Inefficient control of host gene expression by the 2009 pandemic H1N1 influenza A virus NS1 protein

HALE, Benjamin G, et al.

Abstract

In 2009, a novel swine-origin H1N1 influenza A virus emerged. Here, we characterize the multifunctional NS1 protein of this human pandemic virus in order to understand factors that may contribute to replication efficiency or pathogenicity. Although the 2009 H1N1 virus NS1 protein (2009/NS1) is an effective interferon antagonist, we found that this NS1 (unlike those of previous human-adapted influenza A viruses) is unable to block general host gene expression in human or swine cells. This property could be restored in 2009/NS1 by replacing R108, E125, and G189 with residues corresponding to human virus consensus. Mechanistically, these previously undescribed mutations acted by increasing binding of 2009/NS1 to the cellular pre-mRNA processing protein CPSF30. A recombinant 2009 H1N1 influenza A virus (A/California/04/09) expressing NS1 with these gain-of-function substitutions was more efficient than the wild type at antagonizing host innate immune responses in primary human epithelial cells. However, such mutations had no significant effect on virus replication in either human or swine tissue culture substrates. […]

Reference


DOI : 10.1128/JVI.00081-10
PMID : 20444891

Available at:
http://archive-ouverte.unige.ch/unige:75378

Disclaimer: layout of this document may differ from the published version.
In 2009, a novel swine-origin H1N1 influenza A virus emerged. Here, we characterize the multifunctional NS1 protein of this human pandemic virus in order to understand factors that may contribute to replication efficiency or pathogenicity. Although the 2009 H1N1 virus NS1 protein (2009/NS1) is an effective interferon antagonist, we found that this NS1 (unlike those of previous human-adapted influenza A viruses) is unable to block general host gene expression in human or swine cells. This property could be restored in 2009/NS1 by replacing R108, E125, and G189 with residues corresponding to human virus consensus. Mechanistically, these previously undescribed mutations acted by increasing binding of 2009/NS1 to the cellular pre-mRNA processing protein CPSF30. A recombinant 2009 H1N1 influenza A virus (A/California/04/09) expressing NS1 with these gain-of-function substitutions was more efficient than the wild type at antagonizing host innate immune responses in primary human epithelial cells. However, such mutations had no significant effect on virus replication in either human or swine tissue culture substrates. Surprisingly, in a mouse model of pathogenicity, the mutant virus appeared to cause less morbidity, and was cleared faster, than the wild type. The mutant virus also demonstrated reduced titers in the upper respiratory tracts of ferrets; however, contact and aerosol transmissibility of the virus was unaffected. Our data highlight a potential human adaptation of NS1 that seems absent in “classically derived” swine-origin influenza A viruses, including the 2009 H1N1 virus. We discuss the impact that a natural future gain of this NS1 function may have on the new pandemic virus in humans.

In April 2009, an antigenically distinct swine-origin H1N1 influenza A virus was detected in humans (1). This virus has since spread efficiently around the world, leading to the declaration of a global pandemic by the World Health Organization on 11 June 2009. Although infection with the virus is generally associated with a mild, self-limiting influenza-like disease in the majority of people, the young and those with certain underlying conditions (including asthma, diabetes, heart/lung problems, morbid obesity, and pregnancy) seem at greater risk of severe disease progression (25). Lack of significant preexisting immunity to the novel virus in those <60 years old (20, 34) may account for the observation that younger people are more susceptible to this virus (25).

Sequence analysis of the 2009 pandemic H1N1 influenza A virus genome has failed to identify any previously recognized virulence markers (13, 16, 23). Nevertheless, animal studies have indicated that the 2009 H1N1 virus is slightly more pathogenic than contemporary human seasonal H1N1 viruses (23, 33, 37). In ferrets, the pandemic H1N1 virus replicated to titers higher than those of seasonal H1N1 viruses in the upper respiratory tract (23, 33, 37), and it could be detected deeper in the lungs (23, 37) and intestinal tracts (33) of infected animals, which is unusual. In a mouse model, the 2009 H1N1 virus also replicated more efficiently and caused greater morbidity and mortality than seasonal influenza viruses (23, 33). Furthermore, mice infected with the 2009 H1N1 virus produced higher levels of several proinflammatory cytokines and chemokines, including gamma interferon (IFN-γ), interleukin-4 (IL-4), IL-5, IL-12, MIP1α, MIP1β, and RANTES (23). Similar data for the 2009 H1N1 virus with respect to its efficient replication and significant induction of cytokines (together with severe lung pathology) have also been obtained from experimentally infected cynomolgus macaques (23). As might be expected given its swine origin, the 2009 H1N1 virus replicates well in pigs, although whether the virus causes significant disease in this host is unclear (23, 29). In experimental infections of avian species, the 2009 H1N1 virus is unable to replicate efficiently or cause disease (2, 53).

Influenza A virus virulence is a polygenic trait. Multiple unidentified molecular determinants are likely responsible for the ability of the 2009 H1N1 influenza A virus to cause increased disease severity in certain host species. Here, we have focused on the nonstructural (NS1) protein of the 2009 H1N1 virus (2009/NS1). NS1 performs a number of diverse roles in infected cells. However, such mutations had no significant effect on virus replication in either human or swine tissue culture substrates. Surprisingly, in a mouse model of pathogenicity, the mutant virus appeared to cause less morbidity, and was cleared faster, than the wild type. The mutant virus also demonstrated reduced titers in the upper respiratory tracts of ferrets; however, contact and aerosol transmissibility of the virus was unaffected. Our data highlight a potential human adaptation of NS1 that seems absent in “classically derived” swine-origin influenza A viruses, including the 2009 H1N1 virus. We discuss the impact that a natural future gain of this NS1 function may have on the new pandemic virus in humans.

In April 2009, an antigenically distinct swine-origin H1N1 influenza A virus was detected in humans (1). This virus has since spread efficiently around the world, leading to the declaration of a global pandemic by the World Health Organization on 11 June 2009. Although infection with the virus is generally associated with a mild, self-limiting influenza-like disease in the majority of people, the young and those with certain underlying conditions (including asthma, diabetes, heart/lung problems, morbid obesity, and pregnancy) seem at greater risk of severe disease progression (25). Lack of significant preexisting immunity to the novel virus in those <60 years old (20, 34) may account for the observation that younger people are more susceptible to this virus (25).

Sequence analysis of the 2009 pandemic H1N1 influenza A virus genome has failed to identify any previously recognized virulence markers (13, 16, 23). Nevertheless, animal studies have indicated that the 2009 H1N1 virus is slightly more pathogenic than contemporary human seasonal H1N1 viruses (23, 33, 37). In ferrets, the pandemic H1N1 virus replicated to titers higher than those of seasonal H1N1 viruses in the upper respiratory tract (23, 33, 37), and it could be detected deeper in the lungs (23, 37) and intestinal tracts (33) of infected animals, which is unusual. In a mouse model, the 2009 H1N1 virus also replicated more efficiently and caused greater morbidity and mortality than seasonal influenza viruses (23, 33). Furthermore, mice infected with the 2009 H1N1 virus produced higher levels of several proinflammatory cytokines and chemokines, including gamma interferon (IFN-γ), interleukin-4 (IL-4), IL-5, IL-12, MIP1α, MIP1β, and RANTES (23). Similar data for the 2009 H1N1 virus with respect to its efficient replication and significant induction of cytokines (together with severe lung pathology) have also been obtained from experimentally infected cynomolgus macaques (23). As might be expected given its swine origin, the 2009 H1N1 virus replicates well in pigs, although whether the virus causes significant disease in this host is unclear (23, 29). In experimental infections of avian species, the 2009 H1N1 virus is unable to replicate efficiently or cause disease (2, 53).

Influenza A virus virulence is a polygenic trait. Multiple unidentified molecular determinants are likely responsible for the ability of the 2009 H1N1 influenza A virus to cause increased disease severity in certain host species. Here, we have focused on the nonstructural (NS1) protein of the 2009 H1N1 virus (2009/NS1). NS1 performs a number of diverse roles in infected cells. However, such mutations had no significant effect on virus replication in either human or swine tissue culture substrates. Surprisingly, in a mouse model of pathogenicity, the mutant virus appeared to cause less morbidity, and was cleared faster, than the wild type. The mutant virus also demonstrated reduced titers in the upper respiratory tracts of ferrets; however, contact and aerosol transmissibility of the virus was unaffected. Our data highlight a potential human adaptation of NS1 that seems absent in “classically derived” swine-origin influenza A viruses, including the 2009 H1N1 virus. We discuss the impact that a natural future gain of this NS1 function may have on the new pandemic virus in humans.

In April 2009, an antigenically distinct swine-origin H1N1 influenza A virus was detected in humans (1). This virus has since spread efficiently around the world, leading to the declaration of a global pandemic by the World Health Organization on 11 June 2009. Although infection with the virus is generally associated with a mild, self-limiting influenza-like disease in the majority of people, the young and those with certain underlying conditions (including asthma, diabetes, heart/lung problems, morbid obesity, and pregnancy) seem at greater risk of severe disease progression (25). Lack of significant preexisting immunity to the novel virus in those <60 years old (20, 34) may account for the observation that younger people are more susceptible to this virus (25).

Sequence analysis of the 2009 pandemic H1N1 influenza A virus genome has failed to identify any previously recognized virulence markers (13, 16, 23). Nevertheless, animal studies have indicated that the 2009 H1N1 virus is slightly more pathogenic than contemporary human seasonal H1N1 viruses (23, 33, 37). In ferrets, the pandemic H1N1 virus replicated to titers higher than those of seasonal H1N1 viruses in the upper respiratory tract (23, 33, 37), and it could be detected deeper in the lungs (23, 37) and intestinal tracts (33) of infected animals, which is unusual. In a mouse model, the 2009 H1N1 virus also replicated more efficiently and caused greater morbidity and mortality than seasonal influenza viruses (23, 33). Furthermore, mice infected with the 2009 H1N1 virus produced higher levels of several proinflammatory cytokines and chemokines, including gamma interferon (IFN-γ), interleukin-4 (IL-4), IL-5, IL-12, MIP1α, MIP1β, and RANTES (23). Similar data for the 2009 H1N1 virus with respect to its efficient replication and significant induction of cytokines (together with severe lung pathology) have also been obtained from experimentally infected cynomolgus macaques (23). As might be expected given its swine origin, the 2009 H1N1 virus replicates well in pigs, although whether the virus causes significant disease in this host is unclear (23, 29). In experimental infections of avian species, the 2009 H1N1 virus is unable to replicate efficiently or cause disease (2, 53).

Influenza A virus virulence is a polygenic trait. Multiple unidentified molecular determinants are likely responsible for the ability of the 2009 H1N1 influenza A virus to cause increased disease severity in certain host species. Here, we have focused on the nonstructural (NS1) protein of the 2009 H1N1 virus (2009/NS1). NS1 performs a number of diverse roles in infected cells. However, such mutations had no significant effect on virus replication in either human or swine tissue culture substrates. Surprisingly, in a mouse model of pathogenicity, the mutant virus appeared to cause less morbidity, and was cleared faster, than the wild type. The mutant virus also demonstrated reduced titers in the upper respiratory tracts of ferrets; however, contact and aerosol transmissibility of the virus was unaffected. Our data highlight a potential human adaptation of NS1 that seems absent in “classically derived” swine-origin influenza A viruses, including the 2009 H1N1 virus. We discuss the impact that a natural future gain of this NS1 function may have on the new pandemic virus in humans.
and (ii) the global posttranscriptional inhibition of CPSF30-mediated cellular pre-mRNA processing (8, 38). Both of these activities appear to contribute to the replication efficiency of human influenza A viruses, and both likely play roles in determining virulence (8, 11, 12, 55).

There are recently documented examples of nonhuman-adapted virus strains (both laboratory and naturally occurring) that encode NS1 proteins lacking the ability to inhibit CPSF30 (21, 27, 55). The commonly used egg- and mouse-adapted human-derived influenza virus A/Puerto Rico/8/34 (PR/34) NS1 protein has amino acid substitutions at residues 103 and 106 and is consequently unable to bind CPSF30 or block general gene expression (21, 27). The NS1 proteins of two avian-derived influenza A viruses (the highly pathogenic A/Hong Kong/483/97 [H5N1] strain that was transmitted to humans in 1997 and its presumed precursor A/Teal/Hong Kong/W312/97 [H6N1]) are additionally unable to inhibit CPSF30 in either human or avian cells, an intrinsic defect in NS1 function that also maps to substitutions at positions 103 and 106 (55). Sequence analysis indicates that such substitutions are common among avian H6 and H9 hemagglutinin (HA) subtypes, and it has been suggested that these viruses may tolerate (or even be selected for) inefficient CPSF30 binding (55). Furthermore, NS1-CPSF30 complex-destabilizing substitutions at positions 103 or 106 have been reported to arise when viruses are adapted to replicate well in certain new host species (e.g., duck to quail [22] and human to mouse [5]). The biological reasons and apparent selection pressures required for some virus strains to modulate their NS1-CPSF30 binding affinities in particular hosts are unclear.

The ability of swine-derived influenza virus NS1 proteins to function efficiently in human cells has not yet been fully tested. Galvanized by the worldwide spread of swine-origin H1N1 influenza A virus in humans, we assessed the effectiveness of this pandemic virus gene product to antagonize the human innate immune response. Surprisingly, we found that the 2009/2010 swine NS1 protein is unable to block general host gene expression in both human and swine cells. This is a consequence of previously undescribed amino acid changes in NS1 that reduce its binding to CPSF30. Such changes appear to have occurred as soon as the parental virus from which this NS1 is derived (likely the 1918 pandemic H1N1 influenza A virus) was introduced into pigs and established the current “classical” swine H1N1 lineage. Here, we characterize the functional consequences of restoring this lost function in the 2009 pandemic H1N1 virus with specific regard to host innate immune suppression, virus pathogenicity, and virus transmissibility in mouse and ferret animal models.

MATERIALS AND METHODS

Cells. 293T (human), A549 (human), MDCK (canine), and PK-15 (swine) cells were purchased from the American Type Culture Collection (ATCC; VA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Gibco Life Technologies, CA). Cryopreserved primary human tracheobronchial epithelial cells (HTBE; Clonetics; Lonza Walkerville, MD) were cultivated as previously described (14). Briefly, after being thawed, cells were passaged twice in bronchial epithelial growth medium (BEGM; Clonetics) supplemented with rinitic acid. For full differentiation and generation of an air-liquid interface, cells were seeded on collagen-coated 12-mm Transwell clear permeable filters (0.4-µm pores [Corning Inc., MA]; Collagen I from human placenta [Sigma-Aldrich, MO]) at a density of 2 × 10⁴ cells/filter. Cells were submerged for 1 week in a 1:1 mixture of DMEM and BGM (Gray’s medium) containing necessary supplements and growth factors. At confluence, medium was removed from the apical surface, and cells were maintained at the air-liquid interface for at least 2 weeks. Medium was replaced every second day when cells were submerged and daily when cells were incubated at the air-liquid interface.

Plasmids. Mammalian expression constructs for untagged NS1 under the control of the chicken β-actin promoter (pCAGGS vector [40]) have been described previously for A/Puerto Rico/8/34 (PR/34 [54]), A/Texas/36/91 (Tx/91 [27]), A/Brevig Mission/1/18 (BM/18 [3]), and A/Swine/Texas/4199-2/98 (Sw/Tx/98 [11]). To generate an expression construct for A/California/04/09 NS1 (Cal/09), cDNA encoding this NS1 protein was ligated into pCAGGS using the EcoRI and NheI restriction sites. Four-primer overlap PCR was used to introduce site-directed point mutations into the NS1-encoding cDNAs. All NS1-encoding cDNAs also contained silent mutations in the splice acceptor site in order to prevent expression of nuclear export protein (NED)/NS2 (3). A pCAGGS vector expressing glutathione S-transferase (GST) was kindly provided by Luis Martinez-Sobrido (University of Rochester Medical Center, NY). A pCAGGS vector expressing C-terminal FLAG-tagged human CPSF30 has been described previously (27). T7-driven expression constructs for wild-type (WT) or mutant Cal/09 NS1 proteins were generated by ligating the appropriate PCR-amplified cDNAs between the NcoI and BamHI restriction sites of pTMT1 (36). The pcDNA3 plasmid encoding HA-tagged Tx/91 NS1 has been described previously (27). The reporter plasmid carrying the firefly luciferase (FF-Luc) gene under the control of the IFN-β promoter (pL52Luc) was kindly provided by Takashi Fujita (Kyoto University, Japan) (59). The reporter plasmid carrying the Renilla luciferase gene (REN-Luc) under the control of the constitutively active herpes simplex virus thymidine kinase (HSV-TK) promoter (pRL-TK) was purchased from Promega, WI. The identity of each construct generated or used was confirmed by sequencing.

Viruses. Stocks of Sendai virus (SeV; Cantell strain) were propagated in 10-day old embryonated chicken eggs. The reference 2009 pandemic H1N1 influenza A virus isolate, A/California/04/09 (Cal/09), was obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, GA), and propagated in MDCK cells. Recombinant Cal/09 (rCal/09 WT) was rescued according to previously published protocols (10, 16, 46), with minor modifications. Briefly, seven bidirectional pDZ-based Cal/09 RNA expression plasmids (HA, neuraminidase [NA], matrix [M], nucleoprotein [NP], PA, PB1, and PB2 plasmids) were cotransfected with a pRLPL-based Cal/09 NS viral RNA (vRNA) expression plasmid into 293T cells. At 24 h posttransfection, growth medium was replaced with serum-free medium containing 1 µg/ml tunicamycin and chloroform. At 48 h posttransfection, the rescue supernatant was subjected to plaque assay with MDCK cells in order to obtain clonal isolates. For rescue of the rCal/09 TripleMut virus (NS1 amino acid substitutions R108K, E125D, and G189D), site-directed mutagenesis was used to introduce the required mutations into pP01/Cal/09-NS with a QuikChange II site-directed mutagenesis kit (Stratagene, CA). The identity of the generated rCal/09 TripleMut virus was confirmed by DNA sequencing. Rescue of the mutant virus was performed as described for the wild type. Rescued viruses were propagated in MDCK cells, and the genotypes of the rCal/09 WT and TripleMut viruses were confirmed by reverse transcription-PCR (RT-PCR) and sequencing of the entire NS segments. As required by the Mount Sinai School of Medicine biosafety committee, work involving Cal/09 viruses was carried out in either a U.S. Department of Agriculture- and CDC-approved enhanced biosafety level 3 (BSL3+) containment laboratory or a dedicated BSL2 laboratory with personnel adhering to BSL3 working practices.

Reporter assays. For analysis of IFN-β promoter activation, 293T cells in 12-well plates were transfected with 25 ng of pL25Luc and 2 µg of the indicated NS1 (or GST) expression plasmid using FuGene6 (Roche, WI). After 16 h, the cells were infected with approximately 1 PFU/cell of SeV for 12 h. Cells were harvested and lysed in 200 µl of passive lysis buffer (Promega, WI), and FF-Luc activity was determined using a luminometer. To measure gene expression under the control of the constitutively active HSV-TK promoter, 293T or PK-15 cells in 12-well plates were cotransfected with 50 ng of pRL-TK and 2 µg of the indicated NS1 (or GST) constructs using FuGen6 (Roche, WI). Expression of REN-Luc activity was measured 24 h posttransfection as directed by the manufacturer (Promega, WI). All transfections were carried out in triplicate, and experiments were independently repeated at least twice.

SDS-PAGE, Western blotting, and immunoprecipitations. Cells were lysed in disruption buffer (6 M urea, 2 M β-mercaptoethanol, 4% SDS), sonicated to shear nuclear acids, and boiled for 5 min prior to polypeptide separation by SDS-PAGE on 4 to 15% Tris-HCl gradient gels (Bio-Rad Laboratories, CA). Proteins were detected either by Coomassie blue staining or by Western blotting.
following transfection to polyvinylidene difluoride (PVDF) membranes. The rabbit anti-tubulin and mouse anti-FLAG antibodies were from Sigma-Aldrich, MO. Rabbit polyclonal anti-serum to detect NP and rabbit polyclonal anti-serum raised against a GST-NS1 RNA binding domain (RBD) fusion protein (that detects both GST and NS1) have been described previously (49). To assess the interaction of different Cal/09 NS1 constructs with FLA-tagged CPSF30, [35S]methionine-labeled NS1 proteins were synthesized in vitro from pTM1-Cal/09-NS1 expression plasmids using a Tnt transcription/translation kit (Promega, WI). 293T cells transiently expressing FLAG-CPSF30 were lysed in 50 mM Tris-HCl (pH 7.8), 500 mM NaCl, 5 mM EDTA, and 0.5% NP-40, supplemented with a complete Mini protease inhibitor cocktail (Roche, IN). Following sonication, clarified cell lysates expressing FLAG-CPSF30 (or not) were incubated for 2 h at 4°C with the radiolabeled NS1 proteins and 25 µl of anti-FLAG affinity resin (Sigma-Aldrich, MO). After being washed extensively, precipitated proteins were dissociated from the resin using disruption buffer and analyzed by SDS-PAGE followed by Coomassie blue staining or Western blot. The [35S]methionine-labeled NS1 proteins were detected by phosphorimager analysis.

Poly(rI)-poly(rC)-Sepharose coprecipitations. A549 cells were infected with the appropriate virus at a multiplicity of infection (MOI) of 0.001 PFU/cell, and lysates were prepared after 16 h in 50 mM Tris-HCl (pH 7.8), 500 mM NaCl, 5 mM EDTA, and 0.5% NP-40, supplemented with a complete Mini protease inhibitor cocktail (Roche, IN). Following sonication, clarified lysates were incubated for 2 h at 4°C with poly(rI)-poly(rC) (pL-C) Sepharose (generated as described previously [6]) or Sepharose only. After extensive washing, precipitated proteins were dissociated from the resin using disruption buffer and analyzed by SDS-PAGE followed by Western blotting.

Quantitative real-time PCR (qPCR). Cultures of primary differentiated HTBEs were washed extensively with phosphate-buffered saline (PBS) (to remove accumulated mucus) and infected in triplicate with the appropriate virus at a MOI of 0.001 PFU/cell (in the absence of exogenous TPCK-treated trypsin). Following infection, cells were maintained at the air-liquid interface. Culture supernatants were harvested at various times postinfection by the addition of PBS to the apical surface of the cells for 30 min. Growth analysis results for PK-15 cells were essentially the same, albeit no air-liquid interface was established and cells were maintained throughout in Opti-MEM (Gibco Life Technologies, CA) supplemented with 3% bovine serum albumin and 1 µg/ml TPCK-treated trypsin. Virus titers were determined by plaque assay using MDCK cells. Plaques were visualized either by crystal violet staining or by immunostaining with a rabbit polyclonal anti-serum raised against whole inactivated virus (A/Puerto Rico/8/34 [49]).

qRT-PCR. Undifferentiated HTBEs in 12-well plates were infected in triplicate (or mock) with virus at an MOI of 5 PFU/cell. At various times postinfection, cells were lysed and total RNA extracted using an RNaseasy mini kit (Qiagen, CA) according to the manufacturer’s instructions. All samples were subjected to DNase treatment (Qiagen, CA). For reverse transcription, 3 µg of total RNA and 0.5 µg oligo(dT) primer (in a total volume of 12.5 µl) were heated for 10 min at 65°C and the addition of enzyme reaction buffer, 20 units Ribolock RNase inhibitor, 20 nmol deoxynucleoside triphosphates (dNTPs), and 200 units RevertAid M-MuLV reverse transcriptase (Fermentas, Inc., MD), samples were heated at 42°C for 1 h. Reverse transcriptase activity was subsequently inactivated at 72°C for 10 min. The resulting cDNA product acted as the template for quantitative real-time PCR (qPCR) using a Brilliant QPCR SYBR green Master Mix system (Stratagene, TX) and a Roche Light Cycler 480. The assay was performed according to the manufacturers’ instructions, and the fragment of interest was amplified in 40 cycles. Primer sequences used are available upon request. Samples were tested in triplicate, and cDNA levels were normalized to the GAPDH levels (or mock) with virus at an MOI of 5 PFU/cell. At various times postinfection, cells were lysed in 40 cycles. Primer sequences used are available upon request.

Mouse experiments. All mouse procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Maryland and were carried out in a USDA-approved BSL3+ containment laboratory. The basic transmission study scheme consisted of duplicate groups of three 6- to 8-month-old female Fisher ferrets (Triple F Farms, PA): one infected intranasally (with 106 50% tissue culture infective doses [TCID50] of virus in PBS, 600 µl per nostril), one by direct contact, and one by respiratory droplet contact. Nasal washes were collected daily. For tissue collection, infected ferrets were humanely euthanized on day 3 postinfection, and nasal turbinate, trachea, and lung samples were collected. All mouse experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Mount Sinai Laboratory, ME) were anesthetized using ketamine-xylazine and intranasally infected with the indicated dose of virus diluted in 50 µl PBS, 600 µl per nostril. Lungs were excised from euthanized mice and homogenized in 1 ml PBS supplemented with 0.2% bovine serum albumin and penicillin/streptomycin using a mechanical homogenizer (MP Biochemicals, OH). After centrifugation (10,000 × g, 5 min, 4°C), the resulting supernatants were used to determine viral titer, and pelleted tissue was used for RNA extraction using TRIzol (Invitrogen, CA).

Ferret experiments. All ferret procedures were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Maryland and were carried out in a USDA-approved BSL3+ containment laboratory. The basic transmission study scheme consisted of duplicate groups of three 6- to 8-month-old female Fisher ferrets (Triple F Farms, PA): one infected intranasally (with 106 50% tissue culture infective doses [TCID50] of virus in PBS, 600 µl per nostril), one by direct contact, and one by respiratory droplet contact. Nasal washes were collected daily. For tissue collection, infected ferrets were humanely euthanized on day 3 postinfection, and nasal turbinate, trachea, and lung samples were collected. All mouse experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of the University of Maryland and were carried out in a USDA-approved BSL3+ containment laboratory. The basic transmission study scheme consisted of duplicate groups of three 6- to 8-month-old female Fisher ferrets (Triple F Farms, PA): one infected intranasally (with 106 50% tissue culture infective doses [TCID50] of virus in PBS, 600 µl per nostril), one by direct contact, and one by respiratory droplet contact. Nasal washes were collected daily. For tissue collection, infected ferrets were humanely euthanized on day 3 postinfection, and nasal turbinate, trachea, and lung samples were collected. All mouse experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Mount Sinai Laboratory, ME) were anesthetized using ketamine-xylazine and intranasally infected with the indicated dose of virus diluted in 50 µl PBS, 600 µl per nostril. Lungs were excised from euthanized mice and homogenized in 1 ml PBS supplemented with 0.2% bovine serum albumin and penicillin/streptomycin using a mechanical homogenizer (MP Biochemicals, OH). After centrifugation (10,000 × g, 5 min, 4°C), the resulting supernatants were used to determine viral titer, and pelleted tissue was used for RNA extraction using TRIzol (Invitrogen, CA).

RESULTS

IFN-antagonistic properties of the 2009/NS1 protein. We tested the ability of the swine-origin 2009 pandemic H1N1 virus NS1 protein to limit production of IFN-β in human cells and compared it with a panel of other NS1 proteins known to inhibit (or not [21, 27]) general gene expression by binding CPSF30. 293T cells were transfected with a firefly luciferase IFN-β promoter reporter construct together with expression plasmids for the NS1 proteins of A/Puerto Rico/8/34 (PR/34), A/Texas/36/91 (Tx91), A/Brevig Mission/1/1918 (BM/18), A/Swine/Texas/4199-2/98 (Sw/Tx98), or a representative isolate of the 2009 pandemic H1N1 influenza A virus, A/California/04/09 (Cal/09). A GST expression plasmid served as a negative control. Sixteen hours posttransfection, cells were infected with SeV for a further 12 h prior to analysis of FF-Luc activity. As shown in Fig. 1, SeV infection induced robust amounts of IFN-β promoter-driven FF-luciferase activity in GST-expressing cells (set to 100%) but not in cells expressing any of the NS1 proteins (<4%), including the NS1

![FIG. 1. Inhibition of IFN-β induction by different influenza A virus NS1 proteins. 293T cells were cotransfected for 16 h with a pCAGGS expression plasmid encoding the indicated NS1 protein (or GST) together with a FF-Luc IFN-β-promoter reporter plasmid (pL25Luc). After infection with SeV for a further 12 h, FF-Luc activity was determined. The zoomed inset highlights differences between NS1 proteins. Results represent the means and standard deviations of triplicate values (normalized to GST plus SeV) obtained in a single experiment and are representative of results of two independent experiments.](image-url)
Despite this clear inhibition by all NS1 proteins tested, there was a minor (yet statistically significant) difference: while the NS1 proteins of both Tx/91 and BM/18 completely blocked induction of FF-Luc activity, the NS1 proteins of PR/34, Sw/Tx/98, and Cal/09 were unable to inhibit a small fraction of activity (i.e., 3 to 4%) (Fig. 1, inset). Given that a major phenotypic difference between the NS1 proteins of PR/34 and Tx/91 is their ability to bind CPSF30 and thus to limit gene expression (27), we postulated that the incomplete block demonstrated by both Sw/Tx/98 and Cal/09 NS1 proteins was, as with PR/34, due to an inability to inhibit CPSF30. We suspect that pretranscriptional block of TRIM25/RIG-I by NS1 (11, 15, 27, 35, 43, 44, 54) accounts for the majority of IFN-β promoter inhibition in our assay.

The 2009/NS1 protein is unable to block general host gene expression in either human or swine cells. To formally test whether the Cal/09 NS1 protein is unable to block general host gene expression, we cotransfected a constitutively active Renilla luciferase reporter construct (pRL-TK) with each of the indicated NS1 constructs and measured total Renilla luciferase activity 24 h later. Previous data from our laboratory have demonstrated a clear link between the ability of NS1 proteins to bind CPSF30 and their ability to block Renilla luciferase reporter activity in this assay (27). As shown in Fig. 2A, only the NS1 proteins of Tx/91 and BM/18 efficiently inhibited Renilla luciferase activity in human 293T cells, while the NS1 proteins of PR/34 and the swine-derived Sw/Tx/98 and Cal/09 (like GST) were largely deficient in this function. We also analyzed the total amounts of GST or NS1 protein in these transfected cell lysates. As expected, the NS1 proteins that efficiently blocked Renilla luciferase activity (Tx/91 and BM/18) were barely detectable (Fig. 2B), presumably because the small amount of NS1 protein made is sufficient to limit maturation of further RNA polymerase II transcripts from its own transfected plasmid, as well as from the Renilla luciferase plasmid. In contrast, the NS1 proteins of PR/34, Sw/Tx/98, and Cal/09 were expressed well (Fig. 2B), probably because they are unable to block their own expression. These observations are entirely consistent with previous results obtained by comparing NS1 proteins able (or not able) to bind and inhibit CPSF30 (27).

Given that both Sw/Tx/98 and Cal/09 NS1 proteins are derived from swine-origin viruses and therefore may specifically not function in human cells, we tested whether these NS1 proteins were better adapted to block general gene expression in swine cells. Thus, we also performed the Renilla luciferase reporter activity assays with transfected PK-15 cells, a porcine kidney epithelial cell line. However, as shown in Fig. 2C and D, the results obtained were essentially identical to those obtained using a human cell line: Tx/91 and BM/18 NS1 proteins inhibited gene expression (of both the REN-Luc reporter and themselves), while the NS1 proteins of PR/34, Sw/Tx/98, and Cal/09 did not. This result suggests that the inability of swine-origin influenza virus NS1 proteins to block gene expression is not restricted to nonswine cells. Furthermore, in agreement with the hypothesis that failure of these NS1 proteins to block gene expression is related to their inability to bind CPSF30, the CPSF30 amino acid sequences between human and swine are 99.6% identical (243 of 244 residues; G. Pisanelli and A. Garcia-Sastre, unpublished data).

Substitutions of residues 108, 125, and 189 in the 2009/NS1 protein restore its ability to inhibit general gene expression.
and to bind CPSF30. The interaction between NS1 and CPSF30 is mediated primarily by the NS1 effector domain (ED; residues 85 to 203) and two zinc fingers of CPSF30 (the F2F3 region) (8, 56). Based on the assumption that the NS1 phenotypes were due to their differential abilities to bind CPSF30, we aligned the amino acid sequences of the NS1 EDs used in this study (Fig. 3A). Overall, the EDs are highly conserved, and the few amino acid residues of NS1 that have been experimentally implicated in CPSF30 binding to date (e.g., 103, 106, and 184 to 188 [8, 27, 30, 41, 55]) (Fig. 3A, gray shading) are no different between the NS1 proteins of BM/18 or Tx/91 (which block gene expression) and Sw/Tx/98 or Cal/09 (which lack this function). Furthermore, residues 103 and 106 (which determine the lack of CPSF30 binding for PR/34 NS1) are also the human “binding” consensus for the Cal/09 NS1 protein. In fact, there are only nine amino acid positions in the ED that consistently differ between the swine-origin viruses and all the “human-like” viruses (Fig. 3A, black boxes). Notably, three of these residues, 108, 125, and 189 (Fig. 3B and C), are located at the direct interface between NS1 and CPSF30 (8), although none of these NS1 residues have previously been experimentally shown to contribute to CPSF30 binding.

We constructed Cal/09 NS1 expression plasmids with three single-amino-acid substitutions, R108K, E125D, and G189D (Cal/09 residues were changed to human influenza A virus consensus), and tested their ability to block general gene expression in our Renilla luciferase reporter activity assay. In human 293T cells, each mutant Cal/09 NS1 protein was able to limit luciferase activity significantly better than the WT Cal/09 NS1, with the G189D mutant inhibiting approximately 50% of the activity (Fig. 4A). However, compared with the result for Tx/91 NS1, the block was still relatively poor. We therefore...
made combinations of the mutations and tested their effect on gene expression. Although luciferase inhibition with the dual R108K/G189D mutant was better than that with the WT, only the Cal/09 NS1 with all three amino acid substitutions (R108K, E125D, and G189D; TripleMut) completely blocked gene expression to a level comparable to that of Tx/91 NS1 (Fig. 4A). As confirmation, we also observed that NS1 proteins best able to block luciferase activity also limited their own expression (Fig. 4B). This effect was not limited to human cells, as the same overall result was obtained with swine PK-15 cells (Fig. 4C and D, respectively).

In order to assess whether the three mutations, R108K, E125D, and G189D, also increased binding of Cal/09 NS1 to CPSF30, we tested the coprecipitation of in vitro-transcribed and -translated [35S]methionine-labeled NS1 (WT or TripleMut) with transiently expressed FLAG-tagged CPSF30. Under high-stringency conditions (500 mM NaCl), no detectable WT Cal/09 NS1 protein could be coprecipitated with FLAG-CPSF30 (Fig. 5A). However, the TripleMut Cal/09 NS1 clearly bound to FLAG-CPSF30 (Fig. 5A). The interaction of TripleMut Cal/09 NS1 with FLAG-CPSF30 was comparable to that of Tx/91 NS1 (Fig. 5A). We also performed immunoprecipitations using lower salt concentrations (200 mM NaCl), under which conditions a small fraction of WT Cal/09 NS1 was found to coprecipitate with FLAG-CPSF30, although binding efficiency was clearly enhanced for the TripleMut NS1 (Fig. 5B). The three mutations appeared to have a minimal overall effect on the ability of Cal/09 NS1 to bind synthetic double-stranded RNA (dsRNA), as revealed by pl: C pull-down assays (Fig. 5C). Together with data from the Renilla luciferase reporter activity assays, our results indicate that mutation of residues 108, 125, and 189 in Cal/09 NS1 to human consensus restores the ability of this NS1 protein to bind more efficiently to CPSF30 and consequently to inhibit cellular gene expression. Thus, although the amino acid substitutions made are conservative, they clearly have a dramatic impact on a specific NS1 function. It is likely that side chain length, as well as charge, determine overall NS1-CPSF30 complex stability.

In vitro characterization of a recombinant 2009 H1N1 virus expressing NS1 with the R108K, E125D, and G189D amino acid substitutions. We cloned and established a complete reverse genetics system for the Cal/09 influenza virus isolate (16). The rescued recombinant WT Cal/09 virus (rCal/09 WT) grew with kinetics identical to those of the parental nonrecombinant Cal/09 isolate in primary differentiated HTBEs (Fig. 6A). The rescued recombinant WT Cal/09 virus (rCal/09 WT) grew with kinetics identical to those of the parental nonrecombinant Cal/09 isolate in primary differentiated HTBEs (Fig. 6A). We also used this reverse genetics system to generate a recombinant mutant Cal/09 virus expressing NS1 with the R108K, E125D, and G189D amino acid substitutions (TripleMut). Unavoidably, when the G189D amino acid change in NS1 is generated, a V32I amino acid substitution also occurs in NEP/
the three NS1 mutations did not confer a selective growth advantage to rCal/09 TripMut over rCal/09 WT in the primary human cells (Fig. 6D), nor was this mutant significantly attenuated in the swine cell line (Fig. 6E). These data also indicate that the single conservative amino acid substitution (V32I) in NEP/NS2 does not affect the replication of Cal/09 in vitro.

We next tested the ability of the rCal/09 TripleMut virus to limit expression of IFN-β and IFN-stimulated genes during infection. Primary undifferentiated HTBEs were infected at an MOI of 5 PFU/cell with the rCal/09 WT or rCal/09 TripMut virus. At various times postinfection, total RNA was extracted and relative induction levels of the indicated viral and cellular mRNAs were quantified by qRT-PCR. As shown in Fig. 7A, the infectious titers reached after 24 h by the two viruses were identical. In addition, the levels of viral NA mRNA increased at the same rate for both rCal/09 WT and the rCal/09 TripMut viruses (Fig. 7B), consistent with the equal growth kinetics of these viruses observed with both primary differentiated HTBE cells and the swine PK-15 cell line (Fig. 6). Nevertheless, it should be noted that both replication and cytokine responses can differ depending upon the differentiation state of primary cells (7). However, as expected, the amount of IFN-β mRNA produced during infection with the rCal/09 TripMut virus was significantly lower than that produced by the rCal/09 WT virus (Fig. 7C). Furthermore, the mRNA levels of IFN-stimulated genes such as OAS, MxA, and IP10 (Fig. 7D, E, and F, respectively) were also significantly lower in rCal/09 TripMut virus-infected cells, probably as a result of both their direct inhibition (27) and the indirect effect of IFN-β limitation. We attribute this general immune-antagonistic phenotype to the enhanced capability of the rCal/09 TripMut NS1 protein to bind CPSF30 and block cellular gene expression. Such data are in full agreement with our observations using transiently expressed WT and TripMut Cal/09 NS1 proteins in the absence of virus infection (Fig. 4). Furthermore, these results indicate that for WT Cal/09 virus, the viral polymerase is unable to compensate wholly for the WT NS1 deficiency (e.g., by viral cap-snatching activity [26, 45] or virus-induced RNA polymerase II degradation [47, 57]). Nevertheless, as reported for other influenza A viruses with NS1 polymorphisms that also preclude direct CPSF30 binding (28, 55), it may be that the cognate WT Cal/09 viral polymerase complex allows a minor fraction of WT NS1 to associate with CPSF30 during infection, thereby contributing to partial innate immune suppression at the level of pre-mRNA processing. Any such inhibition is likely to be in addition to a pretranscriptional block by NS1 at the level of RIG-I/TRIM25 (11).

In vivo characterization of a recombinant 2009 H1N1 virus expressing NS1 with the R108K, E125D, and G189D amino acid substitutions. To test whether increased ability of NS1 to block general gene expression would affect in vivo viral replication and/or pathogenicity, we compared the rCal/09 WT and rCal/09 TripMut viruses by using a mouse model. Initial studies using 5 × 10⁶ PFU of the rCal/09 WT or rCal/09 TripMut virus showed that these viruses replicated to similar average titers in the lungs of infected congenic B6.A2G-Mx1 mice (day 2, 5.5 × 10⁶ PFU/ml for the WT versus 3.5 × 10⁶ PFU/ml for the TripMut virus; day 4, 4.2 × 10⁵ PFU/ml for the WT versus 2.0 × 10⁵ PFU/ml for the TripMut virus). However, no significant body weight loss after infection was observed in these mice (day 2, 5.5 × 10⁶ PFU/ml for the WT versus 3.5 × 10⁶ PFU/ml for the TripMut virus; day 4, 4.2 × 10⁵ PFU/ml for the WT versus 2.0 × 10⁵ PFU/ml for the TripMut virus). Therefore, we next examined the ability of the rCal/09 TripMut virus to cause body weight loss in mice infected with rCal/09 WT or rCal/09 TripMut virus. As shown in Fig. 7G, the infectious titers reached after 24 h by the two viruses were identical. In addition, the levels of viral NA mRNA increased at the same rate for both rCal/09 WT and the rCal/09 TripMut viruses (Fig. 7B), consistent with the equal growth kinetics of these viruses observed with both primary differentiated HTBE cells and the swine PK-15 cell line (Fig. 6). Nevertheless, it should be noted that both replication and cytokine responses can differ depending upon the differentiation state of primary cells (7). However, as expected, the amount of IFN-β mRNA produced during infection with the rCal/09 TripMut virus was significantly lower than that produced by the rCal/09 WT virus (Fig. 7C). Furthermore, the mRNA levels of IFN-stimulated genes such as OAS, MxA, and IP10 (Fig. 7D, E, and F, respectively) were also significantly lower in rCal/09 TripMut virus-infected cells, probably as a result of both their direct inhibition (27) and the indirect effect of IFN-β limitation. We attribute this general immune-antagonistic phenotype to the enhanced capability of the rCal/09 TripMut NS1 protein to bind CPSF30 and block cellular gene expression. Such data are in full agreement with our observations using transiently expressed WT and TripMut Cal/09 NS1 proteins in the absence of virus infection (Fig. 4). Furthermore, these results indicate that for WT Cal/09 virus, the viral polymerase is unable to compensate wholly for the WT NS1 deficiency (e.g., by viral cap-snatching activity [26, 45] or virus-induced RNA polymerase II degradation [47, 57]). Nevertheless, as reported for other influenza A viruses with NS1 polymorphisms that also preclude direct CPSF30 binding (28, 55), it may be that the cognate WT Cal/09 viral polymerase complex allows a minor fraction of WT NS1 to associate with CPSF30 during infection, thereby contributing to partial innate immune suppression at the level of pre-mRNA processing. Any such inhibition is likely to be in addition to a pretranscriptional block by NS1 at the level of RIG-I/TRIM25 (11).

In vivo characterization of a recombinant 2009 H1N1 virus expressing NS1 with the R108K, E125D, and G189D amino acid substitutions. To test whether increased ability of NS1 to block general gene expression would affect in vivo viral replication and/or pathogenicity, we compared the rCal/09 WT and rCal/09 TripMut viruses by using a mouse model. Initial studies using 5 × 10⁶ PFU of the rCal/09 WT or rCal/09 TripMut virus showed that these viruses replicated to similar average titers in the lungs of infected congenic B6.A2G-Mx1 mice (day 2, 5.5 × 10⁶ PFU/ml for the WT versus 3.5 × 10⁶ PFU/ml for the TripMut virus; day 4, 4.2 × 10⁵ PFU/ml for the WT versus 2.0 × 10⁵ PFU/ml for the TripMut virus). However, no significant body weight loss after infection was observed in these mice (day 2, 5.5 × 10⁶ PFU/ml for the WT versus 3.5 × 10⁶ PFU/ml for the TripMut virus; day 4, 4.2 × 10⁵ PFU/ml for the WT versus 2.0 × 10⁵ PFU/ml for the TripMut virus). Therefore, we next examined the ability of the rCal/09 TripMut virus to cause body weight loss in mice infected with rCal/09 WT or rCal/09 TripMut virus. As shown in Fig. 7G, the infectious titers reached after 24 h by the two viruses were identical. In addition, the levels of viral NA mRNA increased at the same rate for both rCal/09 WT and the rCal/09 TripMut viruses (Fig. 7B), consistent with the equal growth kinetics of these viruses observed with both primary differentiated HTBE cells and the swine PK-15 cell line (Fig. 6). Nevertheless, it should be noted that both replication and cytokine responses can differ depending upon the differentiation state of primary cells (7). However, as expected, the amount of IFN-β mRNA produced during infection with the rCal/09 TripMut virus was significantly lower than that produced by the rCal/09 WT virus (Fig. 7C). Furthermore, the mRNA levels of IFN-stimulated genes such as OAS, MxA, and IP10 (Fig. 7D, E, and F, respectively) were also significantly lower in rCal/09 TripMut virus-infected cells, probably as a result of both their direct inhibition (27) and the indirect effect of IFN-β limitation. We attribute this general immune-antagonistic phenotype to the enhanced capability of the rCal/09 TripMut NS1 protein to bind CPSF30 and block cellular gene expression. Such data are in full agreement with our observations using transiently expressed WT and TripMut Cal/09 NS1 proteins in the absence of virus infection (Fig. 4). Furthermore, these results indicate that for WT Cal/09 virus, the viral polymerase is unable to compensate wholly for the WT NS1 deficiency (e.g., by viral cap-snatching activity [26, 45] or virus-induced RNA polymerase II degradation [47, 57]). Nevertheless, as reported for other influenza A viruses with NS1 polymorphisms that also preclude direct CPSF30 binding (28, 55), it may be that the cognate WT Cal/09 viral polymerase complex allows a minor fraction of WT NS1 to associate with CPSF30 during infection, thereby contributing to partial innate immune suppression at the level of pre-mRNA processing. Any such inhibition is likely to be in addition to a pretranscriptional block by NS1 at the level of RIG-I/TRIM25 (11).
observed at this dose in these mice (data not shown). DBA/2J mice were therefore selected for subsequent experiments, as they are reported to be highly susceptible to influenza virus infection (4, 16, 51). Mice were infected with 10⁵ PFU of each virus (or PBS alone), and body weights were monitored daily for 14 days. Surprisingly, the rCal/09 TripleMut virus caused less weight loss in infected DBA2/J mice than did the rCal/09 WT virus (Fig. 8A). Furthermore, although in the lungs of mice infected with 10⁴ PFU of virus, the rCal/09 TripleMut virus appeared to reach mean peak infectious titers similar to those of the WT (Fig. 8B) (day 2, 2 × 10⁶ PFU/ml for the TripleMut virus versus 4 × 10⁶ PFU/ml for the WT), the rCal/09 TripleMut virus seemed to be cleared slightly faster (Fig. 8B) (day 7, 2.5 × 10³ PFU/ml for the TripleMut virus versus 3 × 10⁴ PFU/ml for the WT). Consistent with our data for infected undifferentiated HTBEs, quantitative analysis of IFN-β mRNA in infected mouse lungs indicated that the rCal/09 TripleMut virus induced less of an innate immune response than the WT (Fig. 8C). However, we cannot exclude the possibility that this is due to a slight attenuation of the TripleMut virus in vivo rather than efficient antagonism of the murine IFN system.

We also studied the replication abilities of the rCal/09 WT and rCal/09 TripleMut viruses in a ferret model. Ferrets were infected with 10⁶ TCID₅₀ of each virus, and the amount of infectious virus present in different parts of the respiratory tract was determined at 5 days postinfection. Consistent with the mouse data indicating that the rCal/09 TripleMut virus is cleared faster than the WT, less rCal/09 TripleMut virus was detected in the nasal turbinate of ferrets at this time point, compared with that of the WT (Fig. 9A). In addition, the amount of rCal/09 TripleMut virus in the lung appeared lower than that of the WT (Fig. 9A). However, similar titers for the two viruses were observed for the trachea (Fig. 9A). As previously described (50), the ferret model also enabled us to assess both the direct contact and aerosol respiratory droplet transmission dynamics of the two viruses. By analyzing daily nasal washes of infected and contact ferrets, we observed that total rCal/09 WT virus shedding was slightly higher than that of the rCal/09 TripleMut virus (Fig. 9B and C), possibly due to faster clearance of the TripleMut virus as seen with the DBA/2J mice. Nevertheless, the rCal/09 TripleMut virus was still transmitted as efficiently as the WT to other ferrets by both direct contact and respiratory droplet routes (Fig. 9B and C). These data suggest that, in contrast to our observations in vivo, gain-of-function mutations in NS1 that
enhance its ability to block general gene expression may reduce the overall replication and pathogenicity of Cal/09 in vivo.

**DISCUSSION**

While characterizing the 2009 pandemic H1N1 influenza A virus, we found that the NS1 protein of this virus lacks the ability to block general host gene expression in both human and swine cells. This is due to its inefficient binding to cellular CPSF30, a property otherwise maintained in circulating human influenza A viruses (55) that is reported to contribute to suppression of host innate immune responses (8, 41). By identifying and making novel human-adaptive mutations in NS1, we were able to introduce this function into a recombinant prototype 2009 H1N1 virus isolate, Cal/09. As with other influenza A viruses shown naturally to be defective in blocking CPSF30 function (e.g., A/Hong Kong/483/97 [H5N1] [55]), we found that restoration of CPSF30
**FIG. 8.** Replication and pathogenicity of the rCal/09 TripleMut virus in mice. Seven-week-old DBA/2J mice were infected intranasally with 10⁵ PFU of the rCal/09 WT or rCal/09 TripleMut virus. PBS-treated mice acted as a negative control. (A) Body weights were determined daily. Data show mean body weights of mice (n = 11). Error bars represent standard deviations. Significance was determined using two-tailed Student’s t test (**, P < 0.01; *, P < 0.05). (B) Lung titers were determined on days 2, 4, and 7 postinfection from three DBA/2J mice infected in parallel with 10⁴ PFU of the rCal/09 WT or rCal/09 TripleMut virus. Bars represent mean values. (C) Murine IFN-β mRNA was quantified from the lung homogenates used in panel B by qRT-PCR as described for Fig. 7.

**FIG. 9.** Replication and transmission dynamics of the rCal/09 TripleMut virus in ferrets. Six- to 8-month-old female Fitch ferrets were infected intranasally with 10⁶ TCID₅₀ of the rCal/09 WT or rCal/09 TripleMut virus. (A) Viral titers in nasal turbinates, trachea, and lungs were determined on day 5 postinfection from two infected ferrets. Bars represent mean values. (B and C) Nasal wash titers for rCal/09 WT (B) and rCal/09 TripleMut (C) viruses were determined from ferrets infected intranasally (n = 2), by direct contact (n = 2), and by aerosol contact (large and small respiratory droplets) (n = 2) on the days indicated. Bars (dark and light gray) represent the raw results of two independent experiments. To limit the number of animals used, the rCal/09 WT-infected ferret data shown are the same as previously described (19). All experiments were performed in parallel to allow fair comparisons.
binding resulted in a virus that induces less IFN-β mRNA (as well as other innate immune mRNAs) during infection.

In order to restore efficient CPSF30 binding, we rationally mutated three residues of Cal/09 NS1 to human H1N1 virus consensus based on the recently published crystal structure of an NS1 ED in complex with CPSF30-F2F3 (8). However, other uncharacterized amino acid changes that also contribute to restoration of CPSF30 binding independently of the three presented here may arise naturally. Nonetheless, these three amino acids (R108, E125, and G189) are highly conserved in the NS1 proteins of “classically derived” swine influenza A viruses, particularly within the H1 subtype (Table 1). The viruses isolated from swine that have other amino acids at these positions are either “spillover” viruses from other species (only a small percentage of human and avian H1 influenza A viruses have the swine consensus) or swine viruses of the “European avian-like” lineage. Based on this conservation, and the poor ability of Sw/Tx/98 NS1 to block general gene expression, we hypothesize that lack of binding and inhibition of CPSF30 is a general feature of classically derived swine influenza A virus NS1 proteins. Indeed, the NS1 protein of the first influenza virus isolated from pigs (A/Swine/Iowa/15/1930) already had R108, E125, and G189. This is in stark contrast to its human relative, the 1918 pandemic influenza virus (e.g., A/Brevig Mission/1/18), which had K108, D125, and D189 and clearly binds/inhibits CPSF30 (this study and references 27 and 55). Thus, it may be that nonbinding amino acid substitutions were selected for in the pig population after introduction of the precursor virus and have been maintained in the classical swine NS lineage until the present day. It should be noted that such nonbinding substitutions have yet to be detected in the European avian-like lineage of swine influenza A viruses that was established in 1979. It is possible that complete swine adaptation has yet to occur for this new lineage or that the classical and European avian-like lineages are evolving independently (9). In the latter regard, it may be that lineage-specific functional adaptation in NS1 is generated by a different set of mutations. We are planning to test the ability of European avian-like NS1 proteins to bind/inhibit CPSF30.

We had hypothesized that an enhanced ability to limit IFN-β production would increase replication of the rCal/09 virus in human cells. However, we were unable to detect an increase in viral replication due to this new function, either in primary human tracheobronchial epithelial cells or in a swine cell line. Furthermore, one might have expected the gain-of-function virus to grow better only in human cells, and worse in swine cells, particularly if the ability of NS1 to bind CPSF30 is a human-adaptive phenotype that is selected against in pigs. Thus, it may be that our experimental setup is not sensitive enough for detecting small phenotypic differences. It is possible that studies using additional primary substrates or cell types (particularly of swine origin) could aid in further dissecting the effect of these NS1 adaptations. We also cannot exclude the notion that the polymorphism changes we made to the Cal/09 NS1 protein negatively affect another of the multiple NS1 functions independently of their clear positive influence on CPSF30 binding. For example, the alterations we made are at one of the proposed dimeric interfaces of the NS1 effector domain (17), which is concomitant with the CPSF30 binding interface (8). Thus, slight alterations in the temporal dimeric packing of the TripleMut effector domain (or even the single-amino-acid change in NEP) may have slightly reduced virus replication, thereby countering any positive effect seen by restoring NS1-CPSF30 binding. Intriguingly, given that we only made naturally occurring polymorphism changes in the NS segment, such a possibility suggests that the potential “unknown function” lost by these mutations may actually be important and selected for in the classical swine lineage.

The contribution of NS1 to the higher replication efficiency and pathogenicity of the 2009 H1N1 virus (compared with those of seasonal H1N1 viruses [23, 33, 37]) is unknown. Nevertheless, the NS1 protein of this pandemic virus lacks previ-

### Table 1. Species- and subtype-specific identity of NS1 residues involved in CPSF30 binding

<table>
<thead>
<tr>
<th>Group</th>
<th>103</th>
<th>106</th>
<th>108</th>
<th>125</th>
<th>189</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>Y</td>
<td>X</td>
<td>M</td>
<td>X</td>
</tr>
<tr>
<td>Avian</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>71</td>
<td>29</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>H3</td>
<td>57</td>
<td>43</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>H6</td>
<td>45</td>
<td>23</td>
<td>33</td>
<td>88</td>
<td>33</td>
</tr>
<tr>
<td>H9</td>
<td>26</td>
<td>3</td>
<td>71</td>
<td>24</td>
<td>76</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>98</td>
<td>0</td>
<td>2</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>H3</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2009 H1N1</td>
<td>98</td>
<td>0</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Swine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>97</td>
<td>0</td>
<td>3</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>H3</td>
<td>97</td>
<td>0</td>
<td>3</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

*a The number of sequences for each group was as follows: avian, 99 H1, 293 H3, 400 H6, and 632 H9 sequences; human, 898 H1, 2,167 H3, and 1,568 2009 H1N1 sequences; swine, 302 H1 and 119 H3 sequences. Values are rounded to the nearest whole percentage point.

*b Percentage of NS1 sequences with certain amino acid residues located at positions 103, 106, 125, and 189. All sequences are derived from publicly available databases (as of November 2009, or April 2010 for the 2009 H1N1 virus) and were aligned prior to analysis. Bold, underlined residues contribute to stable CPSF30 binding. X represents “other” amino acids.
ously postulated virulence markers, such as deletion of residues 80 to 84 (32), a glutamic acid at position 92 (48), or the presence of a consensus C-terminal PDZ-domain ligand motif (24, 42). Furthermore, experimental introduction of naturally isolated specific functional mutations in the NS1 C-terminal tail did not alter replication, pathogenicity, or transmission of the 2009 H1N1 virus (19). In our in vivo DBA/2J mouse model of infection, the gain-of-function (TripleMut) virus was slightly less pathogenic, and appeared to be cleared faster, than the wild-type virus (which lacks the ability to inhibit CPSF30). Prima facie, these data suggest that inefficient CPSF30 inhibition may actually contribute to virulence of this swine-origin virus in mice. Although it has been noted that the wild-type Cal/09 virus induces large amounts of certain proinflammatory cytokines during in vivo infections of mice and cytomolgus macaques (23), it is not known if this is due solely to a defect in NS1 function. In addition, it is unclear whether this exuberant immune response plays any significant role in disease progression. To define the lack of CPSF30 binding as a bona fide virulence factor for the 2009 pandemic H1N1 influenza A virus, we would have to remove this function from an unrelated influenza A virus strain that naturally inhibits CPSF30 and assess its resulting pathogenicity. It may also be that the correlation of poor CPSF30 binding with virulence is a mouse-specific phenotype, particularly as many experimentally derived mouse-adapted viruses have mutations that at least partially destabilize the interaction of NS1 with CPSF30 (5, 27). If this is the case, it is interesting that the TripleMut virus also demonstrated lower viral titers in the nasal turbinates of ferrets. Of note, the mouse-adapted influenza virus strain A/WSN/33 (WSN/33) contains asparagine at position 189, rather than the aspartic acid consensus for efficient CPSF30 binding. Thus, although WSN/33 NS1 has been shown to interact with the F2F3 domain of CPSF30 (55), this interaction may be less efficient than that with NS1 proteins from non-mouse-adapted seasonal human viruses. If enhanced virulence in mice correlates with poor CPSF30 binding, then this may explain the previous observation that introduction of the BM/18 NS1 protein (which binds/inhibits CPSF30 very efficiently) into a recombinant WSN/33 virus significantly reduced its virulence in a mouse model (3).

The biological reasons for different influenza A viruses maintaining (or not) their ability to limit general gene expression via NS1 are unclear. Nevertheless, a pattern is now emerging indicating that this may be a viral strain- and host species-specific phenotype. Our data based on residues 108, 125, and 189 strongly suggest that swine influenza viruses commonly lack this function. Furthermore, an analysis of residues 103 and 106 (two major determinants of CPSF30 binding efficiency [27, 55]) shows that stable CPSF30 binding is also generally lost in avian H6 and H9 viruses (Table 1), particularly in H6 and H9 viruses that infect quail, pheasants, and partridges, rather than ducks and chickens. This observation may have already been confirmed experimentally when adapting a duck influenza virus to quail, as the resulting virus had the M106T amino acid change (22). We note that a surprising proportion of 2009 H1N1 viruses (approximately 2%) isolated since April 2009 also have amino acid substitutions at positions 103 or 106 in NS1 (Table 1). This is likely due to minimal selection pressure to maintain these CPSF30 binding residues given that the protein lacks this function already. Remarkably, most human H3 influenza A viruses appear to have the potential affinity-lowering glutamic acid residue at position 125, although presumably these NS1 proteins can all bind CPSF30 to some extent. Another notable subset of NS1 proteins are those classified as allele B, which are found exclusively in avian influenza A viruses (reviewed in reference 18). Almost all allele B NS1 proteins have tyrosine at position 103 and arginine at position 108. Thus, there may be a sliding scale of CPSF30 binding affinities from strong to weak, with each particular virus (in a particular host) replicating optimally with only a certain level of CPSF30 inhibition.

It is still unclear whether efficient CPSF30 binding by NS1 contributes to the human adaptation of influenza A viruses. However, now that the swine-origin 2009 pandemic H1N1 virus is circulating in humans, we may start to see a human virus consensus emerge at residues 108, 125, and 189. Indeed, nine 2009 pandemic H1N1 viruses with individual mutations at these positions (each with the potential to slightly increase CPSF30 binding) have already been isolated, although whether they are more fit is unknown. From a public health point of view, our in vivo data would suggest that in the current 2009 H1N1 viral genetic background, such amino acid changes in NS1 alone (or others that independently restore even a partial phenotype) will not dramatically increase virus pathogenicity or significantly affect virus transmissibility. Nevertheless, given our data for primary undifferentiated human tracheobronchial epithelial cells, where the rCal09 TripleMut virus seems better than the WT at suppressing the host innate immune response, we cannot exclude the possibility that potential future gain of this NS1 function will enhance replication of the 2009 pandemic H1N1 virus in humans. It may therefore be particularly prudent to survey newly emerging NS segment mutations, determine the corresponding NS1 protein phenotype, and assess any associations with clinical outcome.

ACKNOWLEDGMENTS

We are grateful to Peter Palese (Mount Sinai School of Medicine, NY) for helpful discussions and critical readings of the manuscript. We thank Osman Lizardo for excellent technical assistance and the CDC (Atlanta, GA), Yoshihiro Kawaoka (University of Wisconsin—Madison), Estanislao Nistal-Villan (Mount Sinai School of Medicine), Randy Albrecht (Mount Sinai School of Medicine), Takashi Fujita (Kyoto University, Japan), Peter Stacheli (University of Freiburg, Germany), and Luis Martinez-Sobrido (University of Rochester Medical Center, NY) for some of the reagents used in this study.

This work was partially supported by the National Institute of Allergy and Infectious Diseases (NIAID)-funded Center for Research in Influenza Pathogenesis (CRIP; contract HHSN266200700010C) and by NIAID grants RO1AI46954, U19AI83025, and PO1AI58113 (to A.G.-S.). J.S. is supported by a career development fellowship from the CDC. We thank Osman Lizardo for excellent technical assistance and the CDC (Atlanta, GA), Yoshihiro Kawaoka (University of Wisconsin—Madison), Estanislao Nistal-Villan (Mount Sinai School of Medicine), Randy Albrecht (Mount Sinai School of Medicine), Takashi Fujita (Kyoto University, Japan), Peter Stacheli (University of Freiburg, Germany), and Luis Martinez-Sobrido (University of Rochester Medical Center, NY) for some of the reagents used in this study.

REFERENCES


A virus is mediated by RIG-I which is regulated by the viral NS1 protein.


