HEMATOPOIESIS

C/EBPe Directly Interacts With the DNA Binding Domain of c-myb and Cooperatively Activates Transcription of Myeloid Promoters

By Walter Verbeek, Adrian F. Gombart, Alexey M. Chumakov, Carsten Müller, Alan D. Friedman, and H. Phillip Koeffler

C/EBPe is essential for granulocytic differentiation. We investigated the role of C/EBPe in the transcriptional activation of various myeloid-specific genes. We found that two C/EBPe isoforms, p32 and p30, possessing transcriptional activation domains were coexpressed in myeloid cells. Interestingly, isoform C/EBPe p30 but not p32 was differentially upregulated in NB-4 promyelocytic leukemia cells treated with retinoids. Both isoforms bound specifically to C/EBP sites in myeloid promoters. The kd for C/EBPe binding to the C/EBP site of the neutrophil elastase promoter was 4.2 nM/L. In transfection assays using the nonhematopoietic cell line, CV-1, the p32 isoform activated promoters from the myeloid-specific factor (G-CSF) receptor genes by 2.5-, 1.5-, and 1.6-fold, respectively. The p30 isoform lacked significant transcriptional activity, suggesting that other hematopoietic-specific factors were required for its function. Consistent with this prediction, transfections into the hematopoietic cell line Jurkat showed a 9.0- and 2.5-fold activation of the mim-1 promoter by the p32 and p30 isoforms, respectively.

The additional 32 NH2-terminal residues made p32 a significantly more potent transcriptional activator than p30. T lymphoblasts (Jurkat cells) and immature myeloid cells (eg, Kcl22 cells) expressed high levels of the c-myb hematopoietic transcription factor. Cotransfection of c-myb with either the p32 or p30 isoform of C/EBPe in CV-1 cells cooperatively transactivated the mim-1 promoter by 20- and 16-fold, respectively, and the neutrophil elastase promoter by 10- and 7-fold, respectively. Pulldown assays showed that each C/EBPe isoform interacted directly with the DNA binding domain of the c-myb protein. Further studies showed that Kcl22 myeloid cells only contained active C/EBPe, but not C/EBPp, C/EBPe, or C/EBPe. A mutation of the C/EBP site in the neutrophil elastase promoter markedly decreased the transcriptional cooperation of the promoter in Kcl22 myeloblasts. These results demonstrate a role for C/EBPe in regulating myeloid promoters, such as neutrophil elastase, probably through a direct interaction with c-myb.

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From the Division of Hematology/Oncology, Department of Medicine, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles CA; and the Division of Pediatric Oncology, The Johns Hopkins University, Baltimore, MD.


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Address reprint requests to H. Phillip Koeffler, MD, Cedars-Sinai Medical Center, Becker Building, Room B 213, 8700 Beverly Blvd, Los Angeles, CA 90048.

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activation of myeloid genes. This cooperation was first described for NFM (chicken C/EBPβ) and myb, which induced the expression of myelomonocytic-related genes (eg, the mim-1 and lysozyme genes) even in nonmyeloid cells, underlining their importance as a myeloid differentiation switch. Mamalian C/EBPβ also synergizes with v-myb in the transactivation of mim-1. Mink et al showed that both proteins interact with each other via their DNA binding domains. Recently, the cooperation of the two transcription factors was linked to their interaction with the coactivator protein p300. The coactivators p300 and the closely related CREB binding protein (CBP) are involved in translocation t(11;22) and t(8;16), respectively, which are associated with acute myeloid leukemia. Although C/EBP proteins are presently not known to be directly involved in leukemia-associated translocations, a direct interaction between C/EBPα and the leukemia-specific fusion protein AML1-ETO has been shown to interfere with C/EBPα-mediated transactivation of myeloid genes.

Knock-out murine studies showed that C/EBPα, C/EBPβ, and C/EBPδ are critical for normal myelopoiesis. The C/EBPα−/− mice developed a complex phenotype reflecting the wide expression pattern of C/EBPα. In the hematopoietic system, they exhibit a maturation arrest in myelopoiesis at the stage of the immature myeloblasts. The C/EBPβ−/− mice displayed predominantly defects in macrophage function. The C/EBPδ deletional mice have aberrations in both the neutrophilic and eosinophilic lineages. Granulocytes of C/EBPδ−/− mice showed dysplastic features and are not completely functional. These animals died between 3 and 5 months after birth due to infectious complications.

In this study, we addressed several important questions about the structure and function of C/EBPα. Are both isoforms (p32 and p30) of C/EBPα expressed in myeloid cells? Do they differ in their ability to activate transcription in hematopoietic cells? Do C/EBPδ p32 and p30 bind to and transactivate myeloid promoters such as mim-1 and neutrophil elastase? Finally, does the C/EBPδ p32 or p30 form of C/EBPα directly interact and cooperatively activate transcription with c-myb?

**MATERIALS AND METHODS**

Eukaryotic expression vectors and promoter-reporter gene constructs. The construction of the C/EBPα (p30) expression vector and the amino-terminal truncation mutants were described previously. Eukaryotic expression vectors for the C/EBPα isoforms p32, p217, p14, and murine C/EBPα were a generous gift from Dr K. Xanthopoulos (Aurora Inc, San Diego, CA). The pCMV4 c-myb expression vector was kindly provided by Linda Shapiro (St Jude’s Hospital, Memphis, TN), and the HA epitope-tagged CBP expression vector was from R. Goodman (Vollum Institute, Oregon Health Sciences, University of Oregon, Portland, OR). Neutrophil elastase promoter-luciferase constructs were described previously. The Mim-1 promoter (~240 to +150) luciferase construct was a kind gift from Dr A. Leutz (Max Delbrueck Center for Molecular Medicine, Berlin, Germany).

Preparation of nuclear extracts and Western blotting. For preparation of nuclear extracts, 5 × 10⁶ COS-1 cells were washed three times with ice-cold phosphate-buffered saline (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 mmol/L HEPES, pH 7.9, 20% glycerol, 10 mmol/L NaCl, 0.2 mmol/L EDTA, 1.5 mmol/L MgCl₂, 0.1% Triton X-100, 1 mmol/L diithiothreitol [DTT], 1 mmol/L phenylmethyl sulfonyl fluoride [PMSF], 40 µL/mL Complete [Boehringer, Indianapolis, IN]). After 15 minutes of 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 mmol/L NaCl. Nuclei were rocked for 60 minutes at 4°C. Samples were microcentrifuged at 12,000 RPM and supernatants were frozen at −80°C.

A total of 10⁶ Kcl22, U937, KG-1, and NB4 cells were washed twice in ice-cold PBS and subsequently lysed in RIPA buffer supplemented with protease inhibitors. NB4 cells (5 × 10⁶) were grown in RPMI + 10% fetal bovine serum (FBS) + 10−6 mol/L all-trans-retinoic acid for 24 hours before protein extracts were prepared in RIPA buffer.

Nuclear extracts (15 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel. The gel was electroblotted on a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was blocked with 1% gelatin and incubated with the primary antibody (rabbit polyclonal antibody, dilution 1:1,000 to 1:2,000) and a secondary horseradish peroxidase-conjugated donkey antirabbit antibody (Amersham, Arlington Heights, IL). Detection was performed using the ECL detection kit (Amersham).

The generation of affinity-purified rabbit polyclonal C/EBPα antibody against amino acids 1-115 of C/EBPα p30 was described previously. Rabbit polyclonal antibodies to C/EBPα, C/EBPβ, and murine c-myb, epitope (aa 98-108) of influenza hemagglutinin protein as well as CBP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**In vitro transcription and translation.** C/EBPα and C/EBPδ genes cloned downstream of the T7 promoter in pCDNA I/III (Invitrogen, Carlsbad, CA) were transcribed with T7 RNA polymerase and in vitro translated in rabbit reticulocyte lysate using the TNT coupled Reticulocyte lysate system (Promega, Madison, WI) with 35S methionine (>1,000 Ci/mmol; Amersham) as a label in the translation reaction. The procedure was performed according to the manufacturer.

**Pulldown assays.** A fusion of the gene coding for maltose binding protein (MBP) and C/EBPα was constructed using the pMalc2 plasmid (New England Biolabs, Beverly, MA) as a backbone. C/EBPα cDNA (p30) was cloned in-frame downstream of the MBP sequence. MBP-C/EBPα, GST-C/EBPα-1-115 (amino-acids 1-115 of C/EBPα p30), and GST were expressed in the bacterial host BL21. GST-c-myb and truncation mutants were kindly provided by Dr Timothy Bender and expressed as previously described. Soluble protein was prepared by sonication of the bacterial culture 4 hours after induction with 100 mmol/L isopropyl β-D thiogalactopyranoside (IPTG). Amylose resin (New England Biolabs) or glutathione sepharose beads (Pharmacia Biotech, Uppsala, Sweden) were loaded with equal amounts of the different fusion proteins. The loaded amylose resin and the glutathione sepharose beads were washed three times with column buffer (20 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 0.1mmol/L DTT) and then incubated 2 hours either with COS-1 cell nuclear extract expressing either the c-myb or C/EBPα protein or with in vitro translated 35S-methionine labeled C/EBPα protein. The resin (MBP fusion) or the beads (GST fusions) were washed three times with pulldown assay buffer (150 mmol/L NaCl, 20 mmol/L Tris-Cl, pH 7.5, 0.3% NP40, 0.1 mmol/L EDTA, 1.0 mmol/L DTT, 1.0 mmol/L PMSF, 10 µg/mL leupeptin and pepstatin) and resuspended in 25 µL 1× SDS-PAGE sample buffer. The samples were boiled for 5 minutes and separated by electrophoresis on a 4% to 15% gradient SDS polyacrylamide minigel (Bio-Rad, Hercules, CA). The gel was electroblotted overnight and probed with antibody against either c-myb or C/EBPα. When using 35S-methionine labeled proteins, the gel was directly fixed in 40% methanol/10% acetic acid; dehydrated in 10% methanol, 2.5% acetic acid, and 2.5% glycerol; dried; and exposed overnight to Kodak Biomax X-ray film (Eastman Kodak, Rochester, NY).
Electromobility shift assay (EMSA). Double-stranded oligonucleotides (30 bp) containing the C/EBP consensus site of a specific promoter and adjacent sequences were end-labeled with [γ-32P]ATP. A standard reaction contained 1 ng labeled probe, 10 µg COS-1 nuclear extract expressing either C/EBPα or C/EBPβ, 2 µg polydI:C, and 4.5 µg bovine serum albumin (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. Neutrophil elastase: 5′ TCGAGGC-CAGGATGGGCAATAAC3′; mutant neutrophil elastase: 5′ TCGAGGCGAGACTGGGATACACCG3′; G-CSF receptor: 5′ AAGGTTTGGCAATCCAGC3′.

Transient transfections. For protein expression, 10 µg of eukaryotic expression vector (C/EBPα, C/EBPβ, c-myb, and C/EBP-α HA) was transfected into COS-1 cells (10-cm dish). Transfection was performed with Superfect reagent (Qiagen) as described above. Positive controls. Cells were washed with PBS and fed with DMEM + 10% FCS. Transfection was performed with Superfect reagent (Qiagen) over 3 hours according to the manufacturer’s protocol. Nuclear extracts were prepared after 48 hours, as described above.

For reporter gene assays, CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) + 10% fetal calf serum (FCS). Approximately 5 × 10⁵ cells/dish were plated in 60-mm dishes at 1 day before transfection. Promoter-reporter constructs (5 µg) and expression vectors (100 ng to 1 µg) were added where indicated. Electrophoresis was performed on a 4% polyacrylamide gel at 30 mA. Neutrophil elastase: 5′ TCGAGGC-CAGGATGGGCAATAAC3′; mutant neutrophil elastase: 5′ TCGAGGCGAGACTGGGATACACCG3′; G-CSF receptor: 5′ AAGGTTTGGCAATCCAGC3′.

RESULTS

Myeloid leukemia cell lines express two transcriptionally active C/EBPα isoforms: p32 and p30. Two translation start sites were previously described for C/EBPα. The first generates a protein with a predicted molecular weight of 32.2 kD (p32 isoform); the second in-frame ATG generates a protein that is 32 amino acids shorter (p30 isoform). We examined myeloid leukemia cell lines for the expression of these two isoforms by Western blot analysis and found that both isoforms were present in the myeloid cell lines Kc122, NB4, and U937 at a ratio of approximately 2:1 (p32:p30). Cell line KG-1 did not express C/EBPα (Fig 1). We previously reported the upregulation of C/EBPα during granulocytic differentiation of cell line NB-4 with retinoid treatment. Interestingly, we find that only the p30 isoform of C/EBPα was upregulated 24 hours after treatment of NB4 cells with 10⁻⁶ mol/L all-trans retinoic acid. The level of isoform p32 remained unchanged, indicating that both isoforms are differentially regulated (lane 7). For comparison, C/EBPα p32 (lane 1) and p30 (lane 2) proteins expressed in transfected COS-1 cells were run alongside the nuclear extracts from myeloid cell lines.

C/EBPα binds to C/EBP sites in myeloid promoters. Because the DNA binding domains of C/EBP proteins are highly homologous, we hypothesized that the DNA binding site of C/EBPα is either similar or identical to the known sites of other C/EBP proteins. Double-stranded oligonucleotides representing C/EBPα sites of the neutrophil elastase and G-CSF receptor promoter were used for EMSA. C/EBPα (p32/p30) expressed in COS-1 cells was able to bind to both sites and binding was competed by cold self oligonucleotide and supershifted by antibody to C/EBPα. A similar binding was seen with C/EBPβ expressed in COS-1 cells (Fig 2A). Also, C/EBPα present in nuclear extracts of the myeloid cell line Kc122 bound to the C/EBPα site of the neutrophil elastase promoter (Fig 2B). The complex was supershifted by the affinity-purified C/EBPα antiserum and its formation was inhibited by the commercially available antibody against CRP1 (rat homolog of C/EBPα), demonstrating that the complex contained C/EBPα (Fig 2B, lanes 7 and 8). The complex could not be supershifted by antibodies against C/EBPα, C/EBPβ, and C/EBPδ.
lanes 9 through 11), indicating that these proteins are not present in nuclear extracts of the Kcl22 cell line.

Because the expression of C/EBPα and C/EBPε partly overlaps during myeloid differentiation and because both proteins can bind to the same DNA motif, we studied their relative DNA binding affinities to the C/EBP binding site of the neutrophil elastase promoter. Decreasing amounts of labeled oligonucleotides representing the C/EBP site were titrated with a constant amount of COS-1 cell extract expressing the respective C/EBP protein, and the bound complexes were separated from the free probe by EMSA (Fig 3A). The amounts of bound and free probe were quantitated with an Ambis imaging system (Ambis Inc, San Diego, CA). The values were plotted as bound/free versus bound, and the results were used to determine the dissociation constant, kd (kd = -1/slope). The kd for C/EBPε was 0.65 nmol/L and the kd for C/EBPα was 4.2 nmol/L (Fig 3B and C), showing that C/EBPε had a 6.5-fold higher affinity than C/EBPα to these C/EBP binding sequences.
**C/EBP**

**C/EBPε** p32 is a transcriptional activator in heterologous cells. The transactivation potential of C/EBPε isoforms p32 and p30 were initially examined using the promoters of the neutrophil elastase, G-CSF receptor, and mim-1 genes in heterologous cells. Expression plasmids for either C/EBPε p32 or p30 were cotransfected with promoter-luciferase reporter constructs into CV-1 cells, which do not express endogenous C/EBP proteins. Levels of C/EBPε isoforms p32 and p30 proteins after transfection with these expression vectors were shown to be similar by Western blotting of protein extracts from transfected cells (Fig 1, lanes 1 and 2). C/EBPε isoform p32 activated transcription from the neutrophil elastase promoter by 1.8-fold compared with C/EBP site mutants of the wild-type promoters. The mim-1 promoter was activated by 2.5-fold and the G-CSF receptor promoter by 1.6-fold (Fig 4A). These results are the average of three separate experiments. In all cases, the C/EBPε isoform p30 activated the promoters less efficiently than did the p32 isoform. For comparison, the same experiments were performed with C/EBPα. In CV-1 cells, C/EBPα was more active than C/EBPε for each of the promoter-reporter gene constructs (2- to 4-fold activation; Fig 4A).

Because the overall reporter gene activity for each of the constructs was low in CV-1 cells, we used Jurkat cells, a
hematopoietic cell line, to confirm a potential, concentration-independent, in vivo difference in transcriptional activation potential between C/EBP\textit{e}p32 and p30. We cotransfected Jurkat cells with expression plasmids (330 ng) for either C/EBP\textit{e}p32 or p30 with either the mim-1 or neutrophil elastase promoter luciferase construct. After 48 hours, we measured the reporter gene activity in cytoplasmic lysates and determined the C/EBP\textit{e} expression level in the nuclear lysates (Fig 4B). Reproducibly, C/EBP\textit{e}p32 was threefold to fourfold more active than p30 on the mim-1 promoter and 1.5-fold more active than p30 on the neutrophil elastase promoter. In both cases, the expression levels of p32 and p30 were similar in the transfected samples (Fig 4C). Dose-response experiments using between 125 and 1,000 ng of the expression vector and 1 µg mim-1 reporter plasmid showed that the maximal effect on reporter gene activity was reached with expression plasmid concentrations as low as 125 to 250 ng. The rank order of transactivation efficiency was C/EBP\textit{e}p32 > C/EBP\textit{e}p32 > C/EBP\textit{e}p30 (Fig 4D). C/EBP\textit{e}p32 was more potent than C/EBP\textit{e}p30 at each concentration of the expression vector.

To determine if C/EBP\textit{e} could activate transcription from these promoters in myeloid cells, the activation of the neutrophil elastase was examined in Kcl22 and U937 cells. As demonstrated (Fig 2B), the only C/EBP family member binding to the neutrophil elastase promoter in Kcl22 cells was C/EBP\textit{e}. The wild-type neutrophil elastase promoter construct was 7.7-fold more active than the empty vector, p19Luc, in Kcl22 cells and 5.5-fold more active in U937 cells. In both cell lines, the NE promoter with a mutation in the C/EBP site was 30% to 50% less active than the wild-type promoter. Taken together, these data demonstrate that C/EBP\textit{e} can contribute to the transactivation of the neutrophil elastase promoter in myeloid cells (Fig 5).

Both C/EBP\textit{e} isoforms (p32 and p30) cooperate with c-myb in the transactivation of the neutrophil elastase and mim-1 promoters. c-myb is a hematopoietic transcription factor known to cooperate with C/EBP proteins. It is highly expressed in Jurkat and Kcl22 cells (data not shown). A number of myeloid promoters have c-myb binding sites adjacent to C/EBP sites. We used the promoters from the neutrophil elastase and mim-1 genes to investigate the potential cooperation of C/EBP\textit{e}p32 and p30 isoforms with c-myb. c-myb alone activated the neutrophil elastase promoter about twofold. The C/EBP\textit{e}p32 isoform plus c-myb activated the neutrophil elastase promoter 10-fold in comparison to the empty expression vectors (Fig 6A). The C/EBP\textit{e}p30 plus c-myb activated the same promoter sevenfold. These results indicate that both C/EBP\textit{e} isoforms cooperatively activated the NE promoter with c-myb. The combination of both factors was 1.5-fold to twofold higher in activity than an additive effect. Mutation of either the C/EBP or myb sites in the neutrophil elastase promoter resulted in a loss of cooperativity between the factors (Fig 6A). This demonstrated that binding of C/EBP\textit{e} to the promoter was essential for a significant transcriptional activation. The insertion of 5 bp between the C/EBP and the myb sites to rotate the relative position of the binding sites on the double helix did not change...
the cooperativity. Insertion of 10 bp slightly reduced cooperativity (Fig 6B).

A similar cooperative effect on transactivation of the mim-1 promoter was observed (Fig 6C). The C/EBP\textsubscript{e} isoform p32 plus c-myb activated the promoter 20-fold, C/EBP\textsubscript{e}p30 plus c-myb by 16-fold, and C/EBP\textsubscript{a} plus c-myb by 37-fold. Again, both C/EBP\textsubscript{e} p32 and p30 cooperated with c-myb in transcriptional activation. Together, the combination was twofold to threefold more active than a simple additive effect.

C/EBP\textsubscript{e}p32 and p30 isoforms and c-myb can interact in vitro. To analyze the structural and functional aspects involved in the cooperativity between C/EBP\textsubscript{e} and c-myb, we cotransfected various amino-terminal C/EBP\textsubscript{e} truncation mutants with c-myb and the neutrophil elastase promoter reporter construct (Fig 7). Suprisingly, a truncated C/EBP\textsubscript{e} protein (aa 118-281) that lacked the complete transactivation domain still cooperatively activated transcription from the promoter with c-myb, although at much lower levels than the full-length C/EBP\textsubscript{e} (Fig 7). The truncation mutants alone did not transactivate the neutrophil elastase promoter (data not shown). This result implicates the carboxy-terminal half (DNA binding domain and leucine zipper) of C/EBP\textsubscript{e} in the cooperativity with c-myb.

The transcriptional cooperativity between C/EBP\textsubscript{e} and c-myb could occur via either indirect or direct interactions. We tested whether both factors could physically interact using pulldown assays. c-myb was pulled down by the MBP-C/EBP\textsubscript{e} fusion, but not by MBP alone (Fig 8A). In the reciprocal experiment, C/EBP\textsubscript{e} was pulled down by GST-c-myb but not by GST alone (Fig 8A). The same result was obtained with C/EBP\textsubscript{e} expressed in COS-1 cells or in vitro synthesized, \textsuperscript{35}S-methionine labeled C/EBP\textsubscript{e} p32/p30 protein (data not shown).

To define further the interacting domains, we used three GST fusion proteins representing different regions of c-myb: (1) the DNA binding domain (aa 1-185), (2) the DNA binding domain and the transactivation domain (aa 1-325), and (3) the negative regulatory domain (aa 326-636). Pulldown assays showed that all \textsuperscript{35}S-methionine–labeled C/EBP\textsubscript{e} isoforms (p32, p30, p27, and p14) interacted with the GST-c-myb fusions 1-185 and 1-325 containing the DNA binding domain of c-myb. No interaction occurred with either the GST-c-myb fusion 326-636 or the GST control. Furthermore, interaction of c-myb with all C/EBP\textsubscript{e} isoforms indicated that the carboxy-terminal half of C/EBP\textsubscript{e}, which is common to all isoforms, is sufficient for the interaction (Fig 8B).

C/EBP\textsubscript{e} isoform p30 does not directly interact with coactivator protein CBP/p300 in vitro. Transcriptional cooperativity between v-myb and C/EBP\textsubscript{b} has been attributed to interaction with the coactivator protein, CBP/p300. To investigate a possible interaction between C/EBP\textsubscript{e} p30 and CBP/p300, we performed pulldown assays using MBP-C/EBP\textsubscript{e} as bait and CBP expressed in cos-1 cells as input using the same techniques as those described above. No in vitro interaction between CBP and C/EBP\textsubscript{e} p30 was detected (Fig 8C).

**DISCUSSION**

Regulation of eukaryotic transcription is a highly complex process involving chromatin accessibility, basal transcriptional machinery, and the interplay of ubiquitously expressed and lineage-restricted factors. C/EBP\textsubscript{e} has a highly restricted pattern of expression limited predominately to the granulocytic lineage.\textsuperscript{30} Therefore, it is a good candidate for the regulation of lineage-restricted target genes in granulocytic cells. Because of the high degree of homology of the DNA binding domain between C/EBP\textsubscript{e} and other C/EBP family members and published data for the rat homolog CRP1,\textsuperscript{10} we reasoned that C/EBP\textsubscript{e} can bind to the same C/EBP consensus sites. Although we demonstrated binding to the C/EBP sites of the neutrophil elastase and G-CSF receptor promoters, the binding affinity was lower for C/EBP\textsubscript{e} than for C/EBP\textsubscript{a}. Further studies are needed to clarify whether homodimers or possibly heterodimers of C/EBP\textsubscript{e} can bind with higher affinity to other DNA motifs.

The differential upregulation of C/EBP\textsubscript{e} p30 in NB4 cells and the transactivation domain (aa 1-325), and (3) the negative regulatory domain (aa 326-636). Pulldown assays showed that all \textsuperscript{35}S-methionine–labeled C/EBP\textsubscript{e} isoforms (p32, p30, p27, and p14) interacted with the GST-c-myb fusions 1-185 and 1-325 containing the DNA binding domain of c-myb. No interaction occurred with either the GST-c-myb fusion 326-636 or the GST control. Furthermore, interaction of c-myb with all C/EBP\textsubscript{e} isoforms indicated that the carboxy-terminal half of C/EBP\textsubscript{e}, which is common to all isoforms, is sufficient for the interaction (Fig 8B).

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The differential upregulation of C/EBP\textsubscript{e} p30 in NB4 cells
treated with retinoids suggests a separate functional role for each isoform in myeloid differentiation. Although both C/EBPα p32 and p30 contain the previously mapped transactivation domain, our data show that they differ in their potency by which they activate target promoters. C/EBPα p32 was a significantly stronger transactivator of the mim-1 and neutrophil elastase promoters. We hypothesized that the NH2-terminal 32 amino acids may be important for interacting with additional proteins involved in the transcription of target genes of C/EBPα p32, but not p30.

The overall low transcriptional activation potential of C/EBPα in heterologous cells could be a consequence of either low affinity for the selected sites or an indication that other factors are required either for cooperation with or activation of C/EBPα in myeloid differentiation. In two model systems, we showed that a stronger transactivation can be mediated by the cooperation of C/EBPα with the hematopoietic transcription factor c-myb. For the first time, we demonstrated that a C/EBP protein can directly interact with c-myb in vitro. Interacting regions are the DNA binding domain of c-myb and the carboxy-terminal half of C/EBPα, common to all four C/EBPα isoforms. The significance of this finding is supported by our functional studies using amino-terminal truncation mutants of C/EBPα in transient transfections with c-myb which suggested an important functional role for the carboxy-terminal half of C/EBPα in the cooperation with c-myb. Mink et al previously reported the interaction between the DNA binding domains of avian myeloblastosis virus v-myb and C/EBPα. We conclude that the three mutations in the DNA binding domain of AMV v-myb (aa 91, 106, and 117), although important for differential binding to DNA motifs in comparison to E26 v-myb and c-myb, do not affect their binding to C/EBPα proteins. However, the small
reduction of cooperativity achieved by the insertion of 10 bp between the C/EBP and c-myb binding sites of the neutrophil elastase promoter suggests an additional mechanism. Recently, studies have shown that C/EBP<sub>b</sub> interacted with the coactivator protein p300/CBP, which was important for the cooperation between v-myb and C/EBP<sub>b</sub>.<sup>33</sup> We did not observe an interaction between C/EBP<sub>e</sub> and CBP in pulldown assays. Additional studies will clarify whether differences exist among C/EBP<sub>e</sub> isoforms and their interaction with coactivator proteins.

A cooperation between C/EBP<sub>e</sub> and c-myb may be relevant for target genes expressed in promyelocytes. Our data suggest a role for C/EBP<sub>e</sub> in the transcriptional regulation of the neutrophil elastase promoter. This promoter is transcriptionally regulated by members of at least three transcription factor families: C/EBP, myb, and ets factors. If multiple family members with similar binding capacity are expressed in one cell type, predicting which factor is most important in vivo is difficult. The ets family members GABP and PU.1 are both expressed in myeloid cells. Recently, studies showed that GABP is a significantly more potent activator of transcription from the neutrophil elastase promoter than PU.1, suggesting that GABP is the important ets factor in vivo.<sup>45</sup> Also, C/EBP<sub>a</sub> and C/EBP<sub>e</sub> are coexpressed in myeloid cells expressing neutrophil elastase. Both factors can bind to the promoter and synergize with c-myb.

![Fig 7. C/EBP<sub>e</sub> and c-myb cooperation is mediated by the carboxy-terminal half of C/EBP<sub>e</sub>. Expression plasmids for C/EBP<sub>e</sub> p32 (aa 1-281), C/EBP<sub>e</sub> p30 (aa 33-281), and two amino-terminal truncation mutants of C/EBP<sub>e</sub> (aa 118-281 and aa 218-281) (500 ng) were cotransfected with c-myb and NELuc reporter plasmid (4 μg) into CV-1 cells. Luciferase activity was assayed after 40 hours. The C/EBP<sub>e</sub> 118-281 mutant lacks the transactivation domain but still cooperates with c-myb.](image)

![Fig 8. (A) C/EBP<sub>e</sub> physically interacts with c-myb. MBP-C/EBP<sub>e</sub> (p30) and GST-c-myb were used for in vitro pulldown assays. c-Myb was expressed in COS-1 cells; C/EBP<sub>e</sub> (p32) was 35S methionine-labeled by in vitro translation in rabbit reticulocyte lysate. Pulldown of c-myb by MBP-C/EBP<sub>e</sub> (left); pulldown of C/EBP<sub>e</sub> (p32) by GSTc-myb (right). (B) Pulldown assay with three GSTc-myb truncation mutants: (a) DNA binding domain only, amino acids (aa) 1-185; (b) DNA binding domain + transactivation domain of c-myb, aa 1-325; and (c) amino-terminal, negative regulatory domain only, aa 326-636. 35S-methionine-labeled, in vitro-translated C/EBP<sub>e</sub> isoforms p32, p30, p27, and p14 were used as input for separate pulldown experiments. All C/EBP<sub>e</sub> isoforms were pulled down by GSTc-myb fusion proteins containing the DNA binding domain. (C) MBP C/EBP<sub>e</sub> p30 pulldown assay with CBP as input. CBP does not bind to C/EBP<sub>e</sub> p30 in vitro.](image)
The relative contributions of C/EBPα and C/EBPε to the expression of the neutrophil elastase gene in normal myeloid differentiation remain to be elucidated. Interestingly, forced overexpression of C/EBPε in NB4 cells increased clonal growth, suggesting a finding possibly related to its capacity to cooperate with c-myc, a transcription factor known to be involved in the regulation of hematopoietic cell proliferation.46

In conclusion, p32 and p30 isoforms of C/EBPε act as transcriptional activators; and their potency is markedly enhanced by c-myc. Another partner may be required for efficient transcriptional activation by C/EBPε p30 in late myeloid differentiation.

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