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High Level of Genetic Compatibility between Swine-Origin H1N1 and Highly Pathogenic Avian H5N1 Influenza Viruses▽

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Reassortment is an important mechanism for the evolution of influenza viruses. Here, we coinfected cultured cells with the pandemic swine-origin influenza virus (S-OIV) and a contemporary H5N1 virus and found that these two viruses have high genetic compatibility. Studies of human lung cell lines indicated that some reassortants had better growth kinetics than their parental viruses. We conclude that reassortment between these two viruses can occur and could create pandemic H5N1 viruses.

The influenza virus genome consists of eight single-stranded, negative-sense RNA segments. This segmented genome allows for reassortment, in which coinfection of a cell by two or more different viruses leads to progeny virions with various combinations of segments. Reassortment is an important mechanism for the evolution of influenza viruses because it can lead to antigenic shift and the generation of pandemic viruses (8).

Since 1997, highly pathogenic influenza viruses of the H5N1 subtype have been causing human infections with a high mortality rate. It is feared that such viruses may acquire the ability to spread efficiently among humans, through either adaptation or reassortment or both (12). While H5N1 viruses have not yet acquired pandemic status, a novel swine-origin influenza A virus (S-OIV) of the H1N1 subtype emerged and caused a pandemic. S-OIV contains a unique combination of gene segments from swine, avian, and human viruses and was first identified in humans in April 2009 (4, 15).

Spontaneous reassortment of H5N1 viruses with human influenza viruses has not been reported; however, the emergence and establishment of the S-OIV in the human population may represent a new opportunity for such reassortment and the creation of new viruses with pandemic potential. Therefore, an understanding of the genetic compatibility between these two viruses is of paramount importance.

To assess the likelihood of reassortment between S-OIV and H5N1 viruses, we coinfected Madin-Darby canine kidney (MDCK) cells with A/California/04/2009 (H1N1) (CA04) and a contemporary human H5N1 isolate, A/Vietnam/HN31604/2009 (H5N1) (VN31604), and analyzed the genetic composition of the progeny viruses. Since we would be generating potentially dangerous reassortant viruses that have not been found in nature, we generated an M2-knockout version of each virus [designated CA04(M2KO) and VN31604(M2KO)], using methodology previously described (23). Such viruses show normal growth in M2-expressing cells but grow poorly in unmodified cells and are highly attenuated in animal models.

In preliminary coinfection experiments, we infected M2CK cells [MDCK cells stably expressing the M2 protein derived from A/PR/8/34 (H1N1) virus (6)] with CA04(M2KO) and VN31604(M2KO), both of which encode only the 24 N-terminal amino acids of the M2 protein (i.e., they lack the transmembrane and cytoplasmic tail), at a multiplicity of infection (MOI) of 1 for each virus. Two independent coinfection experiments were performed, but in this case, most of the genes in the progeny viruses were from the H5N1 virus, probably reflecting the faster growth properties of this virus (data not shown). To obtain a better balance of genes from the two viruses, we increased the MOI for CA04(M2KO) to 5, keeping that for VN31604(M2KO) at 1. Supernatants were harvested 8 h postinfection, and progeny virions were either plaque purified or cloned by limiting dilution in M2CK cells. The viral clones were then grown in M2CK cells, viral RNA was extracted from the supernatants and reverse transcribed, and the DNA was genotyped by amplification of the cDNA with sets of primers capable of identifying the gene origins (10).

Three independent coinfection experiments were performed, and 59 viral clones were examined. Among them, there were 33 different genotypes; 85% of the viral clones were reassortants, while the remaining 15% had all of their genes from VN31604(M2KO) (Table 1). Although in theory 254 different reassortants were possible, our results suggest a reasonable degree of genetic compatibility between these two viruses.

The polymerase complex of influenza viruses consists of three subunits, namely, PB2, PB1, and PA. They associate with the nucleoprotein (NP) and viral RNA to form the ribonucleoprotein (RNP) complex, which is required for replication and transcription of the influenza virus genome. The polymerase

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TABLE 1. Genetic composition of progeny virions obtained by coinfection of M2CK cells with the CA04(M2KO) and VN31604(M2KO) viruses[a]

Genetic composition of progeny viruses								Frequency
PB2	PB1	PA	HA	NP	NA	M	NS	9
PB2	PB1	PA	HA	NP	NA	M	NS	1
PB2	**PB1**	PA	HA	NP	NA	M	NS	1
PB2	PB1	**PA**	HA	NP	NA	M	NS	2
PB2	PB1	PA	**HA**	NP	NA	M	NS	1
PB2	PB1	PA	HA	NP	**NA**	M	NS	3
PB2	PB1	PA	HA	NP	NA	**M**	NS	4
PB2	PB1	PA	HA	NP	NA	**M**	NS	2
PB2	PB1	**PA**	HA	NP	**NA**	M	NS	1
PB2	PB1	PA	**HA**	NP	NA	**M**	NS	1
PB2	PB1	PA	HA	NP	**NA**	**M**	NS	4
PB2	**PB1**	PA	**HA**	NP	NA	M	NS	1
PB2	PB1	PA	**HA**	NP	NA	**M**	NS	1
PB2	PB1	PA	HA	NP	**NA**	**M**	NS	2
PB2	**PB1**	PA	**HA**	NP	NA	M	**NS**	1
PB2	**PB1**	PA	HA	NP	**NA**	**M**	NS	1
PB2	PB1	**PA**	HA	NP	**NA**	**M**	NS	2
PB2	PB1	PA	**HA**	NP	**NA**	**M**	NS	1
PB2	PB1	PA	HA	NP	**NA**	**M**	**NS**	5
PB2	PB1	**PA**	HA	NP	**NA**	M	**NS**	1
PB2	**PB1**	**PA**	HA	**NP**	**NA**	M	NS	1
PB2	**PB1**	**PA**	HA	NP	**NA**	**M**	NS	2
PB2	PB1	**PA**	**HA**	NP	**NA**	**M**	NS	1
PB2	PB1	**PA**	HA	NP	**NA**	**M**	**NS**	1
PB2	PB1	PA	HA	**NP**	**NA**	**M**	**NS**	1
PB2	**PB1**	**PA**	HA	**NP**	**NA**	M	NS	2
PB2	**PB1**	**PA**	HA	**NP**	**NA**	M	**NS**	1
PB2	**PB1**	PA	HA	**NP**	**NA**	**M**	**NS**	1
PB2	PB1	**PA**	**HA**	**NP**	**NA**	M	**NS**	1
PB2	**PB1**	**PA**	**HA**	NP	**NA**	**M**	**NS**	1
PB2	**PB1**	PA	**HA**	**NP**	**NA**	**M**	**NS**	1
PB2	**PB1**	**PA**	**HA**	**NP**	**NA**	**M**	NS	1
PB2	**PB1**	**PA**	**HA**	NP	**NA**	**M**	**NS**	1

[a] M2CK cells were infected with M2-knockout CA04 and VN31604 (MOI, 5 and 1, respectively). Culture supernatants were harvested 8 h postinfection, and viral clones were analyzed for their genetic composition. Gene segments derived from CA04 and VN31604 are shown in bold and normal type, respectively. Frequency denotes the number of purified viruses with the indicated genomes; $n = 59$.

subunits play an important role in host range and adaptation (3, 5). However, incompatibility among the RNP genes is a limiting factor for reassortment between two viruses (10, 14). To further characterize the genetic compatibility between the S-OIV and H5N1 viruses, we investigated the compatibility among the RNP components of CA04 and VN31604 in terms of virus performance in human cells. To this end, we used reverse genetics (16) to produce reassortants (again, using M2 genes of these viruses encoding only the 24 N-terminal amino acids) containing all of the possible combinations of RNP genes between CA04 and VN31604, with all of the remaining genes from VN31604, and compared their growth in the human respiratory cell line A549-M2 (A549 cells constitutively expressing the M2 protein derived from A/WSN/33, produced by means of retroviral transduction and antibiotic selection).

Viruses of all possible RNP gene combinations were viable, with high replicative ability; titers in the culture supernatant of transfected 293T cells at 48 h posttransfection ranged from 3×10^6 to 1.3×10^7 PFU/ml. The viruses grew to high titers in M2CK cells (range, 7×10^7 to 6.8×10^8 PFU/ml) and produced large plaques in this cell line, similar to those of wild-type VN31604, indicating a high degree of compatibility among the RNP components of the two viruses. This finding is in sharp contrast to that of Chen et al. (2), who found that seven of nine reassortants containing RNP components from an H3N2 seasonal virus on an H5N1 virus background showed severely impaired replication in cell culture. Similarly, Li et al. (9) found that of 16 reassortants containing all possible RNP gene combinations between another H5N1 and seasonal H3N2 virus on an H5N1 virus background, 5 showed moderate to severe cell culture replication impairment, while 4 were not viable. These studies clearly show that there is limited genetic compatibility between seasonal H3N2 and avian H5N1 viruses, especially with regard to the RNP complex.

Although they varied in their growth kinetics, all of the reassortants produced in our study also grew in A549-M2 cells. Interestingly, some reassortants showed better growth than VN31604(M2KO) (i.e., reassortants containing one or both of the PB2 and PB1 subunits from CA04 and the remaining genes from VN31604 [Fig. 1]). To investigate whether this enhanced growth was cell line specific, we assessed the growth of selected reassortants in another human respiratory cell line, NCI-H358-M2 (NCI-H358 cells constitutively expressing the M2 protein derived from A/WSN/33, produced by transduction with a lentivirus vector [20]). In this cell line, reassortants containing PB2 and PB1 from CA04 also showed faster growth than the other viruses, as evidenced by the higher titers at 12 and 24 h postinfection (Fig. 2).

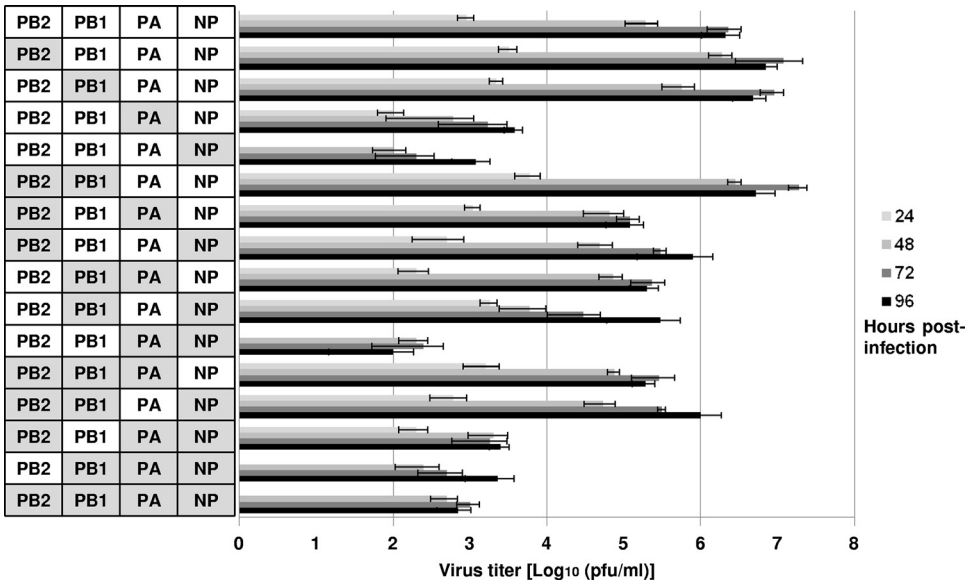

FIG. 1. Viral growth of reassortants in A549-M2 cells. Cells were infected with M2-knockout viruses, produced by reverse genetics, representing all of the possible RNP gene combinations between CA04 and VN31604, with all of the remaining genes (HA, NA, M, and NS) derived from VN31604, at an MOI of 0.0005. RNP complex gene segments derived from CA04 and VN31604 are shown in gray-shaded and clear boxes, respectively. Virus yields at the indicated time points were determined by plaque assay in M2CK cells. Data represent the means of results of three independent infections ± standard deviations.

Nearly all of the possible combinations of RNP genes from CA04 and VN31604 were found in the reassortants obtained by coinfection of M2CK cells (Table 1) (i.e., 15 out of 16 possible combinations). In addition, viruses with all of the possible RNP gene combinations produced by reverse genetics were viable (Fig. 1). To understand the differential growth of the reassortants in these human respiratory cell lines, we assessed the polymerase activity of all of the RNP protein combinations from the two viruses in a luciferase-based minigenome reporter assay. The plasmid pPolINP(0)luc2(0) (100 ng), which contains the firefly luciferase gene flanked by the noncoding regions of the NP gene derived from A/WSN/33, was cotransfected into 293 cells in 24-well plates, along with the protein expression plasmids (pCAGGS/MCS [17]) for PB2, PB1, PA, and NP derived from CA04 and VN31604 (100 ng each) in all 16 possible combinations and pGL4.74[hRluc/TK] (20 ng), by using the TransIT 293 transfection reagent (Mirus) (3 μl/μg of plasmid); 24 h posttransfection, the cells were assayed for luciferase activity by using a Dual-Luciferase reporter assay system and a GloMax 96 microplate luminometer (Promega). Although the activities of the heterogeneous complexes varied, all of the RNP protein combinations showed

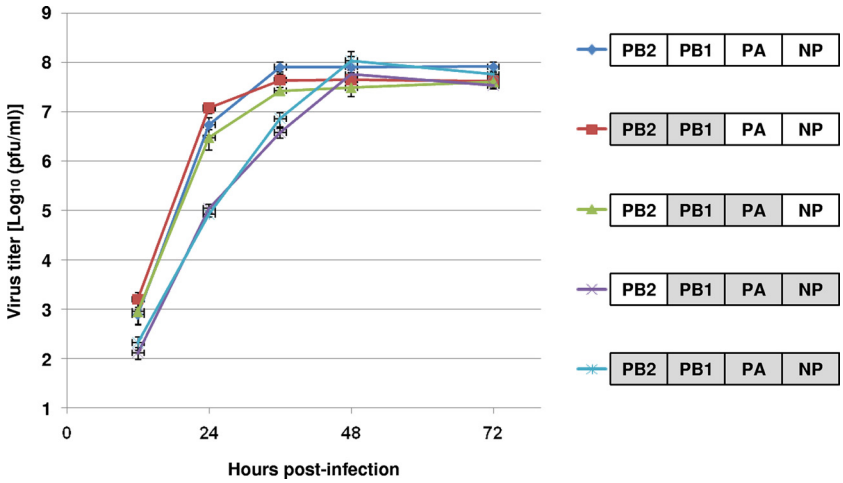

FIG. 2. Viral growth of reassortants in NCI-H358-M2 cells. Cells were infected with M2-knockout viruses, produced by reverse genetics, containing the indicated RNP gene combinations between CA04 and VN31604, with all of the remaining genes (HA, NA, M, and NS) derived from VN31604, at an MOI of 0.001. RNP complex gene segments derived from CA04 and VN31604 are shown in gray-shaded and clear boxes, respectively. Virus yields at the indicated time points were determined by plaque assay in M2CK cells. Data represent the means of results of three independent infections ± standard deviations.

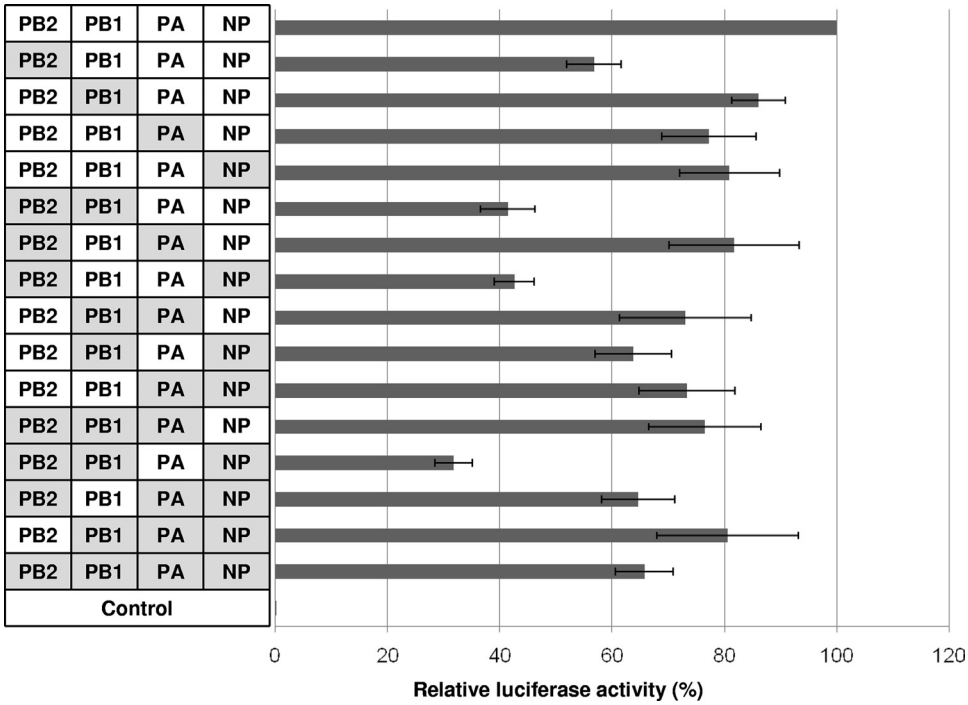

FIG. 3. Polymerase activities of 16 RNP gene combinations measured in a minigenome assay. Four expression plasmids (PB2, PB1, PA, and NP) for the 16 RNP gene combinations between CA04 and VN31604, together with pPolINP(0)luc2(0) for the production of virus-like RNA encoding the reporter luciferase gene, were transfected into 293 cells and assayed for luciferase activity after 24 h of incubation at 37°C. The values shown are means ± standard deviations for results of three independent experiments and are standardized to the activities of the expression plasmids for the VN31604 RNP complex proteins (100%). Genes derived from CA04 and VN31604 are shown in gray-shaded and clear boxes, respectively.

substantial polymerase activity, confirming the high compatibility among the RNP complex proteins of the two viruses (Fig. 3). Intriguingly, the pattern of activity found in the minigenome assay did not match that seen in the viral growth experiments in A549 cells. The reason for this discrepancy is unclear.

In summary, here we demonstrated that reassortment between the pandemic S-OIV and highly pathogenic H5N1 influenza viruses is likely to occur in the event of coinfection in a susceptible host. We used MDCK cells for our coinfection experiments, since they are highly susceptible to both viruses used in this study and thus represent a suitable "mixing vessel" (19). In nature, swine, which are susceptible to both avian and human viruses, have long been considered a potential mixing vessel that may play an important role in the generation of pandemic viruses (19), a concept borne out by the swine origin of the current pandemic H1N1 virus (4, 21). Although replication of some H5N1 viruses in pigs is limited (13), there have been several reports of natural infection of swine by highly pathogenic avian H5N1 influenza viruses (11, 22, 24). Moreover, the susceptibility of swine to the current pandemic virus has been demonstrated and it is likely that the virus will eventually spread to and become enzootic in this species (1, 7, 18). Therefore, appropriate surveillance and containment measures are essential in order to minimize the risks of reassortment between the S-OIV and H5N1 viruses in swine and at the animal-human interface.

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