Expression of interferons-α and -ß, tumor necrosis factor-α and transcription factors, IRF-1 and IRF-2, in leukocytes induced during interferon production

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Analysis of gene expression in peripheral blood lymphocytes is of especial interest because it could reflect physiological conditions. We have examined the expression and compared the relative amounts of specific mRNAs for interferons (IFN-α and IFN-ß), tumor necrosis factor-α (TNF-α) and interferon regulatory factors (IRF-1 and IRF-2) from interferon primed and Sendai virus induced peripheral blood leukocytes. Results obtained showed that IRF-1 was highly inducible by IFN treatment, IFN-α, TNF-α and IRF-2 were weakly induced by IFN treatment, and IFN-ß was not inducible by priming the cells with recombinant human IFN-α2b. The IFN-α, IFN-ß, IRF-2 and TNF-α transcripts increased upon viral infection. The IRF-1 mRNA was rapidly induced by IFN treatment and decreased after Sendai virus infection. Our results show that, in peripheral blood lymphocytes, IFN-α and -ß genes have a different response to IFN induction, thus suggesting different regulatory mechanisms for IFN induction of type I IFN genes in peripheral blood lymphocytes.

Key terms: *gene expression; interferon; IRF; leukocytes; lymphocytes; regulation.*

INTRODUCTION

Interferons (IFNs) form a heterogeneous family of cytokines induced in almost all vertebrate cells in response to virus infection or to immune stimuli. In humans and rodents, type I IFNs are encoded by a superfamily of genes consisting of the IFN-α gene family and the IFN-ß gene, which exists in a single copy. IFNs are characterized by their antiviral activity and have been shown to have antiproliferative and antitumoral properties (Vilcek, 1990). Furthermore, recent evidence indicates a role for IFNs on cell growth and differentiation (reviewed by Vilcek, 1990; Weissmann and Weber, 1986). IFNs are usually not detectable in normally growing tissue cultures or in animals until an appropriate stimulus triggers the induction of the IFN system. However, constitutive IFN expression has been reported in organs from normal individuals (Tovey *et al*, 1987; Khan *et al*, 1989, 1990), in peripheral blood leukocytes (PBLs) [where IFN-α could act in an autocrine fashion (Greenway *et al*, 1995)], in preimplantation mouse embryos and embryonal carcinoma (EC) P19 cells (Riego *et al*, 1995), in 13 day post coitum mouse

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embryos (Barlow *et al*, 1984), in mouse blastocysts (Cross *et al*, 1990; Nieder, 1990) and in ruminant trophoblasts (Imakawa *et al*, 1989). Recently, it has been reported along the regulation of NDV-uninducible mouse IFN-α 11 gene (Civas *et al*, 1991).

The onset of IFN production is due to transcriptional activation of the IFN genes. Several groups have characterized induction-regulatory elements in the 5' flanking regions of human IFN genes (reviewed by Dron and Tovey, 1992) and several transcription factors have been proposed to be involved in IFN gene regulation (Dron and Tovey, 1992; de la Fuente *et al*, 1995).

IRF-1 and IRF-2 were identified as an IFN-ß promoter-binding activity that is present in extracts of uninduced and virus-induced mouse L929 cells (Fujita *et al*, 1988; Miyamoto *et al*, 1988; Harada *et al*, 1989) and their role in IFN-ß gene regulation has recently been documented (Dron and Tovey, 1992; Matsuyama *et al*, 1993; de la Fuente *et al*, 1995). However, IFN-α appears to be, at least in some cell types, differently regulated (MacDonald *et al*, 1990; Ruffner *et al*, 1993; de la Fuente *et al*, 1995). The promoter lacks NF-kB binding sites and binds IRF-1 at least an order of magnitude less tightly than the IFN-ß promoter does. Furthermore, it contains a domain designated as TG element (GAAATG) that, when supported by a SV40 enhancer in a reporter construct, mediates virus inducibility but shows little response to IRF-1 (MacDonald *et al*, 1990; de la Fuente *et al*, 1995). Nevertheless, it has been reported that overexpression of IRF-1 can induce IFN-α genes, at least under special circumstances (Harada *et al*, 1990).

Peripheral blood lymphocytes have been shown to produce a mixture of IFN-α and -ß after viral induction (De la Fuente, 1990; Khan *et al*, 1989; 1990; Greenway *et al*, 1995). It has also been shown that PBLs produce TNF-α mRNA after viral induction (Goldfeld and Maniatis, 1989).

In this study, we show that IRF-1 was highly inducible and IFN-α, TNF-α and IRF-2 were weakly induced by IFN treatment in PBLs, while IFN-ß was not inducible by priming the cells with recombinant human IFN-α 2b. The IFN-α, IFN-ß, IRF-2 and TNF-α transcripts increased upon viral infection. The IRF-1 mRNA was transiently induced by IFN treatment and rapidly decreased after Sendai virus infection.

MATERIALS AND METHODS

Cells and culture conditions

Pooled human peripheral blood leukocytes from buffy coats of healthy donors were prepared by treatment with ammonium chloride and induced for IFN-α production, essentially according to the method of Cantell *et al* (1981). Cultures containing 107 cells/ml were primed at -2 h with 200 IU/ml of recombinant human IFN-α 2b (Heberon-αR, Heber Biotec SA, Havana). At 0 h, Sendai virus was added to a concentration of 150 hemagglutination units (HAU)/ml and incubation of the cultures was continued at 37°C. At the times indicated, cultures were centrifuged and the cells were immediately processed for RNA isolation. Culture supernatants were stored at -70°C.

Message Amplification PhenotyPing (MAPPing)

a. Preparation of RNA. All the materials were treated and reagents prepared using standard methods to eliminate RNAase activity. A microadaptation of the guanidinium thiocyanate/cesium chloride (GuSCN/CsCl) procedure was used to prepare total RNA from PBLs. 10^6 cells were added to 100 µl of GuSCN solution containing 20 µg of *E. coli* tRNA as carrier and then layered over 100 µl of 5.7 M CsCl solution (Brenner *et al*, 1989). RNA was purified by centrifugation at 32,000 rpm for 22 h.

b. Synthesis of cDNA. First strand cDNA was synthesized in a 30 µl reaction volume (1x RT reaction buffer [70 mM Tris-HCl, pH 8.6, 1.4 mM $MgCl_2$, 1.4 mM CaCl2], 2 µg oligo-dT_{12-18} (Boehringer Mannheim), 12.5 U RNAsin (25 U/µl, Promega), 100 mM dithiothreitol (Sigma), dNTPs (0.3 mM each dATP, dCTP, dGTP and dTTP, Boehringer Mannheim). The RNA was incubated at 42°C for 90 min with 35 U (1 µl) of cloned

AMV reverse transcriptase (Heber Biotec SA).

c. Polymerase Chain Reaction (PCR). Ninety µl of the PCR mix were added to 10 µl of first strand cDNA reaction. PCR mix contains: 56 µl sterile water, 10 µl 10x reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.4), 25 mM $MgCl_2$ and 0.1% gelatin], 20 µl dNTP mixture (5 mM each of dATP, dCTP, dGTP, and dTTP, Boehringer Mannheim), 2.5 U of *Thermus aquaticus* DNA polymerase (Heber Biotec SA) and 4 µl primers mixture (1,000 pmoles of each primer). The components were concentrated in the bottom of the tube by centrifugation and overlaid with 50 µl of mineral oil (Sigma). The mixture was subjected to PCR amplification for 30 cycles. The cycles were of 94°C (1 min), 55°C (1 min) and 72°C (1 min). Ethidium bromide-stained 2% agarose gels were used to separate PCR fragments.

Oligonucleotide primer design

Oligonucleotide PCR primers were designed using published sequences (Table I). Sequences within the coding regions were chosen for the 5' primers (+ strand) and 3' primers (- strand). The IFN-α genes are related to IFN-ß gene exhibiting about 45% homology at the nucleotide level and a predicted amino acid sequence homology of about 29% (Weissmann and Weber, 1986). Fidelity of the amplified sequences for IFN-α or -ß genes was clearly identified by Southern blot analysis using ^{32}P-labeled DNA probes for both human genes. Oligonucleotides were synthesized on a Gene Assemble DNA Synthesizer (Pharmacia) and purified by HPLC.

Southern blot analysis

The PCR samples were separated by electrophoresis on a 2% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham International), and crosslinked by UV irradiation for 4 min. The filters were prehybridized during 4 h at 42°C in 50% formamide, 5x Denhardt's solution (1x Denhardt= 0.02% of polyvinylpyrrolidone, Ficoll, and bovine serum albumin), 5x SSC (1x SSC= 0.15 M NaCl, 15 mM sodium citrate, pH 7), 0.1% sodium dodecyl sulfate (SDS) and 200 µg/ml sheared denatured calf thymus DNA. Hybridizations were carried out in the same solution for 16 h at 42°C, in the presence of 10^7 cpm/ml of probes radiolabelled by the random priming technique (Feinberg and Vogetstein, 1984). After hybridization, the filters were washed twice at 25°C in 2x SSC /0.1% SDS for 10 min each, followed by a wash at 60°C for 30

TABLE I

Primers used for MAPPing

GENE	SIZE OF AMPLIFIED FRAGMENT (BASES)	PRIMER
IFN-α	290	5' TCTCTGTCCTCCATGAG(A/C)TGATCCAGCAGA 3'
IFN-α		3' CCCTCCAACAGTCTCGTCTTTAGTACTCT 5'
IFN-ß	310	5' CAGAAGCTCCTGTGGCAATTGAATGGGAGG 3'
IFN-ß		3' GTGGTCCCCTTTTGAGTACTCGTCAGACGT 5'
ß-actin	500	5' ATGGATGATGATATCGCCGCG 3'
ß-actin		3' GGGAGGTAGCAGGTGGCGTTTACGAAGATC 5'

Oligonucleotide primers synthesized according to sequences reported for IFN-α and IFN-ß genes (Pestka, 1983).
(A/C) denotes that either A or C was equally introduced in this position during oligonucleotide synthesis.

min in 0.2x SSC /0.1% SDS, and then subjected to autoradiography.

The probe used for the analysis of IFN-α products was the plasmid pDA8 (de la Fuente *et al*, 1988), which contains the human IFN-α2 cDNA. For IFN-ß, we used the plasmid pAdßIF3 (J de la Fuente, unpublished results) which contains the HincII human IFN-ß gene coding fragment cloned into the expression plasmid pAd25 (Lleonart *et al*, 1991). Plasmids pTNF (A Silva, unpublished results) and pIRF1 (A Pérez, unpublished results) contain the human TNF-α and mouse IRF-1 cDNAs cloned into the plasmid pBluescript, and were used for the screening of TNF-α and IRF-1 transcripts, respectively.

Northern blot Analysis.

Ten µg of RNA were separated on 1% formaldehyde/agarose gel, transferred to a nylon membrane (Hybond-N, Amersham International), and crosslinked by UV irradiation for 4 min. The filters were prehybridized for 4 h at 42°C in 50% formamide, 5x Denhardt's solution, 5x SSC, 0.01% SDS and 200 µg/ml sheared denatured calf thymus DNA. Hybridization was carried out in the same solution with the addition of [^{32}P]-labeled probes (see Southern blot analysis) for 24 h at 42°C. The filters were washed as indicated for Southern blot analysis.

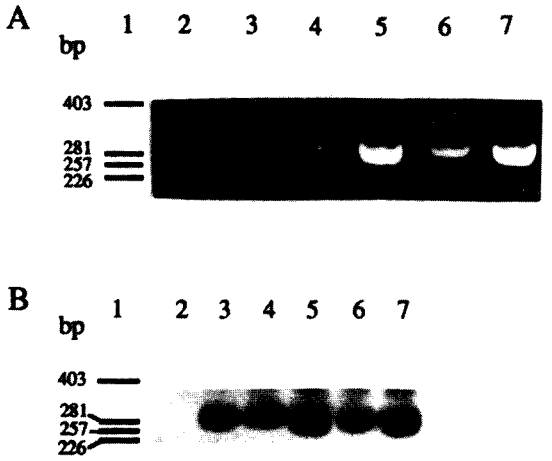

Fig 1. Detection of IFN-α mRNA in PBLs by MAPPing. **A.** Expression of IFN-α transcripts in leukocytes showed by agarose gel electrophoresis of the MAPPing reaction products obtained after 30 cycles of PCR. Lane 1: pBR 322-Alu I fragments; lane 2: leukocyte RNA isolated from uninduced cells; lanes 3, 4 and 6: RNA from leukocytes treated with 200 IU/ml of recombinant human IFN-α 2b for 2, 4, and 8 h respectively; lanes 5 and 7: IFN-α products after 2 h of priming and following the addition of 150 HAU/ml of Sendai virus, during 2 and 6 h, respectively. **B.** Southern blot analysis of the gel shown in A, showing specific amplified products by using a ^{32}P-labeled human IFN-α 2b DNA probe. Lanes numbered as in A.

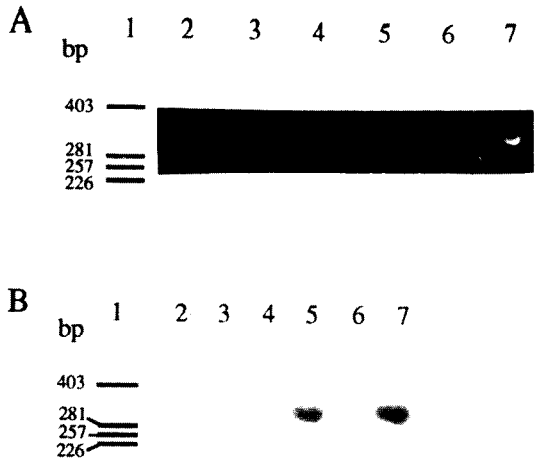

Fig 2. Detection of IFN-ß mRNA in PBLs by MAPPing. **A.** Expression of IFN-ß transcripts in leukocytes showed by agarose gel electrophoresis of the MAPPing reaction products obtained after 30 cycles of PCR. Lane 1: pBR 322-Alu I fragments; lane 2: leukocyte RNA isolated from uninduced cells; lanes 3, 4 and 6: IFN-ß products from the RNA of the leukocytes treated with 200 IU/ml of recombinant human IFN-α 2b for 2, 4 and 8 h respectively; lanes 5 and 7: IFN-ß products after 2 h of priming and following the addition of 150 HAU/ml of Sendai virus during 2 and 6 h, respectively. **B.** Gel shown in A transferred to a nylon membrane and hybridized with a ^{32}P-labeled human IFN-ß DNA probe.

RESULTS

In order to characterize the IFN production through the analysis of the cytokines and other factors produced during leukocyte induction with Sendai virus, we used the MAPPing and Northern blot techniques. RNA was isolated from 10^6 cells and equal amounts of RNA were used for each experiment. IRF-1, IRF-2 and TNF-α gene products were analyzed by Northern blot. IFN-α and -ß transcripts were studied by Northern blot and MAPPing to detect even a low level induction of the corresponding genes. Actin primers were used to monitor for efficient cDNA synthesis.

Analysis of IFN transcripts.

The analysis of IFN-α transcripts showed no detectable mRNA levels in uninduced cells

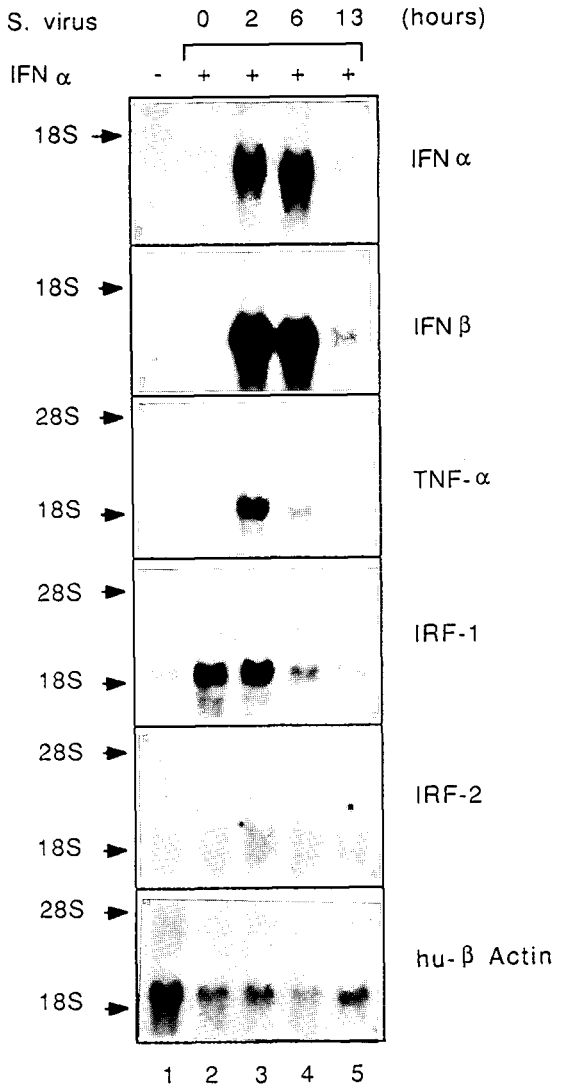

Fig 3. Northern blot analysis of IFN-α, IFN-ß, TNF-α, IRF-1 and IRF-2 transcripts in PBLs after IFN priming and Sendai virus (*S. virus*) infection. Cells primed with 200 IU/ml of recombinant human IFN-α 2b at -2 h (-). At 0 h, cultures induced with 150 HAU/ml of Sendai virus. RNA extracted and analyzed before IFN treatment (lane 1), 2 h after IFN treatment and before virus infection (lane 2), and 2, 6 and 13 h after virus infection (lanes 3-5, respectively). Northern blot hybridized with human IFN-α 2b (IFNα), IFN-ß (IFNß), TNF-α, IRF-1 and IRF-2 probes and subsequently with a human ß-actin probe (hu-ß Actin) as internal standard.

(Fig 1A, line 2; Fig 3). To examine the priming effect on IFN production, leukocytes were treated with recombinant IFN-α 2b (200 IU/ml) for 2, 4 and 8 h (Table II; Fig 1A, lines 3, 4 and 6, respectively; Fig 3). This resulted in the production of increased quantities of IFN-α, and higher amounts at various times (2 and 6 h) after Sendai virus infection (Table II; Fig 1A, lines 5 and 7; Fig 3).

After gel electrophoresis and ethidium bromide staining, IFN-α PCR products were transferred to a nylon membrane and hybridized to a ^{32}P-labeled human IFN-α 2b probe, appearing a single band of the expected size (Fig 1B), thus corroborating the specificity of the amplification reaction.

We have studied IFN-ß messengers synthesized during IFN-α production. Leukocytes produced IFN-ß transcripts at various times (2 and 6 h) after Sendai virus infection (Fig 2 A, lines 5 and 7). Untreated cells (Fig 2A, line 2) and cells treated with 200 IU/ml of recombinant IFN-α2b (priming effect) did not produce detectable levels of IFN-ß mRNA (Fig 2A, lines 3, 4 and 6). Southern blot analysis of products from the MAPPing reaction showed that the amplified bands corresponded to IFN-ß transcripts (Fig 2B). Northern blot analysis gave similar results (Fig 3), thus showing no priming effect for IFN-ß detected in PBLs treated with IFN-α 2b, at least under our experimental conditions.

Analysis of IRF-1 and IRF-2 induction.

We attempted to identify nuclear factors, termed IRF-1 and IRF-2, that specifically binds to upstream regulatory (virus inducible) elements of the IFN-ß gene. As shown in Figure 3, IRF transcripts were present prior to induction. IRF-1 was highly induced by IFN treatment. IRF-2 transcripts had a moderate increase after priming the cells with recombinant human IFN-α2b. After viral infection, IRF-2 transcript levels increased. There was no increase in the IRF-1 mRNA levels after the induction with Sendai virus.

Analysis of the TNF-α transcripts

We also measured the expression of the TNF-α mRNA. As shown in Figure 3, TNF-α mRNA levels increased after priming the cells with recombinant human IFN-α2b, as well as after the infection with Sendai virus. Maximal levels were obtained 4 h after IFN priming and 2 h after virus induction.

TABLE II

Kinetics of IFN-α production

SAMPLE [a]	CONCENTRATION OF IFN-α			
	+ IFN-α 2b		+ IFN-α 2b + virus [b]	
(hours)	(ng/ml) [c]	(IU/ml) [d]	(ng/ml)	(IU/ml)
-2	0	0	0	0
0	20 ± 2	3,913 ± 331	20 ± 2	3,913 ± 331
2	30 ± 4	5,985 ± 714	11 ± 1	2,242 ± 276
4	11 ± 0.5	2,229 ± 91	59 ± 7	11,752 ± 1,390
6	9 ± 5	1,783 ± 1,068	106 ± 5	21,113 ± 909
16	2 ± 0.2	343 ± 34	143 ± 9	28,585 ± 1,708

[a] PBLs prepared and cultured as described in Materials and Methods. At -2 h, cultures primed with 200 IU/ml of recombinant IFN-α 2b.

[b] At 0 h, some cultures induced with 150 hemagglutination units/ml of Sendai virus.

[c] IFN-α protein concentration measured by a specific ELISA (Cruz *et al*, 1990). Values, means ± SD of 4 independent experiments.

[d] IFN activity measured by a cytopathic effect reduction assay using HEp-2 (ATCC) cells and Mengo virus as challenging virus. Activity expressed in international units (IU). Values, means ± SD of 4 independent experiments.

DISCUSSION

Studies on the expression of the IFN genes, coexpressed cytokines and other regulatory genes in PBLs, are of special relevance because they can reflect physiological situations. In this report we have followed the time course of expression of IFN-α and IFN-ß genes, TNF-α and regulatory factors IRF-1 and IRF-2 in PBLs treated with recombinant human IFN-α2b and infected with Sendai virus. The time course selected for the experiments was based on previous studies showing that maximal IFN mRNA levels were observed 3-6 h after Sendai virus induction (Berent *et al*, 1986; Gobl *et al*, 1988).

It is well known that both IFN-α and IFN-ß genes are rapidly induced and then down-regulated after induction by RNA viruses or poly rI:rC (Levy and Salazar, 1992). IFNs are also known to affect their own production. Cells pretreated with low concentrations of IFN before virus induction are able to make more IFN (priming effect) (Abreu *et al*, 1979). However, the finding that IFN-α, but not IFN-ß, mRNA accumulation may be induced by priming the PBLs with recombinant human IFN-α2b constitutes, to our knowledge, a new observation which could have important implications for the understanding of the mechanisms that control IFN gene regulation under physiological conditions.

The expression of IFN-α and IRF-ß genes shows tissue specificities in humans; thus, endogenous IFN-beta gene is expressed efficiently in fibroblasts, but not in other cell types such as leukocytes, which predominantly express classes of IFN-α genes (Hoss *et al*, 1989; Khan *et al*, 1989; de la Fuente, 1990). The differences observed here in the IFN induction of type I IFN genes in PBLs may also reflect differences in the regulation of IFN-α and IFN-ß genes in PBLs.

A number of expression studies with cloned IFN-α and IFN-ß genes have revealed the presence of *cis*-acting DNA transcription elements within the 5'-flanking region (reviewed by Weissmann and Weber, 1986; Dron and Tovey, 1992). Interestingly, such sequence motifs are also found in other virus inducible cytokine genes, such as TNF-α and IL-6 (Fujita *et al*, 1987).

The present study shows that human TNF-α, IFN-α, and IFN-ß genes are induced by virus in PBLs. Previous studies have shown similar results for TNF-α and IFN-α (Berent

et al, 1986; Ronni *et al*, 1995), as well as for TNF-α and IFN-ß (Goldfeld and Maniatis, 1989). The relevance of these observations is not completely understood, but it can have a particular importance in light of the shared and synergistic activities of their gene products (Mestan *et al*, 1986; Wong and Goeddel, 1986).

Interferon regulatory factors IRF-1 and IRF-2 have IFN-ß promoter-binding activity that is present in extracts of uninduced and virus-induced mouse L929 cells (Fujita *et al*, 1988; Harada *et al*, 1989). The mRNA level of these factors rapidly increases within 1 h after IFN treatment of mouse L929 cells (Harada *et al*, 1989), and does not change significantly on viral induction. The results of the experiments presented in this paper show that the IRF-1 and IRF-2 mRNA levels in PBLs are also rapidly induced by IFN, while the IRF mRNA level decreases after Sendai virus infection (Fig 3). However, under our experimental conditions, IRF-2 transcripts increased after 2 h of Sendai virus infection (Fig 3).

We can summarize the results obtained in PBLs in terms that IRF-1 was highly inducible by IFN treatment, IFN-α, TNF-α and IRF-2 were weakly induced after IFN treatment, and IFN-ß was not inducible by priming the cells with recombinant human IFN-α 2b. The IFN-α, IFN-ß, IRF-2 and TNF-α transcripts increased upon viral infection. The IRF-1 mRNA was rapidly induced by IFN treatment and decreased after Sendai virus infection. These results may support previous findings suggesting that IFN-α and -ß genes are not regulated by similar mechanisms, at least in all cell types (MacDonald *et al*, 1990; Ruffner *et al*, 1993; De la Fuente *et al*, 1995). This differential expression of type I IFN genes could be regulated at the transcriptional level, reflecting cell-specific differences in the regulation of the induction of transcription of type I IFN genes (Riego *et al*, 1995; de la Fuente *et al*, 1995).

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