Characterization of an Activation Protein-1-binding Site in the Murine Interleukin-12 p40 Promoter

DEMONSTRATION OF NOVEL FUNCTIONAL ELEMENTS BY A REDUCTIONIST APPROACH*

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Chen Zhu‡, Khatuna Gagnidze‡, James H. M. Gemberling§, and Scott E. Plevy‡‡

From the ‡Immunobiology Center, The Mount Sinai School of Medicine, New York, New York 10029-6574 and the §Howard Hughes Medical Institute, UCLA School of Medicine, Los Angeles, California 90095-1662

Interleukin (IL)-12 is a heterodimeric cytokine produced by macrophages and dendritic cells. Among its biologic activities, IL-12 production is necessary for the differentiation of T-helper-1 (Th1) cells and for the secretion of the Th1 cytokine, interferon-γ (IFN-γ) (1). Accordingly, IL-12 and Th1 cells are required for cell-mediated immunity against intracellular microbes (2–4). The protective role of IL-12 in human infectious diseases including leprosy (5), tuberculosis (6), and leishmaniasis (7) has been well characterized. Conversely, overexpression of Th1 cytokines and IL-12 may contribute to the development of chronic inflammatory disorders (8), including Crohn’s disease and rheumatoid arthritis. Thus, the regulated expression of IL-12 in antigen-presenting cells is a critical event in the pathogenesis of infectious and inflammatory diseases.

IL-12 is composed of two covalently linked glycosylated chains, p40 and p35, that are encoded by separate genes and together form the biologically active p70 heterodimer (9–11). The p35 gene is constitutively expressed in many tissue types (9). p40 mRNA is detected in macrophages and other cells that produce bioactive IL-12 (9) and is strongly induced by intracellular bacteria and bacterial products (12). Therefore, studies of IL-12 transcriptional regulation have focused on the p40 gene. Murphy and colleagues (13) identified an NF-κB site between −122 and −132 relative to the transcription start site in the murine p40 gene that was necessary for induction of promoter activity by LPS. In another series of investigations, Ma and colleagues (14–16) have identified an Ets protein DNA-binding sequence between −212 and −207 in the human p40 gene that was implicated in promoter activation by LPS. We have reported a comprehensive functional analysis of the murine and human IL-12 p40 promoters in RAW264.7 cells (17). The p40 promoter was strongly induced by heat-killed Listeria monocytogenes and LPS. An important control element defined by this analysis, located between −96 and −88 relative to the murine transcription start site, interacts with C/EBP proteins. This element was functionally synergistic with the NF-κB site. However, mutations in several other elements had functional effects on promoter activation. Thus, induction of the IL-12 p40 promoter by bacterial products will be defined by a complex series of events.

In this study, a functional role for three novel cis-acting elements in the p40 promoter is demonstrated in reporter assays by utilizing a combination of multiple substitution and deletion mutants. One of these elements is characterized in detail. Mutations from −79 to −74 in the murine IL-12 p40 promoter significantly reduce lipopolysaccharide-induced promoter activity. Electrophoretic mobility shift assays demonstrate binding of AP-1 family members to this region. Spacing between the C/EBP and AP-1 site is important for promoter activation, suggesting cooperativity between these elements. c-Jun and a mutant c-Jun molecule activate the IL-12 p40 promoter and synergistically activate the promoter when co-expressed with C/EBPβ. Finally, this region of the promoter is demonstrated to be a target for mitogen-activated protein kinase and toll-like receptor signaling pathways.

IL-12β1 is a heterodimeric cytokine produced by macrophages and dendritic cells. Among its biologic activities, IL-12 production is necessary for the differentiation of T-helper-1 (Th1) cells and for the secretion of the Th1 cytokine, interferon-γ (IFN-γ) (1). Accordingly, IL-12 and Th1 cells are required for cell-mediated immunity against intracellular microbes (2–4). The
wise, c-Jun and TAM67 activate the tumor necrosis factor-α (TNF) promoter in RAW264.7 cells. Finally, experiments using inhibitors and activators of signal transduction pathways identify the region of the promoter from −101 to +55 as a target for the p38 kinase, the N-terminal Jun kinase (JNK), and the toll-like receptor (TLR) signal transduction pathways.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The murine wild type IL-12 p40 promoter −350 to +55 region was inserted into the chloramphenicol acetyltransferase (CAT) reporter vector pCAT basic (Promega) modified by addition of KpnI and BglII sites in the polymerase chain reaction (PCR) as described previously (17). All site-directed mutant plasmids described in this paper were generated by two-step polymerase chain reaction using overlapping internal primers containing a mutant site (18). Polymerase chain reaction-generated mutant promoters were cloned into pCAT basic vector (Promega) with a modified polylinker or pGL2B vector (Promega) and sequenced before use in transfection experiments. Mammalian expression plasmids for human wild type c-Jun, c-Fos, JunB (Tom Curran), TAM67 (Michael Birrer), and c/EBPβ (from Steven McKnight) were described previously (17, 19). An expression plasmid for the AP-1 dominant negative protein A-Fos was obtained from Charles Vinson (see Ref. 20). The following plasmids were utilized for signal transduction experiments: constitutively active mouse c-Jun (Roger Davis) (21); constitutively active MKK6, MKK6E (Jiahuai Han) (22); JIP-1 (23); constitutively active MEKK1 (367MEKK1/Dennis Teleman) (24); dominant negative TLR2 (Paul Godowski) (25); constitutively active CD4-TLR4 fusion (26); and dominant negative MyD88, dominant negative TRAF6 (Ruslan Medzhitov) (27). As MAP kinase pathway controls, MEF2e-GALA was obtained from Jiahuai Han. pFA-Jun, and pFR-luciferase were from Stratagene. A multimerized NF-κB element luciferase reporter plasmid and a human TNF-α promoter (for CAT assays) or 20 μg of total protein (for CAT assays) or 20 μl (luciferase assays) from cell extracts, as described previously (17). Cells were harvested by using 1× Reporter Lysis Buffer (Promega). CAT assays were performed with 75 μg of total protein from cell extracts.

**RESULTS**

**Demonstration of Novel Functional cis-acting Control Elements in the IL-12 p40 Promoter**—In our previous analysis of the murine and human IL-12 p40 promoters, the c/EBP element spanning −93 to −88, the NF-κB element at −131 to −120, and the TATA box were the only sites convincingly shown to be important for p40 promoter function (17). However, two results suggested that other functionally important elements exist within the promoter. First, deletion or substitution mutations at other locations appeared to have less significant effects on promoter activity. Second, 25% of the wild type promoter activity was retained following disruption of the NF-κB element. This activity appeared to be too strong to be directed solely by a C/EBP-binding site and a TATA box. In particular, of the 21 clustered substitution mutants analyzed in this previous study, four (−114/−109, −107/−102, −80/−75, and −62/−57) were identified that demonstrated a 2–3-fold decrease in promoter activation (17). These elements, which have relatively minor effects individually, may have additive or synergistic effects when combined with mutations in other sites.

As mutations in the C/EBP site abrogate promoter activity, the functional significance of other potentially important sites (−114/−109, −107/−102, −80/−75, −62/−57) needs to be evaluated in the presence of an intact C/EBP site. The −114/−109, −107/−102, and −62/−57 substitution mutants were described previously (17). However, the −80/−75 mutant from the initial study was re-derived by changing a 13-base pair sequence from −85 to −73. This more extensive mutant may demonstrate more significant functional effects. Furthermore, as random mutants may also have artifactual effects, a different random sequence was inserted to rule out this possibility.

Site-directed mutants in two elements other than the C/EBP site were constructed in the −355 to +55 wild type p40 promoter-CAT reporter (Fig. 1A). The murine macrophage cell line RAW264.7 was transiently transfected with double mutant, single mutant, and wild type plasmids. Consistent with our previously reported results, a mutation in the NF-κB element (Fig. 1B, −132/−127) reduces LPS-induced promoter activation by around 80%, and a mutation in the C/EBP site (Fig. 1B, −93/−88) virtually eliminates promoter activity. Simultaneous disruption of the NF-κB element and the sequence from −85 to −73 markedly reduces promoter activity by LPS compared with the respective single mutations (Fig. 1B; compare the double mutant [−85/−73 and −132/−127] to the single mutant [−85/−73 and the single mutant −132/−127]). Three other mutations (−114/−109, −107/−102, and −62/−57), when combined with mutations in the NF-κB site, also demonstrate significantly diminished p40 promoter activity (Fig. 1B). A double mutant in the −114/−109 and −107/−102 elements does not attenuate promoter activation to a greater extent than the respective single mutants (Fig. 1B). Therefore, one control element likely resides within these two adjacent sequences. In summary, although each of these single mutations had a relatively small effect on promoter activation, their combination with a mutation in the NF-κB site demonstrates dramatic decreases in LPS-induced promoter activity. These results identify three novel elements, from −114 to −102, −82 to −73, and −62 to −57, important for activation of IL-12 p40 transcription by LPS, and provide clear evidence that an intact C/EBP site is not sufficient for promoter activation.

As a second approach, a minimal inducible IL-12 p40 pro-
NF-κB promoter showing the two most important regulatory domains, the elements other than the C/EBP site. The IL-12 p40 promoter are demonstrated by mutation of two induced promoter activity, compared with the respective single mutant B, directed mutations in potentially important elements (B16–18 h. CAT activity was quantitated following thin layer chromatography.

Deletion of all elements upstream of the C/EBP site mediated promoter was defined. From our previous analysis, a promoter deletion of all elements upstream of the C/EBP site mediate strong LPS-inducible promoter activity of greater than 10% of the wild type −355 to +55 promoter (17). Therefore, functional control elements likely exist downstream of the C/EBP element. To test this hypothesis, site-directed mutants were created between −101 to +55 containing 5′ elements beginning at the C/EBP site at position −101 relative to the transcription start site. Within this minimal promoter, 6–10-base pair substitution mutants were created between −93 and −51. The plasmids were transfected into RAW264.7 cells, incubated for 24 h, and then activated with LPS (5 µg/ml) for 16–18 h. Extracts from LPS-activated cells were analyzed by CAT assay. Results (normalized for β-galactosidase activity) are expressed as the percentage of the CAT activity obtained with the −101 to +55 plasmid. Plasmid −101 to +55 and −93/−88 represent a mutation in the C/EBP element. Two mutations in plasmid −101 to +55 (−82/−74 and −62/−57) had significant effects on LPS-induced promoter activity, whereas mutants in the flanking sequences retain wild type activity (Fig. 3B).

Thus, an important regulatory element is localized to the region spanning −79 to −74.

**FIG. 2.** Functionally redundant cis-acting control elements are identified downstream of the C/EBP site in the IL-12 p40 promoter using a minimal LPS-inducible promoter. The p40 promoter-CAT reporter plasmid −101 to +55 contains 5′ elements beginning at the C/EBP site at position −101 relative to the transcription start site. Within this minimal promoter, 6–10-base pair substitution mutants were created between −93 and −51. The plasmids were transfected into RAW264.7 cells, incubated for 24 h, and then activated with LPS (5 µg/ml) for 16–18 h. Extracts from LPS-activated cells were analyzed by CAT assay. Results (normalized for β-galactosidase activity) are expressed as the percentage of the CAT activity obtained with the −101 to +55 plasmid. Plasmid −101 to +55 and −93/−88 represent a mutation in the C/EBP element. Two mutations in plasmid −101 to +55 (−82/−74 and −62/−57) had significant effects on LPS-induced promoter activity. Each result represents the mean ± S.D. of data from four to five experiments.

**FIG. 1.** Functionally redundant cis-acting control elements in the IL-12 p40 promoter are demonstrated by mutation of two elements other than the C/EBP site. A, diagram of the IL-12 p40 promoter showing the two most important regulatory domains, the NF-κB (−132/−127) and C/EBP (−93/−88) elements, and other potentially important regions detected in a previous analysis (17). B, site-directed mutations in potentially important elements (−114/−109, −107/−102, −85/−73, −62/−57) when combined with a mutation in the NF-κB site (−132/−127) demonstrated significant diminution of LPS-induced promoter activity, compared with the respective single mutant promoters (compare mutant −85/−73 and −132/−127 with mutant −85/−73; mutant −114/−109 and −132/−127 with −114/−109; mutant −107/−102 and −132/−127 with −107/−102; mutant −62/−57 and −132/−127 with −62/−57). The single mutant in the NF-κB site (−132/−127), the single mutant in the C/EBP site (−93/−88), and wild type promoter (−350 to +55 p40-CAT) activities are represented at the top of the figure. Two µg of each single and double mutant promoter CAT reporter plasmid and 0.5 µg of HSP-β-galactosidase reporter plasmid were transfected into RAW264.7 macrophage cells. Transfected cells were incubated for 24 h and then activated with LPS (5 µg/ml) for 16–18 h. CAT activity was quantitated following thin layer chromatography by PhosphorImager analysis, and results were expressed as percent conversion of [14C]chloramphenicol to acetylated substrates, normalized to β-galactosidase activity obtained from a co-transfected control reporter plasmid. Each result represents the mean ± S.D. of data from three to five experiments.

### Detailed Characterization of a Functional Element within the −82 to −73 Region

**By two different approaches, the sequence between −82 and −73 is demonstrated to have an important functional role in IL-12 p40 promoter activation by LPS. This control element may contain a binding site for one transcription factor or may represent a composite binding site for two or more proteins. To define better critical nucleotides within this element, 3-base pair site-directed mutants were constructed within this sequence (Fig. 3A). Two mutants, −79/−77 and −76/−74, demonstrate significantly decreased LPS-induced promoter activity, whereas mutants in the flanking sequences retain wild type activity (Fig. 3B).**

Thus, an important regulatory element is localized to the region spanning −79 to −74.

**Determination of DNA-Protein Interactions in the −79 to −74 Region: Identification of an AP-1-binding Element**

To identify DNA-protein interactions in the −79 to −74 region, EMSA probes were constructed that spanned 1) the C/EBP-binding site and −79/−74 (118/69), 2) the C/EBP site alone (112/78), and 3) −79/−74 excluding the C/EBP site (88/63) (Fig. 4A). With the 88/63 probe, a slow mobility DNA-protein complex is detected that is strongly induced in nuclear extracts from RAW264.7 cells activated with LPS (compare Fig. 4B, lanes 9 and 10, upper arrow). This complex is present in EMSAs with probe 118/69 (Fig. 4B, lanes 1–4), containing the C/EBP site and downstream sequences, but not with probe 112/78 (Fig. 4B, lanes 5–8) that spans only the C/EBP site.

The DNA sequence from −81 to −73, CTAGTCAGT, has homology to a canonical AP-1 DNA-binding element (30). The fos/jun proto-oncogenes encode proteins that are major components of the AP-1 transcription factor complex. Several Fos-
related (c-Fos, FosB, Fra-1, and Fra-2) and Jun-related (c-Jun, JunB, and JunD) proteins have been described. These leucine zipper proteins form the transcriptionally active AP-1 complex as Fos/Jun heterodimers or Jun/Jun homodimers (31, 32). Competition with an unlabeled oligonucleotide containing a consensus AP-1-binding site in 100-fold molar excess completely inhibits formation of this complex in EMSAs, strongly suggesting that AP-1 family members interact with this sequence in the IL-12 p40 promoter (Fig. 4B, lanes 4 and 12). A consensus C/EBP-binding site partially competes AP-1 DNA binding activity in gel shift experiments, suggesting that either C/EBP family members are present in this complex or that AP-1 dimers may to some extent bind to a consensus C/EBP element (Fig. 4B, lanes 3 and 11). Competition with a consensus NF-κB DNA-binding site oligonucleotide does not inhibit complex formation on all three radiolabeled probes (data not shown). Mutants within the 88/63 EMSA probe, 79/77m (mutated from −79 to −77) and 76/74m (mutated from −76 to −74) eliminate binding of the AP-1 complex (Fig. 5, lanes 3–6). These specific mutants correlate with decreased promoter activity in functional assays (Fig. 3B).

The presence of AP-1 family members in this DNA-binding complex was confirmed by supershift experiments. A polyclonal antibody to the AP-1 family Fos (Fig. 6, lanes 5 and 15, K-25) strongly inhibits and supershifting the DNA-protein complex. By using antibodies that recognize specific Fos family members, it appears that the most abundant Fos DNA binding activity is attributable to c-Fos (Fig. 6, lanes 6 and 16). Antibodies to Fra-1 and Fra-2 cause minor inhibition of complex formation (Fig. 6, lane 8, 9, 18, and 19). An antibody that recognizes the Jun family member, JunB, strongly inhibits and supershifts the DNA-binding complex (Fig. 6, lanes 3 and 13). Additionally, an antibody to c-Jun causes inhibition and supershift of the complex (Fig. 6, lanes 2 and 12). Antibodies directed against the leucine zipper proteins ATF-1, ATF-2, and C/EBPβ demonstrate little, if any, inhibition of complex formation in nuclear extracts from LPS-treated cells (data not shown). Likewise, antibodies against unrelated transcription factors Pu.1 (Fig. 6, lanes 10 and 20), NF-κB p50, NF-κB p65, and the glucocorticoid receptor (data not shown) do not supershift or inhibit the DNA-binding complex. Thus, the AP-1 DNA-binding complex on the IL-12 p40 promoter is composed of JunB and c-Jun homodimers and/or heterodimers with c-Fos. However, other AP-1 family members and leucine zipper proteins may be present as well.

**Determination of Spacing Requirements between Control Elements in the IL-12 p40 Promoter**—Proper spacing of cis-acting elements may be necessary for the interaction of transcriptional activators with the basal transcription machinery, or it may be required for cooperative binding of transcription factors (33, 34). Spacing restrictions may involve distance or alignment of sites on the DNA double helix. The functional effects of spacing between the TATA box and regulatory elements as well as between control elements within the IL-12 p40 promoter were next determined. Insertion mutants were constructed within the −355 to +55 p40 promoter that increase the distance between the AP-1 and the C/EBP site by 5, 10, and 15 bp,
and 5 and 10 bp between the C/EBP site and the TATA box were created to determine spacing requirements between C/EBP, NF-κB, and the basal transcription machinery. All mutations were inserted within sequences that were not critical for promoter activation (∼45/∼40 for mutants between TATA and C/EBP site, ∼121/117 for mutants between NF-κB site and C/EBP site, and ∼84/∼85 for C/EBP and AP-1 elements) (17). These mutants were tested by transient transfection in unactivated and LPS-activated RAW264.7 cells. Spacing mutations between the TATA box and C/EBP site have little effect on promoter activity (>80% of wild type activity; data not shown). However, mutations between the NF-κB and C/EBP sites, and the C/EBP and AP-1 elements reveal significant effects on promoter induction. A 10-bp insertion between the NF-κB and C/EBP elements decreases LPS-induced promoter activation by 65% (Fig. 7B). A 10-bp insertion between the C/EBP and AP-1 sites diminishes LPS-induced promoter activation by one-third, whereas a 5- and 15-bp insertion decreases activation by 50% (Fig. 7B). As random sequences inserted within the promoter may fortuitously create a protein-binding site, the 5- and 10-bp mutants between the C/EBP and AP-1 elements were re-derived with different sequences, and similar results were demonstrated in reporter assays (data not shown).

As the effect of spacing between the C/EBP and AP-1 sites may have been minimized by redundant control elements, insertion mutants were then constructed within the −101 to +55 minimal promoter. In this context, the effect of 5-, 10-, and 15-bp insertions are more dramatic, reducing promoter activity more than 60% (Fig. 7C).

Based on comparison to consensus sequences, there are two potential AP-1-binding sites within the −79 to −74 region (Fig. 3). To localize better the AP-1 DNA-binding element, and to determine whether the sequence and orientation of this site in the IL-12 p40 promoter influences activity, additional mutants were created. Within each of these two potential AP-1 sites, a canonical AP-1-binding site was created, and the orientation of the AP-1 site was reversed. From −79 to −73, a canonical AP-1-binding site was created by changing the sequence AGTCGAGT to ACTGAGTG. This mutation decreases LPS-induced promoter activation by almost 75% (data not shown). A second mutant reversed the orientation from −79 to −73, by changing AGTCGAGT to TGACTGA. This mutation also diminishes LPS-induced promoter activity by 75% (data not shown). From −81 to −75, a canonical AP-1 site was constructed by changing the sequence CTAGTCAGT to TGAGTCA. When transfected into RAW264.7 cells, this mutation strikingly increases LPS-induced promoter activation to almost 6 times wild type activity (Fig. 7D). Significantly, this mutant demonstrates a strong basal signal (20% of LPS-induced wild type activity) in unactivated cells (Fig. 7D). Next, the IL-12 p40 AP-1 site at −81 to −75 was reversed in orientation from CTAGTCAGT to TGAGTCA. This mutation reduces LPS-induced promoter activity by 5-fold (Fig. 7D). An EMSA probe was created containing this consensus AP-1 sequence from −81 to −75 in the p40 promoter. Compared with the wild type probe 88/63 (see Fig. 4), AP-1 DNA binding activity is markedly increased in unactivated and LPS-treated nuclear extracts from RAW264.7 cells (data not shown). Supershift experiments suggest that DNA binding activities on this mutant probe are attributable to JunB, c-Fos, and c-Jun (data not shown), as demonstrated for the wild type probe. The most plausible interpretation of these results is that the IL-12 p40 AP-1 site lies within the −81 to −75 region. Hence, the consensus AP-1 site created from −79 to −73 would actually weaken the natural AP-1 element at −81 to −75. Also, orientation of this AP-1 site is important for optimal promoter

approximating one-half, one, and one and a half helical turns of DNA, respectively (Fig. 7A). In addition, insertion mutants adding 5 and 10 bp between the C/EBP site and NF-κB element

FIG. 5. AP-1 DNA binding activity is eliminated by mutations that decrease IL-12 p40 promoter activity by LPS in reporter assays. Two mutant EMSA probes (79/77m and 76/74m) were created within the wild type probe 88/63. 32P-Labeled wild type probe 88/63 (lanes 1 and 2), mutant probe 79/77m (lanes 3 and 4), and mutant probe 76/74m (lanes 5 and 6) were incubated with 0.5 μg poly(dI-dC) and 5 μg of nuclear extracts from untreated (lanes 1, 3, and 5) and LPS-activated (lanes 2, 4, and 6) RAW264.7 cells for 30 min prior to electrophoresis. Both mutant probes, which correlate with the decreased promoter activity in CAT assays (see Fig. 3B), do not demonstrate AP-1 DNA binding activity (arrow on left).

FIG. 6. Identification of specific AP-1 family members in the IL-12 p40 DNA-binding complex by supershift assay. 32P-Labeled EMSA probe 88/63 was incubated with 0.5 μg poly(dI-dC), and 5 μg of nuclear extracts from untreated (lanes 1–10) or LPS-treated (lanes 11–20) RAW264.7 cells at room temperature for 30 min. Then 1.5 μl of polyclonal antibodies against c-Jun (lanes 2 and 12), JunB (lanes 3 and 13), JunD (lanes 4 and 14), c-Fos (K-25, lanes 5 and 15), c-Fos (H-125, lanes 6 and 16), Fra1 (lanes 7 and 17), Fra1 (lanes 8 and 18), Fra2 (lanes 9 and 19), and Fu.1 (lanes 10 and 20) were added. Results demonstrate that the IL-12 p40 DNA-binding complex contains Fos family members, JunB and c-Jun, as demonstrated by inhibition and supershift of DNA binding activity. However, other basic ZIP proteins are likely present in smaller amounts, as demonstrated by inhibition of the DNA-binding complex by other antibodies but not by antibodies against non-basic ZIP proteins (lanes 10 and 20, and data not shown). The arrow to the left of the figure denotes the location of the AP-1 DNA-binding complex.
Expression of a dominant negative c-Fos molecule inhibits IL-12 p40 promoter activation by LPS and C/EBPβ. The −101 to +55 p40 minimal promoter-CAT reporter plasmid was co-transfected with an expression plasmid for A-Fos at different concentrations (0, 0.1, 0.25, 0.5, and 0.75 μg). Plasmids were transfected into RAW264.7 cells, incubated for 24 h, and then activated with LPS (5 μg/ml) for 16–18 h. Extracts from LPS-activated cells were analyzed by CAT assay (black bars). In a parallel series of experiments, a constant amount of expression plasmid for the C/EBPβ (0.1 μg) was co-transfected with A-Fos (white bars). In these experiments, promoter activity was determined in untreated cells. Results (normalized for β-galactosidase activity) are expressed as the percentage of the CAT activity obtained with the −101 to +55 wild type promoter co-transfected with 0.1 μg of C/EBPβ expression plasmid (white bars). Results represent the mean ± S.D. of four to five experiments.

Expression of a Dominant Negative c-Fos Molecule Inhibits IL-12 p40 Promoter Activation by LPS—To demonstrate a functional role for AP-1 in IL-12 p40 promoter activity, a dominant negative AP-1 molecule, A-Fos (20), was transfected into RAW264.7 macrophages. A-Fos contains an acidic amphipathic protein sequence appended to the N terminus of the Fos leucine zipper and lacks a DNA binding domain (20). A-Fos inhibits LPS-induced −350 to +55 IL-12 p40 promoter activation in a dose-dependent manner, with maximal inhibition of ~50% (data not shown). A-Fos also inhibits activation of the p40 promoter by C/EBPβ by 50% (data not shown). In the context of the minimal −101 to +55 p40 promoter, the effects of A-Fos are more significant, with inhibition of LPS-induced promoter activation by 70% and inhibition of C/EBPβ-induced activity by greater than 50% (Fig. 8). These inhibitory effects are dependent on the AP-1 DNA-binding element, as LPS- and C/EBPβ-induced promoter activation, as reversal of the sequence of this element significantly diminishes promoter activity.

Fig. 7. Determination of spacing requirements between control elements in the IL-12 p40 promoter. A, 5-, 10-, and 15-base pair insertion mutants were created at −119 and −84 within the IL-12 p40 −350 to +55 promoter-CAT reporter plasmid. B, insertion mutant plasmids were transfected into RAW264.7 cells, incubated for 24 h, and then activated with LPS (5 μg/ml) for 16–18 h. Extracts from LPS-activated cells were analyzed by CAT assay. Results (normalized for β-galactosidase activity) are expressed as the percentage of the CAT activity obtained with the −350 to +55 wild type promoter. These results suggest that spacing between the C/EBP and NF-κB elements, as well as between the C/EBP and AP-1 sites, are important for promoter activation. Each result represents the mean ± S.D. of data from four to five experiments. C, spacing mutants between the C/EBP and AP-1 elements have more dramatic effects on promoter activation by LPS in the context of a minimal deleted promoter, −101 to +55. Results (normalized for β-galactosidase activity) were expressed as the percentage of the CAT activity obtained with the −101 to +55 plasmid. Each result represents the mean ± S.D. of data from three to five experiments. D, the specific sequence and orientation of the AP-1 site in the IL-12 p40 promoter is important for activation by LPS. Within the −350 to +55 p40 promoter-CAT reporter plasmid, the AP-1-binding site (CTAGTCA to TGAGTCA) was mutated to a canonical AP-1 site (CTAGGATC). This mutation strikingly increases LPS-induced promoter activation by 70% and demonstrates a strong basal signal (20% of LPS-induced wild type activity) in unactivated cells. Also, the IL-12 p40 AP-1 site at −81 to −75 was reversed in orientation from CTAGTCAG to ACTGACGTA. This mutation strikingly increases LPS-induced promoter activation to almost 6 times wild type activity (−350 to +55) and demonstrates a strong basal signal (20% of LPS-induced wild type activity) in unactivated cells. Each result represents the mean ± S.D. of data from three experiments.
induced activity of the −350 to +55 p40 promoter with a mutated AP-1 site (−85/−73) is not inhibited by A-Fos (data not shown). This result strongly suggests that A-Fos inhibits IL-12 p40 promoter activation by heterodimerizing with endogenous Jun family members but will not inhibit C/EBP-mediated transcriptional activation in the absence of an adjacent AP-1 element. Furthermore, the inhibitory effect of A-Fos does not represent a nonspecific phenomenon such as transcriptional squelching. A-Fos expression does not decrease the activity of two constitutively active promoters, a cytomegalovirus viral enhancer and an SP-1 multimer upstream of a minimal core promoter (data not shown).

c-Jun and a Mutant c-Jun Molecule Activate the IL-12 p40 Promoter and Synergize with C/EBPβ—To investigate a role for individual AP-1 proteins, mammalian expression plasmids for AP-1 family members were co-transfected with a −101 to +55 p40 promoter-luciferase reporter into RAW264.7 cells. Neither JunB nor c-Fos expression activate the promoter (Fig. 9A). However, expression of c-Jun strongly activates the promoter (Fig. 9A) in a dose-dependent manner (data not shown). Co-transfection of Jun family members with c-Fos does not augment promoter activity (data not shown). Within the TNF promoter, a functional interaction between a C/EBP element immediately adjacent to an AP-1 site from the promoter, a functional interaction between a C/EBP element has not been described (35). Expression of c-Jun and a mutant c-Jun molecule that lacks the C-terminal 67 amino acid transactivation domain, TAM67, were both capable of activating the TNF promoter (35). As TNF and IL-12 are critical mediators of inflammatory responses, we tested whether TAM67 activates the TNF and IL-12 p40 promoters in macrophages. Expression of TAM67 in RAW264.7 cells activates the −101 to +55 IL-12 p40 promoter to the same extent as c-Jun (Fig. 9A). Furthermore, TAM67 activates a −283 to +113 TNF promoter luciferase reporter in RAW264.7 cells (Fig. 9B). The effect of TAM67 on the TNF and IL-12 p40 promoters appears to be specific; c-Jun, but not TAM67, activates a multimerized consensus AP-1 DNA-binding element CAT reporter plasmid (TRE-CAT) in untreated RAW264.7 cells (data not shown). Furthermore, TAM67, originally described as a dominant negative inhibitor of AP-1-mediated transcription (19), strongly inhibits LPS-induced TRE-CAT activity (85%), whereas it augments LPS-induced IL-12 p40 promoter activity by 2-fold in RAW264.7 cells (data not shown).

To assess functionally additive or synergistic effects between C/EBP and AP-1 in IL-12 p40 promoter activation, C/EBPβ was co-expressed with AP-1 family members in RAW264.7 cells. Expression of C/EBPβ with c-Jun results in 8-fold greater promoter activation than with C/EBPβ alone and 4-fold greater promoter activity than with c-Jun alone (Fig. 9C). Similar results are demonstrated with TAM67 (Fig. 9C). However, synergistic activation is not apparent when JunB and C/EBPβ are co-expressed (Fig. 9C).

MAP Kinase and TLR Signaling Pathways Activate the IL-12 p40 Promoter through Control Elements in the −101 to +55 Region—Recently, the p38 MAP kinase and the toll-like receptor (TLR) signal transduction pathways have been linked to IL-12 p40 gene expression (25, 36). However, downstream targets of these pathways that mediate IL-12 p40 promoter activation have not been identified. Therefore, a series of dominant negative and constitutively active molecules from the p38 kinase, JNK, and TLR signal transduction pathways were tested for effects on IL-12 p40 promoter activity. All signal transduction molecules and soluble inhibitors display dose-dependent effects on promoter activity (data not shown). To demonstrate activation and inhibition of the p38 pathway in RAW264.7 cells, activity of a GAL4-luciferase reporter by a MEF2c-GAL4 DNA-binding domain fusion protein (37) was assessed. In RAW264.7 cells, LPS strongly induces p38 activity. The soluble p38 inhibitor SB202190 (1 μM) inhibits LPS-induced p38-mediated transcription by 60%, and constitutively active MKK3 and MKK6 strongly induced this reporter in unactivated cells (data not shown). For the IL-12 p40 promoter, SB202190 inhibits LPS-induced activity of the −101 to +55 promoter, whereas the structurally similar control SB202474 does not demonstrate inhibition (Fig. 10A). Furthermore, expression of constitutively active MKK3 or MKK6 induces −101 to +55 p40

![Fig. 9. c-Jun and a deleted c-Jun mutant, TAM67, activate the IL-12 p40 and the TNF-α promoters in RAW264.7 cells. A. −101 to +55 IL-12 p40 promoter-luciferase reporter plasmid was co-transfected with 0.5 μg of expression plasmids for c-Jun, TAM67, JunB, and c-Fos. Plasmid amounts were normalized to 2.5 μg by the addition of empty expression plasmid. Following transfection, RAW264.7 cells were incubated for 36 h. Experiments with AP-1 expression plasmids were conducted in unactivated cells. As controls, cells were transfected with 2.5 μg of empty expression plasmid and remained unactivated or were activated with LPS (5 μg/ml) for 18 h prior to termination. B, − to TNF-α promoter-luciferase reporter was co-transfected with 0.5 μg of expression plasmids for c-Jun, TAM67, JunB, and c-Fos, as in A. C, c-Jun and TAM67 synergistically activate the IL-12 p40 promoter when co-expressed with C/EBPβ. A −101 to +55 IL-12 p40 promoter-luciferase reporter plasmid was co-transfected with expression plasmids for C/EBPβ (0.1 μg) and AP-1 proteins (0.5 μg), as in A. Results are expressed as fold activation compared with cells transfected with C/EBPβ alone. A-C, results (relative light units normalized for β-galactosidase activity) are expressed as the percentage of luciferase activity obtained with the −101 to +55 wild type promoter activated with LPS. Each result represents the mean ± S.D. of three to five experiments.]
promoter activity in untreated cells, although not to the same extent as LPS (Fig. 10A).

The role of the JNK pathway in IL-12 p40 promoter activation has not been determined, although the description of an AP-1 site suggests that this pathway may be involved. LPS activates the JNK pathway in RAW264.7 cells, as assessed by activation of a GAL4-luciferase reporter by a c-Jun-GAL4 DNA-binding domain fusion protein (data not shown). A dominant negative inhibitor of JNK, JIP-1, inhibits LPS-induced JNK-mediated transcription, while constitutively active MEKK1 strongly up-regulated activity in untreated cells (data not shown). Consistent with involvement of the JNK pathway in IL-12 p40 promoter activation, JIP-1 inhibits LPS-induced −101 to +55 promoter activity, and active MEKK1 strongly activates this minimal p40 promoter (Fig. 10B). As MEKK1 also induces NF-κB (38), it is important to note that this effect occurs with a p40 promoter that lacks an NF-κB element.

Dominant negative signal transduction molecules in the Toll/IL-1 receptor pathway inhibit IL-12 p40 promoter activation by bacterial products (25). As NF-κB is a well described target of TLR signaling, we asked whether this signal transduction pathway affects IL-12 p40 promoter activation through elements other than NF-κB. Expression of a dominant negative TLR2, TRAF6, and MyD88 inhibits LPS-induced −101 to +55 promoter activity (Fig. 10C) to the same magnitude as they inhibit activation of a multimerized NF-κB element luciferase reporter in RAW264.7 cells (data not shown). Similar results were obtained when RAW264.7 cells were activated with heat-killed L. monocytogenes and M. tuberculosis whole cell extract (data not shown). Furthermore, a constitutively active CD4-TLR4 fusion protein activates the −101 to +55 promoter in untreated cells, although not to the same degree as LPS (Fig. 10C). Once again, similar results are obtained for IL-12 p40 promoter constructs containing and lacking the NF-κB site (data not shown), suggesting that the TLR pathway may influence promoter activation through elements other than NF-κB.

**DISCUSSION**

In summary, functional and DNA binding assays reveal complex interactions within a small region of the IL-12 p40 promoter. A C/EBP DNA-binding element was previously characterized at −96 to −88. In the current study, mutations from −79 to −74 in the murine IL-12 p40 promoter significantly reduce LPS-induced promoter activation and correlate with DNA binding of AP-1 by EMSA. AP-1 family members detected in the IL-12 p40 promoter DNA-binding complex include JunB, c-Jun, and c-Fos. Spacing between the C/EBP and AP-1 site and orientation of the AP-1 site are important for promoter activation, suggesting cooperativity between these two sites. Expression of a dominant negative AP-1 protein, A-Fos, in RAW264.7 cells inhibits p40 promoter activation by LPS and C/EBPβ. c-Jun and a c-Jun mutant TAM67 activate the IL-12 p40 promoter and synergize with C/EBPβ in transient expression studies. c-Jun and TAM67 also activate the TNF promoter in RAW264.7 cells. Importantly, this small region of the promoter downstream of −101 is identified as a target for MAP kinase and TLR signal transduction pathways.

This group had reported a comprehensive functional analysis of the murine and human IL-12 p40 promoters in RAW264.7 cells (17). A critical control element for promoter activation by bacterial products, located between −96 and −88, interacts with C/EBP family members. This element was functionally synergistic with the NF-κB site between −131 and −120 (13).
In this analysis, when the C/EBP site was mutated, virtually all LPS-induced promoter activity was lost. Thus, it was possible to conclude that the C/EBP site was both necessary and sufficient for promoter activation. To characterize effects of other cis-acting elements, a strategy was developed to assess the functional role of other elements in the presence of an intact C/EBP site. First, mutations were created in two cis-acting elements other than the C/EBP site. A second approach utilized a minimal promoter where all elements upstream of the C/EBP site were deleted. These experiments demonstrate the existence of three important cis-acting elements at −114 to −102, −82 to −73, and −62 to −57. Of note, the functional regions described in this study correspond to constitutive and bacterial product-inducible DNase I footprints in the previous analysis (17). Furthermore, these regions likely correspond to DNA-protein interactions detected on the endogenous IL-12 p40 promoter in primary macrophages by in vivo genomic DNase I footprinting (39). Taken together, these results provide clear evidence that the C/EBP site may be necessary but is not sufficient for LPS-induced promoter activation.

A cis-acting control element at −207 to −211 in the human IL-12 p40 promoter that binds a multiprotein complex consisting of Ets-2, interferon regulatory factor-1, interferon consensus sequence binding protein, and c-Rel has been extensively characterized (14–16, 40). Although we could not demonstrate a functional effect for a single site-directed substitution mutant in a previous analysis (17), a functional effect may be demonstrable by methodologies employed in the current study. Thus, this analysis and others suggest that at least six control elements within a small region of the IL-12 p40 promoter may be necessary for activated gene expression.

One novel site defined by this analysis from −84 to −73 was characterized in detail. A control element was identified from −79 to −74 that binds AP-1 family members. Supershift experiments detect predominantly c-Fos, JunB, and to a lesser extent, c-Jun in the IL-12 p40 EMSA complex. However, relative amounts of AP-1 family members cannot be accurately deduced from these results, as the degree of inhibition or supershift is a function of the antibody as well as the relative amount of protein in the DNA-binding complex. Furthermore, a recent study demonstrated that c-Rel, a relatively minor component of the IL-12 p40 NF-κB DNA-binding complex, is the relevant activator of p40 transcription in cell line models and in vivo (41). Therefore, from our functional results, c-Jun may be the relevant activator of the IL-12 p40 promoter despite the apparent abundance of JunB and c-Fos in the EMSA complex.

To demonstrate a role for AP-1 in IL-12 p40 promoter activation, a dominant negative AP-1 molecule, A-Fos, was expressed in RAW264.7 macrophages. A-Fos contains an acidic amphipathic protein sequence appended onto the N terminus of the c-Fos leucine zipper. This extension physically interacts with the Jun basic region and prevents the basic region from the c-Fos leucine zipper. This extension physically interacts with the Jun basic region and prevents the basic region from protein-protein interaction with C/EBP or another factor or may be related to the ability of AP-1 to bind DNA, thus facilitating protein-protein interactions (42).

AP-1 family members are prototypic targets for the c-Jun N-terminal kinase (JNK) MAP kinase pathway. Although p38 and extracellular signal-regulated kinases have been implicated in IL-12 p40 gene expression (36, 43), the role of the JNK pathway is unclear. In this study, the JNK pathway is implicated in IL-12 p40 promoter activation using a dominant negative inhibitor of JNK, JIP-1, and a constitutively active MEKK1 molecule. Interaction of conserved bacterial ligands with TLRs is important for activation of IL-12 p40 gene expression in macrophages (25). However, specific promoter region and transcription factor targets that modulate IL-12 p40 gene expression have not been identified. For TLR signaling, NF-κB is a critical downstream target (26), although MAP kinases may be activated through TLRs (44). The p38, JNK, and TLR pathways, in part, appear to influence IL-12 p40 gene expression through elements downstream of −101. However, specific transcription factors activated by these pathways remain elusive. For example, in nuclear extracts from LPS-treated RAW264.7 cells and primary bone marrow-derived macrophages, protein expression and DNA binding for C/EBP or AP-1 family members is not affected by treatment with a soluble p38 inhibitor (data not shown).

The IFN-β (45) and the T-cell receptor α (46) gene enhancers provide important biochemical details of how transcriptional activators are assembled into a nucleoprotein complex called the “enhancosome” that promotes their interaction and cooperative binding to DNA. Extensive analysis of the IL-12 p40 promoter begins to describe an IL-12 p40 enhancosome. In particular, a small region from −96 to −74 of the promoter is a critical focus for protein-DNA and possibly protein-protein interactions. This small sequence that contains a C/EBP and AP-1 DNA-binding element is of particular interest because it appears that the strategy used by macrophages to induce IL-12 p40 expression may define a common mechanism for other important inflammatory genes. As discussed, within the TNF-α promoter, there is a C/EBP element immediately adjacent to an AP-1 site. Functional synergy and cooperative DNA binding between c-Jun and C/EBPβ has been demonstrated in activation of the TNF-α promoter (35). Similarly, AP-1 and C/EBP sites have been characterized at similar locations with respect to a TATA box in the IL-8 gene (47, 48). Thus, transcriptional synergy and cooperativity between the C/EBP and AP-1 families of transcription factors may be involved in the regula-
tion of several important inflammatory genes expressed in macrophages.

These studies, performed in cell lines, suggest mechanisms by which IL-12 p40 may be transcriptionally activated in vivo. Confirming the findings of this analysis in primary cells and in vivo will require a combination of approaches to determine transcription factor occupancy on the endogenous promoter and their responses to biologic inducers and inhibitors of IL-12 gene expression.

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