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Regulation of Interleukin 12 p40 Expression through an NF-κB Half-Site

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Interleukin 12 (IL-12) is an inducible cytokine composed of 35- and 40-kDa subunits that is critical for promoting T helper type 1 development and cell-mediated immunity against pathogens. The 40-kDa subunit, expressed by activated macrophages and B cells, is induced by several pathogens in vivo and in vitro and is augmented or inhibited by gamma interferon (IFN-γ) or IL-10, respectively. Control of IL-12 p40 expression is therefore important for understanding resistance and susceptibility to a variety of pathogens, including *Leishmania major* and perhaps human immunodeficiency virus. In this report, we provide the first characterization of IL-12 p40 gene regulation in macrophages. We localize inducible activity of the promoter to the sequence $^{-122}$GGGGAATTTTA^{-132} not previously recognized to bind Rel family transcription factors. We demonstrate binding of this sequence to NF-κB (p50/p65 and p50/c-Rel) complexes in macrophages activated by several p40-inducing pathogens and provide functional data to support a role for NF-κB family members in IL-12 p40 activation. Finally, we find that IFN-γ treatment of cells enhances this binding interaction, thus potentially providing a mechanism for IFN-γ augmentation of IL-12 production by macrophages.

Interleukin 12 (IL-12) production by macrophages is critical in induction of T helper type 1 (Th1) cells during initial immune responses to pathogens (5, 15, 21, 32, 45). Th1 cells produce gamma interferon (IFN-γ) and IL-2, which promote macrophage activation and cytolytic T-cell maturation, thus generating effective cell-mediated responses to intracellular pathogens (33, 37). The macrophage is the principal source of IL-12 production in responses to certain intracellular pathogens, such as *Listeria monocytogenes* (20, 21, 48). Recently, Chehimi and colleagues described diminished IL-12 production by macrophages in human immunodeficiency virus-infected individuals (2) and proposed that this may contribute to reduced cell-mediated immunity seen in AIDS (6, 7). Thus, understanding IL-12 production by macrophages, and its inhibition in settings of disease, could contribute to immune response-based therapies or vaccine designs.

IL-12 is an inducible, heterodimeric, disulfide-linked cytokine composed of 35- and 40-kDa subunits encoded by separate genes (5, 19, 24, 43, 49). Expression of the 35-kDa subunit is constitutive and ubiquitous. In contrast, the 40-kDa subunit is expressed only by macrophages and B cells; it is strongly induced by several bacterial stimuli and is considered the regulatory component for IL-12 expression (11). Further, we and others have found that some cytokines, notably IFN-γ and IL-10, can exert their effects on T-cell responses by augmenting or inhibiting macrophage production of IL-12 (10, 21, 26). To date, the transcriptional regulation of p40 gene induction by bacteria and by cytokines is uncharacterized.

In this report, we demonstrate several important features of IL-12 p40 gene regulation in macrophages. First, we localize the inducible promoter activity for the p40 gene to a novel sequence not previously recognized to bind Rel family or other transcription factors. Second, we demonstrate binding of this sequence to NF-κB (and c-Rel)-containing complexes in activated macrophages. Third, we show that IFN-γ enhances induced transcription from the IL-12 p40 promoter and increases binding of NF-κB-related factors to this site. These results provide a basis for understanding bacterial induction and IFN-γ augmentation of macrophage IL-12 production.

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MATERIALS AND METHODS

Cell lines. The J774 murine macrophage cell line, obtained from D. Chaplin, Washington University School of Medicine School, St. Louis, Mo., and L929 cells were maintained in Iscove's modified Dulbecco's modified Eagle medium (IDME) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 0.1 mM minimal essential medium nonessential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 mM β-mercaptoethanol. THP-1 human macrophage cell line, obtained from B. Lee, Washington University School of Medicine, was maintained in RPMI with the same supplements.

IL-12 genomic cloning. A 129SV genomic library produced in the vector lambda FIX II (Stratagene, La Jolla, Calif.) was screened by using probes from the p35 and p40 IL-12 cDNAs (43). Three clones positive for the p40 cDNA were isolated and subcloned into the *Eco*RI sites of pBlueScript KS (Stratagene), and structures were determined by restriction mapping, hybridization to p40-specific oligonucleotides, PCR, and sequencing (Fig. 1).

IL-12 measurements. Peritoneal exudate cells (PECs; 10^6), elicited in BALB/c mice with thioglycolate for 4 days, were treated with or without IFN-γ (50 U/ml) and lipopolysaccharide (LPS; 100 ng/ml) for 22 h. IL-12 secreted was quantitated with a sandwich enzyme-linked immunosorbent assay (ELISA) using anti-murine p40 monoclonal antibody Sal E as the primary antibody and biotinylated anti-murine p35 monoclonal antibody Red T as the secondary antibody. Antibodies were generous gifts of C. Tripp and E. Unanue (Washington University School of Medicine). Reverse transcriptase-coupled PCR (RT-PCR) to assess IL-12 p40 mRNA levels was performed essentially as described previously (35). Briefly, total RNA purified by using a Stratagene RNA purification kit was reverse transcribed by using Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) and T_{15} primer (Promega). cDNA was amplified by PCR (Perkin-Elmer Cetus 9600). Aliquots were dot blotted onto nitrocellulose, detected by using ^{32}P-labeled oligonucleotide probes, and quantitated with a PhosphorImager (Molecular Dynamics). PCR primers and probes are shown in Table 1.

Plasmid construction. The control plasmid CMV-CAT and the high-copy-number reporter plasmid pBS-LUC were as described previously (46).

For 5′ deletion mutants, DNA fragments were produced by PCR, using 5′ oligonucleotides tailed with a *Sal*I site annealing over 18 to 22 bp of clone 6 beginning at the indicated base relative to the start site of transcription and the 3′ oligonucleotide m12-6 tailed with a *Bam*HI site annealing over 22 bases of

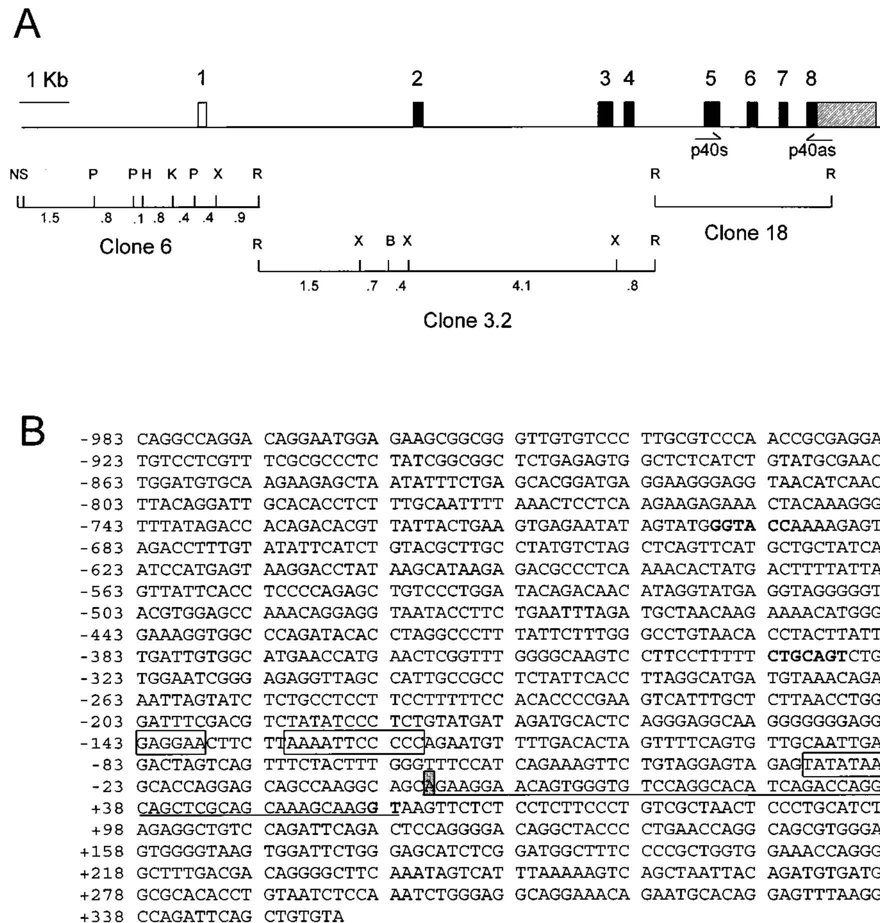

FIG. 1. Structure of the murine IL-12 p40 gene and promoter. (A) Schematic representation of intron/exon organization of murine p40. Numbered boxes, exons in transcribed order; open box, 5′ noncoding sequence; black boxes, coding sequence; hatched box, 3′ noncoding sequence. The binding locations of oligonucleotide primers used for RT-PCR are shown schematically beneath the genomic map. Also shown are the corresponding plasmid subclones used for reporter construction. Genomic restriction sites (approximate locations are shown): H, *Xho*I; N, *Not*I; S, *Sal*I; P, *Pst*I; K, *Kpn*I; X, *Xba*I; R, *Eco*RI. (B) Genomic sequence of the murine p40 promoter region. Numbers are with reference to the start site of transcription. Bold letters indicate *Kpn*I and *Pst*I sites of clone 6. Boxes indicate, respectively, a Pu.1 consensus site, an NF-κB consensus half-site, a TATA box, and the start site of transcription (shaded). Underlined bases are the sequence of the noncoding exon 1.

clone 6 beginning at +54 relative to the start site of transcription. PCR products were *Sal*I-*Bam*HI digested and cloned into the *Sal*I-*Bgl*II sites of pBS-LUC (46).

DM-4300 was produced by first cloning the 1.2-kb *Pst*I fragment from clone 6 into the unique internal *Pst*I site of DM-564. This intermediate plasmid was digested with *Sal*I and *Xho*I, and the 2.5-kb *Sal*I-*Xho*I fragment from clone 6 was inserted.

To produce DM-4300 In1, a DNA fragment was generated by PCR, using clone 3.2 as a template, the 5′ KS oligonucleotide, and a 3′ oligonucleotide tailed with an *Eco*RI site which anneals over 20 bases of clone 3.2 immediately upstream of the ATG of exon 2 and containing the native splice acceptor site of intron 1. The PCR product was digested with *Eco*RI and cloned into the *Eco*RI site of clone 6. The 7-kb *Sal*I fragment containing 4,300 bases of promoter sequence, the first exon, and the first intron was cloned into the *Sal*I site of pBS-LUC.

To produce internal scanning mutations, DNA fragments were generated by PCR, using a 5′ oligonucleotide tailed with a *Bam*HI site which anneals over 20 bases of clone 6 at −703 relative to the start site of transcription with 3′ oligonucleotides tailed with an *Xho*I site and zero or three mutated bases and annealing to clone 6 at −154, −143, −131, and −117. PCR fragments were *Bam*HI-*Xho*I digested and were cloned into the *Bam*HI-*Sal*I sites of DM-148, DM-138, DM-125, and DM-110, respectively. The control plasmid DM-703 was produced by cloning a *Bam*HI-digested PCR fragment generated from clone 6 by using the same 5′ oligonucleotide and m12-6 as the 3′ oligonucleotide into the *Bam*HI site of pBS-LUC.

All plasmids were twice banded in cesium chloride and verified by restriction mapping and by dideoxynucleotide sequencing using Sequenase version 2.

Transient and stable transfections. For transient transfections, J774 cells (10×10^6) were suspended in 1.2 ml of IDME supplemented with 20% fetal calf serum and with 20 μg of the indicated luciferase plasmid per ml and 10 μg of CMV-CAT per ml. Cells were electroporated at room temperature in three 0.4-ml aliquots in 0.4-mm cuvettes (Bio-Rad) in a Bio-Rad Gene Pulser at 300 V and 960 μF. After electroporation, the three aliquots were recombined and then were divided equally among three wells; 18 h later, cells were activated, and cell extracts were prepared by three freeze-thaw cycles in 100 μl of 250 mM Tris-HCl (pH 7.6) followed by 5 min of microcentrifugation. Luciferase activity was determined from 70 μl of cell extract, and chloramphenicol acetyl transferase (CAT) activity was determined from 20 μl of cell extract as described previously (46).

For stable transfections, J774 cells (10×10^6) were electroporated as for transient transfection with 33 μg of the indicated luciferase plasmid per ml and

TABLE 1. Sequences of oligonucleotides used for RT-PCR measurement of murine IL-12 p40

Gene	Oligonucleotide[a]	Sequence
Murine p40	mp40 s	5′GGAGACCCTGCCCATTGAACT
	mp40 as	5′CAACGTTGCATCCTAGGATCG
	mp40 p	5′TGTCTGCGTGCAAGCTCAGGA
HPRT	hprt s	5′TGGGAGGCCATCACATTGT
	hprt as	5′GCTTTTCCAGTTTCACTAATGACA
	hprt p	5′GGTGGAGATGATCTCTCAACTTT

[a] s, sense; as, antisense; p, probe.

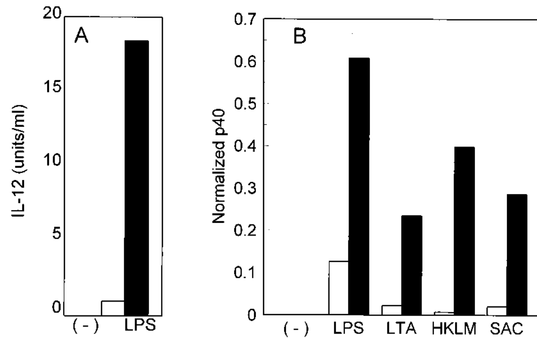

FIG. 2. IFN-γ augments induction of murine IL-12 p40 transcription by bacterial products. (A) Thioglycolate-elicited PECs, 10^6/ml, were left untreated [(−)] or were stimulated for 22 h with LPS (0.1 μg/ml; open bar) or LPS and IFN-γ (50 U/ml; closed bar). Data shown are for IL-12 determined by ELISA. (B) J774 cells were maintained without IFN-γ (open bars) or with 100 U of recombinant murine IFN-γ per ml (closed bars) for 18 h before a 4-h stimulation with *Escherichia coli* LPS (0.1 μg/ml), LTA (0.1 μg/ml), HKLM (10×10^7/ml), or formalin-fixed SAC (0.0075% [wt/vol]) or no stimulation (−). Following RT-PCR analysis, normalized p40 mRNA levels were determined by dividing the PhosphorImager-determined p40 signal by the *HPRT* signal from the same sample.

6.7 μg of the neomycin resistance plasmid pSRαneo72.3 (a gift from A. Shaw, Washington University School of Medicine) per ml. After electroporation, the three aliquots were combined in 6 ml of supplemented IDME in one well of a six-well plate. After 24 h, live cells were suspended at 10^5/ml in medium containing 400 μg of Geneticin (Sigma) per ml. After several passages, cultures were subsequently maintained in 200 μg of Geneticin per ml.

For cotransfection studies, L929 cells grown to 50 to 75% confluence in 24-well plates were transfected with 2 μg of the luciferase reporter plasmid and 0.6 μg of expression vector for the indicated Rel family members by calcium phosphate as described previously (31). Forty-eight hours after transfection, luciferase activity was assessed in extracts prepared by using Promega cell culture lysis buffer.

Oligonucleotides. Complementary oligonucleotides were synthesized with a Beckman Oligo 1000 DNA synthesizer (Beckman Instruments, Fullerton, Calif.). For use in electrophoretic mobility shift assays (EMSAs), complementary oligonucleotides were annealed and gel purified. Double-stranded oligonucleotides with single-stranded ends were labeled with [α-^{32}P]dCTP and the large fragment of DNA polymerase. Double-stranded oligonucleotides without overhangs were end labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. When used as EMSA competitors, double-stranded oligonucleotides with single-stranded ends were filled in with unlabeled nucleotides, using the large fragment of DNA polymerase, prior to gel purification. Sequences of EMSA oligonucleotides from the p40 promoter are indicated in Fig. 5A. Other sequences are as follows: m12-25mut, 5′TAAAATTCCCCCActtTGTTTTG; murine IL-2κB (22), 5′CAAGAGGGATTTCACCTAAATCC; human IgκB (22), 5′AGAGGGGACTTTCCGAGAGGC; murine NF-AT (46), 5′CAAAGAGGAAAATTTGTTTCATACAGAAGGC; human FcγR (25), 5′GTATTTCCCAGAAAAGGAAC; and simian virus 40 Pu.1 (23), 5′CTCTGAAAGAGGAACTTGGTTA.

EMSAs. Nuclear extracts for EMSAs were prepared as described previously (46). EMSAs were performed with 3 μg of nuclear extract, 2 μg of poly(dI-dC) (Pharmacia), and 2.5×10^4 cpm of radiolabeled oligonucleotide probe in a 20-μl reaction mixture on ice for 30 min. Unlabeled oligonucleotide competitors (20 ng) were added before the addition of nuclear extract. For antibody inhibitions, we used the following antimurine reagents: anti-p50 serum 37, anti-p100 serum, anti-p65 serum 34, purified anti-c-Rel, and purified anti-RelB, gifts from W. Sha, Massachusetts Institute of Technology, Cambridge (30); anti-p50 serum, a gift from M. Lenardo, National Institutes of Health, Bethesda, Md. (22); and anti-NF-κB p65 (Santa Cruz Biotechnology, Inc.). Nuclear extracts were incubated with antibodies and poly(dI-dC) in binding reaction buffer for 30 min on ice before the addition of labeled probe.

RESULTS

Genomic organization and promoter sequence of murine IL-12 p40. We used three adjacent genomic regions of the murine p40 gene to define the intron/exon organization of the p40 gene (Fig. 1A). Exon 1 of the p40 gene lies within clone 6 and is noncoding. An intron of 4.1 kb separates exon 1 and exon 2. A consensus splice acceptor site immediately precedes the ATG initiator codon beginning exon 2. We identified the transcriptional start, indicated by the shaded box in Fig. 1B, by primer extension analysis (data not shown). We identified a putative TATA box at −30 bp relative to the transcription start site and several potential transcriptional regulatory sites within the first 1,000 bases upstream of exon 1 identified (Fig. 1B).

p40 transcription in J774 cells models nontransformed macrophages. Since previous studies showed that IFN-γ augments IL-12 production by macrophages (26), we asked whether this effect was at the level of transcription of the p40 gene. We activated murine thyoglycolate-elicited PECs stimulated with LPS in the absence and presence of IFN-γ and measured IL-12 production by ELISA (Fig. 2A). IFN-γ caused

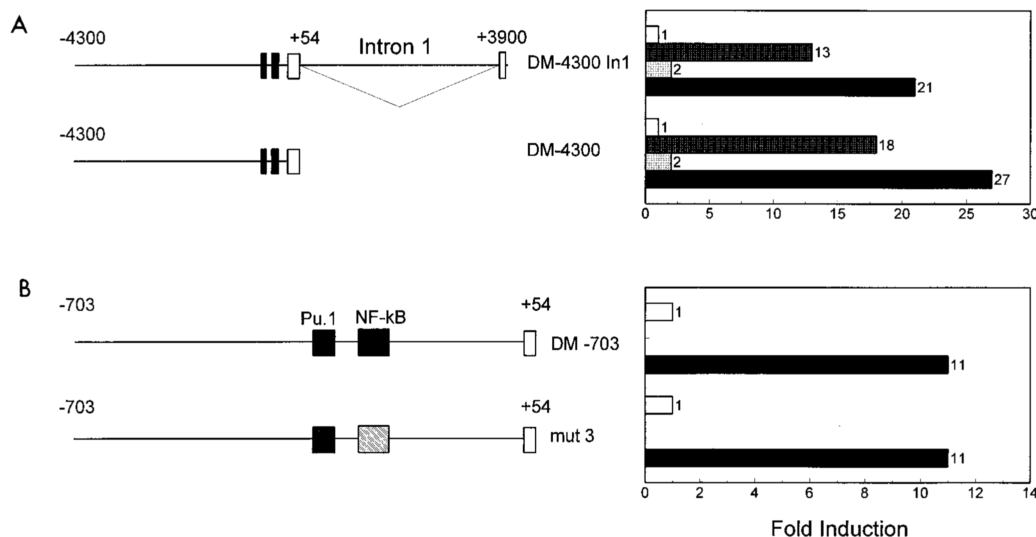

FIG. 3. *cis*-acting elements in a 4.3-kb p40 promoter region confer LPS-inducible, IFN-γ-regulated activity. Stable transfectants of J774 cells were prepared by cotransfection of pSRαneo72.3 and the indicated luciferase plasmid. Black boxes represent potential *cis* elements, $^{-143}$GAGGAA^{-138} and $^{-131}$AAAATTCCCC^{-122}; the hatched box represents mutated site $^{-131}$ActcgaCCCC^{-122}; open boxes represents noncoding exon 1. A total of 10^6 cells from each transfectant pool were treated without or with IFN-γ (100 U/ml) for 1 h and with or without LPS for an additional 4 h and were assayed for luciferase activity. Stimulation conditions: open bar, no treatment; dark hatched bar, LPS alone; light hatched bar, IFN-γ alone; black bar, IFN-γ and LPS. Results shown are fold stimulation over that of untreated cells.

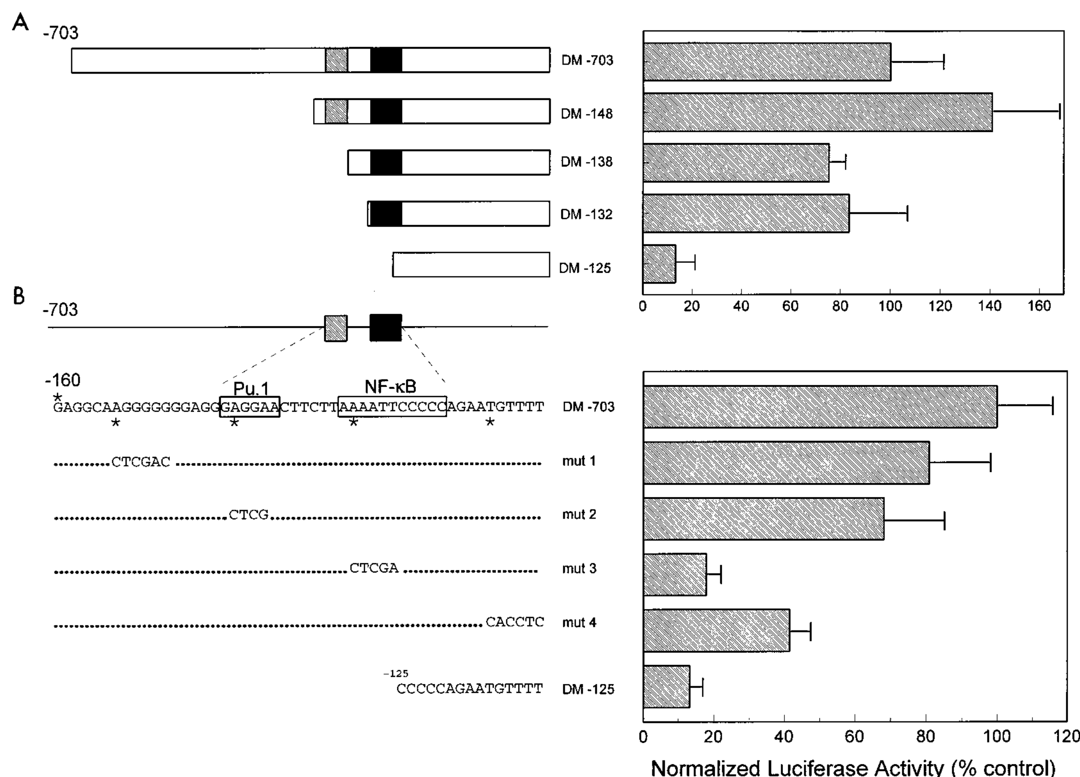

FIG. 4. IL-12 p40 promoter sequences between −132 and −125 mediate induced promoter activity. Transient transfections of J774 cells were prepared by using CMV-CAT and the indicated luciferase plasmid. Shaded boxes represent the Pu.1 consensus sequence $^{-143}$GAGGAA^{-138}; black boxes represent potential NF-κB site $^{-131}$AAAATTCCCC^{-122}. Internal scanning mutations (B) align with * beneath the sequence. At 18 h after transfection, cells were stimulated with 100 U of IFNγ per ml for 1 h and with LPS for an additional 4 h. Normalized luciferase activity was determined by normalizing the relative light units from each transfectant for transfection efficiency, using CAT activity determined in the same cell extracts, and is expressed as a percentage of the activity of the control, DM-703. All constructs contain the native p40 TATA box, initiation site, and 5′ untranslated exon 1. Results are averages of triplicate wells.

a 20-fold increase in the level of IL-12 produced by LPS-stimulated macrophages. This effect of IFN-γ was evident at the level of increased p40 mRNA as well (data not shown). Thus, IFN-γ appeared to augment the LPS-induced transcription of the p40 gene in PECs.

The murine macrophage cell line J774 showed a similar pattern of regulation by LPS and IFN-γ. Thus, IFN-γ markedly augmented p40 mRNA induced by LPS, *Staphylococcus aureus* Cowan (SAC), heat-killed *L. monocytogenes* (HKLM), or lipoteichoic acid (LTA) (Fig. 2B). Furthermore, IFN-γ treatment alone did not induce p40 mRNA. Thus, we used J774 cells as a model of p40 expression in subsequent experiments.

IL-12 p40 promoter sequences between −132 and −125 confer LPS inducibility and IFN-γ augmentation. To determine the contributions of specific genomic regions to inducible p40 gene expression, we prepared J774 cell lines stably transfected with luciferase-based p40 promoter constructs (Fig. 3). J774 cells, stably transfected with constructs containing 4.3 kb of proximal 5′ sequence, first exon, and first intron (DM-4300 In1), showed LPS-inducible IFN-γ-regulated luciferase activity (Fig. 3A). LPS treatment of these cells induced luciferase activity 13-fold above background, which increased to 21-fold above background upon IFN-γ treatment. J774 cells stably transfected with a similar construct lacking intron 1 (DM-4300) also showed 18-fold induction by LPS and 27-fold induction by LPS plus IFN-γ. Notably, IFN-γ treatment alone increased luciferase activity up to twofold, somewhat unlike the effect on native p40 gene expression in nontransformed macrophages. Nonetheless, these results show that *cis*-acting elements in this 4.3-kb proximal promoter region confer LPS-inducible, IFN-γ-regulated transcription to the p40 gene independently of intron 1.

To map these elements, we prepared various luciferase-based reporter constructs containing from 40 to 4,300 bp of 5′ promoter sequence (Fig. 4A). We examined the activity of each of these constructs in transient transfection of J774 cells, normalizing for transfection efficiency by cotransfection with CMV-CAT, a cytomegalovirus promoter-CAT reporter plasmid (46). We found no loss of promoter activity in constructs containing deletions from 4,300 bp (DM-4300) to within 703 bp of the transcriptional start site (DM-703) (Fig. 4A). Deleting sequences between −703 and −148 slightly increased activity, by 20 to 30%. Deleting sequences to within −138 and 132 bp of the start site reduced activity by only 10 to 20% compared with the full-length promoter (Fig. 4A). Further deleting a 7-bp region between −132 and −125 markedly reduced activity to only 15% of the control level, suggesting the presence of an important positive *cis*-acting element. Thus, we next focused within this region to identify specific elements.

We produced a series of scanning mutations in the context of the DM-703 promoter construct (Fig. 4B). A 5-bp scanning mutation between −154 and −150 (mut 1) reduced promoter activity by only 20%, and a 4-bp mutation between −142 and −139 (mut 2) reduced activity by only 30%. In contrast, a 5-bp scanning mutation between −130 and −126 (mut 3) reduced activity by 80%, as great a loss in activity as that upon deletion of the entire promoter region down to −125 bp (DM-125). While the activity of mut 3 was considerably lower than the

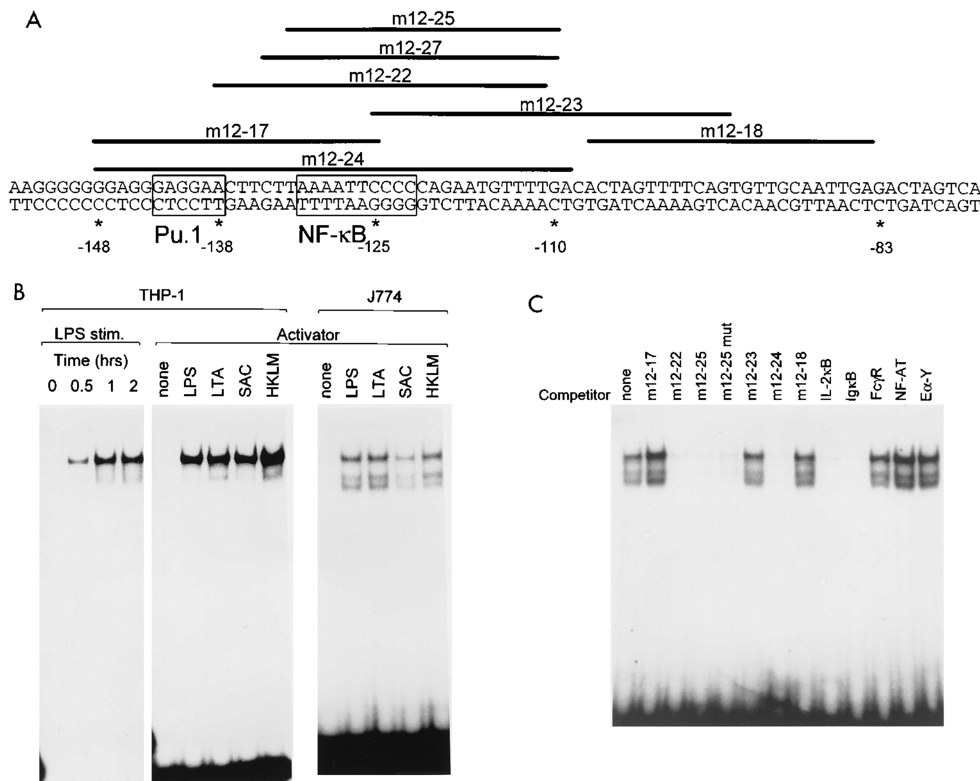

FIG. 5. Inducible factors bind the −132 to −110 p40 promoter region and interact with consensus NF-κB sites. (A) Locations of oligonucleotide probes within the murine p40 promoter. A schematic of the p40 promoter sequence from −155 to −75 relative to the transcriptional start site is shown. Potential factor binding consensus sequences mentioned in the text are boxed and labeled. The various oligonucleotide probes used in EMSA are shown relative to their positions within the promoter. (B) Inducible binding of a factor to probe m12-25. Nuclear extract protein (3 μg) from THP-1 cells, either left untreated (none), treated for the indicated number of hours (0, 0.5, 1, or 2) with LPS, or treated for 4 h with LPS, LTA, SAC, or HKLM as indicated, or from J774 cells treated with IFN-γ for 1 h and for 4 h with the indicated activator was incubated with 25,000 cpm of ^{32}P-labeled m12-25 as described in Materials and Methods and analyzed by nondenaturing 5% polyacrylamide gel electrophoresis and autoradiography. (C) Nuclear extract (3 μg) from IFNγ- and LPS-activated J774 cells was incubated with 25,000 cpm of ^{32}P-labeled m12-25 probe and 20 ng of the indicated competitor oligonucleotide.

control activity, the residual activity was inducible by LPS when measured in stable transfectants (Fig. 3B). This observation could imply residual inducibility from the weak interaction of factors at the mutated site or factors acting at less important sites outside this region. A downstream mutation between −116 and −110 (mut 4) had less effect, leaving approximately 40% of control activity. These results confirm the conclusions from our earlier functional deletion mapping and again suggest that the region between −132 and −120 significantly contributes to overall promoter activity.

Rel family proteins interact with a p40 promoter NF-κB half-site. To identify factors contributing to inducible activity, we prepared overlapping oligonucleotide probes spanning this promoter region (Fig. 5A). First, we examined probe m12-25, which spans the active region, for interactions with nuclear extracts from J774 cells and the human monocytic cell line THP-1 (Fig. 5B). m12-25 formed EMSA complexes with nuclear extracts from activated J774 and THP-1 cells but not from unactivated cells. With THP-1 cells, the predominant EMSA complex formed within 30 min of LPS treatment and was maintained for at least 2 h. Treatment of THP-1 cells with SAC, HKLM, and LTA produced EMSA complexes identical to those from LPS-treated cells. With J774 cells, the predominant EMSA complex had mobility similar to that of THP-1 cells, but a faster-migrating complex was also apparent. Thus, the region −132 to −110 binds induced nuclear factors in both human and murine macrophages.

We next examined the sequence specificity of m12-25 binding to these EMSA complexes, using several competitor oligonucleotides (Fig. 5C). The competitors m12-22, m12-25, and m12-24, which entirely overlap with m12-25, completely inhibited EMSA complex formation with LPS- and IFN-γ-induced nuclear extracts. In contrast, m12-18, an unrelated but adjacent sequence from the p40 promoter, failed to inhibit complex formation. Partially overlapping oligonucleotide competitors m12-17 and m12-23 also failed to inhibit complex formation, providing information on the limits of the binding site for the factor binding site.

While m12-25 lacks established consensus sites for known transcription factors, the sequence $^{-131}$AAAATTCCCC^{-121} ($^{-121}$GGGGAATTTT^{-131}) has 8-of-10-nucleotide identity to the NF-κB consensus. This finding suggests a potential half-site as a target for NF-κB binding, in which two adenine residues, $^{-131}$AA^{-130} (underlined), replace two conserved guanine residues common to reported functional NF-κB sites (1, 18, 29). Testing this possibility, we found that authentic NF-κB sites from the mu heavy-chain enhancer (IgκB) and the IL-2 promoter (IL-2κB) both inhibited complex formation with m12-25 (Fig. 5C). In contrast, three oligonucleotide probes for the distinct transcription factors Stat1 (FcγR), NF-AT (NF-AT), and NF-Y (Eα-Y) failed to inhibit complex formation. These results suggested that m12-25 may interact with members of the NF-κB family.

m12-25 contains an inverted repeat, $^{-128}$ATTCCCCCA

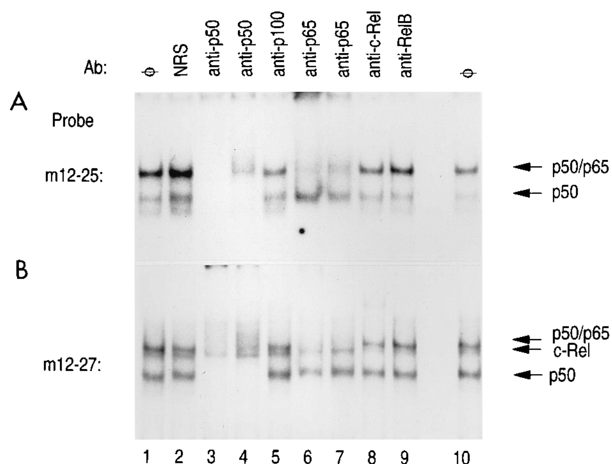

FIG. 6. Factors binding the −134 to −110 promoter region belong to the Rel family of transcription factors. Nuclear extract (3 μg) from J774 cells activated with IFN-γ and LPS was incubated for 30 min on ice without (lane 1 and 10) or with the following antisera against Rel family members: lane 2, nonimmune rabbit serum (NRS; 1 μl); lane 3, anti-p50 rabbit serum 37 (1 μl); lane 4, anti-p50 rabbit serum (1 μl); lane 5, anti-p100 rabbit serum (1 μl); lane 6, anti-p65 rabbit serum 34 (1 μl); lane 7, anti-p65 antibody (10 μg); lane 8, anti-c-Rel antibody (10 μg); and lane 9; anti-RelB antibody (10 μg). Then 25,000 cpm of ^{32}P-labeled probe m12-25 (A) or m12-27 (B) was then added for 30 min on ice. Complexes were analyzed as described in the legend to Fig. 5. Free probe is not shown.

GAAT^{-116}, unrelated to its potential NF-κB site. To test if this motif was responsible for factor binding, we interrupted this repeat without altering the potential NF-κB site (m12-25mut). Interruption of this repeat did not prevent binding to the inducible factor, since m12-25mut completely blocked EMSA complex formation in J774 extracts (Fig. 5C). Identical specificity was observed for complex formation in THP-1 nuclear extracts (data not shown). These results further suggested that the inducible factor that binds m12-25 may be related to NF-κB or other Rel transcription family members.

We next used antisera to various Rel transcription factors to analyze the constituents of the LPS-induced EMSA complexes binding to m12-25 (Fig. 6A). Two distinct p50 antisera, but not preimmune normal rabbit serum, entirely inhibited lower complex formation with the m12-25 probe to LPS-induced J774 extracts. Two distinct p65 antisera reduced formation of the upper but not the lower complex. In contrast, anti-p100, anti-c-Rel, and anti-RelB had no effect on complex formation.

A second probe, m12-27, containing two additional upstream bases, provides finer separation of the upper EMSA complex into a closely spaced doublet (Fig. 6B). Thus, we repeated the above supershift analysis with m12-27. The anti-p50 and anti-p65 antisera inhibited formation of the upper doublet component but not the lower doublet component (lanes 3, 4, 6, and 7). The anti-c-Rel antiserum inhibited formation of only the lower doublet component (lanes 8). Moreover, the anti-p50 antiserum also inhibited the faster-migrating lower complex (lanes 3 and 4), which anti-p65, anti-c-Rel, and anti-RelB antisera left unaltered. These results suggest the faster-migrating complex contains p50 but not p65, c-Rel, or RelB, consistent with p50 homodimers. The more slowly migrating doublet is consistent with p50/p65 heterodimers (upper doublet component) and a c-Rel-containing complex (lower doublet complex).

The NF-κB half-site regulates p40 promoter transcriptional activity. To test the functional role of this NF-κB site for p40 promoter activity, we prepared luciferase constructs with 2-bp mutations of the NF-κB site within the context of DM-138. First, we examined activities of these constructs in stable transfections of J774 (Fig. 7A). The native sequence conferred 15-fold LPS inducibility without IFN-γ pretreatment and 28-fold LPS inducibility with IFN-γ pretreatment. Altering the sequence from $^{-131}$AA^{-130} to $^{-131}$GG^{-130}, converting the half-site to a full NF-κB consensus, markedly increased the level of LPS inducibility from 15- to 274-fold without IFN-γ treatment and from 28- to 450-fold with IFN-γ treatment (Fig. 7A). We also examined promoter activity in transient transfections. Consistent with stable transfections, the $^{-131}$AA^{-130} to $^{-131}$GG^{-130} mutation markedly increased the induced promoter activity (Fig. 7B). As a control, mutation of $^{-131}$AA^{-130} to $^{-131}$CC^{-130}, which leaves the native consensus half-site intact and alters sequences in the nonconsensus half-site, reduced promoter by only 60%. These results suggest that the native sequence $^{-131}$AAAATTCCCC^{-121} directly participates in the LPS inducibility of p40 promoter activity through interaction with Rel family factors.

To test this hypothesis directly, we cotransfected L929 cells with an expression vector for p50, p65, or c-Rel along with a luciferase-based p40 reporter (DM-138) (Fig. 7C). Transfection of L929 cells with the p40 reporter and empty expression vector (GD) produced background levels of luciferase activity, consistent with low p40 expression by fibroblasts. Cotransfection of the p50, p65, or c-Rel expression vector with DM-138 into L929 cells produced only small increases over background luciferase activity. However, cotransfection of p50 and c-Rel expression vectors produced a 30- to 40-fold increase in luciferase reporter activity, suggesting that the p50–c-Rel interaction shown in Fig. 6 may direct transcriptional activation from this site. Notably, transfecting a combination of p65 plus c-Rel or p50 plus p65 did not markedly augment promoter activity in L929 cells. In summary, these results suggest that the half-site $^{-131}$AAAATTCCCC^{-121} regulates promoter induction by LPS and may selectively respond to particular Rel family members, such as p50 and c-Rel, for transactivation.

Potential role of Pu.1 in p40 promoter activity. A consensus binding sequence, $^{-143}$GAGGAA^{-138}, for the B-cell/macrophage-specific transcription factor Pu.1 lies immediately upstream of the NF-κB site. We compared binding of the m12-17 probe, containing this Pu.1 site, with that of an authentic Pu.1 binding site, using EMSA and nuclear extracts from various cells. Constitutive complexes formed with the authentic Pu.1 binding site had the high mobility characteristic of Pu.1-DNA complexes (Fig. 8, lanes 8, 9, and 12). Furthermore, these complexes formed with J774 and TA3 extracts but not with EL4 or L929 extracts, consistent with the restricted B-cell/macrophage expression of Pu.1. In contrast, m12-17 formed two predominant and constitutive complexes; a lower complex comigrating with the Pu.1 complexes, and a more slowly migrating complex (Fig. 8). The faster m12-17 complex also shows cell type specificity similar to that of Pu.1, expressed only by J774 and TA3 cells. In contrast, the slower m12-17 complex forms with extracts from J774, EL4, L929, and TA3 cells, suggesting that it is distinct from Pu.1. Furthermore, the slower m12-17 complex shows a sequence specificity distinct from that of authentic Pu.1, since the simian virus 40 Pu.1 oligonucleotide and m12-17 fail to cross-compete for complex formation (lanes 1 and 2, 7, and 8). Thus, the slower, but not faster, m12-17 complex appears distinct from Pu.1. We next compared activities of p40-luciferase reporter constructs which differ in the region containing the Pu.1 consensus (Fig. 4A). DM-148, containing the Pu.1 consensus, showed activity 40 to 50% higher than that of the full-length promoter construct, DM-703. Deletion of the 10-bp region between −148 and −138 moderately reduced activity to 80 to 90% of that of the

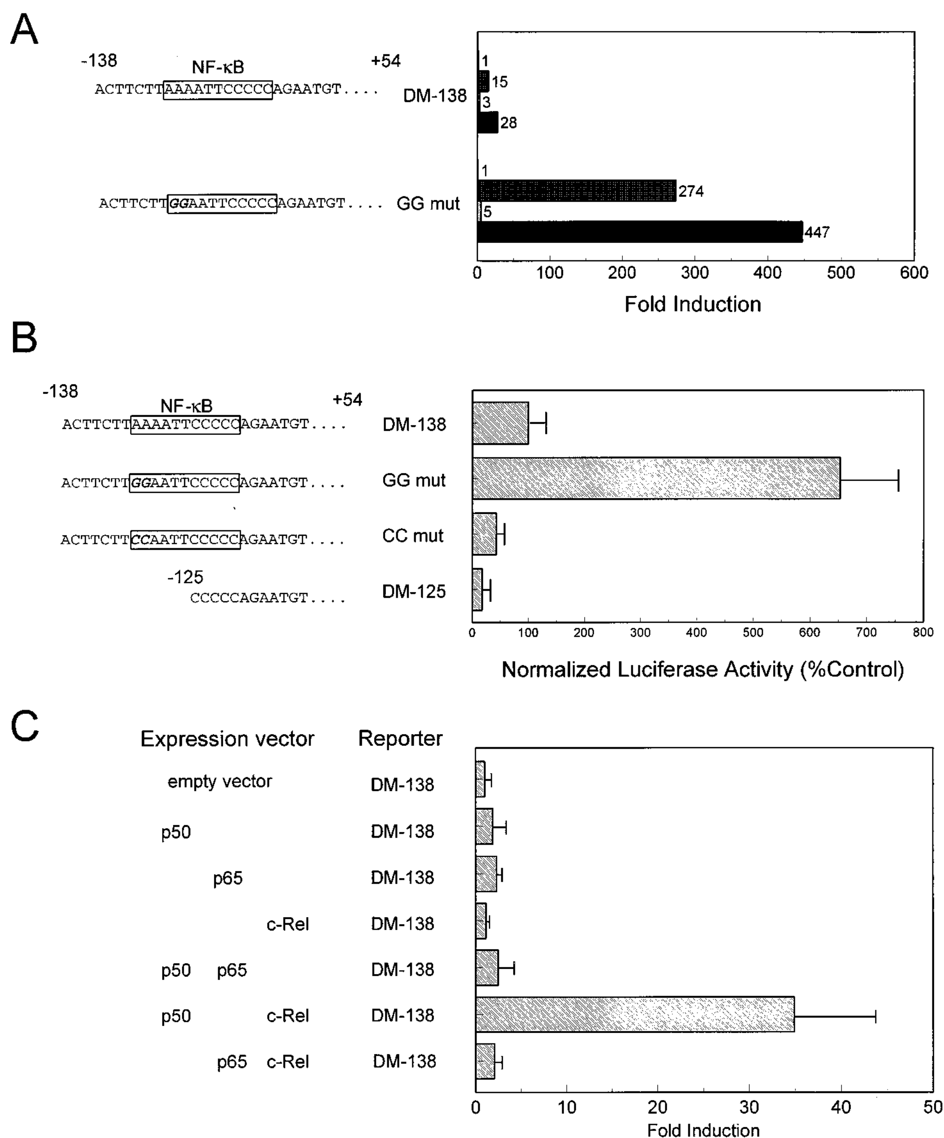

FIG. 7. Rel family transcription factors mediate activation of the p40 promoter via the p40-κB site. (A) Stable transfectants of J774 cells were prepared by using pSRαneo72.3 and either plasmid DM-138 or plasmid GGmut containing a 2-bp mutation at −130 and −131 of DM-138 (bold italicized letters). Following Geneticin selection, 10^6 cells were treated as described in the legend to Fig. 3. (B) Transient transfections of J774 cells were prepared with CMV-CAT and the indicated luciferase reporter plasmid. Two-base-pair point mutations within DM-138, GGmut and CCmut, are indicated with bold italicized letters. At 18 h after transfection, cells were activated with IFN-γ for 1 h and LPS for an additional 4 h. Normalized luciferase activity was determined and is shown as a percentage of the activity of the control, DM-138. (C) Different combinations of p50, p65, and c-Rel expression constructs were cotransfected with DM-138 into L929 cells, using calcium phosphate. After 48 h, cells were assayed for luciferase expression. Results are shown as fold induction over transfections with an empty expression vector.

full-length control. Furthermore, internal scanning mutation of the Pu.1 consensus within the context of DM-703 decreased activity to 80% of the control level (Fig. 4B). Thus, while perhaps contributing somewhat to activity, this site does not appear critical to induced promoter activity. Its potential role in controlling tissue specificity requires further examination.

IFN-γ regulation of p40 promoter activity. IFN-γ treatment augments LPS induction of p40 mRNA in both PECs and J774 cells (Fig. 2). To test if IFN-γ could act via the *cis*-acting elements identified above, we examined promoter activity and factor binding to the p40-κB site and adjacent consensus Pu.1 site in cells treated with or without IFN-γ (Fig. 9). IFN-γ pretreatment of J774 cells stably transfected with DM-138 produced a threefold augmentation of both LPS- and LTA-stimulated luciferase expression (Fig. 9A). This effect of IFN-γ was ablated by concurrent addition of the anti-IFN-γ antibody H22. Thus, IFN-γ can act at a site within 138 bp of the transcriptional start to augment p40 expression. Furthermore, pretreatment of J774 cells with IFN-γ appeared to augment formation of the LPS-induced m12-25 complex. Pretreatment for 1 h before LPS induction produced two- to threefold enhancement (Fig. 9B, top, lanes 3 and 4), while 18-h pretreatment augmented this level still further (Fig. 9B, top, lanes 5 and 6). In contrast, the intensity of the major complex formed with m12-17 was unaffected by IFN-γ pretreatment of cells, arguing for specificity to the effect seen for m12-25. Thus, IFN-γ pretreatment of J774 cells augments activation by LPS of the

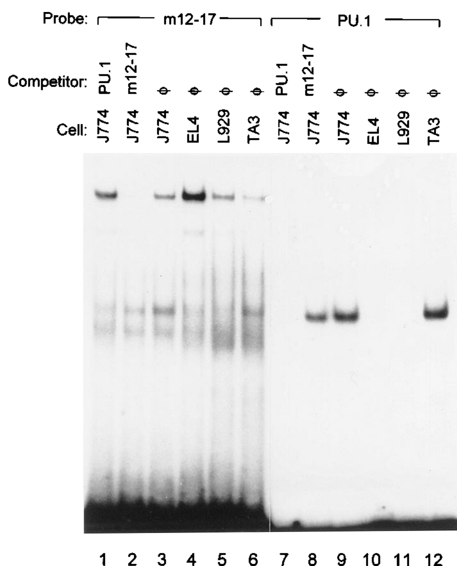

FIG. 8. Constitutive factors bind the −146 to −123 promoter region adjacent to the p40-κB site. Nuclear extract (3 μg) from unstimulated cells as indicated was incubated with either probe m12-17 (lanes 1 to 6) or a consensus Pu.1 binding site from simian virus 40 (Pu.1; lanes 7 to 12) without (ϕ) or with the indicated competitor DNA.

NF-κB-related factors responsible for the induction of p40 message.

DISCUSSION

The aim of this study was to understand IL-12 induction by bacterial products and IFN-γ. We focused on regulation of the 40-kDa IL-12 subunit because it is induced by bacterial stimulation, whereas expression of the 35-kDa subunit is constitutive. Our study identified functional promoter regions that confer LPS inducibility and IFN-γ augmentation. The minimal LPS-inducible promoter, despite lack of recognizable factor binding sites, interacted with Rel dimers p50/p50, p50/p65, and p50/c-Rel. This site mediated the transcriptional induction by both gram-negative bacterial products and gram-positive bacteria (*L. monocytogenes*) and their products (e.g., LTA). Further, the IFN-γ pretreatment of cells increased binding of factors to this site, perhaps contributing to augmentation of promoter activity (26).

Transcription of IL-12 p40 is induced via an NF-κB half-site. It is possible to align the consensus NF-κB sequence GGGRHTYYCC (18) with p40 promoter region sequence $^{-136}$TTCTTAAAATTCCCCC^{-121} in two ways for an 8-of-10-nucleotide match. For example, comparison of lower-strand p40 sequence $^{-123}\text{GGGAATTT}\underline{\text{TA}}^{-132}$ or $^{-122}\text{GGGGAATT}\underline{\text{TT}}^{-131}$ with the NF-κB consensus produces an alignment that replace two consensus CC residues with either $^{-131}$TA^{-132} or $^{-130}$TT^{-131}. While studies have described authentic NF-κB binding sites with mismatch at one of these two terminal CC residues, the p40-κB site is the first to differ at both residues for which regulatory interactions with Rel members have been functionally established. Recently, Muroi et al. (34) showed that the 3′ half-site sequences can influence binding of NF-κB proteins to sites in LPS-inducible genes. One site, located at −850 of the tumor necrosis factor alpha-1 (TNF-α1) gene, whose 3′ half-site TCCTT also differs in both conserved CC termini, competed with authentic NF-κB probes by EMSA. However, this site has not been functionally characterized for interactions with various NF-κB proteins (18). Moreover, others have shown that non-NF-κB proteins can interact with at least one proposed NF-κB site, κ3, in the TNF-α1 gene (16).

Distinct sequences of NF-κB sites offer the potential for selective gene activation by various stimuli. NF-κB mediates induction of several proinflammatory molecules, including induced nitric oxide synthase (50), TNF-α (9, 17), IFN-β (47), IL-1 (8), IL-6 (13), IL-8 (39), and tissue factor (38). However, expression of these molecules is not always coordinately regulated. For example, phorbol esters induce IL-1β and TNF-α in myeloid cell lines but do not induce IL-12 (26). The contribution of distinct adjacent factor binding sites in these genes is another means to provide distinct regulation. Thus, the roles for distinct Rel family members, as well as adjacent sites for Pu.1 and other factors, require further examination.

Roles of Pu.1 and other factors in p40 regulation. The B-cell/macrophage-restricted expression of p40 (11) made the Pu.1 consensus site of particular interest. Promoters for several other macrophage-specific genes, such as CD11b (Mac 1 receptor α chain) and macrophage colony-stimulating factor receptor, use Pu.1 in their transcriptional control (23, 40, 51). Interruption of the Pu.1 site in the p40 gene reduced promoter activity only moderately. The region around the Pu.1 site binds a second factor, currently unidentified, that is distinct in EMSA mobility, tissue distribution, and sequence specificity from Pu.1. We have not yet analyzed the contribution of this second factor to p40 control.

NF-κB can act cooperatively with high-mobility-group

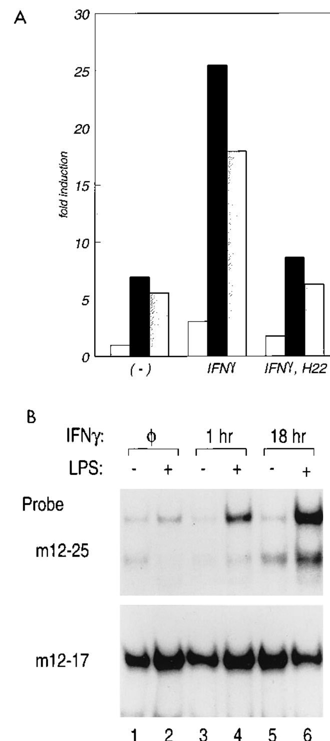

FIG. 9. IFN-γ augments factor binding to the p40-κB site. (A) J774 cells stably transfected with DM-138 were either not treated [(−)] or treated with IFN-γ or with IFN-γ and anti-IFN-γ antibody H22 (20 μg/ml) for 3 h. Cells were then left unstimulated (open bars) or treated with LPS (black bars) or LTA (shaded bars) for 4 h and assayed for luciferase activity. (B) J774 cells were either not treated (lanes 1 and 2) or treated with IFN-γ (100 U/ml) for 1 h (lanes 3 and 4) or 18 h (lanes 5 and 6) and then activated with LPS for 4 h. Nuclear extracts (3 μg) were incubated either with probe m12-25 or with probe m12-17. Complexes were analyzed as described in the legend to Fig. 5.

(HMG) proteins. For example, in the IFN-β promoter/enhancer PRDII element, HMG-I(Y) binds to DNA on the minor groove and contacts the central AT-rich region of the NF-κB site (47). This binding of HMG-I(Y) augments both NF-κB binding and transcriptional activity (47). Also, HMG I(Y) binding can facilitate NF-κB binding to two distinct sites within the E-selectin promoter (28). Another HMG protein, DSP1, binds a negative regulatory element, TCTGAA, to cause both NF-κB and Dorsal, a Rel family member, to switch from transcriptional activators to repressors (27). The sequence immediately 5′ to the p40-κB site, $^{-135}$TCTTAA^{-130}, matches the DSP1 negative regulatory element at five of six residues and may contribute to binding of a constitutive factor (Fig. 8). Thus, there is the potential for additional interactions between the p40-κB site and other factors binding to adjacent sites.

We and others have shown that IFN-γ augments p40 induction in macrophages (26). By increasing the production of IL-12 in response to bacterial stimuli, IFN-γ can critically influence Th1 development (20). Although IFN-γ augments the activity of the promoter region between −138 to +54, we find no binding of IFN-γ-activated Stat (12) or IRF (41) factors in this area (data not shown). IFN-γ could act by inducing the expression of intermediate factors, as it does by inducing CIITA to augment class II major histocompatibility complex gene expression (4). Alternately, IFN-γ treatment could, again either directly or indirectly, enhance IκB degradation or NF-κB translocation to increase effective NF-κB activity and thus p40 expression.

IL-12 expression was recently found to be diminished in asymptomatic human immunodeficiency virus-infected individuals (2). The mechanism whereby human immunodeficiency virus produces this inhibition of IL-12 production is unknown but has been suggested to be important for the progressive development of immunodeficiency (3, 6, 14, 36, 42, 44). Because IL-12 is sufficient to induce development of the Th1 phenotype, knowledge of its regulation is important for understanding cell-mediated immune responses (21). Complete description of IL-12 p40 regulation will require further characterization of the role of NF-κB in macrophage cytokine transcription, thorough identification of other factors involved in p40 transcription, and clarification of the mechanisms for the actions of IFN-γ and IL-10 on this cytokine.

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