FEBS Letters 425 (1998) 112–116 FEBS 19977

Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the *IFN-β* gene

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Received 17 February 1998

Abstract The virus-induced activation of *interferon α/β* (*IFN-α/β*) gene transcription is essential for host defense. The *IFN-β* promoter is controlled primarily by the virus-inducible enhancer elements, the IRF-Es. Here we show that IRF-3, an IRF family transcription factor, translocates to the nucleus from the cytoplasm upon virus infection in NIH/3T3 cells. The nuclear IRF-3 is phosphorylated, interacts with the co-activators CBP/p300, and binds specifically to the *IFN-β* IRF-E. Furthermore, overexpression of IRF-3 causes a marked increase in virus-induced *IFN-β* mRNA expression. Thus, IRF-3 is a candidate transcription factor mediating the activation of the *IFN-β* gene.

Key words: Interferon-β; Interferon regulatory factor; Interferon regulatory factor-3; Transcriptional regulation; IFN response; Protein phosphorylation

1. Introduction

The type I interferons (IFN-αs/β) are a structurally related family of cytokines which are produced in a variety of cells upon viral infection [1,2]. Previous studies have demonstrated that the IFNs have dual functions in antiviral responses: they confer cellular resistance against virus replication in uninfected cells [1,2], while inducing apoptosis in the infected cells [3]. Another class of IFN, the type II IFN (IFN-γ), which is structurally unrelated to type I IFNs, displays similar antiviral function. Expression of IFN-γ is not induced by virus and is restricted to distinct lymphocyte populations, e.g. mitogen-activated Th1-type T cells and interleukin-12-induced natural killer cells [4]. Thus, IFN-α/β primarily control the initial, innate immune response against viral infections, and IFN-γ is presumably more important in the regulation of the late, adaptive immune response [4].

The *IFN-α/β* genes are strictly under transcriptional control. Numerous studies have focused on the mechanisms by which the *IFN-β* gene is transcriptionally activated upon viral infection of the cells [1,2]. It is well established that several *cis*-acting DNA elements exist within the promoter region of the *IFN-β* gene, and that these elements bind transcription factors such as IRF-1, IRF-2, ISGF3, NF-κB, AFT-2, c-Jun and others [5–10]. In particular, much attention has been focused on the elements which function as virus-inducible enhancer elements, which we originally termed IRF-Es [11]. In fact, mutation in the IRF-Es results in a dramatic reduction of the virus inducibility of the *IFN-β* promoter [12]. These IRF-Es overlap with the PRDI and PRDIII elements identified by others [13,14] and show close similarity to the interferon-stimulated response element (ISRE) which is found in many of the IFN-inducible genes [15].

Several factors have been shown to bind specifically to the IRF-Es of the *IFN-β* promoter, which include the IRF family members of the transcription factors, IRF-1, IRF-2 and p48 (ISGF3γ) [6,7,16]. Gene targeting studies have revealed that the regulation of these elements is complex and controlled by multiple factors. Briefly, induction of the *IFN-α/β* genes is dependent on IRF-1 in cells induced by double-stranded RNA, poly(I):poly(C), but independent of this factor in cells infected with the Newcastle disease virus (NDV) [17,18]. On the other hand, gene induction is upregulated, albeit modestly, in cells lacking IRF-2, an observation consistent with the previous notion that IRF-2 functions as a repressor [16,17]. More recently, it has been shown that the virus-induced, full expression of *IFN-α/β* genes requires p48 in the context of ISGF3 (a heterotrimeric complex consisting of p48, Stat1 and Stat2 [15]), which is activated by the initially produced IFNs in virally infected cells [19]. Thus, ISGF3 functions as a mediator of the positive feedback regulation of the IFN system. Given these findings, it is obviously important to elucidate the nature of the factor(s) which initially triggers the induction of these *IFN* genes.

Several other IRF family members have been identified which include IRF-3, IRF-4 and ICSBP (IFN consensus sequence binding protein) [20–23]. Among them, IRF-3 is potentially an interesting candidate since it is constitutively expressed in many cell types and binds to ISRE [20]. However, little is known about the function of IRF-3 in virus-induced *IFN* gene expression.

In this paper, we studied the role of IRF-3 in the expression of the *IFN-β* gene in NIH/3T3 cells. We show that IRF-3 normally resides in the cytoplasm and it is translocated to the nucleus upon NDV infection of the cells. Furthermore, we provide evidence that this translocation is accompanied by phosphorylation of IRF-3, which is essential for specific binding to the *IFN-β*-derived IRF-E. Finally, we demonstrate the activation of the *IFN-β* promoter by IRF-3. These results suggest that IRF-3 plays a key role in the initiation of *IFN-β* gene induction in virally infected cells.

2. Materials and methods

2.1. Construction of expression vectors

HA-IRF-3/pBabe, the retrovirus expression vector for HA-tagged

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Abbreviations: IFN, interferon; IRF, IFN regulatory factor; IRF-E, IRF element; ISRE, IFN-stimulated response element; NDV, Newcastle disease virus; ISGF3, IFN-stimulated gene factor-3; ICSBP, IFN consensus sequence binding protein; CBP, CREB binding protein; EMSA, electrophoretic mobility shift assay

PII S0014-5793(98)00210-5

mouse IRF-3, was constructed as follows. Mouse IRF-3 cDNA was obtained by RT-PCR of the total RNA from mouse embryonic fibroblasts, cloned into the pCRII (Stratagene) vector (pIRF-3), and the nucleotide sequence of the cDNA was confirmed. Sense (5′-AGAGCATGGAAACCCCGAAAC-3′) and antisense primers (5′-TCAGATATTTCCAGTGGCCTG-3′) were used for the RT-PCR. The cDNA was excised by *Bam*HI and *Xba*I digestion and cloned with linker oligo nucleotides (5′-GGCCACGAGGTTCAG-3′ and 5′-GATCCTGAACCTCGT-3′) into the *Not*I and *Xba*I sites of pEF/HA vector which has the EF (elongation factor) promoter followed by a sequence encoding 10 amino acids of influenza virus hemagglutinin peptide (HA). The sequence of the linker DNA was confirmed and the *Eco*RI fragment of HA-IRF-3 cDNA was cloned into the retrovirus vector, pBabe-puro [24]. The *Kpn*I-*Xba*I fragment from pIRF-3 was cloned into the *Hin*dIII-*Xba*I site of pActC [16] to make pAct3. Part of the DNA binding region (from the *Sac*II site to the *Bam*HI site, amino acids 7–36) was deleted in pAct3dN.

2.2. Cell culture and retrovirus infection

NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Transfection of HA-IRF-3/pBabe or control pBabe-puro into Bosc23 cells and infection of the retroviruses were performed as described [25]. 72 h post infection, the retrovirus-infected NIH/3T3 cells were cultured in the presence of 2.5 μg/ml of puromycin to select the cells expressing the viral genes.

2.3. Immunofluorescent staining and immunoblotting analysis

The staining assay was carried out as described previously [26]. Briefly, 4×10^4 NIH/3T3 cells expressing HA-IRF-3 (3T3/IRF-3) were cultured on a chamber slide. After 12 h of NDV infection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. HA-IRF-3 was detected with mouse monoclonal anti-HA antibody (1 μg/ml; clone 12CA5, Boehringer Mannheim) and FITC-conjugated anti-mouse IgG antibody.

Immunoblotting analysis was done with anti-HA antibody (0.8 μg/ml) and horseradish peroxidase-linked anti-mouse IgG antibody (Amersham) according to the published procedure [26]. The signals were detected by enhanced chemiluminescence (Renaissance, NEN Life Science Products).

2.4. EMSA (electrophoretic mobility shift assay)

Preparation of nuclear extracts and EMSA were performed as described [6,19,27]. Binding reaction was incubated for 30 min at 25°C in the presence of 0.35 μg of salmon sperm DNA and 0.2 μg of poly(dI):poly(dC). For some samples, the reaction mixture was preincubated with antibodies at 4°C for 1 h. Anti-human IL-2 receptor γ-chain antibody was used as a control antibody.

2.5. RNA blotting

The RNA blotting method and the probe DNAs for *IFN-β* and *β-actin* have been described [16]. The *Hin*dIII fragment from pIRF-3 was used as IRF-3 probe. Specific activity of each probe was around 1×10^9 cpm/μg.

2.6. Luciferase assay

Two micrograms of reporter plasmid were co-transfected with expression vectors (pAct3 or pAct3dN) and 2 mg of DEAE-dextran into 2×10^5 NIH/3T3 cells. The total amount of expression vector was adjusted to 2.0 μg with pActC. Cells were infected by NDV after 48 h of transfection and harvested 18 h after infection. The luciferase assay was carried out as described previously [19].

3. Results

3.1. Translocation of IRF-3 from the cytoplasm to the nucleus upon NDV infection

We first constructed a retrovirus expressing an HA-tagged mouse IRF-3 to readily monitor the behavior of IRF-3 during the course of NDV infection. The retrovirus was infected into NIH/3T3 cells (referred to as 3T3/IRF-3 cells thereafter). We first examined the localization of the tagged IRF-3 in 3T3/IRF-3 cells before and after NDV infection by immunofluorescence staining. As shown in Fig. 1A, the HA-tagged IRF-3 protein is localized in the cytoplasm in the absence of virus infection. Upon NDV infection, the protein was found to be accumulated in the nucleus (Fig. 1A). To determine the kinetics of this nuclear translocation after NDV infection, we prepared nuclear extracts from 3T3/IRF-3 cells before and after NDV infection, and performed immunoblotting analysis using anti-HA antibody. As shown in Fig. 1B, the amount of IRF-3 protein in the nuclear fraction is dramatically increased 12 h after NDV infection, at which time the *IFN-β* mRNA starts accumulating (see below).

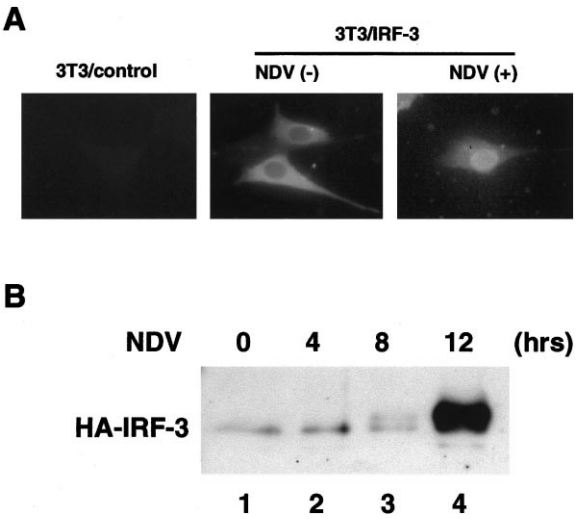

Fig. 1. A: Localization of IRF-3 before and after NDV infection. HA-tagged mouse IRF-3 was detected with an anti-HA monoclonal antibody and visualized by FITC-conjugated anti-mouse IgG antibody. The picture shows some representative cells. B: IRF-3 is translocated to the nucleus during the course of NDV infection. Extracts from the same number of nuclei at indicated times after NDV infection were loaded on 10% SDS-polyacrylamide.

3.2. Specific binding of the nuclear IRF-3 from NDV-infected cells to the IFN-β-derived IRF-E

Next, we tested the binding of IRF-3 in the nuclear fraction of the NDV-infected 3T3/IRF-3 cells to the IRF-E of the *IFN-β* promoter. The nuclear extracts were prepared from the cells expressing HA-IRF-3 12 h after NDV infection or mock infection, and subjected to EMSA using IRF-E from the mouse *IFN-β* gene as a probe. As shown in Fig. 2, a shifted band is detected in the nuclear extract of the NDV-infected cells, but not in mock-infected cells (lanes 1, 2). Importantly, this band was super-shifted by incubation with anti-HA antibody (lane 3), suggesting that it is the HA-tagged IRF-3 which participates in the formation of this complex in response to NDV infection. This virus-inducible factor is presumably complexed to other protein(s), hence tentatively termed V-IRF-3 thereafter (see below). Next, we tested the binding specificity of V-IRF-3 to the *IFN-β*-derived IRF-E by performing competition EMSA. As shown in Fig. 2, the shifted band is no longer detectable when incubated with an excess of unlabeled IRF-E probe or ISRE sequence from the *ISG15* promoter (lanes 6, 9). On the other hand, this competition was not observed with the same amount of unlabeled DNA containing κB or GAS sequence (lanes 8, 10). These results suggest that V-IRF-3 binds to the *IFN-β*-derived IRF-E in a sequence-specific manner. In addition, the IRF-

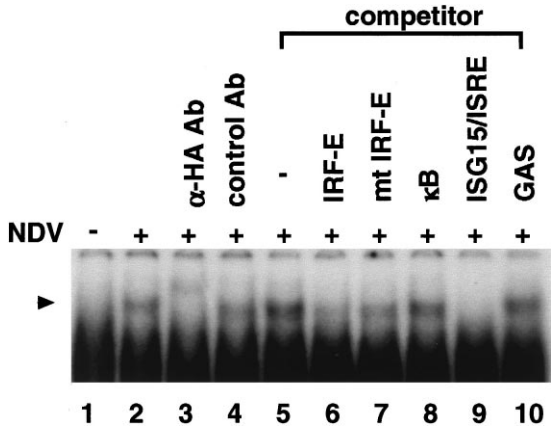

Fig. 2. DNA binding activity of IRF-3 to IRF-E. Nuclear extracts containing 5 μg of protein were used for each binding reaction. Labeled oligonucleotide containing the IRF-E sequence from the mouse *IFN-β* promoter was used as a probe. The 3T3/IRF-3 cells were infected with NDV for 12 h or mock-infected and nuclear extracts were prepared. 0.4 μg of antibody was incubated with extract before the binding reaction (lanes 3, 4). In the competition assay, 100 times molar excess of unlabeled oligonucleotides was included in the binding reaction (lanes 5–10).

E containing two base substitutions within the PRDI region showed a markedly reduced ability to compete with the native sequence for binding to V-IRF-3 (lane 7). It has been shown previously that introduction of these mutations in the *IFN-β* promoter results in a dramatic reduction of the promoter activity [12], an observation consistent with the notion that the binding of V-IRF-3 is critical for virus-induced promoter activation.

3.3. Phosphorylation of IRF-3 and its interaction with the co-activator CBP/p300

Previously, it has been demonstrated that *IFN-β* gene induction by virus requires post-transcriptional events including protein phosphorylation [19]. In this context, the nuclear IRF-3 protein from NDV-infected 3T3/IRF-3 cells was found to migrate more slowly than IRF-3 found in the cytoplasm in SDS-PAGE analysis (Fig. 3A, lanes 1–3), suggesting a modification(s) of IRF-3 in the infected cell nucleus. To test whether or not this modification involves phosphorylation, the nuclear extract from NDV-infected cells was incubated with calf intestinal alkaline phosphatase (CIAP) and the subjected to immunoblotting and EMSA. As shown in Fig. 3A (lane 4), the slowly migrating signal disappeared and the intensity of the fast migrating signal, which is found in the same position as cytoplasmic IRF-3, was significantly increased after CIAP treatment. Moreover, the V-IRF-3 binding activity was not detected after CIAP treatment of the nuclear extract (Fig. 3B, lane 3). These results suggest that IRF-3 is phosphorylated after virus infection and its phosphorylation is essential for the V-IRF-3 binding to IRF-E.

Recently, it was shown that the *IFN-β* promoter activation involves CBP/p300, co-activators of transcription which interact with a variety of transcription factors [28]. We examined whether or not CBP is involved in the formation of V-IRF-3. As shown in Fig. 3C, EMSA revealed that the V-IRF-3/DNA complex was partially reacted with anti-pCBP antibody (clone A-22, Santa Cruz) (lane 2) and the band was eliminated completely with another antibody, which reacts with CBP and p300 (clone 451, Santa Cruz) (lane 4). The result therefore suggests the involvement of CBP/p300 in the formation of V-IRF-3.

3.4. Effect of IRF-3 on IFN-β gene induction by virus infection

The above observations prompted us to investigate the effect of IRF-3 overexpression on the induction of the endogenous *IFN-β* gene by RNA blotting analysis. The 3T3/IRF-3 cells were infected with NDV, and total RNAs were prepared at each time point. Total RNAs were similarly prepared from cells infected by a control retrovirus (referred to as 3T3/control cells). As shown in Fig. 4A, the endogenous *IRF-3* gene is constitutively expressed and it is induced by NDV in both 3T3/IRF-3 and 3T3/control cells. In addition, in the 3T3/IRF-3 cells, the level of HA-IRF-3 mRNA is approximately 12 times higher than that of endogenous *IRF-3* gene. *IFN-β* mRNA induction is detectable in both 3T3/IRF-3 and 3T3/control cells 12 h after virus infection (Fig. 4B), at which nuclear translocation of IRF-3 is also detectable (Fig. 1B). Notably, *IFN-β* mRNA induction levels are much higher in 3T3/IRF-3 cells during the course of infection. In fact, the mRNA level is about 5-fold higher compared to that of 3T3/control cells at 16 h after infection (Fig. 4B). These results strongly suggest that IRF-3 is involved in the activation of *IFN-β* gene transcription. Similar results were also obtained for the *IFN-α* genes, suggesting that IRF-3 also acts on these genes (data not shown). To investigate further the role of IRF-3 in the activation of the *IFN-β* promoter, we employed a transient assay using a reporter gene consisting of the *IFN-β* promoter (containing 125 bp upstream region [12]) and luci-

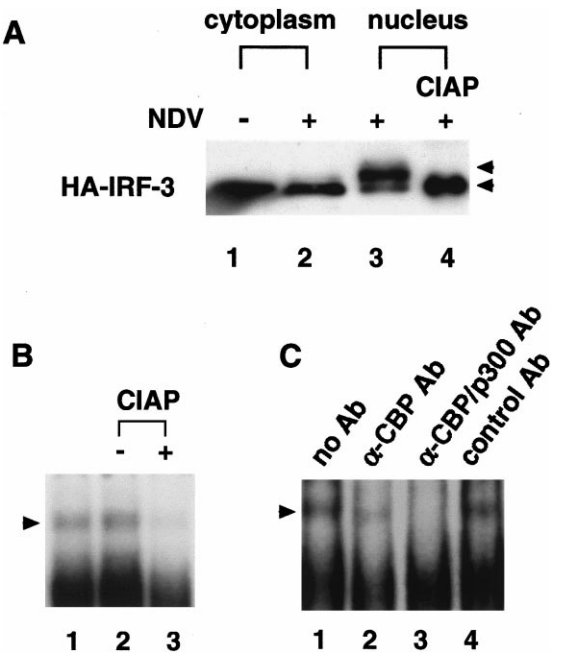

Fig. 3. Effect of CIAP treatment and anti-CBP or CBP/p300 antibodies. A: Western blotting. Nuclear extracts containing 2 μg of protein from NDV-infected cells were treated with 38 units of CIAP. Detection of HA-IRF-3 was done as in Fig. 1B. B: Nuclear extracts from NDV-infected cells were treated with or without CIAP. EMSA was performed using the IRF-E probe as in Fig. 2. One unit of CIAP was used for the treatment of 1 μg protein of nuclear extract. Lane 1: no treatment; lane 2: mock treatment; lane 3: CIAP treatment. C: The nuclear extracts were mixed with 0.4 μg of anti-CBP or CBP/p300 antibodies and subjected to EMSA.

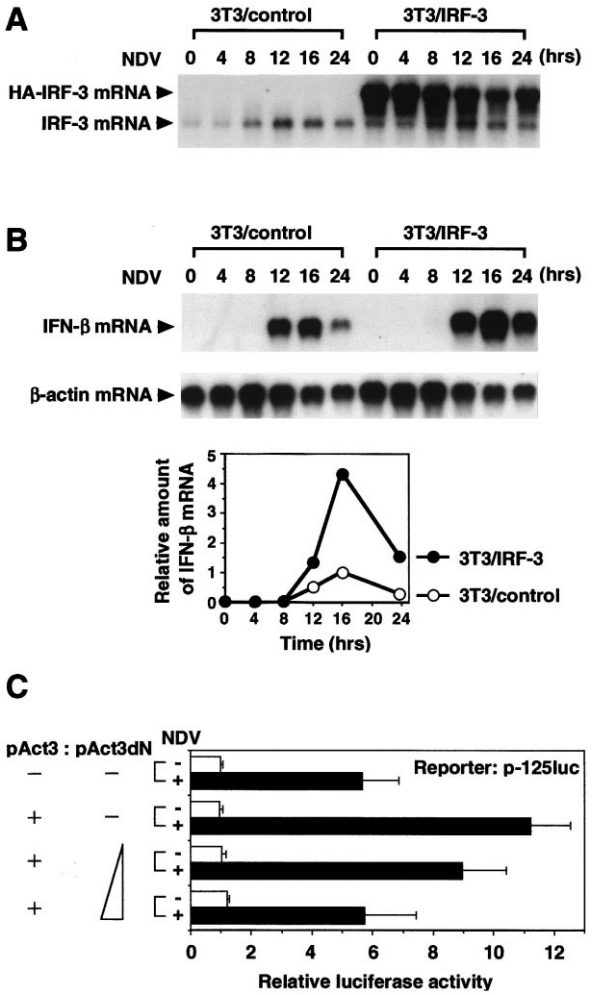

Fig. 4. A: Expression of IRF-3 mRNA. 3T3/control or 3T3/IRF-3 cells were treated with NDV. Total RNA was prepared at each time point indicated and subjected to Northern blotting analysis. B: Effect of IRF-3 overexpression on *IFN-β* mRNA induction. The same RNA was loaded as in A. The mRNA levels were measured using the imaging analyzer (Fujix BAS5000) and the level of *IFN-β* mRNA was normalized by taking the ratio to that of the β-*actin* mRNA. The obtained ratio at the peak point of 3T3/control cells was taken to be 1 and plotted in the graph. C: IRF-3 enhances the luciferase activity driven by the *IFN-β* promoter region. Cell lysates were prepared after 18 h of NDV infection. Protein concentrations of the lysates were adjusted and used for the luciferase assay. In co-expression experiments, 0.02 μg of pAct3 was co-transfected with 0.2 or 2.0 μg of pAct3dN. The histogram shows the mean of two independent experiments, and error bars show standard deviations.

ferase gene (p-125luc). As shown in Fig. 4C, luciferase activity was significantly augmented when p-125luc was co-transfected with an IRF-3 expression vector. Furthermore, this activation was suppressed by co-expressing a cDNA for the inactive form of IRF-3 which lacked a part of the DNA binding domain.

4. Discussion

Virus-induced expression of IFN-α/β is one of the essential aspects of the innate immune response [29]. In fact, the IFNs confer resistance against a wide variety of viruses in uninfected cells [1,2]. In addition, it has been shown recently that IFN-α/β selectively induce apoptosis in virally infected cells [3], revealing a novel antiviral function of these cytokines. The expression of the *IFN-α/β* genes is tightly regulated at the transcriptional level, and virus infection of the cells results in prompt induction of gene transcription. Among many regulatory *cis*-elements found in the promoter of these genes, one element which is of particular interest is IRF-E (PRD-I, PRD-III [13,14]), which is commonly found in the *IFN-α* and *-β* promoters. In this regard, we and others have shown that ISGF3, which is activated by IFN signaling, is a crucial regulator of IRF-E [7,19]. In fact, ISGF3 was found to bind to IRF-E, and significant reduction of NDV-induced *IFN-*α/β mRNA expression was observed in mouse embryonic fibroblasts (EFs) deficient in the *p48*, *Stat1*, or *IFN receptor* genes [6,19]. These results suggest the operation of a positive feedback mechanism, in which ISGF3 is involved in the late phase of gene induction followed by the initial phase of IFN production by an as yet unknown mechanism(s) [19].

In the present study, we have examined the role of IRF-3, an IRF family member which is constitutively expressed in a variety of cell types in the regulation of *IFN-β* gene transcription, by expressing an epitope-tagged IRF-3 (HA-IRF-3) in NIH/3T3 cells. Our results demonstrate that IRF-3 undergoes translocation from cytoplasm to nucleus upon virus infection. Concomitantly with this translocation, *IFN-β* mRNA induction was observed (Figs. 1B and 4B), suggesting a functional role for IRF-3 in *IFN-β* gene transcription. Although NDV induction of *IFN-α* genes is much lower in 3T3/control cells, it is significantly augmented in 3T3/IRF-3 cells (data not shown), suggesting that IRF-3 may also be involved in *IFN-α* regulation.

Unlike the cytoplasmic IRF-3, the virus-induced nuclear IRF-3 is phosphorylated, and appears to interact with the co-activators CBP/p300 (Fig. 3A,C). This nuclear IRF-3, termed V-IRF-3, specifically binds to IRF-E, and it is possible that other factors may also be involved in the formation of the V-IRF-3 complex. Our result that a mutated IRF-E which has lost its function in the context of the *IFN-β* promoter cannot bind V-IRF-3 suggests the critical role of V-IRF-3. In fact, overexpression of IRF-3 in NIH/3T3 cells results in a marked upregulation of *IFN-β* mRNA induction (Fig. 4B). Furthermore, the virus-induced activation of the *IFN-β* promoter is enhanced by the expression of IRF-3 in the transient co-transfection assay, and this enhancement is suppressed by co-expression of the mutant IRF-3 lacking a part of the DNA binding domain (Fig. 4C). We infer that this mutant is functioning as a dominant negative IRF-3 by squelching the critical co-factor(s). During the course of this study, it was reported that a GST-fused IRF-3 protein can bind to the *IFN-α4* promoter and PRDIII of the *IFN-β* promoter, and that IRF-3 cooperates with RelA(p65) [30]. These results are consistent with ours, and one may speculate that the promoter recruits many factors such as IRF-3, CBP/p300, NF-κB and others, so as to form an enhanceosome [28] to ensure efficient activation of this gene.

In conclusion, our present data may reveal a mechanism of the virus-induced activation of *IFN-β* gene transcription, in which IRF-3 interacts with and activates the promoter, through nuclear translocation, phosphorylation and interaction with CBP/p300. Thus, IRF-3 may be the long-thought transcription factor regulating the initial phase of *IFN* gene transcription. Further work is in progress to characterize further the nature of V-IRF-3, to identify the candidate protein

kinase for IRF-3, as well as to generate mice deficient in the *IRF-3* gene to elucidate further the contribution of this factor in the host defense.

Acknowledgements: We thank Dr. Edward L. Barsoumian for critical reading of the manuscript. This work was supported in part by the PFTF Program from JSPS, and by a special grant for Advanced Research on Cancer (to T.T.) from the Ministry of Education, Science and Culture of Japan. M.S. is a JSPS Research Fellow.

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