JOURNAL OF VIROLOGY, Aug. 1986, p. 377–383
0022-538X/86/080377-07$02.00/0

Vol. 59, No. 2

Measurement of the Mutation Rates of Animal Viruses: Influenza A Virus and Poliovirus Type 1

JEFFREY D. PARVIN,[1] ANNE MOSCONA,[2] WAYNE T. PAN,[1] JASON M. LEIDER,[1] AND PETER PALESE[1]*

Departments of Microbiology[1] and Pediatrics, Mount Sinai School of Medicine, City University of New York, New York, New York 10029

Received 1 April 1986/Accepted 21 April 1986

Epidemiologic and genetic evidence suggests that influenza A viruses evolve more rapidly than other viruses in humans. Although the high mutation rate of the virus is often cited as the cause of the extensive variation, direct measurement of this parameter has not been obtained in vivo. In this study, the rate of mutation in tissue culture for the nonstructural (NS) gene of influenza A virus and for the VP1 gene in poliovirus type 1 was assayed by direct sequence analysis. Each gene was repeatedly sequenced in over 100 viral clones which were descended from a single virion in one plaque generation. A total of 108 NS genes of influenza virus were sequenced, and in the 91,708 nucleotides analyzed, seven point changes were observed. A total of 105 VP1 genes of poliovirus were sequenced, and in the 95,688 nucleotides analyzed, no mutations were observed. We then calculated mutation rates of 1.5×10^{-5} and less than 2.1×10^{-6} mutations per nucleotide per infectious cycle for influenza virus and poliovirus, respectively. We suggest that the higher mutation rate of influenza A virus may promote the rapid evolution of this virus in nature.

The variability of animal viruses is a well-recognized phenomenon (for a review, see reference 13). The best-studied example is the influenza A virus in which the antigenic nature is continually changing by reassortment (antigenic shift) and by mutation (antigenic drift). The property of antigenic drift allows virus from a single subtype to persist in the human population despite immunity to strains from previous years (29). It has been observed from sequence analyses that the genes coding for surface as well as nonsurface proteins of influenza A virus evolve at a rate which is approximately 1,000,000-fold greater than that of eucaryotic genes in nature (5a, 20a, 24). Although a high mutation rate of influenza A viruses is most often cited as the cause of the extensive variation (12, 24), a direct measurement of this parameter has never been obtained in vivo.

Mutation rates have been estimated for several bacteriophages. Based on the growth kinetics of spontaneous revertants from a deleterious point mutation, a mutation rate was indirectly calculated for bacteriophage QB (1), and mutation frequencies determined by growth under selective conditions have been estimated for bacteriophages lambda and T4 (7). Attempts have also been made to assess the mutability of animal viruses. For example, the substitution frequency of a specific 5′-terminal nucleotide in the vesicular stomatitis virus genome was determined (28). Similarly, the frequency of variants which have lost the recognition site for a neutralizing monoclonal antibody has been used as a measure of mutation frequency. This method has been used to analyze mutability of RNA- and DNA-containing viruses, including influenza viruses, poliovirus, vesicular stomatitis virus, and herpesviruses (10, 14, 18, 22). Alternatively, reversion frequencies of viral mutants have been used to assess mutation frequencies. For example, the accumulation of thymidine kinase-deficient mutants of herpes simplex virus was used to analyze the roles of mutator and antimutator phenotypes of this virus (11).

In this study we attempted to measure directly the mutation rates of two animal viruses by sequencing genes in randomly selected viral clones. These viral clones were all descended from a single virion after only one plaque generation, which confined the time over which mutations could occur to about five infectious cycles. The mutation rate we measured was a neutral mutation rate, because all lethal and deleterious mutations were not observed. We found that the mutation rate of influenza A virus was higher than that of poliovirus, and we speculate that this difference correlates with the speed of evolution and the lack of success of vaccination against influenza A viruses.

Accurate in vivo measurements of mutation rates should allow a greater understanding of the evolution of viruses in nature and may also provide a new way to test different evolutionary theories with respect to animal viruses. Knowledge of the inherent mutability of a virus may also clarify the mechanism by which variants emerge that are resistant to the immunity conferred by vaccination. The probability of successful vaccination against a particular virus may be determined by the mutation rate of that virus, and thus knowledge of this parameter may influence the strategy of vaccine design. Finally, an understanding of the mutability of a virus may help in studying the development of drug resistance as well as the changes in virulence observed with different RNA- and DNA-containing viruses.

MATERIALS AND METHODS

Viruses and cells. Influenza virus A/WSN/33 was derived from an uncloned viral stock preparation grown in Madin-Darby canine kidney (MDCK) cells in the presence of Eagle minimal essential medium containing 1 μg of trypsin per ml (5). The virus was then plaque passaged twice in MDCK cells, and virus from plaque passage 2 was used for the experiment described. The agar overlay for plaquing contained 0.6% agar (Oxoid Ltd.), minimal essential medium, 0.2% bovine albumin, 0.01% DEAE dextran, and 1 μg of trypsin per ml.

The Mahoney strain of poliovirus type 1 and the HeLa cell line used for its passage were kindly provided by V. Racaniello. Again, the virus was plaque passaged twice

* Corresponding author.

before being used in the experiment. Viral passage was done as described previously (2).

Preparation of influenza A virus clones and purification of viral RNA. Confluent MDCK cells were infected with serial 10-fold dilutions of the plaque-purified virus preparation. At 1 h postinfection (p.i.), the inoculum was removed by aspiration, and the standard agar overlay was added. At 48 h p.i., a well-isolated plaque was identified, and the overlay above the plaque was gently aspirated with a Pasteur pipette. Virus was eluted from the agar plug into 0.5 ml of phosphate-buffered saline containing 0.2% bovine albumin. The cell monolayer from which the plaque was picked was stained with 0.1% crystal violet in 20% methanol to demonstrate that the plaque was indeed discrete.

To clone the individual virions in the plaque, a second plaque passage was done with the virus yield from the first plaque. This time many dishes were prepared to allow the isolation of several hundred discrete plaques. The total PFU in each plaque were about 10^6. One-fifth of the yield from each plaque was used to infect 2×10^6 MDCK cells with a liquid overlay which contained the same components as the agar overlay except for the agar and dextran. The supernatant from the completely lysed monolayer was harvested at 24 to 30 h p.i. The supernatant was then diluted in phosphate-buffered saline containing 0.2% bovine albumin to infect (multiplicity of infection [MOI], ~0.2) seven or eight dishes of MDCK cells, each containing 2×10^7 cells. The medium (65 ml) was harvested approximately 30 h p.i., after complete lysis of the cell monolayer.

The medium containing virus harvested from lysed cells was precleared by centrifugation at $8,000 \times g$ for 30 min. The supernatant was then layered over a 3-ml 20% sucrose cushion, and the virus was pelleted by centrifuging at 25,000 rpm for 2 h in an SW27 rotor (Beckman Instruments, Inc.). The virus pellet was suspended in 4 ml of 100 mM NaCl–10 mM Tris hydrochloride (pH 7.4)–1 mM disodium EDTA. The RNA from the suspended virus was extracted (approximately 100 μg) (21).

Preparation of poliovirus type 1 clones and of viral RNA. Confluent HeLa cell monolayers were infected with serial dilutions of the plaque-purified poliovirus preparation. At 1 h p.i., the virus inoculum was removed by aspiration, and the standard agar overlay containing 1% agar, Dulbecco modified Eagle medium, 5% horse serum, and 0.01% DEAE dextran was added. At 36 h p.i., a well-isolated plaque was identified, and the agar above it was aspirated as before. The monolayer was stained with crystal violet solution to confirm that the plaque was discrete. Virus was eluted from the agar plug into phosphate-buffered saline containing 0.2% horse serum. The total yield from the plaque was 5.2×10^6 PFU.

To clone individual virions from the virus yield of the plaque, a second round of plaque passage was done as before, except that the time allowed for plaque formation was 48 h. One-fifth of the virus yield from each plaque was used to infect 2×10^6 HeLa cells with a liquid overlay which contained the same components as the agar overlay except for the agar and dextran. At 24 h p.i., the medium above the lysed cells was harvested. This supernatant was then diluted for the infection (MOI, 0.5) of five large dishes containing 2×10^7 HeLa cells each. At 24 h p.i., the yield from the lysed cells was harvested.

The medium containing virus (40 ml) was precleared by centrifugation at $9,000 \times g$ for 30 min. The supernatant was centrifuged at $130,000 \times g$ for 90 min. The pelleted virus was suspended in 4 ml of buffer containing 100 mM NaCl, 10 mM Tris hydrochloride (pH 7.4), and 1 mM disodium EDTA, and the RNA (approximately 100 μg) was extracted as was done for the influenza virus.

Sequencing of viral RNA. The viral RNA was sequenced directly by the method of Sanger et al. (26). The hybridization mixture contained 10 μg of viral RNA and 200 ng of a specific oligonucleotide as primer. Three primers were used to sequence each gene. The primers used for the NS gene were complementary to the viral RNA at positions 10 to 29, 293 to 312, and 593 to 612 (5a). The VP1 gene primers were complementary to positions 3422 to 3403, 3136 to 3117, and 2812 to 2793 on the viral RNA (15). The hybridization mix was heated in boiling water for 5 min, cooled to room temperature, and aliquoted to four reaction tubes. The A reaction contained 250 μM each dGTP and dTTP, 100 μM dATP, 12.5 μM dideoxy ATP, and 10 μM dCTP. The G reaction contained 250 μM each dATP and dTTP, 100 μM dGTP, 12.5 μM dideoxy GTP, and 10 μM dCTP. Both the A and G reactions had 15 μCi of [α-^{32}P]dCTP (3,000 Ci/mmole) or [α-^{35}S]dCTP (600 Ci/mmole) added to each reaction. The C reaction contained 250 μM each dGTP and dTTP, 100 μM dCTP, 12.5 μM dideoxy CTP, and 10 μM dATP. The T reaction contained 250 μM each dCTP and dGTP, 100 μM dTTP, 12.5 μM dideoxy TTP, and 10 μM dATP. To both the C and T reactions 15 μCi of [α-^{32}P]dATP (3,000 Ci/mmole) or [α-^{35}S]dATP (600 Ci/mmole) was added. The reactions were started by the addition of 5 U of avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Inc.) and incubated at 42°C for 30 min. The total reaction volume was 10 μl. A cold-chase solution containing 2 mM concentrations of each nucleotide was added (1.5 μl). After a second 30-min incubation at 42°C, the reactions were terminated by the addition of 11 μl of a formamide dye mix. Before gel electrophoresis, the reactions were heated in boiling water for 5 min and then rapidly cooled in an ice water bath.

As a standard procedure, two different gel systems were used to analyze the sequencing results. Gel 1 was a 6% polyacrylamide buffer-gradient gel (3) or a 6% polyacrylamide wedge-shaped gel (CBS Scientific Company, Inc.) which generally permitted resolution of the sequence 10 nucleotides from the primer to at least 180 nucleotides from the primer. Gel 2 was the standard 6% polyacrylamide gel which was used for a long electrophoresis of the reaction products. Gel 2 generally permitted the resolution of nucleotides from 160 to 320 or more bases away from the primer. Gels were fixed in a 10% acetic acid–10% methanol solution, dried, and exposed to Cronex 4 film (E. I. du Pont) for 3 to 10 days.

With this method, bands occasionally appeared in more than one lane at a given position. The secondary bands proved not to be troublesome since their patterns were uniform for positions among the different RNAs.

For the influenza virus NS gene, 91,708 nucleotides were sequenced. Of these, 50 nucleotides could not be positively identified because the bands in the expected lanes were too faint. However, since no other band appeared in any of the other three lanes at the same level, it was assumed that no changes had occurred at these positions. Of the 95,688 sequenced nucleotides for the VP1 gene, 323 could not be positively identified for the same technical reason. Again, none of these positions was scored for mutations because bands were not observed in any of the other three lanes.

Comparison of growth kinetics of NS gene mutants. To determine the relative growth kinetics of each influenza virus NS gene variant, a multicycle infection was done for each mutant, the parental virus, and two randomly picked con-

trols whose NS genes had the wild-type sequence. Samples of each virus were titered in advance, and each was used to infect a dish of MDCK cells at an MOI of 1,000 PFU/3 $\times 10^6$ cells. At 1 h p.i., the inocula were removed from the dish, each monolayer was washed with phosphate-buffered saline, and the standard liquid overlay was added. At 10, 19, 24, 28, 32, 37, 42, and 48 h p.i., 0.3 ml of medium was removed from each dish and was used for plaque titrations. Cytopathic effect was first observed at 37 h.

Conversion of the NS gene mutation rate for comparison. The mutation rate of the NS gene as defined in this paper was calculated as the amount of change which would occur with each cell burst. Drake (7) and Koch and Drake (16) calculated mutation rates from mutation frequencies. By this definition of mutation rate, the number of replications reflected the number of times the genome was copied. Replication number was approximately equal to population size. The formula was: mutation rate = 0.4343 $\times$ mutation frequency/log(population size). With this formula, the NS gene mutation frequency in a plaque was seven changes per 91,708 nucleotides, and the population size was equal to the total nucleotide pool (1.2×10^6 PFU $\times$ 850 nucleotides analyzed per NS gene), which resulted in the converted mutation rate of 3.7×10^{-6} mutations per nucleotide per replication. Alternatively, a correction factor substituting population size with population size times mutation rate could be made since no mutations would be expected until the population size equaled the mutation rate (J. W. Drake, personal communication). The corrected mutation rate was then 8.4×10^{-6} mutations per nucleotide per replication. If the mutation rate ceiling for the poliovirus was similarly converted, it would have been 4.7×10^{-7} mutations per nucleotide per replication or, including the correction factor, 1.2×10^{-6} mutations per nucleotide per replication. By this procedure, the values for the mutation rates of bacteriophage lambda, bacteriophage T4, *Salmonella typhimurium*, *Escherichia coli*, and *Neurospora crassa* were 2.4×10^{-8}, 1.7×10^{-8}, 2.0×10^{-10}, 2.0×10^{-10}, and 0.7×10^{-11}, respectively (7).

RESULTS

Determination of the NS gene mutation rate. The influenza virus clones used to determine the mutation rate in the NS gene were derived from a single plaquing experiment (Fig. 1). Plaque formation occurred under standard tissue culture conditions in the absence of any new selective pressure. Cell monolayers were infected at a high dilution with plaque-purified A/WSN/33 virus, and at 48 h p.i., a single, well-isolated plaque was picked. This plaque, descended from a single virion, contained 1.2×10^6 PFU. To determine the number of mutant NS genes among these viral particles, individual clones were obtained, and viral RNA was prepared after only two amplification steps. The MOI during the amplification steps was maintained at 0.1 for step 1 and 0.2 for step 2 to minimize the enrichment of new mutants. Since RNA obtained from amplified viral preparations was sequenced by using the primer extension protocol, the sequence represented a consensus sequence which should have been identical to that of the individual clone. Any mutants which may have arisen in the amplification protocol would not have been detected.

The NS genes of 108 different clones were sequenced, producing 91,708 nucleotides of information. Comparison of the NS gene sequences revealed seven mutants. The relevant sequencing gels of two of these mutants are shown in Fig. 2. The sequences for five different clones are presented

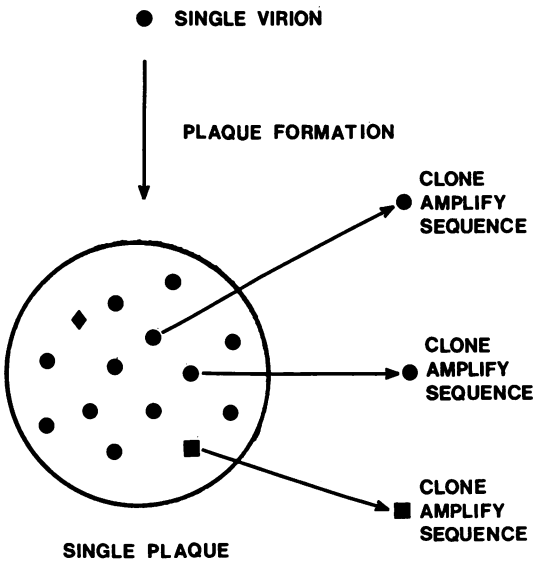

FIG. 1. Experimental design for measuring mutation rates in viral genes. A single virion (the parental virus) formed a plaque after sufficient time for about five infectious cycles. The many progeny virions contained within the plaque carried genes with the parental sequence (●), and a fraction of the virions carried genes with a point mutation (◆ and ■). The individual virions were cloned by plaquing and amplified under conditions that minimized the effect of new mutations on the consensus sequence of the gene in the clone. RNA obtained from purified virus was directly sequenced. The mutation rate was determined by dividing the number of observed point mutations by the number of nucleotides analyzed and by the number of infectious cycles.

for positions 151 to 181 (Fig. 2A). As indicated by the arrows, a G to T transversion in clone 014 was observed at position 169. The sequences for five other clones are shown for positions 751 to 788 (Fig. 2B). On this gel, the G reactions of the five clones were loaded side-by-side as were the A, T, and C reactions. This latter arrangement facilitated the rapid detection of mutants. The A to G transition at position 772 in clone 024 can easily be observed in this gel (arrows). All seven point mutations are listed in Table 1 along with the predicted amino acid coding changes. As can be seen, the mutations were evenly distributed along the gene, and there was only one mutation per variant gene. Two mutants, 050 and 069, which were derived from clones on separate dishes were found to have the same point change. This finding was not unexpected since point mutations which occur early in the development of the plaque would represent a higher fraction of the variants than ones which occur late. Six of the seven variants were found to encode amino acid changes in the NS gene protein products. This observed frequency of coding changes is in agreement with the predicted frequency of coding changes in a randomly mutated gene coding for overlapping NS1 and NS2 proteins.

It is assumed that back-mutations were negligible and that the mutants had a growth fitness similar to that of the wild-type virus (see below). Based on these assumptions, dividing the number of variants by the number of infectious cycles accounted for the effect of mutations occurring over several replications and of mutations being sampled twice.

The mutation rate was calculated by using the formula: mutation rate = 7 mutations/91,708 nucleotides/5 infectious cycles = 1.5×10^{-5} mutations per nucleotide per infectious cycle.

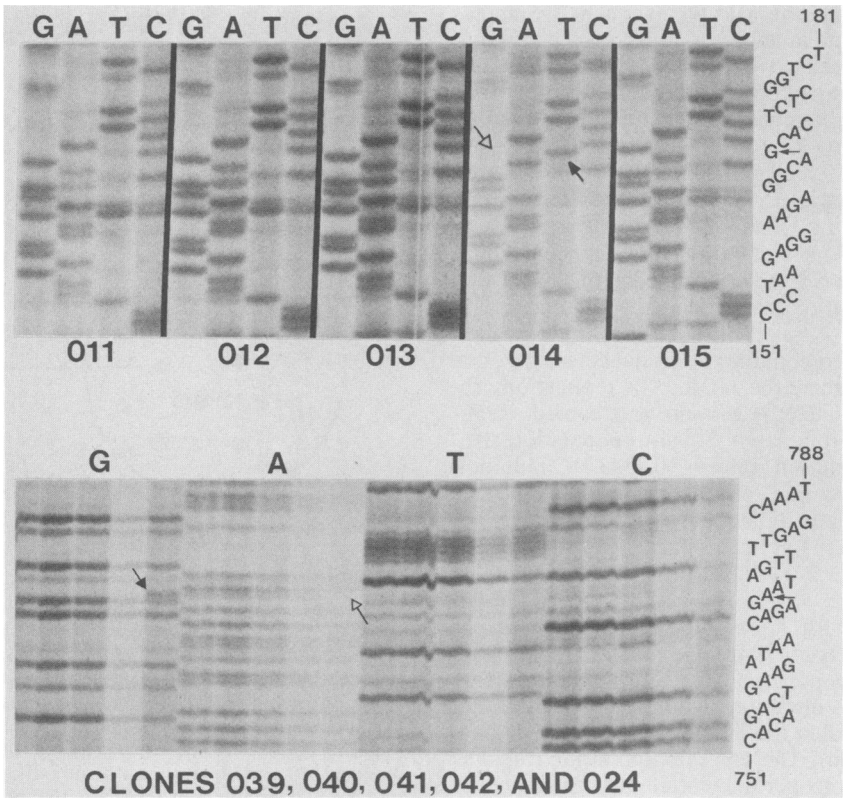

FIG. 2. Detection of point mutations in NS genes of influenza virus clones 014 and 024. (A) Sequencing reactions of the NS genes of clones 011, 012, 013, 014, and 015, were electrophoresed in a 6% polyacrylamide buffer-gradient gel. Positions 151 to 181 are shown, and the G to T change at position 169 in clone 014 is indicated (arrows). (B) Sequencing reactions for the NS genes of clones 039, 040, 041, 042, and 024 were electrophoresed in a buffer-gradient gel. The G reactions for five clones, 039, 040, 041, 042, and 024, were loaded side-by-side as were the A, T, and C reactions. Positions 751 to 788 are shown, and the A to G transition at position 772 in clone 024 is indicated (arrows).

Although plaque growth dynamics are complex and although synchrony in burst cycles may be lost, the number of growth cycles was most probably five. If all the sampled virus clones were derived from only four infectious cycles, the mutation rate would be underestimated by 26%. Similarly, if all the sampled clones resulted from six bursts, the mutation rate would be overestimated by 17% in our calculations. Thus, the error contributed by clones that had different numbers of growth cycles was small.

Characterization of NS gene variants. It was assumed in calculating the mutation rate that the mutants had no significant selective advantage or disadvantage during plaque formation. If the mutations were phenotypically neutral, the percentage of a given mutant would remain constant as the total virus population expanded in the plaque.

TABLE 1. Point mutations detected among NS genes of 108 influenza A/WSN/33 virus clones

Mutant	Mutation[a]	Amino acid change	Position
014	G → U	Ser → Ile in NS1	169
024	A → G	Asn → Asp in NS2	772
031	U → C	Trp → Arg in NS1	72
049	U → C	Silent	68
050	G → A	Arg → Lys in NS1	379
069	G → A	Arg → Lys in NS1	379
082	C → U	Leu → Phe in NS2	856

[a] Mutations detected by RNA sequencing are presented in the message orientation. The parent NS gene sequence is according to Buonagurio et al. (5a).

To test whether the NS gene mutations were neutral, the rate of virus production was assayed in a multicycle infection. The time course of virus production in multicycle infections is very sensitive to small changes in virus fitness. The results are diagrammed in Fig. 3. Included in the study were the parent clone and two randomly selected virus clones whose NS genes did not contain mutations. Samples from the liquid overlay taken at the indicated times were titered for the concentration of PFU. In the diagram, lines connect the time points in the controls. As can be seen, there was no significant difference between the wild-type and variant clones. One virus appeared to lag behind the others at some intermediate time points, but it was the variant carrying the silent mutation, and it is very unlikely that this NS gene mutation was responsible for the small lag. All seven variants were therefore indistinguishable from the wild-type virus, and thus the mutations appeared to be neutral.

Determination of VP1 gene mutation rate. Plaque passage of the Mahoney strain of poliovirus type 1 was done as for the influenza virus experiment. Virus which had been plaque purified twice was infected onto cell monolayers, and at 36 h p.i., a well-isolated plaque containing 5.2×10^6 infectious particles was picked. Since the generation time of poliovirus is about 7 h, this allowed for about five infectious cycles. Again, individual clones from among the 5.2×10^6 PFU were

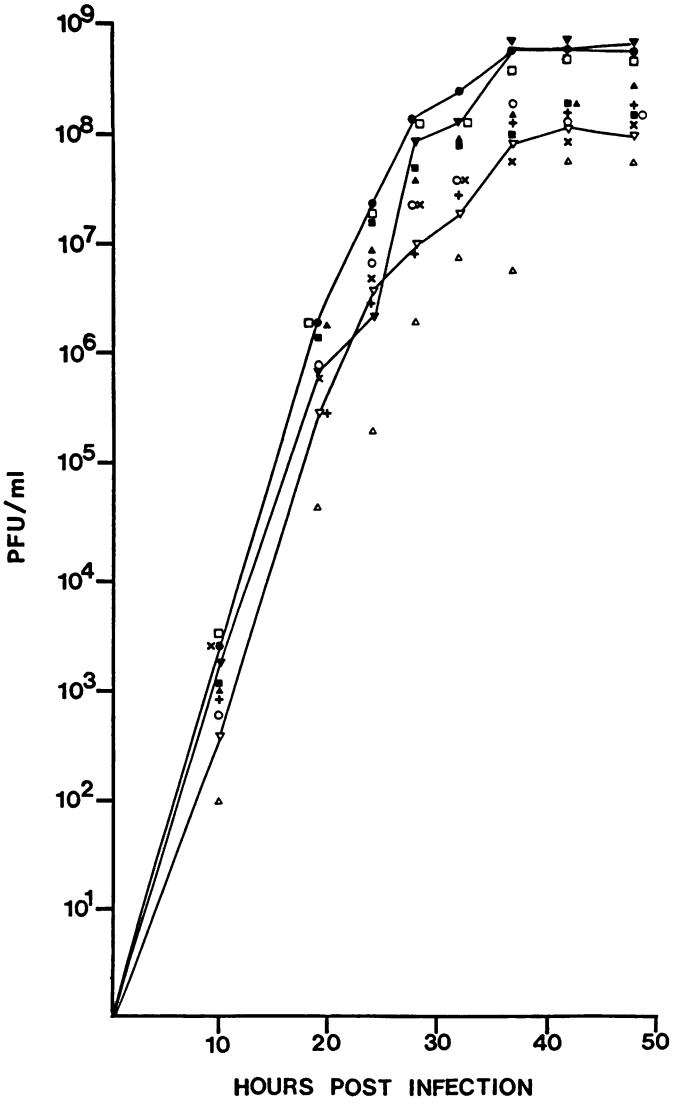

FIG. 3. Comparison of growth kinetics of influenza virus NS gene variants in MDCK cells. The time courses of production of virus after infection at an MOI of 3×10^{-4} were determined for the parental virus, two clones which had the parental NS gene sequence, and the seven variants. The PFU per ml of the parental virus (●) and clones 007 (▼), 014 (■), 024 (□), 031 (○), 049 (△), 050 (▲), 069 (×), 082 (+), and 086 (▽) are shown. The data points for the viruses containing the parental NS gene sequence (the parental virus and clones 007 and 086) are connected by lines for clarity. The PFU per ml of supernatant medium were determined for different time points by plaque titration on MDCK cells.

obtained from this plaque, and viral RNA was prepared following a two-step amplification process.

The sequence of the Mahoney strain VP1 gene is the same as that presented by Racaniello and Baltimore (23) except for three point changes at positions 2837 (A to G), 3139 (C to T), and 3151 (A to T). (Numbering is according to Kitamura et al. [15].) These three positions also differ from the sequence presented by Kitamura et al. and probably represent the changes which randomly occur after repeated passage. Interestingly, two of the three changes were associated with amino acid changes in the VP1 protein. When the sequences of the 105 VP1 genes were compared, no point mutations were observed. Since no mutations occurred in the VP1 genes of the clones analyzed, the upper limit of the mutation rate can be calculated as follows: mutation rate < 1 mutation/95,688 nucleotides/5 infectious cycles $< 2.1 \times 10^{-6}$ mutations per nucleotide per infectious cycle.

Analysis of the statistical significance of the difference between mutation rates in the two experiments was performed by a chi-square test with a two-by-two contingency table of the data. It was found that the mutation rate of the VP1 gene was significantly lower than that of the NS gene ($P < 0.025$). In addition, the difference was statistically significant by a Student t test applied to the binomial standard error of the influenza virus mutation rate ($P < 0.01$). However, since each mutation rate was only measured once, we cannot predict the variation in mutation rates had more than one mutation experiment been performed for each virus.

DISCUSSION

Comparison of influenza A virus and poliovirus type 1 mutation rates. The present study was undertaken to determine the precise mutation rates of two different RNA viruses. The specific strains were chosen because of the technical ease of plaque passaging at 37°C and because of the excellent growth characteristics which facilitated the sequencing of template preparations. The genes were selected based on prior demonstrations that they were capable of variation in nature or in the laboratory. The NS gene of influenza A virus was shown to evolve very rapidly in nature (5a, 17), and the VP1 gene of poliovirus type 1 was shown to tolerate mutations (10, 19). The influenza A virus mutation rate was found to be significantly higher than that of poliovirus type 1. This result was surprising in the light of earlier data involving the selection of antigenic variants with neutralizing monoclonal antibody preparations. The frequency of variants, as measured by the plaque reduction assay, was as high for poliovirus type 1 as it was for influenza A virus in many cases (10, 18). However, this assay may not reflect the true mutation frequency of the viral genomes, since the result may be affected by various other parameters. For example, the avidity and discriminating capabilities of the monoclonal antibody preparations may differ. Also, the genomic target size for the antibody-combining sites is unknown. Further, it has been shown with poliovirus that even mutations outside of the antibody-binding site can inhibit neutralization by the antibody (4). It is thus difficult to predict a precise mutation rate from data on the frequency of antigenic variants.

Is the lower mutation rate in the poliovirus VP1 gene the result of high replicase fidelity or is it due to very high constraints against lethal or deleterious mutations? The data on the selection of antigenic variants (10) demonstrates that there must be regions along the gene which have relaxed constraint, and comparison of the VP1 sequences of the Mahoney and the Sabin 1 strains reveals that many coding and silent changes are allowed in the VP1 gene. The VP1 gene, which represents 12% of the genome, contains 9 of the 57 total point changes and 7 of the 19 coding differences between the Mahoney and Sabin strains (19). It is thus suggested that the absence of poliovirus VP1 mutants among the 105 clones sequenced may not be due to constraints alone but may also be the result of increased fidelity of the viral replicase.

Based on the calculated mutation rates, the following prediction can be made regarding the frequencies of mutants present in influenza virus and poliovirus populations. If all the influenza A virus genes are under the same constraints as

the NS gene and thus have the same mutation rate, then every virus in a plaque would average one point change per genome (mutation rate × 13.6-kilobase genome × 5 cycles). An influenza virus population therefore represents a quasispecies (8, 9) in which there is a consensus sequence, but each individual is unique. A similar calculation suggests that a poliovirus plaque would contain less than 8% mutant infectious particles. Therefore, the diversity in the poliovirus population is not as great as that predicted in the influenza virus population.

Comparisons of mutation rate estimates in other systems. Mutation rates and mutation frequencies have been estimated in a variety of other systems. Recently, a study of the vesicular stomatitis virus substitution frequency revealed a surprisingly high in vitro rate of misincorporation of an extracistronic nucleotide (28). The frequency of misincorporation was about 10^{-4} substitutions per base incorporated at the site. The same site was analyzed in vivo, and the substitution frequency was again found to be about 10^{-4}. Caution must be exercised in comparing this substitution frequency with the mutation rates determined for influenza and polioviruses, since this specific site may not be characteristic for the genome. In this respect, it should be noted that mutation frequencies of specific single bases are often idiosyncratic. In the phage T4rII locus, 10^4-fold differences in mutation frequencies have been observed for single nucleotides at different sites in the gene (25). The indication that VSV might have a high mutation rate would suggest that a direct comparison with the mutation rates measured in this study by using the same methods would be very interesting. The direct analysis of clones derived from a single plaque passage, as was done in the present study, examines the mutation rate of an entire gene and thus appears to give more reliable data.

Mutation frequencies have also been estimated for virus populations after serial uncloned tissue culture passage by using T1 oligonucleotide mapping analysis. After 28 passages, foot-and-mouth disease virus was shown to accumulate virus that consists of particles with two to eight mutations per genome (27), and bacteriophage QB was similarly shown to have one to two changes per genome after 30 or more passages (6). These estimates, based on multiple passages with an unknown number of replication cycles, do not allow us to calculate mutation rates (mutation frequency per infectious cycle) for these viruses, and thus these values cannot be directly compared with the data obtained for influenza and polioviruses during a single plaque generation.

Other estimates of mutation rates for bacteriophages, bacteria, and a fungus were derived from experiments in which the yield of test organisms grown under selective conditions was compared with the yield grown under permissive conditions. Although the molecular basis of the mutation allowing growth under selective conditions was unknown, it was assumed to be a point mutation, and the gene was assumed to have a standard length if the length was unknown (7). To compare our data with these results, the NS gene mutation rate must be converted to fit the definitions of Drake (7). The mutation rate of the NS gene after this conversion is approximately 10^2 times higher than the rates of bacteriophages lambda and T4, approximately 10^4 times higher than the rates of *S. typhimurium* and *E. coli*, and approximately 10^5 times higher than that of *N. crassa*. (For detailed calculations see Materials and Methods.)

Role of mutation rate in viral evolution. There is epidemiologic and genetic evidence that influenza A viruses evolve more rapidly than other viruses in humans (29, 30). Specifically, vaccine strains used against influenza A viruses have to be changed frequently (at least every 2 to 3 years) to protect against an evolving virus population. In contrast, vaccines against poliovirus (and most other human viruses) are based on strains which have been used for the last several decades, ostensibly without loss in efficacy. One might therefore speculate that the higher mutation rates found for influenza A viruses provide a molecular basis for this difference. High mutation rates would be necessary to generate the great amount of diversity required for the extraordinarily rare event of producing a beneficial mutant which would be selected in the host environment. It should be noted, however, that extensive variation has been observed in poliovirus populations circulating in nature (20), and thus, the above model for the role of mutation rate must remain but one hypothesis for explaining the phenomenon of the rapid evolution of influenza A viruses.

Furthermore, one would like to know whether other viruses, for example, the acquired immune deficiency syndrome virus or herpesviruses, show comparably high mutation rates which may then affect the successful use of vaccines. The precise measurement of the mutation rates of other viruses may thus help to dissect the factors which determine the complex genetic interactions of viruses with the natural environment.

ACKNOWLEDGMENTS

We thank M. Eigen and P. Schuster for helping us during the 1985 winter seminar in Klosters to develop the concept for the experiments reported in this paper. We also thank J. W. Drake, J. L. Schulman, M. Krystal, and F. A. Bonilla for helpful suggestions on the manuscript before submission, and we thank V. Racaniello for supplying the Mahoney strain of poliovirus type 1 and the HeLa cell line used for its passage.

J.D.P., W.T.P., and J.M.L. are all supported by Medical Scientist Training grant GM07280 from the National Institutes of General Medical Sciences. A.M. is supported by the Revson Foundation. This work was supported by Public Health Service grants AI-11823 and AI-18998 from the National Institutes of Health.

LITERATURE CITED

1. **Batschelet, E., E. Domingo, and C. Weissmann.** 1976. The proportion of revertant and mutant phage in a growing population, as a function of mutation and growth rate. Gene **1**:27–32.
2. **Bernstein, H. D., N. Sonenberg, and D. Baltimore.** 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol. Cell. Biol. **5**:2913–2923.
3. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and 35S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
4. **Blondel, B., R. Crainic, O. Fichot, G. Dufraisse, A. Candrea, D. Diamond, M. Girard, and F. Horaud.** 1986. Mutations conferring resistance to neutralization with monoclonal antibodies in type 1 poliovirus can be located outside or inside the antibody-binding site. J. Virol. **57**:81–90.
5. **Brand, C., and P. Palese.** 1980. Sequential passage of influenza virus in embryonated eggs or tissue culture: emergence of mutants. Virology **107**:424–433.

5a. **Buonagurio, D. A., S. Nakada, J. D. Parvin, M. Krystal, P. Palese, and W. M. Fitch.** 1986. Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene. Science **232**:980–982.

6. **Domingo, E., D. Sabo, T. Taniguchi, and C. Weissmann.** 1978. Nucleotide sequence heterogeneity of an RNA phage population. Cell **13**:735–744.
7. **Drake, J. W.** 1969. Comparative rates of spontaneous mutation. Nature (London) **221**:1132.
8. **Eigen, M.** 1971. Selforganization of matter and the evolution of

biological macromolecules. Naturwissenschaften **58**:465–523.
9. **Eigen, M., and P. Schuster.** 1977. The hypercycle, a principle of natural self-organization, part A: emergence of the hypercycle. Naturwissenschaften **64**:541–565.
10. **Emini, E. A., S.-Y. Kao, A. J. Lewis, R. Crainic, and E. Wimmer.** 1983. Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. J. Virol. **46**:466–474.
11. **Hall, J. D., D. M. Coen, B. L. Fisher, M. Weisslitz, S. Randall, R. E. Almy, P. T. Gelep, and P. A. Schaffer.** 1984. Generation of genetic diversity in herpes simplex virus: an antimutator phenotype maps to the DNA polymerase locus. Virology **132**:26–37.
12. **Hayashida, H., H. Toh, R. Kikuno, and T. Miyata.** 1985. Evolution of influenza virus genes. Mol. Biol. Evol. **2**:289–303.
13. **Holland, J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol.** 1982. Rapid evolution of RNA genomes. Science **215**:1577–1585.
14. **Holland, T. C., S. D. Marlin, M. Levine, and J. Glorioso.** 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. **45**:672–682.
15. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecaf, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
16. **Koch, R. E., and J. W. Drake.** 1973. Ligase-defective bacteriophage T4. I. Effects on mutation rates. J. Virol. **11**: 35–40.
17. **Krystal, M., D. Buonagurio, J. F. Young, and P. Palese.** 1983. Sequential mutations in the NS genes of influenza virus field strains. J. Virol. **45**:547–554.
18. **Lubeck, M. D., J. L. Schulman, and P. Palese.** 1980. Antigenic variants of influenza viruses: marked differences in the frequencies of variants selected with different monoclonal antibodies. Virology **102**:458–462.
19. **Nomoto, A., T. Omata, H. Toyoda, S. Kuge, H. Horie, Y. Kataoka, Y. Genba, Y. Nakano, and N. Imura.** 1982. Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc. Natl. Acad. Sci. USA **79**:5793–5797.
20. **Nottay, B. K., O. M. Kew, M. H. Hatch, J. T. Heyward, and J. F. Obijeski.** 1981. Molecular variation of type 1 vaccine-related and wild polioviruses during replication in humans. Virology **108**:405–423.
20a. **Palese, P.** 1986. Rapid evolution of human influenza viruses, p. 53–68. *In* S. Karlin and E. Novo (ed), Evolutionary processes and theory. Academic Press, Inc., New York.
21. **Palese, P., and J. L. Schulman.** 1976. Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. Proc. Natl. Acad. Sci. USA **73**:2142–2146.
22. **Portner, A., R. G. Webster, and W. J. Bean.** 1980. Similar frequencies of antigenic variants in Sendai, vesicular stomatitis and influenza A viruses. Virology **104**:235–238.
23. **Racaniello, V. R., and D. Baltimore.** 1981. Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc. Natl. Acad. Sci. USA **78**:4887–4891.
24. **Saitou, N., and M. Nei.** 1986. Polymorphism and evolution of influenza A virus genes. Mol. Biol. Evol. **3**:57–74.
25. **Salts, Y., and A. Rosen.** 1971. Neighbor effects in the mutation of ochre triplets in the T4rII gene. Mutat. Res. **13**:109–113.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
27. **Sobrino, F., M. Davila, J. Ortin, and E. Domingo.** 1983. Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell culture. Virology **128**:310–318.
28. **Steinhauer, D. A., and J. J. Holland.** 1986. Direct method for quantitation of extreme polymerase error frequencies at selected single base sites in viral RNA. J. Virol. **57**:219–228.
29. **Webster, R. G., W. G. Laver, G. M. Air, and G. C. Schild.** 1982. Molecular mechanisms of variation in influenza viruses. Nature (London) **296**:115–121.
30. **World Health Organization.** 1985. New approaches to vaccine development: memorandum from a WHO meeting. Bull. WHO **63**:479–484.