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Genetics

Complexity of the erythroid transcription factor NF-E2 as revealed by gene targeting of the mouse p18 NF-E2 locus

(embryonic stem cells/globin gene expression/maf proteins)

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ABSTRACT **High-level globin expression in erythroid precursor cells depends on the integrity of NF-E2 recognition sites, transcription factor AP-1-like protein-binding motifs, located in the upstream regulatory regions of the α- and β-globin loci. The NF-E2 transcription factor, which recognizes these sites, is a heterodimer consisting of (*i*) p45 NF-E2 (the larger subunit), a hematopoietic-restricted basic leucine zipper protein, and (*ii*) a widely expressed basic leucine zipper factor, p18 NF-E2, the smaller subunit. p18 NF-E2 protein shares extensive homology with the maf protooncogene family. To determine an *in vivo* role for p18 NF-E2 protein we disrupted the p18 NF-E2-encoding gene by homologous recombination in murine embryonic stem cells and generated p18 NF-E2$^{-/-}$ mice. These mice are indistinguishable from littermates throughout all phases of development and remain healthy in adulthood. Despite the absence of expressed p18 NF-E2, DNA-binding activity with the properties of the NF-E2 heterodimer is present in fetal liver erythroid cells of p18 NF-E2$^{-/-}$ mice. We speculate that another member of the maf basic leucine zipper family substitutes for the p18 subunit in a complex with p45 NF-E2. Thus, p18 NF-E2 *per se* appears to be dispensable *in vivo*.**

High-level transcription of globin genes is dependent upon the integrity of AP-1-like protein binding sites, known as NF-E2 motifs, located within the upstream regulatory elements of both the α- and β-globin loci (1–5). The major erythroid-restricted protein that recognizes these sites is the NF-E2 complex, an obligate heterodimer of two novel basic leucine zipper (bZIP) proteins (6, 7). Expression of the larger subunit, p45 NF-E2, is restricted to hematopoietic tissues, whereas the smaller subunit, p18 NF-E2, is more widely expressed. The p45 NF-E2 subunit, and therefore the NF-E2 heterodimer, is required for high-level globin expression in mouse erythroleukemia (MEL) cells (8). In contrast, mice unable to produce p45 NF-E2 exhibit a surprisingly subtle deficit in globin expression (9) but a profound block in platelet biogenesis (10).

p18 NF-E2 shares extensive homology with the chicken oncogene *v-maf* (11) and a human retinal protein, NRL (12). Subsequent to its cloning, additional members of the maf protooncogene family have been identified in the chicken, including mafB, mafF, mafG, and mafK, (13, 14), and in the mouse segmentation gene *kr* (15). The small maf polypeptides, mafF, mafG, and mafK, are most similar to p18 NF-E2. MafK is the chicken homolog of mouse p18 NF-E2 (13, 14). The small maf proteins appear to lack a transactivation domain, but they do have a basic DNA-binding domain and leucine zipper dimerization domains.

Several observations have led to the suggestion that both positive and negative transcriptional regulation may be mediated by NF-E2 recognition sites depending on the relative concentrations of p45 NF-E2 and small mafs present in erythroid cells (13, 14, 16). Consistent with their high degree of homology, all small maf polypeptides can form heterodimers with p45 NF-E2. However, the NF-E2 complex containing p18 NF-E2 displays the highest affinity for the NF-E2 recognition site (13, 14). The p18 NF-E2 subunit specifically recognizes nucleotides lying outside the AP-1 core in the NF-E2 recognition site (7), thus distinguishing the DNA-binding specificity of the NF-E2 heterodimer from that of transcription factor AP-1. These data are consistent with the 13-bp consensus binding site for *v-maf*, (T/C)GCTGA(G/C)TCAGCA, which also contains a core AP-1 element (underlined) and closely resembles the NF-E2 recognition site (17). Heterodimers of p45 NF-E2 and small maf polypeptides activate reporter gene transcription from NF-E2 recognition sites in linked promoter–reporter gene constructs (13, 14).

Homodimers of p18 NF-E2, or other small maf proteins, also bind *in vitro* to the NF-E2 recognition site, although with lower and variable affinities (13). Finally, small maf proteins have also been shown to form heterodimers with c-fos (18) and with a ubiquitously expressed p45 NF-E2-related protein, LCRF1 (8). These observations suggest a complex network of bZIP partner protein interactions that might be involved in regulating transcription in diverse tissues and in developmental stages.

In the experiments reported here we sought to determine the *in vivo* role of p18 NF-E2 by gene targeting in embryonic stem (ES) cells (19). We have determined that p18 NF-E2-null ES cells are able to differentiate into erythroid and myeloid cells upon culture *in vitro*. p18 NF-E2$^{-/-}$ mice are phenotypically normal throughout all stages of development and remain healthy throughout adulthood. Peripheral blood cell counts, blood smears, and red blood cell parameters are also normal in p18 NF-E2$^{-/-}$ mice. Therefore, p18 NF-E2 appears dispensable *in vivo*.

MATERIALS AND METHODS

Genomic Library Screening and Gene Mapping. A 15-kb *Sal* I fragment including the second and third exons of the p18 NF-E2-encoding gene was isolated from a 129 SVJ mouse genomic DNA library (Stratagene) (20). The fragment was digested with *Xho* I, and the resulting 5′ and 3′ fragments were separately subcloned into the *Sal* I site of pUC18 and pBluescript for restriction mapping.

Construction of Targeting Vector. The targeting construct was assembled in the pPNT vector, which contains both a phosphoglycerate kinase (PGK) promoter–neomycin resistance (*neo*) cassette and a herpes simplex virus–thymidine kinase (HSV-tk) cassette (21). A 2.6-kb *Bgl* II–*Bam*HI fragment served as the 3′ homology region. This fragment was subcloned into the *Bam*HI site of pPNT, 3′ to the PGK-*neo*

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Abbreviations: bZIP, basic leucine zipper; ES, embryonic stem; MEL, mouse erythroleukemia; HSV-tk, herpes simplex virus–thymidine kinase; E, embryonic day.

cassette. The 5′ homology region consisted of a 5.3-kb fragment that included exon II. To assemble the 5′ homology region, the pBluescript plasmid containing the 5′ genomic fragment was digested with *Bgl* II, blunt ended, and ligated with *Xho* I linkers. After digestion with Not I and Xho I, the resulting 5.3-kb fragment was subcloned 5′ to the PGK–*neo* cassette in the pPNT vector. The construct was linearized with *Not* I before electroporation.

Transfection and Screen of ES Cells. J1 or D3 ES cells were cultured on primary irradiated embryonic feeder cells as described (22, 23). Clones were selected in G418 (200 μg/ml) with gancyclovir (2 μM). To select for cells that have two copies of the targeted allele heterozygous p18 NF-E2$^{+/-}$ D3, ES cells were cultured in an increased concentration of G418 (1.2 mg/ml) maintained on irradiated STO feeder cells (24).

Genomic DNA was prepared from G418-resistant ES clones and digested with *Bgl* II. Southern blots were probed with a radiolabeled 0.7-kb *Xba* I genomic fragment located 3′ to the targeted sequence (20). A *Bgl* II fragment of 3.7 kb is diagnostic of the gene targeting event; the 3.5-kb *Bgl* II fragment is specific for the wild-type allele.

***In Vitro* ES Cell Differentiation.** Gelatin-adapted ES cells were cultured according to the one-step *in vitro* differentiation method, as described (25). RNA was isolated using the acid/guanidinium/phenol/chloroform protocol (26). Globin transcripts were analyzed by RNase protection assay using probes specific for mouse ζ-globin, β-major, and γ-actin (27). Clones that retained a heterozygous genotype through the selection in G418 (1.2 mg/ml) were used as controls for p18 NF-E2$^{-/-}$ ES cell clones.

Reverse Transcription–PCR. RNA was prepared from fetal livers using the acid/guanidinium/phenol/chloroform protocol (26). First-strand cDNA synthesis was performed using Moloney murine leukemia virus reverse transcriptase. PCRs were performed in the presence of ^{32}P-dCTP as described (10, 28), with the following primers: p18F10, 5′-ATAAGAATGCGGCCGCTTTTCT-GGTGGTTCCGTCCT-3′, and p18R4, 5′-TGTTCTCTCGAGCCAGCTTCTCC-3′. Primer p18R4 encodes a sequence within the p18 NF-E2 third exon. PCR products were separated on a nondenaturing 4% polyacrylamide gel and visualized by autoradiography.

Hematologic Analysis. Peripheral blood cell counts, erythrocyte parameters, and blood smears were analyzed from 2-week-old mice. Samples were prepared and analyzed as described (9).

Histologic Analysis. Tissues from 2-week-old mice were prepared and analyzed as described (9).

Nuclear Extract Preparation and Gel-Shift Assays. Fetal liver erythroid cells were isolated from embryonic day 15.5 (E15.5) fetuses by dissection and washed several times in phosphate-buffered saline; then nuclear extracts were prepared (29). Gel-shift assays were performed according to Andrews *et al.* (6) using the NF-E2 binding site probe (oligonucleotide 1) and a mutant NF-E2 site competitor (oligonucleotide 15). Anti-p45 NF-E2 antisera were described by Andrews *et al.* (6). Anti-p18 NF-E2 (C-16) antibodies were obtained from Santa Cruz Biotechnology.

RESULTS

Targeted Disruption of the Murine p18 NF-E2 Gene. Inactivation of the p18 NF-E2 gene was accomplished using a targeting vector designed to replace the entire third exon of the gene with a *neo* cassette (Fig. 1*A*). Because the majority of the 18-kDa protein, including the basic zipper domain, is encoded by the third exon, the targeted replacement is predicted to create a null mutation. After selection in G418 and ganciclovir, heterozygous p18 NF-E2$^{+/-}$ clones were obtained in the J1 and D3 ES cell lines at a frequency of ≈3%. The targeted allele, indicated by the presence of a 3.7-kb *Bgl* II fragment, was detected by Southern blot analysis (Fig. 1*B*).

Germ line chimeras were obtained from two independent J1 p18 NF-E2$^{+/-}$ ES clones. Heterozygous F_1 animals were interbred to yield homozygous p18 NF-E2$^{-/-}$ mice. As shown in Fig. 1*C*, all genotypes were represented in the F_2 litter. Mutant mice were intercrossed on a C57BL/6 background or maintained on an inbred 129/Sv background. No difference in phenotype was observed between mice derived from these strains or from the different ES cell clones.

Three heterozygous p18 NF-E2$^{+/-}$ D3 ES clones were cultured in an increased concentration of G418 to select for homozygous cell mutants incapable of expressing p18 NF-E2. Southern blot analysis was used to establish the p18 NF-E2$^{-/-}$ genotype (Fig. 1*D*). The faint 3.5-kb fragment corresponding to the wild-type p18 NF-E2 allele present in p18 NF-E2$^{-/-}$ samples was contributed by residual feeder cells.

Characterization of the Gene Disruption as a Null Mutation. To confirm that p18 NF-E2$^{-/-}$ cells do not produce an intact p18 NF-E2 transcript, reverse transcription–PCR was performed on RNA samples isolated from E15.5 fetal liver cells of wild-type, p18 NF-E2$^{+/-}$, and p18 NF-E2$^{-/-}$ fetuses. As expected, no transcript was detected in cells derived from the p18 NF-E2$^{-/-}$ fetus. Therefore the gene-targeting event at the p18 NF-E2 locus constitutes a null mutation (Fig. 2).

***In Vitro* Hematopoietic Differentiation of p18 NF-E2$^{-/-}$ ES Cells.** Hematopoietic development of homozygous p18 NF-E2$^{-/-}$ D3 ES clones was compared with that exhibited by p18 NF-E2$^{+/-}$ D3 ES cells in a one-step *in vitro* differentiation assay. After 10 days in culture, p18 NF-E2$^{-/-}$ clones yielded mixed erythroid and myeloid colonies that were indistinguishable from those derived from p18 NF-E2$^{+/-}$ ES cells (data not shown). Total RNA from each differentiated ES clone was prepared. No differences in either β-major or ζ-globin mRNA transcript levels were observed between p18 NF-E2$^{-/-}$ and p18 NF-E2$^{+/-}$ ES clones as judged by RNase protection assay (Fig. 3).

Analysis of Homozygous p18 NF-E2$^{-/-}$ Mice. Among 132 offspring of F_1 heterozygous matings, wild-type, heterozygous, and homozygous genotypes were represented at the expected frequencies (33 wild type, 64 heterozygous, 35 homozygous mice). At 2 weeks of age, wild-type, p18 NF-E2$^{+/-}$, and p18 NF-E2$^{-/-}$ mice from one F_2 litter were sacrificed for gross and histologic examination. No abnormalities were observed in p18 NF-E2$^{-/-}$ mice (data not shown). Peripheral blood cell counts, red cell parameters, and blood smears from p18 NF-E2$^{-/-}$ mice were indistinguishable from those of wild-type or p18 NF-E2$^{+/-}$ mice (data not shown). Male and female p18 NF-E2$^{-/-}$ mice were fertile, and female mice were able to conceive and nurse their pups.

Formation of NF-E2-Like DNA-Binding Complexes in the Absence of p18 NF-E2. Because no discernible alteration in phenotype was evident in knock-out animals, we examined fetal liver erythroid cells of p18 NF-E2$^{-/-}$ mice to ascertain if NF-E2-like DNA-binding complexes were present. Nuclear extracts were prepared from fetal liver erythroid cells isolated from wild-type, p18 NF-E2$^{+/-}$, and p18 NF-E2$^{-/-}$ fetuses at E15.5 and from cultured MEL cells. DNA-binding activity was determined by gel mobility-shift assay with an NF-E2 recognition site probe. NF-E2-like DNA-binding activities were present in all fetal liver erythroid extracts, irrespective of genotype (Fig. 4). These complexes displayed the same relative mobility as the previously characterized p45–p18 NF-E2 complex in MEL cells. Some proteolytic degradation of the NF-E2-like DNA-binding activity is present in nuclear extracts prepared from fetal liver erythroid cells (Figs. 4 and 5). NF-E2-like DNA-binding activity present in the MEL and the fetal liver erythroid extracts is blocked by preincubation with anti-p45 NF-E2 antisera (Fig. 4, lanes 2, 5, 8, and 11). Addition of polyclonal antibodies

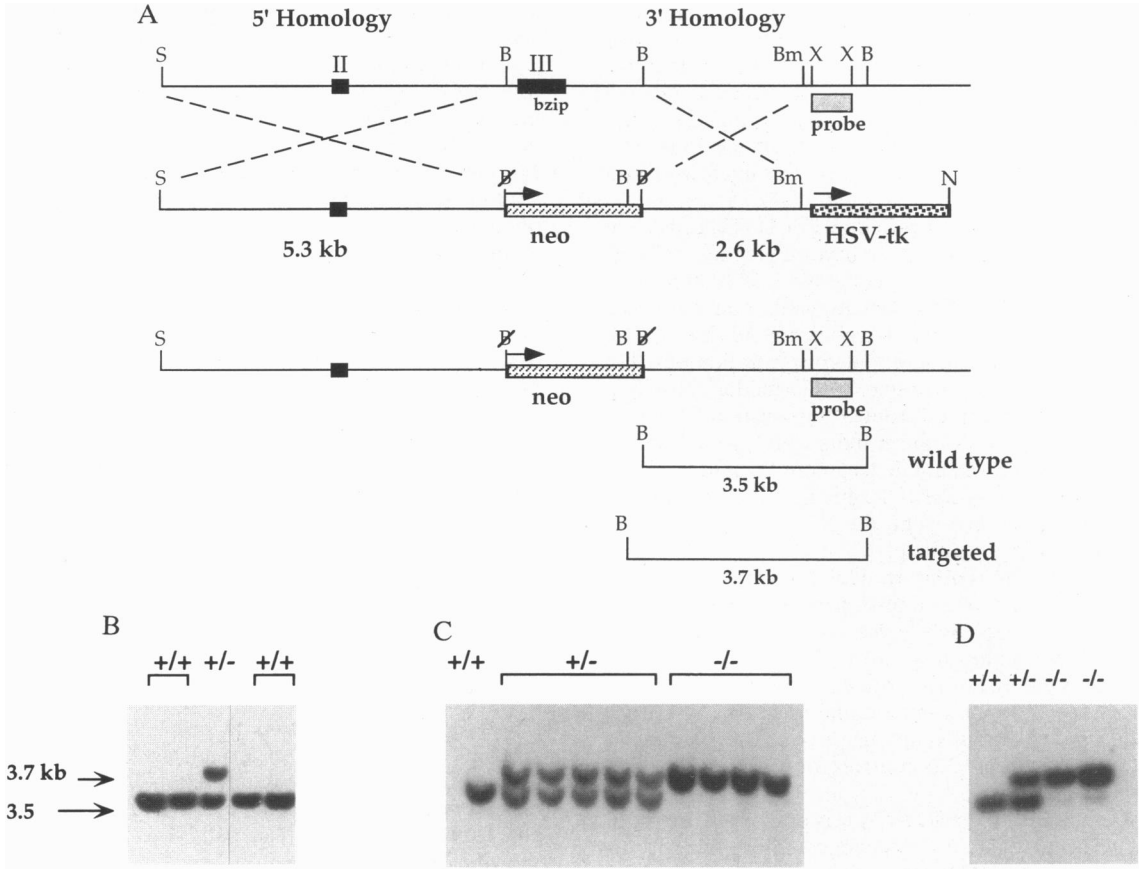

FIG. 1. (*A*) Targeted disruption of the mouse p18 NF-E2 gene. The p18 NF-E2 locus with a partial restriction map is shown (*Upper*) at the top with the targeting construct shown in the middle. Homologous recombination results in the replacement of exon III with the *neo* cassette (*Lower*). Exons II and III are shown as solid boxes. Arrows depict the direction of transcription from the *neo* and HSV-tk cassettes. Restriction enzymes are as follows: B, *Bgl* II; Bm, *Bam*HI; N, *Not* I; S, *Sal* I; X, *Xba* I. (*B*) Southern blot analysis of G418-resistant J1 ES cells. The heterozygous cell line displays both the 3.5- and 3.7-kb fragments corresponding to the wild-type and mutant allele, respectively. (*C*) Southern blot analysis of F_2 offspring. All genotypes were represented among the progeny, as indicated. (*D*) Selection of p18 NF-E2$^{-/-}$ ES cells. Heterozygous D3 ES cell lines were cultured in an increased concentration of G418 to select for a second targeting event. Cell lines were analyzed by Southern blot analysis. Wild-type ES cell genomic DNA has been included as a control.

specific for p18 NF-E2 reduces the intensity but fails to eliminate the gel-shift activity in any sample (Fig. 4, lanes 3, 6, 9, and 12).

Previously, we showed that the p18 NF-E2 subunit imparts DNA-binding specificity to the NF-E2 complex by recognizing the larger portion of the asymmetric 11-bp NF-E2 consensus site (7). To determine whether the NF-E2-like DNA-binding complexes present in p18$^{-/-}$ fetal liver erythroid cells display the same DNA-binding specificity as the native p45–p18 NF-E2 complex, gel-shift assays were performed using various oligonucleotide competitors. An unlabeled mutant NF-E2 site oligonucleotide is unable to compete with the wild-type NF-E2 site probe for binding the p45–p18 NF-E2 MEL cell complex (Fig. 5, lanes 1 and 3). In contrast, the unlabeled wild-type NF-E2 oligonucleotide competes efficiently with the probe for binding to the MEL NF-E2 complex (Fig. 5, lanes 1 and 2). The specificity of NF-E2-like DNA-binding activity present in p18 NF-E2$^{-/-}$ fetal liver erythroid cells is indistinguishable from that of MEL cells, wild-type, or p18 NF-E2$^{+/-}$ fetal liver erythroid cells (Fig. 5). Therefore, in p18 NF-E2$^{-/-}$ erythroid cells, a partner other than p18 NF-E2 forms a heterodimer with p45 NF-E2 to generate a DNA-binding activity that has properties indistinguishable from the previously characterized p45–p18 NF-E2 complex.

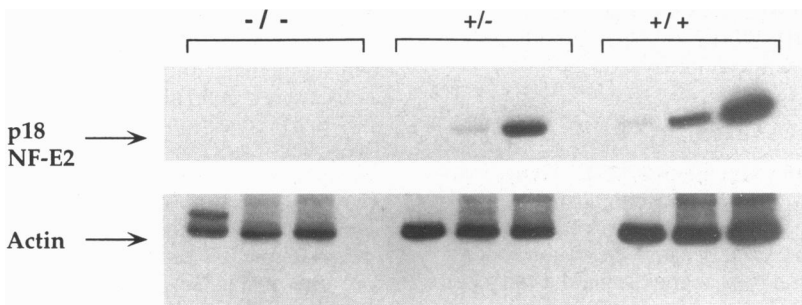

FIG. 2. The p18 NF-E2 gene disruption results in a null mutation. Reverse transcription–PCR was performed on fetal liver erythroid RNA isolated from genotyped F_2 offspring. Aliquots of each PCR were removed for analysis at cycles 23, 27, and 30. The p18 NF-E2 PCR products and the actin PCR products are indicated.

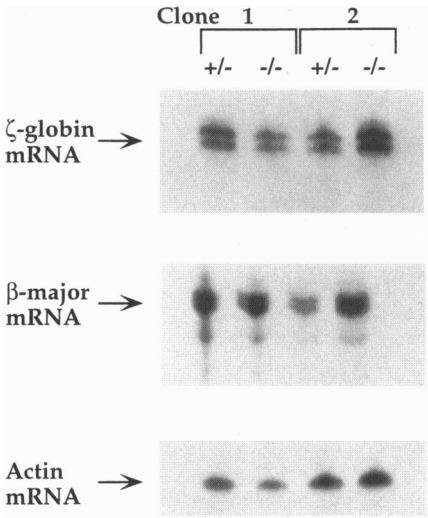

FIG. 3. *In vitro* hematopoietic differentiation of p18 NF-E2$^{+/-}$ and p18 NF-E2$^{-/-}$ ES cells. RNase protection assays for ζ-globin and β-major mRNAs were isolated from embryoid bodies after 10 days in culture. In all samples, actin was assayed in parallel.

DISCUSSION

We have employed homologous recombination in ES cells to produce mice lacking p18 NF-E2. Recently, it was proposed that p18 NF-E2 might act through NF-E2 sites as either an activator of transcription if bound to p45 NF-E2 or as a repressor if bound as a homodimer. If this were the case, the concentration of p18 NF-E2 and other small maf proteins, relative to other bZIP partners, might function as a switch to regulate gene expression through NF-E2 sites (13, 14, 16). These data, coupled with the observation that p45 NF-E2 and p18 NF-E2 are both expressed in various hematopoietic cell lineages, have led to speculation that p18 NF-E2 may play an important regulatory role in the early stages of hematopoiesis (14).

While our results do not identify a specific requirement for p18 NF-E2 *in vivo*, they demonstrate the complexity of proteins acting through NF-E2 sites in hematopoietic cells. We have determined that in the absence of p18 NF-E2, expression of an NF-E2-like DNA-binding complex or complexes that contain the hematopoietic-restricted p45 NF-E2 subunit remains in fetal liver erythroid cells. This NF-E2-like DNA-binding complex has the same DNA-binding specificity as the p45–p18 NF-E2 heterodimer. Since the unique DNA-binding specificity of NF-E2 is imparted by p18 NF-E2, we infer that the remaining NF-E2-like DNA-binding activity also contains a small maf protein in a complex with p45 NF-E2. Therefore, it is likely that mafG or mafF, or an unknown related protein, substitutes for p18 NF-E2 in its absence. The small maf proteins are expressed widely and in overlapping patterns, suggesting that they may have overlapping functions *in vivo* as well (8, 14, 30). Our results are, to our knowledge, the first demonstration that in hematopoietic cells this is, indeed, the case.

Comparison of data obtained from analysis of p18 NF-E2$^{-/-}$ and p45 NF-E2$^{-/-}$ mice has revealed that the complexity of bZIP proteins that may function through NF-E2 sites varies among different hematopoietic cell lineages. Erythroid cells of p45 NF-E2$^{-/-}$ mice have only mildly reduced levels of hemoglobin (9); however, megakaryoctes from these mice are arrested at a stage during terminal maturation, resulting in a block to platelet production (10). These data suggest that either p45 NF-E2 is the only p45-like NF-E2 subunit expressed in megakaryocytes or it is the only p45-like subunit that functions through NF-E2 sites in megakaryocytes to regulate platelet production. In contrast, red cell parameters and platelet counts are normal in p18 NF-E2$^{-/-}$ mice, demonstrating that functional p18 NF-E2-like subunits are present in both erythroid cells and megakaryocytes. Finally, we have interbred p18-null mice with p45-null mice to obtain compound homozygotes to determine if the factors compensating for each NF-E2 subunit are able to functionally reconstitute NF-E2 activity *in vivo*. No dramatic effect on erythropoiesis was observed in the compound homozygotes that were indistinguishable from littermates at E12 to E15 (K.J.K., R. A. Shivdasani, and S.H.O., unpublished data). Together, these results suggest that multiple bZIP partner interactions in various hematopoietic cell lineages are able to function through NF-E2 sites during hematopoiesis.

The complexity of bZIP factors functioning through NF-E2 sites in hematopoietic cells is unexpected, given the strict requirement for the p45–p18 NF-E2 heterodimer in directing high-level globin expression in MEL cells (8). These prior findings may suggest that the repertoire of functional NF-E2-binding factors may be more limited in MEL cells than in hematopoietic cells of the developing mouse. As purification of the NF-E2 complex from MEL cells yielded only p45 NF-E2 and p18 NF-E2 peptide sequences, these subunits appear to

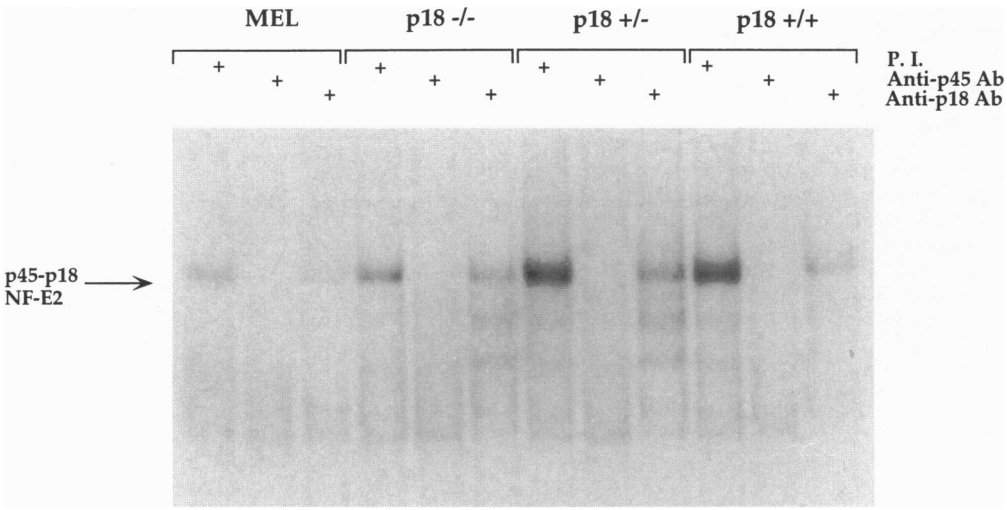

FIG. 4. Formation of NF-E2-like DNA-binding complexes in fetal liver erythroid cells from p18 NF-E2-null mice. Shown is a gel-shift analysis of nuclear extracts prepared from MEL cells and fetal liver erythroid cells from p18 NF-E2$^{-/-}$, p18 NF-E2$^{+/-}$, and wild-type mice using a radiolabeled probe specific for the NF-E2 recognition site. Preincubation of nuclear extracts with either preimmune sera (P.I.), anti-p45 NF-E2 antisera, or anti-p18 NF-E2 antibodies (Ab) is indicated at the top of each lane. The p45–p18 NF-E2 complex from MEL cells is indicated at the left.

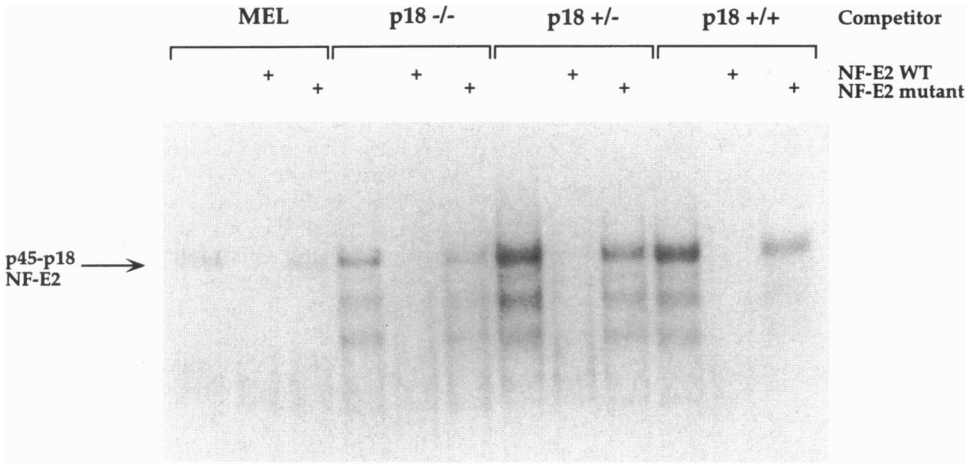

FIG. 5. Specificity of NF-E2-like DNA-binding activity in fetal liver erythroid cells from p18-null mice. Shown is a gel-shift analysis of nuclear extracts prepared from MEL cells and fetal liver erythroid cells from p18 NF-E2$^{-/-}$, p18 NF-E2$^{+/-}$, and wild-type mice using a radiolabeled probe specific for the NF-E2 recognition site. Addition of unlabeled oligonucleotide competitors, corresponding to either the wild-type (WT) NF-E2 or a mutant NF-E2 site, is indicated at the top of each well. The p45–p18 NF-E2 complex from MEL cells is indicated at the left.

represent the predominant species in these cells (6, 7). Also, Lu *et al.* (31) have observed that disruption of the p45 NF-E2 genes in the MEL cell line CB3 was accompanied by a dramatic decrease in globin expression. Hence, in MEL cells p45 NF-E2 plays a unique role in regulating globin gene transcription. Insights into NF-E2 function derived from studies in MEL cells suggest that it may be possible to alter globin gene regulation during development by further limiting the repertoire of bZIP factors operative at NF-E2 sites in intact mice, either by expressing dominant-negative NF-E2 subunits or by additional gene targeting. Defining the subset of transcription factor complexes that operate through NF-E2 sites *in vivo* and determining the developmental stage at which they act will be important steps toward understanding how locus control region–promoter interactions regulate globin gene expression during erythropoiesis.

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