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An "Attenuator Domain" Is Sandwiched by Two Distinct Transactivation Domains in the Transcription Factor C/EBP

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C/EBP is a rat liver DNA-binding protein which can act as a transcription factor. Its N-terminal portion contains three distinct domains. The first domain (amino acids 1 to 107) appears to be a highly potent transactivator. The second domain (amino acids 107 to 170) does not appear to exhibit either activation or repression activity. This domain is defined as an "attenuator domain" because its presence under four different sequence contexts reproducibly decreases the effect of transactivation of C/EBP. The third domain (amino acids 171 to 245) is a relatively weaker transactivator with a striking proline-rich motif. Deletional analysis of this third domain has shown that a 45-amino-acid region is sufficient for transactivation. This region (amino acids 171 to 215) contains 12 proline, 6 histidine, and mainly hydrophobic or noncharged amino acids. Further mutational analysis of a highly conserved proline-octamer region within this domain indicates that a specific proline content is not crucial for transactivation.

C/EBP is a 42-kDa protein initially isolated from rat liver nuclei (for a review, see reference 19). As a DNA-binding protein, it was the first molecule proposed to contain a leucine zipper structure (22). One unusual feature of C/EBP is its versatile DNA-binding specificity. It can bind to a wide variety of sequence motifs including both enhancers (18) and CCAAT promoters (14) of several different animal viruses. A number of cellular genes, including albumin (6, 13, 16), transthyretin, alpha-1 antitrypsin (6), and two adipocyte-specific genes (5), have also been found to contain C/EBP binding sites. Functional studies using a cotransfection assay have demonstrated that C/EBP can function as a transcriptional activator (9, 10, 29). Surprisingly, C/EBP also appears to be capable of acting as a repressor to human hepatitis B virus and simian virus 40 (SV40) (24, 29). Furthermore, C/EBP repression to SV40 appears to be DNA-binding dependent (30). Recently, disruption of a C/EBP binding site in the clotting factor IX promoter has been associated with hemophilia B (8). It appears that transcription of many other viral and cellular genes is likely under the influence of the C/EBP factor.

Despite intensive investigations of the carboxyl-terminal portion of C/EBP (19), which contains the b-ZIP domain with DNA binding activity, very little is known about the functional domains of the N-terminal portion of this molecule. It is possible that important protein-protein interactions between C/EBP and an adjacent transcriptional apparatus or factor are mediated through the N-terminal domains. For example, acidic activator domains have been identified in the yeast GCN4 (17) and Gal4 (11, 25) as well as in the herpes simplex virus VP16 sequences (35). Similarly, glutamine-rich activator domains have been demonstrated in transcription factors such as Sp1 (7) and AP1 (3). A third class of activator domain is rich in proline residues. This kind of proline-rich motif is present in a number of transcription factors including AP2, CTF/NF-1, and FRA-1 (for a review, see reference 26).

Previously, we and others have investigated the mechanism of C/EBP-mediated transcriptional regulation (9, 10, 29). In the study described here, we have conducted a functional characterization of the N-terminal domains of C/EBP. Here, we report that at least three different domains can be discovered. An operationally defined "attenuator domain" is sandwiched by two distinct transactivator domains.

During the review of this manuscript, Friedman and McKnight (10) reported the identification of two domains of C/EBP required for transcriptional activation of the serum albumin gene. Although the existence of a potential attenuator domain in C/EBP was not mentioned in their report, their result is consistent with one of our major conclusions presented in this report.

MATERIALS AND METHODS

Recombinant plasmids. Plasmids E1b-CAT, Gal4×5 E1b-CAT, Gal147, GalE1a, and pGVP16 were obtained from the laboratories of Mark Ptashne and Michael Green at Harvard University. The Gal147 vector consists of the SV40 early promoter-enhancer element, the Gal4 DNA binding domain spanning amino acids 1 to 147 (domain 1–147), followed by a polylinker region and the polyadenylation signal of SV40 (33). The pGCE vector series was constructed by subcloning the various portions of the rat C/EBP cDNA into the polylinker region of the parental effector vector Gal147 (see Fig. 1a, 3a, and 5a). The fusion proteins encoded by the pGCE plasmids therefore contain the Gal4 DNA binding domain 1–147 and the various parts of the N-terminal domain of C/EBP. The reporter plasmids used in this paper include E1b-CAT, Gal4x5 E1b-CAT, TK-hGH, and SV.232.A.CAT.LRS. The first two plasmids consist of a TATAA element of the adenovirus E1b promoter preceded by 0 or 5 copies of the 17-mer oligonucleotide of the Gal4 DNA binding site (23). TK-hGH and SV.232.A.CAT.LRS have been described previously (29).

The Gal4-based plasmids. Thirteen Gal4–C/EBP fusion plasmids (pGCE1–245, pGCE1–35, pGCE1–35/108–245, pGCE1–107, pGCE108–245, pGCE36–245, pGCE36–107, pGCE108–170, pGCE171–245, pGCE1–107/171–245, pGCE1–

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170, pGCE171–215, and pGCE189–222) were constructed according to standard procedures. Detailed descriptions of the constructions will be included in the Ph.D. thesis of D. Pei (28a).

Plasmids R18 and B19. Plasmid R18 has been described (29). Plasmid B19 was made by deleting 111 bp of an *Nco*I-*Sma*I fragment from the C/EBP coding region. A frameshift mutation was created after religation of the large *Nco*I-*Sma*I fragment.

Plasmid R18d(108–170). pGCE1–107/171–245 was first digested with *Eco*RI, followed by Klenow repair to convert *Eco*RI ends into blunt ends. After DNA purification, the plasmid was partially cleaved with *Nco*I. Since C/EBP DNA contains an *Nco*I site at the first methionine codon, the *Nco*I-*Eco*RI fragment containing C/EBP 1–107/171–245 was isolated from a 1% agarose gel by electroelution. R18 was first digested with *Sma*I to completion, followed by *Nco*I partial digestion. The R18 vector, which contains a full-length C/EBP, was deleted of a *Nco*I-*Sma*I fragment (amino acids 1 to 245) and purified from a gel before ligation with a *Nco*I-*Eco*RI (blunted) insert fragment.

Characterization of recombinant plasmids. Most of the recombinant plasmids described above have been characterized by DNA sequencing across the 5′ fusion junction by using an oligo primer derived from Gal4 sequences (5′-ATGCCTCTAACATTGAGACA-3′). All of the C/EBP domain expression plasmids (12 total), except B19, pGCE1–35/108–245, and pGCE36–245, appear to give rise to protein products of predicted molecular weight after transfection and immunoblot analysis (data not shown; see Fig. 3c, 4d, and 5c). It is unclear why these three plasmids do not give rise to detectable or authentic-sized products. It may be related to the stability of the fusion protein. The in-frame fusions at the junctions in plasmids pGCE36–245 and pGCE1–35/108–245 have been confirmed by DNA sequencing across the junctions (data not shown).

Mutagenesis. A *Hin*dIII-*Sst*I fragment containing Gal147 and C/EBP 171–245 was cloned into the M13MP18 vector. Mutagenesis was carried out according to procedures described previously (20, 32). The oligonucleotides used were 5′-CAGCCCCCGCCGTCGTCGCCGCCACCG-3′ and 5′-TATCAGCCCCCGTCGTCGTCGTCGCCACCGCAC-3′. Mutations were screened by DNA sequencing. The *Hin*dIII-*Sst*I fragment isolated from the identified mutants was subcloned back into the expression vector as the pGCE series. Mutant M(−2P) contains two amino acid substitutions changing from proline into serine, while M(−4P) contains four amino acid subbstitutions changing from proline into serine (see Fig. 6).

Cell culture. Huh7 and HepG2 are human hepatoma cell lines (1, 27). They were maintained in high-glucose Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Hyclone Inc., Logan, Utah) in the presence of 5% CO_2 at 37°C.

DNA transfection. Cells were seeded 12 h before transfection at density of 3×10^5 cells per 6-cm dish. A total of 14 μg of DNA, including reporter, effector, and internal reference plasmids and Huh7 genomic DNA as a carrier, was applied to each dish by using the calcium phosphate transfection protocol (29). After an 8-h incubation, the transfected culture was washed and fresh medium was added.

CAT assay. Bacterial chloramphenicol acetyltransferase (CAT) activity was measured as described by Gorman et al. (12).

hGH assay. A radioimmunoassay kit for human growth hormone (hGH) was purchased from Nichols Institute, San Juan Capistrano, Calif. Cell culture media collected 36 h after transfection were assayed by the procedure suggested by the Nichols Institute.

Primer extension analysis. Total RNA was extracted from the transfected culture by the guanidinium denaturation method (4). The sequence of the oligo primer used in this assay is 5′-GTTCTTTACGATGCCATTGGGATA-3′, which is complementary to the coding strand of the CAT gene from amino acids 13 to 20. Briefly, 40 μg of total RNA was hybridized with ^{32}P-labeled CAT primer in 80% formamide overnight. The hybrids were collected by ethanol precipitation and then subjected to reverse transcription with avian myeloblastosis virus reverse transcriptase for 90 min. The cDNA products were extracted with phenol-chloroform-isoamyl alcohol (50:49:1). After alcohol precipitation, the pellets were dried and dissolved in loading buffer containing 99% formamide and analyzed on a 6% polyacrylamide gel containing 7.5 M urea.

Western blot (immunoblot) analysis. The immunoblot procedure was adopted from that of Harlow and Lane (15). The rabbit polyclonal anti-Gal4 antibody was a gift from the laboratories of M. Green and M. Ptashne (Harvard University). The rabbit anti-C/EBP antibody was prepared against a 14-mer synthetic peptide of C/EBP (NH_2-AGPHPDLRTGG GGC-COOH) according to the method of Landschultz et al. (21) by L. Tung of the Protein Chemistry Center, University of Pennsylvania.

RESULTS

Two discontiguous, separable transactivation domains of C/EBP. The yeast transcription factor Gal4 has been well characterized. The N-terminal portion of Gal4, amino acids, 1 to 147, contains both dimerization and nuclear translocation signals (33). By taking advantage of this Gal4 system, domain swap experiments have been conducted.

As shown in Fig. 1a, plasmid pGCE1–245 contains Gal4 from amino acids 1 to 147 fused in frame with the N-terminal portion of C/EBP from amino acids 1 to 245. In this plasmid, the b-ZIP DNA binding domain 272–358 of C/EBP is not included. It was predicted that the protein products produced from this parental construct pGCE1–245 and its eight partially truncated derivatives (Fig. 1a) would be able to recognize and bind to the *cis* element of the Gal4 binding site. One pair of reporter plasmids, E1b-CAT and Gal4x5 E1b-CAT, was used for the following experiments. The former contains no Gal4 binding site, while the latter contains five tandem copies of the Gal4 binding site, positioned in front of the adenovirus E1b promoter TATAA motif. Other than in the Gal4 binding site and the TATAA motif, no other *cis* element is present in these reporters (23; see Fig. 2). Assays of CAT activity were performed after transient cotransfection into the human hepatoma cell line Huh7 (27).

As shown in Fig. 1b, CAT activities were not detectable when E1b-CAT was used as a reporter. By contrast, when Gal4x5 E1b-CAT was used, acetylated chloramphenicol could be detected in plasmids GalE1a, pGCE1–107, pGCE108–245, and the full-length pGCE1–245. The signal generated by pGCE1–107 was consistently stronger than that of Gal-E1a, which is a potent transactivator encoded by adenovirus 5 (23). The signal generated by pGCE108–245 is approximately 20-fold weaker than that of pGCE1–107. Interestingly, the domain containing amino acids 1 to 107 appears to give approximately 2.8-fold-higher activity than

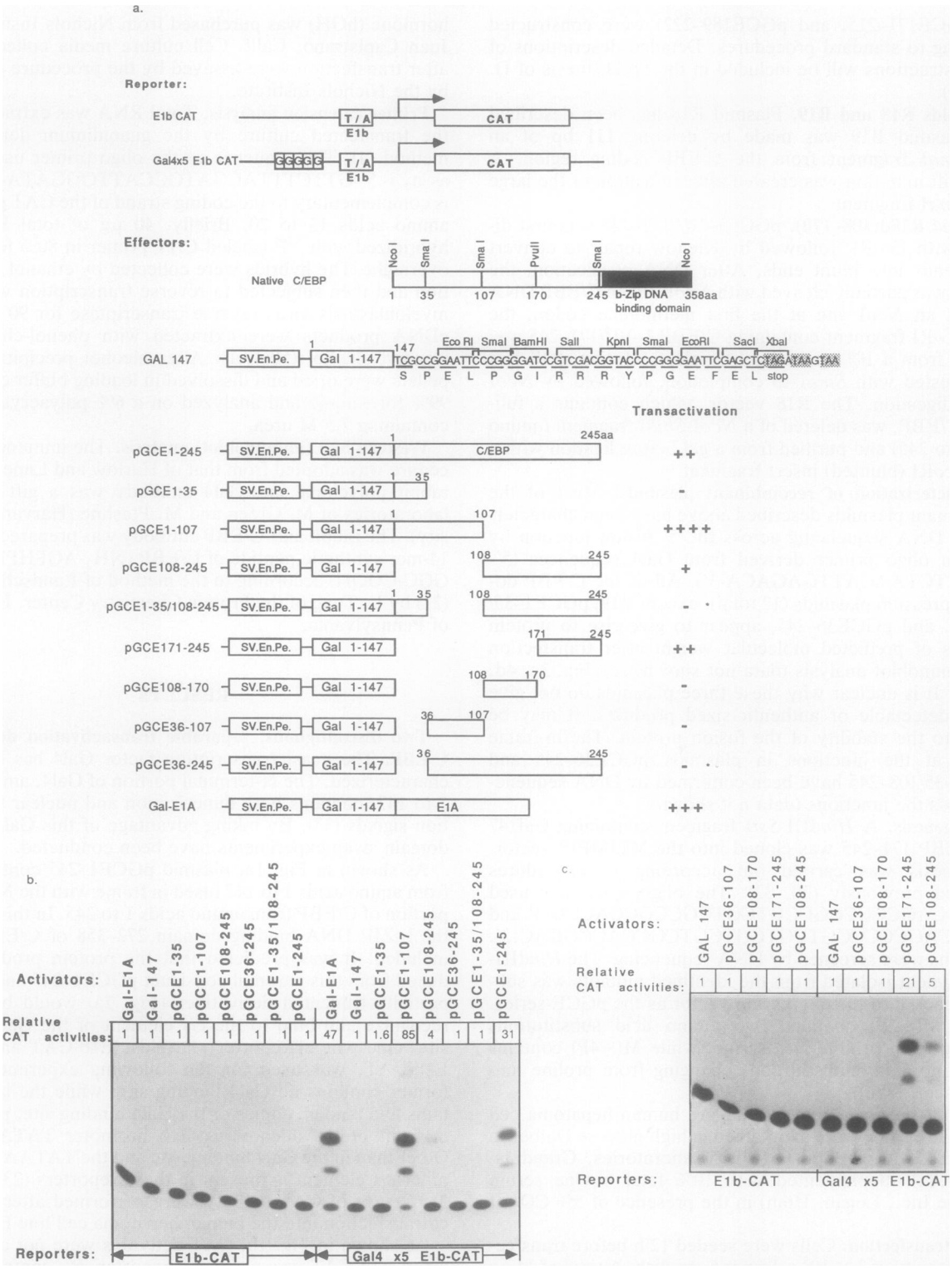

FIG. 1. The N-terminal 245 amino acids of C/EBP contain two separable transactivation domains. (a) Reporter plasmid E1b-CAT contains a synthetic TATA box of the adenovirus E1b promoter in front of the bacterial CAT gene. Gal4x5 E1b-CAT has five copies of a 17-mer oligonucleotide of the Gal4 binding site positioned 5′ upstream of the TATA element as described in reference 23. Effector plasmids: Full-length rat C/EBP consists of 358 amino acids. One-third of the carboxyl portion encodes the b-ZIP DNA binding domain (■). Various parts of the N terminus of the C/EBP protein were fused to the carboxyl end of Gal4 DNA binding domain, resulting in plasmids of the pGCE series (see Materials and Methods). The b-ZIP domain spans from amino acids 272–358. Gal147 serves as a negative control, and Gal-E1a serves as a positive control. +, Positive in transactivation; −, no transactivation observed (+ and − were used to summarize data from panels b and c; for more quantitative comparison, see panels b and c); *, protein products that do not correspond to the predicted molecular weights of the fusion proteins (see Materials and Methods); SV.En.Pe, SV40 enhancer and early promoter. (b) The effector (2 μg) and reporter (2 μg) plasmids as indicated were introduced into Huh7 cells by calcium phosphate cotransfection procedure. Thirty-six hours later, cells were

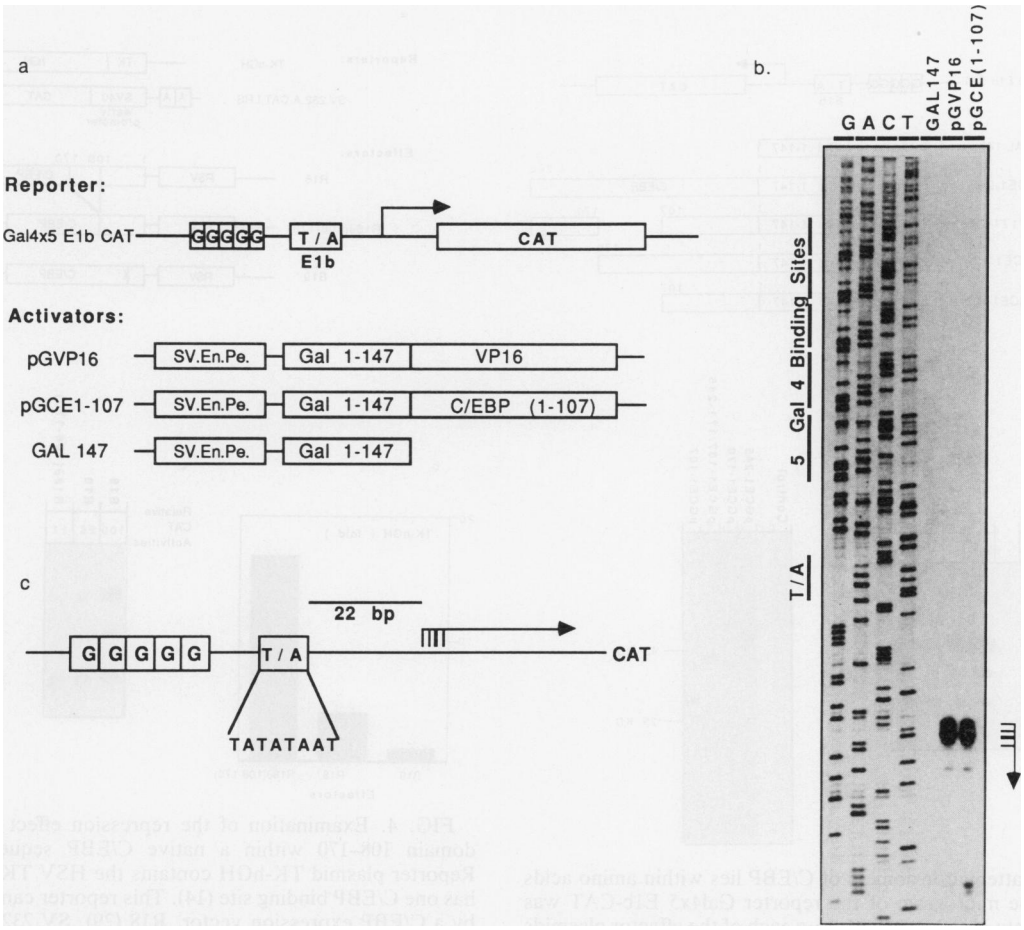

FIG. 2. The transactivation potential of C/EBP domain 1–107 is comparable to that of the immediate-early gene product VP16 of herpes simplex virus. (a) Four micrograms of Gal4x5 E1b-CAT were cotransfected with Gal147 (2 μg), pGVP16 (2 μg), or pGCE1–107 (50 ng). Forty hours posttranssfection, total cellular RNA was isolated and analyzed by primer extension using an oligo primer complementary to the 5′ end of the CAT gene sequence. (b) Primer extended products and the size marker generated from the same primer in a Sanger dideoxy-sequencing reaction were subjected to electrophoresis on a 6% 7.5 M urea polyacrylamide gel. (c) T.A; E1b TATA box (TATATAAT). The distance from TATA to the initiation sites is approximately 22 nucleotides.

the full-length N-terminal domain of C/EBP (amino acids 1 to 245).

To further analyze the weak activity domain containing amino acids 108 to 245, we compared the activities of pGCE108–245 and pGCE171–245. Unexpectedly, there appears to be a fourfold-higher activity observed when domain 108–170 is deleted (Fig. 1c).

Strength comparison of VP16 and pGCE1–107. VP16 is encoded by herpes simplex virus and an extremely potent transactivator (31). We have attempted to compare the strength of transactivation effect mediated by the viral VP16 factor and the cellular C/EBP domain 1–107. When both primer extension (Fig. 2b) and CAT assay (data not shown) were used, the optimum dose of pGCE1–107 (50 ng per transfection) gave rise to an amount of transcriptional stimulation quantitatively similar to the optimum dose of VP16 (2 μg per transfection). The steady-state levels of each protein product encoded by 2 μg of pGVP16 and 50 ng of pGCE1–107 also appeared to be similar (data not shown). The 5′ ends of the mRNAs appeared to initiate correctly, at approximately 22 bp downstream from the TATATAAT element (Fig. 2c).

Attenuator domain 107–170 can decrease the transactivation potential. When plasmids pGCE1–245 and pGCE1–107/171–245 are transfected in parallel, the latter, which has deleted region 108–170, gives a signal fivefold stronger than that of the full-length pGCE1–245. Similarly, when region 108–170 is deleted from pGCE1–170, a 13-fold increase in

assayed for CAT activity. The relative CAT activities were calculated by assigning the activity of Gal147 plus E1b-CAT cotransfection as 1. The data shown are averages of at least two independent experiments. Standard errors for CAT activities: pGCE1–107, ±4.1; pGCE108–245, ±1.3; pGCE1–245, ±7.1; the rest of the pGCE series have standard errors of <0.5. The conversion rate for pGCE1–107/Gal4x5 E1b-CAT cotransfection is over 50% (out of the linear range of the CAT assay), so its activity is likely underestimated in the presentation. (c) Reporter and effector plasmids (4 μg of each) were transfected into Huh7 cells as described above. Standard errors for CAT activities: pGCE108–245, ±1.1; pGCE171–245, ±2.1; the rest have errors of <0.1.

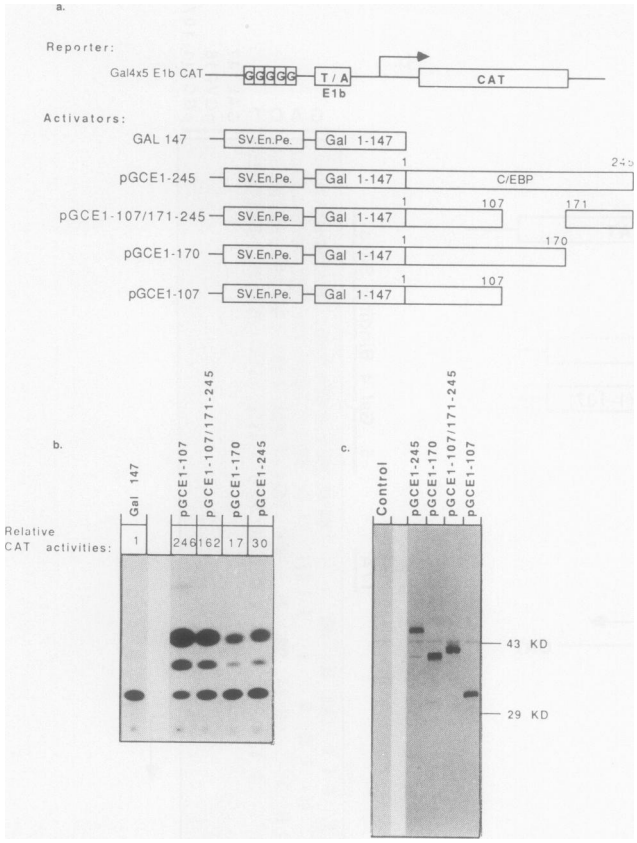

FIG. 3. An attenuator domain of C/EBP lies within amino acids 108 to 170. One microgram of the reporter Gal4x5 E1b-CAT was cotransfected into Huh7 cells with 1 μg each of the effector plasmids as indicated in panel a. Thirty-six hours posttransfection, cells were harvested. One-fifth of the cells were lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for immunoblot analysis (panel c). The rest of the cells were lysed in CAT assay buffer. The results of the CAT assay are shown in panel b (standard errors for CAT activities: pGCE1–107, ±10.7; pGCE1–107/171–245, ±28.5; pGCE1–245, ±9.1; pGCE1–170, ±4.5). The control lane, which exhibits a 43-kDa nonspecific band, is from mock-transfected Huh7 cells (panel c).

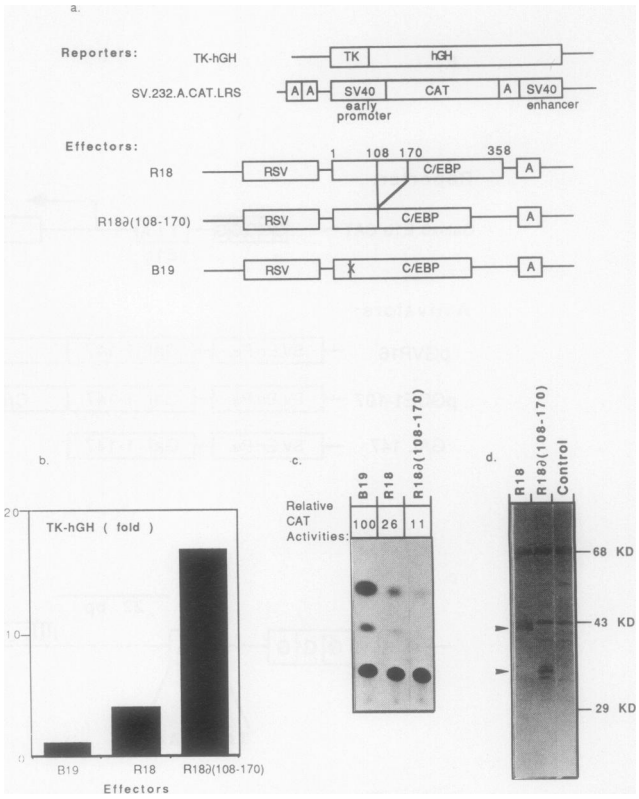

FIG. 4. Examination of the repression effect of the attenuator domain 108–170 within a native C/EBP sequence context. (a) Reporter plasmid TK-hGH contains the HSV TK promoter, which has one C/EBP binding site (14). This reporter can be transactivated by a C/EBP expression vector, R18 (29). SV.232.A.CAT.LRS has the early promoter 5′ upstream of the CAT gene and the enhancer located 3′ downstream. This reporter can be repressed by R18 (29). As illustrated, R18 has a long terminal repeat element of the Rous sarcoma virus which drives the expression of the full-length rat C/EBP cDNA. R18d(108–170) contains an internal deletion from amino acids 108 to 170. B19 is an out-of-frame deletion mutant of R18. (b and c) TK-hGH (2 μg) plus SV.232.A.CAT.LRS (2 μg) were cotransfected into HepG2 cells with each effector plasmid (2 μg) as illustrated in panel a. Forty hours later, cell media were collected for radioimmunoassay of hGH production (panel b, standard errors for hGH activities: R18, ±0.9; R18d(108–170), ±2.5; B19, ±0.1). Cell lysates were measured for CAT activities (panel c). Relative CAT activities were calculated by assigning the activity of B19 cotransfection as 100 (standard errors for CAT activities: R18, ±2.9; R18d(108–170), ±1.0). (d) R18 (5 μg) and R18d(108–170) (5 μg) were transfected separately into Huh7 cells. The protein products encoded by each plasmid were quantitated by Western blot analysis using a rabbit antibody against a 14-mer synthetic peptide of C/EBP (see Materials and Methods). The control lane is from untransfected Huh7 cells. The upper arrowhead indicates the position of the full-length C/EBP protein; the lower arrowhead indicates the one with internal deletion.

CAT activity is again observed in plasmid pGCE1–107. Differences in CAT activity shown in Fig. 3b cannot be attributed to differences in stability of the various protein products encoded by these different plasmids. Western blot analysis using rabbit polyclonal antibody against Gal4 (33) demonstrates no difference in steady-state levels among the various protein products in the transfected culture (Fig. 3c). To minimize potential fluctuation of transfection efficiency from dish to dish, aliquots from the same transfected culture were used for both CAT and Western assays. It therefore appears that whenever the attenuator domain is deleted, transactivation potential is enhanced.

Attenuator domain 107–170 is not part of the repressor domain of C/EBP. Our previous studies have indicated that C/EBP can function as a transcriptional activator to the promoter-enhancer element of thymidine kinase (TK) as well as a repressor to the enhancer elements of human hepatitis B virus and SV40 (29). To see whether domain 107–170 is part of a repressor domain of C/EBP, we constructed plasmids R18d(108–170) and B19 (Fig. 4a). The former has a deletion in domain 108–170, while the latter contains a frameshift mutation near the N terminus of C/EBP. Surprisingly, in a tripartite cotransfection, R18d(108–170) behaves as a superactivator to TK-hGH. Furthermore, the repressor activity of R18d(108–170) to the SV40 enhancer is not significantly altered (Fig. 4b and 4c). Neither transactivation nor repression activities can be seen with plasmid B19. To see whether differences in cotransfected reporter gene activity between R18 and R18d(108–170) are due to the differences in stability of their respective protein products, we performed Western

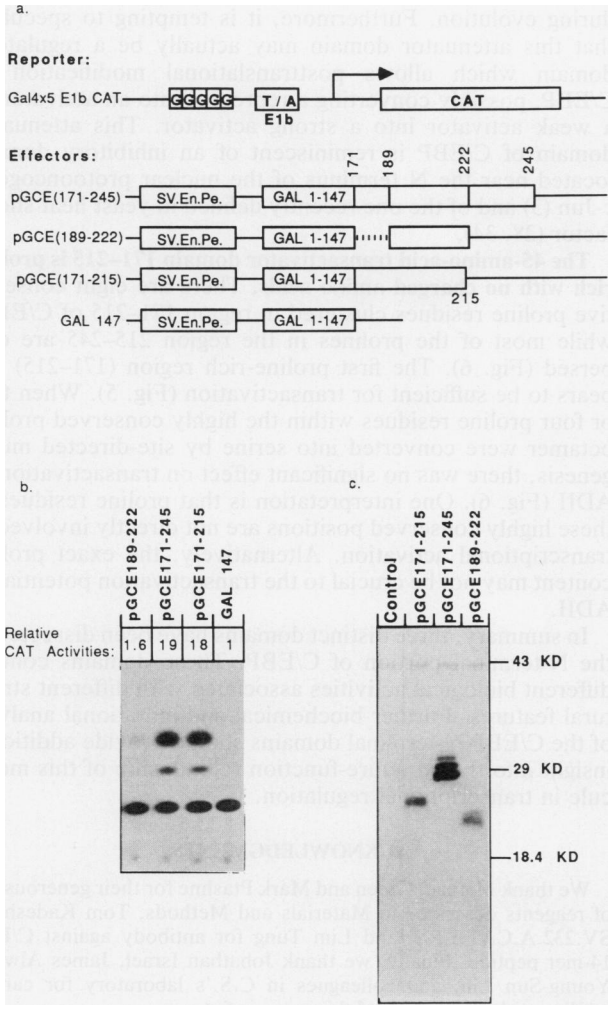

FIG. 5. A 45-amino-acid domain, 171–215, with a proline-rich structure is sufficient for transactivation. Four micrograms of plasmid Gal4x5 E1b-CAT DNA was cotransfected into Huh7 cells with each effector plasmid (2 μg) as indicated (panel a). The assay procedure for CAT activity (panel b) and the quantitation of the steady-state level of each fusion product by Western blot (panel c) were essentially the same as described earlier for Fig. 4. Anti-Gal4 antibody was used for panel c. (Standard errors for CAT activities: pGCE171–245, ±2.3; pGCE171–215, ±3.2; pGCE189–222, ±0.2).

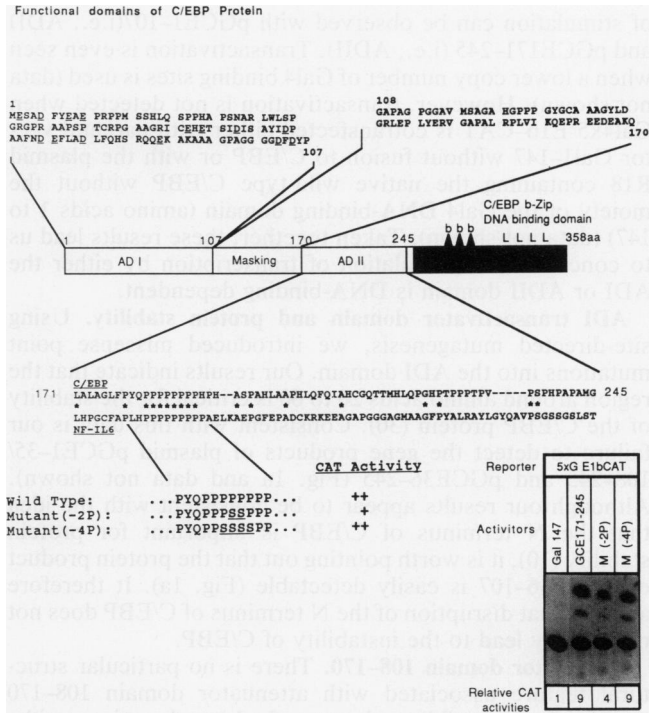

FIG. 6. Summary of the functional domains of the N-terminal 245 amino acids of C/EBP and mutational analysis of the proline-rich domain. ADI, Activation domain I, from amino acids 1 to 107. Acidic amino acids within this domain are underlined. ADII, Activation domain II, from amino acids 171 to 245. The b-ZIP DNA binding motif is bipartite: b, basic amino acid residues; L, adjacent leucine zipper repeats (36). The data of sequence homology between C/EBP and NF-IL6 are taken directly from Akira et al. (2). Mutations changing 2 or 4 consecutive prolines into serine residues within a highly conserved proline octamer do not appear to significantly affect the transactivation potential of ADII. Four micrograms of 5xG E1b-CAT and 2 μg each of Gal147, pGCE171–245, M(−2P), and M(−4P) were transfected and assayed as described in the legend to Fig. 1.

blot analysis using a rabbit antibody prepared against a 14-mer synthetic peptide of C/EBP (see Materials and Methods). The results shown in Fig. 4d indicate that the amounts of C/EBP-specific protein products of R18 at the 42-kDa position and R18d(108–170) at the 36-kDa position are comparable.

The second transactivator domain, 171–215, is proline rich. Fig. 1c indicates the presence of a second transactivator domain from amino acids 171 to 245. To further narrow down the minimal essential region of transactivation within this domain, we compared the CAT activities of pGCE171–245, pGCE189–222, and pGCE171–215 (Fig. 5a). While pGCE171–245 and pGCE171–215 appear to exhibit similar levels of CAT activity, pGCE189–222 gives only a barely detectable signal (Fig. 5b). To control for the potential differences in stability of each gene product produced by these different plasmids, Western blot analysis was performed as described in the legend to Fig. 3. The steady-state levels of proteins encoded by pGCE189–222 and pGCE171–215 are almost indistinguishable (Fig. 5c).

To see whether proline is important for transactivation, we introduced point mutations into a highly evolutionarily conserved proline-octamer region (Fig. 6). To our surprise, we did not see any significant effect of mutation on the transactivation potential of domain 171–245, even when four of eight prolines were changed into serine (Fig. 6).

DISCUSSION

Using a domain-swapping approach, we have constructed and analyzed a number of Gal4–C/EBP fusion proteins. As summarized in Fig. 6, the N-terminal portion of C/EBP can be tentatively divided into at least three functionally distinct domains: NH2-ADI-masking-ADII-COOH (i.e., an attenuator, or masking, domain sandwiched between two separate transactivator domains, ADI and ADII).

Transactivations mediated by ADI or ADII are DNA-binding dependent. Our data (Fig. 1b and c) indicate that no transactivation can be detected when the E1b-CAT reporter alone is used. When five copies of Gal4 binding sites are provided in the plasmid Gal4x5 E1b-CAT, significant levels

of stimulation can be observed with pGCE1–107(i.e., ADI) and pGCE171–245 (i.e., ADII). Transactivation is even seen when a lower copy number of Gal4 binding sites is used (data not shown). However, transactivation is not detected when Gal4x5 E1b–CAT is cotransfected either with parental vector Gal1–147 without fusion to C/EBP or with the plasmid R18 containing the native wild-type C/EBP without the moiety of the Gal4 DNA-binding domain (amino acids 1 to 147) (data not shown). Taken together, these results lead us to conclude that stimulation of transcription by either the ADI or ADII domain is DNA-binding dependent.

ADI transactivator domain and protein stability. Using site-directed mutagenesis, we introduced missense point mutations into the ADI domain. Our results indicate that the region around amino acids 20 to 60 is critical for the stability of the C/EBP protein (30). Consistent with this data is our failure to detect the gene products of plasmid pGCE1–35/108–245 and pGCE36–245 (Fig. 1a and data not shown). Although our results appear to be consistent with the idea that the N terminus of C/EBP is important for protein stability (10), it is worth pointing out that the protein product of pGCE36–107 is easily detectable (Fig. 1a). It therefore appears that disruption of the N terminus of C/EBP does not necessarily lead to the instability of C/EBP.

Attenuator domain 108–170. There is no particular structural feature associated with attenuator domain 108–170 except for a striking cluster of charged amino acids, **KQEPREEDEAKQ**, near the end of this region (amino acids 159 to 170) (Fig. 6). This domain by itself does not exhibit any detectable stimulation of transcription (Fig. 1c, lane pGCE108–170). However, when combined with other domains in four different sequence contexts, it consistently diminishes their transactivation effect (Fig. 1c, 3b, and 4c). For example, a fourfold decrease of CAT activity is detected when this domain is added to the ADII domain (compare pGCE108–245 and pGCE171–245 in Fig. 1c). A 13-fold decrease is observed when this domain is added to the ADI domain (compare pGCE1–107 and pGCE1–170 in Fig. 3b). A 5.5-fold decrease is seen when this domain is added back to the construct of pGCE1–107/171–245 (compare the full-length pGCE1–245 and pGCE1–107/171–245 in Fig. 3b).

One interpretation of the negative effect on CAT activity associated with this domain is that domain 108–170 is part of the repressor domain and, therefore, its presence gives a net effect of reduced transactivation (29). To test this possibility, we deleted this domain from the native C/EBP molecule and assayed its repression activity. Unexpectedly, no significant alteration of repressor activity was observed [compare R18 and R18d(108–170) in Fig. 4c and the protein level in Fig. 4d]. This was not due to any increased toxicity generated by this internally truncated C/EBP since the TK-hGH activity was also increased by 17-fold in this cotransfection system (Fig. 4b). Furthermore, as demonstrated by Western blot analysis, the presence of this domain does not appear to destabilize the resulting protein products (e.g., compare the intensity of signals in different lanes in Fig. 3c or 4d). Finally, missense point mutations introduced into this domain also significantly enhanced the CAT activity (30). Since this domain (108–170) is neither a neutral domain nor a repressor or activator domain, we have tentatively called it an attenuator domain, because of its attenuating effect to transactivation.

Previous studies have suggested that too strong a cellular transactivator may be deleterious to eukaryotic cells (31). We speculate that this domain attenuates a strong nonviral activator like ADI to survive through natural selection during evolution. Furthermore, it is tempting to speculate that this attenuator domain may actually be a regulatory domain which allows posttranslational modification of C/EBP, possibly converting a repressor into an activator or a weak activator into a strong activator. This attenuator domain of C/EBP is reminiscent of an inhibitory domain located near the N terminus of the nuclear protooncogene c-Jun (3) and of the one recently defined in yeast heat shock factor (28, 34).

The 45-amino-acid transactivator domain 171–215 is proline rich with no charged amino acids. There are eight consecutive proline residues clustered in region 171–215 of C/EBP, while most of the prolines in the region 215–245 are dispersed (Fig. 6). The first proline-rich region (171–215) appears to be sufficient for transactivation (Fig. 5). When two or four proline residues within the highly conserved proline octamer were converted into serine by site-directed mutagenesis, there was no significant effect on transactivation of ADII (Fig. 6). One interpretation is that proline residues at these highly conserved positions are not directly involved in transcriptional activation. Alternatively, the exact proline content may not be crucial to the transactivation potential of ADII.

In summary, three distinct domains have been dissected at the N-terminal portion of C/EBP. These domains contain different biological activities associated with different structural features. Further biochemical and mutational analysis of the C/EBP N-terminal domains should provide additional insight into the structure-function relationship of this molecule in transcriptional regulation.

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