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Function of NF-κB/Rel Binding Sites in the Major Histocompatibility Complex Class II Invariant Chain Promoter Is Dependent on Cell-Specific Binding of Different NF-κB/Rel Subunits

ADRIENNE M. BROWN,[1,2]† MICHAEL W. LINHOFF,[1,2] BERND STEIN,[2]‡ KENNETH L. WRIGHT,[2] ALBERT S. BALDWIN, JR.,[2] PATRICIA V. BASTA,[3] AND JENNY P.-Y. TING[1,2]*

Department of Microbiology and Immunology[1] and Lineberger Comprehensive Cancer Center,[2] University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295, and Research Triangle Institute, Research Triangle Park, North Carolina 27709[3]

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The promoter of the human major histocompatibility complex class II-associated invariant-chain gene (Ii) contains two NF-κB/Rel binding sites located at −109 to −118 (Ii κB-1) and −163 to −172 (Ii κB-2) from the transcription start site. We report here that the differential function of each of these NF-κB/Rel sites in several distinct cell types depends on cell-specific binding of NF-κB/Rel transcription factors. Ii κB-1 is a positive regulatory element in B-cell lines and in the Ii-expressing T-cell line, H9, but acts as a negative regulatory element in myelomonocytic and glial cell lines. In vivo protein-DNA contacts are detectable at Ii κB-1 in cell lines in which this site is functional as either a positive or negative regulator. Electrophoretic mobility supershift assays determine that members of the NF-κB/Rel family of transcription factors can bind to this site in vitro and that DNA-binding complexes that contain p50, p52, p65, and cRel correlate with positive regulation whereas the presence of p50 correlates with negative regulation. Ii κB-2 is a site of positive regulation in B-cell lines and a site of negative regulation in H9 T cells, myelomonocytic, and glial cell lines. In vivo occupancy of this site is observed only in the H9 T-cell line. Again, in vitro supershift studies indicate that the presence of p50, p52, p65, and cRel correlates with positive function whereas the presence of only p50 and p52 correlates with negative function. This differential binding of specific NF-κB/Rel subunits is likely to mediate the disparate functions of these two NF-κB/Rel binding sites.

Proper functioning of major histocompatibility complex (MHC) antigens is critical to the immune response. The presence of the MHC class II-associated invariant chain (Ii) is required for the proper structure and function of MHC class II molecules. This was recently illustrated by the analysis of Ii-negative mice produced by gene ablation technology. These mice were deficient in class II function, had altered class II structure, and were deficient in T-cell development (49). The function of Ii is mediated by its binding to MHC class II heterodimers in the endoplasmic reticulum. This blocks peptide binding to class II during transport until the complexes reach endosomes, where Ii dissociates, thereby releasing the heterodimers in the mature and correct conformation for binding antigenic peptides (13). The MHC class II-antigen complex can then be recognized by T-cell receptors which initiate specific helper T-cell functions and cytokine production.

Although the Ii gene and the class II genes are encoded on separate chromosomes and their structures are unrelated, these genes are to a large extent coordinately regulated. They are expressed on the same cell types, generally B lymphocytes and antigen-presenting cells, and they are coordinately induced by cytokines. Coregulation is accomplished through common *cis*-acting DNA sequences in their promoters (11, 17, 19, 52). Specifically, the class II promoters and Ii share three regulatory elements: S (also termed H, W, or Z), X, and Y, which mediate high constitutive expression in B lymphocytes and gamma interferon (IFN-γ)-inducible expression (8, 22). However, in several situations the Ii and class II genes are also differentially regulated (29, 34, 35). A notable instance is that the lymphocytes of patients with a severe immune deficiency, bare lymphocyte syndrome, lack all MHC class II expression but do express Ii (14). The Ii gene contains within its 5′ regulatory region DNA motifs that may regulate Ii expression distinct from the other MHC class II genes. The promoter of the murine Ii gene contains an SP-1-binding site and an NF-κB/Rel site (52). The NF-κB/Rel element in the murine Ii promoter mediates constitutive expression in B cells (52) and mediates the tumor necrosis factor alpha (TNF-α) response in fibroblasts (40). In the human Ii gene, two NF-κB/Rel binding sites at positions −109 to −118 (Ii κB-1) and −172 to −163 (Ii κB-2) from the transcription start site have been noted, but the functional role of these sites remains to be elucidated (Fig. 1) (17).

The NF-κB/Rel family of transcription factors controls the regulation of a variety of cellular and viral genes through NF-κB/Rel DNA-binding site(s) in their promoters (1, 2, 23). Members of this family that have been identified and their genes cloned include the p50 (NFKB1), p52 (NFKB2, p49, or Lyt10), and p65 (RelA) subunits of NF-κB, the proto-oncogene product c-Rel (HIVEN86A), the avian oncogene product v-Rel, and the *Drosophila* morphogen Dorsal (1, 2, 23).

* Corresponding author. Mailing address: Lineberger Comprehensive Cancer Center, CB 7295, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295. Phone: (919) 966-5538. Fax: (919) 966-3015.

† Present address: Molecular Neurobiology Laboratory, The Salk Institute for Biological Sciences, San Diego, CA 92186.

‡ Present address: Signal Pharmaceuticals, San Diego, CA 92121.

NF-κB/Rel family members share a 300-amino-acid sequence homology, termed the Rel domain, in the N-terminal region of the proteins. The Rel domain is required for dimerization, binding to cognate DNA motifs, nuclear localization, and interaction with IκB. NF-κB exists in two forms, either constitutively expressed in the nucleus as in B cells (46) or alternatively located in the cytoplasm in association with an IκB protein. NF-κB translocates to the nucleus upon activation by a number of inducing agents such as phorbol esters, lipopolysaccharides, interleukin-1, TNF-α, calcium ionophores, lectin, antigen, and DNA-damaging agents such as UV light (1). NF-κB expression in the nucleus is regulated by IκB, a family of proteins including IκBα (MAD-3), IκBβ, IκBγ, and the p105 and p100 proteins (the precursors to p50 and p52, respectively). These molecules sequester NF-κB subunits in the cytoplasm and inhibit binding of NF-κB subunits to their cognate DNA-binding sites (9, 38). Induction of NF-κB does not require de novo protein synthesis, is quickly responsive to external signals, and is thus ideal for regulation of genes involved in inflammation and the immune response.

This work shows in vivo genomic footprints of the Ii NF-κB/Rel binding sites in constitutively expressing and IFN-γ-inducible cell lines. Functional analyses of these sites revealed that the Ii NF-κB/Rel sites serve multiple purposes dependent on the cell type. Ii κB-1 is a positive regulatory element in B and T lymphocytes and a negative element in myelomonocytic cells and brain glial cell lines. Ii κB-2 is a positive element in B cells but a negative element in the Ii-expressing T-cell line H9 and brain glial and myelomonocytic cell lines. Nuclear extracts from different cell types formed different complexes with these NF-κB/Rel sites, and these complexes are composed of different NF-κB/Rel transcription factors. The difference in the composition of the Ii κB-1 and κB-2 complexes may explain the opposite function served by these NF-κB/Rel sites in vivo.

MATERIALS AND METHODS

Plasmids. The Ii promoter-CAT plasmid 790IiCAT has been described previously (11), it contains the Ii sequence from positions −790 to +1 cloned immediately upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. Constructs with mutant promoter elements were obtained by oligonucleotide-directed site-specific mutagenesis with a uracil-containing template (32). In plasmid Mut κB-1, the sequence from −109 to −118 bp of the Ii gene was changed from 5′-GGGGTATTTCC-3′ to 5′-TTTTTCTAGAA-3′. In Mut κB-2, the sequence from −172 to −163 bp was changed from 5′-GGGGAGCCCC-3′ to 5′-CCATGGCCCC-3′. The plasmid Ii κB-1m3 mutates Ii κB-1 from 5′GGGGTATT TCC-3′ to 5′-GCACTATTTCC-3′. The plasmids κB-1TKCAT and κB-2TKCAT contain Ii κB-1 and Ii κB-2 sites, respectively, sites inserted between the *Hin*dIII and *Bam*HI restriction enzyme sites upstream of the thymidine kinase (TK) promoter in pBLCAT4 (48). All mutations were confirmed by DNA sequence analysis.

Cell cultures. Raji is an Epstein-Barr virus-transformed human B-lymphoblastoid cell line. H9 (derived from HuT78) is an Ii/MHC class II-positive transformed human T-cell line, and Jurkat is an Ii/MHC class II-negative human T-cell line. U937 is an Ii-positive, class II-negative immature monocytic cell line isolated from a patient with histiocytic lymphoma, and WEHI-3 is a murine myelomonocytic line. All lymphoid and monocytic cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM glutamine. WEHI-3 was additionally supplemented with 5×10^{-5} M β-mercaptoethanol. U-105 MG, previously referred to as U-373 MG in this laboratory, is a human glioblastoma multiforme cell line and was maintained in McCoy's 5A medium–10% fetal bovine serum–2 mM glutamine. Primary cell cultures of rat type I astrocytes were obtained as described previously (44). Briefly, cells were cultured from the cerebral cortex of 1- to 2-day-old neonatal Sprague-Dawley rats. Cells were grown for 2 to 4 weeks, until confluent, and type I astrocytes were purified by a regime of shaking and differential adherence to flasks. Cells were grown in Dulbecco's modified Eagle's medium–10% fetal bovine serum–2 mM glutamine–100 U of penicillin per ml–100 mg of streptomycin per ml. Glioblastoma cells were treated with 500 U of recombinant human IFN-γ per ml, which was generously provided by Biogen, Cambridge, Mass.

Transfection and CAT assay. Transfection of various cell lines was performed by electroporation as previously described (11). Extracts for CAT assays were prepared from the transfected cells, and protein concentrations were determined by the Bradford method (10). Extracts were tested for CAT activity by using [^{3}H]acetyl coenzyme A (37).

In vivo genomic footprinting. The dimethyl sulfate treatment of cell cultures, DNA preparation, and ligation-mediated PCR were performed as described previously (36, 50). The invariant chain-specific primers were as follows (from 5′ to 3′): for the proximal 150 bp, the coding strand (upper strand), primer 1, ATTGTTGGAGATAAGGTCA; primer 2, AT GACTGGCTTCTGATCTTCCCGACAGC; primer 3, ACT GGCTTCTGATCTTCCCGACAGCTCCTG; and the noncoding strand (lower strand), primer 1, GCCCAGAAA CAAGTGATGAG; primer 2, GGATCGTGCTGGCCTTTC TACCTGC; primer 3, GGATCGTGCTGGCCTTTCTACCT GCCTG; for the distal 150 bp, the coding strand (upper strand), primer 1, CTCTTAAAGTCGGTGCTG; primer 2, GCCACTCCGCCCACTTGGTAGATGTG; primer 3, CACTCCGCCCACTTGGTAGATGTGGAAGTG; and the noncoding strand (lower strand), primer 1, TGAGAAGGG GAGACAAAC; primer 2, CAAAGTGCTTTCCTGTCTA GGGAGTGGAC; primer 3, GTGCTTTCCTGTCTAGGG AGTGGACATTTGC.

Nuclear run-on transcription. Nuclear transcription and hybridization were performed as previously described (51). Briefly, the cells were treated with 500 U of recombinant human IFN-γ for 0, 24, 48, and 72 h, harvested, and lysed in high-pH buffer with 0.05% Nonidet P-40. The nuclei were washed and labeled with [^{32}P]UTP (ICN, Boston, Mass.) in a 100-μl reaction for 15 min at 30°C. The labeled nuclei were treated with DNase, extracted with hot (60°C) phenol-chloroform-isoamyl alcohol (1:1:0.01), and concentrated by ethanol precipitation. The labeled RNA was treated with 0.2 M NaOH and hybridized to DNA immobilized on nitrocellulose filters at 40°C. The hybridized material was quantitated with a Molecular Dynamics PhosphorImager.

Nuclear extract preparation and EMSA. The nuclear extracts were prepared by a previously described method (7) with the following modifications. The cell lysis buffer consisted of 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 60 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-*N*′,*N*′-tetraacetic acid (EGTA), and protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl fluoride, pepstatin, and E-64). NP-40 was added to 0.2%, and lysis was monitored by light microscopy. Nuclei were pelleted, washed, and resuspended in an equal volume of nuclear extract buffer (20 mM HEPES [pH 7.9], 60 mM NaCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 25%

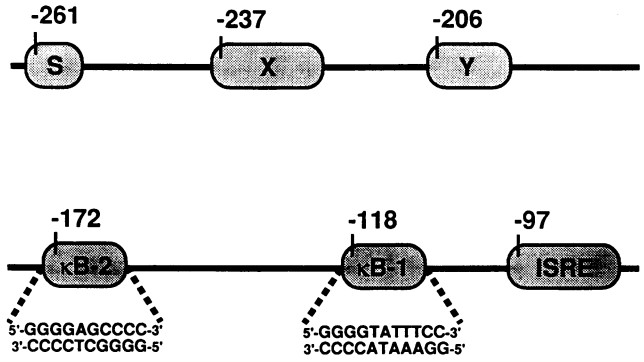

FIG. 1. Ii promoter organization. Schematic diagram of the Ii promoter from −82 to −261 bp shows the Ii regulatory elements (31). The regulatory motifs S, X, Y, κB-1, κB-2, and ISRE are in shaded boxes.

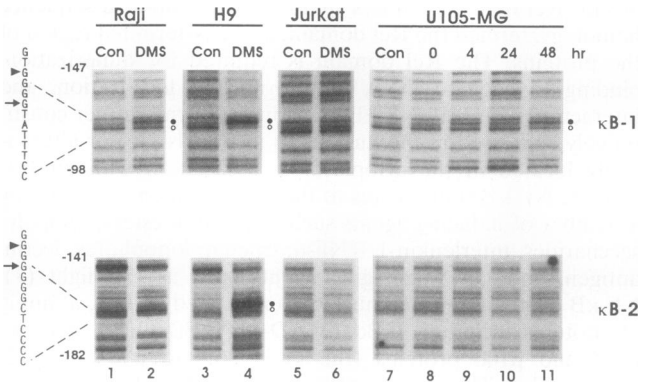

FIG. 2. In vivo genomic footprint of Ii NF-κB/Rel binding sites. Footprint of the Ii κB sites in the region from −98 to −147 (top panel) and −141 to −182 (bottom panel) of the Ii promoter with DNA from Raji (lanes 1 and 2), H9 (lanes 3 and 4), Jurkat (lanes 5 and 6), and IFN-γ-treated U-105 MG at 0, 4, 24, and 48 h (lanes 7 to 11). A footprint of the region surrounding each NF-κB/Rel homolog and the nucleotide sequence of each NF-κB/Rel site are shown. Lanes Con and DMS are DNA methylated in vitro and in vivo, respectively. Open circles mark positions of protections, and solid circles mark positions of enhancements.

glycerol, protease inhibitors). NaCl (5 M) was added to a final concentration of 400 mM NaCl, and the protein concentration was determined (10). The probes were ^{32}P labeled and annealed oligonucleotides that contain the following sequence: 5′-AGCTT**GGGGTATTTCC**AGCCG-3′ (Ii κB-1) and 5′-AGCTTG**GGGGAGCCCC**G-3′ (Ii κB-2) (the binding site is printed in boldface type). Antibodies to NF-κB/Rel proteins were previously described [anti-p65 (7), anti-52 (a gift from M. Karin), and anti-c-Rel (43)]; anti-NF-κB p50 (NL5) was purchased and used as specified by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, Calif.). The bacterially expressed IκBα was the kind gift of Amer Beg. The binding reaction contained 5 to 10 μg of nuclear extract in a buffer consisting of 10 mM Tris (pH 7.6), 75 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 2% Ficoll, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1 μg of poly(dI-dC) per μl. Antibodies and IκB protein were incubated with nuclear extracts for 2 h at 4°C before the addition of the probe. The complexes were resolved on 4 or 5% polyacrylamide gels in Tris-glycine buffer.

RESULTS

Protein-DNA interactions at the Ii NF-κB/Rel sites in vivo. In vivo genomic footprinting is a powerful approach that examines the occupancy of DNA regulatory elements by transcription factors in intact cells. A number of studies have reported that the functional regulatory units in promoters of expressed genes are bound in vivo (5, 16, 27, 33, 36, 50). Our previous analyses by genomic footprinting have revealed that the in vivo protein-DNA interactions and function of the Ii regulatory elements X and Y correlate precisely with the expression of the Ii gene in various cell types (12). We have analyzed in vivo protein binding at two Ii NF-κB/Rel binding sites in B-cell lines, nonexpressing T-cell lines, the Ii-positive T-cell line H9, and IFN-γ-induced and uninduced glioblastoma cells. The NF-κB/Rel site proximal to the Ii transcription start at sequence −109 to −118 is referred to as Ii κB-1, and the distal site at sequence −163 to −172 is referred to as Ii κB-2 (Fig. 1).

Figure 2 shows the results of the in vivo dimethyl sulfate footprinting. Proteins contacted G residues in the Ii κB-1 element in the B-cell line Raji (Fig. 2, top panel, lanes 1 and 2), in H9 T cells (lanes 3 and 4), but not in the Ii-negative Jurkat cells (lanes 5 and 6). Another Ii-positive B-cell line, Namalwa, and another negative T-cell line, HSB, gave identical results to the Raji and Jurkat lines, respectively (data not shown). These observations indicate that the in vivo binding to this site segregates with expression in these cell types and implicates the importance of this element in Ii gene regulation. In addition to the constitutive mode of Ii expression, the occupancy of the Ii κB-1 site was also examined upon induction by IFN-γ in the glioblastoma cell line U-105 MG. Ii in this cell line is inducible to high levels over basal expression by IFN-γ. The Ii κB-1 element was unbound in uninduced U-105 MG glioblastoma cells (Fig. 2, top panel, lane 7). Interestingly, after IFN-γ treatment, a pattern of protections and an enhancement appear over the Ii κB-1 site in the glioblastoma cells (lanes 8 to 11). These bands become most intense by 48 h, whereupon the pattern resembled the pattern observed on Ii κB-1 in Raji and H9 cells.

Ii κB-2 had no detectable protein contacts in Raji cells, Jurkat cells, or U-105 MG cells with or without IFN-γ (Fig. 2, bottom panel). In striking contrast, pronounced binding was detected on Ii κB-2 in H9 cells (lanes 3 and 4). Interestingly, recent findings show in vitro binding to a human immunodeficiency virus κB probe in nuclear extracts from H9 cells (41).

Ii NF-κB/Rel elements have positive or negative regulatory activity depending on the cell type. NF-κB/Rel binding sites control transcription in a number of other immune response genes, and the genomic footprinting results suggest that the Ii NF-κB/Rel sites may have a role in the transcription of the Ii gene. To determine the functional role of Ii κB-1 and Ii κB-2, the two sites were individually mutated in the context of the −790 Ii promoter region (790IiCAT plasmid) and the promoter-CAT constructs were designated Mut κB-1 and Mut κB-2, respectively. The wild-type and mutant plasmids were transfected into cell lines representing various cell types, cell extracts were prepared, and CAT assays were performed. The activity of each mutant was normalized to the wild-type (790IiCAT) construct. Analysis by transfection into Ii-expressing Raji B lymphoblastoid cells was straightforward and showed that mutation of Ii κB-1 resulted in a loss of transcription to 44% of wild-type activity whereas mutation at Ii κB-2 resulted in a loss of 53% of wild-type activity (Fig. 3). Mutation of both sites resulted in 28% of wild-type activity (data not

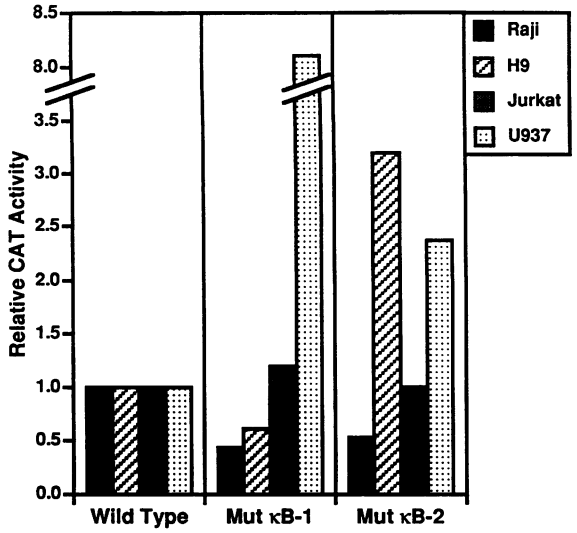

FIG. 3. Ii κB-1 and Ii κB-2 have positive and negative regulatory functions depending on the cell type. Transient-transfection and CAT assays were performed with plasmids containing wild-type Ii promoter and Ii promoter with mutant Ii κB sites in Raji, H9, Jurkat, and U937 cells. Raji, H9, and Jurkat cells were transfected with 5 μg of each plasmid DNA, and U937 cells were transfected with 8 μg of DNA. The mean CAT activity obtained for each mutant ($n > 5$) is normalized to that obtained for the wild-type plasmid (790IiCAT), which is expressed as 100% CAT activity. The mean CAT activity of the wild-type construct in each cell line is as follows: Raji, 130,158 cpm; H9, 222,813 cpm; Jurkat, 26,178 cpm; and U937, 3,591 cpm.

shown). When assayed in Namalwa, another B-lymphoblastoid cell line, similar results were obtained to those obtained with the Raji cell line (data not shown). Interestingly, whereas in vivo occupancy of Ii κB-1 in Raji cells was clearly demonstrated, Ii κB-2 occupancy was not observed (Fig. 2, bottom panel, lanes 1 and 2). It is possible that protein binding occurs at this site but escapes detection because of the nature of the assay or the protein-DNA interaction; this topic is discussed in more detail below (see the discussion of Fig. 8 and 9). These results indicate that in addition to the classical MHC class II promoter elements, S, X, and Y (12), the Ii NF-κB/Rel sites contribute to the high levels of Ii expression in B cells.

Although most T cells do not express the Ii and MHC class II antigens, activated human T cells and the human T-cell line H9 (HUT78) do express these genes. Mutation of Ii κB-1 reduced CAT activity in transfected H9 cells to 61% of control activity (Fig. 3). Similar results were observed when a more conservative Ii κB-1 mutation (κB-1m3; data not shown) was used. Unexpectedly, when Ii κB-2 was analyzed, CAT activity was increased threefold over wild-type levels by mutating this site (Fig. 3). In vivo occupancy of this site was unique to H9 cells (Fig. 2, bottom panel). Thus in the H9 T cells, Ii κB-1 and Ii κB-2 appear to be positive and negative regulatory elements, respectively, and both positive and negative sites are occupied in vivo. Analysis of the Ii-negative T-cell line Jurkat revealed no function for Ii NF-κB/Rel sites in Ii gene promoter regulation since mutation of either site did not significantly change basal levels of expression (Fig. 3). These results agree with the lack of in vivo binding over these sites in Jurkat cells and are expected because NF-κB is cytoplasmic in nonactivated Jurkat cells (1). When these cells are stimulated with phorbol ester, NF-κB activity is detected in the nucleus.

To broadly ascertain the function of the NF-κB/Rel sites, we examined several other cell lines. In the human promonocytic cell line U937 (Fig. 3), the murine myelomonocytic line WEHI-3 (data not shown), and the human cervical carcinoma line HeLa (data not shown), the mutation of the Ii κB-1-binding site resulted in a substantial increase in transcriptional activity and typically increased promoter activity by 8- to 10-fold. In addition, as in the H9 cells, mutation of Ii κB-2 increased the basal levels of expression in U937 cells (Fig. 3). In summary, Ii κB-1 contributes to basal activation in B lymphocytes and H9 T cells and to basal repression in epithelial and monocytic cell types. Ii κB-2 functions as an activator in B cells and as a repressor in H9 cells and U937 cell lines (summarized in Table 1).

The effect of mutation of Ii κB-1 and κB-2 on Ii induction by IFN-γ in glioblastoma U-105 MG cells was also examined. The Ii S, X, Y, and ISRE are required for IFN-γ induction of the Ii gene in U-105 MG cells (4, 11). We were therefore interested in whether the Ii NF-κB/Rel sites play a role in the IFN-γ response. When absolute values were compared, mutation of either Ii κB-1 or Ii κB-2 produced a threefold increase in relative CAT activity for both basal levels and IFN-γ-induced levels (Fig. 4). This increase in Ii gene expression indicates that the Ii NF-κB/Rel binding sites have a negative effect on basal transcription in the glioblastoma cell line. Since there was no significant change in fold induction (Fig. 4), our data demonstrate that the two NF-κB/Rel sites are not necessary for IFN-γ regulation. This is in contrast to the Ii S, X, and Y elements,

TABLE 1. Summary of Ii NF-κB/Rel activities

κB site	Cell line	Presence of antibody supershifted complex[a]				Transcriptional regulation[b]	In vivo occupancy
		α-p65	α-p50	α-p52	α-c-Rel		
κB-1	Raji	+	++	++	++	A	+
	H9	+	+++	++	−	A	+
	U937	−	+	−	−	R	ND[c]
	U105-MG	−	−	−	−	R	−[d]
κB-2	Raji	+	+++	++	++	A	−
	H9	−	+++	++	−	R	+
	U937	−	++	−	−	R	ND
	U105-MG	−	+	−	−	R	−

[a] α indicates "anti."
[b] A, activation; R, repression.
[c] ND, not determined.
[d] In vivo occupancy at this site was observed upon induction with IFN-γ.

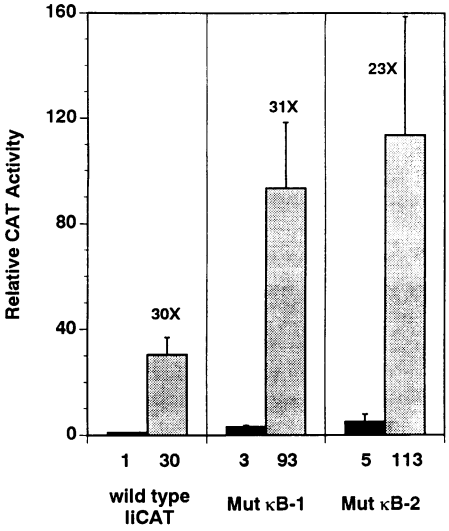

FIG. 4. Effect of mutation of Ii κB-1 and Ii κB-2 on basal and IFN-γ-induced expression in the glioblastoma cell line U-105 MG. Cells were transfected with 5 μg of wild-type or mutant plasmids and treated with IFN-γ or left untreated. CAT activity was determined at 48 h after treatment. The means of the relative CAT activities for each sample are shown below the bars (n = 8). They have been normalized to the basal activity of the wild-type construct (790IiCAT/no IFN-γ equals 1.0). The fold induction for each plasmid is shown above the bar representing the IFN-γ-induced values. The solid and shaded bars represent basal and IFN-γ-induced expression, respectively. The error bars represent standard error of the mean.

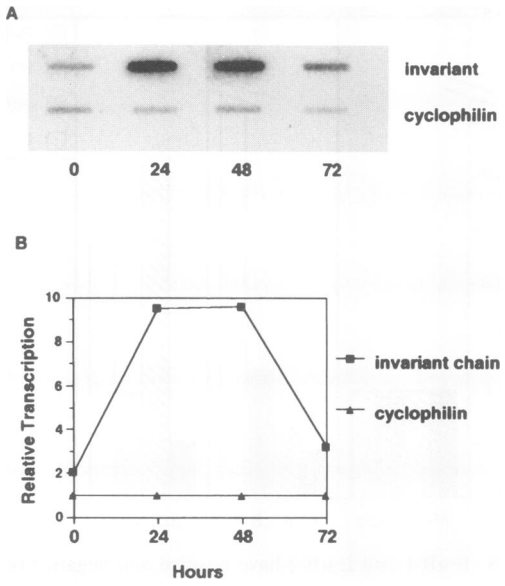

FIG. 5. Rate of transcription of Ii after IFN-γ induction in the glioblastoma cell line, U-105 MG. The glioblastoma cells were treated with IFN-γ for 0, 24, 48, and 72 h. Nascent mRNA was radiolabeled in vivo and hybridized to immobilized Ii or cyclophilin DNA as a control. (A) Results monitored by the PhosphorImager. (B) Values normalized relative to the cyclophilin control and plotted for each time point.

which are all required for IFN-γ-inducible transcription in U-105 MG (11, 12). A similar strong derepression of basal transcription was observed when Mut κB-1 was transfected into CRT cells, a human astrocytoma cell line, and into primary untransformed rat astrocyte cultures (data not shown). These results indicate that the Ii NF-κB/Rel sites also have silencing functions in transformed and primary brain glial cells.

One curious observation is that the Ii κB-1 element is a negative regulatory element in IFN-γ-treated glioblastoma cells (Fig. 4) and, as shown by in vivo footprinting analysis, becomes bound upon IFN-γ induction (Fig. 2, top panel, lanes 7 to 11). This may indicate that the binding of protein(s) at this site is involved in repression during induction. Conceivably this repression occurs after activation because the enhancement and the protections at Ii κB-1 are most intense at 48 h after IFN-γ treatment (lane 11) whereas IFN-γ-inducible binding at X, Y, and ISRE is maximal by 24 h (11). We propose that the timing of this interaction indicates that after transcription is induced, a protein interaction that leads to down-regulation is completed at the Ii κB-1 site. To study this further, we examined the rate of transcription at 0, 24, 48, and 72 h after IFN-γ treatment in a run-on transcription experiment. As seen in Fig. 5, Ii transcription increases up to 24 h, plateaus at 24 to 48 h, and finally returns to near basal levels by 72 h. The timing of down-regulated de novo Ii mRNA synthesis immediately follows the timing of protein binding in vivo at Ii κB-1. Thus changes in in vivo occupancy at this site may be attributed to the binding of repressor protein(s).

To show that the Ii κB-1 mutation did not create a new regulatory site, we tested another mutant plasmid, κB-1m3, in different cell types. κB-1m3 is a more conservative mutation in which described NF-κB contact points (the first three G residues) were mutated (see Materials and Methods). Results of CAT assays in Raji, H9, and U-105 MG cells revealed that κB-1m3 had similar transcriptional activity to that of Mut κB-1 (data not shown). Since two independent mutations of Ii κB-1 resulted in similar changes in Ii basal expression, it is unlikely that Mut κB-1 created another regulatory site for an activator or inhibitor protein.

Ii κB-2 silencer acts in a heterologous promoter, but Ii κB-1 does not. To analyze the two Ii NF-κB/Rel sites in the context of a non-Ii promoter, we made CAT reporter constructs with one of either site inserted upstream of the TK promoter. These reporter plasmids were tested by transfection and CAT assay in

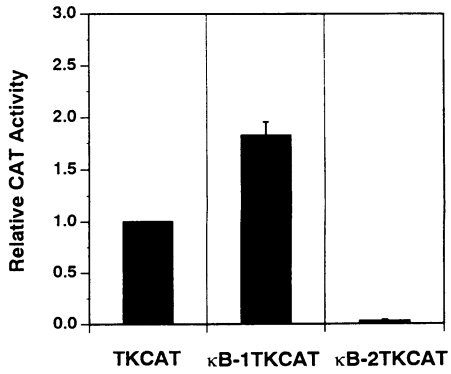

FIG. 6. Effects of Ii κB sites on the TK promoter. U937 cells were transfected with 8 μg of the indicated plasmid. The constructs are the TK promoter upstream of the CAT gene alone (TKCAT), the Ii κB-1 linked to the TKCAT (κB-1TKCAT), and the Ii κB-2 site linked to the TKCAT plasmid (κB-2TKCAT). At 36 h after transfection, the CAT activity was determined. The mean levels of CAT activity of three experiments are shown. The error bars represent standard error of the mean.

U937 cells, in which both sites have a negative effect on Ii gene transcription within the context of its own promoter (Fig. 3). κB-1TKCAT produced slightly increased levels of CAT activity compared with the control TKCAT plasmid (Fig. 6). However, Ii κB-2 exhibited a strong negative effect on the TK promoter, resulting in a complete loss of CAT activity. These experiments suggest that Ii κB-1 may be a position- or context-dependent silencer and that Ii κB-2 is a silencer able to function outside the context of the Ii promoter.

In vitro binding at Ii NF-κB elements and identification of NF-κB/Rel protein subunits. We considered it likely that the positive and negative regulatory effects of the Ii NF-κB/Rel sites in different cell types were due to differential expression or binding of nuclear factors in these cells (39). We examined nuclear extracts from Raji, H9, and U937 cells by EMSA and discovered differences in the protein complexes bound by Ii NF-κB/Rel probes in these cell types. Figure 7 shows protein complexes on Ii κB-1 and Ii κB-2 probes in Raji, H9, and U937 cells; the relatedness of the complexes is demonstrated by competition with unlabeled Ii κB-1 and κB-2 oligonucleotides. Raji nuclear extracts formed four complexes with Ii κB-1 (Fig. 7A, lanes 1 to 3) and three distinct complexes with Ii κB-2 (Fig. 7B, lanes 1 to 3). H9 extracts formed six bands with Ii κB-1 (doublets are resolved upon shorter exposures of the gel; also see Fig. 8B) and two bands with Ii κB-2 (Fig. 7A and B, respectively, lanes 4 to 6). U937 extracts formed two complexes with the Ii κB-1 probe and one complex with the Ii κB-2 probe (Fig. 7A and B, respectively, lanes 7 to 9). All of these complexes were specific as shown by competition with homologous as well as unrelated oligonucleotide sequences. It is striking that each set of protein-DNA complexes is different; although some bands seem to have identical migration, other bands are unique for the corresponding extract. In summary, each Ii NF-κB/Rel site appears to bind a different pattern of complexes in each cell type.

We tested the ability of the Ii κB-1 site to bind cloned recombinant NF-κB proteins that had been expressed in COS cells or in vitro translation systems. The Ii κB-1 probe was able to bind p65 and p50 in the EMSA with the same mobility as that of complexes formed on an HIV-κB probe, whereas extracts containing expression vector alone were not (data not shown). This indicates that Ii κB-1 is able to interact with these combinations if they present. We were also interested in which NF-κB/Rel family members composed the different protein-DNA complexes on activator compared with repressor sites in the various cell lines.

To characterize the factors binding to the Ii NF-κB/Rel sites, we used specific antibodies in EMSA to identify known NF-κB/Rel family proteins (see Table 1 for a summary of the data). A comparison of antibody-treated and untreated reactions with Raji nuclear extracts and Ii κB-1 shows that anti-p65 caused a weak supershifted band (Fig. 8A, lane 3) and addition of the peptide to which the antiserum was made abolished this complex (lane 4). The anti-p65 used in these experiments can effectively shift a TNF-α-induced complex in HeLa nuclear extracts (7), indicating that the weak supershift obtained in these experiments is a reflection of small amounts of p65 as opposed to inadequate recognition of these complexes by the antibody. In addition, anti-p50, anti-p52, and anti-c-Rel all formed specific supershifted complexes (Fig. 8A, lanes 5 to 8). This indicates that Ii κB-1 protein-DNA complexes include all four NF-κB/Rel subunits in Raji cells. A similar analysis of Ii κB-2, the distal site, showed that the same four specific antibodies against p65, p50, p52, and c-Rel reacted with complexes in Raji extracts (Fig. 9A). Anti-p50 eliminated all complexes on this site. In summary, both sites in Raji cells are

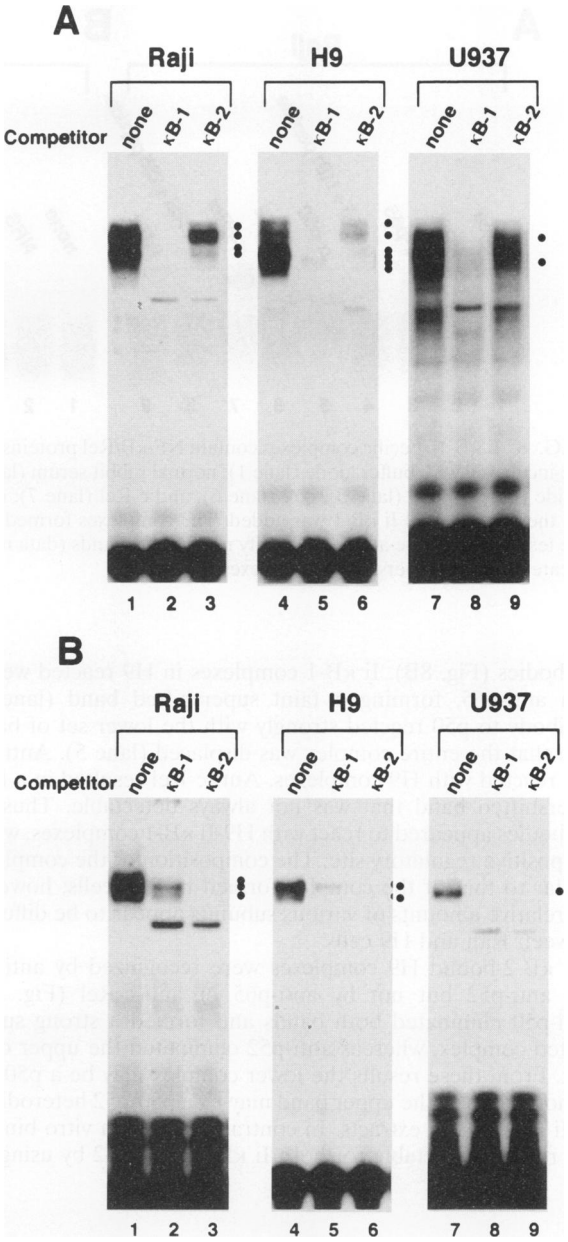

FIG. 7. Ii NF-κB/Rel sites bind complexes in nuclear extracts. Ii κB-1 (A) and Ii κB-2 (B) radiolabeled probes were incubated with nuclear extracts from Raji (lanes 1 to 3), H9 (lanes 4 to 6), and U937 (lanes 7 to 9) cells. Specific complex formation is shown by the addition of competitor oligonucleotides at 100-fold molar excess: Ii κB-1 (lanes 2, 5, and 8) and Ii κB-2 (lanes 3, 6, and 9). Circles indicate specific complexes.

activators and all four NF-κB/Rel subunits were detected in complexes on these sites. It should be noted that binding of NF-κB/Rel factors to Ii κB-1 is significantly more intense than is binding to Ii κB-2. Visible complexes with Ii κB-1 were typically evident after 4 h of exposure, whereas complexes with Ii κB-2 appeared to be significantly weaker and required a threefold longer exposure. This may affect the ability to detect protein-DNA interactions by the genomic footprinting assay (Fig. 2).

Analysis of Ii κB-1 with H9 nuclear extracts resulted in at least six bands which were recognized by NF-κB/Rel-specific

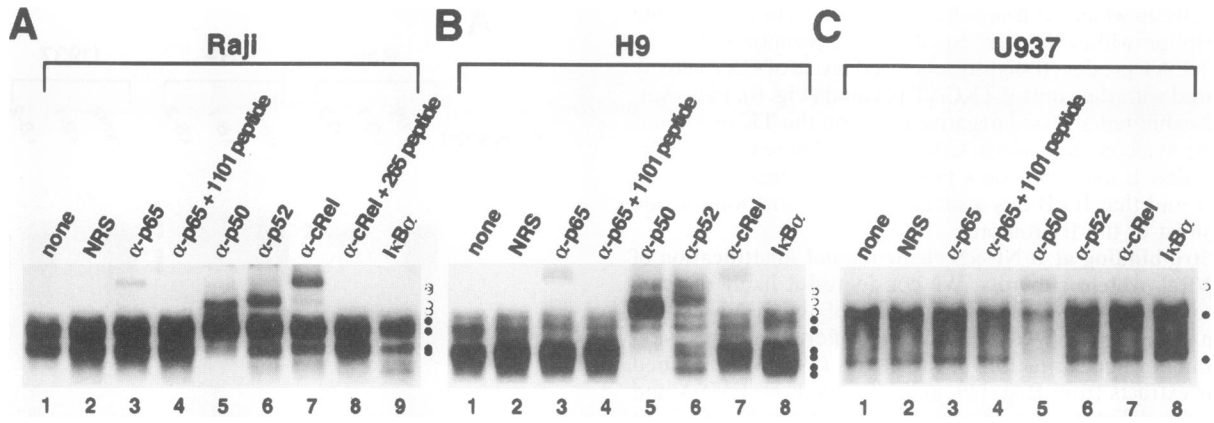

FIG. 8. Ii κB-1-specific complexes contain NF-κB/Rel proteins in different cell lines. Nuclear extracts from Raji (A), H9 (B), and U937 (C) cells were incubated with buffer alone (lane 1); normal rabbit serum (lane 2); specific antibodies against p65 (lane 3), p65 in the presence of competitive peptide (lane 4), p50 (lane 5), p52 (lane 6), and c-Rel (lane 7); or IκBα protein (last lane). These reactions were incubated for 2 h at 4°C, and then the radiolabeled Ii κB-1 was added. The complexes formed were analyzed by EMSA on nondenaturing polyacrylamide gels. All antibodies were tested with probe alone to identify nonspecific bands (data not shown). Solid circles indicate NF-κB/Rel-specific complexes, and open circles indicate antibody supershifted complexes.

antibodies (Fig. 8B). Ii κB-1 complexes in H9 reacted weakly with anti-p65, forming a faint supershifted band (lane 3). Antibody to p50 reacted strongly with the lower set of bands such that the entire complex was displaced (lane 5). Anti-p52 also reacted with H9 complexes. Anti-c-Rel resulted in a faint supershifted band that was not always detectable. Thus, all antibodies appeared to react with H9-Ii κB-1 complexes, which is a positive regulatory site. The composition of the complex is similar to that of the complex formed in Raji cells; however, the relative amounts of various subunits appear to be different between Raji and H9 cells.

Ii κB-2-bound H9 complexes were recognized by anti-p50 and anti-p52 but not by anti-p65 or anti-c-Rel (Fig. 9B). Anti-p50 eliminated both bands and formed a strong supershifted complex, whereas anti-p52 eliminated the upper complex. From these results the lower complex may be a p50-p50 homodimer and the upper band may be a p50-p52 heterodimer on Ii κB-1 in H9 extracts. In contrast to Raji, in vitro binding was readily detectable for both Ii κB-1 and κB-2 by using H9 nuclear extracts, which correlates well with the in vivo occupancy of both sites in H9 cells.

Complexes in U937 were recognized only by the anti-p50 antibody of the four antisera tested (Fig. 8C and 9C). On Ii κB-1 the lower complex was eliminated by anti-p50 while the upper complex was weakly reactive (Fig. 8C). Ii κB-2 bound one complex in U937 cells. This complex was completely eliminated by anti-p50 and was not affected by any other antibodies tested (Fig. 9C). This is similar to findings in a previous study, in which the IL2-Rα enhancer NF-κB/Rel site bound p50 in U937 extracts (28). Nuclear extracts from uninduced and IFN-γ-induced U-105 MG cells were also tested for Ii κB-binding activity. Specific DNA-protein complexes formed in the presence of Ii κB-1 and did not change upon IFN-γ treatment (data not shown). The antibodies used in these experiments were unable to shift any of these complexes. Weak binding to the Ii κB-2 probe was observed for the U-105 MG extracts, requiring 10 times the exposures needed

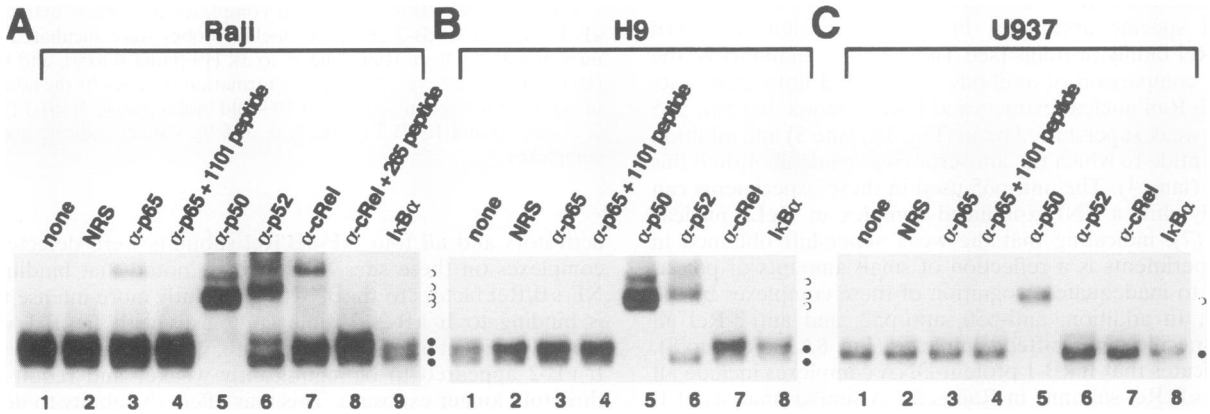

FIG. 9. Ii κB-2-specific complexes contain NF-κB/Rel proteins in different cell lines. Nuclear extracts from Raji (A), H9 (B), and U937 (C) cells were incubated as in Fig. 8 with specific antibodies, controls, or IκBα protein as indicated and reacted with radiolabeled Ii κB-2. Complexes were analyzed by EMSA as described in the text. Solid circles indicate NF-κB/Rel-specific complexes, and open circles indicate antibody supershifted complexes.

for Ii κB-1. Anti-p50 was able to supershift this complex (data not shown).

We also investigated the nature of the DNA-protein complexes by adding purified IκBα (MAD-3) protein to in vitro binding reactions. The addition of IκBα only weakly inhibited the formation of some complexes when Raji nuclear extracts were used; however, other bands remained (Fig. 8A and 9A, last lane of the respective panels). The weak inhibitory effects of IκBα on complex formation suggests the presence of small amounts of NF-κB in these complexes, and the inability to abolish these complexes suggests the presence of small amounts of p65 and c-Rel in these extracts, since it has been observed that IκBα can associate with and prevent DNA binding of complexes containing p65 and c-Rel (6).

Taken together, these experiments show that the Ii NF-κB/Rel sites bind NF-κB/Rel subunits in nuclear extracts. A summary of the data is shown in Table 1. It appears that in cell lines in which Ii NF-κB/Rel sites have a negative regulatory function the complexes formed were recognized only by antisera against p50 and p52, whereas in cell lines in which Ii NF-κB/Rel sites have a positive regulatory function the complexes contained detectable amounts of p65 and c-Rel in addition to p50 and p52. Some DNA-binding complexes were not affected by antibody and may represent unidentified proteins. However, we cannot exclude the possibility that some proteins, when contained in certain complexes, cannot be recognized by these antibodies.

DISCUSSION

In this study we show for the first time the in vivo genomic footprint of two NF-κB/Rel sites present in the human Ii promoter and demonstrate that they have contrasting regulatory functions in different cell types. We also present extensive analysis of the NF-κB/Rel complexes formed on these sites in different cell types. Ii κB-1 is a positive regulatory element in Ii-expressing B- and T-lymphoid cell lines and a negative element in myelomonocytic cell lines, HeLa cells, glioblastoma cells, and primary brain glial cells. Ii κB-2 is a positive regulatory element in B-cell lines and a negative element in H9 cells, myelomonocytic cell lines, and glioblastoma cells. In vivo footprint analysis revealed binding to proteins at NF-κB/Rel sites with both positive and negative activity. In addition, in vitro binding studies revealed that both Ii NF-κB/Rel sites were bound by NF-κB/Rel-containing complexes in nuclear extracts from all expressing cells. However, different NF-κB/Rel subunits bound positive sites compared with those that bound negative regulatory sites. Collectively these results indicate that the function of Ii NF-κB/Rel elements appears to correlate with the binding of different known NF-κB/Rel family members in a cell-type-dependent fashion.

NF-κB/Rel sites are important *cis*-acting elements that regulate the expression of a variety of inducible, tissue-restricted, and viral genes (1). The murine Ii NF-κB/Rel site has previously been shown to be a B-cell-specific activator as well as a TNF-α-responsive element (40, 52). The murine Ii NF-κB/Rel binding site and human Ii κB-1 are nearly identical, having only a one-nucleotide difference. This study presents observations on the cell-type-specific regulation of the human Ii gene and shows that Ii NF-κB/Rel elements have positive and negative regulatory functions that are cell type dependent.

Ii κB-2 dramatically repressed a heterologous promoter when inserted upstream of the TK promoter in TKCAT plasmids in U937, a promonocytic cell line. Ii κB-1 did not repress the heterologous TK promoter. A likely explanation for these results is that the mechanism of repression by Ii κB-1 may be dependent on the context of the Ii promoter. Interestingly, the function of some NF-κB/Rel sites requires the presence of a separate element that acts from a distance. This mechanism has been described for a Dorsal binding site (24, 25) and for an immunoglobulin Ig κB site (42). It is possible that Ii κB-1 functions in this way since it is position dependent; however, Ii κB-2 clearly does not have these restrictions.

In vivo protein-DNA interactions and gene expression tend to correlate, as demonstrated in studies of the MHC class I and class II, invariant-chain, tyrosine aminotransferase, muscle creatine kinase, mouse metallothionein I, and mouse transthyretin genes (5, 16, 27, 33, 36, 50). This correlation is supported by our observations of in vivo interactions on the Ii κB-1 element in expressing B- and T-lymphocytic cell lines and the glioblastoma cell line and the strong interaction on Ii κB-2 in H9 cells. In some cases an observed in vivo protection does not correlate with induced expression of the gene, as noted on the serum response element of c-*fos* during induced expression of the gene (15) and the AP-1 site of the collagenase gene (30). In other situations interactions are not observed on known transcriptional elements such as the S element of MHC class II and Ii genes (12, 28). In vivo binding was also not observed with Ii κB-2 in B cells and the glioblastoma cell line, where the presence of NF-κB/Rel proteins in these extracts was demonstrated by in vitro EMSA (Fig. 2 and 9; also data not shown). The absence of in vivo interactions on known regulatory sites may be attributed to technical limitations of the assay or to the inherent low affinity of protein-DNA interactions at this site. The latter is probably the case because in vitro binding of Ii κB-2 is significantly weaker than at κB-1 and requires 3- and 10-fold longer exposures to rival the intensity of Ii κB-1 binding for Raji and U-105 MG nuclear extracts, respectively.

An intriguing observation is the induction of in vivo protections at the negative regulatory element, Ii κB-1, upon IFN-γ treatment of the glioblastoma cells. The timing of protein binding to Ii κB-1 is such that maximal binding is delayed until 48 h, which coincides with the decrease in transcription detected after this at 48 to 72 h in the run-on experiments. During IFN-γ induction the endogenous promoter becomes available for factor binding and becomes bound at an array of sites including Ii X, Y, and ISRE at 24 h after treatment. These results suggest that occupancy of these positive and negative sites during IFN-γ induction may be sequential, with binding of the positive sites occurring first and binding of the negative site delayed. Therefore, the occupation of Ii κB-1 may lead to subsequent down-regulation of the Ii gene. Another instance of a repressor NF-κB/Rel site which is bound in vivo is the Ii κB-2 site, which has clear protein-DNA interactions in H9 cells (Fig. 2, bottom panel). Thus, NF-κB/Rel binding to a target site can lead to either enhanced or repressed gene expression.

The detection of in vivo interactions on Ii NF-κB/Rel sites that have a negative regulatory role suggests that these elements are bound by proteins that mediate repression. We extensively analyzed complexes formed on Ii NF-κB/Rel sites in in vitro binding studies with nuclear extracts from different cell types to assess whether a specific pattern of protein-DNA interactions was associated with a positive or a negative regulatory function (Fig. 7 to 9; results are summarized in Table 1). In cells in which the Ii NF-κB/Rel sites were activators, the sites are bound by p50, p52, p65, and c-Rel. The binding of p65 and c-Rel was unique to activator κB sites and was not observed on the negative sites. Importantly, p65 has been shown to be a strong transactivator through other κB sites (3, 45, 47). c-Rel has been shown to have an activation domain but functions only weakly through an NF-κB site. The negative Ii κB sites bound proteins identified as p50 and p52, which

correlates with previously published observations. In cotransfection experiments, p50 overexpression resulted in repression of p65 transactivation (through a promoter containing two κB sites linked to a truncated c-*fos* promoter) (45) and repression of an interleukin-2 promoter in EL-4 cells (26). In addition, p50 inhibited induction by TPA (12-*O*-tetradecanoylphorbol-13-acetate) activation through human immunodeficiency virus κB sites, which was reversed by an IκB, Bcl-3 (20). From previous studies, p50 homodimers have negative regulatory roles on known NF-κB/Rel sites (26) whereas on other sites they appear to be transactivators (21). This correlates with our results, which show that p50 is present in complexes on repressor sites in H9 and U937 nuclear extracts as well as on activator sites (Table 1). Although the role of p52 in transcription is less clear, it has been shown to transactivate in the presence of p65 (18, 39). On the Ii NF-κB/Rel sites, p52 is involved in complexes with different functions depending on the cell type. Some complexes were not affected by antibody in these assays, perhaps owing to different protein conformations in complexes with different components. Another possibility is that an undetected or unknown factor(s) is involved in these complexes. Such a factor(s) may determine the cell-type-specific function of the site.

In conclusion, this work demonstrates that the role of Ii κB-1 and κB-2 regulatory elements is cell dependent and probably due to binding of disparate NF-κB/Rel factors in different cell types. In vivo footprinting detected binding of both activator and repressor sites. Activator functions correlated with the presence of binding activity identified as p65, p50, p52, and c-Rel in lymphocytic cell lines. Repressor functions correlated with the presence of only p50 and p52 binding activity in H9 T cells and U937 promonocytic cells. These proteins are involved in the context of a complicated promoter, which differentially regulates Ii expression in a variety of cell types.

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