A Composite C/EBP Binding Site Is Essential for the Activity of the Promoter of the IL-3/IL-5/Granulocyte-Macrophage Colony-Stimulating Factor Receptor βc Gene[1]

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The common β-chain (βc) is the main signaling component of the heterodimeric receptors for IL-3, IL-5, and GM-CSF and is primarily expressed on myeloid cells. The proximal βc promoter is regulated by GGAA binding proteins, including PU.1, a hemopoietic specific member of the Ets family. However, it is not likely that PU.1 alone accounts for the myeloid-restricted expression of the βc subunit. Here we describe the identification of a C/EBP binding enhancer that is located 2 kb upstream of the transcription start site. The enhancer contains two elements that bind C/EBPα and -β in U937 cells, while C/EBPε is also bound in extracts of HL-60 cells. Importantly, deletion of the enhancer or mutation of either of one of the C/EBP sites results in a complete loss of promoter activity in cell lines as well as in primary cells, showing the importance of C/EBP members in βc gene activation. We further show that PU.1 has to cooperate with C/EBP proteins to induce βc transcription. Since the βc is already expressed on CD34$^+$ cells, these results demonstrate that both C/EBP and PU.1 are not only important for the myeloid-specific gene regulation at later stages of myeloid differentiation. *The Journal of Immunology*, **1999, 163: 2674–2680.**

Hematopoiesis is a developmental process in which pluripotent hemopoietic stem cells proliferate and differentiate to lineage-committed progenitors, which eventually generate all kinds of mature, terminally differentiated blood cells (1). The multistep processes leading to the differentiation of CD34$^+$ stem cells toward the erythroid, lymphoid, or myeloid lineages are at least in part regulated by a network of cytokines. Of these, G-CSF, M-CSF, GM-CSF, IL-3, and IL-5 are implicated in the development of myeloid cells, which include monocytes/macrophages and the neutrophilic, eosinophilic, and basophilic granulocytes (2). Where IL-3 and GM-CSF act on early progenitors, IL-5, G-CSF, and M-CSF stimulate the late differentiation of eosinophilic, neutrophilic, and monocytic lineages, respectively (2–4).

Although IL-5, IL-3, and GM-CSF all have distinct effects on different target cells, they elicit similar responses in cells responsive to all three cytokines, and they even cross-compete for binding to the same cell (5, 6). Molecular cloning of the receptor components and reconstitution of the functional receptors have revealed that the receptors for the three cytokines are heterodimers composed of a cytokine-specific α-chain (IL-3Rα, IL-5Rα, and GM-CSFRα) and a common signaling β-chain (βc)[5] (7–9). Both the α- and β-chains are glycosylated proteins and members of the class I cytokine receptor family (10). Although the human βc subunit has no binding capacity by itself, it associates with the low affinity α-chains to form a high affinity receptor (11–13). Besides its role in high affinity binding, the βc chain plays a major role in IL-3-, IL-5-, and GM-CSF-mediated signal transduction, explaining the functional overlap of these cytokines (14). Interestingly, the mouse has two β subunits, known as βc (AIC2B) and βIL-3 (AIC2A) (15, 16). The mouse βc chain is the common β subunit for the mouse IL-3, IL-5, and GM-CSF receptors, in analogy with the human βc. Although βc and βIL-3 have 91% homology at the amino acid level, βIL-3 binds IL-3 with low affinity and does not form a high affinity receptor with IL-5Rα and GM-CSFRα, in contradiction to βc (17). We previously reported the cloning of the human βc gene and the remainders of a βc pseudogene, suggesting a loss of βIL-3 in the human rather than a murine-specific gene duplication (18).

In both the human and murine systems, binding of IL-3/IL-5/GM-CSF induces receptor multimerization, most likely in a ligand:α:β stoichiometry of 2:2:2 (19). This leads to the activation of JAK2 (20), a protein tyrosine kinase that is constitutively associated with the membrane-proximal region of the βc chain (21). Subsequently, signaling molecules of several signal transduction pathways are activated, including STAT5 (22, 23), p21Ras (24), ERK1/2 (25), JNK/SAPK (26), p38 (27), and PI3K (28, 29; for a review, see Ref. 30). Because the βc subunit is involved in IL-3, IL-5, and GM-CSF signaling, expression of the βc chain is important for all myeloid cells and their precursors. Therefore, elucidating the factors that govern its expression will help to understand the events that regulate myeloid lineage commitment.

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[5] Abbreviations used in this paper: βc, common β-chain; wt, wild type; mut, mutant; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; SAPK, stress-activated protein kinase; PI3K, phosphoinositol 3-OH kinase; C/EBP, CCAAT enhancer binding protein; CAT, chloramphenicol acetyltransferase.

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The βc chain is already expressed on hemopoietic CD34$^+$ stem cells (31), while at later stages of differentiation expression of the βc chain is mainly restricted to the myeloid lineages (31). Uncovering the molecular mechanisms that drive its expression will contribute to understanding of the events of myeloid commitment and differentiation. In the last few years it has become clear that PU.1, a hemopoietic-specific member of the Ets family, and the C/EBP family are key transcription factors in myeloid-specific gene activation (32). Receptors that play a significant role in myeloid development, such as GM-CSFRα, G-CSFR, and M-CSFR, have all been shown to be regulated by the combination of PU.1 and C/EBP proteins (33–35). Previous studies described the isolation of the genes for the human and murine βc/βIL-3 subunits (18, 36). We have shown that PU.1 and another, unidentified GGAA binding protein activate the human βc gene by binding to the proximal promoter region (at −65 and −45 bp relative to the transcription start site, respectively) (18). PU.1, a hemopoietic-specific member of the Ets transcription factor family (37), is expressed in myeloid and B cells and has been shown to regulate a number of myeloid specific genes, including M-CSFR (33), G-CSFR (34), GM-CSFRα (35), eosinophil-derived neurotoxin (38), myeloperoxidase (39), and PU.1 itself (40). Other factors that have been shown to be important for myeloid-specific promoter regulation are members of the CCAAT/enhancer binding protein (C/EBP) family. Members of the C/EBP family have been shown to have a changing temporal expression pattern during myeloid differentiation. C/EBPα is expressed at high levels in early progenitors; C/EBPβ and -δ become more prominent at later stages, while C/EBPε is only expressed during granulopoiesis (41–43). C/EBPα, a basic region leucine zipper transcription factor, originally characterized in liver and adipose tissue (44), is expressed in early myeloid precursors (41) and has also been shown to be important for the expression of M-CSFR, G-CSFR, and GM-CSFRα (33–35). Another C/EBP member that is important for myeloid gene expression is C/EBPε, which was recently described to be strongly induced during granulocytic differentiation (42, 43). Gene targeting of both C/EBPα and C/EBPε results in impaired granulopoiesis (45, 46), clearly demonstrating their roles in myeloid development.

Here we show that, analogous to M-CSFR, G-CSFR, and GM-CSFRα, the βc is regulated by C/EBP members. We identified a crucial enhancer located 2 kb upstream of the transcription start site that contains two C/EBP-binding motifs. Both sites are able to bind C/EBPα, -β, and -ε, while mutation of the sites results in a complete loss of promoter activity.

Materials and Methods

Cells, cDNAs, oligonucleotides, and Abs

COS-1 cells were cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 8% heat-inactivated FCS (Life Technologies). The monocytic cell line U937 and the promyelocytic leukemia cell line HL-60 were maintained in RPMI 1640 (Life Technologies) supplemented with 8% FetalClone I (HyClone, Logan, UT).

CD34$^+$ cells were isolated from cord blood and differentiated as described by Caldenhoven et al. (47). In short, mononuclear cells were obtained by centrifugation of the blood on a Ficoll-Hypaque gradient (density, 1.077 g/ml). Mononuclear cells were incubated with CD34$^+$ Ab and magnetic beads before positive selection on a column that was placed in a magnetic field. Cells were cultured in IMDM (Life Technologies) supplemented with 10% FCS, stem cell factor (100 ng/ml), FLT-3 ligand (100 ng/ml), IL-3 (0.1 nmol/l), GM-CSF (0.1 nmol/l), and IL-5 (0.1 nmol/l). After 3 days, cells were cultured in IMDM supplemented with 10% FCS, IL-3, and IL-5. From day 21, IL-3 was also omitted from the medium. Viability remained >95% throughout the 28-day experiment.

Human C/EBPα (a gift from Dr. G. W. M. Swart, University of Nijmegen, The Netherlands), hC/EBPβ (a gift from Prof. S. Akira, Hyogo College of Medicine, Nishinomoya, Japan), human C/EBPγ (cloned by PCR from HL-60 cDNA), rat C/EBPδ (a gift from Prof. S. L. McKnight, Johns Hopkins University, Baltimore, MD), human C/EBPε (cloned by PCR from HL-60 cDNA), and PU.1 (a gift from Prof. R. M. Maki, Burnham Institute, La Jolla, CA) were cloned into the expression vector pLNCX (Clontech, Palo Alto, CA).

The following oligonucleotides were used in this study: for PCR cloning of C/EBPγ, γF (5′-TGGCAAGGGAGAGTGCCCAA-3′) and γR (5′-TGAGGTCTACTG-TCCTGCAT-3′); for cloning of C/EBPε, εF (5′-TCAAGAGCAGTGGGGGCGGG-3′) and εR (5′-TCCACCAGCCAGCCTCAGCT-3′); for site-directed mutagenesis of C/EBP site 1, ENHC1 mut (5′-AACTCAAAGGGGGGCCCACATGAAGGGTA-3′); for C/EBP-site 2, ENHC2 mut (5′-CTCTGTATTTTGCTCGAGTTTCAGAATAAA-3′); and for band-shift analysis (only upper strand is shown), C1 wt (5′-AGCTTCAAAGGGGGTTTCCACATGAAGGGA-3′), C1 mut (5′-AGCTTCAAAGGGGGGGCCCACATGAAGGGA-3′), C2 wt (5′-AGCTTGCTCTGTATTTTGAAATAGTTTCAA-3′), C2 mut (5′-AGCTTGCTCTGTATTTTGCTCGAGTTTCAA-3′), and −65 (5′-AGCTTCCGGCACTGCTTCCTCTTTCTGCTA-3′).

The following Abs were used in this study: anti-C/EBPα (rabbit polyclonal IgG 14AA, sc-061X; Santa Cruz Biotechnology, Santa Cruz, CA), anti-C/EBPβ (rabbit polyclonal IgG C-19, sc-150X; Santa Cruz Biotechnology), anti-C/EBPδ (rabbit polyclonal IgG M-17, sc-636X; Santa Cruz Biotechnology), anti-C/EBPε (rabbit polyclonal IgG C-22, sc-158X; Santa Cruz Biotechnology), and anti-Fli-1 (rabbit polyclonal IgG C-19, sc-356X; Santa Cruz Biotechnology).

Plasmids for promoter analysis, transient transfection, and CAT assay

The 2.7-kb *Hin*dIII fragment of the βc subunit promoter (−2096/+600) was cloned into the *Hin*dIII site of the promoterless pBLCAT3 vector (48). Deletion construct −1967/+600CAT3 was generated by cloning the *Hin*cII-*Hin*dIII fragment (−1967/+600) into *Xba*I and *Xho*I sites of pBLCAT3 via the *Sma*I-*Hin*dIII sites of SK- (Stratagene, La Jolla, CA), The 129-bp *Hin*dIII-*Hin*cII fragment (−2096 to −1967) was cloned into the *Hin*dIII and *Bam*HI sites of pBLCAT2 (48) and the *Hin*dIII and *Pst*I sites of TATACAT (49) to generate HH129CAT2 and HH129TATA, respectively, via the *Hin*dIII and *Sma*I sites of a modified pSG5 (Stratagene). The Bgl2 site at −289 was used to generate −2096/−289CAT2 and −1967/−289CAT2. Site-directed mutagenesis was performed according to the method of Kunkel (50).

For transfection experiments, COS-1 cells were cultured in six-well dishes (Nunc, Copenhagen, Denmark), and 3 h later the cells were transfected with 6 μg of supercoiled plasmid DNA as described previously (51). Following 16- to 20-h exposure to the calcium phosphate precipitate, medium was refreshed. Cells were harvested for CAT assays 24 h later. Transfection of U937, HL-60, eosinophils, and neutrophils was performed by electroporation at 300 V and 960 μF (10^7 cells, 20 μg of supercoiled plasmid DNA). In cotransfection experiments 10 μg of promoter construct was transfected with 10 μg of cDNA. Two days after transfection cells were harvested for CAT assays. CAT assays were performed as follows. Cells were lysed by repeated freeze-thawing in 250 mM Tris (pH 7.4) and 25 mM EDTA. Cellular extract was then incubated in a total volume of 200 μl containing 250 mM Tris (pH 7.4), 2% glycerol, 0.3 mM butyryl coenzyme A (Sigma, St. Louis, MO), and 0.05 μCi of [^{14}C]chloramphenicol (Amersham, Arlington Heights, IL) for 2 h at 37°C. Reaction products were then extracted using 400 μl of xylene/pristane (1/2), and the percentage of acetylated products was determined using liquid scintillation counting. All experiments were performed at least four times. A *lacZ* reporter was used to correct for transfection efficiency.

Gel retardation assay

Nuclear extracts were prepared from U937 and HL-60 cells following a previously described procedure (52). Oligonucleotide probes were labeled by filling in the cohesive ends with [α-^{32}P]dCTP using Klenow fragment of DNA polymerase I. Gel retardation assays were performed as follows. Nuclear extracts (10 μg) were incubated in a final volume of 30 μL containing 10 mM Tris (pH 7.4), 10% glycerol, 2 mM EDTA, 50 mM NaCl, 0.05% Nonidet P-40, 300 μg/ml BSA, 30 μg/ml poly(dI/dC), 0.5 mM DTT, and 1.0 ng of ^{32}P-labeled probe for 20 min at room temperature. Complexes were then separated through nondenaturing 5% polyacrylamide gels and were visualized by autoradiography. In competition experiments, a 10- to 100-fold molar excess of wt or mut oligonucleotide was added to the reaction mix for 5 min before addition of the labeled probe. For supershift analysis, nuclear extracts were preincubated with 2 μg of Ab for 40 min on ice before addition of the labeled probe.

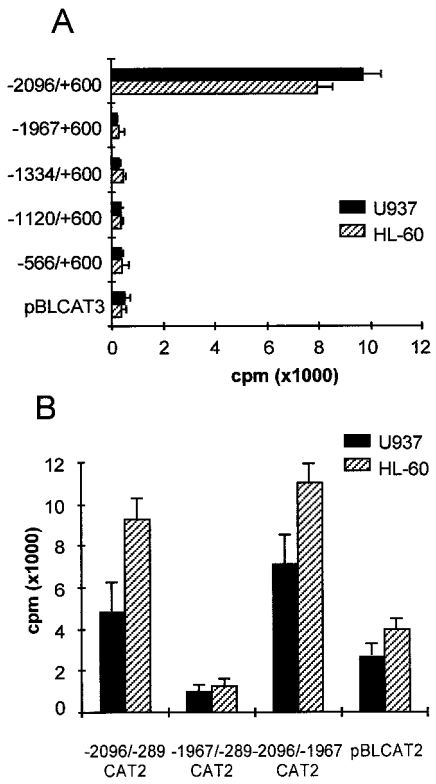

FIGURE 1. An upstream enhancer is crucial for βc promoter activity. *A*, U937 and HL-60 cells were transfected with successive 5′ deletion constructs of the βc promoter cloned in the promoterless pBLCAT3 reporter. Two days after transfection, cells were harvested, and CAT activity was determined. Construct −2096/+600CAT3, which is active in both cell types, contains the 44-bp exon 1, 2096 bp of upstream sequences and 598 bp of the first intron. Deletion of 129 bp at the 5′ end results in the complete loss of promoter activity. *B*, Constructs that lack the proximal promoter, with (−2096/−289) and without (−1967/−289) the enhancer, and the isolated enhancer (−2096/−1967) were cloned into the pBLCAT2 vector, which contains the minimal thymidine kinase promoter. Deletion of the enhancer element again results in the loss of promoter activity, while the isolated enhancer element has maximal CAT activity. This figure demonstrates that the fragment from −2096 to −1967 bp contains a functionally important enhancer element. Bars indicate the mean value of at least three independent experiments. The SD is indicated by error bars.

Results

A 2-kb upstream enhancer regulates βc subunit expression

In a previous study we reported the isolation of a 2.7-kb *Hin*dIII fragment that contains the 44-bp exon 1, 2096 bp of upstream sequences, and 598 bp of the first intron of the human βc subunit gene (18). After cloning into the promoterless pBLCAT3 reporter plasmid (−2096/+600CAT3), transfection experiments in monocytic U937 cells and promyelocytic leukemia HL-60 cells showed that this fragment contains a functionally active promoter. To localize *cis*-activating elements, successive 5′ deletion constructs were generated. The *Nde*I (at −566), *Eco*RI (at −1120), and *Sma*I (at −1334) sites were used to generate −566/+600CAT3, −1120/+600CAT3, and −1334/+600CAT3, respectively. As shown in Fig. 1*A*, these constructs completely lost their activity. Also, construct −1967/+600CAT3, made by using the *Hin*cII site at −1967, has no promoter activity, suggesting that an important positively regulating element has to be present between −2096 and −1967 bp. To test this hypothesis, the 129-bp *Hin*dIII-*Hin*cII fragment, as well as the fragments from −2096 to −289 bp and from −1967 to −289 bp were cloned into the pBLCAT2 vector, a reporter plasmid containing the minimal thymidine kinase promoter (48). The generated constructs, −2096/−1967CAT2, −2096/−289CAT2, and −1967/−289CAT2, were then transfected into U937 cells and HL-60 cells and tested for promoter activity. Fig. 1*B* shows that −2096/−289CAT2 is active in both cell types, while −1967/−289CAT2 has lost this capacity. Indeed, −2096/−1967CAT2 is highly active in both cell types, clearly demonstrating the presence of an enhancer between −2096 and −1967 bp.

The enhancer contains two C/EBP binding motifs

To localize potential transcription factor binding sites, the 129-bp enhancer sequence (Fig. 2*A*) was subjected to the TFMATRIX transcription factor database (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html). As shown in Fig. 2*A*, one NF-κB and two C/EBP binding sites were identified. Because C/EBP family members play an important role in the constitutive and inducible expression of a number myeloid-specific genes, the putative C/EBP sites (C1 and C2, Fig. 2*A*) were studied in further detail. Band-shift analysis was used to investigate whether C1 and C2 were protein binding elements. Oligonucleotide probes, each spanning a single C/EBP site, were end labeled and incubated with 10 μg of U937 and HL-60 nuclear extract. Fig. 2*B* shows that elements C1 and C2 are both able to bind a partially overlapping set of protein complexes (*lanes 1* and *8*). The binding of most complexes could be competed by addition of a 10- to 100-fold molar excess of cold self oligo (*lanes 2* and *3* and *lanes 9* and *10*), but not with mutant C1 (*lanes 4* and *5*), mutant C2 (*lanes 11* and *12*), or nonrelated −65 oligo (*lanes 6* and *7* and *lanes 13* and *14*), a sequence that specifically binds transcription factor PU.1 in HL-60 and U937 cells. One complex (indicated by an arrow) can also be competed with the mutated or nonrelated oligos. These results demonstrate the specificity of the complexes binding to elements C1 and C2, except for the indicated complex. To further characterize the proteins binding to C1 and C2, the nuclear extracts were preincubated with specific Abs against C/EBP members and Fli-1, a member of the Ets family. In HL-60 cells, supershifted complexes appear when extracts are incubated with anti-C/EBPα, -β, and -ε (Fig. 2*C*, *lanes 2, 3*, and *5* for C1; *lanes 8, 9*, and *11* for C2), but not with anti-C/EBPδ (*lanes 4* and *10*) and anti-Fli-1 control Ab (*lanes 6* and *12*). Preincubation with anti-C/EBPδ (*lanes 4* and *10*) results in the disappearance of a complex (indicated by an arrow). It is not clear whether this complex is C/EBPδ, because competition experiments (see above) suggested that this is a nonspecific complex. Almost identical results were obtained with U937 cells, but no supershift was observed when extracts were incubated with anti-C/EBPε (our unpublished observations). Taken together, these results show that different C/EBP proteins, including C/EBPα, -β, and -ε can bind to two distinct elements in the upstream enhancer.

The C/EBP sites are essential for enhancer activity

To elucidate whether the C/EBP binding elements are functionally important, both C1 and C2 were mutated in the −2096/−1967CAT2 construct and tested for promoter activity in U937 and HL-60 cells. Single mutation of either C1 or C2 reduced promoter activity to the background level (Fig. 3*A*), as occurs when both sites are mutated. These data show that both elements are essential for enhancer activity and suggest the cooperation of the proteins binding to the elements. Next, we cotransfected the wt and mut constructs with the cDNAs of several C/EBP family members. Probably due to a cryptic C/EBP site in the pBLCAT2 vector, cotransfection of C/EBP cDNAs with pBLCAT2 resulted in moderately enhanced background values (3). Therefore, the enhancer

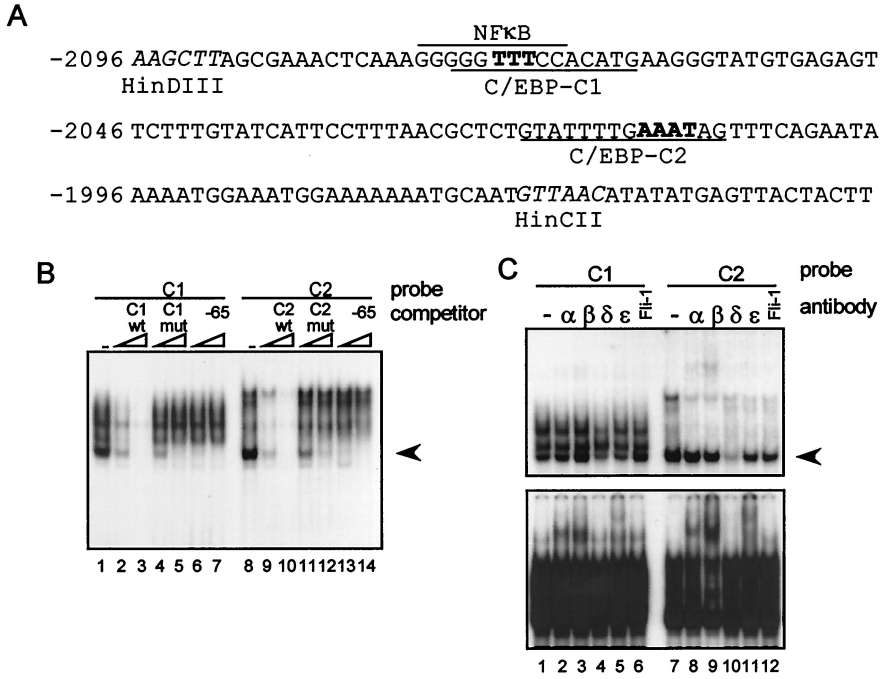

FIGURE 2. Two elements in the enhancer bind C/EBP-containing complexes. *A*, The 150-bp nucleotide sequence of the 5′ end of construct −2096/+600. The 129-bp *Hin*dIII/*Hin*cII fragment was subjected to a transcription factor database; one NF-κB site and two C/EBP binding elements (C1 and C2, underlined) were identified. Nucleotides that were mutated to inhibit C/EBP binding are indicated in boldface. *B*, Nuclear extracts of HL-60 cells were incubated with oligonucleotides spanning a single C/EBP site and analyzed in a band-shift assay. Elements C1 and C2 bind a partially overlapping set of complexes (*lanes 1* and *8*), which can be competed with a 10- to 100-fold molar excess of cold self oligo (*lanes 2* and *3* and *lanes 9* and *10*). When mutant (C1 mut and C2 mut) or nonrelated oligo (−65, a PU.1 binding element) is used, only one complex is competed for binding (indicated by the arrow). *C*, Before addition of the labeled oligo, 2 μg of the indicated Ab was added to nuclear extracts of HL-60 cells. Preincubation with Abs against C/EBPα, -β, and -ϵ, but not with control Ab against Fli-1, results in the appearance of supershifted complexes (*lower panel*). The *upper panel* is a shorter exposure of the same gel. The arrow indicates the complex that disappears when anti-C/EBPδ is added. It is unclear whether this complex contains C/EBPδ, because *A* suggests that this is a nonspecific complex. This figure shows that the enhancer contains two discrete elements that specifically bind C/EBPα, -β, and -ϵ in HL-60 nuclear extracts. In U937 cells similar results were obtained, but no binding of C/EBPϵ was detected.

constructs were cloned into the pLT-G plasmid, a CAT vector with a synthetic TATA box. Fig. 3*B* shows that C/EBPα, -β, -δ, and -ϵ induced a 4- to 5-fold induction of CAT activity when cotransfected with the wt −2096/−1967 construct in HL-60 cells (similar results were obtained in U937 cells; not shown). When C/EBP elements C1 and C2 were mutated, the effect of C/EBP cotransfection was dramatically decreased. The *trans*-activating capacity of C/EBPδ is of interest, because it is unclear whether this protein binds to the enhancer in a band-shift assay (see above). Nevertheless, C/EBPδ binds to C1 and C2 when overexpressed in COS cells (4).

To further demonstrate the importance of C/EBPs for enhancer activity, the −2096/−1967CAT2 construct was also cotransfected with C/EBPγ, a dominant negative C/EBP member (53), and with an antisense construct of C/EBPα. As shown in Fig. 3*C*, cotransfection of antisense C/EBPα reduces promoter activity by 65% in U937 cells and by 50% in HL-60 cells, while C/EBPγ reduces promoter activity by 70% in both cell types. These results clearly demonstrate the role of C/EBP proteins in regulation of the βc chain gene.

To demonstrate that the C/EBP sites also contribute to βc promoter activity in primary cells as opposed to tumor cell lines, we transfected the C1 and C2 double mutant in the −2096/−1967CAT2 construct into in vitro differentiated eosinophils and neutrophils (see *Materials and Methods*). As we found for HL-60 and U937 cells (see Fig. 3*A*), mutation of the C/EBP sites significantly reduces enhancer activity in both eosinophils and neutrophil (Fig. 3*D*). This clearly shows that these C/EBP sites are involved in βc promoter activity in primary cells.

C/EBP and PU.1 cooperate to activate the βc promoter in nonhemopoietic cells

We have previously shown that binding of PU.1 to a GGAA element at −65 bp relative to the transcription start site is crucial for βc chain expression. To more precisely elucidate the roles of PU.1 and C/EBP in βc chain expression, these factors were cotransfected with different promoter constructs in COS fibroblasts. Fig. 4*A* shows that both PU.1 and C/EBPα enhanced the promoter activity of the full promoter construct −2096/+600CAT3 by about 10-fold. However, cotransfection of C/EBPα together with PU.1 resulted in a 30-fold increase in promoter activity. As expected, C/EBPα could no longer activate transcription when the C/EBP enhancer was deleted (construct −1967/+600CAT3; Fig. 4*A*). Surprisingly, PU.1 could no longer activate this construct. This was not due to a PU.1 binding site in the −2096/−1967 sequence, because Fig. 4*B* shows that cotransfection of PU.1 did not affect the activity of construct −2096/−1967CAT2, which is in contrast to cotransfection of C/EBPα. Construct −226/+33CAT3, which contains only the promoter proximal region, can only be induced by PU.1, not by C/EBPα (Fig. 4*B*, *second panel*). If *trans*-activation of the full promoter construct (−2096/+600CAT3) by PU.I is dependent on proteins binding to the C/EBP sites, this would suggest that COS cells contain endogenous binding activity for the C/EBP sites. Therefore, we performed band-shift experiments with

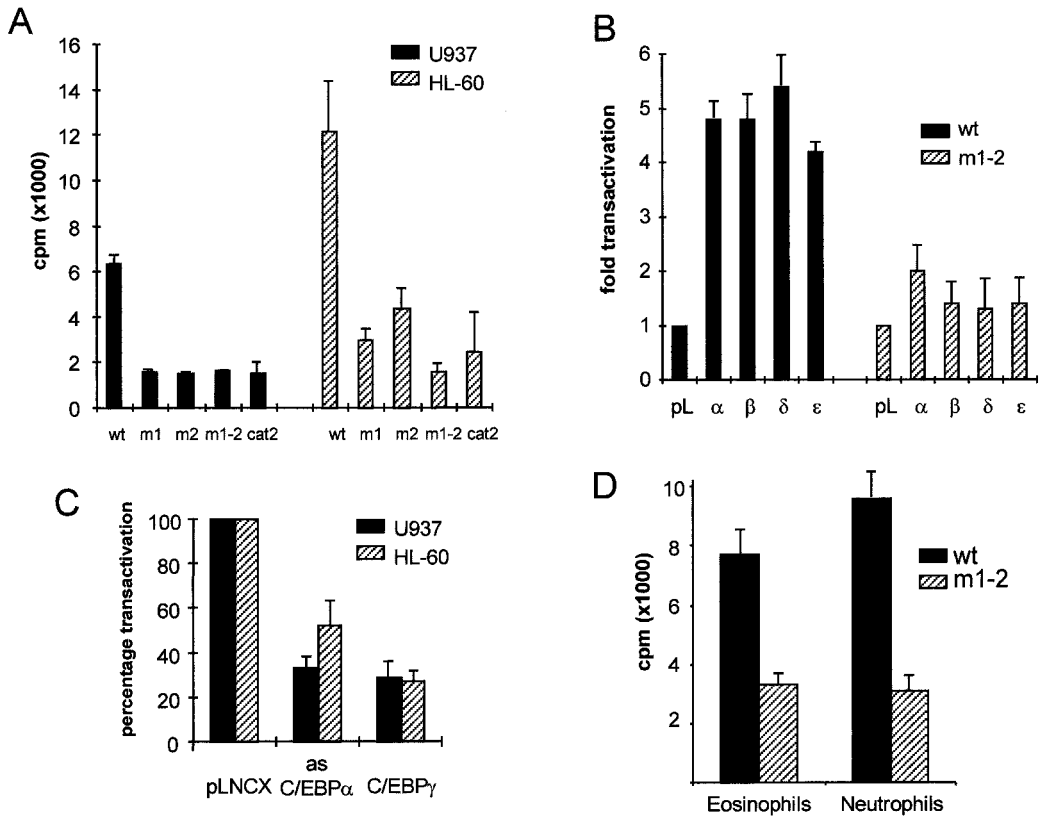

FIGURE 3. Enhancer activity is mediated via the C/EBP binding sites. *A*, C/EBP elements C1and C2 were mutated alone (m1 and m2, respectively) or together (m1–2) in the −2096/−1967CAT2 construct and tested for activity in a CAT assay in both U937 and HL-60 cells. Single mutation of either one of the sites reduces CAT activity to the background level in both cell types. *B*, Cotransfection in HL-60 cells of the wt −2096/−1967 construct with cDNAs encoding C/EBPα, -β, -δ, and -ϵ results in a 4- to 5-fold increase in CAT activity compared with that in the empty pLNCX (pL) vector. The double mutant is not responsive to C/EBP cotransfection. For this experiment the constructs were cloned into the pLT-G reporter, because C/EBP cotransfection has a moderate effect on empty pBLCAT2 vector. Similar results were obtained in U937 cells. *C*, Cotransfection of C/EBPγ, a C/EBP member that lacks the *trans*-activation domain, or antisense C/EBPα significantly reduces promoter activity of −2096/−1967CAT2 in both U937 and HL-60 cells. This figure shows that the C/EBP elements are responsive to C/EBPα and -ϵ and are both crucial in promoter activation. Bars indicate the mean value of at least three independent experiments. The SD is indicated by error bars. *D*, C/EBP elements C1and C2 were mutated both (m1–2) in the −2096/−1967CAT2 construct and tested for activity in a CAT assay in eosinophils and neutrophils differentiated in vitro from $CD34^+$ cells isolated from cord blood. The C/EBP elements clearly contribute to βc promoter activity in primary eosinophils and neutrophils.

COS nuclear extracts. Indeed, binding site C2 binds a specific protein complex in COS cells that can be competed with access unlabeled probe, but not with a mutated C2 sequence or an unrelated oligonucleotide (Fig. 4*C*). We did not observe specific binding activity for C1 in COS cells (data not shown). Taken together, these results indicate that PU.1 and C/EBP functionally cooperate to enhance βc expression, although their respective binding elements are not clustered as is described for the GM-CSFRα, G-CSFR, and M-CSFR promoters.

Discussion

Members of the C/EBP family have been shown to have a changing temporal expression pattern during myeloid differentiation. C/EBPα is expressed at high levels in early progenitors, C/EBPβ and -δ become more prominent at later stages, while C/EBPϵ is only expressed during granulopoiesis (41–43). Alternative splicing and the ability of all members of the C/EBP family to form homo- and heterodimers lead to a variety of possible DNA binding complexes. It is likely that these complexes have specific affinities for particular binding sites and that this mechanism plays an important role in the specific regulation of certain genes by C/EBPs. In the βc subunit enhancer we identified two different binding sites that bind multiple C/EBP containing complexes (Fig. 2, *A* and *B*). Both elements bind C/EBPα, -β, and -ϵ in HL-60, while in U937 cells, which express C/EBPϵ at a very low level, only binding of C/EBPα and -β was observed. Binding of C/EBPδ is unclear. In a band-shift assay, addition of anti-C/EBPδ Ab results in the disappearance of a specific DNA binding complex. However, the same complex can be competed with mut and nonrelated oligonucleotides, suggesting that this is a nonspecific binding complex. On the other hand, C/EBPδ from transfected COS cells can bind both sites (5), and C/EBPδ can *trans*-activate the βc promoter in cotransfection experiments (Fig. 3*B*). Experiments with murine embryonic stem cells and blastocysts have shown that transcription of βc and βIL-3 is induced at the same time as the CD34 gene (54). In $CD34^+$ cells, the C/EBPα is the predominant expressed isoform (55) and, therefore, is likely to be involved in the initial induction of the βc subunit gene(s). Nevertheless, expression of βc/βIL-3 is unaffected in C/EBPα-deficient mice, while expression of the G-CSFR is completely blocked (45). This might indicate that C/EBP members can compensate for each other in some, but not all, situations.

We have shown that both sites can bind C/EBP independently and that both sites are functional. Mutation of either site results in almost a complete loss of promoter activity in cell lines as well as

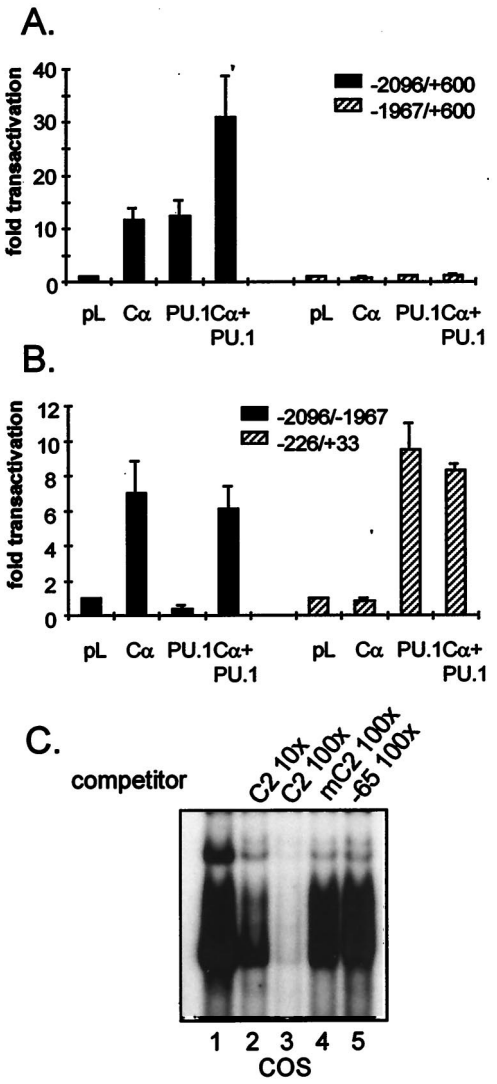

FIGURE 4. C/EBP and PU.1 cooperate in βc gene transcription. *A*, C/EBPα and PU.1 were cotransfected with different promoter constructs in COS fibroblasts using the calcium phosphate precipitation method. C/EBPα and PU.1 each induce a 10-fold increased *trans*-activation when cotransfected with the full promoter construct −2096/+600CAT3. When both factors are transfected, a 30-fold induction is measured, indicating that these proteins cooperate in βc gene activation. The effect of both C/EBP and PU.1 overexpression is completely lost when the C/EBP binding enhancer is deleted (−1967/+600CAT3). *B*, COS fibroblasts were transfected with different promoter constructs, C/EBPα, and PU.1 by the calcium phosphate precipitation method. The upstream enhancer (−2096/−1967) does not contain a functional PU.1 site, while activity of the proximal promoter region (−226/+33) can be enhanced by PU.1, but not C/EBPα, cotransfection. These results demonstrate that PU.1, binding to the promoter proximal region, can no longer activate the βc promoter in the absence of C/EBP in the context of the full promoter. Bars indicate the mean value of at least three independent experiments. The SD is indicated by error bars. *C*, Nuclear extracts of COS cells were incubated with an oligonucleotide spanning C/EBP site C2 and analyzed in a band-shift assay. Element C2 binds a set of complexes, which can be competed with a 10- to 100-fold molar excess of cold self oligo. When mutant (C2 mut) or nonrelated oligo (−65, a PU.1 binding element) was used, no competition was observed.

in primary eosinophils and neutrophils (Fig. 3, *A* and *D*), suggesting that both sites are crucial for transcription activation. However, it is not clear whether both sites are occupied by C/EBP simultaneously in vivo. Transcription could be reduced by cotransfection of C/EBPγ, a dominant negative C/EBP member, and antisense C/EBPα, further demonstrating the specific binding of C/EBP proteins to the enhancer sequence (Fig. 3*C*).

Cotransfection experiments with different deletion constructs showed the cooperation of C/EBP proteins and PU.1 in the regulation of the βc gene (Fig. 4), reinforcing the significance of these transcription factors in myeloid development. The combination of C/EBPs and PU.1 is also important for G-CSFR, GM-CSFRα, and M-CSFR expression. Functional elements for both proteins have been identified in the proximal promoters of these genes. The sites for PU.1 and C/EBP are separated by 15 bp (GM-CSFRα), 35 bp (M-CSFR), or 85 bp (G-CSFR), indicating that both proteins are likely to interact. Furthermore, both elements are close to the transcription start site (<100 bp) (33–35). In the βc subunit promoter the situation is different. Two functional GGAA elements are present in the proximal promoter region, while important C/EBP binding sequences are located 2 kb upstream. When isolated, the PU.1-binding proximal promoter sequence has a high activity (construct −226/+33; Fig. 4). Interestingly, in the full promoter context (construct −2096/+600), this activity is dependent on the C/EBP binding enhancer, because deletion of the enhancer (construct −1967/+600) results in the complete loss of promoter activity. This again suggests an interaction between C/EBPs and PU.1. Why binding of PU.1 is not enough to induce promoter activity of construct −1967/+600 is not known. Initial experiments opened the possibility that additional, yet unidentified proteins play a role in βc gene activation. It is possible that this factor(s), PU.1, and C/EBP all have to be present to obtain a transcriptionally active complex, while in the absence of C/EBP the additional protein blocks transcription by PU.1. Although future experiments are necessary to identify possible additional promoter elements and/or proteins that are involved in βc subunit expression, we have shown that both PU.1 and C/EBP play a critical role in βc chain transcription.

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