

Adaptation of Pandemic H1N1 Influenza Viruses in Mice[∇]

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The molecular mechanism by which pandemic 2009 influenza A viruses were able to sufficiently adapt to humans is largely unknown. Subsequent human infections with novel H1N1 influenza viruses prompted an investigation of the molecular determinants of the host range and pathogenicity of pandemic influenza viruses in mammals. To address this problem, we assessed the genetic basis for increased virulence of A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) isolates, which are not lethal for mice, in a new mammalian host by promoting their mouse adaptation. The resulting mouse lung-adapted variants showed significantly enhanced growth characteristics in eggs, extended extrapulmonary tissue tropism, and pathogenicity in mice. All mouse-adapted viruses except A/TN/1-560/09-MA2 grew faster and to higher titers in cells than the original strains. We found that 10 amino acid changes in the ribonucleoprotein (RNP) complex (PB2 E158G/A, PA L295P, NP D101G, and NP H289Y) and hemagglutinin (HA) glycoprotein (K119N, G155E, S183P, R221K, and D222G) controlled enhanced mouse virulence of pandemic isolates. HA mutations acquired during adaptation affected viral receptor specificity by enhancing binding to α 2,3 together with decreasing binding to α 2,6 sialyl receptors. PB2 E158G/A and PA L295P amino acid substitutions were responsible for the significant enhancement of transcription and replication activity of the mouse-adapted H1N1 variants. Taken together, our findings suggest that changes optimizing receptor specificity and interaction of viral polymerase components with host cellular factors are the major mechanisms that contribute to the optimal competitive advantage of pandemic influenza viruses in mice. These modulators of virulence, therefore, may have been the driving components of early evolution, which paved the way for novel 2009 viruses in mammals.

In early April 2009, a new H1N1 influenza virus with a previously uncharacterized constellation of eight genes (10) was first detected in humans (5). Since then, this newly emerged influenza virus has spread worldwide to 214 countries (more than 503,536 cases [51]) by human-to-human transmission and prompted the World Health Organization to raise the worldwide pandemic alert to phase 6 (52). Sequence analysis of pandemic 2009 isolates revealed the absence of markers associated with high pathogenicity in avian and mammalian species, such as a multibasic hemagglutinin (HA) cleavage site (49) or lysine at position 627 of the PB2 protein (39, 44). Thus, the molecular mechanism by which pandemic 2009 influenza A viruses were able to sufficiently adapt to humans remains unknown. Subsequent human infections with novel H1N1 influenza viruses prompted an investigation of the genetic basis that determines pandemic influenza virus host range and pathogenicity in mammals.

Mice have been shown to be a good mammalian model for studying influenza virus pathogenicity and host range restriction mechanisms (48). Mice are not naturally infected with human or other strains of influenza virus, but most strains can

be experimentally adapted for mouse virulence by serial lung-to-lung passages (15, 34). It is assumed that mouse adaptation results in the acquisition of functions that are critical determinants of virulence. Mouse-adapted influenza virus mutants usually induce pathology in the bronchi or lungs of infected animals (15, 34, 54). Such mutants possess an increased ability to infect alveolar cells, thereby initiating alveolitis, and can cause lethal pneumonitis (34, 54). In addition, all epithelial cells of the bronchi and alveoli are susceptible to infection with fully adapted strains (14, 26). The lung pathology induced by the mouse-adapted viruses shows considerable similarities to that of human influenzal pneumonia (45); thus, the changes that occur in the virus during mouse adaptation may provide insight into factors that affect the development of lung infection in humans.

Several factors that affect influenza virus host range and virulence in mice have been identified. Influenza virus hemagglutinin (HA) is a primary determinant for mouse lung virulence (2, 11, 12, 23, 41, 42). However, seven other genes have also been implicated (3, 4, 21–23, 25, 30, 37, 40, 42, 43, 53–55). For example, studies of the mouse lung-adapted A/FM/1/47 (H1N1) influenza virus revealed that, in addition to the HA protein, the M gene has the capacity to control virus virulence and growth (42). The polymerase basic 2 (PB2) protein is another influenza virus subunit that has been shown to modulate virus virulence and host range in mice (8, 25, 39, 40, 44). PB1, recently identified PB1-F2, and polymerase acidic (PA) proteins have also been implicated in mouse lung virulence but

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have shown no evidence of having acquired mutations during adaptation (4, 7, 23, 37, 43, 46, 53, 55). Other studies have suggested that nonstructural 1 (NS1) protein function is essential for avoidance of an innate cellular immune response against the virus and may be crucial for influenza virus pathogenicity (21, 22).

Although most studies conducted to date have focused on H1, H2, H3, H5, H7, and H9 HA subtypes of influenza viruses, no mouse adaptation experiments on pandemic 2009 H1N1 isolates have yet been reported. We, therefore, investigated the adaptation of two pandemic isolates, A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1), which are not lethal for mice, to a new mammalian host. We used wild-type and mouse-adapted variants to identify molecular determinants of this host adaptation and enhanced virulence in mammals. Our findings suggest that multiple sequence changes within HA and the ribonucleoprotein (RNP) complex of pandemic 2009 influenza A viruses contribute to their adaptation and increased virulence in mice.

MATERIALS AND METHODS

Viruses and cells. The pandemic H1N1 influenza viruses A/CA/04/09 and A/TN/1-560/09 were obtained from the World Health Organization collaborating laboratories. Stock viruses were grown in the allantoic cavities of 10-day-old chicken eggs for 48 h at 37°C, and aliquots were stored at -70°C until used. *In vitro* experiments with pandemic H1N1 influenza viruses were conducted in a biosafety level 2+ containment facility approved by the U.S. Department of Agriculture.

Madin-Darby canine kidney (MDCK) and human embryonic kidney (293T) cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained as previously described (19).

Adaptation of H1N1 influenza viruses in mice. Four mouse-adapted variants of A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) viruses, A/CA/04/09-MA1, A/CA/04/09-MA2, A/TN/1-560/09-MA1, and A/TN/1-560/09-MA2 (MA for mouse adapted), were derived in 2 parallel, independent series of sequential lung-to-lung passages, as described previously (2). Briefly, female 6-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intranasally under light isoflurane anesthesia with 50 μ l of allantoic fluid containing wild-type H1N1 virus. Lungs were harvested after 2 days and homogenized, and 50 μ l of the centrifuged homogenate was used as the inoculum for the next passage. After a total of 9 passages, virus present in the lung homogenate was cloned once by plaque purification in MDCK cells, and the cloned virus was passaged once in the allantoic cavities of 10-day-old chicken eggs for 48 h at 37°C to prepare a virus stock. All animal use was conducted under applicable laws and guidelines, with prior approval of the St. Jude Children's Research Hospital Animal Care and Use Committee.

Infectivity of H1N1 influenza viruses. The infectivity of wild-type and mouse-adapted H1N1 viruses in MDCK cells was determined by plaque assay and expressed as log₁₀ PFU per milliliter (13). Briefly, confluent MDCK cells were incubated at 37°C for 1 h with 10-fold serial dilutions of virus. The cells were then washed and overlaid with minimal essential medium (MEM) containing 0.3% bovine serum albumin (BSA), 0.9% Bacto agar, and 1 μ g/ml L-[tosylamido-2-phenyl]ethylchloromethylketone (TPCK)-treated trypsin. After 3 days of incubation at 37°C, cells were stained with 0.1% crystal violet in 10% formaldehyde solution, and the PFU per milliliter and plaque size were determined using the Finescale comparator (Los Angeles, CA).

The 50% egg infectious dose (EID₅₀) in 10-day-old chicken eggs was determined with 10-fold serial dilutions of virus, incubated for 48 h at 37°C, and calculated by the Reed-Muench method (35).

Replication kinetics. To determine multistep growth curves, MDCK cells were infected with wild-type and mouse-adapted H1N1 viruses at a multiplicity of infection (MOI) of 0.005 PFU/cell. After incubation, the cells were washed and overlaid with infection medium (MEM with 0.3% BSA and 1 μ g/ml TPCK-treated trypsin). Supernatants were collected 12, 24, 36, 48, 60, and 72 h after infection and stored at -70°C for titration by 50% tissue culture infectious dose (TCID₅₀) assay (19).

Pathogenicity and lethality in BALB/c mice. To determine the 50% mouse lethal dose (MLD₅₀), we anesthetized groups of five female 6-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) with isoflurane and intranasally

inoculated them with 50 μ l of 10-fold serial dilutions of H1N1 influenza viruses in phosphate-buffered saline (PBS). The MLD₅₀ values were calculated by the Reed-Muench method (35) after a 21-day observation period and expressed in PFU. To monitor survival, we inoculated groups of 13 mice with H1N1 viruses (5,000 PFU/mouse). Animals that showed signs of severe disease and weight loss of >25% were humanely killed. To evaluate the replication kinetics and pathogenicity of H1N1 viruses *in vivo*, we euthanized three mice on day 3 after inoculation and removed the lungs, brain, spleen, and blood, and the organs were homogenized and suspended in 1 ml of PBS. Virus titers in each of the organ and blood samples were determined by inoculating chicken eggs with serial dilutions of the suspensions. Titers were calculated by the method of Reed and Muench and expressed as mean log₁₀ EID₅₀/ml \pm standard deviation (SD). The limit of virus detection was 0.75 log₁₀ EID₅₀/ml. For calculation of the mean, samples with a virus titer of <0.75 log₁₀ EID₅₀/ml were assigned a value of 0.

Assessment of virus pathogenicity in ferrets. Pathogenicity was tested in 4- to 5-month-old male and female ferrets obtained from Marshall Farms (North Rose, NY). All ferrets were seronegative for influenza A H1N1, H3N2, and H5N1 and influenza B viruses. Groups of three ferrets were inoculated intranasally under light isoflurane anesthesia with 10⁶ EID₅₀ (10^{5.0} to 10^{5.9} PFU/ferret) of H1N1 virus in 1 ml of PBS. Clinical signs of infection, relative inactivity index (36), weight, and temperature were recorded daily. Activity level was assessed by using the following scoring system: 0, alert and playful; 1, alert and playful when stimulated; 2, alert but not playful when stimulated; and 3, neither alert nor playful when stimulated. Body temperature was measured by subcutaneous implantable temperature transponders (Bio Medic Data Systems, Inc.). To monitor virus shedding, nasal washes were collected from ferrets at 3, 5, and 7 days postinoculation (dpi). Virus was titrated in eggs by injecting 0.1 ml of 10-fold serial dilutions of the sample (three eggs per dilution) and expressed as mean log₁₀ EID₅₀/ml \pm SD.

RNA isolation, PCR amplification, and sequencing. Viral RNA was isolated from virus-containing allantoic fluid by using an RNeasy Mini kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. Reverse transcription of viral RNA and subsequent PCR were performed using primers specific for each gene segment, as described previously (17). PCR products were purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. Sequencing was performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. The DNA template was sequenced by using rhodamine or dRhodamine dye terminator cycle sequencing Ready Reaction kits with AmpliTaqDNA polymerase FS (PerkinElmer Applied Biosystems, Waltham, MA) and synthetic oligonucleotides. Samples were analyzed using a PerkinElmer (model 373 or model 377) DNA sequencer. DNA sequences were completed and edited using the Lasergene sequence analysis software package (DNASTAR, Madison, WI).

Hemagglutination inhibition test. A hemagglutination inhibition (HI) test was performed with 0.5% chicken red blood cells by a standard method (33). We used a panel of 4 monoclonal antibodies (MAbs) to HA of 2 H1 strains: 2 antibodies to A/Swine/AR/2976/02 (H1N2), MAbs H5 and G7, and 2 antibodies to A/Swine/NC/18161/02 (H1N1), MAbs E2 and H7.

Receptor-binding assay. The binding of wild-type and mouse-adapted H1N1 influenza viruses to fetuin (containing α 2,3- and α 2,6-linked sialyl receptors) was measured in a direct solid-phase assay using the immobilized virus and horseradish peroxidase-conjugated fetuin, as described previously (9). The affinity of viruses for synthetic 3'- and 6'-sialylglycopolymers obtained by conjugation of a 1-N-glycyl derivative of 3'-sialyllactose (3'SL, "avian-like" α 2,3-containing receptor) or 3-aminopropylglycoside of 6'-sialyllactosamine (6'SLN, "human-like" α 2,6-containing receptor) with poly(4-phenylacrylate) (1) was measured in a competitive assay based on the inhibition of binding to peroxidase-labeled fetuin (28). Association constant (K_{ass}) values were determined as the sialic acid (Neu5Ac) concentration at the point $A_{\text{max}}/2$ on Scatchard plots.

Minigenome assay for polymerase activity. Subconfluent monolayers of 293T cells (7.5 \times 10⁵ cells in 35-mm dishes) were transfected with a luciferase reporter plasmid (enhanced green fluorescent protein [EGFP] open reading frame in pHW72-EGFP substituted with a firefly luciferase gene [16, 38]) and a mix of PB2, PB1, PA, and NP plasmids (wild type or mutated) in quantities of 1, 1, 1, and 2 μ g, respectively. Plasmid pGL4.75[*hRluc*/CMV] vector, which expresses *Renilla* luciferase (Promega, Madison, WI), was used as an internal control for the dual luciferase assay. As a negative control, 293T cells were transfected with the same plasmids, with the exception of the NP expression plasmid. After 24 h, cell extracts were harvested and lysed, and luciferase levels were assayed with a dual luciferase assay system (Promega, Madison, WI) and a BD Monolight 3010 luminometer (BD Biosciences, San Diego, CA). Experiments were performed in triplicate.

Protein gel electrophoresis and Western blot analysis. Subconfluent monolayers of 293T cells were transfected the same way as described for the mini-genome assay (see above). After 24 h, the cells were harvested and whole-cell extracts were prepared by lysis in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% Igepal, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS] [Sigma-Aldrich, St. Louis, MO]) and protease inhibitors (Pierce, Rockford, IL) on ice for 20 min. Extracts were centrifuged ($15,700 \times g$, 15 min, 4°C), and the supernatants were collected. Proteins from cell extracts were denatured by heating at 100°C for 5 min in $2\times$ SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and were then separated by SDS-PAGE. For Western blot analysis, the proteins were transferred to a nitrocellulose membrane, which was then probed with specific primary antibodies and peroxidase-labeled secondary antibody. Equal loading of wells was confirmed by Western blotting with a mouse monoclonal primary antibody against the cellular protein β -actin. The blots were analyzed by chemiluminescence and exposed to X-ray film.

Statistical analysis. Virus yields *in vitro* and *in vivo*, plaque size, mean days to death, weight changes of mice, virus titers in the internal organs (lungs, brain, and spleen) and blood of mice, binding to sialyl receptors, and polymerase activities of RNP complexes of wild-type and mouse-adapted H1N1 influenza viruses were compared by analysis of variance (ANOVA). The probability of survival of infected mice was estimated by the Kaplan-Meier method (47). Weight changes and nasal wash titers of ferrets inoculated with wild-type or mouse-adapted viruses were compared by an unpaired, two-tailed *t* test. A probability value of 0.05 was prospectively chosen to indicate that the findings were not the result of chance alone.

RESULTS

Adaptation of pandemic H1N1 influenza viruses in mice.

Recent studies have shown that the novel 2009 A (H1N1) human influenza viruses are almost avirulent for mice (MLD_{50} of $\geq 10^6$ PFU for A/CA/04/09) (20, 27). To increase the virulence of pandemic H1N1 isolates in mice, we produced mouse-adapted variants of A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) strains (two for each virus) by serial lung-to-lung passages, beginning with intranasal inoculation of $10^{6.2}$ and $10^{5.2}$ EID_{50} of virus per mouse, respectively. Nine subsequent passages involved the inoculation of diluted lung extracts, constituting dosages of $\sim 10^3$ to 10^6 EID_{50} /mouse (data not shown). Survival of infected animals and virus titers of lung homogenates were assayed after each passage. Wild-type A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) influenza viruses generated virus titers of 6.8 and 5.8 \log_{10} EID_{50} /ml of lung homogenates, respectively. However, after 4 to 5 passages, virus titers increased to 7.5 to 8.2 \log_{10} EID_{50} /ml (data not shown). After 6 to 8 passages, mice showed a continuous trend of weight loss and commenced dying at 6 or 7 dpi, indicating that virus populations of mouse-adapted variants of pandemic H1N1 isolates had acquired mutations that profoundly affect virulence.

Growth characteristics of mouse-adapted H1N1 viruses *in vitro*. We studied the growth of A/CA/04/09-MA1, A/CA/04/09-MA2, A/TN/1-560/09-MA1, and A/TN/1-560/09-MA2 variants both in eggs and in MDCK cells. The mouse-adapted variants grew to significantly higher titers in eggs than their respective wild-type viruses ($P < 0.05$) (Table 1). Comparison of virus yields of mouse-adapted variants indicated that A/CA/04/09-MA1 and A/TN/1-560/09-MA1 grew better than their respective MA2 clones ($P < 0.05$). The yields of the mouse-adapted viruses in cell culture were 1.6 to 2.2 logs higher than that of their parental wild-type strains, with the exception that A/TN/1-560/09-MA2 replicated to significantly lower titers than did either A/TN/1-560/09 or A/TN/1-560/09-MA1 ($P < 0.05$). All H1N1 viruses formed homogeneous small plaques

TABLE 1. Growth characteristics of wild-type and mouse-adapted H1N1 influenza viruses^d

H1N1 virus	Virus yield		Plaque size ^c
	Eggs ^a	MDCK cells ^b	
A/CA/04/09	7.5 ± 0.1	6.9 ± 0.1	0.2 ± 0.1
A/CA/04/09-MA1	$8.2 \pm 0.1^{*o}$	$8.1 \pm 0.2^*$	$1.0 \pm 0.2^{*o}$
A/CA/04/09-MA2	$7.8 \pm 0.1^{*o}$	$7.6 \pm 0.4^*$	0.5 ± 0.3^o
A/TN/1-560/09	7.5 ± 0.1	6.6 ± 0.1	0.2 ± 0.1
A/TN/1-560/09-MA1	$9.2 \pm 0.3^{*o}$	$8.2 \pm 0.2^{*o}$	0.6 ± 0.2
A/TN/1-560/09-MA2	$8.5 \pm 0.1^{*o}$	$4.4 \pm 0.1^{*o}$	0.3 ± 0.1

^a Values are mean \log_{10} EID_{50} /ml \pm SD from three independent determinations. The EID_{50} in 10-day-old chicken eggs was determined with 10-fold serial dilutions of virus, incubated for 48 h at 37°C , and calculated by the Reed-Muench method (35).

^b Values are mean \log_{10} PFU/ml \pm SD from three independent determinations. The PFU in MDCK cells was determined by plaque assay after 3 days of incubation at 37°C with 10-fold serial dilutions of virus (13).

^c Values are mean plaque diameter (mm) \pm SD as measured by using the Finescale comparator.

^d *, $P < 0.05$ compared with the value for the respective wild-type virus (one-way ANOVA); ^o, $P < 0.05$ compared with the value for the respective mouse-adapted virus (one-way ANOVA).

(diameter, 0.2 to 0.6 mm) in MDCK cells, although the A/CA/04/09-MA1 plaques were larger than the wild-type A/CA/04/09 plaques ($P < 0.05$) (Table 1).

To further evaluate the replicative ability of the mouse-adapted H1N1 viruses, we assayed their virus yields in comparison with those of the respective wild-type strains after multiple replication cycles in MDCK cells (Fig. 1A and B). Both mouse-adapted variants A/CA/04/09-MA1 and A/CA/04/09-MA2 grew faster and to significantly higher titers than the A/CA/04/09 strain in cell culture (Fig. 1A). However, we observed significant differences in growth rates between A/TN/1-560/09-MA1 and A/TN/1-560/09-MA2 variants at almost all postinfection time points (Fig. 1B). The replication ability of A/TN/1-560/09-MA1 was significantly higher than that of the wild-type virus at 24, 48, 60, and 72 h after infection (>1.5 to 3 logs, $P < 0.05$). In contrast, the yield of A/TN/1-560/09-MA2 was only approximately 0.1 to 16% that of A/TN/1-560/09 at all time intervals except 24 h after infection, when the mouse-adapted virus reached titers comparable to those of the wild-type strain in MDCK cells (Fig. 1B). Taken together, our findings showed that mouse adaptation of pandemic H1N1 influenza viruses selected variants with increased replicative fitness in eggs, MDCK cells, or both *in vitro* systems.

Pathogenicity and lethality of mouse-adapted H1N1 viruses *in vivo*.

Using a BALB/c mouse model, we compared the virulence of generated mouse-adapted variants with that of the original A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) strains. We observed that all mouse-adapted clones were significantly more virulent for mice than their respective wild-type viruses ($\sim 10^3$ - to 10^4 -fold decrease in MLD_{50} values) (Table 2). Furthermore, for a given dose of virus (5,000 PFU), all mice infected with either mouse-adapted isolate exhibited clinical signs of disease, including decreased activity, huddling, hunched posture, and ruffled fur. Mice in these groups lost weight progressively, and all died between 5 and 10 dpi (Fig. 2A and B). We also observed a significant difference in time to death of mice infected with A/CA/04/09-MA1 versus A/CA/04/09-MA2 ($P < 0.05$). In contrast, no morbidity or mortality was

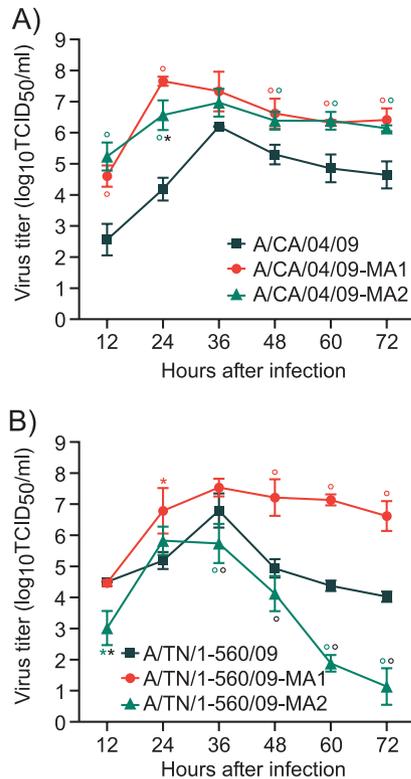


FIG. 1. Multistep growth curves of wild-type A/CA/04/09 (H1N1) (A) and A/TN/1-560/09 (H1N1) (B) viruses and their respective mouse-adapted variants in MDCK cells. Confluent MDCK cells were infected with H1N1 viruses at an MOI of 0.005 PFU/cell. Virus yields (\log_{10} TCID₅₀/ml) at 12, 24, 36, 48, 60, and 72 h after infection were titrated in MDCK cells. Each data point represents the mean virus yield from three individually infected wells \pm the standard deviation. *, $P < 0.05$, and \circ , $P < 0.01$, compared with the value for the wild-type virus (shown in color) or compared with the value for the respective mouse-adapted virus (shown in black) (one-way ANOVA).

observed in mice infected with either parental wild-type virus (Table 2 and Fig. 2).

To determine whether the different levels of virulence of wild-type and mouse-adapted H1N1 influenza viruses were related to a difference in ability to replicate in mouse organs, groups of 3 BALB/c mice were inoculated intranasally with 5,000 PFU of either virus, and virus replication in the lungs,

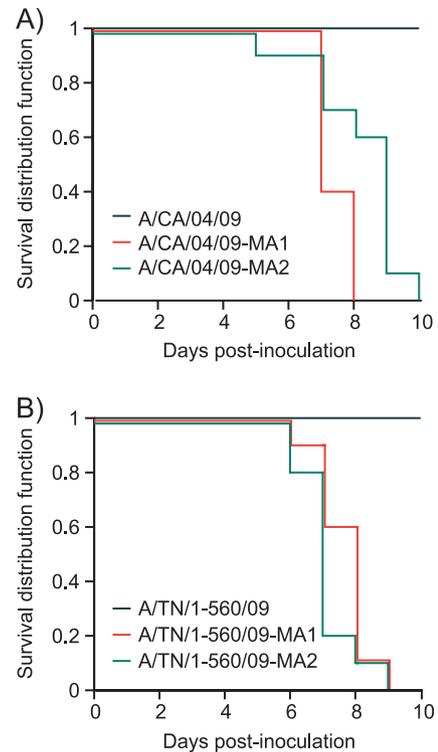


FIG. 2. Survival of BALB/c mice infected with 5,000 PFU/mouse (each in 50 μ l) of wild-type A/CA/04/09, A/CA/04/09-MA1, or A/CA/04/09-MA2 virus (A) and wild-type A/TN/1-560/09, A/TN/1-560/09-MA1, or A/TN/1-560/09-MA2 virus (B).

brains, spleens, and blood of infected animals was determined 3 dpi. Wild-type A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) viruses replicated efficiently in mouse lungs (~ 6.2 \log_{10} EID₅₀/ml) without prior host adaptation and did not spread to other organs tested (< 0.75 \log_{10} EID₅₀/ml) (Table 2). These data were in good agreement with previous reports (20, 27). In contrast, all mouse-adapted H1N1 variants replicated to significantly higher titers in the lungs (up to 8.4 \log_{10} EID₅₀/ml, $P < 0.05$) (Table 2). The virus was also detected in the brains (3.0 to 3.6 \log_{10} EID₅₀/ml), spleens (3.8 to 4.4 \log_{10} EID₅₀/ml), and blood (4.9 to 5.4 \log_{10} EID₅₀/ml) of mice 3 dpi, indicating that mouse-adapted H1N1 viruses had gained the ability to replicate in other organs as well as in the lungs.

TABLE 2. Pathogenicity of wild-type and mouse-adapted H1N1 influenza viruses in BALB/c mice^a

H1N1 virus	MLD ₅₀ (PFU, mean \pm SD)	No. of survivors/total (%)	Day of death (mean \pm SD) ^b	Weight change (%, mean \pm SD) at 4 dpi	Virus titer (\log_{10} EID ₅₀ /ml, mean \pm SD) at 3 dpi			
					Lungs	Brain	Spleen	Blood
A/CA/04/09	10 ^{5.1} \pm 0.1	10/10 (100)	>21.0	+4.7 \pm 3.5	6.4 \pm 0.2	< ^c	<	<
A/CA/04/09-MA1	10 ^{1.8} \pm 0.2*	0/10 (0)	7.4 \pm 0.2* ^o	-16.1 \pm 4.1*	8.4 \pm 0.5*	3.4 \pm 0.7*	4.2 \pm 0.5*	4.9 \pm 0.2*
A/CA/04/09-MA2	10 ^{1.8} \pm 0.4*	0/10 (0)	8.4 \pm 0.5* ^o	-14.3 \pm 5.6*	8.2 \pm 0.5*	3.6 \pm 0.7*	3.8 \pm 0.3*	5.1 \pm 1.3*
A/TN/1-560/09	10 ^{5.0} \pm 0.1	10/10 (100)	>21.0	+6.1 \pm 4.6	6.2 \pm 0.1	<	<	<
A/TN/1-560/09-MA1	10 ^{2.6} \pm 0.3* ^o	0/10 (0)	7.6 \pm 0.3*	-15.1 \pm 4.2*	8.2 \pm 0.3*	3.0 \pm 0.4*	4.2 \pm 0.5*	5.4 \pm 0.5*
A/TN/1-560/09-MA2	10 ^{0.6} \pm 0.2* ^o	0/10 (0)	7.1 \pm 0.3*	-18.0 \pm 2.6*	7.6 \pm 0.9*	3.1 \pm 0.5*	4.4 \pm 0.2*	5.2 \pm 0.3*

^a Groups of 13 BALB/c mice were inoculated intranasally under light anesthesia with 5,000 PFU of H1N1 influenza virus. Three mice from each group were euthanized 3 dpi for virus titration. *, $P < 0.05$ compared with the value for the respective wild-type virus (one-way ANOVA); ^o, $P < 0.05$ compared with the value for the respective mouse-adapted virus (one-way ANOVA).

^b The mean day of death of mice was determined by the Kaplan-Meier method.

^c <, the titer was below the limit of detection (< 0.75 \log_{10} EID₅₀/ml).

TABLE 3. Pathogenicity of wild-type and mouse-adapted H1N1 influenza viruses in ferrets

H1N1 virus	No. of ferrets showing indicated clinical sign of disease/total no.		Nasal wash titer (log ₁₀ EID ₅₀ /ml, mean ± SD) on indicated dpi ^b	
	Weight loss (%) ^a	Sneezing	3	5
A/CA/04/09	3/3 (8.8 ± 1.6)	1/3	5.4 ± 1.1	4.5 ± 0.8
A/CA/04/09-MA1	3/3 (9.7 ± 1.8)	1/3	5.6 ± 0.9	3.6 ± 1.4
A/TN/1-560/09	3/3 (5.8 ± 1.8)	2/3	4.9 ± 0.8	3.9 ± 0.4
A/TN/1-560/09-MA1	3/3 (7.3 ± 1.9)	1/3	5.7 ± 1.0	3.8 ± 0.5

^a The maximum change in the mean value (percentage) ± SD observed over a period of 14 days after inoculation is shown in parentheses.

^b Virus was not detected in any group tested 7 dpi (detection limit, <0.75 log₁₀ EID₅₀/ml).

To determine whether mouse adaptation of pandemic H1N1 isolates resulted in the selection of influenza viruses with increased virulence in other animal models in which influenza virus pathogenesis closely resembles that in humans, we characterized the pathogenicity of the generated mouse-adapted viruses in ferrets. Four groups of three ferrets each were inoculated with 10⁶ EID₅₀ of A/CA/04/09-MA1 or A/TN/1-560/09-MA1 virus, which exhibited better viral growth either *in vitro* or in mice, or their parental wild-type strains (Table 3). Both original wild-type viruses caused mild clinical disease signs without marked changes in body temperature. The mean maximum weight loss was ~6 to 9%, similar to that observed by other groups (20, 27, 29). No significant differences between wild-type and mouse-adapted variants were seen either in clinical features of infection or in virus titers in nasal washes 3 and 5 dpi in a ferret animal model (Table 3). Therefore, our findings showed that changes acquired during mouse adaptation were host specific and did not markedly affect the pathogenesis of pandemic H1N1 influenza viruses in ferrets.

Sequence analysis. To understand the molecular basis for the increased virulence acquired by pandemic H1N1 viruses during mouse adaptation, the complete genomes of the wild-type and mouse-adapted counterparts were sequenced. Sequence analysis mapped the acquired mutations to HA, NP, and polymerase proteins (Table 4). A total of 10 amino acid substitutions affecting 3 or 4 viral proteins were involved in adaptation of the pandemic A/CA/04/09 (H1N1) or A/TN/1-560/09 (H1N1) strain to mice, respectively; however, undetected genetic changes that might have occurred after a single

passage in chicken eggs could not be excluded. Interestingly, the A/TN/1-560/09-MA1 variant had an adaptive change, K119N, in the HA protein (H1 numbering used throughout the text) leading to acquisition of the potential glycosylation site (Table 4).

To investigate whether the amino acid changes observed in mouse-adapted H1N1 variants generated in this study might occur in other pandemic 2009 A (H1N1) isolates, we analyzed the H1N1 sequences deposited in the Influenza Research Database (data were obtained from the National Institute of Allergy and Infectious Diseases database available online at <http://www.fludb.org>). The mouse-adapted viruses were found to share common residues with novel H1N1 isolates in PA (295P) and HA (222G) proteins (Table 4). In fact, all pandemic H1N1 viruses possess proline at residue 295 in PA, and leucine at this position is unique to the original wild-type A/TN/1-560/09 strain. Furthermore, the mutation D222G was found in the HA protein of 1% of contemporary H1N1 isolates. However, the other eight amino acid substitutions acquired during mouse adaptation (E158G/A in PB2; K119N, G155E, S183P, and R221K in HA; and D101G and H289Y in NP [Table 4]) were not present in any H1N1 strains examined (0% or <0.1%).

Effect of HA amino acid substitutions on antigenic and receptor specificity. To test whether in the course of mouse lung adaptation the pandemic H1N1 viruses changed their antigenic specificity due to acquired HA mutations, we determined the antigenic properties of wild-type and mouse-adapted H1N1 variants in an HI test using postinfection goat and ferret antisera and MAbs raised against a selection of currently circulating human, pandemic, and swine H1 isolates (Table 5). We observed that the original A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) strains did not react with the postinfection ferret antisera or MAbs raised against contemporary human or swine H1 viruses, which was in good agreement with previous findings (10, 20). The results of the HI assay showed that the mouse-adapted A/CA/04/09-MA1 and A/CA/04/09-MA2 clones had a reduced ability to react with ferret antisera raised against the parental strains (4- to 8-fold differences), whereas A/TN/1-560/09-MA1 and A/TN/1-560/09-MA2 reacted more strongly with the same antisera. Additionally, mouse-adapted H1N1 variants exhibited a change in reactivity with E2 and H7 MAbs (Table 5).

Since one of the mechanisms by which HA mutations may increase virus virulence is by affecting HA affinity to sialyl receptors (48), we next measured the receptor specificity of

TABLE 4. Amino acid substitutions identified in mouse-adapted H1N1 influenza viruses

H1N1 virus	Amino acid residue at position no.								
	PB2 158	PA 295	HA					NP	
			119	155	183	221	222	101	289
A/CA/04/09	E	P	K	G	S	R	D	D	H
A/CA/04/09-MA1	G	P	K	E	P	R	G	G	H
A/CA/04/09-MA2	E	P	K	E	P	R	G	G	H
A/TN/1-560/09	E	L	K	G	S	R	D	D	H
A/TN/1-560/09-MA1	A	P	<u>N</u> ^a	G	S	K	D	D	Y
A/TN/1-560/09-MA2	E	P	<u>K</u>	G	S	K	D	D	Y

^a The underlined letter indicates the N-glycosylation site.

TABLE 5. Antigenic characterization of wild-type and mouse-adapted H1N1 influenza viruses by hemagglutination inhibition assay

H1N1 virus	Goat antiserum against A/CA/ 04/09	Titer ^a						
		Ferret antiserum against:			MAb against:			
		A/CA/04/09	A/TN/1-560/09	A/Brisbane/59/07	A/Swine/AR/ 2976/02		A/Swine/NC/ 18161/02	
					H5	G7	E2	H7
A/CA/04/09	640	2,560	640	<40	100	<100	200	100
A/CA/04/09-MA1	640	320	80	<40	<100	<100	800	400
A/CA/04/09-MA2	640	320	160	<40	<100	<100	800	200
A/TN/1-560/09	640	2,560	640	<40	<100	<100	400	200
A/TN/1-560/09-MA1	1,280	>5,120	1,280	<40	100	100	800	1,600
A/TN/1-560/09-MA2	1,280	>5,120	1,280	<40	<100	<100	800	800

^a Values are expressed as reciprocals of serum/antibody dilutions that inhibited 8 hemagglutination units of H1N1 influenza virus. Homologous HI titers are shown in bold type.

H1N1 viruses to high-molecular-weight sialic substrates, both natural (fetuin) and synthetic (3'SL and 6'SLN), attached to a polyacrylic carrier (Fig. 3). As shown by the K_{ass} values, pandemic wild-type H1N1 viruses had increased affinity for 6'SLN, whereas the binding to the "avian-type" 3' substrate was negligible (6, 27). Our results revealed that all mouse-adapted H1N1 viruses showed significantly reduced binding to fetuin and the "human-type" 6'SLN receptor (3- to 33-fold difference, $P < 0.01$) (Fig. 3). In contrast, the affinity to the 3' substrate was significantly higher in the mouse-adapted variants than in the respective parental strains, except A/TN/1-560/09-MA2 ($P < 0.05$). The latter exhibited a moderate decrease in affinity toward the 6'SLN-acrylic polymer from that of the wild-type A/TN/1-560/09 (H1N1) virus (2.4-fold difference) (Fig. 3).

Effect of amino acid substitutions in the RNP complex on protein expression and viral polymerase activity. To investigate whether the observed mutations in the PB2, PA, and NP

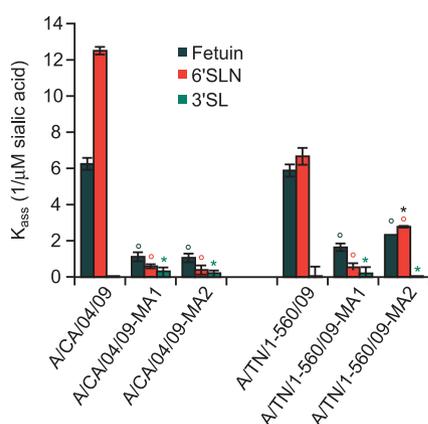


FIG. 3. Receptor specificity of wild-type and mouse-adapted H1N1 influenza viruses. Association constants (K_{ass}) of virus complexes with sialylglycopolymers conjugated to fetuin (containing α 2,3- and α 2,6-linked sialyl receptors), 6'SLN ("human-like" α 2,6-containing receptor), and 3'SL ("avian-like" α 2,3-containing receptor). Higher K_{ass} values indicate stronger binding. Values are the means of three independent experiments \pm standard deviations. *, $P < 0.05$, and o, $P < 0.01$, compared with the value for the wild-type virus (shown in color) or compared with the value for the respective mouse-adapted virus (shown in black) (one-way ANOVA).

proteins of mouse-adapted H1N1 variants changed the total protein expression and/or viral transcription and replication activity, we analyzed reconstituted RNP complexes in 293T cells by Western blotting and a luciferase minigenome assay. Densitometric analysis of protein band intensities showed that the initial expression of all mutant PB2 and NP proteins was 72 to 374% that of the respective wild types, showing that the mutations did not abrogate initial expression levels of these proteins (Fig. 4A and B).

In the minigenome assay, A/CA/04/09-MA1, carrying PB2 E158G and NP D101G mutations, and A/TN/1-560/09-MA1, carrying PB2 E158A, PA L295P, and NP H289Y mutations, had polymerase activities of ~240 to 250% that of their respective parental viruses (Fig. 4C and D). To assign this increase to single mutations, we examined all possible combinations. We observed that the PB2 E158G amino acid substitution increased polymerase activity of the A/CA/04/09 polymerase complex to ~260%, whereas NP D101G did not (A/CA/04/09-MA2 variant), stressing the importance of the PB2 E158G change for the significant enhancement of transcription and replication activity (Fig. 4C). Single point mutations PB2 E158A and PA L295P increased polymerase activity of the A/TN/1-560/09 RNP complex to 200% and 125%, respectively, and their combination further enhanced polymerase activity to 240% in an additive manner (Fig. 4D). In contrast, amino acid change H289Y in the NP protein did not affect enzymatic activity when introduced alone or in any combination tested. Taken together, our findings showed that efficient polymerase activity (2.5-fold increase, $P < 0.01$) was achieved by PB2 E158G or by the combination of PB2 E158A and PA L295P in the A/CA/04/09 (H1N1) or A/TN/1-560/09 (H1N1) RNP complex, respectively.

DISCUSSION

Adaptation is believed to be a driving force in evolution, where organisms are selected in nature because of increased fitness conferred by beneficial mutations. Importantly, until now the molecular basis of adaptation of pandemic influenza A viruses to a new host species has been poorly understood. To address this problem, we assessed the genetic basis for increased virulence of novel H1N1 isolates in a new mammalian host by performing mouse adaptation of these isolates.

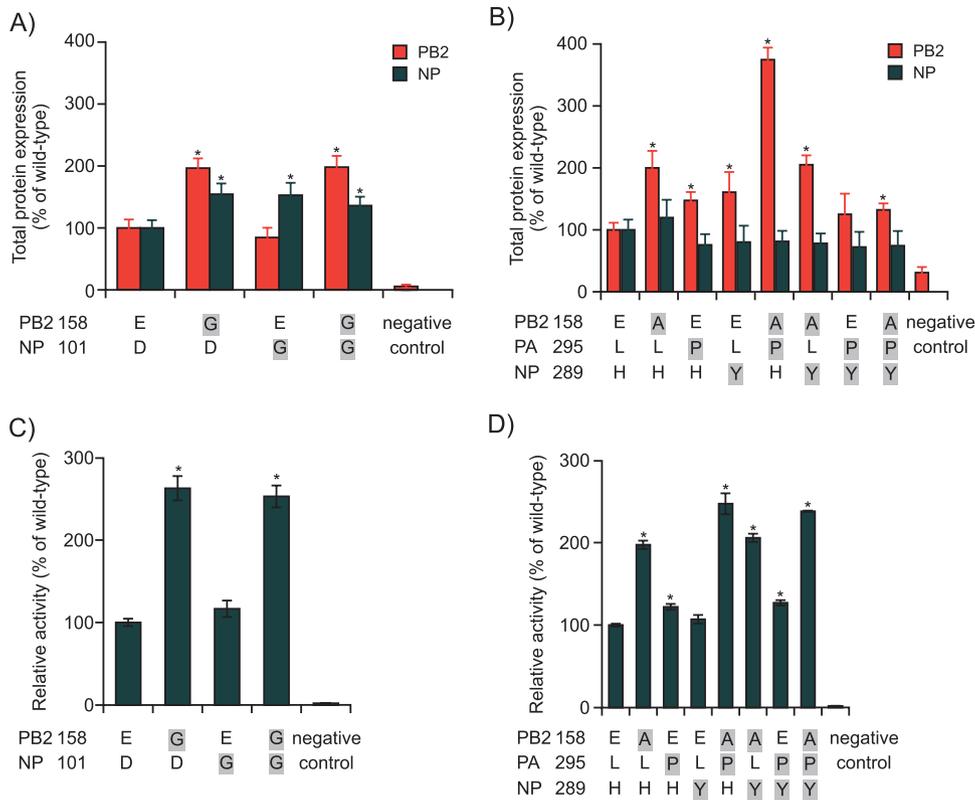


FIG. 4. Total protein expression and viral polymerase activity of RNP complexes. (A and B) Total expression of wild-type and mutant PB2 and NP proteins of A/CA/04/09 (H1N1) (A) and A/TN/1-560/09 (H1N1) (B) viruses in 293T cells. 293T cells expressing wild-type and mutant proteins were processed 24 h posttransfection and analyzed by Western blotting with rabbit polyclonal antibodies raised against PB2 and NP proteins of H1N1 influenza A virus (GenScript, Piscataway, NJ). *, $P < 0.01$ compared with the value for the wild-type virus (one-way ANOVA). (C and D) Polymerase activity of RNP complexes reconstituted from PB2, PB1, PA, and NP plasmids of wild-type A/CA/04/09 (H1N1) (C) and A/TN/1-560/09 (H1N1) (D) viruses and of their single point mutants. The polymerase activities were determined by a dual luciferase reporter assay in three independent experiments. *, $P < 0.01$ compared with the value for the wild-type virus (one-way ANOVA).

To reveal the pattern of positively selected mutations, we performed two independent series of sequential lung-to-lung passages of A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) viruses. Therefore, for each pandemic isolate, two mouse-adapted variants were produced and were subjected to sequence analysis to determine whether they shared common mutations. The A/CA/04/09-MA1 and A/CA/04/09-MA2 viruses differed by one amino acid substitution (E158G) in PB2 and possessed 4 amino acid changes in HA and NP proteins in common, indicating positive selection of these four mutations. Sequencing of A/TN/1-560/09-MA1 and A/TN/1-560/09-MA2 revealed that they differed by two mutations in PB2 and HA (E158A and K119N, respectively) and shared three positive mutations in PA, HA, and NP proteins. The positive selection of 7 out of 10 mutations indicated that they confer a replicative advantage; however, there is reason to believe that the remaining three mutations observed in MA1 clones (namely, PB2 E158G/A and HA K119N) were also adaptive, because they occurred independently at the same residue in two pandemic strains and/or were instrumental in adaptation by increasing viral growth in eggs, MDCK cells, and mice (discussed below).

In the present study, we identified five mutations in the HA protein, K119N, G155E, S183P, R221K, and D222G, that partially and most likely independently controlled the virulence of

pandemic H1N1 viruses in mice. Further characterization of receptor specificity of generated mouse-adapted variants revealed their enhanced binding abilities to $\alpha 2,3$ together with decreased binding to $\alpha 2,6$ sialic acid-linked receptors. It is likely that the observed changes in receptor specificity contributed not only to the increased initial ability of the virus to infect and spread in the lung bronchiolar epithelium (18, 24) but also to the broader tissue tropism in other major mouse organs, which would facilitate consequent pneumonia, systemic spread, and death of infected animals.

The HA protein is a primary determinant for mouse lung virulence, and a number of studies have shown that increase in virulence could be controlled by the changes in important protein functions due to altered biological properties of the virus (48). The HA molecule mediates the attachment of influenza virus to the host cell sialyloligosaccharide receptor via a receptor binding site, which determines the viral binding specificity (50). HA undergoes the cleavage processing that is essential for infectivity of influenza virus particles (49, 50). This permits fusion of the viral envelope with the secondary endosome, a process activated by conformational changes in HA at the acidified pH (50). Previously, it was shown that one of the common structural mechanisms important for acquisition of mouse virulence could be the loss of glycosylation sites, which,

as a consequence, affects the HA receptor binding specificity (30, 48), the pH of HA-catalyzed fusion (4, 42), and/or the ability of mouse serum β inhibitors to inhibit hemagglutination (12, 48). However, these general pathways for adaptation might be achieved not only by the removal of carbohydrate but also by other, yet unidentified structural factors.

Due to the lack of suitable recombinant H1N1 viruses carrying the single HA mutations identified here, it is not yet known which substitutions or combinations of substitutions are necessary for affecting the receptor specificity properties of novel H1N1 viruses and, as a consequence, pathogenesis. However, having two mouse-adapted variants, A/TN/1-560/09-MA1 and A/TN/1-560/09-MA2, which differed from each other only by the presence of the K119N HA mutation associated with the acquisition of a potential glycosylation site, made possible the examination of the role of this particular substitution. Whether or not the HA protein of the A/TN/1-560/09-MA1 virus had acquired an oligosaccharide chain, the K119N change located at the outer region of the HA globular head affected the protein structure such that the binding to α 2,3 sialyl receptors became possible. The K119N HA mutation was also sufficient to significantly reduce α 2,6 binding, compared with the R221K amino acid substitution observed in the A/TN/1-560/09-MA2 variant. Interestingly, our findings also indicated that adaptation of novel H1N1 viruses was accompanied by changes in antigenic reactivity as a result of acquired HA mutations, but not in the elevation of pH for membrane fusion (all H1N1 viruses caused membrane fusion at a pH of \sim 5.6 [data not shown]). However, it would seem likely that the changes in antigenic specificity were not a result of host-induced modification but rather occurred independently of the selection of variants with increased virulence for mice.

We also observed that mutations at four points of the RNP complex, namely, PB2 E158G/A, PA L295P, NP D101G, and NP H289Y, were involved in the mouse adaptation of pandemic H1N1 isolates. Previous studies have shown that the native RNP is a helical structure formed by the association of multiple monomers of NP protein and a single copy of the RNA-dependent RNA polymerase, a heterotrimer composed by the PB1, PB2, and PA subunits (31, 32). Such RNP complexes are independent molecular machines responsible for transcription and replication of each influenza virus gene in the nucleus of infected cells. Here, we found that, of the four mutations that occurred in the RNP complex during adaptation, only substitutions in the PB2 and PA proteins were sufficient to significantly enhance the transcription and replication activity of polymerase complexes of H1N1 influenza viruses. This characteristic resulted in an increased ability to replicate *per se*, which likely was necessary for optimized interaction of the viral polymerase with the host proteins and which allowed the virus to circumvent the host defense barriers, facilitating efficient virus spread and replication in the lower respiratory tract. Thus, our findings confirm and underline that host factors are important for the polymerase action of influenza virus (8).

From the analysis of virus yields in eggs and cell culture and survival of mice infected with the A/CA/04/09-MA1 and A/CA/04/09-MA2 viruses, which differed only by the PB2 E158G mutation, we surmised that the acquisition of this particular substitution provided a replicative advantage both *in vitro* and

in vivo that enabled the pandemic H1N1 virus to become more virulent. Importantly, an amino acid change of the same residue (residue 158) occurred in two pandemic strains in two independent experiments, indicating that this event is not a chance of fluctuation; rather, it reflects a specific character of the variation, most likely connected with identifying one of the strong determinants of virulence and replication of pandemic H1N1 viruses in mice. Furthermore, PB2 E158G/A and PA L295P are localized within the PB1-binding site and the near nuclear localization signal, respectively (32); the NP D101G and NP H289Y substitutions are located within the strands of the NP body domain, which interacts with some cellular or viral proteins (31). Therefore, given the locations of the mutations reported here, they appear to implicate the central processes of influenza virus replication and sites of interaction with host and viral components.

In conclusion, in this study, we identified genes that controlled the virulence of pandemic A/CA/04/09 (H1N1) and A/TN/1-560/09 (H1N1) viruses in a mouse animal model, together with their role in mouse pathogenesis. Our findings showed that increased virulence of pandemic 2009 isolates, seen clinically as increased disease, was associated with accelerated viral growth in mammalian cells and extended tissue tropism *in vivo*. Our results add to the growing body of mutational analysis data defining the structural and functional changes governing new host adaptation, which may provide insights into the development of effective therapeutic intervention strategies. Our findings also suggest that changes optimizing receptor specificity and interaction of viral polymerase components with host cellular factors are the major means of pandemic influenza virus achieving an optimal competitive advantage in a mammalian host. These modulators of virulence, therefore, may have been the driving components of early evolution, which paved the way for pandemic H1N1 influenza A viruses in mammals, including humans.

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