Transcription Factors NF-IL6 and CREB Recognize a Common Essential Site in the Human Prointerleukin 1β Gene

JUNICHI TSUKADA,1 KAZUYOSHI SAITO,1† WAYNE R. WATERMAN,1 ANDREW C. WEBB,2 AND PHILIP E. AURON1,3*

The Center for Blood Research1 and Department of Pathology,3 Harvard Medical School,
Boston, Massachusetts 02115, and Department of Biological Sciences,
Wellesley College, Wellesley, Massachusetts 021812

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A site located between −2782 and −2729 of the human prointerleukin-1β (IL1B) gene functions as a strong lipopolysaccharide (LPS)-responsive enhancer independent of the previously identified enhancer located between −2896 and −2846 (F. Shirakawa, K. Saito, C. A. Bonagura, D. L. Galson, M. J. Fenton, A. C. Webb, and P. E. Auron, Mol. Cell. Biol. 13:1332–1344, 1993). Although these two enhancers appear to function cooperatively in the native sequence context, they function independently as LPS-responsive elements upon removal of an interposed silencer sequence. The new enhancer is not induced by dibutyryl cyclic AMP (dbcAMP) alone but is superinduced by costimulation with LPS-dbcAMP. This pattern of induction depends upon the nature of the sequence, a composite NF-IL6-cAMP response element (CRE) binding site. This pseudosymmetrical sequence is shown to contrast with a classical symmetric CRE which responds to dbcAMP but not LPS. DNA binding studies using in vivo nuclear extract, recombinant proteins, and specific antibodies show that LPS induces the formation of two different complexes at the enhancer: (i) an NF-IL6-CREB heterodimer and (ii) a heterodimer consisting of NF-IL6 and a non-CREB, CRE-binding protein. Cotransfection studies using NF-IL6 and CREB expression vectors show that NF-IL6 transactivates the enhancer in the presence of LPS, whereas CREB acts either positively or negatively, depending upon its cAMP-regulated phosphorylation state. Our data demonstrate that the newly identified enhancer is a specialized LPS-responsive sequence which can be modulated by cAMP as a result of the involvement of NF-IL6-CRE-binding protein heterodimers.

The prointerleukin-1β (proIL-1β) gene coding for the IL-1β precursor protein (referred to here by its genomic locus name, IL1B) has been previously reported (10) to be rapidly and transiently transcribed in monocytes by lipopolysaccharide (LPS). The immediate-early transcription of this gene in the absence of protein synthesis (10) strongly supports a mechanism by which one or more preexisting transcription factors is activated. Such immediate activation is inconsistent with the involvement of AP-1 transcription factors, since unstimulated THP-1 monocytes lack the Jun proteins essential for AP-1 activity (38).

We recently demonstrated (40) that LPS induction of the human IL1B gene requires a 406-bp regulatory sequence located between positions −3134 and −2729, upstream of the transcription start site (now called the upstream induction sequence [UIS]). In particular, two binding sites located between −2896 and −2846 of the UIS were shown to be important for LPS-induced transcriptional activation. The proteins which bind to this sequence are NF-IL6 (C/EBPβ) and an interferon stimulation response element-like binding protein. The role of NF-IL6 as an LPS-responsive transcription factor present in a preexisting inactive form in unstimulated cells has been established previously (2, 22, 28) and is consistent with the immediate-early induction of the IL1B gene.

We now report that an additional binding site in the UIS is important for LPS induction. This site is centered on position −2764 and resembles a cyclic AMP (cAMP) response element (CRE). LPS has been reported to be an efficient immediate-early inducer of IL1B only in the presence of a costimulant (19, 39). The CRE-binding protein (CREB) preexists in cells as an inactive protein which supports transcription following protein kinase A (PKA)-mediated phosphorylation at serine 133 (13, 49). CREB has been variously reported to bind to two classes of CRE as follows: (i) perfectly symmetrical CRE (symCRE) sites consisting of two overlapping CGTCA palindromic half-sites such as those found in the somatostatin (29) and α-chorionic gonadotropin genes (42) and (ii) asymmetrical CRE (asymCRE) sites consisting of the sequence CGTCA, representing a single perfect half-site, such as that present in the tyrosine aminotransferase (3) and enkephalin genes (6). The symCRE can bind CREB in both the phosphorylated and phosphorylated states whereas the asymCRE can bind only phosphoCREB (31). Recent reports have shown that the pseudosymmetrical CRE (pseudCRE), consisting of one perfect and one almost perfect half-site, from the phosphoenolpyruvate carboxykinase (PEPCK) gene, is transactivated by either NF-IL6 or the related C/EBPα as well as CREB (33, 34, 41). Furthermore, it has also been shown that the pseudCRE site preferentially binds a heterodimer between NF-IL6 and C/ATF (a novel ATF-like factor) (46). These results suggest a diverse transcriptional regulation by members of the CREB-ATF family that is dependent upon the nature of the binding site. In particular, the IL1B LPS-responsive CRE-like site contains a core sequence which is identical to the PEPCK

* Corresponding author. Mailing address: The Center for Blood Research, Harvard Medical School, Warren Alpert Bldg., Room 154, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 278-3270. Fax: (617) 278-3131. Electronic mail address (Internet): AURON@CBRSGL.MED.HARVARD.EDU.
† Present address: First Department of Internal Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Kitakyushu 807, Japan.
psymCRE (TTAGGTCA), which can bind NF-IL6, a factor known to respond to LPS in appropriate cell types. Consequently, we examined the enhancer activity of the IL6 psymCRE in transiently transfected monocyte cells and demonstrated that this sequence is extremely sensitive to LPS but does not respond to cAMP in the absence of the LPS costimulant. This activity is observed when the psymCRE is present as either a single copy sequence or a component of the entire UIS regulatory region. Strikingly, the single-copy activity of this sequence is dependent upon its isolation from a silencer sequence which is contained within the UIS. This silencer strongly inhibits function when isolated from the remainder of the UIS, which contains a weaker enhancer that appears to cooperate with the psymCRE to eliminate most of the suppression.

Cotransfection of the IL1B psymCRE chloramphenicol acetyltransferase (CAT) reporter with an expression vector containing a truncated dominant-negative-acting NF-IL6 as well as electrophoretic mobility shift assay (EMSA) studies using specific antibodies (Abs) reveals that the involvement of the psymCRE in LPS induction is likely dependent upon NF-IL6, like the LPS-responsive site between −2896 and −2846 which we previously reported (40). Strikingly, a phosphoCREB protein is observed as a dominant-negative regulator of LPS induction at this site. This is in contrast to phosphoCREB, which is detected on the IL1B psymCRE probe only when LPS-treated cells are treated with a dibutyryl cAMP (dbcAMP) costimulant which acts synergistically to increase expression. Cotransfection of the IL1B psymCRE CAT reporter with a PKA catalytic subunit expression vector also results in increased LPS sensitivity. Binding studies using nuclear extracts and recombinant CREB and NF-IL6 proteins support the existence of complexes between these two proteins that can account for both the dominant-negative effect of CREB in the absence of dbcAMP and the synergy in the presence of either dbcAMP or PKA catalytic subunit. Additional studies demonstrate that a symCRE that binds CREB, but not NF-IL6, is not responsive to LPS. This argues that the IL1B psymCRE is a specialized LPS-responsive sequence which can be modulated by cAMP as a result of its ability to bind NF-IL6-CRE-binding protein heterodimers.

MATERIALS AND METHODS

Endotoxin tests. All materials and solutions including plasmid preparations, dbcAMP, phosphate-buffered saline, media, and fetal bovine serum for tissue culture and transfection were tested for endotoxin by a limulus ameboocyte lysate assay (QCL-1000; Whittaker Bioproducts, Inc., Walkersville, Md.). In particular, we have observed that the source of dbcAMP is critical, since commercial preparations can be contaminated with very high levels of endotoxin as assayed by limulus lysate. The dbcAMP (Boehringer Mannheim) used in these studies contained less than 1 pg of endotoxin per ml. To reduce endotoxin levels, baked glassware, sterile irrigation water (Baxter Healthcare Corp., Deerfield, Ill.), and disposable sterile pipettes (Costar Corp., Cambridge, Mass.) were used.

Plasmids and protein expressions. Human IL-1β genomic DNA fragments were derived from clone BDC454 (5). We have used the same numbering of the sequence as that described in our recent report (40) except that we recently realized that the upstream sequence for the human IL1B gene contains an additional two C nucleotides between positions −2748 and −2747. This error has been corrected in the GenBank entry (accession number L06808). Accordingly, the UIS between positions −3132 and −2729, which had been reported in our previous report (40), is now located between −3134 and −2729. Various portions of the UIS which are located between −3,134 and −2,729 bp upstream of the transcription start site were inserted into CAT gene plasmid vectors containing a minimal (−59 to +105) murine c-fos promoter (fosCAT) (27). All novel constructs were sequenced as described previously (40). The regions in this element were designated B through I (left panel in Fig. 1) as described previously (40). The nomenclature used for various constructs reflects the nature of the inserted fragment. For example, D-G/fosCAT represents a fosCAT vector containing regions D through G. The DNA fragments were generated either by restriction endonuclease digestion or by using PCR primer oligonucleotides to generate deletions or site-directed mutations as described previously (17). The region H modifications resulted in the replacement of 35 bp from −2832 to −2798 of IL1B (Fig. 1, construct 12) with the sequence CCCGGTGCAGAAAATCTTCCAGCTTGA. This sequence is derived from the joining of the downstream end (Sall site) of the IL1B insert in construct 6 of Fig. 1 (B-G) with the upstream end (HindIII site) of a fragment (−2797 to −2729) by ligating a BglII linker after blunting with Klenow enzyme. The pBlue 610 containing the coding region of full-length NF-IL6 and pBlue 620 with a truncated NF-IL6 cDNA lacking the internal sp1-sp1 fragment (amino acid sequence between 41 and 205) were kindly provided by S. Akira (Osaka University, Osaka, Japan). Expression vectors for the full-length NF-IL6 (pcNF-IL6) and for the truncated NF-IL6 with a deletion of the internal sp1-sp1 fragment (pcNF-IL6 (Δsp1)) were generated by inserting the respective coding regions into pcDNA1 (Invitrogen Corp., San Diego, Calif.) and were used in cotransfection studies. For in vitro translations, pBluescript (Stratagene, La Jolla, Calif.) containing the full-length NF-IL6 coding region in a T7 orientation (pBlueNF-IL6) was used. The coding region for the CREB protein was a gift from M. R. Montminy (The Clayton Foundation Laboratories for Peptide Biology, La Jolla, Calif.). CREB expression vector (pcCREB) was also generated by inserting the CREB coding region into pcDNA1 and was used in both cotransfection and in vitro translation studies. The pTKCAT4CRE CAT reporter vector, which contains four tandem repeats of the CRE site of the human somatostatin gene, was kindly provided by S. Ishii (Tsukuba Life Science Center, Tsukuba, Japan) and I. Nishimoto (Massachusetts General Hospital). The pCMVCo expression vector for the α-catalytic subunit of PKA was a gift from M. D. Uhler (University of Michigan). Recombinant full-length NF-IL6 and CREB proteins were produced in a TNT-coupled reticulocyte lysate system (Promega, Madison, Wis.) by using pBlueNF-IL6 and pcCREB, respectively.

Abs. Anti-NF-IL6 Ab was kindly provided by S. Akira. This Ab was raised in rabbits against a synthetic peptide corresponding to the sequence of NF-IL6 between amino acids 224 and 277 (peptide 3) (2). The anti-CREB Ab was kindly provided by J. F. Habener (Massachusetts General Hospital). This Ab can recognize both the CREB and CREM proteins but is more selective for the CREB antigen. The anti-phosphoCREB Ab (anti-phosphoCREB Ab) was specifically directed against phosphorylated CREB on Ser-133 and was a gift from M. E. Greenberg (Harvard Medical School) and does not recognize a-phosphoCREB (12).

Oligonucleotides. The double-stranded oligonucleotides used as competitors were as follows (the core recognition sequence of each oligonucleotide is underlined): blunt-ended competitors, AP-1 binding site, CCCGACCTTGGCCGGAA; NF-κB binding site, AGTTGAGGGGAGTTCCAAGGC; CRE site, AGAGATTGCCCAGCTGACAGAGATGCT.
AG; and mutated CRE site (mCRE), AGAGATTGCTGAGGTCAGAGAGCTAG. The NF-IL6 site from the human IL-6 gene (oligonucleotide IL6) was as described previously (40). The AP-1, NF-kB, and CRE site oligonucleotides were purchased from Promega Corp.

**Transfection and CAT assay.** Transfections and CAT assays were performed as previously described (40). The human THP-1 monocytic cell line (ATCC TIB 202) was transfected by the DEAE-dextran method. This technique was used because it did not generate induction of the endogenous IL1B gene. THP-1 cells (10^7 cells per plate) were transfected with 10 to 20 μg of plasmids. The cells were treated with LPS from *Escherichia coli* O55:B5 (Sigma Chemical Co., St. Louis, Mo.) or dbcAMP (Boehringer Mannheim) at 24 h after transfection and incubated for an additional 24 h before harvesting. The CAT assays were carried out by a liquid scintillation method (43) with 100 μg of THP-1 cell lysate. The protein concentrations for extracts were assessed by Bio-Rad Bradford protein assay kit (Melville, N.Y.). CAT activities were evaluated by calculating slopes from plots of time versus counts per minute within a linear range of the response. The slopes were determined by the use of a polynomial curve fit which allowed the extraction of first order, initial rates, for the reaction.

**EMSA.** Nuclear extracts were prepared from THP-1 cells after a 45-min incubation with 10 μg of LPS per ml or a combination of 10 μg of LPS per ml and 500 μM dbcAMP by the method previously reported (40). In experiments using anti-phosphoCREB Ab, in order to inhibit phosphatase activities in THP-1 nuclear extracts, 10 mM NaF–1 mM orthovanadate–1 mM ZnCl2–5 mM sodium PPi–9 mM β-glycerophosphate was further used for the preparation of THP-1 nuclear extracts. Region I probe was labeled by filling in 3' recessed ends with DNA polymerase Klenow fragment and two [α-32P]deoxynucleoside triphosphates (dATP and dCTP) at 3,000 Ci/mmol (DuPont-NEN). Blunt-ended CRE oligonucleotide was labeled with T4 polynucleotide kinase and [γ-32P]ATP. Unincorporated deoxynucleoside triphosphates were removed by use of G-25 (5 Prime–3 Prime, Inc., Boulder, Colo.). Radiolabeled probes were further purified on a 10% polyacrylamide gel. Binding reaction conditions and polyacrylamide gel electrophoresis were as previously described (40), except that the binding buffer contained 40 mM NaCl and 1.2 μg of poly(dI-dC) (Pharmacia).

**RESULTS**

A 54-bp element (−2782 to −2729) shows higher levels of LPS-induced enhancer activities than the entire UIS. Using transient transfection, we recently demonstrated that the regulatory region which controls the LPS induction-specific expression of the IL1B gene is primarily located between positions −3134 and −2729 (now called the UIS). In order to further characterize specific sequence requirements for LPS induction, various portions of this 406-bp-long region were subcloned into a CAT vector system containing a minimal murine c-fos promoter and assayed for LPS-induced CAT activity following transfection into a human THP-1 monocyte cell line. The full-length UIS CAT construct (Fig. 1, construct
1; B-I) showed a relatively low level of background activity in untreated THP-1 monocyte cells and an eightfold-higher activity in the presence of LPS. As shown in constructs 1 (B-I) and 2 (D-I), deletion of regions B through C from the UIS did not significantly alter LPS-induced CAT activity, whereas a further deletion of regions D through G (construct 3; H-I) reduced the activity to approximately 40% of that of the full-length UIS.

This result agreed well with our previous report (40) that an NF-IL6 binding site in region E and an interferon stimulation response element-like binding site (NF-81 site) in region F are key elements for UIS induction (Fig. 1 schematic). Further deletion of region H from the sequence of construct 3 resulted in a vector which contained only region I (construct 4; −2782 to −2729) and generated a threefold-higher CAT activity following LPS treatment than did the entire UIS. This demonstrated that the short 54-bp-long region I element is a strong LPS-responsive enhancer and that region H acts as a silencer. Furthermore, our finding that deletion of region I from the UIS (construct 5; B-H) resulted in an 80% loss of full-length UIS inducible activity supported this argument. Construct 6 (B-G), which differed from construct 5 by containing an additional deletion of region H, resulted in a partial restoration of activity (50% of UIS). Further removal of either region B (construct 7; C-G) or regions B and C (construct 8; D-G) did not completely abolish LPS-inducible activity. Sequences derived only from region B (construct 10; upB) and from region C (construct 11; C) were inactive. The suppressive effect of region H is also observed when the activity of construct 7 (regions C-G) is compared with that of construct 9 (regions C-H).

Evidence for the suppressive effect of region H in the context of the full-length UIS was further demonstrated by replacing a portion of region H within the UIS with an irrelevant sequence (construct 12; B-I/ΔupH). This resulted in a fourfold increase in inducible activity over that of the wild-type UIS (construct 1; B-I) and an induction fold similar to that observed for the product of regions D-G and I. These data demonstrate that the UIS contains two independent, perhaps cooperative, LPS-responsive enhancer elements, regions I (construct 4) and D-G (construct 8), and further reveal that an interposed element, region H, negatively regulates the LPS induction mediated by each of these two regions.

Activation of region I requires the binding of essential proteins. Our sequence analysis of region I suggested a possible protein binding site (Fig. 2A). This site is a psymCRE site, TTACGTCA, located between positions −2768 and −2761. This core sequence is identical to the psymCRE site in the PEPCK gene promoter, which has been reported to be essential for cAMP responsiveness of this gene. Deutsch et al. (9) have observed that in the 8-bp palindrome consensus sequence for CRE-binding proteins, TGCAGTCA, a single point mutation of the central C residue to a G caused a 90% decrease in transcription activity. Therefore, we inserted this CRE-diagnostic point mutation into region I (Fig. 2A; I/mCRE; TTAgGTCa), resulting in a complete loss of LPS-inducible CAT activity (Fig. 2B). This result demonstrates the importance of this psymCRE site for LPS induction of region I and suggests the possible involvement of a CREB-like protein.

In order to examine specific DNA-protein binding to the psymCRE site within region I, a region I probe was used along with various unlabeled competitor DNA fragments which contained informative wild-type and mutated binding sites. Nuclear extracts were prepared from THP-1 cells stimulated with LPS for 45 min, when nuclear run-on analysis showed that

**FIG. 2.** A single point mutation within a CRE site results in a complete loss of LPS-induced CAT activity for region I (−2782 to −2729). (A) The sequence of wild-type and mutated region I. (B) CAT activity for transfected region I constructs in which 10 μg of either the wild-type (I/wt) or point-mutated I/mCRE reporters were transfected into THP-1 cells. After transfection, cells were either left untreated (open bars) or treated with 10 ng of LPS per ml (solid bars). The CAT data were normalized to the average activity elicited by wild-type I/mCRE in the presence of LPS. Error bars represent SD from three independent experiments.

LPS induction of IL1B transcription is at its maximum (10). Figure 3 (lanes 1 and 7) shows that two specific complexes possessing distinct EMSA mobilities ('Slow' and 'Fast') are formed with wild-type region I probe. Both complexes were effectively inhibited (lane 4) by a 10-fold molar excess of a consensus symCRE (TGAGCTCA) oligonucleotide. In contrast, the mCRE oligonucleotide, which contains the single base mutation of the critical central C residue to a G in the CRE consensus oligonucleotide (TGAGCTCA), could not completely compete for the two complexes (lane 5). Further, region I containing the same point mutation (I/mCRE, containing TTAgGTCa) also showed no significant competition (lane 9). These data confirmed the CAT assay mutation results and strongly suggested the binding of a CREB-like protein to the psymCRE. However, our inspection of this psymCRE site in region I further suggested the possibility that this binding sequence resembled the binding sites for NF-IL6 (see Fig. 13) (2) and AP-1, TGAC(G/T)N(A/C) (36, 37). Consequently, we carried out competition studies with well-known strong binding sites for NF-IL6 and AP-1 proteins as unlabeled oligonucleotides. As shown in lane 6, an oligonucleotide containing the NF-IL6 binding site from the human IL-6 gene competed for both region I complexes, although to a lesser extent than competitors containing either a symCRE (lane 4) or the region I psymCRE (lane 8). In contrast, the AP-1 binding site did not compete (lane 12). These data taken
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important role in the site recognition for both complexes. Furthermore, the 'Slow' complex consisted of both NF-IL6 and CREB, whereas the 'Fast' complex reacted with anti-NF-IL6 Ab, but not with CREB Ab, suggesting that CREB, in contrast to NF-IL6, is not contained within this complex. However, EMSA competition studies (Fig. 3, lanes 4 and 6) showed that the region I factors had a higher affinity for a symCRE than for the NF-IL6 binding site (TTTGTGCAAT), which does not resemble a CRE (i.e., it does not contain a CGTCA motif). Therefore, it is unlikely that the 'Fast' complex is an NF-IL6 homodimer; it may rather involve NF-IL6 and a non-CRE CRE-binding protein.

In addition to the symCRE site, region I also contains a possible NF-κB binding motif, GGGCATTGCC, between −2758 and −2749 (40). Since we have observed that a CAT construct containing tandem repeats of an NF-κB binding site responds to LPS treatment in THP-1 monocytes (not shown) and it has recently been reported that LPS can efficiently activate NF-κB in these same cells (7), it is possible that NF-κB may be involved in region I activity. However, as shown in lane 11 of Fig. 3, a 10-fold molar excess of an oligonucleotide containing a strong p50-type NF-κB binding site capable of binding many forms of the NF-κB/Rel protein family (23) did not compete for any region I complexes. Competition could not be observed even when a 100-fold molar excess of the NF-κB site was used (data not shown). Moreover, a region I oligonucleotide containing an internal deletion of the entire NF-κB site (I/ΔNF-κB) efficiently competed with both complexes (lane 10). Thus, the competition studies did not suggest the involvement of NF-κB protein in the formation of any DNA-protein complexes.

**Nuclear protein binding to the region I CRE is dependent upon LPS treatment.** Because the transfection studies showed a strong LPS-dependent activation of region I, we examined THP-1 nuclear extract binding to region I probe in either untreated or LPS-treated cells. As shown in Fig. 4, only weak binding to radiolabeled region I probe was detected for untreated cells (lane 1). However, after LPS treatment, the intensities of the region I complexes were significantly enhanced and reached a maximum at 1 h poststimulation in good agreement with our previously reported run-on data (10).

**Recombinant NF-IL6 and CREB form a complex which binds to region I.** As described above, EMSA using nuclear extract from LPS-treated THP-1 cells revealed a possible association of NF-IL6 with one or more CRE-binding proteins. Therefore, binding studies of the region I probe using in vitro-translated NF-IL6 and CREB were performed in order to determine whether these proteins can associate in a manner similar to that of the nuclear factors derived from cell extracts. As shown in Fig. 5, both proteins (CREB, lane 1, and NF-IL6, lane 2) could individually bind to region I. However, when a mixture of both proteins was preincubated and then added to the region I probe, a DNA-protein complex with a stronger intensity was detected (lane 3). However, since the migration pattern of this complex was similar to that of CREB homodimer, it was unclear whether this complex was derived from a newly formed NF-IL6-CREB heterodimer or from enhanced CREB homodimer binding. Therefore, we carried out EMSA studies using the non-cross-reacting anti-NF-IL6 and anti-CREB Abs described above. As shown in lanes 4 and 9, both anti-NF-IL6 and anti-CREB Abs reacted, indicating that both NF-IL6 and CREB were involved in this complex. The observation that the anti-CREB Ab-supershifted complex observed in the presence of both NF-IL6 and CREB was eliminated by anti-NF-IL6 Ab (lane 5) further supports the existence of NF-IL6-CREB heterodimers.

![FIG. 3. EMSA analysis of protein binding to region I. The entire 54-bp-long wild-type region I was used as a radiolabeled probe. The THP-1 cell nuclear extract was derived from cells treated for 45 min with 10 μg of LPS per ml. Cold competitor DNA was as described in the text. All competitors were used in 10-fold molar excess. Antibodies were anti-NF-IL6 (3 μl per lane) and anti-CREB (0.5 μl per lane). After incubation of THP-1 nuclear extracts with these Abs at room temperature for 30 min, the radiolabeled region I probe was added.](http://mcb.asm.org/)

Together suggest that the region I psymCRE may be an NF-IL6-CREB composite binding site.

The identity of the proteins which bind to the region I psymCRE was determined by using anti-NF-IL6 and anti-CREB Abs. Anti-NF-IL6 Ab (2) completely eliminated both complexes generated with THP-1 nuclear extracts (lane 2), while anti-CREB Ab supershifted only the 'Slow' complex (lane 3). The abrogation of NF-IL6 binding by the anti-NF-IL6 Ab is consistent with the earlier observation made by Akira et al. (2) with the same Ab and likely relates to the fact that this Ab was raised to a unique peptide located immediately adjacent to the amino side of the DNA binding domain. Furthermore, the anti-NF-IL6 Ab could only immunoprecipitate NF-IL6 out of human SK-MG-4 glioblastoma cell nuclear extract (2) and did not cross-react with either C/EBPα or NF-IL6β, two closely related proteins (1). We tested cross-reactivity between anti-NF-IL6 Ab and anti-CREB Ab by EMSA with recombinant NF-IL6 and CREB proteins. Recombinant CREB protein was supershifted by anti-CREB Ab, whereas anti-NF-IL6 Ab did not block the binding of recombinant CREB. Furthermore, anti-CREB Ab did not supershift the recombinant NF-IL6-DNA complex, whereas anti-NF-IL6 Ab abrogated complex formation (data not shown). Therefore, these two Abs do not cross-react, and the data of Fig. 3 demonstrate that the formation of both complexes was abrogated by the anti-NF-IL6 Ab, arguing that NF-IL6 plays an
LPS-induced transactivation of region I is regulated positively by NF-IL6 and negatively by CREB. In order to examine the functional involvement of NF-IL6 and CREB at region I, we cotransfected pcNF-IL6 (NF-IL6) and pcCREB (CREB) expression vectors along with the wild-type I/fosCAT reporter into THP-1 cells. As shown in Fig. 6, LPS-treated cells showed a pcNF-IL6 dose-dependent increase in CAT activity. The observation that pcNF-IL6 cannot transactivate I/fosCAT in the absence of LPS (Fig. 6, closed squares) is consistent with other reports (30) which demonstrate that NF-IL6 is active only following induction-dependent posttranslational modification. Moreover, the finding that pcNF-IL6 did not transactivate region I/mCRE in the presence of LPS also supports our argument that NF-IL6 acts through the region I psymCRE site (not shown). In contrast, cotransfection of pcCREB inhibited LPS induction of I/fosCAT (Fig. 6, closed circles). In order to test for NF-IL6-CREB synergy, varying amounts (0 to 8 μg) of pcCREB and a constant amount (1 μg) of pcNF-IL6 were both cotransfected along with I/fosCAT. Synergy was not evident (Fig. 6, open squares), but activity inhibition similar to that for the cotransfection of only pcCREB was observed.

Recent studies of NF-IL6 and LAP, the rodent homolog, revealed that NF-IL6 lacking the activation domain between amino acids 1 and 198 functions as a repressor of NF-IL6-dependent transcriptional activation (8, 18). We therefore investigated the effect of expression of a truncated NF-IL6 deficient in amino acid sequence between residues 41 and 205 [mNF-IL6(Δ347)] on region I transcription activation in the presence of LPS. This truncated NF-IL6 harbors intact basic and leucine zipper regions. As shown in Fig. 7, expression of mNF-IL6(Δ347) antagonized the LPS induction in region I in a dose-dependent manner. At 8 μg of the pCMNF-IL6(Δ347), a loss of approximately 80% of the LPS-induced activity was observed. This result clearly shows that LPS-induced activation via the region I psymCRE is dependent upon NF-IL6 protein.

The region I psymCRE is an LPS-responsive enhancer that is modulated by cAMP. By using THP-1 cells, the effects of cAMP and/or LPS on CAT activity for I/fosCAT were examined and compared with the activity of a plasmid, pTKCAT4CRE (4CRE), containing four copies of a classic symCRE similar to that used as a competitor in the EMSA described above (Fig. 3, lane 4). Figure 8 shows that stimula-
with LPS alone resulted in a 10-fold increase in CAT activity for IfosCAT but did not affect 4CRE (compare groups 1 and 2). On the other hand, IfosCAT did not respond to treatment with 100 μM dbcAMP alone, which was capable of eliciting a fivefold induction of 4CRE (group 3). This difference was especially obvious with a higher dose of dbcAMP (500 μM) which resulted in a 13-fold increase for 4CRE (group 4). Treatment with a combined LPS-dbcAMP stimulus, however, resulted in a 61- and 16-fold stimulation, respectively, for IfosCAT and 4CRE (group 5). Therefore, both CRE sites can support synergy between LPS and dbcAMP; however, a single copy of the region I psymCRE appears to be more sensitive to the dual stimulus than do four tandem copies of the symCRE.

In order to confirm the involvement of PKA in the induction of IfosCAT, an expression vector for the PKA catalytic subunit α (pCMVαCα) was used instead of dbcAMP (Fig. 9). This is relevant because increased levels of cAMP are known to cause the activation of PKA, which in turn results in the phosphorylation of CREB protein (13, 24). Cotransfection of pCMVαCα in the absence of stimulation did not result in a significant increase in region I activity, whereas in the presence of LPS the expression of the PKA catalytic subunit enhanced LPS-induced activity for region I in a dose-dependent manner. This result agreed well with the dbcAMP data (Fig. 8), which demonstrates that region I is not responsive to PKA-related induction in the absence of LPS. Consequently, symCREs and psymCREs may differ in their response to a single stimulant in THP-1 cells.

The observation that the dual dbcAMP-LPS stimulus can support a PKA-like effect at region I led us to reexamine the effect of CREB on transcriptional regulation via region I with dbcAMP-LPS as a stimulant. As shown in Fig. 10A with IfosCAT, and in contrast to the data from induction by LPS alone (Fig. 6), cotransfection of 1 μg each of pCREB and pCNF-IL6 in the presence of the dual stimulus resulted in a modest (25%) but reproducible transactivation synergy for region I (open squares). This synergy was reduced at higher ratios of CREB to NF-IL6. The use of either the pCMVαCα PKA catalytic subunit expression vector (Fig. 10B) or a higher concentration (500 μM) of dbcAMP (not shown) confirmed the importance of NF-IL6 and the synergistic effect between CREB and NF-IL6, demonstrating a similar small, but significant, increase in activity. These data suggest that phosphorylated CREB can function as an activator through association with NF-IL6 at region I. In order to address this possibility, we examined the phosphorylation state of CREB protein with either LPS or LPS-dbcAMP dual induction with the aid of an Ab (12) specifically directed against the phospho-Ser-133 CREB protein (anti-phosCREB Ab). As shown in Fig. 11, the dual stimulus resulted in an enhanced formation of both fast and slow complexes (compare lanes 2 and 3). However, in the case of the dual stimulant, only the slow complex was efficiently supershifted by anti-phos-CREB Ab (compare lanes 2 and 4), supporting an argument that the CREB component of the complex is phosphorylated by dbcAMP but not by LPS.
were treated normalized

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radiolabeled complex was generated by complex (lanes 1-7) CREB. That binding. 

significant competition distinguishes symCRE and LPS-treated cells from the human gene.

The involvement of this enhancer (lanes 1-7) is essential for inducible IL6 responsiveness to cAMP. However, this enhancer is not responsible for LPS induction of the human IL1B gene.

The symCRE is an 8-bp palindromic sequence, TGACGTCA, composed of two overlapping palindromic CGTCA half-sites and is recognized by CRE-binding proteins such as CREB and ATF (4, 16). The region I core sequence, TTACGTCA, is a symCRE similar to that which confers cAMP responsiveness upon the PEPCK promoter (35). However, recent reports that NF-IL6 (C/EBPβ), a bZIP transcription factor, can bind the PEPCK CRE (33) suggest an involvement of this LPS-responsive factor in the function of the region I symCRE. In particular, since the region I symCRE site possesses a G residue as the first 3' flanking sequence, the difference between the consensus sequence for NF-IL6 (2) and the region I CRE sequence is two nucleotides (Fig. 13). Furthermore, the upstream half-site, TTACG of the region I core sequence, is a perfect match with the NF-IL6 binding site (−71 to −58) in the haptoglobin gene (2, 32). Because NF-IL6 and the IL-1B gene are both sensitive to LPS, and cAMP can act as a costimulant, it seemed reasonable to explore the possibility that this single composite site could mediate the action of both agents.

FIG. 8. Effect of LPS and/or dbcAMP on CAT activity for UfosCAT and pTKCAT4CRE reporters. A total of 10 μg of either UfosCAT or pTKCAT4CRE was transfected into THP-1 cells. Cells were treated as indicated in each of the five groups. The CAT data were normalized to the average activity elicited by the LPS-induced UfosCAT reporter in the absence of expression vector cotransfection. Error bars represent SD from a minimum of two independent experiments.

Unlike the region I psymCRE, LPS does not induce NF-IL6 to bind to a symCRE. As shown in Fig. 8, the symCRE of the 4CRE reporter, in contrast to the region I symCRE, did not respond to LPS. Therefore, we examined specific protein binding to a symCRE site within THP-1 nuclear extract and a radiolabeled symCRE probe. Figure 12 shows that, unlike the region I probe, nuclear extracts derived from both untreated and LPS-treated cells showed formation of only a single constitutive complex (lanes 1 and 2). The migration of this complex was similar to that generated with in vitro-expressed CREB (lane 7) and was efficiently eliminated by a symCRE oligonucleotide (lane 3). However, in contrast to what was observed with the region I complexes in Fig. 3, an oligonucleotide containing a strong NF-IL6 binding site did not reveal significant competition for the symCRE complex (lane 4). In addition, the use of specific Abs (lanes 5 and 6) demonstrates that CREB, but not NF-IL6, is a component of the symCRE complex generated by THP-1 cell nuclear extract. This further distinguishes symCRE from psymCRE sites and demonstrates the distinct specificity of the region I symCRE for inducible NF-IL6 binding. The involvement of NF-IL6 in the activity of region I, in contrast to that of the symCRE, likely accounts for the LPS sensitivity of the region I symCRE sequence.

DISCUSSION

In this study, we demonstrated that a short 54-bp subregion (region I; −2782 to −2729) of the 406-bp UIS has the capability to function as a strong LPS-responsive enhancer in the human IL1B gene. The activity of this enhancer in isolation is approximately threefold greater than that of the full-length UIS as a result of a silencer (region H) found immediately adjacent in the intact sequence. Region H appears to strongly suppress both region I and the other UIS enhancer core (regions D-G), which we previously reported contains two transcription factor binding sites essential for maximal UIS activity (40). Therefore, the UIS consists of at least two independently acting enhancer regions (regions I and D-G) which appear to cooperate in order to overcome most of the suppression caused by region H. The interplay of the two enhancers and the region H silencer results in the observation that the UIS is strongly dependent upon the integrity of both enhancer sites. Furthermore, our observation that mutation of the psymCRE in region I results in complete activity loss for this sequence argues that the psymCRE is essential for region I-mediated LPS induction of the human IL1B gene.
NF-IL6 AND CREB MEDIATE proIL-1β GENE EXPRESSION

FIG. 10. Region I transcription in THP-1 cells is affected by a dual stimulant involving LPS and the cAMP-PKA pathway which results in the activation of latent CREB activity. A constant amount of IfosCAT reporter was used (10 μg), and the total amount of transfected DNA was kept constant (18 μg) by the addition of pcDNA1 vector. (A) Cells were transfected with expression vectors as indicated along with IfosCAT reporter and were stimulated with a combination of 10 ng of LPS per ml and 100 μM dbcAMP. Symbols: open circles, pcNF-IL6 (NF-IL6); closed circles, pcCREB (CREB); open squares, a constant amount of pcNF-IL6 plus varying amounts of pcCREB as indicated (CREB + 1 μg of NF-IL6). (B) Instead of dbcAMP, a PKA catalytic subunit α expression vector (pCMVα) was used. Various combinations of pCMVα, pcNF-IL6, and pcCREB were introduced into cells along with IfosCAT as indicated. The cells were then stimulated with 10 ng of LPS per ml. The CAT data were normalized to the average activity elicited by the induced IfosCAT reporter in the absence of expression vector cotransfection. Error bars represent SD from three independent experiments for A and two independent experiments for B.

It is well known that many bZIP transcription factors form heterodimers. CREB has been reported to heterodimerize with CREM (25) and ATF-1 (20). NF-IL6 has been reported to dimerize and/or associate with NF-IL6β (21). Fos, Jun (18), C/ATF (46), and NF-κB/Rel transcription factors (26, 44, 45). Interestingly, in the present study, our EMSA data show two distinct LPS-inducible complexes between THP-1 nuclear proteins and the region I psymCRE. The more slowly migrating complex contains proteins that react with both CREB and NF-IL6-specific Abs. Furthermore, the observation that the Ab raised to a unique sequence adjacent to the NF-IL6 DNA binding domain abrogates all binding and that the ILIB psymCRE appears to consist of a composite of NF-IL6 and CREB recognition sites is consistent with the existence of a novel NF-IL6-CREB heterodimer. Moreover, binding studies using recombinant proteins reveal that NF-IL6 and CREB likely associate to form heterodimers in vitro. On the other hand, it has been reported that the anti-phosCREB Ab reacts with two other phosphoproteins which migrate more rapidly than CREB on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12). In this regard, it is noteworthy that the migration of the slow complex (Fig. 5, lane 6) recognized by anti-phosCREB Ab is similar to that generated by a heterodimer between recombinant CREB and NF-IL6.

The faster-migrating EMSA complex formed between region I and THP-1 nuclear extract is recognized by anti-NF-IL6 Ab but not by anti-CREB Ab. This complex probably does not result from the binding of an NF-IL6 homodimer because the protein has a higher affinity for a symCRE than for the NF-IL6 binding site from the human IL-6 gene. In this regard, Akira et al. (2) have reported that NF-IL6 homodimer has a much higher affinity for the NF-IL6 binding site from the IL-6 gene than for a symCRE. Using an NF-IL6 probe and THP-1 nuclear extracts, we also observed anti-CREB Ab-resistant DNA-protein complexes which were efficiently competed for by the NF-IL6 binding site, but not by a symCRE (not shown). This distinct result is likely due to the binding of either NF-IL6 homodimer or another C/EBP protein which binds to the same site. Therefore, it is likely that the ‘Fast’ complex consists of NF-IL6 and a CRE-binding protein other than CREB (NF-X in Fig. 13).

The possibility that an NF-IL6-CREB complex binds to the region I psymCRE caused us to examine functional properties of these factors in cotransfection assays. The observations that cotransfected NF-IL6 expression vector transactivates region I in the presence of LPS and that the Δgpf deletion expression vector acts as a dominant negative argue that NF-IL6 is involved in region I-mediated LPS induction. This argument is supported by the additional observation that a symCRE, which binds CREB, but not NF-IL6, does not respond to LPS. Furthermore, our data show that, in the absence of LPS, NF-IL6 does not transactivate region I. These results are consistent with reports that NF-IL6 requires posttranslational modification to become a competent activator. Examples
The Ab I region
LPS per nuclear
in dbcAMP
bZIP domain
protein (MAP)
in incubated with the Ab under the
ported by
kinase cascade
include phosphorylation by
phosphorylation by
Ziff
and
psymCRE,
because
explains
mechanism
response
activation of
dependent
transfection of
enhance
relatively
poor

FIG. 11. Determination of CREB phosphorylation state within the
region I complex following stimulation either with LPS or with a
combination of LPS and dbcAMP by EMSA using anti-phosphoCREB
Ab. The radiolabeled region I probe was incubated with THP-1
nuclear extracts derived from THP-1 cells treated with either 10 µg of
LPS per ml or a combination of 10 µg of LPS per ml and 500 µM
dbcAMP for 45 min. The anti-phosphoCREB Ab (anti-phosCREB) is
described in Materials and Methods. In lanes 1 and 3, a rabbit pre-
immune serum was added as a control. The THP-1 nuclear extracts were
incubated with the Ab under the same conditions as those for Fig. 3.

include phosphorylation by a ras-dependent mitogen-activated
protein (MAP) kinase at threonine 231 and 235 outside the
bZIP domain (30) and by a calcium-calmodulin-dependent
kinase cascade at serine 325 within the leucine zipper (47).
Consequently, LPS stimulation may activate at least one of
these signal transduction pathways. This argument is sup-
ported by a report which demonstrates that LPS induces MAP
kinase activation in a monocyte cell line (48). In addition, Metz
and Ziff (28) have observed that activation of the serum
response element in the rat c-fos gene following activation of a
cAMP-PKA signaling pathway depends upon NF-IL6 phos-
phorylation and translocation into the nucleus. Although this
mechanism explains the cAMP responsiveness of the c-fos
serum response element, it is unlikely that the cAMP-PKA-
dependent activation of NF-IL6 directly regulates the region I
psymCRE, because neither dbcAMP treatment nor the co-
transfection of a PKA catalytic subunit expression vector
transactivated region I in the absence of LPS (Fig. 8 and 9).
In agreement with this result, PKA catalytic subunit is unable to
enhance NF-IL6-dependent transcription (33) and NF-IL6 is a
relatively poor substrate for phosphorylation by the PKA
catalytic subunit (47). Therefore, NF-IL6 does not likely
respond directly to cAMP in the absence of LPS.

In contrast to NF-IL6, cotransfection of a CREB expression
vector strongly inhibited the induction of region I. However, it
is interesting that in the presence of both dbcAMP and LPS,
transfection of a 1:1 ratio of CREB and NF-IL6 expression
vectors enhanced transcription (Fig. 10). The activation of
region I by CREB was also observed when LPS-treated cells
were cotransfected with a PKA catalytic subunit expression
vector. These data suggest that CREB is involved in both
potential and negative regulation at region I through an associ-
ation with NF-IL6. Such a dual role for CREB as both a
repressor and an activator at the same target site has been
reported for other genes and appears to be dependent upon
the state of CREB phosphorylation (13, 24), where only
phosphoCREB is capable of gene activation. Nichols et al.
have reported that a hemiphosphorylated CREB dimer can
also act as a repressor (31). In EMSA with an Ab specifically
directed against phosphorylated CREB (12), we have demon-
strated that CREB protein in the NF-IL6–CREB heterodimer

FIG. 12. A symCRE, unlike region I, does not bind NF-IL6. The
symCRE oligonucleotide described in Materials and Methods was
used as a radiolabeled probe. Cold competitors described in the
text were used at a 10-fold molar excess. EMSA was carried out under
the same conditions as in Fig. 3. Nuclear extracts were derived from
THP-1 cells left untreated (lane 1) and treated with 10 µg of LPS per
ml for 45 min (lanes 2 to 6). As a control, recombinant CREB was used
(lane 7). Abs and competitors were used as described for Fig. 3.
is phosphorylated by treatment of cells with LPS-dbcAMP but not by LPS alone. Consequently, we can propose a mechanism in which aphosphoCREB inhibits LPS induction through association with NF-IL6 and requires the additional cAMP-PKA phosphorylation in order to act as a positive factor (Fig. 13). This agrees well with the observation that either dbcAMP or PKA catalytic subunit synergizes with the essential LPS stimulus in order to induce region I.

As mentioned above, the region I psymCRE resembles the well-characterized cAMP-responsive element in the PEPCK promoter (35). However, this element has been reported to bind transcription factors other than CREB. For example, both NF-IL6 (C/EBPβ) and the related C/EBPα can bind to the PEPCK psymCRE and induce transcription (33, 34). The AP-1 factor has also been reported to bind to this CRE element (15). However, our EMSA competition studies using THP-1 nuclear extracts and an AP-1 binding site do not reveal any significant inhibition of complexes formed following LPS treatment. The IL1B gene is an immediate-early gene in THP-1 cells, and transcription can be induced in the absence of protein synthesis (10). Consequently, the lack of an involvement of AP-1 is consistent with the observation that Jun, a subunit of AP-1, is not expressed in unstimulated THP-1 cells (38) and should not be abundant in the 45-min-postinduction nuclear extracts used in our studies.

A striking difference between region I and the PEPCK psymCRE site is the absence of cAMP induction for region I. The CREB protein has been shown to constitutively bind to the PEPCK psymCRE and induce transcription following a conversion of inactive aphosphoCREB into the active fully phosphorylated dimer via a cAMP-PKA pathway (31). Although the region I psymCRE can bind in vitro-expressed CREB, neither dbcAMP nor PKA catalytic subunit alone can transactivate region I in our transfection assays. The absence of dbcAMP activation is not due to an inability of PKA to function in these cells, since symCRE sites present in the pTKCAT4CRE vector respond to dbcAMP. These results, as well as the absence of CREB homodimer binding by THP-1 nuclear extract to the region I probe (Fig. 3), suggest that active phosphoCREB homodimers are effectively inhibited by

FIG. 13. Scheme summarizing a possible mechanism for IL1B gene regulation by region I of the UIS. The region I psymCRE is shown to be related to the binding sites for both CREB and NF-IL6 and appears to support the binding of NF-IL6-CREB heterodimers. The data presented support a model in which the activation of region I by LPS stimulant alone (right-hand solid "+") arrow) may be diminished by binding site competition with an inactive NF-IL6-aphosphoCREB heterodimer which is also observed to bind to the region I site (central gray "--" arrow) following LPS-induced NF-IL6 activation. The dominant-negative effect of cotransfected pcCREB expression vector supports this argument and suggests the possibility that aphosphoCREB homodimers may be inhibitory as a result of an overexpression of CREB, which may either bind as a homodimer or promote subunit exchange (dashed lines) of the active NF-IL6-NF-X to form the inhibitory NF-IL6-CREB heterodimer. Neither dbcAMP nor transfected PKA catalytic subunit alone can induce the region I site because of the absolute dependence upon NF-IL6, which requires LPS for activation. The dual stimulus may result in a stronger response, at least in part, because the CREB component of the heterodimer becomes phosphorylated and is likely a competent transactivator (left-hand solid "+") arrow). This results in the binding of only active factors to the psymCRE (i.e., the NF-IL6-phosphoCREB and NF-IL6-NF-X heterodimers). The unknown second partner for NF-IL6 likely responsible for LPS induction in the absence of dbcAMP (NF-X) is probably a non-CREB CRE-binding protein as judged by binding site competition and the absence of anti-CREB Abs reactivity as determined by EMSA.
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