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The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin

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Abstract

Influenza is notable for its evolutionary capacity to escape immunity targeting the viral hemagglutinin. We used deep mutational scanning to examine the extent to which a high inherent mutational tolerance contributes to this antigenic evolvability. We created mutant viruses that incorporate most of the $\approx 10^4$ amino-acid mutations to hemagglutinin from A/WSN/1933 (H1N1) influenza. After passaging these viruses in tissue culture to select for functional variants, we used deep sequencing to quantify mutation frequencies before and after selection. These data enable us to infer the preference for each amino acid at each site in hemagglutinin. These inferences are consistent with existing knowledge about the protein's structure and function, and can be used to create a model that describes hemagglutinin's evolution far better than existing phylogenetic models. We show that hemagglutinin has a high inherent tolerance for mutations at antigenic sites, suggesting that this is one factor contributing to influenza's antigenic evolution.

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Introduction

Epidemic influenza poses an annual threat to human health largely because the virus rapidly evolves to escape the immunity elicited by previous infections or vaccinations. The most potent form of anti-influenza immunity is antibodies targeting the virus's hemagglutinin (HA) protein (Yewdell et al., 1979; Wiley et al., 1981; Caton et al., 1982). The virus evades these antibodies primarily by accumulating amino-acid substitutions in HA's antigenic sites (Smith et al., 2004; Das et al., 2013; Koel et al., 2013; Bedford et al., 2014). Remarkably, HA undergoes this rapid evolution while retaining the ability to fold to a highly conserved structure that performs two functions essential for viral replication: receptor binding and membrane fusion (Wiley and Skehel, 1987; Russell et al., 2004). HA is therefore highly "antigenically evolvable" in the sense that it can accommodate rapid antigenic change without compromising its structural and functional properties.

Two factors that undoubtedly contribute to HA's rapid antigenic evolution are influenza's high mutation rate and the strong selection that immunity exerts on the virus. However, it is unclear whether these factors are sufficient to fully explain HA's antigenic evolution. For instance, while some other error-prone viruses (such as HIV and hepatitis C) also exhibit rapid antigenic evolution of their surface proteins (Burton et al., 2012), other viruses with comparable mutation rates (such as measles) show little propensity for antigenic change (Duffy et al., 2008; Sheshberadaran et al., 1983), despite the fact that evasion of immunity would presumably confer a selective benefit. A variety of explanations ranging in scale from ecological to molecular can be posited to account for these differences in rates of antigenic evolution (Lipsitch and O'Hagan, 2007; Koelle et al., 2006; Heaton et al., 2013). One hypothesis is that HA has a high inherent tolerance for mutations in its antigenic sites, thereby conferring on influenza the evolutionary capacity to escape from anti-HA antibodies with relative ease.

Testing this hypothesis requires quantifying the inherent mutational tolerance of each site in HA. This cannot be done simply by examining variability among naturally occurring viruses, since the evolution of influenza in nature is shaped by a combination of inherent mutational tolerance and external immune selection. For example, the rapid evolution of HA's antigenic sites in nature could reflect the fact that these sites are especially tolerant of mutations, or it could be purely a consequence of strong immune selection. Traditional experimental approaches using site-directed mutagenesis or serial viral passage are also inadequate to quantify inherent mutational tolerance – while such experimental techniques have been used to determine the effect

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of specific mutations on HA, they cannot feasibly be applied to all possible individual amino-acid mutations. Recently Heaton et al. (2013) used transposon mutagenesis to show that HA is tolerant to the random insertion of five to six amino-acid sequences at several locations in the protein. However, the relevance of this tolerance to insertional mutations is unclear, since HA's actual antigenic evolution involves almost entirely point substitutions, with only a very low rate of insertions and deletions.

Here we use the new high-throughput experimental technique of deep mutational scanning (Fowler et al., 2010; Araya and Fowler, 2011) to comprehensively quantify the tolerance of HA to amino-acid mutations. Specifically, we create mutant libraries of the HA gene from the H1N1 strain A/WSN/1933 (WSN) that contain virtually all of the $\approx 4 \times 10^4$ possible individual codon mutations, and therefore virtually all of the $\approx 10^4$ possible amino-acid mutations. We use these mutant libraries to generate pools of mutant influenza viruses, which we estimate incorporate at least 85% of the possible HA codon mutations and 97% of the possible amino-acid mutations. We then passage these viruses to select for functional variants, and use Illumina deep sequencing to determine the frequency of each HA mutation before and after this selection for viral growth. Since these experiments measure the impact of mutations in the absence of immune selection, they enable us to quantify HA's inherent tolerance for each amino acid at each site in the protein. We show that these quantitative measurements are consistent with existing knowledge about HA structure and function, and can be used to create an evolutionary model that describes HA's natural evolution far better than existing models of sequence evolution. Finally, we use our results to show that HA's antigenic sites are disproportionately tolerant of mutations, suggesting that a high inherent tolerance for mutations at key positions targeted by the immune system is one factor that contributes to influenza's antigenic evolvability.

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Results

Strategy for deep mutational scanning of HA

Our strategy for deep mutational scanning (Fowler et al., 2010; Araya and Fowler, 2011) of HA is outlined in Figure 1. The wildtype WSN HA gene was mutagenized to create a diverse library of mutant HA genes. This library of mutant genes was then used to generate a pool of mutant viruses by reverse genetics (Hoffmann et al., 2000). The mutant viruses were passaged at a low multiplicity of infection to ensure a linkage between genotype and phenotype. The frequencies of mutations before and after selection for viral growth were quantified by Illumina deep sequencing of the mutant genes (the **mutDNA** sample in Figure 1) and the mutant viruses (the **mutvirus** sample in Figure 1). An identical process was performed in parallel using the unmutated wildtype HA gene to generate unmutated viruses in order to quantify the error rates associated with sequencing, reverse transcription, and virus growth (these are the **DNA** and **virus** samples in Figure 1). The entire process in Figure 1 was performed in full biological triplicate (the replicates are referred to as #1, #2, and #3). In addition, a repeat of the Illumina sample preparation and deep sequencing was performed for replicate #1 to quantify the technical variation associated with these processes.

Creation of HA codon-mutant libraries

The deep mutational scanning strategy in Figure 1 requires the creation of mutant libraries of the HA gene. We wanted to assess the impact of all possible amino-acid mutations. Most mutagenesis techniques operate at the nucleotide level, and so frequently introduce single-nucleotide codon changes (e.g. `GGA` $\rightarrow$ `cGA`) but only very rarely introduce multi-nucleotide codon changes (e.g. `GGA` $\rightarrow$ `cat`). However, several PCR-based techniques have recently been developed to introduce random codon mutations into full-length genes (Firnberg and Ostermeier, 2012; Jain and Varadarajan, 2014; Bloom, 2014). We used one of these techniques (Bloom, 2014) to create three replicate codon-mutant libraries of the WSN HA gene (Supplementary file 1).

Sanger sequencing of 34 individual clones indicated that the libraries contained an average of slightly over two codon mutations per gene, with a very low rate of insertions and deletions (less than 0.1 per gene). The number of mutations per clone was distributed around this average in an approximately Poisson fashion (Figure 2). The mutations consisted of a mix of one-, two-,

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and three-nucleotide codon changes, and were roughly uniform in their nucleotide composition and location in the gene (Figure 2).

The genes in each mutant library were cloned at high efficiency into a bidirectional influenza reverse-genetics plasmid (Hoffmann et al., 2000). Each of the library replicates contained at least six-million unique clones – a diversity that far exceeds the 10^4 unique single amino-acid mutations and the $\approx 4\times10^4$ unique single codon mutations to the HA gene. The vast majority of possible codon and amino-acid mutations are therefore represented many times in each plasmid mutant library, both individually and in combination with other mutations.

Generation of mutant viruses by reverse genetics

The HA plasmid mutant libraries were used to generate pools of mutant influenza viruses by reverse genetics (Hoffmann et al., 2000). Briefly, this process involves transfecting cells with the HA plasmid mutant library along with plasmids encoding the other seven genes from the WSN strain of influenza. Although the cells were transfected with a very large diversity of HA plasmids, we were uncertain what fraction of the genes encoded on these plasmids would actually be productively packaged into a virus. In an attempt to maximize the diversity in the viral pools, the mutant viruses were generated by transfecting several dozen wells of cells. The logic behind this scheme was to maintain substantial diversity even if only a subset of viral mutants stochastically predominated in each individual well of cells. A different replicate virus pool was generated for each of the three HA plasmid mutant libraries.

The mutant viruses generated for each replicate were passaged at a relatively low multiplicity of infection (0.1) to reduce the probability of co-infection, thereby ensuring a link between viral genotype and phenotype. The passaging was performed in a total of 2.4×10^7 cells, thereby maintaining a diversity of over two-million infectious viral particles.

Deep sequencing reveals purifying selection against many mutations

We used Illumina sequencing to quantify the frequencies of mutations before and after selection for viral growth. For each replicate, we sequenced HA from the unmutated plasmid, the plasmid mutant library, virus produced from the unmutated plasmid, and mutant virus produced from the plasmid mutant library – these are the **DNA**, **mutDNA**, **virus**, and **mutvirus** samples in Figure 1. For the **DNA** and **mutDNA** samples, the HA gene was amplified directly from the plasmids by PCR. For the **virus** and **mutvirus** samples, the HA gene was first reverse-transcribed from

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viral RNA and was then amplified by PCR. In all cases, template quantification was performed prior to PCR to ensure that $> 10^6$ initial HA molecules were used as templates for subsequent amplification.

In order to reduce the sequencing error rate, the HA molecules were sheared to roughly 50 nucleotide fragments and sequenced with overlapping paired-end reads (Figure 3 – figure supplement 1). We only called codon identities for which both paired reads concur – this strategy substantially increases the sequencing fidelity, since it is rare for the same sequencing error to occur in both reads. For each sample, we obtained in excess of 10^7 overlapping paired-end reads that could be aligned to HA (Figure 3 – figure supplement 2). Although the read depth varied somewhat along the primary sequence due to biases in the fragmentation, we obtained well over 2×10^5 unique paired reads for most codons (Figure 3 – figure supplement 3).

Figure 3 shows the frequency of mutations in each sample as quantified by deep sequencing. The **DNA** samples derived from unmutated HA plasmid show a low frequency of apparent mutations which are almost exclusively composed of single-nucleotide codon changes – the frequency of these apparent mutations reflects the rate of errors from the PCR amplification and subsequent deep sequencing. The **virus** samples created from the unmutated plasmid show only a slightly higher frequency of mutations, indicating that reverse-transcription and viral replication introduce only a small number of additional mutations. As expected, the **mutDNA** samples derived from the plasmid mutant libraries show a high rate of one-, two-, and three-nucleotide mutations, as all three types of mutations were introduced during the codon mutagenesis. The **mutvirus** samples derived from the mutant virus pools exhibit a mutation rate that is substantially lower than that of the **mutDNA** samples. Most of the reduction in mutation frequency in the **mutvirus** samples is due to decreased frequencies of nonsynonymous and stop-codon mutations; synonymous mutations are only slightly depressed in frequency. As stop-codon and nonsynonymous mutations are much more likely than synonymous mutations to substantially impair viral fitness, these results are consistent with purifying selection purging deleterious mutations during viral growth.

Most mutations are sampled by the experiments

It is important to assess the completeness with which the experiments sampled all possible HA mutations. It is possible to imagine several problems that could limit mutational sampling: mutations might be absent from the plasmid mutant libraries due to biases in the codon mutage-

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nesis, mutations that are present in the plasmid mutants might fail to be incorporated into viruses due to stochastic bottlenecks during virus generation by reverse genetics, or the sequencing read depth might be inadequate to sample the mutations that are present. The most straightforward way to assess these issues is to quantify the number of times that each possible multi-nucleotide codon mutation is observed in the **mutDNA** and **mutvirus** samples. Restricting the analysis to multi-nucleotide codon mutations avoids the confounding effects of sequencing and reverse-transcription errors, which cause almost exclusively single-nucleotide changes.

Figure 4 shows the number of times that each mutation was observed in the combined sequencing data for the three biological replicates; Figure 4 – figure supplement 1 shows the same data for the replicates individually. More than 99.5% of multi-nucleotide codon mutations are observed at least five times in the combined sequencing data from the plasmid mutant libraries (**mutDNA** samples), and $\approx$ 97.5% of all such mutations are observed at least five times in sequencing of the **mutDNA** for each individual replicate. These results indicate that the vast majority of codon mutations are represented in the plasmid mutant libraries.

In contrast, only 53% of multi-nucleotide codon mutations are observed at least five times in the combined sequencing data for the mutant viruses (**mutvirus** samples), and only $\approx$ 26% of such mutations are observed at least five times in sequencing of the **mutvirus** for each individual replicate (Figure 4, Figure 4 – figure supplement 1). However, these numbers are confounded by the fact that many mutations are deleterious, and so may be absent because purifying selection has purged them from the mutant viruses. A less confounded measure is the frequency of *synonymous* multi-nucleotide mutations, since synonymous mutations are less likely to be strongly deleterious. About 85% of such mutations are observed at least five times in the combined **mutvirus** samples, and $\approx$ 51% of such mutations are observed at least five times in the **mutvirus** samples for the individual replicates (Figure 4, Figure 4 – figure supplement 1). Note that these numbers are only a lower bound on the fraction of codon mutations sampled by the mutant viruses – even synonymous mutations to influenza are sometimes strongly deleterious (Marsh et al., 2008), and so some of the missing synonymous codon mutations may have been introduced into mutant viruses but then purged by purifying selection. Furthermore, the redundancy of the genetic code means that the fraction of possible amino-acid mutations sampled is substantially higher than the fraction of codon mutations sampled. Specifically, if 85% of possible codon mutations are sampled at least five times in the combined libraries (as Figure 4 indicates), then our simulations suggest that $\approx$ 97% of possible amino-acid mutations will have also been sampled at least five times (see Methods).

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Overall, these results indicate that nearly all mutations are represented in the plasmid mutant libraries. Virus generation by reverse genetics does introduce a bottleneck – but fortunately, this bottleneck is sufficiently mild that at least half of all possible codon mutations are still sampled at least five times by the mutant viruses in each individual replicate. Combining the data for the three replicates brings the coverage of possible codon mutations to around 85%, and the coverage of possible amino-acid mutations to 97%. Therefore, the sampling of mutations is sufficiently complete to provide information on the effects of most amino-acid mutations when the data from the three experimental replicates are combined.

Estimation of the effects of each amino-acid mutation to HA

We quantified the effects of mutations in terms of site-specific amino-acid "preferences." These preferences are the expected frequency of each amino acid at each site in the mutant viruses in a hypothetical situation in which all amino acids are introduced at that site at equal frequency in the initial plasmid mutant library (Bloom, 2014). Because many of the HAs in our libraries contain several mutations, these preferences do not simply correspond to the fitness effect of each individual mutation to the WSN HA – rather, they represent the average effect of each mutation in a collection of closely related HA mutants. Mutations to amino acids with high preferences are favored by selection, while mutations to amino acids with low preferences are disfavored. The amino-acid preferences are inferred from the deep sequencing data using a Bayesian statistical framework in which the observed counts are treated as draws from multinomial distributions with unknown parameters representing the initial mutagenesis rate, the various error rates, and selection as represented by the preferences (see Methods for details).

Figure 5 shows the amino-acid preferences for the entire HA gene inferred from the combined data from all three biological replicates. As can be seen from this figure, some sites have strong preferences for one specific amino acid, while other sites are tolerant of a variety of different amino acids. As described in Table 1, the inferred amino-acid preferences are consistent with existing knowledge about mutations and residues affecting HA stability, membrane fusion, proteolytic activation, and receptor binding (Nakajima et al., 1986; Qiao et al., 1999; Stech et al., 2005; Martin et al., 1998). This concordance suggests that the deep mutational scanning effectively captures many of the structural and functional constraints on HA.

Despite the general concordance between the inferred amino-acid preferences and existing knowledge, it is important to quantify the experimental error associated with the deep muta-

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tional scanning. We sought to quantify two factors: *technical* variation due to inaccuracies and statistical limitations during Illumina sample preparation and deep sequencing, and *biological* variation due to stochasticity in the viral mutants that were generated and enriched during each replicate of the experiment. Figure 6A shows the correlation between biological replicate #1 and a technical repeat of the Illumina sample preparation and deep sequencing for this biological replicate. There is a very high correlation between the preferences inferred from these two repeats, indicating that technical variation has only a very minor influence on the final inferred amino-acid preferences. Figure 6B-D show the correlation among the three different biological replicates. Although the biological replicates are substantially correlated, there is also clear variation. Most of this variation is attributable to amino acids which in one replicate are inferred to have preferences near the *a priori* expectation of 0.05 (there are 20 amino acids, which in the absence of data are all initially assumed to have an equal preference of $\frac{1}{20}$), but in another replicate are inferred to have a much higher or lower preference. Such variation arises because the mutant viruses for each biological replicate only sample about 50% of the possible codon mutations (see previous section), meaning that there is little data for some mutations in any given replicate. Fortunately, combining the three biological replicates greatly increases the coverage of possible mutations (see previous section). Therefore, inferences made from the combined data (as in Figure 5) should be substantially more accurate than inferences from any of the individual replicates. This idea is supported by the results of the next section, which quantifies the extent to which the inferred preferences accurately describe natural HA evolution.

Experimental inferences are consistent with HA's natural evolution

Do the results of the deep mutational scanning experiment accurately reflect the real constraints on HA? The comparison in Table 1 uses an anecdotal comparison to a small number of existing experimental studies to suggest that they do. However, a more systematic way to address this question is to compare the inferred amino-acid preferences to the actual patterns of HA evolution in nature.

To make such a comparison, we created an alignment of HA sequences from human and swine influenza viruses descended from a common ancestor closely related to the virus that caused the 1918 influenza pandemic. Figure 7 shows a phylogenetic tree of these sequences. The WSN HA used in our deep mutational scanning falls relatively close to the root of this tree.

The crudest comparison is simply to correlate amino-acid frequencies in the natural se-

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quences to the experimentally inferred amino-acid preferences. Figure 8 shows that the inferred preferences are substantially although imperfectly correlated with the natural amino-acid frequencies. However, this comparison is imperfect because it fails to account for the contingent and limited sampling of mutations by natural evolution. While the deep mutational scanning is designed to sample all possible mutations, only a fraction of theoretically tolerable mutations have fixed in natural H1 HAs due to the finite timespan during which evolution has been exploring possible sequences (in other words, evolution is not at "equilibrium"). Therefore, an amino-acid frequency of close to one among the natural HA sequences in Figure 7 might imply an absolute functional requirement for that amino acid – or it might simply mean that natural evolution has not yet happened to fix a mutation to another tolerable amino acid at that site.

A better approach is therefore to treat natural evolution as a non-equilibrium dynamic process, and ask whether the inferred amino-acid preferences accurately describe this process. This type of analysis can be done using the likelihood-based statistical framework for phylogenetics developed by Felsenstein (1973, 1981). Specifically, we fix the phylogenetic tree topology to that shown in Figure 7 and then assess the likelihood of the natural sequences given a specific evolutionary model after optimizing the branch lengths of the tree. Evolutionary models that more accurately describe HA sequence evolution will have higher likelihoods, and the relative accuracy of models can be quantified by comparing their likelihoods after correcting for the number of free parameters using AIC (Posada and Buckley, 2004). Previous work has described how experimental measurements of amino-acid preferences can be combined with known mutation rates to create a parameter-free phylogenetic evolutionary model from deep mutational scanning data (Bloom, 2014).

Table 2 compares the fit of evolutionary models based on the experimentally inferred amino-acid preferences with several existing state-of-the-art models that do not utilize this experimental information (Goldman and Yang, 1994; Kosiol et al., 2007). The model based on amino-acid preferences inferred from the combined experimental data from the three replicates describes the evolution of the naturally occurring HA sequences far better than the alternative models, despite the fact that the latter have a variety of free parameters that are optimized to improve the fit. Models based on amino-acid preferences inferred from the individual experimental replicates also fit the data better than existing models – however, the fit is poorer than for the model that utilizes the data from all three replicates. This result is consistent with the fact that the individual replicates are incomplete in their sampling of the mutational effects, meaning that aggregating the data from several replicates improves the accuracy of inferred preferences. Overall,

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these comparisons show that the deep mutational scanning reflects the actual constraints on HA evolution substantially better than existing quantitative evolutionary models.

The inherent evolvability of antigenic sites on HA

The amino-acid preferences inferred from the deep mutational scanning reflect the inherent mutational tolerance of sites in HA. In contrast, the evolution of HA in nature is shaped by a combination of HA's inherent mutational tolerance and external selection pressures. Specifically, the evolution of HA in humans is strongly driven by selection for mutations that alter antigenicity (Yewdell et al., 1979; Wiley et al., 1981; Caton et al., 1982; Smith et al., 2004; Das et al., 2013; Koel et al., 2013; Bedford et al., 2014). The fact that such antigenic mutations fix at high frequency implies some degree of mutational tolerance at antigenic sites, since no mutations would fix if these sites were under absolute structural or functional constraint. However, it is not possible to tell from analysis of natural sequences alone whether antigenic sites are unusually mutationally tolerant compared to the rest of HA, or whether their rapid evolution is solely due to the fact that they are under strong external immune selection.

To address this issue, we used the results of the deep mutational scanning to compare the inherent mutational tolerance of antigenic sites to the rest of the HA protein. We classified HA1 (the head domain) into antigenic sites and all other residues, using the antigenic site definitions of Caton et al. (1982) with additional exclusion of a single site that has gained glycosylation in the WSN HA (see Methods). We also performed a similar classification of HA1 into conserved receptor-binding residues and all other residues. These conserved receptor-binding residues represent a control group for our comparison – while we hypothesize that antigenic sites might be inherently more mutationally tolerant than the rest of HA, we strongly expect that receptor-binding residues will be less mutationally tolerant than the rest of HA. The positions of the antigenic sites and conserved receptor-binding residues in the primary sequence are indicated by the top overlay bar in Figure 5. Visual inspection of this figure suggests that the receptor-binding residues are indeed relatively intolerant of mutations (have a strong preference for one specific amino acid), whereas the antigenic sites are relatively tolerant of mutations (have roughly equivalent preferences for many amino acids).

To quantify mutational tolerance, we computed a site entropy from the inferred amino-acid preferences – larger site entropies indicate a higher inherent tolerance for mutations. The site entropies for the antigenic sites and receptor-binding residues are displayed on the HA

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protein structure in Figure 9A. Inspection of this figure reveals that antigenic sites have high site entropies, whereas receptor-binding residues have low site entropies. However, it is well known that sites with higher relative solvent accessibility are typically more mutationally tolerant (Ramsey et al., 2011; Bustamante et al., 2000), so it is necessary to correct for this effect. Figure 9B,C plot site entropy as a function of relative solvent accessibility for all HA1 sites in the crystal structure. These plots suggest that receptor-binding residues are less mutationally tolerant and antigenic sites are more mutationally tolerant than other comparable residues, even after correcting for relative solvent accessibility. This finding is validated at a high level of statistical significance by multiple linear regression (Table 3). Therefore, we can conclude that the antigenic sites in HA have unusually high mutational tolerance, suggesting that this property combines with external immune selection to contribute to HA's rapid antigenic evolution.

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Discussion

A fundamental challenge in studying the natural evolution of influenza is separating the effects of external selection pressures from inherent structural and functional constraints. The evolutionary patterns observed in natural sequences are shaped by a combination of inherent mutational tolerance and external pressures such as immune selection, and the analysis of such sequences is further confounded by the fact that influenza is not at evolutionary equilibrium.

Here we have quantified the inherent mutational tolerance of influenza HA by using deep mutational scanning (Fowler et al., 2010; Araya and Fowler, 2011) to simultaneously assess the impact on viral growth of the vast majority of the $\approx 10^4$ possible amino-acid mutations to influenza HA. The information obtained from the deep mutational scanning is consistent with existing knowledge about the effects of mutations on HA function and structure. For instance, the deep mutational scanning shows strong selection for specific amino acids known to play important roles in HA's receptor-binding activity, fusion activity, and proteolytic activation (Qiao et al., 1999; Stech et al., 2005; Martin et al., 1998). Similarly, at the sites of known temperature-sensitive mutations to HA (Nakajima et al., 1986), the deep mutational scanning identifies the more stabilizing amino-acid as more favorable. Broader trends from the deep mutational scanning are also in agreement with current thinking about mutational effects. For example, the deep mutational scanning finds that there is strong purifying selection against stop-codon mutations and many nonsynonymous mutations, but that there is only weak selection against synonymous mutations. All of these results suggest that the deep mutational scanning faithfully captures both the specific and general effects of mutations on HA.

The comprehensive information generated by the deep mutational scanning can be used to create quantitative evolutionary models for analyzing HA sequence phylogenies. Here we have shown that an evolutionary model constructed from our deep mutational scanning data describes the evolution of human and swine H1 HAs far better than existing state-of-the-art models for sequence evolution. We anticipate that separating HA's inherent mutational tolerance from external selection should also eventually allow the external selection pressures to be studied in greater detail. For example, one might imagine that sites in HA that exhibit evolutionary patterns that deviate from the quantitative model created from our deep mutational scanning are likely to be under external selection. Future work that augments deep mutational scanning with specific experimentally defined selection pressures (such as antibodies against HA) could aid in further elucidation of the forces that shape influenza evolution.

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The deep mutational scanning also enabled us to assess the extent to which HA's inherent mutational tolerance contributes to influenza's antigenic evolvability. It remains a mystery why error-prone RNA viruses differ so widely in their capacity for evolutionary escape from immunity, with some (e.g. influenza and HIV) undergoing rapid antigenic evolution while others (e.g. measles) show little antigenic change on relevant timescales (Lipsitch and O'Hagan, 2007; Koelle et al., 2006; Heaton et al., 2013). Our data demonstrate that the antigenic sites in HA are unusually tolerant to mutations, implying that inherent evolutionary plasticity at sites targeted by the immune system is one factor that contributes to influenza's rapid antigenic evolution. This high mutational tolerance at antigenic sites could itself be a property that influenza has evolved to aid in its antigenic escape – or it might simply be an unfortunate coincidence that the immune system focuses on especially plastic portions of HA. In either case, it is intriguing to speculate whether a high inherent mutational tolerance in antigenic sites is also a feature of other antigenically variable RNA viruses. Application of the deep mutational scanning approach used here to additional viruses should provide a means to address this question.

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Methods

Availability of data and computer code

Illumina sequencing data are available at the SRA, accession SRP040983 (http://www.ncbi.nlm.nih.gov/sra/?term=SRP040983). Source code and a description of the computational process used to analyze the sequencing data and infer the amino-acid preferences is at http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html. Source code and a description of the computational process used for the phylogenetic analyses is available at http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html. Currently the descriptions and source code are available via these links, but the actual processed data for the analysis has not yet been released. The data will be released upon publication of the paper. If you are a reviewer and would like to see this data now, please relay the request to the authors via the editor.

HA sequence numbering

A variety of different numbering schemes for HA are used in the literature. Unless noted otherwise, residues are numbered here using sequential numbering of the WSN HA protein sequence (Supplementary file 1) starting with one at the N-terminal methionine. In some cases, the number of the corresponding residues in the widely used H3 numbering scheme is also indicated. These numbering systems can be interconverted using the Python script available at https://github.com/jbloom/HA_numbering.

Generation of HA codon mutation library

The HA codon-mutant library was generated using the oligo-based PCR mutagenesis protocol described previously by Bloom (2014). The only differences from that protocol were that HA was used as the template rather than NP, and that only two overall rounds of mutagenesis were performed, rather than the three rounds used by Bloom (2014). This reduction in the number of rounds of mutagenesis reduced the average number of codon mutations from the $\approx$ three per clone in Bloom (2014) to the $\approx$ two per clone shown in Figure 2. The libraries were created in full biological triplicate, meaning that each experimental replicate was derived from an independent plasmid mutant library.

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The end primers for the mutagenesis were 5'-cgatcacgtctctgggagcaaaagcaggggaaaataaaaacaac-3' and 5'-gatacacgtctcatattagtagaaacaagggtgtttttccttatatttctg-3' (these primers include BsmBI restriction sites). The mutagenic primers were ordered from Integrated DNA Technologies, and are listed in Supplementary file 2.

The final products from the codon mutagenesis PCR were gel purified and digested with BsmBI (New England Biolabs, R0580L). The BsmBI-digested HA was ligated into a dephosphorylated (Antarctic Phosphatase, New England Biolabs, M0289L) and BsmBI-digested preparation of the bidirectional reverse-genetics plasmid pHW2000 (Hoffmann et al., 2000) using T4 DNA ligase (New England Biolabs, M0202S). Column-purified ligations were electroporated into ElectroMAX DH10B T1 phage-resistant competent cells (Invitrogen, 12033-015) and plated on LB plates containing 100 μg/ml of ampicilin. A 1:4000 dilution of each transformation was plated in parallel to enable estimation of the number of unique transformants – we obtained at least two-million unique colonies per transformation. For each replicate of the codon-mutant library, we performed three transformations to generate approximately six-million independent clones per replicate library. Control ligations lacking an insert yielded at least 100 times fewer colonies, indicating a very low rate of background self-ligation of the pHW2000 plasmid. The transformants from each HA mutant library replicate were pooled, cultured in LB supplemented with ampicillin, and mini-prepped to generate the HA codon mutant plasmid libraries.

For the Sanger sequencing analysis shown in Figure 2, we picked and prepped 34 independent colonies for sequencing. The full analysis of this Sanger sequencing is available at `https://github.com/jbloom/SangerMutantLibraryAnalysis/tree/v0.2`.

Virus rescue and passage in cells

The HA mutant plasmid libraries were used to generate pools of mutant influenza viruses by reverse genetics (Hoffmann et al., 2000). Cocultures of 293T and MDCK-SIAT1 cells were transfected with equal amounts of HA (either unmutated or one of the plasmid mutant libraries) cloned into pHW2000 as described above, plus the seven other WSN genes in bidirectional reverse-genetics plasmids (pHW181-PB2, pHW182-PB1, pHW183-PA, pHW185-NP, pHW186-NA, pHW187-NA, pHW188-NS), which were kind gifts from Robert Webster of St. Jude Children's Research Hospital. Overall, six viral rescues and passages were performed, each using a different HA plasmid preparation: the three HA mutant library replicates (eventu-

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ally yielding the **mutvirus** samples in Figure 1) and three independent unmutated HAs (eventually yielding the **virus** samples in Figure 1).

Each of the viral rescues was performed by transfecting multiple wells of cells in an effort to increase the diversity of the rescued viruses. Specifically, two 12-well dishes were transfected per rescue. Cells were plated at 2×10^5 293T cells and 5×10^4 MDCK-SIAT1 cells per well in D10 (DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml), and then each well was transfected with 1 μg of total plasmid DNA (125 ng of each of the eight plasmids) using the BioT transfection reagent (Bioland B01-02). At 12 to 18 hours post-transfection, the medium was changed to our *WSN viral growth media*: Opti-MEM supplemented with 0.5% heat-inactivated FBS, 0.3% BSA, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 100 μg of calcium chloride/ml. This media does not contain trypsin since viruses with the WSN HA and NA are trypsin independent (Goto and Kawaoka, 1998). Viral supernatants were collected 72 hours post-transfection, and the supernatants from the different wells were pooled for each viral rescue. These pooled supernatants were then clarified by centrifugation at 2000$\times$g for five minutes, aliquoted, and frozen at -80°C. Aliquots were then thawed and titered by TCID50 (see below).

For viral passage, each viral rescue replicate was passaged in four 10-cm dishes. Briefly, 6×10^6 MDCK-SIAT1 cells per 10-cm dish in *WSN viral growth media* were infected with 6×10^5 infectious particles (multiplicity of infection of 0.1). Since there are four dishes for each replicate, this maintains a diversity of 2.4×10^6 TCID50 units per replicate. The passaged viral supernatants were collected at 50 hours post-infection, and the supernatants for the four plates were pooled for each replicate. These pooled supernatants were clarified at 2000$\times$g for five minutes, aliquoted, and frozen at -80°C. Aliquots were then thawed and titered by TCID50.

Virus titering by TCID50

The viruses were titered by TCID50 (50% tissue culture infectious dose). In this assay, 10 μl of a 1:10 dilution of the viral supernatant to be titered was added to the first row of a 96-well tissue culture plate containing 90 μl of *WSN viral growth media*. At least one no-virus control supernatant was included on each plate as a negative control. The virus was then serially diluted 1:10 down the rows of the plates, and then 5×10^3 MDCK-SIAT1 cells were added to each well. The plates were then incubated at 37°C, and scored for cytopathic effects caused by viral growth after for 65-72 hours. Virus titers were calculated by the method of Reed and

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Muench (1938) implemented via the Python script at https://github.com/jbloom/reedmuenchcalculator.

Generation of samples for Illumina deep sequencing

The deep sequencing samples were prepared from PCR amplicons that were generated exactly as described for the **DNA**, **mutDNA**, **virus**, and **mutvirus** samples in Bloom (2014). The viral RNA template for the **virus** and **mutvirus** were isolated using freshly purchased Trizol reagent (Life Technologies; 15596-026) in order to avoid any oxidative damage associated with old reagents. After performing reverse transcription as described in Bloom (2014), quantitative PCR (qPCR) was used to quantify the number of HA cDNA molecules to ensure that there were at least 10^6 unique template molecules before beginning the subsequent PCR amplification. The qPCR primers were designed based on those described by Marsh et al. (2007), and were 5'-taacctgctcgaagacagcc-3' and 5'-agagccatccggtgatgtta-3'.

The PCR amplicons were fragmented and barcoded using the custom modification of Illumina's Nextera kit using the protocol described in Bloom (2014). Samples were barcoded as follows: **DNA** - N701, **mutDNA** - N702, **virus** - N704, and **mutvirus** - N705. For each of the three biological replicates, these four samples were pooled and sequenced on their own Illumina lane with 50-nucleotide paired-end reads as described in Bloom (2014). For the technical sequencing repeat of biological replicate #1, the library preparation and sequencing were repeated from the same viral RNA templates. This technical repeat therefore only quantifies variation associated with sample preparation and sequencing, whereas the biological replicates also quantify variation associated with the processes of codon-mutant library creation, virus generation, and virus passage.

Analysis of deep sequencing data

The deep sequencing data was analyzed using the *mapmuts* computer program (Bloom, 2014). A description of the analysis approach and the resulting data files and figures produced are available at http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html.

Briefly, paired reads were overlapped as illustrated in Figure 3 – figure supplement 1 and then aligned to HA. Reads were retained only if both reads in the pair passed the default Illumina filter, had average Q-scores of at least 25, overlapped for at least 30 nucleotides with no more

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than one mismatch, and the overlap aligned to the HA gene with no more than six mismatches. Figure 3 – figure supplement 2 shows the number of reads for each sample that met these criteria. Most reads that did not meet these criteria failed to do so because they could not be paired with at least 30 nucleotides of overlap – a situation that arises when the HA fragment produced by the Nextera fragmentation produces a fragment smaller than 30 nucleotides or larger than 70 nucleotides. Codon identities were called only if both overlapped paired reads agreed on the identity of the codon. This requirement reduces the error rate, because it is rare for both paired reads to independently experience the same sequencing error.

As shown in Figure 4, we estimated that 85% of possible codon mutations were sampled at least five times by the mutant viruses. To estimate the fraction of amino-acid mutations that would have been sampled, we simulated randomly selecting 85% of the mutant codons from the HA sequence, and determined that these codons encoded $\approx 97\%$ of the amino-acid mutations.

Inference of amino-acid preferences and site entropies

The counts of each codon identity in the deep sequencing data was used to infer the "preference" of each site for each amino acid as described in Bloom (2014). This inference was also done using the *mapmuts* computer program as detailed at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`.

Briefly, the preference $\pi_{r,a}$ of site r for amino-acid a represents the expected frequency of that amino acid in a hypothetical library where each amino-acid is introduced at equal frequency. Specifically, the expected frequency $f_{r,x}^{\textbf{mutvirus}}$ of mutant codon x at site r in the **mutvirus** sample is related to the preference for its encoded amino-acid $\mathcal{A}(x)$ by

$$f_{r,x}^{\textbf{mutvirus}} = \epsilon_{r,x} + \rho_{r,x} + \frac{\mu_{r,x} \times \pi_{r,\mathcal{A}(x)}}{\sum_y \mu_{r,y} \times \pi_{r,\mathcal{A}(y)}},$$

where $\epsilon_{r,x}$ is the rate at which site r is erroneously read to be codon x, $\rho_{r,x}$ is the rate at which site r is erroneously reverse-transcribed to codon x, and $\mu_{r,x}$ is the rate at which site r is mutagenized to codon x in the mutant DNA sample. These unknown error and mutation rate parameters are inferred from the **DNA**, **virus**, and **mutvirus** samples using the Bayesian approach described in Bloom (2014). Inferences of the posterior mean preferences $\pi_{r,a}$ were made separately for each replicate of the experiment, and the correlations among these inferences from different replicates are in Figure 6. The final "best" inferred preferences from the combined data of the

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three biological replicates were obtained by averaging the preferences obtained from the three biological replicates. These final inferred preferences are provided in Supplementary file 3 and displayed graphically in Figure 5.

The site entropies in Figure 9 and Table 3 were calculated from the amino-acid preferences as $h_r = \sum_a \pi_{r,a} \times \log_2 \pi_{r,a}$. These site entropies are therefore in bits. Higher site entropies indicate a higher inherent mutational tolerance.

Alignment of naturally occurring HAs and phylogenetic tree

The inferred amino-acid preferences were compared to amino-acid frequencies in an alignment of naturally occurring H1N1 HAs from swine and human lineages descended from a close relative of the 1918 virus. Briefly, all full-length H1 HAs from these hosts were downloaded from the Influenza Virus Resource (Bao et al., 2008). Up to three sequences per host and year were randomly subsampled and used to build a phylogenetic tree. Clear outliers from the molecular clock (typically lab artifacts or mis-annotated sequences) were iteratively excluded and the trees were rebuilt. The final sequence alignment is in Supplementary file 4. This alignment was used to build the phylogenetic trees in Figure 7 and Figure 7 – figure supplement 1 with *codonPhyML* (Gil et al., 2013) using the codon-substitution model of (Goldman and Yang, 1994) or (Kosiol et al., 2007) with empirical codon frequencies determined using the CF3x4 method (Pond et al., 2010) or the F method, respectively. In both cases, the nonsynonymous-synonymous ratio (ω) was drawn from four gamma-distributed categories (Yang et al., 2000). A description of this process is at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`.

Comparison of evolutionary models

We compared the accuracy with which the naturally occurring HA phylogeny was described by an evolutionary model based on the experimentally measured amino-acid preferences versus several standard codon-substitution models. These comparisons were used made using *HYPHY* (Pond et al., 2005) and *phyloExpCM* (Bloom, 2014). A description of this analysis is at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`.

Briefly, the phylogenetic tree topology was fixed to that shown in Figure 7 or Figure 7 – figure supplement 1. The branch lengths and any free parameters of the evolutionary model were

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then optimized by maximum likelihood. The experimentally determined evolutionary models were constructed from the inferred amino-acid preferences reported here and the experimentally measured mutation rates reported in Bloom (2014). The "fixation probabilities" were computed using either the Metropolis-like relationship described in Bloom (2014) or the relationship proposed by Halpern and Bruno (1998). The results of these comparisons are in Table 2, Table 2 – table supplement 1, Table 2 – table supplement 2, and Table 2 – table supplement 3. All of these comparisons show that the experimentally determined evolutionary models are far superior to the various standard models.

Structural analyses

The WSN HA studied here has a high degree of sequence identity to the HA crystallized in PDB 1RVX (Gamblin et al., 2004). It is this HA structure that is shown Figure 9. The relative solvent accessibilities (RSA) values in Figure 5 and Figure 9 were calculated by first determining the absolute solvent accessibilities of the residues in the full trimeric HA in PDB 1RVX with the DSSP (Joosten et al., 2011) webserver at `http://www.cmbi.ru.nl/hsspsoap/`, and then normalizing by the maximum solvent accessibilities given by Tien et al. (2013).

Classification of antigenic sites and conserved receptor-binding residues

Two sub-classifications of HA residues were performed. Conserved receptor-binding sites were any residues listed in the first table of Martin et al. (1998) that are also conserved in at least 90% of H1 HAs. These residues are listed in Table 1.

Antigenic-site residues are classified based on antigenic mapping of the A/Puerto Rico/8/1934 (H1N1) HA. Specifically, these are any residues listed in the third table of Caton et al. (1982) with the following exceptions: residue 182 (H3 numbering) is not considered for the reason explained on page 421 of Caton et al. (1982), residue 273 (H3 numbering) is not considered for the reason explained on page 422 of Caton et al. (1982), and residue 129 (H3 numbering) is not considered because it has gained a glycosylation site in the WSN HA that is not present in the A/Puerto Rico/8/1934 (H1N1) HA and mutation of this WSN glycosylation site can strongly affect viral growth (Deom et al., 1986). Overall, this gives the following set of antigenic residues, listed by sequential numbering of the HA with the H3 number in parentheses: 171 (158), 173 (160), 175 (162), 176 (163), 178 (165), 179 (166), 180 (167), 169 (156), 172 (159), 205 (192), 206 (193), 209 (196), 211 (198), 182 (169), 186 (173), 220 (207), 253 (240), 153 (140), 156

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(143), 158 (145), 237 (224), 238 (225), 87 (78), 88 (79), 90 (81), 91 (82), 92 (83), and 135 (122).

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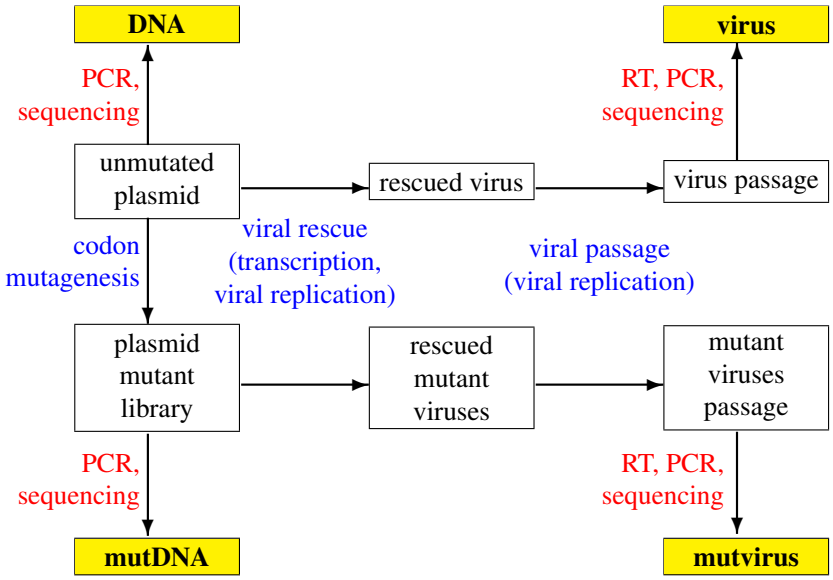

Figure 1: Schematic of the deep mutational scanning experiment. The Illumina deep-sequencing samples are shown in yellow boxes (**DNA**, **mutDNA**, **virus**, **mutvirus**). Experimental steps and associated sources of mutations are shown in blue text, while sources of error during Illumina sample preparation and sequencing are shown in red text. This entire process was performed in biological triplicate.

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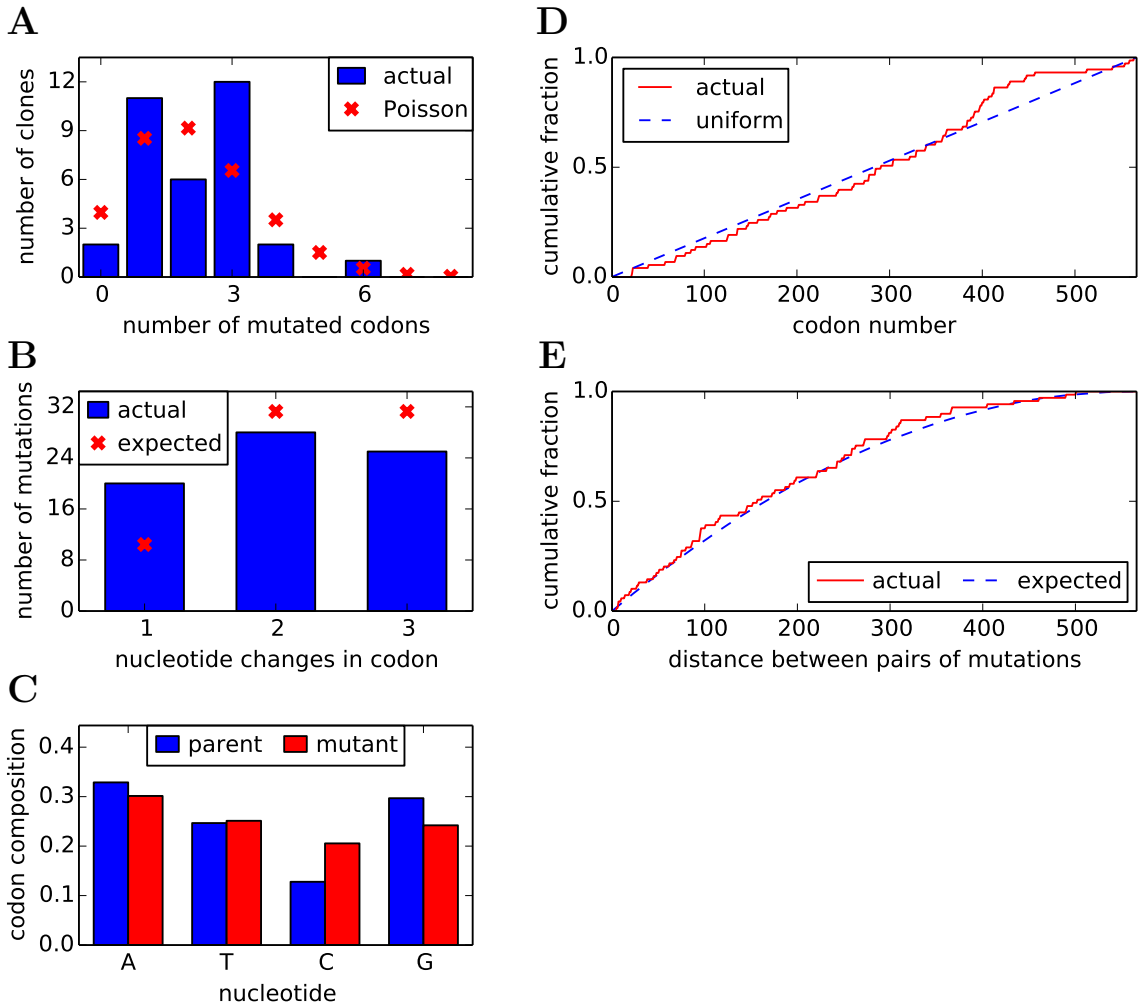

Figure 2: Properties of the HA codon-mutant library as assessed by Sanger sequencing of 34 individual clones drawn roughly evenly from the three experimental replicates **(A)** There are an average of 2.1 codon mutations per clone, with the number per clone following a roughly Poisson distribution. **(B)** The codon mutations involve a mix of one-, two-, and three-nucleotide mutations. **(C)** The nucleotide composition of the mutant codons is roughly uniform. **(D)** The mutations are distributed uniformly along HA's primary sequence. **(E)** There is no tendency for mutations to cluster in primary sequence. Shown is distribution of observed pairwise distances between mutations in multiply mutated clones versus the expected distribution when the mutations are placed independently in the clones. All plots show results only for substitution mutations; insertion / deletion mutations are not shown. However, only two insertion / deletion mutations (0.06 per clone) were identified. The data and computer code used to generate this figure are at `https://github.com/jbloom/SangerMutantLibraryAnalysis/tree/v0.2`.

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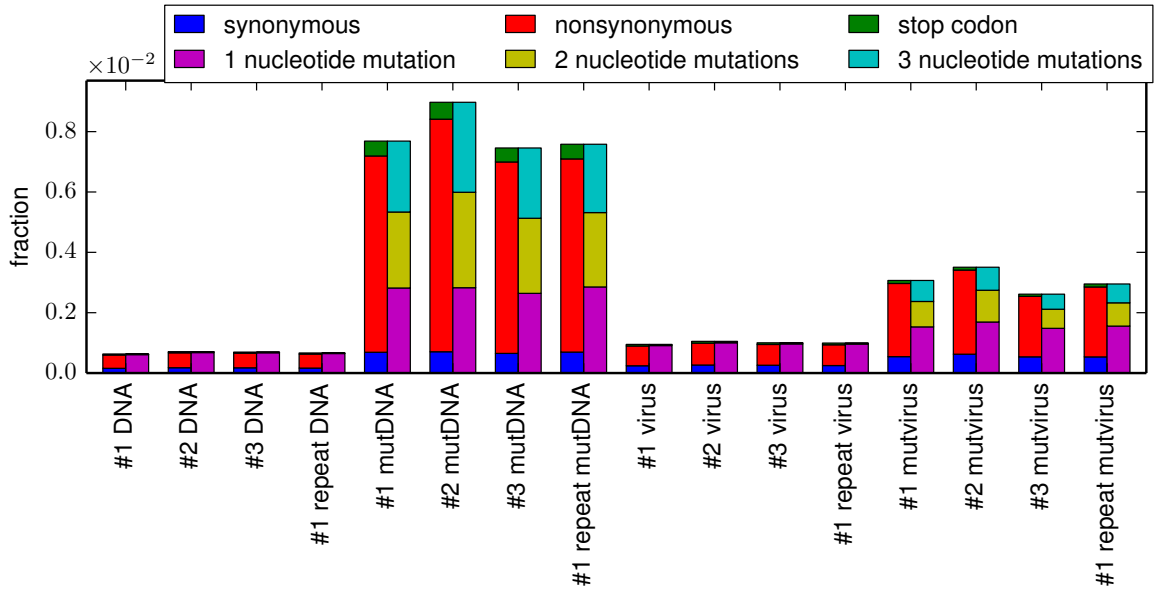

Figure 3: The per-codon frequencies of mutations in the samples. The samples are named as in Figure 1, with the experimental replicate indicated with the numeric label. The **DNA** samples have a low frequency of mutations, and these mutations are composed almost entirely of single-nucleotide codon changes – these samples quantify the baseline error rate from PCR and deep sequencing. The mutation frequency is only slightly elevated in **virus** samples, indicating that viral replication and reverse transcription introduce only a small number of additional mutations. The **mutDNA** samples have a high frequency of single- and multi-nucleotide codon mutations, as expected from the codon mutagenesis procedure. The **mutvirus** samples have a lower mutation frequency, with most of the reduction due to fewer stop-codon and nonsynonymous mutations – consistent with purifying selection purging deleterious mutations. This plot is the file *parsesummary_codon_types_and_nmuts.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`. The sequencing accuracy was increased by using overlapping paired-end reads as illustrated in Figure 3 – figure supplement 1. The overall number of overlapping paired-end reads for each sample is shown in Figure 3 – figure supplement 2. A representative plot of the read depth across the primary sequence is shown in Figure 3 – figure supplement 3.

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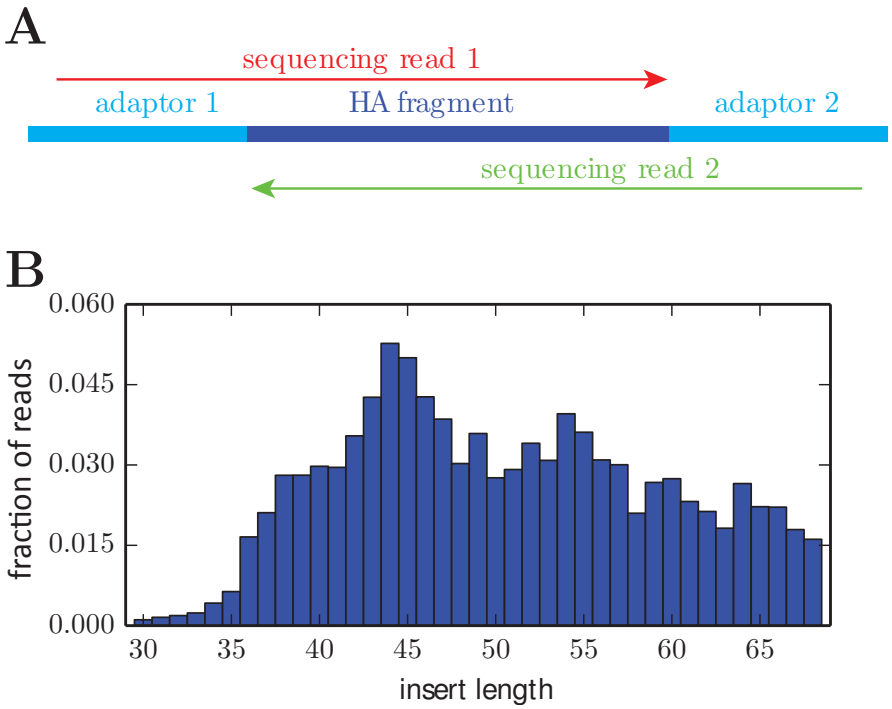

Figure 3 – figure supplement 1: The overlapping paired-end Illumina sequencing strategy. **(A)** Sequencing accuracy was increased by fragmenting the HA gene to pieces roughly 50 nucleotides in length, and then using overlapping paired-end 50 nucleotide Illumina sequencing reads. Codon identities were only called if the reads overlapped and concurred on the codon identity. **(B)** The distribution of actual HA fragment lengths for a representative sample. The plot in **(B)** is the file *replicate_3/DNA/replicate_3_DNA_insertlengths.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`.

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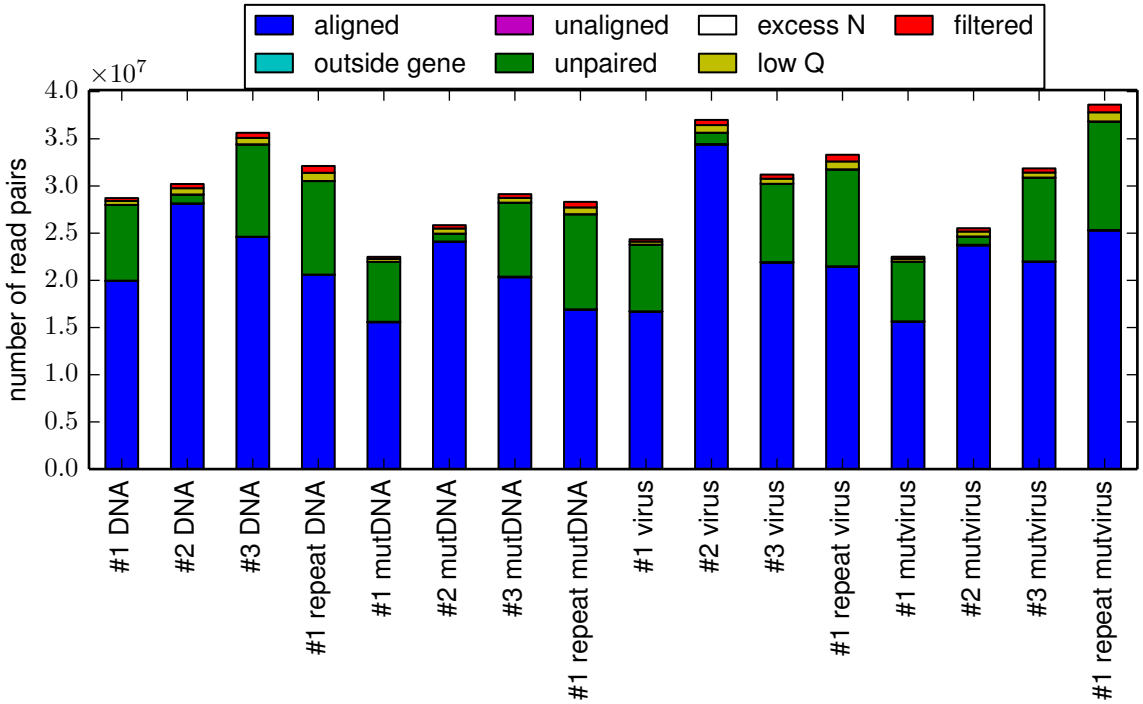

Figure 3 – figure supplement 2: The total number of reads for each sample. For all samples, the majority of reads could be paired and aligned to the HA sequence. However, the exact fraction of reads that could be paired varied somewhat among samples due to variation in the efficiency with which the HA gene was fragmented to the target length of 50 nucleotides. This plot is the file *alignmentsummaryplot.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`.

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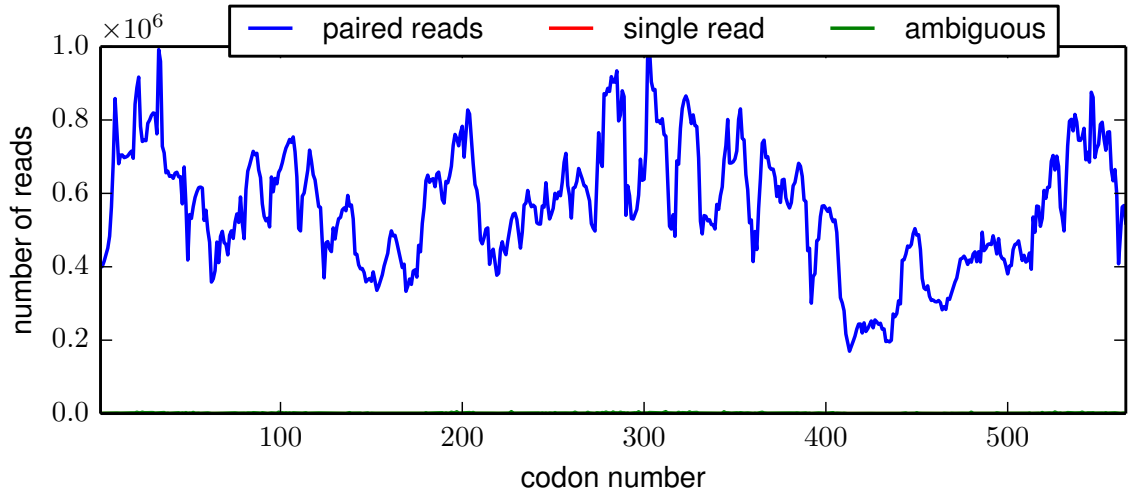

Figure 3 – figure supplement 3: The per-codon read depth as a function of primary sequence. This plot is typical of the samples. The read depth varied fairly consistently as a function of primary sequence, presumably due to biases in the positions at which the HA gene tended to fragment. This plot is the file *replicate_3/DNA/replicate_3_DNA_codondepth.pdf* described at http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html.

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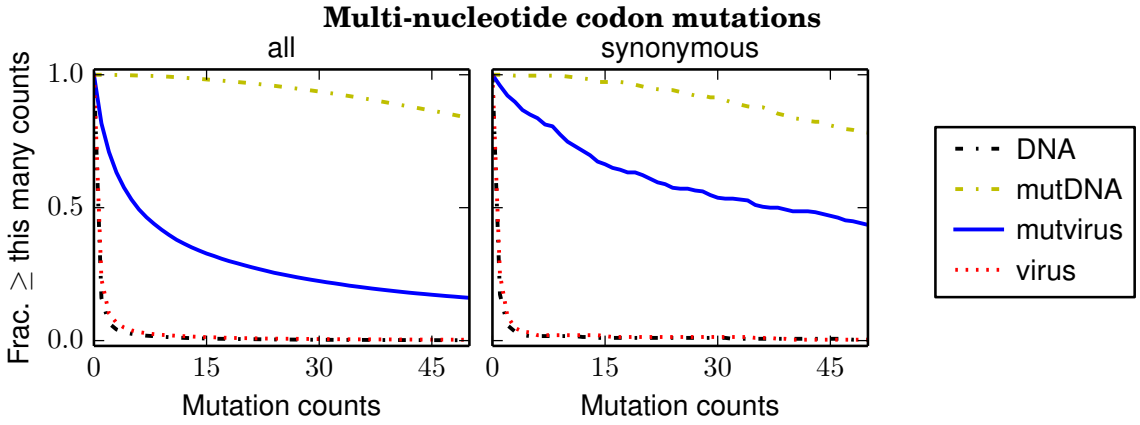

Figure 4: The number of times that each possible multi-nucleotide codon mutation was observed in each sample after combining the data for the three biological replicates. Nearly all mutations were observed many times in the **mutDNA** samples, indicating that the codon mutagenesis was comprehensive. Only about half of the mutations were observed at least five times in the **mutvirus** samples, indicating either a bottleneck during virus generation or purifying selection against many of the mutations. If the analysis is restricted to synonymous multi-nucleotide codon mutations, then about 85% of mutations are observed at least five times in the **mutvirus** samples. Since synonymous mutations are less likely to be eliminated by purifying selection, this latter number provides a lower bound on the fraction of codon mutations that were sampled by the mutant viruses. The redundancy of the genetic code means that the fraction of amino-acid mutations sampled is higher. This plot is the file *countparsedmuts_multi-nt-codonmutcounts.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`. Similar plots for the individual replicates are shown in Figure 4 – figure supplement 1.

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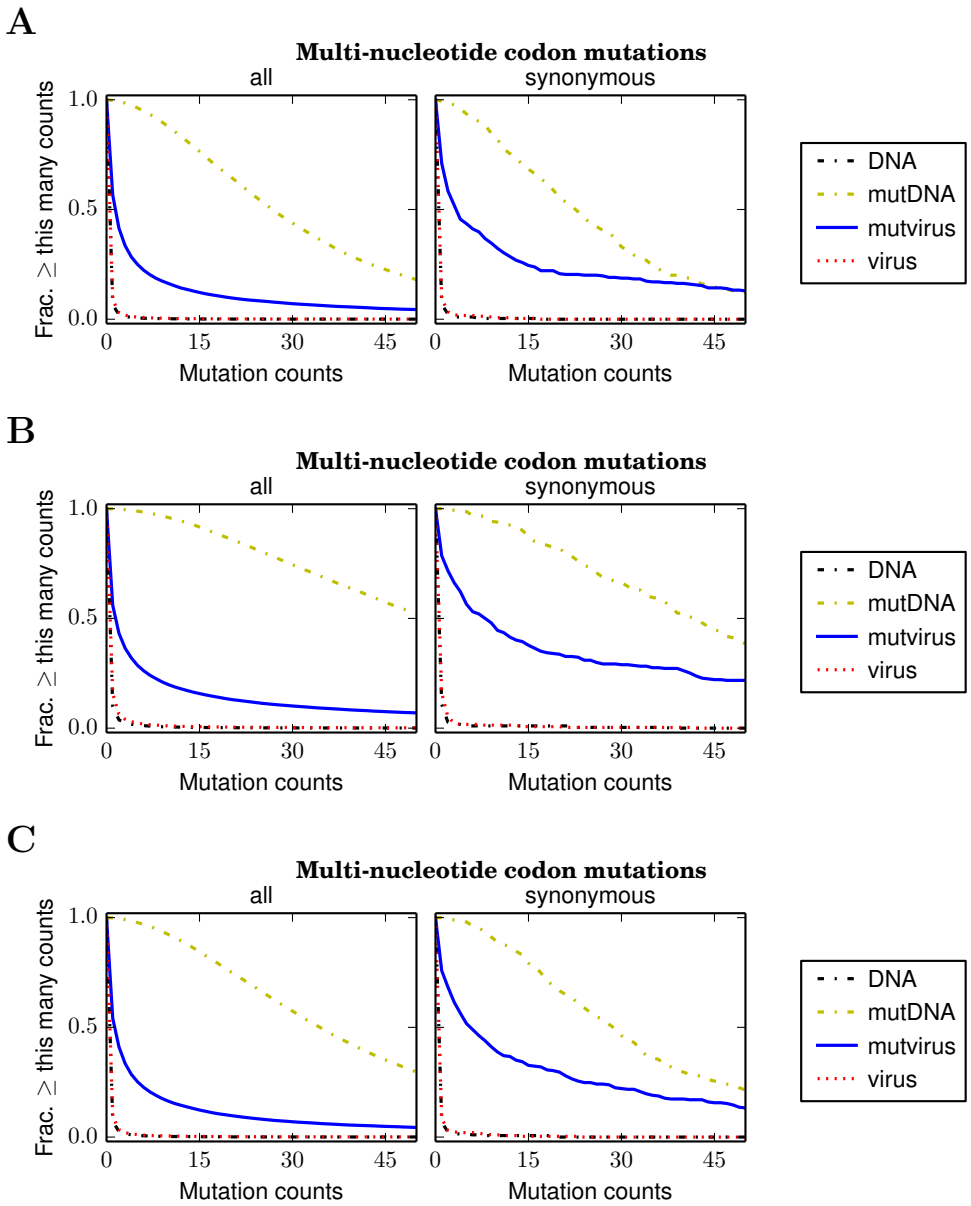

Figure 4 – figure supplement 1: Plots like those in Figure 4 for the individual biological replicates: **(A)** replicate 1, **(B)** replicate 2, and **(C)** replicate 3. These plots are the files *replicate_1/countparsedmuts_multi-nt-codonmutcounts.pdf*, *replicate_2/countparsedmuts_multi-nt-codonmutcounts.pdf*, and *replicate_3/countparsedmuts_multi-nt-codonmutcounts.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`.

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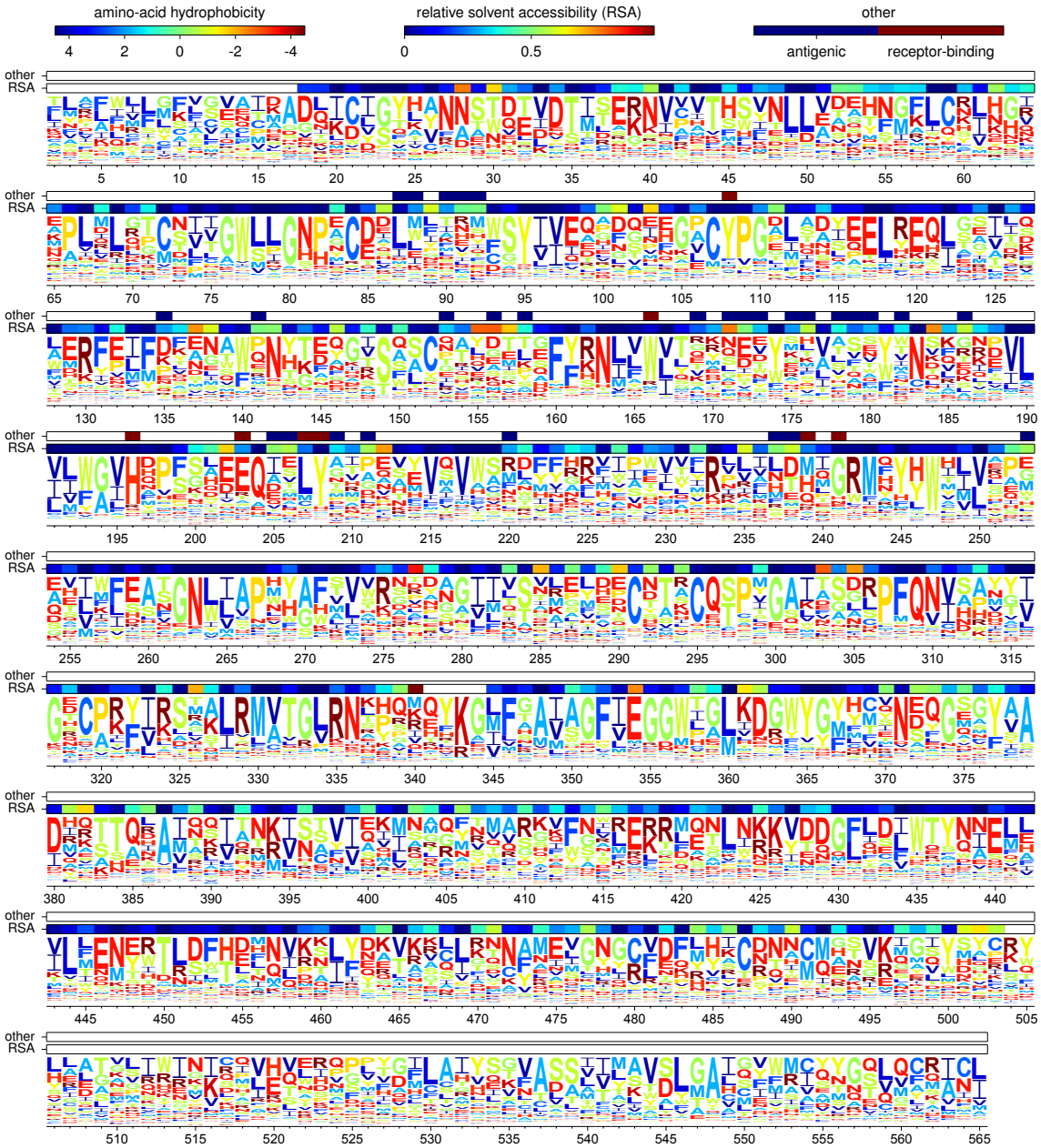

Figure 5: The amino-acid preferences inferred using the combined data from the three biological replicates. The letters have heights proportional to the preference for that amino acid, and are colored by hydrophobicity. The first overlay bar shows the relative solvent accessibility (RSA) for residues in the HA crystal structure. The second overlay bar indicates antigenic sites or conserved receptor-binding residues. The sequence is numbered sequentially beginning with 1 at the N-terminal methionine – however, this first methionine is not shown as it was not mutagenized. Figure 5 – figure supplement 1 shows the same data with H3 numbering of the sequence. This plot is the file *sequentialnumbering_site_preferences_logoplot.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`.

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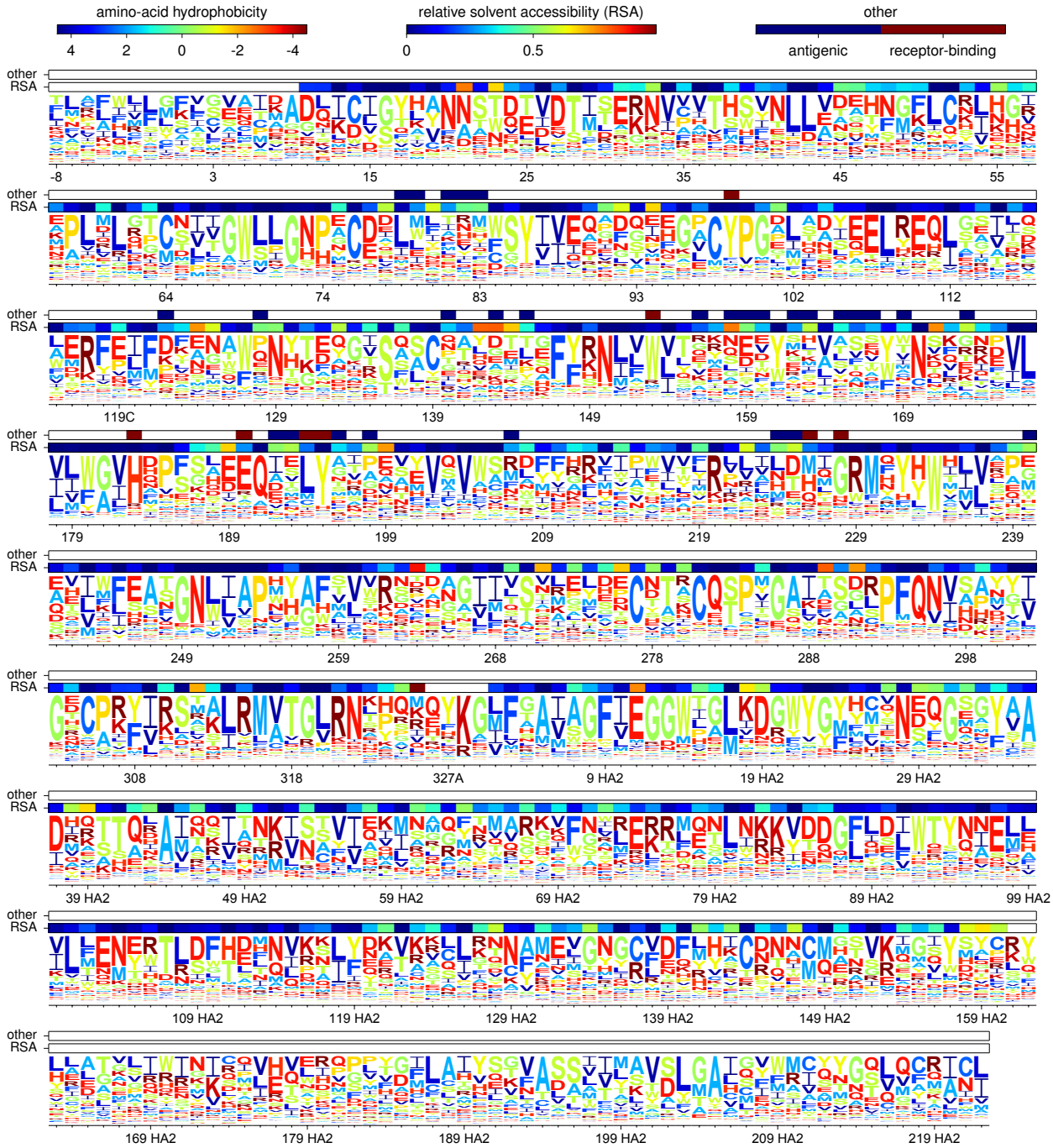

Figure 5 – figure supplement 1: A plot matching that shown in Figure 5 except that the HA sequence is numbered using the H3 numbering scheme. This plot is the file *H3numbering_site_preferences_logoplot.pdf* described at http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html.

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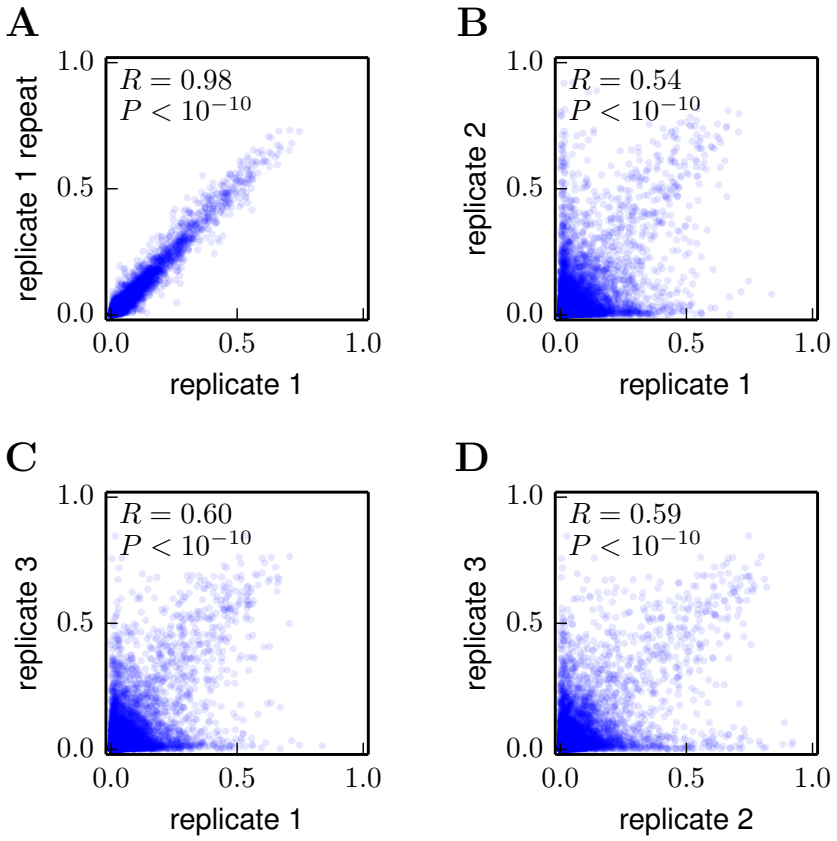

Figure 6: Correlations among the amino-acid preferences inferred using data from the individual biological replicates. **(A)** The preferences from two technical repeats of the sample preparation and deep sequencing of biological replicate #1 are highly correlated. **(B)-(D)** The preferences from the three biological replicates are substantially but imperfectly correlated. Overall, these results indicate that technical variation in sample preparation and sequencing is minimal, but that there is substantial variation between biological replicates due to stochastic differences in which mutant viruses predominate during the initial reverse-genetics step. The Pearson correlation coefficient (R) and associated P-value are shown in the upper-left corner of each plot. These plots are the files *correlations/replicate_1_vs_replicate_1_repeat.pdf*, *correlations/replicate_1_vs_replicate_2.pdf*, *correlations/replicate_1_vs_replicate_3.pdf*, and *correlations/replicate_2_vs_replicate_3.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`.

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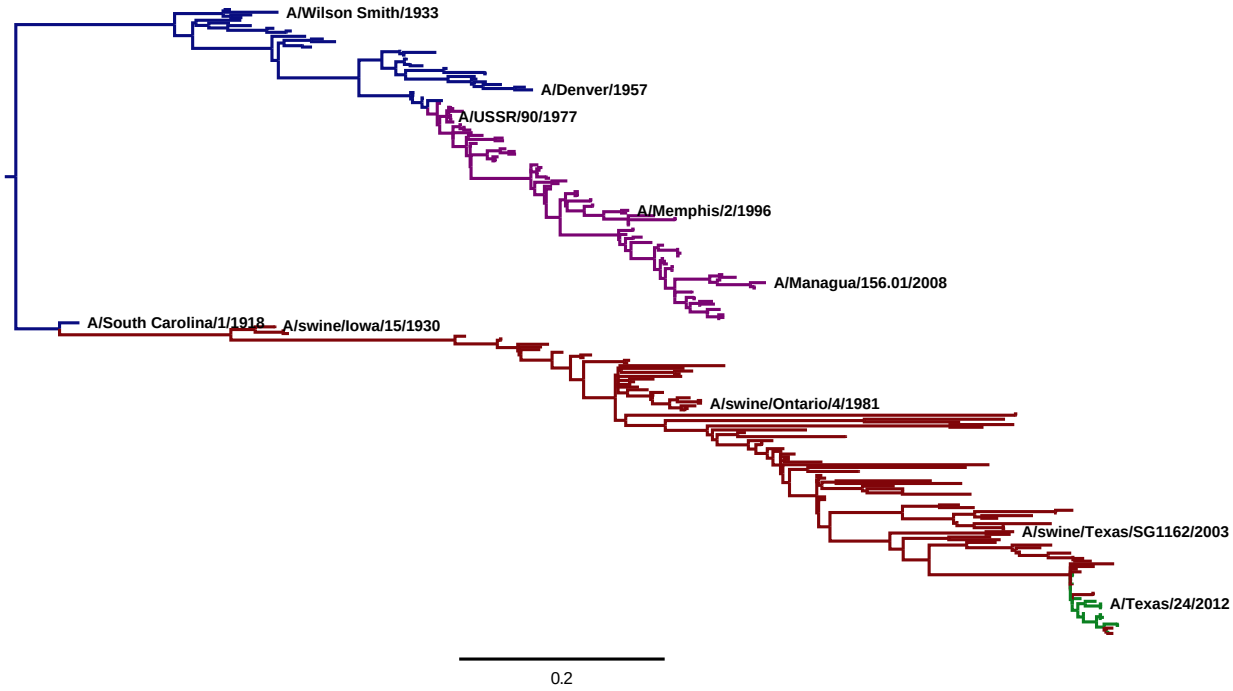

Figure 7: A phylogenetic tree of human and swine H1 HA sequences descended from a common ancestor closely related to the 1918 virus. The WSN virus used in the experiments here is a lab-adapted version of the *A/Wilson Smith/1933* strain. Human H1N1 that circulated from 1918 until 1957 is shown in blue. Human seasonal H1N1 that reappeared in 1977 is shown in purple. Swine H1N1 is shown in red. The 2009 pandemic H1N1 is shown in green. This tree was constructed using *codonPhyML* (Gil et al., 2013) with the substitution model of Goldman and Yang (1994). This plot is the file *CodonPhyML_Tree_H1_HumanSwine_GY94/annotated_tree.pdf* described at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`. Figure 7 – figure supplement 1 shows a tree estimated for the same sequences using the substitution model of Kosiol et al. (2007).

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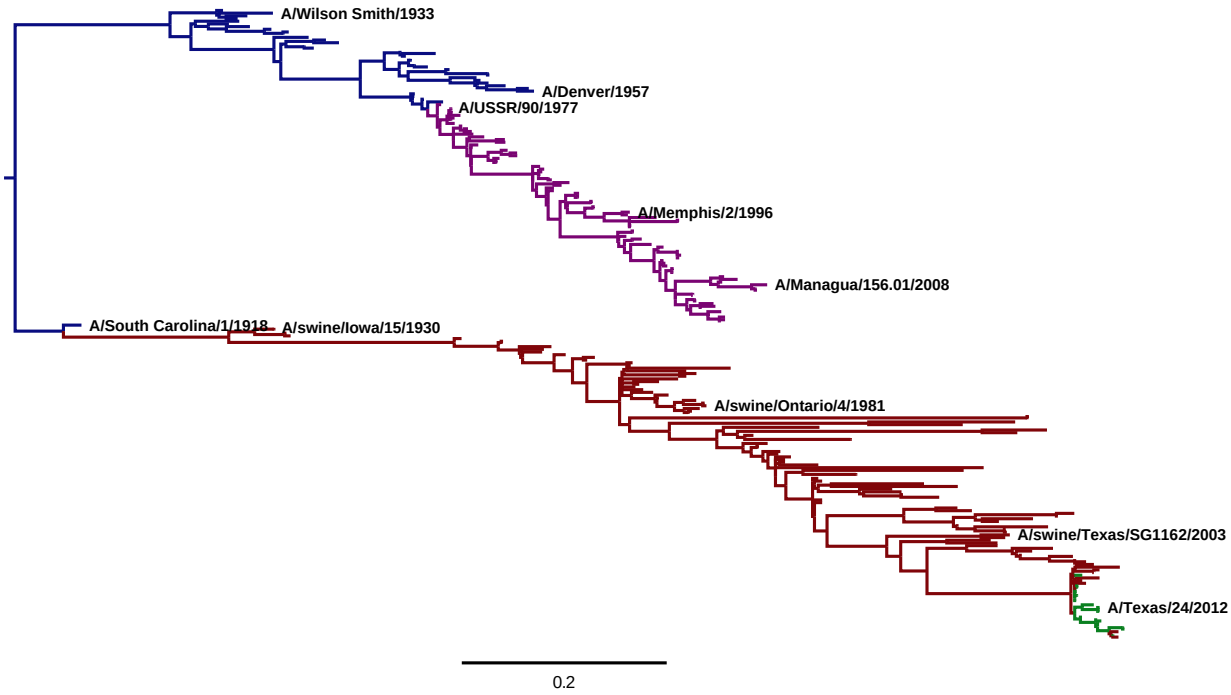

Figure 7 – figure supplement 1: A phylogenetic tree of the same sequences shown in Figure 7, this time inferred using the substitution model of Kosiol et al. (2007). This tree is extremely similar to that in Figure 7, indicating the the inferred topology is robust to the exact choice of codon-substitution model. This plot is the file *CodonPhyML_Tree_H1_HumanSwine_KOSI07/annotated_tree.pdf* described at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`.

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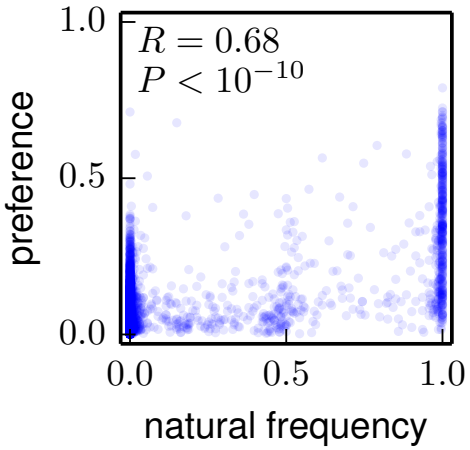

Figure 8: The frequencies of amino acids among the naturally occurring HA sequences in Figure 7 versus the amino-acid preferences inferred from the combined replicate (Figure 5). Note that a natural frequency close to one or zero could indicate absolute selection for or against a specific amino acid, but could also simply result from the fact that natural evolution has not completely sampled all possible mutations compatible with HA structure and function. The Pearson correlation coefficient (R) and associated P-value are shown on the plot. This plot is the file *natural_frequency_vs_preference.pdf* described at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`.

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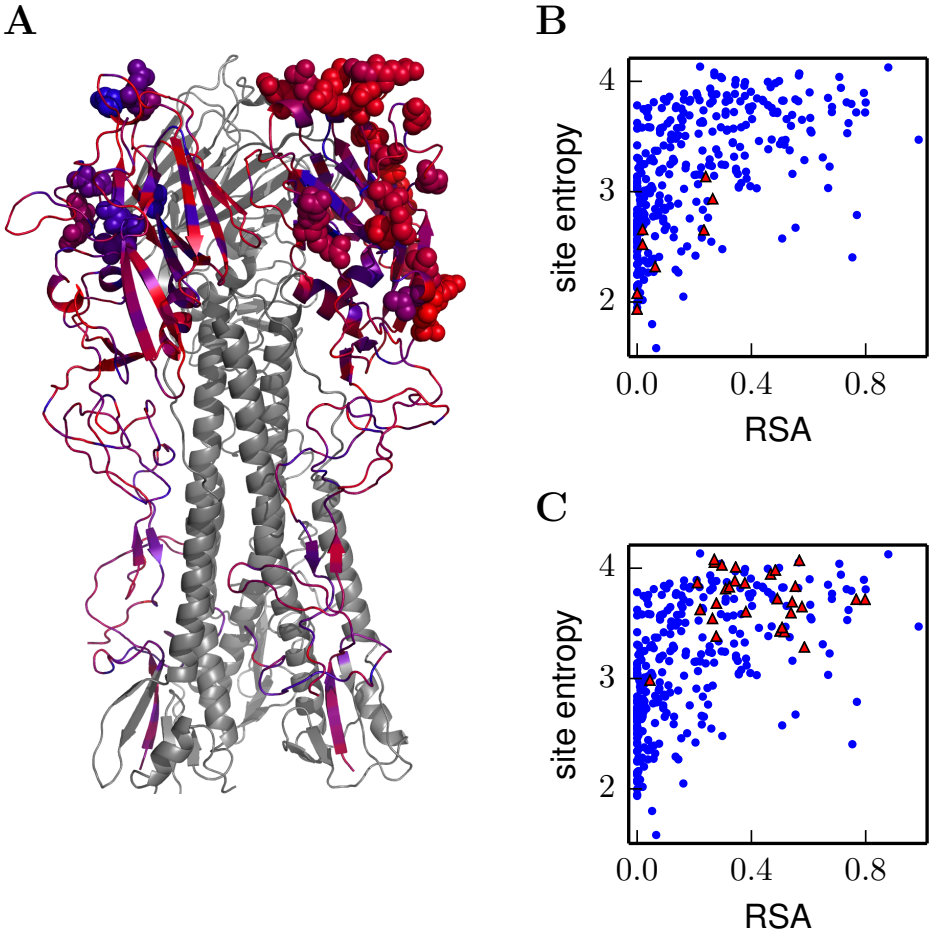

Figure 9: The inherent mutational tolerance of conserved receptor-binding residues and antigenic sites. **(A)** The HA crystal structure with two of the HA1 monomers colored according to site entropy. Blue residues have low site entropy, while red residues have high site entropy. Receptor-binding residues are shown in spheres on the left monomer, while antigenic sites are shown in spheres in the right monomer. **(B)** Site entropy as a function of relative solvent accessibility (RSA) for receptor-binding residues (red triangles) and all other HA1 residues in the crystal structure (blue circles). **(C)** Site entropy as a function of RSA for the antigenic sites (red triangles) and all other HA1 residues in the crystal structure (blue circles). These plots are the files *PDB_structure/entropy_colored_structure.png*, *receptor_binding_entropy_RSA_correlation.pdf*, and *antigenic_entropy_RSA_correlation.pdf* described at `http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html`.

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Site in sequential numbering	Site in H3 numbering	Existing knowledge	Inferred amino-acid preferences
127	117 (HA1)	Mutation from S to P creates a temperature-sensitive defect (Nakajima et al., 1986)	The preference for S is 30 times higher than the preference for P
174	161 (HA1)	Mutation from Y to H creates a temperature-sensitive defect (Nakajima et al., 1986)	The preference for Y is 25 times higher than the preference for H
344	1 (HA2)	Mutation from G to E abolishes HA fusion activity (Qiao et al., 1999)	The preference for G is 11 times higher than for E
343	327 (HA1)	A basic residue (R or K) is required for HA proteolytic activation (Stech et al., 2005)	The combined preferences for R and K (0.87) far exceed those of all other amino acids combined
108	98 (HA1)	Receptor-binding residue, is Y in $> 99\%$ of natural H1 HAs	The preference for Y (0.61) exceeds those of all other amino acids combined
166	153 (HA1)	Receptor-binding residue, is W in $> 99\%$ of natural H1 HAs	The preference for W (0.65) exceeds those of all other amino acids combined
196	183 (HA1)	Receptor-binding residue, is H in $> 99\%$ of natural H1 HAs	The preference for H (0.69) exceeds those of all other amino acids combined
203	190 (HA1)	Receptor-binding residue, is D in 90% of natural H1 HAs	The highest preference is for the chemically similar E
207	194 (HA1)	Receptor-binding residue, is L in 97% of natural H1 HAs	The preference for L (0.55) exceeds those of all other amino acids combined
208	195 (HA1)	Receptor-binding residue, is Y in $> 99\%$ of natural H1 HAs	The preference for Y (0.72) exceeds those of all other amino acids combined
239	226 (HA1)	Receptor-binding residue, is Q in $\approx 99\%$ of natural H1 HAs	Q is one of three amino acids with a high preference
241	228 (HA1)	Receptor-binding residue, is G in $> 99\%$ of natural H1 HAs	The preference for G (0.57) exceeds those of all other amino acids combined

Table 1: The amino-acid preferences inferred from the combined experimental replicates are consistent with existing knowledge about HA structure and function. The conserved receptor-binding residues listed in this table are those delineated in the first table of Martin et al. (1998) that also have at least 90% conservation among all naturally occurring H1 HAs in the Influenza Virus Resource (Bao et al., 2008).

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model	ΔAIC	log likelihood	parameters (optimized + empirical)
combined	0.0	-24088.7	0 (0 + 0)
replicate 3	303.2	-24240.3	0 (0 + 0)
replicate 1	535.4	-24356.4	0 (0 + 0)
replicate 2	876.2	-24526.8	0 (0 + 0)
GY94, gamma ω, gamma rates	882.6	-24517.0	13 (4 + 9)
GY94, gamma ω, one rate	1109.7	-24631.5	12 (3 + 9)
KOSI07 , gamma ω, gamma rates	1620.5	-24834.9	64 (4 + 60)
GY94, one ω, gamma rates	1859.4	-25006.4	12 (3 + 9)
KOSI07 , gamma ω, one rate	1883.0	-24967.2	63 (3 + 60)
KOSI07 , one ω, gamma rates	2378.8	-25215.1	63 (3 + 60)
GY94, one ω, one rate	2544.5	-25350.0	11 (2 + 9)
KOSI07 , one ω, one rate	3040.0	-25546.7	62 (2 + 60)
combined, randomized	5632.8	-26905.1	0 (0 + 0)
replicate 1, randomized	6002.4	-27089.9	0 (0 + 0)
replicate 3, randomized	6138.8	-27158.1	0 (0 + 0)
replicate 2, randomized	6477.8	-27327.6	0 (0 + 0)

Table 2: An evolutionary model derived from the experimentally inferred amino-acid preferences describes the HA sequence phylogeny in Figure 7 far better than a variety of existing state-of-the-art models. The model is most accurate if it utilizes data from the combined experimental replicates, but it also outperforms existing models even if the data are only derived from individual replicates. Models are ranked by AIC (Posada and Buckley, 2004). *GY94* indicates the model of Goldman and Yang (1994), and *KOSI07* indicates the model of Kosiol et al. (2007). The nonsynonymous / synonymous ratio (ω) and the substitution rate are either estimated as a single value or drawn from a four-category gamma distribution. Randomizing the experimentally inferred preferences among sites makes the models far worse. The fixation probabilities are computed from the preferences using the first equation proposed in Bloom (2014). This table shows data from the file *H1_HumanSwine_GY94_summary.tex* described at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`. Table 2 – table supplement 1 shows the results when the fixation probabilities are instead computed using the model of Halpern and Bruno (1998). Table 2 – table supplement 2 shows the results when the tree topology is instead estimated using the substitution model of Kosiol et al. (2007). Table 2 – table supplement 3 shows the results when the fixation probabilities are instead computed using the model of Halpern and Bruno (1998) and the tree topology is instead estimated using the model of Kosiol et al. (2007).

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model	ΔAIC	log likelihood	parameters (optimized + empirical)
combined	500.6	-24339.0	0 (0 + 0)
replicate 3	657.8	-24417.6	0 (0 + 0)
GY94, gamma ω, gamma rates	882.6	-24517.0	13 (4 + 9)
replicate 1	983.2	-24580.3	0 (0 + 0)
GY94, gamma ω, one rate	1109.7	-24631.5	12 (3 + 9)
replicate 2	1190.0	-24683.7	0 (0 + 0)
KOSI07 , gamma ω, gamma rates	1620.5	-24834.9	64 (4 + 60)
GY94, one ω, gamma rates	1859.4	-25006.4	12 (3 + 9)
KOSI07 , gamma ω, one rate	1883.0	-24967.2	63 (3 + 60)
KOSI07 , one ω, gamma rates	2378.8	-25215.1	63 (3 + 60)
GY94, one ω, one rate	2544.5	-25350.0	11 (2 + 9)
KOSI07 , one ω, one rate	3040.0	-25546.7	62 (2 + 60)
combined, randomized	7072.8	-27625.1	0 (0 + 0)
replicate 1, randomized	7795.0	-27986.2	0 (0 + 0)
replicate 3, randomized	7891.8	-28034.6	0 (0 + 0)
replicate 2, randomized	8494.4	-28335.9	0 (0 + 0)

Table 2 – table supplement 1: An evolutionary model derived from the experimentally inferred amino-acid preferences also outperforms existing models when the fixation probabilities are estimated using the equation of Halpern and Bruno (1998). This table shows data from the file *H1_HumanSwine_GY94_summary.tex* described at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`.

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model	ΔAIC	log likelihood	parameters (optimized + empirical)
combined	0.0	-24082.5	0 (0 + 0)
replicate 3	304.8	-24234.9	0 (0 + 0)
replicate 1	534.2	-24349.6	0 (0 + 0)
replicate 2	869.4	-24517.2	0 (0 + 0)
GY94, gamma ω, gamma rates	876.7	-24507.8	13 (4 + 9)
GY94, gamma ω, one rate	1101.0	-24621.0	12 (3 + 9)
KOSI07 , gamma ω, gamma rates	1609.0	-24823.0	64 (4 + 60)
GY94, one ω, gamma rates	1856.2	-24998.6	12 (3 + 9)
KOSI07 , gamma ω, one rate	1867.3	-24953.1	63 (3 + 60)
KOSI07 , one ω, gamma rates	2367.9	-25203.4	63 (3 + 60)
GY94, one ω, one rate	2548.3	-25345.6	11 (2 + 9)
KOSI07 , one ω, one rate	3028.0	-25534.5	62 (2 + 60)
combined, randomized	5628.0	-26896.5	0 (0 + 0)
replicate 1, randomized	5993.6	-27079.3	0 (0 + 0)
replicate 3, randomized	6138.0	-27151.5	0 (0 + 0)
replicate 2, randomized	6475.2	-27320.1	0 (0 + 0)

Table 2 – table supplement 2: An evolutionary model derived from the experimentally inferred amino-acid preferences also outperforms existing models for the tree topology in Figure 7 – figure supplement 1. This table shows data from the file *H1_HumanSwine_KOSI07_summary.tex* described at http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html.

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model	ΔAIC	log likelihood	parameters (optimized + empirical)
combined	494.4	-24329.7	0 (0 + 0)
replicate 3	653.2	-24409.1	0 (0 + 0)
GY94, gamma ω, gamma rates	876.7	-24507.8	13 (4 + 9)
replicate 1	976.8	-24570.9	0 (0 + 0)
GY94, gamma ω, one rate	1101.0	-24621.0	12 (3 + 9)
replicate 2	1180.4	-24672.7	0 (0 + 0)
KOSI07 , gamma ω, gamma rates	1609.0	-24823.0	64 (4 + 60)
GY94, one ω, gamma rates	1856.2	-24998.6	12 (3 + 9)
KOSI07 , gamma ω, one rate	1867.3	-24953.1	63 (3 + 60)
KOSI07 , one ω, gamma rates	2367.9	-25203.4	63 (3 + 60)
GY94, one ω, one rate	2548.3	-25345.6	11 (2 + 9)
KOSI07 , one ω, one rate	3028.0	-25534.5	62 (2 + 60)
combined, randomized	7069.4	-27617.2	0 (0 + 0)
replicate 1, randomized	7786.8	-27975.9	0 (0 + 0)
replicate 3, randomized	7889.2	-28027.1	0 (0 + 0)
replicate 2, randomized	8496.0	-28330.5	0 (0 + 0)

Table 2 – table supplement 3: An evolutionary model derived from the experimentally inferred amino-acid preferences also outperforms existing models for the tree topology in Figure 7 – figure supplement 1 when fixation probabilities are estimated using the equation of Halpern and Bruno (1998). This table shows data from the file *H1_HumanSwine_KOSI07_summary.tex* described at `http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html`.

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Model: **site entropy** $\sim$ **RSA +** $($**receptor binding**$)$ **+ intercept**

Property	Estimate	Standard error	P-value
RSA	1.38	0.11	$< 10^{-10}$
receptor binding	-0.52	0.16	1.7×10^{-3}

Model: **site entropy** $\sim$ **RSA +** $($**antigenic site**$)$ **+ intercept**

Property	Estimate	Standard error	P-value
RSA	1.29	0.12	$< 10^{-10}$
antigenic site	0.29	0.09	1.6×10^{-3}

Table 3: The conserved receptor-binding residue are less mutationally tolerant than other HA1 residues with similar relative solvent accessibility (RSA), while the antigenic sites are more mutationally tolerant than other HA1 residues with similar RSA. The tables show the results of multiple linear regression of the continuous dependent variable of site entropy (as computed from the inferred amino-acid preferences) versus the continuous independent variable of RSA and the binary variable of being a receptor-binding residue or being an antigenic site. Being a receptor-binding residue is significantly associated with lower site entropy, while being an antigenic site is significantly associated with higher site entropy even after including the effect of RSA.

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Supplementary file 1: The coding sequence of the WSN HA gene used in this study is provided in FASTA format.

Supplementary file 2: An Excel file listing the oligonucleotides used for the codon mutagenesis.

Supplementary file 3: The site-specific amino-acid preferences as computed from the averages of the three unique replicates are provided in this supplementary file in text format. This is the file *combined_equilibriumpreferences.txt* described at http://jbloom.github.io/mapmuts/example_WSN_HA_2014Analysis.html.

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Supplementary file 4: The alignment of human and swine HA sequences used to build the phylogenetic trees are provided in this supplementary file in FASTA format. This is the file *H1_HumanSwine_alignment.fasta* described at http://jbloom.github.io/phyloExpCM/example_2014Analysis_Influenza_H1_HA.html.