Secretory Interleukin-1 Receptor Antagonist Gene Expression Requires both a PU.1 and a Novel Composite NF-κB/PU.1/ GA-binding Protein Binding Site*

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The human secretory interleukin-1 receptor antagonist (secretory IL-1Ra) gene is controlled through three lipopolysaccharide (LPS)-responsive promoter elements, one of which was identified as an NF-κB binding site. Sequence analysis of the secretory IL-1Ra promoter identified a potential PU.1 binding site located between positions −80 and −90 on the complementary strand overlapping the NF-κB site. Gel shift analysis using this potential binding site with nuclear extracts from RAW 264.7 macrophages demonstrated the formation of three complexes, one LPS-inducible and two constitutive. The inducible factor was identified as NF-κB, and the constitutive factors were identified as PU.1 and GA-binding protein. Site-directed mutagenesis of the −93 to −79 promoter region demonstrated that mutation of either the NF-κB 5′-half site or the PU.1/GA-binding protein half-site alone did not significantly decrease LPS responsiveness. However, a mutation that disrupted the binding of all three factors resulted in a 50% decrease in LPS responsiveness. A second PU.1 binding site centered at −230 was identified by gel shift and supershift assays. Mutation of the core GGAA region resulted in a 50% decrease in LPS-responsive promoter activity. Mutation of both the distal and proximal LPS response elements led to an almost complete loss of responsiveness. These data therefore suggest that the regulation of IL-1Ra gene expression is a complex event involving the interactions of three different transcription factors with a single cis-acting element and that the two PU.1 binding sites are the major response elements for LPS-induced IL-1Ra gene expression.

Interleukin 1 is an important proinflammatory cytokine produced by a wide variety of cell types and has been implicated in the pathogenesis of acute and chronic inflammatory or autoimmune diseases (1). The discovery of a naturally occurring IL-1 receptor antagonist (IL-1Ra)† has suggested a means through which the pathologic effects of IL-1 can be modulated (2, 3). The human IL-1Ra gene encodes two closely related proteins that act as specific antagonists of the binding of IL-1α and IL-1β to both the type I and type II IL-1 receptors (4, 5). Despite being members of the IL-1 gene family (6) and binding to the same cell surface receptors with approximately equal affinities as IL-1α and IL-1β (7, 8), the IL-1Ra proteins exert no agonist activity.

IL-1Ra was originally described as a 22–26-kDa secreted product of monocytes (9) but is now known to also exist as an 18-kDa intracellular molecule originally found in the cytoplasm of epithelial cells (10). These two isoforms of IL-1Ra, termed sIL-1Ra and icIL-1Ra for the secreted and intracellular variants, respectively, are produced by alternative splicing of two distinct first exons. The first exon for icIL-1Ra is located approximately 9.6 kilobase pairs upstream of the first exon for sIL-1Ra (11) and splices into an internal site with the sequences encoding for the sIL-1Ra signal peptide. Thus, icIL-1Ra contains no secretory leader peptide and remains cell-associated. The sIL-1Ra protein is produced by monocytes, macrophages, neutrophils, hepatocytes, and some fibroblasts, while icIL-1Ra is produced principally by epithelial cells, keratinocytes, monocytes, and macrophages, and some fibroblasts.

The expression of each form of IL-1Ra is controlled by the activity of its own distinct promoter region, which imparts both stimulus and cell-type-restricted expression (11–13). Previously, we reported that the proximal 294 bp of the human sIL-1Ra promoter contains three cis-acting lipopolysaccharide (LPS)-responsive elements (LRE), which act to regulate the expression of the sIL-1Ra gene in monocytes and macrophages in response to stimulation with bacterial LPS (14). One of these elements, LRE1, was identified as an NF-κB binding site, while the transcription factors that bound to the other two LREs were not identified.

The regulation of monocyte and macrophage genes in response to LPS has been a topic of intense interest. A number of transcription factors have been implicated in LPS-inducible gene expression, including NF-κB, NF-IL6 (C/EBPb), AP-1, and members of the Ets gene family including Ets-2, Elk-1, and most recently PU.1 (reviewed in Ref. 15). PU.1, originally identified as the proto-oncogene Spi-1 (16), is a macrophage, B cell, neutrophil, and mast cell-specific member of the Ets gene family (reviewed in Ref. 17) and has been implicated in the tissue-specific regulation of an increasing number of myeloid genes including CD11b, macrophage colony-stimulating factor receptor, neutrophil elastase, FcyR1b, and macrophage scavenger receptor (18–22). Disruption of the PU.1 gene in mice resulted in an embryonic lethal phenotype characterized by defects in complex virus thymidine kinase promoter construct; bp, base pair(s); GABP, GA-binding protein; WT, wild type; IL, interleukin.
the development of both the myeloid and lymphoid lineages (23). The role of PU.1 in LPS-induced expression of macrophage genes is less well understood. Shackelford et al. (24) demonstrated increased PU.1 binding activity in the nuclei of murine peritoneal macrophages following stimulation with IFN-γ or LPS, but this study did not demonstrate a functional role for PU.1 in the response of macrophage genes to LPS. Other reports have shown that LPS does not affect the levels and DNA binding activity of PU.1 in monocytes (25). The only studies to date that have demonstrated a role for PU.1 in the regulation of the LPS response have come from work on the IL-1β gene and the human immunodeficiency virus-1 long terminal repeat. A cap site-proximal region of the human IL-1β promoter was shown to bind a factor originally termed NFβA that was critical for the expression of IL-1β in response to LPS and cytomegalovirus immediate early gene products (26, 27). NFβA was later found to be identical to PU.1 (28). More recently, PU.1 was shown to function in conjunction with NF-κB to mediate LPS-inducible activation of the human immunodeficiency virus-1 long terminal repeat (29).

In this report, we present data indicating that LPS-induced expression of the human sIL-1Ra gene in macrophages is controlled by two PU.1 binding sites. The proximal PU.1 site is located between positions −80 and −90 on the minus strand and binds both PU.1 and GA-binding protein (GABP). This site overlaps the previously identified NFκB binding site and thus represents a novel composite NF-κB/PU.1/GABP binding site. Mutations that disrupted either NFκB or PU.1 binding had no effect on the response to LPS. However, a mutation that disrupted both NFκB and PU.1-binding resulted in a 50% decrease in the response to LPS. GABP appeared to have no effect on the LPS response but did play a role in the regulation of basal promoter activity, thus suggesting that PU.1 and NFκB may be functionally redundant. Mutation of the distal site, located between positions −230 and −220, resulted in an approximately 50% decrease in the activation of the promoter in response to LPS. Interestingly, binding of PU.1 from nuclear extracts of RAW 264.7 cells to either PU.1 site was constitutive and not affected by LPS treatment of the cells. Thus, these studies provide new insights into the regulation of macrophage gene expression and demonstrate for the first time that PU.1 and GABP can also bind to at least some NFκB sites and mediate trans-activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% low endotoxin fetal bovine serum (Summit Biotechnology, Greeley, CO). HT1080/CD14 cells were previously described (30) and cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan UT). 10 mM HEPES, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. RAW 264.7 cells were cultured on bacterial type Petri dishes. RAW 264.7 transfections were performed using the calcium phosphate method as described previously (31, 32). HT1080/CD14 cells were transfected with LipofectAMINE (Life Technologies, Inc.) as described previously (25). Following transfection, cells were cultured for 40 h prior to stimulation for 8 h with or without 1 μg/ml LPS (Escherichia coli 026:B6; Sigma). Cells were harvested and assayed for luciferase activity as described previously (31). In all experiments examining LPS responsiveness, luciferase activity was normalized to total protein concentration in the lysates as determined using a Bio-Rad protein assay kit with bovine serum albumin as standard. To assess basal promoter activity, cells were cotransfected with 2 μg of a cytomegalovirus-β-galactosidase expression plasmid, and luciferase activity was normalized to β-galactosidase activity measured in the same sample. Responses are expressed as the means ± S.D. for at least three experiments.

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared from untreated or LPS-stimulated RAW 264.7 cells by a modification of the procedure of Dignam et al. (33). Nuclei were prepared by lysing the cells in 5 ml of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.3 mM spermine, 0.3 mM spermidine, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 0.1% Triton X-100). Nuclear extracts were harvested and assayed for luciferase activity as described previously (26, 27). Nuclear proteins were extracted by suspending the nuclei in 1 volume of buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 10 mM β-glycerophosphate, 0.1% Triton X-100) and rocking gently at 4 °C for 30 min. Nuclei were pelleted by centrifugation at 4 °C for 30 min in a microcentrifuge. The protein-containing supernatant was removed and stored at −80 °C until use.

**Electrophoretic Mobility Shift Assay (EMSA)**—For EMSA, 5 μg of nuclear extract was incubated in a 20-μl EMSA binding reaction consisting of 20 mM HEPES, pH 7.9, 1 mM dithiothreitol, 0.1 mM EDTA, 50 mM KCl, 5% glycerol, 200 μg/ml bovine serum albumin and 1.5 μg of poly(dI-dC) at 4 °C for 15 min before the addition of 0.5 ng of 32P-labeled oligonucleotide probe. Specific binding was carried out for an additional 20 min at room temperature. The reaction was analyzed by electrophoresis on a 4 or 6% nondenaturing polyacrylamide gel made up with 0.5× Tris borate/EDTA (TBE) buffer. Running buffer was 0.25% TBE. For competition studies, the binding reaction was conducted in the presence of nonspecific DNA together with a 1–100-fold molar excess of unlabeled competitor DNA for 15 min prior to the addition of specific labeled probe. For antibody supershift assays, extracts were preincubated with 1–2 μg of antibody specific for PU.1, c-Ets-2, CCAAT/enhancer-binding protein β, or c-Ets-1/c-Ets-2 (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or 1 μl of specific anti-p50 (NFκB) antisera (34) (a kind gift of Dr. N. R. Rice, NCI-Frederick Cancer Research Facility, Frederick, MD) or rabbit polyclonal antisera to GABP-α and GABP-β (35) (a kind gift of Dr. Shigetaka Kitajima, Tokyo Medical and Dental University, Tokyo, Japan) for 1 h at 4 °C prior to the addition to the EMSA reaction.

**RESULTS**

**LPS Response Element 1 Is a Composite NF-κB/PU.1/GABP Binding Site**—Previously, we demonstrated that LPS treatment of RAW 264.7 macrophage cells induced the binding of NF-κB to a site located between −93 and −84 of the human sIL-1Ra promoter (14). Mutation of this site resulted in a decrease in the promoter response to LPS and was thus termed LPS response element 1 (LRE1). Recent studies that demonstrated a role for PU.1 in the LPS-induced regulation of the IL-1β gene (25, 27) led us to examine the sIL-1Ra promoter for potential PU.1 binding sites. This analysis revealed two potential PU.1 binding sites, one in LRE1 and a second in LRE3. The potential PU.1 site in LRE1 was located between −90 and −80 on the complementary strand.

In order to determine if this region of DNA could bind PU.1, nuclear extracts were prepared from unstimulated (control) or LPS-stimulated (1 μg/ml for 30 min) RAW 264.7 cells and reacted with a probe, designated wild type (WT), which contains both the previously identified NFκB-binding site as well as the potential PU.1 binding site. Fig. 1A shows the sequences of IL-1Ra oligonucleotides used in these experiments and indicates the NFκB-binding site as well as the potential PU.1 binding site. Fig. 1B demonstrates the formation of three complexes, labeled A1, A2, and B, with the WT oligonucleotide. Consistent with our previous findings, the complex A2 was inducible following LPS stimulation (1 μg/ml for 30 min), and binding of the radiolabeled probe to this complex was inhibited by competition with excess consensus NFκB oligonucleotide (lane 4).

Formation of complexes A1 and B were constitutive and not increased following LPS stimulation. Binding of radiolabeled probe could be inhibited by an oligonucleotide derived from the SV40 PU.1 binding site (lane 5), suggesting that the one of the factors responsible for the formation of complexes A1 and B
LPS, lanes 2–9
site-directed mutagenesis experiments. The NF-κB binding site is shown with the solid box, and the putative PU.1 binding site is outlined by a dashed box. B, interactions of nuclear proteins from RAW 264.7 cells with the WT LRE1 oligonucleotide. EMSA reactions were performed as described under “Experimental Procedures.” Radiolabeled WT oligonucleotide was incubated with 5 μg of nuclear extract from unstimulated (C, lane 1) or LPS-stimulated (1 μg/ml LPS for 30 min; LPS, lanes 2–9). Competitions (left), were performed with a 100-fold excess of the indicated unlabeled oligonucleotide. Antibody supershifts (right) were performed with the indicated antibody as described under “Experimental Procedures.” Bands A1, A2, and B represent specific complexes.

may be PU.1. In order to better characterize the DNA sequence requirements for factors A1, A2, and B, oligonucleotides were prepared that were mutated in sequences within either the NF-κB or PU.1 sites or common to both binding sites. Sequences of these competitor DNAAs are shown in Fig. 1A. Mutation of sequences common to both NF-κB and PU.1 binding sites (Mut2, lane 6) disrupted the binding of all three complexes. An oligonucleotide in which the IL-1Ra NF-κB site was changed to resemble the prototypic IgκB site (TA-AC) resulted in a minor decrease in affinity for complexes A1 and B but completely competed complex A2 (lane 7). Lane 8 indicates that a mutation within the NF-κB 5′-half-site (mNF-κB) resulted in a disruption of only A2 binding. Conversely, a mutation outside of the NF-κB binding site but within the PU.1 binding site (mEBS, mutated Ets binding site) disrupted the formation of A1 and B but not A2 (lane 9). These studies therefore indicated that the binding specificity of complex A2 was consistent with that of NF-κB and that the factors responsible for the formation of complexes A1 and B bound to PU.1-like sequences. The antibody supershifts shown in the right part of Fig. 1B confirm that PU.1 is indeed responsible for the formation of complex B. Complex A1 is often obscured by the NF-κB band.

In order to better determine the factors that result in the formation of the three complexes, antibody supershift experiments were performed using oligonucleotides mutated in either the NF-κB (mNF-κB, Fig. 2A) or PU.1 (mEBS, Fig. 2B) binding sites as probes. As shown in Fig. 2A, two complexes were formed using the mNF-κB oligonucleotide. Preincubation of RAW 264.7 nuclear extracts with anti-p50 antibody had no effect on complex formation; however, an antibody to PU.1 resulted in the loss of complex B but had only a small effect on formation of complex A1.

Sequence analysis of the LRE1 PU.1 binding site indicated similarities with that for GABP, another Ets-related transcription factor (36). In order to determine if the factor responsible for the formation of complex A1 is indeed GABP, the EMSA experiment shown in the left part of Fig. 2A was performed. When RAW 264.7 nuclear extracts were preincubated with antisera to either GABP-α or GABP-β, the formation of complex A1 was markedly decreased, while the binding of PU.1 was unaffected. These results thus indicated that PU.1 and GABP can bind to the LRE1 site. We have termed this portion of LRE1 the EBS (Ets family binding site). Notably, we did not detect the formation of a third complex indicative of both PU.1 and GABP binding to the same DNA molecule. This would suggest that the binding to the two factors is mutually exclusive and as reported elsewhere that PU.1 and GABP may compete for binding to the same site on the DNA (37, 38).

Fig. 2B confirms that PU.1 cannot bind to the mutated EBS oligonucleotide but that NF-κB can, as evidenced by the lack of a PU.1 band and the disappearance of complex A2 when nuclear extracts are preincubated with anti-NF-κB (p50) antibody. Anti-PU.1 antibodies had no effect on the formation of any DNA-protein complexes. Likewise, anti-GABP antisera did not affect complex formation (data not shown).

PU.1 or NF-κB Can Mediate the Response to LPS—Previously, we demonstrated that mutation of the NF-κB binding site resulted in a loss of LPS responsiveness of the sIL-1Ra promoter. Unfortunately, the mutation created in those studies was the same one (Mut2) that blocked the binding of NF-κB, PU.1, and GABP. In order to determine which of these factors was the biologically relevant LPS-responsive transcription factor, site-directed mutants were created utilizing the oligonucleotides indicated, which specifically inhibited the binding of NF-κB (mNF-κB) or PU.1 and GABP (mEBS). The transient transfection studies shown in Fig. 3 indicate that, as previously reported (14), mutation of sequences that disrupt binding of all three factors (Mut2) resulted in an approximately 50% loss in LPS responsiveness compared with the wild type 294-bp promoter (10.1-fold for wild type versus 4.8-fold for Mut2). Mutation of the NF-κB 5′-half-site (mNF-κB) resulted in an LPS response that was essentially indistinguishable from that of the wild type promoter (9.5- versus 10.1-fold, respectively).
Likewise, mutation of bases outside of the NF-κB binding site but within the PU.1/GABP site (mEBS) resulted in no significant loss of LPS-responsive promoter activity (10.4-fold for mEBS versus 10.1-fold for wild type). These data therefore indicated that either NF-κB or the Ets family members PU.1 and GABP can mediate transcriptional activation in response to LPS.

In order to evaluate the specific contribution of GABP in the response of the IL-1Ra gene to LPS, the experiments shown in Fig. 4 were performed. A site-directed mutation was created in which the NF-κB 5′ half-site was mutated from GGG to CCC and the PU.1/GABP binding site was altered by a single C to G change at −82. This alteration resulted in a PU.1 site that has the exact same sequence as the PU.1 site identified in LRE3 (see below). The EMSA experiment shown in Fig. 4A demonstrated that competition with the double mutant mNF-κB/GABP oligonucleotide had no effect on the binding of GABP but could compete for PU.1 binding to the mNF-κB probe (left panel). To confirm that GABP could not bind to the mutated oligonucleotide, EMSA was performed using mNF-κB/GABP as a probe. As shown in the right part of Fig. 4A, this probe could bind PU.1 but not GABP. The transient transfection studies shown in Fig. 4B demonstrated that when transfected into RAW 264.7 cells the double mutated mNF-κB/GABP promoter displayed the same degree of LPS responsiveness as the wild-type promoter. These results therefore indicated that PU.1 binding alone is sufficient for conferring an LPS response upon the IL-1Ra gene.

GABP Participates in the Basal (Unstimulated) Expression of the sIL-1Ra Promoter—In order to determine if NF-κB, PU.1, and/or GABP can play a role in the regulation of the basal (unstimulated) activity of the sIL-1Ra gene, the relative activities of the different site-directed mutants were compared following normalization of transcription efficiencies to cotransfected cytomegalovirus-β-galactosidase. As shown in Fig. 5, complete disruption of the NF-κB/PU.1/GABP binding site (Mut2) led to a 50% decrease in basal promoter activity. Restoration of the NF-κB binding site in mEBS resulted in a small but statistically significant (p = 0.026 by two-tailed Student’s t test) increase in promoter activity, perhaps due to a small amount of activated NF-κB within the nucleus. The promoter construct containing only the EBS site (mNF-κB) was found to actually be 50% more active than the wild type promoter, suggesting that the presence of NF-κB may in fact inhibit the ability of the Ets proteins to activate gene expression. Finally, mNF-κB/GABP, which can only bind PU.1, was found to be 50% less active than the wild type promoter. Since the only difference between mNF-κB and mNF-κB/GABP is the inability of the latter to bind GABP, these data suggested that GABP was the factor primarily responsible for the basal activity of the sIL-1Ra promoter.

An LPS-responsive Promoter Element Is Located between −250 and −225 of the sIL-1Ra Promoter—We previously identified a region of the human sIL-1Ra promoter between −250 and −200 that contained a cis-acting DNA element (LRE3) that was required for full responsiveness of the gene to LPS (14). In order to more precisely define the location of that element, another 5′-deletion was created, resulting in a 225-bp promoter fragment upstream of luciferase. To assess the ability of these truncated promoters to induce transcription in response to LPS, the RAW 264.7 murine macrophage cell line was transfected with a series of promoter constructs ranging from −294 to −202 relative to the mRNA start site. Following stimulation of the cultures for 8 h with 1 μg/ml LPS, the cells were harvested, and lysates were analyzed for luciferase activity.

Fig. 6A shows a compilation of the results from four independent experiments. In each experiment, the LPS response of each promoter construct was compared with the fully responsive 294-bp promoter (RA-294). The results shown in Fig. 6A indicated that deletion of sequences between −250 and −225 resulted in an approximately 45% decrease in the ability of the sIL-1Ra promoter to induce luciferase activity in response to LPS. This decrease represented a loss of responsiveness from 9.4-fold for the 250-bp promoter (RA-250) to 4.4-fold for RA-
Fig. 5. Roles of LRE1-binding proteins in the regulation of basal sIL-1Ra promoter activity. RAW 264.7 cells were transfected with 17.5 μg of the indicated LRE1 site-directed mutant and 2.5 μg of cytomegalovirus-β-galactosidase and split into triplicate cultures. Luciferase and β-galactosidase activities were assessed 48 h after transfection. Luciferase activity in each sample was then normalized to β-galactosidase activity. In all experiments, the wild type 294-bp sIL-1Ra promoter construct was transfected in parallel, and data are expressed as activity relative to the wild type promoter. Data represent the means ± S.D. of at least three independent experiments with each construct.

Fig. 6. Mapping of LRE3. A, 5′ deletion mapping. RAW 264.7 cells were transiently transfected with the indicated sIL-1Ra promoter/luciferase construct. Numbers refer to the length of the promoter construct relative to the transcriptional start site. -Fold response to LPS was determined following an 8-h stimulation with 1 μg/ml LPS as described above. B, heterologous promoter mapping. The indicated region of the sIL-1Ra promoter was cloned upstream of the minimal 81-bp HSV-TK promoter as described under "Experimental Procedures" and transfected into RAW 264.7 cells. -Fold response to LPS was determined following an 8-h stimulation as described above. Data represent the means ± S.D. of at least three independent experiments with each construct.

225, indicating the presence between −250 and −225 of a cis-acting element that was required for optimal response of the sIL-1Ra gene to LPS.

In order to confirm the presence an LPS response element between −250 and −225 and to determine if this element can confer LPS responsiveness upon a normally LPS-nonresponsive promoter, the heterologous promoter experiment shown in Fig. 6B was performed. Fragments of the sIL-1Ra promoter were cloned upstream of the minimal, LPS-nonresponsive HSV-TK promoter. Consistent with our earlier reported results (14), the addition of the −294 to −148 sIL-1Ra promoter fragment could confer LPS responsiveness (approximately 5-fold) upon the HSV-TK promoter. Similar to the results of the deletion studies above, removal of sequences between −294 and −250 had no significant effect on the LPS response. In agreement with the above studies, the −225 to −148 promoter fragment could not confer LPS responsiveness upon the HSV-TK promoter, indicating the presence of an LPS-responsive element between −250 and −225. Furthermore, these data demonstrated that this element could act independently of other more proximal promoter elements to mediate a response to LPS.

PU.1 Binds to LRE3—Sequence analysis of the promoter region between −250 and −220 revealed a potential PU.1 binding site located between −230 and −220. In order to determine if PU.1 or other factors from nuclear extracts of RAW 264.7 cells bound to LRE3, EMSA was performed using a radiolabeled probe corresponding to −237/−220 of the sIL-1Ra promoter (termed LRE3.3). As shown in Fig. 7A, a single complex was formed with this oligonucleotide using nuclear extracts from unstimulated or LPS-stimulated RAW 264.7 cells (lanes 1 and 2). The specificity of this complex formation was determined through competition with excess unlabeled oligonucleotides. Lanes 3–5 indicate loss of binding to the radiolabeled probe when increasing amounts of the unlabeled LRE3.3 oligonucleotide were added prior to the formation of the radiolabeled probe. An unlabeled oligonucleotide corresponding to the PU.1 binding site from the SV40 promoter (39) was also able to compete with the radiolabeled LRE3.3 probe. An additional oligonucleotide (mLRE3) that contained base substitutions within the core GGA region of the putative PU.1 binding site could not compete with the complex (lanes 9–11).

These results indicated that the protein that binds to LRE3.3 was likely to be PU.1. In order to confirm that the LRE3.3-binding protein was in fact PU.1 and not another member of the Ets transcription factor family, the antibody supershift experiments shown in Fig. 7B were performed. Nuclear extracts from LPS-stimulated RAW 264.7 cells were preincubated with antibodies specific for PU.1, Ets-2, or CCAAT/enhancer-binding protein β prior to the addition of radiolabeled LRE3.3 probe. Preincubation with anti-PU.1 antibody resulted in a loss of complex formation (closed arrow) and the formation of a
CCAAT/enhancer-binding protein (b) stimulation with 1 μg/ml LPS as described above. -Fold response to LPS was determined following an 8-h stimulation with the indicated sIL-1Ra promoter construct as described above. -Fold response to LPS was determined following an 8-h stimulation with 1 μg/ml LPS as above. Data represent the means ± S.E. of at least three independent experiments with each construct.

Site-directed mutagenesis of LRE3. A, role of LRE3 in the response of the sIL-1Ra promoter to LPS. RAW 264.7 cells were transiently transfected with the indicated sIL-1Ra promoter construct as described above. -Fold response to LPS was determined following an 8-h stimulation with 1 μg/ml LPS as above. B, contributions of distal and proximal LPS response elements to IL-1Ra promoter activity. The LRE3 PU.1 binding site was mutated in the context of the LRE1 site-directed mutant and transfected into RAW 264.7 cells in parallel with the singly mutated promoter constructs. -Fold response to LPS was determined for each construct following an 8-h stimulation with LPS as described above. Data represent the means ± S.D. of at least three independent experiments with each construct.

slowly migrating supershifted complex (open arrow). Specific CCAAT/enhancer-binding protein β or Ets-2 antibodies had no effect on complex formation. GABP antisera had no effect on complex formation (data not shown). These data therefore confirmed that the LRE3-binding factor is PU.1.

Site-directed Mutagenesis of the LRE3 PU.1 Site Decreases sIL-1Ra Promoter LPS Response—The biological significance of the PU.1 binding site was determined by specifically mutating that site within the context of the 294-bp sIL-1Ra promoter to create 294mLre3. The wild type promoter sequence of GAAGGGGAAATA was altered to GAAGGatccATA. The oligonucleotides used to create the mutation were the same ones that were shown in the EMSA experiments in Fig. 7A to be incapable of binding PU.1.

In the transfection experiments shown in Fig. 8A, the wild type 294-bp promoter/luciferase and the 202-bp promoter/luciferase constructs were transfected in parallel with the LRE3-mutated construct (m3.3) into RAW 264.7 cells. As in the studies above, cells were harvested 8 h following LPS stimulation, and luciferase activity in the lysates was assessed. These experiments demonstrated that specific mutation of the PU.1 binding site resulted in an approximate 50% decrease in LPS responsiveness compared with the wild type promoter (4.5- versus 9.9-fold, respectively). The level of LPS-responsiveness of the mutated promoter was nearly identical to that of the −202 promoter construct. Results from three independent experiments indicated that basal activity of mLRE3 was not significantly different from the wild type promoter (data not shown). These studies therefore confirmed that the binding of PU.1 to the region of the sIL-1Ra promoter between −230 and −220 was important for conferring full LPS-responsive activity upon this gene in macrophages.

The 5′ and 3′ LRE Sites Can Act Independently to Regulate the LPS Response—Since both PU.1 sites each contributed approximately 50% to the LPS response, it was of interest to determine the combined role of these two PU.1 binding sites. A promoter construct was created in which both LRE1 and LRE3 sites were mutated in the context of the 294-bp sIL-1Ra promoter and transfected into RAW 264.7 cells. As described above, the response of the doubly mutated promoter was assessed following an 8-h stimulation with LPS and compared with the activities of the wild type and singly mutated promoters. As shown in Fig. 8B, mutation of both LRE sites resulted in an approximately 75% loss of LPS-responsiveness. This loss resulted in a promoter that is almost completely LPS-nonresponsive (2.1-fold increase over unstimulated). In the experiments shown here, the wild type promoter was 8-fold responsive and the two single mutants were 4.2- and 3.0-fold LPS-responsive for m3.3 and Mut2, respectively. These data indicate that activities of the two LRE sites are additive and confirms the independent nature of these two regulatory elements.

LPS-induced Transactivation of the sIL-1Ra Promoter in HT1080/CD14 Cells Requires PU.1—Previously, Delude et al. (30) demonstrated that HT1080 fibrosarcoma cells stably transfected with CD14 exhibited LPS-dependent induction of NF-κ B activity. In addition, we have shown that transactivation of a modified HSV-TK promoter containing six tandem copies of the IL-1β PU.1 binding site in HT1080/CD14 cells required cotransfection of a PU.1 expression plasmid and LPS stimulation (25). To confirm the role of PU.1 in regulating transactivation of the IL-1Ra promoter in response to LPS, HT1080/CD14 cells were transfected with wild type or mutant IL-1Ra promoter constructs with or without cotransfected PU.1 expression plasmid. As shown in Fig. 9, in the absence of PU.1, all of the IL-1Ra promoter constructs were unresponsive to LPS stimulation. However, when HT1080/CD14 cells were cotransfected with PU.1 expression plasmid, LPS stimulation resulted in an 8-fold increase in wild type promoter activity. In contrast to the results observed using the macrophage cell line, mutation of either the LRE3 PU.1 site (m3.3) or the LRE1 PU.1 (Mut2) site resulted in a significant loss of LPS responsiveness. Only in cases where both PU.1 sites were left intact were we able to maintain LPS responsiveness. Thus, in this model it appeared that the two PU.1 sites acted cooperatively and suggested that macrophages may contain another transcription factor that can act in concert with NF-κ B to induce expression of the sIL-1Ra gene in the absence of the LRE1 PU.1 site.
Although these studies indicate that the human IL-1Ra promoter is differentially regulated in macrophages and these LPS-responsive fibroblasts, they clearly demonstrate that the human sIL-1Ra promoter contains two functional PU.1 binding sites.

**DISCUSSION**

The expression of the gene encoding the secretory form of IL-1Ra is restricted to monocytes, macrophages, polymorphonuclear neutrophils, some fibroblasts, and hepatocytes (32, 40). Previously, we identified three regions within the proximal 294 bp of the human sIL-1Ra promoter that are required for LPS-inducible transcriptional activation (14). These elements were termed LRE1, LRE2, and LRE3. The most proximal of these elements (LRE1) was localized to an NF-κB binding site between positions −93 and −84. LRE2 was localized between positions −148 and −200 and LRE3 between positions −200 and −250, although the factors that bind to these elements were not identified. In this report, we demonstrated that one of the transcription factors that plays a role in the tissue-restricted expression of the sIL-1Ra gene is PU.1. However, unlike the role of PU.1 in many other myeloid-specific genes, the binding of PU.1 to the sIL-1Ra promoter does not play a significant role in the regulation of basal activity in macrophages but rather is required for stimulation in response to LPS (Figs. 3 and 5). Two functional LPS-responsive PU.1 binding sites were identified, one between −230 and −220 (LRE3) and a second between −80 and −90 on the complementary strand. Especially interesting is the finding that the proximal −80/−90 site can also bind the Ets family member GABP and overlaps a site originally identified as the LRE1 NF-κB binding site (14).

Further support for the biological significance for these two sites comes from a comparison of the sequences of the human and mouse sIL-1Ra promoters (41). Both elements are nearly perfectly conserved between the two species (15 of 16 bases for LRE1 and 11 of 12 bases for LRE3).

The transcription factor PU.1 was originally identified as a macrophage and B cell-specific factor that recognized a purine-rich sequence in the major histocompatibility complex class II I-Aβ gene (5′-GGGAA-3′) (39). Separately, Moreau-Gachelin et al. (16) cloned the putative oncogene Spi-1, which is specifically activated in erythroblastic Friend tumors. Later, it was determined that PU.1 and Spi-1 are in fact identical proteins and suggested that abnormal expression of this transcription factor in proerythroblastic cells can result in oncogenic transformation (42). Expression of PU.1 is restricted to myeloid and B-lymphoid origin including macrophages, B cells, early granulocytes and erythroid cells, and megakaryocytes. PU.1 is not expressed in mature granulocytes or erythrocytes (43). Thus, PU.1 is a likely candidate for the regulation of macrophage-specific gene expression and indeed has been demonstrated to be required for the optimal expression of an increasing number of genes in macrophages (reviewed in Refs. 15 and 17). The exact mechanisms through which PU.1 activates LPS-inducible gene expression remain to be elucidated; however, earlier studies demonstrated that PU.1 can be inducibly phosphorylated in response to LPS stimulation, and this phosphorylation appears to be required for the transactivation function of PU.1 (25).

The transcription factor GABP was originally identified as a factor that binds to purine-rich sequences required for the expression of herpes simplex virus type I immediate early genes (44). GABP is composed of two subunits; the α subunit has homology to Ets family proteins and confers DNA binding activity, and the ankyrin-containing β subunit confers transcriptional activating activity and nuclear localization upon the complex (45, 46). The human homologs of GABPα and -β were identified as factors that regulate the activity of the adenovirus E4 gene and were termed E4TF1–60 and E4TF1–53, respectively (47). The ability of GABP to bind to NF-κB sites has previously been documented by Flory et al. (48), who demonstrated that the Raf-1 kinase-induced activation of the human immunodeficiency virus type 1 promoter is due to the interaction of GABP proteins with the two NF-κB binding sites within the human immunodeficiency virus type 1 long terminal repeat. Likewise, PU.1 and GABP have been demonstrated to compete for binding to the same site in the CD18 promoter (37). However, to our knowledge, this is the first demonstration that NF-κB, PU.1, and GABP can bind to the same promoter element. Although the binding sites for these transcription factors do share some homology, it should be noted that not all NF-κB sites will be able to bind PU.1 and/or GABP. Likewise, not all PU.1 binding sites will be able to bind GABP. This is in fact evidenced within the sIL-1Ra promoter described here. A comparison of the PU.1 binding sites in LRE1 and LRE3 demonstrated that a single C to G substitution from GGGGAA to GGCGAA is sufficient to create a PU.1-specific binding site within LRE3 (Fig. 7). Previously, Brown and McKnight have demonstrated that a C at the −2 position (relative to GAA) is preferred for GABP binding (36). This base change is also conserved between the human and murine genes.

Although the data presented here indicate that NF-κB, PU.1, and GABP can bind to a single site on the human sIL-1Ra promoter, it is unlikely that more than one factor can bind to a given molecule of DNA at any one time. Support for this concept comes from our own data presented here, in that we were never able to demonstrate, either by oligonucleotide competition or antibody supershifts, the formation of a tertiary complex composed of two transcription factors and the labeled DNA probe. Additionally, the crystal structures of all three transcription factor-DNA complexes have been solved and indicate that all three factors make contacts with the GGAA (TTCC for NF-κB) ets core (49–52). It would thus appear that NF-κB, PU.1, and GABP will compete for binding to the same site on the DNA. This would be consistent with results from studies on the CD18 and neutrophil elastase promoters, which indicated that PU.1 and GABP compete for binding to the same promoter element (37, 38).

If all three transcription factors cannot bind to the same DNA molecule, the following question arises: Which of these three factors are actually regulating the endogenous sIL-1Ra gene? The answer is not likely to be straightforward. The mechanism of activation of a gene within a single cell may depend on the relative affinities of the different transcription factors for a single binding site. In terms of IL-1Ra gene expression, we suggest that it is PU.1 and not NF-κB that is primarily responsible for LPS-induced gene expression in macrophages. Since PU.1 is constitutively present in the nucleus, NF-κB would have to displace PU.1 from the DNA in order to activate transcription. Given that the 5′ NF-κB half-site within the sIL-1Ra promoter does not conform to the consensus site (GGGTA versus GGGRN, respectively) it is likely that this is not a high affinity binding site. In fact, a binding site with a T in the fourth position was not found in any of the 36 DNA sequences selected for binding to p50 or p65 (53). Interestingly, the murine sIL-1Ra gene contains a T in the same position. Thus on the basis of the site-directed mutagenesis studies shown in Figs. 3–5, we propose that in macrophages, LPS-induced expression of the sIL-1Ra gene is driven by PU.1 and that GABP may play a role in the regulation of basal promoter activity. Transfection experiments with the CD14-expressing HT1080 cell line (Fig. 9) further confirm that PU.1 is required for the response of the sIL-1Ra gene to LPS.
Notably, we did not detect the formation of a GABP complex from nuclear extracts of HT1080 cells (data not shown).

Whether or not PU.1 can displace GABP from the sIL-1Ra promoter (or vice versa) remains to be determined. However, an alternative explanation for how these two factors can regulate the expression of the same gene may be that within the population of cells (or in fact within the same cell), some DNA molecules may have PU.1 bound and some GABP. The implication of this would be that those promoters to which PU.1 is bound will respond to the LPS-induced signal, while those occupied by GABP will not. Given the evidence that GABP is a nuclear effector of the Raf-1-induced signal (48) and since we have previously demonstrated that in RAW 264.7 cells LPS does not signal through Raf-1 (31), it is tempting to speculate that GABP may be a critical factor for the response of the sIL-1Ra gene to other stimuli.

In this study, we have demonstrated that the LPS-induced regulation of sIL-1Ra gene expression is a complex event potentially involving the interactions of three different transcription factors, PU.1, GABP, and NF-κB, with a single proximal promoter element. Additionally, we have demonstrated an additional distal, PU.1-specific binding site located further upstream that can act independently of the proximal site. Together, these two cis-acting elements account for 90% of the response of the IL-1Ra gene to LPS. The results of these studies provide further support to the hypothesis that the response of cells to LPS may be a heterogeneous phenomenon and that the interactions of a group of transcription factors with a single cis-acting element may ultimately depend on the relative affinities and concentrations of the proteins as well as their temporal localization within the nucleus.