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Distinct and Essential Roles of Transcription Factors IRF-3 and IRF-7 in Response to Viruses for *IFN*-α/β Gene Induction

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Summary

Induction of the interferon *(IFN)*-α/β gene transcription in virus-infected cells is an event central to innate immunity. Mice lacking the transcription factor IRF-3 are more vulnerable to virus infection. In embryonic fibroblasts, virus-induced *IFN*-α/β gene expression levels are reduced and the spectrum of the IFN-α mRNA subspecies altered. Furthermore, cells additionally defective in IRF-7 expression totally fail to induce these genes in response to infections by any of the virus types tested. In these cells, a normal profile of IFN-α/β mRNA induction can be achieved by coexpressing both IRF-3 and IRF-7. These results demonstrate the essential and distinct roles of the two factors, which together ensure the transcriptional efficiency and diversity of *IFN*-α/β genes for the antiviral response.

Introduction

Cells produce interferon (IFN)-α/β in response to infection by a variety of viruses, an event known to be central to the innate immune response of a host (reviewed in DeMaeyer and DeMaeyer-Guignard, 1988; Vilcek and Sen, 1996). In fact, virus infection results in the transcriptional activation of *IFN*-α/β genes, and numerous studies have been carried out to elucidate the mechanisms of the gene induction in response to virus infections, particularly that of the *IFN*-β gene, which is present as a single copy in humans and mice (reviewed in Weissmann and Weber, 1986; Maniatis et al., 1992; Taniguchi et al., 2000). The *IFN*-β gene contains within its promoter region several regulatory *cis*-elements, consisting of positive and negative elements (reviewed in Taniguchi, 1988; Maniatis et al., 1992). The positive elements constitute a virus-inducible enhancer, which contains at least four regulatory elements, designated as PRDI, -II, -III, and -IV (Goodbourn and Maniatis, 1988; Leblanc et al., 1990; Du and Maniatis, 1992). It has been shown that the PRDII and -IV elements, respectively, bind the transcriptional activators NF-κB and the ATF-2/c-Jun to cooperate with PRDI and -III in the induction of the *IFN*-β gene in virus-infected cells (Fujita et al., 1989a; Lenardo et al., 1989; Visvanathan and Goodbourn, 1989; Du and Maniatis, 1992; Du et al., 1993; Chu et al., 1999). As for the *IFN*-α gene family, which consists of more than a dozen members, sequences similar to PRDII and -IV have not been identified, but sequences similar to PRDI and -III have been found within each of their promoters (Ryals et al., 1985; Raj et al., 1989; Genin et al., 1995). There is also evidence that PRDI or -III alone can function as a virus-inducible enhancer (Fujita et al.,1987; Fan and Maniatis, 1989; Leblanc et al., 1990).

The PRDI and -III elements are known to bind members of a family of transcription factors, termed interferon regulatory factors (IRFs) (Fujita et al., 1988; Miyamoto et al., 1988). Among the nine members of this family (IRF-1, IRF-2, IRF-3, IRF4/Pip/ICSAT, IRF-5, IRF-6, IRF-7, IRF-8/ICSBP [interferon consensus sequence binding protein], and IRF-9/ISGF3γ [interferon-stimulated gene factor 3γ]), four members, IRF-1, IRF-3, IRF-7, and IRF-9/ISGF3γ, have been implicated in the transcriptional induction of the *IFN*-α/β genes (reviewed in Nguyen et al., 1997; Mamane et al., 1999; Taniguchi et al., 2000). Previously, it has been shown that the ectopic expression of IRF-1 results in the activation of *IFN*-α/β promoters in transient assays (Harada et al., 1990) and that induction of the endogenous *IFN*-α/β genes also occurs in some cells (Fujita et al., 1989b). Subsequently, cells lacking IRF-1 have been generated by gene targeting in mice, and it has been shown that the *IFN*-α/β gene induction by Newcastle disease virus ([NDV]; paramyxoviridae) still occurs normally, indicating that either IRF-1 is not involved or that a redundant mechanism(s) may be operating in the gene induction (Matsuyama et al., 1993). Evidence has also been provided that an IFN-activated transcription factor, ISGF3, which is a heterotrimeric complex consisting of IRF-9 (ISGF3γ or p48), signal transducer and activator of transcription (Stat)1 and Stat2, is involved in *IFN*-α/β gene induction (Kawakami et al., 1995; Harada et al., 1996; Yoneyama et al., 1996); however, it is still unclear whether ISGF3 directly acts on the *IFN*-α/β genes (Harada et al., 1996; Yoneyama et al., 1996). Recently, much attention has been focused on two structurally related members, IRF-3 and IRF-7 (reviewed in Mamane et al., 1999; Taniguchi et al., 2000).

IRF-3 and IRF-7 are both expressed in many cell types, and they undergo phosphorylation and nuclear translocation in the event of virus infection of the cell. Furthermore, either of these factors, when expressed ectopically, can enhance the IFN-α/β mRNA induction levels (Marie et al., 1998; Sato et al., 1998a, 1998b). In addition,

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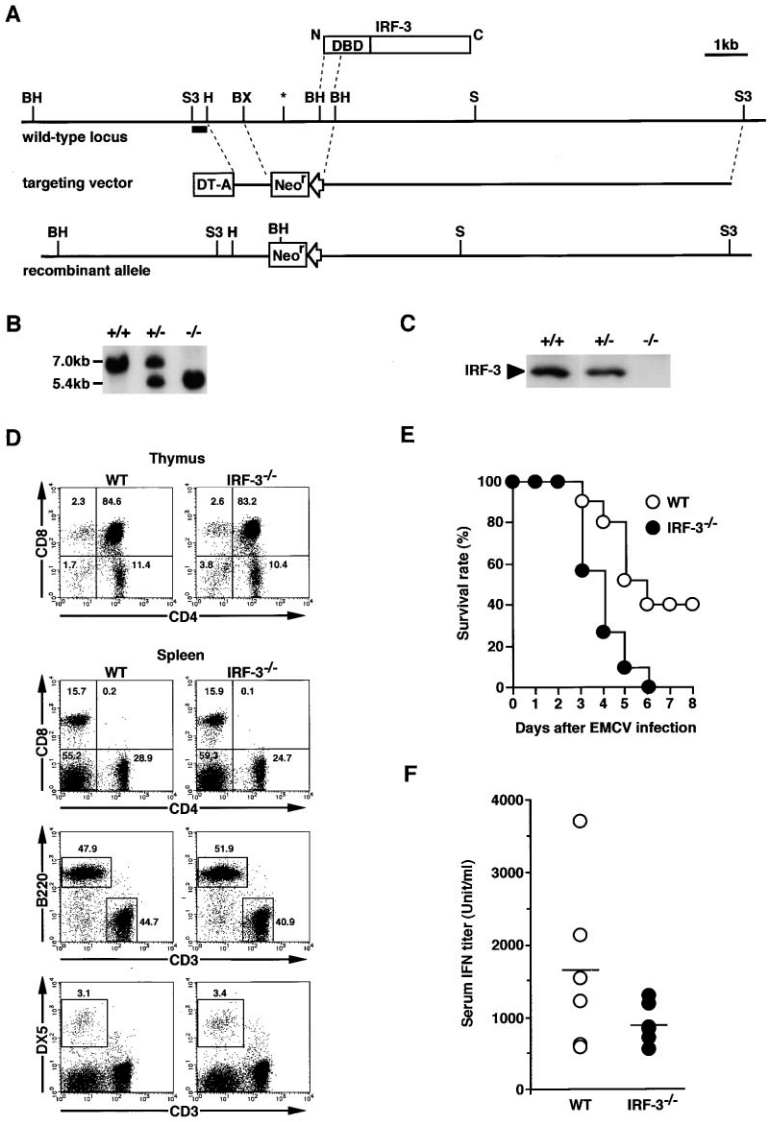

Figure 1. Generation of Mice with *IRF-3* Nullizygosity

(A) Partial restriction map of the wild-type locus and targeting strategy for *IRF-3* gene disruption. The region containing the putative transcriptional initiation site (represented by an asterisk) and the coding exon for the N-terminal part of the DNA binding domain (DBD, shown at the top) were replaced with a pgk-neo cassette. The diphteria toxin A (DT-A) cassette was attached at the 5′ end of the targeting vector as a negative selection marker. The probe for genomic Southern blot analysis is indicated by a thick bar. BH, BamHI; BX, BstXI; S, SalI; S3, Sau3AI.

(B) Southern blot analysis of BamHI-digested genomic DNA isolated from wild-type (+/+), heterozygous mutant (+/−), and homozygous mutant (−/−) mice.

(C) Whole-cell extracts from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) MEFs were subjected to immunoblotting to detect the IRF-3 protein.

(D) Flow cytometric analysis of lymphocyte populations in *IRF-3*$^{-/-}$ mice. Single cell suspensions were prepared from the thymus and spleen from 8-week-old littermate mice and stained with indicated combination of the following fluorochrome-conjugated monoclonal antibodies: anti-CD4 FITC, anti-CD8 PE, anti-CD3 FITC, anti-B220 PE, and anti-DX5 PE. Viable cells (2×10^4) in the lymphocyte gate, as defined according to side and forward scatters, were analyzed. The numbers represent the percentage of cells contained in each region.

(E) Survival rate of WT and *IRF-3*$^{-/-}$ mice after EMCV infection. Ten WT and eleven *IRF-3*$^{-/-}$ mice were infected intraperitoneally with EMCV (10^5 pfu), and the survival of these mice was monitored daily. Four WT mice recovered from infection and remained alive (monitored up to 14 days).

(F) IFN levels in sera from EMCV-infected mice. Six WT and *IRF-3*$^{-/-}$ mice were infected with EMCV as in (E). Serum from each mouse was prepared at day 3 postinfection and the IFN level determined. The bars represent the mean values of the IFN titer. All of the antiviral activity was completely neutralized by adding anti-IFN-α (clone 4E-A1, Yamasa Shoyu) and anti-IFN-β (clone 7F-D3, Yamasa Shoyu) monoclonal antibodies (data not shown).

IRF-3 is also known to bind to the coactivators CBP/p300 (Lin et al., 1998; Sato et al., 1998a; Wathelet et al., 1998; Yoneyama et al., 1998), and it has also been demonstrated that IRF-3 and IRF-7 form a complex, termed virus-activated factor (VAF), that binds to the *IFN*-β promoter (Wathelet et al., 1998). While IRF-3 is constitutively expressed in normally growing cells, the IRF-7 expression is induced by IFN-α/β stimulation, leading to the notion that IRF-3 is primarily responsible for the induction of *IFN*-α/β genes in the early induction phase and that IRF-7 is involved in the late induction phase (Marie et al., 1998; Sato et al., 1998b).

Despite the substantial interest in the roles of these IRF family members in *IFN*-α/β gene induction, it is still unclear as to how these factors actually participate in the gene induction by viruses. In this regard, it is also worth noting that some *IFN*-α members, such as *IFN*-α*4* and *IFN*-α*5*, are preferentially induced in mouse cells (Weissmann and Weber, 1986; Marie et al., 1998). It remains to be clarified how these IRFs actually participate in the regulation of this diversity of gene induction for the *IFN*-α gene family.

To assess the role of IRF-3 and IRF-7, we applied the gene targeting strategy in the present study and generated mice and mouse cells that do not express IRF-3 or both IRF-3 and IRF-7. We demonstrate that both IRF-3 and IRF-7 perform nonredundant and distinct roles from each other for the efficient *IFN*-α/β gene induction, as well as for the diversity of the induction mechanisms within the *IFN*-α gene family. Our present study also indicates that the transcriptional induction of *IFN*-α/β genes against infection by different types of viruses is commonly dependent on these two IRF family members.

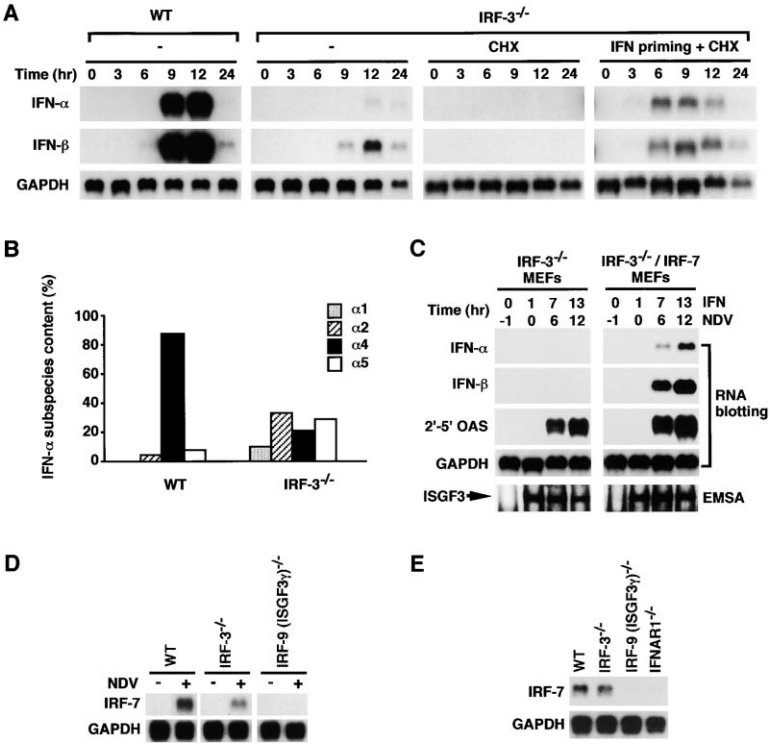

Figure 2. Expression of IFN-α/β and IRF-7 mRNAs in *IRF-3*$^{-/-}$ MEFs

(A) Induction of IFN-α/β mRNA in wild-type (WT) and *IRF-3*$^{-/-}$ MEFs was analyzed by RNA blotting; time after NDV infection is indicated. Five micrograms of total RNA was loaded in each lane. For IFN priming and cycloheximide (CHX) treatment, MEFs were stimulated with 250 U/ml recombinant mouse IFN-β (TORAY) for 6 hr and subsequently with 100 μg/ml CHX 1 hr prior to NDV infection. For blotting control, each membrane was reprobed using the GAPDH probe ([A], [C], [D], and [E]).

(B) Induction profile of the IFN-α mRNA subspecies. Total RNA from the indicated MEFs infected with NDV was subjected to RT–PCR using IFN-α consensus primers (Marie et al., 1998). The obtained cDNA fragments were subcloned, and 50 clones from each of the MEFs were analyzed based on their sequences. In WT cells, none of the 50 clones analyzed encoded IFN-α1 mRNA.

(C) Induction of IFN-α/β mRNA in *IRF-3*$^{-/-}$ MEFs and *IRF-3*$^{-/-}$/IRF-7 MEFs. The retrovirus vector pBabe-hygro or His-IRF-7/pBabe-hygro (IRF-7) was introduced into *IRF-3*$^{-/-}$ MEFs (*IRF-3*$^{-/-}$/IRF-7 MEFs). The cells were treated with 100 μg/ml of CHX for 1 hr and subsequently stimulated with recombinant mouse IFN-β as in (A). After 1 hr, the cells were infected with NDV. Total RNA was extracted at different times, as indicated, and 5 μg of total RNA from each time point was subjected to RNA blotting. The activation of ISGF3 was detected by EMSA using the ISRE (IFN-stimulated response element) probe from the human *2′-5′ OAS* gene (lower panel). Time (hr) after IFN treatment and NDV infection is indicated at the upper part of the panel.

(D) Induction of IRF-7 mRNA in NDV-infected MEFs. WT, *IRF-3*$^{-/-}$, and IRF-9/*ISGF3*$\gamma^{-/-}$ MEFs were mock infected (−) or infected with NDV for 12 hr (+) and total RNA extracted. Five micrograms from each sample was applied for RNA blotting.

(E) Expression of IRF-7 mRNA in normally growing MEFs. Three micrograms of poly(A) RNA prepared from WT, *IRF-3*$^{-/-}$, IRF-9/*ISGF3*$\gamma^{-/-}$, and IFN-α/β receptor 1 (IFNAR1)$^{-/-}$ MEFs was loaded onto each lane.

Results and Discussion

Introduction of *IRF-3* Nullizygosity

The mouse *IRF-3* gene, present as a single copy, was cloned and a targeting vector constructed for the generation of *IRF-3*-deficient (*IRF-3*$^{-/-}$) mice (Figure 1A). The expected nullizygosity was confirmed by Southern blotting (Figure 1B) and immunoblot analysis of *IRF-3*$^{-/-}$ mouse embryonic fibroblasts (*IRF-3*$^{-/-}$ MEFs) using an anti-IRF-3 antibody (Figure 1C). Mutant *IRF-3* heterozygous and homozygous mice showed no difference in size, behavior, and reproductive ability as compared with wild-type (WT) littermates. Since some IRF family members play critical roles for the lymphocyte development in mice (Matsuyama et al., 1993; Ogasawara et al., 1998), we analyzed lymphocyte populations in the thymus and spleen of *IRF-3*$^{-/-}$ mice, but no significant abnormality was detected (Figure 1D). Interestingly, *IRF-3*$^{-/-}$ mice were found to be more vulnerable to virus infection; all of the 11 mice infected with the encephalomyocarditis virus (EMCV) died within 6 days, whereas the similarly infected WT mice were more resistant, and 4 out of 10 mice recovered from infection (Figure 1E). Thus, IRF-3 is critical for the host defense against virus infection. Although the vulnerability of these *IRF-3*$^{-/-}$ mice may involve many events, the IFN levels in serum from EMCV-infected mice were found to be significantly lower in *IRF-3*$^{-/-}$ mice than in WT mice (Figure 1F). On the other hand, when IFN-mediated antiviral response of *IRF-3*$^{-/-}$ MEFs against VSV, EMCV, and herpes simplex virus (HSV) was examined, the response was normal (data not shown).

Impaired *IFN*-α/β Gene Induction in the Absence of IRF-3

The *IRF-3*$^{-/-}$ MEFs were examined for the virus-induced expression of IFN-α/β mRNAs. As shown in Figure 2A, in a typical case, IFN-α/β mRNA expression levels, induced by NDV, dramatically decreased (about 20- to 50-fold 12 hr after the viral infection) in *IRF-3*$^{-/-}$ MEFs, as compared with those in WT MEFs. IFN-α/β levels in the culture medium secreted from *IRF-3*$^{-/-}$ MEFs also decreased (see Table 1). Furthermore, even the residual induction became undetectable when a protein synthesis inhibitor, cycloheximide (CHX), was added to the cell culture prior to the NDV infection. There have been many reports demonstrating that the initial *IFN*-α/β gene induction in normally growing cells occurs in the presence of CHX (Fujita et al., 1981; Harada et al., 1996), and our results indicate that IRF-3 is essential for this induction. In addition, further analysis of the induced mRNA population revealed an abnormality in the spectrum of induction for the IFN-α mRNA subspecies induced in the *IRF-3*$^{-/-}$ MEFs: preferential induction of IFN-α1 and -α5 mRNAs, along with IFN-α4 mRNA, which is the most predominant species in WT MEFs, was observed (Figure 2B). Thus,

Table 1. IFN Production in MEFs

Genotype	IFN titer (unit/ml)
WT	2286
	2685
IRF-3$^{-/-}$	138
	154
IRF-9 (ISGF3γ)$^{-/-}$	608
	548
DKO	<0.1
	<0.1

IFN activities were determined by the procedure described in the Experimental Procedures. MEFs (1×10^5) were seeded in to wells of 12-well plates. After 12 hr of incubation, MEFs were infected with 2.5 hemaggulutinin unit of NDV, and the culture medium was collected after 24 hr of incubation. Virus in the medum was inactivated by incubation at pH 2. All of the antiviral activity was neutralized completely by adding anti-IFN-α (clone 4E-A1, Yamasa Shoyu) and anti-IFN-β (clone 7F-D3, Yamasa Shoyu) monoclonal antibodies.

the absence of IRF-3 expression results in the abnormal expression profile of the IFN-α mRNA species, indicating an additional, novel role of IRF-3 for acquiring normal IFN-α mRNA induction profiles (see below).

Critical Role of IFN Signaling for Residual *IFN*-α/β Gene Induction in *IRF-3* Null Cells

Interestingly, when *IRF-3*$^{-/-}$ MEFs were pretreated with IFN-β (250 U/ml), a process known as "priming" (Stewart et al., 1971), substantial levels of IFN-α/β mRNA induction were observed even in the presence of CHX (Figure 2A). This result indicates that another factor(s) induced by priming can function in the absence of IRF-3. It has been shown that IFN-activated ISGF3 directly binds to the virus-inducible elements of *IFN*-α/β genes (Kawakami et al., 1995; Harada et al., 1996; Yoneyama et al., 1996). More recently, it has been shown that IRF-7, another member of the IRF family that is structurally related to IRF-3, is induced by IFN-α/β via ISGF3 activation (Marie et al., 1998; Sato et al., 1998b).

The availability of *IRF-3*$^{-/-}$ MEFs provided us an opportunity to examine whether ISGF3 is involved directly, or indirectly through IRF-7 induction, in the *IFN*-α/β gene induction. *IRF-3*$^{-/-}$ MEFs, constitutively expressing IRF-7, were prepared by retrovirus-mediated gene transfer, and they were treated with IFN-β in the presence of CHX, followed by NDV infection. As shown in Figure 2C (upper panel), mRNA for the *2′-5′ oligoadenylate synthetase (2′-5′ OAS)* gene, which is directly activated by ISGF3 (Kimura et al., 1996), was induced even in cells infected by a control retrovirus. On the other hand, the induction of IFN-α/β mRNA was observed only in cells ectopically expressing IRF-7. Under these conditions, both cells showed sustained activation of ISGF3 1 hr after IFN-β stimulation, as revealed by the electrophoresis mobility shift assay (EMSA) (Figure 2C, lower panel). These data further lend support to the notion that ISGF3-induced IRF-7, rather than ISGF3 per se, acts on *IFN*-α/β genes (Marie et al., 1998; Sato et al., 1998b). As shown in Figure 2D, IRF-7 mRNA is strongly induced following NDV infection of WT MEFs. Interestingly, IRF-7 mRNA induction by NDV still occurs in *IRF-3*$^{-/-}$ MEFs, albeit inefficiently; however, this induction is completely abolished in MEFs

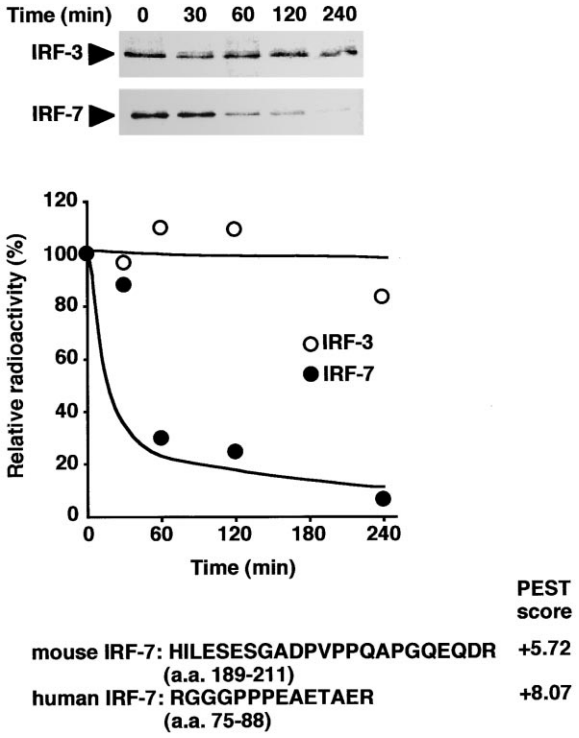

Figure 3. Stability of IRF-3 and IRF-7 Proteins

NIH/3T3 cell lines expressing HA-tagged IRF-3 or IRF-7 were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine. After the chase, cell extracts were prepared at the indicated times and subjected to immunoprecipitation using an anti-HA monoclonal antibody (clone 12CA5, Boehringer Mannheim) by SDS-PAGE analysis. The incorporated radioactivity was measured by BAS5000 (Fujix). PEST sequences of IRF-7 and their PEST-FIND scores were obtained using PEST-FIND (Rechsteiner and Rogers, 1996).

from *IRF-9*-deficient (*IRF-9*$^{-/-}$) mice (Figure 2D) (Kimura et al., 1996; Sato et al., 1998b). Immunoblot analysis using an anti-IRF-7 antibody also revealed similar expression profiles of the IRF-7 protein (data not shown). Furthermore, RNA blot analysis of poly(A) RNA revealed that "constitutive" IRF-7 mRNA expression observed in the WT and *IRF-3*$^{-/-}$ MEFs is barely detectable in MEFs lacking the IFN-α/β receptor 1 (IFNAR1) or IRF-9 (Figure 2E). Similar observations were also seen in splenocytes from the respective mice (data not shown).

These results may be interpreted in relation to the induction mechanism of the *IFN*-α/β genes as follows. In the early phase, upon virus infection, the IFN-independent pathway dominates in WT MEFs due to the constitutive expression of IRF-3 (Figure 1C), and signaling by the newly synthesized IFNs further induces IRF-7 via strong activation of ISGF3 in the subsequent induction phase (Marie et al., 1998; Sato et al., 1998b). In *IRF-3*$^{-/-}$ MEFs, the IFN-dependent pathway, leading to IRF-7 expression, is still operational but inefficient, since the early IFN-α/β induction is solely dependent on low levels of IRF-7 expression. In fact, this low level of IRF-7 expression in uninfected cells is sustained through signaling by IFN-α/β, which are spontaneously expressed at very low levels (Takaoka et al., 2000). Consistently, we found that in *IRF-3*$^{-/-}$ splenocytes, constitutive IRF-7

mRNA expression levels were much higher than that of MEFs and that the IFN-α/β mRNA induction was also higher in these splenocytes than in MEFs (data not shown; see below). It is also known that expression of IRF-7 per se (for example, by IFN priming) is insufficient to induce *IFN*-α/β genes, and it requires about 6 hr after virus infection to undergo modification and nuclear translocation (Au et al., 1998); this would explain the result (shown in Figure 2A) that in IFN-primed cells, it still takes 6 hr before the gene induction.

It is worth noting that the IFN-α/β mRNA expression level is undetectable and low in the "unprimed" and "primed" *IRF-3*$^{-/-}$ MEFs, respectively, in the presence of CHX (Figure 2A). This observation suggests the importance of ongoing de novo protein synthesis for the efficient functioning of IRF-7. In this regard, we found a notable difference in terms of the stability of IRF-3 and IRF-7; IRF-7 has a very short half-life (0.51 ~1 hr), whereas IRF-3 is very stable (Figure 3). Using the algorithm PEST-FIND (Rechsteiner and Rogers, 1996), IRF-7 was found to have a potential proteolytic signal called the PEST sequence, which is rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (Rogers et al., 1986). A potential PEST sequence was found in IRF-7 but not in IRF-3 (Figure 3). Although further work is necessary, it is possible that this sequence accounts for the selective instability of IRF-7, which would explain the low induction level of IFN-α/β mRNA in the presence of CHX in *IRF-3*$^{-/-}$ MEFs.

Complete Loss of *IFN*-α/β Gene Induction in the Absence of Both IRF-3 and IRF-7 Expression

To further examine the contribution of IRF-3 and IRF-7 in the IFN-dependent and -independent *IFN*-α/β gene induction mechanisms, we next generated mice doubly deficient for *IRF-3* and *IRF-9* (DKO mice) by crossing *IRF-3*$^{-/-}$ mice with *IRF-9*$^{-/-}$ mice (Kimura et al., 1996). As shown in Figure 4A, the IRF-7 mRNA induction is totally abolished in DKO cells, whereas IRF-1 mRNA, encoding another transcription factor also implicated in this process, is still inducible (data not shown). Interestingly, the IFN-α/β mRNA induction was completely abolished in the DKO MEFs and splenocytes (Figures 5A and 5B), and, as expected, the IFN-α/β activity was undetectable in the culture supernatant (Table 1). Thus, DKO MEFs are incapable of responding to NDV infection in terms of IFN-α/β mRNA induction. Consistent with this finding, the nuclear run-on assay revealed the absence of any transcriptional induction of *IFN*-β gene in DKO MEFs (M. S., unpublished data). These results further underscore the essential role of IRF-3 for the IFN-independent *IFN*-α/β gene induction and suggest the involvement of IRF-7 in IFN-dependent gene induction. The results also suggest that IRF-3 is not effective in the induction of *IFN*-α genes in the absence of IRF-7 expression (see below).

It has been well documented that infection by a variety of viruses commonly results in *IFN*-α/β gene induction (reviewed in DeMaeyer and DeMaeyer-Guignard, 1988; Vilcek and Sen, 1996). Therefore, IFN-α/β mRNA induction was examined in DKO MEFs infected with three other different types of viruses, i.e., EMCV (picornaviridae), vesicular stomatitis virus (rhabdoviridae), and HSV

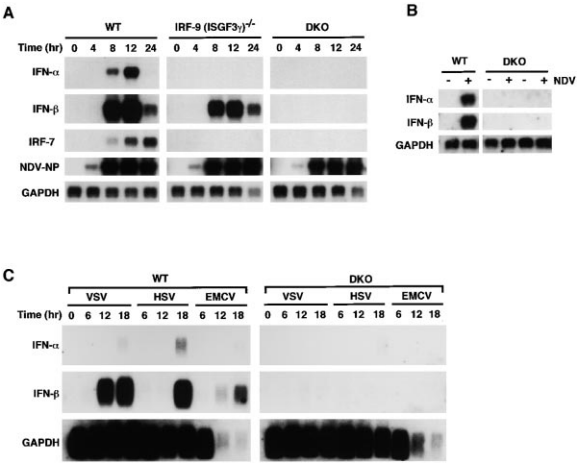

Figure 4. Induction of IFN-α and IFN-β mRNAs in IRF-3/(IRF-9/ISGF3γ) DKO Cells

(A) Induction of IFN-α/β mRNA by NDV infection in MEFs was analyzed by RNA blotting; time after NDV infection is indicated. NDV replication was confirmed by identification of the NDV-nucleocapsid protein (NDV-NP) mRNA expression. Five micrograms of total RNA was loaded onto each lane. For the blotting control, each membrane was reprobed with the GAPDH probe (A–C).

(B) Induction of IFN-α and IFN-β mRNAs in splenocytes. Splenocytes from wild-type (WT) and IRF-3/(IRF-9/ISFG3γ) DKO mice were infected with NDV for 8 hr. Total RNA (0.8 μg) was subjected to RNA blot analysis.

(C) Induction of IFN-α and IFN-β mRNAs by VSV, HSV, and EMCV infections. One microgram of poly(A) RNA was subjected to RNA blotting (lanes at 12 hr and 18 hr after EMCV infection, 0.1 μg of poly(A) RNA was loaded). RNA yield from EMCV-infected MEFs was quite low because of the inhibition of transcription by EMCV (Castrillo and Carrasco, 1986). All of the cells underwent virus-induced cell death (Tanaka et al., 1998) 24 hr after infection.

(herpesviridae). Interestingly, the induction of IFN-α/β mRNA expression by these viruses, which can be demonstrated in WT MEFs, is again not observed in the mutant cells (Figure 4C), indicating the general importance of IRF-3 and IRF-7 in regulating the two-phased *IFN*-α/β gene induction mechanism.

Restoration of *IFN*-α/β Gene Inducibility in DKO Cells by Ectopic Expressions of IRF-3 and/or IRF-7

The generation of DKO MEFs also provided an opportunity to examine the function of IRF-3 and IRF-7 in the absence of either factor in the induction of *IFN*-α/β genes. In these DKO MEFs, the *IFN*-β gene induction by NDV is restored by the retrovirus-mediated expression of IRF-3 (Figure 5A). It is noteworthy that the IRF-3 expression is associated with efficient IFN-β mRNA induction, whereas only a marginal induction was achieved for IFN-α mRNA, an observation consistent with the data shown in Figure 5A. On the other hand, IRF-7 expression resulted in the induction of both *IFN*-α and *IFN*-β genes (Figure 5A), indicating that IRF-7 can function in the absence of IRF-3 (see below). It is worth noting that a similar expression of IRF-1 had little, if any, effect on the gene induction in our assay system (data not shown), indicating further that IRF-1 contributes little, if any, to the virus-induced expression of *IFN*-α/β genes.

Although IRF-7 expression alone can prima facie con-

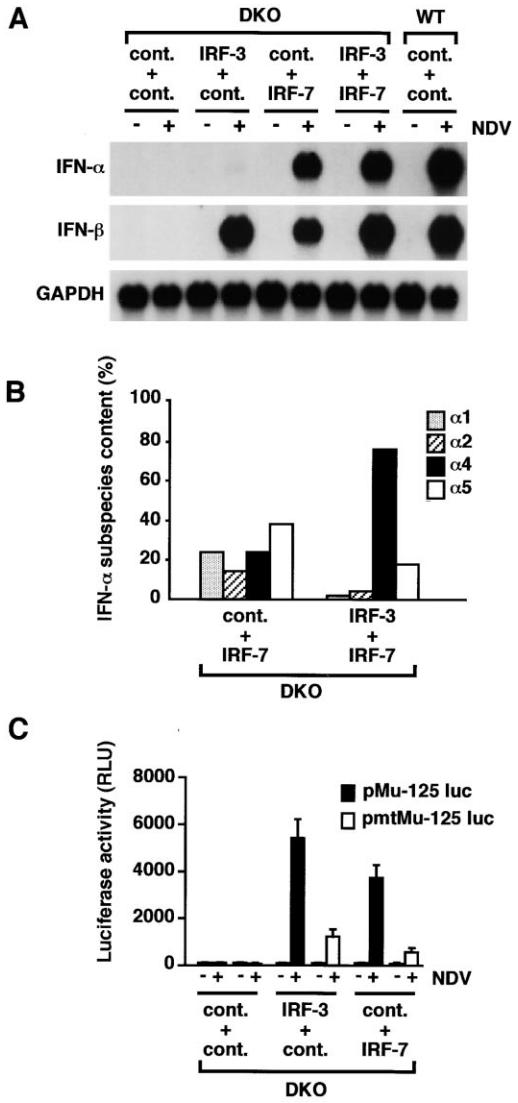

Figure 5. Effects of Retrovirus-Mediated Expression of IRF-3 and IRF-7 in IRF-3/(IRF-9/ISGF3γ) DKO MEFs

(A) The expression of IFN-α and IFN-β mRNAs in DKO MEFs expressing IRF-3 and/or IRF-7. The following retrovirus vectors were introduced into DKO or wild-type (WT) MEFs: pBabe-puro and pBabe-hygro (control + control), HA-IRF-3/pBabe and pBabe-hygro (IRF-3 + control), pBabe-puro and His-IRF-7/pBabe-hygro (control + IRF-7), and HA-IRF-3/pBabe and His-IRF-7/pBabe-hygro (IRF-3 + IRF-7). These MEFs were mock infected (−) or infected with NDV for 12 hr (+) and total RNA prepared. Five micrograms of total RNA was loaded onto each lane. Western blot analysis revealed that the level of ectopically expressed IRF-7 protein was approximately the same as that of the NDV-infected MEFs and that the IRF-3 level was also similar to that of WT MEFs (data not shown). Although IRF-1 has also been implicated in *IFN*-α /β gene induction (Miyamoto et al., 1988; Harada et al., 1990), it had no effect on *IFN*-α/β gene expression (N. H., unpublished observation). As control, the same membrane was reprobed with the GAPDH probe.

(B) Induction profile of the IFN-α mRNA subspecies. Total RNA from the indicated MEFs infected with NDV was subjected to RT–PCR. The obtained cDNA fragments were subcloned, and 50 clones from each of the MEFs were analyzed based on their sequences. Only one clone encoding IFN-α1 mRNA was detected in cells expressing both IRF-3 and IRF-7. (Note that in cells expressing only IRF-7, clones corresponding to IFN-α1, as well as IFN-α5, are dominant.)

fer *IFN*-α gene inducibility on DKO MEFs, an abnormality similar to that observed in *IRF-3*$^{-/-}$ MEFs (Figure 2B) was found in the induction spectrum for the IFN-α mRNA subspecies (Figure 5B). In fact, the preferential induction of IFN-α1 and -α5 mRNAs was again observed in cells expressing solely IRF-7 (Figure 5B). However, this skewing of the mRNA induction profile could be corrected by the concomitant expression of IRF-3, resulting in an mRNA induction spectrum similar to that of the WT cells. In fact, the induction of IFN-α4 mRNA is dominant in cells expressing both IRF-3 and IRF-7, as in the WT cells (Figures 2B and 5B). Taken together with the observation that IRF-3 is ineffective per se to cause a high-level IFN-α4 mRNA induction, it is likely that IRF-3 and IRF-7 must cooperate for the induction of the *IFN-α4* gene. It is also possible that such a cooperation occurs in *IFN*-β gene induction, when both factors are present at high levels (Figure 5A). Thus, a deficiency in the *IRF-9* gene in DKO MEFs can be fully rescued by the ectopic expression of IRF-7; IRF-7 may be the main or only target gene of the IFN-α/β-activated ISGF3, involving IRF-9/ISGF3γ, in terms of the transcriptional induction of *IFN*-α/β genes.

By transient expression of the cDNAs for IRF-3 or IRF-7, we found that both homo- and heteromeric complex formations occur even in the absence of viral infection and that these homo- and heteromeric complexes undergo phosphorylation upon viral infection (N. H., unpublished data). Although further studies are required for elucidating the detailed mechanism, these results indicate that IRF-3 and IRF-7 homomeric complexes have functions distinct from each other, in that the former is active in inducing the *IFN*-β gene but rather inert in the case of the *IFN*-α gene, while the latter preferentially acts on the *IFN-α1* and *-α5* genes. On the other hand, the IRF-3/IRF-7 heteromeric complex, the formation of which has been reported previously (Wathelet et al., 1998), is more critical for the IFN-dependent, late phase *IFN*-α/β gene induction to induce the *IFN*-β and *IFN-α4* genes more efficiently.

Activation of *IFN*-β Promoter in DKO Cells

In view of previous reports indicating that both IRF-3 and IRF-7 act on the virus-inducible *IFN*-β promoter (Au et al., 1998; Juang et al., 1998; Lin et al., 1998; Marie et al., 1998; Sato et al., 1998a; Schafer et al., 1998; Wathelet et al., 1998; Yoneyama et al., 1998), the *IFN*-β promoter activation was examined in these cells by transient expression of the promoter-driven luciferase reporter gene. As shown in Figure 5C, promoter activation no longer occurs in DKO MEFs, whereas it does occur in the cells in which IRF-3 or IRF-7 is ectopically expressed.

The dominant IFN-α subspecies found in cells expressing only IRF-3 (IRF-3 + control) was IFN-α4 (M.A., unpublished observation).

(C) *IFN*-β promoter activation by IRF-3 and IRF-7. The reporter plasmids, which contain the wild-type (pMu-125 luc) or mutant (pmtMu-125 luc) promoter sequence from the *IFN*-β gene, were transfected into the MEFs used in (A). The levels of luciferase activity in indicated MEFs with (+) or without (−) NDV infection were measured. The mean values of five independent samples and standard deviation are presented.

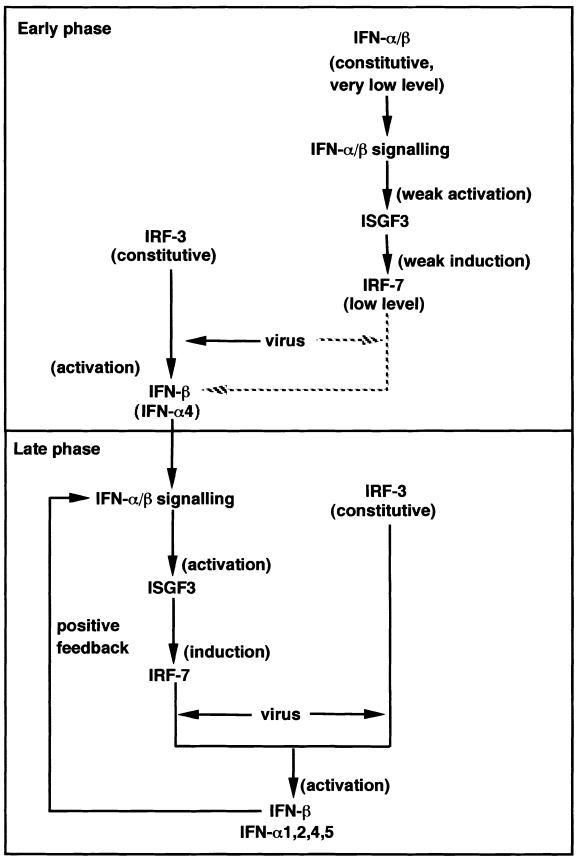

Figure 6. Schematic Representation of the Biphasic Mechanism for *IFN*-α/β Gene Induction, Mediated by IRF-3 and ISGF-3-Induced IRF-7

In the early phase, constitutively expressed IRF-3 is activated by virus infection, and this activation results in efficient and weak activations of *IFN*-β and *IFN*-α*4* genes, respectively. In this phase, IRF-7 is expressed only at very low levels in MEFs by spontaneous IFN-α/β signaling. This induction of IFN-α/β production results in a strong induction of IRF-7 expression through activation of ISGF3 by IFN-α/β signaling. Thus, in the late phase, IRF-3 and IRF-7 cooperate with each other for amplification of *IFN*-α/β gene induction, resulting in the full procurement of the normal mRNA induction profile of the *IFN*-α gene subfamily. It is worth noting that if cells are exposed to higher levels of IFNs prior to virus infection (dashed line), this positive feedback loop starts functioning more promptly and efficiently, even in the absence of IRF-3. Note that this figure is similar but distinct from results of previous reports (Marie et al., 1998; Sato et al., 1998b) in that it incorporates the data in the present work.

Furthermore, mutations introduced in one of the critical IRF binding sequences (i.e., PRDI) (Fujita et al., 1988) resulted in a significant decrease in the promoter activity (Figure 5C). These results suggest that the absence of endogenous *IFN*-β gene induction in mutant MEFs is, at least in part, due to the lack of *IFN*-β promoter activation by IRF-3 and/or IRF-7.

Biphasic Induction of *IFN*-α/β Genes by IRF-3 and IRF-7

Our present study indicates the essential role of IRF-3 in both early and late phases of *IFN*-α/β gene induction (Figure 6). In the early phase, which is mostly IFN independent, IRF-3 functions mainly for the *IFN*-β gene induction. On the other hand, in the late phase IRF-3 is also critical for two reasons: for the potentiation of the overall IFN-α/β mRNA induction and for full procurement of the normal mRNA induction profile for IFN-α subspecies by cooperating with IRF-7. Clearly, IRF-7 is more critical for the late induction phase. However, if the cells are exposed to IFNs prior to virus infection, IRF-7 levels will become upregulated, and it will also participate in the early induction phase. In fact, in the "primed" *IRF-3*$^{-/-}$ MEFs, the mRNA expression levels are not significantly different from those in WT MEFs, and *IFN*-α/β gene induction is more efficient if CHX is not present (M. S., unpublished data). It is interesting to note that the IFN-induced IRF-7 has a very short half-life (Figure 3). The significance of this finding is not totally clear at present, but one may speculate that this labile nature of IRF-7 may represent a mechanism critical in making the entire *IFN* gene induction process transient (see Figure 2A). Obviously, it is important and interesting to examine the phenotype of the mice deficient in the *IRF-7* gene, and work is in progress to generate the mutant mice.

One question that remains to be resolved is the detailed mechanism by which *IFN* genes are constitutively expressed. In fact, reverse transcriptase–PCR (RT–PCR) analysis revealed the expression of both IFN-α and IFN-β mRNAs even in DKO MEFs (M. A., unpublished observation). It is possible that the negative regulation of *IFN*-α/β genes, as proposed previously (Goodbourn et al., 1986; Civas et al., 1991; Nourbakhsh et al., 1993), may not cause complete gene repression, thereby allowing low-level, constitutive IFN production. Whatever the mechanism underlying the constitutive IFN production, the spontaneously produced IFNs may be biologically significant, since their level would have profound effect on the positive feedback loop of IFN production by altering the IRF-7 levels in the cell; that is, spontaneously produced IFNs would ensure a rapid and effective response of the host to effectively induce *IFN*-α/β genes upon virus infection. In fact, this mechanism may have evolved to allow rapid antiviral responses to a variety of viruses to afford a more efficient protection of a host.

Experimental Procedures

Generation of Mice with *IRF-3* Nullizygosity

To construct a targeting vector, a 13 kb genomic fragment was isolated from the C57BL/6N mouse genomic library (Clontech). The vector was designed to disrupt a 2.2 kb BstXI/BamHI region that contains a putative transcription initiation site and amino acids 1 to 35 of the mouse *IRF-3* gene (see Figure 1A). Fifty micrograms of linearized targeting vector was electroporated into CCE ES cells as described previously (Robertson, 1991). Clones that survived selection in 200 μg/ml G418 (GIBCO-BRL) were screened by PCR using the following oligonucleotide primers: IRF-3 sense, 5′-TGACT TCTCATCCTAACCCATGGG-3′ and *Neor* antisense, 5′-CCTGCTT GCCGAATATCATGGTG-3′. Three positive clones were obtained and the homologous recombination confirmed by Southern blot analysis using the 300 bp Sau3AI/HindIII fragment (see Figure 1A) as the probe. The positive clones were injected into C57BL/6J blastocysts. Chimeric male founder mice were crossed with C57BL/6J females to obtain heterozygous F1 offspring. The IRF-3-deficient mice were generated by interbreeding heterozygous mice.

Flow Cytometric Analysis

Cells were incubated on ice with monoclonal antibodies for 30 min and analyzed by flow cytometry (FACScalibur; Becton Dickinson) using CELLQuest software (Becton Dickinson). The monoclonal antibodies used in this study were as follows: anti-mouse CD3ε (fluorescein isothiocyanate [FITC] conjugated, clone 145-2C11), anti-mouse CD8 (phycoerythrin [PE] conjugated, clone 53-6.7), anti-mouse CD4 (FITC conjugated, clone GK1.5), anti-mouse B220 (PE conjugated, clone RA3-6B2), and anti-mouse DX5 (PE conjugated). All monoclonal antibodies were purchased from Pharmingen.

Antibodies and Immunoblot Analysis

Anti-IRF-3 antiserum was obtained from New Zealand white rabbits immunized with a synthetic oligopeptide corresponding to amino acids 147 to 161 of the mouse IRF-3. The antiserum was further purified by peptide affinity column chromatography. Immunoblot analysis was performed as described previously (Takaoka et al., 1999).

Cells and RNA Blot Analysis

MEFs were isolated and cultured as described previously (Harada et al., 1996). All of the experiments were carried out and repeated with several lines of MEFs. The MEFs used in this study were not passaged for more than five times. NDV infection of MEFs or splenocytes was performed at a concentration of 25 hemagglutinin units per 1×10^6 cells. HSV, VSV, and EMCV were allowed to adsorb onto the MEFs for 1 hr at a concentration of 0.1, 0.1, and 1.0 plaque-forming unit per cell, respectively. Total RNA isolation and RNA blotting analysis were performed as described previously (Harada et al., 1990, 1996; Sato et al., 1998b). Poly(A) RNA was prepared using Micro-FastTrack (Invitrogen). To prepare the probe, the following DNAs were labeled by the multiprime labeling reaction (TaKaRa). *IFN-α* is a 690 bp HindIII–EcoRI fragment from the mouse IFN-α genomic clone pBR327(HindIII)/chrMuIFN-α1/pGS3 (Shaw et al., 1983). This IFN-α probe detects multiple IFN-α subspecies. *IFN-β* is a 500 bp BamHI–BglII fragment from mouse IFN-β genomic clone pMGβ3-1 (T. Kuga and T. T., unpublished data). *2′-5′ OAS* is a 1.4 kb EcoRI fragment from pMA25 (Ichii et al., 1986). The 850 bp *NDV-NP* (GenBank AF060483) and 1.0 kb *GAPDH* cDNA probes were prepared by RT–PCR using NDV-NP primers (sense, 5′-ATGTCTTC CGTATTCGACGAGTACG-3′; antisense, 5′-GGCTACTAAGTGCAAG GGCTGATG-3′) and GAPDH primers (sense, 5′-ATGGTGAAGGTCG GTGTGAAC-3′; antisense, 5′-TTACTCCTTGGAGGCCATGTAG-3′).

Electorophoresis Mobility Shift Assay

Cell extracts were prepared as described previously (Harada et al., 1990). ^{32}P-labeled ISRE oligo DNA probe (10 fmol), 3 μg of herring sperm DNA, and 2 μg of poly(dG-dC):poly(dG-dC) were incubated with 15 μg of cell extract at 25°C for 30 min in a final volume of 15 μl of a buffer containing 20 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 50 mM NaCl, and 5% glycerol. The samples were resolved on 4.8% polyacrylamide gels at 4°C. The sequences of ISRE oligonucleotides used in the assay were 5′-AGCTTCTGAG GAAACGAAACCAACAG-3′ and 5′-GATCCTGTTGGTTTCGTTTCCT CAGA-3′.

Detection of Stabilities of IRF-3 and IRF-7 Proteins

HA-tagged mouse IRF-3 (HA-IRF-3) or IRF-7 (HA-IRF-7) cDNA was introduced into NIH 3T3 cells using retrovirus expression vectors and stable cell lines were obtained (Sato et al., 1998a, 1998b). These cells were plated on 60 mm diameter dishes and rinsed twice with methionine/cysteine-free Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL). The cells were overlaid with 1.5 ml of the same medium containing a mixture of 0.2 mCi of [^{35}S]methionine and [^{35}S]cysteine (Amersham, redivue Pro-mix L-[^{35}S] in vitro cell labeling mix). After 20 min of incubation, the cells were rinsed once with DMEM (Nissui) containing 10% FCS (Nippon Bio-Supply Center) and cultured in this medium. The preparation of the cell lysate and immunoprecipitation were performed as described previously (Takaoka et al., 1999).

Retrovirus Vectors and Infection

The retrovirus expression vectors HA-IRF-3/pBabe and HA-IRF-7/pBabe were described previously (Sato et al., 1998a, 1998b). The His-tagged IRF-3 and IRF-7 retrovirus expression vectors His-IRF-3/pBabe-hygro and His-IRF-7/pBabe-hygro were constructed as follows. To obtain the pBabe-hygro vector, the neomycin resistance gene of pBabe-puro was substituted with a 1.2 kb HindIII/BglII hygromycin resistance gene cassette from the pgk-hyg vector. After the ligation of IRF-3 or IRF-7 cDNA fragments into the BamHI site of pET15b (Novagen), His-tagged IRF-3 and IRF-7 cDNA fragments were prepared by EcoRV/XbaI digestion. Each fragment was cloned into the BamHI site of a pBabe-hygro. Retrovirus packaging and infection were performed as described previously (Sato et al., 1998b). To express two different retrovirus vectors, the MEFs were infected with HA-IRF-3/pBabe or pBabe-puro and selected in DMEM containing 3 μg/ml puromycin (Sigma) for 3 days. The MEFs were then subjected to a second infection with His-IRF-3/pBabe-hygro, His-IRF-7/pBabe-hygro, or pBabe-hygro and cultured for 3 days in DMEM containing 100 μg/ml of hygromycin (Sigma).

Transient Transfection Analysis

To construct the reporter plasmid pMu-125 luc, the murine *IFN-β* promoter region (−125 to +55) was amplified by PCR using sense 5′-AGCTTGAATAAAATGCTAGCTAGAAGCTGTTAGAA-3′ and antisense 5′-CAAGATGAGGCAAAGCTTCAAAGGCTGCAGTGAGAAT-3′ primers, and the resulting fragment was ligated into the NheI/HindIII gap in the Picagene luciferase reporter plasmid (Wako). The other reporter plasmid, in which two point mutations were introduced into IRF-E, was constructed as follows. Two synthetic oligonucleotides, sense 5′-GAATAAAATGAATATTAGAAGCTGTTAGAATAAGAGAAAA TGACAGAGGAAAACTGAAAGGGAGAACGGAAAGGGGGAAATTCC TCTGAGGCAG-3′ and antisense 5′-TGAGGCAAAGGCTGTCAAAG GCTGCAGTGAGAATGATCTTCCTTCATGGCCTGGTGCTATTTATA AGGGATGGTCCTTTCTGCCTCAGAGGAATTTCCCCCC-3′, were annealed, and the recessed 3′ termini were filled with Pyrobest DNA polymerase (TaKaRa). The resultant fragment was ligated into the Picagene vector as described above. DNA transfection and luciferase assays were performed as described previously (Sato et al., 1998a). Briefly, MEFs were seeded into wells of 24-well plates and transfected with 0.2 μg of the reporter plasmid (pMu-125 luc or pmtMu-125 luc). After 36 hr, NDV was allowed to adsorb onto the cells for 12 hr and then the levels of luciferase activity measured.

Quantification of IFN Activity

Virus in the culture medium was inactivated by incubation at pH 2 for 4 days at 4°C. L929 cells were incubated in serially diluted culture medium for 24 hr and subsequently challenged with VSV (MOI = 0.01) for 36 hr. The cells were then fixed with 10% formaldehyde and stained with crystal violet. The cytopathic effect was scored and the IFN titer was calculated. A reference mouse IFN-β (TORAY) was used as a standard for measuring IFN activity.

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References

Au, W.C., Moore, P.A., LaFleur, D.W., Tombal, B., and Pitha, P.M. (1998). Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. J. Biol. Chem. *273*, 29210–29217.

Castrillo, J.L., and Carrasco, L. (1986). The inhibition of nucleic acid synthesis in encephalomyocarditis virus-induced L929 cells: effects on nucleoside transport. Mol. Cell. Biochem. *71*, 53–60.

Chu, W.M., Ostertag, D., Li, Z.W., Chang, L., Chen, Y., Hu, Y., Williams, B., Perrault, J., and Karin, M. (1999). JNK2 and IKKβ are required for activating the innate response to viral infection. Immunity *11*, 721–731.

Civas, A., Dion, M., Vodjdani, G., and Doly, J. (1991). Repression of

the murine interferon alpha 11 gene: identification of negatively acting sequences. Nucleic Acids Res. *19*, 4497–4502.

DeMaeyer, E., and DeMaeyer-Guignard, J. (1988). Interferons and other regulatory cytokines (New York: John Wiley and Sons).

Du, W., and Maniatis, T. (1992). An ATF/CREB binding site protein is required for virus induction of the human interferon β gene. Proc. Natl. Acad. Sci. USA *89*, 2150–2154.

Du, W., Thanos, D., and Maniatis, T. (1993). Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. Cell *74*, 887–898.

Fan, C.-M., and Maniatis, T. (1989). Two different virus-inducible elements are required for human β-IFN gene regulation. EMBO J. *6*, 101–110.

Fujita, T., and Kohno, S. (1981). Studies on interferon priming: cellular response to viral and nonviral inducers and requirement of protein synthesis. Virology *112*, 62–69.

Fujita, T., Shibuya, H., Hotta, H., Yamanishi, K., and Taniguchi, T. (1987). Interferon-β gene regulation: tandemly repeated sequences of a synthetic 6 bp oligomer function as a virus-inducible enhancer. Cell *49*, 357–367.

Fujita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y., and Taniguchi, T. (1988). Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN-β gene regulatory elements. EMBO J. *7*, 3397–3405.

Fujita, T., Miyamoto, M., Kimura, Y., Hammer, J., and Taniguchi, T. (1989a). Involvement of a cis-element that binds an H2TF-1/NFκB like factor(s) in the virus-induced interferon-β gene expression. Nucleic Acids Res. *17*, 3335–3346.

Fujita, T., Kimura, Y., Miyamoto, M., Barsoumian, E.L., and Taniguchi, T. (1989b). Induction of endogenous IFN-α and IFN-β genes by a regulatory transcription factor, IRF-1. Nature *337*, 270–272.

Genin, P., Braganca, J., Darracq, N., Doly, J., and Civas, A. (1995). A novel PRD I and TG binding activity involved in virus-induced transcription of IFN-A genes. Nucleic Acids Res. *23*, 5055–5063.

Goodbourn, S., and Maniatis, T. (1988). Overlapping positive and negative regulatory domains of the human β-interferon gene. Proc. Natl. Acad. Sci. USA *85*, 1447–1451.

Goodbourn, S., Burstein, H., and Maniatis, T. (1986). The human beta-interferon gene enhancer is under negative control. Cell *45*, 601–610.

Harada, H., Willison, K., Sakakibara, J., Miyamoto, M., Fujita, T., and Taniguchi, T. (1990). Absence of the type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. Cell *63*, 303–312.

Harada, H., Matsumoto, M., Sato, M., Kashiwazaki, Y., Kimura, T., Kitagawa, M., Yokochi, T., Tan, R.S.-P., Takasugi, T., Kadokawa, Y., et al. (1996). Regulation of IFN-α/β genes: evidence for a dual function of the transcription factor complex ISGF3 in the production and action of IFN-α/β. Genes Cells *1*, 995–1005.

Ichii, Y., Fukunaga, R., Shiojiri, S., and Sokawa, Y. (1986). Mouse 2–5A synthetase cDNA: nucleotide sequence and comparison to human 2–5A synthetase. Nucleic Acids Res. *14*, 10117.

Juang, Y., Lowther, W., Kellum, M., Au, W.C., Lin, R., Hiscott, J., and Pitha, P.M. (1998). Primary activation of interferon A and interferon B gene transcription by interferon regulatory factor 3. Proc. Natl. Acad. Sci. USA *95*, 9837–9842.

Kawakami, T., Matsumoto, M., Sato, M., Harada, H., Taniguchi, T., and Kitagawa, M. (1995). Possible involvement of the transcription factor ISGF3 gamma in virus-induced expression of the IFN-beta gene. FEBS Lett. *358*, 225–229.

Kimura, T., Kadonaga, Y., Harada, H., Matsumoto, M., Sato, M., Kashiwazaki, Y., Tarutani, M., Tan, R.S.-P., Takasugi, T., Matsuyama, T., et al. (1996). Essential and non-redundant roles of p48 (ISGF3γ) and IRF-1 in both type I and type II interferon responses, as revealed by gene targeting studies. Genes Cells *1*, 115–124.

Leblanc, J.F., Cohen, L., Rodrigues, M., and Hiscott, J. (1990). Synergism between distinct enhanson domains in viral induction of the human beta interferon gene. Mol. Cell. Biol. *10*, 3987–3993.

Lenardo, M.J., Fan, C.-M., Maniatis, T., and Baltimore, D. (1989). The involvement of NF-κB in β-interferon gene regulation reveals its role as a widely inducible mediator of signal transduction. Cell *57*, 287–294.

Lin, R., Heylbroeck, C., Pitha, P.M., and Hiscott, J. (1998). Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Mol. Cell. Biol. *18*, 2986–2996.

Mamane, Y., Heylbroeck, C., Genin, P., Algarte, M., Servant, M.J., LePage, C., DeLuca, C., Kwon, H., Lin, R., and Hiscott, J. (1999). Interferon regulatory factors: the next generation. Gene *237*, 1–14.

Maniatis, T., Du Whittermore, L.A.W., Fan, C.M., Keller, A., Palmobella, V., and Thanos, D. (1992). Positive and negative control of human interferon-β gene expression. In Transcriptional Regulation, Part 2, S.L. McKnight and K.R. Yamamoto, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 1193–1220.

Marie, I., Durbin, J.E., and Levy, D.E. (1998). Differential viral induction of distinct interferon-α genes by positive feedback through interferon regulatory factor-7. EMBO J. *17*, 6660–6669.

Matsuyama, T., Kimura, T., Kitagawa, M., Pfeffer, K., Kawakami, T., Watanabe, N., Kundig, T.M., Amakawa, R., Kishihara, K., Wakeham, A., et al. (1993). Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development. Cell *75*, 83–97.

Miyamoto, M., Fujita, T., Kimura, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, T., and Taniguchi, T. (1988). Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. Cell *54*, 903–913.

Nguyen, H., Hiscott, J., and Pitha, P.M. (1997). The growing family of interferon regulatory factors. Cytokine Growth Factor Rev. *8*, 293–312.

Nourbakhsh, M., Hoffmann, K., and Hauser, H. (1993). Interferon-beta promoters contain a DNA element that acts as a position-independent silencer on the NF-kappa B site. EMBO J. *12*, 451–459.

Ogasawara, K., Hida, S., Azimi, N., Tagaya, Y., Sato, T., Yokochi-Fukuda, T., Waldmann, T.A., Taniguchi, T., and Taki, S. (1998). Requirement for IRF-1 in the microenvironment supporting development of natural killer cells. Nature *391*, 700–703.

Raj, N.B., Israeli, R., Kellum, M., and Pitha, P.M. (1989). Upstream regulatory elements of murine alpha 4-interferon gene confer inducibility and cell type-restricted expression. J. Biol. Chem. *264*, 11149–11157.

Rechsteiner, M., and Rogers, S.W. (1996). PEST sequences and regulation by proteolysis. Trends Biochem. Sci. *21*, 267–271.

Robertson, E.J. (1991). Using embryonic stem cells to introduce mutations into the mouse germ line. Biol. Reprod. *44*, 238–245.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science *234*, 364–368.

Ryals, J., Dierks, P., Ragg, H., and Weissmann, C. (1985). A 46-nucleotide promoter segment from an IFN-α gene renders an unrelated promoter inducible by virus. Cell *41*, 497–507.

Sato, M., Tanaka, N., Hata, N., Oda, E., and Taniguchi, T. (1998a). Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-β gene. FEBS Lett. *425*, 112–116.

Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998b). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. FEBS Lett. *441*, 106–110.

Schafer, S.L., Lin, R., Moore, P.A., Hiscott, J., and Pitha, P.M. (1998). Regulation of type I interferon gene expression by interferon regulatory factor-3. J. Biol. Chem. *273*, 2714–2720.

Shaw, G.D., Boll, W., Taira, H., Mantei, N., Lengyel, P., and Weissmann, C. (1983). Structure and expression of cloned murine IFN-α gene. Nucleic Acids Res. *11*, 555–573.

Stewart, W.E., II, Gosser, L.B., and Lockart, R.Z., Jr. (1971). Priming: a nonantiviral function of interferon. J. Virol. *7*, 792–801.

Takaoka, A., Tanaka, N., Mitani, Y., Miyazaki, T., Fujii, H., Sato, M., Kovarik, P., Decker, T., Schlessinger, J., and Taniguchi, T. (1999). Protein tyrosine kinase Pyk2 mediates the Jak-dependent activation

of MAPK and Stat1 in IFN-γ, but not IFN-α, signaling. EMBO J. *18*, 2480–2488.

Takaoka, A., Mitani, Y., Suemori, H., Sato, M., Yokochi, T., Noguchi, S., Tanaka, N., and Taniguchi, T. (2000). Crosstalk between interferon-γ and -α/β signaling components at caveolar membrane domain. Science *288*, 2357–2360.

Tanaka, N., Sato, M., Lamphier, M.S., Nozawa, H., Oda, E., Noguchi, S., Schreiber, R.D., Tsujimoto, Y., and Taniguchi, T. (1998). Type I interferons are essential mediators of apoptotic death in virally-infected cells. Genes Cells *3*, 29–37.

Taniguchi, T. (1988). Regulation of cytokine gene expression. Annu. Rev. Immunol. *6*, 439–464.

Taniguchi, T., Tanaka, N., Ogasawara, K., Taki, S., Sato, M., and Takaoka, A. (2000). The transcription factor IRF-1 and its family members in the regulation of host defense. Cold Spring Harb. Symp. Quant. Biol. *64*, 465–472.

Vilcek, J., and Sen, G.S. (1996). Interferons and other cytokines. In Fields Virology, Third Edition, D.M. Fields, P.M. Knipe, and P.M. Howley, eds. (Philadelphia: Lippincott-Raven), pp. 375–399.

Visvanathan, K.V., and Goodbourn, S. (1989). Double-stranded RNA activates binding of NF-κB to an inducible element in the human β-interferon promoter. EMBO J. *8*, 1129–1138.

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

Weissmann, C., and Weber, H. (1986). The interferon genes. Prog. Nucleic Acid Res. Mol. Biol. *33*, 251–300.

Yoneyama, M., Suhara, W., Fukuhara, Y., Sato, M., Ozato, K., and Fujita, T. (1996). Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). J. Biochem. *120*, 160–169.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., and Fujita, T. (1998). Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. EMBO J. *17*, 1087–1095.