A Short Conserved Motif Is Required for Repressor Domain Function in the Myeloid-specific Transcription Factor CCAAT/Enhancer-binding Protein ε*

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The CCAAT/Enhancer-binding protein ε (C/EBPε) is expressed almost exclusively in the myeloid lineage of the hematopoietic system and functions during terminal differentiation of neutrophils and macrophages, and in the regulation of cytokine gene expression in macrophages and T cells. We have undertaken a series of structure/function studies on the murine C/EBPε polypeptide to investigate the mechanism by which C/EBPε activates transcription. Studies with deletion mutants and fusion proteins consisting of C/EBPε sequences joined to the Gal4 DNA-binding protein identified two transcriptional activation domains in C/EBPε. Removal of sequences between the two activation domains or sequences between the second activation domain and the C-terminal DNA binding domain significantly increased the activity of C/EBPε, suggesting the presence of two separate regulatory domains (designated RD-1 and RD-2). RD-1 behaved as a classic active repressor domain being capable of inhibiting adjacent activation domains irrespective of their origin and when linked to a heterologous DNA binding domain. Mutagenesis studies revealed a short motif in RD-1 that appears to be a target site for protein-protein interactions and is conserved in repressor domains from C/EBPβ, Sp3, c-Fos, and FosB. The juxtaposition of activation and repressor domains may permit C/EBPε to function as a transcriptional activator or repressor at different stages of myeloid differentiation or as an inducible transcriptional activator of cytokine genes.

Transcription factors play important roles in the commitment of precursor cells to particular hematopoietic lineages and the terminal differentiation of specific cell types (1). The CCAAT/Enhancer-binding protein (C/EBP) family of basic region/leucine zipper transcription factors consists of six members that display similar DNA binding specificities and are capable of homo- and heterodimerization in all combinations (reviewed in Ref. 2). Four family members, C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε, are expressed in myeloid cells and appear to play different roles in differentiating and mature cells (3, 4). C/EBPα appears to be critical for granulocytic differentiation (5), whereas C/EBPβ and C/EBPδ appear to function primarily as regulators of cytokine gene expression during inflammatory responses in macrophages (6–8). C/EBPε was first identified based on its relatedness to C/EBPα (9) and is almost exclusively expressed in myeloid cells, with additional sites of expression in lymphoid cells and ovary (10–12). C/EBPε expression is essentially undetectable in uncommitted hematopoietic progenitors but increases during myeloid differentiation resulting in high level expression in mature neutrophils and macrophages (12–14). Mice lacking C/EBPε display defects in granulocytic development (15), a phenotype that is also seen in C/EBPα-deficient mice (5). C/EBPε appears to act later in granulopoiesis than C/EBPα, and its expression may be activated by C/EBPα in this lineage (16, 17).

Although the primary defects observed in C/EBPε-deficient mice were in the development of the granulocyte lineage, there is evidence that C/EBPε also functions in other hematopoietic cell types. C/EBPε mRNA is present in primary murine macrophages and in multiple immortalized monocyctic and macrophage cell lines, and a 34,000 molecular weight C/EBPε polypeptide has been detected in the IC-21 P388D1(IL-1) cell line (12). Ectopic expression of C/EBPε in a lymphoid cell line activated the expression of a number of genes normally expressed in monocytes, including lipopolysaccharide-regulated cytokine genes and the macrophage-CSFR (M-CSFR) gene (12). The lack of defects in monocytic development and function in C/EBPε-deficient mice may be due to functional redundancy among C/EBP family members expressed in this cell lineage (7), C/EBPε mRNA was also detected in the human Jurkat T cell line (10), and the expression of some genes normally expressed in T cells, including interleukin-2 and -4, was diminished or abolished in C/EBPε-deficient mice (15).

The C/EBPε gene contains at least one intron and alternative splice sites and promotors permit the production of multiple polypeptides in humans (14). We have detected a single C/EBPε polypeptide in rodent cells that may direct the synthesis of two forms of C/EBPε due to the presence of at least two in frame translation initiation codons (12). We have carried out a structure/function analysis of the murine C/EBPε polypeptide to identify domains responsible for the transcriptional activity of C/EBPε. We have identified two activation domains in C/EBPε, one at the N terminus and a second close to the center of the protein. In addition, two regions within the C/EBPε polypeptide act as repressor domains and are likely to be sites of protein-protein interactions that modulate the activity of C/EBPε.

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 EXPERIMENTAL PROCEDURES

Plasmid Construction—The murine C/EBPe mRNA contains two in frame initiation codons separated by 96 base pairs (12). PCR was used to introduce NcoI sites at the first of these two sites and a HindIII site following the termination codon, and the resultant cDNA was inserted into the pMEX eukaryotic expression vector (9). N-terminal and internal deletion constructs were prepared by PCR in a manner similar to that previously described for C/EBPβ (18). Briefly, PCR was used to introduce either BgIII or BamHI restriction sites at specific positions within the C/EBP coding sequence, and the resultant fragments were combined to introduce the required mutation. The sequences of the oligonucleotides used in this study are shown in Table I. Each was designed to insert restriction sites at or just following a proline residue and each carried a G-rich sequence at the 5' end to enhance enzyme digestion.

Gal4 fusion genes were constructed in the Gal4 plasmid, which is similar to pSG424 (19). This plasmid encodes amino acids 1–147 of the yeast Gal4 transcriptional activator located downstream of the SV40 early promoter. All C/EBPα- and VP16-derived segments were inserted downstream of the Gal4 sequences as NcoI/Clal fragments as described previously (18). The VP16 activation domain fragment used here encodes amino acids 429–456 (20). Gal4 C/EBPα-(1–108), Gal4 C/EBPβ-(1–83), and Gal4 C/EBPβ-(33–64) (previously named Gal4 CRP1-(33–64)) encode the indicated amino acids from each C/EBP protein and have been described previously (18). The C/EBP-dependent (DE1), 35AlbLUC (which consists of four copies of the DEI element from the serum albumin gene linked to the minimal promoter region of the same gene) and Gal4-dependent G5E1bLUC (which consists of five copies of a Gal4-binding site upstream of the E1b minimal promoter) luciferase reporter plasmids were described previously (18).

Cell Culture and Transfections—The human HepG2 hepatocarcinoma and monkey COS-1 cell lines were cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VI) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). HepG2 cells were transfected at approximately 30–40% confluency in 3.5-cm dishes using a Clontech transfection kit (Pierce). Nuclear extracts were prepared from COS-1 cells as described previously (8). Immune detection was carried out as described previously (22) using either a C/EBP-specific rabbit polyclonal antiserver (C-22, Santa Cruz Biotechnology, Santa Cruz, CA) or a mouse monoclonal antiserver directed against the DNA binding domain of Gal4 (R5K51, Santa Cruz Biotechnology). Immune complexes were detected using the Supersignal chemiluminescence detection kit (Pierce).

RESULTS

The C/EBPα Protein Contains Two Regions That Function as Transcriptional Activation Domains—To identify functional domains that mediate transcriptional activation by C/EBPα, we compared the ability of wild type C/EBPα and two proteins lacking N-terminal segments to activate the (DE1)35AlbLUC reporter construct in HepG2 human hepatocarcinoma cells (Fig. 1A). This construct was activated 45-fold by full-length C/EBPα; however, deletion of either 96 or 139 N-terminal amino acids decreased transcriptional activity to background levels. Thus, C/EBPα is capable of functioning as a potent transcriptional activator. The N-terminal portion of C/EBPα contains most or all of the activation sequences. The N-terminal sequences of C/EBPα, C/EBPβ, C/EBPδ, and C/EBPδ possess three segments of significant sequence similarity that correspond to three activation domain modules (ADM) first identified in C/EBPβ (18) (Fig. 1B). We previously demonstrated that amino acids 33–64 of C/EBPα (which contain ADM2 and -3) functions as a relatively weak activation domain when fused to the DNA binding domain of the yeast transcriptional activator protein Gal4 (18). Therefore, we tested whether inclusion of the first 32 amino acids of C/EBPα, which contain sequences similar to the ADM1 domain of C/EBPβ, would increase the potency of the N-terminal C/EBPα activation domain (ADI). The addition of amino acids 1–32 resulted in a slight but significant (p < 0.01) increase in C/EBPα AD activity (compare Gal4 e(33–64) to Gal4 e(1–64)), indicating that the ADM1 region is likely to be a functional component of ADI of C/EBPα (Fig. 1C). In comparison, the equivalent regions of the C/EBPα (amino acids 1–108) and C/EBPβ(1–83) proteins were 4.5- and 7.5-fold, respectively, more powerful in this assay. The C/EBPα polypeptide also shares a second region of homology (amino acids 140–162) with

| Table I | Oligonucleotides used in the synthesis of C/EBPα segments |
|-----------------|------------------|
| 5'-Oligonucleotide | Amino acid |
| gacgcgagatcGGCCGAAGGCAGG | Pro64 |
| gacgcgagatcCCCATGGAGAGGCTT | Pro67 |
| gacgcgagatcGGCGAGGGACCCGA | Pro67 |
| gacgcgagatcCCTGCCAGTACCAA | Pro67 |
| gacgcgagatcCCCTGGCCAGCTTG | Pro65 |
| gacgcgagatcGGGTCGGCGG | His394 |

| 3'-Oligonucleotide | Amino acid |
|-----------------|------------------|
| gacgcgagatcGGCGTGTTCTCAT | Pro64 |
| gacgcgagatcTCTGGGGCAATGTTG | Asp96 |
| gacgcgagatcTGGGGGCTGACTCC | Arg516 |
| gacgcgagatcTGGCCCTCAGGAGCTC | Glu228 |
| gacgcgagatcGGGTCGGCGGTCG | Pro58 |
| gacgcgagatcGGGTCGGCGGCGG | Pro93 |

*2 N. D. Angerer and S. C. Williams, unpublished results.
*3 A. J. Reinhart, S. C. Williams, B. J. Clark, and D. M. Stocco, submitted for publication.
Repressor Domains in C/EBP\textepsilon

Fig. 1. C/EBP\textepsilon contains two transcriptional activation domains. A, full-length C/EBP\textepsilon-(1–281) and two N-terminal deletion mutants initiating at amino acids 97 and 141 were co-transfected into HepG2 cells with the (DEI)\textsubscript{4}-35AlbLUC reporter plasmids. Luciferase activities were measured and are represented graphically as fold activation (±S.E.) compared with the reporter plasmid alone. The structure of the C/EBP\textepsilon proteins is diagrammed, and shaded regions represent sequence similarity to two transcriptional activation domains (ADI and ADII) previously identified in C/EBP\alpha and C/EBP\beta. B, the N-terminal sequences of C/EBP\alpha, C/EBP\beta, and C/EBP\epsilon were aligned to identify regions of similarity. Amino acid coordinates are shown at the ends of each sequence, and identical amino acids are represented with vertical bars and conservative substitutions with colons.

C/EBP\alpha and C/EBP\beta which includes sequences identified as a second activation domain in C/EBP\alpha (23–25). This region of C/EBP\epsilon functioned as a weak activation domain (referred to as ADII) when fused to the Gal4 DBD (Fig. 1C).

C/EBP\epsilon Contains Two Internal Regulatory Domains—We next tested whether the C/EBP\epsilon polypeptide contains additional sequences outside the activation domains that modulate its transcriptional activity. C/EBP\epsilon proteins lacking internal sequences were tested for their ability to activate (DEI)\textsubscript{4}-35AlbLUC reporter in HepG2 cells (Fig. 2A). The removal of amino acids 65–194 (i.e. all sequences between ADI and the DBD) resulted in a highly significant ($p < 0.01$) 6-fold stimulation of transcriptional activity (compare 1–281 and 1–281 (Δ65–194)). Proteins lacking sequences between ADI and ADII (1–281/Δ65–96) and 1–281/Δ65–139) (Fig. 2A) were also more active than the wild type C/EBP\epsilon protein in this assay, approximately 4- and 7-fold, respectively, identifying a region between amino acids 65 and 140 (termed regulatory domain-1E or RD-1E) that negatively regulates C/EBP\epsilon activity. Removal of amino acids between ADII and the DBD did not significantly affect C/EBP\epsilon activity (1–281/Δ65–194) (Fig. 2A), but combining the deletion of amino acids 163–194 with the deletion of the RD-1E region resulted in further stimulation of C/EBP\epsilon activity. For example, a protein lacking amino acids 65–139 and 163–194 displayed approximately 1.5-fold greater activity than a protein lacking amino acids 65–139 alone (Fig. 2A), and similar results were observed with other constructs lacking sequences in both regions (data not shown). We conclude that a second negatively acting domain, named RD-2E, is located between amino acids 163 and 194 which in this context appears to function in combination with RD-1E. Similar steady-state levels of each recombinant protein were detected by Western analysis (Fig. 2B) indicating that the observed effects were not simply due to differences in protein expression levels or stability.

Fine Mapping of Regulatory Domains Using Gal4 Fusion Proteins—To test whether C/EBP\epsilon regulatory domains could function when attached to a heterologous DNA binding domain, we constructed a series of Gal4-C/EBP\epsilon chimeric genes and tested their ability to activate expression from the Gal4-dependent G\textsubscript{4}E\textsubscript{1}bLUC reporter plasmid in HepG2 cells. Fusion proteins bearing the first 64, 98, or 116 amino acids of C/EBP\epsilon all functioned as strong transcriptional activators (Fig. 3A). However, a significant 34-fold decrease (compared with Gal4\epsilon-(1–64)) in activity was observed when C/EBP\epsilon sequences were extended to amino acid 128, indicating that RD-1E is located N-terminal to this amino acid. A 5-fold increase in activity was observed upon inclusion of sequences up to amino acid 162, presumably due to the inclusion of ADII, and a further decrease (3.3-fold) occurred when the C/EBP\epsilon sequences were extended to amino acid 193 to include the RD-2E-containing region. Finally, we tested whether RD-2E is capable of functioning in the absence of RD-1E by fusing amino acids 162–193 directly to ADI. The addition of this region resulted in a 4-fold decrease in activity (compare Gal4\epsilon-(1–64) to Gal4\epsilon-(1–64)/Δ162–193) indicating that RD-2E is capable of repressing ADI function in a position- and ADI-independent fashion in Gal4 fusions. Unfortunately, we were unable to detect expression of Gal4 fusion proteins in nuclear extracts of HepG2...
cells; however, they were detectable after transient transfection of COS-1 cells. All Gal4 fusions behaved similarly in transactivation assays in COS-1 cells and were expressed at similar levels (Fig. 3B) again indicating that the observed functional differences are not due to variations in protein levels.

RD-1ε and RD-2ε Repress a Heterologous Activation Domain—To study the activation domain specificity of the two regulatory domains in C/EBPε, we tested their ability to repress the activity of Gal4 fusion proteins containing the potent activation domain from the herpes simplex virus VP16 protein (Fig. 4A). Initially we tested whether C/EBPε sequences could repress transactivation when placed downstream of the VP16AD, i.e., in a similar position relative to the activation domain as in C/EBPε. The activity of the Gal4VP16 protein on the G5E1bLUC reporter plasmid in HepG2 cells was set at 100%, and the activity of each fusion protein was calculated relative to this value. The inclusion of amino acids 64–98 or 64–116 did not diminish the activity of the VP16AD. However, a protein containing amino acids 64–128 displayed only 16% of the activity of the control, indicating that RD-1ε is capable of...
repressing the activity of the VP16AD. To map the N-terminal boundary of RD-1e, three additional constructs containing C/EBPε sequences initiating at amino acid 97 were tested. Fusion proteins carrying amino acids 97–162 or 97–193 were 55 and 66% as active as Gal4VP16AD; however, the attachment of amino acids 97–128 repressed VP16AD activity to 4% of control levels. These data further refine the position of RD-1e sequence located between amino acids 97 and 128 (indicated with a hatched box), and RD-2e is located between amino acids 162 and 193 (indicated with a stippled box). B, representative Western blot showing comparable accumulated levels of proteins with significantly different activities.

A second series of chimeric genes was constructed in which the order of the C/EBPε and VP16 sequences was reversed to test for position-dependent effects. Although some differences in the repressive activity of the C/EBPε regulatory domains were observed in the two sets of proteins, the overall pattern of activity was similar (Fig. 4A). The minimal RD-1e and RD-2e segments (amino acids 97–128 and 162–193, respectively) both repressed VP-16AD function, albeit to a slightly lesser but statistically significant (p < 0.01) extent than in the previous set of chimeras. In this second arrangement, RD-2e appeared to be an efficient repressor module even in the presence of ADII of C/EBPε (compare Gal4 ε-(97–162)-V to Gal4 ε-(97–193)-V), which could potentially be explained by its close proximity to the VP16AD in these proteins.

**RD-1e Contains Sequence Motifs That Are Conserved in Other Repressor Domains**—We next compared the sequence of amino acids 64–128 of C/EBPε to the sequence of repression domains identified in other transcription factors. We identified two small conserved motifs (termed conserved motif (CM) 1 and 2 in Fig. 5) in a subset of factors examined. CM1 is located between amino acids 82 and 86 of C/EBPε and is conserved in C/EBPε, rat and human C/EBPβ, and Sp3 but is absent in chicken C/EBPβ, c-Fos, and FosB. A consensus sequence was derived for CM1 as Pro-Ala-Asp-X-B, where X is any amino acid and B is a basic amino acid. CM1 lies outside the region defined as RD-1e and would not be predicted to be critical for RD-1e function. CM2 is located between amino acids 121 and 128 of C/EBPε (i.e., within RD-1e), and related sequences were present in all proteins listed. CM2 has the consensus sequence B-Glu-Glu-X-X-Pro-Glu and is most highly conserved in C/EBPε, C/EBPβ (human and chicken), Sp3, and FosB.

We next tested whether mutating residues in CM1 and/or CM2 would diminish RD-1e function. The mutations focused on the conserved PAD and KEE motifs in CM1 and CM2, respectively (Fig. 5). Both motifs were replaced with alanines in one or both of the Gal4 ε-(1–128) and Gal4 ε-(64–128)-V fusion proteins (see Figs. 3 and 4). Mutation of the CM1 sequence had no effect on the activity of Gal4 ε-(1–128) as proteins thus configured displayed repressed activities similar to the wild-type protein (A82/4 mutant in Fig. 6A). However, mutating CM2 to three alanine residues (A121/3 mutants) resulted in significant increases in the activity of both proteins, 12-fold in Gal4 ε-(1–128) and 29-fold in Gal4 ε-(64–128)-V (Fig. 6, A and B). Mutating pairs of amino acids (A121/2 and A122/3) or the terminal glutamic acid residue alone (A123) all significantly reduced the activity of the regulatory domain in Gal4 ε-(1–128) and, where tested, Gal4 ε-(64–128)-V indicating that residues 121 and 123 within CM2 are critical for RD-1e function. The contribution of the glutamic acid at position 122 remains to be verified. Equivalent levels of each protein were detected by Western blotting (Fig. 6, C and D).

**RD-1e Appears to be a Site for Protein-Protein Interactions**—Finally, we tested whether co-expression of RD-1e might result in derepression of a Gal4-C/EBPε fusion protein by competing for binding of a nuclear protein. Increasing amounts of the C/EBPε-(97–281) expression vector were co-transfected with Gal4 ε-(1–128) into HepG2 cells, and luciferase activities were measured (Fig. 7). At the highest level of C/EBPε-(97–281) expression vector the activity of Gal4 ε-(1–128) was 3-fold higher than in the absence of any competitor molecule. To test whether this effect was attributable to the co-expression of a molecule containing a functional RD-1e element, a second series of transfections were carried out using a competitor molecule carrying the A121/3 mutation, which changes the KEE motif to three alanine residues and abolishes RD-1e activity (see Fig. 6, A and B). In this case relief of inhibition was not observed, in fact luciferase activities decreased as the amount of competitor was increased which we interpret to be the result.
of competition between the promoters in the two expression vectors. Both proteins were efficiently synthesized as measured by Western analysis (Fig. 7B). Taken together, the results of these assays indicate that a co-expressed RD-1e-containing protein is able to relieve inhibition in Gal4 e-(1–128) presumably by competing for a cellular protein that normally binds to RD-1e and mediates the repressive effect of this domain.

**DISCUSSION**

The individual members of the C/EBP family possess a highly conserved bZIP DNA binding domain and are consequently capable of binding to identical recognition sequences in the control regions of target genes (9, 26). Therefore, the unique functions of each protein are likely to be controlled by domains outside the DNA contacting basic region, and numerous structure/function studies have been carried out to identify these domains. In the case of C/EBPε, we have previously identified domains that control DNA binding, dimerization (9), nuclear localization (27), and transcriptional activation (12). To understand more clearly the activity of C/EBPε is regulated we have now identified additional domains that control the activity of C/EBPε. In addition to defining sequences that function as...
transcriptional activation domains, we also identified two separate regulatory domains, RD-1e and RD-2e, that repress C/EBPε activity. RD-1e is located between the two transcriptional activation domains and is wholly contained in the region spanning amino acids 97–281. RD-2e is located N-terminal to the DNA binding domain, and its activity in the context of C/EBPε was only evident when RD-1e was also absent. However, RD-2e was capable of independently inhibiting a linked activation domain in Gal4 fusion proteins. Over the past several years, numerous nuclear proteins have been identified that contain domains that negatively regulate transcription (reviewed in Ref. 28). Two criteria have been used to define these domains as active repressor domains as follows: the ability to function in a modular fashion when attached to a heterologous DNA binding domain, and second to act as the site for interactions with corepressor proteins (29). By these criteria, RD-1e behaves as an active repression domain as it inhibited the activity of C/EBPε and VP16 activation domains when attached to the Gal4 DBD. In addition, our competition data indicate that RD-1e is a site for protein-protein interactions. RD-2e was also capable of inhibiting a linked activation domain when attached to a heterologous DNA binding domain thereby satisfying one criterion for characterization as an active repression domain.

Specific motifs within repressor domains, often comprised of just a few amino acids, have been identified that are critical for repressor domain function. For example, the repressor domains of the Drosophila proteins Hairy and Enhancer of Split contain the four amino acid motif, Trp-Arg-Pro-Trp (WRPW), which mediates interactions with members of the Groucho family of corepressors (30). Our mutation studies identified the sequence Lys-Glu-Glu (KEE) in RD-1 as being required for repressor domain function and for protein-protein interactions. This same motif is required for activity of the repressor domain of Sp3 (31), and similar sequences are present in RD-1 of C/EBPβ (18) and the inhibitory domain (ID-1) of c-Fos and FosB (32).

The corresponding amino acids have not been mutated in C/EBPβ, c-Fos, or FosB; however, the repressor domain of c-Fos has been shown to be a site for protein-protein interactions (32). Therefore, KEE or a similar motif may be a signature for repressor domains from proteins derived from diverse transcription factor families. The KEE motif is not present in RD-2ε; however, as shown in Fig. 8, RD-2e contains multiple copies of the consensus minimal recognition sequence for members of the MAP kinase family, the dipeptide (Ser-Thr)-Pro (underlined) (33). Therefore, it is conceivable that phosphorylation of the RD-2ε element by a member of the MAP kinase family might regulate the activity of C/EBPε. Little is currently known concerning the phosphorylation of C/EBPε; however, support for this hypothesis comes from studies of other C/EBP family members, particularly C/EBPβ. Comparison of this region of RD-2ε to the corresponding regions of C/EBPα, C/EBPβ, and C/EBPδ (i.e. the region immediately N-terminal to the basic region) reveals multiple putative MAP kinase sites in each protein (Fig. 8). Phosphorylation of the threonine residue at position 235 of human C/EBPβ (amino acid 168 of rat C/EBPβ) via a Ras-dependent MAP kinase pathway in NIH3T3 and P19 cells has been shown to stimulate C/EBPβ activity (34). In addition, mutation of this residue to a non-phosphorylatable residue decreased C/EBPβ activity in P19 and neuronal cell lines (34, 35). Mutagenesis studies should begin to define the importance of this region for RD-2ε and C/EBPε activity.

Two previous studies proposed that the activity of C/EBPβ was inhibited by intermolecular interactions between the repressor and activation domains, which served to block access of the activation domain to its target proteins (18, 36). However, the data presented here suggest that the RD-1e, and probably the RD-2e, repressor domains of C/EBPβ act as sites for interactions with accessory proteins that act as corepressors. Multiple proteins have been identified that physically interact with members of the C/EBP family, although most of these proteins interact via the bZIP domain. These proteins are generally transcriptional activators that bind to adjacent sites on promoters and synergize with C/EBP proteins in activating transcription of the target gene and include NF-κB (37, 38), AM11, and PU1 (39), glucocorticoid receptor (40), Fos and Jun (41), Nopp140 (42), and Sp1 (43). In addition, a limited number of
proteins have been identified that interact with regions outside the DNA binding domain of C/EBP proteins, including Sp1 (43) and the retinoblastoma protein (44, 45); however, it is unlikely that any of these proteins interact with RD-1e. The identification of the KEE motif as a critical component of RD-1e should facilitate the identification of its cognate binding proteins and provide clues as to its function in C/EBP.

Two alternative scenarios might explain the role of these repressor domains in determining the biological function of C/EBP. First, interaction of corepressors with the repressor domains may serve to maintain C/EBP in an inactive state until an activating signal is received. This model would explain the ability of C/EBP to participate in the inducible activation of cytokine genes upon lipopolysaccharide stimulation of macrophages and other cell types (12). Further studies will determine whether phosphorylation of potential MAP kinase sites in RD-2e are involved in regulating C/EBP activity. Alternatively, it is becoming apparent that transcriptional repression may be just as important as activation in regulating cell-specific gene expression during development. Therefore, C/EBP could play dual regulatory roles as a transcriptional activator and repressor, depending on its association with corepressors or coactivators. This model would explain the apparent ability of C/EBP to both positively and negatively regulate expression of the M-CSFR gene (12, 15). Detailed characterization of the exact functions of each domain in C/EBP, and their interacting partners, along with analysis of cellular distributions of putative corepressor molecules should aid in determining the activity and specific functions of C/EBP.

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