

Differential viral induction of distinct interferon- α genes by positive feedback through interferon regulatory factor-7

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Interferon (IFN) genes are among the earliest transcriptional responses to virus infection of mammalian cells. Although the regulation of the IFN β gene has been well characterized, the induction of the large family of IFN α genes has remained obscure. We report that the IFN α genes can be divided into two groups: an immediate-early response gene (IFN α 4) which is induced rapidly and without the need for ongoing protein synthesis; and a set of genes that display delayed induction, consisting of at least IFN α 2, 5, 6 and 8, which are induced more slowly and require cellular protein synthesis. One protein that must be synthesized for induction of the delayed gene set is IFN itself, presumably IFN α 4 or IFN β , which stimulates the Jak–Stat pathway through the IFN receptor, resulting in activation of the transcription factor interferon-stimulated gene factor 3 (ISGF3). Among the IFN-stimulated genes induced through this positive feedback loop is the IFN regulatory factor (IRF) protein, IRF7. Induction of IRF7 protein in response to IFN and its subsequent activation by phosphorylation in response to virus-specific signals, involving two C-terminal serine residues, are required for induction of the delayed IFN α gene set.

Keywords: interferon/IRF3/IRF7/phosphorylation/Stat1

Introduction

Infection by viruses triggers transcription of a large number of cellular genes, either directly through activation of cellular transcription factors or indirectly through prior induction of type I interferons (IFN α and IFN β). IFNs synthesized in virus-infected cells are secreted and can act in an autocrine or paracrine manner through binding to a specific cell-surface receptor. IFN binding to the type I receptor stimulates the Jak–Stat signal transduction cascade, leading to induction of IFN target genes responsible for the pleiotropic biological effects of IFN, including antiviral activity.

Signaling from the type I IFN receptor has been

elucidated recently (reviewed in Darnell, 1997). Activation of the cytoplasmic tyrosine protein kinases Jak1 and Tyk2 leads to phosphorylation of Stat1 and Stat2, transcription factors that heterodimerize, translocate to the nucleus and assemble with the DNA-binding protein interferon-stimulated gene factor 3 γ (ISGF3 γ) to form the ISGF3 complex. ISGF3 γ (Veals *et al.*, 1992, 1993) is a member of the IFN regulatory factor (IRF) family (Nguyen *et al.*, 1997) that is characterized by a conserved DNA-binding domain encoding a characteristic DNA sequence preference related to the positive regulatory domain I (PRDI) of the IFN β promoter (Goodbourn and Maniatis, 1988; Harada *et al.*, 1989; Bluysen *et al.*, 1996). The recruitment of Stat1 and Stat2 by ISGF3 γ to form ISGF3 provides a distinct DNA recognition specificity to this complex, allowing it to bind with high affinity to the IFN-stimulated response element (ISRE) found in the promoters of IFN-stimulated target genes but to bind with significantly lower affinity to the PRDI elements found in IFN promoters (Veals *et al.*, 1993; Yoneyama *et al.*, 1996).

Whereas IFN β is encoded by a single gene, IFN α is represented by a large family of structurally related genes localized in a cluster on mouse chromosome 4 (Kelley and Pitha, 1985). More than a dozen IFN α genes have been identified in the mouse and are reported to be coordinately induced in virus-infected cells. However, the primary activation mechanism of these genes by virus infection remains to be elucidated completely. Among the type I IFN genes, activation of IFN β has been studied most extensively. The virus-inducible enhancer of the IFN β gene consists of a complex enhancer composed of different positive and negative regulatory elements that bind different, cooperating transcription factors (Kim and Maniatis, 1997). PRDIV binds a heterodimer of ATF-2 and c-jun, and PRDII binds the inducible transactivator protein NF κ B. PRDI and PRDIII are related sequence elements that bind members of the IRF family. Previous data suggested that IRF1 was the relevant activator protein binding to PRDI and PRDIII (Fujita *et al.*, 1988, 1989; Reis *et al.*, 1992). However, these early observations were disputed by the finding that disruption of the IRF1 gene in mice did not impair the induction of IFN gene expression by virus infection (Matsuyama *et al.*, 1993; Reis *et al.*, 1994). More recent evidence suggests the involvement of other IRF family members, particularly IRF3 (Lin *et al.*, 1998; Sato *et al.*, 1998; Schafer *et al.*, 1998; Wathelet *et al.*, 1998; Yoneyama *et al.*, 1998) and possibly IRF7 (Wathelet *et al.*, 1998), in transcriptional induction of IFN β .

Regulation of the IFN α genes is less well defined. The virus-responsive element in IFN α promoters contains PRDI-like sequences (Näf *et al.*, 1991; Au *et al.*, 1993; Genin *et al.*, 1995; Pitha and Au, 1995; Braganca *et al.*, 1997), suggesting the involvement of IRF proteins. IRF1

is unlikely to be the factor bound to this site in IFN α promoters (Au *et al.*, 1993; Ruffner *et al.*, 1993), and thus the identity of the proteins involved in virus-mediated induction of these genes remains unclear. Here, we show that IFN α 4 plays a unique role in the IFN α family. Based on the observation that induction of IFN α genes by Newcastle disease virus (NDV) was reduced in the absence of Stat1, we show that IFN α 4 is the only species directly induced in response to virus infection. The other species of IFN α were dependent on new protein synthesis for their induction, including the synthesis of IFN itself and of the transcription factor IRF7. In addition, we show that IRF7 requires a virus-specific modification for transcriptional activity, and localize this modification to a phosphorylation event requiring two serine residues near the C-terminus.

Results

Impaired viral induction of IFN α genes in the absence of Stat1

The acute viral sensitivity of Stat1-deficient mice can be at least partially explained by the complete loss of responsiveness to virally induced IFN (Durbin *et al.*, 1996; Meraz *et al.*, 1996). Virus induction of type I IFN mRNA was observed in Stat1-deficient mice using probes for mouse IFN α 4 and IFN β (Durbin *et al.*, 1996). To characterize this response in more detail, we compared the magnitude and kinetics of IFN production in cells from wild-type and Stat1-deficient mice. Although production of IFN γ from virus-stimulated T lymphocytes was not significantly affected by the absence of Stat1 (C.K.Lee, R.Gimeno, R.Gertner and D.E.Levy, manuscript in preparation) and IFN β gene expression was detected in virus-infected fibroblasts (see below), induction of IFN α gene expression was significantly impaired in Stat1 mutant cells (Figure 1A). Immortalized mouse embryo fibroblasts (MEFs) from wild-type and Stat1-deficient mice were infected with NDV and RNA was isolated for quantification of IFN α mRNA levels by RT-PCR. While wild-type cells rapidly produced high levels of IFN α in response to virus infection, with high levels produced within 6–8 h (Figure 1A, lanes 9–12), Stat1 $^{-/-}$ cells produced much lower levels of IFN α mRNA and significant accumulation was not detected until 10–12 h (Figure 1A, lanes 5–6).

The level of IFN α gene induction was quantified by using serial dilution RT-PCR (Figure 1B). At 6 h post-infection (p.i.), IFN α mRNA from wild-type cells was readily detected following 125-fold dilution of cDNA (Figure 1B, lane 4) and at 10 h was detected following 625-fold dilution (Figure 1B, lane 10). In contrast, IFN α mRNA from Stat1 $^{-/-}$ cells was only detected in the undiluted sample 6 h p.i. (Figure 1B, lane 2) and in the 5-fold diluted sample 10 h p.i. (Figure 1B, lane 7). Therefore, the difference in IFN α induction between wild-type and Stat1 $^{-/-}$ cells at peak induction is probably >100-fold.

Murine IFN α is composed of a mixture of isotypes encoded by a family of highly homologous, linked genes tandemly arrayed on chromosome 4 (De Maeyer and De Maeyer-Guignard, 1988). The analysis shown in Figure 1 was designed to detect all members of the IFN α family by using primers corresponding to conserved sequences

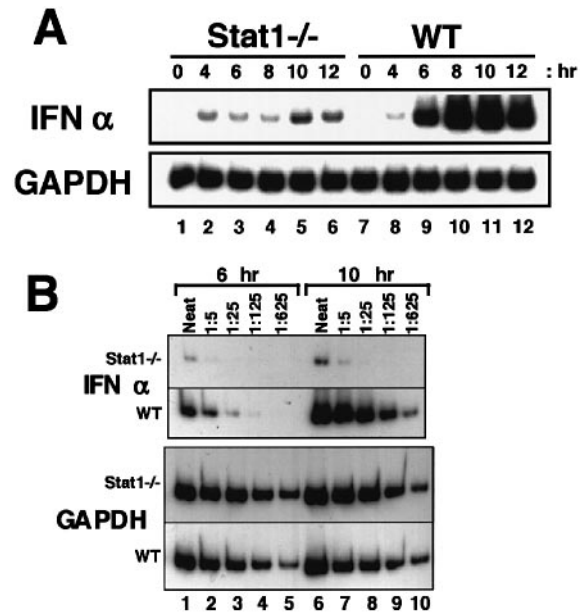


Fig. 1. IFN α induction in response to NDV infection is impaired in Stat1 $^{-/-}$ fibroblasts. (A) Kinetics of IFN α gene expression. Expression of total IFN α mRNA in Stat1 $^{-/-}$ and wild-type (WT) fibroblasts was monitored by RT-PCR using consensus primers that detect all subtypes of IFN α . RNA was extracted from NDV-infected fibroblasts 0, 4, 6, 8, 10 or 12 h post-infection. The lower panel shows the corresponding levels of GAPDH mRNA, as control. (B) Quantitation of IFN α RNA expression. Relative amounts of total IFN α RNA and of GAPDH RNA expressed in wild-type and Stat1 $^{-/-}$ mutant cells 6 and 10 h p.i. were compared by serial dilution RT-PCR, as indicated.

Table I. Representation of IFN α subtypes in virus-infected wild-type and Stat1 $^{-/-}$ fibroblasts

| IFN α species | Wild-type | Stat1 $^{-/-}$ |
|----------------------|-----------|----------------|
| α 2 | 3/19 | – |
| α 4 | 8/19 | 18/18 |
| α 5 | 3/19 | – |
| α 6 | 3/19 | – |
| α 8 | 2/19 | – |

within the coding region (see Materials and methods). In order to distinguish the repertoire of IFN α gene expression from virus-infected wild-type and Stat1 $^{-/-}$ fibroblasts, cDNA fragments of IFN α RNA from infected cells were amplified by RT-PCR using these consensus primers and were cloned into plasmid vectors. Random clones from both wild-type and Stat1 $^{-/-}$ cells were analyzed by DNA sequencing. Sufficient sequence divergence occurs within these segments to distinguish among many of the IFN α isotypes. As expected, IFN α production by virally infected wild-type cells displayed a mixture of distinct isotypes (Table I). IFN α 4 was the most abundant species detected, but IFN α 2, 5, 6 and 8 were also detected in approximately equal abundance to each other. In contrast, only the IFN α 4 isotype was detected in virally infected Stat1 $^{-/-}$ cells. No cDNA clones for other IFN α subtypes were isolated out of 18 clones analyzed (Table I). This result suggests that not only is IFN α production quantitatively affected by the Stat1 gene, but that Stat1 qualitatively influences the pattern of gene expression by differentially affecting distinct IFN α isotypes.

To confirm the observation of differential IFN α gene

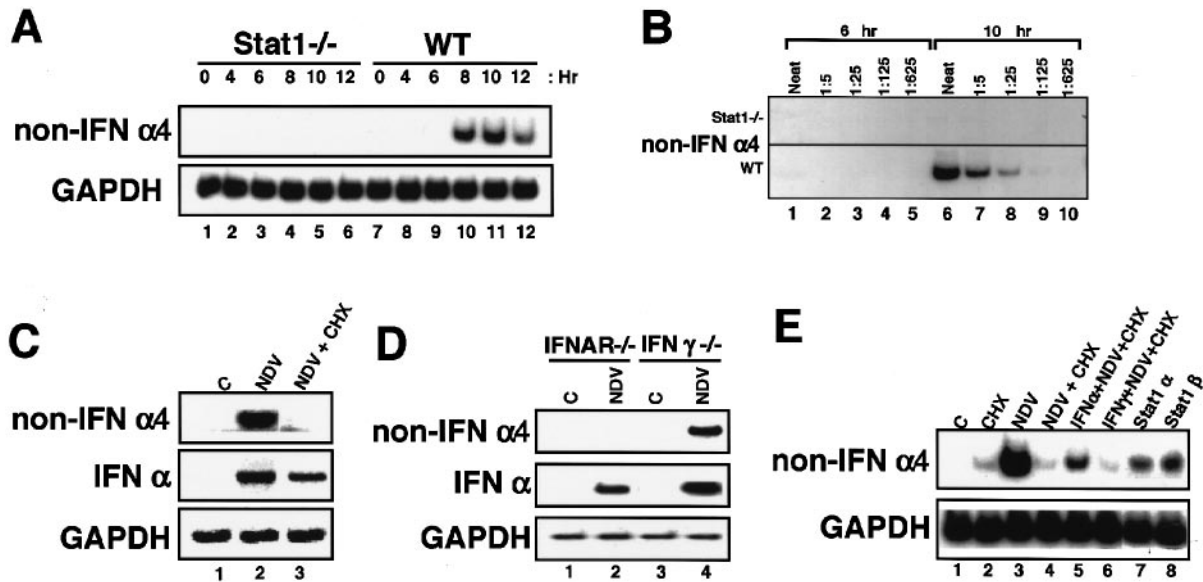


Fig. 2. IFN-dependent positive feedback is implicated in the induction of non-IFN α 4 genes. (A) Stat1-dependent expression of non-IFN α 4 genes. The steady-state level of non-IFN α 4 mRNA in Stat1 $^{-/-}$ and wild-type (WT) fibroblasts was monitored by RT-PCR using a specific set of primers (see Materials and methods). RNA was extracted at the indicated times after NDV infection. As a control, expression of GAPDH mRNA is shown in the lower panel. (B) Quantitation of relative IFN α RNA abundance. Amounts of non-IFN α 4 RNA induced in wild-type and Stat1 $^{-/-}$ cells 6 and 10 h p.i. were compared by serial dilution RT-PCR, as indicated. (C) Protein synthesis is required for expression of non-IFN α 4 genes. Wild-type fibroblasts were mock infected (lane 1), infected with NDV for 9 h (lane 2) or infected with NDV for 9 h in the presence of cycloheximide (lane 3). Expression of non-IFN α 4, total IFN α and GAPDH mRNA was monitored by RT-PCR. (D) Response to type I but not type II IFN is required for non-IFN α 4 expression. IFNAR $^{-/-}$ and IFN γ $^{-/-}$ fibroblasts were infected for 9 h with NDV or left untreated, as indicated. Expression of non-IFN α 4, total IFN α and GAPDH mRNA was monitored by RT-PCR. (E) Exogenous IFN partially rescues CHX block of non-IFN α 4 gene expression. Wild-type fibroblasts were treated as indicated. Lane 1, mock infection; lane 2, 75 μ g/ml CHX for 9 h; lane 3, NDV infection for 9 h; lane 4, NDV infection in the presence of CHX for 9 h; lane 5, pre-treatment with 500 U/ml type I IFN and 75 μ g/ml CHX for 12 h followed by NDV infection for 9 h in the continuous presence of CHX; lane 6, pre-treatment with 50 U/ml type II IFN and 75 μ g/ml CHX for 12 h followed by NDV infection for 9 h in the continuous presence of CHX. Stat1 $^{-/-}$ fibroblasts reconstituted with Stat1 α (lane 7) or Stat1 β (lane 8) were infected with NDV for 9 h. Expression of non-IFN α 4, total IFN α and GAPDH mRNA was monitored by RT-PCR.

expression derived from sequence analysis, an expression assay was devised that specifically detected all species of IFN α other than IFN α 4. PCR primers were designed that excluded IFN α 4 amplification due to a 15 nucleotide difference in this isoform relative to other IFN α species (Kelley and Pitha, 1985). Using this analysis on RNA from virally infected cells, robust gene expression was detected in wild-type cells (Figure 2A, lanes 10–12), consistent with the results from cDNA cloning. In marked contrast, no IFN α gene expression could be detected from Stat1 $^{-/-}$ cells using this assay system, indicating that all the IFN α produced by these cells (see Figure 1) was encoded by the IFN α 4 gene. At 10 h p.i. of wild-type cells, non-IFN α 4 expression was just detectable in 125-fold diluted samples (Figure 2B, lane 9, lower panel). In contrast, no expression was detected in RNA from Stat1 $^{-/-}$ cells even in undiluted samples (Figure 2B, lane 6, upper panel).

Immediate and delayed IFN α genes are differentially regulated

Comparison of the kinetics of total IFN α gene expression with the expression of the non-IFN α 4 subset suggested that IFN α 4 is induced most rapidly. Total IFN α production was detected within 4 h of virus infection (Figure 1, lane 8). However, production of the non-IFN α 4 subset was not detected until 8 h (Figure 2A, lane 10). The difference between these patterns can be accounted for by IFN α 4 since a similar rapid expression of IFN α was detected in Stat1 $^{-/-}$ cells (Figure 1, lane 2) which produce only

IFN α 4. However, the magnitude of IFN α 4 expression was reduced in the absence of Stat1. Comparison of IFN α levels 6 h p.i., when essentially no non-IFN α 4 gene was expressed in either wild-type or Stat1 $^{-/-}$ cells (Figure 2A, lanes 3 and 9; B, lane 1), showed that ~25-fold higher levels of IFN α mRNA were produced by wild-type than by Stat1 $^{-/-}$ cells (Figure 1B). Stat1 therefore regulates two aspects of IFN α gene expression. First, it influences the abundance of IFN α 4 production, and secondly, it is required for expression of all other IFN α subtypes.

The Stat1 requirement for expression of the non-IFN α 4 subset in wild-type cells could be due to a direct involvement of Stat1 in IFN α gene transcription or to an indirect role secondary to production of a Stat1-dependent intermediate. To investigate this notion, the possible requirement for on-going protein synthesis for the regulation of IFN α species was considered. Wild-type cells were infected with NDV in the presence of cycloheximide (CHX) to block new protein synthesis, and the expression of IFN α was measured (Figure 2C). While induction of IFN α 4 was only partially reduced in the presence of CHX, no expression of the non-IFN α 4 subset was detected in CHX-treated cells (lane 3). Therefore, IFN α 4 fits the criteria for an immediate-early response gene while the non-IFN α 4 subset consists of delayed-early genes that require on-going protein synthesis and rely on Stat1 for their expression.

The requirement for both Stat1 and protein synthesis for induction of the delayed, non-IFN α 4 subset of genes suggested that IFN itself might be involved since Stat1 is

known to be activated by IFN. NDV-infected cells produced high concentrations of active type I IFN in culture supernatants and the induction of the non-IFN α 4 genes was partially impaired by treatment with anti-IFN antibodies (data not shown). To confirm a requirement for IFN, induction of IFN α gene expression was investigated in cells from type I IFN receptor-deficient mice (IFNAR $^{-/-}$). The same pattern of IFN α gene expression was observed in NDV-infected IFNAR $^{-/-}$ cells and Stat1 $^{-/-}$ cells (Figure 2D). Only IFN α 4 RNA was detected in infected IFNAR $^{-/-}$ cells while the non-IFN α 4 subset was completely absent. In contrast, IFN α production was normal in cells from IFN γ -deficient mice, showing that only type I IFN is required for expression of the non-IFN α 4 genes.

Delayed-early IFN α genes require IFN-dependent positive feedback

The similarity in phenotype between IFNAR $^{-/-}$ and Stat1 $^{-/-}$ cells suggested that the Stat1 requirement was subsequent to IFN production and that the protein synthesis requirement for IFN α expression might be explained by secretion of IFN. To test this idea, we considered whether exogenously added IFN could replace the requirement for protein synthesis for induction of delayed-early IFN production. Wild-type cells were infected with NDV in the presence of CHX, and type I IFN was added to the culture supernatant (Figure 2E). While CHX fully blocked the production of the non-IFN α 4 subset (Figure 2E, lane 4), addition of type I IFN (Figure 2E, lane 5) but not type II IFN (Figure 2E, lane 6) partially restored the expression of the delayed-early genes, even though IFN treatment alone without subsequent virus infection could not induce the IFN genes in these cells (not shown). Therefore, IFN protein production is at least one of the protein synthetic steps required for expression of non-IFN α 4 genes.

Another indication that IFN is involved in the Stat1 requirement for expression of non-IFN α 4 genes came from analysis of Stat1 isoforms. Two splice variants of Stat1 are normally expressed (Schindler *et al.*, 1992) and the Stat1 β form functions only in type I IFN responses while Stat1 α can function in multiple cytokine responses (Müller *et al.*, 1993). We derived stable cell lines from Stat1-deficient fibroblasts by transfection with cDNA expression constructs for each Stat1 isoform. Infection of cells reconstituted with Stat1 α (Figure 2E, lane 7) or with Stat1 β (Figure 2E, lane 8) were capable of producing non-IFN α 4 isoforms. IFN α induction by reconstituted cells did not equal that from wild-type cells (Figure 2E, compare lanes 7 and 8 with lane 3). However, the level of recombinant Stat1 protein expressed in these cell lines was significantly less than that in wild-type cells (not shown). Taken together, these results strongly suggest that the Stat1 requirement for non-IFN α 4 isoform expression is downstream of a response to type I IFN that is mediated by the type I IFN receptor.

Non-IFN α 4 genes are induced by IRF7

Exogenous IFN only partially restored expression of non-IFN α 4 genes in CHX-treated cells, suggesting that a protein other than IFN was also required to induce these genes. Virus-inducible elements in IFN α gene promoters (Ryals *et al.*, 1985; Au *et al.*, 1993) contain sequences

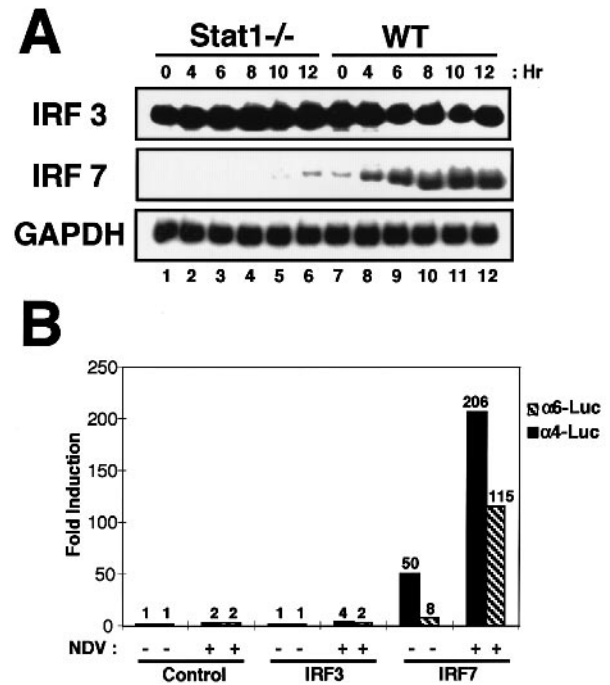


Fig. 3. IRF7 is a potent transactivator of IFN α genes. (A) Stat1-dependent viral induction of IRF7. Stat1 $^{-/-}$ and wild-type (WT) fibroblasts were infected with NDV for the indicated times. Expression of IRF3, IRF7 and GAPDH mRNA was monitored by RT-PCR. (B) Transcriptional induction of IFN α promoters by IRF7. 293T cells were transfected with empty vector or with expression constructs encoding IRF3 or IRF7, as indicated, along with luciferase reporter constructs containing the IFN α 4 or IFN α 6 promoters. After 24 h, cells were mock or NDV infected for 16 h, lysed and assayed for luciferase activity.

similar to the PRDI and PRDIII sequences of the IFN β promoter known to bind members of the IRF family of transcription factors (Genin *et al.*, 1995; Braganca *et al.*, 1997). Therefore, we considered whether any IRF family members that are induced by IFN in a Stat1-dependent manner could be involved in induction of the delayed-early IFN α isoforms. cDNA products were amplified using consensus primers based on the conserved DNA-binding region of IRF proteins. A major product detected using RNA from IFN-treated cells that was not detected using control RNA was sequenced and found to be identical to mouse IRF7 (I.Marié, E.Smith, R.Raz, Y.Wang, D.Ray, H.A.R.Bluysen and D.E.Levy, manuscript in preparation).

IRF7 gene expression was analyzed in wild-type and Stat1 $^{-/-}$ cells infected with NDV (Figure 3A). For comparison, expression of the related IRF3 gene was measured since IRF3 recently has been implicated in virus-induced expression of the IFN β gene (Lin *et al.*, 1998; Sato *et al.*, 1998; Schafer *et al.*, 1998; Wathélet *et al.*, 1998; Yoneyama *et al.*, 1998). IRF7 mRNA was highly induced by virus infection in wild-type cells (Figure 3A, lanes 7–12). In contrast, IRF7 RNA was undetectable in Stat1 $^{-/-}$ cells and its expression was only slightly induced 12 h following virus infection (Figure 3A, lanes 1–6). Dilution analysis demonstrated a >100-fold difference in IRF7 RNA levels between wild-type and Stat1 $^{-/-}$ cells 10 h p.i. (not shown), the time of maximal IFN production (see Figures 1 and 2). Similarly, IRF7 expression was not significantly induced by virus infection of IFNAR $^{-/-}$ cells (data not

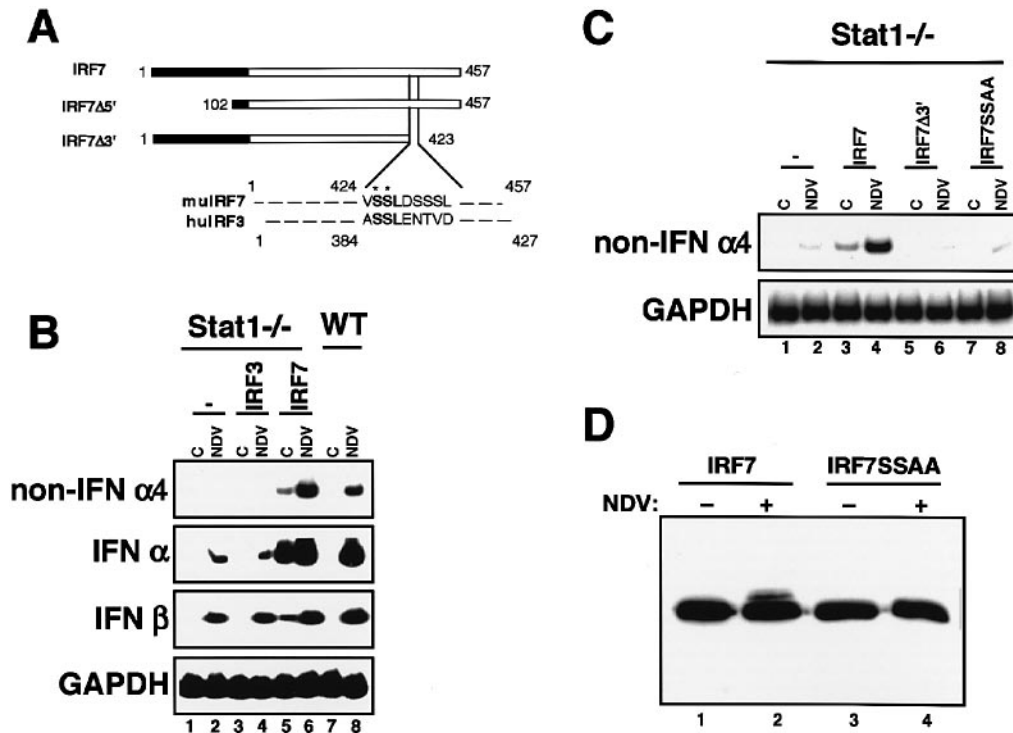


Fig. 4. IRF7 is required for the induction of non-IFN α 4 genes after NDV infection. (A) IRF7 expression constructs. The DNA-binding domain of IRF7 is shown in black. The N-terminal truncation in mutant IRF7 Δ 5' and the C-terminal truncation in mutant IRF7 Δ 3' are indicated, and the sequence context of serine residues 425 and 426 changed to alanine in mutant IRF7SSAA are shown in comparison with the homologous sequence of human IRF3 (huIRF3). (B) Rescue of non-IFN α 4 gene expression by ectopic expression of IRF7. Stat1 $^{-/-}$ fibroblasts (lanes 1–6) were transiently transfected with expression constructs for IRF3 or IRF7, as indicated. After 36 h, the cells were mock treated or infected with NDV for 9 h, as indicated, and total RNA analyzed for IFN and GAPDH expression. IFN expression in untreated and NDV-infected wild-type fibroblasts (lanes 7–8) is shown for comparison. (C) Serine residues are required for IRF7 activity. Cells were treated as in (B) except that mutant IRF7 constructs were transfected, as indicated. (D) Serine phosphorylation of IRF7. 293T cells were transfected with expression plasmids encoding IRF7 or IRF7SSAA, and whole cell extracts were harvested 4 h post-infection, as indicated, and analyzed for IRF7 electrophoretic mobility by Western blotting following SDS-PAGE.

shown), suggesting that induction of this transcription factor in response to virus infection is largely dependent on autocrine production of IFN. Expression of IRF3 RNA was equal in wild-type and Stat1 $^{-/-}$ cells and was unaffected by virus induction. Therefore, it is unlikely that this member of the IRF family is responsible for the IFN and Stat1 sensitivity of delayed-early IFN gene expression. Likewise, IRF1, which was also induced by IFN in a Stat1-dependent manner (Durbin *et al.*, 1996; Meraz *et al.*, 1996), is unlikely to be involved based on results from IRF1 $^{-/-}$ mice (Matsuyama *et al.*, 1993; Reis *et al.*, 1994).

A human homolog of IRF7 previously was suggested to act as a repressor of Epstein–Barr virus (EBV) gene expression (Nonkwelo *et al.*, 1997; Zhang and Pagano, 1997). To test whether IRF7 is responsible for induction of IFN α genes, its ability to induce IFN α 4 and IFN α 6 promoter–reporter constructs was tested by co-transfection. Induction of these constructs by co-transfection of the related protein IRF3 was also measured (Figure 3B). Reporter constructs containing the IFN α 4 promoter were activated 4-fold by NDV infection following co-transfection with IRF3 in 293T cells. However, the IFN α 6 promoter was insensitive to IRF3 co-transfection. In contrast, both IFN α 4 and IFN α 6 promoters responded to co-transfected IRF7, and this induced gene expression was enhanced further in response to virus infection. Thus, the IFN α 6 promoter appears to be insensitive to expression

of IRF3, a factor that is constitutively expressed in both wild-type and Stat1 $^{-/-}$ cells. However, IFN α 6 expression was strongly enhanced by expression of IRF7, the IRF member induced only in wild-type infected cells and expressed in a manner parallel to that of the non-IFN α 4 group.

The virus-independent activity of IRF7 observed with IFN promoter–reporter constructs may be due to the high levels of expression achieved in transfected 293T cells using replicating expression plasmids. The ability of IRF7 expression to rescue the non-IFN α 4 induction defect of Stat1 $^{-/-}$ cells was tested by transfection of non-replicating expression plasmids and measurement of endogenous gene expression (Figure 4B). For comparison, the levels of total IFN α production as well as IFN β production were monitored in cells transfected with either IRF3 or IRF7. Transfection of IRF3 expression constructs had no detectable effect on IFN gene expression in response to NDV infection (Figure 4B, compare lanes 2 and 4), nor did transfection of an N-terminally truncated form of IRF7 (IRF7 Δ 5') that failed to bind DNA (not shown). However, transfection of full-length IRF7 strongly activated non-IFN α 4 gene expression in NDV-infected, Stat1 $^{-/-}$ cells (Figure 4B, lane 6), restoring non-IFN α 4 production to levels comparable with wild-type cells (Figure 4B, lane 8). Although ectopic expression of IRF7 alone induced IFN gene expression (Figure 4B, lane 5), induction was significantly augmented by NDV infection, especially for

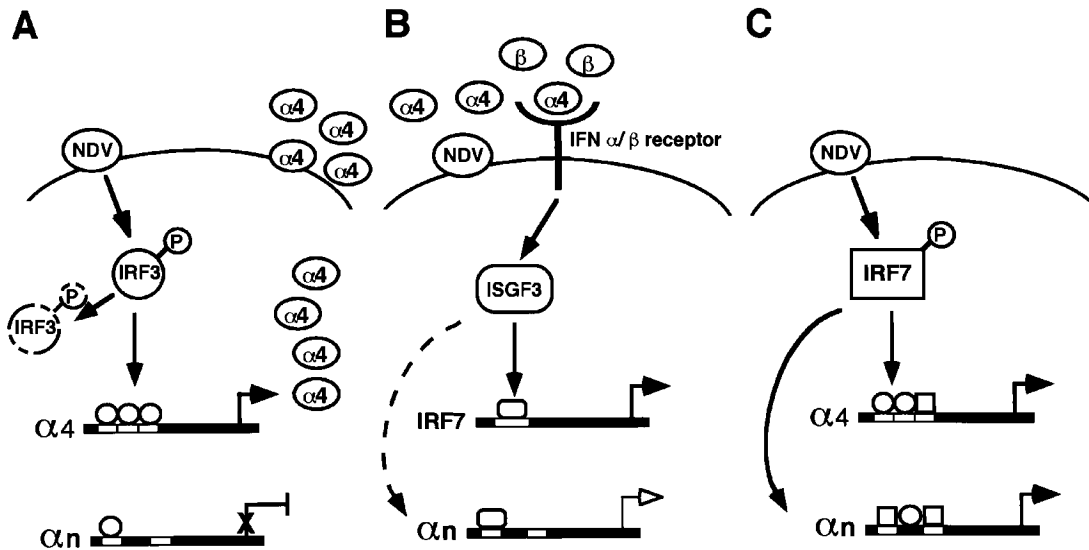


Fig. 5. Model for the multistage induction of the IFN α gene family. (A) Early stage. IFN α 4 and IFN β (not shown) are the only species of type I IFN directly induced by NDV, responding to virus-stimulated phosphorylation of IRF3 (circles). Activated IRF3 may become targeted for degradation (see Discussion). (B) Intermediate stage. Secreted IFN α 4 and IFN β stimulate the IFN α / β receptor in an autocrine fashion, leading to activation of ISGF3 and the transcription of IFN-inducible genes, among them IRF7 and possibly non-IFN α 4 genes (see Discussion). Loss of either Stat1 or IFNAR prevents the priming effect of IFN. (C) Late stage. IRF7 (squares) is synthesized and subsequently activated by serine phosphorylation in response to NDV infection, leading to further and robust induction of IFN α genes by binding promoter elements, perhaps in cooperation with IRF3.

the non-IFN α 4 genes. In response to virus infection, both IRF7 expression and its activation were necessary for full transactivation. The virus infection-independent action of IRF7 may signify that the activation event mediated by virus is also partially mimicked by the transfection process, as has been observed for activation of Stat pathways (Pine *et al.*, 1988), since both constitutive and infection-dependent activity were eliminated by identical point mutations (see below).

C-terminal serine residues are required for IRF7 activation by virus

It has been shown recently that activation of the IFN β promoter by IRF3 requires a virus-induced serine phosphorylation in a C-terminal region (Lin *et al.*, 1998; Yoneyama *et al.*, 1998). Since the C-terminus of IRF7 is serine and threonine rich, we considered whether a similar modification may occur on IRF7. We expressed a truncated form of IRF7 lacking the final 34 amino acids in Stat1 $^{-/-}$ cells (Figure 4C). Ectopic expression of this altered IRF7 construct failed to rescue non-IFN α 4 gene expression in response to virus infection (lane 6) although the altered construct produced a stable protein (not shown). Although IRF7 and IRF3 are only 19% identical outside the DNA-binding region (Nguyen *et al.*, 1997), IRF7 contains two serine residues (amino acids 425 and 426) in similar sequence context to the two serine residues of IRF3 shown to be essential for its virus-induced activity (Yoneyama *et al.*, 1998). Therefore, we mutated these two residues in the context of full-length IRF7 and ectopically expressed this protein in Stat1 $^{-/-}$ cells. Again, this altered IRF7 was incapable of rescuing the induction of the non-IFN α 4 subtypes in response to NDV infection, nor did it affect basal IFN α gene expression. The mutant forms of IRF7 also failed to affect IFN promoter-luciferase reporters, suggesting that both basal and induced transactivation require IRF7 serine phosphorylation (not shown).

Further evidence of virus-induced, serine phosphorylation was indicated by IRF7 electrophoretic mobility. A high level of expression of epitope-tagged IRF7 and serine-mutant IRF7 was achieved in 293T cells, allowing direct detection of the proteins by Western blotting. IRF7 was expressed with and without NDV infection, and protein extracts were analyzed with an antibody to the epitope tag (Figure 4D). Wild-type protein exhibited a distinct mobility shift in response to virus infection (Figure 4C, lane 2) which was reversed by phosphatase treatment (not shown), indicative of protein phosphorylation. In contrast, serine-mutant IRF7 did not display a mobility shift when isolated from either uninfected or virus-infected cells (Figure 4C, lanes 3 and 4). The small fraction of protein that underwent phosphorylation in these experiments probably results from the relative inefficiency of infection of 293T cells, and we were unable to reliably detect IRF7 protein in transfected embryo fibroblasts due to poorer transfection efficiencies and lower expression levels. However, these results strongly suggest that viral induction of the non-IFN α 4 genes requires two changes in IRF7 protein: induction of increased protein abundance (an IFN- and Stat1-dependent event) and protein phosphorylation (a virus infection-dependent event).

Discussion

The above results demonstrate that the mouse IFN α genes are regulated differentially in virus-infected cells. Although it had been noted previously that IFN α genes are not all regulated in the same manner, most attention has been paid to comparisons of IFN α 4, which is strongly induced by virus, and IFN α 11, which is poorly inducible (Pitha and Au, 1995). We show here that the IFN α 4 gene is induced rapidly and directly in response to virus infection while other IFN α subtypes such as α 2, α 5, α 6 and α 8, although also induced, are expressed in a delayed

manner that is dependent on protein synthesis. The protein synthesis-dependent character of the delayed genes results from a requirement for abundant IRF7 accumulation which in turn is dependent on transcriptional induction in response to early IFN synthesis and secretion.

Differential regulation of early and late genes

A model for IFN α gene regulation is illustrated in Figure 5. It has been shown recently that the IFN β gene, which like the IFN α 4 gene is induced in a rapid and direct manner (Maniatis *et al.*, 1987), requires virus-induced modification of the pre-existing transcription factor IRF3 (Lin *et al.*, 1998; Sato *et al.*, 1998; Schafer *et al.*, 1998; Wathelet *et al.*, 1998; Yoneyama *et al.*, 1998). The IRF3 transcription factor may also target other virus-induced cellular genes (Weaver *et al.*, 1998). By analogy, we suggest that the IFN α 4 gene may be regulated similarly by virus-activated IRF3 (Figure 5A) which becomes phosphorylated, transported to the nucleus and engaged in a productive enhanceosome at the IFN α 4 and IFN β promoters in virus-infected cells. The IFN α 4 promoter probably relies on IRF3 for induction while the IFN α 6 promoter is unresponsive even to ectopically expressed IRF3 (Figure 3B), suggesting that IRF3 alone is insufficient to induce this gene. Activation of these immediate-early IFN α 4 and IFN β promoters would lead to production and secretion of type I IFN.

IFN α 4 and/or IFN β secreted from virus-infected cells during the early response would subsequently feed back on the cells through the type I IFN receptor (Figure 5B), stimulating the Jak-Stat pathway to activate the heteromeric transcription factor complex ISGF3 (Levy, 1995). Loss of either the receptor, as in IFNAR $-/-$ cells (Figure 2C), or of Stat1, as in Stat1 $-/-$ cells (Figure 1), abrogates this loop. Active ISGF3 may play two roles in induction of the delayed, non-IFN α 4 genes. First, it may bind directly to PRDI-like elements in these promoters, leading to gene activation, as was suggested for induction of IFN β (Harada *et al.*, 1996; Yoneyama *et al.*, 1996). Secondly, it probably binds to an ISRE sequence in the IRF7 promoter, leading to up-regulation of IRF7 production. Transcription and translation of the IRF7 gene would then lead to abundant IRF7 protein capable of binding to non-IFN α 4 promoters and, following virus-activated phosphorylation, stimulating their activity (Figure 5C). Newly synthesized IRF7 may augment transcription of IFN β and IFN α 4 genes further. However, its activity is dependent on serine phosphorylation, a virus-induced event; therefore, its action is limited to virus-infected cells. This cascade of events would lead to the robust production of type I IFN secreted in response to virus infection.

Recently, it was reported that targeted disruption of the IFN β locus led to impaired expression of IFN α genes in embryonic fibroblasts infected with Sendai virus (Erlandsson *et al.*, 1998). This result would suggest that IFN β rather than IFN α 4 plays an essential role in priming cells for the subsequent induction of IFN α genes. Why no IFN α 4 was detected in this analysis is unclear. Differences due to mouse strain backgrounds, induction by Sendai rather than NDV or the sensitivity of the immunoassay employed for measuring IFN α production may account for the distinct results in our study. Indeed, IFN α

gene expression induced in Stat1 $-/-$ cells was <1% of that detected in wild-type cells (Figure 1) which may score as background by immunoassay.

It has been suggested that IRF3 and IRF7 interact and function cooperatively at the human IFN β promoter (Wathelet *et al.*, 1998). Whether IRF3 is also required along with IRF7 for activation of the non-IFN α 4 promoters as well as of IFN α 4 remains to be determined. It has been reported recently that IRF3 is degraded rapidly in virus-infected cells (Lin *et al.*, 1998; Ronco *et al.*, 1998), resulting in the depletion of IRF3 protein at the time when IRF7 and the delayed IFN α genes are being induced. Therefore, it is possible that IRF7 is the only IRF protein available for induction of the non-IFN α 4 subset, probably cooperating with other promoter-bound factors (Au *et al.*, 1993; Genin *et al.*, 1995). Similarly, it would appear from the inducibility of IFN α 4 and IFN β in Stat1 $-/-$ cells early following virus infection when IRF7 RNA is undetectable that IRF7 may be dispensable for expression of the immediate-early IFN genes. Indeed, IRF7 is expressed at very low levels prior to infection even in wild-type cells (Figure 3A), and ectopic expression of IRF7 has only a modest effect on IFN α 4 and IFN β gene induction although it is essential for expression of the non-IFN α 4 subset (Figure 4). While it is possible that the low levels of IRF7 present prior to infection are sufficient to cooperate with the abundant IRF3 and may be responsible for the higher levels of IFN α 4 expressed by wild-type cells, our results are consistent with the notion that IRF7 is not absolutely necessary for IFN α 4 or IFN β induction while it is indispensable for expression of the non-IFN α 4 genes. Whether IRF3 is required for induction of the non-IFN α 4 subset remains to be determined.

The potential dual role of ISGF3 is more complex. As previously suggested, ISGF3 may bind and activate type I IFN promoters (Yoneyama *et al.*, 1996), and such an activity could explain our finding that non-IFN α 4 genes could be induced in virus-infected cells in the absence of protein synthesis by addition of exogenous IFN (Figure 2D). An alternative explanation for IFN rescue of CHX inhibition is that translation of IRF7 might be relatively resistant to CHX treatment, and thus the added IFN stimulated production of sufficient IRF7 to mediate IFN α late gene expression in response to NDV under the conditions employed. Discrimination between these two possibilities will require an antibody capable of detecting production of IRF7. In either case, ISGF3 alone is not sufficient to activate type I IFN promoters, since no gene expression was induced by IFN in the absence of virus infection in spite of robust activation of IFN-stimulated genes, including IRF7. Therefore, if ISGF3 is capable of functioning at IFN α promoters, it does not act alone and additional virus-dependent factors or activation events must also be required.

Phosphorylation of IRFs

IRF7 is regulated in two ways. It is synthesized in response to IFN and it is activated by phosphorylation in virus-infected cells. The similarity of the phosphorylation site on IRF7 to a virus-dependent phosphorylation site on IRF3 suggests that these two proteins may be phosphorylated by the same or a similar kinase. However, the kinase(s) responsible for this modification is yet to be identified.

One potential kinase would be the double-stranded RNA-dependent protein kinase, PKR. IFN induction is sensitive to the PKR inhibitor, 2-aminopurine (Zinn *et al.*, 1988). However, disruption of the gene for PKR does not prevent IFN induction in response to virus (Yang *et al.*, 1995), making it unlikely that PKR is required for this process. Consistent with the lack of requirement for PKR, we found induction of both IFN α 4 and the non-IFN α 4 gene subset in PKR-null cells infected with NDV (data not shown).

Another candidate kinase would be the I κ B kinase since NF κ B is also activated in virus-infected cells in a serine phosphorylation-dependent manner (Lenardo *et al.*, 1989; Maniatis, 1997), and we have found that the cytokine-induced IKK α kinase (DiDonato *et al.*, 1997; Mercurio *et al.*, 1997; Regnier *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997) is activated in virus-infected cells (data not shown). However, the phosphorylation site on IRF7 does not appear related to the induced phosphorylation sites on I κ B, and we have been unable to observe IRF7 phosphorylation by IKK α *in vitro* (data not shown).

Multiple type I IFN genes

The physiological significance of the large number of IFN α genes has remained obscure. One possibility highlighted by the present results is that multiple genes allow distinct patterns of regulation. The IFN α 4 and IFN β genes may have evolved to provide an immediate response to virus infection, while the sequential induction of delayed genes provides a means of greatly amplifying the protective response to virus. It is possible that an effective response to acute infection requires higher levels of IFN production than can be achieved by expression from a single gene. In addition, some viruses are less effective inducers of IFN than NDV, and the amplification process afforded by IFN feedback may be particularly important for defense against such viruses.

Sequential activation of IFN genes could provide very tight control since a continuous signal from virus infection is required in addition to IRF7 protein induction. Thus, if the initial burst of IFN α 4 and IFN β production successfully eliminated infection, no further IFN would be produced since the newly synthesized IRF7 would be inactive without further modification. In fact, human IRF7 has been suggested as a possible repressor involved in EBV latency (Nonkwelo *et al.*, 1997; Zhang and Pagano, 1997). Perhaps in the absence of a specific activation event, the presence of non-phosphorylated IRF7 could compete for activator proteins. However, cells that were continuously or subsequently infected after IRF7 induction would be primed to continue IFN production because IRF7 would become phosphorylated.

The type I IFN gene family is tandemly arrayed on a single chromosome, and this gene cluster has been conserved throughout mammalian evolution (Nadeau *et al.*, 1986; De Maeyer and De Maeyer-Guignard, 1988). A potential pressure maintaining gene clustering could be to retain global regulation. IRF3 interacts with the histone acetylase p300 (Sato *et al.*, 1998; Wathelet *et al.*, 1998; Weaver *et al.*, 1998; Yoneyama *et al.*, 1998). It is possible that recruitment of p300 to the IFN β gene by IRF3 results in remodeling of the entire IFN gene locus, making the IFN α genes more accessible to transcription factors, such

as IRF7. Such global control of tandemly linked genes could provide a strong evolutionary pressure to retain the linked IFN gene complex.

Materials and methods

Cells culture, transfections and viral infections

Immortalized embryo fibroblasts were derived from wild-type, Stat1 $^{-/-}$ (Durbin *et al.*, 1996), IFNAR $^{-/-}$ (van den Broek *et al.*, 1995) and IFN γ $^{-/-}$ embryos (Dalton *et al.*, 1993), as described (Todaro and Green, 1963), and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. 293T and Cos cells were grown under the same conditions. DNA transfections of 293T, Cos and mouse fibroblasts were performed by standard methods using calcium phosphate. Stable cell lines of Stat1 $^{-/-}$ fibroblasts reconstituted either with Stat1 α or Stat1 β were obtained by co-transfections using expression constructs in which the appropriate cDNA, originally cloned from a mouse brain library (Raz *et al.*, 1994), was driven by a cytomegalovirus immediate-early promoter, along with the pSV2pac selectable marker, followed by selection in medium containing 10 μ g/ml of puromycin. Individual clones demonstrating responsiveness to IFN were obtained by fluorescence-activated cell sorting of IFN-treated cells using antibody M1/42.3 (ATCC) against MHC class I antigens. Expression of Stat1 α and Stat1 β was scored by Western blot using an antibody directed against the mouse Stat1 SH2 domain (Zymed Laboratories).

For viral infections, an NDV, Manhattan (Lee BioMolecular) was suspended at 108 p.f.u./ml in medium containing 0.1% bovine serum albumin (BSA), added to cell monolayers for 1 h at 37°C, and replaced with normal growth medium for 8 h, unless otherwise indicated. Where indicated, cells were treated with type I IFN (Lee BioMolecular) at 500 U/ml or type II IFN (Life Technology) at 5 ng/ml.

Plasmid constructs

Murine IRF7 initially was identified as an IFN-inducible member of the IRF family by RT-PCR using consensus primers derived from the conserved DNA-binding domain (I.Marié, E.Smith, R.Raz, Y.Wang, D.Ray, H.A.R.Bluysen and D.E.Levy, manuscript in preparation). Full-length murine IRF3 (DDBJ/EMBL/GenBank accession No. U75840) and IRF7 (DDBJ/EMBL/GenBank accession No. U73037) were isolated from MEF mRNA by RT-PCR using the following oligonucleotides: IRF3 sense, 5'-ATGGAAACCCGAAACCGC-3'; IRF3 antisense, 5'-GATATTTCCAGTGGCCTGGA-3'; IRF7 sense, 5'-AAACCATAGAG-GCACCAAG-3'; IRF7 antisense, 5'-TTGGGAGTTGGGATTCTGATCAAGGC-3'.

Epitope-tagged versions were prepared by fusing a sequence encoding the FLAG peptide (Kodak) to the C-terminus. IRF3 was cloned into the *NotI* and *XhoI* sites of pBPSRT1 and IRF7 was cloned into the *KpnI* and *XbaI* sites of pcDNA3. N- and C-terminal deletion mutants of IRF7, IRF7 Δ 5' (IRF7¹⁰²⁻⁴⁵⁷) and IRF7 Δ 3' (IRF7¹⁻⁴²³) were designed by PCR and reintroduced into the full-length cDNA using a unique internal *BstEII* site and a flanking cloning site. The double point mutation at serine residues 425 and 426 of IRF7 (IRF7SSAA) was introduced into the full-length IRF7 using standard methods (Deng and Nickoloff, 1992).

Reporter constructs α 4-Luc and α 6-Luc were obtained by substituting the SV40 promoter of pGL3-Promoter (Promega) with the promoter regions of the IFN α 4 or IFN α 6 gene. PCR-generated fragments from mouse genomic DNA encoding -476 to +10 and -432 to +22 relative to the initiation site of IFN α 4 and IFN α 6, respectively, were cloned into the *SmaI* and *NcoI* sites of the vector. Luciferase activities were measured in cell lysates using commercial reagents as recommended by the manufacturer (Promega) and were normalized to the β -galactosidase activity of a co-transfected RSV-lacZ plasmid measured on a luminescent substrate (Tropix).

Expression analysis

Western blots were performed by standard methods using an anti-FLAG antibody (Kodak). RT-PCR was performed by standard protocols using total RNA and the following primers: (i) IFN α (consensus primers annealing with all IFN α subtypes) sense 5'-ATGGCTAGRCT-CTGTGCTTTCT-3', antisense 5'-AGGGCTCTCCAGAYTTCTGCT-CTG-3'; (ii) non-IFN α 4 (5' primer fails to bind IFN α 4 gene) sense 5'-ARSYTGTTGATGCARCAGGT-3', antisense 5'-GGWACACATGTGATCCTGTGG-3'; (iii) IFN β sense 5'-CATCAACTATAAGCAGCTCCA-3', antisense 5'-TTCAAGTGGAGAGCAGTTGAG-3'; (iv)

GAPDH sense 5'-ACCACAGTCCATGCCATCAC-3', antisense 5'-TCCACCACCTGTTGCTGTA-3'; (v) IRF3 sense 5'-CCAGGTCTTCCAGCAGACT-3', antisense 5'-TAGGCTGGCTGTTGGAGATGT-3'; (vi) IRF7 sense 5'-CAGCGAGTGTGTTGGAGAC-3', antisense 5'-AAGTTCGTACACCTTATGCGG-3'.

To estimate relative amounts of specific RNA species, PCRs were performed on serially diluted samples of reverse transcription products, as described (Erlandsson *et al.*, 1998). For sequence analysis of IFN α gene expression, cDNA fragments were amplified by RT-PCR using the IFN α consensus primers, cloned into plasmid vectors, and 19 random clones from wild-type cells and 18 random clones from Stat1 $^{-/-}$ cells were sequenced by standard methods.

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