Distinct activation of murine interferon- α promoter region by IRF-1/ISFG-2 and virus infection

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ABSTRACT

Virus infection in mouse L929 cells activates expression of interferon- α_4 (IFN- α_4), but not IFN- α_6 . The integrity of a symmetrical sequence, GTAAAGAAA-GT (α F1 site); (–103 to –93), present in the 35 nucleotide (nt) long inducible element (IE) (–109 to –75) of the α_4 promoter region is essential for the virus-induced expression. In the present study, we have shown that the interferon regulatory factor 1 (IRF-1) can induce expression of both IFN- α_4 and - α_6 in a transient expression assay. Virus infection cooperates with IRF-1 and further enhances transcription from the α_4 promoter, but inhibits the IRF-1-mediated expression from the α_6 promoter. The virus-mediated induction is determined by both IRF-1 and α F1 sites, while activation by IRF-1 in a cotransfection assay is not greatly influenced by the α F1 sequence. The activation of IFN- α gene promoters by IRF-1 was limited to the transient expression assay. The integrated α_4 promoter or the endogenous IFN- α genes could not be induced by transfection with IRF-1 expressing plasmid and IRF-1 did not up-regulate expression of the endogenous IRF-1 gene. However, expression of IRF-1 alone was sufficient to up-regulate the expression of two IFN stimulated genes, 2',5' oligoadenylate synthetase (OAS) and interferon stimulated (ISG)-15 gene. These results suggest that induction of IFN- α gene expression by virus infection requires cooperation between IRF-1 and another factor(s) that binds to the α F1 sequence.

INTRODUCTION

Interferon (IFN) - α and - β genes consist of a family of nonspliced cellular genes coding for polypeptides with antiviral and anticellular properties (1). Activation of these genes by viral infection or double-stranded (ds) RNA is a transient phenomenon which occurs at the transcriptional level, although the involvement of mRNA stability in the regulation of IFN- β gene expression

has also been implicated (2). Induction of the IFN genes does not require cellular protein synthesis that is needed for both the cessation of IFN- β gene transcription and the rapid degradation of IFN- β mRNA (3–4).

The transcriptional activation of IFN genes is mediated by the interaction of multiple transcriptional factors with the enhancer-like elements present in the promoter region of IFN genes (5, 6). However, several lines of evidence indicates that the *cis* and *trans* factors involved in the induction of the closely related IFN- α and - β genes are not identical (7). Deletion and mutation analysis of the regulatory region of the IFN- β gene have shown that this region consists of three positive regulatory domains (PRD-I, -II and -III) and two negative regulatory domains (NRD-I and -II) (8–10). PRD-I can function both as a positive and negative regulatory domain by interacting with several regulatory proteins that can either enhance or suppress PRD-I-mediated transcription (6, 11–19). The PRD-II domain contains the NF- κ B binding site. Base mutations in this site leads to the loss of inducibility of the IFN- β gene (19–25).

The human IFN- α_1 and murine IFN- α_4 promoters contain regions similar to PRD-I and -III of IFN- β , however, neither of these promoters contain the PRD-II (NF- κ B) site in the region essential for inducibility [virus responsive element (VRE) (26), IE (27)]. Previous studies in our laboratory have shown that the murine IFN- α genes are differentially expressed in NDV-infected mouse L cells (28) and that the different levels of individual IFN- α mRNAs reflect the transcriptional strength of the respective promoter regions (29). We have identified a 35 nt long virus inducible element (IE) in the IFN- α_4 gene promoter (α_4 IE) (27) and have shown that the symmetrical sequence GTAAAGAAA-GT (α F1) is critical for induction by NDV (30). The defect in the inducibility of the IFN- α_6 promoter could be mapped to the 5' and 3' guanosine residues (–103 and –94) in this sequence. However, α_4 IE also contains the putative IRF-1 binding site (–98 to –87) which is similar to the positive response elements in the IFN- β gene promoter region (14, 16, 17) and partially overlaps with the α F1 sequence. This site is also modified in the α_6 IE (G to A replacement at –94), but this replacement may not compromise the function (49).

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The present study was initiated to analyze the role of IRF-1 in the expression of murine IFN- α genes. IRF-1 and the closely related IRF-2 were originally isolated from L929 cells as nuclear proteins that specifically bind to the hexanucleotide repeat (AA-GTGA) $_4$ present in the PRD-I sequence of IFN- β gene (14, 17). It was shown later (25) that the IRF-1 is identical to the ISGF-2 protein that binds to the interferon stimulated response elements (ISRE) of IFN-induced genes, ISG-15 and ISG-54 (31). Recently, IRF-1 was identified as primary response gene in myeloid differentiation (32). Overexpression of IRF-1 in undifferentiated EC cells induced synthesis of the endogenous IFN- α as well as up-regulating the expression driven by cotransfected IFN- α or - β promoter regions (18). In contrast, IRF-2 appears to be a strong inhibitor of IRF-1-mediated activation. Harada et al. (18) suggested that the expression of an IFN gene is determined by the equilibrium between the interaction of the positive (IRF-1) and negative (IRF-2) regulators. However, several observations indicate that the differential induction of IFN- α and - β genes can not be solely explained by the difference in binding of IRF-1 and -2 to the regulatory regions of IFN genes. First, MacDonald et al. (7) demonstrated binding of IRF-1 only to the VRE of human IFN- β but not to the VRE of IFN- α_1 gene, suggesting a difference in affinity of the IRF-1 protein to the IFN- α and - β VRE and demonstrated the importance of binding of another protein (TG) to this element. Second, we have shown (30) that mutation in the α_4 IE outside of the IRF-1 binding site (G at -103) caused a significant decrease in the inducibility and concluded, therefore, that the symmetrical α F1 sequence, rather than the IRF-1 binding sequence is essential for the virus inducibility.

In light of these conflicting observations, we have designed a study which allowed us to directly analyze the ability of a transfected IRF-1 expressing plasmid to up-regulate expression driven by two IFN- α genes promoter regions. One of these promoters (α_4) is transcriptionally active and the other (α_6) inactive in virus-infected mouse L cells. Furthermore, we compared the IRF-1-mediated transactivation in transient assay with its ability to stimulate expression of endogenous IFN genes and genes induced by IFN (2',5' OAS and ISG-15 kD genes). Our results show that, in contrast to Newcastle disease virus (NDV) infection, which was able to activate the expression from α_4 but not from the α_6 promoter, transfected IRF-1 activated expression from both promoter regions in a transient expression assay, but did not activate the expression of the endogenous IFN- α genes. Surprisingly, the transfected IRF-1 also stimulated the expression of two IFN-induced genes 2',5' OAS and ISG-15. The NDV infection further enhanced the IRF-1-stimulated expression from the IFN- α_4 promoter but inhibited the IRF-1-induced activation of the IFN- α_6 promoter. These findings, together with the results of mutation analysis of IRF-1 and α F1 binding sites, suggest that the virus induced activation of IFN- α genes requires cooperation between IRF-1 and α F1 binding factor(s).

MATERIALS AND METHODS

Construction of plasmids

The plasmids containing the various lengths of α_4 and α_6 promoter regions inserted 5' of the CAT gene were described earlier (27). The α_4 IE and α_6 IE plasmids contained a single copy of the respective IE element (-109 to -75) and the plasmids containing base mutations in the α_4 IE inserted

upstream of transcriptionally inactive -88 α_4 chloramphenicol acetyltransferase (CAT) plasmid were described previously (30). Plasmids containing mutations in the putative IRF-1 binding site (-92 and -87) of α_4 IE were constructed in a similar way as described (30). IRF-1 expression plasmid (p94IRS) which contains the murine IRF-1 cDNA inserted behind the cytomegalovirus (CMV) promoter region was a generous gift from Dr. Taniguchi (33).

RNA analysis and the probes

Total RNA was prepared by the guanidine isothiocyanate method (34) and analyzed by S1 mapping with the complementary RNA CAT probe specific for the 5' end of α_4 CAT and α_6 CAT hybrids. The relative levels of correctly initiated CAT mRNA were determined by densitometric scanning. The probe for detection of the endogenous and exogenous IRF-1 was constructed by insertion of a 700-base pair (bp) *Nco*I fragment from the p94IRS plasmid in an antisense orientation into the *Hinc*II site of the SK vector and an RNA probe was prepared by *in vitro* transcription using T7 RNA polymerase. The S1 nuclease mapping using this probe gives a 622-bp long protected fragment when hybridized with the IRF-1 mRNA coded by the transfected CMV-IRF-1 hybrid gene and 500-bp long protected fragment when hybridized with the endogenous IRF-1. The riboprobe for detection of the endogenous IFN- α mRNA by Northern hybridization was constructed by insertion of a 776-bp *Eco*RI/*Bgl*II fragment of α_4 genomic DNA into pSP64 vector. The IFN- β riboprobe was constructed by the insertion of a 525-bp fragment of the β genomic DNA in the pSP64 vector. Both probes were prepared by transcription with Sp6 polymerase.

Figure 1. Schematic representation of the major features of α_4 IE. Sequence of α_4 IE is compared to α_6 IE; mutations in positions -103 and -94 that abolish the inducibility of α_4 IE are shown (mutants 43 and 30); mutations that do not affect the inducibility are presented in the upper boxes (mutants 25 and 38). The regions of α_4 IE that show protection in DNase footprint (30) as well as in *Exo*III protection assay (50) are indicated. The G residue that shows interferences in methylation interference assay (50) are marked by asterisk (*). IRF-1 binding sequence was identified by Taniguchi and Tanaka (personal commun.) and is identical to the inducible sequence identified by Naf et al. (49). The α F1 sequence was determined by mutation analysis (30).

Construction of the riboprobes for the detection of 2',5' OAS and ISG-15 mRNAs was as described previously (35).

Polymerase chain reaction (PCR) amplification of IFN- α mRNA

Two 21 oligonucleotide long primers based on nucleotides between +65 to +85 nt (5' primer) and +487 to +507 nt (3' primer) of the α_4 murine IFN gene were used (36). These primers show a high degree of conservation between all the murine IFN- α genes. For reverse transcription, we used either 10 μ g of total RNA isolated from the uninfected cells or 1.0, 0.1 or 0.01 of total RNA from NDV-induced cells (adjusted to 10 μ g of total RNA with a mixture of tRNAs) or 20 μ g of poly A RNA from IRF-1-transfected cells. RNAs were transcribed into cDNA using avian murine leukemia virus reverse transcriptase and 3' IFN- α specific primer. Then the reaction mixture containing the cDNA was diluted two-fold with PCR buffer and DNA amplified using 5' and 3' IFN- α specific primers. The temperature of the initial denaturation was 93°C and amplification was done using 40 cycles consisting of 1 min at 93°C, 1 min at 60°C, and 1 min at 72°C. A final 5 min incubation at 72°C

followed to ensure completion of all polymerized products. The amplified DNA was purified by phenol extraction and identified by electrophoresis in 5% native acrylamide gel.

Transfection and induction studies

Mouse L929 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Transfections were done as described recently (30). Briefly, cells were seeded at 2×10^6 cells/100-cm plate and 36 hr later plasmid DNA containing the α_4 CAT or α_6 CAT fusion hybrid (10 μ g) was added as a calcium phosphate precipitate. In cotransfection experiments, 5 μ g of IRF-1 plasmid was included in the transfection mixture. Cells were incubated for 4 hr at 37°C, washed with serum-free DMEM, treated with glycerol (15% for 60 sec), washed with phosphate buffered saline, incubated for 16 hr in DMEM with 10% FCS and then induced with NDV.

Figure 2. IRF-1 induces expression of both IFN- α_4 and - α_6 CAT plasmids. Hybrid plasmids containing -152 nt of α_4 or -145 nt of α_6 promoter region respectively inserted in front of the CAT gene were constructed as described previously (30). L929 cells were cotransfected with 10 μ g of respective CAT plasmid and when indicated together with 5 μ g of IRF-1 expression plasmid (p94IRS). Cells were induced with NDV 16 hr after the transfection. A. CAT activity was assayed 16 hr after induction with NDV, or 32 hr after the transfection as described in Materials and Methods. B. Total RNA was isolated from NDV-induced transfected cells at times indicated and the levels of α_4 and α_6 specific transcripts were determined by S1 analysis. Quantitation of the protected fragments was done by densitometric scanning. The α_4 CAT and α_6 CAT are shown by the open boxes and closed circles, respectively.

Figure 3. Minimal 5' upstream sequence of IFN- α_4 and - α_6 promoter region required for IRF-1-mediated activation. Hybrid plasmids (27) were transfected and induced as described in Figure 2. All the samples were collected at 32 hr after transfection with the exception of the IRF-1/NDV cont. sample that was collected at 16 hr after transfection (after NDV adsorption). The percent conversion was calculated by dividing the radioactivity (cpm) present in 3'-acetylchloramphenicol and 1'-acetylchloramphenicol fractions by the sum of radioactivity that included unconverted chloramphenicol and these two fractions. An average value from three independent experiments is given. There was a 15% variability between individual experiments.

For RNA and CAT activity assays, cells were harvested at 8 and 24 hr after NDV infection, respectively.

Assay for CAT activity

Cell lysates were collected for CAT assay either at 32 hr after the transfection or 16 hr after the induction with NDV. Constant amounts of proteins were used for the CAT assay as described recently (30). In the range of protein concentrations used the levels of CAT activity were linear.

RESULTS

Activation and suppression of the transfected α_4 and α_6 CAT hybrid plasmids in L cells

Using mutation analysis of the IFN- α_4 gene promoter region, we have shown (30) that the GTAAAGAAAGT sequence (α F1) present in the inducible element (IE) of this promoter (Fig. 1) is essential for the transcriptional activation of the IFN- α_4 gene in infected L cells and shows a footprint in a DNase I protection assay (30). Mutations in the 5' G residues (-103) (Fig. 1) abolished inducibility of the α_4 IE. The α_4 IE also contains a potential IRF-1 binding site (49 and Taniguchi and Tanaka, personal commun.) that overlaps with the α F1 sequence. To test directly the role of IRF-1 in the inducibility of the IFN- α promoter regions, we performed cotransfection experiments in mouse L cells. The IRF-1 cDNA cloned into a mammalian expression vector (33) was transfected with a reporter plasmids containing the CAT gene under the control of IFN- α_4 or α_6 promoter regions. As shown in Figure 2A, expression of IRF-1 resulted in marked increase in the expression from both -152 α_4 and -145 α_6 IFN gene promoters. In contrast, NDV up-regulated the expression from the α_4 but not from the α_6 promoter region. Since NDV infection in L cells was shown to stimulate expression of IRF-1, we examined whether the overexpression of IRF-1 negates the virus-mediated inducibility from the α_4 promoter. In fact, in α_4 CAT and IRF-1 cotransfected cells, NDV infection further increased CAT levels, indicating an additive effect. In contrast, NDV infection in α_6 CAT and IRF-1 cotransfected cells suppressed the IRF-1 induced CAT activity. Thus, depending on the promoter region, NDV either up- or down-regulated IRF-1-mediated expression.

The IRF-1- and NDV-induced expression of α_4 and α_6 CAT plasmids was also analyzed on the RNA level by S1 mapping. The relative levels of correctly initiated CAT mRNA were about the same in cells cotransfected with either α_4 or α_6 CAT and IRF-1 expression plasmids. Infection with NDV transiently

increased the relative levels of CAT mRNA in cells transfected with α_4 CAT plasmid and IRF-1 plasmids, while it decreased CAT mRNA levels in cells transfected with both the α_6 CAT and IRF-1 (Fig. 2B). The CAT mRNA disappeared from both α_4 CAT and α_6 CAT transfected cells at 14 hr post-NDV-infection.

Sequences required for the IRF-1-mediated activation and NDV induced suppression

Using 5' deletion analysis of the IFN- α_4 promoter region, we have previously shown that the deletions extending up to -109 nt were inducible by both virus and poly rI.rC. (27). In the present study, we examined whether the NDV- and IRF-1-mediated induction have the same sequence requirements. The results of these experiments, summarized in Figure 3, show that all the 5' deletions of the α_4 CAT plasmids that were inducible by virus were also transactivated by IRF-1 and this IRF-1-mediated transactivation was further enhanced by NDV infection. However, neither IRF-1 nor the combination of IRF-1 and NDV could induce the -97 deletion that was not inducible by virus. Since the -97 deletion is missing the 5' G residue that in part of both the IRF-1 and α F1 site, these results indicate that the presence of this residue in the IRF-1 site is required for recognition by IRF-1 and NDV. The IRF-1- and NDV-induced CAT activity decreased gradually as the deletion extended from -464 to -109 , suggesting that additional sequences upstream from the α_4 IE element contribute to the inducible expression of this promoter. In contrast, all the α_6 CAT deletions tested and α_6 IE were inducible only by IRF-1 and not by viral infection. The IRF-1-mediated transactivation was about equal with the -428 α_6 and -145 α_6 CAT plasmids and a sharp decrease in the inducibility was observed between the -145 and -105 deletions (20- and 7-fold stimulation, respectively). This partial loss in inducibility correlates with the deletion of the IRF-1-like site at -111 to -116 nt. However, the replacement of G residue by A at -94 in the putative IRF-1 site of the α_6 promoter did not interfere with the inducibility by IRF-1. In all α_6 CAT deletion plasmids tested, the IRF-1-mediated transactivation was suppressed by infection with NDV, suggesting that the IRF-1-mediated activation and the NDV-mediated inhibition occurs through the recognition of a common or overlapping sequence.

We have previously shown (30) that mutations in the 5' and 3' G residues (-103 and -94 , respectively) of the GTAAAGAAAGT (α F1) sequence abolished the ability of the α_4 IE sequence to confer inducibility by NDV. However, only one of

Table 1. IRF-1 and NDV-mediated inducibility of α_4 IE and its mutated analogues

Mutations	Cont.	CAT activity (% conversion)		IRF-1/NDV	IRF-1	Relative inducibility	
		IRF-1	NDV			NDV	IRF-1/NDV
α_4 IE	0.2	5	6	12	25	30	60
α_4 IE -104	0.4	5	11	25	12	28	62
α_4 IE $-82, -104, -108$	0.5	6	10	15	12	20	30
α_4 IE $-94, -103$	0.3	2	0.3	0.3	7	1	1
α_4 IE -103	0.3	2	0.2	0.4	7	0.6	1
α_4 IE -92	0.2	0.2	0.2	ND	1	1	—
α_4 IE -87	0.4	5	16	ND	12	40	—
α_6 IE	0.3	2	0.2	0.2	7	0.6	1

The α_4 -IE/CAT plasmid and its mutated analogues were transfected in the presence or absence of IRF-1 expressing plasmid (32). The number indicates position of the mutation shown in Figure 1. The average of three independent experiments is given. The variability in relative inducibility among different experiments was about ± 0.2 for controls, 1.5 for IRF-1-transfected cells, and 2.5 for NDV-induced cells, respectively. ND-not done. Induction and time of CAT activity assays are described in Materials and Methods.

these two mutations (–94) is localized within the IRF-1 recognition sequence. To investigate which base substitutions in the α F1 and IRF-1 sites alter the IRF-1-mediated inducibility, we compared the inducibility of the α_4 -IE CAT plasmids containing either the mutations in F-1 or IRF-1 sites. The results show that IRF-1 overexpression increased the basal activity of a majority of the mutated α IE₄ CAT plasmids tested (Table 1). Plasmids with mutations outside of α F1 and IRF-1 sequences (–104 and –108, –104, –82, respectively) were induced only about 2-fold less efficiently by IRF-1 than the unmodified α_4 IE plasmid, indicating that the base substitution outside the α F1 and IRF-1 binding sites did not significantly affect the IRF-1-mediated induction. The base substitution in the α F1 site (–94 and –103) decreased the IRF-1-mediated inducibility of α_4 IE by about 3-fold and similarly, the α_6 IE was induced about 3-fold less efficiently by IRF-1 than α_4 IE. By contrast, the induction by NDV was much more sensitive to changes in the α F1 site and even a single nucleotide change (–103) completely abolished the inducibility by NDV. The coinduction with IRF-1 and NDV was also very sensitive to the alterations in the α F1 site. Both the lack of inducibility and suppression of IRF-1-mediated induction by NDV was seen in mutants containing base replacement either at –103 or –103, and –94 positions as well as in α_6 IE CAT plasmid. These results show that the IRF-1 mediated up-regulation of the α_4 -IFN gene promoter is not abolished by mutations in the α F1 sequence, and that the NDV-mediated activation and suppression occurs through the recognition of the α F1 sequence. The ability of α F1 binding site to confer inducibility was further tested by experiments in which the α F1 sequence was inserted in a single or multiple (three) copies in front of the transcriptionally inactive –88 α_4 CAT plasmid.

Figure 4. The insertion of α_6 IE or α_6 promoter region next to the α_4 IE or α_4 promoter does not suppress NDV-mediated inducibility of the α_4 enhancer region. Hybrid plasmids were prepared as described in Materials and Methods. Transfection, induction, and CAT assays were done as described in Figure 2 and percentage of acetylation was calculated as described in Fig. 3. Average value from three independent experiments that showed 20% of variability are given.

However, neither NDV nor IRF-1 were able to activate the transcription of the α F1 α_4 CAT plasmid in a transient expression assay (50).

To determine whether the putative IRF-1 DNA binding site is essential for the activation of α_4 IE by IRF-1 and by NDV, we introduced two single mutations into this site, outside of the region that overlapped with α F1 binding site. The G to A substitution in position –87 of α_4 IE did not greatly affect inducibility either by IRF-1 or by NDV. In contrast, the substitution in position –92 completely abolished the inducibility of α_4 IE by both IRF-1 and by NDV, suggesting that the –92 G residue is essential for IRF-1-mediated inducibility. These data, together with the results of deletion analyses, indicate that only α_4 IE, which contain both α F1 and IRF-1 binding sites, can be induced by viral infection.

We have previously shown that α_4 IE can confer NDV-mediated inducibility, when inserted 5' of the minimal heterologous (–57 HIV LTR) promoter (30), that alone is transcriptionally inactive. However, an attempt to activate the α_4 IE –57 HIV CAT plasmids by transfection with IRF-1 in a transient expression assay was unsuccessful because the IRF-1 alone inhibited expression of HIV LTR (data not shown). The silencing of constitutively expressed SV40 enhancer by sequences from human IFN- α promoter was previously observed by Kuhl et al. (37).

Figure 5. IRF-1 is unable to activate the endogenous IFN- α promoters. (A) Mouse L cell line (pRB22) containing the integrated hybrid plasmid in which the –464 nt of IFN- α_4 promoter were inserted 5' of the CAT gene (27) or the mouse cell line containing integrated plasmid containing the (AATGAA)₁₂ repeats inserted 5' of –54 α_4 CAT plasmid were either induced with NDV or transfected with IRF-1 (10 μ g); CAT activity in cell lysates was determined 30 hr after the transfection or 20 hr after NDV induction. Induced expression of a transiently transfected (AATGAA)₁₂ –54 α_4 CAT plasmid is shown for comparison. (B) Mouse L929 cells were either transfected with IRF-1 (10 μ g), induced with NDV or treated with murine IFN- α , - β (500 U/ml). Total RNA (15 μ g) isolated from the cells 12 hr post-induction was analyzed by Northern hybridization for the presence of IFN- α and - β specific transcripts. The medium was collected and analyzed for the antiviral activity as described recently (48). (C) Total RNA analyzed by Northern hybridization (in B) was reverse transcribed and amplified by PCR as described in Materials and Methods. The amplified DNA separated on a 5% acrylamide gel was detected by Southern hybridization with ³²P-labeled IFN- α specific probe (28).

The α_6 IE does not repress inducibility of IFN α_4 promoter

Since the α F1 sequence and its mutated analogue (mt α F1) are involved in both virus-induced activation and repression of the α_4 IE and α_6 IE regions respectively, we wanted to determine whether the presence of α_6 IE will interfere with the inducibility of an adjacent α_4 promoter. Others (38) have identified a negative regulatory sequence in the upstream region of IFN- α_{11} gene promoter that was able to suppress the inducibility of IFN- α_4 promoter. We constructed, therefore, reporter genes in which α_4 IE or α_6 IE were inserted 5' to the -145 α_6 CAT or -118 α_4 CAT plasmids, respectively. These plasmids were transfected into L cells, either alone or together with IRF-1 expressing plasmid and induced with NDV. As a control, cells were transfected either with -145 α_6 CAT or -118 α_4 CAT plasmids in the presence or absence of IRF-1 expressing plasmid. The results show that the α_6 IE was not able to suppress the NDV-mediated expression of the α_4 promoter (Fig. 4, lane 4). Furthermore, the construct containing the α_4 IE inserted 5' of the α_6 promoter region was consistently induced much more efficiently by both IRF-1 and NDV (lane 2) than the other plasmids (lane 3 and 4). Surprisingly, however, we did not detect any additive effect between the IRF-1 and NDV induction in this plasmid. Altogether, these results indicate that the activity of α_4 IE in infected cells is not diminished by the presence of α_6 IE *in cis*.

Transfected IRF-1 is not able to activate the endogenous IFN- α genes

In contrast to the transient expression assay, transfection with the IRF-1 expression plasmid was not able to induce CAT activity in the cells containing integrated α_4 CAT plasmid (27), while CAT activity could be easily induced by NDV infection (Fig. 5A). We have previously shown (27) that, in a transient expression assay, NDV infection induces strongly the (AATGAA) $_{12}$ hexanucleotide repeat. This repeat (50), similarly as AAGTGA repeats (18) has high affinity for high affinity for IRF-1. We have, therefore, examined whether transfected IRF-1 can activate the expression of an integrated fusion plasmid containing 12 copies of the AATGAA hexanucleotide inserted in front of transcriptionally inactive -54 α_4 CAT plasmid [(AATGAA) $_{12}$ -54 α_4 CAT] (27). However, transfection with IRF-1 was not able to enhance CAT levels above the constitutive levels. Interestingly, activation of this plasmid by NDV infection was also much less efficient than the activation of α_4 CAT plasmid (Fig. 5A). In transient expression assay both NDV and IRF-1 were effective inducers.

To determine whether transfection with IRF-1 can induce expression of the endogenous IFN genes in L cells, as reported by others (33), we analyzed the levels of IFN- α and - β mRNA and biologically active IFN in cells transfected with the IRF-1 expressing plasmid. Since the biological assay allows us to detect pg quantities of IFN protein, we should have been able to detect IFN synthesis even in a small population of transfected cells. However, no biologically active IFN was induced in IRF-1 transfected cells, while IFN synthesis was induced in NDV-infected cells. Similarly, when the expression of IFN- α and - β genes was examined on the RNA level by Northern blot analysis, we could detect IFN- α and - β mRNAs only in NDV-infected but not in IRF-1 transfected cells (Fig. 5B). To determine unambiguously whether transfection with IRF-1 activates the expression of IFN- α genes, we used PCR analysis. Poly A RNA

isolated from IRF-1 transfected cells was reverse transcribed and the complementary cDNA was amplified by PCR, using primers specific for two conserved regions of IFN- α genes as described in Materials and Methods. However, even by this very sensitive method, no IFN- α mRNA transcripts could be detected in IRF-1 transfected cells. In NDV-infected cells, IFN- α specific transcripts could be identified in even as little as 0.01 μ g of total RNA (Fig. 5C). These data indicate that under the experimental conditions used in this study, transfected IRF-1 plasmid did not induce the expression of endogenous IFN- α genes or the integrated IFN- α promoter regions.

IRF-1 increases levels of ISG-15 and 2',5' OAS mRNA but does not stimulate expression of IRF-1 mRNA

It was suggested that IRF-1 plays a role in the activation of IFN-induced genes. The ISGF-2/IRF-1 binding domains can be found in the ISRE of IFN-induced genes, such as are the major histocompatibility class I genes, 2',5' OAS, ISG-15, and ISG-54 genes. It was further shown that IRF-1 binds with lower affinity to PRD-1 in the VRE of the IFN- β gene than to the ISRE of IFN induced genes (25, 18) and that it can activate the expression of the H-2 promoter linked to a CAT gene in a transient expression assay (16, 39). We examined, therefore, whether transfection of the IRF-1 plasmid is able to enhance the expression of two IFN-induced endogenous genes, ISG-15 and 2',5' OAS. Both of these genes were expressed constitutively at low levels in L cells. The results show that the expression of the ISG-15 gene was enhanced in cells transfected with IRF-1 as effectively as in cells infected with NDV or treated with exogenous IFN (α/β mixture 500 U/ml) (Fig. 6A). Similarly, the relative levels of 2',5' OAS mRNA were greatly enhanced in IRF-1 transfected cells, NDV-infected or IFN-treated cells. These data indicate that the transfected IRF-1 expressing plasmid enhanced expression of two IFN-regulated genes, ISG-15 and 2',5' OAS. The enhanced level of ISG-15 and 2',5' OAS mRNAs could be detected only in cells transfected with IRF-1 expressing vector, but not in cells transfected with plasmid containing the HIV-1

Figure 6. IRF-1 enhances expression of 2',5' OAS and ISGF-15 mRNA but does not up-regulate its own expression. (A) Total RNA was isolated from NDV-infected L cells (8 hr post-infection), IFN-treated cells (500 u/ml for 6 hr) or IRF-1-transfected cells (24 hr after transfection) analyzed by Northern hybridization with 2',5' OAS and ISGF-15 specific riboprobes as described in Materials and Methods. (B) S1 analysis of IRF-1-specific transcripts coded for by the endogenous (e) and transfected (t) IRF-1 genes. The construction of the probe was described in Materials and Methods; the transcripts from the transfected IRF-1 gene were detected as a 622-nt long protected fragment, transcripts from the endogenous genes as a 500-bp long fragment. Times of RNA isolation after induction or transfection were the same as described in A. The amounts of total RNA used for Northern and S1 analysis were 15 and 10 μ g, respectively.

rev gene (40) under the regulation of the CMV promoter region. These data indicate that the induction of ISG-15 and 2',5' OAS is not due to the activation by dsRNA. While this study was in progress Reis et al. (41) have also shown enhanced levels of 2',5' OAS and HLA-B7 mRNAs in human cells constitutively expressing IRF-1.

We were further interested to examine whether the IRF-1 can up-regulate its own expression as suggested by others (41). Using a S1 nuclease analysis with a riboprobe that protected a 622-bp long fragment when hybridized with the IRF-1 mRNA coded by the transfected IRF-1 plasmid, and a 500-bp long fragment when hybridized with the IRF-1 mRNA transcribed from the endogenous IRF-1 gene, we were able to distinguish between the IRF-1 mRNA encoded by the endogenous and the transfected gene (see Materials and Methods). The results have shown (Fig. 6B) the presence of the endogenous IRF-1 mRNA in uninduced L cells, as shown by others (39). The relative levels of IRF-1 mRNA increased about 4-fold after NDV infection while no significant increase was seen in cells treated for 8 hr with exogenous IFN (500 u/ml) or transfected with IRF-1. The lack of enhancement in IRF-1 mRNA levels in IFN-treated cells may be due to our inability to detect a small increase against a high constitutive expression of IRF-1 mRNA. In human cells, treatment with IFN- α and - β was shown to increase the levels of ISGF-2/IRF-1 mRNA or protein (25, 42). The IRF-1 mRNA encoded by the transfected IRF-1 expressing plasmid could be easily detected in transfected cells. No enhancement in the relative levels of CMV driven IRF-1 mRNA was detected in NDV-infected or IFN-treated cells, since CMV early promoter region does not respond to NDV or IFN. These results show that the transfected IRF-1 plasmid does not up-regulate the expression of the endogenous IRF-1 gene. Since the levels of IRF-1 mRNA were relatively high in uninduced cells, we can not exclude the possibility of a small increase in the relative levels of IRF-1 mRNA in transfected cells. However, the absence of IRF-1 binding site in the promoter region of the IRF-1 gene (Taniguchi, personal commun.) correlates with the observed inability of IRF-1 to up-regulate its own synthesis.

DISCUSSION

In this study, we have shown that, in a transient expression assay, IRF-1 can activate expression from the IFN- α_4 and α_6 promoter regions. Surprisingly, however, NDV infection inhibited the IRF-1-mediated induction of the α_6 promoter activity, while it further enhanced transcription from the α_4 promoter. These results suggest that, in addition to IRF-1, virus induces another factor(s) that can either stimulate or repress the IRF-1-mediated activation of IFN- α gene promoters.

Our data demonstrated that, in L cells, IRF-1 binding site is required, but not sufficient, for the NDV-mediated induction of the IFN- α_4 promoter. Mutation in the -92 guanosine residue, but not in the -87 residue of the IRF-1 binding site, abolished the inducibility of α_4 IE mediated either by IRF-1 or by NDV. Interestingly, replacement of the same G residue in the PRD-I sequence abolished inducibility of IFN- β promoter region (43). There are several indications, however, that essentially rule out the possibility that virus induction of α_4 IE occurs only through an increased abundance or post-transcriptional modification of the IRF-1 protein (44). (i.) The difference in the DNA sequence requirement for the IRF-1 and NDV-mediated induction and the fact that the activation of IFN- α_4 promoter or α_4 IE by IRF-1

and NDV is additive, strongly suggest involvement of at least two factors in the induction process. The requirement for activation of two factors, IRF-1 and NF- κ B, has already been well documented in the virus-mediated induction of IFN- β (19–25, 41, 45). (ii.) The fact that the overexpression of IRF-1 from a transfected plasmid was not able to induce expression of an endogenous α_4 promoter or IFN- α genes indicates that, in addition to IRF-1, another binding factor is required for the induction of endogenous IFN- α genes. Others have also shown that constitutive expression of IRF-1 alone was not sufficient to stimulate expression of the endogenous IFN- β gene, but enhanced the virus-mediated inducibility (11, 42, 41). These data suggest that, in the integrated state, the α_4 promoter may not be accessible to interact with IRF-1 and that cooperation with another inducible protein is required. Interestingly, the transient expression assay has been used nearly exclusively to demonstrate the transactivating potential of IRF-1. (iii.) The deletion analysis of the IFN- α promoter region has shown that the presence of sequence between -109 to -97, containing the α F1 site, is essential for both the NDV and IRF-1-mediated induction. Furthermore, in gel shift assays, purified IRF-1/ISGF-2 protein (25) neither binds to α F1 sequence, nor could the binding of an induction-specific nuclear protein to α F1 be competed by the A-GTGAA nucleotide repeats that show a high affinity for IRF-1 (50). The requirement for a second factor that may cooperate with IRF-1 was also shown to be necessary for the induction of human IFN- α_1 promoter. MacDonald et al. (7) identified a TG sequence in the VRE of IFN- α_1 to be essential for induction and suggested that cell type-specific expression of IFN- α and - β sequences may involve cooperation between binding factors that are both common and unique for regulation of the IFN- α and - β genes, respectively.

An unexpected finding was that, while NDV infection further enhanced IRF-1-mediated induction of IFN- α_4 promoter, it inhibited IRF-1-mediated induction of IFN- α_6 promoter. Furthermore changes in the α F1 sequence that negated inducibility of α_4 IE by NDV concomitantly lead to the NDV induced suppression of IRF-1-mediated expression. These results suggest that the absence of IFN- α_6 expression in infected L cells may involve the binding of an NDV-induced repressor that has high affinity for the α_6 α F1 site. A single base replacement in the PRD-I recognition sequence was shown to cause a marked difference in the binding activity of PRD-I-BF repressor protein to the IFN- β gene promoters (16). The regulation of gene expression by competition of positive and negative regulatory proteins for a DNA binding site is not unique to the IFN system and was also recently demonstrated in other systems such as the NF- κ B-directed transcription (46). The question that remains is whether the repression of the α_6 promoter is mediated by one of the previously identified repressors such as the PRDI-BF or IRF-2 repressors. However, both of these repressors bind specifically to PRD-I elements of the IFN- β gene promoter that is different from the α F1 sequence and the PRD-I sequence is not able to compete the binding induced specific nuclear factor to α F1 sequence (50). We believe that it is unlikely that the suppression is mediated by IRF-2, since in a transient expression assay, IRF-2 can inhibit IRF-1-mediated, but not NDV-mediated induction of α_4 IE (50). Furthermore, IRF-2 was shown to be present at high levels in uninduced L929 cells (18) and induced by IRF-1 (Taniguchi, personal commun.). The IRF-2-mediated suppression could, therefore, interfere with the induction of both α_4 and α_6 promoter regions.

In contrast to its inability to induce endogenous IFN genes, IRF-1 was able to up-regulate the expression of two IFN-induced genes 2',5' OAS and ISG-15. In fact elevated constitutive expression of this factor leads to elevated expression of several other endogenous genes (51), and the HLA-B7 gene (41). Why is the overexpression of IRF-1 able to up-regulate expression of IFN induced genes while it is not able to stimulate expression of the endogenous IFN genes? There may be at least two reasons for this difference. The first is that IRF-1 binds with much higher affinity to the ISRE binding site of ISG-15 than to PRD-I (25, 18) or the α_4 IE sequence (50). The second is that IRF-1 may be able to up-regulate only expression of the genes that are constitutively expressed (e.g., 2',5' OAS, ISG-15, H-2) by interacting with other transcriptional factors and enhancing their transcriptional efficiency. Whether one of these mechanisms or both of them are operative remains to be determined. However, the results presented, together with the observations that expression of IRF-1 gene can also be up-regulated by several cytokines (25, 42, 47, 48) and by differentiation inducers (32), suggest that IRF-1 may function by interaction with various transcriptional factors at weak binding sites or may act alone at the strong binding sites and therefore, it can up-regulate transcription from many different promoters. However, the possibility that IRF-1 induces the expression of IFN- α promoter region by an indirect action can not be also excluded.

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