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NOTES

Mutation Frequencies at Defined Single Codon Sites in Vesicular Stomatitis Virus and Poliovirus Can Be Increased Only Slightly by Chemical Mutagenesis

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Mutagenesis by a variety of chemical mutagens conferred only 1.1- to 2.8-fold increases in mutation frequencies at defined single base sites in vesicular stomatitis virus and poliovirus.

RNA viruses usually exhibit high mutation frequencies, but lower values have been calculated for some virus genome regions and single base sites (for recent reviews, see references 6, 7, 18–21, 23; J. Coffin, Appl. Virol. Res., in press). Mutation frequencies (or rate calculations) derived from proportions of selected phenotypes in a virus clone can be greatly influenced by the relative fitness of the mutant vis-à-vis the average virus population. This was documented by Batschelet et al. (1) for Qβ bacteriophage and, more recently, by Coffin (in press) for Rous sarcoma virus. Even quite small differences in relative fitness can give misleading results for mutation rates if mutant neutrality is not demonstrated or relative fitness is not accounted for (1; Coffin, in press). Our analyses (1, 4, 5, 11, 20) of Qβ phage, vesicular stomatitis virus (VSV), and poliovirus at defined single base sites have so far provided mutation frequencies between 10^{-3} and 10^{-5} (averaging about 10^{-4}). Since this approximates the reciprocal of the virus genome length, replicase error rates may often be at or near the maximum tolerable error. To test this possibility, we have analyzed the effects of various mutagens at defined genome sites and at different levels of lethality.

It has long been known that chemical mutagenesis can cause significant increases in mutation frequency in a wide variety of RNA virus genomes. For example, Mundry and Gierer (13) showed that nitrous acid treatment of tobacco mosaic virus increases the spontaneous frequency of necrotic lesion mutants more than 20-fold (and many other new phenotypic characters appeared). Granoff (9) observed a >10-fold increase in small-plaque mutants of Newcastle disease virus after nitrous acid treatment, and Carp and Koprowski observed a similar effect of nitrous acid on poliovirus (2).

Halle (10) reported an increase of up to 220-fold in the frequency of large-plaque mutants of Venezuelan equine encephalitis virus after mutagenesis with 5-azacytidine (5-AZA-C). Fields and Joklik (8) increased temperature-sensitive (*ts*) mutation frequencies of reovirus up to 100-fold after mutagenesis by proflavine, nitrosoguanidine, and nitrous acid, and Pringle (15) observed increases from severalfold to more than 40-fold in the *ts* mutants of VSV after mutagenesis by 5-fluorouracil (5-FU), 5-AZA-C, or ethyl methanesulfonate (EMS) at various levels of survival. In these early studies, the target sites for mutagenesis were not known. Multiple base sites in multiple genes could have been targets, as shown by complementation or reassortment analysis of *ts* mutants (8, 15). We recently reported adjacent single base sites in VSV and poliovirus for which mutation frequencies to a defined, neutral phenotype were approximately 10^{-4} (mutation to monoclonal antibody resistance in the I3 epitope of VSV [11] and mutation from guanidine dependence to guanidine independence [resistance] for poliovirus [4, 14]). We report below the effect of chemical mutagenesis on mutation frequencies at these defined sites for which neutrality of the mutant phenotype has previously been established (4, 11). We tested a variety of mutagens, and the results are shown in Tables 1 and 2.

In agreement with Pringle (15), we have observed increasing percentages of *ts* mutants of VSV with increasing levels of mutagenesis by 5-FU (results not shown). However, 5-FU did not greatly increase the mutation frequencies of different VSV clones and strains at any virus survival level, as measured by frequency of mutation to resistance to I3 monoclonal antibody at defined base sites (11) (Table 1). A two- to threefold increase in frequency was the maximum observed in numerous experiments (of which the data in Table 1 are typical). This was true for three different strains of VSV Indiana: Mudd-Summers (MS), Glasgow (15), and a Panama strain. Equally low increases were observed for resistance to other VSV monoclonal antibodies (not shown). Table 1 also shows similar results with EMS, 5-AZA-C, and nitrous acid (HONO), again regardless of levels of virus survival. These rather small increases in single-site adaptability were usually counterbalanced by decreased virus yields (lethality).

Table 2 shows similar data for another RNA virus, type 1 poliovirus, measuring neutral single-site reversions from guanidine dependence to guanidine independence (4, 14). Single-site mutations in a single poliovirus codon were increased at most two- to threefold by the potent RNA

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TABLE 1. Ratios of single-base mutation frequencies in clones of VSV with and without chemical mutagenesis

Virus strain (multiplicity of infection [PFU/cell])	Mutagen (concn or treatment time)[a]	Virus survival (% of control)	Mutation frequency[b]/site ± SE, 10^{-4}	Mutations with mutagen/mutations without mutagen
MS (400)	None	100	1.0 ± 0.4	
MS (400)	5-FU (10 μg/ml)	93	1.8 ± 0.8	1.8
MS (400)	5-FU (50 μg/ml)	13	2.4 ± 0.5	2.4
MS (10^{-4})	None	100	0.8 ± 0.3	
MS (10^{-4})	5-FU (1 μg/ml)	81	1.8 ± 0.7	2.3
MS (10^{-4})	5-FU (10 μg/ml)	28	1.9 ± 0.8	2.4
Glasgow (10^{-4})	None	100	0.6 ± 0.4	
Glasgow (10^{-4})	5-FU (10 μg/ml)	30	1.2 ± 0.3	2.0
Panama (10^{-4})	None	100	0.5 ± 0.2	
Panama (10^{-4})	5-FU (10 μg/ml)	20	1.1 ± 0.3	2.2
MS (100)	None	100	0.9 ± 0.3	
MS (100)	5-AZA-C (1 μg/ml)	39	1.8 ± 0.5	2.0
MS (100)	5-AZA-C (10 μg/ml)	0.9	1.5 ± 0.8	1.7
MS	None	100	0.8 ± 0.3	
MS	EMS (2 min)	74	1.3 ± 0.6	1.6
MS	EMS (12 min)	10	1.9 ± 0.5	2.4
MS	EMS (20 min)	2.2	1.8 ± 0.7	2.3
MS	None	100	0.7 ± 0.3	
MS	HONO (1 min)	41	1.6 ± 0.5	2.3
MS	HONO (3 min)	9.6	1.3 ± 0.3	1.9

[a] Mutagenesis by 5-FU and 5-AZA-C involved replication of virus in the presence of the indicated concentration of mutagen in BHK-21 cell culture growth medium (15), EMS mutagenesis involved alkylation of purified virions at pH 6.5 (15), and HONO mutagenesis involved nitrous acid treatment of purified virions at pH 4.5 (12). Mutation frequencies are the frequencies (in virus clones) of mutant genotypes (11, 22) resistant to I3 monoclonal antibody (11).

[b] Mutation frequencies observed were twice those shown here. We divided by 2 to correct for the fact that there are two nucleotide sites in VSV at which a single G→A (or C→U) transition prevents neutralization by I3 monoclonal antibody. 5-FU or 5-AZA-C was added to cell culture medium immediately after virus attachment at a low (10^{-4}) multiplicity of infection or 4.5 h prior to virus attachment at a high (400) multiplicity of infection.

mutagen 5-FU, while virus viability decreased with increasing 5-FU concentrations.

The results described above for single codons might appear to contradict earlier work (2, 8–10, 13, 15) in which chemical mutagenesis greatly increased the frequency of *ts*, small-plaque, and other broad mutant phenotypes. There is no contradiction because *ts* and plaque size mutations can occur at numerous different sites in a virus genome. The different mutations at all target sites are additive to provide the overall frequency of that mutant phenotype in the virus population. Therefore, the observed increase in mutation frequency (between 1.1- and 2.8-fold) at single base sites is completely consistent with the studies cited above that showed much larger increases in *ts* and other multitarget phenotypes due to chemical mutagenesis.

TABLE 2. Ratios of mutation frequencies per single base target site in clones of poliovirus with and without mutagenesis by 5-FU

Multiplicity of infection (PFU/cell)	5-FU concn[a] (μg/ml)	Virus survival (%)	Mutation frequency[b]/site ± SE, 10^{-4}	Mutations with 5-FU/mutations without 5-FU ± SE
10^{-4}	0	100	2.0 ± 0.7	
10^{-4}	50	21	4.6 ± 2.4	2.3 ± 0.4
10^{-4}	100	25	5.4 ± 1.9	2.7 ± 0.1
10^{-4}	200	0.5	4.6 ± 1.2	2.3 ± 0.1
10	0	199	9 ± 3.0	
10	50	41	12 ± 0.5	1.3 ± 0.4
10	100	11	25 ± 0.5	2.8 ± 0.4
10	200	0.95	10 ± 0.5	1.1 ± 0.07

[a] 5-FU was added to the HeLa cell culture medium 6 h prior to attachment of cloned poliovirus (virus picked directly from a plaque for a low [10^{-4}] multiplicity of infection or from a clonal pool directly expanded from a plaque for a high [10] multiplicity of infection).

[b] Mutation frequencies are the frequencies of guanidine-independent (resistant) revertants in clones of guanidine-dependent poliovirus (4, 14). The reversion frequencies observed were twice those shown above. We divided by 2 to correct for the fact that there are two nucleotide sites in this guanidine-dependent poliovirus at which an A→G transition or a G→N transition or transversion can cause reversion to guanidine independence (4, 14).

The observed small increment in mutation frequency at these sites is understandable. One would not expect to increase the mutation frequency of a 7- or 11-kilobase genome much beyond the approximately 10^{-4} spontaneous mutations observed at these single base sites. To increase the frequency of spontaneous mutation even 10-fold at a single site would require many random mutations elsewhere in most genomes, thereby significantly increasing virus lethality. On the other hand, if we were able to test a site with a low mutation frequency of 10^{-8}, then the mutation frequency at such a site should be greatly increased in surviving fractions of mutagenized virus. The major result of our analysis is the clear demonstration that spontaneous mutation frequencies approximating 10^{-4} per site are nearly enough to cause virus lethality. Our attempts to increase mutation frequencies greatly met with failure, but not unexpectedly. However, it would not be surprising to observe different results in some DNA-based organisms in which proofreading, mismatch repair, and other DNA repair systems (17) can greatly increase fidelity and reduce mutational

lethality. In fact, Cupples and Miller (3) were able to increase defined reversion frequencies at a number of single base sites in codon 461 of the *Escherichia coli lacZ* gene by several hundred- or thousandfold by using a variety of chemical mutagens, and often with quite high survival levels in treated bacteria. In DNA viruses, however, in which high mutation rates (18) can offer evolutionary advantages, mutagen effects might often resemble those reported here for RNA viruses.

Finally, it is likely that virus mutants exhibiting enhanced mutability, such as the *ts* mutant of VSV reported by Pringle et al. (16), produce a higher than usual proportion of multiply mutated (and lethally mutated) progeny without a great increase in mutation frequency at single base sites.

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