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# Direct Method for Quantitation of Extreme Polymerase Error Frequencies at Selected Single Base Sites in Viral RNA

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**Methods are described which allow direct quantitation and sequence analysis of base substitution levels at predetermined single nucleotide positions in cloned pools of an RNA virus genome or in its RNA transcripts in vitro. Base substitution frequencies for vesicular stomatitis virus (VSV) at one highly conserved site examined were reproducible and extremely high, averaging between $10^{-4}$ and $4 \times 10^{-4}$ substitutions per base incorporated at this single site. If polymerase error frequencies averaged as high at all other sites in the 11-kilobase VSV genome, then every member of a cloned VSV population would differ from most other genomes in that clone at a number of different nucleotide positions. The preservation of a consensus sequence in such variable RNA virus genomes then could only result from strong biological selection (in a single host or multihost environment) for the most fit and competitive representatives of extremely heterogeneous virus populations.**

High mutation frequencies have been observed in RNA viruses since the earliest genetic studies (16, 19, 20, 33, 42). Domingo et al. (11, 12) showed that the mutation rate of RNA phage Qβ can exceed $10^{-4}$ base substitutions per base incorporated. In concert with these high mutation frequencies, RNA animal viruses often exhibit rates of evolution which can exceed by a millionfold the rates of evolution of the chromosomal DNA of their eucaryotic hosts. This rapid evolution of viral RNA genomes has been reviewed in detail (23). The rapid evolution in nature of influenza viruses due to mutations and reassortment of genome segments is a widely recognized phenomenon (38, 56, 58). It is now clear that many other RNA viruses can undergo rapid evolution during outbreaks in their natural hosts. This has been observed with vaccine strain outbreaks of poliovirus during spread from Europe to Canada and the United States (35), in foot-and-mouth disease outbreaks (10, 28), and in enterovirus 70 outbreaks of acute hemorrhagic conjunctivitis which started in Ghana and spread over much of the world (55). In all of these outbreaks, the evolution of viral genomes occurred at the rate of 0.1 to 1% of base substitutions (or more) per year. Kew et al. (27) even observed evidence for more than 100 base changes in poliovirus vaccine genomes during replication in one or two humans.

In contrast to the above, there is evidence for considerable stability of virus RNA genomes under certain environmental conditions. The most remarkable case is the reappearance of H1N1 influenza A viruses in the human population in 1977, which were extremely similar in genome sequence to strains which had circulated in 1950 (26, 34). Yet these reemerged 1977 strains exhibited the expected rapid rates of genome evolution as they spread among humans during 1977 (58). There is also evidence that some plant viruses have not changed greatly between 80-year-old herbarium isolates and present-day isolates (A. Gibbs, Abstr. Sixth Int. Congr. Virol. p. 12, 1984). In laboratory studies with cloned RNA viruses infecting animal cells, either rapid genome evolution or relative genome stability can be observed depending on the virus, the cells, conditions of passage, etc. (2, 22, 45, 53, 54, 59). Pringle et al. (43) described a temperature-sensitive mutant of vesicular stomatitis virus (VSV) with enhanced mutability (i.e., a mutator mutant), and Flamand (18) observed increased mutation frequency when VSV replicated previously at 30°C was passaged at 39.3°C. Therefore, host, virus, and environmental differences may greatly alter rates of RNA virus mutation and rates of virus evolution. Instances of exceptional virus genome stability (such as influenza virus H1N1 reappearance) could result from strongly stabilizing selective pressures in certain hosts or simply from virus recirculation following a period in a dormant (frozen or dried) state.

There are obvious explanations for the very high mutation frequencies observed in RNA viruses. (i) No proofreading enzyme system has been found associated with any viral (or other) RNA polymerase. (ii) The intrinsic error rate for RNA polymerase may be very high (we show below that it is). Drake (13) calculated that mutation rates per base-pair replication are considerably greater in organisms with smaller DNA genomes. He postulated that, whereas larger genomes pay considerable costs to achieve low mutation rates, smaller genomes can devote fewer resources to reducing mutations and might not always benefit from reduced mutation rates. Whereas the rate of base substitution in DNA genomes is in the range of $10^{-11}$ to $10^{-8}$ misincorporations per base replicated (13), RNA virus genetics suggests that it is much higher for RNA viruses (23). Reverse transcriptases of RNA retroviruses exhibit base misincorporation frequencies exceeding $10^{-4}$ in vitro (17, 30), and this high error rate is reflected in high spontaneous base substitution frequencies in vivo (8). Base substitution frequencies for RNA-directed RNA polymerases of nonretrovirus RNA viruses have not been measured directly, but the studies of Domingo et al. (11, 12) indicate that Qβ phage replication involves base substitution rates of about $10^{-4}$ misincorporations per base incorporated.

Sobrino et al. (53) estimated by T1 ribonuclease mapping of clones of foot-and-mouth disease virus that genomes of in vitro-passaged virus pools differ in from two to eight mutations from the average parental sequence. This agrees with the extreme variability observed in field isolates of foot-and-mouth disease virus (10, 28).

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Schubert et al. (46) sequenced multiple overlapping clones of the VSV L protein (polymerase) gene and detected 16 point mutations in its 6,380 nucleotides, although the template RNA came from cloned virus. Many or most of these cDNA clone differences might have arisen from reverse transcriptase error during cDNA synthesis, but at least one of the mutations almost certainly was due to microheterogeneity at one base position in the VSV genomes present in the cloned virus stock. They detected the identical single base deletion in two of four clones covering that segment of the VSV genome, and it is very unlikely that reverse transcriptase made the identical error twice. Since the L protein spans somewhat more than half of the VSV genome, a minimum frequency of $10^{-4}$ to $2 \times 10^{-4}$ misincorporations per base incorporated can be estimated (46). Based on data we present below, it is quite possible that more than one or two of the mutations were due to VSV polymerase error.

E. P. Geiduschek suggested to us that the use of highly specific ribonucleases might allow direct determination of the level of substituted bases at given positions in a viral RNA population. We describe below techniques for direct sequence analysis of site-specific base substitutions in viral RNA and in viral transcriptase product RNA with specific ribonucleases to quantitate and sequence error oligonucleotides present among consensus oligonucleotides.

## MATERIALS AND METHODS

**Cells, virus, and DI particles.** Monolayers of baby hamster kidney (BHK-21) cells grown in Eagle minimum essential medium with 7% calf serum were used for all virus and defective interfering (DI) particle propagation. The wild-type virus used was the Mudd and Summers (32) strain of Indiana VSV cloned nine times consecutively plaque to plaque. Clonal virus pools used here were prepared by one passage of the final plaque virus on BHK-21 cells. DI particles were generated by high-multiplicity passages of cloned virus and were amplified by coinfection of cells with DI particles and wild-type virus. Procedures for virus and DI particle growth and purification have been previously described (24).

**In vitro transcription.** DI particles to be used in transcriptase reactions were subjected to an extra purification step by banding on continuous gradients of 20 to 45% potassium tartrate. Tartrate was removed by dialysis. Ribonucleoprotein cores (nucleocapsids) were prepared essentially as described by Breindl and Holland (3). DI particles at a final concentration of 0.5 mg/ml were incubated in 0.5% Nonidet P-40–50 mM Tris hydrochloride (pH 8.0)–5 mM magnesium acetate–0.1% 2-mercaptoethanol–250 mM potassium acetate at 0°C for 10 min. Samples were layered over discontinuous gradients of 30 and 50% glycerol in 50 mM Tris hydrochloride (pH 8.0) in SW 50.1 tubes. Nucleocapsids were pelleted at 48,000 rpm for 2 h at 4°C and used immediately.

In vitro transcriptase reactions were carried out with purified nucleocapsids in 0.5-ml reaction volumes containing 50 mM Tris acetate (pH 8.2)–8 mM magnesium acetate–3 mM dithiothreitol–1 mM each of ATP, CTP, GTP, UTP, and the $\alpha$-$^{32}$P nucleoside triphosphate label. After incubation for 3 h at 30°C, reactants were digested with proteinase K and extracted with phenol-chloroform. Transcriptase products were then purified by Sephadex G-50 chromatography and 20% polyacrylamide gel electrophoresis.

**Isolation of virus 5′-end RNA.** Wild-type VSV was labeled in vivo with $^{32}$P, and RNA was purified as described by Holland et al. (22). A synthetic DNA complementary to bases 2 through 43 at the 5′ end of the VSV genome was synthesized, purified, and annealed to labeled RNA (and 20 to 100 μg of cold virus RNA) at a 3-to-1 molar excess. Hybridizations were carried out in 100 mM Tris hydrochloride (pH 8.3)–140 mM KCl–16 mM $MgCl_2$. Single-stranded RNA unprotected by DNA was digested for 1 h at 37°C with RNase T1 (20 μg/ml) and (where indicated in the text) pancreatic RNase A (1 to 25 μg/ml). Samples were proteinase K digested, phenol-chloroform extracted, and ethanol precipitated, and DNA-RNA hybrids were purified on a 20% polyacrylamide gel. Double-stranded DNA-RNA hybrids were eluted from the gel, and the DNA was digested by treatment with DNase I (20 μg/ml) for 30 min at 37°C. The temperature was gradually raised to 55°C for the last 5 min of incubation. Remaining RNA was isolated by proteinase K digestion, phenol-chloroform extraction, and multiple ethanol precipitations. It was then digested with T1 RNase and analyzed on polyacrylamide gels as described below.

**Isolation of DI stems.** In vivo labeling and RNA purification of DI particles were done as with wild-type VSV, except that cells were coinfected with infectious virus and DI particles. DI particle RNA in 10 mM Tris hydrochloride (pH 7.6) was heated to 100°C for 10 s, the salt concentration was raised to 300 mM NaCl–5 mM $MgCl_2$, and RNA was quickly cooled on ice to anneal complementary termini. Samples were then digested with nuclease, and stems were purified as described for DNA-RNA hybrids (with the exclusion of DNase I digestion).

**T1 RNase digestion and two-dimensional separation of oligonucleotides.** Labeled RNA samples were digested with 1 mg of RNase T1 per ml in 10 mM Tris hydrochloride (pH 7.6) at 37°C for 30 min. The 20-μl reaction volumes were transferred to new microcentrifuge tubes at the start of digestion and once more during digestion to eliminate sequestering of RNA on tube surfaces not also contacted by T1 RNase. DI particle stem samples were heated momentarily to 100°C following addition of T1 RNase and twice during incubation to denature double strands and allow complete digestion (RNase T1 is resistant to boiling for short periods). B virion 5′ ends were also heated once to denature any regions where possible residual DNA fragments might remain hybridized. Samples were immediately loaded onto the first dimension of the two-dimensional gel system developed by DeWachter and Fiers (9). The modifications of this technique which were used have been reported previously (22).

**Sampling and counting of $^{32}$P-labeled consensus and error oligonucleotides.** All scintillation vials and Eppendorf tubes to be used were carefully precounted. Gel slices of equal size containing the oligonucleotides to be counted and several background samples of equal size were excised from two-dimensional gels and Cerenkov counted in precounted tubes and vials. All samples were counted for at least 50 min to ensure a random error of less than 10% for background samples and 5% for error RNA. All samples were counted at least twice in this manner. Counts in background samples excised from blank adjacent areas of each gel were averaged, and the average was subtracted from counts in gel slices containing oligonucleotides to give the final number used in calculations. Background samples consistently had less than 10 cpm, and in many gels no background counts were detectable.

**RNA sequencing.** RNA to be sequenced was 3′ end labeled by ligation of 5′ [$^{32}$P]Cp with RNA ligase (15). Terminally labeled RNA was purified on preparative gels of 20% acrylamide–8 M urea and sequenced by the chemical method described by Peattie (39).

## RESULTS

**Direct error oligonucleotide approach for separation, quantitation, and sequencing of base substitutions at a selected single site.** A variety of VSV DI particles transcribe a small, unique, A-rich RNA in vitro (14, 44, 49, 50). The sequence of this 46-base endogenous polymerase product has been determined (49, 50) (Fig. 1). Complete digestion of this transcript with RNase T1 (which cleaves specifically at guanine residues) yields two large consensus oligonucleotides, one of 21 and the other of 13 bases. Since these T1 fragments are adjacent in the transcript sequence, misincorporation of any nucleotide other than guanine at the position indicated (Fig. 1) results in a 34-base T1-resistant error oligonucleotide. Separation and quantitation of the relative amounts of error oligonucleotide and the two consensus oligonucleotides allowed us to estimate the in vitro base substitution frequency at this highly conserved position. A sequence exactly homologous to the DI transcript is present at the 5′ end of the DI genome and also at the 5′ terminus of the genome of infectious virus from which this DI particle was generated (29, 31, 36, 37, 40, 48, 49–51, 57). We describe methods for isolating this sequence from complete viral and DI particle genomes with complementary synthetic DNA oligonucleotides, for determining relative amounts of error and consensus oligonucleotides, and for sequencing of error oligonucleotide. We chose this single guanine site for initial analysis because it is present in short DI particle transcripts in vitro and in virus and DI genomes and because it is strongly conserved in VSV Indiana, Cocal, Piry, and Chandipura, serotypes isolated in many areas of the world over many decades (S. T. Nichol and J. J. Holland, unpublished data). Only VSV New Jersey (which has an eight-base insertion at this position) differs among the vesiculoviruses sequenced to date. It is also conserved in evolving VSV Indiana serotype exhibiting extensive base substitutions at termini (36, 37). We show below that, despite its strong conservation, this guanine site is subject to extreme frequencies of base substitution in virus and DI genomes.

**In vitro nucleotide substitution frequencies in DI-particle polymerase product.** DI particles generated by the wild-type Indiana serotype of VSV were used for transcriptase reactions. In vitro reactions were performed in the presence of one or more $\alpha$-$^{32}$P-labeled nucleoside triphosphates, and products were separated by polyacrylamide gel electrophoresis (Fig. 2). Purified 46-base transcripts were digested with RNase T1, and resistant oligonucleotides were separated by gel electrophoresis. The major T1 fragments, as well as 34-base error oligonucleotides, can be seen in Fig. 3. To minimize any problems in quantitation which might arise owing to high or uneven background counts in one-

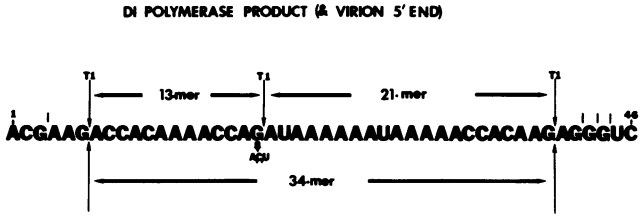


FIG. 1. Sequence of DI particle 46-base in vitro transcript (and 5′ terminal sequence of virus and DI genome RNAs). The arrows show RNase T1 cleavage sites and resulting large oligonucleotides when guanine is correctly incorporated (13-mer and 21-mer) or when a base substitution has occurred at the site studied (34-mer).

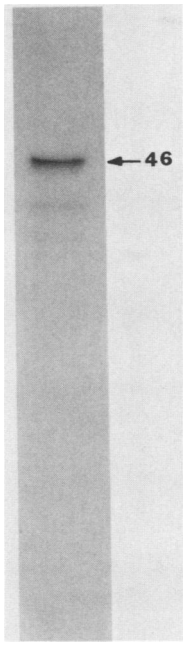


FIG. 2. DI particle in vitro polymerase products. DI particle nucleocapsids were incubated in the presence of [$\alpha$-$^{32}$P] ribonucleotide triphosphates (see Materials and Methods), and transcription products were separated on 20% acrylamide gels. The arrow indicates the main 46-base transcript studied.

dimensional gels, we separated oligonucleotides on large, two-dimensional polyacrylamide gels as described by DeWachter and Fiers (9). Figure 4 shows a normal exposure and an overexposure of films of a T1-digested polymerase product on two-dimensional gels. Normal exposure shows the major consensus spots corresponding to 13- and 21-base oligonucleotides. Overexposure of the gel reveals the 34-base error spot (arrow). Major consensus oligonucleotides and error oligonucleotide were excised from gels and carefully quantitated by Cerenkov counting of $^{32}$P in each (with subtraction of background counts present in adjacent areas of the gels). The proportion of corrected counts in the error oligonucleotide to counts in consensus oligonucleotides was used to calculate the primary data for estimating the nucleotide substitution frequencies at the selected guanine position (Table 1). Primary data alone lead to an overestimate of nucleotide substitution frequencies, because they cannot distinguish between counts in error spot due to polymerase error and counts due to failure of T1 cleavage at correctly incorporated guanine (which possibly results from protective effects of residual polyacrylamide, modification of RNA, or secondary structure).

Several samples of error RNA were 3′ end labeled by ligation to [$^{32}$P]Cp and sequenced by the chemical method of Peattie (39). Sequencing gels of these samples and of undigested 46-base transcript are shown in Fig. 5. Analysis of the sequences at the single base site selected (arrows) show that error RNA is heterogeneous at that position. It contains uncut G residues and misincorporated C, U, and A residues. Note also that the site 2 bases downstream from the selected site shows clear heterogeneity due to a high level of U → C transitions. This may be due to polymerase-template perturbation as the polymerase rides over the template-nascent strand mismatch at the error site, or it may simply be due to repetitive polymerase error.

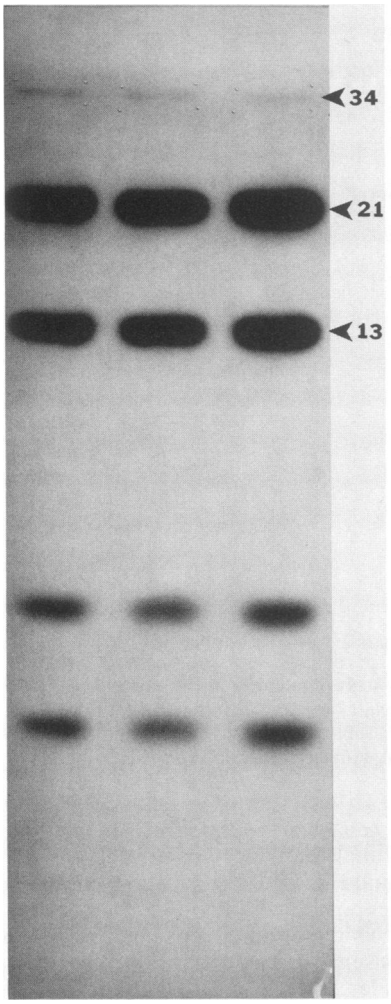


FIG. 3. Products of T1 ribonuclease-digested 46-base DI transcript. Samples of purified DI transcript were digested with RNase T1, and resistant oligonucleotides were separated on 20% acrylamide gels. Major 13- and 21-base consensus oligonucleotides and 34-base error oligonucleotide are indicated in this overexposed gel autoradiograph.

An effort was made to estimate the amount of uncut G in two error oligonucleotides by redigesting with RNase T1 and calculating the proportion of RNA cleaved by the second treatment. Error RNA was 5′ end labeled with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase and gel purified. RNA was then redigested with T1 RNase, and the products were separated by gel electrophoresis (Fig. 6). Bands corresponding to 34-mer and 13-mer (21-mer is not end labeled) were excised from the gel and counted, and ratios were used to estimate the amount of previously uncut G. Calculations on the two samples tested showed that approximately 35 and 50% of error RNAs were cleaved by the second digestion. These data, along with sequence analysis, indicate that the primary data do overestimate the nucleotide substitution frequency, but most often by about a factor of 2. It is clear that base misincorporation frequencies by DI particle polymerase in vitro are between $10^{-3}$ and $10^{-4}$, the average from our data being about $7 \times 10^{-4}$ (Table 1). It is also obvious that the second error U → C transition 2 bases downstream from the selected site occurs at a frequency between $10^{-3}$ and $10^{-4}$.

**In vivo nucleotide substitution frequencies in wild-type virus genomes.** The RNA sequence at the 5′ end of the VSV genome has exact sequence homology to the DI polymerase product (36, 37, 40, 48–51, 57). However, the very complex oligonucleotide maps produced by intact viral and DI genomes (5, 22; see Fig. 8A) cause high backgrounds in greatly overexposed two-dimensional gels, which make it difficult to isolate error oligonucleotides cleanly as was done with DI particle polymerase product. We therefore used synthetic DNA oligonucleotides that are complementary to the genome segment to be examined. These protect against the nuclease used to greatly reduce the background caused by $^{32}$P-labeled nonrelevant oligonucleotides and thus allow accurate determination of error levels at specific sites of virus and DI genomes replicated and labeled in vivo.

VSV was labeled in vivo with $^{32}$P during replication of a first-passage cloned virus pool or during replication of virus picked directly from a plaque, and its RNA was purified. A chemically synthesized oligonucleotide complementary to bases 2 through 43 at the 5′ end of the genome was annealed

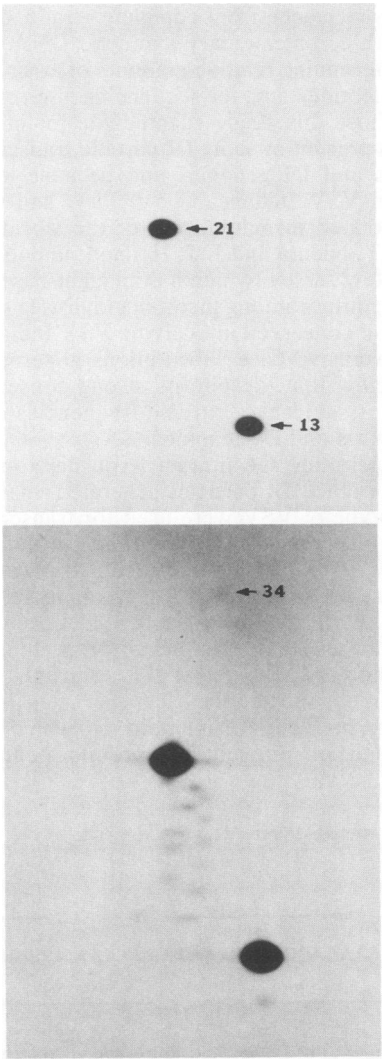


FIG. 4. Two-dimensional gel separation of T1-digested 46-base polymerase product. (A) Normal exposure of this gel shows 13- and 21-base consensus oligonucleotides. (B) Overexposure of the same gel reveals the 34-base error oligonucleotide (arrow).

TABLE 1. Nucleotide substitution frequencies in vitro

| Sample no. | α-$^{32}$P-labeled nucleoside triphosphate | 13-mer cpm | 21-mer cpm | 34-mer cpm | 34-mer cpm / (13-mer + 21-mer cpm) | Substitution frequency[a] |
|---|---|---|---|---|---|---|
| 1 | ATP | 9,500 | 25,500 | 58 | $1.7 \times 10^{-3}$ | $8.5 \times 10^{-4}$ |
| 2 | C, G, UTP | 141,000 | 155,000 | 161 | $5.4 \times 10^{-4}$ | $2.7 \times 10^{-4}$ |
| 3 | C, G, UTP | 80,500 | 94,500 | 262 | $1.5 \times 10^{-3}$ | $7.5 \times 10^{-4}$ |
| 4 | ATP | 55,000 | 131,000 | 215 | $1.2 \times 10^{-3}$ | $6.0 \times 10^{-4}$ |
| 5 | ATP | 83,500 | 157,000 | 210 | $8.7 \times 10^{-4}$ | $4.4 \times 10^{-4}$ |
| 6 | ATP | 58,000 | 105,000 | 176 | $1.1 \times 10^{-3}$ | $5.5 \times 10^{-4}$ |
| 7 | ATP | 67,500 | 158,000 | 459 | $2.0 \times 10^{-3}$ | $1.0 \times 10^{-3}$ |
| 8[b] | ATP | 31,500 | 72,000 | 220 | $2.1 \times 10^{-3}$ | $1.1 \times 10^{-3}$ |

[a] Substitution frequencies were derived from primary data with 50% as an estimate of uncut G residues in error oligonucleotides. Estimates were based on analysis of sequencing gels and experiments in which error oligonucleotide was redigested with RNase T1 (Fig. 5 and 6). Average, $7.0 \times 10^{-4}$; one standard deviation, $3.5 \times 10^{-4}$.

[b] The DI particle used for this transcription reaction was the small ST-1 particle generated by temperature-sensitive mutant *tsG31* (Pringle [42]).

to RNA, protecting that region from degradation by single-strand-specific nuclease(s). Nonannealed single strands were then digested with T1 RNase, either alone or in conjunction with various amounts of pancreatic RNase A. DNA-RNA hybrids were purified on 20% acrylamide gels (Fig. 7) and treated with DNase I to remove DNA. The recovered RNA segment was then T1 digested and run on two-dimensional gels (Fig. 8B and C). Also shown in Fig. 8 is a two-dimensional gel on which T1-digested total genomic RNA was run and consensus oligonucleotides studied are identified. Normal exposure (Fig. 8B) again shows the two major oligonucleotides, and overexposure (C) reveals the error oligonucleotide. These were excised from the gel, $^{32}$P counts were determined, and nucleotide substitution frequencies were calculated as described above (Table 2).

It must be noted that a nucleotide substitution at the guanine position being studied leaves a single base mismatch in the DNA-RNA hybrid. Cleavage of certain single base mismatches is known to occur (52), and it is critical for our purposes to avoid mismatch cleavage which would affect error frequency determinations. Should nuclease cut at the mismatched base of an error oligonucleotide paired to DNA, it would select against this RNA during isolation and ultimately lead to an underestimate of substitution frequency.

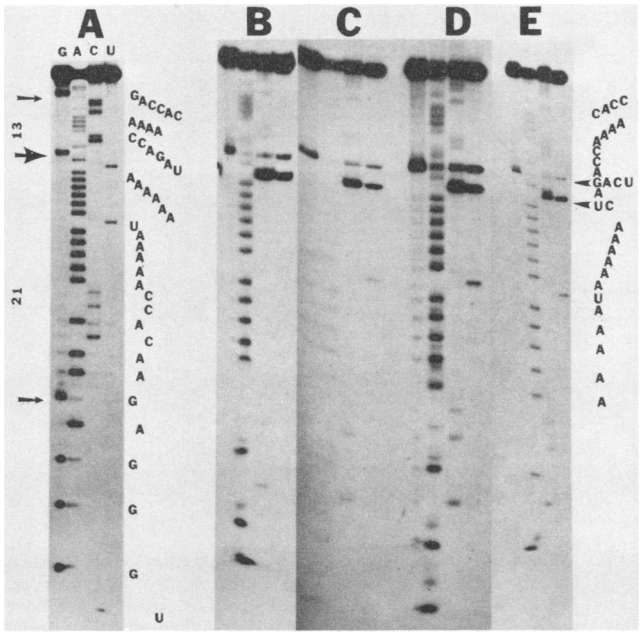


FIG. 5. Sequencing gels of 46-base DI transcript and error oligonucleotides. (A) Sequence of the major DI transcript which has not been treated with RNase T1. Arrows indicate guanine residues available for T1 digestion. The large arrow indicates the guanine site being studied. (B through E) Sequencing gels of several error oligonucleotides. Note the heterogeneity at the guanine site studied (arrows) as well as the U-C heterogeneity 2 bases downstream.

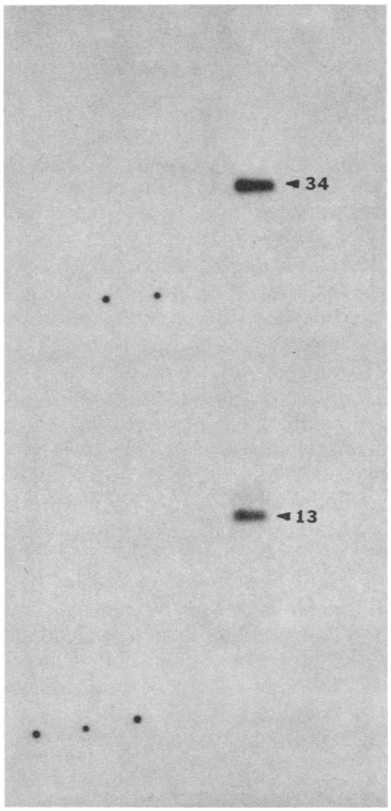


FIG. 6. Error oligonucleotide redigested with RNase T1. Error oligonucleotide was 5′ end labeled with [γ-$^{32}$P]ATP and polynucleotide kinase, gel purified, eluted, and redigested with RNase T1, and the products were separated on 20% acrylamide gels. The proportion of counts in 13-mer to 34-mer was used to estimate the percentage of previously uncut G residues in error oligonucleotide.

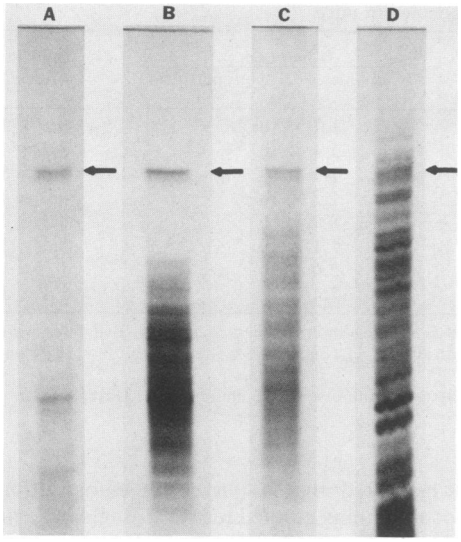


FIG. 7. DNA-RNA hybrid gels for purification of the virus genome RNA sequence to be examined. Genomic RNA was hybridized to a synthetic DNA oligonucleotide complementary to the RNA sequence of interest. Unprotected single strands were digested with RNase T1 (20 μg/ml) and the following amounts of RNase A (lanes): A, 25 μg/ml; B, 8 μg/ml; C, 1 μg/ml; and D, no RNase A (T1 only). DNA-RNA hybrids were then separated on 20% acrylamide gels. When RNase T1 alone was used, a small portion of the sample was removed, digested with RNase A also, and then run in the next lane as a marker for hybrids. The arrows indicate the DNA-RNA hybrid bands.

However, T1 RNase is specific for guanine, so single base mismatches due to misincorporations at this site would not be T1 cleaved. When RNase A (specific for pyrimidines) was used in addition to RNase T1 during isolation of DNA-RNA hybrids, greater purification of the sequence of interest was possible (Fig. 7); however, subsequent recovery of error oligonucleotides was reduced (Table 2). This was likely due to cleavage of error oligonucleotides at the mismatched site of DNA-RNA hybrids by RNase A. Therefore, it is essential that T1 RNase be employed during isolation of RNA-DNA hybrids for error level determinations at a site normally containing guanine. For sites not normally containing guanine, another specific nuclease must be chosen to avoid cleavage at mismatched bases.

$^{32}$P-labeled error RNA from a cloned virus genome preparation was isolated with only RNase T1, 3′ end labeled, and sequenced chemically (Fig. 9). Sequence analysis indicated that approximately 50% of this RNA was due to uncut G residues and that the rest was due to nucleotide misincorporations. Note that misincorporation can be seen 2 bases downstream from the mistake-selected site, mainly owing to U → C transitions. The data from five separate determinations with DNA-RNA hybrids digested only by RNase T1 are presented in Table 2. It is clear that cloned virus genome RNA replicated in vivo exhibited extreme base substitution frequencies (greater than $10^{-4}$). The average of these (after correction for approximately 50% uncut G residues) was $2 \times 10^{-4}$.

**In vivo nucleotide substitution frequencies in DI particle genome RNA.** The DI particle used was a stem-type DI particle derived from the 5′ end of infectious VSV; therefore, the sequence at the 5′ terminus is identical to that of the virus 5′ terminus. We isolated DI particle 5′ termini and again determined base substitution frequencies at the analogous guanine position.

The 3′ end of this DI particle (which codes for DI transcript) is the exact complement of the 5′ end for approximately 50 nucleotides. Heating of DI particle RNA and quick cooling in high-salt solution results in panhandle or stem-type molecules in which the complementary termini are base paired (29, 31, 40, 47, 48). We isolated RNA from

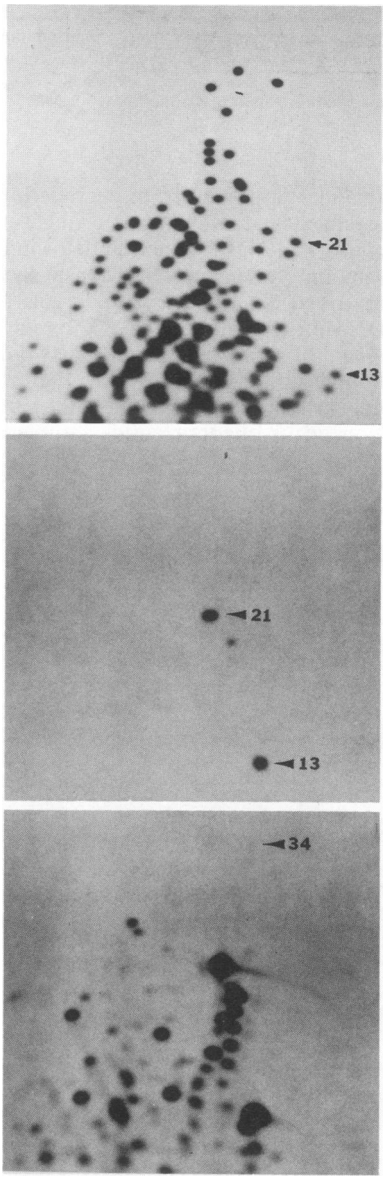


FIG. 8. Two-dimensional gels of T1-digested total genomic RNA and RNA isolated with a synthetic DNA oligonucleotide. (A) Total genomic RNA digested with RNase T1. The 13- and 21-base consensus oligonucleotides being studied are indicated. (B) Normal exposure of T1-digested RNA isolated with synthetic DNA. (C) Overexposure of this gel reveals 34-base error oligonucleotide. Although some RNA from other portions of the genome was present among RNA-DNA hybrids isolated, as can be seen in the overexposure, this procedure eliminated well over 99% of nonrelevant RNA sequences and resulted in greatly reduced background counts in the two-dimensional gel.

TABLE 2. Wild-type VSV nucleotide substitution frequencies in vivo

| Sample | Amt (μg/ml) of RNase A used with RNase T1[a] | 13-mer cpm | 21-mer cpm | 34-mer cpm | 13-mer cpm / (13-mer + 21-mer cpm) | Substitution frequency[b] |
|---|---|---|---|---|---|---|
| A | 25 | 6,700 | 7,700 | <1[c] | $<7.0 \times 10^{-5}$ | $<3.5 \times 10^{-5}$ |
| B | 8 | 36,200 | 42,600 | 7 | $8.9 \times 10^{-5}$ | $4.5 \times 10^{-5}$ |
| C | 1 | 21,800 | 26,500 | 23 | $4.8 \times 10^{-4}$ | $2.4 \times 10^{-4}$ |
| D | T1 only | 41,800 | 47,700 | 25 | $2.8 \times 10^{-4}$ | $1.4 \times 10^{-4}$ |
| E | T1 only | 14,100 | 22,600 | 11 | $3.0 \times 10^{-4}$ | $1.5 \times 10^{-4}$ |
| F | T1 only | 30,000 | 45,000 | 60 | $8.0 \times 10^{-4}$ | $4.0 \times 10^{-4}$ |
| G[d] | T1 only | 24,200 | 41,000 | 32 | $4.9 \times 10^{-4}$ | $2.5 \times 10^{-4}$ |
| H[d] | T1 only | 44,000 | 63,800 | 33 | $3.1 \times 10^{-4}$ | $1.6 \times 10^{-4}$ |

[a] All DNA-RNA hybrids were isolated with 20 μg of RNase T1 per ml. Samples A, B, and C were isolated with RNase A in conjunction with RNase T1.
[b] Substitution frequencies were calculated with 50% as an estimate of uncut G residues (Table 1). Average for samples isolated with T1 only, $2.2 \times 10^{-4}$; standard deviation, $1.3 \times 10^{-4}$
[c] Sample A error oligonucleotide was detected on film after long exposure; however, there were no measurable counts in the excised gel slice.
[d] These samples were labeled in vivo during replication of virus picked directly from a plaque.

DI particles labeled in vivo with $^{32}P$, generated base-paired stems, and treated these with single-strand-specific nuclease(s) as described for DNA-RNA hybrids. The resulting double-stranded RNA stems were then purified on 20% acrylamide gels (41). After elution, RNA was digested with RNase T1, and two-dimensional gels were used to separate the labeled stem oligonucleotides. (RNA double strands must be denatured during T1 digestion to ensure complete cleavage—see Materials and Methods.) The 13-mer, 21-mer, and 34-mer (error) oligonucleotides were cut out of two-dimensional gels, and nucleotide substitution frequencies were calculated as described above (Table 3). Again we observed that treatment with RNase A during preparation of double-stranded RNA can result in decreased recovery of error oligonucleotide because of digestion at mismatch sites.

Error RNA from stem preparation no. 2 was sequenced (data not shown), and again there was clear nucleotide heterogeneity at the selected site and U → C transitions at the site 2 bases downstream from the selected site. Once again, the frequency of base substitutions at the selected site was greater than $10^{-4}$ ($3 \times 10^{-4}$ in the case of stem preparation no. 3, which was cleaved only with T1 RNase).

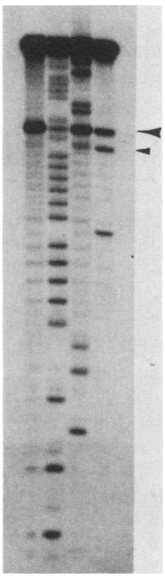

FIG. 9. Sequence of error oligonucleotide isolated from wild-type virus genome. This is a sequence of error oligonucleotide from a two-dimensional gel of T1-digested RNA which was labeled in vivo and isolated with synthetic DNA. The larger arrow indicates the selected error site at which there is clear heterogeneity. Note also the heterogeneity at the site 2 bases downstream (smaller arrow).

We conclude that base substitution frequency at all three sites examined averaged between $10^{-3}$ and $10^{-4}$. It must be noted that, despite the sequence identity at all three of the sites examined here, all three sequences are synthesized from different templates: (i) the DI polymerase product by the 3′ terminus of DI particles, (ii) the virus 5′ terminus sequence by the positive-strand 3′ end, and (iii) the DI particle stem 5′ end by the DI particle positive-strand 3′ end. With all of these templates in vitro and in vivo, the viral polymerase error frequency is extreme.

## DISCUSSION

The results above provide direct sequence evidence for extreme error frequency in animal virus RNA genomes. It should be recognized that the base substitution frequencies obtained are approximations. The largest margin of error likely occurs in our crude estimation of uncut G residues in error oligonucleotides from sequencing gels. Partial modification and cleavage reactions used for sequencing are not always effective to the same degree. Also, film grain exposure is not a linear process, so this can affect estimates of uncut G residues. However, because of the clear heterogeneity in error oligonucleotide sequences and the results obtained when error oligonucleotides were redigested with RNase T1, the base substitution frequency estimates are probably accurate to at least a factor of 2.

The estimates obtained here are for frequencies of base substitution in clonal populations of viral genome, not for rates of substitution. Because the G residue at the site examined here is highly conserved in all vesiculoviruses sequenced to date except VSV New Jersey and is also conserved in virus populations in which nearby residues of VSV termini are rapidly evolving (36, 37), it is likely that a base substitution at this site (or at the complementary C residue in the template sequence or both) is either lethal or very debilitating to these vesiculoviruses. Note also that the error frequencies observed in vivo (Table 2) were very close when the labeled genome populations were prepared by infecting with different clonal pools and even when only a few virus particles picked directly from a plaque were used instead of a high-titered clonal pool. Therefore, base substi-

TABLE 3. DI particle nucleotide substitution frequencies in vivo

| Sample no. | Amt (μg/ml) of RNase A used[a] | 13-mer cpm | 21-mer cpm | 34-mer cpm | 34-mer cpm / (13-mer + 21-mer cpm) | Substitution frequency[b] |
|---|---|---|---|---|---|---|
| 1 | 5 | 13,700 | 23,500 | 10 | $2.7 \times 10^{-4}$ | $1.4 \times 10^{-4}$ |
| 2 | 5 | 58,500 | 97,100 | 40 | $2.6 \times 10^{-4}$ | $1.3 \times 10^{-4}$ |
| 3 | T1 only | 19,600 | 31,800 | 30 | $5.8 \times 10^{-4}$ | $2.9 \times 10^{-4}$ |

[a] RNase T1 (20 μg/ml) was used for all stem samples isolated.
[b] Substitution frequencies were calculated with 50% as an estimate of uncut G residues (Table 1).

tution rates per nucleotide incorporated are probably very close to base substitutions frequencies observed at this site. Obviously, exact base substitution rates will be very difficult to determine in any genome in which error frequency is so extreme, because high error frequencies cause microheterogeneity in the population of template molecules as well as in progeny molecules. Errors during replication of both genome and antigenome RNA molecules can propagate to their complementary sites and affect attempts to determine base substitution rates in either. Therefore, error frequencies at the extreme levels observed here necessitate dealing with probabilities and consensus sequences (11, 12) rather than with the certainties of fixed sequences in template and antitemplates.

Despite the inherent uncertainties, it will be informative to examine base substitution frequencies at homologous sites in a template-antitemplate pair, and we will attempt to do so. We also plan to examine base substitution frequencies at several other highly conserved genome sites and at sites known to be subject to rapid evolution. It will be important to learn whether RNA genome sites vary greatly in base substitution frequency as is known to occur for DNA (1, 7). It will also be interesting to examine homologous sites in various mutant viruses isolated at different times from persistently infected cells (23).

Examination of other sites by the methods used here should also reveal the basis for the interesting phenomenon in which the site 2 bases downstream from the selected site was observed to have greatly increased U → C transitions. If similar mutagenesis is regularly observed a few bases downstream from other selected error sites, then it is likely the result of distorted polymerase-template interactions as the polymerase rides over the selected mismatched base pair during processive synthesis. If enhanced polymerase error is observed at other sites many bases downstream (or upstream) from the selected site, then error-prone subsets of viral polymerase are suggested. Evidence for repetitive, specific VSV replicase error has already been reported (36). Examination of error frequencies at other genome sites will be more technically demanding, because the unusual adenine-rich sequence of the error oligonucleotide examined here separated well away on two-dimensional gels from contaminating large oligonucleotides from other areas of the 11-kilobase VSV genome.

Our results and conclusions agree with those of Domingo et al. (11, 12) with RNA phage Qβ. Our results directly confirm their conclusion that most members of an RNA phage population differ from the average genome sequence in one or more positions. Their RNA phage conclusions were extended to field isolates and multiple clones of foot-and-mouth disease virus by oligonucleotide map comparisons (10, 53), and their estimates of two to eight differences per virus genome (compared with average consensus sequence) fit very well with our direct sequence estimates for VSV. These results also are in accord with the $10^{-3}$ to $10^{-4}$ mutation frequencies estimated for RNA retrovirus reverse transcriptase (4, 6, 8, 17, 30). Our results further suggest that many of the mutations observed among multiple cDNA clones of the VSV L gene by Schubert et al. (46) were due to VSV polymerase error as well as reverse transcriptase error during synthesis of cDNA.

Obviously, if studies at many other sites confirm that uniformly high error frequencies cause an average of two or more base differences per RNA genome, then a consensus sequence can be maintained only by strong biological selective forces. The dominance of a consensus sequence in an equilibrium pool can be maintained by relative replicative efficiences (11, 12) or upset by changing selective forces that promote rapid evolution (23). Extreme error frequencies are probably important for the adaptability of RNA viruses, and the fact that antimutator phenotypes of DNA viruses are not favored in nature (13, 21) strongly supports the evolutionary value of high mutation frequencies for smaller DNA genomes as well.

Finally, if it can be shown that most sites in the RNA genome undergo the high error frequencies observed here, then the multiple-base substitutions introduced into nearly all progeny of an RNA virus clone would be expected to cause many lethal hits in the virus population. This could partly account for the very high particle to infectious unit ratio of most RNA viruses. If a population of virus particles replicated from a clone contains an average of two or three base substitutions per genome, then only 13.5 or 5%, respectively, of the RNA genomes would be identical in sequence to the consensus sequence, based on a Poisson distribution of random error.

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