MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 1231–1240 Vol. 16, No. 3
0270-7306/96/$04.00+0

CCAAT Enhancer-Binding Protein (C/EBP) and AML1 (CBFα2) Synergistically Activate the Macrophage Colony-Stimulating Factor Receptor Promoter

DONG-ER ZHANG,[1]* CHRISTOPHER J. HETHERINGTON,[1] SHARI MEYERS,[2] KRISTINA L. RHOADES,[1] CHRISTOPHER J. LARSON,[3] HUI-MIN CHEN,[1] SCOTT W. HIEBERT,[2] AND DANIEL G. TENEN[1]

Division of Hematology/Oncology, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215[1]; Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101[2]; and The Salk Institute for Biological Studies, La Jolla, California 92037[3]

Received 23 August 1995/Returned for modification 27 September 1995/Accepted 21 December 1995

Transcription factors play a key role in the development and differentiation of specific lineages from multipotential progenitors. Identification of these regulators and determining the mechanism of how they activate their target genes are important for understanding normal development of monocytes and macrophages and the pathogenesis of a common form of adult acute leukemia, in which the differentiation of monocytic cells is blocked. Our previous work has shown that the monocyte-specific expression of the macrophage colony-stimulating factor (M-CSF) receptor is regulated by three transcription factors interacting with critical regions of the M-CSF receptor promoter, including PU.1 and AML1. PU.1 is essential for myeloid cell development, while the AML1 gene is involved in several common leukemia-related chromosome translocations, although its role in hematopoiesis has not been fully identified. Along with AML1, a third factor, Mono A, interacts with a small region of the promoter which can function as a monocyte-specific enhancer when multimerized and linked to a heterologous basal promoter. Here, we demonstrate by electrophoretic mobility shift assays with monocytic nuclear extracts, COS-7 cell-transfected factors, and specific antibodies that the monocyte-enriched factor Mono A is CCAAT enhancer-binding protein (C/EBP). C/EBP has been shown previously to be an important transcription factor involved in hepatocyte and adipocyte differentiation; in hematopoietic cells, C/EBP is specifically expressed in myeloid cells. In vitro binding analysis reveals a physical interaction between C/EBP and AML1. Further transfection studies show that C/EBP and AML1 in concert with the AML1 heterodimer partner CBFβ synergistically activate M-CSF receptor by more than 60-fold. These results demonstrate that C/EBP and AML1 are important factors for regulating a critical hematopoietic growth factor receptor, the M-CSF receptor, suggesting a mechanism of how the AML1 fusion protein could contribute to acute myeloid leukemia. Furthermore, they demonstrate physical and functional interactions between AML1 and C/EBP transcription factor family members.

Transcription factors play a key role in the development and differentiation of specific lineages from multipotential progenitors. This theme has been studied extensively for some hematopoietic lineages, notably erythroid and lymphoid cells, but only recently have some of the major regulators of monocytic cells been identified (25, 58). Identification of these regulators and determining the mechanism of how they activate their target genes not only are important for understanding normal development of monocytes and macrophages, but recent studies have implicated many of these transcription factors in the pathogenesis of the most common form of adult acute leukemia, in which the differentiation of monocytic cells is blocked (33).

An example of a transcription factor which plays an important role in normal monocytic gene expression and in acute myelogenous leukemia (AML) is AML1. AML1 is a member of the core binding factor (CBF) or polyomavirus enhancer-binding protein 2 (PEBP2) family of transcription factors. The members of the CBF family consist of heterodimers between DNA-binding α subunits and a β subunit (CBFβ) which does not bind DNA directly but which enhances the binding of the α subunit (49, 73). Multiple α-subunit genes, including *CBFA1*, *AML1* (*CBFA2*), and *CBFA3*, as well as alternatively spliced isoforms of the α and β subunits, have been detected (31, 39, 49, 65, 68). In particular, a short form (AML1A) and a long form (AML1B) of AML1 have been described, and it is the long form which appears to include the transactivation function (35, 39, 65, 68). All of the CBFα proteins contain a Runt domain, which is similar to the protein product of the *Drosophila* pair-rule gene *runt*, which encodes an early-acting segmentation protein that regulates the expression of other segmentation genes (22, 23). AML1 was identified by studying one of the most frequent chromosomal translocations found in AML, t(8;21)(q22;q22) (11, 34, 38, 40, 47). In this case, the 5′ part of the AML1 gene, including the *runt* domain but lacking the transactivation domain, is fused to almost the entire ETO gene on chromosome 8. Recent studies have indicated that this AML1/ETO fusion protein can act as a dominant negative inhibitor of AML1 transactivation function (12, 35, 65). The expression of some of the normal AML1 gene targets which play a key role in monocytic differentiation might be adversely affected by AML1/ETO, and these include granulocyte mac-

* Corresponding author. Mailing address: Division of Hematology/Oncology, Department of Medicine, Beth Israel Hospital and Harvard Medical School, RE219, 330 Brookline Avenue, Boston, MA 02215. Phone: (617) 667-8930. Fax: (617) 667-3299. Electronic mail address: dzhang@bih.harvard.edu.

rophage colony-stimulating factor (GM-CSF) (12, 65), interleukin-3 (IL-3) (6), the macrophage colony-stimulating factor (M-CSF) receptor (79), and myeloperoxidase (45). AML1 fusion proteins, including AML1/MDS1, AML1/EAP, AML1/Evi-1, and TEL/AML1, are also involved in other forms of leukemia (15, 36, 46, 47, 53). The β subunit of CBF, CBFβ, is also involved in a chromosomal inversion, inv(16)(p13;q22), which is associated with FAB M4eo AML (32). Therefore, each of the two chains of the CBF heterodimer is directly implicated in the pathogenesis of AML.

The CCAAT enhancer-binding proteins (C/EBP) represent a family of transcription factors which have been suggested to play a role in monocytic development. C/EBPα was the first-identified member of one family of basic region-leucine zipper (bZIP) DNA-binding proteins (21, 27, 28). The basic region is highly positively charged and directly interacts with DNA. The leucine zipper domain forms an α-helical coil and is directly involved in homo- and heterodimerization. C/EBPα, C/EBPβ, and C/EBPδ are strongly similar in their C-terminal basic region and leucine zipper domains and diverge in their N-terminal transactivation domains (7, 43, 76, 77). Previous studies have hinted at a role for C/EBP in myeloid development and differentiation. The chicken homolog of C/EBPβ, NF-M, is specifically expressed in the myeloid cell lineage within the hematopoietic system (5, 16, 24, 44, 63). NF-M binds to critical promoter regions of the chicken myelomonocytic growth factor (cMGF), which is distantly related to the mammalian hematopoietic growth factors G-CSF and IL-6. In mammals, C/EBPβ (which is identical to human NF-IL6 [1]) can bind to sites in promoters for the inflammatory cytokines IL-1β, IL-6, tumor necrosis factor alpha, and G-CSF and macrophage lysozyme (42, 51, 69). Mice with targeted disruption of the C/EBPβ gene have shown a lymphoproliferative disorder with overexpression of IL-6 and defective macrophage activation with abnormal bacterial killing and tumor cytotoxicity (57, 67). However, macrophage production and differentiation are apparently not blocked in C/EBPβ$^{-/-}$ mice.

While the C/EBP are expressed in a number of different tissues, studies to date have suggested that their expression may be limited in the hematopoietic system to myeloid (monocytic and granulocytic) cells. It has been shown that C/EBP are specifically expressed in human myelomonocytic cell lines and not in human erythroid cell, B-cell, and T-cell lines (56), in keeping with the myeloid cell-specific expression previously noted for avian C/EBP (16, 24, 44, 63). This indicates the possible importance of C/EBPα during myelomonocytic cell differentiation. In contrast to liver cells and adipocytes, in myeloid cells, C/EBPα is highly expressed in unstimulated and undifferentiated cells (42, 56). When cells were treated with differentiation or activation reagents, such as IL-6, lipopolysaccharide, or G-CSF, C/EBPβ (and C/EBPδ) expression rapidly increased and C/EBPα gradually decreased (42, 56). In sum, these studies suggest the possibility that C/EBPα is more important for directing the commitment of multipotential hematopoietic stem cells to differentiate toward the myeloid cell lineage, while C/EBPβ is more important for the functional activation of differentiated myeloid cells, such as macrophages, and studies of myeloid development in C/EBPα and C/EBPβ knockout mice will be helpful in confirming this hypothesis. However, the key targets of the C/EBP in mammalian monocytic development and their possible role in leukemia have not been elucidated.

One potential key target for monocytic transcriptional regulators is M-CSF (CSF-1), which is involved in the differentiation, proliferation, and survival of cells of the monocytic lineage (59, 62). The M-CSF receptor is encoded by the c-*fms* proto-oncogene, a cell surface tyrosine kinase (TK) receptor (59). Normal expression of the M-CSF receptor is restricted to two tissues; monocytes and placental trophoblasts, in which it is regulated by two different promoters (52, 70). We and others have demonstrated previously that transcriptional regulation is involved in the monocyte-specific expression of the human M-CSF receptor (20, 37, 52, 79, 80). A small region of DNA encompassing the monocytic transcription initiation sites (bp −85 to +71) of the human receptor gene shows tissue-specific promoter activity in transient transfection experiments (79). Several transcription factors have been shown to specifically bind to the human M-CSF receptor promoter in this small region to critically regulate its expression (79, 80). One factor is PU.1, a B-cell- and myeloid cell-specific factor encoded by the *Ets* family *Spi-1* oncogene (26, 41). PU.1 regulates many myeloid cell-specific genes, including the G-CSF receptor and GM-CSF receptors (19, 61), and is essential for myeloid development (55, 71). PU.1 interacts with the human M-CSF receptor promoter at bp −54 to −29 (80). We recently identified AML1 as a second factor which interacts with the M-CSF receptor promoter at bp −75 to −59, therefore showing that the AML1/CBFβ heterodimer regulates the expression of a critical receptor for myeloid cell lineage differentiation (79).

Besides PU.1 and AML1, a third monocyte-enriched factor, Mono A, interacts with the M-CSF receptor promoter at bp −87 to −73, just upstream of the AML1 site (Fig. 1A), and is important for monocyte-specific M-CSF receptor promoter activity. Significantly, the promoter fragment containing only the Mono A- and AML1-binding sites can function as a monocyte-specific enhancer element (79). Here, we demonstrate that Mono A is identical to C/EBP. In addition, we demonstrate physical interaction and synergy between these two factors resulting in stimulation of M-CSF receptor promoter activity.

MATERIALS AND METHODS

Cell culture. Human Mono Mac 6 cells were propagated as described elsewhere (81). African green monkey kidney fibroblast-like CV-1 cells (ATCC CCL 70) and COS-7 cells, which were established from CV-1 cells after transformation with an origin-defective mutant of simian virus 40 which produces wild-type large-T antigen (ATCC CRL 1651), were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 2 mM L-glutamine.

Nuclear extracts. COS-7 cells were transfected by $Ca_3(PO_4)_2$ precipitation with 20 μg of either the C/EBPα expression plasmid, pMSV/EBPα; the C/EBPβ expression plasmid, pMSV/EBPβ; or the C/EBPδ expression plasmid, pMSV/EBPδ (7). Nuclear proteins were harvested 72 h after transfection as described elsewhere (2). Nuclear extracts from Mono Mac 6 cells were prepared by the method described by Dignam et al. (9) in the presence of 1 μg of proteinase inhibitors leupeptin, pepstatin A, chymostatin, antipain, aprotinin, and trypsin inhibitor per ml. Nuclear protein concentrations were assayed by the Bradford method.

EMSA. ^{32}P-labeled double-stranded oligonucleotides for electrophoretic mobility shift assays (EMSA) were prepared as previously described (79). One oligonucleotide probe was the human M-CSF receptor promoter −88 to −73 region; the other probe was 5′-TGCAGATTGCGCAATCTGCA-3′, containing a C/EBP binding site (21). DNA binding conditions were as previously described (79). The reaction mixtures were electrophoresed at 10 V/cm on a 6% polyacrylamide gel (bisacrylamide-acrylamide, 1:29) in 0.5× TBE (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA) at 4°C. The oligonucleotide 5′-TGCAGAgactagtcTCTGCA-3′ (mutations in lowercase) was used as a C/EBP-binding mutant oligonucleotide. For antibody supershift experiments, 1 μl of an antiserum raised against either the carboxyl-terminal four-fifths of murine C/EBPα, the carboxyl-terminal 18 amino acids of murine C/EBPβ, or full-length murine C/EBPδ was added to the reaction mixture (7). Each C/EBP antiserum reacts specifically with both its murine and its human gene products and not with the other two C/EBP proteins (data not shown); for example, the C/EBPα antiserum reacts with murine and human C/EBPα but not with C/EBPβ or C/EBPδ. As a control, 1 μl of a rabbit antiserum raised against the transcription factor Oct-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) was used instead.

Plasmid constructions. The wild-type M-CSF receptor promoter-luciferase constructs p540M-CSF-R-luc and pM-CSF-R-luc, containing bp −416 to +124 and bp −416 to +71 of the human M-CSF receptor promoter, respectively, were described previously (80). The mutated constructs pM-CSF-R(mA)-luc and pM-

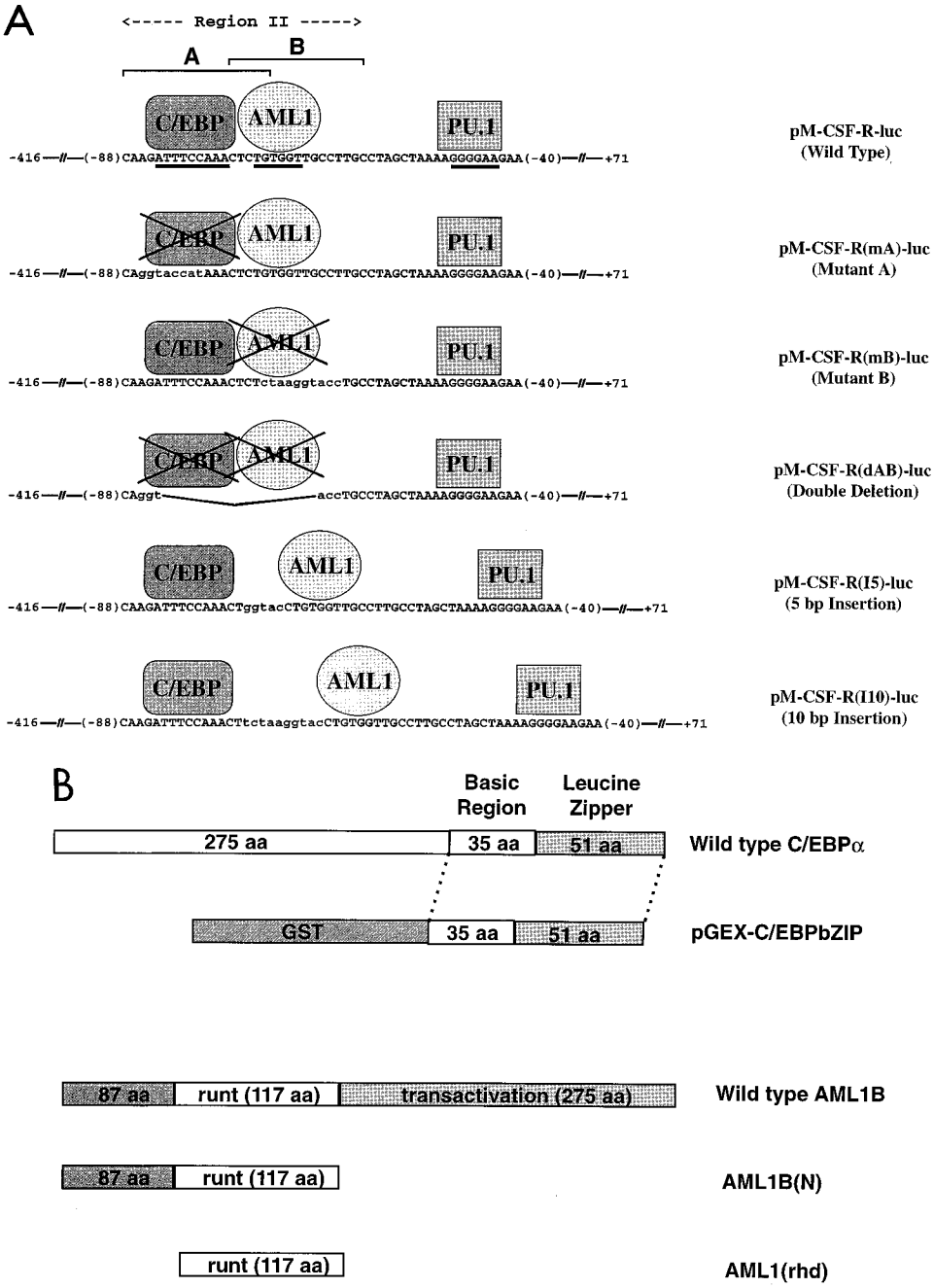

FIG. 1. Schematic of M-CSF receptor promoter, promoter mutants, and GST fusion proteins. (A) Sequences of the wild-type and mutant M-CSF receptor promoters used in this study, as well as the locations of the C/EBP-, AML1-, and PU.1-binding sites. Wild-type promoter sequences are indicated by capital letters. The locations of regions IIA and IIB are indicated by brackets, and the cores of the binding sites are underlined below the wild-type sequence (top line). A crossed out site indicates that the site has been mutated to a nonbinding sequence, with the mutated sequence indicated by lowercase letters. The sequences inserted between the C/EBP and AML1 sites in pM-CSF-R(I5)-luc and pM-CSF-R(I10)-luc are also indicated by lowercase letters. (B) Domain structures of C/EBPα and its GST fusion protein and of AML1B and its deletion constructs. The details of the constructions are described in Materials and Methods. aa, amino acids.

CSF-R(mB)-luc changed the sequence at bp −86 to −79 from AGATTTCC to GGTACCAT and at bp −71 to −62 from GTGGTTGCCT to CTAAGGTACC, respectively (Fig. 1A) (79). To construct pM-CSF-R(dAB)-luc, two DNA fragments were generated by PCR, with the plasmid p540M-CSF-R-luc as a template. Primer A (5′-CGGGATCCAGATATGCATTACTTTGGAGATTCCA AGG-3′) was used with primer B (5′-GGGGTACCTGGGTCTTTAAGAA G-3′) to generate PCR fragment 1. Primer C (5′-GGGGTACCCCTCGGTGG GGAAGTGGCA-3′) was used with primer D (5′-GGGGTACCTGCCTAG CTAAAAGG-3′) to generate PCR fragment 2. PCR fragment 1 was digested with *Bam*HI and *Kpn*I. PCR fragment 2 was digested with *Kpn*I and *Sac*I. Digested PCR fragments 1 and 2 were ligated with *Bam*HI- and *Sac*I-digested vector pXP2 to construct pM-CSF-R(dAB)-luc. pM-CSF-R(dAB)-luc lacks bp −86 to −62 of the wild-type promoter, and therefore deletes both C/EBPα- and AML1-binding sites (Fig. 1A). To construct pM-CSF-R(I5)-luc and pM-CSF-R(I10)-luc, three DNA fragments were generated by PCR with the plasmid p540M-CSF-R-luc as a template. Primer A (5′-CGGGATCCAGATATGCAT TACTTTGGAGATTCCAAGG-3′) was used with primer E (5′-GGGGTAC CAGTTTGGAAATCTTGG-3′) and primer F (5′-GGGGTACCTTAGAGTT TGGAAATCTTGGG-3′) to generate PCR fragments 3 and 4, respectively. Primer C (5′-GGGGTACCCCTCGGTGGGGAAGTGGCA-3′) was used with primer G (5′-GGGGTACCTGTGGTTGCCTTGCC-3′) to generate PCR fragment 5. PCR fragments 3 and 4 were digested with *Bam*HI and *Kpn*I. PCR

fragment 5 was digested with *Kpn*I and *Sac*I. Digested PCR fragments 3 and 5 were ligated with *Bam*HI- and *Sac*I-digested vector pXP2 to construct pM-CSF-R(I5)-luc, which inserts an extra 5 bp between the C/EBPα- and AML1-binding sites. Digested PCR fragments 4 and 5 were ligated with *Bam*HI- and *Sac*I-digested vector pXP2 to construct pM-CSF-R(I10)-luc, which contains an extra 10 bp between the C/EBPα- and AML1-binding sites (Fig. 1A). pGEX-C/EBP-bZIP was constructed by subcloning the human C/EBPα bZIP region (68a) in frame with the glutathione *S*-transferase (GST) moiety in pGEX-2TK (Fig. 1B). pGEX-AML1runt contains the entire AML1 *runt* domain in frame with the GST moiety in the pGEX-2TK vector and was constructed by using PCR to amplify the entire *runt* domain with primer H (5′-CGCGGATCCGGCGAGCTGGT GC-3′) and primer I (5′-CCGATGCGGCCGCGAATTCTGCCGATGTCTTC GAT-3′) with template pCMV5-AML1B (35), by digesting with *Bam*HI and *Eco*RI, and by ligating into *Bam*HI- and *Eco*RI-digested pGEX-2TK (Fig. 1B). pGEX-CBFβ was constructed by inserting the coding region of CBFβ cDNA into pGEX-2T (30). The sequences of these constructs were confirmed by the dideoxy chain termination method.

Transient transfections. Mono Mac 6 cells were transfected by electroporation in RPMI 1640 medium at 960 μF and 230 V. Cells were harvested 5 h posttransfection in 0.5 ml of lysis buffer, and luciferase assays were performed as previously described (50). CV-1 cells were transfected by the $Ca_3(PO_4)_2$ precipitation method with 10 μg of the M-CSF receptor promoter luciferase constructs in the presence or absence of other transcription factor expression constructs, with salmon sperm DNA as carrier, to a total of 25 μg of DNA per ml of $Ca_3(PO_4)_2$ precipitation. Transfection efficiency was normalized to the levels of growth hormone expressed from cotransfected plasmid containing the Rous sarcoma virus promoter directing human growth hormone gene expression (pRSV-hGH) (2 μg for Mono Mac 6 cells and 0.25 μg for CV-1 cells), and the data are given in relative light units (RLU) per nanogram of growth hormone. Growth hormone concentrations were measured by radioimmunoassaying (Nichols Institute, San Juan Capistrano, Calif.).

Expression and purification of recombinant proteins. A 100-ml volume of *Escherichia coli* DH5α cells containing GST fusion protein expression plasmids was cultured at 37°C for 1 h after 1:10 dilution of the cells. The production of GST fusion protein was induced by culturing cells at 32°C for 3 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. GST fusion proteins were prepared as described elsewhere (60). The quality and the quantity of GST fusion proteins were examined by Coomassie blue staining of sodium dodecyl sulfate (SDS) gels with bovine serum albumin as a standard. C/EBPα, AML1B, and CBFβ were in vitro translated from the plasmids pKS-C/EBPα (C/EBPα cDNA in the *Eco*RI and *Hin*dIII sites of pBluescript KS$^-$), pBS-AML1B (35), and pKS-CBFβ5 (35), respectively, with the TnT T7-coupled reticulocyte lysate system (Promega no. L4610) according to the manufacturer's protocols. Approximately 2 μg of GST protein or GST fusion proteins immobilized on glutathione agarose beads was incubated with in vitro-translated ^{35}S-labeled proteins for 2 h in 500 μl of interaction buffer (150 mM NaCl, 20 mM Tris [pH 7.5], 0.3% Nonidet P-40, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μg of leupeptin, pepstatin A, chymostatin, antipain, aprotinin, and trypsin inhibitor per ml) at 4°C with gentle rocking. The protein-GST beads were washed four times with the same buffer at 4°C. SDS-polyacrylamide gel electrophoresis was then used to analyze bound proteins.

RESULTS

C/EBP transcription factors interact with region IIA of the M-CSF receptor promoter. Previous studies have shown that transcriptional regulation is very important for tissue-specific expression of the M-CSF receptor in monocytic cells (20, 37, 52, 80) and that the region from bp −85 to +71 of the human M-CSF receptor contains monocytic promoter activity (79). This region can interact with transcription factors PU.1 and, AML1 and a monocyte-enriched factor, which we named Mono A, as shown in Fig. 1A (79). Mono A specifically binds to region IIA (bp −88 to −73) of the M-CSF receptor. Transient transfections with region IIA-mutated promoter-luciferase gene constructs demonstrated that region IIA was critical for M-CSF receptor promoter activity (79). Heterologous promoter studies showed that region II functions as a monocyte-specific enhancer (79). We compared the DNA sequence in region IIA with the binding region sequences of known transcription factors. The sequence TTTGGAAAT on the noncoding strand of the M-CSF receptor at bp −76 to −84 is similar to the sequence TTNNGNAAT, which is a consensus sequence C/EBP binding site (42). To assay whether C/EBP family members could interact with the M-CSF receptor promoter region IIA, recombinant C/EBPα, C/EBPβ, and C/EBPδ were prepared from COS-7 cells transiently transfected with expression vectors for these C/EBP proteins. These proteins were used in EMSA analysis with a ^{32}P-labeled, double-stranded region IIA oligonucleotide (bp −88 to −73) and an oligonucleotide containing the C/EBP binding site. As shown in Fig. 2, nuclear extracts isolated from untransfected COS-7 cells did not interact with the M-CSF receptor promoter region IIA probe (lane 1) or the C/EBP binding site probe (data not shown). Recombinant C/EBPα (42 kDa), C/EBPβ (34 kDa), and C/EBPδ (33 kDa) bind to both the C/EBP oligonucleotide probe and the M-CSF receptor promoter region IIA.

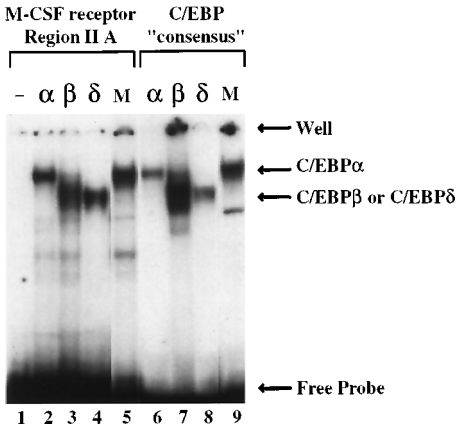

FIG. 2. Transcription factor C/EBP bind to the M-CSF receptor promoter region IIA. Oligonucleotides containing either the M-CSF receptor promoter (bp −88 to −73) (lanes 1 to 5) or the C/EBP-binding site (lanes 6 to 9) were ^{32}P labeled and used as probes in EMSA analysis in the presence of 1 μg of double-stranded poly(dI · dC). Five micrograms of nuclear protein from untransfected COS-7 cells (lane 1) and from COS-7 cells transfected with C/EBPα expression plasmid (pMSV/EBPα [lanes 2 and 6]), C/EBPβ expression plasmid (pMSV/EBPβ [lanes 3 and 7]), or C/EBPδ expression plasmid (pMSV/EBPδ) and 5 μg of nuclear protein from Mono Mac 6 cells (lanes 5 and 9) were used in the binding reaction mixtures. The binding reaction mixtures were assayed by gel mobility shift electrophoresis as described elsewhere (79, 80). C/EBPα, C/EBPβ, and C/EBPδ indicate the complexes formed with oligonucleotides and different full-length C/EBP, respectively.

Nuclear factor Mono A from monocyte nuclear extract which binds to region IIA of M-CSF receptor promoter is C/EBP. As shown in Fig. 2, the DNA-protein complex formed with monocyte nuclear extract migrates at exactly the same position as recombinant C/EBPα. This indicates that C/EBP could be the Mono A factor described in our previous study. To confirm that C/EBP is indeed the transcription factor bound to region IIA in monocytic cells, nuclear extracts from monocytic Mono Mac 6 cells and the region IIA probe were used in EMSA competition and supershift experiments as shown in Fig. 3. The shifted protein-DNA complex can be inhibited specifically by unlabeled region IIA oligonucleotide or a C/EBP-binding oligonucleotide (Fig. 3, lanes 3 and 4) but not by a mutated C/EBP-binding oligonucleotide which no longer binds to C/EBP (Fig. 3, lane 5). When an antiserum directed specifically against C/EBPα was present in the binding reaction mixture, the specific Mono A DNA-protein complex was abolished (Fig. 3, lane 6). Antisera against C/EBPβ, C/EBPδ, or Oct-1 did not have any significant effect on the formation of the DNA-protein complex. These data indicate that monocytic transcription factor Mono A is C/EBP and that C/EBPα is the major form of the C/EBP in the binding complex.

Extending the distance between the adjacent C/EBP- and AML1-binding sites reduces M-CSF receptor promoter activity. The binding sites for C/EBP and AML1 in the M-CSF

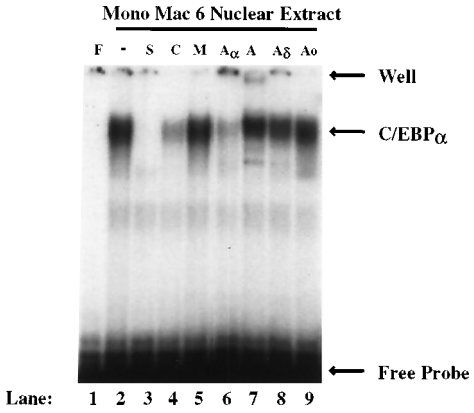

FIG. 3. Identification of C/EBPα binding to the M-CSF receptor promoter region IIA by gel mobility shift analysis. The M-CSF receptor promoter region IIA oligonucleotide (bp −88 to −73) was ^{32}P labeled and incubated with 1 μg of double-stranded poly(dI · dC) in the absence (lane 1) and presence of 5 μg of nuclear protein prepared from Mono Mac 6 cells (lanes 2 to 9). For competition analysis, 50-fold molar excesses of unlabeled region IIA oligonucleotide (lane 3), C/EBP consensus-binding site oligonucleotide (lane 4), and an oligonucleotide containing a mutated C/EBP-binding site which no longer binds C/EBP (lane 5) were used in the binding reaction mixtures. In lanes 6 to 9, 1 μl of anti-C/EBPα serum (lane 6), anti-C/EBPβ serum (lane 7), anti-C/EBPδ serum (lane 8), or anti-Oct-1 serum (lane 9) was added to the binding reaction mixture to identify the transcription factor. The band observed in lane 7 just below the well is a nonspecific band which can be observed in the absence of C/EBPβ protein (data not shown).

receptor promoter are in close proximity. There are only 3 bp between the two consensus binding sites (Fig. 1). The C/EBP site mutation pM-CSF-R(mA) only abolishes C/EBP binding and does not affect the binding of AML1 to the M-CSF receptor promoter and vice versa (79). Mutations at either the C/EBP-binding site or the AML1-binding site in the M-CSF receptor promoter significantly decrease promoter activity. The percentages of decrease resulting from mutation of either site are very similar (79). Both single-site mutations decrease promoter activity specifically in monocytic cells to 13% of the wild-type promoter activity (79). These data suggested that C/EBP and AML1 cooperate with each other. To further study this question, 5- and 10-bp insertion mutations between the C/EBP- and AML1-binding sites in the M-CSF receptor promoter were generated to change the orientation on the helix and the distance between these two factors (Fig. 1). The promoter-luciferase constructs with insertion mutations were then used in transient transfection analysis as shown in Fig. 4. In monocytic Mono Mac 6 cells, both 5- and 10-bp insertions reduced the activity of the M-CSF receptor promoter to 20% of the activity of the wild-type promoter. Thus, changing the relative orientation on the helix by insertion of 5 bp between the two factor-binding sites and changing the distance between the two factor-binding sites by insertion of 10 bp both significantly decreased promoter activity. These results suggested that C/EBP and AML1 cooperate to activate the M-CSF receptor promoter.

C/EBP physically interacts with AML1, but not the AML1 heterodimer partner CBFβ, in the absence of DNA. As noted previously, AML1 belongs to the CBF (PEBP2) family of transcription factors. CBF heterodimers consists of a DNA-binding α subunit (CBFα) and a non-DNA-binding β subunit (CBFβ) (34, 48, 73). Although the β subunit does not bind to DNA, it facilitates the binding of the α/β complex to DNA. Since insertion of 5 or 10 bp between the two binding sites supported the idea that C/EBP and AML1 function in a cooperative manner (Fig. 4), we further asked whether we could detect any physical interaction between these two proteins. We were unable to identify a more slowly migrating complex containing both C/EBP and CBF by EMSA using the M-CSF receptor promoter region II as a probe (data not shown). This indicates either that there is no interaction between the two factors or, alternatively, that the interaction is not stable enough to be detected by EMSA under our experimental conditions. To further study possible physical interactions, we performed in vitro binding experiments in which *E. coli*-expressed GST fusion proteins immobilized on glutathione agarose beads were incubated with in vitro-translated, ^{35}S-labeled proteins (Fig. 1B). As shown in Fig. 5A, in vitro-translated AML1B can be specifically retained on agarose beads containing the fusion protein made from the AML1 heterodimer partner CBFβ (GST-CBFβ) but not on glutathione agarose beads containing only GST. However, in vitro-translated C/EBP was not retained on GST-CBFβ-coated beads. This indicated that there is probably no direct interaction between CBFβ and C/EBP. We then studied the interaction between C/EBP and AML1 using the GST C/EBPα basic region leucine zipper domain fusion protein (GST-C/EBPbZIP) and in vitro-translated AML1B as shown in Fig. 5B. AML1B can be specifically retained on agarose beads containing the GST-C/EBPbZIP fusion protein. To verify that the interaction is not due to nonspecific binding to agarose beads, the same volume of glutathione agarose beads either with the preparation used for GST fusion protein or without any treatment was used in the binding reaction mixture. As shown in lanes 4 and 5 of Fig. 5B, there is no significant interaction of naked beads with AML1B. A total of 16% of the ^{35}S-labeled AML1B added to the binding reaction mixture was bound to GST-bZIP. This result demonstrated a direct physical interaction between C/EBP and AML1B. As a positive control, GST-CBFβ was used in a binding reaction mixture with ^{35}S-labeled AML1B. As shown in Fig. 5B, 32% of the ^{35}S-labeled AML1B added to the binding reaction mixture was bound to GST-CBFβ. Since the Runt domain of AML1 protein plays the major role in the interactions of AML1 with both the CBFβ subunit and the Ets-1 transcription factor (14, 34, 78), we tested the role of the Runt domain in the interaction between C/EBP and AML1. As shown in Fig. 5C, the in vitro-translated AML1 Runt homology domain [AML1(rhd)] showed a specific interaction with both

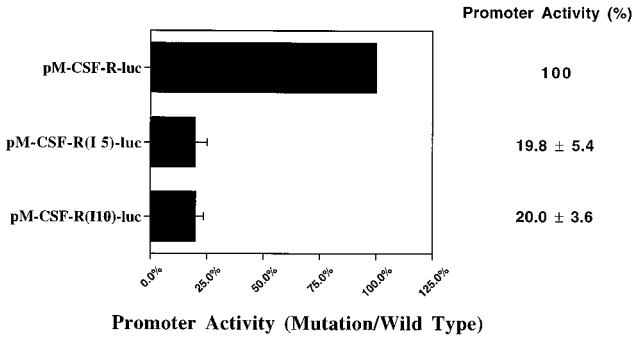

FIG. 4. The relative orientations of and distance between C/EBP and AML1 on the M-CSF receptor promoter are critical for promoter activity. The wild-type M-CSF receptor promoter-luciferase construct pM-CSF-R-luc and 5- and 10-bp insertion mutant constructs pM-CSF-R(I5)-luc and pM-CSF-R(I10)-luc were transfected into the human monocytic cell line Mono Mac 6. The average promoter activities were generated from three separate experiments. The standard deviations of the means are indicated by the error bars. Luciferase activities were normalized for transfection efficiency with the cotransfected growth hormone plasmid RSV-hGH.

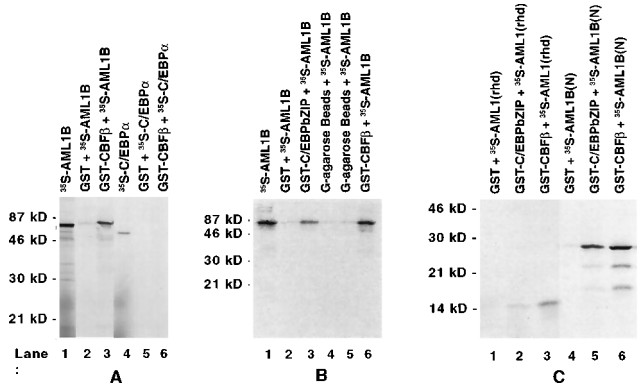

FIG. 5. C/EBP and AML1 physically associate with each other. (A) In vitro translated, ^{35}S-labeled AML1B (lanes 1 to 3) and C/EBPα (lanes 4 to 6) were incubated with *E. coli*-produced GST (lanes 2 and 5, respectively) or GST-CBFβ (lanes 3 and 6, respectively) immobilized on glutathione agarose beads. (B) In vitro-translated, ^{35}S-labeled AML1B was loaded directly on the gel (2 μl [lane 1]) or was incubated (5 μl) with *E. coli*-produced GST (lane 2), GST-C/EBPbZIP (lane 3), or GST-CBFβ immobilized on glutathione agarose beads (lane 6). As a control, the same volume of glutathione agarose beads as in the GST-C/EBPbZIP binding reaction, treated in the same manner as GST fusion protein beads (lane 4), or glutathione agarose beads without any treatment (lane 5) was also used in a binding reaction with ^{35}S-labeled AML1B. (C) The in vitro-translated, ^{35}S-labeled AML1 Runt homology domain, AML1(rhd), was incubated with *E. coli*-produced GST (lane 1), GST-C/EBPbZIP (lane 2), or GST-CBFβ (lane 3) immobilized on glutathione agarose beads. Since AML1(rhd) is a small peptide and has only three methionines to label, the radiolabeled signal is very weak. To confirm this result, the in vitro-translated, ^{35}S-labeled AML1B amino-terminal peptide AML1B(N) (amino acids 1 to 206) was used to perform the same experiment as that with AML1(rhd) (lanes 4 to 6). Bound proteins were analyzed in SDS-polyacrylamide gels and were visualized by autoradiography. Apparent molecular size standards were bovine serum albumin (87 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21.5 kDa). The structures of the GST-AML1B and GST-C/EBPbZIP proteins are shown in Fig. 1B.

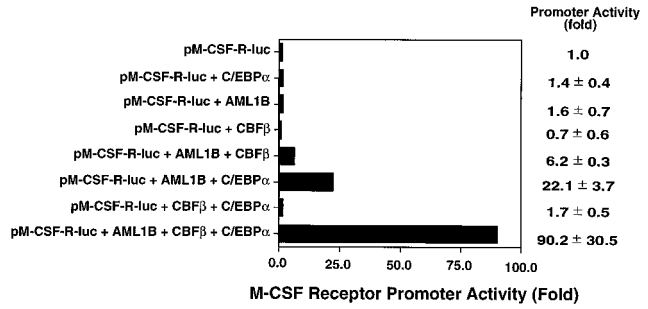

FIG. 6. C/EBP and AML1 synergistically activate the M-CSF receptor promoter. CV-1 cells were transfected by the $Ca_3(PO_4)_2$ precipitation method with 10 μg of the M-CSF receptor promoter luciferase construct pM-CSF-R-luc in the presence or absence of 1 μg of the C/EBPα, AML1B, and CBFβ expression constructs pMSV-C/EBPα, pCMV5-AML1B, and pCMV5-CBFβ, respectively, as indicated. Luciferase activities were normalized for transfection efficiency with the cotransfected growth hormone plasmid RSV-hGH. The data are the averages of three sets of experiments. The standard deviations of the means are indicated by the error bars. Fold increases in promoter activity are relative to that of the wild-type promoter in the absence of any additional transcription factors.

GST-C/EBPbZIP and GST-CBFβ. This indicated that the physical interaction between C/EBP and AML1B is mediated at least in part through the Runt domain of the AML1B protein. This result is further confirmed by using the C-terminally deleted AML1B protein AML1B(N) (Fig. 1B) as shown in Fig. 5C.

Transcriptional synergy between C/EBP and AML1B. C/EBP- and AML1-binding site mutation analysis (Fig. 4) and protein-protein interaction analysis (Fig. 5) both indicated that C/EBP and AML1 may function cooperatively to activate the M-CSF receptor promoter. To obtain further proof of this hypothesis, we performed cotransactivation experiments with monkey kidney CV-1 cells as shown in Fig. 6. In CV-1 cells, basal M-CSF receptor promoter activity is about 3- to 10-fold above background compared with the promoterless construct. When C/EBPα, AML1B, or CBFβ was used alone in transactivation experiments with the M-CSF receptor promoter, there was no significant activation of promoter activity (Fig. 6). There was still no transactivation observed even after increases in the amount of expression vector to 5 or 10 μg in the transfection experiments (data not shown). When AML1B and its heterodimer partner CBFβ were used together, there was a 6-fold induction of M-CSF receptor promoter activity (Fig. 6). This indicates that CBF factors can activate the M-CSF receptor promoter without the presence of C/EBP. When C/EBPα and AML1B were used in the transactivation study in the absence of CBFβ, a 22-fold induction of M-CSF receptor promoter activity could be detected, demonstrating a synergistic effect between AML1B and C/EBPα. In the absence of AML1B, C/EBPα and CBFβ did not show any significant transactivation, in keeping with their lack of physical interactions (Fig. 5). When all three factors, C/EBPα, AML1B, and CBFβ, were used in the same transactivation experiment, M-CSF receptor promoter activity was induced by 90-fold. This demonstrated very strong transcriptional synergy of M-CSF receptor promoter activity in the presence of all three factors.

To assay whether transcriptional synergy with these factors is specifically mediated through the C/EBP- and AML1-binding sites, the M-CSF receptor promoter constructs with mutations which abolished C/EBP or AML1 binding or both were used in transactivation experiments in the presence of all three transcription factors. As shown in Fig. 7, compared with the 55-fold induction of the wild-type promoter, the promoter lacking the C/EBP-binding site or both C/EBP- and AML1-binding sites showed only 2-fold induction with the expression of C/EBPα, AML1B, and CBFβ. Again, loss of only the AML1-binding site caused a much lower (6-fold) induction than that by the wild type. These data demonstrate that the synergistic effect of

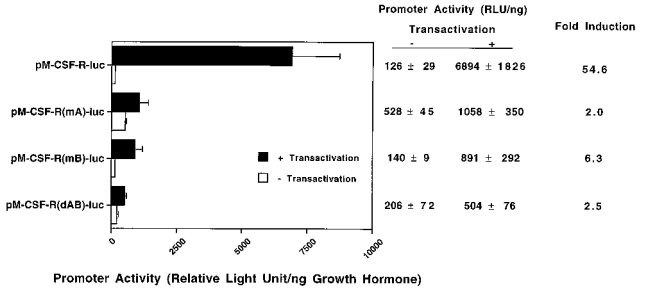

FIG. 7. C/EBP and AML1 activation of the M-CSF receptor promoter depends on interaction with their binding sites on the M-CSF receptor promoter. CV-1 cells were transfected by the $Ca_3(PO_4)_2$ precipitation method with 10 μg of the wild-type M-CSF receptor promoter luciferase construct (pM-CSF-R-luc) or with a promoter with mutations in the C/EBP [pM-CSF-R(mA)-luc]-, AML1 [pM-CSF-R(mB)-luc]-, or C/EBP- and AML1-binding sites [pM-CSF-R(dAB)-luc] in the absence (white bars) or presence (black bars) of 1 μg each of the C/EBPα, AML1, and CBFβ expression constructs pMSV-C/EBPα, pCMV5-AML1B, and pCMV5-CBFβ, respectively. Fold induction is calculated by dividing the promoter activity with the three transactivators by that without the three transactivators for each M-CSF receptor construct. The data are the averages of three sets of experiments. The standard deviations of the means are indicated by the error bars.

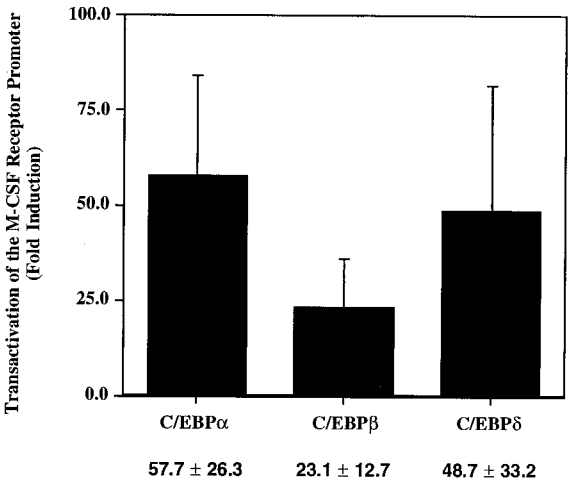

FIG. 8. Different members of the C/EBP family can work synergistically with AML1 and CBFβ to activate the M-CSF receptor promoter. CV-1 cells were transfected by the $Ca_3(PO_4)_2$ precipitation method with 10 μg of the wild-type M-CSF receptor promoter luciferase construct pM-CSF-R-luc in the absence of any transcription factor expression plasmid or in the presence of 1 μg of the AML1B and CBFβ expression constructs pCMV5-AML1B and pCMV5-CBFβ, respectively. One microgram each of the C/EBPα, C/EBPβ, and C/EBPδ expression plasmids pMSV-C/EBPα, pMSV-C/EBPβ, and pMSV-C/EBPδ, respectively, was added in separate transfections. The fold induction is calculated by dividing the promoter activity with transactivation (addition of three factors, AML1B, CBFβ, and one of the three C/EBP proteins) by the promoter activity without transactivation. The data are the averages of three sets of experiments. The standard deviations of the means are indicated by the error bars.

these three transcription factors depends on intact DNA-binding sites.

There are multiple members of the C/EBP family, and all of these C/EBP contain very similar DNA-binding and dimerization basic leucine zipper domains (7, 77). Although C/EBPα was the major form of C/EBP from monocytes which bound to the M-CSF receptor promoter (Fig. 2), recombinant C/EBPα, C/EBPβ, and C/EBPδ can all bind to the M-CSF receptor promoter region IIA (Fig. 2 and 3). The protein interaction results in Fig. 5 had shown that a common bZIP region among C/EBP was critical for the AML1 and C/EBP interaction. To analyze the ability of other C/EBP to effect M-CSF receptor promoter activity, C/EBPβ and C/EBPδ were used in transactivation experiments in the presence of both AML1B and CBFβ. As shown in Fig. 8, in addition to C/EBPα, both C/EBPβ and C/EBPδ could transactivate the M-CSF receptor promoter.

DISCUSSION

In this study, we extended our previous work to identify C/EBP as a critical transcription factor for M-CSF receptor monocytic expression (previously named Mono A [79]). In vitro protein-binding studies demonstrated a physical interaction between C/EBP and AML1, which bind to the M-CSF receptor promoter adjacent to each other. Transient cotransfection experiments showed very strong synergistic coactivation of the M-CSF receptor promoter by coexpressed C/EBP and AML1.

Here, we report that C/EBPα is critical for the activity of the M-CSF receptor promoter. Very recent studies from our group have shown that C/EBPα also plays a significant role in both G-CSF receptor and GM-CSF receptor α-chain promoter activities (19, 61). Signal transduction from these three growth factors (M-CSF, G-CSF, and GM-CSF) inducing multipotential hematopoietic cells to differentiate toward the myeloid cell lineages depends on expression of their specific receptors. Therefore, C/EBPα could play a major role in regulation of myeloid cell development. This hypothesis can be tested by the analysis of myelopoiesis in C/EBPα knockout mice (72).

An important question to be answered is which C/EBP protein is important for M-CSF receptor expression at different stages of macrophage development, from the first stages of myeloid cell commitment from multipotential progenitors to the late stages of macrophage maturation and activation. One possibility is that all three C/EBP proteins (C/EBPα, C/EBPβ, and C/EBPδ) contribute to expression of the receptor, with the important variable being the pattern of C/EBP expression. The observed pattern of C/EBP expression during macrophage commitment and activation suggests that C/EBPα might be important for early expression in progenitors, but that later on C/EBPβ and C/EBPδ may be important during maturation and activation of macrophages (42, 56). However, these studies did not compare absolute amounts of the different C/EBP in myeloid cells at different stages of differentiation (56). In the myeloid cell lines used in our studies, we observed binding only by C/EBPα. Additional studies with unstimulated and stimulated primary macrophages could help answer this question. A second hypothesis is that while all three C/EBP, C/EBPα, C/EBPβ, and C/EBPδ, can bind to and activate the M-CSF receptor promoter (Fig. 2 and 8), differences in affinities will also play an important role. Our studies suggest differences in the binding affinities of different recombinant C/EBP to the M-CSF receptor promoter site relative to a consensus C/EBP binding site oligonucleotide (Fig. 2) (7). C/EBPβ binding is relatively weaker than binding of C/EBPα and C/EBPδ. The different affinities might explain differences in efficiencies of the C/EBP in coactivation of the M-CSF receptor promoter shown in Fig. 8. More definitive affinity studies are necessary to answer this question. Finally, the presence of other interacting proteins, such as AML1, may affect which C/EBP binds to and activates the promoter, although to date in our in vitro EMSA we have not been able to detect cooperativity in DNA binding of C/EBP and AML1 to their adjacent sites.

The identification of C/EBP as the Mono A factor further supports the idea that promoter specificity is mediated by a combination of transcription factors with different tissue specificities. We have previously shown that the activity and specificity of the M-CSF receptor promoter are mediated by a small DNA fragment containing binding sites for PU.1, AML1, and C/EBP (Fig. 1A) (79). PU.1 is myeloid cell and B-cell specific (8, 26), while AML1 is expressed in myeloid cells as well as lymphocytes (4, 31, 35a, 39, 54, 66, 79). In the hematopoietic system, C/EBPα may be specifically expressed in myeloid cells (56) and may be preferentially expressed in macrophages (51a). Therefore, the combinatorial activities of PU.1, AML1, and C/EBPα, all of which are specifically expressed in macrophages, may mediate the specificity of the M-CSF receptor promoter. In our previous studies, a DNA fragment containing both C/EBP- and AML1-binding sites, without the PU.1 site, can function as a monocyte-specific enhancer by using the heterologous basal TK promoter (79). It may be that in the case of the M-CSF receptor, PU.1, which binds to TFIID (17, 74), it is necessary to recruit the TATA-binding protein; however, this function can be substituted by the TATA box in the TK promoter, as was found in studies of the FcγR1b promoter (10). These hypotheses can be tested by mutating and swapping the PU.1 site and the TATA box in the M-CSF receptor and TK promoters. It will be interesting to analyze whether a DNA fragment containing solely the PU.1-, AML1-, and

C/EBP-binding sites can direct gene expression in a monocyte-specific manner in vivo in transgenic mouse studies.

It has been previously suggested that AML1 acts to facilitate the action of other adjacent transcription factors, and it is interesting that the AML1 site is located between the C/EBPα and the PU.1 sites in the M-CSF receptor promoter (Fig. 1A). Significantly, although the C/EBP site is critical for M-CSF receptor activity in transfection experiments (79), C/EBP alone does not transactivate M-CSF receptor promoter activity (Fig. 6). The adjacent factor AML1, along with CBFβ, can by itself activate the M-CSF receptor promoter by 6-fold, but the synergistic activation by C/EBP added to that by AML1 and CBFβ is much greater (more than 60-fold). All C/EBP have highly similar bZIP domains (7, 77), and AML1 interacts with the bZIP fragment of C/EBP (Fig. 5), explaining why C/EBPα, C/EBPβ, and C/EBPδ all can synergistically activate the M-CSF receptor promoter with AML1 (Fig. 8). Either the physical interaction between C/EBP and AML1 could either stabilize binding of both to the M-CSF receptor or their interaction might provide a surface for another transcription factor to bind and activate the M-CSF receptor promoter.

AML1 is a member of the CBF family of proteins, all of which have similar DNA-binding *runt* domains (3, 4, 31, 39, 49, 75). Recent evidence obtained with antisera specific for CBFα3 (AML2) and CBFα1 (AML3) indicates that myeloid cells (and B cells) express both AML1 and CBFα3, whereas the majority of the activity in T cells is composed of AML1 (35a). Since the Runt domain is directly involved in physical interaction with C/EBP (Fig. 5) and the Runt domain is the most conserved region among CBF proteins, other CBF members may substitute for AML1 in activation of the M-CSF receptor. Therefore, we tested this possibility by replacing AML1 with AML2 in the transactivation experiments. The results showed that AML2 can function like AML1 to transactivate the M-CSF receptor promoter.

Previous reports have indicated that AML1 can interact with Ets-1 and Myb (14, 18, 64, 78). Our report is the first to describe physical interaction and cooperativity between AML1 and C/EBP. In contrast to the interaction between AML1 and Ets-1 (14, 64, 78), this interaction is also mediated via the AML1 Runt domain (Fig. 5). The data do not rule out the possibility that other regions of AML1B and C/EBPα in addition to the Runt and bZIP domains are involved in this physical interaction. The activation domains for both C/EBP and AML1 are outside the bZIP and Runt domains, respectively. However, these two domains are critical for the interaction of each factor with DNA and other transcription factors (13, 14, 29, 35, 43, 64). We also studied the function of these two domains in activation of the M-CSF receptor promoter by expressing GST-AML1(rhd) or GST-C/EBPbZIP in transient transfection experiments in a manner similar to the experiments shown in Fig. 6. The results indicate that neither AML1 (rhd) nor C/EBPbZIP can function as full-length AML1 or C/EBP for the activation of the M-CSF receptor promoter (data not shown). Further studies are necessary to elucidate which domains in AML1 and C/EBP are involved in the synergistic activation of M-CSF receptor expression.

ACKNOWLEDGMENTS

We thank H. W. L. Ziegler-Heitbrock for providing Mono Mac 6 cells; Alan Friedman, Steven McKnight, John Papaconstantinou, Gretchen Darlington, and Philip Auron for C/EBP expression plasmids and antisera; and Nancy Speck, Gregory Verdine, and Gerd Blobel for valuable discussions.

This work is supported in part by grants CA/AI59589 (D.E.Z.), CA64140 (S.W.H.), and CA41456 (D.G.T.) and Cancer Center (CORE) support grant P30 CA21765 from the National Institutes of Health and by the American Lebanese Syrian Associated Charities (ALSAC), St. Jude Children's Research Hospital.

REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9:**1897–1906.
2. **Andrews, N. C., and D. V. Faller.** 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. **19:**2499.
3. **Bae, S. C., E. Takahashi, Y. W. Zhang, E. Ogawa, K. Shigesada, Y. Namba, M. Satake, and Y. Ito.** 1995. Cloning, mapping and expression of PEBP2 alpha C, a third gene encoding the mammalian runt domain. Gene **159:** 245–248.
4. **Bae, S. C., Y. Yamaguchi-Iwai, E. Ogawa, M. Maruyama, M. Inuzuka, H. Kagoshima, K. Shigesada, M. Satake, and Y. Ito.** 1993. Isolation of PEBP2 alpha B cDNA representing the mouse homolog of human acute myeloid leukemia gene, AML1. Oncogene **8:**809–814.
5. **Burk, O., S. Mink, M. Ringwald, and K. H. Klempnauer.** 1993. Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. EMBO J. **12:**2027–2038.
6. **Cameron, S., D. S. Taylor, E. C. Tepas, N. A. Speck, and B. Mathey-Prevot.** 1994. Identification of a critical regulatory site in the human interleukin-3 promoter by in vivo footprinting. Blood **83:**2851–2859.
7. **Cao, Z., R. M. Umek, and S. L. McKnight.** 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev. **5:**1538–1552.
8. **Chen, H. M., P. Zhang, M. T. Voso, S. Hohaus, D. A. Gonzalez, C. K. Glass, D. E. Zhang, and D. G. Tenen.** 1995. Neutrophils and monocytes express high levels of PU.1 (Spi-1) but not Spi-B. Blood **85:**2918–2928.
9. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
10. **Eichbaum, Q. G., R. Iyer, D. P. Raveh, C. Mathieu, and A. B. Ezekowitz.** 1994. Restriction of interferon gamma responsiveness and basal expression of the myeloid human FcgammaR1b gene is mediated by a functional PU.1 site and a transcription initiator consensus. J. Exp. Med. **179:**1985–1996.
11. **Erickson, P., J. Gao, K. S. Chang, T. Look, E. Whisenant, S. Raimondi, R. Lasher, J. Trujillo, J. Rowley, and H. Drabkin.** 1992. Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood **80:**1825–1831.
12. **Frank, R., J. Zhang, S. Hiebert, S. Meyers, and S. Nimer.** 1994. AML1B but not the AML1/ETO fusion protein can transactivate the GM-CSF promoter. Blood **84:**229a.
13. **Friedman, A. D., W. H. Landschulz, and S. L. McKnight.** 1989. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes Dev. **3:**1314–1322.
14. **Giese, K., C. Kingsley, J. R. Kirshner, and R. Grosschedl.** 1995. Assembly and function of a TCR alpha enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. Genes Dev. **9:**995–1008.
15. **Golub, T. R., G. F. Barker, S. K. Bohlander, S. W. Hiebert, D. C. Ward, P. Brayward, E. Morgan, S. C. Raimondi, J. D. Rowley, and D. G. Gilliland.** 1995. Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. Proc. Natl. Acad. Sci. USA **92:**4917–4921.
16. **Haas, J. G., M. Strobel, A. Leutz, P. Wendelgass, C. Muller, E. Sterneck, G. Riethmuller, and H. W. Ziegler-Heitbrock.** 1992. Constitutive monocyte-restricted activity of NF-M, a nuclear factor that binds to a C/EBP motif. J. Immunol. **149:**237–243.
17. **Hagemeier, C., A. J. Bannister, A. Cook, and T. Kouzarides.** 1993. The activation domain of transcription factor PU.1 binds the retinoblastoma (RB) protein and the transcription factor TFIID in vitro: RB shows sequence similarity to TFIID and TFIIB. Proc. Natl. Acad. Sci. USA **90:**1580–1584.
18. **Hernandez-Munain, C., and M. S. Krangel.** 1994. Regulation of the T-cell receptor delta enhancer by functional cooperation between c-Myb and core-binding factors. Mol. Cell. Biol. **14:**473–483.
19. **Hohaus, S., M. S. Petrovick, M. T. Voso, Z. Sun, D. E. Zhang, and D. G. Tenen.** 1995. PU.1 (Spi-1) and C/EBPα regulate the expression of the granulocyte-macrophage colony-stimulating factor receptor α gene. Mol. Cell. Biol. **15:**5830–5845.
20. **Jin, D. I., S. B. Jameson, M. A. Reddy, D. Schenkman, and M. C. Ostrowski.** 1995. Alterations in differentiation and behavior of monocytic phagocytes in transgenic mice that express dominant suppressors of *ras* signaling. Mol. Cell. Biol. **15:**693–703.
21. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1:**133–146.
22. **Kagoshima, H., K. Shigesada, M. Satake, Y. Ito, H. Miyoshi, M. Ohki, M. Pepling, and P. Gergen.** 1993. The Runt domain identifies a new family of heteromeric transcriptional regulators. Trends Genet. **9:**338–341.

23. **Kania, M. A., A. S. Bonner, J. B. Duffy, and J. P. Gergen.** 1990. The Drosophila segmentation gene runt encodes a novel nuclear regulatory protein that is also expressed in the developing nervous system. Genes Dev. **4:** 1701–1713.
24. **Katz, S., E. Kowenzleutz, C. Muller, K. Meese, S. A. Ness, and A. Leutz.** 1993. The NF-M transcription factor is related to C/EBP-beta and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. EMBO J. **12:**1321–1332.
25. **Kehrl, J. H.** 1995. Hematopoietic lineage commitment: role of transcription factors. Stem Cells **13:**223–241.
26. **Klemsz, M. J., S. R. McKercher, A. Celada, C. Van Beveren, and R. A. Maki.** 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. Cell **61:**113–124.
27. **Landschulz, W. H., P. F. Johnson, E. Y. Adashi, B. J. Graves, and S. L. McKnight.** 1988. Isolation of a recombinant copy of the gene encoding C/EBP. Genes Dev. **2:**786–800.
28. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:**1759–1764.
29. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science **243:**1681–1688.
30. **Lenny, N., S. Meyers, and S. W. Hiebert.** 1995. Functional domains of the t(8:21) fusion protein, AML-1/ETO. Oncogene **11:**1761–1769.
31. **Levanon, D., V. Negreanu, Y. Bernstein, I. Baram, L. Avivi, and Y. Groner.** 1994. AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics **23:**425–432.
32. **Liu, P., S. A. Tarle, A. Hajra, D. F. Claxton, P. Marlton, M. Freedman, M. J. Siciliano, and F. S. Collins.** 1993. Fusion between transcription factor CBF-beta/PEBP2-beta and a myosin heavy chain in acute myeloid leukemia. Science **261:**1041–1044.
33. **Mastrianni, D. M., N. M. Tung, and D. G. Tenen.** 1992. Acute myelogenous leukemia: current treatment and future directions. Am. J. Med. **92:**286–295.
34. **Meyers, S., J. R. Downing, and S. W. Hiebert.** 1993. Identification of AML-1 and the (8;21) translocation protein (AML-1/ETO) as sequence-specific DNA-binding proteins: the *runt* homology domain is required for DNA binding and protein-protein interactions. Mol. Cell. Biol. **13:**6336–6345.
35. **Meyers, S., N. Lenny, and S. W. Hiebert.** 1995. The t(8:21) fusion protein interferes with AML-1B-dependent transcriptional activation. Mol. Cell. Biol. **15:**1974–1982.

35a. **Meyers, S., N. Lenny, and S. W. Hiebert.** Unpublished data.

36. **Mitani, K., S. Ogawa, T. Tanaka, H. Miyoshi, M. Kurokawa, H. Mano, Y. Yazaki, M. Ohki, and H. Hirai.** 1994. Generation of the AML1-EVI-1 fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. EMBO J. **13:**504–510.
37. **Miyazaki, T., G. Suzuki, and K.-I. Yamamura.** 1993. The role of macrophages in antigen presentation and T cell tolerance. Int. Immunol. **5:**1023–1033.
38. **Miyoshi, H., T. Kozu, K. Shimizu, K. Enomoto, N. Maseki, Y. Kaneko, N. Kamada, and M. Ohki.** 1993. The t(8-21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. EMBO J. **12:**2715–2721.
39. **Miyoshi, H., M. Ohira, K. Shimizu, K. Mitani, H. Hirai, T. Imai, K. Yokoyama, E. Soeda, and M. Ohki.** 1995. Alternative splicing and genomic structure of the AML1 gene involved in acute myeloid leukemia. Nucleic Acids Res. **23:**2762–2769.
40. **Miyoshi, H., K. Shimizu, T. Kozu, N. Maseki, Y. Kaneko, and M. Ohki.** 1991. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc. Natl. Acad. Sci. USA **88:**10431–10434.
41. **Moreau-Gachelin, F., A. Tavitian, and P. Tambourin.** 1988. Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. Nature (London) **331:**277–280.
42. **Natsuka, S., S. Akira, Y. Nishio, S. Hashimoto, T. Sugita, H. Isshiki, and T. Kishimoto.** 1992. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. Blood **79:**460–466.
43. **Nerlov, C., and E. B. Ziff.** 1994. Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-alpha on the serum albumin promoter. Genes Dev. **8:**350–362.
44. **Ness, S. A., E. Kowenzleutz, T. Casini, T. Graf, and A. Leutz.** 1993. Myb and NF-M-combinatorial activators of myeloid genes in heterologous cell types. Genes Dev. **7:**749–759.
45. **Nuchprayoon, I., S. Meyers, L. M. Scott, J. Suzow, S. Hiebert, and A. D. Friedman.** 1994. PEBP2/CBF, the murine homolog of the human myeloid AML1 and PEBP2β/CBFβ proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil elastase genes in immature myeloid cells. Mol. Cell. Biol. **14:**5558–5568.
46. **Nucifora, G., C. R. Begy, P. Erickson, H. A. Drabkin, and J. D. Rowley.** 1993. The 3;21 translocation in myelodysplasia results in a fusion transcript between the AML1 gene and the gene for EAP, a highly conserved protein associated with the Epstein-Barr virus small RNA EBER-1. Proc. Natl. Acad. Sci. USA **90:**7784–7788.
47. **Nucifora, G., and J. D. Rowley.** 1995. AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemia. Blood **86:**1–14.
48. **Ogawa, E., M. Inuzuka, M. Maruyama, M. Satake, M. Naito-Fujimoto, Y. Ito, and K. Shigesada.** 1993. Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. Virology **194:**314–331.
49. **Ogawa, E., M. Maruyama, H. Kagoshima, M. Inuzuka, J. Lu, M. Satake, K. Shigesada, and Y. Ito.** 1993. PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila Runt gene and the human AML1 gene. Proc. Natl. Acad. Sci. USA **90:**6859–6863.
50. **Pahl, H. L., T. C. Burn, and D. G. Tenen.** 1991. Optimization of transient transfection into human myeloid cell lines using a luciferase reporter gene. Exp. Hematol. **19:**1038–1041.
51. **Pope, R. M., A. Leutz, and S. A. Ness.** 1994. C/EBP beta regulation of the tumor necrosis factor alpha gene. J. Clin. Invest. **94:**1449–1455.

51a. **Rhoades, K., and D.-E. Zhang.** Unpublished observations.

52. **Roberts, W. M., L. H. Shapiro, R. A. Ashmun, and A. T. Look.** 1992. Transcription of the human colony-stimulating factor-1 receptor gene is regulated by separate tissue-specific promoters. Blood **79:**586–593.
53. **Romana, S. P., M. Mauchauffe, M. Le Coniat, I. Chumakov, D. Le Paslier, R. Berger, and O. A. Bernard.** 1995. The t(12;21) of acute lymphoblastic leukemia results in a tel-AML1 gene fusion. Blood **85:**3662–3670.
54. **Satake, M., S. Nomura, Y. Yamaguchi-Iwai, Y. Takahama, Y. Hashimoto, M. Niki, Y. Kitamura, and Y. Ito.** 1995. Expression of the Runt domain-encoding *PEBP2*α genes in T cells during thymic development. Mol. Cell. Biol. **15:** 1662–1670.
55. **Scott, E. W., M. C. Simon, J. Anastai, and H. Singh.** 1994. The transcription factor PU.1 is required for the development of multiple hematopoietic lineages. Science **265:**1573–1577.
56. **Scott, L. M., C. I. Civin, P. Rorth, and A. D. Friedman.** 1992. A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood **80:**1725–1735.
57. **Screpanti, I., L. Romani, P. Musiani, A. Modesti, E. Fattori, D. Lazzaro, C. Sellitto, S. Scarpa, D. Bellavia, G. Lattanzio, F. Bistoni, L. Frati, R. Cortese, A. Gulino, G. Ciliberto, F. Costantini, and V. Poli.** 1995. Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. EMBO J. **14:**1932–1941.
58. **Shapiro, L. H., and A. T. Look.** 1995. Transcriptional regulation in myeloid cell differentiation. Curr. Opin. Hematol. **2:**3–11.
59. **Sherr, C. J.** 1990. Colony-stimulating factor-1 receptor. Blood **75:**1–12.
60. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene **67:**31–40.
61. **Smith, L. T., D. A. Gonzalez, and D. G. Tenen.** 1994. The myeloid specific granulocyte colony stimulating factor (G-CSF) receptor promoter contains a functional site for the myeloid transcription factor PU.1 (Spi-1). Blood **84:** 372a.
62. **Stanley, E. R., L. J. Guilbert, R. J. Tushinski, and S. H. Bartelmez.** 1983. CSF-1—a mononuclear phagocyte lineage-specific hemopoietic growth factor. J. Cell. Biochem. **21:**151–159.
63. **Sterneck, E., C. Muller, S. Katz, and A. Leutz.** 1992. Autocrine growth induced by kinase type oncogenes in myeloid cells requires AP-1 and NF-M, a myeloid specific, C/EBP-like factor. EMBO J. **11:**115–126.
64. **Sun, W., B. J. Graves, and N. A. Speck.** 1995. Transactivation of the Moloney murine leukemia virus and T-cell receptor β-chain enhancers by *cbf* and *ets* requires intact binding sites for both proteins. J. Virol. **69:**4941–4949.
65. **Takahashi, A., M. Satake, Y. Yamaguchi-Iwai, S. C. Bae, J. Lu, M. Maruyama, Y. W. Zhang, H. Oka, N. Arai, K. Arai, and Y. Ito.** 1995. Positive and negative regulation of granulocyte-macrophage colony-stimulating factor promoter activity by AML1-related transcription factor, PEBP2. Blood **86:** 607–616.
66. **Tanaka, K., T. Tanaka, S. Ogawa, M. Kurokawa, K. Mitani, Y. Yazaki, and H. Hirai.** 1995. Increased expression of AML1 during retinoic-acid-induced differentiation of U937 cells. Biochem. Biophys. Res. Commun. **211:**1023–1030.
67. **Tanaka, T., S. Akira, K. Yoshida, M. Umemoto, Y. Yoneda, N. Shirafuji, H. Fujiwara, S. Suematsu, N. Yoshida, and T. Kishimoto.** 1995. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macroophages. Cell **80:**353–361.
68. **Tanaka, T., K. Tanaka, S. Ogawa, M. Kurokawa, K. Mitani, J. Nishida, Y. Shibata, Y. Yazaki, and H. Hirai.** 1995. An acute myeloid leukemia gene, AML1, regulates hemopoietic myeloid cell differentiation and transcriptional activation antagonistically by two alternative spliced forms. EMBO J. **14:**341–350.

68a. **Timchenko, N., D. R. Wilson, L. R. Taylor, S. Abdelsayed, M. Wilde, M. Sawadogo, and G. J. Darlington.** 1995. Autoregulation of the human C/EBP gene by stimulation of upstream stimulatory factor binding. Mol. Cell. Biol. **15:**1192–1202.

69. **Tsukada, J., K. Saito, W. R. Waterman, A. C. Webb, and P. E. Auron.** 1994. Transcription factors NF-IL6 and CREB recognize a common essential site

in the human prointerleukin 1β gene. Mol. Cell. Biol. **14:**7285–7297.
70. **Visvader, J., and I. M. Verma.** 1989. Differential transcription of exon 1 of the human c-*fms* gene in placental trophoblasts and monocytes. Mol. Cell. Biol. **9:**1336–1341.
71. **Voso, M. T., T. C. Burn, G. Wulf, B. Lim, G. Leone, and D. G. Tenen.** 1994. Inhibition of hematopoiesis by competitive binding of the transcription factor PU.1. Proc. Natl. Acad. Sci. USA **91:**7932–7936.
72. **Wang, N. D., M. J. Finegold, A. Bradley, C. N. Ou, S. V. Abdelsayed, M. D. Wilde, L. R. Taylor, D. R. Wilson, and G. J. Darlington.** 1995. Impaired energy homeostasis in C/EBPα knockout mice. Science **269:**1108–1112.
73. **Wang, S., Q. Wang, B. E. Crute, I. N. Melnikova, S. R. Keller, and N. A. Speck.** 1993. Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol. Cell. Biol. **13:** 3324–3339.
74. **Weintraub, S. J., K. N. Chow, R. X. Luo, S. H. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature (London) **375:**812–815.
75. **Wijmenga, C., N. A. Speck, N. C. Dracopoli, M. H. Hofker, P. Liu, and F. S. Collins.** 1995. Identification of a new murine runt domain-containing gene, Cbfa3, and localization of the human homolog, CBFA3, to chromosome lp35-pter. Genomics **26:**611–614.
76. **Williams, S. C., M. Baer, A. J. Dillner, and P. F. Johnson.** 1995. CRP2 (C/EBP beta) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. EMBO J. **14:**3170–3183.
77. **Williams, S. C., C. A. Cantwell, and P. F. Johnson.** 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev. **5:**1553–1567.
78. **Wotton, D., J. Ghysdael, S. Wang, N. A. Speck, and M. J. Owen.** 1994. Cooperative binding of Ets-1 and core binding factor to DNA. Mol. Cell. Biol. **14:**840–850.
79. **Zhang, D. E., K. I. Fujioka, C. J. Hetherington, L. H. Shapiro, H. M. Chen, A. T. Look, and D. G. Tenen.** 1994. Identification of a region which directs monocytic activity of the colony-stimulating factor 1 (macrophage colony-stimulating factor) receptor promoter and binds PEBP2/CBF (AML1). Mol. Cell. Biol. **14:**8085–8095.
80. **Zhang, D.-E., C. J. Hetherington, H.-M. Chen, and D. G. Tenen.** 1994. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. Mol. Cell. Biol. **14:**373–381.
81. **Ziegler-Heitbrock, H. W., E. Thiel, A. Futterer, V. Herzog, A. Wirtz, and G. Riethmuller.** 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. Int. J. Cancer **41:**456–461.