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Residue 41 of the Eurasian Avian-Like Swine Influenza A Virus Matrix Protein Modulates Virion Filament Length and Efficiency of Contact Transmission

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ABSTRACT

Position 41 of the influenza A virus matrix protein encodes a highly conserved alanine in human and avian lineages. Nonetheless, strains of the Eurasian avian-like swine (Easw) lineage contain a change at this position: position 41 of A/swine/Spain/53207/04 (H1N1) (SPN04) encodes a proline. To assess the impact of this naturally occurring polymorphism on viral fitness, we utilized reverse genetics to produce recombinant viruses encoding wild-type M1 41P (rSPN04-P) and consensus 41A (rSPN04-A) residues. Relative to rSPN04-A, rSPN04-P virus displayed reduced growth in vitro. In the guinea pig model, rSPN04-P was transmitted to fewer contact animals than rSPN04-A and failed to infect guinea pigs that received a low-dose inoculum. Moreover, the P41A change altered virion morphology, reducing the number and length of filamentous virions, as well as reducing the neuraminidase activity of virions. The lab-adapted human isolate, A/PR/8/34 (H1N1) (PR8), is nontransmissible in the guinea pig model, making it a useful background in which to identify certain viral factors that enhance transmissibility. We assessed transmission in the context of single-, double-, and triple-reassortant viruses between PR8 and SPN04; PR8/SPN04 M, PR8/SPN04 M+NA, and PR8/SPN04 M+NA+HA, encoding either matrix 41 A or P, were generated. In each case, the virus possessing 41P transmitted less well than the corresponding 41A-encoding virus. In summary, we have identified a naturally occurring mutation in the influenza A virus matrix protein that impacts transmission efficiency and can alter virion morphology and neuraminidase activity.

IMPORTANCE

We have developed a practical model for examining the genetics underlying transmissibility of the Eurasian avian-like swine lineage viruses, which contributed M and NA segments to the 2009 pandemic strain. Here, we use our system to investigate the impact on viral fitness of a naturally occurring polymorphism at matrix (M1) position 41 in an Easw isolate. Position 41 has been implicated previously in adaptation to laboratory substrates and to mice. Here we show that the polymorphism at M1 41 has a limited effect on growth in vitro but changes the morphology of the virus and impacts growth and transmission in the guinea pig model.

In 1979, an avian influenza A virus of the H1N1 subtype entered swine populations in Belgium and Germany and established a stable lineage that has since spread across Europe and Asia (1–3). The resultant Eurasian avian-like swine virus (Easw) lineage continues to circulate in Eurasia today (4). Zoonoses of Easw lineage influenza A viruses to humans are typically self-limiting and clinically mild events (5) that do not lead to sustained human-to-human transmission (6–10). Nevertheless, the M and NA segments derived from the Easw lineage (11) are important for the highly transmissible phenotype of the 2009 pandemic H1N1 virus (12–14). Thus, viruses of the Easw lineage are important for animal health, can cause human disease, and have a demonstrated potential to contribute to the emergence of pandemic strains. Still, very little is known about the genetic and molecular factors that influence the transmission and replication of Eurasian avian-like swine viruses (15).

The influenza A virus matrix (M1) protein is encoded in the bicistronic M gene segment. The protein functions in both virus assembly and budding (16–19): it recruits the viral ribonucleoprotein (vRNP) complex to the cell surface during virion assembly (17, 20, 21) and lines the inner surface of the viral membrane. Additionally, M1 provides structural integrity to the influenza vi-
the human isolate, A/Victoria/3/75, and they mapped the phenotypic difference to matrix protein residues 41, 95, and 218. Roberts and colleagues also demonstrated that residue 41 of the matrix protein affects virion morphology (27). In both of these studies, replacement of the alanine at position 41 with valine led to a loss of filamentous morphology.

Interestingly, an alanine-to-valine polymorphism at residue 41 of the matrix has been identified upon adaptation in mice to multiple influenza A strains (30–32). The mutation has been linked to increases in replication and virulence in the mouse model (31, 32).

The SPN04 virus possesses a highly unusual polymorphism at position 41 of the matrix protein, one encoding a proline. A minority of Easw viruses encode a valine at position 41, and the predominant residue is alanine.

We hypothesized that the proline at position 41 of the SPN04 matrix protein alters virion morphology and, although naturally occurring, may attenuate the growth and transmission of the SPN04 virus. By testing this hypothesis, we aimed to shed light on the biological significance of filamentous influenza virions. Our results show that replacement of the widely conserved alanine with proline at position 41 of the matrix protein reduces transmission between guinea pigs, both in the context of the Eurasian avian-like swine virus, A/swine/Spain/53207/04, and in the PR8-based reassortant viruses tested. The A41P mutation concomitantly increased the filament length of each of the viruses.

**MATERIALS AND METHODS**

**Ethics statement.** This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council (33). Animal husbandry and experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee (IACUC protocol 2001001 071214GA).

**Cells.** Human tracheo-bronchial epithelial (HTBE) cells, purchased from Lonza, were cultured on Transwell filters at an air-liquid interface and maintained in basal epithelial growth medium (BEGM) supplied to the basolateral chamber as directed by the manufacturer. Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. 293T cells were maintained in Dulbecco’s MEM supplemented with 10% FBS.

**Guinea pigs.** Female Hartley strain guinea pigs weighing 300 to 350 g were obtained from Charles River Laboratories. Inoculation and nasal lavage were performed as described previously (34). Prior to all procedures, guinea pigs were sedated with a mixture of ketamine and xylazine (30 mg/kg of body weight and 2 mg/kg, respectively).

**Reverse genetics system.** Recombinant virus was generated as previously described (35). Briefly, viral RNA, extracted from A/swine/Spain/53207/04 (H1N1) influenza virus, was reverse transcribed and amplified using influenza virus segment-specific primers (36), resulting in 8 specific cDNA products corresponding to each of the viral gene segments. Six cDNAs (NS, M, NA, NP, HA, and PA) were flanked by LguI restriction enzyme sites. PB1 and PB2 segments were flanked by sequences allowing cloning into the pPol1 plasmid by recombination (37). cDNAs were digested with Lgul as appropriate and subsequently cloned by ligation or recombination into the pPol1 plasmid.

**Viruses.** A/NI/602/09 (H1N1) (a kind gift of Ron Fouchet) virus is an early 2009 pandemic H1N1 strain that was isolated from a patient in the Netherlands. A/Puerto Rico/8/1934 (H1N1) (PR8)-based and A/swine/Spain/53207/04 (H1N1) (SPN04)-based viruses were recovered by reverse genetics following previously described procedures (35, 38). Briefly, 8- or 12-plasmid rescue systems, based on pUZ (39) for PR8 (8 plasmids) and pPol1 for SPN04 (12 plasmids, including pCAGGS WSN PA, PB1, PB2, and NP support plasmids), were used to transfect 293T cells. Reassortant viruses were recovered by using the relevant plasmids from both systems (Fig. 1 depicts virus gene constellations). One day after transfection, 293T cells and associated media were collected and injected into 11-day-old embryonated chicken eggs. Stock titers were determined by plaque assay on MDCK cells. A mutation was introduced into the pPol1 SPN04 M segment plasmid that altered residue 41 from a proline to an alanine, generating the pPol1 SPN04 M 41A plasmid. Viruses containing this mutation were rescued in the same manner as described above.

**Immunostaining and enumeration of plaques.** Characterization of plaque phenotypes on MDCK cells was performed as described previously (40). For immunostaining, polyclonal guinea pig anti-PR8 serum or anti-SPN04 serum (raised in-house) were used as the primary antibodies; horseradish peroxidase-linked anti-guinea pig IgG (Invitrogen) was used as the secondary antibody, and TruBlue (KPL) peroxidase substrate was used for staining. A total of 125 plaques formed by each virus were mea-

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**FIG 1** Schematic diagram of recombinant viruses. Genotypes of each recombinant virus used in the study are depicted. Blue and red bars represent genes from A/PR/8/34 (H1N1) (PR8) and A/Swine/Spain/53207/04 (H1N1) (SPN04) viruses, respectively. The yellow bar indicates the presence of a point mutation encoding a proline-to-alanine change at position 41 of the matrix protein.
sured, using ImageJ software, and statistical analyses were performed on the results using Prism software.

**Multicycle growth experiments.** MDCK cells were inoculated at a multiplicity of infection (MOI) of 0.002 PFU/cell. Following a 45-min incubation at 37°C, inoculum was removed and monolayers were washed with PBS. Dishes were then incubated at 37°C, and samples of growth medium were collected at 1, 12, 24, 48, and 72 h postinfection (hpi). Titters were determined by plaque assay on MDCK cells.

HTBE cells were washed with PBS to remove mucus and then inoculated at an MOI of 0.001 PFU/cell by adding virus in a 100-μl volume of PBS to the apical surface and incubating at 37°C for 45 min. The inoculum was then removed, and the cells were washed with PBS. At 1, 12, 24, 48, and 72 hpi, virus was collected from the apical surface of the cells by adding 200 μl PBS, incubating at 37°C for 30 min, and then collecting 200 μl of supernatant. Titters were determined by plaque assay on MDCK cells.

**Neuraminidase activity.** To compare the neuraminidase activities associated with the viruses, 5 × 10^2 PFU of each variant was incubated in the presence of the fluorescent diosidose, 2-(4-methylumbelliferyl)-d-N-acetylneuraminic acid (MUNANA; Sigma), at a final concentration of 150 μM in a total volume of 100 μl. Fluorescence, which is emitted upon cleavage of the MUNANA substrate by the influenza virus neuraminidase enzyme, was detected using a BioTek Synergy H1 plate reader and recorded every minute for 45 min. The slopes of the resultant fluorescence curves of each pair of viruses were compared. Each experiment included triplicate samples for each virus, and experiments were performed at least three times.

**Transmission experiments.** In order to assess transmission, four guinea pigs were inoculated by the intranasal route with 10^5 or, in some cases, 10^2 PFU of virus in 300 μl PBS. At 24 h postinoculation, infected animals were each placed in a cage with a naive guinea pig. The four cages were subsequently placed within an environmental chamber (Caron 6040) set to 20% relative humidity and 10°C. Nasal washes were collected from all 8 animals on days 2, 4, 6, and 8 postinfection, as previously described (41).

**Electron microscopy.** (i) **Transmission electron microscopy.** For imaging of virions, MDCK cells were inoculated at an MOI of 5. At 18 h postinfection, cells were fixed with 2.5% glutaraldehyde, postfixed with 1% OsO4 and 1.5% potassium ferrocyanide, rinsed with distilled water, and dehydrated through a series of ethanol washes. For transmission electron microscopy (TEM), monolayers were embedded in Eponate 12 resin and stained with 5% uranyl acetate and 2% lead citrate at the Emory Robert P. Apkarian Integrated Electron Microscopy Core facility. After sample preparation, grids were imaged using a Hitachi H-7500 transmission electron microscope and attached charge-coupled device.

(ii) **Scanning electron microscopy.** For scanning electron microscopy (SEM), the chips underwent critical point drying in a Polaron E-3000 unit. After sample preparation, grids were imaged for the influenza virus glycoproteins (19), we evaluated multiple reassortant viruses possessing the HA, NA, and M segments from SPN04, comparing three pairs of viruses possessing the alanine-to-proline substitution in the matrix protein of SPN04, as follows: PR8/SPN04 M and PR8/SPN04 M41A; PR8/SPN04 M+NA and PR8/SPN04 M41A+NA; PR8/SPN04 M+NA+HA and PR8/SPN04 M41A+NA+HA.

**RESULTS**

**In vitro characterization of recombinant viruses.** By using reverse genetics, a set of eight recombinant viruses was generated. The set comprised the recombinant wild-type (WT) SPN04 (rSPN04-P) and SPN04 M41A (rSPN04-A) viruses, which differed in the identity of residue 41 of the matrix protein (proline or alanine), and six reassortants between A/P/PR8/34 (PR8) and SPN04: PR8/SPN04 M, PR8/SPN04 M41A, PR8/SPN04 M+NA, PR8/SPN04 M41A+NA, PR8/SPN04 M+NA+HA, and PR8/SPN04 M41A+NA+HA (Fig. 1). rPR8 virus was rescued as previously described (28). The SPN04 virus rescue system was generated for this study. The cloned SPN04 cDNAs were confirmed to be of the correct size by restriction enzyme digest with XbaI, and each plasmid was fully sequenced to confirm agreement with GenBank accession sequences (CY010584, CY010581, CY010582, CY010583, CY010580, CY010585, CY010586, and CY010587).

(i) **SPN04-based viruses.** Initial characterization of the viruses was performed in vitro. In MDCK cells, the biologic and the recombinant wild-type SPN04 viruses grew with comparable kinetics and to similar peak titers of approximately 1 × 10^6 PFU/ml after 48 h. No significant difference was found between the areas under the curve (AUC) for each virus (log_{10} 9.70 for WT versus log_{10} 9.65 for recombinant virus; P = 0.13 by unpaired t test), confirming that the recombinant virus was not attenuated due to unwanted mutations in the genome (Fig. 2A). Next, the growth of wild-type rSPN04-P and the point mutant rSPN04-A viruses was assayed on fully differentiated HTBE cells cultured at the air-liquid interface and on MDCK cells. In HTBE cells, the average AUC for the rSPN04-A virus was significantly higher than that for the rSPN04-P virus (log_{10} 9.51 versus log_{10} 9.27; P = 0.012 by unpaired t test) (Fig. 2B). Similarly, in MDCK cells the rSPN04-A virus had a significantly higher average AUC than that of the SPN04-P virus (log_{10} 10.15 versus log_{10} 9.81; P = 0.001 by unpaired t test) (Fig. 2C). Finally, during growth in MDCK cells, a reduction in plaque size attributable to the alanine-to-proline mutation was shown to be statistically significant (P < 0.05; one-way analysis of variance) (Fig. 2E). In summary, the alanine-to-proline substitution in the matrix protein resulted in reduced growth phenotypes of the SPN04 virus in multiple in vitro assays.

(ii) **PR8-based reassortant viruses.** Since the lab-adapted PR8 virus does not transmit among guinea pigs, reassortant viruses with a PR8 backbone can be a sensitive platform on which to identify polymorphisms that affect transmission (12, 28). We applied this approach to the SPN04 virus and, because the influenza virus matrix protein is known to interact with the cytoplasmic tails of the influenza virus glycoproteins (19), we evaluated multiple reassortant viruses possessing the HA, NA, and M segments of SPN04, comparing three pairs of viruses possessing the alanine-to-proline substitution in the matrix protein of SPN04, as follows: PR8/SPN04 M and PR8/SPN04 M41A; PR8/SPN04 M+NA and PR8/SPN04 M41A+NA; PR8/SPN04 M+NA+HA and PR8/SPN04 M41A+NA+HA.

Low-multiplicity growth curve experiments performed with MDCK cells demonstrated that viruses possessing the HA, NA, and M segments from SPN04 grew more poorly than other PR8-based viruses, regardless of the nature of the polymorphism at position 41 of the matrix protein (Fig. 2D), and that there was no significant difference between the AUCs for the pair of 5:3 reassortant viruses (log_{10} 7.15 for PR8/SPN04 M+NA+HA versus log_{10} 7.25 for PR8/SPN04 M41A+NA+HA; P = 0.56 by unpaired t test). Those reassortants carrying the PR8 HA were more fit in MDCK cells than those carrying the SPN04 HA. Given that the WT rSPN04 virus grew to high titers in MDCK cells, the data collectively suggest an incompatibility between the SPN04 HA and gene product(s) of the PR8 virus internal genes. Significant differences were observed between the growth phenotypes of the PR8/SPN04 M and PR8/SPN04 M41A, as well as the PR8/SPN04 M+NA and PR8/SPN04 M41A+NA, virus pairs in MDCK cells (Fig. 2D). PR8/SPN04 M41A virus had a significantly higher average AUC than the PR8/SPN04 M41P virus (log_{10} 8.55 versus log_{10} 7.95; P = 0.0003 by unpaired t test). Similarly, PR8/SPN04 M41A+NA virus had a significantly higher average AUC than PR8/SPN04 M41P+NA virus (log_{10} 8.62 versus log_{10} 8.11; P = 0.001 by unpaired t test).
The plaque size of each of the PR8/SPN04 reassortant viruses was assessed. A total of 125 plaques formed by each virus on MDCK cells were measured. For each virus pair, a statistically significant reduction in plaque size was observed with the PR8/SPN04 virus encoding proline, relative to that encoding alanine, at matrix position 41 ($P < 0.05$; one-way analysis of variance) (Fig. 2F to H). In summary, the alanine-to-proline substitution in the matrix protein had an attenuating effect on growth phenotypes of the PR8/SPN04 reassortant viruses in vitro, while PR8-based viruses possessing the SPN04 HA grew more poorly than viruses possessing the PR8 HA in MDCK cells.

Morphology of 41P- versus 41A-encoding viruses. As previous studies demonstrated that an alanine-to-valine polymorphism at position 41 of the matrix protein affects the morphology of multiple influenza A virus strains (23, 24, 27), we wished to examine the effect of the proline-to-alanine change at this position on virion morphology. Using electron microscopy, we studied MDCK cells infected with viruses encoding each amino acid. rSPN04-P exhibited a highly filamentous phenotype in MDCK cells, as observed by either scanning or transmission electron microscopy (Fig. 3A and B). Examples of filaments greater than 10 μm in length were visible by SEM. The mutation of P41 in the rSPN04 virus matrix to A41 altered the morphology of the virion, reducing the length of the filaments from longer than 10 μm to a typical length of approximately 1 μm or less (Table 1). rSPN04-P virus yielded 36% of virions greater than 300 nm in length, as measured by TEM, compared to 26% for rSPN04-A virus. Moreover, only the proline-containing virus displayed filaments greater than 1 μm in length as assayed by TEM. The same trend was observed with two pairs of the PR8/SPN04 reassortant viruses: PR8/SPN04 M41P and PR8/SPN04 M41P/NA possessed 24% and 71% of virions greater than 300 nm in length (Fig. 3A, B, E, and F), as measured by TEM, compared to 4% and 56% for the respective alanine-containing viruses (Fig. 3C, D, G, and H). Again, only the proline-containing viruses possessed filaments greater than 1 μm in length (Table 1). Surprisingly, for the PR8/SPN04 M 41P/NA and PR8/SPN04 M 41A/NA+HA viruses, the same trends in virion morphology were not observed. Similar to the PR8/SPN04M41P and PR8/SPN04M41P+NA viruses, the proline-containing 5:3 reassortant virus possessed virions greater than 1 μm in length, while the PR8/SPN04 M41A+NA+HA virus did not. Nonetheless, the alanine-containing virus possessed a greater proportion of filamentous virions between 300 nm and 1 μm than the proline-containing variant.
Neuraminidase activity of 41P- versus 41A-encoding viruses.

As we previously had observed a correlation between neuraminidase activity of influenza virions and extent of filamentous morphology (42), we tested whether the NA activity of the matrix 41P-encoding viruses differed from those of the 41A-encoding strains. For this purpose, we used an in vitro assay based on the fluorescent substrate MUNANA. Three of the four pairs of viruses (SPN04, PR8/SPN04 M, and PR8/SPN04 M/HA) showed higher neuraminidase activities in

![Image of virion morphologies](http://jvi.asm.org/)

**TABLE 1** Reassortant viruses, measured using TEM, differ in the prevalence of filamentous virions

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Particle length (nm)</th>
<th>SPN04 M1 41P</th>
<th>M1 41A</th>
<th>rPR8/SPN04 M M1 41P</th>
<th>M1 41A</th>
<th>rPR8/SPN04 M + NA M1 41P</th>
<th>M1 41A</th>
<th>rPR8/SPN04 M + HA + NA M1 41P</th>
<th>M1 41A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spherical</td>
<td>&lt;100</td>
<td>36</td>
<td>19</td>
<td>45</td>
<td>49</td>
<td>5</td>
<td>17</td>
<td>43</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>100–200</td>
<td>26</td>
<td>31</td>
<td>21</td>
<td>37</td>
<td>18</td>
<td>21</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>200–300</td>
<td>2</td>
<td>12</td>
<td>9</td>
<td>10</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>64</td>
<td>74</td>
<td>76</td>
<td>96</td>
<td>29</td>
<td>44</td>
<td>89</td>
<td>77</td>
</tr>
<tr>
<td>Filamentous</td>
<td>300–500</td>
<td>10</td>
<td>8.3</td>
<td>6</td>
<td>2</td>
<td>20</td>
<td>15</td>
<td>3</td>
<td>10</td>
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<tr>
<td></td>
<td>500–1,000</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td>2</td>
<td>22</td>
<td>41</td>
<td>4</td>
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<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>36</td>
<td>26</td>
<td>24</td>
<td>4*</td>
<td>71</td>
<td>56</td>
<td>11**</td>
<td>23.3</td>
</tr>
<tr>
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<td>51</td>
<td>82</td>
<td>78</td>
<td>73</td>
<td>73</td>
</tr>
</tbody>
</table>

*Among infected, sectioned MDCK cells. *, the percentage of filamentous virions (>300 nm in length) in proline-containing virus was significantly higher (P > 0.05) than in alanine-containing isogenic virions based on a difference of proportions test; **, the percentage of filamentous virions (>300 nm in length) in proline-containing virions was significantly lower (P > 0.05) than in alanine-containing isogenic virions based on a difference of proportions test.
A proline at residue 41 of matrix protein increases neuraminidase activity of select recombinant viruses in vitro. The neuraminidase activity of each virus (as indicated) was measured through the cleavage of the fluorogenic substrate MUNANA. Virus was incubated in the presence of MUNANA at 37°C for 45 min, and the relative fluorescence was measured at 1-min intervals. Plots displaying (tightly overlapping) triplicate samples of each virus are shown. Each graph is representative of at least three replicate experiments.

FIG 4 A proline at residue 41 of matrix protein increases neuraminidase activity of select recombinant viruses in vitro. The neuraminidase activity of each virus (as indicated) was measured through the cleavage of the fluorogenic substrate MUNANA. Virus was incubated in the presence of MUNANA at 37°C for 45 min, and the relative fluorescence was measured at 1-min intervals. Plots displaying (tightly overlapping) triplicate samples of each virus are shown. Each graph is representative of at least three replicate experiments.

Growth and contact transmission in the guinea pig model. We employed a guinea pig contact model, as previously described (34, 43), in order to assess the effect of the alanine-to-proline mutation on the infectivity, replication, and transmission of the rSPN04 virus. As shown in Fig. 5A, at an inoculum dose of 1,000 PFU, the inclusion of a proline at position 41 resulted in productive infection with rSPN04-P in three of four guinea pigs, and transmission occurred from one of these animals to a cage mate. By comparison, four out of four animals were infected with rSPN04-A virus at this inoculum dose, and two transmission events occurred (Fig. 5B). Due to the small numbers of animals involved in this experiment, the observed differences had no statistical significance, either in terms of the replication levels or transmission efficiency achieved.

At an inoculum dose of 100 PFU, no guinea pigs were infected with the rSPN04-P virus (Fig. 5C), whereas two out of four guinea pigs were infected with the rSPN04-A virus and one accompanying transmission event occurred (Fig. 5D); however, statistically significant differences in replication or transmission were not demonstrated.

Next, we conducted contact transmission experiments involving the PR8-based reassortant viruses. Overall, viruses possessing the SPN04 matrix protein encoding a proline at position 41 transmitted poorly compared to the viruses possessing alanine at this position, broadly reproducing the phenotype observed in the SPN04 genetic background.

The PR8/SPN04 M41P virus did not transmit to any of eight guinea pigs by contact and replicated to approximately 10^4 to 10^5 PFU by day 2 postinoculation, as measured in nasal washes (Fig. 5E). In contrast, PR8/SPN04 M41A virus transmitted to four of eight naive cage mates and replicated to peak titers of approximately 10^6 PFU by day 2 postinfection (Fig. 5F). The PR8/SPN04 M41A virus replicated to significantly higher average titers on day 2 postinoculation (P = 0.0027; unpaired t test) and also had a significantly higher average AUC throughout the period of the infection (P = 0.0189; unpaired t test) than the PR8/SPN04 M41P virus. The cumulative number of transmission events was also significantly different (P = 0.019; unpaired t test). Similarly, the PR8/SPN04 M41P+NA virus did not transmit and replicated to approximately 10^0 to 10^2 PFU by day 2 postinfection (Fig. 3G). In contrast, PR8/SPN04 M41A+NA virus transmitted to four of eight naive cage mates and replicated to peak titers of approximately 10^6 PFU by day 2 postinfection (Fig. 3H). The PR8/SPN04 M41A+NA virus replicated to significantly higher average titers on day 2 postinoculation than the proline-encoding virus (PR8/SPN04 M41P+NA virus did not transmit and replicated to approximately 10^0 to 10^2 PFU by day 2 postinfection (Fig. 3G). In contrast, PR8/SPN04 M41A+NA virus transmitted to four of eight naive cage mates and replicated to peak titers of approximately 10^6 PFU by day 2 postinfection (Fig. 3H). The PR8/SPN04 M41A+NA virus replicated to significantly higher average titers on day 2 postinoculation than the proline-encoding virus (P < 0.0001; unpaired t test), although the average AUC throughout the period of the infection was not significantly different between the two groups of animals (P = 0.78; unpaired t test). The cumulative number of transmission events was, however, significantly different (P = 0.019; unpaired t test).

Consistent with the in vitro data obtained on virus growth, both PR8-based viruses possessing the SPN04 HA replicated to lower levels than the viruses possessing the PR8 HA. Nonetheless, the PR8/SPN04 M41P+NA+HA virus was transmitted to only one of eight cage mates (Fig. 5I), while the PR8/SPN04 M41A+NA+HA virus was transmitted to five out of eight animals (Fig. 5J). The cumulative number of transmission events showed a slight statistical significance (P = 0.04; unpaired t test); however, neither the day 2 nasal wash titers nor the average AUC were significantly different between the two groups of animals. Thus, in each background tested, a lower transmission efficiency was observed for viruses encoding proline at position 41 of the matrix protein than for those encoding alanine.

DISCUSSION

Overall, our results suggest that, dependent upon the specific gene constellation of the virus, the proline residue at position 41 can increase the filamentous nature of the influenza A virion and that this phenotype correlates with a decrease in transmission efficiency, a decrease in replicative capacity, and an increase in neuraminidase activity over an isogenic virus possessing an alanine at matrix position 41.

Among the influenza A virus sequences deposited in GenBank,
the proline at position 41 of the matrix protein is unique, encoded only by the SPN04 isolate. However, a few other Eurasian avian-like swine influenza virus strains encode a valine at this position, suggesting that the lineage tolerates polymorphism at this locus to some extent. We have not tested the effect of valine on the morphology of the SPN04 influenza virion.

The structure of the amino-terminal portion (amino acids 2 to 158) of the matrix protein has been solved using X-ray crystallography (44). The crystallized structure consists of nine α-helices and eight loop regions. Position 41 lies within α-helix 3, consisting of amino acids 39 to 48, and is likely solvent exposed. Based on this positioning, the nature of the residue at position 41 could affect oligomerization of the matrix protein or interaction with other viral or cellular factors. Of note, the cyclic nature of the proline side chain is incompatible with the formation of an α-helix, introducing a kink or a break in the structure; thus, α-helix 3 is most likely disrupted in the SPN04 matrix protein.

Several previous studies examined the effects of mutations in the matrix protein on the morphology of the influenza A virus. Lamb and colleagues examined the morphology of influenza A virions by using the A/Udorn/301/72 (H3N2) (A/Udorn) strain (27). A/Udorn possesses an alanine at position 41 of the matrix, in common with the majority of human H3N2 isolates, and is noted for its filamentous nature, with approximately 15% of virions being long filamentous in nature (27). Interestingly, an A41V change in the A/Udorn matrix protein results in a complete loss of filamentous virions, as assayed in gradient-purified virion preparations (27). Similarly, Ellman and colleagues noted that the filamentous human influenza virus isolate A/Victoria/3/75 (H3N2) (A/Victoria) could be altered to a spherical morphology by introduction of a valine at position 41 of the matrix protein (24). In addition, the commonly used laboratory-adapted strains of influenza virus PR8 and A/WSN/33 also possess a valine at position 41 of the matrix protein and are spherical in nature (24, 28). It should be noted, however, that PR8 and A/WSN/33 also possess several other amino acid changes relative to the seasonal H1N1 human influenza A virus consensus sequence, and morphology may be affected by several of these differences. Nonetheless, overall these data suggest the importance of position 41 in determining the morphology of numerous unrelated influenza A viruses.

The alanine-to-valine change in the matrix has been reported in several strains of influenza A viruses that are virulent to mice: A/Port Chalmers/1/73 (45), A/FPV/34 (46), A/PR/8/34 (31), and A/WSN/33 (30). Indeed, the mutation was observed to arise during mouse adaptation in the A/Port Chalmers/1/73 background (45). Thus, effects of M1 position 41 on virulence have been demonstrated. We note, however, that in those studies, polymorphism at M1 position 41 arose under circumstances where the selective pressure to transmit had been removed. Thus, here we tested the impact of M1 position 41 on transmission. Replacement of the alanine at position 41 of the Eurasian avian-like swine virus matrix protein with proline reduces viral transmission in a contact model. Interestingly, the reliance of efficient transmission on an alanine at M1 position 41 was evident whether or not its replacement with proline resulted in an observable loss in replicative fitness. These findings are consistent with the idea that an alanine at position 41 is optimal for efficient transmission of influenza A virus.

Evidence that molecular determinants of transmissibility reside within the M segment of influenza virus arose following the 2009 swine-origin pandemic, whereupon an influenza virus strain derived from two parental swine virus lineages that do not possess efficient human transmissibility crossed the species barrier and proceeded to infect over 1 billion people worldwide (47). The unusual genotype of the pandemic strain, encompassing 6 gene segments from the North American TRIG lineage and the M and NA segments from the Eurasian avian-like swine lineage, prompted investigators to study the contribu-
tion of the M segment to transmissibility of the pandemic strain. Chou and colleagues demonstrated that the M segment of the pandemic strain A/California/4/2009 restored transmissibility in the guinea pig model to that of the nontransmissible A/Puerto Rico/8/1934 (H1N1) virus (12). Interestingly, A/Puerto Rico/8/1934 encodes valine at position 41 of the matrix protein, whereas A/California/4/2009 encodes an alanine, among several other changes between the two matrix proteins. Further evidence for the importance of the Eurasian origin M segment to transmission of the 2009 pandemic lineage was obtained in ferret (13) and pig (48) models.

Due to the role of the M segment in determining transmission efficiency, we predicted that filamentous influenza virions may be maintained in nature because they are important for transmission. However, our present findings show that viruses carrying M1 41P transmit with lower efficiency than the corresponding, less filamentous variants carrying M1 41A. Nevertheless, we hesitate to conclude that filamentous disfavor transmission or that morphology does not affect transmission. A more plausible explanation is that morphology and transmission are linked, but the relationship between particle length and transmissibility is affected (perhaps even mediated by) other viral factors, such as NA activity and HA avidity (42).

Based on our present results, and also those presented in reference 42, we hypothesize that the pleiomorphic nature of avian influenza virus is necessary to support optimal fitness. Thus, spherical and filamentous virions may play complementary and distinct roles within the respiratory tract of the host to promote spread and transmission. Such a mechanism could exploit putative differences in, for example, attachment and release properties of the virions. We furthermore suggest that the proportion, or length, of filamentous supports optimal replication may vary, depending on strain-specific properties of HA and NA.

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