The EMBO Journal vol.14 no.12 pp.2907–2916, 1995

EBF contains a novel zinc coordination motif and multiple dimerization and transcriptional activation domains

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Communicated by R.Treisman

Early B cell factor (EBF) was identified and cloned as a transcription factor expressed specifically in B lymphocytes and adipocytes. This protein was also identified as olfactory factor 1 (Olf-1) in olfactory neurons. In this study, we analyzed the structural requirements for DNA binding, homodimerization and transcriptional activation by EBF. A carboxyl-terminal region, containing a repeat of α-helices related to the helix–loop–helix motif, is important for dimerization of EBF in solution and can confer dimerization upon a heterologous DNA binding protein. The amino-terminal DNA binding domain by itself is monomeric, but can mediate assembly of dimers on optimized and correctly spaced half-sites. Mutational analysis of the DNA binding domain of EBF indicated that a novel zinc coordination motif consisting of H-X_3-C-X_2-C-X_5-C is important for DNA recognition. Deletion analysis and transfer of regions of EBF onto a heterologous DNA binding domain identified a serine/threonine-rich transcriptional activation domain. Moreover, the DNA binding domain of EBF can mediate transcriptional activation from optimized binding sites. Thus, EBF contains both a complex DNA binding domain that allows for dimerization and transcriptional activation, and additional dimerization and activation domains.

Key words: DNA binding domain/early B cell factor/transcription activation/transcription factor/zinc coordination motif

Introduction

Nuclear regulatory proteins can be classified into families on the basis of shared structural motifs (Pabo and Sauer, 1992). These motifs include domains involved in DNA binding, multimerization and interactions with other proteins. The biochemical characterization of many of these motifs has provided important insights into the mechanisms that underlie protein–DNA and protein–protein interactions. Moreover, the conservation of functional motifs has allowed for the identification of multiple members of protein families, which has provided insight into the diversity of transcriptional regulation during cellular differentiation and development.

Early B cell factor (EBF) was identified as a B lymphocyte-specific protein that recognizes a functionally important site in the *mb-1* promoter (Hagman *et al.*, 1991; Feldhaus *et al.*, 1992). The *mb-1* gene is expressed exclusively within the early stages of B lymphocyte differentiation and encodes the Igα protein, which functions both by anchoring membrane-bound Ig (mIg) in the plasma membrane and as an effector of intracellular signaling through the mIg surface receptor (Hombach *et al.*, 1988, 1990; Campbell and Cambier, 1990; Venkitaraman *et al.*, 1991; Matsuuchi *et al.*, 1992). A role for EBF in the regulation of the *mb-1* gene was inferred from a 4- to 5-fold decrease in *mb-1* promoter activity after mutation of the EBF binding site (Hagman *et al.*, 1991). In addition, multimerized EBF binding sites mediate transcriptional stimulation of heterologous reporter gene constructs specifically in cells containing EBF (Hagman *et al.*, 1991; Feldhaus *et al.*, 1992).

Purification of 65 kDa EBF polypeptides and isolation of cDNA clones encoding this protein revealed that its amino acid sequence was unrelated to other characterized DNA binding proteins, establishing EBF as the founding member of a novel, putative family of transcriptional activators (Hagman *et al.*, 1993; Travis *et al.*, 1993). A limited homology with the amino acid sequence of helix 2 of basic-helix–loop–helix (bHLH) proteins, however, was identified within a short 15 amino acid motif that is repeated and located near the carboxyl-terminus of EBF (Hagman *et al.*, 1993). Deletion of these α-helical repeats in EBF was found to markedly reduce DNA binding and dimerization in solution. DNA binding studies showed that homodimers of EBF recognize specific nucleotide sequences representing variations of an inverted repeat of a 5′-GGGAA/TT half-site separated by a 2 bp spacer (Hagman *et al.*, 1993; Travis *et al.*, 1993). Moreover, gel filtration experiments revealed that native EBF has a radius of gyration of a globular protein of 140 kDa, suggesting that EBF also exists as a dimer in solution (Travis *et al.*, 1993). DNA binding was found to be mediated by an extended domain in the amino-terminal half of EBF that does not resemble other known DNA binding domains.

EBF transcripts are detected in cell lines derived from B cells at the early stages of differentiation, but not in T cells or other hematopoietic cell lineages. Abundant levels of EBF transcripts are found in spleen and adipose tissues, and low levels in several non-lymphoid tissues (Hagman *et al.*, 1993). In olfactory neurons, a DNA binding protein with virtually identical specificity of sequence recognition was independently identified and termed Olf-1 (Kudrycki *et al.*, 1993; Wang and Reed, 1993). Cloning of cDNAs that encode rat Olf-1 revealed complete amino acid sequence

identity with murine EBF, with the exception of an eight amino acid insertion in EBF (Wang and Reed, 1993). Olf-1 was previously shown to bind to variations of the optimized recognition sequence of EBF that are present in the transcriptional control regions of several olfactory-specific genes (Kudrycki *et al.*, 1993; Wang and Reed, 1993; Wang *et al.*, 1993).

With the aim of gaining further insight into the functional and structural organization of EBF, we examined the requirements for DNA binding and transactivation. This analysis suggests that the extended DNA binding domain of EBF contains a novel zinc-coordination motif and sequences that mediate DNA-dependent dimerization and transactivation. Moreover, distinct dimerization and transactivation domains were identified in the carboxyl-terminus of the protein.

Results

EBF contains two distinct dimerization domains

We have previously shown that the carboxyl-terminal half of EBF contains two α-helical repeats that are important for recognition of an imperfect palindromic binding site in the *mb-1* promoter and for dimerization of EBF in solution (Hagman *et al.*, 1993). To determine whether the α-helical repeats solely mediate dimerization or whether other domains within EBF contribute to the formation of homodimers on DNA, we initially examined the binding of various EBF polypeptides to a perfect or imperfect palindromic site (Figure 1A). Wild-type EBF and mutant EBFΔH1 polypeptides lacking one of the α-helical repeats were generated by *in vitro* transcription/translation reactions and examined for DNA binding to ^{32}P-labeled oligonucleotide probes in an electrophoretic mobility shift assay. These and all subsequently used EBF polypeptides lacked amino acids 430–591 which were previously shown to contribute to the formation of higher-order multimers of EBF/DNA complexes (Hagman *et al.*, 1993). Binding of EBF(1–429) to the mb-1 probe, consisting of an imperfect palindrome (Figure 1B), formed a single complex which we have previously shown to consist of homodimers of EBF polypeptides (Hagman *et al.*, 1993). The level of DNA binding by the mutant EBFΔH1 polypeptides was reduced 75-fold relative to the level observed with wild-type EBF (Figure 1C, lanes 1 and 2). Moreover, a small amount of an EBFΔH1/DNA complex with faster mobility was detected, presumably consisting of DNA-bound monomers. This composition of the faster migrating complex was supported by evidence from chemical cross-linking studies which suggested that the EBFΔH1 polypeptides exist as monomers in solution (J.Hagman, data not shown). In contrast, complexes consisting of putative dimers were formed on the perfect palindrome (pal) probe with both the wild-type and EBFΔH1 polypeptides (Figure 1C, lanes 3 and 4).

The efficient formation of EBFΔH1 homodimers on a palindromic binding site is suggested by multiple lines of evidence. First, using a modification of the binding assay, ^{35}S-labeled EBFΔH1 polypeptides formed presumptive dimers on the unlabeled pal DNA probe despite a vast molar excess of the DNA probe relative to the amount of polypeptides as dimers (data not shown). By contrast, inefficient binding of ^{35}S-labeled EBFΔHI polypeptides as presumptive monomers was observed with the mb-1 DNA probe in a parallel experiment. Second, efficient DNA binding by EBF(1–251) polypeptides was observed on a pal DNA probe with correctly spaced (2 bp) half-sites, but not with a pal probe containing an additional 4 bp spacer (Figure 1D). These data suggest that the DNA binding domain of EBF is sufficient to bind DNA as a dimer in a binding site-dependent manner. Finally, with the aim of confirming that the slower migrating complexes contain dimers of EBF(1–251) on the pal probe, we mixed EBF(1–251) with a longer EBF(1–296) polypeptide prior to the addition of DNA. We observed complexes with a mobility intermediate to those formed with either of the two EBF polypeptides (Figure 1E). Together, these data indicate that the DNA binding domain of EBF contains sequences that mediate dimerization on correctly spaced palindromic binding sites, independent of the α-helical repeat domain. However, dimerization of EBF in the absence of DNA or efficient binding to an imperfect palindrome site requires the α-helical repeats.

To determine whether the α-helical repeat region of EBF represents an independent dimerization domain, we examined its potential to confer dimerization upon a heterologous DNA binding protein. Like EBF, the nuclear hormone receptor for estrogen (estrogen receptor, ER) requires dimerization for efficient DNA binding to an inverted repeat of a short nucleotide sequence (ERE). Dimerization of ER was previously shown to be mediated by a carboxyl-terminal 21 amino acid region that is non-overlapping with the DNA binding domain (Lees *et al.*, 1989,1990). We constructed an ER–EBF fusion gene encoding a chimeric polypeptide in which 63 amino acids of EBF (amino acids 367–429) are linked to the carboxyl-terminus of a truncated ER (amino acids 121–384) lacking its own dimerization domain (Figure 2A). We generated chimeric ER–EBF polypeptides by coupled *in vitro* transcription/translation reactions and examined DNA binding to an ERE probe in an electrophoretic mobility shift assay. As controls, we generated ER polypeptides that either lacked a dimerization motif or contained the 21 amino acid dimerization motif of ER. Consistant with previous data (Lees *et al.*, 1989, 1990), the truncated ER did not bind to the ERE at any detectable level (Figure 2B, lane 2). Addition of the ER dimerization motif to the truncated ER polypeptide allowed for DNA binding at a high level (lane 3). Likewise, the addition of the α-helical repeat region of EBF augmented DNA binding by ER to a similar level (lane 4). Mixing experiments performed with ER–EBF(367–429) and ER–EBF(367–591) suggested the formation of dimers (data not shown). Therefore, we conclude that the α-helical repeats of EBF can mediate dimerization of a heterologous DNA binding protein.

Mutagenesis of EBF defines a novel metal binding motif that is required for DNA binding

Deletion of amino acids 1–50 had only a minor effect on DNA binding by EBF (Hagman *et al.*, 1993), suggesting that the minimal DNA binding domain is contained within amino acids 51–251. The carboxyl-terminal 100 amino acids of this domain include several cysteine and histidine residues (Figure 3A). Although the amino acid sequence of EBF could not be aligned with consensus metal binding motifs, e.g. zinc fingers, the array of seven cysteine

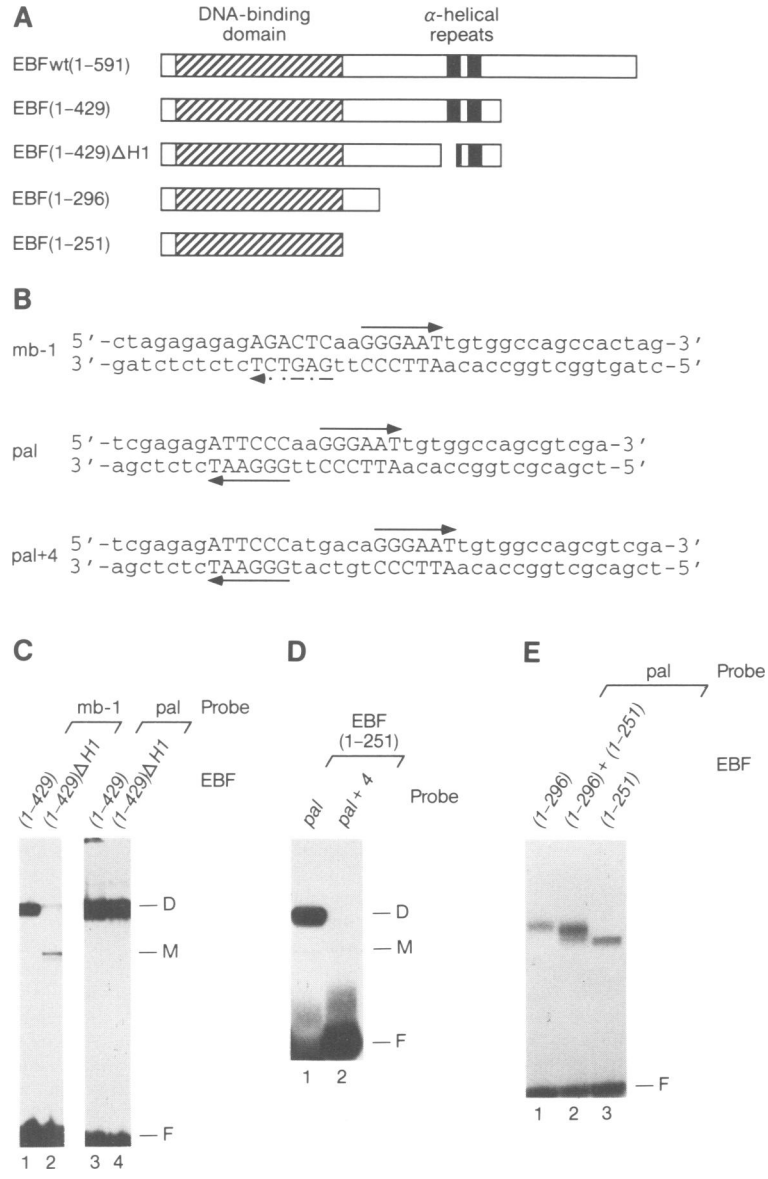

Fig. 1. Dimerization of the DNA binding domain of EBF on a palindromic binding site. (**A**) Schematic structure of full-length EBF and various carboxyl-terminal deletion proteins. The DNA binding domain of EBF is indicated by the hatched box. The two α-helical repeats are indicated by black boxes. The numbers indicate the amino acid positions in the EBF protein. EBF(1–429)ΔH1 contains an internal deletion of amino acids 370–383. (**B**) Sequences of synthetic oligonucleotides used as probes in electrophoretic mobility shift assays. Base pairs that form the half-sites recognized by EBF (Hagman *et al.*, 1993; Travis *et al.*, 1993) are indicated by upper case letters. Non-disrupted arrows indicate optimal EBF binding half-sites, whereas dots indicate nucleotides that differ from the consensus sequence. (C–E) Electrophoretic mobility shift assays with ^{32}P-labeled probes as indicated and truncated forms of recombinant EBF as shown in (A). Recombinant variants of EBF were synthesized by programming rabbit reticulocyte lysate with synthetic RNA transcripts in the presence of unlabeled methionine *in vitro*. The positions of complexes containing dimeric (D) or monomeric (M) forms of EBF protein, and the position of free (F) DNA probe, are indicated at right. (**C**) Requirement for the α-helical repeats for dimerization of EBF is dependent on the binding-site sequence. (**D**) Dimerization of the isolated EBF DNA binding domain requires an optimal spacing of binding half-sites. (**E**) Dimers of the DNA binding domain of EBF assemble on pal probe DNA. Synthesized RNA encoding EBF(1–296) and EBF (1–251) was mixed following separate translation *in vitro* (lane 2).

residues between amino acids 151 and 198 and four histidine residues between amino acids 157 and 240 suggested a possible role of these amino acids for DNA binding by EBF. By oligonucleotide-directed mutagenesis, we changed each cysteine in the DNA binding domain individually to serine, which cannot participate in the coordination of metal ions. Likewise, histidine residues were mutated individually to the neutral amino acid alanine. Each mutation was introduced into a gene construct encoding EBF(1–429), and DNA binding of *in vitro* transcribed/translated polypeptides was examined using both the mb-1 and pal probes (Figure 3B). The relative level of binding of wild-type EBF(1–429) to the pal probe is eight times more efficient than binding to the mb-1 probe and, therefore, the exposure times of autoradiographs were adjusted to facilitate comparison. The levels of *in vitro* synthesis of the polypeptides were shown to be equivalent by SDS–PAGE analysis of parallel translation reactions containing [^{35}S]methionine.

Mutation of the cysteines C161, C164 and C170 to serines and mutation of histidine residues H157 and H235 to alanines abrogated binding to either DNA probe.

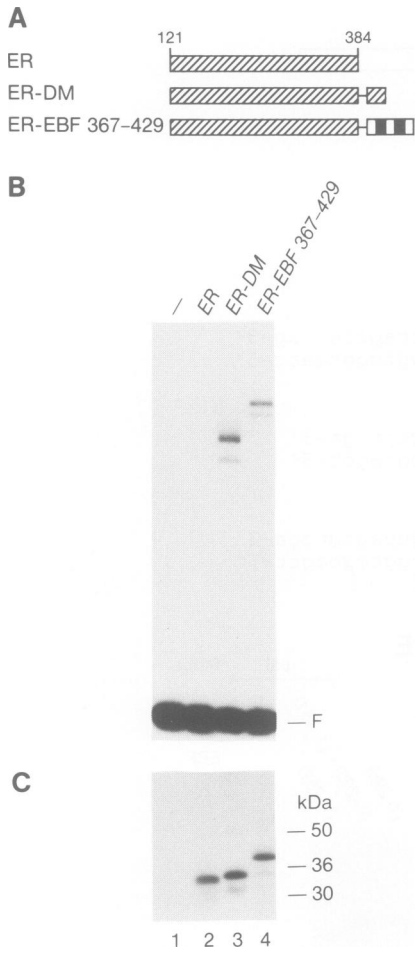

Fig. 2. The α-helical repeats of EBF mediate dimerization of estrogen receptor. (**A**) Schematic structure of truncated ER and ER–EBF fusion proteins. ER sequences are indicated by hatched boxes. The two α-helical repeats are indicated by black boxes. The numbers indicate the amino acid positions in the EBF protein. (**B**) Electrophoretic mobility shift assays with ^{32}P-labeled ERE probe and recombinant proteins as shown in (A). Recombinant ER and ER–EBF proteins were synthesized by programming rabbit reticulocyte lysate with synthetic RNA transcripts in the presence of unlabeled methionine *in vitro*. The position of free (F) DNA probe is indicated at right. (**C**) ER and ER–EBF proteins were synthesized in similar amounts. Translation reactions were performed in parallel with ^{35}S-labeled methionine and labeled proteins were fractionated using SDS–PAGE.

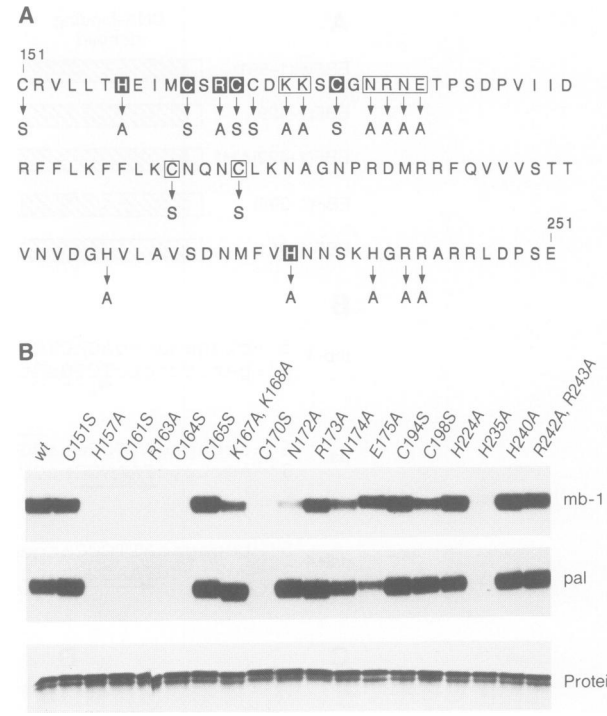

Fig. 3. Mutations in the DNA binding domain of EBF have differential effects on the binding of dimeric EBF to the mb-1 and pal DNA probes. (**A**) Mutations were introduced into EBF in the context of amino acids 1–429. The wild-type EBF amino acid sequence is depicted with individual mutations shown below the altered residues. Boxes indicate the effects of mutations on binding to the DNA probes as shown in (B). Black boxes indicate the complete loss of DNA binding to either DNA probe. Open boxes indicate mutations which show binding site-specific effects on DNA binding. (**B**) DNA binding of dimers of EBF to the mb-1 and pal DNA probes. Upper two panels: electrophoretic mobility shift assay with ^{32}P-labeled oligonucleotide probes as indicated at right and recombinant EBF proteins containing various mutations shown in (A). Lower panel: wild-type and mutated EBF proteins were synthesized in similar amounts. Translation reactions were performed in parallel with ^{35}S-labeled methionine and labeled proteins were fractionated using SDS–polyacrylamide electrophoresis.

These amino acids are likely to have an essential role in maintaining the structural integrity of EBF, or may provide side chains for contacts with DNA. Mutation of C198 to serine reduced binding to the mb-1 probe 2-fold, but binding of the mutant protein to the pal probe was unaffected. Finally, the mutations C151S, C165S, C194S, H224A or H240A had no effect on binding to either DNA probe, indicating that these amino acids have a minimal role for DNA binding by EBF.

The amino acids H157, C161, C164 and C170, which are essential for DNA binding, can be represented as a loop consisting of H-X_3-C-X_2-C-X_5-C stabilized by coordination of a central divalent metal cation(s) (Figure 4A). Although different from the consensus sequences of zinc-finger domains, this putative structural motif shares some similarity with the metal binding domains of nuclear hormone receptors. Because non-coordinating amino acids in zinc binding structures are often involved in protein–DNA and/or protein–protein contacts (reviewed in Pabo and Sauer, 1992), we mutated additional amino acids within or flanking the putative metal coordination motif. We examined the effects of these mutations on binding of EBF(1–429) to either the mb-1 or pal DNA probes. Mutation of arginine 163 to alanine (R163A) decreased the interaction with either DNA probe to <1% of the level observed with wild-type EBF binding (Figure 3B). Mutation of the lysine residues in the putative loop (K167A and K168A) resulted in a 3-fold decrease in DNA binding to the mb-1 probe, but had no effect on binding to the pal sequence. Mutation of asparagine 172 or 174 to alanine decreased DNA binding to the mb-1 probe 7- and 3-fold, respectively, but neither mutation affected binding to the pal probe. Interestingly, mutation of glutamic acid 175 to alanine (E175A) resulted in decreased binding to the pal probe, but not to the mb-1 probe. Mutations of several other amino acids did not affect DNA binding by EBF. Taken together, the mutagenesis data indicated that a novel putative metal coordination motif is required for DNA binding by EBF.

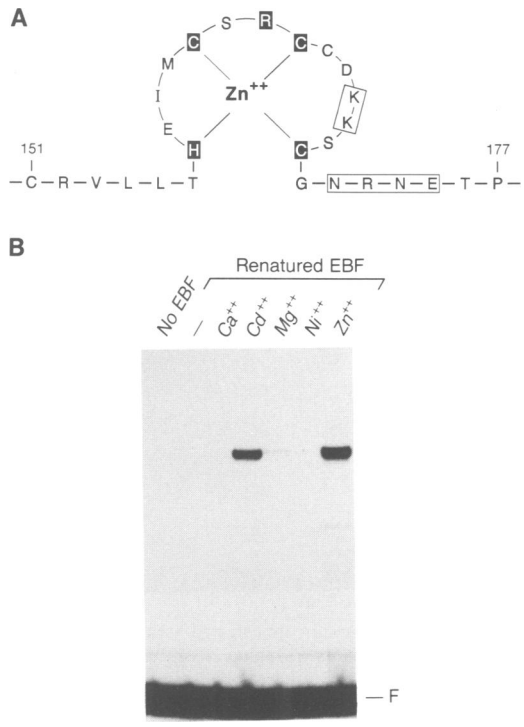

Fig. 4. EBF is a zinc binding protein. (**A**) Hypothetical structure of the zinc binding motif of EBF. The positions of amino acids shown (in Figure 3) to be essential (black boxes) or to have binding site sequence-specific effects (open boxes) on DNA binding are indicated. (**B**) Restoration of DNA binding by EBF requires zinc ions during renaturation *in vitro*. Electrophoretic mobility shift assay of binding to the ^{32}P-labeled pal probe by recombinant EBF subjected to denaturation and renaturation in the absence/presence of divalent metal cations. EBF(1–429) was denatured and renatured in the presence of 10 μM metal ions as indicated above. Relative levels of DNA binding were quantitated using a Molecular Dynamics PhosphorImager. Free probe is indicated at right (F).

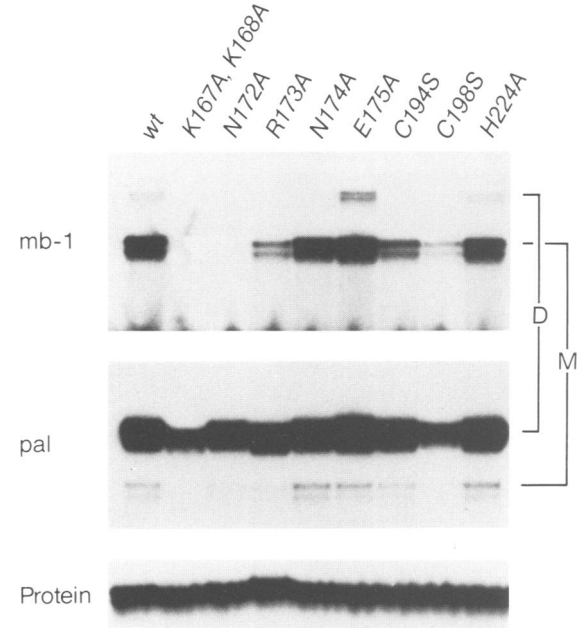

Fig. 5. Differential effects of mutations on the binding of the isolated DNA binding domain of EBF to the mb-1 and pal probes. Upper two panels: recombinant EBF(1–251) containing mutations as indicated above were tested for DNA binding ability using the electrophoretic mobility shift assay as described in Figure 3 and probes as indicated at left. Protein–DNA complexes containing dimers (D) or monomers (M) of EBF are indicated at right. Bands appear as doublets due to the utilization of two closely-spaced translation initiation codons during translation *in vitro*. Lower panel: wild-type and mutated EBF(1–251) proteins were synthesized in similar amounts. Translation reactions were performed in parallel with ^{35}S-labeled methionine and labeled proteins were fractionated using SDS–polyacrylamide electrophoresis.

Zinc is required for DNA binding by EBF

To determine whether DNA binding by EBF requires metal cations, notably zinc, recombinant EBF(15–429) was purified from *Escherichia coli* and denatured in 8 M urea, followed by dialysis against 6 M guanidine hydrochloride in the presence of excess EDTA and EGTA. Renaturation was accomplished by dialysis against the same guanidine solution without EDTA and EGTA and subsequent dialysis against decreasing amounts of guanidine hydrochloride in the presence of various divalent metal cations. The renatured EBF polypeptides were tested for DNA binding to the pal probe (Figure 4B). Abundant DNA binding was dependent upon the inclusion of Cd^{2+} or Zn^{2+} during renaturation (lanes 4 and 7), whereas only a low level of binding was observed with Mg^{2+} (lane 5). Thus, Zn^{2+} ions are essential for DNA binding activity, suggesting the presence of a metal coordination domain in EBF.

Effects of mutations on DNA binding of EBF monomers

The mutational analysis was performed in the context of EBF(1–429). This protein contains the α-helical repeats which may obscure effects of mutations on dimerization of the DNA binding domain. Therefore, we analyzed a select set of mutants in the context of only the DNA binding domain of EBF (amino acids 1–251) for binding to the mb-1 and pal probes (Figure 5). Consistent with the previous experiments, the ratio of DNA binding by dimers versus monomers was 1:15 with the mb-1 probe and 35:1 with the pal probe. Notably, the effect of several mutations on binding to the mb-1 probe was significantly more pronounced in the context of EBF(1–251) than in the context of EBF(1–429). In particular, mutation of asparagine 172 to alanine (N172A) abrogated DNA binding by EBF(1–251) to the mb-1 probe, but had no effect on binding to the pal probe. Likewise, mutation of two lysine residues (K167A, K168A) prevented DNA binding by EBF(1–251) to the mb-1 probe, but decreased binding to the pal probe by only 5-fold. Mutation of arginine 173 (R173A) or cysteine 198 (C198S) decreased binding of EBF(1–251) to the mb-1 probe 4- and 7-fold, respectively, with smaller effects on binding to the pal probe. With the exception of the N174A mutation, which selectively decreased dimer binding to the mb-1 probe, most mutations affected binding of both monomers and dimers to DNA. The E175A mutation, which decreased binding only to the pal probe in the context of EBF(1–429), did not appreciably affect the binding of EBF(1–251). Overall, these data identified amino acids K167, K168, N172 and C198 as residues that are important for the binding of monomers to an imperfect palindromic site, but do not contribute to dimer formation on the perfect palindromic site.

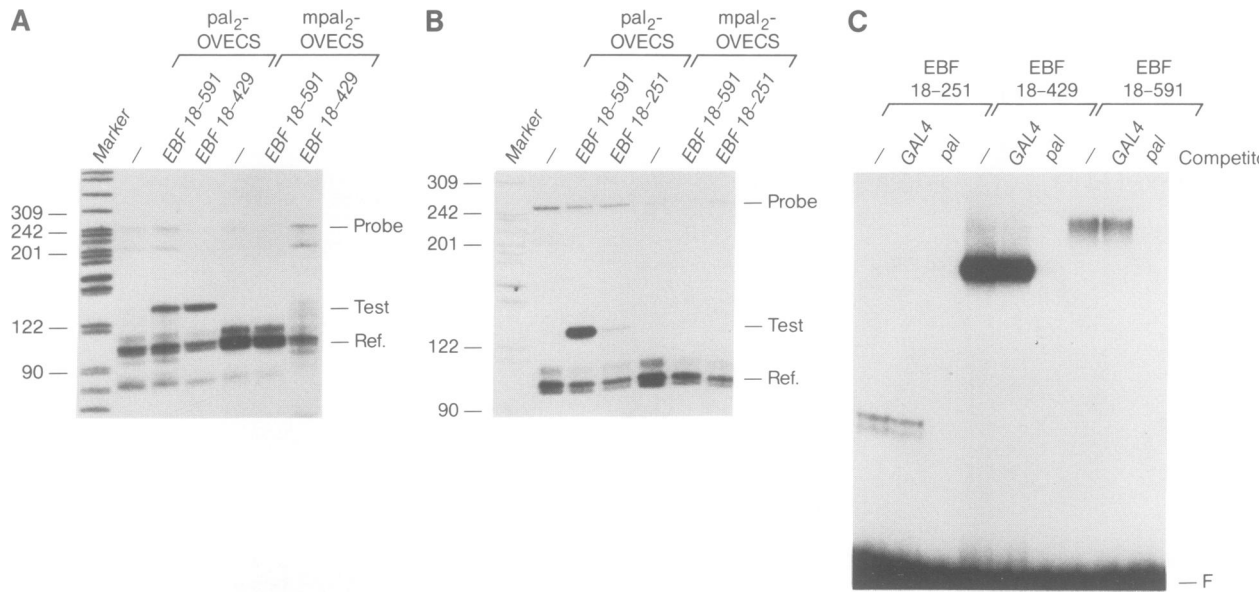

Fig. 6. The DNA binding domain of EBF can activate transcription. (**A**) and (**B**) HeLa cells were transiently cotransfected with effector plasmids for expression of wild-type and mutant EBF polypeptides, OVECS reporter and OVECS-RF reference gene constructs which estimate transfection efficiency. Reporter gene constructs contained either two pal or two mutated mpal EBF binding sites. At 48 h post-transfection, total cellular RNA was isolated and analyzed by S1 nuclease protection using a β-globin probe specific for both the OVECS test and reference transcripts (Westin *et al.*, 1987; Hagman *et al.*, 1993). Correctly initiated β-globin transcripts are designated Test and transcripts from the reference plasmid, Ref. Residual undigested probe is indicated (Probe). (**C**) Wild-type and mutated EBF polypeptides are expressed in transiently transfected cells. Binding of EBF polypeptides was detected by electrophoretic mobility shift assay using a ^{32}P-labeled palindrome (pal) binding-site probe and nuclear extracts of transfected Cos7 cells. The specificty of DNA binding was confirmed by a differential sensitivity of the protein–DNA complexes to competition with an excess of unlabled oligonucleotide comprising the GAL4 binding site or the perfect palindromic EBF binding site (pal). Free probe is indicated at right (F).

EBF contains two transcriptional activation domains

We have previously shown that EBF is a potent activator of gene expression in transfected nonlymphoid cells (Hagman *et al.* 1993). To delineate amino acids that are required for transactivation, we analyzed the ability of EBF-deletion mutants to activate transcription. Effector plasmids encoding wild-type or mutant EBF polypeptides were transiently transfected into HeLa cells together with OVECS reporter plasmids that contained either an optimized palindromic (pal) or a mutated (mpal) EBF binding site upstream of the minimal β-globin promoter (Westin *et al.*, 1987). Two days after transfection, cytoplasmic RNA was isolated and examined for the presence of specific transcripts from the test and reference gene constructs by S1 nuclease protection assays. Deletion of amino acids 430–591 had no significant effect on the activation potential of EBF (Figure 6A). As expected, activation of the OVECS test construct was observed with a duplicated wild-type, but not mutated, palindromic EBF binding site. Interestingly, the DNA binding polypeptide EBF(18–251) also exhibited transactivation potential (Figure 6B). The level of test-gene expression was ~10-fold lower than that observed with EBF(18–429) polypeptide, which could be attributed to observed differences in levels of DNA binding activity of these polypeptides *in vivo* (Figure 6C). Electrophoretic mobility shift assays with extracts from transiently transfected Cos cells indicated weak but specific DNA binding by EBF(18–251) and 20-fold higher levels of DNA binding by EBF(1–429). Thus, the DNA binding domain of EBF, in addition to mediating dimer formation on DNA, also contains sequences that activate transcription.

To search for additional transcriptional activation domains in EBF, we generated effector plasmids in which segments of the EBF cDNA were fused in frame to sequences encoding the heterologous DNA binding domain (amino acids 1–147) of the yeast activator protein GAL4 (Figure 7). Transfection into HeLa cells of these effector plasmids, together with an OVECS test-gene construct containing duplicated GAL4 binding sites, revealed transcriptional stimulation by the chimeric protein containing amino acids 429–591 of EBF. In contrast, the chimeric GAL4–EBF(4–251) and GAL4–EBF(224–429) polypeptides did not activate transcription, although all three chimeric proteins specifically bound to the GAL4 probe in an electrophoretic mobility shift assay (data not shown). The discrepancy between the ability of EBF (18–251) to stimulate transcription and the lack of activation by GAL4–EBF(4–251) can be interpreted to suggest that DNA binding and/or dimerization is important for transcriptional activation by this region of EBF. This interpretation is also consistent with the efficient transcriptional activation by EBF(18–429), which contains the α-helical dimerization domain but lacks the carboxyl-terminal transactivation domain. Thus, EBF appears to contain two functionally independent activation domains: one within the amino-terminal DNA binding domain and the second within the carboxyl-terminal region of the polypeptide.

Discussion

This manuscript describes experiments demonstrating that EBF is comprised of separate modular domains with

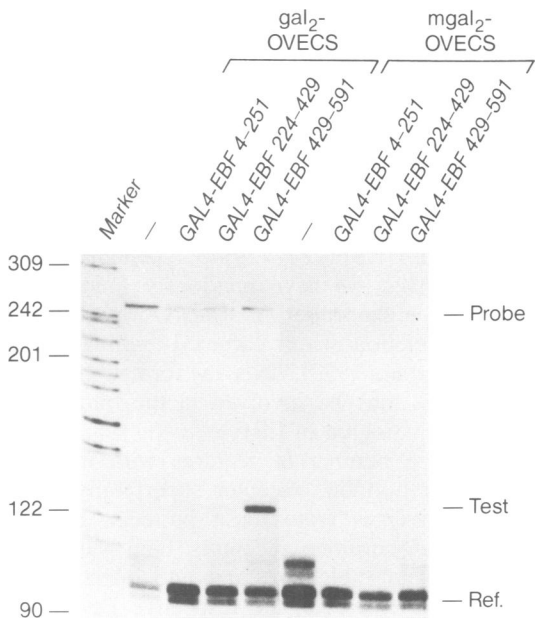

Fig. 7. Carboxyl-terminal sequences of EBF constitute a second transcription activation domain. HeLa cells were transfected with effector plasmids for expression of chimeric GAL4(1–147)–EBF proteins, together with OVECS reporter plasmids and the OVEC-RF reference plasmid to estimate the efficiency of transfection (Westin *et al.*, 1987). Reporter plasmid inserts contained two wild-type (gal) or mutated (mgal) GAL4 binding sites. At 48 h post-transfection, total cellular RNA was isolated and analyzed by S1 nuclease protection using a β-globin probe specific for both the OVECS test and reference transcripts. Correctly initiated β-globin transcripts are designated Test and transcripts from the reference plasmid, Ref. Residual undigested probe is indicated (Probe).

DNA binding, dimerization, and transcriptional activation functions. The amino-terminal half of EBF contains all sequences necessary for DNA binding, dimerization on optimal half-site sequences with appropriate spacing, and transactivation. DNA binding by EBF to natural sites, such as those found in the promoters of the *mb-1* gene and several olfactory specific genes, however, requires a distinct dimerization domain consisting of α-helical repeats. The function of the α-helical repeats may be to stabilize interactions between DNA binding domains of EBF, allowing for the formation of homodimeric complexes in the absence of DNA. Interaction of EBF with DNA requires a novel zinc-coordination motif that is located in the DNA binding domain. In addition, two domains of EBF were found to mediate transcriptional activation, one of which overlaps with the DNA binding domain.

The DNA binding domain of EBF is sufficient to recognize a single half-site (5′-GGGAA/TT) as a monomer, although at a very low level. This DNA binding domain resides between amino acids 50 and 251. Deletion of amino acids 1–50 reduced DNA binding to only one-third of wild-type levels, but DNA binding was completely lost following deletion to serine 60 (Hagman *et al.*, 1993; J.Hagman, unpublished data). The carboxyl-terminal boundary of the DNA binding domain was delineated to glycine 252. However, the actual boundary may lie between glycine 252 and histidine 235, which was identified by point mutagenesis as the most carboxyl-terminal amino acid that is essential for DNA binding. At a minimum of 185 amino acids (R51–H235), this domain is considerably larger than the DNA binding motifs of many other transcriptional regulatory proteins (Pabo and Sauer, 1992) and probably reflects the presence of multiple distinct structures or sub-domains that participate in DNA binding. Analysis of point mutations in the DNA binding domain of EBF identified several amino acids between H157 and C170 that are essential for DNA recognition. By analogy with known metal binding motifs, this region of EBF can be represented as a 14 amino acid motif that coordinates a metal ion, most likely zinc. Metal coordination by this motif was inferred from experiments demonstrating that zinc ions are required for DNA binding by renatured EBF and from mutations of amino acids that could provide coordinating side chains.

The proposed zinc-coordination motif of EBF is unusual in three respects. First, the coordinating amino acids would be arranged as H-X_3-C-X_2-C-X_5-C, and would be the first example of an HCCC metal binding domain (other configurations are reviewed in Berg, 1990, and Coleman, 1992). Numerous metal binding domains that bind metal ions using one histidine and three cysteine residues have been identified in the gag proteins of lentiviruses, yeast transposable element copia, and the mammalian poly-(ADP-ribose) polymerase, but these amino acids are linearly arranged as CCHC (reviewed in Berg, 1990). Second, the mutational analysis suggested that only one zinc binding structure is present in EBF. However, we do not know the precise coordinating residues for zinc and the metal stoichiometry. Therefore, we cannot rule out the existence of a second zinc binding motif that uses alternate residues when one of the cysteines or histidines is mutated. The majority of zinc binding proteins contain two or more metal binding domains. Zinc-finger proteins generally contain multiple repeats of highly related finger structures, such as the nine repeats found within the archetypal zinc-finger protein, TFIIIA (Miller *et al.*, 1985). Nuclear hormone receptors, including the glucocorticoid (Freedman *et al.*, 1988; Hollenberg and Evans; 1988) and estrogen (Green *et al.*, 1988) receptors, contain two metal binding domains that participate in both DNA binding and dimerization. A single zinc finger has been proposed for other DNA binding proteins, including the *E1A* gene of adenovirus type 5 (Culp *et al.*, 1988), the terminus factor of *Drosophila* (Baldarelli *et al.*, 1988), and the yeast transcriptional silencer binding protein ABF1 (Diffley and Stillman, 1989). Third, the overall size of the zinc binding domain of EBF is very small, consisting of as few as 14 amino acids. The amino acids that usually constitute the central portion of the 'finger' (reviewed in Coleman, 1992) are lacking in EBF, which possesses only two amino acids (Ser–Arg) between the first and second presumptive zinc-coordinating cysteines. Formation of this putative zinc binding motif appears to require a specific set of cysteines because C164, but not the adjacent C165, is essential for DNA binding. As reported for metal binding motifs in nuclear hormone receptors, amino acids that are important for DNA binding are located at the apex (R163), side (K167 and K168), or base (N172, N174 and E175) of the putative EBF zinc binding motif of EBF.

A number of mutations, including K167A/K168A, N172A and, to a lesser degree, N174A and C198S, were

found to decrease binding of EBF(1–251) polypeptides to the mb-1 probe, which contains one optimal and one imperfect half-site, but did not affect binding to the pal probe which consists of an inverted repeat of the optimal half-site. In particular, mutation of N172 to alanine resulted in a 100-fold decrease in binding of monomers to the mb-1 probe, but had no effect on binding to the pal probe. To explain these results, we can suggest that binding to the mb-1 probe occurs in an asymmetric manner relative to the pal probe sequence. Determination of specific protein–DNA contacts using methylation interference analysis showed differences in contacts with the two half-sites of the *mb-1* promoter: all six bases of the right-hand strong half-site (GGGAAT), but only the first and last bases of the left-hand weak half-site (GAGTCT) are contacted by EBF (Hagman *et al.*, 1991, 1993). Therefore, based on the relative number of available contacts in the two sequences, mutations in EBF that potentially alter the recognition of specific nucleotides would have more detrimental effects on binding to the mb-1 probe relative to the pal sequence. A potential consequence of this model is that mutations in EBF that affect minor contacts would be more easily compensated by the multiplicity of potential protein–DNA contacts provided by two optimized half-sites.

Several regions of EBF contribute to the multimerization of the protein. The DNA binding domain by itself has an intrinsic potential for dimerization. EBF(1–251) can efficiently assemble dimers on inverted repeats of optimal half-sites separated by 2 bp, but not when the spacing between half-sites is increased to 6 bp. The requirement for appropriate spacing observed with EBF(1–251) suggests that the two DNA binding domains make contacts with one another. In support of this view, the half-life of EBF(1–251) binding to probes with a 2 bp and 6 bp spacer is ~1 h and ~1 min, respectively (J.Hagman, data not shown). Within the DNA binding domain, we did not conclusively identify sequences involved in dimerization. Only the effects of the N174A mutation are consistent with a potential defect in dimer assembly. This mutation decreases binding of EBF(1–251) dimers to the mb-1 probe by a factor of three, but showed little or no effect on binding to the pal probe. Possibly, the effects of this mutation are compensated by the optimal arrangement and composition of the half-sites in the pal probe. EBF also contains a carboxyl-terminal dimerization domain, which is necessary for stable dimerization of EBF in solution (Hagman *et al.*, 1993) and is sufficient to confer dimerization upon a heterologous monomeric ER protein.

Our data also suggest that EBF contains at least two domains that activate transcription in transfected cells. First, the DNA binding domain by itself weakly activated transcription from reporter genes containing optimized palindromic binding sites for EBF. The low level of activation observed for EBF(18–251) may be due to the monomeric state of this protein in solution and the requirement of assembling two monomers on the pal site. However, no transcriptional activation is observed with chimeric polypeptides in which the DNA binding domain of EBF is tethered to the DNA binding domain of GAL4. This suggests that DNA binding and/or dimerization may be important for transcriptional activation by the DNA binding domain of EBF. In addition, the carboxyl-terminal region of EBF contains a transcriptional-activation domain similar to the serine/threonine-rich activation domains previously identified in several transcription factors (reviewed in Struhl, 1991). This transcriptional-activation domain of EBF may also be involved in the formation of higher-order multimers. In electrophoretic mobility shift assays, the GAL4–EBF(429–591)–DNA complex migrates at a slower rate than the nucleoprotein complex containing the larger GAL4–EBF(224–429) polypeptide (data not shown). Moreover, we have previously found that the formation of higher-order EBF–DNA complexes is abrogated by deletion of this carboxyl-terminal region in EBF (Hagman *et al.*, 1993). Thus, the formation of higher-order multimers may be involved in the transcriptional activation by this region of EBF.

EBF shares a number of features with the steroid/thyroid nuclear hormone receptor superfamily, but also significantly diverges from these proteins. First, EBF and the steroid hormone receptors bind variations of palindromic response elements as homodimers (reviewed in Pfahl, 1994). However, the respective DNA binding domains of these proteins have been shown to bind specific half-sites with low affinity as monomers (Tsai *et al.*, 1988; Kurokawa *et al.*, 1993; Perlmann *et al.*, 1993). Similar to EBF, dimerization in the absence of DNA is a function of multiple domains in the nuclear receptors (e.g. estrogen receptor; Fawell *et al.*, 1990; Lees *et al.*, 1990). The similarities between EBF and ER apparently include structural requirements for dimerization, because the α-helical repeats of EBF can mediate dimerization of both polypeptides. Nuclear receptors, such as estrogen receptor (ER), contain a pair of putative zinc binding motifs of the CCCC type, and one or both of these motifs has been shown to be necessary for DNA binding, dimerization and transcriptional activation (Tsai *et al.*, 1988). Amino acids of the 'P box,' which is within and adjacent to the more amino-terminal zinc binding motif of nuclear receptors, provide important contacts for DNA recognition (Danielson *et al.*, 1989; Mader *et al.*, 1989; Umesono and Evans, 1989), and our studies indicated that similarly positioned amino acids in EBF (e.g. K167, K168 and N172) play a role in DNA binding. Moreover, EBF resembles the ER in its requirement of amino acid sequences in addition to the zinc binding motif(s) for DNA binding (Kumar and Chambon, 1988). Our studies do not directly address whether the zinc binding motif of EBF participates in dimerization and transcriptional activation, but the 'finger' structure is a good candidate for both of these functions.

Although structural motifs of EBF may be functionally related to the nuclear receptor motifs, many differences are evident. The overall amino acid sequence of the EBF 'finger' structure is quite different from the nuclear hormone receptor consensus (reviewed in Tsai and O'Malley, 1994). Moreover, EBF contains only a single putative zinc binding structure which would be specified by amino acid side chains arranged as HCCC, the first of its type. Unlike the 70 amino acid zinc binding domains of glucocorticoid receptor, which can alone bind glucocorticoid response elements (Freedman *et al.*, 1988), the putative 'finger' of EBF is probably not sufficient for recognition of the 6 bp half-site, but is required as part of a much larger functional domain (185 amino acids).

Finally, dimerization of EBF polypeptides in solution is dependent upon the presence of the α-helical repeats, a pair of nearly identical structures that are most closely related to the helix 2 sequences of helix–loop–helix proteins (Hagman *et al.*, 1993). Other differences include a lack of evidence for partner proteins that could potentially heterodimerize with EBF, and also the presence of a ligand binding domain that would modulate EBF activity *in vivo*.

Materials and methods

Plasmids and mutagenesis

Plasmids pEBF17, pEBFCΔ430 and pEBFCΔ430(ΔH1) were described previously (Hagman *et al.*, 1993). To remove polylinker sequences that inhibit translation *in vitro*, plasmids pBSCΔ430 and pBSCΔ430(ΔH1) were constructed by insertion of *Eco*RI–*Hin*dIII fragments from pEBFCΔ430 and pEBFCΔ430(ΔH1) between the *Eco*RI and *Hin*dIII sites of the Bluescribe vector (Stratagene, La Jolla, CA).

Plasmids for translation of estrogen receptor polypeptides *in vitro* (pSPMOR 121–384 and pSPMOR 121–384DM) were the gift of M.Parker (Lees *et al.*, 1989). The ER–EBF (367–429) fusion plasmid (pSPMOR 121–384CΔ2) was constructed by amplification of pEBFCΔ430 DNA with the oligonucleotides 5′-CCGGTGAATTCCC-TGGTGACCC AGAGCGCT and 5′-AGCCGGAATTCGTCGACG-GTATCGATAAGC using Pfu DNA polymerase (Stratagene, LaJolla, CA), followed by digestion with *Eco*RI and ligation into the *Eco*RI site of plasmid pSPMOR 121–384 (Lees *et al.*, 1989).

To produce mutated EBF polypeptides, *in vitro* mutagenesis was performed using either the single-stranded mutagenesis method of Kunkel (1985) or by using a modification of the polymerase chain reaction (PCR)-based method of Merino *et al.* (1992). Single stranded mutagenesis was performed on single-stranded pEBFCΔ430 DNA using oligonucleotides as shown to make the following mutations: (C151S), 5′-ACAAGAACCCTGAAATGAGCCGAGTACTGCTCACACACGAG-ATC; (C161S), 5′-CTCACACACGAGATCATGAGCTCTAGATGTTG-TGACAAGAAAAGC; and (K167A, K168A), GTGCAGCCGCTG-TTGTGACGCAGCTAGCTGTGGCAACCGAAATG. Fragments containing mutations were used to replace sequences in pBSCΔ430. PCR mutagenesis was performed by amplifying fragments (which overlap by 10 bp) with mutated and flanking oligonucleotides, then combining and amplifying again using only the flanking oligonucleotides to create mutated fragments. PCR fragments were cleaved with appropriate restriction enzymes and inserted into pBSCΔ430 to analyze mutations in the context of EBF(1–429). Mutated fragments were made using pBSCΔ430 as template and oligonucleotide pairs as shown (only oligonucleotides containing mutations are shown): (H157A), 5′-CACAGCCG-AGATCATGTGCAGCCGC and 5′-CTCGGCTGTGAGCAATACTCGG CAC; (R163A), 5′-CAGCGCCTGTTGTGACAAGAAAAGC and 5′-ACAGGCGCTGCACATGATCTCGTGT; (C164S), 5′-CCGCTC-TTGTGACAAGAAAAGCT and 5′-CACAAGAGCGGCTGCACA-TGATC; (C165S), 5′-CTGTTCTGACAAGAAAAGCTGTG and 5′TGTCAGAACAGCGGCTGCACATG; (C170S), 5′-TGCCAGAGC-TCTTCTTGTCACAACA and 5′-GAAGAGCTCTGGCAACCGAA-ATGAG; (N172A), 5′-TGGCGCCCGA AATGAGACTCCCTCA and 5′-TTCGGGCGCCACAGCTTTTCTTGTC; (R173A), 5′CAACGCC-AATGAGACTCCCTCAGAT and 5′-CATTGGCGTTGCCACAGC-TTTTCTT; (N174A), 5′-ACCGCGCGGAGACTCCCTCAGATCC and 5′-TCTCCGCGCGGTTGCCACAGCTTTT; (E175A), 5′-AAATGCAA-CTCCCTCAGATCCAGTG and 5′GAGTTGCATTTCGGTTGCCAC-AGCT; (C194S) 5′-TTAAAAGCAACCAAAATTGCCTA and 5′-GTTGCTTTTAAGGAAAAACTTCAG; (C198S), 5′-AAAATAGCCTA AAGAATGCAGGAA and 5′-TAGGCTATTTTGGTTGCATTTAAG; (H224A), 5′TGGCGCTGTCCTGGCAGTCTCTGAT and 5′-GAC-AGCGCCATCCACGTTGACTGTG; (H235A), 5′-TGTCGCCAATAA-CTCCAAGCACGGG and 5′-ATTGGCGACAA ACATGTTATCAGAG; (H240A), 5′-CAAGGCCGGGCGGAGGGCTCGGAGG and 5′-CCC-GGCCTTGGAGTTATTGTGGACA; and (R242A, R243A), 5′-GGGC-AGCAGCTCGGAGGCTTGACCC and 5′-GCTGCTGCCCCGTGC-TTGGAGTTAT.

pCΔ2m23.6, which contains a stronger ATG codon (Kozak, 1987) was made by amplification of pBSCΔ2 using the PCR method with oligonucleotides 5′-CCCGAAGAATTCACCATGGGTGGGATCCAG-GAAAGCATCC and 5′-GGGAGTAGCATGTTCCAGATAAG. The resulting fragment was cleaved with *Eco*RI and *Sma*I and inserted in place of the *Eco*RI–*Sma*I fragment of pBSCΔ2. A translation termination codon was inserted in place of Gly152 using the PCR method with oligonucleotides 5′-GGAATGAGCTCCCTCTTATCTGGAACAT and 5′-AGCTCATTCCGAGGGGTCAAGCCTC to make pCΔ2m33.12. Restriction fragments containing many of the mutations generated above were inserted into pCΔ2m33.12 to analyze effects in the context of EBF(1–251).

Plasmid pETCΔ2 was constructed for expression of EBF(15–429) in *E.coli* by amplification of sequences in pEBF17 using PCR and oligonucleotides (5′-GGGAATTCAATGAAGGAAGAGCCGCTG and 5′-CTAGACGAAGTGTTAGCAAG), and ligating the fragment into the *Eco*RV site of BluescriptKS$^+$ (Stratagene, La Jolla, CA). The insert was liberated with *Eco*RI, made blunt with Klenow enzyme, and ligated into the blunted (Klenow) *Nde*I and *Bam*HI sites.

The plasmid CMV-EBF, which allows for expression of full-length EBF in transfected cells, was described previously (Hagman *et al.*, 1993). Plasmid CMV-EBFCΔ2, which allows for expression of EBF(1–429), was made by replacement of the *Asp*718–*Xba*I fragment of CMV-EBF with the *Asp*718–*Xba*I fragment of EBFCΔ2S (Hagman *et al.*, 1993). Similarly, CMV-EBFm33T, which allows for expression of EBF(1–251) was made by substituting the *Nar*I–*Bst*EII fragment of CMV-EBF with the similar fragment from pCΔ2-m33.12.

The pG_5CAT reporter and pBXG1 GAL-4 expression vector (Giniger and Ptashne, 1987; Ma and Ptashne, 1987) were kindly provided by M.Ptashne. The GAL-4/EBF effector plasmid BXGEBF(384–591) was made in three steps. First, the full-length EBF insert of pEBF-17 was inserted as a blunt-ended (Klenow) *Eco*RI fragment into the blunted (Klenow) *Bam*HI site of pBXG1 to make pBxGEBF8. The open reading frame of EBF was placed in frame with GAL-4 (1–147) by replacing the *Eco*RI–*Msc*I fragment of pBXGEBF8 with the *Eco*RI–*Msc*I fragment of pCΔ2m23.6. pBxGEBF(384–591) was constructed by complete digestion of pBXGEBF8 with *Sma*I and partial digestion with *Bgl*II, followed by blunt-ending with Klenow and ligation. pBXGEBF(224–429) was made by replacement of the *Msc*I–*Hin*dIII fragment of pBXGEFL1 with the *Msc*I–*Hin*dIII fragment of pEBFCΔ430 (Hagman *et al.*, 1993) to make pBXGCΔ2, then cleaving pBXGCΔ2 with *Eco*RI and *Msc*I and filling in the ends with Klenow fragment before ligation.

In vitro translation, DNA binding and electrophoretic mobility shift assays

In vitro transcription, purification of transcripts on QIAGEN-tip 20 columns (Qiagen Inc., Chatsworth, CA), and translation using rabbit reticulocyte lysate (Promega, Madison, WI) were described previously (Hagman and Grosschedl, 1992).

Sequence and labeling of the *mb-1* promoter probe was described previously (Hagman *et al.*, 1993). The pal probe (except Figure 1D) was made by annealing oligonucleotides 5′-TCGAGAGATTCCCAA-GGGAATTGTGGCCAGCG and 5′-TCGACGCTGGCCACAATTCC-CTTGGGAATCTC and labeled by filling in the recessed ends with [α-^{32}P]dCTP, cold dNTPs, and Klenow enzyme. The pal and pal+4 probes shown in Figure 1D (Travis *et al.*, 1993), and ERE probe in Figure 2B (Lees *et al.*, 1990) were prepared as described. DNA binding assays were performed as described (Hagman *et al.*, 1993), except that 0.1% Nonidet P40 (NP-40) was included. Plasmids for expression of wild-type and mutated EBF were linearized with *Hin*dIII for transcription with T7 RNA polymerase *in vitro*, except for generation of the EBF(1–296) polypeptide (Figure 1E), which was synthesized using mRNA generated from pBSCΔ2 plasmid linearized with *Asp*718. The plasmid for expression of truncated ER (pSPMOR 121–384) was linearized with *Eco*RI for transcription with SP6 RNA polymerase. PSPMOR 121–384DM and pSPMOR 121–384CΔ2 were linearized with *Apa*LI.

Overexpression and purification of EBF(15–429) from E.coli, denaturation and renaturation

EBF(15–429) was overexpressed in *E.coli* and inclusion bodies were recovered using a modification of the method of Nagai and Thogersen (1987). *Escherichia coli* strain BL-21 was transformed with plasmid pETCΔ2 and grown in L broth to OD_{595} = 0.7. Cells were grown an additional 3 h following addition of IPTG to 1 mM, then harvested by centrifugation and resuspended in 30 ml lysis buffer (50 mM Tris–HCl, pH 8.0/1 mM EDTA/25% sucrose/1 mM phenylmethylsulfonylfluoride/2 mg/ml leupeptin/2 mg/ml aprotinin/2 mg/ml pepstatin). Lysozyme was added to 2 mg/ml and cells were left on ice 30 min. $MgCl_2$ (to 10 mM) and $MnCl_2$ (to 1 mM) were added together with DNase I (10 mg/ml) and incubated 30 min at room temperature to digest DNA. 25 ml of detergent buffer (20 mM Tris–HCl pH 7.5, 0.2 M NaCl, 1% sodium deoxycholate, 1% NP-40, 2 mM EDTA) was mixed with the

lysate and centrifuged at 5000 *g* at 4°C for 10 min. Supernatant was removed and the pellet resuspended in 20 ml of 0.5% Triton X-100/1 mM EDTA and centrifuged as in the previous step. The pellet was resuspended in fresh Triton/EDTA solution and centrifuged four more times. The final pellet was solubilized in 6 M urea and the protein concentration was determined using the Bradford method (Bio-Rad, Richmond, CA).

Thirty eight micrograms of protein were diluted to 2.5 ml in renaturation buffer (20 mM HEPES, 100 μM KCl, 10% glycerol, 3 mM DTT, 100 mg/ml bovine serum albumin) containing 6 M guanidine hydrochloride (GuaHCl) and 3 mM each EDTA and EGTA, and dialyzed as 400 μl aliquots against two changes of the same buffer (without BSA) for 1 h at 4°C. Samples were then dialyzed against renaturation buffer with 6 M GuaHCl, but without EDTA and EGTA, followed by four successive dilutions (1:1) of the dialysate with renaturation buffer (without GuaHCl) and either 10 μM $CaCl_2$, $CdCl_2$, $MgCl_2$, $NiCl_2$, $ZnCl_2$ or without added metal salts. Samples were then dialyzed against renaturation buffers with 10 μM metal salts without GuaHCl. Samples were recovered, NP-40 was added to 0.1% and stored at 4°C for DNA binding analysis.

Transfection assays

Transient transfections of HeLa cells, isolation of total cellular RNA, and S1 nuclease protection analysis of specific transcripts were performed as previously described (Hagman *et al.*, 1993).

Acknowledgements

We thank Tom Alber and Alan Frankel for valuable discussions and James Fraser for technical advice. We are grateful to Malcom Parker and Mark Ptashne for providing plasmids, Erika Heil and Wendy Hodsdon for technical assistance, and Alan Frankel for comments on the manuscript. J.Hagman was supported by a postdoctoral fellowship from the Leukemia Society of America and is currently supported by funds from the National Jewish Center. H.Lin is a postdoctoral fellow of the Arthritis Foundation. This work was supported by funds from the Howard Hughes Medical Institute.

References

Baldarelli,R.M., Mahoney,P.A., Salas,F., Gustavson,E., Boyer,P.D., Chang,M.-F., Roark,M. and Lengyel,J.A. (1988) *Dev. Biol.*, **125**, 85–95.

Berg,J.M. (1990) *J. Biol. Chem.*, **265**, 6513–6516.

Campbell,K.S. and Cambier,J.C. (1990) *EMBO J.*, **9**, 441–448.

Coleman,J.E. (1992) *Annu. Rev. Biochem.*, **61**, 897–946.

Culp,J.S., Webster,L.C., Friedman,D.J., Smith,C.L., Huang,W.-J., Wu,F.Y.-H., Rosenberg,M. and Ricciardi,R.P. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 6450–6454.

Danielson,M., Hinck,L. and Ringold,G.M. (1989) *Cell*, **57**, 1131–1138.

Diffley,J.F. and Stillman,B. (1989) *Science*, **246**, 1034–1038.

Fawell,S.E., Lees,J.A., White,R. and Parker,M.G. (1990) *Cell*, **60**, 953–962.

Feldhaus,A.L., Mbangkollo,D., Arvin,K.L., Klug,C.A. and Singh,H. (1992) *Mol. Cell. Biol.*, **12**, 1126–1133.

Freedman,L., Yamamoto,K.R., Luisi,B.F. and Sigler,P. (1988) *Cell*, **54**, 444.

Giniger,E. and Ptashne,M. (1987) *Nature*, **330**, 670–672.

Green,S., Kumar,V., Theulaz,I., Wahli,W. and Chambon,P. (1988) *EMBO J.*, **7**, 3037–3043.

Hagman,J. and Grosschedl,R. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8889–8893.

Hagman,J., Travis,A. and Grosschedl,R. (1991) *EMBO J.*, **10**, 3409–3417.

Hagman,J., Belanger,C., Travis,A., Turck,C.W and Grosschedl,R. (1993) *Genes Dev.*, **7**, 760–773.

Hollenberg,S.M. and Evans,R.M. (1988) *Cell*, **55**, 899–906.

Hombach,J., Leclercq,L., Radbruch,A., Rajewsky,K. and Reth,M. (1988) *EMBO J.*, **7**, 3451–3456.

Hombach,J., Tsubata,T., Leclercq,L., Stappert,H. and Reth,M. (1990) *Nature*, **343**, 760–762.

Kozak,M. (1987) *J. Mol. Biol.*, **196**, 947–950.

Kudrycki,K., Stein-Izsak,C., Behn,C., Grillo,M., Akeson,R. and Margolis,F.L. (1993) *Mol. Cell. Biol.*, **13**, 3002–3014.

Kunkel,T.A. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 488–492.

Kurokawa,R., Yu,V., Naar,A., Kyakumoto,S., Han,Z., Silverman,S., Rosenfeld,M. and Glass,C. (1993) *Genes Dev.*, **7**, 1423–1435.

Lees,J.A., Fawell,S.E. and Parker,M.G. (1989) *Nucleic Acids Res.*, **17**, 5477–5489.

Lees,J.A., Fawell,S.E., White,R. and Parker,M.G. (1990) *Mol. Cell. Biol.*, **10**, 5529–5531.

Ma,J. and Ptashne,M. (1987) *Cell*, **51**, 113–116.

Mader,S., Kumar,V., de Verneuil,H. and Chambon,P. (1989) *Nature*, **338**, 271–274.

Matsuuchi,L., Gold,M.R., Travis,A., Grosschedl,R., DeFranco,A.L. and Kelly,R.B. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3404–3408.

Merino,E., Osuna,J., Bolivar,F. and Saberón,X. (1992) *BioTechniques*, **12**, 508–510.

Miller,J., McLachlan,A.D. and Klug,A. (1985) *EMBO J.*, **4**, 1609–1614.

Nagai,K. and Thorgersen,H.C. (1987) *Methods Enzymol.*, **153**, 461–481.

Pabo,C.O. and Sauer,R.T. (1992) *Annu. Rev. Biochem.*, **61**, 1053–1095.

Perlmann,T., Rangarajan,P., Umesono,K. and Evans,R. (1993) *Genes Dev.*, **7**, 1411–1422.

Pfahl,M. (1994) *Semin. Cell Biol.*, **5**, 95–103.

Struhl,K. (1991) *Curr. Biol.*, **1**, 188–191.

Travis,A., Hagman,J. and Grosschedl,R. (1991) *Mol. Cell. Biol.*, **11**, 5756–5766.

Travis,A., Hagman,J., Hwang,L. and Grosschedl,R. (1993) *Mol. Cell. Biol.*, **13**, 3392–3400.

Tsai,S.Y., Carlstedt-Duke,J., Weigel,N.L., Dahlman,K., Gustafson,J.-A., Tsai,M.J. and O'Malley,B.W. (1988) *Cell*, **55**, 361–369.

Umesono,K. and Evans,R.M. (1989) *Cell*, **57**, 1139–1146.

Venkitaraman,A.R., Williams,G.T., Dariavich,P. and Neuberger,M.S. (1991) *Nature*, **352**, 777–781.

Wang,M.M. and Reed,R.R. (1993) *Nature*, **364**, 121–126.

Wang,M.M., Tsai,R.Y.L., Schrader,K.A. and Reed,R.R. (1993) *Mol. Cell. Biol.*, **13**, 5805–5813.

Westin,G., Gerster,T., Muller,M.M., Schaffner,G. and Schaffner,W. (1987) *Nucleic Acids Res.*, **15**, 6787–6797.

Received on September 28, 1994; revised on March 16 1995

Note added in proof

Targeted inactivation of the EBF gene in the mouse germline indicated that B lymphocyte differentiation is blocked at a very early stage, whereas no obvious defects were detected in olfactory neurons and other non-lymphoid tissues examined (Lin and Grosschedl (1995) *Nature*, in press).