Influenza A virus nucleoprotein selectively decreases neuraminidase gene-segment packaging while enhancing viral fitness and transmissibility

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Edited by Robert A. Lamb, Northwestern University, Evanston, IL, and approved October 17, 2014 (received for review August 10, 2014)

The influenza A virus (IAV) genome is divided into eight distinct RNA segments believed to be copackaged into virions with nearly perfect efficiency. Here, we describe a mutation in IAV nucleoprotein (NP) that enhances replication and transmission in guinea pigs while selectively reducing neuraminidase (NA) gene segment packaging into virions. We show that incomplete IAV particles lacking gene segments contribute to the propagation of the viral population through multiplicity reactivation under conditions of widespread coinfection, which we demonstrate commonly occurs in the upper respiratory tract of guinea pigs. NP also dramatically altered the functional balance of the viral glycoproteins on particles by selectively decreasing NA expression. Our findings reveal novel functions for NP in selective control of IAV gene packaging and balancing glycoprotein expression and suggest a role for incomplete gene packaging during host adaptation and transmission.

Influenza A virus | genome segmentation | genome packaging | nucleoprotein | host adaptation

Seasonal influenza A virus (IAV) remains a major public health threat, causing tens of thousands of deaths each year in the United States alone (1). Morbidity and mortality rates can increase dramatically when a zoonotic strain adapts to circulate in humans, triggering a pandemic. The continuing toll exerted by IAV stems from its remarkable adaptability, which enables it to move between widely divergent host species and also evade herd immunity within each species. Defining the specific mechanisms that mediate IAV adaptation is essential to improving anti-IAV vaccines, therapeutics, and pandemic surveillance.

The IAV genome consists of eight negative-sense RNA segments, each of which is required for productive infection. Genome segmentation complements the high mutation rate of IAV by facilitating reassortment, which can maximize positive intergenic epistasis (2–5) and allow selective elimination of segments with deleterious mutations (6, 7). Although reassortment is the most obvious and best-characterized benefit of segmentation, there are likely additional evolutionary advantages.

Genome segmentation imposes the substantial constraint of maintaining a gene-packaging mechanism to produce fully infectious virions (8). For IAV, it is widely believed that a single copy of each segment is packaged into progeny virions with nearly perfect efficiency, resulting in an equimolar ratio of the segments at the population level (9–12). Confounding this model, we recently demonstrated that most IAV virions fail to express one or more gene products (13). This finding raises the possibility that in some circumstances incomplete influenza gene packaging is evolutionarily neutral and possibly even advantageous (14).

The viral glycoproteins HA and neuraminidase (NA) are encoded by separate genome segments. HA attaches IAV to terminal sialic acid residues on the host cell surface, enabling viral entry. By hydrolyzing sialic acids, NA detaches budding virions and neutralizes HA-inhibiting glycoproteins and is required for the spread of IAV within and between hosts (15–18). Because the specificity of HA and NA for different sialic acid linkages and contexts can vary substantially, functional alignment between the yin-yang activities of HA and NA represents a major determinant of host adaptation, transmissibility, and immune escape (19–23). Independently controlling levels of HA and NA expression therefore may be critical for fine-tuning their functional balance.

We previously described a single amino acid substitution (F346S) in the nucleoprotein (NP) of mouse-adapted A/Puerto Rico/8/34 (PR8), selected during serial passage in guinea pigs, which enhances replication in the guinea pig respiratory tract and enables contact transmission (4). Here, we report that this adaptive mutation selectively decreases both the expression and packaging of the NA gene segment, thus revealing a surprising role for NP in the regulation of glycoprotein function and demonstrating that decreased gene packaging can be associated with increased in vivo fitness and transmissibility.

Results

NP Selectively Regulates NA Expression and Glycoprotein Functional Balance. While characterizing the NP:F346S mutation, we observed by immunoblotting that purified PR8NP:F346S virions (generated by reverse genetics to ensure a genetic background similar to wild-type virus stocks) contained 10-fold less NA than wild-type virus (PR8WT), as normalized either to absolute virion protein (Fig. 14). Concomitantly, purified PR8NP:F346S Virus exhibited a similar decrease in NA enzymatic activity per particle, as determined by HAU, revealing an unexpected role for NP in modulating virion NA content (Fig. 1B).

Does the decrease in NA virion incorporation stem from decreased cellular expression of NA? We infected Madin–Darby

Significance

The influenza A virus (IAV) genome consists of eight unique RNA segments, each of which is required for productive infection. IAV is believed to copackage its individual gene segments into virions with nearly perfect efficiency to maximize replicative potential. We contradict this view by demonstrating that decreased gene packaging can be associated with increased in vivo fitness and transmissibility.Incomplete packaging likely is facilitated by the extensive coinfection that we demonstrate in vivo, which promotes complementation and explains the frequent reassortment reported previously. We also reveal roles for the viral nucleoprotein in modulating glycoprotein function and gene packaging during host adaptation. These findings necessitate a major shift in how we think about the infectious and evolutionary potential of IAV populations.
Reducing NA Activity Alone Does Not Enhance Fitness in Guinea Pigs. We next examined the phenotype of NP:F346S in vivo, in the context of a guinea pig-adapted PR8 variant virus, which has a single amino acid substitution in M1 (V166M). M1-V166M allows PR8 to replicate to high titer in guinea pigs, with or without NP:F346S (4), but alone has no detectable effect on selective viral gene expression (Fig. S3). We used the M1:V166M background to eliminate the large differences in replication kinetics between PR8WT and PR8NP:F346S in guinea pigs and to allow a more direct assessment of the effect of NP:F346S in vivo.

We inoculated guinea pigs intranasally (i.n.) with a 10^7 50% tissue culture infectious dose (TCID50) of PR8M1:V166M or PR8NP:F346SM1:V166M and collected nasal wash fluids and nasal turbinate cells at 48 hpi, near peak shedding. Importantly, both flow cytometric analysis of viral gene expression in infected nasal turbinate cells and quantitation of viral proteins in nasal wash demonstrated that NP:F346S selectively reduced NA expression in vivo (Fig. 2 A–C).

To examine the relationship between NA activity and enhanced fitness of PR8NP:F346S, we assessed the effects of two independent cis-acting NA substitutions (NA:K239R or NA:G339S, numbering from first ATG) that reduce virion NA content by approximately fivefold on viral replication of PR8 in guinea pigs (Fig. S4) (22, 23). Each mutation diminished PR8 replication, indicating that reducing NA expression/activity alone is insufficient to enhance PR8 replication in guinea pigs (Fig. 2D).

**NP:F346S Selectively Reduces NA Gene-Segment Packaging.** We previously reported that the majority of virions in IAV preparations fail to express one or more viral gene products (13). Low-MOI infection revealed that the fraction of PR8NP:F346S virions that express detectable levels of NA upon infection was reduced by approximately threefold relative to PR8WT, whereas the frequencies of HA, NP, and NS1 expression were essentially unchanged (Fig. 3 A and B).

To determine whether this decrease reflects a reduction in NA gene-segment packaging, we performed segment-specific quantitative RT-PCR on purified particles using primers targeting the flanking regions containing the gene-segment packaging signals (8). PR8NP:F346S populations contained threefold fewer NA vRNA copies relative to M1 and HA, compared with PR8WT (Fig. 3C). Importantly, vRNA extracted from guinea pig nasal wash collected at peak shedding exhibited a similar selective relative decrease in NA segment vRNA (Fig. 3D).

These results clearly establish that NP:F346S reduces relative NA gene packaging in vitro and in vivo, potentially by reducing intracellular NA vRNA abundance.

**PR8NP:F346S Produces More Semi-Infectious Particles per Infected Cell.** The selective reduction in NA vRNA packaging by NP:F346S should increase the fraction of propagation-deficient semi-infected (SI) particles relative to fully infectious (FI) virions, because of the lack of an NA segment (13). Indeed, when we infected cells with equivalent tissue culture infectious doses (TCID50, a measure of FI virions), PR8NP:F346S infected up to eightfold more cells than PR8WT, as measured by cellular NP expression (a measure of both FI and SI virions) (Fig. 3E). This effect was confirmed when we compared the ratios of physical particles (determined by HAU) to TCID50 (Fig. S5).

A decrease in relative NA gene-segment packaging could result in a decrease in FI particle production, an increase in SI particle production, or a combination of both. Thus, we determined the effect of NP:F346S on the number of FI and SI particles produced during a single infection cycle. We infected MDCK cells with PR8WT or PR8NP:F346S at an MOI of 0.01 (TCID50 per cell), collected supernatants at 8 and 9 hpi, and determined the number of FI (measured by TCID50) and SI (measured by single-round flow cytometric infection assay) particles. At the time of harvest, we collected the producer cell monolayer and determined the number of HA*NP*NA*NS1* cells by flow cytometry, which served as a surrogate for productively infected cells. From this study, we found that NP:F346S reduced the number of SI particles produced compared to PR8WT, consistent with our RT-PCR data (Fig. 3F).

### Fig. 1. NP modulates virion glycoprotein balance through epistatic control of NA expression. (A) Relative protein content of HA, NA, and M1 in purified particle preparations. *P < 0.0001 for differences between all NA ratios for PR8WT and PR8NP:F346S (one-way ANOVA, Tukey test). Each data point represents a comparison of independent stocks of PR8WT and PR8NP:F346S grown at the indicated MOIs. (B) NA activity for purified PR8NP:F346S normalized to average HAU of HA or HA or M1 protein as a percentage of the PR8WT control, presented as mean ± SEM; *P < 0.0001 for differences between all NA ratios compared with the matched wild-type control (one-way ANOVA, Tukey test). (C) Relative viral protein expression as determined by geometric mean fluorescence intensity (GMFI) of MDCK cells infected at an MOI <0.05 with PR8WT or PR8NP:F346S that stained positive for the indicated viral proteins. PR8NP:F346S values are presented as the mean percentage of PR8WT ± SEM of three technical replicates. (D) Relative copy numbers of HA and NA vRNA and mRNA in FACS-sorted, singly infected HA particle preparations. Each data point represents a comparison of independent stocks of PR8WT and PR8NP:F346S grown at the indicated MOIs. **:*P < 0.0001 for differences between all NA ratios compared with the matched wild-type control (one-way ANOVA, Tukey test).
result we could calculate the average number of FI and SI particles produced per productively infected cell. We detected a slight decrease in the number of FI particles released from productively infected cells by PR8NP:F346S as compared with PR8WT across multiple independent experiments ($P = 0.09$). Concomitantly, we detected a more significant ($P = 0.04$) increase in the number of SI particles produced by the mutant (Fig. 4A). This result indicates that PR8NP:F346S produces more SI particles per cycle of replication, perhaps at the partial expense of FI particles.

SI Particles Contribute to Replication Through Multiplicity Reactivation. How can decreased NA vRNA packaging and increased SI particle production be associated with increased in vivo replication kinetics? We hypothesized that the increased number of SI particles produced by PR8NP:F346S contributes to productive replication through multiplicity reactivation, a phenomenon in which coinfecting complementary incomplete particles generate a productive infection (24, 25). If this hypothesis is correct, increasing the MOI should have a more pronounced effect on the virus output in MDCK cells infected at low MOI recapitulate what is seen in vivo.

To test this notion, we compared single-cycle infectious virus titers obtained from the nasal wash at 48 hpi to establish the HA/NA coexpression frequencies, demonstrating that coinfection occurs in vivo (4, 26, 27), its actual incidence is unknown. To determine the coinfection frequency in vivo, we exploited the observation that the cellular coexpression frequencies of specific viral gene products predictably increase with MOI because of complementation (Fig. 5). We infected guinea pigs i.n. with PR8M1:V166M, with or without the NP:F346S substitution, and collected nasal wash and turbinates at 9 and 48 hpi. We infected MDCK cells at an MOI ≤0.05 TCID$_{50}$ with virus obtained from the nasal wash at 48 hpi to establish the HA/NA coexpression frequencies for in vivo-produced virus under low-MOI conditions. We then compared these values with the HA/NA coexpression frequencies in NP$^+$ cells isolated from the nasal turbinates of the same animals (Fig. 6A and B).

Nasal turbinates cells collected at 9 hpi exhibited low viral protein coexpression frequencies, demonstrating that (i) the initial round of infection occurs under low-MOI conditions following direct inoculation, and (ii) the incomplete expression patterns observed in MDCK cells infected at low MOI recapitulate what is seen in vivo. Importantly, by 48 h after infection with 10$^5$ TCID$_{50}$, each virus attained coexpression frequencies of $\sim$80–90%, much higher than...
the 40–60% observed during low-MOI ex vivo infection with nasal wash virus and indicative of frequent coinfection.

Together, our results demonstrate that widespread coinfection, and thus the conditions necessary for efficient multiplicity reactivation of SI particles, occurs in guinea pigs by the time of peak shedding. Further, they provide an explanation for how SI particles can contribute to viral replication, thus minimizing the detrimental effects that might be expected from a decrease in gene-packaging efficiency.

**Changes in Population-Level Gene-Segment Abundance Directly Influence Viral Gene Expression in Cells.** Under high-MOI conditions, such as occur in guinea pigs by 48 hpi, cells infected with PR8NP:F346S will receive on average fewer copies of the NA gene segment than of the other gene segments. This disparity results in a divergence in the effective MOIs of the individual gene segments. Because the kinetics of IAV gene expression increase with MOI, this divergence should generate a gene-dosage effect that reduces NA expression relative to other gene products (28). To test this notion, we generated a stock of PR8 lacking the NA vRNA segment (PR8NoNA) using reverse genetics. PR8NoNA, although incapable of NA expression, retained HA, NP, and NS1 expression in infected cells (Fig. 6B).

We coinfected Vero cells with PR8WT along with increasing doses of PR8NoNA to mimic the altered NA-gene segment frequency in the PR8NP:F346S virus population and assessed relative NA expression within the HA\(^+\)NA\(^+\) population by flow cytometry at 15 hpi (Fig. 6C). We observed a dose-dependent decrease in NA expression relative to HA. Ratios of NP and NS1 to HA remained relatively constant, indicating that we were not observing a general effect from increasing input virus. At the 1:2 ratio of wild-type to PR8NoNA, which best approximates the gene-segment frequencies of PR8NP:F346S, we observed a twofold decrease in relative NA expression.

These results demonstrate that the relative abundance of the individual gene segments in an IAV population can directly govern viral gene expression in multiply infected cells.

**Discussion**

Our findings reveal an unappreciated role for genome segmentation in precisely regulating the packaging and expression of individual viral genes through intergenic epistatic interactions. Segment-specific regulation is particularly important for the HA and NA genes, because balancing the relative activity levels of these two molecules is key to maintaining optimal fitness across different host contexts (20, 21, 29). Previously described mechanisms of NA regulation in response to selective pressure entail cis-acting mutations that modulate NA activity in cells or virions (22, 30–32). NA activity on virions also can be influenced in trans by M1 and the viral polymerase proteins PB2 and PB1, although the specific mechanisms involved are uncertain (23, 34).

Our discovery that NP can regulate HA/NA functional balance through selective control of NA gene expression reveals yet another mechanism for modulating this critical viral parameter. Identifying the unique properties of the NA gene that enable its selective regulation is the focus of ongoing studies. Deeper

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**Fig. 3.** NP selectively reduces relative NA gene-segment packaging. (A) Representative flow cytometry plots of viral protein expression in cells infected at an MOI <0.05 with equal HAU. (B) The population expression pattern illustrated as the percentage of cells in A that expressed detectable levels of the indicated proteins. Data represent mean ± SEM of three technical replicates. (C) Relative HA, NA, and M vRNA content in purified PR8NP:F346S virus preparations determined by quantitative RT-PCR as a percentage of the matched PR8WT controls. Data are presented as mean ± SEM (\(P < 0.005\); t test). (D) Relative abundance of vRNA segments as determined by quantitative PCR in nasal wash of PR8M1:V166M-NP:F346S-infected guinea pigs (\(n = 2\)) 48 hpi as a percent of values from PR8M1:V166M-infected guinea pigs. Data are presented as mean ± SEM (\(P < 0.05\); t test). (E) NP expression in MDCK cells infected at an MOI of 0.01 TCID\(_{50}\) per cell with PR8WT or PR8NP:F346S.

**Fig. 4.** SI particles contribute to replication through multiplicity reactivation. (A) Mean TCID\(_{50}\) and SI particle output per HA\(^+\)NP\(^+\)NS1\(^+\) producer cell by 8–9 hpi. Data are pooled from two independent experiments, and each point represents the value obtained from a single culture. \(P = 0.0885\) for TCID\(_{50}\); P = 0.0406 for SI particles; Mann-Whitney test. (B) Virus output (in TCID\(_{50}\)/mL) from MDCK cells infected for 8 h with PR8WT or PR8NP:F346S presented as the fold increase over output at an MOI of 0.01 for three stocks grown in parallel at each MOI ± SEM. Interaction between strain and MOI: \(P = 0.0308\) by two-way ANOVA.
understanding of this intersegment regulatory network is essential for understanding the evolution of HA and NA and for improving sequence-based predictions regarding their function.

The decreased viral replication in guinea pigs infected with either of two cis-acting NA mutants (NA:K239R and NA:G339S) highlights the importance of HA/NA functional balance in viral fitness. It further shows that the increased fitness associated with NP:F346S cannot be explained by simple reduction in per-virion NA activity. Instead, these results raise several other non-mutually exclusive possibilities: (i) Other independent effects of the NP:F346S mutation are sufficiently beneficial to outweigh the detrimental effect of reduced NA expression; (ii) reduced NA activity can be advantageous, but only when combined with other effects of the NP:F346S mutation (i.e., increased SI particle production); (iii) NP:F346S reduces NA on virions in a way that is qualitatively distinct from the NA mutations tested here (e.g., the spatial distribution of NA on the particle surface) and thus has a different effect on host interactions; (iv) selective reduction in NA RNA synthesis, rather than NA content in virions, is critical to the NP:F346S phenotype in guinea pigs.

Regardless of the answer(s), these results highlight the dangers of oversimplification when attempting to attribute a fitness benefit to a given set of measured parameters.

The observation that IAV can selectively reduce the relative packaging frequency of individual gene segments undermines the dogma that IAV genome segments are copackaged in equimolar packaging frequency of individual gene segments undermines the potential of the population (4, 7).

If controlling the efficiency of gene-segment packaging is a common feature of IAV biology, different IAV strains should vary in the frequency with which different gene products are expressed within populations. Indeed, a panel of classic H1N1 viruses (all presumably egg-adapted) exhibited substantial differences in the frequency with which different gene products are expressed that variation in SI particle production, and potentially in gene-packaging efficiency, is widespread among IAV strains. As a practical matter, these findings also highlight the shortcomings of propagation-based infectivity assays (including plaque assays and infectious endpoint assays), which fail to measure SI particles, the predominant output of IAV infection (13). Efforts to compare infectivity between different strains or mutants may need to account for differences in SI particle production.

We have demonstrated that IAV consists of a minor fraction of fully infectious virions supported by a larger pool of SI particles that collectively influence the behavior of the population and whose abundance can be genetically controlled. Thus, the

![Fig. 5.](image)

The frequency of viral protein coexpression increases with MOI. MDCK cells were infected for 9 h with PR8M1:V166M or PR8M1:V166M+NP:F346S at the indicated MOIs, and the percentage of NI + cells that also express HA and NA was determined by flow cytometry. (A) Representative dot plots. (B) Mean ± SEM of two replicates per condition.
complexity of IAV populations extends beyond the classical quasispecies description, and efforts to understand IAV infections also must account for relative gene-segment frequencies in viral populations. Further, our results reveal a possible role for SI particles and incomplete gene-segment packaging in determining viral fitness and transmissibility during species adaptation. In turning a seeming liability into an asset, IAV once again demonstrates the amazing cleverness of viruses, which, with such a limited genetic palate, must exploit every possible trick for maximizing transmission.

Materials and Methods

Animal Care. All animal procedures were carried out in compliance with the Public Health Service policy, Office of Laboratory Animal Welfare guidance, and all guidelines of the National Institute of Allergy and Infectious Diseases (NIAID) Institutional Animal Care and Use Committee (IACUC). Research was conducted under a protocol approved by the NIAID IACUC.

Viruses. The A/Puerto Rico/8/1934 (PR8) strain was generated using an eight-plasmid rescue system (35) (GenBank accession nos. AF389115–AF389122), generously contributed by Adolfo Garcia-Sastre (Mt. Sinai School of Medicine, New York). These clones differ from the published sequence (above) at two positions: PR8 A549C (K175N) and HA A651C (I207L) (H1 numbering). We generated seed virus by transfecting 293T cells with the appropriate materials and methods can be found in SI Materials and Methods.

ACKNOWLEDGMENTS. This work was funded by the Division of Intramural Research, National Institutes of Allergy and Infectious Diseases, National Institutes of Health.
Supporting Information

Brooke et al. 10.1073/pnas.1415396111

SI Materials and Methods

**Virion Protein Quantification.** Viruses were purified by ultracentrifugation on a stepped 15–60% sucrose gradient. Virus was collected from the interface of 15% and 60% sucrose, pelletted, and reconstituted in PBS. Serial dilutions of virus then were boiled in SDS containing DTT (100 mM), resolved by polyacrylamide electrophoresis, and transferred to PVDF membranes. Blots were probed with mouse anti-HA mAb RAS-22, mouse anti-M1 mAb M2-IC6, and rabbit anti-NA C-terminal polyclonal antibody (pAb), followed by incubation with anti-mouse and anti-rabbit secondary mAbs conjugated with 680-nm and 800-nm IRDye (LI-COR). Blots then were visualized simultaneously, and protein was quantitated on an Odyssey infrared scanner using Image Studio v2.0 software (LI-COR).

**ELISA.** Half-area 96-well high-binding ELISA plates (Greiner Bio-One) were incubated with serial dilutions of nasal wash samples overnight at 4 °C. Plates were blocked with 0.1% BSA in PBS for 2 h at room temperature, washed with PBS + 0.05% Tween-20, and incubated for 3 h at room temperature with a mixture of four HA-specific mouse mAbs recognizing distinct epitopes. After extensive washing with PBS + 0.05% Tween-20, bound primary antibodies were detected by incubation with an HRP-conjugated rat anti-mouse IgG kappa light chain antibody for 2 h at room temperature. ELISA plates were washed again with PBS + 0.05% Tween-20, developed with the LumiGlo chemiluminescent substrate (Kirkegaard & Perry Lab, Inc.), and read on a SpectroMax M5 luminometer (Molecular Devices). Signals for dilutions in the linear range were used to calculate the ratio of HA protein to NA activity for each sample.

**NA Activity Assay.** Neuraminidase activity was determined for the same purified serial particle preparations used for the assessment of total protein content. Activity was determined using a fluorogenic substrate, 2′-(4-methylumbelliferyl)-α-N-acetylneuraminic acid (Sigma), as previously described (1). Briefly, virus was serially diluted twofold in assay buffer [33 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 4 mM CaCl2]. Substrate was added to diluted virus samples at a final concentration of 200 μM. Reactions were incubated at 37 °C, and fluorescence was measured every minute over a 30-min period on a SpectraMax M5 microplate reader (Molecular Devices). Michaelis–Menten kinetics were determined for each dilution of virus to obtain the V_{max} value for comparison.

**RNA Quantification.** The vRNA content in purified virus preparations was determined by quantitative RT-PCR. RNA was extracted using the QiaAmp viral RNA extraction Kit (Qiagen) and was “reverse transcribed” with the Verso RT kit (Thermo Scientific) using a universal primer specific for the 3′ end of the vRNA 5′-AGCGAAGGAGCAGG-3′. Quantitative PCR on cDNA was carried out using the Power SYBR Green PCR Master Mix (Invitrogen/Life Technologies) on a Mastercycler ep Realplex thermal cycler (Eppendorf). PCR primer binding sites were within the first 150 bp of the 3′ end (HA and NA segments) or within 170 bp of the 5′ end (M segment). PCR primers used: HA: 3′-AAGGGCAACTCTACTGCTCTGTTTGT-5′, 3′-CAGTGGTCGGGTAGGCTATAC-5′; NA: 3′-AATCTCAGAAAAATCAACACATGGAG-5′, 3′-ATTCCCTATTTGCAATATTAGGCT-5′, 3′-M-ACAGAGACCTGAAAGATGTC-5′, 3′-TTCCTTACAGGCTATCCATGAG-5′. To generate singly infected cells for RNA analysis, MDCK cells were infected with PR8 wt or PR8NP:F346S in triplicate at an MOI of 0.2. At 7 hpi, cells were harvested and surface stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26 and Alexa Fluor 647-conjugated mouse anti-NA mAb NA2-1C1. Approximately 10^6 HA+NA+ cells then were sorted from each replicate using a FACS Aria sorter (BD Biosciences), and RNA was extracted using the RNeasy mini kit (Qiagen).

**Analysis of Single Virion Expression.** MDCK cells were infected with virus diluted to ensure that <5% of cells were infected. For PR8wt and PR8NP:F346S, neutralizing anti-HA mouse mAb H17-L2 (5 μg/mL) was added to cultures at 2 hpi to prevent spread. For other H1N1 strains, spread was blocked by changing to minimal essential medium with 50 mM Hepes, 20 mM NH4Cl, pH 7.2, at 3 hpi. At 16 hpi, cells simultaneously were fixed and permeabilized in foxB3 fix/perm buffer (eBioscience). PR8wt- and PR8NP:F346S-infected cells were stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26 (which does not compete with H17-L2), Pacific Orange-conjugated mouse anti-NA mAb NA2-1C1, Alexa Fluor 647-conjugated mouse anti-NP mAb HB-65, and Pacific Blue-conjugated mouse anti-NS1 mAb NS1-1A7. Other H1N1 strains used were stained with human anti-HA mAb 2G02 (generously provided by Patrick C. Wilson, University of Chicago, Chicago) followed by Alex Fluor 488-conjugated donkey anti-human secondary (Jackson ImmunoResearch), rabbit anti-NA C tail pAb followed by PE-conjugated donkey anti-rabbit secondary (also from Jackson ImmunoResearch), and Alexa Fluor 647-conjugated mouse anti- NP mAb HB-65. After staining, cells were washed, run on a BD LSR II, and analyzed using FlowJo version 9.3 (Tree Star, Inc.). Population expression frequencies were calculated by looking at all infected cells, as determined by positive staining against at least one of the viral proteins examined, and then determining the percentage of infected cells expressing each examined gene product.

**Quantification of Single-Round Virus Output.** MDCK cells in six-well plates were infected with PR8wt or PR8NP:F346S at an MOI of 0.01 TCID50 per cell in the presence of 1 μg/mL TPCK-treated trypsin. At 8–9 hpi, supernatants were collected, clarified, and aliquoted. At the time of collection, producer cell monolayers were disrupted into single-cell suspensions, and live cells were enumerated by Trypan Blue exclusion. Producer cells then were fixed and permeabilized in foxB3 fix/perm buffer (eBioscience), and the frequencies of HA+NP+NA+NS1+ cells were determined by flow cytometry as described above. These frequencies were multiplied by the total cell counts to estimate the number of productively infected cells present in each monolayer. Supernatants then were quantified for TCID50 and SI particle concentrations, using the methods described above, with SI particles measured based on the ability to express HA or NP. The data presented are pooled from two independent experiments using...
independently rescued stocks of the two viruses. Each data point represents the value from a single well of a six-well plate.

**Virus-Free Expression Analysis.** HeLa cells in a 24-well plate were transfected with plasmids expressing PB2, PB1, PA, and NP (wild-type or NP:F346S) from the PR8 eight-plasmid reverse genetics rescue system described in *Materials and Methods* (0.125 μg per well each), along with plasmids expressing PR8 HA and NA vRNA from a human RNA polymerase I promoter (pHHH21 vector generously provided by Andrew Pekosz, Johns Hopkins School of Public Health, Baltimore; 0.25 μg per well each). Transfections were carried out using Lipofectamine 2000 (Life Technologies). Twenty-four hours after transfection, cells were simultaneously fixed and permeabilized in foxP3 fix/perm buffer (eBioscience) and were stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26, Pacific Orange-conjugated mouse anti-NA mAb NA2-1C1, and Alexa Fluor 647-conjugated mouse anti-NP mAb HB-65. After staining, cells were washed, run on a BD LSR II flow cytometer system, and analyzed using FlowJo version 9.3 (Tree Star, Inc.). HA and NA geometric mean fluorescence intensities (GMFI) were determined for the HA⁺ and NA⁺ cell populations, respectively.

**Evaluation of NA Gene Dose on Viral Protein Expression.** Vero cells were infected in triplicate with PR8WT at an MOI of 10 HAU per cell plus 0, 10, 20, 40, or 80 HAU per cell of PR8noNA, and at 2 hpi neutralizing anti-HA mouse mAb H17-L2 (5 μg/mL) was added to prevent spread. At 15 hpi, cells were harvested, fixed, and stained as described for single-virion analysis. Within the HA⁺NA⁺ population, we determined per-cell fluorescence ratios for HA, NP, NA, and NS1 using FlowJo version 9.3 (Tree Star, Inc.).

**Guinea Pig Infections.** Eight-week old SPF female Hartley guinea pigs (Charles River) were inoculated while under isoflurane anesthesia using 300 μL of virus diluted in balanced salt solution supplemented with 0.1% BSA. Virus was applied dropwise into the nares. Nasal washes were performed on isoflurane-anesthetized animals by dispensing 1 mL of PBS supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin into one nostril, and collecting flow-through from the other nostril. Nasal washes were frozen immediately on dry ice and subsequently were quantitated for infectious virus by TCID₅₀ assay on MDCK cells.

**Analysis of Guinea Pig Nasal Turbinate Cells.** At 9 or 48 hpi, guinea pigs were killed by intracardiac pentobarbital injection according to NIAID IACUC guidelines. Nasal turbinate were collected into RPMI + 25 mM Hepes, 7.4% FBS, 800 U/mL type I collagenase (Worthington), and 25 U/mL DNase (also Worthington). Tissues were disrupted further with bone cutters and then were digested at 37 °C with shaking for 1 h. Suspensions were passed through a 70-μm nylon screen and were spun over a lymphocyte separation medium cushion (Lonza). The interface was collected and treated with ammonium-chloride-potassium buffer (Lonza) to remove remaining RBCs. After washing, cells were counted and fixed/permeabilized using foxP3 fix/perm buffer (eBioscience). Cells then were stained with Alexa Fluor 488-conjugated mouse anti-HA mAb H36-26, Alexa Fluor 647-conjugated mouse anti-NA mAb NA2-1C1, and Pacific Blue-conjugated mouse anti-NP mAb HB-65, run on a BD LSR II flow cytometer system, and analyzed using FlowJo version 9.3 (Tree Star, Inc.). Live cells were gated based on the forward scatter A (FSC-A), side-scatter A (SSC-A), and SSC-W profile, followed by the exclusion of autofluorescent cells for some experiments. For viral protein-expression analysis, the per-cell fluorescence ratios of HA, NP, and NA were determined for the HA⁺NP⁺NA⁺ population. To examine coexpression frequency, NP⁺ cells were assessed for HA and NA expression using gates derived from full-minus-one staining controls.


![Fig. S1. NP:F346S decreases NA expression in cells infected at a high MOI. Intracellular HA, NP, and NA expression over time in MDCK cells infected with 20 HAU of PR8WT or PR8NP:F346S, as determined by flow cytometry and presented as the mean percent of mean PR8WT values at 9 h ± SEM for three technical replicates per time point.](www.pnas.org/cgi/content/short/1415396111)
Fig. S2. NP:F346S does not reduce NA expression when NA vRNA is provided in excess. HA and NA expression in HeLa cells, as determined by flow cytometry, 24 h after transfection with expression plasmids encoding PB2, PB1, PA, and NP (wild-type or NP:F346S), along with plasmids that express the HA and NA vRNAs from a human RNA polymerase I promoter. Data are pooled from two independent experiments and represent the mean PR8_{NP:F346S} values as a percentage of PR8_{WT} ± SEM.

Fig. S3. M1:V166M does not affect the expression pattern of PR8. MDCK cells were infected with the indicated viruses at an MOI of 0.05 TCID_{50}. Anti-HA neutralizing Ab H17L2 (5 μg/mL) was added 1 hpi to block spread. At 16 hpi, cells were harvested, fixed, and permeabilized, stained against HA, NA, NP, and NS1, and run on an LSR II flow cytometer. (A) Representative dot plots showing HA and NA expression. (B) The population expression pattern illustrated as the percentage of cells in A that expressed detectable levels of the indicated proteins. Data represent the mean ± SEM of three technical replicates. (C) GMFI of stain against the indicated proteins within cells staining positive for that protein, presented as a percentage of PR8_{WT} values. Data represent mean ± SEM.
Fig. S4. The effect of NA mutations on the incorporation of NA protein into virions. The indicated viruses were purified using a sucrose gradient, and the relative amounts of HA and NA protein were determined by quantitative Western blot. Data represent the mean values for duplicate gel lanes ± SEM.

Fig. S5. NP:F346S increases the particle:TCID$_{50}$ ratio of PR8. The concentrations of physical particles (calculated by determining the furthest dilution of virus capable of agglutinating a predetermined number of turkey RBCs) and TCID$_{50}$ were determined for two independent reverse genetics-generated stocks of PR8$_{WT}$ and PR8$_{NP:F346S}$. Data represent the particle:TCID$_{50}$ ratios for individual stocks of virus.

Fig. S6. Expression pattern of PR8$_{noNA}$. Representative dot plots showing HA, NP, NA, and NS1 expression in MDCK cells 16 h after low-MOI infection with PR8$_{noNA}$. 
Fig. S7. Expression pattern of diverse H1N1 historical isolates. Population expression patterns in the indicated H1N1 strains as determined by flow cytometric analysis of MDCK cells infected at an MOI <0.05 TCID\textsubscript{50} per cell. Data represent mean ± SEM of values from three stocks of each strain.