ATF-2 and C/EBPα Can Form a Heterodimeric DNA Binding Complex in Vitro

FUNCTIONAL IMPLICATIONS FOR TRANSCRIPTIONAL REGULATION*

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We screened an expression cDNA library with a radio-labeled C/EBPα fusion protein and isolated three independent cDNAs encoding ATF-2, a bZIP protein that binds cAMP response elements (CRE). This interaction requires the respective bZIP domains, which form a typical bZIP heterodimer with altered DNA binding selectivity. C/EBPα and ATF-2 homodimers bind CRE sites, but ATF-2:C/EBPα heterodimers do not. Heterodimers bind an asymmetric sequence composed of one consensus half-site for each monomer, and may thus have a unique regulatory function. As predicted, co-transfection of ATF-2 with C/EBPα results in decreased activation of transcription driven from consensus C/EBP-binding sites. In contrast, C/EBPα and ATF-2 function cooperatively to activate transcription driven by the asymmetric sequence. Both factors are expressed in liver, where immunoprecipitation experiments show that ATF-2 co-precipitates with C/EBPα. These results are consistent with the interpretation that C/EBPα and ATF-2 can associate in vivo. Moreover, the formation of ATF-2:C/EBPβ heterodimers suggests that cross-family dimerization with ATF-2 may be a general property for C/EBP family proteins.

CCAAT/enhancer-binding protein, C/EBP,1 was purified as an activity that bound to consensus enhancer elements and to the CCAAT box motif (1). C/EBP is a member of the basic region-leucine zipper (bZIP) class of transcription factors (2, 3). The bZIP domain consists of the leucine zipper, a heptad repeat of leucines, preceded by the basic region, a sequence with net positive charge (4, 5). A combination of molecular and structural studies showed that the heptad repeat region is an amphipathic α helix that mediates dimerization by forming a parallel coiled-coil (4–15). The co-crystal structure of the bZIP domain of GCN4 bound to DNA showed that the coiled helices of the leucine zipper separate, positioning one helix from each chain for sequence specific interaction with DNA (10).

Leucine zippers accommodate both homotypic and heterotypic dimerization. For example, the first C/EBP protein characterized, C/EBPα, is one of at least five gene products comprising the C/EBP gene family (16, 17). These proteins show extensive sequence similarity that is restricted to the bZIP domain, such that each homodimer binds the same DNA element. Additionally, all proteins in the C/EBP family can pair with each other to form DNA binding heterodimers (16, 17). When not bound to DNA, the subunits of bZIP dimers are in a rapid monomer-dimer equilibrium such that the lifetime of dimers is estimated in seconds (18, 19). The ready dissociation of bZIP dimers in vitro suggests that heterodimers with potentially unique regulatory properties may form in vivo. In the case of Fos, a bZIP protein in the API family, no homodimers form. Instead, Fos forms heterodimers with Jun, another API family protein (20).

Sequence-specific interaction with DNA increases the lifetime of bZIP dimers more than 10-fold (18, 19, 21). However, the apparent DNA affinity constants are lower than might be anticipated (18). Surprisingly, GCN4, a protein that binds the core sequence TGAGTCA, exhibited similar affinity for a core motif containing an extra nucleotide, TGACGTCA (18). The structures of GCN4 and Fos:Jun bound to DNA revealed that most of the amino acids involved in base specific contacts are those that are highly conserved among all bZIP proteins (11), including those with distinct DNA binding specificity. Thus, the basic mechanism by which bZIP proteins interact with DNA is known, while the determinants of sequence discrimination are less clearly understood.

C/EBPα activates transcription of several liver and fat cell-specific genes (22–25). Interestingly, over-expression of C/EBPα in cultured cells results in growth arrest (26–29), a finding consistent with the observation that C/EBPα expression commences during the conversion of 3T3L1 preadipocytes into quiescent fat cells in vitro (17, 30, 31). The role of C/EBPα in the adipogenic program was further verified by antisense experiments (32), and by ectopic expression of C/EBPα in a variety of fibroblastic cell lines, where efficient promotion of fat cell differentiation was observed (33).

Support for the notion that C/EBPα plays a central role in regulating energy homeostasis (34) was provided by targeted gene disruption. Homozygous C/EBPα knockout mice are born with apparently normal blood glucose levels, but become severely hypoglycemic within minutes (35). These animals exhibit glycogen storage defects and morphological anomalies in fat and liver tissues. Although these defects are consistent with a role for C/EBPα in energy homeostasis, expression of several putative C/EBP target genes was normal (35). Thus, it has been difficult to identify genes that are C/EBPα targets in vivo.

Activator proteins like C/EBPα bind specific DNA sequences located either upstream or downstream of the core promoter. In response to physiological cues, activators stimulate transcription initiation by interacting with general transcription factors, with TATA-associated factors, or with adapter proteins (36, 37). In the case of C/EBPα, the bZIP domain mediates interaction with the basal transcription machinery, including Mediator and the general transcription factors (38, 39). In vitro, C/EBPα does not stimulate transcription initiation alone, but in the presence of Mediator, and the basal transcription machinery, C/EBPα stimulates transcription initiation with a high level of specificity for DNA sequences capable of forming a bZIP dimer. In vivo, the cDNA of C/EBPα is expected to induce tissue-specific expression of target genes.

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1The abbreviations used are: C/EBPα, CCAAT/enhancer-binding protein α; ATF-2, activating transcription factor-2; CRE, cyclic AMP response element-binding protein; C/EBP, cyclic AMP response element; bZIP, basic region leucine zipper; GST, glutathione S-transferase; RLNE, rat liver nuclear extract; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis.
37). The observation that C/EBPa binds a range of DNA sequences, coupled with the observation that its subunits exchange rapidly, suggests that the monomer may be a target for regulation. We screened a Agt11 expression cDNA library for proteins that physically interact with C/EBPa. Three independent cDNA clones encoding ATF-2, a bZIP protein, were isolated. We mapped the interacting protein domains to the respective leucine zippers, and demonstrated formation of a bZIP heterodimer with restricted DNA binding selectivity, in vitro. C/EBPa and ATF-2 are expressed in liver, and transient transfection analysis shows that co-transfected ATF-2 impacts C/EBPa function. Together with immunoprecipitation results showing that ATF-2 coprecipitates with C/EBPa, these results are consistent with the interpretation that functional ATF-2: C/EBPa heterodimers form in vivo.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids—pGEX-2T (Pharmacia, Uppsala Sweden) was modified by the insertion of a protein kinase A phosphorylation site as described (38). pMSV-C/EBPa1–24 (24) was used for construction of N-terminal GST-C/EBP fusions. Truncated constructs lacking the leucine zipper and the bZIP domain were prepared by placing in-frame stop codons at amino acids 310 and 272, respectively (39). Miu1 digestion and fill-in created the fusion to amino acid 192. Constructs 218–358 and 281–342 have been described (19). A diagram of the fusion proteins is shown (see Fig. 2A).

Our cDNA clones were identical to rat ATF-2 (GenBank accession M56148), with the exception of 294 nucleotides inserted at nucleotide 383 from the 5′ end. A full-length rat ATF-2 cDNA was generated by reverse transcriptase-polymerase chain reaction using the primer pair 5′-GGATCCATGAGTGTGACCAACACCCCTTTCTAGCA-3′ and 5′-ATCGATGCAGTTTTTTTATCTCAGAGG-3′. ATF-2 fusions to GST were prepared by polymerase chain reaction. The primers used to prepare 1–323 were 5′-GCGATCATGAGTGTGACCAACACCCCTTTCTAGCATGACGTTTTTTTATCTCAGAGG-3′ and 5′-GATGTCAGATTGCGCAATCTGCA-3′. The primer pairs for 323–492 were 5′-CAACCTACGTTTTATTTTTATTACGAAAGCCAGCATAAGT-3′ and 5′-CCAATGAGGAGAGAGGCAAGCTTATAAAGAAGACGGAGTGTGTGAATG-3′. The primer pairs for 323–492 and 5′-323–352 were 5′-GCGATGCATGAGTGTGACCAACACCCCTTTCTAGCATGACGTTTTTTTATCTCAGAGG-3′ and 5′-GCGATGGATCCATGAGTGTGACCAACACCCCTTTCTAGCATGACGTTTTTTTATCTCAGAGG-3′. The primer pairs for 323–492 and 5′-C/EBPa antisera were obtained from Dr. Rorth, and C/EBPa antisera do not cross-react against purified recombinant proteins.

Preparation of Antibodies—The ATF-2 cDNA (EcoRI) was subcloned into the vector pMal (Pharmacia, Uppsala, Sweden). Following the removal of lipids with Soroceal (CalBiochem, La Jolla, CA), clarified serum was passed 3 × over a 0.2-ml bed of MBP-ATF-2 equilibrated in phosphate-buffered saline. Column were washed with 10 ml of phosphate-buffered saline, and eluted with 750 μl of 0.1 M glycine (pH 3.0), dripping directly into 750 μl of 0.1 M NaHPO4 (pH 9.2) for neutralization. Affinity purified antibody was dialyzed into phosphate-buffered saline to remove glycin, and biotinylated as described (43). This protocol was adapted for purification and biotinylation of the antisera of intact C/EBPa, kindly provided by Pernille Rorth, and C/EBPa antisera do not cross-react against purified recombinant proteins.

Purification and Biotinylation of Antibodies—2.5 mg of MBP-ATF-2 was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Following the removal of lipids with Soroceal (CalBiochem, La Jolla, CA), clarified serum was passed 3 × over a 0.2-ml bed of MBP-ATF-2 equilibrated in phosphate-buffered saline. Column were washed with 10 ml of phosphate-buffered saline, and eluted with 750 μl of 0.1 M glycine (pH 3.0), dripping directly into 750 μl of 0.1 M NaHPO4 (pH 9.2) for neutralization. Affinity purified antibody was dialyzed into phosphate-buffered saline to remove glycin, and biotinylated as described (43).

Immunoblotting and Modified Western Blotting—Proteins from SDS-PAGE were electrophoblotted to Immobilon P membranes (0.45 μm, Millipore) and developed with polyclonal antisera. Secondary detection utilized horseradish peroxidase-conjugated donkey anti-rabbit serum (Amersham), which was visualized by chemiluminescence (ECL reagent, Amersham). For biotinylated antibodies, secondary detection utilized streptavidin-conjugated horseradish peroxidase (Amersham) and ECL reagent.

To map protein interaction domains, equal amounts of each truncated protein were separated on 10% SDS-PAGE gels, and transferred to nitrocellulose (0.45 μm, Schleicher & Schuell). For biotinylated antibodies, secondary detection utilized streptavidin-conjugated horseradish peroxidase (Amersham) and ECL reagent.

Affinity Chromatography—Equivalent amounts of MBP or MBP-ATF-2 were coupled to Sepharose 4B. 100-μl columns were equilibrated in Hyb150 (50 mM Tris (pH 8.0), 150 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 50% (v/v) glycerol, and 0.1% Nonidet P-40) and washed 2–3 times with 2-mercaptoethanol (0.1% in Hyb150) and 0.1 M glycine (pH 3.0). Columns were washed with 5% nonfat milk, and incubated in Hyb150 containing TGN14 (2 μg/ml), and Agt11 (2 μg/ml). After washing with 15 volumes of Hyb150, Protein was eluted stepwise with 1.8 volumes of buffer containing 0.25, 0.5, and 1.0 M KCl. Equivalent percentages of the unbound and eluted fractions were loaded onto 12% SDS-PAGE gels for Western blot analysis.

Gel Shift Analysis—Protein-DNA complexes were formed in standard TBE gels (42). Binding reactions (10 μl) were incubated at 37 °C in 10 mM Tris (pH 7.5), 1 mM dithiothreitol, 100 mM KCl, 1 mM EDTA, 10% glycerol, 1 mg/ml bovine serum albumin, and 0.05 μg/ml poly(dI-dC). Full-length C/EBP, ATF-2, and CREB were prepared by coupled in vitro transcription/translation (TNT, Promega, Madison, WI). The three DNA probes were radiolabeled to comparable specific activities. One strand of each probe is shown. Consensus C/EBP site: 5′-TGCAGATT-GCGCAATCTGCA-3′; CRE site: 5′-TGCAGATT-GCGCAATCTGCA-3′; from the jun promoter (44); chimeric site: 5′-TGCAGATT-GCGCAATCTGCA-3′. All reactions were performed in probe excess, but free probe was run off the gel to improve the resolution of heterodimeric complexes from homodimeric complexes.

Immunoprecipitation and Co-immunoprecipitation Analysis—Freshly prepared RLNE (45) was pre-cleared with an irrelevant antisum and protein A-agarose beads (Life Technologies). After a brief spin, supernatants were separated and adjusted to 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM MgCl2, 0.1% EDTA, 10% glycerol, 1 mg/ml bovine serum albumin, and 0.05 μg/ml poly(dI-dC). Full-length C/EBP, ATF-2, and CREB were prepared by coupled in vitro transcription/translation (TNT, Promega, Madison, WI). The three DNA probes were radiolabeled to comparable specific activities. One strand of each probe is shown. Consensus C/EBP site: 5′-TGCAGATT-GCGCAATCTGCA-3′; CRE site: 5′-TGCAGATT-GCGCAATCTGCA-3′; from the jun promoter (44); chimeric site: 5′-TGCAGATT-GCGCAATCTGCA-3′. All reactions were performed in probe excess, but free probe was run off the gel to improve the resolution of heterodimeric complexes from homodimeric complexes.
Association of ATF-2 with C/EBPα Requires the bZIP Domains—To map the domains required for interaction between C/EBPα and ATF-2, a sequential deletion strategy was used. C/EBPα deletion constructs were expressed in E. coli as GST fusion proteins, and purified on glutathione-agarose. Equivalent amounts of each protein were separated on SDS-PAGE gels and transferred to nitrocellulose. The blot was incubated with purified GST-ATF-2, washed extensively, and subsequently developed with anti-ATF-2 serum. As shown in Fig. 2A, GST-C/EBP constructs containing the leucine zipper bound soluble GST-ATF-2 efficiently (lanes 1, 4, 5, and 6). Analogously, truncated versions of ATF-2, similarly prepared as GST fusions, were tested for the ability to bind soluble C/EBPα (Fig. 2B). Again, only those constructs containing the leucine zipper efficiently retained soluble C/EBPα (lanes 1 and 3). The weak binding observed in the absence of the leucine zippers (Fig. 2, A and B, lanes 2) is likely due to the putative zinc finger motif in ATF-2 and will be addressed under “Discussion.” As controls, each blot (Fig. 2, A and B, respectively) was incubated with antiserum without prior exposure to soluble proteins, showing that the antisera for C/EBPα and ATF-2 are not cross-reactive (not shown). These results show that the leucine zippers are sufficient to mediate interaction between these bZIP proteins.

ATF-2/C/EBPα Heterodimers Bind DNA—Formation of an ATF-2/C/EBPα heterodimer would bring together different DNA-binding domains, which may lead to a change in DNA binding selectivity. To assay for DNA binding heterodimers, three DNA sites were tested in electrophoretic mobility shift assays (EMSA): a consensus C/EBP-binding site; a consensus CREB-binding site; and a chimeric site consisting of one C/EBP half-site directly abutted to one CRE half-site. To distinguish heterodimeric DNA binding complexes from homodimeric DNA binding complexes, a truncated C/EBPα protein encompassing the bZIP domain (19) was used. DNA binding complexes formed with one short C/EBPα subunit and one full-length C/EBPα, ATF-2, or CREB subunit migrate intermediate to DNA binding complexes composed of two full-length or two short subunits (homodimers), and demonstrate subunit exchange.

As shown in Fig. 3A, both full-length (lane 4) and short (lane 2) C/EBPα homodimers shift the consensus C/EBP-binding site. When full-length and short C/EBPα are mixed, a shift of intermediate migration is observed (lane 3), showing that subunits exchanged. ATF-2 homodimers do not shift this probe (lane 7), and no ATF-2/C/EBPα heterodimers are evident upon co-incubation with the short C/EBPα protein (lane 6). As expected, CREB homodimers bind the probe (lane 10), and no heterodimeric complex forms when CREB and C/EBPα short are mixed (lane 9).

When a radiolabeled CRE site is tested (Fig. 3B), a shift is observed with C/EBPα (lanes 2–4). When C/EBPα short and ATF-2 are mixed, it is predominantly ATF-2 homodimers that shift the probe (lanes 5–7). Although both homodimers bind, no heterodimeric DNA binding complex is observed (see lane 6). Analogous results are obtained when C/EBPα and CREB are mixed (lanes 8–10), where the predominant shift is by CREB homodimers, and no heterodimeric complex is detected (lane 9).

Upon EMSA analysis using the chimeric binding site (Fig. 3C), a gel shift complex of intermediate migration is observed (lane 6), which is consistent with formation of an ATF-2:C/EBPα heterodimer. In fact, C/EBPα, ATF-2, and CREB homodimers (lanes 4, 7, and 10, respectively) all bind the chimeric sequence. The failure of ATF-2:C/EBPα heterodimers to form a gel shift complex on the consensus C/EBP- and CREB-binding sites suggests that these heterodimers have a restricted DNA binding selectivity.

Fig. 1. An MBP-ATF2 affinity column binds C/EBPα from RLNE. Equivalent amounts of MBP-ATF2 or MBP alone were immobilized on maltose beads. 70 μg of rat liver nuclear extract was applied to each column, followed by extensive washing. Equivalent percentages of unbound and eluted protein fractions were separated on 10% SDS-PAGE, electroblotted, reacted with C/EBPα antibody, and developed with horseradish peroxidase-conjugated donkey anti-rabbit serum. The gel shift complexes were visualized by autoradiography. Each lane is from a single column, with 100 μl of elution buffer applied to each lane. The MBP-ATF2 affinity column contains only those complexes that bind to the C/EBPα epitope, whereas the MBP column contains all complexes that bind nonspecifically. The three bands observed between C/EBPα 42 and 29 kDa are internal translation initiation products that have been observed previously (47, 48).
ATF-2 Forms Heterodimers with C/EBPβ—Since C/EBP family proteins dimerize interchangeably, we tested ATF-2 for heterodimer formation with C/EBPβ (Fig. 4). As a control, we show that C/EBPα short and C/EBPβ form a heterodimeric DNA binding complex in the EMSA assay. When C/EBPβ was mixed with ATF-2, a heterodimeric complex formed on the chimeric binding site. Thus, the capability to form heterodimers with ATF-2 is a property that appears to be shared among C/EBP family proteins.

Co-transfection of ATF-2 with C/EBPα Results in Decreased Activation of a Minimal Promoter Driven by C/EBP-binding Sites—To analyze the impact of ATF-2 on C/EBPα function, we transfected the hepatoma cell line Fao with mammalian expression vectors encoding these proteins. Transcription was analyzed with a simplified luciferase reporter vector (2X C/EBP Luc) driven by the minimal thymidine kinase promoter and two copies of the C/EBP-binding site. Hepatoma cells transfected with ATF-2 alone showed basal levels of reporter gene activity (Fig. 5, left panel), consistent with the observation that ATF-2 does not bind the consensus C/EBP site. In contrast, C/EBPα transfectants showed a 7-fold increase in reporter gene activity. When ATF-2 and C/EBPα were co-transfected, activation levels decreased 43 to 55% (Fig. 5, left panel). These results are consistent with the interpretation that formation of ATF-2:C/EBPα heterodimers decreases the pool of C/EBPα homodimers available to bind the reporter construct, resulting in decreased transcription activity.

ATF-2 and C/EBPα Cooperate in the Activation of a Minimal Promoter Driven by Chimeric Binding Sites—The initial characterization of ATF-2 suggested that the protein lacked transactivation activity altogether (49–53). Subsequently, transactivation activity was demonstrated, but was subject to tight control (50, 51). To characterize transcription from the heterodimer binding site, we cloned 3 copies of the chimeric sequence upstream of the minimal thymidine kinase promoter (3X Chimera Luc). Surprisingly, Fao cells transfected with ATF-2 alone showed reporter activity that was 12–18-fold higher than the control (Fig. 5, right panel). Similarly, cells transfected with C/EBPα alone showed 5–7-fold activation of the reporter. When ATF-2 and C/EBPα were co-transfected, the reporter gene was activated approximately 26-fold (Fig. 5, right panel). First, these results show that ATF-2 activates transcription of the minimal promoter in Fao cells, which is surprising in that ATF-2 activity is repressed in many cell types. Second, these results are consistent with the interpretation that ATF-2 and C/EBPα form heterodimers that activate transcription from the chimeric sequence.

ATF-2 Is Expressed in the Liver and Co-Precipitates with C/EBPα—Although ATF-2 mRNA is found in essentially all tissues tested, the protein has been characterized mainly in brain and thymus. Using affinity purified ATF-2 antibodies, we found that ATF-2 is expressed in rat liver, apparently as a doublet of about 68 kDa (Fig. 6A, lanes 2 and 4). ATF-2 is also expressed in thymus, while expression is minimal in L cell and spleen cell nuclear extracts (not shown). As C/EBPα is also expressed in liver, we tested for co-precipitation of ATF-2 with C/EBPα. Freshly prepared rat liver nuclear extracts were subjected to immunoprecipitation with affinity purified C/EBPα antibodies. The 42- and 29-kDa forms of C/EBPα were precipitated by this reagent (Fig. 6A, lane 1). When this immuno-
cipitate was blotted for co-precipitating ATF-2 reactivity, the characteristic doublet at 68 kDa was observed (lane 3). The same result was obtained when C/EBPα was immunoprecipitated with a different antibody, one directed against the COOH-terminal portion of the protein (lane 5). These results are consistent with the interpretation that ATF-2 and C/EBPα can form heterodimers in vivo.

Although the half-life of a bZIP dimer is measured in seconds, binding to a consensus DNA sequence results in a 10–100-fold increase in the lifetime of the dimer (19). We reasoned that inclusion of a specific oligonucleotide-binding site during immunoprecipitation would stabilize heterodimers, facilitating their detection in the co-precipitation assay. The two binding sites we compared were the consensus C/EBP site and the chimeric site (see Fig. 3, A and C, for sequences). As shown previously, ATF-2 is readily detectable in RLNE (Fig. 6B, lane

![Figure 3](https://example.com/figure3.png)

**FIG. 3.** ATF-2:C/EBPα heterodimers bind DNA. A short C/EBPα protein containing only the bZIP domain (19) was mixed with full-length C/EBPα, ATF-2, or CREB in the presence of a radiolabeled DNA probe. A, a radiolabeled C/EBP-binding site; B, a radiolabeled CRE-binding site (Jun2); or C, a radiolabeled chimeric binding site was incubated with proteins as indicated below the figures. The formation of a gel shift complex with migration intermediate to the homodimers is interpreted to indicate heterodimer formation. A reticulocyte endogenous protein binds the CRE probe (Retic., B, arrow). The position of migration of all homodimers is indicated by arrows, as is the ATF2:C/EBPα short heterodimer (C, lane 6). Free probe was run off the gels to better resolve heterodimeric complexes from homodimeric complexes.
ATF-2 and C/EBPα Can Form Heterodimers

1). A control immunoprecipitation with irrelevant antiserum precipitates neither C/EBPα nor ATF-2 (lane 2). Significantly, inclusion of the chimeric binding site during precipitation with C/EBPα antibodies facilitates detection of ATF-2 as a co-precipitant (compare lane 3 to 4). When a C/EBP-binding site is included during immunoprecipitation, the efficiency of ATF-2 co-precipitation (lane 4) is diminished. These precipitates (lanes 3 and 4) show equivalent reactivity with C/EBPα antibodies (not shown), indicating that enhanced co-precipitation of ATF-2 in the presence of the chimeric binding site cannot be explained by a simple difference in the amount of C/EBPα precipitated. These results suggest that the subunits of ATF-2 and C/EBPα are in dynamic equilibrium, and that the composition of dimers is a reflection of the stoichiometry of the individual subunits.

![Fig. 4. ATF-2 forms heterodimers with C/EBPβ. A radiolabeled chimeric binding site was incubated with proteins as indicated below the figure. The ATF-2:C/EBPβ heteromeric shift is indicated by the arrow (lane 5). The C/EBPβ protein sample has a partial degradation product, which is indicated.](image)

**DISCUSSION**

To identify proteins that interact with C/EBPα, we screened an expression cDNA library with a radiolabeled GST-C/EBPα fusion protein. Of the four cDNAs we isolated, three encoded nuclear proteins and one was a novel gene product. Three independently isolated cDNAs encoded the bZIP transcription factor ATF-2. Neither GST-GCN4, a yeast bZIP protein, nor GST itself, interact with ATF-2 under these conditions, indicating that interaction between ATF-2 and C/EBPα is specific. The fact that we did not isolate any abundant cytoskeletal protein that possesses a coiled-coil motif is a further indication of the stringency of the screen.

Although the protein domains mediating association mapped to the respective leucine zippers (see Fig. 2, A and B), weak association of ATF-2 with an immobilized C/EBPα fusion protein containing the DNA-binding domain but lacking the leucine zipper was consistently observed (Fig. 2A, lane 2). Reciprocally, we observed weak association of soluble C/EBPα with immobilized ATF-2 constructs containing only the amino-terminal domain of the protein (Fig. 2B, lane 2). This is likely due to a putative zinc finger motif located near the amino terminus of ATF-2. This motif interacts with bZIP domains (54), and may be involved in negative autoregulation of ATF-2 function (55). Although it is possible that this motif augmented detection during our initial screen, it is clear that ATF-2 interacts with C/EBPα primarily by leucine zipper-mediated dimer formation.

ATF-2:C/EBPα heterodimers form a DNA binding complex with a target specificity that differs from the parental homodimers. The heterodimers do not bind symmetric DNA elements like consensus C/EBP and CRE sites. (see Fig. 3, A and B), but bind to an asymmetric sequence consisting of one consensus half-site for each monomer (Fig. 3C). This is likely that ATF-2:C/EBPα heterodimers do not bind the CRE sequence, considering that both parent homodimers bind this site (Fig. 3B). This suggests that heterodimer formation results in increased DNA binding selectivity, as C/EBPα homodimers bind all three sites, while ATF-2:C/EBPα heterodimers bind only one.

Oppositely charged side chains at specific positions in each helix of a leucine zipper form interhelical electrostatic contacts that stabilize the dimers (56). Comparison of the charges at appropriate positions within the C/EBPα and ATF-2 leucine zippers revealed the occurrence of potential stabilizing contacts. EMSA analysis revealed that such heterodimers can form (Fig. 4), and further suggest that ATF-2 subunits may be
When both ATF-2 and C/EBPα are co-transfected, reporter activity exceeds that observed with either transcription factor alone (Fig. 5, right panel). Taken with the results using the C/EBP site reporter, these findings are consistent with the interpretation that ATF-2:C/EBPα heterodimers form, and affect transcription activity.

Both ATF-2 and C/EBPα are expressed in liver. Using freshly prepared rat liver nuclear extracts, we showed that immunoprecipitates for C/EBPα react with affinity purified ATF-2 antibodies, and that the amount of ATF-2 co-immunoprecipitated could be enhanced or diminished in a predictable fashion by inclusion of specific DNA-binding sites. Together, the results of transient transfection and immunoprecipitation studies are consistent with the formation of ATF-2:C/EBPα heterodimers in vivo. The fact that ATF-2 can associate with C/EBPβ indicates that this association can probably be extended to other C/EBP family proteins. One functional consequence of this interaction is inhibition of transcriptional activity from consensus C/EBP target sites. It is also likely that heterodimers have a positive impact on transcription from promoters containing chimeric DNA elements. A search of primate promoter sequences in the data base using the 8-nucleotide chimeric DNA element indicates that the promoters of the protein disulfide isomerase, glutathione S-transferase, and ornithine decarboxylase genes are candidates for this kind of regulation.

Heterodimer formation is appealing from a regulatory viewpoint because of the asymmetry that is generated. By selecting for asymmetric DNA elements, heterodimers bind their target in an orientation dependent fashion, presenting distinct surfaces for interaction with proteins bound to adjacent DNA sites. Both the position and the orientation of protein-binding sites within some enhancer elements are important for the formation of what has been termed the “sterespecific” complex (57). Although the function of ATF-2:C/EBP heterodimers is not known, it has been proposed that cross-family heterodimers serve as a common target for the integration of signals arising from different extracellular stimuli. In this model, each subunit of the heterodimer would be modified by a unique protein kinase in response to only one of the signals being transduced. Since each subunit of the heterodimer is independently modified in this scenario, signals from two pathways converge, leading to the appropriate changes in the pattern of gene expression (51, 60, 61).

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