High yields of influenza A virus in Madin–Darby canine kidney cells are promoted by an insufficient interferon-induced antiviral state

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Because of their high susceptibility to infection with various influenza virus strains, Madin–Darby canine kidney (MDCK) cells have been widely used as a substrate for influenza virus isolation and vaccine production. However, MDCK cells are also interferon (IFN) competent, and the type I IFN response is commonly thought to be a factor strongly inhibiting virus replication. Therefore, the inhibition of influenza virus replication by IFN signalling was analysed for an adherent MDCK cell line used in vaccine manufacturing. Depending on the respective virus strain, different levels of IFN induction and a corresponding upregulation of the IFN-induced myxovirus resistance protein 1 (Mx1) were observed. Suppression of IFN induction by transient expression of the viral non-structural protein 1 protein enhanced replication of an influenza virus lacking NS1, but not wild-type strains. In agreement with this, stimulation of cells with MDCK cell-derived IFN prior to infection resulted only in a decrease in replication rate, and not in a change of final yields for wild-type influenza viruses. This lack of IFN-induced antiviral activity correlated with missing anti-influenza activity of MDCK Mx proteins. No inhibitory effect on viral polymerase activity was found for canine Mx1 (cMx1) and cMx2 in minireplicon assays. In conclusion, in MDCK cells, IFN expression is not a limiting factor for influenza virus replication and this might partially be caused by a lack of anti-influenza activity of canine Mx proteins.

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INTRODUCTION

Influenza A continues to be a major threat to human health. Madin–Darby canine kidney (MDCK) cells are widely used for the primary isolation of influenza viruses because of their high susceptibility to infection with various influenza strains (Gaush & Smith, 1968; Govorkova *et al.*, 1999). Additionally, cell culture-based influenza vaccine production is becoming increasingly important as an alternative to egg-based processes (Ulmer *et al.*, 2006; Audsley & Tannock, 2008; Genzel & Reichl, 2009). Recently, an MDCK cell culture-derived influenza vaccine has been approved by the European Medicines Agency (Doroshenko & Halperin, 2009). This cell culture-based bioprocess has become one focus of our research. In a previous study, we analysed virus–host cell interactions using a proteomic approach (Vester *et al.*, 2009). Interestingly, strong upregulation of the myxovirus resistance protein 1 (Mx1) was found in influenza virus-infected MDCK cells. Myxovirus resistance (Mx) protein expression is a generally accepted marker for interferon (IFN) activity (Holzinger *et al.*, 2007).

Induction and expression of IFNs are well-characterized parts of the innate immune response directed against viral infections (reviewed by Haller *et al.*, 2006; Randall & Goodbourn, 2008). In the case of influenza, 5′-phosphorylated viral RNA is detected by the cytoplasmic RNA helicase retinoic acid inducible GTPase I (RIG-I). RIG-I activates a signalling cascade leading to activation of the transcription factors interferon regulatory factor 3 (IRF3), nuclear factor κB and activator protein 1. Together, these factors activate the IFN-β promoter and cells start to secrete IFN. IFN binds to the interferon-α receptor (IFNAR), inducing the JAK/STAT signalling pathway in an autocrine and paracrine manner. This results in the transcriptional activation of several hundred IFN-

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A supplementary table is available with the online version of this paper.

stimulated genes (ISGs), some of which possess antiviral activity. An important ISG is IRF7. IRF7 mediates positive-feedback regulation of IFN expression by contributing to the transcriptional activation of IFN-α and -β genes (Honda & Taniguchi, 2006). ISGs with specific anti-influenza activity are the Mx proteins. These are dynamin-like large GTPases and have been described as mediators of natural resistance against orthomyxoviruses in mice (Haller *et al.*, 2009). Direct interaction with the viral nucleoprotein (NP) has been proposed as an antiviral mechanism of Mx proteins (Dittmann *et al.*, 2008).

Many viruses have developed mechanisms to circumvent or counteract IFN signalling in order to proliferate despite the antiviral actions of IFN (Bowie & Unterholzner, 2008). In the case of influenza A virus, the viral non-structural protein 1 (NS1) has been described to act as a powerful antagonist of IFN induction (reviewed by Hale *et al.*, 2008; Wolff & Ludwig, 2009). NS1 directly inhibits primary induction of IFN by interaction with the RIG-I pathway, thereby preventing the sensing of viral RNA by the host. It has also been shown that NS1 can block the post-transcriptional processing of cellular mRNAs, which results in a general shutdown of host protein synthesis. In this manner, the expression of IFN-induced antiviral proteins is reduced. NS1 additionally sequesters dsRNA, which is a by-product of virus replication and functions as an activator of antiviral signalling pathways.

Nevertheless, the outcome of IFN induction during virus infection and its suppression by NS1 as well as the impact on virus replication seem to vary and depend strongly on the virus strain and host cell used. Several studies have reported strain-dependent variation in the IFN-suppressing properties of different NS1 proteins (Hayman *et al.*, 2006; Kochs *et al.*, 2007a). In addition, IFN has been shown to be expressed during infection for several influenza virus strains, indicating that the block of IFN signalling by NS1 is incomplete (Matikainen *et al.*, 2000; Kim *et al.*, 2002; Noah *et al.*, 2003). Furthermore, the amount of IFN expressed due to virus infection also seems to depend on the host cell type (Ronni *et al.*, 1997). Strain-specific differences in sensitivity to Mx proteins were also observed (Dittmann *et al.*, 2008).

For other viruses, a reduction in IFN signalling during infection results in enhanced virus yields, indicating a limiting role of IFN in virus replication (Young *et al.*, 2003; de Vries *et al.*, 2008). In this study, we analysed the impact of IFN signalling on influenza virus replication in MDCK cells, a cell line approved for influenza vaccine production.

RESULTS

IFN signalling in influenza virus-infected MDCK cells

For characterization of IFN signalling during influenza A virus replication in MDCK cells, primary IFN-β induction and the subsequent Mx1 expression were analysed by real-time quantitative RT-PCR (qRT-PCR). Expression of Mx1 was measured as a marker for the induction of ISGs. MDCK cells were infected with A/Puerto Rico/8/34 (PR8) from the National Institute for Biological Standards and Control (PR8-NIBSC), PR8 from the Robert Koch Institute (PR8-RKI), PR8-delNS1 (delNS1) and A/WSN/33 (WSN33) at an m.o.i. of 5. The two PR8 variants were included because they were described previously to vary in apoptosis induction and final virus yields (Schulze-Horsel *et al.*, 2009). To assess virus replication dynamics, transcript levels of viral NS1 were determined in cells infected with wild-type (wt) viruses. In delNS1-infected cells, M1 transcripts were measured for that purpose. The delNS1 strain was included in this experiment to obtain the maximum level of IFN induction. For this virus, an almost immediate induction of IFN-β was found within 1–2 h post-infection (p.i.) (Fig. 1a). IFN-β expression increased until 9–10 h p.i. to a level of about 5 logs higher than in uninfected cells, and then slowly declined. This clearly correlated with the increase in viral transcript levels in the cell (Fig. 1c). As a consequence of the high IFN-β expression, Mx1 was upregulated. Increasing Mx1 expression was first observed at 5 h p.i. (Fig. 1b) and reached a maximal expression level of about 80 times the level of control cells. For wt PR8 virus strains, a much weaker IFN expression was observed compared with delNS1. PR8-NIBSC showed very similar IFN-β induction in the early phase of infection (until 4 h p.i.). However, expression levels then remained about 100-fold below the levels of delNS1 (Fig. 1a). This also resulted in a delayed, tenfold lower expression of Mx1 (Fig. 1b). Interestingly, the second wt PR8 strain, PR8-RKI, showed a further tenfold reduction and delayed induction of IFN-β compared with PR8-NIBSC (Fig. 1a). In addition, a delay of about 1 h was found in the expression of viral NS1, indicating a slower progress of infection for this strain (Fig. 1c). At these low IFN-β expression levels, no increase in expression of Mx1 was detected until 11 h p.i., and only three to four times the level of uninfected cells was subsequently reached (Fig. 1b). For WSN33, IFN-β induction was very similar to that found for PR8-NIBSC and delNS1 until 4 h p.i. However, in contrast to the other virus strains, the induction level stayed relatively constant until 6 h p.i. and then decreased (Fig. 1a). This correlated with a fast increase and a high maximal level of viral NS1 transcripts (Fig. 1c) and no significant rise in Mx1 expression (Fig. 1b).

Effect of IFN suppression on virus replication

In the next step, it was investigated whether suppression of the IFN system could enhance influenza virus replication. The NS1 protein is known to suppress IFN induction, but during the initial period of infection NS1 is not present in cells and IFN expression is induced. We hypothesized that transient expression of NS1 in cells prior to infection could reduce IFN signalling by preventing the induction of IFN in the early phase of infection. NS1 from PR8 was used because this protein has a defect in the domain responsible

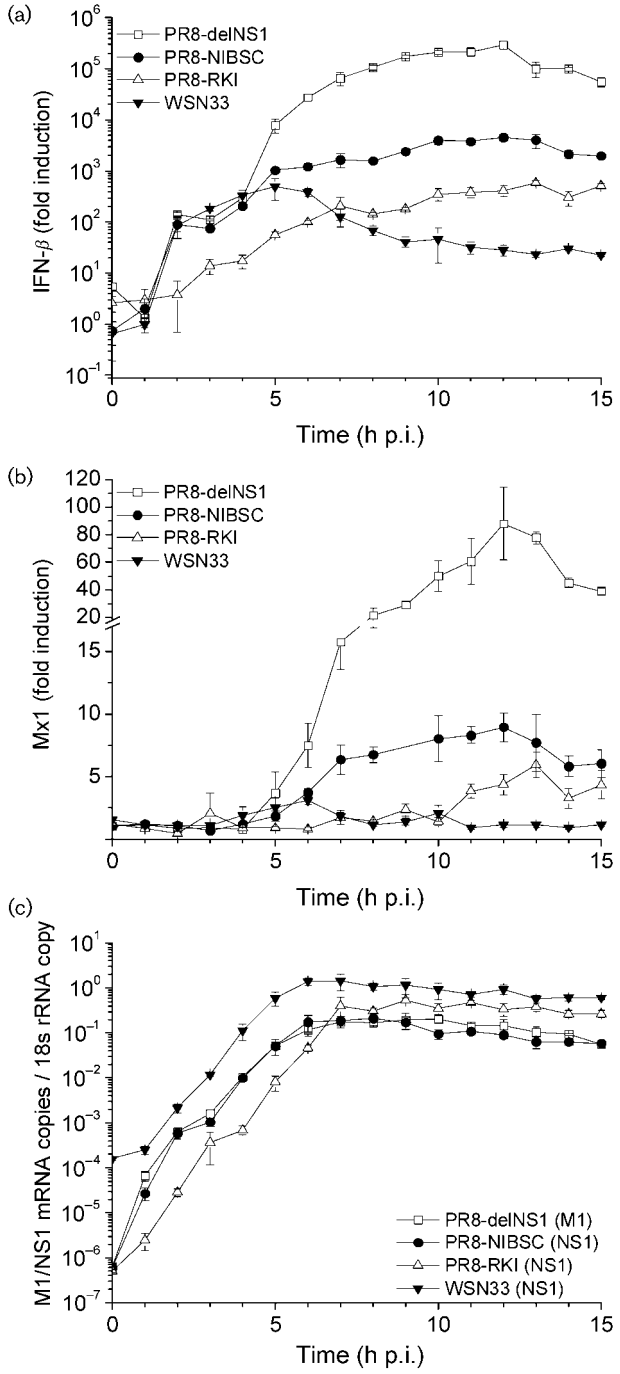

Fig. 1. Induction of IFN signalling in influenza virus-infected MDCK cells. Cells were infected at an m.o.i. of 5 with delNS1, PR8-NIBSC, PR8-RKI and WSN33. Expression of the indicated genes was determined by qRT-PCR. Values represent the mean ± SD of duplicates measured twice in qRT-PCR. The analyses shown are representative of three independent experiments. (a) Relative expression of IFN-β. (b) Relative expression of Mx1. Expression of IFN-β and Mx1 is shown as fold change relative to the mean of three untreated samples. (c) Increase in viral transcript levels (NS1/M1). Viral gene expression (NS1 or M1) is displayed as mRNA copy number of analysed gene per copy of 18S rRNA. For delNS1, the expression of viral matrix protein (M1) was analysed instead of NS1.

for shutdown of host cell mRNA processing and therefore should not be cytotoxic. Cells were transfected with an expression plasmid for PR8 NS1 or empty vector and infected with delNS1 or PR8-NIBSC at 24 h post-transfection (m.o.i. of 0.025). These strains were chosen because both exhibited considerable induction of IFN signalling during infection. Expression levels of IFN-β and Mx1 during infection were compared by qRT-PCR in NS1- and empty vector-transfected cells (Fig. 2a). The expression of both genes was significantly reduced in NS1-transfected cells. For delNS1, maximal IFN-β expression was reduced by 80 % and the resulting Mx1 expression by 60 %. For PR8-NIBSC, IFN-β and Mx1 were expressed at only 5 % and 10 % of the level found in control cells. We concluded that expression of NS1 prior to infection strongly reduced IFN signalling. When virus titres were monitored by haemagglutination (HA) activity and qRT-PCR, higher virus yields and faster replication were observed for delNS1 in NS1-transfected cells (Fig. 2b). Conversely, in the case of PR8-NIBSC, the reduction in IFN signalling did not result in significantly higher virus yields (Fig. 2c). A small increase in virus production speed was observed in NS1-transfected cells up to 24 h p.i., but final virus yields showed no difference. The experiment was also performed with PR8-RKI and WSN33. Although these strains induced only low levels of IFN, a further reduction of at least 80 % was achieved in NS1-expressing cells. However, no significant influence on final virus yield was observed (data not shown). In addition, the experiment was repeated using the phosphoprotein of rabies virus (rP) as an IFN antagonist (Rieder & Conzelmann, 2009). Cells were transfected with the rP plasmid or empty vector and infected with PR8-NIBSC (m.o.i. of 0.025) at 24 h post-transfection. Suppression of IFN signalling was validated by qRT-PCR and suppression efficiencies were comparable to NS1-transfected cells (Fig. 2d). However, identical virus replication dynamics and final virus yields were observed in rP-transfected and control cells (Fig. 2e). Taken together, these data strongly indicated that IFN induction is not a limiting factor for influenza virus replication in MDCK cells.

Effect of IFN signalling activation on virus replication

We then analysed whether IFN treatment prior to infection could inhibit replication of wt influenza viruses in MDCK cells. Supernatants of infected MDCK cells [conditioned medium (CM)] were used as a source of IFN. MDCK cells were infected without the addition of trypsin for 14 h with different influenza virus strains at an m.o.i. of 5. Supernatants were then ultrafiltered (100 kDa cut-off). The absence of virus in ultrafiltered CM was tested by incubating cells for 72 h with CM. No detectable HA titre was found. To test whether the CM contained enough IFN to induce expression of ISGs, Mx1 induction was determined by qRT-PCR in MDCK cells stimulated with CM from influenza virus-infected cells or supernatants of uninfected cells for 5 h (Fig. 3a). As expected, the highest

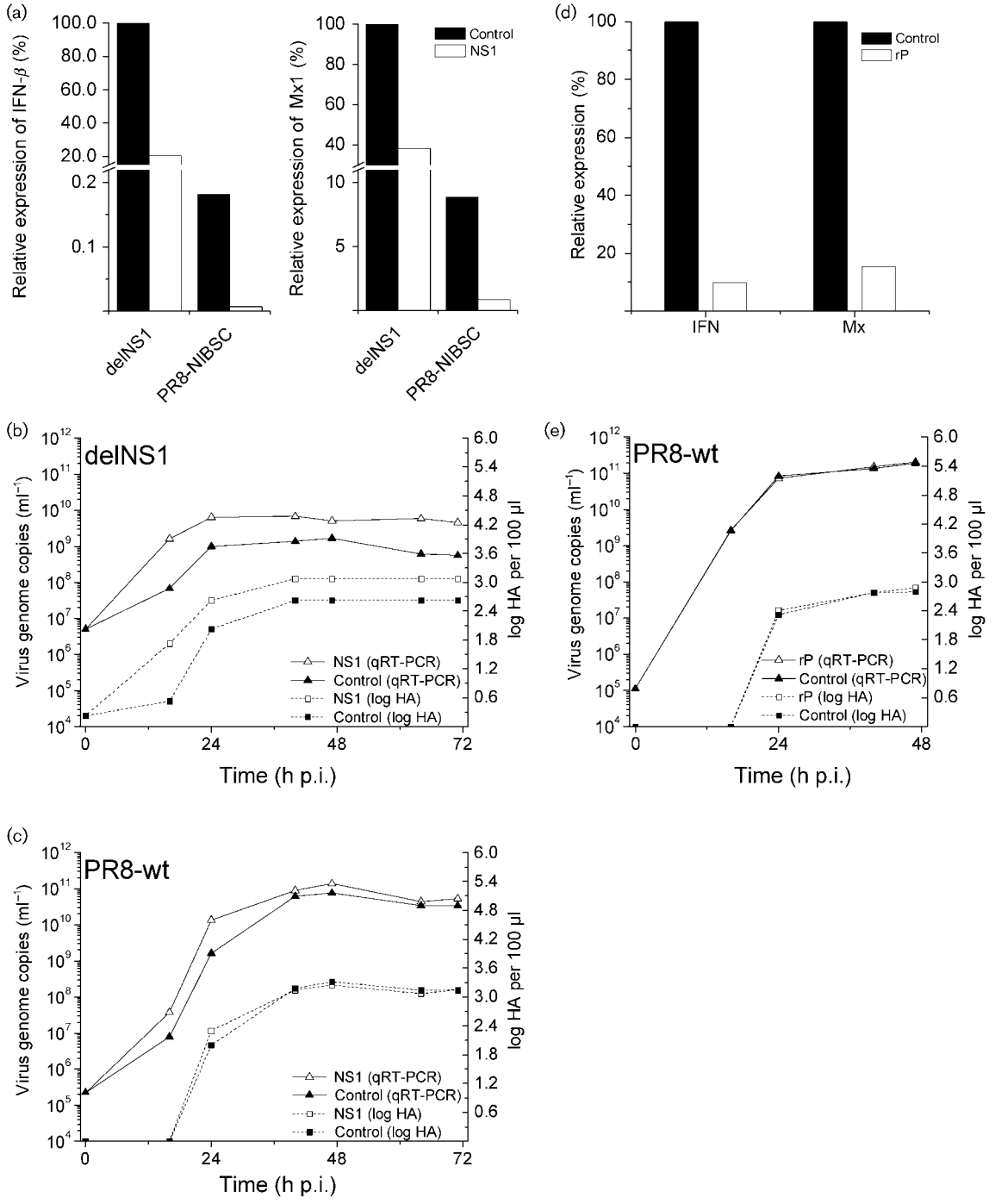

Fig. 2. Effect of transient expression of PR8 NS1 or rP protein on virus replication. MDCK cells were transfected with expression plasmids for PR8 NS1, rP or empty vector 24 h prior to infection. Infections were carried out with delNS1 or PR8-NIBSC using an m.o.i. of 0.025. (a, d) Suppression of IFN signalling in infected NS1-transfected (a) and rP-transfected (d) cells. Expression of IFN-β and Mx1 was analysed by qRT-PCR. Expression levels were determined at 24 h p.i. and normalized to the expression levels induced by delNS1 in control cells, set to 100 % (a) or the levels found in PR8-NIBSC-infected empty vector-transfected cells (d). (b, c, e) Time course of delNS1 (b) and PR8-NIBSC infection in NS1-transfected cells (b, c) and PR8-NIBSC infection in rP-transfected cells (e). Virus yields were estimated by qRT-PCR (segment 7) and a HA activity assay. The data shown are representative of three independent experiments.

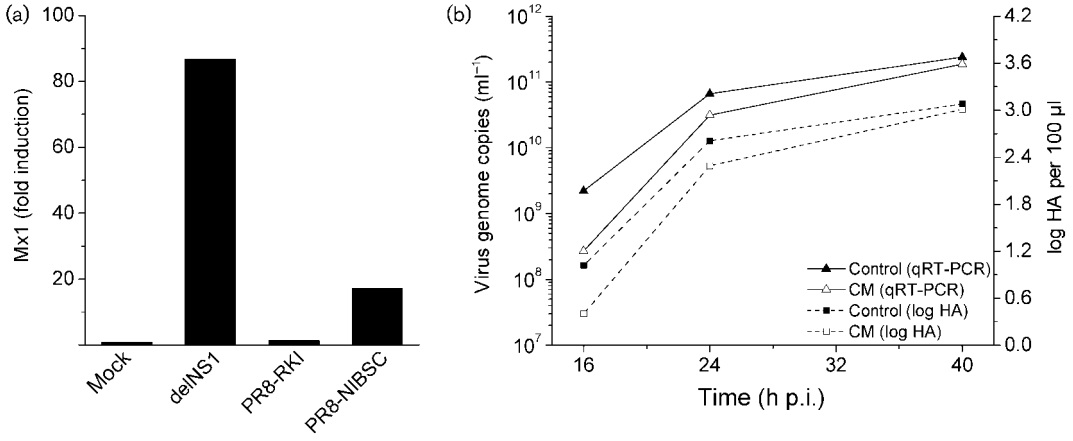

Fig. 3. Influence of IFN stimulation on replication of PR8-RKI. (a) Induction of Mx1 using the CM of different PR8 variants. MDCK cells were infected with the indicated virus strain at an m.o.i. of 5 and CM was taken at 14 h p.i. Virus was removed from the CM by ultrafiltration (100 kDa cut-off). Cells were stimulated for 5 h with the purified CM or with control medium from mock-infected cells. Mx1 expression was determined by qRT-PCR relative to a mock-stimulated sample. (b) Replication of PR8-RKI in cells treated with delNS1 CM. Cells were treated with virus-free CM for 5 h and subsequently infected with PR8-RKI at an m.o.i. of 0.025. Virus yields were estimated by qRT-PCR (segment 7) and a HA activity assay. The results shown are representative of three independent experiments.

induction of Mx1 was found for delNS1 CM. The CM from PR8-NIBSC-infected cells showed significantly less Mx1 induction, and for PR8-RKI, almost no Mx1 induction could be detected. Subsequently, it was analysed whether this induction of the antiviral state could inhibit growth of wt PR8 virus. Cells were treated with delNS1 CM or supernatants of uninfected cells (control) for 5 h and then infected with PR8-RKI at an m.o.i. of 0.025 (Fig. 3b). When the virus titres were monitored by qRT-PCR and HA activity, reduced virus production was seen in the early period of infection (up to 24 h p.i.) for cells treated with CM. However, at the end of infection, almost the same titre was reached in IFN-stimulated and control cells. The experiment was repeated with PR8-NIBSC and WSN33. However, for both strains, no difference in final virus yield was observed between IFN-stimulated and untreated cells (data not shown). The same experiment was performed with PR8-RKI and PR8-NIBSC at lower m.o.i. (0.0025 and 0.00025), but no stronger influence of IFN stimulation was found (data not shown), indicating that IFN treatment had no significant impact on final virus titres.

Antiviral activity of MDCK Mx proteins

Mx proteins are described for many species as key players in the IFN-related innate immune response against influenza virus. To test the impact of canine Mx proteins, canine Mx variants (cMx1 and cMx2) were cloned into a pCAGGS expression vector and analysed for their potential to inhibit replication in a virus-free minireplicon system. Because avian viruses show the highest sensitivity to the antiviral effect of Mx proteins (Dittmann *et al.*, 2008), we used expression plasmids coding for the A/Vietnam/1203/04 (H5N1) polymerase complex. Human embryonic kidney cells (HEK 293T) cells were transfected with the plasmids necessary for the minireplicon assay and 200 ng of expression constructs for different Mx proteins. In addition to cMx1 and cMx2, murine and human Mx homologues, as well as the corresponding inactive mutants and the empty expression vector, were included as controls. Luciferase activity was determined at 24 h post-transfection and Mx expression was confirmed by Western blotting (Fig. 4). For human and murine Mx proteins, a significant reduction in viral polymerase activity was observed compared with empty vector or inactive mutant-transfected cells. However, no inhibition was found for cMx1 and cMx2. Hence, we concluded that canine Mx proteins lack anti-influenza activity.

DISCUSSION

In this study, the impact of IFN induction and subsequent establishment of the antiviral state on influenza virus replication in MDCK cells was determined. Initially, IFN induction and expression of ISGs were analysed for well-characterized virus strains (WSN33, PR8 and its delNS1 mutant) used routinely in influenza-related research. The strain PR8 is also commonly used as a backbone for the development of high-growth reassortants for vaccine production (Wood & Robertson, 2007). In accordance with previous studies, the strongest induction of IFN signalling was found for delNS1 (García-Sastre *et al.*, 1998; Kochs *et al.*, 2009). In contrast, the wt PR8 variants showed significantly less IFN expression. However, significant differences in IFN expression were found between the

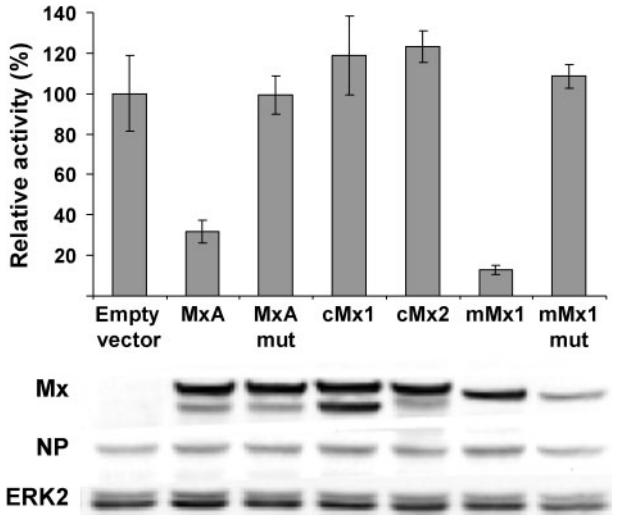

Fig. 4. Anti-influenza activity of canine Mx proteins. HEK 293T cells were transfected with the respective amounts of plasmids for the minireplicon assay and 200 ng of the indicated Mx constructs. Luciferase assays were performed at 24 h post-transfection. The value of empty vector-transfected cells was set to 100 %, and the activities of Mx-transfected cells are expressed as percentages ± SD (n=4). Expression of the indicated proteins was analysed by Western blotting (lower panel). MxA, Human MxA; MxA mut, inactive form of MxA (with T103A mutation); cMx1/2, canine Mx1/2; mMx1, mouse Mx1; mMx1 mut, inactive form of mMx1 (with K49A mutation).

two PR8 isolates. In particular, PR8-NIBSC induced higher levels of IFN and Mx1 expression than PR8-RKI. Only weak stimulation of IFN in PR8-infected A549 cells has been reported previously (Hayman *et al.*, 2006; Kochs *et al.*, 2007a), which is in good agreement with the results obtained for PR8-RKI. On the other hand, the stronger IFN induction by PR8-NIBSC correlated with previous results obtained in our group. PR8-NIBSC infections cause a stronger activation of the general host cell response (B. Heynisch and D. Vester, unpublished results) and an earlier induction of apoptosis, whilst reaching lower virus yields compared with PR8-RKI (Schulze-Horsel *et al.*, 2009). One possible reason for the different levels of IFN induction is amino acid substitutions in the NS1 protein. PR8-RKI carries glutamic acid at positions 55 and 101, whereas PR8-NIBSC possesses lysine at position 55 and aspartic acid at position 101. PR8 strains with glutamic acid at position 55 of NS1 have been reported to induce lower levels of IFN signalling (Murakami *et al.*, 2008). However, differences in viral polymerase activity also could account for strain-specific differences in replication dynamics and IFN induction (Grimm *et al.*, 2007; Murakami *et al.*, 2008). Schulze-Horsel *et al.* (2009) found a higher velocity of virus replication for PR8-NIBSC, which correlates with the results from this study regarding virus expression dynamics. Another difference concerning IFN induction and suppression was observed for WSN33. Whilst both PR8 strains expressed constant levels of IFN in the later phase of infection, decreasing IFN expression was observed during this time period for WSN33. Expression data for viral transcripts indicated very high levels of NS1 in WSN33-infected cells, eventually suppressing IFN induction. A weaker induction of IFN signalling by WSN33 compared with PR8 was also observed in an array analysis of IFN-related genes (Geiss *et al.*, 2002). The authors postulated that differences in the NS1 proteins could be the reason, but that other viral genes might also contribute.

Next, the effects of IFN signalling on influenza virus replication were investigated. It was analysed whether replication of PR8-NIBSC and delNS1, being strong IFN inducing strains, was enhanced by artificial suppression of IFN expression or if the weak IFN inducer PR8-RKI was inhibited by strong IFN stimulation. Suppression of IFN signalling was achieved by transient expression of PR8-NS1 or rP in MDCK cells. Previous studies have already shown that expression of viral IFN antagonist proteins is a simple and efficient way to reduce IFN signalling and increase virus yield (Young *et al.*, 2003; Sherwood *et al.*, 2007; de Vries *et al.*, 2008). In our study, both NS1- and rP-transfected cells showed a significant reduction in IFN signalling. In NS1-transfected cells, this resulted in an increase in virus replication and final virus yield for delNS1. These findings are in good accordance with other reports, which showed that growth of NS1-attenuated influenza viruses was promoted when IFN signalling was absent or inhibited (García-Sastre *et al.*, 1998; Basler *et al.*, 2000; Kochs *et al.*, 2007b). However, PR8-NIBSC and other wt influenza virus strains showed only a slight increase in virus production in the beginning of infection, and no significant differences were observed in final virus titres obtained from NS1-transfected or control cells. As the NS1 protein mediates enhanced translation of viral mRNAs (Hale *et al.*, 2008), this might also contribute to the higher virus production in the early phase of infection. Therefore, rP was applied as another IFN antagonist and this did not enhance PR8-NIBSC virus replication in the early phase. Taken together, these experiments suggest only a low impact of IFN signalling on influenza virus replication. This was unexpected, as several studies previously showed growth enhancement of influenza virus by blocking the expression of IFN. In A549 cells, growth of PR8 increased when the IFNAR receptor was blocked (Ehrhardt *et al.*, 2004) and replication of wt avian and human influenza viruses could be improved by expressing N^{pro} of bovine viral diarrhea virus in the same cell line (Hayman *et al.*, 2007). Additionally, Koerner *et al.* (2007) found higher replication levels of influenza virus strain SC35M in mouse embryo fibroblasts with defective alleles for IFNAR or IFN-β. However, none of these studies was performed using MDCK cells. Hence, the potential of the IFN-induced antiviral state to inhibit influenza virus replication in this host cell line might be limited. This idea was supported by the observation that delNS1 reached moderate virus titres in cells transfected with the empty vector, although these

cells expressed high levels of IFN. To pursue this, the effect of strong IFN stimulation on the replication of PR8-RKI, which induced only low levels of IFN during normal replication, was studied. Using delNS1 CM for IFN stimulation, reduced virus production was observed in the early phase of infection in CM-treated cells. Unexpectedly, the same final virus yields were reached in IFN-stimulated and control cells. Comparable results were also obtained for PR8-NIBSC and WSN33. Even at a low m.o.i. (0.00025), IFN stimulation had no influence on either PR8 strain in MDCK cells. These results contradict previous work describing the sensitivity of PR8 to IFN in other cell lines (Seo *et al.*, 2002; Hayman *et al.*, 2006). However, in the data presented here, neither inhibition nor strong activation of IFN signalling showed significant effects on influenza virus replication. This is in accordance with a report demonstrating delayed replication but an identical final yield of H5N1 and H3N2 strains in human Calu-3 cells pre-treated with IFN-β (Zeng *et al.*, 2007). Therefore, it seems clear that, although IFN is induced in MDCK cells during influenza virus infection, it does not represent a limiting factor for replication of wt virus strains in this cell line.

A possible reason for this might be the missing anti-influenza virus activity of canine Mx proteins observed in this study. For many species, including humans, mice, cotton rats and pigs, Mx proteins are considered a main IFN-dependent effector molecule inhibiting influenza virus growth (reviewed by Haller *et al.*, 2009). However, in this study, no influence of cMx1 and cMx2 on viral polymerase activity was found for an influenza A/Vietnam/1203/04 minireplicon system, although this virus has previously been shown to be highly sensitive to Mx proteins of humans and mice (Dittmann *et al.*, 2008). In contrast to other cell lines (Holzinger *et al.*, 2007), MDCK cells constitutively express moderate levels of cMx1 (Nakamura *et al.*, 2005 and our observations, data not shown). Absent anti-influenza activity of the MDCK Mx1 protein could explain the high permissiveness of this cell line to influenza viruses. To elucidate the reason for the lack of anti-influenza activity, MDCK Mx1 and Mx2 were sequenced, but no differences to *Canis familiaris* reference sequences were identified (data not shown). Canine Mx2 is homologous to human MxB (Haller *et al.*, 2009). The cMx1 protein is phylogenetically related to human MxA and porcine Mx1, which both possess anti-influenza activity. The sequences of the GTPase domain of both cMx proteins are highly conserved when compared with other members of the Mx family, and no obvious inactivating mutations as described for human and murine Mx proteins have been found (Pitossi *et al.*, 1993). Also, critical amino acids in the effector domain such as glutamic acid at position 645 in MxA are present in the canine Mx proteins (Zurcher *et al.*, 1992). cMx1 and cMx2 have been shown to be localized in the cytoplasm, and an inhibitory activity against vesicular stomatitis virus was detected for cMx2 (Nakamura *et al.*, 2005). However, the mechanism of anti-influenza activity of Mx proteins is not well understood (Haller *et al.*, 2009) and species-specific differences in anti-influenza potential have been reported (Stertz *et al.*, 2007; Benfield *et al.*, 2008; Morozumi *et al.*, 2009). Dogs had been regarded as non-susceptible hosts to influenza A (Harder & Vahlenkamp, 2010). This may have prevented the development of influenza-inhibiting properties of canine Mx proteins. Recently, a screen with several cell lines was performed to identify the susceptibility of different cell lines to seasonal influenza A H1N1, swine-origin H1N1 and avian H5N1 (Li *et al.*, 2009). Among all the animal cells tested, MDCK cells best supported the growth of all the influenza viruses. A limited influence of IFN signalling partially caused by a lack of anti-influenza activity of canine Mx proteins found in our study may contribute to the superior growth of influenza viruses in this cell line.

In summary, considerable IFN expression was found in influenza virus-infected MDCK cells, but neither inhibition nor strong stimulation of IFN induction showed a significant impact on final virus titres. A lack of inhibitory potential of canine Mx proteins against influenza virus replication was identified as a possible reason for this unusual observation. Hence, we concluded that IFN signalling has only a minor effect on influenza virus replication in MDCK cells, which makes them an ideal system for high-yield vaccine production.

METHODS

Cells and viruses. HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (high glucose; Biochrom) supplemented with 5 % fetal calf serum and antibiotics. Experiments with adherent MDCK cells (ECACC 84121903) and virus infections were performed in six- or 24-well plates. Culture and infection conditions were as described previously (Genzel *et al.*, 2004). Stocks of influenza viruses PR8-NIBSC, PR8-RKI and WSN33 (a gift from Georg Kochs, University of Freiberg, Germany) were prepared in MDCK cells. The virus delNS1 (AVIR Green Hills Biotechnology) was grown in Vero cells cultured under the same conditions as the MDCK cells. M.o.i. was calculated based on viable cell number at the time of infection and active virus titre (50 % tissue culture infective dose) of virus stocks.

Plasmids. The expression plasmid for PR8 NS1 has been described previously (Talon *et al.*, 2000). The pCR3 vector containing rP was provided by the group of Karl-Klaus Conzelmann (Max von Pettenkofer Institute, Ludwig-Maximilians-University Munich, Germany). Canine Mx proteins were cloned using cDNA from MDCK cells. PCR products were generated using the following primers: Mx1-*Bgl*II-for, 5′-CGGAGATCTTGATGGTTAATTCACA-AGGAAAAATCA-3′; Mx1-*Eco*RV-rev, 5′-CGGGATATCCGGCCTT-AACCAGGGAATTT-3′; Mx2-*Bcl*I-for, 5′-CGGTGATCATCATGT-CTAAGGCCCACGGTTC-3′; and Mx2-*Eco*RV-rev, 5′-CGGGATAT-CGGACCTGCCCCCTTTAACTG-3′. Fragments were cloned into expression vector pCMC-Tag1 (Stratagene) using *Bgl*II and *Eco*RV digestion. For higher expression levels, inserts were introduced into the pCAGGS expression vector (Niwa *et al.*, 1991). Human and mouse Mx expression vectors as well as all plasmids for the minireplicon assay have been described in detail by Dittmann *et al.* (2008).

Transfection. For transient transfection of MDCK cells, a MicroPorator (Digital Bio) was used. Electroporation was carried

out using one pulse of 1700 V for 20 ms. The ratio of plasmid DNA and cells was 4 μg per 1.5×10^6 cells. Cells were infected at 24 h post-transfection.

Stimulation with CM. To prepare CM, MDCK cells were infected with influenza virus at an m.o.i. of 5 without the addition of trypsin to avoid the digestion of secreted cytokines. Supernatant was harvested at 14 h p.i. and centrifuged for 10 min at 300 ***g***. The resulting supernatant was centrifuged for 15 min at 10 000 ***g***, and 14 ml CM was transferred into a Vivaspin 20 100 kDa cut-off ultrafiltration tube (Sartorius) and centrifuged at 5000 ***g*** for 1 h at room temperature. The virus-free CM was used to stimulate MDCK cells. Medium of mock-infected cells was used as a control. Cells were washed twice with PBS and 1.5 ml CM was used per 35 mm diameter dish. Stimulation was carried out for 5 h and cells were subsequently infected with influenza virus at an m.o.i. of 0.025 with the addition of trypsin and gentamicin (0.1 mg ml^{-1} each; Gibco). Samples were taken at 0, 16, 24 and 40 h p.i. and the virus load of the supernatant was determined by qRT-PCR and a HA assay.

HA assay. HA activity was determined as described previously (Kalbfuss *et al.*, 2008). Titres are reported as log HA units per assay volume (log HA per 100 μl).

RNA extraction. Total RNA isolation from infected cells including DNase digestion of genomic DNA was carried out using a NucleoSpin RNA II kit (Macherey-Nagel). Viral RNA from cell culture media samples was extracted with a NucleoSpin RNA Virus kit (Macherey-Nagel). Preparations were carried out according to the manufacturer's instructions.

Reverse transcription. RevertAid M-MuLV reverse transcriptase (Fermentas) was used for reverse transcription of RNA samples. For cellular RNA, 1 μg total RNA was transcribed with an oligo(dT) primer (Invitrogen). Transcription of genomic viral RNA from cell supernatants was carried out using 10 μl viral RNA and a primer binding to the conserved ends of influenza genome segments (UNI12 primer; Hoffmann *et al.*, 2001). Reaction conditions were as recommended by the supplier.

qRT-PCR. qRT-PCR was performed on an iCycler IQ (Bio-Rad) using MESA GREEN Master Mix Plus (Eurogentec). The sequences of primers used are given in Supplementary Table S1 (available in JGV Online). One microlitre of reverse transcription reaction was analysed in a final reaction volume of 25 μl. The reaction set-up and thermal cycling parameters were taken from the technical data sheet of the master mix. Expression of IFN-β and Mx1 was calculated using the $2^{-\Delta\Delta Ct}$ method, using 18S rRNA for data normalization and uninfected cells as the calibrator. NS1 expression was determined as the ratio of NS1 mRNA to 18S rRNA. For analysis of virus loads in cell culture supernatants, a previously described method (Di Trani *et al.*, 2006) was applied with the modification of using plasmid standards (PR8 segment 7 in pGEM; Promega) for absolute quantification of viral genome copies.

Minireplicon assay. To determine the antiviral activity of canine Mx proteins, a minireplicon assay was performed as described by Dittmann *et al.* (2008). In brief, HEK 293T cells were seeded in 12-well plates. Cells were transfected with 1 μg DNA using 3.2 μl Nanofectin (PAA Laboratories) according to the manufacturer's protocol. The following concentrations of plasmids were co-transfected: 10 ng plasmids encoding the polymerases PA, PB1 and PB2 with 100 ng NP of influenza virus A/Vietnam/1203/04 (H5N1); 25 ng reporter construct pPOLI-Luc-RT and 50 ng pRL-SV40-Rluc encoding *Renilla* luciferase; 200 ng plasmids encoding Mx proteins as indicated; supplemented with empty pCAGGS vector to achieve 1 μg DNA per transfection. A Dual Luciferase assay (Promega) was carried out at 24 h post-transfection according to the manufacturer's instructions. Twenty microlitres of these lysates were subjected to Western blotting using antibodies against MxA (clone M143; Flohr *et al.*, 1999), viral NP (clone AA5H; AbD Serotec) and ERK2 (Santa Cruz Biotechnology).

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