MOLECULAR AND CELLULAR BIOLOGY, Dec. 1994, p. 7695–7703
0270-7306/94/$04.00+0

Vol. 14, No. 12

Transcriptional Regulation of NF-κB2: Evidence for κB-Mediated Positive and Negative Autoregulation

SUSANNE LIPTAY,[1]† ROLAND M. SCHMID,[1,2,3]‡ ELIZABETH G. NABEL,[1] AND GARY J. NABEL[1,2,3]*

Departments of Internal Medicine[1] and Biological Chemistry[2] and Howard Hughes Medical Institute,[3] University of Michigan Medical Center, Ann Arbor, Michigan 48109-0650

Received 8 June 1994/Returned for modification 15 July 1994/Accepted 30 August 1994

NF-κB is an inducible transcription factor complex which regulates the expression of a variety of genes which are involved in the immune, inflammatory, and acute-phase responses. The maintenance of NF-κB activity in stimulated cells requires ongoing protein synthesis, suggesting several modes of regulation. In this report, we have characterized the transcriptional regulation of one family member, NF-κB2. The genomic structure and sequence of NF-κB2 revealed the presence of two promoters and at least four κB regulatory elements, which mediate responsiveness to phorbol myristate acetate and tumor necrosis factor alpha. Similar to other NF-κB family members, NF-κB2 is positively autoregulated. In contrast to other family members, we find that κB elements in the NFKB2 promoter can also mediate transcriptional repression in the absence of NF-κB. We identified a nuclear complex which binds specifically to a subset of κB-related sites but not to the canonical κB element. Because of its putative inhibitory or repressive effect, this binding activity has been termed Rep-κB. This mechanism of repressing basal NF-κB2 transcription in an inactivated state enables the cell to tightly control NF-κB2 activity. These data demonstrate that a novel mode of κB-dependent regulation is mediated by specific κB sites in the NFKB2 promoter.

NF-κB was first described as a B-cell-specific transcription factor that bound to the immunoglobulin kappa light-chain enhancer (23). It was shown later that NF-κB is constitutively expressed in mature B lymphocytes, monocytes, and macrophages (7) and is present in an inactive complex in the cytoplasm of T lymphocytes and nonlymphoid cells bound to the inhibitory protein, IκB. Activation of NF-κB involves dissociation of IκB and subsequent nuclear translocation (1). This activation can be mediated by a variety of stimuli, including bacterial lipopolysaccharide, phorbol myristate acetate (PMA), and cytokines such as tumor necrosis factor alpha (TNF-α), lymphotoxin, or interleukin-1. NF-κB is composed of 50- and 65-kDa subunits, which bind to a 10-bp motif found in a variety of cellular genes involved in the immune, inflammatory, and acute-phase responses. At least five independent loci, i.e., NFKB1 (p105), NFKB2 (p100), RelA (p65), RelB, and c-Rel, that encode related proteins that bind κB sites have been isolated (2, 3, 6, 9, 13, 15, 16, 19, 20, 22). NFKB1 and NFKB2 encode precursor molecules, NF-κB1 (p105) and NF-κB2 (p100), respectively, which require proteolytic processing to generate the ~50-kDa subunits.

The initial activation of NF-κB can occur in the absence of de novo protein synthesis (23). Maintenance of NF-κB activity, however, requires ongoing protein synthesis and continuous stimulation, suggesting that regulation also occurs transcriptionally or translationally (8). It has been proposed that PMA and TNF-α, in addition to causing nuclear localization of NF-κB, might stimulate expression of genes encoding NF-κB subunits, as shown previously for NF-κB1 (25). We and others have defined cDNAs encoding NF-κB2, a gene product with similarity to NF-κB1 but with distinct DNA-binding and functional transactivation properties (2, 5, 15, 18, 22). Whether NF-κB2 could be regulated transcriptionally, however, was unknown. To define the regulation of this transcription factor, we have isolated genomic clones and examined its mode of transcriptional regulation. We find that NF-κB2 is autoregulated positively after cell stimulation and negatively in resting cells. Surprisingly, both positive and negative regulation are mediated by κB sites present in the NFKB2 promoter. The definition of a separable negatively acting element within a κB regulatory site provides a novel mechanism that inhibits NFKB2 gene expression in unstimulated cells and helps to maintain strict control of this transcriptional regulatory factor.

MATERIALS AND METHODS

Cloning and sequencing of NFKB2. A human genomic library, kindly provided by David Kurnit, was screened with cDNA probes encoding NF-κB2 as described earlier (12). A 5.5-kb *Hin*dIII fragment was subcloned into Bluescript SK plasmid (Stratagene) and completely sequenced by using a Sequenase kit (United States Biochemical Corp.) and the exonuclease III-mung bean nuclease nested deletion strategy.

Primer extension analysis. A 20-mer (corresponding to positions +55 to +74) and a 30-mer (corresponding to positions +1172 to +1201) antisense oligonucleotide specific for promoters P1 and P2, respectively, were end labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Labeled primer (5×10^4 cpm) was annealed overnight at 55°C (promoter P1) or 42°C (promoter P2) with 20 μg of cytoplasmic RNA from PMA-stimulated (10 ng/ml for 4 h) Jurkat T cells or 20 μg of tRNA in 1 M NaCl–0.17 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5)–0.33 mM EDTA. Primer extension was performed with avian myeloblastosis virus reverse transcriptase at 42°C for 90 min. The reaction mixtures were treated with RNase A (30 μg/ml) for 30 min at 37°C, extracted with phenol-chloroform, ethanol pre-

* Corresponding author. Mailing address: Howard Hughes Medical Institute, University of Michigan Medical Center, 1150 W. Medical Center Dr., 4520 MSRB I, Ann Arbor, MI 48109-0650. Phone: (313) 747-4798. Fax: (313) 747-4730.

† Present address: Department of Pediatrics, Division of Molecular Biology, University of Ulm, 89070 Ulm, Germany.

‡ Present address: Department of Internal Medicine, University of Ulm, 89070 Ulm, Germany.

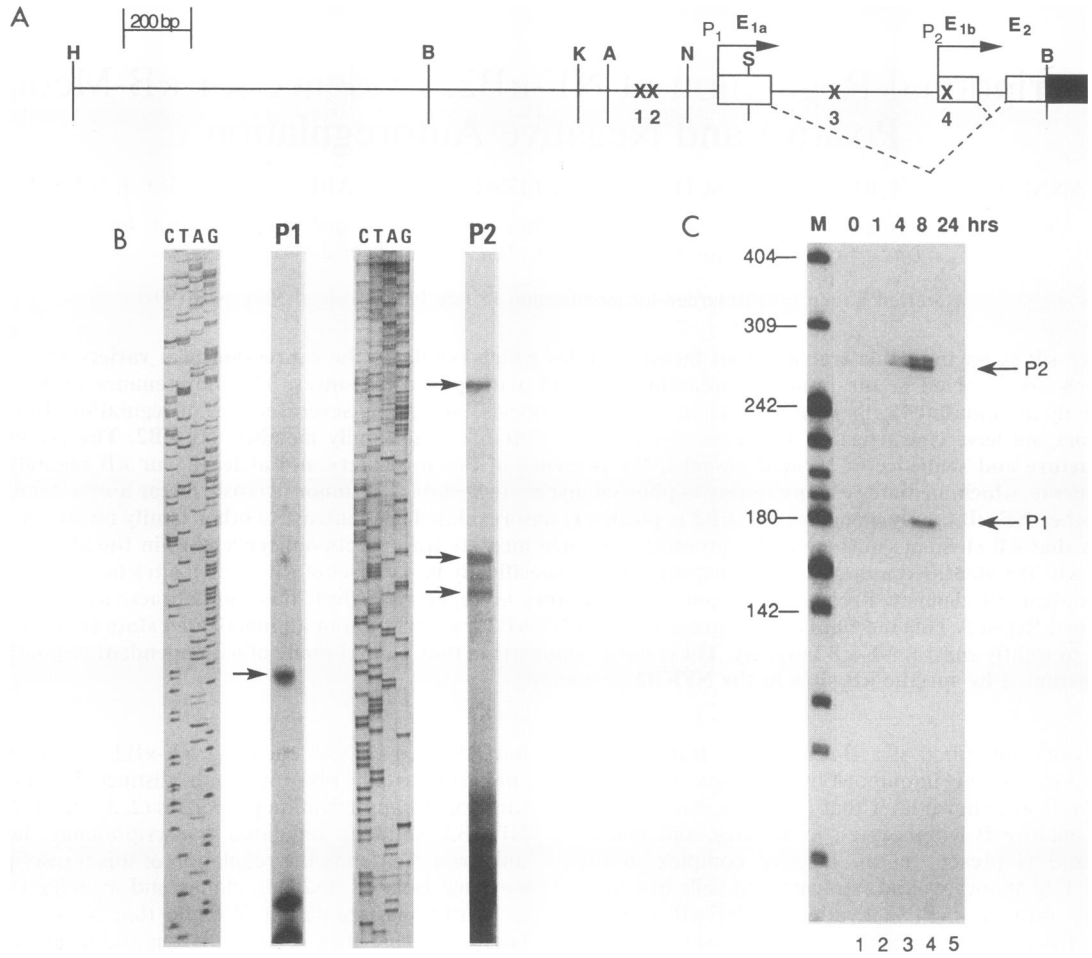


FIG. 1. Structure of the NFKB2 gene. (A) Partial restriction map of the promoter and 5′ untranslated region of the human NFKB2 gene. Open boxes indicate noncoding exons; the filled box indicates the coding exon. The interrupted lines indicates the position of the intron in the untranslated region. Crosses mark the positions of the κB sites. Restriction enzyme sites: H, *Hin*dIII; B, *Bst*XI; K, *Kpn*I; A, *Ava*I; N, *Nru*I; S, *Sal*I. (B) Mapping of the NFKB2 transcription start site by primer extension. Lanes C, T, A, and G contained sequencing reactions primed with the same antisense oligonucleotides as used for mapping and were electrophoresed in parallel. The arrows indicate the major extension products. (C) RNase protection assays verifying promoter utilization in vivo and PMA inducibility of both P1 and P2. Jurkat T cells were treated with PMA (10 ng/ml) over the time period indicated. A radiolabeled molecular weight marker was electrophoresed in parallel. Positions of markers (M) are indicated in base pairs. (D) Nucleotide sequence of the promoter and 5′ untranslated region of the human NFKB2 gene. The most 5′ transcription start site is marked by an arrow (+1). Splice junctions of the intron are indicated. Coding sequence is indicated by corresponding amino acid sequence and alignments with two previously published cDNAs, represented by (a) and (b) (2, 22). Underlined sequences mark potential transcription factor-binding sites; κB sites are double underlined and in boldface.

cipitated, and analyzed on a 6% acrylamide denaturing gel. Molecular weight markers and a sequence ladder were electrophoresed in parallel.

RNase protection assays. A 272-bp *Eco*RI-*Pvu*II fragment of NFKB2 cDNA (22) was subcloned into the *Sma*I and *Eco*RI sites of Bluescript SK+/− plasmid (Stratagene). An antisense RNA probe was generated with T3 polymerase in the presence of [α-^{32}P]UTP. Full-length 334-bp products were gel purified. RNase protection assays were performed with an Ambion RPA II kit (Ambion, Inc., Austin, Tex.). Antisense riboprobe (10^5 cpm) was hybridized to poly(A)$^+$ RNA (1 μg) from PMA-stimulated Jurkat cells or to 40 μg of tRNA at 45°C overnight in 80% formamide–100 mM sodium citrate (pH 6.4)–300 mM sodium acetate (pH 6.4)–1 mM EDTA. The samples were treated with RNase A (5 μg/ml) and RNase T_1 (100 U/ml) for 30 min at 37°C and analyzed on a 6% acrylamide denaturing gel.

Plasmids. The HS-2.0-CAT, BS-0.9-CAT, KS-0.5-CAT, AS-0.36-CAT, and NS-0.2-CAT plasmids were constructed by cloning the ~2.0-kb *Hin*dIII (−1874)-to-*Sal*I (+165), ~0.9-kb *Bst*X1 (−741)-to-*Sal*I (+165), ~0.5-kb *Kpn*I (−292)-to-*Sal*I (+165), ~0.36-kb *Ava*I (−198)-to-*Sal*I (+165), and ~0.2-kb *Nru*I (−37)-to-*Sal*I (+165) fragments of NFKB2, respectively, into the *Hin*dIII and *Sal*I sites of the pCAT basic plasmid (Promega Biotec, Madison, Wis.), immediately 5′ of the chloramphenicol acetyltransferase (CAT) gene. The junctions were verified by DNA sequencing. The RSV-p49, RSV-p52, RSV-p50, and RSV-p65 NF-κB expression vectors were previously described (10, 22). The κB sites at positions −70 and −100 in the mutant plasmids were changed to GCTCAAT CTCC by site-directed mutagenesis as described previously (14). The mutations were verified by sequencing.

Cell cultures, transfections, and CAT assays. Jurkat and YT human T leukemia cells, JY, a human Epstein-Barr virus-transformed B-cell line, and S194 murine B leukemia cells were grown in RPMI 1640 medium supplemented with glu-

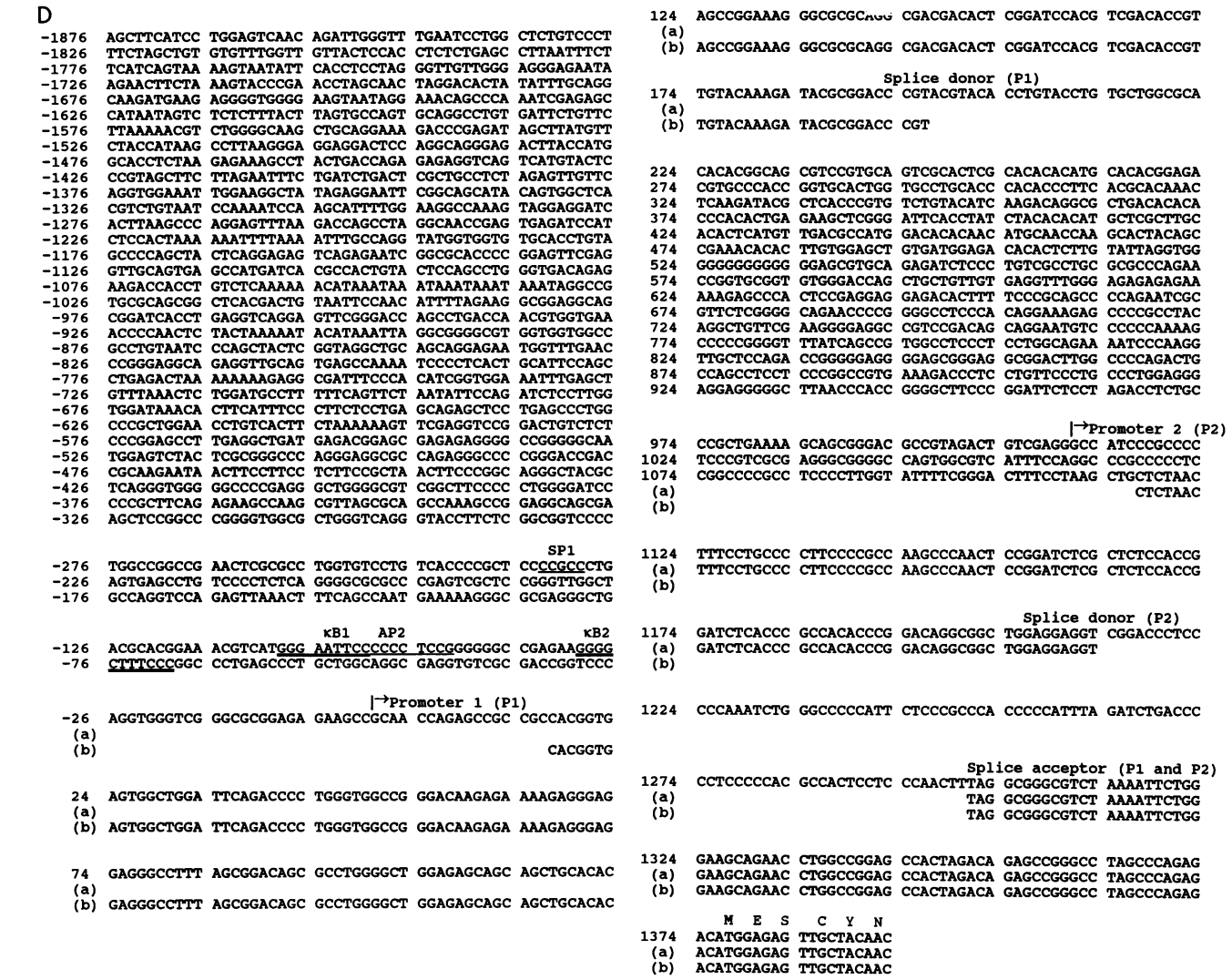


FIG. 1—*Continued.*

tamine, antibiotics, and 10% fetal calf serum. HeLa (cervical epithelial) and 293 (embryonic kidney) cells were cultured in Dulbecco modified Eagle medium containing glutamine, antibiotics, and 10% calf serum or fetal calf serum. Jurkat, YT, JY, and S194 cells were transfected by DEAE-dextran as described previously (22). HeLa and 293 cells were transfected by calcium phosphate precipitation by standard methods (21). CAT assays were performed as previously described (22). Data shown are representative of at least three independent experiments.

Electrophoretic mobility shift assays (EMSAs). Nuclear extracts were prepared as described previously (14). Three to 5 μg of protein was incubated with radiolabeled DNA probe in a 20-μl reaction mixture containing 10 mM Tris (pH 7.5), 100 mM NaCl, and 4% glycerol for 30 to 45 min on ice. The DNA-protein complexes were resolved by electrophoresis on 4% polyacrylamide gels in 1× TGE at 4°C (14).

Partial protein purification. Nuclear extracts of Jurkat, JY, and HeLa cells were applied to DNA-cellulose columns at 100 mM NaCl. After several washes, protein fractions were eluted by stepwise increasing NaCl concentration, analyzed by EMSA, and stored at −70°C.

RESULTS

Structure of the NFKB2 promoter. NFKB2 clones were isolated by screening a human genomic library with a cDNA probe encoding the NF-κB2 (p100) subunit (12). Four of a total of seven positive clones were further characterized by restriction enzyme mapping and Southern blotting. A 5.5-kb *Hin*dIII restriction fragment was subcloned into the Bluescript SK vector (Stratagene) and sequenced. This subclone contained approximately 3.3 kb of sequence upstream of the translational start site. Alignment of the genomic sequence with the cDNA suggested the presence of an intron in the 5′ untranslated portion of the gene, with the splice acceptor site 72 bp upstream of the ATG start codon. Two previously described cDNAs diverge in the nucleotide sequence upstream of this splice acceptor site, which had initially raised the possibility of different untranslated upstream exons converging at a common exon. Comparison of these cDNAs with the genomic sequence confirmed this presumption and suggested that different untranslated first exons were separated by ~1 kb (Fig. 1A and D, E_{1a} and E_{1b}, respectively). The sequences of

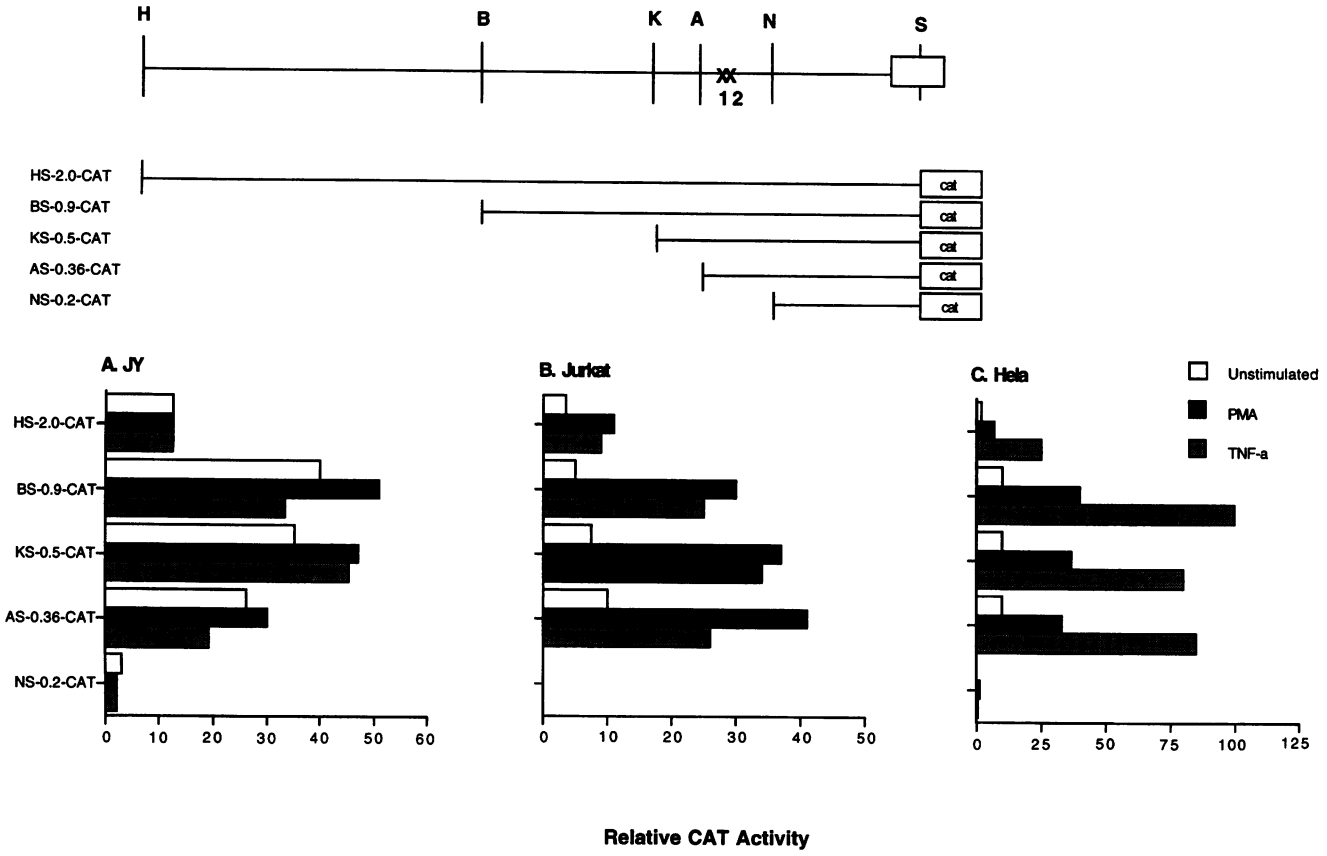


FIG. 2. Identification of *cis*-acting sequences which regulate NFKB2 transcription. The upper scheme shows the partial restriction map of the NFKB2 promoter (see Fig. 1A) and the series of reporter plasmids used for transfections. These plasmids were prepared by ligating different 5′ deleted fragments of the NFKB2 promoter region immediately 5′ of bacterial CAT gene as described in Materials and Methods. Plasmid names are indicated on the left. JY (A), Jurkat (B), and HeLa cells (C) cells were transiently transfected with 10 μg of the indicated reporter plasmid by the DEAE-dextran or calcium phosphate method. Twenty hours after transfection, cells were stimulated with PMA (10 ng/ml) or TNF-α (200 U/ml) for an additional 16 to 20 h. Unstimulated and stimulated CAT activities are shown in bar diagrams. Relative CAT activity refers to CAT activity relative to that of unstimulated cells transfected with the pCAT basic plasmid.

the proposed intron/exon borders were in good agreement with consensus splice sites (17).

To determine the position of the transcriptional initiation sites, primer extension analysis was performed. Cytoplasmic RNA from PMA-stimulated Jurkat T leukemia cells was annealed to an antisense oligonucleotide, complementary to each putative first exon (E_{1a} and E_{1b}, respectively). A predominant transcriptional start site was observed for exon E_{1a}, designated +1 relative to the rest of the sequence. A second promoter, with three possible initiation sites, was identified ~1,025 bp downstream (Fig. 1B and D). Identification of these two promoters, P1 and P2, explained divergent 5′ untranslated sequences isolated from different NF-κB2 clones previously reported (2, 22). These promoter regions lacked classical TATA or CCAAT elements. The promoter sequences were GC rich (approximately 73%) and contained potential Sp1, AP-2, and NF-κB binding sites (Fig. 1D). The two κB motifs of promoter P1 have not been previously described, and the first κB binding site is a perfect palindrome.

To investigate whether both putative promoter regions are utilized in vivo, RNase protection assays were performed (Fig. 1C). Poly(A)$^+$ RNA from unstimulated and PMA-stimulated Jurkat cells protected a 171-bp and a 268-bp fragment corresponding to promoters P1 and P2, respectively. The RNAs derived from both promoters were about equally induced by stimulation with PMA and increased with time, with optimal levels seen after 8 h (Fig. 1C). Similar induction of both promoters was observed after stimulation with TNF-α (data not shown).

The putative promoter region of NFKB2 is functional and inducible by mitogens and cytokine. To identify *cis*-acting sequences that regulate transcription of NF-κB2, a series of reporter plasmids was constructed. Since the activities of P1 and P2 were found to be comparable after mitogen induction, we first analyzed the activity of the P1 promoter in detail. Different 5′ deletion fragments of the putative NFKB2 promoter region were ligated to the bacterial CAT gene in exon 1, downstream of P1 (Fig. 2). Transient transfections into JY, Jurkat, and HeLa cells were performed. In JY cells, transfections of the HS-2.0-CAT, BS-0.9-CAT, KS-0.5-CAT, and AS-0.36-CAT reporter plasmids showed moderate to strong basal activity (Fig. 2A), and stimulation of transfected cells with PMA or TNF-α had only a minor influence on CAT activity. In Jurkat and HeLa cells, the promoter showed less basal activity but could be activated 4- to 10-fold by PMA or TNF-α (Fig. 2B and C). The levels of induction varied in the cell lines tested, with HeLa cells showing the highest induction. No significant differences in activity and inducibility were observed for the BS-0.9-CAT, KS-0.5-CAT, and AS-0.36-CAT reporter plasmids. Similar results were observed with reporter plasmids

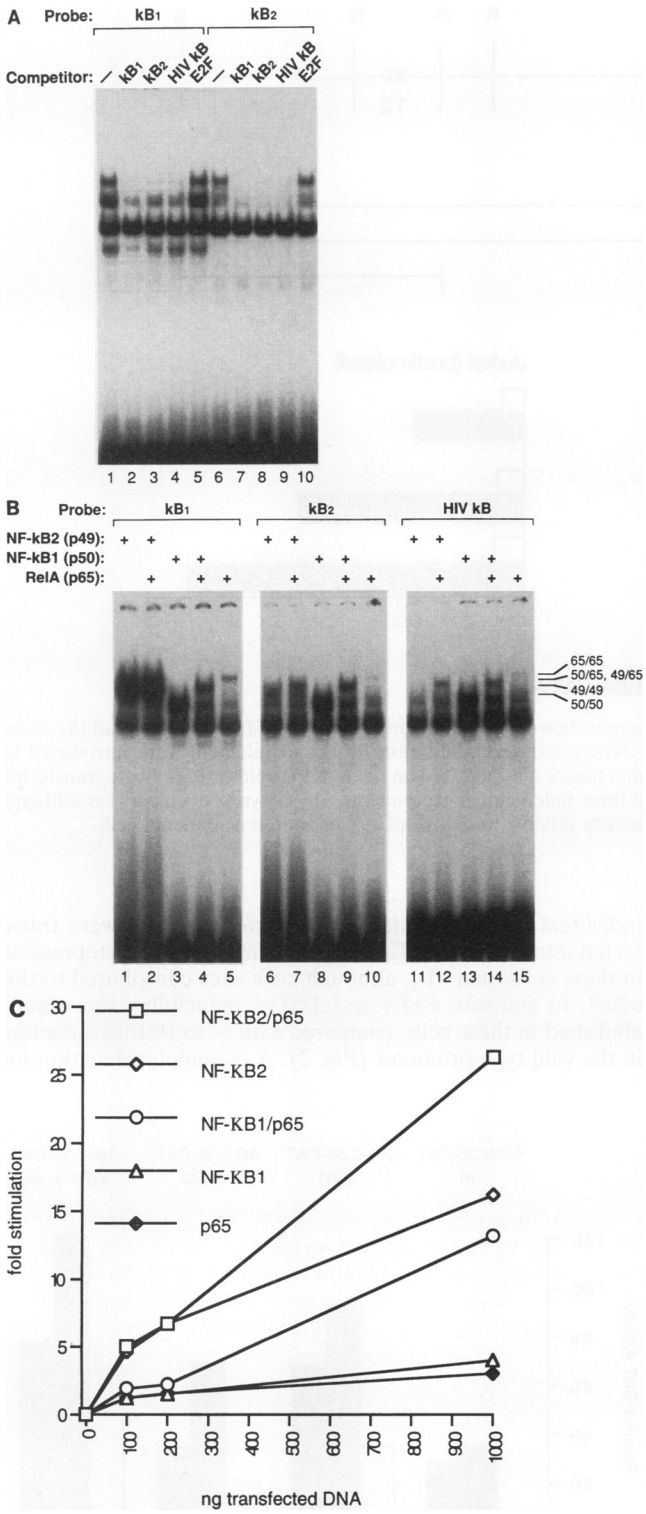


FIG. 3. Analysis of putative κB regulatory sites. (A) Identification of two κB-binding sites in the NFKB2 promoter. EMSAs were performed with nuclear extracts from PMA (10 ng/ml)-stimulated Jurkat T cells and oligonucleotides encompassing the κB_1 (−100 to −109; 5′ AGCTTGGGAATTCCCCACTAGTACG 3′; lanes 1 to 5), and κB_2 (−70 to −79; 5′ AGCTTGGGGCTTTCCCACTAGTACG 3′; lanes 6 to 10) sites of the NFKB2 promoter as radiolabeled probes. Unlabeled double-stranded oligonucleotides at 50-fold molar excess were used as competitors as indicated. (B) Preferential binding of NF-κB2 to κB motifs of its own promoter. EMSAs were performed with nuclear protein extracts of 293 cells transfected with expression vectors for NF-κB2 (p49), NF-κB1 (p50), RelA (p65), or combinations thereof and radiolabeled oligonucleotide probes of κB1 (lanes 1 to 5), κB2 (lanes 6 to 10), and HIV κB (lanes 11 to 15). The mobilities of the different homodimers and heterodimers are indicated. (C) NF-κB2 is regulated in an autoregulative manner. Jurkat T cells were transfected with 10 μg of the AS-0.36-CAT reporter plasmid and the indicated amounts of expression vectors encoding RelA (p65) (16, 19), NF-κB1 (p50) (6, 9, 13), NF-κB2 (p49) (15, 22), or combinations of RelA (p65) plus NF-κB1 (p50) and RelA (p65) plus NF-κB2 (p49). An expression vector encoding β-galactosidase was added in appropriate quantities to keep equal amounts of DNA in all transfections.

inserted downstream of P2 (11). Since PMA and TNF-α stimulation is mediated in part by NF-κB, these results suggested that NF-κB2 might be regulated in a positive autoregulatory manner.

Further 5′ deletion of 160 bp of the NFKB2 promoter region yielded the NS-0.2-CAT construct, which still contained the mapped transcription start site. This construct had almost completely lost its transcriptional activity relative to BS-0.9-CAT, KS-0.5-CAT, and AS-0.36-CAT. Therefore, we concluded that the 160 bp between the *Ava*I and *Nru*I sites contained sequences required for transcriptional activity and inducibility by PMA and TNF-α. Interestingly, two potential κB sites were identified within this region of the NFKB2 promoter.

NFKB2 is regulated by two functional κB-binding sites. To determine whether NF-κB participates in transcriptional regulation of NFKB2, we first tested the ability of these two putative κB motifs to bind NF-κB. EMSAs were performed with nuclear extracts from PMA-stimulated Jurkat cells and double-stranded oligonucleotides encompassing the sequence GGGAATTCCC (−100 to −109) (Fig. 3A, lanes 1 to 5) and GGGCTTTCCC (−70 to −79) (Fig. 3A, lanes 6 to 10). Four DNA-protein complexes could be distinguished. The upper two complexes were specific (lanes 1 and 6), as determined by competition with an excess of unlabeled double-stranded oligonucleotides representing κB_1 (lanes 2 and 7), κB_2 (lanes 3 and 8), and human immunodeficiency virus (HIV) κB (lanes 4 and 9) sites. No competition was observed with an unrelated site, E2F (lanes 5 and 10). These data indicated the presence of two NF-κB-binding sites at positions −100 and −70.

It has been shown that NF-κB subunits bind differentially to variant κB motifs (18). Therefore, binding specificities of different NF-κB subunits to κB_1 and κB_2 sites were determined. 293 cells were transfected with expression vectors encoding NF-κB1 (p50), NF-κB2 (p52 or p49), RelA (p65), or combinations thereof, and nuclear protein extracts were generated. DNA-protein complexes were analyzed in EMSAs using NFKB2 κB_1, κB_2, and HIV κB probes. NF-κB1 (p50) formed complexes of similar intensities with all probes tested (Fig. 3B, lanes 3, 8, and 13). Cotransfection with RelA (p65) resulted in the additional formation of a more slowly migrating, heterodimeric complex, which bound with comparable intensities to these probes (lanes 4, 9, and 14). In contrast, NF-κB2 (p49) displayed different DNA-binding characteristics. Homodimeric (p49/p49) and heteromeric (p49/p65) complexes bound strongly to the κB_1 site (lanes 1 and 2), with less affinity to the κB_2 motif (lanes 6 and 7), and only weakly to the HIV κB probe (lanes 11 and 12). Similar results were observed with NF-κB2 (p52), the processed form of NF-κB2 (p100) which is more widely found physiologically. Thus, NF-κB2 bound as homodimer and heterodimer preferentially to the κB_1 motif in its own promoter.

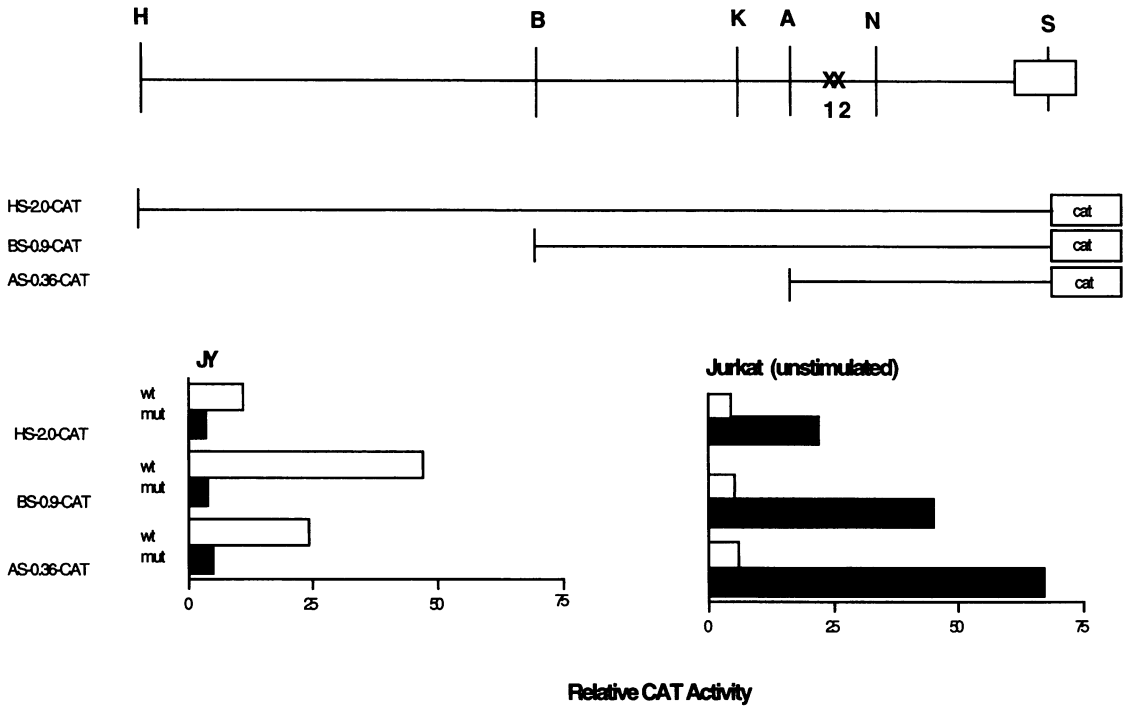


FIG. 4. Function of κB sites in the NFKB2 promoter. The schematic diagram shows the NFKB2 promoter region (see Fig. 1A) and the series of deletion and mutant κB reporter plasmids used for these experiments. Where indicated, mutations of the κB element were introduced by site-directed mutagenesis changing the sequence to GCTCAATCTCC. Plasmid names are indicated on the left. Plasmids (10 μg) were transfected into JY or unstimulated Jurkat cells. CAT activities, representative of at least three independent experiments, are shown as open bars for wild-type and filled bars for mutant plasmids. Relative CAT activity refers to CAT activity relative to that of pCAT basic in unstimulated cells.

Cotransfection experiments were performed to further characterize the functional role of NF-κB subunits in the regulation of the NFKB2 promoter in vivo. Expression vectors encoding truncated NF-κB1 (p50), NF-κB2 (p49), and RelA (p65) were cotransfected with the AS-0.36-CAT reporter plasmid into Jurkat cells (Fig. 2). Transfection of an NF-κB2 expression vector alone with the reporter plasmid resulted in a 15-fold stimulation of CAT activity, which could be increased to 25-fold by cotransfection with RelA (p65) (Fig. 3C). In contrast, NF-κB1 (p50) and RelA, alone or in combination, were less active. These results indicate that the NF-κB2 promoter can be activated in vivo by κB-binding proteins and most strongly by its own gene product. Thus, NF-κB2 is regulated in a positive autoregulatory fashion.

Negative regulatory elements overlap with the κB-binding sites in the NFKB2 promoter. To confirm the functional role of κB-binding proteins in the transcriptional regulation of NFKB2, transfections with several reporter plasmids were performed (Fig. 4 and 5). Wild-type and mutant P1 reporter plasmids were transiently transfected into JY and Jurkat cells. In the mutant reporter plasmids, one or both κB sites at −100 and −70 were changed to GCTCAATCTCC by site-directed mutagenesis. For this type of mutation, a complete loss of NF-κB binding and transactivation was shown previously (18). As expected, in JY cells, which constitutively express NF-κB, NF-κB2 reporters with mutant κB sites had almost completely lost their basal activity. These results supported the notion that the two κB elements can contribute to positive transcriptional activation of NF-κB2. In Jurkat cells, however, in which there is minimal NF-κB in the nuclei of unstimulated cells, mutation of the κB elements unexpectedly increased basal activity 5- to 10-fold (Fig. 4 and 5). To determine the contribution of the individual κB sites, mutant κB reporter plasmids were transfected into these cells. The κB_2 element showed less repression in these cells than κB_1, although both sites contributed to this effect. In contrast, PMA and TNF-α inducibility was almost abolished in these cells, compared with 5- to 10-fold induction in the wild-type promoter (Fig. 2). A possible explanation for

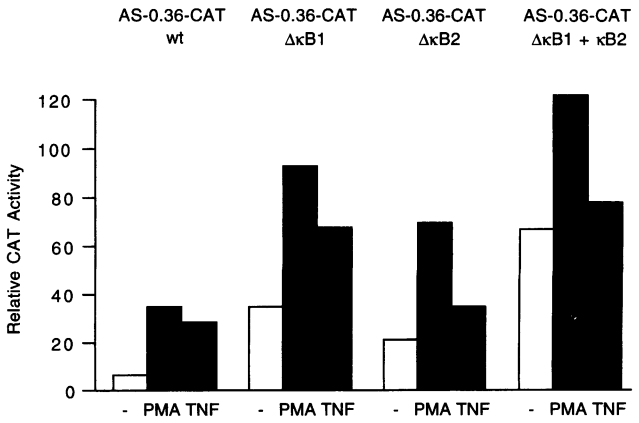


FIG. 5. Repressive elements overlap the κB-binding site in the NFKB2 promoter. Wild-type (wt) or mutant κB reporters (10 μg) with either or both κB sites of the NFKB2 promoter mutated to GCT CAATCTCC were transiently transfected into Jurkat cells. CAT activity was determined as described in Materials and Methods, and the data represent at least three independent transfections. Cells were stimulated as indicated. Relative CAT activity is defined in the legend to Fig. 2.

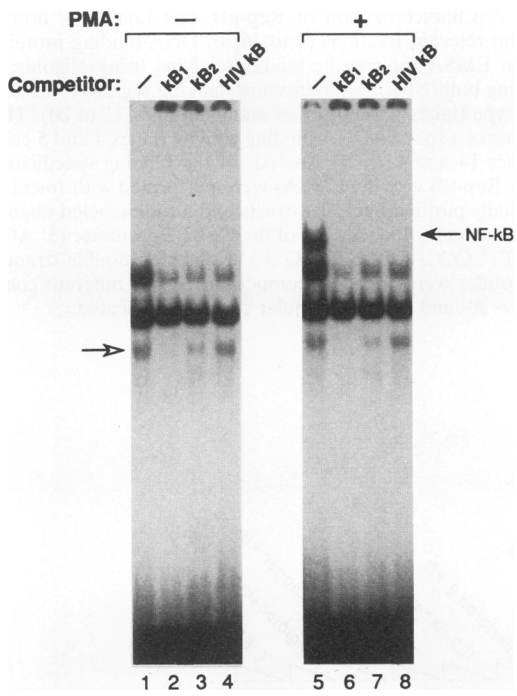


FIG. 6. Identification of a nuclear protein or protein complex in unstimulated cells binding to both NFKB2 κB elements but not to the HIV κB motif. EMSAs were performed with nuclear extracts from unstimulated or PMA (10 ng/ml)-stimulated Jurkat T cells and a radiolabeled oligonucleotide probe containing both NFKB2 κB sites and flanking regions (−63 to −114). Unlabeled oligonucleotides at 50-fold molar excess were used as specific competitors as indicated. The filled arrow indicates the NF-κB complex. The open arrow indicates a newly identified DNA-binding activity.

this phenomenon was that in the absence of NF-κB (e.g., in unstimulated T cells), a repressive protein or protein complex binds to a site overlapping the two κB sites at −100 and −70 and has an inhibitory effect on basal transcriptional activity. Mutation of the κB sites would inhibit binding of the putative repressor, resulting in an increased basal activity. In the presence of NF-κB (e.g., in B cells and stimulated T cells), the putative repressor might be eliminated from the overlapping binding site as NF-κB activates transcription. We therefore examined the abilities of these sites to bind other proteins.

A nuclear protein or protein complex binds the κB elements from NFKB2 in unstimulated T cells. To identify such DNA-binding activity in unstimulated cells, nuclear extracts of Jurkat cells were generated. A probe containing both κB sites from NFKB2 and the flanking regions (−63 to −114) was used (Fig. 1D). NF-κB complexes from PMA-induced Jurkat nuclear extracts bound to κB efficiently and were competed for by an excess of unlabeled κB sites from either of the two NFKB2 or HIV κB elements (Fig. 6, lanes 5 to 8).

In EMSAs with unstimulated and stimulated nuclear extracts, we identified an additional faster-migrating complex (Fig. 6, lanes 1 and 5) which was specifically competed for by κB_1, less effectively by κB_2, but not by canonical κB (lanes 2 and 3 versus lane 4 and lanes 6 and 7 versus lane 8). Further experiments showed that the κB motifs of NFKB2 without native flanking sequences were able to bind this newly identified protein complex. A probe containing the mutated κB site used in functional analysis failed to bind this protein (data not shown). Thus, we could identify a κB-binding activity in unstimulated nuclear extracts from Jurkat cells which preferentially bound the NFKB2 κB_1 and κB_2 elements but not the HIV κB site (lanes 1 to 4, open arrow). The detection of a complex migrating at the same position in EMSAs from stimulated nuclear extracts (Fig. 6, lanes 5 to 8; Fig. 3A, lanes 1 to 5) supports the hypothesis that there is an equilibrium for binding between NF-κB and this novel complex. Because of its putative repressive effect, we designated this factor Rep-κB.

Rep-κB DNA-binding activity was partially purified from unstimulated Jurkat T-cell nuclear extracts by using DNA-cellulose columns. Sixteen fractions were eluted with increasing salt concentration. Fractions were tested in EMSAs, using NFKB2 probes containing both κB sites (sequence from −63 to −114), wild type (Fig. 7A, lanes 1 to 10) and mutant (lanes 11 to 20). A specific binding activity which was eluted at 400 mM NaCl was identified in fractions 10 and 11. DNA-protein complexes were formed with wild-type (lanes 4 and 5) but not mutant (lanes 14 and 15) probes. Rep-κB activity was also identified in JY and HeLa cells (data not shown).

To investigate whether this observed DNA-binding activity was specific for the two NFKB2 κB sites or could also bind to other known κB elements, EMSAs were performed with partially purified Rep-κB and a variety of κB motifs in competition reactions. Two different concentrations of the competitor were used to estimate the affinity to the various sites. Rep-κB activity was competed for strongly by κB sites from NFKB2 (κB_1) (Fig. 7B, lanes 2 and 3), β_2-microglobulin (lanes 10 and 11), and beta interferon (lanes 16 and 17) and less efficiently by NFKB2 (κB_2) (lanes 4 and 5), class II major histocompatibility complex invariant chain (lanes 12 and 13), and both angiotensinogen κB elements (lanes 22 to 25). No competition was observed with sites from HIV (lanes 6 and 7), major histocompatibility complex (lanes 8 and 9), or NFKB1 (p105) (lanes 14 and 15). Thus, previously known κB motifs can be divided into two subsets: those which are capable of binding to the putative Rep-κB activity and those which are not.

Using the beta interferon κB probe (PRD2), we detected Rep-κB activity in EMSAs which was competed for by the sites described above. In supershift assays with antibodies against NFKB1, NFKB2, RelA, and c-Rel, we found no evidence that the putative Rep-κB activity contained any of these NF-κB subunits (data not shown). Recently, Thanos and Maniatis (26) described a high-mobility-group (HMG) I/Y protein binding to the center of the beta interferon κB motif. Although purified HMG I/Y binding to the beta interferon κB element could be detected, no interaction was observed with κB_1 (data not shown). We therefore suggest that this DNA-binding activity is unrelated to previously described κB-binding proteins, although it remains possible that it is related to a different HMG-like protein. This factor is presently undergoing further purification and characterization.

DISCUSSION

The transcription factor NF-κB is a complex composed of multiple, distinct subunits. The genes encoding this activity are immediate-early genes which are activated in response to a wide variety of stimuli, including mitogens, cytokines, and viral proteins. Upon induction, the inhibitory protein, IκB, dissociates from its cytoplasmic complex with p50 and p65, and the active complex is translocated to the nucleus. Induction by NF-κB is followed by rapidly increased expression of its target genes, most of them involved in the immune, inflammatory, and acute-phase responses. Activation of NF-κB does not require de novo protein synthesis, and its regulation occurs at

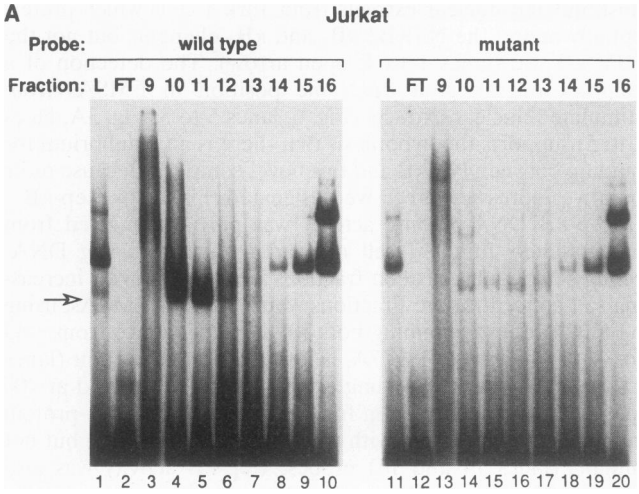
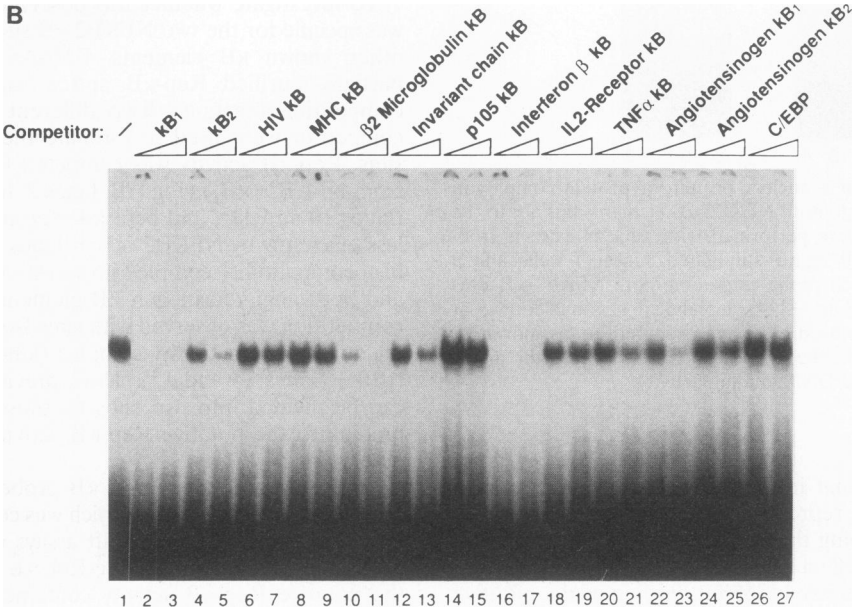


FIG. 7. Characterization of Rep-κB. (A) Load (L), flowthrough (FT), and relevant fractions (9 to 16) of DNA-binding proteins were tested in EMSA for specific binding activity, using oligonucleotides containing both NFKB2 κB sites and flanking regions (−63 to −114) as wild type (lanes 3 to 10) or as mutants (lanes 11 to 20). The open arrow marks a specific DNA-binding activity (lanes 4 and 5 compared with lanes 14 and 15). (B) Analysis of the binding specificity of the putative Rep-κB activity. EMSAs were performed with fraction 10 of the partially purified nuclear extracts and a radiolabeled oligonucleotide encompassing the κB_1 site of the NFKB2 promoter (5′ AGCTTG GGAATTCCCCACTAGTACG 3′). Unlabeled double-stranded oligonucleotides were added as competitors in two different concentrations at ~20- and ~100-fold molar excess as indicated.



a posttranslational level. However, maintenance of NF-κB activity requires ongoing protein synthesis and continuous stimulation, suggesting several modes of regulation.

The NFKB2 promoter showed characteristic features of a housekeeping gene: lack of classical TATA or CAAT elements and a high GC content. Housekeeping genes contain binding sites for ubiquitous transactivators conferring high basal levels of transcription. Two alternative promoters were identified, although no functional differences have yet been noted between them. Upon stimulation with PMA or TNF-α, for example, both promoters were induced. Interestingly, we find that NFKB2 is activated by its own gene product or related NF-κB subunits. The NFKB2 κB_1 site resembles a perfect palindromic sequence. It was previously shown also that NF-κB2 binds preferentially to palindromic sequences (18); therefore, it might be expected that NF-κB2 would bind with stronger affinity to this site compared with NF-κB1. Indeed, these findings were consistent with our functional data. In cotransfection assays, NFKB2 reporter plasmids were transactivated most strongly by NF-κB2/RelA (p49/p65) heterodimers. Thus, the control of NFKB2 transcription is autoregulatory, as shown for other family members, including NF-κB1 and IκB-α (4, 24, 25).

In addition to this positive autoregulatory loop, we have also demonstrated that the κB elements in the NFKB2 promoter mediate transcriptional repression in the absence of NF-κB. Mutations of these κB sites caused an increase in basal activity. This effect is mediated by both κB sites, the κB_1 site being more effective. This mechanism of repressing basal NF-κB2 transcription in an inactivated state enables the cell to tightly control NF-κB2 activity.

We identified a nuclear protein complex which binds specifically to the NFKB2 κB elements in unstimulated cells, with higher affinity to the κB_1 site. Therefore, the binding and functional data were consistent. Because of its putative repressive effect, we have termed this DNA-binding activity Rep-κB. Interestingly, Rep-κB activity was not able to bind to the classical κB sites of the HIV-1 or major histocompatibility complex class I enhancer. In competition experiments, we could demonstrate that Rep-κB can bind to κB sites present in the beta interferon, β_2-microglobulin, invariant chain, and angiotensinogen enhancers. Thus, κB motifs can be divided

into two subsets: those which can bind the putative Rep-κB activity and those which cannot. These data demonstrate the existence of a novel mode of κB-dependent regulation mediated by a repressive activity bound to a specific class of κB sites.

ACKNOWLEDGMENTS

We thank Dimitri Thanos and Thomas Maniatis for helpful discussions and providing HMG I/Y antiserum and protein; Donna Gschwend and Karen Carter for secretarial assistance; Garry Nolan for providing 293 cells and protocols; and Edward Hanley, Kwanyee Leung, Neil Perkins, Bei-yue Wu, Colin Duckett, and other members of the Nabel laboratory for helpful advice and comments.

This work was supported in part by Deutsche Forschungsgemeinschaft fellowships (S.L. and R.M.S.) and grants from Deutsche Forschungsgemeinschaft (Li516/2-1 [S.L.]) and the National Institutes of Health (RO1 AI29179 [G.J.N.]).

REFERENCES

1. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53:**211–217.
2. **Bours, V., P. R. Burd, K. Brown, J. Villalobos, S. Park, R. Ryseck, R. Bravo, K. Kelly, and U. Siebenlist.** 1992. A novel mitogen-inducible gene product related to p50/p105-NF-κB participates in transactivation through a κB site. Mol. Cell. Biol. **12:**685–695.
3. **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.
4. **Chiao, P. J., S. Miyamoto, and I. M. Verma.** 1994. Autoregulation of IκB-α activity. Proc. Natl. Acad. Sci. USA **91:**28–32.
5. **Duckett, C. S., N. D. Perkins, T. F. Kowalik, R. M. Schmid, E. S. Huang, A. S. Baldwin, Jr., and G. J. Nabel.** 1993. Dimerization of NF-κB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an IκB-α (MAD-3). Mol. Cell. Biol. **13:**1315–1322.
6. **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.
7. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. Nature (London) **339:**70–73.
8. **Hohmann, H.-P., R. Remy, C. Scheidereit, and A. P. G. M. van Loon.** 1991. Maintenance of NF-κB activity is dependent on protein synthesis and the continuous presence of external stimuli. Mol. Cell. Biol. **11:**259–266.
9. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. LeBail, M. B. Urban, P. Kourilsky, P. A. 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.
10. **Leung, K., and G. J. Nabel.** 1988. HTLV-I transactivator induces interleukin-2 receptor expression through an NF-κB-like factor. Nature (London) **333:**776–778.
11. **Liptay, S., R. M. Schmid, E. G. Nabel, and G. J. Nabel.** 1994. Unpublished observations.
12. **Liptay, S., R. M. Schmid, N. D. Perkins, P. Meltzer, M. R. Altherr, J. D. McPherson, J. Wasmuth, and G. J. Nabel.** 1992. Related subunits of NF-κB map to two distinct loci associated with translocations in leukemia, NFKB1 and NFKB2. Genomics **13:**287–292.
13. **Meyer, R., E. N. Hatada, H. Hohmann, M. Haiker, C. Bartsch, U. Rothlisberger, H. Lahm, E. J. Schlaeger, A. P. G. M. Van Loon, 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 a. Proc. Natl. Acad. Sci. USA **88:**966–970.
14. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326:**711–713.
15. **Neri, A., C. C. Chang, L. Lombardi, M. Salina, P. Corradini, 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.
16. **Nolan, G. P., S. Ghosh, H. 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.
17. **Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursors. Annu. Rev. Biochem. **55:**1119–1150.
18. **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.
19. **Ruben, S. M., P. J. Dillon, R. Schreck, T. Henkel, C. Chen, M. Maher, P. A. Baeuerle, and C. A. Rosen.** 1991. Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-κB. Science **251:**1490–1493.
20. **Ryseck, R., 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.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 31–57. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **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.
23. **Sen, R., and D. Baltimore.** 1986. Inducibility of kappa immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47:**921–928.
24. **Stein, B., P. C. Cogswell, and A. S. Baldwin, Jr.** 1993. Functional and physical associations between NF-κB and C/EBP family members: a *rel* domain-bZIP interaction. Mol. Cell. Biol. **13:**3964–3974.
25. **Ten, R. M., C. V. Paya, N. Israel, O. LeBail, M. G. Mattei, J. L. Virelizier, P. Kourilsky, and A. Israel.** 1992. The characterization of the promoter of the gene encoding the p50 subunit of NF-kappa B indicates that it participates in its own regulation. EMBO J. **11:**195–203.
26. **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-beta gene. Cell **71:**777–789.