The mutation rates and mutational bias of influenza A virus

Running Title: Influenza mutational spectrum

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Abstract Word Count: 250

Main Text Word Count: 6,762

1 **Abstract**

2 Influenza virus has a high mutation rate, and this low replicative fidelity contributes to its

3 capacity for rapid evolution. Clonal sequencing and fluctuation tests have suggested that the

4 mutation rate of influenza A virus is 7.1 x 10^{-6} - 4.5 x 10^{-5} substitutions per nucleotide per cell

5 infection cycle and 2.7 x 10^{-6} - 3.0 x 10^{-5} substitutions per nucleotide per strand copied (s/n/r).

6 However, sequencing assays are biased toward mutations with minimal impacts on viral fitness

7 and fluctuation tests typically investigate only a subset of the twelve mutational classes. We

8 developed a fluctuation test based on reversion to fluorescence in a set of virally encoded

9 mutant green fluorescent proteins. This method allowed us to measure the rates of selectively

10 neutral mutations representative of all 12 mutational classes in the context of an unstructured

11 RNA. We measured an overall mutation rate of 1.8 x 10^{-4} s/n/r for PR8 (H1N1) and 2.5 x 10^{-4}

12 s/n/r for Hong Kong 2014 (H3N2). The replication mode was linear. The mutation rates of these

13 divergent strains are significantly higher than previous estimates and suggest that each

14 replicated genome will have an average of 2-3 mutations. The viral mutational spectrum is

15 heavily biased toward A to G and U to C transitions, resulting in a transition to transversion bias

16 of 2.7 and 3.6 for the two strains. These mutation rates were relatively constant over a range of

17 physiological temperatures. Our high-resolution analysis of influenza virus mutation rates will

18 enable more refined models of its molecular evolution.

19

20 Key Words – influenza virus, mutation rate, diversity, evolution

21

22 **Significance**

23 The rapid evolution of influenza virus is a major problem in public health. A key factor driving

24 this rapid evolution is the virus' very high mutation rate. We developed a new method for

25 measuring the rates of all 12 mutational classes in influenza virus, which eliminates some of the

26 biases of existing assays. We find that the influenza virus mutation rate is much higher than

27 previously reported and is consistent across two distinct strains and a range of temperatures.

28 Our data suggest that influenza viruses replicate at their maximally tolerable mutation rates,

29 highlighting both the virus' evolutionary potential and its significant constraints.

30

31 **Introduction**

32 The rapid evolution of influenza virus has led to reduced vaccine efficacy, widespread drug

33 resistance, and the annual emergence of novel strains. While complex ecological,

34 environmental, and host demographic factors influence the evolutionary dynamics of influenza

35 virus, the virus' adaptability is driven in large part by its capacity to generate genetic diversity

36 through mutation and reassortment (1). Like other RNA viruses, influenza virus replicates with

37 extremely low fidelity. The influenza virus RNA-dependent RNA polymerase (RdRp) complex,

38 which includes the viral proteins PB1, PB2, PA, and NP, lacks proofreading and repair activity

39 (2). Its mutation rate has been reported to be approximately 10^{-5} to 10^{-6} mutations per nucleotide

40 per cellular infection (3-8).

41

42 An accurate accounting of influenza virus' mutation rate and mutational bias is essential for

43 defining its evolutionary dynamics and for informing control efforts. The mutation rate will

44 determine the probability that a mutation conferring drug resistance, antibody escape, or

45 broadened host range will be generated within a given virus population. It will also define a virus'

46 sensitivity to drug-induced lethal mutagenesis, a broad-spectrum antiviral strategy that exploits

47 the high mutation rate and low mutational tolerance of many RNA viruses (9, 10). We have

48 shown that the antiviral activity of three different nucleoside analogues is due to increased viral

49 mutation rates, and a new anti-influenza drug, favipiravir, has been found to act through a

50 similar mechanism (11-13). As in other RNA viruses, the mutational bias of the influenza

51 polymerase complex is largely undefined. While viral mutation rates are typically reported as a

52 single measurement, each of the 12 mutational classes will have its own rate and determine the

2

53   accessibility of various nucleotide and amino acid substitutions. Many RNA viruses appear to

54   exhibit a pronounced bias toward transition mutations (3). Because transitions are more likely to

55   be synonymous than transversions, this bias can impact models of molecular evolution and

56   inferences of natural selection based on dN/dS ratios.

57

58   Mutations are typically reported as either frequencies or rates (3, 14). Mutation frequency is the

59   number of mutations identified in a sample per nucleotide sequenced. Frequency

60   measurements therefore quantify not only the rate at which a mutation is generated but also that

61   mutation's ability to persist in a population. In contrast, mutation rates measure how many

62   mutations are made in a discrete unit of time (e.g. per infection cycle or strand copied) and are a

63   better representation of polymerase error. Viral mutation rates have often been measured by

64   Sanger sequencing of randomly selected clones obtained through plaque purification or limiting

65   dilutions (6-8, 15-17). Mutation frequencies obtained in this manner can be converted to

66   mutation rates by adjusting for the number of replication cycles prior to sampling (3). With these

67   adjustments, sequencing-based estimates of influenza virus mutation rates range from 7.1×10^{-6}

68   to 4.5×10^{-5} substitutions per nucleotide per cell infection cycle. While sequencing approaches

69   can potentially measure the rate of all substitution classes, they lack precision and have poor

70   power for detecting differences across strains or conditions. They are also biased towards

71   sampling of genomes with higher fitness. Next generation sequencing platforms have increased

72   the throughput and power of clonal sequencing, but in many cases, the impact of reverse

73   transcription error in library preparation has not been thoroughly investigated.

74

75   A more direct way to measure mutation rates is to use a Luria-Delbrück fluctuation test (18-22).

76   In this method, a large number of parallel cultures are infected with small inocula and assessed

77   for a set of newly generated mutants exhibiting a scoreable phenotype after a period of

78   exponential growth. Because the mutations are rare and random, they follow a Poisson

79   distribution in each culture. Mutation rate estimates from a null class model are robust to the

80   mode of replication, which may vary across viral species (20, 21). Using resistance to

81   monoclonal antibodies as a scoreable phenotype, influenza's mutation rate has been estimated

82   to be 2.7 x 10^{-6} to 3.0 x 10^{-5} substitutions per nucleotide per strand copied (4, 5). While

83   fluctuation tests are more precise than sequencing assays, most scoreable phenotypes sample

84   just a few sites or mutational classes.

85

86   Here, we apply two new approaches for measuring the influenza virus mutation rate that

87   overcome the drawbacks of sampling bias and low statistical power inherent to currently

88   available methods. The first relies on measurements of nonsense mutation frequencies within a

89   short segment of the influenza genome using PrimerID, an error-controlled next-generation

90   sequencing approach (23, 24). Because nonsense mutations are lethal and generally not

91   propagated, their frequencies approximate the mutation rate in the prior replication cycle (25).

92   The second is a Luria-Delbrück fluctuation test that scores reversion to fluorescence in virally

93   encoded green fluorescent protein (GFP) mutants (26). The GFP method enabled interrogation

94   of all 12 substitution mutation classes independently and under distinct replication conditions.

95

96   **Results**

97   The vast majority of premature stop codons in RNA virus open reading frames are lethal and

98   are therefore likely to have been generated during the previous replication cycle (e.g. (27)).

99   Eighteen of the 61 sense codons are a single mutation away from a nonsense codon, and the

100   frequency at which these nonsense mutational targets (NSMT) mutate to stop codons

101   approximates the viral mutation rate. When combined with a highly accurate next generation

102   sequencing approach, the NSMT method can provide rate estimates for eight mutational

103   classes (25, 28, 29). We identified a 402 base fragment within the PA gene of

104   A/Wisconsin/03/2007 H3N2 that contains a balanced distribution of 80 NSMT, and used the

4

105   PrimerID method to sequence individual PA clones from an influenza population on the Illumina

106   platform. PrimerID sequencing utilizes a library of barcoded reverse transcription primers to

107   generate consensus sequences for each cDNA template, thereby controlling for the PCR or

108   base-calling errors that plague many next generation sequencing datasets (23, 24). The

109   PrimerID method does not control for errors introduced during reverse transcription (RT), and

110   the mutation rates of reverse transcriptases are similar to those of viral RNA dependent RNA

111   polymerases (RdRp) (3).

112

113   In an attempt to distinguish RT errors from mutations introduced by the influenza RdRp, we

114   compared PrimerID-NSMT estimates of the mutation rate for influenza virus to a control, in

115   which the segment 3 (PA) RNA was expressed from a plasmid pol I promoter in transfected

116   cells (30). Mutations identified in the viral genome are derived from either the influenza RdRp or

117   RT, and the control establishes the background error rate of the assay due to pol I transcription

118   and RT (Figure 1A). We obtained over 449,000 aligned PA fragment consensus sequences for

119   each sample, representing approximately 75% of starting RNA templates. The frequencies of

120   nonsense mutations were similar for 5 of the 8 mutation classes (Figure 1B, Table S1). The

121   frequencies of the other three mutation classes (A to U, C to A, and U to G) were only slightly

122   higher in the samples derived from RNA replicated by the influenza RdRp than those that were

123   not. The G to A mutation rate was highest in both samples (1.3×10^{-4} and 8.5×10^{-5}

124   substitutions per nucleotide for cell-derived and viral-derived samples, respectively), and

125   analyses of the RT mutational spectrum consistently show this to be the most common mutation,

126   with rates of 1×10^{-4} substitutions per nucleotide (28, 31-33). These data demonstrate that the

127   background error rate of reverse transcriptase during sample preparation is equal to or higher

128   than the rate of mutations introduced by the influenza RdRp.

129

130   We also compared the frequency of nonsense mutations to the frequency of all observed

131   mutations. Mutations in the transfected control were evenly distributed across the PA fragment

132   and no more common than the subset of nonsense mutations (Figure 1C). In contrast, the

133   frequency of mutations in the replicated viral RNA was higher than the subset of nonsense

134   mutations. The accumulation of mutations to frequencies above those of nonsense mutations

135   and the background signal indicate the action of selection on newly generated mutations.

136   Together, these data suggest that the high background error rate of reverse transcriptase and

137   issues of selection bias may confound sequencing-based measurements of the basal mutation

138   rate and mutational bias of riboviruses.

139

140   *A fluorescence-based fluctuation test*

141   We developed a Luria-Delbrück fluctuation test for influenza virus mutation rates that scores

142   reversion to fluorescence in a set of 12 mutant green fluorescent proteins (GFP). The

143   fluorescent chromophore of enhanced GFP contains three essential amino acids (T65, Y66, and

144   G67) (34), and nonsynonymous substitution at any of these positions results in a GFP with

145   either absent or altered fluorescent properties (35-37). We used a plasmid that contains GFP

146   instead of hemagglutinin on influenza A virus segment 4 (ΔHA-GFP, (38)), to generate a set of

147   12 recombinant influenza A viruses that each express a mutant GFP protein (Table 1). Each of

148   these mutant GFP proteins has a single nucleotide mutation that, with reversion to fluorescence,

149   will interrogate a specific mutational class introduced by the viral RdRp during replication. These

150   viruses were replicated in cells stably expressing the HA protein *in trans.*

151

152   Because our mutant GFP proteins are not fluorescent, we used anti-GFP antibody staining and

153   immunofluorescence microscopy to verify GFP expression from each of the 12 mutant ΔHA-

154   GFP viruses. In virally infected cultures, we occasionally identified rare cells expressing GFP

155   that was fluorescent at the excitation and emission wavelengths consistent with reversion to

156   fluorescence (Figure 2A). We used antibody staining to titrate the total number of viruses

157   expressing GFP. The growth kinetics of mutant ΔHA-GFP A/Puerto Rico/8/1934 H1N1 viruses

158   were slower than the parental PR8, but similar among the 12 mutants (Figure 2B). In all cases,

159   titers of 1 x 10^5 per milliliter were achieved by 22 hours at 37˚C. This corresponds to 10^4 viruses

160   per well of a 96 well plate, which is the maximum that can be accurately measured by

161   fluorescence microscopy. In subsequent experiments, we used antibody staining of infected

162   cells to titrate the total number of viruses expressing GFP – the mutational target – since a

163   subset of viruses will delete the GFP open reading frame during replication.

164

165   Fluctuation tests are most accurate when the marker is selectively neutral (20, 21). We

166   measured the replicative fitness of viruses expressing the mutant ΔHA-GFP relative to those

167   expressing the wild type ΔHA-GFP. We competed a subset of the mutant viruses against a wild

168   type ΔHA-GFP virus containing a neutral PB1 sequence barcode and used RT-quantitative PCR

169   to measure the frequency of the competitors over serial passage on MDCK-HA cells (27). Each

170   of 6 mutant ΔHA-GFP viruses, which sample mutations in the 3 mutated amino acid positions,

171   maintained stable frequencies over 4 passages. They were just as fit as the wild type ΔHA-GFP

172   virus (Figure 2B, p>0.05, n=3 replicates, one way ANOVA), confirming that the scoreable

173   phenotype and the mutations interrogated are selectively neutral.

174

175   The secondary structure of genomic RNA in positive sense viruses is known to influence

176   mutation rates in a site specific manner (39-42). In influenza virus, the formation of stable RNA

177   structures in the replication complex is limited by the binding activity of the viral nucleoprotein

178   (2). We performed an *in silico* analysis of the ΔHA-GFP RNA to further exclude the possibility

179   that reversion of mutations could be influenced by local RNA structure (Figure 2D). A sliding

180   window analysis of the minimum free energy of RNA folding suggests that the introduced

181   mutations are located within a region of high minimum free energy in the ΔHA-GFP RNA (43,

7

182   44). The rates at which these mutations revert to the wild type sequences are therefore likely to

183   be representative of mutation rates across the influenza virus genome.

184

185   We used a Luria-Delbrück fluctuation test to convert reversion frequencies to viral mutation

186   rates (18, 20). For each of the 12 mutant ΔHA-GFP, we inoculated parallel cultures of MDCK-

187   HA cells and transferred replicated virus to MDCK cells in a 96-well imaging plate (Figure 3A).

188   The replication time and transfer volume were empirically determined for each mutation class,

189   drug, temperature, and virus tested. Because the ΔHA-GFP viruses do not express the HA

190   protein, they only replicate in MDCK-HA cells, and there was no viral spread in the imaging

191   plate. We used a null class model to calculate mutation rates based on the number of parallel

192   cultures without a revertant (green fluorescence) and the degree of viral replication (anti-GFP

193   antibody staining of the inocula and replicated virus). The mutation rates we report using this

194   method are in the coding (+) sense of the RNA, rather than the genomic (-) sense.

195

196   We validated the specificity of our assay for specific mutational classes by performing a set of

197   fluctuation tests in each of three different mutagenic nucleoside drugs. We and others have

198   previously shown that ribavirin increases the frequency C to U and G to A transitions, 5-

199   azacytidine increases the frequency of C to G and G to C transversions, and 5-fluorouracil

200   increases the frequency of all transitions (A to G, C to U, G to A, and U to C) in influenza virus

201   (11, 12). Each of these mutagens increased the rates of only the expected mutation classes

202   (Figure 3B-D). In some cases, the amount of replicated virus was sufficiently low and the

203   mutations were sufficiently rare that we were not able to obtain replicate fluctuation tests in

204   which the null class (P_0) lay within the ideal range of 0.1-0.7 (19, 20). Here and elsewhere,

205   these less precise mutation rate measurements are indicated with open symbols (Figures 3-5).

206

207   *The mutation rates of influenza A virus*

8

208   We used our GFP fluctuation test to measure the mutation rates of two evolutionarily divergent

209   influenza viruses. Influenza A/Puerto Rico/8/1934 H1N1 (PR8) was the second influenza virus

210   isolated and was extensively passaged in various cell culture environments prior to cloning (45).

211   We cloned a circulating seasonal influenza virus, influenza A/Hong Kong/4801/2014 H3N2

212   (Hong Kong 2014), after limited passage in MDCK. The PR8 virus contained the ΔHA-GFP

213   segment with seven PR8 genome segments. The Hong Kong 2014 virus contained the ΔHA-

214   GFP segment, the segments coding for the polymerase complex – PB2, PB1, PA, and NP –

215   from A/Hong Kong/4801/2014 H3N2, and the segments encoding NA, M, and NS from PR8.

216   This chimera was necessary to obtain high titer stocks.

217

218   The mutation rates of the PR8 and Hong Kong 2014 viruses were higher than previously

219   reported for influenza A virus and generally biased toward transitions (6-8). In both viruses,

220   mutation rates were highest for the reciprocal transitions, A to G and U to C (Figure 4 and Table

221   S2). The rates for the other two transitions (C to U and G to A) were approximately six fold lower

222   and similar to the rates of the more common transversion mutations. We note that this G to A

223   mutation rate is much lower than the rate estimated using the PrimerID-NSMT assay (see

224   Figure 1). This discrepancy may reflect differences in mutational bias between the influenza

225   RdRp and retroviral reverse transcriptases. The overall rate and spectrum of mutations for the

226   PR8 and Hong Kong 2014 viruses are very similar, and given the base composition of each

227   virus, we estimate that each replicated 13.5 kb genome contains, on average, 2 to 3 mutations.

228   The transition to transversion ratio is 2.7 in PR8 and 3.6 in Hong Kong 2014.

229

230   We did identify differences between the two viruses in specific mutation classes. The rate of G

231   to A mutations was two-fold higher in Hong Kong 2014 than in PR8 (7.2×10^{-5} vs. 3.1×10^{-5}, p =

232   0.0018, multiple t-test with Holm-Sidak correction), and the Hong Kong 2014 virus also exhibited

233   a marginally increased rate of G to U mutations that was not statistically significant (6.0×10^{-5} vs.

9

234   3.5×10^{-5}, p = 0.083). For both viruses the rates of mutations away from A are symmetrical to

235   the reciprocal mutations away from U. Interestingly, mutations away from C were much less

236   common than the reciprocal mutations away from G. In PR8 G nucleotides are 3.8 times more

237   likely to mutate than C nucleotides. In the Hong Kong virus, this difference is 2.7 fold.

238

239   Because reversion mutations can be introduced during either plus or minus strand RNA

240   synthesis, our fluctuation test estimates the mutation rate per strand copied. To determine

241   whether influenza virus replicates through a linear or a binary mechanism, we calculated the

242   total number of revertants per number of replicated viruses across our parallel cultures. This

243   method estimates mutations per cellular infection cycle and allows for the possibility that cells

244   with reversions that occur early in a replication cycle will produce multiple viruses that express

245   fluorescent GFP. The ratio of the number of mutations per cell cycle and the number of

246   mutations per strand replicated mutation rates therefore approximates the number of strand

247   copying events that occur prior to viral release (3). In linear, or stamping machine replication,

248   there is only one cycle of strand copying per cellular infection cycle and the ratio is two. In

249   binary replication, multiple rounds of stand copying occur, leading to higher rations. Assuming a

250   single cellular replication cycle occurred during each experiment and using all 12 mutation

251   classes, we determined the average ratio to be 1.2 and 1.1 for the PR8 and Hong Kong strains,

252   respectively. These data suggest that influenza largely replicates through a linear mode.

253

254   *Influenza virus mutation rates across physiologic temperatures*

255   Biochemical studies of purified influenza virus RdRp suggest that replication temperature can

256   affect enzyme fidelity (46). Influenza viruses replicate over a range of temperatures in nature

257   from 32˚C and 37˚C across the respiratory tract of humans to 39˚C in febrile illness to 41˚C in

258   birds (47-50). We used the PR8 virus encoding mutant ΔHA-GFP representing the 5 most

259   frequent mutational classes to measure mutation rates at different temperatures. This virus

10

260   replicated reasonably well in MDCK-HA cells at 32°C and 39°C, albeit to lower titers (see Figure

261   2C). The mutation rates for these 5 classes were generally stable over this 7 degree range of

262   physiological temperatures (Figure 5). We were unable to measure mutation rates at

263   temperatures higher than 39˚C due to host cell intolerance.

264

265   **Discussion**

266   We developed two new methods to define the mutation rate and mutational bias of H1N1 and

267   H3N2 influenza viruses. We found that the background error rate of reverse transcriptase may

268   confound measurements of influenza virus mutation rates that are based on sequencing of RT-

269   PCR amplified templates. We therefore developed a high throughput GFP based assay to

270   estimate the mutation rates for all 12 substitution classes. This assay can be easily adapted to

271   any virus that tolerates the addition of the GFP open reading frame. While PR8 (H1N1) and

272   Hong Kong 2014 (H3N2) viruses varied in their mutation rates for individual classes, the overall

273   mutation rate was consistent across these evolutionarily divergent influenza polymerases at a

274   range of temperatures. These mutation rates are considerably higher than previously reported,

275   and given the impact of mutational load, suggest that the virus is replicating at the maximally

276   tolerable mutation rates.

277

278   Sequencing assays for viral mutation rates are plagued by ascertainment and sampling biases.

279   The mutations that are detected in plaque-derived populations represent only the viable fraction

280   and those identified in passaged supernatants are often heavily biased toward mutations with

281   less deleterious fitness effects. While Sanjuan and colleagues have appropriately adjusted for

282   typical viral mutational fitness effects in sequenced-based estimates of mutation rates (3), these

283   fitness effects may not be uniform across viruses or in the genes analyzed (27, 51). Next

284   generation sequencing can minimize these biases by improving the detection of rarer, more

285   deleterious mutations (28, 39, 40, 52). However, our data from PrimerID-controlled next

286   generation sequencing suggest that reverse transcriptase error can be a significant confounder

287   that needs to be considered in studies of RNA viruses. In our experiments, the high background

288   RT error rate made it difficult to distinguish mutations introduced by the influenza polymerase

289   complex from those generated during reverse transcription of the viral genomic RNA. The

290   mutational bias of RT may also differ from that of viral RNA-dependent RNA polymerases.

291   Guanine to adenine transitions are the most common mutation made by RT (28, 31-33) and are

292   the ones found most frequently in our dataset as well as many others that rely on RT-PCR

293   amplification for sequencing (e.g. (11, 12, 53, 54)). In contrast, our fluctuation test suggests that

294   A to G and U to C mutations are the most common classes in influenza.

295

296   Fluctuation tests are sensitive for rare mutational events and avoid many of the issues with

297   sequencing assays (18, 20-22). Our reversion to fluorescence assay has several additional

298   advantages over ones that rely on phenotypic markers such as drug or antibody resistance (26).

299   First, the marker was selectively neutral, as the mutant GFP and revertant wild type GFP

300   viruses had equal fitness. Second, we were able to measure all 12 mutational classes in a

301   format that allowed for sufficient replicates. Third, we were able to control the number of cellular

302   infection cycles by expressing the HA protein in trans (38). Fourth, we used an anti-GFP

303   antibody to measure the number of mutation targets directly. One shortcoming of all fluctuation

304   tests is that genomic mutation rates are extrapolated from data at one specific site. While RNA

305   structures are unlikely to play a major role in mutation rate variability in influenza virus (55), we

306   cannot exclude that sequence context could modulate mutation rates across the genome.

307

308   We found that the mutation rates of the lab adapted PR8 H1N1 strain are similar to those of a

309   recently circulating H3N2 strain, and both sets of measurements are considerably higher than

310   those obtained in previous sequence-based studies. While these earlier works estimated rates

311   between 7.1×10^{-6} and 4.5×10^{-5} mutations per nucleotide per cell infection, our composite

312   mutation rates were 1.8 x 10^{-4} and 2.5 x 10^{-4} mutations per nucleotide per strand replicated for

313   PR8 (H1N1) and Hong Kong/2014 (H3N2), respectively (3, 6-8). Consistent with the biases

314   detailed above, our measurements are closer to those obtained for specific classes obtained in

315   antibody-based fluctuation tests (4, 5). These very high mutation rates mean that each

316   replicated genome has, on average, 2-3 mutations. We have found that 28-31% of randomly

317   selected mutations in influenza virus are lethal (27). Using a 70% probability that a given

318   mutation results in a viable virus, the likelihood of any given genome being able to replicate is

319   only 34 to 49%. We suggest that mutational load accounts for a sizable portion of the 90-99% of

320   genomes in influenza populations that are non-infectious (e.g. (12, 13)). This mutation rate

321   clearly places influenza close to a theoretical maximum rate, and we and others have shown

322   that small increases in the virus' mutation rate leads to considerable losses in genome infectivity

323   (11-13).

324

325   The mode of replication for influenza virus is near linear as the ratios of mutation rates per cell

326   infection to per strand replicated were 1.1 and 1.2 for the strains evaluated. This finding

327   suggests that (-) sense genomic RNA introduced during an infection is copied to (+) sense

328   replication intermediates just once before new (-) sense genomes are synthesized. The fact that

329   our observed ratios are less than 2 – the theoretical minimum for a single stranded RNA virus –

330   may be due to our method of measurement (3). We assessed only a single mutation site in the

331   viral genome for each mutation class. We found that reciprocal mutation classes (e.g. G to U

332   and C to A) do not always occur at the exact same rate. This mutational bias, combined with our

333   measurements of mutation rates at a single nucleotide position, may invalidate this key

334   assumption for determining replication mode and give a ratio less than 2. Additionally, since we

335   allowed approximately one replication cycle and started with very low numbers of infecting virus

336   genomes, our measurements would by strongly biased towards observing mutations generated

337   during synthesis of the (-) sense genomic RNA rather than during synthesis of the (+) sense

13

338　replication intermediates in a system with a linear replication mode. This linear or "stamping

339　machine" replication mode, may actually assist the virus in tolerating its high mutation rate

340　because it allows fewer opportunities for mutations during each viral replication cycle (56-58).

341

342　Cellular replication environments are often hypothesized to influence RNA virus mutation rates,

343　and yet these effects have rarely been documented (22, 33, 42, 59, 60). Here, we found no

344　significant differences in rates of the five most common mutational classes across a seven

345　degree range of physiologic temperatures. Nucleotide pools could potentially influence the

346　observed mutational biases. Intracellular concentrations of nucleotide triphosphates are much

347　higher than those of deoxynucleotides, and cellular pools are typically biased towards ATP and

348　GTP, which have other metabolic functions (61, 62). While it is tempting to speculate that pool

349　bias could lead to the observed asymmetry in mutations away from guanine, this is unlikely to

350　be the case in MDCK cells. The concentrations of all four NTP in MDCK cells are at least ten

351　fold higher the K_m of the influenza polymerase for each (46, 62, 63). We can't exclude that

352　biases in pools could play a role in primary cells where NTPs may be more limiting. However,

353　Combe and Sanjuan found that VSV mutation rates were similar across a range of primary and

354　immortalized cell lines that were cultured under a range of conditions (22). It is also intriguing

355　that A to G and U to C were the most common mutations, as these classes are characteristic of

356　the host enzyme adenosine deaminase acting on RNA (ADAR) (64-66). As ADAR-editing

357　occurs almost exclusively on double stranded RNA, it is not clear that it would contribute to the

358　mutation rates measured on our presumably unstructured GFP messages.

359

360　We expect that our data will lead to improved models of influenza evolution. For example, our

361　estimates of the virus' transition to transversion bias can inform null models for inference of

362　selection in protein coding genes. The availability of a complete nucleotide substitution matrix

363　will also enable studies of selection on codon usage and dinucleotide content. The nucleotide

14

364   frequencies of both PR8 (H1N1) and Hong Kong 2014 (H3N2) are far from what would be

365   predicted by the 12 mutation rates. This suggests either that selection is maintaining the virus'

366   nucleotide content away from the mutational equilibrium or that the virus has not had sufficient

367   time to achieve it. Finally, our measurements for the rate of each mutation class, coupled with

368   recent studies on mutational fitness effects in influenza will also greatly improve our ability to

369   construct more accurate phylogenies.

370

371   **Methods**

372   *Viruses, plasmids, and cells*

373   Madin-Darby canine kidney (MDCK) cells were provided by Arnold S. Monto (University of

374   Michigan School of Public Health) and HEK 293T cells were provided by Raul Andino

375   (University of California, San Francisco). Both cell lines were maintained in Dulbecco's modified

376   Eagle medium (Gibco 11965) supplemented with 10% fetal bovine serum (Gibco 10437) and 25

377   mM HEPES (Gibco 15630). Cells were maintained at 37°C and 5% CO_2 in a humidified

378   incubator except where indicated.

379

380   Influenza A/Puerto Rico/8/1934 H1N1 was obtained from the ATCC (VR-1469). The A/Hong

381   Kong/4801/2014 H3N2 strain was obtained from the Centers for Disease Control and

382   Prevention International Reagent Resource (FR-1483). The A/Wisconsin/03/2007 H3N2 strain

383   was provided by Dr. Arnold S. Monto (University of Michigan School of Public Health). Molecular

384   clones were derived from each of these isolates by reverse transcription polymerase chain

385   reaction (RT-PCR) amplification and insertion of all eight genomic segments into the pHW2000

386   plasmid (30, 67).

387

388   Cells expressing the hemagglutinin (HA) protein of influenza A/Puerto Rico/8/1934 H1N1

389   (MDCK-HA cells) were generated by co-transfection Madin Darby canine kidney (MDCK) cells

15

390   with pCABSD, which expresses a gene for Blasticidin S resistance, and pCAGGS-HA, which

391   expresses the influenza A/Puerto Rico/8/1934 H1N1 HA (38). Pools of cells stably expressing

392   HA were selected in growth media containing 5 μg/mL Blasticidin S. These pools were enriched

393   for cells with high HA expression by staining with an anti-HA antibody (1:1000 dilution, Takara

394   c179) and an Alexa 488-conjugated anti-mouse IgG (1:200 dilution, Life Technologies A11001)

395   followed by fluorescence-activated cell sorting on a FACSAria II (BD Biosciences). Cells were

396   sorted three times over the course of 5 passages and >99% of cells in the final population were

397   positive for high level HA expression.

398

399   A pPOLI vector encoding eGFP with influenza genomic packaging sequences was kindly

400   provided by Luis Martinez-Sobrido (University of Rochester). This construct, which we call ΔHA-

401   GFP, expresses eGFP flanked by the 78 3'-terminal bases (33 noncoding, 45 coding) and 125

402   5'-terminal bases (80 coding, 45 noncoding) of segment 4 from influenza A/WSN/33 H1N1. It

403   lacks the HA translation initiation codon (38). Twelve mutant ΔHA-GFP constructs (Table 2)

404   were generated using the QuikChange II site-directed mutagenesis kit (Agilent Technologies

405   200523) with primers 5'- CTCGTGACCACCCTG<mutant sequence>GTGCAGTGCTTCAGC-3'

406   and 5'- GCTGAAGCACTGCAC< rev comp mutant sequence>CAGGGTGGTCACGAG-3',

407   where mutant sequence corresponds to the sequences in Table 1 and rev comp mutant

408   sequence is the reverse complement of each.

409

410   A neutral genetic barcode was incorporated into the PB1 segment of A/Puerto Rico/8/1934

411   H1N1 in the pHW2000 vector by overlap extension PCR using inner primers 5'-

412   GATCACAACTCATTTCCAACGGAAACGGAGGGTGAGAGACAAT-3' and 5'-

413   ATTGTCTCTCACCCTCCGTTTCCGTTGGAAATGAGTTGTGATC-3', and outer primers

414   containing BsmB1 sites for cloning into the pHW2000 plasmid.

415

416   Recombinant viruses were rescued in 12-well plates after transfection of co-cultures of 2 x 10^5

417   293T cells and 1 x 10^5 MDCK cells with mixtures of pHW2000 plasmids encoding all 8 influenza

418   genome segments (500ng each) using 2 µL of TransIT-LT1 (Mirus 2300) per nanogram of DNA

419   (30). Viruses expressing GFP were rescued in the same manner except that the pPOLI vector

420   encoding ΔHA-GFP or its mutants and pCAGSS-HA were used in place of the pHW2000

421   plasmid encoding influenza HA, and MDCK-HA cells were used in place of MDCK cells.

422

423   *PrimerID Sequencing*

424   A custom R script (https://github.com/lauringlab/NGS_mutation_rate_assay) was used to

425   identify the 402 base region in the A/Wisconsin/03/2007 H3N2 genome (positions 865 to 1266

426   of the PA gene) with the highest concentration of pre-nonsense codons. Total cellular RNA was

427   isolated using Trizol (Life Technologies 15596) from 293T cells 48 hours after transfection with

428   a plasmid expressing A/Wisconsin/03/2007 H3N2 segment 3 (PA). Virus RNA was isolated

429   using Trizol from cell free supernatants of MDCK cells infected with A/Wisconsin/03/2007 H3N2

430   virus at a multiplicity of infection (MOI) of 0.5 for 24 hours. In both cases, the RNA was treated

431   with DNase I (Roche 04716728001) to remove residual plasmid DNA. The copy number of

432   segment 3 (PA) RNA in each sample was determined by reverse transcription with SuperScript

433   III (Invitrogen 18080051) and primer 5'-AGCAAAAGCAGG-3' followed by quantitative PCR on a

434   7500 Fast Real-Time PCR system (Applied Biosystems) with Power SYBR Green PCR Master

435   Mix (Applied Biosystems 4367659) and primers 5'-TCTCCCATTTGTGTGGTTCA-3' and 5'-

436   TGTGCAGCAATGGACGATTT-3'. A plasmid encoding PA was used to generate a standard

437   curve to relate cycle threshold to copy number. The absence of plasmid DNA containing the PA

438   sequence was confirmed by lack of signal in qPCR of RNA that was not reverse transcribed.

439

440   Sequencing libraries were prepared from 2 x 10^5 copies of segment 3 (PA) RNA using

441   Accuscript high fidelity reverse transcriptase (Agilent Technologies 200820) and primer (5'-

17

442   CCTACGGGAGGCAGCAGNNNNNNNNNNNAATTCCTCCTGATGGATGCT-3'), which binds to

443   bases 842 to 861 of the PA gene (positive strand numbering) and contains a degenerate N^{10}

444   barcode sequence (1,048,576 unique sequences). Because the RNA copy number was just

445   one-fifth of the total number of barcode sequences, it is unlikely that the same barcode would

446   prime multiple complementary DNA (cDNA) molecules (25). Three separate reverse

447   transcription reactions were performed for RNA harvested from both transfected and infected

448   cells to increase the total number of RNA templates in the experiment. The resulting PrimerID

449   barcoded cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter A63881) to

450   remove residual primers. The purified cDNA was amplified by PCR for 26 cycles (10 seconds at

451   98°C, 30 seconds at 69°C, and 30 seconds at 72°C) using Phusion high fidelity DNA

452   polymerase (New England Biosciences M0530) and primers 5'-

453   CAAGCAGAAGACGGCATACGAGAT<i7>AGTCAGTCAGTATGGGGCTACGTCCTCTCCAA-3'

454   and 5'-

455   AATGATACGGCGACCACCGAGATCTACAC<i5>TATGGTAATTGGCCTACGGGAGGCAGCA

456   G-3', where i5 and i7 are 8 base Illumina indexing sequences. These primers contain the

457   Illumina flow cell adapters at their 5'-ends. Unique index primers were used in the PCR for each

458   of the three RT replicates. Products were gel purified using a GeneJET Gel extraction kit

459   (Thermo Scientific K0691) and replicates were pooled with each product at 1.5 ng/µL. The two

460   pooled sets (one for transfected cells and one for infected cells) were each sequenced on an

461   Illumina MiSeq with 2 x 250 paired end reads, V2 chemistry, and the sequencing primers 5'-

462   TATGGTAATTGGCCTACGGGAGGCAGCAG-3', 5'-

463   AGTCAGTCAGTATGGGGCTACGTCCTCTCCAA-3', and 5'-

464   TTGGAGAGGACGTAGCCCCATACTGACTGACT-3'. Each pooled set, one derived from

465   transfection and one from infection, made up half of the DNA input on a separate sequencing

466   run with the remaining DNA being composed of bacterial genome libraries. This allowed for

18

467   sufficient sequencing diversity at each base. We obtained over 15 million reads from each of the

468   samples.

469

470   Consensus sequences that met empirically determined count cutoffs were generated for each

471   PrimerID using Ruby scripts kindly provided by Ronald Swanstrom and colleagues (University of

472   North Carolina). We obtained greater than 449,000 consensus sequences for each of the two

473   samples, suggesting that nearly 75% of the original RNA templates (6×10^5 copies among 3

474   separate reactions) were sampled. Consensus sequences were aligned to the

475   A/Wisconsin/03/2007 H3N2 PA sequence using Bowtie2 and analyzed using Samtools. A

476   custom Python script was used to determine the base composition at each position

477   (https://github.com/lauringlab/NGS_mutation_rate_assay) and the number of stop codons within

478   each PrimerID consensus sequence. The mutation frequency for each of the eight mutational

479   classes was determined by dividing the number of stop codons resulting from each class by the

480   number of sites sequenced that could possibly mutate to a stop codon through that same class.

481   Raw sequencing fastq files from this experiment are available at the Sequence Read Archive

482   under BioProject accession number PRJNA347826.

483

484   *Competition assay*

485   Equal quantities ($TCID_{50}$) of selected mutant ΔHA-GFP viruses were mixed with wild type ΔHA-

486   GFP viruses containing a neutral sequence barcode in the PB1 gene, and used to infect 4×10^5

487   MDCK-HA cells in a 6-well plate at an MOI of 0.01. At 24 hours post infection, supernatants

488   were harvested and infectious particles were titered by $TCID_{50}$. The resulting virus was

489   passaged three more times on MDCK-HA cells, maintaining an MOI of 0.01 at each passage.

490   Each viral competition was performed in triplicate. Viral RNA was harvested from the initial

491   mixture and passaged supernatants using a Purelink Pro 96 viral DNA/RNA kit (Invitrogen

492   12280). Complementary DNA was synthesized using Superscript III and random hexamers.

493   Quantitative PCR was used to determine the relative amount of total PB1 (primers 5'-

494   CAGAAAGGGGAAGATGGACA-3' and 5'-GTCCACTCGTGTTTGCTGAA-3'), barcoded PB1

495   (primers 5'-ATTTCCAACGGAAACGGAGGG-3' and 5'-AAACCCCCTTATTTGCATCC-3'), and

496   non-barcoded PB1 (primers 5'-ATTTCCAACGGAAACGGAGGG-3' and 5'-

497   AAACCCCCTTATTTGCATCC-3') in each sample. The relative amounts of barcoded and non-

498   barcoded PB1 at each passage were normalized by subtracting the Ct threshold for the total

499   PB1 primer set from the respective Ct thresholds (e.g. $\Delta Ct = Ct_{competitior} - Ct_{total\ PB1}$). The

500   normalized values at each passage were compared to the initial viral mixture to obtain a relative

501   Ct ($\Delta\Delta Ct = \Delta Ct_{P1} - \Delta Ct_{P0}$). The relative Ct was converted to reflect the fold change in genome

502   copies ($\Delta ratio = 2^{-\Delta\Delta Ct}$). The slope of the differences between the $\log_{10} \Delta$ ratios of the two

503   viruses as a function of the passage number is equal to the $\log_{10}$ relative fitness of the non-

504   barcoded virus ($[\log_{10}\Delta ratio_{non-barcoded} - \log_{10} \Delta ratio_{barcoded}]$/passage) (27).

505

506   *Growth curves*

507   One hundred $TCID_{50}$ of each mutant ΔHA-GFP virus (in 100μL of media) were used to infect 1.2

508   x 10^4 MDCK-HA in a 96-well plate. At two hour intervals between 14 and 26 hours post infection,

509   supernatants from 4 wells were transferred to a black 96-well plate containing 1.5 x 10^4 MDCK

510   cells and 50μL of viral media. Virus equivalent to the initial inoculum was added to 4 wells so

511   that the virus present at 0 hours post infection could be determined. At 14 hours after

512   supernatant transfer, the cells were fixed, stained and imaged as described below.

513

514   *RNA minimum free energy*

515   The minimum free energy of the ΔHA-GFP RNA was determined using the RNA sliding window

516   python script that is included with the CodonShuffle package (44)

517   (https://github.com/lauringlab/CodonShuffle).

518

20

519   *GFP-based Luria-Delbrück Fluctuation Test*

520   Passage 1 (P1) stocks of ΔHA-GFP viruses were made by passing rescued virus once on

521   MDCK-HA cells at an MOI of 0.01 for 48 hours. For each fluctuation test, 24 or more parallel

522   cultures of MDCK-HA cells were infected with P1 influenza viruses encoding one of the twelve

523   ΔHA-GFP mutants in viral media (Dulbecco's modified Eagle medium (Gibco 11965)

524   supplemented with 0.187% BSA, 25 mM HEPES, and 2 µg/mL TPCK treated trypsin

525   (Worthington Biochemical 3740)). Depending on the mutation class, these infections were

526   performed in either 96-well plates (1.2 x 10^4 cells infected with 400 $TCID_{50}$ of virus in 100 µL),

527   48-well plates (3.6 x 10^4 cells infected with 1200 $TCID_{50}$ of virus in 300 µL), or 24-well plates

528   (7.2 x 10^4 cells infected with 2400 $TCID_{50}$ of virus in 600 µL). At 17-30 hours post infection

529   (depending on the mutation class, drug treatment, and assay temperature) supernatants were

530   transferred to black 96-well plates (Perkin Elmer 6005182) containing 1.5 x 10^4 MDCK target

531   cells and 50 µL of viral media. Supernatants from each well of 48-well and 24-well plates were

532   transferred in 150 µL aliquots to 2 or 4 wells of the black 96-well plate, respectively. In addition

533   to the supernatants derived from the parallel replication cultures, two to four wells were infected

534   with the amount of virus used to seed these cultures (see N_i, below)

535

536   At 14 hours post-infection, cells were fixed with 2% formaldehyde for 20 minutes, rinsed with

537   phosphate buffered saline (PBS), and permeabilized with 0.1% triton-X-100 for 8 minutes. Cells

538   were then rinsed again with PBS, incubated at room temperature for one hour in PBS with 2%

539   BSA and 0.1% tween-20 (PBS-T), and stained with 1:5000 Hoechst (Life Technologies 33342)

540   and 1:400 anti-GFP Alexa 647 conjugate (Life Technologies A31852) diluted in 2% BSA in PBS-

541   T for 1 hour. Cells were washed three times with PBS-T, and the plates were sealed with black

542   tape prior to removal of the final wash. Plates were imaged using an ImageXpress Microscope

543   (Molecular Dynamics) using DAPI, Cy5, and FITC specific filter cubes with a 4x magnification

544   lens. Four non-overlapping quadrants were imaged from each well to ensure that the entire

21

545   surface area was captured, Cellular nuclei and antibody stained cells were counted using

546   MetaXpress version 6 software (Molecular Dynamics). Cells expressing fluorescent GFP were

547   manually counted from the collected images.

548

549   Mutation rates were calculated using the null-class model, $\mu_{(s/n/r)} = -\ln(P_0)/(N_f-N_i)$, where $\mu_{(s/n/r)}$ is

550   the mutation rate per strand replicated, P_0 is the proportion of cultures that do not contain a cell

551   infected by a virus encoding fluorescent GFP, and N_f and N_i are the final and initial viral

552   population sizes, as determined by staining with the anti-GFP antibody, which recognizes both

553   fluorescent and non-fluorescent eGFP (20, 21). Cultures that contained a number of green cells

554   greater than or equal to 0.8 (N_f/N_i) were removed from the calculation because they were likely

555   to have contained a pre-existing fluorescent revertant in the inoculum. These events were

556   extremely rare given the low titer inocula. The null class model is most precise when P_0 is

557   between 0.1 and 0.7 (20). Due to the rarity of certain mutation classes and the constraints of the

558   maximum viral population size per culture and per well on the imaging plate, not all of our

559   measurements fell within this range. Measurements where the P_0 was above 0.7 are indicated

560   in the graphical representations of our data.

561

562   Ribavirin (1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1H-1,2,4-triazole-3-

563   carboxamide) (Sigma-Aldrich R9644) was dissolved in PBS at 100 mM. 5-azacytidine (4-Amino-

564   1-(β-D-ribofuranosyl)-1,3,5-triazin-2(1H)-one) (Sigma-Aldrich A2385) and 5-Fluorouracil (2,4-

565   Dihydroxy-5-fluoropyrimidine) (Sigma-Aldrich F6627) were dissolved in dimethyl sulfoxide

566   (DMSO) at 100 mM and 384 mM, respectively. For mutation rate measurements in the presence

567   of drug, MDCK-HA cells were pretreated with viral media containing 2.5 μM ribavirin, 0.625 μM

568   5-azacytidine, or 15 μM 5-fluorouracil for three hours. Mutation rate assays were carried out

569   according to the above protocol except that the viral media for the initial infections contained

570   drugs at the indicated concentrations.

571

572   Mutation rate measurements at different temperatures were carried out as above, except that

573   the initial replication was performed in incubators maintained at 32°C or 39°C. The imaging

574   plates were maintained at 37°C for the 14 hours after the supernatant transfer.

575

585

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744   **Figure Legends**

745   Figure 1. Influenza mutation rates by PrimerID next generation sequencing. (A) Segment 3 (PA)

746   RNA was isolated from either cells transfected with pol I expression plasmids or cell-free

747   supernatants of cells infected with influenza virus. These RNA were reverse transcribed with

748   barcoded PrimerID primers and amplified by PCR for sequencing as described in the methods.

749   We obtained 449,655 PrimerID consensus sequences for the plasmid-derived RNA sample and

750   481,286 consensus sequences for the virus-derived RNA sample. (B) The frequencies of

751   nonsense mutations sequences in pol I transcribed RNA (open circles) and virus-derived RNA

752   (filled circles) were determined by dividing the number of nonsense mutations across the

753   consensus sequences by the total number of nonsense mutation target sites analyzed. See also

754   Table S1. (C) Total mutation frequencies were calculated as the number of observed mutations

755   for a particular mutation class divided by the number of sequenced sites that could mutate by

756   that same class. Shown is the ratio of total mutation frequency to nonsense mutation frequency

757   by mutation class for sequences derived from plasmid-derived RNA (grey bars) and virus-

758   derived RNA (black bars).

759   Figure 2. Characterization of mutant ΔHA-GFP influenza viruses. (A) Fluorescent images of

760   cells infected with mutant ΔHA-GFP (shown are data for the A to C virus, see Table 1) and

761   stained with Hoechst and anti-GFP Alexa 647 conjugate. Images were taken at 4x magnification

762   and these images were magnified to an equal degree in this figure. (B) Growth kinetics of

763   mutant ΔHA-GFP viruses. MDCK-HA cells were infected at an MOI of 0.01 in 96-well plates and

764   incubated at 32˚C (open squares), 37˚C (filled squares), or 39˚C (open circles). At each time

765   point, the supernatants from 4 wells were transferred to a new 96-well plate containing MDCK

766   cells. After 14 hours the cells were fixed and stained using an anti-GFP antibody. The number of

28

767   cells stained were determined by fluorescence microscopy and used to calculate the titer of

768   GFP expressing virus. Data shown are the cumulative mean and standard deviations for 4

769   measurements at each time point for each of two mutant ΔHA-GFP viruses (C to U and U to A

770   viruses). (C) The fitness of 6 of the mutant ΔHA-GFP viruses (x-axis) were compared to wild

771   type ΔHA-GFP through direct competition with a genetically barcoded competitor over 4 serial

772   passages. Quantitative PCR was used to determine the relative changes in the frequency of the

773   two competitors and fitness values were calculated as described in the methods. Mutant viruses

774   are classified by the GFP amino acid mutated, with wild type (black), T65 (gray), Y66 (striped),

775   G67 (white). Shown are the mean and standard deviation for three competitions and fitness

776   measurements for each virus. (D) The minimum free energy of RNA folding for 100 base sliding

777   windows (80 base overlaps) were determined for the ΔHA-GFP construct. The location of the

778   three mutated sites (bases 280-288) are indicated by the dashed line.

779   Figure 3. Fluorescent Luria-Delbruck fluctuation test. (A) General workflow for measuring the

780   mutation rate using mutant ΔHA-GFP viruses. Parallel cultures of MDCK-HA cells were infected

781   with mutant ΔHA-GFP at low multiplicity. The time for initial replication was varied to allow for a

782   number of replicated viruses and revertants adequate to measure the mutation rate for a given

783   class. Supernatants were transferred to 96 well plates of MDCK cells and incubated for 14

784   hours to allow for GFP expression in target cells. The mutation rate for each mutant ΔHA-GFP

785   virus and class was calculated as described in the methods and text based on the initial and

786   final titer (N_i and N_f, anti-GFP positive infected cells) and proportion of cultures with no

787   revertants (P_0, wells without green fluorescence). (B-D) Specificity of the reversion to

788   fluorescence assay. The (B) A to G, (C) G to A, and (D) G to C mutation rates for A/Puerto

789   Rico/8/1934 H1N1 were measured at 37°C in cells pretreated with 0.625μM 5-azacytidine

29

790   (AzaC), 15 µM 5-fluorouracil (5 FU), or 2.5 µM ribavirin (Riba). No data is shown for G to C with

791   2.5 µM ribavirin because large titer decreases upon drug treatment prohibited measurements.

792   Filled symbols represent measurements in which P0 is between 0.1 and 0.69, where the assay

793   is most precise. Open circles represent data with P0 between 0.7 and 0.9. Arithmetic means are

794   indicated. A one-way ANOVA with a Dunnett's correction for multiple comparisons was used for

795   each mutation class to compare each drug treatments to no drug treatment. * = $p < 0.05$, ** = p

796   < 0.01, *** = $p < 0.005$.

797   Figure 4. The mutation rates of influenza viruses replicated at 37°C. (A) Measurements of

798   A/Puerto Rico/8/1934 H1N1 viruses encoding the 12 different mutant ΔHA-GFP constructs. (B)

799   Measurements of viruses encoding the replication complex (PB2, PB1, PA, and NP) from

800   A/Hong Kong/4801/2014 H3N2 and the remaining genes coming from A/Puerto Rico/8/1934

801   H1N1. Filled symbols represent measurements in which P0 is between 0.1 and 0.69. Open

802   circles represent data with P0 between 0.7 and 0.95. The arithmetic means are indicated on the

803   graphs and the means and standard deviations reported in Table S2.

804   Figure 5. The effect of temperature on influenza A virus mutation rates. Mutation rates were

805   determined for A/Puerto Rico/8/1934 H1N1 viruses encoding the indicated mutant ΔHA-GFP

806   constructs replicated at 32°C (blue), 37°C (black) and 39°C (red). Filled symbols represent

807   measurements in which P0 is between 0.1 and 0.69. Open circles represent data with P0

808   between 0.7 and 0.90. The arithmetic means are indicated. A two-way ANOVA revealed no

809   significant differences in mutation rates based upon temperature.
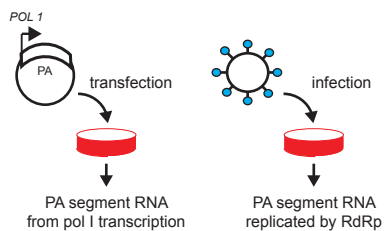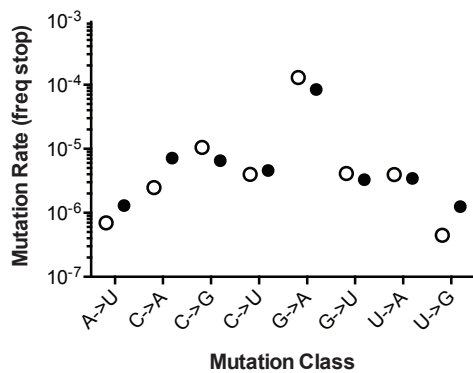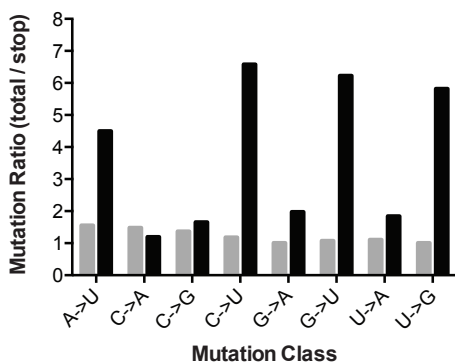
Table 1: Non-fluorescent ΔHA-GFP constructs

Mutation Probed [a]	Nucleotide Sequece [b]	Amino Acid Sequence [c]
WT eGFP	acc uac ggc	T Y G
A -> C	a*Aa* uac ggc	*K* Y G
A -> G	acc uac g*A*c	T Y *D*
A -> U	acc *A*ac ggc	T *N* G
C -> A	acc u*C*c ggc	T *S* G
C -> G	acc uac g*C*c	T Y *A*
C -> U	acc *C*ac ggc	T *H* G
G -> A	acc u*G*c ggc	T *C* G
G -> C	*uGg* uac ggc [d]	*W* Y G
G -> U	acc *G*ac ggc	T *D* G
U -> A	acc u*U*c ggc	T *F* G
U -> C	a*Ua* uac ggc	*I* Y G
U -> G	acc uac g*U*c	T Y *V*

[a] Mutations are in the mRNA coding sense.
[b] Nucleotides 193-201 of the eGFP reading frame are shown. Changes from wild type are in bold and italics. Site that allows reversion to fluorescence is capitalized.
[c] Amino acids 65-67 of eGFP are shown. Changes from wild type are in bold and italics
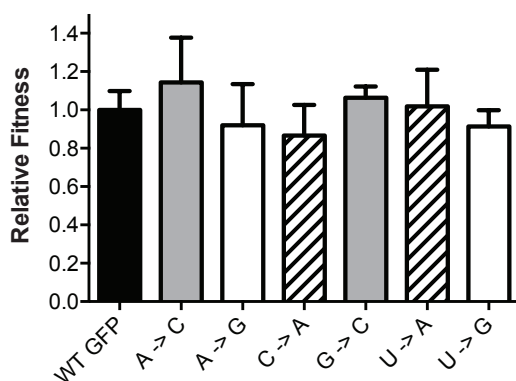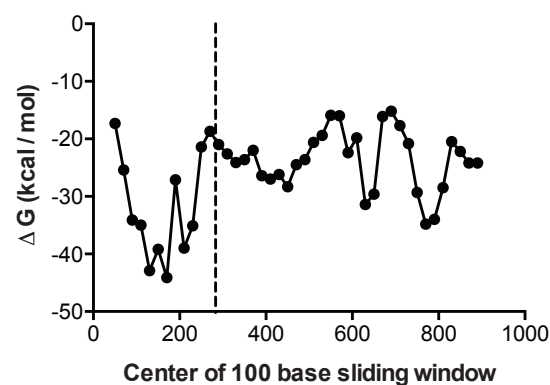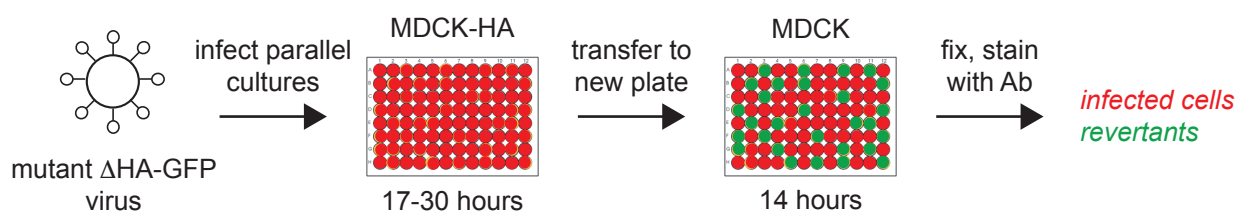[d] This construct is able to revert to wild type GFP (S65)

A

POL 1

PA → transfection

infection

PA segment RNA
from pol I transcription

PA segment RNA
replicated by RdRp

B

C

A

Hoechst   Anti-GFP Ab   GFP

B

C

D

A

B

A -> G

C

G -> A

D

G -> C

A

A/Puerto Rico/8/1934 H1N1

B

A/Hong Kong/4801/2014 H3N2