Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-α on the serum albumin promoter

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We have studied the activation of the serum albumin promoter by transcription factor CCAAT/enhancer binding protein-α (C/EBPα) in the HepG2 hepatoma cell line. We find that three distinct mechanisms determine the ability of C/EBPα to activate this promoter in a cell-type-specific and cooperative manner. First, the *trans*-activating function of C/EBPα is generated through cooperation between three separate domains of the protein that we have named *trans*-activation elements (TE-I through TE-III). The TEs have little or no ability to activate transcription by themselves, but any two can cooperate to do so, both in the C/EBPα protein and when linked to the GAL4 DNA-binding domain. Second, TE-III was found to contain a negative regulatory subdomain, the function of which was alleviated when C/EBPα was bound in the environment of the albumin promoter. This formed the basis for cooperative activation of this promoter by C/EBPα. Finally, we demonstrate that the leucine zipper of C/EBPα participates in determining the cell type specificity of albumin promoter activation, as it exerts a strong negative effect on albumin promoter activation in the nonhepatic HeLa cell line but not in HepG2 cells. These findings shed new light on the mode of action of C/EBPα and show a novel function for a leucine zipper in cell-type-specific gene expression.

[*Key Words:* C/EBP; cooperation; differentiation; serum albumin; *trans*-activation]

Received July 19, 1993; revised version accepted December 8, 1993.

The process by which transcription factors interact to regulate gene expression is of fundamental importance for the process of cellular differentiation, the basic principle in the generation of multicellular organisms. Although critical components of the transcriptional apparatus have been cloned and characterized, little is known about the mechanisms by which regulatory proteins determine the transcription rate of their target promoters. Such understanding will require precise definition of the activating functions of distal transcription factors and the mechanisms by which they interact with each other and with components of the basal transcriptional machinery.

One model system for the study of differentiation-specific activation of gene expression is provided by the CCAAT/enhancer binding protein-α (C/EBPα) transcription factor. C/EBPα is a member of the basic region–leucine zipper class of transcription factors. It was originally purified and cloned as a nuclear factor, enriched in liver cells, that had the ability to bind promoters, such as the albumin and hepatitis B virus promoters, specific for this tissue (Johnson et al. 1987; Landschulz et al. 1988). It is abundant in liver and adipose tissues (Birkenmeier et al. 1989) and will bind to and activate transcription from several liver- and adipose-specific promoters including those of aP2-422, SCD1, GLUT4, serum albumin, and C/EBPα itself (Christy et al. 1989, 1991; Friedman et al. 1990; Kaestner et al. 1989; C. Nerlov and E.B. Ziff, unpubl.). C/EBPα is largely confined to terminally differentiated, nonproliferating cells (Birkenmeier et al. 1989); ectopic expression of C/EBPα in certain cell types is sufficient to induce growth arrest (Umek et al. 1991; Oliviero et al. 1992). Furthermore, antisense inhibition of C/EBPα mRNA will prevent in vitro differentiation of 3T3-422A and 3T3-L1 pre-adipocytes (Samuelsson et al. 1991; Lin and Lane 1992). C/EBPα is thus clearly involved in the process of cellular differentiation at the level of transcription. However, because adipocytes and hepatocytes express largely nonoverlapping differentiated functions, additional levels of regulation must determine which C/EBPα target genes are actually expressed in each cell type. C/EBP-related proteins require cooperation with the NF-Y transcription factor for maximal activation of the liver-specific albumin promoter in vitro (Mantovani et al. 1992; Milos and Zaret 1992). Fur-

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thermore, C/EBPα is a stronger activator of the albumin promoter in the liver-derived HepG2 hepatoma cell line than in the nonhepatic L and HeLa cell lines (Friedman et al. 1989; Williams et al. 1991). These results suggest cell-type-specific cooperation with other transcription factors as a basis for differential activation of genes by C/EBPα in different cell types. Alternatively C/EBPα may contain activation domains that function in a cell-type-specific manner.

The transcription activation function of C/EBPα requires the amino-terminal part of the C/EBPα molecule (Friedman and McKnight 1990), whereas the DNA-binding basic region–leucine zipper motif resides in its carboxyl terminus (Landschulz et al. 1989). Mutational analysis of the C/EBPα protein has revealed two regions of the protein that are involved in activation of transcription from the albumin promoter in hepatoma cells (Friedman and McKnight 1990). However, the amino acid motifs responsible for the activity of these domains have not been delineated. A clue to the identity of one amino acid motif involved in C/EBPα *trans*-activation was obtained by the finding that a homology existed between the amino-terminal C/EBPα *trans*-activation domain and the c-Jun and c-Fos HOB2 motifs. A segment of C/EBPα containing this homology was able to substitute for c-Jun HOB2 in an assay measuring cooperation with the c-Jun HOB1 motif (Sutherland et al. 1992). Whether the C/EBPα HOB2 homology is sufficient for this cooperation is still not clear.

We have analyzed the C/EBPα protein with respect to (1) the functional organization of its *trans*-activation domain, (2) its ability to activate cooperatively the albumin promoter, and (3) the cell type specificity of this cooperative activation. We present evidence that the C/EBPα *trans*-activation domain contains three separable *trans*-activation elements (TE-I, TE-II, and TE-III) that can cooperate with one another to generate *trans*-activation domains both in the context of the C/EBPα protein and when fused to the GAL4 DNA-binding domain. We have mapped the amino acid motifs required for the function of TE-II and TE-III. We have also found that TE-III contains a negative regulatory domain, the function of which is alleviated when C/EBPα is bound to the albumin promoter. This forms the basis of cooperative activation of this promoter by C/EBPα. Furthermore, we have found that the cell type specificity of activation of the albumin promoter by C/EBPα is determined, to a large extent, by its leucine zipper, which appears to have a negative regulatory function in the context of the albumin promoter that is especially pronounced in the nonhepatic HeLa cell line compared with HepG2 hepatoma cells.

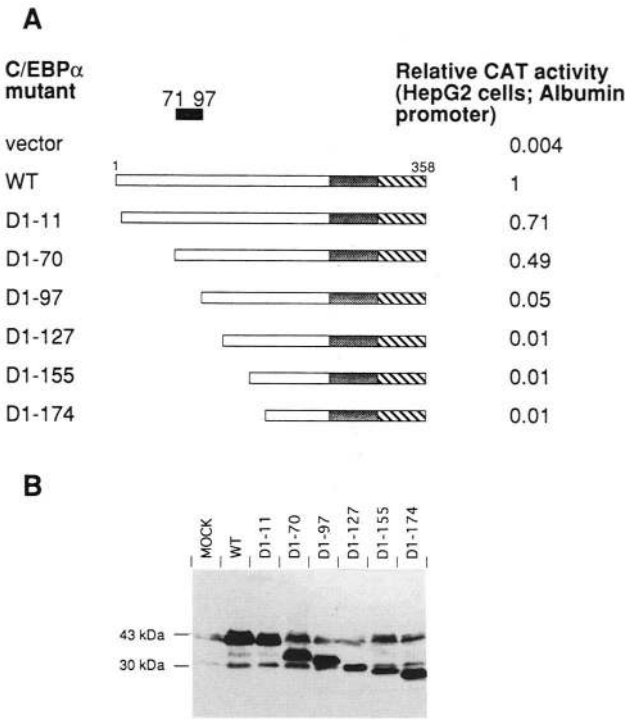

Figure 1. Amino-terminal deletion analysis of C/EBPα. (*A*) HepG2 cells were transfected with a serum albumin promoter–CAT reporter construct (pAlbCAT; 5 μg) and either pcDNAI (vector) or CMV-driven expression vectors encoding wild-type and amino-terminally deleted C/EBPα proteins (2 μg) and the pCMVGH internal control plasmid, using the calcium phosphate coprecipitation technique. The normalized CAT activity (WT = 1) is shown. The region most critical for activity (amino acids 71–97) is indicated above the wild-type protein. (*B*) Western blot of cellular proteins from parallel HepG2 cultures transfected with the same C/EBPα expression vectors as in *A*. The endogenous 43- and 30-kD forms of C/EBPα are indicated.

Results

The C/EBPα trans*-activation domain contains three independent* trans*-activation elements*

We initially performed a 5′ deletion analysis of the C/EBPα *trans*-activation domain (Fig. 1A). In this as well as the following internal deletion analysis, deletion endpoints were located, whenever possible, at or near the position of proline residues to minimize the possibility of interrupting a secondary structure (α-helix or β-strand), an approach similar to that used by Friedman and McKnight (1990). Wild-type and various amino-terminally deleted versions of C/EBPα were expressed from the cytomegalovirus (CMV) promoter in transient transfection assays in HepG2 hepatoma cells. We used the albumin proximal promoter linked to the chloramphenicol acetyltransferase (CAT) gene as target promoter (Mueller et al. 1990); this promoter is strongly activated by C/EBPα in HepG2 cells (Friedman et al. 1989). In this assay, deletion of the amino-terminal 70 amino acids (mutant D1-70) reduces the activity of the protein to ~50% of the wild-type level. The remaining activity is essentially abolished by additional removal of amino acids 71–97, leaving only 5% of the wild-type activity. This identified amino acids 71–97 as important for transcriptional activation and suggested that amino acids 1–70 played a role as well. Similar analysis using internal

deletions showed no significant reduction of *trans*-activation by deletion of amino acids 200–257 (Fig. 2). Removal of amino acids 126–200, however, led to a threefold reduction of *trans*-activation. Further 3′ deletion showed little additional effect of removing amino acids 97-200; however, once amino acids 70–200 were deleted in mutant D70-200, all activity was lost. Thus, in this analysis, amino acids 70–96 appeared to be of critical importance for activity and amino acids 126–200 to play an auxillary role. In these, as well as in all of the following experiments, the expression level of the C/EBPα derivatives was controlled by Western blotting, and it was found that all mutants were expressed at similar levels. Furthermore, all C/EBPα mutants were found by immunocytochemistry to be localized to the nuclei of transfected cells (see Figs. 3C and 8C, below, for representative data).

When amino acids 70–97 were internally deleted the resulting protein still activated transcription with ~50% of wild-type efficiency (Fig. 3A); this domain therefore is only critical for activity once either additional amino- or carboxy-terminal sequences are deleted. The two auxillary domains, amino acids 1–70 and amino acids 126–200, thus are able to maintain high *trans*-activation in the absence of amino acids 70–97, raising the possibility that three redundant elements, any two of which are sufficient for high (35–50% of wild type) activity, exist in C/EBPα. A prediction by this hypothesis is that simultaneous deletion of amino acids 1–70 and 126–200 would lead to an inactive protein, and this was found to be the case (D1-70, D126-200; Fig. 3A). This analysis therefore leads to a model in which three independent elements (amino acids 1–70, 71–97, and 126–200) exist in C/EBPα. None of these is sufficient to generate an active transcription factor, but any combination of the two will. In the following text we call the inactive subunits *trans*-activation elements (TE-I through TE-III) to distinguish them from *trans*-activation domains, which by this definition have *trans*-activating activity by themselves. The term *trans*-activation motif will be used to designate a discrete amino acid segment that has a function within a TE.

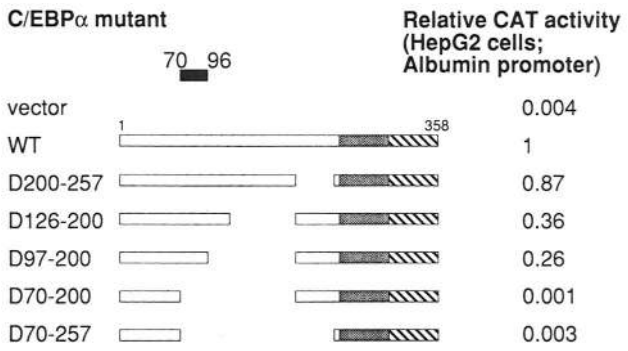

Figure 2. Internal deletion analysis of C/EBPα. HepG2 cells were transfected as described in Fig. 1 with C/EBPα expression vectors encoding internal deletion mutants in the C/EBPα protein and the pAlbCAT reporter. The region most critical to activity (amino acids 70–96) is indicated above the wild-type protein.

To test the above model various fragments of the C/EBPα-coding sequence were fused to the yeast GAL4 DNA-binding domain (Fig. 4). When tested for their ability to *trans*-activate a GAL-4-binding site-containing promoter (5×GAL4–E1B-TATA–CAT), it was found that individual TEs had low *trans*-activating activity and that all combinations of two TEs would cooperatively activate transcription, although quantitative differences were observed relative to their function on the albumin promoter. This confirmed that the TEs could cooperate with each other and that this property was not specific to the albumin promoter. We observed that TE-III had a negative regulatory function when fused to TE-I + TE-II and that TE-I + TE-II activated much better that TE-II + TE-III. This probably reflects the presence of the previously reported attenuator domain inside TE-III (Pei and Shih 1991). We find that TE-III contains a negative regulatory domain, which, however, does not function in the context of the albumin promoter (Figure 7, below; see Discussion), presumably accounting for the observed quantitative differences.

C/EBPα TE-II contains two separable trans-*activation motifs*

TE-II contains a sequence homologous to the c-Fos and c-Jun HOB2 motifs (underlined in Fig. 5A), and it was proposed that this motif contributes to *trans*-activation by C/EBPα (Sutherland et al. 1992). We therefore introduced into the C/EBPα protein a mutation shown previously to inactivate the c-Fos HOB2 motif. However, the effect of mutating the HOB2 motif on the ability of C/EBPα to *trans*-activate the albumin promoter was slight compared with the significant effect of deleting the entire TE-II (Fig. 5B), suggesting that additional motifs within TE-II were responsible for its activity or functioned redundantly with the HOB2 motif. 3′ Deletion analysis implicated the RQQE sequence motif (amino acids 86–89; Fig. 5A) in TE-II activity; however, in the wild-type C/EBPα background, point mutation of this sequence had little effect on *trans*-activation (Fig. 5B, mutant QE; data not shown). The above model for the tripartite structure of the C/EBPα *trans*-activation domain has practical implications for the detailed analysis of the individual TEs. Because removal of one TE will make the protein critically dependent on both the two remaining TEs, mutational analysis of a TE is greatly facilitated in such a deleted background, as a 10- to 50-fold effect of an inactivating mutation would be observed rather than the 2- to 3-fold effect observable in a wild-type background. This reasoning led us to analyze TE-II by introducing point mutations into the HOB2 motif and RQQE-containing sequence motif [designated CTM (C/EBPα *Trans*-activation Motif)] in the D1-70 background and assaying the *trans*-activating activity of the resulting proteins on the albumin promoter (Fig. 5B). As can be seen, both motifs are required for full activity of TE-II. Mutation of the HOB2 motif leads to a two- to threefold

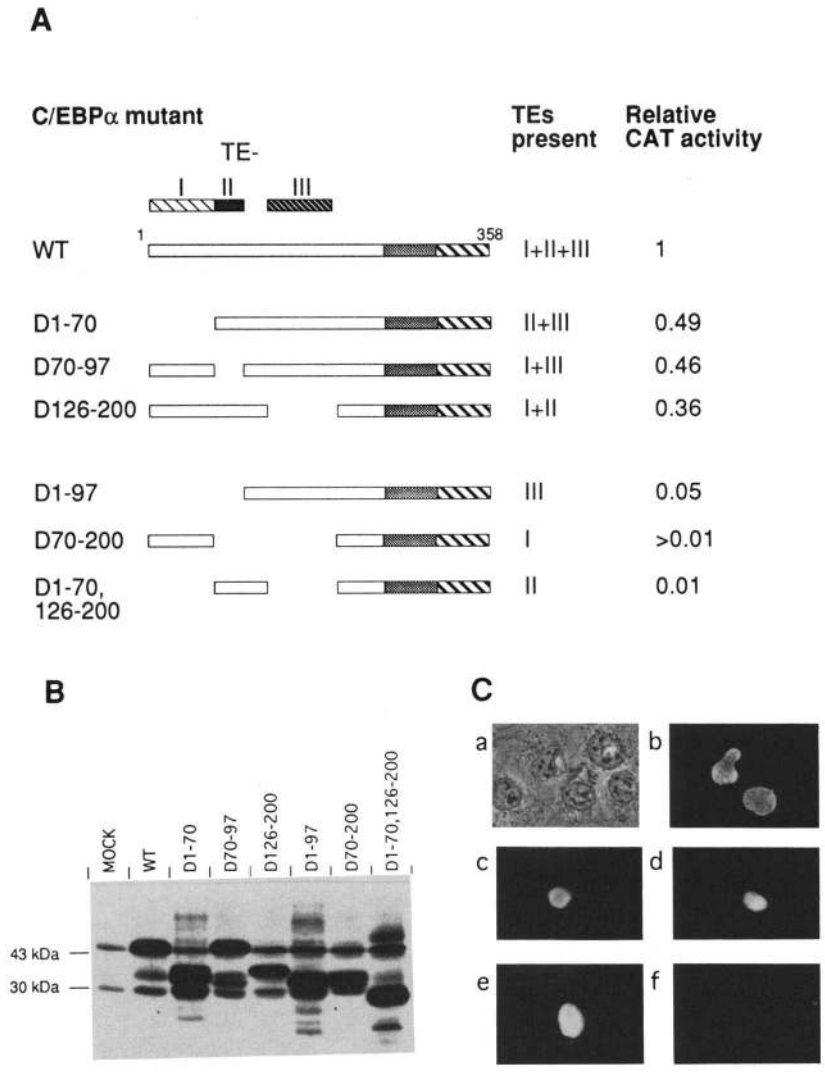

Figure 3. Identification of three independent *trans*-activating regions of C/EBPα. (*A*) CMV-driven expression vectors encoding C/EBPα mutants in which each of the three putative *trans*-activation regions (indicated above as TE-I, -II and -III) or any of their pairwise combinations have been deleted were cotransfected into HepG2 cells with the pAlbCAT reporter as described in Fig. 1. The activation of the albumin promoter relative to wild-type C/EBPα (=1) is shown. Relevant data from Figs. 1 and 2 are included for easier comparison. (*B*) Western analysis of HepG2 cells transfected with the same constructs as above; the endogenous 43- and 30-kD forms of C/EBPα are indicated by arrows; note that the D70-97 mutant, despite its reduced molecular weight, comigrates with the wild-type C/EBPα protein (see also Fig. 8B; Friedman and McKnight 1990). (*C*) Immunofluorescence analysis using anti-C/EBPα antiserum of HepG2 cells transfected with CMV expression vectors for wild-type C/EBPα (*b*), D1-70 (*c*), D70-97 (*d*), and D126-200 (*e*) mutants, and the control vector pcDNAI (*f*). Parallel phase-contrast photograph of the wild-type C/EBPα-transfected cells is shown in *a* to enable comparison with the HepG2 cellular morphology.

reduction in activity; mutation of the CTM motif to an eightfold decrease in the TE-I-deleted background. When both motifs are mutated essentially no activity is observed, in agreement with the 5′ deletion analysis (Fig. 1). Thus, two independent *trans*-activation motifs exist within TE-II, both of which contribute to its activity. The HOB2 homology includes amino acids 73–81 of C/EBPα. We have mapped the carboxy-terminal border

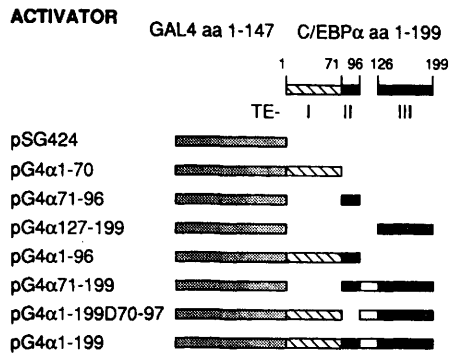

Figure 4. Analysis of C/EBPα TEs fused to the GAL4 DNA-binding domain. HepG2 cells were transfected with SV40 early promoter-driven expression vectors containing the GAL4 DNA-binding domain (pSG424) or the GAL4 DNA-binding domain fused to the three C/EBPα TEs along with a CAT reporter gene containing five GAL4 DNA-binding sites upstream of the E1B minimal promoter (pG_5BCAT) and the pCMVGH internal control plasmid. The numbers in the construct names indicate the C/EBPα amino acids present. The *trans*-activation activity of the fusion proteins was measured as CAT activity relative to the pSG424 vector control. An estimate for the cooperativity between TEs was calculated as the ratio between the activity of the construct containing the TEs fused to each other and the sum of their activities when tested separately.

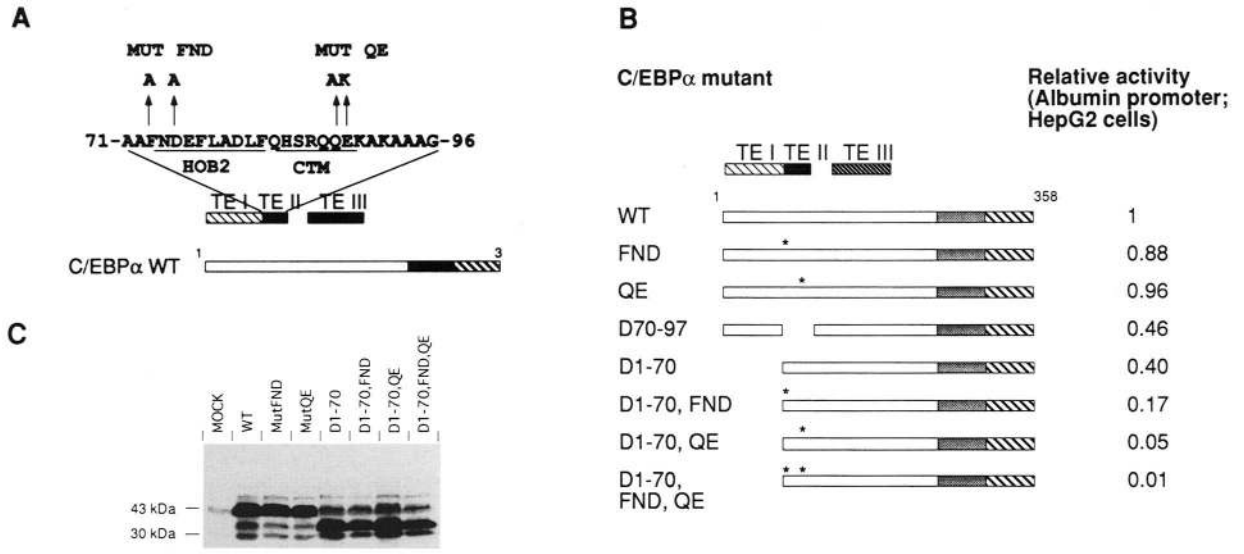

Figure 5. Sequence and mutation analysis of C/EBPα TE-II. (*A*) Amino acid sequence of TE-II. The approximate locations of the HOB2 and CTM motifs are indicated by underlining, and their respective inactivating mutations are shown above the sequence. The FND (F73A,D75A) and QE (Q88A,E89K) mutations are those used in the analysis below. (*B*) The FND and QE mutations were introduced into the wild-type and D1-70 backgrounds of C/EBPα. The resulting proteins were analyzed for their *trans*-activating activity in HepG2 cells as in Fig. 1; activities shown are those relative to wild-type C/EBPα (= 1). The activity of the D70-97 construct (from Fig. 3) is shown for comparison. (*C*) Western blot of HepG2 cells transfected with TE-II mutant proteins. The endogenous 43- and 30-kD forms of C/EBPα are indicated.

of the CTM motif to amino acid 89 (C. Nerlov and E.B. Ziff, unpubl.); thus, barring overlap with the HOB2 motif, it is likely to be contained within amino acids 82–89. Together, these two motifs account for all of the activity of TE-II.

C/EBPα TE-III requires multiple amino acid segments for activity

No information was available to identify critical amino acids within TE-III. Therefore, a more detailed carboxy-terminal deletion analysis of this element was performed (Fig. 6A). As for TE-II the analysis was done in the D1-70 background for increased sensitivity. When increasing amounts of sequence were deleted from the carboxy-terminal end of TE-III and the resulting proteins analyzed as above, it was found that most of the *trans*-activation activity was lost upon deletion of amino acids 181–191. This amino acid segment consists entirely of prolines and histidines. The remaining part of TE-III was not able to maintain *trans*-activating activity in this analysis. However, when a similar analysis was carried out using internal amino-terminal deletions of TE-III (Fig. 6B) two amino acid segments amino-terminal to the proline/histidine-rich region, amino acids 126–155 and 169–180, were found to contribute to *trans*-activation. Deletion of amino acids 126–155 resulted in significant reduction of *trans*-activation (four- to fivefold), leaving ~10% of the wild-type activity. Once both of these regions were deleted, essentially all activity was lost. Therefore, the proline/histidine-rich sequence (amino acids 181–191) did not suffice for TE-III activity in the absence of TE-I. These results showed that, similarly to TE-II, TE-III did not depend on a single amino acid motif for *trans*-activation. At least two, and possibly three, independent segments of TE-III contributed to its activity. Apart from the high proline/histidine content of amino acids 181–191, the only remarkable feature of the amino acid segments implicated in TE-III *trans*-activation function is a preponderance of aromatic amino acids, particularly tyrosine, relative to the rest of the C/EBPα protein.

A negative regulatory subdomain of TE-III mediates cooperative activation of the albumin promoter by C/EBPα

Another function of TE-III was revealed when the cooperative activation of the albumin promoter by C/EBPα was investigated. The *trans*-activation of the mouse albumin proximal promoter by C/EBPα in HepG2 cells is usually 100- to 300-fold. In contrast, a simple promoter, containing only a single copy of the albumin promoter D site (the only high-affinity C/EBP-binding site in the proximal region of the albumin promoter and the critical site for activation by C/EBPα; Lichtsteiner et al. 1987; Friedman et al. 1989), TATA box, and initiator element driving the luciferase reporter gene (hereafter called D site–TATA reporter) is typically activated 20- to 40-fold under the same conditions. On average, activation that is five to six times higher is observed with the complex

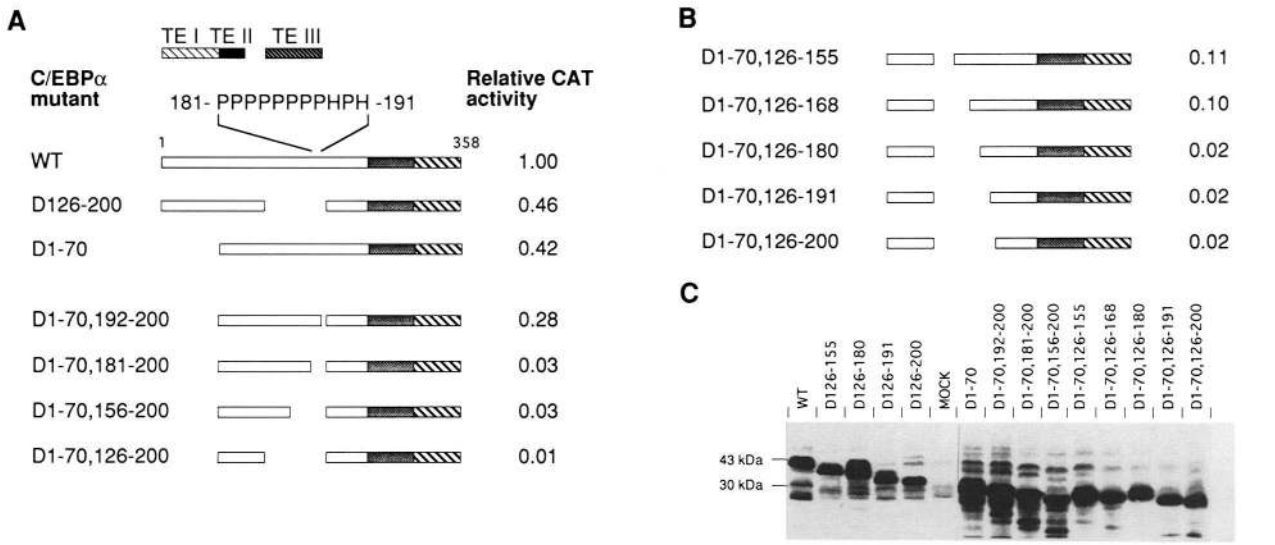

Figure 6. Deletion analysis of C/EBPα TE-III. (*A*) Carboxy-terminal deletion mutants of TE-III were analyzed in the absence of TE-I (D1-70 background). The mutants were analyzed for *trans*-activation in HepG2 cells as in Fig. 1. Activity is relative to wild-type C/EBPα (=1). The sequence of the most critical region of TE-III in this analysis (amino acids 181–191) is shown above the wild-type protein. (*B*) Amino-terminal deletion mutants of TE-III were analyzed as in *A*. (*C*) Western blot analysis of HepG2 cells transfected with TE-III deletion mutants. The endogenous 43- and 30-kD forms of C/EBPα are indicated.

promoter compared with the simple one. When wild-type C/EBPα and C/EBPα mutants individually deleted for the three TEs were tested in comparison on these two promoters, deletion of TE-I or TE-II was found to have similar relative effects on both promoters; however, deletion of TE-III led to a twofold increase in the activity on the simple promoter while leading to the expected two- to threefold decrease in activity on the albumin promoter (Fig. 7A). This indicated that in addition to the previously identified *trans*-activation function, a negative regulatory domain that functions on the simple promoter was contained within TE-III. To clarify this point, a 5′ deletion mapping of TE-III was carried out in the wild-type C/EBPα background (Fig. 7B; to allow comparison of the two reporter constructs, activation is expressed here as fold increase relative to cells transfected with empty expression vector). This experiment showed that negative regulation of C/EBPα *trans*-activation was abolished by deletion of amino acids 126–155 (D126–155), leading to a fivefold increase in the activation of the D site–TATA promoter but no change in the activation of the albumin promoter. This mutant C/EBPα protein showed very similar activation of the albumin and D site–TATA promoters, clearly indicating that the higher activation of the albumin promoter by the wild-type C/EBPα protein could be explained, to a large extent, by alleviation of the function of this negative regulatory domain in the context of the albumin promoter. Further deletion of the rest of TE-III (D126–200) led to the expected decrease in activation of both promoters consistent with the remaining activation function of TE-III residing carboxy-terminal to the negative regulatory domain (amino acids 169–191).

The C/EBPα leucine zipper mediates cell type specificity of albumin promoter activation

The identification of distinct *trans*-activation elements within C/EBPα made it possible to test whether differential activity of any of these would explain the previous observation that C/EBPα *trans*-activated the albumin promoter much more efficiently in HepG2 cells than in the nonhepatic HeLa cell line (Williams et al. 1991). We found that the stimulation was on average 10- to 15-fold in HeLa cells compared with 100- to 300-fold in HepG2 cells. However, deletion of TE-I, TE-II, or TE-III in all cases led to similar relative effects on *trans*-activation as in HepG2 cells (Fig. 8A; cf. Fig. 3A). Thus, inactivity of any particular TE could not explain the difference. Furthermore, when the activation of the D site–TATA-containing promoter by C/EBPα was assayed, similar activation was seen in the two cell lines (see below; Fig. 9A). It therefore seemed that the activity of the C/EBPα transcription factor as such or any of its *trans*-activation elements was not different between the two cell lines to an extent that could explain an ~15-fold higher activation of the albumin promoter in HepG2 cells.

We had previously obtained evidence that a mutant in the nonhydrophobic face of the C/EBPα leucine zipper differentially affected *trans*-activation of the albumin promoter by C/EBPα in HeLa cells, suggesting a role for the zipper in determining cell type specificity. To further investigate this possibility a C/EBPα derivative in which the C/EBPα leucine zipper was substituted with the zipper from the yeast GCN4 protein was constructed (C/EBPα–Gz; Fig. 9A). When this mutant was assayed for its specific DNA-binding activity, a two- to threefold lower

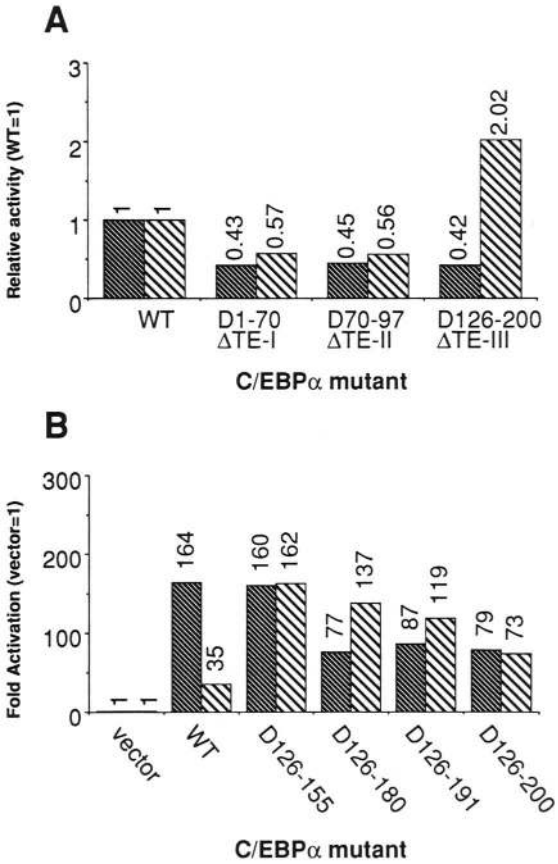

Figure 7. TE-III contains a negative regulatory function. (*A*) The effect of deleting TE-I, TE-II, or TE-III on the ability of C/EBPα to *trans*-activate the albumin promoter (dark hatched bar) or a promoter containing only the albumin promoter D site, TATA-box, and initiator elements driving the luciferase (LC) reporter gene (D site–TATA reporter) (light hatched bar). The albumin–CAT and D site–TATA–LC reporters were cotransfected into HepG2 cells with expression vectors encoding C/EBPα mutants deleted for TE-I, TE-II, or TE-III and the pCMVGH internal control plasmid. The CAT and luciferase activities were measured and are shown relative to those obtained with wild-type C/EBPα (=1). (*B*) Amino-terminal deletions within TE-III (in the wild-type C/EBPα background) were assayed for activity on the albumin and D site–TATA promoters as in *A*, except that activities are given as fold stimulation relative to cells transfected with empty expression vector for direct comparison between the two promoters.

affinity for a C/EBP site was observed (Fig. 9C). The C/EBPα–Gz was tested along with wild-type C/EBPα on the albumin and D site–TATA promoters in both cell lines (Fig. 9A). It was observed that the C/EBPα–Gz protein had about two- to threefold lower *trans*-activating ability on the simple promoter in both cell lines, paralleling its lower DNA-binding ability (again the activation is expressed as fold above vector control). However, on the albumin promoter a slight increase in *trans*-activation was observed in HepG2 cells and a dramatic increase in HeLa cells. The C/EBPα–Gz protein was about equally active in *trans*-activation of the albumin promoter in the two cell lines: Of the ~15-fold difference between the activation potentials of the wild-type C/EBPα protein in the two cell lines, <1.5 fold remains for the C/EBPα–Gz protein (see HepG2/HeLa ratio in Fig. 9A). When assayed on the D site–TATA promoter, neither protein displayed significant cell type specificity of activation. This result clearly showed that the C/EBPα leucine zipper is involved in determining the cell type specificity of albumin promoter *trans*-activation. That the effect was limited to the albumin promoter indicated that this specificity is attributable to a negative interaction between the zipper and other factors bound to this promoter, a negative effect that is particularly pronounced in HeLa cells. The GCN4 zipper does not appear to be subject to this negative regulation, allowing C/EBPα–Gz to function efficiently on the albu-

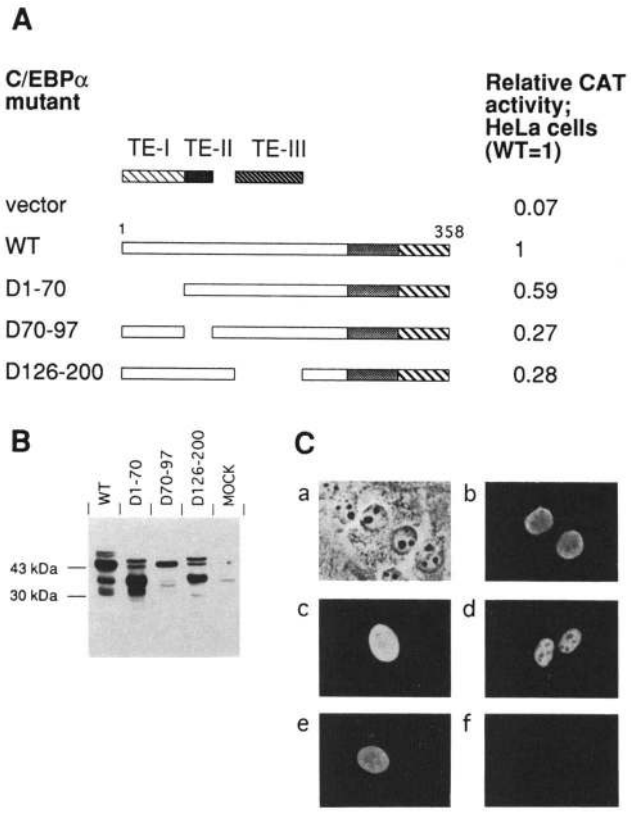

Figure 8. Effect of TE-I, TE-II, and TE-III deletion in HeLa cells. (*A*) The activation of the albumin promoter by wild-type C/EBPα and C/EBPα mutants deleted for TE-I, TE-II, or TE-III was tested in HeLa cells. Cell were transfected as in Fig. 1; *trans*-activation was measured as CAT activity relative to wild-type C/EBPα (=1). Note that wild-type C/EBPα activation is only ~15-fold above vector control (e.g., cf. Fig. 7B). (*B*) Western blot of HeLa cells transfected with the same expression vectors as above. (*C*) Immunofluorescence analysis of HeLa cells transfected as above using anti-C/EBPα antiserum: phase-contrast image of wild-type transfected cells (*a*), immunofluorescence analysis of cells transfected with wild-type C/EBPα (*b*), D1-70 (*c*), D70-97 (*d*), and D126-200 (*e*) mutants, and the control pcDNAI vector (*f*).

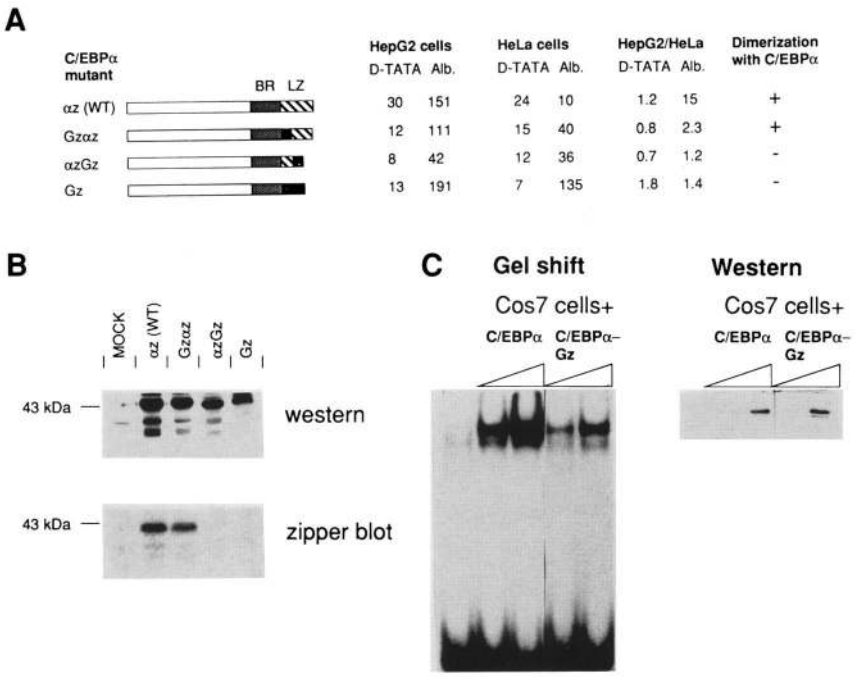

Figure 9. Role of C/EBPα leucine zipper on the albumin promoter. (*A*) The structure C/EBPα–Gz, C/EBPα–Gzαz, and C/EBPα–zGz proteins are shown compared with wild-type C/EBPα. The fold activation obtained with expression vectors for these proteins in HepG2 and HeLa cells, on both the albumin and D site–TATA promoters is shown as fold above vector control. For evaluation of the promoter and cell type specificity of the proteins the ratio between the values obtained in the two cell lines is shown for all activator/promoter combinations. (*B*) Extracts of HeLa cells, either mock-transfected or transfected with expression vectors for the above C/EBPα derivatives, were subjected to Western blotting using the C103 anti-C/EBPα antiserum and subsequent zipper (Far Western) blotting with a C/EBPα leucine zipper probe. (*C*) The C/EBPα and C/EBPα–Gz proteins were expressed in Cos7 cells, nuclear extracts (NEs) were prepared, and identical aliquots were subjected to parallel Western blot (with anti-C/EBP antiserum) and mobility shift (with C/EBP DNA-binding site probe) analysis (~1 mg/ml of protein; 2 μl of mock NEs, 0.2 and 0.6 μl of C/EBPα NEs, and 0.6 and 2 μl of C/EBPα–Gz NEs (left to right); protein concentration was kept constant at 2 μg in all lanes with NEs from mock-transfected cells). The specificity of complex formation was verified by competition and antibody supershifting (data not shown).

min promoter in both cell lines. Further analysis showed that hybrids of the C/EBPα and GCN4 zippers, in which either the two first heptad repeats of the C/EBPα zipper (Gzαz mutant) or the region carboxy-terminal to these two repeats (αzGz mutant) have been replaced by GCN4 sequences, also lost most or all of their cell type specificity (Fig. 9A). When assayed by zipper blotting for their ability to interact with the wild-type C/EBPα zipper, the Gzαz mutant displayed dimerization properties similar to the wild-type C/EBPα protein, displaying an approximate twofold reduction in affinity, whereas the αzGz and Gz mutants showed no binding at all (Fig. 9B). Zipper blotting with two other C/EBP family members, C/EBPβ and CHOP10/C/EBPζ, gave similar results (data not shown). This analysis therefore dissociates the loss of cell type specificity of the Gzαz mutant from its ability to dimerize with C/EBP family members.

Discussion

The trans*-activation domain of C/EBPα has a modular composition*

Our analysis of the C/EBPα *trans*-activation domain defined three independent amino acid sequence elements that contribute to *trans*-activation. We have designated these elements TE-I through TE-III and located them between amino acids 1–70, 71–96, and 126–200, respectively. TE-II and TE-III correspond to regions that have been shown previously to be involved in C/EBPα *trans*-activation of the albumin promoter and were proposed to function as redundant *trans*-activation domains (Friedman and McKnight 1990). Our results indicate that TE-II and TE-III do not function as independent *trans*-activation domains; rather they are both able to cooperate with TE-I to achieve this function. This is based on the observation that neither TE-II nor TE-III is able to activate the albumin promoter by itself when linked to the C/EBPα DNA-binding domain but does so efficiently in the presence of TE-I. Similarly, TE-II and TE-III can cooperate in the absence of TE-I (Figs. 1–3).

We do not believe that differences in stability, expression level, or nuclear localization of C/EBPα derivatives play any significant role in these experiments. By Western blotting and immunofluorescence analysis we observed that all C/EBPα mutants were localized to the nuclei of overexpressing cells, of both cell types analyzed, and were expressed at similar levels. Based on these experiments we estimate that the levels of C/EBPα in transfected HepG2 cells are two orders of magnitude greater than in untransfected cells, consistent with the >100-fold stimulation of promoter activity that can be observed for the albumin promoter in this cell line. All of these observations are in concordance with results obtained previously (Friedman and McKnight 1990). No endogenous C/EBPα is detectable in HeLa cells, which appear to contain mainly C/EBPβ, as we find that all detectable C/EBP-binding activity in HeLa cell nuclear extracts can be supershifted by two independent anti-C/EBPβ antisera (C. Nerlov and E.B. Ziff, unpubl.).

Analysis of fragments of C/EBPα fused to the GAL4

DNA-binding domain has indicated that amino acid sequences overlapping TE-I, TE-II, and TE-III could function as transcriptional activators in a heterologous reporter system (Pei and Shih 1991); however, in this previous study their importance for the activity of C/EBPα was not investigated. The investigators found that a region (amino acids 1–107) containing TE-I and TE-II strongly activated transcription when fused to the GAL4 DNA-binding domain and that a region (amino acids 171–215) overlapping TE-III had weak *trans*-activating activity in this assay system. For the TE-III overlapping region, amino acids 171–189, which are contained within TE-III, were required for activity. We observe that TE-III has weak activity in the GAL4 fusion assay (16-fold stimulation) and that TE-I and TE-II, when combined, have very high activity (>30,000-fold stimulation). A similar observation was made by Friedman and McKnight (1990) using an albumin promoter-based reporter construct. Our results are thus consistent with those obtained previously. Furthermore, we demonstrate that strong (~1000-fold) stimulation can be obtained by fusing TE-III to the GAL4 DNA-binding domain in combination with either TE-I or TE-II, showing that all three TEs function similarly in this heterologous assay system and that their ability to cooperate is a general property of these elements and not one restricted to the albumin promoter.

Multiple amino acid motifs mediate the activity of C/EBPα trans*-activation elements II and III*

The TE-II of C/EBPα contains a homology to the HOB2 motif of c-Fos and c-Jun (C/EBPα amino acids 71–81; Sutherland et al. 1992). The other motif found to contribute to the activity of TE-II (CTM motif; approximately amino acids 82–89) is situated in a region rich in basic amino acids and devoid of hydrophobic amino acids (Fig. 5A). These two motifs appear to be independent of each other and to function redundantly in the wild-type C/EBPα background where mutation of either has little or no effect (Fig. 5B). The importance of a particular motif is increased by inactivation of the other motif or TE-I, indicating functional redundancy also with the rest of the C/EBPα *trans*-activation domain.

Analysis of TE-III led to the identification of an 11-amino acid proline/histidine stretch (amino acids 181–191) as required for its activity (Fig. 7A). Proline-rich domains involved in activation are found in a number of transcription factors, including CTF, AP-2, and the estrogen and progesterone receptors (Gronemeyer et al. 1987; Kumar et al. 1987; Williams et al. 1988; Mermod et al. 1989). However, these domains are usually extended regions containing 20–50% proline, whereas the C/EBPα segment is only 11 amino acids, 9 of these being prolines. This implicates the proline residues of C/EBPα more directly in transcriptional activation. Highly proline-rich amino acid motifs have been implicated in direct protein–protein interactions for SH3 domains involved in signal transduction (Ren et al. 1993). It is interesting to note that the proline-rich domain of CTF, like that of C/EBPα, appears to require cooperation with additional domains for activity (Mermod et al. 1989). In addition to the proline/histidine stretch, amino acids 126–155 and 169–180 were required for TE-III activity; these sequences have no remarkable structural features, except for an elevated content of tyrosine residues.

The main difference between the c-Fos/c-Jun HOB motifs and the C/EBPα TEs seems to be that for both the C/EBPα TEs analyzed in detail it was observed that more than one amino acid motif was required for activity (Figs. 5B and 6A,B), although this has not been definitely ruled out for c-Fos and c-Jun. Despite the presence of multiple motifs, however, the C/EBPα TEs had no ability to *trans*-activate by themselves (Fig. 3). For both TE-II and TE-III at least two distinct amino acid motifs contributed to activity. Our data thus suggest that at least in some cases, a hierachy can be established in which TEs are generated from one or more discrete *trans*-activation motifs and subsequently cooperate to generate active *trans*-activation domains. It is interesting to draw a parallel to the previously proposed model for generation of enhancer activity from enhansons, where those transcription factors that are capable of stimulating transcription from single sites (and therefore supposedly contain a fully functional transactivation domain) correspond to class D enhansons and those requiring multiple binding sites or, in addition, cooperation with other factors (possibly containing the equivalent of a TE or transactivation motif), to class C and A/B enhansons (Fromental et al. 1988; Ondek et al. 1988). While speculative, this parallel provides a synthesis of the mechanisms for generation of active *trans*-activators and enhancers from inactive components. It also raises the possibility of *trans*-cooperation between TEs located on different proteins as a way of generating an active *trans*-activation domain.

What does this tell us about the physical structure of the C/EBPα *trans*-activation domain? That any TE is dispensable for high C/EBPα activity argues against a *trans*-activation domain that is highly organized into a unitary structure; such a structure would presumably be highly dependent on the integrity of each of its subcomponents and be subject to considerable spatial constraints. The observed functional redundancy of the TEs, as well as the observation that single motifs can be eliminated from the wild-type background without significant effect, thus argues that the functional units for *trans*-activation are relatively small and that their cooperation is on a functional rather than structural basis. The data obtained from our analysis of C/EBPα are more compatible with a model in which such discrete motifs, embedded in a protein structure of relatively high plasticity, form multiple contacts with the target(s) for transcriptional activation (the "beads on a string" model). The strength of the individual interactions and their number would then combine to determine the strength of *trans*-activation. This would account for the high malleability of *trans*-activation domains, as well as their

ability to function, even as segments, when fused to a heterologous DNA-binding domain.

A negative regulatory domain within TE-III mediates cooperative activation of the albumin promoter

By comparing the activation of the albumin promoter and a reporter gene carrying the D site from the albumin promoter in front of the albumin minimal promoter (D site–TATA reporter), we found that while wild-type C/EBPα activated the albumin promoter five to six times more efficiently than the D site–TATA construct, a C/EBPα mutant deleted for amino acids 126–155 showed essentially the same high activity on both (Fig. 7B). This indicated that amino acids 126–155 contained a negative regulatory domain that was active primarily when C/EBPα was isolated from the cooperative environment of the albumin promoter. This negative regulatory domain overlapped with a *trans*-activation function, which, however, did not appear to be required for C/EBPα activity in the presence of both TE-I and TE-II (cf. Figs. 6B and 7B). Relief of the negative regulation exerted in the absence of the cooperative environment seems sufficient to account for the difference observed between the two promoters. We therefore propose that cooperative activation of the albumin promoter by C/EBPα is attributable not to cooperation between activation domains on factors bound to adjacent sites in the promoter but primarily to the ability of the cooperative environment of the albumin promoter to alleviate the activity of the TE-III negative regulatory domain. The observation that TE-III has a negative regulatory function active outside the cooperative environment of the albumin promoter may also explain the observation that it, in agreement with previous reports (Pei and Shih 1991), negatively regulated TE-I+TE-II in the GAL4 fusion experiment using a 5×GAL4 site–TATA promoter (Fig. 4). This situation is functionally equivalent to that on the D site–TATA reporter as also there no other factors are present. Two ubiquitous nuclear factors occupy the adjacent sites on the albumin promoter, NF-I (E site) and NF-Y (C site), and both are required for full activity of the promoter in vitro (Lichtsteiner et al. 1987). Although it has not been determined whether any of these, or other factors on the promoter, are involved in the alleviation of negative regulation, the previous demonstration that NF-Y and C/EBP-like factors bound to this region of the albumin promoter can functionally cooperate in transcriptional activation (Milos and Zaret 1992) suggests that NF-Y may function in this manner. Members of the C/EBP family have been shown previously to cooperate with other factors in transcriptional activation. For example, both C/EBPα and NF-M (the chicken homolog of C/EBPβ) will cooperate with Myb in activation of the *mim-1* gene (Burk et al. 1993; Ness et al. 1993). Although the mechanism behind this cooperation is not known, the fact that both C/EBPα and c-Myb have been shown to contain domains that negatively regulate their activity (this study; Sakura et al. 1989; Pei and Shih 1991) raises the possibility that they cooperate by mutual alleviation of these negative regulators.

The leucine zipper provides cell type specificity of albumin promoter activation by C/EBPα

Replacing the leucine zipper of C/EBPα with the GCN4 zipper created C/EBPα–Gz, a protein that was similarly efficient in activation of the albumin promoter in HeLa and HepG2 cells, whereas the wild-type C/EBPα protein is ~15 times more active in HepG2 cells (Fig. 9A). This was due to a much increased activation in HeLa cells, indicating that in this cell line the C/EBPα leucine zipper exerted a strong negative effect in the context of the albumin promoter; the C/EBPα–Gz protein seemingly escapes this negative effect. In HepG2 cells the C/EBPα–Gz protein has activity similar to wild-type C/EBPα, in agreement with previous reports (Friedman et al. 1989). When assayed on the D site–TATA promoter the C/EBPα–Gz protein has similar activation potential in both cell lines, in both cases two- to three-fold lower than the wild-type C/EBPα protein, probably reflecting its somewhat decreased DNA-binding activity, arguing against the possibility that the GCN4 zipper provides an activating function specific for HeLa cells. It is, however, possible that the lower degree of activation observed in HeLa cells is attributable to a promoter-specific effect of heterodimerization with another C/EBP family member. This possibility is argued against by the observation that the C/EBPα–Gzαz mutant, which dimerizes with both C/EBPα, C/EBPβ, and CHOP10/C/EBPζ (Fig. 9B; data not shown), has lost most (>90%; Fig. 9A) of its cell type specificity, primarily because of increased activity in HeLa cells. There is thus no evidence that the GCN4 zipper allows C/EBPα–Gz to escape an inhibitory dimerization partner only found in HeLa cells, such as CHOP10/C/EBPζ, or LIP/C/EBPβ-3 (Descombes and Schibler 1991; Ron and Habener 1992). Furthermore, zipper blots of transfected HeLa cells indicate that other C/EBP-interacting proteins are present in HeLa cell populations at levels at least an order of magnitude lower than that of the transfected C/EBPα proteins (Fig. 9B). This assay will detect proteins of both the C/EBP and CREB/ATF family (Ron and Dressler 1992). Because immunofluorescence analysis shows <10% of the HeLa cells to express C/EBPα we can conservatively estimate that homodimers of transfected proteins comprise >95% of all C/EBP dimers in an expressing HeLa cell, again indicating that the observed biological phenotypes reflect the properties of the various C/EBPα homodimers, as any inhibitory partners would most likely be titrated out. Taken together, these data demonstrate that the cell type specificity of albumin promoter activation by C/EBPα is determined, to a large extent, by the leucine zipper and indicate that this specificity is not related to the dimerization specificity of the zipper. Previous reports have shown that steric interference between C/EBP and NF-Y may take place (Lichtsteiner et al. 1987; Milos and Zaret 1992) and, therefore, could be involved in determining cell type specificity. In this case,

zipper residues both amino- and carboxy-terminal to leucine 324 would be required for the negative interference. The cell type specificity may be determined by the presence of different isoforms of NF-Y and/or NF-I in the two cell lines. It has been shown that both NF-I and NF-Y mRNA exist in different isoforms and splice variants with cell-type-specific distribution (Paonessa et al. 1988; Santoro et al. 1988; Li et al. 1992).

Our results support the notion that in addition to mediating dimerization, leucine zippers participate in interactions with other proteins that determine the biological activity of transcription factors. In the case of C/EBPα, the leucine zipper, together with the negative regulatory domain in TE-III, restricts high activation mediated by C/EBPα to a natural target promoter (serum albumin) in the appropriate cell type (HepG2 hepatoma cells). The basis for the more pronounced inhibitory effect of the C/EBPα leucine zipper in HeLa cells is not yet clear, but based on the above argumentation we presently favor steric interference as the cause. Investigation of which forms of NF-I and NF-Y are present in HeLa and HepG2 cells, respectively, will likely provide further clues to the molecular basis of differential activation of the albumin promoter in the two cell lines.

Materials and methods

Plasmids, oligonucleotides, and DNA manipulations

Plasmids were maintained in bacterial strains MC1061/P3 (Invitrogen) for all pcDNAI (Invitrogen)-based CMV-driven expression vectors and XL-1 BLUE (Stratagene) for all other plasmids and M13 bacteriophage. Plasmid and phage replicative form DNA was prepared by alkaline lysis and purified on Qiagen columns according to the manufacturer's instructions. DNA cloning was performed by standard procedures (Sambrook et al. 1989). C/EBPα cDNA was obtained from Steve McKnight (pMSV–C/EBP; Friedman et al. 1989). Preparation of phage single-stranded DNA and site-directed and deletion mutagenesis were performed by using the Amersham mutagenesis kit according to the manufacturer's instructions or by PCR as described (Higuchi 1990) using *Pfu* polymerase (Stratagene) and appropriate DNA primers. Mutations were verified by DNA sequencing using a Sequenase 2.0 kit (U.S. Biochemical) and restriction analysis. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified on 12–15% denaturing polyacrylamide gels.

C/EBPα 5′ deletion mutants were preceded by methionine and alanine codons; internally deleted fragments were replaced by a *Kpn*I linker encoding glycine and threonine. The deletions are indicated by the amino- and carboxy-terminal amino acids that are deleted. All C/EBPα expression vectors are based on pcDNAI (Invitrogen).

Substitution of the C/EBPα leucine zipper with that of GCN4 was done by overlapping PCR using appropriate primers. The resulting construct, encoding C/EBPα–Gz, replaces amino acid residues 310–358 of C/EBPα with amino acids 253–281 of GCN4, as described previously (Agre et al. 1989). Construction of C/EBPα/GCN4 hybrid zipper mutants was done in the same way. In C/EBPα–Gzαz GCN4, residues 253–267 replace C/EBPα residues 310–324. In C/EBPα–αzGz GCN4, residues 267–281 replace C/EBPα residues 324–358.

GAL4–C/EBPα fusions (pG4α series) were constructed by cloning PCR products generated using appropriate primers and templates into the pSG424 (Sadowski and Ptashne 1989) *Bam*HI and *Xba*I sites.

The reporter plasmids used were pAlb–CAT (Mueller et al. 1990) and pD–AlbMP–Luc. The latter was constructed by PCR amplifying the albumin minimal promoter (base pairs −50 to +22) using the following primers, 5′-GCGCTCGAGGTTATTGGTTAAAGAAG-3′ and 5′-GCGAAGCTTTAGTGGGGTTGATAGG-3′,

and inserting the product into the luciferase vector pGL2–basic (Promega) using *Hin*dIII and *Xho*I, generating pAlbMP–Luc. An oligonucleotide containing the albumin promoter D site (5′-CGTATGATTTTGTAATGGGGTA annealed to 5′-CGCGTACCCCATTACAAAATCATACGGTAC) was then cloned into the *Kpn*I and *Mlu*I sites, generating pD–AlbMP–Luc.

The GAL4 binding site containing reporter pG_5BCAT has been described previously (Lillie and Green 1989). The pCMVGH internal control plasmid was generated by cloning the *Bam*HI–*Eco*RI fragment of pOGH (Selden et al. 1986) into pcDNAI.

Cell culture and transfection analysis

HeLa and HepG2 cell cultures were maintained in DMEM + 10% FCS (GIBCO BRL) and transfected as described (Gorman 1985). For reporter assay transfections, 7 μg of DNA (CAT and/or luciferase reporter at a total of 5 μg, 2 μg C/EBPα expression vector and 0.01–0.1 μg pCMVGH internal control plasmid) was used per 60-mm dish. For cells used in Western blotting analysis, 5–12 μg of expression vector was used alone. Cells were transfected at ~30% confluency, incubated with DNA for 16–18 hr, washed in DMEM, refed with fresh medium (DMEM + 10% FCS), and harvested 48 hr later. Medium was analyzed for human growth hormone (hGH) by radioimmunoassay as described by the manufacturer (Nichols Institute, CA). Cells were harvested in 300 μl of 0.1 M Tris-HCl at pH 7.5, and lysed by freezing in dry ice/ethanol and thawing in a water bath at room temperature (two cycles for analysis of CAT, five cycles for CAT + luciferase). Lysates were obtained by centrifugation (20 min/14,000 rpm at 4°C in an Eppendorf microcentrifuge). Luciferase activity was assayed using a Berthold Lumat LB9501 luminometer in 50 μl of extract essentially as described (Martinez-Salas et al. 1989), except that the final ATP concentration in the reaction buffer was 1 mM. CAT activity was assayed on 150 μl of heat-treated (10 min at 70°C) extract by the two-phase scintillation assay (Neumann et al. 1987). The hGH level was used to normalize the CAT and luciferase activities, correcting for variations in transfection efficiency between dishes. Unless indicated otherwise normalization between experiments was done by setting the activity obtained with the wild-type C/EBPα protein equal to 1. At least 2 and generally 4–12 determinations were made for each reporter/activator combination.

Nuclear extract preparation and mobility shift assay

Cos7 cells were maintained in DMEM + 10% FCS and transfected as above using 5–10 μg of DNA/90-mm dish or 30 μg of DNA/150-mm dish. Forty-eight hours after transfection nuclear extracts were prepared as described (Dignam et al. 1983), except that extracts were stored directly in buffer C, and protein concentrations were determined using the Bio-Rad assay. Mobility shift analysis was performed essentially as described (Nerlov et al. 1991) using a ^{32}P-labeled probe derived from the C/EBPα promoter containing a C/EBP binding site (5′-CCAAAGTTGAGAAATTTCTA-3′ annealed to 5′-TAGAAA-

TTTCTCAACTTTGG-3′ spanning nucleotides −282 to −263 of the C/EBPα gene; Christy et al. 1991).

Western, Far Western, and immunofluorescence analyses

Western blots were performed with the Amersham ECL system according to the manufacturers instructions, except that Immobilon nitrocellulose membranes (Millipore) were used. Equal amounts of total cellular protein, corresponding to ~$2x10^6$ cells per lane, were separated on 12.5% or 15% SDS–polyacrylamide gels and transferred to nitrocellulose by electroblotting. The primary antibody was anti-C/EBPα antiserum C103 (kindly provided by P. Rorth, Carnegie Institute of Washington, Baltimore, MD) used at a 1:3000 dilution in PBS + 0.2% Tween 20. The secondary antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham) at a 1:6000 dilution in the same buffer, which was also used for washing between steps (these and all subsequent manipulations were done at room temperature).

Far Western (zipper) blotting was carried out as described (Ron and Dressler 1992), using a fusion of the C/EBPα leucine zipper to glutathionine *S*-transferase (GST; a kind gift from Dr. David Ron, New York University Medical Center) labeled with ^{32}P as probe on the same filters as were used for Western blotting.

For indirect immunofluorescence, cells were transfected as described above, but on cover slides, fixed in 1.6% paraformaldehyde in PBS for 20 min, permeabilized with methanol/acetone (1:1) for 30 sec, incubated with a 1:3000 dilution of C103 in PBS + 3% BSA for 2 hr, followed by incubation with rhodamine-conjugated goat anti-rabbit immunoglobulin (Cappel) at a 1:100 dilution in PBS + 3% BSA for 2 hr. All steps were followed by extensive washes with PBS.

Acknowledgements

The authors thank Pernille Rorth and Steve McKnight for C/EBPα antibody and cDNA, respectively, David Ron for GST–zipper fusion constructs, Heinz Annus for expert photographic assistance, and Bernie Goldschmidt for oligonucleotide synthesis. We also thank Karen Buchkovich and Robert Hopewell for critical reading of the manuscript and the members of the Ziff laboratory for helpful discussions and suggestions. This work was supported by grant CA44042 from the National Cancer Institute. Computing was supported by the National Science Foudnation under grant number DIR-8908095. C.N. is a Fulbright scholar and a recipient of a candidate scholarship from the University of Copenhagen. E.B.Z. is an investigator of the Howard Hughes Medical Institute.

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References

Agre, P., P.F. Johnson, and S.L. McKnight. 1989. Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. *Science* **246:** 922–926.

Birkenmeier, E.H., B. Gwynn, S. Howard, J. Jerry, J.I. Gordon, W.H. Landschulz, and S.L. McKnight. 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes & Dev.* **3:** 1146–1156.

Burk, O., S. Mink, M. Ringwald, and K.-H. Klempnauer. 1993. Synergistic activation of the chicken *mim-1* gene by v-*myb* and C/EBP transcription factors. *EMBO J.* **12:** 2027–2038.

Christy, R.J., V.W. Yang, J.M. Ntambi, D.E. Geiman, W.H. Landschulz, A.D. Freidman, Y. Nakabeppu, T.J. Kelly, and D.M. Lane. 1989. Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes & Dev.* **3:** 1323–1335.

Christy, R.J., K.H. Keastner, D.E. Geiman, and M.D. Lane. 1991. CCAAT/enhancer binding protein gene promoter: Binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci.* **88:** 2593–2597.

Descombes, P. and U. Schibler. 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67:** 569–579.

Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11:** 1475–1489.

Friedman, A.D. and S.L. McKnight. 1990. Identification of two polypeptide segments of CCAAT/enhancer binding protein required for transcriptional activation of the serum albumin gene. *Genes & Dev.* **4:** 1416–1426.

Friedman, A.D., W.H. Landschulz, and S.L. McKnight. 1989. CCAAT/enhancer binding protein activated the promoter of the serum albumin gene in cultured hepatoma cells. *Genes & Dev.* **3:** 1314–1322.

Fromental, C., M. Kanno, H. Nomiyama, and P. Chambon. 1988. Cooperativity and hierarchical levels of functional organization in the SV40 enhancer. *Cell* **54:** 943–953.

Gorman, C.M. 1985. High efficiency gene transfer into mammalian cells. In *DNA cloning: A practical approach* (ed. D.M. Glover, Vol. 2, pp. 143–190. IRL Press, Oxford, England.

Gronemeyer, H., B. Turcotte, C. Quirin-Stricker, M.T. Bouquel, M.E. Meyer, Z. Krozowski, J.M. Jeltsch, T. Lerouge, J.M. Garnier, and P. Chambon. 1987. The chicken progesterone receptor: Sequence, expression and functional analysis. *EMBO J.* **6:** 3985–3994.

Higuchi, R. 1990. Recombinant PCR. In *PCR protocols* (ed. M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, pp. 177–183. Academic Press, San Diego, California.

Johnson, P.F., W.H. Landschulz, B.J. Graves, and S.L. McKnight. 1987. Identification of a rat liver nuclear protein that binds to the enhancer core of three animal viruses. *Genes & Dev.* **1:** 133–146.

Kaestner, K.H., R.J. Christy, and M.D. Lane. 1990. Mouse insulin-responsive glucose transporter gene: Characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci.* **87:** 251–255.

Kumar, V., S. Green, G. Stack, M. Berry, J.-R. Jin, and P. Chambon. 1987. Functional domains of the human estrogen receptor. *Cell* **51:** 941–951.

Landschulz, W.H., P.F. Johnson, E.Y. Adashi, B.J. Graves, and S.L. McKnight. 1988. Isolation of a recombinant copy of the gene encoding C/EBP. *Genes & Dev.* **2:** 786–800.

Landschulz, W.H., P.F. Johnson, and S.L. McKnight. 1989. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* **243:** 1681–1688.

Li, X.-Y., R.H. van Huijsduijnen, R. Mantovani, C. Benoist, and D. Mathis. 1992. Intron-exon organization of the NF-Y genes. *J. Biol. Chem.* **267:** 8984–8990.

Lichtsteiner, S., J. Wuarin, and U. Schibler. 1987. The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. *Cell* **51:** 963–973.

Lillie, J.W. and M.R. Green. 1989. Transcription activation by

the adenovirus E1a protein. *Nature* **338:** 39–44.

Lin, F.-T. and M.D. Lane. 1992. Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes & Dev.* **6:** 533–544.

Mantovani, R., U. Pessera, F. Tronche, X.-Y. Li, A.-M. Knapp, J.-L. Pasquali, C. Benoist, and D. Mathis. 1992. Monoclonal antibodies to NF-Y define its function in MHC class II and albumin gene transcription. *EMBO J.* **11:** 3315–3322.

Martinez-Salas, E., E. Linney, J. Hassell, and M.L. DePamphelis. 1989. The need for enhancers in gene expression first appears during mouse development with formation of the zygotic nucleus. *Genes & Dev.* **3:** 1493–1506.

Mermod, N., E.A. O'Neill, T.J. Kelly, and R. Tjian. 1989. The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain. *Cell* **58:** 741–753.

Milos, P.M. and K.S. Zaret. 1992. A ubiquitous factor is required for C/EBP-related proteins to form stable transcription complexes on an albumin promoter segment in vitro. *Genes & Dev.* **6:** 991–1004.

Mueller, C.R., P. Maire, and U. Schibler. 1990. DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. *Cell* **61:** 279–291.

Nerlov, C., P. Rørth, F. Blasi, and M. Johnsen. 1991. Essential AP-1 and PEA3 binding elements in the human urokinase enhancer display cell type-specific activity. *Oncogene* **6:** 1583–1592.

Ness, S.A., E. Kowenz-Leutz, T. Casini, T. Graf, and A. Leutz. 1993. Myb and NF-M: Combinatorial activators of myeloid genes in heterologous cell types. *Genes & Dev.* **7:** 749–759.

Neumann, J.R., C.A. Morency, and K.O. Russian. 1987. A novel rapid assay for chloramphenicol acetyl transferase gene expression. *BioTechniques* **5:** 444–446.

Oliviero, S., G.S. Robinson, K. Struhl, and B.M. Spiegelman. 1992. Yeast GCN4 as a probe for oncogenesis by AP-1 transcription factor: Transcriptional activation through AP-1 sites is not sufficient for cellular transformation. *Genes & Dev.* **6:** 1799–1809.

Ondek, B., L. Gloss, and W. Herr. 1988. The SV40 enhancer contains two distinct levels of organization. *Nature* **333:** 40–45.

Paonessa, G., F. Gounari, R. Frank, and R. Cortese. 1988. Purification of a NF1-like DNA-binding protein from rat liver and cloning of the corresponding cDNA. *EMBO J.* **7:** 3115–3123.

Pei, D. and C. Shih. 1991. An "Attennuator domain" is sandwiched by two distinct transactivation domains in the transcription factor C/EBP. *Mol. Cell. Biol.* **11:** 1480–1487.

Ren, R., B.J. Mayer, P. Ciccetti, and D. Baltimore. 1993. Identification of a ten-amino acid proline-rich SH3 binding site. *Science* **259:** 1157–1161.

Ron, D. and H. Dressler. 1992. pGSTag—A versatile bacterial expression plasmid for enzymatic labelling of recombinant proteins. *BioTechniques* **13:** 866–869.

Ron, D. and J.F. Habener. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes & Dev.* **6:** 439–453.

Sadowski, I. and M. Ptashne. 1989. A vector for expressing GAL4(1-147) fusions in mammalian cells. *Nucleic Acids Res.* **17:** 7539.

Sakura, H., C. Kanei-Ishii, T. Nagese, H. Nakagoshi, T.J. Gonda, and S. Ishii. 1989. Delineation of three functional domains of the transcriptional activator encoded by the c-*myb* protooncogene. *Proc. Natl. Acad. Sci.* **86:** 5758–5762.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Samuelsson, L., K. Stromberg, K. Vikman, G. Bjursell, and S. Enerback. 1991. The CCAAT/enhancer binding protein and its role in adipocyte differentiation: Evidence for direct involvement in terminal adipocyte development. *EMBO J.* **10:** 3787–3793.

Santoro, C., N. Mermod, P.C. Andrews, and R. Tjian. 1988. A family of human CCAAT-box-binding proteins active in transcription and DNA replication: Cloning and expression of multiple cDNAs. *Nature* **334:** 218–224.

Selden, R.F., K.B. Howie, M.E. Rowe, H.M. Goodman, and D.D. Moore. 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. *Mol. Cell. Biol.* **6:** 3173–3179.

Sutherland, J.A., A. Cook, A.J. Bannister, and T. Kouzarides. 1992. Conserved motifs in Fos and Jun define a new class of activation domain. *Genes & Dev.* **6:** 1810–1819.

Umek, R.J., A.D. Friedman, and S.L. McKnight. 1991. CCAAT-enhancer binding protein: A component of a differentiation switch. *Science* **251:** 288–292.

Williams, S.C., C.A. Cantwell, and P.F. Johnson. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes & Dev.* **5:** 1553–1567.

Williams, T., A. Admon, B. Lüscher, and R. Tijan. 1988. Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes & Dev.* **2:** 1557–1569.

Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-alpha on the serum albumin promoter.

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Genes Dev. 1994, **8:**
Access the most recent version at doi:10.1101/gad.8.3.350