**PHAGOCYTES**

Myeloid Transcription Factor C/EBPε Is Involved in the Positive Regulation of Lactoferrin Gene Expression in Neutrophils

By Walter Verbeek, Julie Lekstrom-Himes, Dorothy J. Park, Pham My-Chan Dang, Peter T. Vuong, Seji Kawano, Bernard M. Babior, Kleanthis Xanthopoulos, and H. Phillip Koeffler

Targeted mutation of the myeloid transcription factor C/EBPε in mice results in gram-negative septic death at 3 to 5 months of age. This study defines the underlying molecular defects in their terminal granulocytic differentiation. The mRNA for the precursor protein of the cathelin-related antimicrobial peptides was almost completely absent in the bone marrow cells of C/EBPε−/− mice. This finding may help explain their susceptibility to gram-negative sepsis, because both are bacteriocidal peptides with potent activity against gram-negative bacteria. Superoxide production was found to be reduced in both granulocytes and monocytes of C/EBPε−/− mice. While gp91 phox protein levels were normal, p47phox protein levels were considerably reduced in C/EBPε−/− granulocytes/monocytes, possibly limiting the assembly of the NADPH oxidase. In addition, expression of mRNA of the secondary and tertiary granule proteins, lactoferrin and gelatinase, were not detected, and levels of neutrophil collagenase mRNA were reduced in bone marrow cells of the knock-out mice. The murine lactoferrin promoter has a putative C/EBP site close to the transcription start site. C/EBPε bound to this site in electromobility shift assay studies and mutation of this site abrogated binding to it. A mutation in the C/EBP site reduced the activity of the promoter by 35%. Furthermore, overexpression of C/EBPε in U937 cells increased the activity of the wild-type lactoferrin promoter by 3-fold. In summary, our data implicate C/EBPε as a critical factor of host antimicrobial defense and suggests that it has a direct role as a positive regulator of expression of lactoferrin in vivo.

© 1999 by The American Society of Hematology.

**MATERIALS AND METHODS**

Expression vectors and promoter reporter constructs. Eukaryotic expression vectors for the C/EBPε isoform p32 and murine C/EBPε have been described. The lactoferrin promoter (−750 to +39) was amplified using the polymerase chain reaction (PCR) with primers containing KpnI (5′) and BgII (3′) restriction sites. The PCR product was digested with KpnI and BgII, agarose gel purified, and ligated into the KpnI/BgII digested luciferase reporter plasmid pGL3 basic (Promega, Madison, WI). The construct was sequenced to confirm its identity to the published sequence (−750 to +39). A shorter construct (−230 Lac-Luc) was derived from this template by PCR using

From the Division of Hematology/Oncology, the Department of Medicine, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA; Clinical Gene Therapy Branch, National Institutes of Health, Bethesda, MD; the Division of Biochemistry, the Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA; and Aurora Biosciences Inc, San Diego, CA.

Submitted December 3, 1998; accepted June 14, 1999.

Supported in part by the following grants: NIH/C/EBPε, NIH-Genetic Core, Lichtenstein for Leukemia Research, Parker Hughes Grant, and C. and H. Koeffler Grant. W.V. was supported by a Grant of the Deutsche Forschungsgemeinschaft.

Address reprint requests to Dr. H. Phillip Koeffler, MD, Cedars-Sinai Medical Center, Burns and Allen Research-Institute, UCLA School of Medicine, Davis Research Bldg, Room D 5066, 8700 Beverly Blvd, Los Angeles, CA 90048.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

0006-4971/99/9409-0022$3.00/0
Pfu Polymerase. A mutation of the C/EBP site was introduced into the wild-type template by PCR mutagenesis with Pfu polymerase and the mutagenic primer: 5’ GGG TGT CTA TCT GAC GAC AGG GCG GG 3’ according to the method of Picard et al.14 Sequencing primers derived from pGL-3 basic were used as 5’ (pGL-2) and 3’ (RV3) primers for the PCR reaction. The PCR product was digested with KpnI and BglII and religated into the vector. A short construct only containing the proximal 87 bp of the lactoferrin promoter in the KpnI/BglII restriction sites of pGL3 basic was kindly provided by Dr Nancy Berliner (Division of Hematology, Yale School of Medicine, New Haven, CT).

Animals. C/EBPβ−/− mice and control 129/SvEv × NIH Black Swiss mice were bred under sterile conditions in the animal housing facility at the Burns and Allen Research Institute. Mice were killed by cervical neck dislocation. Bone marrow was flushed out of isolated femurs and tibiae with Iscove’s Modified Dulbecco’s Medium (IMDM) + 20% fetal calf serum (FCS) using a no. 26 gauge needle. After depletion of adherent cells by incubation on plastic dishes for 1 hour, cells were spun down and immediately diluted in Trizol reagent. RNA was isolated according to the manufacturer’s protocol.

The bone marrow cells taken for RNA isolation contained less than 1% monocytes and approximately 25% Gr-1 positive cells in wild-type and knock-out mice. Neutrophils were harvested 4 hours after intraperitoneal injection with 2 mL 4% thioglycollate by peritoneal lavage and resuspended in 50 µL DEPC-treated H2O.

Northern blot analysis. Twenty micrograms of RNA of C/EBPβ−/− bone marrow, control bone marrow, and NIH3T3 fibroblasts were run on a denaturing formaldehyde gel at 30 V for 3 hours. RNA was partially hydrolysed by soaking the gel in 0.05 mol/L NaOH/1.5 mol/L NaCl for 30 minutes followed by soaking 30 minutes in 0.5 mol/L TrisCl (pH7.4) for pH neutralization. The gel was blotted in 20X sodium chloride sodium citrate solution (SSC) overnight on a nylon membrane (Magna Charge; Micron Separations Inc, Westborough, MA). The membrane was baked for 1 hour at 80°C in a vacuum oven and crosslinked in a UV crosslinker (Stratagene, La Jolla, CA). Prehybridization and hybridization of the membrane were performed in Rapid Hyb hybridization buffer (Amersham, Arlington Heights, IL) at 65°C for 1 hour and 3 hours, respectively. Posthybridization washes were performed with 2X SSC, 0.1% sodium dodecyl sulfate (SDS) (SSC) for 20 minutes at room temperature and twice with 0.1X SSC, 0.1% SDS at 65°C for 15 minutes each.

Plasmids containing the cDNAs for lactoferrin (probe: PromI/Smal fragment), neutrophil gelatinase (probe: EcoRI/BamHI fragment), and neutrophil collagenase (BglII/PstI fragment) were kindly provided by Dr M. Dinauer (Nrom Institute for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN). A plasmid encoding the cDNA for murine gp91 phox (probe: Ncol fragment) was kindly provided by Dr M. Dinauer (Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN). The probe for murine cathelin-related protein (MCRP) was generated by PCR. The PCR product (200 bp) was gel purified and directly sequenced using the Perkin Elmer BglI and PstII restriction enzymes. After a 15-minute incubation on ice, the nuclei were pelleted at 2500 g for 10 minutes. Nuclei were resuspended in extraction buffer B, and then probed with gp91phox (dilution, 1:2,000) or p47 phox antiserum (dilution, 1:1,000). Antibodies to murine gp91 phox was kindly provided by Dr M. Dinauer.16 Antiserum to murine gp91 phox was described previously.17 Blots were then incubated with biotinylated anti-mouse/anti-rabbit IgG secondary antibody (dilution 1:3,000; Vector Laboratories Inc, Burlingame, CA) followed by peroxidase conjugated streptavidin (BioGenex, San Ramon, CA) as recommended by the manufacturers. All the blots were reprobed with mouse antiarbit GAPDH antibody (1:4,000 dilution) (Research Diagnostics Inc, Flanders, NJ) to ensure equal loading of samples. Signals were detected with Supersignal Chemiluminescent substrate (Pierce, Rockford, IL). Antibody incubations and wash steps were performed with TBS-T (0.05% Tween 20) + 5% nonfat dry milk.

Preparation of nuclear extracts. For preparation of nuclear extracts, 5 × 10⁷ cells were washed 3 times with ice-cold PBS. After the last wash, adherent cells were scraped off the dish with a rubber policeman and resuspended in 500 µL extraction buffer B (20 mM HEPES, pH 7.9, 20% glycerol, 10 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.1% Triton X, 1 mM dithiothreitol [DTT], 1 mM PMSF, 40 µg/mL Complete [Boehringer, Indianapolis, IN]). After a 15-minute incubation on ice, the nuclei were pelleted at 250g for 10 minutes. Nuclei were resuspended in extraction buffer B, and NaCl was added dropwise with mixing to a final concentration of 300 mM NaCl. Nuclei were rocked for 60 minutes at 4°C. Samples were microcentrifuged at 12,000 rpm and supernatants frozen at −80°C.

Electromobility shift assay (EMSA). For protein expression, 10 µg of eukaryotic expression vector (C/EBPβ, C/EBPα) was transfected into pairs listed in Table 1. The annealing temperature for rac-1 amplification was 52°C, and for rac-2 64°C. PCR products were blotted by alkaline transfer in 400 mM NaOH on a nylon membrane and probed with 32P-γATP end-labeled internal oligonucleotides as indicated. In Table 1, primer sequences are given as cDNA sequence positions.

Western blot. 10⁷ cells were collected at either 12 or 72 hours after intraperitoneal injection of 4% thioglycollate into 3-week-old C/EBPβ−/− and wild-type mice. Cells were washed once in ice-cold phosphate-buffered saline (PBS) and then treated with 2.7 µmol/L DFP (diisopropyl fluorophosphate; Calbiochem, La Jolla, CA) for 10 minutes on ice, then washed 3 times in ice-cold PBS and lysed in Triton buffer (20 mM NaCl, 0.5% NP-40, 0.1% SDS, pH 7.4). The lysates were loaded in each lane of a 12% polyacrylamide gel. After electrotransfer to a nitrocellulose membrane, membranes were incubated with biotinylated anti-mouse/anti-rabbit IgG secondary antibody (dilution 1:3,000; Vector Laboratories Inc, Burlingame, CA) followed by peroxidase conjugated streptavidin (BioGenex, San Ramon, CA) as recommended by the manufacturers. All the blots were reprobed with mouse antiarbit GAPDH antibody (1:2,000 dilution) (Research Diagnostics Inc, Flanders, NJ) to ensure equal loading of samples. Signals were detected with Supersignal Chemiluminescent substrate (Pierce, Rockford, IL). Antibody incubations and wash steps were performed with TBS-T (0.05% Tween 20) + 5% nonfat dry milk.

Preparation of nuclear extracts. For preparation of nuclear extracts, 5 × 10⁷ cells were washed 3 times with ice-cold PBS. After the last wash, adherent cells were scraped off the dish with a rubber policeman and resuspended in 500 µL extraction buffer B (20 mM HEPES, pH 7.9, 20% glycerol, 10 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 0.1% Triton X, 1 mM dithiothreitol [DTT], 1 mM PMSF, 40 µg/mL Complete [Boehringer, Indianapolis, IN]). After a 15-minute incubation on ice, the nuclei were pelleted at 250g for 10 minutes. Nuclei were resuspended in extraction buffer B, and NaCl was added dropwise with mixing to a final concentration of 300 mM NaCl. Nuclei were rocked for 60 minutes at 4°C. Samples were microcentrifuged at 12,000 rpm and supernatants frozen at −80°C.

Electromobility shift assay (EMSA). For protein expression, 10 µg of eukaryotic expression vector (C/EBPβ, C/EBPα) was transfected into

== Table 1. cDNA Positions of Primer Sequences Used for RT-PCR ==

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (Sense)</th>
<th>Primer (Antisense)</th>
<th>Internal Oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO</td>
<td>1027-1045</td>
<td>1410-1427</td>
<td>1218-1238</td>
</tr>
<tr>
<td>Cathespin G</td>
<td>255-272</td>
<td>667-690</td>
<td>393-412</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>259-276</td>
<td>814-831</td>
<td>502-524</td>
</tr>
<tr>
<td>CRAMP</td>
<td>49-68</td>
<td>291-310</td>
<td></td>
</tr>
<tr>
<td>B9</td>
<td>190-209</td>
<td>411-430</td>
<td>282-302</td>
</tr>
<tr>
<td>gp91phox</td>
<td>313-331</td>
<td>682-699</td>
<td>501-533</td>
</tr>
<tr>
<td>p22phox</td>
<td>26-44</td>
<td>596-615</td>
<td>316-338</td>
</tr>
<tr>
<td>p40phox</td>
<td>262-282</td>
<td>786-808</td>
<td>561-584</td>
</tr>
<tr>
<td>p47phox</td>
<td>140-160</td>
<td>799-821</td>
<td>500-523</td>
</tr>
<tr>
<td>p67phox</td>
<td>328-349</td>
<td>919-939</td>
<td>566-578</td>
</tr>
<tr>
<td>rac-1</td>
<td>201-220</td>
<td>421-440</td>
<td>303-320</td>
</tr>
<tr>
<td>rac-2</td>
<td>187-208</td>
<td>377-398</td>
<td>305-324</td>
</tr>
<tr>
<td>fMLR P</td>
<td>44-63</td>
<td>235-255</td>
<td></td>
</tr>
<tr>
<td>Interleukin-8 R</td>
<td>222-242</td>
<td>641-660</td>
<td></td>
</tr>
</tbody>
</table>

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
COS-1 cells (10-cm dish). Transfection was performed with Superfect reagent (Qiagen, Valencia, CA) over 3 hours according to the manufacturer’s protocol. Nuclear extracts were prepared after 48 hours. Alternatively, recombinant maltose binding protein (MBP-C/EBPb fusion protein was expressed in B.l.21 bacteria and purified with amylose resin as described previously. Two fragments of the cloned 5’/3’ fusion protein was expressed in Bl 21 bacteria and purified with C/EBPα 4% polyacrylamide gel at 30 mA.

COS-1 nuclear extract expressing either C/EBPα or C/EBPb, 2 µg pdIdC, and 4.5 µg BSA in a 20-µL volume. Competing cold oligonucleotides (shown below at 10- and 100-fold molar excess) or antibodies (1 pdIdC, and 4.5 µg BSA in a 20-µL volume. Competing cold oligonucleotides (shown below at 10- and 100-fold molar excess) or antibodies (1 µg/µL) were added where indicated. Electrophoresis was performed on a 4% polyacrylamide gel at 30 mA.

**RESULTS**

The morphological changes in the neutrophils of C/EBPα/β mice suggest that C/EBPα transactivates target genes important in late myeloid differentiation. Figure 1 compares the ultrastructural morphology of peripheral blood granulocytes from C/EBPα/β and wild-type mice. The EM study shows signs of cytoplasmic immaturity in C/EBPα/β peripheral blood granulocytes. Rod-shaped tertiary granules are completely missing, the ratio of primary to secondary granules is increased, and the number of mitochondria/cell is higher in C/EBPα/β–/– granulocytes.

A striking feature of C/EBPα/β–/– granulocytic cells is their reduced superoxide production. To examine this observation further, we determined the degree of this defect by using a quantitative ferricytochrome c assay. We compared the superoxide production of normal mouse bone marrow cells with C/EBPα/β–/– bone marrow cells. Both samples contained approximately 20% Gr-1–positive granulocytic cells, 2 × 10^6 C/EBPα/β–/– bone marrow cells did not produce detectable superoxide anion levels. Under the same conditions, the identical number of normal mouse bone marrow cells produced 1.3 ± 1.27 nmol superoxide anions/min. Granulocytes and monocytes collected by peritoneal lavage at 12 and 72 hours after injection of 4% thioglycollate into the peritoneal cavity produced 5.95 and 6.5 nmol/min, respectively, in the wild-type and 1.09 and 0.38 nmol/min, respectively, in C/EBPα/β–/– mice. Due to a defect in the migratory function of C/EBPα/β–/– granulocytes, their percentage in the peritoneal cavity of these mice was lower than was found in the wild type (WT) at both 12 hours (C/EBPα/β–/–: 56% v WT: 70%) and 72 hours (C/EBPα/β–/–: 6% v WT: 75%) (Table 2). Normal PMA activated monocytes are known to generate approximately 1/3 the amount of O_2^- compared with PMA-stimulated granulocytes. The difference in cell composition in the samples collected at 12 hours (56% v 70% granulocytes in intraperitoneal [i.p.] lavage from C/EBPα/β–/– v C/EBPα/β+/+, respectively) would not account for the difference in superoxide anion production between the cells of the knock-out and wild-type mice. Interestingly, the superoxide production of a population of 94% pure, C/EBPα/β–/– monocytes/macrophages (2 × 10^6 cells) was only 0.38 nmol/min, indicating that the defect may also affect C/EBPα/β–/– monocytes. (For comparison: O_2^- production of normal murine monocytes/macrophages [2 × 10^6]: 1 to 1.8 nmol/min[20].)

Because the NADPH oxidase system is mainly responsible for superoxide production in phagocytic cells, we examined the expression of its components. Northern blot analysis showed that the expression of the murine gp91 phox gene is not reduced in the monocyte-depleted bone marrow of C/EBPα/β–/– mice (Fig 2A, right panel). Semiquantitative RT-PCR studies with bone marrow cells from mutant and wild-type animals also showed no difference for the other members constituting the NADPH oxidase complex: p22phox, p40phox, p47phox, p67phox (Fig 2A, left panel) as well as rac-1 and -2 (Fig 2B). Protein expression of gp91 phox and p47 phox was measured by Western blot analysis in wild-type and C/EBPα/β–/– granulocytes/macrophages (Fig 2C). Cells collected from the peritoneal cavity of 4 wild-type and 4 C/EBPα/β–/– mice at either 12 or 72 hours after the intraperitoneal injection of 4% thioglycollate were pooled at each time point to determine the mean number of granulocytes and monocytes and to extract protein. Gp 91 phox was found to be normally expressed in C/EBPα/β–/– granulocytes/macrophages collected at 12 hours. At 72 hours the level of gp91phox expression was higher in the C/EBPα/β–/– sample. This sample contained 94% monocytes as opposed to only 6% in the wild type. The differences observed could result from a higher expression of gp91phox in monocytes/macrophages. These experiments were repeated 3 times and each demon...
strated that the protein expression of p47phox was considerably reduced in C/EBP\textsuperscript{e2/2} phagocytes collected at either 12 or 72 hours (Fig 2C). This effect was most prominent in the sample collected at 12 hours, in which the majority of collected cells were granulocytes: wild-type, 70%; C/EBP\textsuperscript{e2/2}, 56%.

To elucidate further the molecular defects in antimicrobial activity of C/EBP\textsuperscript{e2/2} animals, we tested the cathelin protein family that is important for the generation of antibacterial peptides. The peptides CRAMP1 and CRAMP2, both derived from cathelin-related protein (MCRP), have bactericidal activity, especially against gram-negative bacteria.\textsuperscript{15,27} Northern blot analysis shows that the 1-kb mRNA of the cathelin-related protein is virtually absent in C/EBP\textsuperscript{e2/2} bone marrow in comparison to a very strong expression in wild-type mice (Fig 3). Also, the expression of a second murine cathelin homolog (B9 protein) was reduced in the C/EBP\textsuperscript{e2/2} bone marrow (Fig 3). This protein is normally expressed in murine promyelocytes.\textsuperscript{28}

C/EBP\textsuperscript{e2/2} granulocytic cells were shown to exhibit an impaired migration.\textsuperscript{1} Expression of receptors for both interleu-
kin-8 and N-formyl-methionyl-leucyl phenylalanine (fMLP) are required for normal granulocytic migration. Therefore, we examined the expression of these receptors by RT-PCR. Both receptors were expressed in granulocytic cells of C/EBPε−/− mice isolated 4 hours after i.p. thioglycollate injection (data not shown).

Consistent with the morphological conservation of primary granules in C/EBPε−/− granulocytic cells as observed by electronmicroscopy, RT-PCR showed that the primary granule proteins, myeloperoxidase and cathepsin G, were expressed in the bone marrow cells (Fig 4A). The slightly stronger signal detected in C/EBPε−/− bone marrow most likely reflects myeloid hyperplasia. This notion is reinforced by the increased number of myeloperoxidase (MPO)-positive bone marrow cells seen with MPO immunohistochemistry (data not shown).

A hallmark of late myeloid differentiation is the expression of secondary (specific) granules containing lactoferrin, neutrophil gelatinase, neutrophil collagenase, and transcobalamin I32 and tertiary (gelatinase) granules defined by their high concentration of gelatinase. Expression of the secondary granule proteins is at least in part transcriptionally controlled.33 In addition, the downregulation of the binding activity of CCAAT displacement protein (CDP) has been implicated in their coordinate expression.34 Northern blot analysis showed the complete absence of lactoferrin and gelatinase mRNA in C/EBPε−/− bone marrow. The mRNA level of neutrophil collagenase was considerably reduced (Fig 4B).

Therefore, we investigated whether the lactoferrin promoter is a direct target for C/EBPε. We first performed electromobility shift assays with 2 large fragments (−2750 to −300) and (−300 to −139) of the lactoferrin promoter. Recombinant MBP-C/EBPε bound to the proximal, but not the upstream fragment (data not shown). The proximal fragment contains a predicted C/EBP site at position −255. We designed a double-stranded oligonucleotide representing this putative C/EBP site which is conserved between mouse and humans.35 We expressed C/EBPε and C/EBPα protein in cos-1 cells and used 5 µg nuclear extract for the binding assay. Figure 5 shows that C/EBPε and C/EBPα bound specifically to the site. Binding was competed by cold self, but not mutated oligonucleotide. The binding complexes were supershifted by specific antisera against C/EBPε and C/EBPα, respectively. A 32P-γATP-labeled oligonucleotide mutated at the binding site did not bind the C/EBP proteins (Fig 5).

The proximal promoter fragment (−230 to +39) of lactoferrin that contained the putative C/EBP site was fused to a luciferase reporter gene. This construct was 50-fold more active than empty vector when transiently transfected into U937 cells.

| Table 2. Superoxide Anion Production by 2 × 10^6 Wild-Type and C/EBPε−/− Cells Collected From Either the Bone Marrow or Peritoneal Cavity (12 or 72 hours after intraperitoneal injection of 4% thioglycollate) |
|----------------------------------|----------------------------------|-----------------|-----------------|
| Bone marrow                      | Granulocytes (%)                 | Monocytes/ Macrophages (%) | O2 Production (nmol/min) | ± SD  | n  |
| Wild type                        | 21                               | 1                             | 1.3                          | 1.27  | 2  |
| C/EBPε−/−                        | 20                               | 1                             | 0*                           | —     | 2  |
| Peritoneal phagocytes (12 h)     | 70                               | 30                            | 5.95                         | 0.08  | 2  |
| Wild type                        | 56.5                             | 43.5                          | 1.09                         | 0.01  | 2  |
| C/EBPε−/−                        | 75                               | 25                            | 6.5                          | 0.14  | 2  |
| Peritoneal phagocytes (72 h)     | 6                                | 94                            | 0.38                         | 0.27  | 2  |

O2 production was measured by the continuous ferricytochrome c assay. Numbers represent mean and standard deviation of 2 separate experiments.

*Undetectable.
Mutation of the putative C/EBP site reduced this activity by 35% (Fig 6A). Furthermore, an 87-bp lactoferrin promoter-reporter construct was transiently transfected into U937 cells that had been engineered so that they could be induced to overexpress C/EBP because a Zn-inducible C/EBP expression vector was stably integrated into the cells (Fig 6B). Reporter gene activity was more than 3-fold greater in these U937 cells overexpressing C/EBP compared with uninduced U937 cells, suggesting indirectly that C/EBP is a positive regulator of the lactoferrin promoter in myeloid cells (Fig 6C).

**DISCUSSION**

In this study, we examined the expression of a number of myeloid-specific genes in C/EBP−/− mice to elucidate further the molecular basis of their phenotype. Although superoxide production is significantly reduced in phagocytes of C/EBP−/−
mice, we found a normal gp91 phox mRNA expression and no apparent lack of p22, p40, p47, and p67 phox, rac-1, and -2 expression, the major components of the NADPH oxidase complex. Western blot analysis did not show a decrease of gp91phox protein expression. This finding is consistent with our Northern blot data showing the equal expression of gp91phox mRNA in C/EBP\(^e-/-\) mice contrasts to its absence in the granulocytes of PU.1\(^-/-\) mice. The gp91 phox gene is known to be negatively regulated by CDP and was recently shown to be positively regulated by PU.1. The expression of the gp91phox gene in C/EBP\(^e-/-\) mice not only implies that C/EBP\(^e\) is not essential for its expression but, more interestingly, suggests that the mature fraction of C/EBP\(^e-/-\) granulocytic cells probably lacks the repressive binding activity of CDP. The CDP binding activity is.

C/EBP\(^e\) in murine monocytes are consistent with our finding that their capacity to produce superoxide is also reduced.

The normal expression of the gp91phox gene in C/EBP\(^e-/-\) mice contrasts to its absence in the granulocytes of PU.1\(^-/-\) mice. The gp91 phox gene is known to be negatively regulated by CDP and was recently shown to be positively regulated by PU.1. The expression of the gp91phox gene in C/EBP\(^e-/-\) mice not only implies that C/EBP\(^e\) is not essential for its expression but, more interestingly, suggests that the mature fraction of C/EBP\(^e-/-\) granulocytic cells probably lacks the repressive binding activity of CDP. The CDP binding activity is.

C/EBP\(^e\) in murine monocytes are consistent with our finding that their capacity to produce superoxide is also reduced.

The normal expression of the gp91phox gene in C/EBP\(^e-/-\) mice contrasts to its absence in the granulocytes of PU.1\(^-/-\) mice. The gp91 phox gene is known to be negatively regulated by CDP and was recently shown to be positively regulated by PU.1. The expression of the gp91phox gene in C/EBP\(^e-/-\) mice not only implies that C/EBP\(^e\) is not essential for its expression but, more interestingly, suggests that the mature fraction of C/EBP\(^e-/-\) granulocytic cells probably lacks the repressive binding activity of CDP. The CDP binding activity is.

C/EBP\(^e\) in murine monocytes are consistent with our finding that their capacity to produce superoxide is also reduced.

The normal expression of the gp91phox gene in C/EBP\(^e-/-\) mice contrasts to its absence in the granulocytes of PU.1\(^-/-\) mice. The gp91 phox gene is known to be negatively regulated by CDP and was recently shown to be positively regulated by PU.1. The expression of the gp91phox gene in C/EBP\(^e-/-\) mice not only implies that C/EBP\(^e\) is not essential for its expression but, more interestingly, suggests that the mature fraction of C/EBP\(^e-/-\) granulocytic cells probably lacks the repressive binding activity of CDP. The CDP binding activity is.

C/EBP\(^e\) in murine monocytes are consistent with our finding that their capacity to produce superoxide is also reduced.

The normal expression of the gp91phox gene in C/EBP\(^e-/-\) mice contrasts to its absence in the granulocytes of PU.1\(^-/-\) mice. The gp91 phox gene is known to be negatively regulated by CDP and was recently shown to be positively regulated by PU.1. The expression of the gp91phox gene in C/EBP\(^e-/-\) mice not only implies that C/EBP\(^e\) is not essential for its expression but, more interestingly, suggests that the mature fraction of C/EBP\(^e-/-\) granulocytic cells probably lacks the repressive binding activity of CDP. The CDP binding activity is.

C/EBP\(^e\) in murine monocytes are consistent with our finding that their capacity to produce superoxide is also reduced.

The normal expression of the gp91phox gene in C/EBP\(^e-/-\) mice contrasts to its absence in the granulocytes of PU.1\(^-/-\) mice. The gp91 phox gene is known to be negatively regulated by CDP and was recently shown to be positively regulated by PU.1. The expression of the gp91phox gene in C/EBP\(^e-/-\) mice not only implies that C/EBP\(^e\) is not essential for its expression but, more interestingly, suggests that the mature fraction of C/EBP\(^e-/-\) granulocytic cells probably lacks the repressive binding activity of CDP. The CDP binding activity is.
Lack of lactoferrin expression in bone marrow cells of C/EBP\(_e\) and C/EBP\(_a\) knockout mice is due to the lack of these factors and not due to transcriptional inactivity of lactoferrin promoter. We hypothesize that the lack of one of these could prevent the assembly of an active complex and thereby render all contributing factors essential. Our studies using the C/EBP\(_e\) and C/EBP\(_a\) cell line U937 can be driven to express lactoferrin by overexpression of C/EBP proteins. Furthermore, overexpression of C/EBP\(_a\) in U937 cells increases by 3-fold the already high promoter activity of the proximal 87 bp construct, indicating that C/EBP\(_a\) proteins are involved in the regulation of the lactoferrin promoter. Consequently, C/EBP\(_a\) alone is not sufficient for lactoferrin expression in vivo. Together, C/EBP\(_e\) and C/EBP\(_a\) most likely directly contributes to the transcriptional activation of the lactoferrin promoter in vivo.

Secondary granule protein deficiency was reported as a rare genetic disorder in humans. The patients are susceptible to infections, mainly with gram-positive organisms. Secondary granule protein deficiency probably contributes to the complex phenotype of C/EBP\(_e\) and C/EBP\(_a\) mice. Nevertheless, the absence of the potent bacteriocidal peptides CRAMP1 and 2 are also probably extremely important for the phenotype of C/EBP\(_e\) and C/EBP\(_a\) mice. The Northern analysis shows that the mRNA encoding the cathelin-related protein is highly abundant in normal murine bone marrow. Because murine granulocytes lack defensins, cathelins constitute a major source of bactericidal peptides in murine granulocytes. Their predominant activity against gram-positive organisms is due to cathelin-related protein deficiency probably contributes to the complex phenotype of C/EBP\(_e\) and C/EBP\(_a\) mice. Nevertheless, the absence of the potent bacteriocidal peptides CRAMP1 and 2 are also probably extremely important for the phenotype of C/EBP\(_e\) and C/EBP\(_a\) mice. The Northern analysis shows that the mRNA encoding the cathelin-related protein is highly abundant in normal murine bone marrow. Because murine granulocytes lack defensins, cathelins constitute a major source of bactericidal peptides in murine granulocytes. Their predominant activity against gram-positive bacteria matches the infectious spectrum in C/EBP\(_e\) and C/EBP\(_a\) mice. Interestingly, the promoter of the human cathelin homolog Fall 39 has a C/EBP binding site at position −154.45 This raises the possibility of a direct involvement of C/EBP in the transcriptional regulation of the cathelin-related protein.
approach to augment the ability of an individual to fight potentially serious infections.

ACKNOWLEDGMENT

We thank Dr Robert Lehrer for helpful and very thoughtful discussions and Kai Chien for excellent technical assistance.

REFERENCES

8. Antonson P, Stellan B, Yamanaka R, Xanthopoulos KG: A novel human CCAAT/enhancer binding protein gene, C/EBPs, is expressed in...
cells of lymphoid and myeloid lineages and is localized on chromosome 14q11.2 close to the T-cell receptor a/b locus. Genomics 35:30, 1996


23. Itoh T,getString()
Myeloid Transcription Factor C/EBP? Is Involved in the Positive Regulation of Lactoferrin Gene Expression in Neutrophils

Walter Verbeek, Julie Lekstrom-Himes, Dorothy J. Park, Pham My-Chan Dang, Peter T. Vuong, Seji Kawano, Bernard M. Babior, Kleanthis Xanthopoulos and H. Phillip Koeffler