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Virulence Determinants in the PB2 Gene of a Mouse-Adapted H9N2 Virus

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The molecular bases of adaptation and pathogenicity of H9N2 influenza virus in mammals are largely unknown. Here, we show that a mouse-adapted PB2 gene with a phenylalanine-to-leucine mutation (F404L) mainly contributes to enhanced polymerase activity, replication, and pathogenicity of H9N2 in mice and also increases the virulence of the H5N1 and 2009 pandemic H1N1 influenza viruses. Therefore, we defined a novel pathogenic determinant, providing further insights into the pathogenesis of influenza viruses in mammals.

H9N2 avian influenza viruses (AIVs) have been circulating worldwide in multiple avian species and are endemic in poultry populations across Eurasia (1–7). It is noteworthy that H9N2 AIVs have been transmitted from chickens directly to humans and other mammals (8–12), posing a significant threat to public health. Therefore, it is necessary to investigate the molecular basis for pathogenicity of H9N2 AIVs in mammals. We previously passaged a nonpathogenic H9N2 AIV (DK1) in mice and obtained a highly virulent virus (DK1-MA) with eight mutations (PB2-F404L, PA-D3V, PA-S225R, HA-L80F, HA-N193D, NP-V105M, NA-A27T, and M1-A166V) (13). It was not clear, however, from that study which mutations contributed to the high virulence of DK1-MA. Therefore, here we have determined the major genetic determinants associated with the increased pathogenicity of DK1-MA and investigated the contribution of the DK1-MA PB2 to the virulence of highly pathogenic avian influenza (HPAI) H5N1 and pandemic influenza H1N1/2009 (pH1N1/2009) viruses. All experiments involving live viruses were carried out in a biosafety level 3 (BSL3) facility at Yangzhou University, in accordance with the institutional biosafety manual. Animals were housed in negative-pressure isolators with HEPA filters in the BSL3 facility.

Using an eight-plasmid system, we rescued this pair of recombinant viruses (rDK1-MA and rDK1) and generated a set of recombinant viruses by exchanging individual gene between DK1 and DK1-MA (14, 15). We evaluated the pathogenicity of all recombinants by determining their 50% mouse lethal dose (MLD_{50}) values and replication in mice, as previously described (13). rDK1-MA was highly pathogenic to mice, with an MLD_{50} of 2.5 $\log_{10}$ 50% egg infectious doses (EID_{50}), whereas the MLD_{50} of rDK1 was above 7.17 $\log_{10}$ EID_{50} (Fig. 1A). Moreover, rDK1-MA grew to higher titers than rDK1 in mouse lungs and spread sporadically to other organs, whereas rDK1 virus replicated only in the lung (Table 1), indicating that the rescued viruses maintained the same biological properties as the wild-type viruses (13). We then found that the PB2 subunit of the influenza virus polymerase (PB2), the PA subunit of the polymerase (PA), and hemagglutinin (HA) of DK1-MA increased the virulence of DK1, with MLD_{50} values of 3.37, 4.5, and 6.5 $\log_{10}$ EID_{50}, respectively. Moreover, the DK1-MA PB2 and PA caused significant weight loss ($P < 0.05$) as early as days 3 and 5 postinoculation (p.i.) at a dose of 10^5 EID_{50}, and the animals had >25% weight loss by days 6 and 9 p.i. (Fig. 1B). Similarly, viruses carrying DK1-MA PB2 or PA (DK1/MAPB2 and DK1/MAPA) replicated to significantly higher titers than did DK1 in mouse lungs at days 3 and/or 5 postinfection and sporadically spread to the heart and spleen, whereas DK1/MAHA replicated only in mouse lungs and produced viral titers that were not significantly different from those for DK1. The multibasic amino acids in the HA cleavage site play a key role in the virulence and systemic infection potential of influenza virus, especially for the H5 and H7 avian influenza viruses. However, some mouse-adapted influenza viruses, such as H1N1 and H9N2, which do not have multibasic amino acids, also are highly pathogenic and can result in systemic infection in mice, as observed with the mouse-adapted H9N2 virus (PARSSR/G) in this study. Here, the MA virus and recombinant viruses containing the PA or PB2 from the MA virus could replicate in extrapulmonary tissues, which suggested that extrapulmonary replication correlates with the ribonucleoprotein (RNP) polymerase. Moreover, the extrapulmonary replication of virus containing the PB2-MA was higher than that with the PA, so we suggest that the PB2 404L mutation mainly contributes to this phenotype.

Inclusion of the PB2 or HA of DK1 in the DK1-MA background resulted in attenuation of the recombinant viruses (MLD_{50}, 4.17 and 3.62 EID_{50}), with greater attenuation observed with virus harboring the DK1 PB2 gene (47-fold increase in the MLD_{50}). Although all six recombinant viruses containing the single gene for DK1 caused significant weight loss ($P < 0.05$), the MA/DK1 PB2 and MA/DK1 HA viruses caused lower rates of weight loss than other recombinants or DK1-MA (Fig. 1C). Addi-

Received 21 June 2014 **Accepted** 15 October 2014

Accepted manuscript posted online 22 October 2014

Citation Liu Q, Huang J, Chen Y, Chen H, Li Q, He L, Hao X, Liu J, Gu M, Hu J, Wang X, Hu S, Liu X, Liu X. 2015. Virulence determinants in the PB2 gene of a mouse-adapted H9N2 virus. J Virol 89:877–882. doi:10.1128/JVI.01775-14.

Editor: A. García-Sastre

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doi:10.1128/JVI.01775-14

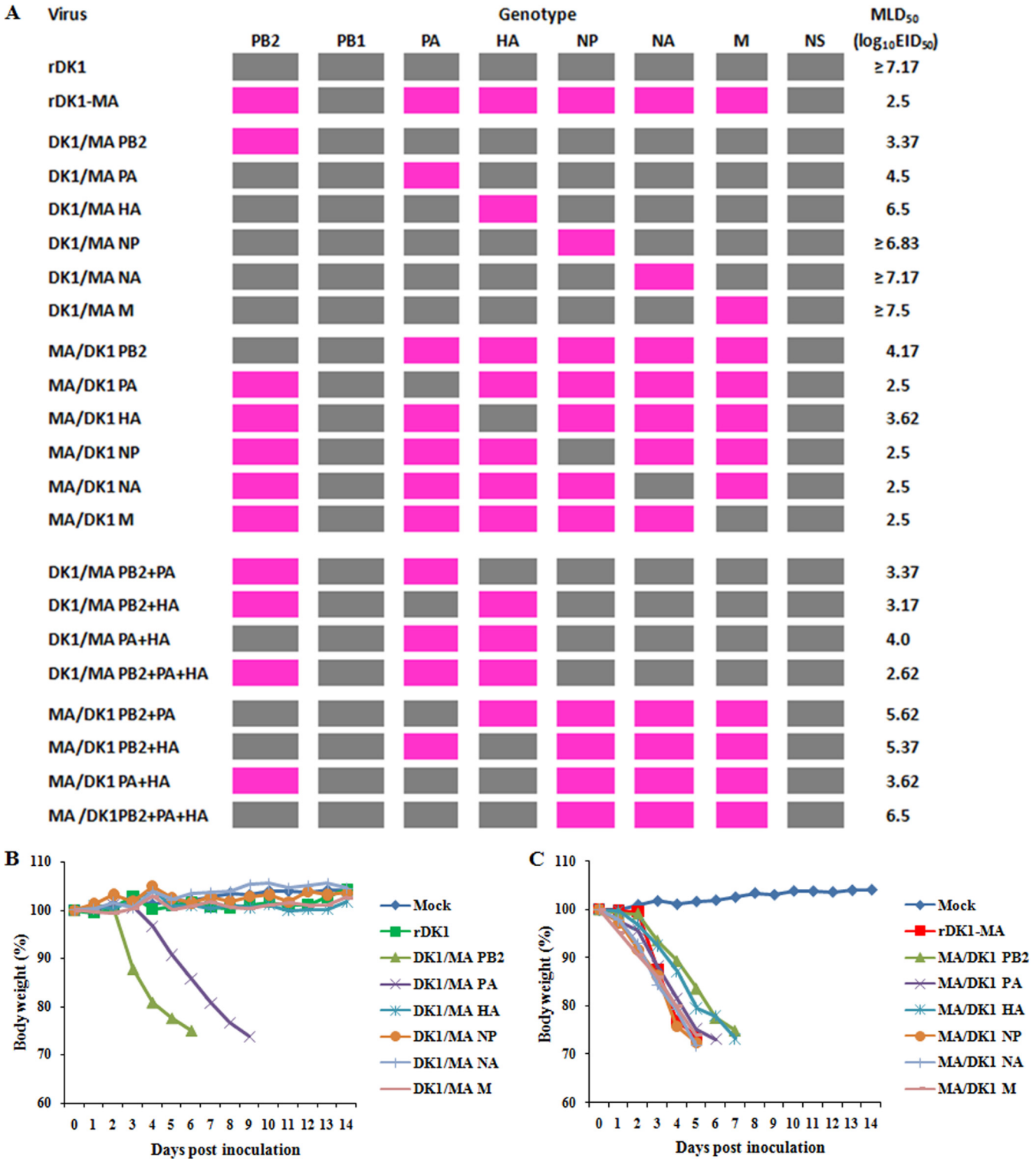

FIG 1 Virulence of the reassortant viruses in mice. The reassortants were generated by exchanging genes between DK1 and DK1-MA viruses. DK1/MA, reassortant DK1 virus carrying the indicated gene of DK1-MA virus, and vice versa. (A) The MLD_{50} was determined by inoculating groups of five 6-week-old female BALB/c mice with 10-fold serial dilutions of the stock reassortant viruses in a 30-μl volume, and result was expressed as the $\log_{10}$ EID_{50}. Gene segments derived from DK1 and DK1-MA viruses are shown in gray and pink, respectively. (B and C) The morbidities caused by single-gene reassortants in the DK1 (B) and DK1-MA (C) backgrounds at a dose of 10^5 EID_{50} were evaluated by monitoring weight changes over a 14-day period and are graphed as the percentage of the animals' body weights on the day of inoculation (day 0). The results are average body weights for each group ($n = 5$).

TABLE 1 Replication of reassortant viruses in mice[a]

Virus	Mean titer (log EID_{50}/ml) ± SD		No. of mice with infection in organ/total no. of mice (titer[s] in individual positive mice)							
	Lung		Heart		Liver		Spleen		Kidney	
	3 dpi	5 dpi	3 dpi	5 dpi	3 dpi	5 dpi	3 dpi	5 dpi	3 dpi	5 dpi
rDK1	5.7 ± 0.4	6.2 ± 0.4	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
rDK1-MA	8.5 ± 0.3	8.0 ± 0.5	2/3 (1.3, 2.3)	2/3 (2.3, 3.5)	0/3	1/3 (2.8)	1/3 (2.0)	1/3 (1.5)	0/3	1/3 (1.8)
DK1/MAPB2	8.1 ± 0.4[b]	7.6 ± 0.3[b]	1/3 (2.5)	2/3 (2.3, 2.9)	0/3	0/3	1/3 (1.5)	1/3 (1.5)	0/3	0/3
DK1/MAPA	6.5 ± 0.4[c]	6.3 ± 0.7	1/3 (2.5)	1/3 (2.5)	0/3	0/3	1/3 (1.5)	0/3	0/3	0/3
DK1/MAHA	6.3 ± 0.4	6.3 ± 0.3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
DK1/MANP	5.5 ± 0.7	5.4 ± 0.4	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
DK1/MANA	5.2 ± 0.3	5.4 ± 0.4	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
DK1/MAM	5.3 ± 0.4	5.7 ± 0.5	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
MA/DK1 PB2	7.3 ± 0.3[b]	7.3 ± 0.5	0/3	1/3 (2.3)	0/3	0/3	0/3	0/3	0/3	0/3
MA/DK1 PA	8.1 ± 0.6	7.5 ± 0.4	1/3 (2.0)	1/3 (0.8)	1/3 (1.3)	0/3	2/3 (1.3, 1.9)	1/3 (0.8)	0/3	1/3 (1.3)
MA/DK1 HA	8.2 ± 0.5	7.4 ± 0.3	2/3 (2.5, 3.3)	2/3 (1.3, 3.3)	0/3	1/3 (1.3)	1/3 (1.3)	1/3 (0.8)	0/3	1/3 (1.5)
MA/DK1 NP	8.3 ± 0.5	8.3 ± 0.4	2/3 (1.2, 1.8)	3/3 (3.8 ± 0.3)[d]	0/3	1/3 (2.0)	1/3 (1.3)	1/3 (1.8)	0/3	1/3 (2.0)
MA/DK1 NA	7.5 ± 0.5	8.3 ± 0.5	1/3 (0.8)	3/3 (3.1 ± 1.0)	1/3 (2.3)	2/3 (2.3, 4.3)	1/3 (1.8)	1/3 (1.3)	0/3	2/3 (1.3, 3.3)
MA/DK1 M	8.1 ± 0.5	8.2 ± 0.8	2/3 (3.0, 3.4)	3/3 (3.7 ± 0.4)	1/3 (2.5)	0/3	1/3 (0.8)	0/3	0/3	1/3 (1.8)
DK1/MA PB2+PA	8.3 ± 0.3[b]	8.0 ± 0.5[b]	1/3 (1.8)	2/3 (1.3, 3.5)	0/3	0/3	0/3	1/3 (2.3)	0/3	0/3
DK1/MA PB2+HA	8.4 ± 0.6[b]	7.5 ± 0.9[c]	1/3 (2.0)	2/3 (2.3, 3.3)	0/3	0/3	0/3	1/3 (1.8)	1/3 (2.3)	0/3
DK1/MA PA+HA	7.8 ± 0.8[b]	7.5 ± 0.5[c]	0/3	1/3 (2.3)	0/3	0/3	1/3 (2.0)	0/3	0/3	0/3
DK1/MA PB2+PA+HA	8.5 ± 0.3[b]	7.8 ± 0.3[b]	0/3	3/3 (2.3 ± 0.4)	0/3	1/3 (1.3)	0/3	1/3 (1. 5)	1/3 (1.8)	0/3
MA/DK1 PB2+PA	6.0 ± 0.3[b]	7.3 ± 0.7	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
MA/DK1 PB2+HA	6.5 ± 0.4[b]	7.3 ± 0.9	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
MA/DK1 PA+HA	7.5 ± 0.7[c]	7.0 ± 0.9	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
MA/DK1 PB2+PA+HA	6.3 ± 0.7[b]	7.3 ± 0.3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

[a] Groups of mice were infected with 10^5 EID_{50} of each virus. Three mice from each group were euthanized, and the lungs, heart, liver, spleen, and kidneys were collected and homogenized in 1 ml of phosphate-buffered saline for virus titration in eggs at 3 and 5 days p.i. The lower limit of detection was $10^{0.8}$ EID_{50}/ml for these samples. dpi, days postinoculation.

[b] Significantly different ($P < 0.01$) from titers in lungs of mice that received the corresponding parental virus.

[c] Significantly different ($P < 0.05$) from titers in lungs of mice that received the corresponding parental virus.

[d] When all three animals in a treatment group had positive virus titration results for an organ, the mean ± standard deviation is reported in parentheses (rather than results for individual animals).

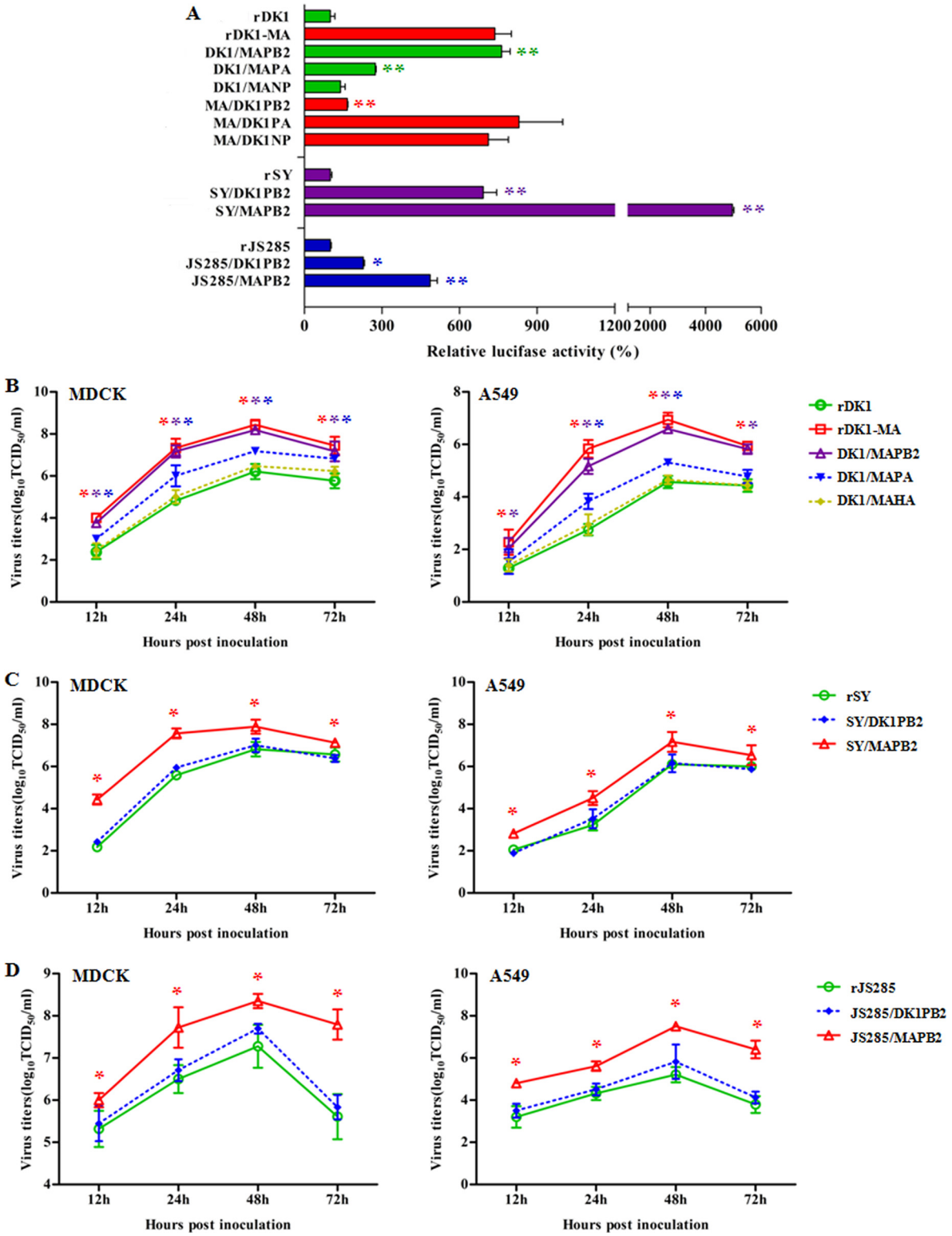

FIG 2 Viral polymerase activity and growth kinetics. (A) Polymerase activities of the viral RNP complex in a minigenome assay. The luciferase reporter plasmid p-Luci and internal control plasmid *Renilla* were transfected into 293T cells, together with PB2, PB1, PA, and NP plasmids. After 24 h, cell lysates were used to measure firefly and *Renilla* luciferase activities. The values shown are means ± standard deviations of results for three independent experiments and were standardized to the activity of rDK1, rSY, or rJS285 (as 100%), respectively. The value of each reassortant virus was compared with that of the corresponding parental virus. *, $P < 0.05$; **, $P < 0.01$. (B to D) Viral growth kinetics in MDCK and A549 cells. Cells were inoculated at a multiplicity of infection of 0.01, and virus titers in supernatants were determined as the number of 50% tissue culture infectious doses ($TCID_{50}$) per ml in MDCK cells at the indicated time points. Averages of triplicate experiments are shown, with error bars representing the standard deviations. *, the value for the corresponding virus was significantly different ($P < 0.05$, based on analysis of variance) from that of the rDK1 (B), rSY (C), or rJS285 (D) virus, respectively.

tionally, the replication of virus bearing DK1 PB2 (MA/DK1PB2) was impaired in mice. To further determine the synergistic effects of PB2, PA, and HA on pathogenicity, we generated an additional eight recombinants by exchanges of genes in various combinations (PB2 plus PA, PB2 plus HA, PA plus HA, and PB2 plus PA plus HA). In mice, recombinant viruses derived from the DK1 backbone showed higher virulence and levels of replication than the corresponding single-gene reassortants. Conversely, recombinant viruses derived from the DK1-MA backbone displayed decreased virulence and replication in mice. These results indicated that mutations in PB2, PA, and HA contribute to the high pathogenicity of the DK1-MA virus in mice and that the mutation F404L in the PB2 gene plays a pivotal role.

To determine the correlation of viral polymerase activity and replication with pathogenicity, the activities of the reconstituted RNP were assessed in a minigenome assay, and the replication kinetics of rDK1, rDK1-MA, DK1/MAPB2, DK1/MAPA, and DK1/MAHA were determined in MDCK and A549 cells, as described previously (16). DK1-MA PB2 increased the polymerase activity of DK1 to a similar extent (736%) as DK1-MA (762%) (Fig. 2A). However, DK1-MA PA increased polymerase activity to 274%, whereas DK1-MA NP did not. Conversely, activity of DK1-MA was significantly decreased by the substitution of DK1 PB2, whereas DK1-PA and DK1-NP did not exhibit such decreases. The rDK1-MA and DK1/MAPB2 viruses grew faster and to significantly higher titers than rDK1 in either MDCK or A549 cells at each time point (Fig. 2B). The viral titers of DK1/MAPA were also significantly higher than that of rDK1 at each time point in MDCK cells and at 24 and 48 h p.i. in A549 cells. However, titers of DK1/MAHA versus rDK1 were not significantly different in MDCK or A549 cells. Collectively, these findings stress the importance of the mouse-adapted PB2 gene for increased viral polymerase activity and replication of DK1-MA *in vitro*, which correlate with its enhanced replication *in vivo*.

We studied the influence of the DK1-MA PB2 gene on the virulence of HPAI H5N1 and pH1N1/2009 viruses in mice by generating recombinants in which the DK1 or DK1-MA PB2 gene was introduced into the background of an H5N1 virus, A/mallard/Huadong/S/2005 (rSY) (17), and a pH1N1/2009 virus, A/Swine/Jiangsu/285/2010 (rJS285) (18). Upon introducing the DK1-MA PB2 gene into the rSY and rJS285 backbones, the pathogenicities of both viruses changed from medium to high, whereas the DK1 PB2 gene did not cause changes in pathogenicity (Table 2). In addition, the DK1-MA PB2 gene significantly enhanced the polymerase activity and replication of rSY and rJS285 in mouse lungs and *in vitro* (Fig. 2C and D). By contrast, the DK1 PB2 gene did not significantly enhance the replication of rSY or rJS285 *in vivo* and or *in vitro*, although activities of both viral polymerases were significantly increased to minor extents. Therefore, the mutation F404L in the PB2 gene of DK1 also contributed to the enhanced virulence of H5N1 and pH1N1/2009 viruses in this study. Based on available sequences in GenBank, we found that a few natural H9N2 and H5N1 AIVs strains also contain the 404L mutation in the PB2 gene, although all strains are avian isolates, which indicates that future molecular epidemiological surveillance of influenza virus reassortants possessing the AIV PB2 segments with L at position 404 is warranted. However, the functional role of the PB2 404L mutation is not fully understood, and additional studies are needed to determine the characteristic role of the PB2 404L mutation on polymerase activity, i.e., whether it directly affects rep-

TABLE 2 Virulence and replication of H5N1 and 2009 pH1N1 recombinant viruses in mice

Virus	MLD_{50} (log_{10} EID_{50})	Viral titer (mean log_{10} EID_{50} ± SD)[a]											
		Lung		Heart		Liver		Spleen		Kidney		Brain	
		3 dpi	5 dpi	3 dpi	5 dpi	3 dpi	5 dpi	3 dpi	5 dpi	3 dpi	5 dpi	3 dpi	5 dpi
rSY	3.62	6.75 ± 0.25	7 ± 0.25	3.67 ± 0.63	4.17 ± 0.63	2.42 ± 0.38	2.75 ± 0.50	4.25 ± 0.25	4.67 ± 0.63	2.42 ± 0.38	4.33 ± 0.76	2.5 ± 0.25	4.25 ± 0.66
SY/DK1PB2	3.5	6.67 ± 0.29	7.33 ± 0.38	3.42 ± 0.14	4.33 ± 0.14	2.25 ± 0.50	2.58 ± 0.63	3.83 ± 0.38	4.67 ± 0.38	2.42 ± 0.14	3.58 ± 0.88	2.58 ± 0.88	3.5 ± 0.50
SY/MAPB2	0.5	7.42 ± 0.29[b,c]	7.67 ± 0.14[b]	3.33 ± 0.38	4.58 ± 0.38	2.58 ± 0.38	3.25 ± 0.25	4.67 ± 0.63	4.5 ± 0.50	2.67 ± 0.88	3.75 ± 0.50	2.75 ± 0.75	3.75 ± 0.50
rJS285	5.5	6.58 ± 0.14	6.5 ± 0.66	—[f]	—	—	—	—	—	—	—	—	—
JS285/DK1PB2	5.36	7.08 ± 0.14	6.83 ± 0.14	—	—	—	—	—	—	—	—	—	—
JS285/MAPB2	1.83	7.75 ± 0.43[d,e]	7.58 ± 0.14[d,e]	—	—	—	—	—	—	—	—	—	—

[a] Groups of mice were infected with 10^5 EID_{50} of each virus. Three mice from each group were euthanized, and the lungs, heart, liver, spleen, kidneys, and brain were collected and homogenized in 1 ml of phosphate-buffered saline for virus titration in eggs at 3 and 5 days p.i. (dpi). The lower limit of detection was $10^{0.8}$ EID_{50}/ml for these samples.

[b] Significantly different ($P < 0.05$) from titers in the corresponding organs of rSY-infected mice.

[c] Significantly different ($P < 0.05$) from titers in the corresponding organs of SY/DK1PB2-infected mice.

[d] Significantly different ($P < 0.05$) from titers in the corresponding organs of rJS285-infected mice.

[e] Significantly different ($P < 0.05$) from titers in the corresponding organs of JS285/DK1PB2-infected mice.

[f] —, no virus was isolated from the sample.

lication, transcription, or both, and the mechanistic basis for how this mutation confers polymerases with the ability to efficiently synthesize RNA.

In summary, we demonstrated here that mutations in PB2, PA, and HA contribute to the high virulence of DK1-MA in mice and that a mutation in the PB2 gene (F404L) is a major virulence determinant. Moreover, the DK1-MA PB2 significantly increased the polymerase activity and replication of DK1, with values similar to DK1-MA, which supports the viewpoint that overwhelming viral replication and polymerase activity contribute to the adaptation and high pathogenicity of AIVs in mice (19–21). Reassortment is an important mechanism for the evolution of influenza viruses and has resulted in the viruses involved in the human influenza pandemics of 1957, 1968, and 2009 (22, 23) and the infection of humans with H5N1 and H7N9 AIVs (24–27), respectively. Therefore, we introduced the DK1-MA PB2 gene in the backbone of H5N1 and pH1N1/2009 viruses and, surprisingly, found that this gene also enhanced the pathogenicity of the two viruses. In addition, the PB2 gene of DK1 did not decrease the pathogenicity of the H5N1 or pH1N1/2009 virus, which indicates that DK1 PB2 has a high degree of genetic compatibility with the two viruses.

ACKNOWLEDGMENTS

This work was supported by the Earmarked Fund for Modern Agro-Industry Technology Research System (grant number nycytx-41-G07), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the National Natural Science Foundation of China (grant number 31101827), and the National High-Tech R&D Program of China (863 Program; grant number 2011AA10A200).

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