Transcription of a Minimal Promoter from the NF-IL6 Gene Is Regulated by CREB/ATF and SP1 Proteins in U937 Promonocytic Cells

Allison Berrier, Gerald Siu and Kathryn Calame

*J Immunol* 1998; 161:2267-2275; ;

http://www.jimmunol.org/content/161/5/2267

---

**References**

This article cites 50 articles, 28 of which you can access for free at:

http://www.jimmunol.org/content/161/5/2267.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Transcription of a Minimal Promoter from the NF-IL6 Gene Is Regulated by CREB/ATF and Sp1 Proteins in U937 Promonocytic Cells

Allison Berrier, Gerald Siu, and Kathryn Calame

NF-IL6 is an important transcriptional regulator of genes induced in activated monocytes/macrophages, and NF-IL6 is the only CCAAT/enhancer-binding protein (C/EBP) family member whose steady-state mRNA levels increase upon activation of monocytes (1). We show that increased transcription of the NF-IL6 gene is responsible, at least in part, for induction of NF-IL6 mRNA following activation of U937 promonocytic cells. We have identified a 104-bp minimal promoter region of the NF-IL6 gene that is sufficient for basal and activation-dependent induction of transcription in U937 cells. This region contains binding sites for the cAMP response element-binding protein/activation transcription factor (CREB/ATF) and Sp1 families of transcription factors. Each site is functionally important and contributes independently to transcription of the NF-IL6 gene in U937 cells. The Journal of Immunology, 1998, 161: 2267–2275.

The NF-IL6 gene was first identified as a transcription factor in monocytes that bound an IL-1 response element in the IL-6 promoter (2). NF-IL6 is a member of the CCAAT/enhancer-binding protein (C/EBP) family of basic-leucine zipper transcription factors that bind as dimers to a consensus C/EBP site (TTNNNGNAAN). NF-IL6 is strongly induced upon activation of monocytes, and it is the only known C/EBP protein induced following monocyte activation (1). In monocyte/macrophages, NF-IL6 is critical for activation-dependent expression of numerous cellular and viral genes. Many cellular genes expressed in activated macrophages, including IL-1β, IL-6, IL-8, macrophage (M)-CSF, TNF-α, granulocyte (G)-CSF, and nitric oxide synthase, contain functionally important C/EBP sites in their transcriptional regulatory regions (2–6). NF-IL6 is also required for the induction of latent HIV-1 provirus and for replication of HIV-1 in monocytes/macrophages (1, 7).

The biologic importance of NF-IL6 in macrophages has been confirmed by gene targeting experiments in mice. NF-IL6−/− mice have impaired macrophage function, including a poor response to bacterial or viral pathogens and severely defective tumortoxicity (8). Cytokine production by macrophages derived from NF-IL6−/− mice is abnormal, confirming the importance of NF-IL6 for regulated transcription of cytokine genes. In addition, splenomegaly due to B cell and monocyte/macrophage hyperplasia is frequently observed in older NF-IL6−/− mice in conjunction with elevations in the serum levels of IL-6 and macrophage (M)-CSF, suggesting that some biologic functions of NF-IL6 in vivo are not completely understood (9, 10).

Although NF-IL6 mRNA is expressed in many tissues, its expression is usually tightly regulated by cellular activation or differentiation. Steady-state NF-IL6 mRNA levels are strongly induced following activation of monocytes/macrophages by a variety of effectors including mitogens and cytokines such as LPS, PMA, IL-1, and IL-6 (2). NF-IL6 is induced upon activation of splenic B cells by the polyclonal B cell mitogen LPS (11). In adipocytes, NF-IL6 mRNA levels decrease during terminal differentiation (12). The molecular basis for the regulated expression of NF-IL6 in these cell lineages is not well understood.

The importance of NF-IL6 in monocytes/macrophages for activation-dependent regulation of cellular genes and of HIV-1 provirus prompted us to investigate the regulation of NF-IL6 mRNA expression in monocytes/macrophages. As a model, we have utilized the U937 promonocytic cell line that can be activated to undergo differentiation by treatment with LPS and/or PMA (13). Our studies demonstrate that in U937 cells steady-state levels of NF-IL6 mRNA increase following cellular activation with LPS and PMA due to increased transcription initiation of the NF-IL6 gene. Analysis of the NF-IL6 promoter showed that a 104-bp region was sufficient to confer strong basal and PMA-responsive promoter activity in U937 cells. Within this region, binding sites for CREB/ATF and Sp1 families of transcription factors were identified and shown to be functionally important for promoter activity. Finally, endogenous CREB/ATF proteins were shown to be important for maximal NF-IL6 promoter activity in U937 cells.

Materials and Methods

Tissue culture

The promonocytic U937 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 supplemented with 10% FCS and gentamicin. LPS (Salmonella typhimurium type w; Difco, Detroit, MI) was resuspended at 10 mg/ml in H2O and used at a final concentration of 10 μg/ml. Phorbol-12-myristate 13-acetate purchased from Sigma Chemicals (catalog number P-8139; St. Louis, MO) was resuspended at 1 mg/ml in DMSO and used at a final

Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032

Received for publication October 16, 1997. Accepted for publication April 23, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

© 1998 by The American Association of Immunologists

This work was supported by National Institutes of Health Grants GM29361 and AI40342 to K.C. and Grant AI34925 to G.S.

1 Address correspondence and reprint requests to Dr. Kathryn Calame, Department of Microbiology, Columbia University College of Physicians and Surgeons, 701 West 168th Street, New York, NY 10032.

2 Current address: Department of Physiology and Cell Biology, Albany Medical College, Albany, NY 12208.

3 Abbreviations used in this paper: C/EBP, CCAAT/enhancer-binding protein; M-CSF, macrophage CSF; CREB/ATF, cAMP response element-binding protein/activation transcription factor; CRE, cAMP response element; AP-1, activator protein-1; wt, wild type; EMSA, electrophoretic mobility shift assay.
concentration of 10 ng/ml. For the stimulations, U937 cells were plated at a density of 1 x 10^6 cell/ml before the addition of LPS or PMA.

**Luciferase assays**

The murine NF-IL6 genomic clone was a generous gift of V. Poli (Istituto Ricerche di Biologia Molecolare, Pomezia, Italy). The 16-kbp genomic DNA Sau3A fragment was cloned into the BamHI site of a LEMBL3. An NcoI fragment containing 2 kbp of the transcription initiation site and 125 bp 3' of the initiation site was end-filled and blunt-end cloned into the Smal site of pG59uc. A plasmid containing 1 kbp of the 5' initiation site was generated by digestion of the 2-kbp luc construct with HindIII and Smal, with subsequent end fill and religation. The HindIII site is a unique restriction enzyme site located on the 5' end of the pG9uc cloning polylinker, whereas the AvrII site is located approximately 1 kbp upstream of the start site of NF-IL6 transcription. The 125-bp NF-IL6 promoter construct was generated by digestion of the 2-kbp luc construct with HindIII and Smal, and with subsequent end fill and religation. The 125-bp NF-IL6 promoter plasmid served as the PCR template for generation of the -97 to +72, -74 to +72, -44 to +72. The primers used for PCR amplification of the minimal promoter constructs were -97 AAG GAA GCT CCG GTT GCC GGG G -74 AAG GAA GCT TGG CCC ACC GCT GAC -44 AAG GAA GCT TGG CCC CAT TAT AAC +72 GGA AAG GCT TCC GAC GCA G. The construct used for transcription efficiency control contains the renilla luciferase gene driven by the thymidine kinase promoter (Promega, Madison, WI). The site I mutant -97 to +72 NF-IL6 promoter plasmid containing 13 nucleotide sequence changes in a 15-bp nucleotide region extending from -53 to -67 was cloned upstream of the firefly luciferase reporter gene through mutagenic PCR using the Smal construct as the PCR template. The sequence of the mutagenic PCR product is as follows: 5' AAG GGC CCG CTT GCC GAC GTC CAC ACC CTT GGG GCC CCT CCG 3'.

**Nuclear run-on assay**

For the nuclear run-on assay, U937 cells were cultured in the presence of LPS/PMA or media alone for 12 h. Cells (5 x 10^6) were set aside for FACS analysis, and the intensity of CD54 (ICAM-1) staining was used to monitor cellular activation. The nascent RNA transcripts within nuclei prepared from the remaining of the cells were eluted in vitro in the presence of the radiolabeled nucleotide [32P]CTP. The labeled RNA transcripts were isolated as described previously (14). A total of approximately 1 x 10^6 cpm of nuclear run-on RNA were hybridized with a filter onto which target DNA had been slotted. The controls used in this study included murine c-myc and glyceraldehyde phosphate dehydrogenase (GAPDH). The filters were air dried and then exposed to a phosphorimager screen for quantitation of the signals using Molecular Dynamics (Sunnyvale, CA) software.

**Transient transfections**

For the transient transfection assays, 6 x 10^6 U937 cells in 0.3 ml of RPMI 1640 and 10% FCS were transfected by electroporation with a Bio-Rad gene pulser set at 240 V, 960 μF. Twenty micrograms of the NF-IL6 promoter-driven firefly luciferase reporter construct were cotransfected with 5 μg of a plasmid containing the renilla luciferase gene driven by the thymidine kinase promoter as a transfection efficiency control. The CREB-2 cotransfection experiments did not include a transfection efficiency control because TK promoter activity is responsive to CREB/ATF family members (15). For the CREB cotransfection experiments, the luciferase activities were corrected for the total amount of protein in the transfected cellular extracts.

Electroporated cells were equally divided into two 6-ml cultures containing complete RPMI with or without PMA. Twelve to sixteen hours following electroporation, the transfected cells were harvested and the levels of luciferase activity were measured as described previously (16). In the renilla luciferase cotransfection experiments, 20× of the transfectant extract prepared from non-PMA-treated cultures were assayed for renilla luciferase activity according to the manufacturer's instructions (Promega Dual Luciferase Assay Kit).

**Transcript stability studies**

U937 cells were cultured without or with LPS and PMA for 12 h. At the end of the stimulation period, cultures of control and activated U937 cells were treated with 5 μg/ml Actinomycin D for variable lengths of time; then total cellular RNA was prepared (17). Total cellular mRNA levels were assessed by Northern analysis. For the Northern analysis, 40 μg of total cellular RNA were loaded per lane in the 1% agarose formaldehyde gel according to the protocol described previously (18). DNA probes for Northern anal-

ysis were labeled by the random hexamer priming method (19). The NF-IL6 hybridization probe was a PstI-EcoRI fragment derived from the pBlue 610 plasmid containing the human NF-IL6 CDNA (kindly provided by S. Akira, Hyogo College of Medicine, Hyogo, Japan). The Northern was hybridized with the NF-IL6 specific probe, stripped (Amersham protocol, Arlington Heights, IL) and then rehybridized with a probe containing sequence of the 2.0-kbp human NF-IL6 genomic clone (kindly provided by R. Della-Favera, Columbia University, New York, NY). The Northern blot signals were quantitated using a phosphorimager (Molecular Dynamics). The RNA signal observed at the 10-min time point was normalized to 100%, and the percentage of signal remaining with time was calculated.

**DNase I protection assay**

A NotI-Nco fragment from the NF-IL6 gene corresponding to -284 to +125 bp relative to the start site of transcription was 5' end labeled on the coding or noncoding strand and subjected to DNase I footprinting as described previously (20). Each reaction contained approximately 50,000 cpm of 5' end-labeled DNA that was subjected to limited DNase I digestion in the presence or absence of nuclear factors. The crude nuclear extracts used for the DNase I reaction were prepared from control and LPS/PMA-activated U937 cells as described elsewhere (20). The nuclear extracts were purified by ammonium sulfate fractionation as previously described (21). Approximately one third of the purified DNase I reaction products were resolved on an 8% polyacrylamide/8 M urea sequencing gel.

**EMSA**

Nuclear extracts from control and 20 h PMA-activated U937 cells were prepared as previously described (22). Site I EMSAs were performed as described previously (20). The binding reactions included 1.5 to 6.0 μg crude nuclear extract, 0.5 μg sheared herring sperm DNA, 10 mM HEPES· KOH pH 7.5, 5 mM NaCl, 5 mM Tris. HCl pH 7.5, 15 mM EDTA pH 8.0, 1 mM DTT, 10% glycerol, 1 mM ZnSO4, and 4 x 10^6 cpm of probe. Binding reactions were incubated at room temperature for 15 min and then loaded directly onto a 4.5% polyacrylamide gel and electrophoresed at 100 V in 1 x Tris glycine electrophoresis buffer (190 mM glycine, 25 mM Tris.HCl pH 8.5, 1 mM EDTA). The binding reaction complexes resolved from free probe on the nondenaturating polyacrylamide gel were dried onto Whatman paper and then exposed to x-ray film overnight. For the competitions, cold oligonucleotides were incubated with extract for 15 min at room temperature before addition of the probe. In the supershift experiments, Abs were added to the binding reaction and incubated for 40 min at room temperature before addition of probe. The CREB/ATF1 mAb was purchased from Santa Cruz Biotechnologies (catalog number sc-270; Santa Cruz, CA). The IgG1 Gag Abs were a kind gift from D. Wong (Columbia University). Site II EMSAs were performed using the binding conditions elsewhere described (see Ref. 28). The binding reaction complexes were resolved from the free probe as described for site I EMSA. For site II supershift experiments, the Sp1 and Sp3 Abs were added to the cold binding reaction for 15 min before the addition of probe.

**Results**

**Activation of U937 promonocytes with PMA and LPS induces NF-IL6 transcription**

Steady-state levels of NF-IL6 mRNA are induced 10- to 15-fold following activation of promonocytes with cytokines or phorbol esters (Ref. 4 and data not shown); however, the molecular basis for this induction is not known. Therefore, we wished to determine whether increased mRNA stability or increased transcription was responsible for the activation-dependent increase in steady-state NF-IL6 mRNA. To determine whether cellular activation alters the stability of NF-IL6 mRNA, the half-life of NF-IL6 mRNA was measured in untreated and activated U937 cells following treatment with actinomycin D. Figure 1A shows results that are representative of two independent experiments. Although activation caused a 14-fold increase in absolute levels of NF-IL6 mRNA, the pattern of mRNA degradation was similar in untreated and activated U937 cells, and NF-IL6 mRNA stability decreased modestly following activation. Therefore, increased stability of NF-IL6 mRNA...
mRNA cannot account for the increase in steady-state levels observed in activated U937 cells.

Nuclear run-on experiments were performed to measure the relative rate of transcription initiation of the NF-IL6 gene in U937 cells before and 12 h after cellular activation with PMA and LPS. Figure 1B shows a representative nuclear run-on experiment where the rate of NF-IL6 transcription initiation was induced approximately threefold. As a control, the relative rate of c-myc transcription was examined and, as reported previously, it decreased following activation of the U937 cells (23). In three nuclear run-on experiments, an average increase of 3.2 ± 1.6-fold in the relative rate of NF-IL6 transcription initiation was observed. We conclude that the induction of NF-IL6 mRNA observed following activation of U937 promonocytes by PMA+LPS can be attributed, at least in part, to increased transcription of the gene.

**Two promoter-proximal regions regulate NF-IL6 gene transcription in U937 promonocytes**

Having shown that the relative rate of NF-IL6 gene transcription initiation is increased in response to cellular activation, we wished to identify the DNA sequences required for this effect. The transcription initiation site of the NF-IL6 gene was mapped previously (24). First we tested whether a portion of the NF-IL6 gene containing the start site of transcription conferred activation-inducible transcription on a luciferase reporter. Transient transfections were conducted in U937 cells using a luciferase reporter where transcription was dependent upon a fragment of the NF-IL6 gene containing 2 kbp 5′ and 125 bp 3′ of the transcription initiation site. Following transfection, the cells were divided and treated with various combinations of activators. As shown in Figure 2A, this
DNA fragment contains promoter and transcriptional regulatory sequences sufficient to confer both basal and activation-responsive transcription on the reporter. The data in Figure 2A confirm the results in Figure 1, showing that transcription from the NF-IL6 promoter increases in response to cellular activation by PMA + LPS. Treatment of U937 cells with PMA alone or PMA + LPS had a similar effect, inducing transcription five- to sixfold; however, treatment with LPS alone induced transcription minimally (1.6-fold). Therefore, in subsequent transfections, PMA alone was used for U937 cell activation, and our studies provide insight into PMA-dependent regulation of NF-IL6.

To define a minimal promoter region and identify regulatory elements required for NF-IL6 promoter activity, a series of truncated promoters was generated and transfected into U937 cells. Deleting sequences 5' of −97 bp had no significant effect on either basal or activated NF-IL6 promoter activity, suggesting that the region from −2 kbp to −97 bp is not required for promoter activity (Fig. 2B). In addition, 3' truncation to +7 bp did not alter promoter activity. Therefore, sequences between +7 and +125 bp are not required for promoter activity. However, 5' truncation to −44 bp resulted in a significant reduction in NF-IL6 promoter activity. Thus, the region from −125 to −7 bp is sufficient to confer basal and PMA-activated promoter activity and can be considered the minimal NF-IL6 promoter in this assay system. Furthermore, the region between −97 to −44 bp was shown to be required for promoter activity in monocytic cells.

To identify protein-binding sites within this functionally defined regulatory region of the NF-IL6 gene, DNase I footprinting reactions were performed with U937 cell nuclear extracts. The DNase I protection pattern conferred by the presence of nuclear factors in the −44 to −97-bp region on both the coding and noncoding strands of the NF-IL6 gene is shown in Figure 3A. Two regions of protection bracketed by hypersensitive sites were observed on the coding strand: −34 to −41 bp, which encompasses the TATA box, and −48 to −69 bp, which we will refer to subsequently as site I. On the noncoding strand, a region corresponding to site I on the coding strand, between −55 to −70 bp, was protected. In addition, another region of protection between −76 to −82 bp was observed only on the noncoding strand, and this region is designated site II. Nuclear extracts prepared from untreated and activated U937 cells gave similar patterns of protection on the coding and noncoding strands (lanes 2 vs 3, and data not shown).

The footprinting results are depicted schematically in Figure 3B. Comparison of the sequences of sites I and II with known transcription factor binding sites revealed that site I contains sequences similar to a CRE or AP-1 site (25, 26). Site II contains a consensus binding site for the Sp1 family of transcription factors (Fig. 3C) (27). Although the region 5' of site II is very GC-rich and contains additional 5' Sp1 consensus binding sites (Fig. 3C), we did not observe protection of these 5' sequences in our DNase I experiments. The DNA sequence of the mouse, rat, and human NF-IL6 promoter region is shown in Figure 3C. The regions of the NF-IL6 gene designated as sites I and II are indicated with a box in Figure 3C. Interestingly, the DNase I protected regions occur within sequences of the NF-IL6 gene, which are highly conserved among the three species.

The contribution of sites I and II to basal NF-IL6 promoter activity in U937 cells was tested by a transient transfection assay. A promoter lacking both site I and site II (−44 to +72 bp) was approximately 20-fold less active than the −97- to +72-bp promoter containing sites I and II (Fig. 4), establishing the importance of the −97 to −44-bp region for NF-IL6 promoter activity. A promoter lacking site II but retaining site I (−74 to +72 bp) displays modest but statistically significant reduction in activity compared with the −97 to +72-bp promoter in untreated cells. Similarly, a promoter containing wild-type site II and a site-directed mutation that alters bases −53 to −67 in site I (mutI −97 to +72 bp) also displays a modest but statistically significant reduction in activity, compared with the promoter containing both sites I and II. Thus, both sites I and II are important for basal NF-IL6 promoter activity in U937 cells.

The contribution of sites I and II to activation-inducible NF-IL6 promoter activity in U937 cells was also determined. The promoter containing sites I and II is reproducibly induced 5.5-fold by activation of U937 cells. The activity of a promoter containing only site I (−74 to +72 bp) is induced 4.2-fold following activation. The activity of a promoter containing site II alone (mutII −97 to +72 bp) is induced 3.5-fold in response to activation. Thus both sites I and II are necessary for full activity in response to activation, but they do not function synergistically. In summary, these data show that sites I and II function as positive regulatory elements for the NF-IL6 promoter in U937 cells and that they are independently required for basal and activation-induced transcription.
CREB/ATF and Sp1 proteins bind to sites I and II in the NF-IL6 promoter

EMSAs were performed to identify proteins from U937 nuclei that bind to sites I and II of the NF-IL6 gene. Table I contains the DNA sequences of the oligonucleotides used in these experiments. In the EMSA shown in Figure 5A, a site I oligonucleotide probe was incubated with PMA-activated U937 cell nuclear proteins. One prominent complex was observed that was competed by the addition of a 20-fold molar excess of unlabeled site I oligonucleotide (Fig. 5A, lanes 9–11) but not by an 80-fold molar excess of a mutant site I oligonucleotide (lanes 12–14). An unlabeled consensus CRE site oligonucleotide (lanes 6–8) competed efficiently for binding to the site I probe at a fivefold molar excess. In contrast, a consensus AP-1 site oligonucleotide did not compete for binding to the site I probe at an 80-fold molar excess (lanes 3–5). These data suggest that nuclear factors that recognize a CRE site bind to site I. We compared the binding of U937 nuclear proteins, before and after activation, to oligonucleotide probes containing consensus binding sites for AP-1 and CRE with site I complexes. The mobility and the presence of a site I complex before and after activation of U937 cells were similar to the CRE consensus probe binding activity, while the AP-1 consensus probe showed a retarded complex with a slower mobility. This result also suggests that CREB/ATF family proteins bind to site I both before and after activation.

To test directly for binding of ATF/CREB proteins to site I, a mAb that recognizes members of the CREB/ATF family was used in a “supershift” experiment. The ability of the CREB/ATF mAb to supershift a consensus CRE site complex was examined in the EMSA binding reactions shown in Figure 5C (lanes 6 and 7). Nuclear factors that recognize the site I probe in untreated and activated U937 cells were supershifted by the CREB/ATF mAb (Fig. 5C, lanes 3 and 5) but not by an isotype-matched control mAb (lanes 2 and 4). The specific site I complex was efficiently supershifted by the CREB/ATF mAb, suggesting that most site I complexes contain CREB/ATF family members. The site I mutation, previously shown to reduce NF-IL6 promoter activity (Fig. 4), was used as a probe in an EMSA that revealed that the site I mutation ablates the binding of nuclear factors to that oligonucleotide (Fig. 5D, compare lanes 2 and 4). We conclude that, in U937 cells, members of the CREB/ATF family bind to site I of the NF-IL6 gene and are likely to mediate site I-dependent transcriptional activation.

Analysis of U937 cell nuclear factors that recognize the NF-IL6 gene site II region is shown in Figure 5E. In the EMSA, binding reactions performed with an excess of unlabeled competitor oligonucleotides corresponding to a consensus Sp1 binding site derived from the p21 promoter (lanes 6–8) or site II from the NF-IL6 gene (lanes 3–5) competed for the binding of U937 cell nuclear factors to the site II probe. An oligonucleotide containing a mutant

![Figure 3](image-url)
Sp1 binding site did not compete for binding to the site II probe (Fig. 5E, lanes 9–11) (28). To examine directly the binding of Sp1 proteins to the site II probe, antisera that recognize Sp1 or Sp3 were added to the binding reactions. Antiserum that recognizes Sp1 supershifted a site II complex formed by nuclear factors from untreated and PMA-activated U937 cells (Fig. 5F, lanes 2 and 5) (29). In contrast, preimmune and antiserum that recognizes Sp3 did not affect the binding of U937 cell nuclear factor(s) to site II (lanes 1, 3, 4 and 6) (30). We conclude that Sp1 family proteins, including Sp1 but not Sp3, bind site II in the NF-IL6 gene and are likely to activate transcription of the NF-IL6 gene in U937 cells.

Endogenous CREB/ATF proteins are required for maximal NF-IL6 promoter activity in U937 cells

Our biochemical studies showed that CREB/ATF protein(s) in U937 nuclear extracts bind site I of the NF-IL6 gene, and transient transfections indicated that site I is functionally important for promoter activity. To test the role of CREB/ATF proteins for regulating NF-IL6 promoter activity in vivo, we performed a transient cotransfection assay using reporters dependent on the NF-IL6 promoter and an expression plasmid encoding a dominant negative form of CREB, CREB-2. CREB-2 exhibits CRE binding activity but does not activate transcription due to changes in amino acid residues of the kinase inducible domain (KID) domain (31). NF-IL6 promoter activity with a cotransfected CREB-2 expression plasmid was compared with promoter activity with a cotransfected expression plasmid lacking the CREB-2 cDNA (Fig. 6). Cotransfection of the CREB-2 cDNA significantly reduced the activity of NF-IL6 promoters containing an upstream CRE site (site I) in untreated U937 cells (Fig. 6, lanes 1 vs 3 and 5 vs 7) and in PMA-activated U937 cells (lanes 2 vs 4 and 6 vs 8). In contrast, the activity of a comparable NF-IL6 promoter containing a mutation in the CRE site, mI-97 to +72 bp, was only slightly reduced by the presence of cotransfected CREB-2 in untreated (lanes 9 vs 11) or PMA-activated (lanes 10 vs 12) U937 cells. We conclude that endogenous CRE-binding proteins are important for basal and PMA-inducible NF-IL6 promoter activity in U937 cells.

Discussion

We have shown that activation of U937 promonocytic cells leads to increased transcription of the NF-IL6 gene. Analysis of the NF-IL6 promoter has revealed that 97 bp 5′ of the transcription initiation site is sufficient to confer both basal and activation-induced activity in this system. Within this region, we have identified functionally important binding sites for CREB/ATF and Sp1 family proteins.

Table I. The sequence of oligonucleotides used in the EMSAs

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt site I</td>
<td>CATCCCCAGGTGAGCGGCCCGGTTGCC</td>
</tr>
<tr>
<td>mut site I</td>
<td>CATCCCCAGGTGAGCGGCCGGCTAG</td>
</tr>
<tr>
<td>Site II</td>
<td>GATCGTGACGTCAGCGCG</td>
</tr>
<tr>
<td>p21 wt</td>
<td>CCGCAGCGCCCGCGCCGCCGCCGAGAGG</td>
</tr>
<tr>
<td>p21 mut</td>
<td>CCGCAGCGCCCGCGCCGCCGCCGAGAGG</td>
</tr>
<tr>
<td>CRE</td>
<td>CACTGAGTCGCGCCTAG</td>
</tr>
<tr>
<td>AP-1</td>
<td>GATCGTACGCGCCGCTAG</td>
</tr>
</tbody>
</table>

FIGURE 4. Site I and site II are required for activity of the NF-IL6 promoter. U937 cells were transiently transfected with reporters dependent on portions of the NF-IL6 promoter. The length and content of the sequences upstream of the NF-IL6 promoter in the panel of constructs are shown schematically at the left. The bars represent the relative activity in untreated (solid) and PMA-activated (open) U937 cells after normalization to a control. The activity of the −97 to +72-bp NF-IL6 promoter construct in untreated U937 cells was set to 1.0. The values shown represent the average of at least five independent transfections. The Student t test for variability was applied to results comparing basal activity of the −97 to +72-bp promoter to that of promoters containing −74 to +72 bp (no site II) and −97 to +72 with a mutation in site I. The reduction in activity of the latter two promoters, compared with the −97 to +72-bp promoter, was statistically significant (p = 0.01).
Our studies show that a conserved CRE site at 262 bp is functionally important for basal and PMA-inducible NF-IL6 promoter activity. U937 cells were cotransfected with a panel of NF-IL6 promoter constructs driving reporter gene expression and control or CREB-2 expression plasmids, as indicated below the bar graph. Following electroporation, the cotransfected cells were equally split into two cultures without (open bars) or with (gray bars) PMA. The luciferase activity was normalized for the protein concentration of the cell extracts. The reporter activity shown for each construct is relative to the reporter activity in untreated U937 cells cotransfected with the control expression plasmid. The constructs used in this experiment include wt site I and II NF-IL6 promoter construct lanes 1–4, wt site I NF-IL6 promoter construct lanes 5–8, mutant site I and wt site II NF-IL6 promoter constructs lanes 9–12.

The role of the CRE site at −62 bp in the NF-IL6 promoter
Our studies show that a conserved CRE site at −62 bp is functionally important for basal and PMA-inducible NF-IL6 promoter activity. A transdominant negative CREB/ATF family member, CREB-2, reduces NF-IL6 promoter activity. U937 cells were cotransfected with a panel of NF-IL6 promoter constructs driving reporter gene expression and control or CREB-2 expression plasmids, as indicated below the bar graph. Following electroporation, the cotransfected cells were equally split into two cultures without (open bars) or with (gray bars) PMA. The luciferase activity was normalized for the protein concentration of the cell extracts. The reporter activity shown for each construct is relative to the reporter activity in untreated U937 cells cotransfected with the control expression plasmid. The constructs used in this experiment include wt site I and II NF-IL6 promoter construct lanes 1–4, wt site I NF-IL6 promoter construct lanes 5–8, mutant site I and wt site II NF-IL6 promoter constructs lanes 9–12.
activity in U937 cells (Fig. 4) and that endogenous CREB/ATF proteins are necessary for maximal activity of the NF-IL6 promoter in U937 cells (Fig. 6). The supershift experiments (Fig. 5) confirm that proteins recognized by CREB antisera (including ATF, CREB-1, and CRE modulator (CREM)), it contains both trans-activators and trans-dominant negative inhibitors of CRE site-dependent transcription (31–35). In the future it will be important to determine which CREB/ATF proteins regulate transcription of the NF-IL6 gene in monocytes/macrophages and how monocyte/macrophage activation may alter the abundance and/or activity of CREB/ATF proteins.

The contribution of CREB/ATF proteins to NF-IL6 promoter activity before cellular activation is consistent with our observation of proteins binding to site I before U937 cell activation (Fig. 5), the observation of others that CREB/ATF proteins can interact directly with the basal transcription machinery (36), and the fact that CREB/ATF proteins are known to regulate the transcription of other genes before cellular activation (37, 38). CREB/ATF proteins are also important for PMA-inducible transcription of the NF-IL6 gene in monocytes. It has been clearly demonstrated that signal-dependent kinases phosphorylate CREB and enhance CREB-mediated trans-activation. In monocytes we observed an increase in transcriptional activation without a change in the binding of nuclear factors to site I, which is consistent with the possibility that PMA may induce kinases that activate CREB/ATF proteins (39–41). The role of the CRE site in cellular activation responsive gene expression is consistent with prior observations that CRE sites are cytokine responsive elements in the myeloid cell line TF-1. Treatment of TF-1 cells with GM-CSF or IL-3 induces erg-1 promoter activity, which is dependent upon CRE and serum response element (SRE) sites (40–42). CRE sites have also been implicated in monocyte differentiation-specific transcription of macrophage inflammatory protein-1B (MIP-1B) (43), MuRANTES, and curr-2 (44). Thus, a functional role for the CRE site in transcriptional regulation of the NF-IL6 gene in activated U937 cells underscores the important role played by CRE-binding proteins in monocyte/macrophage activation-dependent gene expression.

**The role of the −80-bp Sp1 site in the NF-IL6 promoter**

We also demonstrated that a guanosine and cytosine-rich region recognized by the Sp1 family of proteins at −80 is important for basal and activation-induced NF-IL6 promoter activity in U937 cells. Factors known to bind Sp1 sites include Sp1-4, and the Erg family (45–48). Our supershift experiments demonstrated that Sp1, but not Sp3, present in U937 cell nuclear extracts binds to the NF-IL6 gene site II sequence. It is also possible that other Sp1 family members, which cross-react with the Sp1 antisera, bind site II. Consistent with our observations, guanosine and cytosine-rich sequences recognized by Sp1 have been identified as PMA-responsive elements in other genes such as the thromboxane receptor gene (49), proximal platelet-derived growth factor A-chain gene (50), and the HIV-1 long terminal repeat (LTR) (51). Although we do not know how Sp1 proteins confer PMA inducibility to the NF-IL6 promoter, our data add to growing evidence that Sp1 sites can be targets of signal transduction pathways.

**The NF-IL6 promoter is controlled by different regulatory elements in macrophages and hepatocytes**

During completion of this work, a study analyzing NF-IL6 gene expression during liver regeneration was reported (52). In the liver, expression of NF-IL6 is induced at times of physiologic stress such as the acute phase response and liver regeneration (53). There are significant differences in promoter elements required for NF-IL6 transcription in hepatocytic and monocyte cell lines. In hepatocytic lines, basal and protein kinase A (PKA)-inducible NF-IL6 promoter activity required the synergistic activity of two CRE sites, located at −62 and −110 bp relative to the start site of transcription. Although our DNase I footprinting experiments showed that the −110-bp CRE site was occupied by U937 cell nuclear proteins (data not shown), this region was not required for minimal promoter activity in U937 cells (Fig. 2). A 104-bp region of the NF-IL6 gene (−97 to +7 bp), containing a functionally important CRE site at −62 bp and a functionally important Sp1 site at −80 bp, was sufficient to confer full promoter activity in either untreated or activated U937 cells. Our results, together with those of Niehof et al. (52), emphasize the importance of CRE sites for regulation of the NF-IL6 promoter in both cell lineages and show that the −62-bp CRE site alone is not sufficient to provide full promoter activity in either cell lineage. In monocytes, the promoter proximal CRE site requires the Sp1 site, whereas in hepatocytes an upstream CRE site is required. Thus the NF-IL6 gene provides an example of a promoter that is regulated by different cis elements in different cell lineages.

Proteins binding to both CRE and Sp1 sites are ubiquitously present; however, tissue-specific differences in the relative abundance of individual family members, posttranslational modifications or coactivators are likely to exist. It may be that monocytes have increased abundance or activity of proteins that can activate through the Sp1 site relative to hepatocytes. This would be consistent with the finding that the Sp1 site in the macrophage-specific CD11b promoter is occupied in a macrophage-specific way (54). Similarly, hepatocytes may have a cellular activity that functions through the −110-bp CRE site that is less active or abundant in monocytes. Either model could explain why the promoter proximal CRE site requires the Sp1 site in monocytes while the upstream CRE site is required in hepatocytes. As a consequence of being regulated by different cis elements, we speculate that the NF-IL6 promoter is subjected to regulation by different signaling pathways in hepatocytes and monocytes.

**Acknowledgments**

We thank the members of the Calame laboratory for helpful discussions and suggestions during this work and Dr. A. Henderson for critically reading the manuscript. We thank R. Allen for technical help with the DNase I experiments, Dr. R. Prywes for assistance with the EMSAs, Dr. X.-F. Wang for supplying oligonucleotides and antisera used in our work, and Y. Lin for help with the figures. We also thank Dr. V. Poli for the murine NF-IL6 genomic clone, Dr. Denong Wong for supplying the Gag antiserum and suggestions during this work and Dr. A. Henderson for critically reading the manuscript. We thank R. Allen for technical help with the DNase I experiments, Dr. R. Prywes for assistance with the EMSAs, Dr. X.-F. Wang for supplying oligonucleotides and antisera used in our work, and Y. Lin for help with the figures. We also thank Dr. V. Poli for the murine NF-IL6 genomic clone, Dr. Denong Wong for supplying the Gag antiserum, Dr. R. Dalla-Favera for the human β-actin construct, and Dr. J. Leiden for the CREB-2 plasmid.

**References**