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Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2

(locus control region/globin enhancer/hypochromic anemia/gene targeting)

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ABSTRACT **Previous studies in transgenic mice and cultured cells have indicated that the major enhancer function for erythroid cell expression of the globin genes is provided by the heterodimeric basic–leucine zipper transcription factor NF-E2. Globin gene expression within cultured mouse erythroleukemia cells is highly dependent on NF-E2. To examine the requirement for this factor *in vivo*, we used homologous recombination in embryonic stem cells to generate mice lacking the hematopoietic-specific subunit, p45 NF-E2. The most dramatic aspect of the homozygous mutant mice was an absence of circulating platelets, which led to the death of most animals due to hemorrhage. In contrast, the effect of loss of NF-E2 on the erythroid lineage was surprisingly mild. Although neonates exhibited severe anemia and dysmorphic red-cell changes, probably compounded by concomitant bleeding, surviving adults exhibited only mild changes consistent with a small decrease in the hemoglobin content per cell. p45 NF-E2-null mice responded to anemia with compensatory reticulocytosis and splenomegaly. Globin chain synthesis was balanced, and switching from fetal to adult globins progressed normally. Although these findings are consistent with the substitution of NF-E2 function *in vivo* by one or more compensating proteins, gel shift assays using nuclear extracts from p45 NF-E2-null mice failed to reveal novel complexes formed on an NF-E2 binding site. Thus, regulation of globin gene transcription through NF-E2 binding sites *in vivo* is more complex than has been previously appreciated.**

The regulated expression of globin genes within developing erythroid cells is achieved through interactions between a limited repertoire of conserved cis-acting DNA elements and trans-acting nuclear proteins (1). Within the human β-globin gene cluster, critical cis regulatory sequences are located 6–60 kb upstream of the individual β-like globin genes in a segment of chromatin characterized by four erythroid-specific DNase I-hypersensitive sites (HSs) comprising the locus control region (LCR) (2–4). Study of the proteins interacting with specific sequences within the HS cores of the LCR has provided insight into the mechanisms underlying tissue-specific gene expression and erythroid cell differentiation (1, 5).

A substantial portion of the *in vivo* transcriptional enhancer activity of the β-globin LCR depends on the integrity of tandem AP-1-like motifs within HS-2. Mutation of these sites largely abrogates expression of linked genes in transfected cells as well as in transgenic mice (6–8). Similar AP-1-like motifs are present in the upstream α-globin HS-40 regulatory region (9) and within other β-globin HS cores, such as human HS-4 (10). Although ubiquitously expressed transcription factors of the AP-1 family are able to bind these sequences, an additional binding activity, designated NF-E2 (for nuclear factor-erythroid 2), is present in extracts of erythroid cell lines (11–13). NF-E2 has been shown to be an obligate heterodimer between hematopoietic-specific 45 kDa (p45) and widely expressed 18 kDa (p18) subunits, both members of the basic region–leucine zipper (bZip) family of transcription factors (14–16). p45 NF-E2, which together with the *Drosophila* cap'n-collar (CNC) protein defines a bZip subfamily, is expressed in hematopoietic cells of the erythroid, megakaryocytic, and mast cell lineages (14). Two p45 NF-E2/CNC-related polypeptides, Nrf1/LCR-F1 and Nrf2, which are more widely expressed, have been identified by cDNA cloning (8, 17–19).

Studies in mouse erythroleukemia (MEL) cells provide strong support for the proposed role of NF-E2 as an enhancer-binding protein for globin gene expression. MEL cells of the line CB3, which lack NF-E2 protein due to integration of Friend viral sequences within the p45 NF-E2 gene locus, fail to express appreciable β-globin mRNA (20). Forced expression of p45 NF-E2 cDNA partially restores globin expression (20, 21). Moreover, inhibition of NF-E2 function in wild-type MEL cells through expression of a dominant negative mutant of the p18 subunit results in impaired expression of both α- and β-globin which is reversed by expression of a tethered p45/p18 NF-E2 molecule (21). Within MEL cells, therefore, globin gene expression is highly dependent on NF-E2.

To investigate the functions of NF-E2 during development *in vivo*, we used homologous recombination in embryonic stem cells (22) to generate mice lacking p45 NF-E2. As we have reported recently, these mice fail to produce platelets secondary to a maturational arrest in the megakaryocyte lineage and typically die as a result of hemorrhage (23). Further, contrary to predictions based on the findings in MEL cells, effects on the erythroid lineage in these p45 NF-E2 knockout mice are remarkably mild, as described here.

MATERIALS AND METHODS

Hematologic Analysis. Peripheral blood cell counts and erythrocyte parameters were determined with a Technicon H3 instrument. Fifty microliters of blood was collected into microtiter EDTA tubes (Becton Dickinson), diluted 1:4 in preservation buffer (10 mM NaCl/150 mM KCl/10 mM glucose/1 mM $MgCl_2$/2.5 mM potassium phosphate, pH 7.5), and analyzed within 1 hr. Blood smears, prepared prior to dilution, were stained with Wright–Giemsa stain.

Histologic Analysis. Dissected tissues were fixed overnight in buffered 10% formalin and bone samples were decalcified for marrow analysis. Tissues were embedded in paraffin and 5- to 10-μm sections were stained with hematoxylin and eosin for

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Abbreviations: bZip, basic–leucine zipper; E*n*, embryonic day *n*; HS, DNase I-hypersensitive site; LCR, locus control region; MEL, mouse erythroleukemia; PBGD, porphobilinogen deaminase; RT–PCR, reverse transcription–polymerase chain reaction.

histologic examination or with Prussian blue and nuclear fast red (counterstain) for detection of stainable iron.

Measurement of Serum Iron and Iron-Binding Capacity. Serum iron and iron-binding capacity were measured in the clinical laboratories at Children's Hospital (Boston) by standard methods of atomic absorption.

Assessment of Transcript Levels. RNA was prepared (24) from livers dissected from embryonic day 15.5 (E15.5) fetuses or from neonatal peripheral blood. Northern and RNase protection analyses were performed by standard procedures (25) with previously reported probes for ferrochelatase (26), erythroid exon 2 of the human porphobilinogen deaminase (PBGD) (27), and ζ-, α-, and β-globins (28). The probe for Northern analysis of the murine transferrin receptor was derived from a partial PCR clone of the 3′ untranslated region of the human cDNA (bp 3440–4020) which is conserved in the mouse (29).

For quantitative reverse transcription (RT)–PCR analysis, first-strand cDNA synthesis and PCR (cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min) in the presence of [α-^{32}P]dCTP were performed as described (30), for a number of cycles corresponding to the high end of the range in which a linear increase in products could be detected. Reaction products were separated by electrophoresis in 4% polyacrylamide gels. No reaction products were detected for RNA samples from which reverse transcriptase had been omitted (data not shown). Equivalence of mutant and control cDNA was verified by PCRs for actin and hypoxanthine phosphoribosyltransferase (data not shown). Primer sets were Nrf1 (LCR-F1), 5′-CCCTACTCACCCAGTCAGTAT-3′ and 5′-CTGTCGCCGAGCCTGCTGGTC-3′, and Nrf2, 5′-CATTCAAGCCGCTTAGA-3′ and 5′-GGCTATAATTCTGCATA-3′.

Gel Shift Assays. Gel electrophoretic mobility-shift assays were performed as described (14). Nuclear extracts were prepared (31) either from whole fetal liver suspensions or from embryonic red blood cells isolated from single yolk sacs at E10.5 and E11.5. The radiolabeled DNA probe corresponded to the tandem NF-E2 sites within HS-2 of the human β-globin LCR, with the sequence 5′-AGCACAGCAATGCTGAGTCATGATGAGTCATGCTGAGGC-3′ (NF-E2 consensus sequence is shown in bold type).

RESULTS AND DISCUSSION

Erythrocyte Abnormalities in the Absence of p45 NF-E2. The generation of p45 NF-E2-null mice has been described (23). Interbreeding of heterozygous (p45 NF-E2$^{+/-}$) mice yielded homozygotes (p45 NF-E2$^{-/-}$) at the predicted Mendelian frequency of 25%, indicating the absence of fetal loss. However, neonatal mortality of p45 NF-E2$^{-/-}$ mice was high (≈90%) due to extensive hemorrhage (23). Further, in comparison to littermate controls, severe erythrocyte abnormalities, including extensive reticulocytosis, hypochromia, target cells, and dysmorphic cell forms, were detected in p45 NF-E2$^{-/-}$ neonates (Fig. 1 *A* and *B*). Mutant mice that survived beyond the perinatal period exhibited low, sporadic mortality thereafter. Red-cell morphologic abnormalities were less dramatic in surviving adult homozygotes than in the younger mice and included hypochromia, target cell formation and reticulocytosis, with only infrequent dysmorphic red cells (Fig. 1*C*).

Hematologic parameters are summarized in Table 1. Heterozygotes were indistinguishable from wild-type animals; adult homozygotes had a packed red-cell volume (hematocrit) ranging from 50% to 100% of normal, presumably reflecting variation in the degree of recent bleeding. Notably, however, the mean hemoglobin content and concentration of p45-null red blood cells were consistently reduced relative to controls, and the distributions of red cell size and hemoglobin content were considerably wider; these abnormalities were an invariant finding in p45 NF-E2$^{-/-}$ mice regardless of the hematocrit. While these findings may in part reflect increased numbers of reticulocytes, abnormalities intrinsic to mature erythrocytes were suggested by the visual impression of hypochromia (decreased hemoglobin content) and confirmed by automated analysis (Fig. 1 *D* and *E*) of mature red cells and reticulocytes. Thus, examination of erythroid cells in mice lacking NF-E2 revealed abnormalities that are consistent with the proposed role of this factor in enhancing globin gene expression, although the magnitude of the effect was far less than anticipated.

Inspection of hematopoietic tissues revealed active erythropoiesis with normal erythroid maturation (Fig. 2*A*). Consistent with this finding, equal numbers of erythroid (and other hematopoietic) colonies were recovered in *in vitro* colony assays from the bone marrows of p45 NF-E2$^{-/-}$ mice and control littermates (data not shown), and the morphology of the colonies was normal. Further, splenomegaly with active erythropoiesis was detected throughout the life of surviving homozygotes (Table 1).

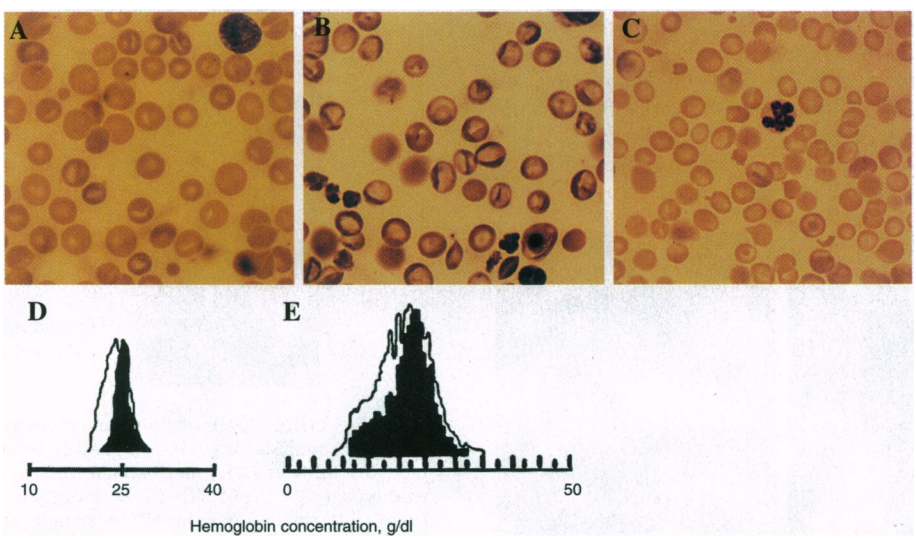

FIG. 1. Hematologic profiles of p45 NF-E2 knockout and control mice. (*A–C*) Peripheral blood smears from control neonatal (*A*), p45$^{-/-}$ neonatal (*B*), and p45$^{-/-}$ adult (*C*) mice, showing dysmorphic red cells, hypochromia, target cells, and reticulocytosis. (×490.) (*D* and *E*) Histograms of hemoglobin concentration (g/dl, *x* axis) per mature erythrocyte (*D*) or per reticulocyte (*E*), with the distribution in control mice shown in black and that in the knockout mice shown in white.

Table 1. Hematologic parameters in adult p45 NF-E2-null (−/−) and control wild-type (+/+) or heterozygous (+/−) mice

Parameter	+/+ or +/−	*n*	−/−	*n*
Hb, g/dl	13.98 ± 1.33	13	10.97 ± 2.07	11
Hematocrit, %	48.67 ± 5.19		39.65 ± 8.05	
Mean red-cell volume, fl	53.52 ± 2.19		52.09 ± 3.39	
Mean cell Hb, pg	15.42 ± 0.83		13.59 ± 0.71	
Mean cell Hb conc., g/dl	28.84 ± 1.46		26.30 ± 1.20	
Red-cell distribution width, %	12.79 ± 1.13		16.94 ± 1.27	
Hb distribution width, %	1.64 ± 0.15		2.22 ± 0.24	
Serum iron, mg/dl (range)	164–256	6	155–272	3
Iron-binding capacity, μg/dl (range)	384–672	4	448–740	2
Spleen weight (average)				
Mass, g	0.106	11	0.635	8
% body weight	0.31		1.58	

Values are expressed as the mean ± SEM, unless otherwise stated, and were measured as described under *Materials and Methods*. Ages of the mice tested ranged from 7 to 12 weeks. Hb, hemoglobin.

Although the pattern of erythrocyte abnormalities observed raises the possibility that these are a secondary consequence of hemorrhage and ensuing iron deficiency, the sustained reticulocytosis and increased erythroid activity are not consistent with this explanation. However, to assess the iron stores of the mutant mice, we tested serum from adults. Serum iron and iron-binding capacity were not different from controls (Table 1), and adequate bone marrow iron stores were evident upon Prussian blue staining (Fig. 2*B*). Thus, although secondary effects of hemorrhage may contribute to the erythrocyte abnormalities, particularly in the neonatal period, mice lacking p45 NF-E2 are not demonstrably iron-deficient.

Adult p45 NF-E2$^{-/-}$ mice consistently exhibited splenomegaly, which most likely reflects a compensatory response to either hemorrhage or decreased hemoglobin accumulation in red cells. Of particular relevance, splenomegaly was evident in p45 NF-E2$^{-/-}$ neonates prior to the onset of clinically significant bleeding (data not shown). Taken together, these findings argue that mice lacking p45 NF-E2 harbor an intrinsic erythroid defect characterized by decreased hemoglobin content. Increased erythropoiesis, evidenced by splenomegaly and reticulocytosis, apparently compensates for the mild deficit so that anemia is insignificant in the absence of bleeding, as judged by the normal hematocrit seen in a number of mutant adults.

Gene Expression in the Absence of p45 NF-E2. We examined globin RNA transcript levels in fetal and adult red cells by RNase protection assays. When samples were standardized for total RNA, no differences were detected between mutant and control samples (Fig. 3*A*). Although the decreased hemoglobin content of red cells (Fig. 1 *D* and *E*) presumably reflects mildly reduced globin RNA levels *per cell*, these data reveal that loss of NF-E2 does not selectively affect the expression of individual globin genes or influence globin gene switching during development.

Besides the globin gene LCRs, NF-E2 sites have been identified in the promoters of the human PBGD (11, 32) and ferrochelatase (33) genes. However, these sites are known only for the human genes, and the functional relevance of individual NF-E2 cis elements has not been established *in vivo*. Northern

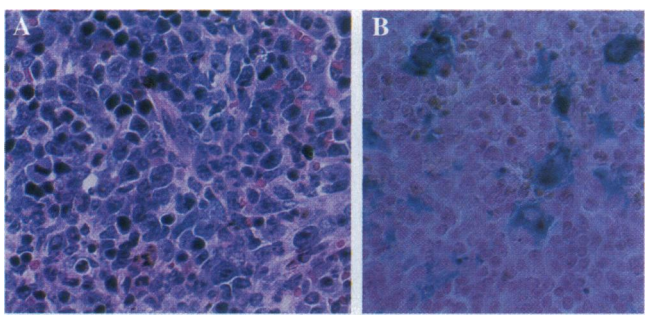

FIG. 2. Bone marrow from mice lacking p45 NF-E2. Sections of the bone marrow were stained with either hematoxylin and eosin (*A*) or Prussian blue (*B*) to reveal active erythropoiesis and adequate body iron stores. (×195.)

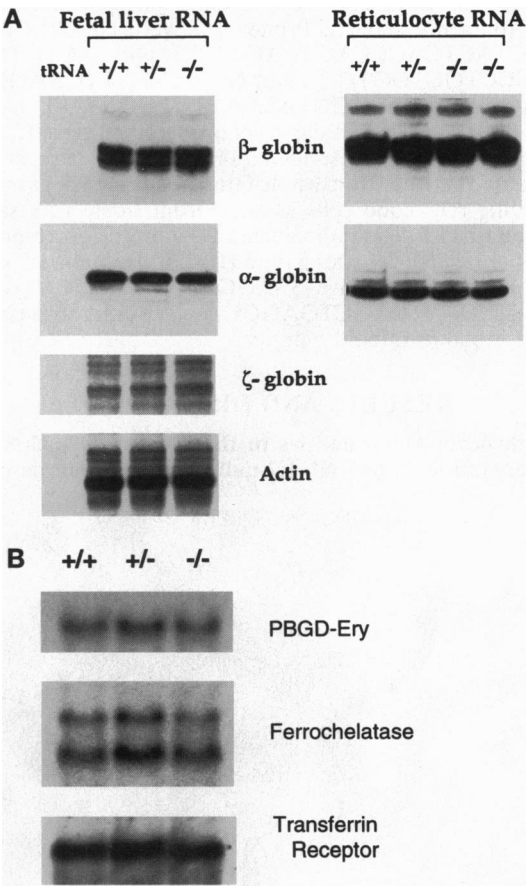

FIG. 3. mRNA levels of globin and other gene products. (*A*) RNase protection analysis for adult (α, β) and embryonic (α, ζ) globin mRNA in E16 fetal liver (left) and neonate reticulocyte (right) RNA from wild-type (+/+), p45 NF-E2 heterozygote (+/−), and knockout (−/−) littermates. A yeast tRNA sample and an actin probe were included as controls, as shown. (*B*) Northern analysis of total RNA from E16 fetal livers for detection of PBGD (erythroid exon 2), ferrochelatase, and transferrin receptor transcripts. Equal loading of samples was confirmed by ethidium bromide staining of 18S and 28S rRNAs and by probing the same blot for actin mRNA (data not shown).

analysis detected no difference between mutant and control samples for the RNAs encoding these proteins (Fig. 3*B*). Hence, *in vivo* loss of the p45 subunit does not result in a significant decrease in expression of the transcripts whose expression is potentially regulated by NF-E2. Moreover, normal levels of transferrin receptor mRNA (Fig. 3*B*) and of all the major erythrocyte cytoskeletal proteins (data not shown) were detected upon Northern analysis and SDS/PAGE analysis of red-cell ghost preparations, respectively.

Although NF-E2 is required for high-level globin gene expression in cultured MEL cells, our findings indicate that the factor is largely dispensable for erythropoiesis and balanced globin gene expression within the intact mouse. One explanation for the apparent disparity is that the function of the p45 subunit in developing erythrocytes *in vivo* may be provided by other cell-specific or ubiquitous factors which are either not expressed or not functional in MEL cells. The presence of consistent, albeit mild, erythrocyte abnormalities further suggests that such compensation is incomplete. Alternatively, NF-E2 binding sites within globin LCR elements may themselves be largely dispensable for high-level gene expression within the context of an intact LCR *in vivo*. To date, studies which have implicated NF-E2 sites in enhancement of globin gene expression in cell lines or transgenic mice have utilized either isolated HSs or artificial LCR constructs, largely tested on the human β-globin gene as a reporter. It remains a formal possibility that other cis-acting DNA elements compensate for the absence of functional NF-E2 sites in an otherwise intact animal. The experimental distinction between these possibilities is critical to a complete understanding of LCR function and awaits the results of studies in which individual LCR subregions are systematically mutated and tested *in vivo* in an appropriate chromosomal context.

Potential Role for Other Factors Binding to NF-E2 Sites. Using nuclear extracts from cultured erythroid cell lines, previous studies have documented that the erythroid-specific p45/p18 heterodimer and ubiquitous AP-1 activity together constitute the dominant proteins binding to NF-E2 sites *in vitro* (12–14). To seek evidence of proteins that might compensate for NF-E2 loss *in vivo*, we performed gel shift assays using nuclear extracts from p45 NF-E2$^{-/-}$ fetal liver cells. In normal fetal liver nuclear extracts, as in MEL cells, the predominant complex formed on an NF-E2 site was the p45/p18 NF-E2 heterodimer (Fig. 4*A*, lanes 1 and 2); less evident, slower-mobility complexes represented AP-1 and undefined AP-1-like proteins. Consistent with the nature of the targeted mutation and with the absence of normal p45 mRNA and protein in the mutants (23), no NF-E2 DNA-binding activity was detected in nuclear extracts of p45 NF-E2$^{-/-}$ fetal liver cells (Fig. 4*A*, lanes 3 and 4). Gel shift assays using nuclear extracts of erythroid cells purified from single yolk sacs at E10 or E11 yielded similar results (Fig. 4*A*, lanes 5–8). Thus, as expected from the composition of NF-E2, loss of the p45 subunit leads to absence of the p45/p18 heterodimer. Notably, no novel gel shift complexes were clearly evident with p45 NF-E2$^{-/-}$ nuclear extracts.

If transcriptional activation mediated through NF-E2 sites in the globin LCRs is indeed essential for high-level globin gene expression *in vivo*, as implied by previous data, then this result suggests that potential compensating proteins are either contained within the undefined AP-1 region or not detected in the gel shift assay. Although cDNAs encoding widely expressed p45 NF-E2-related factors have been cloned, their binding specificities, dimerization preferences, and *in vivo* function are unknown (8, 17–19). We used a quantitative RT–PCR assay to compare the mRNA levels of Nrf1 (LCR-F1) and Nrf2 in fetal erythroid cells lacking p45 NF-E2 with those from wild-type littermates. mRNA levels for these factors (Fig. 4*B*), and protein levels of Nrf1 (immunoblot; data not shown), appeared

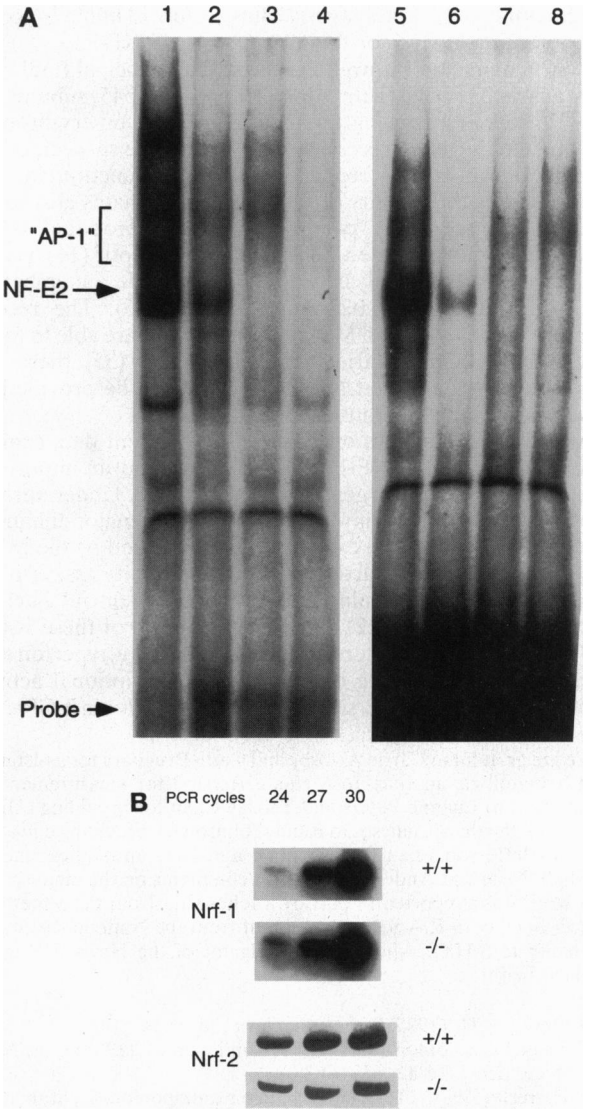

FIG. 4. Search for proteins potentially compensating for *in vivo* absence of NF-E2. (*A*) Gel electrophoretic-mobility shift of NF-E2 binding sites by erythroid nuclear extracts. Extracts were isolated from either MEL cells (lanes 1 and 5), whole fetal livers (lanes 2–4), or red blood cells isolated from single yolk sacs at E10.5 (lanes 6 and 7) or E11.5 (lane 8). NF-E2 activity is seen in extracts derived from heterozygous (p45 NF-E2$^{+/-}$) littermates (lanes 2 and 6) but not from p45-null mice (lanes 3, 4, 7, and 8). The undefined AP-1 activity with slower migration is preserved in control and knockout extracts, and formation of this labeled complex is blocked specifically by excess unlabeled probe (lane 4). (*B*) Assessment of levels of the transcripts encoding p45 NF-E2/CNC-related proteins Nrf1 (LCR-F1) and Nrf2 by RT–PCR. PCR was carried out with [α-^{32}P]dCTP for the indicated number of cycles. Equivalent amounts of p45 NF-E2$^{+/+}$ and p45 NF-E2$^{-/-}$ fetal liver cDNA were used as templates, as judged by RT–PCR for actin and hypoxanthine phosphoribosyltransferase transcripts (data not shown).

essentially the same as in controls, indicating that expression of neither factor is upregulated in the absence of p45 NF-E2.

To test the possibility that redundancy could be attributed to the c-Jun subunit of AP-1, we generated compound heterozygotes with targeted disruptions in each of two genes, p45 NF-E2 and c-*jun* (p45$^{+/-}$/c-*jun*$^{+/-}$), by mating c-*jun*$^{+/-}$ (34) and p45 NF-E2$^{+/-}$ mice. Interbreeding of these compound heterozygotes yielded compound homozygous mice at the expected frequency of 1/16 when the offspring were analyzed *in utero* at E10.5. The compound homozygotes were grossly indistinguishable from their littermates and showed no obvi-

ous anemia (data not shown). Thus, c-Jun is unlikely to be compensating *in vivo* for the absence of p45 NF-E2.

Conclusions and Future Prospects. Our principal finding is that absence of the hematopoietic-specific p45 subunit of NF-E2 leads to surprisingly mild consequences on erythropoiesis and globin gene expression *in vivo*. If, as we suspect, other protein(s) can largely replace p45 NF-E2 function in this circumstance, the identity of such factor(s) remains elusive. A distinct feature of the p45/p18 NF-E2 heterodimer is its specificity for an extended AP-1 recognition motif (14), which is imparted by the widely expressed p18 subunit, a member of the Maf subfamily of bZip proteins (15, 16). The recent demonstration that small Maf-related proteins are able to form heterodimers with a variety of bZip factors (35) therefore raises the possibility that NF-E2 function can be provided *in vivo* by a diversity of transcriptional activators.

As with any knockout experiment, the current data cannot be used to argue that NF-E2 itself does not play an important role in regulating globin gene expression *in vivo*. Under normal circumstances, NF-E2 may indeed provide the major enhancer function for globin gene expression, as suggested by the MEL cell studies (20, 21), particularly since gel shift assays have failed to reveal novel binding activity in erythroid nuclear extracts from p45 NF-E2$^{-/-}$ mice. Resolution of these issues will require a fuller understanding of the entire repertoire of nuclear proteins capable of providing transcriptional activation through the NF-E2 sites located within globin LCRs.

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