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

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We report on the identification of a nuclear protein that serves as a dominant-negative inhibitor of the transcription factors C/EBP and LAP. A ^{32}P-labeled LAP DNA-binding and dimerization domain "zipper probe" was used to isolate a clone that encodes a new C/EBP-homologous protein: CHOP-10. CHOP-10 has strong sequence similarity to C/EBP-like proteins within the bZIP region corresponding to the DNA-binding domain consisting of a leucine zipper and a basic region. Notably, however, CHOP-10 contains 2 prolines substituting for 2 residues in the basic region, critical for binding to DNA. Thus, heterodimers of CHOP-10 and C/EBP-like proteins are unable to bind their cognate DNA enhancer element. CHOP-10 mRNA is expressed in many different rat tissues. Antisera raised against CHOP-10 recognize a nuclear protein with an apparent molecular mass of 29 kD. CHOP-10 is induced upon differentiation of 3T3-L1 fibroblasts to adipocytes, and cytokine-induced dedifferentiation of adipocytes is preceded by the loss of nuclear CHOP-10. Coimmunoprecipitation of CHOP-10 and LAP from transfected COS-1 cells demonstrated a direct interaction between the two proteins, in vivo. Consistent with the structure of its defective basic region, bacterially expressed CHOP-10 inhibits the DNA-binding activity of C/EBP and LAP by forming heterodimers that cannot bind DNA. In transfected HepG2 cells, expression of CHOP-10 attenuates activation of C/EBP- and LAP-driven promoters. We suggest that CHOP-10 is a negative modulator of the activity of C/EBP-like proteins in certain terminally differentiated cells, similar to the regulatory function of Id on the activity of MyoD and MyoD-related proteins important in the development of muscle cells.

[*Key Words*: CHOP-10; nuclear protein; dominant-negative inhibitor; transcription factors]

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The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors play an important role in regulating the expression of various genes. To date, at least five distinct members of the C/EBP-like family of DNA-binding proteins have been identified (Cao et al. 1991; Williams et al. 1991). All contain a conserved carboxy-terminal domain (the bZIP) consisting of a basic region involved in DNA recognition and an adjacent helical structure, the leucine zipper, that mediates subunit dimerization. The restricted nature of the subunit interactions defines the extent of the C/EBP-like family of bZIP proteins. All known members of the C/EBP family bind similar DNA sequences in vitro and activate adjacent promoters in vivo. C/EBP-like proteins are expressed in a cell type-restricted manner and during differentiation of 3T3-L1 fibroblasts to adipocytes (Birkenmeier et al. 1989; Cao et al. 1991).

Conserved *cis*-acting DNA sequences, implicated in cytokine-induced regulation of gene expression, have been used as probes to isolate, by direct expression cloning, a new member of the C/EBP family of transcription factors. Variably referred to as liver-enriched transcriptional activator protein (LAP) (Descombes et al. 1990), NFIL-6 (Akira et al. 1990), IL-6DBP (Poli et al. 1990), and AGP/EBP (Chang et al. 1990), the DNA-binding activity of this protein was found to increase upon cytokine stimulation of responsive cells in culture (Akira et al. 1990;

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Poli et al. 1990). The LAP gene encodes two partially colinear proteins: LAP, an activator of C/EBP sites, and LIP, a competitive inhibitor of activation (Descombes and Schibler 1991). These studies implicate C/EBP-like proteins in mediating both positive and negative cytokine-induced regulation of gene transcription.

C/EBP-like proteins play an important role in regulating the activity of the angiotensinogen gene. These proteins bind to a cytokine-responsive enhancer in the 5′-flanking region of the gene [the acute-phase responsive element (APRE); Brasier et al. 1990a,b; Ron et al. 1990a,b]. C/EBP-like proteins and the cytokine-inducible transcription factor, nuclear factor κB (NF-κB), compete for binding to the APRE, and both classes of proteins mediate cytokine-induced modulation of the activity of the angiotensinogen promoter (Brasier et al. 1990b; Ron et al. 1992).

Treatment of 3T3-L1 adipocytes with cytokines such as tumor necrosis factor (TNF) leads to a significant change in the mobility of the complex of C/EBP-like proteins that bind the angiotensinogen gene APRE. This alteration in mobility is due in part to a reciprocal change in the levels of nuclear C/EBP and LAP induced by TNF (Ron et al. 1992). Southwestern binding assays identified C/EBP and LAP as the major APRE-binding proteins in adipocyte nuclei (Ron et al. 1992). Changes, however, in nuclear levels of C/EBP and LAP DNA-binding activity do not explain fully the spectrum of cytokine-induced changes in the C/EBP-like complex or the observed alteration in functional activity of the APRE. For example, it is difficult to reconcile the observed TNF-induced replacement of C/EBP by LAP with the simultaneously measured increase in APRE transcriptional activity (Ron et al. 1992), when in transfection assays involving cloned recombinant proteins, C/EBP is a more potent *trans*-activator than LAP (Descombes et al. 1990; Poli et al. 1990; D. Ron, and J.F. Habener, unpubl.).

We therefore sought to identify other proteins that might contribute to the activity of the DNA-binding C/EBP-like complex by dimerizing with C/EBP or LAP, without being able to bind the APRE directly as homodimers or even as heterodimers (at least as reflected in the Southwestern blot assays). To isolate cDNA clones that encode such proteins, we modified a detection method that relies on use of the labeled dimerization domain of one protein to detect its immobilized dimerizable partner in a zipper–blot assay (Macgregor et al. 1990; Hoeffler et al. 1991). In this paper we describe the utilization of the ^{32}P-labeled dimerization domain of LAP to isolate CHOP-10, a novel inhibitor of C/EBP-like proteins. We report on characterization of CHOP-10 with respect to its spectrum of action, tissue distribution, and subcellular localization and show that it is a dominant-negative inhibitor of C/EBP and LAP by virtue of forming stable dimers that are rendered incapable of binding to their cognate DNA enhancer elements. Thus, CHOP-10 functions as an inhibitor of the C/EBP-like proteins much in the way that Id serves as an inhibitor of MyoD, a transcriptional activator protein involved in muscle cell development (Benezra et al. 1990).

Results

Specific detection of dimerizing proteins in a zipper–blot

Radiolabeling of proteins by tyrosine iodination with ^{131}I has the potential drawback of structural modification imposed by the harsh oxidizing conditions or the iodotyrosine residues themselves. To avoid these potential difficulties we relied on the ability of purified protein kinase A (PK-A) to specifically phosphorylate bacterially expressed proteins on high-affinity PK-A sites, as a means of creating a labeled protein probe. Chimeric, bacterially expressed proteins consisting of the high-affinity PK-A site of the transcription factor cAMP-responsive element binding (CREB) (Gonzales et al. 1989; Lee et al. 1990) fused to the DNA-binding and dimerization domain of C/EBP or LAP were phosphorylated in vitro with [γ-^{32}P]ATP by purified PK-A. After removal of the unincorporated [γ-^{32}P]ATP by gel filtration, the labeled proteins were used as probes to detect immobilized dimerizing target proteins in a nitrocellulose zipper–blot assay (Fig. 1A) . Both the LAP and C/EBP probes recognized bacterially expressed full-length C/EBP and LAP but not the structurally related CREB, a protein that contains a leucine zipper of the activating transcription factor (ATF)/CREB family (Hai et al. 1989). Both probes recognize essentially indistinguishable sets of proteins from a rat liver nuclear extract in the zipper–blot assay (Fig. 1B). The C/EBP probe binds less well to bacterially expressed C/EBP than to LAP (twofold difference by quantitative densitometry), whereas LAP binds to C/EBP and to itself indistinguishably. The LAP probe was therefore chosen for further study.

Binding of LAP to proteins from rat liver nuclear extract was specifically competed by unlabeled LAP (homologous competition) but not by a similar excess of unlabeled bacterially expressed CREB (Fig. 1C). Because the bacterially expressed DNA-binding domain of CREB does not heterodimerize in solution with C/EBP or LAP (D. Ron and J.F. Habener, unpubl.) we concluded that, by this criteria for specificity of dimerization, the zipper probe preferentially recognizes specifically dimerizing species.

Isolation of a cDNA clone that encodes a novel protein capable of binding C/EBP-like proteins

Using the ^{32}P-labeled LAP probe, we screened a λ Zap (Stratagene, La Jolla, CA) 3T3-L1 adipocyte cDNA expression library for recombinant clones that encode proteins capable of specifically binding to the probe. Positive clones, referred to as CHOPs (C/EBP homologous proteins), were subsequently tested for their ability to encode an inducible protein that would bind the APRE directly in a Southwestern assay. By this criteria we hoped to avoid already characterized C/EBP-like proteins, all of which are presumed to be capable of binding the APRE. Four distinct clones, all of which overlapped in their carboxy-terminal sequence, were found to en-

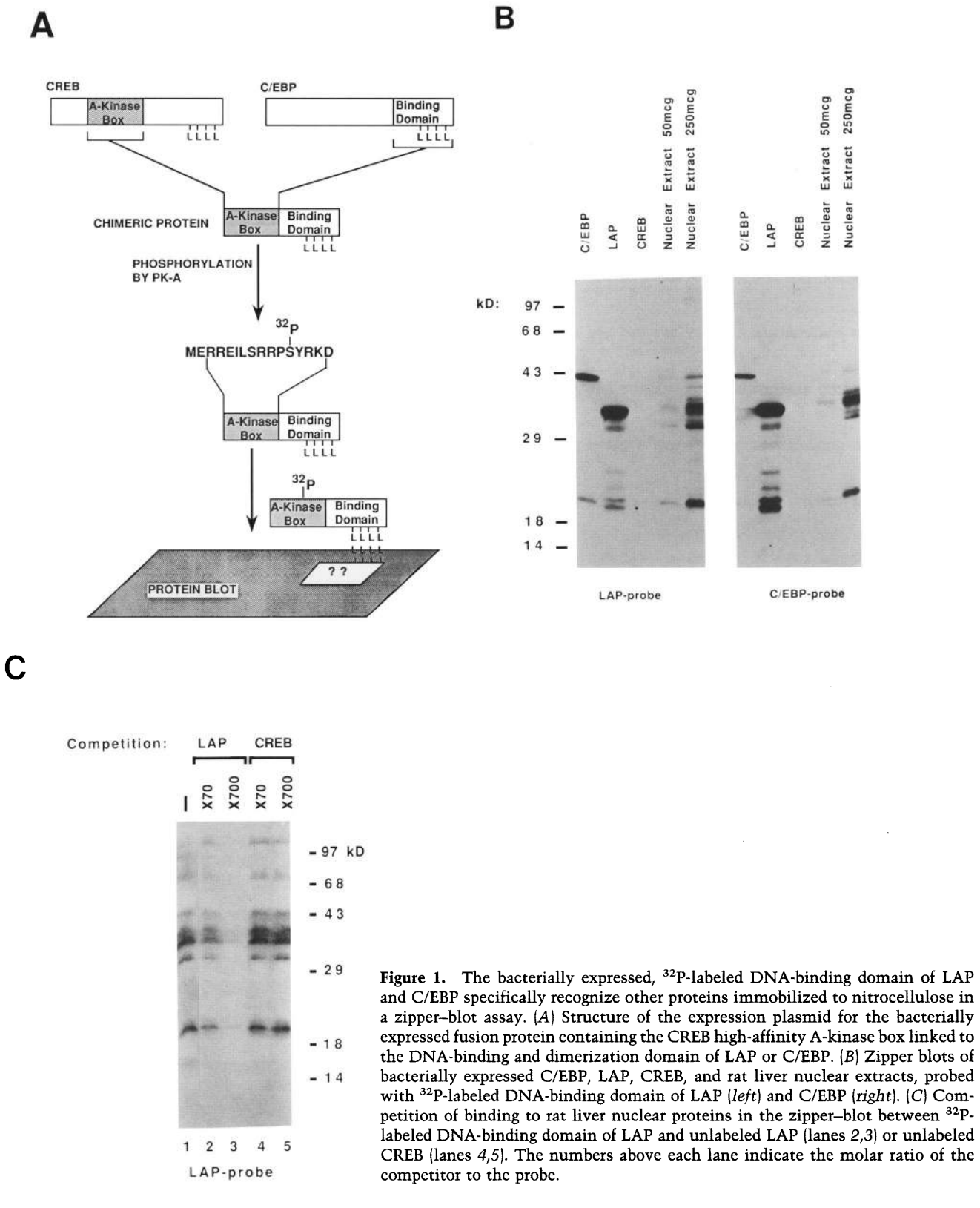

Figure 1. The bacterially expressed, ^{32}P-labeled DNA-binding domain of LAP and C/EBP specifically recognize other proteins immobilized to nitrocellulose in a zipper–blot assay. (*A*) Structure of the expression plasmid for the bacterially expressed fusion protein containing the CREB high-affinity A-kinase box linked to the DNA-binding and dimerization domain of LAP or C/EBP. (*B*) Zipper blots of bacterially expressed C/EBP, LAP, CREB, and rat liver nuclear extracts, probed with ^{32}P-labeled DNA-binding domain of LAP (*left*) and C/EBP (*right*). (*C*) Competition of binding to rat liver nuclear proteins in the zipper–blot between ^{32}P-labeled DNA-binding domain of LAP and unlabeled LAP (lanes 2,3) or unlabeled CREB (lanes 4,5). The numbers above each lane indicate the molar ratio of the competitor to the probe.

code a protein that bound the LAP and C/EBP probe avidly but failed to bind the APRE on a Southwestern blot. Sequencing of both strands of the largest insert (from clone CHOP-10) revealed an uninterrupted open reading frame (ORF) of 168 amino acids that was in-frame with the β-galactosidase gene of the cloning vector (Fig. 2A).

The carboxyl terminus of the predicted protein contained a region, present in all four overlapping clones,

A

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                                        30                                  60
                                        *                                   *
CTG ACG TGT TCC AGA AGG AAG TGC ATC TTC ATA CAC CAC CAC ACC TGA AAG CAG AAC CTG GTC CAC GTG CAG TC

                    90                                  120
                    *                                   *
ATG GCA GCT GAG TCC CTG CCT TTC ACC TTG GAG ACG GTG TCC AGC TGG GAG CTG GAA GCC TGG TAT GAG GAT CTG
 M   A   A   E   S   L   P   F   T   L   E   T   V   S   S   W   E   L   E   A   W   Y   E   D   L>
150                                 180                                 210
*                                   *                                   *
CAG GAG GTC CTG TCC TCA GAT GAA ATT GGG GGC ACC TAT ATC TCA TCC CCA GGA AAC GAA GAG GAA GAA TCA AAA
 Q   E   V   L   S   S   D   E   I   G   G   T   Y   I   S   S   P   G   N   E   E   E   E   S   K>
                    240                                 270
                    *                                   *
ACC TTC ACT ACT CTT GAC CCT GCG TCC CTA GCT TGG CTG ACA GAG GAG CCA GGG CCA ACA GAG GTC ACA CGC ACA
 T   F   T   T   L   D   P   A   S   L   A   W   L   T   E   E   P   G   P   T   E   V   T   R   T>
300                                 330                                 360
*                                   *                                   *
TCC CAA AGC CCT CGC TCT CCA GAT TCC AGT CAG AGT TCT ATG GCC CAG GAG GAA GAG GAG GAA GAG CAA GGA AGA
 S   Q   S   P   R   S   P   D   S   S   Q   S   S   M   A   Q   E   E   E   E   E   E   Q   G   R>
                    390                                 420
                    *                                   *
ACT AGG AAA CGG AAA CAG AGT GGT CAG TGC CCA GCC CGG CCT GGG AAG CAA CGC ATG AAG GAG AAG GAG CAG GAG
 T   R   K   R   K   Q   S   G   Q   C   P   A   R   P   G   K   Q   R   M   K   E   K   E   Q   E>
450                                 480                                 510
*                                   *                                   *
AAC GAG CGG AAA GTG GCA CAG CTA GCT GAA GAG AAC GAG CGG CTC AAG CAG GAA ATC GAG CGC CTG ACC AGG GAG
 N   E   R   K   V   A   Q   L   A   E   E   N   E   R   L   K   Q   E   I   E   R   L   T   R   E>
                    540                                 570
                    *                                   *
GTG GAG ACC ACA CGG CGG GCT CTG ATC GAC CGC ATG GTC AGC CTG CAC CAA GCA TGA A CAG TGG GCA TCA CCT
 V   E   T   T   R   R   A   L   I   D   R   M   V   S   L   H   Q   A
600                                 630                                 660
  *                                 *                                   *
CCT GTC TGT CTC TCC GGA AGT GTA CCC AGC ACC ATC GCG CCA GCG CCA AGC ATG TGA CCC TGC ACT GCA CTG CAC

                      690                                 720
                      *                                   *
ATG CTG AGG AGG GGA CTG AGG GTA GAC CAG GAG AGG GCT CGG CTT GCA CAT AGA CGG TAC ATT GTT TAT TAC TGT

750                                 780
  *                                 *
CCA TGT CCA GTA AAG TGA CTT TGT GTC AAA AAA AA
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B

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                                    *     *     *
CHOP-10                 R T R K R K Q S G Q C P A R P G K Q R M K E K E Q E N E R K V A Q
C/EBP α (C/EBP)         E Y R V R R E R N N I A V R K S R D K A K Q R N V E T Q Q K V V E
C/EBP β (LAP)           E Y K M R R E R N N I A V R K S R D K A K M R N L E T Q H K V L E
C/EBP γ (IgEBP-1)       E Y R Q R R E R N N M A V K K S R L K S K Q K A Q D T L Q R V N Q
C/EBP δ (CRP3)          E Y R Q R R E R N N I A V R K S R D K A K R R M Q E M Q Q K L V E
                                B B - B N - - A A - B - R - B

CHOP-10                 L A E E N E R L K Q E I E R L T R E V E T T R R A L I D R M V S L H Q A
C/EBP α (C/EBP)         L T S D N D R L R K R V E Q L S R E L D T L R G I F R K L P E S S L V K
C/EBP β (LAP)           L T A E N E R L Q K K V E Q L S R E L S T L R N L F K Q L P E P L L A S
C/EBP γ (IgEBP-1)       L K E E N E R L E A K I K L L T R E L S V L K D L F L E M A H S L A A N
C/EBP δ (CRP3)          L S A E N E K L H Q R V E Q L T R D L A G L R Q F F K K L P S P P F L P
                        •           •           •           •
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C

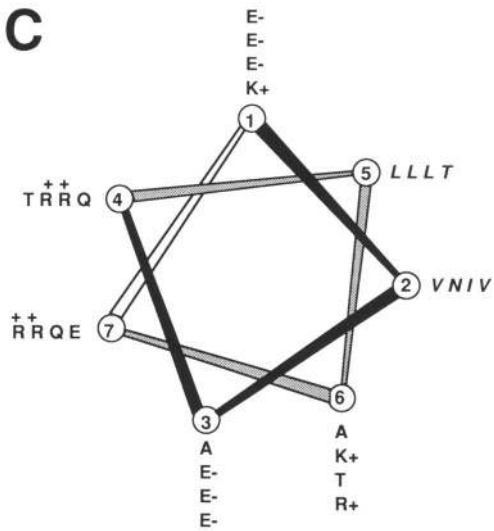

Figure 2. CHOP-10 cDNA and predicted protein sequence. (*A*) Nucleotide sequence of the LAP-binding CHOP-10 cDNA clone and predicted amino acid sequence of the encoded protein. (*B*) Alignment of the carboxyl-terminus of the predicted sequence of CHOP-10 with that of other C/EBP-like proteins. Regions of functionally conserved residues are shaded. The residues comprising the putative leucine zipper are indicated by dots beneath the sequence. The asparagine, aliphatic, and basic residues conserved in other C/EBP family members, but divergent in CHOP-10, are indicated by the asterisks (*) above the sequence. (*C*) "Helical wheel" analysis of the predicted CHOP-10 protein sequence. Residues comprising the hydrophobic face of the putative amphipathic α-helix are italicized (positions 2 and 5 on the wheel).

that exhibited marked sequence similarity to the dimerization surface (the leucine zipper) of previously cloned C/EBP-like proteins (Fig. 2B). This region of CHOP-10 would be predicted to form an amphipathic α-helix (coiled coil), the hydrophobic surface of which would contain one spoke (position 5) composed of a heptad repeat of leucines (Fig. 2C). Notably, position 2 contains the hydrophobic residues valine and isoleucine highly favored in this position of coiled coils, as well as an asparagine found in the leucine zipper of yeast transcription factor GCN4 and believed to be involved in controlling the formation of dimers (O'Shea et al. 1991). In addition, positions 1 and 4 contain polar residues of opposing charges important in the formation of helix-stabilizing ionic bridges (O'Shea et al. 1991). Amino-terminal to this region of the coiled coil the sequence similarity between CHOP-10 and other C/EBP-like proteins weakens, with potentially important deviations in the region corresponding to the DNA-contacting basic region of other C/EBP-like proteins. A glycine residue (108) replaces the invariant asparagine common to all C/EBP-like proteins, and two prolines (111 and 113) substitute for a highly conserved aliphatic residue and a basic residue in the potentially helical, basic region B, present in all bZIP proteins (Vinson et al. 1989). Mutations in this region of C/EBP have been shown to interfere with the capability of the protein to bind DNA (Landschulz et al. 1989). Thus, CHOP-10 would be predicted to encode a protein with functional properties similar to basic region mutants of C/EBP, competent to dimerize but not to bind DNA.

The CHOP-10 insert was labeled and used as a probe to detect hybridizing transcripts in a Northern blot of poly(A)$^+$ RNA from various rat tissues. All tissues tested contained a 1.1-kb transcript, the abundance of which varied by up to fivefold between different tissues (Fig. 3A).

Because other C/EBP-like proteins have been shown to be induced during differentiation of 3T3-L1 fibroblasts to adipocytes, and because we wanted to know whether CHOP-10 might play a role in regulated expression of adipocyte genes during cytokine treatment, we performed Northern blot analysis on RNA from differentiating 3T3-L1 cells. Attainment of the transition from fibroblastic to adipocytic morphology on day 5 correlated with the appearance of detectable CHOP-10 mRNA (Fig. 3B). The significance of the relationship between the adipocytic phenotype and CHOP-10 expression is underscored by the observation that CHOP-10 protein is decreased in the nuclei of 3T3-L1 adipocytes during cy-

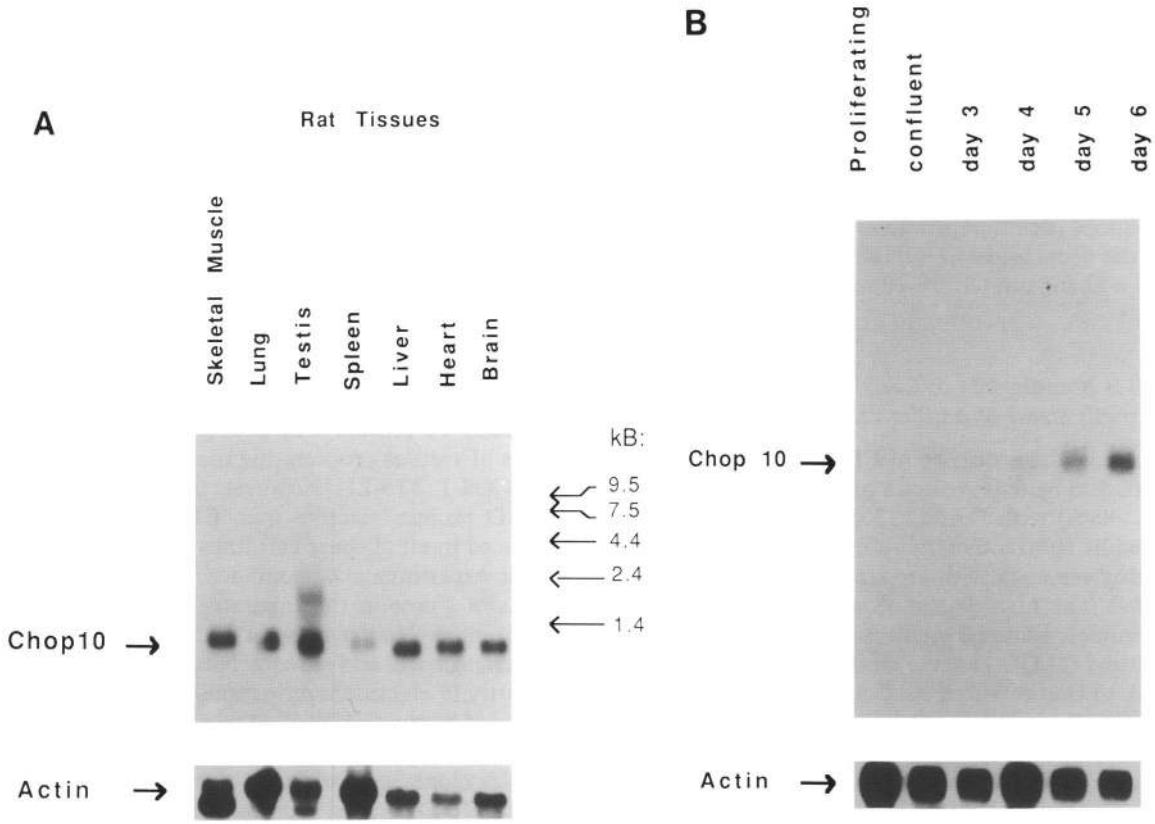

Figure 3. CHOP-10 mRNA is present in many rat tissues and is induced during adipocytic differentiation of 3T3-L1 cells. (*A*) Northern blot analysis of 10 μg of poly(A)$^+$ RNA from different adult rat tissues hybridized to the ^{32}P-labeled CHOP-10 cDNA insert (*top*) or to an actin probe (*bottom*). (*B*) Northern blot analysis of 30 μg of total cellular RNA from 3T3-L1 cells at different time points of the differentiation process. The blot was hybridized to the ^{32}P-labeled CHOP-10 cDNA insert (*top*) or to an actin probe (*bottom*). Morphological differentiation of the cells was complete by day 5.

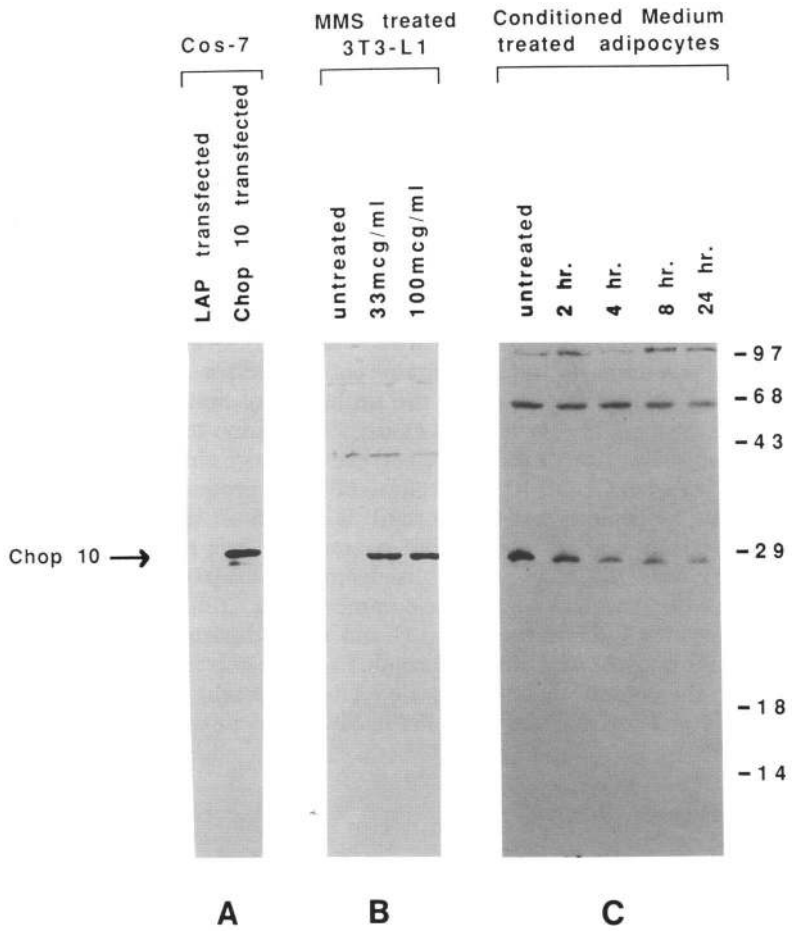

Figure 4. CHOP-10 protein is induced by DNA-alkylating agent treatment of proliferating fibroblasts and is repressed during cytokine mediated dedifferentiation of adipocytes. (*Left*) Anti-CHOP-10 Western immunoblot of 25 μg/lane whole-cell lysates from COS-1 cells transfected with either a control expression plasmid (LAP) or CHOP-10 expression plasmid. (*Middle*) Anti-CHOP-10 Western immunoblot of proliferating 3T3-L1 fibroblasts, treated for 4 hr with the indicated concentration of the DNA-alkylating agent MMS. (*Right*) Anti-CHOP-10 Western immunoblot of 100 μg/lane nuclear extract of fully differentiated 3T3-L1 adipocytes treated, for the indicated period of time, with 0.35% lipopolysaccharide-induced Raw 264.7 monocytic cell-line conditioned medium. Morphological evidence of dedifferentiation was first apparent by 16 hr.

tokine-induced dedifferentiation (see Fig. 4C). Interestingly, the decrease in CHOP-10 protein is not associated with a change in CHOP-10 mRNA levels (data not shown).

CHOP-10 is homologous to GADD 153 and is induced during growth arrest of proliferating cells

Comparison of the sequence of CHOP-10 against existing protein data banks revealed a high degree of sequence identity (>85%) with GADD 153, a hamster gene that was cloned by subtractive hybridization of mRNA from proliferating versus growth-arrested CHO cells (Fornace et al. 1989). The high degree of sequence similarity of both the nucleic acid and predicted protein sequences of CHOP-10 and GADD 153, the similar size of the CHOP-10 mRNA to that reported for GADD 153, and the fact that the CHOP-10 cDNA insert probe detected a single-copy gene on Southern blot analysis of mouse genomic DNA (data not shown) all suggest that CHOP-10 may be the murine analog of GADD 153.

Growth arrest of proliferating 3T3-L1 cells by treatment with the DNA-alkylating agent methylmethane sulfate (MMS, 100 μg/ml) led to the induction of a protein with an apparent molecular mass of 29 kD that reacted strongly in a Western immunoblot with antiserum raised in rabbit against a purified bacterially expressed fusion protein of *S. japonicum* glutathione *S*-transferase (GST) and CHOP-10. The MMS-induced endogenous CHOP-10 of 3T3-L1 cells and the protein induced upon their terminal differentiation to adipocytes were identical in size to the protein encoded by a CHOP-10 cDNA expression plasmid when transfected into COS-1 cells (Fig. 4).

CHOP-10 protein was undetectable in whole-cell lysates of various proliferating mammalian cell lines (PC-12, COS-1, 3T3-L1). However, upon MMS treatment, a 29-kD protein reactive with CHOP-10 antiserum was induced in all of these cell lines (data not shown). From these experiments we conclude that the CHOP-10 gene encodes a protein that migrates on SDS-PAGE with an apparent molecular mass of 29 kD and is conserved between primates and rodents. Furthermore, because of the identity in electrophoretic mobility between the protein encoded by the CHOP-10 cDNA expression plasmid and the endogenous cellular protein, we concluded that the cDNA clone we had isolated contains the full-length CHOP-10 ORF. The reasons for the anomalous migration of the CHOP-10 protein on SDS-PAGE (the predicted mass of CHOP-10, based on its predicted amino acid sequence, is only 19 kD) is not known. The anomalous migration is an inherent feature of the protein, whether expressed in animal or bacterial cells, and is

present regardless of whether the putative stop codon is contributed by the endogenous sequence or by sequences artificially introduced during construction of various expression vectors (see below). These findings imply that neither post-translational events specific to animal cells nor any novel readthrough mechanism unique to the region surrounding the stop codon are responsible for this anomalous migration.

CHOP-10 associates with C/EBP-like proteins in the cell nucleus in vivo

CHOP-10 cDNA was isolated based on the encodement (in bacteria) of a protein that binds to LAP in vitro. Therefore, we sought to determine whether an interaction between the two proteins also occurs in vivo. C/EBP and LAP are both localized to the nucleus and as a first step, we determined whether or not CHOP-10 is present in the nucleus. COS-1 cells, transfected with a CHOP-10 expression plasmid or with a LAP expression vector as a negative control (the anti-CHOP-10 antisera does not recognize LAP protein on Western immunoblot or on immunoprecipitation), were reacted with CHOP-10 antisera or with preimmune serum and then stained with a fluorescent second antibody. A fluorescent signal was detected only in the CHOP-10-transfected cells and only with the CHOP-10 antiserum. In a substantial proportion of the cells the staining is nuclear (Fig. 5). A subpopulation of cells exists in which the staining is diffuse throughout both nucleus and cytoplasm (Fig. 5, lower left), the significance of this observation is not known. Similar results were obtained by performing immunocytochemistry with an avidin–biotin HRP-conjugated second antiserum (Fig 5, lower right). Staining of the LAP-transfected cells with LAP antiserum demonstrated a predominant nuclear localization of the LAP signal (data not shown). Thus, CHOP-10 is found, colocalized in the nucleus, with LAP.

To investigate the possibility of a direct physical interaction between CHOP-10 and LAP, COS-1 cells were transfected with expression plasmids for the two proteins. In vivo ^{35}S-labeled nuclear proteins were immunoprecipitated with antisera directed against the two proteins. Anti-CHOP-10 serum brings down a 29-kD protein present predominantly in the CHOP-10-transfected cells (Fig. 6, lane 2). Trace amounts of the same protein are immunoprecipitated from the LAP-transfected cells (lane 4), presumably reflecting low levels of endogenous CHOP-10 in the COS cells. The LAP antiserum specifically immunoprecipitates a 36-kD protein present in the LAP-transfected cells (lane 3) but not in the CHOP-10-transfected cells (lane 1). The differences in the intensity of the signal between CHOP-10 and LAP may be due to differences in the extent of labeling of the two proteins (CHOP-10 has 3 methionines and 2 cysteines, whereas LAP has 4 methionines and 10 cysteines). Differences, however, in levels of expression of

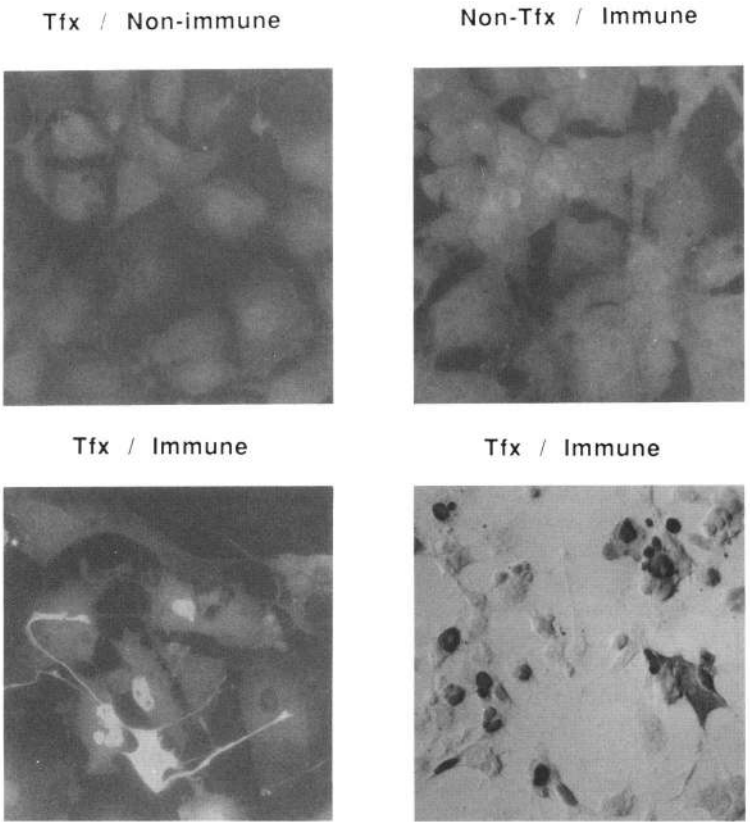

Figure 5. CHOP-10 immunoreactivity is present in the nucleus of transfected COS-1 cells. COS-1 cells transfected with a CHOP-10 expression plasmid were reacted with preimmune serum at a dilution of 1 : 500 (*top left*) or anti-CHOP-10 antiserum at a dilution of 1 : 1000 (*bottom left*) and stained with a fluorescein-conjugated second antibody. The fluorescent material is brightly illuminated against the dark background of the unstained cells. Control cells transfected with a LAP expression plasmid and stained with anti-CHOP-10 antiserum show no reactivity (*top right*). CHOP-10-transfected cells reacted with anti-CHOP-10 antiserum and stained with a biotinylated secondary antibody avidin–biotinylated HRP conjugate are shown in low power with Nomarski optics. The HRP-induced color reaction appears dark against the background of unstained cells (*bottom right*).

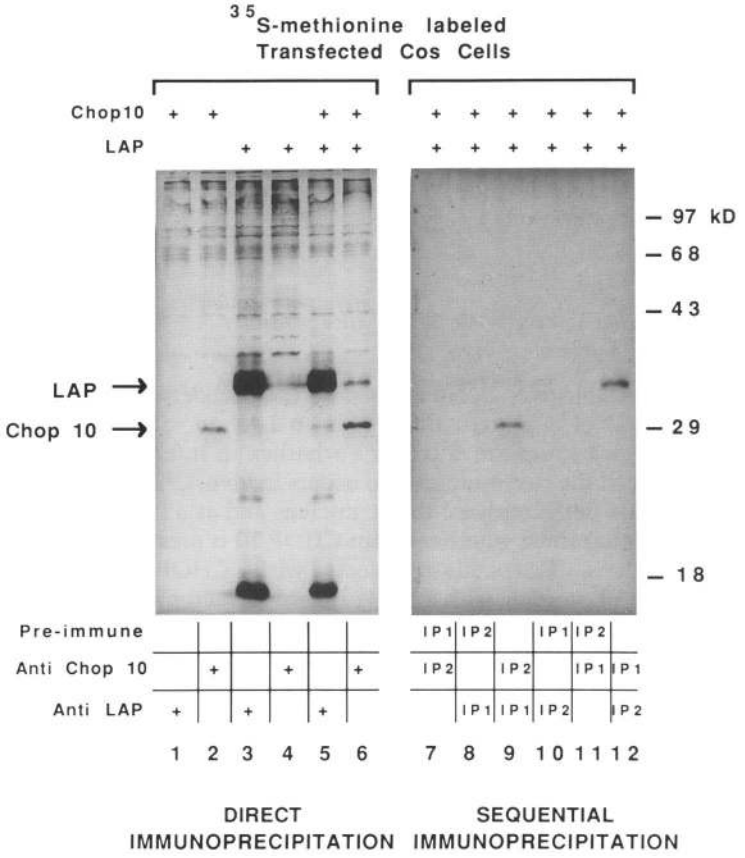

Figure 6. CHOP-10 coimmunoprecipitates with LAP from transfected COS cell lysates. (*Left*) [^{35}S]Methionine-labeled nuclear proteins from COS cells transfected with CHOP-10, LAP, and both expression plasmids were immunoprecipitated with antisera specific to the two proteins. The immunocomplexes were resolved by 12% SDS-PAGE. The positions of migration of LAP and CHOP-10 are indicated by arrows at *left*. (*Right*) [^{35}S]Methionine-labeled nuclear proteins from COS cells cotransfected with LAP and CHOP-10 expression plasmids were sequentially immunoprecipitated, first with one antiserum (IP1) and, following disruption of the immune complex with SDS and DTT, with the second antiserum (IP2). The antiserum used in each step is indicated in the table below the autoradiograph.

LAP and CHOP-10 or affinity of the respective antisera cannot be excluded.

In COS-1 cells cotransfected with both CHOP-10 and LAP, either antiserum immunoprecipitates proteins of both sizes (Fig. 6, lanes 5,6). When nuclear extracts prepared from cells transfected with either LAP or CHOP-10 were mixed and immunoprecipitated with either antiserum, only the homologous protein was immunoprecipitated (data not shown), implying that association of the two proteins occurs in vivo and not as an artifact of the preparation of lysates.

To demonstrate more rigorously an in vivo interaction between CHOP-10 and LAP, labeled nuclear lysates from COS-1 cells cotransfected with both expression vectors were sequentially immunoprecipitated with antisera specific to the two proteins. The first immunoprecipitation, with an antiserum specific to one protein, was performed under mild conditions that would favor the preservation of a putative CHOP-10–LAP complex. Following dissociation of the immune complex with 1% SDS and reducing conditions, the second immunoprecipitation, with an antiserum to the other protein, was performed under conditions that preclude reassociation of CHOP-10 and LAP (RIPA buffer). A similar procedure has been used to demonstrate a physical interaction in vivo between helix–loop–helix proteins (Lassar et al. 1991). Immunoprecipitation with anti-LAP in the first step and anti-CHOP-10 in the second step brought down a labeled 29-kD protein (Fig. 6, lane 9). When the order of antisera addition was reversed, a 36-kD protein, the size of LAP, was brought down (lane 12).

CHOP-10 specifically inhibits the DNA-binding activity of C/EBP and LAP in vitro and blocks transcriptional activation of a reporter gene in vivo

Having demonstrated that CHOP-10 is found in the nucleus and that it can be physically associated with a C/EBP-like protein, it was of interest to examine the functional consequences of this interaction in terms of the DNA-binding characteristics of the CHOP-10–C/EBP complex. CHOP-10 and a mutated version of CHOP-10 (CHOP-10–LZ$^-$), in which the leucine zipper-containing region had been replaced by unrelated plasmid-encoded sequence, were expressed in *Escherichia coli* as GST fusion proteins and purified by affinity chromatography on glutathione–agarose.

Purified, bacterially expressed GST–CHOP-10 fusion protein was added to purified, bacterially expressed C/EBP or LAP fusion protein (50 ng), and binding to the APRE was assayed by means of an electrophoretic mobility-shift assay (EMSA). Increasing amounts of CHOP-10 (50–500 ng) led to a progressive inhibition of DNA-binding activity of C/EBP and LAP (Fig. 7A). Similar results were obtained with a GST fusion protein consisting only of the carboxyl terminus of CHOP-10 and with a

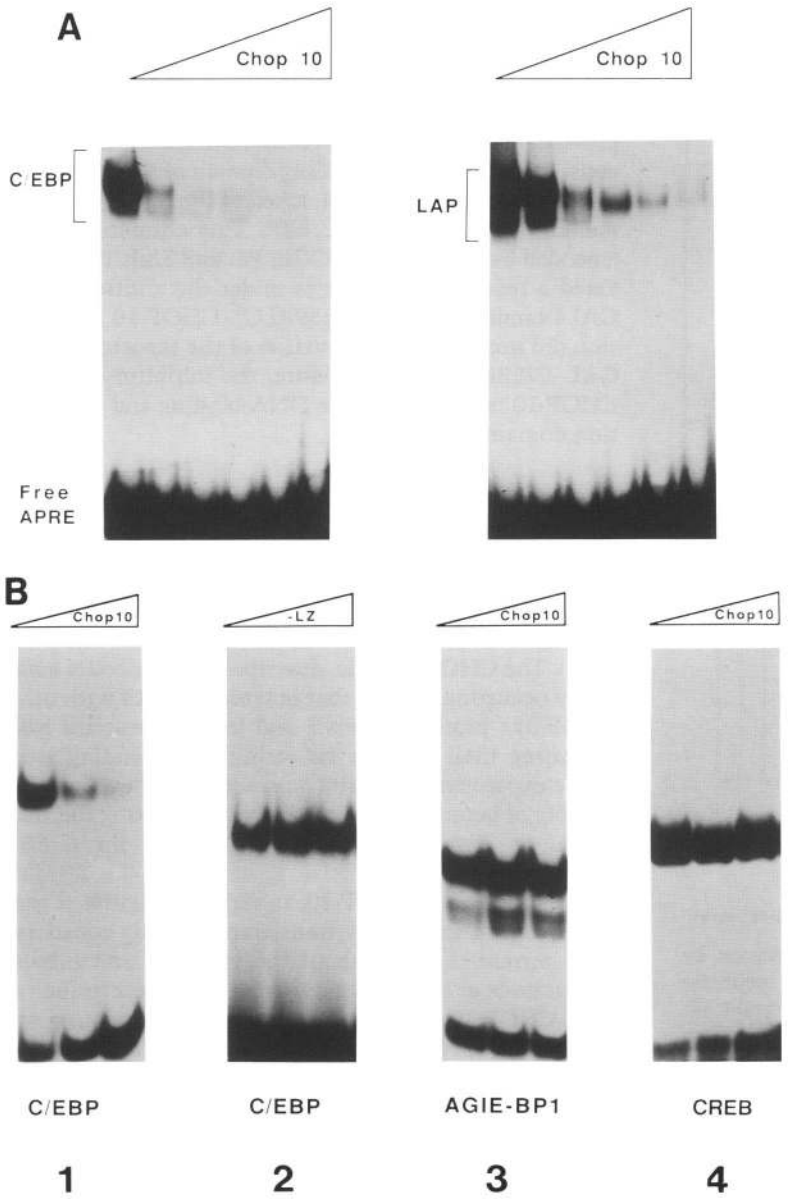

Figure 7. Bacterially expressed CHOP-10 specifically inhibits DNA binding by C/EBP and LAP. (*A*) Increasing amounts of purified, bacterially expressed, GST–CHOP-10 fusion protein (50–500 ng) were added to a constant amount of bacterially expressed C/EBP (*left*) or LAP (*right*), and EMSA, with labeled angiotensinogen gene APRE, was performed. Complexes were resolved on a 6% polyacrylamide nondenaturing gel. The migration of the protein–DNA complex is indicated at *left*. (*B*) Increasing amounts of CHOP-10 fusion protein were added to the bacterially expressed DNA-binding domain of C/EBP, the non-leucine zipper-containing APRE-binding AGIEBP-1, or the leucine zipper-containing CREB (*1,2,4*, respectively). Bacterially expressed CHOP-10–LZ$^-$ was added to the DNA-binding domain of C/EBP (*2*). EMSA was performed with the labeled APRE (*1–3*) or a labeled CRE (*4*).

partially purified preparation of CHOP-10 expressed in bacteria as the native peptide (data not shown).

CHOP-10-mediated inhibition of DNA binding was also apparent when a truncated form of C/EBP, containing only the basic region and leucine zipper of C/EBP, was used (Fig. 7B, panel 1). The bacterially expressed CHOP-10–LZ$^-$ fusion protein, on the other hand, was incapable of inhibiting the DNA binding activity of C/EBP (Fig. 7B, panel 2). These experiments indicate that inhibition of DNA binding by CHOP-10 is dependent on the leucine zipper of CHOP-10 and is independent of sequences lying outside the DNA-binding domain of the target C/EBP-like protein.

CHOP-10 failed to inhibit DNA-binding activity of a zinc finger APRE-binding protein, AGIEBP-1 (Ron et al. 1991). CHOP-10 also failed to inhibit the interaction between the leucine zipper-containing CREB and its cognate binding site, the CRE (Fig. 7B, panels 3,4). These experiments all demonstrated the specificity of CHOP-10 toward a restricted group of APRE-binding leucine zipper-containing proteins.

To study the potential significance of the CHOP-10–C/EBP-like protein interaction in an in vivo model, we cotransfected an expression plasmid for CHOP-10 into HepG2 cells along with a luciferase reporter gene linked to an APRE-driven minimal promoter (APREp59RLG; Brasier et al. 1990b). Activation of the reporter gene by C/EBP and LAP was attenuated when CHOP-10 was coexpressed (Fig. 8). At equal input levels of expression plasmid DNA, C/EBP and LAP accumulate to higher levels than CHOP-10 in the nucleus of transfected cells (Fig. 6; data not shown). Therefore inhibition of C/EBP-like protein-mediated activation of the APRE does not appear to require a vast excess of CHOP-10. Cotransfection of a

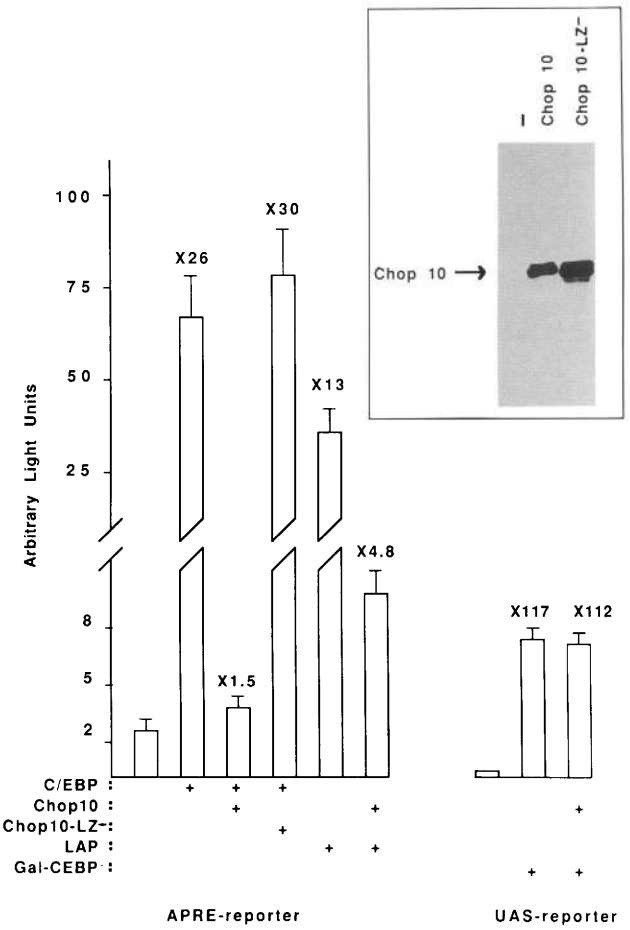

Figure 8. CHOP-10 specifically inhibits *trans*-activation by C/EBP and LAP. HepG2 cells were transfected with a luciferase reporter plasmid containing three copies of the C/EBP and LAP-binding angiotensinogen gene APRE (APRE-reporter) or two copies of the yeast GAL4 transcription factor-binding site (UAS-reporter), both upstream of the minimal angiotensinogen promoter. Expression plasmids encoding C/EBP, LAP, CHOP-10, CHOP-10–LZ$^-$, or a chimeric transcription factor consisting of the DNA-binding domain of GAL4 fused to the *trans*-activation domain of C/EBP (GAL-C/EBP) were cotransfected as indicated. Mean and range of luciferase reporter activity, expressed in arbitrary light units, from transfections performed in triplicate are indicated by the bar diagrams. CHOP-10, but not CHOP-10–LZ$^-$, attenuates activation of the reporter plasmid by C/EBP and LAP. Activation of the UAS site by GAL–C/EBP is not attenuated by CHOP-10. (*Insert*) An anti-CHOP-10 Western immunoblot of lysates from nontransfected COS cells and cells transfected with CHOP-10 and CHOP-10–LZ$^-$, demonstrating comparable levels of expression of the two proteins.

CHOP-10–LZ$^-$ expression plasmid did not inhibit reporter gene activation by C/EBP. Both wild-type CHOP-10 and CHOP-10–LZ$^-$ were expressed to comparable levels in transfected cells, as assayed by Western immunoblot (Fig. 8, insert). The putative leucine zipper region of CHOP-10, essential for inhibition of the DNA-binding activity of C/EBP-like proteins in vitro, is also important for the ability of CHOP-10 to inhibit *trans*-activation by these proteins in vivo.

To study the specificity of the inhibitory effect of CHOP-10 on promoter *trans*-activation in vivo, we expressed, in HepG2 cells, a chimeric transcription factor consisting of the DNA-binding domain of the yeast transcriptional activator, GAL4, fused to the *trans*-activating domain of C/EBP, GAL–C/EBP. This chimeric protein (encoded by the plasmid pGCE; Pei and Shih 1991) activated a reporter gene that is under the control of two GAL4-binding sites (UASp59RLG). CHOP-10 coexpression did not affect the activation of the reporter gene by GAL–C/EBP (Fig. 8). Therefore, the inhibitory effect of CHOP-10 is specific for the DNA-binding and dimerization domain of C/EBP.

Discussion

Heterodimerization between members of the C/EBP family has been postulated to contribute to the complexity of regulation of the activity of promoters capable of binding such proteins (Cao et al. 1991; Williams et al. 1991). The CHOP-10 gene, described here, encodes a naturally occurring protein that heterodimerizes with other C/EBP-like proteins in vivo, and inhibits proteins from activating their cognate *cis*-acting DNA-binding sites. These experimental results represent direct evidence for the role of heterodimer formation in regulating the transcriptional *trans*-activational activities of the C/EBP-like complexes in vivo.

The function of CHOP-10, in terms of regulated gene expression in transiently transfected cells, is consistent with current thinking about the structure and subunit interactions of bZIP proteins. In its carboxy-terminal region, CHOP-10 contains a helical region consisting of a heptad repeat of leucines characteristic of a coiled–coil leucine zipper. It is this region of CHOP-10 that exhibits sequence identity to other C/EBP-like proteins and is demonstrated here as being responsible for the interaction with C/EBP and LAP. Although the precise structural determinants that specify subunit interactions among bZIP proteins are not known, it has been possible to demonstrate, experimentally, restricted interactions between such proteins. This restriction, in terms of heterodimer formation, has provided a basis for grouping proteins into distinct families (Halazonetis et al. 1988; Hai et al. 1989; Cao et al. 1991). CHOP-10 also exhibits restricted interaction with bZIP proteins—binding to C/EBP and LAP but not CREB—and can be provisionally grouped within the C/EBP family of proteins.

CHOP-10 is unique among known members of the C/EBP family because amino-terminal to the putative leucine zipper, the protein contains important substitutions in residues conserved in all other family members. The presence of glycine and proline residues, substituting for conserved aliphatic and basic residues in the otherwise basic region of CHOP-10, may play a determining role in preventing the attainment of the structural conformation necessary for DNA binding (O'Neil et al. 1990; Shuman et al. 1990). The CHOP-10–C/EBP (or

LAP) heterodimer is incapable of binding the APRE, presumably because the defective DNA-contacting surface of the CHOP-10 basic region destabilizes DNA binding; a single DNA-contacting surface is insufficient to allow the "scissor-grip" configuration of the dimeric DNA-binding form of a bZIP protein (Vinson et al. 1989).

Substitution of conserved residues by prolines in the defective DNA-binding domains of known negative regulators of helix–loop–helix proteins (Id, *EMC*) may represent another example of the utilization of this structural modification to create dominant inhibitory regulators of DNA binding (Benezra et al. 1990), with each inhibitor being restricted in its action to a family of transcription factors defined by the specificity of its dimerization surface. Although we cannot formally exclude the possibility that CHOP-10 participates in directing the C/EBP-like protein–CHOP-10 heterodimer to DNA sequences other than the APRE, we note that bacterially expressed CHOP-10 is incapable of significant binding (as a homodimer) to any other labeled DNA fragment tested. CHOP-10 is capable, however, of homodimerization in the zipper–blot assay (data not shown).

CHOP-10 and LAP can be coimmunoprecipitated from the nuclei of expressing COS-1 cells (Fig. 6). By comparing the intensity of the signal obtained in the sequential coimmunoprecipitation assay (lanes 9,12) with the signal obtained when immunoprecipitating CHOP-10 directly from the same lysates (lane 6) it appears that much of the CHOP-10 in the nucleus of transfected COS-1 cells is complexed to LAP. This speaks in favor of a significant physiological role for the interaction of CHOP-10 with other C/EBP-like proteins. In spite of the ability of CHOP-10 to homodimerize in vitro (data not shown), these stoichiometric considerations suggest that it is the C/EBP-like protein–CHOP-10 heterodimer that would be likely to mediate the nuclear effects of CHOP-10.

Dimerization in the zipper–blot assay demonstrates that C/EBP, LAP, and CHOP-10 interdimerize with comparable affinities (data not shown). In vitro, bacterially expressed CHOP-10 inhibits DNA binding by C/EBP and LAP when added to the binding assay at roughly equal amounts (Fig. 7). Furthermore, the stoichiometry of the ambient levels of C/EBP-like proteins and CHOP-10 in the nucleus of transfected cells leaves open the possibility that CHOP-10 exerts part of its inhibitory effect on the activity of a C/EBP-like protein-driven reporter construct through a mechanism that does not depend on direct inhibition of DNA binding by the existing C/EBP-like protein. For example, complexing with CHOP-10 may affect the in vivo stability of the C/EBP-like protein. Such possibilities remain to be explored experimentally.

Not all of the CHOP-10 in transfected COS-1 cells is nuclear. Cellular fractionation experiments and antigen detection, both by Western immunoblot and immunoprecipitation, demonstrate that the cytosolic staining for CHOP-10 in transfected cells is not a fixation artifact. It is therefore possible that regulated subcellular localization of CHOP-10 plays a role in modulating its activity. The recent identification of a phosphorylation-dependent mechanism for regulated nuclear translocation of rat NFIL-6 (LAP) in response to forskolin treatment of PC-12 cells (Metz and Ziff 1991) suggests the intriguing possibility that cytosolic CHOP-10, or a similar molecule, may play a role in this event. CHOP-10 exists in the cell as a phosphoprotein, and bacterially expressed CHOP-10 can be phosphorylated to high specific activity by purified PK-A (data not shown). The functional significance of CHOP-10 phosphorylation may be related to regulation of the subcellular localization of CHOP-10 or its dimerization with other C/EBP-like proteins.

Not all cells tested contain CHOP-10 protein. Most continuously dividing cells in culture contain little if any protein reactive with an antiserum to CHOP-10. Interestingly, CHOP-10 can be induced by manipulations that block cellular proliferation, such as DNA alkylation or terminal differentiation. A hamster homolog of CHOP-10, GADD 153, has been isolated previously by subtractive hybridization of mRNA from growing versus growth-arrested CHO cells (Fornace et al. 1989). These investigators demonstrated that only low levels of GADD 153 mRNA are present in proliferating cells. We cannot detect CHOP-10 protein in lysates of asynchronously dividing cells; it is therefore very likely that CHOP-10 is expressed only at G_0, as is GADD153 (Fornace et al. 1989).

Restriction of CHOP-10 protein and mRNA to nonproliferating cells is a feature shared with C/EBP. C/EBP, when expressed as a conditional mutant in proliferating cells, induces a rapid decrease in cellular proliferation (Umek et al. 1991). It remains to be seen whether CHOP-10 is expressed in C/EBP-arrested cells. The possibility that a CHOP-10–C/EBP heterodimer may play a role in this growth arrest must also be considered. Both C/EBP and CHOP-10 are induced at about the same time during differentiation of 3T3-L1 fibroblasts to adipocytes—day 5 of the differentiation protocol (Fig. 3B; Birkenmeir et al. 1989; Cao et al. 1991). It is difficult to compare the relative abundance of C/EBP and CHOP-10 in adipocyte nuclei by Western immunoblot; but from a functional point of view, it is clear that CHOP-10 is not dominant in these cells. This is attested to by the observation that C/EBP-like DNA-binding activity increases during adipocyte differentiation (Birkenmeir et al. 1989) and by the fact that induction of key adipogenic enzymes, a process that is dependent on C/EBP (Samuelsson et al. 1991; Umek et al. 1991), is not inhibited by the presence of CHOP-10. We suggest that in 3T3-L1 adipocytes CHOP-10 and C/EBP coexist as part of network of interacting C/EBP-like proteins that are present only in the terminally differentiated phenotype. Interactions between CHOP-10 and other C/EBP-like proteins may modulate the activity of C/EBP-binding sites in the promoter region of various genes important for the maintenance of the fat cell phenotype (McKnight et al. 1989).

Nuclear levels of C/EBP and CHOP-10 decrease in parallel during cytokine-mediated dedifferentiation of adipocytes. LAP, on the other hand, increases during cytokine treatment of terminally differentiated adipocytes (Fig. 4; Ron et al. 1992). While the intranuclear stoichiometry of the level of various C/EBP-like proteins is not

known, the observation that CHOP-10 falls during cytokine treatment of adipocytes is consistent with the observation that such treatment is associated with an increase in the C/EBP-like protein-mediated activation of the APRE (Ron et al. 1992).

Not all cell types that express C/EBP coexpress CHOP-10. For example, rat liver nuclear extracts that give rise to easily detectable C/EBP and LAP signals on a Western immunoblot fail to react with the CHOP-10 antiserum (data not shown). This is neither the result of failure of the antiserum to detect the rat protein (MMS-treated PC-12 extracts have easily detectable amounts of CHOP-10) nor of a low sensitivity of anti-CHOP-10 antiserum (assessed by immunoprecipitation of labeled bacterial protein). It remains to be seen whether part of the variation among different tissues in the activity of the C/EBP-like complex is the result of variation in the level of CHOP-10 protein. The level of CHOP-10 mRNA in liver and adipocytes is not very different, suggesting that translational regulation or protein stability may be important in determining the level of CHOP-10 in a given tissue.

A pathological situation that underscores the potential importance of the CHOP-10–C/EBP ratio in the liver to regulation of gene expression is the albino-lethal mutant strain of mice. Animals homozygous for this chromosomal deletion experience severe hypoglycemia in the neonatal period, due in part to a failure to activate a subset of liver genes important for gluconeogenesis. The livers of such animals contain low levels of C/EBP mRNA (Ruppert et al. 1990) and high levels of CHOP-10 mRNA (GADD 153; Fornace et al. 1989). It is likely, in view of the role that C/EBP-binding sites play in regulation of genes important for gluconeogenesis [such as phosphoenolpyruvate carboxy-kinase (PEP-CK); Park et al. 1990], that the excess of inhibitor, CHOP-10, to the activator, C/EBP, plays a role in the failure to activate this set of genes. The nature of the underlying defect in albino-lethal mice is not known. On the basis of a large number of genes affected by the chromosomal deletion, it is unlikely to be a primary *cis*-acting defect in the regulated expression of CHOP-10 or C/EBP. This suggests the existence of a mechanism for discordantly altering the level of C/EBP and CHOP-10 in response to certain changes in the cellular environment. Activation of this mechanism, such as presumably occurs in albino-lethal mice, would be expected to have an important influence on regulated gene expression in the liver.

Materials and methods

Zipper–blot and library screening

For detection of nitrocellulose-immobilized proteins that interact with the LAP and C/EBP dimerization domains, we constructed bacterial expression plasmids encoding chimeric proteins, which contain the CREB A-kinase box peptide (RREILSRRPSYRK), fused in-frame to the C/EBP or LAP DNA-binding and dimerization domains. An oligonucleotide encoding the A-kinase box, with an initiator methionine, was ligated into the unique *Nco*I site of the previously described bacterial expression plasmids that encode the C/EBP and LAP DNA-binding and dimerization domains (Ron et al. 1992). Following introduction of the chimera into pLysS BL.21 *E. coli* (Studier et al. 1990), the sonicated bacterial lysate was heated to 90°C for 10 min and the soluble fraction was purified further by gel-filtration chromatography. Five micrograms of recombinant protein was phosphorylated in a 50-μl reaction volume containing 0.2 U/ml of catalytic subunit of PK-A, purified from bovine heart (Sigma, St. Louis, MO) and 750 μCi of [γ-^{32}P]ATP (6000 Ci/mM) in buffer DK [50 mM potassium phosphate (pH 7.15), 10 mM $MgCl_2$, 5 mM NaF, 4.5 mM dithiothrietol (DTT)] for 30 min at 30°C. Unincorporated label was removed by gel filtration. Binding of the labeled probe to the proteins on the blot was performed following guanidinium-HCl denaturation and washing at room temperature, for 1 hr in buffer DZ [20 mM potassium phosphate, (pH 7.9), 250 mM KCl, 5 mM NaF, 1 mM DTT, 0.2 mM EDTA] that contained 10% nonfat dry milk. The blot was washed extensively with buffer DZ prior to exposure for autoradiography.

The ^{32}P-labeled LAP chimeric protein was used to screen 10^5 recombinant clones from a λ ZAP 3T3-L1 adipocyte cDNA expression library (gift of B. Spiegelman). Recombinant proteins were induced by overlaying the plated library with nitrocellulose filters soaked in 10 mM IPTG. The filters were left in place overnight. The screening protocol was essentially identical to the zipper–blot described above.

Bacterial expression and DNA-binding assays

An *Xba*I–*Sal*I fragment of the CHOP-10 pBS II (KS) phagemid (isolated from the λ ZAP library), containing the full CHOP-10 ORF and 38 amino acids of upstream in-frame vector sequence, was ligated into *Xba*I/*Sal*I-digested pGEX-KG (gift of J. Dixon). Expression of the GST fusion protein and its purification on glutathione–agarose beads (Sigma, St. Louis, MO) was as described previously (Smith and Johnson 1988). The C/EBP and LAP expression plasmids were derived by subcloning the full-length coding region of C/EBP and LAP (NFIL-6) into the aforementioned plasmid. A bacterial expression plasmid for the truncated form of CHOP-10 (CHOP-10–LZ$^-$) was constructed by truncating the CHOP-10-coding sequence at the unique *Nhe*I site (at nucleotide 490, leading to a loss of 34 carboxy-terminal amino acids of CHOP-10) and ligating the truncated fragment liberated by *Xba*I digestion back into an *Xba*I–*Spe*I-digested pBS II (KS), to give rise to the plasmid CHOP-10–LZ$^-$ pBS. Sequencing with a T3 promoter primer confirmed the 3′ truncation of the cDNA. The *Xba*I–*Xho*I fragment of CHOP-10–LZ$^-$ pBS was ligated into the *Xba*I–*Sal*I-digested pGEX KG. Because the *Nhe*I-digested CHOP-10 cDNA does not incorporate an in-frame stop codon at the truncation site, the resulting expression plasmid, CHOP-10–LZ$^-$ pGEX, encodes a protein that is not different in size from full-length CHOP-10. The carboxy-terminal 32 amino acids are contributed by vector sequences.

EMSAs were performed as described previously (Brasier et al. 1989). Fifty nanograms of bacterially expressed DNA-binding protein was mixed with increasing amounts of CHOP-10 fusion protein, the protein mixture was left to stand at room temperature for 15 min after which a labeled oligonucleotide corresponding to the APRE sequence (Ron et al. 1990a) was added, and the binding reaction was allowed to continue for an additional 15 min. The protein–DNA complex was resolved on a 6% acrylamide gel.

Eukaryotic expression plasmids and transfections

A eukaryotic expression plasmid for CHOP-10 was constructed by ligating, in the correct orientation, the *Eco*RI fragment of the

CHOP-10 phagemid into similarly digested pCDNA1 (Invitrogen, La Jolla, CA). A CHOP-10–LZ$^-$ expression plasmid was constructed by ligating the *Eco*RI fragment of CHOP-10–LZ$^-$ pBS into pCDNA1.

To express C/EBP and LAP in eukaryotic cells we constructed expression plasmids in which the initiator methionine of either protein was placed in a context propitious for ribosomal binding. This was accomplished by first transferring the 1.1-kb *Nco*I fragment of C/EBP cDNA (gift of S. McKnight) or the *Nco*I (partial digest)–*Xho*I fragment of the LAP-encoding cDNA (from the plasmid 6.10BS$^+$, gift of R. Cortese and D. Ramji; Poli et al. 1990) into a shuttle vector consisting of pGEM 7Z (Promega, Madison, WI) which had, inserted into its *Hin*dIII–*Eco*RI site, the following oligonucleotide, AAGCTTGCCGCCACCATGGGCAACTGCGCGTGAGAATTC, creating a unique *Nco*I site that further places the initiating methionine-encoding codon in a context favorable for ribosomal binding. From the shuttle vector the coding sequences were transferred to the pECE (in the case of C/EBP) or pCDNA-1 (in the case of LAP) plasmids by *Hin*dIII–*Eco*RI digest. The APRE-containing luciferase reporter plasmid has been described previously (Brasier et al. 1990b). The reporter plasmid UASp59RLG containing the GAL4-binding site was constructed by ligating two copies of an oligonucleotide encoding the GAL4 gene upstream activator sequence (UAS) (Sadowski et al. 1988) upstream of the angiotensinogen minimal promoter—luciferase reporter plasmid p59RLG (Brasier et al. 1989). Triplicate 60-mm plates of HepG2 cells were transfected, as described previously (Brasier et al. 1989), with 10 μg of reporter plasmid, 100 ng of C/EBP LAP or GAL–C/EBP *trans*-activator, and 300 μg of CHOP-10 expression plasmid per triplicate.

Antiserum, Western immunoblots, immunoprecipitation, and immunocytochemistry

Rabbits were immunized with 1 mg of the purified GST CHOP-10 fusion protein on days 0, 14, and 42. The various assays reported were performed with the first immune bleed obtained 7 days after the last booster injection.

Western immunoblots were performed with a 1 : 20,000 dilution of the primary antiserum and a similar dilution of the secondary, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antiserum (Bio-Rad, Richmond, CA). In situ detection of the immune complex was by the ECL system (Amersham, UK).

Immunoprecipitation of in vivo-labeled proteins was performed essentially as described previously (Harlow and Lane 1988). Nuclei, from [^{35}S]methionine/cysteine (Translabel, ICN, Irvine, CA; 800 μCi/ml)-labeled transfected COS-1 cells (2 μg plasmid/100-mm plate), were isolated (Schreiber et al. 1989). For direct immunoprecipitation, nuclear proteins were extracted in RIPA buffer. For the CHOP-10 LAP coimmunoprecipitation experiments the nuclei were extracted for 15 min at 4°C in a buffer containing 20 mM HEPES (pH 7.6), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF. The lysate was subsequently diluted to 200 mM NaCl, in the same buffer lacking the salt. Immunoprecipitation with the first and second antibodies was essentially as described previously (Lassar et al. 1991), except that only 2 μl of antiserum was used.

Immunocytochemical detection of CHOP-10 in transfected COS-1 cells was performed by plating cells that had been transfected 48 hr beforehand onto glass slides with built-on wells. The cells were washed in PBS, fixed in 4% paraformaldehyde 24 hr later, permeabilized with acetone at −20°C, and reacted with the primary antiserum. Secondary detection was performed either with fluorescein-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) or with biotinylated anti-rabbit IgG, followed by avidin-biotinylated HRP conjugate assembly and color formation (Vectastain, ABCD, Vector Laboratories, Burlingame, CA).

Cell culture, RNA isolation, and Northern blot analysis

3T3-L1 fibroblasts were cultured in Dulbecco's modified Eagle medium in 10% calf serum. Semiconfluent cultures were induced to growth arrest by treatment with 100 μg/ml of MMS (Sigma, St. Louis, MO). Poly(A)$^+$ RNA from various rat organs (10 μg/lane) was fractionated on a 1% agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled insert of CHOP-10 cDNA. Total cellular RNA from 3T3-L1 cells, induced to differentiate to adipocytes (Weiss et al. 1980), was harvested at various time points of the differentiation process and analyzed by Northern blot with the CHOP-10 probe.

Fully differentiated adipocytes were treated with 0.35% (vol/vol) conditioned medium from lipopolysaccharide-treated RAW 264.7 monocytic cells in culture, as described previously (Ron et al. 1992). Nuclear extracts were prepared at various time points in the dedifferentiation process and subjected to analysis by Western immunoblotting.

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Note added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank data libraries.

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