Cooperative Interaction between Interferon (IFN) Stimulus Response Element and κB Sequence Motifs Controls IFNγ- and Lipopolysaccharide-stimulated Transcription from the Murine IP-10 Promoter* 

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The transcriptional regulation of the murine IP-10 gene in lipopolysaccharide (LPS) or interferon γ (IFNγ)-treated macrophages was investigated by analysis of regions of the gene that flank the transcription start site. A series of sequence fragments were placed 5′ to the chloramphenicol acetyltransferase (CAT) reporter gene and ability to mediate transcription of CAT in response to IFNγ or LPS treatment was studied following transient transfection in the macrophage-like cell line RAW 264.7. Analysis of larger constructs identified a potential negative regulatory site for IFNγ response in the region between nucleotide positions -2002 and -830 and a positive regulator for LPS response in the region between bases -930 and -676. A 227-base fragment spanning positions -228 to -2 was the minimal sequence able to mediate LPS- and IFNγ-dependent transcription of CAT. Deletion of 24 bases, which included a highly conserved IFN stimulus response element (ISRE) from the -228 construct, abolished response to IFNγ. A 33-base fragment containing the IP-10 ISRE was able to confer both IFNγ and LPS sensitivity upon a heterologous promoter. The ability of LPS to stimulate CAT via the ISRE was apparently mediated by intermediate expression of endogenous IFNα/β. Elimination of bases -204 to -102 abolished sensitivity to LPS. This region contains two κB binding sites. Site-directed mutagenesis of key nucleotides in the ISRE and the two κB sites demonstrated that optimal response to IFNγ required both the ISRE and one of the two κB sites, whereas optimal response to LPS required either both κB sites or one κB site and the ISRE. IFNγ or LPS treatment induced sequence-specific binding activity for the ISRE and the two κB sites. These results indicate that the 230 nucleotides upstream from the transcription start site are important for transcriptional control of the IP-10 gene in response to IFNγ and LPS. The three defined regulatory elements function in distinct fashion for each of the two stimuli; optimal response to either IFNγ or LPS requires cooperation between at least two sites.

The mononuclear phagocyte system participates in immunoregulation, orchestration of inflammation, and maintenance of homeostasis (1-3). Most of these functions are acquired following exposure of the cells to an almost bewildering array of extracellular stimuli (1-3). Among the most frequently encountered signals are bacterial cell wall products such as lipopolysaccharide (LPS) and secretory products of lymphoid cells such as interferon γ (IFNγ) (4, 5). LPS and IFNγ are both well known macrophage activating stimuli that act at least in part by the induction of new gene expression (4-6). These agents act by initiation of multiple intracellular signaling pathways, which culminate in altered gene-specific function in the nucleus (6-8).

IFNγ and LPS have been shown to have overlapping activities with respect to the induction of certain gene products (5, 6). Among these is the inflammatory protein 10-kDa (IP-10) gene, originally identified in human U937 monocyctic leukemia cells as an IFNγ-inducible product (9) and subsequently in murine peritoneal macrophages on the basis of LPS sensitivity (10, 11). Although the physiologic function(s) and significance of the IP-10 gene product have not been clarified, it has significant sequence homology with the superfamily of chemotactic factor genes recently designated the intercrine or small cytokine family (11-13). This superfamily is subdivided into two groups based upon the relative positioning of the first two cysteine residues in the sequence (CC versus CXC). In the mouse the intercrine α family includes IP-10, MIP-2, and KC (10, 11, 13-15). The intercrine β family includes MIP-1α and β and JE (13, 14, 16, 17). Because of the diverse array of extracellular stimuli that can induce expression of IP-10 and the large number of cell types in which expression has been identified (18-21), this gene represents a good model system for analysis of molecular mechanisms involved in the inducible expression of a single gene in response to distinct stimuli.

Many recent studies have focused on the identification and characterization of cis-acting sequence elements present in the region 5′ of the transcription start sites of inducible genes and on trans-acting factors, which recognize and bind such sequences in stimulus-dependent fashion (22-29). As a first step toward understanding the molecular mechanisms involved in regulating expression of the IP-10 gene, we have carried out a functional analysis of the 5′-flanking region of the IP-10 gene using transient transfection of a reporter gene containing this region upstream from the CAT reporter gene.

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1 The abbreviations used are: LPS, lipopolysaccharide; IFN, interferon; CAT, chloramphenicol acetyltransferase; ISRE, IFN stimulus response element; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; bp, base pair(s); PIPES, 1,4-piperazinediethanesulfonic acid; NF, nuclear factor; IL-6, interleukin 6.
Fig. 1. Complete nucleotide sequence of the murine \( \text{IP}-10 \) gene. The transcriptional start site predicted by primer extension analysis is indicated by +1. The exons are underlined, and the deduced amino acid sequence of the exons are also shown below the nucleotide sequence. A potential polyadenylation signal sequence in the 3'-untranslated region is underlined, and a horizontal arrow indicates the polyadenylation motif.
with the indicated doses of IFNγ or LPS for 4 h prior to preparation of total RNA as described under "Materials and Methods." Five pg of total RNA were analyzed in each lane.

(chloramphenicol acetyltransferase, CAT) linked to the IFN-γ and LPS-driven transcriptional activation are mediated by cooperative interaction between distinct cis-regulatory elements; an IFN stimulant response element (ISRE) and two structurally and functionally distinct X box elements, respectively.

MATERIALS AND METHODS

Reagents—NYZ broth and agar were obtained from Difco. Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle's medium, Hank's balanced salt solution, and RPMI 1640 were from Mediatech (Washington, DC). Fetal bovine serum was obtained from HyClone (Logan, UT). All cell culture reagents contained less than 0.125 ng/ml LPS as quantified by the Limulus amebocyte lysate assay (Cape Cod Associates, Woods Hole, MA). Agarose, phenol, guanidine isothiocyanate, and cesium chloride were purchased from GIBCO/BRL. Anhydrous ethanol, sarkosyl, and formamide were obtained from International Biotechnologies, Inc. (New Haven, CT). DEAE-dextran, NAP-10 columns, and deoxyribonucleoside triphosphates (dNTPs) were purchased from Pharmacia LKB Ltd. (Uppsala, Sweden). MAGNA nylon transfer membrane was obtained from Micron Separations Inc. (Westboro, MA). Restriction enzymes, nick translation kits, reverse transcriptase (from avian myeloblastosis virus), polynucleotide kinase, Klenow fragment of Escherichia coli DNA polymerase I, T4 DNA polymerase, acetyl-coenzyme A, and proteinase K were purchased from Boehringer Mannheim. Du Pont-New England Nuclear was the source of [γ-32P]ATP, [α-32P]dCTP, and [γ-32P]ATP. 1-Deoxy[dichloroacetyl-1-C]chloramphenicol was obtained from Amersham Corp. Erase-a-Base kits, site-directed mutagenesis kits, RNase inhibitor, chloramphenocol acetyltransferase (CAT) reporter plasmid (pCAT-Basic, and pCAT-Promoter), and reference plasmid (pSV-β-galactosidase) were purchased from Promega (Madison, WI). pT-K-CAT plasmid was kindly provided by Dr. Games Sen. Tris, sodium dodecyl sulfate (SDS), acrylamide, N,N,N-trimethylenbisacrylamide, urea, and protein assay reagent were obtained from Bio-Rad. Sequencing kits were obtained with GenBank nucleic acid data base (GenBank release 71).

Northern Hybridization Analysis—Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride method (30). Five micrograms of total RNA was separated on a 1% agarose, 2.2 M formaldehyde gel and subsequently blotted onto MAGNA nylon membrane with 20× SSC by capillary transfer according to previously published methods (31). The RNA was cross-linked to the membrane by using UV cross-linker (Stratagene, La Jolla, CA). The blots were prehybridized for 8 h at 12 h at 42 °C in 50% formamide, 1% SDS, 5× SSC, 1× Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.25 mM dithiothreitol, 50 mM sodium phosphate buffer (pH 6.5) and then hybridized with 1× 10^6 cpm/ml [α-32P]dCTP-radiolabeled IP-10 cDNA plasmid probe (specific activity 1× 10^6 cpm/μg DNA) prepared by nick translation at 42 °C for 16 to 24 h. After hybridization, blots were washed with 0.1% SDS, 2× SSC for 30 min at room temperature followed by two washes at 55 °C. The blots were then exposed using XAR-5 x-ray film with screens at ~70 °C.

Isolation of Genomic IP-10—A murine EMBL-3 genomic library was purchased from Clonetech (Palo Alto, CA). This library was prepared by partial MboI digestion of liver DNA from adult DBA/2 mice and cloned into the EcoRI site of the IP-10 gene in pBluescript (Stratagene, and subjected to deoxy sequencing sequence analysis as described below.

Nucleotide Sequence Analysis—Subfragments for sequence analysis were obtained by either restriction enzyme deletion or a nested deletion strategy using the Erase-a-Base kit (Promega). The nucleotide sequence of each of the subclones was determined by the deoxy chain termination method on supercoiled templates using Sequenase DNA sequencing kits (U. S. Biochemical Corp.) and [α-32P]dATP. Sequences were compiled using sequence analysis software (MacVector, International Biotechnologies, Inc., New Haven, CT) and Gene Works (IntelliGenetics, Inc., Mountain View, CA) and compared with GenBank nucleic acid data base (GenBank release 71).

Preparation of Oligonucleotides—Oligonucleotides used in this study (Table I) were synthesized using an Applied Biosystems DNA synthesizer (model 381A). The crude oligonucleotides were purified on NAP-10 columns (Pharmacia LKB). Double-stranded oligonucleotides were prepared by annealing the complementary single strands. For the preparation of probe in the electrophoretic mobility shift assay (EMSA), double-stranded oligonucleotides were diluted in a Klenow fragment of DNA polymerase I and [α-32P]dCTP in fill-in reaction for 5'-protruding ends or with polynucleotide kinase and [γ-32P]ATP for blunt ends.

 Primer Extension Analysis—The position of the 5' terminus of murine IP-10 mRNA was determined by primer extension analysis using an end-labeled synthetic oligonucleotide corresponding to positions 69-85 of the murine cDNA. The radiolabeled oligonucleotide was annealed with 50 μg of total RNA from murine peritoneal macrophages stimulated with LPS (30 ng/ml) for 3 h prepared as described previously (19) in 400 mM KCl, 40 mM PIPES (pH 6.5), 80% formamide, and 1 mM EDTA for 16 h at 30 °C. The annealed primer was extended by reverse transcriptase (30 units, Boehrger Mannheim) in 50 mM Tris·Cl (pH 8.0), 6 mM MgCl2, 10 mM DTT, 100 mM NaCl, 1 mM dNTPs, and 10 units of RNase inhibitor (Promega) for 90 min at 37 °C. The primer-extended hybrids were analyzed on a 6% polyacrylamide gel containing 7 M urea.

Construction of CAT Reporter Plasmids—A series of deletion mutants of the 5'-flanking region of the IP-10 gene were constructed either by restriction endonuclease or exonuclease III digestion. In brief, the 5'-flanking region of the IP-10 gene from −2004 to −2 (EcoRI to XhoI) was excised from a murine genomic IP-10 gene and subcloned into pBluescript (Stratagene). This vector (designated PBSIP2.0) was subsequently linearized with Smal at the polylinker site with the Klenow fragment of DNA polymerase I and [α-32P]dCTP in fill-in reaction for 5'-protruding ends or with polynucleotide kinase and [γ-32P]ATP for blunt ends.
FIG. 3. Functional analysis of murine IP-10 enhancer-promoter in RAW 264.7 cells. Serial 5′-deletion fragments between −2002 and −2 of the IP-10 genomic sequence were cloned into pCAT-Basic (pCAT B0). All CAT constructs (10 μg) were cotransfected with pSV-β-galactosidase reference plasmid (5 μg) using DEAE-dextran. 24 h after transfection, the cells were stimulated with IFNγ (100 units/ml) or LPS (100 ng/ml) for 18 h prior to assay for CAT activity as described under “Materials and Methods.” After autoradiography, acetylated products were estimated by image analysis. A, a representative autoradiogram showing the results of CAT assays of extracts prepared from RAW 264.7 cells transfected with the reporter gene constructs illustrated in B. B, schematic representation of CAT constructs and normalized CAT activity. Potential cis-acting regulatory elements are also shown at the top of the figure. The CAT activity for each construct was normalized to β-galactosidase activity measured in the same sample. The relative CAT activity for each deletion mutants expressed as a percentage of that of pCAT B-243 stimulated with LPS. Values at right indicate the fold induction of CAT activity stimulated with IFNγ or LPS versus untreated but transfected cultures. The values presented are the mean from three independent experiments.
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Fig. 4. Identification of IFN-γ- and LPS- responsive elements in the proximal 5′-flanking region of the murine IP-10 gene. Serial 5′-deletion fragments between -243 and -2 of IP-10 genomic sequence were cloned into pCAT-Basic (pCAT B0) and transfected into RAW 264.7 cells as described in the legend to Fig. 3. A, a representative autoradiogram showing the results of CAT assays using extracts prepared from RAW 264.7 cells transfected with the reporter genes illustrated in B. B, schematic representation of CAT constructs and normalized CAT activity. The diagram at left shows the structure of CAT reporter constructs. Potential cis-acting regulatory elements are shown schematically. The CAT activity for each construct was normalized to β-galactosidase activity measured in the same sample. The relative CAT activity for each deletion mutant is expressed as a percentage of that obtained using macrophages transfected with pCAT B-243 and stimulated with LPS. Values at right indicate the -fold induction of CAT activity stimulated with IFN-γ or LPS versus untreated cells. The values presented are the mean of three independent experiments.

Preparation of Nuclear Extracts—Nuclear extracts were prepared by a modified method of Dignam et al. (36). RAW 264.7 cells were plated at a density of 1 × 10^6 cells in 15-cm diameter dishes, stimulated, harvested, and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min on ice, then vortexed for 10 s with 0.6% Nonidet P-40. Nuclei were separated from cytosol by centrifugation at 12,000 × g for 60 s and were resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and shaken for 30 min at 4°C. Nuclear extracts were obtained by centrifugation at 12,000 × g. Protein concentration was measured by the method of Bradford (37) by using a protein dye reagent (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)—For binding reactions, nuclear extracts (10 μg of protein) were incubated in 25 μl of total reaction volume containing 20 mM HEPES, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 200 μg/ml bovine serum albumin, and 2.5 μg of poly(dI-dC) for 15 min at 4°C. The 32P-labeled oligonucleotide (0.5 ng, 20,000–40,000 cpm) was then added to the reaction mixture and incubated for 20 min at room temperature. The reaction products were analyzed by electrophoresis in a 6% polyacrylamide gel with 0.25 × TBE buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA). The gel was dried and analyzed by autoradiography.

RESULTS

Genomic Sequence of the Murine IP-10 Gene—A clone containing the IP-10 gene was obtained from a murine liver genomic library and subjected to dideoxy sequencing analysis. The complete nucleotide sequence of the IP-10 gene is shown in Fig. 1. Primer extension analysis revealed one potential transcriptional initiation site 65 bp upstream of ATG codon (data not shown). This start site (A) is consistent with the finding that RNA polymerase II-dependent transcription initiates most frequently at adenine nucleotides (38). The predicted nucleotide length of the primary transcript is 2231 bases without a poly(A) tail. Alignment of the murine genomic IP-10 sequence with the human IP-10 genomic sequence indicates that the two genes are organized identically (39).

Analysis of IP-10 mRNA Expression in RAW 264.7 Cells—We have previously reported that LPS, IFN-γ, and IFN-β induce IP-10 transcription in thioglycollate-elicited peritoneal macrophages (19). To study the activity of the IP-10 promoter, various portions of the sequence flanking the transcription
Nucleotide sequences of ISRE and αB oligonucleotides used in this study

The numbers above the sequence refer to the distance from the transcriptional start site (Fig. 1). Lowercase letters represent the bases included for creating restriction sites. Boldface type indicates the substituted bases for mutation. Underlined sequences represent the consensus ISRE (28) and αB (26) region. R: A or G; T: T or C; W: A or T; N: any nucleotide.

| A. ISRE wild type | 5'-gatctCTACGCGTGGAAAGATGAATACCCCTTCTAC-3' |
| ISRE mutant | 5'-CTCCGCTTACGACGCTACGGTTCCGGGTA-3' |
| ISRE consensus | 5'-CTCGGGTTTACAGGTGACTTCCCTCGGGT-3' |
| B. αB wild type | 5'-CCTCAAGGGGAGAGGGAAATTCCCTACGGTTCCGGGTA-3' |
| αB mutant | 5'-CTCCCCCTTCTCCCTTTAAGGTTCAAGTTCC-3' |
| αB consensus | 5'-CTCCCCCTTCTCCCTTTAAGGTTCAAGTTCC-3' |

Functional Analysis of 5' cis-Regulatory Element of the IP-10 Gene—A computer search revealed a large number of potential regulatory elements in the 5' flanking region of the murine IP-10 gene (Figs. 1 and 3). Functional analysis of the IP-10 gene was carried out by cloning the fragment containing positions -202 to -2 into a reporter plasmid (pCAT-B0) that lacks both enhancer and promoter sequences. This plasmid was transfected into the RAW 264.7 macrophage cell line by DEAE-dextran, and 24 h later the cells were stimulated with LPS or IFNγ for 18 h and tested for CAT activity. Cultures transfected with a construct containing 2 kilobase pairs of the IP-10 5'-flanking region exhibited a 9-fold increase in CAT activity in response to LPS treatment, but showed no significant response to IFNγ (Fig. 3). Deletion of the region between -202 and -930 resulted in constructs that showed a 3-fold induction of CAT activity in response to IFNγ while remaining highly responsive to LPS. Removal of the region spanning nucleotides -930 to -676 reduced the sensitivity of the construct to LPS without affecting the sensitivity to IFNγ. Constructs containing the region from -330 to -2 retained sensitivity to both stimuli. When the region from -330 to -243 was deleted both basal and inducible CAT activity increased. Removal of the region from -243 to -102 resulted in a loss of all response to LPS or IFNγ. These results suggested the presence of a distal element between -202 and -930, which negatively regulates response to IFNγ, and an element between -930 and -676, which positively regulates response to LPS. The region between -243 and -102 is essential for LPS or IFNγ inducibility.

In order to more precisely localize the IFNγ and/or LPS response sequence(s) in the region between -243 and -102, we constructed several more 5'-deletion mutants and tested for the inducible CAT activity in response to LPS or IFNγ. pCAT-243 and pCAT-228 gave high inducible CAT activities in response to both LPS and IFNγ (Fig. 4). Deletion of 24 bases from pCAT-228 abolished response to IFNγ. Both basal activity and LPS-inducible activity were also reduced in magnitude. These results indicate that an IFNγ-response element exists in the region between -228 and -204. This region contains a highly conserved ISRE between -212 and -224 (Fig. 2). There was no cooperativity evident between LPS and IFNγ; optimal doses of either agent resulted in maximal expression of IP-10 mRNA that could not be further enhanced in a dose-dependent manner in response to LPS or IFNγ (Fig. 5). When one copy of the IFNγ response sequence was placed in pTK-CAT, basal CAT activity was elevated and treatment with LPS and IFNγ enhanced this 3.6- and 1.7-fold, respectively. These results indicate that an IFNγ-response element exists in the region between -228 and -204. This region contains a highly conserved ISRE between -212 and -224 (Table I). Deletion of nucleotides -204 to -154 did not have an effect on LPS-inducible CAT activity. However, sensitivity to LPS was lost when sequences between -154 and -102 were deleted. These results demonstrate that the region between -154 and -102 is necessary for LPS-inducibility. This region contains a αB site at -113 to -104 (Fig. 1 and Table I).
**Transcriptional Regulation of IP-10**

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**A**

![Diagram showing the TK Promoter and ISRE constructs](image)

**B**

![Table showing fold induction](image)

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**Fig. 5.** The IP-10 ISRE sequence confers LPS and IFNγ inducibility on a heterologous promoter. A, one, two, or three copies of a 33-bp oligonucleotide containing the ISRE from the murine IP-10 gene (see Table 1) were cloned immediately upstream of the enhancerless herpes virus thymidine kinase promoter CAT reporter plasmid (pTK 0) and transfected into RAW 264.7 cells as described in the legend to Fig. 3. The diagram at left shows the structure of the CAT reporter constructs. The ISRE and TK promoter sequence are indicated by the filled arrow and hatched box, respectively. The number of copies and the orientation of ISRE sequence are indicated by the arrows. Values at right indicate the -fold induction of CAT activity in cells stimulated with IFNγ or LPS relative to untreated cells. Similar results were obtained in three separate experiments. B, RAW 264.7 cells were transfected with the pTK CAT plasmid containing three copies of the IP-10 ISRE as described in the legend to Fig. 3. Following a 24-h rest the cells were either stimulated or not with LPS (100 ng/ml) in medium containing antiserum to IFNα/β (sufficient to neutralize 1300 antiviral units of IFNα/β) or an equivalent dilution of preimmune serum prior to assay of CAT activity. Similar results were obtained in two separate experiments.

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CAT expression under control of the ISRE could result from the induced expression of IFNα/β. To test this possibility, RAW 264.7 cells were transfected with the pTK CAT plasmid containing three copies of the ISRE sequence and stimulated with LPS in the absence or presence of anti-serum to murine IFNα/β or preimmune serum (Fig. 5B). Preimmune serum had no effect on the LPS-induced increase in CAT activity but anti-IFNα/β serum markedly reduced induced expression without affecting the levels of basal or constitutive activity.

**ISRE and κB Elements Cooperate in the IP-10 Promoter—**

To examine whether the ISRE and two κB sites cooperate in mediating response to IFNγ or LPS, specific residues in these elements known to be critical for transacting factor recognition were changed by site-directed mutagenesis. Specific mutants utilized are shown in Fig. 6B. Three nucleotide mutations in the ISRE sequence of the -243 CAT construct abolished the IFNγ-inducible CAT. This mutation also resulted in a reduced magnitude for basal and LPS-induced CAT activity although the ratio of induced to basal activity remained high (Fig. 6, pCAT Mu3). The mutation of either κB1 or κB2 sites individually diminished both the LPS-inducible and basal CAT activities; however, mutation of the κB1 site (pCAT Mu1) more effectively reduced the response to LPS than did mutation of the κB2 site (pCAT Mu2). Similar quantitative modulation of IFNγ-inducible CAT activity was reproducibly observed in transfection experiments utilizing plasmids containing an intact ISRE but mutated either in κB1 or κB2. Interestingly, mutation of both κB sites (pCAT Mu1–2), which retains the wild type ISRE sequence, abolished the IFNγ-induced CAT activity. These data indicate that the κB-binding sites cooperate with the ISRE for efficient activation of IP-10 transcription by IFNγ. The combination of the mutations in the ISRE and κB2 site (pCAT Mu2–3) resulted in a loss of LPS inducibility, suggesting the possibilit-
ity that sequences between −243 and −154 may have some negative effect on response to LPS, since deletion of residues −243 to −154, which contains the xB1 sequence, was sensitive to LPS (see Fig. 4, −154 CAT construct). No inducible activity was observed in the CAT constructs containing mutations in the ISRE and one or both xB sites.

IFNγ and LPS Activate Nuclear Factors Binding the ISRE and xB Sequences—In order to determine if IFNγ and/or LPS could induce DNA-binding factor(s) that specifically recognize the IP-10 ISRE or xB sites, double-stranded synthetic oligonucleotides corresponding to the appropriate residues were prepared. An oligonucleotide spanning the sequence from −234 to −202 (Table I) was radiolabeled with [32P]dCTP and used in EMSA with nuclear extracts prepared from untreated LPS- or IFNγ-treated RAW 264.7 cells (Fig. 7). Nuclear extracts from untreated cells displayed a major, rapidly migrating complex. When the cells were stimulated with IFNγ or LPS, a more slowly migrating complex was observed (see arrow). This inducible binding activity was observed as early as 30 min after stimulation with IFNγ but was seen in
treatment strongly induced the formation of complex C2 and an additional slowly migrating complex termed C3.

**DISCUSSION**

Expression of the IP-10 gene is inducible by diverse pro-inflammatory stimuli including LPS, IFNγ, IFNα/β, PDGF, tumor necrosis factor α, and IL-1 in a variety of cell types (19–21). The primary goal of the work presented in this report was to elucidate how such different extracellular signals regulate the transcriptional activation of the IP-10 gene in the same cell population. Structural and functional characterization of the sequence region flanking the transcription start site of the murine IP-10 gene demonstrates that approximately −228 bases upstream from transcription start site are essential for IFNγ inducibility and −154 bases upstream are requisite to LPS sensitivity. Although IFNγ- and LPS-induced transcriptional activation of the IP-10 gene are both influenced by the ISRE and κB sequences, these elements function in distinct ways for each stimulus. These conclusions are based upon the following observations. 1) Deletion of the ISRE sequence from the −228 nucleotide fragment of IP-10 promoter construct abolished the sensitivity to IFNγ without affecting sensitivity to LPS. 2) Deletion of both κB sequences from the −154 nucleotide fragment abolished sensitivity to LPS. 3) IFNγ induced a nuclear factor that specifically recognized and bound an ISRE containing oligonucleotide within 30 min of stimulation while LPS-induced ISRE binding activity required a minimum stimulation time of 3 h. 4) Although both IFNγ and LPS could drive transcription from constructs containing multiple copies of the IP-10 ISRE linked to a heterologous promoter, the response to LPS (but not IFNγ) was blocked by including antibody to murine IFNα/β. These findings suggest that IFNγ and LPS act via distinct (though perhaps overlapping) intracellular signaling pathways. This contention is supported by experimental findings demonstrating the possibility of additional regulatory elements distal to the 243 bp fragment which are functionally distinct. A negative element between −2002 and −930 suppresses response to LPS (but not IFNγ) which is consistent with many previous reports suggesting differences in the signaling responses of macrophages to IFNγ or LPS (4, 6, 7, 19).

Although the ISRE and the κB sequences, respectively, are essential for IFNγ and LPS inducibility in the IP-10 gene promoter, one important characteristic of the transcriptional activation of the IP-10 gene induced by IFNγ or LPS is the cooperative interaction between the ISRE and κB elements. The following observations indicate that the efficient transcriptional activation of the IP-10 gene requires the combined interaction of at least two positive regulatory elements. First, LPS-induced transcriptional activation was completely abolished by the combination of mutation in one κB site (either κB1 or κB2) and in the ISRE or in both κB sites. Thus the ISRE and one κB site or two κB sites are necessary for LPS inducibility. Second, mutation of one κB site reduced or abolished the sensitivity to IFNγ despite of the presence of an intact ISRE sequence. Thus the ISRE and a κB site are both necessary for IFNγ inducibility. Since the ISRE sequence itself was able to confer IFNγ inducibility to heterologous promoter, it is conceivable that another sequence motif between −243 and −154 may play some repressive role which could be overcome by an IFNγ-inducible factor(s) binding to either of the κB sites. The possibility of a negative element in

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**Fig. 7.** INFγ and LPS induce ISRE binding proteins in RAW 264.7 cells. Nuclear extracts were prepared from untreated or from IFNγ-treated (100 units/ml) (A) or LPS-treated (100 ng/ml) (B) cells for the indicated times as described under "Materials and Methods." Binding reactions contained 10 μg of nuclear extract protein and 0.5 ng of radiolabeled ISRE oligonucleotide (Table I). Similar results were obtained in two separate experiments.

LPS-stimulated cells only following 3 h. Several additional minor binding complexes were observed that did not exhibit reproducible stimulus dependence. The binding specificity of the principle stimulus-dependent complex was demonstrated by competition studies with unlabeled oligonucleotides containing the intact ISRE sequence, the mutant ISRE, or unrelated sequences (not shown).

Analysis of κB1 binding activity used a 30-mer corresponding to residues −98 to −123 while the oligonucleotide corresponding to the κB2 site was a 28-residue sequence containing residues −151 to −178 (Table I). Nuclear extracts from untreated cells formed two complexes with the κB1 sequence (Fig. 8). When cells were stimulated with IFNγ for 2 h, the levels of complex 2 moderately increased. A much more marked increase in the formation of both complexes 1 and 2 was observed in LPS-stimulated cells. Both IFNγ- and LPS-inducible complexes could be specifically competed with wild type κB1 oligonucleotide (Fig. 8B) while the mutant κB1 oligonucleotide (Table I) did not compete. Interestingly, when the κB2 sequence was used as a probe, complexes C1 and C2 were detectable at lower levels in untreated cells. IFNγ-treatment caused a modest increase in complex 2 and LPS
FIG. 8. IFNγ and LPS induce βB binding protein in RAW 264.7 cells.

A, RAW 264.7 cells were either untreated (CONT; lanes 1 and 4) or treated with IFNγ (100 units/ml; lanes 2 and 5) or LPS (100 ng/ml; lanes 3 and 6) for 2 h. 10 μg of nuclear extract protein was incubated with 0.5 ng of radiolabeled βB (lanes 1–3) or βB (lanes 4–6) oligonucleotide (Table I) and analyzed by EMSA. Arrows indicate the specific binding complexes. B, competition analysis of βB binding nuclear protein. Nuclear extracts (10 μg of protein) from RAW 264.7 cells treated with IFNγ (100 units/ml) or LPS (100 ng/ml) were incubated with 0.5 ng of radiolabeled βB oligonucleotide in the absence of competitors (lanes 1 and 6) or presence of 5 ng (10-fold excess) or 50 ng (100-fold excess) of wild type βB (lanes 2, 3, 7, and 8) or mutant βB (lanes 4, 5, 9, and 10) oligonucleotide (Table I) and analyzed by EMSA.

the region between −154 and −243 is also supported by the finding that deletion of this region does not affect LPS sensitivity (Fig. 4), whereas mutation of the ISRE and one βB site abolish sensitivity (Fig. 6).

The 33-base sequence of the region between −234 and −202 contains a conserved ISRE sequence that confers responsiveness to IFNγ and LPS in the context of a heterologous promoter. This result indicates that the IP-10 ISRE sequence functions as both IFNγ and LPS responsive enhancer element. Work in multiple cultured cell systems has identified a diverse array of factors that can specifically interact with ISRE-like sequence elements (28, 29, 40–46). For example, interferon-stimulated gene factor 3 is an oligomeric complex that may be activated following stimulation with type 1 IFN (28, 41). Additional ISRE-binding proteins induced in response to IFN have been described (these include interferon-stimulated gene factor 2 (41), interferon regulatory factor −1 (IRF-1, Ref. 42), IRF-2 (43), interferon consensus sequence-binding protein (44), and interferon binding factor 1 (45)). A number of additional ISRE binding activities, which are not responsive to IFN treatment, have also been identified (46). The relationship between the macrophage-derived complexes and those described previously in other cell types cannot be determined on the basis of the available data and will require analysis of the precise protein composition of the complexes themselves. The IFNγ-inducible ISRE binding complex can be detected as early as 30 min after IFNγ treatment and was independent of protein synthesis. This result suggests that induction of ISRE binding is a primary response to IFNγ and involves the activation of preexisting cellular factor(s).

In contrast, the LPS-stimulated ISRE binding activity appeared only after 3 h of stimulation. Since LPS can directly induce IFNα/β in macrophages, the involvement of the ISRE in mediating response to LPS may be indirect and depend upon the intermediate synthesis and secretion of IFNα/β. Indeed this possibility is supported by the finding that anti-
serum against IFNα/β can block the LPS-driven CAT expression from a heterologous promoter containing three copies of the IP-10 ISRE (Fig. 5). Because abundant expression of endogenous IP-10 mRNA occurs with 1–2 h of stimulation with LPS, whereas ISRE binding activity appears only after 3 h, the LPS-dependent activation of IP-10 transcription may not utilize the ISRE.

EMSA showed a different profile of complexes formed with oligonucleotide probes containing either the αB1 or αB2 sequences. These results suggest that the αB1 and αB2 elements are functionally distinct. In this regard, recent studies of the tumor necrosis factor α enhancer have shown that some αB sequence motifs were not active as LPS-inducible enhancers despite their ability to form high affinity complexes with NF-κB (47, 48). These findings are also supported by the observations that NF-κB (p50 and p65) and its related family (Rel-related genes) are functionally different; NF-κB (p50/p65 or p50/p50) acts as a positive factor, whereas Rel (p50/Rel or Rel/Rel) may act as a repressor (49). The αB1 sequence of the IP-10 gene (5′-GGAGTTCCC-3′) has a prototype κB sequence (26), which has been reported to preferentially bind p50/p65 or p50/p50, whereas the κB2 sequence (5′-GGGAAATTCC-3′) is more similar to the Rel-binding motif (59). However, the exact functional relevance of these potential negative repressor elements in this region, including an IRF-2 binding sequence (43, 44), p65 as well as other products (52). Thus it is unclear whether different nuclear factors bind to the αB1 and αB2 sites or same nuclear factor(s) bind to these sites but with different affinity.

We also identified potential regulatory sites more distal to the −2435 position (see Fig. 1). For example, the deletion of nucleotides −930 to −676 reduced the LPS inducibility of the CAT construct (Fig. 3), suggesting that a positive LPS-responsive regulatory region exists in this region. It is of interest to note that this region contains three CK-1 elements and one NF-IL-6 binding sequence. The CK-1 element (5′-GRGRTTCA3′) has been identified in the promoter region of a number of cytokine genes (53, 54) and is recognized by nuclear factor NF-IL-6 (m50, m50) (54). NF-κB is also able to bind this conserved sequence with low affinity (55). The CK-1 motif also appears to act as a LPS-inducible enhancer element in the mouse granulocyte colony stimulating factor gene (56). The NF-IL-6 binding site (5′-TNNNGAAT-3′) has been identified in a number of LPS-inducible cytokine and acute phase protein genes (57), and NF-IL-6 has been shown to be involved in the expression of these LPS-inducible genes (57). Second, a region negatively affecting sensitivity to IFNγ is located between −2002 and −930. There are several potential negative repressor elements in this region, including an IRF-2 binding sequence (43, 58) and a Rel binding sequence (59).

However, the exact functional relevance of these potential elements in the transcriptional regulation of the IP-10 gene remains to be determined.

Luster and co-workers (39) have reported that a IFNγ-inducible DNase I-hypersensitive site is located between 60 and 260 bases upstream of transcriptional start site in human IP-10 gene. The sequence alignment of human and mouse IP-10 gene shows a relatively high degree of conservation of nucleotide sequence (60%) within 300 bases from the transcription start site. In this region, both human and mouse genes contain one ISRE and two κB sites. Taken together with our results, these relationships suggest that this 300-base upstream region is important for the transcriptional regulation of the IP-10 gene in both species.
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