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Activation of IFN-β element by IRF-1 requires a post-translational event in addition to IRF-1 synthesis

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ABSTRACT

Expression of the Type I IFN (i.e., IFN-αs and IFN-β) genes is efficiently induced by viruses at the transcriptional level. This induction is mediated by at least two types of positive regulatory elements located in the human IFN-β gene promoter: (1) the repeated elements which bind both the transcriptional activator IRF-1 and the repressor IRF-2 (IRF-elements; IRF-Es), and (2) the κB element (κB-E), which binds NFκB and is located between the IRF-Es and the TATA box. In this study we demonstrate that a promoter containing synthetic IRF-E, which displays high affinity for the IRFs can be efficiently activated by Newcastle disease virus (NDV). In contrast, such activation was either very weak or nil when cells were treated by IFN-β or tumor necrosis factor-α (TNF- α), despite the fact they both efficiently induce *de novo* synthesis of the short-lived IRF-1 in L929 cells. In fact, efficient activation of the IRF-E apparently requires an event in addition to *de novo* IRF-1 induction, which can be elicited by NDV even in the presence of protein synthesis inhibitor, cycloheximide. Moreover, efficient activation of the IRF-E by NDV is specifically inhibited by the protein kinase inhibitor, Staurosporin. Hence our results suggest the importance of IRF-1 synthesis and post-translational modification event(s), possibly phosphorylation for the efficient activation of IRF-Es, which are otherwise under negative regulation by IRF-2.

INTRODUCTION

Interferons (IFNs) belong to a family of cytokines which elicit anti-viral activity. IFN-α and IFN-β (also referred to as Type I IFNs) are structurally and functionally related. Like other cytokines, IFNs also manifest multiple biological activities on various target cells, particularly affecting the regulation of cell growth and differentiation (1–5). The production of Type I IFNs is induced by a variety of stimuli such as viruses and cytokines, but the efficiency of induction is variable. RNA viruses such as Newcastle disease virus (NDV) are the most efficient inducers, whereas only a modest level of induction can seemingly be achieved by cytokines such as IFNs, TNFs, IL-1s and M-CSF, in various cell lines (6–11).

A number of gene transfection studies have revealed the presence of regulatory sequence elements within the 5′ upstream region of Type I IFN genes. In the IFN-β gene, at least two such elements play an essential role in the virus-induced activation of the 5′ promoter region: the upstream elements which bind both the transcriptional activator, IRF-1 and the repressor, IRF-2 (12–14), and a downstream decamer element which binds NFκB and/or NFκB-like factor (15–17). Although the precise DNA sequences required for the IRF binding have not yet been determined, our previous studies indicated the possible existence of multiple IRF binding sites (14). Hence, these sequence motifs are tentatively referred to as IRF-elements (IRF-Es) (Figure 1a). Two of the IRF-Es (i.e. IRF-E1 and IRF-E2) overlap with PRD-I and PRD-III, respectively, and the downstream element referred to here as the κB-element (κB-E), overlaps with PRD-II (Figure 1a). Both PRD-I and II were originally identified on the basis of the promoter deletion analysis (18, 19), and PRD-III was identified as a virus-inducible element in its multimerized form (20). Mutation analyses of the IFN-β promoter region have revealed the importance of cooperativity between the IRF-Es and κB-E in the efficient induction of gene transcription (15, 20–22). In particular, a deletion in the region containing the IRF-Es results in the complete inactivation of the promoter, suggesting the absolute requirement for the IRF-Es in the IFN-β gene induction (23, 24). By using a transient CAT assay system, it has been shown previously that IFN-α and IFN-β promoters are not activated efficiently by expressing the IRF-1 cDNA in a mouse fibroblast line, L929 (13, 22), but are activated efficiently in an undifferentiated EC cell line, P19 which lacks expression of the endogenous IRF-1 and IRF-2 genes (14). Thus, the failure to achieve activation of the IFN-β promoter (i.e. IRF-Es) by the expression of IRF-1 cDNA is likely to be due to the presence

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of the endogenous repressor, IRF-2 in L929 cells. In fact, expression of IRF-2 in P19 cells by cDNA transfection results in the inhibition of the IRF-1 effects described above (14). These observations thus raise the critical issue of how the IRF-Es become activated in L929 cells by NDV.

In the present study, we examined the effect of NDV and cytokines on a synthetic IRF-E which efficiently binds IRFs, and on $\varkappa$B-E each in multimerized form. We demonstrate that this IRF-E can be efficiently activated by NDV but not by IFN-β and TNF-α; whereas the $\varkappa$B-E can be activated by either NDV or TNF-α. We show here that both NDV and these cytokines induce the biosynthesis of a short-lived pool of IRF-1 to a similar extent in L929 cells, and provide evidence that the cytokine-induced, but not NDV-induced IRF-1 needs a post-translational modification(s) for efficient activation of IRF-E. In fact, our results argue for the potential importance of phosphorylation event(s) in the activation of IRF-Es by IRF-1.

MATERIALS AND METHODS

Cell culture and induction

Mouse L929 cells were cultured in ES medium (Nissui, Tokyo) supplemented with 5% fetal bovine serum. Virus induction was performed by infection with Newcastle disease virus (NDV) as described by Fujita *et al.* (24).

Poly(rI):poly(rC) (100 μg/ml) was added to monolayer cells in the presence of DEAE-dextran (500 μg/ml) in Iscove's modified Dulbecco's medium (Sigma) supplemented with 10% Nu-serum (Collaborative Research Inc.) for 1 hr. Recombinant mouse IFN-β (Toray, Tokyo; 3×10^7 units/mg) and recombinant human TNF-α (Dainippon Pharmaceutical, Osaka; 3.15×10^6 units/mg) were added to the culture medium at the concentration of 10^3 units/ml.

DNA transfection and CAT assay

DNA transfection was performed by DEAE-dextran method (25). CAT activity was measured as described by Fujita *et al.* (24).

Antisera

Antisera used in this study were prepared as described by Harada *et al.* (14).

Gel-shift assay

Whole cell extracts were prepared as described by Harada *et al.* (14). Nuclear extracts were prepared as described by Dignam *et al.* (26) except all buffers were supplemented with 0.5 mM PMSF, 100 μg/ml leupeptin, 10 mM sodium molybdate, 10 mM sodium orthovanadate and 100 mm NaF. Gel-shift assays were carried out as described by Harada *et al.* (14).

Western blot analysis

Whole cell extracts were made as follows; cells were harvested by rubber policeman from cell culture dishes, washed once with PBS (phosphate-buffered saline) and collected by centrifugation. Pelleted cells were suspended with 4 times volume of RIPA buffer (50 mM Tris (pH 8.0), 150 mm NaCl, 1% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 0.1% (w/v) SDS) containing 1 μg/ml leupeptin, 100 μg/ml L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK), 50 μg/ml N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 1 μg/ml pepstatin A and 100 μg/ml PMSF. The suspensions were sonicated for 2 min and centrifuged at 10000 rpm for 10 min. The supernatant was subjected to SDS−PAGE in 10% polyacrylamide gel. After electrophoresis, the proteins were transferred electrophoretically to Nytran membrane (Schleicher and Schuell) in a buffer containing 127 mM Tris, 1 M glycine and 20% (v/v) methanol for 90 min. The membrane was blocked by incubation in PBS containing 1% (w/v) non-fat powdered milk for 1 hr, and then incubated in 2 ml of PBS containing 0.05% tween 20, 1% (w/v) bovine serum albumin and 1:1000 dilution of anti-IRF-1 antiserum for further 12 hr at 4°C. The blots were then probed with ^{125}I-labeled protein G.

Cell labeling and immunoprecipitation

L929 cells (2×10^6) were cultured in 35 mm dishes in 0.5 ml serum-free, methionine-free MEM (Nissui, Tokyo) supplemented with a mixture of ^{35}S-methionine and ^{35}S-cystein (EXPRE^{35}S^{35}S, NEN Research Products) at 37°C for 20 min. After labeling, cells were washed once with PBS, harvested by rubber policeman and collected by centrifugation. Two different systems were used for the preparation of whole cell extracts. In the case of IRF-1, pelleted cells were suspended in 200 μl of complete RIPA buffer, which was supplemented with 100 μg/ml PMSF, 1 μg/ml leupeptin, 10 mM sodium molybdate, 10 mM sodium orthovanadate and 100 mM NaF. The suspensions were sonicated for 2 min and centrifuged at 1×10^5 rpm for 30 min. The supernatant was diluted with 400 μl of complete RIPA buffer. For IRF-2, cells were lysed for 30 min at 4°C with 200 μl of NP-40 lysis buffer (500 mM Tris, (pH 8.0), 500 mM NaCl, 1% (v/v) NP-40, 1 μg/ml leupeptin, 100 μg/ml TPCK, 50 μg/ml TLCK, 1 μg/ml pepstatin A and 100 μg/ml PMSF). The cell lysates were centrifuged at 1×10^5 rpm for 30 min. The supernatant was diluted with 400 μl of 50 mM Tris (pH 8.0) containing 20 mg/ml BSA and 0.05% (v/v) tween 20. Each preparation was then aliquoted, and samples were reacted with the appropriate antiserum for 1 hr at 4°C. Then 100 μl of a 10% (v/v) protein A−Sepharose beads that were suspended in the complete RIPA buffer were added. Incubation proceeded for an additional 1 hr at 4°C. The beads were washed 4 times with 1 ml of complete RIPA buffer. After washing, immune complexes were eluted in SDS sample buffer by boiling in a water bath for 5 min. SDS−polyacrylamide gel electrophoresis was followed by fluorography.

Construction of plasmids

The plasmids p-125cat, p-55C1B and p-55A2 were constructed as described previously (15, 24, 27). The plasmid pC1Btk was constructed by ligating the following four pieces of DNA: (i) EcoRI-BglII fragment from tkM2 (28), whose BglII site was converted to HindIII site: (ii) SalI−BamHI fragment from p-55C1B, whose SalI site was also converted to HindIII site: (iii) the following synthetic DNA

5′ GATCCCCGCCCAGCGTCTTGTCATTGGCG 3′
3′ GGGCGGGTCGCAGAACAGTAACCGCTTAA 5′

(iv) HindIII backbone fragment of pSV00cat (29).

S1 mapping analysis

Total cellular RNA was prepared by the guanidine thiocyanate method (30). S1 mapping analysis was carried out as described by Fujita *et al.* (24). For detection of mRNA transcribed from the HSVtk-CAT fusion gene, the DNA fragment derived from pC1Btk (from the BamHI site at −105 to the PvuII site, which is located within the CAT structural gene) was subcloned between

the BamHI and HincII sites of M13mp18. For an analysis of mRNA transcribed from the RSV-tk fusion gene, the DNA fragment derived from pRSVtk (TaqI site, which corresponds to the −12 from the initiation site of RSV-LTR, to the HaeIII site which is located within the tk structural gene) was also subcloned between the AccI and SmaI sites of M13mp11. The specific activities were $5-10\times10^6$ cpm/pmol. The RNA copy numbers were estimated as described previously (24).

RESULTS

Effect of virus and cytokines on the activation of the IFN-β gene regulatory elements

The gene construct, p-125cat in which CAT gene expression is driven by the IFN-β gene promoter (−125 to +19) (23) was transfected into mouse L929 cells and induction of the reporter gene by NDV or cytokines was examined (Figure 1b). NDV, a potent IFN-α and IFN-β inducer in L929 cells, strongly induced the CAT gene expression, as demonstrated previously (15). However the two cytokines tested, IFN-β and TNF-α, did not induce the gene expression to a significant level. These cytokines are reported to induce IFN production in some cells, albeit at very low levels (8, 10, 11, 31). In fact, the IFN-β mRNA levels induced by IFN or TNF in human FS-4 cells are far lower than those induced by virus or by poly(rI):poly(rC) (11, 31). Thus, the virus but not the cytokines efficiently induces IFN-β promoter activation.

Effect of virus and cytokines on the activation of the IRF-E and κB-E

In order to examine the responses of the regulatory elements in the IFN-β promoter to the above stimuli, we next prepared a set of CAT gene constructs each containing either the multimerized κB-element (κB-E, Figure 2a) or a synthetic IRF-E which consists of the AAGTGA sequence repeated 8 times (IRF-synthetic element, IRF-sE; Figure 3). As shown previously, κB-E binds NFκB and/or NFκB-like factor (15) and IRF-sE binds IRF-1 or IRF-2 (13). p-55A2, which contains the κB-E

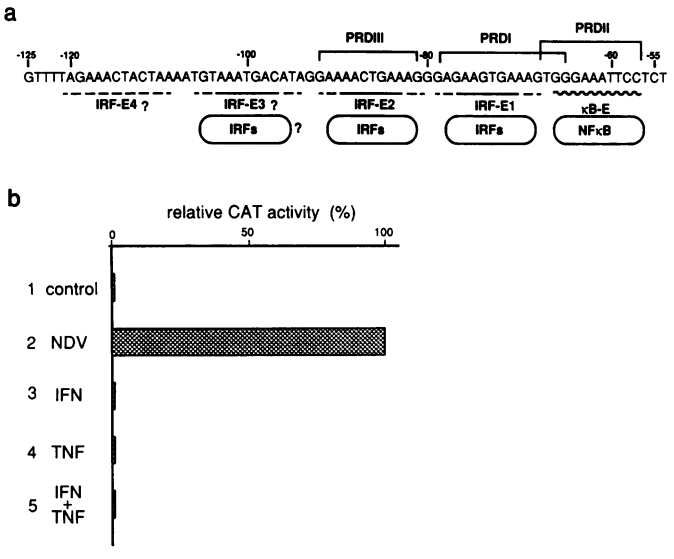

Figure 1. a Regulatory region of IFN-β gene. Numbering is from the cap site of the human IFN-β gene (23). PRD-I and PRD-II are delineated according to Fan and Maniatis (21) and PRD-III according to Leblanc *et al.* (20). Possible core recognition sequences of IRFs (i.e. IRF-Es) are underlined, in which broken lines show tentatively defined regions. Wavy line indicates NFκB or NFκB-like factor(s) binding site (i.e. κB-E). Possible transacting factors are also shown at the bottom of the figure. **b** Activation of natural regulatory element of IFN-β gene by NDV or cytokines. 5×10^6 L929 cells were transfected with 20 μg of p-125cat reporter gene (27). Twenty-four hours after transfection, cells were split to 5 dishes by trypsinization. Therefore, the transfection efficiencies are essentially the same between the samples which are to be compared with one another. Subsequently incubated for 24 hr until cells were infected by NDV or treated with mouse recombinant IFN-β (10^3 U/ml) or human recombinant TNF-α (10^3U/ml). Cell lysates were prepared 12 hr after NDV infection or 8 hr after cytokine treatments and subjected to CAT activity analysis. In calculating the relative CAT activity, CAT activity from the NDV-induced cells (41.1% conversion) was taken as 100%. The transfection experiments were repeated at least three times and the results are highly reproducible.

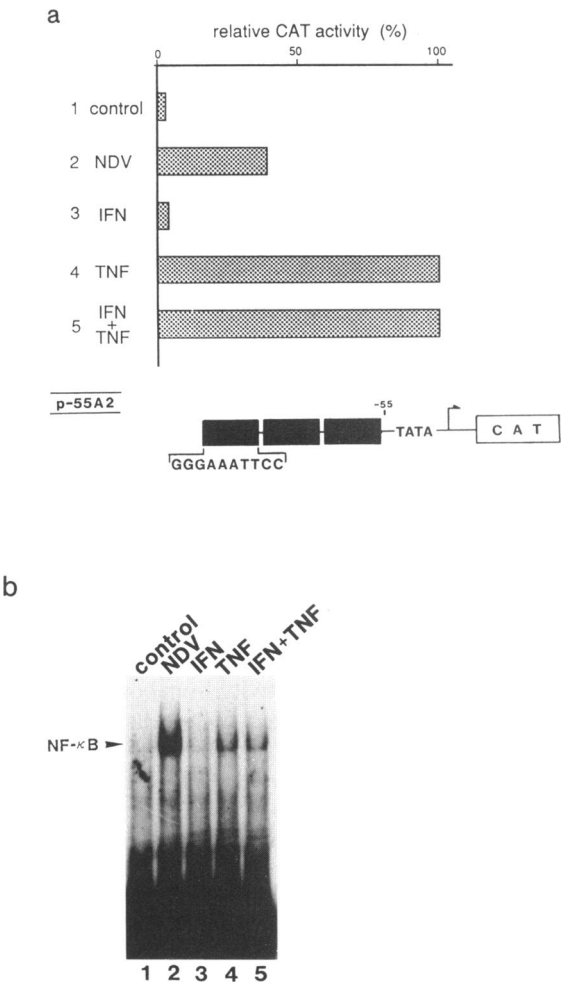

Figure 2. a Activation of κB-element by NDV or cytokines. L929 cells were transfected with p-55A2 reporter gene (15). Subsequent procedures were similar to those of described in Figure 1b. Lower panel indicates the schematic picture of reporter gene. Relative CAT activities were calculated by taking a CAT activity from TNF-induced cells (38.9% conversion) as 100%. The experiments were repeated at least three times and results remained unchanged. Black box shows κB-element, whose sequence is observed within the IFN-β promoter (Figure 1a). **b** Detection of κB-element binding activity in nuclear extracts of L929 cells induced by NDV or cytokines. Cells were infected by NDV for 6 hr or treated with cytokines for 2 hr. Nuclear extracts were prepared as described in Materials and Methods. Three micrograms of each extracts were subjected to gel-shift assay using ^{32}P-labeled A2 oligomor (15) as a probe. The arrowhead indicates the position of NFκB or NFκB-like factor-DNA complex.

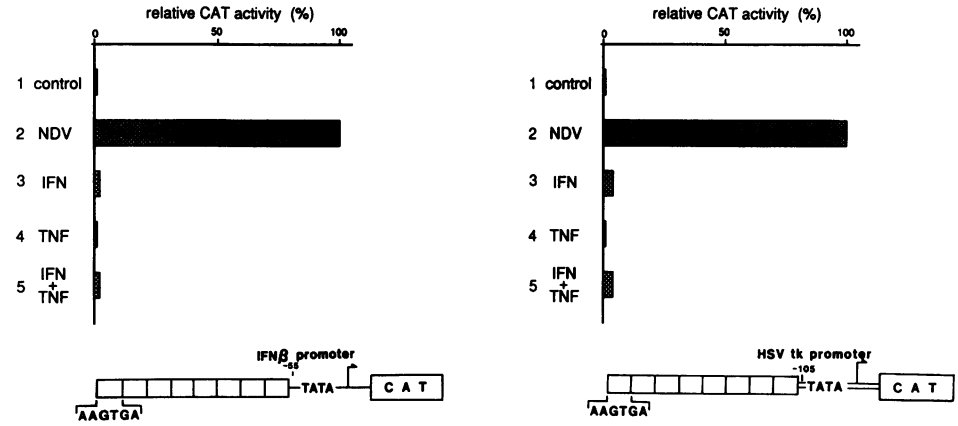

Figure 3. Activation of IRF-synthetic element (IRF-sE) by NDV or cytokines. Each reporter gene, p-55C1B (left panel) and pC1Btk (right panel) contains 8 times repeated hexamer (AAGTGA) upstream of IFN-β or Herpes Simplex Virus (HSV) *tk* gene promoter, respectively. Experiments were carried out in the same way as described in Figure 1b. CAT activity from NDV-induced cells (37.5% and 70.9% conversion for p-55C1B and pC1Btk, respectively) was taken as 100%. The transfection experiments were repeated at least three times and the results are highly reproducible. Lower panel of each side indicates the schematic picture of reporter gene. A white box shows a AAGTGA hexamer sequence.

repeated 3 times in the upstream of the CAT gene, was induced by NDV and even more strongly by TNF-α (Figure 2a). This finding is in agreement with previous reports (15−17, 32). In contrast, IFN-β had no effect on gene induction. Consistent with the measurements of CAT gene induction, the DNA binding activity of NFκB to the multimerized decamer sequence is strongly induced in the nuclear fraction by NDV and TNF-α but not by IFN-β (Figure 2b). The kinetics of the appearance of NFκB activity induced by NDV or TNF-α shows that the times at which the extracts were prepared (9 hr and 2 hr, after exposure to NDV or TNF-α, respectively) corresponded to the peaks of factor accumulation (data not shown).

We next examined the properties of IRF-sE, by using two plasmid constructs p-55C1B and pC1Btk, each containing 8 repeats of the hexamer AAGTGA, i.e. IRF-sE which is abutted upstream of IFN-β (−55 to +19) (23) or Herpes Simplex Virus (HSV) *tk* (−105 to +56) (33) gene promoter, respectively.

As shown in Figure 3, CAT activity from both constructs was similarly induced in NDV-infected L929 cells (50−100 fold). In fact, NDV strongly stimulated the expression of both constructs, whereas IFN-β increased CAT gene expression only 4−6 fold as has been reported by others (21). Unlike the case with κB-E, the IRF-sE in these constructs was not affected by TNF-α (Figure 3).

Expression of IRF-1 and IRF-2 proteins by NDV or cytokines

In order to raise antisera for the quantitation of IRF-1 and IRF-2, they were respectively expressed in *E.coli* to produce 48 kD and 50 kD polypeptides as judged by SDS−PAGE (data not shown). Partially purified recombinant proteins were then used as immunogen to obtain rabbit antisera. The antisera thus obtained specifically precipitated either 48 kD (anti IRF-1) or 50 kD (anti IRF-2) proteins from NDV-infected L929 cell extracts (data not shown). Although these proteins exhibit extensive homology in the primary structure of their DNA binding domain, no cross reactivity was detected with either of the antisera (Figure 4; J.Sakakibara unpublished observation).

Using these antisera, the stability of these factors was first examined by a pulse label-chase experiment (see Materials and Methods). Perhaps surprisingly, a big difference was found between IRF-1 and IRF-2 in the NDV-infected L929 cells. In fact, the results presented in Figure 4a indicate that IRF-1 protein is very unstable (half life ~30 min). On the other hand, IRF-2 protein is apparently stable and its half life is calculated to be more than 8 hr.

In order to monitor the DNA binding activity of IRF-1 and IRF-2, we carried out a gel shift experiment using the above antisera (Figure 4b). Cell extracts prepared from L929 cells infected by NDV or treated with cytokines were subjected to gel shift assay using a ^{32}P-labeled synthetic oligomer, containing 3 repeats of the AAGTGA hexamer, as a probe. The cell extracts from uninduced cells exhibited a single DNA-protein complex, whose formation could be blocked by anti-IRF-2 antiserum, but not by anti-IRF-1 antiserum. This indicates that in uninduced cells the DNA binding activity for IRF-2 is dominant over that for IRF-1. It is worth noting that the mRNA levels for these factors in uninduced L929 cells do not differ significantly; i.e. about 1 and 3 strands per cell for IRF-1 and IRF-2, respectively (13). In the light of the above observation (Figure 4a), the different expression levels of the IRF activities could be explained, at least in part, by the instability of the IRF-1 protein compared to IRF-2 (Figure 4a). Infection by NDV or treatment with IFN-β or TNF-α, resulted in the appearance of a new band that migrates faster than the IRF-2-DNA complex. Formation of this band is specifically inhibited by anti-IRF-1 antiserum, indicating that IRF-1 activity is induced in stimulated cells. This is in agreement with our previous finding that IRF-1 mRNA is induced by these cytokines as well as by NDV (13, 31). In the experimental conditions employed, the level of IRF-1 induced by IFN-β is 3−4-fold higher than that induced by TNF-α as judged from gel shift or Western blotting experiments (see below, Figure 4c). The IRF signals in the NDV infected cells appear to be relatively weaker than those in IFN-β-treated cells (1/2 to 1/4, in repeated experiments). Presumably, certain infected cell populations are already dead or protein synthesis is inhibited by the virus, thereby giving such a low yield of IRFs.

By using the anti-IRF-1 antiserum, we next determined the IRF-1 protein level by Western blotting. As demonstrated in Figure 4c, IRF-1 was below detectable level in the extract from uninduced cells. However, NDV, IFN-β and TNF-α transiently

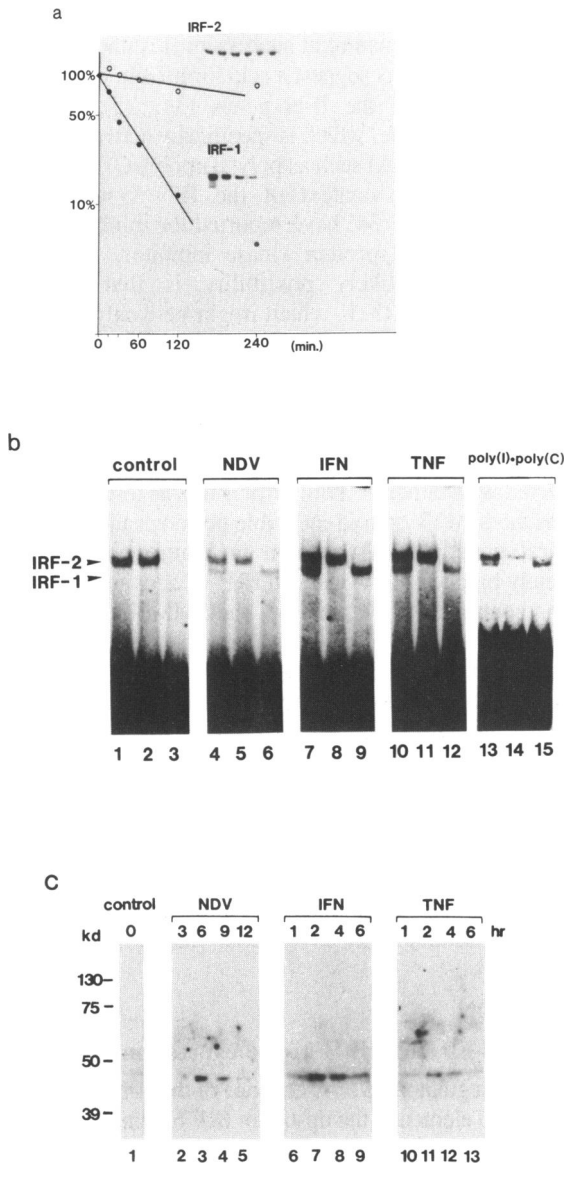

Figure 4. **a** Stability of IRF-1 and IRF-2 proteins. L929 cells (1.9×10^6 cells) infected by NDV for 6 hr were labeled by incubation with a mixture of ^{35}S-methionine and ^{35}S-cysteine (500 μCi) for 20 minutes. After the label, cells were washed and chased in the presence of excess unlabeled methionine and cysteine. After the chase indicated time, cell extracts were prepared and subjected to immunoprecipitation and SDS−PAGE analysis. The relative values of incorporated radioactivity (●; IRF-1, O; IRF-2) were measured by densitometric scanning of the autoradiograms. **b** Induction of IRF proteins by NDV, double-stranded RNA or cytokines. Cells were induced by NDV or IFN-β or TNF-α or poly(rI):poly(rC). Whole cell extracts were prepared 6 hr after NDV-induction or 2 hr after cytokine-induction or 3 hr after poly(rI):poly(rC)-induction. Extracts each obtained from 3×10^5 cells were subjected to gel-shift assay using a ^{32}P-labeled C13 oligomer (27). Lanes 1, 4, 7, 10 and 13, 2 μl of control serum was incubated in the reaction mixture; lanes 2, 5, 8, 11 and 14, 2 μl of anti-IRF-1 antiserum were incubated; lanes 3, 6, 9, 12 and 15, 2 μl of anti-IRF-2 antiserum were incubated. The arrowheads indicate the positions of the factor-DNA complex. **c** Western blot analysis of IRF-1 induced by NDV or cytokines. L929 cells were infected by NDV or treated with IFN-β or TNF-α. At the indicated times after induction, whole cell extracts were prepared. Extracts each obtained from 3×10^5 cells were subjected to Western blot analysis.

induced the accumulation of IRF-1 protein with kinetics very similar to that of the accumulation of IRF-1 mRNA (13, 31). This observation again suggests that the half life of IRF-1 protein, either induced by virus or cytokines is significantly short.

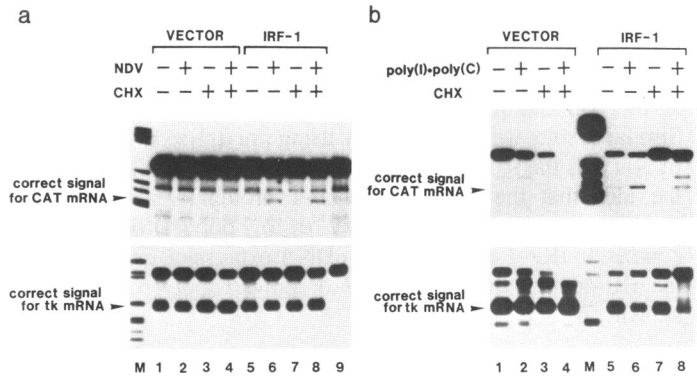

Figure 5. Synergistic effect of IRF-1 synthesis and NDV- or poly(rI):poly(rC)-mediated signal on activation of IRF-sE. **a** L929 cells were co-transfected with pC1Btk reporter plasmid (schematic picture shown in Figure 3) and pCMIRS, a IRF-1 expression vector (lanes 5−8) or CDM8, control vector (lanes 1−4). After incubation at 37°C for 36 hr, cells were induced by NDV in the presence or absence of cycloheximide (100 μg/ml). After further incubation for 6 hr, total RNA were isolated. Forty micrograms of total RNA were subjected to S1 mapping analysis. The arrowheads indicate positions of the protected probes corresponding to the correctly initiated mRNA (reporter gene; 210 bp, reference gene; 61 bp). Lane M; size markers were denatured ^{32}P-labeled, HaeIII-digested fragments of pBR322. Lane 9, probes were annealed with 40 μg of yeast transfer RNA and then subjected to S1 mapping analysis. **b** Each transfected L929 cells were exposed to poly(rI):poly(rC) for 1 hr in the presence or absence of cycloheximide. Incubation proceeded for 2 hr until the cells were harvested to isolate the total RNA. Twenty-eight micrograms of total RNA were subjected to S1 mapping analysis.

Requirement of IRF-1 expression in the NDV-induced activation of IRF-sE

As described above, IRF-1 synthesis is induced by NDV, IFN-β and TNF-α, but only NDV can efficiently induce the activation of IRF-sE (Figure 3). In order to address this issue further, we carried out the following DNA co-transfection experiments. L929 cells were co-transfected with pCMIRS, an IRF-1 expression vector (25) and the pC1Btk reporter plasmid, which contains 8 times repeats of the hexamer AAGTGA, i.e. IRF-sE abutted upstream of HSVtk gene promoter (Schematic picture shown in Figure 3). After incubation at 37°C for 36 hr, these cells accumulated IRF-1 protein in the nucleus (with ~1% transfection efficiency as determined by antibody staining, T.Kimura, unpublished observation). The cells were then induced by NDV in the presence of cycloheximide (100 μg/ml, a concentration that inhibits *de novo* IRF-1 synthesis >99% as determined by radioactive amino acid incorporation followed by immunoprecipitation; T.Fujita, unpublished observation). Under these conditions, NDV should not induce *de novo* IRF-1 synthesis but may be capable of delivering other signal(s) for IRF-1 expressed by the transfected cDNA.

The correctly initiated transcript is detectable by S1 analysis in cells co-transfected with pC1Btk and CDM8 (control vector), and then induced by NDV (Figure 5a, lane 2: 69 copies mRNA/cell). In this assay, the transcription largely depends on *de novo* protein synthesis because the transcript was below detectable levels in the presence of cycloheximide (lane 4: <8 copies mRNA/cell). Increase of the mRNA was also below detectable in cells co-transfected with pC1Btk and pCMIRS (IRF-1 expression vector) without NDV induction (lane 5: <8 copies mRNA/cell). On the other hand, NDV treatment of the transfected cells resulted in an efficient induction of the mRNA (lane 6: 138 copies mRNA/cell). In the presence of cycloheximide

this induction also occurs, and results in the production of higher levels of the mRNA (lane 8: 186 copies mRNA/cell), indicating that in cells accumulating IRF-1, transcriptional activation of IRF-sE by NDV can occur in the absence of *de novo* protein synthesis. Similar results were obtained by using poly(rI):poly(rC) instead of NDV as the inducer (Figure 5b). The above findings support the idea that the activity of IRF-1 protein is a function of modification triggered by NDV or by poly(rI):poly(rC). In contrast to IRF-1 cDNA, IRF-2 cDNA expression did not induce the IRF-sE activation by NDV or poly(rI):poly(rC) (data not shown).

The protein kinase inhibitor Staurosporin blocks IRF-1 mediated activation of IRF-sE

The above results suggest that efficient activation of IRF-sE requires a signal(s) in addition to that for IRF-1 synthesis which can be elicited by NDV or poly(rI):poly(rC), but not by IFN-β or TNF-α. The exact nature of such a signal is unclear at present, but a number of reports suggest a role for protein phosphorylation in the regulation of the IFN genes (34, 35). The role of serine/threonine kinase, which is specifically activated by double-stranded RNA (dsRNA) such as poly(rI):poly(rC), has been well-characterized in the context of the IFN system regulation (36–39). Zinn *et al.* (34) have reported the inhibition of IFN-β gene induction by a protein kinase inhibitor, 2-aminopurine (2-AP). Thus, a likely possibility is that the specific phosphorylation of IRF-1, which might be catalyzed by such a kinase, is required for the IRF-1-mediated activation of IRF-sE.

As an approach to further address this issue, we tested the effect of Staurosporin which is a selective inhibitor for serine/threonine protein kinases. The effective dose (50–100 nM) of Staurospoiin is much lower than 2-AP (10 mM) (34, 40). L929 cells were transfected with either pC1Btk or pGMCS as test gene. pGMCS contains the MMTV steroid-inducible promoter upstream of CAT structural gene (41). As shown in Figure 6a, Staurosporin completely blocked NDV-induced IRF-sE activation (lanes 6 and 8). In contrast, activation of the MMTV promoter by dexamethasone is not inhibited by the drug under similar assay conditions (lanes 1–4, 30 and 10 fold in the absence and presence of Staurosporin, respectively). Staurosporin moderately stimulated the MMTV promoter (lanes 1–4, 2–5 fold). Gel-shift assay revealed that IRF-1 protein can still be induced by NDV in the presence of Staurosporin, albeit the level is reduced by approximately 4–5 fold (Figure 6b). Thus, it is unlikely that complete inhibition of pC1Btk activation by Staurosporin is due to this modest inhibition of IRF-1 synthesis, suggesting that Staurosporin also blocks the IRF-1 modification pathway which may directly or indirectly involve the action of kinase(s).

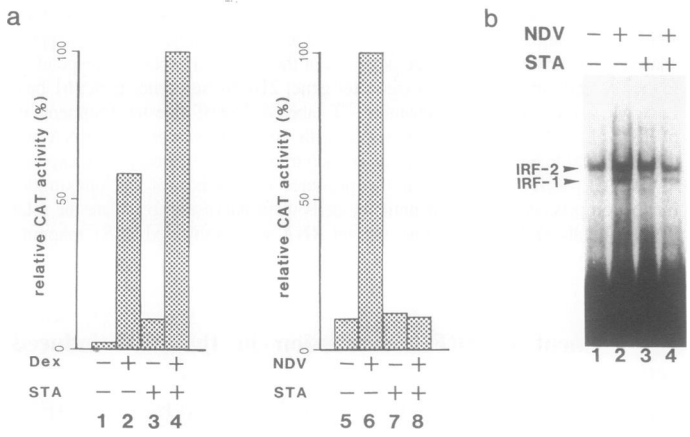

Figure 6. a The effect of protein kinase inhibitor, Staurosporin on activation of IRF-element by NDV. 3×10^6 cells were transfected with 20 μg of either pGMCS (lanes 1–4) or pC1Btk (lanes 5–8) as reporter gene. Twenty-four hours after transfection cells were split into 6 dishes and further cultured 20 hr until the cells were induced by dexamethasone or NDV in the presence or absence of Staurosporin (80 nM). Cell lysates were prepared 12 hr after induction and subjected to CAT activity analysis. In calculating the relative CAT activity, CAT activity from the NDV-induced cells (7.5% conversion) in the absence of Staurosporin was taken as 100% for lanes 5–8, and CAT activity from the dexamethasone-induced cells (32.1% conversion) in the absence of Staurosporin was taken as 100% for lanes 1–4. The transfection experiments were repeated at least three times and the results are highly reproducible. **b** Induction of IRF-1 by NDV in the presence of Staurosporin. L929 cells were induced by NDV in the presence or absence of Staurosporin. Whole cell extracts were prepared 8 hr after induction and 60 μg each of extracts were subjected to gel-shift assay using end-labeled C13 oligomer as a probe. The total cell number (approx. 1×10^5 cells) used for the preparation of each extract remained the same, therefore possible fluctuation between the samples will, if at all, not be large. The arrowheads indicate the positions of the factor-DNA complex.

DISCUSSION

Role of IRF-Es and κB-E in the expression of IFN-β gene

The positive regulatory DNA sequence of the IFN-β gene consists of at least two elements, the upstream IRF binding elements (IRF-Es) and the downstream κB element (κB-E) (Figure 1a). In this study, activation of the individual elements induced by various stimuli was investigated (Figure 1b, 2 and 3). The summary of these results (Table I) shows that the inducer for both elements, i.e. NDV is the only potent inducer of the IFN-β promoter. IRF-sE is activated efficiently by NDV but only weakly by the cytokines, such as IFN-β and TNF-α, whereas κB-E is activated by both NDV and TNF-α. Although we have not examined the properties of IRF-Es other than IRF-sE in the present study, we think it is likely that other IRF-Es also behave in a similar manner in view of previous observations (22, 24). Our results agree with

Table I Activation of IFN-β upstream elements and induction of binding activities by the various stimuli

stimuli	IRF element induction of IRF-1	activation of IRF-sE	κB element induction of NFκB	activation of κB-E	activation of IFN-β *cis* element
NDV	++	+++	+++	++	+++
poly(rI):poly(rC)	++	++	+[a]	+[a]	++
IFN-β	++++	±	−	−	−[b]
TFN-α	+++	−	++	+++	−[c]

[a]Reference for Visvanathan *et al.* (17).
[b]Inducible in certain cell systems (Kohase *et al.*, 8).
[c]Detectable by PCR analysis (Fujita *et al.*, 31; Reis *et al.*, 11).

a previous report that lipopolysaccharide (LPS), a potent inducer of NF$\varkappa$B activity, cannot induce the IFN-β gene whereas Sendai virus infection can efficiently induce the gene in a pre-B cell line (16). These observations are also in agreement with results reported more recently by Leblanc *et al.* (20); i.e. that the concomitant activation of IRF-Es and $\varkappa$B-E is required for the efficient activation of the IFN-β promoter (20).

Regulation of IRF-Es by IRF-1 and IRF-2

In many cell types, the activity of IRF-Es is tightly controlled; its constitutive activity is very low and its induced activity is dependent on the nature of the stimulus. This all-or-none type of regulation is achieved by the interaction of both positive (IRF-1) and negative (IRF-2) transcription factors with the same sequence motifs, i.e. IRF-Es. In uninduced L929 cells, IRF-Es can also function as silencers when they are interposed between viral enhancers and downstream promoters (27, 42). Hence, the activation of the p−55C1B and pC1Btk promoters and the silencing of the enhancers in NDV-induced and uninduced L929 cells, respectively, can be mediated by the same sequence element, IRF-sE. As for silencing, we have shown in the present study that IRF-2 is present at high levels in uninduced L929 cells, and this is compatible with the idea that the IRF-sE binds IRF-2 and thus silences the enhancers (13).

The question then arises as to how the repressing effect of IRF-2 on IRF-sE can be overcome and how in turn IRF-sE can be activated by NDV, but not by TNF-α and IFN-β (Figure 3). In this regard, we have shown that IRF-1 synthesis is highly induced by all of these stimuli (Figure 4). Furthermore, IRF-1 thus induced by NDV or cytokines localizes primarily in the nucleus as examined by cell fractionation (our unpublished data). Therefore, the induction of IRF-1 synthesis alone cannot account for the observed difference between NDV and the cytokines. These observations suggest the requirement for a signal(s) other than that for the induction of IRF-1 synthesis which is elicited by NDV but not by the above cytokines in the activation of IRF-sE. It has been previously reported that DNA elements containing the IRF-sE could respond almost equally to IFN treatment or Sendai virus infection (21). However, our observations demonstrate that NDV is a much stronger inducer of IRF-sE than IFN-β. Are these observations compatible with each other? This difference can probably be explained by the fact that Sendai virus is apparently less than 1/10 as efficient as NDV for inducing the IFN-β gene in mouse L929 cells (M.Tsuneoka and E.Mekada, personal communication). Thus, an increase in IRF-1 protein levels alone is not sufficient for efficient transcriptional activation. In this regard, we have previously shown that a CAT reporter gene which contains the same IRF-sE can be induced by co-transfection with a IRF-1 expression vector (pCMIRS), as monitored by the accumulation of the CAT enzyme activity (13). Presumably, in this case CAT mRNA is expressed due to the high levels of IRF-1, but at relatively low levels. Assuming that this weak expression continues over a long period of time, the relatively stable CAT enzyme may accumulate to significant levels, whereas the relatively unstable CAT mRNA may not increase significantly above detectable levels, as it does in NDV-infected cells.

When cells expressing IRF-1 by cDNA transfection are induced by NDV or poly(rI):poly(rC) in the presence of cycloheximide, efficient gene expression mediated by the IRF-sE is observed (Figure 5). Hence neither IRF-1 expression alone nor induction alone is sufficient for full activation of the gene. Since activation of the IRF-sE can be observed in the presence of cycloheximide in the IRF-1 cDNA-transfected cells, this indicates that NDV infection and poly(rI):poly(rC) treatment may activate a modification reaction(s) which results in the activation of the IRF-1 molecule, presumably resulting in its efficient binding to IRF-Es (14).

It is known that cytokines such as IFNs and TNF can 'prime' the cells to produce increased levels of IFN-β (43). In view of our present study this priming effect is most likely due to the induction of IRF-1 by the cytokines.

Our results with the protein kinase inhibitor, Staurosporin suggest that protein phosphorylation may be involved in the postulated IRF modification. In this respect, it is interesting that *E.coli* derived IRF-1 and IRF-2 can be phosphorylated *in vitro* by the dsRNA-dependent protein kinase in the presence of poly(rI):poly(rC) (our unpublished observation). It remains to be clarified as to whether or not such phosphorylation results in the modification of any IRF properties (e.g. DNA binding affinity, recognition sequence). Metabolic labeling followed by immunoprecipitation indicates that both IRF-1 and IRF-2 proteins induced by either IFN or NDV are phosphorylated and that the bulk level of phosphorylation of both IRFs remains essentially the same for either inducer (T.Fujita, unpublished data; see also 44). However, this does not necessarily exclude the possibility that dsRNA-dependent protein kinase modulates IRF-1 properties by phosphorylating at additional specific amino acid residues. It is also possible that even IRF-2 changes its properties in response to phosphorylation at specific sites. More detailed analysis is required to clarify this issue. Although less likely, we cannot rule out the possibility that the specific protein phosphorylation occurs not on IRFs but on other molecules, in earlier steps of NDV-induced signal transduction, and that this event may induce other kinds of modifications of IRFs. In fact, a similar mechanism has been known to operate in the activation of the AP-1 transcription factor (45).

It has been known that the endogenous IFN-β gene can be transcriptionally induced in the presence of cycloheximide in many different cell types. In L929 cells, NDV infection can trigger the accumulation of IFN-β mRNA in the presence of cycloheximide although to a lower level (1/5 to 1/10) than in the absence of cycloheximide. In view of our previous data showing the low level expression of IRF-1 mRNA in uninduced L929 cells (13), it is likely that low levels of IRF-1 protein are present in cycloheximide treated cells and that this IRF-1 may participate in IRF-E activation. It is worth noting that extended cycloheximide treatment of up to 9 hr prior to virus induction abolished the activation of IRF-sE whereas transcription from CMV promoter was unaffected (C.Weissmann and H.Ruffner, personal communication). The results presented here may further point to the importance of IRF-1 synthesis and post-translational modification signal(s), in particular phosphorylation pathway, which can be elicited by the virus or poly(rI):poly(rC) in the efficient activation of IRF-Es.

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