Comparison of the Mutation Rates of Human Influenza A and B Viruses

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Human influenza A viruses evolve more rapidly than influenza B viruses. To clarify the cause of this difference, we have evaluated the mutation rate of the nonstructural gene as revealed by the genetic diversity observed during the growth of individual plaques in MDCK cells. Six plaques were studied, representing two strains each of type A and B viruses. A total of 813,663 nucleotides were sequenced, giving rates of 2.0×10^{-6} and 0.6×10^{-6} mutations per site per infectious cycle, which, when extended to 1 year, agree well with the published annual evolutionary rates.

Human influenza A and B viruses (A and B viruses) are equally prevalent among human beings, yet most A virus genes evolve two to three times faster than the corresponding genes in B viruses (11, 13, 15). The high evolutionary rate of A viruses has been attributed to positive selection by the human immune system (2–4, 8, 12), and the difference between A and B to an absence of such selection in B viruses (2, 27). However, a possible alternative explanation of the difference in evolutionary rates, a difference in mutation rates at the molecular level, has not been investigated (27). To correct this lack, we have compared the mutation rates of the nonstructural (NS) genes of several circulating A and B viruses during replication in Madin-Darby canine kidney (MDCK) cells, which are unaffected by the human immune system.

Previously, Parvin et al. reported a mutation rate for the NS gene of influenza A virus A/WSN/33(H1N1) (WSN) of 1.5×10^{-5} mutations per nucleotide per infectious cycle during the growth of a single plaque in MDCK cells (23). In their study, Parvin et al. demonstrated that most of the NS mutants they detected replicated with fitness similar to that of the parent virus. This suggests that the MDCK cells did not encourage or discourage the appearance of these mutations, and hence are a suitable medium for the study of mutation rates. We have adopted their method (23), with some modifications.

Since the 1991–1992 and 1992–1993 influenza seasons, the receptor-binding specificity of the A viruses, both H1N1 and H3N2, has changed (19, 20, 21). In particular, the binding affinity of H3N2 viruses to MDCK cells has become weaker (22). To avoid this effect, we have used viruses isolated before this change occurred. All viruses used in this study, A/Aichi/1/87(H1N1) (A/1/87), A/Aichi/12/92(H3N2) (A/12/92), B/Aichi/29/99 (B/29/99), and B/Aichi/44/01 (B/44/01), were directly isolated from clinical samples in MDCK cells.

The mutation rates for the NS genes of A (ANS) and B (BNS) viruses were measured using the procedure illustrated in Fig. 1. In brief, well-isolated parent plaques were picked when they had reached a diameter of approximately 2 mm at

48 and 71 h postinfection for A and B viruses, respectively. The parent plaques were used to produce a series of descendant plaques grown to similar diameters.

The nucleotide sequences of the NS genes in each plaque were determined directly using the reverse transcription-PCR (RT-PCR) technique (Table 1) (21). Mutations were identified by comparing the sequence in randomly selected descendant plaques to the sequence in the corresponding parent plaque using sequence analysis software, GENETYX-MAC ver.11.0 (Genetyx Corporation, Japan). The direct sequencing of RT-PCR products produces an average sequence of many independent copies of the viral cDNA. Although some molecules will be miscopied during the PCR process, they represent a small fraction of the total and do not affect the average sequence (10, 24). All mutations (Table 2) were confirmed by sequencing with the different sense primers listed in Table 1 a total of at least three times.

Most experiments counting the frequency of mutants in viral plaques find a high variability (16, 25, 26). Most of this variability arises from replication errors occurring early in the growth of the plaque. Such random events, when they occur, are amplified to produce large clone lines of identical mutants (Fig. 1), greatly increasing the variance of the number of mutants detected (18, 25). With direct sequencing, it is possible to distinguish each replication error. Counts of the number of unique mutant sequences are related to the number of mutation events, as opposed to the number of progeny resulting from them. Such counts vary only as expected from Poisson statistics. Although no mutant clone lines were detected among the samples taken during the main experiment, in a preliminary test with a B/44/01 parent plaque grown for 96 h, a sample of 169 descendants revealed three mutants. Of these, two exhibited the same mutant sequence, indicating they were very likely clones descended from the same mutation event. By contrast, an A/12/92 parent plaque grown for 72 h contained three different mutant sequences in a sample of 162 descendants.

The results from the main sequencing experiment are listed in Tables 1 and 2. A total of six experiments were performed, three with B viruses (Table 1, experiments 1 to 3) and three with A viruses (Table 1, experiments 4 to 6). For B viruses, a total of two mutations were detected among 461,304 nucleo-

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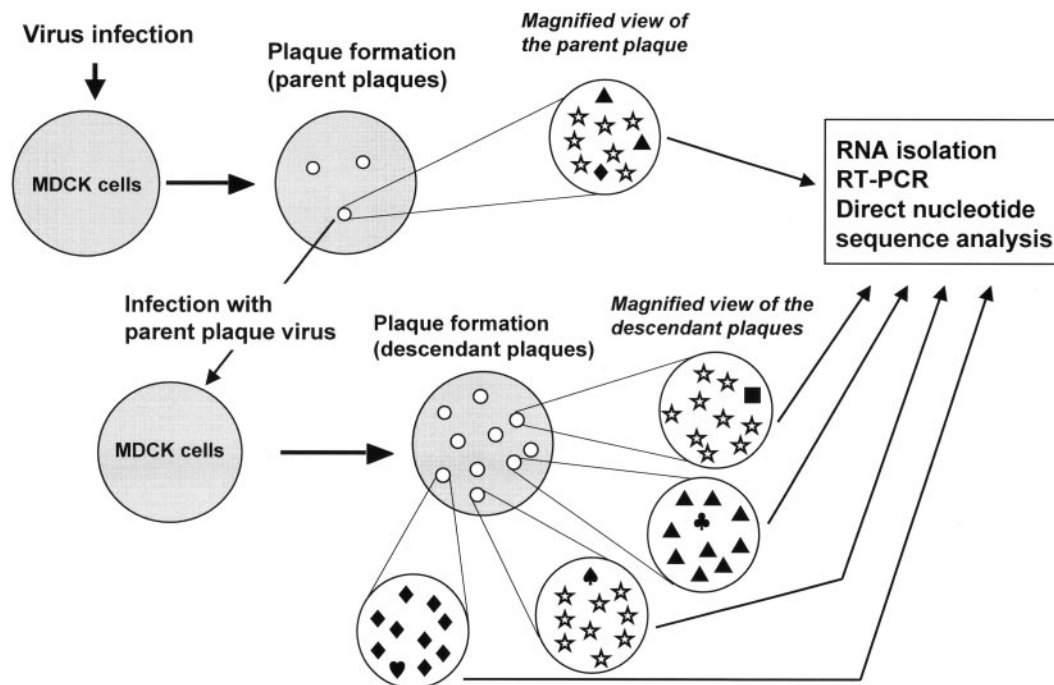
FIG. 1. Measuring the mutation rate during the growth of a single plaque. Plaque formation was performed in confluent monolayers of MDCK cells infected with A and B viruses. A well-isolated parent plaque of approximately 2 mm in diameter was picked at an appropriate time postinfection. To clone selected individual descendant viruses in the parent plaque, a second plaque formation was performed using the virus yield directly from the parent plaque. All plaque viruses were eluted from the agar plug into 300 μl of minimal essential medium containing 0.2% bovine serum albumin, made into aliquots, and stored in a deep freezer (−80°C) until used. The harvested viruses were used without further modification for sequential analysis of their NS genes, which reveals the sequence of the major virus in the plaque. The magnified views of the plaques show the viruses that exist within them. Star, viruses carrying NS gene sequences identical to that of the major virus in the parent plaque; triangle, diamond, spade, club, heart, and square, viruses carrying NS gene sequences different from that of the major virus in the parent plaque. The two triangles in the parent plaque represent a clone line. One of the descendant plaques is descended from a mutant of this type.

tides, giving an observed mutation frequency for BNS of $(2 \pm \sqrt{2})/461{,}304 = 0.4 \times 10^{-5} \pm 0.3 \times 10^{-5}$ mutations/site.

For A viruses, the first two experiments with primary plaques formed at 34°C (Table 1, experiments 4 and 5) indicated a mutation frequency far lower than that reported by Parvin et al. (23), who grew their parent plaque at 37°C. To examine the possible effect of temperature on mutation frequency, we remeasured A/1/87 at 37°C. The number of mutations detected was identical to that at 34°C. Taking all three A virus experiments together, the average mutation frequency for ANS in the parent plaques was $(5 \pm \sqrt{5})/352{,}359 = 1.4 \times 10^{-5} \pm 0.6 \times 10^{-5}$ mutations/site.

The higher mutation frequency of WSN(H1N1) compared to A/1/87(H1N1) may reflect the peculiar character of WSN, a neurotropic virus, produced by repeated pulmonary and intracerebral infection of mice (9). There may also have been some problem with the technology available in 1986. In the absence of PCR and simplified sequence technology, Parvin et al. (23) were unable to repeat the sequence analysis to confirm mutations by reading both strands of the cDNA. For this reason, their reported number of mutations might not be accurate.

To compare the mutation rate per infectious cycle of the A and B viruses, we determined the generation time of each virus in MDCK cells. As shown in Fig. 2, the mean generation time, when the PFU reach 50% of the final value (1, 7), was about 7 and 10 hours for A and B viruses, respectively. As the dynamics of plaque growth are complicated and the budding of progeny viruses could not be synchronized, these generation times were adopted as probable values during plaque formation. Since the 2-mm-diameter parent plaques were picked at 48 h (A viruses) and 71 h (B viruses) postinfection, they correspond to approximately seven infectious cycles in both cases. Using these values, average mutation rates for ANS and BNS were calculated as 1.4×10^{-5} mutations/site/7 infectious cycles $= 2.0 \times 10^{-6}$ mutations/site/infectious cycle, and 0.4×10^{-5} mutations/site/7 infectious cycles $= 0.6 \times 10^{-6}$ mutations/site/infectious cycle, respectively.

It should be noted that some authors interpret "infectious" and "replication" cycle differently (5, 6, 23, 25, 26). The observed difference in mutation rates is (BNS − ANS) = −(1.4 ± 1.0) × 10^{-6} mutations/site/infectious cycle, giving a z-score of −1.4 and $P = 0.08$. This difference in mutation rate presumably reflects a more accurate polymerase in the B virus than in the A virus.

Finally, we attempted to estimate evolutionary rates from the observed in vitro mutation rates in MDCK cells. Since the parent plaques for the A and B viruses were grown for 48 and 71 h, and since a year is 8,760 h long, if the viruses propagated continuously all through the year, we estimate evolutionary rates for the A and B viruses of 1.4×10^{-5} mutations/site/ 48 h × 8,760 h = 2.6×10^{-3} mutations/site/year and 0.4×10^{-5} mutations/site/71 h × 8,760 h = 0.5×10^{-3} mutations/

TABLE 1. Analysis of mutation rate for NS genes of human influenza viruses

Virus type	Expt no.	Virus[a]	PFU of parent plaque	No. of descendant plaques analyzed	Total no. of nucleotides analyzed[b]	No. of nucleotide changes[c]	Mutation frequency/site[d]	Mutation rate Per site per cycle[e]	Mutation rate Per site per year[f]
B	1	B/Aichi/29/99	6.4×10^6	143	147,576[g]	1	0.7×10^{-5}	1.0×10^{-6}	0.8×10^{-3}
	2	B/Aichi/44/01	2.0×10^6	146	150,672[g]	0	0	0	0
	3	B/Aichi/44/01	4.8×10^6	158	163,056[g]	1	0.6×10^{-5}	0.9×10^{-6}	0.8×10^{-3}
	Avg						$0.4 \pm 0.3 \times 10^{-5}$	$0.6 \pm 0.4 \times 10^{-6}$	$0.5 \pm 0.4 \times 10^{-3}$
A	4	A/Aichi/12/92	3.0×10^6	149	124,117[h]	1	0.8×10^{-5}	1.2×10^{-6}	1.5×10^{-3}
	5	A/Aichi/1/87	2.4×10^6	136	113,288[h]	2	1.8×10^{-5}	2.6×10^{-6}	3.2×10^{-3}
	6	A/Aichi/1/87[i]	1.2×10^6	138	114,954[h]	2	1.7×10^{-5}	2.5×10^{-6}	3.2×10^{-3}
	Avg						$1.4 \pm 0.6 \times 10^{-5}$	$2.0 \pm 0.9 \times 10^{-6}$	$2.6 \pm 1.2 \times 10^{-3}$

[a] To avoid a change in character of the viruses by adaptation to MDCK cells, we limited the number of virus amplifications in MDCK cells, prior to the experiment, to five. The overlay for plaquing consisted of minimal essential medium with 0.6% agar (Difco), 0.25% bovine serum albumin, 0.01% DEAE-dextran, 0.1% glucose, and 2.0 μg of acetyl trypsin per milliliter. Viruses were plaque purified twice in MDCK cells before preparing parent plaques. Plaque formation was performed in MDCK cells at 34°C unless otherwise noted. Parent plaques were grown for 48 h for A viruses and 71 h for B viruses.

[b] For the nucleotide sequence analysis, the viral RNA was directly extracted from 100 μl of each descendant plaque virus (300 μl eluant) using Isogen LS (Nippon Gene, Japan). Half of this viral RNA was used to synthesize cDNA of the NS genes by RT-PCR as described previously (21) using primers ANSF(+) (5′-AGCAA AAGCAGGGTGACAAAGACATAA-3′, positions 1 to 27) and ANSR(−) (5′-AGTAGAAACAAGGGTGTTTTTTATCATTAA-3′, positions 861 to 890) for A viruses and BNSF(+) (5′-AGCAGAAGCAGAGCATTTGTTTAG-3′, positions 1 to 24) and BNSR(−) (5′-AGTAGTAACAAGAGGATTTTTATTTT-3′, positions 1073 to 1098) for B viruses. RT-PCR products were used directly for sequence analysis on an ABI 3100 DNA sequencer operated according to the manufacturer's protocols. For sequence reactions, in addition to the primers indicated above, we used primers 153(−) 5′-GTCTCCCATTCTCATTACTGCTTC-3′, positions 637 to 660) and 196(+) (5′-AAGGGCTTTCACCGAAGAGGGAGCAT-3′, positions 467 to 492) for A viruses and 157(−) (5′-CCAACACTCTCAAGGACAACACAT-3′, positions 586 to 609) and 172(+) (5′-GACATGAACAACAAAGATGCAAG-3′, positions 489 to 511) for B viruses.

[c] Number of nucleotide differences between descendant and parent NS genes. Details of each mutation are given in Table 2.

[d] Ratio of number of nucleotide changes detected to number of nucleotides analyzed.

[e] Following Parvin et al. (23), the mutation rate was determined by dividing the observed frequency of nucleotide mutations per site by the number of infectious cycles. This assumes that all mutants continue to replicate as the plaque grows. If mutant lines die out, the mutation rate obtained in this way may underestimate the true mutation rate. We are assuming seven infectious cycles for A and B viruses.

[f] Calculated by correcting observed mutation frequencies over 71 h (B viruses) and 48 h (A viruses) to 365 days (8,760 h).

[g] For each descendant plaque, the 1,032 nucleotides at positions 41 to 1072 of the NS gene were analyzed.

[h] For each descendant plaque, the 833 nucleotides at positions 28 to 860 of the NS gene were analyzed.

[i] Parent plaque formed at 37°C.

site/year, respectively. Evolutionary rates for the NS genes of human influenza A and B viruses have been reported in the range of 1.8×10^{-3} to 2.2×10^{-3} and 0.45×10^{-3} to 0.8×10^{-3} nucleotide substitutions/site/year, respectively, on the ba-

TABLE 2. Mutations observed in NS genes of descendant plaque viruses compared to the parent plaque

Expt no.[a]	Virus	Clone no.	Mutation (position)[b]	Amino acid change (position)[c]	No. of nucleotide differences between parent and reference virus[d]
1	B/29/99	3	G→T (238)	S→I (NS1, 65)	26 (0.75×10^{-3})
3	B/44/01	8	T→A (678)[e]	Y→N (NS1, 212)	42 (1.1×10^{-3})
4	A/12/92	53	C→A (339)	L→I (NS1, 105)	45 (2.25×10^{-3})
5	A/1/87	93	T→C (121)	F→S (NS1, 32)	45 (2.8×10^{-3})
		136	G→A (849)	Silent (NEP, 117)	
6	A/1/87	52	A→G (355)	K→R (NS1, 110)	
		125	A→G (665)	Silent (NS1, 213)	
				H→R (NEP, 56)	

[a] The numbers of the experiments are according to those in Table 1.

[b] Nucleotide number is according to A/Aichi/2/68 (M34829) for A viruses and B/Ann Arbor/1/66 (M20225) for B viruses (the reference viruses). The numbers in parentheses are GenBank accession numbers.

[c] The NS gene codes for two overlapping proteins, NS1 and NEP (nuclear export protein). The amino acids are numbered according to the proteins in reference viruses listed in footnote b.

[d] The numbers in parentheses show the evolutionary rates estimated from the differences between the reference virus and parent plaque, expressed in nucleotide differences per site per year. These rates are close to those reported by others (see text).

[e] B/44/01, like several other B viruses, is missing the noncoding nucleotide at position 40 in B/Ann Arbor/1/66. The remaining nucleotides are numbered as if the omission had not occurred.
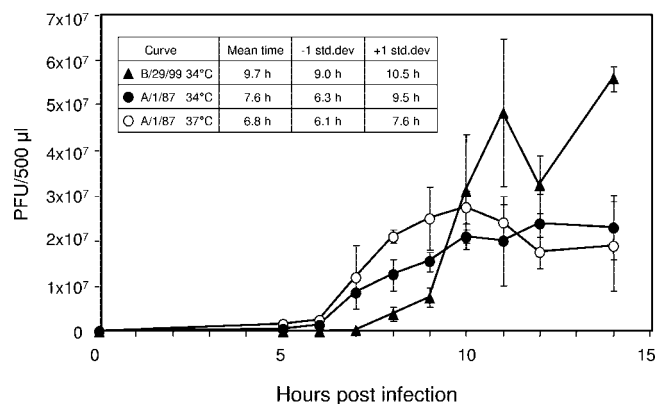

FIG. 2. Comparison of the generation times of A and B viruses in MDCK cells. Confluent monolayers of MDCK cells (5×10^5 cells) were infected with A/Aichi/1/87 and B/Aichi/29/99 at a multiplicity of infection of 8, kept at room temperature for 1 h, washed three times with minimal essential medium, and then incubated in 500 μl of over-lay medium (Table 1, footnote a) excluding agar and DEAE-dextran, at 34 or 37°C. At 0 and 5 h postinfection and at each subsequent hour, a sample of the supernatant was removed and analyzed by plaque titration in MDCK cells. The PFU per 500 μl are shown versus the hours postinfection. (Inset) The generation times were determined as the point of maximum release of PFU per hour in each one-step growth experiment (1, 7). This is generally equivalent to the point at which the PFU reaches 50% of its final value, estimated here by averaging the total PFU at 11, 12, and 14 h. The width of the distribution about the mean was likewise estimated by noting the points at which the PFU reached 16 and 84% of its final value, corresponding to plus and minus 1 standard deviation (1). The change in temperature from 34 to 37°C, tested for A/1/87 only, appeared to have no significant effect on the mean generation time.

sis of nucleotide sequencing of circulating viruses (13, 15, 17). Although the generation time of viruses and the selection factors on NS gene products in vivo may not be the same as those in MDCK cells, our estimated evolutionary rates are in good agreement with the reported actual rates. Whether this agreement is a consequence of the random fixation of neutral mutations (14), of some other origin, or purely an accident remains to be solved.

The current study suggests that one of the reasons for the lower evolutionary rate of influenza B viruses could be a lower mutation rate.

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