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High Nucleotide Substitution Error Frequencies in Clonal Pools of Vesicular Stomatitis Virus

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Nucleotide substitution error frequencies were determined for several specific guanine base positions in the genomes of cloned vesicular stomatitis virus populations. Predetermined sites were examined in coding regions for the N, M, and L proteins and at a site in the genome 5′-end regulatory region. Misincorporation frequencies were estimated to be on the order of 10^{-3} to 10^{-4} at all positions analyzed. Isolates taken from virus populations after disruption of equilibrium conditions displayed replicase fidelity similar to that of cloned wild-type vesicular stomatitis virus. These mutation frequencies apply to all virus genomes present, including viruses rendered nonviable by lethal mutations. At one selected site in the N gene, two of three G → N base substitutions generated lethal nonsense mutations, yet their frequency was also very high. Biological implications for rapid virus evolution are discussed.

Very rapid rates of evolution have now been documented for a variety of viruses with RNA genomes (for reviews, see references 10, 11, 21, 41, and 46). A major factor involved in RNA virus evolution appears to be the error-prone nature of their replicative processes. Although high RNA virus mutation frequencies have been suggested by several genetic studies based on observable phenotypic changes or T_1 fingerprint differences (see reviews referenced above), few investigations have been done which address directly polymerase error frequencies following limited amplification of the original template. The error rate at one position of the RNA bacteriophage Qβ genome was estimated to be about 10^{-4} at that site per genome doubling (1, 9). Loeb and colleagues (2, 20, 48; B. D. Preston, J. D. Roberts, T. A. Kunkel, and L. A. Loeb, Abstr. 7th Int. Congr. Virol. 1987, S32.2, p. 45) have estimated error frequencies per site for avian myeloblastosis virus reverse transcriptase to be greater than 10^{-4} by several in vitro techniques. Ward et al. (47) recently calculated in vitro base misincorporation frequencies per nucleotide incorporated ranging from 7×10^{-4} to 5.4×10^{-3} for purified poliovirus RNA polymerase.

Interestingly, significantly different error frequency estimates can be obtained when studying different viruses or utilizing different approaches. A particular host-range mutant of Sindbis virus was shown to revert at a frequency of less than 5×10^{-7} (14). This is a much lower frequency than is suggested from studies with monoclonal antibody-resistant mutants of Sindbis virus (43). Parvin et al. (32) sequenced the NS gene of 108 influenza A virus isolates generated during the growth of a single plaque. Seven mutations were found in about 92,000 nucleotides sequenced, a frequency of about 7.6×10^{-5} for viable mutants of influenza A virus. In the same study with poliovirus, no mutations were detected in over 95,000 nucleotides analyzed (32). A similarly low error frequency, about 2.5×10^{-6}, was estimated recently for the reversion of a poliovirus amber mutant (40). Considerably higher poliovirus mutation frequencies have been reported for poliovirus mutation to guanidine resistance (34, 35) and mutation to monoclonal antibody resistance (4, 8, 16, 18, 27), but target sizes for mutations and virus passage histories were often not defined in these studies. Dougherty and Temin (13) have calculated the rate of mutation for a specific A · T-to-G · C substitution to be 2×10^{-5} during a single round of replication of a retrovirus vector. Leider et al. (24) recently used denaturing gradient gels to detect single base mutations generated during replication of Rous sarcoma virus. They calculated a misincorporation rate of 1.4×10^{-4} for viable mutants of one clone.

Several investigations have suggested high mutation frequencies for the RNA polymerase of vesicular stomatitis virus (VSV) (39, 45, 49). We previously described methods for direct in vivo polymerase misincorporation frequency determinations and showed it to be greater than 10^{-4} at one VSV genome site (45). Here we expand these studies to include several genome sites of wild-type and mutant VSV and report equally high error frequencies on the order of 10^{-3} and 10^{-4} at each genome site examined.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney (BHK-21) cells were grown in Eagle minimal essential medium with 7% calf serum and used for all virus propagation. The Mudd-Summers strain of Indiana serotype VSV was cloned 10 times plaque to plaque followed by one passage on monolayers of 2×10^7 to 3×10^7 BHK-21 cells. Individual plaques were picked from this clonal pool, and each was amplified during one passage on BHK-21 cells before use as stock for genome labeling with ^{32}P. The *ts*G31 virus is an M gene mutant derived from the Glasgow strain of Indiana VSV. Passage 254 was generated from cloned *ts*G31 virus passaged continuously at high multiplicity as described previously (6, 22, 42). Individual clones were picked from the population of viruses present at passage 254, and one of these, designated high-multiplicity passage 254 clone 1, was amplified by one passage before labeling.

Mutagenized virus was studied with virus which was plaque purified following 23 low-multiplicity passages (of nine-times-cloned wild-type virus) in the presence of 200 μg of 5-fluorouracil (5-FU) per ml (36). To pick plaques, we

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decreased the concentration of mutagen to 5 μg/ml. Error frequency analysis was done after mutagenesis (in the absence of 5-FU because it was found to lower virus titers and labeling efficiency). The temperature shift experiment was done with cloned wild-type virus passaged at low multiplicity 14 times at 30°C. Virus was then plaque purified at 30°C and either amplified by one passage at 30°C for analysis or passaged two times at 39.5°C before error analysis. Virus passaged at 39.5°C was then plaque purified at 37°C, and a clone was picked for base substitution analysis. Again, the error frequency analysis was done at 37°C following high-temperature passages because ^{32}P labeling was inefficient at 39.5°C.

Isolation, digestion, separation, and quantitation of specific T_1 oligonucleotides. Purified in vivo-labeled RNA with a large quantity (100 to 500 μg) of unlabeled viral RNA was annealed to synthetic cDNA at a threefold molar excess of DNA in 500 mM NaCl–4 mM $MgCl_2$ buffered at pH 7.5 with 10 mM Tris hydrochloride. Unprotected single-stranded RNA was digested with 25 μg of RNase T_1 (Calbiochem-Behring, La Jolla, Calif.) per ml and 100 U of RNase T2 per ml at 30°C for 2 h. Neither RNase T_1 nor RNase T2 will digest single-base mismatches in hybridized RNA (28). Samples were proteinase K digested, phenol-chloroform extracted, and ethanol precipitated and then purified on 20% polyacrylamide gels. Alternatively, some of the synthetic DNAs were engineered with poly(dA) tails. This allowed for direct purification of DNA-RNA hybrids on oligo(dT) columns following RNase treatment (details to be published elsewhere [D. A. Steinhauer, D. Kohne, and J. J. Holland, submitted for publication]). Purified hybrids were treated with DNase I (100 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 45 min at 37°C, and then the temperature was raised to 50°C for 5 min. Samples were then proteinase K digested, phenol-chloroform extracted three times (with great care taken not to transfer gel fragments, aggregates, etc., which might interfere with subsequent digestion), and ethanol precipitated two to three times followed by a 70% ethanol wash. Dried RNA was suspended thoroughly in 20 μl of 1-mg/ml RNase T_1 buffered with 10 mM Tris hydrochloride (pH 7.6). Samples were digested at 37°C for 30 min. Once during digestion, samples were heated briefly to 100°C and transferred to new microcentrifuge tubes. RNA was then loaded directly onto the first dimension of the two-dimensional gel system of DeWachter and Fiers (7). Oligonucleotides were excised from the gel along with background gel slices and carefully quantitated by Cerenkov counting as described previously (45).

In an alternative method, viral RNA was not ^{32}P labeled in vivo. Instead, unlabeled RNA was ^{32}P end labeled after T_1 digestion. Hybrids were isolated as described above except that kinased DNA (or very lightly labeled RNA) was used to track the samples. Following T_1 digestion, samples were ethanol precipitated with 25 μg of oyster glycogen as a carrier. Samples were digested with 1 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) at 50°C for 60 min, proteinase K digested, phenol-chloroform extracted, and ethanol precipitated with 25 μg of oyster glycogen as a carrier. RNA 3′ ends were then labeled by ligation with 5′ [^{32}P]Cp (17). RNA ligase was from U.S. Biochemical Corp. (Cleveland, Ohio). RNA was precipitated two times with 10 μg of tRNA and 25 μg of oyster glycogen and loaded onto first-dimension gels. Highly purified oyster glycogen and tRNA carriers were utilized to ensure a high yield during precipitations. When using this alternative method, care must be taken to achieve quantitative ethanol precipitation to avoid biasing the results during steps following T_1 digestion. 5′ end labeling of T_1 digestion products was also employed. After 30 min of T_1 digestion at 37°C, RNA samples were transferred directly to tubes with dried-down [γ-^{32}P]ATP. The concentration of $MgCl_2$ was raised to 10 mM, T4 polynucleotide kinase (U.S. Biochemical Corp.) was added, and reactions were allowed to proceed for an additional 30 min at 37°C before the reaction mixture was loaded directly onto first-dimension gels. This method can sometimes present problems owing to streaking in the first dimension (possibly owing to the presence of $MgCl_2$).

Analysis of error oligonucleotides. Following quantitation, error RNAs were isolated from the gel and 3′ end labeled by ligation to 5′ [^{32}P]Cp. End-labeled error oligonucleotides were then gel purified and sequenced by the chemical method (33). Some were redigested with RNase T_1 (as above) to estimate the amount of previously undigested guanine residues. Error frequency data were then corrected to account for incomplete digestion during the first T_1 treatment.

Preparation of virus clonal pools. For the analyses of individual clones of viruses reported below, we used clonal pools of virus to minimize the number of virus replication cycles following the replication of the original cloned virus particle. To prepare clonal pools, a single well-isolated virus plaque was visualized microscopically and the contents of the plaque were removed with a sterile Pasteur pipette. The virus particles in this agarose plug were withdrawn into 1 ml of Eagle minimal essential medium, and these virus particles were then amplified by further replication in monolayers of about 3×10^7 BHK-21 cells to produce the clonal pools which were analyzed. Therefore, the error frequencies reported below were determined from virus populations which had been expanded through at least 8 to 10 replication cycles, 4 to generate a plaque and at least 4 to amplify each plaque (see Parvin et al. [32]).

RESULTS

The site-specific analysis of base substitution frequencies utilized here relies on the high specificity of RNase T_1 for cleavage immediately following guanine (G) residues. We targeted for analysis guanine sites at the junction between two large T_1-resistant oligonucleotides (guanine residues with no nearby guanine neighbors). Figure 1 shows the four VSV genome sites which were analyzed, one in the 5′-end regulatory region and one site each in the coding regions for the N, M, and L proteins. Complete digestion of sequences such as these normally yields two large consensus oligonucleotides. Nucleotide substitutions at the junction guanine of these sequences result in a larger T_1-resistant error oligonucleotide. These oligonucleotides can be separated and quantitated, and the ratios of error to consensus oligonucleotides can be used to estimate substitution frequencies at the predetermined positions. It should be emphasized that the error oligonucleotides visualized on two-dimensional polyacrylamide gels are not single, clearly defined spots but are laterally diffuse broad bands. This is expected if they are composed of different base substitutions at the selected and nearby sites (see below).

In vivo nucleotide substitution frequencies in clonal pools of VSV. Clonal pools of wild-type VSV Indiana serotype were labeled in vivo with inorganic ^{32}P; then labeled virus was purified and RNA extracted. Synthetic cDNAs were purified and annealed to viral RNA segments selected for misincorporation analysis (Fig. 1). Unprotected RNA was then

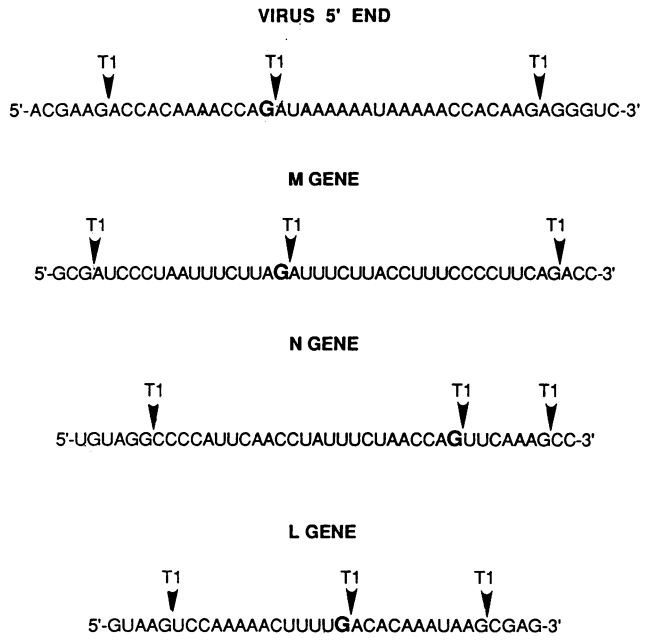

FIG. 1. VSV genome sites analyzed for error frequency estimates. Bold-face G residues just 5′ of central T_1 digestion sites are the positions at which substitutions were selected.

digested with single-strand-specific RNases T_1 and T2. Hybrids were purified and digested with DNase I to yield the RNA sequences to be analyzed. This selected RNA segment was then digested with RNase T_1, and the products were separated on two-dimensional gels. Figure 2A and B shows consensus and error oligonucleotides for a number of VSV genome sites after gel separation. Oligonucleotides were cut out of the gel and Cerenkov counted, and the relative amount of ^{32}P in error versus consensus oligonucleotides was used for approximation of error frequencies (Table 1). These approximations were subsequently corrected to account for uncut guanine residues to give final error frequency estimates (see below). Misincorporation frequencies for all sites examined were high, ranging from about 10^{-3} to 10^{-4}. Although frequencies as high as 1.8×10^{-3} were measured, no true jackpots have been detected to date. Perhaps this is because not enough data points have been examined. Alternatively, errors at the sites we examined might be detrimental to virus replication or competitive ability and therefore quickly outgrown. Indeed, the site in the 5′-end regulatory region is highly conserved among vesiculoviruses (29), and two of three possible substitutions at the N gene site generate UGA or UAA nonsense mutations in the mRNA. This site was selected because most substitutions there would be lethal.

Detecting error oligonucleotides after end labeling. To control against the possibility that in vivo ^{32}P-labeling techniques might affect the error frequency estimates, we used alternative methods to estimate error frequencies of unlabeled viral RNA. Genome segments were purified as described above except that labeled DNA (or lightly labeled RNA) was used to observe DNA-RNA hybrid purification. After treatment with DNase I and RNase T_1, RNA oligonucleotides were end labeled (at either the 5′ or 3′ end) prior to separation on two-dimensional gels. Table 1 (bottom) shows misincorporation frequency estimates obtained in this manner. The results are in agreement with results obtained by in vivo labeling techniques. An advantage to this procedure is that it allows analysis of samples in which high-activity in vivo labeling of RNA is difficult. There are, however, several potential problems with this end-labeling approach. Gel background counts are higher after ^{32}P end labeling; it is also essential to ensure quantitative ethanol precipitation of both the larger error oligonucleotide and smaller consensus oligonucleotides (by the use of excess carrier tRNA and glycogen; see Materials and Methods).

Sequence analysis of error oligonucleotide. Error oligonucleotide spots (broad bands) were eluted from gel slices and 3′ end labeled for chemical sequencing. Figure 3 shows sequence analyses of several error oligonucleotides representing all sites examined. Heterogeneity can clearly be seen at the preselected base positions (arrowheads). Notice that in several of the sequences downstream, and sometimes upstream, U-C heterogeneity can be seen at certain nearby positions other than the selected positions. The molecular mechanisms responsible for generating observed mutations at unselected sites are not known, but they usually involve C → U transitions. Oligonucleotide sequence analysis also shows that a significant proportion of error RNA was due to guanine residues which escaped digestion during a single round of RNase T_1 treatment. Therefore, a number of error RNAs were isolated and redigested with RNase T_1 after end labeling. Figure 4 shows products of second T_1 digestions. Bands were eluted, and their radioactivity was counted to estimate the percentage of the original error RNA which had been due to uncut guanine. The proportion of RNAs digested by the second treatment averaged 25% and ranged from about 10 to 50% (Table 1, in parentheses). Misincorporation frequency approximations were therefore modified to correct for this uncut fraction. In experiments in which redigestion of error spot RNA was not done, 25% was used as the correction factor.

Other interesting observations were noted concerning error RNAs. About 10% of most isolated error RNAs were cleavable with aniline (which is used for cleavage of modified nucleotide sites in chemical RNA sequencing) prior to any deliberate chemical modification reactions. Perhaps this is due to modified bases in RNA or to apurinic sites, which are generated spontaneously in DNA (25) and have been postulated as mutagenic intermediates (26). Attempts to cleave RNA with exonuclease III, which digests apurinic double-stranded DNA (38), were unsuccessful. This was true either with single-stranded error RNA as the substrate (Fig. 5) or with error RNA annealed to DNA to mimic the structure of susceptible apurinic DNA (data not shown). Error RNA which had been reduced prior to aniline treatment revealed no increased cleavage compared with treatment with aniline alone (Fig. 5). This suggests that little if any resistance to T_1 digestion at the selected sites is due to methylation at the N-7 position of guanine. When techniques used in RNA purification prior to selection were prolonged or repeated, no increased misincorporation levels were seen (data not shown), indicating that error RNAs were indeed generated in vivo.

Error frequency estimates for selected isolates following disruption of population equilibrium. It was of interest to determine error frequencies of virus isolates from populations subjected to various selective pressures. Previous experiments have documented rapid genome evolution of VSV mutant strain *ts*G31 following high-multiplicity passages (6, 22, 30, 42). The virus population at passage 254 is very rapidly evolving based on its ability to escape interference by previously generated defective interfering particles

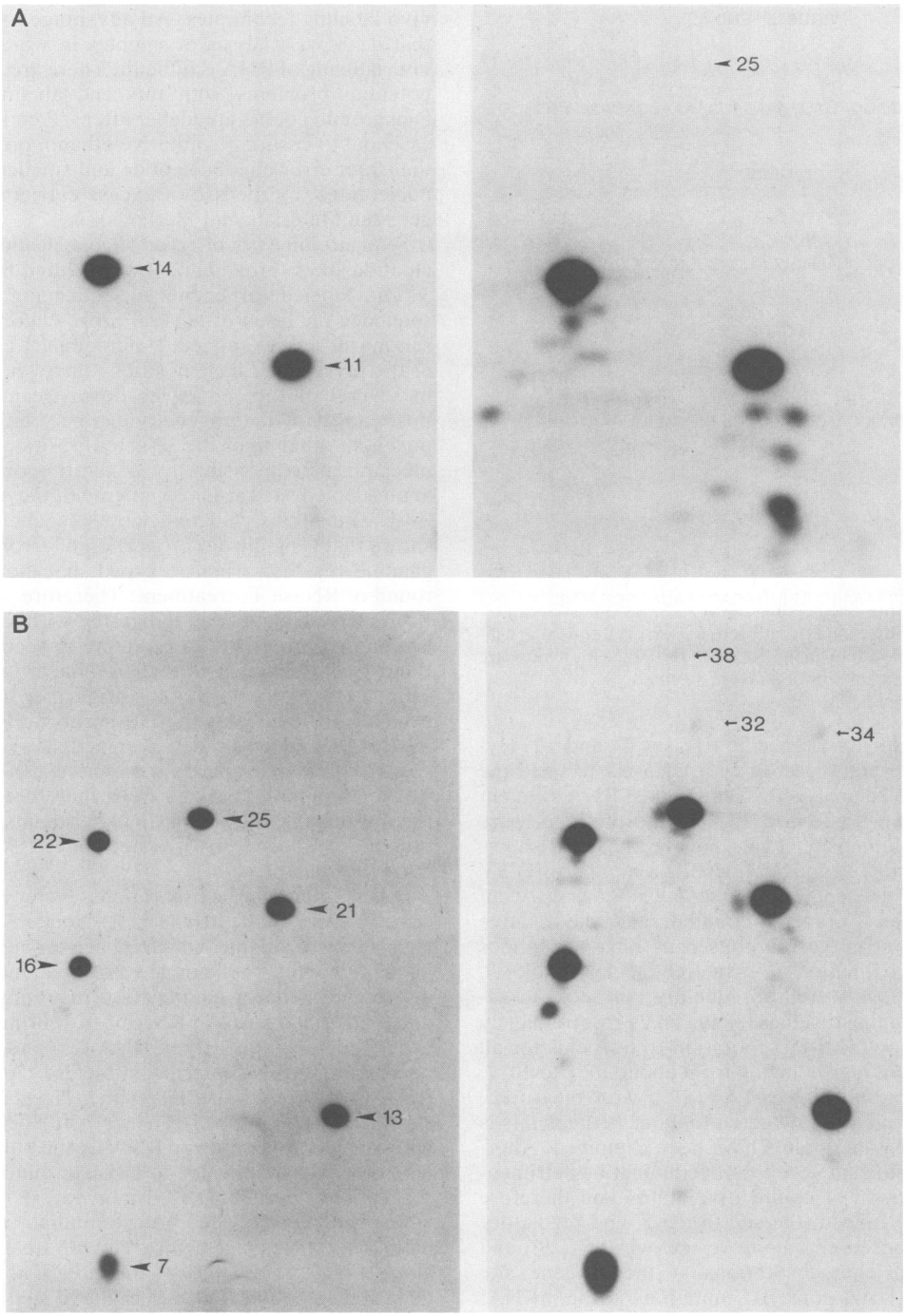

FIG. 2. (A) Normal (left) and very prolonged (right) exposure of two-dimensional gel-separated T_1 digestion products from the L gene site (Fig. 1). Consensus spots are depicted in the normal exposure, and error oligonucleotide is localized in the overexposure. (B) Two exposures of a gel analyzing the T_1 digestion products of three separate VSV genome sites. Consensus oligonucleotides of 21 and 13 bases and their respective 34-base error oligonucleotide are from the 5′-end region. Consensus 25- and 7-mers and 32-base error oligonucleotides are from the N gene sequence, and remaining 22- and 16-mers and 38-base error RNA are from the M gene site.

(6). Oligonucleotide mapping experiments revealed not only that the passage 254 consensus sequence is far removed from that of its progenitor but that individual clones from this population are extremely diverse (see accompanying report [44]). Plaques were isolated from the total population, and one (designated passage 254 clone 1) was chosen for error frequency analysis. Misincorporation frequencies for this clone again averaged between 10^{-3} and 10^{-4}, as did frequencies for its progenitor virus *ts*G31 (Table 1). It would be interesting to analyze the error frequency of the uncloned passage 254 virus population, but this is not possible because defective interfering particle interference depresses virus yields too strongly for such analyses.

Error frequencies were also measured for an isolate from

TABLE 1. VSV polymerase error frequencies[a]

Virus[b]	Genome location	Consensus spot 1 (cpm)	Consensus spot 2 (cpm)	Error oligonucleotide (cpm)	cpm error/cpm consensus	Error frequency estimate
wt clone 1	M gene	26,400	19,500	13	2.8×10^{-4}	2.1×10^{-4}
wt clone 2	M gene	16,600	13,200	9	3.0×10^{-4}	2.3×10^{-4}
wt clone 3	5′ end	11,400	8,400	9	4.5×10^{-4}	3.4×10^{-4}
wt clone 3	M gene	36,000	25,000	32	5.2×10^{-4}	3.9×10^{-4}
wt clone 4	5′ end	25,900	18,200	50	1.1×10^{-4}	8.3×10^{-4}
wt clone 4	M gene	32,600	24,000	58	1.0×10^{-3}	7.5×10^{-4}
wt clone 5	N gene	43,600	12,800	80	1.4×10^{-3}	1.1×10^{-3}
wt clone 6	N gene	113,000	30,800	67	4.6×10^{-4}	3.5×10^{-4}
wt clone 7	N gene	14,900	4,400	10	5.2×10^{-4}	3.9×10^{-4}
wt clone 8	5′ end	20,600	12,800	14	4.2×10^{-4}	3.2×10^{-4}
wt clone 9	5′ end	58,000	36,000	85	9.0×10^{-4}	6.8×10^{-4}
wt clone 9	M gene	26,100	19,200	57	1.3×10^{-3}	9.8×10^{-4}
wt clone 9	N gene	44,900	14,900	29	4.8×10^{-4}	3.6×10^{-4}
wt clone 10	5′ end	31,100	18,600	22	4.4×10^{-4}	3.3×10^{-4}
wt clone 10	M gene	43,500	30,800	180	2.4×10^{-3}	1.8×10^{-3}
wt clone 10	N gene	40,300	11,200	100	1.9×10^{-3}	1.4×10^{-3}
wt clone 11	5′ end	31,100	17,700	72	1.5×10^{-3} (24)	1.1×10^{-3}
wt clone 11	M gene	22,400	15,800	63	1.6×10^{-3} (50)	8.0×10^{-4}
wt clone 11	N gene	29,500	8,300	56	1.5×10^{-3} (12)	1.2×10^{-3}
wt clone 12	5′ end	20,600	13,300	21	6.2×10^{-4}	4.7×10^{-4}
wt clone 12	M gene	23,800	16,100	14	3.5×10^{-4}	2.6×10^{-4}
wt clone 12	N gene	20,800	6,000	30	1.1×10^{-3}	8.3×10^{-4}
*ts*G31 clone 1 (no. 1)	5′ end	34,600	29,000	38	6.0×10^{-4}	4.5×10^{-4}
*ts*G31 clone 1 (no. 1)	M gene	41,100	24,600	68	1.0×10^{-3} (22)	7.8×10^{-4}
*ts*G31 clone 1 (no. 1)	N gene	41,600	10,000	38	7.4×10^{-4}	5.6×10^{-4}
*ts*G31 clone 1 (no. 2)	5′ end	26,400	24,600	29	5.7×10^{-4} (16)	4.8×10^{-4}
*ts*G31 clone 1 (no. 2)	M gene	46,600	25,100	48	6.7×10^{-4} (23)	5.2×10^{-4}
*ts*G31 clone 1 (no. 2)	N gene	36,600	10,800	15	3.2×10^{-4} (34)	2.1×10^{-4}
*ts*G31 clone 1 (no. 3)	5′ end	97,300	64,000	174	1.1×10^{-3} (22)	8.6×10^{-4}
*ts*G31 clone 1 (no. 3)	M gene	84,700	60,500	143	9.8×10^{-4} (29)	7.0×10^{-4}
*ts*G31 clone 1 (no. 3)	N gene	96,100	29,000	142	1.1×10^{-3} (21)	8.7×10^{-4}
*ts*G31 clone 1 (no. 3)	L gene	33,800	24,800	88	1.5×10^{-3} (21)	1.2×10^{-3}
up254 clone 1 (no. 1)	5′ end	16,500	18,400	10	2.9×10^{-4} (23)	2.2×10^{-4}
up254 clone 1 (no. 1)	N gene	23,500	11,600	13	3.7×10^{-4} (15)	3.1×10^{-4}
up254 clone 1 (no. 2)	5′ end	51,100	51,200	48	4.7×10^{-4} (15)	4.0×10^{-4}
up254 clone 1 (no. 2)	M gene	55,300	35,100	51	5.6×10^{-4} (30)	3.9×10^{-4}
up254 clone 1 (no. 2)	N gene	46,900	13,100	24	4.0×10^{-4} (9)	3.6×10^{-4}
up254 clone 1 (no. 3)	5′ end	81,200	54,800	111	8.2×10^{-4} (22)	6.4×10^{-4}
up254 clone 1 (no. 3)	M gene	73,000	53,000	130	1.0×10^{-3} (28)	7.2×10^{-4}
up254 clone 1 (no. 3)	N gene	89,400	28,500	119	1.0×10^{-3}	7.5×10^{-4}
up254 clone 1 (no. 3)	L gene	31,400	24,400	69	1.2×10^{-3} (23)	9.2×10^{-4}
wt 30°C p14 clone 1	5′ end	57,300	42,900	46	4.6×10^{-4}	3.5×10^{-4}
wt 30°C p14 clone 1	M gene	125,300	74,600	173	8.7×10^{-4}	6.5×10^{-4}
wt 30°C p14 clone 1	L gene	43,200	37,600	103	1.3×10^{-3}	9.8×10^{-4}
wt 39°C p2 clone 1	5′ end	49,500	40,300	30	3.3×10^{-4}	2.5×10^{-4}
wt 39°C p2 clone 1	M gene	29,900	20,400	48	9.5×10^{-4}	7.1×10^{-4}
wt 39°C p2 clone 1	L gene	16,400	13,700	26	8.6×10^{-4}	6.5×10^{-4}
5-FU p23 clone 1	5′ end	13,200	10,800	8	3.3×10^{-4}	2.5×10^{-4}
5-FU p23 clone 1	M gene	16,700	12,900	25	8.4×10^{-4}	6.3×10^{-4}
5-FU p23 clone 1	N gene	22,700	8,800	39	1.2×10^{-3}	9.0×10^{-4}
5-FU p23 clone 1	L gene	13,800	13,600	10	3.6×10^{-4}	2.7×10^{-4}
wt clone 4 (5′)	5′ end	1,170,000	1,650,000	1,250	8.9×10^{-4}	6.7×10^{-4}
wt clone 13 (5′)	5′ end	247,000	451,000	63	1.8×10^{-4}	1.4×10^{-4}
wt clone 13 (5′)	N gene	161,000	234,000	154	8.0×10^{-4}	6.0×10^{-4}
wt clone 14 (3′)	5′ end	47,000,000	62,000,000	80,000	1.5×10^{-4} (29)	1.1×10^{-4}
wt clone 14 (3′)	M gene	50,000,000	39,000,000	27,000	6.1×10^{-4} (33)	4.1×10^{-4}
wt clone 14 (3′)	N gene	19,000,000	12,000,000	25,000	1.6×10^{-3} (41)	9.4×10^{-4}

[a] Cerenkov counts in indicated oligonucleotides and calculation of substitution frequencies. Percentages in parentheses indicate amount of uncut G (other approximations were corrected by 25%). The last six samples were labeled after T_1 digestion at the terminus indicated. With these samples, first approximations were determined by dividing counts in the error oligonucleotide by the average number of counts in the two consensus oligonucleotides.

[b] wt, Wild type; up, undiluted passage; p, passage.

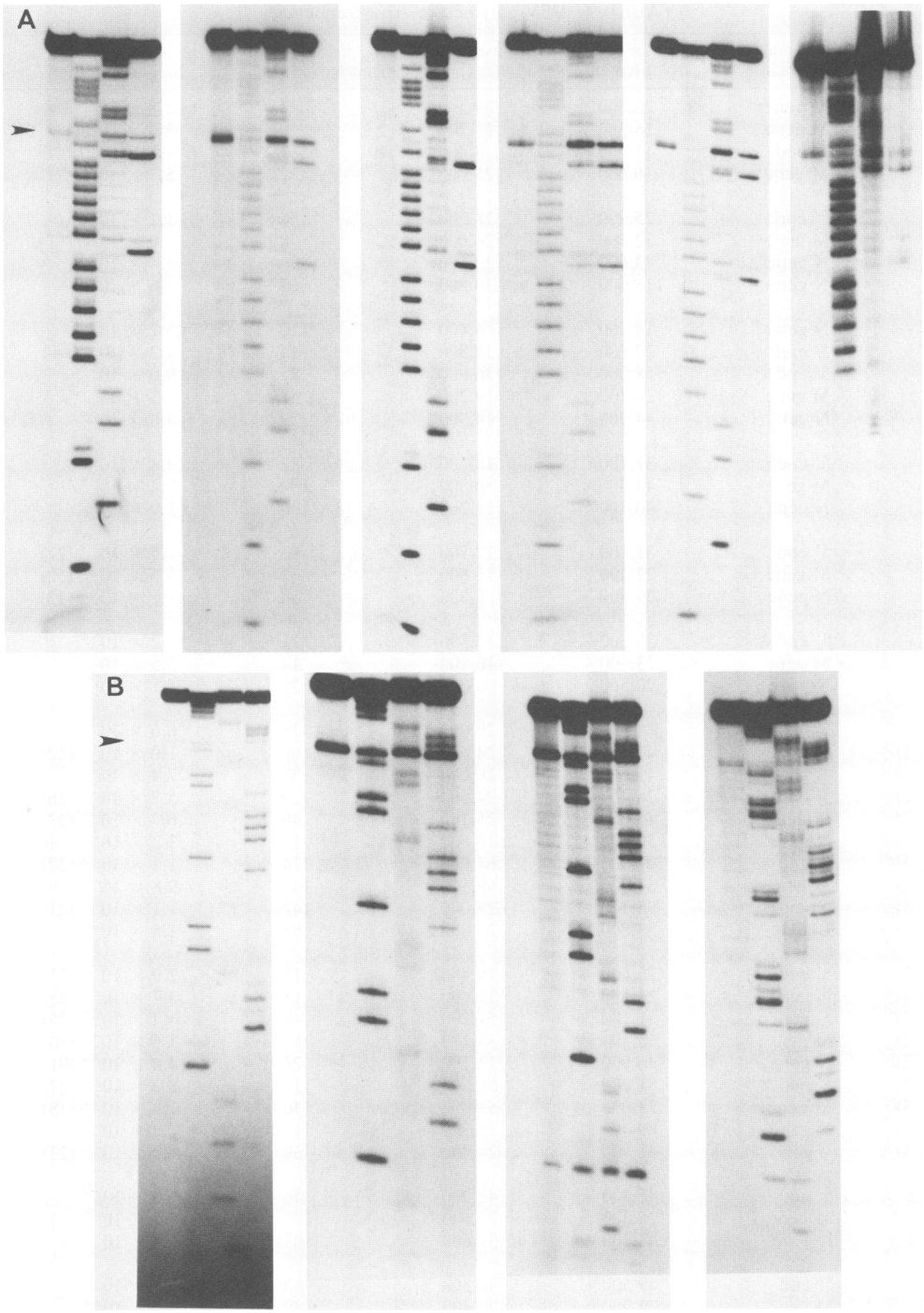

FIG. 3. (A) Chemical sequencing gels of VSV genome 5′-end error oligonucleotides. (B) Sequencing gels of error oligonucleotide from the N gene site. (C) Error oligonucleotide sequences from sites in the M gene and L gene. All sequencing gels were loaded in the order G, A, C, U from left to right. Arrowheads are discussed in the text.

a virus population which had been propagated in the presence of the mutagen 5-FU (36). Wild-type VSV was passaged 23 times in the presence of 200 μg of 5-FU per ml. No substantial difference in error frequency was detected with this clone (Table 1), although various clones from this population revealed oligonucleotide map changes (44).

Flamand (19) has documented a transient increase in temperature-sensitive mutants of VSV following temperature shift from 30 to 39.3°C, peaking at 39.3°C passage 2. We isolated a clone from virus repeatedly passaged at 30°C, passaged it twice at 39.5°C, and cloned an isolate for base substitution frequency measurements. No significant difference was detected between the clone isolated at 30°C and the clone isolated after two passages at 39.5°C (Table 1). In all experiments with isolates from disequilibrated virus populations, the mutagenic chemical or high temperature influences

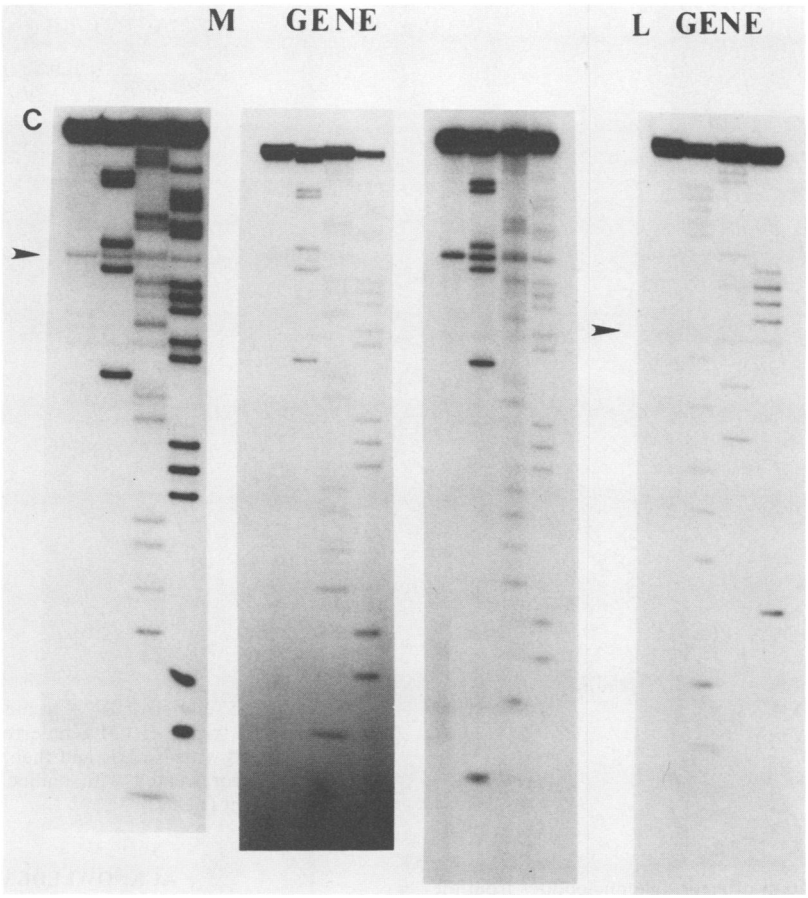

FIG. 3—*Continued*

had to be removed during virus isolation and error frequency analysis (see Materials and Methods). The results do not mean that the base analog or temperature shift is not mutagenic (indeed they are) but that virus isolates studied following such mutagenesis were not detectably more error prone.

DISCUSSION

The results document high nucleotide substitution error frequencies at all sites examined in clonal pools of VSV. Misincorporation frequencies ranged from 1.8×10^{-3} to 1.1×10^{-4} at selected guanine residues in populations of wild-type and mutant virus. At present, we have data only for G residues, and it is not known whether these data reflect mutation frequencies throughout the genome. Mutational hot spots are well known for DNA polymerases (3, 5, 23). We are currently attempting several approaches to analyze mutation frequencies at non-G residues. Experiments with RNase A or RNase U2 have not been successful to date.

If misinsertion frequencies at other VSV genome sites are similar to those analyzed in this study, then any virus clonal population must be composed of an extremely heterogeneous collection of mutants (10, 11). The maintenance of consensus sequences must result from founder effects as well as from selective pressure under given conditions. Results with phage Qβ indicate that RNA virus populations replicating under stable conditions can reach a state of relative equilibrium with mutants being generated at a high rate and then being quickly outgrown (12). Similar results have been seen in our laboratory, where wild-type VSV Indiana serotype has been passaged over 500 times with no changes in the consensus oligonucleotide map (44).

Virus populations sampled for misincorporation frequency analysis in this study would include all virus particles able to mature and bud out of cells during passage 2 of a clone. These would include a spectrum of debilitated and lethal mutants. Particle-to-infectivity ratios for VSV and many other RNA viruses are typically very high. Thus, error frequencies measured by this direct sequence method would be higher than those measured by methods involving characterization of viable, competitive mutant clones. Virus particles which are debilitated under one set of environmental conditions may be quite competitive in a different environment.

It is worth noting that an unknown but possibly significant proportion of mutations observed in this study may have been generated by mutated, error-prone subsets of viral polymerase. This might explain the second site heterogeneity seen in some error oligonucleotides. Mutator mutants of VSV have been isolated (37), and evidence for repetitive polymerase error by a subset of highly mutagenic polymerase molecules has been documented for VSV (31) and measles virus polymerase (4a). It is interesting that when the equilibrium of our virus populations was disrupted by defective interfering particles, temperature shift, or 5-FU, the resulting progeny clones examined exhibited misincorpora-

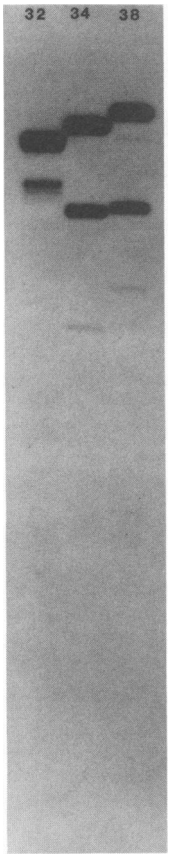

FIG. 4. A 20% gel separation of error oligonucleotides from the N (32-mer) and M (38-mer) genes and 5′ end (34-mer) following a second digestion with RNase T_1. The lower bands represent the proportion of isolated error oligonucleotides which were due to previously undigested G residues.

tion frequencies very similar to those of the wild type. The mutagenic effects of 5-FU and temperature shift have been documented (19, 36), and a role for VSV defective interfering particles in driving virus evolution has been demonstrated (6, 22). However, when mutagenic or selective pressure was removed (as was necessary for error frequency determination), we observed virus polymerase fidelities similar to those of wild-type virus. Exceedingly error-prone polymerases might appear frequently during virus replication, but survival of direct descendants of such a virus would necessitate the reversion of polymerase fidelity to a reasonable level.

Our results appear to support the quantitative treatment of RNA virus "quasispecies" populations by Eigen and Biebricher (15). Virus replicase fidelity may hover near its error threshold—a point at which viability is ensured for a substantial percentage of progeny and sequence information can be maintained, but at which generation of an enormous spectrum of mutants is ensured, thereby allowing for easy adaptation. The composition of these quasispecies populations is determined by competitive fitness under prevailing conditions. This allows RNA viruses to maintain themselves stably in a given ecological niche, while retaining the ability to adapt quickly to exploit new environmental niches or changing conditions.

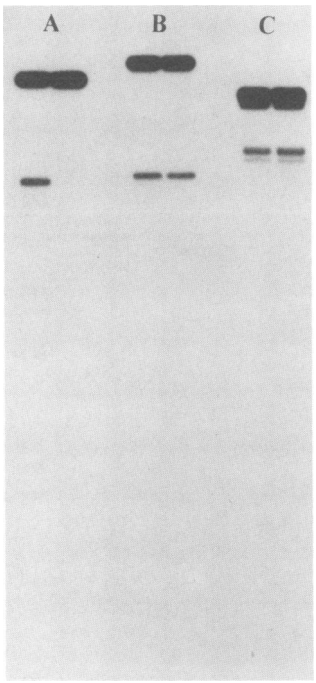

FIG. 5. (A) 5′-end error RNA treated with aniline (left) or with exonuclease III (right). (B) M gene error RNA treated with aniline (left) or reduced with $NaBH_4$ and then treated with aniline (right). (C) N gene error treated with aniline (left) and after prolonged aniline treatment (right).

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