Variation in the ability of human influenza A viruses to induce and inhibit the IFN-β pathway

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Abstract

We investigated the ability of a selection of human influenza A viruses, including recent clinical isolates, to induce IFN-β production in cultured cell lines. In contrast to the well-characterized laboratory strain A/PR/8/34, several, but not all, recent isolates of H3N2 viruses resulted in moderate IFN-β stimulation. Through the generation of recombinant viruses, we were able to show that this is not due to a loss of the ability of the NS1 genes to suppress IFN-β induction; indeed, the NS1 genes behaved similarly with respect to their abilities to block dsRNA signaling. Interestingly, replication of A/Sydney/5/97 virus was less susceptible to pre-treatment with IFN-α than the other viruses. In contrast to the universal effect on dsRNA signaling, we noted differences in the effect of NS1 proteins on expression of interferon stimulated genes and also genes induced by a distinct pathway. The majority of NS1 proteins blocked expression from both IFN-dependent and TNF-dependent promoters by an apparent post-transcriptional mechanism. The NS1 gene of A/PR/8/34 NS1 did not confer these blocks. We noted striking differences in the cellular localization of different influenza A virus NS1 proteins during infection, which might explain differences in biological activity.
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Introduction

The innate immune response is the first barrier a virus encounters after entry into the infected cell and is initiated within hours of receptor binding. It involves the induction of many factors, including the type I IFNs (IFNs-α/β) which induce a range of antiviral processes. The complex mechanisms of these processes are not fully elucidated. Within an infected cell, it is believed that the proximal inducer of IFNs-α/β is intracellular double-stranded RNA (dsRNA) generated as an intermediate during viral replication. The dsRNA triggers a series of events culminating in the activation of PKR and other kinases (TBK-1 and IKKε). Phosphorylation of the substrates of these enzymes results in the translocation of transcription factors NF-κB and IRF-3 from the cytoplasm to the nucleus where, together with factors ATF-2/c-Jun, IRF-7, p300 and CBP, they bind to the IFN-β promoter to form a transcription complex which ultimately drives IFN-β production (Wathelet et al., 1998). Secreted IFN-β subsequently binds to its receptor on the same cell or on neighbouring uninfected cells inducing an antiviral state. This binding step initiates the activation of a JAK/STAT pathway. Consequently, the Interferon Stimulated Response Element (ISRE) in the promoters of many IFN response genes are induced, and an array of genes, including PKR, 2′,5′-oligoadenylate synthetases and Mx proteins, are expressed (as reviewed by Goodbourn et al., 2000).

IFNs were first discovered as factors induced by treatment of cells with heat inactivated influenza virus that could inhibit viral replication (Isaacs and Lindenmann, 1957). Interestingly, many negative strand RNA viruses, for example Sendai Virus, Simian Virus 5 (Didcock et al., 1999a), Ebola Virus (Basler et al., 2003) and human influenza A virus, possess mechanisms of counteracting this host defense system. There is a body of evidence suggesting that the non-structural protein 1 (NS1)

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encoded by influenza A virus segment 8 can have an antagonistic effect on the production of type I IFN. In the context of virus infection, Garcia-Sastre and colleagues showed in 1998 that a recombinant A/PR/8/34 strain lacking the NS1 gene, termed delNS1, was highly attenuated in IFN-competent MDCK cells and 10-day-old chicken embryos. In contrast, the delNS1 virus grew in IFN-deficient systems including Vero cells, young chicken embryos and STAT1−/− mice (Egorov et al., 1998; Garcia-Sastre et al., 1998). The specific mechanisms by which the NS1 protein confers the block in IFN-β production are still not fully resolved. It has been suggested that the NS1 protein might bind to and sequester dsRNA produced during viral infection, thereby inhibiting the downstream events that lead to IFN-β induction. The NS1 protein can bind to both single- and double-stranded RNA, however, the affinity for dsRNA is higher (Hatada and Fukuda, 1992). Amino acids in the amino terminus of NS1 are implicated in this interaction (Qian et al., 1994). Substitution of basic amino acids at position 38 and 41 showed that these two positions are specifically required to mediate dsRNA binding. Structural analyses imply that the arginine at position 38 binds electrostatically to the dsRNA, and that the lysine at position 41 contributes to the affinity (Wang et al., 1999). NS1 expression constructs with mutations R38A and K41A were no longer able to prevent the translocation of IRF-3 to the nucleus (Talon et al., 2000) or the activation of NF-κB (Wang et al., 2000). Furthermore, a recombinant WSN virus with the same two mutations induced higher amounts of IFN-β in A549 cells than wild-type virus and was more attenuated in mice (Donelan et al., 2003), thus supporting a role for a NS1-dsRNA interaction in blocking IFN-β induction.

NS1 also binds two proteins involved with mRNA processing and transport, namely cleavage and polyadenylation specificity factor (CPSF) and poly (A)-binding protein II (PABII) (Chen et al., 1999; Nemeroff et al., 1998). Amino acids 215 to 237 have been identified as the binding site for PABII (Li et al., 2001). The CPSF binding site is located around residue 186 (Li et al., 2001). Krug and colleagues have proposed that this domain of NS1 can prevent the antiviral response by inhibition of post-transcriptional processing of the 3′ ends of cellular antiviral mRNAs (Noah et al., 2003). A recombinant virus with mutations at positions 184 to188 was attenuated in IFN competent cells and infection resulted in high production of antiviral mRNAs (Noah et al., 2003). Consistent with this, Ortín and colleagues had already shown that NS1 expression causes retention of mRNAs in the nucleus (Fortes et al., 1994). Furthermore, incomplete processing of recently induced mRNAs was observed in influenza A virus infected cells and was deficient for a virus mutant with a temperature sensitive defect mapping to the NS1 gene (Shimizu et al., 1999).

Several studies have nevertheless demonstrated that NF-κB and IFN-β mRNA are induced and upregulated respectively upon influenza virus infection in dendritic cells, macrophages and A549 cells, implying that blocks in IFN-β mRNA induction are not complete (Huang et al., 2001; Matikainen et al., 2000; Ronni et al., 1997). Infection with wild-type

influenza A virus (A/Udorn/72) resulted in both the activation of IRF-3 (Kim et al., 2002) and the production of IFN-β mRNA albeit at low levels (Noah et al., 2003). Moreover, the downstream events of IFN signaling have also been detected upon influenza virus infection. This implies that despite the ability of the virus to block several stages of the IFN induction pathway, all of the blocks to IFN induction are incomplete and some IFN is secreted. MxA mRNA was detected by Northern blot analysis in human lung cells (A549s and HFL1) and PBMCs infected with wild-type (A/Beijing/353/89, H3N2) influenza virus (Ronni et al., 1995, 1997), and microarray analysis demonstrated that MxA was upregulated in A549 cells upon infection with both a wild-type A/PR/8/34 strain and the del NS1 virus (Geiss et al., 2002; Ronni et al., 1997). mRNA expression levels of MCP-1, MCP-3 and IP10 were also enhanced in macrophages infected with virus (A/Beijing/353/89) in an IFN-dependent manner (Geiss et al., 2002; Matikainen et al., 2000). Notably, influenza virus induces more IFN gene expression in macrophages than in A549 cells, implying that the level of induction might also be cell type dependant (Ronni et al., 1997).

In previously published studies, various different strains of influenza virus have been used by different groups. These strains contain sequence variation in their viral proteins, including NS1, yet it is often assumed that their properties are typical of all influenza viruses. However, strains which have undergone extensive laboratory passage for more than 70 years, such as A/WSN/33 and A/PR/8/34, may not reflect the characteristics of more recent isolates with a lower passage history or clinical isolates. Indeed, it has been shown that continuous passage in cell culture can lead to viruses which have lost the ability to control IFN induction (Hagmaier et al., 2003). In this study, we therefore wished to address whether different human influenza viruses varied in their IFN-β antagonistic properties. A panel of viruses, including laboratory-adapted strains and recent clinical isolates, was produced, and the ability of each virus to induce and inhibit IFN-β induction was investigated. We have found a great deal of variation in the IFN-β induction profiles of different influenza A strains, although there is no clear distinction between of laboratory-adapted and non-laboratory-adapted strains. The efficiency and mechanism by which the IFN response is blocked are clearly strain-specific.

Results

Wild-type influenza strains vary in their induction of IFN-β and their activation of cellular signaling pathways

To assess the ability of a panel of human influenza A viruses (Table 1) to induce IFN-β, we generated a stable A549 human lung alveolar cell line containing a firefly luciferase gene driven by the IFN-β promoter. Cells were infected at an moi of 5, and induction of the reporter was assessed after 8 h (Fig. 1A). Most of the viruses were poor inducers of IFN-β promoter activity. Unexpectedly, infection with a subset of the strains tested, namely A/England/327/90, A/England/41/96 and A/

Table 1
Panel of viruses

Strain	Subtype	Passage history
Laboratory adapted:		
A/PR/8/34	H1N1	Extensively passaged in eggs, mouse adapted
A/Victoria/3/75	H3N2	Laboratory adapted
A/Sydney/5/97	H3N2	Recent isolate, passaged in eggs
		Antigenic property
Clinical isolate, passaged 3–4 in MDCKs:		
A/England/492/95	H3N2	A/Johannesburg/33/94
A/England/41/96	H3N2	A/Johannesburg/33/94
A/England/919/99	H3N2	A/Sydney/5/97
A/England/24/00	H3N2	A/Panama/2007/99

Sydney/5/97, resulted in significantly higher IFN-β induction. Western blot analysis showed that equivalent amounts of NP and NS1 accumulated in cells infected by viruses regardless of

the extent to which IFN-β had been induced (Fig. 1B). This was also confirmed by biosynthetic labeling of polypeptides with S^{35} (data not shown). IFN-β induction was abolished upon UV inactivation of the virus, implying induction was dependent on replication (Fig. 1C).

In order to establish that the IFN-β promoter induction observed resulted in IFN-β synthesis and its secretion, we measured the levels of endogenous IFN-β mRNA produced during infection with a subset of the viruses (Fig. 2A) and the amount of IFN-β secreted in the supernatant by ELISA (Fig. 2B). The profile was similar to that observed with the reporter construct (Fig. 1A). Low levels of IFN-β were secreted by cells infected with most of the influenza A viruses, and infection with A/Sydney/5/97 resulted in the highest IFN-β secretion into the medium. We note that infection with A/Sydney/5/97 also leads to the induction of the dsRNA- and IFN-responsive ISG56 (Fig. 2A) and ISG15 genes (data not shown).

Since induction of the IFN-β promoter is associated with the activation of NF-κB and IRF-3, we next investigated the status of these factors in infected cells. Following infection with A/Sydney/5/97, NF-κB was detected in increased amounts in the nuclear extracts of infected cells (Fig. 3A). A minor amount was also detectable upon infection with A/PR/8/34, A/Victoria/3/75 and A/England/492/95. To investigate the state of activation of IRF-3, we examined the localization during viral infection. This showed that IRF-3 was translocated from the cytoplasm to the nucleus in many of the cells infected by A/Sydney/5/97 (Fig. 3B). In contrast, infection
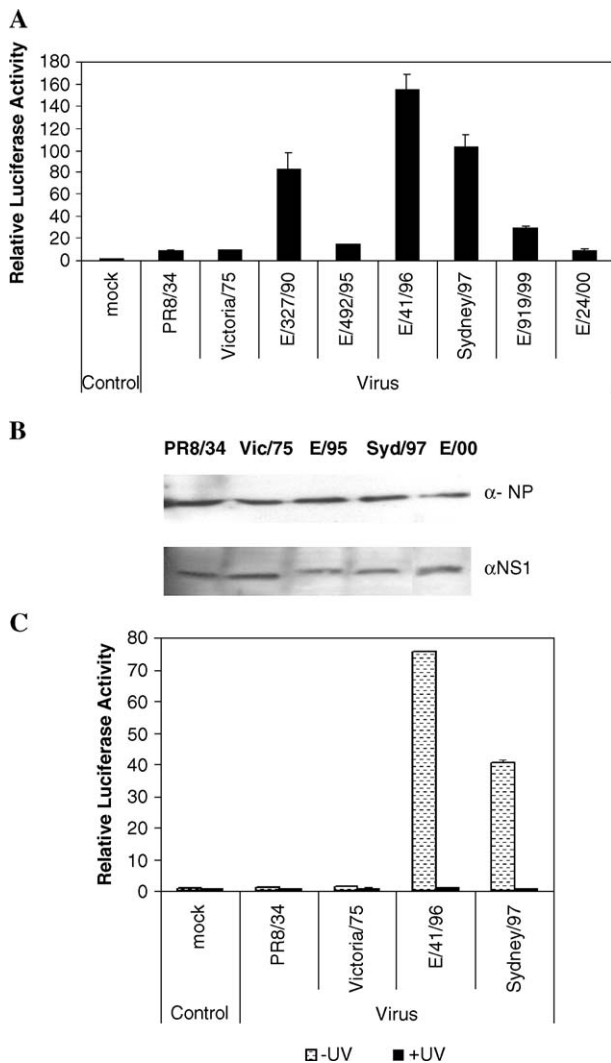

Fig. 1. Influenza A virus strains induce different amounts of IFN-β promoter activity. (A) An A549 IFN-βLuc reporter cell line was infected with a panel of viruses at an moi of 5. Luciferase activity was measured 8 h post-infection. (B) Western blotting was performed to compare the amount of NP and NS1 produced 8 h post-infection. (C) The A549 IFN-βLuc reporter cell line was infected with a selection of viruses at an moi of 5 which had or had not been UV inactivated. Luciferase activity was measured 8 h post-infection.
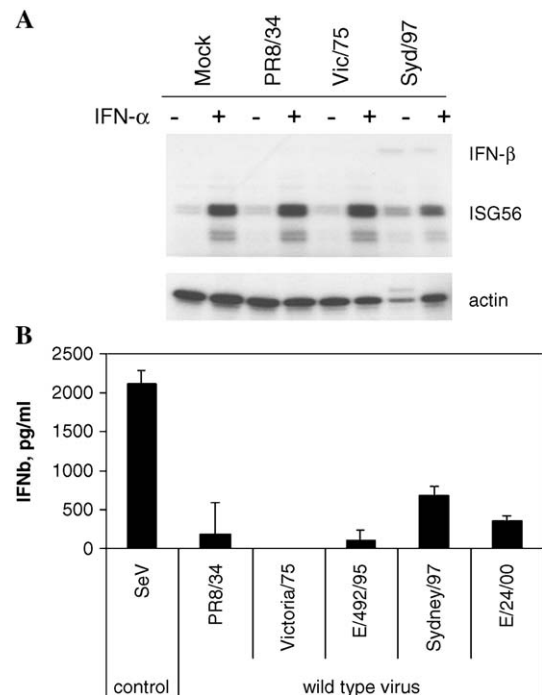

Fig. 2. IFN-β is made and secreted from A549 cells upon influenza A virus infection. (A) A549 cells were infected with virus at an moi of 5 for 2 h and then treated with or without IFN-α 1000 IU/ml for a further 2 h. RNA was extracted and probed for IFN-β or ISG56 mRNA. (B) A549 cells were infected with viruses at an moi of 5. 24 h post-infection, the amount of IFN-β released into the culture supernatant was measured by ELISA.
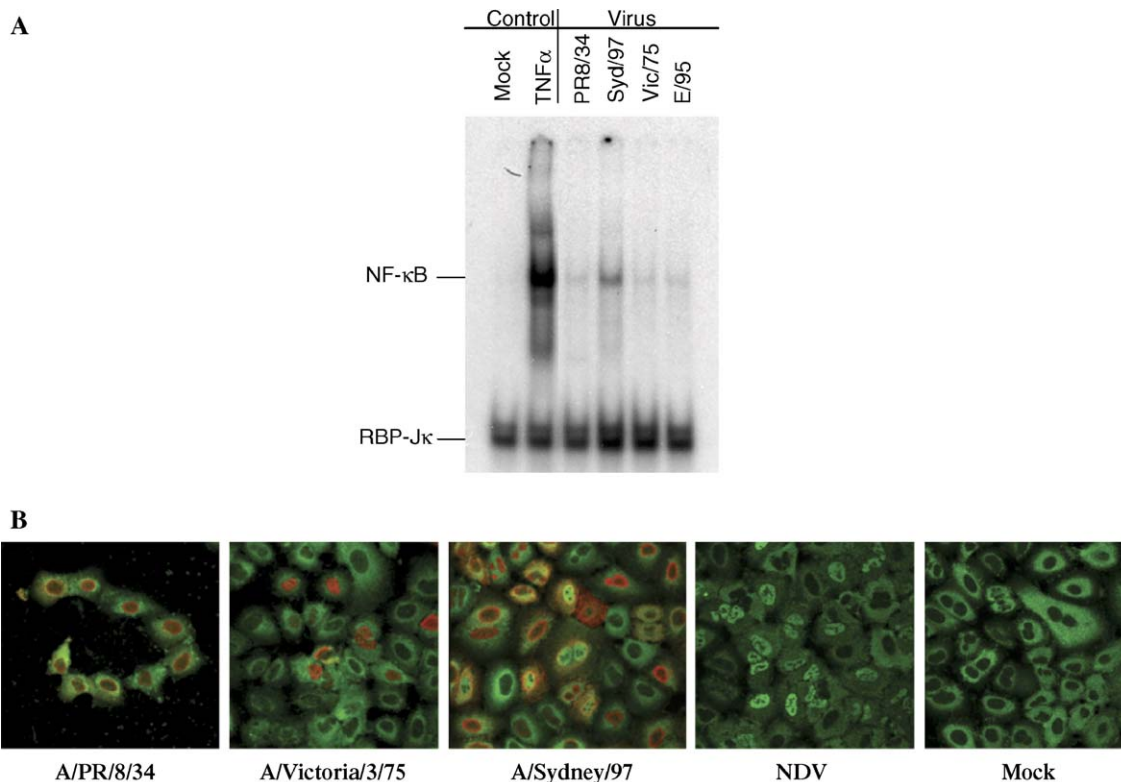
Fig. 3. Infection with influenza virus A/Sydney/5/97 results in increased amounts of NF-κB in the nucleus and the activation of IRF-3 in comparison to infection with A/PR/8/34 or A/Victoria/3/75. (A) A549 cells were infected with virus at an moi of 5 for 4 h, nuclear extracts prepared and an EMSA carried out for NF-κB. (B) A549 cells were infected with virus at an moi of 5 for 8 h and then strained for IRF-3 (green) and virus NP (red).

with A/PR/8/34 or A/Victoria/3/75 did not result in translocation of IRF-3.

A/Sydney/5/97 is less susceptible to pre-treatment of cells with interferon than are other influenza A strains

Despite inducing IFN-β, A/Sydney/5/97 was still able to undergo multiple cycles of replication in the A549 cells (Fig. 4A). We therefore assessed whether A/Sydney/5/97 could replicate in cells previously treated for 24 h with IFN-α. Treatment with IFN-α did not completely block multicycle replication of any of the viruses tested (Fig. 4B); however, A/Sydney/5/97 was less sensitive to the antiviral effects of the exogenous IFN-α, compared with A/PR/8/34 and A/Victoria/3/75.

The NS1 proteins of a range of human influenza A viruses can inhibit dsRNA stimulated IFN-β reporter induction

NS1 has been shown to play an important role in modulating the host IFN-β response. We determined the sequence of the NS1 proteins from each virus and noted several amino acid changes (Table 2). We therefore wanted to establish whether the differences observed in IFN-β induction between the viruses were defined by differences in the sequence of NS1 proteins. In particular, we wanted to test whether the NS1 protein of A/Sydney/5/97 virus was ineffective at limiting IFN-β induction. We cloned and expressed the

NS1 genes derived from representative viruses from the panel and tested their ability to block dsRNA- or SeV-stimulated IFN-β induction. All of the NS1 proteins blocked IFN-β induction to similar extents in both A549 (Fig. 5A) and Vero cells (Fig. 5B). No differences were observed between the different NS1 constructs when compared across a wide range of concentrations (data not shown). Equivalent amounts of NS1 protein were expressed, with the exception of A/PR/8/34 NS1, which showed increased expression (Fig. 5C). We attribute the increased expression of NS1 from A/PR/8/34 to the documented ability of this gene product to enhance translation of proteins expressed from cDNA constructs (Salvatore et al., 2002).

Recombinant influenza A viruses that differ only in their segment 8 genes do not vary in their IFN-β induction in A549 cells

To confirm the observation that NS1 proteins from all the influenza A virus strains in the panel were able to block IFN-β induction, we also used reverse genetics to engineer viruses with heterologous NS genes in a stable genetic background of A/Victoria/3/75. As there were sequence changes in the overlapping region encoding NS1 and NEP, the entire NS gene (RNA segment 8) was exchanged. The cells were infected with the recombinant viruses and IFN-β induction measured after 8 h (Fig. 6A). Strikingly, the recombinant virus with A/Sydney/5/97 NS gene or with A/England/41/96
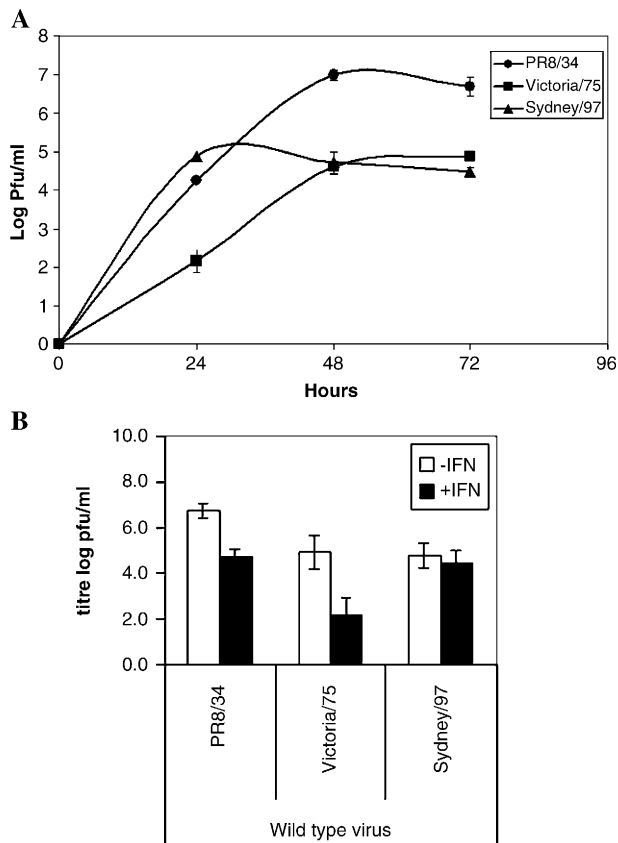
A

B

Fig. 4. A/Sydney/5/97 virus is less susceptible to pre-treatment of cells with interferon than other influenza A virus strains. (A) A549 cells were infected with influenza A viruses at an moi of 0.01. Culture supernatants were then harvested at 24, 48 and 72 h post-infection and titrated on MDCK cells. (B) A549 cells were incubated with 1000 IU/ml IFN-α for 24 h. The cells were then infected at an moi of 0.01. 48 h post-infection, cell culture supernatants were titrated on MDCK cells.

NS gene (both of whose parental viruses were high inducers) induced only low levels of IFN-β, similar to all the other recombinant viruses. A Western blot analysis to quantify NS1 protein accumulation in cells infected with wild-type A/Sydney/5/97 virus or with the recombinant A/Victoria/3/75 bearing the A/Sydney/5/97 NS gene indicated that very similar levels of NP and NS1 were produced in comparison to the other viruses (Figs. 1B and 6B). Thus, the high induction of IFN-β by wild-type A/Sydney/5/97 virus was not explained by a defect in NS1 sequence or NS1 protein accumulation in infected cells.

NS1 effects on ISRE function

Although the A/Sydney/5/97 wild-type virus induced more IFN-β production than the other viruses, it showed increased resistance to the antiviral effects of the IFN-β it induced during infection. We therefore investigated whether influenza A viruses can block the consequences of IFN-β production by inhibiting the IFN-β induced activation of genes under the control of the ISRE (IFN-stimulated response element). A549 cells were transfected with a luciferase reporter construct containing 4 repeats of an ISRE from the 9–27 promoter

(ISRELuc), and then infected with wild-type virus at an MOI of 5. Exogenous IFN-α was applied to induce the ISRE. All of the viruses inhibited the signal in this assay, with the exception of A/PR/8/34 (Fig. 7A). It is notable that all of the viruses induced some luciferase, even in the absence of exogenous IFN-α, probably because the initial block on IFN production is not complete.

To confirm that the apparent block in ISRE induction was conferred by the NS1 protein, cells were co-transfected with the ISRE reporter construct and expression plasmids for each of the NS1 proteins. 48 h post-transfection, cells were incubated with IFN-α, and luciferase activity was measured. Again all of the NS1 proteins, except A/PR/8/34 NS1, blocked the ISRE induced signal in both A549 and Vero cells (Figs. 7B and C). To verify the role of NS1 in the context of viral infection, we also showed that the recombinant virus containing the A/PR/8/34 NS gene in a genetic background of A/Victoria/3/75 behaved like the wild-type virus A/PR/8/34, i.e., neither virus blocked the ISRE induced signal (Fig. 7D).

To investigate whether the block by NS1 to the IFN induced expression from ISRE-luciferase was operating at the level of signal induction, we looked at the formation of ISGF3 in the nucleus of cells infected with A/PR/8/34 virus or with A/Victoria/3/75. In both cases, the formation of ISGF3 was the same as in interferon treated cells which had been mock infected (Fig. 8A).

Although the results of the reporter assays would suggest that the NS1 proteins typified by A/Victoria/3/75 were blocking the signal transduction pathway leading to the activation of the ISRE, the results in Fig. 8A suggested that signaling was not affected. We were mindful of the additional property of NS1 to block post-transcriptional processing and considered that the block exerted by the NS1 protein of A/Victoria/3/75 was mediated after the production of the mRNA transcript. A block of this nature should be effective against a range of transiently induced genes. To test this, we assessed the abilities of the NS1 proteins to block expression of another promoter whose activation pathway was unrelated to that of the ISRE. Each NS1 protein affected the TNFα induction of an NF-κB-dependent reporter (Fig. 8B) in the same way as had been observed for induced expression from the ISRE luciferase reporter. An analysis of the abilities of A/PR/8/34 and A/Victoria/3/75 viruses to block NF-κB activation in response to TNFα confirmed that the block was not operating on the TNFα signal transduction pathway (Fig. 8C), since NF-κB was present in nuclear extracts of treated cells, again consistent with a post-transcriptional effect.

To address the genetic basis of the difference between NS1 proteins of A/PR/8/34 and A/Victoria/3/75 in blocking expression of newly induced genes, we engineered two chimeric NS1 genes which contained either RNA binding domains (amino termini) or effector domains (carboxy termini) from each virus. The ability to block IFN-induced ISRE gene expression mapped to the effector domain (Fig. 9A). Within this domain two regions have been described

Table 2
Amino acid changes in NS1

Position	3	18	21	22	23	26	27	28	41	48	55	56	59	60
Consensus	S	I	Q	V	V	E	I	S	R	N	K	A	H	V
A/PR/8/34	P	V	R	–	A	–	–	G	K	S	E	T	R	A
A/Victoria/3/75	–	V	–	I	–	–	–	G	K	S	E	–	–	–
A/Sydney/97	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/England/492/95	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/England/41/96	–	–	–	–	–	–	–	–	–	–	–	–	–	–
A/England/919/99	–	–	–	–	–	K	–	–	–	–	–	–	–	–
A/England/24/00	–	–	–	–	–	–	K	–	–	–	–	–	–	–

Position	67	78	82	84	90	95	98	101	103	106	107	111
Consensus	K	K	A	T	I	I	L	N	F	M	P	V
A/PR/8/34	R	–	–	V	L	L	M	D	S	I	–	–
A/Victoria/3/75	–	T	–	–	–	T	–	D	–	–	–	–
A/Sydney/97	–	–	–	–	–	–	–	–	–	–	–	–
A/England/492/95	–	–	–	–	–	–	–	–	–	–	–	–
A/England/41/96	–	–	–	–	–	–	–	–	–	–	S	–
A/England/919/99	–	–	V	–	–	–	–	–	–	–	–	G
A/England/24/00	–	–	V	–	–	–	–	–	–	–	–	–

Position	112	118	119	125	129	144	145	152	166	171	178
Consensus	E	R	M	E	M	L	V	E	F	I	I
A/PR/8/34	A	–	–	D	I	–	I	–	L	A	V
A/Victoria/3/75	–	–	I	D	–	–	I	–	–	–	–
A/Sydney/97	–	–	–	–	–	I	–	–	–	–	–
A/England/492/95	–	K	–	–	I	–	–	–	–	–	–
A/England/41/96	–	–	–	–	I	–	–	–	–	–	–
A/England/919/99	–	–	–	–	–	I	–	K	–	–	–
A/England/24/00	–	–	–	–	I	–	–	–	–	–	–

Position	196	197	204	209	211	221	224	226	229	231-37
Consensus	K	N	R	N	G	K	R	A	K	
A/PR/8/34	E	T	–	–	R	E	G	I	E	
A/Victoria/3/75	–	T	G	–	–	–	–	–	–	RRDKMAD
A/Sydney/97	–	–	–	S	–	–	–	–	–	
A/England/492/95	–	–	–	–	–	–	–	–	–	
A/England/41/96	–	–	–	–	–	–	–	–	–	
A/England/919/99	–	–	–	–	–	E	–	–	–	
A/England/24/00	–	–	–	–	–	E	–	–	–	

The NS1 protein from each virus is 230 amino acids long, with the exception of A/Victoria/3/75, which has an additional 7 amino acids at the carboxy terminus. 'dash', same amino acid as consensus.

that bind to host cell proteins involved in mRNA processing. The extreme carboxy terminus from amino acid 215 onwards binds PABII, and another region around amino acid 186 binds CPSF. On analysis of the sequences of the NS1 proteins in this study, we noted that the CPSF binding site was totally conserved, whereas the PABII site differed. We constructed a mutant of A/E/492/95 NS1 that lacked the PABII site. This mutant efficiently blocked IFN-induced ISRE gene expression (Fig. 9B).

NS1 localization in infected cells differs between A/PR/8/34 NS1 and NS1 of other influenza A viruses

We investigated the localization of the different NS1 proteins during infection as this might influence their functional properties. A549 cells were infected with wild-type viruses and stained with anti-NS1 sera at 4, 6 and 8 h post-infection. NS1 protein was not readily detectable at 2 h post-infection for any of the viruses (data not shown). By 6–8 h after infection, the NS1 protein formed characteristic concentrated spots in the nucleus of cells infected with A/Victoria/3/75 and A/Sydney/5/97, indeed this formation was becoming apparent by 4 h in the case of A/Sydney/5/97 (Fig. 10). In contrast, the concentrated spots were not observed in the A/PR/8/34 infection, instead the A/PR/8/34 NS1 appeared to be more evenly spread throughout the cell, but clearly present both in the nucleus and cytoplasm at 4, 6 and 8 h post-infection. The NS1 protein during infection by the recombinant virus containing A/PR/8/34 NS in a genetic background of A/Victoria/3/75 showed a similar pattern to the parental A/PR/8/34 virus.

Discussion

The ability of influenza A virus to block IFN-α/β induction has been demonstrated to be a property of the NS1 protein (Egorov et al., 1998; Garcia-Sastre et al., 1998; Noah et al.,
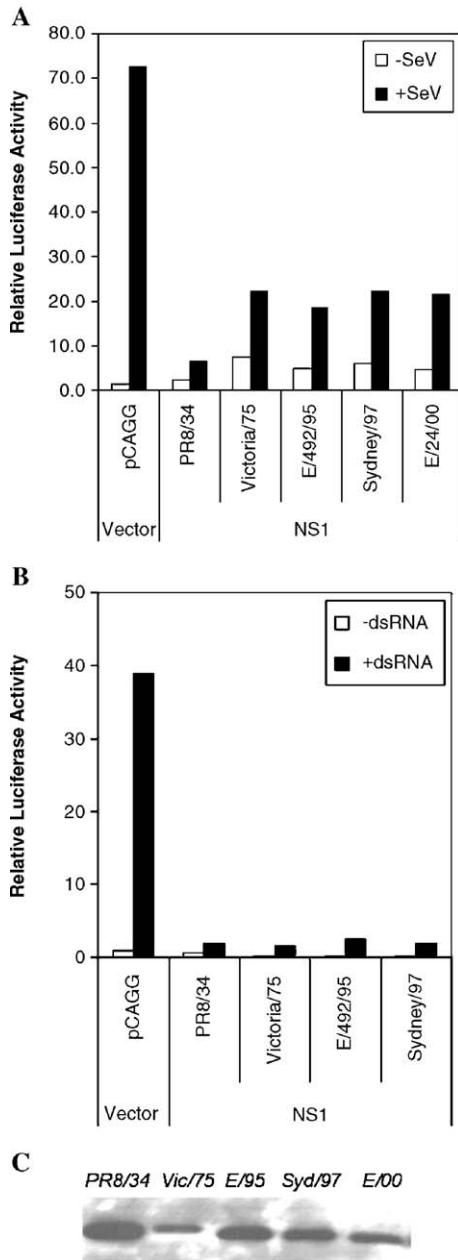
Fig. 5. Influenza A virus NS1 protein blocks induction of IFN-β promoter activation. A549 cells (A) or Vero cells (B) were transfected with 0.67 µg pCAGG-NS1, 0.25 µg β-galactosidase plasmid and 0.5 µg IFN-β Luc construct. 24 h post-transfection, cells were infected with Sendai Virus for 16 h (A) or transfected with dsRNA for 8 h (B). Luciferase activity was measured and normalized to β-galactosidase activity for each transfection to account for differences in translation and transfection efficiencies. (C) A Western blot was performed to compare the amount of NS1 present in the A549 cell extracts, using the SV5 tag for detection.

2003). In this study, we have further confirmed that the NS1 proteins of a number of human influenza A viruses can inhibit IFN-β induction to a similar extent in human alveolar epithelial lung cells (A549) and Vero cells, irrespective of the passage history of the viruses. The recombinant NS1 proteins derived from our panel of viruses could inhibit IFN-β induction stimulated by Sendai Virus infection and dsRNA in both cell types.

Surprisingly, we discovered that certain strains, such as A/Sydney/5/97 and two recent clinical isolates, induced IFN-β following infection, even though their NS1 proteins could block dsRNA induced IFN-β induction. We were able to rule out that the high IFN-β induction by A/Sydney/5/97 was explained by an insufficient production of NS1 during infection. This implies that other viral products of A/Sydney/5/97 can influence IFN-β induction, and, furthermore, that the IFN-β induced by A/Sydney/5/97 may be stimulated through a different mechanism than the presence of dsRNA, since the A/Sydney/5/97 NS1 protein could efficiently block dsRNA signaling. It has recently been shown that recombinant influenza viruses with the HA and NA genes of A/Sydney/5/97 can induce TNFα production in porcine lung epithelial cells (Seo et al., 2004), hence, it is possible these gene segments may influence IFN-β induction in a similar manner.

We also verified that IFN-β was synthesized and released upon infection with our panel of viruses, as it has been suggested that IFN-β might not be secreted from some cell lines because the degree of apoptosis induced by virus infection can prevent cytokines in the cytoplasm from being secreted (Brydon et al., 2003). An ELISA confirmed that IFN-β was present in the cell supernatants of infected cells, although the levels of IFN-β that we measured were relatively low even for our "strong" inducer A/Sydney/5/97 when compared with Sendai virus which was selected here to induce a robust IFN response.

Despite the IFN-β induced during A/Sydney/5/97 infection, this virus was still able to undergo multi-cycle replication in A549 cells, although progeny virus was not produced as rapidly or to as high titers as A/PR/8/34 and A/Victoria/3/75. We
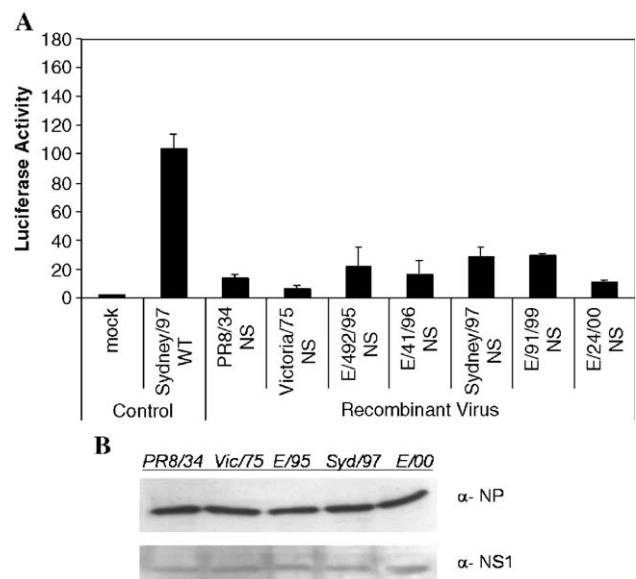

Fig. 6. Recombinant influenza A viruses that differ only in their segment 8 genes do not vary in their IFN-β induction in A549 cells. (A) The A549 IFN-β Luc reporter cell line was infected with recombinant viruses containing different NS genes in a stable genetic background (derived from A/Victoria/3/75) at an moi of 5, and luciferase activity measured after 8 h. (B) A Western blot was performed to compare the amount of NP and NS1 in infected cells at 8 h post-infection.
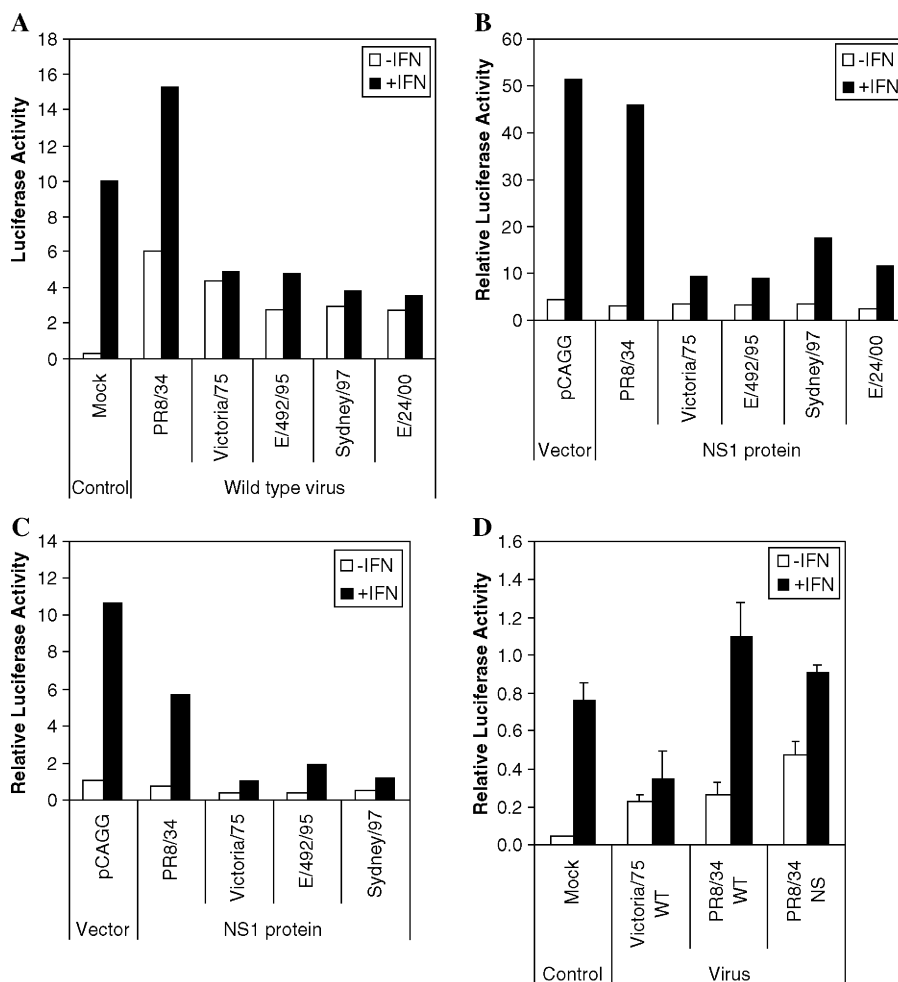
Fig. 7. Influenza A virus NS1 proteins block IFN-induced expression from an ISRE promoter and TNFα−induced expression from a NF-κB promoter. (A) and (D) A549 cells were transfected with 0.5 μg ISRE Luc reporter construct. 24 h post-transfection, cells were infected with influenza A viruses at an moi of 5 for 16 h. 1000 IU IFN-α/ml was then applied for 6 h, and luciferase activity was measured in cell extracts. A549 cells (B) or Vero cells (C) were transfected with 0.67 μg pCAGG-NS1 and 0.25 μg β-galactosidase reporter plasmid, together with 0.25 μg ISRE Luc construct. 24 h post-transfection, cells were incubated with 1000 IU IFN-α/ml for 6 h. Luciferase activity was measured and normalized to β-galactosidase activity for each transfection.

therefore investigated the effect of pre-treating the cells with IFN-α on viral replication. Interestingly, we found that A/Sydney/5/97 was less susceptible to the antiviral effects of IFN-α than were either A/PR/8/34 or A/Victoria/3/75. Indeed, a small fraction of the A/Sydney/5/97 stock (10%) was able to replicate in cells treated with a higher IFN dose, 10,000 IU/ml IFN-α, whereas A/PR/8/34 and A/Victoria/3/75 could not (data not shown). Nevertheless, in contrast to the lethal Hong Kong 1997 H5N1 strains, A/Sydney/5/97 is not completely resistant to the anti-viral effects of cytokines (Seo et al., 2002). It is also possible that packaging defects in A/Sydney/5/97 could make this strain contain resistant fractions (Marcus et al., 2005).

All of the NS1 proteins studied here, with the exception of A/PR/8/34 NS1, blocked the expression of recently induced genes either from an ISRE-containing promoter or from an NF-κB-responsive promoter, and this effect was evident both in the context of virus infection and when NS1 proteins were exogenously expressed. The mechanism was not to inhibit assembly of the transcription complex on the promoter DNA, since this was clearly detected in the context of virus infection

with a typical influenza virus, A/Victoria/3/75, as well as with A/PR/8/34. These observations would support a post-transcriptional mechanism for the inhibition of gene expression by NS1, especially since the ability to block induced gene expression mapped to the carboxy terminus of NS1 wherein lie binding sites for CPSF and PABII.

It has previously been shown that expressed recombinant A/PR/8/34 NS1 protein can enhance translation, possibly by blocking PKR (Salvatore et al., 2002), and we also observed that A/PR/8/34 NS1 enhanced its own translation (Fig. 4B) and that of a co-expressed gene. Generally recombinant A/PR/8/34 NS1 enhanced β-galactosidase expression 3- to 4-fold. It should be noted that this is taken into account in the relative luciferase activity values shown in Figs. 5, 7, 8 and 9. Although all of the other influenza A virus NS1 proteins could inhibit IFN-β induction, none of them enhanced translation. The mechanism of translational enhancement by A/PR/8/34 NS1 is not clear but seems unrelated to the mechanism by which IFN-β induction is inhibited. Importantly, despite its translational enhancement, A/PR/8/34 still blocked IFN-β
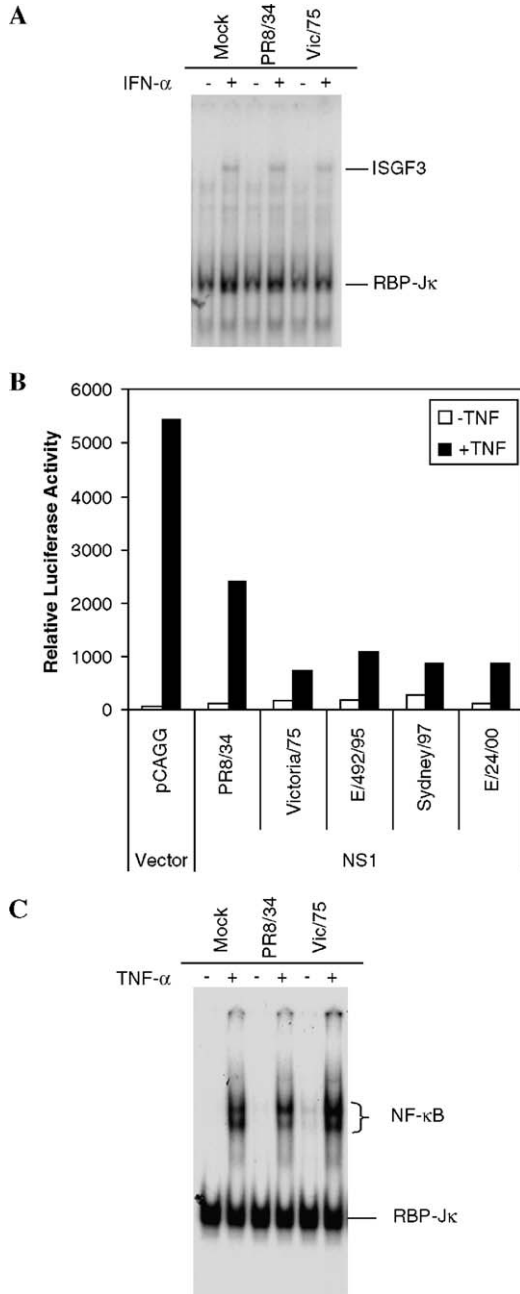
Fig. 8. Influenza viruses A/PR/8/34 and A/Victoria/3/75 do not block IFN-β or TNFα signaling. (A) Cells were infected with virus at an moi of 5 for 4 h and where indicated treated with IFN-α at 1000 IU/ml for a further 1 h before preparing nuclear extracts. EMSAs were then carried out to detect ISGF3. (B) A549 cells were transfected with 0.67 μg pCAGG-NS1, 0.25 μg β-galactosidase reporter plasmid and 0.5 μg NF-κB Luc reporter. 24 h post-transfection, cells were incubated with 10 ng/ml of TNFα for 3 h, and luciferase activity was measured and values were normalized. (C) Cells were infected as described in panel A, and where indicated treated with 10 ng/ml of TNFα. EMSAs were carried out to detect the presence of NF-κB in nuclear extracts.

There are 28-amino-acid differences between the A/PR/8/34 and A/Victoria/3/75 NS1 proteins, which might explain the variations in their functional properties. Moreover, the amino acid sequence of A/PR/8/34 is distinct from the other NS1 sequences in our panel of viruses. This is not surprising as A/PR/8/34 is a considerably older strain. However, the positions which have previously been shown as important for function remain conserved, including positions 38 and 41 which are crucial for dsRNA binding, and likewise the CPSF binding site (Noah et al., 2003). Notably there are 2-amino-acid changes in the PABII binding sites, and A/Victoria/3/75 has an additional 7 amino acids at its carboxy terminal in comparison to A/PR/8/34; hence, these changes may modulate the abilities of these proteins to bind PABII. However, a truncated NS1 protein of 215 amino acids, lacking the PABII binding site, did not lose the ability to block the induction of the ISRE or NF-κB reporter genes (Fig. 9B). Finally, other changes might influence the functional properties through determining the subcellular localization of
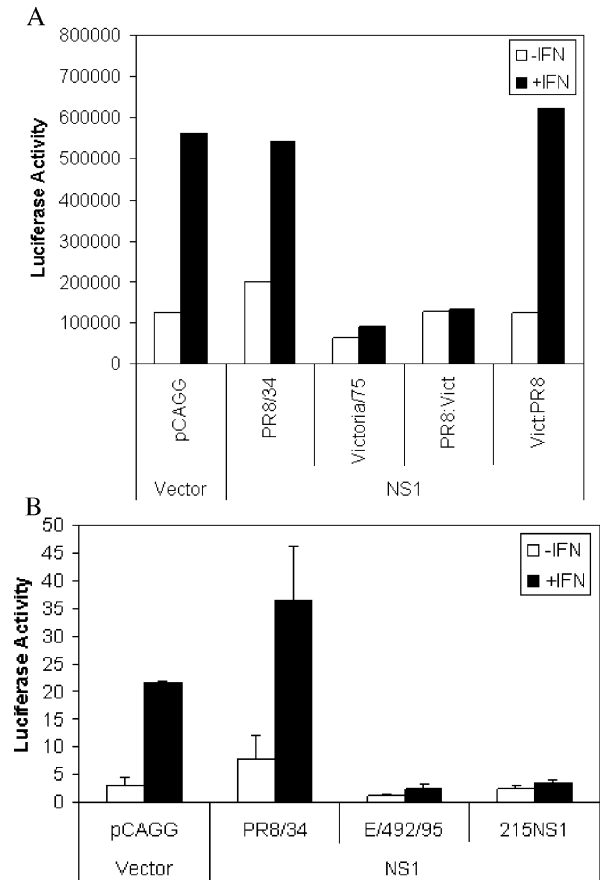

Fig. 9. The effector domain of NS1 determines the ability of the NS1 protein to block induction of an inducible promoter. (A) Vero cells were transfected with wild-type PR8 and Victoria pCAGG-NS1 clones, chimeric NS1 constructs PR8:Vict and Vict:PR8 together with the ISRELuc and β-galactosidase reporter constructs. Cells were subsequently incubated with IFN-α, and lysates assayed for luciferase activity as described previously. (B) A549 cells were transfected with wild-type PR8 or Eng/492/95 pCAGG-NS1 clones or a truncated Eng/492/95 pCAGG 215NS1 construct together with the ISRELuc and β-galactosidase reporter constructs. Cells were subsequently incubated with IFN-α, and lysates were assayed for luciferase activity as described previously.

promoter activation but did not inhibit induced gene expression relative to the controls. It therefore seems likely that this virus does not have the ability to affect events downstream of transcription in the same ways as do the other influenza A virus NS1 proteins studied here. We are currently investigating the genetic basis of this difference.
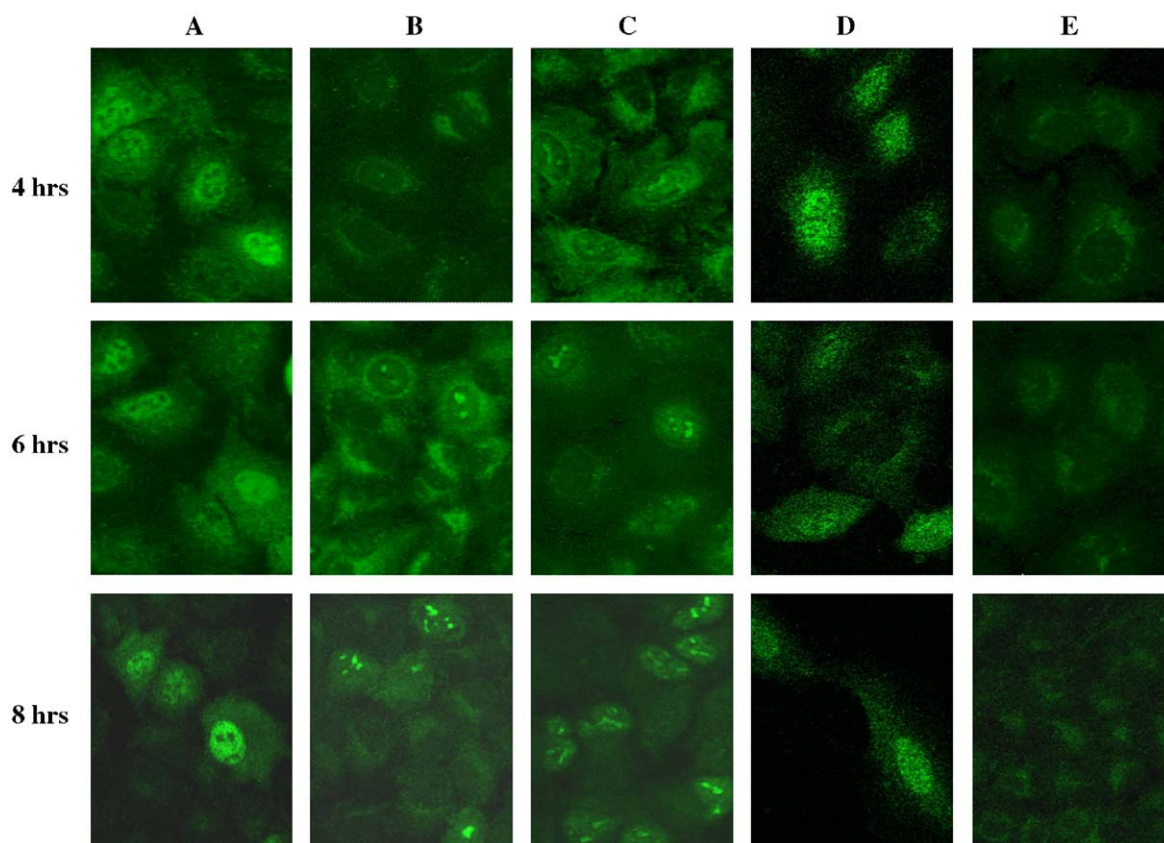
Fig. 10. Localization of NS1 in influenza A virus infected A549 cells. NS1 forms highly localized spots in the nucleus, with the exception of A/PR/8/34 NS1 which localizes more evenly in the nucleus and cytoplasm. Cells were infected at an moi of 5 with wild-type virus or the A/PR/8/34 NS recombinant and stained 4, 6 and 8 h post-infection with anti NS1 polyclonal sera and visualized with anti-rabbit FITC conjugate. (A) A/PR/8/34, (B) A/Victoria/3/75, (C) A/Sydney/5/97, (D) PR8/34 NS recombinant in A/Victoria/3/75 and (E) mock.

the NS1 proteins (Qian et al., 1994). Indeed, the variation in intracellular localization of NS1 we observed further supports the idea that the A/PR/8/34 virus differs from other influenza viruses in the activity of the NS1 protein. Previous reports have shown NS1 to be present in either the nucleus, the cytoplasm or both (Briedis et al., 1981; Falcon et al., 1999; Fortes et al., 1994; Greenspan et al., 1988; Portela et al., 1985; Qian et al., 1994; Talon et al., 2000). Presumably, this depends on the choice of cell type, the time point of infection and whether the protein is expressed as a recombinant protein. In addition, the protein might associate with other host proteins sequestering it to a particular part of the cell.

In the cell type (A549 cells) and time points (4–8 h post-infection) we investigated, A/PR/8/34 NS1 (during infection by both wild-type A/PR/8/34 and the recombinant derivative) was spread evenly throughout the nucleus and cytoplasm, whereas the other NS1 proteins moved to the nucleus where they formed characteristic concentrated spots within 6 h of infection. Thus, initially, when NS1 has first been synthesized and is still present in the cytoplasm, it may dampen the dsRNA induction of the IFN-β promoter. However, the nuclear localization of some NS1 proteins 6 h post-infection might enable interference with processing and export of newly synthesized mRNA, as shown previously for the NS1 protein derived from A/Udorn/72 (Qian et al., 1994), a strain which shares strong sequence similarity to A/Victoria/3/75. In

agreement with this, Ortín and colleagues have also shown that expressed AVictoria/3/75 NS1 formed concentrated spots in the nucleus in COS-1 cells, and resulted in the accumulation of nuclear mRNA (Falcon et al., 1999; Fortes et al., 1994; Portela et al., 1985).

In summary, the mechanisms by which human influenza A virus counters the IFN-α/β response are strain specific. It is perhaps not surprising that the A/PR/8/34 virus which may have been extensively adapted by passage through eggs and mice over many years behaves differently to the other strains. Our studies therefore highlight the importance of bearing in mind the passage history and origin when interpreting studies with viruses whose genes evolve rapidly during interaction with a range of hosts.

Material and methods

Cells and viruses

293-T, MDCK, Vero and A549 cells were grown in 10% Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum, glutamine, pyruvate and penicillin/streptomycin. An A549 cell line containing a stable integrate of the firefly luciferase gene driven by the IFN-β promoter (called IFN-βLuc) was maintained in the same media supplemented with 2 mg/ml G418. Cells were

incubated at 37 °C and 5% CO_2. Clinical isolates were obtained from the Health Protection Agency, Colindale, London, UK, and were passaged a maximum of four times in MDCK cells since recovery from nasal swabs (Thompson et al., 2004). All virus strains were grown in MDCK cells in medium without serum, containing 2 μg/ml trypsin (Worthington). Influenza viruses were quantified by plaque assay on MDCK cells as previously described (Elleman and Barclay, 2004). Sendai virus (SeV) vM3 was prepared in 10-day-old embryonated hen's eggs.

IFN-β induction luciferase assay

A549 cells stably containing the IFN-βLuc construct were incubated with virus diluted in serum-free DMEM at a multiplicity of infection (moi) of 5 pfu/cell in a 24-well plate at 37 °C. After 1 h, the virus was replaced with 2%DMEM, and the cells were incubated for a further 7 h. The cells were washed once in PBS and then lysed in 50 μl CCLR buffer (Promega) for 1 min. Cell debris was removed by centrifugation for 1 min at 13,000 rpm. 20 μl supernatant was added to 100 μl of Luciferase Substrate Reagent (Promega), and the relative luciferase activity was measured on a Turner TD20/20 Luminometer according to the kit manufacturer's instructions (Promega).

To inactivate virus infectivity, virus inoculum was exposed on ice at a distance of 10 cm to the ultraviolet radiation from a germicidal lamp for 5 min. A hemagglutination assay was performed with turkey red blood cells to ensure the virions remained intact.

Measurement of IFN-β secretion by ELISA

A549 cells were infected as described above at an moi of 5 pfu/cell, in a 24-well plate. Sendai virus was used as a positive control. 24 h post-infection, 100 μl of supernatant was directly transferred to a well of an IFN-β ELISA 96-well plate (R&D systems). The ELISA was performed according to the manufacturer's protocol, and the absorbance was measured at 450 nm. The amount of IFN-β was estimated, from the standard curve.

Virus yield assay

A549 cells were incubated with or without 1000 IU/ml of IFN-α (Intron A, Schering-Plough) for 24 h. The cells were then infected at an moi of 0.01 and incubated in serum-free DMEM with a final trypsin concentration of 0.1 μg/ml. Fresh trypsin was added daily. 48 h post-infection, cell culture supernatants were harvested and titrated by plaque assay on MDCK cells.

Generation of NS1 expression constructs

The pCAGG vector was adapted such that cloned genes would be expressed as fusion proteins with a 14-amino-acid long SV5 tag at their carboxy terminus. The new vector, termed pCAGGSV5, was engineered to contain MluI and NotI cloning sites. NS1 was amplified from a full-length cDNA of segment 8 (the pol I NS clone, see below). PCR mutagenesis was used to remove the splice acceptor sequence. PCR 1 included primer pairs NS1NotIF 5′ TATGCGGCCGCACAAAGACATAATG-GAT 3′ and SMAR 5′ ATCCTCATCAGTATGCCCGGGAA-GAGAAGGCAATGG3, which resulted in a ∼ 530-bp product. PCR2 included primers SMAF 5′ CCATTGCCTTCTCTTCC-CGGGCATACTGATGAGGAT 3′ and NS1MluIKR 5′ AGC-CATCTTATCACGCGTAACTTTTG 3′ and gave a ∼ 200-bp product. A different antisense primer, NS1MluIER, was used to amplify A/PR/8/34 segment 8 RNA, to ensure the correct sequence at amino acid position at 229 was incorporated (E rather than a K). A third overlapping PCR3 was then performed to amplify the full-length NS1, using the NS1NotIF and NS1MluIKR or (NS1MluIER for A/PR/8/34), giving a 730-bp product. The PCRs were performed using Bio-X-ACT short (Bioline) according to the manufacturer's instructions. The product from PCR3 was then digested with NotI and MluI and ligated into the pCAGGSV5 vector. Chimeric NS1 clones of A/PR/8/34 and A/Victoria/3/75 were generated using the NcoI site in the NS1pCAGGSV5 clones. Clone 'PR8:Vict' encodes the A/PR/8/34 dsRNA-binding domain (amino acids 1–79) and A/Victoria/3/75 effector domain (amino acids 80–237), whereas clone Vict:PR8 contains the dsRNA-binding domain from A/Victoria/3/75 (amino acids 1–79) and effector domain of A/PR/8/34 (amino acids 80–230). A truncated clone A/E/492/95 pCAGG NS1, encoding the first 215 amino acids of the NS1 construct, was generated by PCR using forward primer NS1NotF, and a reverse primer 215NS1 (5′ AGCCGGTATTAC-GCGTAGTAAGTG GAGGTCCCCCATTCTC 3′) with a MluI site. The PCR product was then cloned into the pCAGGSV5 vector using the NotI and MluI sites as described previously.

Luciferase reporter gene assays

Three firefly luciferase reporter constructs were used containing one of the following inducible promoters: (1) IFN-βLuc containing IFN-β promoter, (2) ISRELuc: contained 4 consecutive ISRE promoter elements, (3) NF-κBLuc containing an NF-κB promoter. A DNA mix containing 0.25 μg of the Luciferase reporter construct, 0.25 μg β-galactosidase plasmid pI and 0.67 μg pCAGGSV5 NS1 or empty vector were transfected into a 6-well plate of A549 s or Vero cells using Polyfect (Qiagen) or Lipofectamine 2000 (LF2000, Invitrogen) according to the manufacturer's instructions. 24 h post-transfection, the reporter genes were induced depending on the construct. The IFN-βLuc reporter construct was induced by transfection with dsRNA for 8 h or infection with a high moi of SeV for 16 h. The ISRELuc construct was induced by treatment with 2000 IU/well of IFN-α for 6 h. The NF-κB promoter was induced by incubation with 20 ng/well of TNFα (R&D Systems) for 3 h. After the induction time had elapsed, cells were lysed in 200 μl CCLR buffer (Promega). β-Galactosidase activity was measured, and the relative luciferase activity was normalized accordingly. Extracts were prepared in RIPA buffer in parallel for Western

blot analysis to determine the amount of NS1 protein made by each construct. A/PR/8/34 NS1 clones with and without the SV5 epitope tag were tested in the IFN-βLuc reporter assay to confirm that the tag did not effect the function of the protein in these assays.

Western blot analysis

A mouse anti SV5 tag antibody (Immunologicals Direct) was used for detection of the SV5 tagged NS1 proteins. Mouse anti-NP antibody (Immunologicals Direct) and anti-NS1 serum (kindly provided by Dr. P Digard, University of Cambridge) were used for detection in virus infected cells. The anti-SV5 and NP antibodies were diluted 1:1000 in 4% dried skimmed powder milk, PBS with 0.1% Tween-20. The anti-NS1 serum was diluted 1:500 in 4% dried skimmed powder milk, 5% horse serum, in PBS with 0.1% Tween-20. Bound antibodies were detected with HRP-labeled secondary antibodies.

Generation of recombinant viruses

vRNA was extracted from 140 μl of viral supernatant (of the virus stocks generated in MDCKs as described above) using a QIAmp Viral RNA mini kit (Qiagen) according to the manufacturers instructions. cDNA was synthesized from 5 to 10 μl RNA, using AMV reverse transcriptase (Promega) and 50 pmol of a primer complementary to the first 12 nucleotides of the 3′ends of all 8 of the vRNA segments. The NS gene was PCR amplified from the cDNA using specific sense and antisense primers complementary to the 3′ and 5′ end of segment 8 respectively which contained BsmBI sites to enable cloning into the pPolIRT vector (originally obtained from Dr. T Zurcher, GlaxoSmithKline). A proof reading enzyme, Bio-X-ACT short (Bioline), was used according to the manufacturer's protocol. The PCR product was digested with BsmBI and cloned into the pPol IRT vector. The NS pol I clones were combined with the remaining 7 plasmids with cDNAs based on an influenza A/Victoria/3/75 virus genetic background. Recombinant viruses were rescued by 12 plasmid transfection into 293-T cells and co-cultivation with MDCK cells, as described previously (Thompson et al., 2004). The rescued viruses were plaque purified, and stocks were grown in MDCK cells. The stocks were subsequently titrated on MDCK cells, and the NS gene sequenced to confirm the correct origin of the segment and that no mutations had arisen.

Immunofluorescence

IRF-3

A549 cells were infected at an moi of 5 for 8 h. Cells were fixed and permeabilized in 4% paraformaldehyde and 0.2% Triton PBS respectively. Cells were incubated with rabbit anti-IRF-3 antibody (FL-425, Santa Cruz) and mouse anti-influenza A NP antibody (Immunologicals Direct) for 1 h. After three washes in PBS, cells were incubated with an anti-rabbit FITC-labeled secondary and anti-mouse Texas Red-labeled secondary respectively for 1 h.

NS1

A549 cells were infected at an moi of 5 for 8 h. Cells were fixed and permeabilized in ice-cold methanol:acetone (1:1) for 10 min and washed in PBS before blocking overnight in PBS containing 5% horse serum and 3% BSA at room temperature. Rabbit anti-NS1 sera were diluted 1:200 in blocking buffer and applied to the cells for 3 h. After three washes, a goat anti-rabbit FITC-labeled secondary antibody (Immunological direct) diluted 1:200 was added for 1 h. Slides were then mounted in Vectashield.

EMSAs

Nuclear extracts were prepared from confluent 9-cm dishes of A549 cells as previously described (Whiteside et al., 1992). Where indicated, cells were infected with virus at an moi of 5 for 4 h and then treated with IFN-α at 1000 IU/ml or TNFα at 10 ng/ml for a further hour before harvesting for extraction. EMSAs were then carried out for either NF-κB (Visvanathan and Goodbourn, 1989) or ISGF3 (Didcock et al., 1999b) as previously described.

RNAse protection assays

Confluent 9-cm dishes of A549 cells were infected with virus at an moi of 5. Two hours post-infection, cells were treated for a further 2 h with IFN-α 1000 u/ml. RNA was prepared using an RNeasy mini kit (Qiagen). 10 μg of RNA was used for each hybridization reaction. Conditions and probes for detection of human IFN-β, human γ-actin and 6–16 (ISG56) have been previously described (King and Goodbourn, 1994).

References

Basler, C.F., Mikulasova, A., Martinez-Sobrido, L., Paragas, J., Muhlberger, E., Bray, M., Klenk, H.D., Palese, P., Garcia-Sastre, A., 2003. The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. J. Virol. 77, 7945–7956.

Briedis, D.J., Conti, G., Munn, E.A., Mahy, B.W., 1981. Migration of influenza virus-specific polypeptides from cytoplasm to nucleus of infected cells. Virology 111, 154–164.

Brydon, E.W., Smith, H., Sweet, C., 2003. Influenza A virus-induced apoptosis in bronchiolar epithelial (NCI-H292) cells limits pro-inflammatory cytokine release. J. Gen. Virol. 84, 2389–2400.

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.

Didcock, L., Young, D.F., Goodbourn, S., Randall, R.E., 1999a. Sendai virus and simian virus 5 block activation of interferon-responsive genes: importance for virus pathogenesis. J. Virol. 73, 3125–3133.

Didcock, L., Young, D.F., Goodbourn, S., Randall, R.E., 1999b. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. J. Virol. 73, 9928–9933.

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.

Egorov, A., Brandt, S., Sereinig, S., Romanova, J., Ferko, B., Katinger, D., Grassauer, A., Alexandrova, G., Katinger, H., Muster, T., 1998. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. J. Virol. 72, 6437–6441.

Elleman, C.J., Barclay, W.S., 2004. The M1 matrix protein controls the filamentous phenotype of influenza A virus. Virology 321, 144–153.

Falcon, A.M., Fortes, P., Marion, R.M., Beloso, A., Ortin, J., 1999. Interaction of influenza virus NS1 protein and the human homologue of Staufen in vivo and in vitro. Nucleic Acids Res. 27, 2241–2247.

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.

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.

Geiss, G.K., Salvatore, M., Tumpey, T.M., Carter, V.S., Wang, X., Basler, C.F., Taubenberger, J.K., Bumgarner, R.E., Palese, P., Katze, M.G., Garcia-Sastre, A., 2002. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc. Natl. Acad. Sci. U. S. A. 99, 10736–10741.

Goodbourn, S., Didcock, L., Randall, R.E., 2000. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J. Gen. Virol. 81, 2341–2364.

Greenspan, D., Palese, P., Krystal, M., 1988. Two nuclear location signals in the influenza virus NS1 nonstructural protein. J. Virol. 62, 3020–3026.

Hagmaier, K., Jennings, S., Buse, J., Weber, F., Kochs, G., 2003. Novel gene product of Thogoto virus segment 6 codes for an interferon antagonist. J. Virol. 77, 2747–2752.

Hatada, E., Fukuda, R., 1992. Binding of influenza A virus NS1 protein to dsRNA in vitro. J. Gen. Virol. 73 (12), 3325–3329.

Huang, Q., Liu, D., Majewski, P., Schulte, L.C., Korn, J.M., Young, R.A., Lander, E.S., Hacohen, N., 2001. The plasticity of dendritic cell responses to pathogens and their components. Science 294, 870–875.

Isaacs, A., Lindenmann, J., 1957. Virus interference: I. The interferon. Proc. R. Soc. London, Ser. B Biol. Sci. 147, 258–267.

Kim, M.J., Latham, A.G., Krug, R.M., 2002. Human influenza viruses activate an interferon-independent transcription of cellular antiviral genes: outcome with influenza A virus is unique. Proc. Natl. Acad. Sci. U. S. A. 99, 10096–10101.

King, P., Goodbourn, S., 1994. The beta-interferon promoter responds to priming through multiple independent regulatory elements. J. Biol. Chem. 269, 30609–30615.

Li, Y., Chen, Z.Y., Wang, W., Baker, C.C., Krug, R.M., 2001. The 3′-end-processing factor CPSF is required for the splicing of single-intron pre-mRNAs in vivo. RNA 7, 920–931.

Marcus, P.I., Rojek, J.M., Sekellick, M.J., 2005. Interferon induction and/or production and its suppression by influenza A viruses. J. Virol. 79, 2880–2890.

Matikainen, S., Pirhonen, J., Miettinen, M., Lehtonen, A., Govenius-Vintola, C., Sareneva, T., Julkunen, I., 2000. Influenza A and Sendai viruses induce differential chemokine gene expression and transcription factor activation in human macrophages. Virology 276, 138–147.

Nemeroff, M.E., Barabino, S.M., Li, Y., Keller, W., Krug, R.M., 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3′ end formation of cellular pre-mRNAs. Mol. Cell 1, 991–1000.

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.

Portela, A., Melero, J.A., Martinez, C., Domingo, E., Ortin, J., 1985. A primer vector system that allows temperature dependent gene amplification and expression in mammalian cells: regulation of the influenza virus NS1 gene expression. Nucleic Acids Res. 13, 7959–7977.

Qian, X.Y., Alonso-Caplen, F., Krug, R.M., 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. J. Virol. 68, 2433–2441.

Ronni, T., Sareneva, T., Pirhonen, J., Julkunen, I., 1995. Activation of IFN-alpha, IFN-gamma, MxA, and IFN regulatory factor 1 genes in influenza A virus-infected human peripheral blood mononuclear cells. J. Immunol. 154, 2764–2774.

Ronni, T., Matikainen, S., Sareneva, T., Melen, K., Pirhonen, J., Keskinen, P., Julkunen, I., 1997. Regulation of IFN-alpha/beta, MxA, 2′,5′-oligoadenylate synthetase, and HLA gene expression in influenza A-infected human lung epithelial cells. J. Immunol. 158, 2363–2374.

Salvatore, M., Basler, C.F., Parisien, J.P., Horvath, C.M., Bourmakina, S., Zheng, H., Muster, T., Palese, P., Garcia-Sastre, A., 2002. Effects of influenza A virus NS1 protein on protein expression: the NS1 protein enhances translation and is not required for shutoff of host protein synthesis. J. Virol. 76, 1206–1212.

Seo, S.H., Hoffmann, E., Webster, R.G., 2002. Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat. Med. 8, 950–954.

Seo, S.H., Webby, R., Webster, R.G., 2004. No apoptotic deaths and different levels of inductions of inflammatory cytokines in alveolar macrophages infected with influenza viruses. Virology 329 (2), 270–279.

Shimizu, K., Iguchi, A., Gomyou, R., Ono, Y., 1999. Influenza virus inhibits cleavage of the HSP70 pre-mRNAs at the polyadenylation site. Virology 254, 213–219.

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.

Thompson, C.I., Barclay, W.S., Zambon, M.C., 2004. Changes in in vitro susceptibility of influenza A H3N2 viruses to a neuraminidase inhibitor drug during evolution in the human host. J. Antimicrob. Chemother. 53, 759–765.

Visvanathan, K.V., Goodbourn, S., 1989. Double-stranded RNA activates binding of NF-kappa B to an inducible element in the human beta-interferon promoter. EMBO J. 8, 1129–1138.

Wang, W., Riedel, K., Lynch, P., Chien, C.Y., Montelione, G.T., Krug, R.M., 1999. RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA 5, 195–205.

Wang, X., Li, M., Zheng, H., Muster, T., Palese, P., Beg, A.A., Garcia-Sastre, A., 2000. Influenza A virus NS1 protein prevents activation of NF-kappaB and induction of alpha/beta interferon. J. Virol. 74, 11566–11573.

Wathelet, M.G., Lin, C.H., Parekh, B.S., Ronco, L.V., Howley, P.M., Maniatis, T., 1998. Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Mol. Cell 1, 507–518.

Whiteside, S.T., Visvanathan, K.V., Goodbourn, S., 1992. Identification of novel factors that bind to the PRD I region of the human beta-interferon promoter. Nucleic Acids Res. 20, 1531–1538.