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Critical role of a common transcription factor, IRF-1, in the regulation of IFN-β and IFN-inducible genes

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Interferon regulatory factor 1 (IRF-1) is a protein that binds to *cis*-elements within the promoter of interferon (IFN)-β and some IFN-inducible genes. We used a human fibroblast line, GM-637, to generate stable transfectants constitutively expressing IRF-1 mRNA in either the sense or antisense orientation. Upon induction with poly(I)·poly(C) or Newcastle disease virus, cells expressing sense IRF-1 mRNA produced significantly higher levels of IFN-β mRNA and protein than control cells, whereas cells expressing antisense IRF-1 mRNA produced little or no IFN-β mRNA and protein. Furthermore, clear differences were seen among the transfectants in the level of expression of two IFN-induced genes (2′-5′-oligoadenylate synthetase and class I HLA). Our data show that IRF-1 is essential for the induced expression of the IFN-β gene. The results also indicate an important role of IRF-1 in the expression of IFN-inducible genes and suggest a role for IRF-1 in many other cytokine actions.
Key words: cytokines/interferons/IRF-1/transcription factor

Introduction

Interferons (IFNs) are pleiotropic cytokines inducible in a variety of cells. Viruses, double-stranded RNA and some cytokines can act as inducers of IFN-α or -β (type I IFN) gene expression (Weissmann and Weber, 1986; Pestka *et al.*, 1987; Taniguchi, 1988; Vilček, 1990). All the information necessary for the regulation of IFN-α and -β gene expression is located within the 5′ flanking regions of these intronless genes (Fujita *et al.*, 1988; Goodbourn and Maniatis, 1988; Hug *et al.*, 1988; Leblanc *et al.*, 1990; MacDonald *et al.*, 1990). In the IFN-β gene, two types of elements in the 5′ promoter region are known to be important in virus-induced activation; these include upstream elements that bind the transcriptional activator, IRF-1, and the repressor, IRF-2 (Miyamoto *et al.*, 1988; Harada *et al.*, 1989, 1990), and a downstream element that binds NF-κB (Fujita *et al.*, 1989b; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989). Multiple IRF binding sites have been identified in the IFN-β promoter (Harada *et al.*, 1990; Watanabe *et al.*, 1991); two of these sites overlap with sites termed PRD-I (Keller and Maniatis, 1988; Goodbourn and Maniatis, 1988) and PRD-III (Leblanc *et al.*, 1990), identified as positive regulatory domains either by deletion analysis or on the basis of virus inducibility after multimerization, respectively. The NF-κB binding element overlaps with a site termed PRD-II, identified by deletion analysis (Goodbourn and Maniatis, 1988). The existence of a potential negative regulatory domain was also proposed on the basis of mutational analysis and *in vivo* footprinting data (Zinn and Maniatis, 1986; Goodbourn and Maniatis, 1988).

Several earlier studies have demonstrated the important roles of IRF-1 and IRF-2 in the regulation of type I IFN gene expression. For example, multimers of AAGTGA or AAATGA, the IRF binding sites, could mediate inducibility by virus (Fujita *et al.*, 1987, 1988; Miyamoto *et al.*, 1988; Raj *et al.*, 1989). Also, a construct containing a reporter gene under control of the IRF binding site as the only enhancer element was inducible by co-transfection with an IRF-1 expression vector (Harada *et al.*, 1990; Leblanc *et al.*, 1990; MacDonald *et al.*, 1991), and such activation could be repressed by expression of IRF-2 (Harada *et al.*, 1990). Furthermore, cDNA-directed expression of IRF-1 (but not IRF-2) led to transcriptional activation of endogenous IFN-α and -β genes in COS cells (Fujita *et al.*, 1989a). Finally cDNA-directed expression of IRF-1 in undifferentiated embryonal carcinoma cells (in which IRF-1 and IRF-2 genes are not functional) efficiently activated transfected IFN-α and -β genes, besides activating the endogenous IFN-α gene (Harada *et al.*, 1990). These findings, together with the observations that all treatments which increase IFN-β mRNA levels also increase IRF-1 mRNA levels (Fujita *et al.*, 1989c) and that full activation of the IFN-β promoter requires the presence of the IRF binding domains (Leblanc *et al.*, 1990), suggest that IRF-1 is necessary for full transcriptional activation of the endogenous IFN-β gene. Available evidence also suggests that IRF-1 may be involved in the regulation of some IFN-inducible genes. For example, it was shown that IRF-1 (and IRF-2) bind to the interferon response sequence (IRS) in the promoter region of the murine H-2D^d class I major histocompatibility antigen gene (Korber *et al.*, 1988; Miyamoto *et al.*, 1988), and that expression of IRF-1 cDNA in undifferentiated embryonal carcinoma cells resulted in the efficient activation of the mouse H-2 gene promoter (Harada *et al.*, 1990). Contrary to the evidence summarized above, Pine *et al.* (1990) concluded that IRF-1 is neither necessary nor sufficient for IFN-β gene expression.

We felt that the most direct way to clarify the issues concerning the role of IRF-1 in the regulation of IFN-β and IFN-induced genes would be to create cell lines that either underexpress or overexpress IRF-1, and to examine their responses to IFN inducers or IFN-β. In the present study, we generated stable transfectants of GM-637 cells, a SV40-transformed human skin fibroblast line, expressing the IRF-1 mRNA in either sense or antisense orientations, and

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we investigated the responses of these cells to inducers of IFN-β as well as to the actions of IFN-β on gene expression. Our results clearly demonstrate that IRF-1 is essential for IFN-β induction by poly(I)·poly(C) and Newcastle disease virus (NDV). In addition, our results reveal a role for IRF-1 in the regulation of two important groups of IFN-induced genes, the 2′-5′ oligoadenylate synthetase and class I major histocompatibility antigen genes. This interesting redundancy in the roles of IRF-1 helps to explain some common features of gene activation observed earlier with viruses, double-stranded RNA, interferons and some other cytokines (Wathelet *et al.*, 1987; Hug *et al.*, 1988; Fujita *et al.*, 1989c; Vilček, 1990).

Results

IFN-β induction in GM-637 cells expressing sense or antisense IRF-1 mRNA

Cloned lines of the SV40-transformed human GM-637 fibroblasts, stably transfected with plasmids expressing IRF-1 mRNA in the sense or antisense orientation, were generated as described in Materials and methods. To assess the capacity of these lines to produce IFN-β, the cells were stimulated either with the double-stranded RNA, poly(I)·poly(C) or with Newcastle disease virus (NDV), and the amounts of IFN-β released into the culture medium were quantified by immunoassay (Table I). None of the cell lines produced detectable IFN-β without exposure to either poly(I)·poly(C) or NDV. Stimulation with poly(I)·poly(C) produced low levels of IFN-β in cells transfected with the control plasmid (C1), significantly higher levels in the two lines expressing sense IRF-1 mRNA (S1 and S7), and no detectable IFN-β in the lines expressing antisense IRF-1 mRNA (AS11 and AS18). Stimulation with NDV (which is a more potent IFN-β inducer in GM-637 cells than double-stranded RNA) led to the production of moderate levels of IFN-β in the C1 line, high levels in the S1 and S7 lines, and low levels in the AS11 and AS18 lines. Treatment of the cells with IFN-β prior to stimulation with poly(I)·poly(C) ('priming') increased the capacity of cells to respond to double-stranded RNA, leading to IFN-β yields similar to those seen with NDV. Hence, under three different conditions of stimulation, cells expressing sense IRF-1 mRNA produced more IFN-β, and cells expressing antisense IRF-1 mRNA produced less IFN-β than control cells.

To analyze the relationship between IRF-1 and IFN-β induction we compared the levels of IRF-1 and IFN-β mRNAs in the C1, S1 and AS11 lines by Northern blot analysis at different times after stimulation with poly-(I)·poly(C) or NDV (Figure 1A and B). Only the S1 cell line expressed detectable IRF-1 mRNA at 0 h, i.e. before stimulation with either poly(I)·poly(C) or NDV. The slower migrating, relatively invariant band hybridizing with IRF-1 cDNA, seen in the S1 cell line, apparently represents IRF-1 mRNA constitutively synthesized off the transfected IRF-1 cDNA, whereas the faster migrating band seen in the same blots evidently corresponds to the inducible endogenous IRF-1 mRNA. It is interesting that the accumulation of the inducible IRF-1 mRNA upon stimulation with either poly(I)·poly(C) (Figure 1A) or NDV (Figure 1B) was more rapid and enhanced in the S1 line, compared with the C1 line. This finding suggests a positive autoregulatory action of IRF-1 on IRF-1 mRNA accumulation. As expected, the levels of IRF-1 mRNA were significantly lower in AS11 cells than in the control cells, especially upon NDV induction (Figure 1B). Levels of IFN-β mRNA showed a similar pattern, i.e. they were not only enhanced but also accumulated more rapidly in the S1 line, while being undetectable (Figure 1A) or very low (Figure 1B) in the AS11 cells. IFN-β mRNA accumulation peaked at 4 and 2 h after poly(I)·poly(C) induction in C1 and S1 cells, respectively. Upon NDV induction, mRNA levels peaked at 12, 9 and 15–18 h in C1, S1 and AS11 cells, respectively. The relative total amounts of mRNA synthesized in the C1, S1 and AS11 cells were about 1:4.7:<0.1, respectively, in the case of poly(I)·poly(C) induction, and 1:1.5:0.2, respectively, in the case of NDV induction, as estimated from the area under the peaks of graphs generated by densitometric analysis of the autoradiograms (data not shown). Inoculation with NDV was cytotoxic for the cells after ~9 h, as seen from the gradual decrease in actin mRNA levels with increasing times of incubation, especially in the S1 cells (Figure 1B). Therefore, if one takes this point into consideration, the relative induction level of the IFN-β mRNA by NDV should be higher for the S1 cells.

IRF-1 protein levels in GM-637 cells expressing sense or antisense IRF-1 mRNA

Gel mobility shift analysis was used to quantify levels of IRF-1 protein in extracts of C1, S1 and AS11 cells, which

Table I. IFN-β production in GM-637 cell lines expressing sense or antisense IRF-1 mRNA

Induction	IFN yield (units/10^6 cells)[a] from cell line				
	C1	S1	S7	AS11	AS18
None	<5	<5	<5	<5	<5
poly(I)·poly(C)[b]	13 ± 3	260 ± 20	834 ± 36	<5	<5
NDV[c]	113 ± 1	617 ± 243	1314 ± 286	19 ± 11	35 ± 5
IFN-β[d]	<5	<5	<5	<5	<5
IFN-β + poly(I)·poly(C)[d]	252 ± 4	505 ± 45	1460 ± 300	37 ± 11	40 ± 8

[a]IFN yields in the culture media were determined by an ELISA specific for human IFN-β (Toray Inc., Tokyo). The values indicate the mean from two independent experiments.
[b]Poly(I)·poly(C) (50 μg/ml) was added to the cells in the presence of DEAE-dextran (500 μg/ml) in medium for 1 h. Six hours after the treatment with poly(I)·poly(C), the supernatants were harvested for determination of the IFN yield.
[c]IFN yields were determined in culture fluids harvested 18 h after inoculation with NDV (Fujita *et al.*, 1985).
[d]Cultures were treated with human IFN-β (1000 U/ml; Toray Inc., Tokyo) for 3 h. Thereafter the cells were washed twice with PBS, and the cultures were exposed to medium with or without poly(I)·poly(C) (50 μg/ml) in the presence of DEAE-dextran (500 μg/ml) for 1 h. Six hours after the treatment with poly(I)·poly(C), the supernatants were harvested for determination of IFN yields.

were either left untreated or treated with poly(I)·poly(C). Since both IRF-1 and IRF-2 form complexes with the oligonucleotide probe used (Harada *et al.*, 1989), antisera to murine IRF-1 and IRF-2 (which cross-react with the human proteins) were used to specifically block complex formation by IRF-1 or IRF-2, respectively. From the gel shift patterns shown in Figure 2, it is apparent that the fainter, slower moving band corresponds to the complex formed with IRF-2, whereas the darker, faster moving band represents the probe complexed with IRF-1 (see Harada *et al.*, 1990). In agreement with the data presented earlier in this paper, S1 cells showed a high constitutive level of expression of IRF-1, whereas the induction of IRF-1 by poly(I)·poly(C) was barely detectable in the AS11 cells.

Reduced responsiveness of AS11 cells to virus induction can be restored by their transfection with a vector expressing sense IRF-1 mRNA

Data shown above indicated that two different GM-637 cell clones expressing IRF-1 mRNA in the antisense orientation produced less IFN-β protein and mRNA upon stimulation with poly(I)·poly(C) or NDV than control cells. Furthermore, IRF-1 protein levels correlated well with IFN-β mRNA and protein levels. Together these results suggested that IRF-1 is an essential factor for IFN-β induction. In order to substantiate further the notion that the reduced inducibility of IFN-β in cells expressing antisense IRF-1 mRNA is a specific result of a reduction in IRF-1 levels, we next addressed the question whether responsiveness of AS11 cells to NDV can be restored by transfection with a vector coding for IRF-1 mRNA in the sense orientation. Responsiveness to NDV was evaluated with the aid of the construct p125cat (Fujita *et al.*, 1987) composed of the 5′ flanking sequence of the IFN-β gene (−125 to +19) linked to the CAT gene. This construct was inducible by NDV upon transient transfection into the C1 line, but was only minimally inducible in the AS11 line (Figure 3), a feature similar to the endogenous IFN-β gene. Inducibility by NDV in the AS11 line was restored by co-transfection with the vector expressing IRF-1 mRNA in the sense orientation (compare lane 4, with lanes 6 and 8). It is possible that the spontaneous small increase in CAT activity observed in lanes 5 and 7 is due to the generation of double-stranded RNA formed by sense and antisense IRF-1 mRNA, which might somewhat affect the p125cat construct. Nevertheless, treatment with NDV induced a much greater increase in CAT activity in these cells, suggesting that it was the presence of sense IRF-1 mRNA that rendered cells responsive to virus. Taken together, these results strongly support the conclusion that the reduced inducibility of the IFN-β gene in cells expressing antisense IRF-1 mRNA is the specific result of a reduction in IRF-1 levels.

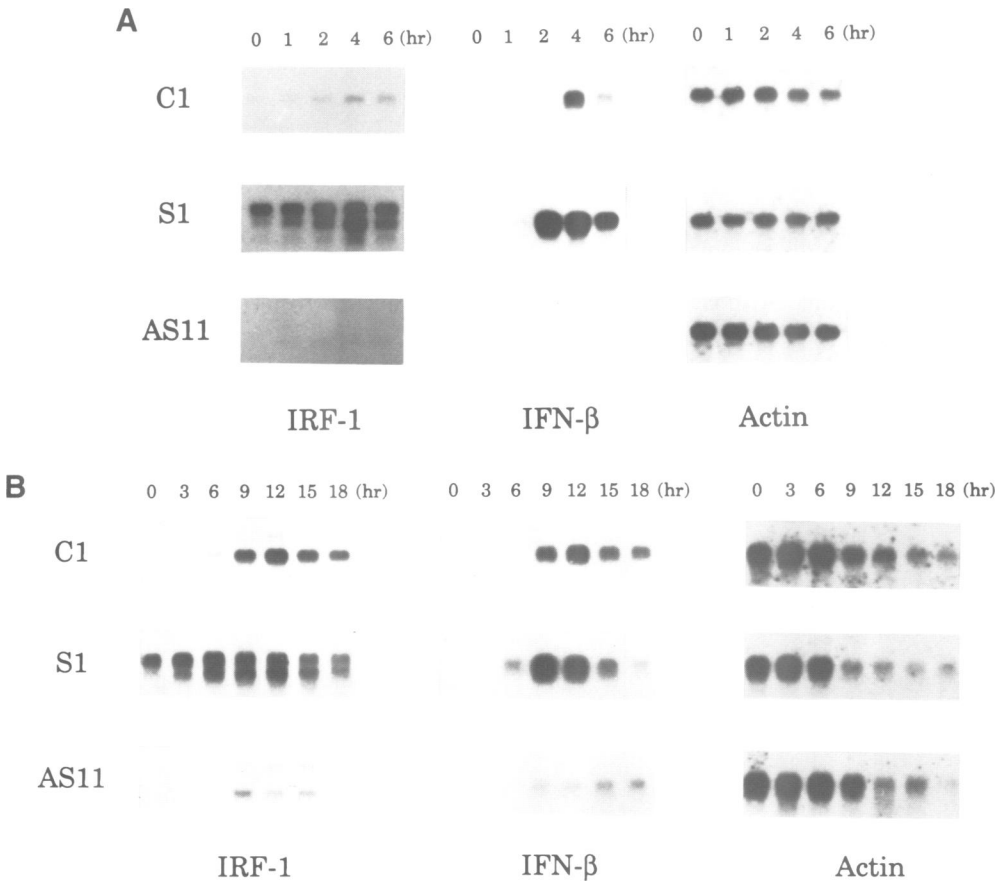


Fig. 1. Induction of IRF-1 and IFN-β mRNAs by poly(I)·poly(C) or NDV. (**A**) Cultures of C1, S1 and AS11 cells were induced by poly(I)·poly(C) as described in Table I. Five micrograms of total RNA isolated from the cells at different times after induction were subjected to Northern blot analysis. The same filters prepared from each cell line were probed with IRF-1, IFN-β and β-actin respectively. (**B**) Cultures of C1, S1 and AS11 cells were induced by NDV (Fujita *et al.*, 1985) and 5 μg of total RNA isolated from the cells at the indicated times was subjected to Northern blot analysis. The same filters prepared from each cell line were probed with IRF-1, IFN-β and β-actin respectively.

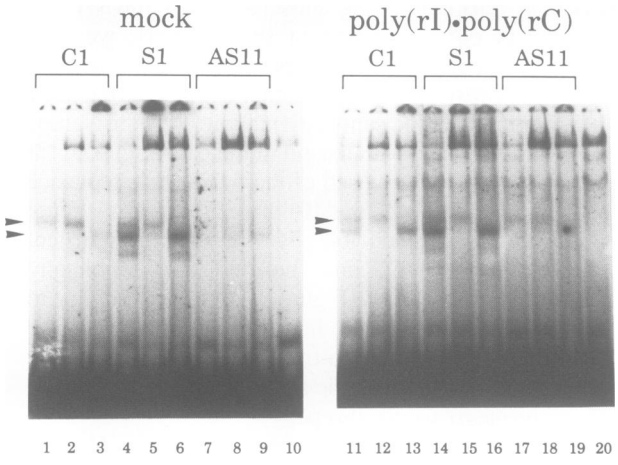


Fig. 2. Detection of IRF-1 protein in transfected GM-637 cells by gel shift analysis. Cells were either left untreated ('mock') or exposed to poly(I)·poly(C) as described in Table I. Whole-cell extracts were prepared 4 h after exposure to poly(I)·poly(C). Gel shift analysis was carried out using 6 fmol of ^{32}P-labeled C13 oligomer (Fujita *et al.*, 1987) as the probe (specific activity 5000 c.p.m./fmol) and whole-cell extracts from 2 × 10^4 cells. In lanes 1, 4, 7, 10, 11, 14, 17 and 20, 3 μl (16.5 μg) of preimmune serum was included in the reaction mixture; in lanes 2, 5, 8, 12, 15 and 18, 3 μl (16.5 μg) of anti-mouse IRF-1 antibodies was included; in lanes 3, 6, 9, 13, 16 and 19, 3 μl (16.5 μg) of anti-mouse IRF-2 antibodies was included; lanes 10 and 20, no extract. The antibodies against mouse IRF-1 and IRF-2 are cross-reactive with the human IRF-1 and IRF-2, respectively. Arrowheads indicate positions of the factor–DNA complexes.

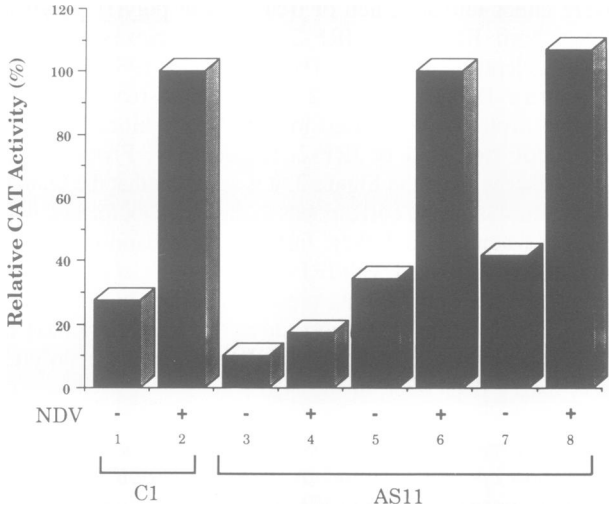


Fig. 3. Functional restoration of virus inducibility in the AS11 line by the expression of IRF-1. The cells were transfected with 7.5 μg of p125cat and 2.5 μg of the effector genes. The transfected effector genes were as follows: lanes 1, 2, 3 and 4, 2.5 μg of pbActneo; lanes 5 and 6, 0.5 μg of pbActneoIRF-1S and 2.0 μg of pbActneo; lanes 7 and 8, 1.5 μg of pbActneoIRF-1S and 1.0 μg of pbActneo. The expression level of p125cat induced by NDV (lane 2) (2.9% conversion) was considered 100%. Similar results were obtained in three separate experiments.

Altered synthesis of IFN-induced gene products in GM-637 cells expressing sense or antisense IRF-1 mRNA

The possible role of IRF-1 in the transcriptional activation of IFN-induced genes was postulated earlier, based mainly on the similarity of IRF binding sites in the promoters of the IFN-β gene and of some IFN-inducible genes (Harada *et al.*, 1989; Fujita *et al.*, 1989c). Among the IFN-inducible genes known to contain IRF binding domains in their promoter regions are the genes for class I major histocompatibility antigens (Miyamoto *et al.*, 1988) and 2′-5′ oligoadenylate synthetase (Wathelet *et al.*, 1987). Availability of cell lines constitutively expressing IRF-1 mRNA in the sense or antisense orientation enabled us to address the role of IRF-1 in the regulation of these genes in a more direct way. C1, S1 and AS11 cells were exposed to IFN-β for different time periods and levels of IRF-1, actin, 2′-5′ oligoadenylate synthetase and HLA-B7 mRNAs were determined by Northern blot analysis (Figure 4A and B). As shown earlier in other cell lines (Harada *et al.*, 1989; Fujita *et al.*, 1989c), IFN-β produced a rapid increase in IRF-1 mRNA levels. Induction of IRF-1 mRNA by IFN-β was reduced in the AS11 line. Induction of 2′-5′ oligoadenylate synthetase mRNA by IFN-β was lower at all time periods in the AS11 line than in the other two lines. In addition, at 6 h after the onset of IFN-β treatment, 2′-5′ oligoadenylate synthetase mRNA levels were higher in the S1 line than in the C1 line (Figure 4B). In comparing HLA-B7 mRNA in the three cell lines, a clear difference was seen in the constitutively expressed mRNA levels at 0 h, with the level being highest in S1 cells, intermediate in C1 and lowest in AS11 (Figure 4A). Similar differences in the constitutively expressed mRNA levels were seen in several other experiments (not shown). Upon treatment with IFN-β, HLA-B7 mRNA levels were consistently much higher in the S1 line than in either C1 or AS11 cells (Figure 4A and B).

To determine whether the differences seen in HLA-B7 mRNA levels are reflected in the levels of protein synthesized, we measured cell surface expression of HLA class I antigen by FACS analysis. The C1, S1, S11 and AS3 lines were incubated in the presence or absence of IFN-β for 24 h. Expression of class I antigen was quantified with the aid of murine mAb W6/32, which detects a monomorphic determinant of HLA-A, B and C antigens (Barnstable *et al.*, 1978). The results obtained (Figure 5A and B) are in accord with the data on HLA-B7 mRNA levels shown in Figure 4. Constitutive class I antigen expression in untreated cells was higher in the S1 and S11 lines than in the C1 or AS3 lines. Upon treatment with IFN-β too, S1 and S11 cells expressed more class I antigen on their surface than C1 or AS3 cells. However, the degree of stimulation of class I antigen expression ('fold induction') was similar in all four cell lines (Figure 5B).

Discussion

Evidence for the role of transcription factors in the regulation of target genes is usually obtained indirectly, either by the analysis of the binding of nuclear proteins to regulatory DNA sequences or on the basis of an intracellular activation of transfected constructs containing gene fragments linked to reporter genes. In the present study we chose a more direct approach to gain new information about the roles of IRF-1. The use of expression vectors coding for IRF-1 mRNA in the sense or antisense orientations made it possible to analyze the effects of IRF-1 on the expression of intact cellular genes *in situ*. Our results provide clear evidence for the important role of IRF-1 in the expression of both the IFN-β gene and of genes inducible by type I IFN.

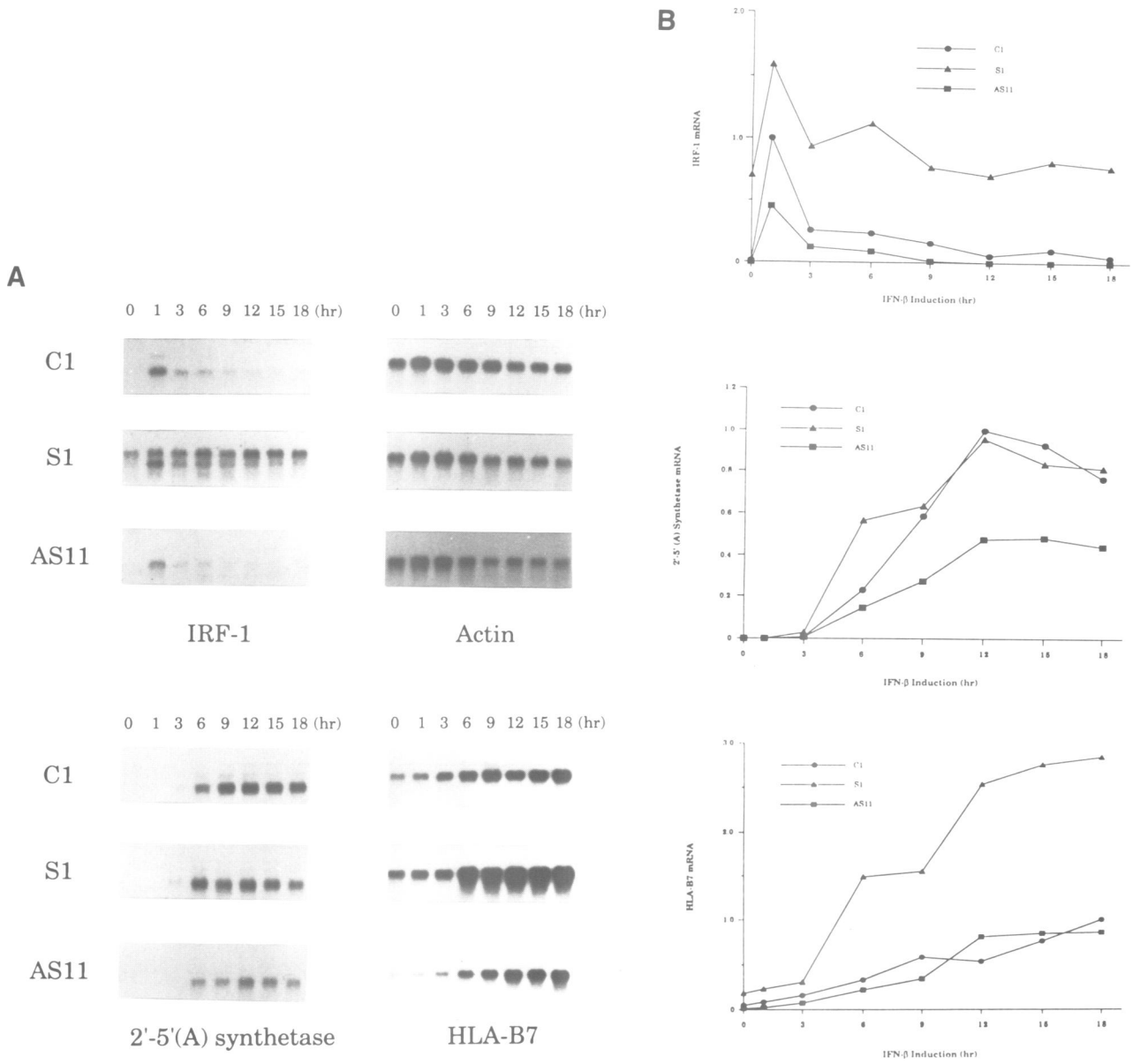
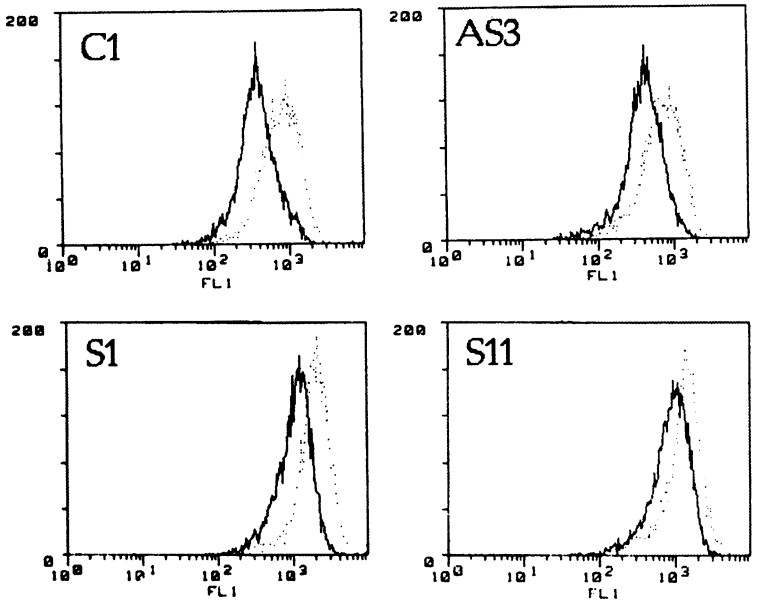


Fig. 4. Induction of IRF-1, 2′-5′ oligoadenylate synthetase, and HLA-B7 mRNAs by IFN-β. (**A**) The cells were induced by IFN-β as described in Table I. Five micrograms of total RNA isolated from the cells at the indicated times was subjected to Northern blot analysis. The same filters from each cell line were probed with IRF-1, 2′-5′ oligoadenylate synthetase, HLA-B7, and β-actin, respectively. (**B**) From the results shown in (A), the IRF-1, 2′-5′ oligoadenylate synthetase and HLA-B7 mRNA levels were quantitated by densitometric analysis. The peak expression level of C1 cells was assigned the value of 1.0 in each graph. For IRF-1 mRNA in the S1 cell line, the results shown in the graph represent the sum of the upper (constitutive) and lower (induced) bands.

IRF-1 is required for IFN-β gene activation

Many earlier studies have provided evidence for the important role of the IRF binding domains and of the IRF-1 and IRF-2 proteins in the regulation of type I IFN gene expression (Fujita *et al.*, 1988; Miyamoto *et al.*, 1988; Fujita *et al.*, 1989a,b,c; Harada *et al.*, 1989, 1990; Raj *et al.*, 1989; Leblanc *et al.*, 1990; MacDonald *et al.*, 1990; Watanabe *et al.*, 1991). A deletion in the region containing the IRF binding domains resulted in the complete inactivation of the IFN-β promoter, suggesting an essential role for this region (Fujita *et al.*, 1985, 1987). The conclusion that IRF-1 acts as a transcriptional activator and IRF-2 as a repressor of the IFN-β gene is supported by earlier studies (Fujita *et al.*, 1989a; Harada *et al.*, 1989, 1990). The role of NK-κB in poly(I)·poly(C)- or virus-induced transcriptional activation of the IFN-β gene was also clearly demonstrated (Lenardo *et al.*, 1989; Fujita *et al.*, 1989b; Visvanathan and Goodbourn, 1989). Recent evidence strongly suggests that both the IRF and the NF-κB binding domains are necessary for the full transcriptional activation of the IFN-β promoter (Fujita *et al.*, 1989b; Leblanc *et al.*, 1990). Yet, in a strict sense none of the earlier work demonstrated directly that IRF-1 was essential for IFN-β induction. As a matter of fact, in a recent study Pine *et al.* (1990) concluded that IRF-1 was not necessary for the expression of the IFN-β gene or of IFN-induced genes. Their conclusion was based on the observation that transcription of the IFN-β gene was induced by treatment of HeLa cells with poly(I)·poly(C) in the presence of the protein synthesis inhibitor cycloheximide, a condition under which they could not detect IRF-1 in the gel shift assay. However, it is possible that amounts of IRF-1 protein sufficient for IFN-β gene

A



B

Cell Clone	Mean Fluorescence Intensity Uninduced	IFN-Induced	Fold Induction
C1	476	930	1.95
AS3	480	815	1.70
S1	1207	2121	1.76
S11	1012	1447	1.43

Fig. 5. Induction of cell surface expression of class I HLA antigens by IFN-β. Cultures of C1, AS3, S1 and S11 cells were treated for 24 h with recombinant human IFN-β (300 U/ml; Betaseron, Triton Bioscience, Alameda, CA) or left untreated. The cells were then incubated with the mouse mAb W6/32, which detects a monomorphic determinant of class I HLA antigens. Thereafter, the cells were incubated with a goat anti-mouse antibody conjugated to FITC. Cell surface staining was quantified using FACS analysis. (**A**) Histograms depicting fluorescence profiles of uninduced (solid lines) and IFN-induced (dotted lines) cells. (**B**) Mean fluorescence intensities for uninduced and IFN-induced cell clones. Fold inductions were calculated by dividing the mean fluorescence intensities of IFN-induced cells by those of uninduced cells.

activation would escape detection in the assay employed by Pine *et al.* (1990). Though less likely, it is also possible that the role of IRF-1 in HeLa cells is different than in other cells.

Our present data show unequivocally that induction of IFN-β mRNA and IFN-β protein by poly(I)·poly(C) or by NDV is strongly reduced in cells expressing the antisense IRF-1 mRNA (Figure 1, Table I). The specificity of the inhibitory effect is supported by the demonstration that reduced levels of sense IRF-1 mRNA (Figure 1) and of IRF-1 protein (Figure 2) are present in the cells expressing antisense IRF-1 mRNA. Moreover, the inhibitory effect on transcriptional activation of the IFN-β gene promoter in the AS11 cell line could be reversed by the transient transfection of cells with the sense IRF-1 mRNA expressing vector (Figure 3). Together, these data strongly support the conclusion that the inhibitory effect on the expression of the IFN-β gene is a specific consequence of reduced levels of IRF-1 in these cells. Although proteins other than IRF-1 and IRF-2 that can bind to the IRF binding domain were found in some cells (Driggers *et al.*, 1990; Keller and Maniatis, 1991), our data indicate that IRF-1 is essential for transcriptional activation of the IFN-β gene and that no other transcription factor can substitute for IRF-1 in this role, at least in the cells employed in our experiments. This apparent lack of redundancy points to a pivotal role for IRF-1 not only in the activation of the IFN-β gene, but also of other genes regulated by IRF-1.

IRF-1 is not sufficient for IFN-β gene activation in most cells

Upon stimulation with poly(I)·poly(C) or NDV, cells transfected with IRF-1 mRNA in the sense orientation produced IFN-β mRNA and protein more rapidly and in greater abundance than control cells (Figure 1, Table I). These results corroborate the importance of IRF-1 as a positive regulator of IFN-β gene expression. On the other hand, the lack of spontaneous production of IFN-β mRNA or protein in otherwise unstimulated cells expressing sense IRF-1 mRNA (Figure 1, Table I) indicates that expression of IRF-1 is not sufficient for the activation of the IFN-β gene promoter in GM-637 cells. In addition, constitutive expression of sense IRF-1 mRNA did not render cells resistant to infection by encephalomyocarditis (EMC) virus, indicating that the presence of IRF-1 in these cells also did not lead to a significant degree of activation of endogenous IFN-α genes (data not shown). The lack of activation of IFN-β (or IFN-α) gene expression in GM-637 cells expressing sense IRF-1 mRNA is in agreement with studies in some other types of cells in which an increase in IRF-1 levels alone was not sufficient to turn on IFN-β gene expression (Fujita *et al.*, 1989c Leblanc *et al.*, 1990). Earlier, IFN activity (mainly IFN-α) was detected, albeit at low levels, in the supernatant of COS cells transfected with IRF-1 expression vectors (Fujita *et al.*, 1989a). Similarly, in undifferentiated embryonal carcinoma (EC) cells, in which both IRF-1 and

IRF-2 genes are not expressed, cDNA-directed expression of IRF-1 led to the induction of endogenous IFN-α genes, as well as to activation of transfected human IFN-α and -β promoters (Harada *et al.*, 1990). It is possible that it is the absence of IRF-2 in EC cells which makes IRF-1 sufficient for induction of transcription, since (i) transfection of EC cells with an IRF-2 expression vector turned off transcription that was induced by IRF-1 and (ii) the IRF-1-induced IFN gene activation in the EC cells becomes suppressed when EC cells accumulate IRF-2 upon their differentiation following exposure to retinoic acid (Harada *et al.*, 1990).

Recent evidence suggests that activation of the promoter elements for the IFN-β gene by IRF-1 requires a post-translational event in addition to IRF-1 synthesis (Watanabe *et al.*, 1991), and this requirement would help to explain why NDV or poly(I)·poly(C) is needed for IFN-β induction even in cells that already express a high level of IRF-1. Whereas NDV and poly(I)·poly(C) can both induce and 'activate' IRF-1 by yet unknown mechanisms, treatment with IFN-β leads to IRF-1 induction but no 'activation' (Watanabe *et al.*, 1991). It cannot be ruled out that the 'activation' involves not only (or not at all) IRF-1 but also other factors, such as IRF-2. The need for activated NF-κB in IFN-β gene induction has also been well documented and this requirement too could help to explain why poly(I)·poly(C) or NDV do induce IFN-β, but treatment with IFN-β alone does not (Table I). Unlike NDV or poly(I)·poly(C), IFN-β does not activate NF-κB (Watanabe *et al.*, 1991).

IRF-1 regulates the expression of IFN-inducible genes

A possible role for IRF-1 and IRF-2 in the regulation of IFN-inducible genes was suggested (Fujita *et al.*, 1989c; Harada *et al.*, 1989) because the IFN response sequences (IRS) present in the promoter regions of genes inducible by IFN are highly homologous to the promoter regions of type I IFN genes, and the homologous regions in the IFN-inducible genes include the IRF binding elements (Wathelet *et al.*, 1987; Cohen *et al.*, 1988; Hug *et al.*, 1988; Korber *et al.*, 1988; Levy *et al.*, 1988; Miyamoto *et al.*, 1988). Direct evidence for the presence of an IRF-1 binding site in the promoter region of the murine H-2D^d class I major histocompatibility antigen gene (Harada *et al.*, 1989) and for the activation of this promoter sequence by co-transfection with an IRF-1 expression vector in undifferentiated EC cells (Harada *et al.*, 1990) has been provided. In addition, IFN-β was shown to act as a potent inducer of IRF-1 and IRF-2 (Fujita *et al.*, 1989c; Harada *et al.*, 1989). However, the question of whether IRF-1 is required and the extent of its contribution to the regulation of IFN-inducible genes have not been critically addressed in the earlier studies.

The first indication that the expression of IFN-inducible genes in the GM-637 cells may be affected by IRF-1 came from the finding that the S1 cell line not only produced high levels of IRF-1 mRNA constitutively, but also showed a much more rapid as well as enhanced induction of IRF-1 mRNA by poly(I)·poly(C) (Figure 1A) or by NDV (Figure 1B). These findings suggested that IRF-1 exerts a positive autoregulatory action on its own synthesis. In addition, as shown earlier in other cells (Fujita *et al.*, 1989c; Harada *et al.*, 1989), treatment with IFN-β-induced IRF-1 mRNA in the GM-637 cells (Figure 4), albeit more transiently than poly(I)·poly(C) or NDV (Figure 1A and B). Interestingly, IFN-induced IRF-1 mRNA levels were not strikingly higher in the S1 cell line than in the C1 line (Figure 4), in contrast to the clear differences seen after induction with poly(I)·poly(C) or NDV (Figure 1A and B). These differences suggest that either a modification of the constitutively expressed IRF-1 protein or some additional factor, not inducible by IFN-β, is needed for the stimulatory action of IRF-1 on IRF-1 gene expression. More work is needed to clarify the regulatory mechanisms of IRF-1 gene expression.

Our results suggest a role for IRF-1 in the regulation of two other IFN-inducible genes, 2′-5′ oligoadenylate synthetase and the class I major histocompatibility antigen gene, HLA-B7. Although the expression of both genes was altered in the sense or antisense IRF-1 mRNA expressing cell lines, they were affected in different ways (Figure 4). The major difference seen in the expression of the 2′-5′ oligoadenylate gene was a decreased mRNA level in the antisense IRF-1 mRNA expressing cells upon induction with IFN-β. This result suggests that IRF-1 is essential for the full induction of the 2′-5′ oligoadenylate gene. In contrast, with the HLA-B7 gene a difference was seen in the basal level of mRNA expression, with the constitutive mRNA levels being higher in the S1 line and lower in the AS11 line (Figure 4A). In addition, upon treatment with IFN-β, HLA-B7 mRNA levels were higher in the S1 line than in the other two lines. Cell surface expression of class I antigen (measured with the aid of an antibody that recognizes products of HLA-A, B and C genes in intact heterodimeric form) showed an increase in both the basal and induced levels in the two sense IRF-1 mRNA expressing cell lines employed (Figure 5). These data support a role of IRF-1 in the control of class I antigen gene expression both in the presence and in the absence of exogenous IFN-β.

It is not clear at present why the 2′-5′ oligoadenylate synthetase gene and the class I HLA gene are affected by the antisense IRF-1 mRNA in different manners. In this regard, it may be worth noting that several *cis*-elements and protein factors have been implicated in the control of these two genes in addition to the IRF element (Cohen *et al.*, 1988; David-Watine *et al.*, 1990). Thus, the contribution of IRF-1 to the expression of the two genes may be variable depending on the surrounding DNA elements and binding factors. In addition, a certain redundancy may exist in the IFN-inducible genes, and factors other than IRF-1 could also act under certain circumstances on the IRS (Levy *et al.*, 1989; Driggers *et al.*, 1990). In any event, the demonstration that IRF-1 affects the expression of 2′-5′ oligoadenylate synthetase and class I HLA genes suggests a role for IRF-1 in the antiviral defenses. Indeed, we have found that GM-637 clones expressing IRF-1 mRNA in the sense or antisense orientation showed increased or decreased sensitivity, respectively, to the antiviral action of IFN-β against encephalomyocarditis virus (L.F.L.Reis, data not shown).

IRF-1 is likely to regulate the expression of a variety of genes induced by double-stranded RNA, viruses and cytokines

Several earlier observations suggested that the regulatory mechansims of type I IFN gene expression partly overlap the mechanisms regulating the expresssion of IFN-inducible genes. Wathelet *et al.* (1987) showed that the 2′-5′ oligoadenylate synthetase gene and the gene for a 56 kDa protein were inducible by poly(I)·poly(C) as well as by IFN-α. Heretofore poly(I)·poly(C) had been known to be an inducer of the IFN-β gene, but not of genes that are

induced by treatment with IFN. A similar dual inducibility by NDV or IFN-α was demonstrated for the murine *Mx* gene (Hug *et al.*, 1988). Our data suggest that activation of the transcription factor IRF-1 is at least in part responsible for the ability of such diverse agents as double-stranded RNA, viruses or IFNs to activate the 2′-5′ oligoadenylate synthetase gene, class I major histocompatibility genes and, very likely, other common target genes. This conclusion is also supported by the demonstration that GM-637 cell clones expressing antisense IRF-1 mRNA showed a much reduced capacity to produce 2′-5′ oligoadenylate synthetase mRNA not only in response to IFN-β (as shown in Figure 4), but also in response to poly(I)·poly(C) (L.F.L.Reis, data not shown).

Inasmuch as they are important in the regulation of cytokine genes (e.g. type I IFN genes), cytokine-induced genes and cytokine receptor genes (Pleiman *et al.*, 1991), IRF-1 and IRF-2 are emerging as transcription factors with significant roles in cytokine network interactions. IRF-1 is likely to be involved in the extensively documented ability of structurally dissimilar cytokines to activate the same target genes. Several IFN-inducible genes are known to be induced also by tumor necrosis factor (TNF) or interleukin-1 (IL-1), e.g. the genes for 2′-5′ oligoadenylate synthetase (Wong and Goeddel, 1986), class I major histocompatibility antigen (Collins *et al.*, 1986; J.D.Wolchok, unpublished data), and several other genes (Rubin *et al.*, 1988; Lee *et al.*, 1990). Earlier we showed that TNF and IL-1, like the IFNs, are potent inducers of IRF-1 and IRF-2 (Fujita *et al.*, 1989c; Reis *et al.*, 1990; Watanabe *et al.*, 1991). IFN-α/β, IFN-γ, TNF and IL-1, though structurally unrelated to each other, exhibit a multitude of overlapping biological actions (reviewed in Le and Vilček, 1987; Vilček, 1990; Vilček and Lee, 1991). The ability of so many structurally dissimilar cytokines to induce IRF-1 genes is likely to play a role in this recently recognized bewildering redundancy in cytokine actions.

Materials and methods

Construction of plasmids expressing IRF-1 cDNA

IRF-1 cDNA (Maruyama *et al.*, 1989) was first subcloned into the plasmid pTZ19U (USB, Cleveland, OH) and pGEM7Z(f+) (Promega, Madison, WI) in order to increase the number of restriction sites. The expression vector, pbActneo, was constructed by replacing the dexamethasone-inducible MMTV-LTR promoter of the plasmid pMAMneo (Clontech, Palo Alto, CA) by the β-actin promoter, excised from the plasmid pbActCAT9 (Fregien and Davidson, 1986). The sense IRF-1 mRNA expressing vector pbActneoIRF-1S was made by subcloning the entire IRF-1 cDNA (*Xba*I−blunt-ended *Hin*dIII fragment) excised from the plasmid pTZ19UIRF-1 into the *Xba*I and blunt-ended *Sal*I sites of pbActneo. The antisense IRF-1 mRNA expressing vector pbActneoIRF-1AS was prepared by subcloning the *Xba*I−*Sal*I fragment of the IRF-1 cDNA (318 bp shorter at the 3′ end) excised from the plasmid pGEMIRF-1 into the same restriction sites of pbActneo. The plasmid pGEM7Z(f+) bearing the entire IRF-1 cDNA was used to generate strand-specific probes by *in vitro* transcription, using either the SP6 or T7 polymerase according to the recommendations of the manufacturer.

Transfection and selection of stably transfected GM-637 cells

GM-637 cells (a SV40-transformed human skin fibroblast line, received from the Human Genetic Mutant Cell Repository, Camden, NJ) were maintained in Eagle's Minimum Essential Medium (MEM) supplemented with 6 mM HEPES, 3 mM Tricine and 10% fetal bovine serum (FBS). Transfections were done as described by Sambrook *et al.* (1989). Individual cell clones were isolated and expanded. Over 10 clones of each sense and antisense IRF-1 mRNA-transfected cells were generated. In addition, two control clones transfected with the expression vector pbActneo, containing the *neo* gene but no IRF-1 sequence, were isolated. The clones generated were screened for the presence of IRF-1 mRNA by Northern blot hybridization or by RNase protection, using sense or antisense strand-specific [^{32}P]UTP-labeled ribonucleoprobes generated by *in vitro* transcription of the IRF-1 cDNA subcloned into the plasmid pGEMIRF-1. Both the sense and antisense transcripts showed a higher than expected molecular size, most likely due to read-through of the first polyadenylation signal on the IRF-1 DNA. The presence of sense or antisense mRNA as well as their sizes were also confirmed by RNase protection analysis. Based on the levels of IRF-1 mRNA present, three sense IRF-1 mRNA expressing clones (S1, S7 and S11) and three antisense IRF-1 mRNA expressing clones (AS3, AS11 and AS18) were selected for further studies. The two control clones, transfected with the *neo* gene but no IRF-1 sequence, were compared in their ability to produce IFN-β mRNA in response to induction. No marked differences were seen and the C1 line was selected for further studies.

RNA isolation and RNA blotting analysis

Total mRNA was isolated by phenol/chloroform extraction as described previously (Fujita *et al.*, 1989c). The procedure of RNA blotting analysis was as described in Harada *et al.* (1990). To prepare probes, the following DNAs were labeled by the multiprime DNA labeling reaction (Amersham, Arlington Heights, IL): IFN-β, a 1.8 kb *Pst*I−*Hin*dIII fragment from pSE-125 (Fujita *et al.*, 1985); 2′-5′ oligoadenylate synthetase, a 1.3 kb *Bam*HI fragment from pE22-1 (Shiojiri *et al.*, 1986), kindly provided by Dr Y.Sokawa, Kyoto Institute of Technology; HLA-B7, a 1.4 kb *Pst*I fragment from pDP001 (Sood *et al.*, 1981), was a gift of Dr S.Weissman, Yale University, New Haven, CT. For detection of IRF-1 mRNA, a strand-specific probe was obtained from the IRF-1 cDNA (*Sac*I−*Kpn*I fragment) subcloned into the *Sac*I−*Kpn*I site of M13mp19. A uniformly labeled probe was synthesized as described previously (Fujita *et al.*, 1986).

Gel mobility shift assay

The assay was performed essentially as previously described (Harada *et al.*, 1990) except that the volumes used were 1.5 times greater.

DNA transfection and CAT assay

The cells (7.5×10^5 cells/6 cm dish) were transfected by the calcium phosphate method (Fujita *et al.*, 1985) with 7.5 μg of p125cat (Fujita *et al.*, 1987) and 2.5 μg of the expression plasmid. The cells were harvested 48 h after transfection. NDV induction was performed 12 h before harvest as described by Fujita *et al.* (1985).

FACS analysis

Cells were grown to confluence in 25 cm^2 plastic flasks and were either treated with IFN-β (300 U/ml) or incubated in control medium for 24 h. Cells were harvested by vigorous pipetting and resuspended in 0.5 ml of tissue culture supernatant from the W6/32 murine hybridoma (purchased from ATCC, Rockville, MD) diluted 1:5 in ice-cold FACS buffer (phosphate buffered saline, with 3% FBS and 0.5% sodium azide). mAb W6/32 detects a monomorphic determinant of HLA-A, B and C antigens (Barnstable *et al.*, 1978). The cells were incubated with the antibody for 30 min at 4°C and then washed three times with FACS buffer. The cells were then incubated in a 1:100 dilution of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (Sigma, St Louis, MO) in FACS buffer for 30 min at 4°C. Thereafter, the cells were washed three times in ice-cold phosphate-buffered saline and analyzed on a Becton-Dickinson FACScan flow cytometer.

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