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A Novel *cis*-Acting Element Required for Lipopolysaccharide-Induced Transcription of the Murine Interleukin-1β Gene

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Regulatory elements important for transcription of the murine interleukin-1β (IL-1β) gene lie within a DNase I-hypersensitive region located >2,000 bp upstream from the transcription start site. We have identified within this region a novel positive regulatory element that is required for activation of an IL-1β promoter–chloramphenicol acetyltransferase (CAT) fusion gene in the murine macrophage line RAW264.7. Electrophoretic mobility shift analysis of the 3′ portion (−2315 to −2106) of the hypersensitive region revealed at least two nuclear factor binding sites, one of which is located between positions −2285 and −2256. Competitive inhibition studies localized the binding site to a 15-bp sequence between −2285 and −2271. Nuclear factor binding was lost by mutation of the 6-bp sequence from −2280 to −2275. The specific retarded complex formed with RAW264.7 nuclear extract was not detected under similar conditions with nuclear extracts from RLM-11, a murine T-cell line which does not express IL-1β RNA. Mutation of the 6-bp sequence (−2280 to −2275) in the chimeric IL-1β promoter −4093 +1 CAT plasmid virtually eliminated the activation of this reporter gene by lipopolysaccharide (LPS) in transfected RAW264.7 cells. Multimerization of the 15-bp sequence containing the core wild-type 6-bp sequence 5′ of minimal homologous or heterologous promoters in CAT reporter plasmids resulted in significant enhancement of CAT expression compared with parallel constructs containing the mutant 6-bp core sequence. This element was LPS independent and position and orientation dependent. The multimerized 15-bp sequence did not enhance expression in RLM-11 cells. Methylation interference revealed contact residues from −2281 to −2271, CCAAAAAGGAA. Because a search of the NIH TFD data bank with the 11-bp binding site sequence found no homology to known nuclear factor binding sites, we have designated this sequence the IL1β-upstream nuclear factor 1 (IL1β-UNF1) target. UV cross-linking and sodium dodecyl sulfate-polyacrylamide electrophoresis identified an IL1β-UNF1-specific binding factor approximately 85 to 90 kDa in size.

Interleukin-1 (IL-1) is produced in many tissues by a variety of cell types. It has a broad range of biological activities which effect immunological, inflammatory, and nonimmunological responses (see reviews in references 4, 12, 29, and 32). The full spectrum of IL-1 bioactivities appears to be elicited individually by at least two distinct polypeptides (IL-1α and IL-1β) which are encoded by separate genes (29). Despite the fact that the two proteins display only 23% homology at the amino acid level, the IL-1 molecules appear to have essentially identical biological activities.

There is growing evidence that IL-1 plays a central and important role in the pathogenesis of many inflammatory diseases (see reviews in references 3, 9, 15, 16, and 23). Sustained production of IL-1 by both resident and migratory macrophages occurs in diseased tissues as a result of infection, traumatic injury, etc. (14). A specific immune component may also be involved in certain pathophysiologic conditions, and this may serve to amplify IL-1 production in these cells (34, 44). Regardless, IL-1 release by these macrophages not only mediates tissue damage (31, 36, 45, 46) but can also activate a cascade of cytokines (1) that include tumor necrosis factor (1) and IL-6 as well as growth factors. These cytokines and growth factors serve to exacerbate the disease by directly evoking responses in target tissue as well as to function as positive feedback regulatory molecules. The latter may result in sustained IL-1 production leading to chronic inflammation. Generally the cytokine network promotes beneficial effects by maintaining normal homeostasis, but in these circumstances it can become a major contributor to inflammatory disease.

Despite numerous reports on IL-1 regulation and the obvious importance of IL-1 in the cytokine network, very little is known regarding the molecular details of IL-1 production, particularly at the transcription level. Information is now emerging for IL-1β. In the human IL-1β gene, Bensi et al. (6) have identified a phorbol 12-myristate-13-acetate (PMA)-responsive enhancer located between positions −2982 and −2795 upstream of the transcription start site. Recent studies have also shown that NFIL-6 sites (19, 20, 41, 49) both proximal and distal to the cap site are important for the lipopolysaccharide (LPS)-induced activation of the human IL-1β gene. Also in the human IL-1β gene, Shirakawa et al. (41) have identified an LPS-responsive region (−3757 to −2729) which consists of at least six discrete subregions essential for maximal LPS induction. One of the subregions containing an NFIL-6 consensus binding site was capable of conferring LPS responsiveness on a reporter plasmid containing a heterologous promoter. In addition, Gray et al. (22) showed that a cyclic AMP (cAMP) response element-like site within the LPS response region (−2761 to −2753) was necessary for induction of the intact promoter in response to a combination of inducers which consisted of LPS, PMA, and dibutyryl cAMP. Very re-

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cently Zhang and Rom have described two NFIL-6-like regulatory motifs near the transcriptional start site (−90 to −82 and −40 to −32) important for human IL-1β gene induction by LPS, tumor necrosis factor, and the *Mycobacterium tuberculosis* cell wall component lipoarabinomannan (49). In the murine IL-1β locus, we have described both proximal (−100 to −50) (19) and distal (−2586 to −2106) (20) LPS response regions containing NFIL-6 regulatory elements that are necessary for IL-1β activation by LPS. By using electrophoretic mobility shift analysis (EMSA) and specific antibodies, these sites were shown to interact with the C/EBPβ (NFIL-6) and C/EBPδ (NFIL-6β) murine transcription factors (7) in macrophage nuclear extracts, suggesting that both C/EBPβ and C/EBPδ are important in IL-1β gene activation. Taken together, the data strongly suggest an important role for C/EBPβ and C/EBPδ transcription factors in human and murine IL-1β gene transcription.

In this report, we describe a novel *cis*-acting element, IL1β-UNF1 (IL1β-upstream nuclear factor 1), located in the distal LPS response region within 50 bp upstream of the distal NFIL-6 site. Mutation of the IL1β-UNF1 site essentially eliminates LPS responsiveness in a reporter construct containing the murine IL-1β upstream promoter. Characterization of the site revealed that it functions in an LPS- and promoter-independent, position- and orientation-dependent manner in the macrophage cell line RAW264.7; no function was detected in the T-cell line RLM-11. Preliminary characterization suggests a factor of approximately 85 to 90 kDa that binds to the enhancer site.

MATERIALS AND METHODS

Cell culture. RAW264.7 cells (27) (American Type Culture Collection, Rockville, Md.) were maintained in flasks in D10 (Dulbecco modified Eagle medium containing 10% heat-inactivated fetal calf serum [HyClone, Logan, Utah], 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml). RLM-11 T cells ($CD3^+$ $CD4^+$ $CD8^-$ J11d heterogeneous; provided by Dennis Y. Loh, Washington University School of Medicine, St. Louis, Mo.) were maintained in flasks in T10 (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.1 mM nonessential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.35], 1 mM sodium pyruvate) supplemented with 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, Mo.). All culture media were from Gibco-BRL (Gaithersburg, Md.) except as indicated and contained less than 6 pg of endotoxin per ml. LPS (*Escherichia coli* serotype O127:B8; Difco Laboratories, Detroit, Mich.) was suspended at 5 mg/ml in sterile phosphate-buffered saline (PBS) (150 mM NaCl, 8.7 mM K_2HPO_4, 1.6 mM NaH_2PO_4) and stored at −20°C.

Plasmid preparation. pBS-CAT was prepared by subcloning a *Hin*dIII-*Bam*HI fragment containing the chloramphenicol acetyltransferase (CAT) gene followed by simian virus 40 splice and polyadenylation sites from pSV2CAT (21) into pBluescript KS+ (Stratagene, La Jolla, Calif.). The *Bam*HI site was destroyed by digestion with *Bam*HI followed by a fill-in reaction using Klenow fragment, polymerase, and religation.

Plasmids for transfection contained various amounts of IL-1β 5′ flanking sequence and extended to the 5′ portion of the second exon, terminating immediately upstream of the translational initiation codon at position +807 (designated +I). We have consistently observed that all of our IL-1β promoter CAT fusion genes require the presence of intron 1 for optimal CAT expression (unpublished observations). For these plasmids containing the first intron, a fragment extending from the *Bam*HI site in exon 1 through the 5′ portion of exon 2 (immediately 5′ to the translational initiation site) was amplified by PCR and cloned into the *Cla*I site of pBS-CAT, using *Cla*I sites that had been included in the amplification primers. Next, a 4,138-bp *Bam*HI genomic DNA fragment extending from −4093 to +45 of the IL-1β gene was inserted in the proper orientation into the *Bam*HI site in exon 1. Oligonucleotides were synthesized with an Applied Biosystems (Foster City, Calif.) model 380 synthesizer using phosphoramidite chemistry. To prepare the 5′ −50 +I CAT plasmid, a *Bam*HI fragment containing the first 50 bp of the IL-1β upstream and the 5′ 45 bp of exon 1 including the natural *Bam*HI site was generated by PCR. The 5′ PCR primer was (−50) TCA CAG GGA TCC ACT TCT GCT TTT TAG GAC, and the 3′ primer was TGG AGA GGA TCC CAG ATG AGC CTA TTA GGC (the *Bam*HI sites are underlined). The amplified *Bam*HI fragment was inserted into the *Bam*HI site at the 5′ end of the partial exon 1 found in the +I CAT plasmid. A plasmid with an altered IL1β-UNF1 site was created by using site-directed mutagenesis (28) with the −4093 +I CAT plasmid. The sequence of the mutagenesis oligonucleotide was GAT ATA CAT GTT GGT ACC CTT GCC TGC TCT.

Plasmids containing oligomers of the IL1β-UNF1 site (GGG ACC AAA AAG GAA) in front of their respective promoters were prepared using synthetic linkers with a *Kpn*I or *Hin*dIII site and a *Sal*I site at the 3′ end. These linkers were introduced into the *Kpn*I and *Sal*I sites of the −50 +I CAT plasmid or into the *Hin*dIII and *Sal*I sites of the thymidine kinase minimal promoter CAT (pBLCAT2 [30]) plasmid. For the −50 +I CAT plasmid, in which a trimer of the IL1β-UNF1 sequence was placed 3′ of the CAT gene, linkers with *Not*I sites at either end were prepared and inserted into the *Not*I sites of the −50 +I CAT plasmid.

The genomic fragment from −2315 to −2106 (−2315/−2106 genomic fragment) used in the initial EMSA analysis was generated from the murine IL-1β cosmid (10) by using PCR. The spleen focus-forming virus (SFFV) long terminal repeat (LTR)-driven β-galactosidase expression plasmid (SFFV-β-gal) and SFFV LTR-driven CAT plasmid (LTR-CAT) were provided by Dennis Y. Loh (Washington University School of Medicine), and the NFκB-TNF CAT plasmid (39) was provided by S. Taffet (SUNY Health Science Center, Syracuse, N.Y.) and C. Victor Jongeneel (Ludwig Institute for Cancer Research, Epalinges, Switzerland).

Cell transfection. Transient transfection of RAW264.7 cells was performed by electroporation with a Bio-Rad Gene Pulser. Twenty micrograms of test plasmid plus 20 μg of the SFFV-β-gal reference plasmid were mixed with 8×10^6 cells in 0.85 ml of complete Iscove's medium containing 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 1 mM sodium pyruvate and transferred into two 0.4-cm cuvettes. Following a single 250-V, 960-μF pulse, the cells were recombined into a 60-mm-diameter dish containing 5 ml of complete D10 medium. Transient transfection of RLM-11 cells was performed with a model BTX600 electroporator (BTX, San Diego, Calif.). Twenty micrograms of reporter plasmid plus 20 μg of the SFFV-β-gal reference plasmid were mixed with 10^7 RLM-11 cells in 1 ml of nonsupplemented RPMI 1640 medium in a 1.8-mm flatpack. Following a single 400-V, 325-μF pulse, the cells were plated in a 60-mm-diameter dish containing 5 ml of T10 medium.

LPS (5 μg/ml) was added 24 h after transfection, and the cells were harvested 24 h later. Cells were washed with ice cold PBS, resuspended in 0.2 ml of 0.25 M Tris-HCl (pH 7.9), and lysed by three cycles of freezing and thawing. Cell debris was removed by centrifugation for 5 min at 12,000 rpm in a microcentrifuge. Half of each supernatant was used for determination of CAT activity, using [^{14}C]chloramphenicol (Amersham) and an organic-phase extraction protocol (38). The remainder of each supernatant was used to measure β-galactosidase activity (37) for normalization of transfection efficiency.

Nuclear extract preparation. Nuclear extracts were prepared as described by Dignam et al. (11), with some modifications (25).

EMSA. Binding reactions were carried out by incubating 4×10^4 cpm of ^{32}P-end-labeled 30-mer oligonucleotides with 4 μg of nuclear extract in binding buffer (50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 10 mM Tris, 0.5 mM dithiothreitol, 4% glycerol) for 20 min at room temperature. Oligonucleotide-protein complexes were resolved from free oligonucleotide by using a 4% polyacrylamide gel in 0.5× TBE (1× TBE is 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA). As indicated, antisera were incubated with the nuclear extract for 10 min at room temperature prior to the addition of the ^{32}P-labeled oligonucleotide. The anti-Ets1/2 and anti-PU.1 antibodies used in attempts to either block or supershift gel retardation were obtained commercially (Santa Cruz Biotech, Santa Cruz, Calif.) and as a gift from R. A. Maki (La Jolla Cancer Research Foundation, La Jolla, Calif.) (40), respectively.

Methylation interference analysis. The methylation interference analysis was performed as described previously (5). The IL1β-UNF1-containing sense or antisense oligonucleotide (5′-CTA CTC ATT GGG GAC CAA AAA GGA AGT GTG GTC TG-3′) was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and partially methylated with dimethyl sulfate for 1 and 3 min at 20°C. Preparative polyacrylamide gel retardation was carried out with nuclear extracts from RAW264.7 cells prepared as described above. The bound and free bands were isolated by electroelution and centrifuged, and the supernatant was passed over G-25 spin columns (Boehringer Mannheim) to remove contaminating polyacrylamide. The flowthrough material was collected, ethanol precipitated, and cleaved with piperidine for 30 min at 90 to 95°C. The products were analyzed on 20% acrylamide–8 M urea sequencing gels and exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, Calif.). Phosphor image scanning and analysis were performed with a PhosphorImager and software provided by Molecular Dynamics.

UV cross-linking. Both in-gel and in-solution UV cross-linking procedures were carried out as described previously (8). The oligonucleotide probe (−2286/−2256) used in these studies contained four 5-bromodeoxyuridine substitutions. The antisense oligonucleotide spanning −2286 to −2256 with four 5-bromodeoxyuridine substitutions was synthesized by Oligos, Etc. (Wilsonville, Oreg.). It had the sequence TCT CTC AGA CCA CAC TTC CUU TUU GGT CCC.

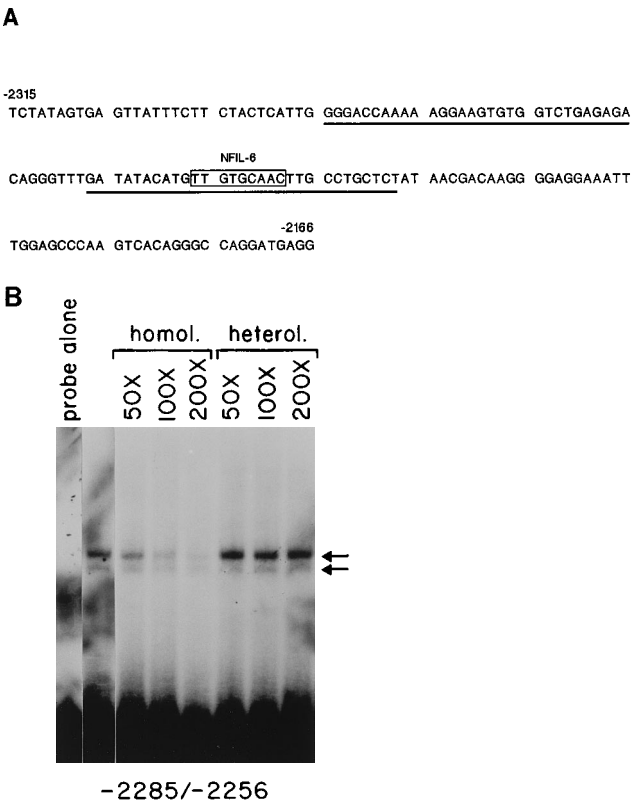

FIG. 1. Interaction of the −2285/−2256 sequence with nuclear proteins. (A) Nucleotide sequence of the region from −2315 to −2166. The locations of the two oligonucleotides which retarded specific complexes are indicated by the solid lines. The NFIL-6-like site is boxed. (B) EMSA using the −2285/−2256 oligonucleotide. EMSA was carried out with end-labeled double-stranded oligonucleotide (−2285/−2256) and nuclear extract from RAW264.7 cells stimulated for 2 h with LPS (5 μg/ml). Reactions were performed in the absence or presence of a 50- to 200-fold molar excess of either unlabeled homologous or heterologous 30-mer oligonucleotide. Probe alone, labeled oligonucleotide without nuclear extract. Arrows indicate the location of the specific complexes.

RESULTS AND DISCUSSION

Localization of a novel specific binding sequence. LPS induces a rapid, usually transient increase in IL-1β gene transcription in murine peritoneal exudate cells and macrophage cell lines (18). Furthermore, this induction of transcription is a direct response to LPS, since transcription occurs in the presence of the protein synthesis inhibitor cycloheximide in both human (13) and murine (data not shown) macrophage/monocyte cell lines.

Our recent findings demonstrated that regulatory elements critical for activation of the murine IL-1β gene by LPS were located between −2315 and −2166 bp upstream of the transcription start site. Within this region, two specific binding sites were identified by EMSA (Fig. 1A). One of these (−2237 to −2229) was identified as a NFIL-6 site which complexed with both C/EBPβ and C/EBPδ DNA-binding proteins from macrophage nuclear extracts (20). In addition, mutation of this site resulted in the loss of LPS responsiveness for a reporter plasmid driven by 4.1 kb of upstream IL-1β promoter sequence plus the entire first intron of the IL-1β gene (−4093 +I CAT) after transfection into RAW264.7 cells. As seen in Fig. 1B, a 30-mer oligonucleotide which spans −2285 to −2256 in the IL-1β gene (Fig. 1A) and includes the second binding site was responsible for specific retarded complexes with nuclear extracts from activated RAW264.7 cells (Fig. 1B). The same complexes were also observed in extracts prepared from unstimulated RAW264.7 cells (data not shown). Significantly, this same specific retarded complex was observed in nuclear extracts prepared from the human monocytic cell line U937 (data not shown).

To better localize the binding site, 15-mer oligonucleotides which spanned the 5′, 3′, and central portions of the −2285/−2256 30-mer oligonucleotide were used as competitors against the homologous 30-mer labeled probe in EMSA. Only the 5′ oligonucleotide (labeled A; −2285/−2271) effectively competed in the binding assays and thus localized the binding site to between −2285 and −2271 (Fig. 2A and B). Mutagenesis of the central sequences (−2280 to −2275) in this 5′ oligonucleotide by replacement with a *Kpn*I restriction site (A.mutant) resulted in its inability to function as a competitor (Fig. 2B and C). These results indicate that the 15-mer contains sequences required for the −2285/−2256-specific gel shift and suggest that some of the nucleotides replaced by the *Kpn*I site are contained in the binding site.

To determine if the binding site and factors responsible for the retarded complexes have some biological relevance, nuclear extracts were prepared from the RLM-11 T cell line (35); we have previously shown that this cell line does not express IL-1 mRNA, nor do various IL-1β promoter constructs function when transfected into this cell line (20). When amounts of RLM-11 nuclear extract similar to those used for RAW264.7 cells were tested, retarded complexes were not observed. Only when the quantity of RLM-11 extract was increased was a similarly migrating complex observed (Fig. 3). The requirement for large quantities of extract to detect the complex was consistent with UV cross-linking studies which compared RLM-11 and RAW264.7 nuclear DNA-binding proteins (discussed below).

The −2280/−2275 sequence is necessary for LPS induction. To determine the functional relevance of this binding sequence, we mutagenized the −2280/−2275 region of the IL-1β −4093 +I CAT construct by insertion of a *Kpn*I restriction site [−4093 (−2280/−2275.*Kpn*I mut) +I CAT] and tested it for LPS inducibility after transfection into RAW264.7 cells. Mutation of this binding site virtually eliminated the induction of CAT activity, while the wild-type −4093 +I promoter, in the presence of LPS, induced CAT activity more than 25-fold over background (Fig. 4). Thus, the binding site, which we designated the IL1β-UNF1 site, appears to be a necessary regulatory element for activation of the IL-1β gene by LPS. Although necessary, the IL1β-UNF1 site is not sufficient for LPS responsiveness, since previous studies showed that mutation of the −2235/−2230 NFIL-6 site alone in the IL-1β −4093 +I CAT construct also was sufficient to abolish LPS responsiveness (20). These results suggest that cooperativity between the NFIL-6 and IL1β-UNF1 upstream sites may be critical for LPS induction. Understanding the molecular details of the cooperativity between the NFIL-6 and IL1β-UNF1 sites must await the isolation and characterization of the factor(s) which interacts with the IL1β-UNF1 site.

Multimerization of the IL1β-UNF1 sequence activates transcription. To further characterize the function of the IL1β-UNF1 sequence, copies of the −2285/−2271 oligonucleotide containing the IL1β-UNF1 site were placed in front of a minimal IL-1β and a minimal heterologous promoter CAT plasmid and transfected into RAW264.7 cells (Fig. 5A). The presence of one or two copies of the IL1β-UNF1 site upstream of the minimal IL-1β promoter CAT plasmid resulted in enhanced CAT activity. There was nearly a threefold-greater enhancement over the activity by one or two copies when the

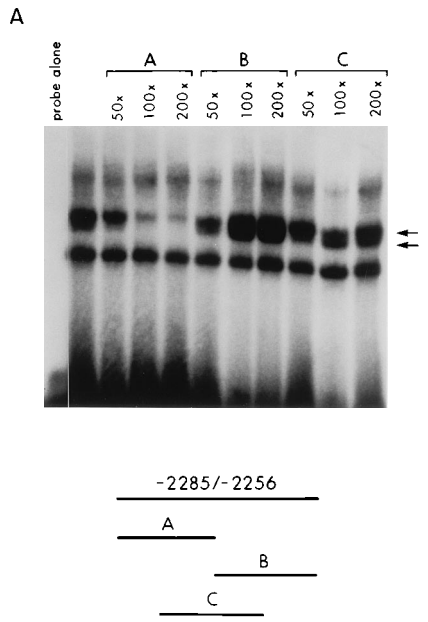

FIG. 2. Localization of binding to the −2285/−2271 sequence. (A) Localization of binding to oligonucleotide A. EMSA was performed as for Fig. 1. The locations of the 15-mer double-stranded oligonucleotides used as cold competitors are as follows: A, −2285 to −2271; B, −2270 to −2256; and C, −2277 to 2263. Arrows indicate the locations of the specific complexes. The more rapidly migrating nonspecific complex was variably present. (B) Nucleotide sequences of the 30-mer oligonucleotide used as the labeled probe (−2285/−2256) and nucleotide sequences of the wild-type 15-mer (oligonucleotide A, −2285/−2271) and mutated (A.mutant, −2285/−2271.mutant) oligonucleotides used as unlabeled competitors. The mutant was created by replacement of the central wild-type sequence (−2280/−2275) with a *Kpn*I restriction site. (C) EMSA with the labeled −2285/−2256 oligonucleotide and the wild-type and mutated 15-mer oligonucleotides as cold competitors. EMSA was performed as for Fig. 1.

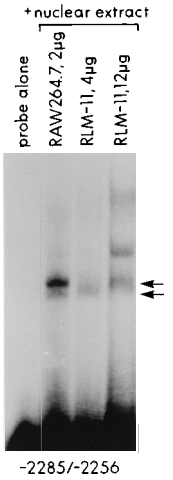

FIG. 3. EMSA of binding reactions with labeled oligonucleotide −2285/−2256 with nuclear extracts prepared from either RAW264.7 or RLM-11 cells. EMSA was performed as for Fig. 1. Note that two- and sixfold more RLM-11 nuclear extract was used in these reactions.

IL1β-UNF1 sequence was trimerized. However, the presence of the IL1β-UNF1 sequence in one, two, or three copies did not alter the degree of LPS inducibility, i.e., the stimulation indices were not altered in the IL1β-UNF1-containing constructs over that of the minimal promoter alone. Enhanced activity was also observed when three copies of the IL1β-UNF1 sequence were placed upstream of the heterologous minimal thymidine kinase promoter. The trimerized sequence was inactive when placed in the antisense orientation upstream [(3×,INV)−50 +I CAT] or in the sense orientation downstream [−50 +I CAT(3×)] of the CAT gene. No enhanced activity was observed when the trimer oligonucleotides, fused to either the homologous or heterologous promoter plasmids, were transfected into the RLM-11 T-cell line (Fig. 5B). These results demonstrate that the IL1β-UNF1 site functions as a position- and orientation-dependent regulatory element in the presence of either a homologous or heterologous promoter. Significantly, it does not function in the non-IL-1β-producing T-cell line RLM-11, nor does it depend on upon activation by LPS.

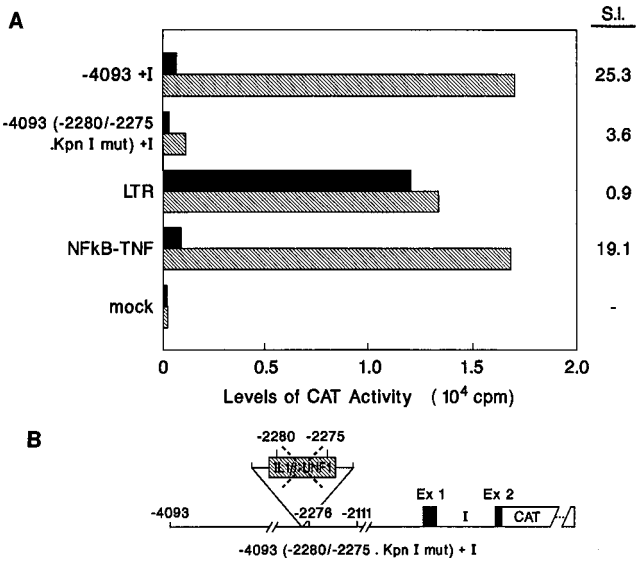

FIG. 4. Site-directed mutagenesis of the IL1β-UNF1 site abolishes LPS inducibility. (A) Results of a transient transfection of the −4093 +I CAT plasmid containing the same *Kpn*I mutation as depicted in Fig. 2B into RAW264.7 cells. S.I., stimulation index. The experiment was repeated three times, and the standard error of the mean was less than 20% after adjusting for the LTR-CAT values. (B) Diagram of the mutated −4093 +I CAT plasmid. Ex, exon.

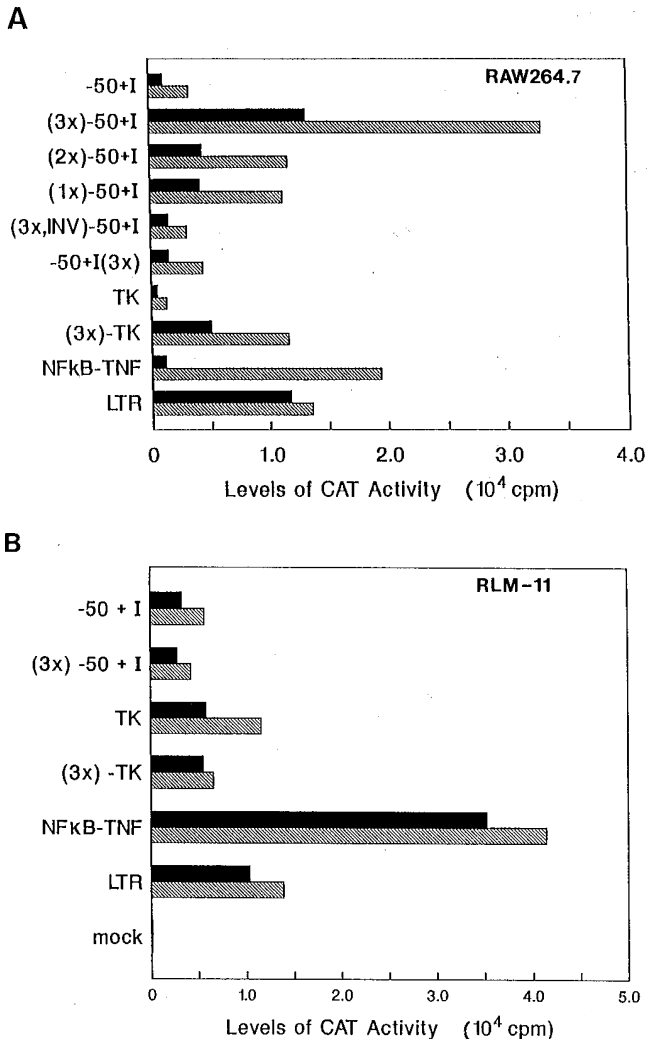

FIG. 5. A multimerized IL1β-UNF1 sequence activates transcription in an LPS-independent and orientation- and position-dependent manner. The IL1β-UNF1 sequence (1× = GGG ACC AAA AAG GAA) was multimerized and placed in front of a minimal IL-1β promoter (−50 +I CAT) and in front of a minimal thymidine kinase (TK) promoter (pBLCAT2) (24). (A) Results of transient transfection in RAW264.7 cells; (B) results of transient transfection in RLM-11 T cells. Each transfection was repeated three times, and the standard error of the mean was less than 25% after adjustment to the LTR-CAT values.

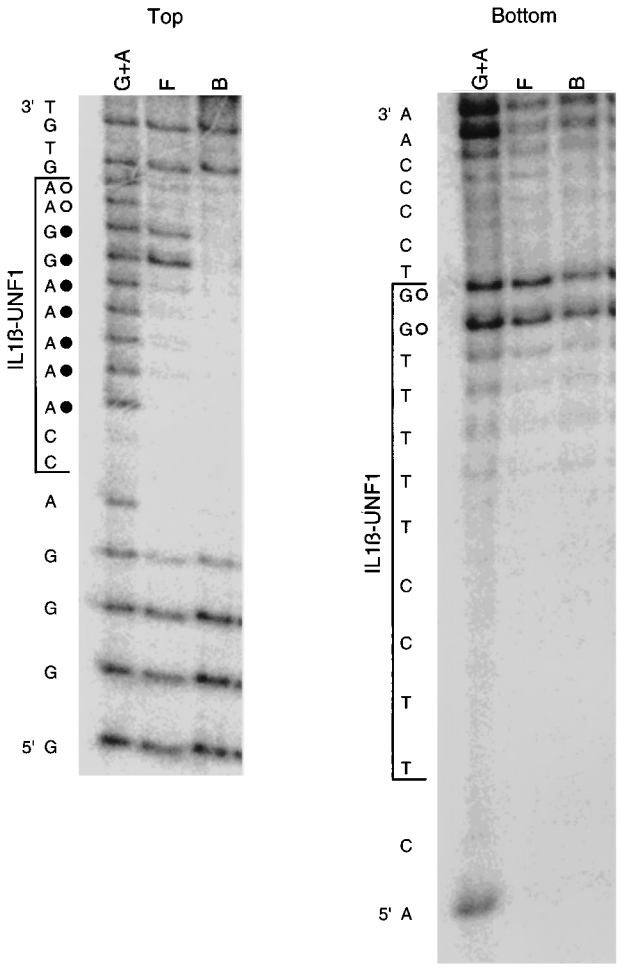

FIG. 6. Methylation interference analysis using RAW264.7 nuclear extract. A 35-mer oligonucleotide containing the IL1β-UNF1 site was labeled on the top or bottom strand, annealed to the complementary strand, and partially methylated with dimethyl sulfate (1-min reaction is shown), and gel retardation was performed with RAW264.7 nuclear extract. Bound (B) and free (F) bands were isolated, cleaved with piperidine, and analyzed on 20% acrylamide–8 M urea gels. A DNA sequencing reaction for guanines and adenines is indicated (G+A). Methylated residues which interfere with binding are indicated by complete (●) and partial (○) interference. The methylated 3′ adenosine residues (top strand) represented 59 and 66% partial interference, respectively (determined by PhosphorImager scanning). The methylated 3′ guanosine residues (bottom strand) represented 42 and 46% partial interference, respectively. Bracketed bases include the contiguous contact residues from upper and lower strands that define the IL1β-UNF1 binding site.

Analysis of the IL1β-UNF1 site by methylation interference. To corroborate the functional data and also to more precisely define the IL1β-UNF1 binding site, methylation interference analysis was performed with a 35-mer oligonucleotide which spanned −2295 to −2261- of the IL-1β gene and encompassed the IL1β-UNF1 site. The results (Fig. 6) revealed that methylation of the adenosines and guanosines in the top and bottom strands within the sequence 5′-CCAAAAAGGAA-3′ interfered with binding to RAW264.7 nuclear extract. Methylation of either of the two guanosines in the top strand resulted in the most dramatic interference; although not as apparent, the quantitation of the 5′ flanking adenosines, when methylated, fully interfered with binding. Only partial interference was noted at the guanosine bases in the bottom strand as well the adenosine bases in the top strand which mark the 5′ and 3′ borders of the binding site, respectively. A search of the NIH TFD database (17) revealed no known sequences homologous to the IL1β-UNF1 regulatory site, CCAAAAAGGAA.

Despite the fact that the AGGAA Ets core motif (48) is present at the boundary of the IL1β-UNF1 site, it is not likely that the Ets family of transcription factors (48) play a role in the IL1β-UNF1 binding site for the following reasons. First, the AGGAA sequence normally is the core sequence in Ets-binding sites, while flanking sequences dictate the specificity of the various family members (47). This is not the case for the IL1β-UNF1 site, where the AGGAA sequence lies at the 3′ boundary. Consistent with this point, the functional IL1β-UNF1-containing enhancer fragment (Fig. 5) terminates with the AGGAA sequence. Second, we have failed to either block or supershift IL1β-UNF1 retarded complexes in EMSA using anti-PU.1 (40) and, in preliminary experiments, anti-Ets1/2 (48) antibodies (data not shown). Since these selected antibodies interact with the most divergent members of the Ets family

(48), which are defined by shared homology in the DNA-binding domains, the results are consistent with the argument that Ets-related factors are not responsible for the retarded IL1β-UNF1 complex. Ultimately, the identification of the factor which interacts at this site must await isolation and characterization at either the gene or protein level. These studies are under way.

UV cross-linking of the IL1β-UNF1-specific complex. To begin the characterization of the protein(s) that binds to the IL1β-UNF1 sequence, UV cross-linking was performed by using two different approaches. The first involved the excision of the UV cross-linked retarded complex from a nondenaturing polyacrylamide gel followed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis under reducing conditions. In the second method, UV cross-linking was performed in solution and the reaction mixture was immediately analyzed by SDS-PAGE. A complex resembling a doublet migrated at approximately 106 kDa and was identified by both approaches (Fig. 7A and B). Considering that a 30-mer oligonucleotide was used for cross-linking, the size of the DNA-binding component of the complex was estimated to be between 85 and 90 kDa. A similar complex was identified in nuclear extracts from the RLM-11 T-cell line and the in-solution method of UV cross-linking (Fig. 7C); however the quantity of the complex observed from these extracts was less than 20% of that observed with nuclear extracts from RAW264.7 cells. As discussed above (Fig. 3), the retarded IL1β-UNF1 complexes were detected at substantially lower levels with RLM-11 nuclear extracts than with RAW264.7 extracts. The discrepancy between the EMSA and UV cross-linking results may just reflect a difference in the sensitivity between the two assays.

The lack of IL1β-UNF1-dependent enhancer activity in RLM-11 cells may be explained by the absence of necessary accessory factors and/or posttranslational modifications of the IL1β-UNF1 complexes in these cells. Alternatively, the lack of activity may be simply due to the subthreshold levels of the IL1β-UNF1 complex needed for transcriptional activation in RLM-11 cells. Future studies will be necessary to clarify the nature of the IL1β-UNF1 complexes between these two cell types. The differences between the two should provide insight into the functionally important aspects of the IL1β-UNF1-associated transcription factors.

Summary and assessment. In this report, we have described a novel site (−2281 to −2271), designated IL1β-UNF1, which is located to within 35 to 45 bp upstream of the previously described NFIL-6 site (20). This IL1β-UNF1 sequence appears necessary but not sufficient for activation of IL-1β gene expression by LPS, since previous findings showed that mutation of the NFIL-6 element alone also resulted in the loss of IL-1β activation (19, 20). The IL1β-UNF1 element, although a necessary component of LPS-induced IL-1β gene activation, functions as an LPS-independent and position- and orientation-dependent element when placed upstream of minimal promoter constructs in RAW264.7 cells. Although this site is active as a monomer when placed upstream of minimal promoter CAT plasmids, its activity was markedly enhanced when it was trimerized, suggesting that the IL1β-UNF1 sequence may function in concert with other elements. These same minimal promoter plasmids containing the IL1β-UNF1 enhancer displayed no activity when transfected into the RLM-11 T-cell line. Consistent with this lack of function are the minimal amounts of IL1β-UNF1-specific complexes detected by UV cross-linking and EMSA with RLM-11 nuclear extracts.

The role of the IL1β-UNF1 site and its associated factors in the overall regulation of IL-1β transcription remains to be elucidated. The importance of the IL1β-UNF1 site and associated factors is strengthened by (i) its presence in the human U937 monocyte cell line (see above) and (ii) the inability of the enhancer to function in the RLM-11 T-cell line, which does not express IL-1 mRNA. This site lies within a tissue-specific DNase I-hypersensitive region which also contains an essential NFIL-6 sequence (20). The proximity of the IL1β-UNF1 sequence to the upstream NFIL-6 site (−2237 to −2229) suggests that there may be cooperative interactions between the two. The C/EBPβ and -δ proteins, which interact with the NFIL-6 site (2, 26), have been shown to interact physically with one another and with other C/EBP family members, as well as with non-C/EBP factors such as NFκB (42). An assessment of the potential interactions between the NFIL-6 and IL1β-UNF1 elements will ultimately require the purification or mo-

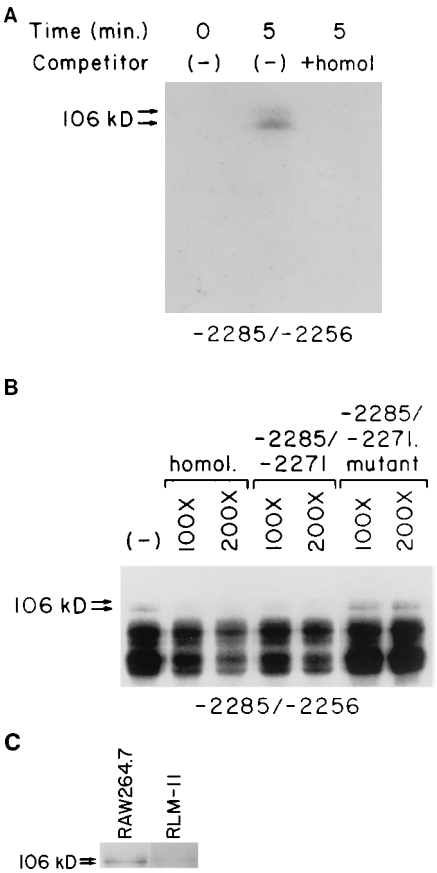

FIG. 7. UV cross-linking. Standard UV cross-linking protocols were carried out as described in Materials and Methods, using a modified −2285/−2256 oligonucleotide with four 5-bromouridines substituted for thymidines in the −2280/−2275 location. (A) Results of a representative UV cross-linking experiment in which the IL1β-UNF1-specific complexes with RAW264.7 nuclear extracts were first run on a 4% nondenaturing polyacrylamide gel, UV cross-linked, cut out of the gel, and loaded onto a reducing SDS-polyacrylamide gel. The arrows indicate the location of the specific complexes. Time indicates the duration of the cross-linking procedure. +homol indicates that the binding reaction was performed in the presence of 100-fold cold homologous competitor. (B) Results of a representative UV cross-linking experiment in which the bromylated, end-labeled oligonucleotide (−2285/−2256) was incubated in solution with LPS-stimulated RAW264.7 nuclear extract in the presence or absence of cold competitor, then UV cross-linked, and finally loaded onto a reducing SDS-polyacrylamide gel. The type and concentration of the cold inhibitor are shown at the top (sequences are given in Fig. 2B). The cold competitors did not contain bromyluridines. (C) Results of a representative in-solution UV cross-linking experiment to demonstrate the quantitative differences of the IL1β-UNF1 complexes between RAW264.7 and RLM-11 nuclear extracts.

lecular cloning of the *trans*-acting factors that interact with the IL1β-UNF1 sequence.

Interestingly, the amount of C/EBPβ and C/EBPδ (7) or the IL1β-UNF1 factors bound to either of their cognate sequences was unchanged following LPS stimulation. Thus, it appears that mechanisms other than DNA binding are involved. For example, activation may depend upon posttranslational modification such as phosphorylation (33, 43) of any of the proteins bound to either of the sequences. Alternatively, activation may depend upon undetected labile proteins or non-DNA-binding adapter proteins whose functions are modulated by LPS stimulation. Finally, there may be LPS-induced changes in chromatin structure surrounding these sequence elements in intact cells. These changes could affect conformation of the proteins bound to either the NFIL-6 or the IL1β-UNF1 site. As a consequence of LPS stimulation, activation domains could be exposed and transcription could proceed.

These and other studies (20, 22, 24, 41, 49) clearly establish that several sequence elements contribute to the regulation of IL-1β transcription. This is not surprising given the numerous stimuli that affect IL-1β transcription and the central role that IL-1β plays in modulating inflammatory processes. Modification of IL-1β transcription by pharmacologic intervention, with the intent of managing inflammatory diseases that manifest IL-1 dysfunction, will require definition of each of the several factors critical to this process.

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