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Inducible expression of nuclear factor IL-6 increases endogenous gene expression of macrophage inflammatory protein-1α, osteopontin and CD14 in a monocytic leukemia cell line

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Abstract

Nuclear factor-IL-6 (NF-IL6) belongs to the CCAAT/enhancer binding protein family of transcription factors. NF-IL6 binds to the regulatory regions of many genes induced in activated macrophages *in vitro*. However, which particular genes are regulated by NF-IL6 *in vivo* is poorly defined. In order to identify the downstream genes of NF-IL6 in a monocytic lineage, we combined an inducible expression system with subtraction cloning in a study of murine M1 monocytic leukemia cells. We demonstrated that inducible expression of NF-IL6 is able to increase endogenous gene expression of macrophage inflammatory protein (MIP)-1α, osteopontin and CD14 in M1 cells. We also showed that NF-IL6 activated murine MIP-1α proximal promoter luciferase construct which contains two NF-IL6 binding sites and a point mutation of either site markedly reduces the luciferase activity. These findings indicate that MIP-1α is a direct target of NF-IL6.

Introduction

Nuclear factor-IL-6 (NF-IL6) was initially identified as a nuclear factor that binds to the IL-1 responsive element in the IL-6 gene (1). Cloned NF-IL6 exhibits homology with CCAAT/enhancer binding protein (C/EBP), a member of the basic leucine zipper (bZIP) family of transcription factors (2). NF-IL6 has been reported by other groups under the names AGP/EBP, LAP, IL-6DBP, rNFIL-6, C/EBPβ and CRP2 (3–8). At present, the C/EBP family consists of six members. These include C/EBP (9), NF-IL6, Ig/EBP (also referred to as GPE-1-BE and C/EBPγ) (10,11), NF-IL6β (C/EBPδ) (7,8,12,13), CRP1 (C/EBPε) (7,14) and CHOP (15). These proteins can both homodimerize and heterodimerize with each other, and bind to the same consensus sequence T(T/G)NNGNAA(T/G). Among them, four members of the C/EBP family, C/EBP, NF-IL6, NF-IL6β and CRP1, have been suggested to be involved in the regulation of a variety of genes in myelopoiesis (16–19). NF-IL6 exhibits low activity until activated by inflammatory stimuli, which induce phosphorylation of NF-IL6 and its translocation to the nucleus (6,20,21). NF-IL6 is dramatically induced during macrophage differentiation and NF-IL6 binding motifs are found in the functional regulatory regions of genes specifically induced in activated macrophages, such as IL-6, IL-1α, IL-8, tumor necrosis factor (TNF)-α, granulocyte colony stimulating factor (G-CSF), nitric oxide synthase and lysozyme (2,17,22). NF-IL6-deficient mice are highly susceptible to facultative intracellular organisms, such as *Listeria monocytogenes* and *Salmonella typhimurium,* due to impairment of bacteria killing by activated macrophages (23). These studies indicate that NF-IL6 activates transcription of a variety of genes in activated macrophages. However, only a few genes, e.g. macrophage chemoatractant protein-1, IL-6 and G-CSF, have been demonstrated to be downstream of NF-IL6 *in vivo*. Overexpression of NF-IL6 confers upon a B lymphoblast cell line the ability to induce macrophage chemoatractant protein-1 and IL-6 in response to lipopolysaccharide (LPS) (24). Target disruption of NF-IL6 reveals

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that G-CSF production is severely impaired in macrophages and fibroblasts from NF-IL6-deficient mice (23). In order to understand the function of NF-IL6 in more detail, it is essential to identify the genes regulated by this transcription factor *in vivo*.

Methods

Cells and cell culture

Murine M1 monocytic leukemia cells used in this study were described previously (17). M1 cells were cultured in Eagle's minimal essential medium (Gibco/BRL, Gaithersburg, MD) supplemented with twice the normal concentration of amino acids and vitamins, and 10% FCS. M1 transfectants were maintained in the same medium supplemented with 400 μg/ml of geneticin (Gibco/BRL) and 50 μg/ml of hygromycin B (Boehringer Mannheim, Mannheim, Germany).

Generation of stable transfectants

To achieve inducible expression of NF-IL6 protein, we used the LacSwitch inducible expression system (Stratagene, La Jolla, CA) in M1 cells as described previously (25). Human NF-IL6 excised from pEF-BOS NF-IL6 (26) was cloned into pOPRSVI vector to construct pOPRSVI-NFIL6. To prepare doubly transfected lines, 1×10^7 M1 cells suspended in K-PBS (31 mM NaCl, 121 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 10 mM $MgCl_2$) were electroporated at 975 μF and 300 V (Gene Pulser II; BioRad, Hercules, CA) with 20 μg of P3′SS *lac* repressor expression vector and 20 μg of pOPRSVI-NFIL6. P3′SS vector and pOPRSVI-NFIL6 vector were linearized by digestion with *Bam*HI and *Aat*II prior to mixing with cells respectively. Cells were placed in the growth medium for 24 h, then transferred to 24-well plates, and placed under selection with 400 μg of geneticin and 50 μg of hygromycin B/ml for 14 days. Single colonies were picked manually, expanded and tested for expression of NF-IL6 by Western blot analysis after 8 h treatment of 1 mM isopropyl-β-D-thiogalactoside (IPTG). We selected three clones expressing NF-IL6 protein at comparable levels (NFIL6 M1 clones 20, 37 and 40). One of them (NFIL6 M1 clone 40) was mainly used for further studies. However, the other two clones were examined for Northern blot analysis against inducible genes, where the results were similar to the presented data. Mock M1 cells were described previously (25).

Western blot analysis

To detect NF-IL6 protein, cells were lysed directly in 1×Laemmli buffer. After boiling for 5 min, cell lysates (1×10^5 cells per lane) were separated by SDS–PAGE (4–20% gradient polyacrylamide gel), electrophoretically transferred to a nitrocellulose membrane and then analyzed for immunoreactivity with anti-C/EBPβ (NF-IL6) antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) by the enhanced chemiluminescence detection system (Dupont, Boston, MA).

Northern blot analysis

DNA for probes was labeled with [α-^{32}P]dCTP by random priming (Megaprime DNA labeling system; Amersham, Little Chalfont, UK). The probes for MIP-1α, osteopontin, CD14, and elongation factor (EF)-1α were *Rsa*I fragments as follows: MIP-1α, GenBank accession no. X12531 nucleotides 110–699; osteopontin, GenBank accession no. J04806 nucleotides 445–1175; CD14, GenBank accession no. M34510 nucleotides 1–435; EF-1α, GenBank accession no. X13661 nucleotides 1–205. Mouse lysozyme and c-*myb* probes were described previously (26).

Total RNA was extracted by TRIZOL Reagent (Gibco/BRL). Total RNA (15 μg/lane) was separated on 1.0% agarose gels containing 6.0% formaldehyde. After transfer to a HybondN+ nylon membrane (Amersham) in 10×SSC (1×SSC is 0.15 M NaCl plus 0.015M sodium citrate), hybridization was performed at 65°C for 12–16 h in a buffer containing 342 mM Na_2HPO_4, 158 mM NaH_2PO_4, 7% SDS, 1 mM EDTA and 1×10^6 c.p.m./ml of denatured DNA probe (27). The membrane was washed twice at 65°C in 2×SSC/0.1% SDS for 10 min and once at 65 in 0.2×SSC/0.1% SDS for 10 min, and exposed to BioMax MS film (Kodak, Rochester, NY) at –80°C. For quantitative analysis, the intensity of the hybridized band of each lane was measured with a BAS 2000 image analyzing system (FUJIX, Fuji Film, Tokyo, Japan) and the densitometric value was normalized to EF-1α.

Construction of subtracted cDNA library and differential screening

NF-IL6 M1 cells were treated for 48 h with 1 mM IPTG or untreated to extract mRNAs. Total RNA was obtained by the guanidine isothiocyanate–cesium chloride gradient centrifugation method, following poly(A)$^+$ RNA selection using Oligotex-dT30 latex beads (TaKaRa, Otsu, Japan). Tester and driver cDNAs were synthesized from 2 μg of poly(A)$^+$ RNA of IPTG-treated and untreated cells respectively. Complementary DNA synthesis and subtraction were done in essence according to the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA) using Gene Taq DNA polymerase (Nippon Gene, Toyama, Japan). To select for NF-IL6 inducible transcripts, *Rsa*I-digested tester cDNA was ligated to oligonucleotide linkers and hybridized with excess driver cDNAs. After hybridization, differential transcripts were selectively amplified by suppression PCR as described (29). The primary PCR was done for 27 cycles and the nested PCR for 10 cycles on a thermal cycler (Gene Amp 9600; Perkin-Elmer, Norwalk, CT). Products from the nested PCR were inserted into pGEM-T vector (Promega, Madison, WI). Independent clones were amplified by direct colony PCR and differential screening against 1400 clones was performed according to the manufacturer's instruction. Briefly, colony PCR products were dot-blotted onto nylon membranes in duplicate. Each DNA dot-blot was hybridized with itself ('+' subtracted probe) or with reverse-subtracted cDNA ('–' subtracted probe). To make the '–' subtracted probe, subtractive hybridization is performed with the original tester cDNA as a driver and the driver cDNA as a tester. Clones that hybridize only with the '+' probe were sequenced by an autosequencer utilizing dye-labeled dideoxynucleotides (Applied Biosystems PRISM 377 DNA Sequencer). Sequence comparisons against DDBJ databases were obtained from the DDBJ blast server (http://www.ddbj.nig.ac.jp/E-mail/homology-j.html) (30).

Luciferase vector construction

Reporter vectors were constructed in the pGL3 basic luciferase vector (Promega). The proximal promoter region of the murine MIP-1α from position –676 to +29 (relative to the transcription start site) was amplified by PCR using genomic DNA extracted from BALB/c mouse tail as a template. The primers for PCR which have additional restriction sites for insertion into the pGL3 basic vector were designed according to the mouse MIP-1α gene (GenBank accession no. M73061, 31): sense primer, 5′-CCGACGCGTGATGGCTTTACATTTGGGTTGTTTC-3′; antisense primer, 5′-GAAGATCTGGTACTCGCTGCTGCTTGTATCC-3′. The PCR fragment was subcloned into pGEM-T vector (Promega) and verified by automatic sequencing. The insert was digested with *Mlu*I and *Bgl*II, and cloned into pGL3 basic vector to produce the construct p-676Luc. For deletion construction p-676Luc was linearized with *Kpn*I and *Mlu*I, which are both located 5′ of the insert and deleted using the Kilo sequence deletion kit (TaKaRa). Briefly, the linearized p-676Luc was digested with exonuclease III. Aliquots (10 μl) were removed at 30 s intervals and mixed with 100 μl of 2×Mung bean nuclease buffer. After heating for 10 min at 65°C, the sample was incubated with Mung bean nuclease for 30 min at 37°C. The 5′-deleted sample was treated with Klenow fragment, followed by T4 DNA ligase reaction. Each mutant was sequenced to determine the extent of deletion into the 5′ end of the promoter. For NF-IL6 binding sites mutation, we used a site-directed mutagenesis kit (QuickChange, Stratagene). The mutagenic primer for position –115 to –114 (with altered nucleotides underlined) was 5′-CCTGGGTTGTGTGGTATCCATCATGACA-3′ and for position –54 to –53 was 5′-CCCAGATTCCATCCCCTCATCTGCTAGGG-3′. The constructs were confirmed by sequencing.

Transient transfections

The cells were maintained at a density of ~5×10^5 cells/ml. Transfection was carried out by electroporation. Briefly, 7×10^6 cells were suspended in 0.7 ml of K-PBS containing 20 μg of luciferase reporter constructs and 2 μg of a sea-pansy luciferase vector (pRL-TK) as a transfection efficiency control. Electroporation was accomplished at 975 μF and 300 V (Gene Pulser II). The cells were split into two equal parts post-transfection and cultured in the presence (1 mM) or absence of IPTG. After 20 h, cells were washed 3 times in PBS, pH 7.4, lysed in 25 μl of 1×passive lysis buffer (Promega) and centrifuged at 14,000 r.p.m. at room temperature, and 20 μl aliquots of the supernatants were tested in the dual luciferase assay system (Promega) using Berthold luminometer LB 9501.

Electrophoretic mobility shift assays (EMSA)

The following single-stranded oligonucleotides were annealed and used for electrophoretic mobility shift assays: A (–158 to –128 bp of the MIP-1α promoter), 5′-GATATCCTGGGCCCCTGTGGTCACTGTGGA-3′ and 5′-GTCCACAGTGACCACAGGGGCCCAGGATAT-3′; B (–127 to –98 bp of the MIP-1α promoter), 5′-CCTGGGTTGTGTAATATCCATCATGACA-3′ and 5′-GGTGTCATGATGGATATTACACAACCCAGG-3′; mB, 5′-CCTGGGTTGTGTGGTATCCATCATGACA-3′ and 5′-GGTGTCATGATGGATACCACACAACCCAGG-3′; C (–117 to –88 bp of the MIP-1α promoter), 5′-GTAATATCCATCATGACACCATTGCTGTG-3′ and 5′-GCACAGCAATGGTGTCATGATGGATATTA-3′; D (–65 to –37 bp of the MIP-1α promoter), 5′-CCAGATTCCATTTCCTCATCTGCTAGGG-3′ and 5′-GCCCTAGCAGATGAGGAAATGGAATCTGG-3′; mD, 5′-CCAGATTCCATCCCCTCATCTGCTAGGG-3′ and 5′-GCCCTAGCAGATGAGGGGATGGAATCTGG-3′; competitor in Fig. 4B (–165 to –138 bp of the human IL-6 promoter), 5′-GGACGTCACATTGCACAATCTTAATAAT-3′ and 5′-ATTATTAAGATTGTGCAATGTGACGTCC-3′.

Complementary DNA oligonucleotides were annealed by heating in a buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 50 mM NaCl at 75°C for 5 min, and cooling at room temperature. Probes were then labeled with [α-^{32}P]dCTP and Klenow fragment. Nuclear extracts were prepared according to Schreiber *et al.* (32). Protein concentration was determined by BCA protein assay reagent (Pierce, Rockford, IL). Nuclear extracts were stored in aliquots at –80°C. For gel shift assays nuclear extract (6 μg) was incubated for 20 min at room temperature with 1×10^4 c.p.m. of the labeled DNA probe in 25 μl of binding buffer containing 10 mM HEPES–KOH, pH 7.8, 50 mM KCl, 1 mM EDTA, pH 8.0, 5 mM $MgCl_2$, 10% glycerol and 3 μg of poly(dI–dC) (Pharmacia, Uppsala, Sweden). Competition assays were carried out in the same manner, except that the above reaction mixture was preincubated with a 50-fold molar excess of unlabeled competitor oligonucleotides for 60 min at 4°C before the addition of the labeled probe. Supershift assays were performed using 200 ng of anti-C/EBPβ antibody (C-19) and preincubated with the above reaction mixture at 4°C for 60 min prior to the addition of the labeled probe. Samples were loaded on native 5% polyacrylamide gels and electrophoresis was carried out at 20 mA in 25 mM Tris, pH 8.5, 190 mM glycine and 1 mM EDTA. Gels were subsequently dried for autoradiography.

Results

Inducible expression of NF-IL6 elevates lysozyme mRNA levels in M1 cells

To achieve inducible expression of NF-IL6, we developed clonal derivatives of M1 cells that contain the human NF-IL6 gene under control of the *lac* repressor. The pOPRSVI-NFIL6 vector and *lac* repressor expression vector were co-transfected into M1 cells, and stable clones were selected in geneticin and hygromycin B. Three clones that produced comparable levels of NF-IL6 protein after exposure to IPTG were selected for further analyses and designated as NFIL6 M1 cells (data not shown). The NF-IL6 gene produces two proteins—LAP (liver-enriched transcriptional activator protein, equivalent to NF-IL6) and LIP (liver inhibitory protein)—by the alternative use of two AUG initiation codons within the same open reading frame (33). LIP contains DNA binding and dimerization domains but is devoid of the N-terminal transcriptional activation domain, and therefore may behave as an antagonist of LAP-induced transcription. As shown in Fig. 1(A), Mock M1 cells did not express both LIP and NF-IL6 proteins, while NFIL6 M1 cells constitutively expressed LIP and

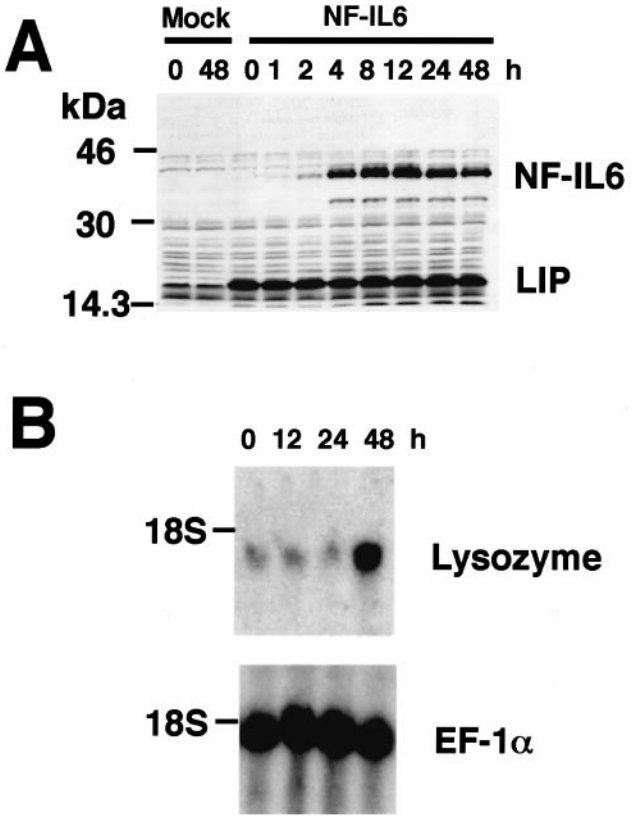

Fig. 1. Induction of lysozyme mRNA by stimulating NF-IL6 expression in M1 cells. (A) M1 cells stably transfected with an IPTG-inducible pOPRSVI-NFIL6 plasmid (NFIL6 M1 cells) were grown at 1.0×10^5/ml and then 1 mM IPTG was added to the medium to stimulate NF-IL6 expression. Cell lysates collected at the indicated time points were tested by immunoblotting with anti-C/EBPβ polyclonal antibody. (B) Total RNA (15 μg/lane) was extracted from NFIL6 M1 cells at the indicated time points following stimulation with 1 mM IPTG and subjected to Northern blot hybridization with mouse lysozyme probe. The bottom panel shows a control hybridization with EF-1α-specific probe.

inducibly expressed NF-IL6 protein to a maximum level within 4 h after IPTG addition. NF-IL6 protein expression remained relatively constant during 4–48 h after IPTG addition. Lac repressor represses transcription from the start of the NF-IL6 gene effectively, but permits transcription from just upstream of the LIP initiation codon in all transfectants (data not shown). To assess the transcriptional activity of NF-IL6 protein, we analyzed the mRNA levels of lysozyme, which is one of the transcriptional targets of NF-IL6 (34). As shown in Fig. 1(B), lysozyme mRNA elevation occurs at 48 h after IPTG addition. This result suggests that inducible expression of NF-IL6 up-regulates NF-IL6 transcriptional targets in M1 cells.

Isolation of NF-IL6 inducible genes in M1 cells

NF-IL6 M1 cells were stimulated for 48 h with 1 mM IPTG, poly(A)$^+$ RNA was isolated and tester cDNA was synthesized. Driver cDNA was synthesized using poly(A)$^+$ RNA extracted from untreated NFIL6 M1 cells. A subtractive plasmid library was constructed and differential hybridization was performed against 1400 colonies as described in Methods. Positive clones were sequenced and compared with DDBJ databases. NF-IL6 inducible genes in this study are listed in Table 1. This is the first study to identify MIP-1α, osteopontin and CD14 as putative downstream genes of NF-IL6. Haptoglobin is a known transcriptional target of NF-IL6 (35), with the level of haptoglobin mRNA increasing 3-fold at 48 h following IPTG addition (data not shown). Isolation of haptoglobin thus demonstrates the validity of our strategy for selecting NF-IL6 downstream genes.

Table 1. List of downstream genes induced by NF-IL6 in M1 cells

Gene	No. of clones out of 1400 total	Fold induction
MIP-1α	20	×27
Osteopontin	9	×14
CD14	15	×7
Haptoglobin	3	×3

Northern blot analyses for selected genes were performed as shown in Fig. 2. Quantitation of blots was performed using a BAS2000 image analyzing system. Values were normalized against EF-1α and each amount of 48 h relative to that at 0 h is shown as fold induction.

Expression profile of the downstream genes in NFIL6 M1 cells

The cells were treated with 1 mM IPTG, and RNA was extracted at 0 and 48 h for Mock M1 cells and 0, 4, 8, 12, 18, 24 and 48 h for NFIL6 M1 cells after IPTG exposure. Stimulation with IPTG did not affect mRNA expression of MIP-1α, osteopontin and CD14 in Mock M1 cells (Fig. 2). MIP-1α belongs to the C–C chemokine family and is secreted by activated macrophages (36). As shown in the time-course experiment in Fig. 2, MIP-1α mRNA was faintly present in untreated NFIL6 M1 cells and the increases were detected as early as 12 h after IPTG addition. The transcript levels showed sequential augmentation up to 48 h after IPTG addition. NFIL6 M1 cells treated for 48 h with IPTG expressed MIP-1α mRNA at levels ~27-fold-higher levels than those of untreated cells. Osteopontin and CD14 mRNA levels also showed an initial increase at 12 h after IPTG addition and successive augmentation up to 48 h following addition of IPTG in NFIL6 M1 cells. Osteopontin mRNA levels increased >14-fold at 48 h after IPTG addition to NFIL6 M1 cells (Fig. 2). CD14 mRNA levels were ~7-fold higher than those of the untreated controls after 48 h exposure to IPTG. When M1 cells differentiate to macrophage-like cells following stimulation with IL-6 or leukemia inhibitory factor, c-*myb* expression dramatically decreases within 6 h (37). In the present study, inducible expression of NF-IL6 augmented the mRNA levels of osteopontin and CD14, which are monocytic differentiation markers, whereas NF-IL6 did not suppress c-*myb* expression (Fig. 2). This suggests that NF-IL6 up-regulates these macrophage-differentiation markers but does not induce macrophage differentiation of M1 cells.

Murine MIP-1α promoter contains two NF-IL6 binding sites

The levels of MIP-1α, osteopontin and CD14 mRNA were elevated at a relatively short time after NF-IL6 expression, at

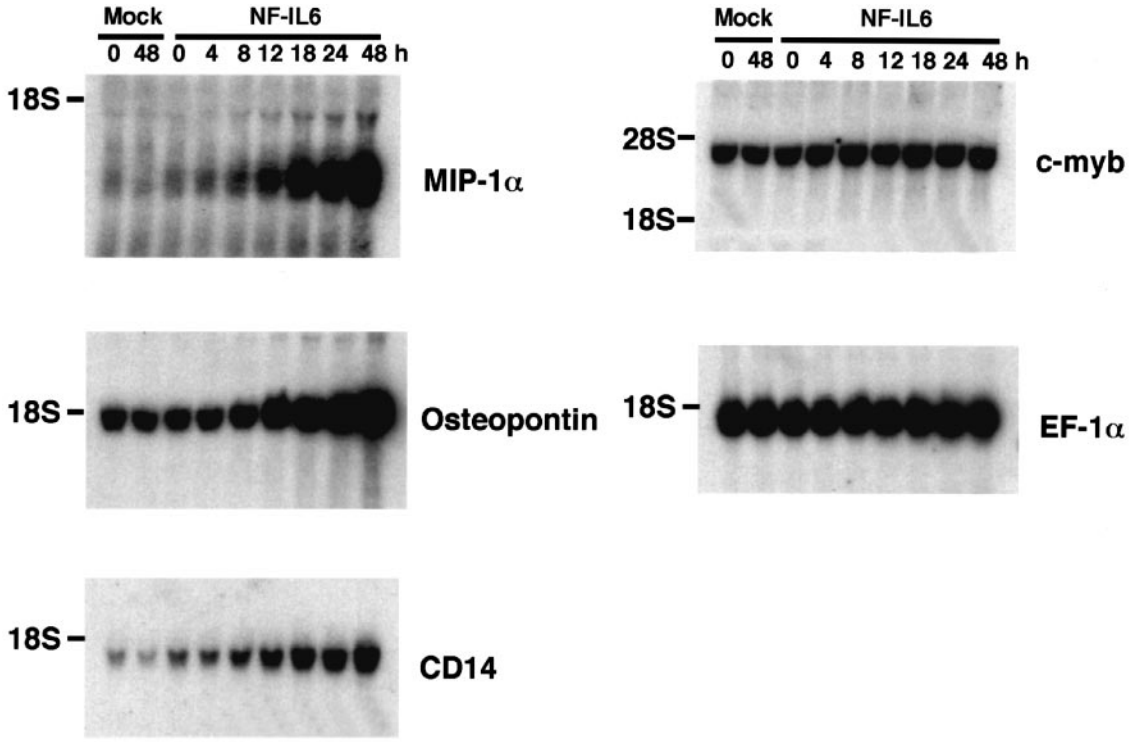

Fig. 2. Levels of MIP-1α, osteopontin, CD14 and c-*myb* mRNA in Mock M1 cells and NFIL6 M1 cells following treatment with IPTG. Total RNA (15 μg/lane) was extracted from Mock M1 and NFIL6 M1 cells at the indicated times following treatment with 1 mM IPTG. Expression was analyzed by Northern blot hybridization using the indicated cDNA probes. EF-1α is a control transcript expressed at relatively constant levels for 48 h after addition of IPTG.

least 12 h following IPTG treatment. This result suggests that these genes are directly induced by the transcriptional activity of NF-IL6. To demonstrate that NF-IL6 inducible genes are direct targets of NF-IL6, we performed reporter gene assays for the MIP-1α, osteopontin and CD14 promoters. MIP-1α chemokine gene expression is rapidly and transiently induced by endotoxin in macrophages (36). Previous studies have shown that the proximal promoter sequences from position –220 to +36 (relative to the transcription start site) are sufficient to confer inducible transcription in transfection assays (38). We linked the murine MIP-1α promoter sequences extending from position –676 to +29 to the luciferase reporter plasmid pGL3basic vector to produce the construct p-676 Luc. p-676 Luc was then transfected into exponentially growing NFIL6 M1 cells by electroporation. The cells were split into two equal parts post-transfection and cultured in the presence (1 mM) or absence of IPTG. After 20 h, the cultures were harvested, cellular extracts were prepared and the promoter activity was determined as relative light units compared with the promoterless pGL3 basic vector. IPTG-induced expression of NF-IL6 led to an approximate 3-fold induction of the MIP-1α promoter activity (Fig. 3). We also examined whether the osteopontin and CD14 promoters were activated by inducible expression of NF-IL6. We inserted the mouse osteopontin promoter from position –979 to +65 and the mouse CD14 promoter from position –647 to +78 in the pGL3basic vector. Following transient transfection into NFIL6 M1 cells, luciferase activities were determined as described above. However, no responsiveness to NF-IL6 was detected in the proximal promoter region of either gene (data not shown).

To identify *cis* elements conferring the responsiveness of the MIP-1α promoter to NF-IL6, a series of 5′-deletion constructs was transiently transfected into NFIL6 M1 cells and their responsiveness to IPTG assayed as described above. The constructs p-676 Luc, p-341 Luc and p-172 Luc were responsive to IPTG, but the truncation to –99 resulted in complete loss of responsiveness to IPTG (Fig. 3). We next tested the interaction of NF-IL6 with potential C/EBP binding elements within the murine MIP-1α promoter. A previous study has suggested that the proximal MIP-1α promoter contains four C/EBP binding sequences, which correspond to positions –145 to –136, –122 to –113, –107 to –96 and –55 to –46 (38). The positions –122 to –113 and –55 to –46 contain completely matched and closely related C/EBP binding elements respectively. On the other hand, both the positions –145 to –136 and –107 to –96 have weak similarities with C/EBP binding consensus sequences. Four double-stranded oligonucleotides (A, B, C and D) that contained putative C/EBP binding sequences (Fig. 4A) were radiolabeled and used in EMSA performed with nuclear protein from NFIL6 M1 cells. As shown in Fig. 4(B), when probe B was incubated with nuclear extracts prepared from IPTG-treated NFIL6 M1 cells, three binding activities (complexes b, c and d) appeared (Fig. 4B, lane 6). A 50-fold molar excess of unlabeled oligonucleotide from the human IL-6 promoter, which contains one C/EBP consensus motif, competed away these nuclear

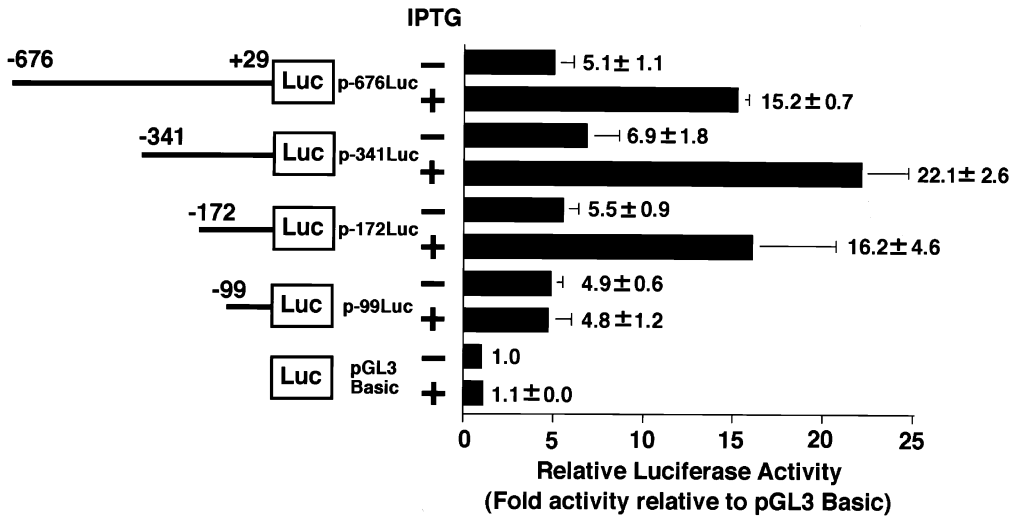

Fig. 3. NF-IL6 stimulates transcription of the MIP-1α promoter in transactivation assays. Murine MIP-1α reporter gene constructs (from top: MIP-1α promoter construct p-676Luc, p-341Luc, p-172Luc, p-99Luc, promoterless luciferase construct pGL3Basic) are shown schematically on the left. Numbers indicate positions relative to the transcription start site. The results of luciferase assays are illustrated on the right. NFIL6 M1 cells were co-transfected with 20 μg of these constructs and 2 μg of a sea-pansy luciferase vector (pRL-TK) as a transfection efficiency control. The cells were split into two equal parts post-transfection and cultured in the presence (1 mM) or absence of IPTG. The cultures were harvested 20 h after transfection and analyzed for luciferase activity. The promoter activity was determined as relative light units compared with the promoterless pGL3 basic vector. The data represent the mean ± SD of three independent experiments.

protein–DNA complexes (Fig. 4B, lane 7). Rabbit polyclonal antibody to NF-IL6 inhibited the formation of these complexes and resulted in supershifted complexes (Fig. 4B, lane 8). These results indicate that NF-IL6 can bind to probe B. Since complex c was detected in control cells (Fig. 4B, lane 5), it is most likely that LIP homodimers form this complex. NF-IL6–LIP heterodimers and NF-IL6 homodimers form a slower-migrating broad complex (complex b) which were strongly induced in nuclear extracts prepared from IPTG-treated NFIL6 M1 cells (Fig. 4B, lane 6). Complex d was also induced like complex b and showed faster-migration than complex c. Complex d may be composed of the degraded NF-IL6 protein. Probe D also forms three complexes (complexes e, f and g) like probe B (Fig. 4B, lane 14). Complexes e, f, and g correspond to complexes b, c, and d respectively. Probe–NF-IL6 binding activities (complexes b and e) were higher than probe–LIP binding activities (complexes c and f) (Fig. 4B, lanes 6 and 14). In contrast, we found that no probe C–nuclear protein complexes were (i) induced following IPTG addition, (ii) competed away by unlabeled C/EBP consensus oligonucleotides or (iii) supershifted by anti-NF-IL6 antibody (Fig. 4B, lanes 9–12). When probe A was incubated with the nuclear extracts, complex a appeared to be induced following IPTG addition (Fig. 4B, lane 2). However, neither unlabeled C/EBP consensus oligonucleotide nor anti-NF-IL6 antibody inhibited this binding activity (Fig. 4B, lane 3 and 4). This result suggests that complex a contains nuclear protein except NF-IL6 and the binding activity of this nuclear protein increases after inducible expression of NF-IL6. Taken together, these results demonstrate that the murine MIP-1α proximal promoter contains at least two NF-IL6 binding sites.

Transactivation of MIP-1α promoter by NF-IL6 requires two NF-IL6 binding sites

To verify the importance of the NF-IL6 binding sites for MIP-1α induction, we mutated these sites and tested for effects on MIP-1α promoter function. Double-stranded oligonucleotides harboring mutated C/EBP binding sites were designed for B and D probes, and referred to as mB and mD respectively. As shown in Fig. 5(A), a 50-fold molar excess of unlabeled oligonucleotides B and D competed away complexes b, c and d and complexes e, f and g respectively. (Fig. 5A, lanes 3 and 7). On the other hand, a 50-fold molar excess of unlabeled oligonucleotides mB and mD could not inhibit the formation of probe-NF-IL6 complexes (Fig. 5A, lanes 4 and 8). Identical mutations were then introduced by site-directed mutagenesis into p-172 Luc to form pMutBLuc and pMutD Luc. These promoter constructs were transiently transfected into NFIL6 M1 cells, and their luciferase activities were compared between untreated and IPTG-treated cells. A point mutation in either NF-IL6 binding site impaired the ability of NF-IL6 to transactivate individual promoter constructs (Fig. 5B). These results clearly demonstrate that NF-IL6 requires both NF-IL6 binding sites to activate the MIP-1α proximal promoter.

Discussion

We developed a strategy to examine the downstream genes of NF-IL6 in murine M1 monocytic leukemia cells. We isolated MIP-1α, osteopontin and CD14 as potential target genes of NF-IL6, which have not previously been recognized as downstream genes of this transcription factor. Using a reporter gene assay, we showed that MIP-1α is a direct target of NF-IL6.

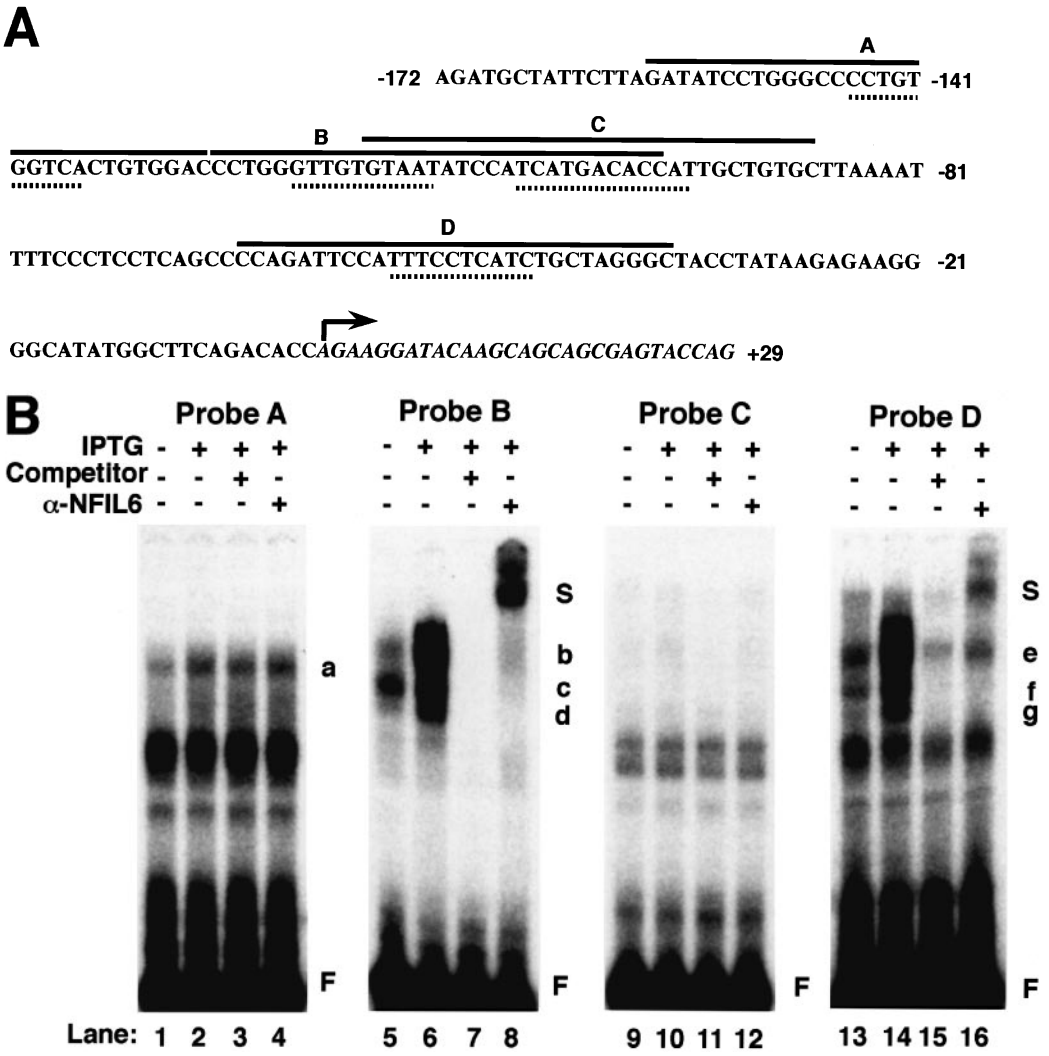

Fig. 4. EMSA of the putative C/EBP binding sequences within the murine MIP-1α proximal promoter. (A) Nucleotide sequences of the murine MIP-1α proximal promoter. The transcriptional start site is *overlined* by a *forward arrow* and designated as position +1. The *dashed underlines* indicate putative C/EBP binding sites described previously (38). Sequences used to generate double-stranded oligonucleotides for EMSA studies are indicated by *lines above* the sequences, and designated A, B, C and D. Each oligonucleotide covers one C/EBP binding site. (B) The double-stranded A–D oligonucleotides were ^{32}P-labeled and incubated with nuclear protein prepared from control NFIL6 M1 cells (lanes 1, 5, 9 and 13) or 12 h IPTG-treated NFIL6 M1 cells (lanes 2–4, 6–8, 10–12 and 14–16). The double-stranded competitor oligonucleotide from the human IL-6 promoter, which contains one NF-IL6 binding site, was added at a 50-fold molar excess over probe oligonucleotides (lanes 3, 7, 11 and 15). In lanes 4, 8, 12 and 16, rabbit polyclonal antibody to NF-IL6 was added to the binding reaction. The positions of free probe (F) and supershifted complex (S) are marked on the *right* side of each panel. The positions of DNA–nuclear protein complexes detected with probe A, B and D are also indicated on the *right* sides.

Inducible expression of NF-IL6 was able to activate the proximal promoter-luciferase construct of the MIP-1α gene. Grove and Plumb (38) revealed four putative C/EBP binding sites (A, B, C and D; Fig. 4A) within the proximal –160 bp promoter region of murine MIP-1α. We showed that NF-IL6 binds to only two of these sites (B and D), both of which are responsible for activation of the MIP-1α proximal promoter–luciferase construct by NF-IL6. Since two unbound sites have weak homology with the NF-IL6 binding consensus sequence, we believe that NF-IL6 is unable to bind to these elements. NF-IL6 protein expression appeared within 4 h after IPTG addition, followed by elevation of MIP-1α, osteopontin and CD14 mRNA within 12 h after IPTG addition. Although the quantity of NF-IL6 protein remained unchanged from 4 to 48 h following IPTG addition, the transcript levels for MIP-1α, osteopontin and CD14 showed sequential augmentation up to 48 h following IPTG addition. NFIL6 M1 cells constitutively express LIP, which works as a negative modulator of NF-IL6 (33). In EMSA using the MIP-1α promoter, probe–NF-IL6 binding activities were higher than probe–LIP bindings (Fig. 4B, lanes 5 and 6 and lanes 13 and 14). NF-IL6 homodimers and NF-IL6–LIP heterodimers may gradually overcome the negative effect of LIP and exert their transcriptional activity.

NF-IL6 up-regulated MIP-1α mRNA expression at levels 27-fold-higher than those of untreated cells after 48 h, but this

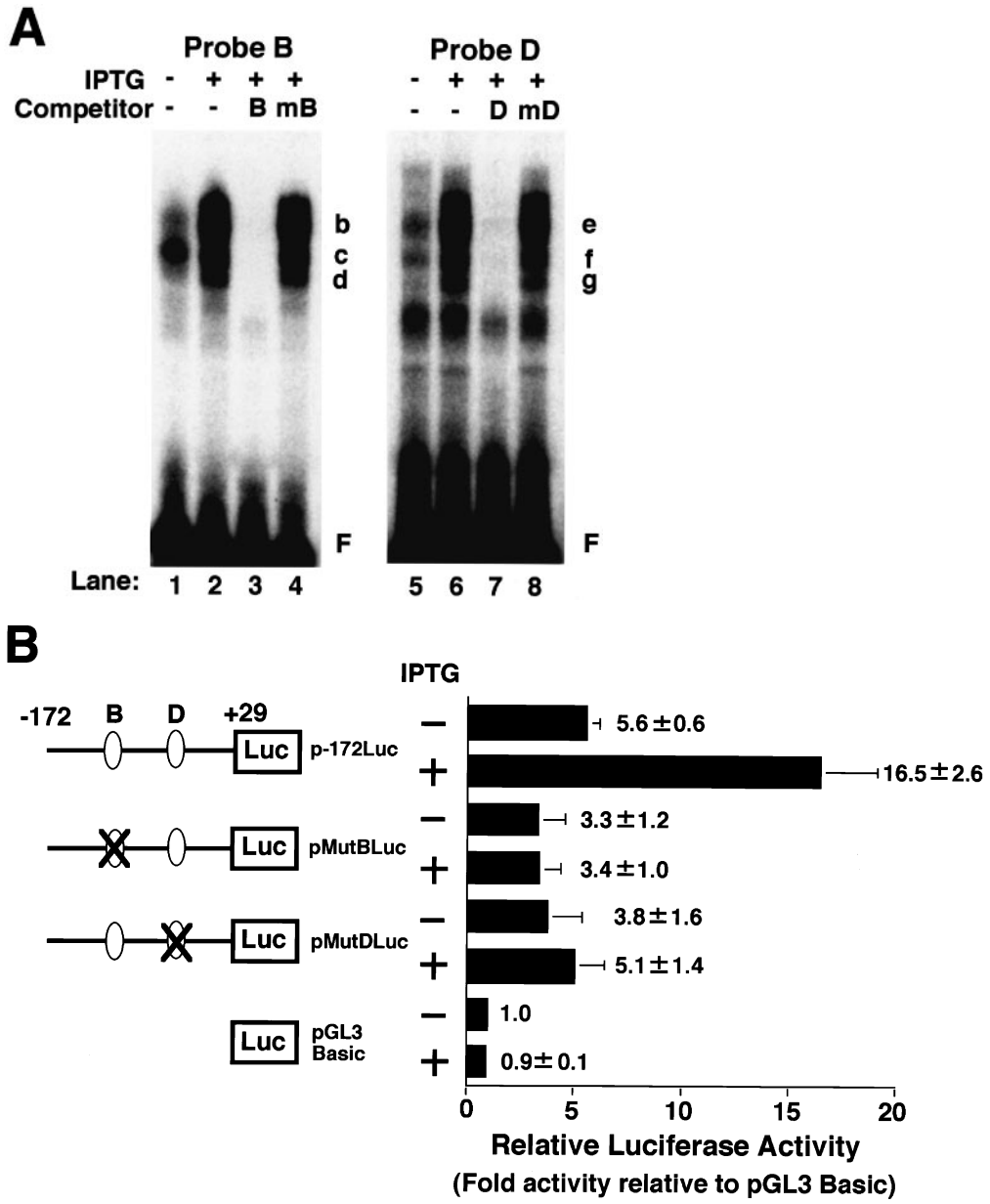

Fig. 5. Transactivation of MIP-1α promoter by NF-IL6 requires two NF-IL6 binding sites. (A) Competition analysis of the mutated NF-IL6 binding sites using an EMSA. The double-stranded B and D oligonucleotides were ^{32}P labeled and incubated with nuclear protein prepared from control NFIL6 M1 cells (lanes 1 and 5) or 12 h IPTG-treated NFIL6 M1 cells (lanes 2–4 and 6–8). A 50-fold molar excess of unlabeled wild-type oligonucleotides B or D (lanes 3 and 7) and NF-IL6 binding site mutant oligonucleotides mB or mD (lanes 4 and 8) were added to the binding reaction. Symbols marked on the *right* side of each panel are as described in the legend to Fig. 4. (B) Murine MIP-1α reporter gene constructs (from top: MIP-1α promoter construct p-172Luc, position –115 to –114 NF-IL6 binding site mutated construct pMutBLuc and position –54 to –53 NF-IL6 binding site mutated construct pMutDLuc, promoterless luciferase construct pGL3Basic) are shown schematically on the left. Numbers indicate positions relative to the transcription start site. The closed circles B and D represent NF-IL6 binding sites located within oligonucleotides B and D used in EMSA respectively. The results of luciferase assays are illustrated on the right. NFIL6 M1 cells were co-transfected with 20 μg of these constructs and 2 μg of a sea-pansy luciferase vector (pRL-TK) as a transfection efficiency control. The cells were split into two equal parts post-transfection and cultured in the presence (1 mM) or absence of IPTG. The cultures were harvested 20 h after transfection and analyzed for luciferase activity. The promoter activity was determined as relative light units compared with the promoterless pGL3 basic vector. The data represent the mean ± SD of three independent experiments.

does not fit well to the level of induction using the MIP-1α promoter–luciferase construct (~3-fold in Fig. 3). NF-IL6 may activate other transcription factors which bind to other *cis*-elements in the surrounding of the MIP-1α promoter or in the distal enhancer. This was suggested by the finding of another binding protein (complex a in Fig. 4B) whose expression was induced by NF-IL6. It is possible that NF-IL6 and another transcription factor synergistically activate transcription of their tar-

get genes. Mutational analysis showed that activation of the MIP-1α promoter by NF-IL6 requires two NF-IL6 binding sites and these sites work collaboratively. Multiple bindings of NF-IL6 are necessary for transactivation of the MIP-1α promoter as in the case of the β-casein promoter which requires multiple binding sites of NF-IL6 for transactivation by lactogenic hormones in mammary epithelial cells (39).

Ritter *et al.* (40) showed that MIP-1α nuclear protein (MNP) is crucial for transcription of the human MIP-1α gene. MNP binds to position –119 to –90 of the human MIP-1α promoter, which corresponds to position –122 to –91 of the murine MIP-1α promoter. This sequence contains one NF-IL6 binding sequence at position –122 to –113 in our experiments and is responsible for the increased promoter activity induced by NF-IL6. However, Ritter *et al.* (40) did not examine the possibility that C/EBP-related proteins may bind to this region. MNP binding activity occurs in non-stimulated U937 cells, but no NF-IL6 proteins appear in non-stimulated U937 cells (17). In contrast, C/EBP expression constitutively occurs in U937 cells (17) and therefore MNP may be C/EBP. Another member of the C–C branch of the chemokine family, MCP-1, contains C/EBP binding elements in its promoter region and LPS-inducible MCP-1 expression is directly regulated by NF-IL6 in a lymphoblast cell line (24). To our knowledge, MIP-1α is the second chemokine gene demonstrated to be a transcriptional target of NF-IL6.

Some macrophage-differentiation markers may be downstream genes of NF-IL6. Osteopontin is a bone sialoprotein presumed to be important in the development and functioning of a number of mammalian organs, and possibly in the progression of malignancies (41–43). This highly phosphorylated protein can function in cell attachment via an Arg–Gly–Asp sequence and is likely involved in cell–matrix interactions (44). Osteopontin expression sequentially increases with macrophage differentiation (45). However, the regulation of the osteopontin gene expression is poorly understood in macrophages. CD14 is a receptor for the complex composed of LPS and LPS-binding protein (46–48). Little or no CD14 is expressed in monocytic precursors and its expression is strongly up-regulated during the course of monocytic differentiation (49,50). A previous study has suggested that a 12-*O*-tetradecanoylphorbol-13-acetate-responsive element-like sequence located at position –255 is responsible for monocyte-specific expression of the murine CD14 gene (51). Another study has shown that the first 128 bp promoter of human CD14 has monocyte-specific promoter activity and that Sp1 transcription factor contributes significantly to monocytic specific expression (52). No previous reports have raised the possibility that monocyte-specific expression of osteopontin and CD14 genes may involve the C/EBP family of transcription factors. Inducible expression of NF-IL6 dramatically increases endogenous expression of osteopontin and CD14 genes, and therefore the simplest interpretation of our results is that NF-IL6 acts directly on these downstream genes. Although the murine osteopontin and CD14 proximal promoter luciferase constructs were not activated by NF-IL6, both mRNAs first increased at 12 h after IPTG addition in NFIL6 M1 cells, as did MIP-1α mRNA. NF-IL6 may interact with far upstream or downstream enhancers of these genes, as NF-M, a chicken NF-IL6-related transcription factor, binds to the far upstream chicken lysozyme enhancer at –6.1 kb (34).

Osteopontin and CD14 are monocytic differentiation markers, and sequential elevation of these transcripts suggests that inducible expression of NF-IL6 induces differentiation of M1 cells to macrophages. NF-IL6 expression increases markedly during differentiation to a macrophage lineage in M1 cells, histiocytic leukemia cells U937 and promyelocytic leukemia cells HL60 (17). M1 cells can be induced to differentiate into macrophages after exposure to IL-6, and this process accompanies expression of the Fcγ receptor on the cell surface and a reduction in proto-oncogene c-*myb* mRNA (53). However, inducible expression of NF-IL6 fails either to induce Fcγ receptor expression (data not shown) or reduce c-*myb* mRNA expression (Fig. 2). Recently, we demonstrated that activation of signal transducer and activator of transcription 3 (STAT3) is the most critical step in the differentiation of M1 cells to macrophages (27). Thus, NF-IL6 increases the expression of several monocytic differentiation markers, but is not able to induce full differentiation of M1 cells to macrophages.

In this study we demonstrate that the combination of an inducible expression system and subtraction cloning is a powerful method to identify downstream genes of NF-IL6. Further examination of this method in a variety of cell lines will identify more target genes, leading to greater understanding of the function of this transcription factor.

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Abbreviations

C/EBP	CCAAT/enhancer binding protein
EF	elongation factor
EMSA	electrophoretic mobility shift assay
G-CSF	granulocyte colony stimulating factor
IPTG	isopropyl-β-D-thiogalactoside
LAP	liver-enriched transcriptional activator protein
LIP	liver inhibitory protein
LPS	lipopolysaccharide
MIP	macrophage inflammatory protein
MNP	MIP-1α nuclear protein
NF-IL6	nuclear factor-IL-6
TNF	tumor necrosis factor

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