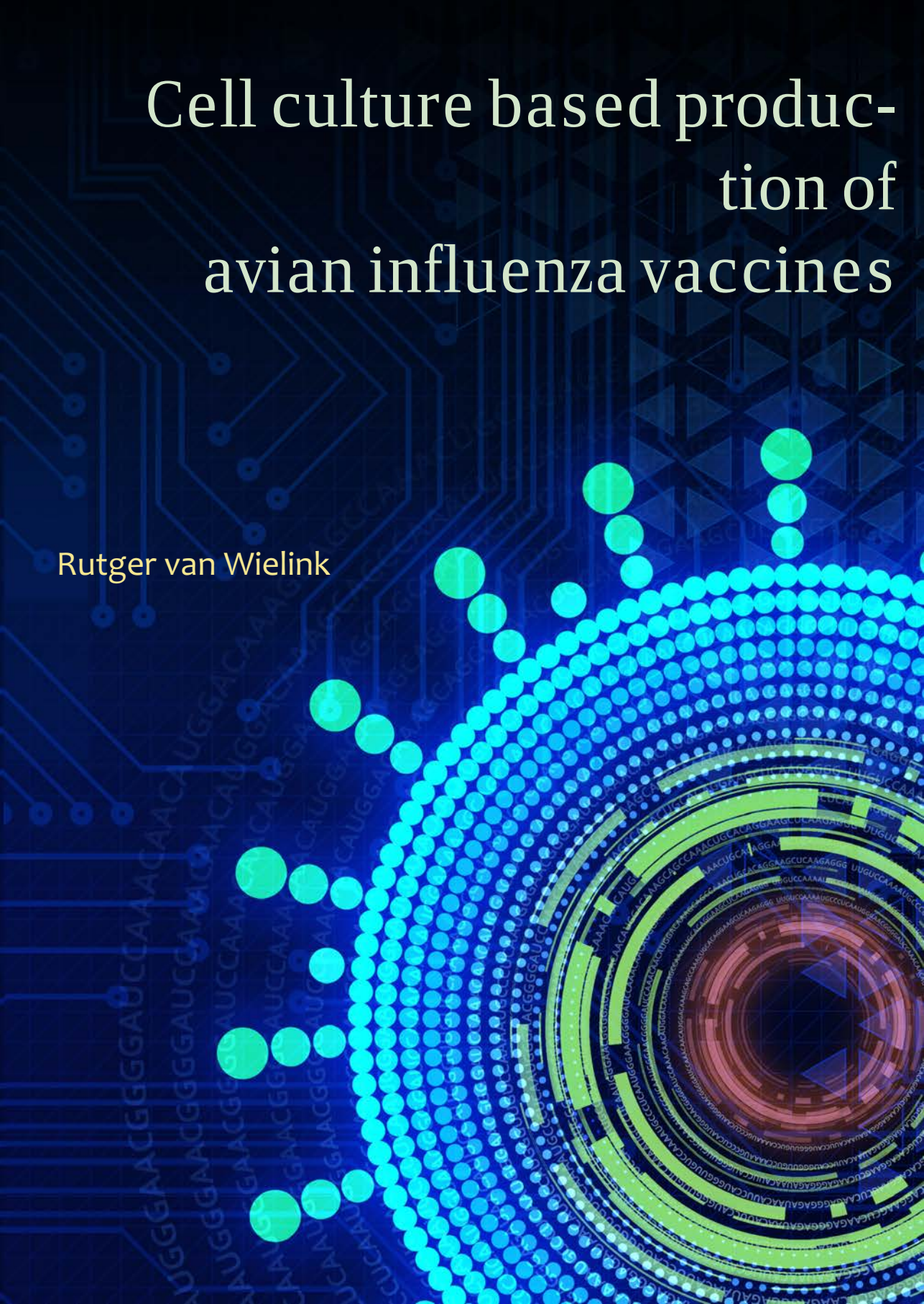
Cell culture based production of avian influenza vaccines

Rutger van Wielink



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Cell culture based production of avian influenza vaccines

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There are some oddities in the perspective with which we see the world. The fact that we live at the bottom of a deep gravity well, on the surface of a gas covered planet going around a nuclear fireball 90 million miles away and think this to be normal is obviously some indication of how skewed our perspective tends to be, but we have done various things over intellectual history to slowly correct some of our misapprehensions.

Douglas N. Adams

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Chapter 1

Introduction

INTRODUCTION

Avian Influenza

Avian influenza (AI) is a contagious disease of birds that is caused by viruses belonging to the influenza A genus within the family Orthomyxoviridae (15, 112). Influenza A viruses are antigenically divided into 16 hemagglutinin (HA; H1-H16) and 9 neuraminidase (NA; N1-N9) subtypes. The primary hosts of AI viruses are wild aquatic birds, in which the virus replicates asymptomatically in the intestinal tract and to a lesser extent in the respiratory tract. All known influenza A virus subtypes have been isolated from birds. Only certain subtypes infect humans and other mammals such as horses, pigs and dogs, in which they cause acute respiratory disease and are efficiently transmitted within the host population (183). Every year, seasonal human influenza causes severe illness in three to five million individuals and can cause up to half a million deaths worldwide (185). These numbers can dramatically increase during occasional pandemic outbreaks.

AI can be transmitted to domesticated birds, such as chickens, ducks and turkeys, through fecal contamination of water. Based on the severity of the disease in poultry, AI can be classified into two clinical forms: a low pathogenic (LPAI) form that causes mucosal infections with variable morbidity and decreased egg production, and a high pathogenic (HPAI) form, which causes high morbidity and mortality in poultry with devastating economic effects (159). HPAI, also known as fowl plague, arises from LPAI subtypes H5 and H7, and it is generally assumed to occur in domestic birds that become infected with LPAI H5 and H7 strains from the wild-bird reservoir (5)

As a zoonosis, influenza A virus can be transmitted between species from animals to humans. Recent outbreaks of HPAI H5N1 and H7N7 strains in poultry resulted in transmission to humans with high fatality among hospitalized individuals (23, 42). Until now, such viruses have yet shown difficult to transmit between humans, although recent studies suggest that they are able to do so (65). Control of outbreaks of AI is therefore not only necessary to prevent economical losses in poultry farming, but also to protect global public health. Migratory behavior of aquatic birds allows the AI virus to spread fast and widely, making outbreaks difficult to prevent or control. Although HPAI outbreaks are generally controlled by culling of the infected flocks, vaccination has been shown to be an effective, additional tool (160).

Influenza A virus anatomy and replication cycle

The influenza A virus (Fig. 1) is characterized by a lipid envelope of spherical or filamentous shape, that contains the negative sense single-stranded RNA genome, divided into eight linear segments (15, 112). These eight segments can encode at least twelve viral proteins, including the three RNA polymerase subunits PB1, PB2 and PA, the nucleocapsid protein NP, the matrix protein M1, the ion channel-like protein M2, the non-structural protein NS1, the nuclear export protein NEP (previously known as NS2) and the earlier mentioned glycoproteins HA and NA. A small, pro-apoptotic protein called PB1-F2 may be expressed from an alternative reading frame by the PB1 gene segment. Furthermore, recent studies revealed an open reading frame in the PA gene that encodes the PA-X protein, which represses cellular gene expression and modulates the host response to infection (71). The host cell derived lipid envelope of the virion is studded with HA trimers and NA tetramers, which form the main influenza virus antigens. In addition to HA and NA, a small number of M2 proteins are present in the viral envelope. Beneath the viral envelope lays a matrix of M1 proteins, which enclose the virion core containing the 8 ribonucleoprotein complexes (vRNP). Each vRNP contains one RNA segment, coated with NP protein and includes the heterotrimeric RNA-dependent RNA polymerase. Furthermore NEP, but not NS1 is present in the virion.

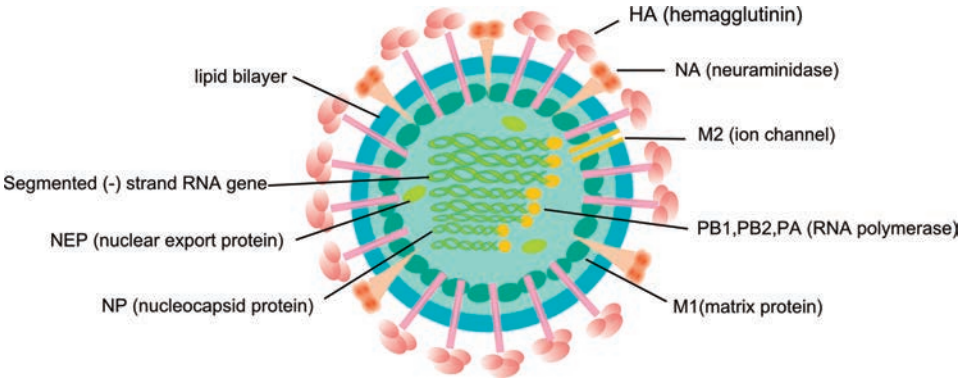

Fig. 1. Schematic overview of the influenza A virus particle.

Infection of a cell starts by attachment of the virion to receptors on the cell membrane (Fig. 2). Human influenza viruses prefer binding to receptors containing sialic acid (SA) with α-2,6 linkage, that are commonly found on human tracheal epithelial cells, whereas avian influenza strains prefer receptors with α-2,3 linkage, that are more common in avian gut epithelium. The HA protein is therefore a determinant for host specificity. Furthermore, HA is also the major virulence factor

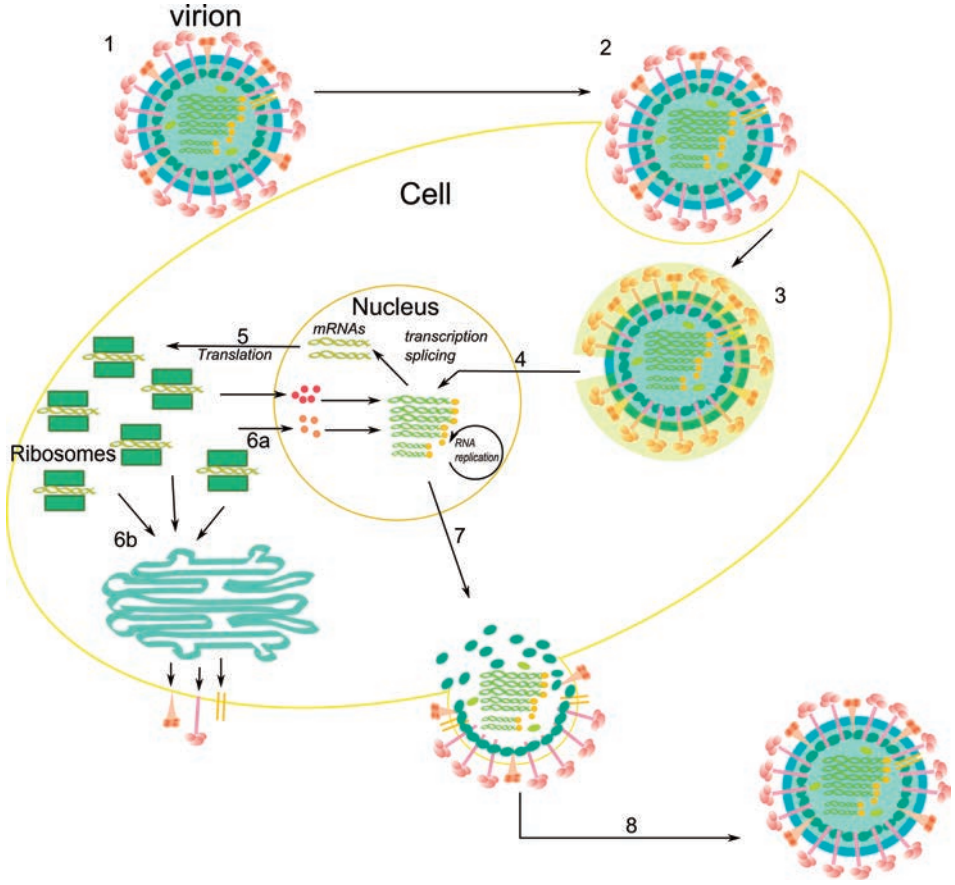

Fig. 2. Schematic overview of the infection of a cell by influenza A virus. Infection starts with binding HA on the virus membrane to sialic acid receptors on the cell membrane (1). Then, the virus particles is internalized by endocytosis (2). Acidification of the endosome results in fusion of the virus membrane with that of the endosome, and release of the viral genome into the cytoplasm (3). The vRNPs are then imported into the nucleus where it is replicated into more vRNA copies, and transcribed into vmRNAs (4). The vmRNAs are exported to the cytoplasm where they are translated (5). PB1, PB2, PA, NP, NS1, M1 and NEP proteins are then imported into the nucleus (6A), whereas HA, NA and M2 are transported to the cell membrane (6B). In the nucleus, progeny vRNPs are formed after binding of NP and polymerase proteins to the new vRNAs. Binding of M1 and NEP to the vRNPs results in export of the viral genome to the cytoplasm, where virus assembly occurs at the cell membrane (7). When a new virus particles is formed, the NA cleaves the cellular sialic acid receptors, thereby facilitating the release of progeny virions from the cell (8).

associated with the HPAI phenotype. HA is build up out of three monomers, each consisting of two disulphide-linked polypeptides, HA1 and HA2, which result from proteolytic cleavage of the precursor HA0 (15). Cleavage is required for the virus to be infectious. HPAI viruses contain multiple basic amino acids at the cleavage site and can therefore be cleaved by a wider range of proteases, thereby allowing the virus to replicate systemically resulting in high pathogenicity. After cellular

attachment, influenza virus particles are internalized by endocytosis. Acidification of the endosome then leads to two essential steps. A pH reduction inside the virion, enabled by the M2 ion-channels, depolymerizes the M1 matrix layer and dissociates M1 from the RNPs (99, 197). Simultaneously, a pH-induced conformational change of HA allows fusion between the viral and the endosomal membrane and release of the viral content in the cytoplasm. The vRNPs are then imported into the nucleus by the host nuclear import machinery. Unlike most other RNA viruses, the influenza virus replicates inside the nucleus of host cells. Each vRNP acts as an independent functional unit that directs the synthesis of two positive-sense RNAs, the viral mRNA (vmRNA) and the complementary RNA (cRNA), by using the negative sense viral RNA (vRNA) as a template. The vmRNAs are exported to the cytoplasm where they are translated. PB1, PB2, PA, NP and NS1 are expressed early in the infection, whereas HA, NA, M1, M2 and NEP are expressed later (112). PB1, PB2, PA, NP, NS1, M1 and NEP are imported into the nucleus, either by the host import machinery, or by diffusion through the nuclear pores (14). The viral polymerase uses the cRNA as an intermediate for the synthesis of more vRNAs, which form the basis of new vRNPs. By associating with M1 and NEP, the vRNPs obtain a nuclear export signal (NES), which leads to transport to the cytoplasm. In the meantime, viral envelope proteins HA, NA and M2 accumulate at the cell surface, whereas M1 accumulates just underneath the cell membrane. Progeny viruses are assembled at the cell surface and bud outward through the cell membrane. Finally, NA cleaves the sialic acid receptors from the cell surface, thereby facilitating the release of progeny virions from the cell.

The NS1 protein does not have a structural function in the virus particle, but instead facilitates virus replication. Cytoplasmic dsRNA and 5′-triphosphate-containing RNA are produced during influenza infection and both are recognized as pathogenic patterns by antiviral proteins such as retinoic-acid inducible gene I (RIG-I), dsRNA-dependent protein kinase R (PKR) and 2′-5′-oligoadenylatesynthetase (OAS). NS1 prevents the initiation of the host antiviral response by binding dsRNA and RIG-I (107), and blocks the activation of PKR and OAS, which could otherwise induce several pathways that lead to type I interferon (IFN) production (132). The IFN response protects cells against invading viral pathogens. The cellular factors that mediate this defense are the products of interferon-stimulated genes (ISGs). Apart from its role as IFN antagonist, NS1 also acts directly to modulate other important aspects of the virus replication cycle, including viral RNA replication, viral protein synthesis (26, 34), and general host-cell physiology. NS1 in-

hibits cellular pre-mRNA processing (including IFN pre-mRNA) and mRNA nuclear export (59). Furthermore, NS1 regulates splicing of M segment mRNA (134), nuclear export of viral mRNA (144) and viral ribonucleoprotein (vRNP) (180).

The influenza RNA polymerase has a high mutation rate of 1.5×10^{-5} mutations per nucleotide (124) or 0.2 mutations per influenza virus genome per replication cycle (129). In theory, a population of 2×10^5 infectious influenza virus particles is expected to possess all possible single amino acid substitutions (129). Such an amount of virus is easily present in 2 μl of the allantoic fluid of embryonated chicken eggs infected with a high yielding influenza strain. The high mutation rate allows the virus to quickly adapt to new environments. The seasonal changes in the antibody binding sites of the viral hemagglutinin protein (antigenic drift) and the occurrence of antiviral resistant virus strains are examples of the adaptability of influenza virus. Because of the high mutation rate, a viral population should be considered as a quasispecies, a dynamic equilibrium of non-identical but related mutants arising from the high mutation rate on the one hand, and a strong selective pressure on the other (28), rather than a multiplicity of viral particles with a defined nucleic acid sequence. The phenotypic plasticity of the influenza virus is further facilitated by the segmented genome. Although it may occur rarely, simultaneous infection of a cell by different influenza subtypes can result in an antigenic shift, when the progeny virus contains a mixture of gene segments from the different virus strains. An influenza pandemic can occur when a reassortant virus arises to which humans are susceptible but immunologically naïve. Examples of such pandemics as a result of antigenic shift are the Spanish flu of 1918-1919 and the recent Mexican/swine flu of 2009. The Spanish flu was infamously lethal as a results of the unique combination of genes, with the HA, NA and PB1 gene contributing to the high pathogenicity (123, 166).

Avian influenza vaccines

Vaccination protects birds against AI by stimulating host immunity, which is largely based on antibodies against the HA protein. These neutralizing antibodies bind to the HA and thereby prevent attachment of the virus to the cell. Due to the large number of HA subtypes, and the antigenic diversity within the subtypes, it is important for the vaccine to immunologically resemble the field strain. The NA protein contributes to a lesser degree to immunity. Moreover, certain vaccines deliberately contain a different NA subtype, allowing infected animals to be differentiated from vaccinated animals (DIVA) (18). Such DIVA vaccines allow for better

surveillance of AI outbreaks and thus may improve worldwide acceptance of vaccination. Historically, vaccines are made of isolated strains that closely resemble the field virus, and which are propagated in embryonated chicken eggs. The development of reverse genetics techniques allowed the rescue of influenza virus from plasmid DNA (40, 67). This has opened up the possibility of virus engineering, allowing the creation of viruses with enhanced productivity yield in eggs or decreased safety concerns associated with HPAI.

Inactivated vaccines are the main type of AI vaccines approved for use and are prepared by either chemical or physical inactivation of the produced virus (160). Whereas influenza vaccines for humans are further purified to create a subunit vaccine containing the HA and NA proteins, avian vaccines generally contain the crude allantoic fluid of the harvested eggs, in order to keep production costs low (73). An adjuvant is used to increase the immunogenicity, often in the form of a water-in-oil emulsion. Live virus vaccines have not been approved yet for use in poultry, although they have been experimentally tested. The main cause of the low acceptance is the potential of such viruses to reassort with genes of other AI viruses, resulting in a virus with increased pathogenicity (73). Infection with a live-virus vaccine stimulates not only the humoral immune response, but also the mucosal and cellular response and thereby gives a better protection against infection with WT virus. Furthermore, they may be administered via aerosol spray or drinking water, thereby reducing the costs of large scale vaccination of commercial poultry. Live influenza virus vaccines have been approved for use in humans and are based on cold-adapted virus, or virus unable to express NS1 (20). Other types of AI vaccines include DNA vaccines and recombinant virus-vectored vaccines (73). The latter group also contains vaccines that have been approved for use in poultry and are based on the in vivo expression of influenza genes through use of a non-influenza virus vector, such as Newcastle disease virus (141). They induce protective immunity as would a live AI virus vaccine but without the risk of reassortment. Both of these recombinant vaccine types do not require the production of influenza virus and therefore fall outside the scope of this thesis.

Production of influenza vaccines

The ability to produce influenza virus in embryonated eggs allowed for large scale production of influenza vaccines. Most influenza vaccines, both human and animal, are still propagated in these systems, which have changed very little since the 1940s (84). As one egg generally produces enough virus for 1 dose of human in-

fluenza vaccine, the egg-based production process requires large quantities of high quality fertilized eggs, which are acquired from specific pathogen-free laying flocks. The supply of these eggs has a long lead time and needs to be planned many months in advance. Furthermore, the egg supply is vulnerable to logistic problems and the availability of eggs can become limiting during an outbreak, when the demand for eggs for human and veterinary vaccines will rise, while laying flocks may be endangered by preventive measures including culling of birds. In case of a pandemic, the egg-based production process is too slow to produce enough vaccine in time. Therefore, animal cell culture has been suggested as an alternative by regulatory agencies and industry for production of human influenza vaccines (8). Cell culture based processes are more robust and flexible, and allow virus to be grown in a closed system. Furthermore, optimization of process conditions and cell line engineering can enhance virus yield and improve product quality.

The production of influenza virus in cell culture consists of two steps (50). First, the cells need to be grown to high cell densities in a short time. A fast growing cell line that has a doubling time of at least 20-30 h is therefore necessary (49). The use of suspension cells simplifies the scale-up of the production process because roller bottles or micro-carriers to provide attachment surface are no longer required and, more importantly, detachment and reattachment is no longer needed between subsequent cultivation steps upon scale-up (47). Furthermore, the ideal production cell line is able to grow in serum-free (SF) media, since the usage of serum is not preferred due to the high costs, lot-to-lot variation and risk of contamination with viruses, mycoplasmas and prions. Additionally, growth on SF media simplifies the production process as medium exchange before infection is no longer required to counter the trypsin inhibition by serum (47). Trypsin is generally added at the start of the virus propagation step, as it cleaves the precursor HA0 and thereby allows multicyclic virus replication. Furthermore, trypsin can cleave extracellular IFN, thereby limiting cellular antiviral defenses (149). Cells are infected at a low multiplicity of infection (m.o.i.) and virus is generally harvested 2-3 days later for further processing. To compete with egg-based processes, cell culture should be able to propagate a wide variety of influenza strains to high virus titers (more than 1×10^8 $TCID_{50}$/ml or more than 7.97 $\log_2$ HAU/100µl (49)). A wide variety of cell lines have been assessed for their suitability for influenza virus production. Vac-

cines produced in three cell lines (MDCK, Vero and PER.C6®) are in clinical trials, and the first licenses for seasonal and pandemic influenza vaccines produced in MDCK and Vero cells have been given (49).

The process requirements for a veterinary vaccine differ from that of human vaccines. Lesser regulatory requirements on culture media, downstream processing and purity (49) allow for a less stringent process. However, the low price of poultry vaccines ($0.05–0.15 per dose) limit companies to invest in research and development, licensing, and scaling-up manufacturing, especially when competition limits the number of doses sold (160). Whereas several animal-cell derived human influenza vaccines are already on the market, veterinary vaccines are still commercially produced in chicken eggs.

Contents of this thesis

In this thesis, the use of several animal cell culture systems as substrate for production of AI vaccines for poultry was assessed and virus and cell line engineering methods were developed, to further enhance virus yield. We started in **Chapter 2** with a comparison of three continuous cell lines that are currently used to produce different virus vaccines. The MDCK, VERO and BHK-21 cell lines were assessed for their ability to replicate a selection of different AI strains to high titers. Furthermore, we tried to adapt VERO and MDCK cells, which normally grow attached, to suspension growth. MDCK-SFS cells, which were adapted to growth in suspension, showed the highest, overall yield of influenza virus and were selected for further studies.

During the comparison of virus replication in the different cell lines, we observed that only a few influenza virus strains were able to replicate in BHK21 cells, a fast growing and high cell density reaching suspension cell line. However, the virus strains that were able to replicate in BHK21 cells, reached virus titers that exceeded those of MDCK and Vero cells. Therefore, we focused in **Chapter 3** on the factors that limit replication in this cell line and suggest a method to use BHK21 cells for the production of virus containing the antigenically important HA1 domain of strains that normally not replicate in this cell line.

In **Chapter 4**, a vaccine candidate virus is introduced from which the gene for the non-structural NS1 protein has been deleted (delNS1). NS1 is important for virus replication in several different ways, including inhibition of the host innate im-

mune response. Without NS1, influenza virus replicates poorly and is therefore considered for use as a live-attenuated vaccine. Furthermore, a delNS1 virus can be used as a DIVA vaccine. To develop a high yielding delNS1 virus production system, we made two MDCK cell lines that are able to express NS1. We then assessed the ability of these cell lines to enhance delNS1 virus replication by complementing the lack of virus-expressed NS1. Furthermore, we assessed the impact of the recombinant NS1 expression on IFN induction and apoptosis.

Whereas the approach used in **Chapter 4** to increase delNS1 virus replication may be described as "intelligent" design, in **Chapter 5** the same goal was tackled by using Darwinian evolution. The delNS1 virus was adapted to MDCK-SFS cells and mutations responsible for the ability of the virus to efficiently replicate in the absence of NS1 were determined. Finally, **Chapter 6** summarizes and discusses the results of the work described in this thesis in relation to current and future influenza vaccine production.

Chapter 2

Adaptation of a Madin-Darby canine kidney cell line to suspension growth in serum-free media and comparison of its ability to produce avian influenza virus to Vero and BHK21 cell lines.

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Abstract

Madin-Darby canine kidney (MDCK) cells are currently considered for influenza vaccine manufacturing. A drawback of these cells is their anchorage dependent growth, which greatly complicates process scale-up. In this paper a novel MDCK cell line (MDCK-SFS) is described that grows efficiently in suspension and retained high expression levels of both α-2,6 and α-2,3 sialic acid receptors, which bind preferably to human and avian influenza viruses, respectively. The production of avian influenza virus by BHK21, Vero and MDCK-SFS cell lines was compared. Although BHK21 cells consisted of two populations, one of which lacks the α-2,3 receptor, they supported the replication of two influenza strains to high titers. However, BHK21 cells are generally not applicable for influenza production since they supported the replication of six further strains poorly. MDCK-SFS cells yielded the highest infectious virus titers and virus genome equivalent concentration for five of the eight influenza strains analyzed and the highest hemagglutination activity for all eight virus strains. Taken together with their suitability for suspension growth this makes the MDCK-SFS cell line potentially useful for large scale influenza virus production.

INTRODUCTION

Avian influenza (AI) virus is a zoonotic pathogen with a natural reservoir entirely in birds. During the recent outbreaks of AI among poultry, transmission of H5, H7, and H9 subtype viruses to humans have occurred, sometimes with fatal outcome (155). Vaccination with inactivated AI vaccine protects chickens from disease and is currently in use in some countries (32). Vaccination of poultry is not only important to protect birds but also to prevent further transmission to humans.

Influenza vaccines are produced traditionally in embryonated eggs. However, the supply of these eggs has a long lead time, is vulnerable to logistic problems and the availability of eggs can become limiting during an outbreak, when the demand for eggs for human and veterinary vaccines will rise, while laying flocks may be endangered by preventive measures including culling of birds. The use of animal cell cultures as an alternative to embryonated eggs is therefore recommended by regulatory agencies and industry (8).

An ideal production cell line is able to grow in serum-free (SF) media as suspended cells. The usage of serum is not preferred due to the high costs, lot-to-lot variation and risk of contamination with viruses, mycoplasmas and prions. Additionally, SF growth simplifies the production process as medium exchange before infection is no longer required to counter the trypsin inhibition by serum (47). The use of suspension cells simplifies the scale-up of the production process because roller bottles or micro-carriers to provide attachment surface are no longer required and, more importantly, detachment and reattachment is no longer needed between subsequent cultivation steps upon scale-up (47).

In the past, various cell lines have been evaluated for their suitability for influenza virus production (49). Madin-Darby canine kidney (MDCK) and Vero cells are considered to be best suited for this purpose as they support the replication of a wide variety of virus strains to high titers (49, 54, 89, 106). However, most of these studies were done with adherent cell lines, which have disadvantages in large scale production processes. In a few studies suspension cell lines were used for influenza virus production. A MDCK cell line able to grow in suspension was generated by transfection with the human *siat7e* gene (21). Another MDCK cell line was adapted to suspension growth by serial passaging (56) and is currently in use by Novartis Vaccines. Furthermore, the PER.C6® (125), AGE1.CR® (91) and

EB14®/EB66® cell lines (104) produce influenza virus to high titers and grow in suspension (49). These latter three continuous cell lines were especially designed for vaccine production. Since they are only available under license, they were not considered for this study. These previous studies focus mainly on production of human influenza viruses.

The aim of this study was to develop SF suspension cell lines that are able to produce a wide variety of AI virus strains, for veterinary vaccine production. For this purpose BHK21, MDCK and Vero cells were used. BHK21 cells grow in suspension and are already used to produce veterinary vaccines against foot-and-mouth disease and rabies. MDCK and Vero cells normally grow adherent and with serum. Therefore, it was first attempted to adapt these cells to SF suspension growth, after which these cell lines were assessed for their suitability for influenza A virus production using seven low-pathogenic AI virus strains and the human PR8 reference strain.

Materials and methods

Cell lines

All parental and adapted cell lines used are shown in Table 1, together with their origin, medium used and whether cells grow adherent or in suspension. Cell culture media were supplemented with 2 to 8 mM glutamine (depending on manufacturer's instructions), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco Invitrogen). Adaptation of cell lines was attempted in four SF cell culture media: Optipro, UltraMDCK, SFM4MegaVir (Hyclone) and SFM4BHK21. Cells were

Table 1: Origin and culture conditions of cell lines used.

Cell line	Species	Origin	Growth conditions	Culture medium[a]	Serum[a]
MDCK	Dog	ATCC, CCL-34	Adherent	MEM	5% FBS
MDCK-SF	Dog	Adapted from MDCK	Adherent	UltraMDCK	None
MDCK-SFS	Dog	Adapted from MDCK-SF	Suspension	SFM4BHK21	None
Vero	Monkey	ATCC, CCL-81	Adherent	MEM	5% FBS
Vero-SF	Monkey	Adapted from Vero	Adherent	Optipro	None
BHK21	Hamster	ATCC, CCL-10 derived	Suspension	K1000	5% FBS
BHK21-SFS	Hamster	Adapted from BHK21	Suspension	SFM4BHK21	None

[a] MEM, K1000 and Optipro were obtained from Gibco, Invitrogen Ltd, Carlsbad, CA; UltraMDCK was obtained from Lonza Biowhittaker, Basel, Switzerland; SFM4BHK21 and Fetal Bovine Serum (FBS) were obtained from Hyclone, Waltham, MA.

grown at 37˚C and 5% CO_2. Suspension cells were grown in shaker flasks at 100 rpm. Total and viable cell densities were determined with a Nucleocounter (Chemometec A/S, Allerød, Denmark).

Virus strains and infection

Seven low pathogenic wild type AI virus strains and the A/Puerto Rico/8/34 (PR8) human reference strain were used in this study (Table 2). The PR8 strain was obtained from the ATCC (VR-95; Manassas, VA, US), whereas all other viruses were kindly provided by Dr. G. Koch (CVI). All viruses were propagated in the allantoic cavity of nine-day-old embryonated chicken eggs and the infectious virus titers were between 7.8 and 8.8 $\log_{10}$ $TCID_{50}$/ml. The viruses were not adapted to individual cell lines prior to analysis of their propagation in cells. Apart from being very laborious, since this should be done for each cell line virus combination, adaptation is also not desired in a vaccine production process because it takes time and may alter the antigenicity of the vaccine.

Table 2: Characteristics of influenza virus strains used for propagation in cell lines.

Influenza strain	Serotype	Origin
A/Puerto Rico/8/34	H1N1	Human
A/duck/Ukraine/1/63	H3N8	Avian
A/chicken/Pennsylvania/21525/83	H5N2	Avian
A/mallard/Denmark/75-64650/03	H5N7	Avian
A/turkey/Wisconsin/68	H5N9	Avian
A/chicken/Italy/1067/99	H7N1	Avian
A/chicken/Netherlands/06022003/06	H7N7	Avian
A/duck/Germany/R113/95	H9N2	Avian

For the infection of adherent cells, the cells were allowed to attach to the surface of 6-well plates (Greiner Bio-One) overnight. The next day, these cells were provided with 5 ml fresh medium without serum, even when cells were earlier grown in serum containing medium (Table 1). Three wells of each adherent cell line were used to count the amount of cells at the start of the infection (0.18×10^6 cells/ml for serum dependent MDCK cells and 0.13×10^6 cells/ml for the other adherent cell lines). The other wells were used to determine the virus titers 72 h post-infection (p.i.).

For the infection of suspension cells the cells were diluted to 0.3 x 10^6 cells/ml in 15 ml medium in 125-ml shaker flasks. Low cell densities were used to prevent depletion of the media as a result of cell growth during slow proceeding infections, and to be able to compare cells grown adherent and in suspension. Before infection, 3 μg/ml trypsin-EDTA (Gibco) was added to all cells. Because of their ability to inactivate trypsin (74), Vero cells were supplied with additional trypsin-EDTA (3 μg/ml), 24 h and 48 h p.i. All infections were performed in triplicate, with a multiplicity of infection (m.o.i.) of 0.01. After 72 h, samples were taken and stored at -70˚C. The amount of virus produced was estimated by measuring the infectious virus particle concentration, the virus genome equivalent concentration and the hemagglutination activity.

Flow cytometric analysis (FCA) of influenza binding sialic acid receptors

The abundance of α-2,3 sialic acid (SA 2,3) and α-2,6 sialic acid (SA 2,6) linkages on the different cell lines was determined as described previously (55). This method was modified by staining cells at the same time with SA 2,3 specific digoxigenin (DIG)-labeled *Maackia amurensis* agglutinin (MAA) lectin and SA 2,6 specific biotinylated *Sambus nigra* agglutinin (SNA) lectin, allowing simultaneous analysis of both receptors. In short, cells were incubated with 10 μg/ml MAA (Roche Applied Science, Mannheim, Germany) and 10 μg/ml SNA (Vector Laboratories, Burlingame, CA), followed by incubation with 5 μg/ml anti-DIG fluorescein (FITC) labeled Fab Fragments (Roche Applied Science) and 0.5 μg/ml Streptavidin R-Phycoerythrin (R-PE) conjugate (Invitrogen, Carlsbad, CA). Cells were fixed in CellFIX (Becton Dickinson, Franklin Lakes, NJ) and analyzed on a FACScan flow cytometer (Becton Dickinson). The contribution of FITC fluorescence to the R-PE signal and R-PE fluorescence to the FITC signal was compensated for. Incubations without lectins and with single lectins were included as controls. Incubations with single lectins gave similar results to incubations with two lectins.

Infectious virus titer assay

The infectious virus titer was measured by determining the tissue culture infective dose required to infect 50% ($TCID_{50}$) of MDCK cells. Tenfold serial dilutions (10^{-1} to 10^{-8}) of the culture supernatant were prepared in medium containing 3 μg/ml trypsin-EDTA and inoculated (six replicates per dilution) in 96-well plates (Greiner Bio-One) grown to confluency with MDCK cells. Plates were incubated at 37˚C for two days and then dried and stored at -20˚C. The end points were determined by immunoperoxidase monolayer assay. Cells were fixed with cold 4% paraformalde-

hyde, followed by a first incubation step with 0.78 μg/ml monoclonal anti-nucleoprotein (NP) antibody (HB65, ATCC) and a second incubation step with 2.6 μg/ml peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark). Cells were stained with 3-Amino-9-ethylcarbazole (AEC) and checked microscopically for viral presence. Virus titers, in $\log_{10}$ $TCID_{50}$/ml were calculated by the method of Reed and Muench (131).

Virus genome equivalent concentration measurement by quantitative RT-PCR

The virus genome equivalent concentration was derived from the amount of viral RNA copies determined by M-gene specific qRT-PCR, as described before (96). RNA was isolated from each sample with a MagNAPure LC system with the MagNAPure LC Total Nucleic Acid Isolation Kit (Roche Applied Science). For the PCR, primers AI-M-F45 (5′-CTTCTAACCGAGGTCGAAACGTA-3′) and AI-M-R251 (5′-CACTGGGCACGGTGAGC-3′) and Taqman probe AI-M-Tqmn1 (5′-6FAM-CT CAAAGCCGAGA TCGCGCAGA-XT-PH) (TIBMolBiol, Berlin, Germany) were used. A calibration curve was used for each virus strain, made by a 3-step serial dilution of the egg-based seeding virus with known infectious virus titer. Titers of the samples are expressed as $\log_{10}$ $TCID_{50}$ equivalent/ml.

Hemagglutination activity assay

A revised hemagglutination activity (HA) assay with improved accuracy was used as described previously (72). This revised assay includes a regression procedure to analyze the HA in a continuous manner. Furthermore, samples are serially diluted 1.5-fold rather than 2-fold for improved accuracy. Plates (Greiner Bio-One) were analyzed at 820 nm and titers are expressed as $\log_2$ HAU/100 μl.

Statistical analyses of virus productivity

An ANOVA general linear model was used to test for differences in average production of the influenza strains by the different cell lines, using the Minitab software package (Minitab Inc., Coventry, UK). Cell lines, virus strains and an interaction term were included in the model. Pair-wise comparisons of the mean were performed with Tukey's simultaneous test. Differences in mean were assumed significant if $p<0.05$.

Results

Adaptation of cell lines to serum free and suspension growth

MDCK, Vero and BHK21 cells were adapted to SF growth, by gradual replacement of the serum containing (SC) medium with SF medium, in 7 to 12 passages (p.). The resulting cell lines are indicated by the suffix –SF (Table 1). Subsequently, attempts were made to adapt MDCK-SF and Vero-SF cells from adherent to suspension growth in shaker flasks. For this purpose, Optipro, UltraMDCK, SFM4MegaVir and SFM4BHK21 cell culture media were used. MDCK-SF cells only grew in suspension in SFM4BHK21 medium, a prototype medium designed specifically for BHK21 cells (Fig. 1A). They grew as single cells in suspension (Fig. 1B). The resulting cell line was named MDCK-SFS. In all other culture media, extensive cellular aggregation was observed before cell growth and viability decreased (data not shown). During the first month of suspended cultivation (12 p.) the growth rate of MDCK-SFS cells increased. Thereafter the growth rate remained stable for over 3.5 months of serial cultivation (43 p.). The cells grow to a maximum cell density of 2.2 x 10^6 cells/ml and have a maximum specific growth rate of 0.029 h^{-1}. MDCK-SFS cells that were cultured statically in SFM4BHK21 medium attached to the surface of the culture flask. This enables comparison of adherent and suspension MDCK cells in the same culture media, as well as direct comparison of MDCK-SFS cells with the adherent Vero-SF cells. To study whether the ability to grow in suspension is connected to the cell line or to the SFM4BHK21 medium the suspension cell line MDCK-SFS was cultured in UltraMDCK medium, which resulted in aggregate formation and attachment to the surface of the shaker flask.

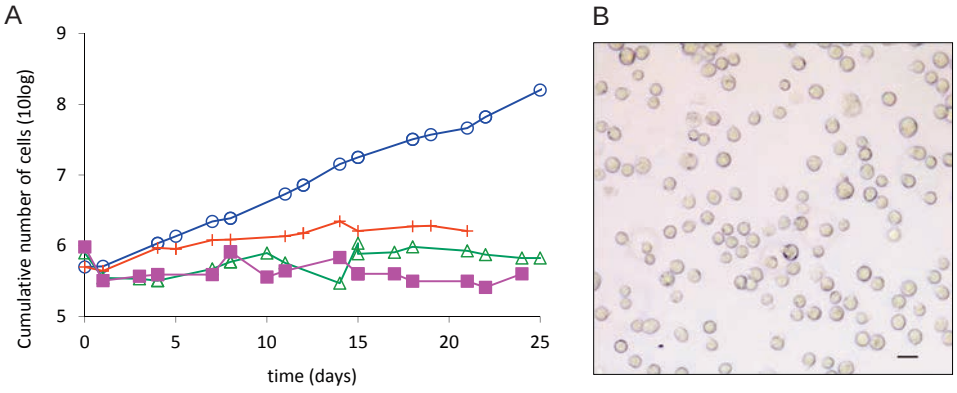

Fig. 1. Adaptation of MDCK-SF cells to suspension growth. Panel A shows the logarithmic cumulative number of cells starting with 1 ml 2 x 10^5 cell/ml, during adaptation in different types of SF cell culture media: SFM4BHK21 (open circle), SFM4MegaVir (+), UltraMDCK (open triangle), Optipro (closed square). Panel B shows a micrograph of single cell suspension MDCK-SFS cells growing in SFM4BHK21 medium (size bar 20 µm).

Unlike MDCK-SF cells, adherent Vero-SF cells formed small aggregates when cultured agitated in SFM4BHK21 medium. Furthermore, despite frequent medium replacement during a period of 45 days, no cell growth was observed and a Vero cell growing in suspension was not obtained. Therefore, adherent Vero-SF cells were used in further studies.

Expression of SA 2,3 and SA 2,6 receptors on cell lines

Influenza viruses enter the cell after recognition and binding to specific cell surface receptors. Most AI virus strains prefer binding to receptors with sialic acid groups having α-2,3 linkages, whereas human influenza strains prefer receptors with α-2,6 sialic acid linkage (15). The availability of the appropriate receptor is therefore in principal a prerequisite for efficient virus replication. The relative abundance of these receptors on the selected cell lines was determined by combining specific SA-2,3-binding MAA and SA-2,6-binding SNA lectins in a single FCA, each linked to a different fluorescent marker. MDCK and Vero-SF cells express both receptors (Fig. 2A and E). The adaptation of MDCK to MDCK-SFS resulted in a broader distribution of SA 2,3 receptors, due to an increase in number of cells with a higher abundance of this receptor (cf. Fig. 2A and B). BHK21 cells consist of two populations of cells with different receptor expression. The majority (76%) of the parental, serum-dependent BHK21 cells express low amounts of SA 2,3 and SA 2,6 receptors, whereas only a few cells (8%) express high levels of SA 2,6 but no SA 2,3 receptors (Fig. 2C). BHK21-SFS cells, however, consist of a larger cell population

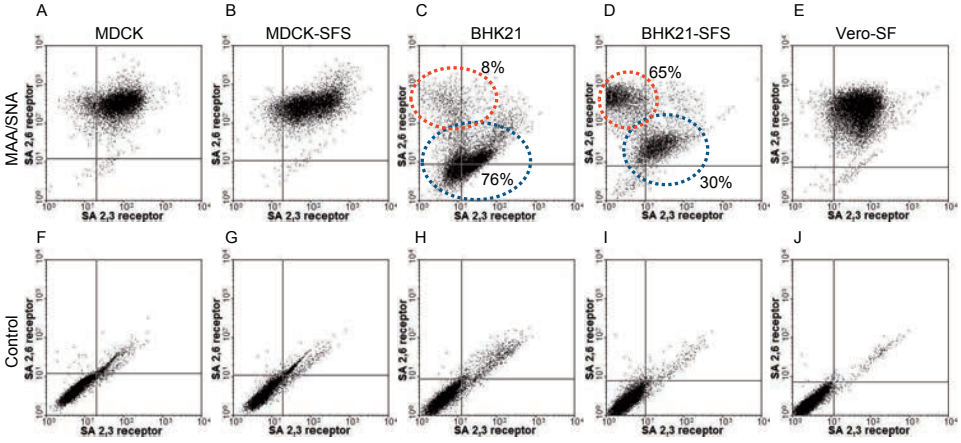

Fig. 2. FCA of influenza binding sialic acid receptors on different cell lines. Cells were incubated with MAA and SNA lectins, binding specifically to SA 2,3 and SA 2,6 receptors, respectively. The different panels represent MDCK (A and F), MDCK-SFS (B and G), BHK21 (C and H), BHK21-SFS (D and I) and Vero-SF (E and J) cells. The control cells (F, G, H, I and J), which were not incubated with the lectins, were for more than 85% within the lower left quadrant of each plot.

(65%) with high levels of SA 2,6 receptors and no SA 2,3 receptors, and a smaller cell population (30%) expressing both receptors at lower levels (Fig. 2D). The difference between BHK21 and BHK21-SFS cells was not caused by serum components binding to the receptors and thereby blocking lectin binding to the receptors during subsequent FCA, since incubation of BHK21-SFS cells for 2 h in SC medium did not affect these results (data not shown).

Comparison of influenza virus production by different cell lines

Two experiments were performed to compare the production of eight influenza virus strains by the different cell lines. Note that virus strains are referred to by their subtype, which are all different (Table 2). In the first experiment MDCK, MDCK-SF, MDCK-SFS and Vero-SF cells were all grown adherently (Fig. 3) in the media as described in Table 1. This allows comparison of the effect of adaptation of MDCK cells to MDCK-SFS cells on virus production without measuring effects of suspension growth itself on virus yield. Virus yields were assessed by virus genome equivalent concentration, infectious virus titers and HA titers, which often gave similar results. However, the virus genome equivalent concentrations are often higher than the infectious virus titers, especially using the three types of MDCK cells, where the difference is 10- to 100-fold (cf. Fig. 3A and B). This probably results from harvesting relatively late in infection, at 72 h p.i., since the infectious virus titer often decreases after reaching its maximum. In contrast, the total viral titer, comprised of both infectious and non-infectious particles as estimated by the hemagglutinin activity (48) and quantitative PCR (our data, not shown) remains constant for a longer period and therefore more likely represents the total amount of virus produced during the 72 h infection period. Furthermore, the virus genome equivalent concentration (Fig. 3A) showed less variation within each triplicate assay as compared to infectious virus titers (Fig. 3B) and HA titers (Fig. 3C). Therefore, virus yields were primarily evaluated based on the virus genome equivalent concentration .

The yields of the eight different viruses did not vary substantially when using the three different MDCK cells (Fig. 3A). When compensated for the 40% higher initial cell density of serum-dependent MDCK cells, this leads to the conclusion that the adaptation to SF and suspension growth did not negatively affect virus yield. In comparison to MDCK-SFS cells grown adherently Vero-SF cells yielded, on average, a 5.1 times lower virus genome equivalent concentration ($p<0.0001$; Fig. 3A) and 3.6 times lower HA titer ($p<0.0001$; Fig. 3C) whereas the average yield of in-

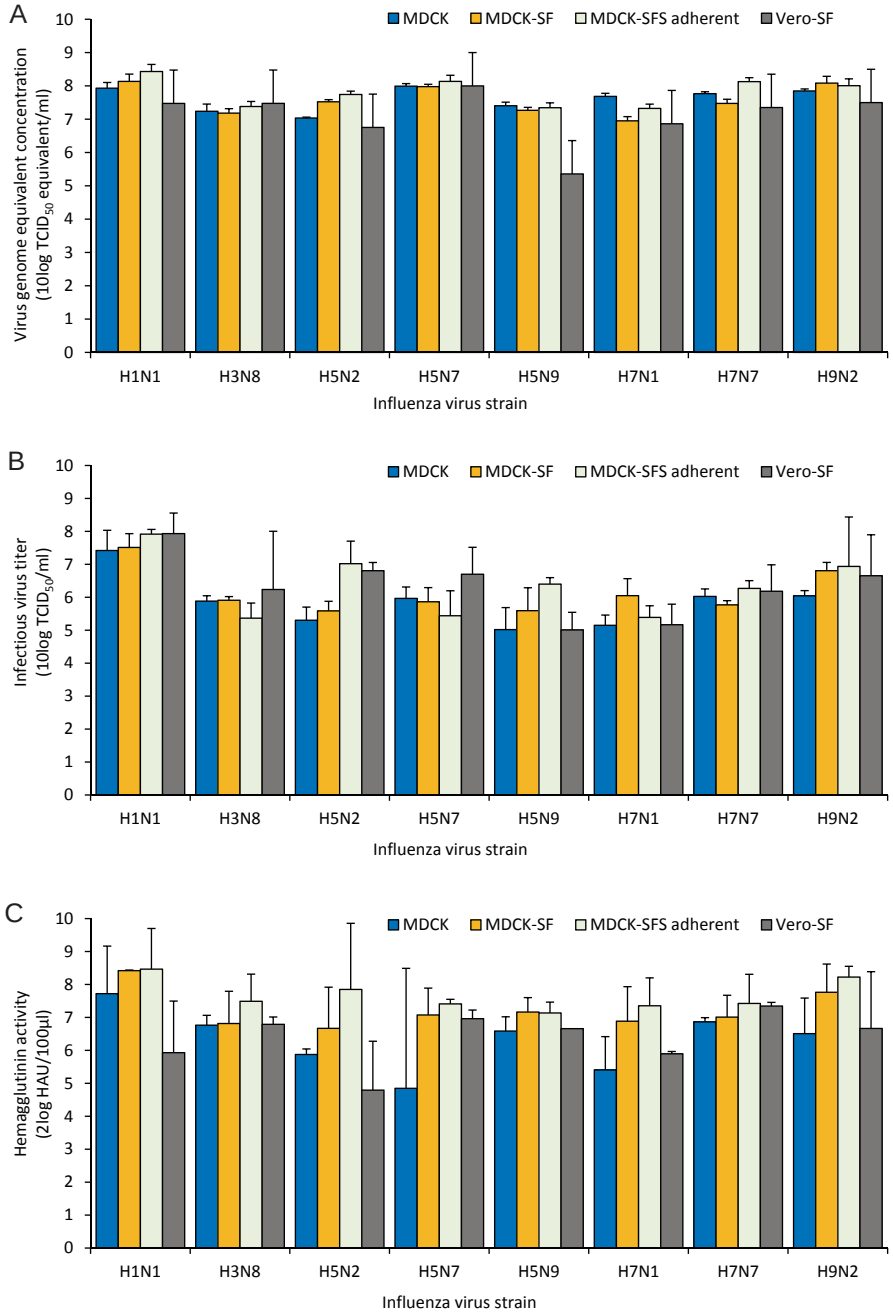

Fig. 3. Yields of different influenza viruses or their HA antigen after propagation in adherent MDCK, MDCK-SF, MDCK-SFS or Vero-SF cell lines. Yields were determined by assessment of virus genome equivalent concentration using M-gene specific quantitative RT-PCR (A), infectious virus concentration by virus titration on MDCK cells (B), or HA titration (C). Geometric mean and 95% confidence interval of virus infections performed in triplicate are presented.

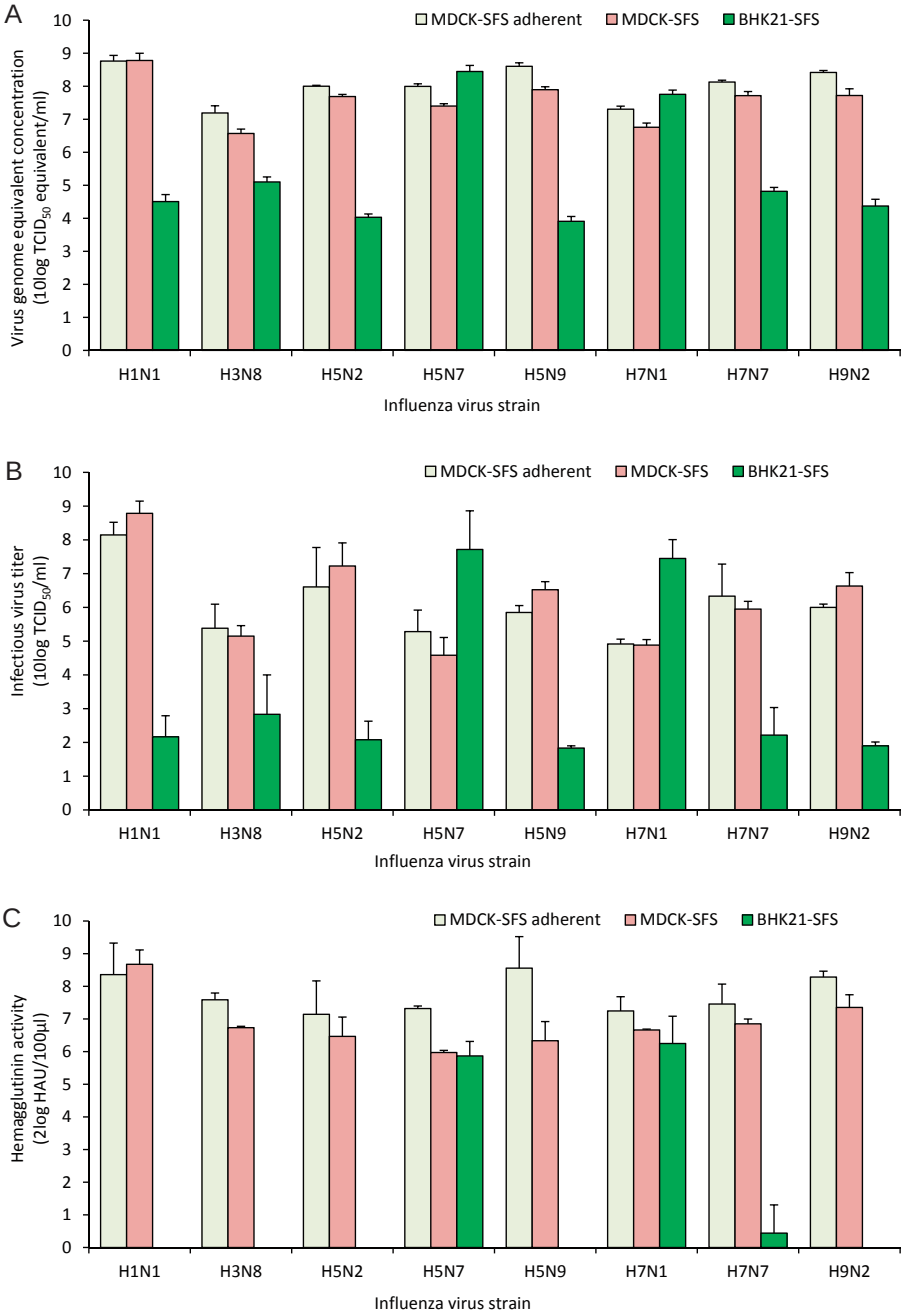

Fig. 4. Yields of different influenza viruses or their HA antigen after propagation in MDCK-SFS or BHK21-SFS cell lines, where the MDCK-SFS cell line was grown both adherent and in suspension. Yields were determined by assessment of virus genome equivalent concentration using M-gene specific quantitative RT-PCR (A), infectious virus concentration by virus titration on MDCK cells (B), or HA titration (C). Geometric mean and 95% confidence interval of virus infections performed in triplicate are presented.

fectious virus did not differ significantly (p=0.98; Fig. 3B). These lower average virus yields by Vero-SF cells primarily reflect the lower yields of H1N1, H5N2 and H5N9 viruses.

In a second experiment virus yields in BHK21-SFS and MDCK-SFS cells were determined. MDCK-SFS cells were grown both in suspension and adherent in SFM4BHK21 medium (Fig. 4). The density of both suspension cell lines at the moment of infection was 2.3 times higher than of the adherent cells, since further dilution of the suspension cells to the density used for the adherent cells seriously hampers their growth. Between the first and second experiment, a significant difference in the virus genome equivalent concentration of the adherent MDCK-SFS was observed (1.7 times, $p<0.0001$), which can be accounted for by a higher H5N9 titer in the second experiment (cf. Fig. 3A and 4A). There were no significant differences between the infectious virus titers (p=0.19) and the HA titers (p=0.92). Adherently grown MDCK-SFS cells yielded on average 3-fold higher virus genome equivalent concentrations ($p<0.0001$) and an almost two times higher HA titer ($p<0.0001$), whereas the yield of infectious virus did not differ significantly (p=0.39). When corrected for the initial cell density, adherent MDCK-SFS cells would have 7-fold higher virus genome equivalent concentrations and 4-fold higher HA titers as compared to the same cells grown in suspension.

BHK21-SFS cells supported replication of the H5N7 and H7N1 strains to titers comparable to those obtained with MDCK-SFS cells (Fig. 4). However, the six other virus strains yielded virus genome equivalent concentrations between 3.9-5.1 $\log_{10}$ $TCID_{50}$ equivalent/ml, which is only slightly higher than the virus genome equivalent concentration that was initially used for infection (3.5 $\log_{10}$ $TCID_{50}$ equivalent/ml). These levels were below the detection limit of the HA assay. Thus, these six strains replicate poorly or not at all on BHK21-SFS cells. Visual inspection showed no lysis of BHK21-SFS cells infected with any of the eight viruses at 72 h p.i. The increase in cell density between 0 and 72 h p.i. was approximately 10-fold for uninfected BHK21-SFS cells, whereas cells infected with the two viruses that replicated efficiently on BHK21-SFS cells, H5N7 and H7N1 virus, showed a lower increase in cell density of 6- and 3-fold, respectively. The other six virus strains that replicate poorly on BHK21-SFS cells did not affect cell growth as the cell density increased to a similar level (factor 10-11) as uninfected cells.

Discussion

MDCK (163), Vero (75) and other epithelial cells require attachment to a surface such as tissue culture flasks or micro-carriers for proliferation. It has been reported that suspended growth of MDCK cells resulted in the formation of multicellular cysts of cells, having a polarized apical outside in which the percentage of replicating cells decreased to 1-4% (176). Others described loose aggregation followed by a slow decrease in viability, caused by aggregation-induced apoptosis (33). These last findings correlate with the aggregation and decreasing viability of MDCK cells when grown in suspension in three of the four culture media used in this study. However, using SFM4BHK21 medium it was possible to isolate a stable, single-cell suspension cell line, called MDCK-SFS.

The ability of MDCK-SFS cells to grow in suspension as single cells appears to be related to the use of the SFM4BHK21 medium, because these cells do not aggregate in this medium whereas the transfer of these cells into UltraMDCK medium resulted in cellular aggregation and surface attachment. SFM4BHK21 medium is designed for BHK21 cells that grow in suspension, whereas the other media are designed for MDCK, Vero and other cell lines which normally grow adherently. Therefore, SFM4BHK21 medium most probably differs from the other media in components that facilitate or prevent attachment to a matrix and to other cells, and which possibly contributed to the success of this medium in supporting suspension growth of MDCK cells. Several medium components are known to have this effect, including calcium which affects the attachment of MDCK cells to micro-carriers (48) and the size and density of cell aggregates (128). However it is difficult to speculate on the nature of the relevant component as the composition of this medium is undisclosed. SFM4BHK21 medium did, however, not support single cell growth in suspension of Vero cells.

Other methods to achieve stably growing suspension MDCK cells have been described. This included adaptation of MDCK cells to culture media containing metalloendopeptidase (165) or by growing cells in selection media in faster than normal rotating roller bottles, resulting in the MDCK 33016 cell line (56). Recently a suspension MDCK cell line was developed through transfection with *siat7e*, a human gene involved in cellular adhesion (21). These suspension MDCK cell lines did support replication of influenza virus. Comparison of virus yield is, however, difficult as different virus strains and assay methods were used. Interestingly,

when infected with influenza B virus, the *siat7e* transfected cells produced 23-fold higher hemagglutinin titers, but 3-fold lower infectious virus titers in comparison to the parental, adherent MDCK cells. However, here an effect of the adaptation to SF and suspension growth on virus yields obtained with MDCK-SFS cells was not observed. The maximum specific growth rate of the MDCK-SFS cells (0.029 h^{-1}) and the MDCK 33016 cells (56) are comparable to those found for adherent MDCK cells (108). The *siat7e* transfected cells, however, grow at an at least 2-fold lower rate.

Avian and human influenza virus strains differ in their binding preference to cell surface receptors. Therefore, a suitable cell line for AI virus production should express at least the avian strain preferred SA 2,3 receptors. MDCK (125) and Vero (29) cells express both receptors and virus binding to these cells is therefore not expected to limit replication. The adaptation of MDCK cells to SF and suspension growth did not affect the expression of both receptors. Similar to the findings described in this paper , others (55) observed low expression of SA 2,6 receptors on serum dependent BHK21 cells and related it to the restricted replication of human influenza virus on these cells and the selection of receptor binding variants of the viruses that bind better to the SA 2,3 receptors. Adaptation to SF media caused a shift in receptor expression towards higher SA 2,6 and lower SA 2,3 receptor levels. This shift could be caused by the direct response of the cell to the absence of serum or by selection of cells having altered receptor expression levels during the adaptation process. The lower SA 2,3 receptor expression levels could cause restricted propagation of AI virus strains in BHK21-SFS cells. However, the H5N7 and H7N1 strains replicate to high titers. Influenza virus receptor expression levels are therefore not determinants for infection of BHK21-SFS cells with these two avian virus strains.

Possibly the different virus titers obtained using BHK21 cells are caused by the presence of different polymerase genes since reassortment studies revealed that the ability of influenza virus to form plaques in BHK21 cell monolayers is dependent on the nature of one of the polymerase genes (6).

Unlike BHK21-SFS cells, MDCK-SFS and Vero-SF cells support the replication of most strains to high titers of about 8 $\log_{10}$ $TCID_{50}$ equivalent/ml. The yields of the eight viruses obtained using Vero-SF cells were on average 5-fold lower as compared to MDCK-SFS cells grown adherently. Such a difference in virus yield

between Vero and MDCK cells has been reported earlier (53, 88, 103). However, by adaptation of the virus to Vero cells the yield could be twofold increased after one passage (103) and 26-fold after 20 passages (53). Thus MDCK-SFS cells appear optimal for influenza virus production, since they propagate a wide variety of strains to high titers without the need for virus adaptation.

The highest titers reached with MDCK-SFS cells already reach the minimum level (> 8 $\log_{10}$ $TCID_{50}$/ml, > 8 $\log_2$ HAU/100 µl) that has been defined for commercial influenza production (49). Furthermore, for the purpose of evaluating the suitability of MDCK-SFS cells for industrial vaccine production it should be noted that many process parameters can still be improved. The cell density at the moment of infection was at least 3-fold lower than generally used (49). This was done here to allow comparison to adherently grown cells and prevent medium depletion. Using higher initial cell densities could possibly increase virus yields. Furthermore, the maximum MDCK-SFS cell density can probably be increased by medium optimization since the medium used was designed specifically for BHK21 cells. The viral yield could be further increased by optimizing process conditions such as m.o.i., trypsin concentration and harvest time, as was shown previously for other cells (91, 147). Alternatively viral yield could possibly be improved by allowing the cells to attach to micro-carriers immediately before virus infection, after their suspension culture during scale up, since it was observed that MDCK-SFS cells produce 4- to 7-fold more virus per cell when grown adherently than when grown in suspension. Finally, the performance of MDCK-SFS cell lines in bioreactor cultures should be studied to evaluate the suitability of these cells for industrial veterinary vaccine production.

Acknowledgements

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Chapter 3

Effect of natural and chimeric hemagglutinin genes on influenza A virus replication in baby hamster kidney cells.

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Submitted for publication

Abstract

Baby hamster kidney (BHK21) cells are used to produce vaccines against various viral veterinary diseases, including rabies and foot-and-mouth-disease. Previously we showed that particular influenza virus strains replicate efficiently in BHK21 cells whereas most strains, including model strain A/PR/8/34 [H1N1] (PR8), replicate poorly. This prohibits the general use of these cells for influenza vaccine production.

We now show that in contrast to PR8, the related strain A/WSN/33 [H1N1] (WSN) replicates efficiently in BHK21 cells. This difference is determined by the hemagglutinin (HA) protein since reciprocal reassortant viruses with swapped HAs behave similarly with respect to growth on BHK21 cells as the parental virus from which their HA gene is derived. The ability of several other influenza virus strains to grow on BHK21 cells appears to be similarly dependent on the nature of the HA gene, since reassortant PR8 viruses containing the HA of these strains often show a similar growth on BHK21 cells as the parental virus from which the HA gene was derived. High virus titers could be obtained with reassortant PR8 strains that contained a chimeric HA consisting of the HA1 domain of PR8 and the HA2 domain of WSN. HA1 contains most antigenic sites and is therefore important for vaccine efficacy. This method of producing the HA1 domain as fusion to a heterologous HA2 domain could possibly also be used for the production of HA1 domains of other viruses to enable the use of BHK21 cells as a generic platform for veterinary influenza vaccine production.

Traditionally, human and veterinary influenza vaccines are produced in embryonated chicken eggs, using reassortant seed virus containing the hemagglutinin (HA) and neuraminidase genes from the epidemic strain and six internal genes from the egg-adapted high-growth A/PR/8/34 [H1N1] (PR8) virus (84). Egg-based manufacturing processes are however logistically complex and vulnerable to changes in egg supply during major influenza outbreaks. Cell culture based production is more robust and flexible, and is therefore suggested as an alternative way to produce influenza vaccines. Indeed, MDCK, Vero and PER.C6® cell lines are nowadays also used for this purpose (49). Baby hamster kidney cells (BHK21) are commonly used for large scale production of virus for veterinary vaccines against foot-and-mouth-disease and rabies. They have favorable growth characteristics as they are easily cultured in suspension (106, 127, 184), have a high specific growth rate of 0.05 h^{-1} and can reach cell concentrations in batch culture as high as 8 x 10^6 cells/ml (105, 184). BHK21 cells appear less suitable for influenza virus propagation as they show an overall low virus yield compared to MDCK and Vero cells (106, 110). However, previously we showed that influenza virus replication efficiency in BHK21 cells is strain dependent (171). Strains that were able to replicate in this cell line did this very efficiently, resulting in virus titers that exceeded those found for MDCK and Vero cells infected with the same strains. Such selective replication in BHK21 cells has been observed previously with two influenza strains and was caused by one of the polymerase genes (6). Based on their favorable growth characteristics, we investigated whether BHK21 cells can potentially be used as a high-yielding, alternative platform to produce a wide variety of avian influenza virus strains for veterinary vaccine production.

Of the eight influenza virus strains used in our previous study (171), six strains replicated poorly in BHK21 cells (titer <3 log10 $TCID_{50}$) whereas two strains replicated efficiently (>7 log10 $TCID_{50}$; Table 1). Similar analysis of three further H1N1 strains revealed that strains A/WSN/1933 (WSN) and A/turkey/NL/543301/1999 replicated efficiently whereas strain A/duck/Alberta/35/1976 replicated poorly (Table 1). Replication in BHK21 cells is not determined by the HA subtype, as both efficient and poor replicating strains are found among the H1, H5 and H7 subtypes (Table 1). Interestingly, a distinct difference in replication efficiency was observed between the two related H1N1 strains PR8 and WSN. For both these strains plasmid-based reverse genetics systems exist that enable the production of reassortant viruses. Reassortant H1(WSN):PR8 virus, containing the HA gene of WSN in a PR8 backbone and H1(PR8):WSN, containing the HA of PR8 in a WSN backbone,

Table 1: Infection of BHK21 cells with wild-type influenza virus strains or PR8-based HA reassortant strains. BHK21-SFS cells (171) were grown in suspension in serum-free SFM4BHK21 culture media (Hyclone, Waltham, MA.) and infected (m.o.i. 0.01) in the presence of 2 µg/ml trypsin-TPCK (Sigma-Aldrich, St. Louis, MO). Infectious virus titres were determined 3 days post infection by TCID50, as previously described (171). Wild-type strains A/Duck/Alberta/35/1976 and A/turkey/NL/543301/1999 were a gift of Dr G. Koch (CVI). All other strains used in the present study were made using reverse genetics. A pHW2000-derived plasmid encoding the PR8 HA genomic RNA (pHW194) was earlier published (66). The HA genomic RNAs of seven further strains were isolated from wild-type virus and amplified by RT-PCR using primers Bm-HA-1 (or derivatives thereof) and Bm-NS-890R (68). When the sequence of the HA gene was available, derivatives of primer Bm-HA-1 were used that contain 6 additional bases at their 3' end to increase PCR specificity. PCR fragments were inserted into pHW2000 using BsmBI restriction endonuclease or BsaI when internal BsmBI sites are present in the HA gene segment (plasmids pROM37 and pROM43), resulting in pROM37-46. The HA genomic RNA of WSN which was available in a pPOL-SAPRIB-derived plasmid (40) was similarly inserted into pHW2000, resulting in pROM1. HA gene sequences were deposited under EMBL accession nrs HE802058 to HE802066. Reassortant viruses were made with PR8 (66) and WSN (40) reverse genetics plasmids as previously described (170). Virus identity was confirmed by sequence analysis.

Influenza virus strain [subtype]	Infectious wild-type virus titre[a] ($TCID_{50}$/ml)	HA reassortant virus in PR8 backbone	Infectious reassortant virus titre[a] ($TCID_{50}$/ml)	pHW2000 derived plasmid	HA gene segment EMBL database Acc. No.
A/PR/8/1934 [H1N1]	2.2[b] (0.6)	-		pHW194	**HE802058**
A/duck/Ukraine/1/1963 [H3N8]	2.8[b] (1.2)	H3(Duck/Ukr):PR8	8,3 (0.2)	pROM37	**HE802062**
A/chicken/Pennsylvania/21525/1983 [H5N2]	2.1[b] (0.6)	-[c]			
A/mallard/Denmark/64650/2003 [H5N7]	7.7[b] (1.1)	H5(Mall/DM):PR8	6,0 (0.2)	pROM40	**HE802063**
A/turkey/Wisconsin/1968 [H5N9]	1.8[b] (0.1)	H5(Ty/Wisc):PR8	2,1 (0.1)	pROM41	**HE802064**
A/chicken/Italy/1067/1999 [H7N1]	7.5[b] (0.6)	-[c]			
A/chicken/NL/06022003/2006 [H7N7]	2.2[b] (0.8)	H7(Ch/NL):PR8	2,4 (0.2)	pROM43	**HE802065**
A/duck/Germany/113/1995 [H9N2]	1.9[b] (0.1)	H9(Duck/Germ):PR8	2,9 (0.9)	pROM44	**HE802066**
A/WSN/1933 [H1N1]	8,6 (0.3)	H1(WSN):PR8	8,1 (0.1)	pROM1	**HE802059**
A/duck/Alberta/35/1976 [H1N1]	<2.1 (0)	H1(Duck/Alb):PR8	<2.1 (0)	pROM38	**HE802060**
A/turkey/NL/543301/1999 [H1N1]	7,9 (0)	H1(Ty/NL):PR8	5,8 (0.3)	pROM46	**HE802061**

[a] Geometric mean titres and 95% confidence interval of the mean (between brackets) of virus infections performed in triplicate are presented.
[b] Infectious virus titres taken from van Wielink et al. [171].
[c] A pHW2000-derived plasmid containing the HA of these strains could not be made, presumably due to plasmid instability in Escherichia coli.

were successfully rescued and could be amplified to high titers in embryonated chicken eggs (as was the case for all other reassortants generated in this study). In BHK21 cells, H1(WSN):PR8 virus replicated efficiently but the reciprocal virus H1(PR8):WSN replicated poorly (Fig. 1A). The inability of PR8 to replicate in BHK21 cells is therefore determined by HA and not by any of the other 7 gene segments.

To assess whether the replication of other strains shown in Table 1 is also determined by the nature of the HA gene segment, seven additional reassortant viruses with the HA of these wild-type strains in the PR8 backbone were made (Table 1). HA reassortants derived from wild-type viruses that replicated efficiently [H1(Ty/NL):PR8 and H5(Mall/DM):PR8] were also able to replicate, although the infectious virus titers were about 100-fold lower than the wild-type virus. Similarly, HA reassortant viruses derived from four wild-type strains that replicated poorly [H1(Duck/Alb):PR8, H5(Ty/Wisc):PR8, H7(Ch/NL):PR8 and H9(Duck/Germ):PR8] also replicated poorly in BHK21 cells. This suggests that the HA of

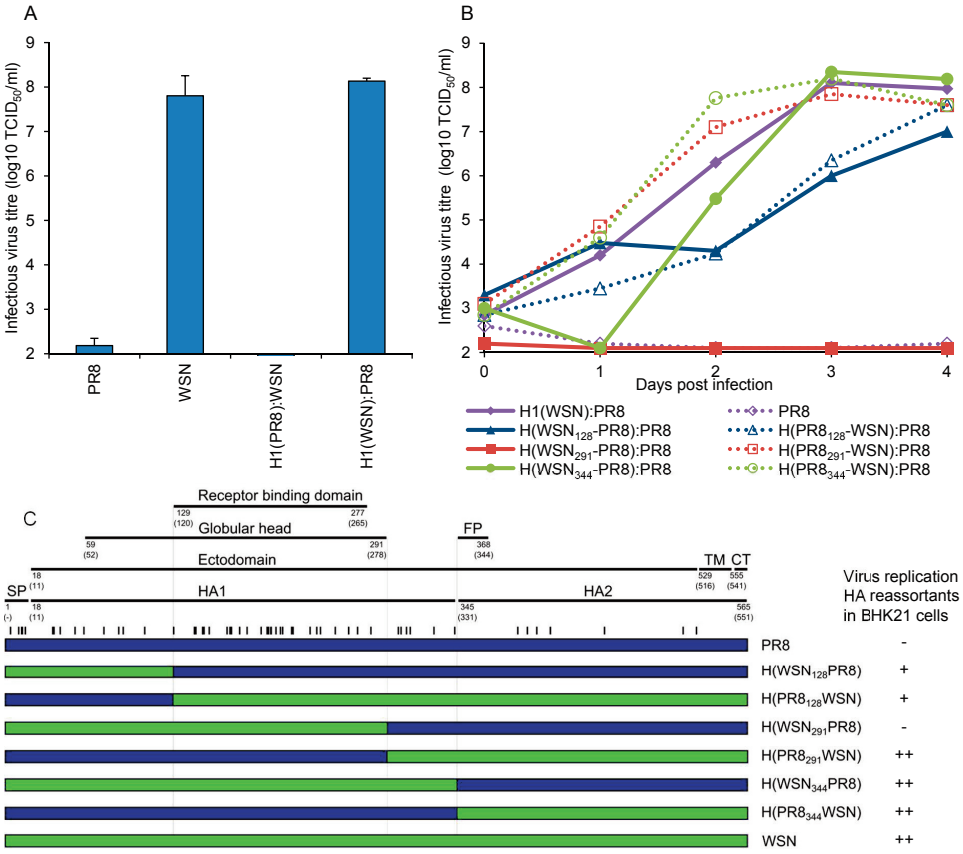

Fig. 1. Replication in BHK21 cells of PR8 and WSN based influenza strains and PR8 reassortants containing cHA. (A) BHK21 cells were infected with recombinant PR8, WSN, or the HA reassortants H(PR8):WSN or H(WSN):PR8 as described in Table 1. Infectious virus titres were determined 3 days post infection by TCID50.Geometric mean titres and 95% confidence interval of the mean of virus infections performed in triplicate are presented. (B) Infection of BHK21 cells with cHA reassortant virus strains. Cells were infected as described in Table 1. The infectious virus titre was determined daily by TCID50. (C) Schematic overview of WSN-PR8 cHA proteins used to make reassortant virus strains. The locations of the signal peptide (SP), HA1, HA2, ectodomain, transmembrane domain (TM), cytoplasmic tail (CT), globular head, fusion peptide (FP) and receptor binding domain are indicated. Amino acid positions are numbered taking the start codon of the signal peptide as 1 and ends at residue 565, with the H3 numbering between brackets (186).Vertical bars represent amino acid differences between PR8 and WSN HA. Replication efficiency of reassortant virus (panel A and B) is indicated with – (no replication), + (delayed replication) and ++ (high yield). Reassortant viruses were made as described in Table 1. PCR fragments encoding chimeras of WSN and PR8 HA genes were created by splice overlap extension PCR, joining the two fragments directly behind nucleotide 416 (amino acid 128), 905 (amino acid 291) or 1114 (amino acid 344). Nucleotide numbering refers to the position in the vRNA template (EMBL accession no. HE802058). Chimeric HA genes were inserted into pHW2000 as described in Table 1, resulting in pROM2-7.

these four strains also limits replication of the wild-type virus in BHK21 cells, although other gene segments may also negatively affect their replication. The importance of the HA gene in determining replication in BHK21 cells has also been illustrated by Govorkova et al, who isolated HA mutants upon serial passage of three influenza strains on these cells (55). Reassortant H3(Duck/Ukr):PR8 reacted differently from the other reassortants since it replicated efficiently whereas the corresponding wild-type virus replicated poorly. This suggests that in this case not the HA but one or several of the other 7 gene segments limits replication in BHK21 cells, possibly one of the polymerase genes (6).

HA is a homotrimeric surface glycoprotein with each monomer consisting of two disulphide-linked polypeptides, HA1 and HA2, that result from proteolytic cleavage of the precursor HA0 (15). HA recognizes sialic acid-linked receptors on the cell surface by the receptor binding domain (RBD), located on HA1 (Fig. 1C). The RBD is also the major antigenic component of the virus as a majority of the antibodies generated after infection with influenza virus is directed against this region. Therefore, HA1 is of primary interest for vaccine production. Structurally, the RBD is located on the globular head that comprises most of HA1 whereas HA2, together with the remainder of HA1, comprises the stalk region. After internalization of the virion in the endosome the fusion peptide, located in the HA2 region (Fig. 1C), undergoes a pH-induced conformational change, resulting in fusion of the viral envelope with the cellular endosomal membrane. The HA proteins of PR8 and WSN share 90% amino acid sequence identity (54 different residues, Fig. 1C). To elucidate which part of the HA protein determines virus replication in BHK21 cells, six plasmids encoding PR8-WSN chimeric HAs (cHAs; Fig. 1C) were generated. Exchanges were made at the start of the RBD (position 129), the end of the globular head (position 291), or the end of the HA1 domain (position 344) (86).

PR8 reassortant viruses encoding these six cHAs were again amplified to high titers in embryonated chicken eggs. Strains H($PR8_{291}$-WSN):PR8, H($PR8_{344}$-WSN):PR8 and H(WSN_{344}-PR8):PR8 replicated in BHK21 cells to high virus titers that were comparable to those obtained with H1(WSN):PR8 (Fig. 1B), although the rise in virus titer of H(WSN_{344}-PR8):PR8 was delayed. Infection with H($PR8_{128}$-WSN):PR8 and H(WSN_{128}-PR8):PR8 resulted in a slower increase in virus titer, but eventually they also reached high virus titers at 4 dpi. H(WSN_{291}-PR8):PR8, together with PR8, did not replicate in BHK21 cells. Based on these results it is not possible to identify a specific region of HA that determines the ability to replicate

in BHK21 cells, as the virus containing WSN HA1 and PR8 HA2 replicated equally well as the strain containing the reciprocal cHA. Also the two strains containing reciprocal cHAs exchanged at amino acid 128 replicated equally well. Nevertheless, these studies show that it is possible to grow a virus encoding the PR8 HA1 domain to high titers by fusion to the WSN HA2 domain.

Others have successfully created reassortants encoding cHAs by either replacing the ectodomain (60, 61), HA1 (178) or the globular head (58) (Fig. 1C) and showed that such viruses behave antigenically similar to the parental strains and are able to induce high levels of HA-specific antibodies. Furthermore, Hai et al. (2012) showed that exchange of the globular head is possible between strains from different subtypes and HA phylogenetic groups. These studies show that HA chimeras can also be produced between highly divergent HAs while retaining antigenicity. Thus, BHK21 cells could potentially provide a high-yielding production platform for PR8-based veterinary influenza vaccines by fusing the HA2 of WSN with the HA1 of strains that themselves do not replicate in BHK21 cells. Further research is required to evaluate this approach and to assess the antigenic and immunological qualities of vaccines resulting from it.

Acknowledgements

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Chapter 4

MDCK cell line with inducible allele B NS1 expression propagates delNS1 influenza virus to high titers.

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Abstract

Influenza A viruses lacking the gene encoding the non-structural NS1 protein (delNS1) have potential use as live attenuated vaccines. However, due to the lack of NS1, virus replication in cell culture is considerably reduced, prohibiting commercial vaccine production. We therefore established two stable MDCK cell lines that show inducible expression of the allele B NS1 protein. Upon induction, both cell lines expressed NS1 to about 1000-fold lower levels than influenza virus-infected cells. Nevertheless, expression of NS1 increased delNS1 virus titers to levels comparable to those obtained with an isogenic virus strain containing an intact NS1 gene. Recombinant NS1 expression increased the infectious virus titers 244 to 544-fold and inhibited virus induced apoptosis. However, NS1 expression resulted in only slight, statistically not significant, reduced levels of interferon-β production. Thus, the low amount of recombinant NS1 is sufficient to restore delNS1 virus replication in MDCK cells, but it remains unclear whether this occurs in an interferon dependent manner. In contrast to previous findings, recombinant NS1 expression did not induce apoptosis, nor did it affect cell growth. These cell lines thus show potential to improve the yield of delNS1 virus for vaccine production.

INTRODUCTION

Influenza A virus is the causative agent of a highly contagious disease that affects both humans and animals. Outbreaks of highly-pathogenic avian influenza (AI) strains among poultry have resulted in transmissions to humans, often with fatal outcome. Vaccination of poultry is an important tool to control such outbreaks. Traditional vaccines consist of inactivated viral particles which closely resemble the circulating strain. Currently vaccine strains are being developed with improved characteristics, which include live attenuation, and the ability to serologically differentiate between naturally infected and vaccinated-only animals (DIVA). Influenza strains lacking the gene encoding the non-structural NS1 protein (delNS1) are able to replicate in a host, albeit with reduced efficiency, indicating that NS1 is dispensable for virus replication. DelNS1 viruses are therefore considered as live-attenuated vaccines (132) for both animals (133, 177) and humans (189). Furthermore, delNS1 virus could be used as DIVA vaccine in poultry, as the presence of antibodies against NS1 in infected animals, but not in vaccinated animals, allows for better surveillance of AI outbreaks and thus may improve worldwide acceptance of vaccination (17).

NS1 facilitates the infection of a cell in many different ways and the reduced replication efficiency of delNS1 virus is a direct effect of the absence of these functions. In cells infected with intact influenza virus, NS1 is expressed at very high levels shortly after infection (59, 148, 172) and it inhibits the cellular antiviral response by reducing the type I interferon's (IFN) expression (such as IFN-α or IFN-β) at various levels. NS1 binds to retinoic-acid inducible gene I (RIG-I) that recognizes both cytoplasmic dsRNA and 5'-triphosphate-containing RNA, products that are generated during influenza infection (107). NS1 also binds directly to dsRNA (132). By binding to these molecules, NS1 inhibits the activation of several pathways that lead to IFN induction. Furthermore, NS1 also limits IFN expression by inhibiting cellular pre-mRNA processing (including IFN pre-mRNA) and mRNA nuclear export (59). The efficiency and mechanism by which different influenza strains block the IFN response is strain dependent (63, 76). NS1 can also block the function of two antiviral proteins, dsRNA-dependent protein kinase R (PKR) and 2'-5'-oligoadenylatesynthetase (OAS) (132), and regulate both viral genome replication and translation (180) and protein synthesis (59).

The NS1 gene is localized on the eighth vRNA segment and splicing of the primary NS1 mRNA results in the generation of a mRNA that encodes the nuclear export protein (NEP) (59), a protein that plays a role in the nucleocytoplasmic export of viral RNP's and regulation of viral genome transcription and replication (135). During development of delNS1 strains it is therefore important to retain the essential NEP protein. The NS1 protein is divided into two distinct functional domains: an N-terminal RNA-binding domain (residues 1–73) and a C-terminal effector domain (residues 74–230), which predominantly mediates interactions with host-cell proteins (59). Phylogenetic analysis of NS1 amino acid sequences indicated that NS1 proteins can be divided into two groups, termed allele A and B (94, 111). The homology within each allele is 93-100%, whereas between the two alleles it can be as little as 62%. Allele B NS1 is found, with a few exceptions, exclusively in avian influenza virus strains, whereas allele A NS1 is found in all human, swine and equine strains and some avian strains.

Infection of cells with delNS1 virus induces higher levels of IFN than infection with a wild-type strain and results in a lower virus yield (45, 77). In cells and animals with a low or absent IFN response, such as Vero cells, STAT1 or PKR knockout mice, and embryonated eggs younger than 8 days, the virus replicates to high titers (30, 45, 77, 161), which indicates that inhibition of the IFN response is the main function of NS1 (132). Influenza strains with C-terminally truncated NS1 genes show intermediate effects on IFN inhibition and virus replication (77), which correlates with the presence of the dsRNA binding protein domain.

In large-scale influenza vaccine production, a serum-free and suspension cell culture based process is preferred above the traditional production in embryonated hen's eggs (171). However, the yield of delNS1 virus, as reported by others (45) is below the minimal yield that is required for commercial vaccine production (49), even with IFN deficient Vero cells. Higher virus titers can be obtained by using a high multiplicity of infection (m.o.i.) (154), but such large amounts of seeding virus are not feasible in large scale production. Another approach to increase virus yield is transient recombinant expression of NS1 in the production cell line before infection (148). However, host-cell encoded NS1 expression induces apoptosis (25, 81, 145, 195). This makes development of stable NS1 expressing cell lines difficult, whereas transient expression is not feasible in large scale production.

The aim of this paper is to develop a stable NS1 expressing Madin-Darby canine kidney (MDCK) cell line for high-yielding delNS1 virus production. To overcome apoptosis induction, an inducible NS1 expression system is used. A commonly used inducible expression system is based on the prokaryotic Tet repressor protein (TetR) which allows expression of a gene of interest (GOI) under control of a tetracycline-response element (TRE) in a cell line that is stably transfected with a transactivator protein (52). Two variants of this transactivator exist that respond in an opposite manner to the doxycycline (Dox) antibiotic used for GOI induction. Using the Tet-off system Dox addition represses GOI expression, whereas in the Tet-on Advanced system Dox addition induces GOI expression. An adherently growing, serum dependent MDCK Tet-off cell line is commercially available. For construction of cell lines stably expressing NS1 with the Tet-on Advanced system, which allows more tight regulation of gene expression, the previously generated MDCK-SFS cell line growing serum-free and in suspension (171) was used. We obtained two MDCK Tet-on Advanced cell lines that showed inducible production of allele B NS1 protein. We subsequently determined whether upon NS1 induction these two cell lines supported replication to high titers of an influenza virus that either contained or lacked an intact NS1 gene. Furthermore, we determined the effect of NS1 induction on IFN-β production and the apoptotic response.

Material and methods

Cell lines and virus strains

MDCK-SFS and Vero-SF cells (171) were grown adherently in serum-free Ultra-MDCK culture media (Lonza Biowhittaker, Basel, Switzerland) and Optipro (Invitrogen, Carlsbad, CA, USA) respectively. MDCK Tet-off cells, obtained from Clontech (Mountain View, CA, USA), were grown in DMEM with 5% Tet-system approved fetal bovine serum (FBS). All culture media were supplemented with 4 mM glutamine and 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). Human embryo kidney (293T) and MDCK cells used for generation of reassortant virus were cultured in Glutamax medium (Invitrogen) supplemented with 10% FBS. Cells were grown at 37˚C and 5% CO_2. Cell density and viability were determined with a Countess automated cell counter (Invitrogen).

The influenza virus strains A/Puerto Rico/8/34 H1N1 (PR8; ATCC VR-95, Manassas VA, USA), A/turkey/Wisconsin/68 H5N9 (kindly provided by Dr. G. Koch, CVI), A/turkey/Turkey/1/05 H5N1 (Veterinary Laboratories Agency, Pirbright, UK) were propagated in embryonated chicken eggs.

Generation of recombinant influenza viruses

Viral RNA of influenza virus strain A/turkey/Turkey/1/05 H5N1 was isolated from allantoic fluid of infected eggs using a high pure viral RNA isolation kit (Roche Applied Science, Penzberg, Germany). Universal influenza genome primer uni12 (66) was used for reverse transcriptase (RT) reactions with the Superscript first strand synthesis system (Invitrogen). HA segment forward (5'-TATT GCTCTTCA GCCAGCAAAAGCAGGGGTWYAATCTGTC-'3) and reverse (5'-ATATGCTCTT CGATTAGTAGAAACAAGGGTGTTTTTAAYTAC-'3) primers and NA segment forward (5'-TATTGCTCTTCAGCCAGCAAAAGCAGGAGTTCAAAATGAA TCC-'3) and reverse (5'-ATATGCTCTTCGATTAGTAGAAACAAGGAGTTTT TTGAACAAAC-'3) primers with Sap I restriction sites were used for PCR reactions with the Expand high fidelity PCR system (Roche). The resulting cDNAs were inserted in vector pPOLISAP1RIB (40). The sequence of each insert was verified (NA, Genbank Acc. nr. EF619973; HA, Genbank Acc. nr. EF619980) by sequence analysis using the BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems**,** Carlsbad, CA, USA).

The sequence of the HA gene encoding the multi basic cleavage site of A/turkey/Turkey/1/05 H5N1 was replaced by the corresponding sequence encoding the consensus cleavage site of a low pathogenic HA gene of subtype H6 by means of PCR mutagenesis as described previously (126). Using this method, the H5 gene sequence CCTCAAGGAGAGAGAAGAAGAAAAAAGAGAGGACTA TTT encoding the multi basic cleavage site PQGERRRRKKRGLF was converted into the sequence CCAGAGATTGAAACTAGAGGACTTTTT encoding the low pathogenic H6 subtype cleavage site PQIETRGLF. The resulting gene is referred to as H5(6).

In order to generate an NS segment that only encodes the NEP protein but not the NS1 protein we used a synthetic gene segment (GenScript Corporation, Piscataway, NJ, USA) corresponding to the NS gene segment of influenza virus strain PR8 (EMBL Acc. nr. AF389122) in which the nucleotide sequence spanning the exact intron sequence (nt 57-528) was deleted. The NS1-deletion gene segment was

inserted in pHW2000 using BsmBI restriction sites as described earlier (66). The bi-directional 8-plasmid reverse genetics system of strain PR8 was a gift of Dr. Hoffman and Dr. Webster (67). To rescue recombinant virus, a mixture of 15 x 10^5 293T and 5 x 10^5 MDCK cells was transfected with equal amounts of the eight plasmids containing the different gene segments, using Lipofectamine 2000 (Invitrogen). At 12 h post transfection (hpt) the transfection mixture was replaced by Glutamax medium supplemented with 0.3 % bovine serum albumin (Chemie Brunschwig AG, Basel, Switzerland) and 250 ng/ml TPCK-trypsin (Sigma-Aldrich, St. Louis, MO, USA). Virus was harvested at 48 hpt and 96 hpt. Virus stocks were generated in 7-9 day old embryonated eggs and virus identity was confirmed by sequence analysis. Using this system we were able to rescue the PR8::H5(6)N1 and PR8::H5(6)N1delNS1 viruses. These strains are referred to as H5(6)N1 and delNS1, respectively.

Generation of stable MDCK cells with inducible NS1 expression

The pTet-on Advanced expression system for inducible gene expression was obtained from Clontech. It includes the improved pTET-on Advanced and pTRE-tight vectors, that make the inducible system more sensitive to Dox while reducing leaky gene expression (167). Synthetic DNA fragments (GenScript Corporation) encoding the allele A NS1 from A/Puerto Rico/8/34 H1N1 (Genbank Acc. no. V01104) and allele B NS1 from A/turkey/Wisconsin/68 H5N9 (Genbank Acc. no. U85378) were inserted in the pTRE-tight vector after mutating the 3'-splice site for generation of NEP mRNA, as described previously (7). Furthermore, a synthetic DNA fragment encoding the N-terminal 99 amino acids of allele A NS1 was inserted into pTRE-tight. The resulting plasmids are pTRE-tight-NS1allA, pTRE-tight-NS1allB and pTRE-tight-NS1allA$_{1-99}$.

After transfection of MDCK-SFS cells with the pTet-On Advanced vector using Fugene HD (Roche) stably transformed cells were selected with 400 µg/ml G418 (Promega, Fitchburg, WI, USA). Functional expression of the rtTA-Advanced transactivator protein was assessed by transient cotransfection of the selected cell lines with pTRE-tight-LUC, that encodes firefly luciferase, and a vector that constitutively expresses Renilla luciferase as a transfection control, followed by induction with different concentration of Dox (Clontech). Two days later both the Renilla and firefly luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega). One MDCK Tet-on Advanced cell line that showed high and inducible firefly luciferase activity was selected for further work.

Both the MDCK Tet-on Advanced and the MDCK Tet-off cell line (Clontech) were cotransfected with either one of the three pTRE-tight derived plasmids and a linear hygromycin marker in a ratio of 20:1. Stably transformed cells were selected with 200 μg/ml hygromycin B (Clontech) and either 300 μg/ml G418 (Tet-on Advanced cells) or 1 μg/ml puromycin (Clontech; Tet-off cells). Cell propagation was performed in the presence of 200 μg/ml G418, 1 μg/ml puromycin and/or 100 μg/ml hygromycin. None of these antibiotics were used during virus infections and other assays.

NS1 mRNA quantification by qRT-PCR

MDCK cells (4×10^5) were incubated in 0, 0.1 and 1 μg/ml Dox for 24, 48 and 72 h in 6-well plates (Greiner Bio-One), after which RNA was isolated with the RNeasy plus kit (Qiagen, Hilden, Germany). The NS1 mRNA concentration was then determined by real-time RT-PCR using the QuantiTect SYBR Green RT-PCR kit (Roche). A calibration curve was used for both allele A NS1 and allele B NS1 genotypes, made by a serial dilution of RNA isolated from cells 24 h post infection (hpi) with H1N1 or H5N9 influenza virus (m.o.i. 5). NS1 mRNA levels were corrected for the amount of actin mRNA. Allele A NS1 forward (5′-CAAAACATGGATCCAAACACTG-3′) and reverse (5′-GAATCCGCTCCACTATCTGCT-3′) primers, allele B NS1 forward (5′-ACAGGGGGTTTGATGGTGA-3′) and reverse (5′-CTTTGGAGGGAGTGGAG GTC-3′) primers, and canine actin forward (5′-GGCATCCTGACCCTGAAGTA-3′) reverse (5′-GGGGTGTTGAAAGTCTCGA A-3′) primers were used.

SDS-PAGE and Western blot analysis

MDCK NS1allAoff, NS1all$A_{1\text{-}99}$on, NS1allBon1, NS1allBon2 and Tet-on Advanced cells were incubated for 24 h with or without 1 μg/ml Dox and then subjected to reducing SDS-PAGE, using NuPAGE® Novex® 12% Bis-Tris precast gels (Invitrogen). MDCK-SFS cells taken 24 h after infection with either H1N1 or H5N9 virus (m.o.i. 5) were used as a source of authentic NS1. As a positive control for the truncated allele A NS1, MDCK Tet-on Advanced cells were incubated for 24 h with 1 μg/ml Dox, transiently transfected with pTRE-tight-NS1all$A_{1\text{-}99}$, and subsequently incubated for another 24 h. Polypeptides were transferred to polyvinylidenedifluoride membranes. NS1 was detected by immunoblotting using a monoclonal mouse antibody against a peptide near the N-terminus of allele A NS1 (SC130568, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a custom-prepared rabbit antiserum (anti-NS1all$B_{217\text{-}230}$) directed against a keyhole limpet hemo-

cyanin conjugated peptide (CKQKRYMARRVESEV; N-terminal acetylation) encompassing the C-terminal 14 amino acids of allele B NS1 of H5N9 virus with an additional N-terminal cysteine for conjugation purposes. The peptide-specific antibodies from the anti-NS1allB$_{217-230}$ antiserum were affinity purified using this same (unconjugated) peptide (GenScript Corporation). After subsequent incubation with, respectively, peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulins (Dako, Glostrup, Denmark), protein levels were visualized with ECL plus (GE Healthcare, Buckinghamshire, UK) and quantified with a Storm840 imaging system (Molecular Dynamics, GE Healthcare).

Virus infection

For analysis of delNS1 virus replication by all four NS1 expressing cell lines, the two control cell lines and the Vero cells, cells were incubated in 96-well plates (Greiner Bio-One) (3 x 10^4 cells/well), for 24 h with or without 1 μg/ml Dox. The following day cells were infected in triplicate with delNS1 virus (m.o.i. 0.001) and after one h, they were washed twice and provided with 120 μl medium with 2 μg/ml trypsin-TPCK, without Dox or selection antibiotics and without FBS for MDCK Tet-off cell lines. Three day post infection (dpi), virus titers were determined by M-gene specific qRT-PCR.

To study the infection kinetics of delNS1 and H5(6)N1 in NS1allBon1 and NS1allBon2, 1 x 10^6 cells per well were incubated in 6-well plates (Greiner Bio-One), with or without 1 μg/ml Dox. After 24 h incubation, one well per cell line was used to determine the cell density. Next, cells were infected in triplicate with either delNS1 or H5(6)N1 virus at an m.o.i. of 0.01. One hour later cells were washed with PBS and supplied with 5 ml UltraMDCK medium containing 2 μg/ml trypsin-TPCK with or without Dox. Supernatant samples were taken 1, 24, 48 and 72 h hpi and stored at -80°C before determining the virus yield.

The infectious influenza virus titer was measured by determining the tissue culture infective dose required to infect 50% ($TCID_{50}$) of MDCK cells. The influenza virus genome equivalent concentration was derived from the amount of viral RNA copies determined by M-gene specific qRT-PCR. These two assays were previously described (171).

Apoptosis measurement

MDCK NS1allBon1, NS1allBon2 and Tet-on Advanced control cells were cultured for 24 h in the presence (1 μg/ml) or absence of Dox, trypsinized and then allowed to adhere to the surface of a 96-well plate (Greiner Bio-One) (1 x 10^4 cells/well) for 1.5 h. To determine inhibition of apoptosis induction by virus infection, cells were infected with 5 m.o.i. of either delNS1, H5(6)N1 or mock-infected. Dox was supplied to cells which were previously cultured with Dox. To determine apoptosis induction by host-cell encoded NS1 expression, culture media with or without 1 μg/ml Dox was added to cells that were previously cultured without Dox. 24 h after infection or NS1 induction caspase-3 and caspase-7 activities were determined using the Caspase-Glo 3/7 Assay kit (Promega). Briefly, supernatant was removed and 25 μl Caspase-Glo reagent and 25 μl PBS was added to each well. After 1 h incubation at room temperature the luminescence of each sample was measured using a GloMax-Multi luminometer (Promega). Results are given as the means of triplicate experiments and represent the increase in caspase activity as compared to control cells (mock infected and non-induced).

IFN-β quantification by mRNA RT-PCR

MDCK NS1allBon1, NS1allBon2 and Tet-on Advanced control cells were incubated for 24 h in 6-well plates (Greiner Bio-One) (6 x 10^5 cells/well), with or without 1 μg/ml Dox. Cells were then infected with delNS1 virus (m.o.i. 3) or mock-infected. After 18 h, RNA was isolated using the RNeasy plus kit (Qiagen). The concentration of IFN-β mRNA was determined by real-time RT-PCR (Onestep RT-PCR kit, Qiagen), using canine IFN-β forward (5'-CCAGTTCCAGAAGGAGGACA-3') and reverse (5'-CCTGTTGTCCCAGGTGAAGT-3') primers and Taqman probe (5'-6FAM-CCTG GAGGACGTCAAAGAGAAGGA-XT-PH). IFN-β mRNA levels were corrected for the amount of actin mRNA. The concentration of actin mRNA was determined by real-time RT-PCR using QuantiTect SYBR Green RT-PCR kit (Roche), using canine actin primers (Section 2.4). A calibration curve was used for both IFN and actin mRNA by a ten-fold serial dilution (10^0 to 10^{-7}) of RNA isolated from delNS1-infected MDCK-SFS cells.

IFN-β quantification bioassay

MDCK NS1allBon1, NS1allBon2 and Tet-on Advanced control cells were incubated for 24 h in 6-well plates (Greiner Bio-One) (6 x 10^5/well), with (1 μg/ml) or without Dox. Cells were then infected with delNS1 virus (m.o.i. 3) or mock-infected. After

25 h, supernatant was harvested and ultra-filtrated (Vivaspin 500, Sartorius, Gottingen, Germany) to remove virus. The absence of virus in the filtrate was confirmed by incubation of adherent MDCK-SFS cells with the filtrate in an immunoperoxidase monolayer assay, as previously described (171). To determine the levels of secreted IFN, MDCK-SFS cells were seeded in 96-well plates (Greiner Bio-One) (5×10^4/well) and incubated with a serial two-fold dilution series of virus-free supernatant for 18 h. Cells were then infected (m.o.i. 3) with Newcastle disease virus (NDV) strain LaSota that was generated by reverse genetics from a Full-Length NDV cDNA copy (NDFL) that expresses a EGFP reporter gene (NDFL-tagEGFP) (4) and incubated for a further 48 h. After removal of supernatant, the fluorescence of each well was measured using a GloMax-Multi fluorimeter (Promega). The amounts of IFN present in the supernatants were estimated based on their ability to induce an antiviral state in MDCK-SFS cells and inhibit NDFL-tag EGFP replication. The supernatant dilution that resulted in the median EGFP signal was determined for each sample. The reciprocal dilution was used as a relative concentration of IFN present in the supernatant.

IFN-β dependent luciferase reporter system

To prepare conditioned medium (CM), MDCK-SFS cells were infected with delNS1 virus (m.o.i. 0.6) without the addition of trypsin. After 15 h, supernatant was ultra-filtrated (Amicon Ultra-15 100k, Millipore, Billerica, MA, USA) to remove virus. This virus-free supernatant was further used to stimulate IFN production in MDCK cells.

MDCK NS1allBon1, NS1allBon2 and Tet-on Advanced control cells were incubated for 24 h in 96-well plates (Greiner Bio-One) (1.5×10^4/well), with or without 1 μg/ml Dox. Next, cells were washed and transiently cotransfected with the reporter plasmid carrying the firefly luciferase gene under the control of the IFN-β promoter (p125Luc, kindly provided by Takashi Fujita, Kyoto University, Japan (193)) and the Renilla luciferase control plasmid pGL4.73 (Promega), using Fugene HD (Roche). Cells were then incubated for 7 h, followed by IFN stimulation by addition of either 10 μg/ml poly-IC or 1% CM, or mock-stimulated. The firefly luciferase activity was measured after 40 h incubation using the Dual Luciferase Reporter Assay System (Promega) and normalized to Renilla luciferase activity. All transfection experiments were conducted in duplicate.

Statistical analysis

Data were analyzed for statistical significance by the Students t-test, using the Minitab software package (Minitab Inc., Coventry, UK). Differences in mean were assumed significant if $p<0.05$.

Results

Generation of MDCK cells with stable NS1 expression

Transfection of MDCK-SFS cells with the pTet-on Advanced plasmid resulted in 98 stable cell lines. These were screened for transactivator activity by transient transfection with a luciferase reporter plasmid. A cell line that showed strong luciferase induction (177-fold) upon Dox addition was selected. Subsequent transfection of this cell line and a commercially available MDCK Tet-off cell line with either one of the pTRE-tight NS1allA, pTRE-tight NS1allA$_{1\text{-}99}$ or pTRE-tight NS1allB plasmids yielded 30 stably transformed cell lines. Fifteen of these cell lines originated from transfection with pTRE-tight NS1allB and ten cell lines from transfection with pTRE-tight NS1allA$_{1\text{-}99}$. Only five (all Tet-off) cell lines were obtained from transfection with pTRE-tight NS1allA. These 30 cell lines were then screened for NS1 mRNA expression and increased production of delNS1 virus upon induction of NS1 expression. Four cell lines, NS1allAoff, NS1allA$_{1\text{-}99}$on, NS1allBon1 and NS1allBon2, that unequivocally showed NS1 mRNA expression and appeared to support growth of delNS1 virus to higher titers upon NS1 induction (results not shown) were selected for further studies.

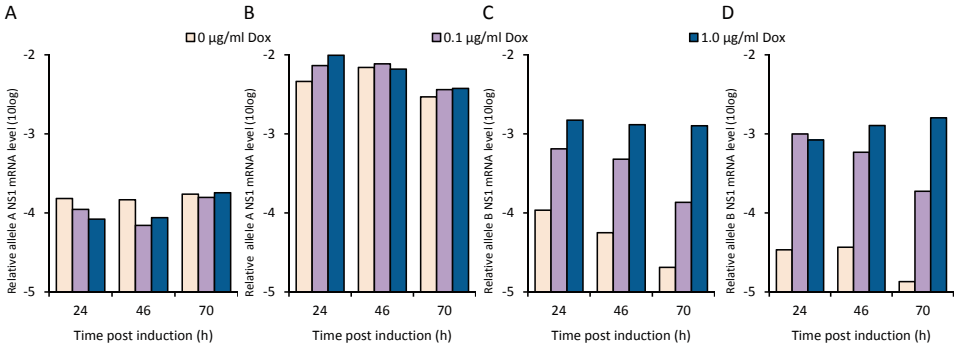

Fig. 1. Inducible expression of NS1 genes in stable MDCK cell lines. The NS1 mRNA levels in MDCK NS1allAoff (A), NS1allA1-99on (B), NS1allBon1 (C) and NS1allBon2 (D) cell lines was determined by qRT-PCR 24, 46 and 70 h after induction with 0, 0.1 or 1.0 µg/ml Dox. The relative NS1 mRNA level as compared to cells infected with H1N1 virus (panels A and B) or H5N9 virus (panels C and D) are presented.

NS1 mRNA and protein expression

The optimal Dox concentration and induction period leading to high NS1 mRNA levels was next determined. Figure 1 gives the ratio of NS1 mRNA levels relative to the NS1 RNA (the sum of mRNA, vRNA and cRNA) levels in cells infected with the corresponding wild type virus. Note that we used the total amount of NS1 RNA present in infected cells as a reference, which comprises viral mRNA, vRNA and cRNA. However, in transfected cells only NS1 mRNA is present. The ratio between NS1 mRNA and total NS1 RNA levels in infected cells is about 0.2 at 24 hpi (172). Thus, the relative NS1 mRNA levels in our stable MDCK cell lines compared to NS1 mRNA levels after virus infection are probably 5-fold higher than reported in Fig. 1.

Each of the four cell lines showed NS1 mRNA expression after 24 h induction, which did not further increase upon prolonged induction (Fig. 1A-D). The NS1allAoff cell line showed an about 10,000-fold lower NS1 mRNA level than cells infected with H1N1 virus in the non-induced state (with Dox). Furthermore the NS1 mRNA level did not change upon induction by Dox omission (Fig. 1A). Similarly, the NS1allA$_{1-99}$on cell line showed no change in NS1 mRNA level upon induction (with Dox) and an about 100-fold lower NS1 mRNA level than H1N1 virus infected cells. (Fig. 1B). However, both NS1allBon1 and NS1allBon2 cell lines showed inducible expression of NS1 mRNA that, in the induced (with Dox) state, was about 1000-fold lower than in H5N9 virus infected cells (Fig. 1C and D). For further studies, NS1 expression was induced by adding 1 μg/ml Dox to Tet-on cell lines or by Dox omission from Tet-off cell lines, both for a period of 24 h.

The NS1 protein expression was analyzed by Western blotting using allele-specific antibodies for detection of allele A NS1 (Fig. 2A) and allele B NS1 (Fig. 2B). Both Western blots show many faint bands representing aspecific binding to proteins of MDCK cells that do not produce NS1 (Fig. 2A, lane 11; Fig. 2B, lanes 11-12). However, a band representing allele A NS1 (Fig. 2A, lane 1-5) and allele B NS1 protein (Fig. 2B, lanes 1-6) is readily observed in samples from MDCK cells 24 hpi with H1N1 or H5N9 virus. Furthermore, a band representing the truncated allele A NS1 is visible near the 6 kDa marker(Fig 2A, lane 6), following transient transfection of Dox induced MDCK Tet-on Advanced cells with pTRE-tight-NS1allA$_{1-99}$.

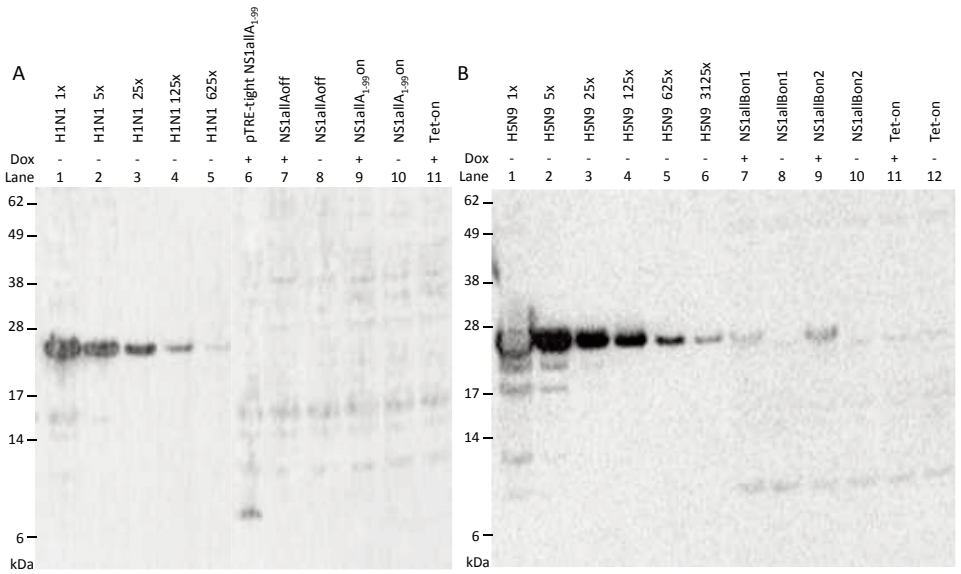

Fig. 2. Western blot analysis of NS1 protein expression. MDCK NS1allAoff, NS1allA1-99on, NS1allBon1, NS1allBon2 and Tet-on Advanced cells were incubated for 24 h in the presence of 1.0 µg/ml Dox or without Dox. Panels A and B represent Western blot analysis of allele A NS1 and allele B NS1, respectively. Cell extracts of MDCK-SFS cells infected with H1N1 (allele A NS1) or H5N9 (allele B NS1) influenza virus at 24 hpi were used as source of authentic NS1. A serial 5-fold dilution series of cell samples infected with H1N1 (panel A, lanes 1-5) or H5N9 (panel B, lanes 1-6) virus was included to allow densitometric NS1 quantification. As a positive control for the truncated allele A NS1, Dox induced MDCK Tet-on Advanced cells were transiently transfected with pTRE-tight-NS1allA1-99 (panel A, lane 6). Non-infected MDCK Tet-on Advanced cells were used as negative control (panel A lane 11 and panel B lane 11-12). Relevant molecular weight markers (kDa) are indicated on the right of each panel.

NS1 bands could not be detected in NS1allAoff and NS1allA$_{1-99}$on cell extracts (Fig. 2A, lanes 7-10). Both NS1Bon1 and NS1Bon2 cell lines show a strong band at the expected position after induction of NS1 expression (Fig. 2B, lanes 9 and 11), which is predominantly absent without induction (Fig. 2B, lanes 8 and 10). The faint band at this position in non-induced cells is not assumed to represent NS1 since it is also visible in cells that do not express NS1 and were incubated with and without Dox (Fig. 2B, lanes 11 and 12). A serial dilution of MDCK cells 24 hpi with H5N9 virus (Fig. 2B, lanes 1-6) was used to quantify the NS1 protein content of the induced NS1allBon cell lines. Densitometric analysis revealed that the NS1allBon1 and on2 cell lines produced, respectively, 1200- and 500 -fold lower NS1 protein levels than H5N9 infected cells.

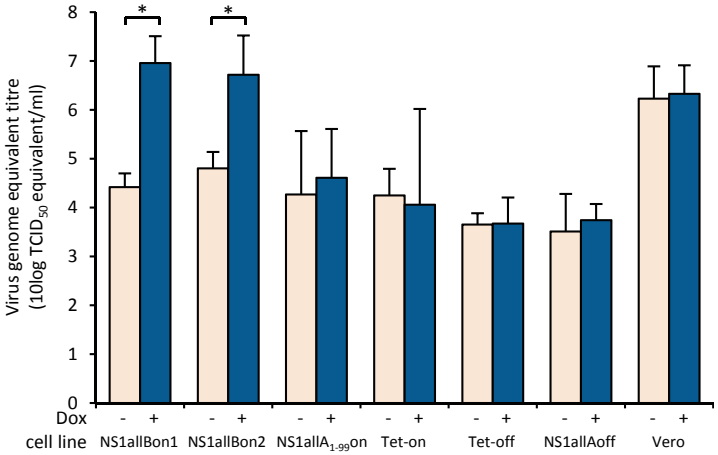

Fig. 3. Effect of NS1 gene induction of four stable MDCK cell lines on production of delNS1 virus. Cells were induced with 1.0 µg/ml Dox (+) or not induced (-) and after 24 h infected with 0.001 m.o.i. delNS1 virus. Three dpi the virus genome equivalent concentration was measured by qRT-PCR. The two MDCK cell lines expressing only the transactivator and Vero cells were included as controls. Geometric mean titres and 95% confidence interval of virus infections performed in triplicate (Vero cells in duplicate) are presented. Differences were significant (*) when $p < 0.05$.

Effect of NS1 on virus replication

The delNS1 total viral particle yield, as determined by the virus genome equivalent titer, after infection of induced and non-induced cells was compared (Fig. 3). NS1 expression increased delNS1 virus yield 346-fold in NS1allBon1 cells (p=0.015) and 82-fold in NS1allBon2 cells (p=0.050). The virus yield obtained with both induced

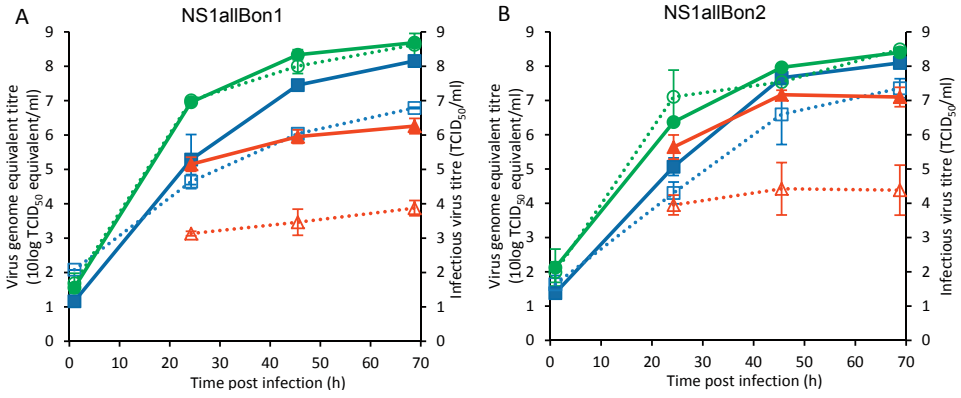

Fig. 4. Replication of delNS1 and H5(6)N1 virus in NS1allBon1 (panel A) and NS1allBon2 (panel B) cell lines. Cells were either induced with 1 µg/ml Dox (closed symbols, full line) for 24 h or not-induced (open symbols, dotted line), followed by infection with 0.01 m.o.i. delNS1 or H5(6)N1 virus. The virus genome equivalent concentration of delNS1 (squares) and H5(6)N1 (circles) virus was determined by qRT-PCR. The titre of infectious delNS1 virus was determined by TCID50 (triangles). Geometric mean titres and 95% confidence interval of the mean are presented of virus infections performed in triplicate.

NS1allBon cell lines was comparable to the virus yield of Vero cells whereas non-induced NS1allBon cells yielded virus levels comparable to the Tet-on control cells, which lack a NS1 gene. However, the virus yield of NS1allA$_{1-99}$on cells and NS1allAoff cells did not differ upon Dox treatment and was comparable to the virus yield of the corresponding Tet-on and Tet-off control cells. Infection at a higher m.o.i. (0.01) resulted in higher maximum titers for all cell lines and a smaller difference between the delNS1 virus yield obtained with induced and non-induced NS1allBon cell lines (data not shown). Thus, only the two NS1allBon cell lines, in which we could demonstrate inducible NS1 expression, showed higher yields of delNS1 virus upon NS1 expression. Therefore only these cell lines were used in further studies.

We next determined the kinetics of delNS1 and H5(6)N1 total viral particle production by induced and non-induced NS1allBon cells (Fig. 4). The H5(6)N1 virus contains an intact NS1 gene but is further isogenic to delNS1 virus. Induction of NS1 expression increased delNS1 total viral particle yield by NS1allBon1 cells at 70 hpi 23-fold ($p=0.001$; Fig. 4A) and by NS1allBon2 cells 5-fold ($p=0.035$; Fig. 4B). The titers at 70 hpi (about 10^8 TCID$_{50}$ equivalent/ml) are comparable to those found after infection with H5(6)N1 virus (Fig. 4A and B), although the H5(6)N1 virus appeared to replicate somewhat faster. Induction of allele B NS1 expression by both cell lines did not increase the replication speed nor the yield of H5(6)N1 virus.

Strikingly, the induction of NS1 expression by NS1allBon1 and NS1allBon2 cells increased the infectious delNS1 virus titer at 70 hpi 244- and 544-fold, respectively (Fig. 4A and B), which is substantially more than the, respectively, 23- and 5-fold increase in total viral particle yield.

Effect of NS1 on IFN-β expression

We next determined the effect of NS1 expression by NS1allBon cells on the induction of IFN-β expression by delNS1 virus infection using three different methods: quantification of IFN-β mRNA levels (Fig. 5A), quantification of secreted IFN using a bioassay (Fig. 5B) and the expression of a luciferase reporter gene under control of the IFN-β promoter (Fig. 5C-E). Production of NS1 by NS1allBon2 cells reduced the IFN-β mRNA expression after delNS1 virus infection 3.5-fold ($p=0.045$), whereas no significant difference was observed for the NS1allBon1 and Tet-on cell lines (Fig. 5A). Furthermore, IFN-β mRNA was not detectable in mock-infected

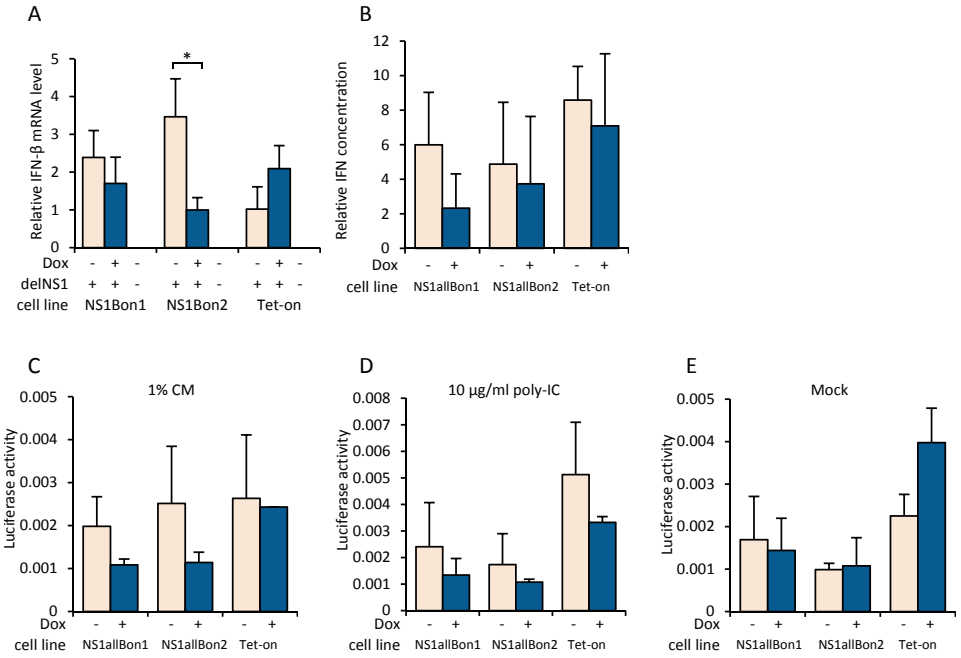

Fig. 5. Effect of NS1 expression on INF-β production determined with three different methods. Before each assay, NS1allBon1, NS1allBon2 and Tet-on Advanced control cells were induced for 24 h with 1.0 µg/ml Dox (+) or not induced (-). The amount of IFN-β mRNA was determined by qRT-PCR, after infection with delNS1 or mock infection and normalized for the amount of actin mRNA (Panel A). The relative amount of excreted IFN was determined after infection of the cells with delNS1, followed by removal of virus from the supernatant 24 h later by ultra-filtration. The IFN in the supernatant was titrated by stimulating MDCK-SFS cells with a serial dilution of the supernatant before infection with NDFLtag-EGFP virus. The relative amount of IFN present in the supernatant was determined by the dilution of the supernatant at 50% EGFP signal (Panel B). Furthermore, the cells were transfected with an IFN-β dependent firefly luciferase reporter plasmid, followed by stimulation of the IFN-β production with 1% CM (Panel C), 10 µg/ml poly-IC (Panel D) or mock (Panel E). The luciferase activity was normalised by the Renilla luciferase activity. The mean and 95% confidence interval of the mean of triplicate experiments (luciferase reporter assay in duplicate) are presented for each assay. Differences were significant (*) when $p < 0.05$.

cells. The concentration of IFN secreted by delNS1 virus-infected cells was determined by titration of MDCK-SFS cells with virus-free supernatant, followed by infection of these cells with NDV that expresses EGFP for its quantification. NDV is known to be highly susceptible to IFN. Thus cells stimulated with IFN produce less NDV virus as assessed by the level of fluorescence. The production of NDV viruses was translated to a relative IFN concentration as described in material and methods. Although NS1 expression by NS1allBon1 cells reduced IFN secretion almost 3-fold, this difference was not statistically significant. The IFN expression of mock-infected NS1allBon1, NS1allBon2 and TET-on cells was below detection limits, since the ultra-filtrated supernatant of these cells did not reduce EGFP expression after infection with NDFL-tag EGFP.

NS1 expression by NS1allBon2 cells reduced IFN secretion to an even lower extent (Fig. 5B). In the third assay, both NS1allBon cell lines showed slightly lower, although not statistically significant, expression of a reporter gene under control of an IFN-β promoter when NS1 expression was induced, as compared to non-induced cells upon stimulation of IFN-β production by either CM (Fig. 5C) or poly-IC (Fig. 5D). These differences were smaller without stimulation of IFN-β production (Fig. 5E). Although the individual assays mostly do not yield statistically significant differences, the combined results of these three assays suggest that induction of NS1 expression by NS1allBon cells slightly reduces IFN production. However, this effect is minimal and therefore not likely to be critical in the process of virus replication.

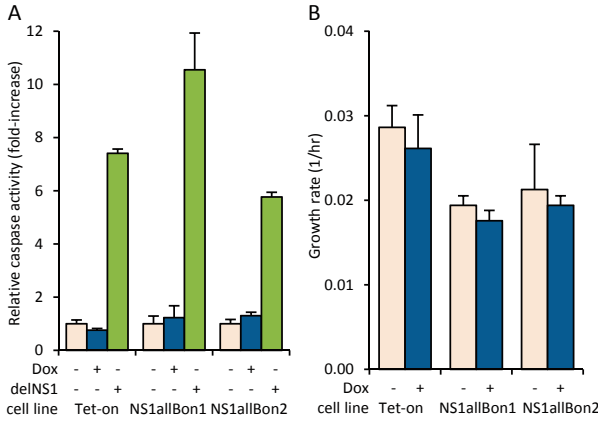

Fig. 6. Effect of NS1 expression on apoptosis and cell growth. NS1allBon1, NS1allBon2 and pTet-on Advanced control cells were induced with 1.0 µg/ml Dox (+), non-induced (-) or infected with delNS1 virus, followed by measurement of the combined caspase-3 and -7 activity 24 h later (panel A). The results show the relative increase in caspase activity as compared to the corresponding non-induced, non-infected cells. The growth rate of these cells during a 48 h cultivation period in the presence or absence of Dox is presented in panel B. Mean and their 95% confidence interval of triplicate experiments are given.

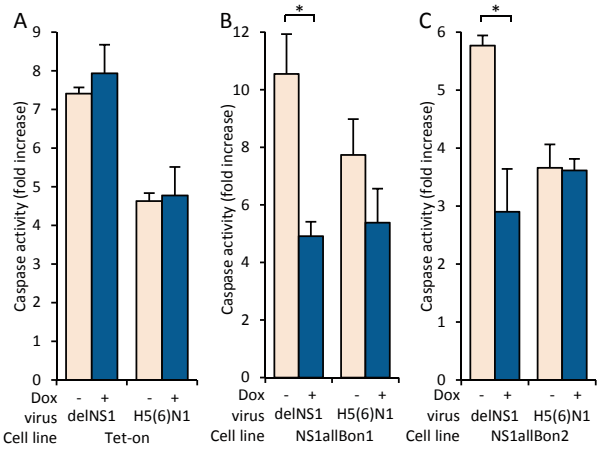

Fig. 7. Inhibition of apoptosis by NS1 expression. Tet-on Advanced control cells (panel A), NS1allBon1 (panel B) and NS1allBon2 (panel C) cells were induced with 1.0 µg/ml Dox (+) or non-induced (-), followed by infection with delNS1 or H5(6)N1 virus. The combined caspase-3 and -7 activity was measured 24 hpi. The relative increase in caspase activity as compared to the corresponding non-infected cells was then calculated. Mean and their 95% confidence interval of triplicate experiments are given. Differences were significant (*) when $p < 0.05$.

Effect of NS1 on apoptosis

Apoptosis induction was determined by measuring the combined activity of capase-3 and caspase-7, two proteins involved in the activation of apoptosis (93). Expression of NS1 in MDCK cells is known to induce apoptosis (81, 145, 195). Furthermore, Infection of cells with delNS1 virus is known to induce apoptosis more efficiently than infection with virus containing an NS1 gene (70, 156, 179, 191, 200), indicating that NS1 can inhibit apoptosis during an infection. With respect to the apoptosis induction by NS1 expression, no significant difference in caspase activity (Fig. 6A) or growth rate (Fig. 6B) was observed between Dox-induced and non-induced NS1allBon1 and NS1allBon2 cell lines, which indicates that allele B NS1 expression in these cells does not induce apoptosis. As a positive control, cells were infected with delNS1, which yielded a four to nine-fold increase in caspase activity (Fig. 6A).

We next analyzed the effect of NS1 expression by NS1allBon cells on apoptosis induction by both delNS1 and H5(6)N1 virus infection. As expected, delNS1 virus induced about 60% higher caspase activity than H5(6)N1 virus in pTet-on Advanced control cells and Dox addition did not affect caspase activity (Fig. 7A). However, expression of NS1 24 h prior to delNS1 virus infection reduced the caspase activity of NS1allBon1 cells 2.1-fold ($p=0.017$; Fig.7 B) and NS1allBon2 cells 2.0-fold ($p=0.018$; Fig. 7C). The caspase activity in the induced cells was comparable to those in cells infected with H5(6)N1 virus. Expression of NS1 prior to infection with H5(6)N1 virus did not affect caspase activity of NS1allBon2 cells (Fig. 7C) but did reduce caspase activity of NS1allBon1 cells 1.4-fold (Fig. 7B) which was, however, not statistically significant ($p=0.074$). Thus, NS1 expression by NS1allBon cells reduces apoptosis induction by a virus lacking an NS1 gene to a level that is comparable with cells that are infected with a virus that contains an intact NS1 gene.

DISCUSSION

Two stable MDCK cell lines that show inducible expression of allele B NS1 from the A/turkey/Wisconsin/68 H5N9 influenza strain were established: NS1allBon1 and NS1allBon2. Upon induction, these cell lines showed 500 and 1200-fold lower allele B NS1 expression levels than virus-infected cells, as assessed by Western blot analysis. Attempts to generate stable cell lines similarly expressing either the full-length allele A NS1 or the allele A NS1 N-terminal domain from the A/PR/8/34 H1N1 influenza strain were not successful. A previous attempt by others to establish a stable MDCK cell line expressing allele A NS1 from A/Ty/Ont/66 was also not successful, which the authors attributed to apoptosis induction due to leaky NS1 expression (145). Transient NS1 expression is known to induce apoptosis in several cell lines, including MDCK (81, 145, 195). However, induction of allele B NS1 expression in the two NS1allBon cell lines did not induce apoptosis nor did it affect cellular growth rate. The relatively low NS1 expression level by these cell lines is likely not the cause of the lack of apoptosis induction, since others previously found apoptosis induction at comparably low allele A NS1 expression levels (145). Noticeably, most of the studies into the effect of transient NS1 expression on apoptosis were done with allele A NS1. There is only one example of an H5N2 strain containing allele B NS1 that does not induce apoptosis in porcine cells (25). The efficiency of apoptosis induction is also known to vary between allele A NS1 genes from different strains (25, 179, 180, 198). Thus, it is plausible that the successful establishment of the NS1allBon cell lines is related to the specific allele B NS1 gene used. One cell line with stable expression of an NS1-GFP protein was developed for the production of delNS1 virus, however no further information on the origin of the NS1 protein, the NS1 expression levels, virus replication efficiency and cell growth were described (78).

Apart from apoptosis induction by recombinant NS1 expression, NS1 can also down-regulate the induction of apoptosis due to virus infection. This is suggested by the lower apoptotic response in cells infected with wild-type influenza virus than in cells infected with the corresponding isogenic strains lacking a functional NS1 gene (70, 156, 191, 200). This inhibition of the apoptotic response was attributed to activation by NS1 of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway, which is known to result in an anti-apoptotic response (31, 198). We observed that recombinant allele B NS1 expression suppresses the apoptotic response induced by delNS1 virus to the same level as observed with NS1-expressing virus,

despite the 1000-fold lower NS1 expression level as compared to virus-expressed NS1. Others similarly observed that low NS1 expression levels are enough to saturate the intracellular Akt signaling pathway, leading to apoptosis suppression (198). Other methods by which NS1 could have downregulated apoptosis are inhibition of PKR, OAS/RNAse L or the JNK/AP-1 stress pathway (59).

NS1 expression in both NS1allBon cell lines increased the delNS1 virus yield comparable to those found for Vero cells infected with the same virus. Restoration of virus production upon recombinant allele B NS1 expression could be related to its function as an IFN antagonist. Organisms and cells with low or absent IFN response, like Vero cells, STAT1 or PKR knock-out mice and young embryonated eggs, are capable of efficient delNS1 virus replication due to their IFN deficiency (30). In addition, IFN expression in MDCK cells was reduced 5-fold after transient expression of allele A NS1 (148). In our studies, however, the NS1 expression by both NS1allBon cell lines had only a minor effect on IFN production, as was observed in three independent assays. Different assays were used to cover multiple levels in the IFN cascade, by looking at either activation of the IFN-β promoter, IFN-β transcription directly or secretion of IFN, and by stimulating IFN induction with either delNS1 virus, poly-IC or CM. Because allele B NS1 expression only has a minimal effect on IFN induction, it appears that the increase in virus production by recombinant NS1 during delNS1 infection is not IFN related. The lack of IFN inhibition could be connected to the origin of the NS1 gene, since inhibition of IFN by NS1 is strain dependent (63, 180) and allele B NS1 was found to be less efficient in the suppression of IFN-β production than allele A NS1 (201). Another possible cause could be the low expression level. During infection, NS1 is expressed in high quantities, suggesting that at least one of the many functions of NS1 requires such a high expression level. This implicates that not all of the NS1 functions, e.g. inhibition of IFN induction, are necessarily complemented by the about 1000-fold lower NS1 expression level in the NS1allBon cell lines. Another function of NS1 that likely does not occur in the NS1allBon cells upon Dox induction is inhibition of cellular mRNA preprocessing. Such a function would most likely affect cellular processes and limit cell division. This was however not observed, as cells grew with comparable high rates, both induced and non-induced.

The delNS1 virus titers from induced NS1allBon cells were comparable to those found for non-induced cells infected with H5(6)N1 virus. This indicates that the NS1 produced by the NS1allBon cells can fully facilitate the viral infection in a

similar manner as the allele A NS1 that originates from the wild-type influenza virus. This is in concordance with the observation that virus replication efficiency is not dependent on the NS1 allele type (180). Replication of H5(6)N1 strain, which yielded high titers already in the absence of Dox, was not improved by Dox-induced NS1 expression. Similar observations were made after transient transfection with NS1, although replication speed of the wild-type strain was somewhat increased (148). Hence there is no significant advantage for NS1 being present at the start of the infection. The efficiency in infectious delNS1 virus particle production, as determined by the ratio of infectious virus particles to the number of virus genome copy equivalent (relative infectious virus titer), is 0.1% for both NS1allBon cell lines in the absence of Dox. Induction of NS1 expression increased the efficiency 11 to 95-fold, due to the strong increase in infectious virus titer (244 to 544-fold). This is especially relevant for live attenuated vaccine production, since these rely on infectious virus particles.

The increased relative infectious titer due to NS1 induction may give a clue to the mechanism by which recombinant NS1 can complement the replication of delNS1 virus. NS1 regulates viral genome replication and transcription (36), and NS1 expression reduces viral RNA accumulation in cells transiently transfected with the influenza polymerase complex genes PB1, PB2, PA and NP, in comparison to cells that do not express NS1 (180). The lack of regulation of the influenza polymerase complex by NS1 could result in an altered ratio between the vRNA, cRNA and mRNA, resulting in lower infectious viral titers while not affecting the number of virus genome copy equivalent titer, which measures all three RNA types. Thus, the low yield of delNS1 virus in normal MDCK cells could be due to the loss of NS1 regulation of viral genome replication and transcription rather than the inability of the virus to interfere with the host cell's antiviral response. This suggestion is further supported by the observation that IFN expression is not a limiting factor for influenza replication in MDCK cells (148).

The inducible expression of NS1 allows for further studies into the timing and level of NS1 expression in relation to its function. Furthermore, these cells are able to replicate delNS1 virus to high titers required in industrial production. The requirement for Dox in the culture medium may however limit their commercial application due to restriction in use of antibiotics in vaccine production. Thus, stable production cell lines with constitutive expression of allele B NS1 are more suited for this purpose. The feasibility of establishing such cell lines is suggested by our

observation that allele B NS1 expression does not affect cell growth (nor induce apoptosis). Finally, the use of allele B NS1 may be advantageous for the production of DIVA vaccines, because of the antigenic difference with the more common allele A protein.

Acknowledgements

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Chapter 5

Changes in the ratio of the M1 and M2 protein levels and in the subcellular distribution of the M1 protein can compensate for the loss of NS1 function in influenza virus NS1 deletion mutants.

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Abstract

Influenza viruses unable to express NS1 protein (delNS1) replicate poorly and induce high amounts of interferon (IFN). They are therefore considered as candidate viruses for live-attenuated influenza vaccines. Their attenuated replication is generally assumed to result from the inability to counter the antiviral host response, as delNS1 viruses replicate efficiently in Vero cells, which lack IFN expression. In this study, delNS1 virus was parallel passaged on IFN competent MDCK cells, which resulted in two strains that were able to replicate to high virus titres in MDCK cells due to adaptive mutations in especially the M-gene segment, but also the NP and NS gene segments. Most notable were clustered U-to-C mutations in the M segment of both strains and clustered A-to-G mutations in the NS segment of one strain, which presumably resulted from host cell mediated RNA editing. The M segment mutations in both strains changed the ratio of M1 to M2 expression, probably by affecting splicing efficiency. In one virus, 2 amino acid substitutions in M1 additionally enhanced virus replication, possibly through changes in the M1 distribution between the nucleus and the cytoplasm. Both adapted viruses induced less IFN and less apoptosis compared to delNS1 virus, but still significantly more than virus able to express NS1. These results indicate that not only inhibition of IFN induction, but also the loss of one or more M1-related functions of NS1 limit delNS1 virus replication. The mutations identified in this paper may be used to enhance delNS1 virus replication for vaccine production.

INTRODUCTION

The non-structural (NS1) protein of influenza A virus is an antagonist of the cellular antiviral response. Infection with virus either not encoding NS1 protein (delNS1) or encoding a truncated NS1 protein results in high levels of type I interferons (IFN) such as IFN-α or IFN-β. Replication in cell culture of such viruses is attenuated, indicating that the NS1 protein is not essential for replication in such hosts (45). In vivo, viruses lacking a fully functional NS1 protein induce IFN in the absence of detectable virus replication (38), which are favourable conditions for use as live attenuated vaccines. The local release of IFN and other cytokines and chemokines appears to be an excellent adjuvant that enhances production of immunoglobulins and contributes to the activation of dendritic cells required for antigen presentation (83, 132). DelNS1 candidate vaccines against influenza A and B have been developed (140, 189) and initial trials in humans showed successful induction of antibody responses (175). Apart from the use in vaccines, delNS1 viruses also show potential as oncolytic agent (113) and viral expression vector (187).

NS1 is expressed at high levels directly after infection and facilitates virus replication in many different ways (reviewed in (59)). Its antiviral properties are focused on reducing the IFN mediated innate immune response and act at several levels. Cytoplasmic dsRNA and 5′-triphosphate-containing RNA are produced during influenza infection and recognized as pathogenic patterns by antiviral proteins like retinoic-acid inducible gene I (RIG-I), dsRNA-dependent protein kinase R (PKR) and 2′-5′-oligoadenylatesynthetase (OAS). NS1 binds both dsRNA and RIG-I, and blocks the activation of PKR and OAS, thereby limiting the onset of several pathways that lead to IFN induction (132). Other functions of NS1 are inhibition of cellular pre-mRNA processing (including IFN pre-mRNA) and mRNA nuclear export (59). Furthermore, NS1 regulates both viral genome replication and translation (180), splicing of M segment mRNA (134), nuclear export of viral mRNA (41) and viral ribonucleoprotein (vRNP) (180), and viral protein synthesis (26, 34). Recently it was found that NS1 binds the human PAF1 transcription elongation complex (hPAF1C) by a histone-mimicking sequence, thereby inhibiting the role of hPAF1C in the antiviral response (98). NS1 mRNA is transcribed from the eighth vRNA segment. It is partially spliced to generate mRNA that encodes the nuclear export protein (NEP) (59). In the nucleus of infected cells, NEP facilitates the export of the vRNP complexes containing the viral genome segments to the cytoplasm,

where assembly of the viral components is completed before virus budding takes place (2). Independent from vRNP export, NEP also regulates viral genome transcription and replication (135). During development of delNS1 strains it is therefore essential to retain the NEP protein.

In cells and animals with a low or absent IFN response, such as Vero cells, STAT1 or PKR knock-out mice, delNS1 virus replicates to high titres (30, 45, 77), whereas replication is attenuated in MDCK cells and other IFN-competent hosts. When Vero cells are externally stimulated with IFN-α before infection, delNS1 virus replication is however also attenuated (38). Moreover, delNS1 only replicated efficiently in embryonated chicken eggs younger than 8 days, when the host immune response is not yet fully developed (161). It is therefore generally assumed that the inability of delNS1 virus to counter the cellular innate immune response is the major cause for its attenuated phenotype (132). In addition to the unimpaired IFN response, the absence of NS1 during influenza virus infection results in enhanced apoptosis induction (156, 200). Activation of caspases, a group of cysteine proteases that play an important role in apoptosis, results in cleavage of viral NP protein and thereby limits the amount of viral protein available for assembly of viral particles (199). The antiviral effect of apoptosis is therefore believed to contribute to the attenuated replication of delNS1 virus. Inhibition of the apoptotic response is attributed to both the activation by NS1 of the phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway, which is known to result in an anti-apoptotic response (31, 198), as well as the inhibition of IFN. IFN sensitizes cells for apoptosis (200) through its transcriptional induction of PKR (162) and activation of the FADD/caspase-8 death signalling pathway (10). The role of apoptosis in influenza infection is, however, still uncertain as several influenza proteins, including NS1, also exhibit pro-apoptotic functions. Furthermore, influenza virus replication is impaired in the presence of caspase inhibitors (190), which appears to be caused by retention of vRNA complexes in the nucleus, preventing formation of progeny virus particles. A possible explanation for this double role of NS1 in apoptosis regulation could be prevention of cell death by inhibition of apoptosis early in the infection, followed by induction at a later stage (198). Ludwig et al. (93) suggested that caspases enhance vRNP export from the nucleus later in the infection by widening of the nuclear pores, thereby allowing diffusion of vRNP out of the nucleus.

Previously we showed that delNS1 virus can efficiently be propagated on a MDCK cell line showing inducible expression of NS1 from a trans-complementing genomic gene (170). A 500-fold increase in infectious virus titre was observed, even though the NS1 level was 1000-fold lower than that in cells infected with wild-type (WT) virus. Furthermore, apoptosis was reduced to similar levels as found in WT virus infected cells, whereas the induction of IFN by delNS1 virus was not significantly reduced in these cells. Because of the limited effect on IFN induction, we then hypothesized that the low yield of delNS1 virus on normal MDCK cells could be caused by loss of other NS1 regulatory function rather than the inability of the virus to interfere with the host cells antiviral response. In this paper we increased the replication efficiency of delNS1 virus by adaptation to IFN competent MDCK cells during serial passage. Next, we determined if the observed increase in virus yield was related to decreased IFN and apoptosis induction. Furthermore, we identified the mutations and partly characterized the mechanism that allowed the virus to efficiently replicate in the absence of the NS1 protein.

Materials and methods

Cell culture and virus strains

MDCK-SFS cells (171) were grown in suspension in serum-free SFM4BHK21 medium (Hyclone, Waltham, MA), supplemented with 8 mM glutamine, 5 mg/L phenol red and 1.5 g/L sodium bicarbonate, or adherent in serum-free UltraMDCK medium (Lonza Biowhittaker, Basel, Switzerland) supplemented with 4 mM glutamine. Adherent NS1Bon2 MDCK cells (170) were also grown in UltraMDCK medium, additionally supplemented with 200 μg/ml G418 (Promega, Fitchburg, WI) and 100 μg/ml hygromycin B (Clontech, Mountain View, CA). G418 and hygromycin were not used during virus infections. Human embryo kidney (293T) cells were cultured in Glutamax medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. All culture media were provided with 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). Cells were grown at 37˚C and 5% CO_2. Suspension cells were grown in shaker flasks at 100 rpm. Cell density and viability were determined with a Countess automated cell counter (Invitrogen).

The influenza delNS1 virus strain (170) used for the adaptation to MDCK-SFS cells was previously passaged 10 times in 7-day old embryonated chicken eggs and is referred to as delNS1^{EA}. Note that in the comparison of reassortant virus replica-

tion, protein expression, apoptosis and IFN-induction, a non-adapted delNS1 virus strain was used, to which we refer as delNS1. The isogenic H5(6)N1 virus (170) strain containing the complete NS segment was passaged once in 9-day old embryonated eggs after virus rescue, and is referred to as wild-type (WT) virus. The infectious influenza virus titre was measured by determining the tissue culture infective dose required to infect 50% ($TCID_{50}$) of MDCK cells, as previously described (171). All virus strains were propagated on the NS1 expressing NS1Bon2 MDCK cell line to generate virus seed stocks with high infectious virus titres (>7 log10 $TCID_{50}$/ml) prior to further viral characterization. For this purpose, NS1 expression was induced in this cell line 24 h before infection by addition of 1 μg/ml doxycycline (Clontech) to the culture medium, followed by infection at multiplicity of infection (m.o.i.) 0.01 and harvesting at 3 days post-infection (dpi).

Virus adaptation

Two independent adaptation experiments were performed. In the first experiment, MDCK-SFS cells in suspension were infected with $delNS1^{EA}$ at m.o.i. 0.1. After 2-3 days the supernatant was collected and used for subsequent infection of fresh MDCK-SFS cells with unknown m.o.i.. The virus was serially passaged 10 times in this manner. In the second adaptation experiment 5 serial passages were performed, starting with infection of adherent MDCK-SFS cells with $delNS1^{EA}$ at m.o.i. 0.01. The infectious virus titre was determined daily and the supernatant with the highest titre was used in subsequent infection of fresh MDCK-SFS cells at m.o.i. 0.01. The two adapted virus strains were cloned 3 times by limiting dilution on MDCK-SFS cells. Of each adapted strain, eight clones were screened for virus replication on MDCK-SFS cells and one clone of each strain with high titre was selected and amplified on NS1Bon2 cells. The resulting strains are referred to as $delNS1^{CA1}$ and $delNS1^{CA2}$ respectively.

Plasmids

Ten plasmids containing single or multiple mutations found in $delNS1^{CA1}$ and $delNS1^{CA2}$ (Table 1) were made. Viral RNA of $delNS1^{CA1}$ and $delNS1^{CA2}$ was isolated from seed virus and HA and M gene segments were amplified by PCR, using primers with BsmBI restriction sites (68). The resulting cDNAs were inserted in plasmid pHW2000 to create pROM33-pROM36. Plasmids containing the mutated PB1 segments of $delNS1^{CA1}$ and $delNS1^{CA2}$ could not be made. pROM16 was made by cloning a synthetic 675 bp BsrGI-NgoMIV fragment (GenScript Corporation, Piscataway, NJ) containing mutation A1381G in pHW195 containing the PR8 NP

Table 1. pHW2000-derived plasmids encoding mutant gene segments from cell-adapted delNS1

Plasmid	Segment	Nucleotide substitutions	Amino acid substitutions[a]
pROM34	HA^{CA1}	U796C	F257L
pROM33	HA^{CA2}	C1326A	None
pROM16	NP^{CA1}	A1381G	R446G
pROM36	M^{CA1}	U640C, U643C, U652C, U688C	None
pROM35	M^{CA2}	U277C, U298C, U315C, U316C, U323C, U325C	V97A, Y100H
pROM51	$M^{CA2.1}$	U315C	V97A
pROM52	$M^{CA2.2}$	U323C	Y100H
pROM53	$M^{CA2.3}$	U315C, U323C	V97A, Y100H
pROM54	$M^{CA2.4}$	U277C, U298C, U316C, U325C	None
pROM13	NS^{CA1}	A148G, A173G, A179G, A180G, A248G, A252G	Y41C, M52V, I76V

[a] Amino acid positions are relative to methionine in the open reading frame.

gene (66). Synthetic fragments with suitable BsmBI restriction sites comprising the complete M segment with one or more of the CA2 mutations or the complete delNS1 NS segment with all six CA1 mutations were inserted in pHW2000 to construct pROM13 and pROM51-pROM54. All plasmid inserts were sequenced to ensure the absence of additional nucleotide substitutions.

Rescue of recombinant influenza virus

To generate recombinant influenza virus, a mixture of 1.5 x 10^6 293T and 5 x 10^5 MDCK-SFS cells were transfected with equal amounts of the eight plasmids containing the different gene segments, using Fugene HD (Roche Applied Science, Penzberg, Germany). DelNS1 viruses were made using plasmids pHW191, pHW192, pHW193, pHW195, pHW197 (66), pPolsabrib H5, pPolsabrib N1 and pHW NEP (170), whereas WT virus was made by replacing pHW NEP with pHW198, which contains the full length NS segment. All other recombinant viruses were made by replacing one or more plasmids with those in Table 1. At 24 h post-transfection (hpt) the transfection mixture was replaced by Glutamax medium supplemented with 0.3 % bovine serum albumin (Chemie Brunschwig AG, Basel, Switzerland) and 1 µg/ml TPCK-trypsin (Sigma-Aldrich, St. Louis, MO). Virus was harvested at 96 hpt. Virus stocks were generated using NS1Bon2 cells as described above and virus identity was confirmed by sequence analysis. Reassortant delNS1 virus strains are referred to as delNS1, followed by the mutated gene segment(s) it contains (Table 1) and the adapted virus from which they originate (e.g. delNS1:[NP M]^{CA1}, which contains the mutated NP and M gene segments from delNS1^{CA1}).

Virus genome sequencing

Viral RNA genomes were isolated using a high pure viral RNA isolation kit (Roche Applied Science). Universal influenza genome primer uni12 (68) was used for reverse transcriptase reactions with the Superscript III first strand synthesis system (Invitrogen), followed by segment specific PCR reactions with an Expanded high fidelity PCR system (Roche Applied Science). DNA sequencing was performed at Baseclear (Leiden, the Netherlands) and sequence analysis was done with Lasergene (DNASTAR Inc, WI).

Comparison of virus replication

To study the infection kinetics of the different virus strains, 10^6 MDCK-SFS cells per well were incubated in 6-well plates (Greiner Bio-One), in 5 ml UltraMDCK medium containing 2 μg/ml trypsin-TPCK. Cells were infected in triplicate at m.o.i. 0.01. Supernatant was sampled at the indicated interval and stored at -80°C before determining the infectious virus titre. The main effect and interactions of the mutated gene segments on the infectious virus titre was analysed by a repeated measures ANOVA with data from two infection experiments, both performed in triplicate, using R statistical software package (R Foundation for Statistical Computing, Vienna, Austria). Analysis was performed separately for delNS1^{CA1} and delNS1^{CA2} reassortants datasets, with the mutated gene segments as explanatory variables, the two infection experiments as random effects and delNS1 virus as the baseline. Non-significant explanatory variables were excluded from the model.

IFN reporter assay

MDCK-SFS cells were allowed to attach to the surface of eight 96-well plates (Greiner Bio-One) for 1 h (4.5×10^4 cells/well) and then transiently cotransfected with a reporter plasmid carrying a firefly luciferase gene under control of the IFN-β promoter (p125Luc, kindly provided by Takashi Fujita, Kyoto University, Japan (193) and a Renilla luciferase control plasmid pGL4.73 (Promega), using Fugene HD. The next day, supernatant was removed and cells were infected in triplicate (m.o.i. 5) with WT, delNS1, delNS1^{CA1} or delNS1^{CA2}, or mock infected. One hour later, supernatant was replaced with fresh medium. At 8, 10, 14, 16, 18, 20, 22 and 24 hour post-infection (hpi) one plate was stored at -20°C without supernatant. The firefly luciferase activity of all plates was measured with a GloMax-Multi luminometer (Promega) using the Dual Luciferase Reporter Assay System (Promega) and normalized to the Renilla luciferase activity.

Apoptosis assay

MDCK-SFS cells were allowed to attach to the surface of six 96-well plates (Greiner Bio-One) for 1 h (10^4 cells/well) and infected in sextuple with WT, delNS1, delNS1^{CA1} or delNS1^{CA2} virus (m.o.i. 5), or mock infected. One hour later, supernatant was replaced with fresh medium. At 10, 14, 16, 19, 22 and 26 hpi, one plate was stored at -20°C without supernatant. Apoptosis was determined by the activity of caspase-3 and caspase-7 using Caspase-Glo 3/7 Assay (Promega). Caspase-Glo reagent, diluted 1:1 with PBS, was added to the frozen cells (50 µl/well). After 1.5 h incubation at room temperature, the luminescence was measured with a Glo-Max-Multi luminometer.

Western blot analysis of M1 and M2 expression

MDCK-SFS cells were allowed to attach to the surface of 24-well plates (Greiner Bio-One) for 1 h (3.3 x 10^5 cells/well) and infected (m.o.i. 5) or mock infected, in triplicate. HEK293T cells (10^6 cells/well in 6-well plates) were transiently transfected in triplicate, using Fugene HD with either 2 µg/well pHW195, pROM35, pROM36, pROM51, pROM52, pROM53, pROM54 (Table 1) or mock transfected. At 10 hpi or 48 hpt, cells were lysed with reducing NuPAGE sample buffer containing in addition complete EDTA-free protease inhibitor cocktail (Roche). Samples were sheared with a 21G needle, incubated for 10 min at 75°C and loaded onto NuPAGE® Novex® 12% Bis-Tris precast gels (Invitrogen). Polypeptides were transferred to polyvinylidenedifluoride membranes and detected by immunoblotting using monoclonal mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against M1 (SC-57881, 0.2 µg/ml) or M2 (SC-32238, 0.4 µg/ml). After subsequent incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulins (0.13 µg/ml; Dako, Glostrup, Denmark), proteins were visualized with ECL plus (GE Healthcare, Buckinghamshire, UK) and quantified with a Storm840 imaging system (Molecular Dynamics, GE Healthcare). The M1/M2 band intensity ratio of each sample was calculated before determining the mean ratio of the triplicate infections or transfections.

Subcellular M1 localization

MDCK-SFS cells were cultured in suspension (6.6 x 10^5 cells/ml) and infected with WT, delNS1 or delNS1:M$^{CA2.3}$ in triplicate (m.o.i. 5). At 6 and 10 hpi, 10^6 cells were harvested and cytoplasmic and nuclear extracts were prepared with the NE-PER nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Rockford, IL), ac-

cording to the manufacturer's description. However, complete EDTA-free protease inhibitor cocktail was added to the extraction reagents. Cytoplasmic and nuclear extractions were subjected to reducing SDS-PAGE and Western blot analysis as described above. Polypeptides were detected by immunoblotting as described in the previous section with monoclonal mouse antibodies (Santa Cruz Biotechnology) against lamin A/C (SC-7292, 0.2 μg/ml), tubulin (SC-5286, 0.2 μg/ml) or M1 (SC-57881, 0.2 μg/ml). Cytoplasmic and nuclear specific proteins tubulin and lamin A/C were used to assess the purity of nuclear and cytoplasmic extracts. The amount of M1 protein was corrected for the extraction efficiency by either tubulin (cytoplasmic extracts) or lamin A/C (nuclear extracts). The ratio of corrected nuclear M1 to corrected cytoplasmic M1 was calculated before determining the mean ratio of the triplicate infections.

RESULTS

delNS1 virus adaptation

The egg-adapted influenza virus delNS1^{EA} replicated poorly in MDCK cells, reaching a maximum infectious virus titre that was 10^4-fold lower than that of WT virus (Fig. 1A). To investigate whether the virus was able to overcome the negative effect of the NS1 deletion by acquiring compensating mutations we serially passaged delNS1^{EA} virus on MDCK-SFS cells in two independent adaptation experiments (see Materials and Methods). In the first cell adaptation experiment (CA1), virus was blindly passaged 10 times. In the second experiment (CA2), virus taken from the time point where the titre was maximal was used to infect cells in the next passage at a controlled m.o.i. of 0.01. Both adaptation experiments on MDCK-SFS cells resulted in virus populations with increased replication rate and increased maximum titres compared to the parent strain. The maximum virus titre of strain delNS1^{CA2} increased during the first 3 to 4 passages, but did not increase further during the fifth passage (Fig. 1B). To obtain clonal virus, the adapted strains were further cultured during 3 limiting dilution steps on MDCK-SFS cells. The infectious virus titres of the resulting cloned cell-adapted virus strains delNS1^{CA1} and delNS1^{CA2} were about 250-fold higher than the parental delNS1^{EA} virus titre, but remained 25-fold lower than the WT virus titre (Fig. 1A).

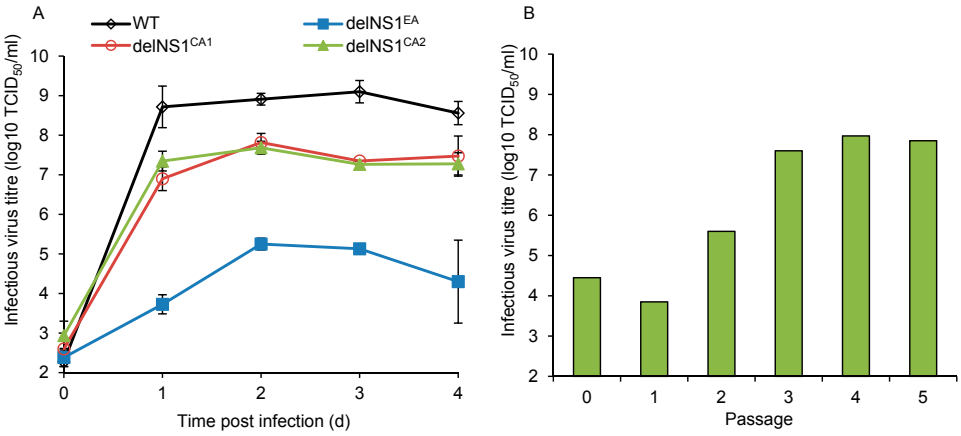

Fig. 1. Replication of delNS1 virus adapted to growth on MDCK-SFS cells. (A) Replication kinetics of the two adapted delNS1^{CA1} and delNS1^{CA2} virus strains in comparison to WT and parental delNS1^{EA} virus after infection of MDCK-SFS cells at MOI 0.01. Geometric mean titres and 95% confidence interval of the mean of virus infections performed in triplicate are presented. (B) Increase in maximum infectious virus titre during each passage step of the second delNS1 adaptation experiment.

Sequence analysis of adapted virus

All eight gene segments of the delNS1^{CA1} and delNS1^{CA2} virus strains and their parental delNS1^{EA} strain were sequenced (Table 2). No mutations were found in the PB2, PA and NA segments. The delNS1^{EA} virus contained two mutations in PB1 and one in HA with all mutations present in approximately 50% of the virus population. These mutations were silent and must have arisen during the passaging in eggs. Both PB1 mutations were present in both cell adapted viruses, whereas the HA mutation was only present in delNS1^{CA2} virus. Due to the presence of mutations in the delNS1^{EA} virus, a non-adapted delNS1 virus was used in the comparison of reassortant virus replication, protein expression, apoptosis, and IFN-induction experiments. We refer to this virus as delNS1.

Compared to delNS1^{EA} virus, delNS1^{CA1} virus had 13 additional mutations, including a silent mutation in PB1, F257L in HA, R446G in NP, four silent U-to-C mutations in M, and six A-to-G mutations in the delNS1 segment. Three of the NS segment mutations resulted in the amino acid substitutions Y41C, M52V and I76V in the NEP protein. The first 4 A-to-G mutations in the NS^{CA1} segment are located on the part that normally encodes NS1, resulting in two amino acid substitutions at the NS1 C-terminal. These mutations may be disadvantageous to the WT virus and removal of the NS1 ORF therefore increased the freedom of this segment to acquire mutations. DelNS1^{CA2} virus had 6 additional mutations that were all lo-

Table 2. Mutations in egg adapted delNS1^{EA} and cell adapted delNS1^{CA1} and delNS1^{CA2} virus strains.

Gene segment	Nucleotide position[a]	Nucleotide substitution[b]			Amino acid substitution[b]
		delNS1^{EA}	delNS1^{CA1}	delNS1^{CA2}	
PB1 (S2)	798	-	G to U	-	-
	1953	A to U 50%[c]	A to U	A to U	-
	2133	U to C 50%[c]	U to C	U to C	-
HA (S4)	796	-	U to C	-	F257L
	1326 (1338)	C to A 50%[c]	-	C to A	-
NP (S5)	1381	-	A to G	-	R446G
M (S7)	277	-	-	U to C	-
	298	-	-	U to C	-
	315	-	-	U to C	V97A
	316	-	-	U to C	-
	323	-	-	U to C	Y100H
	325	-	-	U to C	-
	640	-	U to C	-	-
	643	-	U to C	-	-
	652	-	U to C	-	-
	688	-	U to C	-	-
NS (S8)	148 (620)	-	A to G	-	Y41C
	173 (645)	-	A to G	-	-
	179 (651)	-	A to G	-	-
	180 (652)	-	A to G	-	M52V
	248 (720)	-	A to G	-	-
	252 (724)	-	A to G	-	I76V

[a] Numbering refers to nucleotide positions in the vRNA template [GenBank accession no: EF467819 (PB1), DQ407519 (HA), EF467822 (NP), EF467824 (M), AF389122 (NS)]. Note that the numbering of the HA and NS mutations refers to the recombinant gene segments (170). The numbering in line with the genbank sequences is placed between brackets.

[b] Substitutions as compared to sequence of plasmid used for initial delNS1 virus generation. – No substitution.

[c] Presence of a second nucleotide sequence within the seed virus.

cated in the M segment. These mutations started to appear simultaneously at passage 4 and had increased at passage 5 (Fig. 2A). Again, these 6 mutations were all U-to-C mutations, two of which resulted in amino acid changes V97A and Y100H in M1. Notably, all mutations on the M and NS segments are clustered in regions of 50-100 nucleotides. Both M segment mutation clusters are located on a region of the M1 mRNA that is removed by splicing to generate the M2 mRNA and thus do not affect M2 mRNA structure (Fig. 2B). Apart from the two silent mutations in PB1 which were already present in the initial delNS1^{EA} virus, both parallel adaptations did not lead to identical mutations.

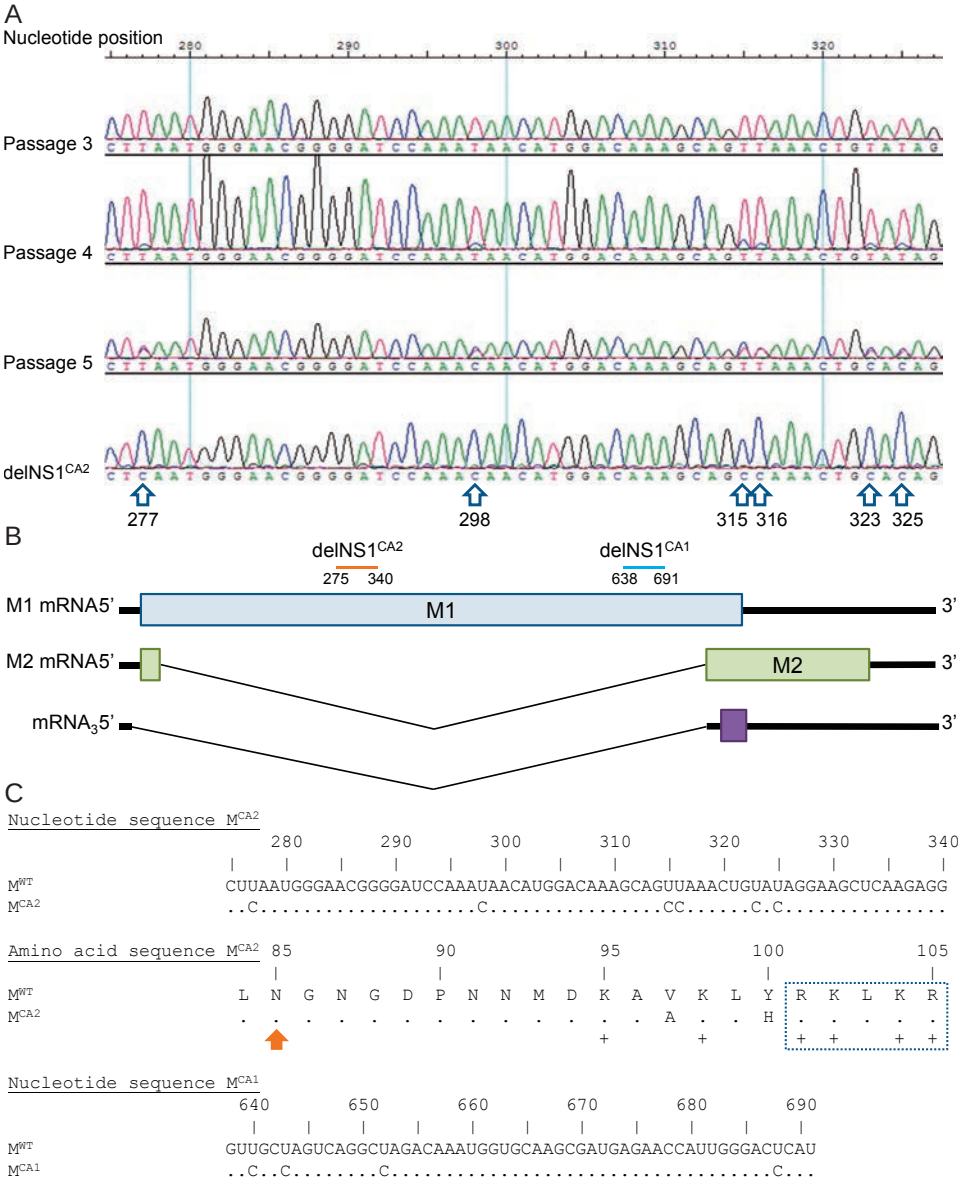

Fig. 2. Nucleotide substitutions in M-gene segments of adapted delNS1 viruses. (A) Sequence analysis of delNS1^{CA2} showing the simultaneous accumulation of six U-to-C substitutions (indicated by arrows) in the M segment vRNA between position 277-325 from passages 3 to 5 on MDCK-SFS cells. Double peaks are visible at passage 4 and 5. Note that in the electropherograms, U is shown as T. (B) Schematic overview of the M segment mRNAs, with the locations of the adaptive M^{CA1} and M^{CA2} mutations and splicing products M2 mRNA and mRNA3. Open reading frames are indicated by thick bars. (C) Nucleotide sequences of the M^{CA1} and M^{CA2} regions shown in panel B, including the amino acid sequence of M^{CA2}, where dots indicate sequence identity of M1^{CA2} to M1^{WT}. The square box indicates the location of the NLS in M1, with positively charged (+) amino acids (192). The arrow indicates the location of the adaptive mutation in influenza B M protein found earlier (189). The amino acid sequence of M1^{CA1} is not shown since it is identical to M1^{WT} (i.e. all mutations were silent).

Interferon-β and apoptosis induction by cell adapted delNS1 virus

To assess if viral adaptation affected IFN-β expression, cells were transfected with a firefly luciferase reporter gene under control of an IFN-β promoter and subsequently infected with WT, delNS1, delNS1^{CA1} or delNS1^{CA2} virus at high m.o.i. (Fig. 3A). The low luciferase activity of cells infected with WT virus as compared to cells infected with delNS1 virus indicates inhibition of IFN induction by NS1. Both cell-adapted viruses induced more IFN than the WT virus, but less than delNS1 virus. Furthermore, IFN induction appeared delayed in comparison to delNS1 virus.

The induction of apoptosis was determined by measuring the activity of caspase-3 and caspase-7, two proteases that are induced late in the apoptosis pathway. Again, the inhibiting effect of NS1 was visible as little caspase activity was seen in cells infected with the WT virus in comparison to cells infected with delNS1 viruses. Furthermore, both cell-adapted viruses induced more caspase activity than WT virus but less than delNS1 virus (Fig. 3B).

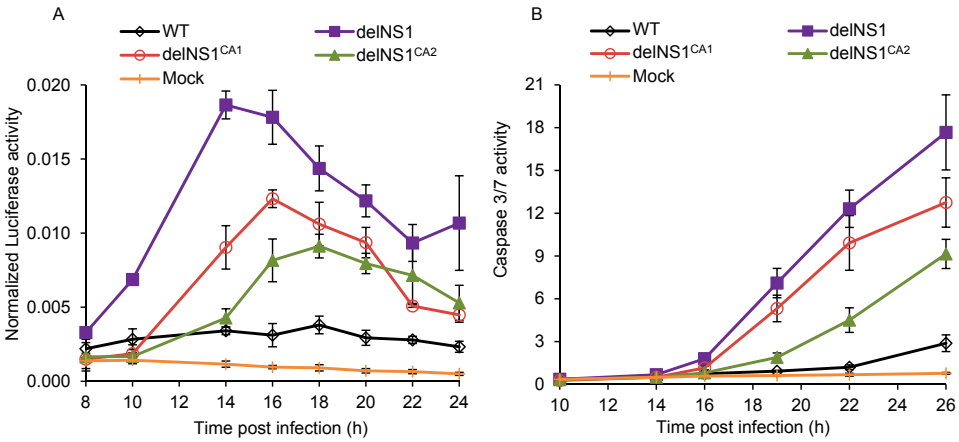

Fig. 3. Induction of IFN-β (A) and apoptosis (B) in MDCK-SFS cells infected with either delNS1, WT, delNS1^{CA1} or delNS1^{CA2} virus, or mock infected. IFN induction was measured with an IFN-β dependent luciferase reporter construct and corrected for transfection efficiency with Renilla luciferase. Apoptosis induction was assessed by measuring the activity of caspase 3 and 7. Mean activities and 95% confidence interval of the mean are presented of experiment performed in triplicate.

Infection with delNS1 viruses containing mutated gene segments

The effect of each mutated gene segment on the infectious virus titre, and possible interactions between the gene segments was determined by employing a full-factorial analytical approach. MDCK-SFS cells were infected with reassortant delNS1

viruses consisting of all 15 possible combinations of delNS1^{CA1} virus HA, NP, M and NS gene segments, or the 3 possible combinations of delNS1^{CA2} virus HA and M gene segments (Fig. 4A). All reassortant viruses contained the WT PB1 segment, as we were unable to generate plasmids containing the mutated PB1 segments. Infectious virus titres were examined using a repeated measures ANOVA, so as to determine the relative importance of the gene segments, and possible interactions between gene segments, on virus replication. Segments with significant effect on the delNS1^{CA1} virus titre were NS^{CA1}, M^{CA1} and NP^{CA1}, with coefficients of respectively 0.68, 0.76 and 0.50 (all $p<0.001$). These coefficients specify the average in-

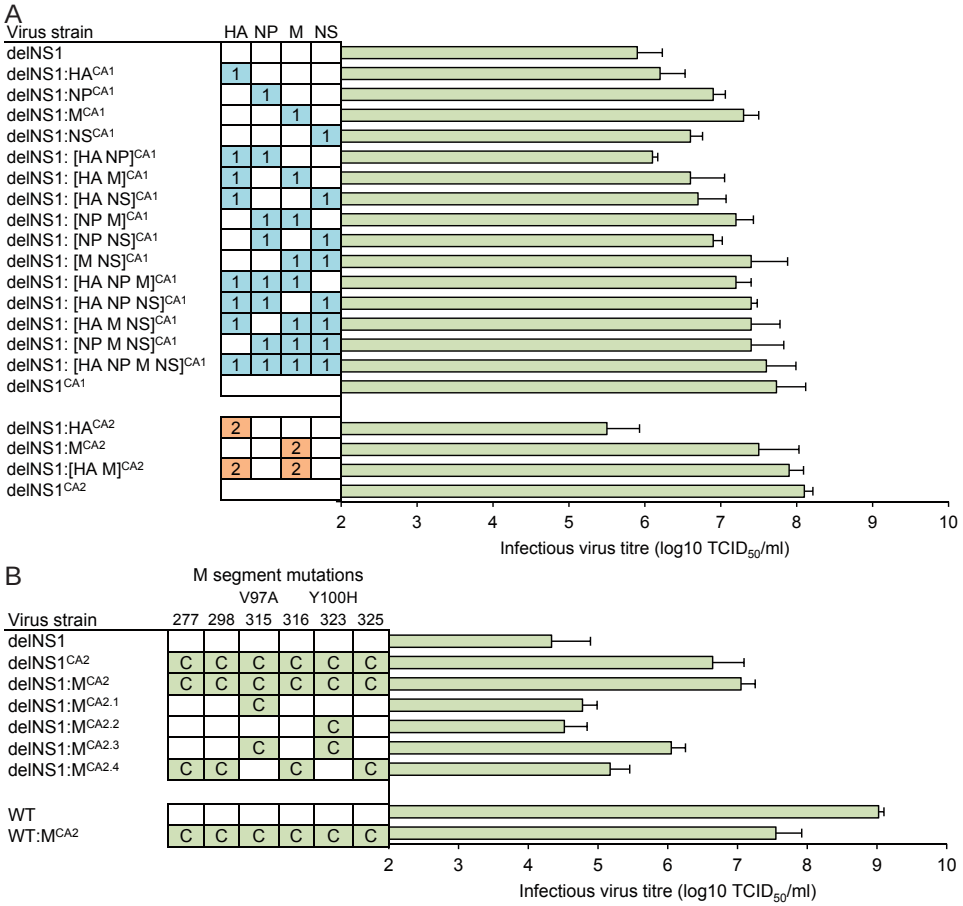

Fig. 4. Comparison of infectious virus titres (3 dpi) after infection of MDCK-SFS cells with the cell-adapted or the various reassortant virus strains. (A) Titres of delNS1 reassortant viruses made with original delNS1 plasmids (not-filled), or plasmids containing single or multiple mutations originating from the adapted virus strains delNS1^{CA1} (filled, marked 1) and delNS1^{CA2} (filled, marked 2). (B) Titres of delNS1 reassortant viruses containing one or more of the delNS1^{CA2} M segment mutations and of WT reassortant virus containing the M^{CA2} mutations. In both panels, geometric mean titres and 95% confidence interval of the mean are presented of triplicate measurements.

crease of the virus titre in log^{10} $TCID_{50}$/ml, when the mutated segment was included in the delNS1 reassortant strain. There was also an interaction effect between the mutated M and NP segments of -0.36 ($p<0.05$), which indicated an average decrease in virus titre when the mutated M and NP segments were combined in the delNS1 virus. Thus, the enhanced delNS1^{CA1} virus replication was the effect of these three mutated gene segments together. The enhanced replication of delNS1^{CA2} was determined by the mutated M segment alone (Fig. 4A). This observation was confirmed by statistical analysis, which appointed a coefficient of 2.0 ($p<0.001$) to M^{CA2}, indicating that the 100-fold increase in virus titre was solely determined by the M segment mutations. Reassortant virus containing all mutated gene segments (delNS1:[HA NP M NS]^{CA1} and delNS1:[HA M]^{CA2}) replicated equally well as the virus from which their segments originated, delNS1^{CA1} and delNS1^{CA2}, indicating that the mutated PB1 gene segments did not contribute to the enhanced virus replication.

Because the M segment plays a major role in increased replication of both adapted viruses, we further focused on the mechanism by which mutations in this segment could overcome the decreased replication in the absence of NS1. To determine which individual M^{CA2} mutation was responsible for virus titre increase, four additional mutant virus strains were made containing either the V97A or Y100H mutation, the combination of V97A and Y100H, or the remaining four silent mutations (Fig. 4B). When compared to delNS1, the two strains with single amino acid substitutions did not replicate more efficiently. However, when V97A and Y100H were combined in delNS1:M$^{CA2.3}$, a 50-fold increase in virus titre was observed. Furthermore, the four silent mutations increased the virus yield approximately 10-fold, as indicated by the comparison of delNS1 to delNS1:M$^{CA2.4}$ and delNS1:M$^{CA2.3}$ to delNS1^{CA2}. Interestingly, when introduced into the WT virus, the M^{CA2} segment decreased replication (Fig. 4B).

Effect of M segment mutations on M1 and M2 protein expression

To determine if the M segment mutations affected splicing of the M1 mRNA (Fig. 2B) we measured the ratio of M1 and M2 protein expression in cells at 10 hpi by Western blot analysis (Fig. 5A). The M1 protein of all Y100H mutant viruses (delNS1:M^{CA2}, delNS1:M$^{CA2.2}$ and delNS1:M$^{CA2.3}$) migrated slightly slower in SDS-PAGE than that of the other virus strains (Fig. 5A, lanes 4, 6 and 7 respectively). Slight changes in mobility in SDS-PAGE due to amino acid changes that affect protein charge, such as Y100H, have been observed before (130).

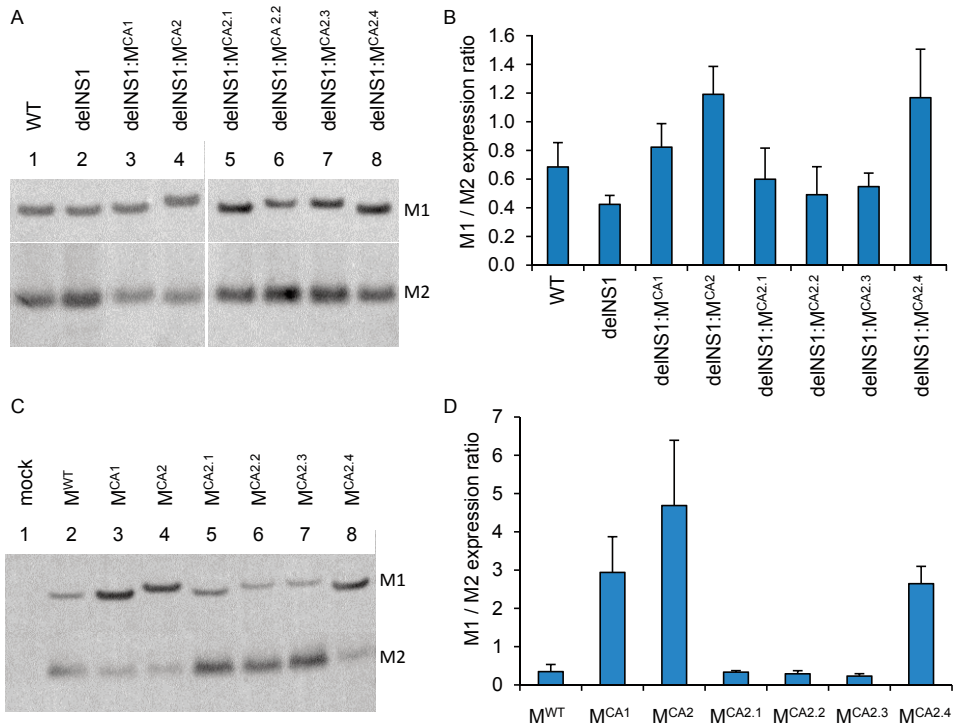

Fig. 5. Western blot analysis of M1 and M2 protein expression 10 h after infection of MDCK-SFS cells (A) or 48 h after transient transfection of HEK293T cells (C) with plasmid (Table 1) containing either the WT M segment (pHW197), the mutated M^{CA1} or M^{CA2} segments (pROM36 and pROM35), or the M segment containing one or more $delNS1^{CA2}$ mutations (pROM51-54). M1 and M2 protein levels of three independent experiments were quantified using densitometry. The M1/M2 ratio for each virus (B) or plasmid (D) is shown as the mean and 95% confidence interval of the mean. Lanes 1-4 and lanes 5-8 in panel A originated from two different blots.

Cells infected with delNS1 virus (Fig. 5A, lane 2) appeared to express more M2 protein than WT virus infected cells (Fig. 5A, lane 1). This difference was consistently observed in several experiments, even though the difference in M1/M2 ratio was not statistically significant from that of the WT virus (Fig. 5B). Infection with virus containing the mutated M segments, delNS1:M^{CA1} (Fig. 5A, lane 3) and M^{CA2} (Fig. 5A, lane 4), resulted in an M1/M2 ratio that was 2- and 3-fold higher, respectively, than that of delNS1 (Fig. 5B). Virus containing only the single or double M1 amino acid mutations (delNS1:$M^{CA2.1}$, $M^{CA2.2}$ and $M^{CA2.3}$, Fig. 5A lanes 5, 6 and 7) showed an M1/M2 ratio similar to delNS1, whereas delNS1:$M^{CA2.4}$ virus (containing the 4 silent M^{CA2} mutations) showed a 2-fold increase (Fig. 5A lane 8 and Fig. 5B). The effect of the mutations on M1 and M2 expression was confirmed by transfection of HEK293T cells with plasmids encoding the different M segments (Fig.

5C). The M1/M2 ratio of cells transfected with WT M segment (0.35; Fig. 5D), is comparable to that of delNS1 infected cells (0.42; Fig. 5B). The M1/M2 expression ratio was higher with segments containing the original mutations acquired during the adaptation (M^{CA1} and M^{CA2}) and with the M^{CA2} segment containing the four silent mutations ($M^{CA2.4}$; Fig. 5C lanes 3, 4 and 8), but not with the M^{CA2} segments containing one or both of the non-silent mutations (Fig. 5C lane 5, 6 and 7). Taken together, these results show that both cell-adapted viruses acquired mutations that increased the M1/M2 protein ratio in infected cells.

Effect of M1 amino acid substitutions on subcellular localization

The M1 mutations V97A and Y100H present in $delNS1^{CA2}$ are located close to the NLS at position 101-105 (Fig. 2C). To determine whether they affected the subcellular localization of M1 protein, cells were infected with WT, delNS1 or

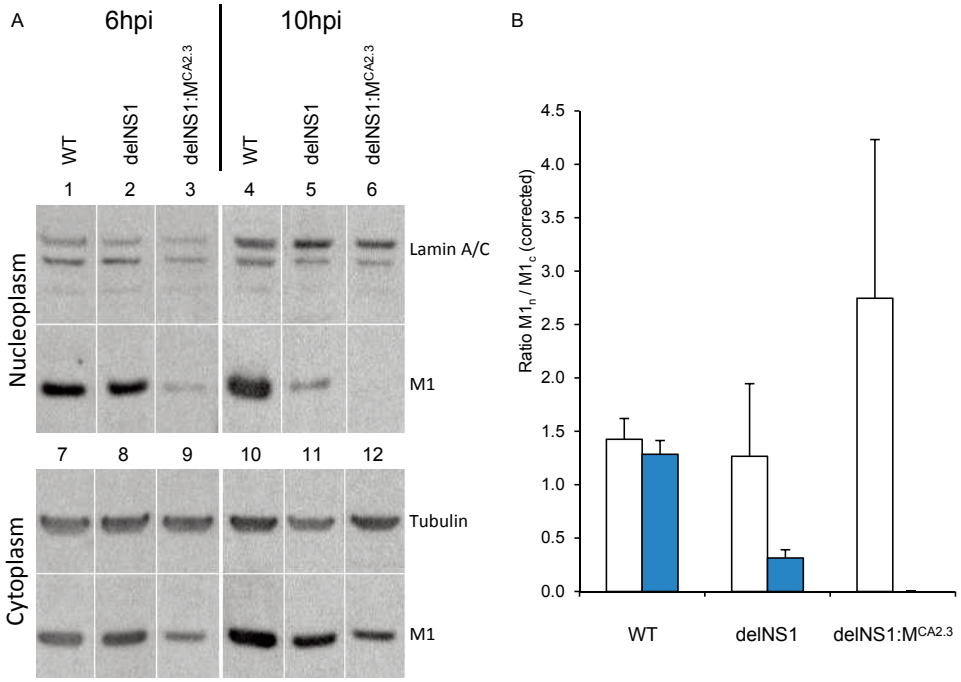

Fig. 6. Subcellular localization of M1 protein. MDCK-SFS cells were infected with WT, delNS1 and delNS1:$M^{CA2.3}$ virus at high MOI in triplicate. Then, cytoplasm and nucleoplasm fractions were prepared at 6 hpi and 10 hpi and M1 protein was quantified by Western blot analysis. Tubulin (Tub) and lamin A/C (Lam) were used as cytoplasm and nucleoplasm specific controls, respectively. Panel (A) shows one representative Western blot. (B) M1 protein levels were quantified by densitometry using tubulin and lamin A/C, respectively, as controls to compensate for the isolation efficiency. The ratio of corrected nuclear M1 ($M1_n$) to corrected cytoplasmic M1 ($M1_c$) was used to determine changes in the distribution of M1 and is shown as the mean and 95% confidence interval of the mean at 6 hpi (empty bars) and 10 hpi (filled bars).

delNS1:$M^{CA2.3}$ virus and the nucleoplasm and cytoplasm was isolated early (6 hpi) and late (10 hpi) in the infection process. The level of M1 present in both compartments was then determined by Western blot analysis and quantified by densitometry (Fig. 6A and B).

At 6 hpi, the ratio of nuclear to cytoplasmic M1 was comparable between WT and delNS1 virus infected cells (Fig. 6B). Four hours later, this ratio remained constant in WT virus infected cells whereas it was 4-fold lower in delNS1 virus infected cells, mainly due to a decrease in nuclear M1. Thus, NS1 appears to affect the concentration of M1 in the nucleus late but not early in the infection. The mutant virus containing only M1 amino acid substitutions V97A and Y100H showed a higher ratio at early stages, although this did not significantly differ from the ratio of the other two viruses. At 10 hpi nuclear M1 was not detectable anymore, thereby reducing the localization ratio below that of delNS1 infected cells.

Discussion

The low yield of delNS1 virus on MDCK cells is assumed to be caused by the inability of this virus to inhibit the antiviral host response (45). In this study, we obtained two delNS1 influenza virus variants that replicated to 250-fold higher infectious virus titres ($TCID_{50}$) after two parallel, serial passages on MDCK cells. Both adapted delNS1 virus variants induced IFN and apoptosis to a somewhat lower level than the original delNS1 virus, but still significantly higher than the WT virus. Apoptosis is linked to IFN induction (10, 162, 200) and early onset of apoptosis limits virus replication. IFN induction results from activation of the innate immune system by pathogen associated molecular patterns, but is inhibited by viral factors such as influenza virus NS1. It is unlikely that the lower IFN induction by the adapted viruses results from the appearance of a new IFN inhibitor and therefore, most probably, results from a lower activation of the IFN response. Enhanced influenza RNA production increases IFN levels (11) and influenza viral RNA accumulates in the absence of NS1 (180). It may therefore be possible that the adapted viruses produce less RNA than the delNS1 virus, resulting in a lower IFN activation. However, the adapted virus strains still induce a significant amount of IFN and lower RNA production may therefore not fully explain the considerable increase in titres. Several recent studies suggest that IFN has a minor effect on influenza replication in MDCK cells because canine myxovirus resistance proteins lack anti-influenza activity (148) and secreted IFN is proteolytically degraded by

trypsin, which is normally present during influenza production on MDCK cells (149). Furthermore, delNS1 virus titres could be increased by recombinant NS1 expression, without lowering IFN induction (170), indicating that IFN induction in MDCK may play a less important role than generally assumed.

The two adapted viruses contained a high frequency of either A-to-G or U-to-C substitutions (19 out of 22) that occur mostly (16 out of 22) in three clusters in M^{CA1}, M^{CA2} and NS^{CA1} gene segments. Furthermore, all mutations in M^{CA2} appeared to be acquired at the same time. Taken together, this suggests that these substitutions result from hyperediting by adenosine deaminases acting on RNA (ADAR), which cause A-to-G substitutions in RNA (143). Hyperediting of the influenza genome by ADAR has previously been reported (158). The occurrence of both A-to-G and U-to-C substitutions can be explained by hyperediting of the positive sense cRNA as well as the negative sense vRNA, respectively. Interestingly, ADAR1 is induced by IFN and is believed to have an antiviral role during influenza infection (143). Furthermore, NS1 interacts with ADAR1, suggesting that it inhibits ADAR1 function (116). Possibly, the high level of IFN induction and the absence of NS1 in delNS1 infected cells resulted in more RNA hyperediting.

By sequence analysis of the two adapted viruses and subsequent analysis of reassortant viruses generated by reverse genetics, we showed that six substitutions in the M segment were responsible for the increase in delNS1^{CA2} virus titres. The increase in delNS1^{CA1} viral titres was caused by substitutions in the NP, M and NS segments, where the M segment was most important. Previously, adaptation to Vero cells yielded an influenza B delNS1 virus with increased titres due to M1 amino acid substitution M86V (189). This is in striking contrast to the many studies on the adaptation of WT virus originating from eggs or clinical specimens to propagation in mammalian hosts or cell lines, which showed that adaptive mutations predominantly accumulated in the HA segment or segments encoding the RNA polymerase (PB1, PB2 and PA), which is assumed to be caused by adaptation to the different host species (65, 129, 138, 164). This suggests that the preferential isolation of M-segment mutations upon delNS1 virus adaptation compensates for the absence of the NS1 protein rather than the replication in a different host species. This conclusion is further supported by our observation that the introduction of the M^{CA2} gene segment into a WT virus (that produces NS1) does not enhance, but even reduces, viral replication.

Therefore, we focused on the mechanism by which the M segment mutations could improve replication in the absence of NS1. The M segment encodes the M1 matrix protein from unspliced M1 mRNA, whereas the M2 ion channel protein is transcribed from a spliced mRNA (Fig. 2B). A second splice product, $mRNA_3$, can arise from an alternative 5′ splice site and encodes a hypothetical and as yet undiscovered 9 amino acid peptide (82). It was previously shown that NS1 expression limits splicing, including that of the M segment-derived mRNA (92, 134), resulting in a higher ratio of M1 to M2 mRNA. Furthermore, M1 expression is reduced in MDCK cells infected with influenza virus expressing truncated NS1 (30, 34). We therefore measured M1 and M2 expression in infected cells and calculated the M1/M2 expression ratio, assuming that this would be dependent on the efficiency of M1 mRNA splicing. Indeed, cells infected with WT virus showed a higher M1/M2 expression ratio than delNS1 infected cells. The M^{CA1} and M^{CA2} segments showed significantly increased M1/M2 expression ratios as compared to the WT M segment, both when expressed using a delNS1 virus backbone and after transfection of cells with M gene-encoding plasmids. The M^{CA1} segment contains four silent mutations whereas the M^{CA2} segment contains four silent and two non-silent mutations. By generating novel reassortant viruses we could show that the altered M1/M2 expression ratio of $delNS1^{CA2}$ was due to the 4 silent mutations. Furthermore, these silent M^{CA2} mutations caused an increase in delNS1 viral titres, although not to the same extent as a segment that also contains the two non-silent M segment mutations. The silent M^{CA1} and M^{CA2} mutations lay in a region that contains the major determinants for M segment splicing (9). Thus it is likely that both these sets of mutations lower M1 mRNA splicing efficiency in a similar manner by restoring a balance that was disturbed due to the absence of NS1. Such a mechanism, aimed at restoration of M1 splicing efficiency may also explain why introduction of the M^{CA2} segment into a backbone of virus that produces NS1 (WT virus) reduces replication efficiency. Surprisingly, NS1 does not affect the M1/M2 expression ratio in Vero cells (142). Furthermore, absence of NS1 causes reduced M1 expression in MDCK but not in Vero cells (30). Taken together with our results this suggests that the improved replication of delNS1 virus in Vero cells as compared to MDCK cells is not only determined by the lack of an IFN response, but also by the ability of Vero cells to retain efficient M segment splicing and M1 expression in the absence of NS1.

The major part of the increase in delNS1^{CA2} virus titre resulted from the combination of M1 amino acid substitutions V97A and Y100H. As single substitutions these mutations did not affect replication efficiency. Mutation V97A was previously introduced into the A/WSN/33 [H1N1] strain (which is able to express NS1) and resulted in a 100-fold lower virus yield (16). Residues 97 and 100 are located on the helix 6 (H6) domain, a positively charged surface region between amino acids 91 to 105 of M1 (150). The influenza B M1 M86V mutation that enhanced delNS1 virus replication (189) is located near this region. The exact mechanism by which this mutation affected viral replication was not further investigated. The H6 domain has multiple functional motifs, including a nuclear localization signal (NLS) between amino acids 101-105 (Fig. 2C) that binds to cellular importin-α (14). Inside the nucleus, M1 binds to the vRNP complex, after which NEP can bind to the NLS of M1 (2). The vRNP-M1-NEP complex can then be exported to the cytoplasm were virus particles are assembled at the cell membrane (2). The localization of V97A and Y100H within the H6 domain suggests that they could affect M1 binding to NEP. Furthermore, Y100H is located immediately next to the NLS and may also affect importin-α binding, as described earlier for a mutation next to a NLS in PB2 (129). In this manner these mutations could affect M1 (and vRNP) subcellular distribution. The absence of NS1 during infection resulted in decreased levels of M1 in the nucleus at late stages of the infection. This may result from increased apoptosis induction by delNS1 virus as widening of the nuclear pores (37) allows diffusion of vRNPs out of the nucleus (93). Amino acid changes V97A and Y100H resulted in full depletion of M1 in the nucleus at 10 hpi, thus these mutations do not restore the M1 localization balance in delNS1 towards infection in the presence of NS1. It is therefore difficult to speculate how these two mutations can cause a 50-fold increase in virus titre.

Interestingly, the increased viral titres of delNS1^{CA1} virus were in part due to six nucleotide substitutions causing three amino acid substitutions in the NEP protein. Especially substitution I76V which is located within the domain that binds to M1 (2) could -similarly to the M1 mutations described above- affect vRNP nuclear export. Similar effects of M1 and NEP mutations that affect their interaction were observed earlier in WT virus. Mutations of NEP glutamate residues 67, 74 and 75 that bind M1, decreased vRNP content of viral particles and caused morphological virion changes similar to those that occur in virus particles with mutated positively charged M1 residues 95, 98, 101 and 102 that bind NEP (3, 16).

In this paper we showed that mutations in the M segment can enhance the replication of delNS1 virus due to both silent mutations that presumably affect the M1 mRNA splicing efficiency and non-silent M1 mutations that presumably affect its subcellular localization. These findings contradict the previous suggestion that the restricted replication of this virus is primarily due to the inability to inhibit the IFN response. The mutations described may have direct applications as they, for example, allow the development of delNS1 based viruses with improved replication efficiency, thereby making it possible to produce such a virus in other cell lines than Vero cells (140) or NS1 expressing MDCK cells (170). Moreover, these mutations may be combined with the G3A and C8U mutations in HA vRNA, which increased the HA expression level of a live attenuated NS1 truncated influenza vaccine strain (95). However, it will be necessary to determine the effect of these mutations on vaccine safety and efficacy.

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Chapter 6

General discussion

General discussion

Cell culture based production of avian influenza virus

Vaccines against influenza virus have been produced in embryonated chicken eggs for over 60 years. Potential difficulties associated with production in eggs include the intensive planning many months in advance of the vast amounts of eggs needed which limits flexibility to expand production, the possible loss of the Specific-Pathogen-Free status of laying flocks during disease outbreak, and sterility problems that may occur during processing of infected eggs (8). Cell culture based production offers greater robustness, flexibility and scalability. In comparison to production in eggs, cell based production is fully contained, thereby reducing the risk of contamination and offering the possibility to produce HPAI virus strains in biosafety level 3 production facilities (84). Moreover, cell lines can genetically be optimized to enhance production and vaccine quality, which is specifically useful for attenuated virus strains. Several commercially available human influenza vaccines are already produced in cell culture, such as Optaflu® by Novartis vaccines (Basel, Switzerland) and Celvapan® and Vepacel® by Baxter vaccines (Vienna, Austria). Veterinary influenza vaccines may also benefit from the development of these alternative production systems. The cost price of current veterinary influenza vaccines is, however, very low and will therefore be a major challenge if the existing egg-based production process is to be replaced by a cell culture based process. Furthermore, vaccines need to be available in time during an outbreak. Since stockpiling of vaccines against the large antigenic diversity of avian influenza strains is costly, a platform production process is preferred that is able to replicate any subtype of avian influenza virus to high enough titers, in a short time period, thereby allowing custom-produced vaccines. Here we discuss the different aspects of a cell culture based process for the production of avian influenza vaccines, to be used in poultry.

Influenza virus cell substrate

In Chapter 2, MDCK, Vero and BHK21 cell lines have been assessed for their ability to propagate a variety of different AI virus strains (171). Overall, the MDCK cell line yields the highest virus titers, although the difference with Vero cells is not very big as MDCK produce on average 3.6-fold more HAU (171). Currently, it is unclear which cell line is a better substrate for influenza virus and the choice for either cell line has been based on other aspects than virus yield, such as the historical use by a specific company (109). Recently, several new cell lines have been de-

veloped by immortalizing primary cells, including the human PER.C6® (125) and the avian AGE1.CR® (91) and EB14®/EB66® (104) cell lines, which all showed to be suitable for vaccine production. The use of avian cell lines above mammalian cell lines may offer additional benefits for the production of avian influenza virus as they are the natural host of these strains. However, unlike Vero, MDCK and BHK21 cells, the use of these proprietary cell lines will add license costs and have therefore not been evaluated in this thesis. Vero cells are appreciated for their long term safety record, in the production of polio and rabies vaccines. Furthermore, Vero cells lack an interferon (IFN) response, due to the absence of the IFN-β gene (27), which makes them especially useful for growth of immune response sensitive viruses, such as influenza virus containing deletions in NS1 (45, 170).

The cells used for vaccine production are often adherent cells (46). At large-scale production, these cells are grown on micro-carriers, which add additional costs to the vaccine. Furthermore, during scale-up cells have to be detached with for example trypsin and re-attached to new carriers, where it is very important to have an even distribution of cells over the new carriers. This not only introduces extra costs, but also extra variation in the process, increases contamination risk and thus makes the whole process less robust. In addition, cells grown on micro-carriers are more sensitive to shear caused by agitation and aeration compared to suspension cells, making scale-up and reaching high cell densities more difficult. Therefore, suspension growth would be preferred in veterinary AI vaccine production. Whereas several studies, including ours, have shown the possibility to adapt MDCK cells to suspension growth (21, 56, 90, 171), only one study showed this to be possible for Vero cells (121), suggesting a difference in the ability to adapt these cell lines to suspension growth. Furthermore, the substrate cell line should preferably be able to grow under serum-free conditions. Not only is serum an expensive medium component and may be a source of extraneous agents such as viruses, mycoplasmas and prions, additional washing steps and medium replacement steps are necessary for serum-dependent cells before infection, as serum components inactivate trypsin (47) that is required for high virus yields. Notably, Vero cells are also known to release a trypsin inhibitor (74), which necessitates repeated trypsin addition during the infection process.

Taken together, the serum-free MDCK-SFS suspension cell line (171) appears a preferred host cell line for commercial, veterinary AI vaccine production, because it grows serum free in suspension and the virus yield with these cells is high and

comparable to adherent MDCK cells. However, adaptation to suspension growth described in this thesis was not performed under GMP conditions and will therefore need to be repeated in order to obtain a GMP approved MDCK suspension cell line. Furthermore, the SFM4BHK21 culture medium is custom made and expensive, and may have to be replaced by a cheaper, generic medium. Our results showed that the ability of MDCK-SFS cells to grow in suspension appears to be linked to the nature of SFM4BHK21 medium, the composition of which is not disclosed (171). Therefore, if these cells were to be adapted to alternative media, it is important to focus on the ability of the cell line to grow in suspension.

In Chapter 3, BHK21 cells were also assessed as a host cell line for AI vaccine production (168). BHK21 cells have favorable growth characteristics as they are easily cultured in suspension, and grow 72% faster and to 3.6-fold higher maximum cell density than MDCK cells (168, 171). Furthermore, BHK21 cells have a compromised innate immune response, due to a deficiency in the RIG-I pathway, resulting in a complete lack of IFN production (57). Although most AI virus strains replicated poorly in these cells, a few strains replicate very efficiently and yielded virus titers that exceeded those of MDCK and Vero cells infected with the same strains (168, 171). This indicates that BHK21 cells have the potential to efficiently propagate influenza virus. The strict, strain dependent replication in BHK21 cells was also observed between the two related H1N1 strains PR8 (poor replication) and WSN (efficient replication). Reassortant viruses in which the HA was exchanged revealed that HA determined replication in BHK21 cells (168). Reassortant virus strains with chimeric HA molecules did not reveal, however, which domain of PR8 HA limits the ability to replicate in BHK21 cells. Nevertheless, the results did show that BHK21 cells could be used to replicate virus containing a chimeric protein consisting of the antigenically important HA1 domain of PR8 in combination with the HA2 domain of WSN. If this approach also allows the production of viruses containing the HA1 domain of other strains that normally do not replicate in BHK21 cells, this may offer a novel production platform for influenza vaccines.

Besides growth, other important aspects for choosing a particular cell line are the quality and the efficacy of the vaccines resulting from the use of these cells. The specific host cell line may, for example, change HA glycosylation patterns (46, 138, 139), which may affect down-stream processing (119), and might be relevant for

the immunogenicity of the resulting vaccine. Before choosing a specific host cell, it is therefore important to assess the efficacy of the viral vaccines produced using that particular host cell.

Optimization of media and process conditions

A major benefit of cell culture based production over the use of embryonated chicken eggs is the possibility to monitor, control and optimize process conditions in order to obtain maximum virus yields and even more important a good quality of the final product (43). Optimizing process conditions such as m.o.i., trypsin concentration and virus harvesting time have shown to increase virus yield (1, 19, 50, 122, 149). Furthermore, optimization of the culture media and process conditions can improve the cell growth rate and maximal cell density as well as the virus yield per cell and through these the final virus concentration reached (19, 51, 152). As stated, even more important than virus yield is the quality of the final produced vaccine. For example, it is important to optimize the pH of the culture media, as a low pH during the virus infection in Vero cells is necessary to prevent the occurrence of mutant virus with lower stability to acidification and elevated temperature (114). These mutants showed significantly lower immunogenicity and this phenotype was caused by an increased pH threshold of the HA conformational change. Finally, fed-batch and perfusion bioreactors can be used to further increase the cell density and final virus concentration (56). In suspension and micro-carrier based batch cultures, MDCK cell densities are normally around 2 x 10^6 cells/ml (13, 90, 171), however concentrations up to 11.2 x 10^6 cells/ml were obtained with MCDK cells adhered to micro carriers (13) and up to 17.5 x 10^6 cells/ml for suspension MDCK cells concentrations (56). However, high cell density may result in a lower cell-specific virus yield, which is called "cell density effect" (97, 188). The mechanism behind this cell density effect is still unknown. Despite this, Bock et al. (13) were able to use fed-batch and perfusion bioreactors to obtain very high virus titers.

Cell line engineering

By resolving factors that limit virus replication in the cell, the cell-specific virus yield can be further enhanced. A logical first approach would be to knock out the cellular antiviral response. Recombinant expression of the V protein of the paramyxovirus simian virus 5 in three human cell lines blocked IFN signaling and enhanced the titer of a variety of different viruses, including slow-growing wild-type viruses and attenuated vaccine candidates (194). However, several of the es-

tablished cell lines used for virus growth, including Vero (27) and BHK21 (57) cells, already have a defective IFN system and this approach will probably not result in increased yields. For MDCK cells this may be a feasible approach. Another target to enhance virus yield may be apoptosis. Influenza virus production starts approximately 4-6 hour post infection (146) with the budding of virus particles from the cell surface and continues until the cell dies by apoptosis. Delay of apoptosis could therefore extend the duration in which an infected cell produces new virion particles and thereby enhance the cell-specific virus yield. Expression of anti-apoptotic genes has been shown to enhance monoclonal antibody production in Chinese hamster ovary cells (39), whereas the addition of anti-apoptotic chemicals to the culture medium can prolong the life of cells infected with a Sindbis virus vector and enhance recombinant protein production (101). Furthermore, delayed apoptosis may also lower the number of lysed cells at the time of harvest, lowering the DNA and protein content of the culture medium and thereby facilitating downstream processing. However, this approach has not yet been successful to enhance influenza virus production as inhibition of influenza-induced apoptosis, either by stable expression of BCL2 (118) or addition of a caspase 3 inhibitor to the culture medium (190), reduced the virus titer. This indicates that apoptosis or parts of the apoptosis machinery are beneficial for virus replication. It was therefore hypothesized that the influenza virus not only acquired the capability to suppress antiviral and pro-apoptotic responses, but also the ability to misuse the remaining antiviral activities to support virus replication (93). Although tools such as proteomic analysis (173) and modeling of the influenza virus replication (64, 147, 153) can hint to what factors limit virus replication, these results demonstrate that our knowledge of the infection process is still insufficient to predict the outcome of a rational cell engineering approach.

Comparison of the transcription profiles of adherent and non-adherent HELA cells using DNA microarrays revealed the human sialyltransferase ST6GalNac V as an important factor in cellular adhesion. Recombinant expression of this protein by inserting the siat7e gene in anchorage-dependent MDCK cells allowed these cells to grow in suspension (21). Surprisingly, the siat7e-expressing MDCK cells also showed a 20-fold higher HA production (22). Others have successfully transfected MDCK, Vero, PER.C6® and CHO cells with the gene for human 2,6-sialyltransferase (siat1), which increases the amount of receptors containing sialic acid (SA) with α-2,6 linkage (12, 62, 79, 85, 102). Most of these cell lines not only showed up to 100-fold increase in virus yield (62), but expression of this gene also resulted in

an improved ability to isolate human influenza virus strains (79, 117) and an increased sensitivity to neuraminidase inhibitors (62, 102). The siat1 gene was successfully introduced in an M2 expressing complementary MDCK cell line, to enhance replication of an influenza virus strain that is unable to encode M2 (delM2) (174). In order to enhance avian influenza virus production in MDCK cells, recombinant expression of a sialyltransferase that specifically increases the presence of SA α-2,3 linkage receptors would probably result in the same increased virus growth as expression of the SA α-2,6 receptors in these cells do for growth of human influenza viruses.

Influenza virus strains unable to express NS1 (delNS1) have potential use as DIVA and live-attenuated vaccines. However, they replicate poorly in most cell lines and are therefore difficult to produce on commercial scale. In Chapter 4, we developed two MDCK cell lines able to express NS1 (170). Complementation of NS1 by these cell lines during infection with delNS1 virus yielded up to 500-fold higher infectious virus titers. Similar complementary cell lines have been described to enhance replication of influenza virus strains unable to express PB2 (120), NA (69) and M2 (181). Due to the reported toxicity of NS1, we used an inducible expression system to selectively express NS1 before infection but not during the cell growth phase. In commercial production systems, however, inducible expression systems are not preferred due to the addition of the expression inducer doxycycline, which is an antibiotic, and because the complicated process is less robust. Therefore, a stable production cell line with constitutive NS1 expression would be preferred. Interestingly, this may be possible as we did not observe apoptosis induction upon NS1 expression in the two developed cell lines, possibly due to the specific NS1 gene used or the low NS1 expression levels.

Virus engineering

Wild type human influenza strains do not usually grow well in eggs or cell culture and are therefore reassorted with a high yielding donor strain (PR8 [A/Puerto Rico/8/34] or a derivative) resulting in a vaccine strain that contains the HA and NA of the wild-type virus and all other segments from the donor strain. With the reverse genetics approach it is now possible to alter the virus more specifically, such as in the creation of NS1, M2, PB2 and NA deletion mutants. Furthermore, the introduction of specific mutations allows to specifically engineer the virus to increase its yield and antigenic quality. Amino acid substitution K58I in HA2 lowers the pH that induces the conformational HA change, thereby increasing the re-

sistance of the virus to acidic pH and high temperature treatment. Live attenuated vaccines are often applied intranasally and thus must overcome the low pH of the mucosal surface. Immunization of mice with a live attenuated delNS1 vaccine strain containing the K58I mutation induced a stronger systemic and local antibody response than immunization with a similar virus lacking this mutation (80). Amino acid substitution E627K in PB2 is a well-known host determining mutation and is frequently observed during adaptation of an avian influenza strain to a mammalian host (100, 196). Inserting this mutation in an avian influenza strain may therefore be used to enhance replication in a mammalian host cell. Furthermore, it will be of great interest to determine if the recently discovered influenza proteins PB1-F2 (24) and PA-X (71) can be used to enhance virus replication in cell culture, e.g. by modulating the protein or removing it from the virus.

It was previously shown that influenza viruses encoding a truncated NS1 express viral proteins such as HA and M1, to lower levels than WT virus (30, 35, 157). HA is the main influenza antigen and low HA expression levels may therefore lower the immunological properties of NS1-truncated influenza vaccines. To overcome this, G3A and C8U "superpromotor" mutations (115) were introduced into the HA vRNA of a NS1 truncated influenza strain, resulting in enhanced HA expression (95). Mice that were immunized with this virus showed enhanced protection from wild-type virus challenge in comparison to mice vaccinated with a similar virus lacking the promoter mutations. Therefore, these promoter mutations may be useful to enhance HA protein production in other delNS1 based virus strains, and possibly in other influenza vaccine strains, as well.

A rational approach to cell line and virus engineering does not always give the required result due to lack of understanding of the complex interplay between the virus and its host. Recent studies revealed that influenza A virus replication requires 1449 host proteins (182), and 87 virus-host and 21 virus-virus protein interactions (151), of which only a minor part of the binding sites have been revealed. An example of a failed rational approach are the attempts to enhance virus yield by inhibition of apoptosis, which resulted in a decrease of virus yield (118, 190). As obligatory parasites, viruses evolve together with their hosts, as both are in a continuing struggle for survival. The high mutation rate of the viral RNA polymerase and the vast amount of progeny virus particles allow the virus to quickly adapt to changes in the environment, such as production of antibodies by the host. In a sense, virology provides us with a window on the mechanism of evolution.

Transmission, entry, host defenses, tissue diversity and anatomical restrictions all are serious obstacles to prevent infection and the influenza virus is optimized not to produce as much infectious virus particles as possible, but to efficiently overcome all these barriers. The environment in which the influenza virus is commercially produced is however very different, as the virus is generally propagated in a different cell type and host species than the one from which it was isolated. Furthermore, secondary defenses such as the adaptive immune response and physical barriers are absent in cell culture. Therefore, vaccine strains often do not replicate well on the production cell line or eggs and require adaptation to their new host (46, 137). The viruses resulting from such an adaptation can thus be used to identify targets for virus engineering to improve virus yield. DelNS1 virus replicates poorly in MDCK cells, which is generally believed to result from the inability of the virus to counter the antiviral response of the cell. The restricted replication of delNS1 introduced a high selective pressure (136) for variants able to replicate efficiently in the absence of NS1, and in Chapter 5 adaptation resulted in 2 strains which showed a 250-fold increase in virus yield (169). In both virus strains, mutations were found that significantly increased virus yield, and these could be used to improve delNS1 virus yield. Interestingly, the mutations appeared not to directly counter the IFN response of the cell, which would logically have been the target in a rational approach. Parallel virus adaptation (129) is therefore a valuable tool to enhance our fundamental understanding of the relationship between the virus and its host, and to identify targets for virus engineering, which are unlikely to be found by rational analysis. However, it is important to specifically focus on the selection mechanism and the resulting mutations. Adaptation of wild-type virus to embryonated eggs and cell lines could increase virus yield, but may also reduce immunological properties, due to mutations in the antigenic sites of the HA protein that were selected during the adaptation process as they enhanced receptor binding (44), virus entry and HA stability (87). Furthermore, it will be necessary to evaluate the safety aspect of these mutations, especially when they are used in a live attenuated vaccine.

Future perspective

Unlike avian diseases such as Newcastle disease, to which poultry are vaccinated, outbreaks of avian influenza are generally dealt with by culling birds at the affected farms. Traditional AI vaccines allow limited differentiation between birds that are vaccinated and birds that carry the virus. Fear for spreading the virus therefore limits the export of vaccinated birds and with that the application of pre-

ventive vaccination. The large scale application of vaccines against AI in poultry is therefore determined by political and economical reasons. Development of cheap and effective DIVA vaccines in combination with the growing societal resistance against large scale culling may pave the way for introduction of vaccination.

Cell culture based human influenza vaccines can financially compete with vaccines produced in eggs. The low cost price of vaccines for poultry will, however, require lower production costs and/or higher yields. Several suggestions have been made regarding virus yield optimization, including via cell line and virus engineering. The use of fed-batch and perfusion bioreactors may increase the yield, if the "cell density effect" can be resolved. Furthermore, the use of a suspension cell line which grows in a generic, serum-free medium will lower production costs. Finally, a generic production platform will allow different AI vaccines to be produced in the same cell line under similar conditions. This will aid the development and registration of new vaccines, thereby further limiting costs and delivery time to the market. A financial analysis will be required to determine which steps are necessary in order to obtain a marketable veterinary AI vaccine.

And finally, avian influenza vaccines may benefit from other developments in the fight against human influenza as well. Current work on universal influenza vaccines appears especially promising. Such a vaccine would induce antibodies that target conserved regions of the influenza virus, such as the M2 protein or the stalk of the HA protein (35) and would protect against a wide variety of serotypes. Stockpiling vaccines against different AI subtypes would thus not be necessary. Furthermore, such universal vaccines could easily be made according to the DIVA principle. The substrate on which these future vaccines will be produced is, however, yet to be determined.

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Summary

Vaccination of poultry can be used as a tool to control outbreaks of avian influenza, including that of highly pathogenic H5 and H7 strains. Such outbreaks do not only cause economical losses in poultry farming, but also pose a serious threat to global public health. Influenza vaccines are traditionally produced in embryonated chicken eggs. Continuous cell lines have been suggested as an alternative substrate to produce influenza vaccines in a more robust and flexible manner, as cell culture based production lacks the long lead time associated with the supply of large amounts of embryonated eggs. Furthermore, cell culture process conditions can be optimized and cell line engineering can increase virus yield and improve product quality.

Madin-Darby canine kidney (MDCK) and African green monkey kidney (Vero) cells are the main cell lines used for influenza virus replication. However, both cell lines normally grow anchorage-dependent. Large-scale virus production with anchorage dependent cell lines is complicated as process scale-up will require detachment and reattachment of the cells from and to micro-carriers or roller bottles between each cultivation step. Furthermore, MDCK and Vero cells require the addition of serum to the culture medium. Serum is not preferred due to the high costs, lot-to-lot variation and risk of contamination with extraneous agents. Furthermore, serum addition necessitates medium exchange before infection, as serum components inhibit trypsin which is required for efficient virus replication. In **Chapter 2** we therefore tried to adapt different cell lines to grow as suspension cells in serum-free (SF) medium. Using a specific culture medium, we were able to obtain an MDCK cell line (MDCK-SFS) that grows efficiently in SF medium in suspension. Vero cells could be adapted to growth in SF medium (Vero-SF), but not to growth in suspension. Baby hamster kidney cells (BHK21), the third cell line used in this study already grew in suspension and was adapted to SF growth (BHK21-SFS).

Next, the production of eight different avian influenza virus strains was compared between MDCK-SFS, Vero-SF and BHK21-SFS cells. On average, MDCK-SFS cells produced the highest virus titers, closely followed by Vero-SF cells. Interestingly, two virus strains replicated to higher titres in BHK21-SFS cells than in MDCK or Vero cells. However, six further virus strains replicated poorly in BHK21-SFS cells. Analysis of the influenza virus binding receptors on the cell membrane revealed that BHK21-SFS cells consists of two populations, with one population lacking the avian influenza virus preferred sialic acid α-2,3 receptors. This could however not

explain the strain dependent influenza virus replication. MDCK-SFS was the only cell line able to grow SF in suspension and replicate a wide variety of influenza strains to high titers and was therefore selected for further studies in **Chapters 4** and **5**.

BHK21 is a fast growing suspension cell line that can reach high cell densities in batch culture and is currently used in the production of veterinary vaccines against foot-and-mouth disease and rabies. However, the selective replication of particular influenza virus strains limits their potential use in influenza vaccine production. The molecular basis of this selective virus replication was further investigated in **Chapter 3**. Model strain A/PR/8/34 [H1N1] (PR8) was found to replicate poorly, whereas the related strain A/WSN/33 [H1N1] (WSN) replicates efficiently in BHK21-SFS cells. This difference is determined by the hemagglutinin (HA) protein since reciprocal reassortant viruses with swapped HA gene segments grow on BHK21-SFS cells to comparable titres as the parental virus from which their HA gene is derived. Furthermore, the ability of several other influenza virus strains to grow on BHK21-SFS cells also appears to depend on the HA gene. To determine which part of the HA protein determines virus replication in BHK21-SFS cells, PR8 based reverse genetics viruses that encode PR8-WSN chimeric HAs (cHAs) were generated. This approach did, however, not allow us to identify a single specific region of HA that determines the ability to replicate in BHK21-SFS cells, as virus containing the WSN HA1 domain and the PR8 HA2 domain replicated equally well as the reciprocal strain containing the WSN HA2 and PR8 HA1. Viruses expressing two other reciprocal cHAs also replicated equally efficient. However, these results do show that it is possible to replicate a virus encoding the PR8 HA1 domain to high titers by fusion to the WSN HA2 domain. The HA1 domain contains most antigenic sites and is therefore important for vaccine efficacy. This method of producing the HA1 domain as fusion to a heterologous HA2 domain could therefore potentially be used to produce HA1 domains of other viruses, and enable the use of BHK21-SFS cells as a generic platform for veterinary influenza vaccine production.

Influenza viruses that are unable to express the NS1 protein (delNS1) replicate poorly on cells and induce high amounts of interferon (IFN). Their attenuated replication is generally assumed to result from the inability to counter the antiviral host response. DelNS1 virus strains are currently considered as candidate viruses for live-attenuated influenza vaccines and veterinary vaccines that allow differen-

tiation of infected from vaccinated animals (DIVA). However, their attenuated growth limits large scale virus production on MDCK cells. To develop a high yielding delNS1 virus production system, we made two MDCK cell lines that are able to express NS1 (**Chapter 4**). Since heterologous NS1 expression can cause apoptosis, we used an inducible expression system. Upon induction, both cell lines replicated delNS1 virus to equally high titers as were obtained with an isogenic virus strain able to express NS1, to which we refer as wild-type (WT) virus. Notably, the NS1 expression by both cell lines was about 1000-fold lower than in influenza virus infected cells. Furthermore, NS1 expression inhibited delNS1 virus-induced apoptosis, but did not significantly lower IFN production. Thus, the enhanced virus replication appeared not to be due to decreased IFN production. In contrast to previous findings, recombinant NS1 expression by itself did not induce apoptosis, nor did it affect cell growth. This may be due to the specific allele B NS1 gene used. As a consequence, it may therefore be possible to develop a stable cell line with constitutive expression of allele B NS1, which can be used to enhance delNS1 yield in large-scale vaccine production.

Another approach to enhance virus replication is virus adaptation to the cell substrate. In **Chapter 5**, the delNS1 virus was adapted in two parallel experiments, which resulted in two virus strains with 250-fold higher infectious virus titers in IFN competent MDCK cells. By sequence analysis of the adapted viruses and subsequent analysis of reassortant viruses generated by reverse genetics, we showed that mutations in the M gene segment of one virus (delNS1^{CA2}), and in the M, NP and NS segments of the other virus (delNS1^{CA1}), caused the significant increase in virus replication. The most notable ones were clustered U-to-C mutations in the M segment of both strains and clustered A-to-G mutations in the NS segment of delNS1^{CA1}. Presumably these mutations resulted from host cell mediated RNA editing. NS1 is known to limit splicing of the M segment mRNA, resulting in a higher M1/M2 mRNA ratio. Interestingly, the silent mutations in the M segment of both adapted virus strains increased the ratio of M1 to M2 expression, probably by affecting splicing efficiency, towards the ratio observed in the WT virus. Furthermore, two amino acid substitutions in the M1 of delNS1^{CA2} virus additionally enhanced virus replication, possibly through changes in the subcellular localization of M1. Both adapted viruses induced less IFN and less apoptosis compared to the delNS1 virus, but still significantly more than the WT virus. It appears that the restricted replication of delNS1 virus is not only caused by the inability to counter the host cell antiviral response, as suggested in the literature, but also by the

loss of one or more M1-related functions of NS1. The mutations identified may have direct applications as they can be used to enhance delNS1 virus replication for vaccine production.

Cell culture based human influenza vaccines can financially compete with vaccines produced in eggs. The low cost price of vaccines for poultry will make it necessary to lower the production costs of a cell culture based production process, for example by increasing the yield of virus per liter medium. In **Chapter 6**, several suggestions have been made regarding virus yield optimization, including engineering of the vaccine virus and substrate cell line. However, large-scale implementation of poultry vaccination is not only determined by the price of the vaccines, but also by macro-economical and political factors. Future developments in veterinary influenza vaccines, such as broadly protecting influenza DIVA vaccines, may further help to implement emergency vaccination as a tool to control and possibly also to prevent outbreaks of avian influenza.

Samenvatting

Vogelgriep (of aviaire influenza) is een besmettelijke vogelziekte die veroorzaakt wordt door het influenza A virus. Tot deze virusgroep behoren ook virussen die griep veroorzaken in andere soorten, zoals mensen, varkens, paarden en zeezoogdieren. Over het algemeen zijn griepvirussen vrij gastheer specifiek, wat betekend dat humane virusstammen alleen mensen infecteren en vogelgriepvirussen alleen vogels. Echter, in sommige gevallen kan het virus van de ene op de andere soort overspringen. Watervogels, zoals eenden en ganzen, zijn de hoofdgastheer het influenza A virus maar vertonen zelf weinig tot geen ziekteverschijnselen. Zij kunnen het virus overbrengen op andere vogelsoorten, zoals kippen en kalkoenen, die wel ziek worden. Een uitbraak van vogelgriep kan daardoor economische schade veroorzaken voor de pluimvee industrie. Er bestaat echter ook de mogelijkheid dat vogelgriepvirussen van het H5 en H7 subtype muteren in dodelijke virusvarianten. In het recente verleden is gebleken dat deze virussen ook mensen kunnen besmetten, met veelal ernstige gevolgen. Om de volksgezondheid te beschermen is het daarom noodzakelijk om uitbraken van vogelgriep te voorkomen of te beheersen. Hiervoor zijn een aantal maatregels beschikbaar, waaronder vaccinatie van pluimvee.

Influenza vaccins worden traditioneel geproduceerd in bevruchte kippeneieren. Deze methode is echter vrij omslachtig omdat zeer grote hoeveelheden bevruchte eieren vele maanden van te voren besteld dienen te worden. Vaccinproductie op basis van dierlijke celkweek is robuuster en flexibeler, en is daarom als alternatief voorgesteld. Voor influenza vaccin productie worden voornamelijk MDCK en Ver cellen gebruikt (respectievelijk honde- en apeniercellen). Echter, beide cellijnen groeien alleen wanneer zij gehecht zijn aan een oppervlak. Voor grootschalige virusproductie is dus een zeer groot oppervlak nodig, in de vorm van rollerbottles of micro-carriers. Daarnaast dienen gehechte cellen gedurende de groei herhaaldelijk losgehaald te worden van het oppervlak. Ook vereisen beide cellijnen toevoeging van serum aan het groeimedium. Serumgebruik heeft echter een aantal nadelen, waaronder hoge kosten, de variatie tussen productiebatches en de kans op besmetting van het vaccin door aanwezigheid van virussen of prionen in het serum. Verder is bij gebruik van serum een wasstap noodzakelijk, omdat serumcomponenten interfereren met de trypsine die nodig is voor infectie. In **Hoofdstuk 2** is daarom geprobeerd om verschillende cellijnen te adapteren naar groei in suspensie in serumvrij medium. Door gebruik te maken van een specifiek medium is het gelukt om een MDCK cellijn te ontwikkelen (MDCK-SFS) die onder deze condities groeit. Vero cellen konden aan serum-vrij condities geadapteerd worden,

maar niet aan groei in suspensie (Vero-SF). Een derde cellijn, BHK21 (hamsterniercellen), groeide al in suspensie en werd geadapteerd naar serumvrije condities. Vervolgens werden deze drie cellijnen vergeleken in hun vermogen om acht verschillende influenza virusstammen te produceren. MDCK-SFS cellen hadden de hoogste virusproductie voor de meeste virusstammen, hoewel de productiviteit van Vero-SF cellen niet veel lager lag. De productie in BHK21-SFS cellen was opmerkelijk omdat zes van de acht virusstammen slecht repliceerde in deze cellijn, terwijl de overige twee virusstammen beter repliceerde dan in MDCK en Vero cellen. Humane en vogelgriepvirussen binden bij voorkeur aan celmembraanreceptoren met, respectievelijk, α-2,6 en α-2,3 siaalzuurreceptoren. Receptor analyse toonde aan dat de BHK21-SFS cellijn bestaat uit twee cel populaties waarvan er één geen α-2,3 siaalzuurreceptoren bezit. De uitkomst van deze analyse kon daardoor niet de virusstamafhankelijke replicatie van influenza in BHK21 cellen verklaren. MDCK-SFS was de enige cellijn die onder serumvrije condities in suspensie kan groeien en tegelijk van verschillende virusstammen grote hoeveelheden virus kan produceren. Deze cellijn is daarom gebruik voor verdere studies in Hoofdstuk 4 en 5.

BHK21 cellen worden gebruikt voor de productie van veterinaire vaccins tegen mond-en-klauwzeer en hondsdolheid, en hebben als voordeel dat ze snel groeien en een hoge celdichtheid kunnen bereiken in batchkweek. Door de virusstam afhankelijke replicatie lijken BHK21 cellen niet geschikt voor de productie van influenza vaccins. De moleculaire basis voor deze selectieve replicatie is onderzocht in **Hoofdstuk 3**. De influenza stam A/PR/8/34 [H1N1] (PR8) repliceert slecht terwijl de verwante A/WSN/33 [H1N1] stam (WSN) zeer goed repliceert in BHK21 cellen. Het verschil blijkt te worden veroorzaakt door het hemagglutinine (HA) eiwit van het influenza virus, want wanneer het HA van het PR8 virus vervangen wordt door het HA van WSN, dan repliceert het virus goed, terwijl een WSN virus waarin het HA vervangen is door het HA van PR8 juist slecht repliceert. Het HA lijkt verder ook de oorzaak te zijn voor de gelimiteerde replicatie van andere influenza virusstammen in BHK21 cellen. Verschillende virussen werden ontwikkeld waarin delen van het HA eiwit zijn uitgewisseld tussen PR8 en WSN. Met behulp van deze chimeren konden we echter niet één specifiek gebied aanwijzen dat de replicatie in BHK21 cellen bepaald, virussen met het HA1 van PR8 en het HA2 van WSN repliceerde namelijk net zo goed als virus met het HA1 van WSN en het HA2 van PR8. Echter, de resultaten lieten wel zien dat het mogelijk is om het PR8 virus efficiënt in BHK21 cellen te produceren door alleen het HA2 eiwit deel te vervan-

gen door dat van WSN. Het HA1 domein bevat de voornaamste antigenen en is daarom van belang voor het vaccin. Deze methode zou het dus potentieel mogelijk maken om het HA1 deel van andere influenza virusstammen, die normaal niet repliceren in BHK21 cellen, te fuseren met het HA2 van WSN. Hierdoor zou de BHK21 cellijn gebruikt kunnen worden als een platform productiesysteem voor veterinaire influenza vaccins.

Influenza virussen die niet in staat zijn om het NS1 eiwit tot expressie te brengen (delNS1) repliceren slecht in de meeste cellijnen en induceren de productie van veel interferon (IFN). IFN is een signaal voor cellen om zich te verdedigen tegen een virusinfectie. Een belangrijke functie van NS1 is om de productie van IFN te remmen. De verzwakte delNS1 virusreplicatie wordt daarom toegewezen aan het feit dat het delNS1 virus niet in staat is deze antivirale reactie te voorkomen. DelNS1 virussen zijn door deze eigenschappen interessant om als basis voor een levend-verzwakt vaccin te dienen. Verder zouden ze ook als DIVA vaccin gebruikt kunnen worden. Dit zijn veterinaire vaccins waarbij gevaccineerde dieren te onderscheiden zijn van geïnfecteerde dieren. Echter, door de verzwakte replicatie is grootschalige vaccin productie moeilijk. Om toch hoge virusproductie te behalen hebben we twee MDCK cellijnen ontwikkeld in **Hoofdstuk 4**, die in staat zijn om het virale NS1 eiwit te maken. Beide cellijnen produceerden delNS1 virus tot hoge concentraties, vergelijkbaar met normale MDCK cellen die geïnfecteerd waren met een wild-type (WT) virus dat wel een functioneel gen voor NS1 bezat. Opmerkelijk genoeg produceerden de twee cellijnen ongeveer 1000 keer minder NS1 dan normale MDCK cellen die geïnfecteerd waren met het WT virus. Verder beschermde de heterogene NS1 expressie de cellen tegen delNS1 virus geïnduceerde celdood (apoptose). Er is was echter geen significant effect op de IFN expressie, wat erop wijst dat de verhoogde virusreplicatie niet veroorzaakt werd door een verlaging van de cellulaire antivirale reactie. Omdat uit eerder onderzoek was gebleken dat NS1 expressie in cellen tot apoptose kan leiden, hebben we een induceerbaar expressiesysteem gebruikt. Echter, in tegenstelling tot deze eerdere vindingen, had de NS1 expressie in beide cellijnen geen effect op celgroei en veroorzaakt het geen apoptose. Dit houdt mogelijk verband met het specifieke NS1 allel dat gebruikt is, en maakt het potentieel mogelijk om een cellijn te ontwikkelen die NS1 constitutief tot expressie brengt. Een dergelijke cellijn zou potentieel gebruikt kunnen worden voor grootschalige influenza vaccin productie op basis van het delNS1 virus.

Adaptatie van een virus aan een cellijn is een bekende methode om de replicatie efficiëntie te verhogen. In **Hoofdstuk 5** hebben we het delNS1 virus in twee parallelle experimenten geadapteerd aan MDCK cellen. Dit resulteerde in twee virusstammen die tot 250 keer meer infectieuze virusdeeltjes produceerden. Door analyse van het virale genoom en het maken van reverse genetics virussen hebben we vervolgens aangetoond dat mutaties in het M segment (beide virussen) en in het NP en NS segment (een virus) verantwoordelijk zijn voor de toename in virus replicatie. Opmerkelijk waren de geclusterde U-naar-C mutaties in de M segmenten van beide virussen en de A-naar-G mutaties in het NS segment. Deze mutaties zijn waarschijnlijk het gevolg van RNA editing door het cellulaire ADAR eiwit. Mutaties in het M segment bleken het belangrijkste te zijn voor de toename in virus replicatie in afwezigheid van NS1. Het is bekend dat NS1 een remmend effect heeft op de splicing van het M segment mRNA, waardoor de verhouding M1/M2 mRNA stijgt. De stille mutaties die werden gevonden in het M segment van beide virussen verhoogden het M1/M2 mRNA ratio. Hierdoor lijkt de ratio van de geadapteerde virussen meer op de ratio van het WT virus en dit komt waarschijnlijk doordat deze mutaties de splicing efficiëntie van het M1 mRNA beïnvloeden. Twee additionele M1 eiwit aminozuur mutaties bleken ook een significant effect te hebben op de virusreplicatie en mogelijk houdt dit verband met de lokalisatie van het M1 eiwit in de cel. Beide geadapteerde virussen induceerden minder IFN en minder apoptose dan het oorspronkelijke delNS1 virus, maar nog altijd meer dan het WT virus. Het lijkt daarom dat de lagere delNS1 virusreplicatie niet alleen veroorzaakt wordt doordat het virus niet in staat is om de IFN expressie te onderdrukken, zoals gesuggereerd in de literatuur, maar ook door het gebrek aan 1 of meer M segment gerelateerde functies van NS1. De gevonden mutaties bieden meer inzicht in de werking van het NS1 eiwit en zouden verder gebruikt kunnen worden om delNS1 virusproductie te verhogen in een commercieel vaccin productie proces.

De productie van influenza vaccins op basis van celkweek kan financieel competeren met vaccins die geproduceerd zijn op de klassieke manier in bevruchte eieren. Door de lage prijs van vaccins voor pluimvee is het noodzakelijk om de productiekosten van een celkweek proces zo laag mogelijk te krijgen, bijvoorbeeld door het verhogen van de opbrengst per liter medium. In **Hoofdstuk 6** worden verschillende suggesties gegeven om de virusproductie te verbeteren, waaronder het aanpassen van de vaccin virus stam en de substraat cellijn. Echter, het grootschalig vaccineren van pluimvee tegen vogelgriep wordt niet alleen bepaald

door de prijs van vaccins, maar ook door macro-economische en politieke factoren. Toekomstige ontwikkelingen in veterinaire influenza vaccins, zoals een breed beschermend DIVA vaccin moet het mogelijk maken om vaccins vaker toe te passen als methode om uitbraken van vogelgriep te beheersen en mogelijk ook te voorkomen.

摘要

Chinese summary

禽流感的爆发不仅仅会为家禽养殖造成经济损失，同时也会严重威胁全球公众的健康。因此为家禽接种疫苗，由此抵御病毒的传播，包括预防高致病性H5、H7菌株，是当前控制疫情的发生和传播的有效手段之一。传统的禽流感疫苗是在鸡胚胎蛋中繁殖和培育的。然而由于鸡胚胎蛋的局限性：例如等待胚胎蛋生产和供应的时间过长，繁琐的操作；使病毒的培育以及疫苗的制作过程变得复杂。因此，另一种培育方式：在可连续传代的细胞系中繁殖禽流感病毒，从而制作禽流感疫苗，已经被建议作为一种可以代替传统工艺的方式。相比于鸡胚胎蛋，细胞培养更易于操作，并且细胞培养的工艺条件可以被尽可能的优化，从而提高病毒的产量和产品的质量。

犬肾传代细胞（MDCK）和非洲绿猴肾细胞（Vero细胞）是当前主要用于禽流感病毒复制的细胞系列。然而，这两种细胞株生长的局限性和依赖性使病毒的生产过程复过于复杂。在生产病毒的过程中，细胞对于微载体的附属和分离要在不同的步骤之间转换。而相对于细胞自身来讲，这两种细胞在繁殖的过程中都需要在培养基中额外添加血清。血清具有成本高，生产批量存在差异的缺点，并且血清极易被外界因素污染。以上种种都为成功生产病毒从而制作疫苗带来不可预知的风险。此外，众所周知，血清有抑制胰蛋白酶的作用。而胰蛋白酶却是病毒有效复制的关键因素之一，因此在病毒感染细胞前加入血清会在一定程度上影响病毒的复制和繁殖。为了有效解决这个问题，在本论文的第二章中，我们尝试培养适应无血清培养基、并且能够在悬浮状态中繁殖的细胞。使用特殊的培养基，我们得到可以在无血清培养基的悬浮介质中有效增长的MDCK细胞（MDCK-SFS）。虽然Vero细胞也可以适应无血清培养基（Vero-SF），但是Vero细胞无法在悬浮介质中增长。此外，我们也成功培养了仓鼠崽肾细胞（BHK21）适应无血清培养基并以悬浮状态生长（BHK21-SFS）。

在获得可用于病毒繁殖且不需要血清参与的细胞的基础上，八个不同的禽流感病毒株被分别培养在MDCK-SFS，Vero-SF和BHK21-SFS细胞上进行比较。平均而言，MDCK-SFS细胞产生的病毒浓度最高，其次是Vero-SF细胞。有趣的是，在BHK21-SFS细胞上，两种病毒株各自复制后的浓度要远远大于这两种病毒株在MDCK-SFS或是Vero-SF细胞上复制后的浓度。然而，其余六种病毒株在BHK-21SFS细胞上复制的结果却不理想。针对于这个现象，我们对细胞膜表层的流感病毒的结合受体进行了分析。分析后我们发现，BHK21-SFS细胞包含两种群体，其中一种缺少禽流感病毒倾向于结合的sialic acid α-2,3受体。然而这个发现并不足以解释为什么部分病毒株可以繁殖而另一部分不能。

MDCK-SFS是目前所测试的细胞中唯一可以在无血清培养基中悬浮生长、且适合大多数禽流感病毒株的复制和高效繁殖的细胞。因此，MDCK-SFS细胞被选择进

行进一步研究。详情请见第四章和第五章。

BHK21细胞可快速的以悬浮状态增长并且在分批培养中达到需求的细胞密度。因此，具有这种特性的BHK21细胞当前被用来生产对抗口蹄疫的兽用疫苗。然而，禽流感病毒不同病毒株在BHK21细胞上的选择性复制局限了这种细胞在禽流感疫苗生产上的应用。因此，在第三章中，我们详细讨论了在分子水平上我们对于这种病毒选择性复制特性的研究。我们选择A/PR/8/34[H1N1](PR8)和A/WSN/33[H1N1]（WSN）作为研究的模板。结果指出，前者不能在BHK21-SFS细胞上进行正常的复制，后者却可以在该细胞上完成有效的复制。由于在病毒复制过程中，子代所携带的血凝素（HA）基因片段是来自于父代和母代的HA基因交换以及重组，而HA蛋白质是复制的关键因素之一，因此这两种病毒株复制能力上的差异取决于血凝素（HA）蛋白质。

为了证实HA蛋白质是决定因素之一，我们用另外几种流感病毒株做了实验，结果指出，其他几种流感病毒株在BHK21-SFS细胞上的繁殖能力似乎也取决于HA基因。为了确定具体哪一部分的HA蛋白质决定了病毒的复制能力，我们在PR8的基础上合成了携带反向遗传因子的病毒。虽然这种病毒可以编码并复制PR8-WSN嵌合HAs，由此为我们提供大量的可用信息; 然而根据这些信息，却不足以让我们识别与鉴定具体是哪个HA上的单一区间在以BHK21-SFS细胞为载体的病毒复制上起了决定性的作用。这是由于包含WSN HA1域的病毒与包含PR8 HA1的病毒所表现出来相近的复制能力，以及当病毒表达另外两种cHAs时，复制能力几乎没有变化。虽然如此，结果依旧指出，倘若将PR8 HA1域与WSN HA2域相融合，病毒就可以增强复制能力。这是由于HA1域中包含了大量的抗原位点，大量的抗原位点对于疫苗的有效性起到了至关重要的作用。虽然携带HA1域的流感病毒在BHK21-SFS上并没有非常显著的复制与繁殖能力，但是携带HA2域的流感病毒却可以在该细胞上进行非常高效的繁殖与复制。于是我们提出，将HA1域融合入杂合HA2域，并依赖于携带HA2域的病毒在BHK21-SFS细胞上高效复制繁殖的特性，使得融合后的HA1域同时进行了复制和繁殖。这样的方式，可以提高病毒的复制能力，疫苗的有效性，并且可以将BHK21-SFS细胞引入流感疫苗的制作基底细胞之一。

不表达NS1蛋白（delNS1）的流感病毒在细胞上的复制率会降低，同时会诱导产生大量的干扰素（IFN）。降低的复制率通常由于这类病毒无法抵抗具有抗病毒能力的宿主。基于这些特性，目前DelNS1病毒株正在被考虑作为制作减毒流感活疫苗和兽用疫苗的候选病毒。然而，在MDCK细胞上，病毒减活的生长限制了大规模的病毒生产。为了研制高收益的delNS1病毒生产系统，我们合成了两种可以表达NS1蛋白的MDCK细胞（第四章）。由于异源性NS1表达可以导致细胞凋亡，因此我们使用诱导表达系统。诱导后，delNS1病毒在两种合成细胞上的复制和繁殖产

量显著增高并且与同源病毒株（WT病毒，自身表达NS1蛋白）的生产浓度几近持平。同时值得注意的是，这两种合成表达NS1蛋白的细胞，NS1的表达量是普通流感病毒感染后细胞的千分之一。此外，NS1蛋白表达抑制delNS1病毒诱导的细胞凋亡，但没有显著降低干扰素的产生。因此，增强病毒复制并不是由降低IFN的产量而造成的。与先前研究结果相反，重组后的NS1蛋白表达本身并不诱导细胞凋亡，也不影响细胞的生长。这可能是由于特定的等位基因B NS1基因。因此，若细胞可以稳定表达等位基因B的NS1蛋白，那么他就可以被用来大规模生产delNS1病毒，并且由此生产减活高效流感疫苗。

另一种加强病毒复制能力的方式是让病毒适应细胞基底。第五章中，两个并列实验同时测量适应后的delNS1病毒。结果显示，当感染干扰素主导的MDCK细胞时，两个实验中病毒分别感染细胞的感染率为普通MDCK细胞感染率的250倍。通过分析改编后的病毒序列以及随后产生的基因重组，我们发现，显著增长的病毒复制是由一个病毒中M基因片段上的突变（delNS1 CA2）和其他病毒中M,NP,NS片段上的突变（delNS1 CA1）造成的。其中最显著的是同时在两个病毒株的M片段上发现了U到C的突变和在delNS1 CA1病毒株中NS片段上A到G的突变。据推测，这些突变导致宿主细胞被感染到修改后的RNA。NS1蛋白通常限制M片段中mRNA的剪接，从而使M1/M2的mRNA的比值增大。有趣的是，两个适应后病毒中的M片段中的无表达突变使得M1/M2表达的比值增大，这个可能是由于剪接的效率造成的。此外，通过改变M1的亚细胞定位，两个delNS1 CA2 M1中置换的氨基酸也同时增强病毒的复制能力。和delNS1病毒比较，两种适应后的病毒都具有较低的IFN的诱导和细胞凋亡率，但是依旧明显高出WT病毒。通过这样的比较得出，限制delNS1病毒的复制不只由于病毒本身对宿主抗病毒反应的不耐，也由于病毒丧失一个或多个NS1中有关M1蛋白的功能。相关的突变也因而可以直接应用于加强delNS1的病毒复制能力，从而应用于疫苗的生产。

细胞培养的流感疫苗在经济效益上比鸡胚胎卵生产的疫苗更有竞争力。由于本身禽流感疫苗的价格并不高，于是对于成本造价的要求就更为突出。因此基于细胞培养的流感病毒在降低成本上体现出来了更多的优势。例如，用细胞培养流感病毒可以方便的提升每升培养基中病毒的产量。第六章介绍了几种优化病毒产量的建议，其中包括疫苗病毒工程和基底细胞工程。然而，若实施大规模的家禽接种疫苗，将不仅仅取决于疫苗的价格，同时也取决于宏观的经济和政治因素。兽用流感疫苗未来的研究和发展，例如使用广谱流感疫苗DIVA，会进一步帮助落实紧急防御接种的必要性，并将作为一种常规方案来控制甚至预防禽流感疫情的发生和扩散。

实际内容以英文版本为准。中文翻译仅作为参考。

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Mooi boekje he?! Ik zal je iets verklappen. Ik heb het niet alleen gedaan. Mijn naam staat dan wel op de voorkant, maar zonder de directe of indirecte hulp van veel mensen was ik nooit zo ver gekomen. En al die mensen wil ik hierbij graag bedanken….. **Vinaka vaka levu!**

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D., you did truly amazing work designing the cover. 非常感谢

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Curriculum vitae

My life started on the 22th of June 1976 in Amsterdam. I was a curious child, interested in everything that was related to nature or technology. In 1994, the year that I received my VWO diploma, I heard for the first time about DNA and biotechnology, which made me decide to study Biology at the University of Amsterdam. However, after one year I realised I wanted a more technical study and therefore changed to the Wageningen University to study Bioprocess Engineering. During this study, I performed two thesis research projects at the department of Bioprocess Engineering. Under the supervision of Dr. R. Osinga I assessed the potential use of marine sponges as biocatalysts by growing their cells as aggregates (primmorphs). Following this project, I worked on a dynamic model of the glycolysis in lactic acid bacteria, under supervision of Dr. M. Hoefnagel. Around this time, I also grew an appetite for travel and therefore grabbed the opportunity to live in Australia for a half year, where I performed a research project at the Royal Melbourne Institute of Technology, on the optimization of the production of a veterinary mycoplasma-vaccine, under guidance of Dr. J Power.

After obtaining my Master degree in the beginning of 2002, I worked at the biopharmaceutical company Centocor in Leiden (now Janssen biologics), initially as a Trainee at the Upstream and Downstream Production departments and at the Quality Assurance department. After 2 years, I started as Technical Specialist at the DSP Process Development department on the improvement of large-scale chromatography column packing. In 2005, I had another opportunity to work abroad, now as Marine Project Officer for Coral Cay Conservations (Puttenham, UK) in Fiji. While experiencing a tropical half year, I assisted and coached a local, government endorsed team in setting-up marine protected reserves around the island of Kandavu, with the aim to protect the marine ecosystem.

After return to the Netherlands in 2006, I started as an Assistant Researcher at the Animal Science Group Products Division (now Merial Lelystad) of the Wageningen University and Research Centre (WUR). Here, I characterized and optimized both cell culture and virus propagation of the foot-and-mouth disease vaccine production process. In 2008 I decided to further develop my scientific skills by applying for the PhD position, of which this thesis is the final result. In October 2012, I will start as Scientist Process Development at UniQure, an Amsterdam-based biotech company that develops viral vectors for gene therapy.

Discipline specific courses and activities
Bioreactor design and operation, Wageningen, 2008
Statistics for life science, Wageningen, 2009
Waters Millenium/empower fundamentals, 2008
BD FACS operation course, 2010

Bird flu congress, Oxford, UK, 2008
Minisymposium MPI[2], Magdeburg, DE, 2008
European Society for Animal Cell Technology meeting[2], Vienna, AT, 2011
ACTIP fellowship[2], Glasgow, UK, 2011
ESWI Influenza conference[1], Malta, 2011
NBC workgroup day[2], Ede, 2011
European Network on Viral Vaccine Processes[2], Frankfurt, DE, 2011
Dutch Annual Virological Symposium, Amsterdam, 2012
Netherlands Biotechnological Congress[2], Ede, 2012

General courses
Project and time management, Wageningen, 2008
PhD competence assessment, Wageningen, 2009
Writing for academic publications, Lelystad, 2009
Teaching and supervising thesis students, Wageningen, 2010
Career assessment, Wageningen, 2012

Optional courses and activities
PhD excursion MSD animal health & Synthon 2008,2012
PhD study trip USA, 2010
Brainstormday Process Engineering, 2012

[1] Poster
[2] Presentation

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