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Stress-Induced Binding of the Transcription Factor CHOP to a Novel DNA Control Element

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CHOP (GADD153) is a mammalian nuclear protein that dimerizes with members of the C/EBP family of transcription factors. Absent under normal growth conditions, CHOP is induced by the stress encountered during nutrient deprivation, the acute-phase response, and treatment of cells with certain toxins. The basic region of CHOP deviates considerably in sequence from that of other C/EBP proteins, and CHOP-C/EBP heterodimers are incapable of binding to a common class of C/EBP sites. With respect to such sites, CHOP serves as an inhibitor of the activity of C/EBP proteins. However, recent studies indicate that certain functions of CHOP, such as the induction of growth arrest by overexpression of the wild-type protein and oncogenic transformation by the TLS-CHOP fusion protein, require an intact basic region, suggesting that DNA binding by CHOP may be implicated in these activities. In this study an in vitro PCR-based site selection assay was used to identify sequences bound by CHOP-C/EBP dimers. These sequences were found to contain a unique core element PuPuPuTGCAAT(A/C)CCC. Competition in DNA-binding assays, DNase I footprint analysis, and methylation interference demonstrate that the binding is sequence specific. Deletions in the basic region of CHOP lead to a loss of DNA binding, suggesting that CHOP participates in this process. Stress induction in NIH 3T3 cells leads to the appearance of CHOP-containing DNA-binding activity. CHOP is found to contain a transcriptional activation domain which is inducible by cellular stress, lending further support to the notion that the protein can function as a positively acting transcription factor. We conclude that CHOP may serve a dual role both as an inhibitor of the ability of C/EBP proteins to activate some target genes and as a direct activator of others.

Members of the C/EBP family of transcription factors have been implicated in regulating processes relevant to cellular proliferation, differentiation, and expression of cell-type-specific genes (7, 28, 38, 44). C/EBP proteins bind DNA exclusively as dimers and are characterized by the presence of a conserved C-terminal structure, the bZIP domain, that contains a DNA-contacting basic region and a leucine zipper dimerization module. Both are essential for binding to DNA control elements (22, 45). CHOP is a small nuclear protein that dimerizes avidly with members of the C/EBP family. However, the basic region of CHOP deviates significantly from the consensus defined by other members of the C/EBP family in that it contains proline and glycine substitutions in conserved residues believed to be essential to the interaction of these proteins with DNA-binding sites (29, 40). Indeed, CHOP-C/EBP heterodimers fail to bind several known C/EBP sites in vitro. And when expressed in cells, CHOP attenuates the ability of other C/EBP proteins to activate promoters containing such sites (35). CHOP is identical to GADD153 and is markedly induced by a variety of stressful conditions (8, 9, 15, 26, 31, 35). These observations led us to propose that CHOP regulates the activity of C/EBP proteins by serving as a stress-inducible inhibitor of their ability to bind DNA (35). However, new findings have led us to consider a parallel mode for CHOP action.

CHOP is induced during the differentiation in vitro of 3T3-L1 fibroblasts to adipocytes (8, 35), yet C/EBPα and C/EBPβ, the targets for the inhibitory activity of CHOP, are important components in this differentiation process (7, 16, 17, 25, 38), making it unlikely that CHOP is a significant physiological inhibitor of C/EBP proteins in this setting. We have recently found that microinjection of a CHOP expression plasmid or protein into proliferating NIH 3T3 cells induces arrest at the G_1/S boundary; this arrest phenotype is maintained when CHOP is comicroinjected with C/EBPα and -β. C/EBPα and -β also induce growth arrest (3, 6, 44), and growth arrest by C/EBPα has been shown to require that the protein bind DNA (44). It is therefore hard to reconcile the fact that CHOP shares the ability to induce growth arrest with C/EBPs with a putative role for CHOP as an inhibitor of C/EBP DNA-binding activity.

Further evidence that the role of CHOP is not restricted to that of an inhibitor of other C/EBP proteins comes from an experiment of nature. In human myxoid liposarcoma, a tumor of adipose tissue, the *CHOP* gene is fused to *TLS*. The product of the fusion oncogene, TLS-CHOP, is an abundant nuclear protein that contains the full-length CHOP coding region and a portion of the N terminus of TLS (13, 32). In contrast to CHOP, TLS-CHOP does not arrest cells, even though it dimerizes avidly with C/EBP proteins in vivo and in vitro (3, 13, 49). TLS-CHOP stably introduced in NIH 3T3 cells causes transformation to a tumor phenotype, a property which is negated by deletion of the CHOP basic region (49). Moreover, the ability of CHOP to induce growth arrest is also abrogated

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by lesions in the basic region of the protein (3). These observations suggest that CHOP activity amounts to more than merely the formation of inactive dimers with C/EBP proteins and suggests the possibility that DNA binding by CHOP may be important under some circumstances.

The Id proteins, potent inhibitors of DNA binding by helix-loop-helix transcription factors, are present in considerable molar excess with respect to their inhibition targets (4, 20). CHOP in most circumstances appears to be much less abundant than its dimerization targets. This is true even under conditions of induction of the *CHOP* gene, as occurs during the acute-phase response (see below) or in the liver in albino-lethal mice (15, 19a). In these situations the intracellular stoichiometry of CHOP and the C/EBP proteins is inconsistent with a mechanism by which CHOP acts solely as a dominant inhibitor of DNA binding by C/EBP proteins. An alternative function for the CHOP-C/EBP heterodimer is thus suggested. Such activity could entail regulation of target gene expression by a DNA-binding activity of CHOP. This possibility is supported by the observation that, apart from the aforementioned proline and glycine substitutions, the overall structure of the basic region is conserved between CHOP and other C/EBP family members (15, 30, 35). This conservation in structure of the CHOP basic region is in marked contrast to proteins that exclusively inhibit DNA binding such as the Ids and EMC, which have no DNA-binding basic region (4). Herein, we report on the identification of a DNA-binding-site motif for CHOP-C/EBP heterodimers and demonstrate that CHOP DNA-binding activity is induced in stressed cells in vivo.

MATERIALS AND METHODS

Immunoprecipitation, zipper blotting, and electrophoretic mobility shift assay (EMSA) experiments. CHOP and C/EBP proteins were immunoprecipitated from rat liver nuclear extracts that were prepared by the method of Gorski et al. (18). To induce the acute phase, adult rats were injected with bacterial lipopolysaccharide as previously described (5). Three milligrams of nuclear protein was used as the input in each immunoprecipitation reaction, carried out essentially as described elsewhere (35), except that 10 μl of rabbit antiserum was used in each reaction and the immunoprecipitations were carried out in buffer D (18) rather than RIPA buffer, to reduce dissociation of the CHOP-C/EBP heterodimers. The antisera to CHOP, C/EBPα, and C/EBPβ have been described previously (34, 35). Following immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins in the gel were transferred to a nitrocellulose filter and probed with a ^{32}P-labeled C/EBPβ zipper probe as described previously (35). The C/EBPβ zipper probe recognizes bacterially expressed CHOP and C/EBP proteins with similar affinities (data not shown). The inability of C/EBPα and -β to coprecipitate in this assay is a reproducible observation that we have confirmed by studies in transfected COS1 cells and suggests that C/EBPα and -β heterodimers are not stable under these conditions.

To study the relative affinities of dimerization of CHOP, C/EBPα, and C/EBPβ with a ^{32}P-labeled CHOP zipper probe, glutathione *S*-transferase (GST) fusion proteins that contain the leucine zipper domain of C/EBPα and -β were constructed. The fusion proteins contained residues 304 to 359 and residues 294 to 345 of the rat C/EBPα (21) and human C/EBPβ (1) proteins, respectively. The full-length GST-CHOP was used both as a target on the zipper blot and as the ^{32}P-labeled ligand. CHOP was labeled on its high-affinity protein-kinase A site at serine 107. Identical amounts of target protein were loaded into each comparable lane of the zipper blot, which was then probed with the ^{32}P-labeled CHOP.

Analysis of C/EBP proteins' DNA-binding activities in acute-phase rat liver nuclei was performed by EMSA using the APRE-M6 mutant that binds only C/EBP proteins, exactly as previously described (5). Where indicated, 100 ng of purified bacterially expressed CHOP was added to the DNA-binding reaction mixture prior to the addition of the radiolabeled probe.

To analyze CHOP DNA-binding activity in stressed mammalian cells, mouse NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Confluent cells were treated with tunicamycin (Sigma) at a concentration of 50 μg/ml. After 3 h, nuclear extracts were obtained by the method of Schreiber et al. (39).

Monoclonal antibodies to CHOP were raised against the bacterially expressed GST-CHOP fusion protein. One microliter of the hybridoma supernatant was added to the EMSA. Immunodepletion of nuclear extracts was performed by incubating the extracts with a protein A-Sepharose column containing specific monoclonal antibodies to CHOP; the control column was constructed with an unrelated monoclonal antibody directed against human Myc.

PCR-based site selection and analysis of sequence specificity of DNA binding. PCR-based site selection was performed as described previously (42). The selection EMSA was performed with approximately 100 ng of partially purified bacterially expressed rat C/EBPα DNA-binding domain (amino acids 216 to 359) and 1 μg of pure CHOP-GST fusion protein. The region of the gel corresponding to the migration of the GST-CHOP-C/EBPα heterodimer was excised, avoiding the faster-migrating C/EBPα homodimer and the selected DNA amplified by PCR. Where indicated, CHOP immune rabbit serum or nonimmune rabbit serum was added to the EMSA. Following five cycles of selection and amplification, the pool of oligonucleotides was digested with *Bam*HI and *Xho*I and ligated into the pBS plasmid (Stratagene). The plasmids from 39 randomly chosen colonies representing 39 independent insert ligation events were analyzed by dideoxy nucleotide sequencing of the insert.

Methylation interference and DNase I footprint analysis were performed with the same concentrations of C/EBPα and CHOP that were used in the EMSAs. Probe preparation and assay conditions were as previously described (5). The probe consisted of the SAAB35 sequence ligated into the *Xba*I site of pBS-KS. The 117-bp fragment was labeled on the unique *Sal*I site and liberated by *Sac*I digestion.

Mutagenesis of the CHOP basic region. PCR primers were designed to introduce *Eco*RI sites into defined points of the basic region of CHOP. Digestion and religation of the PCR-generated *Eco*RI fragments led to deletion of the desired regions of the basic region with preservation of the open reading frame. Mutant A is missing residues 101 to 109, mutant B is missing residues 101 to 122, and mutant Cb is missing residues 109 to 122. The mutant forms of CHOP were expressed in bacteria as GST fusion proteins and purified. In the analysis of DNA binding by the bacterially expressed mutant forms of CHOP, the C/EBP dimerization partner consisted of 100 ng of purified, bacterially expressed fragment of rat C/EBPβ (residues 153 to 297). One microliter of normal rabbit serum was added to the EMSA, as we found that nonspecific carrier protein enhanced the DNA-binding activity of the purified bacterially expressed proteins. The mutant form of CHOP that is lacking the leucine zipper (CHOP-LZ$^-$) has been described previously (35).

Transactivation of reporter genes. The DNA-binding domain of the yeast transactivator Gal4 (amino acids 3 to 147) (37) was amplified by PCR and ligated in frame with murine CHOP cDNA at the unique *Nhe*I site, deleting the CHOP dimerization domain. The chimeric cDNA was transferred to the pCDNA1 expression plasmid. Integrity of the protein was evaluated by immunoblot with CHOP antisera and by its ability to gel shift a UAS oligonucleotide (not shown).

Transfection experiments were carried out in NIH 3T3 cells, plated in 60-mm plates. Seven micrograms of the reporter gene, UASp59RLG that contains two Gal4 binding sites upstream of a minimal promoter (35), or a plasmid (CamRE-41TK-CAT) containing the CaMRE sequence (36, 46) ligated upstream of the thymidine kinase minimal promoter driving the chloramphenicol acetyltransferase structural gene was transfected along with 5 to 10 ng of the CHOP-Gal-BD plasmid or 0.5 μg of the CHOPpCDNA1 expression plasmid. Twenty-four hours following transfection the cells were switched to media containing 0.5% serum and stimulated with 100 μg of methyl methanesulfonate (MMS) per ml for 1.5 h (Sigma) (35) or tunicamycin, 50 μg/ml, for 3 h. The stress inducer was removed, and the cells were incubated for an additional 16 h in low-serum media until harvest and assay of reporter gene activity.

RESULTS

CHOP is significantly less abundant than its dimerization partners. CHOP is normally expressed at very low levels in both dividing and postmitotic cells (8, 15, 35). The acute-phase response is associated with induction of *chop* and other *gadd* genes in rat liver (48). We utilized this experimental system to study the relative abundance of CHOP protein and its dimerization partners under conditions in which *chop* is induced. CHOP, C/EBPα, and C/EBPβ proteins were immunoprecipitated from acute-phase rat liver nuclear extracts and quantified by a zipper-blot assay using the ^{32}P-labeled C/EBPβ dimerization domain as a probe. CHOP protein is directly precipitated by saturating amounts of CHOP antiserum in a quantity similar to that coimmunoprecipitated by saturating quantities of an antiserum to C/EBPβ (Fig. 1A, lanes 1 and 2). C/EBPα level is reduced in the acute phase (2, 34), and no detectable CHOP was coprecipitated with C/EBPα (lane 3). Comparison of the intensities of the CHOP and C/EBPβ signals reveals that CHOP is more than 10-fold less abundant than its C/EBPβ dimerization partner (lane 2). Of note, the C/EBPβ zipper probe recognizes CHOP and C/EBPβ with similar affinities

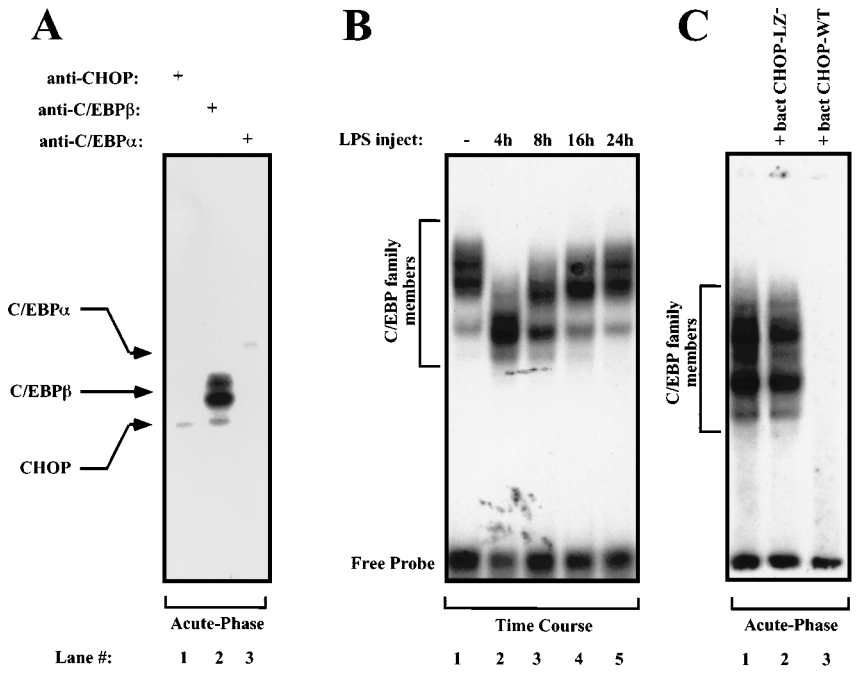


FIG. 1. CHOP is less abundant than its C/EBP dimerization partners. (A) Estimation of the relative abundance of CHOP and C/EBP by zipper blotting of proteins coimmunoprecipitated from acute-phase rat liver nuclei. The blot was probed with a ^{32}P-labeled C/EBPβ zipper probe. The precipitating antisera are indicated at the top. The positions of the proteins detected by the zipper probe are indicated by arrows. (B) Analysis of C/EBP DNA-binding activity from rat liver nuclei at various time points following induction of the acute phase by lipopolysaccharide (LPS) injection. Ten-microgram aliquots of rat liver nuclear extract were used to shift a C/EBP-binding-site oligonucleotide (APRE M6). The induction of CHOP during the acute phase does not lead to a decrease in total C/EBP DNA-binding activity. (C) An excess of bacterially expressed CHOP, added to the DNA-binding reaction mixture, inhibits the DNA-binding activity of C/EBP proteins from acute-phase rat liver. WT and LZ$^-$, bacterially expressed wild type and leucine zipper deletion mutant of the CHOP protein, respectively. The differences in mobility of the complex in panels B and C are due to differences in concentration of acrylamide in the gels used to resolve the complexes.

(data not shown); therefore, the difference between the CHOP and C/EBPβ signals is not due to differences in the affinities of interaction of the proteins with the dimerization probe.

We have previously found that binding of C/EBP proteins to certain C/EBP sites such as the angiotensinogen gene acute-phase response element (APRE) (5) is inhibited in vitro by an excess of CHOP (35). The low ratio of CHOP to its C/EBP dimerization partners, noted above, is therefore consistent with the observation that induction of the acute phase is not associated with a global attenuation of C/EBP APRE-binding activity (Fig. 1B). The increased mobility of the C/EBP-DNA complex, observed during the acute phase, correlates with the induction of the smaller C/EBPβ and C/EBPγ isoforms and the level of the larger C/EBPα protein (2, 5, 34). In interpreting these results, it is important to be sure that CHOP has the capacity to inhibit all the APRE-binding activity. This is revealed by the ability of bacterially expressed CHOP to block all such binding when added to the DNA-binding reaction mixture in vitro (Fig. 1C; compare lane 1 and 3). CHOP that is missing the leucine zipper dimerization domain does not inhibit DNA binding (lane 2). Results similar to these have been obtained in liver in albino-lethal mice, another in vivo model system associated with marked induction of CHOP protein (19a).

On the basis of these observations we conclude that CHOP is unlikely to serve as a quantitative inhibitor of C/EBP DNA-binding activity in these circumstances. However, the possibility that in other circumstances, yet to be discovered, the stoichiometry of CHOP and C/EBP proteins may be such that CHOP is in excess and can serve as a dominant negative regulator of DNA-binding activity is not excluded.

CHOP-C/EBP heterodimers are capable of sequence-specific DNA binding. If, as the experiments described above suggest, CHOP is not a significant inhibitor of C/EBP proteins, then perhaps it has a role as a DNA-binding protein. To evaluate this possibility, we wished to characterize the theoretical binding sites for CHOP proteins in an in vitro PCR-based site selection assay in which bacterially expressed proteins are confronted with a complex library of possible binding sites from which only those with high affinity are selected (42). Before setting up our selection scheme, we needed to know if we should be looking for binding sites for CHOP homodimers or sites for CHOP-C/EBP heterodimers.

Zipper-blot analysis of bacterially expressed C/EBPα, C/EBPβ, and CHOP with a ^{32}P-labeled CHOP dimerization probe demonstrated that CHOP does not form stable homodimers (Fig. 2A). This, coupled with the observation that CHOP and TLS-CHOP exist in vivo as dimers with other C/EBP proteins (Fig. 1a in the present paper and Fig. 3 in reference 49) led us carry out the site selection assay using heterodimers of CHOP and C/EBP. To be able to resolve the CHOP-C/EBP heterodimer from the C/EBP homodimer, we used a mixture of a larger protein, bacterially expressed full-length GST-CHOP fusion protein, and a partially purified preparation of a smaller protein consisting of an active truncated version of C/EBPα that contains only the basic region and leucine zipper. In this setting the heterodimers exhibit reduced mobility in EMSA compared with the smaller C/EBPα homodimer.

After three cycles of selection the amplified pool of oligonucleotides began to exhibit detectable interaction with the heteromeric complex of CHOP-C/EBPα. By the fifth round of selection robust DNA-binding activity of the complex was seen

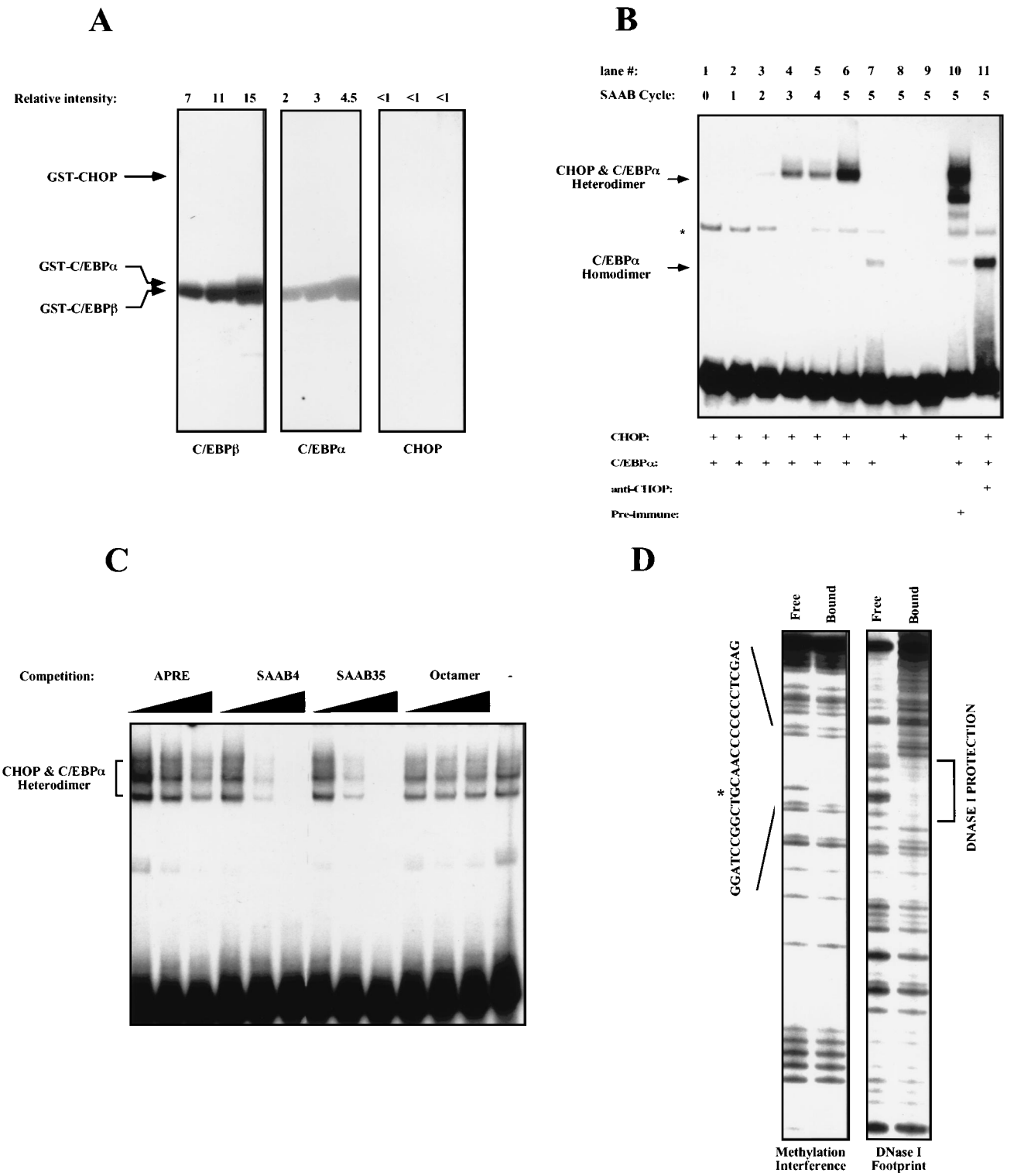
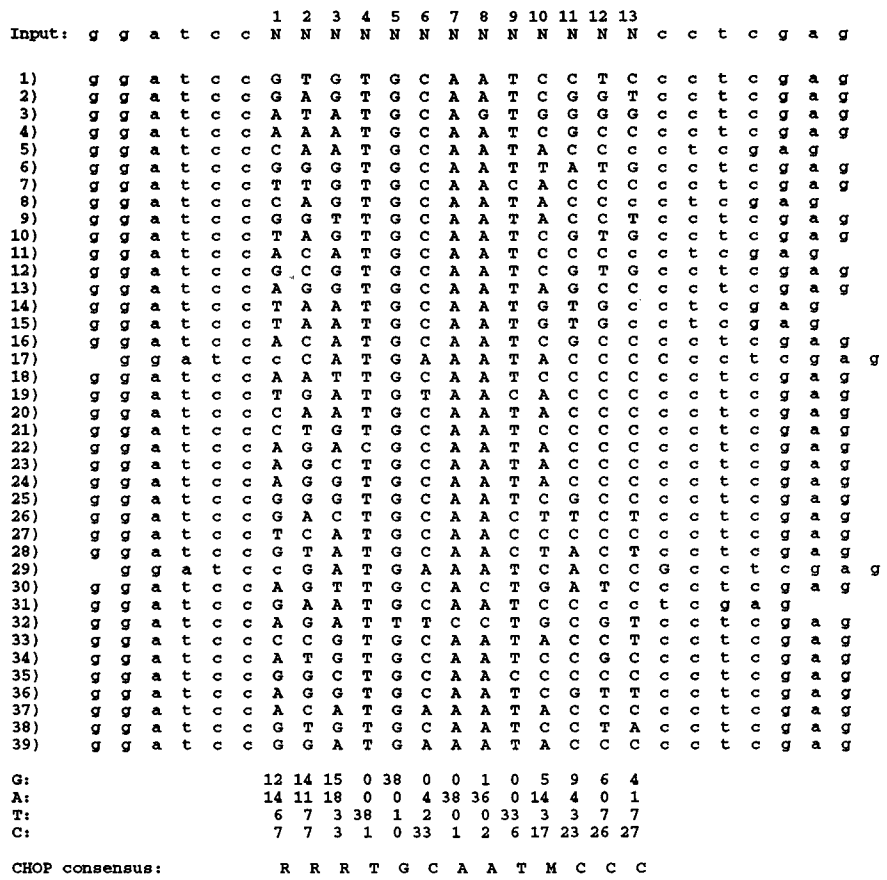


FIG. 2. Sequence-specific DNA binding by CHOP-C/EBPα heterodimers. (A) CHOP does not form stable homodimers. A zipper blot with equal quantities (1, 1.5, and 2.5 μg) of purified, bacterially expressed GST fusion proteins containing the C/EBPβ zipper, C/EBPα zipper, and CHOP, probed with a ^{32}P-labeled CHOP zipper probe, is shown. The positions of the proteins are indicated by arrows. The relative intensities of the binding signals, based on densitometric scanning of the blot, are indicated at the top. (B) EMSA by CHOP-C/EBPα heterodimers of a labeled pool of random oligonucleotides following successive rounds of selection by binding to CHOP-C/EBPα and PCR amplification. The composition of the protein mix used in the EMSA is indicated below the autoradiogram; the positions of the protein-DNA complexes are indicated on the left, and the selection cycle used as a probe in each lane is indicated at the top (SAAB cycle). One microliter of CHOP antiserum or preimmune control serum was added, respectively, to the reaction mixtures in the last two lanes. Asterisk, nonspecific band contributed by the partially purified C/EBPα protein solution. (C) Competition for CHOP-C/EBPα binding by unlabeled double-stranded oligonucleotides in EMSA using labeled SAAB35 as a probe. Increasing amounts (2-, 20-, and 200-fold molar excesses) of the indicated unlabeled oligonucleotides were added to the EMSA. (D) Methylation interference and DNase I footprint analysis of CHOP-C/EBPα heterodimer binding to the SAAB35 sequence. The position of the SAAB35 binding site in the 117-bp DNA probe is indicated, as is the region protected from digestion by DNase I. The single guanine residue, methylation of which interferes with CHOP-C/EBPα binding, is marked by an asterisk.



							1	2	3	4	5	6	7	8	9	10	11	12	13								
Input:	g	g	a	t	c	c	N	N	N	N	N	N	N	N	N	N	N	N	N	c	c	t	c	g	a	g	
1)	g	g	a	t	c	c	G	T	G	T	G	C	A	A	T	C	C	T	C	c	c	t	c	g	a	g	
2)	g	g	a	t	c	c	G	A	G	T	G	C	A	A	T	C	G	G	T	c	c	t	c	g	a	g	
3)	g	g	a	t	c	c	A	T	A	T	G	C	A	G	T	G	G	G	G	c	c	t	c	g	a	g	
4)	g	g	a	t	c	c	A	A	A	T	G	C	A	A	T	C	G	C	C	c	c	t	c	g	a	g	
5)	g	g	a	t	c	c	C	A	A	T	G	C	A	A	T	A	C	C	c	c	t	c	g	a	g		
6)	g	g	a	t	c	c	G	G	G	T	G	C	A	A	T	T	A	T	G	c	c	t	c	g	a	g	
7)	g	g	a	t	c	c	T	T	G	T	G	C	A	A	C	A	C	C	C	c	c	t	c	g	a	g	
8)	g	g	a	t	c	c	C	A	G	T	G	C	A	A	T	A	C	C	c	c	t	c	g	a	g		
9)	g	g	a	t	c	c	G	G	T	T	G	C	A	A	T	A	C	C	T	c	c	t	c	g	a	g	
10)	g	g	a	t	c	c	T	A	G	T	G	C	A	A	T	C	G	T	G	c	c	t	c	g	a	g	
11)	g	g	a	t	c	c	A	C	A	T	G	C	A	A	T	C	C	C	c	c	t	c	g	a	g		
12)	g	g	a	t	c	c	G	C	G	T	G	C	A	A	T	C	G	T	G	c	c	t	c	g	a	g	
13)	g	g	a	t	c	c	A	G	G	T	G	C	A	A	T	A	G	C	C	c	c	t	c	g	a	g	
14)	g	g	a	t	c	c	T	A	A	T	G	C	A	A	T	G	T	G	c	c	t	c	g	a	g		
15)	g	g	a	t	c	c	T	A	A	T	G	C	A	A	T	G	T	G	c	c	t	c	g	a	g		
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19)	g	g	a	t	c	c	T	G	A	T	G	T	A	A	C	A	C	C	C	c	c	t	c	g	a	g	
20)	g	g	a	t	c	c	C	A	A	T	G	C	A	A	T	A	C	C	C	c	c	t	c	g	a	g	
21)	g	g	a	t	c	c	C	T	G	T	G	C	A	A	T	C	C	C	C	c	c	t	c	g	a	g	
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27)	g	g	a	t	c	c	T	C	A	T	G	C	A	A	C	C	C	C	C	c	c	t	c	g	a	g	
28)	g	g	a	t	c	c	G	T	A	T	G	C	A	A	C	T	A	C	T	c	c	t	c	g	a	g	
29)		g	g	a	t	c	c	G	A	T	G	A	A	A	T	C	A	C	C	G	c	c	t	c	g	a	g
30)	g	g	a	t	c	c	A	G	T	T	G	C	A	C	T	G	A	T	C	c	c	t	c	g	a	g	
31)	g	g	a	t	c	c	G	A	A	T	G	C	A	A	T	C	C	c	t	c	g	a	g				
32)	g	g	a	t	c	c	A	G	A	T	T	T	C	C	T	G	C	G	T	c	c	t	c	g	a	g	
33)	g	g	a	t	c	c	C	C	G	T	G	C	A	A	T	A	C	C	T	c	c	t	c	g	a	g	
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G:							12	14	15	0	38	0	0	1	0	5	9	6	4								
A:							14	11	18	0	0	4	38	36	0	14	4	0	1								
T:							6	7	3	38	1	2	0	0	33	3	3	7	7								
C:							7	7	3	1	0	33	1	2	6	17	23	26	27								
CHOP consensus:							R	R	R	T	G	C	A	A	T	M	C	C	C								

FIG. 3. Frequencies of selected residues in CHOP-C/EBP sites. Residues selected from the random pool are uppercased; linker sequence found in the input oligonucleotide are lowercased. The input sequence is shown at the top. The frequency with which a given residue occurs at a given position and a CHOP-C/EBP consensus are presented below. N, all four nucleotides; R, purine; Y, pyrimidine; M, adenine or cytosine.

(Fig. 2B). Participation of both CHOP and C/EBPα in binding is demonstrated by the dependence of complex formation on both constituents (Fig. 2B, lanes 7 to 8). The selected pool of oligonucleotides could also interact with C/EBPα homodimers (Fig. 2B, lanes 7 and 11). However, the binding of the selected sites to the CHOP-C/EBP heterodimer was much stronger than that to the C/EBPα homodimer. Under conditions of equal affinity one would expect the heterodimer lane, which contains the same amount of C/EBPα as the homodimer lane, to have no more than twice the binding activity. We cannot, however, distinguish between different affinities of the dimeric combinations for the probe and differences in stability between the two types of dimers.

To confirm that CHOP is present in the heteromeric complex that binds DNA, anti-CHOP rabbit serum was added to the EMSA reaction mixture; this was found to prevent the formation of a CHOP-C/EBPα DNA-binding complex but does not interfere with the binding of C/EBPα homodimers (Fig. 2B, lanes 10 and 11). In fact, an enhancement of the activity of the C/EBPα homodimer was found (lane 11). That and the alteration in the mobility of the CHOP-C/EBPα heterodimer (lane 10) are due to the effects of serum on the DNA-binding activity of the bacterially expressed proteins, effects that are not related to the immune reactivity of the serum (see Materials and Methods).

To analyze the sequence(s) selected for by CHOP-C/EBPα binding, the pool of oligonucleotides was ligated into a plasmid and random independent insert ligation events were chosen for sequence analysis. Figure 3 shows the sequences of 39 randomly chosen inserts (these sequences are referred to as SAABs). The sequences have been aligned around centrally recurring T-G residues. Most of the oligonucleotides selected conform to a remarkably tight consensus. This tight consensus contrasts markedly with the redundancy of C/EBP binding sites culled from the literature (Fig. 4). The fact that the CHOP-C/EBP consensus sequence retained a fixed orientation with respect to the linkers used in oligonucleotide construction suggests that the sequences flanking the 13 redundant residues in the original pool significantly influenced DNA-protein interaction.

Several of the oligonucleotides selected were individually labeled and used as probes in EMSAs. All bound to the heteromeric complex (Fig. 2C and data not shown). To analyze the sequence specificity of the interaction between the CHOP-C/EBPα heterodimer and the selected sites, two randomly chosen sites (SAAB4 and SAAB35) were used as competitors in EMSA. For comparison, the APRE (a sequence that binds C/EBP proteins but not CHOP-C/EBP heterodimers) and an unrelated oligonucleotide (Octamer) were used. The SAAB oligonucleotides competed better for binding to the CHOP-C/EBPα heterodimer than the APRE or Octamer sequences (Fig. 2C). A higher concentration of APRE than the SAAB oligonucleotides is required to compete for binding of CHOP-C/EBPα heterodimers to the SAAB35 probe. This is consistent with the fact that in these reactions CHOP protein is in molar excess to C/EBPα; therefore, C/EBPα is driven into a hetero-

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	(Reference:)
Gene:														
rAPRE-1:	A	A	T	C	C	C	A	A	C	T	G	T	G	(5)
rAPRE-2:	T	T	T	C	C	C	A	A	C	C	T	G	A	(5)
rC/EBP promoter:	T	G	G	C	G	C	A	A	C	G	C	C	C	(10)
ra AGP-HA:	T	T	G	T	G	C	A	A	G	A	C	A	T	(33)
ra AGP-HB:	T	G	G	C	A	C	A	A	T	C	T	C	A	(33)
rAlb-D1:	T	T	T	T	G	T	A	A	T	G	G	G	G	(27)
hNF-IL6-1:	T	T	G	C	A	C	A	A	T	C	T	T	A	(1)
hNF-IL6-2:	T	T	G	T	G	C	A	A	T	G	T	G	A	(1)
hC3:	T	T	T	C	T	C	A	A	T	A	C	C	A	(47)
hFIX:	T	T	T	C	A	C	A	A	T	C	T	G	C	(12)
rPEPCK-D:	T	T	A	C	A	C	A	A	C	G	T	T	T	(43)
rPEPCK-E:	A	T	C	A	G	C	A	A	C	A	G	T	C	(43)
mSAA3-1:	T	G	G	A	G	C	A	A	T	C	C	C	T	(23)
mSAA3-11:	T	T	A	T	G	C	A	A	G	A	T	C	A	(23)
rSAA1-1:	A	C	A	G	G	A	A	A	T	G	A	C	A	(24)
rSAA1-III:	T	T	T	T	G	C	A	A	T	C	A	G	G	(24)
maP2:	T	T	T	C	T	C	A	A	C	T	T	T	G	(11)
mSCK1:	T	G	A	G	G	A	A	A	T	A	C	T	G	(11)
RSVLTR:	T	T	A	T	G	C	A	A	T	A	C	T	C	(36)
C/EBP "optimal":	T	T	G	C	G	C	A	A	T	C	T	G	C	(44)
Frequency:														
G:	0	4	7	2	12	0	0	0	2	5	3	6	5	
A:	3	1	5	2	4	2	20	20	0	6	2	1	7	
T:	17	14	7	6	2	1	0	0	12	2	9	7	3	
C:	0	1	1	10	2	17	0	0	6	7	6	6	5	
C/EBP dimers:	T	T	N	R	R	C	A	A	Y	N	Y	N	N	
CHOP-C/EBP:	R	R	R	T	G	C	A	A	T	M	C	C	C	

FIG. 4. Frequencies of residues in C/EBP sites from known C/EBP target genes. N, all four nucleotides; R, purine; Y, pyrimidine; M, adenine or cytosine. RSVLTR, Rous sarcoma virus long terminal repeat.

meric configuration in which it does not bind APRE. The CHOP-C/EBP complex variably migrates as a broad band (Fig. 2B and 5B) or a doublet (Fig. 5C); the reason for this is not known.

Further evidence for the sequence-specific nature of binding by CHOP-C/EBPα dimers to the SAAB sequences is provided by results of DNase I footprinting and methylation interference analysis. Protection from digestion by DNase I and interference with binding by methylation localize to the region of the probe that contains the selected sequence (Fig. 2D). Methylation of the highly conserved guanosine residue on the coding strand of the binding site interferes with DNA binding by CHOP-C/EBPα dimers. Methylation of guanosine residues on the opposite strand did not have a measurable effect on DNA binding by CHOP-C/EBPα heterodimers (data not shown).

DNA binding by CHOP-C/EBP heterodimers is dependent on an intact CHOP basic region. The primary amino acid sequence of the CHOP basic region and predicted secondary structure differ markedly from that of other C/EBP proteins. It was important therefore to determine if the basic region of CHOP plays a role in DNA binding by the CHOP-C/EBP heterodimer. A gel shift assay was performed with mutant forms of the CHOP protein in which various parts of the basic region had been deleted (Fig. 5A). Deletions that remove the whole basic region (mutant B) or the portion of the basic region that is adjacent to the leucine zipper (Cb) are deleterious to DNA binding, whereas a deletion of the N-terminal portion of the basic region (mutant A) has only a minimal effect on DNA binding (Fig. 5B).

The basic region mutant forms of the CHOP protein, incapable of binding DNA, nonetheless retain the ability to inhibit C/EBPβ homodimer binding (Fig. 5B, lanes 5 and 6). This provides evidence that they are functional and capable of dimerizing with the C/EBP partner. The LZ^- version of CHOP is completely inert, consistent with previous observations (35). Identical results were obtained with C/EBPα (not shown). The integrity of the bacterially expressed proteins is demonstrated by the Coomassie blue-stained gel (Fig. 5C). Despite its unusual structure, the basic region of CHOP is necessary for DNA binding by the CHOP-C/EBP heterodimer, suggesting an active role for CHOP in this process.

Cellular stress induces a specific CHOP/DNA complex in NIH 3T3 cells. To investigate further the possible physiological relevance of CHOP-C/EBP heterodimer binding to the in vitro-selected sequences, we sought to determine if such binding could occur when the binding site was presented in the context of a natural promoter. A calmodulin-responsive element (CaMRE) in the Rous sarcoma virus long terminal repeat (36, 46) matches the selected SAAB core sequence (Fig. 4). A double-stranded oligonucleotide, containing the reported CaMRE sequence, was found to specifically bind CHOP-C/EBPβ heterodimers in EMSA (Fig. 6A). The bacterially expressed C/EBPβ DNA-binding domain bound very poorly to the CaMRE (lane 1), whereas robust DNA binding was apparent following addition of bacterially expressed CHOP (lane 3).

Having found that bacterially expressed CHOP can participate in sequence-specific DNA binding, we next wanted to know if CHOP, induced in vivo by stress, shares the DNA-binding properties of the bacterially expressed protein. Stress was induced in NIH 3T3 cells by treatment with either the alkylating agent MMS or tunicamycin, and induction of CHOP protein was revealed by immunoblot (Fig. 6B). Because of the extreme toxicity of MMS, subsequent experiments were done with tunicamycin-treated cells. Nuclear extracts of tunicamycin-stressed cells contain a new EMSA retardation complex not present in the uninduced nuclear extract. Competition of binding with excess unlabeled DNA demonstrated that binding is specific for sequences containing the core recognition motif for CHOP-C/EBP heterodimers (Fig. 6C). A monoclonal antibody, 3H8, specifically reactive with the C-terminal portion of CHOP, supershifted this induced complex, indicating that it contains CHOP immunoreactivity (Fig. 6C). Participation of CHOP in the tunicamycin-induced protein-DNA complex was confirmed by the finding that immunodepletion of nuclear extracts with the anti-CHOP monoclonal antibody resulted in

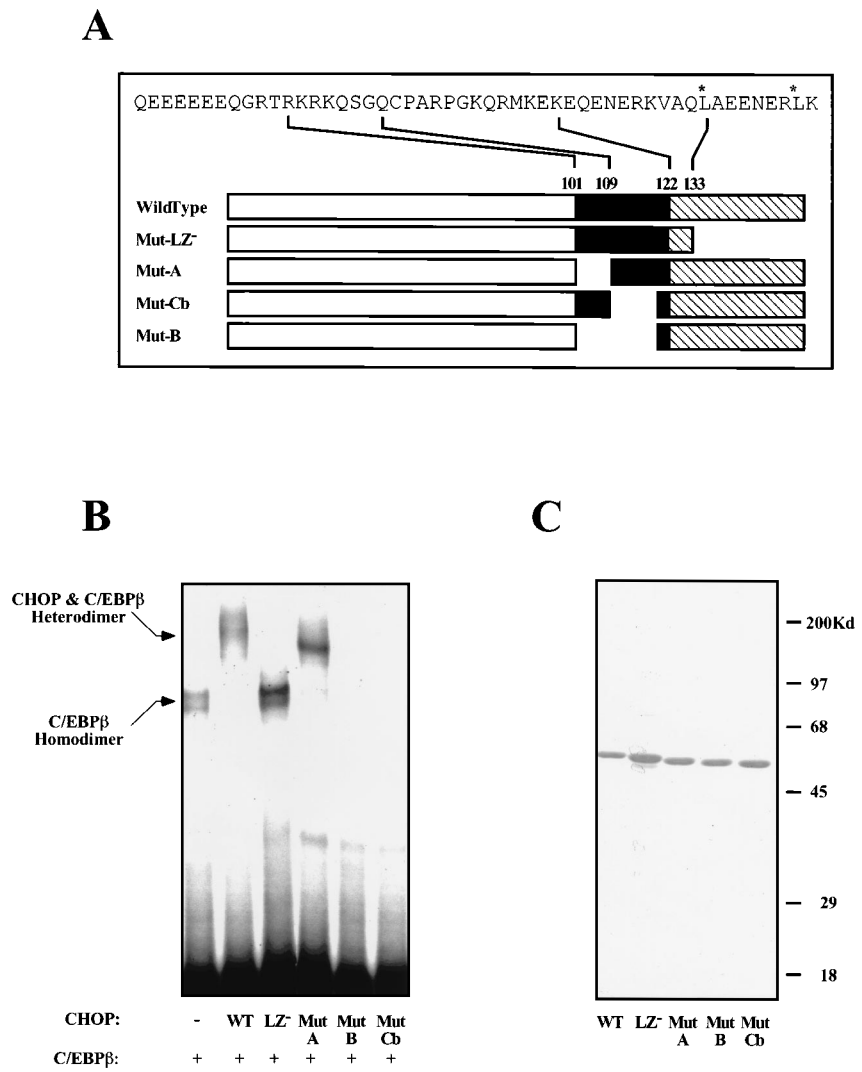

FIG. 5. Deletions in the C-terminal portion of the CHOP basic region abolish DNA binding. (A) Linear map of the structure of the CHOP deletion mutant (Mut) proteins. Solid box, basic region; hatched box, leucine zipper. The residues defining the endpoints of the deletions are indicated at the top. Asterisks, first two leucines of the heptad repeat that forms the CHOP leucine zipper. (B) EMSA of CHOP-C/EBPβ heterodimers that contain wild-type (WT) and mutant forms of CHOP protein using the SAAB4 sequence as a probe. (C) Coomassie blue-stained gel of purified, bacterially expressed GST-CHOP wild-type and mutant fusion proteins.

elimination of the tunicamycin-inducible complex (Fig. 6D). Identical results were obtained with a different monoclonal antibody that recognizes a domain in the N terminus of CHOP (data not shown).

Transcriptional regulation by CHOP. A CAT reporter gene driven by CaMRE has relatively high basal activity, which is increased further upon cotransfection of a C/EBPβ expression plasmid (data not shown). This suggests that a substantial contribution to the activity comes from the endogenous C/EBP proteins that are interacting with CaMRE in vivo (46). Cotransfection of CHOP leads to attenuation of the reporter gene activity (eightfold decrease; Fig. 7A). However, when the CHOP-expressing cells were stressed, reporter gene activity increased to ~45% of basal activity. This stress-induced increase in the activity of the reporter gene was not observed in cells that do not express CHOP. These findings are consistent with CHOP having two opposing effects on the activity of CaMRE: in the absence of stress the CHOP-C/EBP dimers are less active than the C/EBP dimers that occupy the site; hence, the reduction of activity induced by ectopic expression of CHOP. However, when the CHOP-containing cells are stressed, CHOP is activated, leading to some recovery of reporter gene activity.

To study the transcriptional activity of CHOP in isolation from associated C/EBP dimerization partners, a fusion protein of CHOP and the DNA-binding domain of the yeast transcription factor Gal4 was used. The chimeric protein was constructed in such a way that the Gal4 DNA-binding domain replaces the C-terminal CHOP dimerization domain, mimicking the normal configuration of CHOP and eliminating the possibility of recruitment of a CHOP dimerization partner to the DNA-binding complex. At high input plasmid level (>0.1 μg per plate) CHOP-Gal4-BD markedly activates a reporter gene driven by Gal4 binding sites, 20- to 50-fold over baseline, with no further increase if the cells are stressed (data not shown). However, if the input plasmid levels are kept very low (<10 ng per plate), activation of the reporter gene by CHOP is dependent on induction of cellular stress either by treatment with MMS (Fig. 7B) or by tunicamycin (not shown). The specific role of CHOP in mediating this stress-induced activation

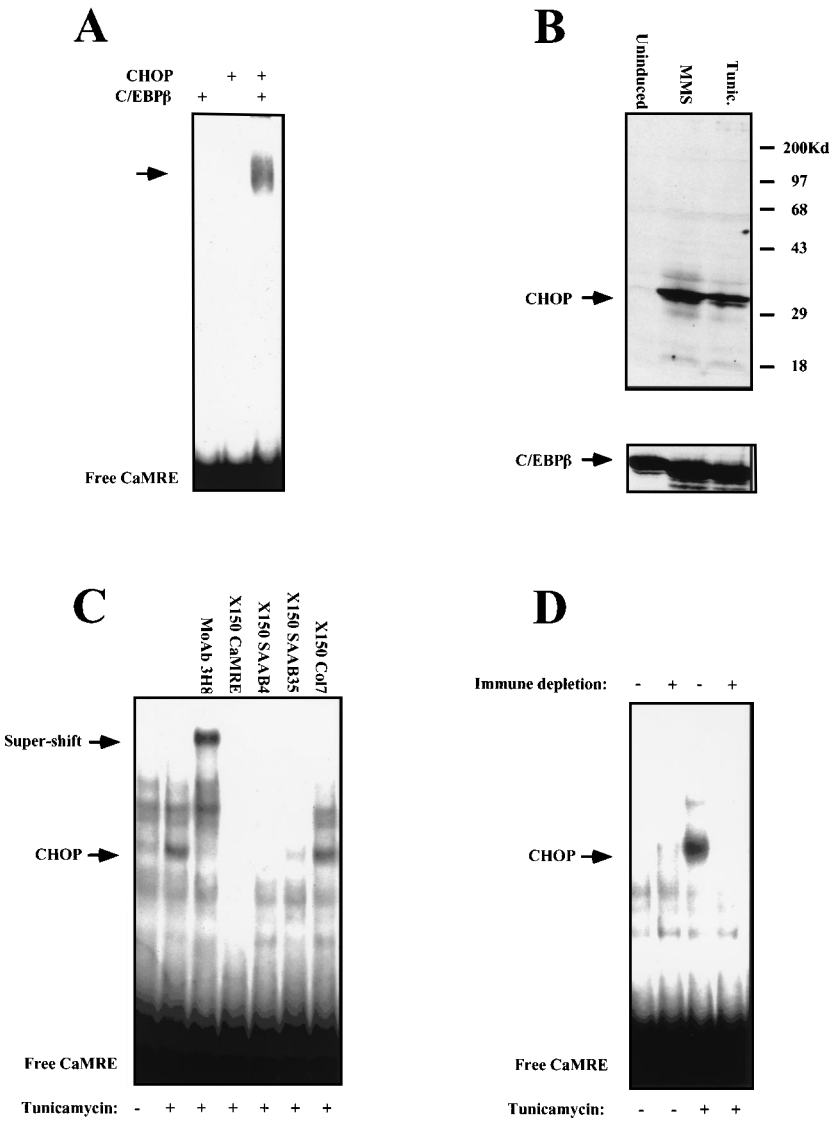

FIG. 6. CHOP, induced in vivo by stress, binds to a naturally occurring promoter element. (A) Heterodimers between CHOP and C/EBPβ bind to CaMRE, a naturally occurring region of the Rous sarcoma virus long terminal repeat that contains a CHOP-C/EBP heterodimer core motif. The bacterially expressed proteins used in the assay are indicated above the autoradiogram. Arrow, CHOP-C/EBPβ heterodimer. (B) Stress induces CHOP proteins in NIH 3T3 cells. Western blot (immunoblot) with the 3H8 monoclonal antibody to CHOP (upper panel) and polyclonal antiserum to C/EBPβ (lower panel) of extracts from uninduced cells, cells treated for 4 h with 100 μg of MMS per ml and 50 μg of tunicamycin (Tunic.) per ml. The positions of the proteins are indicated. (C) EMSA performed with nuclear extracts from untreated cells and cells treated with tunicamycin (50 μg/ml). The CHOP-containing complex is supershifted by the 3H8 monoclonal antibody (MoAb) to CHOP and is inhibited by an excess of the unlabeled CaMRE probe and other oligonucleotides containing the CHOP-C/EBP core binding motif (SAAB4 and SAAB35) but not the unrelated Col7 oligonucleotide. (D) Nuclear extracts from untreated and tunicamycin-stressed cells were immunodepleted with an unrelated monoclonal antibody affinity column (−) or an anti-CHOP, 3H8 affinity column (+) prior to EMSA with the CaMRE probe.

of the reporter gene is supported by the fact that mutation of key residues implicated in stress-induced phosphorylation of the protein abolishes transactivation by stress (data not shown). This line of experimentation supports the notion that CHOP has stress-inducible transactivation potential.

DISCUSSION

Despite an unusually structured basic region, CHOP is capable of binding as a heterodimer with C/EBPα and -β to specific DNA sequences. DNA binding by CHOP is dependent on the presence of a suitable C/EBP dimerization partner and requires the basic region of CHOP. The DNA sequences bound by CHOP-C/EBP heterodimers in vitro bear considerable resemblance to other known C/EBP sites, the sequence requirements of which are rather redundant (see below). The observation that C/EBPα and -β homodimers are also capable of binding to the sites selected by binding CHOP-C/EBPα heterodimers therefore comes as no surprise. Not all C/EBP sites, however, are functionally equivalent with respect to CHOP-C/EBP binding. We have previously demonstrated that a class of C/EBP sites such as the angiotensinogen gene APRE, the albumin promoter D site, and the fos promoter SRE, while serving as excellent target sequences for C/EBP proteins, will not bind CHOP-C/EBP heterodimers and, by dimerizing to C/EBP proteins, CHOP inhibits their binding to these target sequences (35). A different class of C/EBP sites, exemplified by the sequences selected in our assay can be bound both by

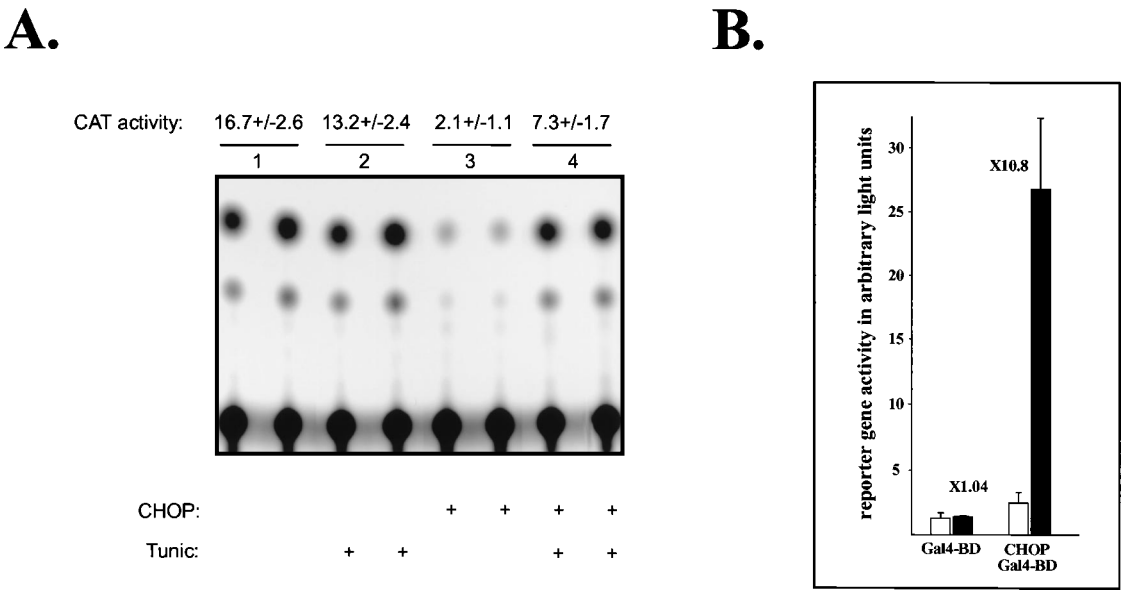

FIG. 7. Transcriptional activation by CHOP. (A) NIH 3T3 cells were transfected with a reporter gene consisting of the CaMRE linked to a minimal promoter driving CAT. In the absence of cotransfected CHOP, stressing the cells with tunicamycin (Tunic) has little effect on reporter gene activity (compare lanes 1 and 2). A cotransfected CHOP expression plasmid leads to a marked basal attenuation of the activity of the reporter gene (lane 3) and the acquisition of inducibility by stress (lane 4). Shown is a fluorogram of a typical experiment performed in duplicate and reproduced three times. (B) NIH 3T3 cells were transfected with a luciferase reporter gene under the control of two Gal4 binding sites. In the presence of the cotransfected CHOP-Gal4 reporter gene, activity is induced 10-fold by stress (compare open bars with solid ones). The Gal4 DNA-binding domain without CHOP had no such effect. The data are means and ranges of a typical experiment performed in duplicate and reproduced three times.

dimeric forms of C/EBP proteins and by CHOP-C/EBP heterodimers. We expect, therefore, that the occupant of such sites will depend, at any one time, on the relative affinities of the different dimers for the site and the relative quantities of the different dimeric forms in the nucleus.

In our in vitro binding assays CHOP-C/EBP heterodimers generally exhibited stronger binding to the selected sequences than C/EBP homodimers (Fig. 2B and 6B). This may be due to increased affinity of the heterodimer for the site but is also consistent with increased formation of C/EBP-CHOP heterodimers compared with C/EBP homodimerization. Coupled with the relative stability of CHOP-C/EBPβ heterodimers in vivo, these observations suggest that, if the natural binding sites of CHOP resemble the ones we have selected, they would, under conditions in which CHOP is induced, preferentially be occupied by CHOP-C/EBPβ heterodimers. The 39 DNA sequences randomly chosen from a pool selected by their binding to CHOP-C/EBP heterodimers show striking similarity to each other (Fig. 3). They have an invariant thymidine at position 4, a high preference for cytosine at positions 5, 11, 12, and 13, and a high frequency of purine residues at positions 1, 2, and 3. This finding is notable because C/EBP dimers are known to bind to a variety of sequence motifs that are remotely related and form only a general consensus motif (19). When a selection scheme similar to ours was recently applied to DNA binding by C/EBPα homodimers, a rather wide variety of sequences were identified, and they did not match the consensus defined by our CHOP-C/EBP dimers (19). Thus, we suggest that CHOP-C/EBP heterodimers are targeted to a select and relatively specific subset of genes that contain special C/EBP sites. The fact that all of the sequences selected for in our scheme bore a fixed relationship to the linkers used in constructing the oligonucleotides suggests that these linker sequences influenced the binding significantly. While this does not detract from the evidence in favor of the existence of CHOP target genes, the natural sites for CHOP-C/EBP heterodimers may be very different in sequence from the ones we selected.

Alignment of the basic region of CHOP with that of other bZIP proteins reveals that residues found to impart sequence-specific DNA recognition in the case of other bZIP proteins are the ones predicted to break the basic-region helical structure in CHOP (19, 41; also, see Fig. 2 in reference 35). Additional evidence that the mechanism of binding of CHOP-C/EBP heterodimers to their cognate motifs may differ from that of C/EBP dimers comes from the results of the methylation interference analysis. Alignment of the C/EBP site of the angiotensinogen gene (which does not bind CHOP-C/EBP heterodimers) with the CHOP-C/EBP selected sites, using the common CAA triplet as a reference point, demonstrates that the methylation interference patterns for the two classes of dimers are different. Methylation of the conserved guanosine on the sense strand of the SAAB consensus interfered with CHOP-C/EBP binding (Fig. 2D) but did not affect C/EBP dimer binding to APRE (Fig. 2a in reference 5). CHOP-C/EBP binding on the other hand, is indifferent to methylation of guanosine residues on the antisense strand, whereas C/EBP dimer binding is sensitive to methylation of that strand of APRE (Fig. 2a in reference 5). These results are consistent with different modes of protein-DNA interaction by the two kinds of dimers.

Analysis of the CHOP protein demonstrates that deletion of the leucine zipper or defects in the basic region block its ability to participate in DNA binding. This result suggests that the CHOP basic region plays a role in contacting DNA and in stabilizing the protein-DNA complex. The basic region of GCN4, a typical bZIP protein, has been demonstrated to traverse the major groove of the DNA as an uninterrupted alpha helix contiguous with the leucine zipper (14). In CHOP, the presence of proline and glycine residues in the center of the basic region makes it very unlikely that this domain retains the

structure of an uninterrupted alpha helix. However, it is notable that deletions in the portion of the basic region that is adjacent to the leucine zipper (mutants B and Cb) are deleterious to DNA binding, whereas deletion of the N-terminal portion of the basic region (*mutA*) had a less profound effect on binding (Fig. 5B). These findings are consistent with the possibility that the portion of the basic region between the leucine zipper and the helix-breaking prolines and glycines may in fact retain a structure that is similar to that found in other bZIP proteins and may be orienting the otherwise unusual structure of the CHOP basic region to a correct interaction with the target sequence. Further support for this idea comes from recent studies demonstrating that in the case of C/EBPα and GCN4 it is the C-terminal portion of the basic region that mediates the interaction with specific DNA residues (19, 41). The resemblance of a significant portion of this domain of CHOP to that of other bZIP proteins is notable (35).

In light of the fact that the CHOP basic region has a primary sequence so very different from all other known bZIP proteins, it remains possible that most of the contacts between the CHOP-C/EBP heterodimer and DNA are made by the C/EBP partner. In this respect the selected sequences may be thought of as containing a high-affinity C/EBP half-site in a context that is permissive for binding. In this scenario the CHOP component of the dimer may be contributing to only weak, backbone interactions with the DNA. Resolution of these questions will require a more detailed structural analysis of the interaction between CHOP and DNA.

Sequence specificity of DNA binding, demonstrated by competition in EMSA, methylation interference, and DNase I footprint analysis, as well as the observation that DNA binding by CHOP is dependent on an intact basic region, all point to a process with structural specificity and, hence, potential biological importance. The fact that CHOP-containing DNA-binding activity is induced in vivo in stressed cells indicates that DNA binding is not merely a property of the high concentration of bacterially expressed proteins in vitro. A functional role for DNA binding by CHOP is supported by the observation that mutations in CHOP that affect the basic region and consequently interfere with DNA binding also abolish the ability of the protein to induce cell cycle arrest (3). Similar deletions in the basic region of the oncogenic TLS-CHOP fusion protein abolish transformation (49). Finally, the fact that CHOP possesses a transcriptional activation domain which is inducible by stress suggests that, when brought into the proximity of an active promoter, CHOP is transcriptionally active. These results are most consistent with the existence of downstream effector genes that mediate CHOP action. Their identity and the mechanism by which they are regulated by CHOP and TLS-CHOP remain to be determined.

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