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Autocrine Amplification of Type I Interferon Gene Expression Mediated by Interferon Stimulated Gene Factor 3 (ISGF3)[1]

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Interferon regulatory factor (IRF)-1 and IRF-2 have been implicated for the virus-induced expression of the interferon-α and β (type I IFN) genes. However, recent gene disruption studies in mice suggested the presence of other factor(s) interacting with overlapping promoter elements. In the present paper, we describe the characterization of a DNA binding factor which is strongly induced after virus infection and recognizes these promoter elements. After extensive purification, the factor was revealed to be identical to IFN-stimulated gene factor 3 (ISGF3), a transcription factor complex activated by IFN treatment. ISGF3 binds to the promoter element of IFN-β, positive regulatory domain I (PRDI), with significantly higher affinity than IRF-1, 2, and mutational analysis of PRDI showed that the gene expression and binding of ISGF3, but not of IRF-1, 2, are highly correlated. Furthermore, our functional analysis involving a dominant negative inhibitor for ISGF3 activation and an anti-IFN neutralizing antibody clearly demonstrated the presence of a positive feedback pathway for type I IFN genes mediated by ISGF3.

Key words: gene regulation, IRF, ISGF3, type I interferon, viral induction.

The type I interferon (IFN) system is considered to be an important element of the host defense mechanism against certain viral infections. Type I IFNs (IFN-α and IFN-β) are efficiently produced upon a variety of virus infections, and cells treated with IFNs develop an antiviral state in which the replication of a wide variety of viruses is repressed. Type I IFNs also exhibit other biological activities, such as an antiproliferative effect on certain susceptible cell types, immune response modulation, and induction of a variety of cellular proteins with physiological functions (*1*).

IFN genes are normally silent in unstimulated cells but can be activated transcriptionally by viruses or other inducers. The regulatory elements of the IFN-α and IFN-β genes have been defined within the 5′ upstream region of the genes (*2-4*). IFN-β gene expression is regulated by multiple factors: NF-κB, a regulator of many cellular and viral genes recognizes the κB motif (-66 to -57) or the positive regulatory domain (PRD) II (*5-7*), and disruption of the κB site reduces the gene expression by 3- to 5-fold (*5*). However, tumor necrosis factor (TNF)-α treatment, which induces NF-κB strongly, triggers very inefficient (*8*) or insignificant (*9*) IFN-β gene expression in fibroblasts. These observations indicate that NF-κB is necessary but not sufficient for full induction of the gene. Another element, PRDI, essential for the induction of the gene lies within the -79 to -66 region (*10, 11*). PRDI contains a binding site for interferon regulatory factor-1 and -2 (IRF-1 and -2) (*12, 13*). The virus-responsive element of IFN-α (-109 to -64), VRE-α, also contains IRF binding sites (*13*). IRF-1 and IRF-2 are an activator and repressor of transcription, respectively (*12-14*), and because of their differential protein stability, IRF-2 is constitutive whereas IRF-1 is transient, and detected only in stimulated cells (*9*). Disruption of IRF elements by mutation revealed a significant reduction in gene expression (*15*). Moreover, it has been shown that overexpression of IRF-1 results in low levels of endogenous IFN-α and IFN-β gene expression in the absence of virus infection (*16*). The upstream region of the IFN-β promoter (-101 to -93) is the binding site for the dimeric complex ATF-2/c-Jun (PRDIV) (*17, 18*). Also, the chromatin proteins, HMG-I/Y, may play a role in the activation of the promoter, presumably by being involved in the complex of regulatory proteins and the promoter DNA (*18*). Of these *cis*-elements, only the IRF element is commonly found in the IFN-α and IFN-β genes.

Recently, mice with a null mutation in either the IRF-1 or IRF-2 gene were generated and their phenotypes were analyzed (*19, 20*). Interestingly, the mice or the embryonic fibroblast cells from these mice produced nearly normal levels of IFN-α and IFN-β upon virus infection. The only defect observed with IRF-1 deficient fibroblasts was poor induction of IFN-α and IFN-β on treatment with the synthetic interferon inducer, poly(rI):poly(rC). However, this defect could be overcome by treating the cells with IFN

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prior to inducer treatment (priming) (*19*) or administering the synthetic inducer directly to the mice (*20*). The above observations strongly suggest the presence of *trans*-acting factor(s) other than IRF-1 and IRF-2 which activate the type I IFN gene by interacting with VRE-α/PRDI in induced cells.

In the present paper we report that ISGF3 recognizes a DNA sequence motif overlapping that of IRF-1/2. The results obtained on functional analyses of ISGF3 clearly demonstrate the presence of strong feedback regulation of type I IFN genes mediated by ISGF3.

MATERIALS AND METHODS

Cell Culture, and Preparation of Mutant Cell Lines Expressing p48 and Δp48—L929 cells were maintained in Minimum Essential Medium Eagle (MEM) supplemented with 5% fetal bovine serum (FBS). Stimulation with Newcastle disease virus (NDV) and treatment with poly(rI):poly(rC) were performed as described previously (*9*). Sf9 cells were maintained in Grace's medium containing 10% FBS, 0.2% tryptone, and 0.2% yeast extract. The recombinant baculoviruses were propagated following the MAXBAC manual (Invitrogen). P19 cells were cultured in alpha-MEM supplemented with FBS (10%). 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS. To obtain stable transformants expressing p48 or Δp48, L929 cells (3×10^6 cells) were co-transfected with 50 μg of pEFp48 or pEFΔp48 (see below) with the selectable marker gene, pCDM8neo (0.5 μg). The colonies grown in medium containing 1 mg/ml of G418 (GIBCO) were further selected for the expression of p48 (F1) or Δp48 (M1 to M4) by immunoblotting analysis of lysates with an anti-p48 antibody (see below).

Plasmid Construction—To construct p-125Luc, the *Eag*I-*Hin*dIII fragment containing the promoter region of the murine IFN-β gene excised from p-125cat (*15*) was inserted between the *Not*I and *Hin*dIII sites of pBL (*21*). p-125AALuc and p-125TTAALuc were obtained by site-directed mutagenesis using the following two oligonucleotides on p-125Luc, respectively: p-125AALuc: 5′-TGAAAGTGGGAAATTAATCTGAATAGAGAGAG-3′; p-125TTAALuc: 5′-AGGAAAACTGAAAGGTATAAGTGAAAGTGGGAAATTAATCTGAATAGAGAGAG-3′ (the mutated nucleotides are underlined).

Murine ISGF3γ (p48) cDNA was isolated from an NDV-induced L929 cDNA library using human ISGF3γ cDNA as a probe (*22*). To obtain the mammalian expression vector for p48, the murine cDNA was inserted into the *Xba*I site of pEF-BOS (*23*) (pEFp48). The cDNA for Δp48 was obtained by PCR using two appropriate oligonucleotides designed so as to initiate translation from Met37. The resultant DNA fragment was inserted into the *Xba*I site of pEF-BOS (pEFΔp48).

Reporter constructs containing repeated PRDI (p-55C-1BLuc) and repeated PRDII (p-55A2Luc) were prepared by implanting the regulatory elements from the corresponding CAT constructs (*5, 10*) into *Not*I-*Hin*dIII cleaved pBL (*21*). A construct containing VRE-α (pαLuc) was prepared by inserting the synthetic VRE-α of the IFN-α1 gene (-109 to $+7$) (*24*) between the *Not*I and *Hin*dIII sites of pBL. pActLuc was constructed by inserting the regulatory element of the β-actin gene excised from pActcat (*14*) into the *Hin*dIII site of pBL.

Preparation of Cell Extracts, and Partial Purification of ISGF3, IRF-2, and p48—NDV-treated L929 cells were suspended in lysis buffer (20 mM HEPES, pH 7.9, 50 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM DTT, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml leupeptin) and clarified by ultra-centrifugation. The resultant supernatant was recovered and used as the crude extract. The ISGF3/X protein was partially purified by Heparin Sepharose (Pharmacia), DNA-affinity, and Mono Q (Pharmacia) column chromatography, in that order. For DNA-affinity chromatography, the following oligonucleotides containing the binding sequences for IRFs (*10*) were coupled with cyanogen bromide-activated Sepharose 4B (Pharmacia):

5′-(AAAGTG)$_6$AAACCCCC-3′
3′-(TTTCAC)$_6$TTT-5′

Silver staining of the fractions containing DNA binding activity of ISGF3/X showed the existence of major polypeptides of 110, 90, 80, and 48 kDa (purity: ~70%) not present in the fractions similarly purified from control cells.

For preparation of the recombinant IRF-2 protein, we used the MAXBAC baculovirus expression system. Murine IRF-2 cDNA (*13*) was inserted into the *Xba*I site of pVL1393, and then transfected into Sf9 cells with AcNPV viral DNA. The recombinant baculovirus clone was selected by morphology screening of plaques. A crude extract was prepared from virus-infected Sf9 cells as described previously (*25*). The recombinant IRF-2 protein was partially purified by Heparin Sepharose, Blue Sepharose (Pharmacia) and Mono Q column chromatography, in that order.

The recombinant baculovirus encoding human p48 cDNA (*26*) was prepared using the MAXBAC system essentially as described above. The crude extract was fractionated first on Heparin Sepharose. The fractions containing p48 were pooled and further purified on Mono Q using a FPLC system (Pharmacia).

Antibodies—Anti-IRF-2 and anti-p48 antisera were obtained by immunization of rabbits with the purified recombinant murine IRF-2 or human p48. The anti-IRF-1 antiserum was prepared in a rabbit using a peptide corresponding to human IRF-1 as an antigen (*27*). The anti-p91/84 monoclonal antibody was obtained from Transduction Lab. The anti-p113 antisera were kindly provided by Dr. X.-Y. Fu (*28*). The anti-mouse IFN antiserum was described previously (*29*).

Electrophoresis Mobility Shift Assay (EMSA)—The following oligonucleotides containing the self-complementary sequences of the IFN-α or IFN-β promoter region were end-labeled with ^{32}P, self-annealed, and used as probes. (The underlined sequences correspond to the promoter region): IFN-α, 5′-GAGAAAGCAAAAACAGAAATGGAAAGTGGCCCAGAAATTAGCTTTTCTGGGCCACTTTCCATTTCTGTTTTTGCTTTCT-3′; IFN-β, 5′-GAGAAAACTGAAAGGGAGAAGTGAAAGTGATTAGCTTCACTTTCACTTCTCCCTTTCAGTTTTCT-3′.

To detect NF-κB binding activity, a self-complimentary oligonucleotide containing PRDII was used (*25*). The binding mixture comprised 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.3 mg/

ml BSA, 2 to 100 fmol of labeled probe, and 1 μl protein sample [when protein samples were crude, herring sperm DNA (0.175 mg/ml) was added]. The mixture was incubated at 25°C for 10 min and then subjected to PAGE at room temperature as described previously (*25*). The antisera were mixed with extracts before the DNA-protein binding reaction for 1 h at 0°C. For competition analysis, cold wild-type or mutant oligonucleotides were added to the reaction: mutant of IFN-β, 5′-GAGAAAACTGAAAGGTATAAGT-GAAAGTGATTAGCTTCACTTTCACTTATACCTTTCA-GTTTTCT-3′ (the mutated nucleotides are underlined).

Northern Analysis—Northern analysis was performed as described previously (*14*). To detect murine IFN-β and IFN-α mRNAs, the *Bam*HI-*Bgl*II fragment excised from pMGβ3-1 (*30*), and the *Hin*dIII-*Eco*RI fragment from pBR327(*Hin*dIII)/chrMuIFNα1/pGS3 (*31*) were used as probes, respectively. For actin mRNA, the *Bam*HI-*Pvu*II fragment from 204pBV2.1 (*12*) was used.

Methylation Interference—The probe for EMSA was also used for methylation interference. The protocol was the same as that described previously (*13*).

DNA Transfection and Luciferase Assay—P19 cells (2.5×10^5 cells) were transfected with 10 μg of plasmids (4 μg, reporter plasmids; 6 μg, pAct1 or control vector) by the calcium phosphate method (*21*), incubated at 37°C for 48 h, and then subjected to the luciferase assay. L929 cells (5×10^5 cells) were transfected with 2.5 μg of plasmids (1.25 μg, reporter plasmids; 1.25 μg, effector plasmids) by the DEAE-dextran method (*21*). After incubation at 37°C for 12 h, the cells were divided into two aliquots, which were treated with NDV and mock-infected at 24 h post-transfection for 12 h, respectively. In Fig. 7B, the cells were divided into three aliquots and incubated for 9 h with or without anti-IFN antiserum after mock- or NDV-infection. The luciferase assay was performed as described previously except for quantitation of luminescence with a Lumiphotometer (*21*).

Scatchard Analysis—Scatchard analysis was performed as described previously (*25*).

Western Blotting—p48 and Δp48 in crude lysates of L929-derived transformants were subjected to 10% SDS-PAGE by standard methods, and then transferred to a Immobilon membrane (Millipore). The membrane was reacted with anti-human p48 antisera. For detection of the antigen-antibody complex on the membrane, an alkaline phosphatase-conjugated antibody was used as a secondary reagent, and visualized with the NBT/BCIP substrate (Promega).

Reconstitution of ISGF3 In Vitro—HeLa cells were treated with human IFN-α (1,000 U/ml) for 15 min and then the ISGF3α fraction was prepared. 293T cells (3×10^6 cells) were transfected with pEF-BOS, pEFp48, or pEFΔp48 (10 μg) by the calcium phosphate method (*21*). The cells were harvested at 72 h after transfection to prepare a lysate for a control, p48 or Δp48, respectively.

Assay for Antiviral Activity—For the analysis of antiviral activity, cells seeded in 96-well plates (5×10^4 cells/well) were treated with serial dilutions of recombinant murine IFN-β or IFN-γ for 12 h at 37°C, and then infected with vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) at an input multiplicity of 0.04 and 0.22 pfu/cell, respectively. After adsorption of the virus, the cells were incubated for 24 h at 37°C. The cytopathic effect of VSV or the EMCV hemagglutinin (HA) yield was determined as described previously (*32, 33*).

RESULTS

ISGF3 Specifically Recognizes VRE-α and PRDI—In order to characterize previously unidentified virus-inducible factor(s) that binds to the promoter element, VRE-α and PRDI, we carried out electrophoresis mobility shift assays (EMSA) under different conditions. It appeared that the most critical parameters are the source and amount of carrier DNA (data not shown). One of the conditions, as described under "MATERIALS AND METHODS," was finally adopted. The probes used were oligonucleotides containing VRE-α or PRDI (Fig. 1, C and D). Extracts of Newcastle disease virus (NDV)-infected mouse L929 cells, which produce high levels of IFN-α and IFN-β, were prepared. Both probes detected two apparent virus-inducible complexes: a fast migrating band, which was identified as a mixture of IRF-1 and IRF-2, respectively, and bound with the probe (data not shown), and a relatively slow migrating species. Because the formation of the latter complex was not affected by antibodies against IRF-1 or -2, it was designated as X. The formation of all these complexes was specifically inhibited in the presence of a molar excess of cold oligonucleotides containing VRE-α, PRDI, or the repeated IRF motif (*10*), but not by an unrelated oligonucleotide containing the NF-κB motif (*25*) (data not shown). The DNA binding activity of X appeared to be transient, with a peak around 9-12 h, after NDV infection (Fig. 1, C and D), preceding the accumulation of IFN-α and IFN-β mRNAs (Fig. 1, A and B). The EMSA signals of IRF-1, 2, and X were significantly weaker when the VRE-α probe was used. This is likely due to the lower binding affinity of these factors to the sequence, because the probes for IFN-α and IFN-β were labeled with comparable specific activities. Interestingly, the amount of accumulated IFN-α mRNA was also lower than that of IFN-β, irrespective of the fact that the probe used for Northern analysis may detect several mRNA species derived from the IFN-α gene family. It is worth noting that the complex shown by an open triangle detected with an uninfected cell extract started to decrease at 12 h post-infection and had virtually disappeared at 15 h. The complex also decreased with poly(rI):poly(rC) treatment but not with IFN-β or TNF-α, and the complex did not show reactivity to antibodies to IRF-1 or -2 (unpublished observation).

The above results indicate that X interacts with a DNA sequence similar to the IFN-α/β-stimulated response element (ISRE), to which IRF-1 and 2, and ISGF3 commonly bind (*34*). ISGF3 is a multi-subunit transcription factor and has been implicated in the activation of the genes induced by IFN treatment (*35-37*). When the cell surface IFN receptor is stimulated by its homologous ligand, certain tyrosine kinases are activated, resulting in the phosphorylation of the cytoplasmic ISGF3α subunits, the signal transducers and activators of transcription (STAT) 1 (p91, p84) and STAT2 (p113) (*28, 38-40*). Then the subunits associate with DNA binding subunit ISGF3γ (p48) (*26*) to form the heterotrimer, ISGF3. The above series of events can occur in the absence of *de novo* protein synthesis, and thus is considered as a primary response to IFN treatment.

EMSA showed the indistinguishable mobilities of X and ISGF3 induced by IFN treatment (Fig. 1E, lanes 2 and 3). Antibodies against the components of ISGF3 caused supershifts of X and ISGF3 in the same assay (Fig. 1E). Also, ISGF3 extracted from HeLa cells induced by the published protocol using IFN-γ and IFN-α (*35*) gave a clear complex with PRDI with comparable mobility to the ISGF3/ISRE complex in EMSA (unpublished observation). Moreover, the partially purified X fraction from NDV-infected L929 cells contained polypeptides reactive to anti-p113, p91/84, and p48 antibodies on Western blotting and these reactivities were not observed in the fractions purified similarly from untreated L929 cells (data not shown). We conclude therefore that X is identical to ISGF3.

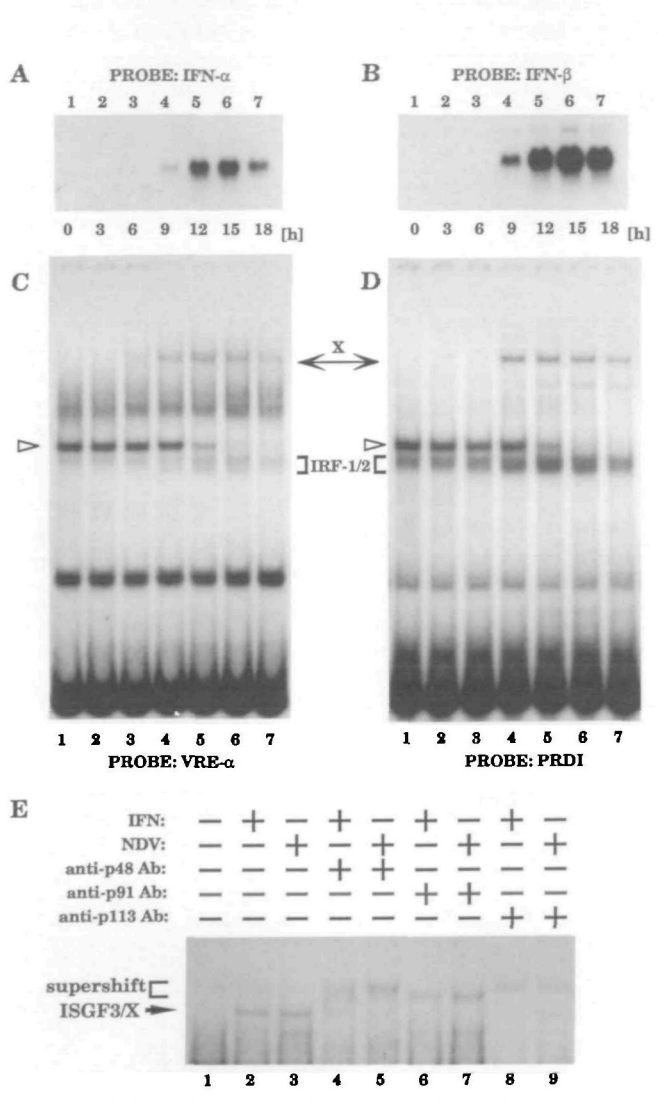

Fig. 1. **Viral induction of IFN-α and IFN-β mRNA, and *binding factors to their promoters.*** *L929 cells were mock-treated* or infected with NDV, and then harvested every 3 h after infection for total cellular RNA and protein extractions. The extracted RNA samples were subjected to Northern blotting analysis for IFN-α mRNA (A) and IFN-β mRNA (B). The protein extracts were subjected to EMSA using VRE-α (C) and PRDI (D) as probes. The positions of specific probe DNA complexes are indicated by arrows. Lanes: 1, mock-treated; 2 to 7, infected with NDV for 3, 6, 9, 12, 15, and 18 h, respectively. (E) Characterization of X with antibodies against subunits of ISGF3. Crude cell extracts of control (lane 1), IFN-β treated (for 3 h, lanes 2, 4, 6, and 8) or NDV-infected (for 6 h, lanes 3, 5, 7, and 9) cells were reacted without (lanes 2 and 3) or with anti-p48 (lanes 4 and 5), anti-p91 (lanes 6 and 7), or anti-p113 antibodies (lanes 8 and 9), and then subjected to EMSA using PRDI as a probe. The position of ISGF3/X is indicated.

Overlapping but Distinct DNA Sequence Recognition by ISGF3 and IRF-2 on PRDI—Because ISGF3 and IRF-1, -2 recognize common short DNA motifs, we further mapped the contact sites for these factors on PRDI. We performed methylation interference analysis using partially purified ISGF3 (see "MATERIALS AND METHODS") and PRDI (Fig. 2A). For a comparison, purified IRF-2 produced in insect cells was used. Methylation of the guanine residues at −72 and −74 was strongly inhibitory for the binding of both ISGF3 and IRF-2. These residues were shown to be critical for interaction with bacterially produced IRF-1 and IRF-2 (*13*). However, methylation of upstream (−77 and −79) guanine residues strongly interfered with the DNA binding of ISGF3, whereas the binding of IRF-2 was only slightly affected. This was in agreement with the previous observations that bacterially produced IRF-1 and -2 interfered weakly with these residues (*13*), and that ISGF3 recognizes a broader sequence motif than IRF-1 (ISGF2) (*41, 42*). These results suggest that ISGF3 and IRF-1, -2 recognize distinct but overlapping sequence motif(s) present in PRDI. The results also suggest that the interaction of these

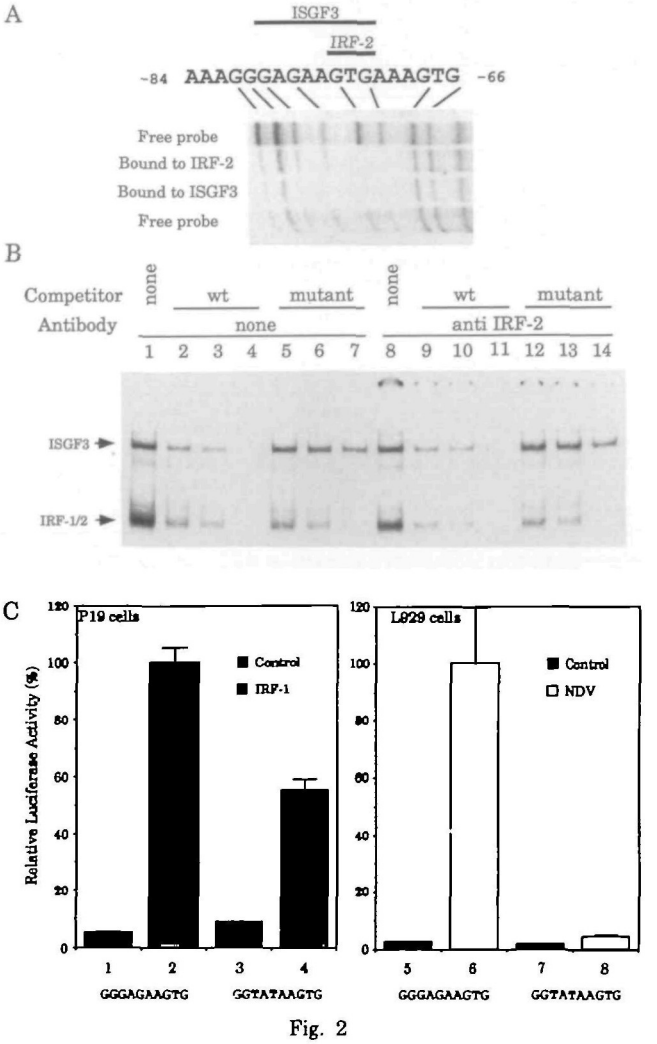

Fig. 2

proteins with PRDI occurs in a mutually exclusive manner.

The above findings prompted us to create a mutant binding site that allows discrimination between ISGF3 and IRF-1, -2. These G residues, that preferentially interact with ISGF3 but weakly with IRF-1, -2 (−77 and −79), were replaced by T residues. The mutagenized PRDI competed poorly with the binding of the wild type IRF element to ISGF3 (Fig. 2B, lanes 5, 6, 7, 12, 13, and 14). However, the mutant was nearly as effective as the wild type in competing with binding of the native probe to a mixture of IRF-1 and -2 (Fig. 2B, lanes 5 to 7), or binding to IRF-1 (lanes 12 to 14). These results indicate that the above mutation allows quantitative discrimination between the binding of IRF-1, -2, and ISGF3.

To analyze further the properties of this mutation, reporter genes containing the IFN-β regulatory region (−125 to +19) upstream of the luciferase structural gene were prepared. To exclude the effect of the NF-κB motif (PRDII) present in the regulatory sequence, base substitutions were introduced (−58: C to A; −57: C to A; p-125AALuc). This mutation completely inactivated the NF-κB motif, but the IFN-β gene was still virus-inducible due to the presence of other elements (*5*). The other reporter, p-125TTAALuc, contained base substitutions (−79: G to T; −77: G to T) in addition to the above mutation. It has been shown that the IFN-α and IFN-β genes can be activated efficiently by the ectopic overexpression of IRF-1 in undifferentiated mouse teratocarcinoma P19 cells, which lack IRF-1 and -2 (*14*). First, we examined the above reporter constructs in this system to determine their reactivity with IRF-1. The wild type IRF element (p-125AALuc) was efficiently activated by IRF-1, and the mutant (p-125TTAALuc) showed reduced (50%) but significant activation by IRF-1 (Fig. 2C, lanes 1 to 4). This reduction was likely due to the slightly inefficient interaction of the mutant with IRF-1 (Fig. 2B, lanes 9 to 11, and 12 to 14). In order to monitor their response to viral induction, the constructs were transfected into L929 cells and then induced by NDV. p-125AALuc was highly inducible by virus infection, however, induction of mutant p-125TTAALuc was almost completely abolished (Fig. 2C, lanes 5 to 8). Similar results were reproducibly obtained in repeated experiments. These findings indicate that the binding specificity of ISGF3 as to the regulatory element of IFN-β

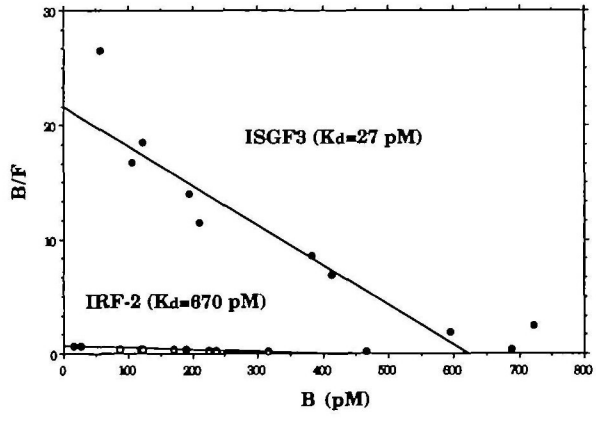

Fig. 3. **DNA binding affinity of IRF-2 and ISGF3.** Constant amounts of partially purified ISGF3 (●) and recombinant IRF-2 (○) were subjected to EMSA using various amounts of ^{32}P-PRDI as a probe. Because both protein fractions were essentially free of non-specific DNA binding proteins ("MATERIALS AND METHODS"), the assay was carried out in the absence of carrier DNA. The free probe and the probe in the specific complex were quantitated with a Phosphor Image Analyzer (Fuji). K_d values were determined by Scatchard plotting of the data.

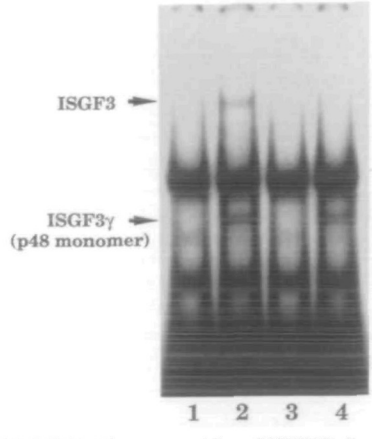

Fig. 4. **Δp48 inhibits the assembly of ISGF3 *in vitro*.** A crude HeLa cell lysate containing ISGF3α but not detectable ISGF3 was reacted with extracts of 293T cells which had been transfected with the control vector (lanes 1 and 3) or p48 expression vector (lanes 2 and 4) in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of Δp48 produced by 293T cells (20-fold molar excess over p48). The formation of ISGF3 was analyzed by EMSA using PRDI as a probe.

Fig. 2. **The two G residues within PRDI are critical for its interaction with ISGF3 and gene activation by NDV infection.** (A) Methylation interference analysis of the DNA contact sites of ISGF3 and IRF-2 on PRDI. Partially purified ISGF3 and recombinant IRF-2 were subjected to EMSA using partially methylated end-labeled PRDI as a probe. The specific complexes or free probes were extracted from the gel and processed as described (*13*). The bands were correlated to the G residues in the PRDI sequence. (B) DNA binding affinity of IRF-1, -2, and ISGF3 to the wild type or mutant PRDI. Cell extracts of NDV-infected L929 cells were subjected to EMSA using wild type PRDI as a probe. Anti-IRF-2 antibody was added to lanes 8 to 14 in order to show the binding of IRF-1 to DNA. Cold oligonucleotides with either the wild type PRDI sequence or mutant PRDI, in which the two G residues at −77 and −79 were exchanged for T residues, were included in the reaction. Lanes 1 and 8, no competitor DNA; lanes 2 and 9, 10-fold molar excess of the wild type competitor; lanes 5 and 12, 10-fold molar excess of the mutant competitor; lanes 3 and 10, 20-fold excess of the wild type competitor; lanes 6 and 13, 20-fold excess of the mutant competitor; lanes 4 and 11, 50-fold excess of the wild type competitor; lanes 7 and 14, 50-fold excess of the mutant competitor. Arrows indicate specific DNA-protein complexes. (C) Effect of the mutation within PRDI on IRF-1-induced or NDV-induced activation of the IFN-β promoter. P19 cells (lanes 1 to 4) were transfected with p-125AALuc (lanes 1 and 2) or p-125TTAALuc (lanes 3 and 4) with equal amounts of a vector, pCDMAct (lanes 1 and 3) or pAct1, the expression construct for mouse IRF-1 (lanes 2 and 4). The cells were harvested 48 h after transfection for the luciferase assay. L929 cells (lanes 5 to 8) were transfected with p-125AALuc (lanes 5 and 6) or p-125TTAALuc (lanes 7 and 8). 24 h after transfection, the cells were either mock-treated (lanes 5 and 7) or infected with NDV (lanes 6 and 8). The cells were harvested for the luciferase assay at 12 h after infection. Relative luciferase activities were calculated by taking the luciferase activity in lane 2 or 6 as 100% for each set of experiments. Error bars represent SE for quadruplicate transfection trials.

was correlated highly with the gene inducibility by virus infection.

ISGF3 Binds to PRDI with Higher Affinity than IRF-2— To address further the regulatory role of ISGF3, its DNA binding affinity was quantitated and compared with that of cellular repressor IRF-2 (Fig. 3). ISGF3 bound to PRDI with dramatically high affinity (K_d = 27 pM) compared to recombinant IRF-2 (K_d = 670 pM). The partially purified fraction containing IRF-1 and IRF-2 from L929 cells exhibited a comparable binding affinity (K_d = 310 pM) to the recombinant IRF-2 (data not shown). The determined K_d values are 1/100 to 1/1,000 of the previous ones (*13*). This is likely because of the different assay conditions, particularly the omission of carrier DNA and the adoption of EMSA rather than a filter binding assay in the present study. Also, IRF-1 and IRF-2 produced in eukaryotic cells may exhibit different characteristics compared to those produced in prokaryotic cells as insoluble aggregates and renatured (*13*).

These results suggest that ISGF3 can dominate the constitutive repressor, IRF-2, in binding to PRDI in induced cells. This explains our observation that although IRF-2 efficiently repressed IFN-β gene expression by IRF-1 overproduction in undifferentiated P19 cells (*14*), overexpression of IRF-2 did not affect IFN-β promoter activation induced by virus infection in L929 cells (unpub-

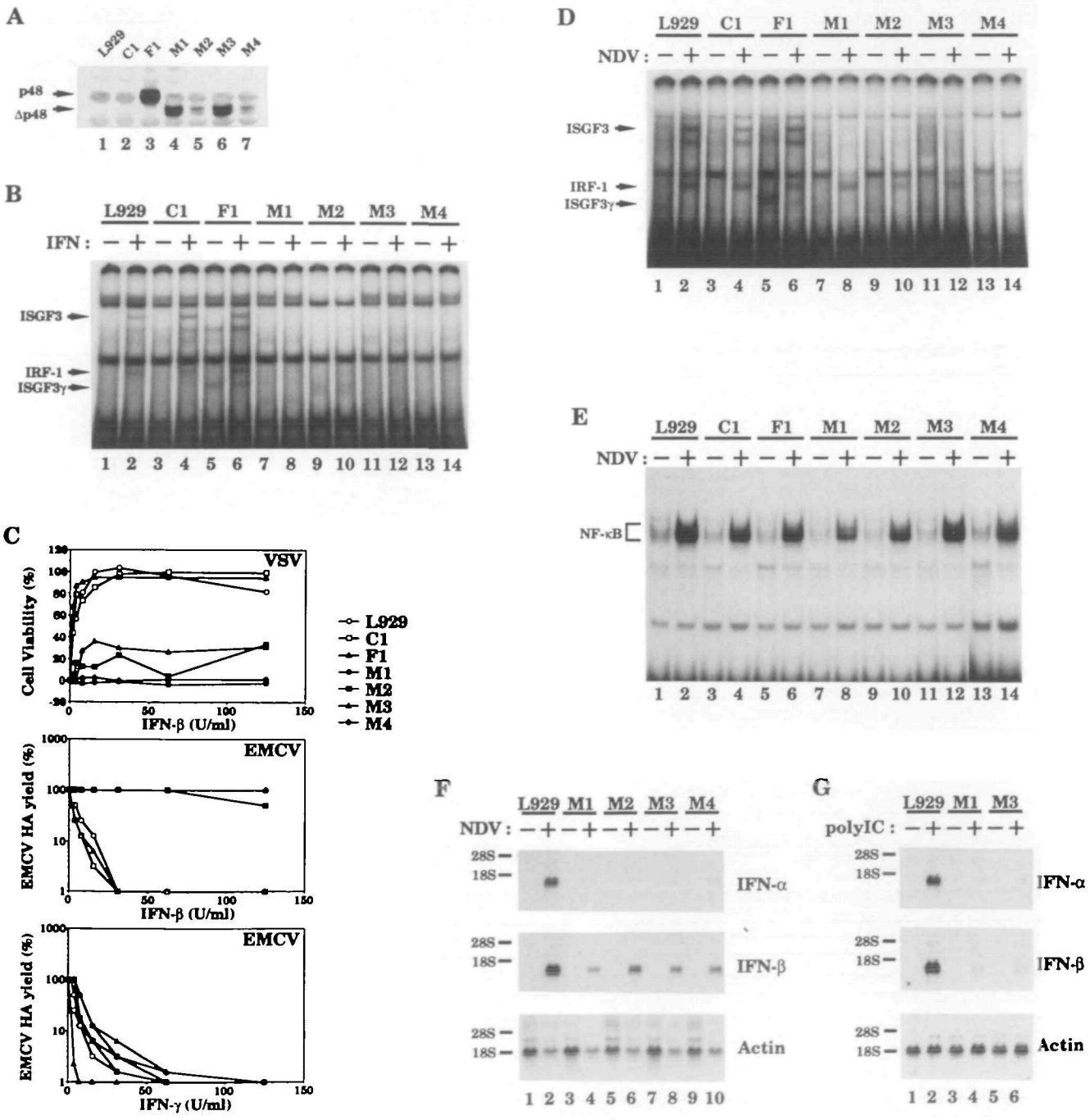

Fig. 5

lished observation).

In Vivo Functional Analysis Using a Dominant Negative Inhibitor for ISGF3 Activation—To investigate the function of ISGF3, we generated a deletion mutant of p48, Δp48, which lacks a portion of the DNA binding domain, but retains the domain for interaction with ISGF3α (*43*), expecting that such a mutant protein may squelch ISGF3α. The *in vitro* assembly of ISGF3 observed when the activated ISGF3α fraction and recombinant p48 were mixed was dramatically inhibited by the addition of excess Δp48 (Fig. 4). However, the DNA binding properties of monomer p48 were unaffected, demonstrating that Δp48 can specifically interfere in the assembly of ISGF3.

To assess the dominant negative nature of Δp48 in cultured cells, we obtained L929 cells stably expressing high levels of Δp48 by immunoblot screening of transfected cells (clones M1 to M4, Fig. 5A). For comparison, a clone expressing the full length p48 (F1) and a clone transfected with the empty vector (C1) were prepared (Fig. 5A). First, ISGF3 induction by IFN-β treatment was examined (Fig. 5B). The untransfected L929, C1, and F1 cells showed clear induction of ISGF3 and IRF-1 following IFN-β treatment. However, clones expressing Δp48 displayed no detectable ISGF3 indicating that Δp48 inhibited the assembly of ISGF3. Interestingly, IRF-1 induction, which normally takes place after IFN treatment, was also abolished in these cells. Concomitantly with the absence of ISGF3 and IRF-1, these cells failed to enter an antiviral state as to vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) infection (Fig. 5C). This is in contrast with the observation that *IRF-1−/−* fibroblasts exhibit an almost normal antiviral state as to these viruses after IFN-β treatment (*44*). The results are complementary to the findings for IFN-resistant mutant human cell lines (*45*), and demonstrate that functional ISGF3 is critical for the establishment of an antiviral state following type I IFN treatment. Interestingly, Δp48 transformants normally responded to type II IFN for the establishment of an antiviral state (Fig. 5C).

Next, viral induction of the ISGF3 and IFN genes in these mutant cells was investigated. The generation of ISGF3 was also inhibited by Δp48 (Fig. 5D). However, IRF-1 (Fig. 5D) and NF-κB (Fig. 5E) were independently induced at normal levels. Concomitant with the absence of ISGF3, induction of the IFN-α gene was dramatically inhibited (>20-fold), whereas the IFN-β gene was partially but significantly inhibited (3- to 5-fold) (Fig. 5F). Because Δp48 may affect viral replication, we also examined the effect of the non-viral inducer, poly(rI):poly(rC). When

Fig. 5. **Δp48 inhibits both the action and production of type I IFN.** (A) Immunoblot analysis of extracts of parent L929 cells (lane 1), and control (C1, lane 2), p48 (F1, lane 3), and Δp48 (M1 to M4, lanes 4 to 7) clones. The positions of p48 and Δp48 are indicated. The band common to all lanes slightly below the p48 band is a background signal and does not correspond to endogenous p48 (data not shown). (B) Δp48 inhibits IFN-β-induced activation of ISGF3 and IRF-1 in L929 cells. L929 cells or a stable transformant transfected with a vector (C1) or a transformant expressing high levels of p48 (F1) or Δp48 (M1 to 4) were mock-treated (1, 3, 5, 7, 9, 11, and 13) or treated with 1,000 U/ml of IFN-β for 3 h (2, 4, 6, 8, 10, 12, and 14). Cell lysates were prepared and analyzed by EMSA using PRDI as a probe in the presence of anti-IRF-2 antibodies to visualize the IRF-1/probe complex. The positions of ISGF3, IRF-1, and ISGF3γ (p48), respectively, complexed with the probe are indicated. (C) L929 transformants expressing Δp48 failed to enter an antiviral state upon IFN-β treatment. L929 cells (○) and transformants (C1, □; F1, △; M1, ●; M2, ■; M3, ▲; M4, ◆) were treated with serially diluted IFN-β (upper and middle panels) or IFN-γ (lower panel) for 12 h. The cells were then challenged with VSV (upper panel) or EMCV (middle and lower panels). The antiviral state induced by IFN was determined by measuring the cytopathic effect or viral HA yield for VSV or EMCV, respectively. (D) Δp48 inhibits NDV-induced ISGF3 but not IRF-1 in L929 cells. The L929 cells and transformants used in (B) were mock-infected (1, 3, 5, 7, 9, 11, and 13) or infected with NDV (2, 4, 6, 8, 10, 12, and 14) for 12 h, and then cell lysates were prepared and analyzed as in (B). (E) Δp48 does not affect NDV-induced activation of NF-κB in L929 cells. The cell lysates described in (D) were analyzed by EMSA using the κB motif (PRDII) as a probe. (F) L929 cell transformants producing Δp48 express barely detectable levels of IFN-α and IFN-β mRNA after induction by NDV. Total RNA extracted from L929 cells and transformants (M1 to 4), which had been mock-infected (1, 3, 5, 7, and 9) or infected with NDV (2, 4, 6, 8, and 10), was subjected to Northern blotting analysis using ^{32}P-labeled IFN-α, -β, or actin as a probe, as indicated. (G) Δp48 transformants express low levels of IFN-α and IFN-β mRNA after induction by poly(rI):poly(rC). Cells were treated with poly(rI):poly(rC) for 12 h, and then the IFN-α and IFN-β mRNA levels were determined as in (F). Lanes: 11, 13, and 15, mock-treated; 12, 14, and 16, treated with poly(rI):poly(rC).

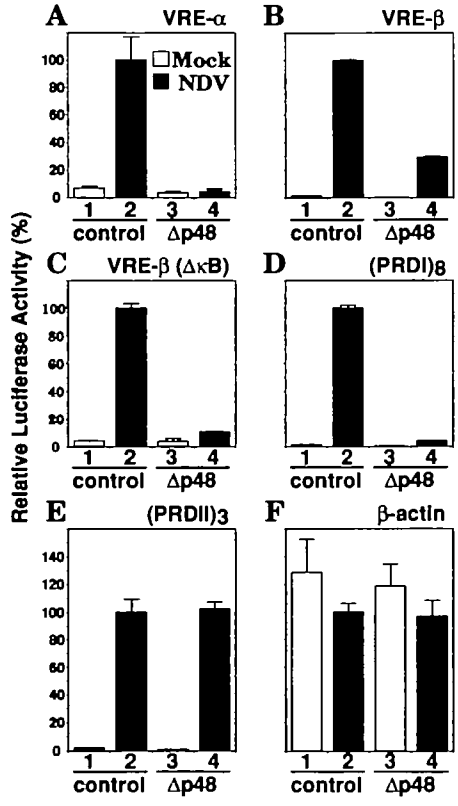

Fig. 6. **Mapping of the target sequence for virus-induced ISGF3.** L929 cells were transiently transfected with luciferase reporter genes driven by VRE-α (A), VRE-β (B), VRE-β with the disrupted κB motif (C), the repeated PRDI motif (D), the repeated PRDII motif (E), and the β-actin enhancer/promoter (F), with the control pEF-BOS vector (lanes 1 and 2) or the expression vector for Δp48 (lanes 3 and 4). Cells were mock-treated (lanes 1 and 3) or infected with NDV (lanes 2 and 4). The luciferase activity in lane 2 in each figure was taken as 100%. Error bars represent SE for quadruplicated transfection trials.

poly(rI):poly(rC) was used as an inducer, ⊿p48 almost completely inhibited the induction of IFN-α and -β gene expression (Fig. 5G). The results demonstrate that the induction of ISGF3 is necessary for the efficient induction of type I IFN by virus infection or double-stranded (ds) RNA. A similar effect of ⊿p48 was demonstrated in transient assays (Fig. 6), and the effect was mediated specifically by the virus response element of IFN-α (VRE-α) (*2*) (Fig. 6A) and PRDI (*10*) (Fig. 6D), with which ISGF3 specifically interacts. The differential effects of ⊿p48 on NDV-induced IFN-α and -β gene expression (Figs. 5E, and 6A and 6B) are likely due to the κB motif present in the promoter of IFN-β (VRE-β) but not in VRE-α (*5-7*), because the κB motif (PRDII) (*25*) is strongly stimulated by a virus in the presence of ⊿p48 (Fig. 6E), and the induction of VRE-β with a disrupted κB motif is strongly repressed by ⊿p48 (Fig. 6C). The constitutive actin promoter/enhancer was not affected by ⊿p48, excluding the possibility that the observed inhibition was the result of an enhanced cytotoxic effect of virus infection (Fig. 6F).

Positive Feedback Activation of PRDI by Autocrine IFN—Because NDV-infected L929 cells produce high levels of IFN, which potentially activates ISGF3 through an autocrine mechanism, we examined the effect of removal of IFN activity from the culture medium by adding a neutralizing antibody. The addition of the antiserum resulted in a significant decrease in the ISGF3 level (Fig. 7A), with a concomitant decrease in gene activation mediated by PRDI (Fig. 7B, $PRDI_8$ and VRE-$\beta\Delta\kappa$B). A similar effect was observed with VRE-α (data not shown). The inhibitory effect was less prominent on the intact VRE-β, presumably because NF-κB was normally induced in these cells (Fig. 7A). These results indicate that the positive feedback activation by IFN is necessary for efficient induction of the IFN gene in L929 cells.

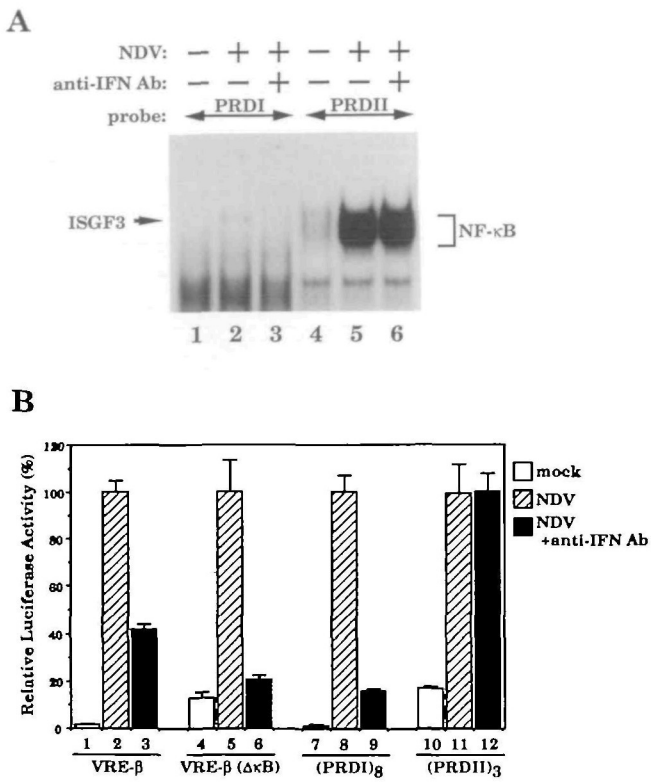

Fig. 7. **Existence of an auto-regulatory loop for virus-inducible activation of ISGF3 and the IFN-β gene promoter.** (A) A neutralizing antibody to type I IFN inhibits virus-inducible activation of ISGF3. Mock-treated (lanes 1 and 4) or NDV-infected (lanes 2, 3, 5, and 6) L929 cells were cultivated in the absence (lanes 1, 2, 4, and 5) or presence (lanes 3 and 6) of anti-mouse IFN antiserum for 9 h. Crude extracts of those cells were subjected to EMSA using PRDI (lanes 1 to 3) or κB (PRDII) oligonucleotides (lanes 4 to 6) as probes. The complexes of ISGF3 and NF-κB with the probe DNA were indicated. (B) Induction of the IFN-β gene promoter by NDV infection is inhibited by the anti-IFN antibody. L929 cells transfected with the reporter constructs (VRE-β, lanes 1 to 3; VRE-$\beta\Delta\kappa$B, lanes 4 to 6; repeated PRDI, lanes 7 to 9; repeated PRDII, lanes 10 to 12) were mock-treated (lanes 1, 4, 7, and 10) or NDV-infected (lanes 2, 3, 5, 6, 8, 9, 11, and 12) in the absence (lanes 2, 5, 8, and 11) or presence (lanes 3, 6, 9, and 12) of the anti-IFN antiserum. The cells were subjected to the luciferase assay at 9 h after infection.

DISCUSSION

ISGF3 Complex Specifically Interacts with VRE-α/PRDI—In the present paper we described the identification of factors interacting with VRE-α/PRDI in virus-induced cell extracts. Technical improvements (see "MATERIALS AND METHODS") allowed us to demonstrate that ISGF3 binds to these sequence motifs. This is rather surprising because it has been considered that the ISGF3 complex recognizes ISRE but not PRDI (*43*). Recently, Kawakami *et al.* demonstrated that p48 interacts with PRDI, but failed to show the interaction between ISGF3 and PRDI (*46*). However, as our analysis involving ⊿p48 demonstrates clearly, it is trimeric ISGF3 that actually participates in the gene activation. Also, overexpression of p48 neither activated IFN genes nor led to an antiviral state (Fig. 5C and unpublished result).

ISGF3 Is Secondarily Induced by Virus Infection—Regarding the possibility of direct induction of ISGF3 by virus infection, we examined human cell lines which are non-responsive (HEC-1) (*47*) or deleted the whole loci for both the IFN-α and -β genes (U87MG, U118MG, Reh, K562, and RS4:11) (*48, 49*). None of these cells responded to NDV infection by inducing ISGF3 even though these cells, other than HEC-1, induced ISGF3 on IFN-α treatment (unpublished observation). The results suggest that ISGF3 is secondarily induced in L929 cells after virus infection. However, this induction of ISGF3 participates in the expression of IFN genes *per se* because impairment of this feedback loop by a neutralizing antibody to type I IFN (Fig. 7) or inhibition of ISGF3 assembly by ⊿p48 (Figs. 5 and 6) resulted in reduced gene expression. Thus, it appears that the autocrine ISGF3 induction pathway is essential for the amplification of type I gene expression initially triggered by a virus or dsRNA.

Primary Response to Virus Infection or dsRNA Treatment—Which transcription factor triggers the initial activation of type I IFN genes? The putative transcription factor must be activated directly by virus infection or dsRNA. In this regard, we observed the generation of PRDI binding activity distinct from that of ISGF3 in the above mentioned cell lines deficient in the IFN receptor or IFN locus after virus infection (unpublished observation). The DNA binding activity exhibits similar mobility in a gel to DRAF1, an ISRE binding activity induced by dsRNA (*50*).

Although the natures of these binding activities have yet to be elucidated in detail, these are candidates for the putative primary activator. It is possible that these factors are responsible for the residual IFN gene induction in Δp48-expressing cells (Fig. 6).

Autocrine Amplification of Type I IFN Gene Expression—Once the IFN gene is activated by the putative primary transcription factor, the produced IFN triggers the activation of ISGF3. We demonstrated that the accumulation of ISGF3 in cells is necessary for the maximum level of PRDI activation, and that the PRDI residues necessary for interaction with ISGF3 are also essential for gene activation. These results argue for the direct involvement of ISGF3 in activation of the type I IFN promoter. However, we can not rule out the possibility that protein factors which are secondarily induced by ISGF3 participate in the gene activation. These hypothetical proteins may include signaling molecules such as protein kinases and components of transcription factors. This issue needs to be investigated further.

The above phenomenon resembles the priming effect of IFN: treatment of cells with IFN prior to inducer treatment enhances the IFN yield or accelerates the induction kinetics. Although the molecular mechanism of priming is poorly understood, it has been suggested that *de novo* protein synthesis induced by IFN is required for the establishment of a primed state (*33*). A priming effect has been observed for many cell types, including primary fibroblasts, suggesting that the autocrine amplification constitutes general physiological regulation of IFN genes. It has been reported that some cell lines, such as U937, are resistant to cycloheximide treatment (*51*). In such cells, autocrine amplification may be dispensable and then may rely more on the primary response.

Why Type I IFN Does Not Induce IFNs—Generally, type I IFN treatment does not induce IFN genes, this being partly because promoter elements other than IRF binding sites are required for full induction of the genes. The IFN-β promoter is composed of multiple factor binding sites: NF-κB motif (PRDII) (*5-7*), IRF motif (PRDI) (*10*), and ATF site (PRDIV) (*17*), from proximal to distal positions. Disruption of either the NF-κB motif (PRDII) or ATF site (PRDIV) reduces the expression level to 1/5 or 1/3, respectively (*5, 17*). Although these motifs have not been identified in the IFN-α promoter, we cannot exclude the possibility of the presence of other types of elements. Concomitant induction of these activators is required for the maximum level of IFN-β gene activation. Thus, virus infection may trigger multiple activators, whereas IFN treatment primarily induces ISGF3, which alone is insufficient to activate type I IFN genes.

However, the observation that the repeated PRDI motif is strongly induced by NDV, but only weakly by IFN treatment (*9*), suggests additional mechanisms. One such mechanism is the involvement of transcriptional co-activators which do not interact directly with the promoter elements that could not be detected with the conventional EMSA method (*21*). It has been shown that the IFN-β gene is under negative control by a putative repressor, which is inactivated following gene induction (*52*). In this regard, we reproducibly observed a PRDI binding activity which disappears following virus infection or dsRNA treatment (Fig. 1 and unpublished observation). The possibility of the involvement of a negative regulatory factor need to be investigated further.

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