Synergism of type I and type II Interferons in stimulating the activity of the same DNA enhancer

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Type I and type II interferons (IFNs) can act synergistically to activate the transcription of the 2-5A synthetase gene. We used in vivo functional assays of sequences from the gene promoter region to determine which DNA segment mediates the gene induction by IFNγ and the synergistic effect. We found that the type I IFN-inducible enhancer (or IRS) of the 2-5A synthetase gene also confers inducibility by type II IFN to a reporter CAT gene, though the time course and dose response of the induction by the two IFNs are quite different. A clear synergism of the two IFNs in stimulating the IRS is observed at low doses of the two IFNs.

2-5A Synthetase; Type I interferon-inducible enhancer synergistic activation type I interferon; Type II interferon

1. INTRODUCTION

The 2-5A synthetase enzymatic activity is inducible by type I(α,β) interferons (IFNs) and type II(γ) IFNs in most cell lines [1]. The two types of IFNs differ, however, notably in their structure, their cell surface receptors, and their biological activities [2,3]. It is therefore logical that the characteristics of induction of the cloned 2-5A synthetase mRNAs [4] by the two IFNs are not similar [5,6]. The mRNAs are induced much less by IFNγ than by type I IFNs [5,6]. In HeLa cells, their induction by IFNγ can be abolished by the protein synthesis inhibitor cycloheximide, while their induction by IFNα is, in general, not sensitive to cycloheximide [5] and considered as a direct effect on transcription [7]. At the same time, it was described that the two IFNs can synergize to produce antiviral and antigrowth effects [8,9] and induce the 2-5A synthetase [10–14]. The synergistic effect is particularly visible in cells partially resistant to type I IFNs [12], and has been shown to occur at the level of gene transcription [13,14]. That the synergistic effect could result from a change in IFN receptors is not likely [11,12].

It was therefore important to assess whether the activation of the gene by the two types of IFNs is mediated by the same or by distinct DNA sequence elements. To answer this question, we have tested the ability of different segments of the 2-5A synthetase promoter, coupled to a reporter gene, to mediate the induction by either type of IFN or their combination upon transfection in HeLa cells.

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2. MATERIALS AND METHODS

2.1. *Cell culture, transfections and CAT assays*

HeLa cells were grown as monolayers. For transient transfection experiments, cells were transfected at half-confluency with supercoiled plasmid DNA by the $CaPO_4$ precipitation method, as previously described [15–17]. After 12 h, the medium was changed, and the cells were induced for 24 h by 200 IU/ml of recombinant human IFNβ1, 500 IU/ml recombinant human IFNγ, or left untreated. To obtain permanently transformed HeLa cells, 50% confluent cells in 10-cm plates were transfected simultaneously by 19 μg of XhE-IRS TK-CAT [15] and 1 μg of pSV_2-neo plasmid [18], the latter conferring resistance to the antibiotic neomycin (G-418, Sigma). After 3 weeks of selection in the presence of 500 μg/ml G418, 100 G-418 resistant clones were pooled. The integration of the plasmid XhE-IRS TK-CAT was checked by Southern blotting [19]. 24 h after induction the cells were collected and CAT activity (expressed as percent acetylation of chloramphenicol) measured [13].

2.2. *Plasmids*

The pGEM CAT-E and 5Δ31 vectors containing the −748/+82 and −159/+82 fragments of the 2-5A synthetase promoter, respectively, fused in frame to the CAT coding sequences in the pGEM CAT-0 vector were constructed as described [16]. pGEM TK CAT-0 was obtained by cloning the HSV TK gene −109/+51 promoter fragment in front of the CAT gene in pGEM CAT-0 [16,17]. hE-IRS TK-CAT or moE-IRS TK-CAT were obtained by cloning synthetic IRS regions (−102/−87 of the human 2′-5′ A synthetase gene, TGAGGAAACGAAACCA, or −74/−59 of the mouse 2-5A synthetase gene, TCGGGAAATGGAAACT) in the multilinker of the pGEM TK CAT-0 plasmid, upstream of the TK promoter [17]. The XhE-IRS TK-CAT contains six copies of the synthetic human IRS, forming the following nucleotide sequence cloned in the *Bam*H1 site of the polylinker in front of the TK gene [17]: ggatCTGAGGAAACGAAACCAg{gaTCCTGGGCGTTTCCTCA}$_5$gatccaga.

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2.3. *RNA preparation and S1-nuclease analysis*

Total cellular RNA was prepared from HeLa cells by the hot phenol method [17] from two 9-cm plates, 36 h after transfection, and 12 h after induction of 200 IU/ml IFN-β1, 500 IU/ml IFNγ, or no IFN, in the presence or absence of 50 μg/ml of cycloheximide for 12 h. To prepare a labelled probe, the *Eco*RI fragment of the plasmid pGEM TK-CAT-0 was first inserted in the *Eco*RI site of the M13$^+$ Bluescript vector (Stratagene). The non-coding strand was then labelled with α^{32}P-dCTP by the Klenow enzyme from the T7 primer, cut with *Hin*dIII, and isolated from a denaturing polyacrylamide gel. The annealing to the RNA and S1 nuclease analysis were as described [17].

3. RESULTS

3.1. *Sequences governing the transcriptional response to β and γ IFNs*

The CAT activities were measured in extracts of cells transfected by various plasmid DNAs, and treated by IFNs β or γ (section 2). The ratio of the CAT activities in the presence of IFNs relative to the activities in the absence of IFN are shown in Fig. 1. We observe that the segments $-748/+82$ (pGEM CAT E) or $-159/+82$ (5Δ31 CAT) of the 2-5A synthetase promoter both have the ability to render the CAT gene inducible by IFNβ and IFNγ, IFNγ being much less efficient in this process. Inducibility by IFNγ is also visible when only a 14-bp segment of the human 2-5A synthetase promoter ($-102/-87$), hE IRS TK CAT), or of the mouse promoter [17] (($-74/-59$), mE IRS TK CAT), was cloned in front of the TK-CAT chimeric construct (Fig. 1). This element has been identified as a type I IFN-responsive sequence or IRS [16,17].

A higher induction ratio, due to a higher expression of the CAT, was observed with both IFNs when a multimer of the hE IRS was in front of the TK-CAT chimeric construct (XhE IRS TK CAT) (Fig. 1). No IFN effect was observed with the control vectors pGEM CAT-0, or pGEM TK CAT-0 (Fig. 1), or other vectors including a 2-5A synthetase promoter fragment which did not contain the IRS ($-206/-109$, not shown). In the constructs where only the IRS fragment is present, as when the whole promoter is used, IFNγ induces less CAT activity than IFNβ.

3.2. *The induction of CAT mRNA by IFNγ is inhibited by cycloheximide*

We isolated RNA from HeLa cells transfected by the XhE IRS TK CAT plasmid and treated by IFNs in the presence or absence of cycloheximide. After hybridization of the RNAs with a radiolabelled DNA probe complementary to the 5′ regions of the CAT gene and TK

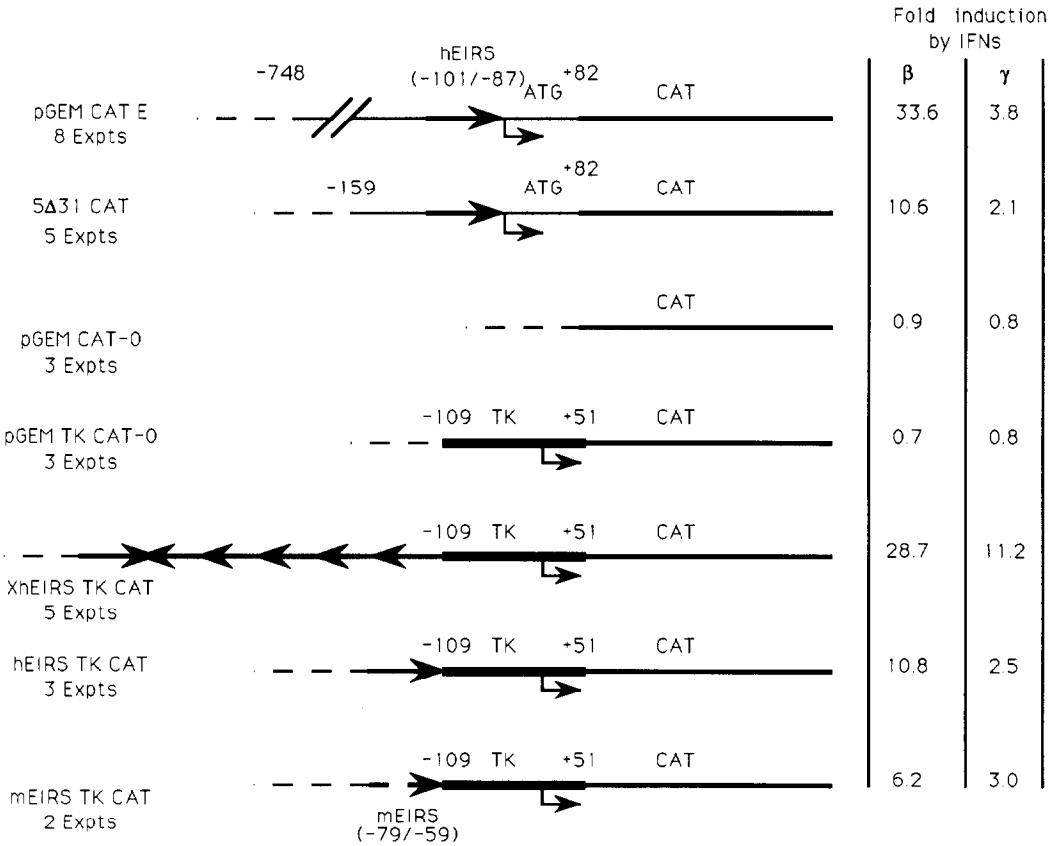

Fig. 1. Two variants of the IRS are able to mediate the effects of both types of IFNs. Subconfluent HeLa cells were transiently transfected by the constructs described in section 2. The thick arrows indicate the orientations of the IRS. The dashed arrows correspond to the murine IRS. The thin arrows show the location of RNA start sites. The values given are the average of several transfections experiments. The differences from the control plasmids (pGEM CAT-0 and pGEM TK CAT-0) are significant at the threshold of 1% with the Student's *t*-test.

promoter [17], the CAT mRNA concentration was determined by its ability to protect this probe from S1 nuclease digestion [19]. We see in Fig. 2 that in the absence of cycloheximide, the CAT mRNA was induced by IFNβ (8.4-fold) and by IFNγ (3.0-fold). The mRNA, like the CAT activity was less induced by IFNγ, showing that the differential effect is really transcriptional. The CAT mRNA was also increased by cycloheximide treatment alone (Fig. 2). This increase does not involve the IRS, since it is observed also in cells transfected by the pGEM TK CAT-0 vector (not shown). In cells treated with IFN and cycloheximide, IFNβ induced the CAT mRNA above the basal level (4.5-fold stimulation) while IFNγ did not stimulate, or even depressed, the CAT mRNA level.

3.3. *Combined effects of the two IFNs on the activation of the IRS*

To perform this study, which involves a large number of assays at different doses of IFN, the use of HeLa cells permanently transformed by the XhE IRS TK CAT vector was preferred to transient transfections. Fig. 3A shows the ratios of induction of the CAT activity brought by each IFN separately, after 48 h of treatment. At 24 h of treatment, the induction by IFNγ was lower than after 48 h treatment (by 2-fold), while the induction by IFNβ was similar or higher (not shown). IFNγ is therefore unable by itself to stimulate the IRS to the levels of maximal induction obtained with IFNβ. When the two IFNs were mixed, however, a synergistic effect could be obtained, depending on the

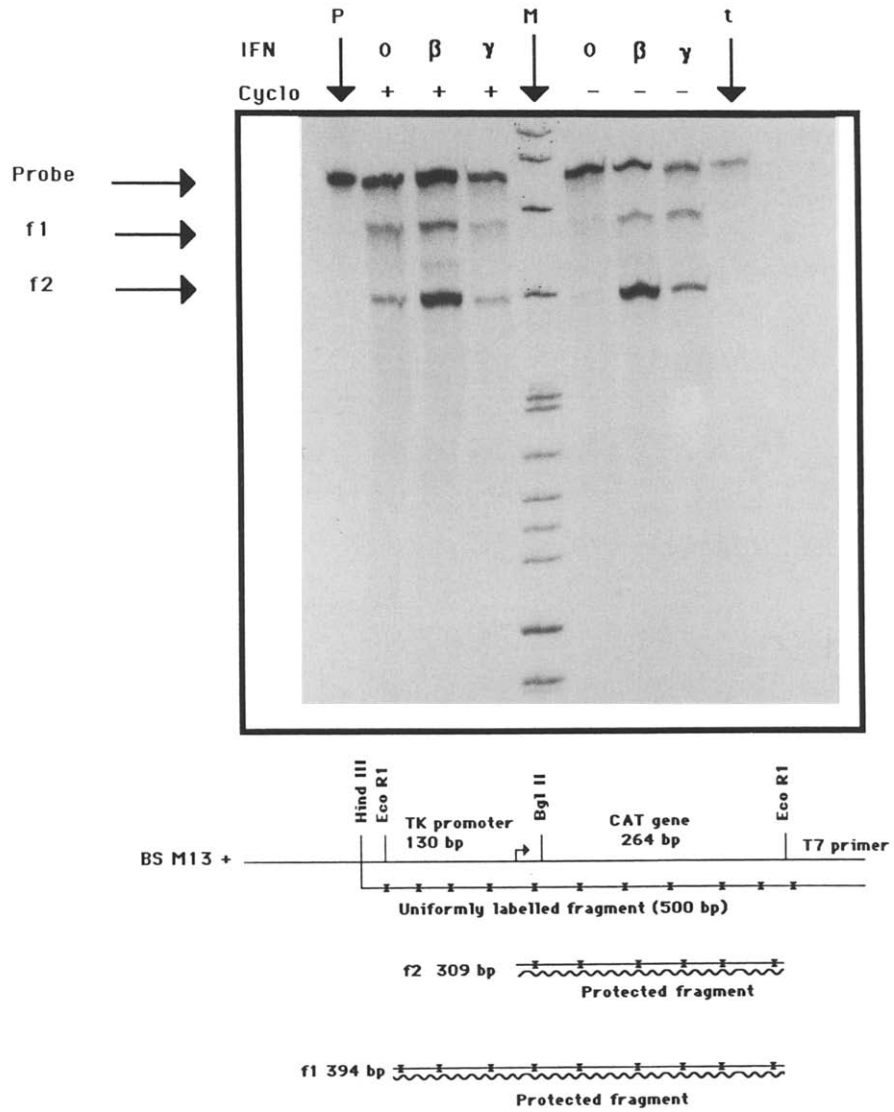

Fig. 2. Activation of the XhEIRS TK promoter by IFNγ but not by IFNβ requires protein synthesis, S1 nuclease analysis of the transcripts in HeLa cells transiently transfected by the XhEIRS TK-CAT plasmid and treated (+) or not (−) with cycloheximide in the absence (0) or presence of 200 U/ml IFNβ or 500 U/ml IFNγ, for 12 h. Fragments f1 and f2 protected by transcripts are schematized. f2 corresponds to transcripts starting at the TK cap site. P = probe without S1. M = *Msp*II-cut PBR 322 size markers, ^{32}P end-labelled. t = Probe annealed to tRNA before S1.

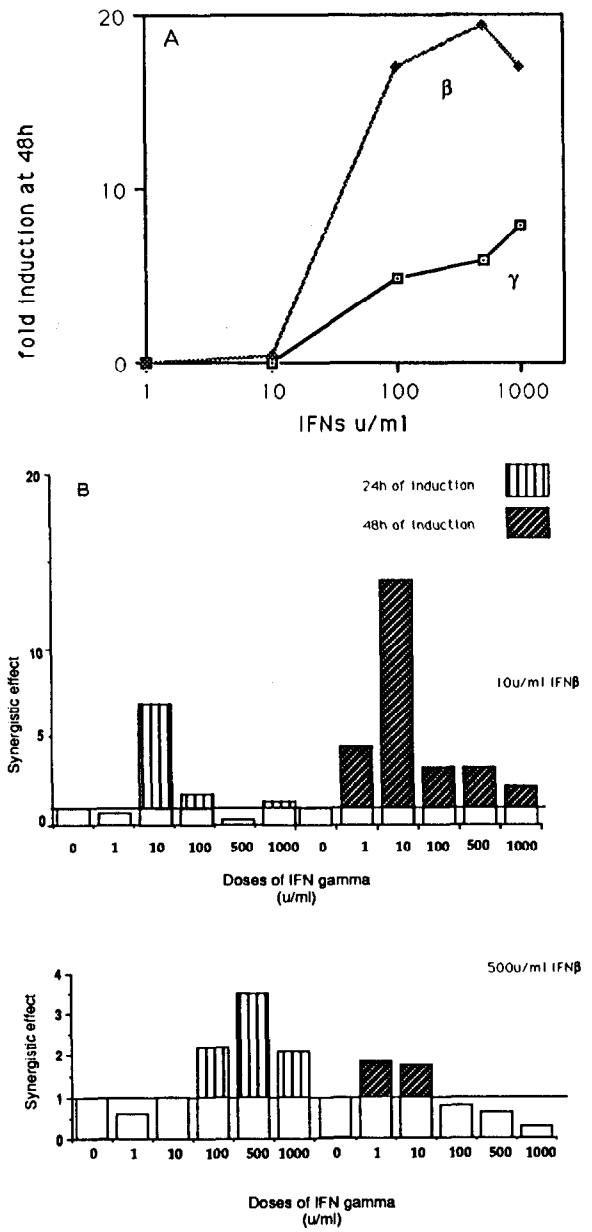

Fig. 3. Dose- and time-dependent effects of the IFNs, added separately or simultaneously, on CAT activity in XhE-IRS TK-CAT transformed cells. Subconfluent HeLa cells permanently transformed by XhEIRS TK-CAT were treated for different times with several doses of IFNβ or IFNγ, or a combination of both. (A) Effects of increasing doses of each IFN on the induction of CAT activity in triplicate cell cultures was determined, and the ratio of CAT activity in extracts treated by IFN relative to non-treated samples was calculated. (B) Synergistic effect of simultaneous treatment by IFNβ and IFNγ. The CAT activity of control cultures was subtracted from all the corresponding values obtained with IFN. The ratio between the CAT activity in the presence of both IFNs and the sum of CAT activity obtained with each IFN added at the same doses represents the synergistic effect of the combination of the two IFNs. Average CAT activity in non-treated samples: 24 h 1.1%, 48 h 2.8%.

dose of IFN and length of treatment (Fig. 3B). A synergistic effect of 2- to 13-fold is observed at 10 IU/ml of IFNβ (Fig. 3B) the effect being stronger at 48 h. At higher concentrations of IFNβ (100–500 IU/ml), and low concentrations of IFNγ (1–10 IU/ml), synergism (2–3-fold) is seen at 48 h. At high concentrations of IFNβ and IFNγ, the synergism is seen at 24 h rather than at 48 h (Fig. 3B).

4. DISCUSSION

We have established that, as we have briefly reported before [20], the same DNA element of the 2-5A synthetase gene mediates transcriptional induction by type I and type II IFNs. An element homologous to the 2-5A synthetase IRS, also present in the 5'-flanking region of other IFN-induced genes (6-16, and 9-27 [21], H_2K^d [22]), was able to confer inducibility by the two types of IFNs on a reporter gene. This statement can thus be now generalized.

In addition, we show here that IFNγ is less efficient than IFNβ in stimulating the IRS, at all the doses examined, and that the induction of the reporter gene IFNγ is obtained by long treatments. The same remarks can be made when the induction of the endogeneous 2-5A synthetase activity [11,12,23] or mRNA is measured [5,6,13,14], meaning that the difference comes mainly from the transcriptional effects of IFNs. The delay in obtaining the maximal response to IFNγ and the effect of cycloheximide suggest that the synthesis of a new protein is needed to obtain the transcriptional effect of IFNγ, and that the two IFNs stimulate the IRS by different mechanisms.

Furthermore, the combination of the two IFNs is not merely additive (Fig. 3), which might suggest that the two IFNs could also mutually modify the factors used by each of them. That the synergistic effects of the two IFNs is better seen after 48 h than 24 h raises the possibility that: (i) a protein factor induced by IFNγ is also needed in the process of activation of the enhancer by IFNβ; or (ii), as previously suggested [24], down regulation of transcription, occurring normally at later times with type I IFN, is blocked by IFNγ.

Progress has been made in the characterization of proteins interacting with the type I IFN-inducible enhancer. The induction of gene transcription by type I IFNs has been correlated with the formation of a new IRS-protein complex within minutes of IFN treatment (ISGF3 [25], E [26]). Most interestingly, in vitro, a protein present in extracts of HeLa cells treated by IFNγ is able to complement the factors activated by type I IFNs for formation of this IFN-inducible complex [27].

Our results indicate that the synergistic effect that we observe here by functional assays in living cells could well be provided by such a mechanism.

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