

Attenuation of Equine Influenza Viruses through Truncations of the NS1 Protein

Michelle Quinlivan,^{1,2} Dmitriy Zamarin,¹ Adolfo García-Sastre,¹ Ann Cullinane,³
Thomas Chambers,⁴ and Peter Palese^{1*}

Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029¹; Department of Life Sciences, University of Limerick, Limerick, Ireland²; Virology Unit, Irish Equine Centre, Johnstown, Naas, County Kildare, Ireland³; and Department of Veterinary Science, Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546⁴

Received 17 November 2004/Accepted 7 March 2005

Equine influenza is a common disease of the horse, causing significant morbidity worldwide. Here we describe the establishment of a plasmid-based reverse genetics system for equine influenza virus. Utilizing this system, we generated three mutant viruses encoding carboxy-terminally truncated NS1 proteins. We have previously shown that a recombinant human influenza virus lacking the NS1 gene (delNS1) could only replicate in interferon (IFN)-incompetent systems, suggesting that the NS1 protein is responsible for IFN antagonist activity. Contrary to previous findings with human influenza virus, we found that in the case of equine influenza virus, the length of the NS1 protein did not correlate with the level of attenuation of that virus. With equine influenza virus, the mutant virus with the shortest NS1 protein turned out to be the least attenuated. We speculate that the basis for attenuation of the equine NS1 mutant viruses generated is related to their level of NS1 protein expression. Our findings show that the recombinant mutant viruses are impaired in their ability to inhibit IFN production in vitro and they do not replicate as efficiently as the parental recombinant strain in embryonated hen eggs, in MDCK cells, or in vivo in a mouse model. Therefore, these attenuated mutant NS1 viruses may have potential as candidates for a live equine influenza vaccine.

Equine influenza virus belongs to the *Orthomyxoviridae* family and is an enveloped, negative-sense RNA virus with a segmented, single-stranded genome. There are two distinct subtypes of equine influenza virus: subtype 1, H7N7, first isolated in Prague in 1956 (32), and subtype 2, H3N8, first isolated in Miami in 1963 (36). It is believed that subtype 1 is no longer in circulation, as the last confirmed outbreak caused by this virus was in 1978 (38).

Equine influenza has been recognized as a common malady of the horse for centuries and is considered the most economically important respiratory disease of the equine in countries with substantial breeding and racing industries. In a 1998 study of infectious upper respiratory tract disease in 151 horses in Colorado, it was found that the pathogen was responsible for two-thirds of equine viral respiratory infections (18).

Vaccination is the most effective method of prophylaxis against influenza, designed to elicit a protective antibody response and resistance to reinfection. The most widely used vaccines are inactivated (killed) whole equine influenza virus preparations. However, the ability of some of these vaccines to provide protection against disease has been proven to be quite poor in efficacy studies. In one study, Morley et al. (17) demonstrated that horses vaccinated with an inactivated aluminum phosphate-adjuvanted vaccine did not differ significantly from those given a placebo in the severity of the clinical disease they suffered during an influenza epidemic. A cold-adapted, modi-

fied-live attenuated influenza virus vaccine (Flu-Avert I.N.; Heska Corp.) has shown more promising results. Efficacy trials of this vaccine showed animals were clinically protected 3 months after vaccination (14) and had reduced severity of disease with significant clinical protection 6 months after vaccination (35).

Live vaccines which are administered intranasally may have advantages over their inactivated counterparts. First, live vaccines are thought to induce improved cross-reactive cell-mediated cytotoxicity as well as a humoral antibody response, providing better protection than inactivated vaccines (6, 7). Second, protective immunity to equine influenza is likely to involve a mucosal immunoglobulin A (IgA) response which is not seen with traditional intramuscularly administered vaccines (19). Equine influenza virus replicates in the nasal mucosa, and thus an intranasally administered vaccine may be a preferable route of inoculation to elicit this response (31). Finally, live vaccines also have the advantage of intranasal administration, which avoids the swelling and muscle soreness occasionally associated with the intramuscular administration of inactivated adjuvanted vaccines.

Influenza viruses undergo continual antigenic variation of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Thus, in order to be effective, influenza vaccines require frequent updating to include relevant circulating strains of equine influenza virus. Using reverse genetics, influenza viruses can be created entirely from cloned plasmid DNA (4, 8, 21). This technology could potentially allow rapid generation of vaccine candidates by employing a master strain that can be rescued easily. This strain could then be updated with relevant surface antigens (HA and NA) of circulating virus, an

* Corresponding author. Mailing address: Dept. of Microbiology, Box 1124, Mount Sinai School of Medicine, 1 Gustave Levy Place, New York, NY 10029. Phone: (212) 241-7318. Fax: (212) 534-1684. E-mail: peter.palese@mssm.edu.

adaptation to the classical "6:2 reassortant" approach used to create cold-adapted live vaccines for humans (15).

The NS1 protein of influenza A virus is a multifunctional protein with three domains that have been reported to have a number of regulatory functions during influenza virus infection. The amino-terminal 73 amino acids are responsible for binding to RNAs (26), in particular double-stranded RNAs, conferring on the virus the ability to escape the alpha/beta interferon (IFN) response (2). The central portion of the protein interacts with the eukaryotic translation initiation factor 4GI, facilitating preferential translation of viral mRNAs over host mRNAs (1). The carboxy terminus or the effector domain has been shown to inhibit host mRNA processing, specifically host mRNA polyadenylation (20), binding to poly(A) tails of mRNA preventing nuclear export (27), and pre-mRNA splicing (13).

Studies of a human recombinant influenza virus lacking the NS1 gene (delNS1) showed that this virus could only replicate in IFN-incompetent systems such as STAT1^{-/-} mice or Vero cells, and thus the NS1 protein is responsible for IFN antagonist activity (5). Also, it has been shown that human influenza viruses with truncated NS1 proteins are attenuated in mice (3) and provide protection against wild-type challenge (34). Alternatively, mutations in the full-length NS1 protein may increase viral pathogenicity. For example, it was recently shown that the NS1 protein plays a role in virulence of the highly pathogenic H5N1 influenza virus strains via a glutamic acid at position 92 which confers on the virus resistance to antiviral cytokines (30).

Here, we describe the establishment of a reverse genetics rescue system for equine influenza virus and the construction of three recombinant equine influenza viruses with truncations in their NS1 genes. These recombinant viruses are attenuated in their ability to inhibit IFN production and in their ability to replicate both *in vitro* and *in vivo*. Therefore, these viruses may be good candidates for a live equine influenza vaccine.

MATERIALS AND METHODS

Cells and viruses. Fetal equine kidney (FEK) cells (S. Cook, University of Kentucky) and Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA) were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Human embryonic kidney 293T cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium, also supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The equine influenza virus A/eq/KY/5/02 was isolated in 2002 from a nasopharyngeal swab from a diseased horse in Lexington, Kentucky. A stock of virus was propagated following a total of three passages in embryonated hen eggs (SPAFAS Avian Product Services, CT) and had an HA titer of 128. Recombinant vesicular stomatitis virus tagged with green fluorescent protein (VSV-GFP) (33) was kindly provided by John Hiscott, McGill University, Montreal, Canada.

Gene end sequence determination. Viral RNA was extracted from 300 μ l of allantoic fluid using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the protocol provided. To determine 3' viral RNA end sequences, an adenosine tail was added to viral RNA using a poly(A) polymerase enzyme (Ambion, Austin, TX) and then reverse transcribed using an oligo(dT) primer (primer sequences are available upon request). Reverse transcription reactions were carried out with Superscript II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA). PCRs were carried out using the same oligo(dT) primer and a conserved gene-specific primer for each viral segment. For sequence determination of the 5' viral RNA ends, PCRs were carried out using a primer recognizing the 5' viral RNA noncoding region common to all eight influenza virus segments and a conserved gene-specific primer for each viral segment. PCR products were sequenced (Mount Sinai DNA Sequencing Facility), and based on the sequences

obtained, cloning primers for each segment were designed that included a SspI recognition sequence and the first 15 to 25 nucleotides of the viral RNA ends.

Construction of plasmids. Full-length cDNAs were generated for each viral segment by reverse transcribing viral RNA using a Forward SspI primer for the noncoding region of each segment followed by PCR with the designed cloning primers. The ambisense cloning vector pDZ was derived from protein expression plasmid pCAGGS, provided by J. Miyazaki, Osaka University, Osaka, Japan (22). The pCAGGS vector was modified to additionally produce viral RNA from the cDNA template by insertion of a human RNA polymerase I promoter sequence and a mouse terminator sequence with adjacent SspI recognition sequences.

Purified vector pDZ and the individual SspI-digested cDNA inserts (molar ratio of 1:3 except for PB2 1:7) were ligated using the Roche Rapid DNA ligation kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations. A genetic tag was inserted into the NS-encoding plasmid by silent mutagenesis. Nucleotides 447 to 452 (5'-CTGGA-3') were replaced with 5'-CTCAG-3', resulting in two silent mutations and the creation of a novel XhoI restriction enzyme site. *Escherichia coli* DH5 α cells (Invitrogen) were transformed and screened for correct constructs using the Qiagen miniprep system. All constructs were sequenced at the Mount Sinai DNA Core Sequencing facility.

Stocks of DNA were prepared by transforming bacterial cells with approximately 20 ng of the miniprep DNA and purifying with the Qiagen plasmid maxiprep system. Plasmid pPOLI has a truncated human polymerase I promoter and a hepatitis delta virus ribozyme separated by two SspI sites (24). This plasmid was used for construction of the three NS1 mutants pPOLI-Eq-NS1-126, pPOLI-Eq-NS1-99, and pPOLI-Eq-NS1-73. The coding region of A/eq/KY/5/02 NS segment is 657 nucleotides long (219 amino acids). The name of the mutated NS1 construct corresponds to the length of the protein that is expressed from the plasmid. Desired deletions were introduced by PCR of the pDZ-Eq-NS construct, and the primers used were designed to introduce stop codons in each reading frame and unique restriction sites into each segment (Fig. 1a). The Eq-NS1-126 construct has a deletion of nucleotides 405 to 482, an introduction of four stop codons, a BglII site, and a PacI site. The Eq-NS1-99 construct has a deletion of nucleotides 324 to 482, an introduction of three stop codons, an AseI site, and a PacI site. The Eq-NS1-73 construct has a deletion of nucleotides 246 to 482, an introduction of three stop codons, a HindIII site, and a PacI site. The three constructs were sequenced across the entire NS gene to confirm the novel changes.

Rescue of infectious virus from cDNA. We diluted 0.5 μ g of each of the eight ambisense plasmids (pDZ-Eq-PB2, pDZ-Eq-PB1, pDZ-Eq-PA, pDZ-Eq-HA, pDZ-Eq-NP, pDZ-Eq-NA, pDZ-Eq-M, and pDZ-Eq-NS) and 1.0 μ g of each of five protein expression plasmids (pCAGGS-WSN-PB1, pCAGGS-WSN-PB2, pCAGGS-WSN-PA, pCAGGS-WSN-NP, and pCAGGS-Eq-NS1) to a total volume of 50 μ l in OptiMEM (GIBCO, Invitrogen). Expression plasmids for the H1N1 virus A/WSN/33 were included to improve rescue efficiency. The DNA solution was added to a mix of 10 μ l of cationic liposomal reagent Lipofectamine 2000 (Invitrogen) in 240 μ l of OptiMEM. The transfection mixture was incubated at room temperature for 30 min. One milliliter of OptiMEM was added to the transfection mixture and then mixed with 250 μ l of freshly trypsinized 293T cells resuspended in OptiMEM, enough for \approx 50% confluence in 35-mm dishes. After 6 h of incubation at 37°C, 5% CO₂, the transfection supernatant was removed and 1 ml of freshly trypsinized MDCK cells resuspended in OptiMEM with 0.3% bovine albumin (Sigma-Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (GIBCO) were added. Twelve hours later, 1 ml of OptiMEM containing 1 μ g/ml of TPCK (tosylsulfonil phenylalanyl chloromethyl ketone)-trypsin was added to the dish. Another 1 μ g of TPCK-trypsin was added 24 h later. Seventy-two hours posttransfection, 100 μ l of the supernatant was used to inoculate 7- to 10-day-old embryonated chicken eggs at 37°C, 5% CO₂, for 48 h. NS1 mutant viruses were propagated in 7-day-old eggs which do not have a competent interferon system; full-length recombinant A/eq/KY/5/02 virus was propagated in 8- to 11-day-old eggs. Harvested allantoic fluid was serially diluted in phosphate-buffered saline (PBS; GIBCO) and assayed for hemagglutination of 0.5% Cox hen red blood cells (CBT Farms, Chestertown, MD) in v-bottomed, 96-well plates.

Western blot analysis. Cell extracts were made in a lysis buffer containing 0.5% NP-40, 150 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10% glycerol, and Complete protease inhibitor cocktail (Roche). Ten or twenty microliters of protein lysate was loaded onto a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Separated proteins were probed for analysis of the NS1 protein with a rabbit polyclonal antibody generated against the first 73 amino acids of the protein (unpublished data). A mouse monoclonal antibody

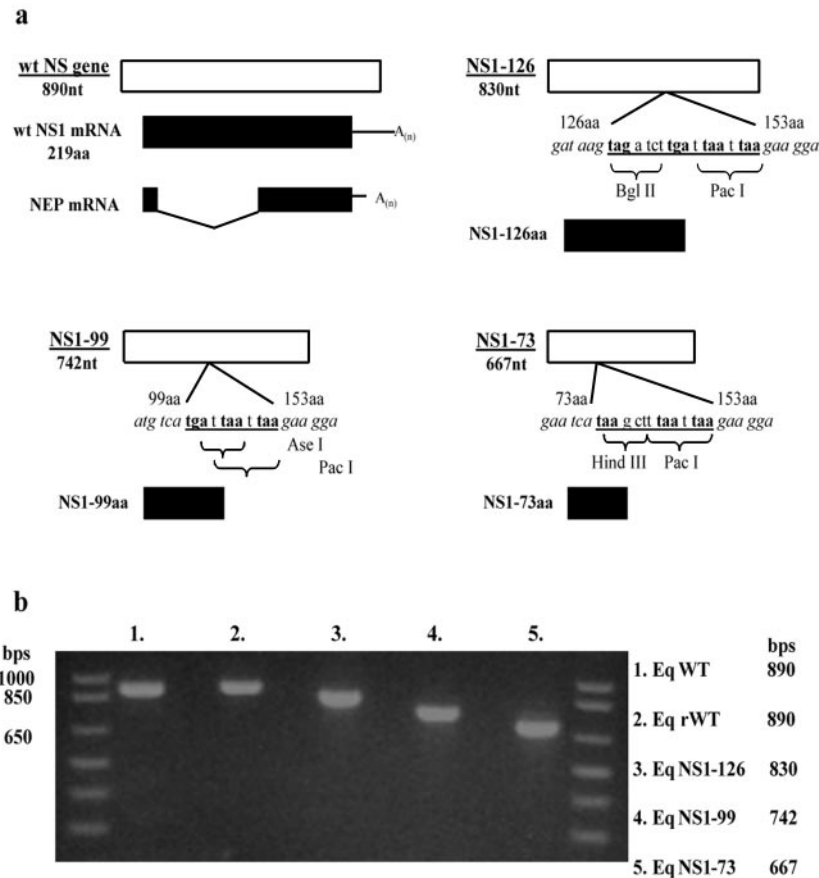


FIG. 1. Generation of equine influenza viruses with truncated NS1 proteins. (a) Schematic diagram of the wild-type and mutated influenza virus NS gene segments. The coding region of the A/eq/KY/5/02 NS1 is 219 amino acids long. C-terminal truncations were made to this segment, resulting in three constructs encoding the first 126, 99, or 73 amino acids of the NS1 protein. The nuclear export protein (NEP) open reading frame was not altered in these constructs. The underlined sequences were introduced to generate stop codons and restriction sites in each construct. (b) RT-PCR of NS segments of wild-type (WT) and recombinant (r) equine (Eq) influenza viruses. RNA was extracted from allantoic fluid of HA-positive eggs. The sizes of the cDNAs generated confirmed the rescue of the specific viruses. The sizes of the DNA markers are indicated on the left side of the figure, and the lengths of the RT-PCR products for each of the viruses are shown on the right.

against the viral M1 protein (ATCC hybridoma M2-1C6-4R3, 0.8 mg/ml, used at 1:1,000) was also included as a positive control.

Bioassay for IFN production. To determine the amount of IFN produced in cells infected with the different recombinant viruses, we modified a bioassay described previously (23). Six-well dishes of confluent FEK cells were infected at a multiplicity of infection (MOI) of 1.5 with each of the four equine recombinant viruses Eq-rWT, Eq-NS1-126, Eq-NS1-99, and Eq-NS1-73 for 11 h. The infected cells were maintained in MEM supplemented with 0.3% bovine albumin, 1% penicillin/streptomycin, and 6% allantoic fluid. At 2-hour intervals postinfection, 100 μ l of supernatant was collected, and any virus present was UV inactivated in plates 6 in. (ca. 15 cm) from an 8-watt UV lamp (UVP, Upland, CA) for 20 min. Flat-bottomed, 96-well plates of confluent FEK cells were treated with the inactivated supernatants for 24 h and then infected with VSV-GFP at an MOI of 0.1. For controls, the cells were also mock treated or treated with 1 unit/ml of universal alpha/beta interferon (PBL Biomedical Laboratories, NJ). At 20 h postinfection, the cells were examined for GFP under a fluorescence microscope (Olympus IX70).

Real-time quantitative PCR. To quantify the relative levels of IFN- β mRNA produced in FEK cells infected with the recombinant viruses, we used a real-time quantitative PCR assay for IFN- β . Thirty-five-millimeter dishes of confluent FEK cells were infected with each of the recombinant viruses at an MOI of 1.5. At different time points postinfection, the infected cells were collected and RNA was extracted using the RNA miniprep kit (Stratagene) according to the manufacturer's protocol. For real-time reverse transcription (RT)-PCR analysis, two separate reactions were carried out in the LightCycler (Roche) utilizing oligonucleotides specific for equine IFN- β and equine β -actin. The 20- μ l reaction

mixture volume in a glass capillary tube contained 2 μ l of RNA (50 ng), SYBR green I (Molecular Probes), 0.5 μ M of each primer, 400 μ M dUTP, 200 μ M each dCTP, dATP, and dGTP, 4 mM MgCl₂, 4 μ l of 5x AccuRT buffer, and 0.7 μ l of AccuRT enzyme (Applied Biosciences).

Reaction conditions were chosen according to a standard LightCycler protocol and were 30 min at 60°C, followed by denaturation at 95°C for 9 min and 45 cycles of 30 s at 95°C and 30 s at 60°C. Fluorescence increase, i.e., creation of specific product, was measured during the extension step at 60°C. A melting curve analysis was performed after the last amplification cycle with 95°C for 0 s, 60°C for 30 s, and 95°C for 0 s. Temperature change rates were 20°C/s for all but the last step, where the rate was 0.1°C/s. Relative levels of IFN- β mRNA were normalized to β -actin mRNA. Final results are presented as relative induction times, where the sample with the highest IFN- β mRNA induction was arbitrarily set at 1,000. The results represent a mean of two separate experiments.

Viral growth kinetics. A comparison of viral growth kinetics for the recombinant wild-type and the three NS1 mutant viruses was undertaken in MDCK cells and embryonated hen eggs. Nearly confluent 12-well plates of MDCK cells were infected with each of the viruses at MOIs of 0.1, 0.01, and 0.001. Infected cells were maintained in MEM supplemented with 0.3% bovine albumin, 1% penicillin/streptomycin, and 1 μ g/ml TPCK-trypsin. The viral titer in the supernatant of infected cells was examined daily for 5 days by the hemagglutination assay as previously described. The allantoic cavities of 7-, 9-, and 11-day-old embryonated eggs were also infected with 1,000 PFU of each of the recombinant equine viruses. Six eggs were infected with 100 μ l of each virus per egg age group. Allantoic fluid was withdrawn from each of the eggs at 24, 48, and 72 h postinfection and examined for hemagglutination.

Viral replication in mouse lungs. Six-week-old female C57BL/6 mice (Jackson Laboratories) were sedated with ether and infected intranasally with 50 μ l of 10^5 PFU of equine influenza recombinant virus. Influenza A/WSN/33 virus and sterile PBS were included as controls. Mice were sacrificed on days 2 and 4 postinfection: five mice per equine virus, four for WSN, and 1 for PBS on each day. Lungs were removed, homogenized, and resuspended in sterile PBS. Viral lung titers were determined by immunofluorescence. Briefly, a 96-well plate of confluent MDCK cells was infected with 20 μ l of limiting dilutions of virus. Seven hours postinfection, the cells were fixed with 5% paraformaldehyde in PBS. A rabbit polyclonal antibody against PR8 virus was used for detection of infected cells, which were counted at the most limiting dilutions to determine the virus titer. All procedures were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals.

RESULTS

Construction of NS1 mutants. Plasmid pPOLI was used for construction of the three equine influenza virus NS1 mutants pPOLI-Eq-NS1-126, pPOLI-Eq-NS1-99, and pPOLI-Eq-NS1-73, while plasmid pDZ was used for the construction of the wild-type NS virus. The coding region of the A/eq/KY/5/02 NS segment is 657 nucleotides long (219 amino acids). Sequential C-terminal truncations were made to this segment, resulting in three constructs encoding the first 126, 99, or 73 amino acids of the NS1 protein. The nuclear export protein (NEP) open reading frame was not altered in these constructs (Fig. 1a). Desired deletions were introduced by PCR using the pDZ-Eq-NS construct as the template. The primers used were designed to introduce stop codons in each reading frame and unique restriction sites into each segment.

Rescue of recombinant equine influenza viruses. To generate an equine influenza virus using reverse genetics, the eight viral RNAs from A/eq/KY/5/02 were cloned into the ambisense plasmid pDZ. Upon transfection of these plasmids along with five protein expression plasmids encoding WSN-PB1, WSN-PB2, WSN-PA, WSN-NP, and Eq-NS1 in 293T/MDCK cocultured cells and subsequent passage into embryonated eggs, infectious virus was recovered. The three viruses with truncated NS1 proteins were rescued in the same manner as the full-length recombinant equine virus. NS1 mutant viruses were propagated in 7-day-old eggs, which do not have a competent interferon system, whereas full-length recombinant A/eq/KY/5/02 was propagated in 8- to 11-day-old eggs. RT-PCR was carried out on RNA extracted from allantoic fluid of HA-positive rescued virus preparations.

The sizes of the generated cDNAs confirmed the rescue of the specific viruses (Fig. 1b). The genetic tag in the recombinant wild-type virus was used to confirm equine influenza virus rescue from cloned cDNA and to rule out the possibility of contamination with wild-type virus (data not shown). As previously described, each of the mutant viruses has a unique restriction site. NS DNA from the mutant viruses was also digested with these specific enzymes for additional proof that each virus contained the correct NS segment (data not shown).

Expression of NS1 proteins. Thirty-five-millimeter dishes of confluent MDCK cells were infected at an MOI of 1.5 with each of the four equine recombinant viruses Eq-rWT, Eq-NS1-126, Eq-NS1-99, and Eq-NS1-73 for 2, 4, 6, and 8 h. A polyclonal antibody against the amino terminus of the NS gene, common to the parental wild-type virus and the three derived NS1 mutants, was used to probe for the protein. Expression of the viral M1 protein was also examined as a control (Fig. 2a).

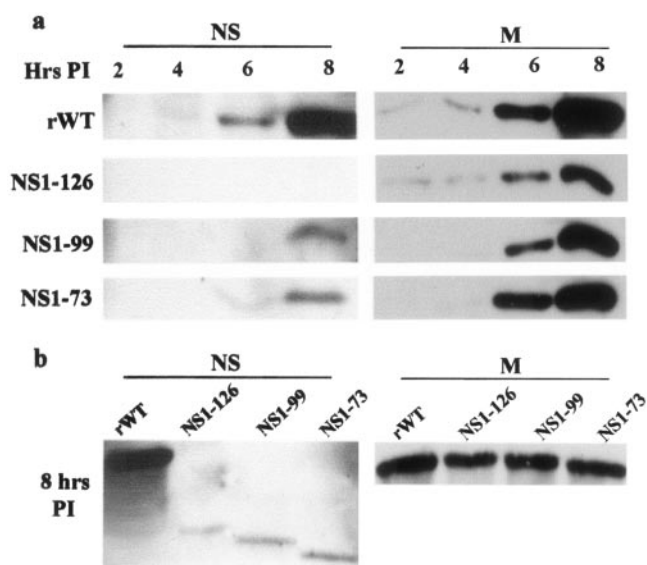


FIG. 2. Expression of NS1 proteins in MDCK cells. (a) Cells were infected with each of the recombinant viruses at an MOI of 1.5. Ten microliters of 1.4 mg/ml total protein was loaded onto a 12% SDS-polyacrylamide gel. Separated proteins were probed for analysis of the NS1 protein with a rabbit polyclonal antibody generated against the first 73 amino acids of the protein. A mouse monoclonal antibody against the viral M1 protein was also included as a positive control to monitor viral infection. (b) Expression of NS1 proteins in MDCK cells. Cells were infected with each of the recombinant viruses at an MOI of 5. Twenty microliters of 2.0 mg/ml total protein was loaded onto a 12% SDS-polyacrylamide gel. Separated proteins were probed for analysis of the NS1 and M1 proteins. PI, postinfection.

Expression of the recombinant wild-type NS1 protein can be seen as early as 4 hours postinfection, with levels increasing up to 8 h. NS1-73 virus protein expression was detectable at 6 h postinfection. Cells infected with the NS1-99 virus had visible NS1 protein expression at 8 h postinfection. Expression of the NS1-126 protein was undetectable at 8 h postinfection at this MOI. Expression of the M1 protein at 6 and 8 h postinfection was comparable for all recombinant mutant viruses, however, cells infected with the NS1-126 virus were expressing M1 protein at earlier time points compared to NS1-99 and NS1-73. In addition, cells were infected with each of the recombinant viruses at a higher MOI (MOI = 5) for 8 h in order to see expression of the NS1-126 protein (Fig. 2b). There was a clear reduction in the level of NS1 protein expression in cells infected with the mutant viruses compared to cells infected with the recombinant wild-type virus. These data indicate that the mutated NS1 viruses still express their truncated NS1 proteins, albeit at lower levels compared to the recombinant wild-type virus.

Induction of IFN by recombinant equine viruses. Next we sought to compare the mutated equine viruses in their ability to counteract the antiviral cytokine response (IFN) in equine primary cells infected with equine influenza virus. For this purpose, a bioassay which measured the production of IFN in infected cells was employed (Fig. 3a). FEK cells were induced to produce interferon by infection with the recombinant viruses. Supernatants of the infected cells were collected at different time points postinfection and UV inactivated. Fresh

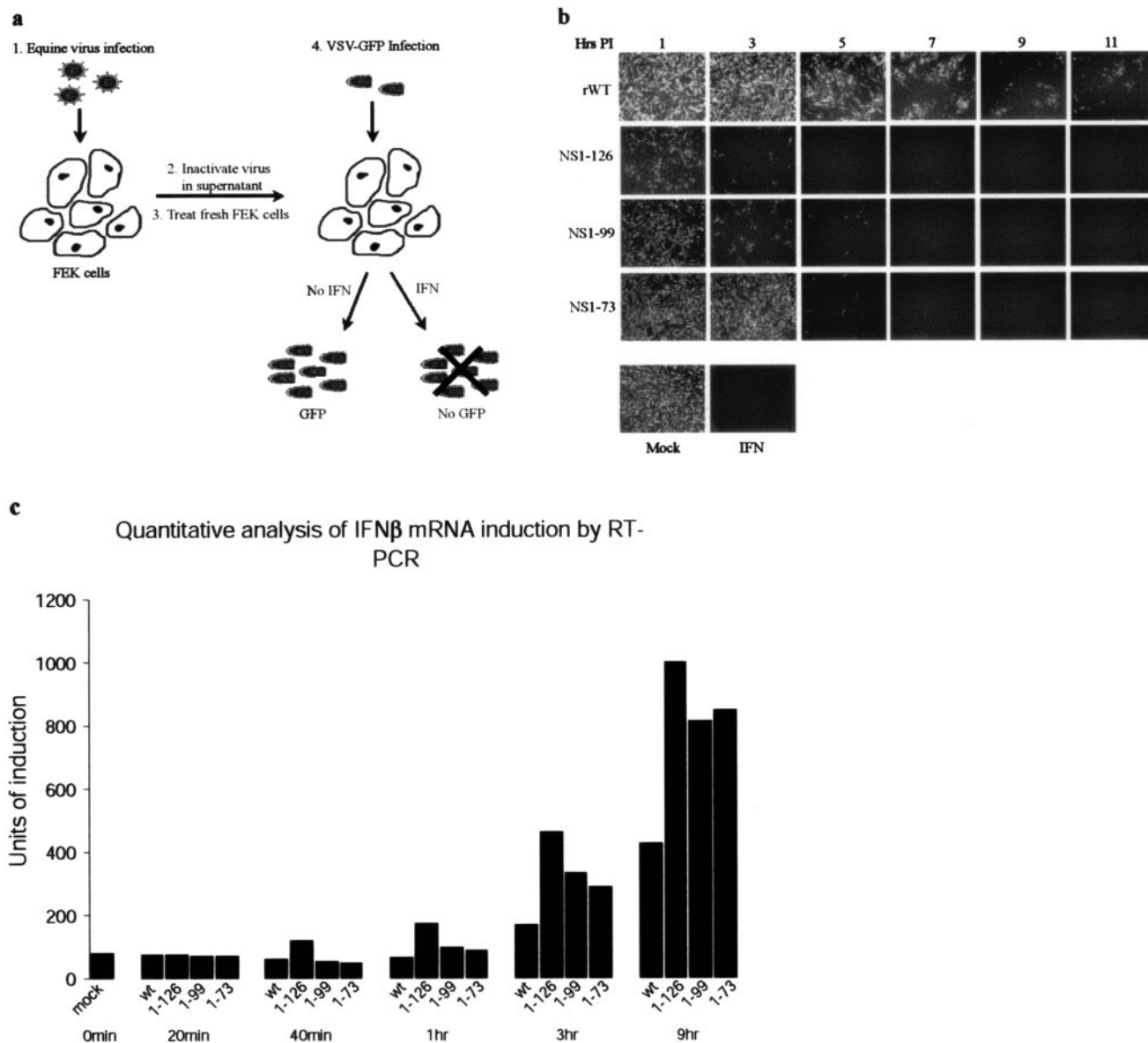


FIG. 3. Bioassay for production of IFN in FEK cells infected with recombinant wild-type (rWT) and NS1 mutant viruses. (a) Schematic diagram of the bioassay for IFN production. FEK cells were infected with each of the four equine recombinant viruses. Supernatants were collected, and any virus present was UV inactivated. Fresh FEK cells were treated with the inactivated supernatants and then infected with VSV-GFP at an MOI of 0.1. (b) Time course of IFN production. Over an 11-h infection period, supernatants were taken at 2-h intervals as indicated. VSV-GFP-infected cells were examined by fluorescence microscopy, and pictures of representative fields were taken. (c) Quantification of IFN mRNA synthesis by real-time RT-PCR. Infected cells were collected at the time points indicated, and RNA was processed for real-time RT-PCR analysis for the β -actin and IFN- β genes. The results represent relative IFN- β mRNA levels normalized to levels of β -actin mRNA.

cells were then treated with the supernatants for 24 h to induce an antiviral state.

We characterized the antiviral cytokine response induced by the different viruses by looking at VSV-GFP replication in the cells treated with supernatants (Fig. 3b). The mutant viruses were expected to induce different levels of IFN in the infected FEK cells and subsequently promote an antiviral state in treated fresh FEK cells. The level of inhibition of VSV-GFP replication in these cells is a reflection of the amount of IFN produced in cells infected with the equine viruses. VSV-GFP replication was inhibited when cells were treated with as little

as 1 unit/ml of universal IFN. When comparing the levels of IFN produced in the equine virus-infected cells, the recombinant wild-type virus did not induce IFN until 9 h postinfection, as shown by the ability of VSV to replicate in the treated cells up until this point. In comparison to the recombinant wild-type virus, the NS1-73 virus was attenuated in its ability to block the antiviral response, as IFN was clearly being produced at 5 h postinfection. The NS1-99 and NS1-126 viruses were also attenuated in this respect, the latter more severely so.

To further determine whether the induction of the antiviral state in the treated cells is correlated with the level of IFN- β

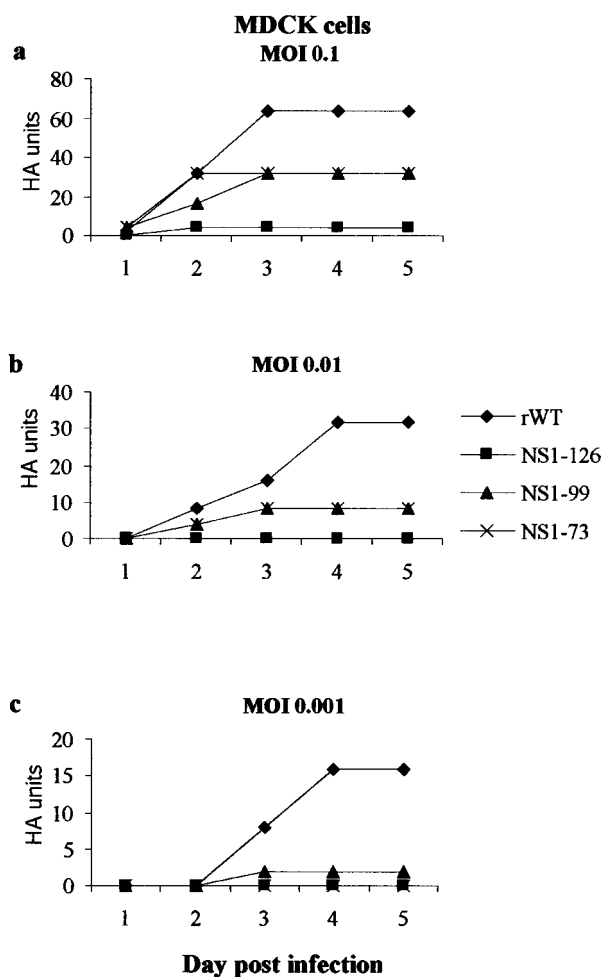


FIG. 4. Characterization of viral growth in MDCK cells. A comparison of viral growth was done for the recombinant wild-type (rWT) and the three NS1 mutant viruses in MDCK cells. MDCK cells were infected with each of the viruses at an MOI of 0.1 (a), 0.01 (b), or 0.001 (c). Viral growth in the supernatant of infected cells was examined daily for 5 days by the hemagglutination assay.

induction in FEK cells infected with the recombinant viruses, we quantified the relative levels of IFN- β mRNA in the infected cells by quantitative RT-PCR (Fig. 3c). Cells were collected at different time points, and RNA was extracted and analyzed for IFN- β and β -actin expression as described in Materials and Methods. Induction of IFN- β expression was seen as early as 40 min for the NS1-126 mutant virus and increased for all viruses with time. At all time points the NS1-126 mutant appeared to induce the highest levels of interferon, followed by the NS1-99 and NS1-73 mutant viruses. At 9 h postinfection the levels of IFN- β mRNA induced by the wild-type virus are comparable to the levels of IFN- β mRNA induced by the NS1-126 mutant at 3 hours (Fig. 3c). This reflects the ability of the wild-type virus to block interferon induction until 9 h, as was demonstrated by the bioassay (Fig. 3b).

Viral replication in MDCK cells. Growth of the recombinant wild-type and the three NS1 mutant viruses was examined in MDCK cells. Cells were infected with each of the viruses at MOIs of 0.1, 0.01, and 0.001 (Fig. 4). Growth in the superna-

tant of infected cells was examined daily for 5 days by the hemagglutination assay. At a higher MOI (0.1), the recombinant wild-type virus replicated to the highest titers, with the NS1-99 and NS1-73 viruses reaching lower HA titers. The NS1-126 virus was severely attenuated in its replication. At an MOI of 0.01, the same trend was seen with the NS1-99 and NS1-73 viruses, which replicated at significantly lower titers compared to the wild type. The NS1-126 virus did not replicate at this MOI. Finally, at the lowest MOI of 0.001, the NS1 mutant viruses were highly attenuated in comparison to the recombinant wild-type virus. The parental strain grew to an HA titer of 16, but the mutant viruses did not grow to significant titers or were below the detection limit.

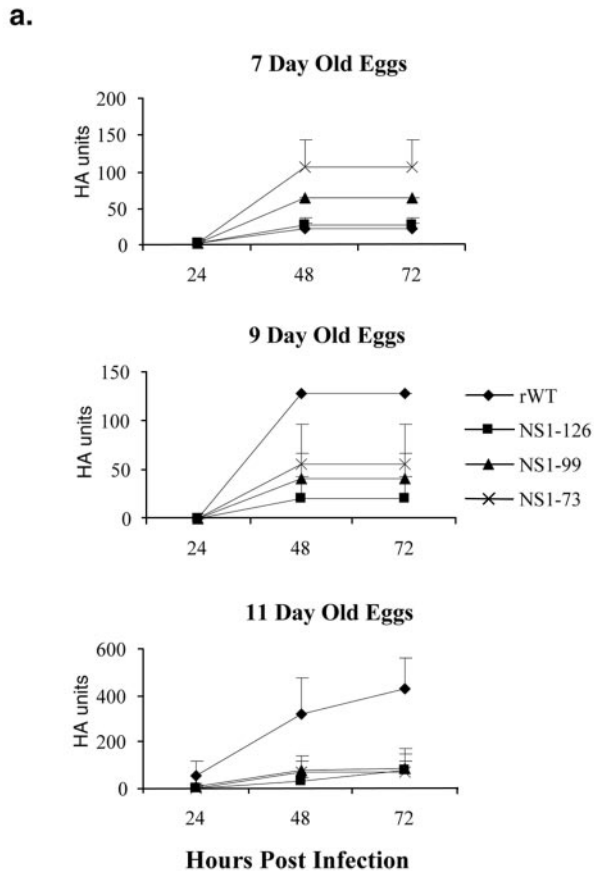
Viral replication in embryonated hen eggs. To assess viral replication in embryonated eggs, the allantoic cavities of 7-, 9-, and 11-day-old eggs were infected with 1,000 PFU of each of the recombinant equine viruses. Allantoic fluid was withdrawn from each of the eggs at 24, 48, and 72 h postinfection, and the average HA titer from six eggs for each group was calculated, as shown in Fig. 5a. Interestingly, in 7-day-old eggs, the mutant viruses NS1-73 and NS1-99 actually grew better (HA titers of 107 and 64, respectively) than the recombinant wild-type virus, which grew to an HA titer similar to that of NS1-126. However, in 9- and 11-day-old eggs, the mutant viruses were much more attenuated in their ability to replicate in comparison to the recombinant wild-type virus. In these eggs the recombinant wild-type virus grew to HA titers of 128 and 472, respectively, which was significantly higher than the values for the best-growth mutant virus, NS1-73.

To determine whether the viral HA titers accurately reflected the actual number of infectious viral particles, we further examined the infectious titers of the viral stocks by immunofluorescence. The difference in HA units between the recombinant viruses directly correlated with the actual viral titers, suggesting that truncations in the NS1 protein did not result in enhanced formation of defective viral particles (Fig. 5b).

Viral replication in mice. Lastly, we looked at the viral growth kinetics for the recombinant wild-type and the three NS1 mutant viruses in vivo by infecting 6-week-old female C57BL/6 mice with 10^5 PFU of each of the viruses. Influenza A/WSN/33 virus and sterile PBS were included as controls. Viral titers in the lungs of the mice on days 2 and 4 postinfection were measured by immunofluorescence, and a mean titer per group is shown in Fig. 6. All of the recombinant equine viruses except for NS1-126 replicated in mouse lungs with a decrease in titer from day 2 to day 4. As with the viral growth kinetics seen in vitro, NS1-73 virus was the least attenuated of the three mutant viruses, with ≈ 1.5 log lower growth in comparison to the recombinant wild-type virus. The NS1-99 virus was more attenuated than the NS1-73 virus, being over 5 logs lower than the recombinant wild-type virus on day 2 postinfection. The NS1-126 virus was the most attenuated of the mutant viruses, as it was not detectable in mouse lungs on either day lung titers were measured.

DISCUSSION

We have established a plasmid-based reverse genetics system for equine influenza virus. Utilizing this method, we gen-



b.

virus	HA titer (dilution units)	Infectious titer (pfu/ml)
wt	256	3x10 ⁷
1-126	128	1x10 ⁷
1-99	512	5x10 ⁷
1-73	128	1.5x10 ⁷

FIG. 5. Characterization of viral growth in embryonated hen eggs. (a) The allantoic cavities of 7-day-old (a), 9-day-old (b), and 11-day-old (c) embryonated eggs were infected with each of the recombinant equine viruses. The mean viral titer (\pm SEM) was determined by hemagglutination assay of allantoic fluid at 24, 48, and 72 h postinfection. (b) Recombinant viruses grown in eggs were titered by hemagglutination assay and immunofluorescence. Viral titers are represented in hemagglutination units and PFU/ml.

erated three recombinant equine influenza viruses encoding carboxy-terminally truncated NS1 proteins of 73, 99, or 126 amino acids. Our previous studies of human influenza virus lacking the entire NS1 gene showed it could only replicate in IFN-incompetent systems, implicating the protein in IFN antagonism (5). Thus, we made these mutations with the expectation that influenza viruses with NS1 proteins of different lengths would have different levels of IFN antagonist activity. Indeed, our data show that in comparison to the recombinant

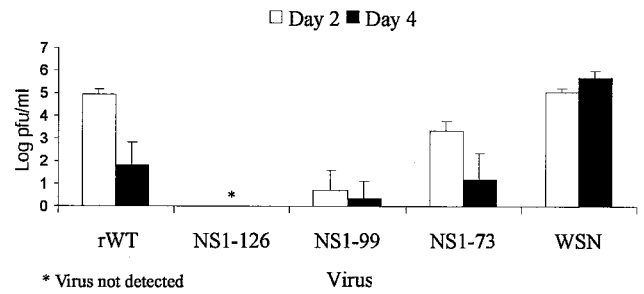


FIG. 6. Viral replication in mouse lungs. Six-week-old female C57BL/6 mice were infected intranasally with 10⁵ PFU of each recombinant equine influenza virus and A/WSN/33 virus. Mice were sacrificed on days 2 and 4 postinfection, and viral titers in the lung were determined by immunofluorescence in MDCK cells. The graph represents the mean viral titer (\pm SEM) for each group.

wild-type parental virus, the mutant NS1 viruses induce IFN production at earlier time points in a primary equine cell culture system. Specifically, the NS1-126 virus induces IFN production earliest, followed by the NS1-99 and NS1-73 viruses.

We also found that the three mutated equine influenza viruses were attenuated in their replication in comparison to wild-type virus in cell culture and in embryonated eggs. Again, this attenuation of mutant NS1 viruses was seen as NS1-73 being least attenuated, followed by NS1-99 and NS1-126 being most attenuated. In the growth curves in embryonated eggs, it was expected that the older eggs would provide more of a challenge to replication for the mutant viruses because the IFN- α/β system in older eggs is more mature (16, 28, 29). Consistent with our hypothesis, it was found that the mutant viruses were attenuated in comparison to the wild-type virus in 9- and 11-day-old eggs. Interestingly, in 7-day-old eggs the recombinant wild-type virus grew to lower titers in comparison to the mutant viruses. We speculate that this reduction in HA titer may have been due to the recombinant wild-type virus killing the younger eggs early in infection, as they do not have a competent IFN system. A similar phenomenon has been observed in highly pathogenic H5 influenza virus strains, which cannot be grown successfully in embryonated hen eggs for vaccine production (11). The same pattern of attenuation in replication seen with the three mutants (NS1-126 > NS1-99 > NS1-73) in eggs and tissue culture was also observed in vivo in the mouse model. In this system the NS1-126 virus was unable to replicate to detectable levels in the mouse lung. Our data suggest that the NS1 protein of equine influenza virus plays a role in virulence in vivo and may be useful for vaccine development.

In our previous studies of human influenza viruses with carboxy-terminally truncated NS1 proteins, we have demonstrated that the length of the NS1 protein correlates with the level of attenuation of the virus, i.e., the shorter the NS1 protein, the less virulent the virus (37). However, in the case of equine influenza virus, we find that attenuation does not follow the same pattern, in that the NS1-73 virus was the most virulent of the mutants, followed by NS1-99 and NS1-126. We speculate that the basis for attenuation of the equine NS1 mutants is related to their level of NS1 protein expression, as

seen from the Western blot analysis (Fig. 2). Cells infected with NS1-73 and NS1-99 viruses had higher levels of NS1 protein expression than cells infected with the NS1-126 virus. The different amounts of NS1 protein expressed by the mutant viruses may be due to decreased protein stability as a result of the C-terminal truncations that were made. Additional studies to determine the precise mechanism of decreased protein expression in the mutant viruses will be required.

Similar truncations of the NS1 protein in swine influenza viruses generated comparable results, in that viruses with shorter NS1 proteins were least attenuated (Solórzano et al., in press). Kittel et al. demonstrated that influenza virus may circumvent the antiviral IFN system via the NS1 protein's RNA binding domain (RBD) (9). Viruses with an intact RBD (wild-type influenza virus and NS1-125) replicated to normal titers in Vero cells treated with IFN- α , whereas influenza viruses with mutations in the RBD (delNS1 and NS1-del40-80) were attenuated. This suggests that there may be other viral factors or domains besides the RBD that are responsible for influenza virus virulence to explain the attenuation effect seen in our study. Alternatively, the recombinant wild-type NS1 protein is in a more stable conformation than the mutant virus proteins, which may be subject to differential degradation.

Equine influenza is a worldwide disease adversely affecting the economies of countries with significant equine industries through yearly epidemics. Although inactivated equine influenza vaccines are available, the protective response they elicit is short-lived and repeated vaccination of horses is necessary to reduce the risk of influenza. It is anticipated that a live attenuated vaccine would be more effective than a killed vaccine, as it would stimulate both humoral and cell-mediated immunity. Moreover, a live vaccine generated by reverse genetics would allow for more standardized production and may require less virus per dose than the conventional equine influenza virus vaccines. The vaccine master strain would be precisely defined and could be easily updated with relevant circulating influenza virus surface antigens.

Here, we propose equine influenza vaccine candidates that are attenuated through truncations to the NS1 protein. Previous findings suggest that viruses with the entire NS1 gene deleted or expressing only NS 1 to 99 are immunogenic in mice (34). In addition, interferon-inducing influenza virus mutants have been shown to be potent inducers of dendritic cell maturation (12), which is an important event involved in initiation of specific immune responses. Finally, the studies that examined the role of IFN in generating an antigen-specific adaptive immune response showed that IFN possesses adjuvant activity (10, 25). This implies that IFN-inducing influenza viruses such as the NS1 mutants that we have generated would generate a stronger immune response than an inactivated or cold-adapted virus. Should these viruses also prove to be attenuated and immunogenic in horses, they could become potential vaccine candidates against equine influenza.

ACKNOWLEDGMENTS

We thank Alicia Solórzano for providing the NS1 polyclonal antibody. Real-time quantitative PCR was performed in James Wetmur's laboratory at the Mount Sinai School of Medicine and was supported by NIH grant P42ES007384.

This work was supported by NIH training grant AI007647 (D.Z.) and partially supported by NIH grants to P.P. and A.G.-S. P.P. is an

Ellison Medical Foundation scholar in global infectious diseases. T.C. was supported in part by USDA-CSREES Project No. KY014014. M.Q. was funded by the Irish Research Council for Science, Engineering and Technology under the supervision of Sean Arkins at the University of Limerick.

REFERENCES

- Aragón, T., S. de la Luna, I. Novoa, L. Carrasco, J. Ortín, and A. Nieto. 2000. Eukaryotic translation initiation factor 4G1 is a cellular target for NS1 protein, a translational activator of influenza virus. *Mol. Cell. Biol.* **20**:6259–6268.
- Donelan, N. R., C. F. Basler, and A. García-Sastre. 2003. A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. *J. Virol.* **77**:13257–13266.
- Egorov, A., S. Brandt, S. Sereinig, J. Romanova, B. Ferko, D. Katinger, A. Grassauer, G. Alexandrova, H. Katinger, and T. Muster. 1998. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. *J. Virol.* **72**:6437–6441.
- Fodor, G., L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, and A. García-Sastre. 1999. Rescue of influenza A virus from recombinant DNA. *J. Virol.* **73**:9679–9682.
- García-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster. 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* **252**:324–330.
- Gorse, G. J., and R. B. Belshe. 1990. Enhancement of anti-influenza A virus cytotoxicity following influenza A virus vaccination in older, chronically ill adults. *J. Clin. Microbiol.* **28**:2539–2550.
- Gorse, G. J., M. J. Campbell, E. E. Otto, D. C. Powers, G. W. Chambers, and F. K. Newman. 1995. Increased anti-influenza A virus cytotoxic T cell activity following vaccination of the chronically ill elderly with live attenuated or inactivated influenza virus vaccine. *J. Infect. Dis.* **172**:1–10.
- Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster. 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. USA* **97**:6108–6113.
- Kittel, C., S. Sereinig, B. Ferko, J. Stasakova, J. Romanova, A. Wolkerstorfer, H. Katinger, and A. Egorov. 2004. Rescue of influenza virus expressing GFP from the NS1 reading frame. *Virology* **324**:67–73.
- Le Bon, A., G. Schiavoni, G. D'Agostino, I. Gresser, F. Belardelli, and D. F. Tough. 2001. Type I interferons potentially enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity* **14**:461–470.
- Li, S., C. Liu, A. Klimov, K. Subbarao, M. L. Perdue, D. Mo, Y. Ji, L. Woods, S. Hietala, and M. Bryant. 1999. Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. *J. Infect. Dis.* **179**:1132–1138.
- Lopez, C. B., A. García-Sastre, B. R. Williams, and T. M. Moran. 2003. Type I interferon induction pathway, but not released interferon, participates in the maturation of dendritic cells induced by negative-strand RNA viruses. *J. Infect. Dis.* **187**:1126–1136.
- Lu, Y., X. Y. Qian, and R. M. Krug. 1994. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. *Genes Dev.* **8**:1817–1828.
- Lunn, D. P., S. Hussey, R. Sebing, K. E. Rushlow, S. V. Radecki, P. Whitaker-Dowling, J. S. Youngner, T. M. Chambers, R. E. Holland, Jr., and D. W. Horohov. 2001. Safety, efficacy, and immunogenicity of a modified-live equine influenza virus vaccine in ponies after induction of exercise-induced immunosuppression. *J. Am. Vet. Med. Assoc.* **218**:900–906.
- Maassab, H. F., and M. L. Bryant. 1999. The development of live attenuated cold-adapted influenza virus vaccine for humans. *Rev. Med. Virol.* **9**:237–244.
- Morahan, P. S., and S. E. Grossberg. 1970. Age-related cellular resistance of the chicken embryo to viral infections. I. Interferon and natural resistance to myxoviruses and vesicular stomatitis virus. *J. Infect. Dis.* **121**:615–623.
- Morley, P. S., H. G. Townsend, J. R. Bogdan, and D. M. Haines. 1999. Efficacy of a commercial vaccine for preventing disease caused by influenza virus infection in horses. *J. Am. Vet. Med. Assoc.* **215**:61–66.
- Mumford, E. L., J. L. Traub-Dargatz, M. D. Salman, J. K. Collins, D. M. Getzy, and J. Carman. 1998. Monitoring and detection of acute viral respiratory tract disease in horses. *J. Am. Vet. Med. Assoc.* **213**:385–390.
- Nelson, K. M., B. R. Schram, M. W. McGregor, A. S. Sheoran, C. W. Olsen, and D. P. Lunn. 1998. Local and systemic isotype-specific antibody responses to equine influenza virus infection versus conventional vaccination. *Vaccine* **16**:1306–1313.
- Nemeroff, M. E., S. M. Barabino, Y. Li, W. Keller, and R. M. Krug. 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3' end formation of cellular pre-mRNAs. *Mol. Cell* **1**:991–1000.
- Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. USA* **96**:9345–9350.

22. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**:193–199.
23. Park, M. S., A. García-Sastre, J. F. Cros, C. F. Basler, and P. Palese. 2003. Newcastle disease virus V protein is a determinant of host range restriction. *J. Virol.* **77**:9522–9532.
24. Pleschka, S., R. Jaskunas, O. G. Engelhardt, T. Zürcher, P. Palese, and A. García-Sastre. 1996. A plasmid-based reverse genetics system for influenza A virus. *J. Virol.* **70**:4188–4192.
25. Proietti, E., L. Bracci, S. Puzelli, T. Di Pucchio, P. Sestili, E. De Vincenzi, M. Venditti, I. Capone, I. Seif, E. De Maeyer, D. Tough, I. Donatelli, and F. Belardelli. 2002. Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. *J. Immunol.* **169**:375–383.
26. Qian, X. Y., C. Y. Chien, Y. Lu, G. T. Montelione, and R. M. Krug. 1995. An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure. *RNA* **1**:948–956.
27. Qiu, Y., and R. M. Krug. 1994. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J. Virol.* **68**:2425–2432.
28. Sekellick, M. J., W. J. Biggers, and P. I. Marcus. 1990. Development of the interferon system. I. In chicken cells development in ovo continues on time in vitro. *In Vitro Cell Dev. Biol.* **26**:997–1003.
29. Sekellick, M. J., and P. I. Marcus. 1985. Interferon induction by viruses. XIV. Development of interferon inducibility and its inhibition in chick embryo cells “aged” in vitro. *J. Interferon Res.* **5**:651–667.
30. Seo, S. H., E. Hoffmann, and R. G. Webster. 2004. The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. *Virus Res.* **103**:107–113.
31. Soboll, G., K. M. Nelson, E. S. Leuthner, R. J. Clark, R. Drape, M. D. Macklin, W. F. Swain, C. W. Olsen, and D. P. Lunn. 2003. Mucosal co-administration of cholera toxin and influenza virus hemagglutinin-DNA in ponies generates a local IgA response. *Vaccine* **21**:3081–3092.
32. Sovinová, O., B. Tümová, F. Pouska, and J. Nemeč. 1958. Isolation of a virus causing respiratory disease in horses. *Acta Virol.* **2**:52–61.
33. Stojdl, D. F., B. D. Lichty, B. R. tenOever, J. M. Paterson, A. T. Power, S. Knowles, R. Marius, J. Reynard, L. Poliquin, H. Atkins, E. G. Brown, R. K. Durbin, J. E. Durbin, J. Hiscott, and J. C. Bell. 2003. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **4**:263–275.
34. Talon, J., M. Salvatore, R. E. O’Neill, Y. Nakaya, H. Zheng, T. Muster, A. García-Sastre, and P. Palese. 2000. Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. *Proc. Natl. Acad. Sci. USA* **97**:4309–4314.
35. Townsend, H. G., S. J. Penner, T. C. Watts, A. Cook, J. Bogdan, D. M. Haines, S. Griffin, T. Chambers, R. E. Holland, P. Whitaker-Dowling, J. S. Youngner, and R. W. Sebring. 2001. Efficacy of a cold-adapted, intranasal, equine influenza vaccine: challenge trials. *Equine Vet. J.* **33**:637–643.
36. Wadell, G. H., M. B. Teigland, and M. M. Siegel. 1963. A new influenza virus associated with equine respiratory disease. *J. Am. Vet. Med. Assoc.* **143**:587–590.
37. Wang, X., C. F. Basler, B. R. Williams, R. H. Silverman, P. Palese, and A. García-Sastre. 2002. Functional replacement of the carboxy-terminal two-thirds of the influenza A virus NS1 protein with short heterologous dimerization domains. *J. Virol.* **76**:12951–12962.
38. Webster, R. G. 1993. Are equine 1 influenza viruses still present in horses? *Equine Vet. J.* **25**:537–538.