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Induction of Human Interferon Gene Expression Is Associated with a Nuclear Factor That Interacts with the NF-κB Site of the Human Immunodeficiency Virus Enhancer

J. HISCOTT,* D. ALPER, L. COHEN, J. F. LEBLANC, L. SPORTZA, A. WONG, AND S. XANTHOUDAKIS

Lady Davis Institute for Medical Research, Sir Mortimer B. Davis-Jewish General Hospital, and Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3T 1E2

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The relationship between transcription of alpha and beta interferon (IFN-α and IFN-β) genes and the interaction of IFN promoter-binding transcription factors has been examined in monoblastoid U937 cells following priming with recombinant IFN-α2 (rIFN-α2) and Sendai virus induction. Pretreatment of U937 cells with rIFN-α2 prior to Sendai virus infection increased the mRNA levels of IFN-α1, IFN-α2, and IFN-β as well as the final yield of biologically active IFN. Analysis of nuclear protein-IFN promoter DNA interactions by electrophoretic mobility-shift assays demonstrated increased factor binding to IFN-α1 and IFN-β regulatory domains, although no new induction-specific complexes were identified. On the basis of competition electrophoretic mobility-shift assay results, factors interacting with the IFN-α1 and IFN-β promoters appear to be distinct DNA-binding proteins. U937 factor binding was localized to the P2 domain (−64 to −55) of the IFN-β regulatory element, a sequence motif with 80% homology to the recognition site of transcription factor NF-κB. Protein-DNA interactions within the IFN-β P2 domain were, in fact, specifically competed by either excess homologous P2 fragment or the human immunodeficiency virus enhancer element which contains two duplicated NF-κB recognition sites. Hybrid promoter-chloramphenicol acetyltransferase fusion plasmids, containing either the IFN-β regulatory element or the human immunodeficiency virus enhancer element linked to the simian virus 40 promoter, were analyzed for virus and phorbol ester inducibility in epithelial and lymphoid cells, respectively. In the 293 cell line, both plasmids were constitutively expressed but not virus inducible, while in Jurkat cells, chloramphenicol acetyltransferase activity from these plasmids was induced by tumor-promoting agent treatment. These experiments suggest that induction of IFN gene expression may be controlled in part by transcription regulatory proteins binding to an NF-κB-like site within the IFN-β promoter.

Interferons (IFNs) comprise a family of structurally and functionally distinct cytokines possessing a variety of antiviral, immunoregulatory, and antiproliferative activities (28, 35, 44, 46). The IFNs are classified into three immunologically distinct groups, designated alpha, beta, and gamma IFNs (IFN-α, IFN-β, and IFN-γ, respectively). IFN-α is encoded by a multigene family containing more than 20 individual genes and pseudogenes, while only single genes corresponding to human IFN-β and IFN-γ have been identified (52).

IFN treatment of cells leads to the coordinate induction of specific genes involved in the generation of an antiviral state, including those encoding major histocompatibility complex class I antigens, 2′5′-oligoadenylate synthetase, Mx, 6-16, ISG54, and ISG15 (7, 22, 23, 26, 29, 36, 37, 42, 49). The action of IFN upon gene activity is mediated initially by binding to high-affinity receptors at the cell membrane and ultimately by the interaction of cellular transcription factors with an IFN-responsive sequence which is conserved upstream of IFN-inducible genes. Extensive homology to the IFN-responsive sequence is also found in the 5′-flanking regions of other transcriptionally regulated genes, including *fos*, *HSP70*, and type I IFNs (IFN-α and IFN-β) (7, 36, 37).

Genes encoding the type I IFNs are normally silent but can be activated rapidly by a diverse group of natural and synthetic agents, such as viruses, synthetic duplex polyribonucleotides, and foreign antigens (20, 35, 44, 47). Transcriptional activation of IFN gene expression is mediated by the association of transcriptional regulatory proteins with a virus-inducible enhancer element that is composed of multiple *cis*-acting positive and negative regulatory domains (9–11, 13–15, 18, 25, 27, 32, 38). Transcription factors interact with two positive regulatory domains (PRDI and PRDII, −77 to −64 and −66 to −55, respectively) within the interferon-β regulatory element (IRE, −77 to −37 relative to the mRNA start site) in vitro and with a negative regulatory domain (−63 to −37) in vivo (25, 57). Fujita et al. (9) identified a more extensive inducible enhancer region (−105 to −65) that contains repetitive hexanucleotide units which, when multimerized, behave as a virus-responsive element (11). The multimerized hexamers, the region between −94 and −78 (P5 domain in this study), and the PRD I domain (P1 domain) appear to bind the same nuclear factor (10, 54). Miyamoto et al. (33) cloned and characterized a gene encoding a factor, termed IFN regulatory factor 1 (IRF-1), and suggest that IRF-1 may mediate virus-induced IFN-β transcription, as well as the regulated expression of IFN-α and other inducible genes. IFN-α1 gene transcription is controlled by a 46-base-pair virus-responsive element which contains two imperfect direct repeats (repA and repB) (27, 38).

Interestingly, production of IFN in response to virus induction can be increased by pretreating cells with IFN itself, a phenomenon referred to as priming (44–46). IFN does not act as an inducer per se, but it can both increase the amount and alter the kinetics of IFN synthesis following induction (19, 45, 46, 54). In fibroblastic cells, priming with

* Corresponding author.

IFN increases the transcription of IFN genes after virus infection and is mediated by a factor that is itself IFN inducible (6).

The extensive sequence homologies between the promoter regions of IFN-responsive genes and type 1 IFN genes, together with the biological similarities between IFN-activated gene expression and induction of IFN genes, prompted us to examine the relationship between IFN priming, transcription of IFN-α and IFN-β genes, and the interaction of IFN promoter-binding transcription factors. In myeloid U937 cells, pretreatment with rIFN-α2 prior to Sendai virus induction increased the mRNA levels of IFN-α1, IFN-α2, and IFN-β and the final yield of biologically active IFN. Electrophoretic mobility-shift assays (EMSA) demonstrated increased factor binding to IFN-α1 and IFN-β promoter domains, although no new induction-specific complexes were identified. U937 factor binding to the IFN-β promoter was localized to the −64 to −55 region (P2 domain) and was specifically competed by the enhancer domain of the human immunodeficiency virus type 1 (HIV-1). Together with functional studies on IFN and HIV hybrid promoter inducibility, these experiments suggest that induction of IFN gene expression may be controlled in part by NF-κB-like proteins that can also interact with the HIV-1 long terminal repeat (LTR).

MATERIALS AND METHODS

Cell culture. 293 cells, a human cell line established by transformation with adenoviral DNA, and U937, a monoblastoid cell line, were grown at 37°C in RPMI 1640 medium supplemented with 5% bovine serum, glutamine, and antibiotics. rIFN-α2 was a gift from Schering Canada.

Construction and sequencing of hybrid promoter plasmids. The construction of SV2CAT, SV1CAT, HIV/CAT, IFNβ/CAT ($SV_0\beta$), and IFNα/CAT ($SV_0\alpha$) has been described previously (16, 34, 53). Synthetic double-stranded oligonucleotides corresponding to the IFN-β IRE (−79 to −35) and the HIV-1 enhancer element (−105 to −80) were blunt-end ligated into the *Sph*I-*Acc*I-cleaved SV1CAT vector. The resulting IRE/SV1 and κB/SV1 plasmids were sequenced by using Sequenase (United States Biochemical Corp.), following manufacturer instructions, and an oligonucleotide primer to the SV1CAT plasmid (5′-AGTACAATCTCCTCTG ATGC-3′). The IRE/SV1 plasmid contained one copy of IRE in the 5′→3′ orientation; κB/SV1 contained three copies of the HIV enhancer, all in the 5′→3′ direction relative to the mRNA start site of the plasmid.

Transfections and chloramphenicol acetyltransferase (CAT) assays. The 293 cells were transfected with 10 μg of plasmid DNA by the calcium phosphate method as previously described (17, 21, 53) and virus induced 20 h later. Jurkat cells were transfected in a volume of 1 ml with 10 μg of plasmid per 10^7 cells by a DEAE-dextran procedure (39); after 20 min at room temperature, the cells were diluted with 10 ml of complete medium containing 100 μM chloroquine and incubated for 45 min at 37°C. Cells were pelleted by low-speed centrifugation, the medium was aspirated, and the cells were replated at 10^6 cells per ml in complete medium. At 20 to 24 h after transfection, phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) were added to the culture medium to final concentrations of 5 μg/ml and 25 ng/ml, respectively. Analysis of CAT activity was performed at 40 to 44 h after transfection by the procedure of Gorman et al. (16) and as previously described (21, 53). A constant amount of protein was used in each analysis, determined beforehand by microassay (Bio-Rad Laboratories).

RNA isolation and S1 analysis. Total cellular RNA was isolated from uninduced, Sendai virus-induced, and IFN-α2-primed (1,000 IU/ml for 8 h) and Sendai virus-induced U937 cells at different times postinduction by a modified guanidinium isothiocyanate procedure (5). Virus induction of U937 cells was performed by concentrating U937 cells to 1×10^7 to 2×10^7 cells per ml by low-speed centrifugation; cells were infected with Sendai virus in RPMI 1640 (250 to 500 hemagglutinating units per ml) for 90 min and repelleted, the virus-containing supernatant was removed, and the cells were suspended at 10^6 cells per ml in complete medium. RNA was prepared at 2, 6, and 10 h after induction. IFN-α- and IFN-β-specific mRNA was measured with 5′-[γ-^{32}P] ATP-end-labeled probes as previously described (20, 50).

Induction of U937 cells and preparation of nuclear extracts. U937 cultures were grown to a density of 10^6 cells per ml in RPMI 1640 medium supplemented with 5% fetal calf serum and antibiotics. High-efficiency nuclear extracts were prepared from 2 liters of uninduced cells following the protocol described by Shapiro et al. (41). The induced extract was prepared from the same volume of cells following priming or priming and induction. Briefly, cells were treated for 8 h with 1,000 IU/ml of rIFNα2 (a kind gift from Schering Canada), pelleted by centrifugation at 1200 rpm for 15 min, suspended in a volume of 200 ml in complete medium, and infected with Sendai virus (200 hemagglutinating units per ml) or mock infected for 4 h. At the end of the induction period, cells were processed for nuclear extract as previously described. The nuclear extracts (final protein concentration, 3 to 5 mg/ml) were quick-frozen in liquid nitrogen and stored in aliquots at −70°C.

Preparation of IFN probes. Synthetic oligonucleotides spanning the IFN-α1 and IFN-β regulatory regions (see Fig. 2) were prepared by a Gene Assembler (Pharmacia) and purified by separation on a 10% polyacrylamide gel followed by Sephadex G-25 chromatography. Complementary strands were annealed and repurified by gel electrophoresis to separate single- from double-stranded material.

Fragments were 5′ end labeled by using [γ-^{32}P]ATP (3,888 Ci/mmol; ICN Pharmaceuticals, Inc.) and polynucleotide kinase (Pharmacia). Incorporation of label was monitored by measuring radioactivity in trichloroacetic acid-insoluble material.

Gel electrophoresis DNA-binding assay. The gel retardation assay (12) was performed as previously described (54, 55). Briefly, probe DNA (30,000 to 50,000 cpm; 0.1 ng) was incubated with 5 μg of poly(dI)-poly(dC) as nonspecific competitor and 0 to 12 μg of nuclear extract as described in individual experiments. Unlabeled specific competitors were used at 0 to 80 ng. Reactions were carried out on ice for 30 min in buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 5% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*-*N*-*N*′-*N*′-tetraacetic acid], and 2 mM dithiothreitol and immediately loaded on a 6% native polyacrylamide gel (60:1 cross-link), prepared with Tris-glycine (25 mM Tris, 195 mM glycine [pH 8.5]). The gels were run at 150 V for 1.5 to 2.5 h, after which the gels were dried and exposed to autoradiographic film at −70°C. The relative intensities of the protein-DNA complexes were measured by using the 2200 scanning laser densitometer (LKB Instruments, Inc.); scans were integrated by using the 2202 Gelscan software package (LKB).

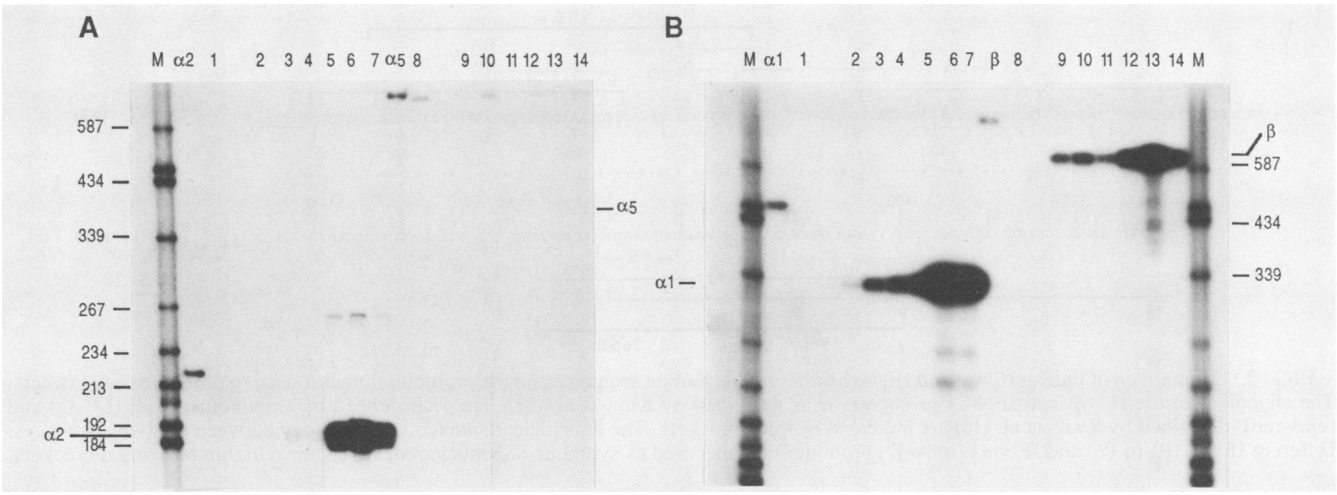


FIG. 1. IFN mRNA levels in IFN-treated, Sendai virus-infected U937 cells. Total RNA from U937 cells (10^8 cells per sample) was analyzed by using 5'-end-labeled IFN DNA fragments corresponding to IFN-α2 (A, lanes 1 to 7), IFN-α5 (A, lanes 8 to 14), IFN-α1 (B, lanes 1 to 7), and IFN-β (B, lanes 8 to 14) (20). The RNA samples were derived from uninduced cells (lanes 1 and 8), Sendai virus-infected cells at 2, 6, and 10 h (lanes 2 and 9, 3 and 10, and 4 and 11, respectively) and rIFN-α2-treated, Sendai virus-infected cells at 2, 6, and 10 h (lanes 5 and 12, 6 and 13, and 7 and 14, respectively). The positions of the DNA-RNA hybrids are indicated. M, γ-^{32}P-labeled fragments of pAT153, digested with *Hae*III; α2, 220-base-pair *Bgl*II-*Eco*RI IFN-α2 probe; α5, 750-base-pair *Eco*RI-*Hin*dIII IFN-α5 probe; α1, 460-base-pair *Bgl*II-*Bam*HI IFN-α1 probe; β, 922-base-pair *Bgl*II-*Eco*RI IFN-β probe.

RESULTS

Pretreatment with IFN increases IFN gene activity. The yield of biologically active IFN can be substantially augmented in many cell types by the pretreatment of cells before induction with inhibitors of protein synthesis or by priming with IFN itself (19, 44–46). To examine the effect of IFN priming on the activation of individual IFN genes, U937 cells were pretreated with rIFN-α2 for 8 h prior to Sendai virus induction, and the amounts of IFN-specific mRNA produced at 2, 6, and 10 h after induction in primed and unprimed cultures were compared. Figure 1 illustrates that IFN priming of U937 cells resulted in a dramatic increase in the steady-state levels of IFN mRNA measured by S1 mapping from individual IFN genes, corresponding to IFN-α1, -α2, -α5, and -β. Figure 1A illustrates that neither the IFN-α2 nor the -α5 genes were expressed at high levels following Sendai virus infection; however, priming with rIFNα2 for 8 h prior to virus induction resulted in a greater than 20-fold increase in the amount of IFN-α2 mRNA. Priming did not similarly augment expression of IFN-α5, a gene which is poorly inducible in several hematopoietic cell types (20, 52). Figure 1B demonstrates that virus induction of IFN-α1 and IFN-β genes was stimulated 20-fold and 5-fold, respectively, by priming with rIFNα2. Priming did not alter RNA stability, as determined by an actinomycin D chase experiment, suggesting that priming affected mainly the IFN promoter (data not shown). The amount of IFN antiviral activity was three times higher in supernatants from primed and induced cells compared with those from induced cells (30,000 IU/ml versus 10,000 IU/ml; N. Roberts, personal communication). These experiments are in agreement with previous biological studies which have shown that priming of hematopoietic cells increased predominantly the amount of IFN-α antiviral activity (1).

The influence of IFN priming on the IFN-β and IFN-α1 promoters was next examined by transfection experiments using the previously described IFNβ/CAT- and IFNα/CAT-expressing plasmids (53). Following transfection of the IFN/CAT plasmids, 293 cells were treated with either cycloheximide or rIFN-α2 prior to IFN promoter induction by Sendai virus infection. Pretreatment with both compounds increased the final yield of Sendai virus-inducible CAT activity (Table 1). The relative virus inducibility of the IFN-β promoter was about 13-fold, while pretreatment with IFN or cycloheximide increased CAT activities more than 5-fold above the levels obtained with virus induction alone. The IFN-α1 promoter is not efficiently expressed in 293 cells (53), and IFN or cycloheximide had no influence or IFN-α/CAT activity. Together, these experiments demonstrate that pretreatment of cells with IFN can increase virus-inducible transcriptional activation of the IFN promoter in different cell types.

U937 factor binding to the IFN-α1 promoter. Are changes in the expression of IFN genes accompanied by modulation of transcriptional factors associated with the IFN regulatory domains? To examine the protein-DNA interactions that accompany activation of type 1 IFN gene transcription, nuclear protein extracts were prepared from untreated cells,

TABLE 1. Effect of priming on IFN-CAT gene activity[a]

Treatment	Relative induction[b]	
	$SV_0\beta$	$SV_0\alpha$
Uninduced	1.0 (0.3)	1.0 (0.6)
Sendai virus	13.3	1.5
Cycloheximide	1.3	1.2
Cycloheximide-virus	93.7	1.7
rIFN-α2	2.0	0.7
rIFN-α2-virus	71.6	2.5

[a] Cells were transfected as described in Materials and Methods. At the end of transfection, IFN priming was initiated with rIFN-α2 (1,000 IU/ml) for a period of 20 h before addition of virus; cycloheximide (50 μg/ml) was added to cultures for 4 h, and during the last 2 h virus was added. Cells were induced with Sendai virus (250 hemagglutinating units per ml) for 2 h and harvested for CAT assay 18 h later.

[b] The average percent conversion of [^{14}C]chloramphenicol produced by CAT enzyme from induced samples divided by the percent conversion from uninduced samples. These values in parentheses represent the average percent conversions obtained in uninduced cells.

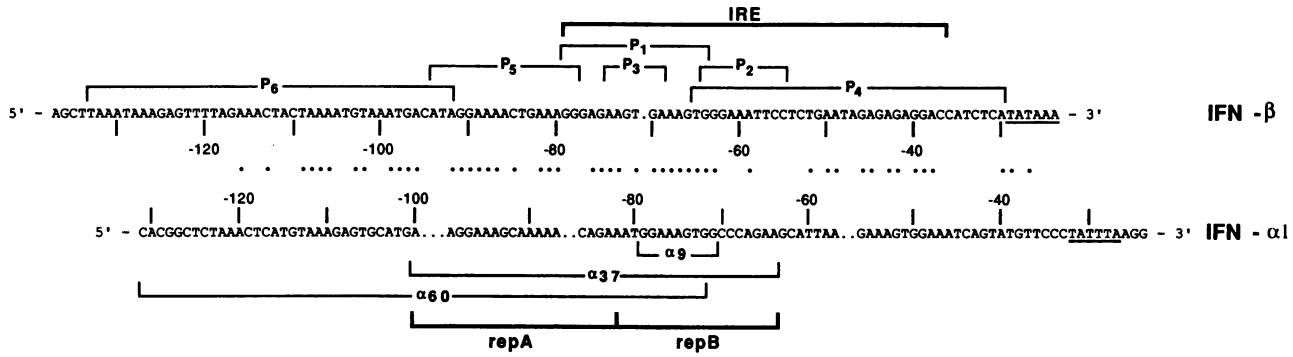


FIG. 2. Comparison of human IFN-β and IFN-α1 promoter nucleotide sequence and the promoter domains used to probe nuclear extracts. The alignment of the IFN-β and IFN-α1 promoters is as described by Kuhl et al. (27). The IRE defined by Goodbourn et al. (13, 15) and repA-repB described by Ryals et al. (38) are indicated by thick brackets. The dots indicate nucleotide homology between the two sequences. Different IFN-β (P1 to P6) and IFN-α1 (α9, α37) promoter domains used as synthetic oligonucleotides are shown in thin horizontal brackets.

IFN-treated cells, or IFN-treated, virus-infected U937 cells. A gel EMSA was used to identify factors binding to the IFN promoter (12). In this assay, ^{32}P-labeled DNA probe is incubated with nuclear protein extracts and analyzed by polyacrylamide gel electrophoresis; migration of specific protein-DNA complexes formed during incubation is retarded compared with the migration of noncomplexed probe. 5′-End-labeled synthetic oligonucleotides, corresponding to different domains of the IFN-α1 and IFN-β promoter (Fig. 2), were used to probe these extracts for factors binding to the IFN promoters.

A titration experiment using a fixed concentration of α37 fragment, corresponding to the virus-responsive element of the IFN-α1 promoter (repA-repB domain) (27, 38), and increasing amounts of nuclear extract identified several discrete protein-DNA complexes by EMSA (Fig. 3). At low nuclear extract concentration (1 to 2 μg), the predominant complex resolved by EMSA was complex A; however at higher protein concentration the amount of complex A decreased and a higher-molecular-weight complex, B, became the predominant band. At protein concentrations greater than 8 μg, several higher-molecular-weight complexes, C to E, were also observed. The relative changes in complexes A and B suggest independent binding of proteins with various affinities for the DNA elements. Alternatively, the binding of one protein to the α37 probe may facilitate the binding of a second, distinct or similar, factor to the repA-repB domains. Also, as clearly illustrated in Fig. 3, more protein was bound to the α37 probe in IFN-primed or IFN-primed and Sendai-induced extracts than in uninduced extracts. Interestingly, complex αB was increased by priming but undergoes little or no additional increase after

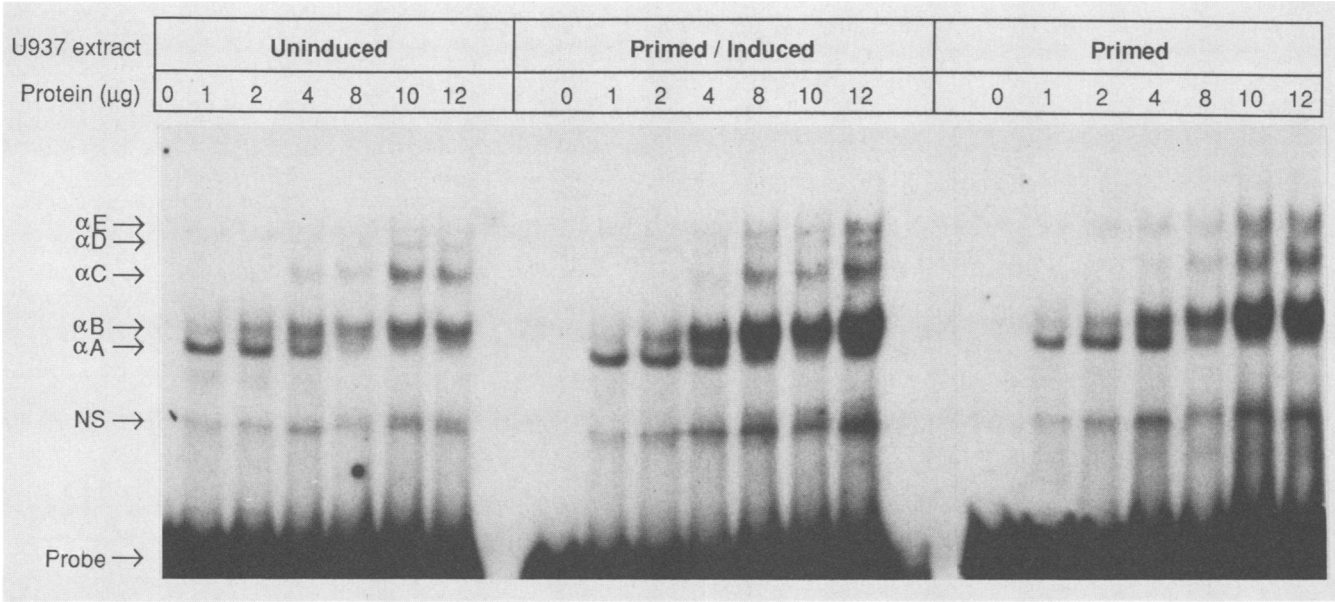


FIG. 3. U937 factor binding to the IFN-α1 promoter. The ^{32}P-labeled α37 probe (0.1 ng), corresponding to IFN-α1 promoter sequences between −100 and −64, was incubated with different amounts (0 to 12 μg) of nuclear protein from uninduced, IFN-treated, Sendai virus-induced, and IFN-treated U937 cells, together with 5 μg of poly(dI)-poly(dC) as nonspecific competitor DNA. Differentially migrating complexes (αA to αE) are indicated by arrows; NS, noncompeting complex; Probe, noncomplexed probe DNA. Samples were electrophoresed through a low-ionic-strength 6% polyacrylamide gel, dried down, and exposed to autoradiographic film. The type and amount of nuclear extract in each lane are indicated above the gel.

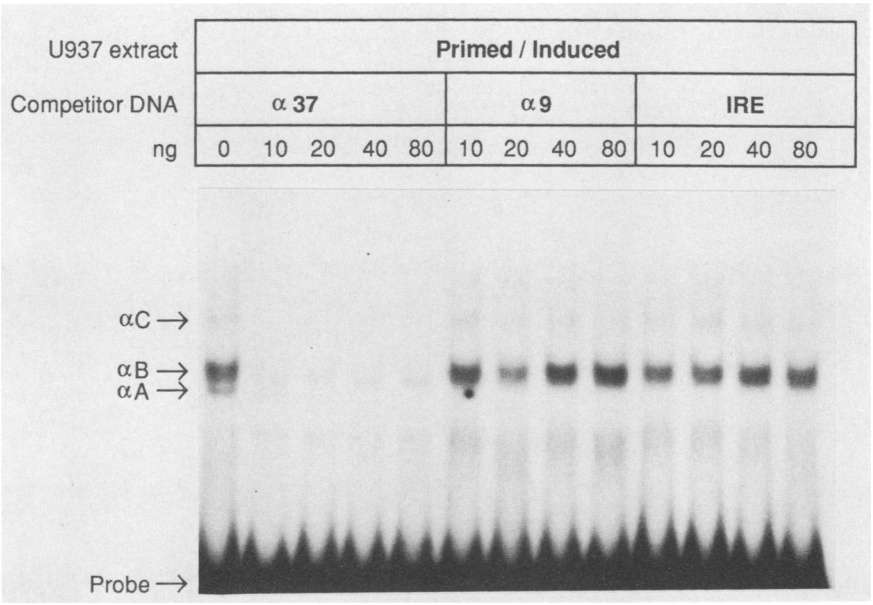


FIG. 4. Competition for U937 factor binding to α37. The binding reactions were set up in a 25-μl volume containing nuclear dialysis buffer, 0.1 ng of ^{32}P-labeled α37 probe, 4 μg of nuclear extract from IFN-primed, Sendai-induced U937 cells, 5 μg of poly(dI)-poly(dC), and 0 to 80 ng of unlabeled DNA fragments, corresponding to α37, α9 and IFN-β IRE. The positions of complexes αA to αC and the noncomplexed probe (Probe) are indicated by arrows; the type and amount of competing DNA are shown above the lanes of the gel.

priming and induction. αB binding may thus be necessary but not sufficient for IFN induction.

A competition analysis using a constant amount of labeled α37 probe and nuclear extract from primed and induced cells and different amounts of the homologous α37 fragment, or heterologous fragments—the IRE and a duplicated domain from within repB, the α9 fragment—showed that only the homologous α37 acted as an efficient competitor for complex B, while α9 and IRE did not compete, even at a 400-fold excess (Fig. 4). This was surprising, since the IRE shares considerable sequence homology with repA-repB and α9. When α9 domain was used directly as a factor-binding probe, no complex formation was observed (data not shown) indicating that, under these conditions, duplicated α9 alone did not represent a factor-binding domain or that factors interacting with α9 were in low concentration in these extracts.

U937 factor binding to the IFN-β promoter. When the IRE fragment (−79 to −35; Fig. 3) was used to probe for factor binding in U937 nuclear extracts, a distinct pattern of high-molecular-weight complexes was formed that differed from the pattern observed with α37 (Fig. 5). In uninduced or IFN-primed cell extracts, relatively little binding was detected (Fig. 5, lanes 4 and 5), but in IFN-primed, virus-infected extracts, a 10-fold increase in factor binding to IRE was observed (Fig. 5, lane 6). In contrast, a three- to fourfold increase in the αB complex of the α37 probe was detected in IFN-primed U937 extracts (compare Fig. 5, lanes 1 and 2). This result would suggest that IFN priming has a greater effect on the IFN-α1 promoter than on the IFN-β promoter, since no detectable increase in priming factor binding was observed with the IRE. It is also possible that sequences outside the IRE probe may be required to observe the binding of such a factor.

Figure 6 illustrates that IRE complex formation generated in U937 extracts was competed by the homologous IRE fragment, an oligonucleotide representing a duplication of the P2 domain (−64 to −55) of the IFN-β promoter (Fig. 3), and the enhancer element of HIV-1 (−105 to −80) (34, 48). Protein-DNA complexes were not competed with an oligonucleotide representing the P1 domain (−79 to −64) nor with a tetrahexamer of the sequence AAGTGA (11, 33, 54), a

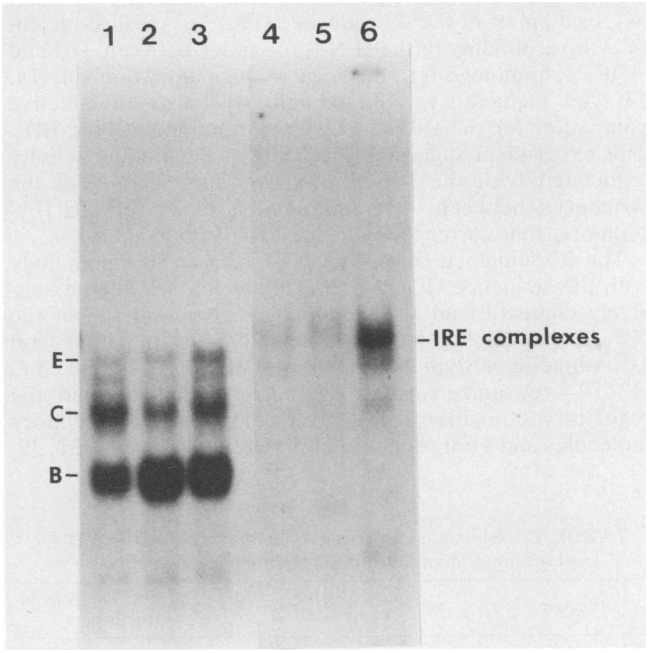


FIG. 5. U937 protein-DNA interactions with the IRE and α37 probes. U937 nuclear extracts (10 μg per reaction) from uninduced (lanes 1 and 4), IFN-primed (lanes 2 and 5), or IFN-primed, Sendai virus-induced (lanes 3 and 6) cells were incubated with either the IFN-α1 α37 probe (lanes 1 to 3) or the IFN-β IRE probe (lanes 4 to 6) and 5 μg of poly(dI)-poly(dC) as nonspecific DNA. Samples were electrophoresed through a 6% polyacrylamide gel, dried down, and exposed to autoradiographic film. The positions of the IRE and α37 complexes B, C, and E are indicated.

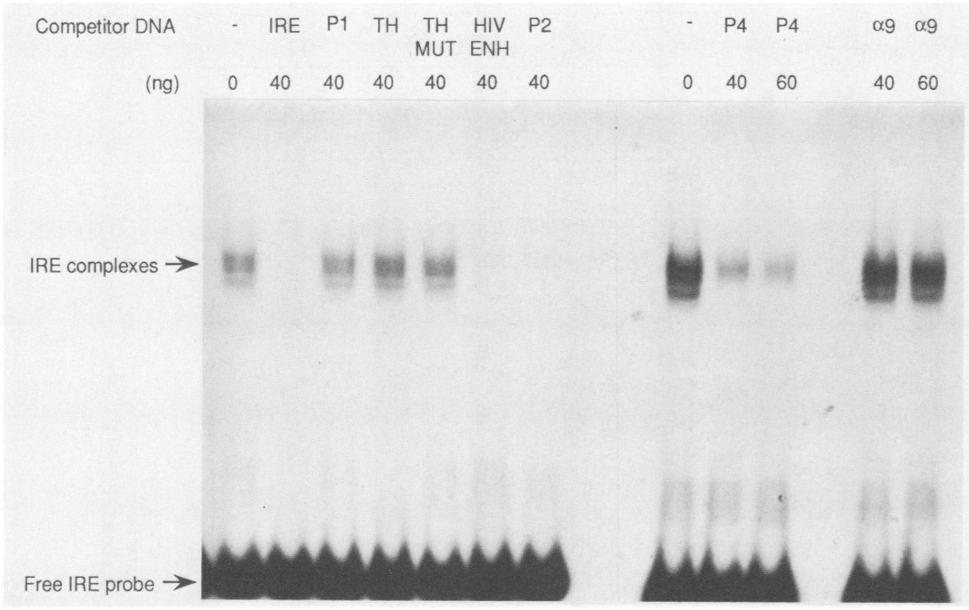


FIG. 6. U937 nuclear protein binding to the IFN-β IRE. The ^{32}P-labeled IRE probe (0.1 ng) was mixed with 10 μg of primed and induced nuclear protein, 5 μg of poly(dI)-poly(dC) and 0 to 60 ng of different competitor DNA fragments in a total volume of 20 μl. The type and amount of competitor DNA are shown above the lanes of the gel. IRE, IFN-β promoter −79 to −35; P1, IFN-β promoter −79 to −64; TH, a tetramer of the hexameric sequence AAGTGA (11, 33, 54); TH MUT, a permutation of the tetrahexamer AAAGGA; HIV ENH, an oligonucleotide representing the HIV-1 enhancer element from −105 to −80; P2, a direct duplication of the sequence in the IFN-β promoter −64 to −55; P4, the region of the IFN-β promoter from −65 to −30; α9, a direct duplication of the sequence of the IFN-α1 promoter from −79 to −71. The positions of these oligonucleotides relative to the IFN-α1 and IFN-β promoters are shown in Fig. 3. The positions of the IRE complexes and the noncomplexed IRE probe are indicated by arrows.

tetrahexameric permutation of that sequence AAAGGA (11, 54), or a dimer of the α9 sequence (Fig. 3). An oligonucleotide corresponding to the IFN-β promoter between −65 and −30 (P4, homologous to the negative regulatory domain) (14, 57) and including the P2 domain was also an effective competitor for the U937 factor(s) interacting with the IRE. This experiment suggested that most of the binding activity associated with the IFN-β promoter interacted with the sequences between −64 and −55, a region of the IFN promoter that corresponds to the PRDII domain (14).

The P2 sequence GGGAAATTCC shares 80% homology with the sequence GGGACTTTCC, which has been extensively characterized as the binding recognition site of the NF-κB transcription factor (3, 4, 34, 39, 40). In fact, several DNA-binding activities—NF-κB, HIVEN86A, KBF-1, and H2TF1—recognize similar sequence elements within the regulatory domains of genes encoding immunoregulatory molecules and viral promoters (Table 2) (2, 4, 24, 30, 31, 39, 40, 43, 56). To assess whether the P2 domain and the HIV enhancer, which contains two copies of the NF-κB site and binds protein in vitro (4, 34, 48), interacted with similar nuclear proteins from U937 cells, a constant amount of radiolabeled P2 probe was incubated with a fixed amount of U937 extract (8 μg) derived from uninduced or from primed and induced cells (Fig. 7). Increasing amounts of either homologous P2 competitor DNA or heterologous HIV competitor DNA were used for competition of binding to P2.

TABLE 2. Sequence comparisons between NF-κB-like DNA binding domains of different enhancer elements[a]

Element	DNA-binding domain sequence
IFN-β	gTGGGAAATTCCg
HIV (5′)	tAGGGACTTTCCa
HIV (3′)	tGGGGACTTTCCa
κ enhancer	aGGGGACTTTCCg
β2-microglobulin	aAGGGACTTTCCc
H-2K^b	tGGGGATTCCCCg
IL2Rα	aGGGGAATCTCCc

[a] Sequences derived from data presented in references 2, 4, 24, 30, 31, 39, 40, 43, and 56.

Two points can be made from the data used for Fig. 7: protein-DNA complex formation was about 20-fold greater in primed and induced extracts than in uninduced extracts, and competition analysis using increasing amounts of unlabeled P2 or HIV enhancer demonstrated that both DNA fragments competed with equal efficiency for factors associated with the P2 probe. When the HIV enhancer was used to probe U937 extracts for DNA-binding proteins, similar protein-DNA complexes were generated and both homologous HIV enhancer and P2 DNA competed for factor binding to the HIV enhancer (data not shown). Furthermore, an HIV enhancer fragment mutated in the κB sites to the sequence CATGGTTTCC was unable to compete for factors binding to P2 or HIV enhancer fragments (data not shown). These experiments indicate that the factor(s) binding to a duplicated P2 domain of the IFN-β promoter also interacted with the HIV enhancer elements.

Functional activity of IFN-β and HIV enhancer domains in epithelial and lymphoid cells. To determine whether inducible gene expression could be conferred upon a noninducible heterologous promoter by distinct regulatory domains (which appear to bind similar *trans*-acting factors), IFN-β and HIV hybrid promoters were transfected into epithelial and lymphoid cells. Hybrid plasmids were generated by

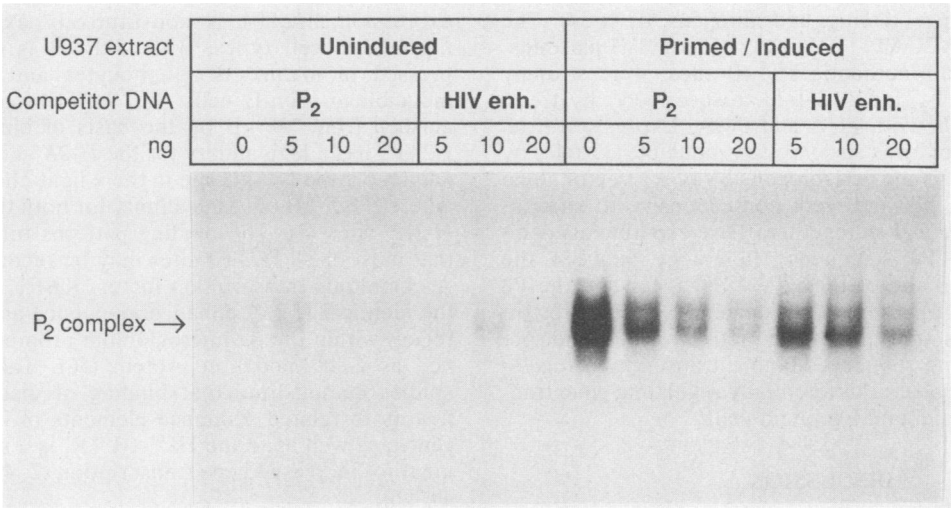
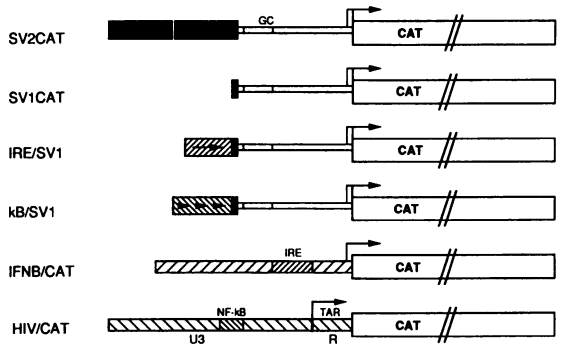


FIG. 7. Factor binding to the IFN-β P2 domain and the HIV enhancer. ^{32}P-labeled P2 probe (0.1 ng) was incubated with 8 μg of nuclear protein from uninduced or IFN-primed, virus-induced U937 cells, 5 μg of poly(dI)-poly(dC), and 1 to 20 ng of unlabeled P2 or HIV enhancer fragment and analyzed as described in Materials and Methods. The type of extract and the amounts of the competitor DNA are indicated above the lanes of the gel.

subcloning synthetic double-stranded oligonucleotides corresponding to the IRE (Fig. 2) or the HIV-1 enhancer (34, 48) into the SV1CAT vector, which contains the simian virus 40 (SV40) early promoter but lacks SV40 enhancer activity. The plasmids were assessed for Sendai virus inducibility following transfection into 293 epithelial cells and also examined for phorbol ester inducibility in Jurkat ($CD4^+$, $CD8^-$) T lymphoid cells (Fig. 8). The inducibility of CAT expression from the IRE/SV1 and κB/SV1 plasmids was compared with that of CAT activity generated by SV2CAT (which contains the SV40 enhancer element), the enhancerless SV1CAT plasmid, and the IFNβ/CAT plasmid which contains the entire IFN-β regulatory region from −281 to +19 (relative to the mRNA start site) in place of the SV40 promoter (53). In 293 cells, CAT activity from the SV2CAT plasmid was constitutive and not inducible by Sendai virus infection; removal of the SV40 enhancer by *Sph*I-*Acc*I cleavage decreased the constitutive level of CAT activity 12- to 20-fold, as previously documented (21, 53). In contrast, the IFN-β/CAT plasmid was strongly virus inducible for CAT enzyme activity. Interestingly, a single copy of the IRE linked to the SV40 promoter did not confer virus inducibility but generated instead a strong constitutive promoter that was only slightly inducible when compared with SV2CAT. Activity of the IRE/SV1 plasmid was similar to that of the κB/SV1 plasmid, which also produced a high constitutive level of CAT activity in 293 cells (Fig. 8).

These plasmids displayed distinct regulatory properties when analyzed in Jurkat cells, a T-cell line which can be induced to express NF-κB in response to tumor-promoting



CAT ACTIVITY (% Conversion of 14 C Chloramphenicol)

	293 CELLS			JURKAT CELLS		
	uninduced	induced	relative induction	uninduced	induced	relative induction
SV2CAT	53.9	59.0	1.1	7.4	65.0	8.8
SV1CAT	4.6	2.8	0.6	2.3	2.3	1.0
IRE/SV1	82.1	87.8	1.1	1.9	71.1	37.4
κB/SV1	71.5	97.0	1.4	4.8	58.1	12.1
IFNB/CAT	1.8	52.7	29.3	0.2	0.2	1.0
HIV/CAT	ND	ND		3.4	49.1	14.4

FIG. 8. Inducibility of IFN and HIV hybrid promoters in epithelial and lymphoid cells. The illustrated hybrid plasmids were transfected into 293 or Jurkat cells and induced with Sendai virus or PHA-PMA, respectively, at 20 h after transfection, as described in Materials and Methods. Cell lysates were prepared 20 h later, and 100 μg of protein was analyzed in a 60-min CAT assay (16, 53). Relative induction was determined by dividing percent conversion in the induced sample by that in the uninduced sample. Values represent the average of 2 to 4 separate measurements. The arrows within the boxes indicate the number and orientation of the inserted oligonucleotides corresponding to IRE or NF-κB. The IRE and NF-κB sequences are shown schematically within the IFN-β promoter and HIV LTR. The mRNA initiation sites within the plasmids are indicated by the arrows over the boxes. GC, GC-rich region of the SV40 early region; TAR, U3, and R, regulatory regions within the HIV LTR (48). ND, Not done.

agents such as PMA, lectins, and mitogens (3, 4, 34). The activities of the SV2CAT, HIV/CAT, and κB/SV1 plasmids, all of which contain authentic NF-κB sites, were strongly induced (8.8-, 14.1-, and 12.1-fold, respectively) by treatment of Jurkat cells with PHA and PMA. Expression from the IRE/SV1 plasmid was also strongly inducible (35-fold) by PHA-PMA, whereas the heterologous SV1CAT vector alone or the IFNβ/CAT plasmid were nonresponsive to stimulation by PMA-PHA. Taken together, these experiments demonstrate that the 80% sequence homology between the NF-κB recognition site of the HIV-1 enhancer and the P2 domain of the IFN-β promoter is accompanied by protein-DNA interactions and functional activities that indicate these two *cis*-acting sequences bind a transcription regulatory protein(s) capable of differentially regulating gene transcription in epithelial and lymphoid cells.

DISCUSSION

The functional importance of interactions between *trans*-acting factors and *cis*-acting DNA sequences in transcriptional regulation has been demonstrated for a number of inducible and constitutive genes (31). Three types of evidence suggest that one of the transcription factors involved in the regulation of the IFN-β promoter is related to the NF-κB binding proteins: (i) the sequence to which factor binding is detected in U937 cells (a direct duplication of the sequence 5′-GGGAAATTCC-3′) is 80% homologous to the recognition sequence of NF-κB (5′-GGGACTTTCC-3′); (ii) factor binding to the duplicated P2 (PRDII) domain is competed by the HIV-1 enhancer element which contains two copies of the NF-κB recognition site; and (ii) functional studies indicate that hybrid plasmids containing the IRE or HIV enhancer domains linked to an unresponsive heterologous promoter are similarly regulated relative to one another, when reintroduced into epithelial or lymphoid cells. Both hybrid promoters are constitutively expressed in epithelial cells, while in lymphoid cells both promoters are inducible by PMA.

Several DNA-binding activities have been identified that are capable of interacting with the recognition sequences described in Table 2. NF-κB, a 51-kilodalton protein, was recently purified (24) and shown to activate transcription from the enhancer-containing HIV-1 promoter in vitro. Other studies have demonstrated that NF-κB is normally complexed in the cytoplasm with a protein inhibitor (IκB); induction is a posttranslational event that involves two steps—dissociation of the κB-IκB complex and nuclear translocation of NF-κB prior to DNA binding. An 86-kilodalton inducible protein, termed HIVEN86A, which binds to the HIV-1 enhancer and the IL2Rα promoter, has been characterized by a microscale DNA precipitation assay (4, 30). NF-κB and HIVEN86A appear identical on the basis of DNA-binding specificity and inducibility; the molecular weight disparity suggests that these proteins may be related members of a family of proteins or distinct proteins capable of recognizing the same DNA-binding site. H2TF1 recognizes the palindromic sequence 5′-GGGGATTCCCC-3′ and stimulates transcription from the mouse *H-2K*^{b} class I major histocompatibility complex gene enhancer (2). Although the recognition sequence is very similar to that recognized by NF-κB, H2TF1 and NF-κB are distinguishable by three criteria. (i) H2TF1 contacts eight symmetrically positioned guanine residues of the recognition site, whereas NF-κB contacts the six internal guanines of the H2TF1 site. (ii) NF-κB and H2TF1 are also distinguishable by cell-specific expression. H2TF1 is constitutively expressed in many fibroblastic cell types, while NF-κB is constitutively expressed in mature B lymphocytes and is phorbol ester inducible in non-B cells. (iii) H2TF1 can also be distinguished from NF-κB on the basis of binding site affinity; H2TF1 has a high affinity for the *H-2K*^{b} binding site but low affinity for the NF-κB site in the κ light-chain gene enhancer whereas NF-kB has high affinity for both the κ enhancer and *H-2K*^{b} sites (2). The binding patterns furthermore suggest that most or all H2TF1 sites may be recognized by NF-κB. A ubiquitous transcription factor, KBF1, which recognizes the identical *H-2K*^{b} enhancer sequence as well as the same region within the β2 microglobulin promoter has been purified as a 48-kilodalton protein (56). Taken together, the studies demonstrate that binding of distinct transcription factors to related sequence elements of immunoregulatory genes, as well as to the HIV-1 LTR, is a necessary requirement for increased gene transcription (2, 4, 8, 30, 34, 39, 40, 48, 56).

Cell specificity of IFN production may be mediated in part by the association of NF-κB-like proteins with the PRDII domain of the IFN-β regulatory region. Introduction of single-base-pair mutations to the PRDII domain (P2) produce complex transcriptional phenotypes, consistent with the involvement of this domain in both negative and positive regulation (14). Mutations at −63, −62, and −61 (G, G, and A residues, respectively) within PRDII increase base-level transcription of the IFN-β gene in uninduced cells, suggesting that interaction of a negative regulatory factor may be impaired. Mutations throughout the −64 to −55 region also decrease relative IFN-β transcription following induction (14). The present study demonstrates increased binding of a factor to the P2 domain of the IFN-β promoter, implicating the region in positive regulation. In support of this idea, distinct patterns of gene expression were observed in heterologous cells when the IRE or the HIV-1 enhancer was linked to an SV40 promoter-CAT reporter plasmid-constitutive expression in epithelial cells and TPA-inducible expression in T cells. It is possible that the same regulatory element interacts with distinct DNA binding activities in a cell-type-dependent manner. A posttranslational modification of a protein binding to the PRDII domain may positively activate transcription, a mechanism in which different states of the same protein would control gene activation. Alternatively, in some cells an inducible transcription factor similar to NF-κB may displace, by virtue of higher binding affinity or transiently higher concentration, a ubiquitous factor such as H2TF1 from the PRDII domain.

Regulation of IFN-β gene expression is not controlled solely by factors interacting with the PRDII domain (9, 11, 13–15). The adjacent sequences of the IFN-β promoter between −109 and −65 also bind a transcription factor termed IRF-1 that binds to multimerized permutations of the hexameric sequence AAGTGA, a sequence element present seven times in the region of the IFN-β promoter between −109 and −65 (10, 11, 33). Since multimers of the hexamer confer inducibility to a truncated IFN promoter and also silence heterologous enhancer elements, IRF-1 binding to PRDI (−79 to −64) and adjacent sequences may confer both inducibility and silencing properties to the IFN promoter (10, 27, 33). The observation that the gene encoding IRF-1 is activated by IFN treatment furthermore implies that IRF-1 may be one of the proteins binding to the IFN-responsive sequences in IFN-activated genes (7, 22, 23, 26, 29, 36, 37, 42, 49) as well as the protein responsible for the priming phenomenon (6). Cooperative interactions between IRF-1,

κB-like proteins, and perhaps other factors, all of which may vary in terms of DNA affinity and quantity in different cell types, may control inducibility and cell specificity of the type 1 IFN gene family. Minor differences in the promoter sequence of distinct members of the IFN multigene family (52) may also significantly alter binding of transcription factors and, hence, inducibility (20).

Does IFN-α possess recognition sequences similar to the NF-κB-like site found in IFN-β? We were unable to compete binding to the IFN-β promoter by using the α37 fragment and vice versa which would suggest that the proteins interacting with IFN-α and IFN-β are unique or that great differences in DNA binding affinities exist. However, there is extensive sequence homology between these two promoters (Fig. 3), and in a recent study, Miyamoto et al. (33) demonstrated that at high molar excesses of competitor DNA (2,000- to 6,000-fold molar excess) competition for factor binding to the IFN-β hexameric sequences could be achieved with repA-repB sequences. Within the repB domain of the IFN-α1 promoter the sequence GGAAAGTG GCCCC at −79 to −68 is the complement in the opposite orientation of the NF-κB site, with the exception of two guanine residues inserted at positions −71 and −72. Two circumstantial pieces of evidence suggest the importance of this entire sequence in regulation of IFN-α transcription. (i) The sequence GGAAAGTGG, which corresponds to the enhancer core sequence (51), was used as a probe in this study and found to be an ineffective protein-binding sequence. This observation is similar to the findings of Fromental et al. (8), who used a related sequence domain from the SV40 enhancer region and found no factor binding. (ii) A natural IFN-α promoter mutant in the IFN-α6 gene is deleted in the region −61 to −72 and is transcriptionally inactive (20, 52).

Finally, these studies suggest that the IFN family may be added to a growing list of genes involved in immune responsiveness—including immunoglobulins, class I major histocompatibility complex, and the interleukin-2 receptor—that are regulated in part by interaction with NF-κB-like transcription regulatory proteins. The capacity of HIV-1 to utilize similar host cellular factor(s) to increase LTR-mediated transcription of the viral genome illustrates the complex interdependence between cellular and viral factors in the regulation of latent versus lytic states during HIV-1 infection (48).

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