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C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements

MARIO VALLEJO, DAVID RON*, CHRISTOPHER P. MILLER, AND JOEL F. HABENER

Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02114

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ABSTRACT **Members of the C/EBP family of basic–leucine zipper (bZip) transcription factors form heterodimers and bind to the CAAT box and other sequence-related enhancer motifs. Using a ^{32}P-labeled protein probe consisting of the bZip domain of C/EBPβ, we isolated a clone encoding C/EBP-related ATF (C/ATF), a bZip protein that heterodimerizes with C/EBP-like proteins but belongs to the CREB/ATF family. C/ATF homodimers do not bind to typical C/EBP DNA sites. Instead they bind to palindromic cAMP response elements such as that of the somatostatin gene. In addition, C/ATF–C/EBPβ heterodimers bind to a subclass of asymmetric cAMP response elements exemplified by those in the phospho*enol*pyruvate carboxykinase and proenkephalin genes. Transient transfection studies indicate that interactions between C/ATF and C/EBPβ are the basis for a functional cross talk between these two families of transcription factors that may be important for the integration of hormonal and developmental stimuli that determine the expression of subsets of genes in specific cellular phenotypes.**

A predominant mechanism of control of gene expression in response to extracellular or intracellular cues occurs at the transcriptional level by the binding of cell-specific and ubiquitous transactivating nuclear proteins to their cognate DNA regulatory elements. Transcription factors that are widely distributed amongst cells of different phenotypes utilize a combinatorial mechanism that ultimately determines the transcriptional activities of specific genes.

Distinct classes of transcription factors have been defined based on their structural relatedness. One of these classes is characterized by conserved basic–leucine zipper (bZip) domains required for DNA recognition and binding and for protein dimerization (1). The bZip transcription factors can be grouped into three major families based on the similarities of their structures, their capability to associate as homodimers or as heterodimers with other members of the same family, and their DNA-binding specificities. One of these families is composed of CREB/activating transcription factor (ATF) proteins, which bind to cAMP response element (CRE) sequences and are involved in the modulation of the transcriptional responses induced by cAMP or by certain viral proteins (2, 3). Another family is that of Fos- and Jun-related oncoproteins (4). The third family is composed of CAAT box/enhancer-binding proteins (C/EBPs) (5, 6), which bind to CAAT-box and related enhancer motifs and regulate the expression of genes during cellular differentiation or in response to inflammatory cytokines. Interactions of bZip proteins within each subfamily are restricted and dictated by a specific and complex dimerization code.

Here we report the identification of C/EBP-related ATF (C/ATF),† a bZip protein that heterodimerizes with C/EBP-like proteins but fails to bind to typical C/EBP sites as a homodimer. The DNA-binding specificities of C/ATF–C/EBP heterodimers are different from those of the corresponding homodimers, inasmuch as they bind to and activate transcription from both asymmetric and palindromic CRE sites. Amino acid sequence analysis and the observation that C/ATF homodimers bind efficiently to palindromic CRE sites indicate that C/ATF is more closely related to the CREB/ATF than it is to the C/EBP family of DNA-binding proteins. These observations point to the existence of cross talk between C/EBP and ATF proteins.

MATERIALS AND METHODS

Screening. A λZAP (Stratagene) 3T3-L1 mouse adipocyte expression library (gift of B. Spiegelman, Dana–Farber Cancer Institute, Boston) was screened by the zipper-blot method using a ^{32}P-labeled peptide consisting of the bZip region of C/EBPβ (7). Positive clones encoding proteins that bound to an oligonucleotide containing a canonical C/EBP binding site (8) were discarded.

Northern Blots. Poly(A)$^+$ RNA from various rat organs was fractionated in a 1% agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled insert of C/ATF cDNA.

Immunoprecipitations and Immunocytochemistry. Pulse–chase experiments were carried out with HepG2 human hepatoma cells or COS-1 monkey kidney cells transfected with a C/ATF expression plasmid (2 μg). Cells were incubated with [^{35}S]methionine/cysteine (Tran^{35}S-label, ICN; 800 μCi/ml; 1 μCi = 37 kBq) for 45 min, washed with medium containing nonradioactive methionine, and left in that medium for 30-, 90-, or 330-min "chase" periods. Lysates were prepared from the cells for immunoprecipitation of ^{35}S-labeled proteins as described (7), using serum from a rabbit immunized with purified glutathione *S*-transferase–C/ATF fusion protein. RNAs encoding C/ATF or C/EBPβ were translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methioine. Immunoprecipitations were carried out as described (7). Immunocytochemical detection of C/ATF was carried out (7) in COS-1 cells transfected with pcDNA-C/ATF 48 hr earlier.

Bacterial Expression and Electrophoretic Mobility-Shift Assays. The C/ATF cDNA containing the complete open reading frame and a fragment of cDNA encoding only the bZip domain of C/EBPβ (7) were cloned into the *Bam*HI–*Eco*RI

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Abbreviations: ATF, activating transcription factor; APRE, acute-phase response element; bZip, basic–leucine zipper; CAT, chloramphenicol acetyltransferase; C/EBP, CAAT box/enhancer binding proteins; CRE, cAMP response element; PEPCK, phospho*enol*-pyruvate carboxykinase.

*Present address: Department of Cell Biology, New York University Medical Center, 550 First Avenue, New York, NY 10016.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L13791).

site of plasmid pGEX-KG (gift of J. Dixon, University of Michigan Medical School, Ann Arbor). Approximately 50 ng of purified bacterially expressed glutathione *S*-transferase fusion protein (9) was used for each mobility-shift assay (10). The oligonucleotides used correspond to the somatostatin gene CRE (5′-GATCCGGCGCCTCCTTGGCTGACGTCA-GAGAGAGAGA-3′) (11), COL-8 (5′-GATCCGGCT-GACGTCATCAAGCTA-3′) (12), the phospho*enol*pyruvate carboxykinase (PEPCK) gene CRE-1 (5′-GATCCCCT-TACGTCAGAGGCGA-3′) (13), the enkephalin gene CRE (5′-GATCCGGCGCGGGGCTGGCGTAGGGCCTGCGT-CAGCTGCA-3′) (14), and the angiotensinogen gene acute-phase response element (APRE-M6) (5′-GATCCACAGT-TGTGATTTCACAACCTGACCAGA-3′) (8).

Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. Eukaryotic expression vectors for C/EBPβ (7) and C/ATF were constructed by inserting cDNA encoding the entire protein into pcDNA-I (Invitrogen). CAT reporter plasmids were constructed by inserting the aforementioned oligonucleotides in front of the minimal promoter of the herpes simplex virus thymidine kinase (TK) gene through the *Bam*HI site of −41TKCAT (15). HepG2 cells were transfected (10) with CAT reporter plasmids (10 μg) and the expression plasmids pcDNA-C/ATF (5 μg) and pcDNA-C/EBPβ (0.5 μg). The amount of DNA was kept constant (20 μg) by adding pBluescript (Stratagene). CAT activity was measured by a solution assay 48 hr after transfection (16).

RESULTS

We identified several clones encoding proteins containing leucine zippers that dimerized with that of C/EBPβ but that did not bind to the angiotensinogen gene APRE-M6 (a C/EBP-binding oligonucleotide; ref. 8) in a Southwestern blot assay. Sequencing of one of these clones revealed an open reading frame of 349 amino acids (Fig. 1*A*) including a predicted bZip domain that contains a leucine zipper region with a significant degree of sequence similarity with known C/EBP proteins (Fig. 1*B*). However, the basic region amino-terminal to the leucine zipper diverges significantly from that of other C/EBPs and shows the most similarity with the ATF family (Fig. 1*B*). For this reason the encoded protein was named C/EBP-related ATF (C/ATF).

Northern analysis of poly(A)$^+$ RNA revealed a single 1.6-kb C/ATF transcript in all the rat tissues analyzed except testis (Fig. 2*A*). Similarly, the C/ATF message was present in the HepG2 human cell line (data not shown).

Western immunoblotting with a specific antiserum detected C/ATF in some but not all liver cell lines tested (Fig. 2*B*), suggesting that it may be relatively unstable. Therefore, we evaluated the rates of synthesis and turnover of C/ATF protein. Pulse–chase labeling of proteins with [^{35}S]methionine in COS-1 cells transfected with pcDNA-C/ATF, followed by immunoprecipitation, showed that C/ATF is a relatively labile protein with a half-life of <30 min (Fig. 2*C*). Similar results were obtained with the endogenous protein in HepG2 cells (data not shown).

Immunocytochemical detection of C/ATF in transfected COS-1 cells revealed intense fluorescence staining in the nuclei of ≈1% of cells (Fig. 3), a finding not due to poor transfection efficiency, because ≈30% of COS-1 cells similarly transfected with pcDNA-CHOP-10 (7) showed positive staining with a specific antiserum (Fig. 3).

To investigate whether C/ATF and C/EBPβ form heterodimers in solution, both proteins were synthesized by *in vitro* translation with [^{35}S]methionine and immunoprecipitated with antiserum directed against C/ATF or C/EBPβ. The anti-C/ATF and anti-C/EBPβ antisera individually immunoprecipitated 50-kDa and 33-kDa proteins, respectively (Fig. 4*A*). When cotranslation reactions were carried out with

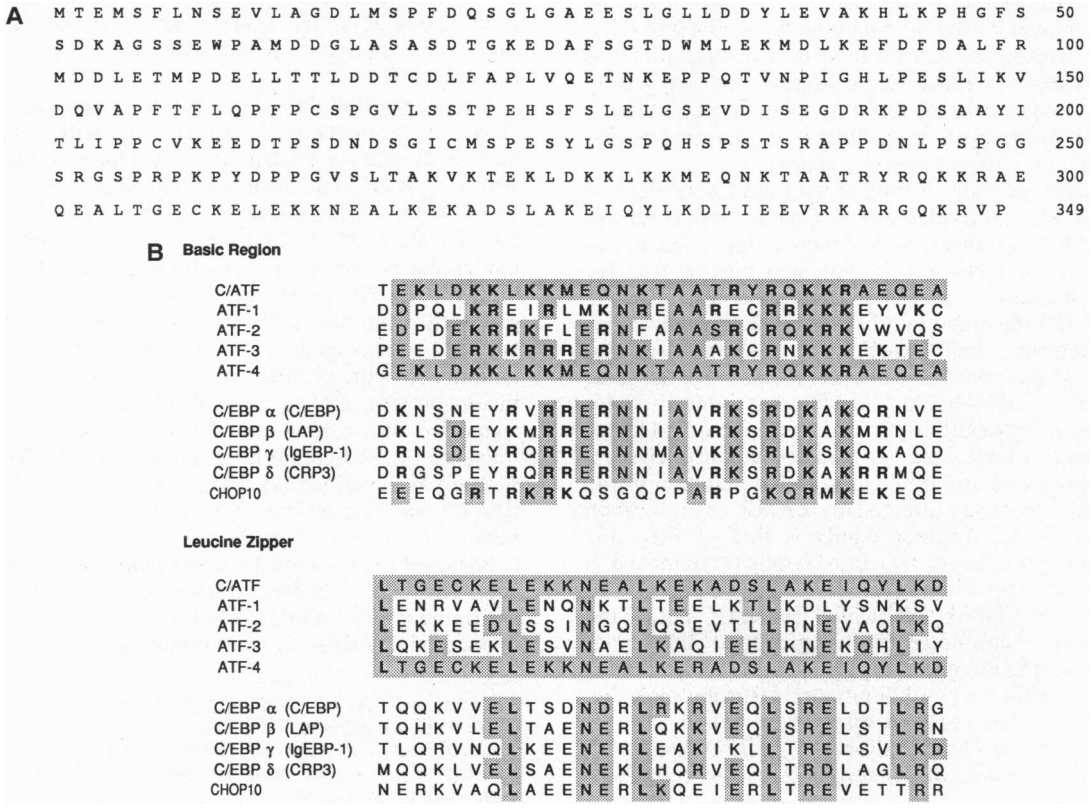


FIG. 1. (*A*) Predicted amino acid sequence of C/ATF. (*B*) Alignment of the sequence corresponding to the bZip domain of C/ATF with those of other ATF and C/EBP proteins. Conserved residues are shaded. Basic amino acids and residues comprising the putative leucine zipper are shown in boldface type.

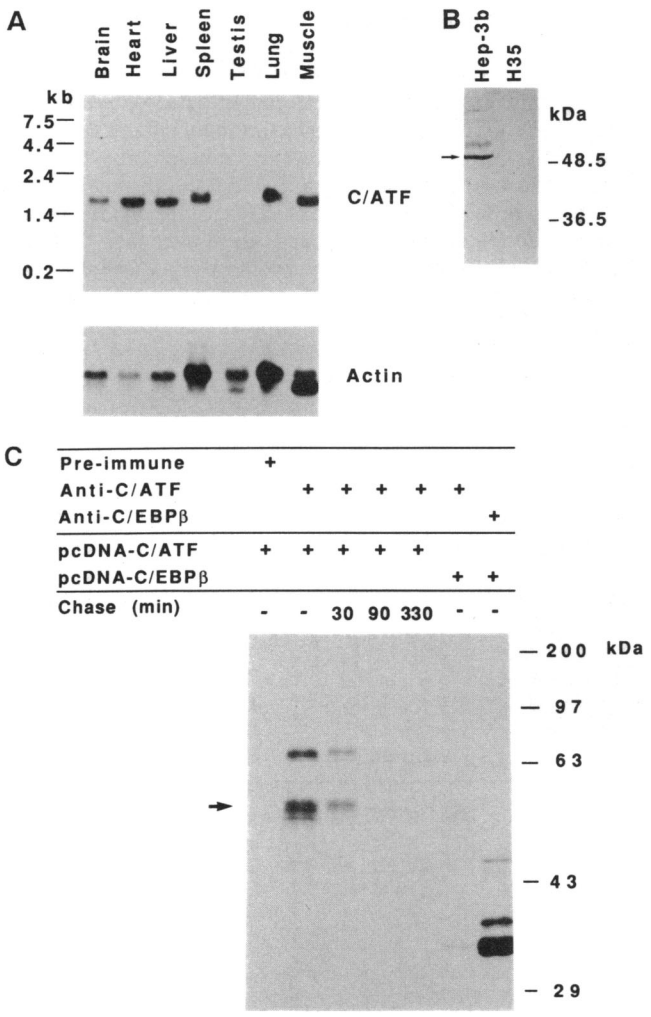


FIG. 2. (*A*) Distribution of C/ATF mRNA as assessed by Northern analysis. The blot was hybridized to the ^{32}P-labeled C/ATF cDNA insert (*Upper*) or to an actin probe (*Lower*). (*B*) Western immunoblot of nuclear proteins from liver cell lines, probed with a C/ATF antiserum. The immunoreactive band corresponding to C/ATF present in nuclear extract from Hep-3b cells is indicated by an arrow. (*C*) Pulse–chase experiment to determine the half life of the C/ATF protein. ^{35}S-labeled C/ATF from COS-1 cells transfected with pcDNA-C/ATF expression plasmid was immunoprecipitated immediately after an [^{35}S]methionine pulse, or after incubation (chase) in [^{35}S]methionine-free medium following the pulse. The band corresponding to C/ATF is indicated by arrow. Specificity was monitored by lack of immunoprecipitation with C/ATF antiserum from cells transfected with pcDNA-C/EBPβ.

both C/ATF and C/EBPβ RNAs added to the same reticulocyte lysate mix, each antiserum immunoprecipitated proteins of both 50 and 33 kDa, indicating that C/ATF and C/EBPβ proteins exist in heterodimeric form in solution (Fig. 4*A*). Similar experiments indicated that C/ATF did not heterodimerize with *in vitro* translated CREB (12) (data not shown), attesting to the specificity of the dimerization.

Because of the differences in the structure of their basic regions, we speculated that ATF and C/EBP may redirect one another to bind to different DNA regulatory elements, thereby expanding the repertoire of DNA-binding specificities of these proteins. To test this hypothesis, we expressed recombinant C/ATF, C/EBPα, C/EBPβ, and CREB in bacteria and used them to test the binding of homo- and heterodimers to different CRE motifs by EMSA. Four oligonucleotides containing different CRE sequence motifs were chosen: the palindromic CRE present in the promoter

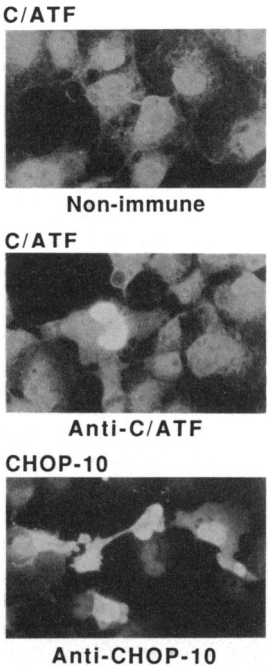


FIG. 3. Immunocytochemical detection of C/ATF by fluorescence microscopy. COS-1 cells were transfected with pcDNA-C/ATF (*Top* and *Middle*) or pcDNA-CHOP-10 (*Bottom*) and incubated with nonimmune serum at a dilution of 1:500 (*Top*), C/ATF antiserum at a dilution of 1:1000 (*Middle*), or CHOP-10 antiserum at a dilution of 1:1000 (*Bottom*). The fluorescent material is shown as a brightly illuminated signal against the dark background of the unstained cells. (×170.)

of the somatostatin gene, with core sequence 5′-TGACGTCA-3′; asymmetric CREs found in the enkephalin and phospho*enol*pyruvate carboxykinase genes, with core sequences 5′-TGACGCAG-3′ and 5′-TGACGTAA-3′, respectively; and COL-8, which contains a core sequence identical to that of the somatostatin CRE, but with different flanking sequences (12). Binding of the recombinant proteins to these oligonucleotides was compared with their binding to an oligonucleotide containing the APRE-M6 from the angiotensinogen gene, a C/EBP binding site with core sequence 5′-AGGTTGGG-3′ (8).

Recombinant C/ATF homodimers bound efficiently to the somatostatin CRE but failed to bind to the enkephalin CRE, PEPCK CRE, and APRE-M6 (Fig. 4*B*). Homodimers of recombinant C/EBPβ did not bind to the enkephalin CRE but did bind to the somatostatin and PEPCK CREs, although less efficiently than to APRE-M6 (Fig. 4*B*). Thus, both homodimers selectively recognized distinct CRE sequences with different specificities. In marked contrast, C/EBPβ-C/ATF heterodimers bound efficiently to all CRE-containing oligonucleotides tested, but not to the C/EBP site in APRE-M6 (Fig. 4*B*). In addition, C/ATF formed heterodimers with C/EBPα that bound to the somatostatin CRE (data not shown). Identical results to those obtained with the somatostatin CRE were observed with the COL-8 CRE (Fig. 4*B*), thus supporting the notion that the palindromic CRE motif is the site where both homodimers and the heterodimer bind. C/ATF and CREB did not form heterodimers as assessed by mobility shift (data not shown), demonstrating the specificity of these C/EBP–C/ATF heterodimers.

To investigate whether the binding of C/ATF and C/EBPβ homo- and heterodimers to the APRE-M6 and CRE sequences correlated directly with transcriptional activity, we tested the relative transactivational activities of C/EBPβ, C/ATF, and a combination of C/EBPβ and C/ATF in directing transcription from reporter plasmids in a cell trans-

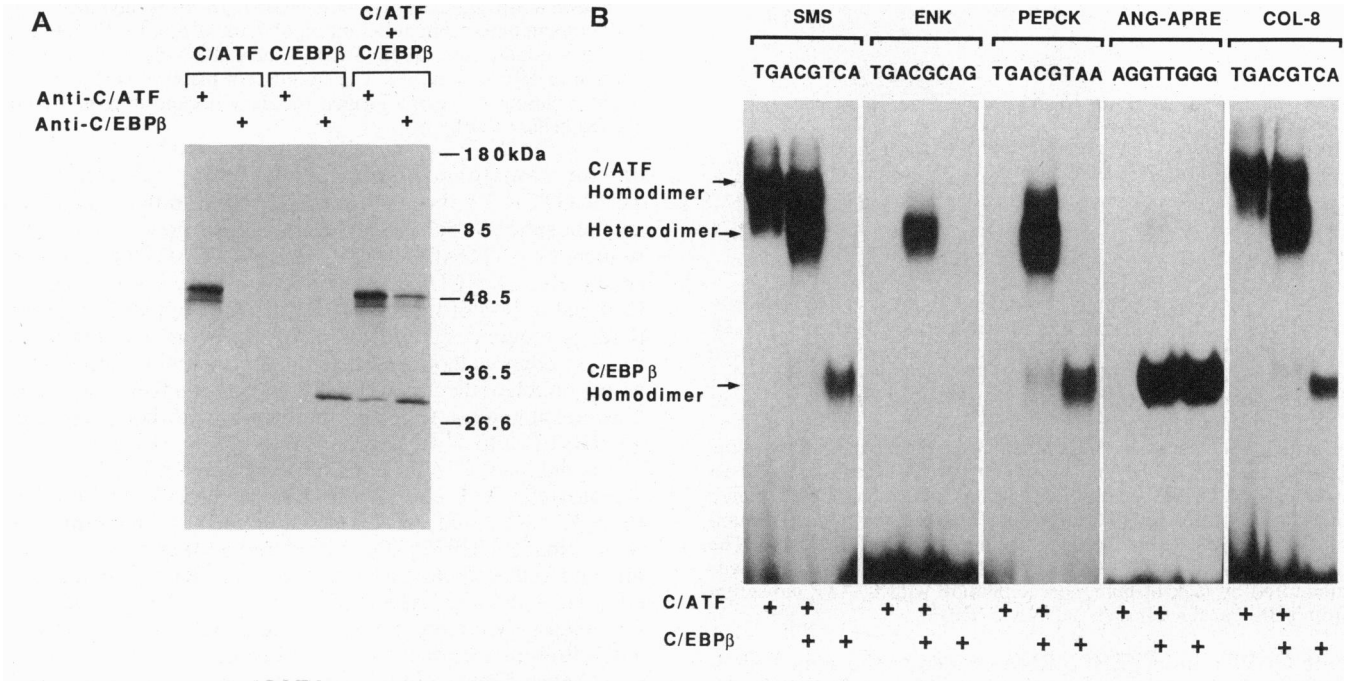
fection assay. CAT reporter plasmids with somatostatin, PEPCK, or enkephalin CRE or APRE-M6 were tested by transient transfection assays in HepG2 cells. The CAT activity generated by these plasmids was normalized to that generated by RSVCAT, a reporter plasmid containing the Rous sarcoma virus promoter/enhancer fused to the CAT-coding sequences.

Cotransfection of the somatostatin CRE reporter plasmid with expression plasmids encoding either C/ATF or C/EBPβ increased CAT activity significantly (Fig. 4*C*). When both C/ATF and C/EBPβ expression plasmids were cotransfected with this reporter, the maximal level of expression was higher than that obtained with either one of the transactivators alone (Fig. 4*C*), indicating that either protein can activate this enhancer and that C/ATF–C/EBPβ heterodimers act synergistically. On the other hand, the level of expression of the enkephalin CRE reporter was not stimulated by either C/ATF or C/EBPβ alone, but when both transactivators were present, a significant increase in CAT activity was detected (Fig. 4*C*). These functional results correlate with the *in vitro* binding studies (Fig. 4*B*).

Cotransfection of C/ATF expression vector with either PEPCK CRE or APRE-M6 reporter plasmid resulted in no detectable increase in CAT activity, consistent with the lack of binding of C/ATF to the PEPCK CRE or to the APRE-M6 observed *in vitro*. However, cotransfection of C/EBPβ expression plasmid with either PEPCK CRE or APRE-M6 reporter resulted in marked increases in CAT activity (Fig. 4*C*). When both C/ATF and C/EBPβ expression plasmids were cotransfected together, different effects were observed: with the PEPCK CRE reporter, the level of CAT activity elicited by the two transactivators simultaneously was no different from that elicited by C/EBPβ alone, but with the APRE-M6 reporter, the level of CAT activity elicited by the two transactivators simultaneously was lower than that elicited by C/EBPβ alone (Fig. 4*C*). These data are also consistent with those found in the *in vitro* binding experiments, in that C/ATF–C/EBPβ heterodimers did not bind to the APRE, and in this case C/ATF acted as a dominant negative inhibitor of C/EBPβ transactivation.

DISCUSSION

The bZip transcription factor C/ATF was isolated by way of its heterodimerization with C/EBPβ. Yet, based on analysis of sequence similarities with other bZip proteins, C/ATF clearly resides within the ATF family, not within the C/EBP family. The bZip domain of C/ATF closely resembles that of human ATF-4 (2). Over the length of the entire protein, C/ATF is homologous to TAXREB67, a human protein that has a bZip domain identical to that of ATF-4 and interacts with the Tax/CRE of human T-cell leukemia virus 1 (3).



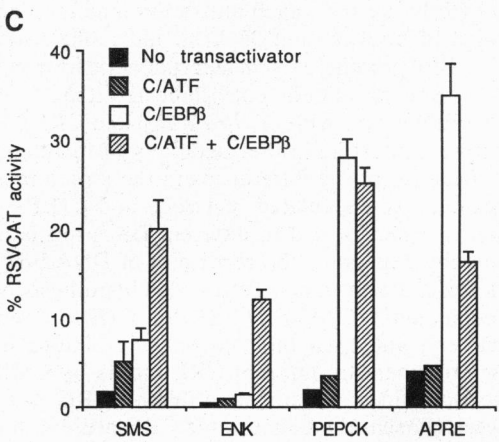


FIG. 4. (*A*) Interaction of C/ATF and C/EBPβ. ^{35}S-labeled *in vitro* translation products of RNAs encoding C/ATF, C/EBPβ, or both were immunoprecipitated and resolved by SDS/10% PAGE. Note the absence of crossreactivity (inability of the C/ATF antiserum to immunoprecipitate *in vitro* translated C/EBPβ, and vice versa). (*B*) DNA binding specificity as assessed by mobility-shift assay. Aliquots of bacterially expressed full-length C/ATF or a truncated version of C/EBPβ corresponding to its bZip region were incubated, alone or in combination, with ^{32}P-labeled oligonucleotides containing the CREs of the somatostatin (SMS), proenkephalin (ENK), and PEPCK genes or the C/EBP-binding APRE of the angiotensinogen (ANG) gene. The core motifs are depicted at the top. Bands corresponding to C/ATF and C/EBPβ homodimers or to C/ATF–C/EBPβ heterodimers are indicated. (*C*) CAT activities after transient transfections of HepG2 cells with reporter plasmids containing the CREs of the SMS, ENK, and PEPCK genes or APRE-M6 of the ANG gene, cloned in front of the thymidine kinase minimum promoter. Transfections were carried out in the absence or presence of either 5 μg of pcDNA-C/ATF or 0.5 μg of pcDNA-C/EBPβ, or both. Values (mean ± SEM) are percentages of the activities elicited by the Rous sarcoma virus–CAT (RSVCAT) fusion gene transfected in the same experiments. Results from duplicate transfections in at least three experiments are shown.

Recently, a human cDNA encoding a protein named CREB-2, identical to TAXREB67, has been reported (17). We cannot determine with certainty whether C/ATF is the mouse homologue of human ATF-4 or the product of a distinct but related gene, because the full-length sequence of ATF-4 has not been reported. Comparison of the sequences of mouse C/ATF and human TAXREB67/CREB-2 suggests that they are products of different genes. The amino acids of these two proteins are only 85% conserved, and many of the changes in residues would not be expected to occur in the evolutionary time span that separates mice and humans (18). Further, the nucleotide sequences differ in several regions by insertions and deletions of bases. This situation is characteristic of the conservation of sequences observed in distinct but closely related gene pairs such as those encoding CREB and CREM [78% (19)], c-Fos and FosB [82% (20)], and c-Jun and JunD [86% (4)] and is not consistent with the conservation found between mouse and human homologues of the same genes: CREB, 99.7%; c-Jun, 98.2%; and c-Fos, 99.1%. The highest degree of similarity exists in the bZip domains, a situation similar to that found between ATF-4 and C/ATF.

In the present study, we demonstrate that transcription factors from two related but different bZip families (C/EBP and CREB/ATF) can associate with significant functional consequences. By forming a complex with C/EBPβ, C/ATF directs binding of C/EBPβ away from C/EBP sites onto palindromic CREs. In addition, the C/ATF–C/EBPβ heterodimer binds to a particular subclass of asymmetric CREs, exemplified by the PEPCK and enkephalin CREs, that are not recognized by homodimers of C/ATF or C/EBPβ.

Our results suggest a hypothetical model involving a dimerization code important for the regulation of cell-specific expression of target genes. Such a code would be dictated by the ambient cellular concentrations of C/EBPβ and C/ATF, determined by relative rates of production and turnover of the individual proteins. Thus, when the levels of C/EBPβ are higher than those of C/ATF, the formation of C/EBPβ homodimers will be favored and transcription from genes containing canonical C/EBP recognition sequences, such as the angiotensinogen gene APRE, will be activated preferentially. When the relative amounts of C/EBPβ and C/ATF are similar, heterodimers will predominate, and the transcription of genes such as those encoding somatostatin, enkephalin, or PEPCK will be activated through the interaction of the heteromeric complex with the CREs contained in their promoters. When the relative concentrations of C/ATF are higher than those of C/EBPβ, the formation of C/ATF homodimers will be favored, and transcription of genes bearing palindromic CREs, such as that encoding somatostatin, will be activated.

The notion that ambient protein concentrations are important in determining the relative proportion of homo- and heterodimeric complexes is suggested by the observation of the short half-life of C/ATF, reflected in low or undetectable levels in most cell types as assessed by Western immunoblotting. This finding is in contrast with the relatively high steady-state levels of C/ATF mRNA in tissues and cell lines. Therefore, the rates of translation and/or degradation of C/ATF appear subject to regulation by extra- or intracellular signals depending on the functional needs of the cell at a particular time or during certain stages of the cell cycle. Specific postranslational modifications, such as phosphorylation of C/EBPβ on a threonine located in its leucine zipper domain (21), may also affect dimerization with other bZip proteins.

Previous studies have shown that C/EBPα and -β can bind to CREs (22, 23). Interactions of C/EBPβ or ATF-4 with members of different families of transcription factors have been reported (24, 25), but the functional consequences of these interactions have not been elucidated. Our data indicate the existence of functional cross talk between the C/EBP and CREB/ATF families, providing a basis for the integration of hormonal stimuli at the level of transcriptional regulation.

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