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 (μ EBP-E). We call the gene encoding this protein Ig/EBP-1. A fusion protein encoded by the cDNA binds specifically to μ EBP-E-binding sites (E sites) in both the IgH enhancer and the V_H 1 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_H 1 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_H promoters early in B-cell development.

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Transcription of immunoglobulin heavy chain (IgH) genes is controlled by two B-cell-specific transcriptional elements: an enhancer, located between I_H and C_μ gene segments in the first intron, and a promoter, located 5' of each heavy-chain variable (V_H) gene segment (Calame and Eaton 1988; Calame 1989). The IgH enhancer and V_H promoters contain multiple positive and negative regulatory elements that contribute to their B-cell-specific and developmental stage-specific activity (Grosschedl and Baltimore 1985; Mason et al. 1985; Ballard and Bothwell 1986; Kadesch et al. 1986; Wasylyk and Wasylyk 1986; Eaton and Calame 1987; Kiledjian et al. 1988; Tsao et al. 1988; Weinberger et al. 1988; Scheuerman and Chen 1989). Several proteins that bind

to these elements have been identified and characterized (Ephrussi et al. 1985; Landolfi et al. 1986; Peterson et al. 1986, 1988; Schlokat et al. 1986; Sen and Baltimore 1986; Staudt et al. 1986; Weinberger et al. 1986; Lenardo et al. 1987; Peterson and Calame 1987, 1989) and cDNAs encoding two of them, Oct-2 (Clerc et al. 1988; Staudt et al. 1988) and TFE3 (μ EBP-C2) (Beckman et al. 1990; C. Roman and K. Calame, in prep.) have been isolated. Here, we report the isolation and characterization of cDNA clones encoding a polypeptide with the same DNA binding properties as a third IgH enhancer binding protein, μ EBP-E (Peterson and Calame 1987; Peterson et al. 1988).

μ EBP-E is one of three known positive activators that bind with high affinity to both the IgH enhancer and V_H promoters (Fig. 1; Peterson et al. 1988). A second lower affinity site for μ EBP-E has also been mapped to one of

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Figure 1. Schematic representation of positive acting protein binding sites in the murine IgH enhancer and V_H1 promoter. Numbering for the V_H1 promoter is from Eaton and Calame (1987) and for the enhancer is from Ephrussi et al. (1985). Solid ovals (labeled Ig/E) represent mapped Ig/EBP- μ EBP-E binding sites (Peterson et al. 1988); the high affinity enhancer E site is centered on position 330, and the lower affinity site (E') maps to the most 3' SV40 enhancer core homology at position 475. Sites μ E1, μ E2, and μ E3 are protein-binding sites mapped originally in vivo on the IgH enhancer (Church et al. 1985; Ephrussi et al. 1985). Cloned proteins are represented by shaded ovals; other proteins are represented by empty ovals. Known proteins included in the enhancer diagram are μ EBP-B, which binds to the μ E1 site (Peterson and Calame 1987); μ E5-binding proteins (E12/E47, Murre et al. 1989a; ITF-1 and ITF-2, Henthorn et al. 1990); TF-E3 (Beckman et al. 1990; C. Roman and K. Calame, in prep.); μ B, a newly discovered B-cell-specific element (Libermann et al. 1990; Nelson et al. 1990); and Oct-2, octamer binding protein which binds the octamer site, 8, in the enhancer.

the SV40 enhancer core homologies in the IgH enhancer (site E', Fig. 1; Peterson et al. 1988). A deletion of the high affinity binding site for μ EBP-E in the IgH enhancer, subsequently referred to as the IgH enhancer E site, reduces enhancer activity to 36% of wild-type levels (Tsao et al. 1988). A mutation in the E site of the V_H1 promoter, which ablates μ EBP-E binding, reduces promoter activity $\sim$ 35% (C. Roman and K. Calame, unpubl.). Our goal is to understand how μ EBP-E contributes to the synergistic ability of IgH enhancer and promoter to activate transcription.

μ EBP-E was identified originally by in vitro DNA binding assays in which a portion of the IgH enhancer was used as a probe. Subsequently, μ EBP-E was purified to homogeneity and shown to be a heat-stable polypeptide of $\sim$ 45 kD. Purified μ EBP-E also bound with high affinity to two V_H promoters, in the immunoglobulin κ (Ig κ) intron enhancer just upstream of KE1 (Peterson et al. 1988), and in the T-cell receptor β enhancer (McDougall et al. 1988). μ EBP-E binding activity is present in all tissues and cell types examined (Peterson and Calame 1987; Peterson et al. 1988). The ubiquitous expression of the protein suggests it may play an important role in regulating transcription of genes in addition to IgH and Ig κ . We wish to identify such genes so that we may begin to assess the general importance of this transcription factor.

Here, we report the isolation and characterization of cDNA clones encoding Ig/EBP-1, a polypeptide that displays μ EBP-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-EFII 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 EFII 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 μ EBP-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 λ gt11 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 1.6×10^6 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' *Eco*RI 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

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 λ gt11. (A)_n denotes the poly(A) tail. (B) A map of the overlapping *Eco*RI 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 V_H promoter

It was important to determine whether the protein encoded by our cDNAs had the same DNA binding properties as purified μ EBP-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 μ E1 and μ E3. 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 V_H1 promoter probe that contains the E site (second frame; the V_H1 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 μ EBP-E (Peterson and Calame 1987). The LE1 fusion protein also footprints the E site in the V_H1 promoter (data not shown). Although the LE1 fusion protein binds specifically to E binding sites, until we have the protein sequence of μ EBP-E we cannot formally conclude that μ EBP-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

Figure 3. Ig/EBP-1 and C/EBP bind to E sites in the IgH enhancer and V_H1 promoter. (A) EMSA of LE1 induced lysogen extract using IgH enhancer and V_H1 promoter probes. All lanes contain 4 μg of induced lysogen extract and 50 ng of the indicated competitors. The EMSA shown in the first frame was carried out with the IgH enhancer 200-bp *Dra*-*Dde* probe. The EMSA in the second frame was carried out with the 130-bp *Hinf*I fragment from the V_H1 promoter as a probe. (B) OP/Cu chemical nuclease protection pattern of Ig/EBP-1 and C/EBP fusion proteins on the IgH enhancer. Lanes 1–3 show the nuclease protection pattern on the bottom strand of the enhancer that resulted for the 220-bp *Pst*I-*Rsa*I fragment of the IgH enhancer. Lanes 4–6 show the protection pattern on the top strand of the enhancer using a 112-bp probe that comprises 52 bp of pUC19 and the 60-bp *Dra*I-*Pst*I fragment of the enhancer. Lanes 1 and 4, free probe; lanes 2 and 5, probe complexed with Ig/EBP fusion protein; lanes 3 and 6, probe complexed with C/EBP fusion protein. Sequences of the footprints were derived from G and G + A ladders (Maxam and Gilbert 1977) of the probes run in adjacent lanes (not shown).

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 μ EBP-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). EFII 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 EFII site (Ryden and Beemon 1989). Figure 5C shows the results of an EMSA in which the probe was a fragment containing the RSV EFII site. These results demonstrate that the Ig/EBP-1 fusion protein binds specifically to the RSV LTR. Ig/EBP-1 fusion protein binding to the IgH enhancer is

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 begins at nucleotide 142 and ends at nucleotide 913, where it is polyadenylated. The polyadenylation signal begins at nucleotide 893. (B and C) Amino acid sequence comparison of Ig/EBP-1, C/EBP, c-fos, and c-jun. Identical or conserved amino acid residues are highlighted. (B) Comparison of the basic regions. (C) Comparison of the leucine-zipper regions.

decreased efficiently by competition with both E site and EFII site competitors (Fig. 5D). In addition, Ig/EBP-1 and C/EBP fusion proteins give indistinguishable footprints on the EFII site of the LTR (data not shown). Thus we conclude that cloned Ig/EBP-1 binds with similar af-

finity to the IgH enhancer E site and the RSV LTR EFII site.

Finally, we tested the ability of a C/EBP fusion protein (from a lysogen of phage L20, Landschulz et al. 1988b) to bind to the E site in the IgH enhancer. Cloned C/EBP gives an OP/Cu endonuclease protection pattern nearly identical to that of Ig/EBP-1 fusion protein (Fig. 3B, lanes 3 and 6), although the C/EBP fusion protein does not protect the top strand of the enhancer probe as well as does Ig/EBP-1 (cf. lanes 5 and 6). The C/EBP fusion protein also footprints the E site in the V_{H1} promoter in a manner indistinguishable from that of Ig/EBP-1 (data not shown). We conclude that C/EBP binds specifically to IgH E sites, although these data do not allow us to compare the affinity of the two proteins for these sites.

IgEBP and C/EBP form 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). 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 IgH enhancer E site, a high-mobility complex was observed ("–IPTG" lanes); two slower mobility complexes were obtained when the same probe was bound by Ig/EBP-1 fusion protein expressed by a LE1 lysogen (LE1 4 μ g, 0 C/EBP, lane 0). 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 μ EBP-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 μ EBP-E (data not shown). These results demonstrate that both cloned Ig/EBP-1 and endogenous μ EBP-E are able to form heteromultimers with C/EBP and that these heteromultimers bind to the IgH enhancer E site. Because C/EBP (Landschulz et al. 1989) and other members of this family (Hope and Struhl 1985; Gentz et al. 1989; Turner and Tjian 1989) have been shown to form dimers and because the mobility of the μ EBP-E, Ig/EBP-1, and heteromultimeric complexes relative to the C/EBP complex is consistent with their being dimers, we assume that these heteromultimeric complexes are heterodimers. The demonstration that endogenous μ EBP-E as well as cloned Ig/EBP-1 is capable of complexing with C/EBP

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 μ E1 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*–*Bsu36I* 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 μ E1 (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 μ E1 site competitor as indicated above each lane.

lends additional support to the proposition that Ig/EBP-1 encodes μ EBP-E.

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, μ EBP-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 μ g; -IPTG), and 2 and 4 μ g 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 μ g of P3X extract was incubated with increasing amounts of BR-A peptide, as shown. Control lanes were BR-A mixed with 4 μ g of crude bacterial extract in the indicated amounts. (C) A schematic α -helical representation of amino acid residues within the leucine zippers of Ig/EBP-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.

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 μ EBP-E binding activity was ubiquitous (Peterson and Calame 1987).

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, μ EBP-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 V_H 1 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 V_H promoters

In early B cells, low-level transcription occurs from unrearranged germ line V_H genes (Yancopoulos and Alt 1985); this transcription is believed to be important for targeting the locus for DNA rearrangement by making the V_H gene segments accessible to the VDJ recombinase machinery. Upon rearrangement, transcription from the rearranged V_H gene is activated by the IgH enhancer, and in later B cells there is no evidence of enhancer-independent germ line V_H transcription. Three factors, μ EBP-E, μ EBP-C2, and Oct-2, bind in both the IgH enhancer and V_H 1 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 V_H promoters, whereas limiting factor levels in later B cells would make V_H 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 V_H 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 (Ramakrishnan and Rosenberg 1988), additional factors must also affect enhancer-independent transcription.

Figure 7. Expression of Ig/EBP-1 RNA in murine cell lines and adult tissues. (A) Northern analysis of Ig/EBP-1 mRNA in representative tissues. Lanes 1–3 represent 5 μ g of poly(A)⁺ RNA from P3X63-Ag8 (P3X), L cells (L), and adult murine liver (Liver) hybridized with the Ig/EBP-1 cDNA and glyceraldehyde phosphate dehydrogenase cDNA (GAPDH; Fort et al. 1986) as a control. (B) Schematic representation of Ig/EBP-1 riboprobe and predicted protected products for RNase protection assay. Plasmid BE1 was linearized with *Bgl*III, and antisense run-off transcript riboprobe was produced by T7 polymerase. (C) Representative RNase protection assay showing both protected fragments from HPCM2 and EL4 cell line RNA with GAPDH control. (D) Representative RNase protection assay showing 480-bp protected fragments and GAPDH from various murine adult tissues and cell lines. (E) Bar graph summarizing RNase protection data. Values represent the average of two experiments except from sources followed by (1) where one experiment was performed; the average difference in values for two experiments was 15%. Filled bars represent levels of the long mRNA, and stippled bars represent the short RNA. For cells in which short mRNA represents 10% or less of the long form, no data is shown. Tissues: (A) adrenal gland; (B) brain; (F) adipose tissue; (H) heart (1); (K) kidney; (L and LC) liver; (Lu) lung (1); (M) muscle; (S) spleen. T cells: (a) EL4 (1); (b) BW5147 (1). L cells: (1). B cells: (a) 300-10; (b) 300-18; (c) 22D6; (d) 38B9; (e) 40E3; (f) WEHI 279; (g) HPCM2; (h) P3X63-Ag8 (1); (i) M603.

The possible role of Ig/EBP-1 in retroviral enhancer activity

Our data show that Ig/EBP-1 binds to the EFII 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). EFII-like sites occur in a number of other avian retroviral LTRs including ALV and in an internal RSV enhancer (*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 EFII 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 (*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 *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 EFII 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, pers. comm.; C.J. Chang and S.C. Lee, pers. comm.) that are unrelated to Ig/EBP-1 except in the region of DNA binding and dimerization. This is similar to the ATF and POU/homeo box families of DNA-binding proteins, which are related in amino acid sequence to each other and in the nucleotide sequences to which they bind (Herr et al. 1988; Hai et al. 1989). The existence of these clones suggests that the purified μ EBP-E described previously (Peterson et al. 1988) could be a mixed population of heterodimers. These data also suggest the potential complexity of how transcriptional regulation may be achieved through Ig/EBP-1 binding sites. It is likely that the members of the C/EBP family are able to bind related sequences with varying affinities; they may form a variety of heterodimers with differential binding affinities, transcriptional regulating activities, and stabilities. Thus it will be necessary ultimately to understand the tissue distribution, the ability to form heterodimers, and the functional activity of heterodimers formed by all the members of this growing family. As more genes are cloned, it will become possible to address these complex issues.

Methods

Isolation of Ig/EBP-1 recombinant phage

We used the screening strategy of Singh et al. (1988) with modifications introduced by Vinson 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*-*SstI* 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 *SstI* 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 μ g 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). $1-3 \times 10^4$ and 3×10^5 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 μ g of lysogen extract, 2 μ g of poly[d(I-C)] (Pharmacia), and 50 ng of sheared salmon sperm DNA in μ EBP-E binding buffer (10 mM Tris, 80 mM NaCl, 8 mM $MgCl_2$, 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 $1 \times$ TBE (Peterson et al. 1988). Preparative gel mobility-shifts for footprinting were performed with 40 μ g of lysogen extract and 40 μ g of poly[d(I-C)] in binding buffer in a total volume of 75 μ l 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 (Peterson and Calame 1987), and the IgH enhancer μ E1 and μ E3 competitors contained 4 copies of the murine IgH enhancer sequence 344 to 365 and 390 to 409, re-

spectively. The EFII 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 μ EBP-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 μ g) or lysogen extract (2 μ g) was incubated in the presence of 0, 1, 5, or 10 ng of the C/EBP peptide BR-A and 1 μ g 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, 5 mM KCl, 0.1 mM EDTA, 0.25 mM DTT, and 2 mM $MgCl_2$] for 5 min at room temperature prior to the addition of probe. Electrophoresis was performed in a 6% polyacrylamide gel in $0.5 \times$ 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 *HincII*.

Sequence analysis

Sequencing 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 N_2 by the guanidiniumisothiocyanate 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 μ g) 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*-*SstI* 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 transcripts according to the manufacturer's instructions essentially as described (Eaton and Calame 1987; Melton et al. 1984). Band intensities of autoradiographs with linear range exposures were quantitated by scanning densitometry with the LKB Ultrascan Densitometer XL. The rat GAPDH 1.2-kb *PstI* fragment cDNA (Fort et al. 1985) was inserted into pKS-(Stratagene) and also used as a template for T7 polymerase. Hybridization to this GAPDH probe yielded several bands due to cuts at polymorphic sites. The bands were shown to be specific for GAPDH, and band intensity increased linearly with increasing amounts of RNA within the context of the assay. The two major GAPDH bands were quantitated and averaged and did not vary significantly with respect to one another between cell types or experiments. BE1 (50,000 cpm) and GAPDH (5000 cpm) riboprobes were added from a mix of both probes to 50–100 μ g of total RNA.

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Note added in proof

We have demonstrated subsequently that the Ig/EBP-1 fusion protein also footprints the 3' SV40 core homology (site E').

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