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NF-κB Subunit-Specific Regulation of the Interleukin-8 Promoter

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Interleukin-8 (IL-8), a chemotactic cytokine for T lymphocytes and neutrophils, is induced in several cell types by a variety of stimuli including the inflammatory cytokines IL-1 and tumor necrosis factor alpha TNF-α. Several *cis* elements, including a binding site for the inducible transcription factor NF-κB, have been identified in the regulatory region of the IL-8 gene. We have examined the ability of various NF-κB subunits to bind to, and activate transcription from, the IL-8 promoter. A nuclear complex was induced in phorbol myristate acetate-treated Jurkat T cells which bound specifically to the κB site of the IL-8 promoter and was inhibited by addition of purified IκBα to the reaction mixture. Only antibody to RelA (p65), but not to NFKB1 (p50), NFKB2 (p50B), c-Rel, or RelB was able to abolish binding, suggesting that RelA is a major component in these κB binding complexes. Gel mobility shift analysis with in vitro-translated and purified proteins indicated that whereas the κB element in the human immunodeficiency virus type 1 long terminal repeat bound to all members of the κB/Rel family examined, the IL-8 κB site bound only to RelA and to c-Rel and NFKB2 homodimers, but not to NFKB1 homodimers or heterodimers of NFKB1-RelA. Transient transfection analysis demonstrated a κB-dependent expression of the IL-8 promoter in a human fibrosarcoma cell line (8387) and in Jurkat T lymphocytes. Cotransfection with various NF-κB subunits indicated that RelA and c-Rel, but neither NFKB1 nor heterodimeric NFKB1-RelA, was able to activate transcription from the IL-8 promoter. Furthermore, cotransfection of NFKB1 and RelA, although able to support activation from the human immunodeficiency virus type 1 long terminal repeat, failed to activate expression from the IL-8 promoter. Antisense oligonucleotides to RelA, but not NFKB1, inhibited phorbol myristate acetate-induced IL-8 production in Jurkat T lymphocytes. These data demonstrate the differential ability of members of the κB/Rel family to bind to, and activate transcription from, the IL-8 promoter. Furthermore, while providing a novel example of a κB-regulated promoter in which the classical NF-κB complex is unable to activate transcription from the κB element, these data provide direct evidence for the role of RelA in regulation of IL-8 gene expression.

Infiltration of leukocytes is a hallmark of the inflammation process and is mediated by several chemotactic factors, one of which is interleukin-8 (IL-8). Although the constitutive production of IL-8 appears to be limited in most cell types examined, a wide variety of cells including lymphocytes, monocytes/macrophages, fibroblasts, keratinocytes, and endothelial cell types can be induced to produce IL-8 by a variety of stimulatory agents including IL-1, tumor necrosis factor, and tumor promoters (28, 37, 45, 61). IL-8 has diverse biological properties including chemotaxis of neutrophils and T lymphocytes both in vitro and in vivo (45), regulation of cell adhesion properties (21), activation of neutrophils (13, 35), and modulation of histamine release (11, 26). These observations implicate IL-8 as a key factor in the pathogenesis of inflammatory diseases (21, 37); however, the molecular mechanisms which govern the regulation of the IL-8 gene are presently unclear.

Previous studies have identified a minimal region in the IL-8 promoter that is necessary for IL-8 gene activation (36). Nucleotide sequence analysis of this region demonstrated potential binding sites for several nuclear factors including C/EBPβ (NF-IL6), NF-κB, AP-1, and octamer binding proteins (38). Studies have suggested that the AP-1 and octamer binding motifs are dispensable for IL-8 gene activation; however, the NF-κB and the NF-IL6 binding sites appear to be essential for activation (36). Gel mobility shift analysis has indicated that a NF-IL6-like factor constitutively binds to the region between −94 and −81, whereas an inducible factor binds to the NF-κB-like binding element in the region between −80 and −71 of the IL-8 gene (36).

NF-κB is an inducible transcription factor that was originally identified as a heterodimeric complex consisting of a 50-kDa subunit (originally called p50 and now designated NFKB1) and a 65-kDa subunit (originally called p65 and now designated RelA) (reviewed in references 1, 18, and 30). NF-κB was initially characterized by its ability to bind to a *cis* element in the immunoglobulin light chain enhancer (56) and has subsequently been shown to bind to slight variations of this sequence in the regulatory regions of several other genes (20). The cloning of the genes for both NFKB1 (5, 17, 24, 33) and RelA (44, 50) revealed a high degree of homology to the proto-oncogene product c-Rel (7, 48). Other members of the NF-κB/Rel gene family include NFKB2 (previously designated p50B [4, 32, 43, 54]), RelB (51, 53), an alternatively spliced variant of RelA (42, 52), v-Rel (3, 59), and the *Drosophila* maternal morphogen dorsal (23, 60). All of the members of the κB/Rel family share homology within a 300-amino-acid region responsible for DNA binding and dimerization (1).

Initially it was postulated that only the NFKB1 subunit of NF-κB conferred DNA-binding activity and the RelA subunit provided transcriptional activation properties. Now it is apparent that all of the members of the κB/Rel family (as either homo- or heterodimeric complexes with other κB/Rel members) can bind to DNA. In addition to RelA, transcriptional activation properties have been identified with c-Rel (8) and RelB (14). Although not demonstrated in mammalian

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cells, NFKB1 also has transcriptional activation properties in vitro (15, 25) and in *Saccharomyces cerevisiae* (34). Because the members of the κB/Rel family can form various complexes with each other, one level of regulation imparted by this family of transcription factors likely involves the formation of different heterodimeric complexes. The identification of optimal binding sites for homodimers of NFKB1 and RelA led to the observation that κB binding elements which bind selectively to certain κB/Rel family members exist (27, 62, 63). Consequently, it is likely that differential binding of certain κB/Rel subunits to *cis*-acting elements in the regulatory region of genes represents an additional mechanism by which this family of transcription factors can selectively regulate gene expression.

Although binding of nuclear factors to the κB element in the IL-8 promoter has been observed (36), it has not been directly tested as to whether these factors constitute members of the κB/Rel family. Furthermore, the abilities of various members of the κB/Rel family to bind to this site and regulate expression from the promoter have not been addressed. Inspection of the κB binding element within the IL-8 promoter revealed the sequence 5′-GTGGAATTT CC-3′ that was identical to a site which we previously identified as being unable to bind to NFKB1 homodimers (27, 38). This suggested that the IL-8 promoter may bind differentially to various members of the κB/Rel family and that its expression may be regulated in response to only a subset of κB/Rel factors. The findings reported here demonstrate that unlike most other well-characterized κB binding elements, the IL-8 κB motif is unable to bind NFKB1 homodimers and NFKB1-RelA heterodimers, although it does bind RelA, NFKB2, and c-Rel homodimers. Transcriptional activation studies support the in vitro binding data. These observations suggest that prototypical NF-κB is not involved in κB-regulated expression of the IL-8 gene and provide direct support for the selective involvement of RelA in IL-8 gene expression.

MATERIALS AND METHODS

Cell culture and transfection. Jurkat T lymphocytes and U937 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and L-glutamine (2 mM) in a humidified incubator containing 5% CO_2. The human fibrosarcoma cell line 8387 (a generous gift of Alberto Mantovani) was grown in Dulbecco modified Eagle medium containing 5% fetal bovine serum and 100 U of penicillin per ml. For transient transfection of Jurkat T lymphocytes, 10^7 cells were transfected by the DEAE-dextran procedure (47) and harvested 48 h post-transfection. In some samples, cells were stimulated with phorbol myristate acetate (PMA) 12 h prior to harvest. 8387 cells were grown in individual 100 mM culture plates to approximately 80% confluency, at which time they were transfected by a modified calcium phosphate procedure (GIBCO/BRL). Cells were collected by scraping, suspended in 200 μl of 0.25 M Tris-Cl (pH 8.0), and lysed by three cycles of repeated freeze-thaw. Chloramphenicol acetyltransferase (CAT) assays were then performed by the standard method of Gorman et al. (19). In some cases, CAT assays were quantitated by Betascope analysis (Betagen). All assays were performed at least three times, and representative results are shown.

ELISA for IL-8. To measure the level of secreted IL-8 in the culture medium of Jurkat T lymphocytes, cells were either untreated or treated for 48 h with PMA (50 ng/ml). The sense and antisense phosphorothioate analogs of oligonucleotides were prepared as previously described (41). Oligonucleotides were added to the culture medium at a final concentration of 40 μM 5 h prior to stimulation with PMA. Supernatants were collected and IL-8 concentration was measured by enzyme-linked immunosorbent assay (ELISA; Amersham). The DNA sequences of the oligonucleotides were as follows: RelA antisense, 5′-GGGGAACAGTTCGT CCATGGC-3′; RelA sense, 5′-GCCATGGACGAACTGTT CCCC-3′; NFKB1 antisense, 5′-TGGATCTTCTGCCATT CT-3′; and NFKB1 sense, 5′-AGAATGGCAGAAGATC CA-3′.

Oligonucleotides and preparation of radiolabeled probes. Oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer. To prepare ^{32}P-labeled double-stranded oligonucleotides, a short, complementary oligonucleotide (5′-CATAGGATCTCGAGC-3′) was annealed and extended with reverse transcriptase (U.S. Biochemicals) and 20 μCi of each [^{32}P]deoxynucleoside triphosphate (dNTP) (27). Unincorporated nucleotides were removed by column chromatography over a Sephadex G-25 column (5′→3′, Inc.). The DNA sequences of the oligonucleotide probes used are as follows: Ig/HIV, 5′-GTAGGG GACTTTCCGCTCGACATCCTATG-3′; 65-9, 5′-GTAGTG GAATTTCCGCTCGAGATCCTATG-3′; IL-8 (−93/−66), 5′-AGTTGCAAATCGTGGAATTTCCTCTGACGCTCGA GATCCTATG-3′; and IL-8 (−κB) mut., 5′-AGTTGCAAA TCCTGCAATGTCGTCTGACGCTCGAGATCCTATG-3′.

Gel mobility shift assay and nuclear extract preparation. Bacterially expressed NFKB1, RelA, c-Rel, and IκBα (MAD-3) were prepared and purified as previously described (16, 51, 52). NFKB2 was purchased from Promega. In vitro-translated proteins were prepared as previously described (9) by standard in vitro transcription and translation in a rabbit reticulocyte lysate (Promega). Nuclear extracts were prepared by a modification of the method described by Dignam et al. (12). Briefly, after collection and washing of cells in phosphate-buffered saline, the cell pellet was suspended in buffer A (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF]) plus 0.1% Triton X-100 and incubated at 4°C, and the nuclei were collected by centrifugation. The nuclear pellet was then washed in buffer A without Triton X-100, resuspended in buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 2 mM PMSF), and incubated for 30 min at 4°C. After centrifugation at top speed in a microcentrifuge (Eppendorf) the supernatant was dialyzed against modified buffer D (20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.5 mM PMSF) and stored at −80°C. Binding reactions were carried out in a 10- or 20-μl binding reaction mixture consisting of 20 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 1 mM DTT, 0.1% Nonidet P-40, 0.5 mM PMSF, 1 mg of bovine serum albumin per ml, and 2 μg of poly(dI-dC). Approximately 4 μg of nuclear extract and 25 ng of purified protein were used. ^{32}P-labeled oligonucleotide probe (~0.5 ng; 50,000 cpm) was incubated with the protein for 20 min at room temperature. In some samples unlabeled competitor (used at 80-fold molar excess) or antisera were added 10 min after the addition of probe and incubated for an additional 10 min. Anti-NFKB1, RelA, and c-Rel antisera were obtained from Santa Cruz Biotechnology, Inc. Anti-NFKB2 is a anti-peptide antisera and was generously supplied by U. Siebenlist (National Institutes of Health), and anti-RelB is a polyclonal antisera

raised against bacterially expressed human RelB. The samples were electrophoresed through a 4% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA. All experiments were performed two or three times, and results of a representative experiment are shown.

Plasmid constructions. The eukaryotic expression vectors used to encode NFKB1, RelA, and the chimeric proteins NFKB1-RelA and c-Rel–RelA have been described previously (27, 52). The reporter plasmids $p(HIV/Ig)_4CAT$ and $p(65\text{-}9)_4CAT$ contain four tandem copies of the individual DNA sequences cloned upstream of a minimal human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) and fused to the coding region of the bacterial CAT gene (27). To prepare the plasmid containing the promoter region of the IL-8 gene, primers were designed according to the published sequence (38) and used to amplify human genomic DNA by polymerase chain reaction (PCR). The resulting amplicon was cloned into the vector pBLCATPA which contains the simian virus 40 polyadenylation signal cloned downstream of the CAT gene. Point mutations within the IL-8 promoter sequence were generated by PCR-mediated mutagenesis (22). All plasmid constructions were confirmed by automated dideoxy sequencing with dye terminators on an Applied Biosystems automated DNA sequencer.

RESULTS

κB element-dependent binding and transcriptional activation of κB/Rel subunits. The unbiased selection of DNA sequences that bind to homodimers of NFKB1, RelA, and c-Rel has been previously described (27). Analysis of the binding specificities of these DNA sequences revealed that many bind differentially to other members of the κB/Rel family. For example, several sequences that were selected for binding to RelA are unable to bind to NFKB1. One sequence that was selected for binding to RelA was identical to the κB motif found in the regulatory region of the IL-8 gene (GTGGAATTTCC), and initial analysis of this site indicated that NFKB1 homodimers were unable to bind to this site (27). To examine in more detail the binding of κB/Rel subunits to the κB motif found in the IL-8 promoter, gel mobility shift assays were performed with in vitro-translated proteins and by using a minimal binding element as a probe. As shown in Fig. 1A, the DNA probes for the binding experiments contained an 11-nucleotide κB site either from the immunoglobulin light chain enhancer and the HIV LTR (designated Ig/HIV) or from the site present in the IL-8 promoter (designated 65-9). As shown in Fig. 1B, both NFKB1 (lane 1) and RelA (lane 4) bound to the Ig/HIV site. When both NFKB1 and RelA were cotranslated together, an intermediate complex (representing heterodimeric NFKB1-RelA) bound to the Ig/HIV site with a mobility intermediate to either of the individual homodimers (lane 2). This complex is apparent in all of the translation lysates with binding to the Ig/HIV site and represents binding of endogenous NF-κB. In contrast, neither NFKB1 homodimers nor NFKB1-RelA heterodimers bound to the κB element from the IL-8 promoter (lanes 5 and 6). However, RelA homodimers were able to bind to this site (lane 8). The differential binding affinities of these two κB elements is also apparent by the binding of endogenous factors present in unprogrammed reticulocyte lysate. Whereas binding of NFKB1-RelA heterodimers and NFKB1 homodimers is apparent with the Ig/HIV site (lane 3), neither of these complexes bound to the 65-9 probe (lane 7). Only binding of endogenous RelA was observed with probe 65-9. These data provide further evidence that the κB binding motifs from in the immunoglobulin enhancer–HIV LTR or the IL-8 promoter bind selectively to different κB/Rel complexes.

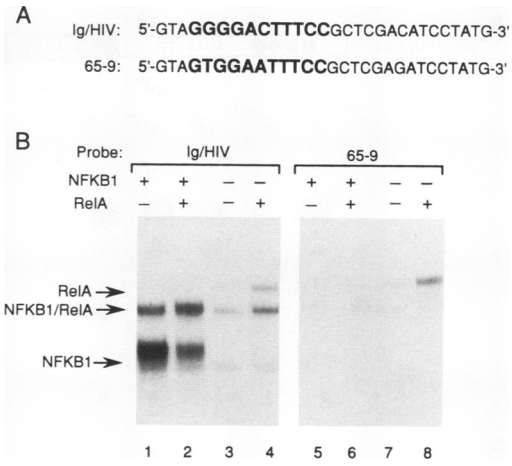

FIG. 1. Gel mobility shift assay. (A) DNA sequence of oligonucleotides used for the gel mobility shift assay. The specific κB element is indicated in boldface. Oligonucleotides were made double stranded by annealing a short oligonucleotide complementary to the 3′ end and extension with [^{32}P]dNTPs and reverse transcriptase. (B) NFKB1 (lanes 1 and 5) and RelA (lanes 4 and 8) were translated alone or together (lanes 2 and 6) and bound to ^{32}P-labeled probes containing the κB element found in the immunoglobulin light chain enhancer and HIV-1 LTR (Ig/HIV) (lanes 1 to 4) or the κB element in the IL-8 promoter (65-9) (lanes 5 to 8). Binding reactions and electrophoresis were performed as described in Materials and Methods. Equivalent amounts of translated protein and radiolabeled oligonucleotide were used for each sample. The formation of specific complexes is indicated on the left.

To examine the abilities of these two κB elements to activate NF-κB-dependent transcriptional activation, transient transfections were performed in Jurkat T lymphocytes. For these experiments, four tandem copies of the individual κB elements were cloned to replace the authentic κB elements present in the HIV-1 LTR (49) and fused upstream of the bacterial CAT gene. Previous studies have demonstrated that the HIV-1 LTR is responsive to NF-κB and that induction is mediated through the NF-κB motif (40, 49). To assay for transcriptional activation, Jurkat T cells were transfected with an expression vector encoding NFKB1, RelA, or the DNA-binding domains of NFKB1 or c-Rel fused the carboxy-terminal activation domain of RelA (27). These chimeric proteins contain the DNA-binding specificity of NFKB1 or c-Rel, while the transcriptional activation functions are provided by the C terminus of RelA. The NFKB1-RelA protein was necessary to demonstrate NFKB1-dependent activation, since little to no transcriptional activation is seen with the wild-type NFKB1 protein in mammalian cells (2, 5, 53, 55). As shown in lanes 2 of Fig. 2, induction of Jurkat T lymphocytes with PMA activated transcription from the Ig/HIV κB motif (Fig. 2A) and to a much lesser extent the 65-9 motif (Fig. 2B). CAT expression directed by both the Ig/HIV and 65-9 sites was activated by RelA (lanes 5) and c-Rel/RelA (lanes 6); however, NFKB1-dependent activation was only observed with the Ig/HIV site (lane 4). The ability of the c-Rel DNA-binding domain to activate transcription from both sites supports in vitro binding studies with c-Rel to these sites (Fig. 3C) (27). Activation was specific for the κB motif, as no CAT expression was

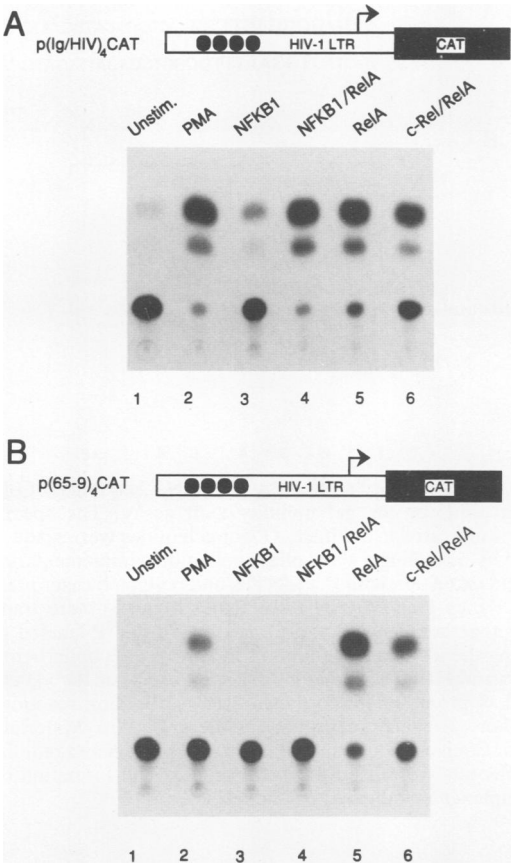

FIG. 2. Differential transcriptional activation mediated by the IL-8 κB element. Jurkat T lymphocytes were transfected with 1 μg of p(Ig/HIV)$_4$CAT (A) or p(65-9)$_4$CAT (B) and 2 μg of expression vectors encoding NFKB1 (lanes 3), the chimeric NFKB1-RelA (lanes 4), RelA (lanes 5), or the chimeric c-Rel–RelA (lanes 6). See text for a description of the expression vectors encoding the chimeric proteins. In addition, Jurkat T lymphocytes were either unstimulated (unstim.; lanes 1) or stimulated with 50 ng of PMA per ml 36 h after transfection. Cells were harvested at 48 h posttransfection, and CAT assays were performed as described in Materials and Methods.

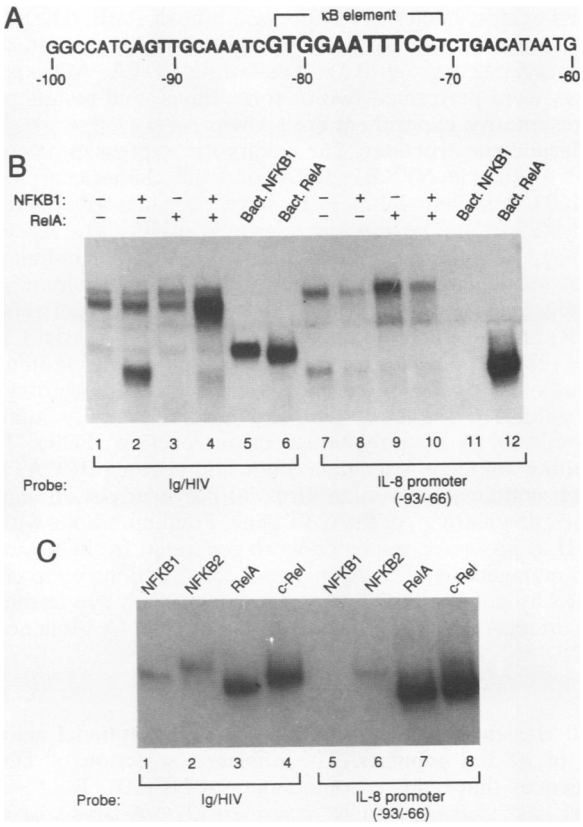

FIG. 3. Differential binding of κB/Rel complexes to the IL-8 promoter. (A) DNA nucleotide sequence of the IL-8 promoter. An oligonucleotide containing nucleotides −93 to −66 (boldface) of the IL-8 promoter was used in the gel mobility shift assays. (B) NFKB1 (lanes 2 and 8), RelA (lanes 3 and 9), or NFKB1 and RelA (lanes 4 and 10) were translated in a rabbit reticulocyte lysate and used in electrophoretic mobility shift assays. Purified, bacterially expressed NFKB1 (lanes 5 and 11) or RelA (lanes 6 and 12) were also used. (C) Purified, bacterially expressed NFKB1 (lanes 1 and 5), NFKB2 (lanes 2 and 6), RelA (lanes 3 and 7), and c-Rel (lanes 4 and 8) were tested for binding to the IL-8 promoter. The DNA probes consisted of oligonucleotides containing the Ig/HIV κB site (lanes 1 to 6 [B] and lanes 1 to 4 [C]) or the region from −93 to −66 of the IL-8 promoter (lanes 7 to 12 [B] and lanes 5 to 8 [C]). Binding reactions were performed as described in Materials and Methods.

observed with a reporter plasmid which contains several point mutations within the κB motif that abolish binding to NF-κB (27). These data support the in vitro observations demonstrating differential binding of the NF-κB subunits to the Ig/HIV and the IL-8 κB motif. Furthermore, the observation that a differential activity is seen following PMA stimulation suggests that PMA-inducible κB factors bind differentially to these two κB motifs.

Differential binding and transcriptional activation of the IL-8 promoter by κB/Rel subunits. By using a minimal κB element, the above observations suggested that differential binding of κB/Rel subunits to the κB element in the IL-8 promoter allows selective regulation of the IL-8 gene. Since it has yet to be determined whether the κB/Rel family of proteins can directly bind to, and regulate transcription of, the IL-8 promoter, we analyzed the abilities of recombinant κB/Rel proteins to bind to the intact IL-8 promoter sequence. Gel mobility shift assays were performed with both in vitro-translated and bacterially expressed, purified proteins. A 28-bp fragment from positions −93 to −66 which encompasses the κB element of the IL-8 promoter/enhancer was used as a probe (Fig. 3A). As shown in Fig. 3B, in vitro-translated NFKB1, RelA, and heterodimeric NFKB1-RelA were able to bind to the Ig/HIV κB site (lanes 2, 3, and 4, respectively). In addition, purified NFKB1 and RelA bound well to this site (lanes 5 and 6). In contrast, neither NFKB1 homodimers (lanes 8 and 11) nor NFKB1-RelA heterodimers (lane 10) were able to bind the −93/−66 IL-8 promoter fragment. In vitro-translated (lane 9) and bacterially expressed (lane 12) RelA, however, were able to bind to the IL-8 promoter fragment. The binding of these κB/Rel factors is similar to that observed with the minimal κB element (Fig. 1B), suggesting that flanking sequences of the IL-8 promoter do not contribute significantly to the binding specificity of κB/Rel proteins. The faint band observed in the NFKB1-translated lane (lane 8) does not represent binding of NFKB1 because there is no increase in binding compared with that in the unprogrammed lysate control (lane 7). In fact, translation of NFKB1 resulted in a decrease in the binding of endogenous RelA in the lysate, presumably by

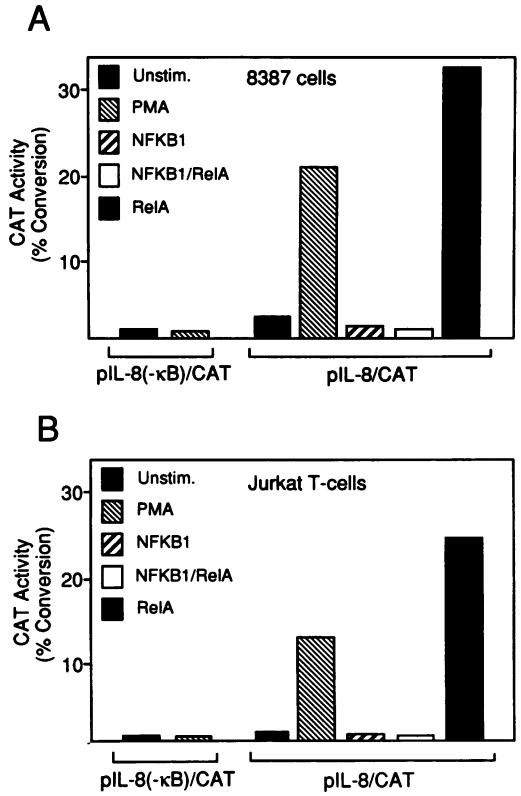

FIG. 4. κB/Rel-specific transcriptional activation of the IL-8 promoter. 8387 cells (A) or Jurkat T lymphocytes (B) were transfected with 10 μg (A) or 3 μg (B) of pIL-8/CAT or pIL-8(−κB)/CAT containing a 4-nucleotide mutation in the κB element. Cells were cotransfected with 1 μg of expression vectors encoding NFKB1, RelA, or the chimeric NFKB1-RelA proteins. Cells were also stimulated with PMA 36 h after transfection. Cells were harvested 48 h after transfection, and CAT assays were performed as described in Materials and Methods. CAT activity is expressed as percent conversion of the nonacetylated to the acetylated form.

creating heterodimeric complexes of NFKB1/RelA which are unable to bind to this probe. The slower-mobility band observed in lanes 7 to 10 represents the binding of endogenous RelA to the IL-8 promoter fragment. This is demonstrated by the increase in intensity of this band in the RelA-translated sample (lane 9) and the ability of anti-RelA antibody to supershift this complex (data not shown). In addition to the binding of NFKB1 and RelA, NFKB2 and c-Rel were able to bind to the κB element in the IL-8 promoter (Fig. 3C). Taken together, these data demonstrate that (i) selected members of the κB/Rel family can directly bind to the IL-8 promoter independent of other nuclear factors and (ii) only a subset of the κB/Rel complexes analyzed are able to bind to this promoter element.

To determine the ability of the NFKB1 and RelA subunits of NF-κB to regulate transcription from the IL-8 promoter, transient transfections were performed in Jurkat T lymphocytes and a human fibrosarcoma cell line (8387) which has previously been shown to express IL-8 mRNA and protein after induction (31). The reporter construct contained a 518-nucleotide fragment (−420 to +101) of the IL-8 gene cloned upstream of the bacterial CAT gene (pIL-8/CAT). As shown in Fig. 4, minimal amounts of basal expression from the promoter were observed in unstimulated 8387 (Fig. 4A) and Jurkat T cells (Fig. 4B). A mutant reporter which contains a 4-nucleotide substitution in the κB site of the promoter [pIL-8(−κB)/CAT] and eliminates binding of RelA to the promoter (data not shown) resulted in slightly decreased basal levels of expression. Stimulation of either 8387 or Jurkat T cells with PMA resulted in a substantial increase in CAT activity directed by pIL-8/CAT which was abolished by a mutation in the κB element. In addition, stimulation of 8387 cells with recombinant IL-1 and tumor necrosis factor alpha resulted in significant CAT activity (data not shown). These results indicate the requirement of an intact κB element for PMA-induced expression of the IL-8 gene in 8387 cells and Jurkat T lymphocytes.

Since the in vitro binding experiments (Fig. 1 and 3) indicated that RelA, but neither NFKB1 nor NFKB1-RelA heterodimers, could bind to the κB site in the IL-8 promoter, transient transfections were performed with the individual κB/Rel members to determine if, in vivo, a differential effect of NFKB1 and RelA is observed in transcriptional activation (Fig. 4). As expected, transfection with the NFKB1 expression vector resulted in little to no transcriptional activation in either cell type. We next tested the ability of the chimeric NFKB1-RelA protein to elicit NFKB1 binding-dependent transcriptional activation. Transfection with this chimeric expression vector did not result in activation of the IL-8 promoter in either cell type. This construct was, however, able to support activation from the Ig/HIV binding site in 8387 cells (data not shown) and Jurkat T lymphocytes (Fig. 2). Transfection with the RelA expression vector resulted in substantial activation of the IL-8 promoter in both 8387 and Jurkat T cells. The activation by RelA was specific for the κB element in the IL-8 promoter, since no activation was observed with the pIL-8(−κB)/CAT reporter (data not shown). Transfection of an expression vector encoding the chimeric c-Rel–RelA fusion protein and a full-length c-Rel clone also resulted in transcriptional activation (although less than RelA), suggesting that overexpression of c-Rel can activate transcription from the IL-8 promoter.

Since NF-κB (NFKB1-RelA heterodimers) is unable to bind to the κB element in the IL-8 promoter in vitro (Fig. 1 and 3), we wished to determine the contribution of NFKB1-RelA heterodimers in vivo on the transcriptional regulation of the IL-8 gene. Transfection of increasing amounts of the NFKB1 subunit with a constant amount of RelA resulted in a substantial decrease in the RelA-dependent transcriptional activation from the IL-8 promoter in 8387 cells (Fig. 5A). However, when similar cotransfections were performed with $p(Ig/HIV)_4CAT$ (Fig. 5B), no decrease in transcriptional activation was observed with the ratio of NFKB1 to RelA that resulted in complete loss of activation of the IL-8 promoter. Transfection of increasing quantities of NFKB1 expression vector did result in decreased expression from the $p(Ig/HIV)_4CAT$ reporter (data not shown). These results recapitulate the observed in vitro binding data, indicating that overexpression of RelA can activate transcription from the IL-8 promoter. However, NFKB1 homodimers or NFKB1-RelA heterodimers are unable to bind to, or support transcriptional activation from, the IL-8 promoter.

Binding of nuclear factors to the κB element in the IL-8 promoter. To characterize the nuclear factors which bind to the κB/Rel-responsive element in the IL-8 promoter, gel mobility shift assays were performed with nuclear extracts from Jurkat T cells. PMA stimulation of Jurkat T cells and 8387 cells (data not shown) resulted in an enhancement of binding of primarily a single complex to both the Ig/HIV κB element and the IL-8 promoter fragment (Fig. 6, lane 2).

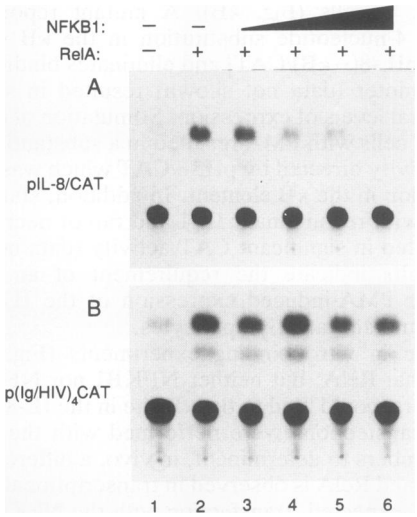

FIG. 5. NFKB1 inhibition of RelA-dependent transcriptional activation of the IL-8 promoter. 8387 cells were transfected with 10 μg of pIL-8/CAT (A) or p(Ig/HIV)$_4$CAT (B) and 1 μg of RelA expression vector alone (lane 2) or 1 μg of RelA plus 0.2, 1.0, 2, or 4 μg of NFKB1 (lanes 3 to 6, respectively). Cells were harvested 48 h posttransfection, and CAT assays were performed as described in Materials and Methods.

Binding of these complexes to either the Ig/HIV site or the IL-8 promoter was completely eliminated by inclusion of excess cold Ig/HIV oligonucleotide as competitor (Fig. 6, lane 3). Inclusion of unlabeled, excess IL-8(−93/−66) promoter fragment effectively competed for binding to the labeled IL-8 probe but competed less effectively for binding to the labeled Ig/HIV probe (lane 4), suggesting that the binding affinity of the induced Ig/HIV κB binding complex for the IL-8 κB site is less than that for the Ig/HIV site. An oligonucleotide which contains a mutation in the κB element of the IL-8 promoter was unable to compete for binding of these nuclear factors to both the Ig/HIV κB site and the IL-8 promoter (lane 5), suggesting that these nuclear factors bind specifically to the κB site of the IL-8 promoter.

To identify the individual components of the induced κB binding complex in PMA-stimulated Jurkat T cells, antibody supershift experiments were performed with antisera to individual members of the κB/Rel family (Fig. 6, lanes 6 to 10). Inclusion of antisera to RelA resulted in a supershift in complexes with both the Ig/HIV site and the IL-8 promoter fragment (Fig. 6, lane 6), indicating that RelA, or an immunologically related protein, is a component of these binding complexes in Jurkat T cells. Whereas anti-NFKB1 antisera resulted in a supershift with the Ig/HIV site, very little if any supershifted complex was seen with the IL-8 probe (Fig. 6, lane 7). Although purified NFKB2 and c-Rel were able to bind to the IL-8 site with affinities comparable to the Ig/HIV site (Fig. 3C), antisera to either of these proteins or RelB did not result in a supershifted complex. However, the antisera to NFKB2 and c-Rel were able to supershift the corresponding purified proteins binding to the Ig/HIV site (data not shown). Antisera to RelB also were unable to supershift the induced binding complex (Fig. 6, lane 10).

To further characterize the induced binding complex in Jurkat T cells, purified IκBα (MAD-3) was included in the binding reactions. IκBα completely inhibited the binding of the induced complex to both the Ig/HIV site and the IL-8 promoter fragment (Fig. 7, lanes 3 and 7). Since IκBα has been shown to have a preferential binding affinity for RelA-containing complexes, this observation further supports the conclusion that the PMA-inducible binding complex contains RelA. Addition of purified IκBα to the binding reaction with the Ig/HIV site resulted in formation of a faster-migrating complex (Fig. 7, lane 3) which could not bind to the IL-8 site (Fig. 7, lane 7). This complex could be completely supershifted by addition of anti-NFKB1 (Fig. 7, lane 4) but not anti-RelA or anti-c-Rel antisera (data not shown). On the basis of these data, the faster-migrating complex most likely represents NFKB1 homodimers and demonstrates that NFKB1 homodimers synthesized in vivo are unable to bind to the IL-8 promoter. Further support for this is the inability of the IL-8 promoter fragment to recognize NFKB1 homodimers in U937 cell extracts (Fig. 6, lane 11). This faster-migrating κB binding activity is recognized by NFKB1 antisera (Fig. 6, lane 12) but not anti-RelA or anti-c-Rel antisera (data not shown).

Inhibition of IL-8 production by antisense RelA oligonucleotide. To further assess the role of RelA in regulation of IL-8 gene expression, sense and antisense oligonucleotides to either NFKB1 or RelA were tested for their ability to inhibit IL-8 production in Jurkat T lymphocytes. These oligonucleotides have been used previously to demonstrate the involvement of RelA in cell adhesion properties with several cell types, and it has been demonstrated that these oligonucleotides specifically inhibit RelA mRNA expression and κB

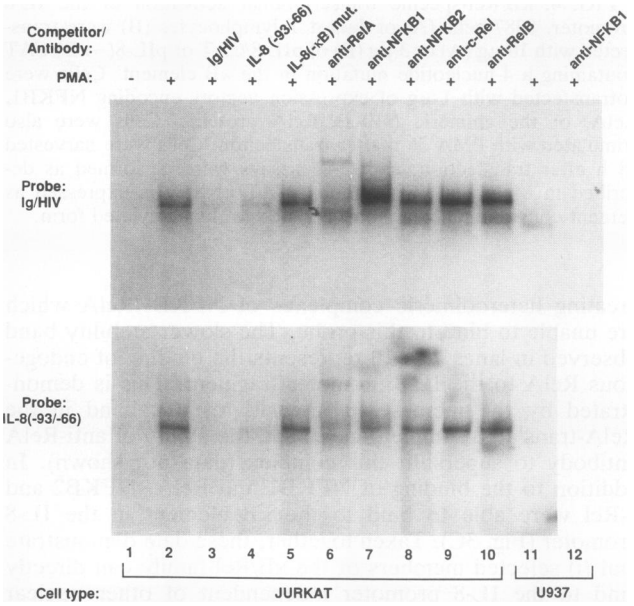

FIG. 6. Binding of nuclear factors from Jurkat T lymphocytes to the IL-8 promoter. Nuclear extracts from unstimulated (−) or Jurkat T cells stimulated for 2 h with PMA (+) (lanes 1 to 10) or U937 (lanes 11 and 12) cells were analyzed by electrophoretic mobility shift assay with ^{32}P-labeled probe containing the Ig/HIV κB element (top panel) or nucleotides −93 to −66 of the IL-8 promoter (bottom panel). An 80-fold molar excess of unlabeled Ig/HIV oligonucleotide (lane 3), IL-8 (−93/−66) (lane 4), or the mutant [IL-8(−κB) mut.] IL-8 promoter fragment (lane 5) were used as competitors. Antisera to RelA (lane 6), NFKB1 (lanes 7 and 12), NFKB2 (lane 8), c-Rel (lane 9), or RelB (lane 10) were added prior to the addition of the radiolabeled probe. Binding conditions and electrophoresis are described in Materials and Methods.

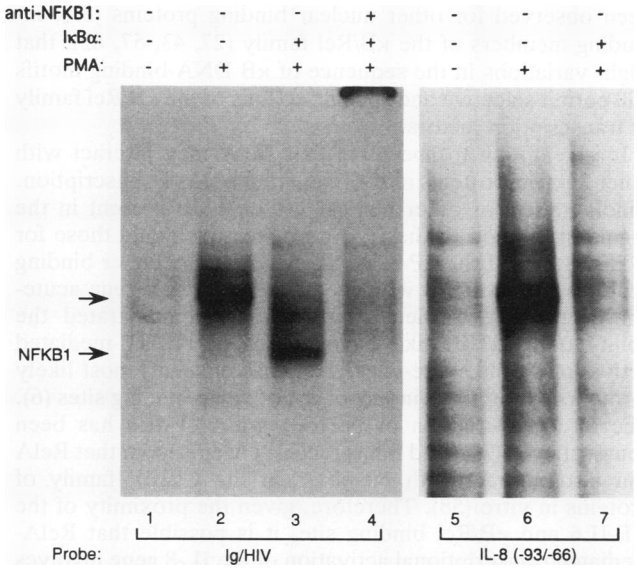

FIG. 7. Inhibition of nuclear factor binding by IκBα (MAD-3). Nuclear extracts from unstimulated (−) or PMA-stimulated (+) Jurkat cells were analyzed by electrophoretic mobility shift assay with ^{32}P-labeled probe containing the Ig/HIV κB element (lanes 1 to 4) or nucleotides −93 to −66 of the IL-8 promoter (lanes 5 to 7). After addition of nuclear extract, approximately 100 ng of bacterially expressed and purified IκBα was added to the reaction mixture (lanes 3 and 7). Antisera to NFKB1 were added after addition of purified IκBα (lane 4). Binding conditions and electrophoresis are described in Materials and Methods.

binding activity (41). As shown in Fig. 8, undetectable levels of IL-8 were secreted from unstimulated Jurkat T lymphocytes. However, PMA stimulation induced IL-8 secretion to a level of approximately 20 pg/ml. Antisense oligonucleotide to RelA resulted in complete inhibition of the PMA-stimulated IL-8 production in these cells. The inhibition was specific for RelA, as neither antisense oligonucleotide to NFKB1 nor the corresponding sense oligonucleotides to NFKB1 or RelA were capable of inhibiting PMA-induced IL-8 expression. These results provide further evidence for

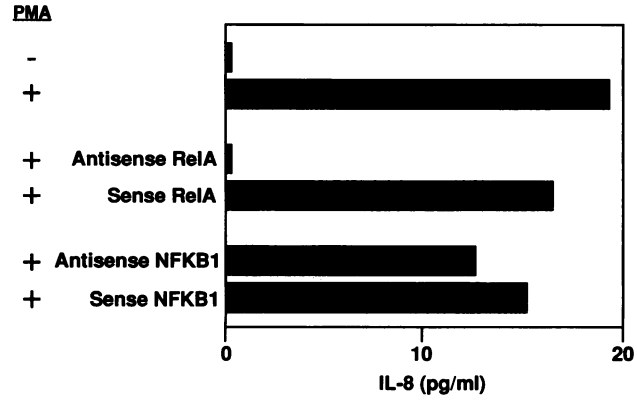

FIG. 8. Antisense oligonucleotide inhibition of IL-8 production in Jurkat T lymphocytes. Jurkat T lymphocytes were subcultured at an approximate density of 10^6 cells per ml. Cells were treated with the indicated oligonucleotides for 5 h prior to stimulation with PMA for 48 h. Culture supernatants were collected, and the level of IL-8 was determined by ELISA.

the involvement of RelA and not NFKB1 in IL-8 gene expression and suggest that modulation of the level of RelA can dramatically affect IL-8 expression.

DISCUSSION

Since expression of IL-8 is limited in most cell types and is induced by various stimuli, the control of its expression represents an interesting model system to study inducible gene expression. Very little is known concerning the mechanisms which control IL-8 gene expression; however, analysis of the 5′ regulatory region of the gene have suggested that several *cis* elements may be involved (36). Although a previous study has suggested the importance of the κB binding element for inducible expression of the IL-8 gene, we have directly assessed the ability of various members of the κB/Rel family to bind to, and activate transcription from, the IL-8 promoter. Using purified κB/Rel subunits and nuclear extracts from PMA-treated Jurkat T cells, we observed selective association of κB/Rel complexes with the κB element in the IL-8 promoter and have demonstrated the direct involvement of RelA in inducible nuclear factor binding, transcriptional activation, and regulation of endogenous IL-8 gene expression.

Analysis of the 5′ flanking region of the IL-8 promoter revealed that the sequence of the κB binding site is 5′-GTGGAATTTCC-3′. Using a DNA-binding site selection strategy to select for DNA sequences which bind to RelA homodimers, we have previously identified this element as one which binds exclusively to RelA homodimers but not to NFKB1 homodimers (27). Consistent with that observation, we found that neither NFKB1 nor heterodimeric NFKB1-RelA complexes could bind to the IL-8 κB element alone or in the context of the authentic IL-8 promoter. However, RelA, NFKB2, and c-Rel were capable of binding to this element. Although purified NFKB2 and c-Rel were able to bind to the IL-8 promoter, antibody supershift experiments suggested that neither of these proteins represent a major component in the inducible nuclear protein-DNA binding complex in Jurkat T cells. However, c-Rel and NFKB2 may play a role in IL-8 expression in other cell types or under different physiological conditions.

The abilities of (i) RelA to bind to the IL-8 promoter, (ii) anti-RelA antibody to supershift a PMA-inducible protein-DNA binding complex to the IL-8 promoter, (iii) overexpression of RelA to activate IL-8 gene expression, and (iv) antisense oligonucleotide to RelA to inhibit IL-8 production clearly implicate RelA as a regulator of IL-8 gene expression. Although RelA can bind as a homodimer to the IL-8 promoter in vitro, whether the observed RelA-containing complexes in induced cells represent homodimeric RelA or RelA in association with another cellular protein(s) remains to be determined. However, the mobility of the PMA-inducible, IL-8 binding complex is slightly faster than the mobility of in vitro-translated RelA homodimers (data not shown), suggesting that the binding complex is not composed solely of RelA homodimers. Preliminary UV cross-linking analysis and immunoprecipitation with anti-RelA antisera suggest that a DNA-protein adduct of the expected size for RelA (~70 kDa) was cross-linked to the IL-8 promoter fragment (data not shown). In addition, a smaller DNA-protein adduct that had a mobility of approximately 50 to 55 kDa, a size consistent with NFKB1 or NFKB2, was detected. The ability to detect this smaller DNA-protein adduct was inconsistent, however, and appeared to be dependent on the specific binding conditions. From these

preliminary data and given the similar molecular sizes of some members of the κB/Rel family, we cannot conclude for certain the exact identity of the protein species which are cross-linked to the IL-8 promoter. However, as discussed below, we feel that heterodimers of NFKB1 and RelA do not bind to any significant extent. Whether RelA forms a specific binding partner with an as-yet-unidentified member of the κB/Rel family remains to be determined.

An interesting aspect of these studies was the observation that classical NF-κB (heterodimeric NFKB1-RelA) was unable to bind to the κB element in the IL-8 promoter. This supports previous conclusions that DNA contact of both the NFKB1 and RelA subunits of NF-κB is required for DNA binding and transcriptional activation of the NF-κB complex (27). The observation that expression of NFKB1 and RelA together in 8387 cells resulted in decreased transcriptional activation compared with the expression of RelA alone supports the notion that in vivo heterodimers of NFKB1 and RelA cannot bind to and activate transcription. This most likely results from the formation of heterodimeric NFKB1-RelA complexes which cannot bind to the IL-8 promoter. Another possible explanation would be that expression of NFKB1 may be sequestering another cellular factor(s) which is necessary for RelA-mediated activation. However, this is less likely, as expression of NFKB1, at the levels used, did not change the level of expression from the $p(Ig/HIV)_4CAT$ reporter which is capable of binding NFKB1-RelA heterodimers. Antibody supershift experiments further support the conclusion that NFKB1 is most likely not a major component of the PMA-inducible binding complex. If, perhaps, a small amount of heterodimeric NFKB1-RelA does not bind to the IL-8 promoter, we believe that this binding results in a nonfunctional binding complex, as the transient transfection assays suggest that heterodimeric NFKB1-RelA complexes are transcriptionally inactive on the IL-8 promoter and antisense oligonucleotides to NFKB1 do not affect the level of IL-8 expression following PMA stimulation. Since it has previously been shown that the major κB binding complex to the Ig/HIV site in PMA-stimulated Jurkat T cells is composed primarily of NFKB1-RelA heterodimers, the inability of the IL-8 site to compete as well as the Ig/HIV site for binding of nuclear factors to the Ig/HIV site (Fig. 6) and the observation that PMA stimulation results in different levels of activation from reporter plasmids containing the different κB elements (Fig. 2) suggest that the IL-8 site does in fact have a reduced binding affinity for NFKB1-RelA heterodimers and may recognize an entirely different set of induced κB factors than those recognized by the Ig/HIV site. These results suggest that the mere existence of a κB element in the regulatory region of a gene does not imply that classical NF-κB (NFKB1-RelA) can bind to the site.

A notable feature of the κB sequence in the IL-8 promoter is the lack of three or four consecutive G residues in the 5′ half of the binding site. Consecutive G's in the 5′ half-site have been shown to be important for binding of NFKB1 to DNA (27, 63, 64). Except for the single G → T change in the second position of the binding element, this sequence is identical to the κB elements found in the invariant chain Ii (46) and tumor necrosis factor alpha (10) genes. A T → G substitution at the second position in the IL-8 motif allowed binding of NFKB1 and NFKB1-RelA heterodimers (26a). Therefore, κB binding elements in the regulatory region of immune response genes that differ by only a single nucleotide can result in profound alterations in κB/Rel subunit binding characteristics. This supports the notion, as has been observed for other nuclear binding proteins (39), including members of the κB/Rel family (27, 43, 57, 62), that slight variations in the sequence of κB DNA-binding motifs will permit selective and specific actions of the κB/Rel family of transcription factors.

It is tempting to speculate that RelA may interact with other nuclear proteins in the regulation of IL-8 transcription. Binding sites for other nuclear proteins are present in the regulatory region of the IL-8 promoter, including those for C/EBPβ (NF-IL6), AP-1, AP-2, AP-3, and octamer binding proteins (37). Studies with the angiotensinogen gene acute-phase response element have directly demonstrated the ability of a C/EBP-like factor to block NF-κB-mediated activation of the acute-phase response element, most likely through competitive binding of overlapping binding sites (6). The direct interaction of NFKB1 with NF-IL6 has been demonstrated (29), and it has recently been shown that RelA can also interact with members of the C/EBP family of proteins in vitro (58). Therefore, given the proximity of the NF-IL6 and κB/Rel binding site, it is possible that RelA-mediated transcriptional activation of the IL-8 gene involves interaction with NF-IL6 or a closely related protein. In fact, recent data from our laboratory suggest that C/EBPβ and RelA cooperatively activate transcription from the IL-8 promoter (26a).

Recently a model for regulation of IL-8 expression which suggests that following stimulation of cells, heterodimers of NFKB1 and RelA translocate from sequestered IκB-containing complexes in the cytoplasm to the nucleus has been proposed (37). In the nucleus, it was proposed that the NFKB1 subunit of NF-κB contacts the κB element of the IL-8 gene, while the RelA subunit acts as a bridging factor to contact NF-IL6 without directly contacting DNA on its own. On the basis of our results, this model can be modified and expanded. Our observations suggest that stimulation of cells results in an increase of RelA-containing complexes which bind specifically to the κB site in the IL-8 promoter. By using recombinant subunits of κB/Rel proteins, we have shown that heterodimeric NFKB1-RelA is likely not to be involved in regulation of the IL-8 gene. Rather it appears that RelA can directly bind the κB element as either homodimers or as a complex with other cellular proteins (perhaps NF-IL6) and regulate transcription from the IL-8 gene. In addition, modulation of RelA expression will likely directly affect the level of IL-8 production. While providing direct evidence for a role of κB/Rel members in the regulation of IL-8 gene expression, these findings suggest that other κB-regulated genes might not always be regulated by the prototypical NFKB1-RelA heterodimeric complex. Rather it is likely that distinct subsets of κB/Rel family members will mediate transcriptional activation through binding to slight variations in the DNA sequence of the κB element. Furthermore, these data suggest that regulation of κB/Rel DNA binding plays a role in the inflammation process through regulation of IL-8 production.

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