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Mice devoid of interferon regulatory factor 1 (IRF-1) show normal expression of type I interferon genes

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The transcription factor interferon regulatory factor 1 (IRF-1) binds tightly to the interferon (IFN)-β promoter and has been implicated in the induction of type I IFNs. We generated mice devoid of functional IRF-1 by targeted gene disruption. As reported by others, IRF-1-deficient mice showed a discrete phenotype: the CD4/CD8 ratio was increased and IFN-γ-induced levels of macrophage iNO synthase mRNA were strongly diminished. However, type I IFN induction *in vivo* by virus or double-stranded RNA was unimpaired, as evidenced by serum IFN titers and IFN mRNA levels in spleen, liver and lung. There was also no impairment in the response of type I IFN-inducible genes. Therefore, IRF-1 is not essential for these processes *in vivo*.
Key words: iNO synthase induction/interferon gene induction/interferon-inducible genes/ IRF-1 knockout

Introduction

Interferons (IFNs) are inducible, pleiotropic cytokines that are characterized by their ability to induce an antiviral state against a variety of viruses. As in the case of many other cytokines, expression of the type I IFN genes (IFN-α and -β) is tightly regulated. While most of the information necessary to control the transcription of these intronless genes appears to be located within the 5′ flanking region, sequences located in the 3′ untranslated region of IFN-β mRNA may contribute to the regulation of the levels of the cognate protein by modulating mRNA turnover. It is generally accepted that IFN-β gene transcription is achieved by derepression as well as by activation of one or more transcription factors that act on different regulatory domains within the IFN-β promoter (for reviews, see deMaeyer and deMaeyer-Guinard, 1988; Vilcek, 1990).

Two DNA-binding proteins, NF-κB and HMG Y/I, interact with the positive regulatory domain II (PRDII) and are required for full activation of the IFN-β promoter in cultured cells (Clark and Hay, 1989; Fujita *et al.*, 1989b; Hiscott *et al.*, 1989; Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989; Thanos and Maniatis, 1992). Another sequence, PRDIV, which is the binding site for the transcription factor ATF-2, is also required for full activation of the IFN-β promoter (Du and Maniatis, 1992; Du *et al.*, 1993). PRDI and PRDIII are binding sites for interferon regulatory factor 1 (IRF-1), which was originally identified by its ability to bind multimers of double-stranded AAGTGA or AAATGA (Fujita *et al.*, 1988; Miyamoto *et al.*, 1988; Harada *et al.*, 1989). Interestingly, similar sequences appear within some promoters of IFN-α genes, as well as in several IFN-stimulated genes (ISGs) (Miyamoto *et al.*, 1988) to some of which IRF-1 also binds (Harada *et al.*, 1989), raising the question as to whether it is also involved in the control of their expression (Reis *et al.*, 1992). More recently, it has been shown that ISGF-3 (ISG factor-3), a multimeric DNA-binding protein that is assembled upon stimulation of cells with type I IFNs and comprises ISGF-3γ, a member of the IRF family (Veals *et al.*, 1992), plays a central role in the induced expression of the ISGs (for reviews, see Pellegrini and Schindler, 1993; Hunter, 1993).

The role of IRF-1 in regulating the expression of the IFN-β gene is still controversial. Whereas overexpression of IRF-1 elicits expression of the IFN-β gene in at least some cell lines (Harada *et al.*, 1989; 1990), and reduction of IRF-1 protein was associated with impaired poly(I)·poly(C)- and Newcastle Disease virus (NDV)-induced IFN-β gene expression (Reis *et al.*, 1992), other experiments suggested that the IFN-β gene can be activated under conditions where IRF-1 was not detectable (Pine *et al.*, 1990; Whiteside *et al.*, 1992).

In order to evaluate the role of IRF-1 in the induction of type I IFNs, as well as in the induction of ISGs, we generated clones of embryonic stem (ES) cells in which both IRF-1 alleles were disrupted by two rounds of homologous recombination *in vitro* (Ruffner *et al.*, 1993). Treatment of differentiated ES cells with NDV elicited expression of IFN-β as well as IFN-α mRNAs in both IRF-$1^{o/o}$ and IRF-$1^{+/+}$ cells, albeit at levels 1.3- to 7-fold lower in IRF-$1^{o/o}$ cells. We concluded that IRF-1 is not essential for the expression of type I IFN genes, at least in ES cells, but that it can enhance gene expression.

Matsuyama *et al.* (1993) recently reported on the generation of mice lacking functional IRF-1 and investigated the inducibility of IFN genes in embryonic fibroblasts (MEFs) derived from IRF-$1^{o/o}$ and IRF-$1^{+/+}$ mice. Whereas treatment with NDV revealed no difference in the inducibility of IFN genes in the two types of MEFs, poly(I)·poly(C) induction led to a 3- to 10-fold lower level of type I IFN mRNAs in IRF-$1^{o/o}$ MEFs. This difference in inducibility by poly(I)·poly(C) was abrogated by priming the MEFs with type I IFN (Matsuyama *et al.*, 1993) prior to induction. It was concluded that IRF-1 is necessary for normal expression of type I IFN genes.

We have also generated mice with disrupted IRF-1 genes and report here the characterization of the IFN system *in vivo* and in cell culture. We confirmed the

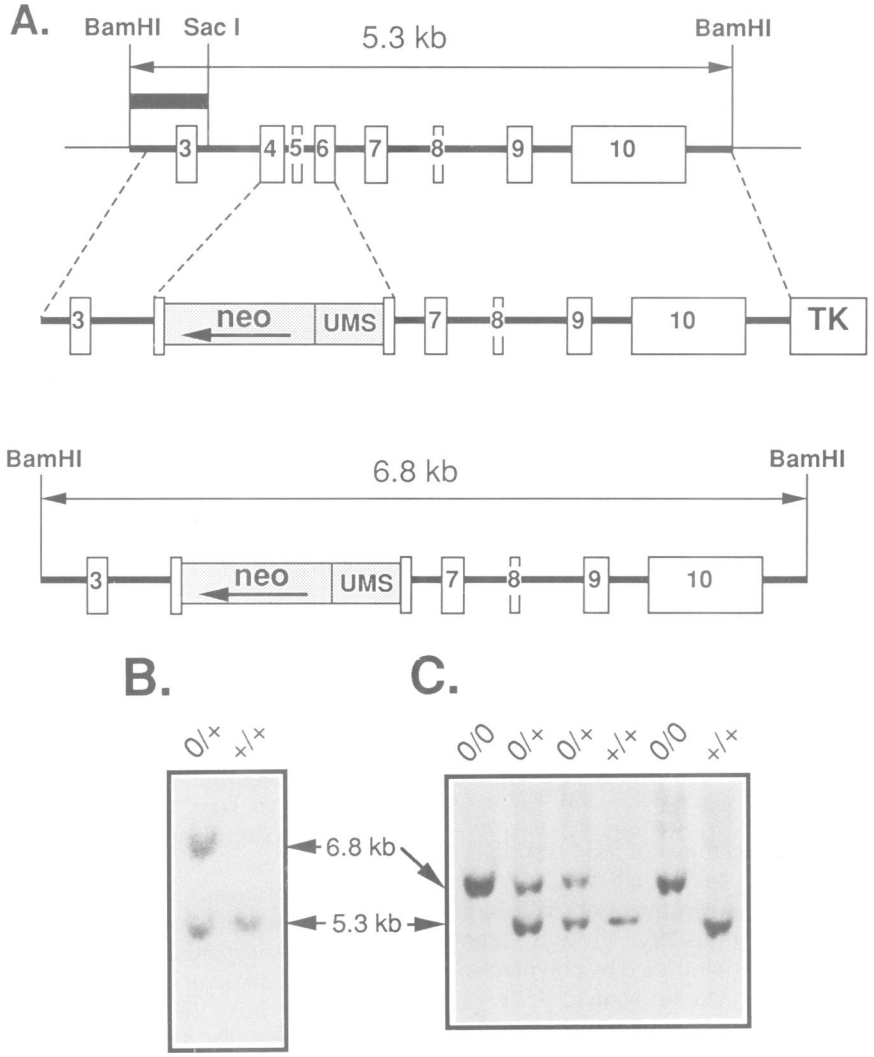

Fig. 1. Disruption of the IRF-1 gene. (**A**) Top: scheme of the IRF-1 locus (the scale is not exact); open boxes represent exons. Middle: the pIRFneo targeting vector (Ruffner *et al.*, 1993) in which 308 bp of coding sequence were replaced by the PGK-neo/UMS sequences. The arrow indicates the direction of neo transcription. Bottom: the disrupted IRF-1 allele after homologous recombination. Southern blots of *Bam*HI-cleaved genomic DNA from (**B**) wild type and IRF$^{o/+}$ (GS1-32.4) ES cells, and (**C**) tail DNA of F2 animals were hybridized with the ^{32}P-labeled *Bam*HI−*Sac*I probe [black bar in (A), top]. The 6.8 and the 5.3 kb fragments correspond to the disrupted and wild type alleles, respectively. There was no ectopic integration of the targeting vector in the GS1-32.4 clone.

findings of Matsuyama *et al.* (1993) regarding the inducibility of IFN genes in cultured MEFs; however, we found that *in vivo* there was no difference in the inducibility of type I IFN genes by either NDV or poly(I)·poly(C), as measured by steady-state levels of IFN mRNA in different organs or by antiviral activity in the serum. We conclude that *in vivo* IRF-1 is completely dispensable for normal inducibility of type I IFN genes. Moreover, inducibility of several ISGs by type I IFN was also unimpaired.

Results

Generation of mice with disrupted IRF-1 genes

Mice with disrupted IRF-1 genes were obtained essentially as described previously (Doetschman *et al.*, 1987; Thomas and Capecchi, 1987; Capecchi, 1989; McMahon and Bradley, 1990). In short, ES cells with one disrupted IRF-1 allele (IRF-1$^{o/+}$ cells) were generated by homologous recombination with a targeting vector in which a gene segment comprising exon 5 and parts of exons 4 and 6 (308 bp of coding sequence) was replaced by an extraneous DNA sequence (Ruffner *et al.*, 1993). This sequence contained a neomycin resistance expression cassette in the opposite transcription orientation (Figure 1A) and the UMS sequence, described as a transcription termination sequence (Heard *et al.*, 1987). Injection of ES cells with a disrupted IRF-1 allele (Figure 1B) into blastocysts gave rise to chimeric males which showed a high frequency of germ line transmission. Mating of heterozygous offspring yielded IRF-1$^{o/o}$ homozygous pups with a frequency of ~25%. IRF-1$^{o/o}$ mice appeared anatomically normal, were fertile and healthy up to at least 4 months. Southern analysis of tail DNA (Figure 1C) showed the pattern typical for the disrupted IRF-1 genes and the absence of wild type genes in some progeny.

Northern blot analysis of poly(A)$^+$ RNA isolated from organs of mice with the disrupted IRF-1 gene after treatment with poly(I)·poly(C) revealed a mRNA species (Figure 2) that hybridized with antisense probes corresponding to cDNA position 413–702 (upstream of the

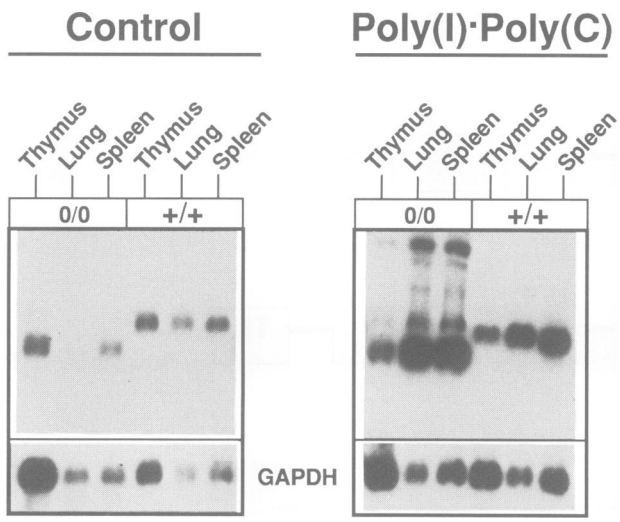

Fig. 2. IRF-1-specific mRNA in organs of wild type or IRF-$1^{o/o}$ mice. Total RNA was isolated from organs of control or poly(I)·poly(C)-treated animals. Poly(A)$^+$ RNA was purified from 100 μg total RNA using oligo(dT)-containing magnetic beads (Dynabeads, Dynal, Oslo, Norway), according to the recommendations of the manufacturer. Northern blots were hybridized with a ^{32}P-labeled, antisense RNA probe complementary to IRF-1 nucleotide sequences 413–702, located 5′ of the PGK-neo/UMS insertion. After autoradiography, filters were stripped and hybridized with the GAPDH cDNA probe to monitor loading.

insert) with a mobility greater than that of wild type IRF-1 mRNA. The same band was visualized with a probe covering positions 1123–1363 (downstream of the insert; data not shown), suggesting that this RNA arose by exon skipping. This interpretation was confirmed by polymerase chain reaction (PCR) analysis (data not shown). Using a sense primer located 5′ of the insert (cDNA position 433–453) in combination with antisense primers located in exons 7, 8 or 10, PCR products were obtained which in all cases were ~360 bp shorter for IRF-$1^{o/o}$ than for wild type RNA. Exon 3 was present in all PCR products, as shown by the presence of an *Nco*I site. Therefore, exons 4, 5 and 6, comprising 360 bp, had been spliced out. This conclusion was confirmed by sequence analysis of a cloned reverse PCR product generated with primers located 5′ of the insert (cDNA position 433–453) and in exon 8 (data not shown).

Because the skipping of exons 4–6 removed sequences reported to comprise the DNA binding region of IRF-1 (Matsuyama *et al.*, 1993), translation of the abbreviated RNA, if it occurred, would be expected to yield an inactive protein. We prepared and translated *in vitro* wild type as well as abbreviated IRF-1 mRNA lacking exons 4–6. The resulting ^{35}S-labeled proteins (Figure 3A) were incubated with and without unlabeled oligonucleotides and analyzed on a neutral polyacrylamide gel. As shown in the 'reverse band shift' experiment of Figure 3B, double-stranded $(GAAAGT)_4$, which contains two copies of the IRF-1 binding site PRDI (Näf *et al.*, 1991), and to a slight extent the mutated oligonucleotide $(GACAGT)_4$, increased the mobility of the radioactive wild type IRF-1. No shift was observed with the abbreviated IRF-1, showing that the DNA binding site had indeed been obliterated. The increased mobility of the labeled protein on binding of an oligonucleotide is due to the increased ratio of negative charge to mass.

It has been reported by Kamijo *et al.* (1994) that macrophages of the IRF-$1^{o/o}$ mice generated by Matsuyama *et al.* (1993) show a striking reduction in the level of inducible nitric oxide synthase (iNOS) RNA after induction by IFN-γ and lipopolysaccharide (LPS). As represented in Figure 4, macrophages from our IRF-$1^{o/o}$ mice showed the same phenotype. Moreover, our IRF-$1^{o/o}$ mice had an 85% reduction of the CD8$^+$ T lymphocytes (data not shown), as reported previously (Matsuyama *et al.*, 1993). Thus, we conclude that our IRF-$1^{o/o}$ mice had no functional IRF-1 and exhibited essentially the same phenotype as the mice described earlier, as far as a comparison can be made. It is evident from Figure 1 of Matsuyama *et al.* (1993) that the mice described earlier also produced an abbreviated IRF-1 mRNA, presumably lacking exons 4–7.

In vivo induction of type I IFN genes

Three independent experiments were performed for NDV and two for poly(I)·poly(C) induction. One to three mice were injected i.p. with poly(I)·poly(C) or i.v. with NDV in each experiment. Control animals were untreated. No significant difference in the levels of antiviral activity in the sera of wild type or IRF-1-deficient mice could be observed (Figure 5) in either case. IFN-α and IFN-β mRNA levels were determined in organs of IRF-$1^{o/o}$ and wild type control mice. Considerable differences in the mRNA levels of the individual animals of each group were found, stressing the importance of examining several animals for each experimental condition. After NDV challenge, substantial levels of IFN-β mRNA were detectable in spleen, liver and lung, in both wild type and IRF-1-deficient mice (Figure 6A). Lower levels of IFN-β mRNA were also detected in thymus, but not in kidney, heart or brain (determined in wild type animals and in one IRF-$1^{o/o}$ mouse; data not shown). In the case of IFN-α mRNA, spleen samples gave a strong and liver a weak signal (Figure 6B); virtually no signals were detectable in lung or in the other organs mentioned above. Overall, there appeared to be no significant difference between wild type and mutant animals.

Challenge with poly(I)·poly(C) led to high levels of mRNA for both IFN-β and IFN-α in spleen and liver, whereas in lung there was lower expression of both IFN-β and IFN-α mRNA (Figure 6C and D). Again, overall, there was no significant difference between wild type and mutant mice.

Induction of ISGs in mice deficient in IRF-1

Our IRF-1-deficient mice had a 2- to 3-fold lower constitutive level of MHC class I, as evaluated by FACS analysis in splenocytes and thymocytes, at variance with the report by Matsuyama *et al.* (1993) who found no difference. However, upon treatment with 5×10^5 U of type I IFN/day for 3 days, cells from both wild type and IRF-$1^{o/o}$ mice showed increased expression at the cell surface, suggesting that this molecule can be upregulated in the mutant mice (data not shown). We also evaluated the inducibility of some ISGs at the mRNA level. MHC class I, 2′,5′-oligoadenylate synthetase (OAS) and 1-8 genes (Flenniken *et al.*, 1988) were all induced to the same

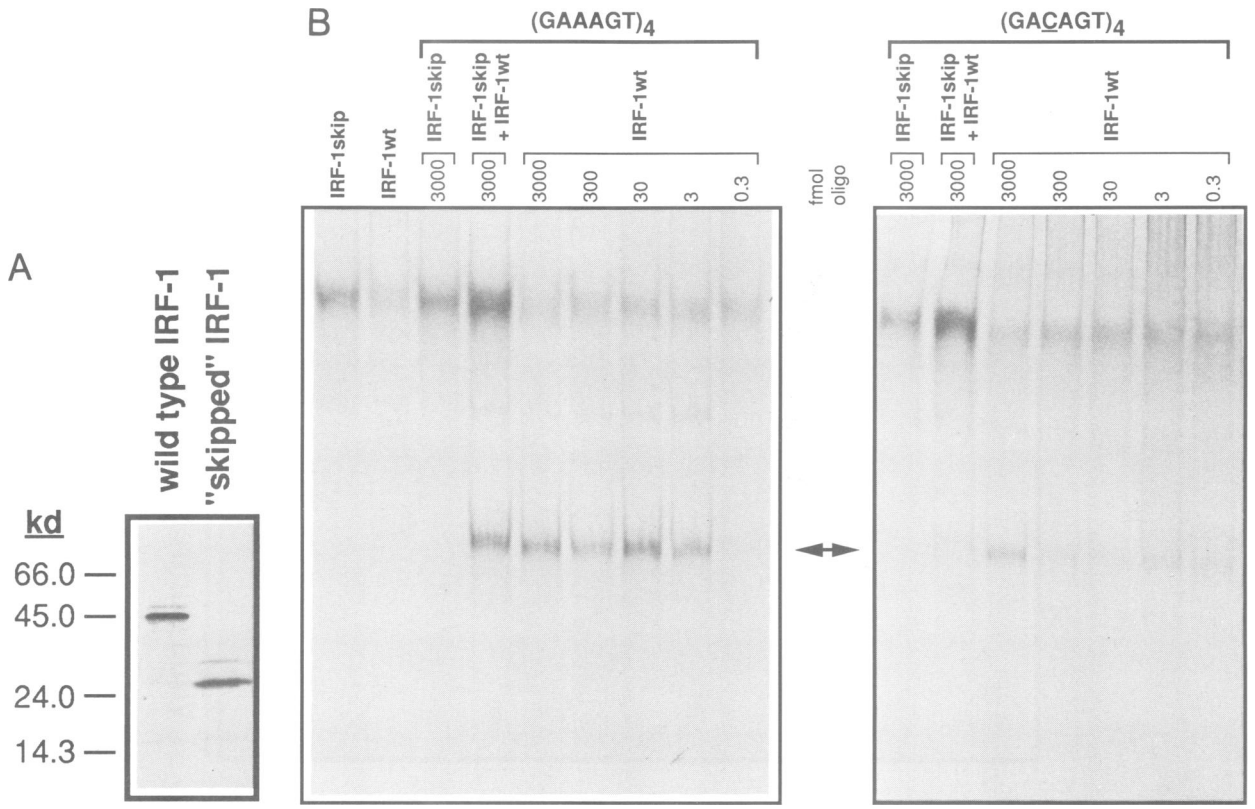

Fig. 3. Reverse band shift assay of IRF-1 lacking amino acids 63–182 and wild type IRF-1. mRNA for wild type IRF-1 and for IRF-1 lacking amino acids 63–182 due to exon skipping was prepared by *in vitro* transcription and used as a template for protein synthesis in a reticulocyte lysate system with [^{35}S]methionine. SDS–PAGE (**A**) showed the presence of a major 45 kDa band in the case of IRF-1 and a 26 kDa band in the case of the deletion mutant. Reverse band shift analysis (**B**) was performed by incubating the radioactive protein with the oligonucleotides indicated and analysing the product on a 4% polyacrylamide gel. IRF-1wt, wild type IRF-1; IRF-1skip, IRF-1 lacking amino acids 63–182.

extent in spleen, liver, lung and thymus of IRF-1$^{o/o}$ as of wild type animals after a single injection of 5×10^5 U of type I IFN (data not shown).

Induction of type I IFN genes in MEFs

As reported by Matsuyama *et al.* (1993), both IFN-α and IFN-β mRNAs were induced to about the same extent by NDV in mutant and wild type MEFs, while induction by poly(I)·poly(C) resulted in lower levels of both mRNAs, particularly for muIFN-α, in IRF-1$^{o/o}$ MEFs (Figure 7). Also in agreement with Matsuyama *et al.* (1993), the reduced IFN gene expression observed in poly(I)·poly(C)-induced IRF-1$^{o/o}$ cells was overcome by pre-treating, i.e. priming the cells with type I IFN (Stewart *et al.*, 1971; Raj and Pitha, 1981; Enoch *et al.*, 1986; Dron *et al.*, 1990) (Figure 7).

To determine whether the restoration of full inducibility of MEFs by priming with IFN involved protein synthesis, the effect of cycloheximide was determined. As shown in Figure 7, poly(I)·poly(C)-induced levels of IFN-α and IFN-β mRNA were, if anything, slightly increased by cycloheximide; however, the priming effect of IFN was completely abolished by the protein synthesis inhibitor in both wild type and mutant cells. On the other hand, induction by NDV in the presence of cycloheximide led to vastly increased levels of both IFN-α and IFN-β mRNA (Figure 7), a phenomenon known as superinduction (Tan *et al.*, 1970; Vilcek and Havell, 1973; Dinter and Hauser, 1987), in both wild type and mutant cells.

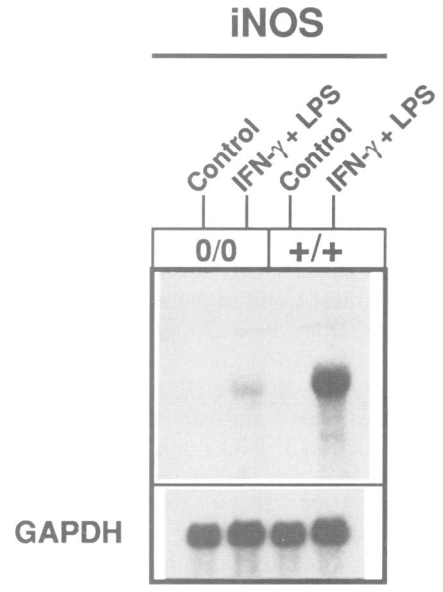

Fig. 4. Detection of iNOS RNA in peritoneal macrophages.Wild-type and IRF-1$^{o/o}$ animals were injected i.p. with 2 ml of 4% thioglycolate. After 72 h, peritoneal exudate was harvested, macrophages were isolated and induced with IFN-γ (100 U/ml) in the presence of LPS (Difco; 100 ng/ml) for 12 h, as described by Kamijo *et al.* (1994). Northern blots (10 μg total RNA/lane) were hybridized with a ^{32}P-labeled 648 bp *Hin*cII–*Bam*HI iNOS cDNA probe (Xie *et al.*, 1992). After autoradiography, the filter was stripped and rehybridized with the GAPDH cDNA probe.

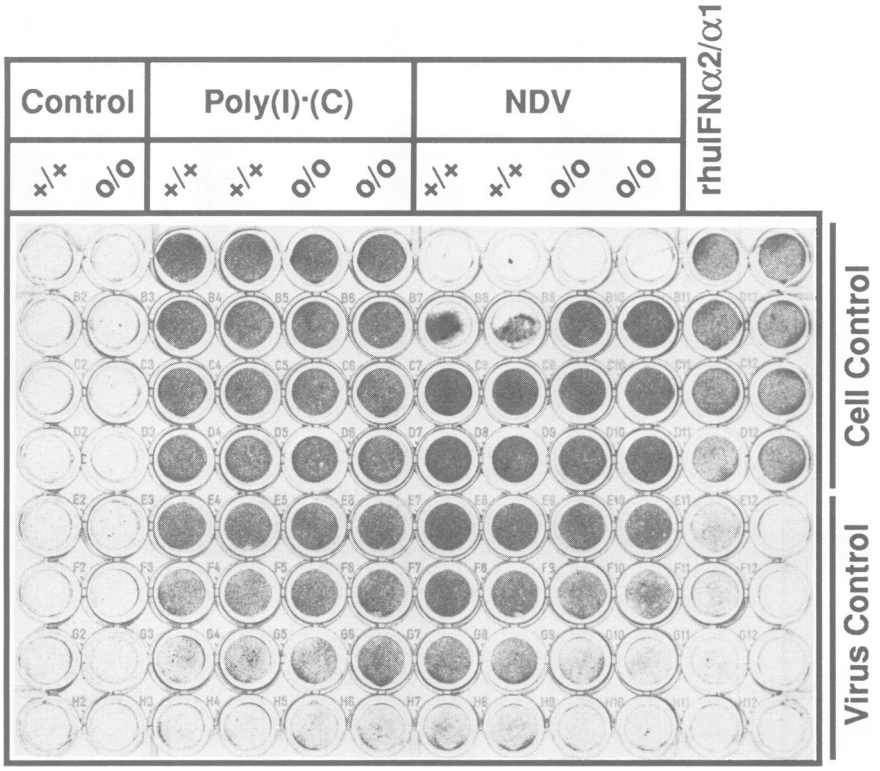

Fig. 5. Titration of type I IFN in sera from wild type or IRF-$1^{o/o}$ mice. Animals were injected i.p. with 200 μg of poly(I)·poly(C) in 200 μl phosphate-buffered saline (PBS) or injected i.v. with 10^7 p.f.u. of NDV in 200 μl BSS (Hank's balanced salt solution) and bled after 6 or 4 h, respectively. Virus was inactivated by acid treatment and IFN titers were determined as described in Materials and methods. Sera were initially diluted 1:20 (first row) and then serially 1:2. Owing to inaccurate neutralization, cells were destroyed in the lowest dilution of the acidified samples. Controls (right column) include four uninfected (top) and four VSV-infected wells.

Discussion

It has been suggested that IRF-1 plays an important role in the expression of the IFN-β and possibly also in that of the IFN-α genes. To test this hypothesis, we generated mice homozygous for disrupted IRF-1 genes and determined the inducibility of the type I IFN genes by virus and poly(I)·poly(C) *in vivo* and in cultured embryo fibroblasts.

Disruption of the IRF-1 gene by replacement of a DNA segment comprising exon 5 and parts of exon 4 and 6 with an extraneous DNA sequence resulted in the abolition of full-length mRNA and in the accumulation of a shorter RNA resulting from exon skipping, i.e. from a splicing event joining exon 3 to exon 7. This abbreviated mRNA lacked exons 4–6, so that the DNA binding domain, which lies between residues 1 and 188 (Fujita *et al.*, 1989a), was disrupted. Induction of iNO synthase mRNA transcription in macrophages, which is dependent on functional IRF-1 (Kamijo *et al.*, 1994), is strongly reduced in our IRF-$1^{o/o}$ mice. Moreover, in agreement with Matsuyama *et al.* (1993), IRF-$1^{o/o}$ mice had a dramatically reduced level of $CD8^+$ T cells. Thus, we found the same phenotype for our animals as the previous workers, as far as comparisons are possible. However, in addition, we have established that the levels of type I IFN activity in serum, and of IFN-α or IFN-β mRNA in the various organs tested after induction with virus or poly(I)·poly(C), are similar in IRF-$1^{o/o}$ and in wild type mice. These results show that the inducibility of type I IFN genes *in vivo* is intact in the absence of IRF-1.

As regards the expression of IFN-stimulated genes, our results showed that IRF-1 is dispensable for the induction by type I IFN of at least some of the ISGs, such as MHC class I, OAS and 1-8 *in vivo*, as is the case for MEFs and ES cells in tissue culture (Matsuyama *et al.*, 1993; Ruffner *et al.*, 1993). Type I IFN-treated MEFs from mutant mice were protected against vesicular stomatitis virus (VSV) (data not shown) and IRF-1-deficient mice were as resistant to infection by VSV as wild type animals (Matsuyama *et al.*, 1993). Resistance to VSV is highly dependent on a functional IFN system, as shown by the finding that mice lacking type I IFN receptor succumb after infection with as little as 50 p.f.u. of VSV, whereas wild type animals survive and clear infection elicited with 10^6 p.f.u. (Müller *et al.*, 1994). We conclude that the expression of whichever genes are responsible for the establishment of the antiviral state against VSV, both in cell culture and *in vivo*, is also independent of IRF-1. However, as mentioned above, IFN-γ-induced expression of the inducible form of NO synthase is severely impaired in macrophages derived from IRF-$1^{o/o}$ mice, suggesting that IRF-1 might be necessary for the activation of some ISGs by type II IFN.

IRF-$1^{o/o}$ MEFs in cell culture, while responding normally to NDV induction, show diminished expression of IFN-α and IFN-β mRNA after poly(I)·poly(C) induction (Matsuyama *et al.*, 1993; this paper). Priming with type I IFN, which acts at the transcriptional level (Dron *et al.*, 1990), increases the expression in both normal and mutant cells, bringing them to about the same level. An important conclusion of this finding is that even in MEFs a direct interaction of IRF-1 with the IFN promoter is not essential.

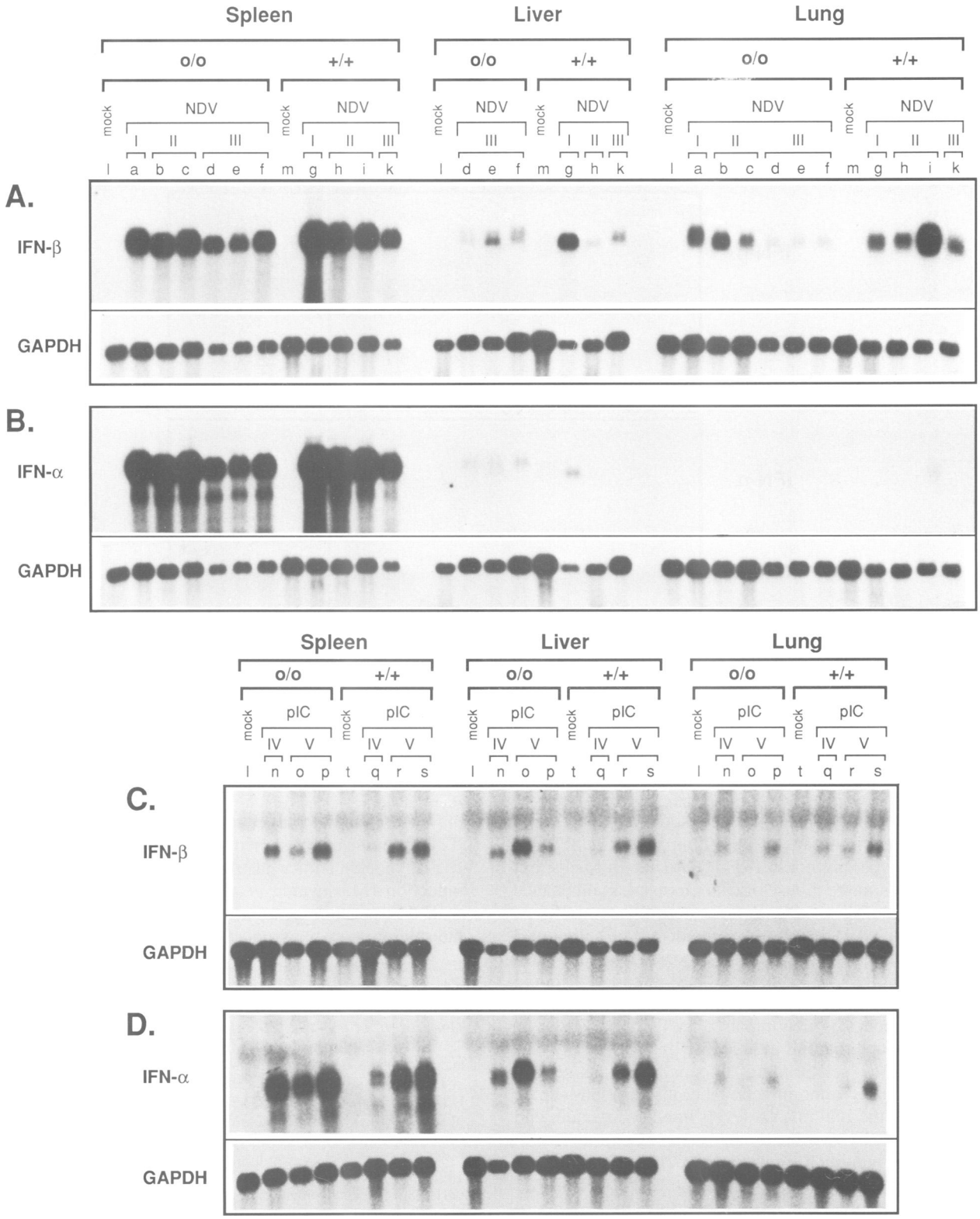

Fig. 6. Detection of muIFN-α and muIFN-β RNA in organs of poly(I)·poly(C)- or virus-induced mice. Animals were injected i.v. with 10^7 p.f.u. of NDV in 200 µl BSS and killed after 4 h (**A** and **B**) or injected i.p. with 200 µg of poly(I)·poly(C) (pIC) in 200 µl PBS and killed after 6 h (**C** and **D**). Total RNA from the organs indicated was subjected to Northern blotting (10 µg total RNA/lane) and hybridized with a ^{32}P-labeled 430 bp *Bam*HI−*Kpn*I muIFN-β (A and C) or a 690 bp *Hin*dIII−*Eco*RI muIFN-α1 (B and D) cDNA probe. After autoradiography, filters were stripped and hybridized with the GAPDH cDNA probe. I−V, five experiments carried out on different occasions; a−t, individual mice.

The priming effect most likely requires protein synthesis because it is abolished by cycloheximide.

Our results can be interpreted (Figure 8) by assuming that some factor X [which could be one of the known factors implicated in the induction of IFNs, other than IRF-1, or one of the proteins identified by band shift analysis by Whiteside *et al.* (1992)] is essential for the induction of both IFN-α and IFN-β genes by poly(I)·

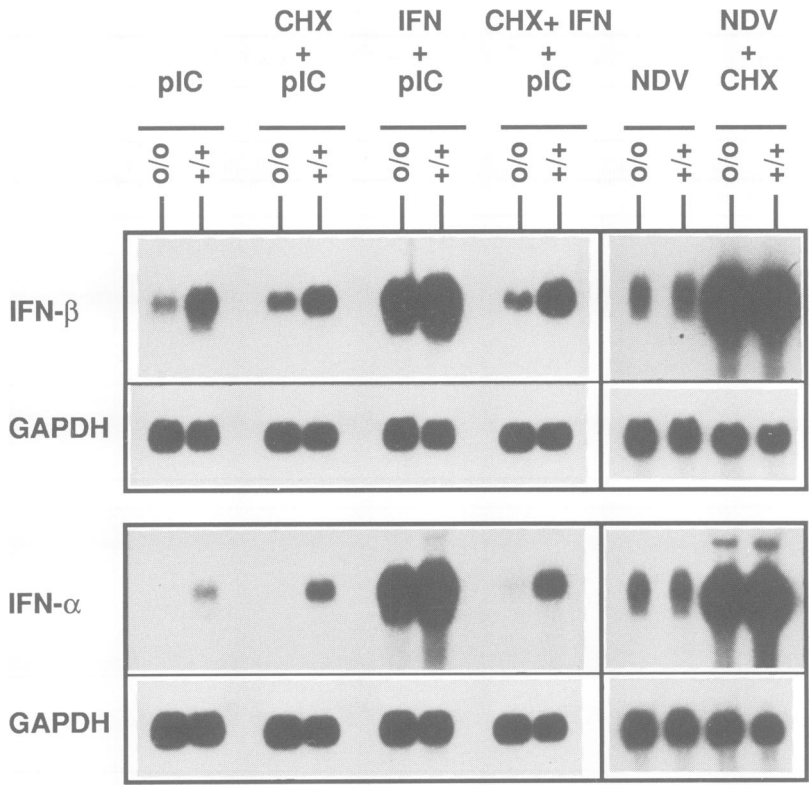

Fig. 7. Detection of muIFN-α and muIFN-β RNA in poly(I)·poly(C)- or virus-induced MEFs. MEFs were cultured in 5 cm Petri dishes and induced with poly(I)·poly(C) (100 μg/ml) in the presence of DEAE–dextran (500 μg/ml) or with an optimized dose of NDV. Where indicated, cells were primed with rhuIFNα2/α1 (500 U/ml) for 3 h prior to induction and, where indicated, cycloheximide (CHX; 75 μg/ml) was added 1 h before NDV induction or 4 h before poly(I)·poly(C) induction and remained present to the end of the experiment. Northern blots (13 μg total RNA/lane for poly(I)·poly(C)-induced and 10 μg total RNA/lane for NDV-induced samples) were hybridized with ^{32}P-labeled IFN-β (upper panel) or IFN-α (lower panel) cDNA probes as described in the legend to Figure 6. After autoradiography, filters were stripped and hybridized with the GAPDH cDNA probe to monitor loading. Identical results were obtained with MEFs derived from an independent set of embryos.

poly(C), and that this factor is constitutively present at effective levels in organs of wild type and IRF-1$^{o/o}$ mice and in wild type, but not in IRF-1$^{o/o}$ MEFs. In MEFs, but apparently not in the mouse organs surveyed, maintenance of effective levels of factor X is dependent on IRF-1. Moreover, in MEFs synthesis of factor X can be induced by IFN in an IRF-1-independent process. It would seem that induction by NDV utilizes, at least in part, a different pathway (designated 'Y' in Figure 8) because induction of the IFN genes is not impaired in IRF-1$^{o/o}$ MEFs. It cannot be argued that full inducibility by NDV in IRF-1$^{o/o}$ MEFs is due to 'self-priming' by small amounts of IFN synthesized early after infection because cycloheximide superinduces the IFN mRNA levels in NDV-infected wild type and IRF-1$^{o/o}$ MEFs to the same extent. Different pathways for induction by virus and poly(I)·poly(C) have been proposed earlier (Kelley and Pitha, 1985). Enoch *et al.* (1986) have reported that in some cell lines inducibility of the IFN-β gene by both poly(I)·poly(C) and virus is dependent on priming by IFN.

The mechanism of superinduction is not entirely clear, but it involves the IFN gene promoter and may be due to an increased level of a transcription factor resulting from stabilization of its mRNA by cycloheximide (Dinter and Hauser, 1987), rather than to stabilization of IFN mRNA as suggested by Sehgal *et al.* (1977).

In summary, we conclude that although IRF-1$^{o/o}$ mice show some distinct phenotypic changes, IRF-1 neither plays an essential, direct role in the induction of type I IFN nor in the establishment of the antiviral state. Because IRF-2 knockout mice also show no impairment of IFN induction (Matsuyama *et al.*, 1993), we are now faced with the question as to which transcription factor plays the role previously assigned to IRF-1.

Materials and methods

Generation of IRF-1$^{o/+}$ ES cells

The GS1 ES cell line was derived from 129/SV blastocysts by G.Stark and M.Aguet as described previously (Bradley, 1987; Robertson, 1987) and grown on irradiated SNL cells (McMahon and Bradley, 1990) in DMEM supplemented with 20% fetal calf serum (D-20). The construction of the targeting vector pIRFneo, in which ~0.5 kb of genomic sequence, corresponding to 308 bp of cDNA sequence (753–1060), were replaced by a PGK-neo/UMS cassette has been described earlier (Ruffner *et al.*, 1993). The linearized plasmid pIRFneo (10 μg) was electroporated into GS1 ES cells and colonies resistant to neomycin and 1-(2-deoxy,2-fluoro-β-D-arabinofuranosyl)-5 iodouracil (FIAU) were selected in D-20 containing 400 μg G418/ml and 0.2 μM FIAU. Individual colonies were picked and analyzed for homologous recombination by PCR using a primer complementary to the 3′ end of the neomycin cassette and a primer corresponding to a genomic sequence 5′ from the targeting vector [Figure 1 in Ruffner *et al.* (1993)]. Five of 528 clones were heterozygous for the disrupted IRF-1 gene and had no extra ectopic insertion, as shown by Southern analysis of genomic DNA (data not shown).

Generation of IRF-1$^{o/o}$ mice

ES cells heterozygous for the disrupted IRF-1 gene were injected into 3.5-day-old C57BL/6 embryos which were implanted in the uteri of pseudo-pregnant ICR mice. One of the clones (GS1-32.4) generated

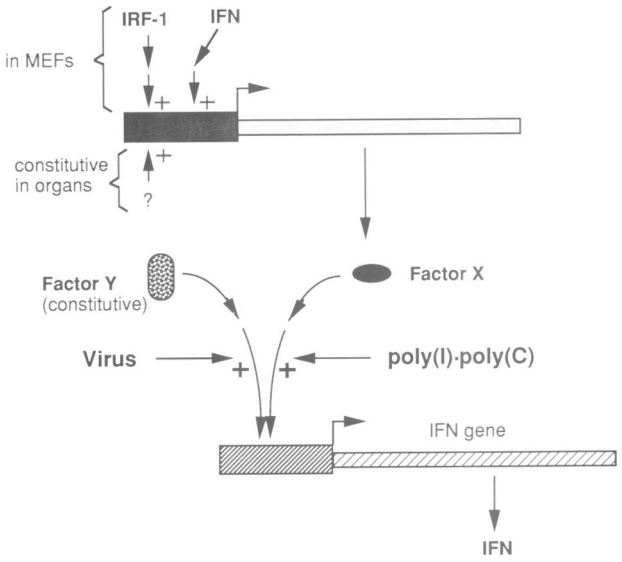

Fig. 8. Generalized scheme for the induction of IFN genes by poly(I)·poly(C) and virus. The scheme postulates that poly(I)·poly(C) and virus induction do not utilize congruent signal transmission pathways, at least in MEFs. Induction by poly(I)·poly(C) is impaired in IRF-$1^{o/o}$ MEFs, but after priming with IFN it is increased in both wild type and mutant MEFs to the same level. Because this enhancement is blocked by cycloheximide, we postulate that synthesis of a factor X is induced by IFN and mediates the poly(I)·poly(C)-induced signal. In mouse organs, factor X (or a factor with similar function) is assumed to be present constitutively because poly(I)·poly(C) induction is not impaired in IRF-$1^{o/o}$ animals, whereas in MEFs its level is dependent on IRF-1. Because virus-mediated induction is not impaired in IRF-$1^{o/o}$ MEFs and not inhibited by cycloheximide, we postulate that it is mediated by a latent, constitutively expressed pathway Y, which is activated by virus infection.

chimeric males with a high degree of chimerism, as evidenced by the presentation of agouti coat patches derived from the ES cell line. Mating of chimeras with C57BL/6 females yielded agouti offspring, of which ~50% carried the disrupted IRF-1 gene, as evidenced by PCR and Southern blot analysis of genomic tail DNA (data not shown). Heterozygous animals were mated and generated ~25% each of homozygous IRF-$1^{o/o}$, as well as of IRF-$1^{+/+}$ offspring, which were used as wild type controls.

Southern and Northern blot analysis

Genomic DNA from tail biopsies (Laird *et al.*, 1991) was digested with *Bam*HI (BioLabs) and electrophoresed through a 0.8% agarose gel. Total RNA (Chomczynski and Sacchi, 1987) was fractionated on 1% denaturing agarose gels. Nucleic acids were transferred by capillarity to a Hybond membrane (Amersham). Hybridization was carried out with ^{32}P-labeled probes prepared by the random primer method (PrimeIt, Stratagene) as described previously (Ruffner *et al.*, 1993). After autoradiography, Northern blots were stripped and rehybridized with a ^{32}P-labeled 490 bp *Xho*II fragment of rat GAPDH cDNA (obtained from G.R.Müller, deceased, Institute of Molecular Biology II, Zürich).

Induction of IFN genes and ISGs in mice and MEFs

Four- to 11-week-old mice were injected i.p. with 200 µg poly(I)·poly(C) (Sigma) and killed after 6 h or injected i.v. with 10^7 p.f.u. NDV and killed after 4 h. Sera were collected for determination of antiviral activity. In the case of NDV induction, sera were diluted 1:10 in 0.2 M KCl–HCl buffer (pH 2) and kept for 48 h at 4°C to inactivate virus. After neutralization, titrations were performed on mouse embryonic fibroblast cells deficient for the IFN-γ receptor (Huang *et al.*, 1993) to exclude antiviral activity due to type II IFN. Organs were kept frozen at − 80°C until RNA extraction. For induction *in vitro*, MEFs were infected with an optimized amount of NDV for 8 h or treated with 100 µg poly(I)·poly(C)/ml plus 500 µg DEAE–dextran/ml for 1 h in the presence or absence of 75 µg cycloheximide/ml; RNA was harvested 4 h after starting poly(I)·poly(C) induction. Where indicated, cells were primed with 500 U/ml of rhuIFN-α2/α1 (which is fully active on mouse cells; Weber *et al.*, 1987) for 3 h before induction by poly(I)·poly(C). For induction of ISGs *in vivo*, animals were injected i.p. with 500 000 U of rhuIFN-α2/α1 and organs were collected 7.5 h later.

Synthesis in vitro of IRF-1 and IRF-1 lacking amino acids 63–182

An IRF-1 cDNA segment (position 330–2401 according to the EMBL nucleic acid data bank, accession number JO3160) containing the complete IRF-1 coding sequence (535–1524) was excised as an *Xba*I fragment from pIRF-L (Miyamoto *et al.*, 1988) and inserted into the *Xba*I site of pBluescript (KS+) to yield pBluesIRF-1. To obtain an expression plasmid for the 'exon skipped' IRF-1, a reverse PCR product was generated from NDV-induced IRF-$1^{o/o}$ lung RNA using a sense primer located 5′ of the insert (cDNA position 433–453) and an antisense primer located in exon 8 (position 1233–1252). Amplification was for 35 cycles with *Taq* polymerase. The 460 bp product was cleaved with *Bsp*EI (exon 3) and *Nde*I (exon 7), and the resulting 62 bp (plus overhangs) fragment inserted into the *Bsp*EI- and *Nde*I-cleaved IRF-1 expression plasmid to yield pBluesIRF-1skip. Sequence analysis confirmed that exon 3 was joined in-frame to exon 7.

In vitro transcription was carried out with T7 RNA polymerase using the expression plasmids cleaved with *Eco*RV in the downstream linker or, for control purposes, with *Cla*I within the coding sequence at position 1287 or with *Dra*I at position 1809 in the 3′ non-coding region. The resulting RNA was precipitated with 2 M ammonium acetate.

Protein synthesis was carried out *in vitro* using reticulocyte lysate (Promega) under the conditions recommended by the manufacturer using 60 µCi ^{35}S-labeled methionine and cysteine, and ~1.5 µg RNA/50 µl lysate, for 90 min at 30°C. The translation products were freed of ^{35}S-labeled amino acids by diluting to 550 µl and concentrating to ~50 µl three times, in Centricon 10000 centrifugation tubes (Amicon).

Reverse band shift

A 10 µl reaction mixture contained 0.4 µl of the protein synthesis mixture with wild type IRF-1 (430 c.p.m., ~0.04 fmol) or 0.8 µl of the protein synthesis mixture with exon-skipped IRF-1 (320 c.p.m., ~0.04 fmol), and 0.3–3000 fmol double-stranded $(GAAAGT)_4$ or $(GACAGT)_4$ in 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA (pH 7.5), 5% glycerol. After 1 h at 25°C, 1.5 µl 10×loading buffer [0.625 M Tris–HCl (pH 7.5), 25% glycerol, 0.1 mM DTT] were added and the products were analyzed on a 4% polyacrylamide gel (20×20×0.1 cm) in 25 mM Tris, 25 mM boric acid, 1.25 mM EDTA (pH 7.5) for 3 h at 250 V.

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