Aus dem Biomedizinischen Forschungszentrum, Institut für Virologie
Geschäftsführender Direktor: Prof. Dr. Stephan Becker
des Fachbereichs Medizin der Philipps-Universität Marburg

in Zusammenarbeit mit dem Universitätsklinikum Gießen und Marburg GmbH,
Standort Marburg

Identification and characterization of host specificity factors of a lethal human influenza H5N1 isolate

Inaugural-Dissertation
zur Erlangung des Doktorgrades der Naturwissenschaften
dem Fachbereich Medizin der Philipps-Universität Marburg
vorgelegt von

Benjamin Mänz aus Eschwege

Marburg, 2012

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg
am: 09.03.2012

Gedruckt mit Genehmigung des Fachbereichs.

Dekan:	Prof. Dr. Matthias Rothmund
Referent:	Prof. Dr. Hans-Dieter Klenk
1. Korreferent:	Prof. Dr. Eberhard Weihe

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This thesis is in the form of a cumulative dissertation.

List of publications and manuscripts:

Peer-reviewed articles that build the basis for the underlying thesis:

3.1. page 29:

Mänz, B., Matrosovich, M., Bovin, N. & Schwemmle, M. A polymorphism in the hemagglutinin of the human isolate of a highly pathogenic H5N1 influenza virus determines organ tropism in mice. *Journal of virology* **84**, 8316-8321, (2010).

3.2. page 36:

Mänz, B., Brunotte, L., Reuther, P. & Schwemmle, M. Adaptive mutations in NEP compensate defective RNA replication of H5N1 influenza viruses in humans. (Submitted for publication)

3.3. page 62:

Zimmermann, P., **Mänz, B.**, Haller, O., Schwemmle, M. & Kochs, G. The viral nucleoprotein determines mx sensitivity of influenza a viruses. *Journal of virology* **85**, 8133-8140, (2011).

3.4. page 71:

Mänz, B., Götz, V., Wunderlich, K., Eisel, J., Kirchmair, J., Stech, J., Stech, O., Chase, G., Frank, R. & Schwemmle, M. Disruption of the viral polymerase complex assembly as a novel approach to attenuate influenza A virus. *The Journal of biological chemistry* **286**, (2011).

See chapter 3 for personal contribution.

Further list of publications and manuscripts:

Peer-reviewed articles that additionally resulted from the work of the underlying thesis:

Reuther, P., **Mänz, B.**, Brunotte, L., Schwemmle, M. & Wunderlich, K. Targeting of the influenza A virus polymerase PB1-PB2 interface indicates strain-specific assembly differences. *Journal of virology*, (2011).

Bogs, J., Kalthoff, D., Veits, J., Pavlova, S., Schwemmle, M., **Mänz, B.**, Mettenleiter, T. C. & Stech, J. Reversion of PB2 627E to 627K during replication of an H5N1 clade 2.2 virus in mammalian hosts depends on origin of the nucleoprotein. *Journal of virology*, (2011).

Wunderlich, K., Juozapaitis, M., **Mänz, B.**, Mayer, D., Götz, V., Zohner, A., Wolff, T., Schwemmle, M. & Martin, A. Limited compatibility of polymerase subunit interactions in influenza A and B viruses. *The Journal of biological chemistry* **285**, 16704-16712, (2010).

Wunderlich, K., Mayer, D., Ranadheera, C., Holler, A. S., **Mänz, B.**, Martin, A., Chase, G., Tegge, W., Frank, R., Kessler, U. & Schwemmle, M. Identification of a PA-binding peptide with inhibitory activity against influenza A and B virus replication. *PloS one* **4**, e7517, (2009).

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List of abbreviations

A	alanine
aa	amino acid
cRNA	copy RNA
D	aspartic acid
DNA	desoxyribonucleic acid
E	glutamic acid
e.g.	exempli gratia
ELISA	enzyme-linked immunosorbent assay
Fig.	figure
FluA	influenza A virus
HA	hemagglutinin
HPAI	highly pathogenic avian influenza
HPAIV	highly pathogenic avian influenza virus
IFN	interferon
ISG	interferon stimulated genes
K	lysine
LD50	lethal dose 50
LPAIV	low pathogenic avian influenza virus
M1	matrix protein
M2	proton channel
MOI	multiplicity of infection
Mx	myxovirus resistance
mRNA	messenger RNA
N	asparagine
NA	neuraminidase
NEP	nuclear export protein
NES	nuclear export sequence
NLS	nuclear localization sequence
NP	nucleoprotein
NS1	non structural protein 1
ORF	open reading frame
PA	polymerase acidic protein
PAMP	pathogen associated molecular pattern
PB1	polymerase basic protein 1

PB2	polymerase basic protein 2
PCR	polymerase chain reaction
PRR	pathogen recognition receptor
RdRP	RNA-dependent-RNA-polymerase
RNA	ribonucleic acid
RNA Pol II	cellular RNA polymerase II
RNP	ribonucleoprotein
SOIV	swine origin influenza virus
T	threonine
TLR	toll-like receptor
vRNA	viral RNA
wt	wildtype

Abstract

Influenza A viruses are major human and avian pathogens. Despite a species barrier, subtypes of influenza A can transmit from the avian reservoir to humans and widely spread in the population. Since H5N1 viruses circulate in the avian reservoir and cause high lethality rates when transmitted to humans, infections with the H5N1 subtype pose an ongoing threat. Although human-to-human transmission is a rare event, rapid evolution of the virus might result in a strain, which gains the ability to spread in the human population, leading to high morbidity and mortality. Advanced surveillance by understanding the mechanisms by which influenza viruses acquire the ability to cross the species barrier from birds to humans and new strategies to improve current vaccines are needed to control future pandemics. In this study, the fatal human case isolate A/Thailand/1(KAN-1)/2004 (H5N1) (KAN-1) was analyzed to examine mechanisms of H5N1 viruses to overcome host range restriction.

• We were able to identify an adaptive mutation in KAN-1 hemagglutinin (HA). A polymorphism leading to an amino acid change in the HA that was previously reported to be positively selected during replication in humans altered the organ tropism of KAN-1 in mice and ferrets. In the mouse model we found an increased replication of the selected variant in the lung.

• In a genetic analysis of the KAN-1 virus, we identified further mutations crucial for adaptation to the mammalian host. Interestingly, the KAN-1 polymerase was poorly adapted to human cells, in contrast to other H5N1 viruses isolated from humans. We identified the NEP protein as a new pathogenicity factor of H5N1 viruses in humans, which is able to overcome this incomplete adaptation of the KAN-1 and avian H5N1 polymerases in human cells. Furthermore, functional studies revealed that the restriction of avian influenza polymerases in mammals is due to a general defect in RNA-replication and not transcription.

• Since the human MxA GTPase is an important factor in the immune response against influenza viruses, we analyzed its antiviral activity against KAN-1. KAN-1 proved to be sensitive, while an isolate of the 2009 pandemic was relatively resistant to MxA. We were able to determine the viral nucleoprotein (NP) as the determinant for MxA sensitivity *in vitro* and *in vivo*. In addition, we identified mutations in NP responsible for resistance against MxA and could draw conclusions about the evolution of NP.

• Based on our knowledge about protein-protein interactions in the polymerase complex, we developed a new strategy to create polymerase assembly mutants as a basis for live attenuated vaccines against H5N1. Vaccination of mice with these mutants showed protection against homologous and heterologous challenge with lethal doses of H5N1 viruses including KAN-1, therefore providing new options for live attenuated vaccine design.

Zusammenfassung

Influenza-A-Viren sind wichtige humane und aviäre Krankheitserreger. Trotz einer Speziesbarriere können aviäre Influenza-A-Viren von Wasservögeln auf Menschen übertragen werden. Sporadisch kann es dabei zu einer Etablierung des Virus in der menschlichen Bevölkerung kommen, woraus eine Pandemie resultieren kann. Humane Infektionen mit aviären Influenza-A-Viren des Subtyps H5N1 stellen dabei eine stete Bedrohung dar. Das Verständnis der Mechanismen, durch die Influenza-A-Viren die Spezies-Barriere zwischen Vögeln und Menschen überwinden und Verbesserungen der derzeitigen Impfstrategien sind essentiell für die Kontrolle von zukünftigen Pandemien. Das in dieser Arbeit verwendete H5N1 Virus A/Thailand/1(KAN-1)/2004 (H5N1) (KAN-1) wurde aus einem Menschen isoliert, der an dieser Infektion starb. Es lässt sich an diesem Beispiel detailliert untersuchen, welche Veränderungen ein H5N1 Virus benötigt, um die Speziesbarriere vom Vogel zum Menschen zu überschreiten.

• Wir konnten eine Adaption im KAN-1 Hämagglutinin (HA) nachweisen, indem wir für eine im Menschen positiv selektionierte Mutation im HA-Protein einen veränderten Gewebetropismus in der Maus und im Frettchen beobachten konnten. Im Mausmodel konnten wir eine erhöhte Replikation dieser Virusvariante in der Lunge feststellen.

• Durch eine genetische Analyse des KAN-1 Virus konnten wir entscheidende Mutationen für die Adaption an den Säuger identifizieren. Interessanterweise zeigte sich, dass im Gegensatz zu anderen humanen H5N1-Viren die KAN-1-Polymerase nur schwach an den Menschen angepasst ist. Mit dem Nachweis, dass eine Mutation im NEP Protein von KAN-1 die nicht genügend angepasste Polymerase kompensiert identifizierten wir NEP als neue Pathogenitätsdeterminante der H5N1-Viren im Menschen. Zudem zeigten mechanistische Studien einen Replikationsdefekt der aviären Influenza-Polymerasen im Menschen.

• Da die menschliche GTPase MxA ein wichtiger Faktor der Immunantwort gegen Influenza-Viren ist, analysierten wir ihre antivirale Aktivität gegen KAN-1. KAN-1 erwies sich als sensitiv, während ein Isolat der Pandemie von 2009 relativ resistent gegenüber MxA war. Wir konnten das virale Nukleoprotein (NP) als die Determinante für MxA-Sensitivität *in vitro* und *in vivo* bestimmen. Zudem identifizierten wir Mutationen in NEP, die verantwortlich für die Resistenz gegenüber MxA sind und konnten Rückschlüsse auf die Evolution von NP ziehen.

• Basierend auf unserem Wissen über Protein-Protein-Wechselwirkungen innerhalb des Polymerase Komplexes entwickelten wir eine neue Strategie zur Entwicklung von Polymerase-Assembly-Mutanten als Basis einer Lebendvaccine gegen H5N1. Die Impfung von Mäusen mit diesen Mutanten vermittelte einen Schutz vor homologer und heterologer Infektion mit tödlichen Dosen von H5N1 Viren inklusive KAN-1. Diese neue Strategie bietet daher eine Ergänzung zu bereits verfügbaren Lebend-Impfstoffen.

1 Introduction

1.1 Relevance of influenza research

Influenza A viruses are major human and avian pathogens, infecting the respiratory system or the gastrointestinal tract of the host. In humans, influenza virus infection leads to febrile respiratory illness. The disease is characterized by a sudden onset of high fever, cough, headache, sore threat and muscle pain from which most patients recover after several days up to two weeks. However, influenza may produce life-threatening complications, such as pneumonia or acute lung failure especially in children, elderly or immuno-compromised persons. Damage to the respiratory mucosa even allows the entry of other pathogens, which can result in secondary bacterial infections. Incomplete protection by vaccines and the emergence of resistant viruses to currently used antiviral drugs pose a challenge in the fight against influenza infections.
Recurring epidemics and sporadic pandemics account for significant morbidity and mortality among humans as exemplified by the 1918 pandemic with up to 50 million deaths worldwide (156). Influenza outbreaks have been recorded since the middle ages (99) and the 2009 pandemic once more demonstrates the topicality. The pandemic threat is caused by zoonotic infections of immunologically naïve humans with avian or swine derived influenza viruses. The mechanisms by which influenza viruses stably adapt to humans are still largely not elucidated. Research in this field and the development of improved vaccines are needed to prepare for future pandemics.

1.2 The influenza A virus

1.2.1 Taxonomy

Influenza viruses, with the genera A, B, C, the Thogoto- and the Isavirus form the family *orthomyxoviridae*. This family is characterized by a single-stranded, segmented RNA genome with negative orientation. While influenza C viruses circulate in humans and pigs, influenza B viruses have been isolated only from humans and rarely from seals (8). Influenza A viruses show the greatest host range and infect humans as well as many other mammals, e.g. pigs, horses and whales and many species of birds. Wild waterfowl is the primary host reservoir of the influenza A viruses (61).
Influenza viruses differ by the number of genome segments (eight for influenza virus A

and B, seven for influenza virus C). Influenza A viruses can also be distinguished by the variability of the surface antigen proteins. 16 HA subtypes and 9 NA subtypes are known (2, 35). The World Health Organization standardized the nomenclature for influenza viruses in 1980. The isolate is designated in the order genus, host species (not required for human influenza A viruses), place of isolation, isolate number and year of isolation. In the case of influenza A viruses, the subtype is also added in parentheses, for example: A/Thailand/1(KAN-1)/2004 (H5N1).

1.2.2 Morphology and encoded proteins

Influenza viruses are spherical or filamentous structures, with a diameter of about 100 nm (15). The segmented RNA genome, encapsidated by many molecules of the nucleoprotein, forms a helical capsid (6). At its ends, the trimeric RNA dependent RNA polymerase (RdRP) is bound. The complex, consisting of RdRP, RNA and NP is known as ribonucleoprotein (RNP) (Fig. 1). Another protein found in virus particles in low copy number is the nuclear export protein NEP (NS2) (127, 177). The nucleoproteins of the RNP interact with the matrix protein (M1), which forms a layer inside of the plasma membrane of the host cell derived lipid envelope (139). The M2 protein, which forms a homotetramer and functions as a proton channel, is embedded as a transmembrane protein in the envelope membrane in small quantities (77). As virus-encoded glycosylated surface proteins (spikes) the hemagglutinin (HA) and neuraminidase (NA) are incorporated into the lipid envelope and protrude about 10 nm with a diameter of about 3 nm (136). The HA exists as a homotrimer and mediates receptor binding and membrane fusion. The NA exists as a homotetramer and mediates cleavage of the receptor (162).

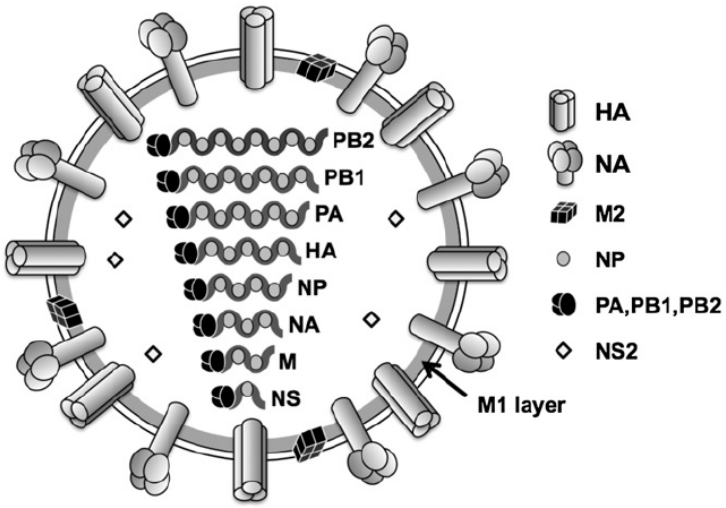

Fig. 1: Schematic drawing of an influenza virion
(from (154)) The viral RNPs are enclosed by the M1 protein layer inside of the cell derived lipid bilayer. The HA, NA and M2 proteins are integral membrane proteins.

1.2.3 Replication cycle

Adsorption, penetration, uncoating

The replication cycle of the influenza virus begins with the adsorption of infectious virions on permissive host cells, initiated by binding of the hemaglutinin to the cellular receptor sialic acid. For successful virus infection a cleavage of the hemagglutinin precursor HA_0 into the subunits HA_1 and HA_2 that stay connected via a disulfide bridge is essential (73). The adsorption of the virus particle to the cell is mediated by binding of the receptor-binding site of the hemagglutinin to sialic acid (140). Sialic acids are sugars that occur as modifications of membrane proteins and glycolipids on cell surfaces of most animal tissues, explaining the wide host range of influenza viruses. The hemagglutinin of avian influenza viruses binds preferentially to α2,3-linked sialic acids, whereas human influenza isolates recognize α2,6-linked sialic acids (91, 129). Receptor-mediated endocytosis leads to penetration of the cell membrane (Fig. 2). A subsequent acidification of the endosomal vesicles by cellular H^+-ATPases causes an irreversible conformational change in the hemagglutinin (138, 170). The hydrophobic fusion peptide at the amino terminus of HA_2 is stretched and integrated into the endosomal membrane, which causes the membrane fusion by further conformational changes (169). The viral proton channel M2 transfers the acidification of the endosome to the interior of the virion (120), which dissolves the RNP complexes from the M1 matrix protein (58). Ensuing membrane fusion, the RNP complexes reach the cytoplasm and are actively transported through the nuclear pore complex into the nucleus of the host cell, utilizing nuclear localization signals (NLS) of the proteins PB1, PB2, PA and NP (171, 172).

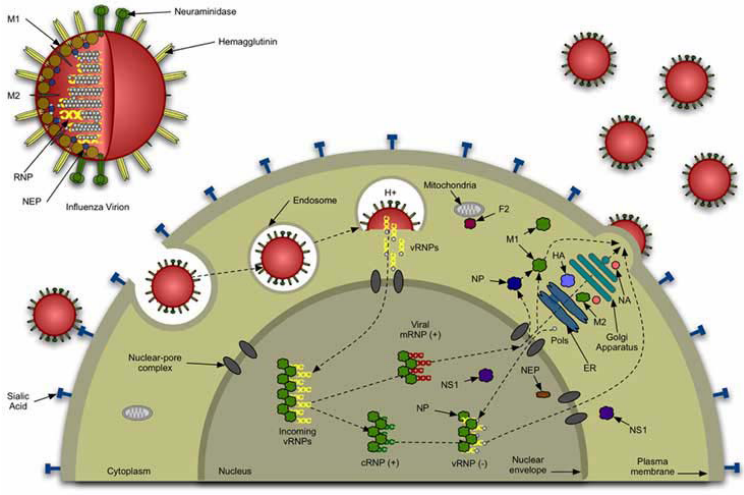

Fig. 2: The influenza virus life cycle (from (142))

Transcription, translation and protein modification

Since influenza viruses possess an RNA genome of negative polarity, which cannot be translated from the cellular ribosomes, the viral RNA dependent RNA polymerase (RdRP) starts by transcribing mRNA from the viral RNA (vRNA) template of the RNP. To initiate trancription, the RdRP needs 5'-cap structures that serve as a primer for the

viral polymerase. For this purpose, influenza viruses make use of the so-called cap-snatching. In this process, the PB2 protein binds cap structures of cellular mRNAs (50, 159) and thereby enables the endonuclease within PA to cleave the mRNA 9-17 nucleotides behind the 5'-end (29, 178). By adding a guanine complementary to the vRNA, PB1 initiates transcription. The elongation proceeds up to the uridine-rich region approximately 15-22 nucleotides in front of the 5'-end of the vRNA (78), where the RdRP reiterate the incorporation of A residues thereby synthesizing the 3'-polyA tail.
The mRNAs derived from the gene segments 7 and 8 are processed post-transcriptionally by the cellular spliceosome. For the following translation, all viral mRNAs are exported to the cytoplasm (172), where the virus exploits the cellular translation machinery. At the beginning of the infection, the proteins of the polymerase (PB2, PB1, PA), NP, NS1, NS2, and M1 are synthesized as early proteins on free ribosomes and transported by means of their nuclear localization signal, or their small size into the nucleus (96, 103, 107, 172). The integral membrane proteins (HA, NA, M2) are synthesized in the later stages of infection and in contrast to the other viral proteins at ribosomes of the rough endoplasmic reticulum (rER). These proteins undergo post-translational modifications on their way from the rER via the cis-and trans-Golgi network to the plasma membrane of the cell. HA and NA are glycosylated in the ER and Golgi apparatus and further processed. The N-terminal signal sequence of the HA is cleaved in the ER lumen by a signal peptidase. The terminal sialic acids of the attached oligosaccharides are removed by neuraminidases to prevent cross-linking of the HAs with the carbohydrate side chains (11). In the case of a multibasic cleavage site in the amino acid sequence of the HA_0 precursor protein, HA can already be activated in the Trans-Golgi network by the ubiquitous protease furin (149). Multibasic cleavage sites are a characteristic of highly pathogenic avian influenza viruses of subtypes H5 and H7. Further post-translational modifications for example are palmitoylation (152) and phosphorylation of M2 (59), sumoylation of NS1 and NEP (111, 176) and NEP phosphorylation (127).

Genome replication

In contrast to mRNA-transcription, the replication of the viral genome is primer independent. First, a full length copy RNA (cRNA) of the vRNA is synthesized and serves as a template for new viral genomes (vRNAs) (76). Early in the infection only mRNA synthesis is observed, whereas increasing amounts of cRNA are found later in infection. It is still controversially discussed, whether there is a switch from transcription to replication. A first hypothesis says that the polymerase performs full-length cRNA synthesis from the beginning of the infection. However, the cRNA transcripts are

shorter due to impaired elongation in the absence of newly synthesized NP (60, 135). Additionally the cRNA needs to be stabilized by NP and the viral polymerase, in order not to be degraded (163). The second hypothesis favors the model of a switch from transcription to replication mediated by binding of free NP molecules to the polymerase (105). Furthermore, the NEP (NS2) protein was shown to alter the relative efficiency of transcription and replication (128) possibly via the generation of small viral RNAs which correspond to the first 22-27 nt of each vRNA 5'end and which accumulate to high numbers during viral infection (118, 160).

The increased accumulation of vRNA to cRNA points towards differences in generation of these two full-length RNA species. For vRNA synthesis, one current model describes that the reaction is carried out by a non-resident polymerase in trans. This might result in initiation of several polymerases during one polymerization cycle and explain the higher abundance of vRNA above cRNA during the infection.

The structures of vRNA and cRNA promoter regions determined by NMR demonstrated some remarkable differences that might indicate two distinct RNP conformations (115). In fact, there is evidence that different regions in the polymerase recognize the vRNA or cRNA promoter. In the current model, cRNA sythesis is believed to initiate at nucleotide position 2 on the vRNA, while the first nucleotide is subsequently added. The underlying mechanism for vRNA sythesis is suggested to be a prime and re-align mechanism, in which the first two bases are transcribed at the internal positions 4 and 5 of the cRNA and then shifted to the 3' end of the template terminus to allow generation of a full length vRNA.

Morphogenesis and release of virus particles

Since the replication of influenza viruses, in contrast to most RNA viruses, takes place in the nucleus, the newly synthesized vRNPs must be transported from the nucleus to the cell membrane. For export, a nuclear accumulation of the M1 protein is necessary (87). Nuclear export is mediated by the NEP protein, which has a nuclear export signal (NES) and interacts with cellular CRM1 and nucleoporin (109). The interaction between NEP and the vRNPs is mediated by M1 (165, 177). The assembly of the virions takes place at the plasma membrane of infected cells in specific plasma membrane domains, the so-called lipid rafts, where the surface proteins HA, NA and M2 are accumulated (133, 180). HA and M2 are present as tetramers to form complexes, NA is arranged as a trimer. Despite a strong intracellular expression, M2 is of low abundance in both the cell membrane, as well as in virions (179). A crucial role in the morphogenesis plays the M1 protein, since it interacts with the vRNPs, as well as the cell membrane. It is believed that the process of release is initiated by a direct interaction of M1 with the

cytoplasmic domains of HA and NA (10, 43, 44). To ensure production of infectious virus particles, packaging of each of the eight genome segments is required. Although the mechanism for efficient genome packaging is not clearly understood, it was shown that it doesn't take place randomly, but rather by selective incorporation via packaging sequences in the vRNAs (37). During the budding process, the viral neuraminidase cleaves off terminal sialic acids from the viral surface as well as from the host cell and thus prevents agglutination of virions with the host cell and each other (112). Moreover, uncleaved HA_0 is cleaved by extracellular proteases in HA_1 and HA_2.

1.3 Human Influenza

Influenza A viruses that have efficiently adapted to humans can be transferred easily through airborne transmission and spread quickly. In the upper respiratory tract, epithelial cells are infected first (92, 93) and newly formed viruses are released at the apical side of the plasma membrane into the airway. The virus can spread to the lower respiratory tract, where the ciliated cells and mucus-producing layers of skin are destroyed. Macrophages, neutrophils and T-cells migrate into the infected areas to eliminate the virus, where especially macrophages secrete pro-inflammatory cytokines, which are responsible for the systemic symptoms of influenza. In exceptional cases this might result in a cytokine storm that leads to death (117).

Every 1-2 years new epidemic influenza A strains appear under the pressure of the adapted immune system. The strains differ mainly by positively selected mutations in the surface proteins HA and NA. This process is called antigenic drift, which is generated by the large error rate and the lack of a proofreading function of the viral polymerase (167, 173). Another way to evade the immune response is the exchange of entire gene segments (reassortment) between different Influenza A subtypes. Such reassortant viruses may arise, when two different influenza viruses infect a single cell. When the exchange of gene segments involves surface proteins, this reassortment is called antigenic shift. The coexistence of humans, pigs and poultry under poor hygienic conditions favors such processes, because pigs are highly susceptible for human and avian influenza viruses. Due to the processes of antigenic drift and antigenic shift, there is no lasting immunity against influenza viruses, neither after natural infection or vaccination against a former strain.

In contrast to mild to moderate epidemics occurring almost every winter season, pandemics are rare events, taking place every 10 to 50 years for at least 500 or more years back in the past (99). Typically for influenza pandemics is the shift in mortality

from the elderly to younger age groups. In the last hundred years, four pandemics with different severity were recorded. Remarkably, all human influenza pandemics originated from avian influenza. The 1918 pandemic has been the most devastating pandemic in human history, claiming up to 50 million lives (66). From archived formalin-fixed lung autopsy materials and from frozen unfixed lung tissue of a flu victim, who was buried in November 1918 in the permafrost, the entire genome of the pandemic strain has been reconstructed (155) and the virus was identified as an H1N1 virus, which appeared to be a descendant of a bird virus (125). The two following pandemics were caused by reassortant viruses containing three (H2N2 1957) or two (H3N2 1968) genes of avian origin (69). The most recent pandemic was the H1N1 pandemic in 2009. The virus was identified to be a triple reassortant with genes of swine, human and avian origin (104, 141). It contains none of the hallmarks of highly pathogenic avian influenza viruses and only some human aa signatures that were already present in the swine viruses (22). Noteworthy, pandemic influenza seem to have a strong dependence on viruses of avian origin. Thus, in the recent past, serious illness monitored in people infected with viruses of avian origin raised public health interest and concern for a future pandemic.

1.4 Avian Influenza

The entire range of influenza A virus subtypes is found in wild aquatic birds (166), which therefore form the natural reservoir of all influenza A viruses (Fig. 3). The most abundant low pathogenic avian influenza viruses (LPAIV) generally do not cause disease in their natural hosts. Viral replication takes place in the epithelia of the gastrointestinal tract and virus particles are excreted in high titers (168). However, domesticated poultry is considered particularly vulnerable to influenza, as LPAIV infection can already reduce growth rates in broiler poultry (20). When adapting to poultry, LPAIV of subtypes H5 and H7 also possess the potential to mutate into a highly pathogenic avian influenza virus (HPAIV) form (20, 90). These highly pathogenic variants are distinguished mainly by a multibasic cleavage site in the

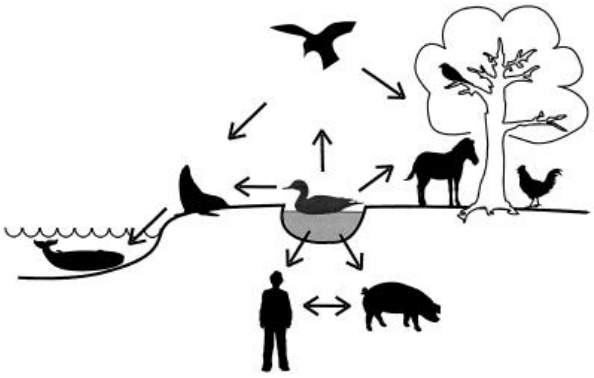

Fig. 3: Host spectrum of influenza A viruses (from (61)) Influenza A viruses have a broad host spectrum with wild waterfowl as their natural reservoir.

HA precursor protein, which may be cleaved by ubiquitous proteases such as Furin, enabling the systemic spread of the virus (62, 63, 73). In contrast, the monobasic cleavage site of most LPAIV is cleaved by trypsin-like proteases, which leads to restricted organ tropism.
HPAIV infections are not only devastating for poultry farms, but also possess a threat on public health, as documented transmission of avian influenza to humans predominantly occurred in association with HPAI epizootics (154).

1.4.1 The H5N1 epizootic

In 1997 the first disease in humans caused by an H5N1 strain was documented (1). Within a year, this epizootic caused 18 human cases and six deaths in Hong Kong (101). H5N1 continued to circulate in birds in China and reappeared in epizootic form in 2003. Rapid evolution of viruses in birds, acquisition of mutations that were also found in the genome sequence of the 1918 isolate and growing numbers of human zoonotic infections suggest H5N1 as a possible base for another pandemic (148). Although the H5N1 influenza remains largely a disease in birds, infection of humans still occurs frequently through direct contact with diseased poultry. To date (2011-12-06) the WHO confirmed 571 human H5N1 cases resulting in 335 deaths (59%). The virus analyzed in this study, A/Thailand/1(KAN-1)/2004 (KAN-1), was isolated from a fatal case in Thailand in the beginning of 2004, when widespread H5N1 infections were recognized in Thailand and Viet Nam (123). The patient was a 6 year old before healthy boy, who suffered approximately 4-5 weeks from the H5N1 infection (25). Subsequently, the KAN-1 virus was shown to be highly pathogenic for mice and ferrets (47, 86).
Although H5N1 is the most devastating influenza zoonosis in humans, other avian influenza A viruses were also able to infect humans and cause disease, such as H7N7 and H9N2 (36, 80).

1.4.2 Host specificity factors

Many biological properties affect the host specificity of influenza viruses. The combination of host and viral factors determines the virulence of a virus isolate in a particular host.
Few mutations in an apathogenic LPAIV H7N1 virus in Italy resulted in 1999 in a HPAIV version that reached mortality rates up to 100% in domestified poultry, while humans beeing in close contact, did only rarely show seroconversion and never got diseased (9, 70, 124). On the other hand, infections with HPAIV influenza viruses of the H5N1

subtype lead to high mortality rates in humans (151), while the virus often causes mild disease in wild waterfowl (119). Despite significant research, the molecular mechanism of how influenza viruses switch hosts remains poorly understood.

Every single viral protein fulfills a variety of functions for effective propagation of the viral infection. The question of which factors determine the viral pathogenicity, virulence and host range of influenza A viruses is not yet fully understood. However, comprehension of the function of individual proteins is steadily increasing. The hemagglutinin plays a central role in tissue tropism and systemic spread of the virus. It is responsible for receptor binding and membrane fusion, two essential functions for successful infection of a cell. For the membrane fusion, the proteolytic cleavage of HA_0 into HA_1 and HA_2 is essential and the expression of proteases mediating this cleavage is important for organ tropism of the virus, exemplified by the efficient activation of HPAIV hemagglutinin by ubiquitous furin-like proteases (72). Furthermore, the tissue tropism and host specificity are significantly influenced by the receptor specificity of the hemagglutinin. The different distribution of α2,3- and α2,6-linked sialic acids in a particular host and between different species is an important factor that determines the spread of a virus isolate (12, 64).

The neuraminidase is another important virulence factor. The transfer of an avian influenza A virus from wild birds to domestified poultry results in most cases in a deletion in the NA stalk region, which decreases the enzyme activity. For H5N1 isolates from chickens it has been shown that this stalk deletion restores a balance between haemagglutinin and neuraminidase-binding activity that was disturbed by the reduced receptor-binding ability of the HA in the new host (90, 164). It was also shown that the mouse-adapted A/WSN/33 neuraminidase specifically contributes to activation of the hemagglutinin by recruitment of the protease plasminogen (45, 46).

Besides the glyocproteins, especially the polymerase genes have been shown to contribute to pathogenicity and host range (3). A replacement of the PB1 gene of a human isolate with the one of an avian virus (as occurred in the 1957 pandemic) increases pathogenicity in mice (23). Furthermore, single amino acid changes, which will be described in the following, have been shown to affect pathogenicity and host range. While avian influenza A virus isolates almost exclusively contain a glutamic acid at position 627 of the polymerase PB2 protein (PB2-E627), this position is frequently mutated to a lysine in human-derived isolates, including H5N1 isolates that cause a high morbidity in humans (56, 150). Mutation to PB2-E627K is accompanied by improved viral replication at low temperatures (57) and enhanced binding of the viral polymerase to the nucleoprotein in human cells (75). However, fatal infections of humans with avian viruses of the H5N1 subtype that retained the avian like glutamic

acid at position 627 of PB2 are frequent. Interestingly, the 2009 H1N1 pandemic virus also harbors 627E, but additionally possesses a lysine at position 591 (PB2-K591), which is able to partially, but not completely, compensate for the lack of the PB2-E627K mutation (94). Other mutations known to play a role in host adaptation processes are the exchange of aspartic acid with asparagine at position 701 in PB2 (PB2-D701N) (38, 79), which can confer high pathogenicity to avian H5N1 and H7N7 viruses in mice and the substitution of a threonine to an alanine residue at position 271 (PB2-T271A) which has been suggested to enhance polymerase activity in mammalian cells (18). The PA protein of the 2009 pandemic H1N1 was shown to carry three mutations, conferring high activity to the polymerase in human cells (19). Furthermore, adaptation to human importin α by the influenza polymerase proteins, but also the nucleoprotein was suggested to play a crucial role in interspecies transmission and pathogenicity (39, 40).

During an infection, the host innate immune system has to be countered by influenza A viruses. The influenza nucleoprotein was shown to determine the level of sensitivity to the mammalian antiviral protein Mx (30). The small viral protein PB1-F2 induces cell-specific apoptosis, thereby eliminating cells of the innate immune system (24). In addition to the clear role of the NS1 protein in interferon antagonism, PB1-F2 and PB2 contribute to the inhibition of innate immunity pathways (34, 48, 161).

Furthermore, a C-terminal PDZ-binding-motif, elevating pathogenicity was proposed for avian influenza NS1. Also the NS1 localization seems to play a major role in the infection cycle (70, 110). However, only little experimental work has been performed to evaluate the potential as a pathogenicity determinant for the viral proteins M1, M2 and NEP. However, a mutation in NEP was suggested to be positively selected in a mouse-adapted virus, but experimental data are lacking (17).

1.5 Influenza vaccines

Influenza vaccination protects from infection and disease, although the frequent immune escape of influenza viruses requires annual updating of the vaccine strains to adapt to changes in the HA and NA genes. Emerging viruses like H5N1 pose a serious threat to the human population, as it is immunologically naïve to these viruses. The annual vaccine consists of two different FluA and one FluB strain. Strains are chosen, which are thought to most likely represent the majority of circulating strains in the following season. Two types of vaccines are currently used: a chemically inactivated virus delivered by injection, and a live-attenuated influenza virus vaccine based on a

cold-adapted master strain, delivered as a nasal-spray (4, 83). The inactivated vaccine elicits an antibody response recognizing the respective HA and NA subtypes. It has proven to be of suboptimal efficacy in the elderly population, the same population that is most endangered by seasonal influenza (137). In contrast, it is believed that the live vaccine not only induces humoral, but also a cellular immune response that confers long-lived and heterosubtypic immunity (13, 33, 88, 145). However, for safety reasons, administration is restricted to healthy persons between 2 and 49 years of age (4). The degree of attenuation is crucial for the efficacy of the live-attenuated vaccine. A strong attenuation reduces risk at administration, but this is accompanied with decreased protection. In the currently used live vaccine, the attenuation is fixed due to the use of a specific master strain. Identification of new viral target sites to vary the degree of attenuation would contribute to safety and efficacy of live attenuated viruses.

1.6 The interferon system and the antiviral protein Mx

As a component of the innate immune response, the interferon (IFN) system can react rapidly against an infection by inducing mechanisms to counteract spread of the infection, thereby offering the adaptive immune system enough time to develop a highly specific response (102). Type I Interferons whose main representatives are IFN-α and IFN-β and the recently discovered type III interferons including IFN-λ1, -λ2 and -λ3 are important factors interfering with viral replication (5, 98, 147). They are induced upon recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (153) such as RIG-I, MDA-5 and toll-like receptors (TLRs) that activate the transcription factor IRF-3 and lead to elevated expression of IFN by different mechanisms. Alternatively the transcription factor NF-κB induces the synthesis of IFN. The secreted interferons bind their respective membrane-

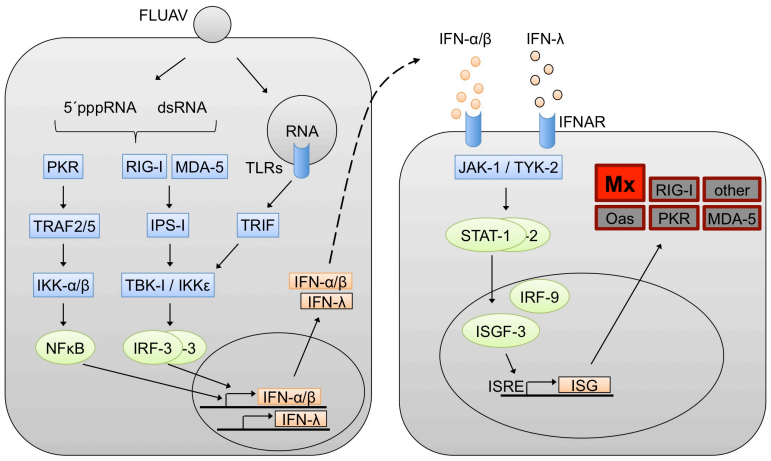

Fig. 4: The Interferon-system (adapted from (52))
Viral target structures are recognized by the cellular receptors MDA5, RIG-I and PKR, which results in the synthesis and secretion of type I and III interferon (IFN). The binding of IFN-β and IFN-λ to their respective receptor activates the JAK / STAT signaling cascade, thereby inducing the expression of interferon-stimulated genes (ISGs).

bound receptor and induce the expression of various IFN-stimulated genes (ISG) (146) via the JAK-STAT signaling pathway, such as RIG-I, MDA5, the 2'-5'-oligoadenylate synthetase (OAS), PKR and Mx proteins (132). While many ISGs interact with other cellular factors and exert relatively non-specific effects (26, 55, 68), Mx proteins have an intrinsic antiviral activity and act autonomously without the need for additional IFN-induced factors (7, 53, 144) (Fig. 4). The resistance to influenza A viruses observed in specific inbred mice carrying the Mx gene coined the nomenclature Mx (myxovirus resistance) (81, 82). Mx proteins belong to the family of large dynamin-like GTPases and have been identified in many vertebrates, including fish, birds, pigs, rodents and humans (54). Mx proteins contain three domains, the N-terminal GTPase domain, which binds and hydrolyzes GTP, a middle domain, which is involved in homo-oligomerization and a C-terminal GTPase effector domain, which serves primarily to detect viral target structures (65, 183). The murine Mx1 has a nuclear localization signal and therefore localizes in the nucleus, in contrast to the cytoplasmic human MxA (183). The antiviral mechanism of Mx has been extensively investigated by using primarily murine Mx1, as Mx1-positive mice have proven to be a suitable model organism for in vivo studies with influenza (49, 51, 182) and since Mx1-expressing swiss 3T3 cells efficiently interfere with primary transcription of influenza A viruses (143). However, despite its different localization, human MxA is able to inhibit early steps of influenza A virus replication, but primary transcription seems not to be affected (116). Despite these differences, Mx1 and MxA seem to recognize the same viral target structures. By insertion of a nuclear localization signal, MxA localizes into the nucleus and is able to interfere with the primary transcription, as Mx1 (183). The general mechanism of action thus seems to be indistinguishable between Mx1 and MxA. The N-terminal GTPase domain is crucial, as point mutations in Mx1 (K49A) and MxA (T103A) that prevent GTP hydrolysis, diminish the antiviral activity (121, 122). Mx1 and MxA interfere with the replication of all influenza A viruses. Nevertheless the degree of antiviral effect differs strongly between different strains. Influenza A viruses of avian origin show a Mx-sensitive phenotype, whereas human influenza A viruses show a relatively resistant phenotype (30). The artificial exchange of gene segments in a polymerase reconstitution system co-transfected with Mx expression plasmid revealed that NP is the determinant of Mx-sensitivity, which is further underscored by a physical interaction of MxA with the influenza NP (158). The strong sensitivity conferred by avian influenza NP might be explained by the observation that avian Mx appears to possess no protective function against influenza A viruses (134). Porcine Mx on the other hand is highly polymorphic, but so far tested porcine Mx only shows intermediate antiviral activity against infuenza A viruses (100, 113, 114).

1.7 Objectives of the underlying thesis

Zoonotic transmission poses a constant risk for the introduction of novel avian influenza viruses into the human population. Therefore, to identify and characterize host specificity determinants that drive the emergence of new pandemic influenza strains and to develop strategies to fight newly emerging influenza A viruses is of upmost importance. In this study we were analyzing the human H5N1 isolate A/Thailand/1(KAN-1)/2004 (KAN-1) of a fatal case to identify adaptive mechanisms and the potential of KAN-1 to serve as a basis for a live attenuated vaccine.

- In a first approach, we wanted to investigate and characterize adaptive mutations in KAN-1 enabling efficient infection and replication in human cells. We especially focused on a polymorphism in the hemagglutinin of this human H5N1 isolate and wanted to determine viral growth and organ tropism in mice. Based on a predicted avian precursor virus of KAN-1, we intended to identify adaptive mutations in the polymerase and NEP and analyze the principle defect of avian influenza polymerases restricting virus replication in human cells.

- As H5N1 viruses are to date unable to efficiently transmit in the human population, we hypothesized that KAN-1 might be insufficiently adapted to the innate immune system of humans, particularly to the human antiviral protein MxA. We therefore compared the sensitivity of the KAN-1 polymerase towards Mx proteins in cell culture and suitable mouse models to the pandemic strain from 2009.

- The pandemic potential of H5N1 viruses requires a fast and flexible vaccine development. By targeting the highly conserved PB1-PA binding domain in the influenza A polymerase, we wanted to provide proof of principle for a new strategy to attenuate influenza viruses for the optimization of live attenuated influenza vaccines. This strategy might be attributable for seasonal as well as H5N1 and other influenza A viruses with pandemic potential.

2 Results and discussion

2.1 Identification and characterization of a polymorphism in the HA

The objective of this thesis was, to characterize the human H5N1 isolate A/Thailand/1(KAN-1)/2004 (KAN-1) to gain increased understanding of adaptive mechanisms that enable avian influenza viruses to cross the species barrier to humans. In a first step, we started sequencing the open reading frames of the complete viral genome of the KAN-1 virus stock passaged three times in MDCKII cells. As the hemagglutinin is a fundamental factor for adaptation of avian viruses to mammals, we analyzed the sequence of the hemagglutinin gene of the KAN-1 virus stock first. Interestingly, we identified a non-synonymous adenine / guanine polymorphism with similar nucleotide ratios present in the HA segment (Figure 5A). This nucleotide heterogeneity either encodes a lysine or a glutamate at amino acid position 222 (H3 nomenclature) of the HA protein. The H5N1 consensus sequence has a lysine at this position. The polymorphism at position 222 of the HA was also identified in a report, where the authors analyzed viral quasispecies in tissues of H5N1 infected persons (74), including the patient infected with KAN-1. Since the published crystal structure of the HA revealed that position 222 is located in the receptor binding domain (148) and the hemagglutinin protein has been previously described to be crucial for host switch (42), we addressed the potential biological function of the identified heterogeneity and infected BALB/c mice with the KAN-1 virus stock. The polymorphism was retained in the extracted lung RNA, while only the lysine variant of KAN-1 was found in the brain (Figure 5B), suggesting a different organ tropism of the two virus variants. To further investigate this point, we generated recombinant viruses and tested these for pathogenicity and organ tropism in BALB/c mice. rKAN-1 HA_{222K} revealed a higher pathogenicity with a LD_{50} of 3 PFU compared to rKAN-1 HA_{222E} with a LD_{50} of 200 PFU.

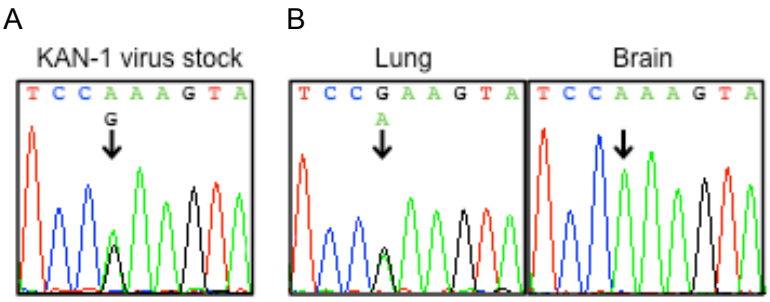

Fig. 5: Sequence analysis of the KAN-1 hemagglutinin. Nucleotide position 725-733 of the hemagglutinin gene sequence obtained from RT-PCR products are shown of **(A)** the KAN-1 isolate virus stock and **(B)** viral RNA isolated from indicated mouse organs 6 days post infection with an infectious dose of 1000 PFU of the KAN-1 isolate.

Thus, HA_{222E}, which was positively selected in the human lung, showed to be less virulent in the mouse model, whereas HA_{222K} showed to be more virulent. This is in line with the strong brain tropism and high viral titers in the brain of rKAN-1 HA_{222K} infected animals **(86, manuscript 3.1)**. Human H5N1 infections are also associated with viral dissemination to the brain (41), which is unusual for influenza infections in general. Mimicking these infections in humans, H5N1 viruses were also reported to have a strong brain tropism in mice (84).

Surprisingly, lung titers of mice infected with rKAN-1 HA_{222E} were higher at all investigated time points **(86, manuscript 3.1)**. Viruses isolated from mouse brain tissue infected with this virus showed a HA_{E222K} reversion, indicating that the HA_{K222E} mutation indeed increases replication efficiency in the lung, while lowering replication efficiency in the brain. Thus, we could show a positive effect of the HA_{K222E} mutation on replication kinetics in the lung of mice, while the mutation resulted in a disadvantage in replication in the brain. Concluding, the heterogeneity in HA222 was likely retained by KAN-1 to maintain tissue tropism for efficient replication in both the lung and the brain.

Although the mouse model is discussed controversially for influenza research because mice are not a natural influenza virus host and several parameters are different to humans, e.g. receptor distribution and immune system (108), we could demonstrate that the positive effect of the mutation selected in the human lung can be also shown in the mouse lung. However, the finding that increased replication efficiency in the lung does not correlate with higher virulence is rarely observed. Possibly, it reflects the limitations of the mouse model, since heavy brain infections as seen in the mouse, were only rarely found in humans (41). To address this, we tested the KAN-1 virus in the ferret model, which is considerd a more suitable animal model to mimic influenza disease than the mouse model (108). In the ferret model, KAN-1 replicated to high titers in the brain and sequencing of viral RNA in the brain again only revealed the HA_{222K} variant of KAN-1 (Holznagel et al., submitted, data not shown). Sequence analysis of the lung showed to be heterogeneous for aa position 222, as seen in the mouse lung. Thus, the ferret model confirmed our mouse data, suggesting that aa position 222 in the hemagglutinin indeed is responsible for an altered organ tropism.

Remarkably, seasonal human strains exhibit high replication efficiency in humans, while not causing strong tissue damage like observed with H5N1 infections. An adaptation to a new host that is accompanied with lower pathogenicity is a widespread phenomenon and seems to be important for co-evolution (14). Adaptational mutations in the HA are frequently associated with a switch in receptor binding preference. Whereas avian influenza viruses bind preferentially to α-2,3 linked sialic acids, seasonal human isolates bind α-2,6 linked sialic acids (12, 64). Receptor-binding

studies revealed that, as expected, the common avian HA_{222K} variant bound efficiently to α-2,3 whereas there was no binding detected to α-2,6 linked sialic acids. The HA_{222E} variant did not increase α-2,6 binding, but even reduced binding to α-2,3 linked sialic acids, except for the Su-SLe^x analogue **(86, manuscript 3.1)**. Reduced binding to α-2,3 linked sialic acids is an advantage in the human lung, as these receptors are often found on airway mucus (27) and binding of the virus to mucins would impair viral spread. Although the increased binding property to the analogue Su-SLe^x might have just happened by coincidence, a positive effect for the virus can't be excluded. For instance, cells from the immune system could be targeted and neutralized by this altered receptor preference, resulting in lower virus clearance.

Concluding from this part of the data, we studied the mutation HA_{K222E} that was positively selected in the human lung and could show increased replication in the mouse lung and human airway cells. The data of Konchanagul et al. show additional positive selected mutations in the HA segment isolated from the KAN-1 infected patient (74). However, none of these mutations show a frequency high enough to be present in the consensus sequence of KAN-1. These quasispecies were most likely counterselected, as the virus stock we were analyzing was passaged multiple times in MDCK cells. The viral quasispecies are important for propagation of mutant viruses and often do not show up in sequence databases. As human infections with H5N1 are mostly infections where the virus doesn´t transmit to other humans (dead end), these mutant viruses are lost before they can further adapt and propagate in the human population. Nevertheless it is important to study these early stages of adaptation to humans, to predict emerging viruses with the potential to spread from human to human.

2.2 Adaptation of an avian precursor of KAN-1 to humans

The mutation HA_{K222E} in the HA protein increased replication of the KAN-1 virus in the lung, but did not account for the high pathogenicity of the virus in mice. Therefore, we wanted to investigate the adaptation process that conferred the high virulence of rKAN-1 HA_{222K} (as follows named KAN-1) in mammals. Analysis of the complete KAN-1 genome revealed no further heterogeneities, but a comparison to avian H5N1 strains revealed possible adaptive mutations. To identify pathogenicity determinants usually an isolate being highly pathogenic in a certain animal model is compared to a virus comprising low pathogenicity in this system. In recent studies, these viruses differed often by 32-50 mutations (79, 130), mostly due to divergent evolution in avian hosts. In

the case of KAN-1, we wanted to investigate the mutations that were acquired by the virus to gain the ability to replicate efficiently in mammals. To understand the evolution of this virus isolate a direct avian precursor virus of KAN-1 needed to be identified. However, due to the rapid evolution of avian viruses in birds (181), it is difficult to isolate a direct precursor of a human isolate and in the case of KAN-1 no direct precursor is available. Therefore we used the influenza sequence database to model a potential avian precursor virus. By analyzing the available genome sequences, we could show that KAN-1 differs from the consensus sequence of avian H5N1 isolates in Thailand in 2003-2004 by only 9 amino acids. Seven of these amino acids were unique for KAN-1 and not common for avian isolates and therefore mutating these to avian-like aa most likely represents the avian precursor virus of KAN-1 (AvianPr). However, we were not able to identify an avian isolate with exactly the same sequence, most likely due to the high mutation rate of influenza viruses, but several isolates share a very similar sequence pattern e.g. VSMU (A/open-bill stork/Thailand/VSMU-20-AYA/2004 (H5N1)) with only three different amino acids, one in HA, M1 and M2, respectively **(Mänz et al., submitted, manuscript 3.2)**.

2.2.1 Identification of adaptive mutations in the polymerase

We hypothesized that AvianPr would be attenuated in mammals. Indeed, infection of BALB/c mice revealed that AvianPr is attenuated by about 3 $\log_{10}$ compared to KAN-1 **(Mänz et al., submitted, manuscript 3.2)**. The possible mutations responsible for this difference in pathogenicity are spread over the five genes PB2, PB1, PA, NA and NS. To find out which genes contribute to the high pathogenicity of KAN-1, we created reassortant viruses between KAN-1 and AvianPr. Besides the hemagglutinin, the polymerase was shown to play a crucial role in adapting avian influenza viruses to mammals (3, 56, 130). As the three polymerase proteins PB2, PB1 and PA build a functional complex, we examined the four amino acid differences in the polymerase in

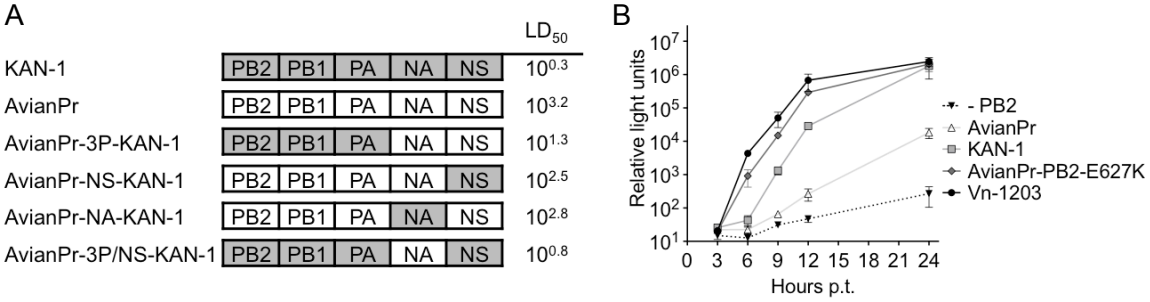

Fig. 6: The KAN-1 virus carries multiple adaptive mutations beside the hemagglutinin
(A) Characterization of reassortant viruses of KAN-1 and AvianPr in BALB/c mice.
(B) Reporter activity of indicated polymerases in the polymerase reconstitution assay.
See also Manuscript 3.2. Fig. 1b and 2a

conjunction. Additionally, we substituted amino acids of the NA- and NS-segment. Determination of the LD_{50} of the reassortant viruses revealed as expected a strong contribution of the four mutations found in the KAN-1 polymerase, as AvianPr-3P-KAN-1 showed a reduction of LD_{50} by 1.9 log_{10} (Figure 6A). Interestingly, also the single mutations in NS and NA reduced the LD_{50} by 0.7 and 0.4 log_{10} respectively. To dissect the role of the single mutations in the polymerase, we were taking advantage of the polymerase reconstitution assay (minireplicon). Avian polymerases were demonstrated to comprise only low activity, while human isolates as shown for the isolate A/Viet Nam/1203/2004 (H5N1) (Vn-1203) show high polymerase activity in human cells (130). The mutation E627K in PB2 was shown to restore efficient replication in humans. Interestingly, KAN-1 is lacking the adaptive mutation E627K, which suggests the presence of other compensatory mutations in the polymerase. Thus we compared the AvianPr, AvianPr-PB2-E627K, KAN-1 and Vn-1203 polymerases in the minireplicon system to estimate their activity level (Figure 6B). Although an increase in activity of the KAN-1 polymerase compared to AvianPr polymerase could be observed, the activity stayed one to two log_{10} below the activity of the Vn-1203 or AvianPr-PB2-E627K polymerases at 6, 9 and 12 hours post transfection. This suggested that the adaptive mutations in the KAN-1 polymerase are far less efficient in increasing the polymerase activity in human cells than for the Vn-1203 polymerase. In the avian cell-line LMH, all polymerases exhibited high polymerase activity in the polymerase reconstitution assay, proving that the AvianPr polymerase is active in avian cells **(Mänz et al., submitted, manuscript 3.2)**. The lack of enhanced activity of the KAN-1 and Vn-1203 polymerases in avian cells might be attributed to mutations that increase or decrease interaction with cellular factors that differ between human and avian cells, as suggested by others (95, 97), or that the experimental system does not allow for discrimination, as the AvianPr polymerase already exhibits high activity in avian cells. Interestingly, when examining the single mutations that were responsible for increased activity in mammalian cells, we identified PB2-E627K and PA-K142E for Vn-1203 and PB2-D701N and PA-K142R for KAN-1 **(Mänz et al., submitted, manuscript 3.2)**. Both viruses acquired an adaptive mutation in the C-terminus of the PB2 Protein and additionally a mutation at the same aa position (K142) of the PA Protein. PA-K142E was reported before to increase virulence of an H5N1 virus in mice (71), hence here we show that the increased virulence might result from the higher polymerase activity in mammals. Surprisingly KAN-1 carries a mutation at exactly the same position as Vn-1203, but this position is changed to a completely different amino acid. As the N-terminus of PA influences promoter binding (67), the position 142 might be involved in the regulation of the synthesis of the three different RNA species.

2.2.2 Avian influenza polymerases compensate a replication defect to facilitate efficient replication in mammals

As previously described, avian influenza polymerases comprise high activity in avian cells, while in mammalian cells the activity is strongly reduced. We wanted to know, if the synthesis of all three viral RNA species is inhibited in mammalian cells, or if only a sub-step is affected. Others examined before the levels of the three viral RNA species and observed a strong reduction of all RNA species in human cells with an avian influenza polymerase compared to a human influenza polymerase (94). Because of the strong interdependency of the viral RNAs (cRNA and mRNA levels are dependent on the vRNA template, vRNA levels are dependent on cRNA levels), no conclusion could be drawn weather the defect of the avian influenza polymerase is a general defect in all three steps of viral RNA synthesis, or just an intermediate step in RNA replication. There were several hints towards a defect being restricted to RNA replication. Mutations at positions 3, 5, 8 in the vRNA (up-promoter) resulted in almost 100% activity of an avian polymerase in a polymerase reconstitution system (28). Furthermore *in vitro* experiments mimicking primed transcription did not show significant difference between a polymerase containing PB2-627E or PB2-627K (unpublished data). Therefore, we speculated that the low level of mRNA transcripts in human cells produced by avian polymerases is due to low availability of vRNA

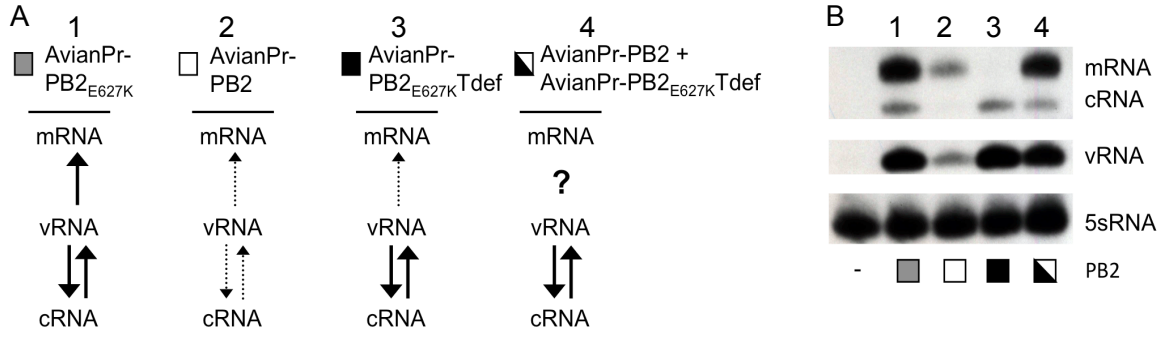

Fig. 7: Complementation assay. (A) Schematic overview of the assay. The transcription inactive polymerase containing PB2-E627K-Tr-def was complemented with the AvianPr polymerase in the polymerase reconstitution assay. **(B)** Primer extension assay was performed to analyse the three viral RNA species.
See also Manuscript 3.2 Fig. 3a,b

template. To prove this hypothesis, we used a polymerase complementation assay. The replication competent polymerase AvianPr-PB2-E627K was mutated in the cap-binding domain (PB2-361A, PB2-404A) to abrogate cap-binding and therefore lacks mRNA synthesis (Figure 7A,B, panel 3). The AvianPr polymerase that only shows very poor activity (Figure 7A,B, panel 2) was able to complement the transcription deficient polymerase and lead to full activity (Figure 7A,B, panel 4). Thus an avian polymerase

is able to transcribe a provided encapsidated vRNA template to mRNA. If the avian polymerase can also utilize cRNA provided by the transcription deficient polymerase has to be further analyzed. To prove this observation in a setting of a viral infection, we infected cycloheximide treated HEK-293T cells with the recombinant viruses AvianPr, or AvianPr-PB2-E627K, respectively. As both polymerases showed the same mRNA transcription level **(Mänz et al., submitted, manuscript 3.2)**, we conclude that PB2-E627K does not increase the transcription rate, but very efficiently boosts the replication rate of the polymerase. Additionally, substantial amounts of cRNAs are generated by the AvianPr polymerase. However, these transcripts are not bona fide templates for generating vRNA copies. Most strikingly, the avian and even the AvianPr-PB2-E627K polymerase are unable to use these cRNAs, highlighting the intrinsic defect of these transcripts. Interestingly, the AvianPr polymerase is able to synthesize vRNA from cRNA transcripts provided by infection with the AvianPr-PB2-E627K virus, indicating that the AvianPr polymerase is able to synthesize all three viral RNA species, when an adequate template is provided **(Mänz et al., submitted, manuscript 3.2)**. Adaptive mutations in the KAN-1 polymerase also increase RNA replication **(Mänz et al., submitted, manuscript 3.2)**. Thus, after crossing the species barrier to humans, the defect in replication has to be compensated by adaptive mutations in the polymerase or other proteins as discussed below.

2.3 Identification of NEP as a new pathogenicity factor

Although we previously showed that the mutations in the KAN-1 polymerase compensate partially for the lack of PB2-E627K (Figure 6B), we observed a strong delay of the KAN-1 polymerase in reporter activity compared to AvianPr-PB2-E627K or Vn-1203 polymerases (Figure 6B). As KAN-1 is an extremely virulent virus, comparable to Vn-1203 (47), it was unlikely that its polymerase activity in the reconstituted polymerase system is sufficient to confer this high pathogenicity in a viral infection. The observation that the KAN-1 NS segment was needed to prevent adaptive mutations in infected mice makes the possibility likely that NS1 or NEP is a cofactor for efficient KAN-1 polymerase activity **(Mänz et al., submitted, manuscript 3.2)**. Due to the data presented in 2.2.2 that avian polymerases are forced to compensate a replication defect in human cells, we considered the NEP protein as an interesting target, as it was shown by others to increase the replication capability of influenza polymerases (128). Coexpression of KAN-1 NEP in the minireplicon assay indeed increased the reporter activity (Figure 8B). AvianPr NEP reduced the reporter activity

slightly at 25ng coexpressed NEP expression plasmid. To investigate if there is a replication enhancing effect of NEP on the AvianPr polymerase, we performed a primer extension assay to measure mRNA, cRNA and vRNA levels (Figure 8A). Coexpression of AvianPr NEP showed an increase in cRNA level, but no change in mRNA and vRNA level, indicating that AvianPr NEP is active, but lacks the ability to efficiently boost AvianPr polymerase activity. Astonishingly, KAN-1 NEP strongly increased all three RNA species, but most prominently cRNA and vRNA levels. Although we cannot exclude a direct effect of KAN-1 NEP on mRNA levels, we favor the hypothesis that the increase in mRNA level is due to an increase in vRNA template. Thus, KAN-1 NEP can efficiently compensate the replication defect of avian polymerases in mammals. To investigate if the positive effect of KAN-1 NEP can also be seen in a viral infection, we infected human cells with the recombinant viruses AvianPr and AvianPr-NS-KAN-1. Primer extension analysis revealed a strong increase in all three RNA species of AvianPr-NS-KAN-1 infected cells, compared to AvianPr infected cells **(Mänz et al., submitted, manuscript 3.2)**. To investigate, if adaptive mutations in NEP are a general mechanism for H5N1 viruses to adapt to humans, we used the sequence database and screened for mutations that were present in human H5N1 isolates, but absent in avian isolates. We were able to identify several amino acids with adaptive potential. AvianPr NEP with the potential adaptive mutations 41C and 75G was tested in the minireplicon system. Additionally we included the mutation NEP-7L that is common in the Indonesian H5N1 lineage that caused most of the human H5N1 infections while retaining PB2-627E to 89%. We were also interested in the NEP of the 2009 pandemic H1N1 (NEP-CA/04), as these viruses comprise PB2-627E. Interestingly all human isolates possessing PB2-627E were shown to code for an NEP with reporter activity enhancing function, indicating a common adaptive mechanism (Figure 8B). In contrast, avian isolates and the human isolate Vn-1203 possessing PB2-627K did not show an enhancing activity compared to KAN-1 NEP.

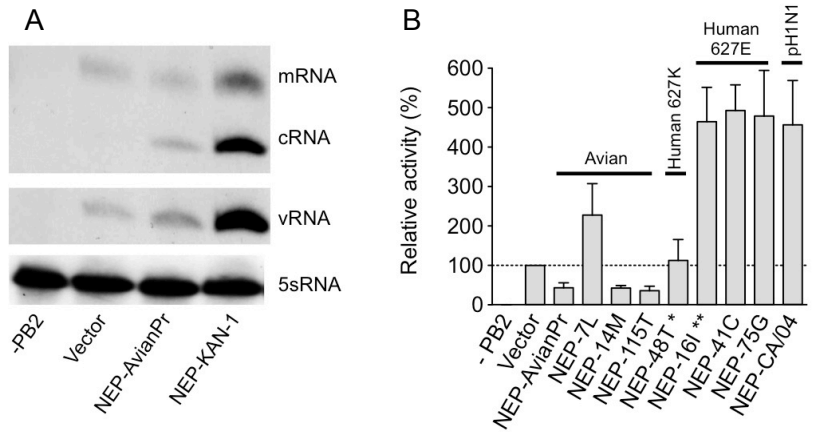

Fig. 8: Adaptive mutations in NEP increase polymerase activity. Polymerase reconstitution assay performed using AvianPr polymerase and co-expression of 25 ng empty vector or the indicated NEP expression plasmid. **(A)** mRNA, cRNA and vRNA levels were measured by primer extension assay. Co-expression of KAN-1 NEP leads to enhanced levels of all three viral RNAs. **(B)** Adaptive mutations in NEP of human PB2-627E viruses increase reporter activity. * NEP-Vn-1203, ** NEP-KAN-1

Interestingly, the NEP-7L mutation that is found in all avian and human isolates of the Indonesian H5N1 lineage increased AvianPr polymerase activity to intermediate levels. As infections of humans in Indonesia occurred relatively often, the NEP-7L mutation might contribute to the infectivity of these virus strains, by enabling the virus a basal replication in humans. This enhanced replication would raise the probability of the H5N1 virus to acquire adaptive mutations and therefore increase the chance of a productive infection and disease in the human host.

Our data indicate for the first time that the NEP protein of influenza can act as a pathogenicity factor. Although we can only speculate about the mechanism by which NEP increases the replication activity of influenza polymerases, we could demonstrate that NEP specifically associates with the PB1 and PB2 subunits **(Mänz et al., submitted, manuscript 3.2)**. In our model, this interaction might also help to stabilize cRNPs by direct contact with the polymerase. Furthermore, we provide a link between the adaptive mutation E627K in PB2 and adaptive mutations in NEP, since both increase the replication capability of avian influenza polymerases in human cells to overcome the restriction in the new host.

The restriction phenotype has been studied in detail, however many aspects remain elusive. As influenza viruses replicate in the intestine of the avian host at 42°C and in the human upper respiratory tract at 33°C, there might be a great discrepancy in optimal temperature of avian and human influenza polymerases (57). Indeed, shifting the temperature in human cells changes the relative polymerase activities of human and avian polymerases, but the general defect of avian polymerases in human cells remains (89). Inefficient binding of the polymerase to NP was suggested to hamper avian polymerases in mammalian cells (75), but the inefficient NP-polymerase interaction might be just the result of a low polymerase activity, as described by Resa-Infante et al. (126). Additionally, insufficient adaptation to human importin α was found to be a reason for the restriction of avian influenza polymerases in humans (39, 40). Importin α might be required for the formation of an active polymerase complex (16). Interestingly, the mechanistical function of the first and still most frequent occurring mutation discovered to overcome the restriction of avian influenza polymerases in human cells (PB2-E627K) is not known. Polymerases change their conformation substantially during their phases of transcription as exemplified by the T7 polymerase (32). It is therefore likely that the influenza polymerase exists in different conformations for mRNA, cRNA and vRNA synthesis. One of these conformational changes that are needed to fulfill all functions necessary for a complete replication cycle might be restricted in human cells. This would explain, why avian polymerases are still able to perform primed transcription, but are unable to synthesize bona fide cRNA templates.

The NEP protein might be a cofactor enabling the influenza polymerase to change its conformation suitable for replication. The interaction of NEP with RNPs (21) together with the finding that NEP binds to PB1 and PB2 **(Mänz et al., submitted, manuscript 3.2)** strongly supports this hypothesis. If the interaction is direct or indirect and whether binding to PB1 or PB2 is important for the replication enhancing function still has to be proven. However, we favor the model of a direct interaction of NEP with PB2 that influences the conformation of the RNP and subsequently stabilizes the viral RNA.
Adaptive mutations in the polymerase like PB2-E627K might also change the preference of the influenza polymerase to change its conformation. This would suggest a more intrinsic effect of the adaptive mutations on the viral polymerase in avian as well as human cells. Another possibility as the defect of avian polymerases in humans might be a lack of efficient encapsidation of the newly synthesized cRNA. This would render the resulting cRNPs unsuitable targets for further replication and even would make the cRNA prone for rapid degradation.

2.4 Role of the antiviral protein Mx in influenza evolution

Although the KAN-1 virus like other human H5N1 isolates is highly virulent in mammalian animal models and a boy succumbed to the KAN-1 infection, H5N1 viruses are to date very inefficient in spreading in the human population. We showed that the adaptive mutations in the KAN-1 genome mainly influence polymerase activity (PB2, PA and NEP) or are found in the glycoproteins (HA, NA). These mutations are sufficient for replication, but seem to be insufficient for transmission. Thus, the adaptation process of human H5N1 viruses is not completed. It is of importance to identify the missing link to a completely human adapted virus.
One of these missing factors might be adaptation to the antiviral protein Mx, which counteracts influenza virus replication efficiently. This antiviral effect was shown to be most prominent against avian influenza viruses, while seasonal human isolates and the pandemic virus A/Brevig Mission/1/1918 (H1N1) (1918) showed to be relatively resistant (30). The viral factor that is decisive for resistance or sensitivity to Mx in the minireplicon system has been identified to be the nucleoprotein NP (30).
Although the human H5N1 isolate Vn-1203 harbors several adaptive mutations, this virus was shown to be highly sensitive to the antiviral activity of Mx (131, 157). As Vn-1203 and KAN-1 share the same amino acid sequence in the NP, KAN-1 was expected to be sensitive to Mx as well. We therefore used KAN-1 as a tool to identify the determinants of Mx resistance *in vitro* and *in vivo*.

2.4.1 The viral NP determines Mx sensitivity *in vivo*

We hypothesized that viruses that spread efficiently in the human population would prove Mx resistant *in vivo*, in contrast to avian viruses, which should be sensitive. Therefore, to analyze whether the viral NP is the determinant of Mx sensitivity *in vivo*, we first compared KAN-1 (H5N1) and the pandemic H1N1 virus from 2009 (pH1N1) in the minireplicon. We chose pH1N1 since its NP gene originated from classical swine flu viruses and we speculated that it should have acquired mutations that would confer Mx resistance. Furthermore, since the pH1N1 has only circulated in humans for a restricted timeframe, additional mutations acquired during this short time might have increased its Mx resistance. This would put us in a position to not only confirm NP as a determinant for Mx sensitivity *in vivo* but to additionally identify the specific amino acid residues that confer resistance or sensitivity.

Indeed, the H5N1 polymerase and NP showed to be sensitive, while the pH1N1 polymerase and NP were relatively resistant towards the antiviral activity of Mx (Figure 9A). Exchange of polymerase subunits and the NP confirmed that the NP is the determinant of Mx sensitivity *in vitro* for this virus pair (Figure 9B).

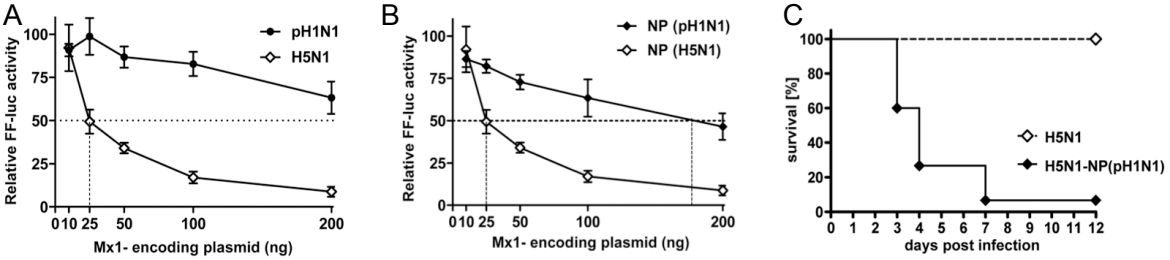

Fig. 9: The viral nucleoprotein is the determinant of Mx-sensitivity *in vitro* and *in vivo*. (A) Reporter activity of H5N1 is strongly restricted by Mx1 in the minireplicon system, while reporter activity of pH1N1 is only slightly reduced by the antiviral activity of Mx1. **(B)** The nucleoprotein of pH1N1 renders H5N1 relatively resistant to Mx1 *in vitro*. **(C)** BALB/A2G/-Mx1 mice were infected with $2*10^6$ PFU of H5N1 or H5N1-NP(pH1N1). While all mice infected with KAN-1 survived the infection, 88% of mice infected with H5N1-NP(pH1N1) succumbed the infection.
See also Manuscript 3.3 Fig. 1A,D and Fig. 4G.

To test if this is also the case in a viral infection, we created the recombinant viruses H5N1 and H5N1 coding for the NP of pH1N1 (H5N1-NP(pH1N1)) and infected cells stably expressing the antiviral protein Mx1, MxA or an empty vector (NEO). Western blot analysis revealed efficient expression of viral proteins of both viruses in Neo cells. PA levels were strongly reduced in Mx1 and MxA expressing cells infected with H5N1, while PA levels were only slightly reduced in H5N1-NP(pH1N1) infected cells **(182, manuscript 3.3)**. We next infected MDCKII cells lacking an antiviral active Mx-protein with H5N1 or H5N1-NP(pH1N1), respectively. H5N1-NP(pH1N1) showed a delayed growth rate, indicating that the NP of pH1N1 might attenuate the H5N1 virus. Astonishing, despite the attenuated phenotype of H5N1-NP(pH1N1) in cells lacking the

antiviral protein Mx and reduced titers in Mx negative mice, this virus showed increased pathogenicity in the Mx positive mouse strain BALB/A2G/-Mx1 (Figure 9C). This confirmed that the viral NP is the determinant of Mx sensitivity not only *in vitro*, but also *in vivo* and that in contrast to pH1N1, H5N1 viruses such as KAN-1 did not acquire Mx-resistance enhancing mutations. Interestingly the reassortant KAN-1 virus carrying the NP of pH1N1 was attenuated **(182, manuscript 3.3)** making it unlikely that this reassortant virus will evolve in nature.

2.4.2 Identification of mutations in NP conferring Mx-resistance

Our data in 2.4.1 together with the work of others (30) indicate that adaptation to the human antiviral protein MxA is very important for the propagation of a virus in the human population. We also wanted to know whether the attenuation of KAN-1 carrying the pH1N1 NP is due to the Mx-resistance enhancing mutations. This might explain, why H5N1 viruses did not efficiently acquire Mx-resistance enhancing mutations and did not yet spread efficiently in the human population. Obtaining knowledge about these mutations would improve our understanding of the adaptation process of avian influenza viruses that cross the species barrier to humans and potential dangerous viruses could be identified at an early stage of adaptation. Additionally, these mutations might provide a powerful tool to investigate the molecular mechanism of Mx. We showed before that the KAN-1 virus is sensitive to the antiviral effect of Mx and therefore might be a good tool to study these mutations. The pH1N1 NP is able to render KAN-1 Mx resistant. Thus, some of the amino acid differences between KAN-1 and pH1N1 NP should be responsible for the difference in Mx sensitivity. Unfortunately these two viruses differ in 32 amino acids. As it is unlikely that the resistance conferred by pH1N1 NP is caused by only one amino acid, we were looking for a more suitable virus possessing an NP that confers high resistance to Mx and displays less amino acids differences. We identified the NP of the 1918 pandemic virus as a suitable target, as its amino acid sequence is still very avian like (155), but can confer high Mx resistance in the minireplicon system (30). The 14 amino acid differences between KAN-1 and 1918 NP are spread over the whole protein. Exchanging amino acids in the KAN-1 background towards 1918 NP, we identified the mutations R100I/V, L283P and F313Y to be crucial in mediating resistance to Mx1 in the minireplicon system (31). To investigate the importance of these mutations in a viral infection, we first created recombinant A/Puerto Rico/8/1934 (H1N1) (PR8) virus that is highly virulent for Mx positive mice (49), and the two mutant viruses PR8-NP-V100R, P283L and PR8-NP-P283L, Y313F that each lack two Mx-resistance conferring mutations. Infection of Mx-

negative B6 mice revealed no change in virulence of the mutant viruses. Astonishingly, infection of Mx-positive mice revealed an increased LD_{50} value of 2,5 and 3,8 log_{10} of the mutant viruses compared to PR8 WT (Figure 10A). This further highlights these mutations as essential for Mx-resistance of PR8. To investigate, if these mutations can also confer Mx-resistance to the sensitive KAN-1 virus, we created recombinant KAN-1 viruses harboring the 100V, 283P, 313Y triple mutations, the 100V, 313Y mutations, or only the 283P mutation. A growth curve performed in MDCKII cells revealed a one to two log_{10} attenuation due to the introduced mutations for KAN-1-NP-283P and KAN-1-NP-100V,283P,313Y (Figure 10B), as examined before with the H5N1-NP(pH1N1) virus. KAN-1-NP-100V,313Y showed a delayed growth phenotype, but catches up with wt 24 hours post infection (Figure 10B). As KAN-1-NP-100V-313Y showed only slight attenuation, we infected BALB/A2G/-Mx1 mice with 10^6 PFU and analyzed weight changes for 12 days. The KAN-1-NP-100V-313Y virus containing two amino acids that confer partial resistance to Mx1 exhibited higher pathogenicity than the wild type KAN-1 virus (Figure 10C). These data confirm the importance of Mx-resistance enhancing mutations in the case of an infection under Mx pressure. Sequence analysis in the influenza database revealed that mutations E53D, F313V and I316M are unique for NP of pH1N1 viruses. We created recombinant viruses with these three positions mutated to the consensus in the classical swine influenza viruses pH1N1-NP-53E,313F,316I and the single mutant pH1N1-NP-53E, to see if the NP mutations in pH1N1 also attenuate the virus as observed for KAN-1. Indeed, growth curves in MDCKII cells revealed enhanced growth rate with both mutant viruses compared to pH1N1 (Figure 10D). Unlike the mutations observed in the polymerase, NEP and glycoproteins of KAN-1 that are often found in human isolates, the mutations conferring Mx-resistance are only rarely found in human H5N1 isolates. As KAN-1 carrying one or several of these mutations showed to be attenuated in the absence of antiviral active Mx, we speculate

A

	LD_{50}	
	B6-$Mx^{-/-}$	B6-$Mx^{+/+}$
PR8	$10^{0,5}$	$10^{1,5}$
PR8-NP-V100R, P283L	$10^{0,5}$	10^{4}
PR8-NP-P283L, Y313F	$10^{0,5}$	$10^{5,3}$

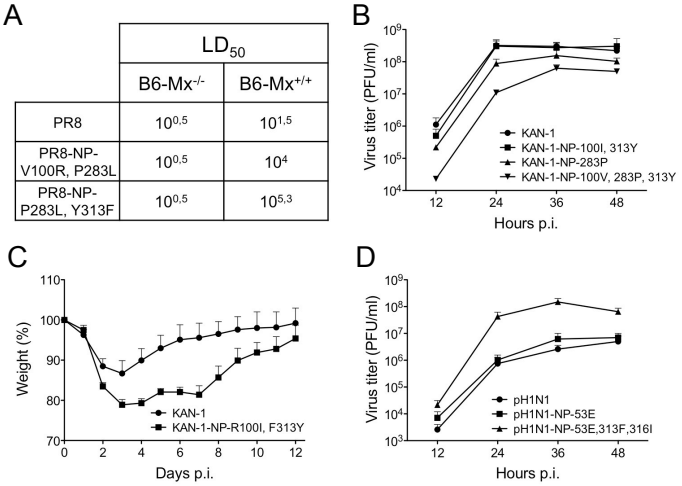

Fig. 10: Identification of Mx-resistance conferring amino acids. (A) A/Puerto Rico/8/1934 (H1N1) WT virus (PR8) and the two mutants PR8-NP-V100R, P283L and PR8-NP-P283L, Y313F were tested for pathogenicity in Mx1 negative (B6-$Mx^{-/-}$) and Mx1 positive mice (B6-$Mx^{+/+}$). **(B)** Growth kinetics of KAN-1 mutant viruses in MDCKII cells. Cells were infected with an MOI of 0.001. **(C)** Mx1 positive mice (BALB/A2G/-Mx1) were infected with 10^6 PFU oft he two indicated viruses. Weight was analysed for 12 days p.i. **(D)** Growth kinetics of pH1N1 mutant viruses in MDCKII cells. Cells were infected with an MOI of 0.001.

that it is far more difficult for an avian influenza virus to adapt to Mx than to have adaptive mutations in the polymerase. Mutations in the polymerase immediately improve viral growth, in contrast to mutations in NP that confer resistance to Mx. These mutations have an intrinsic negative effect on viral growth and only a positive effect in the presence of Mx. Thus it needs a fine balancing to acquire suitable mutations in NP that do not attenuate the virus too much, but are able to confer high Mx-resistance. Fortunately to date, H5N1 viruses were not able to mutate efficiently in this direction.

2.5 Attenuation of influenza A viruses by targeting polymerase assembly

The rapid evolution of influenza viruses poses a constant threat to human health. It remains difficult to predict a new pandemic with the emergence of dangerous viruses all of a sudden. Thus, it is of great concern to improve the primary defense against influenza viruses, the vaccines. Live attenuated viruses were shown to often produce a better and longer lasting immunity than inactivated vaccines. However, live attenuated viruses have to deal with serious safety issues, as the virus still has to replicate in the vaccinated person. It is difficult to generate a virus that is adequately attenuated for save administration, but still replicates sufficiently to be immunogenic. We wanted to use our findings about the polymerase complex to contribute to safety and efficiency of live attenuated viruses. Thereby, the creation of a live vaccine with desirable attenuation would be ideal. Additionally the introduced mutations should remain stable in the viral genome. We decided to follow up our detailed analysis of the highly conserved PB1-PA binding domain (174, 175). Weakening the binding between PB1 and PA resulted in less efficient polymerase complex formation and reduced polymerase activity *in vitro* of the model virus SC35M **(85, manuscript 3.4)**. While we identified several mutations with the potential to create a stable attenuated virus, we concentrated on the mutation PA-W706E to test its feasibility in a life attenuated KAN-1 vaccine. As expected, introduction of the mutation PA-W706E into the genome of KAN-1 resulted in low level of polymerase activity in the minireplicon system (data not shown). Recombinant viruses were strongly attenuated in cell culture and also in animal experiments (**(85, manuscript 3.4)**, Figure 11A and 11B). Notably, vaccinated BALB/c mice with the attenuated virus KAN-1 PA_{706E} survived an otherwise lethal challenge with 100 LD_{50} of wildtype KAN-1 virus (Figure 11C).

These data indicate that our approach for generation of attenuated viruses can be used to improve available live attenuated vaccines or to generate a new live attenuated

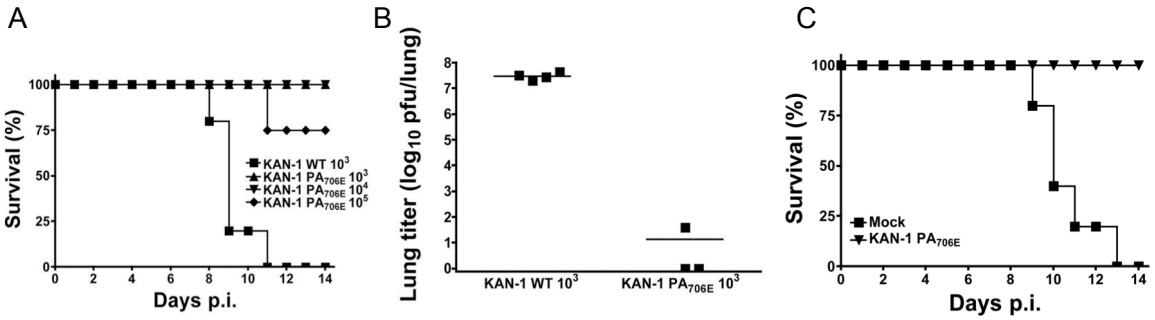

Fig. 11: Polymerase assembly mutants can be used as life attenuated vaccines. **(A,B)** KAN-1 PA_{706E} is attenuated in mice (**C**) Mice vaccinated with KAN-1 PA_{706E} survive an otherwise lethal challenge with wildtype KAN-1. See also Manuscript 3.4 Fig. 4E

vaccine by combining available strategies with our new approach. As the emergence of new pandemic strains is still difficult to predict, a rapid strategy to create influenza vaccines with high potential against all kind of influenza strains is needed. Targeting a highly conserved region like the PB1-PA binding domain would provide such a rapid strategy feasible for all known influenza A strains.

2.6 Conclusions

In summary, we have shown that adaptation of avian influenza viruses to humans is a multigenic process that we are just starting to understand. The human H5N1 isolate KAN-1 proved to be a powerful tool to investigate adaptive mechanisms. In this study we could show that an avian virus gained adaptive mutations to increase fitness in distinct parts of the viral life cycle. The positive selected mutation K222E in the hemagglutinin changes its receptor binding properties, thereby increasing replication of the KAN-1 virus in the lung. Adaptive mutations in the NEP protein or the polymerase increase genome replication and subsequently also genome expression. The observation that a virus can mutate either position 627 in the PB2 protein or the NEP protein demonstrates the flexibility of influenza viruses. However, even after enabling a fully competent replication cycle, influenza viruses are restricted by the human innate immune system. Mx resistance enhancing mutations, which seem to be a prerequisite of pandemic and seasonal influenza viruses, are difficult to acquire by H5N1 viruses, most likely one reason why H5N1 viruses did not spread efficiently in the human population. However, it might be that only few additional mutations enable an H5N1 virus to efficiently spread in the human population. We therefore created a new strategy to attenuate influenza viruses by disruption of the polymerase formation. Due to its high conservation, the PB1-PA binding-domain could be a suitable target to improve live attenuated vaccines to be rapidly produced after emergence of a new dangerous influenza strain.

3 Original publications and manuscripts

3.1 A polymorphism in the hemagglutinin of the human isolate of a highly pathogenic H5N1 influenza virus determines organ tropism in mice

Published in: Journal of virology

Own contributions:

I contributed to design of the experiments and writing of the manuscript. I performed all experiments shown in figure 1, 2 and 3. I created the inactivated viruses used for the Elisa shown in table 1.

JOURNAL OF VIROLOGY, Aug. 2010, p. 8316–8321 Vol. 84, No. 16
0022-538X/10/$12.00 doi:10.1128/JVI.00850-10

NOTES

A Polymorphism in the Hemagglutinin of the Human Isolate of a Highly Pathogenic H5N1 Influenza Virus Determines Organ Tropism in Mice[▽]

Benjamin Mänz,[1,2] Mikhail Matrosovich,[2] Nicolai Bovin,[3] and Martin Schwemmle[1]*

Department of Virology, Institute for Medical Microbiology and Hygiene, University of Freiburg, Germany[1]; Institute for Virology, University of Marburg, Germany[2]; and Shemyakin Institute of Bio-organic Chemistry, Russian Academy of Sciences, Moscow, Russia[3]

Received 21 April 2010/Accepted 21 May 2010

We characterized a human H5N1 virus isolate (KAN-1) encoding a hemagglutinin (HA) with a K-to-E substitution at amino acid position 222 that was previously described to be selected in the lung of the infected patient. In mice, the growth of the HA_{222E}-encoding virus was mainly confined to the lung, but reversion to 222K allowed virus to spread to the brain. The HA_{222E} variant showed an overall reduced binding affinity compared to that of HA_{222K} for synthetic Neu5Ac2-3Gal-terminated receptor analogues, except for one analogue [Neu5Acα2-3Galβ1–4(Fucα1–3)(6-HSO_3)GlcNAcβ, Su-SLe^x]. Our results suggest that human-derived mutations in HA of H5N1 viruses can affect viral replication efficiency and organ tropism.

The H5N1 avian influenza A virus is a highly contagious and deadly pathogen in poultry (13). Although rare, infections of humans occur and cause severe disease symptoms associated with a high morbidity (13). Human and avian influenza A viruses differ in the recognition of the host cell receptor (10). Whereas human influenza A viruses preferentially bind to 2,6-linked sialic acids, avian influenza A viruses preferentially bind to 2,3-linked sialic acids. Although H5N1 viruses can infect humans, they do not bind to 2,6-linked sialic acids, a property believed to be one of the reasons for inefficient human-to-human transmission (10). However, there is a recent report indicating that adaptation involving changes in receptor specificity can occur in infected humans (9). In this study, the authors analyzed viral quasispecies found in lung tissue, nasopharyngeal secretions, and intestinal tissue of three H5N1-infected patients (9). Several variants with distinct mutations in the receptor-binding site of hemagglutinin (HA) were observed, including the variant with the mutation K222E (H3 numbering system), which is part of the 220 loop of the receptor-binding site of HA. The aim of this study was to characterize the replication efficiency and organ tropism in mice and the receptor binding properties of the H5N1 virus with this mutation.

The virus variant with K222E represented 45% and 6% of the quasispecies present in the lung tissues of two out of three patients (9). The virus A/Thailand/KAN-1/2004 (KAN-1), isolated from the first patient in cell culture and passaged several times in MDCK cells, was kindly provided to us by Pilaipan Puthavathana (Mahidol University, Bangkok, Thailand). We sequenced the HA gene of the virus stock and indeed revealed the polymorphism at amino acid position 222 (Fig. 1A). Sequence analysis of all other open reading frames, including the neuraminidase-encoding gene segment, did not reveal any heterogeneity (data not shown). Because H5N1 viruses are known to be neurotropic (8, 18), we infected BALB/c mice with this virus stock, collected lung and brain tissue 5 days postinfection (p.i.), and determined the sequence of the viral HA. Whereas both HA variants were present in the lung, we only identified the HA_{222K} variant in the brain (Fig. 1A), suggesting that the polymorphism in HA affects organ tropism. To confirm these findings, we used reverse genetics (7) to generate recombinant KAN-1 virus encoding either HA_{222K} (rKAN-1 HA_{222K}) or HA_{222E} (rKAN-1 HA_{222E}). For other gene segments, consensus sequences were used that were determined from the virus stock described above by sequencing overlapping reverse transcription (RT)-PCR products. Comparisons of the growth characteristics in various cell lines revealed that rKAN-1 HA_{222E} replicated slightly more efficiently in MDCK cells and A549 human lung carcinoma cells than rKAN-1 HA_{222K}, whereas both virus variants showed similar growth kinetics in avian hepatoma cells (LMH) (Fig. 1B). In BALB/c mice, infection with 100 PFU of either virus variant resulted in a pronounced weight loss; however, in contrast to mice infected with rKAN-1 HA_{222K}, 70% of all mice survived infection with rKAN-1 HA_{222E} (Fig. 1C to F). The 50% lethal doses (LD_{50}) from the experiments whose results are shown in Fig. 1C to F were determined as described previously (16) and revealed a 67-fold difference between rKAN-1 HA_{222E} (LD_{50} = 200 PFU) and

* Corresponding author. Mailing address: Department of Virology, University of Freiburg, Hermann-Herder-Strasse 11, D-79104 Freiburg, Germany. Phone: 49-761-203-6526. Fax: 49-761-203-6639. E-mail: martin.schwemmle@uniklinik-freiburg.de.

▽ Published ahead of print on 2 June 2010.

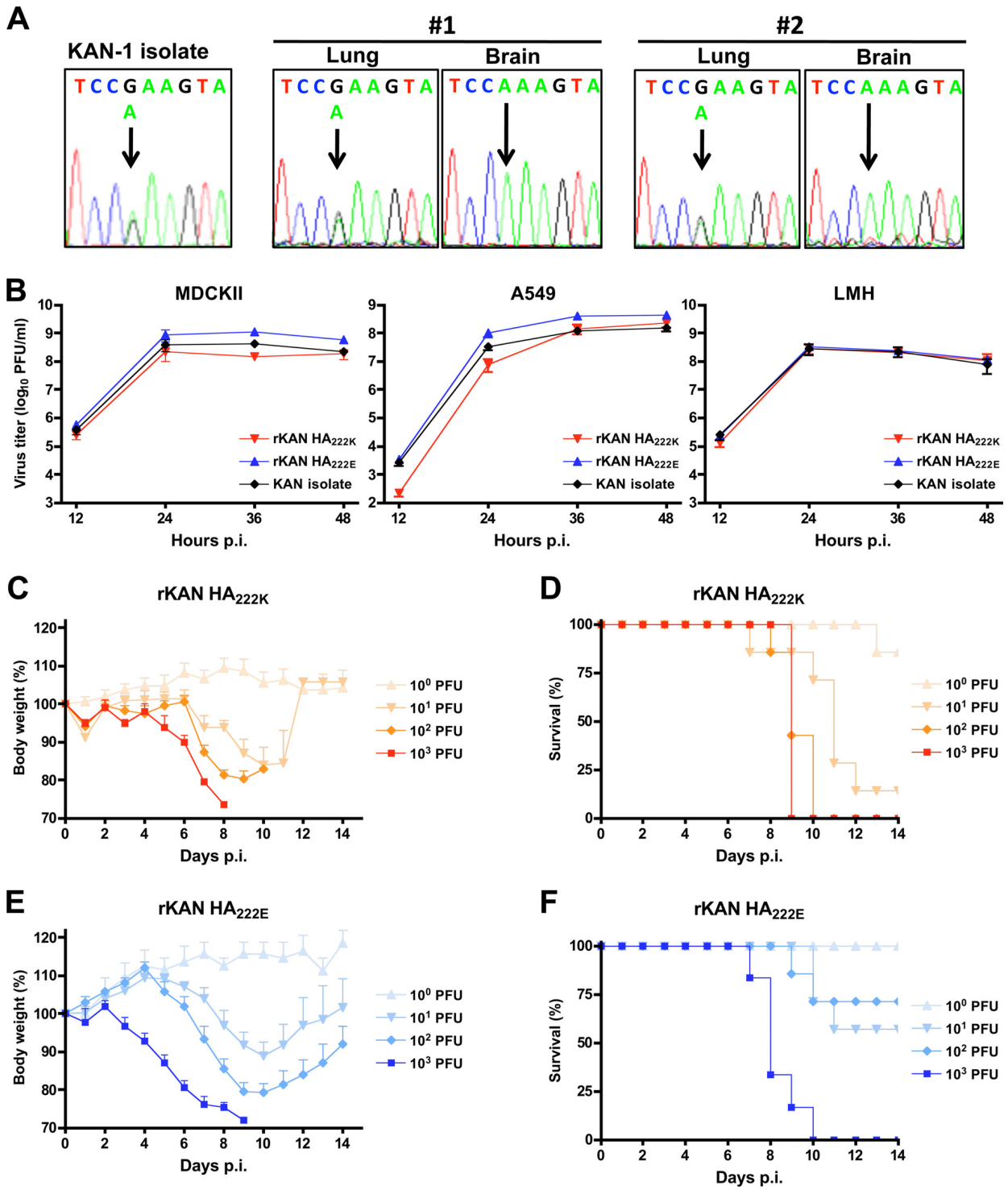

FIG. 1. Growth properties of KAN-1 variants with a polymorphism in HA. (A) Two 8-week-old female BALB/c mice (#1 and #2) were infected intranasally with 10^3 PFU of the virus isolate A/Thailand/KAN-1/2004. Five days postinfection (p.i.), the sequences of the HA genes from lung and brain tissue were determined and compared to the sequence obtained for the virus isolate used for infection. Sequences shown represent nucleotide positions 725 to 733 (aa 221 to 223) of the HA gene. Arrows indicate the site of the nucleotide polymorphism. (B) Cells were infected with a multiplicity of infection of 0.001 with rKAN-1 HA_{222K}, rKAN-1 HA_{222E}, or the virus isolate A/Thailand/KAN-1/2004 used in the experiment whose results are shown in panel A. (C to F) Eight-week-old female BALB/c mice were infected intranasally with either rKAN-1 HA_{222K} (C and D) or rKAN-1 HA_{222E} (E and F) using the indicated virus doses and were monitored for body weight loss and survival. Error bars show standard deviations.

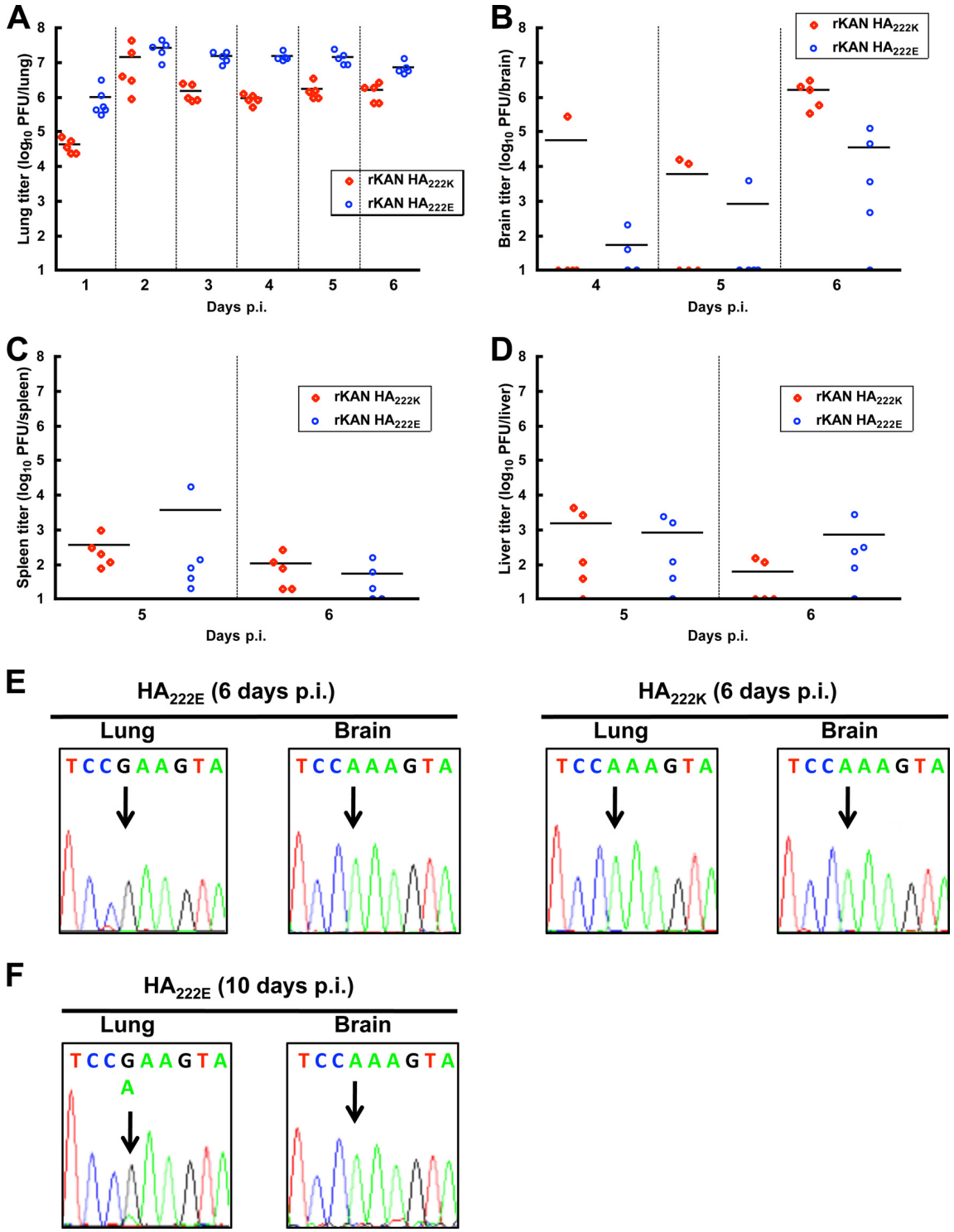

FIG. 2. Organ tropism of KAN-1 variants in mice. (A to D) Eight-week-old female BALB/c mice were infected intranasally with 1,000 PFU of either rKAN-1 HA_{222K} or rKAN-1 HA_{222E}. At the indicated time points p.i., virus titers were determined in lung (A), brain (B), spleen (C), and liver (D). Horizontal lines show the means of the results. (E) HA sequences obtained 6 days p.i. from lung and brain tissues of the BALB/c mice that were infected with either rKAN-1 HA_{222E} or rKAN-1 HA_{222K} as described for panels A to D. (F) HA sequences obtained from lung and brain tissues of a BALB/c mouse 10 days after infection with 10 PFU rKAN-1 HA_{222E} as described for Fig. 1F. Arrows indicate the site of the nucleotide polymorphism.

rKAN-1 HA_{222K} (LD_{50} = 3 PFU), suggesting that rKAN-1 HA_{222K} is more pathogenic for mice. Surprisingly, determination of the viral titers in the lung showed that rKAN-1 HA_{222E} replicated to higher titers than rKAN-1 HA_{222K} at all time points investigated (Fig. 2A). On the contrary, at 6 days postinfection, lower viral titers could be detected in the brain of rKAN-1 HA_{222E}-infected animals (Fig. 2B). Consistent with earlier reports on H5N1-infected mice (15), only low viral

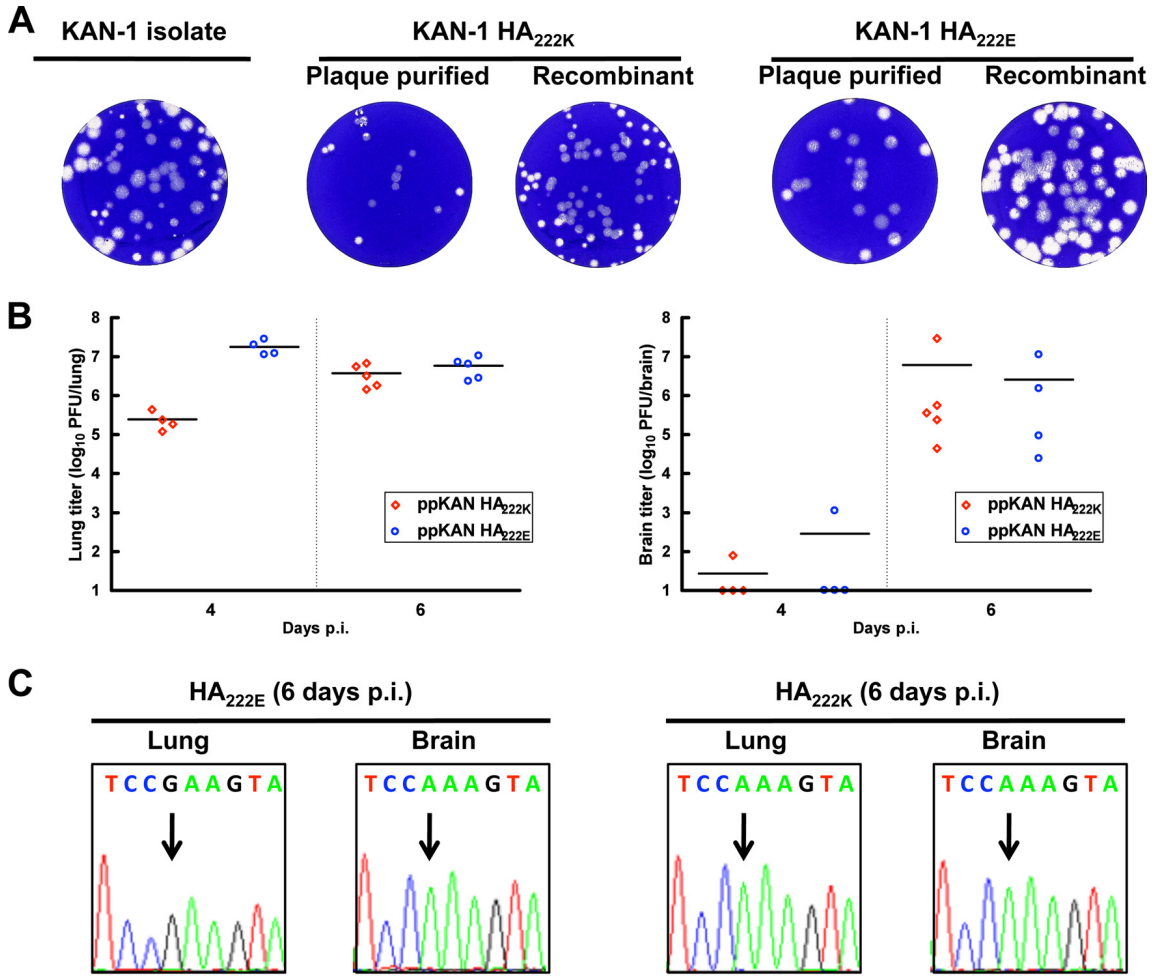

FIG. 3. Growth properties of plaque-purified KAN-1 variants. (A) Comparison of the plaque sizes of the virus isolate A/Thailand/KAN-1/2004 and its plaque-purified and recombinant variants. (B) Eight-week-old female BALB/c mice were infected as described in the Fig. 1 legend with 1,000 PFU of the indicated plaque-purified viruses. At 4 and 6 days p.i., virus titers were determined in lung and brain. Horizontal lines show the means of the results. (C) HA sequences obtained from lung and brain tissues of two animals that were infected with either ppKAN-1 HA_{222E} or ppKAN-1 HA_{222K} 6 days p.i. as described for panel B. Arrows indicate the site of the nucleotide polymorphism.

titers were observed for both virus variants in liver and spleen of mice at 5 and 6 days postinfection (Fig. 2C and D). Interestingly, sequencing analysis revealed that in all animals infected with rKAN-1 HA_{222E}, the virus isolated from the brain was represented by a revertant with HA_{222K} (Fig. 2E and data not shown). However, no reversion was found in the lung of rKAN-1 HA_{222E}-infected animals. These results further highlighted the restriction of rKAN-1 HA_{222E} to the lung. As expected, no changes in HA were observed in the lung and brain of mice infected with rKAN-1 HA_{222K} (Fig. 2E and data not shown). In the lung of one mouse infected with 10 PFU, we also observed the appearance of the mutation E222K in HA and, as expected, only the HA_{222K} variant in the brain (Fig. 2F). We therefore speculate that reversion to HA_{222K} occurs in the lung, thereby allowing virus to spread to the brain.

To verify these results with isolates of the original virus stock, we plaque purified two viruses (ppKAN-1 HA_{222E} and ppKAN 1 HA_{222K}). Both plaque-purified variants showed characteristic differences in their plaque sizes, which resembled those of the rescued viruses (Fig. 3A). With the exception of the polymorphism in HA, both plaque-purified isolates revealed complete sequence identity in all genes compared to the rescued viruses (data not shown). Similar to the results observed with the recombinant KAN-1 variants, the lung titers in mice infected with ppKAN-1 HA_{222E} were significantly higher ($P < 0.05$, Student's t test) than the lung titers of ppKAN-1 HA_{222K} at 4 days postinfection (Fig. 3B). At 6 days postinfection, high viral titers were observed in both ppKAN-1 HA_{222E}- and ppKAN-1 HA_{222K}-infected animals. Consistent with the results obtained with rKAN-1 HA_{222E}, we only observed the HA_{222K} variant in the brain of all the ppKAN-1 HA_{222E}-infected animals (Fig. 3C and data not shown).

To assess the effect of K-to-E substitution in the viral HA on the receptor-binding properties, we determined the affinity of the viruses for synthetic sialylglycopolymers (SGPs) bearing distinctive sialyloligosaccharide moieties as described previously (1, 5, 6, 12). The structures and designations of the oligosaccharide parts of the SGPs are shown in Table 1. rKAN-1 HA_{222K} bound preferentially to Neu5Ac2-3Gal-terminated receptors and displayed the highest binding affinity for

TABLE 1. Association constants of virus complexes with sialylglycopolymers

Structure of sialyloligosaccharide moiety	Abbreviation	Association constant[a] (mM^{-1} Neu5Ac) of HA variant:	
		222K	222E
Neu5Acα2-3Galβ1-3GalNAcα	3′STF	0.41 (0.069)	0.16 (0.014)
Neu5Acα2-3Galβ1-3GlcNAcβ	SLe^c	0.38 (0.093)	0.21 (0.045)
Neu5Acα2-3Galβ1-3(6-HSO_3)GlcNAcβ	Su-SLe^c	0.47 (0.089)	0.12 (0.02)
Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ	SLe^a	0.11 (0.089)	0.07 (0.018)
Neu5Acα2-3Galβ1-4GlcNAcβ	3′SLN	0.29 (0.073)	0.20 (0.032)
Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ	SLe^x	0.13 (0.031)	0.1 (0.017)
Neu5Acα2-3Galβ1-4(6-HSO_3)GlcNAcβ	Su-3′SLN	2.1 (0.22)	0.82 (0.28)
Neu5Acα2-3Galβ1-4(Fucα1-3)(6-HSO_3)GlcNAcβ	Su-SLe^x	0.46 (0.15)	1.3 (0.29)
Neu5Acα2-6Galβ1-4GlcNAcβ	6′SLN	<0.05[b]	<0.05
Neu5Acα2-6Galβ1-4(6-HSO_3)GlcNAcβ	Su-6′SLN	<0.05	<0.05
Neu5Acα2-6GalNAcα	S-Tn	<0.05	<0.05

[a] The constants were determined using solid-phase fetuin binding inhibition assays as described previously (1, 5, 6, 12) and are expressed in μM^{-1} with respect to sialic acid. The data are the mean values and standard deviations (in parentheses) from 2 to 4 replicate samples in one experiment that is representative of three independent experiments.

[b] A value of <0.05 indicates that there was no significant inhibition in the fetuin binding inhibition assay at the highest concentration of SGP used.

the sulfated trisaccharide Su-3′SLN, which is a typical binding pattern for Asian H5N1 viruses isolated from birds and humans in 1999 to 2004 (4, 17). The binding to other Neu5Ac2-3Gal-containing receptors varied depending on the structure of the oligosaccharide core. In particular, fucosylation of the GlcNAc residue of Su-3′SLN decreased the binding affinity (compare Su-3′SLN and Su-SLe^x), suggesting that the fucose moiety does not fit into the receptor-binding site (4). There was no binding to any of the Neu5Ac2-6Gal-terminated SGPs.

rKAN-1 HA_{222E} bound more weakly than rKAN-1 HA_{222K} to most of the analogues tested. This result explains the reduced hemagglutinin activity of H5N1 virus with the K222E mutation as observed by others (2). We have looked into the prevalence of 222E in the HAs of H5 viruses in the influenza sequence database (Influenza Virus Resource on http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html, accessed on 6 March 2010). Among about 2,500 available nonredundant H5 HA1 sequences, only three sequences of avian viruses and one sequence of the human isolate studied here had E at position 222. Nineteen HAs of the H5N2 viruses from South America and Japan contained 222Q. All other sequences contained either K or (much less frequently) R. This analysis suggests that a positively charged amino acid at position 222 is essential for the survival of H5N1 virus in avian and occasional mammalian host species.

It is interesting that rKAN-1 HA_{222E} is less pathogenic in mice than the HA_{222K} variant despite the higher lung titers of the former virus. We speculate that the higher receptor-binding affinity of HA_{222K} allows efficient infection of cells of the central nervous system, resulting in enhanced pathogenicity. This is consistent with the findings that only the HA_{222K} variant is found in the brain of KAN-1 infected mice and that the receptor distribution is different in brain and lung tissue (14). We speculate that the increased LD_{50} of the HA_{222E} variant most likely reflects decreased probability of mutation to 222K at lower infection doses.

The reasons for the apparent selection of HA_{222E} in two cases of human infection (9) are not clear. As noticed previously (11), a common feature of the human and swine viruses as compared to their avian precursors is the substantially decreased affinity of mammalian viruses for Neu5Ac2-3Gal-containing receptors. This feature may indicate that binding to such receptors is disadvantageous for the virus's replication in humans, for example, owing to neutralization by decoy receptors present on airway mucins (3). If this hypothesis is correct, it could provide an explanation for the accumulation in humans of the variant with the mutation 222E and decreased binding affinity to Neu5Ac2-3Gal-terminated oligosaccharides. This could also explain the higher viral lung titers in mice infected with rKAN-1 HA_{222E} compared to the lung titers seen with infections with the HA_{222K} variant.

Interestingly, despite its overall reduced binding affinity, rKAN-1 HA_{222E} showed enhanced binding to one analogue tested, Su-SLe^x, a receptor determinant commonly recognized by poultry viruses with different HA subtypes (6, 10). It remains to be determined whether enhanced binding of HA_{222E} to Su-SLe^x plays any specific role in H5N1 virus adaptation to humans or if this effect is merely coincidental with the mutation that serves to decrease viral binding to a majority of Neu5Ac2-3Gal-containing receptors.

We thank Pilaipan Puthavathana (Mahidol University, Bangkok, Thailand) for providing the virus isolate A/Thailand/KAN-1/2004 (KAN-1) and Geoffrey Chase for critical reading of the manuscript.

This work was funded by the Bundesministerium für Bildung und Forschung (BMBF, FluResearchNet) and the European Union FP6 projects FLUPATH and FLUINNATE.

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3.2 Adaptive mutations in NEP compensate defective RNA replication of H5N1 influenza viruses in humans

Submitted to: Nature Communications

Own contributions:

I contributed to design of the experiments and writing of the manuscript. I performed all experiments except parts of the primer extension assays and the co-immunoprecipitations.

Adaptive mutations in NEP compensate defective RNA replication of H5N1 influenza viruses in humans

Benjamin Mänz[1,2], Linda Brunotte[1], Peter Reuther[1] and Martin Schwemmle[1,*]

[1]Department of Virology, Institute for Medical Microbiology and Hygiene, University of Freiburg, Germany, [2]Institute for Virology, University of Marburg, Germany

Running title: Pathogenicity factor NEP rescues polymerase defect

*Correspondence to:

Dr. Martin Schwemmle
Department of Virology
Institute for Medical Microbiology and Hygiene
University of Freiburg
Hermann-Herder-Strasse 11
D-79104 Freiburg, Germany
Phone: +49-761-203-6526
Fax: +49-761-203-6639
e-mail: martin.schwemmle@uniklinik-freiburg.de

Abstract
Zoonotic transmission of avian influenza viruses into the human population requires adaptive mutations to achieve high-level replication in the new host. We provide evidence that this requirement for adaptation is caused by the inability of avian polymerases lacking the human signature lysine 627 in PB2 to generate bona fide cRNA templates in human cells. Characterization of the highly pathogenic human H5N1 isolate A/Thailand/1(KAN-1)/2004, which contains E627, revealed that the RNA replication defect is only partially compensated by adaptive mutations in the KAN-1 polymerase, and that mutations in the nuclear export protein (NEP) were crucial for efficient polymerase activity. KAN-1 and other human isolates, acquired adaptive mutations in NEP which strongly enhanced the polymerase activity of avian polymerases in human cells, whereas NEP of avian isolates did not. Thus, when crossing the species barrier, avian influenza viruses acquire adaptive mutations in NEP to escape restricted viral genome replication in the mammalian host.

Introduction
Transmission of avian influenza viruses to mammals is dependent on the acquisition of adaptive mutations that allow efficient viral replication in the new host. Besides amino acid changes in the hemagglutinin and NS1 proteins[1-3], mutations in the viral polymerase proteins have been shown to be major determinants of adaptation[2,4,5]. While avian influenza A virus isolates almost exclusively contain glutamic acid at position 627 in the polymerase PB2 protein (PB2-E627), this position is frequently mutated to lysine in human-derived isolates (PB2-E627K), including H5N1 isolates that cause a high morbidity in humans. Mutation to PB2-E627K was shown to increase viral polymerase activity in mammalian cells[2,6], is accompanied by improved viral replication at low temperatures[6,7] and enhanced binding of the viral polymerase to the nucleoprotein in human cells[8]. Intriguingly, fatal viral infections of humans with avian viruses of the H5N1 subtype that have retained the avian like PB2-E627 have been reported[9]. Moreover, the 2009 H1N1 pandemic virus also harbors PB2-E627, but additionally possesses a lysine at position 591 (PB2-E591K), which is able to partially, but not completely, compensate for the lack of the PB2-E627K mutation[10,11], suggesting that other adaptive factors may account for the efficient replication of these viruses in humans. Other mutations in PB2 known to play a role in host adaptation are the exchange of aspartic acid with asparagine

at position 701 (PB2-D701N), which can confer high pathogenicity to avian H5N1 and H7N7 viruses in mice[4,12] and the substitution of threonine with alanine at position 271 (PB2-T271A) which has been suggested to enhance polymerase activity in mammalian cells[13]. Furthermore, adaptation to human importin α might play a crucial role in interspecies transmission and pathogenicity[15,16].

The human H5N1 isolate KAN-1 (A/Thailand/1(KAN-1)/2004)[17], originating from an avian influenza outbreak in Thailand in 2003, also encodes PB2-E627, yet nonetheless is highly pathogenic in different animal models, including mice and ferrets[14,15]. To understand the molecular basis of this pathogenicity, we generated a probable avian precursor virus (AvianPr) of KAN-1, devoid of adaptive mutations. Using AvianPr, we could show that an avian influenza polymerase containing PB2-E627 exhibits a distinct defect in vRNA replication in mammalian cells. This malfunction in vRNA replication is manifested by the generation of defective cRNPs, which cannot be utilized as templates for efficient vRNA synthesis. Furthermore, we provide evidence that this defect can be compensated by the PB2-E627K mutation or adaptive mutations in the nuclear export protein (NEP).

Results

Generation of an avian precursor virus of KAN-1 and identification of pathogenicity determinants of KAN-1

The rapid evolution of H5N1 influenza in birds[16] usually prevents isolation and assignment of a direct avian precursor to highly pathogenic human viruses. Discrimination between non-adaptive amino acid changes in avian isolates that have been acquired during circulation in the avian host and adaptive mutations that specifically facilitate infection in humans is a difficult task. To circumvent this problem, we used the influenza Virus Resource Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) and compared the amino acid sequences of various H5N1 viruses of avian or human origin (Supplementary Table 1). Reversion of non avian-like amino acids in the highly pathogenic human-derived H5N1 strain A/Thailand/1(KAN-1)/2004 (KAN-1) to avian-like residues allowed us to generate the sequence of a probable low pathogenic avian precursor virus (AvianPr) of KAN-1 that differs in 7 positions in PB2, PB1, PA, NA, NS1 and NEP (Fig. 1a). To evaluate the pathogenicity of AvianPr in mammals, recombinant KAN-1 and AvianPr viruses were generated using reverse genetics[17]. Both viruses showed comparable growth kinetics in avian cells, whereas viral growth of AvianPr was strongly impaired in mammalian MDCK cells at 12 h p.i. by about three $\log_{10}$ compared to KAN-1 (Supplementary Fig. S1). Similarly, infection of BALB/c mice revealed a strong attenuation of the AvianPr virus, demonstrated by an increase in the 50% lethal dose 50 (LD_{50}) of approximately three $\log_{10}$ compared to the KAN-1 virus (Fig. 1b). These results suggest that the identified mutations in KAN-1 indeed contribute to the pathogenicity of this virus in mammalian hosts.

To dissect the contribution of the individual mutations in more detail, reassortant viruses of the two strains were generated (Fig. 1b). Introduction of the KAN-1 polymerase into the low pathogenic avian precursor (AvianPr-3P-KAN-1) resulted in reduction of the LD_{50} from $10^{3.2}$ to $10^{1.3}$ (Fig. 1b), indicating that the mutations in the polymerase proteins are crucial for the high pathogenicity of the KAN-1 virus in mice. However, introduction of the KAN-1 NS (AvianPr-NS-KAN-1) or NA segments (AvianPr-NA-KAN-1) also enhanced the pathogenicity of AvianPr, leading to a decrease in the LD_{50} by 0.7 and 0.4 $\log_{10}$, respectively (Fig. 1b). Interestingly, AvianPr containing both the KAN-1 polymerase together with the KAN-1 NS segment (AvianPr-3P/NS-KAN-1) was highly pathogenic with an LD_{50} of $10^{0.8}$, suggesting that either NS1 or NEP or both are important co-factors for KAN-1 pathogenicity.

Although mice infected with 10^4 plaque forming units (pfu) of AvianPr succumbed to infection, there was a considerable delay in weight loss (Supplementary Fig. S2), suggesting the emergence of virus

mutants harboring disease-enhancing adaptive mutations. Interestingly, sequencing of the C-terminus of PB2 from infected lung tissue revealed that all viruses containing the avian polymerase acquired adaptive mutations in PB2 during mouse infection, as well as 63 % of the AvianPr-3P-KAN-1 viruses containing the KAN-1 polymerase and 78 % of the AvianPr-NS-KAN-1 viruses containing the NS segment of KAN-1 combined with the AvianPr polymerase (Table 1). However, in mice infected with the reassortant virus AvianPr-3P/NS-KAN-1 harboring the KAN-1 polymerase and NS segment, no adaptive mutations in PB2 appeared, indicating that this virus might be sufficiently adapted to replicate in the mammalian host.

Mutations in the KAN-1 polymerase only partially increase its activity in human cells

Although introduction of the KAN-1 polymerase increased the pathogenicity of AvianPr in mice, the appearance of compensatory mutations in PB2 during mouse infection suggests an incomplete adaptation of the KAN-1 polymerase to the mammalian host., We therefore compared the polymerase activity of KAN-1 with the human H5N1 isolate A/Viet Nam/1203/2004 (Vn-1203), a highly active prototypical human H5N1 strain which encodes PB2-K627, using a polymerase reconstitution assay in human cells. For comparison we included both the AvianPr and an AvianPr polymerase containing PB2-E627K (AvianPr-PB2-E627K). Measuring reporter gene activity at 3, 6, 9, 12 hours post transfection (p.t.), we found that the KAN-1 polymerase activity was approximately one $\log_{10}$ lower compared to the Vn-1203 and AvianPr-PB2-E627K polymerases. However, after 24 hours all three polymerases reached the same activity (Fig. 2a; Supplementary Fig. S3b), indicating that the KAN-1 polymerase has a functional disadvantage in human cells, but is able to reach high productivity at later time points (Fig. 2a). As expected, the AvianPr polymerase lacking the adaptive mutations displayed the lowest polymerase activity, at least 2 $\log_{10}$ less than the PB2-E627K containing polymerases at 9, 12 and 24 hours p.t. (Fig. 2a). In contrast, all polymerases exhibited comparable activity in avian LMH cells (Supplementary Fig. S4a), thereby confirming the functionality of the AvianPr polymerase, and indicating that the enhancing effect of the adaptive mutations is specific to mammalian cells. Detailed analysis of single amino acid substitutions highlighted the importance of the mutations PB2-D701N and PA-K142R for KAN-1 and PB2-E627K and PA-K142E for Vn-1203 polymerase activity in mammalian but not avian cells (Supplementary Fig. S3a-e; Supplementary Fig. S4a-d). The increase in polymerase activity observed in human cells additionally correlated with enhanced formation of viral RNPs (Supplementary Fig. S5), as previously described[8].

Next, we performed a primer extension assay to analyze the accumulation of the three viral RNA species (mRNA, cRNA and vRNA) produced by the AvianPr, KAN-1 and AvianPr-PB2-E627K polymerases. In agreement with the results from the reporter assay (Fig. 2a), the AvianPr polymerase produced very low amounts of vRNA and mRNA and no detectable cRNA (Fig. 2b). Interestingly, the KAN-1 polymerase produced higher amounts of vRNA and mRNA than AvianPr, but,also only minor amounts of cRNA. In comparison, the highly active AvianPr-PB2-E627K polymerase produced high amounts of all three viral RNA species, correlating with the strong reporter gene activity in the reconstitution assay (Supplementary Fig. S3d). In summary, these results indicate that the adaptive mutations in the KAN-1 polymerase are not able to fully compensate for the lack of PB2-E627K and are insufficient to reach the activity of the PB2-E627K containing polymerases.

PB2-E627K compensates a defect in RNA replication of avian viruses in human cells

According to the primer extension analysis, the AvianPr polymerase, which is identical to several avian isolates (Supplementary Table S1, data not shown) produces substantially lower amounts of viral cRNA, vRNA and mRNA compared to AvianPr-PB2-E627K

polymerase. However, it remains unclear if this impairment is a result of a deficiency in c/vRNA synthesis (replication), mRNA synthesis (transcription), or a combination thereof. In order to clarify which function of the polymerase is impaired in avian strains, we performed a PB2 complementation assay using the polymerase reconstitution system. In this assay, we co-expressed a modified AvianPr PB2 subunit (PB2-E627K-Tdef) that harbors both the adaptive mutation E627K as well as mutations in the cap-binding region that diminish the cap-binding activity[18] and therefore leads to inefficient transcription of mRNA from vRNA. If AvianPr is only defective for RNA replication in human cells, the avian precursor polymerase should be able to complement the transcription defect of PB2-E627K-Tdef, whereas no complementation should occur if the AvianPr polymerase also has a defect in transcription (Fig. 3a). As shown in Fig. 2b, polymerase containing AvianPr PB2 (PB2-AvianPr) produced only low levels of all three RNA species (Fig. 3b). In contrast, the transcription-deficient polymerase (PB2-E627K-Tdef) produced high amounts of vRNA and cRNA, but no detectable amounts of mRNA. Co-expression of equal amounts of PB2-AvianPr and PB2-E627K-Tdef polymerases resulted in the restoration of full RNA synthesis and the production of all three RNA species to levels resembling the PB2-E627K phenotype. These results suggest that the AvianPr polymerase is not defective for mRNA synthesis and can synthesize mRNA when the vRNA template is provided in trans. Using the luciferase reporter construct, we could similarly observe strongly reduced reporter gene activity for PB2-AvianPr and PB2-E627K-Tdef, while the activity was restored by co-expression of both polymerases (Fig. 3c), confirming the results observed by primer extension analysis. We therefore conclude that the non-adapted AvianPr polymerase does not harbor a defect in viral mRNA synthesis. Supporting this hypothesis, AvianPr and AvianPr-E627K recombinant viruses exhibited equal primary mRNA synthesis after infection of 293T cells in the presence of cycloheximide (Supplementary Fig. S6), conditions which prevent viral RNA replication.

We then investigated whether the replication defect of AvianPr is caused by impairment in cRNA synthesis, since no cRNA production by AvianPr was detectable in the primer extension assay (Fig.2b). Since this could be caused by impaired cRNA synthesis and/or rapid degradation of synthesized cRNA, we perfomed a cRNA stabilization assay[19]. As described by others, cRNA synthesis can be observed by pre-expression of NP and a catalytically inactive polymerase, containing an inactive PB1 subunit[19] prior to viral infection in the presence of cycloheximide[19]. As shown in Fig. 3d (lane 1 and 2), in AvianPr and AvianPr-E627K-infected cells polymerases produce substantial and equivalent amounts of cRNA. To further determine whether the cRNA transcripts can be utilized as templates for vRNA synthesis, we overexpressed functional AvianPr or AvianPr-PB2-E627K polymerase subunits and NP prior to infection and cycloheximide-treatment. Intriguingly, in cells infected with AvianPr, both polymerases failed to produce vRNA (Fig.3d, lanes 3 and 5) but synthesized substantial amounts of vRNA in AvianPr-PB2-E627K infected cells (Fig.3, lanes 4 and 6). Thus, avian polymerases lacking PB2-E627K appear incapable of providing bona fide cRNA templates in mammalian cells (see Discussion).

These data suggest a general requirement of avian influenza polymerases to increase their RNA replication efficiency in mammals, which can be achieved by the adaptive mutation E627K in PB2. Based on the observation that the KAN-1 polymerase is not fully adapted to mammalian cells, we speculated that an additional co-factor is necessary for efficient RNA replication by KAN-1.

The NEP protein of KAN-1 is involved in host adaptation.

As we could show that the NS segment contributes to viral pathogenicity of KAN-1, we speculated that either NS1 or NEP, both encoded by this segment, could have a stimulatory effect on viral

polymerase activity in human cells. Due to overlapping open reading frames of the two proteins, the single mutation in the NS segment of the KAN-1 virus results in an amino acid change at position 174 (valine to isloleucine) in the NS1 protein and at position 16 (methionine to isoleucine) in NEP (Fig. 4a). To elucidate if these amino acid changes in NS1 or NEP stimulate the activity of a polymerase lacking adaptive mutations, we determined the polymerase activity of AvianPr in the presence of either protein in human cells. Co-transfection of either KAN-1 or AvianPr NS1 expression plasmids resulted in a reduction of AvianPr reporter activity (Fig. 4b), suggesting that the mutation in KAN-1 NS1 is not important for viral polymerase function. In contrast, we could observe an enhancement of the AvianPr reporter activity by the NEP proteins of both the AvianPr and KAN-1 virus at low plasmid concentrations (Fig. 4c). However, reporter gene activity was up to 51-fold higher upon co-expression of the KAN-1 NEP compared to NEP of AvianPr. This indicates that the mutation M16I in the KAN-1 NEP leads to an enhancement of the polymerase stimulating property of the avian NEP (Fig. 4c). On the other hand, we observed an inhibition of reporter gene activity at high plasmid concentrations for both NEP proteins. This inhibitory effect was much more pronounced for the AvianPr NEP upon co-transfection, leading to a reduction of reporter gene activity to background levels (Fig. 4c). Expression levels of the AvianPr and KAN-1 NEP in human HEK293T cells were comparable (Fig. 5c). At low levels of transfected NEP expression plasmids, primer extension analysis revealed that AvianPr NEP slightly increased the level of cRNA but not mRNA (Fig. 4d). In contrast, KAN-1 NEP greatly enhanced the synthesis of all viral RNA species, including cRNA. At 10-fold higher amounts of transfected NEP-expression plasmids viral polymerase was inhibited by NEP of both KAN-1 and AvianPr, however, the inhibitory effect of KAN-1 NEP was less pronounced. Next, we tested whether the stimulatory effect of KAN-1 NEP is able to compensate the inefficient adaptation of the KAN-1 polymerase in a polymerase reconstitution assay, as observed in Fig. 2a. Co-expression of KAN-1 polymerase with KAN-1 NEP resulted in similar reporter activity as AvianPr-PB2-E627K co-expressed with AvianPr-NEP (Fig. 4e). As expected, the AvianPr NEP did not lead to a comparable increase of the AvianPr polymerase activity.

We recently showed that NEP is associated with purified viral RNPs[20]. Since NEP appears to compensate for a defect in the viral polymerase, we tested whether these proteins physically associate using co-immunoprecipitation. KAN-1 NEP interacted with both PB1 and PB2, whereas no complex formation was detected with PA (Fig. 4f). Moreover, KAN-1 NEP associated with PB2 of KAN-1, AvianPr as well as Avian-Pr-E627K (Fig. 4g), suggesting that the protein interaction domain on PB2 is conserved in all three PB2 subunits. Using PB2 truncation mutants we could further map the interaction of NEP to the N-terminal part of PB2 comprising amino acids (aa) 1-534, containing the Cap-binding domain, but not to a shorter fragment (aa 1-299) or the C-terminal domain of PB2 (aa 535-759)(Fig. 4h).

Due to the overlapping open reading frames coding for NS1 and NEP, we cannot formally exclude a contribution of the NS1 protein of KAN-1 in the adaptation process to humans. However, we observed no difference in type I interferon induction between both strains after infection of a reporter cell line coding for firefly luciferase under the control of the interferon beta promoter with either AvianPr or AvianPr-NS-KAN-1 (Supplementary Fig. S7).

Together, these results indicate that the adaptive mutation M16I in the NEP protein of the KAN-1 virus enhances the capacity of NEP to stimulate viral polymerase activity, rendering NEP an essential co-factor in the adaptation process of the KAN-1 virus to a mammalian host.

The KAN-1 NS segment enhances viral RNA synthesis of the AvianPr virus

To investigate whether the RNA synthesis enhancing effect of the KAN-1 NEP is also relevant in the context of a viral

infection, HEK293T cells were infected with the recombinant viruses AvianPr, KAN-1, AvianPr containing the KAN-1 NS segment (AvianPr-NS-KAN-1) and AvianPr with the polymerase harboring the adaptive mutation E627K in the PB2 protein (AvianPr-PB2-E627K), respectively. To monitor early phases of viral RNA synthesis, total RNA from cells harvested at 1, 2, 2.5 and 3 hours post infection (p.i.) were used for measuring the accumulation of vRNA, cRNA and viral mRNA by primer extension analysis. AvianPr failed to synthesize viral RNAs to levels comparable to KAN-1 and AvianPr-PB2-E627K at any time point measured (Supplemental Fig. S8). However, introduction of the KAN-1 NS segment (AvianPr-NS-KAN-1) could rescue this defect and lead to enhanced synthesis of all three viral RNA species by the avian precursor polymerase (Supplemental Fig. S8). Together, these results indicate that the non-adapted avian polymerase has a disadvantage to replicate efficiently in mammalian cells and that an adaptive mutation in either PB2 (E627K) or in NEP (M16I) can compensate this defect.

NEPs of different human influenza isolates containing PB2-E627 enhance viral polymerase activity in mammals

We could show that the adaptive mutation in the KAN-1 NEP protein increases its capacity to enhance the avian viral polymerase activity. This polymerase enhancing activity is comparable to the effect mediated by the introduction of the PB2-E627K mutation into the AvianPr polymerase, suggesting that NEP may be an important pathogenicity factor for human influenza viruses that have retained the avian-like PB2-E627. We therefore investigated whether other H5N1 viruses encoding PB2-E627 contained adaptive mutations in NEP. Through sequence alignment, we identified additional avian and human clade 1 and clade 2 H5N1 isolates that contain PB2-E627 and differ by only a few amino acids from the AvianPr NEP, which resembles the consensus sequence of H5N1 viruses (Fig. 5a). Whereas the mutations V14M, A115T, A48T and S7L are found in avian and human isolates, the mutations Y41C and E75G are restricted to human isolates. The mutation S7L is mainly restricted to H5N1 strains from Indonesia and, all 80 human isolates analyzed of this subtype contain this mutation. These mutations were introduced into the AvianPr NEP protein to investigate their polymerase enhancing capacity in the polymerase reconstitution assay. As the pandemic H1N1 virus also possesses PB2-E627 and is the dominant influenza A virus strain currently circulating in the human population, we also included the NEP of A/California/04/2009 (H1N1). In agreement with our assumption that only mutations in human-adapted strains lead to the enhanced NEP phenotype, the avian NEP variants harboring the mutations V14M and A115T retrieved from the avian isolates did not lead to an increase in reporter activity (Fig. 5b). The mutation A48T found in the human H5N1 isolate A/Viet Nam/1203/2004 (Vn-1203), which harbors PB2-K627, only weakly enhanced the reporter activity. In contrast, the human-derived mutations Y41C and E75G in avian NEP and also the pandemic H1N1 NEP protein were able to increase reporter gene activity to a similar extent as KAN-1 NEP (Fig. 5b). Western blot analysis confirmed comparable expression of these proteins (Fig. 5c). The increased enhancing activity of the pandemic H1N1 NEP and the NEP variants containing the human-specific mutations Y41C and E75G was further supported by primer extension analysis, which showed a strong increase in all three viral RNA species (vRNA, cRNA and mRNA) upon co-expression (Supplemental Fig. S10). However, none of the NEP proteins encoding the avian-derived mutations V14M, A115T or A48T was able to raise viral RNA levels to the same extent as KAN-1 NEP. Notably, the S7L mutation found in avian and human H5N1 isolates (Fig. 5a) also stimulated the AvianPr polymerase (Supplemental Fig. S9). Thus the increased polymerase-enhancing phenotype of NEP can be achieved by many different amino acid changes. Therefore the utilization of NEP as a co-factor in the viral adaptation process to the human host is most likely not restricted to the KAN-1 virus, as it can also be observed for other highly

pathogenic human H5N1 isolates and the pandemic H1N1 strain containing the avian-like PB2-E627 signature.

Discussion

Zoonotic transmission of influenza A viruses is a constant threat to the human population. Although viral replication of avian viruses, including highly pathogenic avian H5N1 strains, is impaired in humans, incorporation of adaptive mutations enables the virus to overcome this defect. In this study, we characterized the supply of bona fide cRNA as the principle defect of avian polymerases in human cells. Furthermore, we provide evidence that the polymerase of the human H5N1 isolate KAN-1, which encodes PB2-E627, is only partially able to compensate for this defect, and that the NEP of KAN-1 acquired an adaptive mutation (M16I) to stimulate the inefficient polymerase activity of this virus in human cells. We further show that NEPs of circulating avian H5N1 viruses fail to compensate for the defect of avian viruses in human cells, while NEPs of human-derived H5N1 viruses encoding PB2-E627 contain adaptive mutations which enhance polymerase activity. Thus, we identified adaptive mutation of NEP as a strategy by which avian H5N1 viruses facilitate transmission from birds to humans.

Our results suggest that the major defect of avian polymerases in human cells is an impairment in vRNA replication. We observed that an avian polymerase (AvianPr), which normally produces only very low levels of viral mRNA, synthesized much higher amounts of mRNA when cRNA and vRNA were provided by an alternative polymerase (Fig. 3b), suggesting that the avian polymerase is specifically impaired in either cRNA or vRNA synthesis. Interestingly, both avian and human-like (AvianPr-PB2-E627K) polymerases synthesized cRNA to an equivalent extent (Fig. 3d). However, cRNA produced by AvianPr was unable to support robust vRNA synthesis in the presence of an active polymerase (Fig. 3d lanes 5), while cRNA produced by AvianPr PB2-E627K supported robust vRNA synthesis (Fig. 3d, lanes 4 and 6). Based on this finding, we speculate that avian polymerases do not have an impaired rate of RNA synthesis in human cells, but rather synthesize defective cRNPs which are not bona fide templates further RNA replication. This hypothesis is supported by our observation that AvianPr could efficiently synthesize vRNA when provided with competent cRNPs (Fig. 3d, lane 4), and by a previous report which showed that recombinant, purified polymerase is active in vitro regardless of the identity of PB2 residue 627[21]. Moreover, we observed reduced levels of cRNA after providing a competent polymerase compared to an incompetent polymerase (Fig. 3d, compare lane 3 and 5 to lane 1). This suggests a decreased stability of cRNPs created by an avian influenza polymerase (Fig. 6, upper panel), as observed before for cRNPs at high temperature[22]. In summary, the well-known adaptive mutation PB2 E627K appears to confer the ability to correctly synthesize cRNPs to avian polymerases in human cells.

Intriguingly, 71% of the human H5N1 virus isolates (127/179, Influenza virus research database) have not acquired the PB2-E627K mutation, likely reflecting an incomplete adaptation to human hosts. Nonetheless, many of these viruses are highly pathogenic, even fatal, in humans. Our results suggest that these viruses utilize NEP as a polymerase cofactor to elevate their low RNA replication capacity in humans (Fig. 6, lower panel). Adaptive mutations in NEP are required to efficiently stimulate avian and avian-like influenza polymerases. Interestingly, human H5N1 isolates that harbor the adaptive mutation E627K in PB2 do not contain adaptive mutations in NEP which enhance the polymerase activity to a similar degree as KAN-1 NEP (Fig. 5). We therefore speculate that viruses such as Vn-1203, which possess highly active polymerases due to the PB2-E627K mutation, are not dependent on the additional replication-enhancing property of NEP (Fig. 6, middle panel).

Interestingly, our data also suggest that NEP directly interacts with the viral polymerase in human cells, as we could show an association with the PB1 and PB2 subunits. Since both subunits have

been implicated in promoter binding[23], we speculate, that association with NEP might also lead to a stabilization of cRNPs, thereby increasing the synthesis of vRNA transcripts, as suggested by others[24].

Although the KAN-1-specific adaptive mutation M16I is located within the nuclear export signal (NES) of NEP (aa 12-21)[25], the replication enhancing property of NEP is most likely independent of its export function[24]. In addition, treatment of cells with leptomycin B, an inhibitor of nuclear export, had no effect on the reporter enhancing function of either KAN-1 NEP or AvianPr NEP (data not shown). Furthermore, other adaptive mutations found to enhance the polymerase activity (Y41C and E75G) are located outside the NES motif. The latter mutation (E75G) is known to be involved in M1 binding[35], however since M1 is not included in the polymerase reconstitution assay, it is unlikely to be relevant.

Interestingly, we observed that the mutation S7L promoted the polymerase enhancing activity of NEP. Sequence analysis revealed that this mutation is present in NEP of all avian and human H5N1 isolates from the Indonesian lineage. Correspondingly, 89% (80/90, Influenza virus research database) of the human isolates originating from this lineage have retained the avian like PB2-E627. We hypothesize that the acquisition of the mutation S7L in NEP is responsible for the extraordinarily high frequency of PB2-E627 in this lineage. Through an increased stimulatory effect of NEP on polymerase activity, this mutation possibly lowered the selective pressure on these viruses to obtain the PB2-E627K mutation in humans.

Our identification of NEP as a novel pathogenicity factor suggests that avian influenza viruses have developed multiple strategies to adapt to human hosts. The recognition of adaptive mutations in NEP might help to evaluate the potential risk of circulating avian viruses to the human population.

Materials and Methods

Plasmid constructions. The previously described pHW2000 vectors[36,37] were used for influenza A virus rescue. To obtain rescue plasmids containing segments that encode mutant genes, site directed mutagenesis was performed. To create the pCAGGS expression plasmids encoding for mutant PB2, PB1 or PA, the required mutations were introduced by assembly PCR. The resulting PCR fragments were cloned into vectors expressing PB2 and PB1 with the restriction enzymes NotI and XhoI or PA using NotI and NheI. The NS1 and NEP coding plasmids were cloned using the gene-specific forward and reverse primers for NS1 and NEP and cloned into pCAGGS vectors using NotI and XhoI. The pCAGGS expression plasmids encoding the respective C-terminally HA-tagged polymerase subunites (PA, PB1, PB2) or PB2 truncation mutants were generated by PCR and ligated into pCAGGS PA_{SC35M}-HA vector[38] linearized with XmaI and NotI. The N-terminal Strep-tag[20] was cloned in pCAGGS-HA-NEP_{KAN-1} expression plasmid linearized with EcoRI and NotI .

Animal experiments. BALB/c mice were obtained from Janvier (Straßburg). Six to eight week-old mice were anesthetized with a mixture of ketamin (100 µg per gram body weight) and xylazine (5 µg per gram) administered intraperitoneally and inoculated intranasally with the indicated doses of viruses in 50 µl phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA). Animals were euthanized if severe symptoms developed or body weight loss approached 25% of the initial value. Lung homogenates were prepared using the FastPrep24 system (MP Biomedicals). Briefly, after addition of 800 µl of PBS containing 0.2% BSA, lungs were subjected to two rounds of mechanical treatment for 10 s each at 6.5 m/s. Tissue debris was removed by low-speed centrifugation. The LD_{50} values were calculated based on the infectious dose (PFU).

Sequencing of viruses. RNA purification was carried out using 800 µl of Trizol™ reagent and 200 µl of virus stock or lung homogenate. Purified RNA was reversed transcribed using the one step RT-PCR

kit (Roche) and PCR was performed with KAN-1 specific primers.

Cells. HEK293T, 3T3 and MDCK II cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1% Penicillin/Streptomycin. Chicken hepatocellular epithelial cell line (LMH) was grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum 2% chicken serum, 2 mM L-glutamine and 1% penicillin/streptomycin. All cells were maintained at 37°C and 5% CO_2. Co-precipitation experiments were essentially carried out as described[26] using Strep-Tactin sepharose beads (IBA).

Reconstitution of the influenza virus polymerase. As described[27], HEK293T cells seeded into a 12-well plate were transiently transfected with a plasmid mixture containing either influenza A virus PB1-, PB2-, PA- (50 ng each), NP-expression plasmids (200 ng), and a human polymerase I (Pol I) expression plasmid (25 ng) expressing an influenza virus-like RNA (vRNA-luciferase) coding for the reporter protein firefly luciferase to monitor viral polymerase activity. vRNA-luciferase was flanked by non-coding sequences of segment 8 of FluA. The transfection mixture also contained a plasmid constitutively expressing renilla luciferase (25 ng), which served to normalize variation in transfection efficiency. Reconstitution of the viral polymerase complex in avian cells was performed as above with the exception that avian LMH cells were transfected and the minigenome RNA was expressed under the control of a chicken Pol I promoter (kindly provided by Daniel Mayer).

cRNA stabilization assay. The experiment was essentially carried out as described in[23]. Briefly, human 293T cells were transiently transfected with the pCAGGS expression plasmids coding for various polymerase subunits (250 ng) and NP (1000 ng) for 16 to 18 hours and subsequently infected with the indicated virus strains at an MOI of 5 in the presence of 100µg of cycloheximide/ml.

Primer extension analysis. For determination of viral transcript levels of reconstituted polymerases, 6-well plates with confluent HEK293T cells were transiently transfected with a plasmid mixture containing either FluA PB1-, PB2-, PA- (125 ng each), NP-expression plasmid (500 ng), and a polymerase I (Pol I)-expression plasmid (25 ng) expressing an influenza virus RNA coding for the segment 6 of FluA. 25 ng of empty vector or NEP expression plasmid was contransfected for experiments involving NEP. For determination of viral transcript levels in virus-infected HEK293T cells, cells were seeded in 6-well plates. Infection was carried out with infection media (Dulbecco's modified Eagle's medium supplemented with 0.2% BSA, 2 mM L-glutamine and 1% Penicillin/Streptomycin). For analyzing primary viral transcription, cycloheximide (100 µg/ml) was added to the infection media. After the indicated time point post transfection or infection, cells were harvested in Trizol™ and RNA was purified according to the manufacturer's protocol (Invitrogen). Primer extension analysis was performed using specific primers for the NA and PB2 segment (mRNA, cRNA and vRNA) and cellular 5sRNA as described[41,42].

Ethics statement. All animal experiments were performed in compliance with the German animal protection law (TierSchG). The animal welfare committees of the University of Freiburg, as well as the local authorities approved all animal experiments.

Acknowledgements

We thank Georg Kochs for providing the 3T3 cells stable transfected with a plasmid coding for Firefly luciferase under the interferon beta promoter, Thorsten Wolff for providing the NEP specific antibody and Geoffrey Chase, Veronika Götz and Mindaugas Juozapaitis for critically reading the manuscript. This study was funded by the Bundesministerium für Bildung und Forschung (FluResearchNet) and the Deutsche Forschungsgesellschaft (SCHW 632/11).

Figure legends

Figure 1. Characterization of KAN-1 and AvianPr reassortant viruses in mice. (**a**) Amino acid differences between indicated viral proteins of KAN-1 and the avian precursor AvianPr. (**b**) Lethal dose 50 (LD_{50}) of KAN-1, AvianPr, and the indicated AvianPr reassortant viruses after intranasal infection of BALB/c mice (n=5/group). Reassortant viruses include AvianPr harboring either all three polymerase segments (AvianPr-3P-KAN-1), NS (AvianPr-NS-KAN-1), NA (AvianPr-NA-KAN-1) or both the NS and polymerase segments (AvianPr-3P/NS-KAN-1) of KAN-1. Segments originating from KAN-1 virus are highlighted in grey.

Figure 2. Activity of KAN-1 polymerase. (**a**) To determine the polymerase activity of the indicated viruses in a time-dependent manner, HEK293T cells were transiently transfected with expression plasmids coding for the corresponding PB2, PB1, PA and NP proteins, a human polymerase I-driven vRNA-luciferase reporter plasmid and a renilla-expressing plasmid. Omission of PB2 (-PB2) was used as a negative control. Renilla activity was used to normalize variation in transfection efficiency. Error bars indicate the standard error of the mean of three independent experiments. (**b**) Determination of the mRNA, cRNA and vRNA levels by primer extension analysis after reconstitution of the AvianPr, KAN-1 and AvianPr-PB2-E627K polymerases 24 hours post transfection using segment 6. Determination of the 5sRNA levels served as an internal loading control.

Figure 3. Polymerase complementation assay. (**a**) Cartoon depicting the various PB2 mutants and the expected polymerase activity in the presence of these mutants. AvianPr-PB2-E627K-Tdef represents the AvianPr PB2 harboring the mutation E627K and two mutations (E361A, F404A) in the cap-binding domain. Black solid arrows refer to synthesis of high RNA levels by the indicated polymerase; arrows with dotted lines indicate synthesis of low viral RNA amounts. The question mark indicates unknown levels of RNA synthesis. (**b**) Determination of the mRNA, cRNA and vRNA levels by primer extension analysis after reconstitution of the AvianPr with the indicated PB2 subunits using specific primers for segment 6. Determination of the 5sRNA levels served as an internal loading control. For reconstitution the total amount of 50 ng of PB2-expressing plasmids were transfected. The complementation assays included 25 ng of AvianPr-PB2 and 25 ng AvianPr-PB2-E627K-Tdef. Omission of PB2 (-PB2) was used as a negative control. (**c**) Luciferase reporter activities of mutant polymerases 24h post transfection after reconstitution of the indicated polymerases as described in Fig. 2. For each reconstitution a total amount of 50 ng of PB2-expressing plasmids were used. The complementation assays included 25 ng of AvianPr-PB2 and 25 ng AvianPr-PB2-E627K-Tdef. Error bars indicate the standard error of the mean of three independent experiments. Student's *t*-test was performed to determine the *P* value. **P*<0.05; ***P*<0.01. (**d**) Rescue of cycloheximide-mediated inhibition of influenza virus replication. Human 293T cells were transiently transfected with the indicated polymerase subunits and viral nucleoprotein (NP) and subsequently infected with the indicated virus strain at an MOI of 5. Viral RNA species were analyzed 6 hours post infection by primer extension using specific primer for segment 1.

Figure 4. Influence of NS segment encoded proteins on polymerase activity.
(**a**) Cartoon depicting the amino acid mutations in NS1 and NEP of KAN-1 caused by a single nucleotide mutation in the KAN-1 NS segment. (**b**) Reporter activities after reconstitution of the AvianPr polymerase as described in Fig. 2 and co-transfection of the indicated amounts of NS1-AvianPr, or NS1-KAN-1 encoding plasmids. Vector, empty plasmid. (**c**) Reporter activities after reconstitution of the AvianPr polymerase as described in Fig. 2 and co-transfection of the indicated amounts of NEP-AvianPr, or NEP-KAN-1 encoding plasmids. Student's *t*-test was performed to determine the *P* value. **P*<0.05, ***P*<0.01. Vector, empty plasmid. (**d**) Determination of the mRNA,

cRNA and vRNA levels by primer extension analysis after reconstitution of the AvianPr polymerase in human cells and co-transfection of either empty plasmid (Vector), NEP-AvianPr or NEP-KAN-1 using primers specific for segment 6. Determination of the 5sRNA levels served as an internal loading control. (**e**) Time course of reporter activities of AvianPr, KAN-1 and Vn-1203 polymerases in human cells as described in Fig. 2a after co-transfection of 10 ng of the indicated NEP-expressing plasmids. (**f**) Complex formation of NEP with either PB1, PA or PB2. Human 293T cells were transiently transfected with Strep-tagged KAN-1 NEP and the indicated HA-tagged polymerase subunits and subjected to co-immunoprecipitations (IP). Precipitated polymerase subunits were identified by Western blot analysis using specific antibodies against the HA- and the Strep-tag. (**g**) Binding of Strep-tagged KAN-1 NEP to the indicated PB2 variants and (**h**) to PB2 truncation mutants.

Figure 5. Identification of adaptive mutations in NEP of other human isolates. (**a**) Sequence alignment of NEPs of human and avian H5N1 viruses as well as the NEP of the 2009 pandemic H1N1 virus to the H5N1 consensus sequence. Bold letters indicate amino acids exclusively found in human H5N1 isolates. (**b**) Reporter activities after reconstitution of the AvianPr polymerase as described in Fig. 2 in human cells and co-transfection of 10 ng of expression plasmids coding for AvianPr NEP harboring the indicated amino acid substitution. (**c**) Western blot analysis of the NEP expression levels after transient transfection of HEK 293T cells with equal amounts of expression plasmids (500 ng) encoding the indicated NEP variants.

Fig. 6 Model of adaptive mutations contributing to the replication of avian H5N1 isolates in human cells. See discussion for details**.**

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Supplementary figure legends

Supplementary Table 1: Alignment of human H5N1 isolates from Thailand used for the generation of the KAN-1 precursor virus AvianPr. NCBI influenza database was used to identify the amino acid sequence of the potential avian precursor virus of the human H5N1 isloate KAN-1. Amino acids of the clade 1 human H5N1 isolates from Thailand (KAN-1, Thai16, SP-33, KK-494 and SP83) that differ to the amino acids of the avian H5N1 consensus sequence (H5N1 consensus) were identified. Full-length sequence information (PB2: n=999, PB1: n=1059, PB1-F2: n=682, PA: n=1105, HA:

n=2638, NP: n=1081, NA: n=1668, M1: n=1584, M2: n=1229, NS1: n=1777, NEP: n=1220) was used to identify the avian H5N1 consensus. Amino acids shown in brackets represent common mutations found in avian isolates. Mutations that are exclusively found in human isolates and therefore most likely acquired after transmission to humans are highlighted in grey. * The mutation E627K in PB2 (PB2-E627K) is also found in avian isolates, but not in isolates of the analyzed clade 1 viruses. The amino acid sequence of the AvianPr virus was obtained by exchanging the exceptional mutations in the KAN-1 virus to common avian amino acids (H5N1 consensus). Comparison of AvianPr to the closely related avian isolate VSMU-20 (A/open-bill stork/Thailand/VSMU-20-AYA/2004 (H5N1)) revealed only 3 amino acid differences (HA-R341K, M1-T168I, M2-V86I). The virulence of the individual H5N1 strains (HP, highly pathogenic; LP, low pathogenic) in mammals, as demonstrated by others is indicated[1,2]. KAN-1: A/Thailand/1(KAN-1)/2004 (H5N1); Thai16: A/Thailand/16/2004 (H5N1); SP-33: A/Thailand/2(SP-33)/2004 (H5N1); KK-494: A/Thailand/5(KK-494)/2004 (H5N1); SP83: A/Thailand/SP83/2004 (H5N1).

Supplementary Fig. S1: Growth curves of infected cell culture. Confluent canine MDCKII **(a)** and avian LMH **(b)** cells were infected with an MOI of 0.001 and incubated at 37°C. At the indicated time points virus titer in the supernatant was analyzed by plaque assay. Error bars indicate the standard error of the mean of three independent experiments. Student's *t*-test was performed to determine the *P* value. ***$P<0.001$.

Supplementary Fig. S2: Weight curves of infected BALB/c mice. (a-d) 6-8 weeks old female BALB/c mice (n=5/group) were infected intranasally with indicated infectious doses of either AvianPr or KAN-1 virus. Weight loss was monitored for 14 days.

Supplementary Fig. S3: Reporter activities of reconstituted polymerases in human 293T cells. (**a**) Amino acid differences between the polymerase subunits PB2, PB1 and PA of the avian precursor virus (AvianPr) and the H5N1 isolates KAN-1 and Vn-1203. Bold Amino acids represent mutations that differ from the AvianPr sequence. (**b**) Polymerase activity of the AvianPr, the AvianPr harboring a lysine at position 627 in PB2 (AvianPr-PB2-E627K) KAN-1 and Vn-1203 viruses in human cells. To determine the polymerase activities, HEK293T cells were transiently transfected with expression plasmids coding for PB2, PB1, PA and NP of the annotated viruses, a vRNA-luciferase reporter plasmid under the control of a human Pol I-promoter and for normalization, a renilla-expressing plasmid. Omission of PB2 (-PB2) was used as a negative control. Luciferase reporter activity was normalized to renilla activity and the levels obtained with the AvianPr polymerase was set to 100%. Error bars indicate the standard error of the mean of three independent experiments. (**c**) Relative polymerase activity as described in (b) after exchange of the indicated polymerase subunits.
(**d,e**) Relative AvianPr polymerase activity as described in (b) after mutation of the indicated amino acid positions in PB2 (**d**) or PA (**e**).

Supplementary Fig. S4: Reporter activities of reconstituted polymerases in avian LMH cells. (**a**) Polymerase activity of the AvianPr, the AvianPr harboring a lysine at position 627 in PB2 (AvianPr-PB2-E627K), KAN-1 and Vn-1203 viruses in avian cells. To determine the polymerase activity avian LMH cells were transiently transfected with expression plasmids coding for PB2, PB1, PA and NP of the annotated viruses, a vRNA-luciferase reporter plasmid under the control of the chicken Pol I promoter and for normalization, a renilla-expressing plasmid. Omission of PB2 (-PB2) was used as a negative control. Luciferase reporter activity was normalized to renilla activity and the levels obtained with the AvianPr polymerase was set to 100%. Error bars indicate the standard error of the mean of three independent experiments. (**b**) Relative polymerase activity as described in (a) after exchange of the indicated polymerase subunits. (**c,d**) Relative polymerase activity of the AvianPr as described in (a) after mutation of the indicated amino acid positions in PB2 (**c**) or PA (**d**).

Supplementary Fig. S5: RNP formation of reconstituted polymerases. HEK293T cells were transiently transfected with expression plasmids coding for PB2-HA with the annotated mutation, AvianPr-PB1, AvianPr-PA and AvianPr-NP, a vRNA-luciferase reporter plasmid under the control of a human Pol I-promoter and for normalization, a renilla-expressing plasmid. Luciferase reporter activity was normalized to renilla activity and the levels obtained with the AvianPr-PB2-HA was set to 100%. Error bars indicate the standard error of the mean of three independent experiments. Immunoprecipitation was carried out as described[3].

Supplementary Fig. S6: Analysis of primary viral transcription. HEK293T cells were treated with 100 µg/ml cycloheximide and infected with an MOI of 5 of either AvianPr or AvianPr-PB2-E627K. Cells were harvested 4 hours p. i. and mRNA, cRNA and vRNA levels were determined using primer extension analysis with primers specific for segment 6. Levels of cellular 5sRNA served as internal loading control.

Supplementary Fig. S7: Interferon induction in infected cells. Mouse 3T3 cells stably transfected with a plasmid coding for firefly luciferase under the control of the interferon beta promoter were infected with AvianPr or AvianPr harboring the NS-segment of KAN-1 (AvianPr-NS-KAN-1) at the indicated MOI. Mock infection or infection with either SC35M-WT or SC35M-delNS1 at a MOI of 0.5 known to induce β-interferon served as internal controls. Luciferase activity was determined after 16 hours post infection.

Supplementary Fig. S8: Accumulation of viral RNAs during infection with recombinant viruses. (**a**) Human HEK293T cells were infected with an MOI of 5 with AvianPr, KAN-1, AvianPr-NS-KAN-1 and AvianPr-PB2-E627K. After the indicated time points post infection (p.i.), cells were lysed and RNA levels were determined by primer extension analysis using primers specific for segment 6. Determination of the 5sRNA levels served as an internal loading control. -, primer extension analysis using uninfected cells. (**b**) Quantification of the radioactive signals shown in panel (a). Signals were normalized to 5sRNA level. Increase in mRNA and cRNA levels was normalized to the strain-specific vRNA levels at 1 hour p.i.

Supplementary Fig. S9: Mutation S7L in NEP enhances reporter activity of the AvianPr polymerase. Reporter activities after reconstitution of the AvianPr polymerase as described in Fig. 2 in human cells and co-transfection of 25 ng of expression plasmids coding for an empty vector, AvianPr NEP, or the S7L mutant NEP. Error bars indicate the standard error of the mean of three independent experiments. Student's *t*-test was performed to determine the *P* value. **P*<0.05.

Supplementary Fig. S10: Influence of co-expressed mutant NEPs on the accumulation of viral RNAs. Determination of the mRNA, cRNA and vRNA levels by primer extension analysis after reconstitution of the AvianPr polymerase in human cells and co-transfection of either empty plasmid (Vector) or AvianPr NEP harboring the indicated amino acid substitution using primers specific for segment 6. Determination of the 5sRNA levels served as an internal loading control.

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Table 1. Adaptive mutations in mice

Strain	Mutation in PB2/ number of mice		Mutation rate (%)
	E627K	D701N	
KAN-1	0/5	-	0
AvianPr	6/7	5/7	100
AvianPr-3P-KAN-1	5/8	-	63
AvianPr-NS-KAN-1	7/9	0/9	78
AvianPr-3P/NS-KAN-1	0/8	-	0

Table-1 Schwemmle

a

Protein	PB2	PB1	PA		NA	NS1	NEP
AA-Position	701	568	142	628	429	169	16
KAN-1	N	V	R	M	T	I	I
AvianPr	D	I	K	V	N	V	M

b

						LD_{50}
KAN-1	PB2	PB1	PA	NA	NS	$10^{0.3}$
AvianPr	PB2	PB1	PA	NA	NS	$10^{3.2}$
AvianPr-3P-KAN-1	PB2	PB1	PA	NA	NS	$10^{1.3}$
AvianPr-NS-KAN-1	PB2	PB1	PA	NA	NS	$10^{2.5}$
AvianPr-NA-KAN-1	PB2	PB1	PA	NA	NS	$10^{2.8}$
AvianPr-3P/NS-KAN-1	PB2	PB1	PA	NA	NS	$10^{0.8}$

Figure-1 Schwemmle

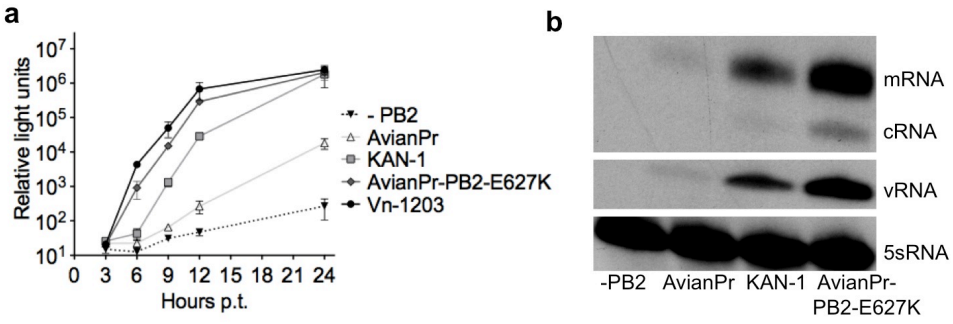
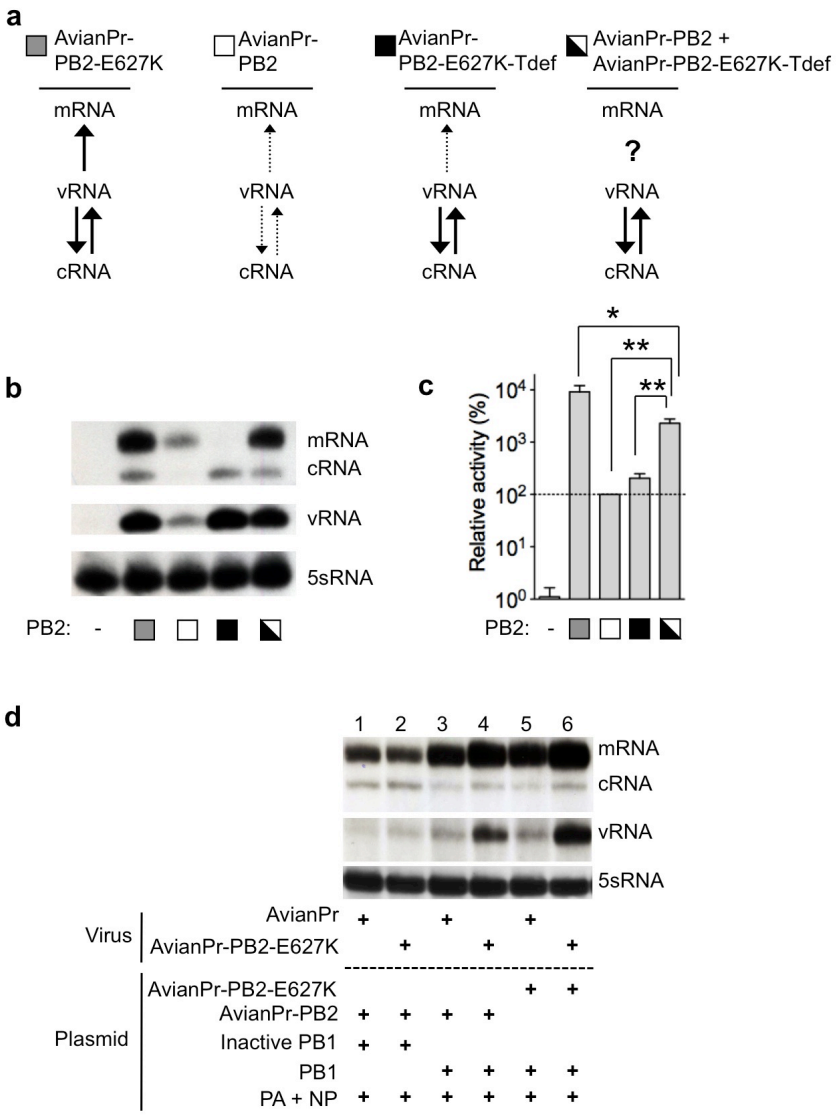


Figure-2 Schwemmle



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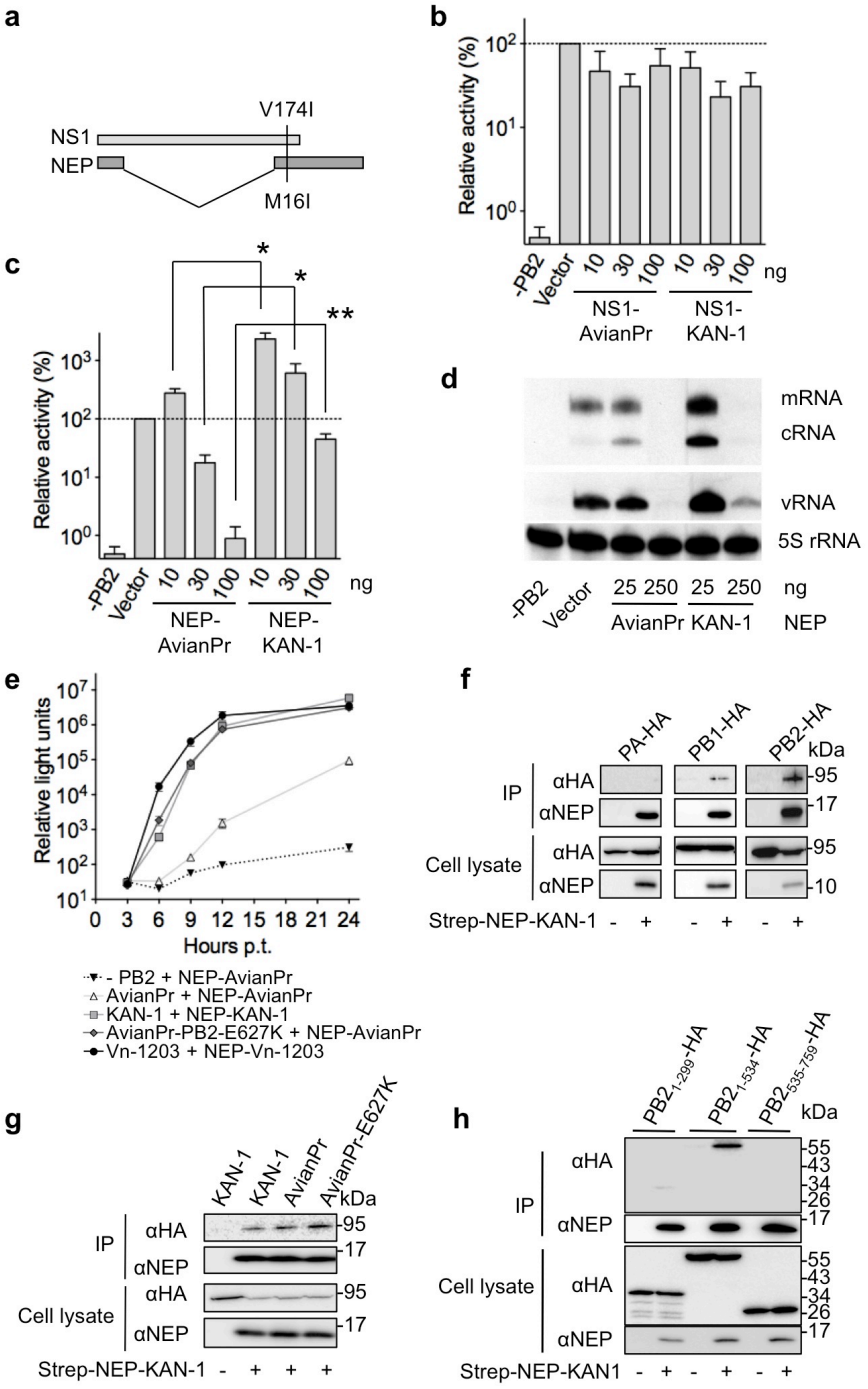

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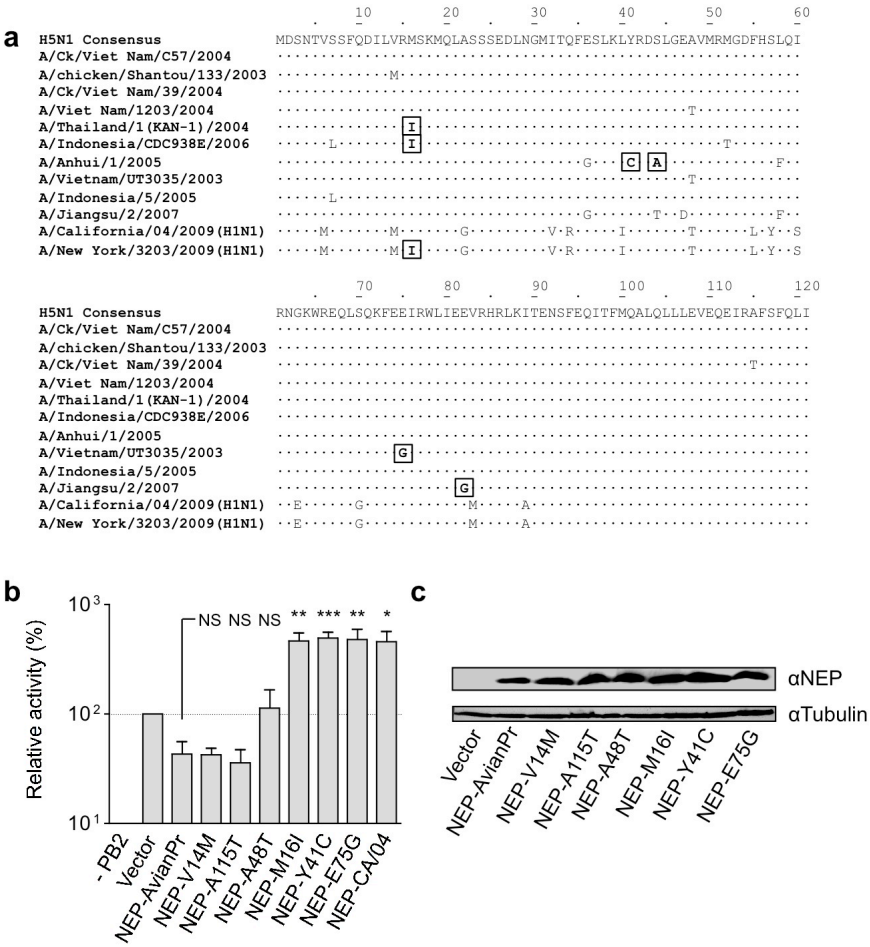

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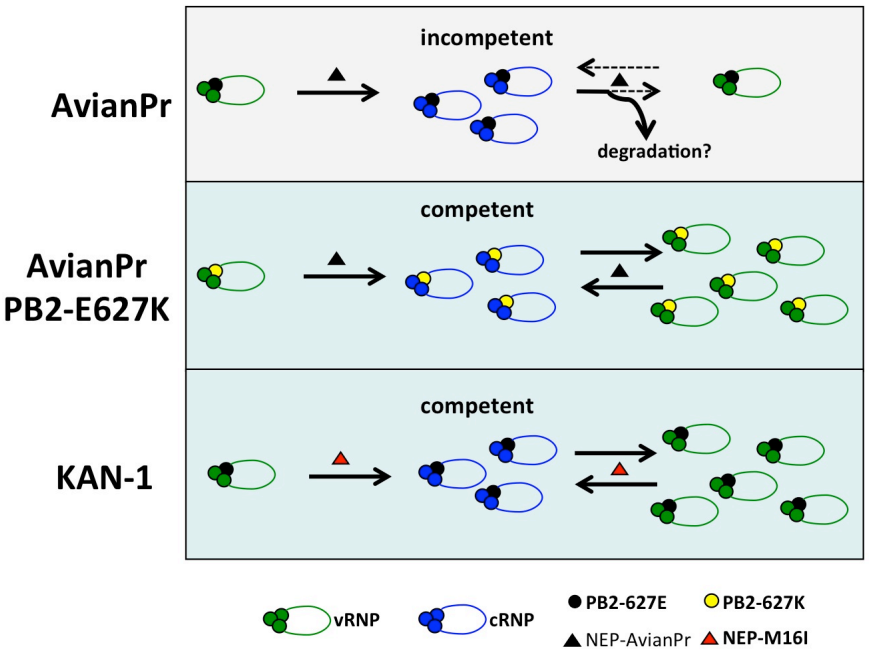
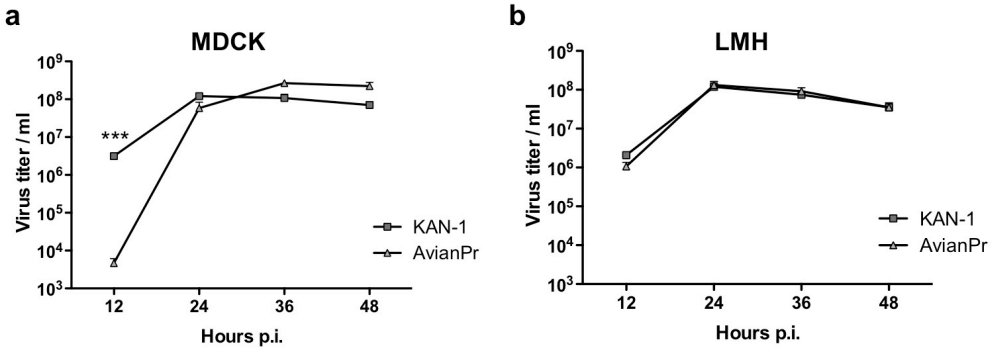


Figure-6 Schwemmle

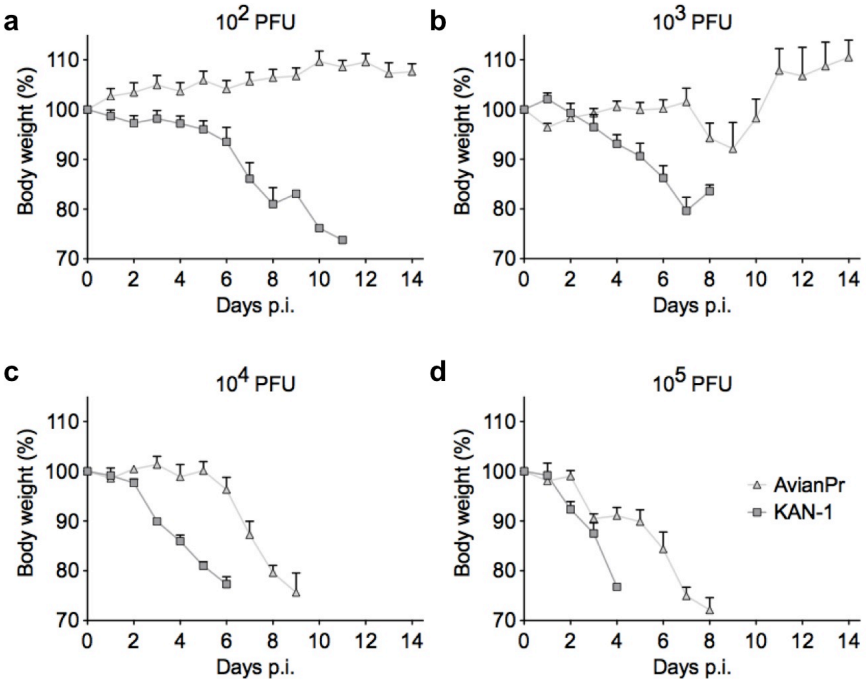
Sup. Table 1. H5N1 sequence alignment

Isolated from		Human	Human	Human	Human	Human	Avian	-	Avian
		KAN-1	Thai16	SP-33	KK-494	SP83	H5N1 consensus	AvianPr	VSMU-20
Virulence in mammals		HP	HP	?	?	LP	?	?	?
Protein	AA								
PB2	148	N	N	N	H	N	N	N	N
	189	K	K	R	K	K	K	K	K
	192	E	E	E	E	D	E	E	E
	443	K	R	R	K	R	K / R	K	K
	492	E	E	G	E	E	E	E	E
	627	E	K	K	K	E	E / K*	E	E
	701	N	D	D	D	D	D	D	D
	734	V	V	G	V	V	V	V	V
PB1	177	E	D	D	E	E	E / D	E	E
	499	Y	Y	Y	S	Y	Y	Y	Y
	568	V	I	I	I	I	I	I	I
PB1-F2	-	-	-	-	-	-	-	-	-
PA	21	M	M	M	M	L	M	M	M
	22	K	K	K	K	I	K	K	K
	31	E	E	E	E	G	E	E	E
	44	V	V	V	I	V	V	V	V
	45	C	C	S	C	C	C	C	C
	142	R	K	K	K	K	K	K	K
	548	M	M	M	I	M	M	M	M
	627	G	R	R	G	G	G / R	G	G
	628	M	V	V	V	V	V	V	V
	712	A	T	A	A	A	A / T	A	A
HA	143	A	V	V	A	A	A	A	A
	341	R	R	R	R	R	R / K	R	K
	383	D	D	A	D	D	D	D	D
	473	R	K	K	R	R	R / K	R	R
NP	357	Q	Q	Q	K	Q	Q	Q	Q
	454	E	E	E	E	D	E	E	E
NA	44	H	H	H	H	Q	H / Q	H	H
	80	Y	H	H	Y	Y	Y / H	Y	Y
	180	N	N	N	N	S	N / S	N	N
	309	N	N	N	H	N	N	N	N
	361	T	T	T	T	I	T	T	T
	418	T	A	A	T	T	T	T	T
	429	T	N	N	N	N	N	N	N
M1	168	T	I	I	I	I	I / T	T	I
	213	V	V	V	C	V	V	V	V
	226	S	S	S	R	S	S	S	S
M2	86	V	V	V	V	V	V/I	V	I
NS1	36	L	F	L	L	L	L	L	L
	123	I	V	V	I	I	I / V	I	I
	169	I	V	V	V	V	V	V	V
NEP	16	I	M	M	M	M	M	M	M
	110	E	E	E	G	E	E	E	E

Supplementary Table-1 Schwemmle



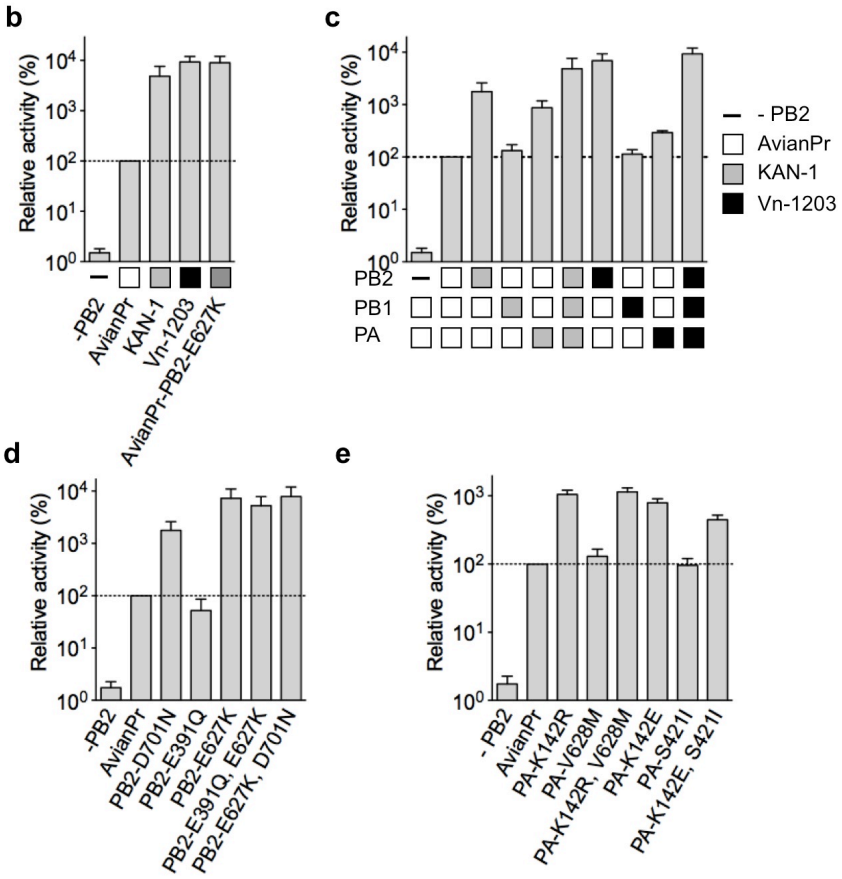
Supplementary Figure-1 Schwemmle



Supplementary Figure-2 Schwemmle

a

	AA Position	AvianPr	Kan-1	Vn-1203
PB2	391	E	E	**Q**
	627	E	E	**K**
	701	D	**N**	D
PB1	568	I	**V**	I
	591	V	**I**	V
PA	142	K	**R**	**E**
	421	S	S	**I**
	628	V	**M**	V



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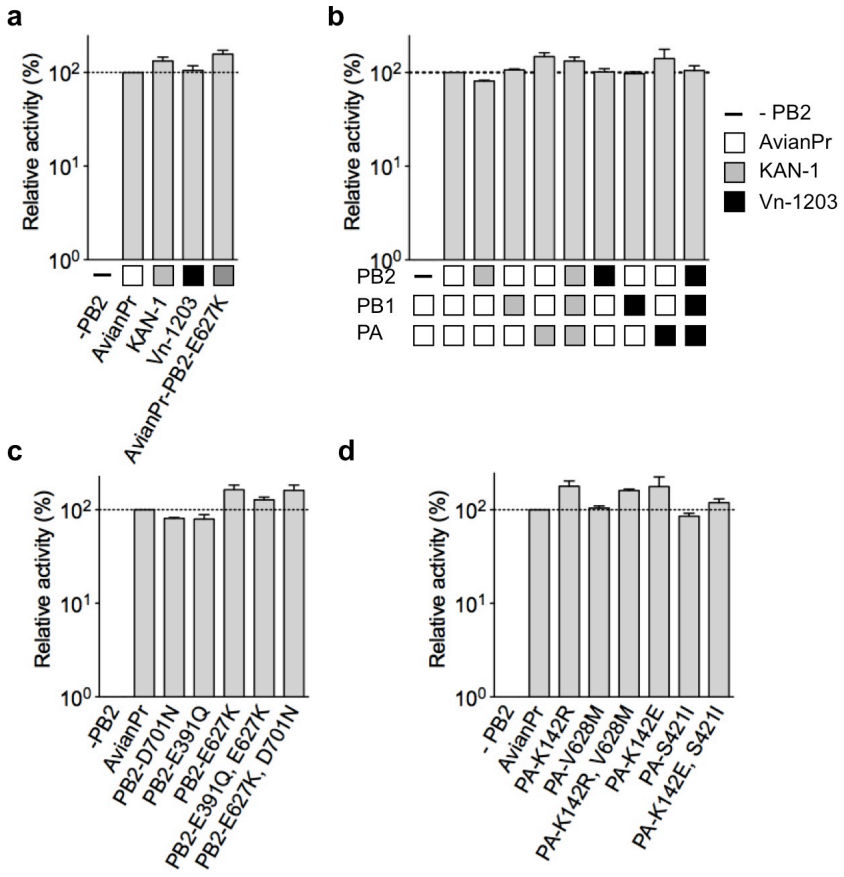

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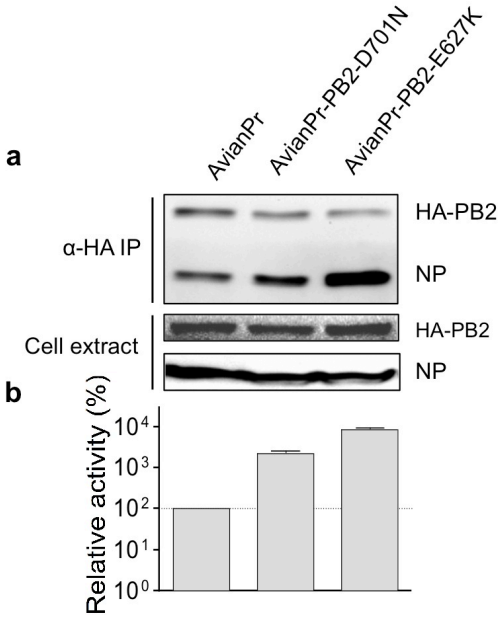

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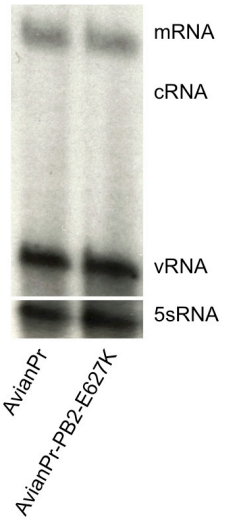
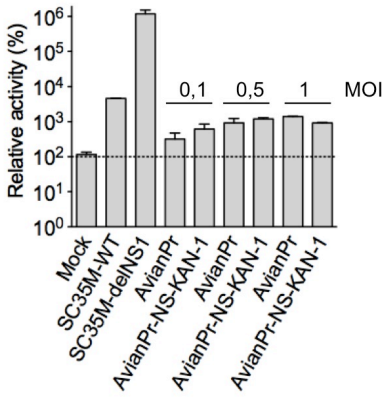
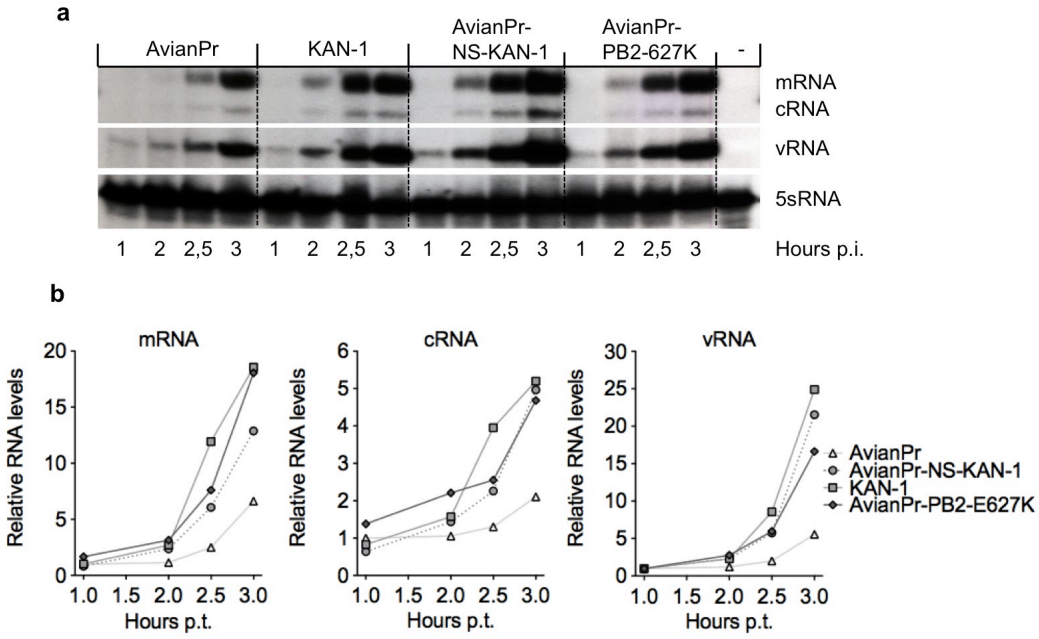


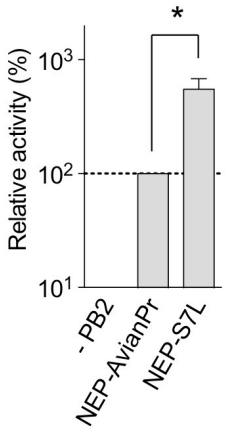
Supplementary Figure-6 Schwemmle



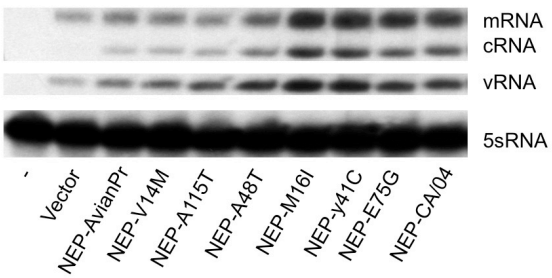
Supplementary Figure-7 Schwemmle



Supplementary Figure-8 Schwemmle



Supplementary Figure-9 Schwemmle



Supplementary Figure-10 Schwemmle

3.3 The viral nucleoprotein determines Mx sensitivity of influenza A viruses.

Published in: Journal of virology

Own contributions:

I contributed to design of the experiments. I performed the KAN-1 virus specific experiments and all animal experiments together with Petra Zimmermann.

JOURNAL OF VIROLOGY, Aug. 2011, p. 8133–8140 Vol. 85, No. 16
0022-538X/11/$12.00 doi:10.1128/JVI.00712-11

The Viral Nucleoprotein Determines Mx Sensitivity of Influenza A Viruses[▽]

Petra Zimmermann, Benjamin Mänz, Otto Haller, Martin Schwemmle,* and Georg Kochs*

Department of Virology, Institute for Medical Microbiology and Hygiene, University of Freiburg, Hermann-Herder-Strasse 11, 79104 Freiburg, Germany

Received 8 April 2011/Accepted 1 June 2011

Host restriction factors play a crucial role in preventing trans-species transmission of viral pathogens. In mammals, the interferon-induced Mx GTPases are powerful antiviral proteins restricting orthomyxoviruses. Hence, the human MxA GTPase may function as an efficient barrier against zoonotic introduction of influenza A viruses into the human population. Successful viruses are likely to acquire adaptive mutations allowing them to evade MxA restriction. We compared the 2009 pandemic influenza A virus [strain A/Hamburg/4/09 (pH1N1)] with a highly pathogenic avian H5N1 isolate [strain A/Thailand/1(KAN-1)/04] for their relative sensitivities to human MxA and murine Mx1. The H5N1 virus was highly sensitive to both Mx GTPases, whereas the pandemic H1N1 virus was almost insensitive. Substitutions of the viral polymerase subunits or the nucleoprotein (NP) in a polymerase reconstitution assay demonstrated that NP was the main determinant of Mx sensitivity. The NP of H5N1 conferred Mx sensitivity to the pandemic H1N1 polymerase, whereas the NP of pandemic H1N1 rendered the H5N1 polymerase insensitive. Reassortant viruses which expressed the NP of H5N1 in a pH1N1 genetic background and vice versa were generated. Congenic Mx1-positive mice survived intranasal infection with these reassortants if the challenge virus contained the avian NP. In contrast, they succumbed to infection if the NP of pH1N1 origin was present. These findings clearly indicate that the origin of NP determines Mx sensitivity and that human influenza viruses acquired adaptive mutations to evade MxA restriction. This also explains our previous observations that human and avian influenza A viruses differ in their sensitivities to Mx.

Zoonotic transmissions pose a constant risk for the introduction of novel influenza A viruses into the human population (25). At the beginning of a pandemic, humans are normally immunologically naïve to the newly introduced viruses. Therefore, innate host defenses play a major role in preventing virus infection and spread (30). Influenza viruses trigger the synthesis of type I and type III interferons (IFNs), which in turn activate the expression of numerous IFN-inducible genes, including the Mx genes (1, 29, 30). Mx proteins belong to the family of dynamin-like large GTPases and are found in many species (10). The human MxA protein and the murine Mx1 protein have antiviral properties and also protect cells from infection with influenza A viruses (10, 11). The protective role of Mx proteins is best illustrated in mice carrying the wild-type *Mx1* resistance gene (8, 9). Mx1-positive ($Mx1^{+/+}$) mice are highly resistant to influenza virus infection and survive large challenge doses, whereas Mx1-negative ($Mx1^{-/-}$) mice with defective Mx1 alleles are susceptible and die (24, 31).

Mx proteins are known to block an early step in the influenza virus replication cycle. After entering a cell, the viral nucleocapsids (also called vRNPs) are imported into the cell nucleus, where the associated viral RNA polymerase becomes active and starts primary transcription. This process is inhibited by the murine Mx1 protein, which is located in the nucleus (18). Human MxA is predominantly cytoplasmic and interferes with secondary transcription and viral replication (22). A variant of MxA carrying a foreign nuclear localization signal moves into the nucleus and blocks primary transcription, exactly as does murine Mx1 (5, 35). This nuclear MxA variant was shown to form a complex with the influenza virus nucleoprotein (NP), and NP could also be coimmunoprecipitated with wild-type MxA under cross-linking conditions (32). Influenza virus strains were recently found to differ in their sensitivities to the antiviral effect of Mx proteins. Avian influenza viruses proved to be more sensitive than human strains, and this was influenced by the viral NP in vRNP reconstitution assays (4).

Here we compared the Mx sensitivity of the 2009 pandemic influenza virus A/Hamburg/4/09 (pH1N1) strain with that of the highly pathogenic avian influenza virus A/Thailand/1(KAN-1)/04 (H5N1) strain. Moreover, we determined the role of the NP in Mx sensitivity, using reconstituted polymerase assays as well as reassortant viruses for infection of Mx-positive cells and mice. Our results demonstrate that the viral NP determines Mx sensitivity and that the NP of the pandemic 2009 virus is relatively Mx insensitive. They also suggest that human MxA may provide a natural barrier to transmission of influenza A viruses from avian reservoirs and that a degree of Mx resistance may develop due to natural selection in the human population.

(This work was conducted by Petra Zimmermann in partial fulfillment of the requirements for a Ph.D. degree from the Faculty of Biology of the University of Freiburg, Freiburg, Germany.)

* Corresponding author. Mailing address: Department of Virology, Institute for Medical Microbiology and Hygiene, University of Freiburg, Hermann-Herder-Strasse 11, 79104 Freiburg, Germany. Phone for Georg Kochs: 49-761-2036623. Fax: 49-761-2036562. E-mail: georg.kochs@uniklinik-freiburg.de. Phone for Martin Schwemmle: 49-761-2036526. Fax: 49-761-2036639. E-mail: martin.schwemmle@uniklinik-freiburg.de.

▽ Published ahead of print on 15 June 2011.

MATERIALS AND METHODS

Cells and viruses. Mouse 3T3 cell lines expressing murine Mx1 (3T3-Mx1), human MxA (3T3-MxA), or Mx-negative control cells (3T3) have been described previously (22). 3T3 cells, MDCK cells, and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin.

Generation of recombinant influenza A viruses. Recombinant viruses, including pH1N1 (A/Hamburg/4/09), H5N1 [A/Thailand/1(KAN-1)/04], and the NP-reassortant viruses H5N1-NP(pH1N1) and pH1N1-NP(H5N1), were generated under biosafety level (BSL) 3 conditions by the eight-plasmid reverse-genetics system as described previously (12, 20, 26). For generation of the pH1N1 viruses, the HA rescue plasmid was mutated by quick-change site-directed mutagenesis to encode the mouse-adapted mutations D131E and S186P (34). Successful introduction of these mutations was confirmed by sequencing. All recombinant viruses were plaque purified on MDCK cells in the presence of 1 μg/ml of tosylsulfonyl-phenylalanyl-chloromethyl keton (TPCK)-treated trypsin. Virus stocks were prepared on MDCK cells, and titers were determined by plaque assay.

Mice. BALB/c mice with defective *Mx1* alleles and congenic BALB.A2G-*Mx1* mice (designated BALB-Mx1) carrying the functional *Mx1* allele (28) were bred locally. Six- to eight-week-old mice were anesthetized with a mixture of ketamine (100 μg per gram body weight) and xylazine (5 μg per gram) administered intraperitoneally (i.p.) and inoculated intranasally (i.n.) with the indicated doses of viruses in 50 μl phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (BSA). Mice were monitored daily for weight loss until 12 days postinfection (p.i.). Animals with severe symptoms or more than 25% weight loss were euthanized. For determination of lung titers and histopathological analysis, mice were euthanized at the indicated time points. All animal work was conducted under BSL 3 conditions in accordance with the guidelines of the local animal care committee.

Titration of virus in lungs. Mice were infected i.n. with 1,000 PFU of the indicated viruses. Lungs from infected mice were collected at 48 h p.i. and homogenized using the FastPrep24 system (MP Biomedicals). Tissue debris was removed by low-speed centrifugation, and virus titers in supernatants were determined by performing 10-fold serial dilutions in PBS with 0.3% BSA followed by plaque assay on MDCK cells.

Virus growth curves. MDCK cells seeded in 6-well plates were incubated with virus at a multiplicity of infection (MOI) of 0.001 in PBS containing 0.3% BSA for 1 h at 37°C. The inoculum was removed, and 2 ml infection medium (DMEM supplemented with 0.3% BSA), additionally containing 1 μg/ml TPCK-treated trypsin for pH1N1 viruses, was added. Virus titers in cell culture supernatants were determined at the indicated time points by plaque assay and are expressed as PFU per ml.

Tissue culture infection experiments. Mouse 3T3 cells were seeded at 10^6 cells per well in 6-well plates and infected at an MOI of 5 for 1 h at 37°C. Subsequently, the inoculum was replaced by infection medium. Cells were lysed in buffer containing 50 mM HEPES (pH 7.3), 125 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol (DTT), 25 U/ml of Benzonase, and protease inhibitors (Roche). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western blotting. The blots were probed with the monoclonal antibody M143 to detect Mx proteins (6), monoclonal mouse anti-PA antibody (G. Chase et al., unpublished data), and a rabbit antiactin antibody (Sigma). Horseradish peroxidase-labeled secondary antibodies and a chemiluminescence detection system (Pierce femto kit) were used to detect primary antibodies. Signal quantification was performed using ChemiDoc XRS equipment (Bio-Rad).

Influenza A virus polymerase reconstitution assay (minireplicon assay). HEK 293T cells seeded in 12-well plates were transfected using the Nanofectin transfection reagent (PAA Laboratories) according to the manufacturer's protocol. Ten nanograms of pCAGGs plasmids encoding PB2, PB1, and PA and 100 ng of NP-encoding plasmid were cotransfected with 50 ng of the firefly luciferase-encoding viral minigenome construct pPolI-FFLuc-RT, which is flanked by the noncoding regions of segment 8 of influenza A virus (4). The transfection mixture also contained 25 ng of pRL-SV40, a plasmid constitutively expressing *Renilla* luciferase under the control of the simian virus 40 promoter, used to normalize variations in transfection efficiency. To evaluate the antiviral potential of Mx1, we cotransfected increasing amounts of Mx1-encoding plasmid. Cotransfection of the antivirally inactive mutant Mx1 (K49A) (23) was used as a control. To achieve equal amounts of transfected DNA, an empty vector plasmid was added. Twenty-four hours posttransfection, cells were lysed and firefly and *Renilla* luciferase activities were determined using the dual luciferase reporter assay (Promega) according to the manufacturer's protocol. Relative polymerase activity was calculated as the ratio of luciferase to *Renilla* luminescence.

Histopathological analysis. Lungs were collected from BALB-Mx1 mice 3 and 4 days p.i. Lungs were inflated and fixed in 4% formaldehyde overnight at 4°C and subsequently embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin (H&E) and examined under the light microscope for histopathological changes. Representative images were obtained on a Nicon Eclipse E400 microscope at magnification ×10.

RESULTS

Mx sensitivity differs between human and avian influenza A virus strains. Since Mx1 interferes with the transcriptional activity of vRNPs (4), we determined the polymerase activities of influenza virus strains A/Hamburg/4/09 (pH1N1) and A/Thailand/1(KAN-1)/04 (H5N1) in a minireplicon system in the presence or absence of Mx1. The H5N1 polymerase activity decreased to almost 10% at the largest amount (200 ng) of cotransfected Mx1-expressing plasmid, whereas the pH1N1 polymerase activity was reduced to only 60% (Fig. 1A). The dose of Mx1-expressing plasmid necessary to achieve 50% inhibition of the H5N1 polymerase activity (ID_{50}) was found to be 25 ng (Fig. 1A). These results demonstrate that the polymerase of the pandemic H1N1 isolate is less sensitive to Mx1 inhibition than the polymerase of the avian H5N1 strain.

Mx sensitivity correlates with the nature of the NP. To identify the viral factor determining Mx sensitivity, we exchanged each protein component of the resistant pH1N1 vRNP (i.e., the NP and the three polymerase subunits PA, PB1, and PB2) with the corresponding components of the sensitive H5N1 strain and determined the activity of the reassorted vRNPs in the minireplicon assay. The NP of H5N1 origin increased the Mx1 sensitivity of the pH1N1 polymerase (Fig. 1B and C), resulting in a drop in the ID_{50} from over 200 ng to 36 ng of transfected Mx1 expression plasmids. In contrast, substitutions with the polymerase subunit PA, PB1, or PB2 of H5N1 did not result in enhanced Mx1 sensitivity (Fig. 1B). Conversely, the pH1N1 NP rendered the H5N1 polymerase activity relatively resistant to Mx1 inhibition (Fig. 1B and D), as demonstrated by an increase in the ID_{50} from 25 ng to 173 ng of transfected Mx1 expression plasmids. In the absence of Mx1, the activities of both the pH1N1 and H5N1 polymerases were not affected by the source of the NP (data not shown), excluding the possibility that subunit incompatibilities were affecting Mx1 sensitivity. Together, these data indicate that the nature of the NP is the main determinant of Mx sensitivity.

The NP of avian H5N1 origin increases Mx sensitivity of the reassortant pH1N1 virus. To verify the importance of NP as a determinant of Mx1 sensitivity *in vivo*, we generated two recombinant strains, namely, wild-type strain A/Hamburg/4/09 (H1N1) (here designated pH1N1) and a reassortant A/Hamburg/4/09 strain which carries the NP gene of A/Thailand/1(KAN-1)/04 (H5N1) and is here designated pH1N1-NP(H5N1). Since pandemic H1N1 viruses are almost avirulent in BALB/c mice (14), we introduced two mutations (D131E and S186P) into the hemagglutinin (HA) protein, which have been described as increasing virulence (13, 34). Both pH1N1 and pH1N1-NP(H5N1) grew to comparable titers in MDCK cells infected with an MOI of 0.001. However, infection with the latter virus

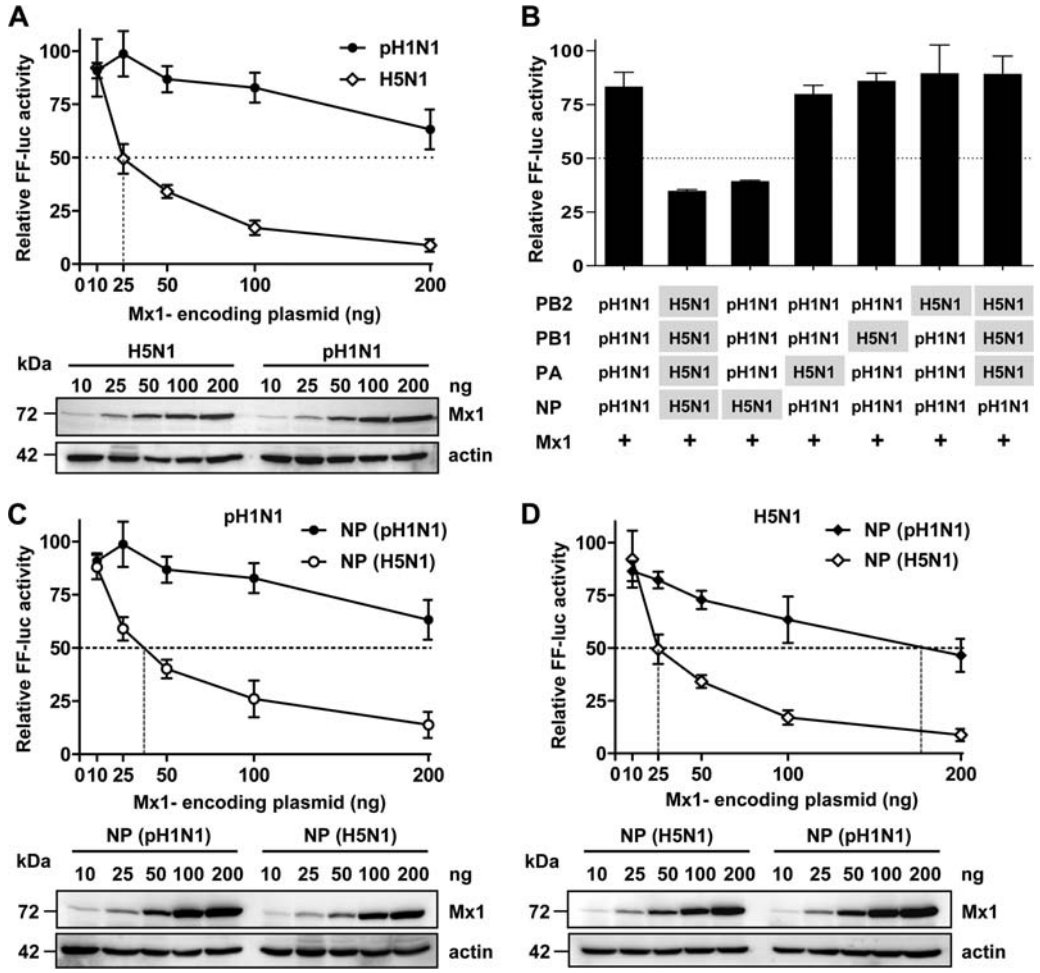


FIG. 1. The origin of NP determines Mx1 sensitivity of influenza A virus polymerase activity. The viral polymerase of A/Hamburg/4/09, designated pH1N1, and that of A/Thailand/1(KAN-1)/04, designated H5N1, were reconstituted in 293T cells by transfection with 50 ng of a firefly luciferase (FF-luc)-encoding viral minigenome construct, 10 ng of expression constructs encoding the viral polymerase subunits PB2, PB1, and PA of the respective strain, and 100 ng of the indicated NP-encoding plasmids. In addition, the indicated amounts of Mx1-encoding plasmids were cotransfected. Twenty-four hours posttransfection, cells were lysed and luciferase activities were measured. Cotransfection of the inactive mutant Mx1 (K49A) was used as a control, and polymerase activity in the presence of Mx1 (K49A) was set to 100%. Mean values of data from three independent experiments are shown. Mx1 expression was monitored by Western blot analysis. Actin was used as a loading control (lower panels in A, C, and D). Dotted lines indicate the amount of Mx1-encoding plasmid necessary for 50% inhibition of polymerase activity. (A) H5N1 and pH1N1 polymerase activity in the presence of increasing concentrations of Mx1. (B) Polymerase activity after exchange of single vRNP components in the presence of 100 ng of Mx1-encoding plasmid. (C) pH1N1 polymerase activity in the presence of increasing concentrations of Mx1 and either NP of pH1N1 [NP (pH1N1)] or H5N1 [NP (H5N1)]. (D) H5N1 polymerase activity in the presence of increasing concentrations of Mx1 and NP of either H5N1 [NP (H5N1)] or pH1N1 [NP (pH1N1)].

resulted in slightly higher titers 24 h p.i. (Fig. 2A). To test the replication efficiency of both viruses in Mx-expressing cells, we took advantage of stably transformed Swiss 3T3 cells that express high levels of murine Mx1 or human MxA under the control of a constitutive promoter (22) (Fig. 2B). The cells were infected with either pH1N1 or pH1N1-NP(H5N1) at an MOI of 5, and viral replication was monitored 10 or 12 h later by determining viral protein expression. The two viruses replicated with comparable efficiencies in Swiss 3T3 cells that do not express Mx proteins, as indicated by equal amounts of PA protein levels (Fig. 2C, lanes 1 to 4). The two types of viruses replicated less well in both Mx1- and MxA-expressing cells, as expected (Fig. 2C and D). However, their replication efficiencies differed significantly in the presence of either Mx1 or MxA. The PA antigen signals were much weaker following infection with the reassortant pH1N1-NP(H5N1) virus than with parental pH1N1 (Fig. 2C, lane 5 to 12). Quantification of PA signal intensities from three independent experiments confirmed the differential reduction in antigen expression levels in infected cells (Fig. 2D). These results indicate that the NP is responsible for the relative resistance of pH1N1 viruses toward the antiviral action of mouse and human Mx GTPases.

To evaluate the *in vivo* growth capacities of pH1N1 and pH1N1-NP(H5N1), Mx1-negative BALB/c mice were infected i.n. with 1,000 PFU. For both viruses, comparable lung titers were obtained after 48 h which were in the range of 10^7 PFU/ml (Fig. 3A, left panel). These results indicated that the NP of H5N1 is well tolerated and does not compromise fitness

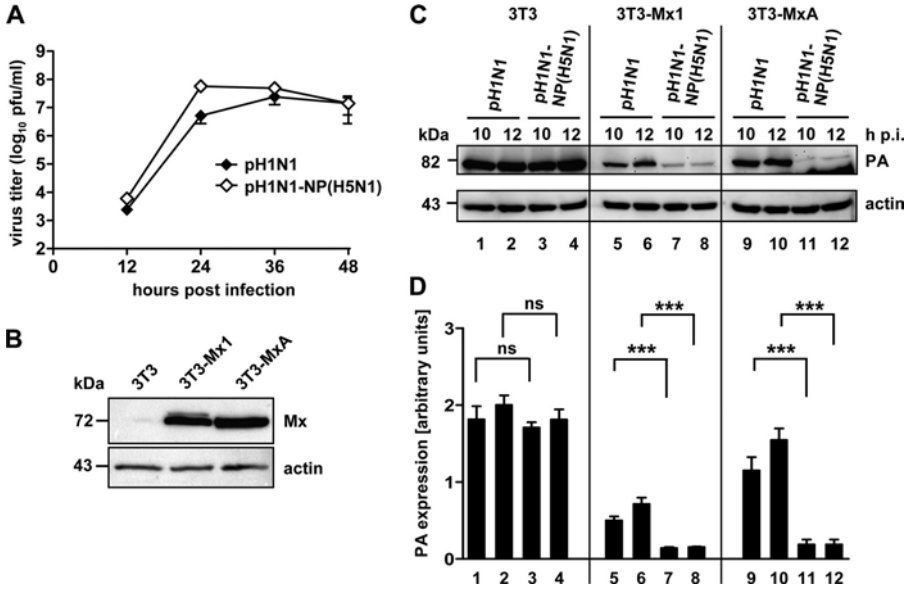


FIG. 2. The NP of avian H5N1 origin increases Mx sensitivity of a reassortant pH1N1 virus. (A) Growth kinetics of pH1N1 and pH1N1-NP(H5N1) in MDCK cells after infection at an MOI of 0.001. Culture supernatants were collected at the indicated time points, and virus titers were determined by plaque assay. Error bars represent the standard errors of the means of data from three independent experiments. (B) Mx expression in Swiss 3T3 cells expressing either recombinant murine Mx1 (3T3-Mx1), human MxA (3T3-MxA), or no Mx proteins (3T3) was monitored by Western blot analysis. (C) The indicated Swiss 3T3 control and Mx-expressing cells were infected with 5 MOI of pH1N1 or pH1N1-NP(H5N1). At the indicated time points p.i., cells were lysed and viral PA protein expression was monitored by Western blot analysis. Cellular actin was detected as a loading control. (D) PA signals of the experiment shown in panel C and two additional independent experiments were quantified and normalized to actin. The *P* values were calculated by Student's *t* test comparing individual time points. ***, $P < 0.001$; ns, not significant.

of the reassortant pH1N1 virus. Next, Mx1-positive BALB-Mx1 mice were challenged with a high dose of either parental pH1N1 or reassortant pH1N1-NP(H5N1) virus. All animals infected with the parental virus showed dramatic body weight loss, and 70% of the animals succumbed within 4 days (Fig. 3B and C). In contrast, infection with the reassortant pH1N1-NP(H5N1) virus caused only moderate weight loss, and all animals survived (Fig. 3B and C). To exclude the possibility that the high virulence of pH1N1 resulted from a general suppression of the interferon response, Mx1 expression in animals was monitored 48 h after infection. Western blot analyses of lung homogenates demonstrated that both viruses induced the same amount of Mx1 protein (Fig. 3D). To determine whether the clinical signs correlated with enhanced virus replication, BALB-Mx1 mice were infected with 1,000 PFU of either pH1N1 or pH1N1-NP(H5N1), and lung titers were determined 48 h after infection. Viral titers were 10-fold higher in animals infected with pH1N1 than in animals infected with pH1N1-NP(H5N1) (Fig. 3A, right panel). To determine whether the difference in lethality between pH1N1 and pH1N1-NP(H5N1) correlated with altered lung pathology, histological tissue sections of Mx-positive mice were examined 4 days after infection (Fig. 3E). Lungs of mice infected with pH1N1 had a greater number of lesions and displayed more massive infiltration of inflammatory cells than lungs of mice infected with pH1N1-NP(H5N1). Together, these data indicate that the nature of NP determines viral replication efficiency and lung pathology in Mx1-positive mice.

The NP of the 2009 pandemic H1N1 virus confers increased Mx resistance to an avian virus. The NP of the pandemic H1N1 isolate A/Hamburg/4/09 negatively affected the Mx sensitivity of the H5N1 polymerase of A/Thailand/1(KAN-1)/04, as assessed in a minireplicon system (Fig. 1C). We therefore anticipated that an A/Thailand/1(KAN-1)/04 reassortant virus equipped with the pH1N1 NP gene should gain increased Mx resistance. To prove this, we generated recombinant wild-type A/Thailand/1(KAN-1)/04, designated H5N1, and a reassortant of this parental virus, designated H5N1-NP(pH1N1), coding for the NP protein of A/Hamburg/4/09. Growth of the reassortant virus in MDCK cells infected at a low MOI of 0.001 was significantly impaired by up to 2 $\log_{10}$ compared to that of the parental H5N1 strain (Fig. 4A). However, after infection at a high MOI of 5, the two viruses replicated with comparable efficiencies in Swiss 3T3 cells that do not express Mx proteins, as demonstrated by the accumulation of equal amounts of PA protein (Fig. 4B, lanes 1 to 4). Importantly, the replication efficiency of H5N1 and H5N1-NP(pH1N1) clearly differed in Mx-expressing cells. Compared to cells infected with H5N1-NP(pH1N1), expression of the viral PA protein was strongly reduced in H5N1-infected cells, irrespective of whether they expressed murine Mx1 or human MxA (Fig. 4B, lanes 5 to 12). Quantification of PA signal intensities from three independent experiments confirmed that the observed differences between H5N1-NP(pH1N1) and H5N1 were significant (Fig. 4C). These findings indicated that the NP of the human pH1N1 strain is able to confer partial Mx resistance to the otherwise sensitive avian H5N1 virus.

Next, we determined the growth capacity of H5N1 and H5N1-NP(pH1N1) in lungs of Mx1-negative BALB/c mice. Viral lung titers of animals infected with 1,000 PFU of reas-

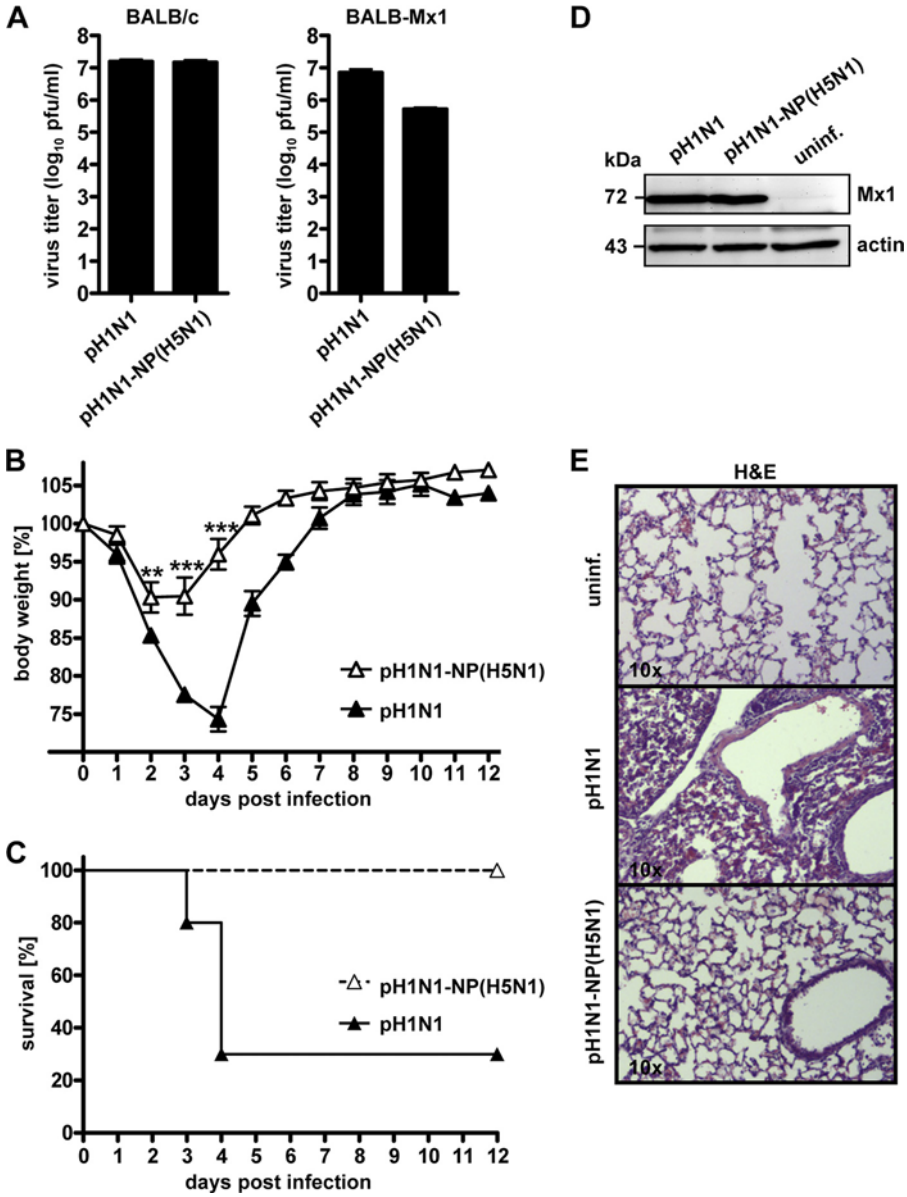


FIG. 3. NP determines virulence of pH1N1 in Mx1-positive mice. (A) BALB/c (left columns) and BALB-Mx1 (right columns) mice were inoculated i.n. with 1,000 PFU of the indicated viruses. Lungs from infected mice ($n = 5$) were collected 48 h p.i. and homogenized, and virus titers were determined by plaque assay. (B and C) Changes in body weight (B) or survival (C) of BALB-Mx1 mice ($n = 10$/group) intranasally infected with 2×10^6 PFU of pH1N1 and pH1N1-NP(H5N1). Survival and weight loss were monitored daily for 12 days. The P values for changes in weight loss between the two groups were calculated by Student's t test comparing individual time points. ** and ***, $P < 0.01$ and 0.001, respectively. (D) Mx1 expression levels in lungs of mice infected with 2×10^6 PFU were determined 48 h p.i. by Western blot analysis of lung homogenates. (E) Histopathological analysis of lungs from BALB-Mx1 mice intranasally infected with 2×10^6 PFU of the indicated viruses 4 days p.i. Serial sections were examined for histopathological changes following hematoxylin-and-eosin (H&E) staining, and representative images were captured at magnification ×10. Sections are representative of three mice per group. uninf., uninfected.

sortant H5N1-NP(pH1N1) were 100-fold lower than the titers observed for mice infected with parental H5N1 virus, in full agreement with the decreased viral replication found in cell culture (Fig. 4D, left panel). Similar differences in viral lung titers between the two virus strains were also observed in BALB-Mx1 mice (Fig. 4D, right panel), indicating that the reassortant H5N1-NP(pH1N1) virus is attenuated in both Mx1-positive and Mx1-negative mice. However, despite these differences in viral growth, the induction of Mx1 in the lungs at 48 h after infection with 10^6 PFU was comparable (Fig. 4E). Notably, intranasal infection of BALB-Mx1 mice with 10^6 PFU of H5N1-NP(pH1N1) virus caused rapid weight loss (Fig. 4F) and almost 100% mortality (Fig. 4G). In contrast, all H5N1-infected animals recovered rapidly (Fig. 4F) and survived (Fig. 4G). Consistent with the clinical outcome, multiple foci of extended inflammatory cell infiltrates and thickening of bron-

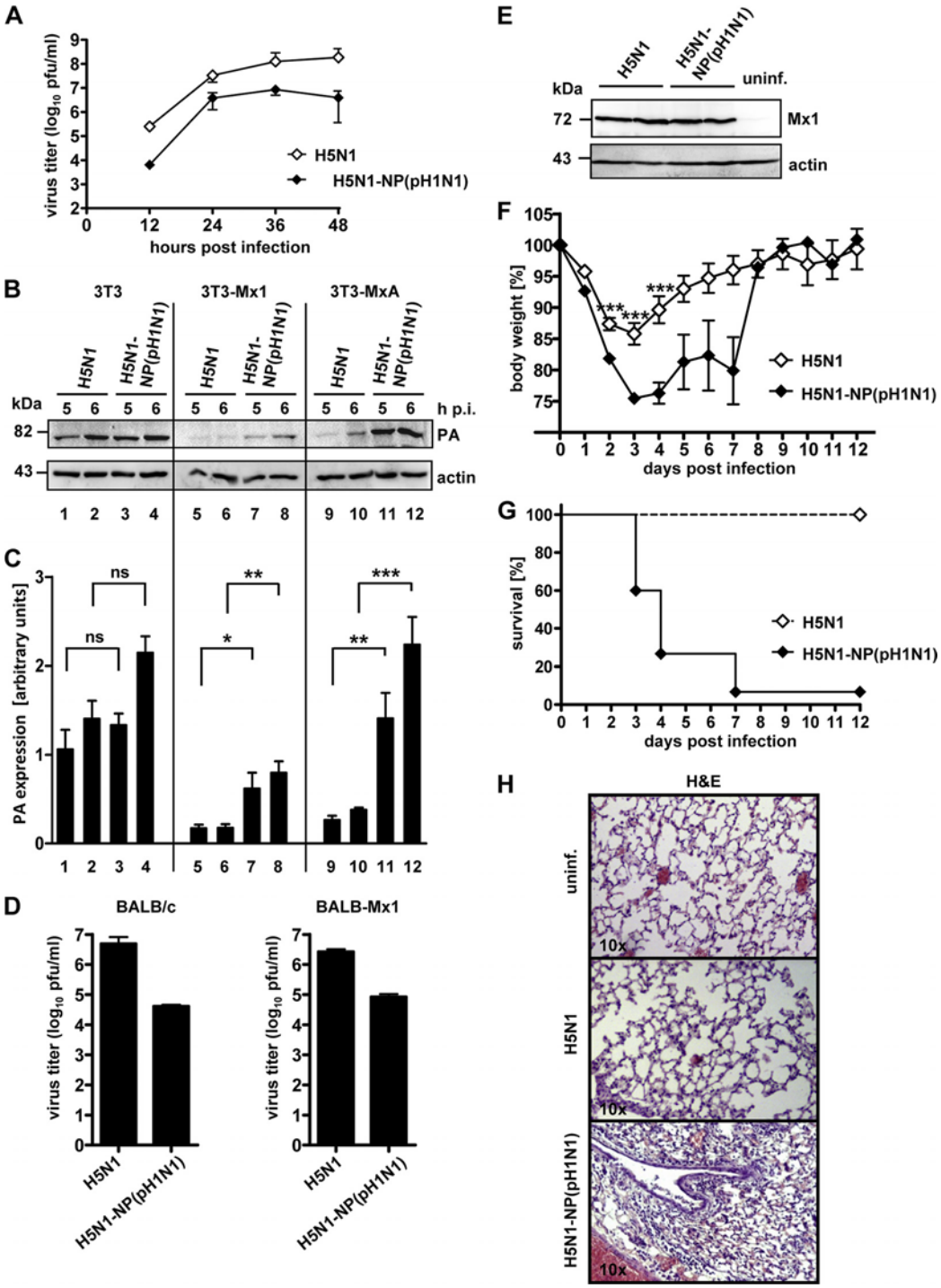


FIG. 4. The NP of the 2009 pandemic H1N1 virus confers increased Mx resistance to an avian virus. (A) MDCK cells were infected at an MOI of 0.001 with the indicated viruses. Virus titers in the culture supernatants were determined by plaque assay at the indicated time points. Error bars represent the standard errors of the means of data from three independent experiments. (B) Swiss 3T3 control and Mx1- or MxA-expressing cells were infected with 5 MOI of the indicated viruses. At 5 and 6 h p.i., cells were lysed, and viral PA protein expression was monitored by Western blot analysis. Actin was used as a loading control. (C) Signals of PA obtained for panel B and in two additional independent experiments were quantified and normalized to actin expression. The *P* values were calculated by Student's *t* test, comparing individual time points. *, **, and ***, $P < 0.05$, 0.01, and 0.001, respectively. ns, not significant. (D) Viral lung titers from BALB/c and BALB-Mx1 mice infected i.n. with 1,000 PFU of the indicated viruses. Lungs from infected mice ($n = 5$) were collected 48 h p.i. and homogenized, and virus titers were determined by plaque assay. (E) Mx1 expression levels in lungs of BALB-Mx1 mice ($n = 2$) infected with 10^6 PFU were determined 48 h p.i. by Western blot analysis of lung homogenates. (F and G) BALB-Mx1 mice ($n = 14$/group) were infected i.n. with 10^6 PFU of the indicated viruses and monitored daily for weight loss (F) and survival (G). (H) Histopathological analysis of lungs from BALB-Mx1 mice infected i.n. with 10^6 PFU of the indicated viruses 3 days p.i. Serial sections were examined for histopathological changes following hematoxylin-and-eosin (H&E) staining. Images were captured at magnification ×10 and are representative of three mice per group.

chiolar and alveolar walls were observed 3 days after infection with H5N1-NP(pH1N1) virus (Fig. 4H). On the contrary, the pulmonary pathology in mice infected with H5N1 was only moderate (Fig. 4H). Thus, despite causing considerable attenuation in mice, NP of pH1N1 rendered the H5N1 virus less sensitive to Mx1 restriction, thereby resulting in increased lung pathology and death of infected animals.

DISCUSSION

Here we have shown that the pandemic H1N1 virus A/Hamburg/4/09 is less sensitive to the inhibitory actions of the human MxA and mouse Mx1 proteins than the highly pathogenic avian H5N1 virus A/Thailand/01/04. Introduction of the avian H5N1 NP into the pandemic H1N1 virus abrogated its partial Mx resistance. We also show that the NP of pandemic H1N1 virus was able to render the avian H5N1 virus less sensitive to Mx restriction both in cell culture and in mice. These results indicate that NP is a major viral determinant of Mx sensitivity.

Infection of Mx1-positive mice with the pandemic H1N1 virus resulted in increased titers in lungs compared to infection with the reassortant pH1N1-NP(H5N1) virus and caused more-severe histopathological changes in the lung. Similar severe lung pathology was observed after infection with the reassortant H5N1-NP(pH1N1) virus. The lung viral titers, however, were significantly lower than the lung viral titers of mice infected with the parental H5N1 virus. Thus, increased replication efficiencies in the lung do not necessarily correlate with lung pathology and disease outcome. This discrepancy between reduced replication efficiency and enhanced morbidity and mortality in influenza A virus-infected mice has already been noticed by others (2, 19). We can only speculate that other factors, such as enhanced replication in specific cell types, dysregulation of pulmonary cytokines, or other deleterious effects on tissue integrity might have contributed to the severe pathogenicity of pH1N1 and H5N1-NP(pH1N1).

The present data with the reassortant viruses clearly suggest that the viral NP is the target of Mx action. A direct physical interaction between MxA and influenza A virus NP was previously demonstrated in immunoprecipitation assays under cross-linking conditions (32). Furthermore, there is compelling biochemical evidence for recognition and binding of the nucleocapsids of Thogoto virus by human MxA (16, 17, 33). Thogoto virus is an influenza virus-like virus transmitted by ticks. It is highly sensitive to the antiviral action of MxA (17) and may display high-affinity binding sites for the MxA GTPase. We have previously shown that MxA has to form highly ordered oligomers to exert its antiviral function (7). The structural details of MxA oligomerization have recently been elucidated (7). They suggest that oligomerization is a stepwise process. The model predicts that MxA tetramers oligomerize on the well-ordered surface of viral nucleocapsids into filamentous or ring-like structures, thereby inhibiting the transcriptional activity of the viral polymerase complex (10). Processivity may largely depend on appropriate binding sites on the viral NP. Even small changes in the amino acid composition of NP may greatly affect the strength of MxA binding, oligomerization, and antiviral activity.

Introduction of the NP of pH1N1 origin into the H5N1 virus caused considerable attenuation of the reassortant virus in cell culture and in mice. This was interesting, because the polymerase activity of the reassortant strain remained unchanged in a minireplicon system. The present findings are in agreement with those of a recent study for which a high degree of compatibility between components of the viral polymerase complex of avian H5N1 and pandemic H1N1 strains, resulting in normal polymerase activity but strong attenuation of viral growth, was reported (21). Importantly, the reassortant H5N1-NP(pH1N1) virus used in the present study showed increased Mx resistance in tissue culture and *in vivo*, in addition to its partial attenuation. It is conceivable that a gain of Mx resistance may reduce virus fitness. If this were the case, successful adaptation of avian viruses would require additional mutations to compensate for the growth defects caused by Mx escape mutations in the NP. Surprisingly, introduction of avian NP into the pandemic H1N1 strain was well tolerated and did not affect viral fitness in the absence of Mx, indicating that the polymerase complex of this pandemic H1N1 strain has gained a remarkable flexibility in the cooperation with NPs of distantly related strains. This capacity may have enabled the pandemic H1N1 progenitor virus to gain Mx resistance without obvious attenuation, facilitating its successful transmission and spread in 2009.

A picture is beginning to emerge which has implications for influenza virus evolution and epidemiology. Virtually all NPs of human influenza virus strains studied so far (including the pandemic H1N1 isolate from 2009 described in this study or the pandemic virus of 1918) show increased resistance to the antiviral action of mammalian Mx proteins, whereas the NPs of avian origin [including the H5N1 strain A/Thailand/(KAN-1)/04 and others] are highly Mx sensitive (4; this study). Mx genes are also present in avian species, and some antiviral activity for chicken Mx linked to a serine-to-asparagine polymorphism at position 631 has been described (15). However, recent studies could not detect any antiviral effect of chicken Mx proteins against different strains of influenza A viruses (3, 27). In any case, it is conceivable that newly emerging influenza A viruses acquire adaptive mutations in the mammalian host which allow them to escape Mx restriction. As yet, the amino acids that determine Mx resistance are not known. We are currently investigating the 32 amino acids of NP which differ between the pandemic H1N1 and the avian H5N1 strain to identify the crucial amino acid patch required for Mx sensitivity.

ACKNOWLEDGMENTS

We thank Daniel Mayer for helpful discussions and suggestions, Geoffrey Chase and Linda Brunotte for critical reading, and H.-D. Klenk for providing the A/Hamburg/4/09 rescue plasmids.

Parts of this work were supported by grants from the Deutsche Forschungsgemeinschaft (Ko1579/8-1) to G.K., from the Mueller-Fahnenberg-Stiftung at the Medical Faculty of the University of Freiburg to G.K., and from the Bundesministerium für Bildung und Forschung (BMBF) (FluResearchNet) to M.S. and by EU grant FLUINNATE 044161 to O.H.

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3.4 Disruption of the viral polymerase complex assembly as a novel approach to attenuate influenza A virus

Published in: The Journal of biological chemistry

Own contributions:

I designed the experiments and supervised a diploma student (Veronika Götz) performing most of the experiments. I was involved in plasmid cloning and virus rescue, performed all animal experiments and contributed to writing of the manuscript.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 10, pp. 8414–8424, March 11, 2011

Disruption of the Viral Polymerase Complex Assembly as a Novel Approach to Attenuate Influenza A Virus*[S]

Received for publication, November 22, 2010, and in revised form, December 23, 2010 Published, JBC Papers in Press, December 23, 2010, DOI 10.1074/jbc.M110.205534

Benjamin Mänz[‡1], Veronika Götz[‡1], Kerstin Wunderlich[‡], Jessica Eisel[‡], Johannes Kirchmair[§], Jürgen Stech[¶], Olga Stech[¶], Geoffrey Chase[‡], Ronald Frank[‖], and Martin Schwemmle[‡2]

From the [‡]Department of Virology, University of Freiburg, 79104 Freiburg, Germany, the [§]Department of Pharmaceutical Chemistry, University of Innsbruck, A-6020 Innsbruck, Austria, the [¶]Friedrich-Loeffler-Institut, D-17493 Greifswald-Insel Riems, Germany, and the [‖]Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany

To develop a novel attenuation strategy applicable to all influenza A viruses, we targeted the highly conserved protein-protein interaction of the viral polymerase subunits PA and PB1. We postulated that impaired binding between PA and PB1 would negatively affect trimeric polymerase complex formation, leading to reduced viral replication efficiency *in vivo*. As proof of concept, we introduced single or multiple amino acid substitutions into the protein-protein-binding domains of either PB1 or PA, or both, to decrease binding affinity and polymerase activity substantially. As expected, upon generation of recombinant influenza A viruses (SC35M strain) containing these mutations, many pseudo-revertants appeared that partially restored PA-PB1 binding and polymerase activity. These polymerase assembly mutants displayed drastic attenuation in cell culture and mice. The attenuation of the polymerase assembly mutants was maintained in IFNα/β receptor knock-out mice. As exemplified using a H5N1 polymerase assembly mutant, this attenuation strategy can be also applied to other highly pathogenic influenza A virus strains. Thus, we provide proof of principle that targeted mutation of the highly conserved interaction domains of PA and PB1 represents a novel strategy to attenuate influenza A viruses.

Infection with influenza viruses annually claims 250,000–500,000 lives worldwide (1). As exemplified by the 1918 influenza pandemic, which resulted in more than 50 million deaths (2), global spread of a pandemic influenza virus strain can lead to high morbidity and mortality. Besides inactivated vaccines, a live attenuated influenza virus vaccine (FluMist) (3, 4) is currently used for vaccination. Importantly, the degree of attenuation is critical for the safety of the live vaccines, but it also affects their efficacy. For safety reasons, children below the age of two and immunocompromised persons are excluded from vaccination with these live vaccines (3). Furthermore, the live vaccine used in the 2007–2008 season was 50% less efficacious than the inactivated vaccine that was used (5). In the currently used influenza A virus (FluA)[3] live vaccine, the degree of attenuation is fixed because a specific master strain is used (3). Thus, to vary the degree of attenuation and to identify new sites that contribute to the safety of live vaccines, new attenuation approaches applicable for all FluA strains are desirable.

A promising target for attenuation of all known FluA strains is the trimeric polymerase complex. Its assembly from the subunits PA, PB1, and PB2 is crucial for polymerase activity and thus virus replication (6–8). PB1 represents the central scaffold protein that binds to PA and PB2 (6, 9–11). Several recently published crystal structures have defined the highly conserved protein-protein-binding domains for PA-PB1 and PB1-PB2 (11–13). Alteration of these conserved residues abrogates subunit interactions accompanied by restricted assembly of polymerase heterotrimers, resulting in decreased polymerase activities (6, 14).

We therefore hypothesized that inefficient polymerase assembly would lead to impaired viral growth and thus attenuation. We identified mutations in the PA-binding domain of PB1 ($PB1_{L8N}$), in the PB1-binding domain of PA (PA_{W706E}), or in both domains simultaneously ($PB1_{L8I}PA_{W706E}$), all of which reduced PA-PB1 binding affinities, leading to impaired polymerase assembly and activity. We observed a correlation between reduction in polymerase activity and increased attenuation in cell culture and mice. Importantly, the attenuated phenotype of the polymerase assembly mutants was maintained in the IFNα/β receptor knock-out mice. Thus, targeting of the highly conserved protein-protein interaction domains of PA and PB1 represents a novel strategy to attenuate influenza A viruses.

EXPERIMENTAL PROCEDURES

Generation of Recombinant Influenza A Viruses—Generation of the recombinant viruses A/SC35M (H7N7) (15) and A/Thailand/KAN-1/2004 (H5N1) (16) was performed as described (14, 16) in 6-well tissue plates with 10^6 293T cells per well using the eight pHW2000 plasmids (300 ng of each) expressing the individual segments and the four pCAGGS plasmids (150 ng of each) coding for PA, PB1, PB2, and NP. After

* This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) and the Bundesministerium für Bildung und Forschung (BMBF, FluResearchNet). This study was also supported by a grant from the Excellence Initiative of the German Research Foundation (GSC-4, Spemann Graduate School) (to K. W.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Hermann-Herder Str. 11, 79104 Freiburg, Germany. Tel.: 49-7612036526; Fax: 49-7612036639; E-mail: martin.schwemmle@uniklinik-freiburg.de.

[3] The abbreviations used are: FluA, influenza A virus; MDCK, Madin-Darby canine kidney; IFNAR, IFNα receptor; aa, amino acid(s); p.i., post infection.

24 h, released virus was used to infect MDCK cells. Sequences of viral genomes were determined from particles released in the supernatant of the MDCK cells 48 h post infection. Rescued viruses were plaque-purified, and MDCK cells were infected for propagation of virus stocks.

Virus Infections and Growth Assays—MDCK cells or human lung cells (A549) were infected at a multiplicity of infection of 0.001 at 37 °C. Virus titers in the cell supernatants were determined at the indicated time points by plaque assay and expressed as plaque-forming units (pfu)/ml. For passaging the mutant viruses 10 times in cell culture, MDCK cells were infected with $\sim 10^3$ pfu corresponding to a multiplicity of infection of 0.001 for 24–36 h.

Mouse Experiments—BALB/c mice were obtained from JANVIER SAS (Strasbourg, France). IFNα receptor knock-out ($IFNAR^{0/0}$) mice (17) backcrossed 12 generations to C57BL/6 were generated by J. Sprent and M. Rubinstein (The Scripps Research Institute, La Jolla, CA) and kindly provided by A. Diefenbach (Institute of Medical Microbiology and Hygiene (IMMH), Freiburg, Germany). 6–8-week-old animals were used for all infection experiments, which were performed in accordance with the guidelines of the local animal care committee. Animals were euthanized if severe symptoms developed or body weight loss approached 25% of the initial value. Lung homogenates were prepared using the FastPrep24 system (MP Biomedicals). For passaging the mutant viruses *in vivo*, 6–8-week-old BALB/c mice were infected with 10^3 pfu intranasally. 48 h post infection, total lung was homogenized, and an infectious dose of 10^3 pfu was used for another round of infection of BALB/c mice. In total, three passages were performed.

Molecular Modeling—Based on the structural model of PB1 (aa 1–15) bound to PA (Protein Data Bank (PDB) 2znl), a homology model was derived for SC35M using Prime (Schroedinger Suite 9.1.107) on an Intel 6600 Core2Duo processor machine (3 GB of RAM) running openSUSE 11.2. To accurately reflect conformational shifts in PA and PB1 induced by the V12I exchange, the side chains of amino acids forming the proximate shell around this residue were refined using Prime Refinement with default settings. Thereby, mainly a shift of the side chain of Met-595 is observed, which is displaced by the bulkier substituent of V12I.

Plasmid Constructions—The pHW2000 plasmid system (18) was used for influenza A virus rescue. To obtain rescue plasmids containing the mutant PB1 or PA genes, site-directed mutagenesis was performed. To generate the pCAGGS expression plasmids encoding PA or PB1 mutants, parts of the corresponding open reading frames were PCR-amplified, digested with AgeI and NotI (PB1) or CFR9I and Bsp119I (PA), and subsequently cloned into pCAGGS-SC35M-PB1-HA and pCAGGS-SC35M-PA-HA.

Co-immunoprecipitation and Immunoblot Analysis—293T cells were transfected with the indicated plasmids in 6-well plates using METAFECTENE (Biontex, Martinsried, Germany). Cells were incubated 24 h post transfection with lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 1% protease inhibitor mix G, (Serva, Heidelberg, Germany), 1 mM DTT) for 15 min on ice. After centrifugation by 13,000 rpm at 4 °C, supernatant was incubated with HA- or FLAG M2-specific antibodies (Sigma) coupled to agarose beads, respectively, for 1 h at 4 °C. After three washes with 1 ml of washing buffer (lysis buffer without protease inhibitor mix), bound material was eluted under denaturing conditions, separated on SDS-PAGE gels, and transferred to PVDF membranes. Viral polymerase subunits were detected with antibodies directed against the HA (Covance, Berkeley, CA) or His (Qiagen) or FLAG tag (Sigma).

Enzyme-linked Immunosorbent Assay (ELISA)—ELISA was essentially performed as described previously (19) using biotinylated $PB1_{1-15}$ peptides bound to streptavidin-coated microwell plates and cell extract containing HA-tagged PA WT or mutant proteins.

Cells—293T, A549, and MDCK cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin/streptomycin. All cells were maintained at 37 °C and 5% CO_2.

Peptides—Peptides were synthesized as described in Ref. 19 using a Pioneer automatic peptide synthesizer (Applied Biosystems, Foster City, CA).

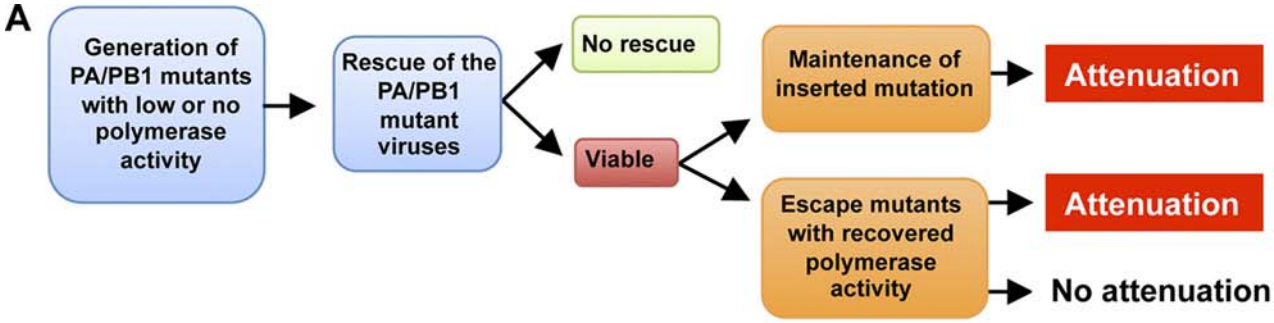
Reconstitution of the Influenza Virus Polymerase Activity—293T cells were transiently transfected with a plasmid mixture containing the influenza A virus-derived PB1, PB2, PA, and NP expression plasmids and a polymerase I-driven plasmid transcribing an influenza A virus-like RNA coding for the reporter protein firefly luciferase to monitor viral polymerase activity (14). The transfection mixture also contained a plasmid constitutively expressing *Renilla* luciferase, which served to normalize variation in transfection efficiency. The reporter activity was determined 24 h post transfection and normalized using the Dual-Glu® luciferase assay system (Promega).

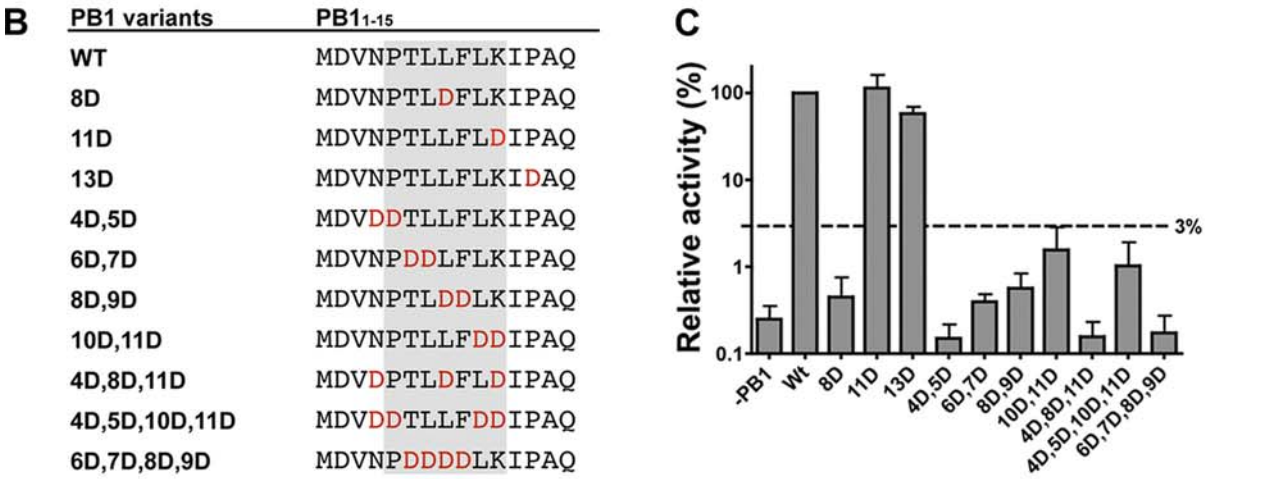
Statistical Analysis—With the exception of the mouse experiments, *error bars* represent S.E. from at least three independent experiments. For the animal experiments, the indicated number of mice was used for calculating S.E.

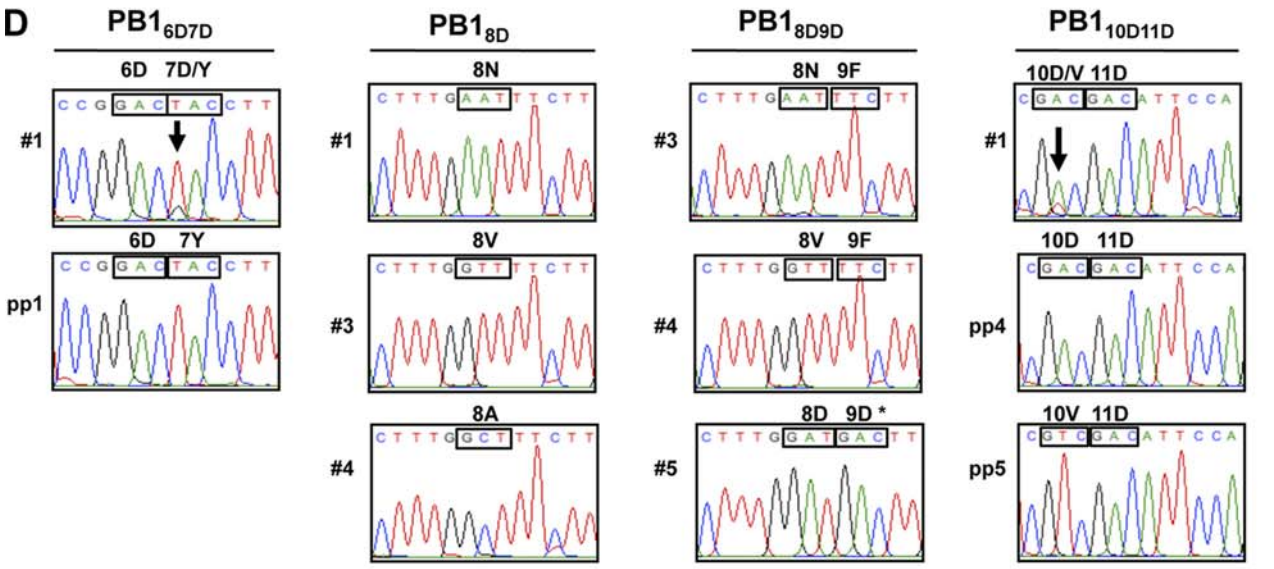
Ethics Statement—All animal experiments were performed in compliance with the German animal protection law (Tierschutzgesetz). The mice were housed and handled in accordance with good animal practice as defined by the Federation of Laboratory Animal Science Associations (FELASA) and the national animal welfare body GV-SOLAS. The animal welfare committees of the University of Freiburg (Regierungspräsidium Freiburg) approved all animal experiments.

RESULTS

Mutation at the PA-PB1-binding Interface Leads to Escape Mutants with Impaired Viral Replication—To identify mutations in PA or PB1 that attenuate FluA to a desirable level, we devised an experimental procedure to force spontaneous escape mutants (Fig. 1*A*). The first step is the generation of PA or PB1 mutant proteins with substantially impaired polymerase activity and subunit interaction. In a second step, viruses expressing these mutant proteins are created by reverse genetics techniques. Taking into account the rapid mutability of FluA, we expected, dependent on the severity of the introduced mutations, the following possible outcomes: (i) mainte-


A
Generation of PA/PB1 mutants with low or no polymerase activity
Rescue of the PA/PB1 mutant viruses
No rescue
Viable
Maintenance of inserted mutation
Attenuation
Escape mutants with recovered polymerase activity
Attenuation
No attenuation


B
PB1 variants
PB1 1-15
WT MDVNPTLLFLKIPAQ
8D MDVNPTLDFLKIPAQ
11D MDVNPTLLFLDIPAQ
13D MDVNPTLLFLKIDAQ
4D,5D MDVDDTLLFLKIPAQ
6D,7D MDVNPDDLFLKIPAQ
8D,9D MDVNPTLDDLKIPAQ
10D,11D MDVNPTLLFDDIPAQ
4D,8D,11D MDVDPTLDFLDIPAQ
4D,5D,10D,11D MDVDDTLLFDDIPAQ
6D,7D,8D,9D MDVNPDDDDLKIPAQ
C
Relative activity (%)
100
10
1
0.1
3%
-PB1
Wt
8D
11D
13D
4D,5D
6D,7D
8D,9D
10D,11D
4D,8D,11D
4D,5D,10D,11D
6D,7D,8D,9D


D
PB1 6D7D
6D 7D/Y
#1
6D 7Y
pp1
PB1 8D
8N
#1
8V
#3
8A
#4
PB1 8D9D
8N 9F
#3
8V 9F
#4
8D 9D *
#5
PB1 10D11D
10D/V 11D
#1
10D 11D
pp4
10V 11D
pp5

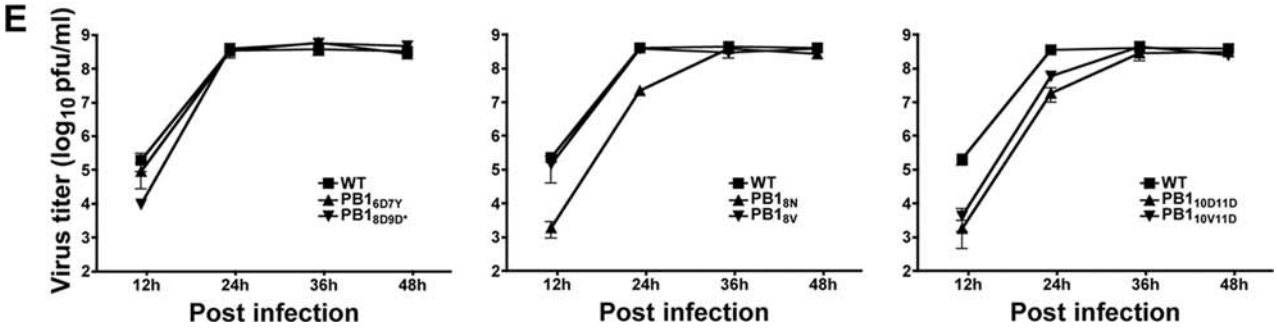

E
Virus titer (log10 pfu/ml)
WT
PB1 6D7Y
PB1 8D9D*
PB1 8N
PB1 8V
PB1 10D11D
PB1 10V11D
12h
24h
36h
48h
Post infection

TABLE 1
Characteristics of SC35 M mutant viruses
ND, not determined; −. no mutations.

Mutation	Minireplicon activity	Successful rescue attempts/attempts	Compensatory mutations
	%		
PB1			
L8D	0.5	4/6	D8N (1×); D8V (2×); D8A (1×)
L8N[a]	67	2/2	No
K11D	113	2/2	No
L4D,L5D	0.2	0/4	−
T6D,L7D	0.4	3/4	D7Y(3×)
L8D,F9D	0.6	2/5	L8N,D9F (1×); L8D,F9D (1×)
L10D,K11D	1.6	6/8	D10V (2×)
L4D,L8D,K11D	0.2	0/3	−
L4D,L5D,L10D, 11D	1.0	0/4	−
L6D,L7D,L8D,F9D	0.2	0/3	−
PA			
W706E	10	2/2	No
W706R	2	1/2	W706G (1×)
PB1 + PA			
L8N + W706E	12	2/2	PB1 N8I (1×); PB1 N8L (1×)
L8I + W706E[a]	ND	2/2	No

[a] Direct rescue.

nance of the inserted mutations, resulting in attenuated viruses; (ii) maintenance of the inserted mutations, leading to non-viable viruses; or (iii) introduction of compensatory mutations. These mutations could restore wild-type (WT) viability or display an attenuated phenotype (Fig. 1*A*).

To efficiently disrupt the highly hydrophobic binding interface between PA and PB1 (12, 13), we replaced either single or multiple aa in the PA-binding domain of PB1 by the charged aa aspartic acid (Fig. 1*B*) and determined viral polymerase activity in human 293T cells (Fig. 1*C*). Single substitutions by Asp outside (P13D) or at the border (K11D) of the PA-binding core region (12, 13, 19) resulted in a moderately decreased or unchanged polymerase activity (Fig. 1*C*). In contrast, replacement of the leucine at position 8 (L8D), which lies within the core-binding 3_{10} helix and is known to be essential for binding to PA (9, 19), abrogated polymerase activity to a level below 1% of WT activity (Fig. 1*C*). Double substitutions at other positions in the N terminus of PB1 ($PB1_{1-15}$) reduced the polymerase activity below 3% of WT activity (Fig. 1*C*). Next, rescue experiments were performed to generate recombinant SC35M viruses encoding for the above mentioned PB1 mutants. To prevent simple reversion to the WT sequence, 2–3 nucleotide exchanges were performed to mutate the individual aa positions in PB1 (supplemental Fig. S2). As expected, we could generate the mutant virus coding for $PB1_{K11D}$ without compensatory mutations (Table 1) and impaired growth (supplemental Fig. S1*A*). However, besides the $PB1_{K11D}$ mutant, we were also able to generate recombinant SC35M mutated to $PB1_{T6D,L7D}$, $PB1_{L8D}$, $PB1_{L8D,F9D}$, and $PB1_{L10D,K11D}$, respectively (Table 1, Fig. 1*D*). However, the majority of these viruses represented pseudo-revertants, which we defined as viruses containing a non-introduced, non-WT mutation at the site of the mutated residue, which partially or fully restores the WT phenotype. Specifically, during the rescue of SC35M coding for $PB1_{T6D,L7D}$, the pseudo-revertant $PB1_{T6D,D7Y}$ emerged frequently (Fig. 1*D*), whereas rescue of the $PB1_{L10D,K11D}$ mutant virus produced two viruses coding for $PB1_{L10D,K11D}$ and $PB1_{L10D,D11V}$ (Fig. 1*D*, Table 1). Importantly, several independent attempts to rescue mutant viruses coding for $PB1_{L8D}$ resulted exclusively in virus mutants harboring changes to $PB1_{D8A}$, $PB1_{D8V}$, or $PB1_{D8N}$ (Fig. 1*D*, Table 1). $PB1_{D8V}$ or $PB1_{D8A}$ mutant viruses showed only slightly reduced viral growth (Fig. 1*E*, data not shown), whereas the mutant virus coding for $PB1_{D8N}$ displayed a pronounced attenuation at 12 h p.i. (Fig. 1*E*). No additional mutations were found in the polymerase genes of this virus (Table 1, supplemental Fig. S2*A*). Independent attempts to generate SC35M coding for $PB1_{L8D,F9D}$ yielded three virus mutants: $PB1_{D8N,D9F}$, $PB1_{D8V,D9F}$, and $PB1_{L8D,F9D^*}$ (Fig. 1*D*). Because $PB1_{D9F}$ represents the wild-type aa at this position, the $PB1_{D8N,D9F}$ and $PB1_{D8V,D9F}$ mutants were phenotypically indistinguishable from the $PB1_{D8V}$ or $PB1_{D8N}$ mutants, respectively. However, the $PB1_{L8D,F9D^*}$ mutant represents a recombination event that resulted in restoration of the PB1 open reading frame downstream of the mutated PB1 site (supplemental Fig. S3). As shown in Fig. 1*E*, the $PB1_{L8D,F9D^*}$ mutant showed no attenuation in cell culture (Fig. 1*E*).

Pseudo-reversions in PB1 Partially Restore Polymerase Activity and Trimeric Polymerase Complex Assembly—We reasoned that the pseudo-reversions $PB1_{D8N}$ and $PB1_{D8V}$ would

FIGURE 1. **Characterization of influenza A viruses with mutations in the PA-binding domain of PB1.** *A*, flowchart of the experimental procedure used to identify attenuated influenza A virus mutants. *B*, graphic depicting the PB1 mutants used in *panel C* with single or multiple Asp substitutions within or near the core PA-binding domain (*gray box*). *8D*, L8D; *11D*, K11D; *13D*, P13D; *4D,5D*, *N4D,P5D*; *6D,7D*, T6D,L7D; *8D,9D*, L8D,F9D; *10D,11D*, L10D,K11D; *4D,8D,11D*, *N4D,L8D,K11D 4D,5D,10D,11D*, *N4D,P5D,L10D,K11D*; *6D,7D,8D,9D*, T6D,L7D,L8D,F9D. *C*, determination of FluA polymerase activity in the presence of the indicated PB1 mutants. The activity observed with PB1 WT was set to 100%. The omission of PB1 (*−PB1*) in the transfection mixture served as a negative control. *D*, emergence of revertants after rescue of PB1 mutants with impaired polymerase activity. PB1 sequences were determined from individual rescue attempts, indicated by #. Virus mixtures with polymorphisms in PB1 were further subjected to plaque purification (indicated by *pp* and the number of the picked plaque). The *arrows* indicate the site of polymorphism in the PB1 sequence. $PB1_{6D7D}$, $PB1_{T6D,L7D}$; $PB1_{8D}$, $PB1_{L8D}$; $PB1_{8D9D}$, $PB1_{L8D,F9D}$; $PB1_{10D11D}$, $PB1_{L10D,K11D}$. *E*, viral growth of PB1 mutant viruses. MDCK cells were infected with the indicated SC35M mutants at a multiplicity of infection of 0.001, and virus titers in the cell supernatant were determined by plaque assay. $PB1_{8N}$, $PB1_{D8N}$; $PB1_{8V}$, $PB1_{D8V}$.

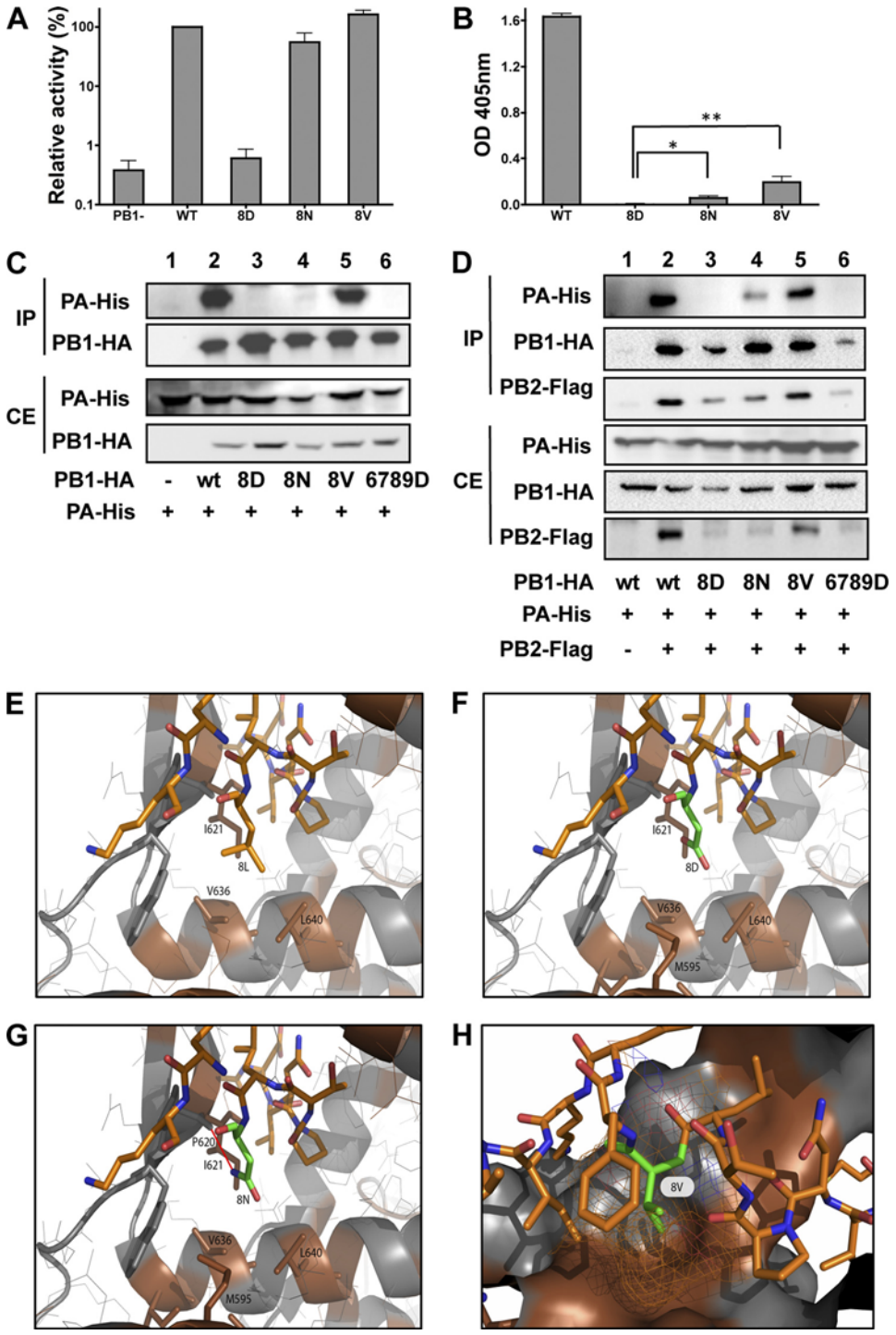


FIGURE 2. **Effect of PB1 mutations on polymerase activity and formation of the trimeric polymerase complex.** *A*, determination of FluA polymerase activity in the presence of the indicated PB1 mutants. The activity observed with PB1 WT was set to 100%. The omission of PB1 (*PB1*−) in the transfection mixture served as a negative control. *8D*, L8D; *8N*, D8N; *8V*, D8V. *B*, determination of the PA binding affinities of the PB1 mutants. An ELISA-based binding assay was carried out with biotinylated peptides corresponding to the N-terminal 15 aa of the indicated PB1 mutants and a cell extract containing HA-tagged PA. * and ** indicate *p* values (Student's *t* test) of <0.05 and <0.01, respectively. *OD*, optical density. *C* and *D*, dimer (*C*) and trimer (*D*) formation in the presence of the indicated PB1 mutants was assayed by immunoprecipitation (*IP*). *CE*, cell extract. The PB1 mutant 6789D, T6D,L7D,L8D,F9D served as a negative control. *E–H*, four helices of PA (*gray/brown drawings*) are involved in the formation of the binding interface for PB1; nitrogen atoms are in *blue*, and oxygen atoms are in *red*. *E*, crystal structure of the WT $PB1_{8L}$. *F–H*, model of the interface containing the mutations $PB1_{L8D}$ (*F*), $PB1_{D8N}$ (*G*), and $PB1_{D8V}$ (*H*). In *H*, the surface complementarity between the binding partners is illustrated by visualization of the surface of PA in *opaque style* and of the surface of $PB1_{8L}$ in *mesh style*.

rescue polymerase activity through restoration of PA-PB1 binding and thus polymerase complex assembly. Indeed, 60% of WT polymerase activity was achieved with $PB1_{D8N}$, whereas $PB1_{D8V}$ even exceeded WT activity (Fig. 2*A*). Using an ELISA-based binding assay (19) and peptides corresponding to the 15 N-terminal aa of PB1 ($pPB1_x$), we determined

that PA did not bind to $pPB1_{L8D}$, whereas binding was observed to both $pPB1_{D8N}$ and $pPB1_{D8V}$, although with significantly lower affinity than $pPB1_{WT}$ (Fig. 2*B*). To test whether the pseudo-reversions affect the formation of PA-PB1 dimers, we performed co-immunoprecipitation studies. Although $PB1_{D8N}$ weakly interacted with PA, no interaction was detected between $PB1_{L8D}$ and PA (Fig. 2*C*). In contrast, complex formation between PA and $PB1_{D8V}$ was comparable with that of PA and WT PB1 (Fig. 2*C*). When co-immunoprecipitation was performed with all three polymerase subunits, both $PB1_{D8N}$ and $PB1_{D8V}$ supported trimeric polymerase complex assembly, whereas $PB1_{L8D}$ did not (Fig. 2*D*). Consistent with earlier observations (14), lack of trimeric polymerase assembly was accompanied by reduced levels of PB2 (Fig. 2*D*, cell extract). We speculate that the reduced levels of PB2 are the result of an enhanced degradation of the unbound PB2. To better understand the reason for the diminished binding, we modeled the PA-binding domain of $PB1_{L8D}$, $PB1_{D8V}$, and $PB1_{D8N}$ into the PA-PB1 crystal structure. Within the hydrophobic groove of PA (12, 20), Leu-8 plays a key role in protein binding affinity by mediating contacts to Ile-621, Val-636, and Leu-640 (Fig. 2*E*). Most strikingly, mutation to L8D causes total affinity loss by repulsion of the charged amino acid from this non-polar region (Fig. 2*F*). L8N still places hydrophilic chemical functionalities unfavorably in this binding groove but may establish a hydrogen-bond interaction with Pro-620 that likely increases the binding affinity (Fig. 2*G*). In contrast, the D8V mutation apparently causes a reduction of the hydrophobic interaction area due to its smaller dimensions when compared with 8L (Fig. 2*H*), resulting in weaker binding but not in a complete loss of binding affinity.

Molecular Modeling Reveals Attenuating Mutations in the PB1-binding Site of PA—To test whether mutations in the PB1-binding domain of PA have the potential to cause attenuation of FluA, we mutated the highly conserved tryptophan at aa position 706 (Fig. 3*A*) to either glutamine (PA_{W706E}) or arginine (PA_{W706R}). Molecular modeling suggests that the negatively charged carboxylate moiety of W706E will attract Lys-643 of PA to form a salt bridge (Fig. 3*B*). This mutation abrogated binding of PA to PB1 in a biochemical pulldown assay (12). We also expected that the mutation W706R would abrogate the interaction with PB1 because W706R positions a positively charged group in the close vicinity of Lys-643 of PA, resulting in the destabilization of the PA protein conformation (Fig. 3*C*). Both mutants, PA_{W706E} and PA_{W706R}, failed to bind to peptides comprising the N-terminal 15 aa of WT PB1 (Fig. 3*D*). When compared with WT PA, the polymerase activity was reduced by ~90% for PA_{W706E} and 98% for PA_{W706R} (Fig. 3*E*). SC35M coding for PA_{W706E} could be generated by reverse genetics without further compensatory mutations in PA (Fig. 3*F*) or both PB1 and PB2 (Table 1). As expected, this mutant virus was significantly attenuated by 2.5 $\log_{10}$ 24 h p.i. in MDCK (Fig. 3*G*) and A549 cells (supplemental Fig. S1*B*). Attempts to rescue SC35M-PA_{W706R} revealed a pseudo-reversion (PA_{W706G}) (Fig. 3*F*), which was barely impaired in viral growth in MDCK cells (Fig. 3*G*) and attenuated by 2 $\log_{10}$ 24 h p.i. in A549 cells (supplemental Fig. S1*B*).

To test the feasibility of generating viruses harboring mutations in both PB1 and PA, we tried to rescue a virus coding for both PA_{W706E} and $PB1_{D8N}$. In one rescue attempt, we obtained a virus mutant harboring a reversion to WT PB1 and no changes in PA (Table 1). In a second independent rescue attempt, we obtained a virus mutant (SC35M-$PA_{W706E}PB1_{L8I}$) harboring a mutation in PB1 to N8I without changes in PA (Fig. 3*H*), which displayed considerably impaired growth in MDCK (Fig. 3*I*) and A549 cells (supplemental Fig. S1*C*) with 3–4 $\log_{10}$ titer reduction 24 h p.i., corresponding to a small plaque phenotype of SC35M-$PA_{W706E}PB1_{L8I}$ when compared with WT virus (supplemental Fig. S4). Thus, virus mutants with aa changes in both PB1 and PA can be generated and show impaired growth properties.

Polymerase Assembly Mutants Are Attenuated in Mice—To evaluate the degree of attenuation of our mutant viruses *in vivo*, we infected BALB/c mice with SC35M mutants. Following intranasal infection, we monitored for weight loss and survival and determined viral lung titers. Although WT-infected mice succumbed to death following infection with 10^3 pfu, all mice infected with 10^4 pfu of SC35M-$PB1_{D8N}$ survived despite an initial weight loss (Fig. 4*A*). Consistent with the lack of attenuation in cell culture, infection with SC35M-$PB1_{D8V}$ was as pathogenic as WT SC35M (supplemental Fig. 5). Mice infected with 10^3 or 10^4 pfu of SC35M-PA_{W706E} (Fig. 4*B*) or SC35M-$PA_{W706E}PB1_{L8I}$ (Fig. 4*C*) survived with only marginal reduction of body weight, whereas at 10^5 pfu, 25 and 37.5% of the animals survived infection with SC35M-PA_{W706E} and SC35M-$PA_{W706E}PB1_{L8I}$, respectively. The degree of attenuation observed with the polymerase assembly mutant viruses correlates with decreased viral lung titers (Fig. 4*D*).

To demonstrate that our findings apply to other influenza A viruses, we introduced the W706E mutation in PA of the highly pathogenic H5N1 virus A/Thailand/(KAN-1)/2004 (clade 1) (21). When compared with infection with WT KAN-1, this mutant virus (KAN-1-PA_{W706E}) was attenuated by 4 $\log_{10}$ 24 h p.i. in MDCK cells (supplemental Fig. 1*D*) and showed an increase in LD_{50} of ~10,000-fold (Fig. 4*E*, data not shown) as well as significantly lower lung titers (>5 $\log_{10}$) in BALB/c mice (Fig. 4*F*).

To determine the safety of our mutant viruses, we infected $IFNAR^{0/0}$ mice, which are highly susceptible to virus infection (17, 22, 23), with up to 10^3 pfu of the attenuated strain SC35M-PA_{W706E} or 10 pfu of WT virus. All SC35M-PA_{W706E}-infected mice survived, whereas all animals infected with WT virus succumbed to infection (Fig. 4*G*). At an infection dose of 10^4 pfu, 66% of the SC35M-PA_{W706E}-infected mice survived. As expected, the viral lung titer of mice infected with 10^3 pfu SC35M-PA_{W706E} for 48 h was at least 1000-fold reduced when compared with WT-infected mice (Fig. 4*H*).

Polymerase Assembly Mutants Remain Attenuated after Passaging in Cell Culture and Mice—To determine whether the polymerase assembly mutants can easily revert to WT, we performed three serial lung passages of SC35M-$PB1_{D8N}$, SC35M-PA_{W706E}, and SC35M-$PA_{W706E}PB1_{L8I}$ in BALB/c mice. No changes were observed in PA, whereas mutations to either V92M or V92L outside the PA-binding domain (12, 13,

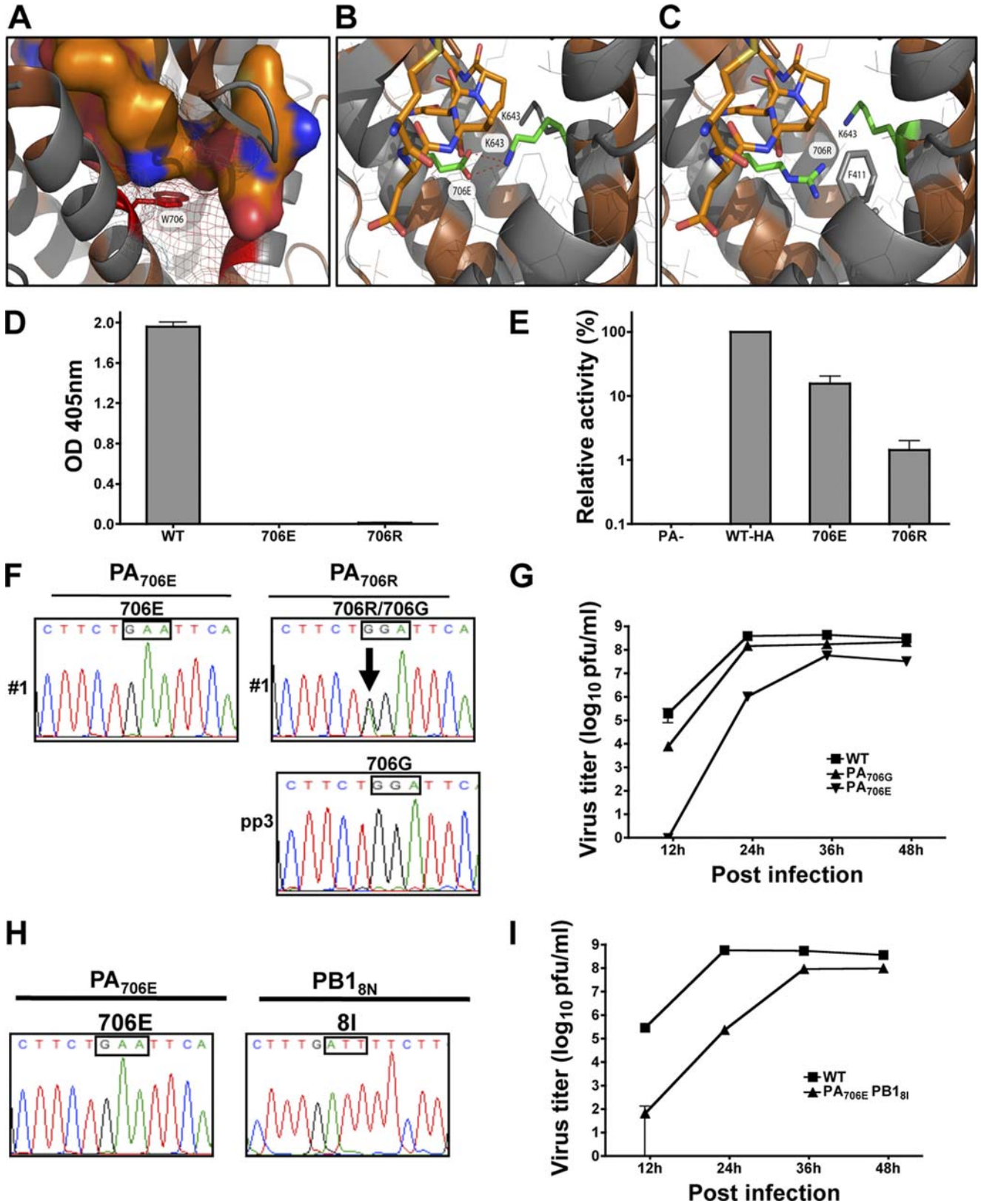
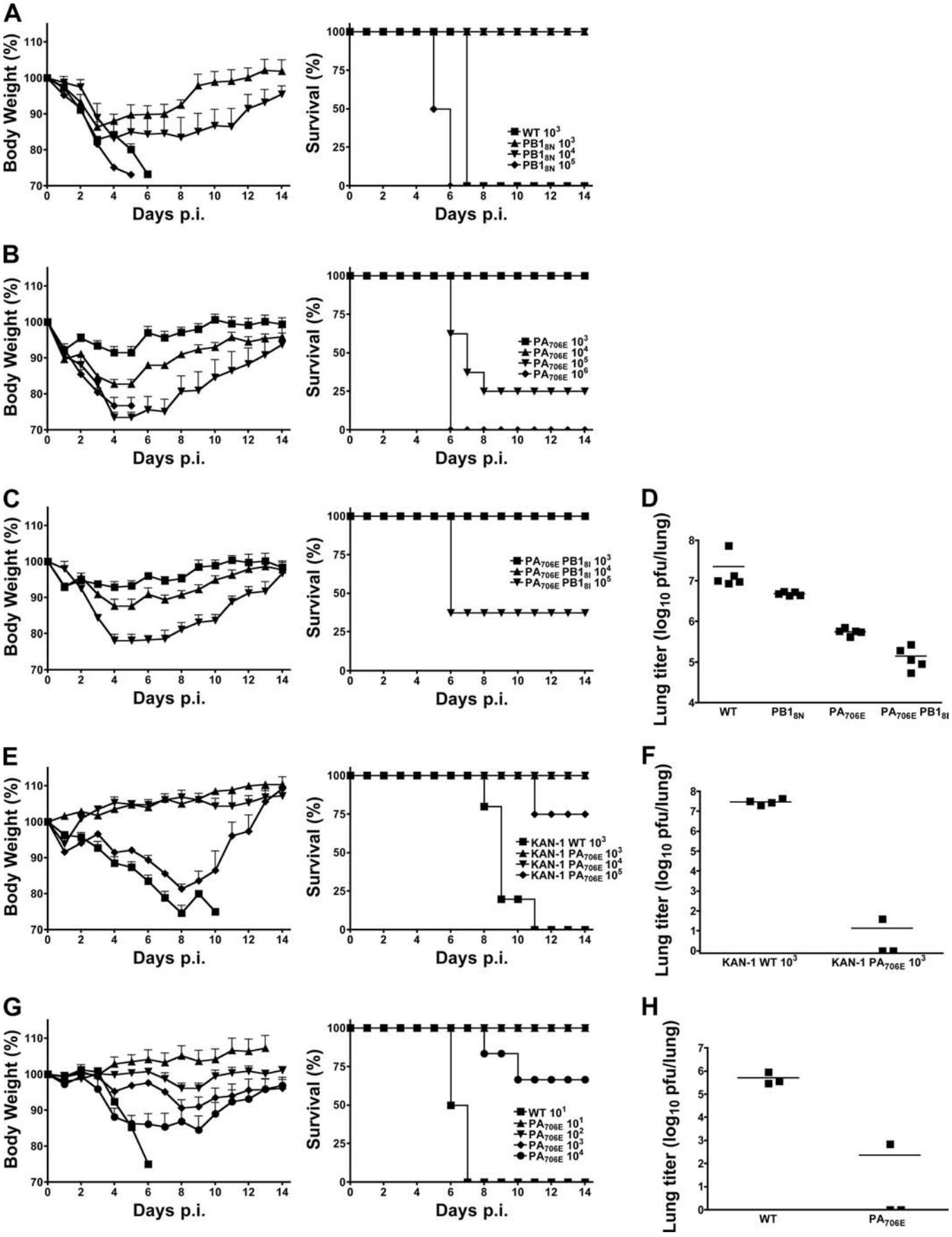


FIGURE 3. **Mutations in the PB1-binding site of PA affect viral growth properties.** *A–C*, Trp-706 contributes to PA-PB1 binding by formation of surface contacts with PB1 Asn-4 and PB1 Pro-5 to PA. *A*, a model of these contacts as in Fig. 2*H*. The surface of PA around Trp-706 is shown in *mesh style*, and the surface of PB1 is shown in *opaque style*, respectively. *B*, the negatively charged carboxylate moiety introduced by the PA W706E mutation may attract PA Lys-643 to form a salt bridge (wild-type model in *gray*; conformation predicted for the mutant in *green*). *C*, the positive charge introduced by the W706R mutation is likely to have a repulsive effect due to the positive charge of Lys-643. *D*, determination of the PB1 binding affinities of the PA mutants by ELISA using cell extracts containing mutant PA-HA. *OD*, optical density; *706E*, W706E; *706R*, W706R. *E*, determination of the FluA polymerase activity in the presence of the indicated PA mutants. *PA−*, omission of PA. *F*, rescue of SC35M mutants coding for PA mutants. The *arrow* indicates the site of polymorphism. *706G*, W706G. *G*, viral growth of PA mutant viruses in MDCK cells. *H*, rescue of a SC35M mutant with mutations in PA and PB1. *8N*, D8N; *8I*, L8I. *I*, viral growth curve of SC35M-$PA_{W706E}PB1_{L8I}$ in MDCK cells.


A
Body Weight (%)
Days p.i.
Survival (%)
Days p.i.
WT 10^3
PB1 8N 10^3
PB1 8N 10^4
PB1 8N 10^5
B
Body Weight (%)
Days p.i.
Survival (%)
Days p.i.
PA 706E 10^3
PA 706E 10^4
PA 706E 10^5
PA 706E 10^6
C
Body Weight (%)
Days p.i.
Survival (%)
Days p.i.
PA 706E PB1 8I 10^3
PA 706E PB1 8I 10^4
PA 706E PB1 8I 10^5
D
Lung titer (log10 pfu/lung)
WT
PB1 8N
PA 706E
PA 706E PB1 8I
E
Body Weight (%)
Days p.i.
Survival (%)
Days p.i.
KAN-1 WT 10^3
KAN-1 PA 706E 10^3
KAN-1 PA 706E 10^4
KAN-1 PA 706E 10^5
F
Lung titer (log10 pfu/lung)
KAN-1 WT 10^3
KAN-1 PA 706E 10^3
G
Body Weight (%)
Days p.i.
Survival (%)
Days p.i.
WT 10^1
PA 706E 10^1
PA 706E 10^2
PA 706E 10^3
PA 706E 10^4
H
Lung titer (log10 pfu/lung)
WT
PA 706E

A

Strain	SC35M-PB1$_{8N}$			SC35-PA$_{706E}$			SC35-PA$_{706E}$PB1$_{8I}$		
Passage	mP1	mP2	mP3	mP1	mP2	mP3	mP1	mP2	mP3
PB1	-	-	-	-	-	V92M	-	-	V92L
PA	-	-	-	-	-	-	-	-	-

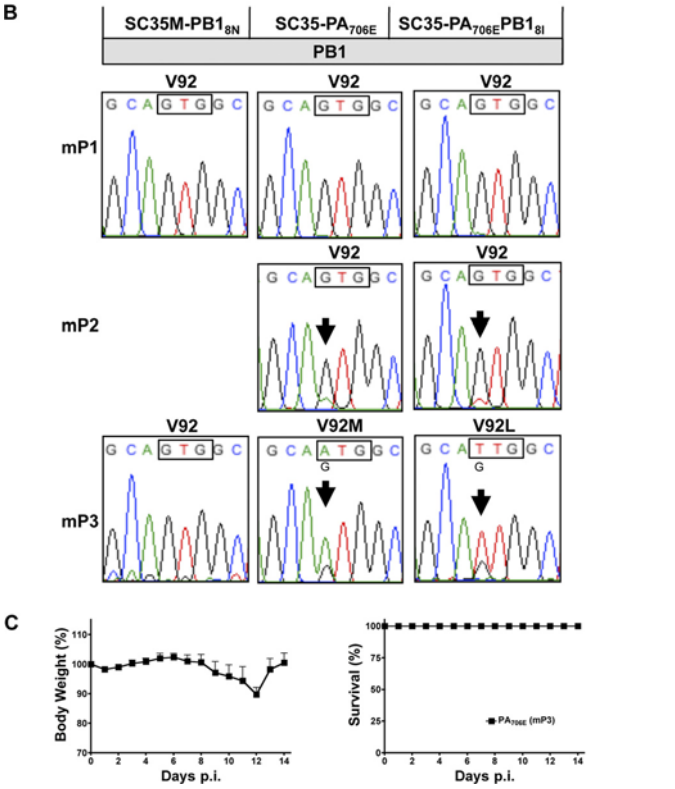


FIGURE 5. **Characterization of polymerase assembly mutants after serial passages in mice.** *A*, mutations in PB1 and PA observed after serial passages of the indicated virus mutants in BALB/c mice. *mP1–3*, mouse passage 1–3. −, no mutations found. *B*, electropherogram of PB1 and PA sequences after serial passages of the indicated viruses in BALB/c. The *arrows* indicate the site of mutation or polymorphism. *SC35M-PB1$_{8N}$*, SC35M-PB1$_{D8N}$; *SC35M-PA$_{706E}$*, SC35M-PA$_{W706E}$. *C*, survival and weight loss of 8–10-week-old IFNAR$^{0/0}$ mice ($n = 6$) infected with the SC35M-PA$_{W706E}$ passaged three times in mice (*PA$_{706E}$ (mP3)*).

A

Strain	SC35-PA$_{706E}$		SC35-PA$_{706E}$PB1$_{8I}$	
Passage	cP5	cP10	cP5	cP10
PB1	-	V92M	-	V92M
PA	-	-	T639A	T639A

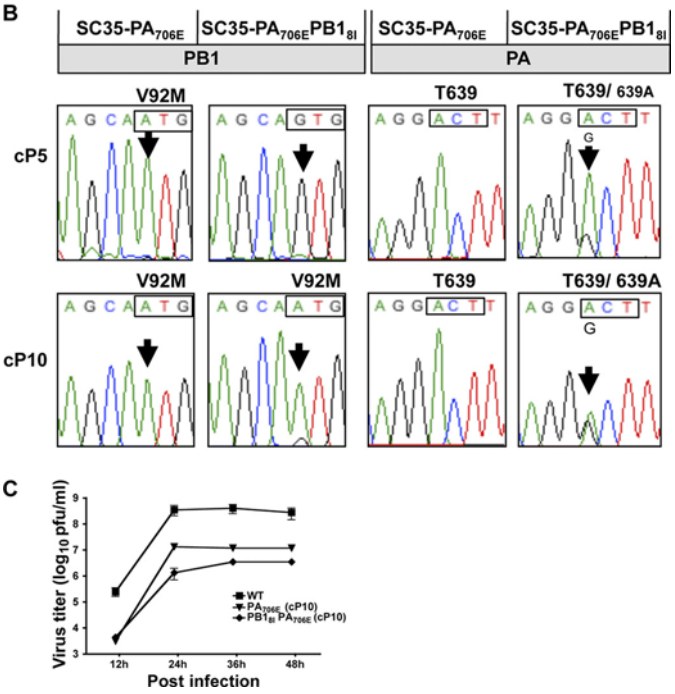


FIGURE 6. **Characterization of polymerase assembly mutants after serial passages in cell culture.** *A*, mutations in PB1 and PA observed after serial passages of the indicated virus mutants in MDCK cells. *cP5* and *cP10*, cell passage 5 and 10. −, no mutations found. *B*, electropherogram of PB1 and PA sequences after serial passages of the indicated viruses in MDCK cells. The *arrows* indicate the sites of mutation or polymorphism. *SC35M-PA$_{706E}$*, SC35M-PA$_{W706E}$; *SC35M-PA$_{706E}$*PB1$_{8I}$, SC35M-PA$_{W706EO}$PB1$_{L8I}$; *C*, viral growth of the indicated mutant viruses in MDCK cells.

19) appeared in PB1 of SC35M-PA$_{W706E}$ and SC35M-PA$_{W706E}$PB1$_{L8I}$, respectively (Fig. 5, *A* and *B*). Infection of IFNAR$^{0/0}$ mice with 100 pfu of SC35M-PA$_{W706E}$ passaged three times in mice resulted only in a transient weight loss (Fig. 5*C*), demonstrating that this virus was still strongly attenuated. Furthermore, serially passaging SC35M-PA$_{W706E}$ and SC35M-PA$_{W706E}$PB1$_{L8I}$ 10 times in MDCK cells also led to a mutation to PB1$_{V92M}$ (Fig. 6, *A* and *B*), as well as PA$_{T639A}$. Importantly, growth of both passaged viruses remained impaired in MDCK cells (Fig. 6*C*). Together, these results indicate that the polymerase assembly mutants can acquire mutations in the viral genome during the passages in mice or cell culture but that these mutations do not reverse their attenuated phenotype. Therefore, the polymerase assembly mutants are phenotypically stable.

DISCUSSION

The objective of this study was to generate attenuated influenza A viruses based on defects in the assembly of the polymerase complex. Accordingly, we reasoned that alteration of key amino acids involved in the subunit interactions would force the virus to escape by acquisition of new mutations, some of which would fail to restore efficient polymerase complex assembly and activity. Indeed, the majority of the rescued mutant viruses represent pseudo-revertants with various degrees of attenuation. Only a few intentionally introduced mutations (*e.g.* PB1$_{L10D,K11D}$ and PA$_{W706E}$) were tolerated and, as predicted, recombinant viruses expressing these proteins were attenuated. However, the occurrence of compensatory mutations at other aa positions in

FIGURE 4. **Polymerase assembly mutants are attenuated in mice.** *A–C*, survival (*right*) and weight loss (*left*) of 6–8-week-old female BALB/c mice ($n =$ 6/group) after intranasal infection with the indicated doses of SC35M mutant viruses encoding PB1$_{D8N}$ (*PB1$_{8N}$*) (*A*), PA$_{W706E}$ (PA$_{706E}$) (*B*), or both PA$_{W706E}$ and PB1$_{L8I}$ (PB1$_{8I}$) (*C*). *WT*, wild-type virus SC35M. *D*, BALB/c mice ($n = 5$) were infected with 10^3 pfu of the indicated virus mutants. 48 h p.i., lung titers were determined by plaque assay. *E*, weight loss and survival of 6–8-week-old female BALB/c mice ($n = 10$/group) after intranasal infection with the indicated doses of KAN-1 or KAN-1-PA$_{W706E}$. *F*, lung titers in mice ($n = 5$) infected with 10^3 pfu of either KAN-1 or KAN-1-PA$_{W706E}$ at 48 h p.i. *G*, weight loss and survival of 8–10-week-old IFNAR$^{0/0}$ mice ($n = 7$/group) infected with the indicated doses of WT virus or SC35M-PA$_{W706E}$. *H*, lung titer in IFNAR$^{0/0}$ mice ($n = 3$) infected with 10 pfu of either WT or SC35M-PA$_{W706E}$ at 48 h p.i.

PA or PB1 was not observed. This strongly suggests that only few aa exchanges are tolerated within these binding domains without loss of affinity. The lack of compensatory mutations especially in the relatively small core PA-binding domain might be caused by the involvement of several aa of the 3_{10} helix in mediating contact to PA (12, 13, 19).

The amino acids within the PA-binding domain of PB1 (*e.g.* Leu-8) and the PB1-binding domain of PA (*e.g.* Trp-706), which are essential for polymerase subunit interaction, are highly conserved and identical among all known influenza A virus strains (12–14, 24). We therefore infer that targeting these key residues results in attenuation of virtually all influenza A virus strains. As exemplified by two unrelated viruses, the highly pathogenic H5N1 strain KAN-1 and the mouse-adapted strain SC35M (H7N7) mutation in PA (W706E) resulted in severe attenuation of both viruses in mice. However, the degree of attenuation is higher for the H5N1 mutant virus. This might reflect differences in intrinsic PA/PB1 binding affinities between these virus strains, as described recently for FluA and influenza B viruses (14).

We also observed that targeting different key residues within the PA- or PB1-binding domains could vary the degree of attenuation because the mutation L8N in PB1 resulted in a less attenuated phenotype of SC35M in cell culture and mouse models when compared with the W706E mutation in PA or a combination of both mutations. The possibility to fine-tune the individual degree of attenuation by defined substitutions in PA or PB1 represents a unique tool to avoid over-attenuation. The repertoire of suitable mutations could possibly be extended to the similarly conserved PB2-PB1-binding site (11).

The polymerase assembly mutant viruses are also drastically attenuated in mice with immune system defects, as demonstrated in IFNAR$^{0/0}$ mice, which are highly susceptible to virus infection (17, 22, 23). We attribute this observation to the host-independent polymerase assembly defect of these viruses, which forces attenuation under all conditions. Our findings suggest that live vaccines harboring these mutations should possess a good safety profile in patients with impaired antiviral defense mechanisms.

Several reasons might account for the genetic stability of the polymerase assembly mutants in mice and cell culture. The observed reduction in growth kinetics of these mutants further decreases the population size and thus the likelihood that viable escape mutants will emerge. Furthermore, the PB1/PA-binding interface is highly conserved and cannot tolerate many aa changes without reducing the binding affinity (12–14, 24). Thus, we speculate that point mutations, which cause reversions to wild-type polymerase activity, are unlikely.

Live attenuated viruses confer several advantages as vaccines over traditionally used inactivated viruses, including induction of cellular as well as humoral immunity, broader protection among influenza virus subtypes, and better protection in children (3). However, the currently used cold-adapted influenza vaccine (FluMist) is not approved for children under the age of two or immunocompromised patients (3). Our attenuation strategy might contribute to a better safety profile for these viruses, as demonstrated by infection of IFNAR$^{0/0}$ mice. Furthermore, the cold-adapted master strain A/AnnArbor/6/60 H2N2 contains a fixed degree of attenuation (3), whereas we have developed a strategy to easily identify new mutations, which allow attenuation to be fine-tuned.

Overall, generation of polymerase assembly mutants can be applied to all influenza A viruses, resulting in adjusted degrees of attenuation. Thus, this novel approach may also contribute to the development of phenotypically stable live vaccines against seasonal and pandemic influenza. Furthermore, this attenuation strategy could also be used to make successful reassortment between attenuated live vaccines and circulating influenza A viruses less probable. Such reassortment events are of great concern because the resulting viruses might have an increased pathogenicity. By targeting a protein-protein interaction domain encoded on two segments, exchange of only one segment should maintain attenuation. However, our mutations could be combined with several other attenuation strategies, which have been described (3, 25–28).

We provide proof of principle that disruption of a protein-protein interaction site essential for polymerase complex formation results in an attenuation of influenza viruses. Thus, targeting the interaction domains of viral proteins that are essential for productive infection may represent a universal approach that could be applied to many other viruses.

Acknowledgment—We thank Friedemann Weber for critical reading of the manuscript.

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Supplementary figure legends:

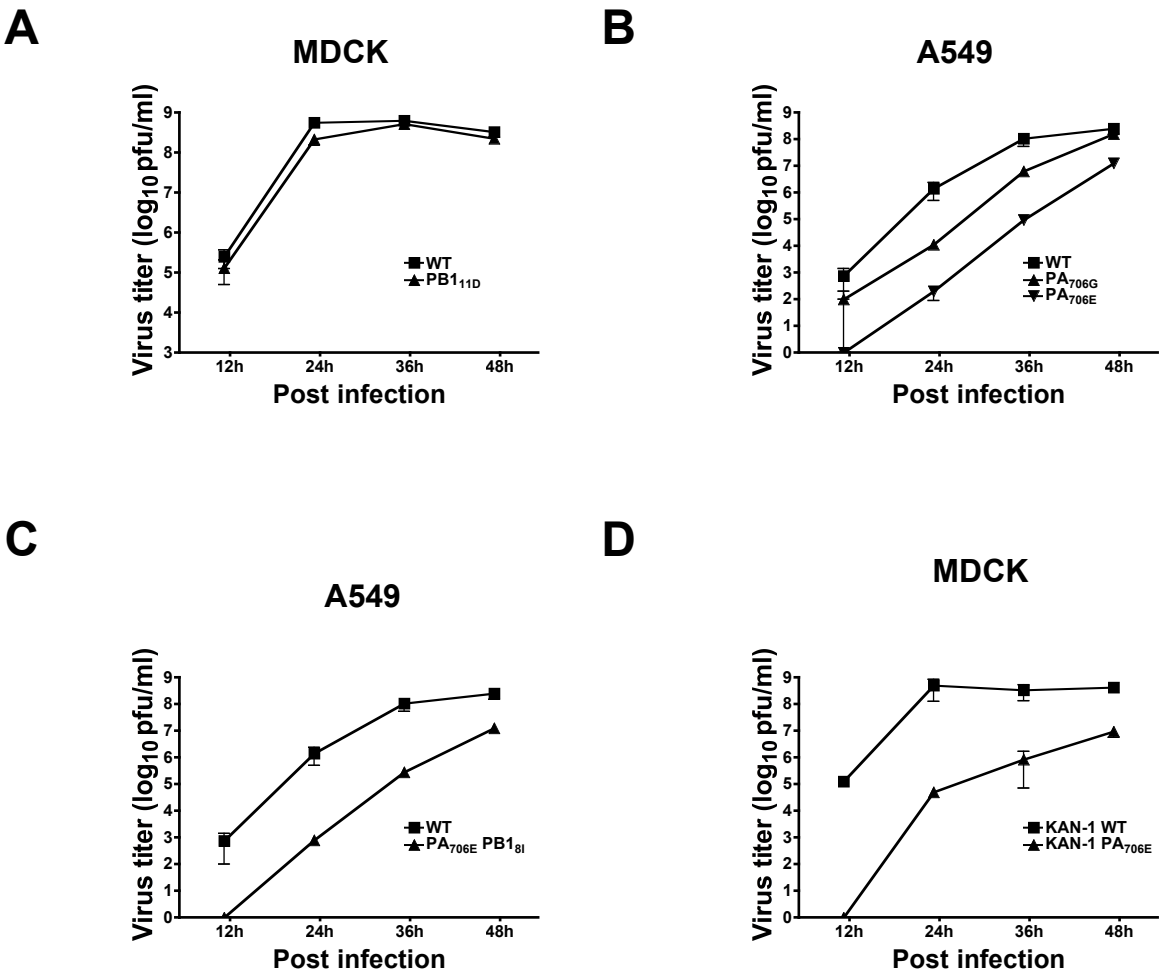
Fig. S1 Growth properties of polymerase assembly mutants in MDCK and A549 cells
(A to C) MDCK or A549 cells were infected with the indicated SC35M virus mutants at a MOI of 0.001 and virus titers in the cell supernatant were determined by plaque assay.
(D) MDCK cells infected with either KAN-1 or KAN-1-PA_{706E} at a MOI of 0.001 and virus titers in the cell supernatant were determined by plaque assay.

Fig. S2 Sequence changes obtained in the escape mutants
(A) Nucleotide changes in PB1. Nucleotides highlighted in blue represent the changes introduced into the rescue plasmid coding for $PB1_{6D7D}$, $PB1_{8D}$, $PB1_{8D9D}$ and $PB1_{10D11D}$. Nucleotides marked in red are the nucleotide changes observed in the escape mutant viruses coding for the indicated PB1 mutants. *, sequence changes observed after the rescue attempt of SC35M-$PB1_{8N}PA_{706E}$.
(B) Nucleotide changes in PA. Nucleotides highlighted in blue represent the changes introduced into the rescue plasmids coding for PA_{706E} and PA_{706R}. Nucleotide marked in red represent the changes observed in the escape mutant viruses coding for PA_{706G}.

Fig. S3 Rescue of SC35M-$PB1_{8D9D*}$ is a result of a recombination event
During rescue of SC35M-$PB1_{8D9D}$ a virus mutant emerged that maintained both D substitutions (Fig. 2A, 8D9D*). Sequencing of the complete PB1 segment revealed a recombination event, which inserted a complete wt PB1 open reading frame.
(A) RT-PCR amplification products corresponding to a fragment of the PB1 open reading frame (nt 1 - 863) from SC35M viruses generated by transfection with the rescue plasmids coding for either wt PB1 or $PB1_{8D9D*}$. Note the difference in length of the amplification products.
(B) Cartoon depicting the sequences involved in the recombination event. Red highlights the region of the helper plasmid (pCA-PB1 (wt)) found in the viral PB1 genome of SC35M-$PB1_{8D9D*}$. The recombination site shortly after a residual $PB1_{8D9D}$ open reading frame could be identified by a partial NotI restriction site. The complete Not1 restriction site is only present in the helper plasmid but not in the rescue plasmid pHW2000-$PB1_{8D9D}$. The site of the downstream recombination event, indicated by a dashed arrow cannot be identified due to the identical sequence of helper and rescue plasmid.

Fig. S4 The plaque size of the polymerase assembly mutants differ from wt SC35M.
Plaque assay of SC35M coding for PA_{706E} (PA_{706E}) or PA_{706E} and $PB1_{8I}$ ($PA_{706E}PB1_{8I}$) and wt SC35M 48h p.i.

Fig. S5 Pathogenicity of SC35M-$PB1_{8V}$ in mice
Survival and weight loss of 6-8 week old female BALB/c mice (n=5/group) after intranasal infection with SC35M mutant viruses encoding $PB1_{8V}$.


A
MDCK
Virus titer (log_{10} pfu/ml)
Post infection
12h
24h
36h
48h
WT
$PB1_{11D}$
B
A549
WT
PA_{706G}
PA_{706E}
C
A549
WT
PA_{706E} $PB1_{8I}$
D
MDCK
KAN-1 WT
KAN-1 PA_{706E}

Suppl. Fig. 1

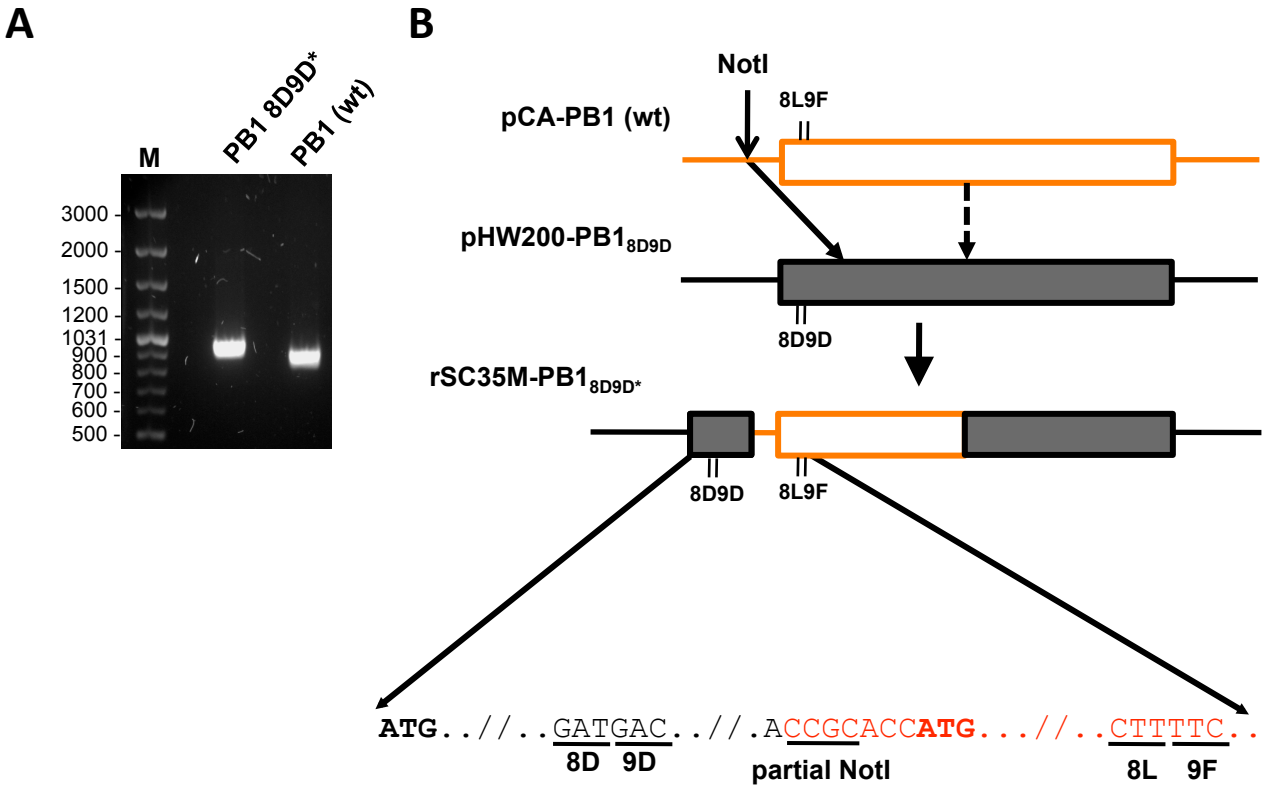
A

PB1 (nt 37 - 60)	
wt	ccgactttgcttttcttgaagatt
6D7D	...gacgac..............
6D7Y	...gactac..............
8D	.........ga.............
8N	.........aa.............
8V	.........gt.............
8A	.........gc.............
8I*	.........at.............
8L*	.........act............
8D9D	.........ga.ga..........
8V9F	.........gt.tt.........
8N9F	.........aa.tt.........
10D11D	..............gacg.c...
10V11D	..............gtcg.c...

B

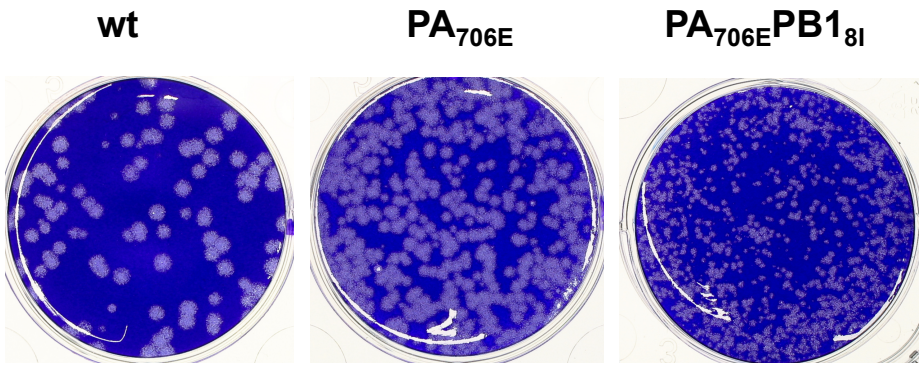
PA (nt 2128 - 2153)	
wt	cttaatgcatcttggttcaactcc
706E	............gaa.........
706R	............a.a.........
706G	............g.a.........

Suppl. Fig. 2

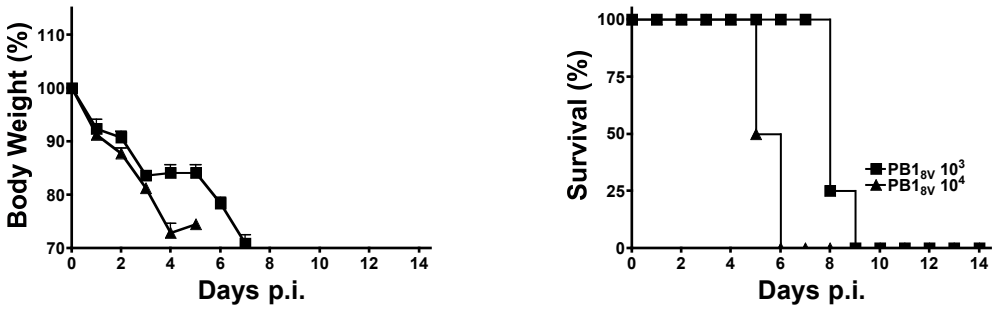

A
M
PB1 8D9D*
PB1 (wt)
3000
2000
1500
1200
1031
900
800
700
600
500
B
NotI
8L9F
pCA-PB1 (wt)
pHW200-PB1 8D9D
8D9D
rSC35M-PB1 8D9D*
8D9D
8L9F
ATG..//..GATGAC..//.ACCGCACCATG..//..CTTTTC..
8D 9D
partial NotI
8L 9F

Suppl. Fig. 3

rSC35M



Suppl. Fig. 4


Body Weight (%)
110
100
90
80
70
0
2
4
6
8
10
12
14
Days p.i.
Survival (%)
100
75
50
25
0
PB1$_{8V}$ 10^3
PB1$_{8V}$ 10^4
Days p.i.

Suppl. Fig. 5

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5 Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren Damen/Herren in Marburg:

Herr Batschauer
Herr Becker
Herr Bölker
Herr Bremer
Herr Buckel
Herr Dörnemann
Herr Galland
Herr Garten
Frau Hassel
Herr Heldmaier
Herr Homberg
Frau Kahmann
Herr Klenk
Frau Maisner
Herr Matrosovich
Herr Mösch
Frau Renkawitz-Pohl
Herr Stech

6 Danksagung

Als erstes möchte ich den Personen danken, die zu dieser Doktorarbeit möglich geführt haben. Prof. Klenk, der diese Arbeit erst möglich gemacht hat und mich die ganze Zeit über selbstlos unterstützt hat. Bei Björn Keiner und Prof. Klenk habe ich alle Grundlagen der Influenza-Virologie gelernt, die essenziell für diese Arbeit waren. Ganz besonderer Dank gilt Prof. Martin Schwemmle, für die Möglichkeit in seinem Labor zu arbeiten, für die enthusiastische und mitreißende Motivation, durch die ich es immer wieder geschafft habe an die Arbeit mit Begeisterung zu gehen.

Besonders freut mich, dass ich durch diese Arbeit gleichzeitig Kontakt mit der Virologie von Marburg und Freiburg halten konnte.

Vielen Dank an Veronika und Dominik. Dass ihr so fleißig und geduldig gearbeitet habt und sogar manchmal gemacht habt, was ich gesagt habe.

Without our two Taiwanese, Chia Ching and Yuan Ju, work in the lab would have been not half as funny! Thanks a lot also for the most helpful information ever: Everything will be fine!

Danke an Petra für die schöne und gute Zusammenarbeit in dem Mx-Projekt und an Linda, du hast mich bei meinem Hauptprojekt so gut und lange unterstützt. Ohne deine Hilfe wäre es nur halb so gut geworden.

Die ganze Squashgruppe hat immer sehr dazu beigetragen mich auszupowern und mal den ganzen Frust aus dem Labor zu vergessen.

Auch den ganzen Leuten, denen ich eine schöne lange Zeit in Freiburg zu verdanken habe, Nadja, Peter, Annegrit, Geoff, Jessi, Mindaugas, Arnold, Kerstin, Barbara, Christel, Sonja, Anne-Sophie, Alex, Corinna..... und vielen mehr.

Ebenso den Freunden die ich noch aus Marburg habe und auch den nicht virologischen Freunden aus Freiburg: Danke! Ihr habt mich immer wieder etwas aus der Wissenschaft in die Realität zurück geholt.

Ganz besonderer Dank gilt meinen Eltern Rita und Walter und meiner Schwester Melanie, dafür, dass ihr mich die ganzen Jahre so toll unterstützt habt und immer an mich geglaubt habt.

Und zum Schluss die Person, die wohl am meisten zu den letzten Jahren beigetragen hat. Danke, dass du mich aus einem tiefen Loch rausgeholt hast, für deine Geduld von der du mehr hast als du glaubst und einfach für die schöne Zeit, Kerstin.