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Interferon-β promoters contain a DNA element that acts as a position-independent silencer on the NF-κB site

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The human interferon-β (IFN-β) promoter contains several functional domains that contribute to its virus-inducible regulation. One of them, PRDII, an NF-κB-binding sequence, can function as a constitutively activating element. Due to the presence of a negative regulatory domain that mediates a constitutive repression the natural IFN-β promoter is silent in the non-induced state. Within this domain we have delimited an 11 bp element that acts as a negative regulatory element (NRE) of PRDII. Although the NRE is physically overlapping with PRDII in the IFN-β promoter, it acts as a position-independent silencer of PRDII. Virus infection, which leads to the transcriptional activation of the IFN-β promoter, does not alter the negative activity of the NRE on an isolated PRDII. It is the cooperative effect of PRDI and PRDII that is able to overcome the NRE function after virus infection. By UV cross-linking analysis using uninduced and virus-induced nuclear extracts, we show that two factors with molecular masses of ~95 and 100 kDa bind to the NRE.

Key words: interferon-β/negative regulatory element/NF-κB/silencer

Introduction

Interferon-β (IFN-β) genes from different mammalian species are absolutely silent, but can be activated in nearly any differentiated type of cells. Viruses and double-stranded RNA (dsRNA) are potent inducers of the transient IFN-β gene expression. Many agents, such as the biological response modifiers Il-1, TNF-α and PDGF, are also able to induce the expression of IFN-β, however, to a lower extent. Furthermore, during differentiation of several cell lines the transient production of low amounts of IFN-β can be detected (for review see De Maeyer and De Maeyer-Guinard, 1988). The mechanisms by which IFN-β genes are differentially induced and shut off are not known in detail, but the major regulatory elements within the human IFN-β promoter have been identified: three basic elements are responsible for its regulation. They are found within the virus response element (VRE), which is localized from −74 to −37 with respect to the transcription start site. Two positive virus-inducible elements, PRDI and PRDII (positive regulatory domains), and a negative regulatory domain (NRD) constitute the minimal VRE (Goodbourn *et al.*, 1986; Goodbourn and Maniatis, 1988; Fan and Maniatis, 1989). In some cell lines, for example mouse L929 cells, sequences up to −104 are required for a functional VRE (Fujita *et al.*, 1985; Dinter and Hauser, 1987). These sequences contain variants of PRDI and a binding site for ATF/CREB (Du and Maniatis, 1992). The VRE in all known mammalian IFN-β promoters is highly conserved, which is in accord with the similarity of expression of the IFN-β genes within the different species.

Experiments with synthetic promoters have shown that PRDI or related sequences are not able to exert virus-inducible transcription (Fujita *et al.*, 1989). Multimers of PRDI (GAGAAGTGAAAGT) or the hexanucleotide GAAAGT, which represents an idealized half-site of PRDI, confer virus inducibility to heterologous promoters (Fujita *et al.*, 1987). On the other hand, PRDI acts as a silencer for upstream positioned activators like the SV40 enhancer in uninduced cells (Kuhl *et al.*, 1987; Fan and Maniatis, 1989).

PRDII is functionally related to PRDI: monomers of PRDI do not function as virus-inducible elements, but oligomers do (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989). The tandem arrangement of PRDI and PRDII in IFN-β promoters constitutes a virus-inducible sequence which is achieved by a synergistic cooperation of both domains (Fan and Maniatis, 1989).

Oligomers of PRDI and PRDII as well as monomeric PRDI−PRDII act as constitutive transcriptional activators in several cell lines (Fan and Maniatis, 1989). Therefore, other sequence elements silencing this activity must be present in the original IFN-β promoter. This is confirmed by deletion studies reported by Goodbourn *et al.* (1986). They showed that a deletion of the sequence between the TATA box and −65 (NRDI) results in a constitutive activity of the IFN-β promoters in C127 cells. The authors have concluded that this region constitutes an NRD preventing the enhancing activity of the PRDs. Accordingly, Dirks *et al.* (1989) could derepress the human IFN-β promoter in DNA competition experiments with DNA sequences covering the NRDI. Further evidence for a repressor that blocks the positive activity of the PRDs was obtained by the introduction of single base mutations within the NRDI. Some of these mutations lead to a derepression of the human IFN-β promoter (Goodbourn and Maniatis, 1988).

Promoter sequences from isolated mammalian IFN-β genes contain stretches of high homology. However, sequences between PRDII and the TATA box, the major part of NRDI, are not well conserved. We have tried to define a short negative regulatory element (NRE) by comparing the sequences of mammalian IFN-β promoters around the VRE (Figure 1). A core sequence from −77 to −50 is almost completely conserved. This sequence covers PRDI, PRDII and extends for further 5 bp 3′ to PRDII. With the experiments presented here we demonstrate that these conserved 5 bp belong to the NRE. An extension of the positive regulatory domains I and II for these 5 bp completely

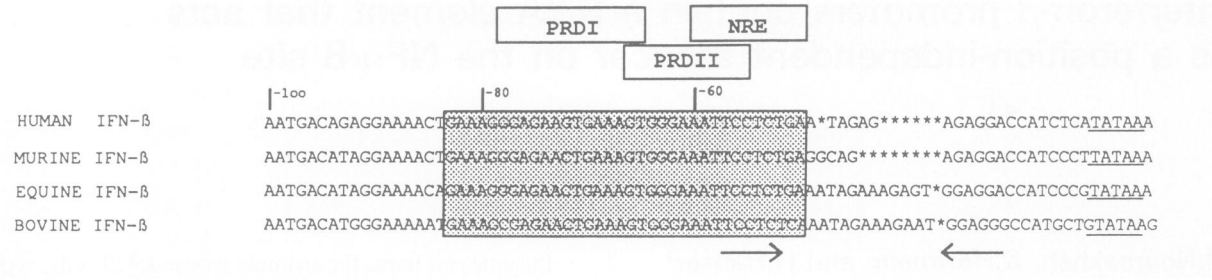

Fig. 1. Sequence comparison of IFN-β promoters from different mammalian species. Sequences are aligned to give the maximum homology. The dotted frame indicates the region with highest homology. Numbering refers to the transcription initiation site of the human IFN-β. The TATA boxes are underlined. PRDI and PRDII are indicated according to Fan and Maniatis (1989). The NRE is boxed according to the results obtained here. The arrows indicate a sequence of dyad symmetry (see Discussion).

eliminates the constitutive activation of a heterologous promoter, which is exerted by PRDI combined with PRDII. We also show that an 11 bp sequence covering the 3′ half-site of PRDII plus the 3′-adjacent 5 bp is able to act as a position independent silencer of PRDII.

Results

Negative regulatory sequences are adjacent to PRDII

In our experimental approach we have used the core herpes simplex virus thymidine kinase (TK) promoter fused to the human IFN-β gene, which serves as a reporter. This construct gives rise to a low constitutive expression of human IFN-β on transfection into different murine cell lines. DNA elements derived from the human IFN-β promoter were integrated either 5′ proximal or distal to the TK promoter and tested for secretion of human IFN-β and accumulation of specific mRNA in stably transfected murine Ltk$^-$ and C243 cells.

In order to elucidate the function of the 5 bp that are located 3′ adjacent to PRDII, the highly conserved sequence (shaded in Figure 1) with (-77 to -50) and without (-77 to -55) the NRE (AATTCCTCTGA) was integrated into the reporter plasmid p0 close to the TATA box of the TK promoter (Figure 2). The resulting plasmids were tested for expression of human IFN-β in mouse Ltk$^-$ cells.

The plasmid that contains PRDI and PRDII (p12) gives rise to a >10-fold higher constitutive expression compared with the reporter plasmid p0. Single PRDs do not lead to constitutive activation (data not shown). The activation is the result of a synergism between PRDI and PRDII as had been shown earlier (Fan and Maniatis, 1989). The 3′ extention of PRDI and PRDII by the conserved 5 bp (TCTGA) (p12n) eliminates the activation of the TK promoter by PRDI and PRDII (Figure 2). The same results were obtained in C243 cells (data not shown). We conclude that the 5 bp constitute or are part of a NRE.

Since it had been shown that nucleotides contributing to the NRD are located within PRDII (Goodbourn *et al.*, 1986; Goodbourn and Maniatis, 1988) we have tested the possibility that an 11 bp element (AATTCCTCTGA), which includes the 5 bp shown to contribute to the NRE, is sufficient to mediate this repressive effect out of its normal context of surrounding sequences. The element includes 6 bp of the 3′ half-site of PRDII which is not active in the modulation of transcription per se. This element is able to reduce the constitutive PRDII-activated TK promoter (Figure 3A, p22n).

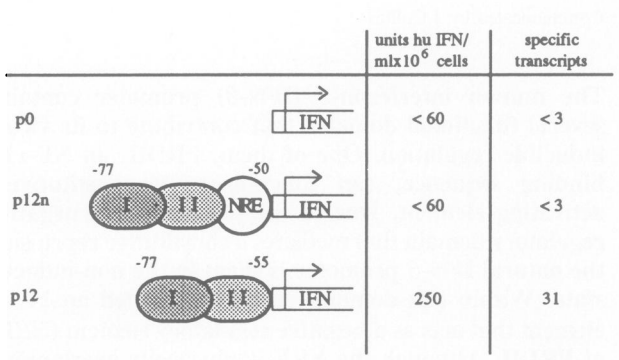

Fig. 2. Differential activation from IFN-β promoters. p0 contains the reporter gene with TK promoter and IFN-β cDNA gene (open box). p12 and p12n contain the regulatory elements of IFN-β proximal to the TK promoter (hatched oval and circle). In the hatched ovals, I corresponds to PRDI and II to PRDII. NRE in the open circle depicts the negative regulatory sequence. Numbering refers to the cap site of the natural human IFN-β gene. Pools of stable LTK$^-$ transfectants were tested for constitutive expression of the reporter (human IFN-β) by measuring secreted human IFN and the steady-state amount of human IFN-β specific transcripts. The human IFN values were normalized to 10^6 cells per ml supernatant (units human IFN/ml $\times 10^6$ cells). The indicated numbers represent mean values of at least three independent tests. The standard deviation is within the accuracy of the antiviral assay. The copy number of human IFN-β specific transcripts were normalized to 20 pg total mRNA.

Definition of the target sequence of the NRE

The negative effect mediated by the NRE seems to dominate over the activation of transcription brought about by dimeric PRDII or PRDI−PRDII. This could be due to a reduction in the strength of the truncated TK promoter or an inhibitory interaction with a component of RNA polymerase II. To distinguish between these possible alternatives the effect of NRE on the truncated TK promoter was tested (Figure 4). Expression from the reporter construct is not altered by the presence of the NRE. These results indicate that the NRE does not affect the promoter directly but exerts its negative activity via PRDI and/or PRDII.

The results shown in Figure 3B indicate that the activity of trimeric AAGTGA, which contains an idealized PRDI (Fujita *et al.*, 1985), is not affected by the NRE. This is indicative of a specific interaction of the NRE with PRDII. This is in fact demonstrated in Figure 3A where only a PRDII is present. PRDII is an NF-$\varkappa$B binding sequence. The human CMV enhancer contains four NF-$\varkappa$B elements that contribute to its constitutive transcriptional strength (Boshart *et al.*, 1985; Lenardo *et al.*, 1989). The negative effect of

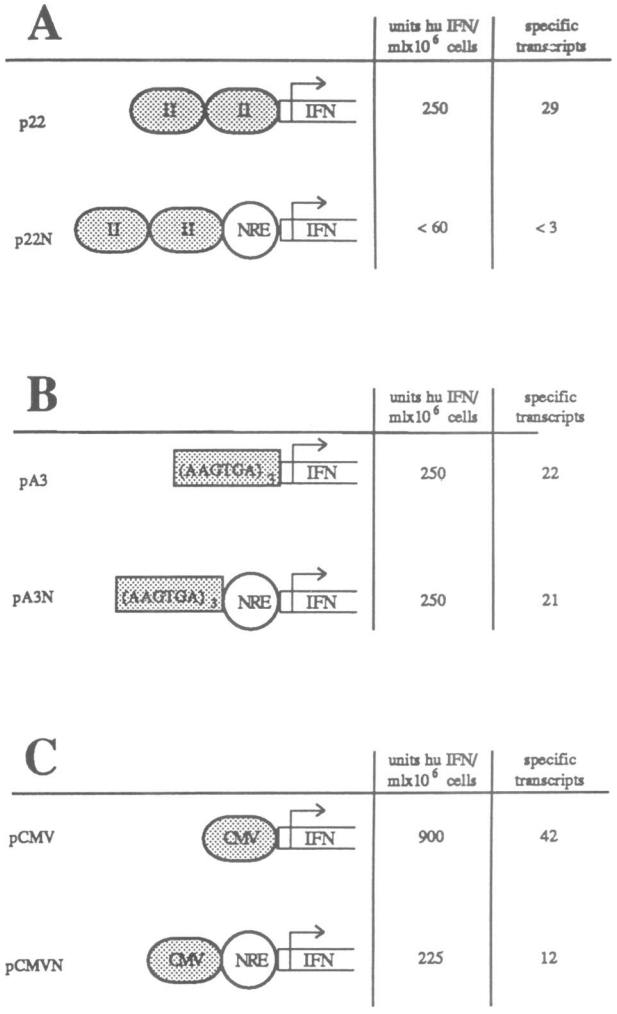

Fig. 3. The effect of the NRE on constitutive transcriptional activators. Three different transcriptional activating elements were tested: PRDII (**A**), AAFTGA trimer (**B**), CMV enhancer fragment (**C**). Experimental outlines are as in the legend to Figure 2. Dotted ovals represent either single copies of PRDII or the human, i.e. CMV enhancer. Dotted rectangles correspond to the trimer of AAGTGA. The open circles correspond to the 11 bp NRE.

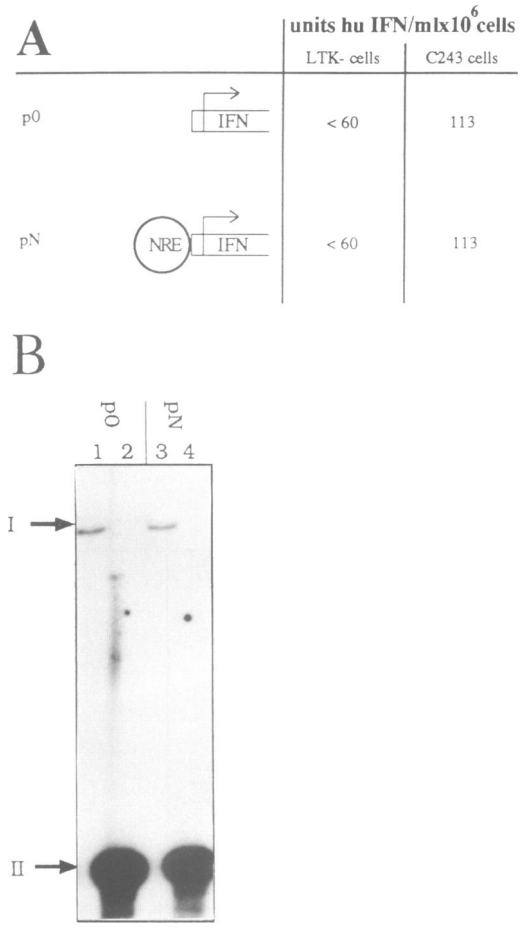

Fig. 4. Effect of the NRE on the truncated promoter. Experimental outlines are as in the legend to Figure 2. **A.** LTK$^-$ and C243 cells were tested. **B.** Transcript levels in LTK$^-$ cells were quantitated by S1 analysis as outlined in the legend of Figure 6C and visualized by 72 h exposure. I: Human IFN-β specific probe corresponding to correctly initiated transcription. II: Pyruvate kinase specific transcripts.

we have tested the function of the NRE in a different the NRE on other NF-κB binding sequences was confirmed by juxtaposing it to the human CMV enhancer (Figure 3C). As expected, the activity of the CMV enhancer is reduced but not eliminated by the NRE.

Mutations within the NRE abolish its negative activity

A series of point mutations in the human IFN-β promoter, including the NRE site, have been tested by Goodbourn and Maniatis (1988). Significant effects within the NRE leading to the enhancement of basic transcriptional level were only observed upon transition of T residues −52 and −54 to C. We have mutated the NRE by introducing both transitions. The mutated NRE (N_m) no longer significantly affects constitutive transcriptional activity of dimeric PRDII and the CMV enhancer (Figure 5A and B). This confirms the functional homology between the NRE as it is found within the complete IFN-β promoter and as used here experimentally as an isolated element.

In the reporter gene constructions shown here the NRE is always in the same context. In order to eliminate artefacts sequential context (i.e. GTCGAC-NRE-CCCGGG instead of AATTCG-NRE-CGAACA). The results of these experiments were not significantly distinguishable from those shown in Figures 3 and 4 (data not shown). We conclude that the NRE is sufficient for exertion of the negative effect on PRDII.

The NRE is a position-independent silencer

The dimeric PRDII not only acts as a positive promoter element but also as a constitutive enhancer. This is demonstrated in Figure 6A and C (p22-//-). The enhancer function is still silenced by the NRE even when the enhancer is positioned 1.4 kb away (p22-//-N). The mutated NRE does not exert this effect (p22-//-N_m). From our previous conclusion, that the NRE specifically interacts with PRDII and not with the TK promoter and the fact that they interact over long distances, one could expect that the NRE should also silence the dimeric PRDII from a distal position. This was tested in constructs in which the NRE is in a distal position and the dimeric PRDII is either juxtaposed to it or in proximity to the TK promoter (Figure 6B and C; p22n-//-, pN-//-22). In both cases the enhancer activity of PRDII is not detectable. This confirms that the NRE is a typical silencer of the PRDII enhancing activity.

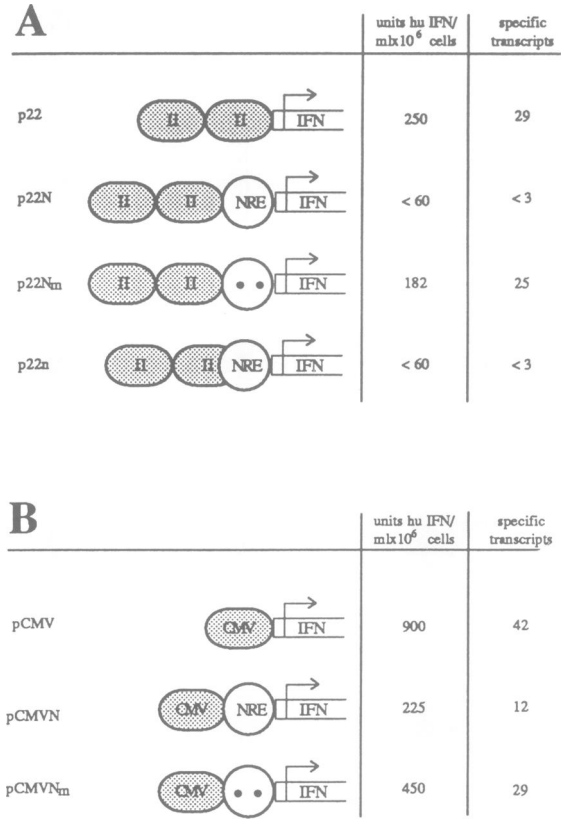

Fig. 5. Effect of substitution of two nucleotides in the NRE. The effects of the mutant NRE on the PRDII dimer (**A**) and the CMV enhancer fragment (**B**) were tested. The NRE sequence was altered by substitution of T-52 and T-54 to C (open circles with two dots). The open circle (NRE) overlapping the dotted oval (PRDII) corresponds to the overlap of both elements by common sequences.

The effect of virus induction on the silencing activity of the NRE

Oligomeric PRDII constitutes a virus-inducible element. This has been shown earlier (Lenardo *et al.*, 1989; Visvanathan and Goodbourn, 1989) and is reproduced in our experimental system (Figure 7A; p22). However, the virus induction of dimeric PRDII is not detectable in the distal position (p22-//-). We investigated the interaction of dimeric PRDII with the NRE upon virus induction. In all constellations in which the NRE silences the dimeric PRDII, virus induction could not overcome its negative effect (Figure 7; p22N, pN-//-22, p22-//-N). We conclude that the silencing capacity of the NRE is maintained during virus infection.

Dimeric PRDII plus NRE (p22N) is not representative of the complete IFN-β promoter, in that it cannot be activated by virus infection. It is possible that the cooperation of other elements with PRDII is required to overcome the negative effect of the NRE after virus induction. The most obvious partner for such a cooperation is PRDI. The cooperation of PRDI and PRDII during virus induction was demonstrated earlier (Fan and Maniatis, 1989). This possibility was tested by viral induction of cells transfected with p12n, which contains PRDI and PRDII overlapping the NRE. The results are depicted in Figure 7B, C. p12n is clearly inducible by virus and reaches the same extent of expression as p12, which lacks the NRE.

The results shown in Figure 3 indicate that the NRE only reduces the PRDII-mediated constitutive transcription level, but not the constitutive level, dependent on the PRDI-like sequence. The linear arrangement of both elements, PRDI and PRDII, as found in the wild type IFN-β promoter, gives rise to some constitutive expression which is more than additive with respect to the contribution of the separated elements (Fan and Maniatis, 1989). Our results obtained with p12n demonstrate that this constitutive expression, which is based on the cooperation of PRDI and PRDII, is eliminated by the NRE.

With our results we have defined a new minimal virus responsive element (VRE) which is inactive before induction and mediates a strong transcriptional activation to the TK promoter after virus treatment. The fragment is only 28 bp long and is conserved in all known mammalian IFN-β promoters (Figure 1).

Binding of nuclear factors to the NRE

We have tried to identify DNA binding factors in nuclear extracts from HeLa and Ltk$^-$ cells that specifically recognize the NRE. In this paper we only show the results obtained from HeLa nuclear extracts, but the results found with Ltk$^-$ nuclear extracts are similar. The results of the gel retardation experiments with the NRE oligonucleotide (N) are shown in Figure 8A. The two major retarded bands can be competed by a surplus of unlabelled NRE, however, to a different extent. The results are interpreted as the lower band (II) having a lower binding affinity than the upper band, although this might be an over-simplification if, for example, there is low concentration of factors involved in complex II formation. As expected the non-functional mutated NRE (NRE_m) is a poor competitor.

From the reverse genetics experiments (Figure 7) we have concluded that the NRE is a silencer of PRDII before and after virus induction. Assuming that the effect of the silencer is identical before and after virus induction, the same nuclear factor(s) should bind to the NRE in the virus-induced state as well as in the non-induced state. We have compared the binding properties of the NRE using nuclear extracts from mock- and virus-induced cells. Figure 9A shows that the retardation of both complexes is maintained after virus induction. However, gel retardation with PRDII showed significant changes with the same nuclear extracts from control and virus-induced cells (data not shown). To determine whether the protein components involved in these complexes have changed upon virus induction, we have performed UV cross-linking experiments using BrdU-substituted labelled NRE. Figure 9B shows that complex I contains a factor with an apparent molecular weight of 95 kDa. Complex II contains two factors of 90 and 105 kDa. The size and intensity of factors from virus-induced cells are not distinguishable from those of control cells. Obviously, the factors are not removed or replaced by virus induction.

Since the NRE contains the 3′ half-site of PRDII we have tested whether PRDII could compete for binding of nuclear factors to the NRE (Figure 8A). The result demonstrates that the dimeric PRDII competes preferentially for complex Ia indicating that the 95 kDa factor should bind to PRDII. A complex formed with an oligonucleotide consisting of PRDII with the overlapping NRE migrates with the same retardation as complex Ia from the NRE, suggesting that the same factor is binding (complex Ib in Figure 8B).

Complex IIa, which contains at least two different nuclear

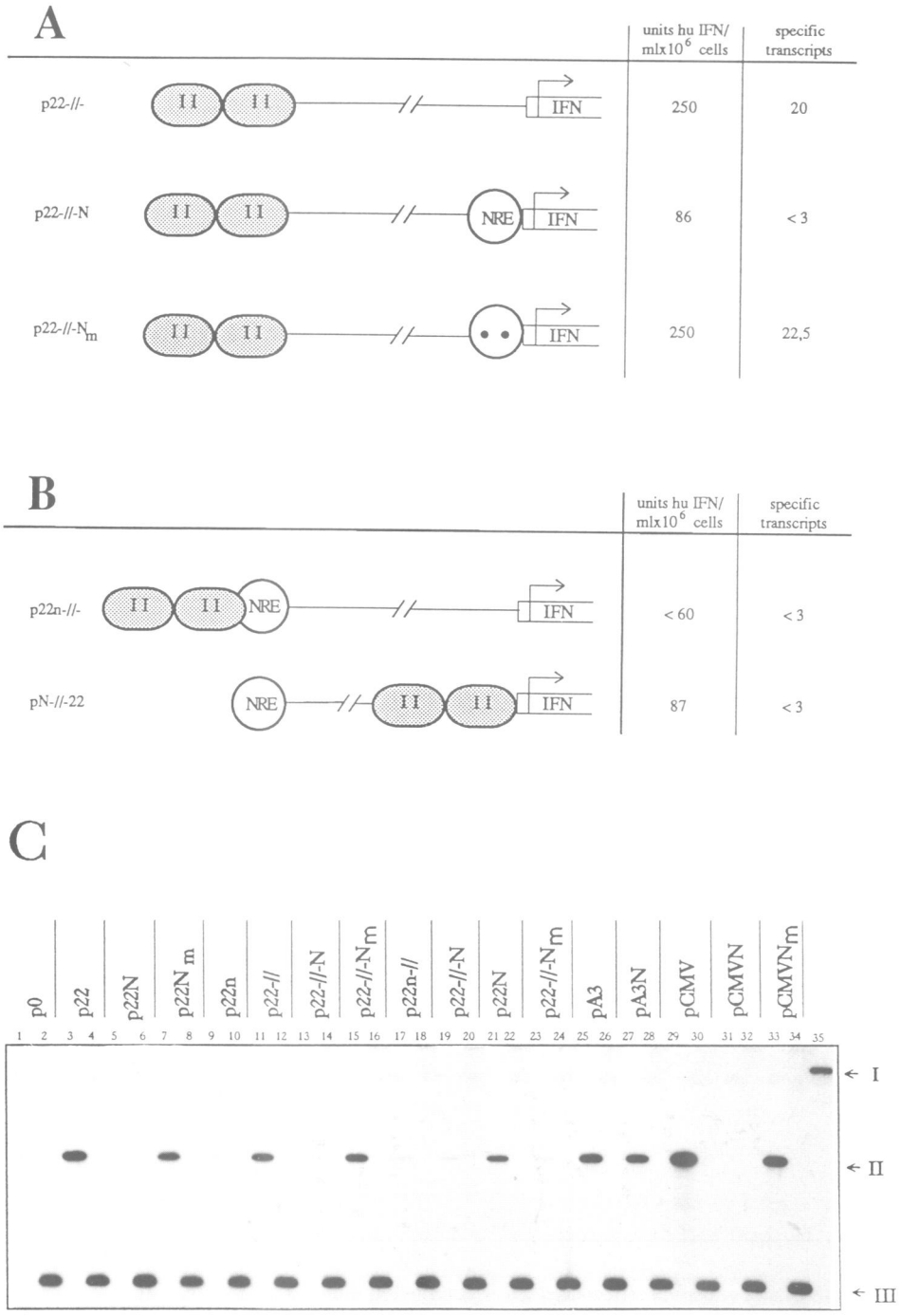

Fig. 6. The action of distantly located NRE. Experimental outlines for **A** and **B** are as in the legend of Figure 2. The interrupted line corresponds to 3 kbp of pBR322 sequence. **C.** S1 analysis. Total RNA from the indicated transfectants was prepared. The human IFN-β mRNA and pyruvate kinase were detected separately. Arrows indicate the positions of the undigested probe (I); the digested probe corresponding to the correctly initiated human IFN-β mRNA (II in the lanes with odd numbers); and the reference signal of pyruvate kinase (III in lanes with even numbers).

factors, seems to be more specific for the NRE. Both factors can bind simultaneously as shown by extending the time of UV cross-linking: a band of ~200 kDa appears in SDS–PAGE (data not shown). These proteins bind to the same DNA strand since a BrdU substitution of the mRNA strand reveals the same results obtained with both strands when labelled with BrdU. Cross-linking with the opposite strand does not give rise to the coupling of nuclear factors (Figure 9B). These results indicate that in complex II both factors can bind on one strand of the NRE.

Discussion

The experiments reported here indicate that the NRE is acting as a specific silencer of PRDII from mammalian IFN-β promoters. The activation of DNA sequences, trimeric AAGTGA and the SP1-binding GC box of the TK promoter are not affected by the presence of the NRE. The PRDII element from the IFN-β gene and the NF-κB binding site from the Igκ light chain gene are interchangeable (Lenardo *et al.*, 1989). Although variations of the sequences affected

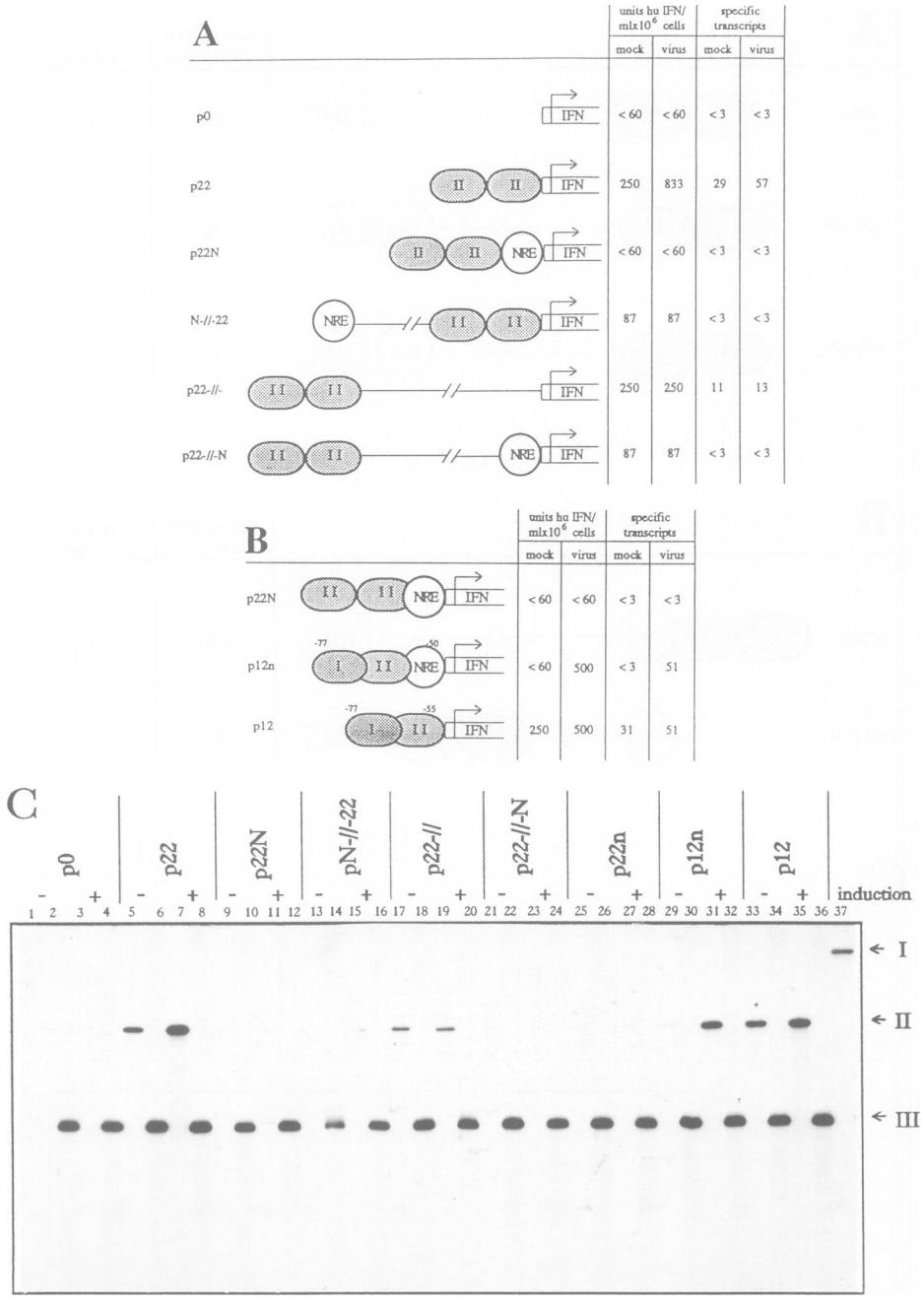

Fig. 7. NRE activity after virus induction. Experimental outlines are as in the legend to Figure 2, except that aliquots of transfectants were induced with NDV. Supernatants were subjected to test of the antiviral activity 15 h after induction. **A.** Represents different constellations of NRE with respect to dimeric PRDII. **B.** Compares the interaction of NRE with dimeric PRDII and PRDI–PRDII. **C.** S1 analysis. Total mRNA was prepared from mock- (−) and virus-treated (+) transfectants 8 h after induction. For further details see Figure 6C and Materials and methods.

by the NRE have not been examined extensively, it is assumed that not only PRDII but also other NF-$\varkappa$B-binding elements are silenced. This is demonstrated by partial silencing of the CMV enhancer by the NRE. In this latter case effects of the NRE on promoter or enhancer elements other than NF-$\varkappa$B-binding sites have not been excluded.

Although the NRE is overlapping the PRDII element it can exert its silencing effect from the distance of >1.4 kbp independently of the constellation of the NRE or the PRDII with respect to the TATA box. The nature of this silencing mechanism has not been investigated. Interaction of the NRE and PRDII with the transcriptional complex could be considered by looping mediated by their DNA binding factors.

The NRE or very closely related sequences are found in only a few well characterized promoters for which, however, a negative function has not been reported. IFN-$\varkappa$ promoters have neither NF-$\varkappa$B binding sites nor NRE-related elements. Upstream sequences of genes encoding the MHC class I complex contain activating sequences that are closely related to PRDI and PRDII of the IFN-β promoters: a so-called interferon stimulated response element, IRS, which is homologous to PRDI, is in close proximity to enhancer A, which is similar to PRDII. Both sequences cooperate

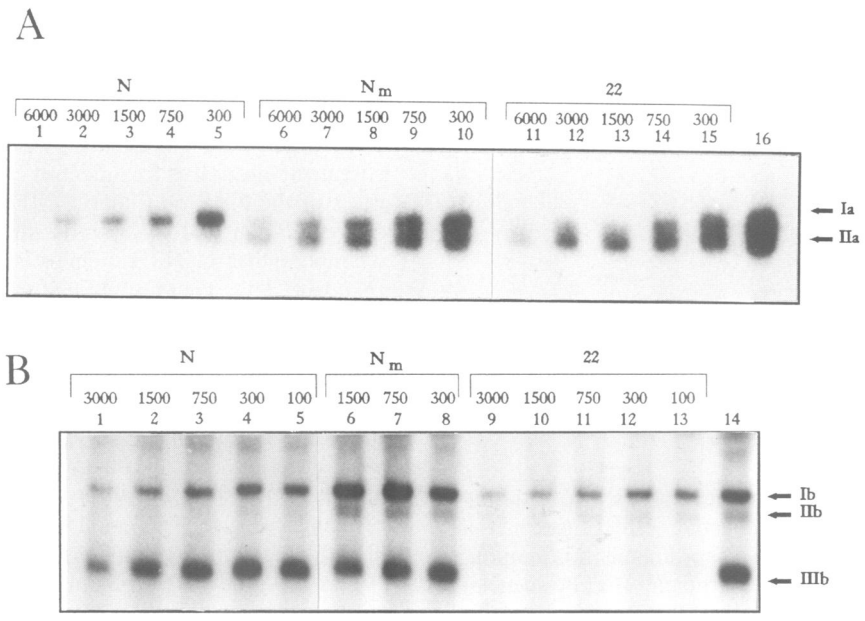

Fig. 8. Detection of nuclear factors that bind to the NRE by gel retardation assays. 2 fmol (20 000 c.p.m.) of the respective ^{32}P-labelled oligonucleotide were incubated with 5 μg of nuclear extract from HeLa cells and competitor DNA. The molar excess of competitor compared with the labelled probe is indicated. The competitor DNAs are indicated: N, NRE; N_m, mutated NRE; 22, dimeric PRDII. Arrows indicate the position of complexes. **A.** The oligonucleotide N was labelled, containing the NRE and the same flanking sequences as after insertion in the reporter gene (see Materials and methods). **B.** The oligonucleotide 2n, which contains PRDII overlapped with the NRE, was labelled.

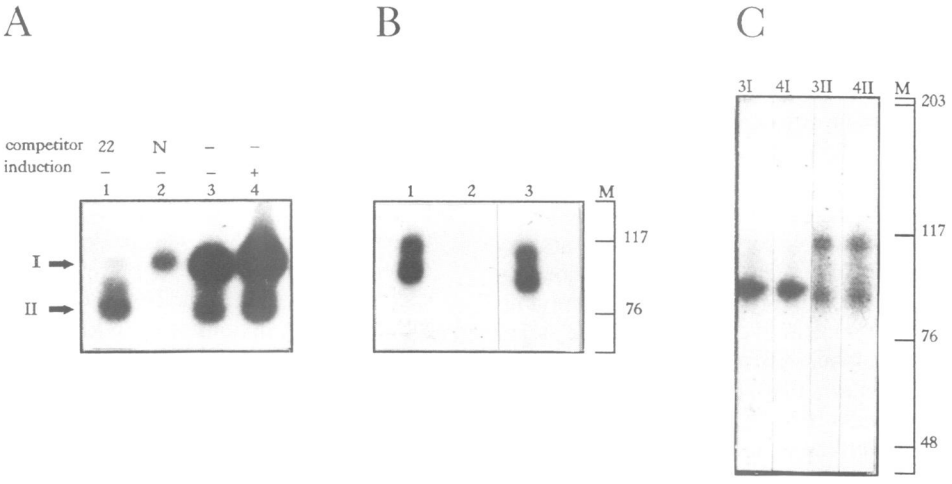

Fig. 9. Analysis of NRE-binding nuclear factors from mock- and virus-induced cells. **A.** Gel-shift analysis with BrdU-labelled NRE. NBrdU was incubated as indicated with nuclear extracts from mock- (lanes 1, 2 and 3) and virus-induced HeLa cells (lane 4) and 3000-fold molar excess of competitor DNAs 22 (lanes 1) and N (lane 2). **B.** SDS−PAGE analysis of proteins cross-linked to the NRE. N′ oligonucleotides in which the sense (NBrdU) (lane 1), antisense (lane 2) or both (lane 3) strands are BrdU-labelled were incubated with nuclear extracts from uninduced HeLa cells. The complexes were subjected to gel-shift analysis and UV cross-linked as described in Materials and Methods. Complexes corresponding to I and II from panel A) were eluted together and analysed on SDS−PAGE. Identical results were obtained with nuclear extracts from virus-induced cells. **C.** SDS−PAGE analysis of the NRE-specific complexes I and II from sense-BrdU-labelled NRE (NBrdU). Gel-shift analysis corresponds to A. Complexes I of lane 3 from mock-induced (lane 3I) and lane 4, from virus-induced nuclear extracts (lane 4I), complex II of lane 3 from mock-induced (lane 3II) and lane 4, using virus-induced nuclear extracts (lane 4II) were subjected to SDS−PAGE. Molecular weights of markers are indicated (M).

synergistically to activate MHC class I transcription constitutively and in an interferon-inducible manner (Israel *et al.*, 1986). As expected from the constitutive expression, no sequence related to the NRE is found in the MHC class I promoters.

Further promoters with NF-κB binding sites were examined. In two cases a negative regulatory element was found in conjunction with an NF-κB site (Boehnlein *et al.*, 1988). Lu *et al.* (1990) have defined a short sequence between positions −173 and −159 from the transcription initiation site of HIV-1 that contributes to the negative regulation of transcription. Smith and Greene (1989) localized a negative regulatory element between nucleotides −400 and −368 relative to the major distal transcription start site of the interleukin-2 receptor α-chain (IL-2Rα) gene. A comparison of this negative control region with the NREs from HIV-1 and IFN-β reveals a core element that shares a significant sequence similarity (Figure 10). At least for the

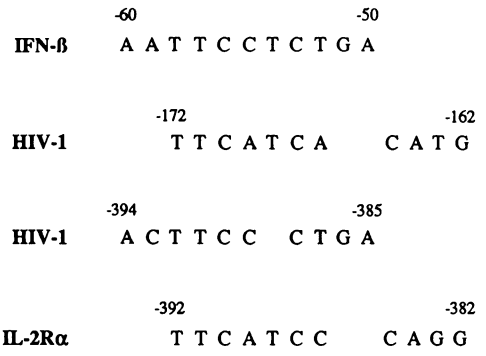

Fig. 10. Sequence similarity between the NREs of HIV-1, IFN-β and IL-2α.

IL-2 Rα NRE it was shown that it requires a specific *cis*-acting element to exert its negative effect, since it is insufficient to downregulate the TK promoter. It is tempting to speculate that all three NREs exert their effect by silencing the NF-$\varkappa$B sites in their respective promoters.

In examining the negative regulatory domain of the human IFN-β promoter, Goodbourn *et al.* (1986) and Goodbourn and Maniatis (1988) found a repressing activity that is located between -50 and -36. They have discussed the duplication of sequences as being responsible for this activity. The area between $-65/-48$ and $-47/-30$ of the human IFN-β promoter contains an interrupted dyad symmetry (Figure 1; Figure 10).

This constitutive activity of PRDII and related sequences was demonstrated in several cell types (Fan and Maniatis, 1989; Hiscott *et al.*, 1989; Visvanethan and Goodbourn, 1989). NF-$\varkappa$B should not be responsible for this effect since it is not found in the nucleus of most cells without treatment with specific agents like virus, TNF or TPA (Blank *et al.* 1992). The factor(s) responsible for the constitutive activity of PRDII is (are) not known. Most probably, members of the rel-family are involved. This is supported by previous studies showing that the constitutively active NF-$\varkappa$B site of MHC class I promoter binds KBF-1 (Israel *et al.*, 1986), a dimer of p50 (Kieran *et al.*, 1990) and cross-linking studies on the PRDII of the human IFN-β promoter (Xanthoudakis and Hiscott, 1990). Nevertheless, in the activated state when NF-$\varkappa$B is thought to bind PRDII, the NRE is still able to silence its activity. We assume that the PRDII binding factors of the uninduced and the induced state share homology with respect to the ability to be silenced by the NRE.

The factor(s) involved in the silencing by the NRE is/are of great interest. We have shown that one of these factors must be functionally available only in a limited concentration in that the human IFN-β promoter can be derepressed in competition experiments with DNA fragments containing the NRE sequences (Dirks *et al.*, 1989). *In vitro* studies with nuclear extracts showed that the NRE can form two complexes that can be separated by gel retardation. The dominant complex (I) can be specifically competed with PRDII and seems to contain one DNA binding factor of $\sim$95 kDa. Because of the competition with the PRDII sequence, we assume that this factor is located on the PRDII-specific half-site of the NRE. The less abundant complex contains two proteins of $\sim$90 and 105 kDa. The molecular weights of the factors that bind to the NRE, as shown by cross-linking studies, have not yet been found in published studies with IFN-β promoter sequences or PRDII/NF-$\varkappa$B binding sequences. We assume that their binding is distinct from the already described factors and might be involved in mediating the NRE-specific function.

The negative effect mediated by the NRE-binding factor(s) is retained after virus treatment of cells, since the silencing effect of the NRE on dimeric PRDII is not relieved. The gel retardation and DNA cross-linking data support the assumption that the NRE complex is not changed after virus treatment. This would imply that the negative effect of the NRE is relieved by changes at other sites in the IFN-β promoter. The data from this paper indicate that PRDII must cooperate with PRDI in order to overcome the negative effect of the NRE after virus induction. Therefore, the presence of PRDI is important for the virus-inducible relief from the NRE silencing. In a speculative model the NRE function eliminates promoter activation exerted by PRDI$-$PRDII in the uninduced state. This could be due to 'activator masking', which is the complexing of the transactivators that bind to PRDI$-$II with the NRE binding factor(s) thus being unable to activate transcription. Alternatively, 'locking' of the transcription complex by the NRE-binding factor(s) would lead to the inability of the PRDI$-$II binding factors to activate transcription. The model further implies that virus induction, which eliminates the action of the NRE leads to a modification or replacement of the factor(s) binding to PRDI$-$PRDII in the uninduced state. This requires an intimate interaction of PRDI and PRDII-binding factors, which is in agreement with the fact that PRDI and PRDII interact cooperatively to constitute virus-inducibility. The induced 'new' transactivator on PRDI$-$PRDII would either escape the 'masking' or activate the transcriptional complex at another site that is not locked by the repressor.

Induction of IFN-β is inducible in nearly all cell types, but is normally absolutely silent (De Maeyer and De Maeyer-Guinard, 1988). The results presented in this paper are from experiments with different murine cell lines. Based on the specific properties of the IFN-β expression we assume that the silencing functions of the NRE are active in many if not all murine tissues. The sequence of the NRE is identical in different mammalian species (Figure 1). The function might also be identical. The composition of DNA binding factors on the NRE from murine and human cells show the same size of NRE-bound compounds (data not shown). We therefore propose that the virus-reversible silencing mediated by the NRE on PRDI$-$PRDII is an evolutionarily conserved mechanism.

Materials and methods

Plasmid constructions and transfections

Constructions were carried out by standard procedures (Sambrook *et al.*, 1989). p0 includes a fusion of a 116 bp *Eco*RI$-$*Hin*cII fragment containing the truncated promoter and the first 38 bp of the transcribed region of the TK of herpes simplex virus I (HSV) with the 767 bp *Hin*cII fragment containing the IFN-β structural gene (reporter). This gene hybrid was fused to the 4.1 kbp *Bam*HI$-$*Hin*cII fragment of pBR322. The *Sfu*I (proximal) and *Nar*I (distal) sites (positions -75 and $+1437$ with respect to the cap site of the reporter gene) of p0 were used to insert synthetic oligonucleotides representing IFN-β promoter sequences flanked by *Sfu*I and *Nar*I compatible overhangs: 12 5′-CGAATGAGAAGTGAAAGTGGGAAATTCC-3′; 12n 5′-CGAATGAGAAGTGAAAGTGGGAAATTCCTCTGA-3′; 22 5′-CGAATCGATGTGGGAAATTCCGTGGGAAATTCCGG-3′; 2n 5′-CGCGAGTGGGAAATTCCTCTGAGG-3′; A3 5′-CGAATAAGTGA-AAGTGAAAGTGA-3′; N 5′-CGAATTCCTCTGA-3′; N_m 5′-CGAAT-TCCCCCGA-3′; N′ 5′-CGAATTCGAATTCCTCTGACGAACA-3′.

pCMV was created by insertion of the 476 bp *Xba*I–*Bam*HI fragment of the CMV enhancer into the *Eco*RI (−79) site of p0. pGEP was constructed by ligation of the *Eco*RI–*Pvu*II fragment of p0 containing the TK promoter and IFN-β coding sequences into the polylinker of pGEM-1 (Promega).

Cell lines and DNA transfection

LTK⁻ and C243 cells, maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, were stably transfected by calcium phosphate coprecipitation. 5 μg of reporter plasmid, 10 μg of LTK⁻ high molecular weight DNA and 0.5 μg of selection plasmid pTKcos2 (Lindenmaier *et al.*, 1982) per 3×10^5 LTK⁻ or 0.5 μg pAG60 (Colbère-Garapin *et al.*, 1981) per 2×10^5 C243 cells were transfected. More than 100 HAT- or G418-resistant clones were pooled.

Induction protocols and expression analysis

After expansion of the pools, aliquots of 10^5 cells were induced with Newcastle disease virus (NDV) or mock induced (Dirks *et al.*, 1989). Induction was carried out by incubation of the cells with 10 plaque-forming units of NDV per cell in serum-free DMEM for 1 h. After induction the cells were washed and incubated for a further 15 h for interferon production or 8 h for mRNA preparations in fresh DMEM plus 10% fetal calf serum. Human interferon was measured using the anti-viral assay on Vero cells and vesicular stomatitis virus (VSV) as challenging virus. The absolute amount of the reporter gene products differs slightly from one transfection series to the other. However, the induction ratio between virus-treated and control cultures was essentially identical in different transfection experiments. This was tested by several transfection experiments with each plasmid construct. The results presented in this article are based on a single experiment in which all plasmids were transfected in parallel. Virus inductions were carried out at least in triplicate. Quantitative IFN mRNA determinations are from cell pools 10 days after selection of stable transfectants, i.e. ~17 days after transfection.

Total cellular RNA was prepared as described by Sambrook *et al.* (1989). RNA probes were synthesized *in vitro* from pGEP. The specific activity was 10^{-5} c.p.m./copy. After *Pvu*II cleavage, run-off transcription generated a probe complementary to the sequences from the cap site to +242 of the human IFN-β mRNA. The reference probe was an *in vitro* transcribed product of pBSPK containing the murine pyruvate kinase gene (V.Schwaab, Heidelberg). S1 analysis was performed according to Sambrook *et al.* (1989). The protected fragments were subjected to 6% polyacrylamide−8 M urea gel analysis. Densitometric analysis of bands from the protected probes of the autoradiogram allowed a quantitative analysis of reporter gene expression using the following relationship: $A = a \times B/b$, where A is the radioactivity of the protected band, B is the intensity of the protected band, a is the input radioactivity of the undigested probe and b is the intensity of the undigested probe.

Cell extracts and gel retardation assays

Nuclear extracts from HeLa cells were prepared according to Dignam *et al.* (1983) with modifications introduced by Wildeman *et al.* (1984).

Gel-shift analysis was carried out according to the protocol of Fried and Crothers (1981) with the following modifications: ~5 μg of protein were incubated with 20 000 c.p.m./fmol of labelled oligonucleotide in the presence of 0.01 units poly(dIC) in 10 mM HEPES pH 8.0, 5 mM $MgCl_2$, 50 mM KCl, 0.025% bromphenol blue, 0.025% xylene cyanol and 10% Ficoll for 10 min at room temperature. The samples were loaded on pre-electrophoresed 8% native polyacrylamide gels. The gels were run for at least 8 h at 100 V. After drying, the gels were autoradiographed overnight.

UV cross-linking analysis

For UV cross-linking analysis gel retardation assays were performed as described before (Ausubel *et al.*, 1990; Xanthoudakis and Hiscott, 1990). NBrdU is a BrdU-substituted oligonucleotide probe of N′(5′-CGAATTC-GAABBCCBCBGACGAACA-3′). The opposite strand was labelled in the following way: 3′-TTAAGCBBAAGGAGACBGCTTGTGC-5′. Protein–DNA complexes were resolved by electrophoresis through 8% polyacrylamide gels. The complexes were cross-linked by putting the wet gel on a Spectroline transluminator (Reprostar II, Cammag) and exposing it to UV light at 302 nm with an intensity of 7000 μW/cm² for 20 min. The shifted complexes were identified by autoradiography, excised and soaked in sample buffer (2% v/v 2-mercaptoethanol, 20% glycerol, 10 μg/ml bromophenol blue and 62 mM Tris–HCl pH 6.8). The complexes were analysed on 12% SDS–polyacrylamide gels with a 4% stacking gel (Schägger and von Jagow, 1987).

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