PB2 Protein of a Highly Pathogenic Avian Influenza Virus Strain A/chicken/Yamaguchi/7/2004 (H5N1) Determines Its Replication Potential in Pigs[▽]

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It has been shown that not all but most of the avian influenza viruses replicate in the upper respiratory tract of pigs (H. Kida et al., J. Gen. Virol. 75:2183–2188, 1994). It was shown that A/chicken/Yamaguchi/7/2004 (H5N1) [Ck/Yamaguchi/04 (H5N1)] did not replicate in pigs (N. Isoda et al., Arch. Virol. 151:1267–1279, 2006). In the present study, the genetic basis for this host range restriction was determined using reassortant viruses generated between Ck/Yamaguchi/04 (H5N1) and A/swine/Hokkaido/2/1981 (H1N1) [Sw/Hokkaido/81 (H1N1)]. Two in vivo-generated single-gene reassortant virus clones of the H5N1 subtype (virus clones 1 and 2), whose PB2 gene was of Sw/Hokkaido/81 (H1N1) origin and whose remaining seven genes were of Ck/Yamaguchi/04 (H5N1) origin, were recovered from the experimentally infected pigs. The replicative potential of virus clones 1 and 2 was further confirmed by using reassortant virus (rg-Ck-Sw/PB2) generated by reverse genetics. Interestingly, the PB2 gene of Ck/Yamaguchi/04 (H5N1) did not restrict the replication of Sw/Hokkaido/81 (H1N1), as determined by using reassortant virus rg-Sw-Ck/PB2. The rg-Sw-Ck/PB2 virus replicated to moderate levels and for a shorter duration than parental Sw/Hokkaido/81 (H1N1). Sequencing of two isolates recovered from the pigs inoculated with rg-Sw-Ck/PB2 revealed either the D256G or the E627K amino acid substitution in the PB2 proteins of the isolates. The D256G and E627K mutations enhanced viral polymerase activity in the mammalian cells, correlating with replication of virus in pigs. These results indicate that the PB2 protein restricts the growth of Ck/Yamaguchi/04 (H5N1) in pigs.

Influenza A viruses have been isolated from a variety of species, including humans, birds, pigs, horses, minks, seals, whales, cats, dogs, and tigers (23, 50, 51, 55). Indeed, influenza A viruses exhibit a restricted host range with efficient replication in their natural hosts and poor or no replication in other host species (3, 12, 13, 35); however, influenza viruses may cross this species barrier. Interspecies transmission of human, swine, and avian influenza viruses has been documented on several occasions (4, 6, 36, 54). The causative viruses of both the 1957 (Asian) and the 1968 (Hong Kong) pandemics were reassortant viruses which acquired the polymerase basic protein 1 (PB1), hemagglutinin (HA), and neuraminidase (NA) genes and the PB1 and HA genes, respectively, from avian influenza viruses (22, 26, 45, 56, 58). The role of pigs in the generation of new influenza viruses is well documented (25). It was shown that the H3 HA gene of the Hong Kong pandemic strain A/Hong Kong/1968 (H3N2) was of a migratory duck origin and was acquired as a result of reassortment with the precedent human H2N2 influenza virus in pigs (26, 58). Furthermore, avian-human reassortant viruses were isolated from Italian pigs (4), and those isolated from children in The Neth-

erlands in 1993 were found to be avian-human reassortants circulating in pigs in Europe (6). These findings indicate that pigs can support the growth of both avian and human influenza viruses and are therefore termed “mixing vessels” (44). Nevertheless, not all influenza viruses replicate in pigs, as demonstrated by Kida et al. (25) in a study of the replication potential of 38 different H1 to H13 subtypes of avian influenza viruses.

The molecular bases for influenza virus host-range restriction and adaptation to a new host species are poorly understood. The first host range barrier is offered at the cell surface where receptor-mediated entry into cells starts (20). After cell entry, a second level of host range barrier is offered where the interaction between viral and cellular proteins takes place. In addition to surface glycoproteins, influenza virus internal proteins also harbor determinants for host range and virulence (7, 29, 53). Among these internal proteins, PB2 is a well-documented component of the viral polymerase complex required for virus replication. The PB2 protein has been shown to be involved in host range restriction and pathogenicity (1, 52).

In late December 2003, there was an influenza outbreak in a layer chicken farm in Yamaguchi Prefecture, Japan. The causative agent was identified as the highly pathogenic avian influenza virus A/chicken/Yamaguchi/7/2004 (H5N1) [Ck/Yamaguchi/04 (H5N1)] (32). This virus was shown to be highly pathogenic to chickens, quails, budgerigars, and ducklings and less virulent for mice, while miniature pigs were resistant to infection with the virus (19). This virus offers a good subject with which to study the mechanism underlying interspecies

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transmission to a new host. The classical swine influenza viruses or avian-human reassortant viruses have been reported to be circulating in pigs in Europe and Asia (4, 6, 11). These viruses can contribute genes to viruses like Ck/Yamaguchi/04 (H5N1) and enable them to replicate in new host species, thereby facilitating the interspecies transmission. Therefore, the present study was conducted to address the molecular basis of restricted replication of Ck/Yamaguchi/04 (H5N1) in pigs by using classical swine influenza virus, A/swine/Hokkaido/2/1981 (H1N1) [Sw/Hokkaido/81 (H1N1)].

MATERIALS AND METHODS

Cells and viruses. Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (Nissui, Japan) supplemented with 5% calf serum. Human embryonic kidney cells (293T) were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum. Both cell lines were maintained at 37°C in a 5% CO₂ atmosphere. Two viruses (designated parent viruses) were used in this study. Sw/Hokkaido/81 (H1N1), a classical swine influenza virus (42), was obtained from the virus repository of our laboratory, while Ck/Yamaguchi/04 (H5N1), a highly pathogenic avian influenza virus, was provided by the National Institute of Animal Health, Ibaraki, Japan (32).

All viruses in the present study were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C. Before the infectious allantoic fluid was harvested, the eggs were chilled at 4°C overnight, and the harvested allantoic fluid was stored at -80°C until use.

Experimental infection of pigs. Three- to four-week-old, crossbred (Landrace × Duroc × Yorkshire) specific pathogen-free pigs, free of antibodies against influenza A viruses (Takikawa swine station, Hokkaido, Japan), were housed in the biosafety level 3 facility of the Graduate School of Veterinary Medicine, Hokkaido University, Japan. The serum antibody titers against influenza A viruses were determined by enzyme-linked immunosorbent assay (24).

The pigs were inoculated intranasally with 500 µl of infectious allantoic fluid containing 10^{7.0} to 10^{7.5} 50% egg infectious doses (EID₅₀) of viruses, except for Ck/Yamaguchi/04 (H5N1). The Ck/Yamaguchi/04 (H5N1) strain was inoculated intranasally with 500 µl of infectious allantoic fluid containing 10^{8.4} EID₅₀ of virus. The nasal swabs were collected either for 7 days postinoculation (p.i.) from pigs inoculated with infectious allantoic fluid prepared from coinoculated eggs or for 10 days p.i. from pigs inoculated with other viruses used in this study. The nasal swabs were collected in 1 ml of virus transport medium (30). Preinoculation blood samples and blood sampled at 14 days p.i. for serum were collected, and antibody titers were determined using enzyme-linked immunosorbent assay (24). The infectivity titers of the different viruses in the nasal swabs of pigs were calculated in embryonated chicken eggs by the 50% end-point method (41) and were expressed as EID₅₀/ml of swab.

All animal experiments were conducted in accordance with the guidelines of the institutional animal care and use committee of Hokkaido University, Japan.

In vivo selection of H5N1 reassortant viruses, generated between Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1) capable of replication in pigs. The virus inoculum containing reassortant viruses was produced by coinoculation of 10-day-old embryonated chicken eggs with 100 µl of inoculum containing Ck/Yamaguchi/04 (H5N1) (10^{7.4} EID₅₀/50 µl) and Sw/Hokkaido/81 (H1N1) (10^{3.0} EID₅₀/50 µl) viruses. The harvested infectious allantoic fluid was used as the inoculum for pigs, to select the H5N1 reassortant viruses capable of replication in the pigs. The inoculum contained parental H5N1, H1N1, and reassortant viruses. The nasal swabs were collected for 7 days p.i. and were used for selecting virus clones by plaque cloning.

Virus clones were selected from nasal swabs by plaque cloning on MDCK cells as described by Kida et al. (25). Individual virus clones were selected and propagated in 10-day-old embryonated chicken eggs at 35°C. The eggs were chilled at 4°C overnight, and allantoic fluid was harvested. The HA subtype of virus clones was determined by hemagglutination inhibition assay (46).

Evaluation of replicative potential of H5N1 subtype virus clones recovered from pigs. Eleven H5N1 subtype virus clones were isolated by plaque cloning. All gene segments of these virus clones were amplified and partially sequenced. It was found that two virus clones were single-gene reassortants, while the gene constellation of the remaining nine virus clones was like that of parental Ck/Yamaguchi/04 (H5N1) virus. For the determination of their replicative potential, two single-gene reassortant virus clones and two virus clones of the Ck/Yamaguchi/04 (H5N1)-like gene constellation were reinoculated into pigs. Nasal swabs

were collected for 10 days p.i., and infectivity titers were measured as described above.

Generation of viruses by reverse genetics. Eight genes from each of the Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1) viruses were cloned to produce viruses by reverse genetics (rg) as described by Hoffmann et al. (14). In brief, the RNA of viruses was extracted using TRI reagent LS (Sigma). The cDNAs were amplified by reverse transcription of viral RNA, using Uni 12 primer (5'-AGC AAA AGC AGG-3'). Full-length genes of Ck/Yamaguchi/04 and Sw/Hokkaido/81 were amplified by using gene-specific universal primer sets (17). The amplified genes were then sequenced using a GenomeLab DTCS Quick Start kit (Beckman Coulter) according to the manufacturer's instructions and analyzed with a CEQ 2000XL sequencer (Beckman Coulter). The amplified genes were first cloned into the pCR 2.1 TOPO cloning vector (Invitrogen) and then into the pHW2000 expression vector (kindly provided by E. Hoffmann, St. Jude Children's Research Hospital), except for the PA, HA, and NA genes of Ck/Yamaguchi/04 (H5N1) and all eight genes of Sw/Hokkaido/81 (H1N1), which were directly cloned into the pHW2000 expression vector. Genes cloned into pCR 2.1 TOPO or pHW2000 were sequenced, and only those clones with sequences identical to the consensus sequence were selected. Ligation of the genes into the pHW2000 expression vector was carried out using a DNA ligation kit (version 2.1; Takara, Japan) according to the manufacturer's instructions.

For generating viruses by reverse genetics using eight plasmids, MDCK and 293T cells were used as described previously (16). The rg-Ck/Yamaguchi/04 (H5N1) and rg-Sw/Hokkaido/81 (H1N1) viruses were inoculated into pigs to compare their potential to replicate in pigs with that of parental viruses. The rg-Ck-Sw/PB2 virus [the PB2 gene from Sw/Hokkaido/81 (H1N1) and seven genes from Ck/Yamaguchi/04 (H5N1)] was inoculated into pigs to evaluate the replicative behavior of virus clones 1 and 2 in pigs. The rg-Sw-Ck/PB2 virus [the PB2 gene from Ck/Yamaguchi/04 (H5N1) and seven genes from Sw/Hokkaido/81 (H1N1)] was inoculated into pigs to study the host range-restrictive effect of the PB2 gene of Ck/Yamaguchi/04 (H5N1) on seven genes of Sw/Hokkaido/81 (H1N1) in pigs.

Site-directed mutagenesis. The E627K and D256G mutations were introduced into the PB2 gene of Ck/Yamaguchi/04 (H5N1) cloned into the pHW2000 expression vector, using a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The presence of the desired mutations and the absence of unwanted mutations were confirmed by sequencing the full length of the cloned PB2 genes.

Luciferase assay. The luciferase assay was conducted as described by Salomon et al. (43). The luciferase reporter plasmid (pHW72-Luc) was constructed by replacing the open reading frame of the enhanced green fluorescent protein (EGFP) in the pHW72-EGFP plasmid (kindly provided by R. Webby, St. Jude Children's Research Hospital) with the luciferase gene (15). Sixty percent-confluent 293T cell monolayers (in 12-well tissue culture plates; Nunclon) were transfected with 2 µg of pHW72-Luc, 1 µg of pHW2000-PB2, 1 µg of pHW2000-PB1, 1 µg of pHW2000-PA, and 2 µg of pHW2000-NP, using Trans-IT-293 (Mirus) according to the manufacturer's instructions. After 24 h of transfection, cell extracts were prepared in 250 µl of passive lysis buffer, and luciferase levels were assayed with a dual-luciferase assay system (Promega) using a Lumat LB 9507 (Berthold, Germany) instrument. The results were recorded from two independent experiments, and each experiment was run in triplicate.

RESULTS

Selection of in vivo-generated reassortant viruses capable of replication in pigs. The nasal swabs, collected from pigs intranasally administered inoculum prepared from embryonated chicken eggs coinoculated with Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1), were used for picking virus clones by plaque cloning. A total of 119 virus plaque clones were picked from nasal swabs collected from days 1 to 3 p.i. (Table 1). Of the total, 11 virus clones were of the H5N1 subtype. The H5N1 subtype virus clones were plaque purified on MDCK cells. Partial genome sequencing of these virus clones revealed that two of these (virus clones 1 and 2) were single-gene reassortants deriving the PB2 gene from Sw/Hokkaido/81 (H1N1) virus and the remaining seven genes from Ck/Yamaguchi/04 (H5N1). The remaining 9 virus clones (clones 3 to 11) derived all of their genes from Ck/Yamaguchi/04 (H5N1) (Table 2).

TABLE 1. Recovery and subtyping of virus clones from nasal swab samples

Day postinoculation	Total no. of plaques picked	No. of plaques of HA subtype:	
		H5	H1
1	61	11	50
2	38	— ^a	38
3	20	—	20

^a —, No H5 subtype virus clone was recovered.

Virus clones recovered by in vivo selection in pigs are capable of replication in pigs. Virus clones 1 and 2 [the PB2 gene of Sw/Hokkaido/81 (H1N1) and the remaining seven genes of Ck/Yamaguchi/04 (H5N1) origin] and virus clones 3 and 4 [all eight genes of Ck/Yamaguchi/04 (H5N1) origin] were reinoculated into pigs to assess the virus clones' replicative potential (Table 2). Virus clones 1 and 2 were recovered from nasal swabs, and virus shedding occurred from days 1 to 5 p.i., while virus clones 3 and 4 were not recovered from nasal swabs. The sera collected 14 days p.i. from pigs inoculated with virus clones 1 and 2 showed seroconversion, while sera from pigs inoculated with virus clones 3 and 4 did not show any seroconversion (Table 2). This finding suggested that the PB2 gene of Sw/Hokkaido/81 (H1N1) conferred replicative potential to the reassortant virus possessing the seven genes of Ck/Yamaguchi/04 (H5N1) origin.

The replication potential of rg-Ck-Sw/PB2 is similar to that of virus clones 1 and 2. To confirm the importance of the PB2 gene of Sw/Hokkaido/81 (H1N1) for the replicative potential in pigs, reassortant viruses were produced by reverse genetics. The replicative potential of rg-Ck/Yamaguchi/04 (H5N1) and rg-Sw/Hokkaido/81 (H1N1) was similar to that of the parental viruses (Table 3) (2, 19). A single-gene reassortant virus (rg-Ck-Sw/PB2) possessing a gene constellation like that of virus clones 1 and 2 (Table 2) was generated by reverse genetics and inoculated intranasally into the pigs. The rg-Ck-Sw/PB2 was shed for 2 to 3 days, as were the parent virus clones 1 and 2 (Table 3). This finding further supported the results obtained by reinoculation of in vivo-selected reassortant virus clones into pigs.

Amino acid substitutions found in the PB2 protein of rg-Sw-Ck/PB2 after a single passage in pigs. If the PB2 gene of

Ck/Yamaguchi/04 (H5N1) restricted viral replication in pigs, it should also restrict the replication of Sw/Hokkaido/81 (H1N1) in pigs. Therefore, rg-Sw-Ck/PB2, possessing seven genes from Sw/Hokkaido/81 (H1N1) and the PB2 gene from Ck/Yamaguchi/04 (H5N1), was inoculated intranasally into two pigs (Table 3, pigs 7 and 8). Interestingly, rg-Sw-Ck/PB2 virus was first recovered on day 3 p.i. from nasal swabs, in contrast to rg-Sw/Hokkaido/81 (H1N1), which was recovered on day 1 p.i. During the first 3 days (days 3 to 5 p.i.) of rg-Sw-Ck/PB2 virus shedding, virus titers were 2 to 4 logs lower than those of rg-Sw/Hokkaido/81 (H1N1) (Table 3). Moreover, the duration of rg-Sw-Ck/PB2 virus shedding was 3 to 4 days shorter than that of rg-Sw/Hokkaido/81 (H1N1). The full-length genes of virus isolates (Table 4, Pig 7-day 3 and Pig 8-day 3) recovered from pigs on day 3 p.i., as well as from rg-Sw-Ck/PB2 (inoculum), were sequenced and compared. Predicted amino acid sequences of all genes, except for the PB2 gene, were identical to those of rg-Sw-Ck/PB2 (inoculum) (Table 4). The PB2 proteins of both isolates, Pig 7-day 3 and Pig 8-day 3, had amino acid substitutions of glutamic acid to lysine at position 627 (E627K) and glycine to aspartic acid at position 256 (D256G), respectively.

To reconfirm the replicative potential of recovered virus, isolates Pig 7-day 3 and Pig 8-day 3 were inoculated into the pigs. The viruses were recovered from the nasal swabs from day 1 p.i., in contrast to rg-Sw-Ck/PB2 which was recovered on day 3 p.i. (Table 3). This finding suggested that the E627K and D256G mutations must have played important roles in host adaptation.

The D256G and E627K amino acid substitutions enhance polymerase activity. In order to assess the polymerase activity, a luciferase reporter gene construct was used. The polymerase activity of Sw/PB2-PB1-PA-NP was approximately twice that of Ck/PB2-PB1-PA-NP. However, there was a considerable increase in the polymerase activity of ribonucleoprotein (RNP) expressed by the Sw/PB2-Ck/PB1-PA-NP polymerase complex, achieved by replacing the PB2 gene of Ck/Yamaguchi/04 (H5N1) with that of Sw/Hokkaido/81 (H1N1) (Table 5). This finding correlates with the replication of in vivo-isolated virus clones 1 and 2 or in vitro-generated rg-Ck-Sw/PB2 virus in pigs. Interestingly, the RNP expressed by Ck/PB2-Sw/PB1-PA-NP, produced by replacing the PB2 gene of Sw/Hokkaido/81 (H1N1) with that of Ck/Yamaguchi/04

TABLE 2. Genome segment origin and susceptibility of pigs to H5N1 subtype virus clones recovered from nasal swabs of inoculated pigs

Virus clone(s)	Genome segment origin ^a								Susceptibility of pigs to virus clone ^b	
	PB2	PB1	PA	HA	NP	NA	M	ND	Replication	Antibody response
1	Sw	Ck	Ck	Ck	Ck	Ck	Ck	Ck	+	+
2	Sw	Ck	Ck	Ck	Ck	Ck	Ck	Ck	+	+
3	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	—	—
4	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	—	—
5–11 ^c	Ck	Ck	Ck	Ck	Ck	Ck	Ck	Ck	ND	ND

^a Genome segment origins were determined by partial sequencing. Sw, swine; Ck, chicken.

^b Inoculum contained 10^7 to $10^{7.5}$ EID₅₀/500 µl of viruses. ND, not determined.

^c Virus was recovered from days 2 to 5 p.i., and titers ranged from $10^{1.5}$ to $10^{3.8}$ EID₅₀/ml.

^d Virus was recovered from days 1 to 3 p.i., and titers ranged from $10^{1.5}$ to $10^{2.8}$ EID₅₀/ml.

^e Susceptibility of pigs to virus clones 5 to 11 was not determined.

TABLE 3. Virus titers in nasal swabs of pigs inoculated with viruses produced by reverse genetics

Virus	Pig	Virus titer (log ₁₀ EID ₅₀ /ml) at p.i. day ^a										Antibody response
		1	2	3	4	5	6	7	8	9	10	
rg-Ck/Yamaguchi/04 (H5N1)	1	—	—	—	—	—	—	—	ND	ND	ND	—
	2	—	—	—	—	—	—	—	ND	ND	ND	—
rg-Sw/Hokkaido/81 (H1N1)	3	5.8	≥6.5	6.3	5.5	3.8	4.8	—	1.8	1.8	1.3	+
	4	5.3	≥6.5	6.3	5.8	2.5	3.3	—	—	3.5	2.3	+
rg-Ck-Sw/PB2	5	3.5	3.8	1.3	—	—	—	—	ND	ND	ND	+
	6	—	1.5	1.3	—	—	ND	ND	ND	ND	ND	+
rg-Sw-Ck/PB2	7	—	—	3.8 ^b	5.8	4.3	4.8	2.5	2.8	1.8	—	+
	8	—	—	1.3 ^b	3.5	2.3	4.8	3.5	2.8	—	—	+
Pig 7-day 3	9	3.8	4.8	5.8	4.5	2.8	2.8	—	—	—	ND	+
	10	2.5	2.5	2.8	2.8	2.8	3.3	1.3	—	—	ND	+
Pig 8-day 3	11	4.3	≥4.5	5.5	5.5	3.8	2.3	—	—	—	ND	+
	12	2.8	4.8	5.3	4.8	4.5	3.3	—	—	—	ND	+

^a ND, not determined; —, virus titer determined to be <1.5 log₁₀ EID₅₀/ml.^b Virus isolates were sequenced and results are shown in Table 4.

(H5N1), showed lower polymerase activity than that of Sw/PB2-PB1-PA-NP (Table 5); conversely, Ck/PB2_{D256G}-Sw/PB1-PA-NP and Ck/PB2_{E627K}-Sw/PB1-PA-NP showed 3 to 15 times higher polymerase activity than that shown by Ck/PB2-Sw/PB1-PA-NP. These findings also correlate with the replicative behavior of virus isolates Pig 7-day 3 and Pig 8-day 3, which were isolated on day 3 p.i. from pigs inoculated with rg-Sw-Ck/PB2. After reinoculation into pigs, both virus isolates were isolated on day 1 p.i. The effect of the D256G and E627K amino acid substitutions on polymerase activity was further evaluated by using RNP expressed by homologous Ck/PB2_{D256G}-PB1-PA-NP and Ck/PB2_{E627K}-PB1-PA-NP polymerase complexes. There was a 43 to 175 times increase in the polymerase activity of RNP expressed by Ck/PB2_{D256G}-PB1-PA-NP and Ck/PB2_{E627K}-PB1-PA-NP compared to that of Ck/PB2-PB1-PA-NP and a 12 to 14 times increase compared to that of Ck/PB2_{D256G}-Sw/PB1-PA-NP and Ck/PB2_{E627K}-Sw/PB1-PA-NP, respectively. These findings suggest that the D256G and E627K amino acid substitutions in the PB2 protein of Ck/Yamaguchi/04 (H5N1) counteracted the suppressive effects of the naïve PB2 protein of Ck/Yamaguchi/04 (H5N1).

DISCUSSION

It has been shown that avian and human H5N1 viruses isolated in 1997 (48) and 2004 (5) replicated to moderate levels

in the upper respiratory tracts of experimentally infected pigs. There is also evidence of cocirculation of avian and human influenza viruses in pigs in China (39). Therefore, it is reasonable to think that pigs can provide opportunity for the reassortment and subsequent emergence of new reassortant influenza viruses.

Kida et al. (25) inoculated pigs with A/duck/Hokkaido/8/1980 (H3N8) (nonreplicating strain) and Sw/Hokkaido/81 (H1N1) (replicating strain). They recovered both the viruses possessing the parental gene constellation and the reassortant viruses. The recovered H3N8 subtype viruses, after reinoculation, did not replicate in the pigs, while reassortant viruses replicated. Similarly, in the present study, viruses with the

TABLE 5. Viral polymerase activity correlates with the virus replication potential in pigs^a

Genome segment origin ^b				Luciferase activity ± SD (10 ⁴ RLU)
PB2	PB1	PA	NP	
Ck	Ck	Ck	Ck	38.3 ± 2.2
Sw	Sw	Sw	Sw	63.3 ± 2.3
Sw	Ck	Ck	Ck	234.2 ± 5.4
Ck	Sw	Sw	Sw	38.4 ± 1.2
Ck _{E627K}	Sw	Sw	Sw	579.7 ± 78.5
Ck _{D256G}	Sw	Sw	Sw	120.3 ± 9.0
Ck _{E627K}	Ck	Ck	Ck	6696.8 ± 233.0
Ck _{D256G}	Ck	Ck	Ck	1654.1 ± 146.5
Control ^c				2.2 ± 0.7

^a Polymerase activity was assayed by a viral untranslated region-driven luciferase reporter gene. 293T cells were transfected with the pHW2000 plasmid containing the cloned PB2, PB1, PA, and NP genes of Ck/Yamaguchi/04 (H5N1) and Sw/Hokkaido/81 (H1N1). After 24 h of transfection, luciferase activity (in relative light units [RLU]) was assayed in cell lysates. Results are the means ± standard deviations (SD) of two independent experiments, and each experiment was conducted in triplicate (*n* = 6).

^b Sw (swine) and Ck (chicken) indicate that the respective genes are derived from Sw/Hokkaido/81 (H1N1) or Ck/Yamaguchi/04 (H5N1), respectively.

^c 293T cells were transfected with pHW2000 and pHW-Luc as the negative control.

TABLE 4. Comparison of amino acid sequences of PB2 gene products of isolates recovered from pigs inoculated with rg-Sw-Ck/PB2

Virus isolate	PB2 amino acid at:	
	Position 256	Position 627
rg-Sw-Ck/PB2 (inoculum)	D	E
Pig 7-day 3	D	K
Pig 8-day 3	G	E
rg-Sw/Hokkaido/81 (H1N1)	D	K

parental gene constellation were recovered, and two of these virus clones (virus clones 3 and 4) were found to be identical to the parental virus and rg-Ck/Yamaguchi/04 (H5N1) in that all three had the capability to replicate in pigs; therefore, the isolation of entire H5N1 virus clones could be due to concurrent infection of cells lining the upper respiratory tract of the inoculated pigs, with different reassortant viruses present in the inoculum which might have provided all eight gene segments of Ck/Yamaguchi/04 (H5N1).

The role of the PB2 protein in determining the host range has been studied extensively using squirrel monkeys (7), mice (9, 29), and mammalian cells (57). In the present study, we found that the PB2 gene of Ck/Yamaguchi/04 (H5N1) restricted its replication in pigs, since its replacement by the PB2 gene of Sw/Hokkaido/81 (H1N1) enabled it to replicate in the pigs, as observed for naturally selected virus clones 1 and 2 and rg-Ck-Sw/PB2 virus. Kida et al. (25) isolated triple-gene reassortants deriving the NP, NA, and M or NP, NA, and NS genes from the replicating strain Sw/Hokkaido/81 (H1N1) and the remaining five genes from the nonreplicating strain A/duck/Hokkaido/8/1980 (H3N8). In the present study, single-gene reassortant virus clones deriving the PB2 gene from Sw/Hokkaido/81 (H1N1) were isolated. It could be due to differences in the gene constellations of nonreplicating influenza virus strains bearing different host range determinants (28, 49), as used by Kida et al. (25) and in the present study.

The restrictive effect of the PB2 gene of Ck/Yamaguchi/04 (H5N1) virus was evaluated by studying the replication of rg-Sw-Ck/PB2 virus in pigs. Interestingly, the viruses were recovered on day 3 p.i. and replicated to moderate levels for a shorter duration than rg-Sw/Hokkaido/81 (H1N1) (Table 3). These findings indicate that during the first 2 days p.i., the virus might have undergone adaptive changes. This assumption was supported by examining the predicted amino acid sequences of the two virus isolates, Pig 7-day 3 and Pig 8-day 3, whose PB2 proteins had E627K and D256G amino acid substitutions, respectively. Amino acid substitution at position 256 in the PB2 protein has not been reported previously, while amino acid substitution at position 627 has been reported to be a host range determinant. Li et al. (29) inoculated mice with two duck isolates of contrasting pathogenicity for mice. They found that more than 50% of the virus isolates recovered from mouse lungs had E627K substitutions in the PB2 protein. Similarly, viruses recovered from mice inoculated with Ck/Yamaguchi/04 (H5N1) had the E627K substitution in the PB2 protein (31); therefore, these studies suggested that the presence of E or K at position 627 is host dependent and is an indicator of avian-to-mammalian adaptation. The finding that the Pig 7-day 3 and Pig 8-day 3 isolates were isolated from pigs on day 1 p.i. and previous findings suggest that the E627K and D256G substitutions enabled the Pig 7-day 3 and Pig 8-day 3 isolates to replicate in pigs like that of parental or rg-Sw/Hokkaido/81 (H1N1) virus.

The *in vivo* replicative behavior of virus clones 1 and 2 or of virus isolates Pig 7-day 3 and Pig 8-day 3 was further supported by the luciferase assay. The E627K amino acid substitution has been shown to increase the polymerase activity (9), while the D256G amino acid substitution found in the present study has not been reported previously. The findings suggest that replication of virus clones 1 and 2 or virus isolates Pig 7-day 3 and

Pig 8-day 3 in pigs may be due to enhancement of viral polymerase activity in the epithelial cells lining the upper respiratory tract of pigs.

The PB2, PB1, and PA proteins make up the viral RNA polymerase complex. The presence of overlapping PB1 and NP functional regions on the PB2 protein has suggested their role in switching the transcriptase to replicase activity (40, 47). The D256G substitution is located in the functional domain of the PB2 protein. This region has been shown to be related to a cap binding function (18, 40), interaction with NP protein (40), and interaction with PB1 protein (38). Similarly, the E627K substitution is located in the C-terminal region of the PB2 protein, which interacts with both the PB1 and NP proteins (40). Labadie et al. (27) suggested that the presence of K at position 627 in the PB2 protein helps to stabilize the PB2-NP interaction in human cells through an unknown host cellular factor, while K at this position impairs this interaction in avian cells. Many host cell proteins have been shown to interact with different subunits of influenza virus polymerase complex, and some of these were involved either in translocation of viral RNPs such as importin α (10), Ran binding protein 5 (8), or heat shock protein 90 (37) or in regulation of polymerase activity (33, 34). Recently, Jorba et al. (21) identified many influenza virus polymerase-interacting nuclear and cytosolic proteins involved in transcription, modification, and translocation. Those findings suggest that interaction of polymerase components with each other to carry out transcription or replication involves host cellular factors; thus, adaptive changes to host cellular factors might play an important role in host range determination.

The role of the D256G and E627K amino acid substitutions in the adaptation of influenza viruses to new hosts is reflected by a significant increase in the polymerase activity of both homologous and heterologous polymerase complexes (Table 5). This result indicates that the D256G and E627K amino acid substitutions might be critical changes to control polymerase activities independently, not only for the reassortant virus rg-Sw-Ck/PB2 but also for the original Ck/Yamaguchi/04 (H5N1). It was interesting to find that out of 3,146 predicted amino acid sequences of the PB2 gene obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>), only one isolate, A/swine/Wisconsin/1/1967 (H1N1), had the D256G amino acid substitution, while one swine and one duck isolate had the D256R and D256I amino acid substitutions, respectively. This finding indicates that D256G might not be a common mutation in the process of virus evolution. In any case, it is speculated that the mutations D256G and E627K might have appeared as a result of the interaction of the PB2 protein of Ck/Yamaguchi/04 (H5N1) with pig cellular proteins, resulting in enhanced replication of virus isolates Pig 7-day 3 and Pig 8-day 3 in pigs.

In light of earlier and present findings, it is reasonable to conclude that the PB2 protein of Ck/Yamaguchi/04 (H5N1) determined its host range. However, the molecular events which lead to the appearance of D256G and E627K substitutions have yet to be elucidated.

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