MOLECULAR AND CELLULAR BIOLOGY, Oct. 1993, p. 6231–6240
0270-7306/93/106231-10$02.00/0

Vol. 13, No. 10

Characterization of a Functional NF-κB Site in the Human Interleukin 1β Promoter: Evidence for a Positive Autoregulatory Loop

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Received 5 March 1993/Returned for modification 6 April 1993/Accepted 9 July 1993

The −300 region of the interleukin 1β (IL-1β) promoter contains a functional NF-κB binding site composed of the decamer sequence 5′-GGGAAAATCC-3′. Probes representing the −300 region or the NF-κB site alone interacted with NF-κB proteins present in phorbol myristate acetate-, lipopolysaccharide-, or Sendai virus-induced myeloid cell extracts as well as recombinant NFKB1 (p50) and RelA (p65); furthermore, NF-κB protein-DNA complex formation was dissociated in vitro by the addition of recombinant IκBα. Mutation of the NF-κB site in the context of the IL-1β promoter reduced the responsiveness of the IL-1β promoter to various inducers, including phorbol ester, Sendai virus, poly(rI-rC), and IL-1β. A 4.4-kb IL-1β promoter fragment linked to a chloramphenicol acetyltransferase reporter gene was also preferentially inducible by coexpression of individual NF-κB subunits compared with a mutated IL-1β promoter fragment. When multiple copies of the IL-1β NF-κB site were linked to an enhancerless simian virus 40 promoter, this element was able to mediate phorbol ester- or lipopolysaccharide-inducible gene expression. In cotransfection experiments, RelA (p65) and c-Rel (p85) were identified as the main subunits responsible for the activation of the IL-1β NF-κB site; also, combinations of NFKB1 (p50) and RelA (p65) or c-Rel and RelA were strong transcriptional activators of reporter gene activity. The presence of a functional NF-κB binding site in the IL-1β promoter suggests that IL-1 positively autoregulates its own synthesis, since IL-1 is a strong inducer of NF-κB binding activity. Thus, the IL-1β gene may be considered as an important additional member of the family of cytokine genes regulated in part by the NF-κB/*rel* family of transcription factors.

Interleukin 1 (IL-1) is a member of the family of inflammatory cytokines that possess an essential role in responsiveness to infection, immunoregulation, and immunological homeostasis (1, 17); other members of this group of polypeptide hormones include tumor necrosis factor, IL-6, and the interferons (IFNs). A rapid increase in IL-1 levels in serum in response to bacterial or viral infection produces a cascade of biological effects detectable in many tissues. IL-1 activity is encoded by two distinct genes (α and β) that share about 25% homology at the amino acid level. Both IL-1α and -β are initially synthesized as 31-kDa precursor polypeptides that are processed in the cytoplasm to secreted or cell-associated forms of 17 kDa (2, 11, 38, 39). IL-1 is produced by multiple cell types, including cells of the monocytic lineage, T and B lymphocytes, fibroblasts, neutrophils, and nervous system microglia (1, 17).

Recent studies have begun to characterize the regulatory pathways that contribute to the activation of IL-1β transcription in response to viruses, lipopolysaccharide (LPS), phorbol ester, and other inducers of IL-1β production (8, 12, 20, 30). The IL-1β gene is regulated by an inducible promoter element containing both positive and negative regulatory elements (20). These *cis*-acting DNA sequences are the ultimate targets of numerous transcription factors activated by distinct signal transduction pathways. A phorbol myristate acetate (PMA)-responsive enhancer element located between positions −2982 and −2795 upstream from the transcriptional start site (8) which contains DNA motifs similar to those of the AP-1 binding site of the collagenase gene and the PRDI region of the human IFN-β promoter has been identified (19, 43, 59). Additional LPS-responsive elements that overlap and extend upstream of the PMA-inducible enhancer have also been localized (59). Hunninghake et al. recently characterized a novel protein termed NFIL-1βA interacting with the cap site-proximal −49 to −38 promoter region, a domain that is uniquely conserved in the human and murine IL-1β promoter (30).

NF-κB is a family of structurally and functionally related peptides that regulate transcription of immunoregulatory genes coding for cell surface receptors, cytokines, transcription factors, and viral genes including the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (for a review, see references 3 and 24). The consensus recognition site is a decamer (5′-GGGANNYYCC-3′) with two pentameric half-sites, each of which participates in the recognition and stabilization of binding of the NF-κB dimer (3). NF-κB was originally characterized as two proteins of 50 and 65 kDa (3, 4). Cloning of these factors revealed significant amino-terminal homology with the *rel* family of oncoproteins and with the *Drosophila* developmental morphogen *dorsal* (3, 23, 33). These proteins preexist in the cytoplasm coupled to an inhibitor protein, IκB (4, 22, 63, 68). Activation of cells by a variety of agents (PMA, radical oxygen

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intermediates, LPS, viruses, and cytokines) liberates the DNA-binding proteins from IκB, leading to translocation to the nucleus and interaction with DNA (3, 4, 29, 32, 44, 63, 68).

The NF-κB family now consists of multiple NF-κB/*rel* peptides ranging in molecular mass from ~100 to 49 kDa. In addition to the proto-oncogene c-*rel* (10), the gene family consists of the p105 gene product or NFKB1, which is the precursor of the p50 DNA-binding subunit of NF-κB (9, 23, 33, 42); a p65 DNA-binding subunit, now termed RelA (47, 54), that possesses a strong transcriptional activation domain (5, 53, 58); a distinct gene encoding a 100-kDa precursor (now termed NFKB2) and a p52 product that is identical to the *lyt*-10 gene (46, 57); and the recently cloned gene encoding the 68-kDa RelB (I-Rel) product (55, 56). Four distinct forms of the ankyrin repeat containing IκB proteins have also been identified: IκBα (MAD3 or pp40), cloned as an immediate-early response gene in phorbol ester-induced macrophages (28, 32); IκBβ, purified as a distinct inhibitory activity (68); *bcl3*, identified initially as a gene translocated in B-cell lymphoma (48); and IκBγ, a unique 70-kDa gene product encoded by the carboxy-terminal 607 amino acids of the p105 gene (31). The association of IκBα with NF-κB proteins occurs via the nuclear localization sequence; in the uninduced state IκB masks the nuclear localization sequence to prevent nuclear translocation (7). As shown recently, phosphorylation and rapid degradation of IκBα are the first detectable changes in NF-κB–IκB complexes after treatment of cells with different inducers (6); loss of IκBα results in the translocation of NF-κB to the nucleus, where RelA (p65) has been shown to stimulate IκBα transcription de novo by an autoregulatory mechanism (62).

In this study we have characterized the interactions of NF-κB proteins with a putative NF-κB site located at −297 to −288 in the IL-1β promoter. This site interacted with recombinant and native NF-κB proteins present in extracts from PMA-, LPS-, or Sendai virus-induced myeloid cells. Mutation of the NF-κB site within the IL-1β promoter decreased responsiveness to different inducers and to NF-κB subunit coexpression. In transfection studies using a reporter construct with two IL-1β NF-κB sites, activation or repression of reporter gene activity was achieved by distinct combinations of NF-κB subunits. These experiments demonstrate the presence of a functional NF-κB binding site in the IL-1β promoter and suggest that IL-1 positively autoregulates its own synthesis, since IL-1 itself is a strong inducer of NF-κB binding activity.

MATERIALS AND METHODS

Cell culture and transfection. Myeloid cell lines U937 and PLB-985, as well as Jurkat, a T-lymphoid cell line, and 293, an adenovirus-transformed human embryonic kidney cell line, were grown in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% fetal calf serum, glutamine, and antibiotics. Exponentially growing U937 and Jurkat cells were transfected by the DEAE-dextran method, as described previously (35); 293 cells were transfected by calcium phosphate coprecipitation, as described previously (35). All transfections contained equivalent amounts (10 μg) of DNA; in those assays in which less chloramphenicol acetyltransferase (CAT) reporter plasmid was used, additional pUC8 DNA was added. At 24 h after transfection, cells were induced with PMA (25 ng/ml; Sigma), LPS (15 μg/ml; Sigma), IL-1β (100 U/ml; R&D Systems, Inc.), poly(rI-rC) (50 μg/ml; Pharmacia), or Sendai virus (250 hemagglutinating units/ml) for 24 h prior to lysis. For individual CAT assays, 50 to 100 μg of total protein extract was assayed for 4 to 6 h at 37°C (described in each experiment). The percentage of acetylated chloramphenicol was determined by excising the spots containing nonacetylated and acetylated forms of chloramphenicol on the thin-layer chromatography plates and measuring the amount of ^{14}C radioactivity by liquid scintillation counting. All transfections were performed at least three times for each cell type.

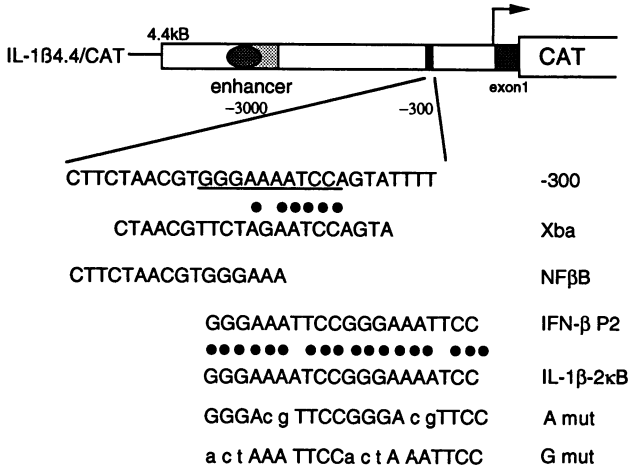

FIG. 1. Schematic of the IL-1β promoter. The schematic illustrates the IL-1β promoter present in the IL-1β4.4 CAT plasmid (8) and identifies the sequence of the −300 region containing the putative NF-κB binding site located at positions −297 to −288 (underlined). The enhancer element (59) located between −3757 and −2729 (enhancer −3000) and exon 1—all present in the IL-1β4.4CAT plasmid—are illustrated. The sequences of oligonucleotides used in this study are also indicated. The dots indicate homology between the −300 region and the Xba oligonucleotide at the NF-κB site (upper) and between the P2 element and the IL-1β element (lower).

Plasmid construction and oligonucleotide synthesis. Plasmids SV_2CAT, SV_1CAT, P2(1)CAT, and P2(2)CAT have all been previously described (35, 36) and are derivatives of pSV_2CAT (26). pIL-1β×1 κBCAT, pIL-1β×2-κBCAT, and pIL-1β×3-κBCAT plasmids were obtained by subcloning synthetic oligonucleotides containing one, two, or three copies of the IL-1β NF-κB sequence with *Acc*I-*Sph*I ends into the *Acc*I-*Sph*I site of SV_2CAT. The IL-1β4.4CAT plasmid was previously described (8) and is shown diagrammatically in Fig. 1. The IL-1β4.4 mutant was produced by overlap polymerase chain reaction (PCR) mutagenesis and contains an *Xba*I recognition sequence (TCTAGA) in place of the GGGAAA nucleotides at positions −297 to −292. The sequences and positions of other oligonucleotides used in this study are shown in Fig. 1. The NF-κB expression plasmids were produced by subcloning different NF-κB genes into the SVK3 vector as follows: (i) p50—a 1,381-bp *Eco*RI-*Rsa*I fragment from KBF-1 (33) was subcloned into the *Eco*RI-*Sma*I site of SVK3L; (ii) p65Δ—a 2,572-bp *Xba*I-*Xho*I fragment from plasmid BL-SK (54) was subcloned into the *Bam*HI-*Xho*I site of SVK3; (iii) c-Rel—the 2,340-bp *Eco*RI fragment of c-*rel* cDNA (10) was cloned into the SVK3 *Eco*RI site; (iv) IκB—a 1,190-bp *Eco*RI fragment from pGEX-2T (see below) was subcloned into the *Eco*RI site of SVK3. The CMIN-p65 vector (53) was used to express the p65 subunit.

Extract preparation and gel retardation assay. Whole-cell

extracts (WCE) were prepared from exponentially growing U937, THP-1, and 293 cells at different times after induction (described in individual experiments). Cell extracts were prepared essentially as described previously (34, 35). A 5- to 15-μg sample of WCE was preincubated with 5 μg of poly(dI-dC) in nuclear dialysis buffer (14) for 10 min at 4°C. [γ-^{32}P]ATP-labelled double-stranded oligonucleotides containing a duplicated NF-κB site from the IL-1β promoter (5′-GGGAAAATCCGGGAAAATCC-3′), the −300 region of the IL-1β promoter (5′-CTTCTAACGTGGGAAAATC CAGTATTTT-3′), or the PRDII domain of the IFN-β promoter (5′-GGGAAATTCCGGGAAATTCC-3′) were incubated with the extracts at room temperature for 30 min. The sequences of other oligonucleotides used in competition experiments are as follows: mutant NF-κB sites 5′-act-AAATTCCactAAATTCC-3′ (the G mutant), 5′-GGGAcgTT CCGGGAcgTTCC-3′ (the A mutant), 5′-CTAACGTTCTAG AATCCAGTA-3′ (the Xba oligonucleotide), and 5′-CTTCT AACGTGGGAAA-3′ (NF-κB oligonucleotide). In competition assays, excess unlabelled wild-type and mutant oligonucleotides were added during preincubation. Samples were analyzed on a 6% native Tris-glycine-polyacrylamide gel, run at 150 V for 5h, dried, and exposed overnight to Dupont Cronex film. All oligonucleotides were 5′ end labelled with [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham) by using T4 polynucleotide kinase (Pharmacia). The relative intensities of the protein-DNA complexes were measured with the LKB Ultroscan XL scanning laser densitometer.

Recombinant NF-κB and IκBα proteins. The gene encoding the NF-κB p50 subunit (NFKB1) was a kind gift from Alain Israel, Pasteur Institute. The NFKB1 (p105) coding sequence was cleaved from Bluescript by *Stu*I-*Eco*RI cleavage and religated into pGEX-3X. Cleavage of pGEX-3X with *Xba*I and *Eco*RI liberated a fragment which left the p50 coding sequence intact and under the control of the *lacZ* promoter, inducible by isopropyl-β-D-thiogalactopyranoside. Purified p50 was obtained by chromatography on glutathione-agarose, and the glutathione *S*-transferase (GST) moiety was removed by factor Xa cleavage (61). The N terminus of RelA (p65) was also expressed as a GST fusion protein in pGEX-3X. Recombinant IκBα cDNA was generated by reverse transcriptase-PCR amplification with total myeloid cell RNA from PLB-985 (52) stimulated with PMA for 2 h and IκBα-specific primers (28) located at positions 81 to 99 (5′-ACGTGAATTCAGCTCGTCCGCGCC-3′) and 1151 to 1171 (5′-ATATAGGTGTGACGTGTGACCTTAAG-3′). The 5′ end of each oligonucleotide primer contained an *Eco*RI site (underlined) used for ligation of the amplified product into pGEX-2T. Recombinant IκBα was liberated from the GST-IκB fusion protein by thrombin cleavage (61).

RESULTS

Mutation of a putative NF-κB site in the IL-1β promoter. Examination of the IL-1β promoter sequence revealed a sequence located at positions −297 to −288 (5′-GG-GAAAATCC-3′) with 90% similarity to the PRDII/NF-κB sequence in the IFN-β promoter (5′-GGGAAATTCC-3′) and differing in a T-to-A transition at position 7 in the IL-1β sequence (Fig. 1). To evaluate the role of this putative NF-κB site in IL-1β promoter activity, overlap PCR mutagenesis was used to modify the −300 region to include an *Xba*I site (TCTAGA) in place of the GGGAAA nucleotides (Fig. 2). CAT reporter constructs containing the wild-type and mutant 4.4-kb IL-1β promoter fragments were transfected into U937 cells, and the inducibility of the reporter plasmids was measured; the enhancerless SV1CAT and HIV LTR-CAT plasmids were used as negative and positive controls, respectively. Figure 2 illustrates the inducibility of the IL-1β4.4 wild-type promoter and the corresponding IL-1β4.4 mutant. Activity of the IL-1β4.4 wild-type construct was four- to fivefold higher when induced with PMA and fourfold higher when induced with poly(rI-rC) compared with the uninduced level of activity. The IL-1β4.4 promoter was also induced by Sendai paramyxovirus infection and, interestingly, by IL-1β. Mutation of the putative NF-κB site in the −300 region of the IL-1β4.4 promoter fragment generated a promoter that was not inducible by PMA, poly(rI-rC), or IL-1β but retained a reduced level of Sendai virus inducibility (Fig. 2). By comparison, the enhancerless SV1CAT construct was neither PMA nor poly(rI-rC) inducible, whereas the HIV LTR which contains two adjacent NF-κB sites in the −100 region was induced 12-fold by PMA and 10-fold by poly(rI-rC). This initial experiment suggested that the −300 region of the NF-κB promoter was an important regulatory element involved in NF-κB gene activation.

Proteins interacting with an IL-1β NF-κB site. Recent experiments have demonstrated that rapid induction of NF-κB binding activity occurs in response to LPS, IL-1, or other inducers (6). By using the LPS-responsive myeloid cell PLB-985, IL-1β transcription and NF-κB induction were shown to be temporally related; IL-1β transcription and NF-κB binding activity were induced within 30 min of LPS treatment (data not shown). To examine the possibility that NF-κB proteins are capable of interacting with the IL-1β sequence, extracts from U937 or PLB-985 stimulated with PMA or LPS for 6 h were analyzed for protein-DNA complex formation by electrophoretic mobility shift assay; as demonstrated previously (35) and shown in Fig. 3A, PMA-induced extracts (Fig. 3A, lanes 2 and 4) contained about 20-fold-higher levels of DNA-binding proteins than uninduced extracts (Fig. 3A, lanes 1 and 3), and these proteins interacted with the putative NF-κB site in the IL-1β promoter as well as the PRDII/NF-κB (P2) site (Fig. 3A and B, lanes 1 and 7, respectively). The IL-1β NF-κB probe also detected a strong protein-DNA complex of higher mobility similar to NF-βB (45; discussed below). By using a probe that spanned the −300 region of the IL-1β promoter, similar induced complexes were identified by using either PMA-induced or Sendai virus-induced extracts. In competition analysis (Fig. 3B), binding of NF-κB proteins from PMA-induced U937 cells was completely abolished by the addition of a 200-fold excess of IL-1β or P2 competitor DNA (Fig. 3B, lanes 2, 4, and 5) while mutation of the first three G residues of the P2 site or mutation of the A residues at positions 5 and 6 (G mutant and A mutant oligonucleotides) eliminated the ability of the P2 site to compete for IL-1β complex formation (Fig. 3B, lanes 3 and 6). In contrast, binding to the IFN-β P2 site (Fig. 3B, lane 7) was about 80% inhibited by the addition of a 200-fold excess of oligonucleotide containing one, two, or three copies of the IL-1β site (Fig. 3B, lanes 9 to 11). Binding to the −300 region probe (nucleotides −307 to −280) (Fig. 4, lane 1) was inhibited by a 200-fold excess of NF-κB oligonucleotide (Fig. 4, lane 2) or the homologous −300 region (Fig. 4, lane 7) but not by oligonucleotides corresponding to the Xba, NF-βB, G mutant, or A mutant sequences (Fig. 4, lanes 3 to 6). The −300 region probe also interacted efficiently with recombinant NFKB1 (p50) and RelA (p65) (Fig. 4, lanes 8 and 9), as well as with a T-cell extract containing high levels of c-Rel (Fig. 4, lane 10) (35a). Competition EMSA analyses with increasing concentrations of unlabelled competitor DNA were used

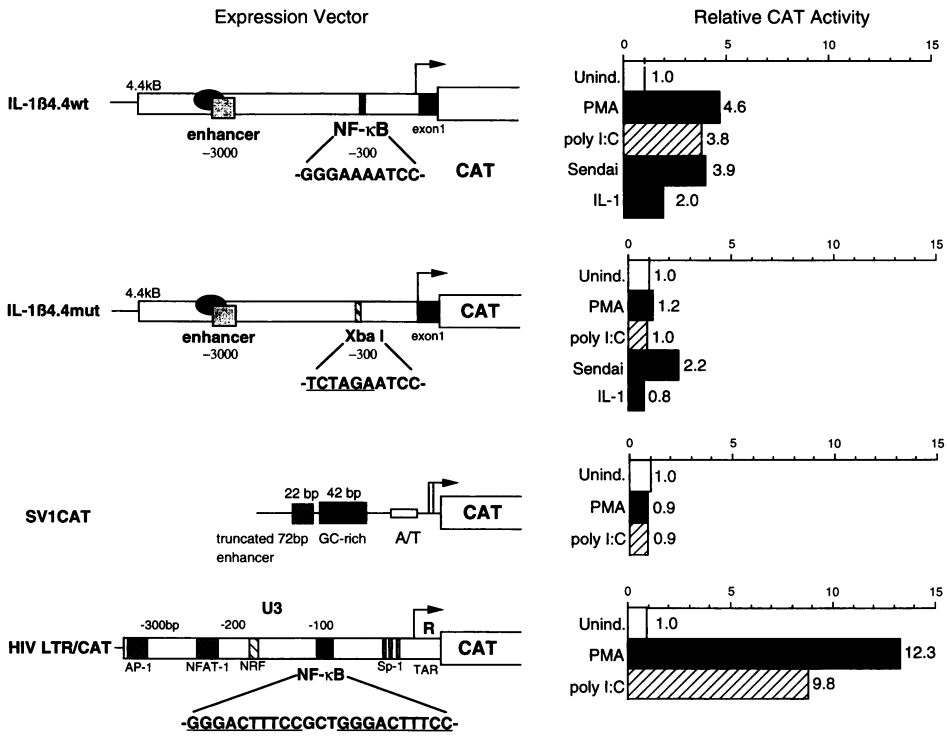

FIG. 2. Inducibility of the IL-1β construct. The structures of the wild-type and mutant (with the inserted *Xba*I site) IL-1β4.4 CAT construct, HIV LTR (including binding sites for AP-1, NFAT-1, NRF, and Sp1) and SV1CAT (including the AT-rich region [A/T], the GC-rich region, and the truncated 72-bp enhancer element) plasmids are also illustrated. U937 cells were transfected by the DEAE-dextran method, and induced 24 h later with PMA (25 ng/ml), poly(rI-rC) (50 μg/ml), Sendai virus (250 hemagglutinating units/ml for 90 min), or IL-1β (100 U/ml). Cells were harvested 48 h after transfection, and 200 μg of total cell lysate was assayed for CAT enzyme activity for 4 to 6 h. Transfections were performed in triplicate, and the average results of triplicate measurements of CAT activity are shown. The uninduced activities of the CAT plasmids were as follows: HIV LTR, 5.4%; SV1, 1.1%; IL-1β4.4, 1.0%; and IL-1β4.4 mutant, 1.4%.

to examine the relative affinities of the IL-1β and the IFN-β NF-κB binding sites; multiple experiments indicated that the single T-to-A transition at position 7 of the IL-1β NF-κB site resulted in a fourfold-weaker binding site compared with that at the PRDII/NF-κB site (data not shown).

Dissociation of the NF-κB complex by recombinant IκB. Recombinant IκBα was used together with PMA-induced U937 extracts to examine the involvement of RelA (p65) in IL-1β NF-κB complex formation. Previous studies have demonstrated that IκBα interacts preferentially with the RelA (p65) subunit of the NF-κB complex to cause the dissociation of DNA-bound RelA (p65)-NFKB1 (p50) complexes and forms the basis of a functional assay for IκBα activity and RelA (p65) DNA-binding activity (63, 68). Addition of recombinant IκBα to protein-DNA complexes generated with either IFN-β (Fig. 5, lanes 1 and 2) or IL-1β sites (Fig. 5, lanes 3 to 6) dissociated the NF-κB complex (Fig. 5, lanes 2, 4, and 6); addition of IκBα to protein-DNA complexes generated with recombinant NFKB1 (p50) did not decrease complex formation, indicating the specificity of IκBα for RelA (p65)-containing complexes (data not shown). Furthermore, addition of anti-NF-κB subunit antibodies (a gift from N. Rice) produced supershift complexes, indicating the presence of NFKB1 (p50), RelA (p65), a small amount of c-Rel, but no NFKB2 (p52) in the complexes (data not shown). Also, IκBα did not affect the faster-migrating NF-βB-like complex (Fig. 5, lanes 4 and 6); the nature of this complex, which does not form to a significant extent with the P2 probe (Fig. 5, lanes 1 and 2) is currently under investigation (45; see Discussion). This complex is also not related to the NFKB1 (p50) or RelA form of NF-κB, since only the NF-κB protein-DNA complex formed in the presence of recombinant proteins (Fig. 4, lanes 8 and 9).

Functional activity of IL-1β NF-κB-dependent promoters. To examine the transcriptional activity of the IL-1β NF-κB site, one, two, or three copies of the IL-1β site were subcloned into *Acc*I-*Sph*I-cleaved SV_1CAT and compared with the activity of reporter plasmids containing two and four copies of the PRDII/NF-κB sequences, P2(1)CAT and P2(2)CAT (36), respectively, following transient transfection into U937 (Fig. 6), 293, or Jurkat T cells (data not shown). The upper panel of Fig. 6 illustrates a representative CAT analysis of the inducibility of the IL-1β–κB-dependent constructs in U937 cells in response to PMA or LPS; with all the NF-κB-containing constructs, low basal level activity was observed in U937 cells (Fig. 6, open bars). Induction with PMA led to increases in IL-1β NF-κB-dependent CAT activity ranging from 5- to 50-fold (Fig. 6, solid bars); the level of PMA inducibility of the constructs was directly dependent on the number of IL-1β NF-κB enhanson sites present. As demonstrated previously (19, 36), multimerization of the PRDII element in CAT-based reporter plasmids also led to a synergistic stimulation of transcriptional activity (Fig. 6, lower panel). In all experiments, the PRDII sequences were stronger transcriptional elements than the IL-1β sequences, indicating that differences in the binding affinities of the two sites were also reflected in distinct levels of functional transcriptional activity. LPS treatment of trans-

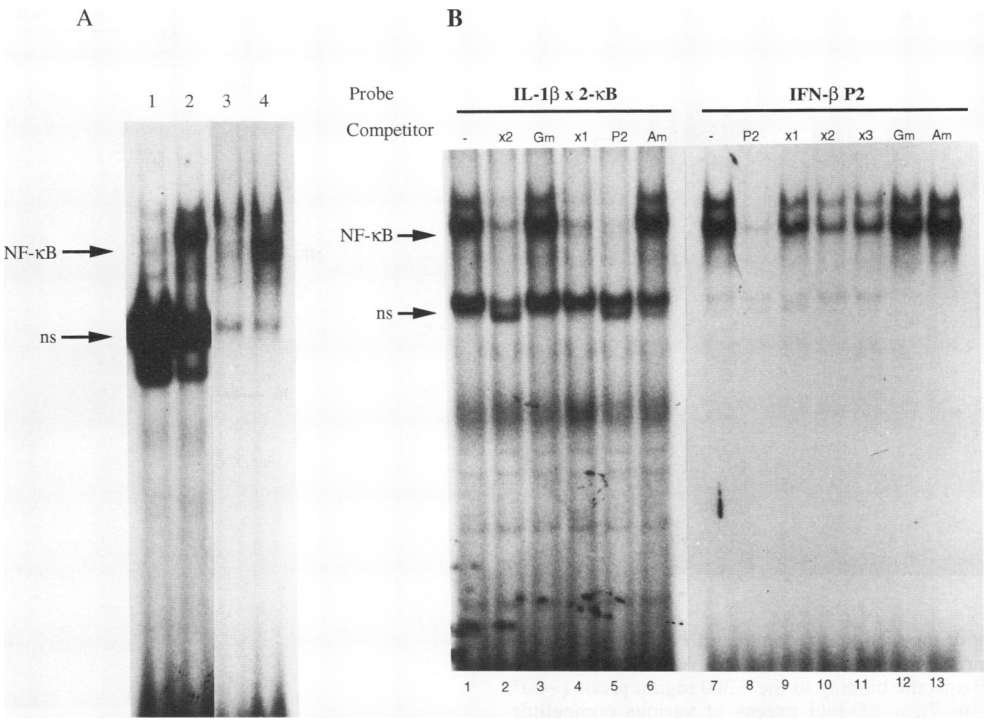

FIG. 3. Binding of NF-κB proteins to the IL-1β NF-κB sequence. (A) WCE were prepared from uninduced (lanes 1 and 3) and PMA-induced U937 cells (lanes 2 and 4); 10 μg of protein was assayed for NF-κB-specific binding to the IL-1β × 2-κB (lanes 1 and 2) and IFN-β P2 (lanes 3 and 4) sequences. (B) PMA-induced extracts were preincubated without (lanes 1 and 7) or with a 200-fold molar excess of various competitor oligonucleotides (lanes 2 to 6 and 8 to 13, competitors described above the gel lane) and analyzed on a 6% native Tris-glycine-polyacrylamide gel. ×2, IL-1β × 2κB oligonucleotide; Gm, G mutant (actAAATTCCactAAATTCC); Am, A mutant (GGGAcgT TCCGGGAcgTTCC); × 1, IL-1β × 1κB oligonucleotide; ×3, IL-1β × 3κB oligonucleotide; P2, IFN-β PRDII × 2 oligonucleotide; ns, nonspecific.

fected U937 cells did not significantly stimulate NF-κB-dependent reporter constructs containing one or two copies of either the IL-1β or the PRDII elements (Fig. 6). Multimerization of IL-1β or the PRDII sites— to three and four copies, respectively—led to a 5- to 10-fold increase in CAT activity following LPS stimulation (Fig. 6, shaded bars). The fact that multimerization of the κB elements was necessary to detect any LPS-inducible activity indicates that the NF-κB site alone in a heterologous construct is not sufficient to mediate LPS responsiveness but rather suggests that LPS inducibility requires other elements.

Activation and repression of the IL-1β NF-κB site by NF-κB subunits. To determine which combinations of the NF-κB subunits were involved in the stimulation of the IL-1β NF-κB binding site, various NF-κB expression vectors were transfected into Jurkat cells together with the IL-1β × 2κBCAT reporter construct (Fig. 7). Expression of NFKB1 (p50) subunit alone had a twofold stimulatory effect on the IL-1β × 2κBCAT construct (Fig. 7, lane 2); expression of individual RelA (p65) and c-Rel proteins produced a threefold and sixfold stimulation of the reporter gene, respectively (Fig. 7, lanes 3 and 5). The coexpression of either the naturally occurring splicing variant ΔRelA (p65Δ) or IκBα (MAD3) failed to stimulate gene activity and in fact depressed basal level activity two to threefold (Fig. 7, lanes 4 and 6). In contrast, the combinations of RelA (p65) plus NFKB1 (p50) and RelA (p65) plus c-Rel produced a strong 10- to 13-fold activation of the NF-κB site (Fig. 7, lanes 7 and 9). Strikingly, the combination of ΔRelA (p65Δ) and NFKB1 (p50) reduced *trans* activation to basal levels (Fig. 7, lane 8), demonstrating the *trans*-dominant negative effects of the ΔRelA (p65Δ) subunit (59); likewise, the combination of ΔRelA (p65Δ) and c-Rel decreased reporter gene activity about threefold compared with RelA (p65) plus c-Rel (Fig. 7, compare lanes 9 and 10). IκBα also functioned as a *trans*-dominant negative subunit to block RelA (p65) plus NFKB1 (p50) and RelA (p65) plus c-Rel-mediated *trans* activation of the IL-1β NF-κB site (Fig. 7, lanes 11 and 12).

To examine the effect of NF-κB subunits on IL-1β *trans* activation, a similar cotransfection experiment was performed using the wild-type and mutant 4.4kb IL-1β promoter fragments (Table 1). Interestingly, expression of NFKB1 or RelA alone stimulated the wild-type IL-1β4.4 promoter about 5- to 10-fold, whereas c-Rel coexpression induced the wild-type promoter about 20-fold; mutation of the NF-κB site in the IL-1β4.4 construct increased the basal level of expression of the construct two- to threefold but reduced the inducibility of the IL-1β promoter to basal levels; furthermore, c-Rel-mediated *trans* activation of the IL-1β4.4 mutant was reduced to less than twofold. Coexpression of RelA plus NFKB1, NFKB1 plus c-Rel, and RelA plus c-Rel *trans* activated the wild-type IL-1β4.4 construct between 5- and 10-fold, whereas again the mutant construct was not activated. These results suggest that the −300 region contributes to both positive and negative regulation of the IL-1β promoter.

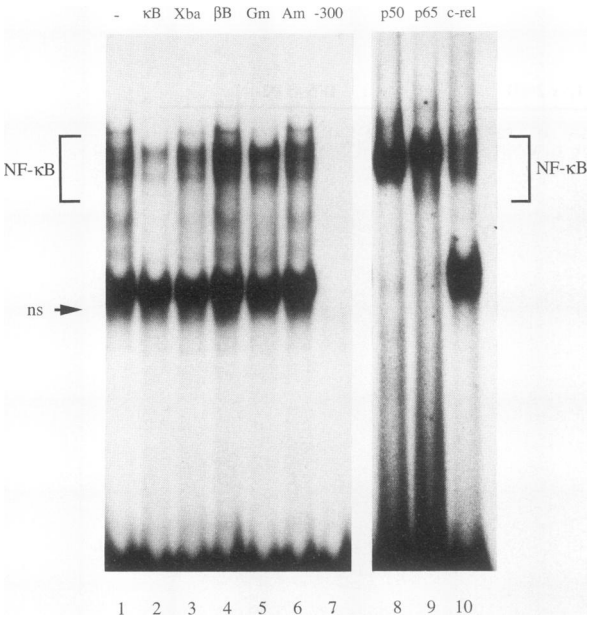

FIG. 4. Binding of induced and recombinant NF-κB proteins to the −300 region probe. Sendai virus-induced WCE (10 μg) were assayed for NF-κB-specific binding to the −300 region probe (−307 to −280) (lanes 1 to 7); a 200-fold excess of various competitor oligonucleotides (indicated above the lane) was preincubated with the extract in lanes 2 to 7. Partially purified recombinant p50 (NFKB1) and p65 (RelA) (isolated as described in Materials and Methods) were incubated with the −300 region probe (lanes 8 and 9); a WCE from MT-2 cells (10 μg) containing high levels of c-Rel was also incubated with the −300 probe (lane 10). ns, nonspecific.

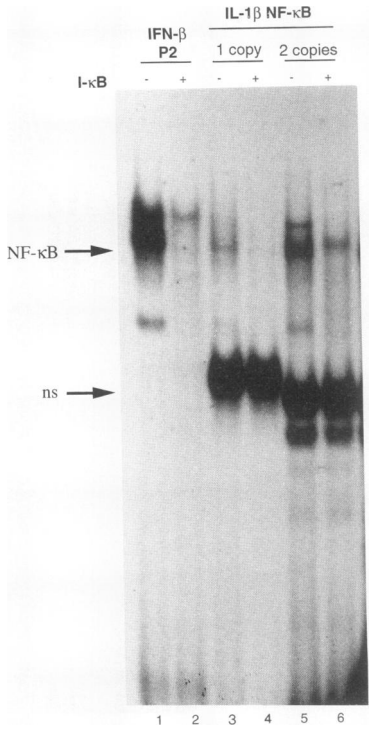

FIG. 5. Recombinant IκB-induced inhibition of NF-κB binding. WCE from PMA-induced U937 cells (10 μg) was preincubated with or without 5 ng of recombinant IκB (indicated above the lanes) obtained from bacterial extracts and analyzed for binding to 0.2 ng of [γ-^{32}P]ATP end-labelled probes. Lanes 1 and 2, IFN-β P2; lanes 3 and 4, IL-1β × 1κB probe; lanes 5 and 6, IL-1β × 2κB. ns, nonspecific.

DISCUSSION

The posttranslational release of cytoplasmic NF-κB proteins via degradation of the IκB molecule provides a general mechanism by which the convergence of different inductive signals may result in the rapid nuclear translocation of NF-κB and activation of NF-κB-dependent genes (6, 7, 62). With regard to IL-1β gene regulation, the induction of NF-κB by cytokines such as tumor necrosis factor and IL-1 also implies the existence of an autoregulatory loop whereby IL-1 release as a consequence of virus or bacterial infection may further upregulate IL-1 transcription. The presence of a positive IL-1 loop may in turn provide a physiologically important mechanism for upregulation of IL-1-mediated inflammatory responses (1, 17, 69). In this study, we provide three types of evidence to link the NF-κB pathway with the activation of the IL-1β promoter. (i) Directed mutation of the putative NF-κB binding site in the −300 region of the IL-1β promoter (composed of the decamer sequence 5′-GG GAAAATCC-3′) reduced the responsiveness of the IL-1β4.4-kb fragment to different inducers, including phorbol ester, poly(rI-rC), Sendai virus infection, and IL-1β. (ii) By using probes corresponding to the −307 to −280 region of the IL-1β promoter or to the IL-1β NF-κB site, inducer-specific NF-κB binding activity was identified. Anti-NF-κB subunit-specific antibodies detected the presence of NFKB1 (p50), RelA (p65), and a small amount of c-Rel in protein-DNA complexes from PMA-induced U937 cells. Furthermore, complex formation was largely inhibited by the addition of recombinant IκBα (28), thus supporting evidence that the extracts contained mainly p50/p65 heterodimers and some p50 homodimers that were resistant to IκBα. Since IκBα at high concentrations can also inhibit p50 DNA binding, p50 homodimers may also be affected by the recombinant IκBα (7). (iii) In cotransfection experiments, individual NF-κB subunit expression plasmids or combinations of NF-κB subunits differentially *trans* activated reporter gene activity in constructs regulated by the IL-1β4.4 promoter or the IL-1β NF-κB site. Interestingly, p65 (RelA) and c-Rel were the strongest activators, possibly reflecting the binding site preference of these subunits for the NF-κB site in the −300 region (34, 60) or possibly elsewhere within the IL-1β promoter (59). A fourth piece of evidence linking NF-κB activation and IL-1 comes from the recent studies of Beg et al. (6, 7): rapid NF-κB induction after treatment with LPS, IL-1, and other inducers was shown to be mediated by phosphorylation and subsequent degradation of IκBα. Induction of IL-1β transcription and NF-κB binding are also temporally related; both IL-1β transcription and NF-κB activation were induced within 30 min of LPS treatment in the PLB-985 myeloid cell model (39a). In previous studies, the importance of the NF-κB sequence was implied by the observation that deletion of the −300 region containing the IL-1β NF-κB binding site reduced the ability of the IL-1β promoter to direct CAT expression in transient transfection assays (12, 20, 59). Thus, the IL-1β promoter may be considered as an additional member of the family of cytokine genes regulated at least in part by the NF-κB/*rel* family of transcription factors (1, 3).

These results imply that rapid induction of NF-κB is important for cooperative interactions among transcription factors involved in IL-1β promoter activation but do not

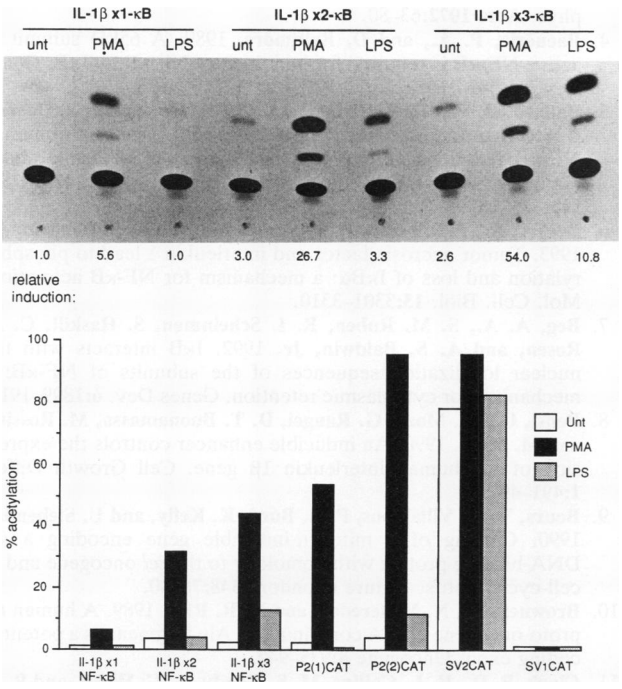

FIG. 6. Induction of IL-1β and IFN-β NF-κB hybrid promoters in U937 cells. U937 cells were transfected by the DEAE-dextran method and induced 24 h later with PMA (25 ng/ml) or LPS (10 μg/ml). Cells were harvested 48 h after transfection, and 50 μg of total cell lysate was assayed for CAT enzyme activity for 4 h. Transfections were performed in triplicate; one representative thin-layer chromatography plate is shown above, and the average CAT values are plotted below. Average inducibility values are listed below the autoradiograph; the values were calculated relative to the percent conversion obtained for the untreated (unt) IL-1β × 1κB cell transfections. Each transfection contained SV_1CAT and SV_2CAT controls.

suggest that NF-κB is the sole transcriptional protein responsible for IL-1β activation. The importance of NF-IL6 transcription factors in the activation of IL-1 has been demonstrated previously (59); NF-IL6 is composed of two C/EBP-like proteins, at least one of which is capable of interacting with the NFKB1 (p50) subunit (37). A 180-bp PMA-inducible enhancer element located between −2982 and −2795 upstream of the mRNA start site of IL-1β has been identified (8); interestingly, this element contained motifs related to the AP-1 site of the collagenase promoter and to the PRDI/IRF-1 domain of the IFN-β promoter (19, 43, 66). The presence of NF-κB binding sites and putative IFN regulatory factor (IRF) binding domains have now been observed in several cytokine promoters, including IFN-β, IL-1, and tumor necrosis factor (8, 20, 25, 36, 59). In the case of IFN-β, it was shown that synergistic interaction between PRDI/IRF-1 and PRDII/NF-κB motifs was required for maximal stimulation of IFN-β gene expression (19, 36). Recent detailed analysis of the IL-1β upstream element has extended the inducible region to include sequences from −3757 to −2729 (59). This complex enhancer element contained discrete cooperative regions which provide tissue-specific expression in the context of the cap site-proximal pro-IL-1β promoter sequences. Three essential factors were shown to bind within this region, and mutations that decreased the binding efficiency of two of these factors resulted

TABLE 1. Activation of the IL-1β promoter by NF-κB subunits

Subunit	IL-1β4.4		IL-1β4.4mut	
	% CAT activity[a]	Relative induction[b]	% CAT activity	Relative induction
None	4.8	1.0	11.3	1.0
NFKB1 (p50)	41.6	8.7	8.7	0.8
RelA (p65)	36.4	7.6	7.6	0.6
c-Rel	77.9	16.2	20.1	1.8
NFKB1 + RelA	25.7	5.4	4.4	0.4
NFKB1 + c-Rel	18.1	3.8	8.0	0.7
RelA + c-Rel	32.8	6.8	12.4	1.1

[a] Percent CAT activity is an average of triplicate experiments with average deviations of 30%.
[b] Induced value/uninduced value.

in a corresponding decrease in LPS-dependent gene expression. The proteins corresponded to NF-IL6 and NF-β1, an IRF-1-like protein (59).

A DNA-binding protein complex termed NF IL-1βA or (NF-βA) that binds to a 12-bp highly conserved sequence within the cap-proximal promoter region of IL-1β and binds to the sequence ACTTCTGCTTT, located adjacent to the IL-1β TATA box at position −49 to −38, was characterized recently (30). Activation of monocytic cells with PMA or LPS rapidly and transiently affects the relative levels of NF-βA activity, suggesting that this protein also contributes to IL-1β induction (30). The absence of an NF-βA site in the IL-1α promoter furthermore suggests a mechanism of differential regulation of the two genes in a cell-type-specific fashion. Thus, at least two DNA elements, widely dispersed within the IL-1β promoter, mediate inducibility.

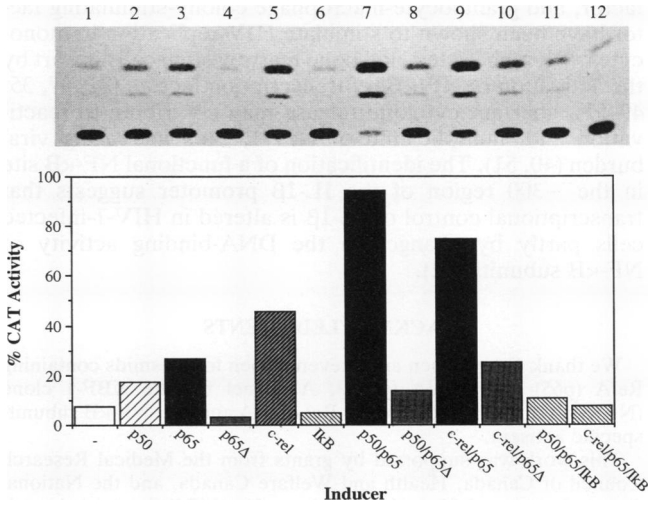

FIG. 7. Activation of the IL-1β NF-κB site by distinct NF-κB subunits. The IL-1β × 2κB CAT plasmid (5 μg) was transfected into Jurkat cells by the DEAE-dextran method together with different SVK3-derived plasmids expressing the IκBα, NFKB1 (p50), ΔRelA (p65Δ), or c-Rel proteins; the RelA (p65) subunit was expressed from the CMIN-p65 vector; expression vectors were used at 5 μg, with the exception of CMIN-p65, which was used at 2 μg. Cultures were harvested at 48 h after transfection and assayed for CAT activity (50 to 100 μg of protein for 4 h). The bar graph was coded to reflect the presence of different NF-κB subunits: ▨, p50 alone; ■, p65-containing transfections; ■, c-Rel alone; ▩, p65Δ-containing transfections; ▧, IκB-containing transfections.

In addition to NF-κB binding, a novel DNA-binding activity termed NF-βB that overlaps significantly with the IL-1β NF-κB site has been characterized (45). By UV cross-linking analysis, this complex was generated by a distinct polypeptide of 61 kDa (45). With the IL-1β NF-κB probe, an additional, higher-mobility complex that was insensitive to competition by PRDII/NF-κB and only partially sensitive to competition by homologous IL-1β NF-κB oligonucleotides was formed. As demonstrated in Fig. 3 and 4, this protein lacks apparent specificity for the PRDII/NF-κB binding site and was unrelated to NFKB1 (p50) or RelA (p65). It has not been possible to determine with certainty if this complex is NF-βB or an unrelated binding activity.

The fact that multimerization of the κB elements was necessary to detect any LPS-inducible activity indicates that the NF-κB site alone in a heterologous construct is not sufficient to mediate the LPS response. Shirakawa et al. demonstrated that the LPS-responsive element maps to the −3000 upstream enhancer region and functions in a tissue-specific manner only in the context of a simultaneous interaction with the 5′ cap site-proximal pro-IL-1β promoter (59). Cooperative interactions between upstream and promoter-proximal transcription factors other than NF-κB appear to be necessary to mediate the LPS response.

Preliminary evidence suggests that the IL-1β promoter is more active in HIV-infected myeloid cells treated with PMA or LPS than in uninfected cells (39a). IL-1β production is dramatically increased in HIV-infected myeloid cells, and elevated levels of IL-1 have been detected in the serum of HIV-1-infected individuals (13, 15, 16, 41, 64, 65, 67). This phenomenon is particularly important since cytokine production by HIV-1-infected cells may differentially activate HIV replication and may contribute to viral pathogenesis (18, 21, 27, 35, 40, 49–51). Since cytokines such as tumor necrosis factor alpha, IL-1, macrophage colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor have been shown to stimulate HIV replication in monocytes, T lymphocytes, and bone marrow stem cells in part by the activation of NF-κB/*rel* transcription factors (21, 27, 35, 49–51), aberrant cytokine release may contribute to reactivation and multiplication of HIV-1, thus increasing viral burden (40, 51). The identification of a functional NF-κB site in the −300 region of the IL-1β promoter suggests that transcriptional control of IL-1β is altered in HIV-1-infected cells partly by changes in the DNA-binding activity of NF-κB subunits (52).

ACKNOWLEDGMENTS

We thank Craig Rosen and Steven Ruben for plasmids containing RelA (p65) and ΔRelA (p65Δ), A. Israel for the KBF-1 clone (NFKB1), and N. Rice for the c-Rel cDNA and anti-NF-κB subunit-specific antisera.

This work was supported by grants from the Medical Research Council of Canada, Health and Welfare Canada, and the National Cancer Institute. J.H. is the recipient of an MRC Scientist Award; A.R. is the recipient of a studentship from FCAR.

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