JOURNAL OF VIROLOGY, June 1992, p. 3784–3793
0022-538X/92/063784-10$02.00/0

Vol. 66, No. 6

Determination of the Poliovirus RNA Polymerase Error Frequency at Eight Sites in the Viral Genome

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Received 26 November 1991/Accepted 5 March 1992

The poliovirus RNA polymerase error frequency was measured in vivo at eight sites in the poliovirus genome. The frequency at which specific G residues in poliovirion RNA changed to another base during one round of viral RNA replication was determined. Poliovirion RNA uniformly labeled with $^{32}P_i$ was hybridized to a synthetic DNA oligonucleotide that was complementary to a sequence in the viral genome that contained a single internal G residue. The nonhybridized viral RNA was digested with RNase T_1, and the protected RNA oligonucleotide was purified by gel electrophoresis. The base substitution frequency at the internal G residue was measured by finding the fraction of this RNA oligonucleotide that was resistant to RNase T_1 digestion. A mean value of $2.0 \times 10^{-3} \pm 1.2 \times 10^{-3}$ was obtained at two sites. A modification of the above procedure involved the use of 5′-end-labeled RNA oligonucleotides. The mean value of the error frequency determined at eight sites in the viral genome by using this technique was $4.1 \times 10^{-3} \pm 0.6 \times 10^{-3}$. Sequencing two of the RNase T_1-resistant RNA oligonucleotides confirmed that the internal G was changed to a C, A, or U residue in most of these oligonucleotides. Thus, our results indicated that the polymerase had a high error frequency in vivo and that there was no significant variation in the values determined at the specific sites examined in this study.

The central role of both RNA and DNA polymerases is to catalyze the accurate template-directed incorporation of nucleotide substrates in a 5′→3′ direction into a growing strand of nucleic acid by Watson-Crick base pairing. The large genomes of eukaryotic organisms are replicated with high fidelity to ensure their perpetuation as a homogeneous species. Rates of evolution of cellular genes average 10^{-9} substitutions per site per year, in part because of elaborate proofreading and repair mechanisms (2, 13). On the other hand, viral RNA genomes are on average only 3 to 30 kb in length, and RNA viruses are known to evolve at rates a millionfold higher than their hosts with DNA genomes (1, 8, 9). It is important when discussing the fidelity of polymerases to distinguish between error frequencies and evolutionary rates. Error frequencies, or mutation frequencies, are the frequencies of a mutation event that would be represented by single base substitutions during one round of viral replication. In most studies, only the viable mutation frequency is measured. Evolutionary rates are defined as the rate at which viable mutations accumulate in the genome. Poliovirus evolution rates have also been measured many times by RNase T_1 oligonucleotide mapping. In one study, clinical isolates were monitored during a 13-month epidemic (16). These isolates showed continual mutation and selection during replication in humans. About 100 nucleotide changes, or 1 to 2% of the genomic bases, were fixed in the viral genome. Similarly, changes in RNase T_1 oligonucleotide maps have been measured in vaccine-associated cases of paralytic poliomyelitis (11). Most isolates were multisite mutants that ranged from less than 10 base changes to greater than 100 base changes. These changes again represented up to 1 to 2% of the genomic bases but presumably occurred during replication in only one or two individuals.

Many methods have been used to measure the viable mutation frequencies of RNA viruses. Common methods include the frequency of mutation to monoclonal antibody resistance, drug resistance mutation frequencies, reversion frequencies of point mutations, and direct RNA sequencing. Poliovirus variants resistant to monoclonal antibody neutralization arise at a frequency of 10^{-4} to 10^{-5} for Mahoney type 1 (6, 7). Similar measurements have been made for Leon type 3 virus and the attenuated Sabin type 3 vaccine strain derived from it (14). These variants were recovered at the rate of 10^{-4} to 10^{-5} for the Sabin strain, whereas mutants from the Leon strain arose 10 times more frequently. Most of these mutations were point mutations in the capsid proteins. Poliovirus mutants resistant to guanidine have been seen to arise at the rate of approximately 3×10^{-5} (10). This may, however, represent changes at more than one site in the genome and thus underestimate the frequency of a single mutation. Recently, measurements were made for the reversion of a guanidine-dependent poliovirus mutant to guanidine resistance (3). This represents the reversion of a single amino acid and was found to occur at a frequency of 6.5×10^{-4}. In contrast, lower viable mutation frequencies were found in two other studies. In one study, a single-base revertant of a poliovirus amber mutant arose at approximately 10^{-5} (20). In a second study, Parvin et al. sequenced a segment of the VP1 gene of poliovirus type 1 from multiple individual virus plaques that had all descended from a single plaque (19). A mutation rate of less than 2.1×10^{-6} was calculated for this site compared with 1.5×10^{-5} for the NS gene of influenza virus. It is difficult to know whether the lower mutation frequency observed in the last two studies was due to a lower polymerase error frequency or an increased level of selection at the sites examined.

What is now needed is a direct measurement of the error frequency of the poliovirus RNA polymerase in vivo, and this was the primary objective of this study. We have adopted and modified the technique developed by Steinhauer and Holland (22, 24) and used it to measure the substitution frequency of specific G residues in purified poliovirion RNA. There were some limitations on which

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nucleotides could be used in this assay, but it was possible to select specific nucleotides from different regions of the viral genome. The advantage of this approach was that there was no requirement that viable virion RNA be used in the assay, unlike most in vivo techniques that only measure mutation frequencies in viable virus. Although there was no requirement that the RNA be infectious, there was an obvious requirement that the RNA be packaged during the infection since virion RNA was assayed in this study. Our measurement of the error frequency yielded mean values of $2.0 \times 10^{-3} \pm 1.2 \times 10^{-3}$ at two sites and $4.1 \times 10^{-3} \pm 0.6 \times 10^{-3}$ at eight sites in the viral genome by using the two methods described in this article.

MATERIALS AND METHODS

Cell culture and purification of poliovirion RNA. HeLa cells maintained in suspension culture were infected with poliovirus type 1 (Mahoney strain) as previously described (25). Briefly, HeLa cells were infected with poliovirus (multiplicity of infection, 20) and were incubated for 6 h at 37°C. Infected cell cultures contained 4×10^6 cells per ml in Eagle's modified minimal media with 5% bovine calf serum and 2% fetal calf serum. Poliovirus was purified from cytoplasmic extracts of the infected cells by banding the virus in a CsCl equilibrium density gradient as previously described (27). Poliovirion RNA was isolated from the purified virus by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation.

DNA oligonucleotides. All DNA oligonucleotides were synthesized on an Applied Biosystems model 380A or 380B automated DNA synthesizer by using phosphoramadite chemistry in the ICBR DNA Core Facility at the University of Florida and were purified by electrophoresis on a 20% polyacrylamide–7 M urea gel. The DNA oligonucleotides used in this study were complementary to poliovirion RNA sequences (see Fig. 2) and each contained a 3′-terminal $poly(A)_{15}$.

Poliovirion RNA uniformly labeled with $^{32}P_i$. HeLa cells growing in suspension culture were washed three times with phosphate-free modified Eagle's medium buffered at pH 7.2 with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]. The cells were infected with poliovirus (multiplicity of infection, 20) and the virus was allowed to adsorb for 30 min at 25°C. The cells were then diluted to 4×10^6 cells per ml in phosphate-free media with 5% dialyzed bovine calf serum and 2% dialyzed fetal calf serum. After 15 min, actinomycin D was added at a concentration of 5 μg/ml. At 30 min, 600 μCi of $^{32}P_i$ per ml was added. At 6 h the cells were centrifuged and washed with Earle's saline. The poliovirion [^{32}P]RNA was then purified as described above.

Determination of polymerase error frequency with ^{32}P-labeled poliovirion RNA. Poliovirion RNA (10 μg) uniformly labeled with $^{32}P_i$ was hybridized with 0.3 μg of a DNA oligonucleotide in 30 μl of 10 mM Tris (pH 7.5), 500 mM NaCl, and 4 mM $MgCl_2$ for 3 h at 50°C. RNA sequences not hybridized with the DNA oligonucleotide were digested with 6 U of RNase T_1 (Calbiochem Corporation) for 2 h at 50°C. The resulting RNA-DNA duplex was treated with proteinase K (Boehringer Mannheim) by diluting the sample with 120 μl of 0.5% sodium dodecyl sulfate (SDS) buffer (100 mM NaCl, 10 mM Tris [pH 7.5], 1 mM EDTA, 0.5% SDS) and by adding 75 μg of proteinase K for 1 h. The RNA-DNA duplex was extracted twice with phenol-chloroform-isoamyl alcohol, ethanol precipitated, and gel purified by electrophoresis on a nondenaturing 20% polyacrylamide gel. The RNA-DNA duplex was denatured in 80% formamide, and the protected RNA oligonucleotide was purified on a 20% polyacrylamide–7 M urea gel to separate it from the DNA oligonucleotide and from the poly(A) that comigrated with the RNA-DNA duplex on the first gel. The purified RNA oligonucleotide was then digested with RNase T_1 (see below) for 1 h at 50°C, and the resulting digestion products were analyzed by electrophoresis on a 20% polyacrylamide–7 M urea gel. To determine the error frequency, the radioactivity (in counts per minute) in the RNase T_1-resistant RNA oligonucleotide was compared with the total radioactivity in the digestion products of this reaction (Fig. 1B). The amount of radioactivity in each band was quantitated by using an AMBIS or Betagen automated gel scanner.

Determination of polymerase error frequency with RNA oligonucleotides 5′ end labeled with ^{32}P. Poliovirion RNA (10 μg) was hybridized with 0.03 μg of [^{32}P]DNA oligonucleotide and 0.27 μg of unlabeled DNA oligonucleotide (Fig. 2) and digested with RNase T_1 as described above. The RNA-DNA hybrid was electrophoresed on a nondenaturing 20% polyacrylamide gel, located by autoradiography, and eluted from the gel fragment. The RNA oligonucleotide in the RNA-DNA duplex was 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase and was subsequently treated with 2 U of DNase (Boehringer Mannheim) in 50 μl of 100 mM $NaCH_3CO_2 \cdot 3H_2O$ (pH 4.5)–5 mM $MgSO_4$ to remove the DNA. The 5′-end-labeled RNA oligonucleotide was then gel purified by electrophoresis on a denaturing 20% polyacrylamide–7 M urea gel. The purified 5′-end-labeled RNA oligonucleotide was digested with RNase T_1 as described below. The digestion products were treated with proteinase K and analyzed by electrophoresis on a 20% polyacrylamide–7 M urea gel (Fig. 1A). The labeled digestion products were quantitated with a Betagen gel scanner, and the resulting values were used to determine the error frequency as described above.

RNase T_1 digestions. Isolated labeled RNA oligonucleotides were ethanol precipitated with 20 μg of glycogen (Boehringer Mannheim) and dissolved in 6 μl of a solution of 25 mM sodium citrate (pH 3.5), 7 M urea, 1 mM EDTA, 0.035% xylene cyanol, and 0.035% bromophenol blue. RNase T_1 (6 U; Calbiochem Corporation) was added and incubated at 50°C for 1 h. The sample was then boiled for 1 min, and another 6 U of RNase T_1 was added and incubated for 1 h at 50°C. Proteinase K (1 μg) was added and incubated at 37°C for 1 h. The reaction mixtures were heated to 100°C, quick-chilled on ice, and loaded directly onto a 20% polyacrylamide–7 M urea gel.

Poliovirus RNA transcripts synthesized in vitro. Plasmid DNA pOF1205 was used to generate labeled poliovirus-specific transcripts. The plasmid pOF1205 contains the 3′-terminal nucleotides 6516 to 7440 and a $poly(A)_{83}$ sequence (17). This plasmid was cut at the 3′ end of the poly(A) sequence with *Eco*RI and was transcribed with SP6 polymerase by the method supplied by Promega Biotechnologies, Inc. The transcript RNA was labeled by including 10 μCi of either [^{32}P]UTP or [^{32}P]ATP in the reactions. The RNA was treated with DNase to remove the plasmid DNA, phenol-chloroform extracted, and ethanol precipitated. The amount of labeled RNA synthesized was determined by counting of a small sample of each reaction after precipitation in trichloroacetic acid. The labeled RNA transcripts were used in hybridization and RNase T_1 digestion experiments as described for the ^{32}P-labeled poliovirion RNA.

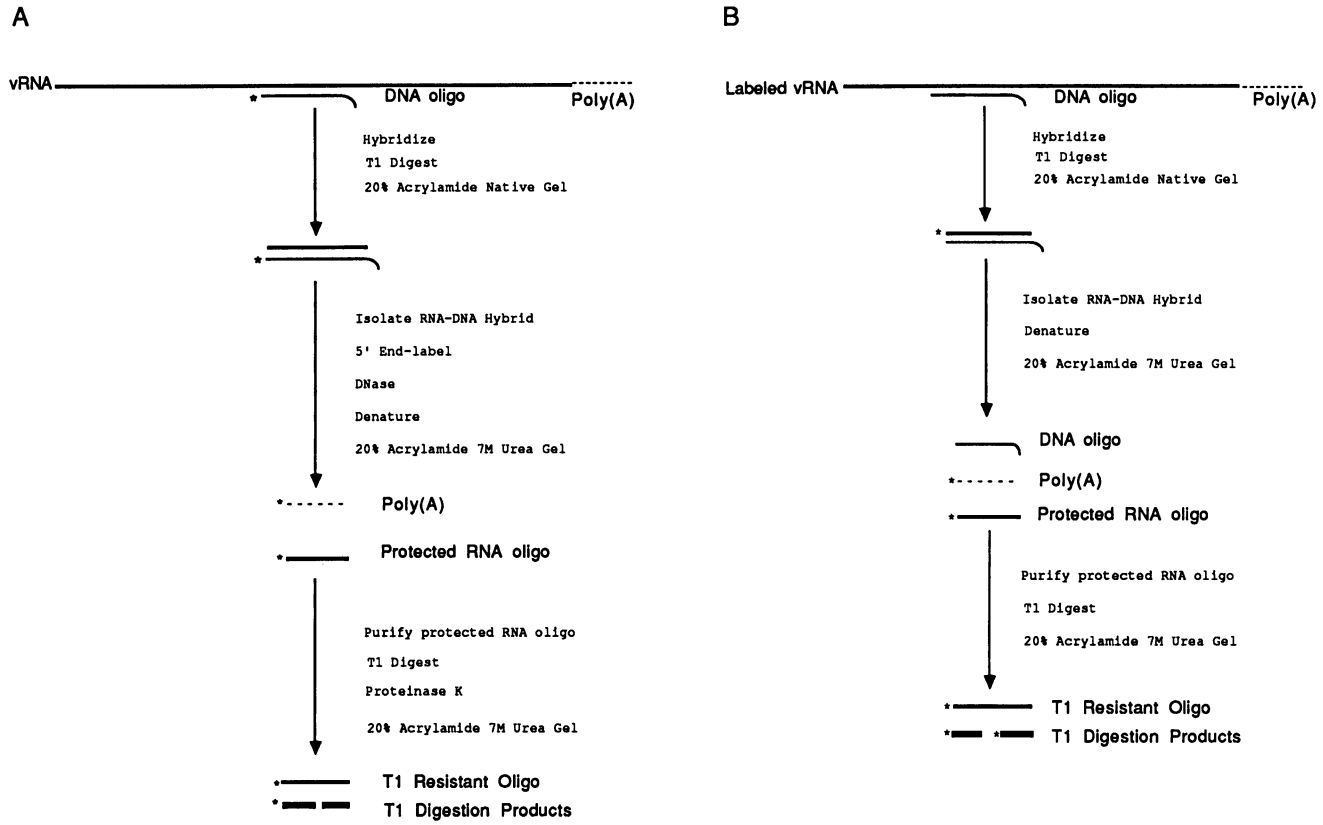

FIG. 1. Diagram of the two procedures used to determine the polymerase error frequency at specific G residues in poliovirion RNA. (A) Purified poliovirion RNA (vRNA) was annealed to a ^{32}P-labeled synthetic DNA oligonucleotide and digested with RNase T_1. The RNA-DNA duplex was located by autoradiography after electrophoresis in a nondenaturing 20% polyacrylamide gel. The protected RNA oligonucleotide was 5′ end labeled and gel purified by electrophoresis on a denaturing 20% polyacrylamide–7 M urea gel to separate it from poly(A). The purified RNA was then digested to completion with RNase T_1. The error frequency at the single internal G residue in the RNA was determined by quantitating the fraction of the purified RNA that was resistant to digestion. Oligonucleotides that were 5′ end labeled with ^{32}P are denoted by an asterisk in this panel. (B) The procedure shown in this panel was similar to that described for panel A except that poliovirion RNA uniformly labeled with $^{32}P_i$ in infected cells was used in this assay. Oligonucleotides uniformly labeled with $^{32}P_i$ are denoted with an asterisk in panel B. Refer to Materials and Methods for additional details.

Gel purification of oligonucleotides. All oligonucleotides that were gel purified were located either by autoradiography or by UV shadowing. Gel fragments containing the oligonucleotides were cut out, crushed, and eluted overnight at 37°C in 150 μl to 1 ml of sterile H_2O, depending on the size of the gel fragment. Large pieces of polyacrylamide were removed by centrifugation in an Eppendorf centrifuge, and the supernatant was passed through a sterile disposable polypropylene column with a paper disc (Isolabs). The oligonucleotide was then ethanol precipitated with 20 μg of glycogen. Oligonucleotides eluted from very large gel fragments were extracted three or four times with absolute ethanol to remove any urea.

5′ End labeling of oligonucleotides with ^{32}P. RNA and DNA oligonucleotides were 5′ end labeled with 10 μCi of [γ-^{32}P]ATP by using 10 U of T4 polynucleotide kinase (New England BioLabs) for 30 min at 37°C in 70 mM Tris (pH 7.6)–10 mM $MgCl_2$–5 mM dithiothreitol. When RNA oligonucleotides were sequenced, the amount of [γ-^{32}P]ATP was increased to 50 μCi.

Sequencing of RNase T_1-resistant RNA oligonucleotides. RNase T_1-resistant RNA oligonucleotides that were 5′ end labeled were extracted from the gel and ethanol precipitated with 15 μg of tRNA. The RNA was divided into five aliquots which were digested with RNase T_1 (Calbiochem Corporation), RNase U2 (Bethesda Research Laboratories), RNase PhyM (Bethesda Research Laboratories), RNase B. cereus (Bethesda Research Laboratories), or RNase CL3 (Pharmacia). RNase T_1 and U2 digestion conditions were 25 mM sodium citrate (pH 3.5)–7 M urea–1 mM EDTA–0.035% dyes, with 2 and 0.5 U of RNase per ml, respectively. Digestion conditions for PhyM were 25 mM sodium citrate (pH 5.0)–7 M urea–1 mM EDTA–0.035% dyes, with 100 U of RNase per ml. Digestion conditions for B. cereus were 25 mM sodium citrate (pH 5.0) with 200 U of RNase B. cereus per ml. Digestion conditions for CL3 were 10 mM sodium phosphate (pH 6.5)–10 mM EDTA, with 50 U of RNase per ml. All digestions were for 15 min at 55°C, except CL3 which was done at 37°C. Digestions with RNase B. cereus and CL3 were stopped by the addition of 7 M urea and 0.035% dyes and quick-freezing on dry ice. All other digestions were stopped by direct-freezing on dry ice. Samples were then boiled for 3 min and quick-chilled before being loaded onto a 20% polyacrylamide–7 M urea gel.

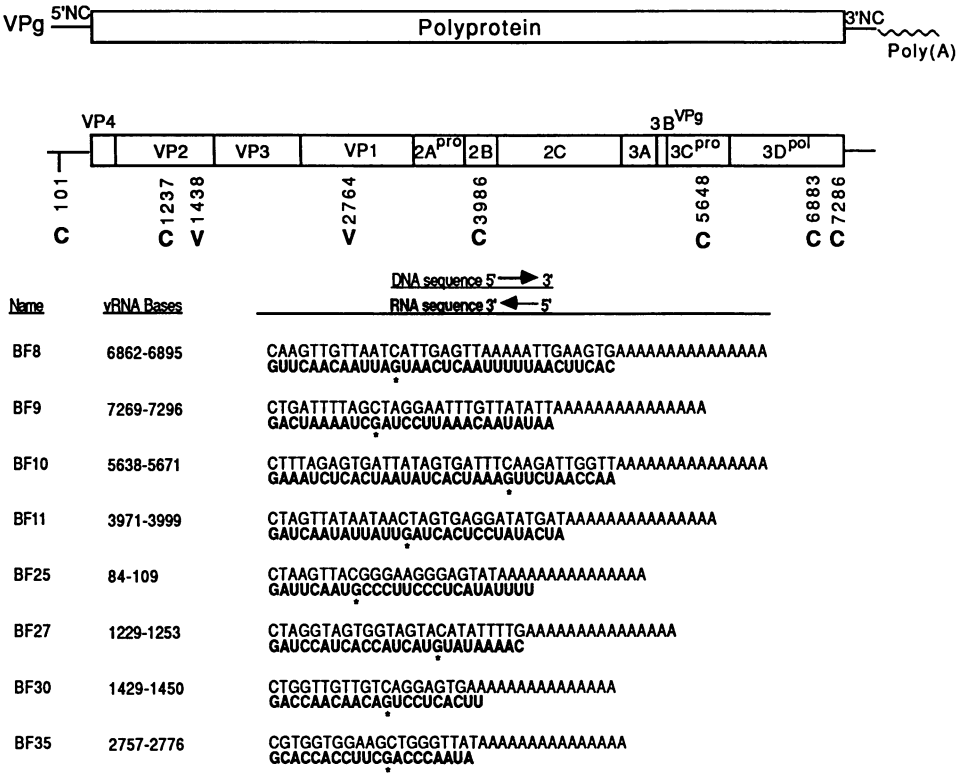

FIG. 2. Sequences of eight RNA oligonucleotides protected from RNase T_1 digestion by a complementary synthetic DNA oligonucleotide. Eight sequences in the poliovirus genome which contained a single internal G residue which was spaced from 8 to 23 bases from the next G in either the 5′ or the 3′ direction were selected. These sequences along with their exact location in the viral genome are listed. The 5′-terminal G is not included since it is removed during the RNAse T_1 digestion step. The numbers at the top of the diagram indicate the location of the internal G residue in each RNA oligonucleotide. C and V, constant and variable regions, respectively, as defined in the text. The position of the G in each RNA is denoted by an asterisk. vRNA, poliovirion RNA.

RESULTS

Polymerase error frequency determined with poliovirion RNA uniformly labeled with $^{32}P_i$. The basic approach used to determine the polymerase error frequency was to measure the frequency at which specific G residues in poliovirion RNA changed to another nucleotide during viral RNA replication in infected cells. Briefly, virion RNA uniformly labeled with $^{32}P_i$ was hybridized to a synthetic DNA oligonucleotide which was complementary to a sequence containing a single internal G residue (Fig. 2). The nonhybridized single-stranded RNA was then digested with RNase T_1. The protected RNA oligonucleotide was isolated by two steps of gel purification and then digested to completion with RNase T_1 (Fig. 1B). The single-base substitution frequency at the internal G residue in the protected RNA oligonucleotide was determined by quantitating the amount of the oligonucleotide that was resistant to RNase T_1 (G-specific) digestion. The error frequency was defined as the fraction of the radioactivity (in counts per minute) recovered in the RNase T_1-resistant oligonucleotide relative to the total radioactivity recovered in the bands that represented the RNase T_1 digestion products plus the RNase T_1-resistant oligonucleotide (Fig. 1B).

The above procedure was initially developed by using pOF1205 transcript RNA, which consists of the 3′-terminal 1,000 bases of poliovirus RNA. The transcript RNA was labeled with either [^{32}P]UTP or [^{32}P]ATP, hybridized to a synthetic DNA oligonucleotide, digested with RNases T_1 and U2 or RNase T_1 alone, and run on a 20% polyacrylamide–7 M urea gel. A protected RNA oligonucleotide of the expected size (34 bases) that was not present when the RNA was digested in the absence of the synthetic DNA oligonucleotide (data not shown) was recovered from this gel. When the transcript RNA was labeled with [^{32}P]ATP, the same protected RNA oligonucleotide was recovered. In this case, however, it was apparent that a ladder of labeled poly(A) fragments was also present. This suggested that a protected RNA oligonucleotide that was isolated by using this approach would be contaminated with a poly(A) fragment of the same size. This was confirmed in subsequent experiments in which the protected RNA oligonucleotide was isolated from the gel, 5′ end labeled, and sequenced by using an enzymatic sequencing procedure. The original Steinhauer and Holland procedure (24) did not deal with this problem since vesicular stomatitis virus virion RNA is not polyadenylated.

The coisolation of poly(A) with the protected RNA oligonucleotide was avoided by using a two-step gel purification procedure. Initially the RNA-DNA duplex was isolated on a nondenaturing 20% polyacrylamide gel (Fig. 3). An RNA-DNA duplex was recovered from [^{32}P]UMP-labeled pOF1205 transcript RNA hybridized to BF8 DNA and digested with RNase T_1 (Fig. 3, lane 2). As expected, the duplex was not present when the RNA was digested in the absence of the BF8 DNA (Fig. 3, lane 1). The RNA-DNA duplex comigrates on this gel with some poly(A) fragments

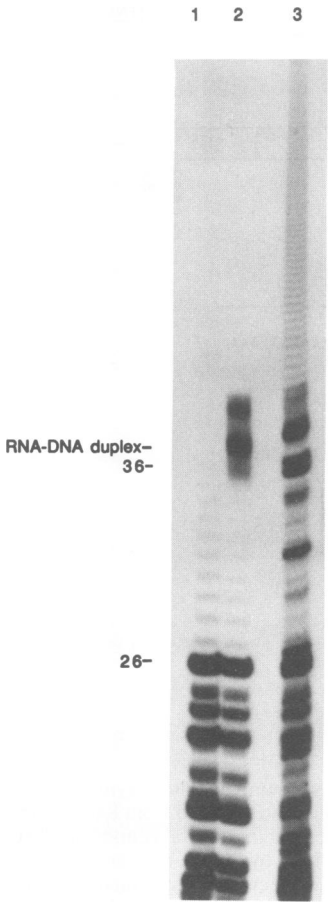

FIG. 3. Gel electrophoresis of the RNase T_1 digestion products of either pOF1205 transcript RNA or poliovirion RNA hybridized to BF8 DNA. [^{32}P]UMP-labeled pOF1205 RNA was digested with RNase T_1 either before (lane 1) or after (lane 2) being hybridized to BF8 DNA and was analyzed by electrophoresis on a nondenaturing 20% polyacrylamide gel. Poliovirion RNA uniformly labeled with $^{32}P_i$ was annealed to BF8 DNA, digested with RNase T_1, and run on the same gel (lane 3).

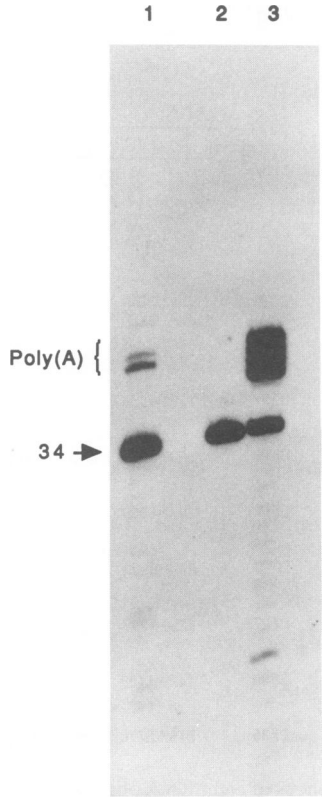

FIG. 4. Purification of RNA oligonucleotides protected by BF8 DNA from contaminating poly(A) by a second step of gel electrophoresis. RNA-DNA duplexes were isolated as described in the legend to Fig. 3, denatured, and run on a 20% polyacrylamide–7 M urea gel. Shown are RNA oligonucleotide (34-mer) that was isolated from poliovirion RNA labeled in vivo with $^{32}P_i$ (lane 1) and RNA oligonucleotides that were isolated from pOF1205 transcript RNA labeled in vitro with either [^{32}P]UMP (lane 2) or [^{32}P]AMP (lane 3).

which are longer than the protected RNA which is part of the duplex. Thus, the RNA-DNA duplex was isolated from the nondenaturing gel, denatured with urea, and run on a 20% polyacrylamide–7 M urea gel to separate the protected RNA from the BF8 DNA and any contaminating poly(A) (Fig. 4, lanes 2 and 3). The separation of the protected RNA oligonucleotide from the contaminating poly(A) was clearly demonstrated when the transcript RNA was labeled with [^{32}P]AMP (Fig. 4, lane 3). It should be noted that the DNA was engineered to have a poly(A)$_{15}$ tail so that it would separate from the RNA oligonucleotide on a denaturing gel. In earlier studies, we found that it was not possible to quantitatively remove all of the DNA from the duplex by a digestion with DNase. Thus, the two-step gel purification procedure was adopted. The purified RNA oligonucleotide was isolated from the second gel and then digested with RNase T_1 to determine the error frequency.

The error frequency was measured at two sites in poliovirion RNA uniformly labeled with $^{32}P_i$. The first site was at the G at position 6883 in the 3D^{pol} coding sequence and the second was at the G at position 5648 in the 3C^{pro} coding sequence. DNA oligonucleotides BF8 and BF10 were used to isolate RNA oligonucleotides that contained these two sites (see Fig. 2 for the exact sequences). The RNA oligonucleotide protected by BF8 was isolated as an RNA-DNA duplex on a nondenaturing gel. The duplex migrated more slowly than the two longest RNase T_1-generated oligonucleotides (36 and 37 bases long) derived from ^{32}P-labeled poliovirion RNA (Fig. 3, lane 3). The BF8-protected RNA oligonucleotide was purified on a denaturing gel to remove contaminating poly(A) (Fig. 4, lane 1) and then digested to completion with RNase T_1 and analyzed by gel electrophoresis (Fig. 5). A small but detectable amount of the RNA was resistant to digestion (Fig. 5, lane 1). The average amount of radioactivity (in counts per minute) recovered in this resistant band represented 3.2×10^{-3} of the total radioactivity recovered in all three bands (i.e., the two major bands representing the digestion products and the resistant band). Thus, the polymerase error frequency at this site (i.e., base 6883) was 3.2×10^{-3} (Table 1). The error frequency at the second site (base 5648) was determined by using the same procedure and was slightly lower, with a value of 0.7×10^{-3} (Table 1).

Polymerase error frequency determined with RNA oligonucleotides 5′ end labeled with ^{32}P. Disadvantages of the previous method for determining the error frequency were the relatively low specific radioactivity of the uniformly labeled virion RNA and the amount of $^{32}P_i$ (e.g., 50 mCi) that

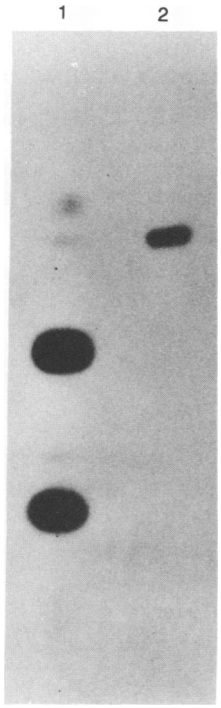

FIG. 5. Electrophoresis of RNase T_1 digestion products of purified RNA oligonucleotide protected by BF8 DNA. The RNA oligonucleotide protected by BF8 (34-mer) was isolated from ^{32}P-labeled poliovirion RNA by two steps of gel purification as described in the legends to Fig. 3 and 4. The purified RNA was digested with RNase T_1 and run on a 20% polyacrylamide–7 M urea gel (lane 1). Undigested RNA was run as a marker (lane 2).

TABLE 1. Error frequency of the poliovirus polymerase at specific sites in viral genome

DNA oligonucleotide[a]	Site on genome[b]		Error frequency[c] (10^{-3})	
	Nucleotide	Protein	Method 1	Method 2
BF8	6883	$3D^{pol}$	3.2	4.7
BF9	7286	$3D^{pol}$		4.4
BF10	5648	$3C^{pro}$	0.7	3.2
BF11	3986	2B		3.5
BF25	101	5′ NC		3.8
BF27	1237	VP2		5.0
BF30	1438	VP2		4.6
BF35	2764	VP1		3.2
Mean ± SE			2.0 ± 1.2	4.1 ± 0.6

[a] Sequences of DNA oligonucleotides and protected RNAs are given in Fig. 2.

[b] $3D^{pol}$, $3C^{pro}$, 2B, VP2, and VP1 refer to the genes encoded at these sites on the poliovirus genome. 5′ NC, 5′ noncoding region. The nucleotide numbers listed are the positions of the G residues that were assayed for base substitution.

[c] The error frequencies represent an average of three or more experiments. The numbers shown were corrected as described in the text. Method 1 used RNA uniformly labeled with $^{32}P_i$. Method 2 used RNA oligonucleotides 5′ end labeled with ^{32}P. An example of raw data from Fig. 8A, lane 2, that was used to calculate the error frequency at nucleotide 101 (BF25) is as follows: band 26 = 11,182 counts (186 cpm), band 18 = 2,215,749 counts (36,929 cpm), and background = 281 counts (4.7 cpm). These results were from a 1-h Betagen scan.

was required in these experiments. For these reasons, a second approach was used to determine the in vivo error frequency of the poliovirus RNA polymerase. The error frequency at eight different G residues in the viral genome was determined by using RNA oligonucleotides 5′ end labeled with ^{32}P. These nucleotides were located in constant and variable regions of the poliovirus genome (Fig. 2). Constant sites were defined as nucleotides which are conserved among the three serotypes of poliovirus. Any change in these nucleotides would result in a change in the amino acid encoded. Variable sites either are not conserved between the three serotypes of poliovirus or have been observed to change rapidly when the virus is grown under selective pressure. The technique used is summarized briefly here and in Fig. 1A. Poliovirion RNA was hybridized to a DNA oligonucleotide 5′ end labeled with ^{32}P and then was digested with RNase T_1. The resulting RNA-DNA duplex was purified on a nondenaturing 20% polyacrylamide gel (Fig. 6, lanes 2 and 3 for BF27 and lanes 4 and 5 for BF10). The RNA in the duplex was 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase and then gel purified on a denaturing 20% polyacrylamide–7 M urea gel (Fig. 7). This separated the 5′-end-labeled RNA from the contaminating poly(A) and a small amount of residual DNA (Fig. 7). The band representing the protected RNA oligonucleotide was isolated from the gel, digested with RNase T_1, and run on a denaturing 20% polyacrylamide–7 M urea gel. The digestion products were quantitated by using a Betagen gel scanner to determine the error frequency.

Detectable amounts of the purified RNA oligonucleotides protected by the eight DNA oligonucleotides were resistant to RNase T_1 digestion. For example, a small but reproducible amount of the BF25- and BF9-protected RNA oligonucleotides were resistant to digestion (Fig. 8A and B). Figure 8A, lane 1, contains the undigested RNA oligonucleotide (26

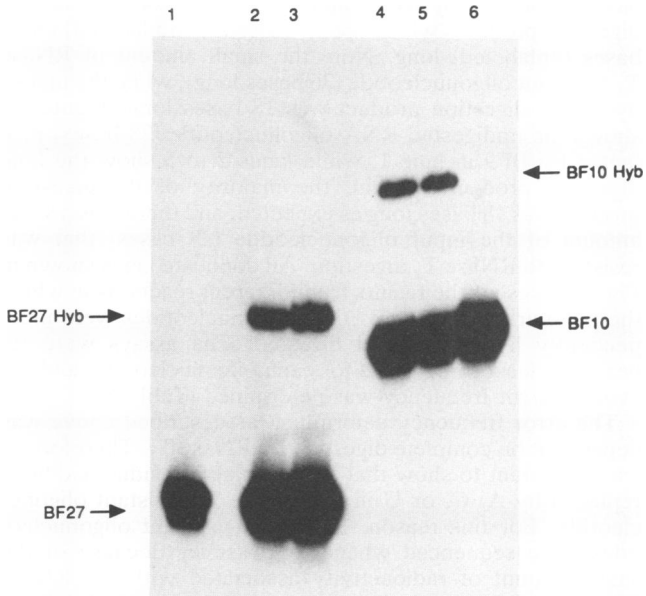

FIG. 6. Isolation of RNA-DNA duplexes after RNase T_1 digestion of poliovirion RNA hybridized to BF27 DNA or BF10 DNA. Unlabeled poliovirion RNA was annealed with either ^{32}P-labeled BF27 or BF10 DNA, digested with RNase T_1, and run on a native 20% polyacrylamide gel. Lanes 2 and 3, BF27 DNA; lanes 4 and 5, BF10 DNA. Labeled BF27 and BF10 were also run directly in the gel as markers (lanes 1 and 6).

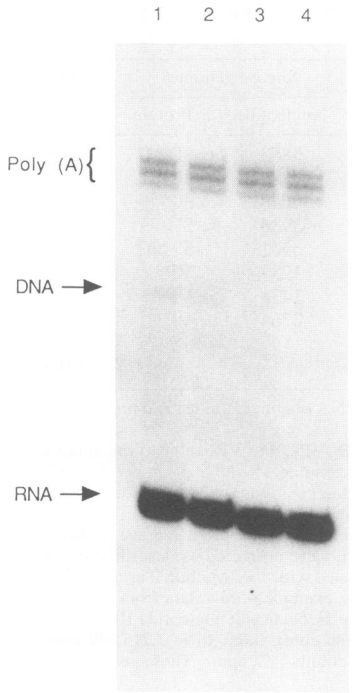

FIG. 7. Gel purification of 5′-end-labeled RNA oligonucleotide from RNA-DNA duplex to remove poly(A) and DNA contaminants. RNA-DNA hybrids were isolated as described in the legend to Fig. 6, labeled with polynucleotide kinase and [γ-^{32}P]ATP, digested with DNase, denatured in urea, and run on a 20% polyacrylamide–7 M urea gel. The results represent the results from independent hybridization and digestion experiments (lanes 1 to 4). The positions of the contaminating poly(A) and DNA in the gel are indicated.

bases) protected by BF25, and lanes 2 to 5 contain the final digestion products which are 18 bases (5′ end labeled) and 8 bases (unlabeled) long. Note the small amount of RNase T_1-resistant oligonucleotide (26 bases long), while the majority of the digestion product was 18 bases long. Figure 8B shows the undigested RNA oligonucleotide (28 bases) protected by BF9 in lane 1, while lanes 2 to 5 show the final digestion products. Again, the majority of the digestion product was 18 bases long as expected, and there was a small amount of the input oligonucleotide (28 bases) that was resistant to RNase T_1 digestion. All duplicate lanes shown in Fig. 8 represent the results from different reactions in which the poliovirion RNA and DNA oligonucleotides were independently hybridized and digested. The assays were repeated at least three times for each oligonucleotide, and the average error frequency was determined (Table 1).

The error frequency determined as described above was dependent on complete digestion by RNase T_1. Therefore, it was important to show that the internal G residue had been replaced by A, C, or U in the RNase T_1-resistant oligonucleotide. For this reason, RNase T_1-resistant oligonucleotides were sequenced whenever possible. Because of the small amount of radioactivity associated with the RNase T_1-resistant oligonucleotides, it was only possible to obtain sequence information on the oligonucleotides protected by BF8 and BF11 (Fig. 9A and B, respectively). However, both of these sequencing gels clearly show the presence of A, C, and U in addition to some remaining G in these RNase T_1-resistant oligonucleotides (Fig. 9A and B, left side). The remaining G appears to represent approximately 25% of the

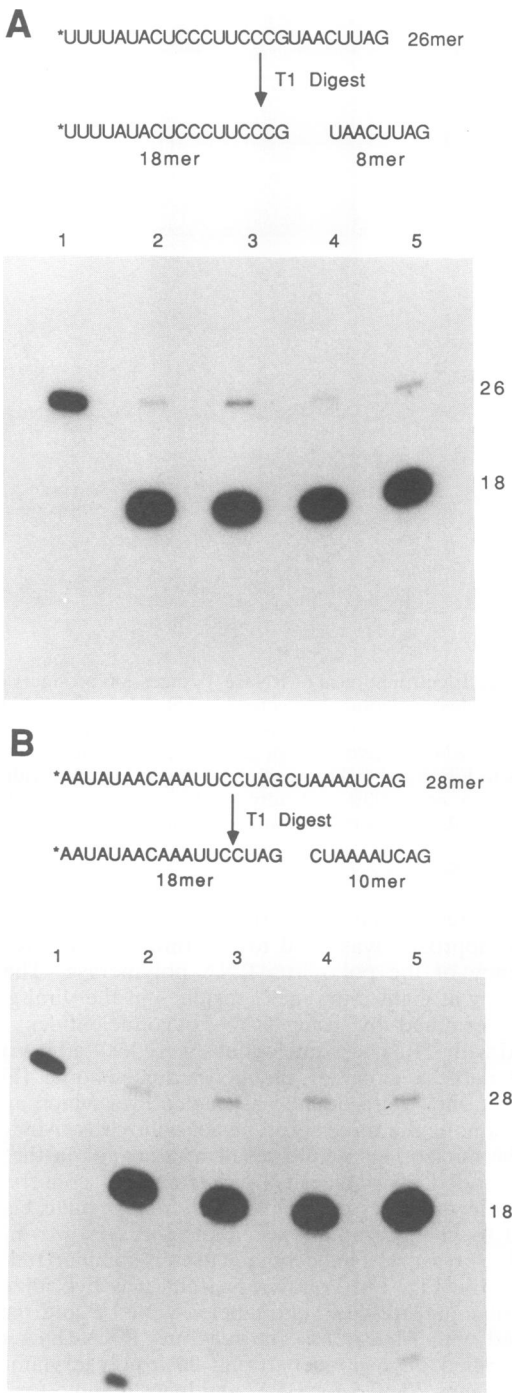

FIG. 8. Electrophoresis of final RNase T_1 digestion products of 5′-end-labeled RNA oligonucleotides protected by BF25 DNA and BF9 DNA. (A) The RNA oligonucleotide (26-mer) protected by BF25 DNA was labeled and purified by two steps of gel purification as described in the legends to Fig. 6 and 7. The purified RNA isolated in four independent hybridization and digestion experiments was digested with RNase T_1 and run on a 20% polyacrylamide–7 M urea gel (lanes 2 to 4). Undigested RNA was run as a marker (lane 1). (B) The RNA oligonucleotide (28-mer) protected by BF9 was analyzed as described for panel A.

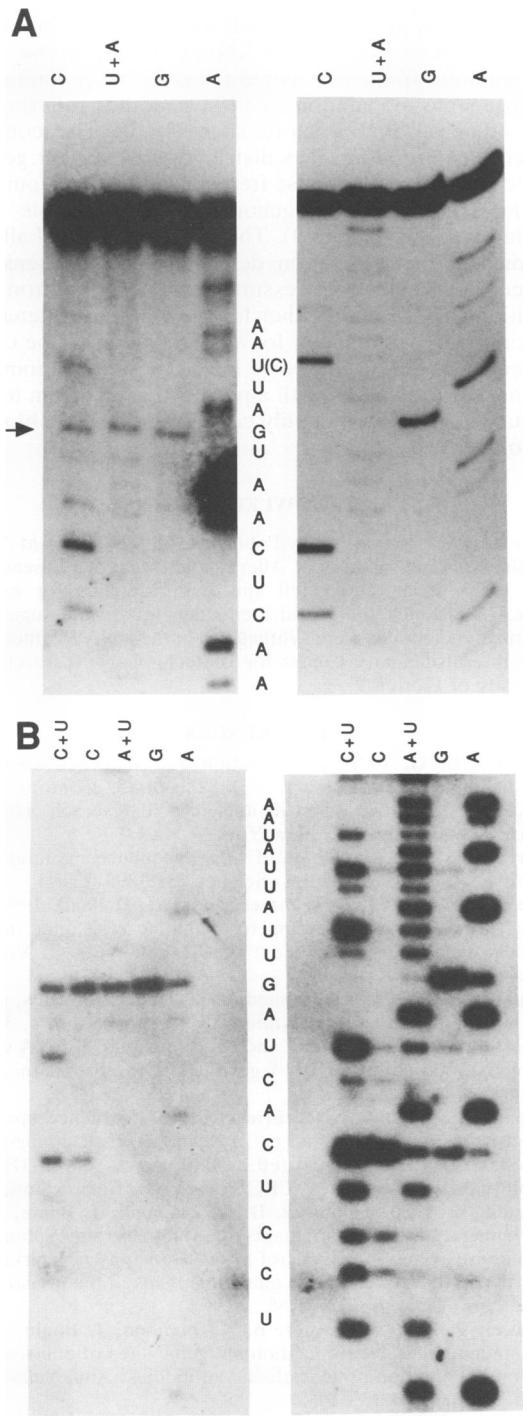

FIG. 9. Enzymatic sequencing of RNA oligonucleotides that were resistant to RNase T_1 digestion. (A) The small fraction of the RNA oligonucleotide protected by BF8 DNA that was resistant to RNase T_1 digestion was isolated by procedures identical to those described in the legend to Fig. 8. The sequence of the RNase T_1-resistant RNA was determined as described in Materials and Methods (left four lanes). The sequence of the original RNA oligonucleotide (i.e., prior to the final RNase T_1 digestion step) is shown for comparison (right four lanes). (B) The sequence of the RNase T_1-resistant RNA protected by BF11 DNA (left four lanes) and the sequence of the original RNA oligonucleotide (right four lanes) were determined as described for panel A.

total radioactivity in the bands at this position in the sequencing gels. In other experiments, the amount of the resistant oligonucleotide still containing a G residue was estimated by redigestion with RNase T_1. The amount of RNase T_1-resistant fragment that could be redigested with T_1 ranged from 10 to 50%. For this reason, an average correction factor of 25% was used. This average correction factor was taken into account in calculating the values shown in Table 1.

DISCUSSION

The results of this study indicated that the poliovirus RNA polymerase had a very high base substitution frequency in vivo. The techniques used and the results obtained were similar to those previously reported for the vesicular stomatitis virus RNA polymerase (22, 24). Because the poliovirus genome contains a 3′-terminal poly(A) sequence, some unexpected problems were encountered. It was previously shown that the average size of the 3′-terminal poly(A) sequence in poliovirion RNA is 75 nucleotides long (21). We found, however, that a small fraction of virion RNA molecules contain short poly(A) sequences. Thus, in our initial isolation of the RNase T_1-resistant RNA oligonucleotides, we found that they were contaminated with a significant amount of poly(A). For this reason, a two-step gel purification procedure to remove contaminating poly(A) from the RNase T_1-resistant RNA oligonucleotides that were used to measure the error frequency of the polymerase was developed in this study.

The polymerase error frequency at several different sites in the poliovirus genome was determined by using two types of labeled RNA. The RNA oligonucleotides used to measure the error frequency were either uniformly labeled with $^{32}P_i$ or 5′ end labeled with polynucleotide kinase and [γ-^{32}P] ATP. An average value of $2.0 \times 10^{-3} \pm 1.2 \times 10^{-3}$ was found for two sites by using uniformly labeled RNA. A total of eight sites were tested by using the 5′ end labeling method, with an average value of $4.1 \times 10^{-3} \pm 0.6 \times 10^{-3}$ being found. The error frequency did not vary significantly between the eight different sites measured on the poliovirus genome, nor did the error frequency vary significantly with the two different techniques used. These values were also similar to the error frequencies (10^{-3} to 10^{-4}) that we previously measured in vitro on homopolymeric templates (26). Thus, it appears that the poliovirus RNA polymerase error frequency is quite high, both in vitro and in vivo.

The eight sites that were measured were referred to as constant and variable sites. Two of the sites examined are known to mutate rapidly under selective pressure and these were thus referred to as variable sites. Site 1439 (BF30) is located within the E-F loop of VP2, and this site has been observed to change rapidly when the virus is grown in the presence of specific neutralizing monoclonal antibodies (18). Site 2765 (BF35) is located in the B-C loop of VP1 in which host range mutants have been mapped (15). This G is not conserved between serotypes 1 and 3, which results in an amino acid change from alanine to proline. The other six sites are conserved between the three serotypes of poliovirus. Site 101 is located in the 5′ noncoding region of poliovirus. Sites 6883 (BF8) and 7286 (BF9) are located within $3D^{pol}$, and any change in these G residues would result in an amino acid change. Site 5648 (BF10) is located within $3C^{pro}$ and site 3986 (BF11) is located within 2B, and any change in these two G residues would result in an amino acid change at these sites as well. The sixth conserved site,

1236 (BF27), is located within the β barrel B of VP2. All of the β barrels of the capsid proteins are highly conserved between the serotypes of poliovirus. A change in this G residue would result in an amino acid change from methionine to isoleucine. The error frequency determined at these eight different sites varied less than twofold. The only exception to this was the slightly lower value determined at site 5648 by using uniformly labeled vRNA. The very small differences observed did not correlate with conserved and variable regions of the viral genome. Thus, it appears that the error frequency of the poliovirus polymerase is relatively constant at G sites across the poliovirus genome.

The error frequency of this technique is dependent on the complete digestion of the protected RNA oligonucleotides by RNase T_1. For this reason RNase T_1-resistant oligonucleotides were sequenced when possible. It was found that the G residue did change to all three other ribonucleotides. However, about 25% of the RNase T_1-resistant band was due to undigested G residues. Thus, a correction factor of 25% was used when determining the error frequency. There did not appear to be a preference for one ribonucleotide over another. This was also previously observed in our in vitro assays, which directly measured the misincorporation frequency of an incorrect nucleotide by using a homopolymeric template. However, this differed from what was observed by Kuge et al. (12), who saw a strong bias toward transition mutations during poliovirus replication. We plan to investigate this point further by measuring the poliovirus polymerase error frequency in vitro on heteropolymeric templates.

The polymerase error frequency estimates determined in this study were consistent with an in vivo error frequency of 1.8×10^{-3} to 1.1×10^{-4} obtained by Steinhauer et al. (22, 24) for the vesicular stomatitis virus polymerase. In addition, a mutation frequency of 6.5×10^{-4} was reported by de la Torre et al. (3) for the reversion of a single amino acid in a poliovirus guanidine-dependent mutant. The estimated minimal base substitution frequency in this codon was 2.1×10^{-4}. The authors suggested that this was a minimal value, since the target size in this codon was closer to 1.2 than 3 and because they were scoring only for viable mutants which represent only a fraction of the total mutants (viable and nonviable). In contrast, error frequency estimates of 10^{-6} were reported in two other studies (19, 20). As discussed in more detail by de la Torre et al. (3), these values are not necessarily in conflict with the above results since different assays were used, different sites in the genome were examined, and only the viable mutation rate was determined. Obviously, additional work is now required to measure the single-base substitution frequency at a larger number of sites in the poliovirus genome by using the same assay. In addition, the difference between the total mutation frequency and the viable mutation frequency should be quantitated for specific bases.

Overall, our results suggest that the poliovirus RNA polymerase has a high error frequency, on the order of 10^{-3} to 10^{-4}. The polymerase did not appear to have a bias for a higher than average error frequency at specific sites in the genome, on the basis of our current data. This does not rule out the possibility that the error frequency could be higher at other nucleotides or at G residues that were not tested in this study. The low mutation rate measured in cell culture compared with the high mutation rate observed when picornaviruses replicate in their natural hosts is probably due to strong selection pressure to either maintain or change the viral genome. A high random polymerase error frequency and the selection for mutation at specific sites would explain in part how poliovirus and other picornaviruses can rapidly change as they replicate in their natural hosts. In addition, the quasispecies concept for RNA viruses proposes that a high mutation frequency would result in the generation of a heterogeneous population of viral genomes differing from each other at one or more sites (4, 5). The consensus sequence of a quasispecies distribution of variant genomes can be defined as the most frequent nucleotide found at a specific position in the genome when a sample of the population is sequenced (4). The relative fitness of all of the genomes in the population determines the consensus sequence. Thus, selective pressure provided by environmental conditions can provide either for the stable maintenance of the consensus sequence or for a rapid change in the consensus sequence during the evolution of the viral genome (23). This provides the virus with a powerful mechanism to adapt and survive in either a rapidly changing or a stable biological environment.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI15539 from the National Institute of Allergy and Infectious Diseases.

We thank Brian O'Donnell and Joan Morasco for excellent technical assistance and David Steinhauer for helpful suggestions. DNA oligonucleotides were synthesized in the DNA Synthesis Core of the Interdisciplinary Center for Biotechnology Research at the University of Florida.

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