

## **Properties of H7N7 influenza A virus strain SC35M lacking interferon antagonist NS1 in mice and chickens**

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**Non-structural protein NS1 of influenza A virus counteracts the host immune response by blocking the synthesis of type I interferon (IFN). As deletion of the complete NS1 gene has to date been reported only in the human H1N1 strain A/PR/8/34, it remained unclear whether NS1 is a non-essential virulence factor in other influenza A virus strains as well. In this report, the properties of NS1-deficient mutants derived from strain SC35M (H7N7) are described. A mutant of SC35M that completely lacks the NS1 gene was an excellent inducer of IFN in mammalian and avian cells in culture and, consequently, was able to multiply efficiently only in cell lines with defects in the type I IFN system. Virus mutants carrying C-terminally truncated versions of NS1 were less powerful inducers of IFN and were attenuated less strongly in human A549 cells. Although attenuated in wild-type mice, these mutants remained highly pathogenic for mice lacking the IFN-regulated antiviral factor Mx1. By contrast, the NS1-deficient SC35M mutant was completely non-pathogenic for wild-type mice, but remained pathogenic for mice lacking Mx1 and double-stranded RNA-activated protein kinase (PKR). Wild-type SC35M, but not the NS1-deficient mutant virus, was able to replicate in the upper respiratory tract of birds, but neither virus induced severe disease in adult chickens. Altogether, this study supports the view that NS1 represents a non-essential virulence factor of different influenza A viruses.**

## INTRODUCTION

Non-structural protein NS1 of influenza A virus is a virulence factor that interferes with innate immune responses of infected hosts by inhibiting virus-induced synthesis of type I interferon (IFN) (Garcia-Sastre *et al.*, 1998; Krug *et al.*, 2003; Noah *et al.*, 2003). NS1 appears to prevent IFN synthesis in several ways. It inhibits IFN gene transcription specifically by preventing virus-induced activation of IFN-regulatory factor 3 by multiple mechanisms (Talon *et al.*, 2000; Donelan *et al.*, 2003; Pichlmair *et al.*, 2006; Cauthen *et al.*, 2007; Mibayashi *et al.*, 2007). By inhibiting post-translational processing of the 3' ends of cellular mRNAs, NS1 further inhibits cytokine gene expression in a less specific manner (Fortes *et al.*, 1994; Chen *et al.*, 1999; Krug *et al.*, 2003). In certain influenza A virus strains, NS1 not only inhibits the production of IFN, but also seems to block its action (Seo *et al.*, 2002; Hayman *et al.*, 2006).

If the sole role of NS1 was to inhibit antiviral host responses, mutant influenza virus lacking NS1 should be viable in suitable cell-culture systems and should exhibit a highly attenuated phenotype in IFN-competent hosts. A mutant derived from virus strain A/PR/8/34 (PR8) that completely lacks the NS1 gene was recently shown to possess such properties (Garcia-Sastre *et al.*, 1998; Bergmann *et al.*, 2000). However, as no other influenza virus strain with complete NS1 deletion has been described to date, it remained unclear whether PR8 is exceptional and whether NS1 might perform additional essential functions in other virus strains. In fact, essential functions of NS1 in the influenza A virus life cycle were indicated by the observation that temperature-sensitive mutants of A/Victoria/3/75 with specific defects in NS1 exhibit pronounced defects in viral genome replication and particle formation (Falcon *et al.*, 2004; Garaigorta *et al.*, 2005) and that binding of NS1 to a regulatory subunit of phosphatidylinositol 3-kinase generates signals in infected cells that have a positive effect on influenza virus replication (Hale *et al.*, 2006).

To solve these questions and to determine whether NS1 might help to evade the innate immune response of non-mammalian hosts, we generated NS1 mutants of influenza A virus strain SC35M, which was reported to replicate in both chickens and mice (Scheiblaue *et al.*, 1995; Gabriel *et al.*, 2005). We found that a mutant of SC35M that completely lacks the NS1 gene was viable, supporting the view that NS1 is a non-essential virulence factor in most, if not all, influenza A virus strains.

## METHODS

### Cells.

Madin–Darby canine kidney (MDCK), Vero and A549 cells were maintained in Dulbecco's modified Eagle's high-glucose medium (DMEM high glucose) containing 10 % fetal bovine serum. Chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryonated chicken eggs by trypsin digestion. They were suspended in DMEM high glucose containing 7 % normal chicken serum. After one passage, the cells were used for infection experiments.

### Viruses.

Recombinant wild-type SC35M generated from cDNA was described previously (Gabriel *et al.*, 2005). To generate mutants with partial or complete deletions of the NS1 gene, specific deletions were introduced into segment 8 by using a PCR-based cloning strategy (Quinlivan *et al.*, 2005). The presence of the introduced changes and absence of unwanted mutations were confirmed by sequencing. For rescue of the NS1 mutants, the modified plasmids were combined with the remaining seven plasmids of SC35M and transfected into 293T/MDCK cell mixtures. Stocks of SC35M-deINS1 were prepared in Vero cells, whereas stocks of all other viruses were made in embryonated chicken eggs.

### Virus titrations.

Titres were determined by performing plaque assays on either MDCK cells or Vero cells as described previously (Gabriel *et al.*, 2005).

### Mice.

Standard C57BL/6 mice, which carry defective alleles of the influenza virus resistance locus *Mx1* (Staeheli *et al.*, 1988), were purchased from Harlan Netherlands. Congenic B6.A2G-Mx1 mice (Staeheli *et al.*, 1985) carrying intact *Mx1* alleles were bred locally. Mice of strain 129/SvJ with a targeted disruption of the protein kinase R gene ( $PKR^{0/0}$ ) (Yang *et al.*, 1995) (which carry defective *Mx1* alleles; Staeheli *et al.*, 1988) were bred in the facilities of the University of Zürich. Six- to ten-week-old animals were used for the challenge experiments, which were performed in accordance with the local Animal Care Committee. Animals were euthanized if severe symptoms developed. Lethal dose 50 ( $LD_{50}$ ) values were calculated as described by Reed & Muench (1938).

### Infection of mice.

Animals were anaesthetized by intraperitoneal injection of a mixture of ketamine [ $100 \mu\text{g}$  ( $\text{g body weight}^{-1}$ )] and xylazine [ $5 \mu\text{g}$  ( $\text{g body weight}^{-1}$ )] and infected intranasally with the indicated doses of virus in  $50 \mu\text{l}$  PBS containing 0.3 % BSA.

## **Chickens.**

Six-week-old commercial Lohmann selected leghorn (LSL) chickens were obtained from Lohmann Tierzucht GmbH. The birds were housed in groups of six animals in embellished cages.

## **Infection of chickens.**

Chickens were infected intratracheally. The indicated doses of virus were applied in 500  $\mu$ l cell-culture medium by using a blunt-ended canula. Animals were killed by intraperitoneal injection of barbiturate.

## **ELISA for mouse IFN- $\beta$ .**

Lungs were removed, frozen immediately in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. Lung homogenates were prepared by grinding the tissue in liquid nitrogen using a mortar and sterile quartz sand. Two-thirds of each lung homogenate was suspended in 300  $\mu$ l PBS and tissue debris was removed by low-speed centrifugation. Dilutions of supernatant were assayed for IFN- $\beta$  content by ELISA (PBL Biomedical Laboratories).

## **RT-PCR for IFN- $\beta$ .**

Lungs were removed, frozen immediately in liquid nitrogen and total RNA was isolated from one-third of each lung homogenate by using 1 ml TriFast according to the protocol of the manufacturer (peQLab). The RNA was further purified by using RNeasy mini kit columns (Qiagen). Reverse transcription (RT) was performed by using random-hexamer primers and Revert Aid H<sup>-</sup> murine leukemia virus reverse transcriptase (Fermentas). The RT sample was used to amplify cDNAs by *Taq* DNA polymerase (Roche) for 30 cycles using the indicated primer pairs for mouse IFN- $\beta$  [GenBank accession no. NM\_010510, primers from positions 21 to 42 and 145 to 124 as described by Petro (2005)] and mouse  $\beta$ -actin (GenBank accession no. X03672, primers from positions 1374 to 1396 and 1585 to 1564). RT-PCR products were separated by agarose electrophoresis, stained with ethidium bromide and visualized under UV light.

MDCK cells were infected with the different viruses at an m.o.i. of 0.5. At 10 h post-infection, RNA was extracted from  $1 \times 10^6$  cells and reverse-transcribed. PCR was performed by using the indicated primer pairs for cDNAs encoding canine IFN- $\beta$  (GenBank accession no. XM\_538679, primers 1–18 and 594–575), canine  $\beta$ -actin (accession no. XM\_536230, primers 378–401 and 805–781), NP of SC35M (accession no. DQ266096, primers 863–883 and 1276–1255) and NS1 of SC35M (accession no. DQ266101, primers 41–64 and 752–730).

## **Bioassay detecting type I IFN in MDCK cells.**

Supernatants of infected MDCK cells were collected, dialysed against 0.1 M glycine (pH 2.0) and subsequently against PBS (pH 7.5). A reporter assay was then used to measure type I IFN in these samples. For this purpose, MDCK cells were transiently transfected with a pGL3 plasmid encoding firefly luciferase under the control of the murine Mx1 promoter (Jorns *et al.*, 2006) before they were incubated for 20 h with the acid-treated supernatants. The cells were

then lysed and luciferase activity in the lysates was determined according to the protocol of the manufacturer (Promega). Baseline luciferase activity of transfected cells exposed to supernatants of uninfected MDCK cells (control) was defined as a 'relative IFN activity' of 1.

### **Bioassay detecting chicken type I IFN.**

Chicken embryo cell cultures were infected with either SC35M or SC35M-deINS1 at an m.o.i. of 2. At 10 h post-infection, supernatants were harvested and analysed for IFN activity by using a bioassay as described previously (Schwarz *et al.*, 2004).

### **Western blot analysis.**

Cells were lysed in passive lysis buffer (Promega) and denatured by using SDS and  $\beta$ -mercaptoethanol. The proteins were separated by SDS-PAGE (12 % gel) and transferred onto a PVDF membrane (Millipore). The blots were probed with polyclonal rabbit antibodies directed against influenza A virus NP and NS1 (Solorzano *et al.*, 2005) (kindly provided by Alicia Solórzano, Department of Microbiology, Mount Sinai School of Medicine, New York, USA) and a mouse monoclonal antibody against  $\beta$ -tubulin (Sigma). Horseradish peroxidase-labelled secondary antibodies and the ECL detection system (Roche) were used to detect the primary antibodies.

### **Real-time RT-PCR to quantify influenza virus.**

Influenza A virus-specific RNA was quantified with a one-step real-time RT-PCR detecting the viral M gene, essentially as described previously (Spackman *et al.*, 2002). Briefly, a conserved fragment of the M gene was amplified with primers FLU\_panAmod\_F (5'-AGATGAGYCTTCTAACCGA-3') and FLU\_panAmod\_R (5'-GCAAAGACATCTTCAAGTYTC-3') in combination with the 5'FAM/3'TAMRA-labelled probe FLU\_panA-P [identical to probe M+64 (Spackman *et al.*, 2002)]. Quantitative RT-PCR was carried out with a QuantiTect Probe RT-PCR kit (Qiagen) on a 7500 Fast Real-Time PCR system (Applied Biosystems). Mean  $C_t$  values were determined from triplicate amplifications of each sample.

### **Detection of influenza A virus-specific antibodies in chicken serum.**

Analysis of chicken sera for influenza A virus-specific antibodies was performed with the FLOCKTYPE recAIV Screening ELISA (Labor Diagnostik Leipzig). Titres were expressed as percentage reactivity of the positive-control serum according to the manufacturer's protocol.

## RESULTS

### SC35M-derived mutants with partial or complete deletions of the NS1 gene

By transfecting suitable cDNA constructs into 293T/MDCK cell mixtures, two mutants of SC35M were generated that express truncated versions of NS1 consisting of aa 1–99 and 1–126, respectively. We further rescued a mutant (designated delNS1) in which the complete open reading frame of the NS1 gene is deleted. All three mutant viruses were able to multiply in IFN-deficient Vero cells. Wild-type SC35M and the mutants (1–99) and (1–126) grew to very high titres (Fig. 1, upper panel), whereas growth of the delNS1 mutant was impaired substantially. A strikingly different picture emerged when the various viruses were grown in IFN-competent human A549 cells. Wild-type SC35M grew to approximately  $10^8$  p.f.u. ml<sup>-1</sup>, whereas the NS1 mutants (1–99) and (1–126) reached titres of only approximately  $10^2$  p.f.u. ml<sup>-1</sup> (Fig. 1, lower panel). Mutant virus delNS1 failed to multiply in A549 cells altogether. These results matched predictions from work with similar mutants of other influenza A virus strains (Garcia-Sastre *et al.*, 1998; Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005; Cauthen *et al.*, 2007) and demonstrated further that NS1 is essential for efficient multiplication of influenza A virus in IFN-competent cell lines.

### Induction of IFN by SC35M-derived NS1 mutants

The IFN-inducing potential of wild-type and NS1-deficient SC35M variants was first assessed in MDCK cells. Semiquantitative RT-PCR performed at 16 h post-virus infection (Fig. 2a) clearly indicated enhanced transcription of the IFN- $\beta$  gene in cells infected with the delNS1 mutant. Wild-type SC35M and mutants (1–99) and (1–126) did not differ substantially in this analysis. When supernatants of the infected cultures were analysed for IFN activity by using a bioassay, it became clear that the delNS1 mutant prompted the secretion of approximately eightfold higher amounts of IFN than wild-type SC35M (Fig. 2b). Mutants (1–99) and (1–126) were clearly less powerful inducers of IFN than delNS1 in MDCK cells, but they still induced slightly more IFN than wild-type SC35M (Fig. 2b). Western blot analysis demonstrated that wild-type and mutant viruses replicated comparably well in MDCK cells under the conditions used for this comparison (Fig. 2c). The IFN-inducing capacity of the NS1-deficient SC35M mutant was also studied in cultured chicken embryo cells by using a bioassay that measures type I IFN activity. Supernatant of chicken embryo cell cultures infected with wild-type SC35M did not contain detectable levels of IFN, whereas supernatants of cells infected with the delNS1 mutant contained about 300 U type I IFN ml<sup>-1</sup> (data not shown).

The IFN-inducing capacity of the various viruses was next analysed in mouse lungs (Fig. 3). Groups of C57BL/6 mice ( $n=5$ ) were infected by the intranasal route with  $5 \times 10^4$  p.f.u. of either wild-type SC35M or the various NS1 mutants. At 10 h post-infection, the animals were killed and virus-induced IFN- $\beta$  in the lung tissue was analysed by semiquantitative RT-PCR (Fig. 3a) or ELISA (Fig. 3b). Although IFN- $\beta$  mRNA accumulated in all virus-infected lungs, the signals were strongest in lungs of mice that were infected with the delNS1 mutant. The differences

between the viruses became clearer when lung extracts were analysed for the presence of IFN- $\beta$  by ELISA. Substantial levels of IFN- $\beta$  could only be measured in lungs of delNS1-infected mice (Fig. 3b).

### **Growth behaviour of SC35M-derived NS1 mutants in mice and chickens**

We next set out to measure the biological consequences of NS1 gene deletion in SC35M by employing mammalian and avian model systems. A first series of infection experiments was performed in C57BL/6 mice that either carry ( $Mx1^{+/+}$ ) or lack ( $Mx1^{-/-}$ ) functional alleles of the influenza virus resistance gene *Mx1*. As expected, wild-type SC35M was highly pathogenic for  $Mx1^{-/-}$  mice ( $LD_{50}=7\times 10^2$  p.f.u.), but not for  $Mx1^{+/+}$  mice ( $LD_{50}=2\times 10^5$  p.f.u.) (Table 1). The NS1 mutants (1–99) and (1–126) were only slightly less pathogenic than wild-type SC35M in  $Mx1^{-/-}$  mice (Table 1). In  $Mx1^{+/+}$  mice, mutant (1–126) induced disease in only a small fraction of animals if used at  $10^6$  p.f.u. The (1–99) and delNS1 mutants were non-pathogenic at all doses that were tested (Table 1). These results are compatible with the view that attenuation of the mutant viruses resulted from enhanced synthesis of IFN in the infected lungs, and they support the notion that the IFN-regulated *Mx1* gene greatly determines the degree of virus resistance in mice (Haller *et al.*, 1979). If this interpretation is correct, the NS1-deficient virus should be pathogenic for  $Mx1^{-/-}$  mice that have an additional defect in IFN-regulated genes which contribute to influenza virus resistance, such as the double-stranded RNA-activated protein kinase (PKR). As shown in Table 2, we found that all *Mx1*-deficient, *PKR*<sup>0/0</sup> mice infected with the delNS1 mutant of SC35M became severely ill within 5–7 days post-infection.

Infection experiments with wild-type SC35M and mutant delNS1 were next performed in chickens. Groups of five adult animals were infected by the intratracheal route with  $10^6$  p.f.u. wild-type or mutant virus and the animals were observed for 10 days. Birds infected with wild-type SC35M showed reduced activity around day 3 post-infection, but none of the infected chickens developed clear signs of disease (Table 3 and data not shown). When pharynx swabs taken between days 2 and 6 post-infection were examined for viral nucleic acid by real-time RT-PCR, we observed that two of five pharynx swabs became positive on day 3 post-infection. On day 4, all five samples were positive, and three of five samples were still positive on day 6 (Table 3). All pharynx swabs taken from animals infected with the delNS1 mutant of SC35M remained negative during the complete observation period (Table 3). On day 8 post-infection, high anti-influenza serum titres were observed in all five animals that were infected with wild-type SC35M (Fig. 4). Similarly, all animals infected with the delNS1 mutant of SC35M seroconverted by day 8 post-infection, but antibody titres were substantially lower than in animals infected with wild-type virus (Fig. 4).

## DISCUSSION

In this study, we demonstrated that SC35M with a complete deletion of the NS1 gene is viable and able to replicate fairly well in IFN-deficient cell lines and in mice deficient for the antiviral factors Mx1 and PKR, supporting the view that NS1 is a non-essential virulence-determining factor of influenza A viruses. We further showed that the NS1-deficient mutant of SC35M is attenuated in adult chickens.

SC35M-delNS1 represents the second reported case of a viable influenza A virus mutant with a complete deletion of the NS1 gene. The first example was a mutant derived from strain A/PR/8/34 (Garcia-Sastre *et al.*, 1998). As none of our attempts to introduce complete NS1 deletions into the WSN strain of influenza A virus were successful (J. Stech, unpublished results), it was conceivable that NS1 of A/PR/8/34 lost some of its many activities during species or cell adaptation, and that in A/PR/8/34 (but not in other virus strains), the NS1 deficiency is compensated at least in part by other viral proteins. The fact that we managed to delete the complete NS1 gene in SC35M argues against the view that the NS1 of A/PR/8/34 exhibits exceptional features. SC35M and A/PR/8/34 have few properties in common. The progenitor of SC35M was isolated from a seal and probably originates from a sea bird (Webster *et al.*, 1981; Scheiblaue *et al.*, 1995). In contrast, A/PR/8/34 was isolated from a human case in 1934. Our data support the assumption that NS1 in most, if not all, influenza A virus strains is a non-essential virulence factor that can modulate host-cell functions. A similar conclusion was reached in studies with influenza virus B/Lee/40, showing that a mutant strain with complete NS1 deletion was viable (Dauber *et al.*, 2004). The observation that the delNS1 mutant of SC35M grew about 100 times less well than the parental virus in IFN-deficient Vero cells shows clearly that NS1 of influenza A virus is not simply involved in counteracting the IFN response. Interestingly, similar growth differences were observed between wild-type and NS1-deficient A/PR/8/34 (Garcia-Sastre *et al.*, 1998), indicating that NS1 has additional effects on the host cell that are not related to IFN signalling or IFN action. It should be noted that we cannot exclude the possibility that other cytokines slowed down virus replication in Vero cells. Furthermore, it remains possible that the strong activation of transcription factor IRF3 induced expression of antiviral genes in an IFN-independent manner (Haller *et al.*, 2006). NS1 was recently found to activate signalling through the phosphatidylinositol 3-kinase pathway, which promotes virus replication (Hale *et al.*, 2006). Furthermore, NS1 was shown to modulate apoptosis induction in infected cells (Zhirnov *et al.*, 2002; Li *et al.*, 2006). It is thus conceivable that production of virus particles by infected cells is reduced severely if NS1 is not modulating these particular cellular pathways in favour of the virus. We would like to suggest that generating NS1-deficient mutants might be particularly difficult if virus strains are used that depend very strongly on the host cell-activating functions of NS1.

Wild-type SC35M was a poor inducer of IFN- $\beta$  in cultured cells and even more so in mouse lungs. By contrast, the delNS1 mutant of SC35M was an excellent inducer in both systems.

Interestingly, viruses with C-terminally truncated NS1 suppressed IFN- $\beta$  synthesis in mouse lungs with an efficacy similar to that of wild-type virus, and the degree of attenuation of these viruses was modest in standard mice that lack functional alleles of the influenza virus resistance gene *Mx1*. As expected, attenuation of these viruses was more prominent when *Mx1*<sup>+/+</sup> mice were used, in which IFN can develop its full activity against influenza A virus due to the presence of the antiviral factor Mx1 (Haller *et al.*, 1979; Staeheli *et al.*, 1986). From the fact that the delNS1 mutant was non-pathogenic in *Mx1*<sup>-/-</sup> mice, but pathogenic in mice lacking Mx1 and PKR, it further became clear that PKR contributes substantially to IFN-mediated resistance against influenza viruses of mice. This view is in good agreement with earlier observations from mouse infection studies with the NS1-deficient mutant of influenza virus strain A/PR/8/34 (Bergmann *et al.*, 2000).

The effects of C-terminal truncations of NS1 were studied previously in viruses that are pathogenic for pigs, horses or chickens (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005). A significant degree of attenuation was observed when these mutant viruses were compared with their wild-type counterparts in IFN-competent cell lines and in animals, which correlated with reduced ability to suppress IFN synthesis. In the pig and horse viruses, C-terminal deletions of NS1 to position 126 resulted in reduced stability of the NS1 proteins in infected cells. Consequently, viruses carrying such truncated forms of NS1 were attenuated more strongly than viruses carrying NS1 proteins with larger deletions (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005). In the SC35M mutants described here, we found no evidence for reduced stability of truncated versions of NS1, as reported previously for NS1 deletions of virus strain WSN (Wang *et al.*, 2002). Accordingly, in our set of virus mutants, the degree of attenuation in IFN-competent cells and mice correlated directly with the extent of truncation.

Unlike non-recombinant SC35M, which was reported to kill mice and chickens (Scheiblauer *et al.*, 1995), we found that cDNA-derived SC35M has a low-pathogenic phenotype in adult chickens. The virus replicated in the upper respiratory tract, but did not induce overt disease. In contrast, the NS1-deficient SC35M was not detected in the upper respiratory tract of any infected animal, indicating that it is attenuated substantially in birds. However, due to the low-pathogenic phenotype of wild-type SC35M, a more complete assessment of the role of NS1 for influenza A virus virulence in chickens was not possible. To clarify this issue, it will be necessary to continue these studies with a pair of NS1-competent and -deficient viruses that originate from a highly pathogenic avian influenza A virus strain. To date, such virus mutants have not been described.

## ACKNOWLEDGEMENTS

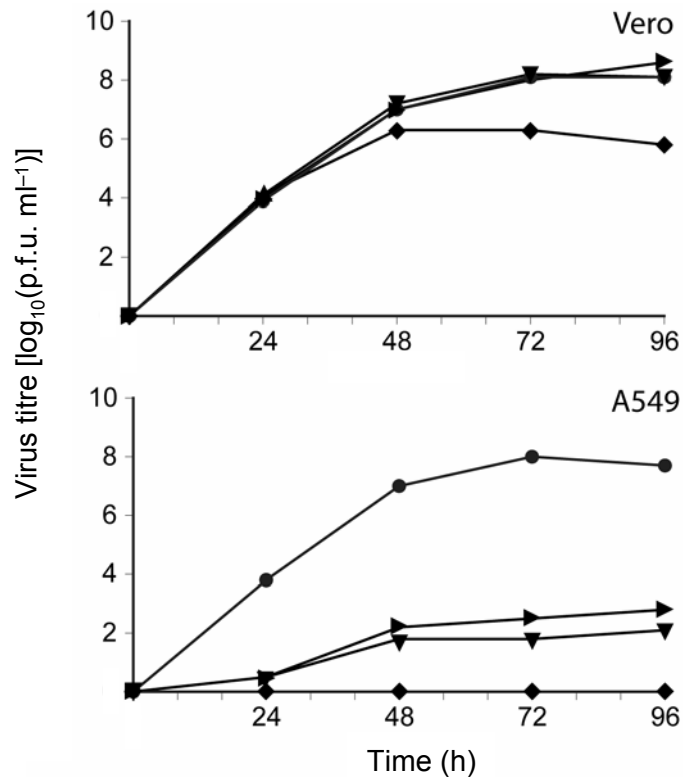
We thank Anette Baumer and Martin Hofmann for analysis of influenza A virus RNA by real-time RT-PCR, Barbara Thür and Markus Mader for help with serological tests and Kathrin Summermatter and Ruth Riechsteiner for help with biosafety issues. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

## REFERENCES

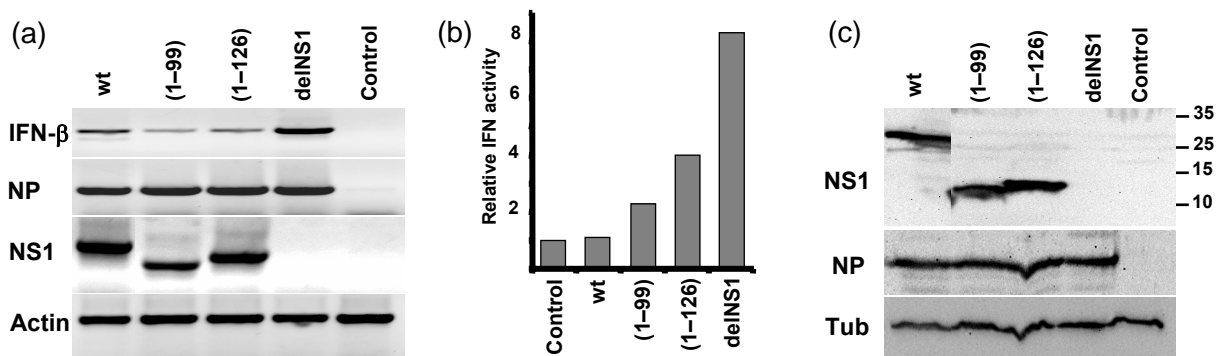
- Bergmann, M., Garcia-Sastre, A., Carnero, E., Pehamberger, H., Wolff, K., Palese, P. & Muster, T. (2000).** Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. *J Virol* **74**, 6203–6206. [Medline](#)
- Cauthen, A. N., Swayne, D. E., Sekellick, M. J., Marcus, P. I. & Suarez, D. L. (2007).** Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. *J Virol* **81**, 1838–1847. [Medline](#)
- Chen, Z., Li, Y. & Krug, R. M. (1999).** Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery. *EMBO J* **18**, 2273–2283. [Medline](#)
- Dauber, B., Heins, G. & Wolff, T. (2004).** The influenza B virus nonstructural NS1 protein is essential for efficient viral growth and antagonizes beta interferon induction. *J Virol* **78**, 1865–1872. [Medline](#)
- Donelan, N. R., Basler, C. F. & Garcia-Sastre, A. (2003).** A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. *J Virol* **77**, 13257–13266. [Medline](#)
- Falcon, A. M., Marion, R. M., Zurcher, T., Gomez, P., Portela, A., Nieto, A. & Ortin, J. (2004).** Defective RNA replication and late gene expression in temperature-sensitive influenza viruses expressing deleted forms of the NS1 protein. *J Virol* **78**, 3880–3888. [Medline](#)
- Fortes, P., Beloso, A. & Ortin, J. (1994).** Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. *EMBO J* **13**, 704–712. [Medline](#)
- Gabriel, G., Dauber, B., Wolff, T., Planz, O., Klenk, H. D. & Stech, J. (2005).** The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc Natl Acad Sci U S A* **102**, 18590–18595. [Medline](#)
- Garaigorta, U., Falcon, A. M. & Ortin, J. (2005).** Genetic analysis of influenza virus NS1 gene: a temperature-sensitive mutant shows defective formation of virus particles. *J Virol* **79**, 15246–15257. [Medline](#)
- Garcia-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D. E., Durbin, J. E., Palese, P. & Muster, T. (1998).** Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* **252**, 324–330. [Medline](#)
- Hale, B. G., Jackson, D., Chen, Y. H., Lamb, R. A. & Randall, R. E. (2006).** Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. *Proc Natl Acad Sci U S A* **103**, 14194–14199. [Medline](#)
- Haller, O., Arnheiter, H., Gresser, I. & Lindenmann, J. (1979).** Genetically determined, interferon-dependent resistance to influenza virus in mice. *J Exp Med* **149**, 601–612. [Medline](#)

- Haller, O., Kochs, G. & Weber, F. (2006).** The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* **344**, 119–130. [Medline](#)
- Hayman, A., Comely, S., Lackenby, A., Murphy, S., McCauley, J., Goodbourn, S. & Barclay, W. (2006).** Variation in the ability of human influenza A viruses to induce and inhibit the IFN-beta pathway. *Virology* **347**, 52–64. [Medline](#)
- Jorns, C., Holzinger, D., Thimme, R., Spangenberg, H. C., Weidmann, M., Rasenack, J., Blum, H. E., Haller, O. & Kochs, G. (2006).** Rapid and simple detection of IFN-neutralizing antibodies in chronic hepatitis C non-responsive to IFN-alpha. *J Med Virol* **78**, 74–82. [Medline](#)
- Krug, R. M., Yuan, W., Noah, D. L. & Latham, A. G. (2003).** Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology* **309**, 181–189. [Medline](#)
- Li, S., Min, J. Y., Krug, R. M. & Sen, G. C. (2006).** Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* **349**, 13–21. [Medline](#)
- Mibayashi, M., Martinez-Sobrido, L., Loo, Y. M., Cardenas, W. B., Gale, M., Jr & Garcia-Sastre, A. (2007).** Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J Virol* **81**, 514–524. [Medline](#)
- Noah, D. L., Twu, K. Y. & Krug, R. M. (2003).** Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAs. *Virology* **307**, 386–395. [Medline](#)
- Petro, T. M. (2005).** Disparate expression of IL-12 by SJL/J and B10.S macrophages during Theiler's virus infection is associated with activity of TLR7 and mitogen-activated protein kinases. *Microbes Infect* **7**, 224–232. [Medline](#)
- Pichlmair, A., Schulz, O., Tan, C. P., Naslund, T. I., Liljestrom, P., Weber, F. & Reis e Sousa, C. (2006).** RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997–1001. [Medline](#)
- Quinlivan, M., Zamarin, D., Garcia-Sastre, A., Cullinane, A., Chambers, T. & Palese, P. (2005).** Attenuation of equine influenza viruses through truncations of the NS1 protein. *J Virol* **79**, 8431–8439. [Medline](#)
- Reed, L. J. & Muench, H. (1938).** A simple method of estimating fifty percent endpoints. *Am J Hyg* **27**, 493–497.
- Scheiblaue, H., Kendal, A. P. & Rott, R. (1995).** Pathogenicity of influenza A/Seal/Mass/1/80 virus mutants for mammalian species. *Arch Virol* **140**, 341–348. [Medline](#)
- Schwarz, H., Harlin, O., Ohnemus, A., Kaspers, B. & Staeheli, P. (2004).** Synthesis of IFN-beta by virus-infected chicken embryo cells demonstrated with specific antisera and a new bioassay. *J Interferon Cytokine Res* **24**, 179–184. [Medline](#)
- Seo, S. H., Hoffmann, E. & Webster, R. G. (2002).** Lethal H5N1 influenza viruses escape host antiviral cytokine responses. *Nat Med* **8**, 950–954. [Medline](#)

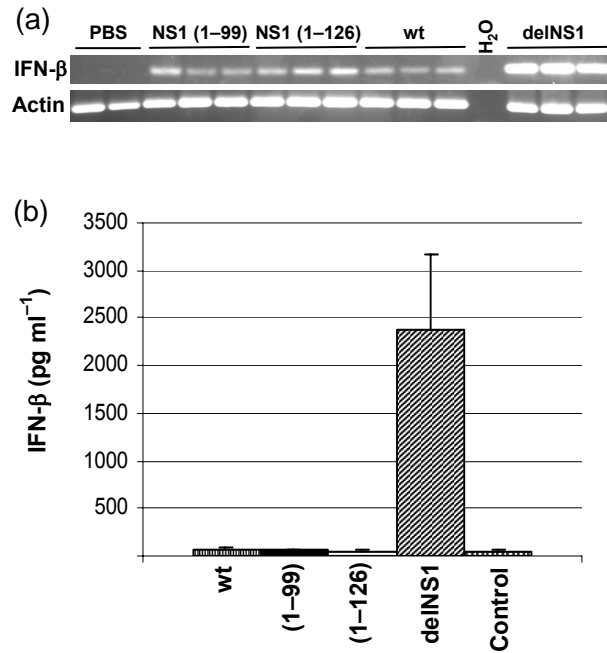
- Solorzano, A., Webby, R. J., Lager, K. M., Janke, B. H., Garcia-Sastre, A. & Richt, J. A. (2005).** Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. *J Virol* **79**, 7535–7543. [Medline](#)
- Spackman, E., Senne, D. A., Myers, T. J., Bulaga, L. L., Garber, L. P., Perdue, M. L., Lohman, K., Daum, L. T. & Suarez, D. L. (2002).** Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* **40**, 3256–3260. [Medline](#)
- Staeheli, P., Dreiding, P., Haller, O. & Lindenmann, J. (1985).** Polyclonal and monoclonal antibodies to the interferon-inducible protein Mx of influenza virus-resistant mice. *J Biol Chem* **260**, 1821–1825. [Medline](#)
- Staeheli, P., Haller, O., Boll, W., Lindenmann, J. & Weissmann, C. (1986).** Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. *Cell* **44**, 147–158. [Medline](#)
- Staeheli, P., Grob, R., Meier, E., Sutcliffe, J. G. & Haller, O. (1988).** Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol Cell Biol* **8**, 4518–4523. [Medline](#)
- Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., Palese, P. & Garcia-Sastre, A. (2000).** Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* **74**, 7989–7996. [Medline](#)
- Wang, X., Basler, C. F., Williams, B. R., Silverman, R. H., Palese, P. & Garcia-Sastre, A. (2002).** Functional replacement of the carboxy-terminal two-thirds of the influenza A virus NS1 protein with short heterologous dimerization domains. *J Virol* **76**, 12951–12962. [Medline](#)
- Webster, R. G., Hinshaw, V. S., Bean, W. J., Van Wyke, K. L., Geraci, J. R., St Aubin, D. J. & Petursson, G. (1981).** Characterization of an influenza A virus from seals. *Virology* **113**, 712–724. [Medline](#)
- Yang, Y. L., Reis, L. F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B. R., Aguet, M. & Weissmann, C. (1995).** Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J* **14**, 6095–6106. [Medline](#)
- Zhirnov, O. P., Konakova, T. E., Wolff, T. & Klenk, H. D. (2002).** NS1 protein of influenza A virus down-regulates apoptosis. *J Virol* **76**, 1617–1625. [Medline](#)



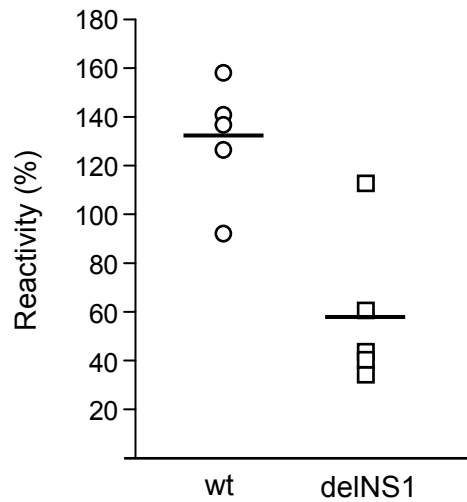
**Fig. 1.** Growth behaviour of wild-type SC35M and NS1-deficient mutants in IFN-deficient Vero (top) and IFN-competent A549 (bottom) cells. Cells were infected at an m.o.i. of  $10^{-3}$  with either wild-type SC35M (filled circles), mutant (1–99) (right-pointing triangles), mutant (1–126) (inverted triangles) or mutant delNS1 (diamonds). Virus titres in the culture supernatants were determined at the indicated times post-infection. Two independent experiments yielded similar results. Representative data from one experiment are shown.



**Fig. 2.** Induction of IFN by wild-type (wt) SC35M and NS1-deficient mutants in MDCK cells. (a) Cells were infected with the various viruses at an m.o.i. of 0.5 and analysed at 10 h post-infection by RT-PCR for IFN- $\beta$ , NP, NS1 and actin. Other cultures were harvested at 20 h post-infection and analysed for (b) IFN activity in the supernatant by using a bioassay or (c) the presence of viral proteins by Western blotting (size markers are shown on the right in kDa). Two independent experiments yielded similar results. Representative data from one experiment are shown.



**Fig. 3.** IFN- $\beta$  in lungs of C57BL/6 mice infected for 10 h with  $5 \times 10^4$  p.f.u. of either wild-type (wt) SC35M or NS1-deficient mutants. Lung extracts were analysed for IFN- $\beta$  by (a) RT-PCR or (b) ELISA.



**Fig. 4.** Anti-influenza titres in sera of chickens at day 8 post-infection with  $10^6$  p.f.u. wild-type (wt) SC35M and mutant delNS1. Titres of individual animals as determined by ELISA are plotted. Serum titres before infection were below 6 % reactivity.

**Table 1.** LD<sub>50</sub> of wild-type and mutant SC35M in *Mx1*<sup>+/+</sup> and *Mx1*<sup>-/-</sup> mice

Virus strain	C57BL/6 ( <i>Mx1</i> <sup>-/-</sup> )	B6.A2G-Mx1 ( <i>Mx1</i> <sup>+/+</sup> )
wt	7×10 <sup>2</sup>	2×10 <sup>5</sup>
(1-126)	3×10 <sup>3</sup>	>10 <sup>6</sup>
(1-99)	5×10 <sup>3</sup>	>10 <sup>6</sup>
delNS1	>5×10 <sup>4</sup>	>5×10 <sup>4</sup>

**Table 2.** Disease in *Mx1*-deficient, *PKR*<sup>0/0</sup> mice infected with SC35M-delNS1

Data are no. severely ill animals/total no. infected animals.

Virus dose (p.f.u.)	<i>Mx1</i> <sup>-/-</sup> , <i>PKR</i> <sup>+/+</sup>	<i>Mx1</i> <sup>-/-</sup> , <i>PKR</i> <sup>0/0</sup>
10 <sup>4</sup>	0/4	5/5
5×10 <sup>4</sup>	0/4	6/6

**Table 3.** Disease and viral parameters in adult chickens infected with wild-type or NS1-deficient SC35M

Virus	Disease*	Viral RNA in pharynx swabs by RT-PCR†			
		Day 2	Day 3	Day 4	Day 6
wt	0/5	0/5	2/5	5/5	3/5
delNS1	0/5	0/5	0/5	0/5	0/5

\*Animals were infected with 10<sup>6</sup> p.f.u. of either virus and observed daily for 10 days. Data are no. severely ill animals/total no. animals.

†Samples were scored as negative if no signal was observed after 50 cycles. Data are no. positive samples /total no. samples.