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The M Segment of the 2009 New Pandemic H1N1 Influenza Virus Is Critical for Its High Transmission Efficiency in the Guinea Pig Model[▽]

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A remarkable feature of the 2009 pandemic H1N1 influenza virus is its efficient transmissibility in humans compared to that of precursor strains from the triple-reassortant swine influenza virus lineage, which cause only sporadic infections in humans. The viral components essential for this phenotype have not been fully elucidated. In this study, we aimed to determine the viral factors critical for aerosol transmission of the 2009 pandemic virus. Single or multiple segment reassortments were made between the pandemic A/California/04/09 (H1N1) (Cal/09) virus and another H1N1 strain, A/Puerto Rico/8/34 (H1N1) (PR8). These viruses were then tested in the guinea pig model to understand which segment of Cal/09 virus conferred transmissibility to the poorly transmissible PR8 virus. We confirmed our findings by generating recombinant A/swine/Texas/1998 (H3N2) (sw/Tx/98) virus, a representative triple-reassortant swine virus, containing segments of the Cal/09 virus. The data showed that the M segment of the Cal/09 virus promoted aerosol transmissibility to recombinant viruses with PR8 and sw/Tx/98 virus backgrounds, suggesting that the M segment is a critical factor supporting the transmission of the 2009 pandemic virus.

A hallmark of the 2009 pandemic influenza virus is its efficient transmission in humans. The virus was first reported in Mexico, followed by 2 confirmed cases in children from Southern California in April 2009 (2). In the months following its emergence, nearly 180,000 cases of 2009 H1N1 virus had been confirmed around the globe (35). In June 2009, the World Health Organization (WHO) formally declared an H1N1 pandemic. The virus was determined to be a reassortant, with 6 viral genes from a triple-reassortant classical swine virus and 2 genes from a Eurasian swine virus (7, 24). Interestingly, triple-reassortant classical swine virus infections in humans had been sporadically reported and mostly self-limiting until this time (4, 22, 29). Even though several studies have determined the viral virulence factors for the 2009 pandemic influenza virus (8, 9, 12, 19, 26), the viral factor(s) contributing to the sustained transmission in humans, a most intriguing question about this virus, remains unresolved.

Herein, we used the well-established guinea pig model of influenza virus transmission in mammals (14, 15) to investigate the viral genetic determinants of the 2009 pandemic virus that confer its efficient transmission. We hypothesized that through exchanging the 2009 pandemic virus gene segments with other influenza viruses that transmit with low efficiency, we could identify the critical genetic factors responsible for viral transmission. A/California/04/2009 (Cal/09) virus is a representative strain of the 2009 pandemic, and it has been shown by multiple laboratories that the Cal/09 virus transmitted efficiently through the aerosol route in both ferrets and guinea pigs (16, 28).

We first evaluated the effect on viral transmission of introducing different viral segments of the Cal/09 virus into the poorly transmissible influenza A/Puerto Rico/8/34 (PR8) virus background. Phylogenetic studies showed that the 2009 new pandemic H1N1 virus originated with reassortment between the North American triple-reassortant classical swine influenza viruses and the Eurasian avian-like swine influenza viruses (7, 24). To better recapitulate the emergence of the pandemic virus, we further confirmed our findings with reassortant viruses between the Cal/09 virus and influenza A/Swine/Texas/4199-2/1998 (H3N2) virus, a representative virus of the triple-reassortant classical swine influenza virus lineage (34). Our data showed that only the reassortant viruses harboring the Cal/09 M segment were able to transmit with high efficiency in guinea pigs, suggesting that the M segment is important for enhancing viral transmission through the air.

Through the study with reassortant viruses in two different influenza A virus backgrounds, we have determined that the M segment is a critical viral genetic factor conferring efficient transmission to the 2009 pandemic influenza virus.

MATERIALS AND METHODS

Cells, bacteria, and viruses. 293T and MDCK cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in Dulbecco's minimal essential medium (DMEM) or in Eagle minimum essential medium (MEM) (Gibco, Invitrogen), respectively, supplemented with 10% fetal calf serum (HyClone; Thermo Scientific) and penicillin-streptomycin (Gibco, Invitrogen).

All influenza A/California/04/2009(H1N1) (Cal/09) recombinant viruses and influenza A/swine/Texas/1998 (H3N2) (sw/Tx/98) viruses were propagated in MDCK cells for 3 days at 35°C. All PR8 recombinant viruses were grown in MDCK cells at 37°C for 2 days. Experiments involving 2009 H1N1 viruses were

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conducted under biosafety level 2+ conditions for both *in vitro* and *in vivo* experiments, in accordance with the 2009 Centers for Disease Control and Prevention guidelines H1N1 Influenza Virus Biosafety Guidelines for Laboratory Workers and with Mount Sinai School of Medicine (MSSM) Institutional Biosafety Committee approval, no. 24a.

Construction of plasmids. The reverse-genetics plasmids used for rescue of recombinant Cal/09 viruses were previously described (8). The pDZ plasmids encoding eight viral segments of PR8 virus were also described in an earlier study (21). The eight pHW plasmids for rescue of recombinant sw/Tx/98H3N2 influenza viruses were constructed as described previously (25).

Rescue of recombinant influenza A viruses. The rescue of influenza A recombinant viruses was performed as described previously (5, 18). Briefly, 293T cells were transfected with eight ambisense pDZ vectors or pHW vectors encoding viral genomic RNA segments. At 24 h posttransfection, the supernatant and the 293T cells were inoculated into 8-day-old embryonated chicken eggs. The allantoic fluid was harvested after 3 days of incubation at 35°C. The rescued viruses were further isolated by plaque purification in MDCK cells.

Virus growth kinetics assay. To analyze the multicycle replications of the viruses, confluent MDCK cells were infected with the PR8 reassortant viruses at a multiplicity of infection (MOI) of 0.001, and the sw/Tx/98 reassortant viruses were infected at an MOI of 0.005. The PR8 reassortant virus-infected cells and the sw/Tx/98 reassortant virus-infected cells were incubated in serum-free minimal essential medium containing 0.3% bovine albumin and 1 μg of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin/ml at 37°C and 35°C, respectively. Supernatants were collected at selected time points postinfection, and the viral titers in the supernatants were determined by plaque assays in MDCK cells. Characterization of plaque phenotype on MDCK cells was performed as previously described (6).

Animals. Six-week-old female Hartley strain guinea pigs were obtained from Charles River Laboratories (Wilmington, MA), weighing 300 g to 350 g. Prior to all experimental procedures, including inoculation, nasal wash, and blood collections, guinea pigs were anesthetized through intramuscular injections of a ketamine-xylazine mixture. All animal experiments were performed according to the guidelines of the Mount Sinai School of Medicine Institutional Animal Care Use Committee.

Aerosol transmission experiments. Aerosol transmission, via small or large respiratory droplets, was investigated as previously described (14, 15). Briefly, four guinea pigs were intranasally inoculated with 10^3 PFU (for PR8-based viruses) or 10^4 PFU (for sw/Tx/98-based viruses) in 300 μl phosphate-saline buffer (PBS). Following infection, animals were then kept in individual cages with filter tops to prevent cross contamination. At day 1 postinoculation, naive and infected animals were moved to individual cages with wire meshes on one side and without filter tops; these cages allow free airflow but prevent contact among guinea pigs. Four naive guinea pigs were each paired with four inoculated guinea pigs in an environmental chamber. Conditions in the chamber were maintained at 20°C and 20% relative humidity. Nasal lavage samples were collected from each guinea pig on days 2, 4, 6, and 8 postinfection. Collected samples were stored at −80°C before plaque assays were performed to determine the viral titers.

RESULTS

Recombinant wild-type (rWT) Cal/09 virus transmits efficiently, while rWT PR8 virus does not transmit through the aerosol route. We first compared transmission of the rWT Cal/09 virus to that of another H1N1 virus, A/Puerto Rico/8/34. As shown in Fig. 1, when naive guinea pigs were exposed to guinea pigs shedding PR8 virus, virus was not detected in nasal lavage fluid collected from these animals throughout the 7-day exposure period. Conversely, 7 out of 8 animals were infected following exposure to guinea pigs infected with rWT Cal/09 virus, which was further verified by hemagglutination inhibition assay of paired sera for each guinea pig (Table 1). The data indicated that rWT PR8 virus does not transmit via aerosol in guinea pigs, while rWT Cal/09 transmits efficiently. Based on these observations, we hypothesized that we could determine which viral segments are essential for the transmission phenotype of the Cal/09 virus by swapping the gene segments between the rWT PR8 and rWT Cal/09 viruses.

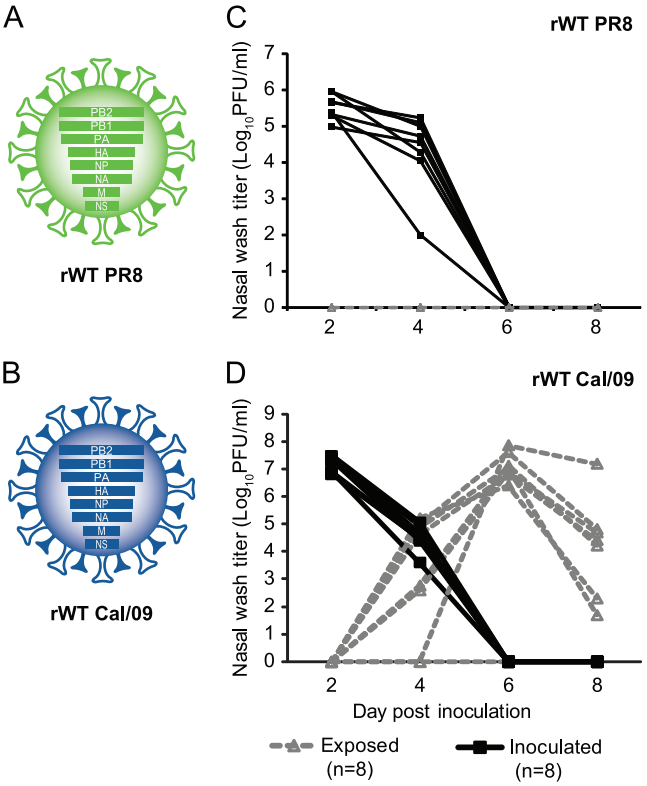

FIG. 1. PR8 virus does not transmit in guinea pigs through the aerosol route. Aerosol transmission experiments were performed with rWTPR8 virus (A) or rWT Cal/09 virus (B). Nasal wash titers are plotted against the time postinoculation, and the titers of the inoculated animals are represented by solid black lines and filled squares, while the titers of the exposed animals are represented by dashed gray lines and open triangles. Cumulative results of two independent experiments for the rWT PR8 virus (C) or rWT Cal/09 virus (D) are shown.

The M segment of the Cal/09 virus promotes aerosol transmission of reassortant PR8:Cal/09 M virus. First, we generated a set of recombinant PR8 viruses containing viral segments from the Cal/09 virus (Fig. 2A). To test whether the introduction of different Cal/09 viral segments into the PR8 virus background would affect viral replication, growth kinetics of all of the reassortant PR8 viruses were compared to those of the rWT Cal/09 and rWT PR8 viruses by multicycle growth assay in MDCK cells. As shown in Fig. 2A, all of the reassortant viruses exhibited replication patterns similar to those of the two rWT viruses and reached similar peak titers at 24 h postinfection, except for the PR8:Cal/09 HA virus. This virus showed a delay in replication and reached its peak titer at 48 h postinfection. In addition, it formed smaller plaques than the others (Fig. 2B). These data indicated that the reassortment of the Cal/09 viral segments into the PR8 genetic background did not result in viruses with defective replication capacity *in vitro*.

Six viral gene segments, the PB2, PB1, PA, HA, NP, and NS segments of the 2009 pandemic viruses, originated with the triple-reassortant swine influenza virus lineage. Viruses of this lineage have been circulating in pigs in North America since approximately 1997, when they were introduced following multiple reassortment events involving avian, human, and swine

TABLE 1. Hemagglutination inhibition assay for infection of animals[a]

Expt no.	Sample	Result for virus					
		rWT Cal/09		PR8:Cal/09 M		sw/Tx/98:Cal/09 HANAM	
		Virus detection	Seroconversion	Virus detection	Seroconversion	Virus detection	Seroconversion
1	Inoculated 1	+	+	+	+	+	+
	Inoculated 2	+	+	+	+	+	+
	Inoculated 3	+	+	+	+	+	+
	Inoculated 4	+	+	+	+	+	+
	Exposed 1	+	+	−	−	−	−
	Exposed 2	+	+	+	+	+	+
	Exposed 3	+	+	−	−	+	+
	Exposed 4	+	+	+	+	+	+
2	Inoculated 1	+	+	+	+	+	+
	Inoculated 1	+	+	+	+	+	+
	Inoculated 1	+	+	+	+	+	+
	Inoculated 1	+	+	+	+	+	+
	Exposed 1	+	+	−	−	−	−
	Exposed 2	−	−	+	+	+	+
	Exposed 3	+	+	+	+	+	+
	Exposed 4	+	+	+	+	+	+

[a] Limit of detection was 20 PFU/ml. Seroconversion was defined as a ≥6-fold increase in hemagglutination inhibition titer.

viruses. The other two segments of the 2009 H1N1 virus, NA and M, are most closely related to those in the Eurasian avian-like swine influenza viruses (3, 24). While the triple-reassortant North American swine viruses commonly infect swine, these viruses lead only to sporadic infection in humans (22) and are inefficiently transmitted in guinea pigs (28). Therefore, we hypothesized that the NA and/or M segment from the Eurasian avian-like swine viruses might contribute to the efficient transmission of the 2009 pandemic viruses. To evaluate this hypothesis, we tested whether the introduction of the M or NA segment of the Cal/09 virus into the PR8 virus background would confer aerosol transmission. In two independent experiments for the PR8:Cal/09 M virus, recombinant virus could be isolated from nasal wash fluid of 5 out of 8 aerosol contact animals (62.5% increased transmission rate; $P < 0.05$, Student's t test) (Fig. 2C) compared to the lack of transmission by the wild-type PR8 virus (Fig. 1C). A hemagglutination inhibition assay was performed to further confirm the infection of the exposed animals by the PR8:Cal/09 M virus (Table 1). In contrast, virus was detected in nasal wash fluid from only 1 out of 8 animals exposed to guinea pigs inoculated with the PR8:Cal/09 NA virus (Fig. 2D). This suggested that the M segment contributes to the efficient transmission phenotype of the 2009 pandemic viruses.

To further understand whether other viral segments were also contributing to viral transmission, the transmission properties of the other PR8 reassortant viruses (the PR8:Cal/09 HA, PR8:Cal/09 NS, and PR8:Cal/09 3PNP viruses) were assessed in the guinea pig model. No aerosol contact animals were infected following exposure to guinea pigs that were directly infected with either PR8:Cal/09 HA or PR8:Cal/09 NS viruses (Fig. 2E and F). Following exposure to guinea pigs infected with PR8:Cal/09 3PNP virus, only one out of the four exposed guinea pigs was infected (Fig. 2G). This enhancement in the transmission rate is minimal compared to the lack of transmission by the rWT PR8 virus and was not statistically significant ($P > 0.05$, Student's t test). Hence, the data shown here demonstrated that the six segments originating from the triple-reassortant classical swine viruses did not enhance the transmission of the PR8 reassortant viruses. Thus, it is suggested that the PB1, PB2, PA, NP, HA, and NS segments play a minimal role in the efficient transmission of the 2009 pandemic viruses.

The Cal/09 M segment promotes aerosol transmission of a triple-reassortant classical swine virus. To further confirm the role of the M segment in enhancing transmission, we tested whether the Cal/09 M segment improves the transmission of the sw/Tx/98 virus. Because triple-reassortant viruses belonging to the North American lineage do not readily transmit (22) and because it is known that sw/Tx/98 does not transmit as efficiently as Cal/09 in the guinea pig model (28), we hypothesized that adding the M segment from Cal/09 virus would increase transmission efficiency and recapitulate the reassortment events that led to the emergence of the pandemic swine viruses of 2009. It is of note, however, that sw/Tx/98 virus is an H3N2 subtype virus, while Cal/09 virus is of the H1N1 subtype. To avoid incompatibility between the glycoproteins and the M1/M2 proteins (1, 30), the transmission efficiency of a recombinant virus harboring Cal/09 M, HA, and NA and sw/Tx/98 PB2, PB1, PA, NP, and NS was investigated in the guinea pigs. Thus, two reassortant sw/Tx/98 viruses containing either Cal/09 HA and NA (sw/Tx/98:Cal/09 HANA) or Cal/09 HA, NA, and M (sw/Tx/98:Cal/09 HANAM) were generated (Fig. 3). The multicycle growth kinetics analysis of MDCK cells showed that the replication of Tx/98:Cal/09 HANA and that of Tx/98:Cal/09 HANAM viruses were comparable to that of the wild-type sw/Tx/98 virus, reaching similar peak titers at 24 h postinfection (Fig. 3A). This indicated that there is no defect in viral replication upon the introduction of Cal/09 segments into the sw/Tx/98 virus background. Morphologies of the plaques formed by the reassortant viruses were also similar to those of the rWTsw/Tx/98 viruses (Fig. 3B). Aerosol transmission of the two reassortant viruses was assessed in the guinea pig model under the same conditions as previously described. For the

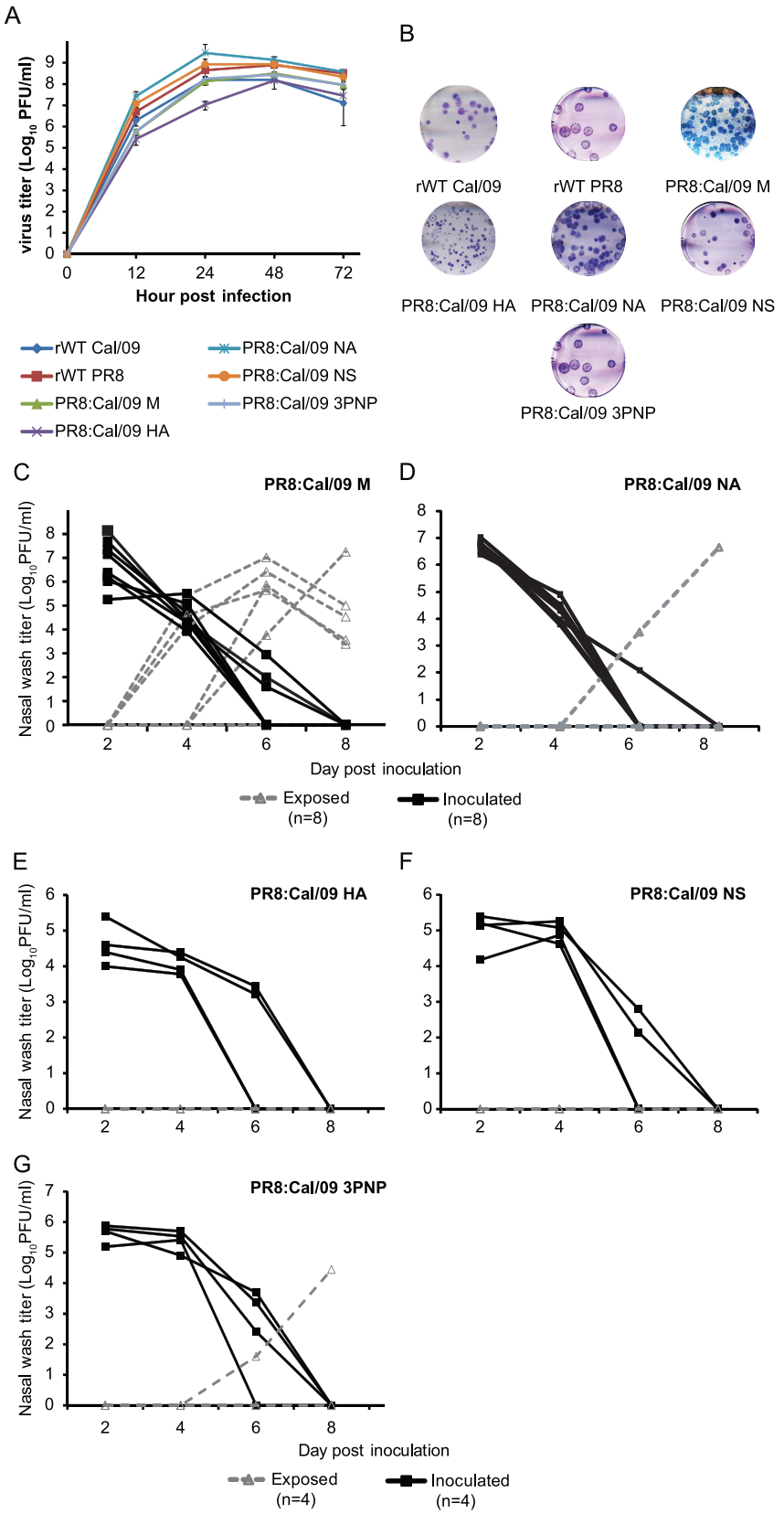

FIG. 2. The M segment of Cal/09 virus confers transmissibility in the PR8 background. (A) Multicycle growth kinetics analysis of the rWT viruses and the PR8 reassortant viruses in MDCK cells are shown with an MOI of 0.001. The viral titers are plotted as a function of time postinfection, with each data point representing the average of biological triplicates. The error bars represent the standard deviations of the triplicates. Titers for each virus are represented with different color lines as shown under the figure. (B) Plaque morphologies of each PR8 reassortant viruses are shown. Viral genotypes are indicated under each image. (C to H) Aerosol transmission experiments for each PR8 reassortant virus were performed under the conditions described in Materials and Methods. Virus titers of the nasal lavage fluid are plotted as a

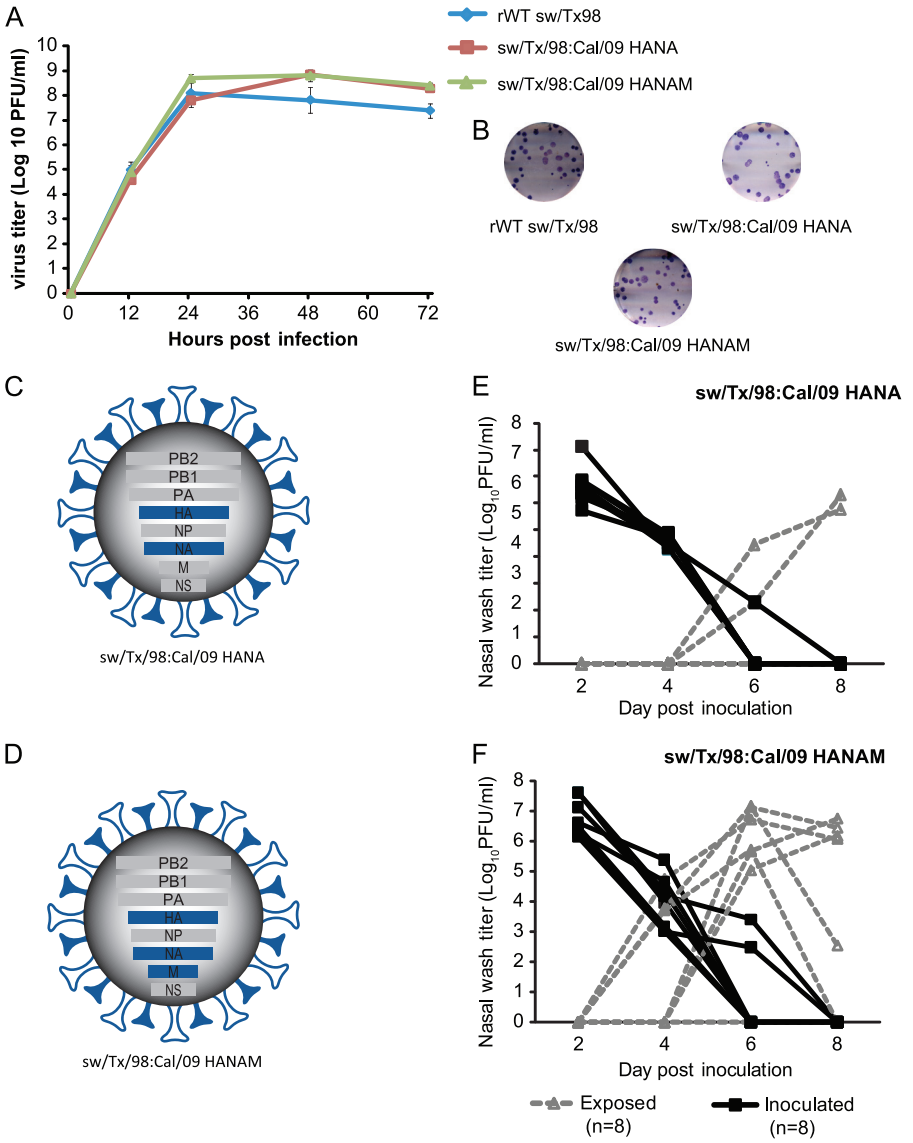

FIG. 3. The M segment of the Cal/09 virus promotes aerosol transmission of sw/Tx/98 virus. (A) Multicycle-growth kinetics analysis with MDCK cells was performed for the rWTsw/Tx/98, sw/Tx/98:Cal/09 HANA, and sw/Tx/98:Cal/09 HANAM viruses with an MOI of 0.005. The viral titers are plotted as a function of time postinfection, with each data point representing the average of biological triplicates. The error bars represent the standard deviations of the triplicates. The titers of rWT sw/Tx/98 are represented by blue lines; the titers of the sw/Tx/98:Cal/09 HANA virus are represented by red lines; the sw/Tx/98:Cal/09 HANAM virus is represented by green lines. (B) Plaque phenotypes of the reassortant sw/Tx/98 viruses are shown. The strain shown in each image is labeled individually. (C and D) The genetic constellation of sw/Tx/98 reassortant virus sw/Tx/98:Cal/09 HANA (C) or sw/Tx/98:Cal/09 HANAM (D) is shown. (E and F) Cumulative results of two independent aerosol transmission experiments with the sw/Tx/98:Cal/09 HANA virus (E) or sw/Tx/98:Cal/09 HANAM virus (F) are shown. The nasal wash titers of the inoculated animals are represented as solid black lines and filled squares, while the nasal wash titers of the exposed animals are represented as dashed gray lines and open triangles.

sw/Tx/98:Cal/09 HANA virus, 2 out of 8 exposed animals were infected by day five postexposure, resulting in a 25% transmission rate (Fig. 3E). This low transmission efficiency of sw/Tx/98:Cal/09 HANA is the same rate that had been previously published for the wild-type sw/Tx/98 virus (28) and further demonstrates that the expression of the glycoproteins from the Cal/09 virus does not contribute to the high transmissibility of the 2009 H1N1 pandemic virus. Six out of eight guinea pigs

function of time postinoculation. The black solid lines and the filled squares represent the viral titers of the inoculated animals; the dashed gray lines and open triangles represent the viral titers of the exposed animals. Cumulative results of two independent experiments are shown for the following reassortant strains: PR8:Cal/09 M viruses (C), PR8:Cal/09 NA viruses (D), PR8:Cal/09 HA viruses (E), PR8:Cal/09 NS viruses (F), and PR8:Cal/09 3PNP viruses (G).

became infected by day five following exposure to guinea pigs infected with the sw/Tx/98:Cal/09 HANAM virus (Fig. 3F), which was further verified by hemagglutination inhibition assay (Table 1). This 75% transmission rate is three times higher than that for the sw/Tx/98:Cal/09 HANA virus ($P < 0.05$, Student's t test). These results demonstrate that the M segment of the Cal/09 virus is capable of enhancing transmission of a triple-reassortant swine influenza virus by the aerosol route, further solidifying the role of the Cal/09 M segment in supporting efficient transmission of the 2009 pandemic virus. From the data collected with reassortant viruses in two different influenza virus backgrounds, we showed that the M segment of the Cal/09 virus is critical to the efficient transmission of this virus in the guinea pig model.

DISCUSSION

Gaining the capability of efficient aerosol transmission in humans is a prerequisite for the emergence of pandemic influenza viruses. Some zoonotic influenza viruses, such as the highly pathogenic H5N1 avian influenza viruses, have not caused pandemics to date because these viruses lack the ability to transmit from human to human. The first report of influenza virus infection in pigs was published in 1931 (23). Since then, human infections with swine influenza viruses have been reported sporadically in the United States (11, 17). The 2009 swine-origin pandemic influenza virus, which transmits efficiently in humans, therefore served as a valuable model for the study of viral factors conferring efficient aerosol transmission. Here, by reassorting the Cal/09 viral segments into two poorly transmissible viruses, PR8 and sw/Tx/98, we identified the Cal/09 viral M segment as a major determinant of transmission in the guinea pig animal model. Studies using both the guinea pig and the ferret models of influenza virus transmission have demonstrated differences among viruses in their ability to transmit through the aerosol route, including viruses of different strains or genetic compositions (14, 15, 33). Nevertheless, the essential players, including the viral genetic factors, governing efficient aerosol transmission are not fully understood. In this study, the aerosol transmission rate of a virus was measured as the percentage of susceptible guinea pigs infected following exposure to inoculated animals. The guinea pigs were housed in separate cages under well-defined temperature and relative humidity conditions which have been shown to allow the distinction between viruses with differing capacities to transmit through the air (14). Using this experimental setup, viruses carrying the Cal/09 M segment were found to have increased transmission efficiency compared to parental strains with the wild-type PR8 and sw/Tx/98 backgrounds. In turn, exchanging the M segment of the Cal/09 virus with that from the PR8 virus led to reduced transmissibility (1 out of 4 exposed animals shed virus; unpublished).

Similar studies that identified genetic determinants for the aerosol transmission of the 1918 pandemic influenza virus have been done using the ferret model (31, 33). It was found that the human-like α-2,6-sialic acid binding specificity of the hemagglutinin was required for viral transmission among ferrets (31). The 1918 PB2 gene was also shown to contribute to efficient aerosol transmissibility (32). Since the 2009 H1N1 pandemic virus originated from viruses of the swine reservoir, the genetic determinants for efficient aerosol transmission may be different from those of viruses of avian origin. Glycan microarray and structural analysis studies have shown that HA of the 2009 pandemic virus displayed α-2,6-sialic acid binding specificity, suggesting a human-like receptor binding of the virus (3, 36). The introduction of the E627K and D701N mutations into the PB2 gene of the pandemic isolate A/Netherlands/602/2009, which had been previously reported to affect viral transmission in other viruses (27), did not affect the transmission rate of the 2009 pandemic H1N1 strain (10). This implied that additional genetic elements other than the known viral factors are required for the aerosol transmission of swine-origin viruses. The identification of the M segment as an important factor for aerosol transmission of the 2009 pandemic virus suggested that adaptation of the M segment may be necessary for the development of human aerosol transmission for swine influenza viruses. This finding further highlights the complexity inherent in the transmission mechanism of different influenza virus strains.

Our current findings fit nicely within the context of the 2009 new pandemic virus evolution. The virus emerged following a reassortment event in which the NA and M segments of a Eurasian avian-like influenza virus were combined with a triple-reassortant swine virus background (24). Despite their circulation in pigs since 1997, prior to 2009, triple-reassortant swine viruses caused only sporadic human clinical cases in the United States (22). The data presented here, showing that the M segment of the pandemic virus promoted aerosol transmission of the triple-reassortant sw/Tx/98 virus, suggested that the introduction of viral segments from the Eurasian avian-like swine viruses was important to the development of viral transmissibility in humans. However, our findings ruled out a significant contribution of NA to transmission.

The M segment of influenza virus encodes two highly conserved proteins, the matrix protein M1 and the ion channel M2 (20). These multifunctional proteins are involved in different stages during viral replication, including the processes of viral entry, assembly, and budding (20). The exact mechanism by which the M segment enhances aerosol transmission of the 2009 pandemic virus is unclear. The independent effects of the M1 and M2 proteins on viral transmission need to be further explored. While our results support the role of the Cal/09 M segment in driving viral transmission, we cannot exclude the involvement of other Cal/09 viral factors in this process. Interestingly, the Eurasian avian-like swine viruses, which possess an M segment that is closely related to that of the Cal/09 virus, do not transmit efficiently in humans (13). It is possible that other genetic requirements must be fulfilled in order for transmission to take place. This hypothesis is further strengthened by a recent report from M. Peiris's group (37). It is therefore possible that the entire viral genetic constellation retains an influence on viral transmission.

In conclusion, the data presented here indicate that the M segment is a genetic determinant of the efficient transmissibility of the Cal/09 pandemic virus. These findings broaden our view of the viral factors contributing to transmission and provide valuable information for the identification through surveillance and containment of influenza virus strains with pandemic potential.

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