Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP

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We report the isolation and characterization of cDNA clones that encode a protein with the same DNA binding specificity as the immunoglobulin heavy chain enhancer binding protein E (μEBP-E). We call the gene encoding this protein Ig/EBP-1. A fusion protein encoded by the cDNA binds specifically to μEBP-E-binding sites (E sites) in both the IgH enhancer and the V_{HH} promoter. Sequence analysis reveals that Ig/EBP-1 is a member of the “basic-zipper” family of DNA-binding proteins that are characterized by basic regions and heptad repeats of leucine residues. Among known family members, Ig/EBP-1 demonstrates highest homology to C/EBP throughout the DNA-binding domain and leucine repeat region. Ig/EBP-1 and C/EBP have highly overlapping binding specificities; both cloned proteins bind to the IgH enhancer and the V_{HH} promoter E sites, and Ig/EBP-1 binds to previously characterized C/EBP binding sites in the Rous sarcoma virus (RSV) LTR and the murine albumin promoter. Consistent with their homology in the leucine repeat region, Ig/EBP-1 and C/EBP form heterodimers; Ig/EBP-1 is the first member of this family that has been found to heterodimerize with the well-characterized C/EBP. Ig/EBP-1 mRNA is present in all tissues and cell lines examined, although its levels vary almost 20-fold from different sources, with highest levels in early B cells. In tissues where Ig/EBP-1 and C/EBP are both present, heterodimers may be functionally important. The presence of Ig/EBP-1 in fibroblasts and other tissues where C/EBP is not expressed suggests that Ig/EBP-1 may be functionally important for the activity of the RSV enhancer in these cell types. Finally, elevated expression of Ig/EBP-1 in early B cells may explain in part the enhancer-independent activity of V_{HH} promoters early in B-cell development.

[Key Words: Immunoglobulin transcription; C/EBP; DNA-binding protein]

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Here, we report the isolation and characterization of cDNA clones encoding Ig/EBP-1, a polypeptide that displays μEBP-E binding specificity. Sequence analysis reveals that Ig/EBP-1 is a member of the basic-zipper family of DNA-binding proteins by virtue of the conservation of two motifs: a "basic region" and a "leucine heptad repeat" [Landschulz et al. 1988a]. Mutational analyses of other members of this family have shown these regions to be critical for DNA binding and protein dimerization, respectively (Gentz et al. 1989; Landschulz et al. 1989; Turner et al. 1989). Ig/EBP-1 contains striking sequence homology to the CCAAT/enhancer binding protein C/EBP in the basic and leucine repeat regions (Johnson et al. 1987; Landschulz et al. 1988b). Consistent with this homology, we show that both Ig/EBP-1 and C/EBP fusion proteins bind to the E sites in the IgH enhancer and that Ig/EBP-1 binds to the C/EBP–EII site in the Rous sarcoma virus (RSV) long terminal repeat (LTR; Sealy and Chalkley 1987; Ryden and Beemon 1989) and the C/EBP–DBP site in the murine albumin promoter (Lichtsteiner et al. 1987). We also show that Ig/EBP-1 forms heterodimers with C/EBP, it is the first protein known to be capable of doing so. Northern and RNase protection analyses show that, unlike C/EBP, which is expressed in only a few tissues [Birkenmeier et al. 1989], Ig/EBP-1 mRNA is present in all cell lines and tissues examined. The occurrence of Ig/EBP-1, but not C/EBP, in fibroblasts and other tissues suggests that Ig/EBP-1 may be an important activator binding to the EII site in the LTR of Rous sarcoma virus (RSV) and could help explain the activity of RSV in these cell types.

Results

Isolation and sequencing of cDNA clones for Ig/EBP-1 from L-cell and B-cell cDNA libraries

Because native μEBP-E is present in low abundance and the purified protein has a blocked amino terminus [C. Peterson and M. Bond, unpubl.], we chose to screen a L-cell cDNA expression libraries with IgH enhancer E site oligonucleotide probes for phage plaques expressing a β-galactosidase fusion protein with IgH enhancer E site-specific DNA binding activity [Singh et al. 1987; Vinson et al. 1988]. Initially an oligo(dT) and random-primed cDNA library from the plasmacytoma line P3XAg8.63 was screened; however, only nonspecific DNA-binding clones were obtained. We then screened 1.6 × 10⁶ recombinant phage from an unamplified cDNA library derived from murine L cells and found one clone, LE1, which was positive. Upon plaque purification, LE1 bound specifically to the IgH enhancer E site probe but not to heterologous probes (data not shown).

A map of phage LE1 is shown in Figure 2A. The clone is unusual in that 516 bp of the β-galactosidase gene has been deleted [ends at Val 834] and the 5' EcoRI restriction site has been lost. The cDNA insert is 780-bp long and contains a 3' poly(A) segment preceded by a canonical polyadenylation signal [AAUAAA] [Proudfoot and
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Figure 2. The structure of clones LE1, BE1, and the Ig/EBP-1 fusion protein. (A) The LE1 cDNA insert shown in the context of phage λgt11. (A) denotes the poly(A) tail. (B) A map of the overlapping EcoRI insert from pSK-plasmid BE1. Coding sequences are shown in cross-hatched box; 3′-untranslated sequence is a single line; vector sequences are represented in a dotted line. (C) Southwestern analysis of the Ig/EBP-1 fusion protein produced from phage LE1. The probe is concatamerized synthetic IgH enhancer E site oligonucleotide. LC21 denotes an extract made from a lysogen that expresses a fusion protein with an unrelated binding specificity. (+ and −) The presence or absence of IPTG induction.

Brownlee 1976]. The sequence predicts an open reading frame of 513 bp (171 amino acids) and a fusion protein of 115 kD; this was confirmed by Southwestern analysis (Fig. 2C).

Additional overlapping cDNA clones for Ig/EBP-1 were obtained by screening amplified P3X and ED20 (pre-B-cell) cDNA libraries with a subfragment of LE1. Several overlapping clones were isolated; one from the ED20 library [BE1] contained additional 5′ sequence (see Fig. 2B). We have not yet cloned the 5′ end of the mRNA or the translational initiation codons. The two cDNAs are identical within the overlapping sequence. However, they have different 3′ ends, apparently generated by differential polyadenylation; clone LE1 has 250 bp of 3′-untranslated sequence followed by a poly(A) tail, whereas clone BE1 has 480 bp of 3′-untranslated sequence and is not polyadenylated.

Recombinant Ig/EBP-1–β-galactosidase fusion protein binds specifically to E sites within the IgH enhancer and V₄₅ promoter

It was important to determine whether the protein encoded by our cDNAs had the same DNA binding properties as purified μEBP-E. Therefore, we tested the ability of the LE1 fusion protein to bind to the IgH enhancer. Figure 3A shows the results of an electrophoretic mobility shift assay [EMSA] in which LE1 fusion protein was used with an IgH enhancer probe. DNA binding activity is present only in IPTG-induced lysogen extracts and can be specifically decreased by competition with excess nonradiolabeled IgH enhancer E site oligonucleotide [E, Fig. 3A] but not heterologous oligonucleotides μE1 and μE3. We believe the high mobility bands from the fusion protein extract represent proteolytic fragments of the fusion protein that retain DNA binding activity because all the complexes are specifically decreased by competition and give the same footprint [Fig. 3B and data not shown]. The LE1 fusion protein also binds specifically to a V₄₅ promoter probe that contains the E site [second frame; the V₄₅ probe had lower specific activity than enhancer probe causing the shifted complex to appear fainter].

A more detailed analysis of the DNA–protein interactions was achieved with orthophenanthroline/copper (OP/Cu) chemical nuclease footprint analysis [Fig. 3B]. The LE1 fusion protein protects the same site in the enhancer [lanes 2 and 5] as purified μEBP-E [Peterson and Calame 1987]. The LE1 fusion protein also footprints the E site in the V₄₅ promoter [data not shown]. Although the LE1 fusion protein binds specifically to E binding sites, until we have the protein sequence of μEBP-E we cannot formally conclude that μEBP-E and the protein produced by the gene we have cloned are the same; therefore we refer to the gene we have cloned and the protein it encodes as “Ig/EBP-1” [Ig/Enhancer binding protein-1].

Ig/EBP-1 is a unique single copy gene

The copy number of Ig/EBP-1 was examined by Southern analysis using the LE1 cDNA insert as a probe; a single fragment hybridized to the probe in three different restriction digests [data not shown]. We estimate that the limit of homology detected under our conditions is 80% and conclude that there are no other genes closely related to Ig/EBP-1 on the nucleotide level.

The sequence of Ig/EBP-1 predicts a polypeptide which is a member of the “basic-zipper” family of DNA-binding proteins with particularly high homology to C/EBP

The predicted amino acid sequence of cloned Ig/EBP-1 is shown in Figure 4A. It is a member of the family of DNA-binding proteins characterized by a basic region and a heptad repeat of leucine residues, which includes Fos, Jun, C/EBP, ATF 1-8, CREB, GCN4, and many
Cloned Ig/EBP-1

Others (for review, see Landschulz et al. 1988a; Ziff 1990; Busch and Sassone-Corsi 1990 and references therein). Outside of these conserved domains, Ig/EBP-1 has no recognizable homology to any known member of this family. Mutagenesis of several members of this family has shown that the basic region is necessary for mediating DNA binding, and the leucine zipper is necessary for dimerization (Gentz et al. 1989; Landschulz et al. 1989; Turner et al. 1989); by analogy, we expect that this will also be true for Ig/EBP-1.

Cloned Ig/EBP-1 shares the highest amino acid sequence homology with C/EBP throughout the basic and zipper regions (Fig. 4B,C). The leucine zipper regions of Ig/EBP-1 and C/EBP bear striking sequence homology outside of the conserved leucine residues, particularly at the fourth and fifth amino acid residues. In an α-helical structure, these residues would flank the conserved leucine residues on one face of the α-helix; this surface has been proposed to form a hydrophobic dimerization interface between parallel helices (Landschulz et al. 1988a; O’Shea et al. 1989). There is also striking homology between Ig/EBP-1 and C/EBP in the basic region, where we note 20 of 26 amino acid identity and 22 of 26 identity, allowing for conservative changes (Fig. 4B). We also note that the consensus binding sequence for purified μEBP-E [TTNNGCAAT; Peterson et al. 1988] is similar to the consensus binding site of C/EBP [see Fig. 5A; Ryden and Beemon 1989]. We wished to explore this similarity between C/EBP and Ig/EBP-1 further by testing the ability of Ig/EBP-1 to bind to sites originally identified as C/EBP sites and vice versa.

Ig/EBP-1 and C/EBP bind to the same regulatory sites

We tested the ability of Ig/EBP-1 to bind to the well-characterized DBP site (-83/-117) in the murine albumin promoter [Maire et al. 1989] by competition in an EMSA with an IgH E site probe [Fig. 5B]. The Ig/EBP-1 fusion protein binding to the IgH enhancer site is decreased by competition with an oligonucleotide containing the C/EBP-DBP site, although ~10-fold less efficiently than by an IgH enhancer E site competitor. We conclude that Ig/EBP-1 binds to the C/EBP-DBP site in the albumin promoter but with lower affinity than when it binds to the IgH enhancer E site.

We also wished to test the ability of cloned Ig/EBP-1 to bind to the enhancer factor II (EFII) site in the RSV LTR [Sealey and Chalkley 1987; Ryden and Beemon 1989]. EII was identified originally as a protein from quail fibroblasts [Sealey and Chalkley 1987] that interacts with sequences between -193 and -231 in the RSV LTR that are critical for wild-type LTR function [Laimins et al. 1984; Cullen et al. 1985a,b; Norton and Coffin 1987; T. Ryden and K. Beemon, in prep.]. It was demonstrated subsequently that C/EBP binds to the EII site [Ryden and Beemon 1989]. Figure 5C shows the results of an EMSA in which the probe was a fragment containing the RSV EII site. These results demonstrate that C/EBP binds to the EII site [Ryden and Beemon 1989].
Figure 4. Sequence of Ig/EBP-1 and comparison to related proteins. (A) The sequence of Ig/EBP-1 from phage BE1. The open reading frame spans 218 amino acids. The sequence of LE1 protein also footprints the E site in the V.I promoter in a manner indistinguishable from that of Ig/EBP-1 fusion protein (Fig. 3B, lanes 1 and 5) for Ig/EBP-1 and C/EBP are nearly identical and the chemical nature of some of the flanking amino acid residues is also conserved (Fig. 6C). This observation and the fact that C/EBP forms homodimers prompted us to determine whether these two proteins were capable of forming heterodimers in vitro. Figure 6A shows that when a truncated C/EBP polypeptide was used in an EMSA with a probe containing a single Ig/EBP-1 enhancer E site, a high-mobility complex was observed (Fig. 3B, lanes 5 and 6). The C/EBP fusion protein also footprints the E site in the V.D promoter in a manner indistinguishable from that of Ig/EBP-1 (data not shown). We conclude that C/EBP binds specifically to IgE sites, although these data do not allow us to compare the affinity of the two proteins for these sites.

IgEBP and C/EBP from heteromultimeric complexes

When aligned in an α-helical wheel representation, the putative dimerization interfaces (formed by amino acids at positions 1 and 5) for Ig/EBP-1 and C/EBP are nearly identical and the chemical nature of some of the flanking amino acid residues is also conserved (Fig. 6C). These results demonstrate that both cloned Ig/EBP-1 and C/EBP fusion proteins are capable of forming heterodimers in vitro. Figure 6B shows that when the truncated C/EBP peptide was mixed with the Ig/EBP-1 fusion protein, a new gel mobility-shift complex (marked by an arrowhead) of intermediate mobility was observed in addition to the complexes for C/EBP and Ig/EBP-1 alone. Because intermediate complex formation is dependent on the presence of both C/EBP and Ig/EBP-1, it appears to represent a gel mobility-shift complex formed by a heteromultimer of the two proteins. Figure 6B shows that similar results were obtained using crude nuclear extracts from plasmacytoma P3X as a source of endogenous μEBP-E. Again, an intermediate gel mobility-shift complex is formed only when both proteins are present. Similar results were obtained using L-cell crude nuclear extracts as a source of endogenous μEBP-E [data not shown]. These results demonstrate that both cloned Ig/EBP-1 and endogenous μEBP-E are able to form heteromultimers with C/EBP and that these heteromultimers bind to the IgH enhancer E site. Because C/EBP (Landshulz et al. 1989) and other members of this family (Hope and Struhl 1985; Gentsz et al. 1989; Turner and Tjian 1989) have been shown to form dimers and because the mobility of the μEBP-E, Ig/EBP-1, and heteromultimeric complexes relative to the C/EBP complex is consistent with their being dimers, we assume that these heteromeric complexes are heterodimers. The demonstration that endogenous μEBP-E as well as cloned Ig/EBP-1 is capable of complexing with C/EBP
Cloned Ig/EBP-1

Figure 5. Ig/EBP-1 and C/EBP bind to the same sequences. (A) A comparison of sequences to which Ig/EBP-1 and C/EBP bind, the consensus sequence is shown underneath. (B) EMSA of the Ig/EBP-1 fusion protein with IgH PstI-RsaI 220-bp fragment. The top line indicates the competitors included in the binding reactions; the amount of IgH enhancer E, DBP, and μE1 site competitor included in each reaction is indicated above each lane. See Methods for more complete description of competitors. (C) EMSA of the Ig/EBP-1 fusion protein and the RSV LTR. Ig/EBP-1 fusion protein was incubated with the 210-bp EcoRI-Bsm36I fragment from RSV-SRD LTR. Binding reaction included: 0, no competitor; E, 10 ng of IgH E site competitor; R, 10 ng of EFII site (RSV) competitor; H, 50 ng of μE1 (Heterologous competitor). (D) EMSA of the Ig/EBP-1 fusion protein and the 220-bp RsaI-PstI IgH enhancer probe incubated with: 0, 1, 5, and 10 ng of EFII and IgH E site and 50 ng of μE1 site competitor as indicated above each lane.

Ig/EBP-1 is expressed ubiquitously

The similarity in binding specificity of Ig/EBP-1 and C/EBP and their ability to form heterodimers emphasizes the importance of determining the expression pattern of Ig/EBP-1 and comparing it with that of C/EBP. C/EBP mRNA is known to be present in a limited number of nonproliferating tissues [liver, adipose, small intestine, placenta, and lung]; its levels are low or absent in dividing cells [Birkenmeier et al. 1989; Xanthopoulos et al. 1989; S.L. McKnight, pers. comm.]. To determine steady-state levels of Ig/EBP-1 mRNA, the cDNA insert from LE1 was used as a probe in a Northern analysis [Fig. 7A]. The probe hybridized to an mRNA species ~4.75 kb in length, which is present in adult murine liver, L cells, and the plasmacytoma line P3X.

Subsequently, we used an RNase protection assay to better quantify the relative steady-state levels of Ig/EBP-1 mRNA from various sources. The design of the probe allowed 3' ends corresponding to both cDNAs to be individually quantitated [Fig. 7B]. Two protected fragments were anticipated—one of 480 bp, representing the longer 3'-untranslated form and one of 250 bp, representing the shorter 3'-untranslated form [Fig. 7C]. The results from a typical experiment showing the 480-bp fragment and control GAPDH are shown in Figure 7D. A summary of the levels of Ig/EBP mRNA is shown in Figure 7E.

The data confirm that Ig/EBP-1 RNA is present in all tissue types and cell lines examined; the levels vary almost 20-fold between the lowest (muscle) and the highest (pre-B line 22D6). There was, on average, a 4- to 5-fold higher level of Ig/EBP-1 mRNA in pre-B cells than in plasmacytomas. The protected fragment in WEHI 279 is slightly smaller than that seen from other tissues; we believe this is likely to be the result of a polymorphism in the Ig/EBP-1 gene in this cell line. The amount of the 250-bp fragment [shorter 3'-untranslated form] in different tissues varies, from nearly undetectable levels in some early B-cell lines to as much as 40% of the 480-bp Ig/EBP-1 fragment in HPC M2 plasmacytoma. It is not known if different 3'-untranslated ends for Ig/EBP-1 mRNA are functionally important although it is possible that they confer differential stability to the mRNAs.
Figure 6. Ig/EBP-1, μEBP-E, and C/EBP from heteromers. (A) EMSA of the Ig/EBP-1 fusion protein and the BR-A C/EBP peptide with an IgH enhancer E site probe. Uninduced LE1 lysogen extract (4 μg, -IPTG), and 2 and 4 μg of induced LE-1 lysogen extract (LE1) were mixed with 0, 1, 5, or 10 ng of BR-A C/EBP peptide as indicated. The probe used was a synthetic IgH enhancer E site oligo within the polylinker of pUC19. Arrows mark positions of new complexes. (B) EMSA showing titration of BR-A peptide into P3X crude nuclear extract. In the first four lanes, 4 μg of P3X extract was incubated with increasing amounts of BR-A peptide, as shown. Control lanes were BR-A mixed with 4 μg of crude bacterial extract in the indicated amounts. (C) A schematic α-helical representation of amino acid residues within the leucine zippers of Ig/EPB-1 and C/EBP. Identical or conserved amino acid residues are shown in capital letters, dissimilar amino acids in C/EBP are in lower case.

Discussion

We report the isolation and characterization of cDNA clones encoding a protein whose DNA binding characteristics and expression pattern correspond to that of a previously described transcription factor, μEBP-E. The gene encoding this protein, which we call Ig/EBP-1, appears to be a single-copy gene that encodes a member of the basic-zipper family of DNA-binding proteins. Ig/EBP-1 has high homology to C/EBP in the basic region, which contacts DNA, and the leucine zipper region, which is a dimerization domain. Consistent with the amino acid sequence conservation in the basic region, both Ig/EBP-1 and C/EBP bind to the IgH enhancer and Vh1 promoter E sites, the C/EBP–DBP site in the albumin promoter, and the EFII site in the RSV LTR. Consistent with the sequence similarities in the leucine zipper, Ig/EBP-1 and C/EBP form heterodimers in vitro. In contrast to C/EBP, Ig/EBP-1 mRNA is present in all cells tested, although relative levels vary by about 20-fold; highest levels are found in early B cells.

Ig/EBP-1 levels in B cells and the possible role of Ig/EBP-1 in enhancer-independent transcription from Vh promoters

In early B cells, low-level transcription occurs from unrearranged germ line Vh genes (Yancopoulos and Alt 1985); this transcription is believed to be important for targeting the locus for DNA rearrangement by making the Vh gene segments accessible to the VDJ recombinase machinery. Upon rearrangement, transcription from the rearranged Vh gene is activated by the IgH enhancer, and in later B cells there is no evidence of enhancer-independent germ line Vh transcription. Three factors, μEBP-E, μEBP-C2, and Oct-2, bind in both the IgH enhancer and Vh1 promoter. We speculated previously (Peterson et al. 1988) that elevated levels of one, or a combination, of these three factors in early B cells may allow enhancer-independent activity of Vh promoters, whereas limiting factor levels in later B cells would make Vh promoters enhancer dependent. This model assumes that enhancers function to concentrate required transcription proteins near the transcription initiation site, although it makes no predictions as to how this is achieved or how the proteins subsequently activate transcription.

By use of sensitive RNase protection studies, we now show that levels of Ig/EBP-1 mRNA are on average four to five times higher in early B cells than in plasmacytomas. Although we do not yet know the relative levels of Ig/EBP-1 protein or required threshold levels of the protein for IgH gene transcription, the elevated mRNA levels in early B cells are consistent with the possibility that increased levels of Ig/EBP-1 could be important for the enhancer-independent activity of Vh promoters in early B cells. Because Ig/EBP-1 mRNA levels are similar in pre-B-cell lines which do [40E3, 22D6, 38B9] and do not [300-10, 300-18] express germ line transcripts (Rakrishnan and Rosenberg 1988), additional factors must also affect enhancer-independent transcription.

Our data make it clear that Ig/EBP-1 is expressed very differently from C/EBP. Ig/EBP-1 mRNA is present ubiquitously, both in dividing cells and proliferating tissues as well as other tissues. These data are consistent with earlier EMSAs showing that μEBP-E binding activity was ubiquitous (Peterson and Calame 1987).
The possible role of Ig/EBP-1 in retroviral enhancer activity

Our data show that Ig/EBP-1 binds to the EII site in the LTR of RSV, a positive regulatory site that is essential for LTR enhancer activity (Laimins et al. 1984; Cullen et al. 1985a, b; Norton and Coffin 1987; T. Ryden and K. Beemon, in prep.). Although RSV is an avian retrovirus, the activity of the viral LTR in murine tissues is well documented (Luciw et al. 1983; Overbeek et al. 1986). EII-like sites occur in a number of other avian retroviral LTRs including ALV and in an internal RSV enhancer \(\text{gag}\) [Carlberg et al. 1988], and proteins have been reported that interact with these elements [Karnitz et al. 1987, 1989; Ruddel et al. 1988, 1989]. C/EBP has been shown to bind to the EII site in the RSV LTR, to related sites in several other retroviral LTRs [Johnson et al. 1987; Ryden and Beemon 1989], and to the RSV internal \(\text{gag}\) enhancer [Carlberg et al. 1988]. However, the limited pattern of C/EBP mRNA expression is inconsistent with the possibility that it mediates the enhancer activity of the RSV LTR or \(\text{gag}\) enhancers in all the murine tissues and cell types where these enhancers are known to be active [Overbeek et al. 1986; T. Ryden and K. Beemon, in prep.]. Given the ability of Ig/EBP-1 to bind the EII site and the fact that Ig/EBP-1 mRNA is expressed in cell types where the RSV LTR is active [Luciw et al. 1983], we postulate that Ig/EBP-1 may be important for the enhancer activity of RSV and murine retroviral LTRs [Johnson et al. 1987].

The relationship between Ig/EBP-1, C/EBP, and other family members

The overlapping DNA binding specificities of Ig/EBP-1 and C/EBP are striking and raise the problem of assessing which protein or proteins are functionally important in the regulation of genes where two or more proteins bind to one important regulatory site. Similar questions are posed by the growing number of proteins that bind to ATF sites [Hai et al. 1989], homeotic protein-binding sites [Han et al. 1989], and octamer sites [Scholer et al. 1989].

We have shown that Ig/EBP-1 is expressed in liver and
that it binds to the DBP site of the albumin promoter in vitro; however, we do not know if Ig/EBP-1 can activate albumin gene transcription in vivo. At least two other proteins, C/EBP and DBP (Mueller et al. 1990), are also known to bind to and activate the albumin promoter at this site; their expression is partially restricted to liver. Albumin promoter activity in vivo and occupation of the DBP site in vitro is liver specific and correlates with C/EBP mRNA expression (Lichtsteiner et al. 1987; Friedman et al. 1989); this indirectly suggests that Ig/EBP-1 by itself may not form a stable interaction at the albumin promoter in vivo. However, all the data are consistent with the possibility that Ig/EBP–C/EBP heterodimers bind at the DBP site to activate the albumin promoter. We have demonstrated the ability of Ig/EBP-1 and C/EBP to form heterodimers in vitro; Ig/EBP-1 is the only protein known to interact with C/EBP. Ig/EBP-1–C/EBP heterodimers may be functionally important in the context of the albumin promoter and other genes that appear to depend on C/EBP (for review, see Costa et al., 1988; Christy et al. 1989; Kaestner et al. 1989; McKnight et al. 1989) because we have found that heterodimers have a higher affinity for the albumin promoter DBP site than C/EBP homodimers (C. Roman and K. Calame, unpubl.). This may be similar to the situation reported recently for MyoD1, a tissue-specific master regulator of myogenesis (Weintraub et al. 1989), which forms heterodimers with ubiquitously expressed E47 (Murre et al. 1989b). Further exploration of the role of Ig/EBP-1 in the function of the albumin promoter may reveal important aspects of how transcription factors interact with one another to modulate transcription.

In tissues such as lymphoid tissues where C/EBP is not expressed, Ig/EBP-1–C/EBP heterodimers could not form, and Ig/EBP-1 alone may activate genes dependent on Ig/EBP-1 sites. However, C/EBP is not the only protein that shares considerable homology to Ig/EBP-1. There have been reports of other members of this C/EBP family of DNA-binding proteins [S. Akira et al. 1990; S. McKnight et al. 1989; McKnight et al. 1989] because we have found that heterodimers have a higher affinity for the albumin promoter DBP site than C/EBP homodimers (C. Roman and K. Calame, unpubl.). This may be similar to the situation reported recently for MyoD1, a tissue-specific master regulator of myogenesis (Weintraub et al. 1989), which forms heterodimers with ubiquitously expressed E47 (Murre et al. 1989b). Further exploration of the role of Ig/EBP-1 in the function of the albumin promoter may reveal important aspects of how transcription factors interact with one another to modulate transcription.

Methods

Isolation of Ig/EBP-1 recombinant phage

We used the screening strategy of Singh et al. [1988] with modifications introduced by Vines et al. [1988] to obtain one clone [LE1], which bound specifically to an oligomerized IgH enhancer E site oligonucleotide from a primary screen of 1.6 × 10^6 unamplified recombinant phage containing oligo(dT) and random-primed L-cell cDNA [Clontech]. An overlapping clone [BE1] was obtained by conventional nucleic acid hybridization by use of an EcoRI–StuI 0.6-kb fragment from the LE1 cDNA insert to screen an oligo(dT)-primed lambdaZAP [Stratagene] cDNA library from the pre-B-cell line ED20, subline of 18-81 [gift of Paul Rothman]. This fragment spans the sequences between an StuI site at nucleotide 366 [Fig. 3A] and the EcoRI site 3′ of the poly[A] tail. The phage L20 contains the DNA-binding and dimerization domain of C/EBP [Landschulz et al. 1988b; gift of S.L. McKnight].

Protein preparation

Lysogen extracts for LE1 and L20 were made as described in Landschulz et al. (1988b).

Southwestern analysis

Proteins in 50 μg of lysogen extract were separated by SDS/5%-PAGE [Laemmli 1970] and transferred to nitrocellulose in 0.025 M Tris base, 0.192 M glycine. Filters were then processed and incubated with oligomerized E site probe in the same manner as were the plaque lifts for DNA binding activity.

EMSAs and OP/Cu footprinting

Probes for footprinting were end-labeled by T4 polynucleotide kinase and gel-purified as described [Maxam and Gilbert 1977]. 1–3 × 10^4 and 3 × 10^4 cpm of probe [0.3–1.0 ng and 10 ng] were used per analytical and preparative EMSAs, respectively. Analytical gel mobility-shifts were performed by use of 2–4 μg of lysogen extract, 2 μg of poly[d1-C] [Pharmacia], and 50 ng of sheared salmon sperm DNA in μEBP-E binding buffer [10 mM Tris, 80 mM NaCl, 8 mM MgCl2, and 10% glycerol]. Competitors or water were preincubated for 10 min with protein at room temperature prior to the addition of probe. Binding reactions were incubated at room temperature for 20 min and loaded directly onto a 6% polyacrylamide gel and electrophoresed at 20 mA constant current in 1 × TBE [Petersen et al. 1988]. Preparative gel mobility-shifts for footprinting were performed with 40 μg of lysogen extract and 40 μg of poly[d1-C] in binding buffer in a total volume of 75 μl and incubated at room temperature for 30 min. After electrophoresis, gels were treated in situ with the chemical endonuclease OP/Cu as described [Kuwabara and Sigman 1987]. Free and bound complexes were visualized by autoradiography, cut out, and eluted from the gel. The DNA was then run out on a 10% sequencing gel with G and G + A ladders [Maxam and Gilbert 1977] and visualized by autoradiography.

The IgH enhancer and murine albumin promoter competitors used in this study were all ~200-bp fragments synthesized by PCR amplification using the M13 universal and pUC reverse primers to amplify multimerized binding site oligonucleotides inserted into the polylinker of a pUC plasmid. The DBP site competitor [the kind gift of S. Tilghman] contained 3 copies of the murine albumin DBP site (~83′–117′), the IgH enhancer E site oligo contained 4 copies of the sequence 324 to 343 of the murine IgH enhancer [Petersen and Calame 1987], and the IgH enhancer μE1 and μE3 competitors contained 4 copies of the murine IgH enhancer sequence 344 to 365 and 390 to 409, re-
spectively. The EFlI competitor oligo (the kind gift of M.G. Rosenfeld) was a 44-bp double-stranded oligonucleotide spanning sequences –231 to –193 from the RSV–SRD LTR.

Heterodimer formation between native μEBP-E or Ig/EBP-1 fusion protein with the C/EBP-BR-A peptide was monitored by the method of Hope and Struhl (1987). The BR-A peptide spans amino acids Asn281 through Ala358 (Landschulz et al. 1988a) and was overproduced and purified from Escherichia coli [J. Shuman and S.L. McKnight, in prep.]. Crude nuclear extract (4 μg) or lysozyme extract (2 μg) was incubated in the presence of 0, 1, 5, or 10 ng of the C/EBP peptide BR-A and 1 μg of acetylated BSA. The BR-A peptide and the indicated protein extracts were incubated in BR-A binding buffer [10 mM HEPES (pH 8.0), 10% glycerol, 0.1 mM EDTA, 0.25 mM DTT, and 2 mM MgCl2] for 5 min at room temperature prior to the addition of probe. Electrophoresis was performed in a 6% polyacrylamide gel in 0.5 × TBE at 10 V/cm constant voltage. For these experiments, the probe was an end-labeled 104-bp PCR fragment spanning the polylinker of pUC with one IGH enhancer E site oligo inserted into the HinClI.

Sequence analysis

Sequenceing of M13, pUC [BRL], and pSK-Stratagene containing subclones of the cDNA inserts was performed by Ulla Beauchamp in the DNA Sequencing Facility (Columbia University) using the dideoxy chain-termination technique with dye-conjugated primers and an ABI sequenator.

Northern and Southern analysis

Total RNA was isolated from pelleted tissue-culture cells or tissues frozen in liquid N2 by the guanidinium isothiocyanate method as described (Chirgwin et al. 1979). Polyadenylated RNA was selected on an oligo(dT) resin [BMB]. Formaldehyde gel electrophoresis was performed as described (Maniatis et al. 1982).

Genomic DNA [15 μg] from 18-81 DNA was digested to completion and run on a 1% agarose gel and blotted according to standard protocols. Northern and Southern blots were probed with the EcoRI–Stul 0.6-kb fragment of Ig/EBP-1 cDNA labeled by random priming [Feinberg et al. 1984].

RNase protection assays

The clone BE1 was used as a template for T7 polymerase [Stratagene] to generate an antisense riboprobe for Ig/EBP-1 expressed in nuclei. The EFII competitor oligo was an end-labeled 104-bp PCR fragment spanning the polylinker of pUC with one IGH enhancer E site oligo inserted into the HinClI.

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Note added in proof

We have demonstrated subsequently that the Ig/EBP-1 fusion protein also footprint the 3' SV40 core homology [site E:].

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