Possible involvement of the transcription factor ISGF3γ in virus-induced expression of the IFN-β gene

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Abstract Two virus-inducible transcription factors, IRF-1 and IRF-2 have been identified as an activator and a repressor, respectively, of the type I interferon (IFN) genes. Recent studies with mice carrying null mutations for the IRF-1 or IRF-2 alleles have revealed the existence of IRF-1-dependent and -independent pathways mediating IFN-β gene induction. Here we report that the expression of an IRF family member ISGF3γ is induced upon viral infection in IRF-1−/−, IRF-2−/− embryonic fibroblasts. Furthermore, ISGF3γ can bind to a virus-inducible promoter element in the IFN-β gene. These results suggest that ISGF3γ or complex containing ISGF3γ is involved in the IRF-1-independent pathway mediating IFN-β gene regulation.

Key words: Interferon β; Transcription factor; Viral infection; ISGF3; IRF

1. Introduction

Interferons (IFNs) are pleiotropic cytokines that possess several biological activities, such as anti-viral and anti-proliferative actions, on a variety of target cells [1,2]. Expression of the genes for type I (IFN-α, -β) and type II (IFN-γ) IFN is regulated primarily at the transcriptional level [3–5]. Type I IFN genes are efficiently induced in a variety of cell types upon viral infection or treatment by double-stranded RNA, whereas the expression of type II IFN, i.e. IFN-γ is restricted to mitogen-activated T lymphocytes [1,2].

During the course of our study on the regulation of the IFN-β gene expression, two transcription factors, termed IRF-1 (IFN regulatory factor-1) and IRF-2 were isolated [6,7]. The IRF-1 and IRF-2 genes are induced by virus and IFNs [7]. IRF-1 and IRF-2 each binds to the same DNA sequence elements (IRF-Es) found within the virus-inducible promoters of the type I IFN genes and the ISREs (IFN stimulated response elements) of IFN-inducible genes [7,8]. A series of transfection studies showed that IRF-1 can activate type I IFN and IFN-inducible genes and that IRF-2 represses the action of IRF-1 [7,9–11].

As an approach to study further the role of IRFs in the regulation of the IFN system, we generated mice which lack functional IRF-1 and/or IRF-2 using gene disruption technology [12–14]. We have shown that induction of IFN-β mRNA by double-stranded RNA, poly(rI):poly(rC), is markedly reduced in embryonic fibroblasts (EFs) from IRF-1-deficient mice, as compared to EFs from wild type animals. On the other hand, IFN-β mRNA expression levels were similar in mutant and wild-type EFs following induction by Newcastle disease virus (NDV). These results indicate the existence of IRF-1-dependent and -independent pathways of IFN-β gene induction [12]. Thus, in addition to IRF-1, other transcription factor(s) must be acting upon the virus-inducible elements.

Besides IRF-1 and IRF-2, two other factors, ISGF3γ (IFN stimulated gene factor 3γ) and ICSBP (IFN consensus sequence binding protein) have been found whose amino terminal sequences are highly homologous to those found in the amino terminal regions of the IRFs. This region has been shown to be the DNA binding domain [15,16], and these factors have been designated as members of the 'IRF-family' [16]. ISGF3γ was found to be the DNA binding subunit of a trimeric complex containing two other proteins, STAT1 and STAT2. This complex, termed ISGF3, is found upon cellular stimulation by type I IFNs. ISGF3 in turn participates in the transcriptional activation of IFN-inducible genes by binding to the ISRE [17]. ICSBP also binds to similar DNA sequence elements, however, the expression of ICSBP is restricted to cells of lymphoid and macrophage lineages [15].

In the present study, we report our findings on the potential role of ISGF3γ in IFN-β gene regulation. First, we demonstrate that the expression of ISGF3γ mRNA is induced by NDV and that this induction correlates with the induction of IFN-β mRNA in cells which lack both IRF-1 and IRF-2. We also show that ISGF3γ by itself binds to the virus-inducible element of the IFN-β promoter, although the binding of the larger ISGF3 complex to the element is weak, if occurring at all. Furthermore, the binding affinity of ISGF3γ is decreased when mutations which cause reduced NDV inducibility are introduced into the IFN-β promoter sequences. These results suggest that ISGF3γ participates in the regulation of the transcription of the IFN-β gene in response to viral infection.

2. Materials and methods

2.1. Cell culture

The IRF-1−/− and IRF-2−/− mice used in this study have been previously described [12]. The method of isolation and culture of mouse

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Abbreviations: IFN, interferon; IRF, IFN regulatory factor; IRF-E, IRF-element; ISRE, IFN stimulated response element; EFs, embryonic fibroblasts; NDV, Newcastle disease virus; ISGF3, IFN stimulated gene factor 3; ICSBP, IFN consensus sequence binding protein; dKO, double knock-out; PCR polymerase chain reaction; EMSA, electrophoretic mobility shift assay; CHX, cycloheximide; RRL, rabbit reticulocyte lysate.

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primary EFs were as described [13,14]. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. NDV infection was performed as previously [18].

2.2. Preparation and screening of cDNA library

Total RNA was prepared from EFs derived from mice deficient for both IRF-1 and IRF-2 genes (IRF-dKO EFs) [13,14] 8 hours after NDV infection. Poly(A)$^+$ RNA was purified using mRNA Purification Kit (Pharmacia). cDNA was synthesized from the poly(A)$^+$ RNA using random hexanucleotides as primers. To prepare the cDNA library, the cDNA was cloned into the λgt11 vector (Stratagene). To clone the murine ISGF3γ cDNA, the library was screened using a ^{32}P-labeled DNA probe containing a part of the sequence of murine ISGF3γ obtained by the polymerase chain reaction (see below).

2.3. Polymerase chain reaction (PCR) and sequencing

The cDNA mentioned above was used as the template. Primers used were as follows; sense primer, ATICCITGGAA(A/G)CA(T/C)GC, antisense primer, GCACAICGTA(A/G)IC(G/T)IGT(T/C)TTCCA PCR was performed for 30 cycles with the conditions; 94°C 30 s (denaturation), 37°C 60 s (annealing), 72°C 60 s (extension).

PCR products were subcloned into the pCRII vector (Invitrogen) and sequenced via a dideoxy method (Sequenase, USB).

2.4. RNA blotting analysis

The procedure for RNA blotting analysis was as previously described [10]. The probe for the IFN-β mRNA was prepared as described [6]. The probe for the murine ISGF3γ mRNA was prepared from a 165 bp *Eco*RI fragment of the pCRII vector containing the ISGF3γ PCR product. The probes were labeled with [α-^{32}P]dCTP by the random prime method (Amersham).

2.5. Electrophoretic gel mobility shift analysis (EMSA)

The binding reaction for EMSA was performed as previously described [10]. The samples were then resolved on 4.8% polyacrylamide gels run in TBE buffer [19].

The double-stranded oligonucleotide probes used in the assays were as follows,

IFN-β (−95 to −54 [18]);

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GACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTCT
    ATCCTTTTGACTTTCCCTCTTCACTTTCACCCTTTAAGGAGACTTA
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IFN-β (−95 to −63 [18]);

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GACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGG
    ATCCTTTTGACTTTCCCTCTTCACTTTCACCCTTTA
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ISRE (from human 2′-5′ oligoA synthetase; −104 to −83 [20]);

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AGCTTCTGAGGAAACGAAACCAACAG
    AGACTCCTTTGCTTTGGTTGTCCTAG
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Probes were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Protein sources were prepared as follows. The full-length human ISGF3γ cDNA (clone 38–1) cloned into pBluescript SK(+) [16] was transcribed using T7 RNA polymerase (Stratagene). The full-length human IRF-1 cRNA was prepared as described [21]. The RNAs were subjected to in vitro translation with rabbit reticulocyte lysate (Amersham). The procedure for preparing whole cell extracts was as described [10].

Anti-ISGF3γ serum was as described [16]. Anti-IRF-1 and anti-IRF-2 sera were as described [11]

3. Results

3.1. Correlation of ISGF3γ and IFN-β mRNA expression upon viral infection

As an approach to identify a factor(s) involved in the IRF-1-independent induction of IFN-β gene expression, we utilized cells from mice lacking the expression of both IRFs [13,14], thus allowing us to search for a factor(s) that is active in the absence of IRF-1 and IRF-2. The IRF-dKO EFs expressed IFN-β mRNA upon NDV infection (see Fig. 2). The induction kinetics, as well as the expression level of the IFN-β mRNA in the IRF-dKO EFs was indistinguishable from those of IRF-1-deficient EFs from a littermate animal (data not shown), indicating that a similar IRF-1-independent pathway is at work in both the IRF-dKO EFs and IRF-1-deficient EFs.

We initially hypothesized that the putative factor(s) involved in the IRF-1-independent pathway may belong to the IRF-family, and that such a factor would compensate for the action of IRF-1 on the IRF-Es in the IFN-β gene promoter. We attempted to clone this candidate factor(s) by PCR amplification of cDNA prepared from IRF-dKO EFs during the peak of IFN-β gene expression following NDV infection. For primers, we used a set of degenerate synthetic oligonucleotides whose sequence was inferred from the amino acid sequences of the conserved IRF-family DNA binding domain [16] (see section 2). The PCR produced a single fragment of the size predicted from the known IRF-family members. The products were subcloned into an appropriate vector and sequenced. The nucleotide sequence of all 33 clones analyzed was identical, and the deduced amino acid sequence resembled that of human ISGF3γ. To confirm that these PCR products were derived from the murine homologue of ISGF3γ, we cloned the 5′ upstream nucleotide sequences by screening a cDNA library with the PCR product as a probe. The predicted amino acid sequences of these upstream sequences were again found to be homologous to human ISGF3γ (Fig. 1). In fact, a 90% homology in amino acids 1–87 exists between murine and human ISGF3γ. These observations indicate that the clones obtained by PCR did indeed correspond to murine ISGF3γ and raised the possibility that ISGF3γ, which is known to function in the regulation of IFN-inducible genes, may also contribute to the regulation of the IFN-β gene.

To examine this possibility, we first analyzed the expression of the ISGF3γ and IFN-β mRNAs in IRF-dKO EFs during the course of viral infection by RNA blotting analysis. As shown in Fig. 2A, the expression of ISGF3γ mRNA was increased after NDV infection. This increase in ISGF3γ mRNA expression preceded the induction of the IFN-β mRNA. The induction levels of the ISGF3γ mRNA after NDV infection were essentially the same in wild type and IRF-1-deficient EFs (data not shown). As shown in Fig. 2B, ISGF3γ mRNA was induced by NDV infection even in the presence of a protein synthesis inhibitor, cycloheximide (CHX). This result indicates that ISGF3γ mRNA induction is mediated directly by the virus,

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          1        10        20        30        40        50        60        70        80
mouse MASGKVRCTR KLRSWIVEQV ESGHFPGVCW DDAAKTMFRI PWKHAGKQDF REDQDAAIFK AWALFKEKHK DGDIGHPAVW KTRLRCA
      ||||* |||| ||| |*|||| ||| |||||| || ||||||| |||||||||| ||||||| || |||*|| | | *|| | |||| |||||||
human MASGRARCTR KLRNWVVEQV ESGQFPGVCW DDTAKTMFRI PWKHAGKQDF REDQDAAFFK AWAIFKGKYK EGDTGGPAVW KTRLRCA
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Fig. 1. The deduced amino acid sequence of mouse ISGF3γ and the comparison with the sequence of human ISGF3γ [16]. Identical residues are marked by bars (|), and residues that are conservative substitutions are marked by asterisks (*).

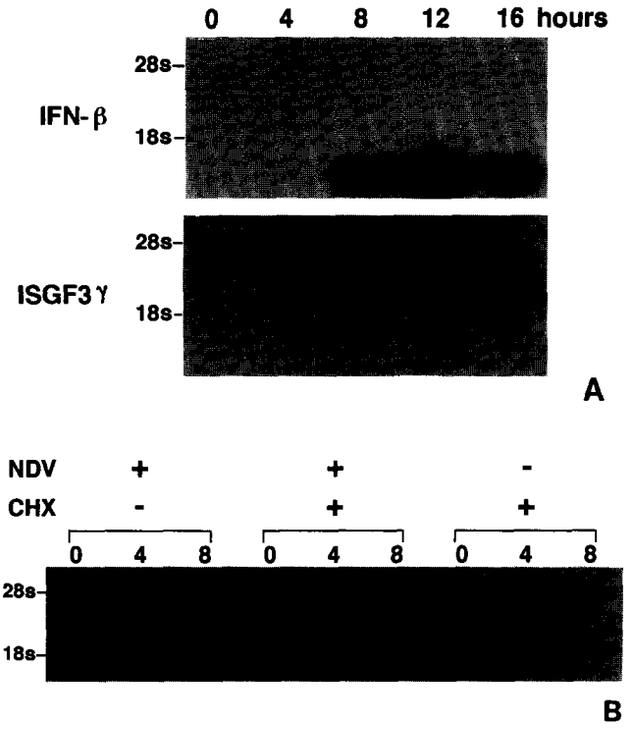
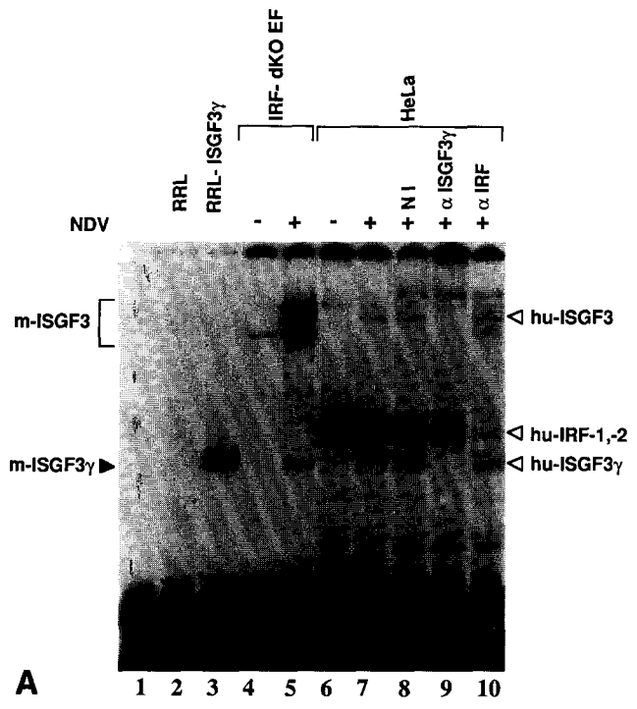


Fig. 2. (A) Expression of IFN-β and ISGF3γ mRNA induced by NDV infection. (B) Expression of ISGF3γ mRNA after the infection or mock-infection of NDV in the presence or absence of CHX (100 μg/ml). Total RNA was extracted at the indicated times, and 5 mg of RNA was subjected to RNA blotting analysis. Every filter was reprobed with the β-actin probe to confirm the RNA content in each slot.

rather than indirectly, e.g. by virus-induced IFNs. The expression level of IFN-β mRNA was dramatically decreased in the presence of CHX (25-fold; data not shown), an observation consistent with the idea that the induction of ISGF3γ expression is a prerequisite event for maximal IFN-β gene induction.

Next we tried to detect the activity of ISGF3γ in cell extracts after viral infection by EMSA using an ISRE sequence as a probe. As shown in Fig. 3A, infection with NDV induced ISRE binding activity in dKO-EF extracts. With HeLa cells, we also detected the induction of binding activity with similar mobility following NDV infection. Addition of an anti-ISGF3γ serum resulted in the disappearance of two bands; the lower of which



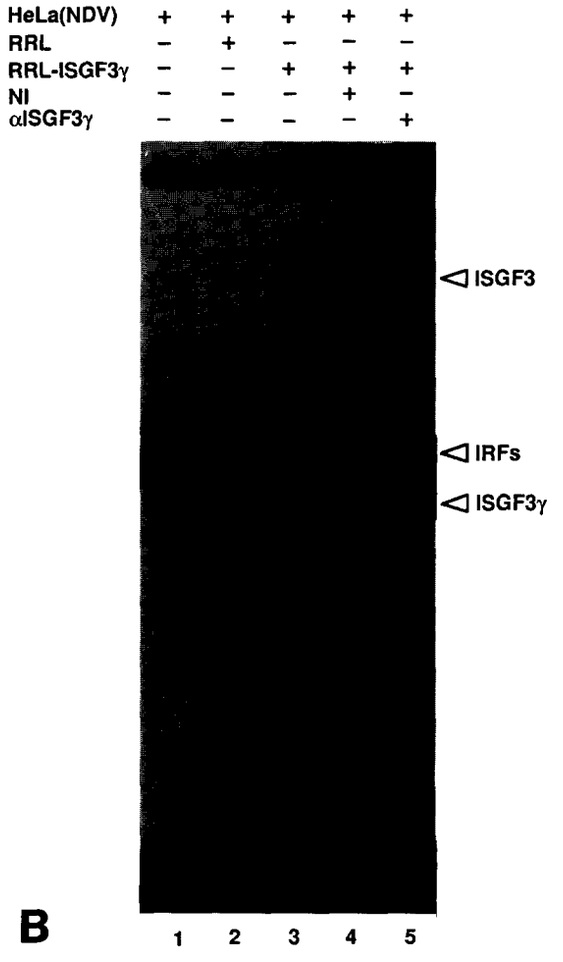
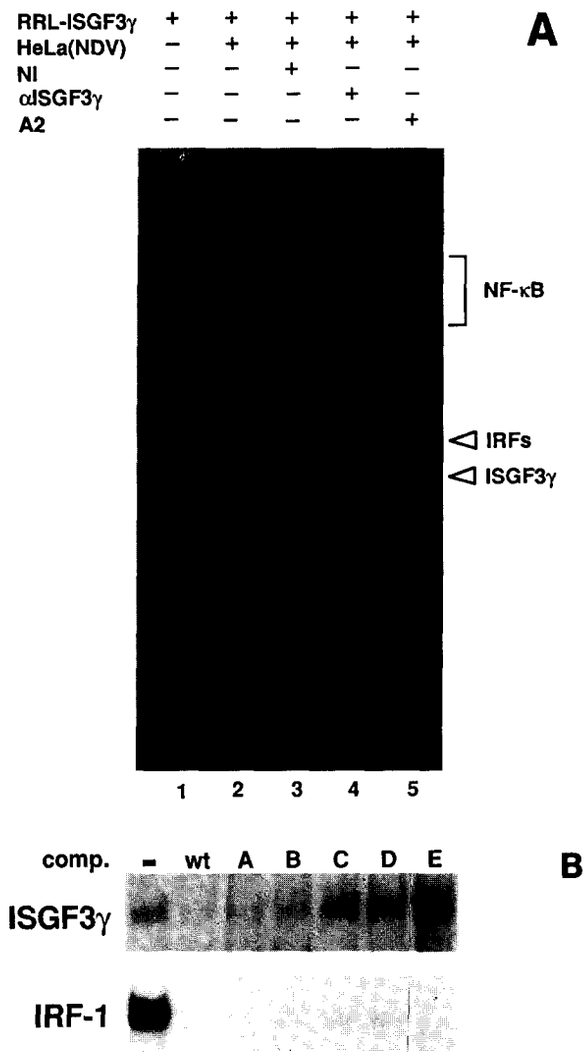


Fig. 3. (A) Induction of ISGF3γ and ISGF3 activities by NDV infection as detected by an ISRE probe. Proteins or extracts examined were as follows. Lanes: 1, no protein; 2, rabbit reticulocyte lysate (RRL); 3, RRL programmed with human ISGF3γ mRNA; 4, mock-infected IRF-dKO EFs; 5, IRF-dKO EFs 12 hours after NDV infection; 6,mock-infected HeLa cells; 7–10, HeLa cells 8 hours after NDV infection. In lane 8–10, extracts were preincubated with the following rabbit antisera. Lanes: 8, non-immune serum; 9, anti-ISGF3γ serum [16]; 10, a mixture of anti-IRF-1 and -IRF-2 sera [11]. (B) Enhancement of ISGF3 activity in HeLa cell extract by the addition of in vitro translated ISGF3γ. The extract prepared as that used in lane 5 of (A) (lane 1) were supplemented with RRL (lane 2) or RRL programmed with human ISGF3γ mRNA (lane 3–5). In lanes 4–5, proteins were preincubated with the non-immune rabbit serum (lane 4) or anti-ISGF3γ serum.



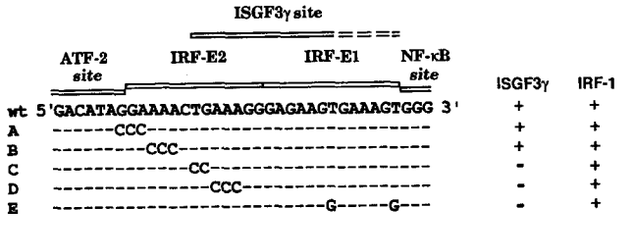


Fig. 4 (A) ISGF3γ binds to the promoter element of the IFN-β gene. Rabbit reticulocyte lysate (RRL) programmed with ISGF3γ mRNA (lane 1) or after mixing with the extract of HeLa cells infected with NDV (lanes 2–5) were incubated with a promoter element of the IFN-β gene (−95 to −54). In lanes 3–4, proteins were preincubated with the non-immune rabbit serum (lane 4) or anti-ISGF3γ serum. In lane 5, a 200-fold molar excess of cold A2 oligomer (a sequence which can bind to NF-kB [27]) was included in the binding reaction. (B) Competition EMSA. The binding of in vitro translated ISGF3γ or IRF-1 to the radiolabeled probe (−95 to −63 of the IFN-β gene promoter) were challenged by a 2000-fold molar excess of each cold competitor as depicted in the figure.

we assume to be ISGF3γ and the higher the ISGF3 complex containing ISGF3γ. The fact that addition of in vitro translated ISGF3γ into the HeLa cell extract enhanced the activity of ISGF3 (Fig. 3B), due to the presence of pre-existing ISGF3 components in this cell as described in [22] further verifies this assignment. Since the anti-ISGF3γ serum available do not cross-react with mouse proteins, we were not able to conduct a similar analysis using IRF-dKO EFs, but we observed that complexes of similar mobilities appeared using extracts from NDV-infected IRF-dKO EFs. Thus both ISGF3γ and ISGF3 complex binding activities are induced by NDV infection in HeLa and IRF-dKO cells. Addition of anti-IRF sera resulted in the inhibition of a band of intermediate mobility. This band is absent in the IRF-dKO EFs lane and therefore presumably corresponds to IRF-1 and IRF-2.

Collectively, these results prompted us to further pursue the possibility that ISGF3γ and/or the ISGF3 complex may be a candidate(s) for the putative factor involved in IFN-β gene induction. In fact, extensive sequence similarity has been noted previously between one virus-inducible element in the IFN-β gene promoter and the ISREs of IFN-inducible genes [7].

3.2. *ISGF3γ can bind to the IFN-β promoter*

We next tested whether ISGF3γ can bind to the IFN-β promoter. EMSA was performed with the labeled oligonucleotide spanning the two IRF-Es and the NF-kB binding site in the IFN-β promoter as a probe [8,24]. As shown in Fig. 4A, ISGF3γ, produced by in vitro translation with rabbit reticulocyte lysate, formed a complex with the IFN-β promoter sequence. Although the affinity to this sequence is weaker than to ISRE by 20-fold (data not shown), these results indicate that ISGF3γ has the potential to bind to the IFN-β promoter.

We then assessed the binding of the ISGF3 complex to the IFN-β promoter sequence using HeLa cell extract supplemented with in vitro translated ISGF3γ. As shown in Fig. 4A, ISGF3γ could bind to the probe under these conditions, however ISGF3 complex was not detectable in this EMSA assay. Complexes with a similar mobility to ISGF3 complex are probably NF-κB because they were competed out by excess amounts of cold κB sequence. Reconstituted ISGF3 made of in vitro translated ISGF3γ and partially purified STAT1/STAT2 complex from HeLa cell extracts provided similar results (data not shown).

In an attempt to define the recognition sequence of ISGF3γ in the IFN-β promoter, we performed competition EMSA using a series of mutated versions of a region of the IFN-β promoter containing two IRF-Es. As the labeled probe, we used a shortened version of the wild-type sequence containing only the two IRF-Es, which can still bind to ISGF3γ, and as a protein source we used in vitro translated ISGF3γ. As shown in Fig. 4B, sequences with mutations in the 3′ half of IRF-E2 region lost their ability to compete with the natural sequences for binding to ISGF3γ. In contrast, sequences with mutations in the 5′ half of IRF-E2 region were still effective competitors with the natural sequence. These results suggest that the 5′ boundary required for the binding of ISGF3γ is at least the −86 or −85 nucleotides 5′ to the start of the IFN-β gene transcription. In contrast, all of the mutants could compete with the binding of in vitro translated IRF-1 to the natural sequence, consistent with previous findings that IRF-1 can bind to either the IRF-E1 or IRF-E2 element within the IFN-β promoter [8]. In addition, one probe containing a mutation which causes decreased inducibility of the IFN-β promoter by NDV (mutant E) [24] does not compete with ISGF3γ, but does compete with

IRF-1 for binding to the natural promoter. This and the above results together support the idea that the sequence requirements of ISGF3γ and IRF-1 are different and that the binding of ISGF3γ is important for the induction of the IFN-β gene.

4. Discussion

In order to identify a factor(s) required for the IRF-1-independent pathway of IFN-β gene regulation, we performed PCR to clone other IRF-family members from IRF-dKO EFs induced to express IFN-β mRNA. In this study, we were able to obtain only ISGF3γ. Although the sequence of the ICSBP cDNA could justify its amplification by the PCR, this result was not seen, consistent with the fact that ICSBP is expressed only in lymphoid and macrophage cells [15].

In addition, we have shown by EMSA that ISGF3γ can bind to the IRF-Es of the IFN-β promoter. Significantly, the binding affinity is decreased by a mutation which reduces the inducibility by NDV infection [24]. Taken together, these results indicate ISGF3γ as an attractive candidate for the factor involved in the IRF-1 independent activation pathway of IFN-β gene transcription. If this is true, then ISGF3γ is another factor which is involved in the regulation of both the IFN and IFN-inducible genes, a role which is reminiscent of that of IRF-1 [12,13,25].

In contrast to our results, it has been previously reported that ISGF3γ cannot bind to the promoter element of the IFN-β gene [16,26]. However the DNA probe used in that study spanned nucleotides −79 to −57 of the IFN-β promoter and covered only the NF-kB binding site and IRF-E1. Our results from competition EMSA studies suggests that the 5′ boundary required for the binding of ISGF3γ is located at least 86 or 85 nucleotides upstream of the transcription start site.

It is known that ISGF3γ is the main DNA binding subunit of the ISGF3 complex and that upon formation this complex displays higher affinity towards the ISRE than that of ISGF3γ alone [17]. At present, it is not clear whether ISGF3γ functions on the IFN-β promoter alone or in combination with other factors. Although our EMSA failed to detect the binding of the larger ISGF3 complex to the IFN-β promoter, we cannot rule out the possibility that ISGF3 participates in the IFN-β gene regulation. In addition, the possibility also exists that ISGF3γ may form a complex with proteins other than STAT1 and 2 in its regulation of the IFN-β gene.

Obviously, the best way to elucidate the functional role of ISGF3γ in the regulation of the IFN-β gene would be through a gene knock-out study. We are now in the process of producing mice with disrupted ISGF3γ alleles.

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