An Eight-Segment Swine Influenza Virus Harboring H1 and H3 Hemagglutinins Is Attenuated and Protective against H1N1 and H3N2 Subtypes in Pigs

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Swine influenza virus (SIV) infections continue to cause production losses in the agricultural industry in addition to being a human public health concern. The primary method of controlling SIV is through vaccination. The killed SIV vaccines currently in use must be closely matched to the challenge virus, and their protective efficacy is limited. Live attenuated influenza vaccines (LAIV) provide strong, long-lived cell-mediated and humoral immunity against different influenza virus subtypes with no need for antigen matching. Here we report the generation of a new potential LAIV, an eight-segment SIV harboring two different SIV hemagglutinins (HAs), H1 and H3, in the genetic background of H1N1 SIV. This mutant SIV was generated by fusing the H3 HA ectodomain from A/Swine/Texas/4199-2/98 (H3N2) to the cytoplasmic tail, transmembrane domain, and stalk region of neuraminidase (NA) from A/Swine/Saskatchewan/18789/02 (H1N1) SIV. While this H1-H3 chimeric SIV, when propagated *in vitro* in the presence of exogenous neuraminidase, showed kinetics and growth properties similar to those of the parental wild-type virus, *in vivo* it was highly attenuated in pigs, demonstrating a great potential for serving as a dual LAIV. Furthermore, vaccination with the H1-H3 virus elicited robust immune responses, which conferred complete protection against infections with both H1 and H3 SIV subtypes in pigs.

Swine influenza virus (SIV) is the causative pathogen of swine influenza (SI), a highly contagious acute respiratory viral disease of swine. The mortality of SIV-infected pigs is usually low, while morbidity may reach 100%. SI is characterized by a sudden onset, coughing, respiratory distress, weight loss, fever, nasal discharge, and rapid recovery (1).

Three major SIV subtypes, H1N1, H1N2, and H3N2, with multiple genetic and antigenic variants within each subtype, currently circulate in swine populations in North America (2, 3). The primary method for the control of SIV infections on swine farms is vaccination. The SIV vaccines currently used are inactivated mono-, bi-, or trivalent vaccines containing antigens of SIV H1N1 and H3N2 subtypes. The application of these vaccines reduces the severity of disease but does not provide consistent protection from infection (4–7). In addition, these vaccines primarily generate antibody responses with limited or no cell-mediated immunity (CMI). In contrast, live attenuated influenza vaccines (LAIV) provide strong, long-lived cell-mediated and humoral immunity against different influenza virus subtypes with no need for perfect antigen matching (8–11). Previously, it was shown that SIVs generated by genetic modifications within the hemagglutinin (HA) or nonstructural (NS) genes are attenuated in pigs (12, 13). These viruses demonstrated the ability to induce strong cell-mediated and humoral immunity and to provide full protection against homologous SIVs and partial protection against heterologous SIVs (11, 14–17). In this study, we describe a novel approach that generates a LAIV candidate containing an H3 subtype of HA derived from a circulating SIV in the genetic background of H1N1 SIV. This genetically engineered vaccine candidate expresses two antigenically different HAs (H1 and H3) on its surface and maintains eight RNA segments with their original packaging signals within its genome. Theoretically, such a virus can serve as a bivalent

LAIV, providing complete protection against broader SIV strains in the field.

SIV is a member of the family *Orthomyxoviridae* and belongs to the *Influenzavirus A* genus. The genome contains eight single-stranded RNA segments of negative polarity (18). Each RNA segment encodes at least one viral protein. The HA and neuraminidase (NA) proteins project through the viral envelope and interact with the host immune system (18). HA is a type I integral membrane glycoprotein that binds to sialic acid-containing receptors on the host cell surface and mediates the fusion of the viral envelope with the endosomal membrane after receptor-mediated endocytosis (18–20). In addition, HA is the major antigen against which neutralizing antibodies are synthesized (19). In contrast, NA protein is a type II integral membrane glycoprotein (19, 21, 22) that plays a crucial role late in the virus life cycle by removing sialic acid from sialyloligosaccharides, thus releasing newly assembled virions from the cell surface and preventing the self-aggregation of the virus (19, 21, 23). The abundance of each protein differs among virus subtypes; the HA/NA ratio ranges approximately from 4:1 to 10:1 (24). A balance of competent HA and NA activities appears to be critical for efficient completion of the virus life

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cycle; however, exogenous bacterial sialidases could be used as substitutes for altered NA activity (23, 25).

For efficient virus replication, all of the eight viral RNA (vRNA) segments must be packaged into the influenza virus virions. However, the exact mechanism by which these viral RNA segments are incorporated is still not completely understood (18, 26). The packaging of viral genomes into virions typically involves recognition by viral components of a *cis*-acting sequence in the viral nucleic acid, the so-called “packaging signal.” Such a packaging signal, which is necessary for viral genome segment-specific packaging, was believed to reside in both the 3′ and 5′ ends of the noncoding regions (NCRs) and the adjacent terminal coding region of each RNA segment (26–34). It was demonstrated that foreign genes or influenza virus genes from different types and subtypes could be efficiently cloned between the packaging signals of different influenza virus genes and expressed in the form of proteins (34–36). Most of the previous studies were based on the incorporation of reporter proteins (green fluorescent protein [GFP], chloramphenicol acetyltransferase [CAT]) or different foreign proteins as an additional ninth segment flanked by influenza virus packaging signals (35, 37, 38). These approaches resulted in the generation of replication-deficient recombinant influenza virus.

Here we report a strategy of generating a recombinant SIV in the background of A/Swine/Saskatchewan/18789/02 (H1N1) (SK02) that possesses eight RNA segments yet expresses the H1 and H3 subtypes of HA. All eight original packaging signals were kept, while the antigenic properties of the virus were changed and improved by replacing the NA segment with the H3 HA coding sequence flanked by the NA packaging signal. The NA activity was supplemented exogenously by the addition of bacterial sialidase (neuraminidase). When propagated in cell culture containing exogenous neuraminidase, this recombinant SIV showed growth properties similar to those of the parental wild-type (WT) SIV. *In vivo*, the recombinant SIV was highly attenuated and immunogenic and provided complete protection against infection with both H1N1 and H3N2 SIVs.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby canine kidney (MDCK) cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen). 293T (human embryonic kidney) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. The wild-type A/Swine/Saskatchewan/18789/02 (H1N1) (SIV/SK02) and A/Swine/Texas/4199-2/98 (H3N2) (SIV/TX98) viruses were propagated at 37°C in the allantoic cavities of 11-day-old embryonated chicken eggs. The mutant virus SIV/606 (described below) was propagated in tissue culture in the presence of 5 mU/ml *Vibrio cholerae* neuraminidase (N6514; Sigma-Aldrich). Virus titers were determined on MDCK cells by plaque assays as described previously (39). Challenge and vaccine stock viruses were purified and prepared as described previously (12).

Plasmid construction. Plasmid pHW-SIV-NA-H3HA, encoding H3 HA flanked by NA packaging signals, was constructed by modifying an original plasmid, pHW-SIV/SK-NA (12). Briefly, the NA segment-specific packaging signals at the 3′ and 5′ ends (202 nucleotides [nt] and 185 nt, respectively) were amplified by PCR using pHW-SIV/SK-NA as the template with the following sets of primers: for amplifying the 3′ NA packaging signal, 5′-TAATACGACTCACTATAGGG-3′ and 5′-GT CATT TCC GGGAAGTTTTGCACCAAGTATTGTTTTCGTAG-3′; for amplifying the 5′ NA packaging signal, 5′-GCTGAAATCAGGATAC AAAGATTGAGGCCTTGCTTCTGGGTG-3′ and 5′-ACAGGTGTCC

GTGTCGCG-3′. The H3 HA ectodomain (excluding the signal peptide sequence, transmembrane domain, and cytoplasmic tail) from SIV/TX98 was amplified by PCR using pHW-Tx98 HA as the template with primers 5′-CTACGAAAACAATACTTGGGTGCAAAAACCTCCCGGAAAT GAC-3′ and 5′-CAACCCAGAAGCAAGGCCTCAATCTTTGTATCCTG ATTTCAGC-3′. The three pieces of PCR products were joined by overlapping PCR. Finally, this PCR product was digested by *NaeI*/*NheI* and was used to replace the corresponding part in pHW-SIV/SK-NA (see Fig. 1). The DNA of the constructed plasmid was sequenced to ensure that additional mutations were not introduced during the PCR.

Rescue of the recombinant SIV by reverse genetics. The recombinant SIV bearing H1 and H3 was rescued using an eight-plasmid reverse genetics system described by Hoffmann et al. (40). Briefly, 293T and MDCK cells were cocultured at the same density (2.5×10^5 cells/well) in a 6-well plate and were maintained in DMEM containing 10% FBS at 37°C under 5% CO₂ for 24 h. One hour prior to transfection, the medium containing FBS was replaced with fresh Opti-MEM (Invitrogen). Cells were transfected with eight plasmid constructs (pHW-SIV/SK-PB2, pHW-SIV/SK-PB1, pHW-SIV/SK-PA, pHW-SIV/SK-HA, pHW-SIV/SK-NP, pHW-SIV-NA-H3HA, pHW-SIV/SK-M, and pHW-SIV/SK-NS) by using TransIT-LT1 transfection reagent (Mirus). Six hours later, the transfection mixture was replaced with 1 ml of fresh Opti-MEM. Twenty-four hours posttransfection, 1 ml of Opti-MEM containing 0.4% bovine serum albumin (BSA), 2 µg/ml of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin, and 20 mU/ml *Vibrio cholerae* neuraminidase were added to the transfected cells. Supernatants were collected at 96 h posttransfection. Cytopathic effect (CPE) was observed after the third consecutive passage on MDCK cells, and the presence of virus was confirmed by a hemagglutination test. This recombinant virus was designated SIV/606.

RT-PCR. Viral RNA was extracted from 100 µl of purified SIV/SK02, SIV/TX98, or SIV/606 by using an RNeasy extraction kit (Qiagen) according to the manufacturer’s instructions. Ten nanograms of RNA was used in one-step reverse transcription-PCR (RT-PCR) (Express One-Step SYBR GreenER kits; Invitrogen). To detect H1 HA, primers 5′-TGGCCA AACCATGAGACAAC-3′ and 5′-GGCGTTATTCCTCAGTTGTG-3′ were used in PCR. To detect H3 HA, 5′-CAACCCAGAAGCAAGGCCTC AATCTTTGTATCCTGATTTCAGC-3′ and 5′-CGCAATCGCAGGTTT CATAG-3′ were used.

Immunoelectron microscopy. Purified SIV/606 particles were applied to a 400-mesh copper grid coated with Formvar carbon film (Electron Microscopy Sciences) and were stained with a mouse monoclonal antibody against influenza virus H1N1 HA (AB128412; Abcam) or a mouse monoclonal antibody against influenza virus A H3 antigen (OBT1560; AbD Serotec). After washing, the grids were incubated with goat anti-mouse IgG conjugated with 10-nm gold particles (Electron Microscopy Sciences). The grids were negatively stained with 2% phosphotungstic acid (Sigma-Aldrich) as described previously (41).

Western blotting. Western blotting was performed as described previously with minor modifications (42). MDCK cells (7×10^5) were plated onto 35-mm dishes and were either mock infected or infected with SIV/SK02 (H1N1), SIV/TX98 (H3N2), or SIV/606 at a multiplicity of infection (MOI) of 0.01. Forty-eight hours postinfection (hpi), cell monolayers were lysed, and 30 µg of total protein was resolved on sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with a mouse anti-HA (A/California/06/2009 [H1N1]) monoclonal antibody (MIA-0013; eEnzyme), a rabbit anti-multi-HA (H3N2) polyclonal antibody raised against the H3 proteins of A/Wyoming/3/03, A/Wisconsin/67/X-161/2005, and A/Brisbane/10/2007 (IA-PAN4-0100; eEnzyme), or a rabbit polyclonal antibody against M1 (in house) (42). Infrared (IR) dye-labeled anti-rabbit IgG or anti-mouse IgG (both from Li-Cor Biotechnology, Lincoln, NE, USA) was used as a secondary antibody for detection. The immunoblots were visualized by using an Odyssey IR scanner according to the manufacturer’s instructions (Li-Cor Biotechnology, Lincoln, NE, USA).

TABLE 1 Assignment of pigs for viral infection

Group (n)	Inoculum	Concn of virus (PFU/ml)
1 (4)	MEM	
2 (5)	SIV/SK02	10 ⁵
3 (5)	SIV/SK02	10 ⁶
4 (5)	SIV/TX98	10 ⁵
5 (5)	SIV/TX98	10 ⁶
6 (5)	SIV/606	10 ⁵
7 (5)	SIV/606	10 ⁶

Design of animal experiments and sampling. For the purpose of the study, we designed and executed two animal trials. The first animal trial was carried out to evaluate the attenuation of the mutant virus in pigs. Thirty-four 4-week-old SIV-seronegative pigs (Cudworth Pork Investors Group Inc., Cudworth, Saskatchewan, Canada) were randomly selected and were divided into seven groups (Table 1). All animals were kept separately in isolation rooms until the end of the study. After a week of acclimatization, pigs in all groups were challenged intratracheally (i.t.) with 4 ml of MEM (group 1) or 4 ml of MEM containing either 4×10^5 or 4×10^6 PFU of SIV/SK02, SIV/TX98, or SIV/606 (groups 2 to 7, respectively) (Table 1). Pigs were monitored daily for 5 consecutive days for the presence of SIV clinical signs and were then humanely euthanized. At necropsy, lung lesions corresponding to SIV infection were examined, recorded, and scored as described previously (12). Tissue samples from the right apical, cardiac, and diaphragmatic lobes were taken for virus isolation and histopathology (12, 15). One pathologist scored all slides and was blinded for the experimental groups.

The second animal trial was designed to evaluate whether SIV/606 could protect pigs from viral challenge with homologous parental H1N1 and heterologous H3N2 viruses. Twenty-three 4-week-old SIV-negative pigs were assigned to five groups (Table 2). Pigs in groups 1 and 2 were mock vaccinated i.t. with 4 ml of MEM. Pigs in groups 3 and 4 were vaccinated i.t. twice, 3 weeks apart, with 4 ml of MEM containing 4×10^6 PFU of SIV/606. Ten days after the second vaccination (day 31), pigs were challenged i.t. with 8×10^5 PFU of either the homologous parental virus SIV/SK02 (H1N1) (groups 1 and 3) or the heterologous virus SIV/TX98 (H3N2) (groups 2 and 4). The three pigs in group 5 were used as nonvaccinated and nonchallenged controls. Pigs were monitored daily for clinical signs of SIV infection and were then euthanized on day 5 postchallenge (day 36). Serum and nasal swab samples were collected before and after each vaccination and after the viral challenge. At necropsy, bronchoalveolar lavage fluid (BALF) was collected; lungs were examined for the presence of lesions characteristic of SIV and were scored; and tissue samples from apical, cardiac, and diaphragmatic lung lobes were collected for virus isolation and histopathology. All animal experiments were conducted at the Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, according to the ethical guidelines of the University of Saskatchewan and the Canadian Council of Animal Care.

Virus isolation and virus neutralization test (VNT). Lung samples were weighed, minced, and homogenized in 1 ml of MEM. Samples were clarified by centrifugation, and supernatants were subjected to plaque assays. Virus titers were expressed as PFU per gram of tissue.

The VNT was conducted according to the *WHO Manual on Animal Influenza Diagnosis and Surveillance* (43).

Enzyme-linked immunosorbent assay (ELISA). Antigen-specific antibody titers (IgG and IgA) from serum, nasal swabs, and BALF were determined as described previously (11).

Statistical analysis. Statistical analysis of macroscopic lung lesion scores, virus loads in lung tissues, and antibody titers was performed using GraphPad Prism 5 statistical software (GraphPad Software Inc., San Diego, CA, USA). Differences between the two groups were determined by using the nonparametric Mann-Whitney *t* test. If the *P* value was lower than or equal to 0.05, the difference between the groups was considered statistically significant.

TABLE 2 Assignment of pigs for virus challenge

Group (n)	Vaccination		Challenge (day 31)
	1 (day 0)	2 (day 21)	
1 (5)	MEM	MEM	SIV/SK02
2 (5)	MEM	MEM	SIV/TX98
3 (5)	SIV/606	SIV/606	SIV/SK02
4 (5)	SIV/606	SIV/606	SIV/TX98
5 (3)	None	None	None

RESULTS

Generation of eight-segmented SIV/606 expressing H1 and H3 HAs. Previously, Liu and Air reported that influenza A virus with a large deletion in the NA segment was viable when bacterial neuraminidase was provided in the culture (44), while Fujii et al. identified a distinct NA packaging signal (28). On the basis of the results of these studies, it was hypothesized that mutant SIV with the NA segment replaced by the HA open reading frame (ORF) flanked by the NA packaging signal would be able to replicate in cell culture in the presence of exogenous bacterial neuraminidase and would be attenuated *in vivo*. In this study, a recombinant SIV possessing a genome composed of eight RNA segments was constructed (Fig. 1B) in the background of the SIV/SK02 (H1N1) strain with the following gene modification: segment 6 (NA) of the viral genome was modified by the insertion of the ORF of the HA ectodomain (excluding the signal peptide, transmembrane domain, and cytoplasmic tail sequences) from SIV/TX98 (H3N2) flanked by the NA segment-specific packaging sequences originating from SIV/SK02 (H1N1). The NA sequence included the 3' 19-nt NCR and 183-nt terminal coding region and the 5' 28-nt NCR and 157-nt terminal coding region (Fig. 1A). After transfection using the eight-plasmid system (40) and three consecutive passages on MDCK cells in the presence of exogenous neuraminidase, a recombinant virus, SIV/606, was rescued. The optimal concentration of exogenous neuraminidase for virus propagation was later adjusted to 5 mU/ml.

Characterization of SIV/606 *in vitro*. In order to confirm that the recombinant virus SIV/606 possesses both H1 and H3 HA segments in its genome, viral RNA was isolated from purified virions and was subjected to reverse transcription-PCR (RT-PCR) using primers specific for H1 and H3 HAs. As shown in Fig. 2A, PCR products corresponding to the H1 HA segment were detected in the SIV/SK02 and SIV/606 genomes, while PCR amplicons corresponding to the H3 HA segment were observed only in the RNA samples originating from SIV/TX98 and SIV/606. These data demonstrated that chimeric segment 6 is successfully incorporated into the genome of the recombinant virus SIV/606.

To further examine whether both H1 and H3 HAs are expressed, lysates of SIV/606-infected cells were subjected to Western blotting using antibodies specific for H1 HA, H3 HA, and M1 proteins. As shown in Fig. 2B, M1 protein was detected in all virus-infected cells, H1 HA was present in SIV/SK02- and SIV/606-infected cells, and H3 HA was detected in SIV/TX98 and SIV/606 samples.

In order to demonstrate that both H1 and H3 HAs are incorporated into SIV/606 virions, purified virions were subjected to immunogold microscopy. Figure 2C shows that the majority of SIV/606 virions exhibit spherical enveloped particles with a diameter of approximately 100 nm, resembling the wild-type virions in

FIG 1 Schematic representation of segment 6 and the genome of the chimeric virus SIV/606. (A) Segment 6 is composed of the open reading frame of the HA ectodomain from SIV/TX98 (H3N2) flanked by the NA segment-specific packaging sequences derived from SIV/SK02 (H1N1). The 183-nt region derived from NA consists of the cytoplasmic tail (CT), transmembrane domain (TMD), and stalk region (SR). (B) Combination of the eight segments for virus generation.

morphology. Both anti-H1 HA and anti-H3 HA antibodies could react with the SIV/606 virion. However, SIV/SK02 and SIV/TX98 virions were stained only with their respective antibodies.

The replication potential of SIV/606 was investigated in cell culture. In the presence of exogenous bacterial sialidase, SIV/606 formed plaques similar in size to those of the parental WT virus SIV/SK02. In contrast, SIV/606 was not able to replicate in the absence of exogenous sialidase (Fig. 3A) indicating that replication of the recombinant SIV/606 is highly dependent on the presence of exogenously added sialidase. As shown in Fig. 3B, the growth kinetics of SIV/606 was similar to that of the parental virus SIV/SK02. Both viruses reached plateaus at 24 hpi; however, the titer of SIV/606 was approximately 1 log unit lower than that of the parental virus SIV/SK02 (6×10^7 PFU/ml for the WT virus; $6.6 \times$

10^6 PFU/ml for SIV/606). These results indicated that although SIV/606 is slightly attenuated, it is still able to reach a relatively high titer in cell culture, justifying its use as a vaccine seed candidate.

To ensure that modification of the NA segment does not alter virus stability, we propagated mutant SIV/606 by as many as 5 consecutive passages on MDCK cells in the presence of exogenous neuraminidase. RNA was isolated from passage 5 supernatants and was subjected to RT-PCR to confirm the presence of the chimeric H3 segment as well as the H1 segment in the virion. SIV/606 was able to maintain both the H1 HA and H3 HA segments for 5 consecutive passages (data not shown).

SIV/606 is attenuated in pigs. In order to evaluate the pathogenicity of SIV/606, 34 4-week-old SIV-seronegative pigs were randomly selected and were divided into seven groups with 5 pigs per group, except for group 1, which had 4 pigs. Pigs were challenged i.t. with 4 ml MEM containing either 1×10^5 (low dose) or 1×10^6 (high dose) PFU/ml of SIV/SK02, SIV/TX98, or SIV/606. The animals in the control group were mock infected with medium only (MEM) (Table 1). Following challenge, pigs were monitored daily for the presence of clinical signs and for elevation of body temperatures. Five days postchallenge, pigs were euthanized, and necropsies were performed. During the 5-day observation period, respiratory symptoms (sneezing, coughing, and increased respiration) were observed on day 1 in all groups infected with either WT virus, SIV/SK02 or SIV/TX98 (low-dose and high-dose groups). Animals in the control, unchallenged group, as well as animals in both groups that received SIV/606, showed no clinical signs associated with SIV infections. In addition, only animals that were challenged with wild-type SIV (SIV/SK02 or SIV/TX98) showed elevations in body temperatures at 24 h postchallenge (data not shown). These data were consistent with our previous studies (12, 15).

At necropsy, lungs were removed *in toto* and the percentage of areas affected with pneumonia was estimated visually for each lung lobe. Total percentage for the entire lung was calculated based on weight proportions of each lung lobe to the total lung volume (45). The final score represents accumulative scores from all lobes originating from same animal (Fig. 4A). All pigs infected with SIV/SK02 developed typical SIV lung lesions. These lesions

FIG 2 Characterization of the chimeric virus SIV/606. (A) RT-PCR of H1 HA and H3 HA. Viral RNAs purified from SIV/606, SIV/SK02, and SIV/TX98 were transcribed and were amplified with an H1 HA- or H3 HA-specific primer set. (B) Detection of H1 and H3 in infected-cell lysates by Western blotting. MDCK cells were either mock infected or infected with SIV/606, SIV/SK02, or SIV/TX98 at an MOI of 0.01, and whole-cell lysates were prepared at 48 hpi. (C) Transmission immunoelectron microscopy of SIV/606. Purified SIV/606 virions were stained with monoclonal antibodies against H1 and H3, followed by negative staining. Black dots represent 10-nm gold particles.

FIG 3 Growth properties of SIV/606. (A) Plaques formed by SIV/SK02 and SIV/606 on MDCK cells in the presence or absence of neuraminidase. (B) Multiple-cycle growth curve of SIV/606 on MDCK cells. Cells were infected either with SIV/SK02 or with SIV/606 at an MOI of 0.001. SIV/SK02 was propagated in the presence of trypsin, while SIV/606 was grown in the presence of trypsin and neuraminidase. Supernatants were collected at the indicated time points until 72 hpi, and titers were determined by plaque assays on MDCK cells.

presented as purple to dark red consolidated areas predominant in the cardiac and apical lobes, regardless of the dose used (median lung scores were 9 and 10 in groups 2 and 3, respectively). Pigs infected with SIV/TX98 (H3N2) also developed gross lesions characteristic of SIV in the lungs (median scores were 5 and 5.5 in groups 4 and 5, respectively). In contrast, both groups of SIV/606-infected pigs (groups 6 and 7) showed no or minimal lung lesions characteristic of SIV. Pigs in the control group (group 1) were free of gross lesions.

Tissue samples from the apical, cardiac, and diaphragmatic lobes were collected and processed for virus isolation and histopathology. Viruses were recovered from the lung samples of all

animals infected with the wild-type SIVs (groups 2 to 5). The median virus titers in the lungs of pigs infected with SIV/SK02 at low and high doses (groups 2 and 3) were 2.4×10^4 PFU/g and 2.6×10^4 PFU/g, respectively. As shown in Fig. 4B, the median viral titers in the SIV/TX98-infected groups reached 1×10^4 PFU/g (group 4) and 4×10^4 PFU/g (group 5). In contrast, no virus was isolated from 4 out of 5 pigs infected with a low dose, and from 2 out of 5 pigs infected with a high dose, of SIV/606. The remaining pigs in these groups had very low virus titers in their lungs (the median titer in group 7 was 2.0×10^1 PFU/g). No virus was detected in the control group.

Histopathological lesions were examined using lung tissues

FIG 4 Macroscopic lung lesion scores (A) and virus titers in lung tissues (B) of pigs infected with a high (H) (4×10^6 PFU) or low (L) (4×10^5 PFU) dose of virus. (A) On day 5 postinfection, pigs were euthanized, and the lungs were scored according to the presence and severity of lung lesions. (B) Lung tissues were collected and homogenized, and virus titers were determined by plaque assays on MDCK cells. Each symbol represents the lung score or virus titer for an individual pig within an experimental group. Horizontal lines indicate median values.

FIG 5 Microscopic lung lesions. (A) Lung of a control pig inoculated with MEM only. (B to G) Representative histopathology lung samples from pigs infected with SIV/SK02 at a low (B) or high (C) dose, with SIV/TX98 at a low (D) or high (E) dose, or with SIV/606 at a low (F) or high (G) dose. Magnification, $\times 20$; bars, 100 μM .

from the right apical, cardiac, and diaphragmatic lobes. The most severe microscopic lesions were observed in groups challenged with SIV/SK02 (groups 2 and 3). The dominant histopathology observations in these groups were necrotizing bronchiolitis and interstitial pneumonia (Fig. 5B and C). Pigs infected with SIV/TX98 (groups 4 and 5) developed similar histopathological changes in the lungs (Fig. 5D and E), although they were less severe than those in the SIV/SK02-infected groups. SIV/606-infected pigs (groups 6 and 7) showed minor histopathological changes, consisting of mild interstitial thickening and peribronchial neutrophil infiltration (Fig. 5F and G). Lung sections from mock-infected pigs showed no microscopic lesions (Fig. 5A). These results indicated that the mutant virus SIV/606 is highly attenuated in pigs.

Antibody titers after SIV/606 vaccination. To determine whether SIV/606 is immunogenic and able to provide protection against homologous and heterologous SIV infections in pigs, we proceeded with a vaccination and viral challenge study. Twenty-three H1N1- and H3N2-seronegative pigs were randomly selected and were divided into five groups (Table 2). Pigs in groups 1 and 2 were mock vaccinated with MEM, while pigs in groups 3 and 4 were vaccinated i.t. with 4×10^6 PFU of SIV/606. Pigs in group 5 served as nonvaccinated and nonchallenged controls. Three weeks after the first vaccination, pigs in all groups were revaccinated with the same vaccine material. Ten days after the second vaccination (day 31), pigs were challenged i.t. with either WT SIV/SK02 or WT SIV/TX98; they were euthanized on day 5 postinfection (day 36). Serum and nasal swab samples were collected prior to each vacci-

nation and before challenge (days 0, 21, and 31). Antigen-specific serum IgG and nasal IgA titers were determined by ELISA. The first vaccination with SIV/606 resulted in significant increases in the titers of serum IgG specific to SIV/SK02 (median antibody titer, 158.5; P , <0.0001). The antibody titers continued to rise and reached a median value of 1,323 (P , <0.0001) after the second vaccination with SIV/606 (Fig. 6A). The titers of IgG against SIV/TX98 in serum did not show significant increases after one vaccination; however, antibody titers rose significantly after the second vaccination (median antibody titer, 614.5; P , <0.0001) (Fig. 6B).

To assess the presence of IgA antibodies specific to H1N1 and H3N2 influenza A viruses at the mucosal surfaces in the upper and lower respiratory tracts, nasal swabs and BALF samples from pigs in all groups were collected and tested by ELISA. As seen in Fig. 6C and D, the first vaccination with SIV/606 induced low to moderate increases in IgA levels at nasal mucosal surfaces, while after the second vaccination, levels of IgA antibody specific to SIV/SK02 and SIV/TX98 were significantly increased, with median titers of 302 and 137, respectively (P , <0.0001 and 0.003, respectively). BALF samples were collected at necropsy on day 5 after the viral challenge (Fig. 6E and F). SIV/SK02- and SIV/TX98-specific IgA titers from BALF were significantly induced in both SIV/606-vaccinated and challenged groups (median titers, 3,050 for group 3 and 915 for group 4 [P , 0.0079 in both cases]).

Vaccination with SIV/606 induced neutralizing antibodies against both H1N1 and H3N2 SIVs in serum. Neutralizing antibodies in serum against SIV/SK02 (H1N1) and SIV/TX98 (H3N2)

FIG 6 Antibody titers induced after i.t. vaccination with SIV/606. Serum IgG (A and B), nasal IgA (C and D), BALF IgA (E and F), and neutralizing antibody (G) titers specific to SIV/SK02 and SIV/TX98 were detected in samples collected before vaccination and after the first and second vaccinations with SIV/606. Horizontal lines indicate median values. **, $P < 0.01$; ****, $P < 0.0001$.

were evaluated using samples prior to the challenge with wild-type viruses (day 31). As seen in Fig. 6G, two vaccinations with SIV/606 induced considerable levels of neutralizing antibodies against H1N1 and H3N2 SIVs (median titers, 640 and 480, respectively). None of the pigs in the MEM control group had detectable neutralizing antibodies against either of the SIVs tested.

Vaccination with SIV/606 provides protection against H1N1 and H3N2 SIV infection in pigs. Following viral challenge (day 31), pigs in all groups were monitored daily for the presence of clinical signs characteristic of SIV and were humanely euthanized on day 36. In agreement with our previous observations, pigs in the mock-vaccinated and challenged groups (groups 1 and 2) showed increases in body temperatures at 24 h postchallenge (mean temperatures, 40.9°C and 40.1°C). In contrast, the pigs in SIV/606-vaccinated and challenged groups (group 3 and 4) had temperatures within the normal range ($39.5 \pm 0.5^\circ\text{C}$) during the 5-day observation period (Fig. 7A and B). No other clinical signs were observed in any experimental groups.

At necropsy, lungs were examined and were scored according to the presence and severity of gross lesions characteristic of SIV (Fig. 7C). All unvaccinated pigs challenged with either SIV/SK02 or SIV/TX98 (groups 1 and 2) had gross lesions typical for SIV, consisting of clearly demarcated dark purple consolidated areas found mostly in the apical and cardiac lobes. The median lung scores for these two groups were 7 and 8, respectively. Pigs vac-

inated with SIV/606 and challenged with either SIV/SK02 or SIV/TX98 had no detectable gross lesions in the lungs at necropsy.

Viral loads in the lungs of infected pigs were determined from the tissue samples of the right apical, cardiac, and diaphragmatic lobes collected at necropsy (Fig. 7D). SIV/SK02 was detected in the lung tissues from all pigs in the unvaccinated, SIV/SK02-challenged group (median viral titer, 2×10^4 PFU/g). Virus was recovered from 1 of 5 unvaccinated, SIV/TX98-challenged pigs (group 2) (Fig. 7D). The poor recovery of SIV/TX98 can be attributed to the lower pathogenicity of this strain than of SIV/SK02 and the increased immunity of the pigs, which correlated with their age. This observation was consistent with those from previous studies using SIV/TX98 (46). No virus was detected in the lung samples of pigs in the SIV/606-vaccinated, SIV-challenged groups (groups 3 and 4) (Fig. 7D).

The histopathology findings were consistent with the necropsy observations. Microscopic lesions were examined using lung tissue samples taken from the right apical, cardiac, and diaphragmatic lobes at necropsy. As shown in Fig. 8B and D, severe to moderate histopathological lesions were observed in the lung samples of unvaccinated pigs challenged with SIV/SK02 or SIV/TX98. The dominant microscopic observations in these two groups were moderate to severe necrosis of bronchiolar epithelium, hypertrophy and hyperplasia of bronchiolar epithelium, peribronchiolar and perivascular lymphocyte and neutrophil infiltration, interstitial thickening, and

FIG 7 Vaccination with SIV/606 provides protection against SIV infection in pigs. (A and B) Body temperature changes in pigs for 5 days after infection with SIV/SK02 (A) or SIV/TX98 (B). The daily mean body temperatures ($\pm$ standard deviations) of pigs in the MEM and SIV/606 vaccine groups were compared. (C and D) Macroscopic lung lesion scores (C) and virus titers in the lungs (D) of pigs vaccinated with SIV/606 and challenged with SIV/SK02 or SIV/TX98. Each symbol represents the lung score or virus titer of an individual pig within an experimental group. Horizontal lines indicate median values. **, $P < 0.01$.

proliferation of bronchiole-associated lymphoid tissues (BALT). In contrast, no histopathological changes were observed in the lung sections of SIV/606-vaccinated groups challenged with SIV/SK02 or SIV/TX98 (Fig. 8C and E). These two vaccinated groups maintained healthy bronchiolar epithelium and alveolar structures, with mild microscopic changes predominantly presenting as interstitial thickening. Taking our findings together, even though virus could be detected in only one pig in the mock-vaccinated group challenged with the H3N2 virus, the body temperature and macroscopic and microscopic lung pathology results showed that vaccination with SIV/606 provided protection against challenge with H1N1 and H3N2 viruses.

DISCUSSION

LAIV are recognized as an effective alternative to currently used inactivated-SIV vaccines. Commercially available LAIV in North America are registered for use in humans and horses only. These LAIV are attenuated based on the incorporation of cold-adapted (ca) and/or temperature sensitive (ts) phenotypes into vaccine strains by recombination with a donor strain or by multiple passages of the vaccine strain at low temperatures (47–49). Several different experimental live attenuated SIV vaccines have been demonstrated to induce strong cell-mediated immunity (CMI) and humoral immunity, as well as cross-protection against homologous heterotypic and heterologous SIV isolates (11, 16). Even though these experimental SIV LAIV have advantages over their inactivated counterparts, no LAIV is currently registered for use against SIV infections worldwide.

The mechanism of influenza virus genome packaging has been debated for a long time. However, an increasing number of reports provide convincing evidence to support the selective pack-

aging model (28, 50, 51). The selective packaging model claims that each of eight vRNA segments possesses a segment-specific packaging signal sequence, allowing the selective incorporation of eight vRNA segments into a fully functional virion. These packaging signal sequences comprise noncoding regions and parts of coding regions at both ends of each RNA segment. Along with extensive studies on packaging signals, there have been several experimental attempts to generate a mutant virus carrying a chimeric segment replacing the original ORF with other genes flanked by packaging sequences (28, 34, 52, 53). A mutant virus carrying a foreign gene in the different influenza virus segments was generated to prove that there are segment-specific packaging sequences for the specific viral RNA to be incorporated into a virion. Segments encoding HA and/or NA, the two major surface glycoproteins, were commonly used as targets for the manipulation and introduction of foreign genes (34, 53). A seven-segmented influenza A virus in which the HA and NA segments were replaced with the hemagglutinin-esterase fusion (HEF) segment of influenza C virus flanked by HA packaging sequences was generated by Gao et al. (52). Watanabe et al. described the use of the coding region of vesicular stomatitis virus G glycoprotein flanked by the HA packaging sequence to replace the HA ORF (34). In addition, Gao et al. reported the generation of a nine-segment virus containing two HAs, one of which was incorporated by replacing the ORF of PB2 with the H3 HA ORF (35). Very recently, Pena et al. generated a recombinant influenza virus by rearranging the genome of an avian H9N2 virus and expressed the entire H5 HA ORF from the NS segment RNA (54). All these results suggest the possibility and feasibility of rescuing influenza A viruses by replacing the coding region of HA, NA, or other influenza virus

FIG 8 Lung histopathology results. Shown are representative lung samples from the unvaccinated and unchallenged control group (A), the groups challenged with SIV/SK02 after vaccination with MEM (B) or SIV/606 (C), and the groups challenged with SIV/TX98 after vaccination with MEM (D) or SIV/606 (E). Magnification, $\times 20$. Bars, 100 μM .

segments with the gene of interest flanked by segment-packaging sequences originating from the original gene segment.

Previous studies demonstrated that an elastase-dependent SIV could be used as a LAIV against homologous, homologous heterotypic, and heterologous SIV isolates (11, 14). Despite the significant protective efficacy against parental and homotypic SIVs, only partial protection was elicited against the heterologous H3N2 subtype. The partial protection was most likely attributable to the low levels of neutralizing antibodies despite the induction of strong CMI and cross-reactive antibodies in serum and BALF. In an effort to increase the efficacy of protection against heterologous SIV infections in pigs, it was hypothesized that a live virus expressing two HAs of different subtypes would be able to induce potent CMI and humoral immunity, resulting in high titers of neutralizing antibodies that would provide protection against the two dominant circulating strains within the swine population. To overcome the stability issues for mutant viruses where an additional segment is incorporated into the virion, we utilized an approach retaining

the eight original packaging signals while replacing the ectodomain of NA with the ectodomain of H3 HA. It is well documented that the enzymatic function of influenza virus NA can be replaced by the addition of exogenous sialidases (44, 55). Therefore, in the present study, we generated a mutant SIV that expresses two HAs, H1 and H3, by replacing the NA segment with the H3 HA coding sequence flanked by NA packaging sequences in order to ensure the incorporation of all eight segments into the virion.

SIV/SK02 (H1N1) is an influenza virus of avian origin isolated from Canadian pigs that possesses genetic and antigenic differences from classical SIV (2). The H3 HA coding region originated from the triple-reassortant H3N2 virus SIV/TX98 (56). Using molecular techniques and reverse genetics, we successfully rescued a mutant virus bearing a chimeric NA-HA segment in the presence of an exogenously added neuraminidase substitute (bacterial sialidase). The incorporation of the H1 HA segment and the NA segment containing the H3 HA coding region into the virion was confirmed by PCR (Fig. 2A). The expression and incorporation of

both the H1 and H3 HAs was further confirmed by Western blotting and immunoelectron microscopy (Fig. 2B and C). The replacement of the NA ORF with the H3 HA ORF resulted in the loss of the natural influenza virus neuraminidase enzymatic activity and reduced the replication potential of the mutant virus SIV/606. Without the NA activity, progeny virions will remain attached to the cellular membrane, unable to cleave sialic acids and initiate a new replication cycle, resulting in virus attenuation. In addition, these attached virions will serve as targets for the host immune system, inducing both CMI and humoral immune responses in animals. The replication ability of the mutant virus SIV/606 was highly dependent on exogenously provided neuraminidase (Fig. 3A), as evidenced by the fact that the mutant virus was able to replicate and form visible plaques only in the presence of exogenous neuraminidase (Fig. 3A). However, the kinetics and virus yield of SIV/606 were slightly lower than those of the WT virus SIV/SK02 (Fig. 3B). These results suggested that the mutant virus SIV/606 was able to maintain its modified genome after multiple passages and to replicate, with a considerable yield, in tissue culture when propagated in the presence of exogenous neuraminidase.

Based on previous experience with elastase-dependent SIV, which exhibited restricted replication *in vivo* due to the lack of elastase (12), it was anticipated that SIV/606 would behave in a similar manner, displaying altered replication due to the lack of neuraminidase activity *in vivo*. The pathogenicity and attenuation level of the mutant virus SIV/606 were evaluated in pigs. Necropsy and histopathology results indicated that the mutant virus SIV/606 was attenuated *in vivo*. Minimal gross lesions in the lungs and low virus titers in 4 of 10 challenged pigs corresponded to histopathology findings characterized by mild peribronchial infiltration of polymorphonuclear cells and neutrophils. Taken together, these findings suggested limited viral replication and limited induction of inflammatory responses.

The presence of neuraminidase in the respiratory organs of swine is not well defined. However, Lillehoj et al. reported sialidase activity in human airway epithelial cells derived from lysosomal neuraminidase (NEU1) (57). *Clostridium perfringens* neuraminidase, which is highly homologous to NEU1 (58), was unable to support SIV/606 replication *in vitro* (data not shown), possibly because the neuraminidase activity of *Clostridium perfringens* has optimal pH and calcium ion requirements different from those of *Vibrio cholerae* (59).

To evaluate the efficacy of the mutant virus SIV/606, pigs were vaccinated twice, 3 weeks apart, by the i.t. route and were challenged with either the homologous H1N1 virus SIV/SK02 or the heterologous H3N2 virus SIV/TX98. We based our choice of the i.t. route of vaccination on the following rationale: the vaccine efficacy would be dependent largely on the first cycle of SIV/606 infection; thus, for a proof-of-concept study, we wanted to ensure that 100% of the SIV/606 would be delivered to the respiratory tract. We are aware that this is not an optimal route for field application; we are currently pursuing an intranasal vaccination study. As in our previous studies with elastase-dependent LAIV (11), SIV/606 was evaluated for efficacy following SIV challenge by monitoring clinical signs, evaluating macroscopic and microscopic lung lesions, and determining virus loads and antibody responses in serum, nasal mucosae, and BALF. SIV/606-vaccinated animals showed no clinical signs of SIV, had no or minimal macroscopic and microscopic lesions, and demonstrated sterile

immunity to the challenges with the homologous H1N1 virus SIV/SK02 and the heterologous H3N2 virus SIV/TX98. Two vaccinations with SIV/606 induced antigen-specific IgG and IgA in serum and mucosal surfaces of the upper and lower respiratory tracts. In addition, SIV/606 was able to induce neutralizing antibodies in serum against both the homologous H1N1 virus SIV/SK02 and the heterologous H3N2 virus SIV/TX98, suggesting that the presence of neutralizing antibodies is required to provide sterile immunity against heterologous viruses.

In this study, we described a novel strategy to generate LAIV against SIV infections. The mutant virus we constructed was able to express two dominant antigenic forms of HA on its virions without altering its stability and replication ability under optimal experimental conditions. This novel mutant virus was highly attenuated *in vivo* but capable of inducing strong immune responses, which resulted in complete sterile protection against two antigenically distinct circulating SIVs. To better understand the mechanism of protection by this novel SIV vaccine candidate, future studies will include more-detailed examination of immune responses, as well as of the intranasal route of vaccination, the vaccine dosage, and the regimen.

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REFERENCES

1. Straw BE, D'Allaire S, Menegeling WL, Taylor DJ. 1999. Diseases of swine, 8th ed. Iowa State University Press, Ames, IA.
2. Karasin AI, West K, Carman S, Olsen CW. 2004. Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. *J. Clin. Microbiol.* 42:4349–4354.
3. Olsen CW. 2002. The emergence of novel swine influenza viruses in North America. *Virus Res.* 85:199–210.
4. Brown GB, McMillen JK. 1994. MaxiVac-Flu: evaluation of the safety and efficacy of a swine influenza vaccine, p 37–39. *In* Proceedings of the American Association of Swine Practitioners, 25th Annual Meeting, Chicago, IL.
5. Kobinger GP, Meunier I, Patel A, Pillet S, Gren J, Stebner S, Leung A, Neufeld JL, Kobasa D, von Messling V. 2010. Assessment of the efficacy of commercially available and candidate vaccines against a pandemic H1N1 virus. *J. Infect. Dis.* 201:1000–1006.
6. Kyriakis CS, Gramer MR, Barbe F, Van Doorselaere J, Van Reeth K. 2010. Efficacy of commercial swine influenza vaccines against challenge with a recent European H1N1 field isolate. *Vet. Microbiol.* 144:67–74.
7. Powers DC, Smith GE, Anderson EL, Kennedy DJ, Hackett CS, Wilkinson BE, Volvovitz F, Belshe RB, Treanor JJ. 1995. Influenza A virus vaccines containing purified recombinant H3 hemagglutinin are well tolerated and induce protective immune responses in healthy adults. *J. Infect. Dis.* 171:1595–1599.
8. Heinen PP, de Boer-Luijtz EA, Bianchi AT. 2001. Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. *J. Gen. Virol.* 82:2697–2707.
9. Kappes MA, Sandbulte MR, Platt R, Wang C, Lager KM, Henningson JN, Lorusso A, Vincent AL, Loving CL, Roth JA, Kehrli ME, Jr. 2012. Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. *Vaccine* 30:280–288.
10. Loving CL, Vincent AL, Pena L, Perez DR. 2012. Heightened adaptive immune responses following vaccination with a temperature-sensitive,

- live-attenuated influenza virus compared to adjuvanted, whole-inactivated virus in pigs. *Vaccine* 30:5830–5838.
11. Masic A, Booth JS, Mutwiri GK, Babiuk LA, Zhou Y. 2009. Elastase-dependent live attenuated swine influenza A viruses are immunogenic and confer protection against swine influenza A virus infection in pigs. *J. Virol.* 83:10198–10210.
 12. Masic A, Babiuk LA, Zhou Y. 2009. Reverse genetics-generated elastase-dependent swine influenza viruses are attenuated in pigs. *J. Gen. Virol.* 90:375–385.
 13. Solorzano A, Webby RJ, Lager KM, Janke BH, Garcia-Sastre A, Richt JA. 2005. Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. *J. Virol.* 79:7535–7543.
 14. Babiuk S, Masic A, Graham J, Neufeld J, van der Loop M, Copps J, Berhane Y, Pasick J, Potter A, Babiuk LA, Weingartl H, Zhou Y. 2011. An elastase-dependent attenuated heterologous swine influenza virus protects against pandemic H1N1 2009 influenza challenge in swine. *Vaccine* 29:3118–3123.
 15. Masic A, Lu X, Li J, Mutwiri GK, Babiuk LA, Brown EG, Zhou Y. 2010. Immunogenicity and protective efficacy of an elastase-dependent live attenuated swine influenza virus vaccine administered intranasally in pigs. *Vaccine* 28:7098–7108.
 16. Richt JA, Lekcharoensuk P, Lager KM, Vincent AL, Loiacono CM, Janke BH, Wu WH, Yoon KJ, Webby RJ, Solorzano A, Garcia-Sastre A. 2006. Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. *J. Virol.* 80:11009–11018.
 17. Vincent AL, Ma W, Lager KM, Janke BH, Webby RJ, Garcia-Sastre A, Richt JA. 2007. Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. *Vaccine* 25:7999–8009.
 18. Palese P, Shaw ML. 2007. *Orthomyxoviridae: the viruses and their replication*, p 1648–1679. In Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (ed), *Fields virology*, 5th ed, vol 2. Lippincott Williams & Wilkins, Philadelphia, PA.
 19. Gamblin SJ, Skehel JJ. 2010. Influenza hemagglutinin and neuraminidase membrane glycoproteins. *J. Biol. Chem.* 285:28403–28409.
 20. Skehel JJ, Wiley DC. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* 69:531–569.
 21. Air GM, Laver WG. 1989. The neuraminidase of influenza virus. *Proteins* 6:341–356.
 22. Colman PM, Laver WG, Varghese JN, Baker AT, Tulloch PA, Air GM, Webster RG. 1987. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature* 326:358–363.
 23. Palese P, Tobita K, Ueda M, Compans RW. 1974. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology* 61:397–410.
 24. Mitnaul LJ, Castrucci MR, Murti KG, Kawaoka Y. 1996. The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. *J. Virol.* 70:873–879.
 25. Liu C, Eichelberger MC, Compans RW, Air GM. 1995. Influenza type A virus neuraminidase does not play a role in viral entry, replication, assembly, or budding. *J. Virol.* 69:1099–1106.
 26. Hutchinson EC, von Kirchbach JC, Gog JR, Digard P. 2010. Genome packaging in influenza A virus. *J. Gen. Virol.* 91:313–328.
 27. Fujii K, Fujii Y, Noda T, Muramoto Y, Watanabe T, Takada A, Goto H, Horimoto T, Kawaoka Y. 2005. Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. *J. Virol.* 79:3766–3774.
 28. Fujii Y, Goto H, Watanabe T, Yoshida T, Kawaoka Y. 2003. Selective incorporation of influenza virus RNA segments into virions. *Proc. Natl. Acad. Sci. U. S. A.* 100:2002–2007.
 29. Liang Y, Hong Y, Parslow TG. 2005. *cis*-Acting packaging signals in the influenza virus PB1, PB2, and PA genomic RNA segments. *J. Virol.* 79:10348–10355.
 30. Liang Y, Huang T, Ly H, Parslow TG. 2008. Mutational analyses of packaging signals in influenza virus PA, PB1, and PB2 genomic RNA segments. *J. Virol.* 82:2295–2304.
 31. Marsh GA, Rabadan R, Levine AJ, Palese P. 2008. Highly conserved regions of influenza A virus polymerase gene segments are critical for efficient viral RNA packaging. *J. Virol.* 82:2295–2304.
 32. Muramoto Y, Takada A, Fujii K, Noda T, Iwatsuki-Horimoto K, Watanabe S, Horimoto T, Kida H, Kawaoka Y. 2006. Hierarchy among viral RNA (vRNA) segments in their role in vRNA incorporation into influenza A virions. *J. Virol.* 80:2318–2325.
 33. Ozawa M, Maeda J, Iwatsuki-Horimoto K, Watanabe S, Goto H, Horimoto T, Kawaoka Y. 2009. Nucleotide sequence requirements at the 5' end of the influenza A virus M RNA segment for efficient virus replication. *J. Virol.* 83:3384–3388.
 34. Watanabe T, Watanabe S, Noda T, Fujii Y, Kawaoka Y. 2003. Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes. *J. Virol.* 77:10575–10583.
 35. Gao Q, Lowen AC, Wang TT, Palese P. 2010. A nine-segment influenza A virus carrying subtype H1 and H3 hemagglutinins. *J. Virol.* 84:8062–8071.
 36. Gao Q, Palese P. 2009. Rewiring the RNAs of influenza virus to prevent reassortment. *Proc. Natl. Acad. Sci. U. S. A.* 106:15891–15896.
 37. Ozawa M, Victor ST, Taft AS, Yamada S, Li C, Hatta M, Das SC, Takashita E, Kakugawa S, Maher EA, Neumann G, Kawaoka Y. 2011. Replication-incompetent influenza A viruses that stably express a foreign gene. *J. Gen. Virol.* 92:2879–2888.
 38. Zhou Y, König M, Hobom G, Neumeier E. 1998. Membrane-anchored incorporation of a foreign protein in recombinant influenza virions. *Virology* 246:83–94.
 39. Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y. 2007. Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. *J. Gen. Virol.* 88:13–18.
 40. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. U. S. A.* 97:6108–6113.
 41. Pyo HM, Masic A, Woldeab N, Embury-Hyatt C, Lin L, Shin YK, Song JY, Babiuk S, Zhou Y. 2012. Pandemic H1N1 influenza virus-like particles are immunogenic and provide protective immunity to pigs. *Vaccine* 30:1297–1304.
 42. Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y. 2007. Effect of the phosphatidylinositol 3-kinase/Akt pathway on influenza A virus propagation. *J. Gen. Virol.* 88:942–950.
 43. World Health Organization. 2002. WHO manual on animal influenza diagnosis and surveillance, 2nd ed. World Health Organization, Geneva, Switzerland.
 44. Liu C, Air GM. 1993. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase genes. *Virology* 194:403–407.
 45. Halbur PG, Paul PS, Meng XJ, Lum MA, Andrews JJ, Rathje JA. 1996. Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. *J. Vet. Diagn. Invest.* 8:11–20.
 46. Richt JA, Lager KM, Janke BH, Woods RD, Webster RG, Webby RJ. 2003. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. *J. Clin. Microbiol.* 41:3198–3205.
 47. Maassab HF. 1967. Adaptation and growth characteristics of influenza virus at 25 degrees C. *Nature* 213:612–614.
 48. Maassab HF, Bryant ML. 1999. The development of live attenuated cold-adapted influenza virus vaccine for humans. *Rev. Med. Virol.* 9:237–244.
 49. Murphy BR, Coelingh K. 2002. Principles underlying the development and use of live attenuated cold-adapted influenza A and B virus vaccines. *Viral Immunol.* 15:295–323.
 50. Chou YY, Vafabakhsh R, Doganay S, Gao Q, Ha T, Palese P. 2012. One influenza virus particle packages eight unique viral RNAs as shown by FISH analysis. *Proc. Natl. Acad. Sci. U. S. A.* 109:9101–9106.
 51. Noda T, Kawaoka Y. 2012. Packaging of influenza virus genome: robustness of selection. *Proc. Natl. Acad. Sci. U. S. A.* 109:8797–8798.
 52. Gao Q, Brydon EW, Palese P. 2008. A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. *J. Virol.* 82:6419–6426.
 53. Shinya K, Fujii Y, Ito H, Ito T, Kawaoka Y. 2004. Characterization of a neuraminidase-deficient influenza A virus as a potential gene delivery vector and a live vaccine. *J. Virol.* 78:3083–3088.
 54. Pena L, Sutton T, Chockalingam A, Kumar S, Angel M, Shao H, Chen H, Li W, Perez DR. 2013. Influenza viruses with rearranged genomes as live-attenuated vaccines. *J. Virol.* 87:5118–5127.
 55. Hughes MT, Matrosovich M, Rodgers ME, McGregor M, Kawaoka Y. 2000. Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. *J. Virol.* 74:5206–5212.

56. Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG. 2000. Evolution of swine H3N2 influenza viruses in the United States. *J. Virol.* 74:8243–8251.
57. Lillehoj EP, Hyun SW, Feng C, Zhang L, Liu A, Guang W, Nguyen C, Luzina IG, Atamas SP, Passaniti A, Twaddell WS, Puche AC, Wang LX, Cross AS, Goldblum SE. 2012. NEU1 sialidase expressed in human airway epithelia regulates epidermal growth factor receptor (EGFR) and MUC1 protein signaling. *J. Biol. Chem.* 287:8214–8231.
58. Hinek A, Bodnaruk TD, Bunda S, Wang Y, Liu K. 2008. Neuraminidase-1, a subunit of the cell surface elastin receptor, desialylates and functionally inactivates adjacent receptors interacting with the mitogenic growth factors PDGF-BB and IGF-2. *Am. J. Pathol.* 173:1042–1056.
59. Cassidy JT, Jourdain GW, Roseman S. 1965. The sialic acids. VI. Purification and properties of sialidase from *Clostridium perfringens*. *J. Biol. Chem.* 240:3501–3506.