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The ubiquitous subunit of erythroid transcription factor NF-E2 is a small basic–leucine zipper protein related to the v-*maf* oncogene

(globin gene expression/locus control region/AP-1 motif)

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ABSTRACT **Erythroid transcription factor NF-E2 is a tissue-restricted heterodimeric protein which recognizes an extended AP-1 motif [(T/C)TGCTGA(C/G)TCA(T/C)] found in the upstream locus control regions of the α- and β-globin gene clusters. A cDNA clone encoding a cell-type-specific subunit of NF-E2, designated p45 NF-E2, has previously been characterized and shown to encode a basic–leucine zipper DNA-binding protein. Here we describe protein purification and cloning of cDNA that encodes the second basic–leucine zipper subunit of the native NF-E2 heterodimer. This polypeptide, designated p18, is widely expressed. It displays extensive homology to the v-*maf* oncogene product and a human retinal-specific protein, NRL. Unusual features in the basic region shared by v-Maf, NRL, and p18 place them in a distinct subfamily of AP-1-like proteins.**

Mammalian globin genes are organized in multigene complexes which are subject to tissue-specific and developmental regulation. Segments of chromatin exhibiting erythroid-specific DNase I hypersensitivity coincide with powerful, far-upstream enhancers for globin transcription, termed locus control regions (LCRs) (1, 2). Linkage of LCR sequences to test genes confers erythroid-specific, high-level expression in transgenic mice, independent of integration site (3). Fine-structure mapping of the LCRs has revealed core units of 200–300 bp that contain binding sites for general and tissue-specific transcription factors (4–12).

Two of the transcription factors implicated in LCR function, GATA-1 and NF-E2, are expressed nearly exclusively in hematopoietic cells of the erythroid, megakaryocyte, mast cell tri-lineage. GATA-1, a zinc finger protein, is thought to be involved in control of virtually all erythroid genes (13, 14) and is essential for normal erythroid differentiation (15). In contrast, NF-E2 motifs have been found only in the globin LCRs and the promoters of two heme biosynthetic enzyme genes (porphobilinogen deaminase and ferrochelatase) (8, 16–20). The importance of NF-E2 in LCR function has been inferred from mutagenesis experiments. Specifically, mutation of dimeric NF-E2 sites within the HS2 DNase I-hypersensitive region of the β-globin LCR leads to marked reduction of its potent enhancer activity in transfected cells and transgenic mice (6, 7, 16, 21–23).

We recently reported the purification of NF-E2 DNA-binding activity, and the cDNA cloning and characterization of p45 NF-E2, a basic–leucine zipper (bZIP) protein involved in the NF-E2 complex (20, 24). The expression pattern of p45 NF-E2 parallels the tissue distribution of NF-E2 DNA-binding activity, and antibodies directed against p45 block formation of the NF-E2 complex. As p45 appears to be the site of the defect in mice homozygous for microcytic anemia (*mk/mk*), it may regulate intestinal iron metabolism, as well as globin and heme biosynthetic enzyme genes (25).

p45 NF-E2 belongs to a large family of bZIP proteins. As all previously described bZIP proteins bind DNA as dimers, we investigated the possibility that p45 binds the NF-E2 motif as a homodimer and showed that this was not the case (20). However, a small protein of 18 kDa coimmunoprecipitates with p45 (24). We hypothesized that the native NF-E2 complex is a heterodimer of p45 and a smaller protein, designated p18. In the current study, we have isolated and characterized a cDNA clone encoding p18.‖ We demonstrate that p18 is an authentic component of the NF-E2 heterodimer and bears striking similarity to a human retinal protein, NRL (26), and a chicken oncoprotein, v-Maf (27). Coexpression of p18 and p45 cDNA clones should facilitate studies of the role of NF-E2 in LCR function and experimental attempts to reconstitute LCR activity in nonerythroid cells.

MATERIALS AND METHODS

Purification of NF-E2 DNA-Binding Activity and Isolation of cDNA Clones. NF-E2 DNA-binding activity was purified from mouse erythroleukemia (MEL) cells as described (20). Three peptide sequences were obtained from an 18-kDa polypeptide observed in stoichiometric equivalence to p45—SKYEALQTFA, EAGENAPVL, and AELSSTSVPFSAA. Nested degenerate oligonucleotides were designed spanning the first and second peptide sequences and used in all combinations for PCR amplification of cDNA derived from reverse transcription of MEL RNA (28). The product was cloned, sequenced, and used to isolate sequences from a 129 SVJ mouse genomic DNA library (Stratagene; ref. 28). Analysis of the genomic clones established 3′ cDNA sequence and located the third peptide. The 5′ end of the cDNA was characterized with the 5′ RACE (rapid amplification of cDNA ends) AmpliFINDER procedure (Clontech), and the entire cDNA was assembled by PCR.

Northern Analysis. Northern blot analysis was performed according to standard procedures (28).

Preparation of Anti-p18 Antibodies. Female New Zealand White rabbits were immunized with a glutathione *S*-transferase fusion protein containing amino acids 77–156 of p18. Antiserum was tested in parallel with an unrelated anti-glutathione *S*-transferase serum to confirm that effects seen were due to anti-p18 specificity.

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Abbreviations: LCR, locus control region; bZIP, basic–leucine zipper; MEL, mouse erythroleukemia.

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‖The sequence presented in this paper has been deposited in the GenBank data base (accession no. U01036).

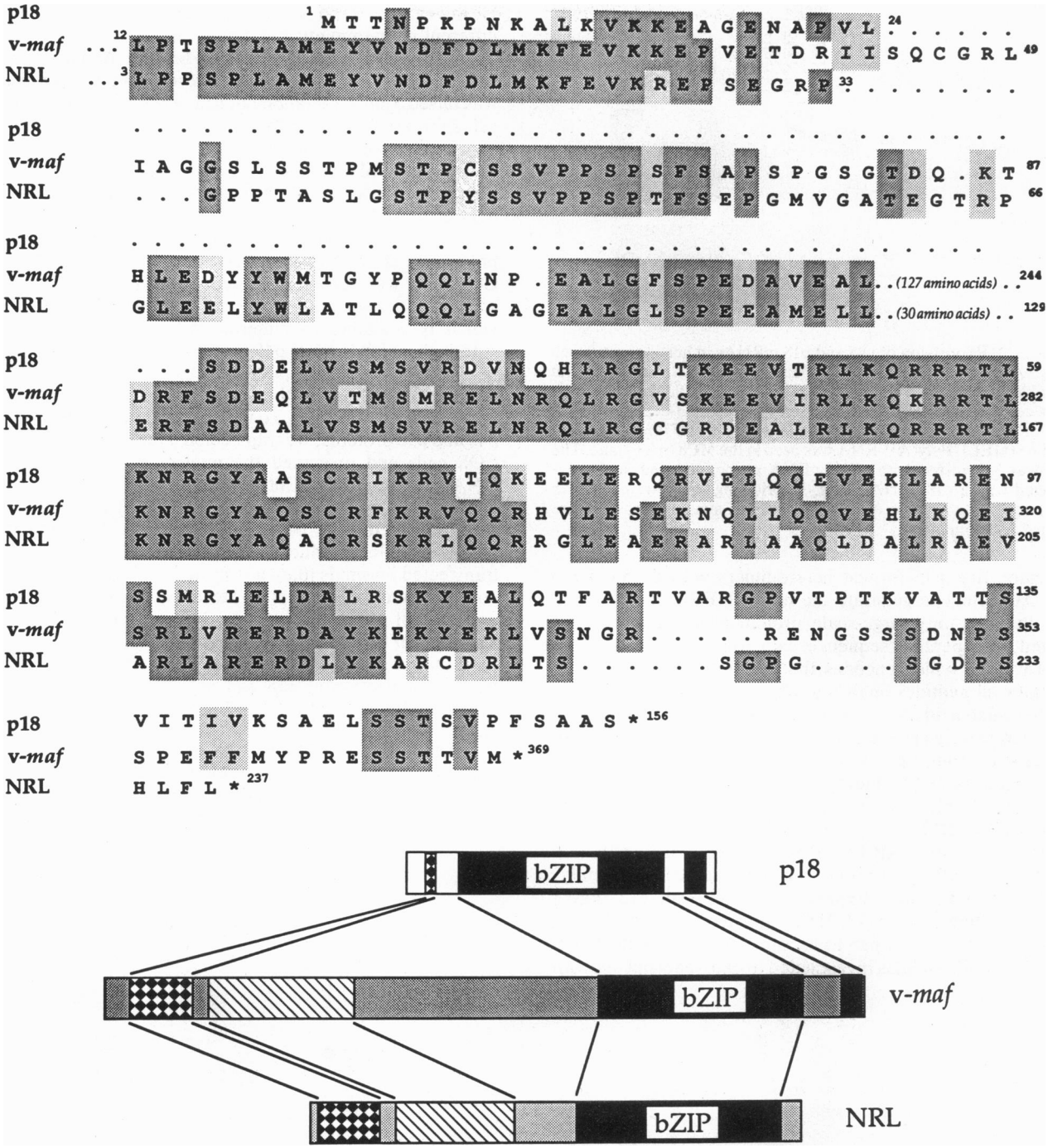

FIG. 1. p18 is related to v-Maf and NRL. Predicted p18 amino acid sequence is aligned with v-Maf and NRL sequences. Numbers indicate amino acid positions. The start codon was assigned based on the apparent molecular weight of p18 and absence of other in-frame methionine residues upstream. Nonhomologous portions of v-Maf and NRL were omitted as indicated. Darker shading indicates identical residues; lighter shading indicates conserved residues. Homologies are shown schematically at the bottom.

Gel Shift Assays. Gel shift assays were carried out (8) using an NF-E2 binding-site oligonucleotide (top strand, 5′-TGGGGAACCTGTGCTGAGTCACTGGAG-3′) or a large half-site palindrome probe ("PAL;" top strand, 5′-CCTGTGCTGACTCAGCACAGGTTCCCCAG-3′). Preimmune or immune serum (1 μl) was added to the reaction mixture as indicated. Unlabeled, competitor oligonucleotides were added to 0.5 ng/μl. Sequences of these oligonucleotides were reported previously (20); in this study the NF-E2 oligonucleotide corresponds to oligonucleotide 1; AP-1 to oligonucleotide 15; αLCR to oligonucleotide 2, band 3 to oligonucleotide 5, ALA-S to oligonucleotide 6, mut AP-1 to oligonucleotide 18, and CREB to oligonucleotide 14. Nuclear extracts were prepared from COS cells (29).

COS Transfection Experiments. cDNA encoding p18 or p45 NF-E2 in the pXM expression vector (14) was transfected into COS cells by the DEAE-dextran procedure (28).

RESULTS

p18 Is a bZIP Protein with an Unusual Basic Region Related to v-Maf. Previously we reported purification of NF-E2 DNA-binding activity from MEL cells and isolation of a cDNA clone encoding p45 NF-E2. Several lines of evidence

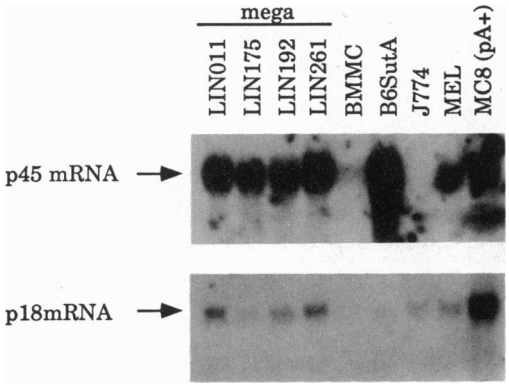

FIG. 2. Expression of p45 and p18 mRNAs in hematopoietic cell lines. A Northern blot was prepared with RNA samples from megakaryocytic cell lines (LIN011, LIN175, LIN192, and LIN261), mast cell lines (BMMC and MC8) an interleukin-3-dependent progenitor cell line (B6SutA), a macrophage cell line (J774), and a MEL cell line (MEL). Poly(A)$^+$ RNA was used in the MC8 (pA+) lane. The blot was hybridized with a p45 cDNA probe, stripped, and rehybridized with a p18 cDNA probe. Hybridizing bands were ≈1.8 kb (p45) and 2.8 kb (p18). Autoradiographs of similar exposures are aligned for comparison.

indicated that p45 formed heterodimers with a ubiquitous 18-kDa protein to generate the native NF-E2 complex (20, 24). In this study large-scale protein purification was repeated, and peptide sequence data were used to obtain a cDNA clone which encodes the complete p18 protein and contains all peptides analyzed.

The amino acid sequence of p18 is shown in Fig. 1, aligned with two closely related bZIP proteins, NRL and v-Maf (26, 27). Homologous regions are diagrammed at the bottom. The basic, putative DNA-binding regions (p18 residues 51–72) are nearly identical, though the leucine zipper domains (p18 residues 79–121) differ significantly. The shared basic region diverges from other AP-1 bZIP proteins (compiled in ref. 30), placing p18, NRL and v-Maf in a separate bZIP subfamily.

p18 mRNA Is Widely Expressed. The tissue distribution of NF-E2 binding activity (17, 31) is regulated at the level of p45 transcription (20, 25). p18 transcripts have been detected in all tissues and cell lines investigated to date (data not shown).

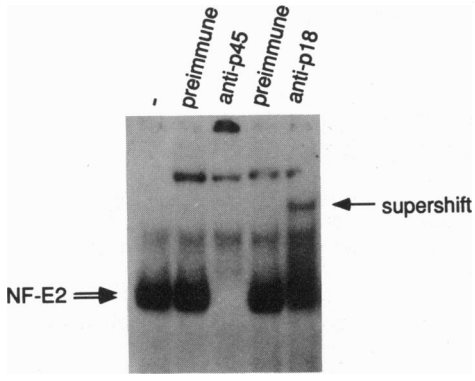

FIG. 3. Supershift of NF-E2 by anti-p18 antibody. Gel shift assays were carried out with MEL nuclear extract and a radiolabeled NF-E2 binding-site probe in the absence or presence of rabbit sera as indicated. The bottom portion of the gel, containing the unbound probe, has been omitted from the figure. Preimmune sera from rabbits immunized with p45 protein (second lane) or p18 protein (fourth lane) had no effect on the NF-E2 DNA-protein complex (indicated by double arrows). Anti-p45 serum blocked the NF-E2 complex (third lane). Anti-p18 serum supershifted a portion of the NF-E2 complex (single arrow, fifth lane).

However, the ratio of p45 to p18 RNAs varies among different hematopoietic cell lines (Fig. 2).

p18 Contributes to NF-E2 DNA-Binding Activity. To test NF-E2 binding activity for the presence of p18, antiserum was raised against recombinant p18 protein. Upon addition to gel shift assays, anti-p18 antiserum, but not preimmune serum, supershifted the DNA–protein complex formed between native MEL NF-E2 and a test DNA probe (Fig. 3). Therefore, the cloned cDNA encodes an authentic component of NF-E2 DNA-binding activity.

Coexpression of p45 and p18 Generates NF-E2 Activity. We previously showed that expression of p45 NF-E2 cDNA in COS cells was sufficient to generate NF-E2 DNA-binding activity (20). Apparently COS cells express endogenous p18, which is available to dimerize with the introduced p45. Although a high level of p45 protein was detected in extracts of the transfected cells, NF-E2 DNA-binding activity was modest, suggesting that the endogenous p18 was limiting (ref. 20, and data not shown). To test dimerization of recombinant p45 and p18 proteins, we transfected expressible cDNAs into COS cells and examined the nuclear extracts for NF-E2 DNA-binding activity. Cotransfection of p45 and p18 cDNAs generates appreciably greater NF-E2 DNA-binding activity than transfection of p45 alone (Fig. 4*a*). The amount of NF-E2 complex produced is related to the amounts of both transfected plasmids (data not shown). Binding specificity of cotransfected cell extracts was tested by gel shift assay in the presence of various competitor oligonucleotides (Fig. 4*b*). The pattern of competition observed was indistinguishable from that previously observed for NF-E2 (20). We conclude that cotransfection of p18 and p45 cDNAs into COS cells generates the authentic NF-E2 complex found in erythroid cells.

p18 Recognizes the Larger Portion of the NF-E2 Binding Site. NF-E2 recognizes and binds to an unusually long sequence of 11 bp (20). The consensus site, (T/C)GCTGA(C/G)TCA(C/T), contains an asymmetrically positioned AP-1

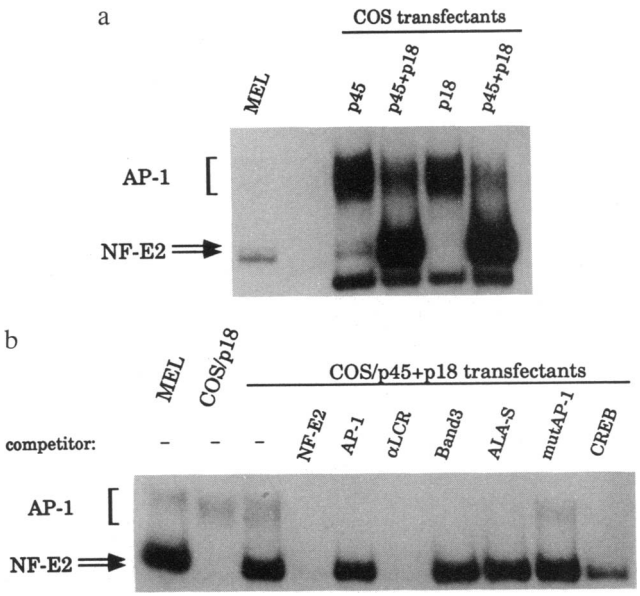

FIG. 4. Reconstitution of NF-E2 DNA-binding activity in COS cells. Extracts from MEL cells and COS transfectants were incubated with NF-E2 binding-site probe and assayed for gel shifts. COS transfectants used are identified above the lanes. In *a* lanes 2 and 4 show results with two different p45 expression constructs. AP-1 and NF-E2 complexes are indicated at left. In *b*, unlabeled competitor oligonucleotides were added to incubation mixtures prior to addition of the probe. Oligonucleotide sequences are given in *Materials and Methods* and in ref. 20.

core palindrome (underlined). Based on studies of other AP-1 site-binding bZIP proteins, one would predict that the binding site represents two half-sites, flanking a central C·G base pair—that is, (T/C)GCTGA(C/G) on the left (reading top strand, 5′ to 3′), and (A/G)TGA(C/G) (reading bottom strand, 5′ to 3′) on the right. To determine which subunit binds to the larger half-site, an oligonucleotide containing a 13-bp palindrome of the larger half-site (TGCTGAGTCAGCA) was incubated with nuclear extracts of COS cells transfected with p45 NF-E2 or p18 cDNAs. The extracts expressing high levels of p45 did not produce a specific gel shift with this probe (data not shown). The extracts expressing high levels of p18 did demonstrate specific binding (Fig. 5). The p18 gel-shift complex migrated faster than both AP-1 and NF-E2 gel-shift complexes (lane 4) and was specifically retarded by anti-p18 antibodies (lane 6). Its formation was inhibited by excess unlabeled palindromic probe (lane 8) but not competed by excess unlabeled NF-E2 binding-site probe (lane 7). This complex most likely represents the specific binding of p18 homodimer, though we cannot rule out binding of a heterodimer involving p18 and a small protein endogenous to COS cells. We infer that p18 is involved in recognition of the extended portion of the 11-bp NF-E2 binding site.

DISCUSSION

High-level, erythroid-specific globin gene regulation is mediated by cis-acting sequences in the LCRs which are recognized by both general and tissue-restricted transcription factors (reviewed in ref. 1). *In vivo* studies of LCR function have implicated erythroid transcription factor NF-E2 as a primary effector of LCR enhancer activity (6, 7, 16, 21–23). Little is understood of the mechanisms by which NF-E2 and associated proteins interact with the LCR to activate expression of globin genes at a distance. To approach this problem, we undertook purification and cDNA cloning of the NF-E2 DNA-binding complex. With the cloning of p18 cDNA reported here, the structure of heterodimeric NF-E2 is now complete.

Several lines of evidence support our conclusions regarding the association of p18 with p45 NF-E2. p18 copurifies with p45 and NF-E2 activity and comigrates with a polypeptide which coimmunoprecipitates with p45 (ref. 24, and data not shown). p18 belongs to the bZIP protein family, all members of which bind DNA as dimers. Gel shift migration of the NF-E2 complex is consistent with an apparent protein molecular mass of 60 kDa (data not shown). Antibodies directed against p18 supershift the NF-E2 gel shift complex (Fig. 3). Moreover, coexpression of p45 and p18 in eukaryotic cells generates binding activity indistinguishable from NF-E2 in competitive gel shifts (Fig. 4).

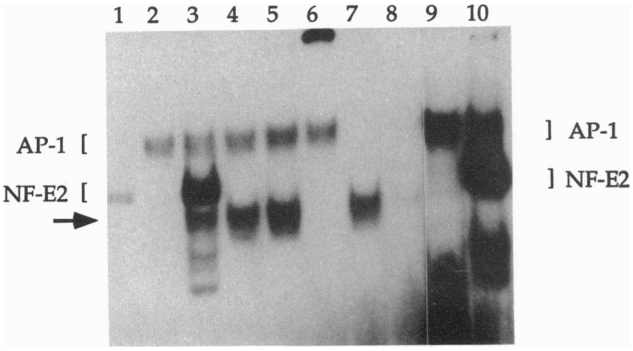

FIG. 5. COS cells transfected with p18 cDNA express a DNA-binding activity. Extracts from MEL cells and COS transfectants were incubated with radiolabeled probes in the absence or presence of antiserum or unlabeled competitor oligonucleotides and assayed by gel shift. Probes used were PAL (lanes 1–8) and standard NF-E2 site (lanes 9 and 10). Extracts used were from MEL cells (lanes 1 and 10) COS cells transfected with p45 alone (lane 2), COS cells transfected with p18 plus p45 (lane 3), or COS cells transfected with p18 alone (lanes 4–9). Preimmune serum was added to lane 5; anti-p18 immune serum was added to lane 6. NF-E2 competitor oligonucleotide was added to lane 7; PAL competitor oligonucleotide was added to lane 8. Positions of AP-1 and NF-E2 complexes are marked with brackets; the arrow indicates a new complex formed by p18 COS transfectants on the PAL probe.

Transcription factors which bind DNA as obligate dimers are subject to an additional level of regulation of activity (32). Leucine zipper-mediated dimerization is selective, restricted by structural features of individual zippers (reviewed in ref. 33). By dimerizing with different partners under diverse circumstances, a limited repertoire of polypeptides may be mixed and matched to assume a greater role in gene regulation. Variation in dimer pairing may differentially affect target gene transcription (34, 35), titrate complex formation, allow recognition of different DNA-binding sites (36–39), promote interaction with different coactivators and corepressors (40), or result in different subcellular localization (41).

Several observations hint that p45 and p18 may each participate in other dimer pairings. First, in contrast to p45, p18 is expressed in all cell types examined. We speculate that p18 plays other roles in other tissues, either as a homodimer or as a heterodimer with other partners. Moreover, the relative amounts of p45 and p18 mRNA vary among hematopoietic cell lines (Fig. 2), suggesting differing ratios of p45 and p18 polypeptides. We speculate that p45 and p18 interact with other proteins during hematopoietic differentiation. Perhaps modulation of dimer pairing plays a role in lineage commitment or allows p45 NF-E2 to function in cell-specific transcription in three different lineages (erythroid, mast, and megakaryocytic cells).

The DNA binding site for NF-E2 is unusual among bZIP-binding sites in that at least 11 bp are specifically recognized—the 7 bp comprising the AP-1 motif and an additional flanking 4 bp (20). Indeed, this feature led to the initial identification of NF-E2 as a complex distinct from transcription factor AP-1 and allowed discrimination between NF-E2 activity and AP-1 activity in functional assays (6, 16, 17, 22, 23). The recently described crystal structure of GCN4, a prototypical bZIP protein, accounts only for recognition of the AP-1 core itself (30). Extended binding specificity of NF-E2 implies the presence of unique features in its basic DNA-binding domain. Inspection of amino acid sequences shows that the basic region of p45 more closely resembles other AP-1 site-binding bZIP proteins than does p18. Together with the related proteins NRL (26) and v-Maf (27), p18 appears to belong to a distinct subfamily (Fig. 1). This may be a clue as to how, in spite of its smaller size, p18 recognizes 7 of the 11 bp specifying the NF-E2 binding site (Fig. 5). It appears that the contribution of p18 to the complex is to increase binding-site specificity, whereas the contribution of tissue-specific p45 is to interact with other cell-specific effector molecules to regulate transcription.

p18 is the smallest member of the bZIP family described to date. Indeed, its size suggests that it may not contain a transcriptional activation domain. p18 is reminiscent of Max, a similarly small member of the basic region–helix–loop–helix–leucine zipper family, which forms heterodimers with Myc to activate transcription and homodimers to repress transcription (42). We speculate that cell-specific modulation of p45 and p18 dimer combinations may play an important role in regulating transcription throughout red-cell differentiation.

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