Identification of Transcriptional Activation and Repression Domains in Human CCAAT/Enhancer-binding Protein ε*

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Human CCAAT/enhancer-binding protein ε (C/EBPε), a new member of the C/EBP family, significantly up-regulates both the mim-1 and human myeloperoxidase promoters, suggesting an important role for C/EBPε in the transcriptional regulation of a subset of myeloid-specific genes. To elucidate the structure and function of C/EBPε in transcriptional activation, amino acid residues 1–115, 147–249, or 1–249 of C/EBPε were fused to the yeast GAL4 DNA binding domain. These expression vectors were cotransfected with a chloramphenicol acetyltransferase reporter gene and, in all cell lines tested, only the GAL-C/EBPε-(1–115) fusion protein significantly activated expression from the chloramphenicol acetyltransferase reporter gene. Sixteen deletion mutants of C/EBPε mapped the transactivation domain to amino acids 1–18 at the N terminus and revealed the presence of a transcription repression element between amino acid residues 116 and 162. Expression vectors containing the repression domain of C/EBPε strongly inhibited gene transcription from TK, SV40, and adenoviral major late promoters bearing GAL4 binding sites. Fusion of this repression domain to the VP16 activation domain inhibited the transactivation function of VP16. Deletion of this repression domain increased gene transcription from a neutrophil elastase promoter-luciferase reporter. Taken together, these data suggest that C/EBPε regulates transcription by utilizing both activation and repression functions.

The CCAAT/enhancer-binding protein (C/EBP)† family of transcriptional regulators currently includes six members: C/EBPα (1), C/EBPβ (2–7), C/EBPγ (8), C/EBPδ (3, 9, 10), C/EBPε (11, 12), and C/EBPζ (13). This family of transcription factors regulates several cytokine genes (14), liver-specific genes involved in acute phase response (7, 15, 16), adipocyte-related genes (17, 18), the regulatory elements of many viruses (19), and control sequences in the Ig heavy chain and κ loci (20).

The C/EBP proteins interact with other proteins such as the transcription factors c-Myb (21), cAMP response element-binding protein (22–24), c-Jun (25), glucocorticoid receptor (26), AMML1 (27), NF-κB (28), and the hypophosphorylated form of the cell cycle regulatory protein retinoblastoma (pRB) (29).

The C/EBP family members share a highly conserved C-terminal basic amino acid-rich region and a flanking leucine zipper domain that are essential for DNA binding and dimer formation (30). In contrast, the N terminus is more diverse. Transcriptional activation domains from C/EBPα (31, 32), C/EBPβ (33), and C/EBPε were localized to this region. However, C/EBPγ and C/EBPζ lack these N-terminal activation domains and can function as trans-dominant repressors of transcription (34, 35). Additional negatively acting regulatory domains were identified in C/EBPα (32, 36) and C/EBPβ (33). These “attenuator” domains not only regulate transcription but define cell-specific transcriptional activity. The rat homologue of C/EBPε, CRP1, shares significant homology at its N terminus with other C/EBP family members (33).

The human C/EBPε gene was cloned recently (11, 12). Differential splicing and the use of alternative promoters generates a total of four proteins with calculated molecular masses of 32, 30, 27, and 14 kDa (12, 37). This use of alternative splice sites has also been demonstrated for the C/EBPβ gene (5). Unlike other C/EBP proteins, C/EBPε is primarily expressed in the myeloid and lymphoid cell lineages (11, 12, 38, 39). The human C/EBPε protein strongly and specifically binds to double-stranded DNA containing consensus C/EBP sites and significantly up-regulates transcription from both the myeloid-specific c-mim and human myeloperoxidase promoters. This suggests an important role for C/EBPε in the transcriptional regulation of a subset of myeloid-specific genes that contain these sites (12).

To understand further the mechanisms of transcriptional regulation of myeloid-specific genes by C/EBPε, we used molecular genetic methods to perform a systematic structure/function analysis of the C/EBPε protein. The ultimate goal of our experiments was to identify regions of C/EBPε that contribute to its ability to regulate myeloid gene expression. In this report, we demonstrate that the transcriptional activation function of C/EBPε is mediated through a bipartite activation domain localized in the N terminus. This domain functions as a transcriptional activator both in the context of the C/EBPε protein and when fused to the GAL4 DNA binding domain. In addition, we identified an intrinsic repression domain in C/EBPε that, when deleted, allowed C/EBPε to enhance transcription of gene transcription compared with the wild-type C/EBPε protein. Furthermore, this inhibitory signal conferred its repression function on a heterologous transcriptional activator. We propose a model in which C/EBPε regulates myeloid-

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specific gene expression through transcriptional activation and repression that is dependent on its associated factor(s).

EXPERIMENTAL PROCEDURES

Plasmid Constructions

GAL4-C/EBP\(\alpha\) Hybrid Constructs—Cloning vector pGALM (a gift from Drs. Gregory Kato and Chi Dang) was used for all recombinant plasmids in this study. An EcoRI-SalI DNA fragment, obtained from Drs. Gregory Kato and Chi Dang, was used for all recombinant plasmids. PCR was used to introduce restriction sites, EcoRI and SalI, at the beginning and the end, respectively, of each C/EBP\(\alpha\) fragment. The oligonucleotide primers for introduction of restriction sites consisted of a 5′-clamp sequence (6 base pairs), the restriction site, and 18 bases of homology to the target sequence at the 3′-end. PCR (25 cycles of 1 min at 94 °C, 1.5 min at 55 °C, and 1.5 min at 72 °C) using Pfu polymerase (Stratagene, La Jolla, CA) was carried out under standard conditions as recommended by the manufacturer. The DNA fragments treated with restriction enzymes EcoRI and SalI were inserted into pGALM. All recombinant constructs were confirmed by sequencing.

GAL4-VPI16-C/EBP\(\alpha\) Hybrid Constructs—Restriction sites, EcoRI/HindIII and HindIII/SalI, were introduced by PCR into the VP16 activation domain from herpes simplex virus (40) and DNA fragments encoding C/EBP\(\alpha\) amino acids 1–45, 1–115, 115–162, 162–199, and 115–199, respectively. After proper treatment with restriction enzymes, the VP16 AD and the C/EBP\(\alpha\) fragments were inserted into the cloning vector pGALM by a three way ligation and confirmed by sequencing.

CMV-C/EBP\(\alpha\) Expression Constructs—All PCR-derived C/EBP\(\alpha\) DNA fragments were digested with EcoRI/SalI enzymes and inserted into a CMV expression vector, pCMVSPORT (Life Technologies, Inc.). A translational initiation codon, ATG, present in the 5′-primer was introduced into all N-terminal deletions during PCR reactions. Expression plasmids containing internal deletions of C/EBP\(\alpha\) were made in two ways. (i) For CMV-e\(\alpha\)116–234, PCR-derived DNA fragments encoding amino acids 1–70 and 235–249 of C/EBP\(\alpha\) were digested with restriction enzymes SalI and EcoRI and then ligated together after the two fragments were blunted by a fill-in reaction using Klenow. This produced an in-frame fusion of the two C/EBP\(\alpha\) fragments that removed residues 116–234. The oligonucleotide primers for introduction of restriction sites into PCR reactions, was introduced at the junction of all internal deletions. After proper treatment with restriction enzymes, a three way ligation was performed to insert these DNA fragments into the cloning vector pGALM.

Reporter Constructs—Several GAL4-responsive reporter constructs were used in this study: (i) G5E1bCAT, a gift from Drs. Gregory Kato and Chi Dang (42), which contains a chloramphenicol acetyltransferase (CAT) reporter gene regulated by the adenoviral E1b promoter bearing five GAL4 binding sites; (ii) G5TKCAT, G5TKCAT, G5MKCAT, GSV40CAT, and G5MLPCAT, gifts from Dr. Arnold Berk (43), which contain zero, one, or five GAL4 binding sites upstream of the promoters for herpes simplex virus thymidine kinase (TK), SV40, or adenoviral major late promoter. The neutrophil elastase promoter containing a consensus C/EBP binding site 1 base pair upstream of a Myb binding site (44) was fused to the luciferase gene (NE-Luc) for use as a reporter gene.

Transient Transfections and Reporter Assays

COS-1, CV-1, and 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were transfected at 50–60% confluency in 60-mm dishes as described (12). For CAT assays, performed as described (45), a total of 20 µg of DNA was introduced, consisting of 5 µg of reporter plasmid, 5 µg of GAL4-C/EBP\(\alpha\) hybrid constructs, and pUC19 carrier DNA. The plasmid pCMV-Luc (0.5 µg) was included as a monitor of transfection efficiency. Lysates were prepared 48–60 h after DNA was added to the cells.

The HL60 (myeloblast/prolymphocytic), U937 (myelomonocytic), and NB4 (promyelocytic) were maintained in RPMI with 20% fetal bovine serum. A total of 50 µg of DNA, including 10 µg each of the reporter and effector plasmids and 5 µg of pCMV-Luc and pUC19 carrier DNA, were introduced into the cells by electroporation as described (12). Cells were harvested 24–48 h after transfection. All effector plasmids were tested at least three times. Levels of transactivation or repression were all compared with that of the reporter plasmid in the absence of any effector plasmid.

RESULTS

The C/EBP\(\alpha\) Activation Domain Is Localized in the N Terminus—We previously demonstrated that C/EBP\(\alpha\) can activate transcription from the myeloid-specific mim-1 and human myeloperoxidase promoters (12). To map the transcriptional activation domains as they function in mammalian cells, expression plasmids were constructed to give the following fusions of the GAL4 DNA binding domain with C/EBP\(\alpha\): GAL-C/EBP\(\alpha\), GAL-\(\alpha\)-(1–115); and GAL-\(\alpha\)-(147–249). Fig. 1A shows a schematic representation of these constructs. Sequence analysis revealed that the basic region and the leucine zipper (bZIP) domains are located between amino acid residues 162 and 235 (11, 12). We used the adenoviral E1b promoter bearing five GAL4 binding sites that was linked to the CAT gene as the target promoter.

Transient cotransfections of these GAL4-C/EBP\(\alpha\) expression plasmids with the GAL4–5E1bCAT reporter construct into COS-1 cells demonstrated that the GAL-\(\alpha\)-(1–115) fusion protein activated expression of the CAT reporter gene 12-fold when compared with the level of reporter expression alone (Fig. 1A). The GAL-\(\alpha\)-(1–115) chimeric protein also activated CAT expression to a similar level in three hematopoietic cell lines (HL-60, U937, NB4) (data not shown). In contrast, neither GAL-\(\alpha\)-(147–249) nor GAL-C/EBP\(\alpha\) significantly activated CAT expression in any of these cell lines (Fig. 1, A and B). Western blotting demonstrated that the three protein fusions were expressed at similar levels (data not shown). Taken together, these data indicate that amino acids 1–115 of the C/EBP\(\alpha\) protein are sufficient to confer the C/EBP\(\alpha\) transcriptional activation function, which is independent of the bZIP domain, and suggest that a repressive effect is mediated by amino acids 116–249.

Mapping of the C/EBP\(\alpha\) Regulatory Domains—To define regulatory domains within the C/EBP\(\alpha\) gene, we constructed two sets of nested deletions that removed various amino acid residues from either the N or C terminus of C/EBP\(\alpha\) (Fig. 2A). These deletion mutants were fused to the GAL DBD and tested for their abilities to transactivate the CAT reporter gene in COS-1 cells. The N-terminal deletions consisted of eight truncations beginning from the first 18 amino acids and extending varying distances toward the C terminus. As shown in Fig. 2, A and B, all eight hybrid proteins were completely inactive in transactivation of the CAT reporter gene. The expression of all the fusion proteins was confirmed by Western blotting (data not shown).

The C-terminal deletion mutants also comprised a total of eight truncations starting with deletion of the C-terminal bZIP domain (Fig. 2A). Deletion mutants lacking amino acid residues 200–249, 180–249, and 163–249 were unable to activate transcription (GAL-\(\alpha\)-(1–199), GAL-\(\alpha\)-(1–179), and GAL-\(\alpha\)-(1–162), respectively) (Fig. 2A). Removal of amino acids 116–249 (GAL-\(\alpha\)-(1–115)) generated, as before, a 12.8-fold increase in transactivation in our CAT reporter assays. Further deletion of 45 amino acids, resulting in a GAL-\(\alpha\)-(1–70) fusion, produced a 21.2-fold increase in the level of CAT activity (Fig. 2A). Fusions GAL-\(\alpha\)-(1–45) and GAL-\(\alpha\)-(1–18) both activated CAT expression; GAL-\(\alpha\)-(1–18) activated the CAT reporter gene to 80% of the level observed with GAL-\(\alpha\)-(1–70) (13.7- versus 17.2-fold) (Fig. 2, A and B).

Amino acid sequence comparisons have revealed the presence of two homologous domains at the N termini of C/EBP\(\alpha\) and C/EBP\(\beta\) that are also present in C/EBP\(\epsilon\) (amino acids 1–18 and 19–27) (33). CRP1, the rat homologue of C/EBP\(\alpha\), has been
shown to contain a second activation domain (amino acids 19–27), which corresponds to a motif shared by Fos and Jun called homology box 2 or HOB2 and AMD3 of C/EBPb (33, 46). However, a construct containing this domain alone, GAL-e-(19–45) showed little activity in our CAT reporter assays (Fig. 2A). Likewise, another fusion containing this second domain, GAL-e-(19–70), as well as the constructs GAL-e-(39–70) and GAL-e-(39–115), did not activate CAT expression beyond basal levels (data not shown). Similar results were obtained from transient transfections in U937 cells. Therefore, the essential element involved in C/EBPε transcriptional activation is localized within amino acids 1–18, and maximal transactivation function is conferred by amino acids 1–70.

C/EBPε has also been identified with an ATG site 32 amino acids upstream from our designated start site (11, 12). However, we have demonstrated that both isoforms of C/EBPε are present in cells transfected with a construct containing both ATG sites, suggesting that both proteins may have a role within the cell. Since these 32 N-terminal amino acids contain sequences related to the ADM1 region of C/EBPβ (33), fusions were constructed to include these 32 amino acids either alone (GAL-e32N) or with amino acids 1–18 (GAL-e32N18), 1–45 (GAL-e32N45), or 1–70 (GAL-e32N70). In our CAT reporter assays from transiently transfected COS-1 cells, GAL-e32N did not activate CAT expression beyond basal levels, and the presence of the upstream 32 amino acids did not increase the activation of CAT gene expression further compared with those sequences (amino acids 1–18, 1–45, and 1–70) previously identified as having transactivation function in C/EBPε (Fig. 2B). Western blotting confirmed that all fusions were expressed at similar levels (data not shown). These results indicate that the additional 32 amino acid residues do not significantly alter the transcriptional activity of C/EBPε.

2 A. M. Chumakov, unpublished observations.

FIG. 1. The C/EBPε activation domain is localized in the N terminus. A, schematic representation of expression and reporter plasmids used in transfection assays. The GAL4-(1–147) DNA binding domain was fused to either amino acids 1–115 (GAL-C/EBPε-(1–115)), 147–249 (GAL-C/EBPε-(147–249)), or full-length C/EBPε (GAL-C/EBPε). GAL-VP16 and GAL4DBD were included as positive and negative controls, respectively. The positions of the basic region (BR) and leucine zipper (L-ZIP) of the C/EBPε protein are indicated. The reporter plasmid (G5E1bCAT) consists of the CAT gene downstream of the adenoviral E1b promoter and five artificial GAL4 binding sites. Relative CAT activities are an average of three independent transfections in COS-1 cells. The basal level of the CAT activity with the reporter gene alone was set to 1, and transactivation was calculated as the ratio of activator-stimulated to basal CAT expression (× fold activation). B, representative CAT assays using the E1b reporter cotransfected with expression plasmids into COS-1 cells.
Identification of a Repressor Domain in C/EBPe—Our results suggest that the inability of GAL-C/EBPe to activate the CAT reporter gene may result from an intrinsic inhibitory domain in the protein. We observed little transactivation from GAL-e-(1–199), GAL-e-(1–179), and GAL-e-(1–162) fusion proteins from which the leucine zipper and basic regions were deleted sequentially (Fig. 2A). Further deletion of amino acids 116–162 (GAL-e-(1–115)), however, produced at least a 10-fold increase in CAT activity, thus suggesting the presence of a transcriptional inhibitory element within this segment (residues 116–162) of C/EBPe (Fig. 2A).

To test this hypothesis, we first cotransfected our GAL-C/EBPe fusion constructs with reporter plasmids that express intermediate levels of CAT activity to permit detection of either up- or down-regulation of transcription. The reporter constructs used in this study contained zero (G0TKCAT), one (G1TKCAT), or five (G5TKCAT) GAL4 binding sites upstream of the herpes simplex virus TK promoter (−105 to +51) controlling CAT expression. Cotransfection of the GAL-C/EBPe constructs with the reporter plasmid G5TKCAT into COS-1 cells showed that GAL-C/EBPe, GAL-e-(147–249), GAL-e-(40–249), and GAL-e-(111–249) repressed CAT gene expression up...
to 90% (Fig. 3). Also, constructs GAL-e-(1–199), GAL-e-(1–179), and GAL-e-(185–249) showed no significant effect on CAT activity, thus indicating that the observed repression was not due to the basic region and leucine zipper domains of C/EBPε (Fig. 3). In contrast, constructs GAL-e-(1–115) and GAL-e-(1–70) activated transcription in this reporter assay (Fig. 3). The above constructs produced similar levels of transcriptional repression or activation with G1TKCAT, but CAT expression was unchanged with G0TKCAT as the reporter plasmid, indicating that repression required binding of the fusion proteins to the reporter (data not shown).

In addition, the GAL-e-(40–249) fusion protein significantly inhibited CAT reporter genes downstream from the strong SV40 early control region (complete enhancer plus promoter) and the relatively weak (in uninfected cells) adenovirus 2 major late promoter (data not shown).

We next examined whether the repression domain of C/EBPε could inhibit the activational function of a known transcriptional activator. PCR-derived DNA fragments encoding C/EBPε amino acids 1–45, 1–115, 115–162, 115–199, or 162–199 were fused individually in frame downstream of the VP16 activation domain (Fig. 4A). Each construct was cotransfected into U937 cells with the G5E1bCAT reporter and tested for its ability to repress the transactivation of CAT by VP16. The chimeras GAL4-VP16ε-(115–162) and GAL-VP16ε-(162–199) repressed VP16 transactivation function 60 and 65%, respectively, when compared with GAL-VP16 (Fig. 4B). The GAL-VP16ε-(115–199) hybrid inhibited VP16 activation by 85%, suggesting a slight cooperative effect by the repression domain and basic region domain of C/EBPε (Fig. 4B). However, fusion of the VP16 activation domain to regions of C/EBPε previously shown to activate CAT gene expression (amino acids 1–45 and 1–115) had no significant effect on the transactivation function of VP16 (Fig. 4B). Our results demonstrate that C/EBPε possesses an intrinsic repressor domain that is capable of attenuating the strong transcriptional activator VP16.

Deletion of the Repression Domain of C/EBPε Increases Transactivational Capability on a Neutrophil Elastase Promotor—Our group and others have demonstrated that c-Myb, together with C/EBPα, C/EBPβ, and C/EBPε, synergistically activates the promoters of a subset of myeloid specific genes including the neutrophil elastase gene (44). Therefore, we next investigated the function of the transactivation and repression domains of C/EBPε in regulating a myeloid-specific gene promoter in the absence of GAL4DBD. The two full-length forms of C/EBPε (1–249 and PE32, the latter a gift from Dr. Kleanthis

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**Fig. 3.** GAL4-C/EBPε fusion proteins repress activities of promoters containing upstream GAL4 binding sites. The effect of GAL4-C/EBPε fusions on a TK promoter with five upstream GAL4 binding sites is shown. All GAL-C/EBPε fusions were cotransfected with the G5TKCAT reporter gene into COS-1 cells. The levels of CAT activity were quantitated, normalized to the level of the reporter alone (lane 1), which was set at 100%. These values are representative of two experiments for each fusion protein.
FIG. 4. The intrinsic repression domain of C/EBPε inhibits a heterologous transcriptional activation domain. A, GAL-VP16-C/EBPε repression domain fusions. PCR-derived C/EBPε gene segments encoding amino acids 1–45, 1–115, 115–162, 162–199, or 115–199 were fused to VP16 activation domain, which were then ligated in frame to the GAL4 DBD. B, CAT assay using G5E1bCAT reporter gene. All GAL-VP16-C/EBPε repression domain fusions driven by SV40 early promoter were cotransfected with G5E1bCAT reporter gene into U937. The level of CAT activity was quantitated and normalized to the CAT activity of GAL-VP16, which was set at 100%.

Xanthanopoulos) and various C- and N-terminal deletions were placed under the control of the CMV promoter, and four internal deletions were generated that removed the repression domain and its surrounding residues (Fig. 5A). These constructs, in the presence and absence of c-Myb, were cotransfected with the reporter NE-Luc into COS-1 cells, and the activity of the NE promoter was measured. Both full-length forms of C/EBPε activated the NE promoter 2-fold (Fig. 5B). Each N-terminal deletion mutant failed to activate transcription from the NE-Luc reporter gene (Fig. 5B). However, two of the internal deletions, CMV-cΔ71–161 and CMV-cΔ116–161, demonstrated a 2-fold higher luciferase activity over that seen with the full-length forms. We also observed that while C/EBP with c-Myb synergistically activated NE-Luc, deletion of the repression domain further increased transcription 2-fold in the presence of c-Myb (Fig. 5B).

DISCUSSION

Our results indicate that C/EBPε contains a distinguishable polypeptide element that can function in efficient transcriptional activation both in the context of the C/EBPε protein and when fused to a heterologous DNA binding domain. Moreover, we have identified an internal region of the C/EBPε protein that can negatively regulate gene transcription from three different promoters, as well as attenuating the function of a known potent transcriptional activator VP16. Furthermore, when the repression domain was deleted from C/EBPε, the altered C/EBPε protein enhanced transcription of gene transcription 2-fold when compared with that of the wild-type form of C/EBPε. Taken together, both transactivation and repression domains exist within the C/EBPε gene (Fig. 6A).

The activation domain of C/EBPε was mapped to the N terminus of the protein with a minimal requirement of the first 18 amino acids, while the highest activity of transactivation was conferred by amino acid residues 1–70. Two predicted activation domains within the N terminus of CRP1 (rat C/EBPε) were identified based on sequence comparison between C/EBP family members (33). When the first domain, amino acids 1–18, was fused to the GAL4DBD, its transactivation potential retained 80% of the peak activity. Amino acid residues 19–45 of C/EBPε, containing the second domain (amino acids 19–27), conferred only weak activating potential to the GAL4DBD; however, when both domains were fused to the GAL4DBD (GAL-ε(1–45)), potent activity was observed. The potent activity observed with GAL-ε(1–45) does not reflect synergy between these domains in human C/EBPε. Deletion of these two domains in the construct CMV-ε(40–249) abolished the transactivation potential on the NE promoter and reduced the synergistic stimulation of gene transcription with c-Myb.

The major difference in the transactivation domains between C/EBPε and other C/EBP proteins is that only one activation domain, amino acids 1–18 of C/EBPε, corresponding to ADM2 of C/EBPβ (Fig. 6A), is sufficient to confer a potent transcriptional activation function to a heterologous GAL4DBD (33). In contrast, the activation domains identified in either C/EBPα or C/EBPβ were unable to confer transcriptional activation when fused individually to the GAL4DBD (31, 32, 33, 36). Unlike C/EBPε, combinations of these individual domains resulted in synergistic activation, indicating that more than one domain was required for full activating potential to be realized.

Two forms of C/EBPε have been identified that differ by 32 amino acids containing sequences related to the ADM1 region of C/EBPβ (11, 33). However, this sequence alone was inactive, and in combination with the identified activation domains it did not increase the transactivating potential on the GAL4DBD. Further, both forms activated the NE promoter to the same level in the absence or presence of c-Myb. In C/EBPβ,
two residues, Phe$^6$ and Tyr$^7$, were identified as being essential for the function of ADM1, and mutation of these residues yielded a protein with activity similar to a protein lacking this domain (33). The aforementioned 32 amino acids of C/EBP$\text{e}$ contain tyrosine residues at both sites, suggesting a possible reason for the lack of activity in this domain.

Among the six C/EBP family members characterized, C/EBP$\alpha$, C/EBP$\beta$, C/EBP$\delta$, and C/EBP$\varepsilon$, are activators of transcription for a variety of target genes. However, C/EBP$\alpha$ (32, 36) and C/EBP$\beta$ (33) both also contain extra regulatory domains, called the attenuator domain in C/EBP$\alpha$ and RD1 and RD2 in C/EBP$\beta$ (Fig. 6A). In C/EBP$\beta$, RD1 and RD2 have independent functions. While RD1 modulates the activation domain of the protein, RD2 controls cell type-specific transactivation function through regulation of its DNA binding activity; together, RD1 and RD2 inhibit transcription 50-fold and decrease VP16 transactivation function by over 95% (33). The attenuator domain identified in C/EBP$\alpha$, however, exhibits neither detectable stimulation nor repression of transcription. When combined with other activation domains of C/EBP$\alpha$, the

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**FIG. 5.** Deletion of C/EBP$\varepsilon$ repression domain enhances activation of the neutrophil elastase gene promoter by c-Myb. **A**, schematic diagram of CMV-driven C/EBP$\varepsilon$ constructs. Full-length C/EBP$\varepsilon$ and its deletions were inserted into the pCMVSPORT expression vector. **B**, these plasmids were cotransfected into COS-1 cells together with (filled bars) or without (open bars) a c-Myb expression plasmid, which was also driven by the CMV promoter. The target gene was the neutrophil elastase gene promoter, which contains a C/EBP consensus binding site and a Myb binding site, fused to the luciferase gene. Forty-eight hours after DNA transfection, cell extracts were prepared, normalized to the levels of $\beta$-galactosidase activity used to monitor transfection efficiency, and analyzed for luciferase activity. All luciferase activities were normalized to the level of the reporter plasmid alone. These values were the average of two independent transfections. **BR**, basic region; **L-ZIP**, and leucine zipper.
attenuator domain diminishes their transactivation effect (32, 36). Deletion of these sequences causes only a modest increase in transcriptional activity (33). The attenuator domain may actually be a regulatory domain that allows post-translational modification of C/EBPα, possibly converting a repressor into an activator or a weak activator into a strong activator (36).

C/EBPε is distinct from other C/EBP family members in that, although C/EBPε expression occurs exclusively within the myeloid and lymphoid lineages, the identified domains show little difference in cell type-specific regulation of transcription. As for C/EBPα and C/EBPβ, we have identified a negative regulatory element in C/EBPε localized to amino acids 116–162, downstream of the activation domain and upstream of the DNA binding and dimerization domain (Fig. 6A). In contrast to C/EBPβ, deletion of the C/EBPε repression domain does not give the same magnitude of increase in transactivation, and this effect may be more similar to the increase observed with deletion of the attenuator domain of C/EBPα. Also, the inhibition of VP16 transactivation function by the repression domain of C/EBPε is significantly less than that mediated by RD1 and RD2 of C/EBPβ. Therefore, the repression domain identified in C/EBPε may be functionally more similar to the attenuator domain of C/EBPα rather than RD1 and RD2 of C/EBPβ. We cannot determine from these experiments whether the GAL4-C/EBPε fusions repress transcription directly or whether additional factors interacting with the repression domain are required.

What role do the transcriptional activation or repression domains play in the function of C/EBPε? Recent studies indicate that it is quite common for a protein to serve either as an activator or a repressor, depending on the other gene regulatory proteins present in the cell (47). In the case of C/EBPε, this may reflect its specific role in regulating myelopoiesis (48, 49).

C/EBPε has been demonstrated to have a remarkable effect on myeloid cell proliferation (12). Transient transfection of a promyelocyte cell line (NB4) with a C/EBPε expression plasmid increased cell growth by 7-fold, but proliferation was significantly decreased upon transfection of the C/EBPε antisense expression vector (12). Cells may depend on a negatively regulatory domain (C/EBPε itself) or associated factors that interact with this domain to control cell proliferation at a certain growth stage. Therefore, the repression domain (and its associated factors) probably acts as a buffer for the activation function of C/EBPε.

Our simplified and speculative model shown in Fig. 6B proposes that C/EBPε interacts with different partners in the varied contexts of cellular signaling, which induces exposure of the activation or repression domain. These domains may actually represent oligomerization surfaces that allow C/EBPε to form higher order aggregates either with itself or with other proteins that play a more direct role in transcriptional regulation of myeloid genes. In support of this model, our data indicate that Myb is such a potential activation partner. Many other proteins have been suggested as potential C/EBP-interacting factors (21–29), and likewise, we anticipate that C/EBPε interacts with other factors. It will be interesting to identify factor(s) that interact with the repression domain to dissect further the repressive function of C/EBPε. Although no common signature has been found within the repression domain of C/EBPε when searched in the data base, it contains a relatively high content of prolines and other hydrophobic amino acid residues. Application of the two-hybrid cloning system by using the repression domain of C/EBPε as bait may lead to new information about the network of transcription factors that specifically modulate genes important in hematopoiesis.

Fig. 6. A model for C/EBPε function. A, a comparison of the mapped regulatory domains of C/EBPα, C/EBPβ, and C/EBPε. B, we propose that C/EBPε modulates myeloid gene expression by cooperating with either transactivation partner(s) or trans-dominant repressive factor(s). BR, basic region; L-ZIP, and leucine zipper.
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REFERENCES

1. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. (1988) Genes Dev. 2, 786–800
2. Akira, S., Ishihaki, H., Suguita, T., Tanaba, S., Kinoshita, S., Nishio, Y., Hirane, T., and Kishimoto, T. (1990) EMBO J. 9, 1897–1906
3. Cao, Z., Umek, R. M., and McKnight, S. L. (1990) Genes Dev. 5, 1520–1532
4. Chang, C. J., Chen, T. T., Lei, H. Y., Chen, D. S., and Lee, S. C. (1990) Mol. Cell. Biol. 10, 6642–6653
5. Desombe, P., and Schibler, U. (1991) Cell 67, 569–579
6. Katz, S., Kowen-Leutz, E., Muller, C., Meese, K., Ness, S. A., and Leutz, A. (1993) EMBO J. 12, 1321–1322
7. Poli, V., Mancini, P. P., and Cortese, R. (1990) Cell 63, 643–653
8. Roman, C., Platero, J. S., Shuman, J. D., and Calame, K. (1990) Genes Dev. 4, 1404–1415
9. Kinoshita, S., Akira, S., and Kishimoto, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1473–1476
10. Williams, S. C., Cantwell, C. A., and Johnson, P. F. (1991) Genes Dev. 5, 1553–1567
11. Antonson, P., Stellen, B., Yamanaka, R., and Xanthopoulous, K. G. (1996) Genomics 35, 30–38
12. Chumakov, A. M., Grass, I., Chumakova, E., Chih, D., Slater, J., and Koeffler, H. P. (1996) Mol. Cell. Biol. 17, 3375–3386
13. Ron, D., and Habener, J. F. (1992) Genes Dev. 6, 439–453
14. Akira, S., and Kishimoto, T. (1992) ImmunoL Rev. 127, 25–50
15. Lichtsteiner, S., Wurin, J., and Schibler, U. (1987) Cell 51, 963–973
16. Olivier, S., and Cortese, R. (1989) EMBO J. 8, 1145–1151
17. Freytag, S., and Geddes, T. (1992) Science 256, 379–382
18. Umek, R. M., Friedman, A. D., and McKnight, S. L. (1991) Science 251, 288–292
19. Johnson, P., Landschulz, W., Graves, B., and McKnight, S. (1987) Genes Dev. 1, 133–146
20. Leinwand, M., Larson, C., Fomin, A., Xu, M., Stavnezer, J., and Severinson, E. (1994) J. Immunol. 153, 2983–2995
21. Mink, S., Kerber, U., and Klemmnaeuer, K.-H. (1996) Mol. Cell. Biol. 16, 1316–1325
22. Leclair, K. P., Blanar, M. A., and Sharp, P. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8145–8149
23. Tsukada, J., Saito, K., Waterman, W. R., Webb, A. C., and Auron, P. E. (1994) Mol. Cell. Biol. 14, 7285–7297
24. Vallejo, M., Ron, D., Miller, C. P., and Habener, J. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4679–4683
25. Hsu, W., Kerppola, T. K., Chen, P.-L., Curran, T., and Chen-Kiang, S. (1994) Mol. Cell. Biol. 14, 268–276
26. Nishio, Y., Ishihaki, H., Kishimoto, T., and Akira, S. (1983) Mol. Cell. Biol. 13, 1854–1862
27. Zhang, D. E., Hetherington, J., Meyers, S., Rhoades, K. L., Larson, C. J., Chen, H. M., Siebert, S. W., and Tenen, D. G. (1996) Mol. Cell. Biol. 16, 1211–1240
28. Ray, A., Hannink, M., and Ray, B. K. (1995) J. Biol. Chem. 270, 7365–7374
29. Chen, P.-L., Riley, D. J., Chen-Kiang, S., and Lee, W.-H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 465–469
30. Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1989) Science 243, 1681–1688
31. Friedman, A. D., and McKnight, S. L. (1990) Genes Dev. 4, 1416–1426
32. Ferlov, C., and Ziff, E. B. (1994) Genes Dev. 8, 350–362
33. Williams, S. C., Baer, M., Diller, A. J., and Johnson, P. F. (1995) EMBO J. 14, 3170–3183
34. Barone, M. Y., Crozat, A., Tabaea, A., Phillipson, L., and Ron, D. (1984) Genes Dev. 8, 453–464
35. Cooper, C., Henderson, A., Artandi, S., Avitahl, N., and Calame, K. (1995) Nucleic Acids Res. 23, 4371–4377
36. Pei, D., and Shih, C. (1991) Mol. Cell. Biol. 11, 1480–1487
37. Yamanaka, R., Kim, G.-D., Radomska, H. S., Lekstrom-Himes, J., Smith, L. T., Antonson, P., Tenen, D. G., and Xanthopoulos, K. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6462–6467
38. Chih, D. Y., Chumakov, A. M., Park, D. J., Silla, A. G., and Koeffler, H. P. (1997) Blood 90, 2987–2994
39. Morosetti, R., Park, D. J., Chumakov, A. M., Grillier, I., Shiouara, M., Gombart, A. F., Nakaokaki, T., Weinberg, K., and Koeffler, H. P. (1997) Blood 90, 2091–2096
40. Sadowski, I., and Ptashne, M. (1989) Nucleic Acids Res. 17, 7539
41. Cress, W. D., and Tiedzenberg, S. J. (1991) Science 251, 87–90
42. Sadowski, I., Ma, J., Tiedzenberg, S., and Ptashne, M. (1988) Nature 335, 559–562
43. Yew, P. R., Xuan, L., and Berk, A. J. (1994) Genes Dev. 8, 190–202
44. Oegeschlagler, M., Nuchprayoon, I., Luscher, B., and Friedman, A. (1996) Mol. Cell. Biol. 16, 4717–4725
45. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) Current Protocols in Molecular Biology, pp. 7.9.1–7.9.4, John Wiley and Sons, Inc., New York
46. Sutherland, J. A., Cook, A., Bannister, A. J., and Kouzarides, T. (1992) Genes Dev. 6, 1810–1819
47. Johnson, A. D. (1995) Cell 81, 655–658
48. Scott, L. M., Civin, C. I., Rorth, P., and Friedman, A. D. (1992) Blood 80, 1725–1735
49. Shivdasani, R. A., and Orkin, S. H. (1996) Blood 87, 4025–4039