Interaction of CCAAT/enhancer-binding protein α and β with the rat caeruloplasmin gene promoter

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To determine the mechanisms of expression of the rat caeruloplasmin gene, the promoter region was analysed by DNAase I footprinting. Using nuclear extract from rat liver, a prominent site of protein–DNA interaction was detected from –93 to –48 upstream of the caeruloplasmin gene transcription start and sequence analysis of this region revealed three potential CCAAT/enhancer-binding protein (C/EBP) consensus elements. Mobility-shift analysis using an oligonucleotide encoding this region identified specific binding of proteins from rat liver nuclear extract, and some of these complexes were supershifted using antisera to the C/EBP α and β family members. Mobility-shift studies using a polypeptide encoding the DNA-binding domain of C/EBP α also revealed a specific interaction with this region of the caeruloplasmin promoter, and DNAase I footprinting

using this polypeptide protected the identical region from –93 to –48. Co-transfection of expression plasmids encoding C/EBP α or a related leucine-zipper factor D-binding protein (DBP) revealed a C/EBP-specific increase in reporter gene activity in HepG2 cells transfected with caeruloplasmin–chloramphenicol acetyltransferase containing the –93 to –48 region. A similar result was obtained when these constructs were co-transfected into mouse L cells which were shown not to express the endogenous caeruloplasmin gene. Taken together, these data indicate a role for C/EBP α and β in mediating transcription from the caeruloplasmin gene promoter and suggest that this region of the promoter is not responsible for tissue-specific expression.

INTRODUCTION

Caeruloplasmin is an abundant blue copper oxidase present in the sera of all vertebrate species where it accounts for more than 95% of copper found in plasma [1]. The precise biological roles of caeruloplasmin are unknown but may include functions in angiogenesis, coagulation, antioxidant defence, amine oxidation and copper and iron metabolism [2,3]. The protein is synthesized in the liver as a single-chain polypeptide and secreted as an α_2 -glycoprotein after the intracellular incorporation of 6 atoms of copper early in the biosynthetic pathway [3]. Although copper is essential for caeruloplasmin oxidase activity, hepatocyte copper content does not affect caeruloplasmin gene expression or biosynthesis and copper incorporation is not rate-limiting for the synthesis or secretion of apocaeruloplasmin [4,5]. In Wilson disease, a failure to incorporate copper into newly synthesized caeruloplasmin results in a marked reduction of serum holocaeruloplasmin concentration [6].

Although hepatocytes are a prominent site of caeruloplasmin biosynthesis, recent studies have demonstrated extrahepatic caeruloplasmin gene expression in several tissues [7]. Consistent with a proposed function in host defence, caeruloplasmin is an acute-phase protein and the serum concentration increases during inflammation, infection and malignancy. Although the contribution of each tissue site to the serum caeruloplasmin concentration is not known, this acute-phase response is regulated at the level of gene transcription in multiple tissues. Moreover, caeruloplasmin gene expression during development and inflammation is tissue-specific with regard to mRNA abundance and the response to cytokines [8]. This complexity of caeruloplasmin gene expression extends to the level of cell-type specificity where distinct endodermal and mesodermal derivatives have been shown to express the gene in liver and lung tissue.

To elucidate the mechanisms determining the regulation of caeruloplasmin gene expression, the rat gene was cloned and sequenced [9]. These studies revealed that the caeruloplasmin mRNA found in different tissues is identical in nucleotide sequence and the product of a single transcription start site. DNAase I footprinting, gel mobility-shift studies and transient expression analysis indicated that sequences 732 bp upstream from the start site were sufficient to confer tissue-specific expression. This specificity was in part conferred by a *cis*-acting element from –393 to –367 with partial similarity to the rat albumin D site. During the course of these studies additional regions of protein–DNA interaction were noted proximal to this sequence. The current study was undertaken to define further the role of these regions in caeruloplasmin gene expression.

MATERIALS AND METHODS

Materials

Chemicals and reagents used in this study were obtained from the following sources: seakem agarose from FMC Bioproducts; nylon membranes (Hybond-N) from Amersham (Arlington Heights, IL, U.S.A.); poly(dI-dC) and acetyl-CoA from Pharmacia (Alameda, CA, U.S.A.); silica plates from Baker (Phillipsburg, NJ, U.S.A.); protein assay reagents from Bio-Rad (Richmond, CA, U.S.A.); [¹⁴C]chloramphenicol from Du Pont–New England Nuclear (Boston, MA, U.S.A.); ³²P-labelled nucleotides from ICN Biochemical (Costa Mesa, CA, U.S.A.); lipofectin from Gibco BRL (Gaithersburg, MD, U.S.A.); DNA-modifying enzymes from Stratagene (San Diego, CA, U.S.A.); restriction enzymes from Promega Biotech (Madison, WI, U.S.A.); and additional chemicals from Sigma (St. Louis, MO, U.S.A.).

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; DBP, D-binding protein; NF κ B, nuclear factor κ B; Cp, caeruloplasmin.

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Figure 1 DNAase I protection of caerulein promoter using rat liver nuclear extracts

DNAase I footprinting was performed as described in the Materials and methods section using the caerulein gene sequence from -280 to $+17$ labelled on the 5' end of the non-coding strand and using increasing amounts of rat liver nuclear extracts (12.5–100 ng). After digestion, the reaction products were resolved on an 8% acrylamide sequencing gel, fixed, dried and autoradiographed. Dideoxynucleotide termination sequencing reactions were used as size standards, and the corresponding rat caerulein nucleotide sequences are shown diagrammatically.

Plasmids and construction of chimeric genes

Construction of the pOCAT-BS and pSV2CAT-BS vectors has previously been described [9]. Specific regions of the rat caerulein 5'-flanking sequence were amplified by PCR using oligonucleotide primers with synthetically introduced *Bam*HI sites and then subcloned into the *Bam*HI site of pOCAT-BS [10]. All subcloned DNA was sequenced by the dideoxy chain-termination method to determine orientation and to confirm the fidelity of the amplification [11].

Transfections and chloramphenicol acetyltransferase (CAT) assays

HepG2 cells were obtained as stock clones from the American Tissue Culture Collection and murine L cells were a gift from Dr. David Chaplin. Cells were maintained as monolayers in RPMI medium containing 10% (v/v) fetal calf serum, 100 units/ml

penicillin and 150 μ g/ml streptomycin. HepG2 cells were transfected as 70% confluent monolayers using 2.5 μ g of chimeric caerulein-CAT constructs and equal amounts of carrier DNA pBS KS (Stratagene) and 20 μ l of lipofectin. Lipofectin/DNA mixtures were left on cells for 5 h, then the cells were washed three times with PBS and re-fed with complete growth medium [12]. In some experiments, carrier DNA was replaced with expression plasmids for CCAAT/enhancer-binding protein (C/EBP) α [13] or D-binding protein (DBP) [14]. To control for efficiency of transfection, 1 ng of pSV- β gal was co-transfected into cells during all transfections. L cells were transfected using DEAE-dextran at 50% confluency as described [15].

Report gene assays

Cells were prepared 56 h after transfection by three cycles of freeze-thawing. Extracts were centrifuged and protein concentration was determined using the Bradford assay [16]. CAT activity was assayed as described [17] and percentage acetylation was determined by scintillation counting after chromatographic separation of acetylated and unacetylated chloramphenicol on silica plates. Under these conditions, percentage acetylation varied linearly with the amount of transfected construct DNA. Assays for β -galactosidase were performed using 30 μ l of extract in assay buffer containing *O*-nitrophenyl β -D-galactopyranoside, and substrate conversion was quantified by A_{420} [18].

Nuclear extracts and mobility-shift assays

Nuclear extracts were prepared from nuclei isolated from rat liver as described [19]. After dialysis, the protein concentration was determined and the extracts were divided into portions, frozen in liquid N_2 and stored at -80°C . Freshly thawed extracts were used for all experiments. Overlapping complementary oligonucleotide sequences were synthesized on an Applied Biosystems oligonucleotide synthesizer, annealed, labelled by filling in the overlapping regions using Klenow polymerase and [^{32}P]dCTP and used as probes in gel mobility-shift assays [20]. Binding reactions were performed at room temperature in 20 μ l of reaction solution containing 0.5 μ g of poly(dI-dC), 1 mg/ml BSA, 10% (v/v) glycerol, 10 mM Tris/HCl (pH 7.5), 50 mM KCl, 1 mM EDTA, 5 μ g of rat liver nuclear extract or 25 ng of the C/EBP90 polypeptide [21]. The labelled probe (1 ng) was added to the reaction last. Some reactions additionally contained 100 ng of unlabelled competitor DNA as indicated. Competitor DNA consisted of annealed oligonucleotides corresponding to sequences encoding the rat albumin D site (5'-TGGTATGATTTTGTAATGGGGTGGTA-3') [22] and the nuclear factor κ B (NF κ B) site of the human immunoglobulin light chain gene (5'-ACAGAGGGGACTTTCCGAGAGG-3') [23]. In some experiments, 1 μ l of diluted antiserum specific for C/EBP-related proteins [24] or non-immune serum was added to the reactions on ice and incubated at 4°C for 20 min before the addition of the labelled probe.

DNAase I footprinting analysis

Regions of the rat caerulein gene from -280 to $+17$ were prepared from recombinant plasmids [9], and the 5'-ends were labelled with T4 polynucleotide kinase and [γ - ^{32}P]ATP after phosphatase treatment of linearized vector [9]. All radiolabelled DNA fragments were separated by electrophoresis on native polyacrylamide gels, eluted in a buffer containing 1 M NaCl, 10 mM Tris/HCl (pH 7.5) and 1 mM EDTA, and purified by filtration through 0.2 μ m membranes and ethanol precipitation.

Figure 2 Gel mobility-shift assays using rat liver nuclear extracts and the caeruloplasmin promoter

Gel mobility shifts were performed with 5 μ g of rat liver nuclear extract as described using a 32 P-labelled -103 to -51 caeruloplasmin (lanes 1–12) or albumin-D-site (lanes 13–20) oligonucleotide. The reactions were performed as probe alone (lanes 1, 13), in the absence (lanes 2, 14) or presence of specific (lanes 3, 15) or non-specific (lanes 4, 16) competitor oligonucleotides. For antibody supershifts 1 μ l of diluted specific antiserum to C/EBP α (lanes 5, 9, 17), C/EBP β (lanes 6, 10, 18), C/EBP δ (lanes 7, 11, 19) or non-immune (N) sera (lanes 8, 12, 20) was added to the reaction before the addition of the probe.

Binding reactions were performed using 10000 c.p.m. of labelled probe and C/EBP90 or rat liver nuclear extract as indicated. After DNAase I digestion, the reaction was stopped and the digested fragments were electrophoresed on 8% polyacrylamide/urea sequencing gels [25]. Dideoxynucleotide termination sequencing reaction mixtures were co-electrophoresed for use as size standards.

RNA isolation and analysis

Total RNA was isolated from rodent tissue and cell lines by caesium chloride gradient centrifugation after dissolution in guanidinium isothiocyanate [26]. RNA samples were electrophoresed, transferred to nylon membranes and analysed by hybridization using [32 P]cRNA antisense probes as described [5,6].

RESULTS

Identification of a region of DNA–protein interaction by liver nuclear extracts

To identify potential *cis*-acting elements determining caeruloplasmin gene expression, DNA fragments corresponding to -280 to +17 of the promoter were analysed by DNAase I footprinting using rat liver nuclear extract. As can be seen in Figure 1, a prominent site of protein–DNA interaction was detected from -93 to -63 (Figure 1, lane 2). When the amount of nuclear protein was increased, the protected region extended to -48 (Figure 1, lanes 3–5). This same protected region was detected using nuclear extracts from newborn rat lung and HepG2 cells and was abolished by the addition of excess unlabelled DNA from this same region but not by an excess of an unrelated DNA region (results not shown). Analysis of the nucleotide sequence in this region of the caeruloplasmin gene promoter identified three regions with conservation of sequences shown to be essential for binding of the C/EBP-related proteins [24]. These specific regions are located at -93 to -83, -75 to

-66 and -60 to -51 from the transcription start site as indicated previously [9].

Interaction of C/EBP- α and β with the caeruloplasmin promoter

To determine which specific proteins were responsible for the DNAase I protection of the caeruloplasmin sequence, gel mobility-shift assays were performed using a 32 P-labelled oligonucleotide corresponding to -103 to -51 (Cp-103). Gel mobility-shift reactions with rat liver nuclear extracts resulted in the formation of complexes (Figure 2, lane 2) which were specifically competed for by an excess of the unlabelled probe (Figure 2, lane 3) but not by an unrelated competitor (Figure 2, lane 4). When the same reactions were performed in the presence of specific antibodies to C/EBP α , β and δ , supershifted complexes were observed with antisera to both C/EBP α and β (Figure 2, lanes 5 and 6) but not with antiserum to C/EBP δ or non-immune serum (Figure 2, lanes 7 and 8). Increasing the concentration of the antiserum in each reaction resulted in an increase in the amount of the supershifted complexes but still failed to produce a supershift with the C/EBP δ antiserum (Figure 2, lanes 9–12). A similar pattern was observed when the albumin-D-site oligonucleotide was used as a probe (Figure 2, lanes 13–20), confirming the specificity of these antibodies.

Caeruloplasmin sequences between -103 and -52 bind C/EBP *in vitro*

To directly demonstrate interaction of C/EBP with the caeruloplasmin promoter, gel mobility-shift experiments were performed using a C/EBP peptide (C/EBP90) containing the leucine zipper and DNA-binding domains of C/EBP α . Initially it was confirmed that this protein would specifically interact with a 32 P-labelled oligonucleotide corresponding to the rat albumin gene D site (Figure 3a, lanes 1 and 2). C/EBP90 was next used in gel mobility-shift reactions using 32 P-labelled oligonucleotides corresponding to regions of the caeruloplasmin promoter. Consistent with the footprinting data, specific complexes were

Figure 3 Mobility-shift analysis using the C/EBP90 polypeptide

(a) Gel mobility shifts were performed as described in the Materials and methods section using the indicated 32 P-labelled caerulein or albumin-D-site oligonucleotides with (+) or without (-) 25 ng of C/EBP90. After incubation, the reaction products were electrophoresed, fixed, dried and exposed to film. (b) Gel mobility shifts were performed as described in the Materials and methods section using the 32 P-labelled albumin-D-site (lanes 1–4) or caerulein -103 to -52 oligonucleotides as probes in the presence of 25 ng of C/EBP90. Competition studies were performed by including a 100-fold molar excess of the indicated competitor sequences in the binding reactions.

observed with oligonucleotides corresponding to -103 to -52 and from -76 to -25 of the caerulein gene (Figure 3a, lanes 3–6) but not using an oligonucleotide from -51 to +1 (Figure 3a, lanes 7 and 8). In each case the specificity of these mobility shifts was determined by competition with excess unlabelled oligonucleotide. Furthermore, binding of C/EBP90 to the albumin D site and to the -103 to -52 region was effectively competed for by an excess of unlabelled D site oligonucleotide, but not by an excess of the unrelated NF κ B

sequence (Figure 3b, lanes 1–8). These mobility shifts produced with C/EBP90 were also competed for by an excess of the unlabelled -103 to -52 caerulein sequence (results not shown).

C/EBP protects the identical site on the caerulein promoter

To further define the sequences within the caerulein promoter that interact with C/EBP, the region was subjected to DNAase I footprinting using a concentration range of C/EBP90. As seen previously using liver nuclear extract, at the lowest concentration only, the region from -93 to -63 was protected from DNAase I digestion (Figure 4a, lane 2). At higher concentrations of C/EBP90, the region of protection increased to include the sequence from -63 to -48 (Figure 4a, lanes 3–5). The specificity of this reaction was confirmed by using an excess of unlabelled caerulein sequence which effectively competed with binding (Figure 4a, lane 6). A nearly identical pattern of protection was found using C/EBP90 and 32 P-labelled probes for both the coding (Figure 4b, lanes 1 and 2) and non-coding strands (Figure 4b, lanes 3 and 4).

Caerulein-CAT gene constructs are transactivated by C/EBP

Thus far the data have demonstrated that C/EBP was capable of interacting with a specific region of the caerulein promoter *in vitro*. To determine if C/EBP was capable of functionally interacting with this region, transfection experiments were performed in HepG2 cells with a series of Cp-CAT constructs and an expression plasmid encoding C/EBP α (pMSV-C/EBP). As noted in previous studies, basal activity of these constructs in HepG2 cells was observed only when the region containing -393 to -367 was included (Figure 5a, lanes 1, 3 and 5). In contrast, a marked increase in reporter-gene activity was observed with each construct after co-transfection of C/EBP (Figure 5a, lanes 2, 4 and 6). In each case, this increase was entirely accounted for by sequences contained within the -103 region (results not shown). Surprisingly, transfection of this same -103 construct into mouse L cells resulted in a similar increase in reporter activity after co-transfection of C/EBP (Figure 5b, lanes 1 and 2). Despite the activity of the -103 construct in murine L cells, no endogenous caerulein gene expression was detected in these cells by RNA blot analysis under conditions that permitted detection of human and murine caerulein transcripts (Figure 6a, lanes 1–3). This remained so upon prolonged exposure of the blot (Figure 6b).

To examine the specificity of the C/EBP transactivation, control constructs containing a minimal simian virus 40 promoter (pA₁₀CAT), a promoterless plasmid pOCATBS or Cp constructs with the -103 to -52 region deleted (-42 Cp-CAT) or intact (-103 Cp-CAT) were co-transfected into HepG2 cells with expression plasmids for C/EBP or a related leucine-zipper transcription factor DBP (pCMV-DBP). As seen previously, no reporter-gene activity was observed in HepG2 cells transfected with C/EBP in the absence of the -103 to -52 sequence (Figure 7, lanes 1–8). Furthermore, whereas activation of the -103 construct was observed with C/EBP co-transfection, no such activation was observed after co-transfection with DBP (Figure 7, lane 10 compared with lane 11). Control experiments using the rat albumin D site confirmed that the pCMV-DBP plasmid was active in these cells (results not shown). In addition, transfections performed with expression plasmids for C/EBP β and C/EBP δ showed that these two proteins were also able to functionally transactivate the -103 construct in HepG2 cells (results not shown).

Figure 4 DNAase I protection analysis using the C/EBP90 polypeptide

(a) DNAase I footprinting was performed as described in the text using caeruloplasmin gene sequence from -280 to $+17$ labelled on the 5' end of the non-coding strand. Indicated amounts (ng) of C/EBP90 were included in the binding reactions. The reaction represented in lane 6 included 100-fold molar excess of unlabelled caeruloplasmin sequence. Dideoxynucleotide termination sequencing reaction mixtures were used as size standards, and the corresponding rat caeruloplasmin gene sequences are shown diagrammatically. (b) DNAase I footprinting was performed as described in the text using caeruloplasmin gene 5'-flanking sequences from -280 to $+17$ labelled on the 5' end of the coding (lanes 1 and 2) and non-coding (lanes 3 and 4) strands. Dideoxynucleotide termination sequencing reactions were used as size standards and the corresponding rat caeruloplasmin gene sequences are shown diagrammatically.

Figure 5 Transient expression of chimeric caeruloplasmin-CAT gene constructs in HepG2 and L cells

HepG2 (a) and L cells (b) were transfected with the indicated chimeric gene constructs and assayed for CAT activity as described in the Materials and methods section. The length of each construct is indicated, as is the absence (—) or presence (+) of co-transfected pMSV-C/EBP.

DISCUSSION

Previous study of the caeruloplasmin gene revealed that sequences 732 bp upstream of the start nucleotide were sufficient to confer tissue-specific gene expression [9]. Although few similarities to previously characterized *cis*-acting elements were found in this

region, a specific region of DNA-protein interaction from -393 to -363 was detected by DNAase I footprinting, mobility-shift assay and transient expression. Analysis indicates that this *cis*-acting element binds a nuclear protein present only in tissues expressing the caeruloplasmin gene. The present study extends the analysis of the caeruloplasmin gene promoter, examining a region of protein-DNA interaction closer to the transcription start site. As can be seen from Figure 1, this region from -93 to -48 is protected from DNAase I digestion by nuclear proteins from rat liver. The titration studies suggest that multiple proteins of differing affinities within the nuclear extract interact with this region; however, such analysis does not distinguish between protein-protein and protein-DNA interactions [27]. The nucleotide sequence of this region indicates multiple potential regions with similarity to the C/EBP consensus sequence [24,28], and the specific antibody studies reveal interaction of at least two of the C/EBP family members with this region (Figure 3).

C/EBP is a heat-stable DNA-binding protein which belongs to the basic domain-leucine-zipper (bZIP) class of transcription factors [29]. This protein was initially characterized from rat liver nuclear extracts and has subsequently been shown to be present in terminally differentiated cells of several tissues including liver, adipose tissue and lung [30]. A number of tissue-specific genes from these tissues contain *cis*-acting elements which interact with C/EBP, and co-transfection studies have demonstrated cell-specific transcriptional activation of reporter genes when C/EBP is overexpressed [31,32]. Subsequent to these initial studies,

Figure 6 RNA blot analysis of caeruloplasmin gene expression

Total RNA (15 µg) was isolated from the indicated sources, electrophoresed on a 0.7% agarose/formaldehyde gel, transferred to nylon and hybridized with a 32 P-labelled antisense cRNA probe for human caeruloplasmin. The blots were exposed to autoradiograph film for 4 h (a) or 18 h (b).

additional proteins were isolated with a high degree of similarity in the C-terminal DNA-binding domains. To date six unique gene members have been characterized including: C/EBP α [29]; C/EBP β [24] also identified as LAP [33], IL6DBP [34], AGP/EBP [35], NF-IL6 [36], CRP-2 [28] and LIP [37]; C/EBP δ [24] also identified as NFIL6 β [38], CRP-3 [28] and CELF [39]; C/EBP γ [4] also identified as Ig/EBP-1 [40]; CRP-1 [28]; and CHOP [41]. Ribosomal scanning of the C/EBP- β mRNA results in alternative start-site usage and thus two distinct protein transcripts from this gene [37]. On the basis of their ability to recognize the same DNA sequences and to form heterodimers as well as the amino acid homology in the bZIP regions, these proteins are referred to as the C/EBP family. Recently a C/EBP homologue has been characterized in *Drosophila* and shown to be essential for embryogenesis [42,43]. Multiple forms of C/EBP family members can be present in a single tissue or cell type

permitting formation of inhibitory and stimulatory heterodimers and allowing a functional redundancy and amplification of transcriptional control of any given gene by these factors [24,28].

The data presented in this study indicate that C/EBP α and β interact with *cis*-acting sequences within -103 to -52 of the caeruloplasmin promoter. The titration studies and the fact that mobility shift with the C/EBP90 polypeptide resulted in the formation of two specific complexes suggested that multiple functional binding sites are present in this region (Figure 3). Consistent with this observation, previous studies with oligonucleotides containing multiple C/EBP sites and recombinant C/EBP or nuclear extract have shown the formation of multiple retarded complexes in gel mobility-shift assays each of which can be supershifted with specific antibody [44]. The current data do not permit any conclusions about which C/EBP isoform interacts with which region or the precise role each sequence may play in regulating transcription from the caeruloplasmin promoter. Nevertheless, the lack of interaction of C/EBP δ with the same regions of DNA confirms the specificity of interaction with certain isoforms and is consistent with data indicating that C/EBP δ is expressed at low levels in liver tissue [24]. In view of published observations that C/EBP-binding sites are often localized in close proximity to binding sites for other transcription factors, it is likely that additional transcription factors may interact with this region either alone or by interacting with C/EBP directly [45,46]. Consistent with such a hypothesis, the complexes observed from rat liver nuclear extract (Figure 2) were not all supershifted by the C/EBP antibodies, and preliminary studies indicate functional HNF-3 sites localized to this same region (C. D. Bingle and J. D. Gitlin, unpublished work).

Although the -103 Cp-CAT construct has very minimal basal activity in HepG2 cells, this region was shown to be activated when co-transfected with the C/EBP α expression plasmid. This observation is consistent with previous studies indicating very low endogenous levels of C/EBP α in HepG2 cells [13]. The lack of additional activation by sequences upstream of -103 is also consistent with previous studies indicating that the *cis*-acting elements identified in this region do not interact with C/EBP *in vitro* [9]. The activation of this -103 construct in L cells suggests that this minimal promoter has lost tissue specificity and that a hepatocyte-specific interaction is not required for C/EBP-induced expression of the caeruloplasmin promoter. These data suggest that C/EBP activation of tissue-specific genes is highly promoter-context-specific and not due in all circumstances to cell-specific co-activators or inhibitors [47,48]. The regulation of caeruloplasmin gene transcription by

Figure 7 Effect of C/EBP and DBP on expression of chimeric gene constructs in HepG2 cells

HepG2 cells were transfected with the indicated chimeric gene constructs and assayed for CAT activity as described in the Materials and methods section. The specific constructs are indicated and, in some experiments, cells were co-transfected with pMSV-C/EBP (lanes 2, 4, 6, 8 and 10) or 10 µg of pCMB-DBP (lane 11) as indicated.

the C/EBP family members is specific in the region characterized here because the related liver-enriched transcription factor DBP did not induce reporter gene activation despite the ability of this factor to bind to and activate the albumin D site [14] (Figure 7). Studies using the individual C/EBP consensus sequences and site-directed mutagenesis will be needed to define further the precise role of each region in determining C/EBP-mediated caeruloplasmin gene transcription.

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