MOLECULAR AND CELLULAR BIOLOGY, Jan. 1995, p. 152–164 Vol. 15, No. 1
0270-7306/95/$04.00+0

Identification of the rel Family Members Required for Virus Induction of the Human Beta Interferon Gene

DIMITRIS THANOS† AND TOM MANIATIS*

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

Received 15 July 1994/Returned for modification 13 September 1994/Accepted 22 September 1994

We have carried out experiments to determine which members of the rel family of transcription factors are involved in virus induction of the beta interferon (IFN-β) gene. First, we examined the inducibility of artificial DNA binding sites that preferentially interact with different homo- or heterodimeric combinations of rel proteins in vitro. We found that only those sites capable of binding the p50/p65 heterodimer are virus inducible. Second, we analyzed a series of mutant rel DNA-binding sites in the context of the intact IFN-β promoter. We found a correlation between (i) sites capable of binding both the p50/p65 heterodimer and the high-mobility-group protein HMG I(Y) and (ii) virus inducibility. Third, cotransfection of the IFN-β gene enhancer/promoter with plasmids capable of expressing several different rel proteins revealed that only the combination of p50 and p65 efficiently activated transcription. Finally, we have used antibodies directed against different rel proteins to show that virus-inducible protein-DNA complexes assembled on the IFN-β enhancer in vitro contain both p50 and p65. We conclude that the p50/p65 heterodimer is responsible for the NF-κB-dependent activation of the IFN-β gene promoter in response to virus infection.

The human beta interferon (IFN-β) gene is transiently induced by virus infection or treatment of cells with synthetic double-stranded RNA (dsRNA) (for a review, see reference 10). The DNA sequences required for activation and postinduction repression have been localized to the first 104 bp immediately upstream from the start site of transcription. This region, which contains four positive regulatory domains (PRDI to PRDIV), can function as a virus-inducible transcriptional enhancer (reviewed in reference 34). Although none of these regulatory elements function in isolation, two or more copies of each element or pairwise combinations of two elements are capable of conferring virus inducibility on a heterologous promoter (34). Additional studies have shown that virus induction requires combinatorial interactions between transcription factors that specifically bind to the four positive regulatory domains of IFN-β gene promoter (12, 57).

PRDII-dependent virus induction requires both the transcription factor NF-κB and the high-mobility-group protein HMG I(Y) (60). NF-κB recognizes the G/C-rich sequences at each end of PRDII through contacts in the major groove, while HMG I(Y) interacts with the central A/T-rich region of PRDII, contacting DNA in the minor groove (60). Mutations in PRDII that decrease the affinity of either NF-κB or HMG I(Y) result in a substantial decrease in the level of virus induction in vivo. Moreover, antisense RNA for p65, p50, or HMG I(Y) separately inhibits virus induction from PRDII (60).

In uninfected cells, NF-κB is sequestered in the cytoplasm by at least two mechanisms. First, NF-κB is bound to an inhibitory protein called IκB-α (for reviews, see references 1 and 20). Virus infection or treatment of cells with dsRNA leads to the release of IκB-α from NF-κB, resulting in the translocation of NF-κB to the nucleus, where it binds to the PRDII element and activates transcription (16, 22, 31, 63). Second, the p65 subunit of NF-κB is bound to the p105 precursor of p50, and this heterodimer remains in the cytoplasm. Induction leads to the processing of p105 to p50, generating active NF-κB (reviewed in reference 5).

Although NF-κB was initially characterized as a heterodimeric complex consisting of a p50 and a p65 subunit, the subsequent cloning of several other related proteins led to the discovery of the rel family of transcription factors. The members of this family have an extensive region of sequence similarity with the product of the proto-oncogene c-*rel* (rel homology domain). One class of proteins in this family includes p50 (7, 19, 26, 37) and the related p52 (6, 35, 40, 50) protein, both of which are generated by proteolytic processing of larger precursor proteins (13, 36).

The other class of rel proteins includes c-rel (8), p65 (RelA) (3, 41, 46), RelB (47, 49), the *Drosophila* morphogen dorsal (55), and the dorsal related immunity factor dif (23). This class of rel proteins contains transcriptional activation domains at their carboxyl termini, and they are not generated by protein processing. Members of both classes of rel proteins can form homodimers or heterodimers with members of the same or different class of rel proteins through amino acid sequences in the rel homology domain.

Regulation of transcription by rel proteins presents an interesting problem in subunit mixing and DNA recognition. A number of observations suggest that specific combinations of rel proteins regulate transcription from several distinct, naturally occurring binding sites (κB motifs) (44). These studies suggested that the DNA sequence of the binding site determines which combination of rel proteins binds and activates transcription. In addition, conformational changes in the rel protein complexes may be induced by the DNA binding site (17).

Our in vitro binding studies with recombinant proteins have shown that all members of the rel family of transcription factors can bind to the PRDII element of the human IFN-β gene promoter either as homodimers or as various heterodimeric combinations. This observation raises the interesting question of whether all of these forms of rel proteins can activate transcription from PRDII or whether binding is necessary but not sufficient for activation. In this study, we have addressed this

* Corresponding author. Mailing address: Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Ave., Cambridge, MA 02138. Phone: (617) 495-1811. Fax: (617) 495-3537.

† Present address: Department of Biochemistry and Molecular Biophysics, Columbia University, New York 10032.

question by using three different approaches. First, we generated artificial NF-κB binding sites that preferentially bind p50 or p65 homodimers or the p50/p65 heterodimer. When these sites were tested in multiple copies in synthetic promoters, we found that only those capable of binding the p50/p65 heterodimer can function as virus-inducible enhancers. Furthermore, site-directed mutagenesis of the PRDII element in the context of the intact IFN-β promoter revealed that binding of both the p50/p65 heterodimer and HMG I(Y) is required for virus induction. Second, when the whole panel of rel proteins was tested in cotransfection experiments, we found that only the p50/p65 heterodimer efficiently activates the PRDII element. Finally, analysis of the composition of virus-inducible protein-DNA complexes assembled on PRDII reveal that they are mainly composed of a p50/p65 heterodimer. Taken together, these results indicate that the PRDII element of the IFN-β gene promoter is activated by the p50/p65 heterodimer of NF-κB.

MATERIALS AND METHODS

Cell culture and transfection. HeLa or MG63 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, antibiotics, and L-glutamine (2 mM) in a humidified incubator containing 5% CO_2. Transient transfection, virus induction, and chloramphenicol acetyltransferase (CAT) assays were performed essentially as described before (60). Cells were induced with phorbol myristate acetate (PMA; Sigma) at 100 ng/ml and with tumor necrosis factor alpha (TNF-α; Boehringer) at 1,000 U/ml. CAT activity was normalized for transfection efficiency by using a CMVβ-gal expression vector (33) in all transfection experiments.

Plasmid constructions. All oligonucleotides were synthesized with *Bam*HI ends, phosphorylated, annealed, and cloned at the *Bam*HI site of the −40IFN-βCAT vector (60). Clones containing one or two tandem copies were selected, sequenced, and used in transfection experiments.

The expression vectors for p50, p65, p52, and RelB have been described before (11, 15, 60). A cDNA encoding human c-rel was cloned at the *Eco*RI site of the mammalian expression vector pcDNAI (Invitrogen). Site-directed mutagenesis in the IFN-β promoter was performed as described before (60), using the degenerate PRDII oligonucleotide 5′ GG(A/G)A(A/T/C)(A/T/G)T(C/T)CC 3′.

Electrophoretic mobility shift assay (EMSA) and extract preparation. Bacterially expressed and purified p50, p65, and HMG I(Y) proteins are as described before (60). To construct the c-rel expression plasmid, the entire open reading frame of c-rel was cloned in the bacterial expression vector pRSETA (Invitrogen) in frame with the 6-His moiety. c-rel protein was prepared and purified as described previously (60). Binding reaction mixtures with purified proteins contained 0.1 to 0.5 ng of probe (50,000 cpm), 10 mM Tris (pH 8), 50 mM NaCl, 1 mM EDTA, 1 mg of bovine serum albumin (BSA) per ml, and 0.5% Nonidet P-40. Protein-DNA complexes were resolved on 5% polyacrylamide (30:1)–0.5× Tris-borate-EDTA gels. Bound and free probes were quantitated with a PhosphorImager.

Whole cell extracts from MG63 cells were prepared as follows. Briefly, after collection and washing of the cells in phosphate-buffered saline, the cell pellet was resuspended in lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 300 mM KCl, 0.2 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 15% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of pepstatin and leupeptin per ml) at 400 μl per 150-mm-diameter plate. The cells were lysed by three rounds of freeze-thawing. Insoluble material was removed by centrifugation at 14,000 rpm for 10 min. The supernatant was adjusted to 1 mg of total protein per ml and used for EMSA.

Binding reactions with whole cell extracts (5 μg) were carried out on ice at 20-μl final volume consisting of 10 mM Tris (pH 8), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly(dI-dC) · poly(dI-dC), and 20,000 cpm of PRDII oligonucleotide as the probe. Antisera were added 10 min after the addition of probe (postincubation) and were allowed to interact with the DNA-protein complexes for 1 h. The binding mixture was then loaded on a polyacrylamide gel. For preincubation experiments, antisera were incubated with the extract in binding buffer for 1 h, after which the probe was added for another hour.

DNase I footprinting and methylation interference. Methylation interference was performed exactly as described previously (60). For the DNase I footprinting experiment, IFN-β promoter fragments (20,000 cpm) from −110 to +20 were end labeled at the noncoding strand and incubated with the indicated proteins in 20 μl of binding buffer consisting of 10 mM Tris (pH 8), 15 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mg of BSA per ml on ice for 30 min. DNase I (Worthington) was diluted in 50 mM $CaCl_2$–20 mM HEPES (pH 7.9) to 2 ng/μl, and 2 μl was added directly to the binding reaction mixture. Digestion was allowed to proceed for 5 min on ice, and then 200 μl of stop buffer (2.5 M ammonium acetate, 25 μg of sonicated salmon sperm DNA per ml) was added. The DNA was ethanol precipitated, dried, resuspended in 3 μl of formamide dye, and loaded on a 6% sequencing gel.

RESULTS

High-affinity binding sites for p50 and p65 homodimers. NF-κB binds to a number of different DNA recognition sequences (1, 20), and previous studies suggested that the p50 and p65 subunits of NF-κB contact these binding sites asymmetrically. Specifically, the p50 subunit preferentially interacts with the more conserved 5′ half site, while the p65 subunit interacts with the more divergent 3′ half site (62). To obtain direct evidence for differential recognition of natural NF-κB sites by p50 and p65, we performed methylation interference experiments by using oligonucleotides containing the immunoglobulin κ light-chain (IgκB) or the IFN-β (PRDII) site with bacterially produced and purified p50 and p65 homodimers. The results are shown in Fig. 1A and summarized in Fig. 1B. Purified p50 homodimers make specific contacts with the 5′ half sites of both IgκB and PRDII oligonucleotides, since methylation of the G residues at positions 1, 2, and 3 strongly interferes with p50 binding to both sites. Surprisingly, p50 homodimers do not specifically contact the 3′ half site of either binding site. The methylation interference pattern of purified p65 is clearly distinct from that of p50, since bases in the 3′ half site are required for binding (Fig. 1). Overall, the p65 methylation interference pattern is more extensive than that of p50, since p65 also contacts most of the nucleotides within the 5′ half site, especially with PRDII. The methylation interference pattern of the p50/p65 heterodimer appears to be a simple combination of the individual p50- and p65-specific contacts (31) (data not shown).

To generate high-affinity binding sites for either p50 or p65, we synthesized decameric oligonucleotides containing the major contact points for these proteins in reverse orientation. Thus, the IgκB55 (GGGACGTCCC) and IgκB33 (GGAAA TTTCC) oligonucleotides are perfectly symmetric 5′ and 3′ half sites of the IgκB sequence and should preferentially interact with p50 and p65, respectively. Similarly, oligonucleotides PRDII55 (GGGAATTCCC) and PRDII33 (GGAAT ATTCC) represent the palindromic half sites for the PRDII element. To verify that these artificial sites preferentially interact with p50 or p65, we used an EMSA to determine the ability of each of these oligonucleotides to compete for the binding of p50 or p65 homodimers, or the p50/p65 heterodimer, to an oligonucleotide containing the PRDII site. Consistent with our methylation interference results, p50 interacts with the IgκB55 oligonucleotide better than with the IgκB33 sequence (Fig. 2A). The affinity of p50 homodimers is 10-fold higher for the artificial IgκB55 than for the natural IgκB site. Furthermore, p50 binds only weakly to the IgκB33 oligonucleotide (Fig. 2A) (65). In contrast, p65 binds with high affinity to the IgκB33 oligonucleotide and relatively weakly to the IgκB55 site (Fig. 2B). Finally, the affinity of p65 homodimers is higher for the IgκB33 than for the natural IgκB site.

In summary, the 5′ half palindromic site IgκB55 binds preferentially to p50 homodimers, while the 3′ half palindromic site IgκB33 interacts preferentially with p65 homodimers. It is important to note that both IgκB55 and IgκB33 conform to a recently derived consensus for p50 and p65 homodimers, respectively (28). Both artificial sites represent high-affinity sites for the respective proteins. Similar competition experiments using both half palindromic sites from PRDII revealed that the 5′ site PRDII55 interacts strongly with both p50 and p65 homodimers. Surprisingly, the 3′ site PRDII33 is a low-affinity site for both p50 and p65 homodimers (Fig. 2A and B). The

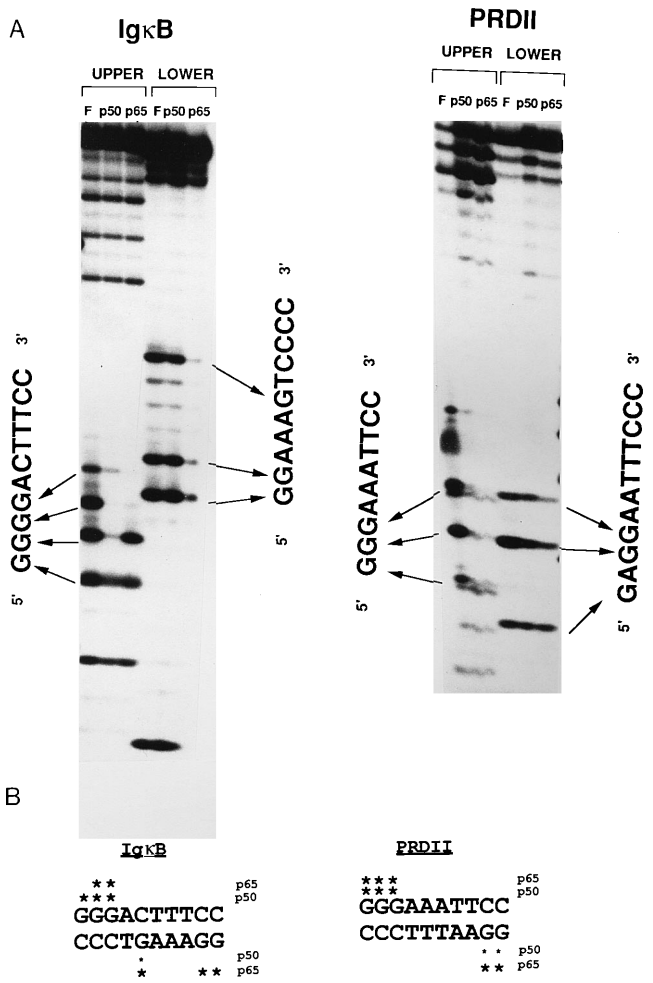

FIG. 1. Methylation interference studies of the IgκB and PRDII elements. (A) The p50 and p65 subunits of NF-κB asymmetrically contact natural κB elements. The IgκB and the PRDII oligonucleotides were end labeled with [γ-^{32}P]ATP in either the upper or lower strand and then partially methylated with dimethyl sulfate. The modified oligonucleotides were incubated with p50 or p65 homodimers, and the bound and free DNAs were separated by EMSA, purified, cleaved, and analyzed on a 15% denaturing polyacrylamide gel. The free DNA is indicated by F above the corresponding lanes and is compared with the pattern of cleavage of p50 and p65 homodimer-bound oligonucleotides. The sequence of each strand is shown beside of the gels; the arrows correspond to the G residues of the κB motifs. (B) Summary of the methylation interference data shown in panel A for both PRDII and IgκB with the p50 and p65 proteins. The large asterisks indicate almost complete interference; the small asterisks indicate partial interference.

observation that these artificial sites can specifically interact with either p50, p65, or both raises interesting questions regarding the specificity of the NF-κB activity. For example, is the binding of both subunits required for high-affinity interaction of NF-κB with its cognate recognition sequence?

To determine whether these artificial sites can also be recognized by the p50/p65 heterodimer, we performed competition binding experiments using in vitro reconstituted p50/p65 heterodimer (60). In agreement with our previous results (60), the p50/65 heterodimer binds with higher affinity to the IgκB site than to the PRDII site (Fig. 2C). Surprisingly, the high-affinity binding sites for p50 and p65 homodimers, IgκB55 and IgκB33, respectively, are very weak competitors for the p50/p65 heterodimer (Fig. 2C). In contrast, the PRDII55 site, which interacts with both p50 and p65 homodimers, competes very efficiently for binding of the p50/p65 heterodimer. Interestingly, the PRDII55 site (GGGAATTCCC) is identical to the palindromic NF-κB site (PD) used by others as a high-affinity NF-κB site (4). As expected, the PRDII33 site does not bind the p50/p65 heterodimer (Fig. 2C). These results suggest that both subunits of NF-κB must contact DNA for high-affinity binding of the heterodimer. This conclusion is further supported by the methylation interference pattern of NF-κB (p50/p65 heterodimer), which is identical to the combination of the pattern obtained with the individual p50 and p65 homodimers.

In vivo activity of the p50- and p65-specific DNA binding sites. To examine the activities of the NF-κB artificial binding sites in vivo, two copies of each oligonucleotide were separately cloned immediately upstream of the IFN-β gene promoter TATA box at −40 from the start point of transcription. This artificial promoter was in turn fused to the bacterial CAT gene. These reporter constructs were transfected into HeLa cells, and CAT activity was determined from extracts prepared from either uninduced cells, cells infected with virus, or cells treated with PMA or TNF-α. The results obtained in a representative experiment are shown in Fig. 3. The natural PRDII and IgκB sites confer significant activation (about 10-fold) on the reporter plasmid in response to all of the NF-κB inducers tested (Fig. 3, lines 7 and 8). Significantly, reporter constructs containing the p50- and the p65-specific sites (IgκB55 and IgκB33, respectively) did not respond to NF-κB activation (Fig. 3, lines 3 and 4). By contrast, the PRDII55 site, which binds both p50 and p65, and therefore the p50/p65 heterodimer, is activated by all of these NF-κB inducers (Fig. 3, line 6). These results show that NF-κB sites that interact preferentially with p50 or p65 homodimers do not function in vivo as inducible enhancer elements. Thus, the binding of both subunits to these sites is required for NF-κB activity in vivo, at least when tested in a minimal promoter context.

To test the possibility that p50 and p65 homodimers can cooperate to activate transcription when bound to adjacent sites, we synthesized oligonucleotides containing a p50-specific site immediately adjacent to a p65-specific site. These oligonucleotides were used in EMSA experiments with recombinant p50 and p65 homodimers as well as with the reconstituted p50/p65 heterodimer. As expected, both p50 and p65 homodimers bind efficiently to this oligonucleotide (Fig. 4, lanes 4 and 6, respectively). By contrast, the p50/p65 heterodimer failed to interact with this oligonucleotide (Fig. 4, lane 5). When the in vivo activities of these binding sites were tested in the minimal promoter context, we found that both the p50- and p65-specific sites (κB55-κB33) in one or two copies did not respond to any of the NF-κB inducers tested (Fig. 3, lines 1 and 2). Thus, the p50 and p65 homodimers do not appear to be able to cooperate to activate transcription. Of course, these experiments do not exclude the possibility that p50 or p65 homodimers can function in the context of the intact IFN-β promoter, since either or both could cooperate with other transcription factors that bind to this promoter.

To examine the activities of various combinations of p50 and p65 in the context of the intact promoter, we altered the sequence of PRDII within the promoter to create p50 or p65 homodimer binding sites. These binding sites were tested in EMSA and DNase I footprinting experiments for the ability to bind p50, p65, and c-rel homodimers as well as for the ability to bind the p50/p65 heterodimer and the high-mobility-group protein HMG I. A representative quantitative DNase I footprinting experiment with one of these mutated PRDII elements is shown in Fig. 5. In this experiment, we compared the affinity of p50 or p65 homodimers with that of the p50/p65

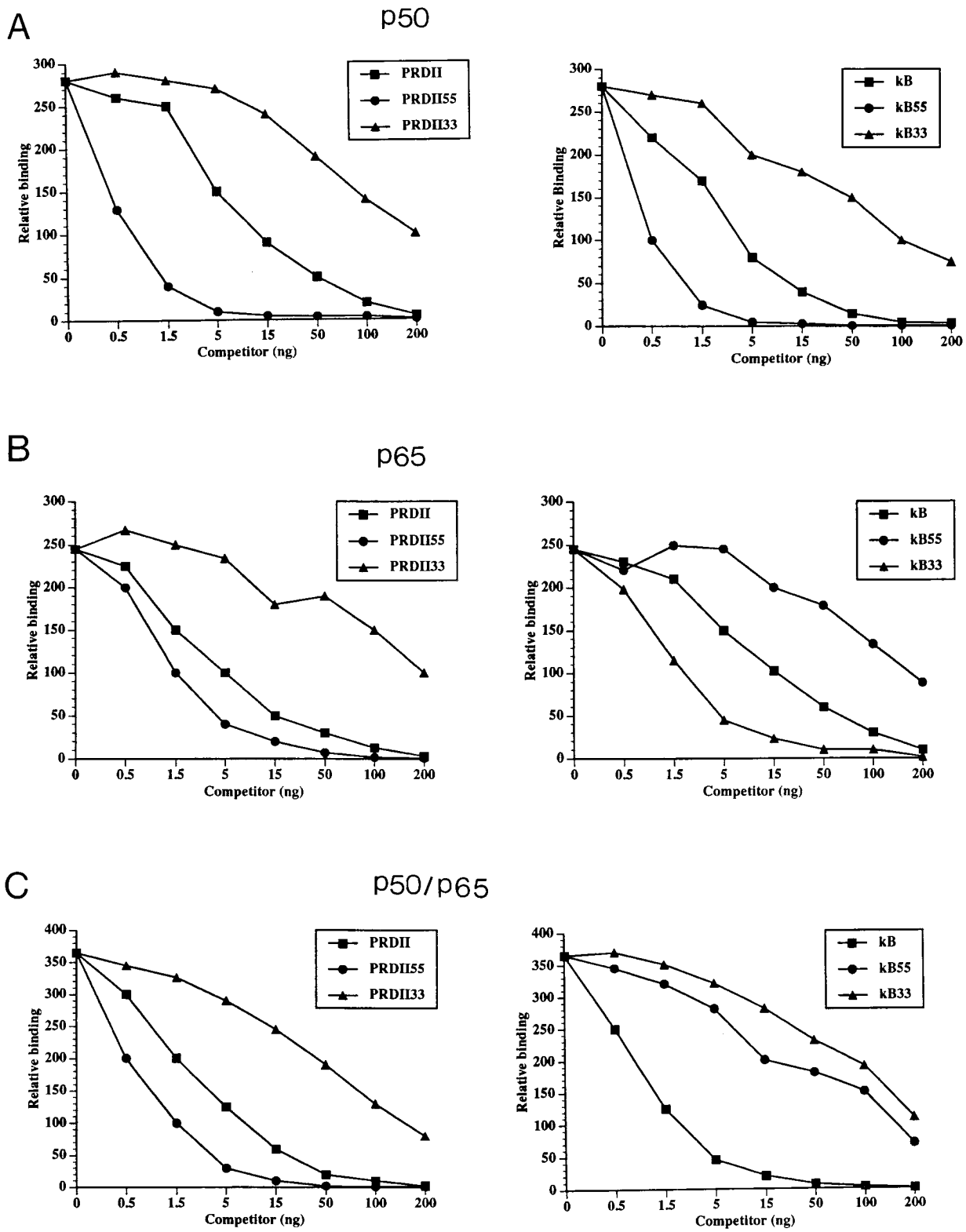

FIG. 2. Comparison of the DNA binding specificities of p50 and p65 homodimers and the p50/p65 heterodimer. A radiolabeled oligonucleotide (100,000 cpm, 0.5 ng) containing the PRDII element of the IFN-β promoter was incubated with purified p50 (A) and p65 (B) homodimers and the reconstituted p50/p65 heterodimer (C) in the presence of increasing amounts of the indicated unlabeled oligonucleotides. The binding reaction mixtures were loaded on a native polyacrylamide gel, and the bound and free DNAs were quantitated with a PhosphorImager. One of two independent experiments is shown. The sequences of the oligonucleotides were as follows: PRDII, GGGAAATTCC; κB, GGGACTTTCC; PRDII55, GGGAATTCCC; PRDII33, GGAATATTCC; κB55, GGGACGTCCC; and κB33, GGAAATT TCC. The p50 homodimer binding is efficiently competed for by the κB55 oligonucleotide (A), whereas the p65 homodimers interact preferentially with the κB33 sequence (B). Significantly, none of these binding sites efficiently compete for p50/p65 heterodimer binding (C).

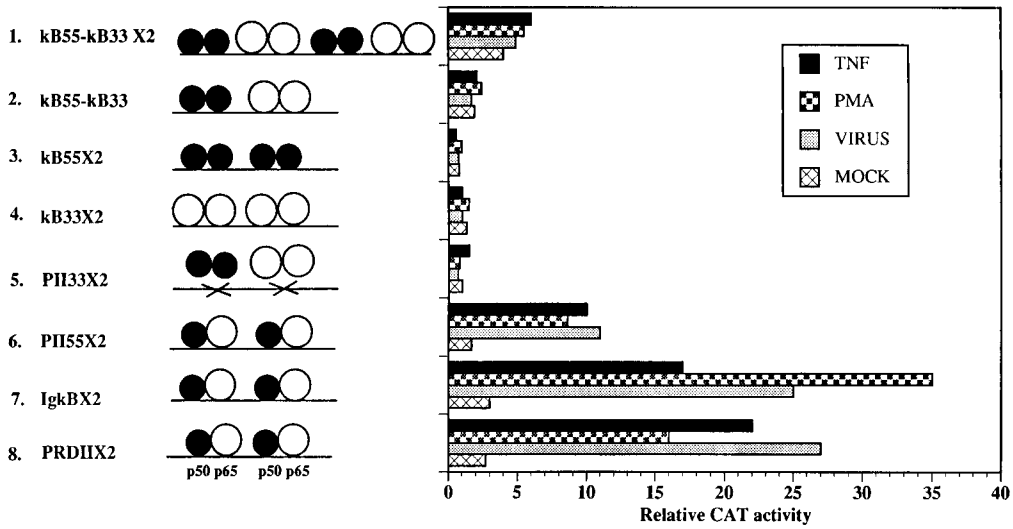

FIG. 3. Activation of various κB binding sites in response to TNF-α, PMA, or virus requires binding of both the p50 and p65 subunits of NF-κB. HeLa cells were transfected with reporter constructs containing two copies of PRDII (line 8), two copies of the IgκB (line 7), two copies of the PRDII55 (line 6), two copies of the PRDII33 (line 5), two copies of the κB33 line 4), two copies of the κB55 (line 3), one and two copies of the κB55-κB33 (lines 2 and 1, respectively) elements. The DNA binding specificity of the NF-κB subunits to these sites is illustrated with filled (p50) or open (p65) circles. Twenty-four hours after transfection, the cells were either mock, virus, PMA, or TNF-α treated for an additional 18 h. Shown are the relative CAT activities of a representative experiment.

heterodimer for binding to the wild-type promoter containing the PRDII element (GGGAAATTCC) and to the mutant SD24 (GGGATATCCC). Increasing amounts of recombinant p50 protect both PRDII and SD24 from cleavage from DNase I in quantitatively similar manners (compare lanes 2 to 5 in Fig. 5A and B). In contrast, recombinant p65 does not bind to the SD24 mutation (Fig. 5B, lanes 6 to 9), although p65 binds with high affinity to the PRDII element (Fig. 5A, lanes 6 to 9). Similarly, the p50/p65 heterodimer does not interact with the SD24 promoter (Fig. 5B, lanes 10 to 13), confirming our previous observation that both the p50 and p65 subunits of NF-κB must contact DNA for high-affinity binding. As a negative control, we showed that binding of interferon regulatory factor 1 (IRF-1) to the PRDI and PRDIII elements is not affected by this mutation (Fig. 5A and B, lanes 14 to 17 and 15 to 18, respectively). It is important to mention that the SD24 sequence (GGGATATCCC) differs at two positions from the recently described consensus (GGGRNTTTCC) for p65 binding (29). Of note, the high-affinity p65 binding site PRDII55 (GGGAATTCCC) differs from the SD24 by alteration of the AT run to TA in SD24. This mutation results in complete

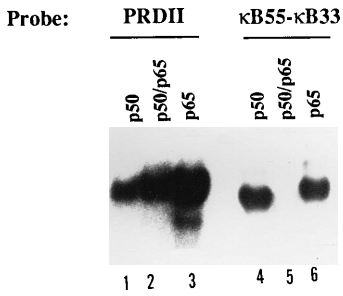

FIG. 4. The p50/p65 heterodimer does not bind to the κB55-κB33 oligonucleotide. An EMSA was performed with the PRDII (lanes 1 to 3) and κB55-κB33 (lanes 4 to 6) oligonucleotides as probes with p50 homodimers (lanes 1 and 4), p65 homodimers (lanes 3 and 6), and the p50/p65 heterodimer (lanes 2 and 4). Both p50 and p65 homodimers bind to the κB55-κB33 sequence. By contrast, the p50/p65 heterodimer interacts only with the PRDII probe (lane 2).

inhibition of the binding of both p65 and the p50/p65 heterodimer.

The in vivo functions of all of these mutated IFN-β promoters were then tested in transient transfection experiments as described above. The data from these experiments are presented in Table 1. Consistent with our previous observations (60), conversion of the PRDII element to either the IgκB or H-2 site results in a dramatic decrease of virus induction of the IFN-β promoter. The failure to activate IFN-β promoters containing these sites correlates with the failure of HMG I(Y) to bind to these elements (60) (Table 1). Interestingly, conversion of the PRDII element to the high-affinity p65 site (construct SD9) also results in complete loss of virus induction of the IFN-β promoter even though HMG I(Y) binds. In fact, this mutated promoter binds HMG I and c-rel homodimers as well as the c-rel/p65 heterodimer. In contrast, the p50/p65 heterodimer does not bind to this site (Fig. 2 and Table 1). Similar results were obtained with the SD45 site, which selectively binds p50 homodimers or p50/c-rel heterodimers. However, in this case no binding of HMG I was detected. The SD10 promoter, which contains an NF-κB binding site identical to that of the PRDII55 site, was inducible, but the level of induction was fourfold less than with the PRDII site. The reduced activity of this construct correlates with a decreased level of binding of HMG I to this site (Table 1).

The results of the in vitro binding experiments in conjunction with the in vivo experiments can be summarized as follows. The combination of either the endogenous p65, c-rel homodimers, or p65/c-rel heterodimer is not sufficient for virus induction of the intact IFN-β promoter even in the presence of HMG I (constructs SD9, SD11, SD21, and SD38). The combination of either the endogenous p50, c-rel homodimers, or p50/c-rel heterodimers again results in completely inactive promoters (SD15, SD16, SD24, and SD45). Therefore, binding of a p50/p65 heterodimer in the presence of HMG I(Y) is required for virus induction of the intact human IFN-β promoter. Our studies do not provide conclusive evidence for the role of c-rel in the regulation of the IFN-β gene (see below), since mutations that inhibit binding of c-rel and not of p50/p65

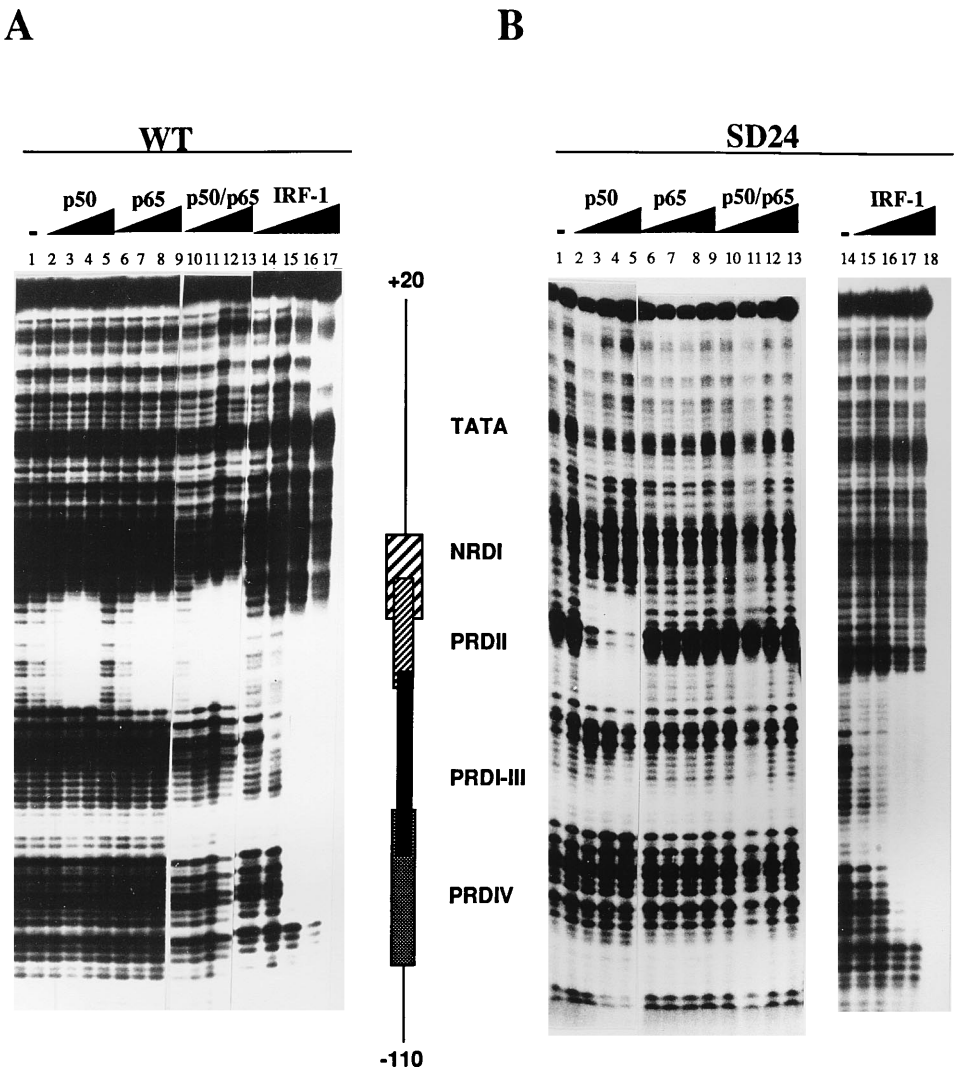

FIG. 5. Quantitative DNAse I footprinting of different NF-κB subunits on the wild-type and mutant IFN-β promoters. The wild-type (WT; A) and the mutant SD24 (B) IFN-β promoter fragments (−110 to +20) were end labeled at the −110 position (*Xba*I) and incubated with increasing amounts of purified p50 homodimers (lanes 2 to 5), p65 homodimers (lanes 6 to 9), p50/p65 heterodimers (lanes 10 to 13), and IRF-1 (lanes 14 to 17 and 15 to 18 in panels A and B, respectively). The DNA-protein complexes were treated with DNase I, and the digested DNA was analyzed on a sequencing gel. Relative positions of the IFN-β positive regulatory domains are depicted at the side of the gel. In contrast to p50 homodimers, the p50/p65 heterodimer and p65 homodimers do not bind to the SD24 site. Amounts of proteins used: lanes 2 to 5, 0.2 0.5, 1, and 2.5 ng of p50; lanes 6 to 9, 1, 2.5, 5, and 10 ng of p65; lanes 10 to 13, 0.1, 0.2, 0.5, and 1 ng of p50/p65; lanes 14 to 17 in panel A and 15 to 18 in panel B, 2, 5, 10 and 20 ng of IRF-1.

have not been identified. This is due to the more relaxed DNA binding specificity for c-rel (29).

In conclusion, analysis of a large number of mutations has revealed that NF-κB sites that bind both p50 and p65 homodimers and therefore the p50/p65 heterodimer are functional both in the context of a minimal artificial promoter and in the context of the intact IFN-β promoter. Furthermore, and in agreement with our previous work, binding of HMG I(Y) correlates with the activity of certain of these NF-κB motifs in the context of the intact IFN-β promoter.

In vitro binding of NF-κB to the artificial sites parallels their in vivo activities. We have shown that artificial binding sites that are specifically recognized in vitro by p50 or p65 homodimers do not respond in vivo to inducers of NF-κB. To determine whether the binding specificities for the various forms of NF-κB established in vitro correlate with activation in vivo, we cotransfected the reporter constructs described in Fig. 3 with expression vectors for p50 and p65 separately or together. Transfection of p65 alone strongly activated a reporter plasmid containing either the PRDII or the IgκB site (Fig. 6, lines 8 and 7) because p65 contains a potent transcriptional activation domain that is largely responsible for its biological activity (3, 48, 51). Strong activation by p65 homodimers is also observed with the PRDII55 site (line 6). The p65 homodimers also activated transcription from the p65-specific site-containing plasmids κB33X2 and κB55-κB33X2 (lines 1 and 4). In contrast, no activation was observed with reporter constructs containing the p50-specific site (line 3). Thus, activation of transcription by p65 correlates with its in vitro binding properties. Consistent with previous observations (3, 14, 15, 29, 49–51), transfection of p50 homodimers only slightly activated transcription from reporter plasmids containing NF-κB sites (Fig. 6).

To examine the effect of the p50/p65 heterodimer on these

TABLE 1. Analysis of the effects of PRDII mutations on in vitro binding and virus induction[a]

Construct	Sequence	Fold induction	DNA binding				
			p50	p65	p50/p65	rel	HMG I(Y)
PRDII	GGGAAATTCC	135	+	+	+	+	+
IgκB	GGGACTTTCC	6	+	+	+	+	−
H-2	GGGATTCCCC	9	+	+	+	+	−
SD9	GGAAATTTCC	2	−	+	−	+	+
SD10	GGGAATTCCC	35	+	+	+	+	+/−
SD11	GGAATTTTCC	3	−	+/−	−	+	+
SD12	GGAATTTTTC	4	−	−	−	+/−	+
SD15	GGGATGTTCC	10	+	−	−	+/−	−
SD16	GGATATCCC	3	+/−	−	−	+	+
SD21	GGAATTATCC	5	−	+/−	−	+	+
SD24	GGGATATCCC	4	+	−	−	+	+/−
SD31	GGGAATTTCC	127	+	+	+	+	+
SD38	GGAATATTCC	2	−	+/−	−	+	+
SD42	GGGAATGTCC	6	+	+/−	+/−	+	−
SD45	GGGACGTCCC	3	+	−	−	+	−

[a] HeLa cells were transfected with reporter constructs containing the wild-type intact IFN-β promoter (PRDII) or with constructs containing PRDII mutations in the context of the intact promoter (SD series). The IgκB and H-2 mutations have been described previously. Twenty-four hours after transfection, the cells were either mock or virus infected for an addition 18 h. The effects of these mutations on in vivo activity are shown as fold induction. The DNA-binding properties of the different rel proteins and HMG I(Y) to the mutant PRDII sequences are also shown. For simplicity, all mutations are compared with PRDII as + (binding in the range of 3-fold), +/− (reduced binding in the range of 3- to 10-fold), and − (reduced binding 10-fold or more).

reporter constructs, we cotransfected a mixture of the p50 and p65 expression plasmids. Under these conditions, transcriptional activation is primarily elicited through the heterodimer (see below). In vitro binding of the p50/p65 heterodimer strongly correlates with in vivo activation by transfected p50 and p65 to the respective sites (Fig. 6, lines 6 to 8). However, no activation is observed with the reporter constructs which preferentially bind p50 or p65 homodimers, although the latter are activated by p65 (Fig. 6). The specificity of these effects is further demonstrated by the fact that no activation is observed with reporter plasmids containing the p50- and p65-specific sites linked to each other (lines 1 and 2). Taken together, these results show that the binding specificity observed in vitro for the different homo- and heterodimeric NF-κB subunits closely parallels their in vivo activities.

Transcriptional synergism between p50 and p65 at the PRDII element. Previous studies have suggested that different members of the rel family display distinguishable transcriptional activities both in vivo and in vitro (17, 44). To examine the effect of different rel family homodimers at the PRDII site, expression vectors for p50, p65, p52, RelB, and c-rel were cotransfected along with the PRDIICAT reporter construct into HeLa cells, and CAT activity was determined after mock or virus induction. Consistent with previous studies, p50 or p52 homodimers are very poor activators, resulting in only a two- to threefold activation of the PRDII element (data not shown). Interestingly, c-rel does not activate the PRDII site, although it can activate a reporter construct containing the NF-κB element from the interleukin-2 receptor α-chain gene (56) (data not shown). By contrast, transfection of p65 homodimers results in a very efficient activation of both the basal and the virus-induced levels of CAT activity with the PRDII reporter (Fig. 7; compare column 1 with columns 2 to 11).

The role of p50 in PRDII activation was established in similar transfection experiments in which equimolar amounts of the p50 and p65 expression vectors were cotransfected. As shown in Fig. 7, columns 12 to 21, p50 strongly synergizes with p65 in transcriptional activation, especially when small

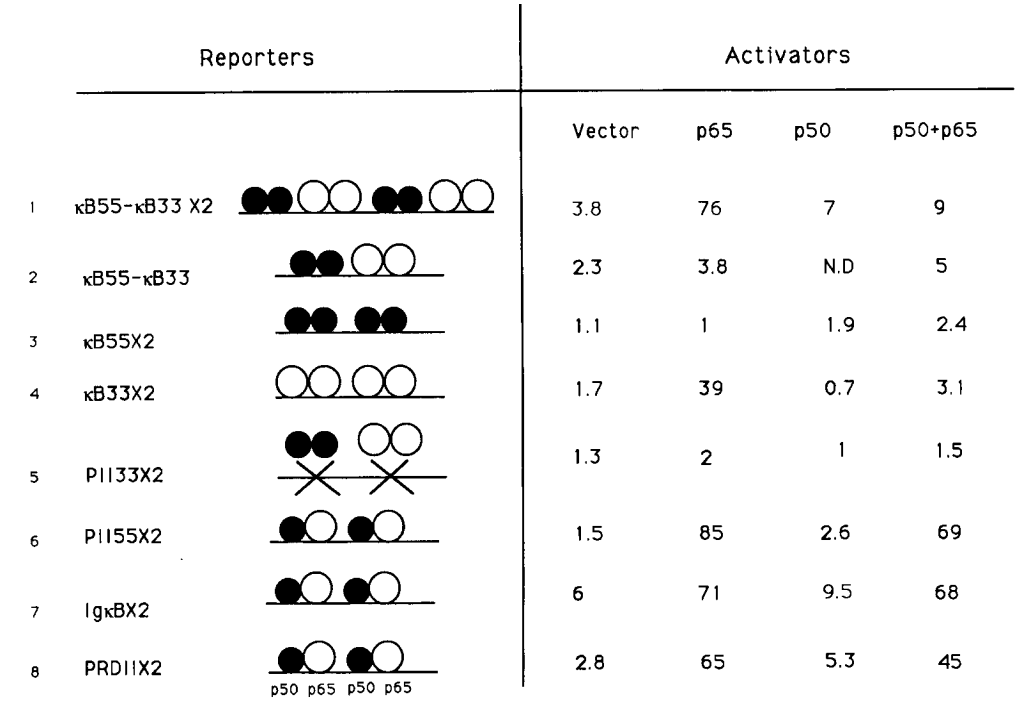

FIG. 6. Transactivation of artificial NF-κB binding sites by p50 and p65 homo- and heterodimers. HeLa cells were cotransfected with the reporter constructs (2 μg) indicated at the left and either the expression vector alone (5 μg), 500 ng or p65 or p50 expression vector, or a mixture of the two (100 ng). The total amount of DNA transfected was brought to 5 μg by including the empty expression vector. The numbers indicate the percentage conversion of [^{14}C]chloramphenicol.

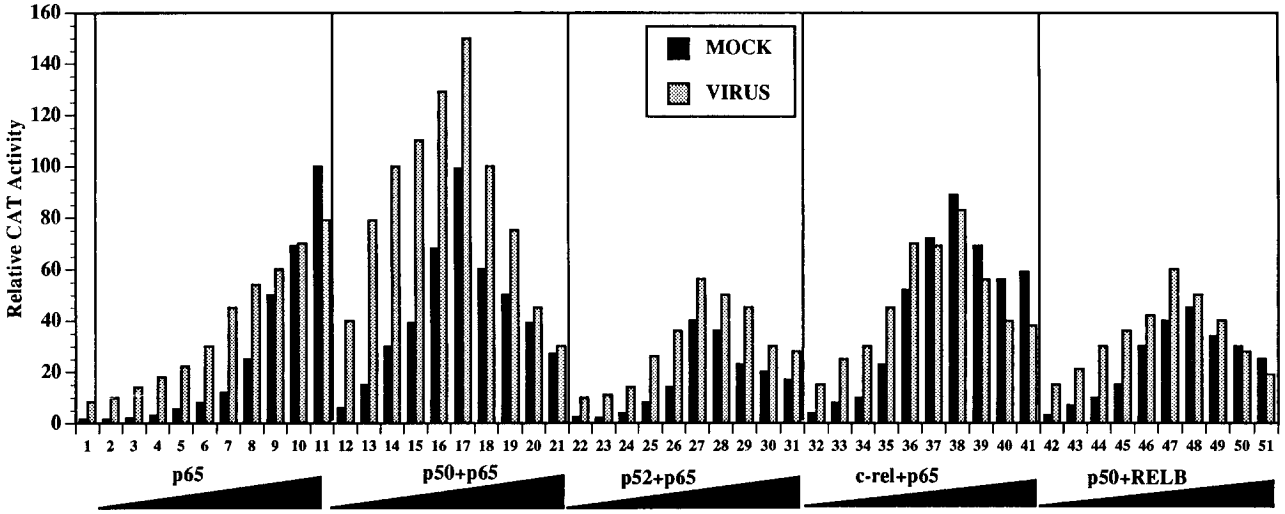

FIG. 7. The p50/p65 heterodimer is a potent transcriptional activator of the PRDII element. HeLa cells were cotransfected with the PRDII reporter construct plus increasing amounts of the indicated expression vectors for rel proteins. Twenty-four hours posttransfection, the cells were either mock or virus infected for an additional 18 h. Lanes: 1, empty expression vector (5 μg); 2 to 11, increasing amounts of p65 expression plasmid (2.5 ng, 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, 250 ng, 500 ng, 1 μg, and 2.5 μg); 12 to 21, increasing amounts of an equimolar mixture of p50 and p65 expression plasmids (2.5 ng, 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, 250 ng, 500 ng, 1 μg, and 2.5 μg); 22 to 31, increasing amounts of an equimolar mixture of p52 and p65 expression plasmids (2.5 ng, 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, 250 ng, 500 ng, 1 μg, and 2.5 μg); 32 to 41, increasing amounts of an equimolar mixture of c-rel and p65 expression plasmids (2.5 ng, 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, 250 ng, 500 ng, 1 μg, and 2.5 μg); 42 to 51, increasing amounts of an equimolar mixture of p50 and RelB expression plasmids (2.5 ng, 5 ng, 10 ng, 25 ng, 50 ng, 100 ng, 250 ng, 500 ng, 1 μg, and 2.5 μg).

amounts of the plasmids were transfected. The overall activity of the p50/p65 combination is 5- to 10-fold higher than the activity of equal amounts of the p65 plasmid. However, transfection of higher amounts of the p50 and p65 expression plasmids inhibits transcription, resulting in levels of expression which are lower than for the corresponding p65 homodimers. This observation has been reported previously, and it was proposed that the p50 homodimers formed under these conditions exhibit an inhibitory function on the p50/p65 heterodimer or on p65 homodimers (15). The strong synergism between p50 and p65 can be explained if one assumes that the p50/p65 heterodimer binds to DNA with a higher affinity than the p65 homodimer. However, a direct contribution of p50 in the activation process cannot be excluded. In contrast to p50, transfection of p52 with p65 results in a modest degree of synergism with the PRDII element (Fig. 7, columns 22 to 31). However, the p52/p65 heterodimer appears to activate the IgκB site more efficiently than the p50/p65 heterodimer (42) (data not shown), ruling out the possibility that p50 and p52 are not synthesized at comparable levels in the transfected cells. Similarly, cotransfection of p50 and RELB activates transcription three- to fivefold less than does cotransfection of p50 and p65 (Fig. 7, columns 42 to 51). The ability of c-rel to synergize with p65 is demonstrated in Fig. 7, columns 32 to 41. Thus, cotransfection of c-rel and p65 activates PRDII-dependent transcription to levels three- to fivefold higher than p65 alone. In sharp contrast with the p50-plus-p65 cotransfection, higher levels of c-rel do not significantly inhibit transcription (Fig. 7).

Cotransfection of plasmids expressing the combination of p50/c-rel, p52/c-rel, p52/RelB, or RelB alone does not significantly activate transcription (data not shown).

The experiments described above show that of all possible combinations of rel proteins capable of binding to PRDII, the p50/p65 heterodimer is the most active in stimulating transcription from reporter constructs containing two copies of PRDII. However, activation of the IFN-β gene by virus infection requires synergistic interactions between transcription factors bound to PRDIV, PRDIII-I, and PRDII (12, 57, 61). We have previously shown that an ATF-2/c-Jun heterodimer binds specifically to PRDIV and activates transcription from the intact IFN-β promoter (12). In addition, the transcription factor IRF-1 has been implicated in IFN-β gene regulation through the PRDIII-I element (38). To provide direct evidence that the p50/p65 heterodimer preferentially synergizes with these transcriptional activators, we attempted to activate the intact IFN-β promoter by transfecting increasing amounts of expression plasmids encoding ATF-2, c-Jun, IRF-1, and different combinations of rel proteins in mouse embryonal cells which are devoid of endogenous IRF-1 and NF-κB proteins. As shown in Fig. 8, transfection of increasing amounts of IRF-1, ATF-2/c-Jun, and p50/p65 heterodimers activates transcription from the intact IFN-β promoter to a level that is almost 2,000 times higher than the basal level. By contrast, when equal amounts of p65 homodimers or the p52/p65 heterodimer were transfected, the levels of transcriptional synergism were significantly reduced. We conclude that in the context of the intact IFN-β promoter, the p50/p65 heterodimer is the optimum combination of rel proteins that can synergize with transcription factors bound to the PRDIV and PRDIII-I elements.

Virus infection activates distinct rel proteins. The results described above implicate p50, p65, and c-rel in the induction of PRDII by virus or dsRNA. All three proteins are expressed constitutively in most cell types, and induction of the IFN-β gene does not require new protein synthesis. To determine whether these proteins bind to PRDII in whole cell extracts and whether their binding activities change upon virus infection, we carried out EMSAs. Whole cell extracts were prepared from mock-treated human MG63 cells, from MG63 cells infected with Sendai virus in the presence or absence of cycloheximide, or from cells treated with dsRNA in the presence of cycloheximide. A single DNA-protein complex was detected with extracts from uninduced cells and was also observed after induction with virus or treatment with dsRNA (Fig. 9, complex

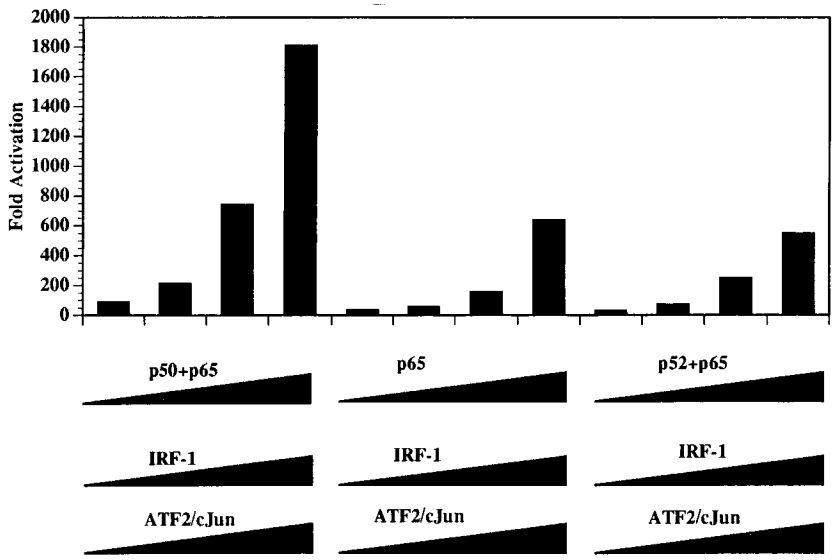

FIG. 8. The p50/p65 heterodimer preferentially synergizes with ATF-2/c-Jun and IRF-1 in the context of the intact IFN-β promoter. P19 embryonal carcinoma cells were cotransfected with a reporter construct containing the −110 IFN-β promoter fused to the CAT gene and increasing amounts (100 ng, 300 ng, 1 μg, and 3 μg) of the indicated expression vectors. Forty-eight hours posttransfection, the cells were harvested and CAT activities were determined.

D). The identity of the protein(s) responsible for this complex is not known. By contrast, virus infection leads to the appearance of three complexes (A, B, and C; Fig. 9, lane 2), all of which were specifically competed for by unlabeled PRDII oligonucleotide and not by an unrelated sequence (data not shown).

Complexes A and B were still observed after virus infection in the presence of cycloheximide or after the combined treatment of dsRNA plus cycloheximide (lanes 8 and 14). In contrast, the inducible binding activity of complex C was blocked by cycloheximide. Thus, the assembly of complexes A and B does not require new protein synthesis, while the formation of complex C does.

To identify the proteins present in the inducible complexes, we examined the effects of various antibodies directed against specific rel proteins on their formation. Anti-p50 antiserum was raised against recombinant p50 produced in bacteria. The specificity of this antiserum was established by EMSA with recombinant proteins and by Western blot (immunoblot) analysis (data not shown). As shown in Fig. 9, the anti-p50 antiserum completely abolished the formation of complex C and drastically decreased the formation of complex B, without affecting the integrity of complex A. In this experiment, the antibodies were added after the formation of the complexes (postincubation). However, when the p50 antiserum was incubated with the cellular extract before the addition of the probe, all of the B complex was supershifted (data not shown). These observations indicate that complexes B and C both contain p50.

Antiserum against the p65 subunit completely abolished the formation of complexes A and B but had no effect on complex C (Fig. 9). An antiserum against c-rel had no effect on complexes B and C but slightly decreased the formation of complex A, giving rise to a supershifted complex, especially in the extracts prepared from dsRNA-plus-cycloheximide-treated cells (Fig. 9, lane 18). Finally, antiserum against RelB had no effect on the formation of these complexes (not shown). We conclude that infection of cells with virus or treatment with dsRNA results in the formation of three distinct protein-DNA complexes on PRDII. Complex C contains p50 homodimers, and its formation requires new protein synthesis. Complex A contains a c-rel/p65 heterodimer and/or a p65 homodimer, while complex B contains a p50/p65 heterodimer. Neither of these complexes requires new protein synthesis for formation.

DISCUSSION

The transcription factor NF-κB can be activated by a variety of extracellular signals. In addition, NF-κB or other members of the rel family of transcription factors have been implicated in the control of a remarkably large number of different genes that respond to distinct inducers. Thus, a variety of mechanisms must exist for achieving the observed high level of specificity of NF-κB-dependent gene activation. For example, rel proteins can form homodimers or heterodimers that display distinct DNA recognition properties and transcriptional activities. Specific homo- or heterodimers can therefore selectively regulate gene expression by preferentially binding to distinct regulatory sequences. Additional specificity is achieved by synergistic interactions between NF-κB and other transcription factors that bind to adjacent DNA sequences. For example, NF-κB has been shown to associate with NF-IL6, ATF-2, AP-1, and Sp1 in solution and to transcriptionally synergize with each of these transcription factors in specific promoter contexts (12, 25, 30, 44, 53, 54). NF-κB also associates with the high-mobility-group protein HMG I(Y) (58), and the PRDII element of the IFN-β promoter is regulated through synergistic interactions between these two proteins (60). An important consequence of these mechanisms for specific gene activation is that small changes in the level of only one of the monomeric components could result in profound changes in the pattern of gene expression. This change could in turn drastically alter the cellular phenotype. For example, abnormally high levels of expression of the p100 (the precursor for p52) or *BCL-3* gene are associated with certain types of leukemia (40, 42).

Identification of rel family members required for IFN-β gene expression. In this study, we carried out experiments to identify which members of the rel family are involved in virus

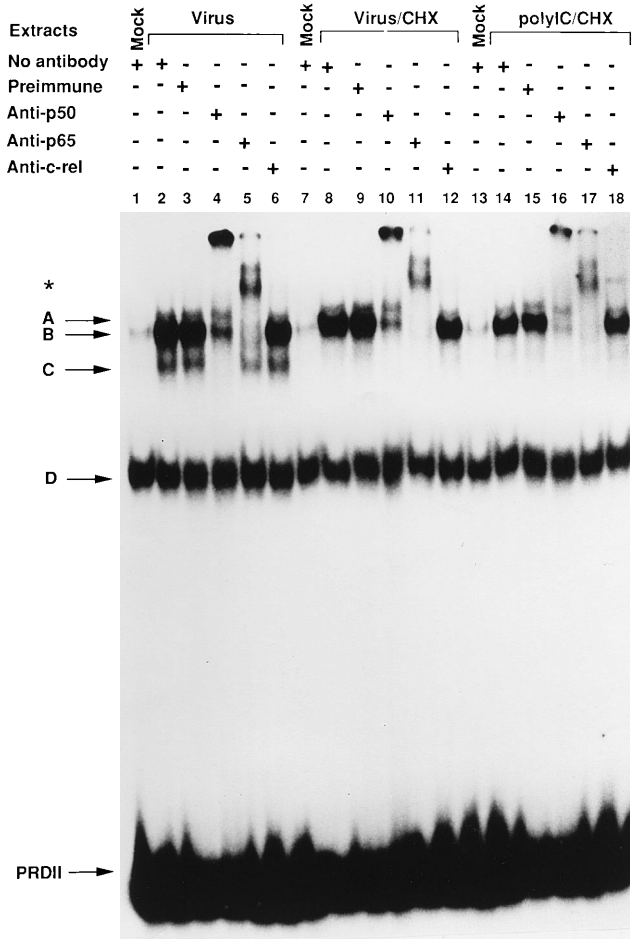

FIG. 9. Virus-inducible protein-DNA complexes assembled on PRDII in vitro contain both the p50 and p65 subunits of NF-κB. Shown is an EMSA using the PRDII as a probe and extracts prepared from mock-treated (lanes 1, 7, and 13), virus-infected (lanes 2 to 6), virus-plus-cycloheximide (CHX)-treated (lanes 8 to 12), and dsRNA-plus-cycloheximide-treated (lanes 14 to 18) cells. Complexes A, B, and C are indicated at the left. The protein-DNA complexes were reacted with preimmune serum (lanes 3, 9, and 15), anti-p50 serum (lanes 4, 10, and 16), anti-p65 serum (lanes 5, 11, and 17), and anti c-rel serum (lanes 6, 12, and 18). The asterisk indicates the supershifted complexes.

induction of the human IFN-β gene. This is an important question, since PRDII and most other naturally occurring NF-κB binding sites are recognized by several different combinations of rel homo- and heterodimers in vitro. If all of these combinations were equally active on all recognition sequences, specificity of gene activity would have been achieved entirely by the constraints of promoter context. Alternatively, it is possible that many different combinations of rel family members can bind to a promoter, but only one species is capable of activating transcription because of constraints imposed by the sequence of the binding site.

Initially, our approach was to generate high-affinity NF-κB sites which preferentially interact with either the p50 or p65 heterodimers. The distinct methylation interference pattern of p50 and p65 homodimers, especially on the IgκB site, was used as a guide for the design of these high-affinity sites. Previous studies failed to establish differential sequence specific contacts of p50 homodimers on asymmetric NF-κB binding sites, presumably as a result of the presence of contaminated rel proteins in the cellular fractions used in these studies (2, 9). However, UV cross-linking studies indicated that p50 is preferentially bound to the 5′ half site of PRDII, whereas p65 interacts with the 3′ half site (62). We have verified and extended these results by identifying the sequence-specific contacts for both p50 and p65 homodimers by using the methylation interference method.

Palindromic oligonucleotides containing two half sites that specifically contact p50 or p65 homodimers are high-affinity binding sites for the respective homodimers, but neither of these palindromic sequences is efficiently recognized by the p50/p65 heterodimer. Thus, specific binding of NF-κB requires that both subunits of NF-κB specifically contact DNA. Information regarding the in vivo transcriptional activities of p50 or p65 homodimers was obtained by introducing the palindromic sites immediately upstream of a minimal promoter. Significantly, neither of these palindromic sites could be activated in vivo by virus or other inducers of NF-κB such as PMA or TNF-α. Thus, DNA binding is not sufficient for transcriptional activation.

After these elements were tested in the context of a minimal promoter, they were examined in the context of the intact IFN-β gene promoter in the place of PRDII. In addition, we tested minimal promoters containing one p50- and one p65-specific palindromic site to determine whether p50 homodimers can synergize with p65 homodimers. However, none of these reporter constructs were functional in vivo, even though they clearly bind to p50 and p65 homodimers in vivo. The latter point was demonstrated by cotransfecting these reporter genes with plasmids that express either p50 or p65. Taken together, these results strongly suggest that the p50/p65 heterodimer is the particular combination of rel proteins required for virus induction of the IFN-β gene.

An intriguing finding of this study is that the p65 homodimer binding site is not activated by endogenous p65 but is activated by cotransfected p65. The latter observation is consistent with previous transfection studies using p65 expression plasmids or GAL4-p65 fusions, indicating that p65 contains potent transcriptional activation domains (3, 39, 48, 51). The failure to detect transcriptional activation of p65 homodimer binding sites by activated endogenous proteins may be a consequence of the relatively low affinity of the homodimer for these sites and/or low levels of activated p65 homodimers. In fact, the abundance of complex A compared with complex B in our EMSA supports this conclusion. The existence of p65 homodimers has also been demonstrated in PMA-activated T cells (18), and a central role for p65 homodimers has been proposed for the regulation of the interleukin-8 gene (28). However, another report argues for the involvement of p50/p65 or p52/p65 heterodimers in the regulation of this gene (52).

Transcriptional activities of p50 homodimers. Previous studies suggested that p50 homodimers (known as KBF1) are responsible for the basal-level expression of the class I histocompatibility gene, *H2K*^{b} (24). However, a more recent analysis shows that transfected p50 homodimers actually repress expression of the *H2K*^{b} gene (45). Similarly, p50 homodimers have been shown to inhibit the activity of NF-κB on the interleukin-2 gene promoter (24a), and endogenous or transfected p50 homodimers can inhibit NF-κB activity on certain κB binding sites (14). Thus, in at least some circumstances, p50 homodimers appear to function as transcriptional repressors. However, p50 homodimers can function as weak transcriptional activators in vivo and in vitro (17, 17a, 27), and we find that transfected p50 weakly stimulates transcription (two- to threefold) from either the PRDII- or the p50 homodimer-specific binding site. Surprisingly, p50 homodimers function as

a potent transcriptional activator in yeast cells (39). Thus, p50 homodimers appear to have the intrinsic capacity to function as a transcriptional repressor or activator, and this activity may be binding site specific (17) or may be regulated by other proteins (30a). In both cases, it is possible that the change in activity is a result of conformational changes that expose repressor or activator domains.

We have shown that p50 homodimers can inhibit the transcriptional activity of the p50/p65 heterodimer in cotransfection experiments, suggesting the interesting possibility that p50 homodimers are involved in the postinduction repression of the IFN-β gene. Virus induction of the IFN-β gene is transient, and previous studies have shown that induction does not require new protein synthesis, while postinduction repression does (34). One mechanism proposed for postinduction repression is that virus induction induces the synthesis of repressor proteins that bind specifically to the promoter and prevent the binding of activators (34, 64). Postinduction turnoff is an intrinsic property of the PRDII element, since reporter genes containing two copies of PRDII display the same transient kinetics of virus induction as the intact IFN-β gene promoter (64). Thus, the putative virus-inducible repressor of PRDII must compete with the p50/p65 heterodimer for PRDII binding.

We have shown that virus infection induces the binding activity of p50 homodimers in a cycloheximide-sensitive manner, which implies that the gene for p50 (NF-κB_1, p105) is activated by virus. In fact, we have recently shown that the p105 gene is activated by virus infection (59). Thus, p50 may be a postinduction repressor of the IFN-β gene. Several lines of evidence are consistent with this possibility. First, p50 homodimers do not significantly activate transcription from the PRDII site. Second, maximal levels of p50 homodimers correlate with the postinduction repression. Third, p50 homodimers efficiently compete with NF-κB for binding at the PRDII element.

Function of p50 in the p50/p65 heterodimer. Although p50 does not work as a strong activator in many different contexts, it does appear to synergize with the p65 transcriptional activity in vivo. We found that transfection of small amounts of equimolar p50 and p65 expression plasmids activate transcription of the IFN-β gene promoter 10-fold better than equal amounts of p65. The strong synergism between p50 and p65 is probably a consequence of the increased DNA binding affinity of the p50/p65 heterodimer relative to the p65 homodimers. However, it is also possible that the formation of heterodimers with p65 on the DNA alters the structure of p50, exposing an otherwise cryptic transcriptional activation domain which synergizes with p65. In fact, Fujita et al. (17) proposed that specific binding sites induce a conformational change in the bound p50 to expose a cryptic transcriptional activation domain. Alternatively, the interaction between p50 and p65 increases the strength of the p65 transcriptional activation domain. Further experiments are required to test these possibilities. Significantly, transfection of the p50/p65 heterodimer also increases the virus-inducible expression to levels much higher than with p65 homodimers.

In similar cotransfection experiments, we established that the c-rel/p65 heterodimer is also a potent activator of the PRDII element but weaker than the p50/p65 heterodimer. In contrast, transfection of p52 with p65 does not result in significant synergistic activation from the PRDII site although this heterodimer is a stronger activator for the IgκB element. These experiments further illustrate the selectivity of distinct combinations of rel proteins for different κB elements.

Virus-inducible rel protein-DNA complexes. Virus infection or treatment of cells with dsRNA induces the binding activity of several NF-κB proteins. Two of the complexes formed (A and B) do not require new protein synthesis, whereas the third complex (C) is dependent on protein synthesis. By using specific polyclonal antisera, we have identified complex C as p50 homodimers, whereas complexes A and B represent mainly p65 homodimers with some p65/c-rel and p50/p65 heterodimers, respectively. Since activation of the IFN-β gene is not dependent on new protein synthesis, it is reasonable to assume that transcriptional activation is mediated through complexes A and B. However, the high abundance of the p50/p65 heterodimer (complex B) and its ability to behave as a potent transcriptional activator underscore the dominant role for this heterodimer in the regulation of the human IFN-β gene, confirming our mutagenesis analysis.

What is the role of the c-rel/p65 heterodimer or p65 homodimers? We have shown that sites which bind only these dimeric forms do not respond to virus infection. It appears that the low abundance of these complexes in the induced cells is not sufficient for transcriptional activation. However, these results do not exclude the possibility that the c-rel/p65 heterodimer or p65 homodimers do contribute to the overall level of IFN-β gene expression. Another possibility is that other rel heterodimers like p50/RelB play a role in IFN-β gene regulation in specific cell types. In fact, the p50/RelB heterodimer has been identified as the constitutive form of NF-κB in lymphoid cells (32). However, the inducible form of NF-κB is the p50/p65 heterodimer.

Previous studies and the results reported here clearly demonstrate that different dimeric combinations of rel family members display distinct DNA recognition properties. The p50 subunit preferentially interacts with the more conserved 5′ half site, whereas p65 specifically binds to the more divergent 3′ half site. Interestingly, this asymmetry is reflected also in the nature of the NF-κB binding sites. It appears that most of the natural NF-κB sites are preferentially recognized by heterodimers and not by homodimers. The simplest explanation of this observation is that regulation of gene expression by heterodimers has significant advantages compared with homodimers. We can imagine several reasons to account for this.

First, the DNA affinity of the p50/p65 heterodimer is at least 10-fold higher than that of the p65 homodimer, even with sites which bind the p65 with relative high affinity—for example, the PRDII element (our unpublished observations and reference 17). Thus, most natural NF-κB sites will not be activated by p65 homodimers unless these proteins are present at very high levels (e.g., after transfection). Second, the p50/p65 heterodimer is more stable than the p65 or p50 homodimers (21). This observation is consistent with the report that most of the p50 and p65 molecules in the cell are present as heterodimers, and the homodimers are detected only when high-affinity binding sites are used as probes (18). Third, the primary mechanism for controlling the activity of NF-κB involves the association of the heterodimer with IκB or the association of p65 with the p105 precursor of p50 (for a recent review, see reference 5). Finally, regulation of transcription by heterodimeric forms like the p50/p65 heterodimer provides more possibilities for combinatorial regulation due to specific protein-protein interactions. For example, c-Jun preferentially interacts with p65, where ATF-2 interacts with higher affinity with p50 than with p65. Thus, an ATF-2/c-Jun heterodimer interacts with higher affinity with the p50/p65 heterodimer than with the homodimeric forms separately. It is obvious that interaction between distinct families of transcription factors results in synergistic activation of transcription (for a review, see reference 61).

ACKNOWLEDGMENTS

We thank U. Siebenlist for the p52 expression plasmid and R. Bravo for the RelB antibody and expression vector. We also thank S. C. Chan and R. Majeti for help in quantitating the CAT assays and members of the Maniatis and Ptashne laboratories for valuable discussions and critical reading of the manuscript.

This work was supported by NIH grant AI 20642 to T.M. D.T. was supported by the Lucille P. Markey Charitable Trust.

REFERENCES

1. **Baeuerle, P. A.** 1991. The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim. Biophys. Acta **1072:**63–80.
2. **Baeuerle, P. A., and D. Baltimore.** 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3:**1689–1698.
3. **Ballard, D. W., E. P. Dixon, N. J. Peffer, H. Bogerd, S. Doerre, B. Stein, and W. C. Greene.** 1992. The 65-kD subunit of human NF-κB functions as a potent transcriptional activator and a target for v-Rel-mediated repression. Proc. Natl. Acad. Sci. USA **89:**1875–1879.
4. **Ballard, D. W., W. H. Walker, S. Doerre, P. Sista, J. A. Molitor, E. P. Dixon, N. J. Peffer, M. Hennick, and W. C. Greene.** 1990. The v-rel oncogene encodes a κB enhancer binding protein that inhibits NF-κB function. Cell **63:** 803–814.
5. **Beg, A. A., and A. S. Baldwin.** 1993. The IκB proteins: multifunctional regulators of rel/NF-κB transcription factors. Genes Dev. **7:**2064–2070.
6. **Bours, V., P. R. Burd, K. Brown, J. Villalobos, S. Park, R. P. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist.** 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in *trans* activation through a κB site. Mol. Cell. Biol. **12:**685–695.
7. **Bours, V., J. Villalobos, P. R. Burd, K. Kelly, and U. Siebenlist.** 1990. Cloning of a mitogen-inducible gene encoding a κB DNA-binding protein with homology to the rel oncogene and to cell cycle motifs. Nature (London) **348:**76–80.
8. **Brownell, E., N. Mittereder, and N. R. Rice.** 1989. A human rel proto-oncogene cDNA containing an Alu fragment as a potential coding exon. Oncogene **4:**935–942.
9. **Collart, M., P. Baeuerle, and P. Vassalli.** 1990. Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. Mol. Cell. Biol. **10:** 1498–1506.
10. **DeMaeyer, E., and J. DeMaeyer-Guignard.** 1988. Interferons and other regulatory cytokines. John Wiley, New York.
11. **Dobrzanski, P., R. P. Ryseck, and R. Bravo.** 1993. Both N- and C-terminal domains of RelB are required for full transactivation: role of the N-terminal leucine zipper-like motif. Mol. Cell. Biol. **13:**1572–1582.
12. **Du, W., D. Thanos, and T. Maniatis.** 1993. Mechanisms of transcriptional synergism between distinct virus inducible enhancer elements. Cell **74:**887–898.
13. **Fan, C.-M., and T. Maniatis.** 1991. Generation of p50 subunit of NF-κB by processing of p105 through an ATP-dependent pathway. Nature (London) **354:**395–398.
14. **Franzoso, G., V. Bours, V. Azarenko, S. Park, M. Tomita-Yamaguchi, T. Kanno, K. Brown, and U. Siebenlist.** 1993. The oncoprotein Bcl-3 can facilitate NF-κB mediated transactivation by removing inhibiting p50 homodimers from select κB sites. EMBO J. **12:**3893–3901.
15. **Franzoso, G., V. Bours, S. Park, M. Tomita-Yamaguchi, K. Kelly, and U. Siebenlist.** 1992. The candidate proto-oncogene Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. Nature (London) **359:**339–342.
16. **Fujita, T., M. Miyamoto, Y. Kimura, J. Hammer, and T. Taniguchi.** 1989. Involvement of a cis element that binds an H2TF1/NF-κB like factor, in the virus induced interferon-β gene expression. Nucleic Acids Res. **17:**3335–3346.
17. **Fujita, T., G. P. Nolan, S. Ghosh, and D. Baltimore.** 1992. Independent modes of transcriptional activation by the p50 and p65 subunits of NF-κB. Genes Dev. **6:**775–787.

17a. **Fujita, T., G. P. Nolan, H.-C. Liou, M. L. Scott, and D. Baltimore.** 1993. The candidate proto-oncogene bcl-3 encodes a transcriptional coactivator that activates through NF-κB p50 homodimers. Genes Dev. **7:**1354–1363.

18. **Ganchi, P. A., S. C. Sun, W. C. Greene, and D. W. Ballard.** 1993. A novel NF-κB complex containing p65 homodimers: implications for transcriptional control at the level of subunit dimerization. Mol. Cell. Biol. **13:**7826–7835.
19. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolan, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell **62:**1019–1029.
20. **Grilli, M., J. J.-S. Chiu, and M. J. Lenardo.** 1993. NF-κB and rel-participants in a multiform transcriptional regulatory system. Int. Rev. Exp. Cytol. **143:** 1–62.
21. **Grimm, S., and P. A. Baeuerle.** 1993. The inducible transcription factor NF-κB: structure-function relationship of its protein subunits. Biochem. J. **290:**297–308.
22. **Hiscott, J., D. Alper, L. Cohen, J.-F. LeBlanc, L. Sportza, A. Wong, and S. Xanthoudakis.** 1989. 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. Virol. **63:**2557–2566.
23. **Ip, Y. T., M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, S. Gonzalez-Crespo, K. Tatei, and M. Levine.** 1993. Dif, a dorsal related gene that mediates an immune response in Drosophila. Cell **75:**753–763.
24. **Israel, A., O. Le Bail, D. Hatat, J. Piette, M. Kieram, F. Logeat, D. Wallach, M. Fellous, and P. Kourilsky.** 1989. TNF stimulates expression of mouse MHC class I genes by inducing an NF-κB like enhancer binding activity which displaces constitutive factors. EMBO J. **8:**3793–3780.

24a. **Kang, S. M., A. C. Tran, M. Grilli, and M. J. Lenardo.** 1992. NFκB subunit regulation in non transformed $CD4^+$ T lymphocytes. Science **256:**1452–1456.

25. **Kaszubska, W., R. H. vanHuijsduijnen, P. Ghersa, A.-M. DeRaemy-Schenk, P. B. C. Chen, T. Hai, J. F. DeLemarter, and J. Whelan.** 1993. Cyclic AMP-independent ATF family members interact with NF-κB and function in the activation of the E-selectin promoter in response to cytokines. Mol. Cell. Biol. **13:**7180–7190.
26. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. LeBail, M. B. Urban, P. Kourilsky, P. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product. Cell **62:**1007–1018.
27. **Kretschmer, M., M. Meisterernst, C. Scheidereit, G. Li, and R. G. Roeder.** 1992. Transcriptional regulation of the HIV-1 promoter by NF-κB in vitro. Genes Dev. **6:**761–774.
28. **Kunsch, C., and C. A. Rosen.** 1993. NF-κB subunit-specific regulation of the inerleukin-8 promoter. Mol. Cell. Biol. **13:**6137–6146.
29. **Kunsch, C., S. M. Ruben, and C. A. Rosen.** 1992. Selection of optimal κB/Rel DNA-binding motifs: interaction of both subunits of NF-κB with DNA is required for transcriptional activation. Mol. Cell. Biol. **12:**4412–4421.
30. **LeClair, K. P., M. A. Blanar, and P. A. Sharp.** 1992. The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. Proc. Natl. Acad. Sci. USA **89:**8145–8149.

30a. **Lehming, N., D. Thanos, J. M. Brickman, J. Ma, T. Maniatis, and M. Ptashne.** 1994. An HMG-like protein that can switch a transcriptional activator to a repressor. Nature (London) **371:**175–179.

31. **Lenardo, M., C.-M. Fan, T. Maniatis, and D. Baltimore.** 1989. The involvement of NF-κB in β-interferon gene regulation reveals its role as a widely inducible mediator of signal transduction. Cell **57:**287–294.
32. **Lernbecher, T., U. Muller, and T. Wirth.** 1993. Distinct NF-κB/Rel transcription factors are responsible for tissue specific and inducible gene activation. Nature (London) **365:**767–770.
33. **MacGregor, G. R., and C. T. Caskey.** 1989. Construction of plasmids that express E. coli β-galactosidase in mammalian cells. Nucleic Acids Res. **17:** 2365.
34. **Maniatis, T., L.-A. Whittemore, W. Du, C.-M. Fan, A. D. Keller, V. Palombella, and D. Thanos.** 1992. Positive and negative regulation of the human interferon-β gene expression, p. 1193–1220. *In* S. L. McKnight and K. Yamamoto (ed.), Transcriptional regulation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1992. Molecular cloning and characterization of a novel Rel/NF-κB family member displaying structural and functional homology to NF-κB p50. DNA Cell. Biol. **11:**523–527.
36. **Mercurio, F., J. A. DiDonato, C. Rosette, and M. Karin.** 1993. p105 and p98 precursor proteins play an active role in NF-κB-mediated signal transduction. Genes Dev. **7:**705–718.
37. **Meyer, R., E. N. Hatada, H. P. Hohmann, M. Haiker, C. Bartsch, U. Rothlisberger, H. W. Lahm, E. J. Schlaeger, A. P. G. M. vanLoon, and C. Scheidereit.** 1991. Cloning of the DNA-binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. Proc. Natl. Acad. Sci. USA **88:**966–970.
38. **Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi.** 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. Cell **54:**903–913.
39. **Moore, P. A., S. M. Ruben, and C. A. Rosen.** 1993. Conservation of transcriptional activation functions of the NF-κB p50 and p65 subunits in mammalian cells and *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**1666–1674.
40. **Neri, A., C. C. Chang, L. Lombardi, M. Selina, A. T. Maiolo, R. S. K. Chaganti, and R. Dalla-Favera.** 1991. B cell lymphoma associated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. Cell **67:**1075–1087.
41. **Nolan, G. P., S. Ghosh, H. C. Liou, P. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a rel-related polypeptide. Cell **64:**961–969.
42. **Ohno, H., G. Takimoto, and T. W. McKeithan.** 1990. The candidate proto-oncogene Bcl-3 is related to genes implicated in cell lineage determination and cell cycle control. Cell **60:**991–997.
43. **Perkins, N. D., N. L. Edwards, C. S. Duckett, A. B. Agrenoff, R. M. Schmid, and G. J. Nabel.** 1993. A cooperative interaction between NF-κB and Sp1 is required for HIV-1 enhancer function. EMBO J. **12:**3551–3558.
44. **Perkins, N. D., R. M. Schmid, C. S. Duckett, K. Leung, N. R. Rice, and G. J.**

Nabel. 1992. Distinct combinations of NF-κB subunits determine the specificity of transcriptional activation. Proc. Natl. Acad. Sci. USA **89:**1529–1533.
45. **Plaksin, D., P. A. Baeuerle, and L. Eisenbach.** 1993. KBF1 (p50 NF-κB homodimers) acts as a repressor of H-2K^b gene expression in metastatic tumor cells. J. Exp. Med. **177:**1651–1662.
46. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C. H. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a rel-related cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251:**1490–1493.
47. **Ruben, S. M., J. F. Klement, T. A. Coleman, M. Maher, C. H. Chen, and C. A. Rosen.** 1992b. I-Rel: a novel rel-related protein that inhibits NF-κB transcriptional activity. Genes Dev. **6:**745–760.
48. **Ruben, S. M., R. Narayanan, J. F. Klememt, C. H. Chen, and C. A. Rosen.** 1992. Functional characterization of the NF-κB p65 transcriptional activator and an alternatively spliced derivative. Mol. Cell. Biol. **12:**444–454.
49. **Ryseck, R. P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo.** 1992. RelB, a new rel family transcription activator that can interact with p50-NF-κB. Mol. Cell. Biol. **12:**674–684.
50. **Schmid, R. M., N. D. Perkins, C. S. Duckett, P. C. Andrews, and G. J. Nabel.** 1991. Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature (London) **352:**733–736.
51. **Schmitz, M. L., and P. A. Baeuerle.** 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-κB. EMBO J. **10:**3805–3817.
52. **Stein, B., and A. S. Baldwin.** 1993. Distinct mechanisms of regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. Mol. Cell. Biol. **13:**7191–7198.
53. **Stein, B., A. S. Baldwin, D. W. Ballard, W. C. Greene, P. Angel, and P. Herrlich.** 1993. Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potentiated biological functions. EMBO J. **12:**3879–3894.
54. **Stein, B., P. C. Cogswell, and A. S. Baldwin.** 1993. Functional and physical association between NF-κB and C/EBP family members: a rel domain-bZip interaction. Mol. Cell. Biol. **13:**3964–3974.
55. **Steward, R.** 1987. Dorsal, an embryonic polarity gene in Drosophila, is homologous to the vertebrate proto-oncogene, c-rel. Science **238:**692–694.
56. **Tan, T.-H., G. P. Huang, A. Sica, P. Ghosh, H. A. Young, D. L. Longo, and N. R. Rice.** 1992. κB site-dependent activation of the interleukin-2 receptor α-chain promoter by human c-rel. Mol. Cell. Biol. **12:**4067–4075.
57. **Thanos, D., W. Du, and T. Maniatis.** 1993. The high mobility group protein HMG I(Y) is an essential structural component of a virus inducible enhancer complex. Cold Spring Harbor Symp. Quant. Biol. **58:**73–81.
58. **Thanos, D., S. C. Chan, and T. Maniatis.** Unpublished data.
59. **Thanos, D., R. Majeti, and T. Maniatis.** Unpublished data.
60. **Thanos, D., and T. Maniatis.** 1992. The high mobility group protein HMG I(Y) is required for NF-κB-dependent virus induction of the human IFN-β gene. Cell **71:**777–789.
61. **Tjian, R., and T. Maniatis.** 1994. Transcriptional activation: a complex puzzle with few easy pieces. Cell **77:**5–8.
62. **Urban, M. B., R. Schreck, and P. A. Baeuerle.** 1991. NF-κB contacts DNA by a heterodimer of the p50 and p65 subunit. EMBO J. **10:**1817–1825.
63. **Visvanathan, K. V., and S. Goodbourn.** 1989. Double stranded RNA activates binding of NF-κB to an inducible element in human β-interferon promoter. EMBO J. **8:**1129–1138.
64. **Whittemore, L.-A., and T. Maniatis.** 1990. Postinduction repression of the β-interferon gene is mediated through the positive regulatory domains. Proc. Natl. Acad. Sci. USA **87:**7799–7803.
65. **Zabel, U., R. Schreck, and P. Baeuerle.** 1991. DNA binding of purified transcription factor NF-κB. J. Biol. Chem. **266:**252–260.