# Genetic compatibility between the pandemic (H1N1) 2009 and contemporary influenza viruses: implications for the generation of the next pandemic.

（パンデミック（H1N1）2009 ウイルスと季節性及び高病原性鳥インフルエンザウイルスの遺伝子適合性の解析－新たなパンデミック発生におけるその意義）

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Influenza is a major public health concern. Annual epidemics cause approximately 5 million cases of severe illness and between 250,000 and 500,000 deaths worldwide (68). Sporadic pandemics have been more devastating: for example, the Spanish influenza of 1918-1919 caused the deaths of at least 20 million people with some estimate as high as 50 to 100 million people worldwide (21, 51).

Influenza viruses belong to the family *Orthomyxoviridae* of enveloped, negative sense, single-stranded RNA viruses. They are divided into three genera, influenza A, B and C viruses. Of these three genera, influenza A viruses present the greatest public health concern, because they are associated with the most morbidity and mortality and are the only genus known to cause pandemics (1, 73).

Influenza A viruses are subtyped based on the antigenic characteristics of their hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. Currently, there are 16 different HA and 9 different NA subtypes for influenza A viruses. Influenza A viruses have been isolated from a variety of animal species, including humans, swine, horses, sea mammals, dogs, and birds. All known HA and NA subtypes have been found in aquatic birds, and phylogenetic studies have revealed that birds are the main reservoir and source of all influenza viruses in other species. While they generally cause benign enteric infection in their natural reservoir of wild aquatic birds, influenza A viruses that cross the host species barrier

can cause catastrophic disease in humans, poultry, and horses.

The influenza A virus genome comprises eight RNA segments that encode up to 11 viral proteins. The structure of the influenza A virion is represented in Figure 1. The segmented nature of the influenza virus genome allows the phenomenon of reassortment, in which co-infection of a cell by two or more different viruses leads to the generation of progeny virions of mixed genetic composition.

Influenza pandemics are caused by antigenic shift, that is, the emergence of novel viruses that transmit efficiently among humans, but to which humans are immunologically naive. This requires the virus to acquire the surface glycoprotein HA from a non-human source and therefore to cross the species barrier. However, influenza viruses do not transmit freely among different host species, and when cross-species transmission does occur, it tends to be self-limiting. Nevertheless, such viruses may acquire the ability to spread efficiently in their new host through adaptation, reassortment, or a combination of both (8, 30, 73). The importance of reassortment for the generation of pandemic viruses is highlighted by the fact that the last three influenza pandemics, that is, the 1957 "Asian influenza", the 1968 "Hong Kong influenza" and the pandemic (H1N1) 2009 influenza, were caused by viruses that were generated by reassortment (31, 57) . However, reassortment does not occur freely in influenza viruses and the factors that govern it remain poorly understood.

Since 1997, highly pathogenic avian H5N1 influenza viruses have frequently crossed

the species barrier and infected humans with a high lethality rate, raising concerns that these viruses could acquire the potential to spread efficiently among humans and cause a pandemic. While this has not yet happened, a novel swine-origin influenza A virus of the H1N1 subtype, containing a unique combination of gene segments from swine, avian, and human viruses, emerged in April 2009 and caused the first influenza pandemic of the 21st century.

The control of influenza relies primarily on vaccination and treatment with antivirals. Moreover, in the face of the economic and public health threat posed by outbreaks of avian influenza, measures have also been instituted to control the spread of highly pathogenic influenza in poultry, such as culling during outbreaks and movement restrictions.

Recently, much effort has been directed towards the characterization of molecular markers of pathogenicity and host range in influenza viruses to help guide surveillance and control measures against influenza (8, 73). Similarly, a thorough understanding of the factors that govern reassortment in influenza viruses may provide insights into the future of pandemic influenza, allowing effective surveillance, preparedness, prevention, and control measures.

With the ultimate goal of shedding light on the factors that may shape the emergence of the next pandemic viruses, I have studied different aspects of reassortment in influenza virus, with special attention to the genetic compatibility between the pandemic (H1N1) 2009 influenza virus and contemporary seasonal viruses and highly pathogenic avian H5N1 viruses.
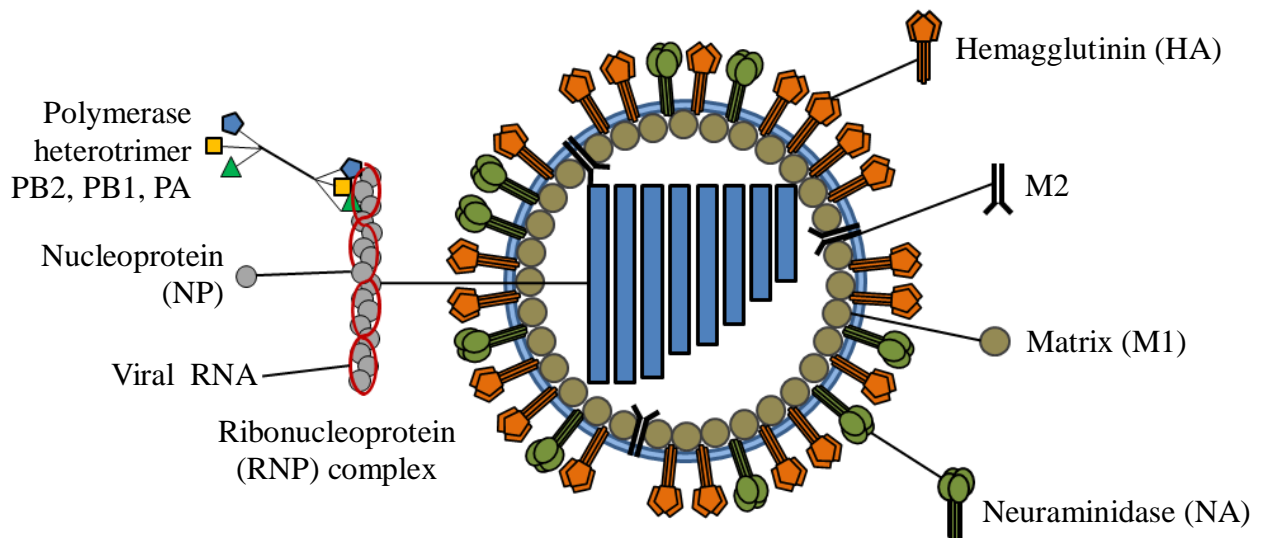
**Figure 1. Overview the influenza A virus particle.** The major surface glycoprotein hemagglutinin (HA) is responsible for receptor binding (attachment to host cells) and membrane fusion. The second-major glycoprotein spike neuraminidase (NA) has receptor-destroying activity. The matrix protein (M1) underlies the lipid envelope. M2 is a proton-specific ion channel, important for virion disassembly. In the virion core are the ribonucleoprotein (RNP) complexes (represented in the diagram as blue bars), which are composed by the nucleoprotein (NP), viral RNA and the polymerase heterotrimer (PB2, PB1 and PA). The RNP complex is responsible for the replication and transcription of the influenza virus genome.

# CHAPTER I


**High level of genetic compatibility between pandemic (H1N1) 2009 and**

**highly pathogenic avian H5N1 influenza viruses**

# ABSTRACT

Reassortment is a crucial factor for the antigenic shift that generates pandemic influenza viruses. Avian H5N1 influenza viruses have since 1997 been crossing the species barrier and infecting humans with a high lethality rate, and it is feared that they could acquire the ability to transmit efficiently among humans, possibly through reassortment, thus acquiring pandemic potential. The emergence of the pandemic (H1N1) 2009 influenza A virus (pdm2009) may represent a new opportunity for such reassortment; therefore, an understanding of the genetic compatibility between these two viruses is of paramount importance. Here, I co-infected cells with pdm2009 and a contemporary human H5N1 isolate and found that the two viruses have a high level of genetic compatibility, since about 85% of the progeny virions were reassortants. A replicon assay showed that all possible combinations of ribonucleoprotein complex components between the two viruses have substantial activity, and studies in human airway epithelium cell lines revealed that reassortants between pdm2009 and H5N1 have high growth capability, with some reassortants showing better growth kinetics in human lung cell lines than their parental viruses. It is concluded that in the event of co-infection of a susceptible host with pdm2009 and H5N1 viruses, reassortment is likely to occur, with the possibility of generating pandemic H5N1 viruses.

**INTRODUCTION**


The influenza A virus genome consists of eight single-stranded, negative-sense RNA segments. This segmented genome allows for reassortment, in which co-infection 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 (27).

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, either through adaptation or reassortment or both (30), thereby acquiring the potential to cause a devastating pandemic. While H5N1 viruses have not yet acquired pandemic status, a novel swine-origin influenza A virus of the H1N1 subtype emerged and caused a pandemic. The virus, named pandemic (H1N1) 2009 (pdm2009), contains a unique combination of gene segments from swine, avian, and human viruses and was first identified in humans in April 2009 (11, 40, 58).

Spontaneous reassortment of H5N1 viruses with human influenza viruses has not been reported; however, the emergence and establishment of the pdm2009 in the human population may represent a new opportunity for such reassortment and the creation of new viruses with pandemic potential.

Reassortment does not occur freely between distinct influenza viruses, since some

gene combinations between two different viruses fail to produce viable virus (6, 17, 28, 29).

Therefore, in the assessment of the risk of generation of reassortants between pdm2009 and

H5N1, an understanding of the genetic compatibility between these two viruses is of

paramount importance.

To shed light on the genetic compatibility between pdm2009 and contemporary

highly pathogenic avian H5N1 influenza viruses, I performed co-infection experiments,

analyzed the polymerase activity of hybrid ribonucleoprotein complexes, and the growth

capability of reassortant viruses.


**MATERIALS AND METHODS**


**Cells and viruses.** Hygromycin-resistant Madin-Darby canine kidney cells stably expressing

M2 protein derived from A/Puerto Rico/8/34 (H1N1) (M2CK) have been previously

described (19). Human lung adenocarcinoma epithelial A549 cells stably expressing M2

protein, which is derived from A/WSN/33 (H1N1) virus (A549-M2), were established by

means of retroviral vector transduction. Human bronchioalveolar carcinoma NCI-H358 cells

stably expressing M2 protein derived from A/WSN/33 (NCI-H358-M2) were prepared by use

of a lentiviral vector transduction, as described previously (55). M2 expression was confirmed

by immunofluorescent assay using anti-M2 monoclonal antibody.

Human embryonic kidney (HEK) 293 and 293T and A549-M2 cells were maintained in

Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

M2CK cells were maintained in minimal essential medium (MEM) containing 10% fetal calf

serum and 0.15 mg/ml of hygromycin. NCI-H358-M2 cells were maintained in RPMI-1640

medium supplemented with 10% fetal calf serum and antibiotics. All cells were maintained in

a humidified incubator at $37^{o}C$ with a 95% air, 5% $CO_2$ atmosphere.

M2-knockout (M2KO) versions of viruses A/California/04/2009 (H1N1) (CA04) and a

contemporary human H5N1 isolate, A/Vietnam/HN31604/2009 (H5N1) (VN31604),

designated CA04(M2KO) and VN31604(M2KO) respectively, as well as reassortants

between these two viruses, were used in this study.


**Construction of plasmids**. The plasmid constructs for vRNA production, containing the

genes of CA04 and VN31604 flanked by the human RNA polymerase I promoter and the

mouse RNA polymerase I terminator (referred to as pPolI plasmids) were produced as

previously described (41).

Plasmids pPolI CA04 M(M2KO) and pPolI VN31604 M(M2KO), for production of M2

knockout viruses, were produced as previously described (64). Briefly, by use of sets of

primers containing BsmBI sites in a back-to-back PCR reaction, followed by digestion and

ligation, two stop codons (TGA TGA) were introduced downstream of the open reading frame

of the M1 protein in the pPolI plasmids for the generation of either M segments, thus

eliminating the transmembrane and cytoplasmic tail domains of the resulting M2 proteins.

Plasmids for expression of RNP complex proteins were produced by cloning of the full-length PCR-amplified cDNA of PB2, PB1, PA and NP derived from CA04 and VN31604 into pCAGGS/MCS (23, 43).

Plasmid pPolINP(0)luc2(0) allows the synthesis of a negative-sense vRNA encoding the firefly luciferase gene flanked by the 3' and 5' non-coding regions of the WSN NP vRNA. To generate this plasmid, pPolI-WSN-NP (a plasmid for the production of wild-type WSN NP vRNA) was amplified in a back-to-back PCR reaction using primers which contain NotI and XbaI restriction endonuclease sites. The luciferase gene was also PCR-amplified using primers containing the same restriction sites, and plasmid pGL4.1 (Promega, Madison, USA) as template. The two fragments were then digested with NotI and XbaI, and ligated to produce pPolINP(0)luc2(0).

**Generation of M2KO viruses by plasmid-based reverse genetics.** Viruses CA04(M2KO) and VN31604(M2KO) were generated by a plasmid-based reverse genetics system, as previously described (64). Briefly, pPolI plasmids for each viral gene derived from CA04 or VN31604, where the M gene was replaced by pPolI CA04 M(M2KO) or pPolI VN31604 M(M2KO), together with pCAGGS/MCS for expression of PB2, PB1, PA and NP proteins derived from A/WSN/33 (H1N1), were transfected into subconfluent HEK 293T cells in OPTI-MEM, supplemented with 0.3% bovine serum albumin (BSA), by using the Trans IT 293 transfection reagent (Mirus, Madison, USA). Culture supernatants were harvested at 48

hours post-transfection, and the virus contents were assessed by means of plaque assay in M2CK cells. Virus stocks were produced by infection of M2CK cells in MEM supplemented with 0.3% (v/v) BSA and 0.5 mg/l TPCK-treated trypsin (MEM/BSA/trypsin). All experiments with live H5N1 viruses were performed in a biosafety level 3 containment laboratory. The biological containment of M2KO viruses was confirmed by the inability to grow in unmodified MDCK, A549 and NCI-H358 cells.

**Co-infection and genotyping of progeny viruses.** CA04(M2KO) and VN31604(M2KO) viruses were used to co-infect confluent M2CK cells. After adsorption for one hour, the inoculum was removed, the cells washed 5 times with MEM supplemented with 0.3% BSA (MEM/BSA), and then covered with MEM/BSA/trypsin. Progeny virions were harvested at 8 hours post-infection and purified either by plaque purification or limiting dilution using M2CK cells. Purified viruses were then propagated in M2CK cells, vRNA was extracted from the culture supernatant by using a QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), and reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, USA). To distinguish the gene origins of the purified viruses, 16 pairs of strain-specific, gene-specific identifying primers were synthesized based on the sequence diversities between CA04 and VN31604 (primer sequences are available upon request), and used for PCR amplification of the reverse-transcribed viral RNA. The origin of each viral gene was determined by the presence or absence of the corresponding band after ethidium

bromide-stained agarose gel electrophoresis.

**Infection of A549-M2 and NCI-H358-M2 cells.** Confluent cell monolayers in MEM/BSA/trypsin were infected with viruses at an MOI of 0.0005 or 0.001 (for A549-M2 and NCI-H358-M2, respectively). Culture supernatants were harvested at various time points, and the viral titers assessed by plaque assay in M2CK cells.

**Replicon assay.** The plasmid pPolINP(0)luc2(0) was co-transfected into HEK 293 cells along with the pCAGGS/MCS protein expression plasmids for PB2, PB1, PA, and NP derived from CA04 and VN31604 in all 16 possible combinations, and pGL4.74[hRluc/TK], by using the TransIT 293 transfection reagent (Mirus, Madison, USA); 24 hours post-transfection, the cells were assayed for luciferase activity by using a Dual-Luciferase Reporter Assay System and a GloMax 96 microplate luminometer (Promega, Madison, USA).

## RESULTS

To assess the likelihood of reassortment between pdm2009 and H5N1 viruses, I co-infected M2CK cells with CA04(M2KO) and VN31604(M2KO), and analyzed the genetic composition of the progeny viruses.

In preliminary co-infection experiments, cells were infected with CA04(M2KO) and VN31604(M2KO) at a multiplicity of infection (MOI) of 1 for each virus. Two independent

co-infection 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, I increased the MOI for CA04(M2KO) to 5, keeping that for VN31604(M2KO) at 1. Supernatants were harvested 8 hours post-infection, and the genetic composition of purified progeny virions determined.

Three independent co-infection experiments were performed, and 59 viral clones were examined; among them, there were 33 different genotypes; approximately 85% (50 out of 59) of the viral clones were reassortants, while the remaining 15% had all of their genes from VN31604(M2KO) (Table1). 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 subunits play an important role in host range and adaptation (9, 17, 25, 60, 61). However, incompatibility among the RNP genes is a limiting factor for reassortment between two viruses (29, 37). To further characterize the genetic compatibility between the pdm2009 and H5N1 viruses, I investigated the compatibility among the RNP components of CA04 and VN31604 in terms of virus performance in human

**Table 1.  Genetic composition of progeny virions obtained by co-infection of M2CK cells with the CA04(M2KO) and VN31604(M2KO) viruses.***

| \ | 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 |

*M2CK cells were infected with M2-knockout CA04 and VN31604 (MOI 5 and 1, respectively), and progeny viruses were analyzed for their genetic composition. Gene segments derived from CA04 and VN31604 are shown in bold and italic type, respectively. Frequency denotes the number of purified viruses with the indicated genomes; *n* = 59.

cells. To this end, M2-knockout reassortants were produced by reverse genetics, containing all of the possible combinations of RNP genes between CA04 and VN31604, with all of the remaining genes from VN31604, and their growth properties were compared in the human respiratory cell line A549-M2.

Viruses containing all possible RNP gene combinations were viable with high replicative capability; titers in the culture supernatant of transfected 293T cells at 48 hours post-transfection ranged from $3 \times 10^6$ to $1.3 \times 10^7$ plaque-forming units (pfu)/ml. The viruses grew to high titers in M2CK cells (range $7 \times 10^7$ to $6.8 \times 10^8$ pfu/ml) and produced large plaques in this cell line, similarly to those of wild-type VN31604, indicating a high degree of compatibility among the RNP components of the two viruses.

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 higher 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. 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 hours post-infection (Fig. 2).
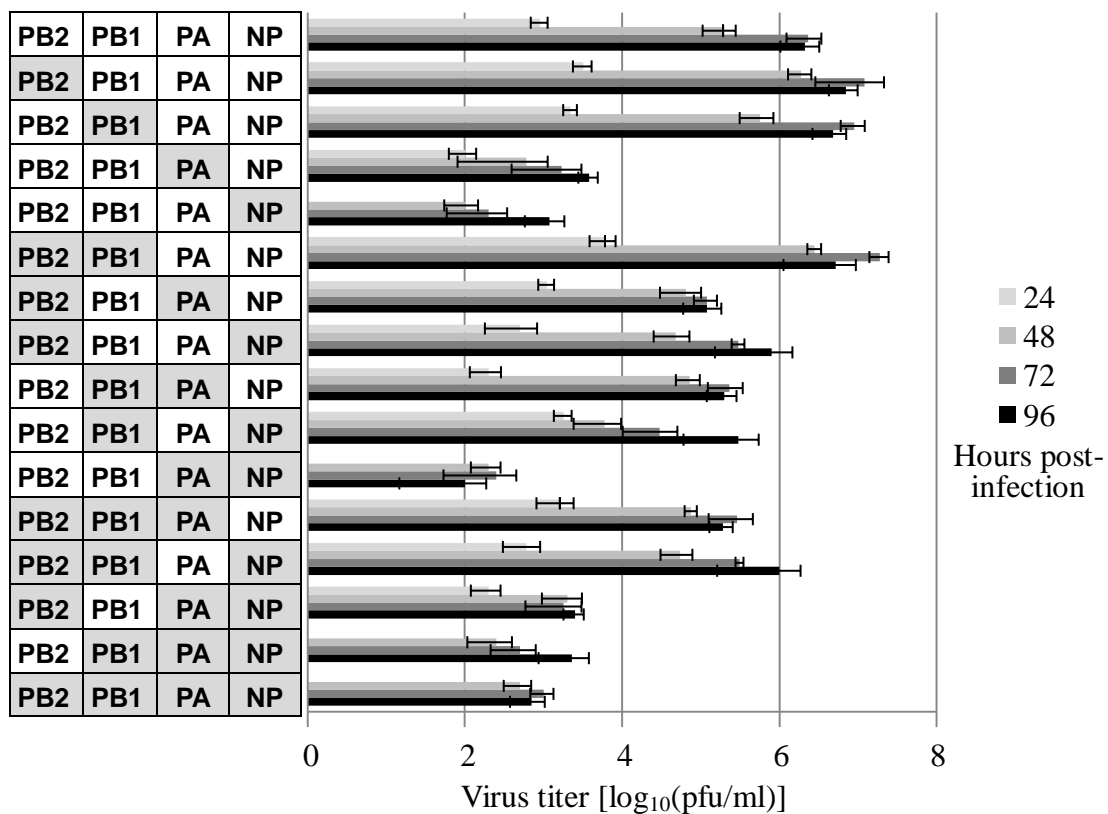
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| | | | |
|---|---|---|---|
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |
| PB2 | PB1 | PA | NP |

Hours post-infection
- 24
- 48
- 72
- 96

Virus titer [$\log_{10}$(pfu/ml)]

**Figure 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 mean of three independent infections ± standard deviations.
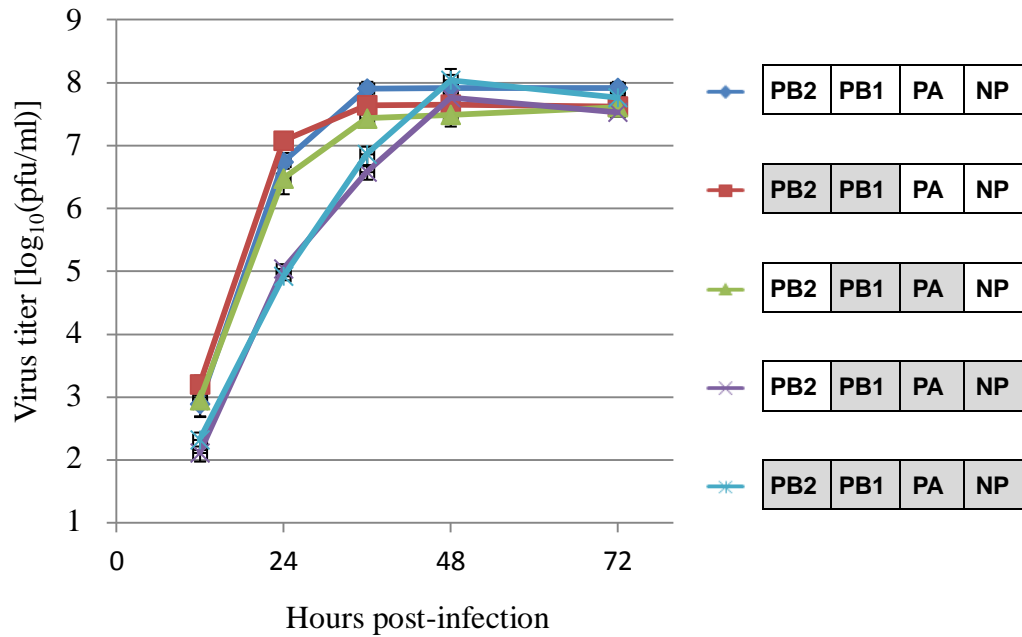
**Figure 2. Viral growth of reassortants in NCI-H358-M2 cells.** Cells were infected with M2-knockout viruses, produced by reverse genetics, containing the indicated RNP gene combinations between CA04 and VN31604, with all of the remaining genes (HA, NA, M, and NS) derived from VN31604, at an MOI of 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 mean 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 co-infection 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, the polymerase activity of all of the RNP protein combinations from the two viruses was assessed in a luciferase-based replicon assay. Although the activity of the heterogeneous complexes varied, all of the RNP protein combinations showed 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 replicon assay did not match that seen in the viral growth experiments in A549 cells. The reason for this discrepancy is unclear.
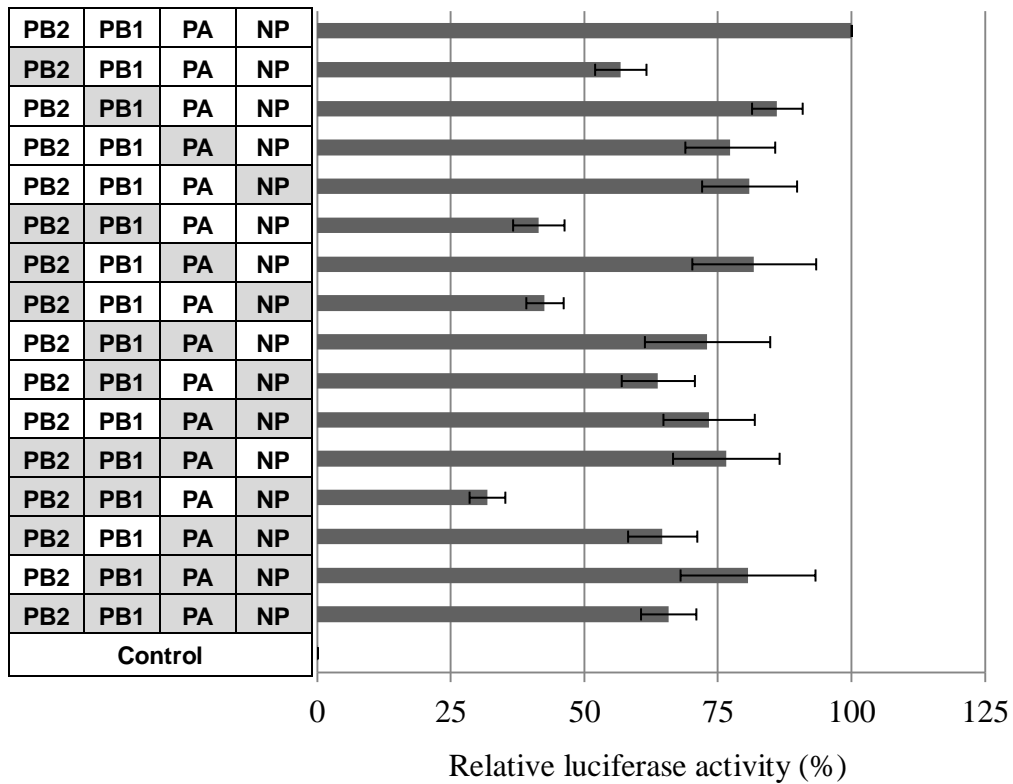
**Figure 3. Polymerase activities of 16 RNP gene combinations measured in a replicon assay.** Four expression plasmids (PB2, PB1, PA, and NP) for the 16 RNP gene combinations between CA04 and VN31604, together with pPolINP(0)luc2(0) for the production of virus-like RNA encoding the reporter luciferase gene, were transfected into 293 cells and assayed for luciferase activity after a 24h incubation at 37$^{\circ}$C. The values shown are means $\pm$ standard deviations for 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.

**DISCUSSION**

In addition to biosafety level 3 containment, we chose to use biologically contained M2-knockout viruses in this study, since we would be generating potentially dangerous reassortant viruses that have not been found in nature. M2-knockout viruses show normal growth in M2-expressing cells, but grow poorly in unmodified cells and are highly attenuated in animal models (64).

The experiments described here demonstrated that reassortment between the pdm2009 and highly pathogenic H5N1 influenza viruses is likely to occur in the event of co-infection in a susceptible host: co-infection of cells yielded reassortants at a high rate, approximately 85% of progeny virions analyzed being reassortants, and displaying a great variety of genetic compositions; moreover, all genes from CA04 and VN31604 were present in the reassortants, suggesting a substantial level of genetic compatibility between the two viruses.

Also of great importance is the fact that all possible combinations of RNP complex proteins between CA04 and VN31604 showed substantial polymerase activity in the replicon assay, and reassortant viruses produced by reverse genetics containing all of the RNP protein combinations were viable with high replicative ability. These findings are in sharp contrast to those of Chen et al (6), who found that 7 of 9 reassortants containing RNP components from an H3N2 seasonal virus on an H5N1 virus background showed severely impaired replication

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in cell culture. Similarly, Li et al (28) 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.

MDCK cells were used in our co-infection experiments since they are highly susceptible to both viruses used in this study and thus represent a suitable "mixing vessel" (54, 56). 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 (5, 54, 57), a concept borne out by the swine origin of the pandemic (H1N1) 2009 virus (11, 15, 58). Although replication of some H5N1 viruses in pigs is limited (32), there have been several reports of natural infection of swine by highly pathogenic avian H5N1 influenza viruses (30, 42, 63, 76). Moreover, the susceptibility of swine to pdm2009 has been demonstrated, and the virus has already spread to and caused numerous epizootics in this species (4, 26, 50, 66, 72). Therefore, appropriate surveillance and containment measures are essential in order to minimize the risks of reassortment between the pdm2009 and H5N1 viruses in swine and at the animal-human interface.

# CHAPTER II


# Reassortment between seasonal H1N1 and pandemic (H1N1) 2009 influenza viruses generates viruses with enhanced growth capability in cell culture

# ABSTRACT

Two years after the emergence of the pandemic (H1N1) 2009 influenza virus (pdm2009), most cases have been relatively mild. In August 2010 the World Health Organization declared that the pandemic period had finished; however, the virus continues to circulate worldwide. In spite of its relatively low pathogenicity in humans, the possibility of enhanced viral growth ability by reassortment with contemporary viruses cannot be overlooked. Here, it is demonstrated that reassortant viruses containing a hemagglutinin derived from a seasonal H1N1 virus and NA and M genes derived from pdm2009 virus have enhanced virus growth capability over their wild-type parental viruses. The emergence of such viruses in nature could, therefore, represent a threat.

# INTRODUCTION

The influenza virus genome comprises eight single-stranded RNA segments of negative polarity, which allows genetic reassortment among different viruses when they co-infect a single cell. Reassortment is important for influenza virus evolution, contributing to the generation of novel viruses, such as those that cause influenza pandemics (27).

In March 2009, a swine-origin influenza A virus of the subtype H1N1 emerged in North America, spreading rapidly through the human population (58), and in June 2009, the World Health Organization announced the first influenza pandemic of the twenty-first century (69). Seventeen months after the emergence of pdm2009, the World Health Organization declared the end of the pandemic (67). In contrast to the three previous influenza pandemics, the pdm2009 was mostly associated with mild disease and a low mortality rate (39). Although there has been concern that, as the virus adapts to the human host, virulence might increase (35), this has not yet been observed. The introduction of known pathogenicity markers, such as the PB1-F2 protein and the E627K and D701N mutations in the PB2 gene, as well as the introduction of human virus-like features in the pdm2009 NS1, have failed to increase virus growth in cell culture, or virulence in animal models in most studies (12, 13, 14, 18, 59, 75), although some NS1 and PB1-F2 mutations have been suggested to increase the replication or pathogenicity of the virus (49). Nevertheless, the possibility of acquisition of enhanced replicative ability or pathogenicity through reassortment with contemporary seasonal H1N1

and H3N2 viruses cannot be overlooked (18).

Here, I investigated whether reassortment between pdm2009 and seasonal H1N1 influenza viruses results in viruses with increased growth capacity. To this end, reassortants were produced by reverse genetics (41), and had their growth properties analyzed in cell culture. Further, the mechanisms for enhanced growth capability of reassortant viruses were investigated.

## MATERIALS AND METHODS

**Cells and viruses.** Human embryonic kidney (HEK) 293 and 293T, human lung adenocarcinoma epithelial A549, and porcine kidney CPK (24) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) containing 5% newborn calf serum and antibiotics. All cells were maintained in a humidified incubator at $37^{o}C$ with a 95% air, 5% $CO_2$ atmosphere.

Viruses A/California/04/2009 (H1N1) (CA04), A/Kawasaki/UT-SAI-k23/2008 (H1N1) (k23), A/Norway/3487/2009 (H1N1) (Nw3487) and A/Utah/42/2009 (H1N1) (Ut42), as well as all reassortants used in this study, were produced by reverse genetics as previously described (41), and propagated and titrated by plaque assay in MDCK cells.

**Construction of plasmids.** The plasmid constructs for generation of viruses by reverse genetics were produced as previously described (41).

Plasmid pPol1-CA04HA(k23RBS), for the production of vRNA of a chimeric CA04 HA, in which the amino acid sequences corresponding to the structures that form the rim of the receptor binding site (RBS), the 130 loop, 190 helix and 220 loop (10), were replaced by those of k23 HA (Fig. 3), was prepared as follows. Using a forward primer that introduced the mutations A448T and G454T, and a reverse primer that introduced the mutation A707G, a PCR reaction was performed using the pPol1-CA04 HA plasmid as a template. The PCR product was then purified and used as a megaprimer in a back-to-back PCR reaction to amplify the entire pPol1-CA04 HA plasmid. This second PCR product was digested with HindIII and DpnI and ligated, thus introducing k23 HA-like mutations into the CA04 130 loop and 220 loop regions of pPol1-CA04 HA. Finally, a new set of primers containing BsmBI sites was used to introduce k23 HA-like mutations into the region corresponding to the 190 helix, thus generating the plasmid pPol1-CA04HA(k23RBS).

**Determination of growth kinetics of viruses.** MDCK, A549 and CPK cells were infected with viruses at an MOI of 0.001 in MEM supplemented with 0.3% bovine serum albumine (BSA) and 0.5 mg/l TPCK-treated trypsin (MEM/BSA/trypsin). Culture supernatants were harvested at various time points, and had their viral contents assessed by plaque assay in MDCK cells.

Plaque morphology in MDCK was done by measuring the diameter of plaques (100 for each virus) at 48 hours post-infection, in a Biozero BZ-8000 microscope (Keyence, Osaka, Japan).

**Virus wash away assay.** Confluent MDCK cells grown in 12 well plates were washed twice with MEM supplemented with 0.3% BSA (MEM/BSA), then covered with 100 μl of MEM/BSA containing serial tenfold dilutions of viruses. Cells were maintained at $37^{o}C$ for periods of 5, 10, 20, or 40 minutes, with tilting every 10 minutes, to allow adsorption of viruses, after which they were washed three times with MEM/BSA and covered with MEM/BSA/trypsin containing 1% melted agarose, and incubated for 48 hours at $37^{o}C$, after which the plaques were counted.

**Hemagglutination assay.** Hemagglutination assays were performed as previously described (70), using red blood cells from chickens, turkeys and guinea pigs (Nippon Biotest Labo, Tokyo, Japan).

**Electron microscopy.** Ultrathin section electron micrography was performed as previously described (44).

# RESULTS

To determine if reassortment between pdm2009 and seasonal H1N1 influenza viruses results in viruses with increased growth capacity, we produced reassortants by reverse genetics, and analyzed their growth in cell culture. In preliminary experiments, we found that a reassortant virus containing the HA gene from k23 on a CA04 background produced plaques in MDCK cells that were distinctly larger than those of either parental virus (Table 1). This prompted us to compare the growth properties of this reassortant (designated k23HA-CA04) with that of the parental viruses (k23 and CA04) in three different cell lines: MDCK, A549, and the porcine kidney cell line CPK. Interestingly, k23HA-CA04 had significantly enhanced growth over either parental virus in these three cell lines (Fig. 1).

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**TABLE 1. Rescue efficiency, stock titers and plaque size of viruses.**

| Virus | Rescue efficiency [$\log_{10}$(pfu/ml)]* | Titer of stock virus grown in MDCK cells [$\log_{10}$(pfu/ml)] | Plaque size in MDCK cells (mm)† |
|---|---|---|---|
| CA04 | 4.26 | 7.88 | 0.26 ± 0.08 |
| k23 | 2.48 | 7.68 | 0.58 ± 0.22 |
| k23HA-CA04 | 6.04 | 9.00 | 1.57 ± 0.35 |
| CA04NA-k23 | 5.27 | 8.08 | 1.01 ± 0.24 |
| CA04M-k23 | 4.26 | 7.72 | 0.91 ± 0.29 |
| CA04NA,M-k23 | 6.00 | 8.73 | 1.41 ± 0.28 |
| Nw3487 | 5.23 | 8.70 | 1.01 ± 0.36 |
| k23HA-Nw3487 | 6.15 | 9.30 | 1.76 ± 0.31 |
| Nw3487NA,M-k23 | 5.95 | 8.73 | 1.25 ± 0.34 |
| Ut42 | 4.15 | 7.68 | 0.29 ± 0.09 |
| k23HA-Ut42 | 6.08 | 9.08 | 1.42 ± 0.36 |
| Ut42NA,M-k23 | 6.00 | 8.83 | 1.27 ± 0.31 |

*Measured by determining the viral titer in the supernatant of transfected 293T cells at 48 hours post-infection, by plaque assay using MDCK cells. Pfu: plaque-forming units.

†Values represent mean ± standard deviations, measured at 48 hours post-infection; $n = 100$. The plaque size of CA04NA,M-k23 differed significantly from that of CA04NA-k23 and CA04M-k23 ($p < 0.001$).
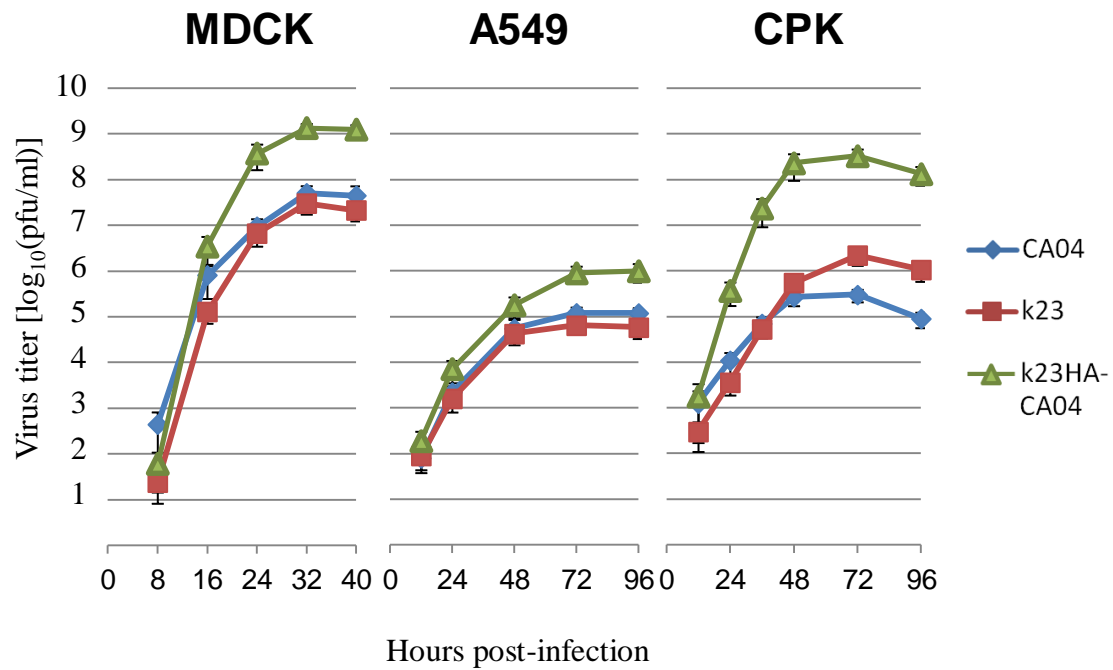
**Figure 1. Viral growth of CA04, k23, and k23HA-CA04 in MDCK, A549, and CPK cells.**
Cells were infected with viruses at a multiplicity of infection of 0.001. Virus yields at the indicated time points were determined by plaque assay in MDCK cells. Data represent the mean of three independent infections ± standard deviations.

Next, I wished to investigate whether the enhanced growth of k23HA-CA04 was due to a difference in the receptor binding properties of the k23 and CA04 viruses. To this, I devised a "virus wash away assay", which consists of a plaque assay, in which the adsorption times are progressively shortened, and the relative reduction in the number of plaques provides a measure of the affinity between the viruses and cells. The results of this assay indicated that CA04 has slower attachment to cells than does k23HA-CA04, as evidenced by the fewer plaques produced when adsorption times were limited to 5 or 10 minutes (t-test, $p < 0.05$) (Fig. 2A).

To gain insight into the relative binding activities of the CA04 and k23 hemagglutinins, hemagglutination assays (HA) were performed with the two viruses, using red blood cells (RBC) from three different species: chicken, turkey, and guinea pig. As shown in Fig. 2B, the reassortant k23HA-CA04 showed higher hemagglutination activity on chicken and to some extent on turkey RBCs, whereas CA04 had slightly higher hemagglutination activity on guinea pig RBCs.

To further investigate the importance of receptor-binding to the growth of k23HA-CA04 and CA04, I produced a virus containing a chimeric CA04 HA, in which the amino acid sequences corresponding to the structures that form the rim of the receptor binding site (RBS), the 130 loop, 190 helix and 220 loop (10), were replaced by those of k23 HA (Fig. 3).
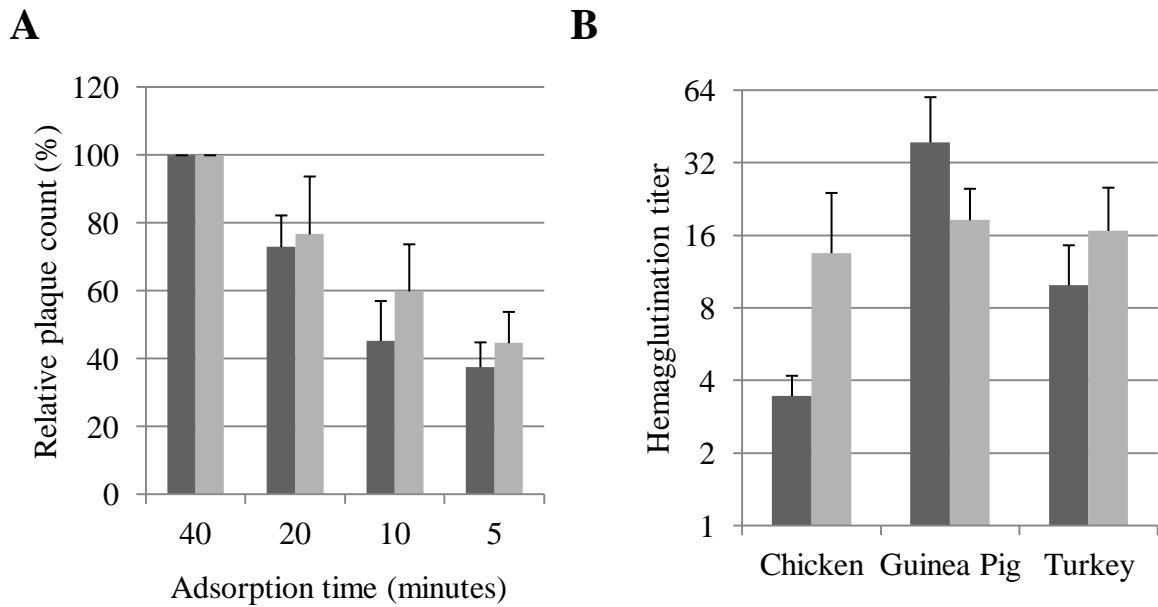
**Figure 2. (A) Virus wash away assay.** CA04 (dark grey) and k23HA-CA04 (light grey) were subjected to a plaque assay in MDCK cells, in which the adsorption periods varied from 5 to 40 minutes. Data represent the relative plaque count obtained for the indicated adsorption periods, normalized to values of the 40 minute adsorption time (100%). Data represent the mean of three independent experiments ± standard deviations. Plaque counts of CA04 and k23HA-CA04 differed significantly with adsorption times of 5 and 10 minutes (t-test, $p < 0.05$). **(B) Relative hemagglutination titers of CA04 and k23HA-CA04.** Virus stocks were adjusted to a titer of $7.6 \times 10^7$ pfu/ml, before hemagglutination assays using chicken, guinea pig, and turkey erythrocytes was performed. CA04 is shown in dark grey; k23HA-CA04 is shown in light grey. Data represent the mean of three independent experiments ± standard deviations.
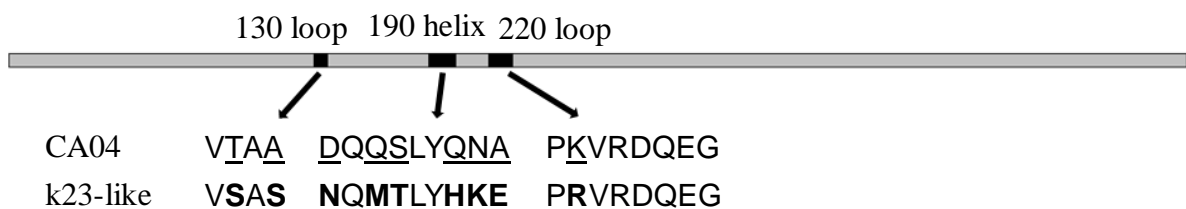


**Figure 3. Schematic diagram of the chimeric CA04 HA.** Black columns represent sequences encoding the structures that form the rim of the HA RBS (130 loop, 190 helix and 220 loop: amino acids 149-152, 204-212 and 235-242, respectively). Mutations were introduced into the pPol1-CA04HA plasmid to substitute the original CA04 (underlined) with k23-like (bold) amino acids in the encoded HA protein.

This virus, designated CA04HA(k23RBS)-CA04, could be rescued, but failed to grow to higher titers, or produce larger plaques in MDCK cells than wild-type CA04 (data not shown). Although the possibility of structural constraints cannot be overlooked, this result suggests that the receptor binding region alone is not responsible for the enhanced growth of k23HA-CA04.

The reassortant k23HA-CA04 showed remarkable enhanced growth over either parental virus. I therefore sought to identify the genes of CA04 that, together with the k23 HA, were responsible for this increased growth. To this end, I used reverse genetics to generate a series of single gene reassortants, containing each of the CA04 genes on a k23 genetic background. It was found that viruses containing either NA or M from CA04 on a k23 background (designated CA04NA-k23 and CA04M-k23) had better rescue efficiency (as measured by determining viral titers in the supernatant of transfected 293T cells) than k23, and produced larger plaques than either CA04 or k23, although neither reassortants reached the levels of k23HA-CA04 (Table 1). Therefore, another reassortant virus was produced, containing both the NA and M genes from CA04 on a k23 background; this reassortant, designated CA04NA,M-k23, had high rescue efficiency, produced plaques significantly larger than those produced by CA04NA-k23 and CA04M-k23 ($p < 0.001$) (Table 1), and had enhanced growth in MDCK cells (Fig. 4), approaching the characteristics of k23HA-CA04, indicating that cooperation between the HA, NA, and M genes is essential for enhanced virus
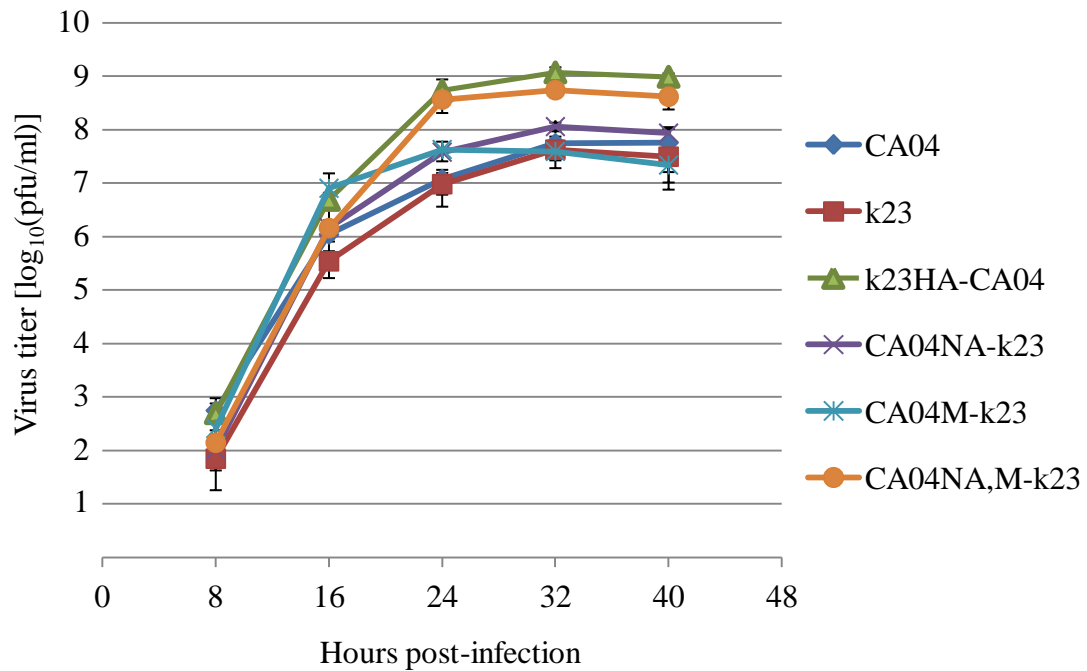
**Figure 4. Viral growth of CA04, k23, k23HA-CA04, CA04NA-k23, CA04M-k23, and CA04NA,M-k23 in MDCK cells.** Cells were infected with viruses at a multiplicity of infection of 0.001. Virus yields at the indicated time points were determined by plaque assay in MDCK cells. Data represent the mean of three independent infections ± standard deviations.

growth.

To determine whether these findings also apply to other pdm2009 isolates, we used A/Norway/3487/2009 (H1N1) (Nw3487) and A/Utah/42/2009 (H1N1) (Ut42) to produce reassortants containing the k23 HA on the pdm2009 background (k23HA-Nw3487, k23HA-Ut42), and NA and M genes from the pdm2009 on the k23 background (Nw3487NA,M-k23; Ut42NA,M-k23). These experiments repeated the pattern seen with CA04, since both the introduction of the seasonal HA into the Nw3487 and Ut42 background and the introduction of NA and M from Nw3487 and Ut42 into the k23 background led to the generation of viruses with higher rescue capability, increased plaque size, and enhanced virus growth in MDCK cells, relative to the parental viruses (Table 1), thus confirming that the finding is conserved among pdm2009.

The HA, NA, and M gene products play central roles in the assembly and budding of influenza viruses at the plasma membrane (38). To gain insight of the effect of the introduction of a seasonal HA into a pdm2009 virus background on virus morphogenesis, we assessed the virion morphology of CA04, k23 and k23HA-CA04 by ultrathin section electron microscopy (44). The electron microscopic analysis of the viruses showed that, while CA04 and k23 were predominantly filamentous viruses, k23HA-CA04 particles were distinctly shorter, with a predominance of quasi-spherical virus particles (Fig. 5). This finding may suggest that a cooperation between the k23 HA and the CA04 NA and M proteins may lead to

more efficient viral morphogenesis and, thereby, contribute to increased viral production in
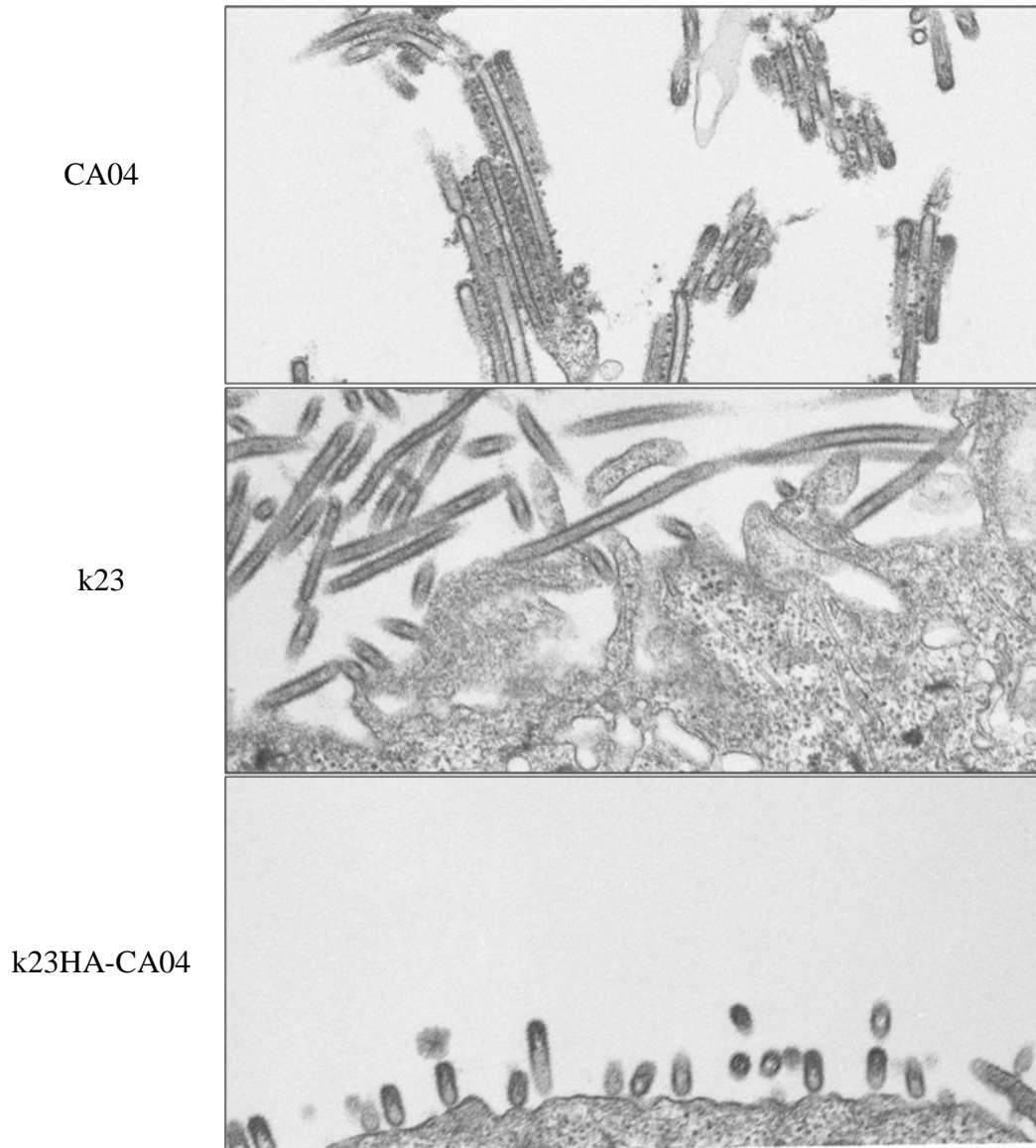
cell culture.



CA04

k23

k23HA-CA04

**Figure 5. Ultrathin section electron micrographs of CA04, k23 and k23HA-CA04 viruses.**
MDCK cells were infected with the viruses at a multiplicity of infection of 0.1. Samples were
fixed 20 hours post-infection and analyzed as previously described (44).

**DISCUSSION**

In summary, the experiments described here demonstrated that reassortment between pdm2009 and seasonal H1N1 viruses generates viruses with enhanced growth properties, when there is a combination of a seasonal HA, and swine-origin NA and M genes.

The virus wash away assay and relative hemagglutination activity experiments indicate that differences in the efficiency of virus attachment to cells may play a role in the enhanced growth kinetics of the reassortants in this study. While an accurate interpretation of the relative hemagglutination activities of these viruses may be complex, the results do show a difference in receptor binding between the two viruses. Glycan array analyses of the receptor specificity of pdm2009 have yielded contrasting results; while Maines et al. (35) found that pdm2009 exhibited dose-dependent binding to a single α2-6 glycan, with minimal binding to α2-3 glycans, Childs et al. (7) observed broader specificity, with binding to both α2-6 and α2-3-linked receptors. It should be noted, however, that those are results from in vitro assessments of HA binding to synthetic glycans, and that the actual availability of the various species of carbohydrate in different cell types, and their exploitation by influenza viruses, is still poorly understood.

Considering the cooperation between HA, NA, and M for virus morphogenesis (38), it is also possible that a suitable combination of these genes may somehow facilitate this

process, thus contributing to viral fitness. Influenza virions have a pleomorphic morphology that ranges from spherical to filamentous. Previous studies have mapped the determinants of virion morphology mainly to the M1 protein, although M2 has also been shown to play a role (3, 19, 33, 53). Moreover, spherical virion morphology is associated with high virus growth (33). Our findings are in agreement with this latter report, since the high growth properties of our reassortants also correlated with spherical virion morphology. However, further investigation is needed to fully understand the functional basis for the enhanced growth of these reassortants.

It is interesting that, while the introduction of well-known virulence markers to pdm2009 failed to influence the replication of the viruses (12, 13, 14, 18, 59, 75), the introduction of the HA gene from a seasonal virus greatly enhanced viral growth. This highlights how little we know of the molecular mechanisms that control the replicative ability of influenza viruses, and make clear that further efforts are needed in order to achieve a thorough understanding of such mechanisms, as it will empower surveillance and control measures against influenza.

The findings described here have important implications for human health, as they may indicate a reemergence potential for the "old" H1 HA, especially after the human population acquires immunity to the new pdm2009 HA. The seasonal H1 HA, which descends from the 1918 Spanish influenza virus HA, already reemerged once, after over two decades,

causing the so-called "Russian flu" (74). It is, therefore, of fundamental importance that

continuous surveillance and containment measures be implemented, in response to this risk.

# CHAPTER III


# Reassortment between seasonal H1N1 and pandemic (H1N1) 2009 influenza viruses is restricted by limited compatibility among polymerase subunits.

## ABSTRACT

Reassortment is important for influenza virus evolution and the generation of novel viruses that cause pandemics; however, the factors influencing reassortment are still poorly understood. To identify the factors that restrict the occurrence of reassortment between the pandemic (H1N1) 2009 influenza virus (pdm2009) and seasonal H1N1 viruses, I employed a replicon assay, and produced reassortant viruses using reverse genetics to analyze the function of hybrid ribonucleoprotein (RNP) complexes between the two viruses. These experiments demonstrated that a mixed polymerase complex containing a pdm2009 PB2 on a seasonal H1N1 virus background has reduced polymerase activity, leading to impaired virus viability. Adaptation of viruses containing the mixed polymerase complex resulted in compensatory mutations in PB1 that were able to partially restore the functionality of the hybrid RNP complex and virus growth capability. Taken together, our results identify the cooperation between PB2 and PB1 as an important restricting factor for reassortment of influenza viruses.

# INTRODUCTION

Influenza A viruses are enveloped viruses of the family *Orthomyxoviridae* that cause disease in humans, pigs, horses, dogs, sea mammals and a multitude of avian species (52, 65, 73). The genome of influenza A viruses is composed of eight single-stranded, negative sense RNA segments, which allows the phenomenom of genetic reassortment between viruses that co-infect the same cell. Reassortment is an important mechanism for the generation of new viruses, such as those that sporadically cause influenza pandemics (27).

Influenza pandemics are caused by the introduction of a virus with an HA subtype that is new to human populations, which can result from reassortment (73). However, at least the three last pandemic viruses, i.e. the 1957 "Asian influenza", the 1968 "Hong Kong influenza" and the pandemic (H1N1) 2009 influenza viruses contained, besides the HA gene, one or more polymerase subunits from a non-human source (22, 45, 58), which suggests the importance of these genes for the generation of pandemic viruses through reassortment. Nonetheless, limited compatibility among polymerase subunits from different viruses is also a restricting factor for reassortment (6, 29, 36, 37).

The emergence and establishment of the pandemic (H1N1) 2009 influenza virus in the human population has created a new scenario of possibilities of reassortment with the contemporary, co-circulating viruses, including seasonal H3N2 and H1N1 viruses (18, 46). The aim of this study was to further our knowledge of the factors that govern reassortment

between the pandemic (H1N1) 2009 influenza virus and contemporary human viruses, as this is important for a complete understanding of the factors that may shape the evolution and emergence of future epidemic and pandemic viruses, thus allowing the institution of appropriate surveillance and control measures.

## MATERIALS AND METHODS

**Cells and viruses.** Human embryonic kidney (HEK) 293 and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) containing 5% newborn calf serum and antibiotics. All cells were maintained in a humidified incubator at $37^{o}C$ with a 95% air, 5% $CO_2$ atmosphere.

Viruses A/California/04/2009 (H1N1) (CA04) and A/Kawasaki/UT-SAI-k23/2008 (H1N1) (k23) as well as all reassortants used in this study were produced by reverse genetics, as previously described (41), and propagated and titrated by plaque assay in MDCK cells.

**Construction of plasmids**. The plasmid constructs used for generation of viruses by reverse genetics were produced as previously described (41).

Plasmid pPolINP(0)luc2(0) has been described in Chapter I.

Plasmids for the expression of chimeric PB2s were constructed by making use of restriction

endonuclease sites naturally present in the k23 PB2 open reading frame (ORF). Briefly, primers were designed that introduced restriction endonuclease sites at positions 460, 836, 1318 and 1849 (SalI, PstI, NsiI and Eco0109I, respectively) of the CA04 PB2 gene. These primers were used together with primers that introduced ClaI and XhoI endonuclease sites to the 5' and 3' ends respectively of the ORF of CA04 PB2 in PCR reactions to clone different lengths of the CA04 PB2 gene. The k23 PB2 gene was amplified with primers introducing ClaI and XhoI endonuclease sites to the 5' and 3' ends respectively. The amplified CA04 and k23 PB2 genes were digested with the relevant restriction endonucleases and ligated in combination, between the ClaI and XhoI sites of pCAGGS/MCS vector (23, 43) to generate a series of CA04/k23 PB2 chimeras (Figure 3). All constructs were fully sequenced to ensure the absence of unwanted mutations.

The plasmid pCAGGS k23PB1-FLAG, for expression of a k23 PB1 with a C-terminal fused FLAG tag, was constructed by cloning the PCR-amplified PB1 gene derived from k23 into a ClaI- and NotI-digested pCAGGS-FLAG expression vector, which was constructed by adding the FLAG epitope sequence upstream of the BglII site of pCAGGS/MCS (16).

**Infection of cells.** MDCK cells were infected with viruses at an MOI of 0.001 in MEM supplemented with 0.3% bovine serum albumine (BSA) and 0.5 mg/l TPCK-treated trypsin (MEM/BSA/trypsin). Culture supernatants were harvested at 48 hours post-infection, and the viral titers assessed by plaque assay in MDCK cells.

**Replicon assay.** The plasmid pPolINP(0)luc2(0) was co-transfected into HEK 293 cells along with the pCAGGS/MCS protein expression plasmids for PB2, PB1, PA and NP as indicated, by using the TransIT 293 transfection reagent (Mirus, Madison, USA); 24 hours post-transfection, the cells were assayed for luciferase activity by using a Bright-Glo Luciferase Assay System and a GloMax 96 microplate luminometer (Promega, Madison, USA).

**Immunoprecipitation assay.** HEK 293 cells grown in 6 well plates were co-transfected with 750 ng each of expression plasmids for k23 PA, k23 PB1-FLAG, and k23 PB2 or CA04 PB2. At 24 hours post-transfection, cells were harvested, washed twice with phosphate-buffered saline, resuspended in lysis buffer of the indicated compositions (Fig. 5) and incubated for 30 minutes at 4$^{\circ}$C under rotation. The cell lysates were then clarified by centrifugation at 12,000 $\times$ *g*, and ANTI-FLAG M2 affinity gel (Sigma, St. Louis, USA) was added to the cleared lysate, followed by incubation at 4$^{\circ}$C under rotation for 2 hours. The ANTI-FLAG M2 gel was washed three times with lysis buffer, and the agarose-bound proteins were analyzed by sodium dodecyl sulfate polyacrilamide gel electrophoresis, and Western blotted with mouse monoclonal anti-PB2, or anti-FLAG antibodies.

# RESULTS

In previous studies, when I used reverse genetics to generate a series of single gene reassortants containing each of CA04 genes on a k23 genetic background, it was found that some of the reassortants could not be rescued after three independent attempts; notably, reassortants containing the PB2, PA, or NP genes derived from CA04 on a k23 background could not be generated. As this suggested a limited level of genetic compatibility among ribonucleoprotein (RNP) components between the two viruses, I wished to investigate further this finding, by means of a luciferase-based replicon assay. Plasmid pPolINP(0)luc2(0), which contains the firefly luciferase gene flanked by the non-coding regions of the NP gene derived from A/WSN/33, was co-transfected into HEK 293 cells along with protein expression plasmids containing the PB2, PB1, PA, and NP genes derived from k23 and CA04 in all possible 16 combinations; 24 hours post-transfection, the cells were assayed for luciferase activity. It was found that the activities of the heterogeneous RNP complexes varied substantially. Although the introduction of CA04 PB1, PA, or NP genes in the k23 RNP background caused some reduction in polymerase activity relative to the all k23 RNP complex, the introduction of CA04 PB2 caused the greatest reduction in the polymerase activity (Fig. 1). Interestingly, all mixed RNP complexes that contained a PB2 derived from CA04 in combination with a PB1 derived from k23 showed markedly reduced activity, indicating a possible incompatibility between these two genes.
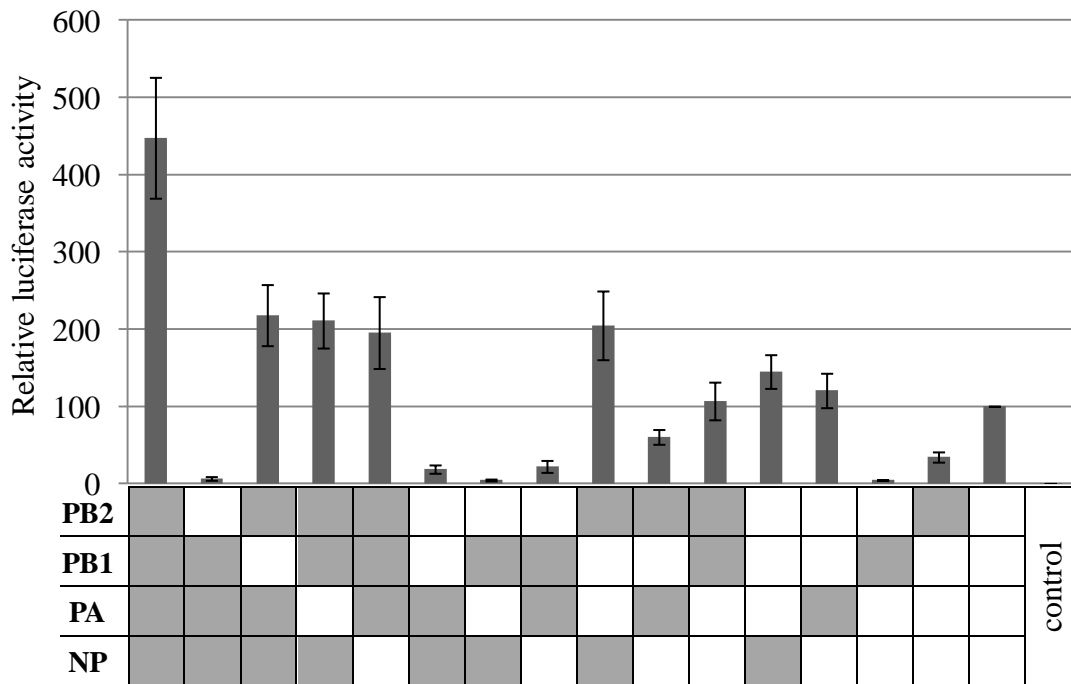
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**Figure 1. Polymerase activities of 16 RNP gene combinations measured in a replicon assay.** Four expression plasmids (PB2, PB1, PA, and NP) for the 16 RNP gene combinations between k23 and CA04, together with pPolINP(0)luc2(0) for the production of virus-like RNA encoding the reporter luciferase gene, were transfected into HEK 293 cells and assayed for luciferase activity after 24h of incubation at 37°C. The values shown are means ± standard deviations for three independent experiments and are standardized to the activities of the expression plasmids for the CA04 RNP complex proteins (100%). Genes derived from k23 and CA04 are shown in gray-shaded and clear boxes, respectively.

Next, I wanted to investigate the relevance of this genetic incompatibility in the context of virus growth. However, most of the single gene reassortants containing CA04 RNP components on a k23 background could not be rescued. This led me to try a different approach for the generation of the reassortant viruses, by using the HA, NA, M and NS genes derived from WSN, which is a virus with high rescue efficiency by reverse genetics.

Reassortant viruses containing different combinations of RNP components between k23 and CA04 on a WSN background could be rescued, and had their rescue efficiency by reverse genetics as well as growth capability and plaque morphology in MDCK cells assessed (Fig. 2). Intriguingly, the pattern of polymerase activity found in the replicon assay did not match that of the virus growth of the corresponding viruses; the reason for this discrepancy is unclear. Interestingly, however, the virus containing the PB2 gene from CA04 with the remaining RNP components from k23 (designated CA04PB2-k23RNP-WSN) showed substantially lower rescue efficiency by reverse genetics, growth capability and plaque size in MDCK cells than all other reassortant viruses.
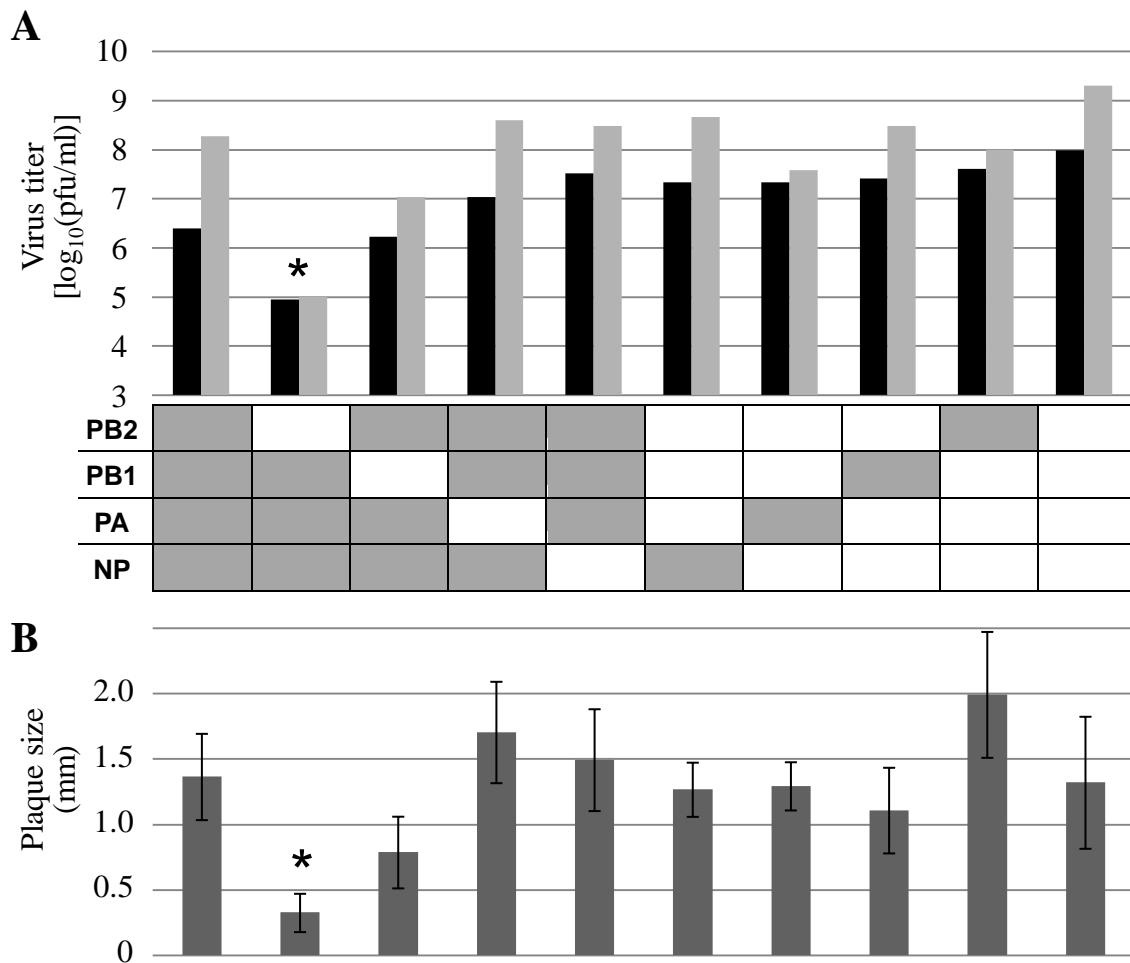
**A**

Virus titer [log$_{10}$(pfu/ml)]

| | PB2 | PB1 | PA | NP |
|---|---|---|---|---|

**B**

Plaque size (mm)

**Figure 2. (A) Rescue efficiency and titers of stock viruses.** Virus titers in the supernatant of transfected HEK 293T cells at 48 hours post-transfection (black columns), and in the supernatant of MDCK cells at 48 hours post-infection (multiplicity of infection = 0.001) (grey columns), measured by plaque assay in MDCK cells. Genes derived from k23 and CA04 are shown in gray-shaded and clear boxes, respectively; the remaining genes are derived from WSN. **(B) Plaque size in MDCK cells.** Plaques were measured at 48 hours post-infection; values represent means ± standard deviations ($n = 50$). The genetic composition of viruses is the same as indicated in panel A.

*Virus CA04PB2-k23RNP-WSN.

The results from the replicon assay, combined with the differential growth capability of reassortant viruses, point to a limited compatibility of the CA04 PB2 with the remaining polymerase subunits from k23. To determine the region in PB2 responsible for this deficiency, we constructed a series of chimeric PB2 expression plasmids in which different extentions of the genes derived from k23 and CA04 were exchanged (Fig. 3). The chimeric plasmids, along with expression plasmids for PB1, PA and NP derived from k23, were used in a replicon assay. Recombinant RNP complexes containing the chimeric PB2s on a k23 background showed substantial variation in polymerase activity: while substitution of either the N- or C-terminal region of the k23 PB2 by CA04 did not reduce appreciably the activity, constructs containing the central region derived from CA04 PB2 showed a substantial loss of activity of the recombinant RNP complexes, suggesting that this region of CA04 PB2 is responsible for its relative inability to function in combination with the remaining RNP components derived from k23.

Reassortant viruses containing the CA04 PB2 with the remaining RNP components derived from k23 showed substantially impaired growth capability, compared with viruses containing either pure k23 or CA04 RNP complexes (Fig. 2). Therefore, we wished to investigate the possibility of adaptation of viruses containing the hybrid RNP complexes. To this end, we subjected the virus CA04PB2-k23RNP-WSN to serial passage in MDCK cells. After five passages, partial restoration of virus growth capability and plaque formation ability
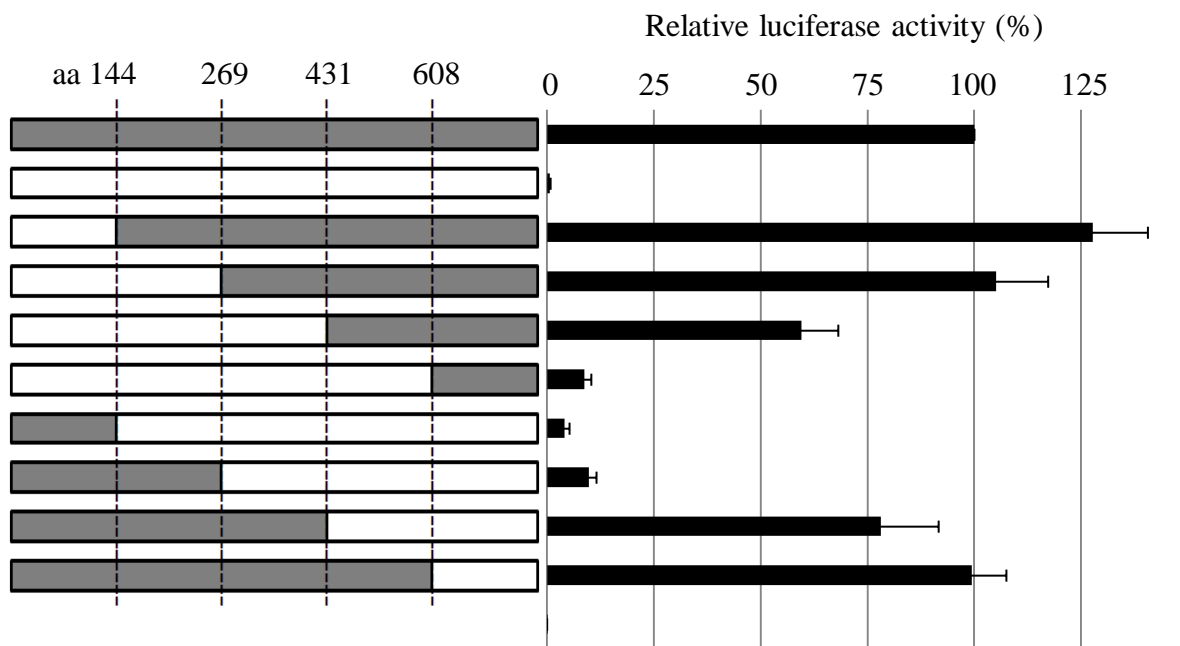
**Figure 3. Schematic diagram of CA04/k23 PB2 chimeras and their effects on RNP activities in a replicon assay.** pCAGGS/MCS plasmids for the expression of chimeric PB2s were constructed, making use of restriction endonuclease sites naturally present in the k23 PB2 ORF corresponding to the amino acid (aa) positions indicated above the diagram. The coding regions of k23 and CA04 PB2 are represented by gray and white colors, respectively. Plasmids for expression of k23 PB2, CA04 PB2, or chimeric PB2 plasmids, together with expression plasmids for PB1, PA, and NP derived from k23 were used in a replicon assay. The values shown are means ± standard deviations for three independent experiments and are standardized to the activities of the expression plasmids for the k23 RNP complex proteins (100%).

was observed (Fig. 4 A and B). Sequencing analysis of the adapted viruses showed compensatory mutations in the PB1 gene in three independent experiments (V285I in one experiment, I423T in two experiments), and also a mutation in NP (T423R) in one experiment (Fig. 4A). No mutation was observed in any other genes. To identify the relevance of each of the compensatory mutations for the polymerase activity, we cloned each of the adapted genes into pCAGGS/MCS, and used them in combination with the plasmids for the remaining RNP components of CA04PB2-k23RNP-WSN in the replicon assay. As shown in Fig. 4C, only the mutation PB1 I423T corresponded with a significant increase in polymerase activity of the mixed RNP complex. Moreover, a reassortant virus containing the CA04 PB2 on a k23 background containing the PB1 I423T mutation (designated CA04PB2-k23PB1[I423T]-k23) could be generated by reverse genetics. These findings, combined with the severely impaired activity of all mixed RNP complexes containing a CA04 PB2 in combination with a k23 PB1 (Fig. 1), indicate that the interaction between PB2 and PB1 is an important restricting factor for influenza virus reassortment, and that an I423T mutation in PB1 can overcome the restriction of polymerase activity of the hybrid RNP complex. Intriguingly, the mutation PB1 V285I, despite improving the growth of CA04PB2-k23RNP-WSN, did not confer substantial polymerase activity in the replicon assay. The mechanism for the partial restoration of viral growth by this mutation remains unclear.
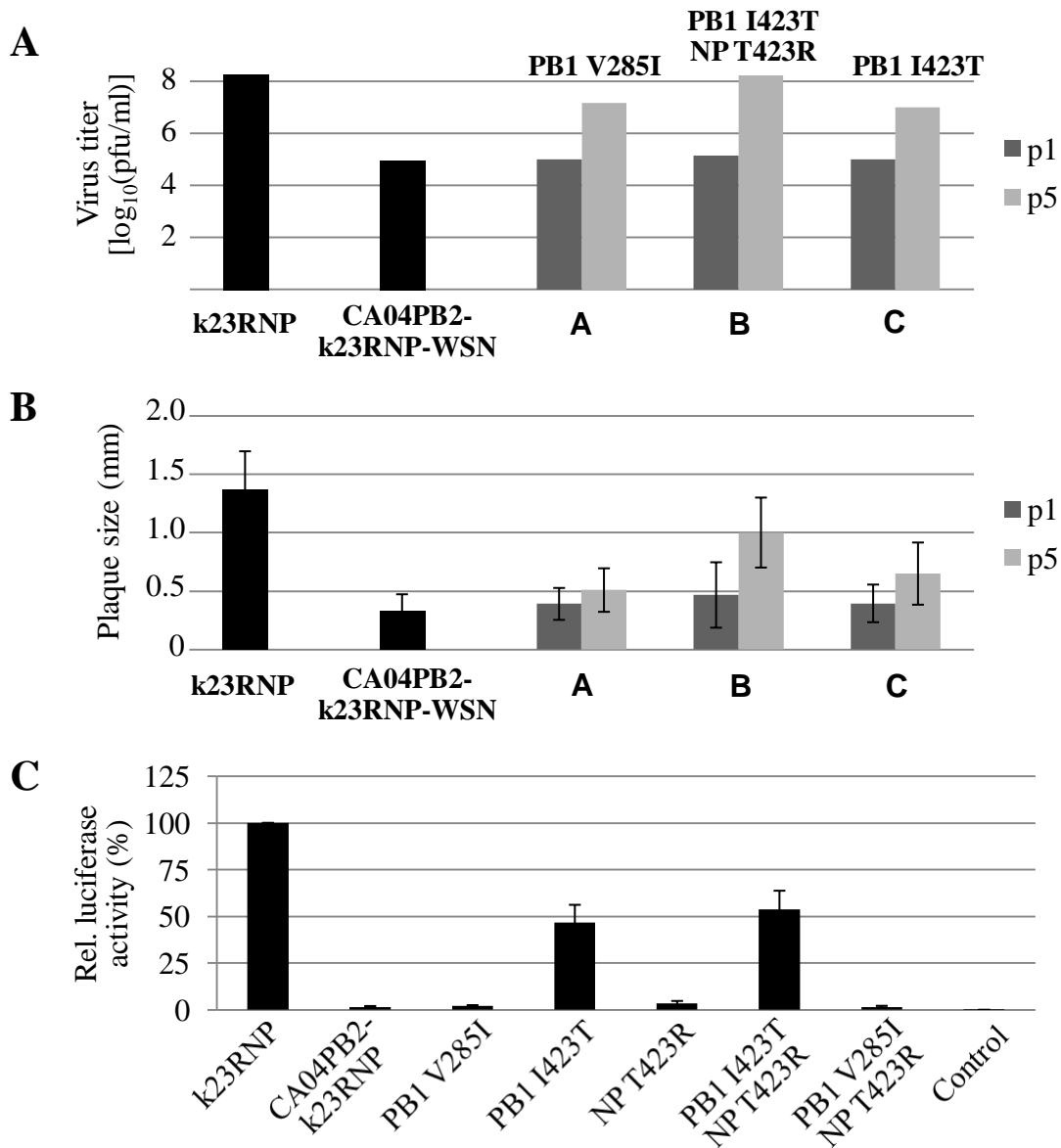
**Figure 4. (A) Virus titers of serially passaged CA04PB2-k23RNP-WSN.** Virus titers in supernatants of infected MDCK cells at passage 1 and 5 are represented in dark gray and light gray respectively. The mutations observed at passage 5 for each virus are indicated above the relevant columns. A, B, and C represent three independent adaptation experiments. Stock titers of k23RNP (RNP components derived from k23 with the remaining genes derived from WSN) and CA04PB2-k23RNP-WSN are given for reference. **(B) Plaque size of serially passaged CA04PB2-k23RNP-WSN in MDCK cells.** Values represent means $\pm$ standard deviations, measured at 48 hours post-infection; $n = 50$. **(C) Effect of mutations observed in serially passaged CA04PB2-k23RNP-WSN on RNP activity in a replicon assay.** Genes with mutations acquired upon five passages in MDCK cells were cloned into pCAGGS/MCS expression vector and used in conjunction with plasmids for remaining RNP components of CA04PB2-k23RNP-WSN in a replicon assay. The mutations tested are indicated below the relevant columns.

Inability to form a heterotrimer is one possible mechanism for incompatibility among polymerase subunits between different viruses (29), so I wondered whether this could explain the incompatibility between CA04 PB2 and k23 PB1. To investigate this, immunoprecipitation assays were performed, using different salt and detergent concentrations, to verify whether CA04 PB2 has reduced affinity for k23 PB1 . However, no difference in the co-immunoprecipitation pattern could be seen between CA04 PB2 and k23 PB2 (Fig. 5). Our results suggest that the functional incompatibility between the polymerase subunits cannot be explained by lack of ability to form a heterotrimer, at least as demonstrated by the methods employed here.
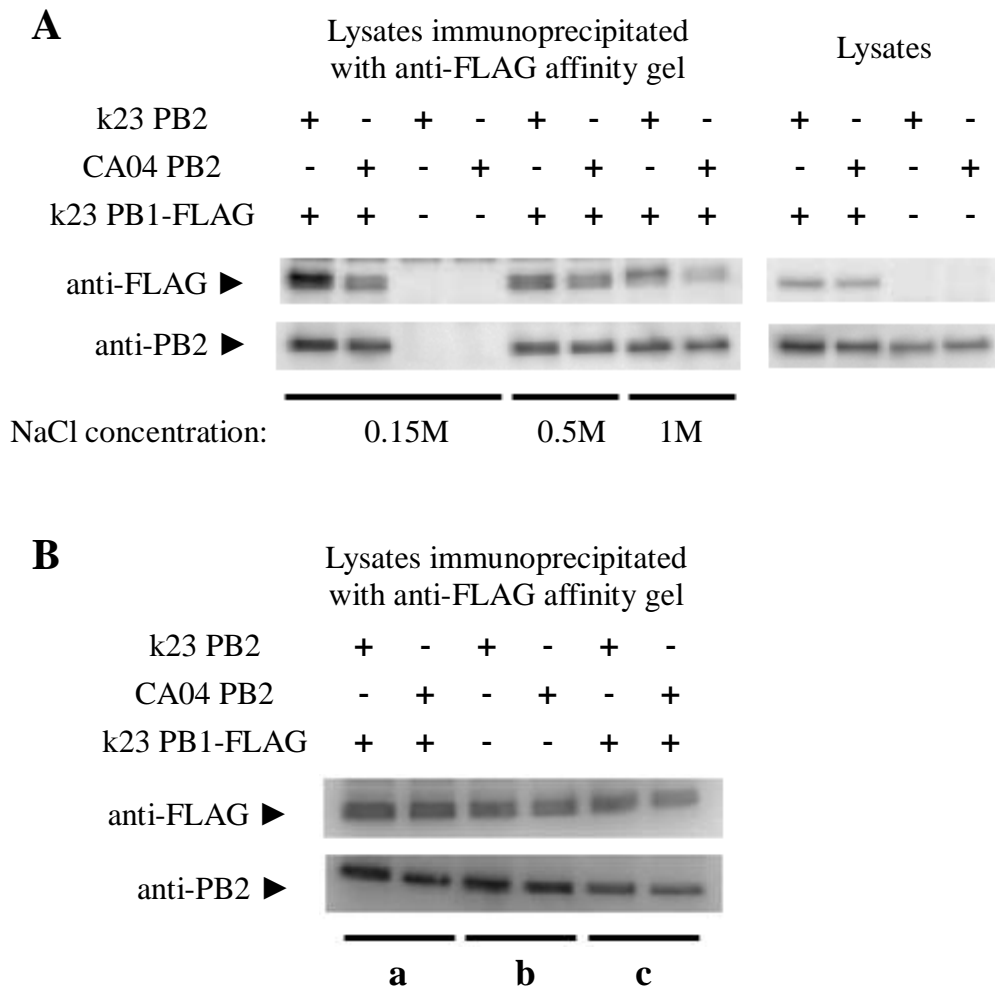
**Figure 5. Immunoprecipitation of polymerase subunits at different salt and detergent concentrations.** HEK 293 cells were co-transfected with expression plasmids for k23 PA, k23 PB1-FLAG, and k23 PB2 or CA04 PB2, as indicated (+). At 24 hours post-transfection, cells were lysed and the cleared lysates were immunoprecipitated with ANTI-FLAG M2 affinity gel. The agarose-bound proteins were analyzed by sodium dodecyl sulfate polyacrilamide gel electrophoresis and Western blotted with mouse monoclonal anti-PB2, or anti-FLAG antibodies. **(A)** Cell lysis and immunoprecipitation were carried out in buffer (50 mM Tris-HCl, pH 7.8, 1% NP40, 1 mM EDTA, protease inhibitor cocktail) with different salt concentrations, as indicated. The cell lysates were also directly Western blotted with mouse monoclonal anti-PB2, or anti-FLAG antibodies. **(B)** Cell lysis and immunoprecipitation were carried out in buffer containing 0.15 M NaCl, and 1% NP40, 0.1% SDS (a); 2% NP40, 0.2% SDS (b); or 5% NP40, 0.5% SDS (c), as indicated.

**DISCUSSION**

In summary, here it was demonstrated that limited compatibility among polymerase subunits is a restricting factor for reassortment between pdm2009 and a contemporary seasonal H1N1 virus, since mixed RNP complexes containig the pdm2009 PB2 gene with other components derived from the seasonal virus showed severely impaired function in a replicon assay, which reflected in poor growth capability of reassortant viruses containing such hybrid RNP. Our mapping of the region of CA04 PB2 responsible for the functional incompatibility with k23 RNP components using chimeric CA04/k23 PB2s pointed to the central region of the gene (Fig. 3), while adaptation of virus CA04PB2-k23RNP-WSN caused a compensatory mutation in amino acid 423 of PB1 that was able to restore the functionality of the mixed RNP complex to some extent (Fig. 4) further evidencing the functional interaction between PB2 and PB1 as a factor limiting the reassortment between these two viruses. However, this position has not been identified as an interaction site between PB1 and PB2 in immunoprecipitation experiments, as reported by other groups (reviewed in reference 2), which is in agreement with our finding that CA04 PB2 interacted with k23 PB1 as well as k23 PB2 (Fig. 5). The reason for the limited activity of the mixed RNP complex therefore remains unclear, and is probably due to subtler functional constraints, that is, the level of incompatibility between the two proteins was not explained by the lack of heterotrimer formation, yet the resulting hybrid complex was nonetheless functionally impaired. Isoleucine at position 423 is highly conserved (approximately 99.7%) among influenza A viruses, and although analysis of 3156 unique influenza A virus PB1 sequences, including H1N1, H3N2, and H5N1 subtypes, showed two amino acid substitutions at this position (leucine and valine), threonine was not found. While the significance of this is not clear, the high degree of conservation of isoleucine at this position may indicate that adaptation through an I423T

mutation could be restricted due to additional constraints.

The findings reported here contrast with previous observations by our group, in which a high degree of genetic compatibility was found between pandemic (H1N1) 2009 influenza virus and a contemporary H5N1 virus, with all combinations of RNP components between those two viruses showing substantial polymerase activity, and producing viruses with high growth capability (47). Further efforts are required for a thorough understanding of the factors governing reassortment in influenza viruses, as this will improve our ability to understand, predict and control influenza epidemics and pandemics in the future.

## CONCLUDING REMARKS

In recent years, increased attention has been given to the study of factors that govern the reassortment of influenza viruses, and to the implications of reassortment for viral growth capability, pathogenicity, transmissibility, and drug resistance (6, 20, 28, 29, 34, 37, 48, 59, 62); in fact, studies that built upon the findings described here have already been published (59, 62). Although promising and exciting, this field of study is also challenging, given the complexity of the genetic diversity of influenza viruses. Nonetheless, I believe that this continuing investigation will provide knowledge that will allow better control of influenza in the future.

The findings presented here represent my contribution to this field of study. Although countless questions remain, these studies have already provided knowledge that may be useful in guiding future influenza surveillance and control efforts. In particular, the results presented in Chapter I indicate that the emergence of the pandemic (H1N1) 2009 influenza virus may have brought us one step closer to an H5N1 pandemic. Considering the role of swine as mixing vessels in the generation of pandemic viruses, and their susceptibility to both H1N1 and H5N1 viruses, it is imperative that efforts be made to closely monitor and control influenza in pigs; although prospective surveillance of influenza viruses is done extensively in wild birds, such activity is lacking in swine. A surveillance system for influenza in swine similar to the Global Influenza Surveillance Network for human influenza conducted by the

WHO has been suggested for the rapid detection of novel viruses in this species. (8). Besides surveillance, measures to control and prevent influenza infection in pigs must be intensified. Vaccination is recommended by the World Organization for Animal Health (OIE) as part of the strategy to control highly pathogenic avian influenza in poultry, in conjunction with intense surveillance and biosecurity measures (71). Clearly, the same level of proactive surveillance and control is required in swine to effectively prevent future pandemics. Biosecurity measures should be implemented to restrict contact between pigs and wild waterfowl; also movement restrictions and vaccination of pigs against influenza, including pdm2009 and H5N1 viruses, should be considered.

The studies presented here also show that reassortment between seasonal and pdm2009 viruses may represent a threat, especially if the resulting viruses contain a seasonal H1HA, and NA and M derived from pdm2009 (Chapter II). This finding also has implications for surveillance and containment measures, especially considering the remarkable history of this seasonal virus HA, which originates from the Spanish influenza pandemic of 1918-1919, and has spanned most of the 20th century, including a puzzling reemergence in the "Russian" influenza outbreak of 1977, after a hiatus of nearly 20 years. Finally, the results presented in Chapter III show the limitations in reassortment between pdm2009 and seasonal H1N1 viruses, with respect to the polymerase complex; they identify the cooperation between PB2 and PB1 as a restricting factor for reassortment in influenza viruses.

Further efforts are needed to thoroughly understand the factors that govern reassortment in influenza viruses, including the genetic compatibility between co-circulating viruses. Such knowledge will provide tools to improve our ability to predict, prevent, and control future influenza pandemics.

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