C/EBPε Is a Myeloid-specific Activator of Cytokine, Chemokine, and Macrophage-Colony-stimulating Factor Receptor Genes*

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Simon C. Williams‡‡, Yang Du‡, Richard C. Schwartz§, Sarah R. Weiler¶, Mariaestela Ortiz**, Jonathan R. Keller**, and Peter F. Johnson‡‡

From the §Department of Cell Biology & Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430, the ¶Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1101, the **Laboratory of Leukocyte Biology-Division of Basic Science, " Intramural Research Support Program, Scientific Applications International Corporation, Frederick, Maryland 21702-1201, and the ‡‡Advanced Biosciences Laboratories-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201

C/EBPε is a member of the CCAAT/enhancer binding protein family of basic region/leucine zipper transcriptional activators. The C/EBPε protein is highly conserved between rodents and humans, and its domain structure is very similar to C/EBPa. In mice C/EBPε mRNA is only detected in hematopoietic tissues, including embryonic liver and adult bone marrow and spleen. Within the hematopoietic system, C/EBPε is expressed primarily in myeloid cells, including promyelocytes, myelomonocytes, and their differentiated progeny. To identify potential functions of C/EBPε, cell lines over-expressing the C/EBPε protein were generated in the P388 lymphoblastic cell line. In contrast to the parental cell line, C/EBPε-expressing cell lines displayed lipopolysaccharide-inducible expression of the interleukin-6 and monocyte chemoattractant protein 1 (MCP-1) genes as well as elevated basal expression of the MIP-1α and MIP-1β chemokine genes. In the EML-C1 hematopoietic stem cell line, C/EBPε mRNA levels increased as the cells progressed along the myeloid lineage, just preceding activation of the gene encoding the receptor for macrophage-colony-stimulating factor (M-CSFR). M-CSFR expression was stimulated in C/EBPε-expressing P388 cell lines, when compared with either the parental P388 cells or P388 cells lines expressing either C/EBPa or C/EBPβ. These results suggest that C/EBPε may be an important regulator of differentiation of a subset of myeloid cell types and may also participate in the regulation of cytokine gene expression in mature cells.

The CCAAT/enhancer binding protein (C/EBP)† family of transcription factors consists of four highly related members, named C/EBPa, C/EBPβ, C/EBPδ, and C/EBPε, along with the less closely related Ig/EBP (C/EBPγ) and CHOP (gadd153) proteins (reviewed in Ref. 1). The C/EBP proteins are basic region/leucine zipper transcription factors that bind to the consensus sequence 5’ ATTGCGCAAT 3’ and variants thereof (2). Individual members of this family have been implicated as regulators of differentiation in multiple cell types, including hepatocytes, adipocytes, and certain blood cell types. In the hematopoietic system, C/EBPε is expressed primarily in myeloid cells, including monocytes, macrophages, granulocytes, and their precursors (3, 4). C/EBPε mRNA has been detected in primitive (CD34+ and CD38+ ) progenitor cells, and its expression is specifically up-regulated when these cells are induced to differentiate along the granulocytic lineage but not the monocytic lineage (5). Analysis of granulocytic differentiation of the 32DCl.3 hematopoietic progenitor cell line suggested that C/EBPa acts prior to the terminal stages of differentiation in this cell lineage (4). In support of this hypothesis, C/EBPa nullizygous mice lack mature granulocytes and contain large numbers of immature myeloid blast cells in their blood (6).

Expression of both C/EBPβ and C/EBPδ is up-regulated during terminal differentiation of cells in both the granulocytic and monocytic lineages, and C/EBPβ appears to be the predominant C/EBP protein in mature granulocytes and macrophages (3, 4). Differentiation-specific functions for C/EBPβ and C/EBPδ have not been clearly defined as yet, although both are capable of collaborating with wild type and oncogenic forms of the Myb transcription factor to activate macrophage-specific genes in heterologous cell types (7). The primary functions of C/EBPβ and C/EBPδ within the hematopoietic system appear to be in the regulation of a number of cytokine and other genes during inflammatory responses (3, 8–11). Mice carrying a null mutation in the C/EBPβ gene display essentially normal myeloid development but exhibit marked defects in bacterial killing and cytokstatic functions (12, 13). Despite the fact that C/EBPβ has been proposed to be a critical regulator of cytokine gene expression in activated macrophages, LPS-dependent induction of cytokine gene expression was essentially identical in macrophages isolated from wild type and mutant mice. The relatively normal pattern of cytokine gene expression in C/EBPβ null mice is likely to be due to functional redundancy among family members (14).

A detailed molecular analysis of the roles of individual...
C/EBP proteins over the entire spectrum of myeloid differentiation has been hampered by technical difficulties in isolating and culturing progenitor cells from bone marrow and by the transformed phenotype of most immortalized cell lines. An alternative approach is now possible with the recent establishment of an immortalized hematopoietic progenitor cell line, named EML-C1 (15). The EML-C1 cell line was derived by infecting mouse bone marrow cells with a retrovirus expressing a dominant negative retinoic acid receptor α molecule and proliferates in culture in the presence of stem cell factor (SCF). The dominant negative retinoic acid receptor molecule specifically blocks differentiation along the myeloid lineage; however, activation of the gene encoding the receptor for macrophage-LPS-stimulated expression of multiple cytokine genes and activation of the gene encoding the receptor for macrophage-colony-stimulating factor.

**EXPERIMENTAL PROCEDURES**

**Cloning and Sequencing**—Isolation of the rat C/EBPα genomic clone was described previously (16). A mouse C/EBPα cDNA was isolated by PCR amplification of cDNA generated from total RNA isolated from the P388D1(IL-1) macrophage cell line using the amplifiers CRP1 ATG-4 (GACGGCCC CATGCCAGGAGGACGTAC) and 5′-end primers. The PCR product was subsequently used to screen a mouse 129SV genomic library (Stratagene, La Jolla, CA), and nine positive clones were isolated which together spanned 18 kilobase pairs of the C/EBPα locus, including the complete coding sequence. DNA sequencing was carried out using the Sequenase kit (Amersham Pharmacia Biotech) under standard conditions, except that in double-stranded sequencing reactions, nonspecifically terminated fragments were extended by incubation in the presence of terminal deoxynucleotidyltransferase and all four nucleotides prior to addition of the stop solution. DNA sequences were analyzed using the SEQUED, BESTFIT, PILEUP, and GELASSEMBLE programs of the University of Wisconsin Genetics Computer Group package.

The 5′ end of C/EBPα mRNA molecules was mapped by 5′-RACE using Marathon-Ready cDNA from mouse spleen (CLONTECH, Palo Alto, CA). The first round of PCR reactions (see below for details) was carried out using 0.1 ng of cDNA and 150 ng of a C/EBPα gene-specific primer (CRP1 100–80, CTCCAGGCCTGGTAGCTCATAG) and adaptor primer 1 provided with the cDNA. A second round of PCR was then carried out on an aliquot of the first reaction using two nested primers (CRP1 64–44, TAGCTGTTCTTCCAGAGCAGTAC and AP2), and the products were subcloned into the pGEM-T vector (Promega, Madison, WI), and positive subclones were sequenced. Although several potential cap sites were identified by this procedure, only those that appeared in several (n > 3) independent clones were considered to represent bona fide transcriptional start sites.

**Total RNA Isolation and Northern Analysis**—Total RNA was prepared from tissues and cells using a modified guanidine isothiocyanate/phenol extraction procedure and analyzed by Northern blotting and hybridization as described previously (17). The C/EBPα probe was an 850-bp NcoI/HindIII fragment containing the complete murine cDNA, and the MCP-1 probe was a 580-bp murine CDNA (18).

**Reverse Transcription-PCR**—RT-PCR was carried out essentially as described (19). Briefly, 2 μg of total RNA was reverse-transcribed using 0.2 μg of oligo(dT)₁₄₉ and 400 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in a total volume of 50 μl for 1 h at 37°C. 3 μl of the RT reaction was then mixed with 1 μl of 10 × Buffer (500 mM Tris-HCl (pH 8.3), 2.5 mg/ml crystalline bovine serum albumin, and MgCl₂ at 10, 20, or 30 mM (Idaho Technology Inc., Idaho Falls, ID), 0.2 mM each dNTP, 150 ng of each primer, and 1.25 units of Taq DNA polymerase (Fisher) in a total volume of 10 μl. Reactions were loaded into a capillary tube, and PCR cycles were carried out using the cycles of “Thermal Cycler” (Idaho Technology). Annealing temperatures and Mg²⁺ concentrations were initially optimized for each primer pair. In most cases, PCR was carried out at 2 mM MgCl₂ with the following cycle parameters: 94°C, 15 s, 1 cycle; 94°C, 0 s, 60°C, 0 s, 72°C, 25 s, 30 cycles; 72°C, 30 s, 1 cycle. Reaction products were visualized in ethidium bromide-stained agarose gels, and images were captured as PICT files using UVP ImageStore 7500 Gel Documentation software, cropped in Adobe Photoshop version 3.0.4, and annotated using Macromedia Freehand version 7.0. Accuracy and linearity of RT-PCR results was confirmed by varying input cDNA concentration, cycle number, and by comparing results with those from quantitative Northern blots (see Fig. 2 for example). The primers used in this study are shown in Table I.

**Purification of Primary Blood Cells**—Normal murine bone marrow cells were aspirated from BALB/c mouse femurs with Iscove's modified Dulbecco's medium containing 20% fetal bovine serum (Hyclone, Logan, UT), penicillin, and streptomycin. 32DC1.3, FDCP-1, NFS-58, and DA-3 cell lines were maintained in the same medium supplemented with 20% WEHI-3-conditioned medium as a source of M-CSF. GG2EE and WEHI-274.1 were maintained in RPMI containing 10% fetal bovine serum, and 5% fetal bovine serum (Hyclone, Logan, UT), penicillin, and streptomycin.

**Cell Cultures**—GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### Table I

| Gene      | Sense primer | Antisense primer | Ref.   |
|-----------|--------------|------------------|--------|
| C/EBPα    | TGGCCGCTGAAAGGAGGAGCT | GGTCAGCTGCAAGCCCC | 16     |
| IL-6      | GCGGCTCGAGTTGAGGAGAGG | GTGAGAAGATGACAGG | 38     |
| MCP-1     | AGACCGGTCCCAAGAGAGAAGG | ACAAAGTTCACCATCCAT | 39     |
| M-CSF     | ACTCCCTTTAACAGTGGCTTC | GGTGCAATATTCCAGCC | 40     |
| MIP-1α    | ATGCGCTGTTCTTCCATCACC | AGGCAACTGTTCCAGGTCGAGT | 13     |
| MIP-1β    | GGAGCGGTGGGAGGAAGAGG | ATAGAATAATGACCTCCC | 41     |
| GAPDH     | AGAGCCTGCCAGGCTGAGGATG | TTGTAGCAAGGCTCCGTGTT | 42     |

* GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
FIG. 1. Characterization of the C/EBPβ gene. A, sequence of the rat C/EBPβ genomic locus. The sequence of 2391-bp of a rat C/EBPβ genomic clone is shown with the deduced amino acid sequence of the C/EBPβ translation protein. The position of the splice donor site and two potential splice acceptor sites are underlined, and a consensus polyadenylation signal is double underlined. The position of three transcriptional initiation sites identified in multiple 5′ RACE products are indicated in reverse print. This sequence has been submitted to the GenBankTM data base under accession number AF034716. B, comparison of rat (R), mouse (M), and human (H) C/EBPβ protein sequences. The rat C/EBPβ sequence is from A and the mouse sequence from our unpublished results. The primary human sequence is from Antonson et al. (27), and disparities in the human sequence of Chumakov et al. (26) are indicated in parentheses below. Identities between species are indicated with dots, and changes are indicated by the single letter amino acid replacement. The residues that characterize the leucine repeat are indicated with asterisks. C, structural comparison of C/EBP family members. Domain structures of each C/EBP protein were aligned based on the position of the C-terminal, bipartite DNA-binding RDII site identified in multiple 5′ splice acceptor sites (indicated as underlined AG dinucleotides). Sequences of each C/EBP protein sequence were compared with the predicted peptide sequence of the C/EBPα protein. The translation product. The position of the splice donor site and two potential splice acceptor sites (indicated as underlined AG dinucleotides). Sequence revealed an in frame translation initiation codon located 96 bp upstream of the AUG codon described previously.

Western Blotting and Electrophoretic Mobility Shift Assays (EMSA)—Total cellular protein was prepared from selected cell lines, and Western blotting was performed as described previously (17). Western blots were probed with a C/EBPβ-specific antiserum (C-22, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Preparation of nuclear extracts and EMSA procedures have been described previously (20), and the oligonucleotide probe used here contains a consensus C/EBP DNA-binding site. Supershift assays were carried out by preincubating the nuclear extract with a rabbit polyclonal C/EBPβ-specific antiserum directed against amino acids 266–279 of the rat polypeptide.

Plasmid Construction—The complete C/EBPβ coding sequence, including the intron, was amplified from the rat genomic clone by PCR using two oligonucleotide primers (CRP1 ATG-4 and S1CRP1, see above) that overlap the initiation codon and termination codon, respectively. The resultant PCR product was digested with NcoI and HindIII (incorporated as part of the amplimers) inserted into the pMX eukaryotic expression vector (16) to generate pMEX/C/EBPβp34. The amplified region was sequenced to check for errors and was shown, after transient transfection into a number of cell types, to direct the synthesis of a 34,000 molecular weight protein that was recognized by a C/EBPβ-specific antiserum (data not shown). pMEX/C/EBPβp34 was digested with HindIII, and the 5′ overhangs were made blunt using Klenow, and a BamHI linker was added. This construct was then digested with BamHI, and the full coding sequence was inserted into the pSVx expression vector (20) to generate pSVx/C/EBPβp34. Inserted genes in pSVx are expressing from the Moloney murine leukemia virus long terminal repeat with a subgenomic splicing product expressing the neomycin resistance gene for selection of stably transfected cell lines.

Generation of C/EBPβ Expressing P388 Cell Lines—106 P388 cells were transfected with 5 μg of pSVx/C/EBPβp34 expression vector using 40 μg of Lipofectin (Life Technologies, Inc.) as described previously for the generation of C/EBPβ expressing P388 cell lines (20). Selection was carried out at three concentrations of G418 (150, 250, and 350 μg/ml), and four independent pools of resistant cells (named P388-C1 to C4) were established. Expression of the C/EBPβ transgene was confirmed by RT-PCR, Western blotting, and EMSA.

RESULTS

Characterization of the C/EBPβ Gene—The sequence of 2391 bp of a rat C/EBPβ genomic clone is presented in Fig. 1A along with the predicted peptide sequence of the C/EBPβ protein. The C/EBPβ gene contains a single intron with two potential splice acceptor sites (indicated as underlined AG dinucleotides). Sequencing of RT-PCR products generated using C/EBPβ-specific primers confirmed that the more 5′ splice acceptor sequence is apparently exclusively utilized, at least in hematopoietic cells (see below). Examination of the 5′ end of the C/EBPβ coding sequence revealed an in frame translation initiation codon located 96 bp upstream of the AUG codon described previously.
C/EBPα and Myeloid Gene Expression

(16). Consequently, conceptual translation of the longest open reading frame predicts that the full-length C/EBPα protein is 281, rather than 249, amino acids in length. The 5′ end of C/EBPα mRNA was determined by sequencing of 5′-RACE products generated from adult mouse spleen cDNA. Several potential start sites were identified; however, only three of these appeared in multiple subclones and are included in Fig. 1A. These sites are likely to be bona fide start sites as they map close to start sites identified for the Pβ promoter of the human C/EBPα gene (21). The sequences around the cap sites are purine-rich and lack common promoter elements such as TATA and CCAAT boxes, a feature shared with many genes expressed in myeloid cells.

The complete coding sequence of the C/EBPα gene has now been determined in three species, rat, mouse, and human, and the sequences of these three proteins are compared in Fig. 1B. The sequence of the C/EBPα protein is highly conserved, displaying 98% identity between rat and mouse and 93% between rat and human. The most highly conserved regions are the first 50 amino acids, which contain sequences that function as a transcriptional activation domain (22), and the C-terminal sequences that contain the DNA-binding domain (16). A structural comparison of the four members of the C/EBP family is shown in Fig. 1C, with regions of significant similarity indicated by shading. The structure of C/EBPα displays greatest similarity to C/EBPδ and C/EBPβ, containing an N-terminal tripartite activation domain and a second activation domain close to the center of the protein.

C/EBPα is Expressed in Hematopoietic Tissues—Previously, we were unable to detect C/EBPα mRNA in a number of adult mouse tissues, suggesting that C/EBPα is expressed in a limited temporal or spatial pattern or in tissues omitted from our original survey. In an expanded Northern blot survey, we detected a strong signal at approximately 1.8 kilobase pairs in RNA prepared from adult bone marrow (Fig. 2A, lane 5), with weak signals in embryonic liver and adult spleen (lanes 1 and 3). Because C/EBPα appears to be expressed at low levels, even in positive tissues, we used reverse transcription-PCR (RT-PCR) to carry out a more extensive survey. Primers were chosen to flank the single intron within the C/EBPα gene to ensure that PCR products generated from cDNA could be distinguished from products synthesized from a genomic template. In agreement with the Northern analysis, C/EBPα mRNA was detected in embryonic liver and adult bone marrow and spleen, each of which are hematopoietic tissues. C/EBPα mRNA was essentially absent from all other tissues analyzed although low levels were detected in adult lung, which may be due to infiltration of this tissue with blood cells. In this and all subsequent RT-PCR assays, a single amplified product was observed which corresponds to a spliced mRNA utilizing the 5′ splice acceptor site described above.

C/EBPα Is Primarily Expressed in Myeloid Cells—By having shown that C/EBPα is expressed in hematopoietic tissues, and at highest levels in bone marrow, RNA from a panel of rodent cell lines was analyzed by RT-PCR to determine which cell types within the hematopoietic system express C/EBPα (Fig. 3). C/EBPα mRNA was detected in 12 out of 13 cell lines of myeloid origin, the lone exception being the immature myeloid FDCP-1 cell line (Fig. 3A). The highest level of C/EBPα mRNA was detected in cell lines arrested at the promyelocyte stage of myeloid development (EPRO and MPRO) and two IL-3-dependent myeloid cell lines (NFS-58 and DA-3), whereas lower levels were detected in two myelomonocytic leukemia cell lines (M1 and WEHI-3). C/EBPα mRNA was also detected in five monocytic/macrophage cell lines including M-CSF-dependent M-NFS-60 cells and the factor-independent cell lines WEHI-264.1, GG2EE, P388D1(IL-1), and IC-21. C/EBPα mRNA was not detected in cell lines representative of erythroid, mast cell, basophil, or T cell lineages and was present in only one cell line (P88) derived from the B cell lineage (Fig. 3B). As further confirmation of the restricted pattern of expression of C/EBPα, RNA was prepared from purified populations of primary murine blood cells (Fig. 3C). High levels of C/EBPα mRNA were detected in granulocytes and macrophages but not in B cells, T cells, or erythrocytes.

We next performed Western blot analysis to examine the expression of C/EBPα at the protein level in selected rodent cell lines. Whole cell protein extracts from a subset of the cell lines described above were analyzed by Western blotting using a C/EBPα-specific antisera (Fig. 4). A single Mr 34,000 protein was detected in M1 myelomonocytic cells, and P388D1(IL-1) macrophage cells but not in other cell lines, including some that contain C/EBPα mRNA. The inability to detect C/EBPα protein in all cell lines containing C/EBPα mRNA may simply be due to the fact that the amount of C/EBPα protein in these samples is below the level of sensitivity of our Western blotting procedure or may indicate that C/EBPα expression is regulated at a post-transcriptional stage. Comparison of the size of the C/EBPα protein detected in P388D1(IL-1) cells with recombinant C/EBPα proteins confirmed that the methionine residue...
indicated as amino acid 1 in Fig. 1A is the primary site of translational initiation (data not shown).

**Establishment of C/EBPε-expressing P388 Cell Lines—**P388 is a murine B lymphoblastic cell line that does not express detectable levels of any C/EBP proteins, despite the presence of C/EBPε mRNA (see Fig. 4 and below). This cell line was previously used to analyze the functions of C/EBPε and C/EBPβ (14, 20). We established four G418-resistant lines using a C/EBPε expression vector. C/EBPε mRNA levels in three of the four cell lines (P388-Cε-1, Ce-3, and Ce-4) were elevated compared with those in the control P388-Neo cell line as assessed by RT-PCR (Fig. 5A) and Northern blotting (data not shown). In addition, C/EBPε mRNA levels were unchanged in C/EBPβ-expressing P388-Cβ cells (Fig. 5A, compare lanes 1 and 2) or in P388 cell lines expressing either C/EBPε or C/EBPβ (data not shown), consistent with our previous findings that overexpression of one C/EBP protein in P388 cells does not activate expression of other family members (14). To test whether the elevated mRNA levels resulted in expression of C/EBPε protein in these cell lines, an EMSA was performed using a radiolabeled oligonucleotide containing a consensus C/EBP-binding site, and a representative result showing P388-Cε-3 is shown in Fig. 5B. A number of protein-DNA complexes were observed in nuclear extracts from control P388-Neo cells; however, none were affected by addition of a C/EBPε-specific antiserum (compare lanes 1 and 2) or antisera recognizing other C/EBP family members (14). Two new complexes were observed using nuclear extracts from P388-Neo-3 cells (open arrowheads, lane 3). The upper complex was completely supershifted by addition of C/EBPε antiserum (closed arrowhead, lane 4) and, based on its co-migration with bacterially produced, recombinant C/EBPε (data not shown), appears to correspond to a C/EBPε homodimer. The mobility of the lower complex, which presumably represents a heterodimer with an unidentified C/EBP partner (20, 22), was only slightly affected by the C/EBPε antiserum. Similar binding patterns were observed in both P388-Cε-1 and P388-Cε-4 (data not shown).

**C/EBPε Participates in LPS-regulated Expression of IL-6 and MCP-1 Genes**—The expression of a number of inflammatory cytokine genes is up-regulated following lipopolysaccharide treatment of macrophage cell lines such as P388D1(IL-1) while these genes are not normally induced in P388 lymphoblasts. To test whether C/EBPε might participate in the regulation of these genes, cultures of each of the cell lines described above were exposed to LPS for 18 h, and RNA was prepared and analyzed by RT-PCR using oligonucleotides specific for MCP-1 and IL-6. IL-6 and MCP-1 mRNAs were barely detectable in control P388-Neo cells and were essentially unresponsive to LPS stimulation (Fig. 6A, lanes 1 and 2). Basal expression levels of IL-6 or MCP-1 were elevated in each C/EBPε-expressing cell line with the relative level of target gene mRNA being approximately proportional to the level of C/EBPε mRNA and protein (i.e. higher in lanes 5, 9, and 11 than in lane 7). C/EBPε-expressing lines also displayed LPS-dependent increases in both cytokine mRNAs, a feature that is most evident in the P388-Cε-2 cell line for IL-6 (lane 8) and P388-Cε-3, Ce-3, and Ce-4 for MCP-1 (lanes 8, 10, and 12). Finally, in support of our previous results (20), both genes were also induced in the P388-Cε cell line (lanes 3 and 4).

We next compared the kinetics of MCP-1 induction in control, C/EBPε, and C/EBPε-expressing lines and P388D1(IL-1) macrophages. P388-Cε-4 was chosen as the representative C/EBPε-expressing line as it displayed the most robust increase in MCP-1 mRNA levels (Fig. 6A). Each cell line was incubated in the presence of LPS for various times up to 24 h, and MCP-1 expression was analyzed by Northern blotting (Fig. 6B). In agreement with the RT-PCR data, MCP-1 mRNA was essentially undetectable in P388-Neo cells under all conditions.
The plasmids used to establish each line were as follows: P388-Neo, pSV(x); P388-Cβ, phage dehydrogenase (GAPDH) potential targets, we determined that both C/EBP activation of MIP-1 αV(x)C/EBP σion of MIP-1 a in either the absence (incubated with an oligonucleotide containing a perfect C/EBP binding 2h( expression in stable P388-derived cell lines. From a survey of several potential targets, we determined that both C/EBPα and C/EBPβ were capable of activating expression of the genes encoding the β-chemokines, MIP-1α and MIP-1β (Fig. 7). MIP-1α mRNA was detected at low levels in control P388-Neo cells but was significantly increased after exposure to LPS for 2 h (lanes 1 and 2). The basal level of MIP-1α mRNA was significantly increased in both P388-Cα-4 and P388-Cβ in comparison to P388-Neo cells but was relatively unaffected by LPS treatment, a pattern similar to that seen in P388D1(IL-1) cells (lanes 3–8). MIP-1β mRNA was undetectable in P388-Neo cells and slightly increased by LPS treatment. Again basal rather than LPS-dependent mRNA levels were primarily affected by ectopic C/EBPα and C/EBPβ, although levels did not approach those observed in P388D1(IL-1) cells (compare lanes 3–6 with 7 and 8). Similar results were obtained in four independent experiments.

Regulation of C/EBPα Expression during Myeloid Differentiation—Having shown that C/EBPα is capable of activating the expression of genes expressed primarily in mature cells, we next examined whether it might also play a role in regulating gene expression during myeloid differentiation. As a first step, we determined the temporal pattern of C/EBPα expression during myeloid differentiation using the EML-C1 hematopoietic progenitor cell line. EML-C1 cells were incubated in the presence of SCF, IL-3, and retinoic acid, which induces their differentiation along both the macrophage and neutrophil lineages. Cells were harvested for RNA preparation at various time points over a 6-day period, and C/EBPα mRNA levels were determined by RT-PCR (Fig. 8). Low levels of C/EBPα mRNA were detected in the uninduced parental cells and remained low during the first 8–16 h after retinoic acid addition. C/EBPα mRNA levels began to increase after approximately 16 h and reached maximal levels at the 120-h time point, which were similar to those seen in MPRO promyelocyte cells. A similar pattern of C/EBPα expression has been observed in multiple independent induction experiments and was found to be unaffected by the use of either pure recombinant SCF or conditioned medium from an SCF-overexpressing cell line (data not shown). The low level of C/EBPα mRNA at early time points may be explained either by weak basal expression of C/EBPα in hematopoietic progenitor cells or by the presence of a small number of myeloid precursors that escape the dominant negative retinoic acid receptor block. However, because the initial rapid proliferation of EML-C1 cells slows and appearance of myeloid progenitors occurs around 24 h after retinoic acid addition, the increase in C/EBPα at this time suggests that it may activate the expression of genes required for establishment of certain myeloid cell lineages.

To identify potential C/EBPα target genes that might support its putative role in regulating myeloid differentiation, we analyzed the expression of other myeloid-specific or -restricted genes in the same EML-C1 RNA samples. The best candidate to arise from these studies was the gene encoding the receptor for macrophage-colony-stimulating factor (M-CSFR) (Fig. 8). M-CSFR mRNA levels displayed a transient increase at 2 and 4 h after retinoic acid addition, a feature that is shared by a number of other genes.2 At later times M-CSFR exhibited a pattern that was similar, but slightly delayed, to that of C/EBPα. As expected, M-CSFR mRNA was undetectable in MPRO cells, which are restricted to the granulocytic lineage and are arrested just prior to terminal differentiation, but was present at high levels in P388D1(IL-1) macrophages.

M-CSFR Expression Is Specifically Activated by C/EBPα—Based on their similar expression patterns, and the fact that the promoter of the M-CSFR gene is known to contain a functional C/EBP-binding site (23), we tested whether M-CSFR expression was affected by ectopic C/EBPα expression in P388 cells. For comparison, we analyzed the P388-Cβ cell line described above and four independent cell lines expressing C/EBPα (P388-Ca-1 to Ca-4). M-CSFR mRNA levels were as

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2 Y. Du and S. C. Williams, unpublished observations.
sessed by RT-PCR using RNA prepared from unstimulated cells (Fig. 9). M-CSFR mRNA was undetectable in control P388-Neo cells but, in two independent experiments, was present in each of the four C/EBP\(\text{e}\)-expressing cell lines at levels proportional to the level of C/EBP\(\text{e}\) mRNA (see Fig. 6A), albeit at lower levels than in P388D1(IL-1) cells. This effect was essentially specific to C/EBP\(\text{e}\) as M-CSFR mRNA levels were only slightly elevated over background levels in the P388-C\(a\) or P388-C\(b\) cell lines, despite the fact that both cell lines attain the ability to activate other C/EBP-dependent genes (see Figs. 7 and 8 and Ref. 14).

**DISCUSSION**

Our understanding of the molecular mechanisms controlling the development of mature blood cells from uncommitted pro-
ing with C/EBPα the ability to confer lipopolysaccharide-responsive expression of certain cytokine genes onto a lymphoid cell line, C/EBPε is also capable of activating the expression of the M-CSFR gene. This finding indicates that C/EBPε may be required for both the function and development of macrophages.

Structural Analysis of the C/EBPε Gene in Rodents—Unlike in humans, where multiple C/EBPε mRNA species have been detected (21, 26, 27), Northern analysis has revealed the presence of a single C/EBPε mRNA species of approximately 1.8 kilobase pairs in murine hematopoietic tissues and cell lines. The simpler pattern in mice appears to be due to lack of alternate splicing, combined with the use of a single promoter, albeit one that uses multiple transcriptional start sites. In addition, despite the existence of internal translational initiation codons in the C/EBPε coding sequence, we have only detected a single C/EBPε protein in extracts from murine cells. This protein is 32 amino acids longer than previously reported due to the presence of an in frame methionine codon located 96 nucleotides upstream of the site predicted in our earlier study (16). Although a detailed examination of the structure of the C/EBPε protein has not been reported, our preliminary data indicate that it is an efficient transcriptional activator, and its domain structure is very similar to C/EBPα.3 The differential expression of C/EBPε mRNA and protein in P388 cells suggests that expression of the C/EBPε gene may be controlled at the post-transcriptional levels in certain cell types. The mechanism underlying this mode of regulation is unclear but does not appear to involve short open reading frames such as those proposed to modulate translation of both the C/EBPα and C/EBPβ mRNAs (28).

Myeloid Specific Expression and Functions of C/EBPε—The highly restricted pattern of expression of C/EBPε in both mouse and human strongly supports the idea that its primary function is within the myeloid lineage of hematopoietic cells. However, before detailing the putative myeloid-specific functions of C/EBPε, it may be worthwhile to consider the possibility that it may also be expressed, and function, outside the myeloid lineage. For example, C/EBPε mRNA has been detected at low levels in tissues such as mouse lung or human ovary (27), although these signals could theoretically be due to the presence of blood cells. To date, there are two exceptions to the myeloid-specific expression of C/EBPε in hematopoietic cell lines, namely the human Jurkat T cell line (27) and murine P388 lymphoblasts. We have confirmed the presence of C/EBPε mRNA in Jurkat cells; however, we have not detected C/EBPε mRNA in any other human lymphoid cell lines (data not shown), and it is not expressed at significant levels in the thymus. The C/EBPε gene has been mapped in both human and mouse and is located on human and mouse chromosome 14 close to a compound locus containing the T cell receptor α/δ genes and other genes expressed in T cells (27, 29). Multiple rearrangements within this region have been associated with T cell leukemias or lymphomas in humans, and it is possible that the presence of C/EBPε mRNA in Jurkat cells is related to the origin or activation state of this T cell line (30). The significance of C/EBPε mRNA in P388 cells is unclear, particularly as Western analysis, gel shift assays, and functional tests indicate that C/EBPε protein is not expressed in these cells. However, the strong expression of C/EBPε in mouse spleen, a major source of B cells, and the reported existence of a common precursor for both macrophages and B cells (31), may indicate a role for C/EBPε in B cell development and/or function.

Redundant Functions of C/EBP Proteins: the Involvement of C/EBPε in Cytokine and Chemokine Gene Expression—The promoters of many cytokine genes contain composite DNA elements consisting of binding sites for C/EBP proteins and NFκB transcription factors that are required for activation of these promoters during inflammatory responses (32). Functional cooperativity between C/EBP proteins and NFκB requires physical interaction between their bZip and Rel homology domains, respectively (33, 34). A number of lines of evidence, including the demonstration that ectopic expression of C/EBPβ in the P388 lymphoblastic cell line conferred LPS responsiveness onto the IL-6 and MCP-1 genes (20), suggested that C/EBPβ was the primary family member involved in cytokine gene regulation. However, it now appears that other family members must be capable of participating in cytokine gene regulation in vivo because LPS-inducible expression of most cytokine genes, including IL-6, is not greatly affected in C/EBPε null mice (13). C/EBPε is a good candidate for performing these functions in mice because, like C/EBPβ, it is capable of conferring LPS inducibility on both IL-6 and MCP-1 genes in P388 cells. The generation of mice carrying targeted mutations in multiple C/EBP genes should address the relative importance of each protein in cytokine gene regulation.

C/EBPε and C/EBPβ were also both capable of activating expression of the genes encoding the chemokines MIP-1α and MIP-1β. The chemokines are a family of small secreted molecules that attract white blood cells to sites of inflammation (35) and are expressed by a number of hematopoietic cell types including macrophages and activated B and T cells (36). Expression of MIP-1α and MIP-1β is activated in macrophages by inflammatory inducers, including LPS and interferon-γ (37), and C/EBP-binding sites have been identified in the promoter of the MIP-1α gene (10). Ectopic expression of C/EBPε and C/EBPβ increased basal level expression of both MIP-1α and MIP-1β genes in P388 cells but did not affect LPS-dependent regulation. These results implicate C/EBP proteins, including C/EBPε, as regulators of basal chemokine gene expression in specific cell types but suggest that LPS-dependent stimulation of chemokine genes versus other cytokines such as IL-6 and MCP-1 is regulated via different mechanisms.

C/EBPε as a Critical Regulator of Macrophage Development: Activation of M-CSFR Expression—The specific functions of each C/EBP protein within the hematopoietic system are rapidly being determined through the combinatorial use of experimentally manipulatable cell culture systems and the disruption of C/EBP genes in mice. From these studies, it has been shown that the C/EBP proteins are expressed in temporally

3 N. Angerer and S. C. Williams, unpublished observations.
distinct patterns during myeloid differentiation and have implicated C/EBPα as a critical regulator of neutrophil development and C/EBPβ as an important factor for macrophage function (3, 4, 6, 21). The activation of C/EBPβ expression in EML-C1 cells at a critical time point during myeloid differentiation when colony-forming units (granulocyte/macrophage) first become evident in the culture, along with the coordinate activation of M-CSFR expression, has led us to predict that C/EBPβ may be a critical regulator of macrophage development. This hypothesis is further supported by the specific activation of M-CSFR gene expression by C/EBPβ in P388 cells, when compared with either C/EBPα and C/EBPβ. The promoter of the M-CSFR gene also contains binding sites for PU.1, AML-1, and probably other unidentified factors, and the activation of M-CSFR expression may be dependent upon selective interactions between C/EBPβ and other promoter-bound transcription factors. Elucidation of the specific functions of C/EBPβ during myelopoiesis should be achieved through analysis of cellular systems such as EML-C1 in combination with the disruption of the C/EBPβ gene in mice.

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C/EBPα and Myeloid Gene Expression

13501