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Qiang Tian

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Richard C. Schwartz
Major professor

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IDENTIFICATION AND CHARACTERIZATION OF C/EBP REGULATED GENES IN MYELOMONOCYTIC DIFFERENTIATION

By

Qiang Tian

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF C/EBP REGULATED GENES IN MYELOMONOCYTIC DIFFERENTIATION

By

Qiang Tian

The CCAAT/enhancer binding proteins (C/EBP) comprise a family of basic leucine zipper (bZIP) transcription factors that dimerize through their leucine zippers and bind to a consensus DNA motif through their adjacent basic regions. The involvement of C/EBPs in myelopoiesis has been demonstrated by their temporal pattern of expression in differentiating 32D clone 3 myeloblasts and by the complete lack of neutrophils associated with the targeted disruption of C/EBPα and C/EBPε in transgenic mice. However, the currently identified target genes of C/EBP regulation seem inadequate to fully explain the role of these transcription factors in myelomonocytic differentiation. We sought to elucidate the regulatory scheme in which C/EBPs act in differentiation by identifying new targets of their regulation in immature myeloid cells by utilizing differential display PCR. C/EBPβ was overexpressed in 32D clone 3 myeloblasts and differential display RT-PCR was performed to identify genes induced by C/EBPβ overexpression. Among the four genes identified, two were endogenous retroviral elements, one encoded a cathepsin G, a primary granule protein of neutrophils and monocytes, and one encoded a novel gene that we have named CDDP-1 for C/EBP Differential-Display Product-1. A CDDP-1 cDNA containing a 453 amino acid open reading frame was isolated from a λgt11 human bone marrow cDNA

library. The expression of CDDP-1 mRNA is upregulated by the overexpression of C/EBPα, β, δ, or ε. An examination of sequences immediately upstream of the CDDP-1 cDNA reveals several potential C/EBP binding sites suggesting that CDDP-1 may be a direct target of C/EBP regulation. CDDP-1 displays a biphasic pattern of expression in the granulocytic differentiation of myeloid progenitor cell lines: an early period of elevated expression just prior to and continuing through the induction of myeloperoxidase mRNA and a later second period coincident with the expression of lactoferrin mRNA. It is also induced upon granulocytic differentiation of HL-60 cells, but is downregulated upon monocytic differentiation. This pattern of expression coupled with the finding that CDDP-1 is most highly expressed in adult bone marrow and fetal liver suggests a role in myelopoiesis. Constitutive overexpression of CDDP-1 in MPRO promyelocytes promoted their differentiation toward both the neutrophil and monocyte lineages. Retinoic acid treatment can further facilitate the neutrophilic differentiation of CDDP-1 transductants. Subcellular localization studies suggested that CDDP-1 is predominantly a cytoplasmic protein. CDDP-1 mRNA is also modulated during the course of erythroid and mast cell differentiation suggesting roles for this gene in the differentiation of these other lineages.

In Chapter 3, I conducted transient transfection assays to confirm that truncated forms of C/EBPβ which retain only the bZIP regions can confer LPS induced activation of an IL-6 promoter. An intact NF-κB binding site on the IL-6 promoter is required for this activation. These results implicate the bZIP domain in some function other than dimerization with known C/EBP family members.

To my family:

My great parents, lovely wife Danbin and wonderful daughter Elaine.

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Introduction

CCAAT/enhancer binding proteins (C/EBPs) comprise a family of sequence-specific DNA-binding proteins that are critical in the regulation of various aspects of cellular differentiation and function in multiple tissues (reviewed by Williams and Johnson 1994). To date, six different C/EBP isoforms have been isolated and characterized, designated C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε, and C/EBPζ.

All of the C/EBP family members share a highly conserved basic region and leucine zipper domain at the carboxyl terminus whereas the amino terminal transactivation domains are more diverse (Fig. 1). The basic region is located immediately to the N-terminal side of the zipper. It is highly positively charged and responsible for DNA binding. C/EBPs bind DNA as dimers. Dimerization is required for DNA binding (Landschulz et al., 1989). The sequence recognition properties of C/EBPs are identical in vitro. They can recognize the same consensus sequence on the target promoter and form either homodimers or heterodimers between family members through the leucine zipper region (Vinson et al., 1993). The transactivation domain is responsible for interacting with proteins of other transcription factor families as well as the basal transcription apparatus. However, the unique and sometimes redundant function of each family member is determined by its tissue- and stage- specific expression,

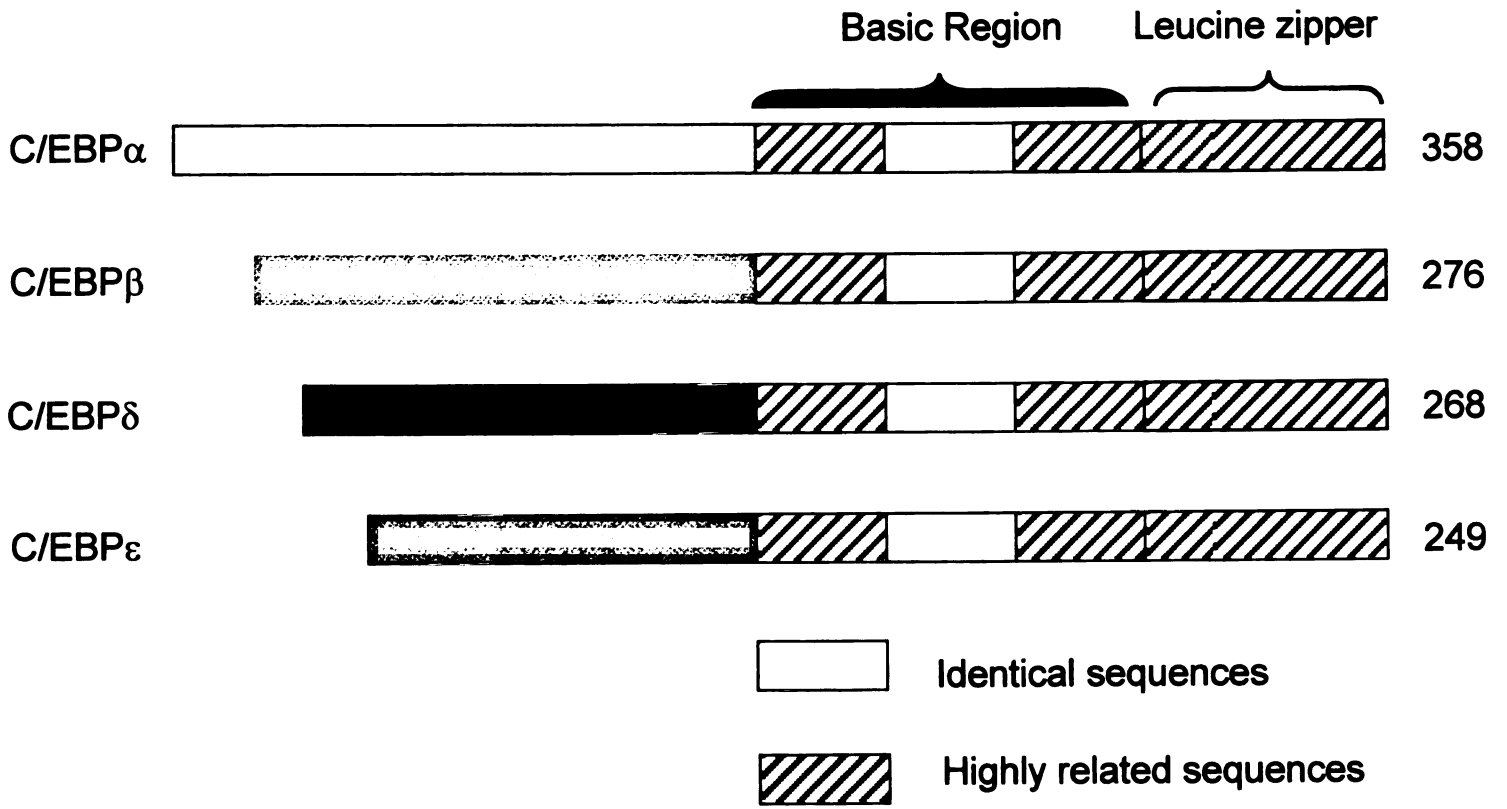

Figure 1. Schematic representation of four trans-activator proteins of the C/EBP family.

alternative ribosomal reading, post-transcriptional modifications, and differential abilities to interact with other DNA binding proteins on a given promoter. Here, I will review the characteristics of all the C/EBP isoforms with emphasis on their roles in myeloid differentiation.

1.1 Overview of C/EBP isoforms

1.1.1 C/EBPα

C/EBPα is the founding member of the C/EBP family. It was first identified by Steven McKnight's group in 1986 from adult rat liver in a search for cellular DNA-binding factors that can recognize viral gene regulatory sequences, such as the CCAAT box and "enhancer core" elements of retroviral LTRs, thus named CCAAT/Enhancer Binding Protein (C/EBP) (Graves et al., 1986; Johnson et al., 1987; Landschulz et al., 1988). C/EBPα is highly expressed in liver and adipose tissue where it transactivates hepatocyte and adipocyte specific gene expression, such as albumin, 422/aP2 (Christy et al., 1989), PEPCK (Park et al., 1990). It functions in fully differentiated non-proliferative cells and regulates adipogenesis. Overexpression of C/EBPα in immature adipocytes induces differentiation whereas anti-sense to C/EBPα mRNA blocks adipocyte differentiation (Freytag and Geddes, 1992; Lin and Lane, 1992). As will be discussed later, C/EBPα also plays a critical role in myelopoiesis (Zhang et al., 1997; Radomska et al., 1998).

C/EBPα can undergo autoregulation. The murine C/EBPα promoter contains a functional C/EBP binding site that can be transactivated by C/EBPα

(Christy et al., 1991). Two isoforms of C/EBPα can be generated from its mRNA by a ribosomal scanning mechanism. One is a 42 kd full-length protein which contains three transactivation domains (Nerlov and Ziff 1994; Nerlov and Ziff 1995). The other one is a 30 kd truncated protein which retains its dimerization and DNA-binding domains (Lin et al., 1993; Ossipow et al., 1993). Two activation domains (ADI and ADII) have been identified in the N-terminal region of C/EBPα that are required for transcriptional activation of the serum albumin gene (Friedman and McKnight, 1990).

1.1.2 C/EBPβ

C/EBPβ, also known as NF-IL6, CRP2, LAP, AGP/EBP, IL-6DBP, and NF-M in chicken, is an important mediator of the inflammatory response. Lipopolysaccharide (LPS), Interleukin 6 (IL-6), Interleukin 1 (IL-1), dexamethasone, glucagon, and some other stimuli can strongly induce C/EBPβ expression. C/EBPβ was originally identified as a nuclear factor that binds to the IL-1 response element of the human IL-6 gene (Isshiki et al., 1990; Akira et al., 1990). It can bind to IL-6-responsive elements in the promoters of several acute phase response genes including those encoding IL-8, TNF, and G-CSF. TNFα can facilitate nuclear localization of C/EBPβ and C/EBPδ in response to inflammatory stress (Yin et al., 1996). Cytokine stimulation can further increase C/EBPβ transcriptional activity by enhanced DNA binding (Poli et al., 1990). The activation of the MAP kinase (Tramley-Stein et al., 1996), protein kinase A and protein kinase C (Trautwein et al., 1994), and calcium-dependent signal

transduction pathways (Wegner et al., 1992) appears to activate transcription through the post-translational phosphorylation of C/EBPβ. Most recently, Buck et al showed that phosphorylation of rat serine 105 or mouse threonine 217 in C/EBPβ is required for hepatocyte proliferation induced by TGFα (Buck et al., 1999).

C/EBPβ also has two isoforms due to leaky ribosomal scanning. The longer form called LAP and the shorter form termed LIP. LIP initiates at Met 132 and lacks activation domains and has been shown to be a transdominant negative inhibitor of C/EBPβ mediated transcriptional activity (Descombes and Schibler, 1991). However, our own experiments show that LIP can also confer LPS-induced IL-6 and MCP-1 expression to a lymphoblastoma cell line p388D1. This indicates that the leucine zipper region of C/EBPβ may have some other functions in addition to dimerization (See Chapter 3 for more detail). In at least some instances, LIP may be a product of certain lysis procedures (Baer et al., 1998) rather than the aforementioned mechanisms. Thus, the physiological significance of LIP is unclear.

1.1.3 C/EBPγ

C/EBPγ, also known as Ig/EBP, is a short intronless gene which encodes a 16.4 kd protein. It was first discovered by Calame's group in a search for transcription factors from B lymphocytes that bind to a cis-regulatory site in the immunoglobulin heavy chain enhancer (Roman et al., 1990). Its mRNA is ubiquitously expressed and C/EBPγ protein functions as a dominant negative

inhibitor of C/EBP family transactivators (Cooper et al., 1995). C/EBPγ contains only a leucine zipper dimerization domain and DNA binding region but no transactivation domain. Although C/EBPγ can heterodimerize with C/EBP family members, it attenuates rather than activates target gene expression.

1.1.4 C/EBPδ

C/EBPδ is also known as CRP3 and NF-IL6β. Low amounts of C/EBPδ mRNA can be detected in mouse tissues under normal conditions. However, high level expression occurs in many organs following stimulation by LPS or inflammatory cytokines, such as IL-6 (Alam et al., 1992; Kinoshita et al., 1992; Ramji et al., 1993). The transactivating ability of C/EBPδ is comparable to that of C/EBPα and C/EBPβ. It can also confer LPS-induced expression of IL-6 and MCP-1 when ectopically expressed in a lymphoblastic cell line P388D1, suggesting functional redundancy with C/EBPβ in regulating inflammatory cytokines (Hu et al., 1998). The DNA binding affinity of C/EBPδ is somehow diminished compared to other C/EBP isoforms (Cao et al., 1991).

1.1.5 C/EBPζ

C/EBPζ, also known as CHOP and Gadd153, is another dominant negative inhibitor of the C/EBP family. It was originally cloned in hamster and named growth arrest and DNA damage-inducible gene (Gadd153) (Fornace et al., 1989). It is also induced as a result of glucose deprivation in differentiating adipocytes. C/EBPζ can form heterodimers with the other members of the C/EBP

family. However, the presence of two prolines in the DNA-binding region disrupts its helical structure and prevents the binding of C/EBPζ-containing heterodimers to classic C/EBP binding sites (Ron and Habener, 1992). A subsequent study showed that CHOP-C/EBP dimer can actually bind to some other specific DNA sequences and CHOP possesses a transcriptional activity which is inducible by cellular stress (Ubeda, et al., 1996). Also of note is that CHOP can specifically block C/EBPα activity and induce increased apoptosis of myeloid 32D cells (Friedman, 1996).

1.1.6 C/EBPε

C/EBPε is the human homologue of rat CRP1 (Williams et al., 1991). The full-length cDNA of C/EBPε has only been isolated recently. It is expressed exclusively in hematopoietic lineages (Antonson et al., 1996; Chumakov et al., 1997; Williams et al., 1998). The human C/EBPε gene is transcribed from two alternative promoters. Four C/EBPε mRNA isoforms have been identified due to alternative splicing and differential use of promoters. They encode four proteins of 32, 30, 27, and 14 kDa (Yamanaka et al., 1997). The 32 kDa protein is an activator of transcription of granulocyte colony stimulating factor receptor (GCSF-R), while the 14 kDa protein is not. The transcriptional activation function of C/EBPε is mediated through a bipartite activation domain located in the N terminus with a minimal requirement of the first 18 amino acid residues. The highest activity of transcription was conferred by the amino acid residues 1-77 (Williamson et al., 1998). An intrinsic repression domain was also identified

between amino acid 116 and 162. A form of C/EBPε in which this region is deleted can enhance gene transcription from a neutrophil elastase promoter-luciferase reporter. Thus, C/EBPε may regulate gene expression by utilizing both transcriptional activation and repression depending upon its associated factors.

1.2 HEMATOPOIESIS

The main question that will be addressed in this thesis is: what are the roles of C/EBP family of transcription factors in the regulation of hematopoiesis, especially myelopoiesis? Hematopoiesis is the process by which blood cells acquire a defining phenotype as a result of coordinated, cell specific gene expression. During this process, pluripotent hematopoietic stem cells undergo a series of progressive restriction of lineage potential, and acquire the characteristics of mature, terminally differentiated cells. In 1961, Till and McCulloch first demonstrated the existence of pluripotent hematopoietic stem cells (HSCs) that had the property, at the single cell level, of (i) self-renewal as well as (ii) multilineage differentiation (Till and McCulloch, 1961). Then in the 1980s, Irving Weissman's group first identified and isolated HSCs by cell sorting using monoclonal antibodies against cell surface markers (Muller-Sieburg et al., 1986; Spangrude et al., 1988). A subset of this stem cell population perpetually self renews, and these cells are long term stem cells (LT-HSCs). In mice, LT-HSCs give rise to short-term HSCs (ST-HSCs), which give rise to multipotent progenitors (MPPs), whose further progeny are oligolineage-restricted (Weissman, 2000). HSCs give rise alternatively to the clonal common

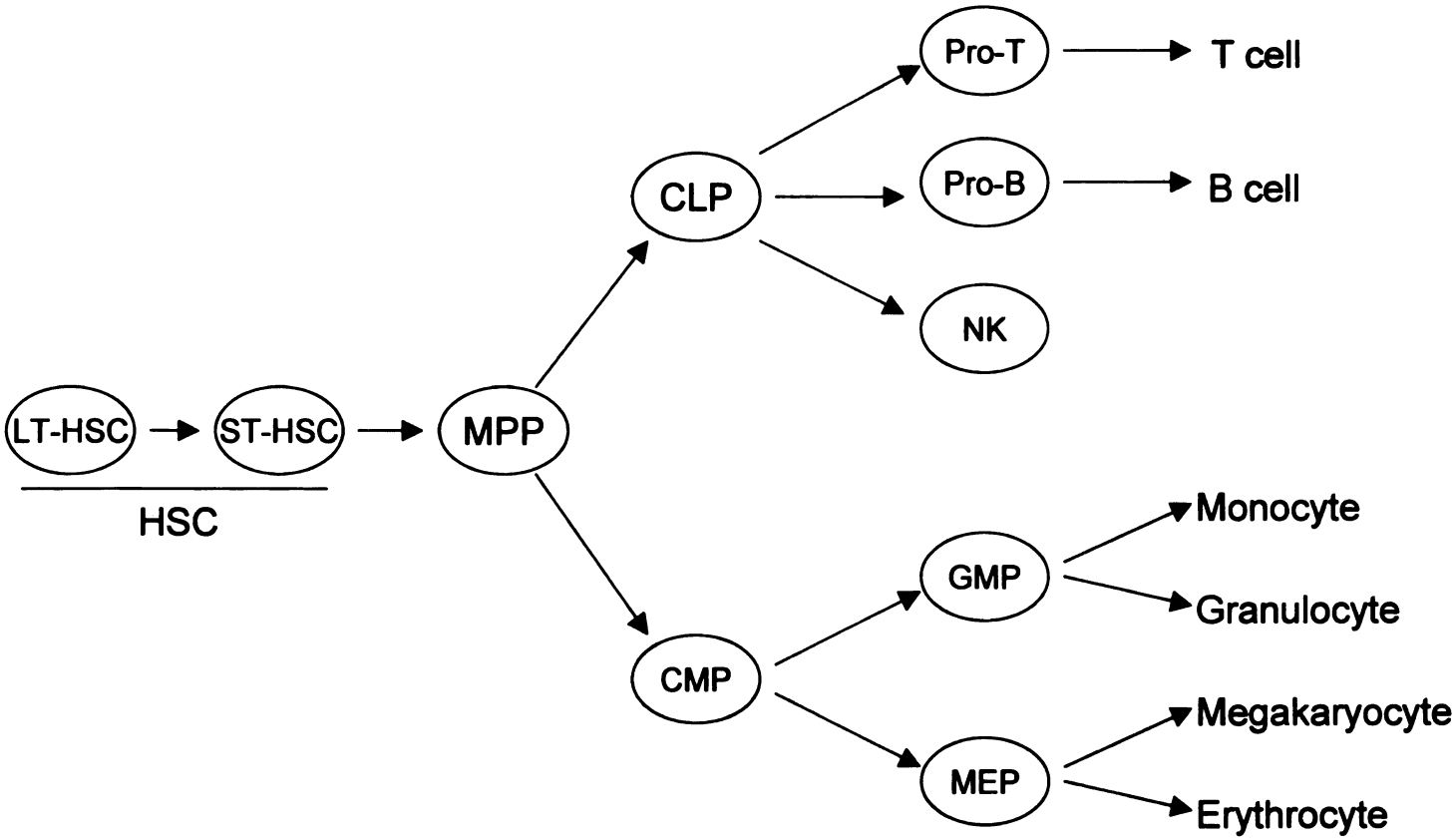

Figure 2. Model of major hematopoietic differentiation pathways from hematopoietic stem cells (HSCs). Long term HSCs give rise to short term HSCs, which give rise to multi-potent progenitors (MPPs). MPPs further divide into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CLPs give rise to T cell, B cell and natural killer cell lineages. CMPs give rise to granulocyte/monocyte progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs). GMPs differentiate into granulocyte and monocyte lineages, whereas MEPs give rise to megakaryocyte and erythrocyte lineages.

lymphocyte progenitor (CLP), or the clonogenic common myeloid progenitor (CMP). CLPs are restricted to give rise to T lymphocytes, B lymphocytes, and natural killer (NK) cells. CMPs are progenitors for the myeloerythroid lineages. Single CMPs give rise to myelomonocytic progenitors (GMPs) and megakaryotic/erythoid progenitors (MEPs) (Akashi et al., 2000). All of these populations are separable as pure populations using cell surface markers (Fig. 2).

1.2.1 Overview of myelomonocytic development

In the case of myeloid differentiation, the terminal stages include granulocytes (neutrophils, eosinophils, and basophils), monocytes/macrophages, as well as erythrocytes and megakaryocytes. The main focus of my research has been placed on the development of myelomonocytic lineages that eventually give rise to neutrophils and monocytes/macrophages, both of which play crucial roles in host defense against bacterial infections. Interruption of their normal developmental process may also cause myelogenous leukemia.

Bone marrow is the major site of de novo myelopoiesis. Cells of the monocytes/macrophage series and neutrophilic granulocytes are derived from a common progenitor cell, namely the colony-forming unit granulocyte-macrophage (CFU-GM). The development of neutrophlic granulocytes proceeds through a series of intermediate stages. Morphologically recognizable myeloid precursor cells can only be found in the bone marrow under steady-state conditions. They include myeloblasts, promyelocytes and myelocytes which are all still capable of

cell division. Cells that mature beyond the myelocyte stage no longer have the capacity of cell division. These include metamyelocytes, band forms, and mature neutrophils. Each of these developmental stages has a distinctive morphology with the most striking morphological features of mature neutrophils being multilobed nuclei, an abundance of cytoplasmic granules, prominent deposits of particulate glycogen, and the paucity of other cytoplasmic organelles (Fig. 3). In addition to morphological features, each stage can also express a distinct set of molecular markers. For example, the promyelocyte stage of granulocyte development extends from the initiation of primary (azurophil) granule formation to its completion. The major components of the primary granules include acid hydrolases (β-glucuronidase, β-galactosidase), neutral proteases (cathepsin G, elastase, collagenase), cationic proteins, myeloperoxidase, and some lysozymes. The myelocyte stage extends from the initiation of secondary (specific) granule formation to its completion. The secondary granule contains lysozyme, lactoferrin, and in some species, alkaline phosphatase. The most commonly used molecular markers for granulocytic differentiation are myeloperoxidase and neutrophil elastase of primary granules for classification of promyelocytes and lactoferrin of secondary granules for classification of myelocytes.

The development of the monocytic lineage involves three different stages: monoblast, promonocyte, and monocyte. The monocytes migrate from circulatory system to various tissues and become tissue macrophages. Several genes primarily expressed in macrophages have been used as markers for the

Marrow development, 14 days

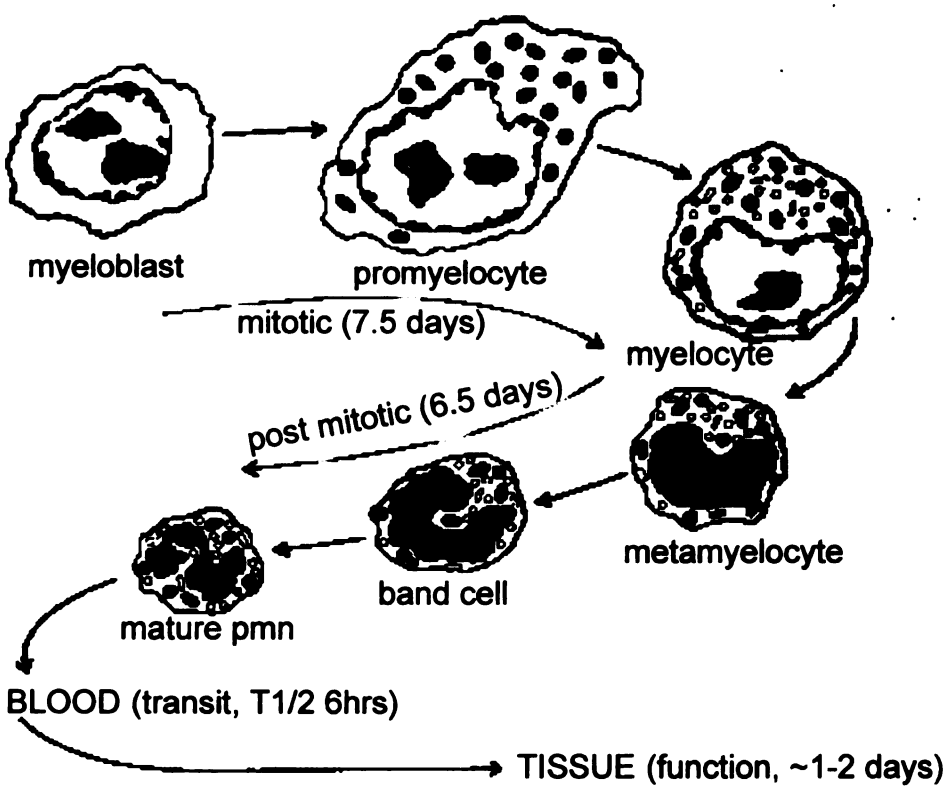

Figure 3. Maturation process of neutrophilic granulocyte.

classification of this lineage. These include M-CSF receptor (c-fms), macrophage scavenger receptor (MSR), LPS receptor (CD14), monocyte specific esterase, and macrophage metalloelastase (Reviewed in Clarke and Gordon).

Several myeloid specific cell lines have been established, representing different stages of myeloid development. 32Dcl3 is a murine myeloblastic cell line. Its growth depends on interleukin-3 and can be induced to differentiate into mature neutrophils by treatment with G-CSF (Valtieri et al., 1987). MPRO is a murine promyelocytic cell line that grows in the presence of GM-CSF. Treatment with high concentration of all-trans retinoic acid (ATRA) can induce its terminal differentiation into neutrophils (Tsai et al, 1993). HL-60 is a human bipotential promyelocytic leukemia cell line that can differentiate into either neutrophils or monocytes/macrophages following treatment by ATRA or TPA, respectively (Collins et al, 1987). U937 and M1 are promonocytic cell lines that can be induced to differentiate into monocytes/macrophages by treatment with TPA or IL-6, respectively (Sundstrom and Nilsson, 1976).

1.2.2 Hematopoietic growth factors in hematopoiesis

Hematopoietic growth factors play important roles at different stages of development (Fig. 4). The cytokines interleukin 3 (IL-3), interleukin 6 (IL-6) and stem cell factor (SCF) have been shown to act on stem cells and multipotential progenitors (reviewed in Watowich et al., 1996). IL-3 has the broadest specificity of any known cytokines, as it stimulates survival, proliferation and differentiation of hematopoietic stem cells (HSCs) and progenitor cells of all known

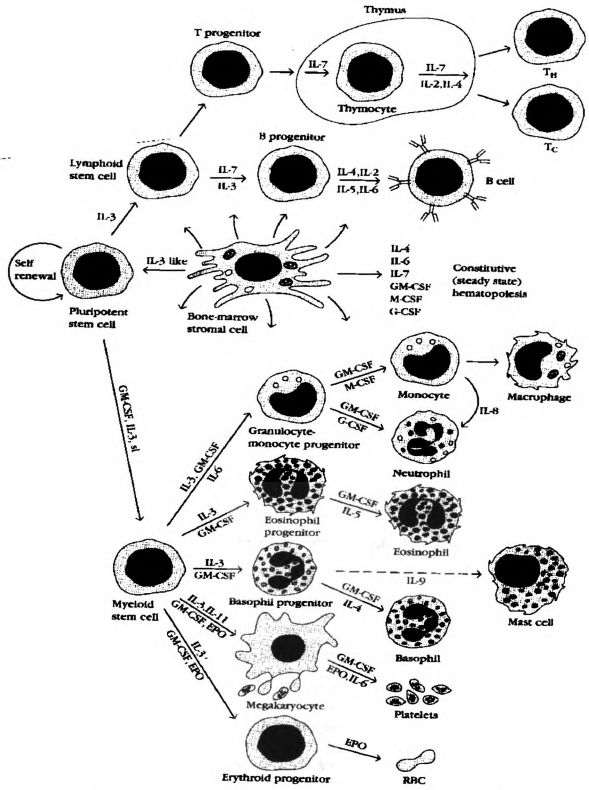

Figure 4. Regulation of hematopoiesis by cytokines that stimulate the proliferation and/or differentiation of various hematopoietic cells.

hematopoietic lineages (reviewed in Schrader, 1994). However, targeted disruption of IL-3 or its receptor revealed that IL-3 is not required for steady-state hematopoiesis, but is needed for proliferation of mast cells during parasitic infections (Nishinakamura et al., 1996; Lantz et al., 1998). SCF probably provides the missing IL-3 activity in these knock-out mice. IL-6 stimulates survival, proliferation and differentiation of HSCs and at high concentrations can induce proliferation of committed granulocytic progenitors (reviewed in Kishimoto, 1989; Liu et al., 1996). Targeted mutation of IL-6 causes selective reduction in granulocytic progenitors (Liu et al., 1997). SCF promotes survival, proliferation and differentiation of stem cells and progenitors of multiple lineages (reviewed in Lyman and Jacobsen, 1998).

Within the myeloid lineages, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and macrophage colony stimulating factor (M-CSF) represent a set of myeloid specific cytokines that are important for the development of granulocytes and macrophages (reviewed in Metcalf, 1995). GM-CSF stimulates the survival, proliferation and differentiation of bipotential granulocyte-macrophage progenitors as well as unipotent macrophage or granulocyte progenitors. However, targeted disruption of GM-CSF or its receptor does not disrupt hematopoiesis in vivo (Dranoff et al., 1994; Stanley et al., 1994; Nishinakamura et al., 1995) suggesting functional redundancy with other growth factors of similar function. G-CSF stimulates survival, proliferation and maturation of granulocytic progenitors (Suda et al., 1987). Targeted mutation of G-CSF or its receptors

causes a selective and profound reduction in the number of circulating granulocytes in mice (Lieshke et al., 1994; Liu et al., 1996). M-CSF stimulates survival, proliferation and maturation of committed monocytic progenitors (Stanley, 1985). It is required for development of macrophages and monocyte-derived lineages such as osteoclasts. Targeted mutation of M-CSF gene results in greatly reduced numbers of tissue macrophages, as well as osteopetrosis (Yoshida et al., 1990). Similarly, erythropoietin (EPO) and EPO receptor are crucial for erythropoieisis (reviewed in Adamson 1988), whereas thrombopoietin (TPO) is important for megakaryocytic development (Murone et al., 1998).

Currently, there are two models for lineage commitment and hematopoietic differentiation. The first model is the "instructive model", which proposes that lineage commitment is regulated by external signals such as hematopoietic growth factor and/or progenitor-stromal cell interactions. The second one is the "stochastic model" which proposes that the fate of lineage commitment is intrinsically determined; growth factors can only expand already committed cell types but do not bias the cells toward a specific lineage. A third one that combines the first two models has also been proposed. Whichever of these models is correct, undoubtedly, transcription factors are likely to play pivotal roles in specifying lineage commitment and differentiation.

1.3 Transcription factors in hematopoiesis and leukemia

Hematopoiesis is a highly regulated process in which the growth and differentiation of pluripotent hematopoietic stem cells into specific cell lineages

are controlled by the coordinated regulation of gene expression. The genetic switches which control blood cell development are obvious sites of malfunction in the formation of leukemia. Transcription factors make up many, if not most, of these genetic switches. Abnormality in either the abundance or activity of these factors can result in inappropriate expression of genes critical to the process of growth and differentiation. The characterization of these transcription factors is of great significance in the study of leukemia.

Several important questions need to be addressed when studying transcriptional regulation of hematopoiesis: How is gene expression restricted in certain lineages? What controls the temporal expression of lineage-specific genes? Why does a common progenitor cell differentiate into one cell type but not another? The answers to all of these questions involve transcription factors. Several transcription factor families have been found to be involved in hematopoiesis. Those that are thought to be important in myelopoiesis include the C/EBP family, the GATA family, the PU-1 family, Egr-1, retinoic acid receptors (RARs), myeloid zinc finger factor (MZF-1), AML-1, and Myb. Since the question that interests us most is the role of the C/EBP family of transcription factors in myelomonocytic differentiation which gives rise to monocytes and neutrophils, emphasis will be placed on reviewing how C/EBPs specify myeloid lineage and regulate terminal differentiation.

1.3.1 C/EBPs in myelopoiesis

Early experiments performed by Alan Friedman's group showed the temporal expression of different C/EBP isoforms during granulocytic differentiation of 32D clone 3 myeloblast cells (Scott et al., 1992). In this study, C/EBPα was highly expressed in proliferating myelomonocytic cells upon induction of differentiation and down regulated with maturation. In contrast, C/EBPβ and C/EBPδ were upregulated upon differentiation. However, a recent study showed that C/EBPα mRNA was maintained during granulocytic differentiation, but markedly down-regulated with monocytic or erythroid differentiation (Radomska et al., 1998). These patterns of expression suggest that different C/EBP isoforms may play a distinct role during myelomonocytic differentiation.

Several lines of evidence suggest that C/EBPβ is an important regulator for the activation and/or terminal differentiation of macrophages. C/EBPβ expression is dramatically induced during macrophage differentiation (Natsuka et al., 1992; Scott et al., 1992). Several genes commonly expressed by activated macrophages contain functional C/EBP binding sites in their promoter regions, such as IL-6, IL-1α, IL-8, TNFα, G-CSF, NO synthase, and lysozyme genes (Natsuka et al., 1992; Zhang and Rom, 1993; Mukaida et al., 1990; Lowenstein et al., 1993, Pope et al., 1994). Previous studies by our lab have shown that ectopic expression of C/EBPβ, as well as C/EBPα and C/EBPδ, in a lymphoblast cell line P388D1, which has no endogenous C/EBP expression, can result in LPS-induced expression of IL-6 and MCP-1 (Macrophage Chemoattractant

Protein 1) (Bretz et al., 1994; Hu et al., 1998). Chicken C/EBPβ was shown to cooperate with Myb to induce transcription of the myeloid-specific mim-1 gene in heterologous cell types. (Ness et al., 1993). Most recently, it was found that the N-terminus of the full-length C/EBPβ isoform, which is essential for induction of the mim-1 gene in chromatin, interacts specifically with the SWI/SNF complex to activate myeloid genes (Kowentz-Leutz et al. 1999). Overexpression of chicken C/EBPβ in multi-potential myeloid progenitor cells promotes eosinophil as well as myeloid differentiation (Muller et al., 1995; Nerlov et al., 1998). More evidence has come from murine knock-out analyses. The C/EBPβ-deficient mice showed defective bacterial killing and tumor cytotoxicity by macrophages, as well as lymphoproliferative disorders (Tanaka et al., 1995; Screpanti et al., 1995). Bone marrow B cell expansion was impaired, and stromal cell IL-7 mRNA expression was decreased (Chen et al., 1997). However, myelopoiesis was not adversely affected. Taken together, the data suggest that C/EBPβ is essential for mature macrophage function but less important for the differentiation of various hematopoietic lineages including granulocytes.

On the other hand, C/EBPα has been shown to be a myeloid specific switch toward granulocyte differentiation. First, several myeloid specific genes have C/EBP binding sites in their promoter and can be transactivated by C/EBPα in transient transfection studies. These include myeloperoxidase (MPO) and neutrophil elastase (NE) (Ford et al., 1996; Oelgeschlager et al., 1996). Secondly, several important hematopoietic growth factor receptors, such as granulocyte colony stimulating factor (G-CSF) receptor, macrophage colony

stimulating factor (M-CSF) receptor, and GM-CSF receptor are regulated by C/EBPα, in conjunction with other hematopoietic specific transcription factors such as PU.1 (Hohaus et al., 1995; Smith et al., 1996). Most importantly, overexpression of C/EBPα in bipotential promyelocytic cell lines (U937 and HL60) can promote neutrophilic differentiation and inhibit monocytic differentiation (Radomska et al., 1998). Even more compelling evidence for the critical role of C/EBPα in neutrophilic differentiation came from targeted disruption experiments. C/EBPα knock-out mice display a complete lack of mature neutrophils in both embryonic and newborn mice. This is due, in part, to the absence of G-CSF signaling because of a significant reduction in G-CSF receptor mRNA expression (Zhang et al., 1997). More recently, it was found that the IL-6 receptor mRNA expression was also greatly reduced in the C/EBPα deficient mice (Zhang et al., 1998). The G-CSF and IL-6 receptors are clearly major target genes of C/EBPα. All the above evidence has established that C/EBPα is a key regulator of granulocyte differentiation.

Recently, accumulating evidence has suggested that the newly cloned C/EBP family member, C/EBPε, is essential for terminal differentiation and functional maturation of already committed granulocyte progenitor cells. First of all, C/EBPε is preferentially expressed in myeloid lineages. It is upregulated during neutrophil differentiation and downregulated upon monocytic differentiation (Chin et al., 1997; Morosetti et al., 1997). C/EBPε significantly upregulates mim-1 and human myeloperoxidase (MPO) promoters (Chumakov et al., 1997). It directly interacts with the DNA binding domain of c-myb and

cooperatively activates transcription from myeloid promoters including those of mim-1, neutrophil elastase (NE), and GCSF receptor (Verbeek et al., 1999). C/EBPε is also involved in the positive regulation of lactoferrin gene expression in neutrophils (Verbeek et al., 1999). Specific disruption of the C/EBPε gene in mice prevented the maturation of neutrophils in blood (Yamanaka et al., 1997). The fact that C/EBPε is also a putative retinoic acid target gene suggests that C/EBPε is a downstream effector of RA-induced granulocytic differentiation of acute promyelocytic leukemia (APL) cells (Park et al., 1999). Consistent with the above described data, overexpression of C/EBPε in immature myeloid U937 cells can induce neutrophilic differentiation (Park et al., 1999). C/EBPε is another important regulator of granulopoiesis and probably acts downstream of C/EBPα because C/EBPα-deficient animals accumulate cells of a less mature phenotype than C/EBPε-deficient animals (Yamanaka et al., 1997).

1.3.2 Other important transcription factors that play a role in myelopoiesis

Egr-1 can induce monocytic differentiation: Another transcription factor with an important role in myelomonocytic differentiation is the early response gene Egr-1. Egr-1 was cloned as an early response gene during TPA treatment of human myeloid cell lines and was noted to be up-regulated during monocytic but not granulocytic differentiation of these bipotential lines (Nguyen et al., 1993). The Egr-1 gene was found to be transcriptionally silent in HL-60, but active in U-937 and M1 cells. The latter two cell lines preferentially differentiate toward the

monocyte/macrophage lineages. Overexpression and anti-sense studies implicated Egr-1 as an inducer of monocytic differentiation.

Egr-1 can block granulocytic differentiation of HL-60 cells, restricting differentiation to the monocytic lineage (Nguyen et al., 1993). It can also block G-CSF induced differentiation of IL-3-dependent 32Dcl3 hematopoietic precursor cells, endowing the cells with the ability to be induced by GM-CSF for terminal differentiation along the macrophage lineage (Krishnaraju et al., 1995). Ectopic expression of Egr-1 in the M1 myeloblastic leukemic cell line resulted in activation of the macrophage differentiation program in the absence of a differentiation inducer (Krishnaraju et al., 1998). The observed function of Egr-1 as a positive modulator of monocyte/macrophage differentiation varies, depending on the state of lineage commitment for differentiation of the hematopoietic cell type being examined. Surprisingly, targeted-disruption of Egr-1 in mice showed no abnormalities of monocytic differentiation or function (Lee et al., 1996). In this mouse model, it is possible that other members of the Egr-1 family can compensate for loss of the Egr-1 gene.

RARα in Myeloid Differentiation and Acute Promyelocytic Leukemia (APL): Retinoic acid receptor (RAR) has long been known as a key regulator of myeloid differentiation (reviewed in Lawson and Berliner, 1999). In early 1980s, Breitman et al (1980) showed that all-trans retinoic acid (ATRA) can induce granulocytic differentiation of the HL-60 cell line which was derived from a patient with acute promyelocytic leukemia (APL) and of primary leukemia cells. Retinoic acid (RA)

exerts its function through RARs that belong to the nuclear receptor superfamily of transcription factors. RARs control specific gene expression in a ligand dependent manner. Three APL associated rearrangements of RARα genes have been identified: t(15:17) results in a fusion protein PML-RARα (Kakizuka et al., 1991), t(11:17) results in PLZF-RARα (Chen et al., 1993) and t(5:17) results in NPM-RARα (Redner et al., 1996). Both experimental and clinical data indicate that RARα function is required for the myeloid differentiation process. When a dominant negative RARα was introduced into mouse bone marrow cells and cultured in stem cell factor and IL-3, hematopoietic differentiation was blocked at the hematopoietic stem cell stage. The resultant cell line EML can be induced to differentiate into erythroid, myeloid and lymphoid lineages depending upon the exposure to various cytokines and ATRA (Tsai et al., 1994). Treatment with supra-normal levels of ATRA and IL-3 with stem cell factor leads to the development of GM-CFU. When further treated with GM-CSF, EML evolves into promyelocytic cells as represented by the cell line called EPRO whose growth is GM-CSF dependent. A second ATRA-dependent step in myelopoiesis blocks EPRO differentiation at the promyelocyte stage. Treatment with supra-physiological concentrations of ATRA can fully induce neutrophilic differentiation of EPRO producing polymorphonuclear and "band form" cells (Tsai et al. 1994).

The RARs bind to RA response elements (RAREs) within the promoter of target genes as heterdimers with RXRs to mediate the RA response. In the absence of ligand, RAR and RXR can bind to RAREs but repress transcription from target genes. In this unliganded state, RAR:RXR is associated with co-

repressors (SMRT or N-CoR), which serve to recruit histone deacetylase-1 (HDAC-1) into a ternary complex with RAR and RXR. HDAC-1 can deacetylate histones providing a closed chromatin structure not accessible to the basal transcription apparatus, thus inhibiting transcription (Nagy et al., 1997).

1.3.3 Cross-talk between transcription factors determines lineage

Although individual transcription factors have been shown to be crucial for lineage commitment, myelopoiesis is a more complicated process that is orchestrated by multiple regulator proteins. The cross-talk between transcription factors is very important for lineage determination and the regulation of lineage specific genes by means of either synergism or antagonism. For example, c-Myb and chicken C/EBPβ can combinatorially activate the myeloid specific mim-1 gene. AML-1 is a transcription factor and one of the most frequent targets of chromosomal translocation in acute myeloblastic leukemia. The resultant fusion protein AML-1-ETO can inhibit C/EBPα-dependent activation of the myeloid cell specific rat defensin NP-3 promoter (Westendorf et al., 1998). Since C/EBPα is important for differentiation, AML-1-ETO may generally block differentiation through its antagonism of C/EBPα, and thus may contribute to leukemogenesis. PU.1 controls both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors (Dekoter et al., 1998). Knock-out studies demonstrated that PU.1 is required for the development of both the myeloid (granulocyte, macrophage) and lymphoid (B, T) lineages (Scott et al, 1995). PU.1 can physically interact with C/EBPα and synergistically activate expression of

myeloid growth factor receptors such as G-CSF, M-CSF and GM-CSF receptors (Hohaus et al., 1995; Smith et al., 1996; Zhang et al., 1994). PU.1 can also antagonize GATA-1 in mast cell lineage determination (Singh, personal communication).

The GATA family of transcription factors has been shown to be important for erythroid differentiation. However, in myeloid cells, GATA-1 is not expressed at significant levels, and both GATA-1 and GATA-2 are downregulated during myeloid differentiation. In contrast to monocytic and neutrophilic cells, high levels of GATA-1 are detected in eosinophils. It appears that GATA proteins do not play an important positive role in myelomonocyte development, however, their downregulation may be critical. Consistent with this, activation of GATA-1 represses myelomonocytic gene expression (Kulessa et al., 1995). Since eosinophils derived either from myelomonocytic cells or from MEP (a chicken myleloid progenitor celll line) cells showed concomitant expression of c-Myb, in addition to NF-M (chicken C/EBPβ) and GATA-1, while myelomonocytic cells lack GATA-1 (Kulessa et al.,1995), it is speculated that the balance between these three transcription factors plays a pivotal role in lineage determination (Muller et al., 1995). The lineage commitment of MEP cells (and possible also of normal progenitor cells) is influenced by the presence or absence of NF-M and GATA-1 on a background of Myb expression. If GATA-1 is expressed in the absence of NF-M, progenitors can differentiate into the thrombocytic and erythroid lineage. However, when NF-M is expressed in the absence of GATA-1,

myelomonocytic differentiation is favored. Furthermore, the combination of both GATA-1 and NF-M would allow eosinophilic differentiation.

The relative expression levels of an individual transcription factor may add another level of regulation for lineage determination. One example is PU.1. Induction of PU.1 gene expression may induce macrophage differentiation since PU.1 is expressed at significantly higher levels in the monocytic versus B lineage (Singh and Glimcher, 1999). Graded levels of PU.1 expression may participate in cell fate decisions with high levels favoring macrophage development, intermediate levels favoring B cell development, and an absence of PU.1 favoring T cell development.

1.3.4 A model for myelopoiesis

Given all of the above data, a simplified model of induction of hematopoietic differentiation by specific transcription factors has been proposed (Daniel Tenen et al., 1997). In this model, some yet unidentified signaling events upregulate specific transcription factors in the hematopoietic stem cells. These transcription factors undergo autoregulation and upregulate lineage specific growth factor receptors, such as PU.1 for M-CSF receptor, GATA-1 for EPO receptor, and C/EBPα for G-CSF receptor. Lineage specific growth factors then in turn lead to further maturation of corresponding lineages (figure 5).

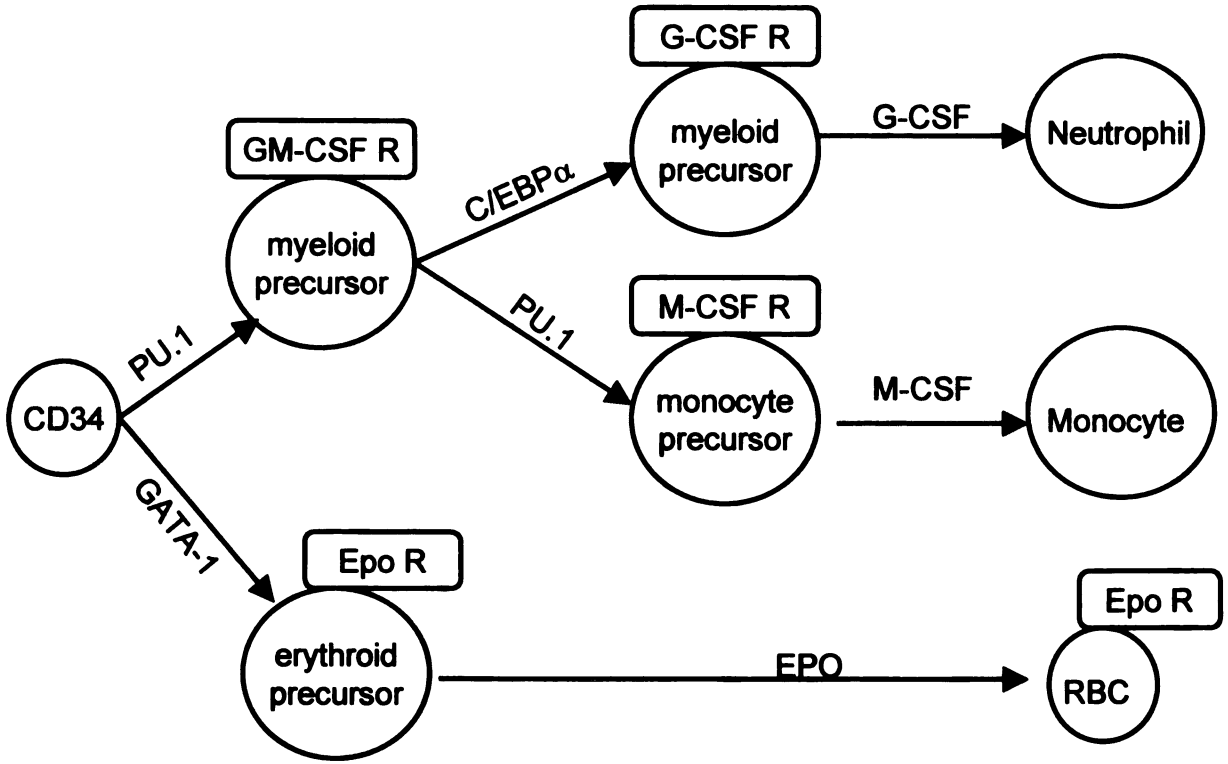

Figure 5. A simplified model of myeloid and erythroid differentiation regulated by lineage specific transcription factors

1.3.5 C/EBPs in erythroid and lymphoid lineages

C/EBP family members have also been implicated in the differentiation of the erythroid and B cell lineages. Erythropoietin upregulates expression of CHOP, a C/EBP family member that acts as a dominant negative regulator of other C/EBP family members (Coutts et al., 1999). Overexpression of CHOP in Rauscher cells enhanced erythropoietin and DMSO-induced differentiation, while inhibition of CHOP expression reduced CFU-E formation. In C/EBPα deficient mice, the erythropoietin receptor was upregulated, albeit erythroid differentiation was not adversely affected (Zhang et al., 1997). C/EBPβ is also upregulated in the course of erythroid differentiation.

Mice deficient in C/EBPβ expression are impaired in their generation of bone marrow B lymphocytes (Chen et al., 1997). These mice show decreased expression of IL-7 by bone marrow stromal cells as well as defective IL-7 signaling. C/EBPβ has also been shown to be a component of a DNA binding complex that activates the Id1 gene, a negative regulator of differentiation in pro-B cells (Saisanit et al., 1997). This suggests that C/EBPβ participates in blocking differentiation and promoting proliferation at the pro-B cell stage, but promotes differentiation through the IL-7 pathway at later stages of differentiation.

1.4 OBJECTIVES

Given the fact that the C/EBP family of transcription factors are important regulators of myelomonocytic differentiation, one legitimate question that many investigators in the field are trying to answer is:"What are the target genes of

C/EBPs that are involved in this differentiation process?". Over the past several years, numerous genes have been shown to be regulated by C/EBPs. Among myeloid lineages, the most noteworthy ones were the genes encoding the G-CSF and IL-6 receptors which are regulated by C/EBPα. However, unlike C/EBPα knock-out mice, both G-CSF receptor and IL-6 receptor knock-out mice, and even the double knock-out of both receptor genes, can still generate mature granulocytes, although the number is much reduced (Zhang et al., 1998). This leads to the hypothesis that some other genes regulated by C/EBPα that play a role in myelopoiesis remain to be identified. The objective of my research is to identify target genes regulated by C/EBPs in the course of myeloid differentiation. By doing this, we can gain a better understanding of the role of the C/EBP family of transcription factors in normal myeloid development, as well as in acute myelogenous leukemia in which a block in differentiation of myeloid precursors is a key feature of the disease.

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Chapter 2

A Novel C/EBP-Regulated Gene with a Role in Myelomonocytic Differentiation

Qiang Tian and Richard C. Schwartz

ABSTRACT

The C/EBP family of transcription factors are important regulators of myelopoiesis and the expression of genes associated with the differentiated function of myeloid cells. The involvement of C/EBPs in myelopoiesis has been demonstrated by their temporal pattern of expression in differentiating 32D clone 3 myeloblasts and by the complete lack of neutrophils associated with the targeted disruption of C/EBPα and C/EBPε in transgenic mice. We have utilized differential display PCR to identify novel C/EBP-regulated genes that play a role in myeloid differentiation. C/EBPβ was overexpressed in 32D clone 3 myeloblasts and differential display RT-PCR was performed to identify genes induced by C/EBPβ overexpression. Among the four genes identified, two were endogenous retroviral elements, one encoded a cathepsin G, a primary granule protein of neutrophils and monocytes, and one encoded a novel gene that we have named CDDP-1 for C/EBP Differential-Display Product-1. The expression of CDDP-1 mRNA is upregulated by the overexpression of C/EBPα, β, δ, or ε. CDDP-1 displays a biphasic pattern of expression in the granulocytic differentiation of myeloid progenitor cell lines: an early period of elevated expression just prior to and continuing through the induction of myeloperoxidase mRNA and a later second period coincident with the expression of lactoferrin mRNA. It is also induced upon granulocytic differentiation of HL-60 cells, but is downregulated upon monocytic differentiation. This pattern of expression coupled with the finding that CDDP-1 is most highly expressed in adult bone marrow and fetal liver suggests a role in myelopoiesis. Constitutive overexpression of CDDP-1 in

MPRO promyelocytes promoted their differentiation toward both the neutrophil and monocyte lineages. Retinoic acid treatment further facilitated the neutrophilic differentiation of CDDP-1 transductants. Subcellular localization studies showed that CDDP-1 is predominantly a cytoplasmic protein. CDDP-1 mRNA is also modulated during the course of erythroid and mast cell differentiation suggesting roles for this gene in the differentiation of these other lineages.

INTRODUCTION

The CCAAT/enhancer binding proteins (C/EBP) comprise a family of basic region-leucine zipper (bZIP) transcription factors that dimerize through their leucine zippers and bind to a consensus DNA motif through their adjacent basic regions (reviewed in Johnson and Williams, 1994). They are involved in the regulation of differentiation and function in multiple tissues. Within hematopoietic lineages, C/EBPα, β, δ, and ε have been implicated in both the regulation of differentiation and differentiated function in myelomonocytic cells. C/EBPα, C/EBPβ, and C/EBPδ are temporally regulated in the granulocyte colony-stimulating factor (G-CSF)-stimulated differentiation of 32D clone 3 myeloblasts into neutrophils (Scott et. al., 1992). C/EBPα is expressed at high levels early in differentiation and decreases to an undetectable level in terminally differentiated cells, while C/EBPβ and C/EBPδ are upregulated as cells differentiate. Similarly, C/EBPβ is upregulated in macrophage differentiation (Natsuka et. al., 1992). Observations in primary human cells and in bipotential precursor cell lines yield a somewhat different pattern of C/EBPα expression (Radomska et. al., 1998): C/EBPα is first expressed on the commitment of multipotential cells to the myeloid lineage, then its expression is upregulated and maintained during neutrophilic differentiation, while being rapidly downregulated during monocytic differentiation. Consistent with its early appearance and high level of expression in granulocyte differentiation, conditional expression of C/EBPα in transfected bipotential cells induces neutrophilic differentiation and blocks monocytic differentiation (Radomska et. al., 1998). C/EBPα-deficient mice

are completely blocked in the development of neutrophils and eosinophils (Zhang et. al., 1997).

Consistent with peak expression late in myelomonocytic differentiation, C/EBPβ and C/EBPδ are effectors in the induction of proinflammatory genes responsive to lipopolysaccharide (LPS), interleukin-1 (IL-1), or IL-6 stimulation (Akira et. al., 1990; Kinoshita et. al., 1992). The DNA binding activity of C/EBPβ is increased by a post-transcriptional mechanism when cells are exposed to LPS, IL-1 or IL-6 (Akira et. al., 1990; Poli et. al., 1990) and C/EBPβ and C/EBPδ mRNA levels can be induced by LPS, IL-1 or IL-6 (Akira et al., 1990; Kinoshita et al., 1992). C/EBPβ expression is capable of enhancing transcription from promoters containing elements responsive to those factors (Akira et al., 1990; Poli et al., 1990; Chang et al., 1990; Descombes et al., 1990). Both C/EBPβ and C/EBPδ can transactivate a reporter gene regulated by the IL-6 promoter in transient expression assays (Akira et al., 1990; Kinoshita et al., 1992). Furthermore, the promoter regions of the genes for IL-6, IL-1α, IL-1β, IL-8, tumor necrosis factor α, G-CSF, macrophage inducible nitric oxide synthase and lysozyme (Akira et al., 1990; Furutani et al., 1986; Lowenstein et al., 1993; Natsuka et al., 1992; Shirakawa et al., 1993; Zhang et al., 1993) all contain C/EBP binding motifs. Our own lab has shown that ectopic expression of C/EBPα, β, or δ confers LPS-inducibility of IL-6 and monocyte chemoattractant protein-1 expression to a cell line that normally lacks C/EBP expression (Bretz et al, 1994; Hu et al., 1998).

C/EBPε is unique among the C/EBP family in being myeloid-specific in its expression (Williams et al., 1998; Chumakov et al., 1997). C/EBPε is most highly expressed in maturing neutrophils (Chih et al., 1997; Morosetti et al, 1997; Yamanaka et al., 1997). C/EBPε-deficient mice fail to develop functional neutrophils and eosinophils (Yamanaka et al., 1997a) with a block later in differentiation than that seen in C/EBPα-deficient animals. Consistent with a block later in the differentiation process, C/EBPε has been found to transactivate in cooperation with c-myb the promoters for mim-1 and neutrophil elastase in transient expression assays (Verbeek et al., 1999), and to upregulate endogenous expression of a number of genes encoding proinflammatory cytokines including IL-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β (Chumakov et al., 1997).

A major issue still incompletely resolved in our understanding of the critical role of C/EBPα in the differentiation of neutrophils is the identity of target genes that are necessary in the differentiation process. While C/EBPα-deficient mice no longer express the C/EBPα-regulated G-CSF receptor, this is not a full explanation of their inability to produce neutrophils because mice deficient in G-CSF receptor expression are still capable of producing reduced numbers of mature neutrophils (Liu et al., 1996). The IL-6 receptor is either directly or indirectly regulated by C/EBPα and IL-6 signaling is certainly important for granulopoiesis, but mice deficient in both IL-6 receptor and G-CSF receptor expression are similar to mice deficient in expression of the G-CSF receptor

alone (Zhang et al., 1998). Therefore, there must be important C/EBPα target genes other than those encoding the G-CSF and IL-6 receptors.

Since the currently identified target genes of C/EBP regulation seem inadequate to fully explain the role of these transcription factors in myelomonocytic differentiation, we sought to elucidate the regulatory scheme in which C/EBPs act in differentiation by identifying new targets of their regulation in immature myeloid cells. To that end, we produced retroviral transductants of 32D clone 3 myeloblasts that overexpress C/EBPα, C/EBPβ, C/EBPδ and C/EBPε and performed differential display reverse transcription-PCR (RT-PCR) between C/EBP-overexpressing transductants and control transductants. In this paper, we have characterized one product of this analysis, CDDP-1 (C/EBP Differential-Display Product-1). The CDDP-1 gene encodes a novel protein displaying little relationship to any proteins of known function. CDDP-1 expression is modulated in the course of myelomonocytic differentiation and is a positive effector of myelomonocytic differentiation.

MATERIALS AND METHODS

Cell lines and cell culture. 32D clone 3 murine myeloblasts (Valteri et al., 1987) were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (FCS) and 10% WEHI-3 conditioned medium as a source of interleukin-3. For induction of granulocytic differentiation, cells were washed twice with phosphate-buffered saline and placed in IMDM supplemented with 20% FCS supplemented with 5 ng/ml murine G-CSF (R&D Systems). WEHI-3 cells (ATTC TIB 68) were maintained in RPMI 1640 medium supplemented with 5% FCS. EML-C1 murine hematopoietic progenitor cells (Tsai et al., 1994) were maintained in IMDM supplemented with 20% horse serum, and 15% conditioned medium from BHK cells transfected with an expression vector for rat stem cell factor (BHK/MKL cells). For induction of myelomonocytic differentiation, EML-C1 cells were transferred to IMDM supplemented with 20% horse serum, 15% BHK/MKL conditioned medium, 10% WEHI-3 conditioned medium, and 5 μM all-*trans* retinoic acid (ATRA). For induction of erythroid differentiation, EML-C1 cells were transferred to IMDM supplemented with 20% horse serum, 15% BHK/MKL conditioned medium, 0.5% WEHI-3 conditioned medium, and 8 units/ml human erythropoietin (R&D Systems). For induction of mast cell differentiation, EML-C1 cells were transferred to IMDM supplemented with 20% horse serum, 15% BHK/MKL conditioned medium, and 10% WEHI-3 conditioned medium. MPRO murine promyelocytes (Tsai et al., 1993) were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS and 10ng/ml

murine granulocyte-macrophage colony-stimulating factor (R&D Systems). For induction of granulocytic differentiation, ATRA was added to 20μM. HL-60 human promyelocytic leukemia cells (ATTC CCL 240) were maintained in RPMI 1640 medium supplemented with 20% FCS. HL-60 cells were treated with either 10 μM ATRA or 100nM phorbol myristate acetate to induce granulocytic or monocytic differentiation, respectively. BB88 Friend erythroleukemia cells (ATTC TIB 55) were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS and 2-mercaptoethanol. For induction of erythroid differentiation, dimethyl sulfoxide was added to a final concentration of 15% (vol/vol). U937 human histiocytic lymphoma cells (ATTC CRL 1593) were grown in RPMI 1640 supplemented with 10% FCS. U937 cells were treated with either 10 μM ATRA or 100 nM phorbol myristate acetate to induce granulocytic or monocytic differentiation, respectively. 293T cells (Pear et al., 1993) and CV-1 cells (ATCC CCL 70) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS. All cells were grown at 37^0C in the presence of 5% CO_2.

Differential display RT-PCR, cDNA cloning and sequencing. Differential display RT-PCR was performed with the Delta RNA Fingerprinting Kit (Clontech) according to the manufacturer's instructions. The full-length cDNA of CDDP-1 was isolated from a λgt11 human bone marrow cDNA library (Clontech). The library was screened according to manufacturer's protocol using the 230bp CDDP-1 differential display product, as well as a probe generated by 5' rapid amplification of cDNA ends, as probes. Two rounds screening were performed to eventually isolate the 2.3 kb CDDP-1 cDNA. Automated dye terminator

sequencing was performed by the Michigan State University DNA Sequencing Facility.

RNA isolation and analysis. Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's directions. RNA's were electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to a stringency of 0.1x SSPE at 65°C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[α-^{32}P]dATP (3000 Ci/mmol; DuPont-New England Nuclear). The C/EBPα and C/EBPβ probes were rat genomic fragments derived from the pSV(X)C/EBPα and pSV(X)C/EBPβ expression vectors (16, 17). The C/EBPδ probe was a murine genomic fragment derived from the pSV(X)C/EBPδ expression vector (17). The C/EBPε probe was a rat genomic fragment derived from the pSV(X)C/EBPεp34 expression vector (18). The myeloperoxidase probe was a 0.8 kb PstI fragment derived from a 2.73 kb murine cDNA (from G. Rovera, The Wistar Institute, Philadelphia, PA). The lactoferrin probe was a 0.56 kb murine cDNA (from G. Rovera, The Wistar Institute, Philadelphia, PA). The fms probe was a 2.7 kb ClaI/BamHI fragment of the McDonough strain of feline sarcoma virus (Donner et al., 1982). The lysozyme probe was a 1.0 kb human cDNA (Peters et. al., 1989). The cathepsin G probe was a 0.5 kb murine cDNA cloned through the differential display RT-PCR process described in this paper. The glyceraldehyde-3-phosphate dehydrogenase probe was a 1.3 kb rat cDNA (Fort et al., 1985).

Expression vectors. pSV(X)C/EBPβ expresses C/EBPβ from the Moloney murine leukemia virus promoter and its construction from the parent vector, pSV(X)Neo, has been described previously (Bretz et al., 1994; Hu et al., 1998), pBABE-CDDP-1 expresses CDDP-1 from the Moloney murine leukemia virus promoter and was constructed by inserting the CDDP-1 cDNA into the BamHI site of the murine retroviral vector, pBABE-Puro (Morgenstern and Land, 1990). pMTD-CDDP-1 was constructed by inserting the CDDP-1 cDNA into the BglII site of pMTD (Sompayrac and Danna, 1994), which placed CDDP-1 under the regulation of a modified metallothionein promoter. pMEX-CDDP-1 was constructed by inserting the CDDP-1 cDNA into the EcoRI site of pMEX (Williams et al., 1991), which utilizes the Moloney murine sarcoma virus promoter. β-RE2-Luc (from Ron Evans, The Salk Institute, San Diego, CA) contains 2 copies of retinoic acid response elements. pCMV-RARα (from David Talmage, Columbia University, New York, NY) expresses the gene encoding human retinoic acid receptor α from the cytomegalovirus promoter. The SV40 early promoter-reporter is a commercial product, pβgal-Control (Clontech), where the SV40 early promoter and enhancer sequences are cloned upstream and downstream , respectively, of the *lacZ gene.*

Retroviral infection. In order to prepare retrovirus stocks derived from pSV(X)Neo, pSV(X)C/EBPβ, pBABE-Puro and pBABE-CDDP-1, 10 cm plates of 70% confluent 293T cells were co-transfected with 3ug of pMOV-ψ$^-$ (Mann et al., 1983), a packaging construct, and 3μg pBABE-CDDP-1 using DMRIE-C reagent (Life Technologies) according to manufacturer's directions. 60 hr after

transfection, viral supernatants were collected by centrifugation at 1000 rpm for 5 min and passed through 0.4 μm filters. $2x10^6$ cells in normal growth medium were mixed with an equal volume of viral stock and 8 μg/ml polybrene (Sigma) and then incubated at 37^0C for 3 hr. 24 hr after infection, cell cultures were split 1:4 and puromycin was added into the media at a final concentration of 1 μg/ml for a period of 1 to 2 wk.

Transient Transfection. Transient transfections of CV-1 cells were performed using DMRIE-C reagent (Life Technologies). β-RE2-Luc and pCMV-RARα were cotransfected in the presence and absence of pMEX-CDDP-1 in the quantities indicated. Total DNA was made up to 3 μg for each transfection with pMEX. Transfectants were either untreated or treated with 10μM ATRA. A SV-40 early promoter-reporter was used as a control for transfection efficiency. After 48 hrs, cells were lysed and analyzed for luciferase activity by using the Luciferase Reporter Gene Assay Kit (Roche Molecular Biochemicals) and for β-galactosidase activity by using the Luminescent β-Galactosidase Genetic Reporter System II (Clontech).

Western blotting. Whole cell protein extracts were prepared using TRIzol reagent (Life Technologies) following the manufacturer's protocol. For cytoplasmic and nuclear fractionation, $5x10^6$ cells were washed in phosphate-buffered saline and lysed in 200μl buffer A (15 mM KCl, 10 mM HEPES [pH 7.6], 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% [vol/vol] NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin) for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000 x *g*

for 20 sec. The supernatant lysate was the cytoplasmic fraction. Proteins were extracted from nuclei by incubation at 4°C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 25% [vol/vol] glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin). Nuclear debris was pelleted by centrifugation at 14,000 x *g* for 15 min at 4°C. This supernatant extract was the nuclear fraction. Protein samples were electrophoresed through sodium dodecyl sulfate-12% polyacrylamide gels, transferred to a Protran membrane (Schleicher and Schuell), and antigen-antibody complexes were visualized with the Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech).

Antisera. A 540 bp BamHI fragment of CDDP-1 was subcloned into pGEX-2 (Pharmacia Biotech) to encode a GST-CDDP-1 fusion protein. The CDDP-1 fusion protein was purified from bacterial extract by chromatography with a glutathione Sepharose 4B column (Amersham Pharmacia Biotech). The purified CDDP-1 fusion protein was used to immunize rabbits to produce a polyclonal antiserum specific for CDDP-1. Immunizations and serum preparation were performed by Alpha Diagnostic International (San Antonio, TX). Antisera specific to cytoplasmic lactate dehydrogenase and nuclear Sm protein were obtained from R. Patterson (Michigan State University, East Lansing, MI).

Cell staining. MPRO transductant cells were cytocentrifuged onto glass microscope slides and stained in Wright-Giemsa Stain (Sigma) according to the manufacturer's directions.

RESULTS

Identification and sequence analysis of CDDP-1. A series of differential display RT-PCR experiments were performed comparing 32D clone 3 myeloblasts infected with a retroviral vector expressing C/EBPβ to cells infected with an "empty" control vector. 64 sets of primer pairs were used to identify 27 differential display products potentially representing both gains and losses of expression. Four of these products were confirmed as being differentially expressed by Northern blot analysis of RNA's from the original transduced cell lines that were used to generate the differential display. Sequence analysis revealed that 2 of these products were retroviral elements (most murine cell lines contain multiple copies of endogenous retroviruses which have C/EBP binding sites in their long terminal repeats). A third product encoded a portion of cathepsin G, a neutral serine protease found in the primary granules of neutrophils (Heusel et al., 1993). The fourth product, a 230 bp cDNA, is shown in Fig. 1.

The mRNA for this product was verified as being overexpressed in 32D clone 3 cells transduced for C/EBPβ expression, as well as in cells transduced for C/EBPα, C/EBPδ and C/EBPε expression (Fig. 2), suggesting a C/EBP-regulated gene. Sequence analysis found this cDNA to represent a novel gene, previously unreported to GenBank. We refer to this gene as CDDP-1 for C/EBP Differential-Display Product-1. Several highly homologous murine, rat and human expressed sequence tag (EST) clones of unknown function were identified. A CDDP-1 cDNA containing a 453 amino acid open reading frame was isolated from a λgt11 human bone marrow cDNA library using the original 230 bp

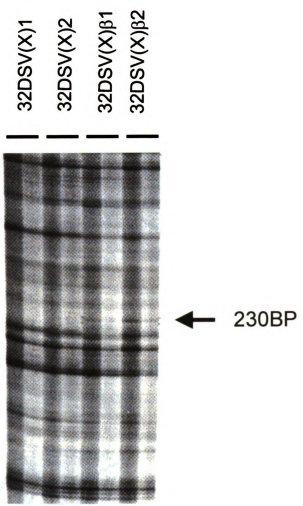


Figure 1. Differential display between control and C/EBPβ–overexpressing cells. Two control populations [32DSV(X)1 and 2] and two C/EBPβ-overexpressing populations [32DSV(X)β1 and 2] were examined at two concentrations of initial cDNA products.

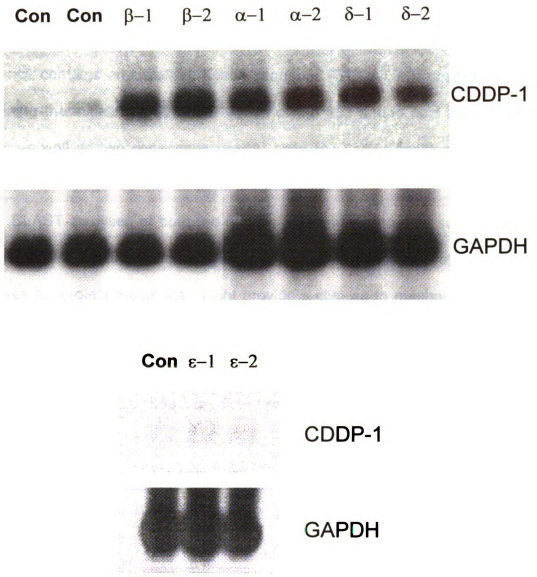


Figure 2. Overexpression of C/EBP isoforms in 32Dclone3 induces expression of a differential display RNA species. A Northern blot confirmed that the gene represented by the 230 bp differential display product (CDDP-1) was indeed expressed at a higher level in C/EBPß-expressing cells (β1 and β2) than in controls. It also revealed that this gene was induced by C/EBPα (α1 and α2), C/EBPδ (δ1 and δ2), and C/EBPε (ε1 and ε2) expression as well.

cDNA and a probe generated by 5' rapid amplification of cDNA ends (RACE). The nucleic acid sequence of this clone and the amino acid translation of that sequence (Accession No. AF123073) are presented in Fig. 3. The 2330 bp sequence contains an excellent Kozak sequence (GCCATGG; positions 28-34) containing the initiating methionine codon of the 453 amino acid open reading frame, as well as two consensus poly(A) sites (AATAAA; positions 2291-2296 and 2299-2304) upstream of an apparent poly(A) tail.

BLAST analyses of both the nucleic acid and amino acid sequences did not reveal any significant homologies to any known genes or proteins (NCBI). Analyses for protein motifs that might provide a clue as to mechanistic function were unrevealing: several potential protein kinase C and casein kinase 2 sites were found, as well as a potential N-glycosylation site (PROSITE). Interestingly, a protein BLAST search finds a predicted protein of unknown function (GenBank Accession No. AAD50514) supported by several human EST's with 43% identity to amino acid sequence of CDDP-1. This raises the possibility of CDDP-1 being a member of a larger family of as yet uncharacterized proteins. A sequence tagged site (sts-W37771) identical to CDDP-1 sequence maps to human chromosome 17q21-q22 of the GB4 radiation hybrid map, a region with a number of mapped chromosomal abnormalities observed in leukemias (NCBI). While some chromosomal abnormalities in this region involve the genes encoding retinoic acid receptor α, hepatic leukemia factor and AF17, others are not defined.

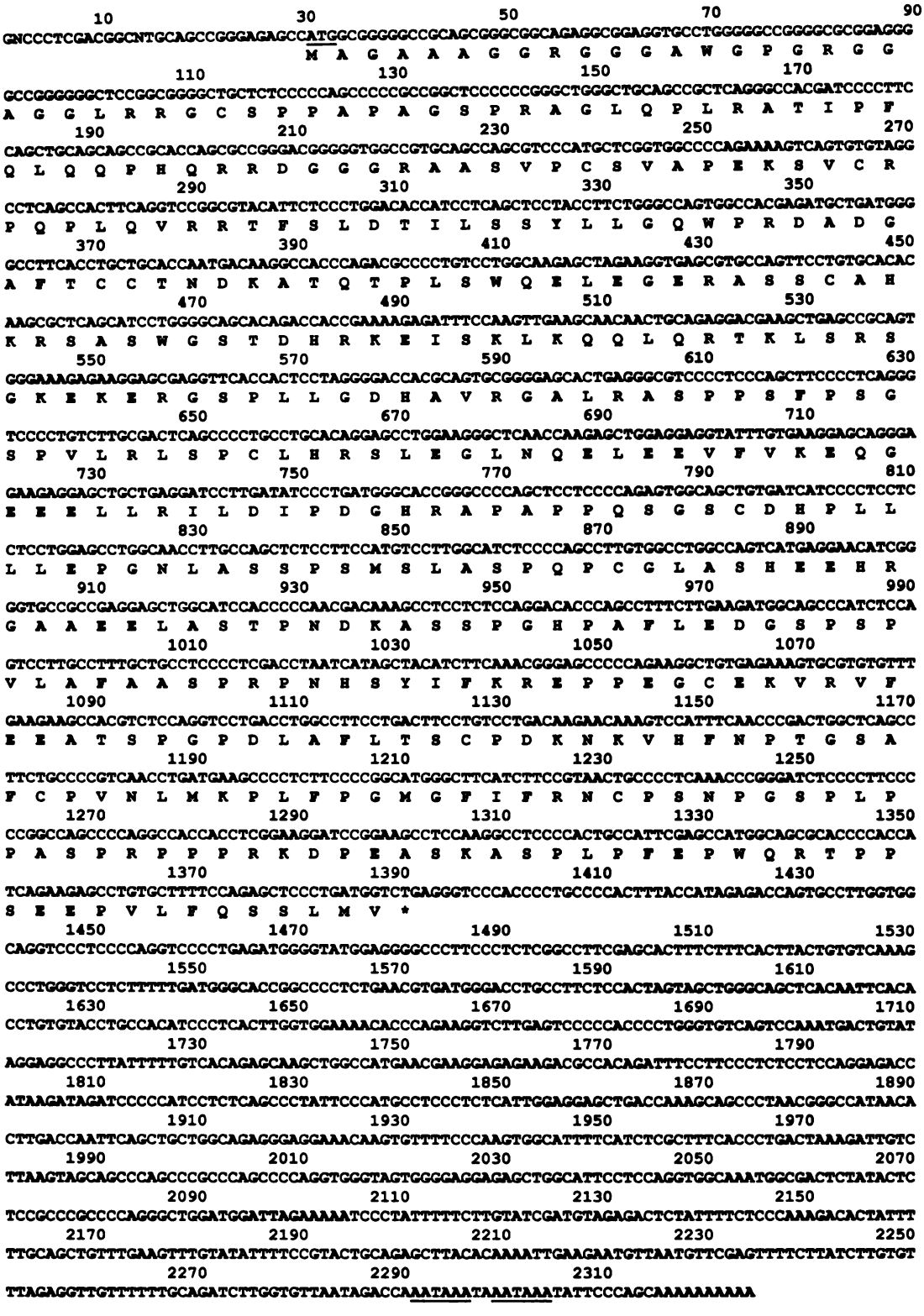


Figure 3. The nucleic acid and the deduced amino acid sequences of CDDP-1.

The CDDP-1 cDNA sequence was identical to sequences reported to GenBank as "unfinished High Throughput Genomic Sequences" (Accession No. AC015795). Comparison to these unordered sequences from human chromosome 17 reveal the CDDP-1 gene to be organized into 8 exons: 16-226, 227-393, 394-491, 492-602, 603-738, 739-939, 940-1093, 1094-2314. An examination of sequences upstream of the first exon found several potential C/EBP binding sites consistent with the upregulation of CDDP-1 by overexpression of C/EBP isoforms (Fig 4).

CDDP-1 is most highly expressed in fetal liver and adult bone marrow. Hybridization of the CDDP-1 cDNA to an RNA dot blot of human tissues (RNA Master Blot, Clontech) found CDDP-1 to be expressed most highly in fetal liver (4.2-fold that seen in peripheral leukocytes) and adult bone marrow (3.0-fold that seen in peripheral leukocytes) as would be expected of a gene with a function in hematopoiesis (Figure 5). Northern analysis of a diverse panel consisting mostly of hematopoietic cell lines (Figure 6) found expression of CDDP-1 RNA in myeloblast cell lines other than 32D clone 3 (M1 and HL60) as well as a monocytic cell line (WEHI 274.1), but lacking in mature macrophage cell lines (IC21 and P388D1[IL-1]). Expression was also observed in a B cell line (M12), a pre-B cell line (70Z/3), a CD4+ pre-T cell line (EL4), a mastocytoma (P815), and an erythroblast cell line (BB88). In the case of BB88, expression increased upon DMSO-induced differentiation. CDDP-1 was neither observed in a CD8+ T cell line (CTLL-2) nor NIH3T3 cells.

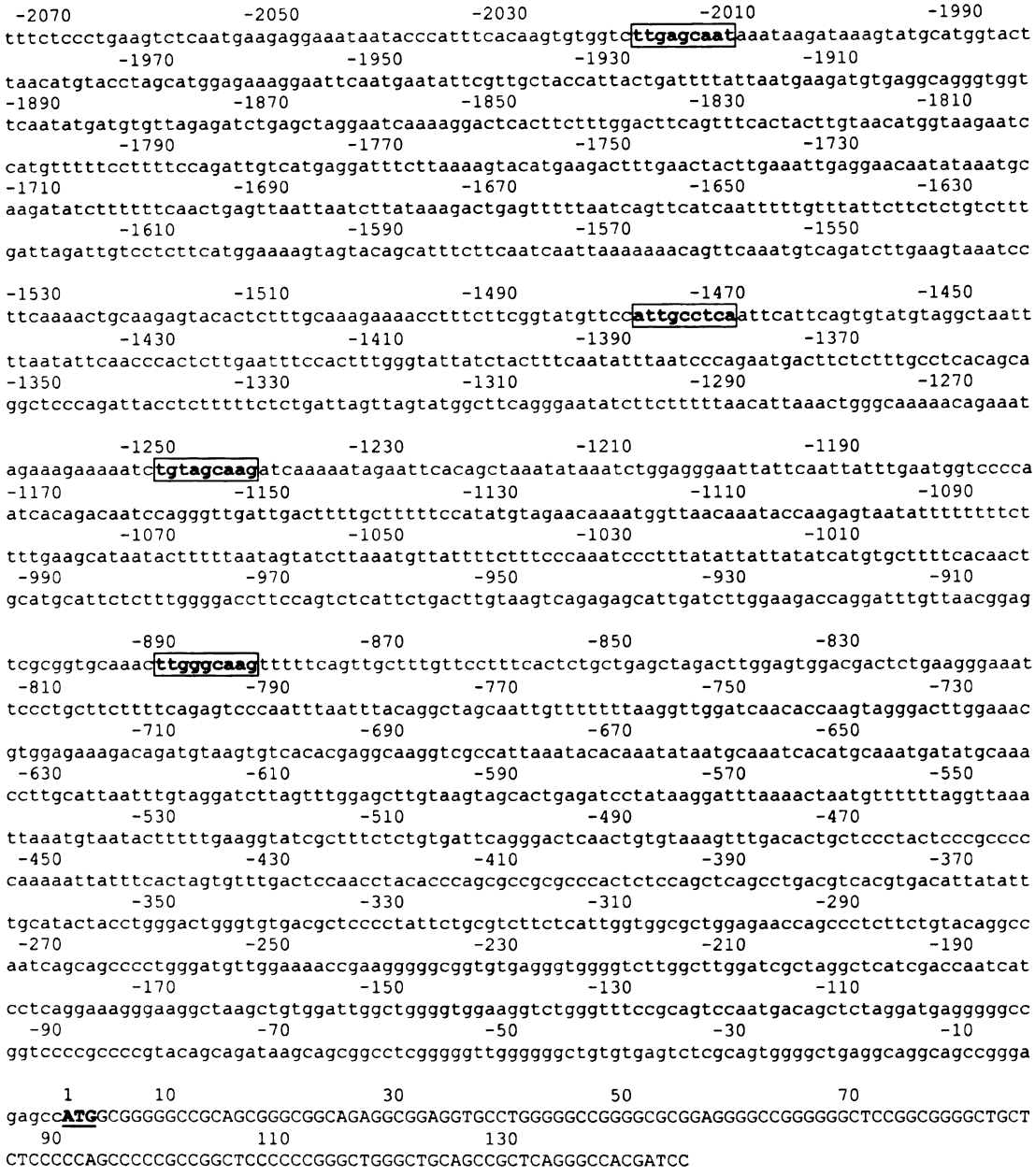
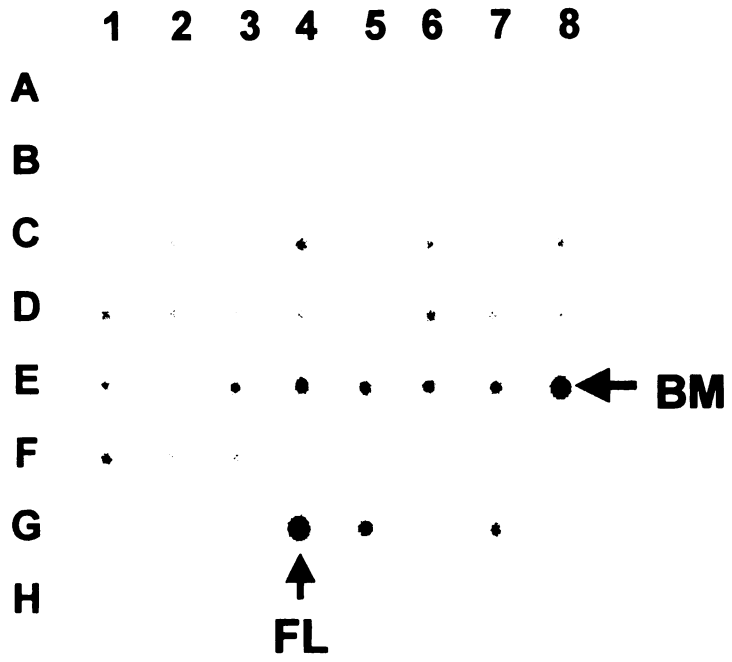


Figure 4. The approximate 2kb upstream sequence of CDDP-1 cDNA contains four copies of putative C/EBP binding sites (boxed regions). The adenine residue of the first ATG codon in CDDP-1 open reading frame was arbitrarily numbered +1.



	1	2	3	4	5	6	7	8
A	whole brain	amyg-adala	caudate nucleus	cere-bellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
B	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	acumens	spinal cord	
C	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
D	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
E	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
F	appendix	lung	trachea	placenta				
G	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
H	yeast total RNA	yeast tRNA	E coli rRNA	E.coli DNA	Poly r(A)	human DNA	human DNA	human DNA

Figure 5. CDDP-1 RNA is expressed most highly in adult bone marrow and in fetal liver A multiple tissue RNA dot blot (Clontech) showed that CDDP-1 was expressed in a variety of tissues with highest expression in fetal liver and bone marrow.

Figure 6. Northern blot of a panel of hematopoietic cell lines.

CDDP-1 is predominantly a cytoplasmic protein. In order to verify that the open reading frame observed in the CDDP-1 cDNA was expressed as protein and to examine the subcellular localization of its expression, an antiserum specific for CDDP-1 was generated. A Western blot showed the apparent molecular weight of CDDP-1 to be 48 kDa (Fig. 7). This is in good agreement with the calculated weight of the CDDP-1 protein from its translated sequence. An examination of cellular localization of CDDP-1 in cytoplasmic versus nuclear fractions of MPRO, BB88, and U937 cells revealed a largely cytoplasmic localization (Fig. 8). The localization of CDDP-1 protein followed that of lactate dehydrogenase, an exclusively cytoplasmic protein. The small amount of CDDP-1 observed in the nuclear fraction of MPRO cells is likely an artifact since lactate dehydrogenase contaminates the nuclear fraction to the same extent. To examine whether the subcellular localization of CDDP-1 changes as cells differentiate, the U937 cells were treated with ATRA to induced neutrophilic differentiation. Both cytoplasmic and nuclear extracts were prepared over a time course of four days. CDDP-1 protein could only be detected in the cytoplasmic fractions. As a control for nuclear protein, SM protein, which is a component of spliceosome, could only be found in the nuclear fractions. As an additional control, RARα, a transcription factor that appeared in both the cytoplasmic and the nuclear fractions in untreated cells, translocated into nucleus upon ATRA treatment. All the above evidence strongly suggestes that CDDP-1 is predominantly a cytoplasmic protein. Immuno-fluorescent studies using CDDP-1 specific anti-serum in MPRO

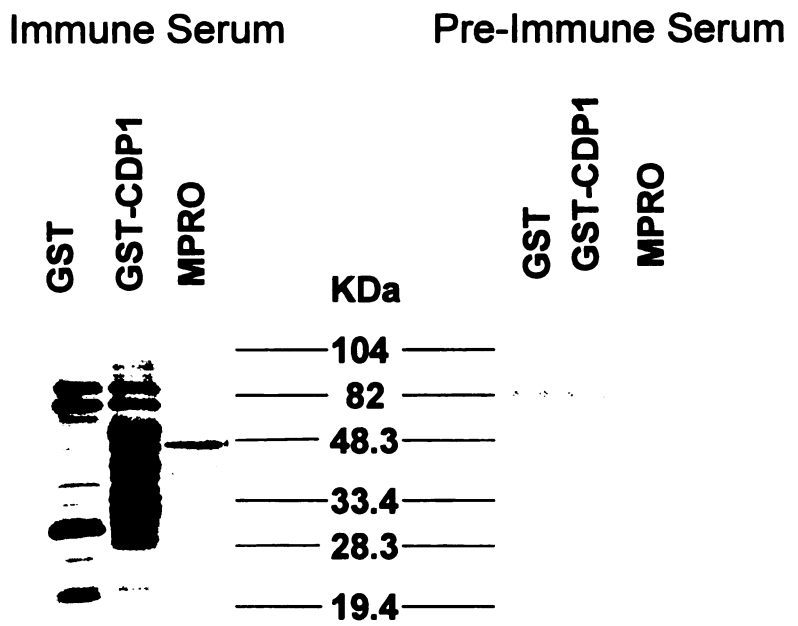


Figure 7. Western analysis comparing CDDP-1 antiserum to pre-immune serum. The samples examined were a control bacterial lysate with GST protein (GST), a bacterial lysate containing the GST-CDDP-1 fusion protein (GST-CDDP-1), and a cellular lysate MPRO cells.

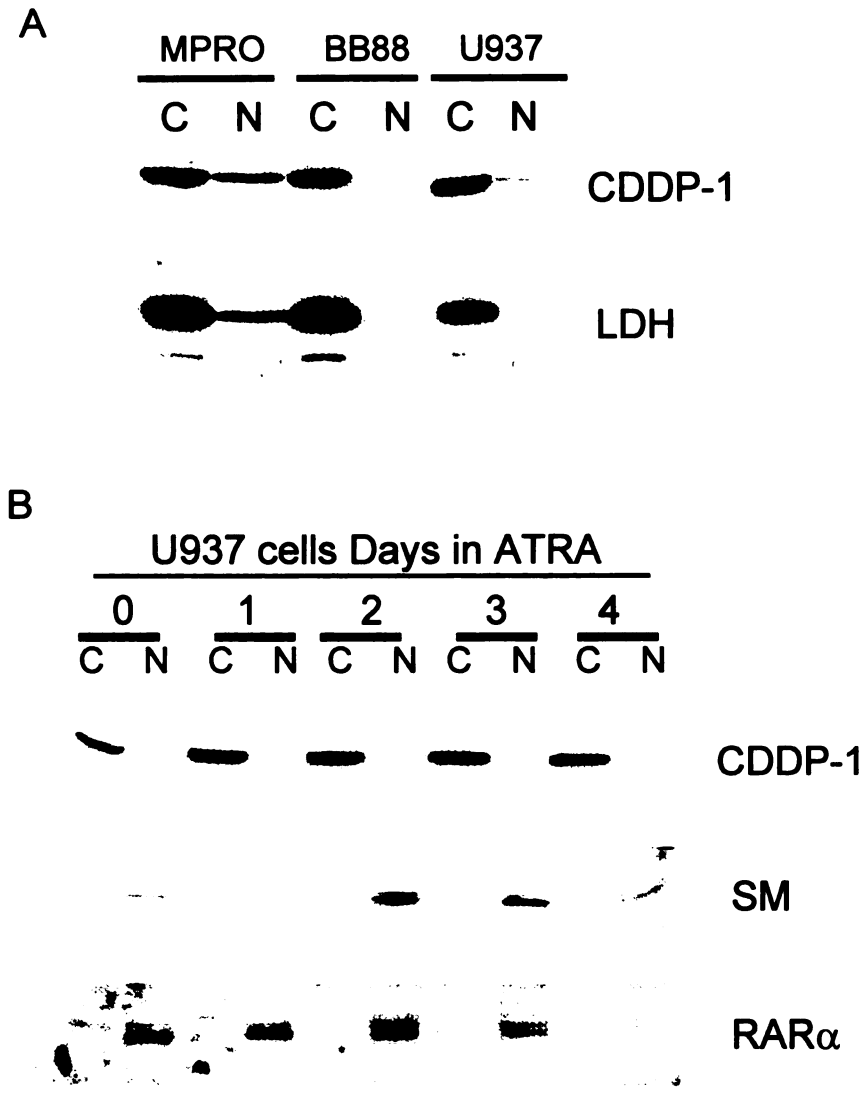


Figure 8. Western analysis of cytoplasmic (C) and nuclear (N) fractions from MPRO, BB88, U937 (A) and a time course of U937 neutrophilic differentiation induced by ATRA for four days (B). Porteins were detected with antiserum specific to CDDP-1, lactate dehydrogenase (LDH), SM, and retinoic acid receptor α (RARα).

promyelocytes showed that CDDP-1 protein existed mainly in the cytoplasm (data not shown).

CDDP-1 mRNA is up-regulated during neutrophilic differentiation and down-regulated during monocytic differentiation. The expression pattern of CDDP-1 mRNA was next examined in time courses of neutrophilic differentiation of several immature myeloid cell lines: 32D clone 3 myeloblasts (Figure 9), MPRO promyelocytes (Figure 10), and bipotential HL-60 late myeloblasts (Figure 11). In all three cell lines, CDDP-1 RNA was induced by 24 hours. In 32D clone 3 cells, CDDP-1 reached peak levels of expression ahead of myeloperoxidase, and then is downregulated while myeloperoxidase expression is still robust. In this experiment, viability could not be maintained past 5 days and, thus, the temporal relationship to lactoferrin expression could not be examined. In MPRO cells, peak CDDP-1 expression is coincident with lactoferrin expression, which would seem later than its expression in 32D clone 3 cells. However, CDDP-1 initially shows a decrement in RNA levels followed later by induction. Myeloperoxidase mRNA levels decrease with CDDP-1 levels suggesting that the expression of CDDP-1 may be biphasic: a period of elevated expression associated with the transcription of primary granule protein-encoding genes (myeloperoxidase) and a period of elevated expression associated with secondary granule protein-encoding genes (lactoferrin). In HL-60 cells, myeloperoxidase is expressed at a relatively high level in uninduced cells and lactoferrin expression is not observed (data not shown), making it difficult to place CDDP-1 expression in a temporal sequence with these markers of neutrophilic differentiation. However, CDDP-1

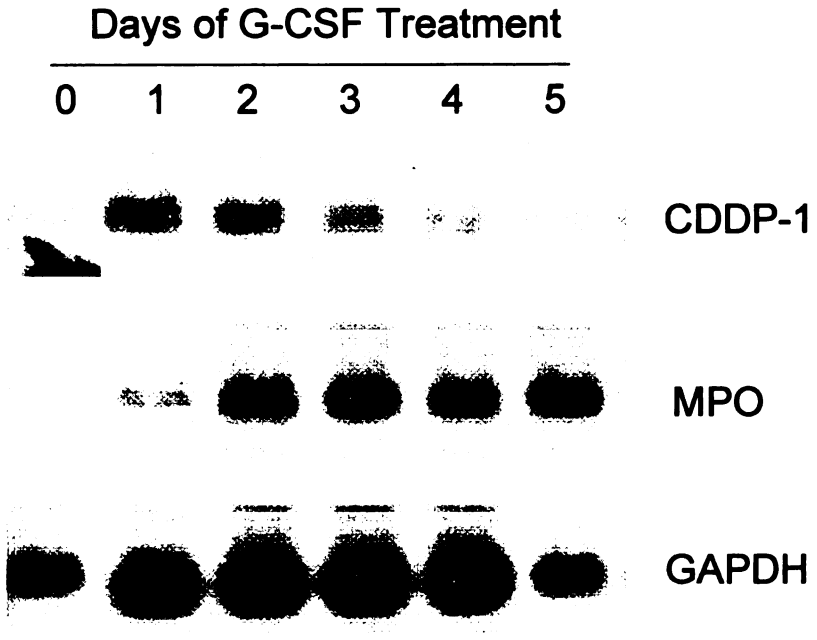


Figure 9. RNA was isolated from a time course of G-CSF-induced differentiation of 32D clone 3 cell. A northern blot was successively hybridized with probes for CDDP-1, myeloperoxidase (MPO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

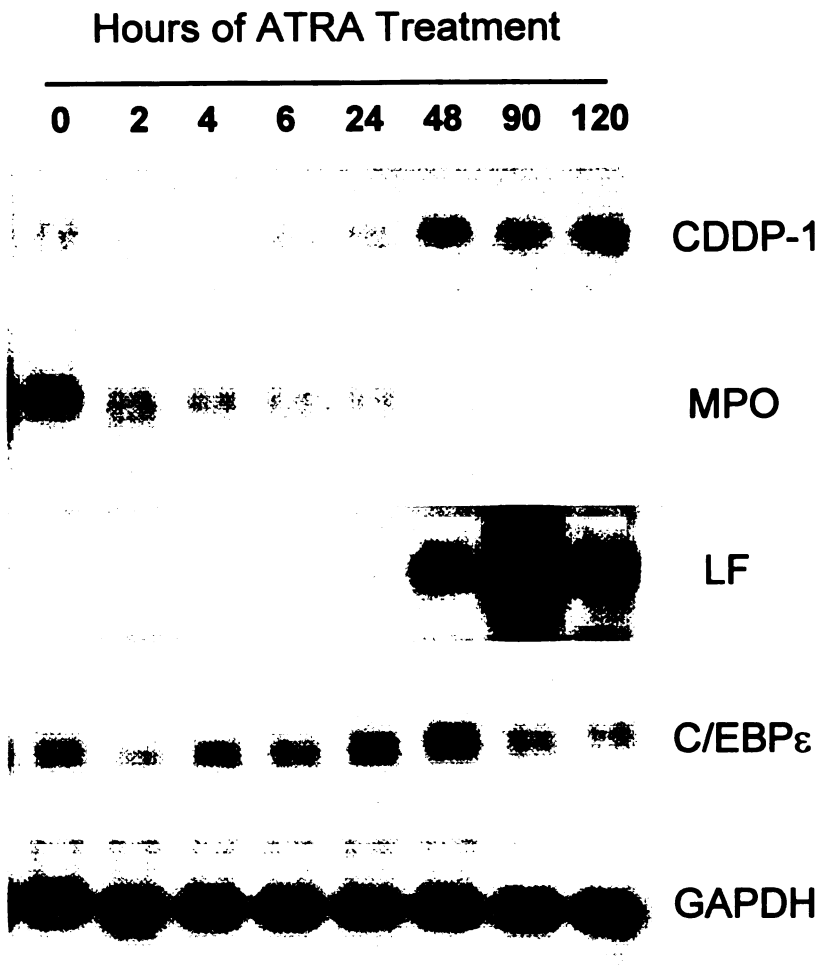


Figure 10. RNA was isolated from a time course of all trans tetinoic acid (ATRA)-induced differentiation of MPRO cells. A northern blot was successively hybridized with probes for CDDP-1, myeloperoxidase (MPO), lactoferrin (LF), C/EBPε and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

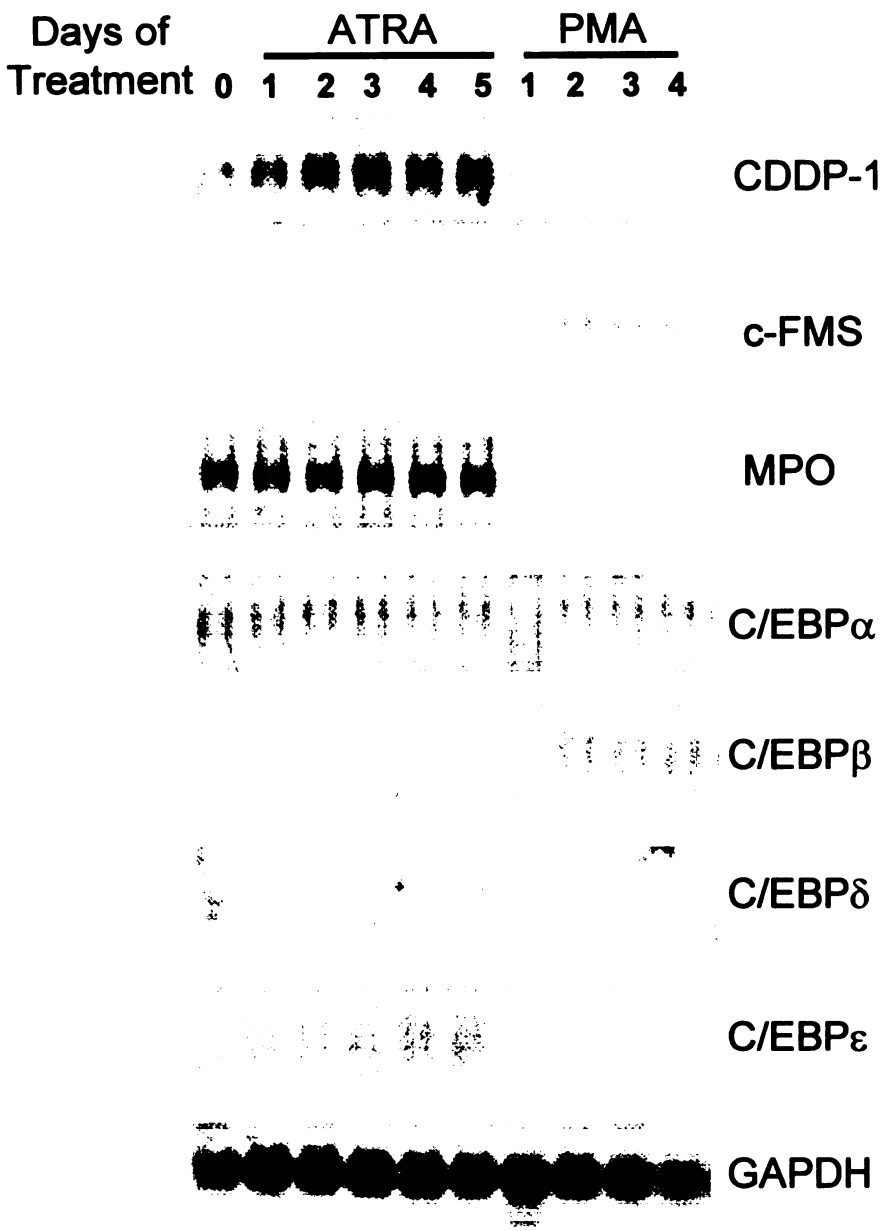


Figure 11. RNA was isolated from a time course of all trans retinoic acid (ATRA)-induced differentiation and PMA-induced differentiation of HL-60 cells. A northern blot was successively hybridized with probes for CDDP-1, c-fms, myeloperoxidase (MPO), C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

induction parallels that of C/EBPε in these cells placing its expression after acquisition of C/EBPα expression, but prior to peak expression of C/EBPβ and C/EBPδ. In contrast to the induction of CDDP-1 RNA with neutrophilic differentiation in the three cell lines we examined, CDDP-1 RNA was downregulated with monocytic differentiation of HL-60 cells (Fig. 11). This is consistent with the lack of CDDP-1 expression in mature macrophage cell lines that was observed in Fig. 6. CDDP-1 expression is thus associated with neutrophilic as opposed to monocytic differentiation. The data collectively suggest a role in the later stages of neutrophilic differentiation, being similar to that of C/EBPε (MPRO, Fig. 10; HL-60, Fig. 11). In relation to the expression of genes encoding granule proteins, the timing of CDDP-1 expression may be biphasic: expression is associated with induction of myeloperoxidase expression (32D clone 3, Fig. 9) and again with the later induction of lactoferrin expression (MPRO, Fig. 10). CDDP-1 expression is thus first observed at the promyelocyte stage of differentiation.

In order to better explore earlier timepoints in the course of myelomonocytic differentiation, CDDP-1 expression was examined during the myeloid differentiation of the EML-C1, a hematopoietic stem cell line. Northern analysis of RNAs isolated over a time course of ATRA-induced differentiation showed CDDP-1 to be transiently expressed in parallel to myeloperoxidase (Fig. 12). This timing is slightly later than the observations obtained from the differentiation of 32D clone 3 myeloblasts (Fig. 9). Granulocyte-macrophage colony-stimulating factor-treated products of EML-C1 differentiation are blocked

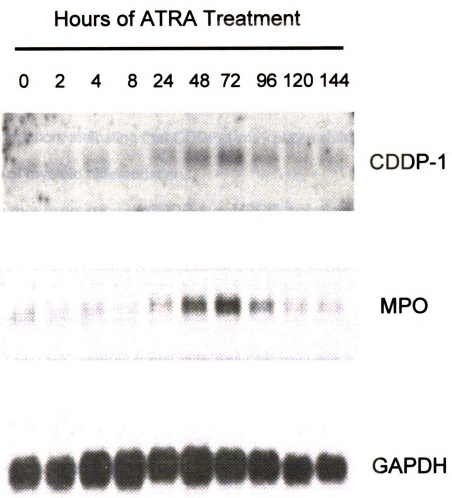


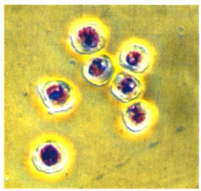
Figure 12. RNA was isolated from a time course of ATRA-induced differentiation of EML-C1 cells. A northern blot was successively hybridized with probes for CDDP-1, myeloperoxidase (MPO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

at the promyelocyte stage unless further treated with retinoic acid (Tsai et al., 1993). MPRO, which displayed induction of CDDP-1 RNA at the point of lactoferrin induction (Fig. 10), is analogous to such promyelocytes (Tsai et al, 1994) and its differentiation can be considered an extension of the EML-C1 time course. Thus, the results from EML-C1 are consistent with a biphasic induction of CDDP-1 occurring first with myeloperoxidase induction and then again with lactoferrin induction, indicating that CDDP-1 may play a role in both the early and later stages of myeloid differentiation.

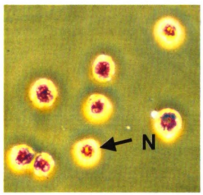
Constitutive overexpression of CDDP-1 in MPRO promyelocytes promotes their differentiation. The close temporal association of CDDP-1 with genes encoding granule proteins led us to ask whether CDDP-1 encodes a novel granule protein or a novel regulator of granule protein expression. In order to address that question, we produced stable transductants of MPRO cells with either a retroviral vector constitutively expressing CDDP-1 or an "empty vector" control. The vector used, pBABE-Puro, allowed selection of stably transduced cells by puromycin selection (Morgenstern and Land, 1990). Three independent retroviral transduction experiments were carried out generating a total of 10 cell populations overexpressing CDDP-1, as well as 12 control populations. RNAs were isolated from all of these cell populations and analyzed by Northern blots. All of the CDDP-1 transductants showed high levels of vector-derived CDDP-1 expression. Significantly, six of the populations transduced for CDDP-1 expression showed a more differentiated neutrophilic phenotype than the control populations. They showed a higher proportion of band form and

polymorphonuclear cells (Fig. 13) and elevated levels of the mRNAs encoding the primary granule proteins, myeloperoxidase, cathepsin G, and lysozyme. Four of the CDDP-1 transductants also showed elevated levels of lactoferrin mRNA (Fig. 14). Lactoferrin is a secondary granule protein that is only expressed after the more mature myelocyte stage of neutrophilic differentiation. A western blot confirmed that CDDP-1 transductants also expressed elevated levels of CDDP-1 protein in comparison to control transductants (Fig. 15). Furthermore, when the neutrophilic CDDP-1 transductants were treated with ATRA to induce differentiation, they all displayed a more rapid morphological differentiation than control populations with band cells and polymorphonuclear cells consistently appearing a day ahead of controls (Fig. 16).

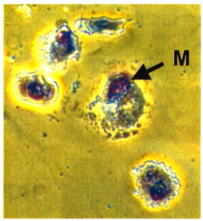
Interestingly, two of the CDDP-1 transductants showed morphological features suggesting monocyte/macrophage differentiation. These transductants had become very adherent to their culture plates with a typical monocytic morphology: higher cytoplasm/nucleus ratio, pseudopods, and a foamy cytoplasm with many vesicles (Fig. 13). A Northern blot analysis showed diminished levels of myeloperoxidase expression compared to controls (Fig. 17). This is consistent with monocytic differentiation. Two of the CDDP-1-overexpressing populations showed no dramatic differences from the control populations. Taken together, these data point to a role for CDDP-1 in promoting both the neutrophilic and monocytic differentiation of MPRO promyelocytes.



MPRO-puro



MPRO-CDDP-1 #1



MPRO-CDDP-1 #9

Figure 13. Wright-Giemsa staining of representative CDDP-1 transductants of MPRO cells with either neutrophilic (N) or monocytic (M) morphology. MPRO-puro represents transductants infected with a control retrovirus.

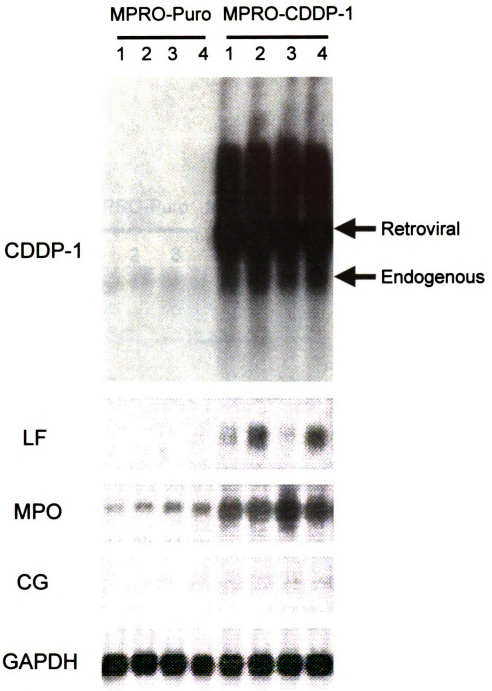


Figure 14. RNA was isolated from 4 independent populations of MPRO cells infected with a CDDP-1 expressing retrovirus. A northern blot was successively hybridized with probes for CDDP-1, lactoferrin (LF), myeloperoxidase (MPO), cathepsin G (CG), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

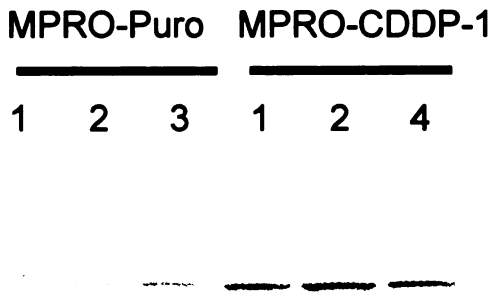


Figure 15. A western blot using CDDP-1-specific antibody showed elevated levels of CDDP-1 protein expression in three independent MPRO transductants of CDDP-1 (MPRO-CDDP-1) in comparison to control transductants (MPRO-Puro).

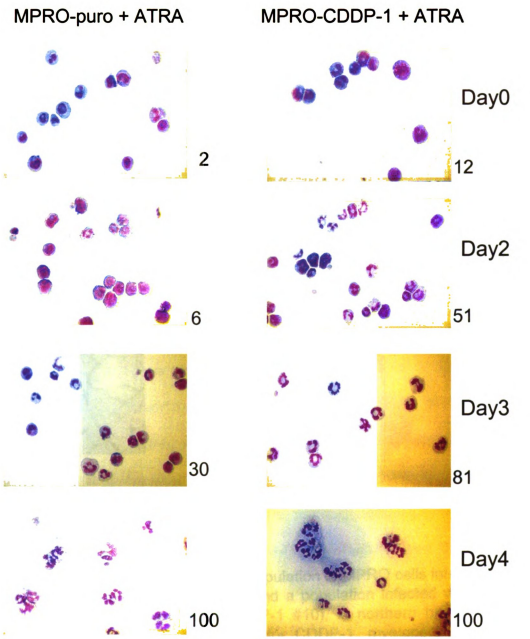


Figure 16. Wright Giemsa staining of a population of control MPRO cells and a population of CDDP-1 MPRO transductants over a time course of differentiation after treatment with ATRA. The numbers at the lower right corner of each figure represent percentage of mature band form and polymorphonuclear cells. The examples shown are representative.

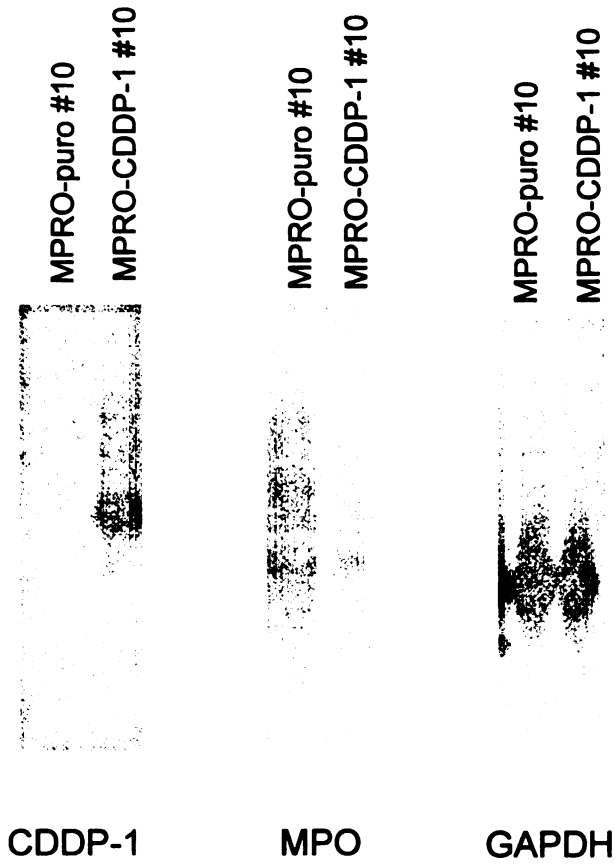


Figure 17. RNA was isolated from a population of MPRO cells infected with a control retrovirus (Puro #10) and a population infected with a CDDP-1 expressing retrovirus (CDDP-1 #10). A northern blot was successively hybridized with probes for CDDP-1, myeloperoxidase (MPO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

CDDP-1 was upregulated upon ATRA-induced differentiation of HL60, MPRO and EML-C1 cell lines (Figs. 10, 11, 12). When overexpressed in MPRO cells, CDDP-1 facilitated ATRA-induced neutrophil differentiation (Fig. 16). In addition, a weak homology (31% identity over a region of 101 amino acids) was found between CDDP-1 and SMRT (Silencing Mediator of Retinoid and Thyroid hormone action), a transcriptional co-repressor protein for retinoic acid receptor (NCBI). These findings led to the hypothesis that CDDP-1 might be an effector protein in retinoic acid signaling. To explore this, transient transfection assays of CDDP-1 using a retinoic acid response element (RARE) promoter-reporter were conducted in CV-1 cells. However, no consistent activation of this reporter by CDDP-1 was observed either in the presence or absence of retinoic acid receptor α and retinoid-X receptor α expression, and in the presence or absence of treatment with ATRA (data not shown).

To further explore the mechanism of CDDP-1 in promoting myelomonocytic differentiation, we performed a series of transient transfection experiments in P388T lymphoblastic cells using promoter-reporter constructs driven by several promoters that are active in myeloid cells including those for the myeloperoxidase, G-CSF receptor, M-CSF receptor, and IL-6 genes. However, none of these promoter-reporter constructs could be activated by CDDP-1 (data not shown). Since myeloperoxidase was upregulated in several instances of CDDP-1 overexpression in MPRO cells, that induction is likely not a direct result of CDDP-1 expression. Rather, it is more likely that myeloperoxidase is upregulated as part of a more general differentiation program that is induced

by CDDP-1 expression. The failure of CDDP-1 to activate the other promoters demonstrates that CDDP-1 is likely not a direct activator of myeloid gene expression.

CDDP-1 expression is also modulated in erythroid and mast cell differentiation. Northern analysis of a panel of hematopoietic cells (Fig. 6) found that CDDP-1 was expressed in a broad range of hematopoietic cells, being absent only in mature macrophage cell lines and a $CD8^+$ T cell line. Since the EML-C1 cell line can easily be induced toward erythroid and mast cell differentiation, the pattern of CDDP-1 expression was examined in time courses of differentiation of these two lineages. A Northern analysis of RNAs isolated over a time course of erythropoietin (EPO)-induced differentiation of EML-C1 cells along the erythroid lineage showed CDDP-1 mRNA to be upregulated prior to β-globin, after one day treatment in EPO. The highest level of expression occurred at day three. Then CDDP-1 was downregulated to basal levels as β-globin expression kept increasing until day seven of EPO treatment (Fig. 18). A more complete time course of dimethyl sulfoxide-induced differentiation of BB88 cells (initially examined in Fig. 6) revealed CDDP-1 to be induced by 10 hours post-induction, prior to induction of β-globin (Fig. 19). Northern analysis of RNAs isolated over a time course of differentiation of EML-C1 cells along the mast cell lineage showed CDDP-1 to be upregulated after one day of induction in WEHI-3 conditioned media (as a source for IL-3). High levels of CDDP-1 expression were maintained until 6 days of treatment (Fig. 20).

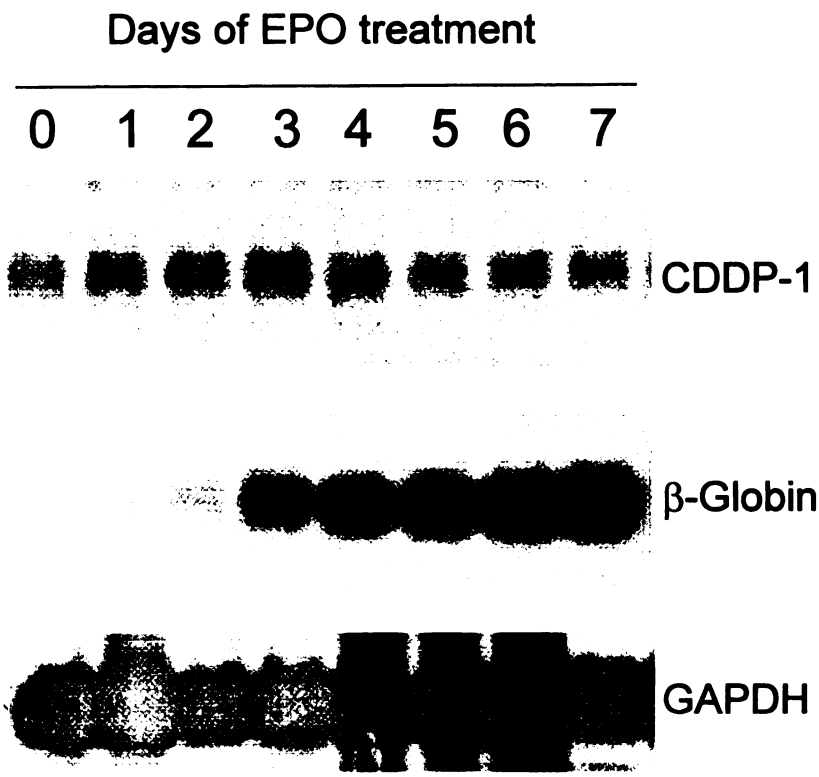


Figure 18. RNA was isolated from a time course of EPO-induced differentiation of EML-C1 cells. A northern blot was successively hybridized with probed for CDDP-1, β-globin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

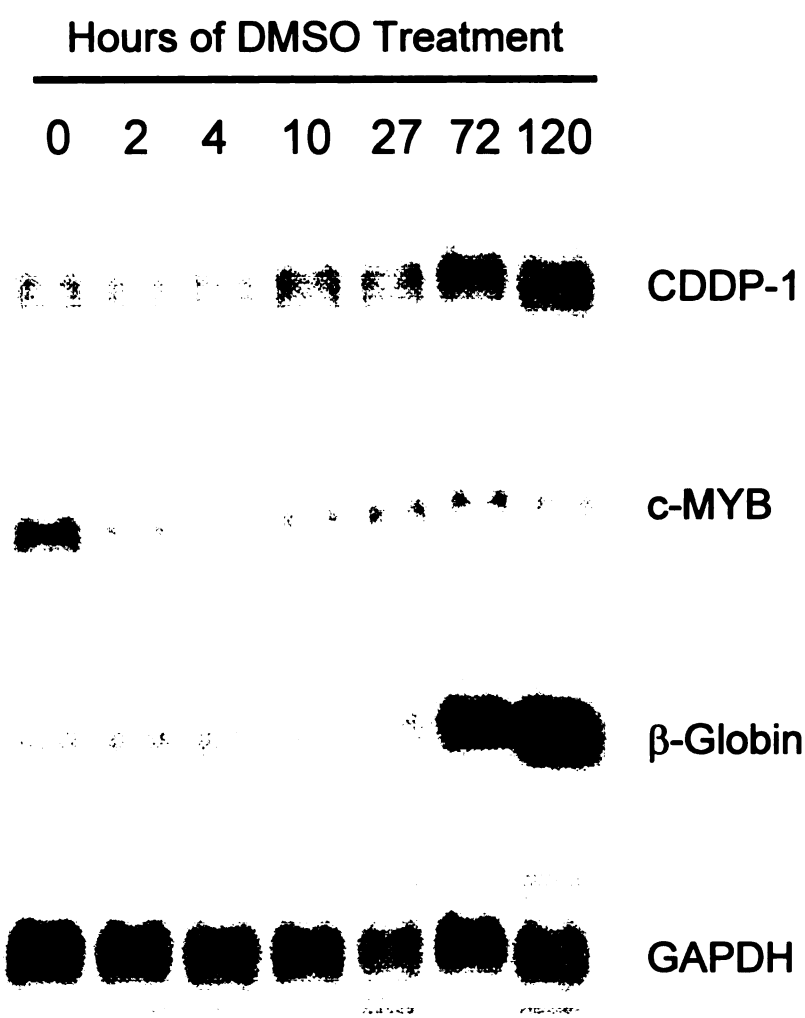


Figure 19. RNA was isolated from a time course of DMSO-induced erythroid differentiation of BB88 cells. A northern blot was successively hybridized with probes for CDDP-1, β-globin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

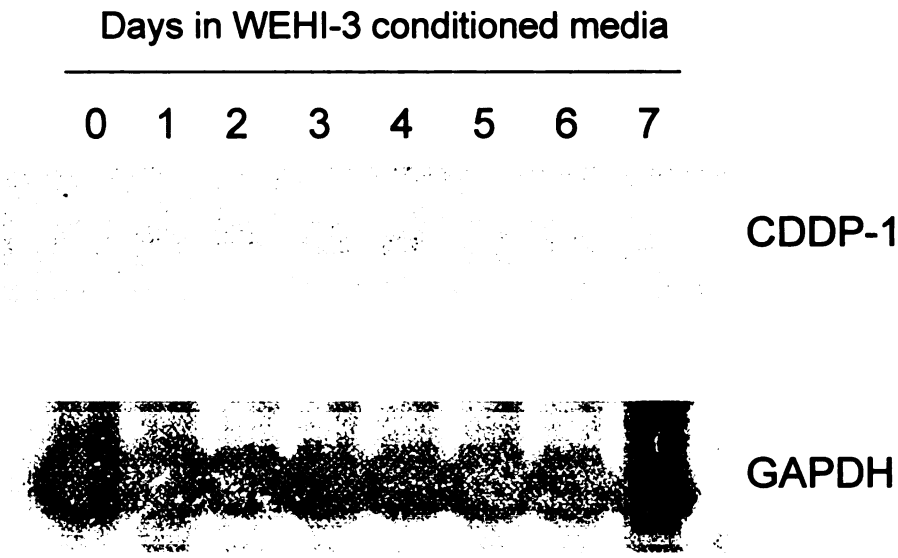


Figure 20. RNA was isolated from a time course of IL3-induced mast cell differentiation of EML-C1 cells. A northern blot was successively hybridized with probes for CDDP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). WEHI-3 conditioned medium provides a source for IL-3.

The EML-C1 cell line does not yield adequate numbers of lymphoid cells for Northern analysis. In order to examine differential expression of CDDP-1 in the course of B cell differentiation, RNAs were isolated from a panel of B cell lines representing several stages of differentiation: 70Z is a pre-B cell line that is positive for cytoplasmic IgM but negative for cell surface IgM; M12 is a B cell line negative for cell surface IgM; R1 and R2, are pre-B cell lines that are negative for surface, as well as being negative for cytoplasmic IgM heavy chain. Northern analysis revealed that while CDDP-1 is expressed in pre-B (70Z) as well as in B (M12) cells (Fig. 6), there may be differential expression in regard to neoplastic transformation (Fig. 21). R1 and R2, two v-H-ras-transformed pre-B cell lines (Schwartz et al., 1986), expressed a higher molecular weight form of CDDP-1 mRNA than that observed in the various myeloid cell lines, the M12 B cell line, and the 70Z/3 pre-B cell line (See Fig. 6). On the other hand, non-transformed clone 1 and clone 3 pre-B cell lines (Whitlock et al., 1983) did not express CDDP-1 mRNA (Fig. 21).

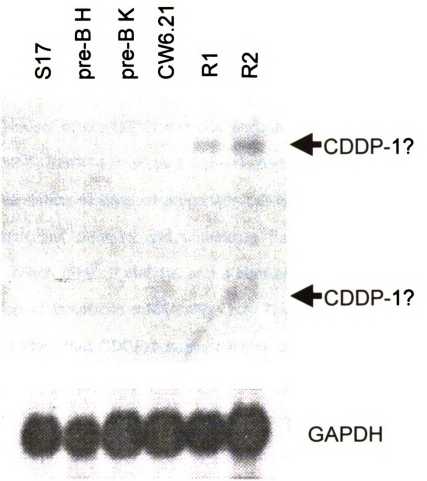


Figure 21. Two mRNA species in v-H-ras-transformed pre-B cell lines (R1 and R2) can be detected using CDDP-1 probe. Non-transformed control pre-B cell lines (pre-B H and pre-B K) do not express CDDP-1 mRNA. S17 and CW6.21 are stromal cell lines used to culture the pre-B cells.

DISCUSSION

C/EBPβ was overexpressed in 32D clone 3 myeloblasts and differential display RT-PCR was performed to identify genes induced by C/EBPβ overexpression. Among the four genes identified, two were endogenous retroviral elements, one encoded a cathepsin G, a primary granule protein of neutrophils and monocytes (Heusel et al., 1993), and one encoded a novel gene that we have named CDDP-1. CDDP-1 displays a biphasic pattern of expression in the granulocytic differentiation of myeloid progenitor cell lines: an early period of elevated expression just prior to and continuing through the induction of myeloperoxidase mRNA (Figs. 9 and 12) and a later second period coincident with the expression of lactoferrin mRNA (Fig. 10). This pattern of expression coupled with the finding that CDDP-1 is most highly expressed in adult bone marrow and fetal liver (Fig. 5) suggests a role in myelopoiesis. Indeed, the overexpression of CDDP-1 in MPRO promyelocytes promoted their differentiation toward both the neutrophil and monocyte lineages (Figs. 13, 14, 17).

The biochemical function of CDDP-1 remains unknown. Bioinformatic approaches have not been informative as the predicted protein structure of CDDP-1 bears little homology to proteins of known function. CDDP-1's largely cytoplasmic localization (Fig. 8) provides scant evidence of its molecular function other than eliminating the possibility of its being a transcription factor. CDDP-1 is upregulated in several cell lines where differentiation is induced by treatment with retinoic acid, such as EML-C1, MPRO, HL-60 and U937. Additionally, when

CDDP-1 is overexpressed in MPRO cells, it can facilitate their retinoic acid-induced differentiation into neutrophils (Fig. 16). This may simply be a reflection of CDDP-1 being one of many genes that are part of the process of myeloid differentiation that is induced in these cells by retinoic acid. On the other hand, CDDP-1 may have a functional relationship to retinoic acid signaling. A weak homology was observed between CDDP-1 and SMRT (Silencing Mediator of Retinoid and Thyroid receptor) (Chen and Evans, 1995). Transient transfection assays with a retinoic acid response element (βRE_2)-dependent promoter-reporter failed to find any enhancement of promoter activity by CDDP-1 (Data not shown) suggesting no direct involvement in retinoic acid dependent signaling.

C/EBPα-deficient mice are completely lacking in neutrophils and eosinophils (Zhang et al., 1997), while C/EBPε-deficient mice lack functional neutrophils and eosinophils (Yamanaka et al., 1997). The G-CSF and the IL-6 receptors have been proposed as critical genes for neutrophilic differentiation that are regulated by C/EBPα, but targeted gene disruption experiments have shown that mice lacking the G-CSF and IL-6 receptors are still capable of producing reduced numbers of mature neutrophils (Zhang et al., 1998). The targets of C/EBPα and C/EBPε that are responsible for the defects observed in animals that fail to express these transcription factors are incompletely identified. Representational difference analysis has been applied to the RNAs of fetal livers from wild type and PU.1 or C/EBPα knockout mice to identify eight novel genes regulated during myeloid differentiation (Iwama et al., 1998). As with the case of CDDP-1, the functions of these genes are unknown. The ability of CDDP-1 to

promote differentiation of MPRO cells suggests that CDDP-1 may be one of the critical genes in the differentiation of neutrophils that is regulated by C/EBPα and/or C/EBPε. Though originally isolated through overexpression of C/EBPβ, CDDP-1 expression is also upregulated by overexpression of C/EBPα, C/EBPδ, and C/EBPε (Fig. 2). Analysis of the sequence upstream of the translational start codon for CDDP-1 reveals several potential C/EBP binding sites (Fig. 4) suggesting that CDDP-1 may be a direct target of C/EBP regulation.

Interestingly, the overexpression of CDDP-1 in MPRO cells yielded both neutrophilic and monocytic differentiation (Figs. 13). This seems to be at odds with the reduced CDDP-1 expression observed upon monocytic differentiation of HL-60 cells (Fig. 11). Perhaps, the initial elevated expression of CDDP-1 prior to myeloperoxidase expression that was observed in 32 D clone 3 cells reflects an early role for CDDP-1 prior to neutrophil versus monocyte lineage commitment. The overexpression of CDDP-1 in a cell line with stem cell properties, such as EML-C1, may help to resolve this issue. For instance, CDDP-1-driven differentiation toward both lineages would strengthen the case for a role in both neutrophil and monocyte differentiation. The eventual production of CDDP-1-deficient mice may also help to resolve this issue by determining whether one or both lineages would be affected in such animals. The modulation of CDDP-1 expression in both erythroid (Figs. 18, 19) and mast cell (Fig. 20) differentiation, as well as the observation of a uniquely sized RNA species in transformed pre-B cells (Fig. 21) suggests roles for this gene in the differentiation of these other

lineages. Again, overexpression in EML-C1 and the development of CDDP-1-deficient animals will be helpful in elucidating this finding.

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Chapter 3

The C/EBP bZIP Domain Can Mediate Lipopolysaccharide Induction of the Proinflammatory Cytokines Interleukin-6 and Monocyte Chemoattractant Protein-1

Hsien-ming Hu, Qiang Tian, Mark Baer,

Chauncey J. Spooner, Simon C. Williams, Peter F. Johnson, and

Richard C. Schwartz

ABSTRACT

C/EBPα, β, and δ are all expressed by bone marrow-derived macrophages. Ectopic expression of any of these transcription factors is sufficient to confer lipopolysaccharide(LPS)-inducible expression of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) to a B lymphoblast cell line, which normally lacks C/EBP factors and does not display LPS induction of proinflammatory cytokines. Thus, the activities of C/EBPα, β, and δ are redundant in regard to expression of IL-6 and MCP-1. Surprisingly, the bZIP region of C/EBPβ which lacks any previously described activation domains, can also confer LPS-inducible expression of IL-6 and MCP-1 in stable transfectants. Transient transfections reveal that the bZIP regions of C/EBPβ C/EBPδ and, to a lesser extent, C/EBPα can activate the IL-6 promoter and augment its induction by LPS. Furthermore, the transdominant inhibitor, LIP, can activate expression from the IL-6 promoter. The ability of the C/EBPβ bZIP region to activate the IL-6 promoter in transient transfections is completely dependent upon an intact NF-κB binding site, supporting a model where the bZIP protein primarily functions to augment the activity of NF-κB. Replacement of the leucine zipper of C/EBPβ with that of GCN4 yields a chimeric protein that can dimerize and specifically bind to a C/EBP consensus sequence, but shows a markedly reduced ability to activate IL-6 and MCP-1 expression. These results implicate the leucine zipper domain in some function other than dimerization with known C/EBP family members, and

suggest that C/EBP redundancy in regulating cytokine expression may result from their highly related bZIP regions.

Note: This paper has been accepted for publication by *The Journal of Biological Chemistry*. In this paper, Hsien-ming Hu, a former graduate student in our lab made the original finding that the bZIP region of C/EBPβ, which lacks the transactivation domain, can still confer LPS induced expression of IL-6 and MCP-1 in stably transfected P388D1 cells. My work was to further confirm this finding and to explore the mechanism for this phenomenon using transient transfection assays. My contribution for this study was to establish and optimize the transient transfection systems in our lab and perform most of the transient transfection experiments used in this paper (with the exception of Fig. 8).

INTRODUCTION

CCAAT/enhancer binding protein (C/EBP) α, β, and δ are members of a family of basic region-leucine zipper (bZIP) transcription factors (reviewed in Johnson and Williams, 1994). These proteins dimerize through their leucine zippers and bind to a consensus DNA motif through their adjacent basic regions. C/EBPβ and C/EBPδ have been implicated in the regulation of proinflammatory cytokines as well as other gene products associated with macrophage activation and the acute phase response. For example, the promoter regions of the genes for interleukin-6 (IL-6), IL-1α, IL-1β, IL-8, tumor necrosis factor α, granulocyte-colony stimulating factor, nitric oxide synthase, and lysozyme (Akira et al., 1990; Furutani et al., 1986; Lowenstein et al., 1993; Natsuka et al., 1992; Shirakawa et al., 1993; Zhang et al., 1993.) contain C/EBP binding sites. Furthermore, C/EBPβ and C/EBPδ have both been shown to activate a reporter gene controlled by the IL-6 promoter in transient expression assays (Akira et al., 1990; Kinoshita et al., 1992). We have previously demonstrated that the stable expression of C/EBPβ in a murine B lymphoblast cell line is sufficient to confer lipopolysaccharide (LPS) inducibility of IL-6 and monocyte chemoattractant protein 1 (MCP-1) expression (Bretz et al., 1994).

There have been some reports demonstrating specificity of a particular C/EBP family member for a given promoter. One example is the rat *CYP2D5* gene, which encodes a cytochrome P450 protein. It is transactivated cooperatively by C/EBPβ and SP1, but not C/EBPα (Lee et al., 1994). The

specificity of this cooperativity is determined by both the leucine zipper and activation domains of C/EBPβ (Lee et al., 1997). Another case is promoter P1 of furin (Ayoubi et al., 1994). It is transactivated by C/EBPβ but not C/EBPα or C/EBPδ. Recently, it has been reported that C/EBPδ but not C/EBPβ, can transactivate the promoter for nerve growth factor (Colangelo et al., 1998). Conversely, C/EBPα, β and δ are all functional in a heterologous transgenic rescue assay for a *Drosophila* C/EBP mutant, *slow border cells* (Rorth, 1994). Our own studies have demonstrated redundancy in the abilities of C/EBPα, β and δ to support LPS induction of IL-6 and MCP-1 gene transcription (Hu et al., 1998).

A simple hypothesis for the redundancy of C/EBPs that we have observed is that only their highly homologous bZIP regions (Williams et al., 1991) are required for activation of the IL-6 and MCP-1 genes. Against this hypothesis stand results obtained with a truncated form of C/EBPβ that initiates at Met 132 and lacks activation domains. This protein, referred to as LIP, could not activate transcription and, in fact, inhibited C/EBPβ-mediated transcriptional activation of an artificial promoter derived from the DE-I site of the albumin gene (Descombes and Schibler, 1991). In this report, we have unexpectedly found that a truncated form of C/EBPβ lacking all known activation domains and consisting solely of the C/EBPβ bZIP region is capable of supporting LPS induction of IL-6 and MCP-1 expression. Furthermore, the transdominant inhibitor LIP, as well as a truncated form of C/EBPδ and, to a lesser extent, a truncated form of C/EBPα that similarly lack conventional activation domains have transcriptional activity on the IL-6

promoter. The transcriptional activity of both C/EBPβ and the C/EBPβ bZIP region is completely dependent on the presence of an intact NF-κB binding site in the IL-6 promoter, implying that these proteins primarily act by augmenting NF-κB activity. Replacement of the leucine zipper of C/EBPβ with that of GCN4 yields a chimeric protein that can dimerize and specifically bind to a C/EBP consensus sequence, but has reduced ability to activate IL-6 and MCP-1. Furthermore, the majority of both full length and truncated C/EBPβ exists as heterodimers with C/EBPγ (Ig/EBP), a C/EBP family member lacking any known activation domains. These results imply that the leucine zipper domain has a function beyond dimerization to known C/EBP family members, and that the basis for C/EBP redundancy in regulating cytokine expression resides in their highly related bZIP regions.

EXPERIMENTAL PROCEDURES

Cells and cell culture-P388 cells are murine B lymphoblasts (4) (American Type Culture Collection; CCL46). P388-Cβ cells and P388-Neo cells have been described previously by Hu et al. (1998). Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and 50 μM β-mercaptoethanol. Inductions were conducted with LPS derived from *Escherichia coli* serotype 055:B5 (Sigma) added to 10 μg/ml.

Transfections-Stable transfections were conducted with $2x10^6$ cells, 5 μg of DNA, and 10 μl of DMRIE-C reagent (Life Technologies) in 1.2 ml of Opti-MEM I medium (Life Technologies). Cells were incubated in the transfection mixture for 16 h followed by addition of 2.8 ml of standard growth medium. After 24 h, the medium was replaced with standard growth medium supplemented with G418 (Life Technologies) at 0.67 mg/ml.

Transient transfections were conducted with $2x10^6$ cells, 4 μg of DNA, and 8 μl of DMRIE-C reagent (Life Technologies) in 1.2 ml of Opti-MEM I medium (Life Technologies). The DNA was comprised of either 1μg of IL-6 promoter-reporter or albumin DE-I promoter-reporter, 1μg of SV40 early promoter-reporter, 0.5 μg of C/EBP expression vector, and pMEX plasmid to total 4 μg. Cells were incubated in the transfection mixture for 5 h followed by addition of RPMI 1640 medium supplemented to 15% with fetal calf serum. After 24 h, the medium of certain transfections was supplemented with 10 μg/ml LPS. After 4 h in the presence or absence of LPS, transfected cells were harvested, lyzed, and

analyzed for luciferase activity by using the Luciferase Reporter Gene Assay Kit (Boehringer Mannheim) and for β-galactosidase activity by using the Luminescent β-Galactosidase Genetic Reporter System II (Clontech).

Expression vectors and promoter-reporters-For stable transfections, C/EBPs were expressed from pZIP-NEO SV(X)1 constructs (18). C/EBP sequences were inserted into the BamHI site of the vector. Inserted sequences were transcribed from the Moloney murine leukemia virus promoter and the gene conferring G418-resistance was expressed from a subgenomic splicing product from the same promoter. For transient transfections, C/EBPs were expressed from pMEX (Williams et al., 1991), which utilizes the Moloney murine sarcoma virus promoter. The construction of C/EBP deletions and C/EBPβ:G_{LZ} have been described previously (Williams et al., 1991).

The IL-6 promoter-reporter consists of the murine IL-6 promoter (Tanabe et al., 1988) (-250 to +1) inserted into the luciferase vector, pXP2 (Nordeen, 1988). The IL-6 promoter-reporter with a mutated NF-κB site replaces GGGATTTTCCC with AAGATTTTCTT. The albumin DE-I promoter-reporter is $(DEI)_4$(-35alb)LUC (Williams et al., 1995) which is derived from pXP2 (Nordeen, 1988) and contains four copies of the DE-I element upstream of the albumin minimal promoter. The SV40 early promoter-reporter is a commercial product, p□gal-Control (Clontech), where the SV40 early promoter and enhancer sequences are cloned upstream and downstream, respectively, of the *lacZ gene.*

RNA isolation and analysis-Total RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's directions. RNA's were

electrophoresed through 1% agarose/formaldehyde gels. Transfers to membranes were hybridized and washed to a stringency of 0.1x SSPE at 65°C. Hybridization probes were prepared with a random priming kit (Life Technologies) with the incorporation of 5'-[α-^{32}P]dATP (3000 Ci/mmol; DuPont-New England Nuclear). The IL-6 probe was a 0.65 kb murine cDNA (from N. Jenkins and N. Copeland, National Cancer Institute-Frederick Cancer Research and Development Center). The MCP-1 probe was a 0.58 kb murine cDNA (Rollins et al., 1988). The glyceraldehyde-3-phospahate dehydrogenase (GAPDH) probe was a 1.3 kb rat cDNA (Fort et al., 1985).

Western analysis-Nuclear extracts were prepared as described below. The extracts (50 µg) were adjusted to 1x Laemmli sample buffer (Laemmli, 1970) and processed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis. The gel was transferred to a Protran membrane (Schleicher and Schuell), and antigen-antibody complexes were visualized with the Enhanced Chemiluminescence Kit (Amersham).

Electrophoretic mobility shift assay (EMSA)-Nuclear extracts were prepared as follows. Cells were washed in phosphate-buffered saline and lysed in 15 mM KCl, 10 mM HEPES [pH 7.6], 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% [vol/vol] NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 µg/ml leupeptin, 5 µg/ml antipain, and 5 µg/ml aprotinin for 10 min on ice. Nuclei were pelleted by centrifugation at 14,000 x *g* for 20 sec at 4°C. Proteins were extracted from nuclei by incubation at 4°C with vigorous vortexing in buffer C (420 mM NaCl, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 25% [vol/vol] glycerol, 1

mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin). Nuclear debris was pelleted by centrifugation at 14,000 x *g* for 15 min at 4°C and the supernatant extract was collected and stored at -70°C.

The EMSA probe was a double-stranded oligonucleotide containing an optimal C/EBP binding site (5'-GATCCTAGATATCCCTGATTGCGCAATAGG CTCAAAGCTG-3' annealed with 5'-AATTCAGCTTTGAGCCTATTGCGCAATCA GGGATATCTAG-3') labeled with the incorporation of 5'-[α-32P]dATP (3000 Ci/mmol; DuPont-New England Nuclear) and Klenow DNA polymerase. A probe containing a CTF/NF-1 biding site (Landshulz et al, 1988) (5'-GATCCTT TGGCATGCTGCCAATATG-3' annealed with 5'-AATTCATATTGGCAGCATG CCAAAG-3') was used as a nonspecific competitor in some assays. Underlined sequences correspond to the binding motifs of the specified transcription factors.

DNA binding reactions were performed at room temperature in a 25 μl reaction mixture containing 6μl of nuclear extract (1mg/ml in buffer C) and 5 μl of 5x binding buffer (20% [wt/vol] Ficoll, 50 mM HEPES [pH 7.9], 5mM EDTA, 5 mM dithiothreitol). The remainder of the reaction mixture contained 1 μg poly(dI-dC), 1.25 ng of probe, bromophenol blue to a final concentration of 0.06% [wt/vol], and water to volume. For supershifts, nuclear extracts were preincubated with antibodies for 30 min at 4°C prior to the binding reaction. Samples were electrophoresed through 5.5% polyacrylamide gels in 1x TBE (90 mM Tris base, 90 mM boric acid, 0.5 mM EDTA) at 160 V.

Antibodies-Rabbit anti-C/EBPβ specific to the carboxyl terminus (product C-19) and normal rabbit IgG were purchased from Santa Cruz Biotechnology. Rabbit anti-C/EBPβ specific to the amino terminus has been described (Williams et al., 1991). Rabbit anti-C/EBPγ (Ig/EBP) was prepared against a synthetic peptide corresponding to the carboxyl terminus of C/EBPγ and will be described elsewhere (S. Parkin, M. B., and P. F. J., unpublished).

RESULTS

Ectopic expression of the bZIP domain of C/EBPβ---- We previously found that C/EBPα, β and δ were all effective in supporting the LPS-induced transcription of the genes encoding IL-6 and MCP-1 (Hu et al., 1998). To test whether this redundancy was based on the bZIP domain which is highly conserved among these C/EBP family members (Williams et al, 1991), we examined the expression of a truncated form of C/EBPβ (amino acids 192-276; C/EBP$\beta_{192\text{-}276}$) that lacks all conventional activation and regulatory domains and simply consists of the bZIP domain of C/EBPβ (Williams et al., 1995) (Fig. 1). It was expected that the activity of C/EBP$\beta_{192\text{-}276}$ would be similar to that of LIP, which initiates at Met 132 and lacks transcriptional activation domains (Descombes and Schibler, 1991) (Fig. 1).

We performed stable transfections of P388 cells with a murine retroviral vector expressing C/EBP$\beta_{192\text{-}276}$, and compared those transfectants to P388-Cβ cells that had been transfected with a vector expressing C/EBPβ as well as a control population transfected with the same vector lacking an expressed insert (P388-Neo) (Bretz et al., 1994; Hu et al., 1998). P388 is a murine B lymphoblastic cell line (Bauer et al., 1986) that lacks C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε expression and has been a useful system for analyzing C/EBP protein function (Bretz et al., 1994; Hu et al., 1998). The population of cells transfected for C/EBP$\beta_{192\text{-}276}$ expression was designated P388-C$\beta_{192\text{-}276}$.

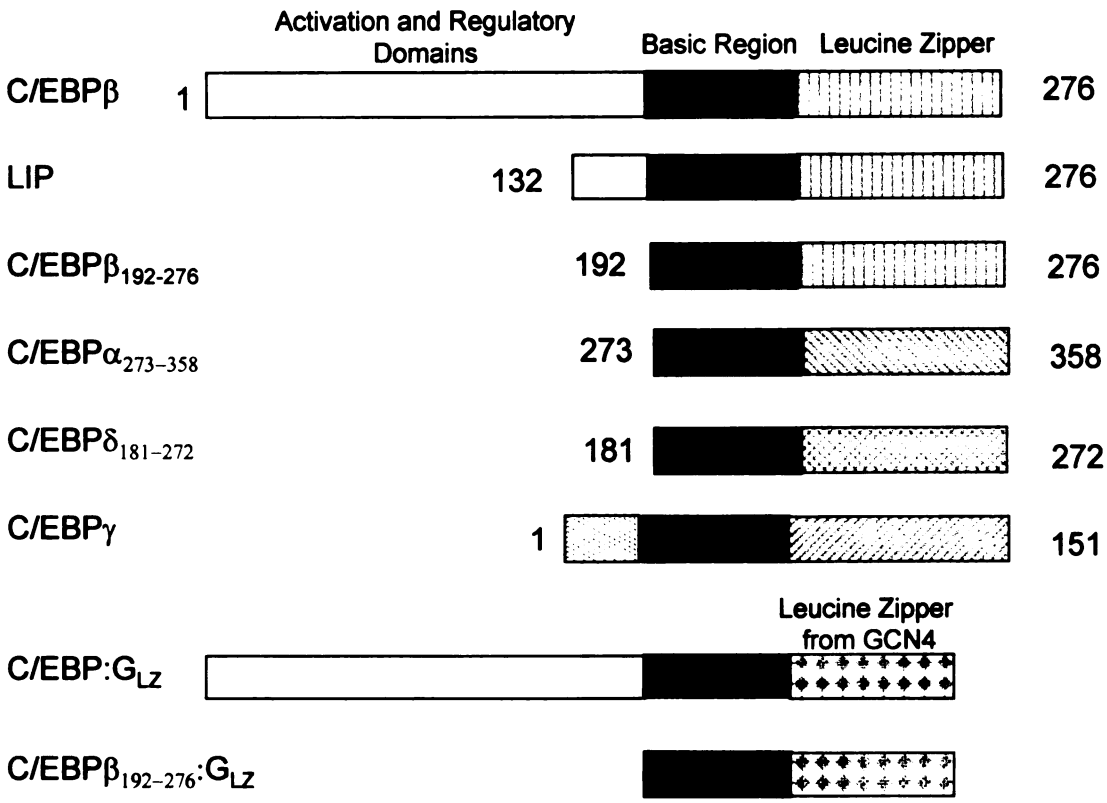

FIG. 1. Structures of the various altered C/EBP isoforms used in the studies described in this paper.

EMSA of nuclear extracts from transfected populations, as well as western blot analyses, verified proper expression of stably transfected C/EBPβ genes. In comparison to nuclear extracts from P388-Neo, nuclear extracts from P388-Cβ and P388-$C\beta_{192\text{-}276}$ yielded supershifted protein-DNA complexes upon incubation with an antibody specific for C/EBPβ (Fig. 2A, lanes 5, 6, and 9). The EMSA species that gave rise to the supershifts were also evident in the samples incubated with normal IgG (Fig. 2A, lanes 4 and 7). As expected, the C/EBPβ-specific EMSA species from P388-$C\beta_{192\text{-}276}$ cells were of greater mobility than those of P388-Cβ cells reflecting their truncated structure. Additionally, the C/EBP$\beta_{192\text{-}276}$ EMSA species could only be supershifted with antibody specific to the carboxyl-terminus of C/EBPβ (Fig. 2A, lanes 8 and 9), while EMSA species of intact C/EBPβ could be supershifted by both amino- and carboxyl-terminus specific antibodies (Fig. 2A, lanes 5 and 6).

Western analysis of nuclear extracts from transfected populations examined actual levels of protein expression of C/EBPβ and P388-$C\beta_{192\text{-}276}$ (Fig. 2B). The level of C/EBP$\beta_{192\text{-}276}$ expression in transfected cells was much lower than that of C/EBPβ. Interestingly, although the level of protein expression for P388-$C\beta_{192\text{-}276}$ was much lower than that of intact C/EBPβ, its binding activity in the EMSA was quite high. We note that C/EBP$\beta_{192\text{-}276}$ has been reported to have enhanced affinity for its binding site (Williams et al., 1995).

To ensure that the supershifted species observed in Figure 2A specifically bound to the C/EBP binding site, competitions were performed with the addition

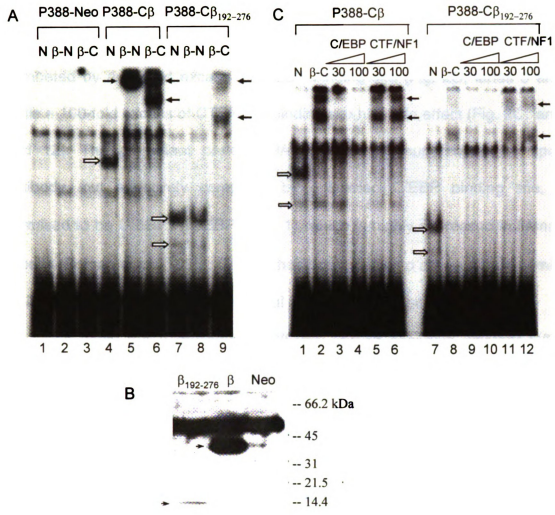

FIG.2. Ectopic expression of C/EBPβ and C/EBP$\beta_{192\text{-}276}$. (A) EMSA of nuclear extracts of P388-Neo, P388-Cβ and P388-C$b_{192\text{-}276}$ cells. Cell line nomenclature is explained in *Results*. Binding reactions included normal rabbit IgG (N), amino-terminal-specific C/EBPβ antibody (β-N), or carboxyl-terminal-specific C/EBPβ antibody (β-C). The positions of C/EBP:DNA complexes are indicated by open arrows and C/EBP-specific antibody supershift species are indicated by solid arrows. (B) Western analysis of C/EBP$\beta_{192\text{-}276}$ ($\beta_{192\text{-}276}$) compared to C/EBPβ (β) in the P388 transfectants. Control P388-Neo (Neo) cells are included as a control. Arrows mark the position of the C/EBP proteins. The proteins were detected with a carboxyl-terminal-specific C/EBPβ antibody. The positions of protein standards are noted. (C) EMSA of nuclear extracts of P388-Cβ and P388-C$\beta_{192\text{-}276}$ cells in the presence of unlabeled competing oligonucleotide binding sites. Binding reactions are as described for panel A except some binding reactions, in addition to specific antibody, included 30-fold and 100-fold excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides.

supershifted samples (Fig. 2C). All of the supershifted protein-DNA complexes observed upon incubation with C/EBPβ-specific antibody were effectively competed by a 30-fold excess of C/EBP binding site (Fig. 2C, lanes 3 and 9) while a 100-fold excess of CTF/NF-1 binding site had little effect (Fig. 2C, lanes 6 and 12). The prominent protein-DNA complex not supershifted by specific antibody, but effectively competed by unlabeled C/EBP binding site, was surmised to be C/EBPγ (Ig/EBP) (Fig. 1), which is highly expressed in immature B cells (Roman et al., 1990). C/EBPγ has been shown to act as a transdominant inhibitor of C/EBP family transcriptional activators (Cooper et al., 1995).

To verify the expression of C/EBPγ and to ascertain whether C/EBPγ might be forming heterodimers with either C/EBPβ or C/EBP$\beta_{192\text{-}276}$, EMSA was performed in the presence of antibodies specific for C/EBPβ and antibodies specific for C/EBPγ (Fig. 3). Nuclear extracts from P388-Neo cells clearly showed a C/EBPγ:DNA complex that could be supershifted with C/EBPγ-specific antibody (Fig. 3, lanes 1 and 2). In nuclear extracts from P388-Cβ cells, a single C/EBPβ:DNA complex of lower mobility than that for C/EBPγ was observed and it could be supershifted both by antibodies specific to C/EBPβ and C/EBPγ (Fig. 3, lanes 4, 5 and 6). Analysis of extracts from P388-C$\beta_{192\text{-}276}$ cells found two C/EBPβ:DNA complexes, both more rapidly migrating than that for C/EBPγ (Fig. 3, lane 7). The more abundant of the two C/EBPβ:DNA complexes could again be supershifted by both C/EBPβ- and C/EBPγ-specific antibodies (Fig. 3, lanes 8 and 9). Surprisingly, the majority of both C/EBPβ and C/EBP$\beta_{192\text{-}276}$ was found to

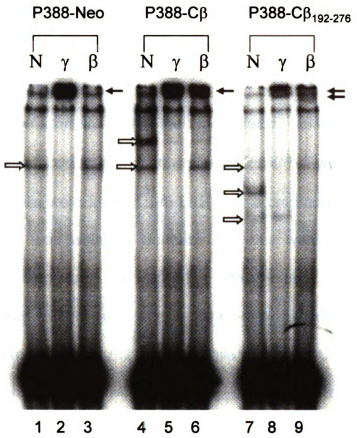

FIG. 3. Ectopically expressed C/EBPβ and C/EBP$\beta_{192-276}$ form heterodimers with C/EBPγ (Ig/EBP). An EMSA was performed upon nuclear extracts of P388-Neo, P388-Cβ and P388-C$\beta_{192-276}$ cells. Binding reactions included normal rabbit IgG (N), carboxyl-terminal-specific C/EBPβ antibody (β), or C/EBPγ-specific antibody (γ). The positions of C/EBP:DNA complexes are indicated by open arrows and C/EBP-specific antibody supershift species are indicated by solid arrows.

be in heterodimers with C/EBPγ (Fig. 3 and S. Parkin, M. B., and P. F. J., unpublished data). Since we had previously shown that ectopic expression of C/EBPβ was sufficient to confer LPS-inducibility of IL-6 and MCP-1 to P388 cells (Bretz et al., 1994), it is clear that C/EBPγ is not strongly inhibitory to C/EBPβ activity on the IL-6 promoter.

A truncated form of C/EBPβ lacking activation domains retains the ability to support LPS-induced transcription of IL-6 and MCP-1 genes---- P388-Cβ and P388-$C\beta_{192\text{-}276}$ cells were treated with LPS over a time course of 0, 2, 4, 8, and 24 h, and RNA was isolated. Northern analyses were performed to detect transcripts encoding IL-6 and MCP-1. Unexpectedly, P388-$C\beta_{192\text{-}276}$ cells behaved similarly to P388-Cβ cells in their ability to induce IL-6 and MCP-1 transcription in response to LPS (Fig. 4). While IL-6 and MCP-1 mRNAs were not induced to as high a level with C/EBP$\beta_{192\text{-}276}$ as with intact C/EBPβ and the kinetics of their expression were somewhat altered, the activity of a truncated form of C/EBPβ lacking all known activation domains is surprising. The level of C/EBP$\beta_{192\text{-}276}$ expression in P388-$C\beta_{192\text{-}276}$ cells is much lower than that of C/EBPβ in P388-Cβ cells (Fig. 2B) making its ability to support expression of IL-6 and MCP-1 all the more remarkable. A second population of P388 cells independently transfected for expression of C/EBP$\beta_{192\text{-}276}$ behaved similarly (data not shown).

A chimeric form of C/EBPβ with a heterologous leucine zipper has no activity in stable transfectants---Since C/EBP$\beta_{192\text{-}276}$ retains only the DNA binding

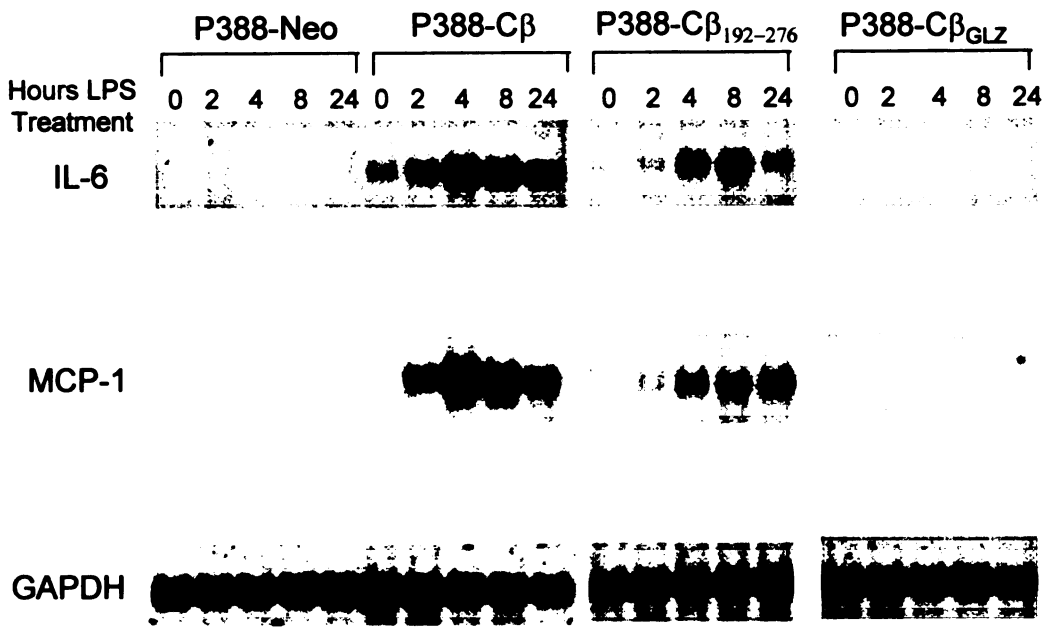

FIG. 4. Transfectants of P388 that stably express C/EBPβ (P388-Cβ) and C/EBPβ$_{192-276}$ (P388-Cβ$_{192-276}$) are stimulated by LPS to produce IL-6 and MCP-1 mRNAs, while cells that express C/EBPβ:G$_{LZ}$ (P388-Cβ$_{GLZ}$) are not induced to produce these mRNAs. Northern analyses of IL-6 and MCP-1 expression in P388 transfectants. RNA was isolated over time courses of LPS treatment as indicated. Twenty micrograms of RNA analyzed on northern blots that were hybridized in parallel to probes for IL-6, MCP-1, and GAPDH.

and leucine zipper domains of C/EBPβ yet is still active on the IL-6 promoter, we decided to examine whether the leucine zipper domain contains determinants for activation other than those necessary for homo-dimerization. To that end, we tested a chimeric C/EBPβ protein in which the C/EBPβ leucine zipper was replaced with that of yeast GCN4 (C/EBPβ:G_{LZ}) (Williams et al., 1991) by stable expression in P388 cells (P388-$C\beta_{GLZ}$) (Fig. 1). C/EBPβ:G_{LZ} retains wild type amino terminal activation and regulatory domains as well as the DNA binding domain, and has previously been shown to activate transcription from an albumin DE-I site-driven reporter (Williams et al., 1995). We verified the ability of C/EBPβ:G_{LZ} to activate a DE-I promoter in P388 cells (data not shown). In contrast to that result, P388-$C\beta_{GLZ}$ cells did not induce IL-6 or MCP-1 mRNA in response to LPS (Figure 4).

To verify that C/EBPβ:G_{LZ} was properly expressed in P388-$C\beta_{GLZ}$ cells, both EMSA and western blot analyses were performed. While a C/EBPβ:G_{LZ} protein-DNA complex was not obvious in a simple EMSA, the addition of C/EBPβ-specific antibodies revealed a supershifted protein-DNA complex with amino-terminal specific but not carboxyl-terminus specific antibodies (Fig. 5A, lanes 8 and 9). This result is consistent with replacement of the leucine zipper at the carboxyl-terminus of the C/EBPβ protein. These data also demonstrate that C/EBPβ:G_{LZ} retains the capacity to dimerize and bind to the optimal C/EBP binding site. Competition experiments were performed with unlabeled C/EBP binding site and unlabeled CTF/NF-1 binding site to verify the specificity of C/EBPβ:G_{LZ} binding (Fig. 5C). The supershifted protein-DNA complexes were

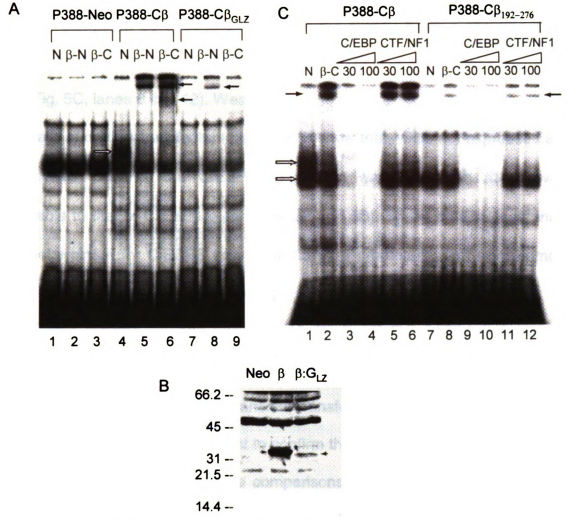

FIG. 5. Ectopic expression of C/EBPβ and C/EBPβ:G_{LZ}. (A) EMSA of nuclear extracts of P388-Neo, P388-Cβ and P388-Cβ_{GLZ} cells. Cell line nomenclature is explained in *Results*. Binding reactions included normal rabbit IgG (N), amino-terminal-specific C/EBPβ antibody (β-N), or carboxyl-terminal-specific C/EBPβ antibody (β-C). The positions of C/EBP:DNA complexes are indicated by open arrows and C/EBP-specific antibody supershift species are indicated by solid arrows. The C/EBPβ:G_{LZ}:DNA complex is not observable. (B) Western analysis of C/EBPβ:G_{LZ} (b:G_{LZ}) compared to that of C/EBPb (β) in the P388 transfectants. Control P388-Neo (Neo) cells are included as a control. Arrows mark the position of the C/EBP proteins. The proteins were detected with an amino-terminal-specific C/EBPβ antibody. The positions of protein standards are noted. (C) EMSA of nuclear extracts of P388-Cβ and P388-Cb_{GLZ} cells in the presence of unlabeled competing oligonucleotide binding sites. Binding reactions are as described for panel A except some binding reactions, in addition to specific antibody, included 30-fold and 100-fold excess quantities of unlabeled C/EBP or CTF/NF1 binding oligonucleotides.

effectively competed by a 30-fold excess of the C/EBP binding site (Fig. 5C, lanes 3 and 9), while a 100-fold excess of the CTF/NF-1 binding site had little effect (Fig. 5C, lanes 6 and 12). Western analyses of nuclear extracts from P388-Cβ_{GLZ} cells and other transfectant populations show that, while not expressed as efficiently as C/EBPβ, the C/EBPβ:G_{LZ} protein was expressed at a level comparable to that of C/EBP$\beta_{192\text{-}276}$ (Figs. 2B and 5B). These data suggest that some determinant for C/EBPβ activity on the IL-6 promoter, apart from homodimerization, is located in the leucine zipper.

Both C/EBP$\beta_{192\text{-}276}$ and LIP can activate the IL-6 promoter in transient transfections---We further examined the dispensability of the amino terminal activation domains of C/EBPβ in transient transfections with an IL-6 promoter-driven luciferase reporter. We sought to confirm the surprising results obtained in stable transfections and to facilitate comparisons of C/EBPβ with its structural variants and analogous truncations of other C/EBP family members. P388 cells were cotransfected with an IL-6 promoter-luciferase reporter and expression vectors for C/EBPβ, C/EBP$\beta_{192\text{-}276}$, or LIP. These transfections were carried out with and without LPS treatment. As with the endogenous IL-6 promoter in stably transfected cells, both C/EBPβ and C/EBP$\beta_{192\text{-}276}$, and additionally LIP, were capable of activating the IL-6 promoter-reporter to varying degrees in transient transfections (Fig. 6). While LPS treatment of C/EBPβ transfectants induced luciferase expression by a mean value of 12-fold over an untreated, "reporter-only" control, LPS-treatment of C/EBP$\beta_{192\text{-}276}$ and LIP transfectants induced luciferase expression 6.2-fold and 8.3-fold, respectively. LPS stimulation alone

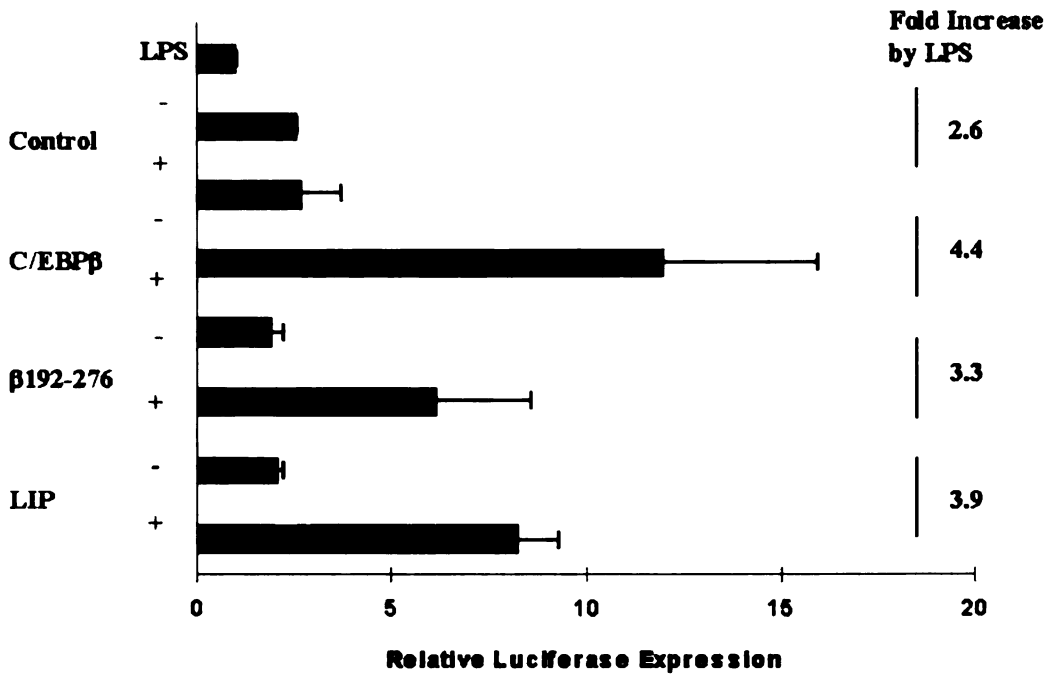

FIG. 6. C/EBP$\beta_{192\text{-}276}$ (β192-276) and LIP, although lacking activation domains, can activate the IL-6 promoter in transient transfections of P388 cells. Transient transfections were carried in out duplicate with and without LPS treatment. Luminometer values were normalized for expression from a cotransfected SV40 early promoter-β-galactosidase reporter. These values were then normalized to a relative value of 1 for the cells not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean of 3 experiments with their standard error.

(without transfection of a C/EBP expression vector) produced a mean value of only 2.6-fold over the control value. Interestingly, the transient transfections show C/EBPs to generally elevate the level of transcription rather than mediate LPS induction; LPS induction of luciferase expression occurs to a similar extent in either the presence or absence of C/EBP expression (between 2.6 and 4.4-fold [Figs. 6 and 8]). This is in contrast to stable transfection experiments where LPS stimulation fails to induce IL-6 mRNA expression in the absence of C/EBP expression (see P388-Neo in Fig. 4). The greater contribution of C/EBP expression to the LPS induction of IL-6 in stable transfection experiments may be a reflection of the more complete endogenous IL-6 promoter, or the more repressed basal activity of the gene in a native chromatin environment.

We previously showed that LPS stimulation induces NF-κB activity in P388 cells (Hu et al., 1998). C/EBPβ and NF-κB can synergistically activate the IL-6 promoter requiring the cognate binding sites of both factors (Matsuaka et al., 1993). If this synergy is largely based on the capacity of the bZIP domain by itself to modify NF-κB activity, then the activity of C/EBP$\beta_{192\text{-}276}$ on the IL-6 promoter should be dependent upon NF-κB. To test this notion, we repeated the transient transfection of expression vectors for C/EBPβ and C/EBP$\beta_{192\text{-}276}$ with an IL-6 promoter-reporter containing a mutated NF-κB binding site (Fig. 7). Not only was the promoter no longer LPS-responsive in the presence of either C/EBPβ and C/EBP$\beta_{192\text{-}276}$ expression, but C/EBPβ and C/EBP$\beta_{192\text{-}276}$ showed a complete lack of activity in the absence of a functional NF-κB binding site. Presumably, the

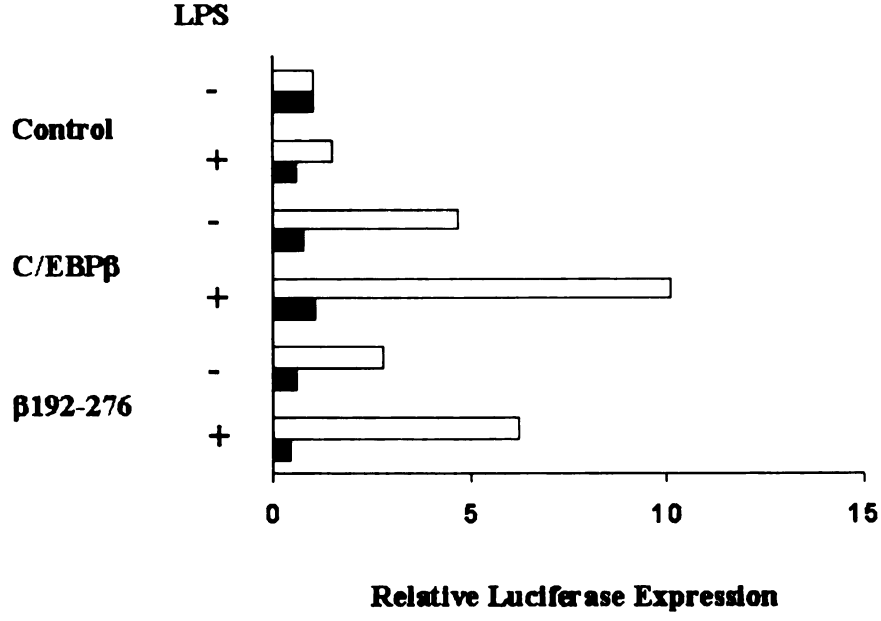

FIG. 7. C/EBPβ and C/EBP$\beta_{192-276}$ (β192-276) fail to activate the IL-6 promoter-reporter in the absence of a functional NF-kB binding site. Transient transfections were carried in out duplicate with and without LPS treatment. Luminometer values were normalized for expression from a cotransfected SV40 early promoter-β-galactosidase reporter. These values were then normalized to a relative value of 1 for the cells not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean values of one representative experiment. Open bars are values obtained with a wild type IL-6 promoter-reporter and solid bars are values obtained with a IL-6 promoter-reporter containing a mutated NF-κB site.

activity of C/EBP isoforms on the wild type IL-6 promoter in the absence of LPS stimulation (Figs. 6, 7, and 8) reflects a low level of constitutive NF-κB activity.

The expression of both C/EBPβ and C/EBP$\beta_{192\text{-}276}$ in P388 cells as heterodimers with C/EBPγ (Fig. 3) presents the possibility that C/EBPγ as a heterodimer with other C/EBP family members is a critical factor in activation of the IL-6 promoter. However, cotransfection of an expression vector for C/EBPγ with one for C/EBP$\beta_{192\text{-}276}$ did not augment the activity of an expression vector for C/EBP$\beta_{192\text{-}276}$ alone and was, in fact, mildly inhibitory both with and without LPS stimulation (Fig. 9). Thus, it is unlikely that C/EBPγ is responsible for the observed activity.

Other investigators may not have observed significant activity of C/EBP$\beta_{192\text{-}276}$ and LIP because they were tested on artificial promoter-reporter constructs based on the DE-I site of the albumin promoter and were thus solely C/EBP-dependent (Descombes and Schibler, 1991; Williams et al., 1995). We performed transient transfections in P388 cells with a multimeric albumin DE-I site promoter-reporter and found that C/EBP$\beta_{192\text{-}276}$ and LIP are, in fact, inactive on the simpler DE-I promoter, both in the presence and absence of LPS stimulation (Fig. 10); presumably their activity on the IL-6 promoter is dependent on interactions with other transcription factors including NF-κB that are available on the more complex promoter.

A truncated form of C/EBPδ analogous to C/EBP$\beta_{192\text{-}276}$ is capable of activating the IL-6 promoter---We previously showed that C/EBPα, β, and δ have virtually redundant activities in regard to the IL-6 promoter (Hu et al., 1998).

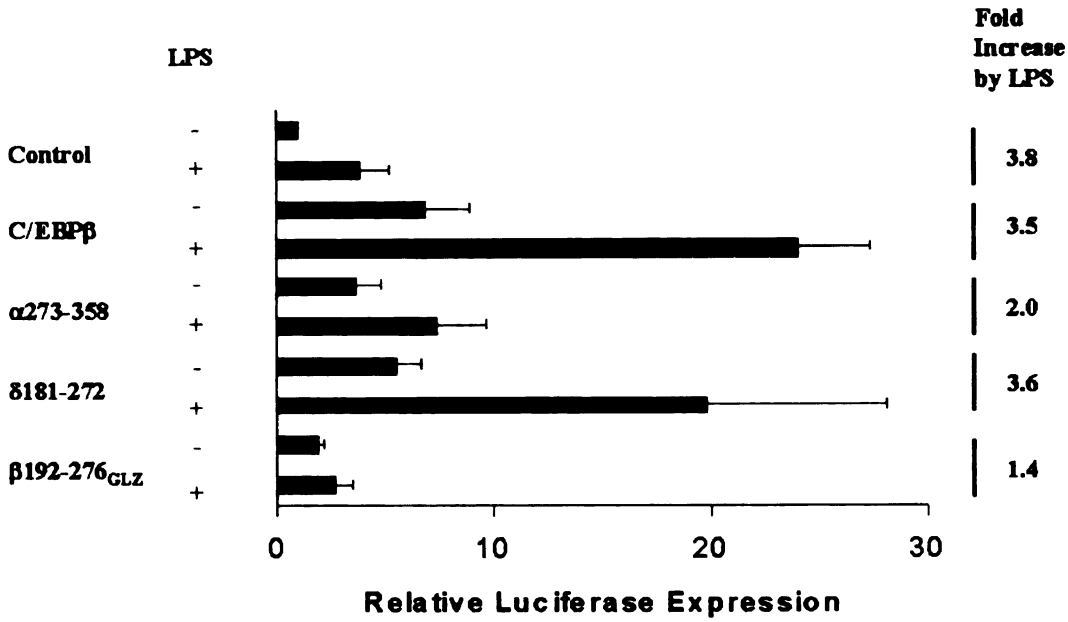

FIG. 8. C/EBP$\delta_{181\text{-}272}$ (δ181-272), although lacking an activation domain, can support the LPS induced activation of the IL-6 promoter in transient transfections of P388 cells. C/EBP$\alpha_{273\text{-}358}$ (α273-358) has modest activity in comparison to C/EBPβ. C/EBP$\beta_{192\text{-}276}$:G_{LZ} (β192-276$_{GLZ}$) is inactive. Transient transfections were carried in out duplicate with and without LPS treatment. Luminometer values were normalized for expression from a cotransfected SV40 early promoter-β-galactosidase reporter. These values were then normalized to a relative value of 1 for the cells not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean of 3 experiments with their standard error.

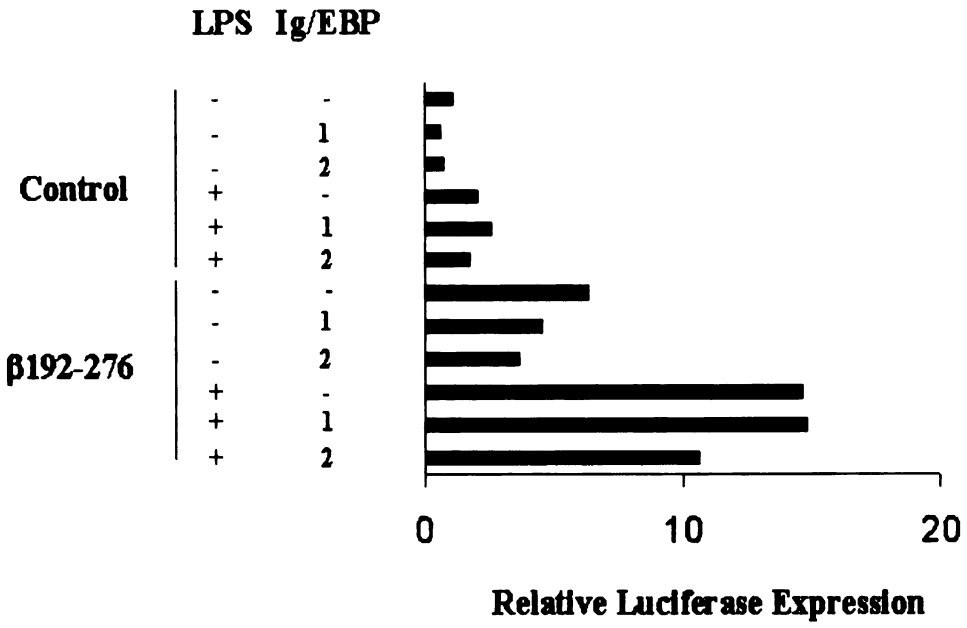

FIG. 9. C/EBPγ (Ig/EBP) is mildly inhibitory to C/EBP$\beta_{192-276}$ activity on the IL-6 promoter-reporter. Transient transfections were carried in out duplicate with and without LPS treatment. Transfected cells received 0 mg (Control) or 1 mg of C/EBP$\beta_{192-276}$ expression vector (β192-276), and 0, 1, or 2 mg of C/EBPγ expression vector as indicated. Luminometer values were normalized for expression from a cotransfected SV40 early promoter-b-galactosidase reporter. These values were then normalized to a relative value of 1 for the cells not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean values of one representative experiment.

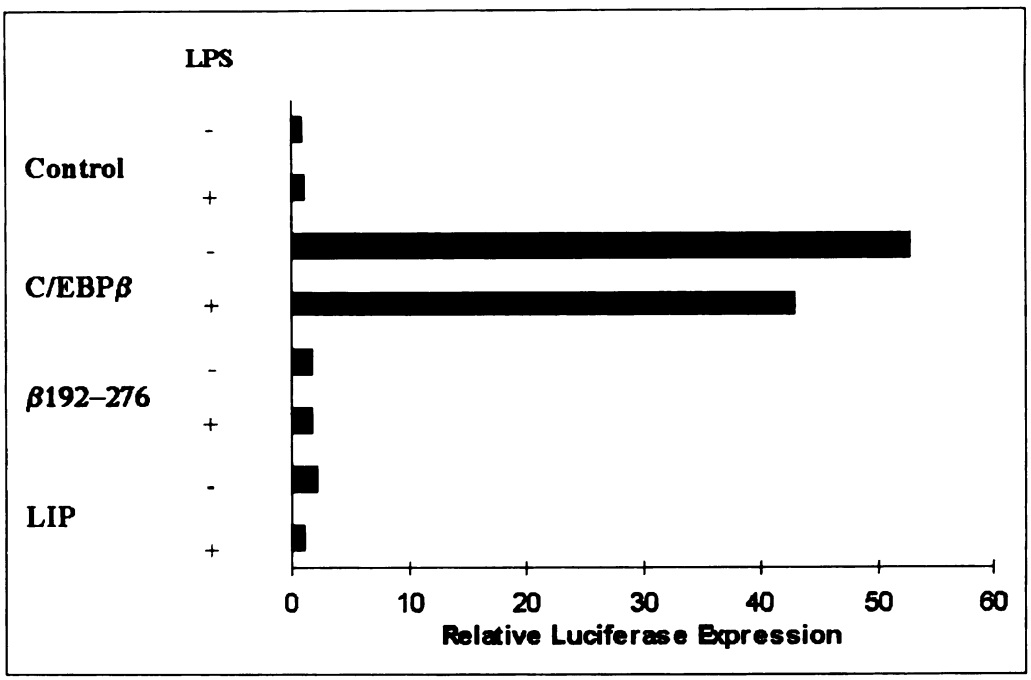

Fig.10. C/EBP$\beta_{192-276}$ (β192-276) and LIP fail to activate an albumin DEI site-reporter in transient transfections of P388 cells with and without LPS stimulation. Transient transfections were carried in out duplicate with and without LPS treatment. Luminometer values were normalized for expression from a cotransfected SV40 early promoter-β-galactosidase reporter. These values were then normalized to a relative value of 1 for the cells not receiving a C/EBP expression vector and untreated with LPS. The data presented are the mean values of one representative experiment.

These earlier results suggested that whatever structural feature allows activity of C/EBP$\beta_{192-276}$ might be a general feature of C/EBPs. To test this hypothesis, we performed transient transfections with vectors expressing truncated forms of C/EBPα and C/EBPδ (C/EBP$\alpha_{273-358}$ and C/EBP$\delta_{181-272}$; Fig. 1) in comparison to intact C/EBPβ in P388 cells (Fig. 8). To more directly examine the contribution of the leucine zipper to C/EBP activity, we also transfected a vector expressing a truncated form of C/EBPβ in which the C/EBPβ leucine zipper was replaced with that of yeast GCN4 (C/EBP$\beta_{192-276}$:G_{LZ}; Fig. 1). The transfections were performed with and without LPS stimulation, and the expression vectors were cotransfected with an IL-6 promoter-luciferase reporter. C/EBP$\delta_{181-272}$ was almost as active as intact C/EBPβ. LPS treatment of C/EBPβ transfectants induced luciferase expression to a mean value of 24-fold over an untreated, "reporter-only" control, while LPS treatment of C/EBP$\delta_{181-272}$ transfectants induced luciferase expression to 20-fold over the control value. C/EBP$\alpha_{273-358}$ transfectants treated with LPS had levels of luciferase expression 7-fold over the control value and LPS treatment by itself without transfection of a C/EBP expression vector induced a mean value of luciferase expression only 3.8-fold of the control value. Confirming the critical role of the leucine zipper, C/EBP$\beta_{192-276}$:G_{LZ} transfectants treated with LPS had levels of luciferase expression only 2.7-fold over the control value, less than that observed without transfection of a C/EBP expression vector. Once again, LPS induction of luciferase expression was observed in either the presence or absence of C/EBP expression, although in the case of C/EBP$\beta_{192-276}$:G_{LZ} this induction is slight at best (1.4-fold). EMSA and western blot analysis

did not detect C/EBP$\alpha_{273\text{-}358}$, C/EBP$\delta_{181\text{-}272}$, or C/EBP$\beta_{192\text{-}276}$:G_{LZ} species (data not shown), so their level of expression relative to C/EBPβ could not be assessed. C/EBP$\alpha_{273\text{-}358}$ expression increases the basal level of IL-6 promoter activity without augmenting LPS induction. This modest level of activation by C/EBP$\alpha_{273\text{-}358}$ is consistent with the reduced activity of intact C/EBPα observed in LPS inductions of the endogenous IL-6 promoter in stable transfectants (Hu et al., 1998).

DISCUSSION

The data presented in this paper demonstrate that the conventional activation domains of C/EBPβ (Williams et al., 1995) and C/EBPδ are dispensable for their roles in the LPS-induced activation of IL-6 and MCP-1 expression. C/EBP$\beta_{192\text{-}276}$, a truncated form of C/EBPβ lacking its first 191 amino acids, is capable of activating transcription of IL-6 and MCP-1 genes after LPS stimulation of both stably and transiently transfected P388 lymphoblasts. Transient transfections showed that LIP, a form of C/EBPβ lacking its first 131 amino acids, and C/EBP$\delta_{181\text{-}272}$, a truncated form of C/EBPδ were also effective in activating the IL-6 promoter. A truncated form of C/EBPα, C/EBP$\alpha_{273\text{-}358}$, also showed modest activity.

The activity of the C/EBP bZIP domains and of LIP was unexpected. LIP has been found to possess little or no transcriptional activity (Descombes and Schibler, 1991; Williams et al., 1995; Cooper et al., 1995; Cooper et al., 1994). Previous investigators may not have observed this activity because they used different truncations of C/EBPβ, different reporters and/or different cell types in their transfection systems. Using an embryonic carcinoma cell line, an internally deleted form of C/EBPβ failed to activate the IL-6 promoter (Matsuaka et al., 1993). This mutant, however, would have retained regulatory sequences that inhibit transactivation potential and mediate cell specificity (Williams et al. 1995; Kowentz-Leutz et al., 1994). Others have assayed LIP and C/EBP$\beta_{192\text{-}276}$ on promoter-reporter constructs based on the DE-I site of the albumin promoter

(Descombes and Schibler 1991; Williams et al, 1995) or other tandem arrangements of C/EBP binding sites (Cooper et al, 1995; Cooper et al., 1994), all of which are solely C/EBP-dependent. We also found that these forms of C/EBPβ are inactive on an albumin DE-I site-based promoter in P388 lymphoblasts (Fig. 10). Presumably, the activity that we have observed on induction of IL-6 and MCP-1 is dependent on interactions with other transcription factors, such as NF-κB, that bind to these promoters.

It is interesting that the majority of both C/EBPβ and C/EBP$\beta_{192\text{-}276}$ are present as heterodimers with C/EBPγ (Ig/EBP) in P388 cells (Fig. 3). Although C/EBPγ has been characterized as a transdominant inhibitor of C/EBP transcriptional activation (Cooper et al., 1994), it does not act in that manner in the context of the IL-6 promoter. On the other hand, C/EBPγ does not appear to be an activator of IL-6 transcription either. Endogenous expression of C/EBPγ at a level affording DNA binding activity similar to that that of C/EBPβ and C/EBP$\beta_{192\text{-}276}$ (Fig. 3) is not sufficient to allow LPS induction of IL-6 expression. In addition, co-transfection of a C/EBPγ expression vector with a vector for C/EBP$\beta_{192\text{-}276}$ expression was only mildly inhibitory to LPS activation of the IL-6 promoter (Fig. 9). Since C/EBPγ lacks any known activation domains and is essentially a C/EBP bZIP domain (Cooper et al., 1995), the apparent ability of a C/EBP$\beta_{192\text{-}276}$:C/EBPγ heterodimer to activate the IL-6 promoter with LPS stimulation is consistent with the C/EBPβ activation domains being dispensable for LPS-induced transcription of the IL-6 gene.

There is good evidence that C/EBPβ and NF-κB synergistically activate the IL-6 promoter (Matsuaka et al., 1993). C/EBPα, β, and δ have all been shown to synergize with NF-κB in activating the IL-8 promoter (Kunsch et al., 1994; Stein et al., 1993). This synergy may involve direct physical association through their respective basic region-leucine zipper (bZIP) and Rel homology domains (Stein and Baldwin 1993, Stein et al., 1993). Thus, the basis for the ability of truncated C/EBPs to activate the IL-6 promoter may involve the capacity of the bZIP domain alone to synergize with NF-κB. Supporting this notion, we found that an intact NF-κB binding site is required for C/EBP activity in transient transfections of the IL-6 promoter-reporter (Fig. 7). Furthermore, robust activation of the IL-6 promoter by C/EBPs is only observed under conditions of LPS stimulation (Bretz et al., 1994, Hu et al., 1998, this paper), and LPS stimulation induces NF-κB activity in P388 cells (Hu et al., 1998). Future experiments will examine the ability of truncated forms of C/EBPβ as well as point mutants within the bZIP domain, to synergize with NF-κB in activating the IL-6 promoter.

Regardless of the mechanism by which the C/EBP bZIP domain mediates LPS induction of IL-6 and MCP-1 expression, our findings suggest that the basis for C/EBP redundancy in the activation of these genes (Hu et al., 1998) resides in this well-conserved region that is shared by all C/EBP isoforms. Our experiments replacing the leucine zipper of C/EBPβ with that of GCN4 suggest that the critical structural feature for activity of bZIP domains may be further localized to, or at least require, the leucine zipper. While the leucine zipper swap mutant C/EBPβ:G_{LZ} can transactivate a DE-I promoter-reporter in P388 cells

(Fig. 10), it shows no activity compared to intact C/EBPβ in the LPS induction of IL-6 and MCP-1. There is some uncertainty in the interpretation of this result because the levels of C/EBPβ:G_{LZ} expression are below that for intact C/EBPβ. On the other hand, the level of C/EBPβ:G_{LZ} expression is similar to that of C/EBP$\beta_{192\text{-}276}$ (Figs. 2B and 5B), which is a far more potent transcriptional activator (Fig. 4). Consistent with these findings, the truncated zipper swap mutant C/EBP$\beta_{192\text{-}276}$:G_{LZ} displays very little activity in transient expression assays and may even suppress LPS-inducibility (Fig. 8). It is possible that the leucine zipper possesses critical determinants for the activity of C/EBPs on the IL-6 promoter in addition to mediating dimerization to known C/EBP family members. For example, the leucine zipper might mediate dimerization to an as yet unknown dimerization partner with inherent activation potential or, as proposed above, it might mediate synergistic interaction with NF-κB. The leucine zippers of C/EBP proteins have previously been implicated in functions beyond dimerization. The leucine zipper of C/EBPα has been shown to mediate cell type specificity of albumin promoter activation (Nerlov and Ziff, 1994). Another instance of a non-dimerization function residing in the leucine zipper is that of serine 276 of human C/EBPβ (Wegner et al., 1992). Phosphorylation of this serine residue confers calcium-regulated transcriptional stimulation to a promoter that contains binding sites for C/EBPβ. The experiments reported here show C/EBP$\alpha_{273\text{-}358}$ to have quite modest activity in comparison to the truncated forms of C/EBPβ and C/EBPδ in transient transfections, as well as being the least able to support LPS induction of the IL-6 promoter-reporter. It would be useful to

evaluate the activity of a chimeric C/EBPα with the leucine zipper of C/EBPβ. Such a mutant would be expected to have enhanced activity in our model. In future experiments, it will be useful to examine single amino acid substitutions in the leucine zipper not only because they may more sharply delineate critical structural features, but because these altered forms of C/EBPβ may provide levels of expression more comparable to that of intact C/EBPβ and thus allow more direct comparisons of activity.

Interestingly, we have found that the effects of C/EBP expression are more profound in stable than in transient transfection assays. While C/EBP expression activated the IL-6 promoter in both assay systems, the level of IL-6 induction and synergy with LPS stimulation is much more dramatic in stably transfected cells. This is probably not a function of C/EBP overexpression, since EMSA assays of P388 lymphoblasts with stable ectopic C/EBP expression do not reveal abnormally high levels of DNA binding activity in comparison to bone marrow-derived macrophages (Hu et al., 1998). It seems likely that stable C/EBP transfectants with intact IL-6 promoters at normal diploid abundance in chromatin are a more accurate reflection of C/EBP activity on the IL-6 promoter. The IL-6 promoter would be expected to be in a more repressed basal state in chromatin and more dependent upon the activity of C/EBP to achieve activation. In either system, however, the surprising activity of truncated forms of C/EBP is plainly evident.

Finally, our results show a significant capacity for LIP to activate the IL-6 promoter. LIP has previously been proposed to be a transdominant inhibitor of

transcription (Descombes and Schibler, 1991). It is proposed that high levels of LIP observed in fetal liver constitute a mechanism for inhibiting the activity of other C/EBP isoforms in hepatocytes that are not yet terminally differentiated. Furthermore, it has been proposed that LIP could block C/EBPβ mediated inhibition of hepatocyte proliferation (Buck et al., 1994). The regulation of LIP expression has also been proposed to play a role in the regulation of lactation-associated genes such as β-casein (Raught et al., 1995). More recently, LIP expression has been correlated with neoplastic transformation of mammary tissue and has been proposed as a prognostic indicator for breast cancer because of its overexpression in breast tumors that were negative for the estrogen and progesterone receptors (Raught et al., 1996; Zhanow et al., 1997). The central theme of these models is that the expression of LIP in immature proliferating cells suppresses the activity of C/EBPβ and other isoforms in activating the expression of gene products associated with terminal differentiation. It is clear from the findings reported here that conditions favoring LIP expression would not universally down-regulate C/EBPβ regulated genes, but would be permissive for the expression of IL-6 and other genes with a similar promoter structure.

Among tissues particularly noted for IL-6 production, high levels of LIP expression have been observed in fetal liver (Descombes and Schibler, 1991), as well as transiently in adult liver following partial hepatectomy (Timchenko et al.,1998) or LPS treatment (An et al., 1996). While LIP might repress the activation of C/EBP-dependent gene products that inhibit hepatocyte

proliferation, it could at the same time support the induction of IL-6 expression as well as several other acute phase genes that show synergistic activation by C/EBP and NF-κB family members (reviewed in Poli, 1998). Indeed, the observation of LIP expression in LPS-treated liver that would produce IL-6 is consistent with our findings. Any model for the physiological significance of LIP must be tempered, however, by the finding that LIP can be the product of proteolysis associated with hypotonic lysis procedures (Baer et al., 1998). The findings reported here of LIP activity in the expression of IL-6, as well as uncertainties associated with the generation of LIP, call for a reexamination of its role as a physiological repressor of C/EBP-mediated transcription.

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Chapter 4

Summary and Future Plans

The data presented in chapter 2 showed that we have identified and molecularly cloned a novel gene (CDDP-1) from a myeloblastic cell line 32D cl3 overexpressing C/EBPβ. CDDP-1 is apparently upregulated by the C/EBP family of transcription factors including C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε. It is a potential target gene of C/EBPs because the promoter region of CDDP-1 has several putative C/EBP binding sites. CDDP-1 was induced early in myelomonocytic differentiation and was further upregulated as the immature myeloid cells differentiate into neutrophils but downregulated as cells advance into monocytes/macrophages. When constitutively overexpressed in an immature myeloid cell line MPRO, CDDP-1 can promote both neutrophilic and monocytic differentiation programs as evidenced by morphological studies and molecular markers. CDDP-1 was also modulated during the differentiation processes of other hematopoietic lineages such as erythroid, mast, and B lymphoid cells indicating a role in the respective lineages. However, transient co-transfection assays using promoter-reporter constructs driven by several myeloid specific promoters including those for the myeloperoxidase, G-CSF receptor, M-CSF receptor, retinoic acid receptor, and IL-6 genes, failed to pinpoint a specific pathway that CDDP-1 may act upon in regulating myelomonocytic differentiation.

To further explore the mechanisms of CDDP-1 function, an immediate future experiment would be to characterize the proteins with which CDDP-1 interacts. This will help to identify downstream elements in a regulatory scheme. Since we already generated a polyclonal antibody against a GST- CDDP-1 fusion protein, this antibody can be used for co-immune precipitation experiments. The

myelomonocytic cells where CDDP-1 is induced will be metabolically labeled with ^{35}S-methionine and their extracts immune-precipitated with anti-CDDP-1. Co-precipitated proteins will be visualized by fluorography of SDS-PAGE and further characterized by protein microsequencing analyses. Alternatively, "GST pull-down" chromatography can be used to identify CDDP-1 interacting proteins. CDDP-1 would be expressed from a bacterial glutathione S-transferase (GST) fusion protein expression vector. The GST fusion protein would be bound to glutathione-Sepharose beads. Cellular extracts prepared from cells at a stage of myelomonocytic differentiation where CDDP-1 is induced would then be incubated with control GST beads and GST-CDDP-1 beads. Bound proteins will be eluted and analyzed by SDS-PAGE and subsequent sequencing analyses.

To find out a more definitive function of CDDP-1 in hematopoiesis in vivo, mice deficient in CDDP-1 will be generated by electroporation of the CJ7 embryonic stem cell line with a replacement-type targeting vector. The targeting vector will consist of between 2 and 5 kb of 5' homologous and 3' homologous sequences flanking the CDDP-1 locus. We have isolated a mouse genomic clone which contains the 3' flanking region of CDDP-1 from a 129SvJ mouse genomic library. The 5' flanking sequence will be isolated by screening the same library using the 5' region of the cloned CDDP-1 cDNA. Once obtained, CDDP-1-deficient mice will be analyzed in comparison to wild type and heterozygous mice for the composition of their peripheral blood and bone marrow. Hemoglobin will be measured with a Hemoglobinometer. Leukocytes will be cytocentrifuged, stained with Wright-Giemsa, and evaluated on the basis of their morphology.

Leukocytes from bone marrow and peripheral blood will also be analyzed by flow cytometry for the following hematopoietic markers: Mac-1, myeloid; Gr-1, granulocyte; F4/80, macrophage; B220, B lymphoid; Thy 1.2, T lymphoid; TER-119, erythroid; CD41, megakaryocyte. If expected abnormality are observed, such as decreased levels of granulocytes and/or erythroid cells, bone marrow cells will also be tested for GM-CFU, M-CFU, E-CFU, and GEMM-CFU.

Potential downstream targets of CDDP-1 activity can be identified using cDNA microarray analyses by comparing CDDP-1 deficient mice with wild type littermate. RNAs will be isolated from either bone marrow or fetal liver. Radiolabeled cDNA will be used as probe on the microarray chips. Alternatively, MPRO or U937 cells would be transiently transfected with CDDP-1 expressing vectors. After 24 hours of transfection, RNA will be isolated and radiolebeled cDNA prepared for hybridization to "Atlas" cDNA expression arrays (Clontech). Any RNAs upregulated or downregulated at this early time point after CDDP-1 expression would be considered a potential target gene and will help to understand the mechanism of CDDP-1 function.

To confirm whether CDDP-1 is a direct target of C/EBP family members and to place CDDP-1 in a regulatory scheme, the promoter region of CDDP-1 will be identified. The 2000 bp region proximal to the 5' coding sequence will be placed upstream of luciferase in the pXP2 reporter construct. Transient co-transfection with C/EBP isoforms will be performed in 32D clone 3 cells to verify the functionality of the constructs and to compare the effectiveness of various C/EBP isoforms to stimulate CDDP-1 transcription.