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Synergism between Distinct Enhanson Domains in Viral Induction of the Human Beta Interferon Gene

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This study demonstrates distinct virus-inducible enhanson properties for three regions of the human beta interferon (IFN-β) promoter; maximum virus inducibility required syngerism among all three enhansons. Expression of the IRF-1 transcription factor differentially increased the expression of plasmids containing $(AAGTGA)_4$ or PRDIII (−94 to −78) motifs but was inefficient in the induction of the intact IFN-β promoter. The human T-cell lymphotropic virus type I Tax protein was a strong positive activator of PRDII (−64 to −55)-containing plasmids but was also unable to stimulate the IFN-β promoter. Induction of the intact IFN-β promoter linked to a reporter plasmid was achieved in lymphoid and epithelioid cellular backgrounds by a triple transfection with IRF-1 and Tax expression plasmids or a combination of IRF-1 and phorbol ester, indicating that at least two *trans*-activating events and the association of two proteins on the promoter template are required for IFN-β activation.

Inducible, tissue specific, and temporal-developmental control of gene expression is mediated by the interplay of transcriptional regulatory proteins with two types of functionally interdependent DNA sequences, promoters and enhancers. Transcriptional enhancers possess a modular organization composed of short overlapping 8- to 12-base-pair sequences, called enhansons, that serve as recognition sites for DNA-binding proteins and function to increase transcription of RNA polymerase II-dependent genes by a mechanism relatively independent of orientation and distance (11, 14, 38, 41). The contribution of an individual enhanson to overall enhancer activity may vary in a cell-specific manner, reflecting the relative abundance and/or activity of specific transcription factors. Four distinct classes of enhansons have been described. Class A enhansons display strict spacing requirements and exhibit enhancer activity when tandem repeats of the motif are oligomerized. Class B enhansons exhibit no enhancer activity when multimerized on their own but can generate enhancer activity when juxtaposed with class A motifs (to form a proto-enhancer) and then oligomerized. Class C enhansons possess intrinsic proto-enhancer activity and, when oligomerized, form a functional enhancer element without strict spacing requirements between enhansons. Class D enhansons, exemplified by steroid response elements, display enhancer activity once the receptor is bound (14, 31, 36, 41).

Virus-induced activation of beta interferon (IFN-β) transcription is mediated by the interaction of regulatory proteins with enhanson elements in the IFN-β promoter. In particular, a hexameric sequence, AAGTGA, permutations of which are present throughout the IFN-β promoter between −107 and −65 relative to the mRNA start site, can function as a virus-inducible or constitutive enhancer when present in tandem repeats (12, 20). Multimers of the AAGTGA hexamer generate the sequence GAAAGT, which is thought to represent a high-affinity site for two DNA-binding proteins, IRF-1 and IRF-2 (15, 18, 19, 26, 39). Recently, different types of $(GAAANN)_4$ sequences mediating virus inducibility have been described, indicating that different hexameric sequences are not equivalent and that other IRF-like proteins are involved in alpha interferon and IFN-β induction (37).

The natural IFN-β promoter contains an interferon regulatory element (located at −77 to −37) which includes two positive regulatory domains (PRDI and PRDII) and one negative regulatory domain, as defined by mutational analysis and protein-DNA interactions (21, 22, 33, 48, 49). The PRDI domain (−77 to −64) is thought to interact with the IRF proteins (19, 26, 39, 48), as well as with other constitutive and inducible factors (33, 37, 48). The PRDII domain (5′-GGGAAATTCC-3′; −64 to −55) binds NF-κB, a cellular factor involved in the transcriptional activation of several viral and cellular genes (3, 6, 30, 32, 40, 43). Virus or double-stranded RNA treatment of fibroblastic or monocytic cells induces IFN-β gene transcription and is accompanied by the binding of a protein indistinguishable from NF-κB to PRDII (16, 28, 34, 46).

The aim of the present study was to characterize the relative virus inducibility of distinct IFN-β enhanson domains and to examine the contribution of IRF-1 protein and inducers of transcription factor NF-κB to overall enhancer activity. Maximum virus responsiveness required combined synergism among three distinct enhanson motifs. Significant induction of the intact IFN-β promoter was achieved in lymphoid and T lymphoid and epithelial cells by coexpression of IRF-1 and the human T-cell lymphotropic virus type I (HTLV-I) Tax *trans*-activator protein.

MATERIALS AND METHODS

Oligonucleotide preparation and plasmid construction. Complementary oligonucleotide strands were synthesized on a Pharmacia LKB Biotechnology Inc. Gene Assembler, annealed, and purified on a 20% nondenaturing polyacrylamide gel. The SV2CAT, SV1CAT, HIV/CAT, and IFN-

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β/CAT ($SV_0β$) plasmids have been previously described (23, 39, 47). SV1CAT-derived constructs were made by directional subcloning of synthetic oligonucleotides (see Fig. 1) into the *Acc*I-*Sph*I site of the enhancerless SV1CAT vector. The structures of the hybrid genes were determined by sequencing (United States Biochemical Corp.) by the collapse plasmid method (27).

Transient expression assays. Subconfluent human 293 cells were transfected with 5 μg of CsCl-purified chloramphenicol acetyltransferase (CAT) reporter plasmid DNA by the calcium phosphate coprecipitation method (24). Jurkat lymphoid T cells (10^6 cells per ml) were transfected by the DEAE-dextran procedure (25). For cotransfection experiments, pHIRF31 (15), a plasmid constitutively expressing human IRF-1, and pTat-I (41), a plasmid constitutively expressing the HTLV-I Tax protein, were used at 10 and 5 μg, respectively. 293 cells were infected 18 to 20 h posttransfection with 500 to 1,000 hemagglutinating units of Sendai virus per ml for 90 min (29). Phorbol ester induction was performed by the addition of phorbol 12-myristate 13-acetate (PMA) to a final concentration of 25 ng/ml. Cells were harvested 16 to 20 h postinduction, and soluble protein extracts (usually 100 μg) were assayed for 1 h for CAT activity as previously described (23).

RESULTS

Enhanson motifs of the IFN-β promoter. To assess the virus-inducible enhancer activity of distinct IFN-β promoter regions, we subcloned synthetic oligonucleotides representing specific IFN-β regulatory domains (Fig. 1) into the enhancerless SV1CAT vector and transfected them into human epithelioid (293 and HeLa) and lymphoid (Jurkat) T cells. The interferon-CAT transient expression system has been shown to accurately reflect transcriptional activation of the IFN-β promoter (12, 20, 28, 46, 47). The reporter gene activities of the plasmids and the SV1CAT parental vector were compared with those of other plasmids containing (i) the simian virus 40 enhancer, SV2CAT (a constitutive control); (ii) the entire IFN-β promoter from −278 to +19, IFN-β/CAT (47); (iii) the human immunodeficiency virus (HIV) enhancer (−105 to −80); and (iv) the entire HIV long terminal repeat (LTR) (−350 to +80) (Fig. 1) (40).

The results of a representative transfection and Sendai virus induction are shown in Fig. 2. Several observations can be made from these data. (i) Multimers of the P5 and P1 oligonucleotides had strikingly different activities; three copies of PRDI in plasmid P1(3) were virtually inactive, while four copies of the P5 region [plasmid P5(4)] functioned as a strong virus-inducible enhancer. Interestingly, a tandem repeat of P5 [P5(2)] was not induced by virus, suggesting that the activity of a P5-linked promoter depends on the number of motifs. Two copies of the tetrahexamer $(AAGTGA)_4$ (TH) were also virus inducible, while the $(AAAGGA)_4$ tetrahexamer [TH(m)] had a higher basal activity and similar virus inducibility. These results suggest that in human cells the P5 region may be a third positive regulatory domain (PRDIII) which has the properties of a class A enhanson (14) and which becomes a virus-responsive enhancer when a tandem repeat is oligomerized. The hexameric sequences likewise exhibit properties of a virus-responsive class A enhanson (12, 14, 20). In contrast, intact PRDI behaves as a class B enhanson (14), exhibiting no intrinsic enhancer activity when multimerized. (ii) Duplication of the PRDII domain in plasmid P2(1) generated a virus-responsive enhancer (10-fold inducibility), while plasmid P2(2) was a strongly constitutive, weakly virus-inducible enhancer (2- to 3-fold inducibility). Plasmid P2(2) and plasmid κB(3) possessed identical enhancer properties, further supporting observations that both domains interact with similar proteins (16, 28, 34, 36). Clustered mutations within the NF-κB-binding sites (Fig. 1) of the HIV enhancer abrogated all the inducibility of the κB(m) plasmid. The PRDII and κB motifs exhibit properties of a class C enhanson (proto-enhancer), since these motifs do not have strict spacing requirements for functional activity (14, 31, 36). (iii) Maximum virus-responsive enhancer activity was obtained when the three domains were linked. The combination of PRDIII and PRDI domains in plasmid P51 exhibited almost no enhancer activity, while the PRDI-PRDII combination in plasmid P12 exhibited enhancer activity that was weakly (two- to threefold) virus inducible. However, when the three domains were arranged in their natural order, plasmid P512 behaved as a regulated virus-responsive enhancer, similar to the entire IFN-β promoter. The linkage of PRDIII (P5) to PRDI and PRDII not only augmented the response to the virus but also reduced the basal activity of the PRDI and PRDII domains. Together, these results imply a strong synergistic effect of three contiguous enhansons in IFN-β gene regulation.

Activation of IFN-β promoter domains by IRF-1. To investigate the role of the IRF-1 protein (19, 39) in positive regulatory domain enhanson activity, we cotransfected hybrid promoter constructs together with a human IRF-1 cDNA expression vector (15). IRF-1 coexpression significantly increased the basal activity of a reporter plasmid containing the entire IFN-β promoter and of reporter plasmids P1(3), P5(4), and TH (Fig. 3). Although IRF-1 increased the basal activity of the intact IFN-β promoter 10-fold, induction by virus further increased promoter activity by 10- to 15-fold. Similarly, IRF-1 augmented P5(4) activity about 10-fold but was 3- to 4-fold less effective than was virus as an inducer of this hybrid promoter. In contrast, virus and IRF-1 increased P1(3) activity only about two- and fourfold, respectively. Plasmid TH was unique in that expression was activated more effectively by IRF-1 than by virus induction. Interferon treatment alone had no effect on these plasmids (data not shown). These results show that IRF-1 positively interacts with distinct IFN-β enhanson domains but is not sufficient for maximal induction of the natural promoter. IRF-1 alone is, however, sufficient for maximal induction of the tetrahexamer $(AAGTGA)_4$-containing promoter.

***trans* activation of the intact IFN-β promoter.** The observation that IRF-1 partially activated the IFN-β promoter (Fig. 3) suggested that maximal induction of the IFN-β promoter may also require simultaneous activation of the PRDII domain. Since no NF-κB cDNA was available, we sought to induce NF-κB binding to PRDII in Jurkat lymphoid T cells by phorbol ester treatment or by *trans* activation with the Tax protein of HTLV-1. The Tax protein has been shown to indirectly stimulate transcription of the interleukin-2 gene, the interleukin-2 receptor α-chain gene, and the HIV LTR via the NF-κB element (4, 10, 35, 42, 45). Tax protein coexpression or phorbol ester treatment similarly activated PRDII-containing constructs [P2(1) and P2(2)], as well as other constructs with NF-κB recognition sites, including SV2CAT, HIV/CAT, and κB(3) (Fig. 4). Plasmids such as SV1CAT, TH, P1(3), and P5(4) were not induced by Tax or phorbol ester in either Jurkat cells (Fig. 4) or 293 cells (data not shown). Importantly, the entire IFN-β promoter was not activated by phorbol ester or Tax, demonstrating that the NF-κB motif in the context of the natural IFN-β promoter was not sufficient for activation.

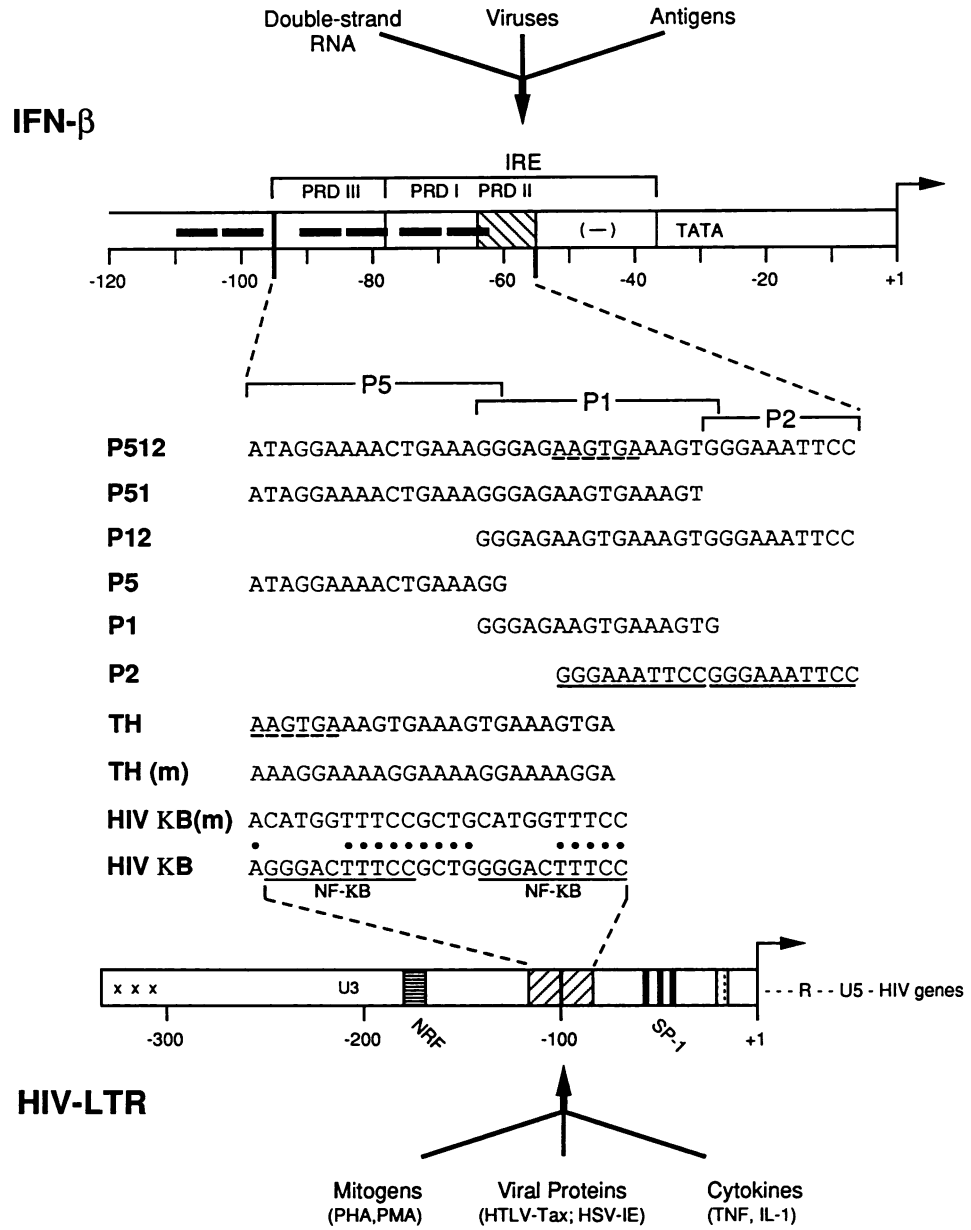

FIG. 1. Organization of the IFN-β promoter and the HIV LTR. The IFN-β promoter (12) is illustrated schematically above the list of oligonucleotides used in this study to generate SV1CAT-derived plasmids. The oligonucleotides were directionally cloned into the *Acc*I-*Sph*I-cleaved SV1CAT vector (23). The solid boxes within the IFN-β promoter represent the locations of the hexamer repeats; the dashed box indicates the region of the promoter with homology to the NF-κB recognition sequence; IRE, interferon regulatory element; (−), negative regulatory domain. The locations of three positive regulatory domains (PRD) are shown. The solid line beneath the oligonucleotides indicates NF-κB binding sites; the broken line indicates the hexameric sequence AAGTGA within the IFN-β promoter. The dots between oligonucleotides indicate the homology between the two sequences. The HIV LTR is illustrated schematically below the oligonucleotides, and the region of the HIV enhancer containing the NF-κB motifs is indicated by the area bracketed by broken lines (40). The positions of binding sites for other transcription factors (SP-1, negative regulatory factor [NRF], AP-1 (×), and TATA region [┆]) are indicated. PHA, Phytohemagglutinin; TNF, tumor necrosis factor; IL-1, interleukin-1.

The combined effect of IRF-1 and Tax coexpression or IRF-1 and phorbol ester treatment on the intact IFN-β promoter was next examined. IRF-1 expression increased the basal level of IFN-β promoter activity about 15-fold in 293 cells (Fig. 5A), whereas Tax expression or phorbol ester treatment did not significantly activate the promoter. However, when both *trans*-activator proteins were expressed, IFN-β promoter activity was increased almost 100-fold without virus induction.

The same experiment was performed in Jurkat cells, which do not normally produce IFN-β (J.-F. Leblanc, unpublished observations). In this cell background, IRF-1 expression alone did not enhance IFN-β promoter activity, while Tax expression alone increased the activity about threefold. However, a triple transfection that included Tax, IRF-1, and the intact IFN-β reporter plasmid significantly increased IFN-β promoter activity. A similar level of inducibility was obtained when phorbol ester was used in combi-

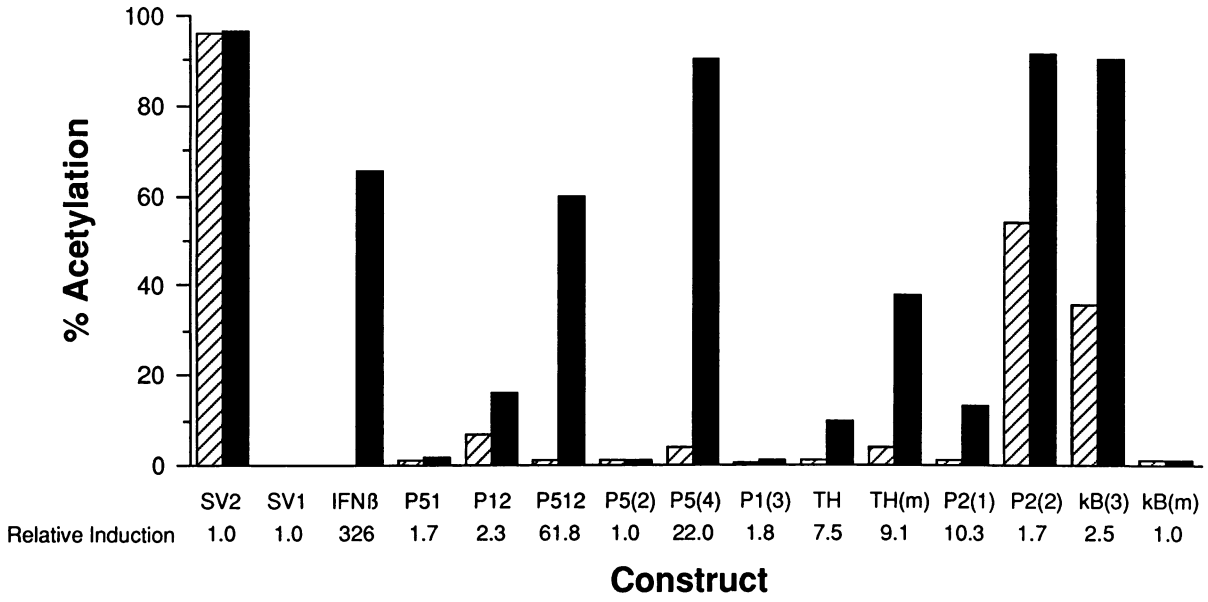

FIG. 2. Activity of IFN-β/CAT hybrid constructs. 293 cells were transfected and induced as described in Materials and Methods. The structures of SV2CAT (SV2), SV1CAT (SV1), and IFN-β/CAT (IFNβ) plasmids have been described previously (47). SV1CAT-derived plasmids were named according to the inserted oligonucleotides shown in Fig. 1; the number in parentheses after the plasmid name indicates the number of inserts. The bar graphs show the percentage of acetylated chloramphenicol in extracts from uninduced (hatched bars) or Sendai virus-induced (solid bars) extracts. Relative induction was obtained by dividing percent conversion in the induced sample by that in the uninduced sample.

nation with IRF-1. These results, obtained in two different cellular backgrounds, indicate that IFN-β induction requires at least two distinct activation signals, mediated by combined synergism between IRF-1 and NF-κB.

DISCUSSION

We have shown that three distinct IFN-β enhanson domains are required for a maximal response to virus induction. (i) The PRDIII region (−94 to −78), which exhibited properties of a virus-responsive class A enhanson, increased virus inducibility when combined with the PRDI and PRDII domains (−77 to −55) and also reduced the basal activity of PRDI and PRDII in the uninduced state. (ii) The PRDII region, which represents the binding site for NF-κB, exhibited the characteristics of a class C enhanson, as previously shown for the simian virus 40 TCII motif (31, 36). (iii) The PRDI region behaved as a class B enhanson, lacking intrinsic enhancer activity when multimerized.

The region of the IFN-β promoter adjacent to the interferon regulatory element and specified by the P5 oligonucleotide (−94 to −78) has been implicated previously in virus inducibility in human cells (9, 17, 20, 21). Four copies of P5 exhibited strong virus-inducible enhancer activity when linked to a heterologous promoter, whereas two copies were not virus responsive. Furthermore, the linkage of a single P5 domain to PRDI and PRDII dramatically increased the activity of the promoter. These results demonstrate that in human cells the P5 region represents a third positive regulatory domain (PRDIII) which synergistically contributes to virus inducibility when combined with PRDI and PRDII.

The minimal enhancer activity observed with PRDI was unexpected, since PRDI and the tetrahexameric sequences are often considered interchangeable elements; previous experiments with the $(AAGTGA)_4$ sequences demonstrated virus-inducible or constitutive enhancer activity, depending on the cell line used (12, 20). We also demonstrated that the $(AAGTGA)_4$ and $(AAAGGA)_4$ sequences were both virus inducible. However, multimers of the natural PRDI domain have not been previously examined for inducibility, and on the basis of the present results, PRDI and the tetrahexameric sequences do not appear to be equivalent elements. PRDI may also be a class A enhanson which, in plasmid P1(3), does not preserve the strict spacing requirements.

The present study also demonstrated that the IRF-1 protein can differentially increase the activity of plasmids containing the intact IFN-β promoter, the $(AAGTGA)_4$ sequences, the PRDIII domain and, to a limited extent the PRDI domain. However, two distinct patterns of response were observed. The activity of the IFN-β and P5 (4) plasmids was increased about 10-fold by the IRF-1 protein, but virus induction was still significantly more effective as an inducer; in contrast, TH plasmid activity was increased more effectively by IRF-1 coexpression than by virus induction. Therefore, while IRF-1 may be required for IFN-β induction, it is not sufficient, indicating that other proteins are also necessary for maximal activity. On the other hand, IRF-1 is sufficient for maximal TH responsiveness. This may not be surprising, since the IRF-1 cDNA clone was originally selected on the basis of high-affinity binding to the TH sequence. Interestingly, plasmid THm was only twofold inducible by IRF-1 (data not shown), suggesting that it may be a type II hexameric sequence (37).

An additional important region involved in IFN-β regulation is the PRDII domain, which exhibits the properties of a class C enhanson and interacts with NF-κB (14, 16, 28, 31, 34, 36, 46). In the present study, we demonstrated that the expression of the HTLV-1 Tax protein or phorbol ester treatment *trans*-activated PRDII and HIV enhancer-containing plasmids up to 100-fold. However, Tax or PMA treatment alone did not activate transcription from the

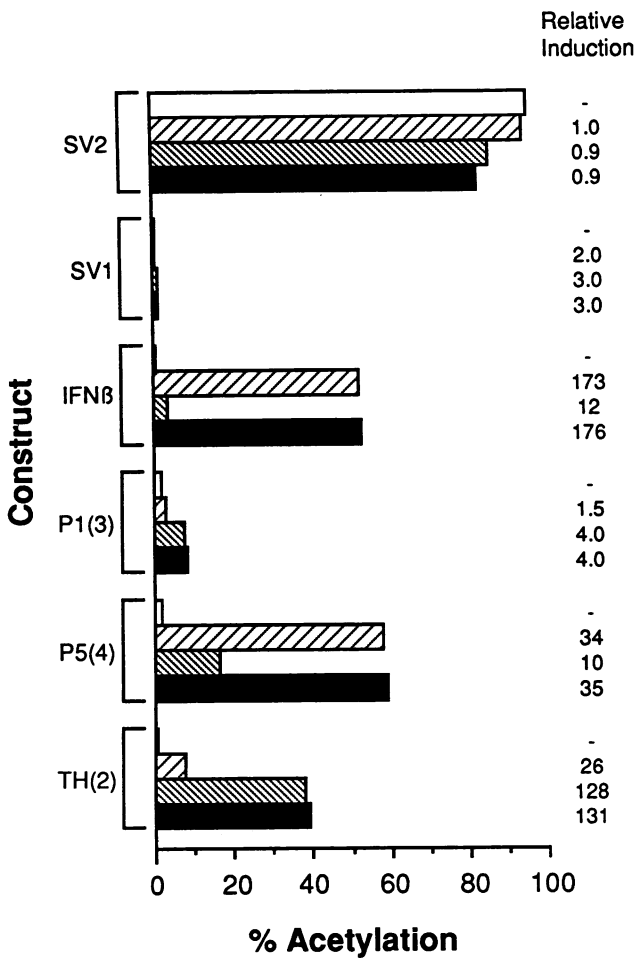

FIG. 3. Effect of IRF-1 coexpression on IFN-β/CAT hybrid plasmids. 293 cells were cotransfected with 5 μg of reporter plasmid (indicated on the left) and, in some samples, with 10 μg of pHIRF31. Cells were induced with Sendai virus after 4 h of pretreatment with recombinant alpha-2 interferon (250 IU/ml) and assayed as described in Materials and Methods. The basal percent acetylation of the plasmids was as follows: SV2CAT (SV2), 95.0; SV1CAT (SV1), 0.3; IFN-β/CAT (IFNβ), 0.6; P1(3), 1.9; P5(4), 5.0; and TH(2), 2.0. Symbols: □, uninduced; ▨, induced by interferon and Sendai virus; ▧, induced by IRF-1; ■, induced by IRF-1, interferon, and Sendai virus.

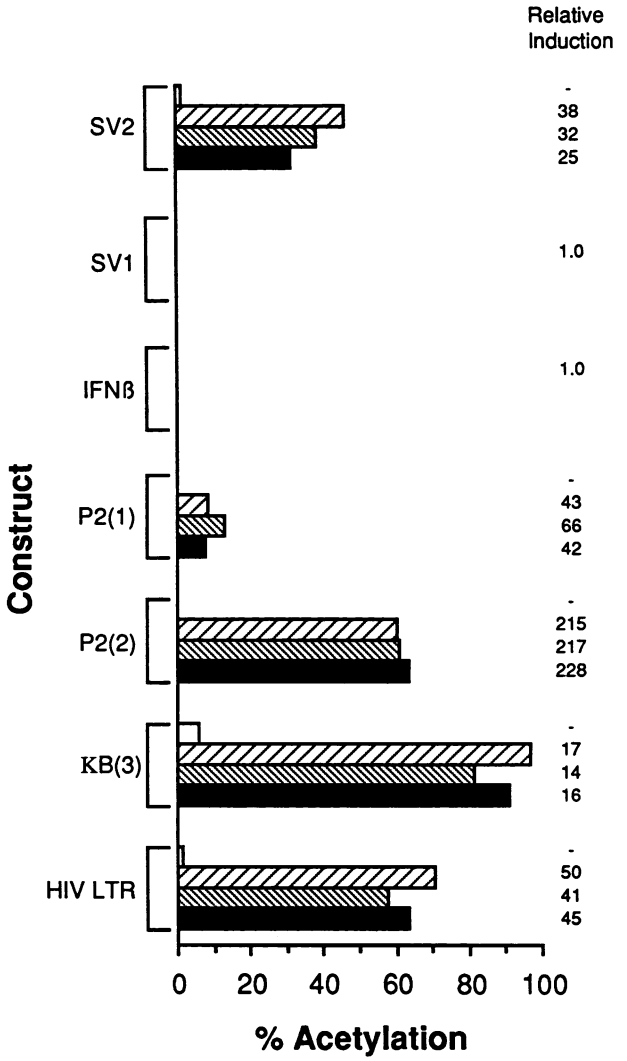

FIG. 4. HTLV-1 Tax protein and phorbol ester induction of IFN-β- and HIV LTR-derived plasmids in lymphoid T cells. Jurkat cells were cotransfected with 5 μg of reporter plasmid (indicated on the left) and, in some cases, with 5 μg of pTat-1 by DEAE-dextran-mediated transfection (25). Cells were induced with PMA (25 ng/ml) 24 h later and harvested 18 h after induction. Relative induction was determined as described in the legend to Fig. 2. The basal percent acetylation of the plasmids was as follows: SV2CAT (SV2), 1.2; SV1CAT (SV1), 0.2; IFN-β/CAT (IFNβ), 0.2; P2(1), 0.2; P2(2), 0.3; κB(3), 5.9; and HIV LTR, 1.4. Symbols: □, uninduced; ▨, treated with PMA; ▧, treated with Tax; ■, treated with PMA and Tax.

natural IFN-β regulatory region. Tax was nonetheless able to induce the HIV LTR in lymphoid cells (Fig. 4) and, to a lesser extent (about fivefold), in epithelial cells (J.-F. Leblanc, unpublished observations). Thus, activation of NF-κB binding to the PRDII domain also appears to be a necessary but not sufficient requirement for IFN-β gene expression and illustrates the importance of factor binding context in determining gene activation events (8). A strong repression mechanism must be operative to control IFN-β transcription, since NF-κB-binding activity can be stimulated by compounds such as PMA which do not in themselves activate IFN-β transcription (18; this study).

Using two different cellular backgrounds, inducible epithelioid and noninducible lymphoid T cells, we demonstrated that triple transfection with the IFN-β/CAT reporter and the IRF-1 and Tax expression vectors synergistically activated the natural IFN-β promoter better than did either *trans* activator alone. Induction with phorbol ester in combination with IRF-1 was also capable of inducing IFN-β promoter activity. The simplest explanation of these results is that IFN-β gene activation requires the combined interaction of at least two transcription factors, IRF-1 and NF-κB, and that these proteins are activated by two distinct signal transduction pathways. As demonstrated previously, induction of NF-κB binding to DNA is a posttranslational event involving cytoplasmic dissociation of the NF-κB p65-p50 heterodimer from an inhibitor protein, IκB, and nuclear translocation of the DNA-binding activity (1, 2, 36, 43). Induction is mediated by multiple inducers, including virus infection, double-stranded RNA, activators of protein kinase C and protein kinase A, and protein synthesis inhibitors (2,

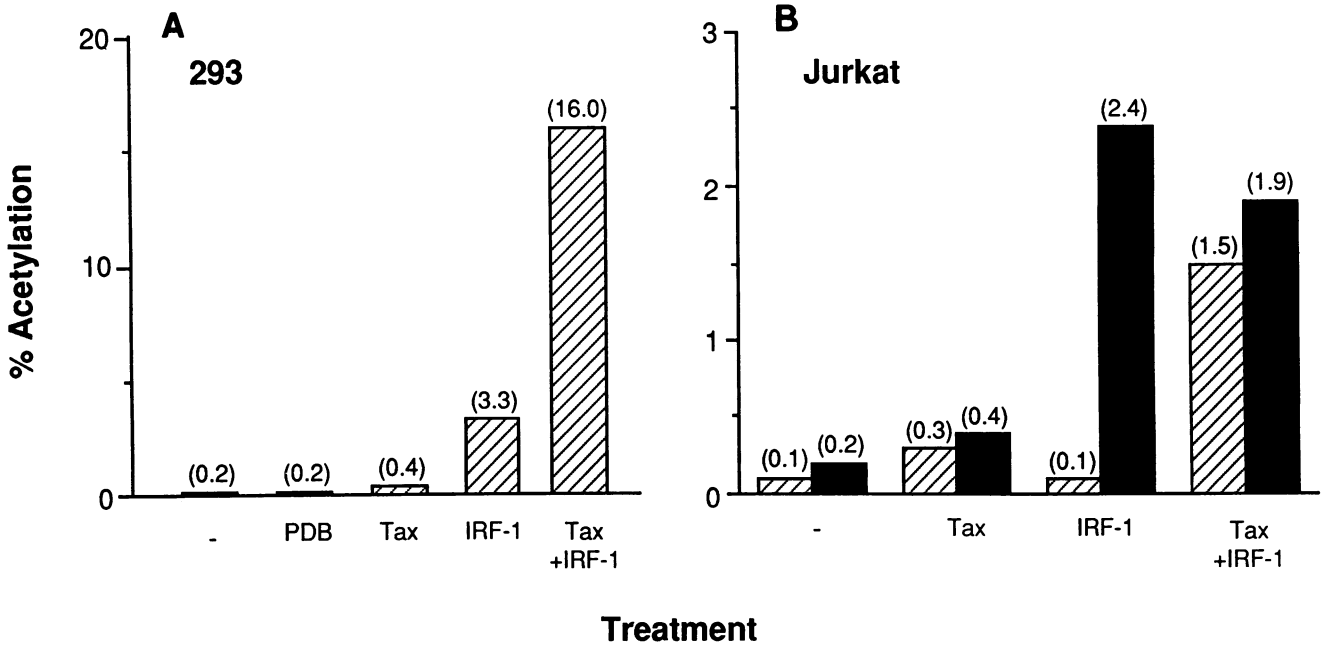

FIG. 5. *trans* activation of the IFN-β promoter by IRF-1 and Tax proteins. Plasmid IFN-β/CAT (5 μg) was transfected into 293 (A) or Jurkat (B) cells. Expression vectors pHIRF31 and pTat-1 were used at 10 and 5 μg, respectively; phorbol dibutyrate (PDB) (25 ng/ml) was added to Jurkat cells 16 h after transfection, and cells were harvested and assayed for CAT activity 40 h after transfection. The different treatments are indicated below the bar graph. Symbols in panel B: ▨, untreated; ■, treated with phorbol dibutyrate.

16, 28, 34, 44, 46). Nuclear protein IRF-1 is one of several proteins inducible by alpha and gamma interferons and virus infection (5, 18, 39). The association of these factors on the DNA template appears to be the necessary and sufficient requirement for significant IFN-β gene activation, whereas activation of either pathway independently is not adequate for activation.

Induction of the IFN-β promoter by Tax and IRF-1 coexpression in 293 cells was still about fourfold lower than was activation by virus, suggesting that other proteins may also be required for optimal IFN-β induction. Proteins such as H2TF1, EBP-1, and PRDII-BF1 are capable of interacting with the PRDII domain (3, 7, 13, 16, 31, 36), while proteins such as IRF-1, IRF-2, PRDI-BFc, PRDI-BFi, and IBP-1 (5, 19, 26, 33, 39) bind to the PRDI domain. In addition, the IEFga and TG proteins have been found to interact with different types of $(GAAANN)_4$ sequences (37). As yet it remains to be determined how these proteins function in vivo to control activation and repression of the IFN-β promoter.

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