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Journal Title: Nature biotechnology

Volume: 28 **Issue:** 7
Month/Year: July 2010 **Pages:** 723 - 726

Article Author:

Article Title: Steffen Mueller, J Robert Coleman, Dimitris Papamichail, Charles B Ward, Anjaruwee Nimnual, Bruce Fu; Live attenuated influenza virus vaccines by comp

Imprint: New York ; Nature America, Inc., c1996-

ILL Number: 71011756

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Live attenuated influenza virus vaccines by computer-aided rational design

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Despite existing vaccines and enormous efforts in biomedical research, influenza annually claims 250,000–500,000 lives worldwide[1], motivating the search for new, more effective vaccines that can be rapidly designed and easily produced. We applied the previously described synthetic attenuated virus engineering (SAVE)[2] approach to influenza virus strain A/PR/8/34 to rationally design live attenuated influenza virus vaccine candidates through genome-scale changes in codon-pair bias. As attenuation is based on many hundreds of nucleotide changes across the viral genome, reversion of the attenuated variant to a virulent form is unlikely. Immunization of mice by a single intranasal exposure to codon pair–deoptimized virus conferred protection against subsequent challenge with wild-type (WT) influenza virus. The method can be applied rapidly to any emerging influenza virus in its entirety, an advantage that is especially relevant when dealing with seasonal epidemics and pandemic threats, such as H5N1- or 2009-H1N1 influenza.

Influenza viruses are negative-stranded, enveloped orthomyxoviruses with eight gene segments, each encoding one or two proteins[3]. The signature antigenicity of the A and B types of influenza viruses is determined by the viral glycoproteins hemagglutinin (HA) and neuraminidase (NA). The annual genetic drift in antigenicity, which is driven by point mutations, is responsible for seasonal influenza epidemics[1,3,4]. Swapping of gene segments by reassortment between viruses of aquatic birds, swine and humans (genetic shift) produces new type A influenza viruses with novel antigenicity that may cause devastating pandemics[1,3,4].

The capacity of influenza viruses for immune escape requires that vaccine strains be updated annually to reflect changes in the HA and NA genes within the impending seasonal strains. Two types of vaccines are currently used: a chemically inactivated virus delivered by injection, and a live attenuated influenza virus vaccine of cold-adapted virus[5], delivered as a nasal-spray (FluMist) (http://www.cdc.gov/flu/protect/keyfacts.htm). Both vaccines have limitations. Whereas cell-mediated responses are increasingly recognized as a major determinant of influenza immunity[6–9], traditional, killed influenza virus vaccines act mainly by inducing neutralizing antibodies. Unfortunately the killed vaccine appears to have suboptimal efficacy in the elderly population (>65 years old)[10], which is the same population most prone to morbidity and mortality from seasonal influenza epidemics. In contrast, live attenuated influenza virus vaccine induces both humoral and cellular immunity but its administration remains restricted to healthy children, adolescents and adults (nonpregnant females), ages 2–49. It works better in immunologically naive young children than in adults[11,12].

Here we illustrate the use of SAVE[2] to rationally design live attenuated influenza virus vaccines.

The central idea of SAVE is to recode and synthesize a viral genome[13] in a way that perfectly preserves the WT amino acid sequence, while rearranging existing synonymous codons to create a suboptimal arrangement of pairs of codons[2]. For reasons that are not understood, some pairs of codons occur more frequently, and others less frequently, than expected[14]. This codon-pair bias, which is found in every species examined[15], evolves slowly. Yeast and humans have a radically different codon-pair bias, but all mammals share essentially the same codon-pair bias (unpublished results).

Codon-pair bias is independent of codon bias. For example, consider the amino acid pair Arg-Glu. As there are six codons for arginine and two for glutamic acid, there are twelve possible codon combinations that encode this pair of amino acids. Taking into account the frequency of the two contributing codons (codon bias), the pair CGC-GAA is expected 2,397 times in the annotated human ORFeome. However, it is instead observed only 268 times (observed/expected = 0.11). This is an infrequently used codon pair. In contrast, the Arg-Glu pair encoded by AGA-GAA is expected 2,644 times, but is observed 4,195 times (observed/expected = 1.59); this is a frequently used codon pair. By whole genome synthesis[13,16] we previously recoded poliovirus to contain 'poor' (that is, infrequently used) codon pairs, and found that this dramatically attenuated the virus[2]. Although the mechanism of attenuation is unclear, preliminary evidence suggests that translation is affected[2]. Attenuation can be 'titrated' by adjusting the extent of codon-pair deoptimization[2]. Because codon-pair deoptimization results from miniscule effects at each of hundreds or thousands of nucleotide

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Received 12 March; accepted 21 April; published online 13 June 2010; doi:10.1038/nbt.1636

Table 1 Characteristics of deoptimized influenza genome segments

Gene segment	Deoptimized coding region[a]	CPB of WT segment[b]	CPB of deoptimized segment[c]	Number of silent mutations
NP^{Min}	125–1426	0.012	−0.421	314
$PB1^{Min}$	519–1494	0.007	−0.386	236
HA^{Min}	157–1654	0.019	−0.420	353

[a]Nucleotide position within the genome segment that underwent the codon pair–deoptimization algorithm. [b]Original codon-pair bias (CPB) of the corresponding WT sequence. [c]CPB of the synthetic, codon pair–deoptimized gene segment.

mutations (without changing amino acid sequences), reversion to virulence is extremely unlikely[2]. Aided by computer algorithms[2], codon pair–deoptimized viral genomes can be rapidly designed and synthesized, and live virus can be generated by reverse genetics.

To attenuate influenza virus, we redesigned large parts of the coding regions of the polymerase subunit B1 (PB1), nucleoprotein (NP) and HA genes of influenza virus A/PR/8/34 (PR8), using our deoptimization computer program[2]. Along with other viral genes, these genes play important roles in replication and assembly of influenza virus. Without altering either amino acid sequence or codon bias, the program rearranged existing synonymous codons to deoptimize codon pairs. This resulted in hundreds of silent mutations per genome segment without any amino acid changes. The characteristics of the synthetic genome segments and their changes in codon-pair bias are summarized in **Table 1** and **Supplementary Figure 1**.

The deoptimized segments were synthesized *de novo* and cloned into a standard ambisense, eight-plasmid system[17,18]. To generate influenza viruses carrying one or more deoptimized segments, the plasmids carrying the recoded, synthetic segments, together with the complement of the remaining PR8 WT plasmids, were transfected into susceptible cells. Each deoptimized segment $PB1^{Min}$, NP^{Min} and HA^{Min} in the background of the complementing seven WT segments yielded a viable virus, as did any combination thereof, including the virus simultaneously containing all three deoptimized segments (PR8-$PB1^{Min}$/NP^{Min}/HA^{Min}, abbreviated $PR8^{3F}$) (**Fig. 1** and data not shown).

Several of these new synthetic viruses were analyzed for their *in vitro* growth characteristics in Madin-Darby canine kidney epithelial (MDCK) cells. All mutant viruses formed plaques that were either indistinguishable from, or only slightly smaller than that of the WT virus (**Fig. 1a**). The mutant viruses did not grow as well as WT, but typically only to about tenfold lower titers (**Fig. 1b**). The plaque and growth properties of viruses carrying combinations of synthetic segments other than those depicted in **Figure 1** fall in between those of PR8 and $PR8^{3F}$ (data not shown). The deoptimized virus $PR8^{3F}$ had about the same response to changes in temperature (to either 33 °C or 39.5 °C) as the WT virus (**Supplementary Fig. 2**).

Previously, we found that codon- or codon pair–deoptimized polioviruses had a reduced specific infectivity[2,19]. This was not the case for deoptimized influenza viruses, as their ratio of plaque-forming units (PFU) to HA units was nearly identical to that of WT virus (data not shown).

Our working hypothesis postulates that deoptimization of viral open reading frames reduces protein synthesis, which in turn generates an attenuated virus[2]. Therefore we used western blot analysis of infected whole-cell lysates to test protein synthesis driven from the deoptimized gene segments in PR8-NP^{Min}, PR8-HA^{Min} and PR8-$PB1^{Min}$ (**Fig. 1c**). In all three viruses, the deoptimized viral gene product was specifically reduced compared to other proteins from the same virus, or to the same protein in WT-infected cells, or to the actin control in the same cells (**Fig. 1c**). Protein synthesis from the WT segments in a deoptimized virus was apparently not affected. The exact molecular mechanisms responsible for the reduced protein production remain to be determined.

Despite their reasonably robust growth, codon pair–deoptimized influenza viruses proved to be remarkably attenuated in mice (**Table 2**). Each individual deoptimized segment had a demonstrable attenuating effect, reducing the median lethal dose (the dose killing half the animals; LD_{50}) ~10-, 30- and 500-fold, for PR8-NP^{Min}, PR8-HA^{Min}, and PR8-$PB1^{Min}$, respectively. Combinations of two deoptimized genes have not been tested, but combining all three attenuating genes into one virus ($PR8^{3F}$) led to a cumulative attenuation of about 13,000-fold (**Table 2**).

To test the codon pair–deoptimized viruses in animals, BALB/c mice were infected intranasally with 10^4 PFU of $PR8^{3F}$ or PR8, and monitored for disease symptoms (ruffled fur, lethargy, weight loss, death). At this dose, mice infected with PR8 WT virus developed severe symptoms with rapid weight loss and did not survive more than 5 d after infection (**Fig. 2a**). Mice infected with $PR8^{3F}$, on the other hand, experienced no observable symptoms or weight loss, except for a small, transient delay in weight gain as compared to mock-infected animals (**Fig. 2a**).

Live attenuated vaccines in general depend on a limited, yet safe, degree of replication within the host to stimulate the immune system. To assess the replicative potential of our influenza vaccine candidate in an immune competent host, we monitored viral load in the lungs of BALB/c mice infected intranasally with either 10^3 PFU of $PR8^{3F}$ or PR8 WT virus. Within 24 h, WT-infected mice had 3,000-fold higher viral load in their lungs compared to $PR8^{3F}$, leading to death in less than 6 d (**Fig. 2b**). Conversely, in $PR8^{3F}$-infected animals, amplification of the vaccine virus progressed slowly and peaked at a lower viral load than the WT virus, resulting in a controlled infection with no overt disease symptoms, and virus clearance after 9 d (**Fig. 2b**).

Infection by a sub-lethal dose of WT virus can in principle provoke protective immunity, which is the usual course of natural human

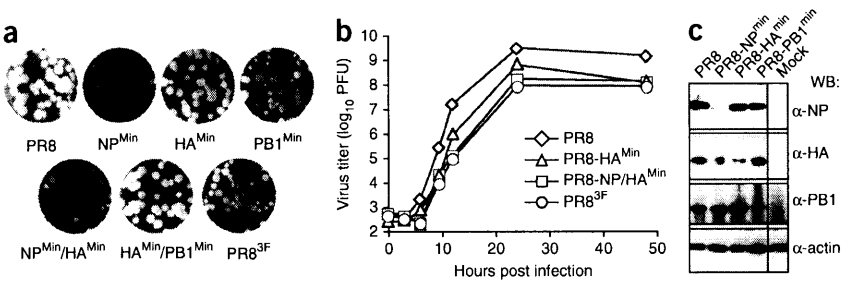

Figure 1 Characterization in tissue culture of synthetic codon pair–deoptimized influenza viruses. (**a**) Plaque phenotypes on MDCK cells of PR8 WT virus and synthetic PR8 derivatives, carrying one (NP^{Min}, HA^{Min} or $PB1^{Min}$), two (NP^{Min}/HA^{Min} or HA^{Min}/$PB1^{Min}$) or three ($PR8^{3F}$) deoptimized gene segments. (**b**) Growth kinetics of PR8 WT virus and three synthetic PR8 derivatives in MDCK cells after infection with 0.001 MOI of the indicated viruses. All combinations grew within log 8–9 although only PR8-HA^{Min}, PR8-NP/HA^{Min} and $PR8^{3F}$ are shown as compared to WT. (**c**) Analysis of influenza virus protein expression in infected cells. MDCK cells were infected with PR8 WT virus or synthetic PR8 derivatives, carrying one deoptimized gene segment each (NP^{Min}, HA^{Min} or $PB1^{Min}$), as indicated. Western blot (WB) analysis of proteins extracted from whole cell lysates was carried out with PB1, NP, HA or actin antibodies. Actin was used to indicate equal protein loading.

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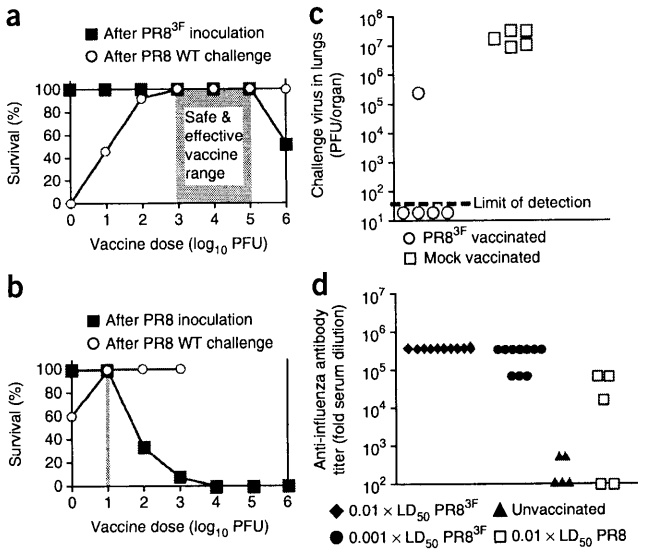
Table 2 LD_{50} and PD_{50} of deoptimized influenza viruses

Virus	LD_{50} (PFU)[a]	PD_{50} (PFU)[b]
PR8 (WT)	6.1×10^1	$\sim 1.0 \times 10^0$ [c]
PR8-NP^{Min}	5.0×10^2	n.d.[d]
PR8-$PB1^{Min}$	3.2×10^4	n.d.
PR8-HA^{Min}	1.7×10^3	n.d.
PR8-$NP^{Min}/HA^{Min}/PB1^{Min}$ ($PR8^{3F}$)	7.9×10^5	1.3×10^1

[a]The dose required to result in lethal disease in 50% of inoculated mice[24]. [b]The dose of vaccine required to protect 50% of mice with a single vaccination from a challenge infection with 1,000 LD_{50} of the PR8 WT virus on day 28 post vaccination. [c]At the lowest level of inoculum tested (1.0×10^0 PFU), 60% of mice were protected. [d]Not determined.

infections. The Chinese scholar Li Shizhen described the art of inoculating humans with live smallpox[20]. This method of smallpox vaccination, practiced in China for centuries, was very dangerous because the difference between the lethal dose and the immunizing protective dose of WT smallpox is very small. To address the issue of safety margin quantitatively with our influenza viruses, we determined the LD_{50} and the protective dose 50 (the dose providing protective immunity to half the animals; PD_{50}) for PR8 WT virus and also for the attenuated strain, $PR8^{3F}$ (**Fig. 3**). PR8 had a very low PD_{50} of 1 PFU, which is equivalent to ~40 virus particles when titered on MDCK cells[21]. The LD_{50} of PR8 was 61 PFU, resulting in an LD_{50}/PD_{50} ratio of about 60. This ratio between the LD_{50} dose and the PD_{50} dose is the 'safety margin' of a given virus if it were to be used as a vaccine. The narrow safety margin of WT ($LD_{50}/PD_{50} = 60$) compromises its suitability for use as a vaccine. In contrast, the attenuated virus $PR8^{3F}$ had a PD_{50} of 13 PFU, which although higher than the PD_{50} of WT, is still very low. The attenuated $PR8^{3F}$ had an LD_{50} of 790,000 PFU and, thus, an LD_{50}/PD_{50} ratio (safety margin) of 60,000, which is 1,000-fold better than the WT virus (**Fig. 3a** versus **Fig. 3b**, shaded areas under the curve). Thus, it is easy to determine a dose of the attenuated virus $PR8^{3F}$ that is both safe to administer and effective in provoking immunity (**Supplementary Fig. 3**).

To characterize the protective immunity in more detail, we immunized mice with a single intranasal vaccination of 10^4 PFU of $PR8^{3F}$. After 28 d, these mice were challenged with 1,000× LD_{50} of WT PR8. Three days after challenge, PR8 titers in lung homogenates were determined. In 80% of the mice, challenge virus was below the level of detection (suppressed at least 10^6-fold) (**Fig. 3c**; open circles), whereas titers of ~10^7 PFU were found in lungs of mock-vaccinated animals (**Fig. 3c**; open squares).



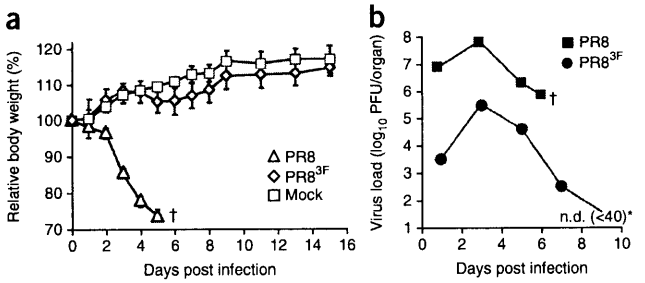


Figure 2 Attenuation of codon pair–deoptimized influenza virus $PR8^{3F}$ in BALB/c mice. (**a**) Body weight curve after intranasal infection with 10^4 PFU of PR8 WT virus (triangles), 10^4 PFU of deoptimized $PR8^{3F}$ virus (diamonds) or mock-infected (saline; squares). Each time point indicates the average of five mice, with error bars indicating s.d. WT-infected mice did not survive beyond day 5 (indicated by a cross). (**b**) Virus titer in whole lung homogenate after infection with either 10^3 PFU of PR8 WT virus (squares) or deoptimized $PR8^{3F}$ (circles). Average of three mice per time point. *On day 9 post infection, $PR8^{3F}$ was no longer detectable (below 40 PFU/lung).

The mean anti-influenza serum IgG titer in mice immunized with 0.01× LD_{50} of the respective viruses was 312,500 for $PR8^{3F}$ and 27,540 for PR8 (**Fig. 3d**). At an even lower, and thus safer, vaccine dose of 0.001× LD_{50}, the immune response toward $PR8^{3F}$ was nearly unchanged with an antibody titer of 237,500 (**Fig. 3d**). Thus, at identical doses relative to their respective LD_{50}, $PR8^{3F}$ is a more potent inducer of influenza-specific antibodies than WT. These findings attest to the strong immunizing potential of a low-grade influenza virus infection in general, and to the safety profile of codon pair–deoptimized influenza viruses in particular.

Vaccines created in this way encode all viral proteins with the amino acid sequences found in WT viruses. Thus the viruses express the entire WT repertoire of antigenic sites and would have the maximum chance of inducing both cellular and humoral immunity against all epitopes. As attenuation results from hundreds or thousands of nucleotide changes, the probability of reversion to virulence is extremely low, an advantage over other approaches to making live vaccines. Co-infection of the vaccine recipient with a naturally circulating WT virus could lead to gene reassortment, but this is unlikely to produce variants more virulent than the co-infecting virus. The deoptimized segments of the vaccine strain would 'poison' any such reassortant.

Figure 3 Immune responses and protection. (**a**,**b**) Vaccine margin of safety for PR8 WT and deoptimized $PR8^{3F}$ viruses. The left ordinate indicates the percentage of animals surviving the primary inoculation (black squares) with (**a**) $PR8^{3F}$ or (**b**) WT PR8, at doses ranging between 10^0 to 10^6 PFU. After 28 d, the surviving, vaccinated animals were challenged with a single 1,000× LD_{50} of PR8 WT virus. Disease and survival were monitored (right ordinate; open circles) for (**a**) $PR8^{3F}$- and (**b**) PR8-vaccinated mice. (**c**) Virus load in mouse lungs after PR8 WT virus challenge of $PR8^{3F}$-vaccinated animals. Twenty-eight days after a single intranasal vaccination with 10^4 PFU $PR8^{3F}$ mice were challenged with 1,000 × LD_{50} of PR8 WT virus. Three days thereafter the level of challenge virus in lung homogenates was determined. (**d**) ELISA determination of influenza-specific serum antibodies. Twenty-eight days after a primary infection, serum was collected, and anti-influenza IgG serum titers were determined from animals that had received a primary inoculation of 0.01× LD_{50} (black diamonds) or 0.001× LD_{50} of $PR8^{3F}$ (black circles), 0.01× LD_{50} of PR8 (white squares) or saline (black triangles). ELISA antibody titer against PR8 virus antigen is expressed as the lowest reciprocal serum dilution that resulted in a positive ELISA signal (5 s.d. above background). In **c** and **d** each symbol represents the data from one animal.

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In spite of striking genetic dissimilarities, codon-pair deoptimization has given similar results with both influenza virus and poliovirus[2]. Poliovirus is a plus-stranded RNA virus whose genome encodes a single polyprotein. Codon-pair deoptimization in the 5′-region of the poliovirus genome disturbs the synthesis of all viral proteins with drastic effects on replication[2]. In contrast, influenza virus, a minus-stranded RNA virus, has a genome divided into eight segments and its proteins are expressed independently from individual mRNAs. The fact that two such extremely different viruses each are highly sensitive to codon pair deoptimization suggests that the strategy may operate quite generally.

As with any new technology, there are issues to resolve before applying this approach for human use. These include our incomplete knowledge of the molecular mechanism(s) involved in attenuation, an in-depth assessment of the genetic stability of the attenuation phenotype and a need to find the right balance between attenuation (for provoking an immune response) and robust replication (for purposes of production). For seasonal epidemics, SAVE may allow a different strategy than the existing live vaccines, such as FluMist. For FluMist, the attenuating mutations (obtained by lengthy selection procedures) map to six viral 'backbone' genes, which therefore must be kept constant every year, with only HA and NA being updated annually (6:2 recombinants). Individuals immunized yearly could develop immunity against 'backbone' proteins, limiting the replication and thus the efficacy of the vaccine (possibly helping to explain the relatively poor efficacy of FluMist in previously immunized army personnel[22]). In contrast, use of SAVE could attenuate the entire genome of an impending seasonal or pandemic strain, providing a perfect antigenic match between the vaccine and target virus. However, this approach would require an appropriate regulatory environment that might, for instance, approve a method of attenuating each new strain to a certain standardized degree with a standardized change in codon-pair bias (just as the current inactivated virus vaccine uses a standardized method of inactivation). To attenuate in a standardized and predictable way will require many more experiments to exhaustively explore the variables of SAVE.

The recoded influenza viruses described here present a useful paradigm for vaccines, as attenuation is the result of hundreds or thousands of nucleotide changes without the change of a single amino acid. The attenuated phenotype results from large-scale rearrangements of existing synonymous codons, producing under-represented pairs of codons[2]. Considering (i) the expected high genetic stability of the attenuating genetic changes ("attenuation by a thousand cuts"[23]), (ii) the possibility of systematically designing such viruses, (iii) the favorable growth kinetics in tissue culture (10^8 PFU/ml), (iv) the small protective dose of 'deoptimized' influenza viruses, (v) the efficacy of $PR8^{3F}$ as revealed in challenge experiments and, particularly, (vi) the wide safety margin, the SAVE technology sets the stage for making efficient live attenuated influenza vaccines. Although it is difficult to extrapolate to human use, in our system 10 ml of culture supernatant apparently contains enough virus to vaccinate and protect ~1 million mice with a single vaccination of 100 PD_{50} doses of $PR8^{3F}$ (**Fig. 3a** and **Supplementary Fig. 3**).

Vaccines based on changes in codon-pair bias could be generated within weeks for any emerging influenza virus once its genome sequence is known, although of course a further period of testing would be required before the vaccine could be used. The margin of safety appears to be unusually high. This new strategy will be applicable to the rapid development of human vaccines against seasonal flu epidemics and pandemics.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturebiotechnology/.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

The authors are indebted to A. García-Sastre and P. Palese for sharing with us their 8-plasmid system for PR8, antibodies, and information. We thank A. Paul and J. Cello for comments on the manuscript. Supported by National Institutes of Health grants AI075219 and AI15122 (EW) and a TRO-Fusion Award by Stony Brook University (S.M. and S.S.).

AUTHOR CONTRIBUTIONS

S.M. designed and carried out the majority of the study and wrote the paper. J.R.C. and A.N. carried out experiments contributing to **Figures 1c** and **2**. D.P., C.B.W. and S.S. developed computer design and analysis algorithms. B.F. and E.W. contributed to the design of the study and writing of the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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1. Salomon, R. & Webster, R.G. The influenza virus enigma. *Cell* **136**, 402–410 (2009).
2. Coleman, J.R. *et al.* Virus attenuation by genome-scale changes in codon-pair bias. *Science* **320**, 1784–1787 (2008).
3. Palese, P. & Shaw, M.L. in *Field's Virology*, vol. 2 (eds. D.M. Knipe *et al.*) 1647–1689, (Lippincott Williams & Wilkins, Philadelphia, 2007).
4. Steinhauer, D.A. & Skehel, J.J. Genetics of influenza viruses. *Annu. Rev. Genet.* **36**, 305–332 (2002).
5. Maassab, H.F. Adaptation and growth characteristics of influenza virus at 25 degrees c. *Nature* **213**, 612–614 (1967).
6. Rimmelzwaan, G.F., Fouchier, R.A. & Osterhaus, A.D. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. *Curr. Opin. Biotechnol.* **18**, 529–536 (2007).
7. Sant, A.J. *et al.* Immunodominance in CD4 T-cell responses: implications for immune responses to influenza virus and for vaccine design. *Expert Rev. Vaccines* **6**, 357–368 (2007).
8. Hikono, H. *et al.* T-cell memory and recall responses to respiratory virus infections. *Immunol. Rev.* **211**, 119–132 (2006).
9. Swain, S.L. *et al.* CD4+ T-cell memory: generation and multi-faceted roles for CD4+ T cells in protective immunity to influenza. *Immunol. Rev.* **211**, 8–22 (2006).
10. Simonsen, L. *et al.* Impact of influenza vaccination on seasonal mortality in the US elderly population. *Arch. Intern. Med.* **165**, 265–272 (2005).
11. Belshe, R.B., Van Voris, L.P., Bartram, J. & Crookshanks, F.K. Live attenuated influenza A virus vaccines in children: results of a field trial. *J. Infect. Dis.* **150**, 834–840 (1984).
12. Belshe, R.B. *et al.* The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *N. Engl. J. Med.* **338**, 1405–1412 (1998).
13. Cello, J., Paul, A.V. & Wimmer, E. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. *Science* **297**, 1016–1018 (2002).
14. Gutman, G.A. & Hatfield, G.W. Nonrandom utilization of codon pairs in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**, 3699–3703 (1989).
15. Moura, G. *et al.* Large scale comparative codon-pair context analysis unveils general rules that fine-tune evolution of mRNA primary structure. *PLoS ONE* **2**, e847 (2007).
16. Wimmer, E., Mueller, S., Tumpey, T.M. & Taubenberger, J.K. Synthetic viruses: a new opportunity to understand and prevent viral disease. *Nat. Biotechnol.* **27**, 1163–1172 (2009).
17. Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G. & Webster, R.G.A. DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. USA* **97**, 6108–6113 (2000).
18. Schickli, J.H. *et al.* Plasmid-only rescue of influenza A virus vaccine candidates. *Phil. Trans. R. Soc. Lond. B* **356**, 1965–1973 (2001).
19. Mueller, S., Papamichail, D., Coleman, J.R., Skiena, S. & Wimmer, E. Reduction of the rate of poliovirus protein synthesis through large-scale codon deoptimization causes attenuation of viral virulence by lowering specific infectivity. *J. Virol.* **80**, 9687–9696 (2006).
20. Shizhen, L. *Compendium of Materia Medica (Bencao Gangmu,* 1593) (Foreign Language Press, 2004).
21. Hutchinson, E.C., Curran, M.D., Read, E.K., Gog, J.R. & Digard, P. Mutational analysis of cis-acting RNA signals in segment 7 of influenza A virus. *J. Virol.* **82**, 11869–11879 (2008).
22. Wang, Z., Tobler, S., Roayaei, J. & Eick, A. Live attenuated or inactivated influenza vaccines and medical encounters for respiratory illnesses among US military personnel. *J. Am. Med. Assoc.* **301**, 945–953 (2009).
23. Coffin, J.M. Attenuation by a thousand cuts. *N. Engl. J. Med.* **359**, 2283–2285 (2008).
24. Reed, L.J. & Muench, M. A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493–497 (1938).

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ONLINE METHODS

Cell lines. 293T and MDCK cells were obtained from the American Type Culture Collection (ATCC). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% FBS (HyClone) and penicillin-streptomycin (Invitrogen).

Reverse genetics and generation of synthetic influenza viruses. All synthetic influenza viruses used here are based on the strain A/PR/8/34 (Mt. Sinai variant, short PR8). The reference sequences of the eight gene segments for this strain are available under GenBank accession numbers AF389115, AF389116, AF389117, AF389118, AF389119, AF389120, AF389121, AF389122). An eight-plasmid ambisense system for this strain cloned in the vector pDZ[25] was kindly made available by Peter Palese and Adolfo García-Sastre (Mt. Sinai School of Medicine).

Codon pair–deoptimized genome segments were designed using a computer algorithm previously described[2]. Coding regions of the segments PB1, HA and NP were targeted to be recoded. A minimum of 120 nucleotides at either segment terminus were left unaltered because these sequences contain packaging signals[3]. All of the codon changes were synonymous; that is, none of them introduced any amino acid changes. The precise extent of recoded sequence for each target segment and the resulting changes in codon-pair bias are summarized in **Table 1**.

The recoded gene segments were synthesized *de novo* (Mr. Gene) and introduced into WT plasmids to replace the respective WT counterpart sequence. Codon pair–deoptimized viruses were derived by DNA transfection of either one, or combinations of two, or three deoptimized segments together with the remaining WT plasmids. For this purpose a total of 2 μg plasmid DNA (250 ng of each of 8 plasmids) was transfected into co-cultures of 293T and MDCK cells in 35-mm dishes using Lipofectamine 2000 (Invitrogen) according to manufacturers recommendations. After 6 h of incubation at 37 °C, the serum free Opti-MEM containing the transfection mix was replaced with DMEM containing 0.2% Bovine Serum Albumin (BSA). After a further 24 h of incubation, 1 μg/ml TPCK-Trypsin was added to the dishes. Two days thereafter virus containing cell supernatants were collected and amplified on MDCK cells.

***In vitro* growth characteristics and titration of synthetic influenza viruses.** The growth characteristics of codon pair–deoptimized synthetic viruses were analyzed by infecting confluent monolayers of MDCK cells in 100-mm dishes with 0.001 multiplicities of infection (MOI). Infected cells were incubated at 37 °C in DMEM, containing 0.2% BSA and 2 μg/ml TPCK-Trypsin (Pierce). At the given time points 200 μl of supernatant was removed and stored at −80 °C until titration. Viral titers and plaque phenotypes were determined by plaque assay on confluent monolayers of MDCK cells in 35-mm six-well plates using a semisolid overlay of 0.6% tragacanth gum (Sigma-Aldrich) in minimal Eagle medium (MEM) containing 0.2% BSA and 4 μg/ml TPCK-trypsin. After 72 h of incubation at 37 °C plaques were visualized by staining the wells with crystal violet.

Mouse pathogenicity, *in vivo* virus replication and vaccination. A minimum of five BALB/c mice (5–6-weeks-old) per group were infected once by intranasal inoculation with doses ranging from 10^0 to 10^6 PFU of $PR8^{3F}$ or of PR8 WT virus. Inoculum virus was diluted in 25 μl PBS and administered evenly into both nostrils. A control group of 5 mice was inoculated with PBS only (mock). Venous blood from the tail vein was collected from all animals before initial infection for subsequent determination of pre-vaccination antibody titers.

Morbidity and mortality (weight loss, reduced activity, death) was monitored. LD_{50} of the WT virus and the vaccine candidates was calculated as described[24]. Mice experiencing severe disease symptoms (rapid, excessive weight loss over 25%) were euthanized and scored as a lethal outcome.

For vaccination experiments mice were infected as above. Twenty-eight days after the initial infection (vaccination), venous blood from the tail vein was drawn for subsequent determination of post-vaccination antibody titers.

The mice were then challenged with 10^5 PFU of the PR8 WT virus corresponding to >1,000 times the LD_{50}. Mortality and morbidity (weight loss, reduced activity, death) was monitored. The PD_{50} of codon pair–deoptimized $PR8^{3F}$ versus that of the PR8 was determined as the dose required to protect 50% of mice from a challenge with 1,000× LD_{50} of the WT virus, 28 d after a single inoculation with the vaccine virus.

To assess virus replication in the lungs of infected animals, BALB/c mice were infected intranasally with 10^3 PFU of either PR8 or $PR8^{3F}$. At 1, 3, 5, 7 and 9 d after infection the lungs of three mice each were collected (WT infected mice did not survive beyond day 6). Lungs were homogenized in 1 ml of PBS and the virus load per organ was determined by plaque assay on MDCK cells, as described above. Similarly, replication of PR8 challenge virus in lungs of $PR8^{3F}$-vaccinated mice were determined. Twenty-eight days after a single intranasal inoculation with 10^4 PFU of $PR8^{3F}$ or saline, five animals each were challenged with 1,000× LD_{50} of PR8. Three days after challenge (the usual peak of viral replication), lungs were processed and viral load per organ determined as described above.

Western blot analysis of influenza virus proteins in infected cells. MDCK cells were infected with virus at an MOI of 5 and incubated for 4 h at 37 °C. Subsequently, the cells were harvested in Laemmli buffer. The proteins were resolved by SDS-PAGE, analyzed by western blots with α-HA, α-PB1 and α-actin (Santa Cruz Biotechnology), and α-NP (generous gift from Peter Palese) and HRP-conjugated secondary antibodies, and detected by autoradiography.

Enzyme-linked immunosorbent assay (ELISA) determination of influenza-specific serum IgG-antibodies after vaccination. Nunc Maxisorp ELISA 96-well plates were coated over night with 100 ng purified influenza PR8 virus in 100 μl PBS followed by blocking with 100 μl 1% BSA in PBS. Serial fivefold dilutions in PBS/1% BSA of mouse sera obtained before and 28 d after a single intranasal vaccination were incubated for 2 h at 22 °C. Mice were previously vaccinated with ~0.01 or 0.001× LD_{50} of $PR8^{3F}$ (10^3 PFU or 10^4 PFU, respectively), 0.01× LD_{50} of PR8 WT (10^0 PFU) or mock vaccinated. After four washes with PBS the wells were incubated with 1:500 of anti mouse-alkaline phosphatase conjugated secondary anti-mouse IgG antibody (Santa Cruz) for another 2 h at 22 °C. After four washes with PBS and brief rinsing with distilled water 100 μl of a chromatogenic substrate solution containing 9 mg/ml *p*-nitrophenylphosphate in 200 mM diethanolamine, 1 mM $MgCl_2$, pH 9.8 was added. The color reaction was stopped by addition of an equal volume of 500 mM NaOH. Absorbance at 405 nm was read using a Molecular Devices ELISA reader. The endpoint antibody titer was defined as the highest dilution of serum that gave a signal >5 s.d. above background. Background level was determined from wells processed identically to experimental samples, in the absence of any mouse serum.

25. Quinlivan, M. *et al.* Attenuation of equine influenza viruses through truncations of the NS1 protein. *J. Virol.* **79**, 8431–8439 (2005).

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