate agreement with the report of Portner et al. (31). Simultaneously with each neutralization test, we conducted parallel reconstruction experiments on mixtures of wt VSV with known ratios of added I3 MARM mutants (wt/MARM ratio was 1,000/1 in all cases). To get multiple rounds of replication, these 1,000/1 mixtures were passaged once on BHK₂₁ cell monolayers at a very low MOI (10^{-4}) to obtain phenotypically mixed virus progeny. These progeny virions were then neutralized by I3 MAB in parallel with (and in an identical manner to) the matched wt clonal pools. Once again neutralization (phenotype) frequencies in the reconstruction experiments were much lower (10- to 37-fold) than were genotype (MAB in overlay) frequencies. In each experiment in Table 2, the observed genotype/phenotype ratio was then used as a correction factor to estimate the MARM genotype frequency for each of the five VSV clonal pools. In each case, the (corrected) estimate of MARM genome frequency was approximately 2×10^{-4} to 3×10^{-4} at the I3 epitope. We reported earlier that there are two independent mutations which can produce MARMs at the I3 epitope (44). Both occur in the GGC codon encoding the glycine at position 54 of the VSV glycoprotein. One is a GGC $\rightarrow$ GAC transition substituting an aspartate for the glycine, and the other is a GGC $\rightarrow$ AGC transition substituting a serine for the glycine at position 54 (44). Thus, the estimated frequency of each specific G $\rightarrow$ A transition would be twofold lower, or about 10^{-4} in these VSV clonal pools. This estimate is independently supported by direct estimation of MARM genome frequencies below.

Direct measurement of I3 MARM genome frequencies in wt clonal pools of VSV. As demonstrated above, MAB in agar

TABLE 2. Estimates of I3 MARM genotype frequencies by correction of neutralization phenotype frequencies (using phenotype/genotype correction factors from reconstruction experiments carried out simultaneously)

Clonal pool no.	MARM phenotype frequencies (by neutralization of VSV virions) ^a	Genotype/phenotype ratio correction factors (from reconstruction experiments) ^b	Estimated MARM genotype frequencies (approximate)
1	1.5×10^{-5}	20	3×10^{-4}
2	1.9×10^{-5}	13	2×10^{-4}
3	0.5×10^{-5}	37	1.8×10^{-4}
4	2×10^{-5}	17	3×10^{-4}
5	2×10^{-5}	10	2×10^{-4}

^a MARM phenotype frequencies in five different clonal pools of VSV were determined by using standard virion neutralization tests as described in Materials and Methods.

^b Genotype/phenotype ratio correction factors (ratios) were determined by using the methods in Table 1 (lower section), except that reconstruction experiments were done simultaneously with the neutralization tests. In each of the five experiments, the reconstruction experiments used low-input multiplicities (10^{-4}) of wt virus and an input ratio of wt/MARM of 1000/1. The single-passage yield ratios of wt/MARM were calculated as for Table 1, and these ratios were used as the correction factor to derive the estimated MARM genotype frequency from phenotype frequency.

overlay medium can allow quantitative detection of viable MARM genomes (but only if attached virus is allowed to penetrate beyond the extracellular influence of antibody before overlay addition). We next determined whether this MARM genome assay is capable of quantitating directly the frequency of I3 MARM genomes present in VSV clonal pools. Since the results in Table 2 suggest the frequency of I3 MARM genomes to approximate 10^{-4} , we compared plaque numbers in control plaque assay flasks with plaque numbers in MARM genome assays (at 10^{-3} and 10^{-4} lower dilutions under MAb in overlay medium). Figure 1 shows a photograph of two such direct MARM genome assays on wt clonal pools. The flask on the left contains relatively few plaques, and both the large plaques and the small plaques are distinctly defined. The flask on the right also contains discrete, countable plaques, but there are more of them and the background of normal BHK₂₁ cells is less distinct. This erosion of the background BHK₂₁ monolayers becomes severe if too many MARM plaques are present. This occurs, of course, because there are approximately 10,000-fold more wt genomes than MARM genomes present. Therefore, the numbers of MARM plaques which can be counted are limited. For example, if there are 200 MARM genomes plated on 3×10^6 BHK₂₁ cells and the wt genomes are approximately 10^4 -fold more abundant, then most of the cells in the monolayer will be infected by wt virus prior to MAb overlay addition and most cells will be killed by wt virus replication even without cell-to-cell transmission. Therefore, this assay is most effective in quantitating MARM genomes when about 20 to 50 MARM plaques are produced. As a result, virus dilutions for direct MARM assays must be adjusted appropriately to avoid monolayer destruction at high levels of virus and statistically insignificant numbers of plaques at low virus levels. At appropriate dilutions, MARM plaque numbers are rather reproducible (see below), but accurate MARM frequency estimates require repeated assays. MARM viruses can be picked directly from plaques in genotype assay flasks, mixed with I3 MAb (to neutralize wt viruses present), and amplified for sequence analysis (and other analyses). We have sequenced 10 I3 MARM viruses picked directly from I3 MARM genotype (MAb overlay) assays. Each of them contained either the

FIG. 1. Direct plaque assays for I3 MARM genomes present at about a 10^{-4} frequency in wt VSV clonal pools. The large plaque MARM phenotypes are due to GGC $\rightarrow$ GAC transitions, and the small plaques are due to GGC $\rightarrow$ AGC transitions in the GGC codon for glycine at position 54 in wt VSV glycoprotein mRNA (44). These specific single-site mutations substitute aspartate or serine, respectively, for the glycine present in the wt at amino acid position 54. Note that the flask on the right with a greater number of MARM plaques shows erosion of the cell monolayer background due to 10,000-fold greater numbers of wt virus (which infected the monolayer cells prior to addition of MAb in the overlay medium).

glycine $\rightarrow$ aspartate or the glycine $\rightarrow$ serine mutation (G $\rightarrow$ A transitions) in the codon for position 54 (44).

This direct MARM genotype plaque assay method was then used for estimation of I3 MARM frequencies in eight independently prepared clonal pools of wt VSV. As shown in Table 3, the total MARM genome frequency (i.e., at both codon 54 sites) averaged approximately 2×10^{-4} , therefore (correcting for two alternate sites) the frequency of G $\rightarrow$ A

TABLE 3. Frequencies of I3 epitope monoclonal antibody-resistant VSV (MARM) genomes (phenotypic masking circumvented^a) in clonal pools of wt VSV

Clonal pool number	Number of assays	Total MARM genome frequency	MARM frequency/site ^b
1	14	$9 \pm 7 \times 10^{-5}$	4.5×10^{-5}
2	10	$1 \pm 0.7 \times 10^{-4}$	5×10^{-5}
3	15	$2 \pm 1.2 \times 10^{-4}$	1×10^{-4}
4	20	$4.6 \pm 2.8 \times 10^{-5}$	2.3×10^{-5}
5	18	$2.1 \pm 1.8 \times 10^{-4}$	1×10^{-4}
6	9	$4.1 \pm 1 \times 10^{-4}$	2×10^{-4}
7	18	$1.2 \pm 0.9 \times 10^{-4}$	6×10^{-5}
8	32	$2.2 \pm 1.5 \times 10^{-4}$	1.1×10^{-4}
Mean	17	1.7×10^{-4}	8×10^{-5}

^a Phenotypic masking of MARM genomes was circumvented by adsorbing all virions to BHK₂₁ monolayers and allowing penetration into cells at 37°C. Then control monolayers (for total infectious virus plaques) were incubated under agarose medium without antibody, whereas MARM assays were carried out under agarose medium containing very high concentrations of I3 monoclonal antibody (see Materials and Methods).

^b All I3 epitope MARM plaque clones (44) that were picked, amplified, purified, and sequenced exhibited a single G $\rightarrow$ A transition at either the first or second base of the GGC (glycine) codon at amino acid position 54 of the VSV glycoprotein mRNA (causing Gly $\rightarrow$ Ser or Gly $\rightarrow$ Asp substitutions, respectively).

TABLE 4. Frequencies of I3 monoclonal antibody-resistant VSV genomes (phenotypic mixing circumvented) in virus clones isolated directly from plaques

Plaque isolate	Number of assays	Total MARM frequency	MARM frequency/site ^a
A	8	$1.8 \pm 0.8 \times 10^{-4}$	9×10^{-5}
B	9	$3.2 \pm 1.6 \times 10^{-4}$	1.6×10^{-4}
C	13	$3.8 \pm 1.7 \times 10^{-4}$	1.9×10^{-4}
D	10	$2.1 \pm 0.7 \times 10^{-4}$	1×10^{-4}
E	7	$1.1 \pm 0.5 \times 10^{-4}$	5.5×10^{-5}
F	8	$2.1 \pm 0.7 \times 10^{-4}$	1×10^{-4}
Mean	9	2.3×10^{-4}	1.1×10^{-4}

^a All procedures were as described for Table 3, except that MARM frequencies were determined by using virions isolated directly from wt VSV plaques. Virus was aspirated from 2-mm plaques and suspended in 1 ml of Eagle MEM prior to assays.

transitions averages about 10^{-4} for viable genomes. Note that there is no evidence for large "jackpots" of I3 MARM mutants in the pools examined in Table 3. Likewise, we generally observed both large and small plaque phenotype MARM mutants in each clonal pool (we did not attempt differential counting), so the frequency of G → A transitions at each of the two adjacent codon sites did not appear to be greatly divergent.

Next, we directly examined the I3 MARM genome frequency in clones of VSV by picking virus directly from 2-mm plaques, suspending them in 1 ml of MEM, and assaying in the presence and absence of MAb in agarose overlays. Table 4 shows the results of these MARM genotype frequency estimates. Once again, the total MARM genome frequencies averaged around 2×10^{-4} , so the G → A reversion frequencies at each of the two codon sites approximates 10^{-4} . This indicates that the average I3 MARM frequency does not change greatly during expansion of virus clones to large virus pools. We have also observed (data not shown) that low-multiplicity (0.01) passages of clonal pools did not greatly affect the I3 MARM frequency, which remained approximately 10^{-4} . As shown in Table 1, these MARMs are neutral mutants with no growth advantage or disadvantage obvious at either high or low multiplicities.

Finally, it was important to determine whether the direct (MAb in agarose overlay) assay for I3 MARM frequency might be greatly overestimating MARM frequencies due to early arising MARM genomes produced by mutation within initially wt-infected cells of the assay monolayer. This was easily tested by carrying out infectious center assays of BHK₂₁ cells infected at a high MOI with a 3/1 ratio of wt/I3 MARM viruses. Infectious centers produced at these ratios mimic wt-infected cells with very early arising MARM genomes. When we plated approximately 100 such infectious centers on plaque assay monolayers without antibody, we obtained an average of 84 plaques. However, when we plated the same number of infectious centers under agarose overlays containing I3 MAb (MARM genome assays), we observed no plaques. When we plated 10 times as many of these infectious centers (approximately 1,000) under MAb-containing agarose overlays, we observed an average of 13 very small, turbid plaques. These probably reflect the small percentage (less than 2%) of infectious centers which adsorbed a high proportion of MARM virus and a low proportion of wt virus in a Poisson distribution (the input MOI for infectious centers was approximately 30 for wt and 10 for MARM viruses). Regardless, it is clear that those rare I3 MARMs that arose almost immediately during replication of

wt virions under MAb overlay medium generally did not produce a visible plaque. Presumably, this is because phenotypic masking by only slightly more numerous wt viruses prevents efficient cell-to-cell spread under antibody.

DISCUSSION

The mutation frequencies estimated above for viable mutants appear to be slightly lower than we have estimated previously at other sites in the VSV genome for total (viable and nonviable) genomes by using a direct sequence analysis approach (40). However, at the two adjacent sites examined in the present study, only C → U transitions in the genome RNA (G → A transitions in the mRNA) produce detectable MARM viruses (44). Therefore, C → A and C → G transitions are not scored here, so we have not examined the frequency of all mutations at each site.

It is expected that total mutation frequencies (which include nonviable mutants) might exceed viable mutation frequencies. However, we cannot attempt accurate comparisons of frequencies by the two methods, since different sites have been examined and the inherent variability of each method precludes very accurate comparisons. It should also be noted that by both methods, we have thus far examined only half of all possible types of genome mutations of VSV (G-C pair to A-U transitions, and G-C to U-A or C-G transversions). It is, of course, not possible to determine whether a G → N mutation in the VSV genome (or mRNA) was due to mutational events in the genome or in its antigenome template. Obviously both can occur, but relative differences in frequency (if any) are not known as yet.

The methods used here can allow estimation of neutral mutation frequencies at other epitopes of VSV (where other than G-C → N-N mutations are scored) and in epitopes of many other RNA viruses. It appears that many previous estimates of MARM mutation frequencies for enveloped viruses may require revision. This will probably also be true for icosahedral and helical naked capsid viruses, since they too exhibit phenotypic masking and mixing (2, 13, 16, 19, 20, 28). It should be emphasized that the two I3 MARM mutants studied above are neutral (or nearly neutral) mutations and that their rates of replication are not significantly inhibited by the MAb used. Obviously, debilitated (nonneutral) mutants or mutants that are partly inhibited by the MAb used, would not be suitable for estimates of mutation frequency. MARM genome assays should be useful for comparisons of neutral mutation frequencies in different cells, at different temperatures, and in the presence and absence of mutagens, etc. The absence of significant jackpots of MARM analogous to jackpots originally analyzed in bacteria by Luria and Delbrück (25) is not surprising because our virus populations are very large, the mutation frequencies are very high, large MARM jackpots due to very early mutations during clone expansion should be rather infrequent, and we have examined a rather limited number of wt clones. If hundreds of clones were analyzed, a number of large jackpots of I3 MARM mutants would be expected because these are neutral (or nearly neutral) mutants.

Finally, we have learned that Valcárcel and Ortín have also recently observed phenotypic masking of MARM mutants in influenza virus clones (43). In conclusion, it is clear that phenotypic masking or mixing can cause order-of-magnitude underestimates of MARM frequencies when virion neutralization tests are used. However, this may vary depending upon the antibody-epitope combination used, the virus studied, and other factors.

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