MOLECULAR AND CELLULAR BIOLOGY, Aug. 1997, p. 4572–4588 Vol. 17, No. 8
0270-7306/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Multiple Control Elements Mediate Activation of the Murine and Human Interleukin 12 p40 Promoters: Evidence of Functional Synergy between C/EBP and Rel Proteins

SCOTT E. PLEVY,[1,2] JAMES H. M. GEMBERLING,[1] SANG HSU,[3] ANDREW J. DORNER,[3] AND STEPHEN T. SMALE[1]*

Howard Hughes Medical Institute and Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90095-1662[1]; Inflammatory Bowel Disease Center, Cedars-Sinai Medical Center, Los Angeles, California 90048[2]; and Genetics Institute, Inc., Cambridge, Massachusetts 02140[3]

Received 29 January 1997/Returned for modification 11 March 1997/Accepted 15 May 1997

Interleukin 12 (IL-12) is a heterodimeric cytokine whose activity is critical for T-helper 1 responses. The gene for the IL-12 p40 subunit is expressed in macrophages following induction by bacterial products, and its expression is augmented by gamma interferon. In this study, we performed a functional analysis of the murine and human p40 promoters in the murine macrophage cell line RAW 264.7. Transcription from the murine p40 promoter was strongly induced by lipopolysaccharide and heat-killed *Listeria monocytogenes* (HKLM), but promoter activity was not enhanced by gamma interferon. Multiple *cis*-acting elements involved in activated transcription were identified through an extensive mutant analysis. The most critical element, whose activity is conserved in mice and humans, is located between positions −96 and −88 relative to the murine transcription start site. This element exhibits functional synergy with a previously described NF-κB half-site which interacts with Rel proteins. DNase I footprinting and electrophoretic mobility shift assays demonstrated that C/EBP proteins interact with the critical element, but in nuclear extracts, cooperative binding of C/EBP and Rel proteins to their respective sites was not observed. Interestingly, promoter activity was induced by HKLM in the presence of cycloheximide, consistent with induction by posttranslational mechanisms. The results suggest that C/EBP and Rel proteins play important roles in the activation of IL-12 p40 transcription by bacteria. However, many complex interactions will need to be clarified to fully understand p40 regulation.

Interleukin 12 (IL-12) is a heterodimeric cytokine produced by macrophages in response to intracellular pathogens and bacterial products. Among its many biological activities, IL-12 provides an obligatory signal for the differentiation of effector T-helper 1 (Th1) cells and for the secretion of the Th1 cytokines, gamma interferon (IFN-γ) and IL-2 (59). Th1 cells are required for cell-mediated immunity and host defense against intracellular microbes (1, 34). The importance of IL-12 in the generation of a Th1 response against human pathogens, including *Mycobacterium leprae* (50), *Mycobacterium tuberculosis* (65), and *Leishmania* species (41), and during clinical progression of human immunodeficiency virus infection (6, 7, 9) has been well characterized. Because of the ability of IL-12 to promote protective immunity, its expression may be critical for successful development of vaccines against these and other intracellular pathogens (2). Although the induction of IL-12 by intracellular organisms is necessary for a protective host Th1 response, overexpression of Th1 cytokines and IL-12 may contribute to the development and perpetuation of chronic inflammatory and autoimmune diseases (49). Thus, an understanding of the regulated expression of IL-12 in macrophages may provide insight into the pathogenesis of infectious and inflammatory diseases and may suggest novel approaches for altering immune responses.

IL-12 is composed of two covalently linked glycosylated chains, p40 and p35, which are encoded by separate genes and together form the biologically active p70 heterodimer (18, 46, 63). The p35 gene is expressed in many tissue types (18). p40 mRNA is detected in macrophages and other cells that produce bioactive IL-12 (18), and it is strongly induced by intracellular bacteria and bacterial products (10). Accordingly, previous studies of IL-12 regulation have focused on the p40 gene. Murphy and colleagues reported an analysis of murine p40 gene regulation in the J774 murine macrophage cell line (35). They identified a nonconsensus NF-κB half-site, between positions −122 and −132 relative to the transcription start site, which was necessary for induction of promoter activity by lipopolysaccharide (LPS). They also demonstrated that IFN-γ, which augments bacterially induced IL-12 expression in animals and in cultured cells, enhanced binding of Rel family members to the p40 promoter, thus providing a potential mechanism for IFN-γ-mediated upregulation of IL-12 gene expression (35). In another study, Ma and colleagues performed a human p40 promoter analysis in the murine macrophage cell line RAW 264.7 (29). They identified a consensus Ets protein recognition sequence between nucleotides −212 and −207 which, by mutational analysis and Ets-2 overexpression, was implicated in promoter activation by LPS and IFN-γ (29). These results suggested that the murine and human p40 promoters may be regulated by different factors and mechanisms.

In this paper, we present the results of a comprehensive functional analysis of the murine and human IL-12 p40 promoters in the RAW 264.7 cell line. A detailed analysis of mutants revealed that multiple control elements are involved in the induction of promoter activity by LPS and heat-killed *Listeria monocytogenes* (HKLM). IFN-γ did not enhance p40 promoter activity in this cell line, although a strong promoter-independent activation of luciferase reporter plasmids was ob-

* Corresponding author. Mailing address: Howard Hughes Medical Institute, 6-730 MRL, 675 Circle Dr. South, UCLA School of Medicine, Los Angeles, CA 90095-1662. Phone: (310) 206-4777. Fax: (310) 206-8623. E-mail: steves@hhmi.ucla.edu.

served. The most important control element, located between positions −96 and −88 relative to the murine transcription start site, interacts with C/EBP proteins. This element was functionally synergistic with a previously reported NF-κB half-site between nucleotides −131 and −122, although cooperative binding of C/EBP and Rel proteins was not observed in nuclear extracts. DNase I footprinting experiments identified proteins which interact with other elements that appear to be functionally relevant. Finally, new protein synthesis was not required for activation of the p40 promoter by HKLM, and, interestingly, mRNA from the endogenous p40 gene could be strongly induced by cycloheximide alone. These results provide an initial and critical step toward a detailed dissection of the regulatory mechanisms for this important cytokine.

MATERIALS AND METHODS

Plasmids. A 10-kb murine IL-12 p40 genomic clone was isolated in pBluescript KS+ (Stratagene). Intron-exon structure and the location of the promoter region were determined by restriction mapping and DNA sequencing. One kilobase of the promoter region was sequenced (Sequenase, version 2) and was found to be identical to that reported previously (35). The wild-type promoter (position −800 to +55 or −350 to +55) was inserted into the luciferase reporter vector pGL2B (Promega) by PCR, using an upstream primer containing a *Kpn*I restriction site and a downstream primer containing a *Bgl*II site. Deletion mutants were generated by PCR using upstream primers containing either a *Sac*II, *Nsi*I, or *Pst*I site 3′ of the *Kpn*I site. Most substitution mutants were derived from the deletion mutants by inserting a second PCR product extending from position −350 to the deletion end point, using an upstream primer containing a *Kpn*I site and the −350 sequence and a downstream primer containing a *Sac*II, *Nsi*I, or *Pst*I site and the sequence adjacent to the deletion end point. The control promoters and enhancers described in Results were in pGL2B, pGL3B (Promega), or pXP2 (38). Several p40 deletion mutants were also tested in pGL3B. All p40 promoters used in chloramphenicol acetyltransferase (CAT) assays were cloned from pGL2B into pCAT basic (Promega) which had been modified to contain a *Kpn*I site and a *Bgl*II site in the polylinker. The human p40 promoter was amplified from human genomic DNA by PCR. Human promoter substitution mutants were generated by a two-step PCR procedure using overlapping internal primers which contain a mutant sequence (20). Mutants with 3-bp mutations in the murine IL-12 p40 promoter were also generated by a two-step PCR method. A pCAT vector backbone containing the luciferase gene was created by replacing the CAT gene at the *Bam*HI and *Sal*I sites with the luciferase gene from *Bam*HI- and *Xho*I-digested pGL2B. All PCR-generated plasmid inserts were sequenced in full prior to their use in transfections. Expression plasmids for the C/EBPβ isoforms LAP (pSCT-LAP) and LIP (pSCT-LAP from which an *Nco*I fragment has been deleted), containing a cytomegalovirus (CMV) promoter/enhancer, were obtained from Andrew Henderson and Kathryn Calame and were originally constructed by Patrick Descombes and Ueli Schibler (11). All plasmids used in transient transfections were purified by using an endotoxin-free plasmid purification system (Qiagen).

Cell lines and reagents. The RAW 264.7 murine macrophage line (American Type Culture Collection) was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco) (assayed for low endotoxin activity). *L. monocytogenes* (provided by Jeffery F. Miller, UCLA) was grown in brain heart infusion broth to an optical density of 1.5 and then heat killed by autoclaving for 45 min. HKLM cells were washed extensively with phosphate-buffered saline (PBS) and stored at 4°C. To avoid LPS contamination, new glassware or sterile disposable containers were used to grow, wash, and store the HKLM. LPS and cycloheximide were from Sigma Chemicals, and murine recombinant IFN-γ was from Genzyme.

RT-PCR of the endogenous IL-12 p40 gene. Total RNA was isolated from RAW 264.7 cells (Qiagen RNeasy Mini Kit). p40 cDNA was derived from 5 μg of total RNA by reverse transcription (RT) using Superscript (Life Technologies, Inc.) and an oligo(dT) primer. PCR was performed on 0.3 μg of cDNA with specific primers, yielding a 327-bp product. IL-12 p40 PCR primers were designed to span the first intron. The following primer sequences were used: 5′ TCGCAGCAAAGCAAGATGTG 3′ and 5′ GAGCAGCAGATGTGAGT GGC 3′. For IL-12 p40, PCR was carried out by a standard protocol for 35 cycles. An equal aliquot of cDNA was amplified with β-actin primers for 35 cycles (26). The specific β-actin PCR product was 623 bp in size. Aliquots of PCR reactions were separated on a 1% agarose gel and visualized with UV light after ethidium bromide staining.

Transfections. RAW 264.7 cells were transiently transfected by electroporation by a previously described protocol with modifications (54). For luciferase transfections, 5×10^6 cells were suspended in 200 μl of DMEM with 10% FBS plus 20 μg of luciferase plasmid and 1 μg of HSP–β-galactosidase reporter (provided by Bradley Cobb, UCLA). Cells were electroporated in a 0.4-mm-long cuvette (Bio-Rad) in a Bio-Rad Gene Pulser at 250 V and 960 μF. After a 10-min incubation (room temperature), cells were washed with 5 ml of PBS and resuspended in 1 ml of DMEM plus 10% FBS. Each transfection product was divided into 200-μl aliquots and plated in a total of 1 ml of medium in a 24-well plate. Twenty-four hours after electroporation, cells were activated. Following activation, cell extracts were prepared by using 1× cell lysis buffer (Promega). Luciferase activity was determined from 20 μl of cell extract, and β-galactosidase activity was determined from 30 μl of cell extract, as per the Promega protocol. Transient transfections of CAT constructs were performed by the same protocol with the following exceptions: (i) for electroporation, 7.5×10^6 cells were suspended in growth medium; (ii) after the PBS wash, cells were resuspended in 1 ml of medium; and (iii) each transfection product was divided into two 500-μl aliquots, each of which was diluted to 2.5 ml with medium in a six-well plate. CAT assays were performed with 50 μg of total protein from cell extracts, as per the Promega protocol. Quantitation of the conversion of [^{14}C]chloramphenicol to its acetylated forms was performed with a PhosphorImager (Molecular Dynamics). Cell lines containing stably integrated luciferase reporter plasmids were generated by the transient-transfection protocol, except the cells were cotransfected with 2 μg of pSV2-His, containing the gene for histidinyl transferase. After electroporation, cells were resuspended in 10 ml of DMEM plus 10% FBS, and 100 μl was plated in each well of a 96-well plate. After 24 to 48 h, cells were selected by addition of 200 μl of histidine-free RPMI 1640 (Selectamine) plus 10% FBS with 1 μM histidinol (Aldrich). After multiple medium changes over 2 weeks, cells were screened for activated luciferase activity, as well as for histidinyl transferase gene expression from total RNA by primer extension analysis.

Nuclear extracts and DNA binding assays. RAW 264.7 nuclear extracts were prepared by a modification of the method of Dignam et al. (12) as previously described (27). Extraction and dialysis buffers were supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma), 0.5 μg of leupeptin (Sigma) per ml, 1 μg of aprotinin (Sigma) per ml, and 1 μM pepstatin (Sigma). Electrophoretic mobility shift assay (EMSA) probes were made by annealing single-stranded oligonucleotides with 5′ GATC overhangs (Operon) that had been gel purified. Two hundred nanograms of probe was labeled by filling in with [α-^{32}P]dGTP and [α-^{32}P]dCTP by using the Klenow enzyme. The labeled probes were purified with a NucTrap purification column (Stratagene). Sequences of IL-12 p40 wild-type and mutant EMSA probes are displayed below (see Fig. 8A). Sequences of other probes used are as follows: C/EBP consensus, 5′ AAGCTGCAGATTGCGCA ATCTGCAGCTT 3′, C/EBP mutant, 5′ AAGCTGCAGAGACTAGTCTCTG CAGCTT 3′ (61); and murine immunoglobulin kappa NF-κB consensus, 5′ CAGAGGGGACTTTCCGAGA 3′ (40). For NF-κB EMSAs, probe (10^5 cpm) was added to 5 μg of nuclear extract plus 2 μg of poly(dI-dC) in binding buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol), and binding was performed on ice for 45 min; EMSA products were separated on a 5% polyacrylamide–0.4× Tris-borate-EDTA gel run at room temperature for 1.5 h at 150 V. For C/EBP EMSAs, binding was optimized at room temperature for 45 min, and EMSA products were separated on a 5% acrylamide–1× Tris-glycine-EDTA gel run at 4°C for 3 h at 150 V; unlabeled oligonucleotide competitors were added to nuclear extracts, poly(dI-dC), and binding buffer at a 100-fold molar excess 30 min prior to addition of labeled probe. For supershift experiments, 2 μl of antibody was added to extracts, poly(dI-dC), and binding buffer, at room temperature, 30 min prior to addition of labeled probe. Polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. Probes for DNase I footprinting spanning positions −174 to −24 relative to the transcription start site were generated by PCR using ^{32}P-, 5′-end-labeled upstream (coding strand) or downstream (noncoding strand) primers, with the −350-to-+55 p40-luciferase plasmid and the 6-bp-substitution mutants −132/−127s, −93/−88s, and −68/−63s (which have substitutions from nucleotide −132 to −127, from −93 to −88, and from −68 to −63, respectively) as templates. Ten picomoles of PCR primer was 5′-end labeled with [γ-^{32}P]dATP and T4 polynucleotide kinase (New England Biolabs) for 1 h at 37°C. Labeled primer was added to the PCR reaction, and 25 cycles of PCR were performed. The probes were purified on a 6% nondenaturing polyacrylamide gel and eluted overnight in 0.5 M ammonium acetate–1 mM EDTA at room temperature. A total of 6,000 cpm of probe and 25 μl of nuclear extract were used per binding reaction. DNase I footprinting reactions were performed as previously described (27). Gels were dried and exposed to Kodak X-AR 5 film at −80°C in the presence of an intensifying screen.

Primer extension analysis of luciferase mRNA. Primer extension analysis was performed as previously described (52) with the following modifications. Thirty micrograms of total RNA from stably transfected cells was used for primer extension analysis with a ^{32}P-, 5′-end-labeled primer complementary to the luciferase gene, 5′ ACCAACAGTACCGGAATGCC 3′. Hybridization was performed at 37°C for 90 min. Primer extension products were separated on an 8% denaturing polyacrylamide gel. Gels were dried and exposed to Kodak X-AR 5 film at −80°C in the presence of an intensifying screen. Primer extension bands were quantitated by densitometry.

RESULTS

IL-12 p40 mRNA is induced in the RAW 264.7 macrophage cell line. IL-12 p40 protein and mRNA are upregulated in animals, in primary macrophages, and in macrophage cell lines by treatment with bacterial products such as LPS, *Staphylococ-*

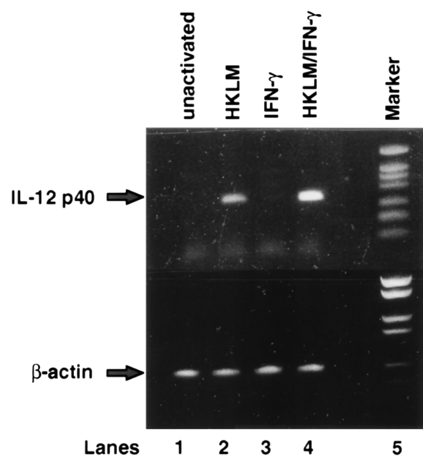


FIG. 1. IL-12 p40 mRNA production in RAW 264.7 macrophages is induced by HKLM and augmented by pretreatment with IFN-γ. RAW 264.7 macrophages either were not activated (lane 1) or were activated with 1.5×10^8 HKLM per ml for 4 h (lane 2), with 100 U of recombinant murine IFN-γ per ml for 5 h (lane 3), or with 100 U of IFN-γ per ml for 5 h plus HKLM for 4 h (lane 4). Total RNA (2.5 μg) was reverse transcribed with an oligo(dT) primer; this was followed by PCR with either IL-12 p40 primers or β-actin primers. Products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The IL-12 p40 and β-actin products are indicated with arrows. Molecular size markers were run in lane 5.

cus aureus, and HKLM (10, 21, 24, 29, 51). IFN-γ augments p40 mRNA induction and protein secretion (10, 24, 29, 35, 51). To determine whether the murine macrophage line RAW 264.7 is appropriate for an analysis of IL-12 p40 gene regulation, p40 mRNA levels were determined by RT-PCR following activation with HKLM and IFN-γ. In unactivated cells (Fig. 1, lane 1) and in cells activated with IFN-γ for 5 h (Fig. 1, lane 3), p40 mRNA was not detectable by RT-PCR. Following activation with HKLM for 4 h, an RT-PCR product derived from IL-12 p40 mRNA was readily observed (Fig. 1, lane 2). This signal was augmented by a 1-h pretreatment with IFN-γ (Fig. 1, lane 4). These results are consistent with previous studies (10, 24, 29) and demonstrate that RAW 264.7 cells are appropriate for analyzing IL-12 p40 gene regulation.

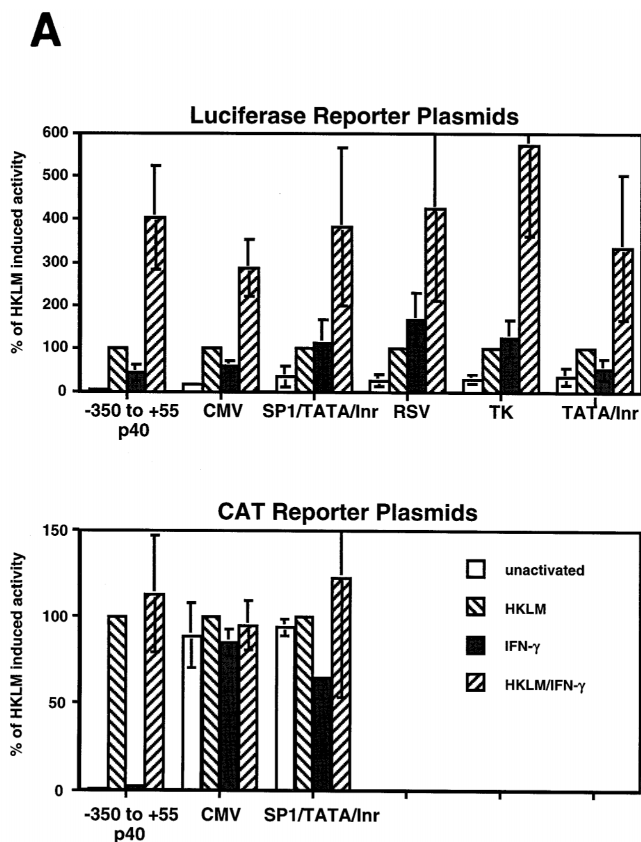
An IL-12 p40 promoter-luciferase reporter plasmid is active following transient transfection of RAW 264.7 cells. To determine whether the IL-12 p40 promoter is induced by bacterial products and IFN-γ in RAW 264.7 cells, promoter activity was analyzed by a transient transfection assay. The p40 promoter from position −350 to +55 with respect to the transcription start site was amplified from a murine IL-12 p40 genomic clone by PCR and inserted into the luciferase reporter vector pGL2B (Promega). Following transfection of RAW 264.7 cells by electroporation and incubation for 48 h, weak basal luciferase activity was detected (Fig. 2A, top). The luciferase activity was strongly induced by addition of HKLM 24 h posttransfection, with luciferase activity being measured 48 h posttransfection (Fig. 2A, top). HKLM was added at a concentration of 1.5×10^8 organisms/ml, which was the concentration determined to yield the optimal luciferase signal (data not shown). IFN-γ also induced luciferase activity, and a combination of HKLM and IFN-γ resulted in synergistic induction (Fig. 2A, top). All of the induced luciferase signals were much stronger than those observed with the promoterless pGL2B vector (see the legend to Fig. 2). Kinetic analyses following induction with HKLM and IFN-γ revealed that luciferase activity was maximal after 24 h, although induction could be detected as early as 4 h (data not shown). Results similar to those obtained with HKLM were obtained with LPS, either alone or in combination with IFN-γ (data not shown).

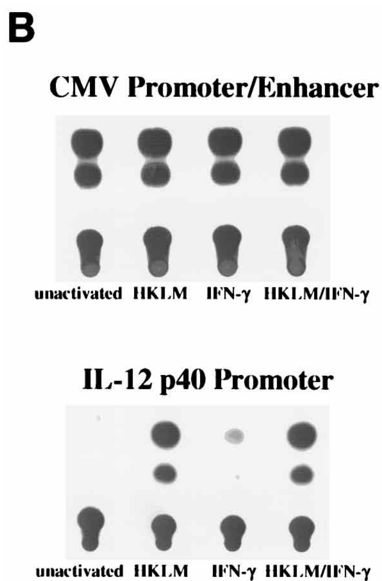
To determine whether induction of the p40-luciferase plasmid was specific for the p40 promoter, several luciferase reporter plasmids containing other promoters were tested. Synergistic induction of luciferase activity with HKLM and IFN-γ was observed with all promoters and enhancers evaluated, including the CMV promoter/enhancer (Fig. 2A), a synthetic promoter containing multiple SP1 binding sites upstream of consensus TATA and initiator elements (Fig. 2A), the Rous sarcoma virus promoter/enhancer (Fig. 2A), the herpes simplex virus thymidine kinase promoter (Fig. 2A), a synthetic promoter containing consensus TATA and initiator elements (Fig. 2A), the simian virus 40 promoter/enhancer (data not shown), the terminal transferase promoter (data not shown), a synthetic promoter containing a consensus TATA box (data not shown), the IL-10 promoter (data not shown), and all IL-12 p40 substitution and deletion mutants (see Fig. 3 and 4) (data not shown). These promoters and enhancers were tested in one of three different luciferase vectors, pGL2B (Promega), pGL3B (Promega), or pXP2 (38). The weak luciferase activities obtained with the promoterless pGL2B and pGL3B vectors were also synergistically induced by HKLM and IFN-γ (data not shown). These results suggest that responsiveness to HKLM and IFN-γ, as determined by luciferase activity, may be promoter independent.

The luciferase coding region was the only common sequence among all of the plasmids tested, raising the possibility that the luciferase gene itself may mediate induction by IFN-γ and/or HKLM. To eliminate possible effects of the luciferase gene, the IL-12 p40 promoter and two control promoters were cloned into a CAT reporter plasmid (pCAT basic; Promega). Following transient transfection, the IL-12 p40-CAT reporter was strongly induced by HKLM but was only weakly induced by IFN-γ (Fig. 2A, bottom, and 2B) (the weak IFN-γ induction was variable and was observed in only a few experiments). Moreover, no synergistic induction of CAT activity by HKLM and IFN-γ was detected. Interestingly, no induction by either HKLM or IFN-γ was observed with CAT reporter plasmids containing the CMV promoter/enhancer (Fig. 2A, bottom, and 2B) or a synthetic promoter consisting of multiple SP1 binding sites upstream of consensus TATA and initiator elements (Fig. 2A, bottom). IFN-γ pretreatment of the cells for 20, 8, 4, 1, or 0 h prior to activation by HKLM did not augment the activity observed with any of the CAT reporter plasmids (data not shown). Thus, in RAW 264.7 cells, the IFN-γ responses of the luciferase plasmids appear to be unrelated to the augmentation of endogenous p40 expression by IFN-γ.

Multiple vector backbones and promoters containing a luciferase reporter gene demonstrated promoter-independent activation by IFN-γ and HKLM. Therefore, the luciferase gene itself may contain the elements that are responsive to HKLM and IFN-γ. To test this hypothesis, the luciferase gene was excised from the pGL2B vector and cloned into the pCAT basic vector along with the CMV promoter/enhancer or the IL-12 p40 promoter. Luciferase activities observed with both plasmids were synergistically induced by HKLM and IFN-γ (CMV-luciferase, Fig. 2C; p40-luciferase, data not shown). This result confirms that the luciferase transcription unit confers promoter-independent responsiveness to HKLM and IFN-γ in RAW 264.7 macrophages.

Luciferase reporter plasmids have been utilized to study the induction of several genes by IFN-γ and bacterial products in macrophages (28, 31, 33, 37). Therefore, it is important that the mechanism of this promoter-independent induction be discerned. Primer extension analyses of luciferase mRNA re-





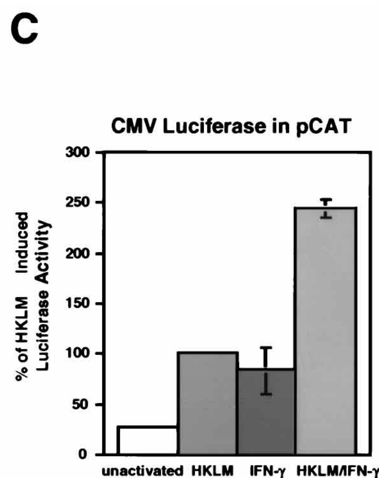


FIG. 2. Promoter-independent and promoter-dependent induction of luciferase and CAT reporter plasmids. (A) Luciferase (upper panel) and CAT (lower panel) reporter plasmids with various promoters (denoted beneath the graphs) were transiently transfected into RAW 264.7 cells. Twenty-four hours after transfection, cells were activated with HKLM (1.5×10^8/ml), murine recombinant IFN-γ (100 U/ml), or both HKLM and IFN-γ for 24 h. Luciferase and CAT results are expressed as the percentage of HKLM-induced activity (relative light units or relative percent conversion of [^{14}C]chloramphenicol) for each promoter tested. Standard deviations are also indicated (error bars). For comparison, the mean numbers (± standard deviations) of relative light units obtained in six experiments involving transfection of the empty pGL2B plasmid into RAW 264.7 cells were 485 ± 109 for unactivated cells, 1,116 ± 797 for HKLM-activated cells, 672 ± 229 for IFN-γ-activated cells, and 1,453 ± 798 for cells activated with both IFN-γ and HKLM. The mean relative light unit values obtained for five experiments with the −350-to-+55 p40 luciferase reporter were 3,272 ± 1,467 for unactivated cells, 78,484 ± 21,395 for HKLM-activated cells, 32,735 ± 9,446 for IFN-γ-activated cells, and 310,616 ± 100,212 for cells activated with both IFN-γ and HKLM. For the CMV promoter/enhancer, the luciferase and CAT data presented were derived from five and two experiments, respectively. For the Sp1/TATA/Inr promoter, luciferase and CAT data are from four and two experiments, respectively. For other promoters and enhancers, data were derived from three experiments. RSV, Rous sarcoma virus; TK, thymidine kinase. (B) A thin-layer chromatogram of a representative CAT assay is shown. RAW 264.7 cells were transfected with CAT reporter plasmids containing either the CMV promoter/enhancer (upper panel) or the −350-to-+55 p40 promoter (lower panel). Prior to the CAT assay, the cells were activated with HKLM and/or IFN-γ for 24 h, as indicated beneath samples. (C) A plasmid which contains the CMV promoter/enhancer and the luciferase coding sequence and 3′ untranslated region inserted into the pCAT basic plasmid (Promega), from which the CAT gene had been removed, was analyzed. RAW 264.7 cells transfected with this plasmid either were unactivated or were activated with HKLM (1.5×10^8/ml) and/or recombinant murine IFN-γ (100 U/ml) for 24 h, as indicated. Results (relative light units normalized for β-galactosidase activity) are expressed as the percentage of HKLM-induced luciferase activity and represent the means ± the standard deviations of data from two experiments.

vealed a synergistic induction by HKLM and IFN-γ that paralleled the induction of luciferase enzymatic activity (data not shown). Furthermore, synergistic induction of luciferase protein by HKLM and IFN-γ was observed by immunoblot analysis with an antiluciferase antibody (data not shown). Although this effect was not explored in greater detail, these data indicate that HKLM and IFN-γ induce luciferase activity by increasing steady-state mRNA abundance, which is most consistent with the regulation of transcription initiation, elongation, or mRNA stability. We speculate that the promoter-independent induction is likely to occur at the level of transcription elongation or mRNA stability, as it is improbable that the luciferase gene contains an element that is powerful enough to significantly influence the activities of all the promoters tested in this study, some of which are very strong (e.g., CMV and Rous sarcoma virus). Perhaps RAW 264.7 activation is asso-

ciated with a general stabilization of mRNA or a general increase in the efficiency of RNA polymerase II elongation. The luciferase gene may fortuitously contain an element that subjects it to regulation. Alternatively, the CAT gene may be unusually resistant to regulation during macrophage activation. In either case, the CAT gene is the preferred reporter for studying promoter activation in the RAW 264.7 cell line and perhaps in other inducible cell lines.

Deletion analysis of the murine IL-12 p40 promoter identifies potential regulatory regions. As a first step toward localizing important control elements within the p40 promoter, 25 5′ deletion mutants were constructed and analyzed. The mutant promoters were inserted upstream of the luciferase gene in pGL2B before the promoter-independent activation of luciferase reporters was discovered. These plasmids therefore cannot be used to determine the effect of each mutation on the magnitude of promoter induction. However, they can be used to define the control elements required for activated transcription, as mutations which disrupt critical elements within the promoter should reduce luciferase activity despite the nonspecific-induction phenomenon. Accordingly, our overall strategy was to localize important control elements by using the luciferase plasmids and then to confirm the roles of these elements in promoter induction by studying the relevant mutants in CAT reporter plasmids.

A plasmid containing the sequence from position −800 to +55 was used to generate the serial-deletion mutants (see Fig. 3). RAW 264.7 cells were transfected with the mutants, incubated for 24 h, and activated with LPS or HKLM for 24 h. Luciferase activities were measured and normalized to the β-galactosidase activities obtained with a cotransfected control plasmid. Gradual deletion of the sequence from nucleotide −800 through −150 resulted in relatively minimal changes in promoter activity (Fig. 3A). Overall, a twofold reduction in promoter activity was observed when this region was deleted, with the elements responsible for the reduction residing primarily between positions −330 and −150. Further decreases in promoter activity were observed with deletions past nucleotide −150. Deletion of 8 bp from −143 to −136 resulted in a twofold reduction in promoter activity. Deletion of 3 bp from −126 through −124 resulted in another twofold loss of activity. With deletions past position −102, HKLM- and LPS-induced luciferase activities were not significantly higher than the activity of the promoterless luciferase vector (Fig. 3B). An additional plasmid contained the promoter from nucleotide −350, along with the first exon and the 4.1-kb first intron of the p40 gene. The luciferase activity observed with this plasmid was twofold lower than the activity observed with the plasmid containing sequences from nucleotide −350 to +55 (Fig. 3A). This difference is unlikely to be significant, since the intron-containing plasmid is much larger. Thus, the deletion analysis identified several potential regulatory regions, primarily between positions −330 and +55.

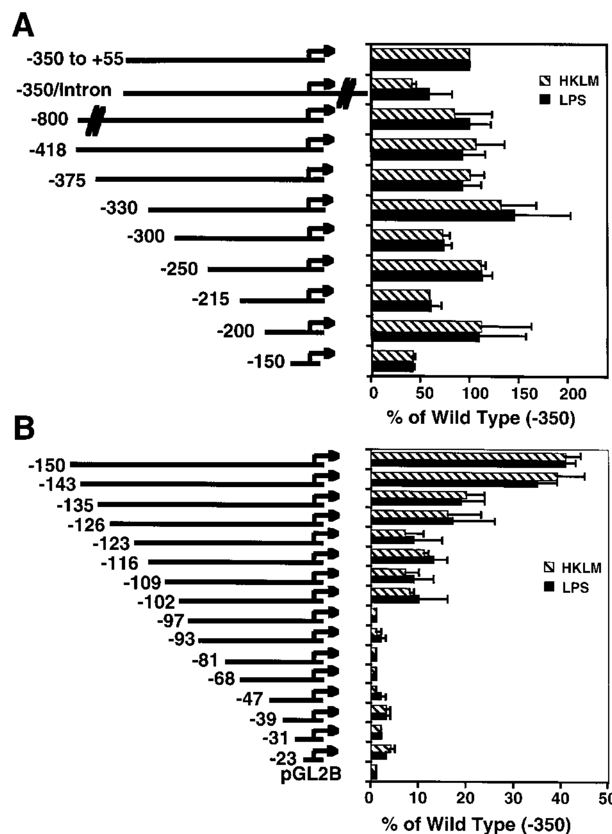


FIG. 3. Deletion-mutant analysis of the IL-12 p40 promoter demonstrates that induction by HKLM and LPS is mediated by elements downstream of nucleotide −330. Deletion mutants were constructed from a fragment of the p40 gene extending from positions −800 to +55 and are represented to the left. A plasmid that contained the promoter from position −350, exon 1, and the 4.1-kb first intron fused upstream of the luciferase reporter gene was also tested. Mutants were transiently transfected into RAW 264.7 cells with 1 μg of HSP–β-galactosidase reporter plasmid. Twenty-four hours after transfection, cells were activated with 5 μg of LPS per ml or 1.5×10^8 HKLM cells per ml for 24 h. Results (relative light units normalized for β-galactosidase activity) are expressed as the percentage of the HKLM- or LPS-activated signal obtained with the −350-to-+55 plasmid. The result for each mutant represents the mean ± the standard deviation of data from two to four experiments. Note that the *x* axes on the upper and lower panels are different.

Substitution mutants reveal promoter elements critical for activated transcription. The deletion analysis revealed strong promoter activity within the −150-to-+55 region of the IL-12 p40 gene, with promoter activity being abolished by 5′ deletions to position −97. Thus, the control elements that are essential for promoter activity are located within this 205-bp region. To localize the important control elements, 20 substitution mutants which systematically altered 5- to 10-bp sequences between positions −150 and +17 were prepared (Fig. 4A). These mutants were constructed in the context of the −350-to-+55 promoter. As with the deletion mutants, the substitution mutants were inserted into the luciferase vector before the nonspecific induction of luciferase activity was detected. Thus, these plasmids are useful for localizing control elements necessary for induced promoter activity but not for studying the extent of induction.

This analysis revealed several regions involved in LPS- and HKLM-activated transcription (Fig. 4B). The element that was most critical for promoter activity was disrupted by the −99/−94s and −93/−88s mutations, which reduced promoter activity by over 90%. The importance of this element was also apparent from the deletion analysis, since deletion to nucleotide −97 (Fig. 3B; compare mutants −102 and −97) reduced promoter activity to background levels. Interestingly, the sequence disrupted by the two substitution mutations, 5′ GTGT TGCAAT 3′, exhibits homology to consensus binding sites for the CCAAT enhancer binding protein (C/EBP) family of transcription factors (62), which have been implicated in the regulation of several proinflammatory genes in macrophages (30, 42).

A second critical element was disrupted by the −132/−127s mutation, which reduced activity by 80% (Fig. 4B). This element contains an NF-κB half-site previously demonstrated to

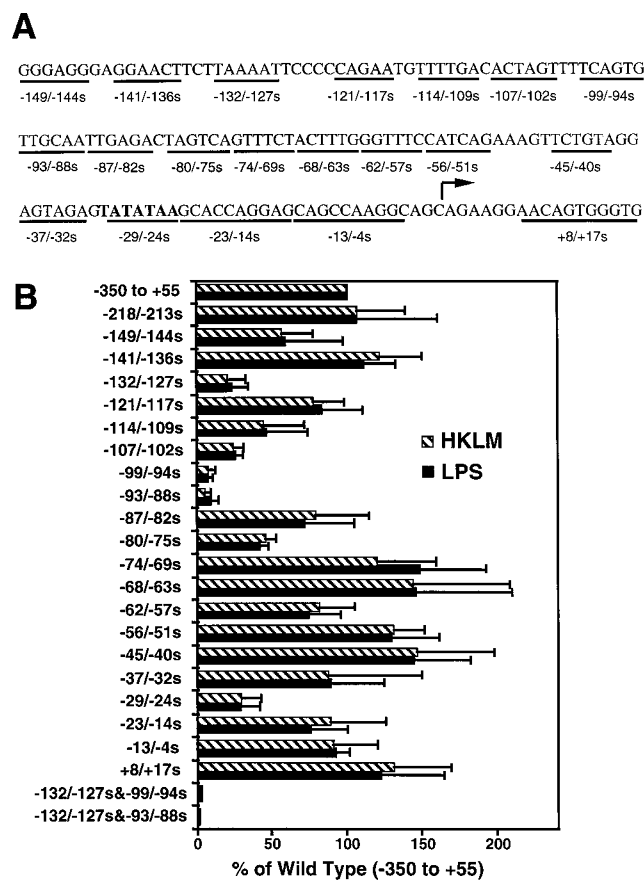


FIG. 4. Substitution-mutant analysis of the IL-12 p40 promoter identifies multiple control elements involved in HKLM- and LPS-induced activity. (A) The sequence of the murine IL-12 p40 promoter is shown. Underlined sequences signify the substitution mutants analyzed. One mutant, −218/−213s, is not represented but corresponds to the murine sequence homologous to an Ets site in the human p40 promoter. All substitution mutants were in the context of the −350-to-+55 promoter. (B) Mutants were transiently transfected into RAW 264.7 cells with 1 μg of HSP–β-galactosidase reporter plasmid. Cells were activated as described in the legend to Fig. 3. Results are expressed as described in the legend to Fig. 3. Each result represents the mean ± the standard deviation of data from three to five experiments. The two plasmids represented at the bottom of the graph are double mutants, combining −132/−127s with −99/−94s and −93/−88s, respectively.

be important for p40 promoter activity (35). Interestingly, the activity of deletion mutant −126, which contains a disrupted NF-κB site, was not significantly diminished relative to that of deletion mutant −135, which appears to retain the NF-κB site (Fig. 3B). These results suggest that the activity of the NF-κB site may depend on one or more control elements located further upstream, perhaps the elements disrupted in deletion mutants −150 and −135 (Fig. 3). In a previous study, the sequence from position −143 to −138 was reported to bind PU.1 (35), but as observed here, mutations in this region had a relatively small effect on promoter activity (35). Further studies will be required to elucidate the potential relevance of these upstream regions to the function of the NF-κB half-site.

Several mutations were found to have smaller effects on promoter activity (Fig. 4B). Two mutations downstream of the NF-κB site, −114/−109s and −107/−102s, reduced promoter activity by two- and threefold, respectively. Two other mutations reduced promoter activity by greater than 50%: −80/−75s and −29/−24s, which disrupts the TATA box. Since residual promoter activity was observed with all of the substitution mutants, mutants in which the putative C/EBP and NF-κB elements were jointly disrupted were created. With the −132/−127s mutation coupled to either the −99/94s mutation or the −93/−88s mutation, promoter activity was reduced to background levels (Fig. 4B).

In conclusion, the substitution mutations from nucleotide −150 to +17 of the p40 promoter revealed the presence of multiple putative regulatory elements. The two most important elements appear to contain recognition sites for C/EBP and Rel proteins. These two elements appear to functionally synergize with each other, as disruption of either element greatly weakens the promoter. Finally, LPS, a gram-negative bacterial product, and HKLM, a gram-positive intracellular organism, activated the p40 promoter through the same control elements.

Three-base-pair substitution mutations further define the critical control elements. The critical control elements defined above may each represent a binding site for one transcription factor monomer or dimer or may represent a composite binding site for two or more factors. Insight into the number of proteins that interact with each element can be gained by defining more precisely the functionally critical nucleotides. Therefore, 3-bp mutations spanning the putative C/EBP element (Fig. 5A) and the NF-κB half-site (Fig. 5B) were introduced into the p40 promoter, and the resulting mutants were analyzed by transient transfection of RAW 264.7 cells.

At the putative C/EBP element, three mutations, −96/−94s, −93/−91s, and −90/−88s, reduced promoter activity by approximately 10-fold (Fig. 5A). In contrast, three surrounding mutations, −102/−100s, −99/−97s, and −87/−82s, had no significant effect on promoter activity (Fig. 5A). These results demonstrate that the critical element encompasses a minimum of 5 bp (nucleotides −94 to −90) and a maximum of 9 bp (nucleotides −96 to −88), which is most consistent with the binding of one protein monomer or dimer. It is noteworthy that the sequence that is homologous to the C/EBP consensus extends from position −95 to −88 (61), providing further evidence that C/EBP proteins functionally interact with this element.

At the NF-κB half-site, four mutations, −131/−129s, −128/−126s, −125/−123s, and −122/−120s, reduced promoter activity by greater than 60% (Fig. 5B). In contrast, two surrounding mutations, −134/−132s and −119/−117s, did not reduce p40 promoter activity (Fig. 5B). The 5-bp mutation −121/−117 also did not reduce promoter activity (Fig. 4). These results demonstrate that the critical element at this location encompasses a minimum of 8 bp (nucleotides −129 to −122) and a maximum of 10 bp (nucleotides −131 to −122). The size of this element is most consistent with the binding of one protein monomer or dimer, although multiple proteins could interact at adjacent or overlapping sites within a 10-bp region. As reported previously (35), Rel proteins are likely to be required for the activity of this element, as promoter activity was enhanced by a 2-bp mutation (−131/−130s) that generates a stronger NF-κB consensus sequence (Fig. 5B).

Analysis of IL-12 p40 substitution mutants with a CAT reporter. Because of the promoter-independent induction of the luciferase reporter plasmids (Fig. 2), the analysis described above was useful for defining control elements needed for the activity of the induced promoter but not for determining the effect of each mutation on promoter induction. In other words, with the luciferase assay, ratios between the uninduced and induced promoter activities are meaningless. To compare the effects of the mutations on uninduced and induced promoter activities, the −800-to-+55 and −350-to-+55 wild-type promoters, along with several of the substitution mutants, were analyzed in the context of the CAT reporter plasmid. This

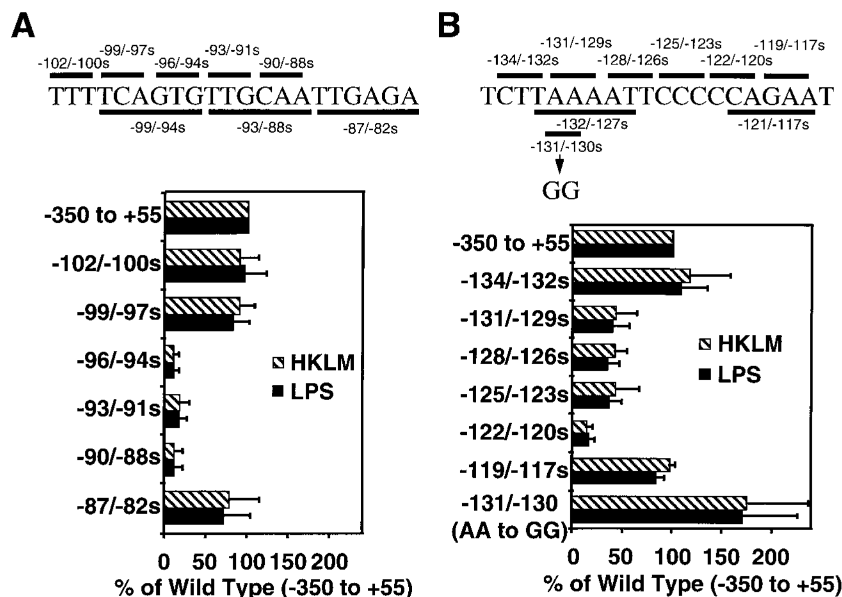


FIG. 5. Three-base-pair substitution mutants through the −102-to-−88 region and the −134-to-−117 region of the IL-12 p40 promoter further delineate important control elements. (A) At the top, the sequence from nucleotide −102 to −82 is shown. Six-base-pair substitution mutants analyzed in Fig. 4 are indicated below the sequence, and 3-bp mutants are indicated above the sequence. Mutants were transfected into RAW 264.7 cells and analyzed as described in the legend to Fig. 3. Results are expressed as described in the legend to Fig. 3. Each result represents the mean ± the standard deviation of data from three to four experiments. Transfection results for the −87/−82s mutant are presented at the bottom of the graph to demonstrate the boundary of the functional element. (B) At the top, the sequence of the NF-κB half-site from position −135 to −116 is shown. Six- and 3-bp mutants are indicated below and above the sequence, respectively. One 2-bp mutation, −131/−130s (AA to GG), created a consensus NF-κB binding site (35). Mutants were analyzed, and results are represented as described in the legend to Fig. 3. Each result represents the mean ± the standard deviation of data from three to four experiments.

analysis (Fig. 6) confirmed the key results obtained with the luciferase plasmids, in that promoter activity was greatly reduced by mutations in the NF-κB (−132/−127s) and the putative C/EBP (−99/−94s and −93/−88s) elements. Interestingly, when uninduced and induced promoter activities were compared, the NF-κB mutant promoter (−132/−127s) was found to remain strongly inducible, demonstrating that proteins interacting with other elements can mediate induction in the absence of Rel interactions. The importance of the C/EBP element for promoter induction could not be assessed because the mutations in this element reduced the HKLM-induced promoter activity to background levels. Thus, although the C/EBP element is likely to be a primary mediator of promoter inducibility, its actual role cannot be discerned from these experiments. It also should be noted that with the wild-type and all of the mutant promoters, the precise fold induction calculations are meaningless because, in each case, the uninduced promoter activity was not significantly greater than background levels.

Five other mutations which had moderate effects (−114/−109s, −107/−102s, −80/−75s, and −62/−57s) or no effect (−56/−51s) in the context of the luciferase reporter plasmids were tested in the context of the CAT reporter (Fig. 6). Two of these mutations, −80/−75s and −56/−51s, had similar effects with the luciferase and CAT reporter genes, but the other three mutations had somewhat different effects. One mutation, −62/−57s, reduced promoter activity to a greater extent with the CAT reporter. The other two, −114/−109s and −107/−102s, reduced promoter activity to a lesser extent with this reporter, although these differences are insignificant, given the standard deviations. Importantly, all of these mutants remained strongly inducible, suggesting that none of them disrupted an essential mediator of promoter induction.

The human IL-12 p40 promoter is regulated through the same elements as the murine promoter in RAW 264.7 cells. By transient transfection analysis of luciferase reporters in RAW 264.7 cells, the human IL-12 promoter was previously reported to require a consensus binding site for Ets proteins between positions −211 and −205 for activation by LPS and IFN-γ (29). In the present study, a deletion mutation (Fig. 3A) and a substitution mutation (Fig. 4B and 6) that specifically alter the homologous Ets sequence in the murine promoter (from −218

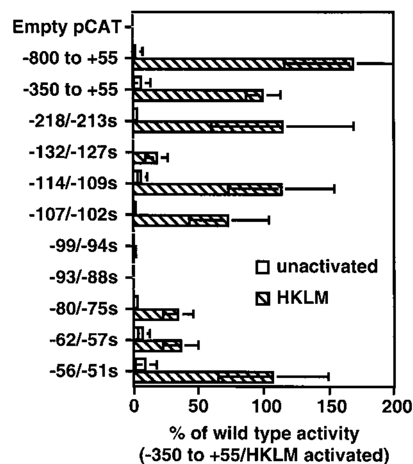


FIG. 6. Analysis of CAT reporter plasmids confirms that the control elements identified influence promoter induction. CAT plasmids containing the promoter sequences indicated to the left were transiently transfected into RAW 264.7 cells with 1 μg of HSP–β-galactosidase reporter plasmid. Cells were activated as described in the legend to Fig. 3. Thin-layer chromatograms from CAT assays using cell extracts were quantitated with a PhosphorImager (Molecular Dynamics). Results (percent conversion of [^{14}C]chloramphenicol to acetylated forms) were corrected for β-galactosidase activity and are expressed as the percentage of HKLM-activated values for the −350-to-+55 promoter. Results obtained with the promoterless (empty) pCAT plasmid are also displayed. Each result represents the mean ± the standard deviation of data from four to six experiments.

A

```
-364
tgaactcggtttggggcaagtccttccttttctgcagtctgtggaatcgggagaggttagccattgccgcctctattcaccttaggcatgatgtaaac.
tcccgtctgaccgccccttggccttccttcttcctcgatctgtggaacccagggaatctgcctagtgctgtctccaagcaccttggccatgatctaaacc
-360

                                              Ets  -218/-213
...agaaattagtatctctgcctccttccttttccacaccccgaagtcatttcctctta.......acctgggatttcgacgtctat.atccctctgta
cagagaaattagcatctccatctccttccttattccccacccaaaagtcatttcctcttagttcattacctgggatttgatgtctatgttccctcctcg
                                               -210/-205

                                          NF-κB  -131/-122                    C/EBP  -96/-88
tgatagatgcactcagggaggcaagggggggagggaggaacttcttaaaattcccccagaatgttttgacactagttttcagtgttgcaattgagactag
ttattgatacacacacagagagagacaaacaaaaaggaacttcttgaaattccccagaaggttttgagagttgttttcaatgttgca.....acaag
                                             -121/-106                         -80/-72

tcagtttctactttgggtttccatcagaaagttctgtaggagtagagtatataagcaccaggagcagccaaggcagcagaaggaacagtgggtg.tccag
tcagtttctagtttaagtttccatcagaa........aggagtagagtatataagttccagtaccagcaacagcagcagaagaaacaacatctgtttcag

                                      +61
gcacatcagaccaggcagctcgcagcaaagcaaggtaag <-Murine
ggccattggactctccgtcctgc.ccagagcaaggtaag <-Human
                                      +63
```

B

-747 to +56

-378 to +56

-378(-210/-205) (Ets)

-378(-116/-111) (NF-κB)

-378(-77/-72) (C/EBP)

HKLM

LPS

0 50 100 150 200 250

% of Wild Type (-747 to +56)

FIG. 7. The activity of the human IL-12 p40 promoter relies on the same control elements as the murine promoter. Human p40 promoter-luciferase plasmids were generated by PCR using human genomic DNA and specific primers to the reported sequence (29). Substitution mutants were created in the context of a −378-to-+56 fragment. The mutations disrupt a previously reported Ets site, −223/−218s (−210/−205) (29), the NF-κB half-site (−116/−111), and the putative C/EBP site (−76/−71). (A) The murine and human IL-12 p40 promoter sequences are aligned; the two functionally important control elements from the murine promoter are underlined. The Ets site previously identified in the human promoter is also underlined. (B) Mutants were transfected into RAW 264.7 cells. Cells were activated as described in the legend to Fig. 3. Results (relative light units normalized for β-galactosidase activity) are expressed as the percentage of the signal obtained with the HKLM- or LPS-activated −747-to-+56 plasmid. Each result represents the mean ± the standard deviation of data from three to five experiments.

to −213) did not affect induction of promoter activity by LPS or HKLM, suggesting that the human and murine promoters may be regulated by different factors and mechanisms. Nevertheless, the Ets site identified in the previous study, as well as the two critical elements identified in this study, are highly conserved in the murine and human promoters (Fig. 7A).

To determine whether the NF-κB and C/EBP elements contribute to the activity of the human p40 promoter, the human promoter from nucleotide −747 to +56 (29) was first amplified from human genomic DNA by PCR and cloned into pGL2B (Promega). Six-base-pair substitution mutations through the Ets site (−210/−205), the NF-κB half-site (−116/−111), and the putative C/EBP element (−76/−71) were then introduced in the context of the human promoter extending from position −378 to +56. RAW 264.7 cells were transfected with the wild-type and mutant plasmids and, after 24 h, were induced with HKLM or LPS. The induced promoter activity that was detected was not affected by a mutation in the Ets element (Fig. 7B). However, mutations in the C/EBP element or the NF-κB element substantially diminished promoter activity (Fig. 7B). (The CAT vector could not be used for this analysis because the human promoter was very weak in the murine RAW 264.7 cells, making the enhanced sensitivity of the luciferase reporter necessary.) These results reveal that in our experiments, the activities of both the human and murine IL-12 p40 promoters depend on the NF-κB and C/EBP elements but not the Ets element. Further investigation will be required to determine the reason for the discrepancy between this study and the previous study.

EMSAs reveal the binding of HKLM-inducible C/EBP proteins to the p40 promoter. To identify the proteins that interact with the putative C/EBP element between nucleotides −96 and −88 of the murine p40 promoter, a radiolabeled double-stranded oligonucleotide probe spanning the sequence from position −110 to −69 was prepared and used for EMSAs (Fig. 8A). Nuclear extracts from unactivated and HKLM-activated RAW 264.7 cells generated four protein-DNA complexes with this probe (Fig. 8B, lanes 1 and 2). The uppermost two complexes were strongly induced by HKLM. Complexes 1 to 3 were not detected with comparable oligonucleotides containing mutations at positions −93 through −88 (Fig. 8B, lanes 3 and 4) or −96 through −94 (Fig. 8B, lanes 5 and 6), which are critical

for promoter function (Fig. 4 and 5) and which contain the putative C/EBP recognition sequence.

To test the hypothesis that the protein-DNA complexes contain C/EBP proteins, oligonucleotide competition and antibody inhibition experiments were performed. Consistent with the hypothesis, the three complexes were not detected with either the extract from unactivated cells or that from the HKLM-activated cells when a 100-fold excess of an unlabeled competitor oligonucleotide containing a consensus C/EBP sequence was included in the binding reaction (Fig. 8C, lanes 6 and 13). In contrast, a mutant competitor oligonucleotide had no effect on complex formation (Fig. 8C, lanes 7 and 14). Also consistent with the hypothesis, polyclonal antibodies directed against specific C/EBP family members disrupted or altered the mobilities of the three complexes. In extracts of unactivated cells, a C/EBPα antibody altered the mobility of a portion of complex 1 (Fig. 8C, lane 2). This antibody also altered the mobility of a portion of one of the complexes which formed with extracts of activated cells, as was evident from the appearance of a slow-mobility complex (Fig. 8C, lane 9). In extracts of unactivated cells, a C/EBPβ antibody altered the mobilities of complexes 2 and 3 and a portion of complex 1 (Fig. 8C, lane 3). In extracts of activated cells, this antibody altered the mobilities of all three complexes, including almost all of complex 1 (Fig. 8C, lane 10). The C/EBPβ DNA-binding activity in complexes 1 and 2 was strongly induced by HKLM. A C/EBPδ antibody had no effect with unactivated-cell extracts but altered the mobilities of complexes obtained with extracts of HKLM-activated cells, based on the appearance of slow-mobility complexes (Fig. 8C, lanes 4 and 11). In the absence of the antibody, the complexes containing C/EBPδ appear to be very weak and are obscured by the C/EBPβ complexes. Antibodies to Rel family members p50 (Fig. 8C, lanes 5 and 12), p65, and c-Rel (data not shown) did not affect these complexes, suggesting that Rel-C/EBP heterodimers do not stably interact with this probe. Nuclear extracts from another murine macrophage cell line, J774, also demonstrated HKLM- and LPS-inducible binding of C/EBP proteins to this promoter region (data not shown). Furthermore, 20- and 1-h pretreatments of RAW 264.7 and J774 cells with IFN-γ prior to a 4-h activation with HKLM did not augment the C/EBP DNA-binding activities (data not shown).

Previous studies have described three EMSA complexes attributed to two alternatively translated forms of C/EBPβ, the larger one called LAP (or NF-IL-6) and the smaller one called LIP (11). LIP appears to be a competitive inhibitor of LAP because it contains DNA-binding and dimerization domains but not a transcriptional activation domain (11). In previous studies, the fastest-migrating C/EBPβ EMSA complex was composed of LIP homodimers, the intermediate complex consisted of LIP-LAP heterodimers, and the slowest-migrating complex contained LAP homodimers. C/EBPα and C/EBPδ are able to heterodimerize with LAP, as well as with each other (39, 44). Based on this information, a simple and plausible model for the EMSA results shown in Fig. 8B and C is that complexes 1 to 3 formed with the extracts of HKLM-activated cells are composed primarily of LAP homodimers, LAP-LIP heterodimers, and LIP homodimers, respectively. Complex 1 is likely to be a mixture of two independent complexes, one containing the LAP homodimers and the other containing either C/EBPα homodimers or heterodimers with an unknown partner. The induced C/EBPδ complexes, which can only be visualized following antibody addition, may contain C/EBPδ homodimers, heterodimers with C/EBPα or C/EBPβ, or heterodimers with other proteins. Additional experiments will be needed to clarify and confirm these assignments and, more importantly, to determine which complex or complexes contribute to p40 promoter function.

Interactions between Rel proteins and the IL-12 p40 promoter. In a previous study performed in the J774 macrophage cell line, Rel proteins were found to bind to an NF-κB half-site within the IL-12 p40 promoter, with a heterodimer of two Rel proteins, p50 and c-Rel, implicated as the functional activator (35). Furthermore, the binding of Rel proteins was strongly induced by LPS and was synergistically induced by pretreatment with IFN-γ (35). To investigate the interactions between Rel proteins and the p40 promoter in RAW 264.7 cells, EMSAs were performed with a radiolabeled DNA probe spanning nucleotides −146 through −107 (Fig. 8A). Four protein-DNA complexes were readily detected in unactivated-cell extracts, and two of these complexes (complexes 2 and 3) were enhanced to a moderate degree in extracts of HKLM-activated cells (Fig. 8D, lanes 1 and 6). A probe containing the −132/−127 mutation eliminated all four protein-DNA complexes (data not shown), suggesting that all of the DNA-binding activities depend on the functionally important element within the probe. An antibody directed against the p50 protein completely disrupted complex 3 and partially disrupted complex 2 in both unactivated- and activated-cell extracts (Fig. 8D, lanes 2 and 7). A c-Rel antibody altered the mobility of complex 2, particularly in activated-cell extracts (Fig. 8D, lanes 4 and 9). Antibodies directed against another Rel protein, p65 (Fig. 8D, lanes 3 and 8), or against the C/EBP proteins (data not shown) had no noticeable effect on the four complexes. An antibody directed against PU.1 partially inhibited the formation of complex 4, consistent with the presence of a PU.1 recognition site between nucleotides −147 and −142, a region whose importance for p40 promoter function remains to be established (Fig. 3 and 4) (35).

The simplest interpretation of these results is that complex 3 contains p50 homodimers and that complex 2 primarily contains p50–c-Rel heterodimers. These results, from experiments performed with RAW 264.7 cells, are therefore consistent with the previous proposal, from studies performed with J774 cells, that a p50–c-Rel heterodimer may be responsible for the activity of the NF-κB half-site. Unlike the previous study, however, we have been unable to augment the induction of Rel DNA-binding activities in RAW 264.7 or J774 cells by pretreatment with IFN-γ for 1 or 20 h prior to activation by HKLM (data not shown). Also, the degree to which complexes 2 and 3 were enhanced by HKLM in RAW 264.7 and J774 cells was small relative to the previous studies (35). The reason for this small enhancement will require further investigation, but it appears to result from the relatively abundant Rel protein DNA-binding activity in uninduced extracts.

DNAse I footprinting reveals multiple DNA-protein interactions within the IL-12 p40 promoter. Our functional studies have suggested that multiple transcription factors contribute to p40 promoter activity in RAW 264.7 cells. C/EBP and Rel proteins appear to play prominent roles and act in a synergistic, rather than an additive, manner. In other promoters, functional synergy between C/EBP and Rel proteins involves cooperative binding or heterodimerization between family members (4, 25, 30, 56). To address the mechanism by which these proteins synergistically activate the p40 promoter and to identify other protein-DNA interactions that may contribute to promoter function, DNAse I footprinting experiments were performed.

Footprinting experiments performed with a 5′-end-labeled probe extending from position −174 to −24 revealed four protein-DNA interactions (Fig. 9A), three of which (interactions B, C, and D) are qualitatively different in extracts pre-

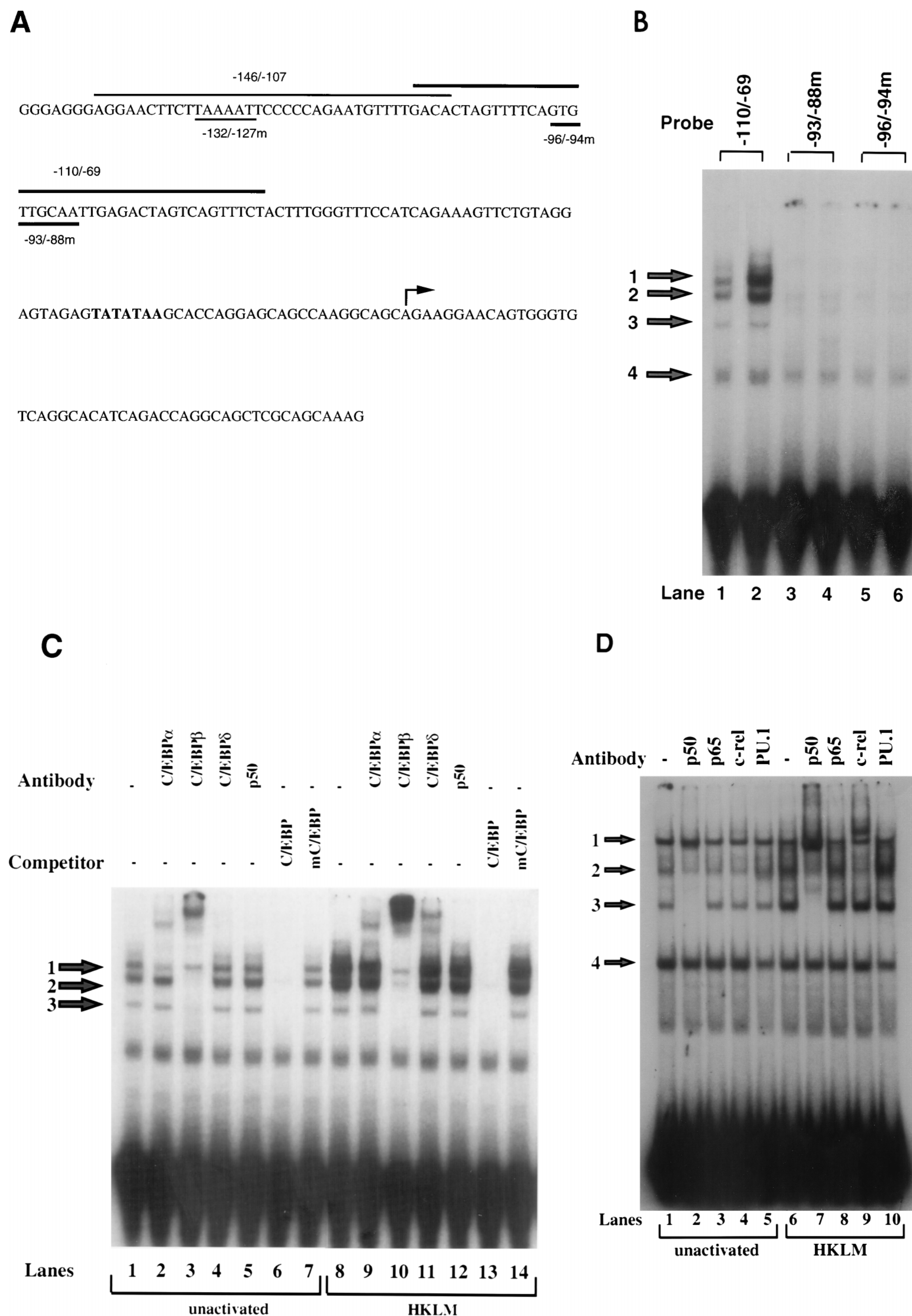


FIG. 8. EMSAs demonstrate HKLM-inducible binding of C/EBP and Rel proteins to the critical p40 promoter elements. (A) Locations of oligonucleotide probes within the p40 promoter. The probe represented by a thin line (−146/−107) spans the region containing the NF-κB half-site. A mutant probe contains the 6-bp mutation −132/−127m, as denoted. The probe represented by the thick line (−110/−69) spans the putative C/EBP site. The sequences altered in two specific mutant probes, −96/−94m and −93/−88m, are indicated. The arrow indicates the location of the transcription start site, and boldfaced letters indicate the TATA box. (B) EMSAs were performed with nuclear extracts from unactivated RAW 264.7 cells and from cells activated with HKLM (1.5×10^8/ml) for 4 h. Labeled probe was incubated with 5 μg of nuclear extract and 2 μg of poly(dI-dC) at room temperature for 45 min. Extracts from unactivated (lanes 1, 3, and 5) and activated (lanes 2, 4, and 6) cells were added to probes −110/−69 (lanes 1 and 2), −93/−88m (lanes 3 and 4), and −96/−94m (lanes 5 and 6). The four protein-DNA complexes are indicated by the arrows on the left. (C) Five micrograms of extract from unactivated (lanes 1 to 7) or HKLM-activated (lanes 8 to 14) cells was incubated without (lanes 1 and 8) or with 2 μl of the following polyclonal antibodies at room temperature for 30 min: anti-C/EBPα (lanes 2 and 9), anti-C/EBPβ (lanes 3 and 10), anti-C/EBPδ (lanes 4 and 11), and anti-NF-κB p50 (lanes 5 and 12). Unlabeled oligonucleotides (100-fold molar excess) containing a C/EBP consensus sequence (lanes 6 and 13) or a mutant consensus sequence (mC/EBP) (lanes 7 and 14) were incubated with nuclear extracts at room temperature for 30 min. Following this incubation, labeled probe −110/−69 was added for 45 min prior to electrophoresis. (D) EMSAs were performed with 5 μg of nuclear extract from unactivated (lanes 1 to 5) and HKLM-activated (lanes 6 to 10) cells. Extract and 2 μg of poly(dI-dC) were incubated at 4°C for 30 min without (lanes 1 and 6) or with 2 μl of the following polyclonal antibodies (Santa Cruz Biotechnology); anti-p50 (lanes 2 and 7), anti-p65 (lanes 3 and 8), anti-c-Rel (lanes 4 and 9), and anti-PU.1 (lanes 5 and 10). After this incubation, labeled probe −146/−107 was added to the mixture, and then incubation was continued for 45 min at 4°C prior to electrophoresis. Four protein-DNA complexes (1 to 4) are indicated by arrows on the left.

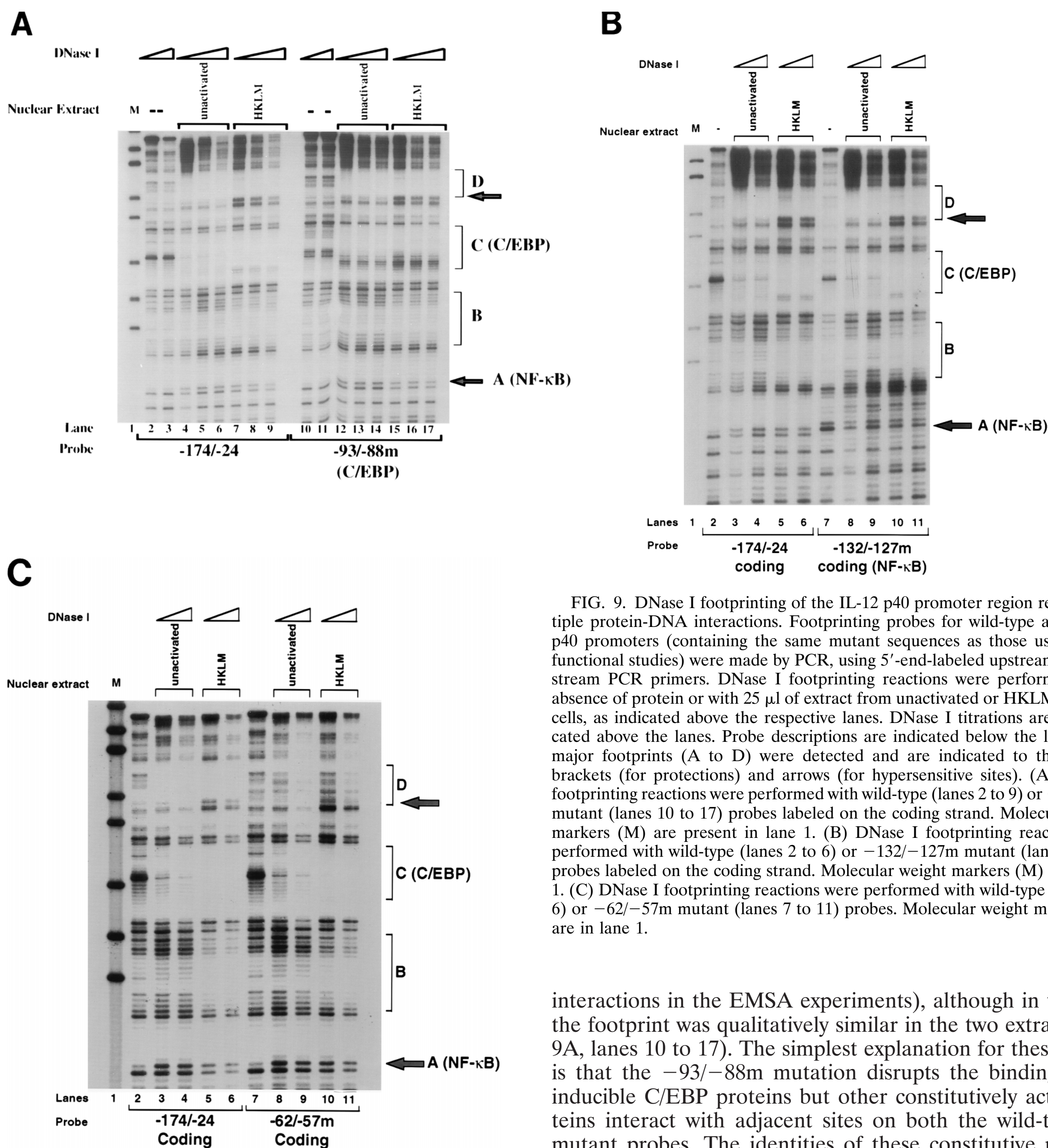


FIG. 9. DNase I footprinting of the IL-12 p40 promoter region reveals multiple protein-DNA interactions. Footprinting probes for wild-type and mutant p40 promoters (containing the same mutant sequences as those used for the functional studies) were made by PCR, using 5′-end-labeled upstream or downstream PCR primers. DNase I footprinting reactions were performed in the absence of protein or with 25 μl of extract from unactivated or HKLM-activated cells, as indicated above the respective lanes. DNase I titrations are also indicated above the lanes. Probe descriptions are indicated below the lanes. Four major footprints (A to D) were detected and are indicated to the right by brackets (for protections) and arrows (for hypersensitive sites). (A) DNase I footprinting reactions were performed with wild-type (lanes 2 to 9) or −93/−88m mutant (lanes 10 to 17) probes labeled on the coding strand. Molecular weight markers (M) are present in lane 1. (B) DNase I footprinting reactions were performed with wild-type (lanes 2 to 6) or −132/−127m mutant (lanes 7 to 11) probes labeled on the coding strand. Molecular weight markers (M) are in lane 1. (C) DNase I footprinting reactions were performed with wild-type (lanes 2 to 6) or −62/−57m mutant (lanes 7 to 11) probes. Molecular weight markers (M) are in lane 1.

pared from unactivated and HKLM-activated RAW 264.7 cells (compare lanes 4 to 6 with lanes 7 to 9). Interaction A was evident from a single hypersensitive site immediately upstream of the NF-κB site. This hypersensitive site was not detected with a probe containing the −132/−127m mutation (Fig. 9B, lanes 7 to 11), suggesting that it may result from interactions by the Rel family members that were detected by EMSA. Footprint C, which spans the functionally critical C/EBP element, was readily detected in both unactivated- and activated-cell extracts (Fig. 9A, lanes 2 to 9). However, this footprint was qualitatively different in the two extracts, based on the increased protection of the central region in the activated-cell extracts and the appearance of a hypersensitive doublet at the distal end (Fig. 9A; compare lanes 4 to 6 with lanes 7 to 9). Interestingly, this region was strongly protected on a probe containing the −93/−88m mutation (which abolished C/EBP interactions in the EMSA experiments), although in this case the footprint was qualitatively similar in the two extracts (Fig. 9A, lanes 10 to 17). The simplest explanation for these results is that the −93/−88m mutation disrupts the binding of the inducible C/EBP proteins but other constitutively active proteins interact with adjacent sites on both the wild-type and mutant probes. The identities of these constitutive proteins, and their relevance to p40 protein activity, remain to be elucidated. Of note, the mutation in the NF-κB site (−132/127m [Fig. 9B]) had no effect on footprint C and the mutation in the C/EBP site (−93/−88m [Fig. 9A]) had no effect on hypersensitive site A, suggesting that C/EBP and Rel proteins do not bind cooperatively in these experiments. A similar absence of cooperative binding was observed in EMSA experiments performed with probes spanning both elements (data not shown).

In addition to the interactions at the NF-κB and C/EBP elements, two other protein-DNA interactions were noteworthy. One interaction, footprint B, was relatively weak but was consistently enhanced in the extracts from HKLM-activated cells (Fig. 9). The second interaction, footprint D, which occurred downstream of the C/EBP site, was quite strong in both unactivated- and activated-cell extracts, with two strong hypersensitive sites at the distal end of the footprint apparent only in the activated-cell extracts (Figs. 9). Interestingly, this footprint was abolished by the −62/−57 mutation (Fig. 9C, lanes 7 to

11), which reduced promoter activity in the CAT reporter assay by threefold (Fig. 6).

In summary, the DNase I footprinting experiments revealed multiple protein-DNA interactions, suggesting that proteins other than the Rel and C/EBP proteins detected by EMSA may contribute to the regulation of p40 promoter activity. An in-depth analysis will be needed to identify these proteins and to determine their functional relevance. These data also suggest that the functional synergy between Rel and C/EBP proteins may not result from cooperative binding. This issue will require further investigation, however, since we have not yet employed in vitro transcription experiments to determine if synergistic activation can be observed with the conditions and protein concentrations used for the footprinting experiments.

Modulation of IL-12 p40 promoter activity by transient overexpression of LIP and LAP. To provide additional evidence that C/EBP family members may contribute to p40 gene transcription, the functional C/EBPβ isoform LAP and the dominant negative isoform LIP (11) were transiently overexpressed in RAW 264.7 cells with the −355-to-+55 p40 promoter-CAT reporter. Overexpression of LIP essentially ablated LPS-induced promoter activity (Fig. 10, lanes 1 to 10). This effect was specific for the p40 promoter, as cotransfection of 5 μg of the LIP expression plasmid reduced the activity of a CMV promoter/enhancer-CAT reporter by less than fourfold (data not shown). Overexpression of LAP yielded strong p40 promoter activity in uninduced cells (Fig. 10, lanes 11 to 18). This promoter activity was dependent on the C/EBP binding site, as no activity was observed when LAP was overexpressed with the −99/−94s promoter mutant (data not shown). Interestingly, p40 promoter activity in LPS-activated cells was slightly diminished in the presence of overexpressed LAP (Fig. 10, lanes 11 to 18), possibly because of nonspecific squelching.

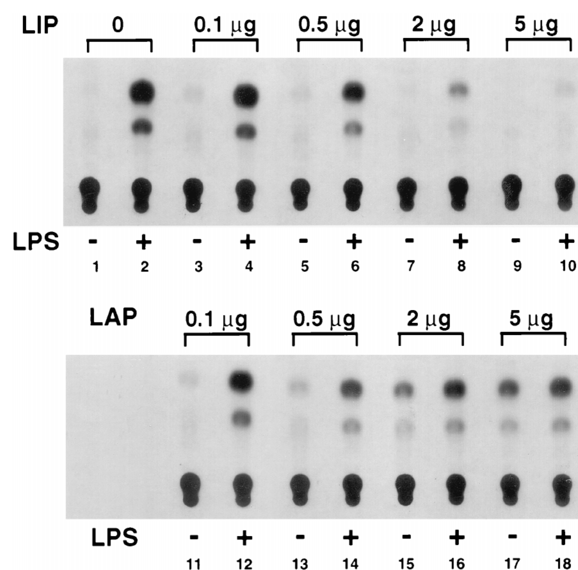


FIG. 10. Modulation of p40 promoter activity by overexpression of the C/EBP-β isoforms LIP and LAP. Expression plasmids for LAP and LIP (11) containing a CMV promoter/enhancer were cotransfected in transient assays into RAW 264.7 cells with 15 μg of the −355-to-+55 IL-12 p40 promoter-CAT reporter. The amounts of the LIP and LAP plasmids cotransfected are indicated above the respective lanes. Plasmid amounts were normalized to 20 μg per transfection by addition of a CMV promoter/enhancer-luciferase plasmid. Twenty-four hours after transfection, cells, either were not activated (lanes 1, 3, 5, 7, 9, 11, 13, 15, and 17) or were activated with 5 μg of LPS per ml for 24 h (lanes 2, 4, 6, 8, 10, 12, 14, 16, and 18). A thin-layer chromatogram from one representative experiment is shown. Similar results were obtained in three other experiments.

These results demonstrate that overexpressed C/EBP family members are capable of binding to the critical p40 promoter element in vivo. The results are also consistent with the hypothesis that C/EBP family members are functional activators of p40 transcription through the critical element. However, like most experiments involving overexpressed DNA-binding proteins, these experiments must be interpreted with caution. Overexpressed LIP, for example, could ablate promoter activity by preventing the functional activator from binding to the critical promoter element, regardless of whether the functional activator is a member of the C/EBP family. Similarly, LAP might transactivate the promoter through the critical element when overexpressed yet play no role in p40 gene transcription when present at normal levels. Indirect effects of LIP and LAP overexpression on p40 promoter activity also cannot be ruled out. Thus, although intriguing, these results only marginally advance our knowledge of p40 regulation relative to the studies of mutants and the DNA-binding studies described above.

Activation of the IL-12 p40 promoter by cycloheximide. During the innate immune response against bacterial pathogens, the rapid induction of IL-12 expression is necessary to induce NK cell IFN-γ production (14, 22, 60). Therefore, it is not surprising that bacterial activation of the p40 gene utilizes NF-κB, as this cytoplasmic protein is rapidly translocated to the nucleus following degradation of the cytoplasmic inhibitor known as IκB (19). Less is known about the kinetics of C/EBP activation, but these proteins have been reported to transactivate genes following posttranslational modification and in the absence of new protein synthesis (8, 32, 44, 58, 64).

To determine whether new protein synthesis is required for induction of the endogenous IL-12 p40 gene, RAW 264.7 cells were treated with cycloheximide for 15 min prior to activation with HKLM for 2 or 4 h. As detected by RT-PCR, the amount of endogenous p40 mRNA produced in the presence of HKLM and cycloheximide was comparable to the amount produced in the presence of HKLM alone (Fig. 11A; compare lanes 4 and 7 with lanes 5 and 8). Interestingly, a similar amount of p40 mRNA was produced following cycloheximide treatment in the absence of HKLM (Fig. 11A, lanes 3 and 6).

To extend this analysis, RAW 264.7 cell lines containing stably integrated p40 promoter-luciferase reporter plasmids were prepared (see Materials and Methods). Transcripts synthesized from the integrated p40 promoters were analyzed by primer extension, using a radiolabeled primer complementary to the 5′ end of the luciferase gene. Stable cell lines were used for this analysis because the reporter mRNA levels were too low to monitor by primer extension following transient transfection. Furthermore, RT-PCR assays yield unacceptably high background signals when used to analyze mRNA derived from transiently transfected plasmids. Stable cell lines containing luciferase reporter genes, rather than CAT reporter genes, were used because stable cell lines containing p40 promoter-CAT reporter plasmids did not yield detectable primer extension signals (data not shown).

Figure 11B shows the primer extension results obtained with a representative cell line containing a stably integrated p40 promoter (−350 to +55)-luciferase reporter plasmid. A luciferase primer extension signal could not be detected with this cell line in the absence of cell activation (lane 2). However, treatment with cycloheximide alone for either 2 or 4 h resulted in weakly detectable signals (lanes 3 and 6). Following a 2-h cycloheximide treatment, the signal detected was comparable to that detected following a 2-h activation with HKLM (lanes 3 and 4). Superactivation of the specific mRNA was observed with a combination of cycloheximide and HKLM (lane 5).

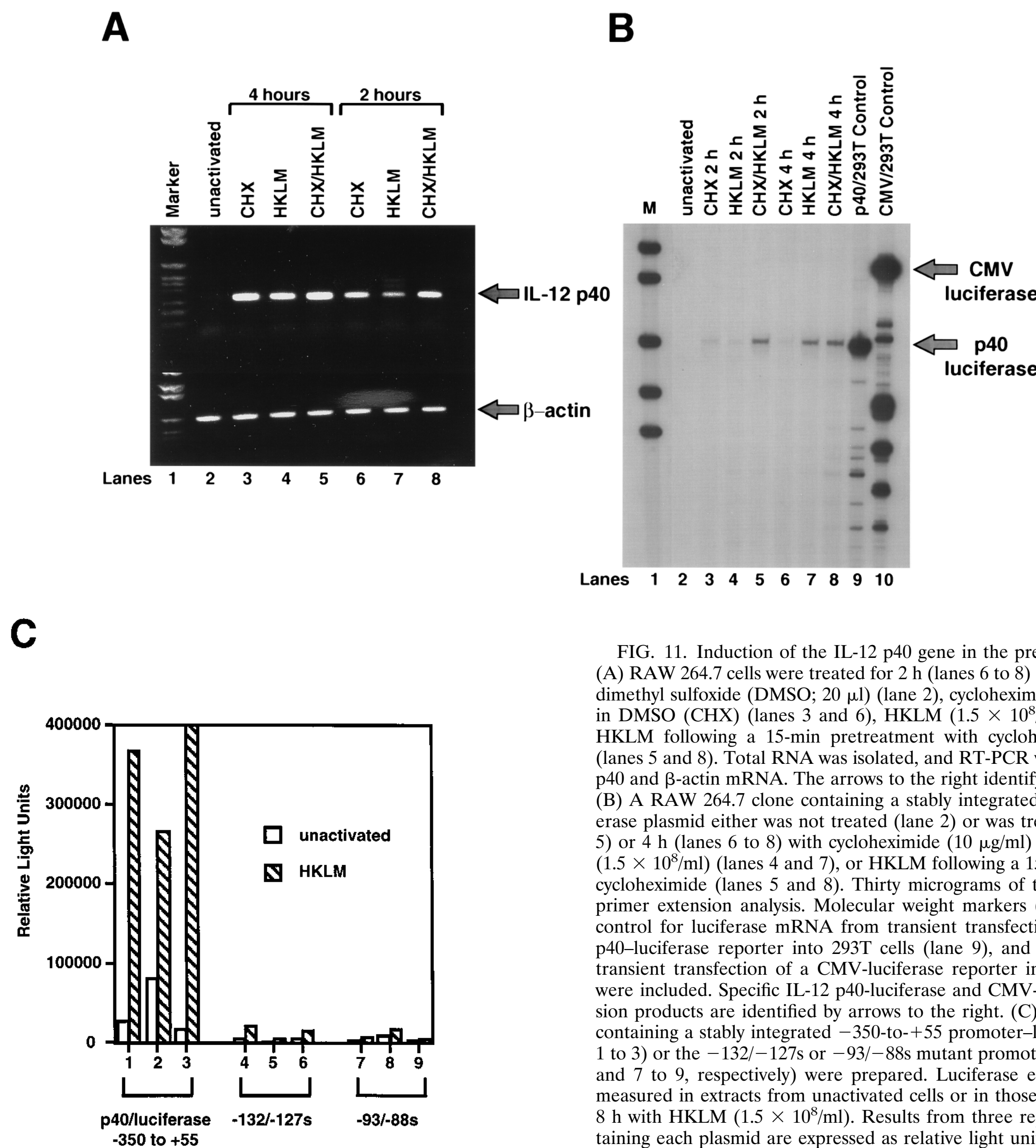


FIG. 11. Induction of the IL-12 p40 gene in the presence of cycloheximide. (A) RAW 264.7 cells were treated for 2 h (lanes 6 to 8) or 4 h (lanes 2 to 5) with dimethyl sulfoxide (DMSO; 20 μl) (lane 2), cycloheximide (10 μg/ml) dissolved in DMSO (CHX) (lanes 3 and 6), HKLM (1.5×10^8/ml) (lanes 4 and 7), or HKLM following a 15-min pretreatment with cycloheximide (CHX/HKLM) (lanes 5 and 8). Total RNA was isolated, and RT-PCR was performed for IL-12 p40 and β-actin mRNA. The arrows to the right identify specific PCR products. (B) A RAW 264.7 clone containing a stably integrated −350-to-+55p40–luciferase plasmid either was not treated (lane 2) or was treated for 2 h (lanes 3 to 5) or 4 h (lanes 6 to 8) with cycloheximide (10 μg/ml) (lanes 3 and 6), HKLM (1.5×10^8/ml) (lanes 4 and 7), or HKLM following a 15-min pretreatment with cycloheximide (lanes 5 and 8). Thirty micrograms of total RNA was used for primer extension analysis. Molecular weight markers (M) (lane 1), a positive control for luciferase mRNA from transient transfection of the −350-to-+55 p40–luciferase reporter into 293T cells (lane 9), and a positive control from transient transfection of a CMV-luciferase reporter into 293T cells (lane 10) were included. Specific IL-12 p40-luciferase and CMV-luciferase primer extension products are identified by arrows to the right. (C) RAW 264.7 cell clones containing a stably integrated −350-to-+55 promoter–luciferase plasmid (lanes 1 to 3) or the −132/−127s or −93/−88s mutant promoter plasmids (lanes 4 to 6 and 7 to 9, respectively) were prepared. Luciferase enzymatic activities were measured in extracts from unactivated cells or in those from cells activated for 8 h with HKLM (1.5×10^8/ml). Results from three representative clones containing each plasmid are expressed as relative light units.

Following a 4-h cycloheximide treatment, the signal detected was significantly weaker than that obtained with HKLM alone (lanes 6 and 7), and cycloheximide did not superactivate the HKLM-activated mRNA (lane 8). Importantly, the promoter activity detected in these experiments was dependent on the NF-κB and C/EBP elements; three independently generated cell lines containing the wild-type promoter exhibited strong luciferase activity following induction with HKLM, whereas very little luciferase activity was detected in cell lines containing integrated plasmids with mutations in the NF-κB or C/EBP elements (Fig. 11C).

These results suggest that the induction of p40 promoter activity by HKLM does not require new protein synthesis, consistent with the previously reported activation of both Rel and C/EBP proteins by posttranslational mechanisms. Furthermore, the results suggest that an inhibition of protein synthesis may be sufficient for induction of the p40 promoter. An important caveat about this result, however, is that we have not eliminated potential effects of cycloheximide on p40 mRNA stability and on the promoter-independent activation of luciferase reporter plasmids. Indeed, cycloheximide has been reported to stabilize mRNAs to a considerable extent (43, 66). Nevertheless, given the short, 2-h activation period used in these experiments and the superactivation observed with cycloheximide and HKLM (Fig. 11B, lane 5), it appears likely that the p40 promoter can be activated in the absence of new protein synthesis.

To further explore the importance of new protein synthesis for p40 promoter induction, Rel and C/EBP DNA-binding activities were analyzed. EMSA analyses using a consensus NF-κB probe or a probe containing the p40 NF-κB element revealed that complex 2, which is believed to contain the p50–c-Rel heterodimer, was strongly induced by a combination of HKLM and cycloheximide (Fig. 12B, lanes 4 and 8). This complex was also slightly induced by cycloheximide alone (Fig. 12B, lanes 3 and 7). In contrast, the level of protein-DNA complexes containing C/EBP proteins was diminished by treatment with cycloheximide alone (Fig. 12B, lanes 3 and 7) and, relative to the unactivated extracts (Fig. 12A, lanes 1 and 5),

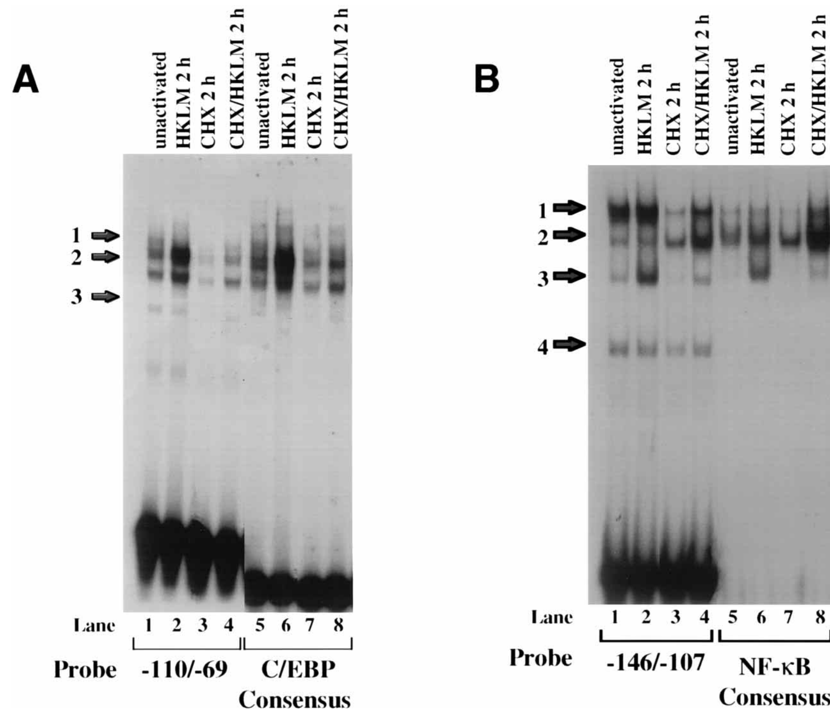


FIG. 12. DNA-binding activities in extracts from cycloheximide-treated cells. (A) EMSAs were performed with nuclear extracts from unactivated RAW 264.7 cells (lanes 1 and 5), cells activated with HKLM for 2 h (lanes 2 and 6), cells treated with 10 μg of cycloheximide (CHX) per ml for 2 h (lanes 3 and 7), and cells treated with cycloheximide for 15 min prior to treatment with HKLM for 2 h (lanes 4 and 8). Radiolabeled probes contained the IL-12 p40 −146-to-−107 sequence (lanes 1 to 4) or a consensus NF-κB sequence (lanes 5 to 8). Labeled probes were incubated with 5 μg of nuclear extract and 2 μg of poly(dI-dC) at 4°C for 45 min. (B) EMSAs were performed with nuclear extracts from unactivated RAW 264.7 cells (lanes 1 and 5), cells activated with HKLM for 2 h (lanes 2 and 6), cells treated with 10 μg of cycloheximide per ml for 2 h (lanes 3 and 7), and cells treated with cycloheximide for 15 min prior to treatment with HKLM for 2 h (lanes 4 and 8). Radiolabeled probes used in this experiment contained the IL-12 p40 −110-to-−69 sequence (lanes 1 to 4) or a consensus C/EBP sequence (lanes 5 to 8). Labeled probes were incubated with 5 μg of nuclear extract and 2 μg of poly(dI-dC) at room temperature for 45 min.

was not induced by HKLM in the presence of cycloheximide (Fig. 12A, lanes 4 and 8).

Further experiments will be needed to explain these complicated results. However, the induction of p40 mRNA by HKLM in the presence of cycloheximide, with no induction of the C/EBP binding activities, suggests that induction of these binding activities may not be critical for the induction of p40 promoter activity. A simple hypothesis, which is consistent with previous studies, is that excess amounts of the relevant C/EBP proteins may be present prior to induction, with p40 induction being due to phosphorylation of the transcriptional activation domains within the C/EBP proteins. An alternative hypothesis is that the superactivation of Rel proteins by the combination of cycloheximide and HKLM may compensate for the lack of C/EBP activation. Numerous alternative explanations can be envisioned to explain the effects of the combination of cycloheximide and HKLM as well as the effects of cycloheximide alone.

DISCUSSION

A systematic analysis of the murine IL-12 p40 promoter and a confirmatory analysis of the human promoter have been performed, providing an initial step toward a detailed understanding of the regulation of IL-12 expression. This knowledge will contribute to our understanding of the molecular events that lead to a Th1 immune response. The p40 promoter was strongly induced by HKLM, but IFN-γ did not augment induction in transient transfection experiments performed in RAW 264.7 cells. Multiple control elements involved in HKLM- and LPS-activated p40 transcription were identified. The most critical element binds C/EBP proteins and functions synergistically with a previously reported binding site for Rel proteins. Multiple criteria suggest that additional control elements contribute to optimal p40 promoter activity. In contrast to a previous study (29), the human IL-12 p40 promoter required the same control elements as the murine promoter. Finally, the p40 promoter was induced in the presence of cycloheximide, consistent with previous reports that C/EBP and Rel proteins are activated by posttranslational mechanisms.

The C/EBP recognition sequence was the only element that was essential for induction of promoter activity in the transient-transfection assay. Substitution mutations in this element reduced promoter activity to undetectable levels with CAT reporter plasmids and to less than 10% of wild-type levels with luciferase reporter plasmids. The functional element spans 5 to 9 bp, suggesting that it is not a composite element but is recognized by one protein monomer or dimer. In nuclear extracts, multiple DNA-binding activities involving C/EBPβ were induced by HKLM. DNA-binding activities involving C/EBPδ were induced to a lesser extent. C/EBPβ has previously been shown to be induced in monocytes and macrophages (47, 62) and has been implicated in the activation of the genes for several cytokines expressed in myeloid cells, including IL-1, IL-6, IL-8, tumor necrosis factor alpha, and monocyte inflammatory protein 1α (MIP-1α) (5, 15, 17, 30, 42, 55). Several potential mechanisms for regulating the activity of C/EBPβ have been reported, although the relative importance of each mechanism remains to be established (3, 11). Mice with a disrupted C/EBPβ gene generated in one laboratory demonstrated markedly decreased IL-12 production and increased

susceptibility to infection with intracellular organisms, consistent with the hypothesis that C/EBPβ plays a direct role in IL-12 induction (48). Mice with a disrupted C/EBPβ gene generated in another laboratory were also susceptible to infection but did not manifest a defect in IL-12 gene expression, suggesting that other proteins, perhaps C/EBPδ, may compensate for the absence of C/EBPβ (57). Despite these supporting results, the identities of the C/EBP homo- and/or heterodimers that contribute to IL-12 p40 regulation, and the mechanisms by which the activities of these dimers are modulated, remain to be determined.

Rel proteins also have been implicated in the induction of several proinflammatory genes produced by macrophages, including tumor necrosis factor alpha, IL-8, MIP-1α, and IL-6 (17, 30, 55). Our results confirm those of Murphy and colleagues (35), who demonstrated that Rel proteins contribute to p40 promoter activity. Transactivation experiments performed in that study suggested that the functionally active protein is a p50–c-Rel heterodimer (35), consistent with our observation that a complex containing these proteins is induced in RAW 264.7 extracts. As with the C/EBP proteins, however, further experiments will be needed to confirm the identity of the protein complex that actually contributes to p40 promoter activity. Another critical issue that remains to be clarified is the importance of Rel protein induction for the induction of p40 promoter activity. In our experiments, Rel proteins bound constitutively to the p40 promoter, their DNA-binding activities were only moderately induced, and the p40 promoter could be strongly induced in transient-transfection assays in the absence of the NF-κB half-site. Constitutive Rel DNA-binding activities have previously been observed in macrophage cell lines, primary peripheral blood monocytes, and peritoneal macrophages (13, 16). It therefore remains possible that the Rel DNA-binding activities modulate promoter induction but are not the primary mediators of induction. It is worth noting that some Rel DNA-binding activities correlate with macrophage differentiation, in that they are found only in mature cells and are induced as promonocytes differentiate into monocytes (13, 16). Perhaps the degree to which the induction of Rel proteins contributes to the induction of the p40 promoter will be found to depend on the differentiation state of the uninduced cells.

Another biologically relevant event that may be linked to the differentiation state of the cells is the augmentation of IL-12 expression by IFN-γ. In many systems, IFN-γ is necessary for the production of bioactive IL-12 (51, 53). With CAT reporters, IFN-γ did not enhance p40 promoter activity in RAW 264.7 cells, although expression of the endogenous gene was augmented. These results differ from those of Murphy and colleagues, who found that IFN-γ greatly enhanced p40 promoter activity in J774 cells and that Rel DNA-binding activities were enhanced by IFN-γ (35). The J774 functional studies were performed with luciferase reporter genes, which exhibited nonspecific effects in RAW 264.7 cells; nevertheless, the effect of IFN-γ on the DNA-binding activities in J774 cells is intriguing and clearly different from the results obtained in RAW 264.7 cells. Perhaps J774 cells are less differentiated than RAW 264.7 cells; IFN-γ may facilitate differentiation, which may facilitate Rel protein induction and, therefore, induction of the p40 promoter. RAW 264.7 cells may exist in a more terminally differentiated state, which may explain why IFN-γ has no effect on Rel DNA-binding activities. The augmentation of p40 mRNA by IFN-γ in RAW 264.7 cells may be mediated through a DNA sequence element that is upstream or downstream of the promoter region analyzed or through another mechanism, such as mRNA stabilization.

C/EBP and Rel proteins appeared to activate the p40 promoter in a synergistic manner, as specific mutations in either recognition site reduced promoter activity considerably. In other words, the additive contributions of the two elements to promoter activity total more than 100%. Several proinflammatory genes expressed in macrophages are synergistically regulated by C/EBP and NF-κB, including IL-8, MIP-1α, and IL-6 (17, 30, 55). One mechanism for functional synergy is through cooperative binding (55), which was not observed in our experiments. Direct associations between Rel and C/EBP proteins also contribute to synergistic activation of some genes (25, 30, 56), but again they were not observed in our studies. Perhaps the nuclear extracts used for our experiments were inappropriate for detection of physical interactions between Rel and C/EBP proteins. Alternatively, specific interactions between these proteins and the basal transcription machinery (23, 36, 45) may account for the functional synergism at the p40 promoter.

The C/EBP element, the NF-κB element, and the TATA box were the only elements convincingly shown to be important for p40 promoter function, but three results suggest that other functionally important elements exist within the promoter. First, deletion or substitution mutations at other locations appeared to have significant yet modest effects on promoter activity. Second, other DNA-binding activities were detected by DNAse I footprinting and EMSAs, at least one of which correlates with an element that appears to be functionally significant. Third, 25% of the wild-type promoter activity was retained following disruption of the NF-κB element; this activity appears to be too strong to be directed solely by a C/EBP dimer and a TATA box. Since no other mutations had dramatic effects on promoter activity, the other important control elements may be redundant with each other or may function in an additive rather than a synergistic manner. Despite the large number of mutations tested in this study, even more mutants, with two or more potential control elements simultaneously disrupted, will need to be tested to clarify their locations and mechanistic roles.

In summary, this study and others provide a point of departure for characterizing IL-12 p40 gene expression in biological situations. The expression of IL-12 p40 is a proximal event in the development of a protective immune response to intracellular pathogens, and its unregulated expression may be a driving force behind the Th1 response in chronic inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis. The regulated expression of p40 by transcriptional and posttranscriptional mechanisms will be defined by a complicated series of events. Its detailed elucidation will provide new insights into the pathogenesis of infectious and autoimmune diseases and may delineate novel targets for manipulating an immune response at the level of macrophage activation.

ACKNOWLEDGMENTS

We are extremely grateful to Robert Modlin, Jeffery F. Miller, Xiaojing Ma, Giorgio Trinchieri, Andrew Henderson, and Kathryn Calame for helpful discussions and to Amy Weinmann and Hans Brightbill for critical reading of the manuscript. We also thank Daniel Deming for assistance with some of the experiments. Finally, we thank Stephan Targan for his continued support.

S.E.P. was supported by a Crohn's and Colitis Foundation of America fellowship and by PHS grant K11 DK02358-02. S.T.S. is an Associate Investigator with the Howard Hughes Medical Institute.

REFERENCES

1. **Abbas, A. K., K. M. Murphy, and A. Sher.** 1996. Functional diversity of helper T lymphocytes. Nature **383:**787–793.
2. **Afonso, L. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. The adjuvant effect of interleukin-12 in a vaccine against

Leishmania major. Science **263:**235–237.

3. **An, M. R., C.-C. Hsieh, P. D. Reisner, J. P. Rabek, S. G. Scott, D. T. Kuninger, and J. Papaconstantinou.** 1996. Evidence for posttranscriptional regulation of C/EBPα and C/EBPβ isoform expression during the lipopolysaccharide-mediated acute-phase response. Mol. Cell. Biol. **16:**2295–2306.
4. **Betts, J. C., J. K. Cheshire, S. Akira, T. Kishimoto, and P. Woo.** 1993. The role of NF-κB and NF-IL6 transactivating factors in the synergistic activation of human serum amyloid A gene expression by interleukin-1 and interleukin-6. J. Biol. Chem. **268:**25624–25631.
5. **Cao, Z. D., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of 3 C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. **5:**1538–1552.
6. **Chehimi, J., S. E. Starr, I. Frank, A. D'Andrea, X. Ma, R. R. MacGregor, J. Sennelier, and G. Trinchieri.** 1994. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. J. Exp. Med. **179:**1361–1366.
7. **Chougnet, C., T. A. Wynn, M. Clerici, A. L. Landay, H. A. Kessler, J. Rusnak, G. P. Melcher, A. Sher, and G. M. Shearer.** 1996. Molecular analysis of decreased interleukin-12 production in persons infected with human immunodeficiency virus. J. Infect. Dis. **174:**46–53.
8. **Clarkson, R. W., C. M. Chen, S. Harrison, C. Wells, G. E. Muscat, and M. J. Waters.** 1995. Early responses of trans-activating factors to growth hormone in preadipocytes: differential regulation of CCAAT enhancer-binding protein-beta (C/EBPβ) and C/EBPδ Mol. Endocrinol. **9:**108–120.
9. **Clerici, M., D. R. Lucey, J. A. Berzofsky, L. A. Pinto, T. A. Wynn, S. P. Blatt, M. J. Dolan, C. W. Hendrix, S. F. Wolf, and G. M. Shearer.** 1993. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. Science **262:**1721–1724.
10. **D'Andrea, A., M. Rengaraju, N. M. Valiante, J. Chehimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young, E. Nickbarg, et al.** 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. J. Exp. Med. **176:**1387–1398.
11. **Descombes, P., and U. Schibler.** 1991. A liver-enriched transcriptional activator protein, Lap, and a transcriptional inhibitory protein, Lip, are translated from the same messenger RNA. Cell **67:**569–579.
12. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
13. **Frankenberger, M., A. Pforte, T. Sternsdorf, B. Passlick, P. A. Baeuerle, and H. W. Ziegler-Heitbrock.** 1994. Constitutive nuclear NF-κB in cells of the monocyte lineage. Biochem. J. **304:**87–94.
14. **Gazzinelli, R. T., M. Wysocka, S. Hayashi, E. Y. Denkers, S. Hieny, P. Caspar, G. Trinchieri, and A. Sher.** 1994. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. J. Immunol. **153:**2533–2543.
15. **Godambe, S. A., D. D. Chaplin, T. Takova, and C. J. Bellone.** 1994. An NFIL-6 sequence near the transcriptional initiation site is necessary for the lipopolysaccharide induction of murine interleukin-1β. DNA Cell Biol. **13:**561–569.
16. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-κB. Nature **339:**70–73.
17. **Grove, M., and M. Plumb.** 1993. C/EBP, NF-κB, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1α immediate-early gene. Mol. Cell. Biol. **13:**5276–5289.
18. **Gubler, U., A. O. Chua, D. S. Schoenhaut, C. M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A. G. Wolitzky, P. M. Quinn, P. C. Familletti, and M. K. Gately.** 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc. Natl. Acad. Sci. USA **88:**4143–4147.
19. **Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P. A. Baeuerle.** 1993. Rapid proteolysis of IκB-α is necessary for activation of transcription factor NF-κB. Nature **365:**182–185.
20. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
21. **Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science **260:**547–549.
22. **Hunter, C. A., R. Chizzonite, and J. S. Remington.** 1995. IL-1β is required for IL-12 to induce production of IFN-γ by NK cells. A role for IL-1β in the T cell-independent mechanism of resistance against intracellular pathogens. J. Immunol. **155:**4347–4354.
23. **Kerr, L. D., L. J. Ransone, P. Wamsley, M. J. Schmitt, T. G. Boyer, Q. Zhou, A. J. Berk, and I. M. Verma.** 1993. Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-κB. Nature **365:**412–419.
24. **Kubin, M., J. M. Chow, and G. Trinchieri.** 1994. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor alpha, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. Blood **83:**1847–1855.
25. **LeClair, K. P., M. A. Blanar, and P. A. Sharp.** 1992. The p50 subunit of NF-κB associates with the NF-IL6 transcription factor. Proc. Natl. Acad. Sci. USA **89:**8145–8149.
26. **Li, Y. S., K. Hayakawa, and R. R. Hardy.** 1993. The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. J. Exp. Med. **178:**951–960.
27. **Lo, K., N. R. Landau, and S. T. Smale.** 1991. LyF-1, a transcriptional regulator that interacts with a novel class of promoters for lymphocyte-specific genes. Mol. Cell. Biol. **11:**5229–5243.
28. **Lowenstein, C. J., E. W. Alley, P. Raval, A. M. Snowman, S. H. Snyder, S. W. Russell, and W. J. Murphy.** 1993. Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. Proc. Natl. Acad. Sci. USA **90:**9730–9734.
29. **Ma, X., J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzialo, and G. Trinchieri.** 1996. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. J. Exp. Med. **183:**147–157.
30. **Matsusaka, T., K. Fujikawa, Y. Nishio, N. Mukaida, K. Matsushima, T. Kishimoto, and S. Akira.** 1993. Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc. Natl. Acad. Sci. USA **90:**10193–10197.
31. **McDowell, M. A., D. M. Lucas, C. M. Nicolet, and D. M. Paulnock.** 1995. Differential utilization of IFN-γ-responsive elements in two maturationally distinct macrophage cell lines. J. Immunol. **155:**4933–4938.
32. **Metz, R., and E. Ziff.** 1991. cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to *trans*-locate to the nucleus and induce c-*fos* transcription. Genes Dev. **5:**1754–1766.
33. **Miller, L., E. W. Alley, W. J. Murphy, S. W. Russell, and J. S. Hunt.** 1996. Progesterone inhibits inducible nitric oxide synthase gene expression and nitric oxide production in murine macrophages. J. Leukocyte Biol. **59:**442–450.
34. **Mossman, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. **7:**145–173.
35. **Murphy, T. L., M. G. Cleveland, P. Kulesza, J. Magram, and K. M. Murphy.** 1995. Regulation of interleukin 12 p40 expression through an NF-κB half-site. Mol. Cell. Biol. **15:**5258–5267.
36. **Nerlov, C., and E. B. Ziff.** 1995. CCAAT/enhancer binding protein-alpha amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cells. EMBO J. **14:**4318–4328.
37. **Nicolet, C. M., and D. M. Paulnock.** 1994. Promoter analysis of an interferon-inducible gene associated with macrophage activation. J. Immunol. **152:**153–162.
38. **Nordeen, S. K.** 1988. Luciferase reporter gene vectors for analysis of promoters and enhancers. BioTechniques **6:**454–458.
39. **Ossipow, V., P. Descombes, and U. Schibler.** 1993. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc. Natl. Acad. Sci. USA **90:**8219–8223.
40. **Pierce, J. W., M. Lenardo, and D. Baltimore.** 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. Proc. Natl. Acad. Sci. USA **85:**1482–1486.
41. **Pirmez, C., M. Yamamura, K. Uyemura, M. Paes-Oliveira, F. Conceicao-Silva, and R. L. Modlin.** 1993. Cytokine patterns in the pathogenesis of human leishmaniasis. J. Clin. Invest. **91:**1390–1395.
42. **Pope, R. M., A. Leutz, and S. A. Ness.** 1994. C/EBPβ regulation of the tumor necrosis factor alpha gene. J. Clin. Invest. **94:**1449–1455.
43. **Raj, N. B., and P. M. Pitha.** 1981. Analysis of interferon mRNA in human fibroblast cells induced to produce interferon. Proc. Natl. Acad. Sci. USA **78:**7426–7430.
44. **Ramji, D. P., A. Vitelli, F. Tronche, R. Cortese, and G. Ciliberto.** 1993. The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBPδ/NF-IL6β, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. Nucleic Acids Res. **21:**289–294.
45. **Schmitz, M. L., G. Stelzer, H. Altmann, M. Meisterernst, and P. A. Baeuerle.** 1995. Interaction of the COOH-terminal transactivation domain of p65 NF-κB with TATA-binding protein, transcription factor IIB, and coactivators. J. Biol. Chem. **270:**7219–7226.
46. **Schoenhaut, D. S., A. O. Chua, A. G. Wolitzky, P. M. Quinn, C. M. Dwyer, W. McComas, P. C. Familletti, M. K. Gately, and U. Gubler.** 1992. Cloning and expression of murine IL-12. J. Immunol. **148:**3433–3440.
47. **Scott, L. M., C. I. Civin, P. Rorth, and A. D. Friedman.** 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood **80:**1725–1735.
48. **Screpanti, I., L. Romani, P. Musiani, A. Modesti, E. Fattori, D. Lazzaro, C. Sellitto, S. Scarpa, D. Bellavia, G. Lattanzio, et al.** 1995. Lymphoproliferative disorder and imbalanced T-helper response in C/EBPβ-deficient mice. EMBO J. **14:**1932–1941. (Erratum, **14:**3596.)
49. **Seder, R. A., B. L. Kelsall, and D. Jankovic.** 1996. Differential roles for IL-12 in the maintenance of immune responses in infectious versus autoimmune disease. J. Immunol. **157:**2745–2748.
50. **Sieling, P. A., and R. L. Modlin.** 1994. Cytokine patterns at the site of mycobacterial infection. Immunobiology **191:**378–387.

51. **Skeen, M. J., M. A. Miller, T. M. Shinnick, and H. K. Ziegler.** 1996. Regulation of murine macrophage IL-12 production. Activation of macrophages in vivo, restimulation in vitro, and modulation by other cytokines. J. Immunol. **156:**1196–1206.
52. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57:**103–113.
53. **Snidjers, A., C. M. Hilkens, T. C. van der Pouw Kraan, M. Engel, L. A. Aarden, and M. L. Kapsenberg.** 1996. Regulation of bioactive IL-12 production in lipopolysaccharide-stimulated human monocytes is determined by the expression of the p35 subunit. J. Immunol. **156:**1207–1212.
54. **Stacey, K. J., I. L. Ross, and D. A. Hume.** 1993. Electroporation and DNA-dependent cell death in murine macrophages. Immunol. Cell Biol. **71:**75–85.
55. **Stein, B., and A. S. Baldwin, Jr.** 1993. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF-κB. Mol. Cell. Biol. **13:**7191–7198.
56. **Stein, B., P. C. Cogswell, and A. S. Baldwin, Jr.** 1993. Functional and physical associations between NF-κB and C/EBP family members: a Rel domain-bZIP interaction. Mol. Cell. Biol. **13:**3964–3974.
57. **Tanaka, T., S. Akira, K. Yoshida, M. Umemoto, Y. Yoneda, N. Shirafuji, H. Fujiwara, S. Suematsu, N. Yoshida, and T. Kishimoto.** 1995. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. Cell **80:**353–361.
58. **Trautwein, C., C. Caelles, P. van der Geer, T. Hunter, M. Karin, and M. Chojkier.** 1993. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature **364:**544–547.
59. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **13:**251–276.
60. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. Reversal by IFN-γ. J. Immunol. **152:**1883–1887.
61. **Vinson, C. R., P. B. Sigler, and S. L. McKnight.** 1989. Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science **246:**911–916.
62. **Wedel, A., and H. W. Ziegler-Heitbrock.** 1995. The C/EBP family of transcription factors. Immunobiology **193:**171–185.
63. **Wolf, S. F., P. A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, K. Kelleher, S. H. Herrmann, S. C. Clark, L. Azzoni, S. H. Chan, G. Trinchieri, and B. Perussia.** 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J. Immunol. **146:**3074–3081.
64. **Yin, M., S. Q. Yang, H. Z. Lin, M. D. Lane, S. Chatterjee, and A. M. Diehl.** 1996. Tumor necrosis factor alpha promotes nuclear localization of cytokine-inducible CCAAT/enhancer binding protein isoforms in hepatocytes. J. Biol. Chem. **271:**17974–17978.
65. **Zhang, M., M. K. Gately, E. Wang, J. Gong, S. F. Wolf, S. Lu, R. L. Modlin, and P. F. Barnes.** 1994. Interleukin 12 at the site of disease in tuberculosis. J. Clin. Invest. **93:**1733–1739.
66. **Zubiaga, A. M., E. Munoz, and B. T. Huber.** 1991. Superinduction of IL-2 gene transcription in the presence of cycloheximide. J. Immunol. **146:**3857–3863.