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Dependence of Globin Gene Expression in Mouse Erythroleukemia Cells on the NF-E2 Heterodimer

KAREN J. KOTKOW AND STUART H. ORKIN*

Division of Hematology/Oncology, Children's Hospital, Department of Pediatrics, Dana-Farber Cancer Institute, and Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts

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High-level, tissue-specific expression of the β-globin genes requires the presence of an upstream locus control region (LCR). The overall enhancer activity of the β-globin complex LCR (β-LCR) is dependent on the integrity of the tandem NF-E2 sites of HS-2. The NF-E2 protein which binds these sites is a heterodimeric basic leucine zipper protein composed of a tissue-specific subunit, p45 NF-E2, and a smaller subunit, p18 NF-E2, that is widely expressed. In these studies, we sought to investigate the role of NF-E2 in globin expression. We show that expression of a dominant-negative mutant p18 greatly reduces the amount of functional NF-E2 complex in the cell. Reduced levels of both α- and β-globin were associated with the lower levels of NF-E2 activity in this cell line. Globin expression was fully restored upon the introduction of a tethered p45-p18 heterodimer. We also examined CB3 cells, a mouse erythroleukemia (MEL) cell line that does not express endogenous p45 NF-E2, and demonstrated that the restoration of globin gene expression was dependent upon the levels of expressed tethered NF-E2 heterodimer. Results of DNase I hypersensitivity mapping and in vivo footprinting assays showed no detectable chromatin alterations in β-LCR HS-2 due to loss of NF-E2. Finally, we examined the specificity of NF-E2 for globin gene expression in MEL cells. These experiments indicate a critical role for the amino-terminal domain of p45 NF-E2 and show that a related protein, LCRF1, is unable to restore globin gene expression in p45 NF-E2-deficient cells. From these results, we conclude that NF-E2 is specifically required for high-level globin gene expression in MEL cells.

Vertebrate globin genes are organized in small multigene complexes that are subject to cell-specific and developmental regulation. High-level, tissue-specific expression of globin genes depends on far-upstream regulatory regions (locus control regions [LCRs]) which coincide with segments of chromatin displaying erythroid-cell-specific DNase I hypersensitivity (14; for a review, see reference 11). The β-globin complex LCR (β-LCR) is divided into four distinct 200- to 300-bp core regions (HS-1, HS-2, HS-3, and HS-4) (12, 14, 46). An analogous erythroid-cell-specific DNase I-hypersensitive core, termed HS-40, functions as an upstream enhancer in the α-globin gene cluster (15, 19). Linkage of LCR or HS-40 sequences to test genes confers high-level erythroid-cell-specific expression in transgenic mice, largely independent of the chromosomal integration site. Three types of DNA-binding sequences, GATA, Sp1 (GTGG), and AP-1- or NF-E2 (nuclear factor-erythroid 2)-like motifs, are consistently found within the core elements of the DNase I-hypersensitive sites (21, 34, 36, 37, 41, 42, 44, 45). Previous studies have associated the GATA and Sp1 (GTGG) sites with position-independent activation (44), whereas the overall enhancer activity of the LCR requires the integrity of tandem AP-1-like sequences of β-LCR HS-2 (27, 28, 31, 32, 44, 45). Similarly, enhancer activity of the α-locus HS-40 element is also dependent on AP-1–NF-E2 sequences (35). Through mutational analysis of these sites in the porphobilinogen deaminase promoter and β-LCR HS-2, an erythroid-activity-enriched AP-1-like DNA-binding activity termed NF-E2 was detected (23).

Purification of the NF-E2 protein from mouse erythroleukemia (MEL) cells has allowed elucidation of its preferred binding site, an asymmetric 11-bp element containing a central AP-1 core [(C/T)GCTGA(G/C)TCA(T/C)] (1). Previous studies have also revealed that NF-E2 is an obligate heterodimer of two novel basic leucine zipper (b-zip) polypeptides (1, 3). The expression of the larger subunit (p45 NF-E2) is largely restricted to hematopoietic tissues, whereas that of the smaller subunit (p18 NF-E2) is widespread. p45 NF-E2 shares extensive homology in its b-zip region with the *Drosophila cnc* (cap 'n collar [25]) protein and the *Caenorhabditis elegans* protein skn-1 (7). As such, these factors define the NF-E2–CNC subfamily of b-zip proteins. More recently, additional proteins related to p45 NF-E2 in their b-zip domains, designated Nrf1/LCRF1 and Nrf2, have been characterized by cDNA cloning (8, 9, 26). p18 NF-E2, on the other hand, shares close homology with a human retinal protein (NRL) and the chicken oncogene v-*maf* (33, 43). Additional members of the maf subfamily include other maf polypeptides (mafB, mafG, and mafF) (18) and the mouse segmentation gene *kr* (10). The small chicken polypeptide known as mafK appears to be equivalent to mouse p18 NF-E2. The presence of p18 NF-E2 in the NF-E2 heterodimer increases binding specificity for the bases lying outside the AP-1 core of the NF-E2 consensus binding site (3). Accordingly, it has been suggested that the role of p45 NF-E2 in the complex is to interact with other cell-specific effector molecules to activate transcription (3).

In the studies reported here, we sought to investigate the role of NF-E2 in globin gene expression. As a first approach, we used a dominant-negative mutant of p18 NF-E2 to inhibit NF-E2 activity in MEL cells and demonstrate the dependence of α- and β-globin gene expression on the cellular NF-E2 level. To show formally that a p45-p18 complex is the active species of NF-E2 in vivo, we generated a tethered p45-p18 heterodimer which rescues globin gene expression in cells harboring dominant-negative p18 NF-E2. Our findings were compared with those obtained with a variant MEL cell line, CB3,

* Corresponding author. Mailing address: Division of Hematology, Children's Hospital, 300 Longwood Ave., Boston, MA 02115. Phone: (617) 355-7910. Fax: (617) 355-7262.

which fortuitously lacks NF-E2 because of retroviral integration of the Friend leukemia virus genome in the p45 NF-E2 gene (22). Finally, we investigated the specificity of NF-E2 for rescue of globin gene expression. These experiments reveal a critical role for the amino-terminal domain of p45 NF-E2 and show that the related polypeptide LCRF1, though reported to be a potent activator of HS-2–β-globin constructs in erythroid cells (8), is unable to rescue globin gene expression in NF-E2-deficient cells. Therefore, NF-E2 is specifically required for globin gene expression in vivo.

MATERIALS AND METHODS

Plasmid constructions. pEF1α-neo and pEF1α-puro expression vectors (48) were constructed as follows. A *Hin*dIII-*Not*I fragment containing the promoter region of human EF1α (translational elongation factor 1α) (24) was subcloned into the polylinker region of pBluescript KS$^+$ (Stratagene). cDNAs encoding the selectable markers neomycin phosphotransferase and puromycin *N*-acetyltransferase under the control of the phosphoglycerate kinase promoter were subcloned into the *Xho*I-*Cla*I and *Sal*I sites, respectively.

The dominant-negative p18 NF-E2 mutant was constructed by overlap extension using PCR (16). *Not*I and *Eco*RI sites (boldface) were incorporated into the primers as follows to facilitate subcloning: p18F12, 5′-ATAAGAAT**GCGGCCGCGAATTC**TTTTCTGGTGGTTCCGTCCT-3′; p18R12, 5′-ATAAGAAT**GCGGCCGCGAATTC**GCAGACACTAGGAGGCGG-3′. Mutant oligonucleotide primers used in the PCR were as follows: p18MF1, 5′-GCACACTCGCCGCGGCTGGCTACGC-3′; p18MR1, 5′-GCGTAGCCAGCCGCGGCGAGTGTGC-3′ (mutated residues are underlined). Thirty cycles of PCR were performed under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min, using *Pfu* polymerase (Stratagene). cDNAs encoding the mutant p18 were subcloned into the *Not*I site of the expression vector pEF1α-neo.

The p45-p18 tethered heterodimer was similarly constructed. In this heterodimer, the carboxy terminus of p45 is linked to the amino terminus of p18 via an in-frame flexible polypeptide [p45-RS$(GGGS)_4$GGRS-p18]. *Not*I and *Eco*RI sites (boldface) were specified 5′ of the initiation codon in the forward p45 primer, NFE2-F1 (5′-ATAGAAT**GCGGCCGCGAATTC**TGGCACAGTAGGATGCCC-3′), and 3′ of the translation termination codon of the reverse p18 primer, NFE2-R2 (5′-ATAGAAT**GCGGCCGCGAATTC**CAGCAGACACTAGGAGGC-3′). Other primers were designed to overlap the sequence encoding the polypeptide linker (underlined; *Bgl*II sites that flank the linker are boldface): NFE2-F2, 5′-GGGGTCTGGAGGTGGGAGCGGCGGAGGGTCCGGCGGA**AGATCT**ATGACGACTAATCCCAAG-3′; and NFE2-R1, 5′-CCGCTCCCACCTCCAGAACCCCCGCCGGATCCACCCCC**AGATCT**ATCTGTAGCCTCCATTTT-3′. PCR conditions were as described above. The tethered LCRF1-p18 heterodimer was similarly constructed except that the following primers specific for the LCRF1 cDNA sequence were used together with primers NFE2-F2 and NFE2-R2 (8): 14F, 5′-ATAGAAT**GCGGCCGCGAATTC**CCATTTGATTTGGAACAG-3′; and 14R, 5′-CCGCTCCCACCTCCAGAACCCCCGCCGGATCCACCCCC**AGATCT**CTTTCTCCGGTCCTTTGG-3′. cDNAs encoding the p45-p18 heterodimer or the LCRF1-p18 heterodimer were subcloned into the *Not*I site of the expression vectors pEF1α-neo and pEF1α-puro, respectively.

A truncated form of p45 NF-E2 was constructed by PCR. A primer (6F) incorporating an *Eco*RI site (boldface) followed by a Kozak translation consensus sequence and the initiation of the p45 NF-E2 sequence (encoding amino acid 207) was utilized: 5′-CCG**GAATTC**AACCACCATGGAGTCATCCTCCGGTCCA-3′. Another primer (3R) used in the PCR incorporated a translation termination codon followed by an *Xba*I site (boldface): 5′-TGC**TCTAGA**TCAATCTGTAGCCTCCAT-3′. The *Eco*RI-*Xba*I cDNA fragment was subcloned into the expression plasmid pEF1α-neo.

Cell culture and transfections. MEL cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Approximately 10^7 cells were electroporated with 20 μg of linearized DNA in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline (Bio-Rad Gene Pulser; 280 V, 960 μF). Stable transfectants were selected in puromycin (1 μg/ml) (Sigma) and/or G418 (1.0 mg/ml) (Gibco/BRL). Induction of differentiation was performed by diluting cells to 10^5/ml in 1.8% dimethyl sulfoxide (DMSO). Cells were harvested after 72 h of incubation. For each set of experiments, cultures of the various stable transfectants were divided and nuclear extracts and cytoplasmic RNA were prepared in parallel.

Gel shift and Western analysis. Approximately equal numbers of cells from each stably transfected MEL cell clone were harvested and washed once with phosphate-buffered saline, and nuclear extracts were prepared (2). Gel shift assays were performed by the method of Andrews et al., using the NF-E2 binding site probe (oligonucleotide 1) (3). The NF-E2 and AP-1 complexes were identified by using the oligonucleotide competitors described by Andrews et al. (3). Western blots (immunoblots) were developed by using anti-p18 NF-E2 antibodies (3) and either alkaline phosphatase reagents (Promega) or an enhanced chemiluminescence detection system (Amersham).

Northern analysis and RNase protection assay. Total cytoplasmic RNA was prepared from stably transfected MEL cells, and Northern (RNA) blot analysis was performed according to standard procedures (38). Antisense riboprobes for mouse β-major globin, α-globin, and γ-actin were synthesized as described elsewhere (4).

DNase I hypersensitivity site mapping. Nuclei from 4×10^7 cells were purified by the method of Wu and resuspended in nuclear buffer with 5% glycerol to 3×10^7 nuclei per ml (47). Aliquots of 3×10^6 nuclei each were incubated with various concentrations of DNase I (0 to 2.0 U/ml; Boehringer-Mannheim) at 25°C for 3 min. The reaction was stopped by the addition of sodium dodecyl sulfate and EDTA to final concentrations of 0.5% and 10 mM, respectively. Each sample was digested with proteinase K (100 μg/ml) and incubated overnight at 37°C. Genomic DNA was isolated by repeated extraction with phenol-chloroform and ethanol precipitation. A 20- to 30-μg sample of DNA was digested with *Pst*I and subjected to Southern blotting. A 359-bp *Ssp*I-*Bgl*II fragment corresponding to nucleotides −14239 to −13880 of the mouse β-LCR (kindly provided by T. Ley) was radiolabeled and used as a probe for Southern analysis (17). This probe hybridizes to a 13-kb *Pst*I fragment that contains both HS-1 and HS-2. Digestion with DNase I yields a 6.5-kb band (*Pst*I–HS-2 fragment) and an 11.5-kb band (*Pst*I–HS-1).

Ligation-mediated in vivo footprinting. A total of 4×10^8 cells from each MEL cell line were harvested. Methylation, isolation of genomic DNA, guanine-adenine-specific cleavage of methylated DNA, and in vivo footprinting were all performed by the method of Strauss et al. (41). Oligonucleotide primers specific for the sense strand of mouse β-LCR HS-2 were as follows: 1, TTCTAGGTTATGTCACACAGC; 2, ATGTCACACAGCAAGGCAGGGTC; and 3, CACAGCAAGGCAGGGTCCCCTTTTC. The sequences of the double-stranded linker (AS.1) and 25-mer linker primer (S.1) are as follows: S.1, 5′-GCAGTGACTCGAGAGATCTGAATTC-3′; AS.1, 5′-GAATTCAGATC-3′.

RESULTS

A dominant-negative mutant p18 NF-E2 reduces NF-E2 DNA-binding activity and impairs globin gene expression in MEL cells. In an effort to inhibit NF-E2 function in vivo, we constructed a mutant form of p18 NF-E2 in which three conserved amino acids (lysine 59, asparagine 60, and arginine 61) in the basic domain were changed to alanine residues. On the basis of the structure of other b-zip proteins (13, 20), we predicted that heterodimers containing this mutation would be unable to bind DNA and therefore function as dominant-negative molecules. Stably transfected MEL clones harboring the mutant p18 NF-E2 cDNA under the control of the strong ribosomal EF1α promoter were selected in the presence of G418 and screened for high-level expression of p18 protein by Western blotting using anti-p18 polyclonal antibodies.

Initial study of numerous clones revealed that increasing expression of the mutant p18 NF-E2 transgene was directly correlated with reduced NF-E2 DNA-binding activity in nuclear extracts, as determined by a gel mobility shift assay (data not shown). No significant variation in GATA-1 DNA-binding activity was detected in these nuclear extract samples (data not shown). Thus, as illustrated by a representative clone in Fig. 1A and B (lanes 1 to 4), abundant expression of mutant p18 NF-E2 led to a marked reduction in binding activity in extracts of both uninduced and DMSO-treated MEL cells. Even at the highest levels of transgene expression, some residual NF-E2 gel shift activity was observed, particularly following DMSO treatment. As a consequence of mutant p18 NF-E2 expression, expression of α- and β-globin mRNAs was reduced (Fig. 1C, lanes 1 to 4). α-Globin expression was only modestly reduced (~50%), whereas a dramatic decrease in β-globin RNA (~5% of the wild-type level) was observed. Thus, inhibition of NF-E2 activity in vivo by expression of a dominant-negative form of p18 NF-E2 is associated with reduced globin gene expression. Moreover, the relative sensitivities of α- and β-globin gene expression to reduced levels of NF-E2 differ. These findings were confirmed with four other independent MEL clones expressing mutant p18 NF-E2.

A tethered p45-p18 heterodimer restores NF-E2 activity and globin gene expression. We next sought to show that reduced NF-E2 activity and globin gene expression were indeed attrib-

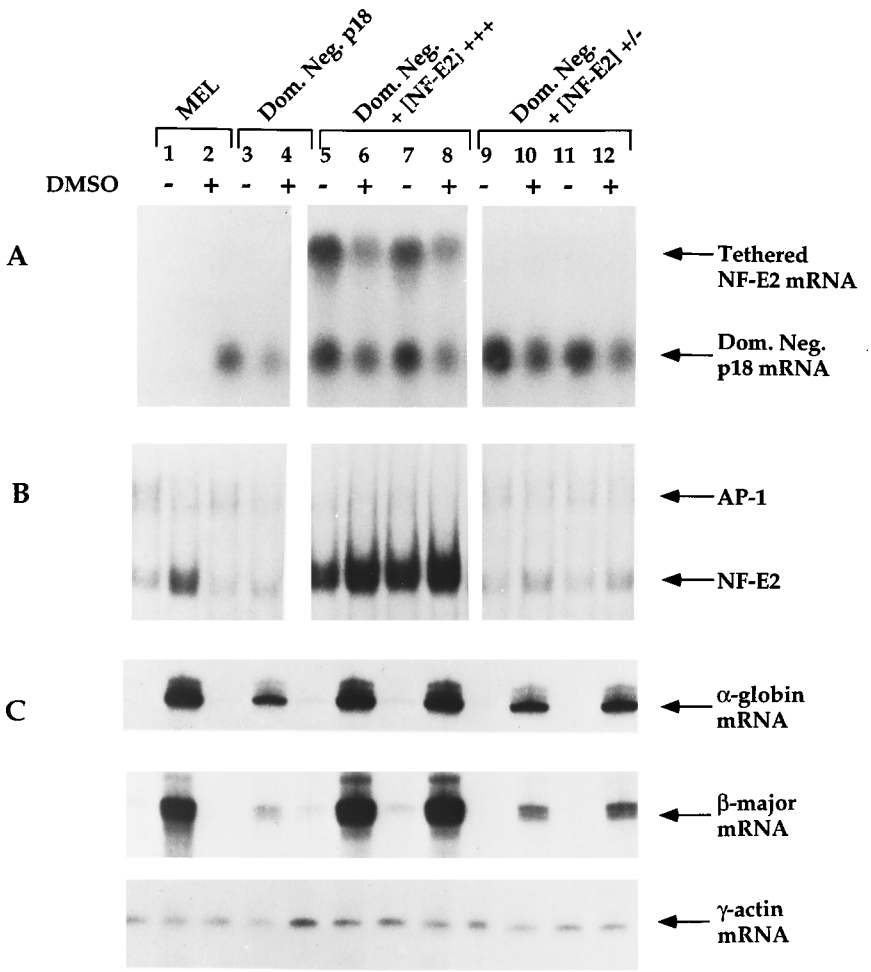

FIG. 1. The presence of NF-E2 is correlated with globin gene expression in MEL cells. (A) Northern blot analysis of cytoplasmic RNAs from MEL cells and MEL derivatives expressing transgenes as shown. A 15-μg sample of RNA was loaded in each lane. The blot was hybridized with a p18 cDNA probe. mRNA species corresponding to the dominant-negative (Dom. Neg.) p18 transgene and the tethered NF-E2 transgene are indicated on the right. (B) Gel shift analysis of NF-E2 binding activity in nuclear extracts prepared from MEL cells and MEL derivatives. Oligonucleotide-bound AP-1 and NF-E2 complexes are indicated on the right. The tethered NF-E2 heterodimer-DNA complex migrates slightly more slowly than the NF-E2–DNA complex. (C) RNase protection assays for α-globin and β-major mRNAs from MEL cells and MEL derivatives. In all samples, γ-actin mRNA was assayed in parallel.

utable to expression of the dominant-negative mutant p18 NF-E2 molecule and not due merely to clonal variation. On the basis of previous work demonstrating that a tethered form of the myoD-E47 complex is resistant to inhibition by Id (30), we constructed a tethered form of NF-E2 in which the carboxy terminus of p45 NF-E2 and the amino terminus of p18 NF-E2 were linked by a soluble polypeptide chain [p45-RS$(GGGS)_4$ GGRS-p18]. Tethering the p45-p18 NF-E2 heterodimer complex provides control over the specificity of multiple b-zip partner interactions. The tethered p45-p18 NF-E2 heterodimer was introduced into cells expressing dominant-negative p18 NF-E2, and stable clones were selected in the presence of puromycin. Western analysis of nuclear extracts from cells expressing the tethered heterodimer construct showed that the protein is maintained in the tethered form (data not shown). Clones expressing abundant tethered NF-E2 mRNA exhibited prominent NF-E2 DNA-binding activity with a mobility slightly slower than that of native NF-E2 (Fig. 1A and B, lanes 5 to 8). Concomitantly, α- and β-globin RNA expression was restored to wild-type levels despite the continued expression of dominant-negative p18 NF-E2 RNA (Fig. 1C, lanes 5 to 8). In clones expressing little or no detectable tethered NF-E2 RNA, gel shift activity and globin RNA expression were unaffected (Fig. 1, lanes 9 to 12).

These experiments permit us to draw several conclusions. First, inhibition of NF-E2 activity by the dominant-negative mutant demonstrates that the vast majority of the DNA–NF-E2 complex detected in gel shift assays includes a protein species (presumably p45 NF-E2) that dimerizes with p18 NF-E2. This finding is entirely consistent with the biochemical characterization of NF-E2 (1). Second, globin gene expression closely parallels the level of NF-E2 activity. Thus, NF-E2 in MEL cells appears rate limiting, and not merely permissive, for globin gene expression. Finally, the dependence of α- and β-globin RNA expression on NF-E2 differs.

Rescue of globin gene expression in NF-E2-null CB3 MEL cells. During the course of our studies, Lu et al. reported the absence of p45 NF-E2 expression in CB3 MEL cells and the partial restoration of depressed β-globin gene expression levels upon reintroduction of p45 NF-E2 cDNA (22). In agreement with their findings, we observed no detectable α- or β-globin mRNA in these cells following treatment with DMSO (Fig. 2A, lane 2). We next examined rescue of globin gene expression by the tethered p45-p18 heterodimer. Globin mRNA levels from

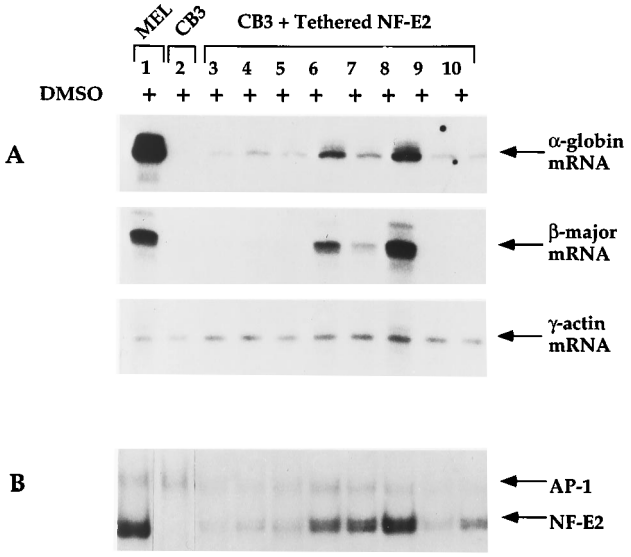

FIG. 2. Rescue of globin gene expression is dependent on NF-E2 expression in CB3 cells. (A) RNase protection assays for α-globin and β-major mRNAs from MEL cells, CB3 cells, and CB3 clones expressing various concentrations of tethered NF-E2. All cell lines were induced with DMSO. In all samples, γ-actin mRNA was assayed in parallel. (B) Gel shift analysis of NF-E2 binding activity in nuclear extracts prepared from MEL cells, CB3 cells, and CB3 clones expressing various concentrations of tethered NF-E2. Oligonucleotide-bound AP-1 and NF-E2 complexes are indicated on the right. The tethered NF-E2 heterodimer-DNA complex migrates slightly more slowly than the NF-E2–DNA complex.

several independent CB3 cell clones expressing various concentrations of the tethered heterodimer are shown in Fig. 2A, lanes 3 to 10. Of note, α-globin mRNA was detected in all clones which exhibited detectable NF-E2 DNA-binding activity, with the levels of α-globin mRNA paralleling the gel shift activity (Fig. 2A and B, lanes 3 to 10). However, the maximal amount of α-globin gene expression was far less than that of the wild-type MEL cell line, even in the presence of approximately equivalent levels of functional NF-E2 complex (Fig. 2A and B, compare lanes 1 and 8). This differs from our findings with the rescued dominant-negative p18 cells in which α-globin levels were restored to wild-type levels (Fig. 1A, lanes 2, 6, and 8).

Contrary to the data obtained for α-globin, β-globin gene expression was detected in only three of eight clones expressing the tethered heterodimer (Fig. 2A, lanes 3 to 10). Again, these clones displayed the highest levels of NF-E2 gel shift activity, and β-globin gene expression was greatest in that with the most prominent DNA-binding activity (Fig. 2B, lanes 3 to 10). In this instance, expression was comparable to that in wild-type MEL cells (Fig. 2A and B, lanes 1 and 8). Similar results were obtained from experiments in which untethered p45 NF-E2 was introduced into the CB3 cells (data not shown).

These findings extend those of Lu et al. (22) and further suggest two additional features of globin gene expression rescue in CB3 cells. First, full rescue of β-globin gene expression is possible but requires high-level expression of NF-E2. Second, though low-level rescue of α-globin gene expression occurs in nearly all clones, full rescue does not. The implications of this conclusion are addressed below.

β-LCR HS-1 and HS-2 are unaltered by inhibition or loss of NF-E2 activity. We next examined the role of the NF-E2 complex in maintaining an active chromatin structure in β-LCR HS-2, where tandem NF-E2 binding sites are functionally important, and HS-1, a site not known to bind NF-E2. DNase I hypersensitivity mapping was performed with wild-type MEL cells, MEL cells expressing a high level of dominant-negative p18 NF-E2, and CB3 cells. As shown in Fig. 3, HS-1 and HS-2 were present in all lines. Therefore, we conclude that NF-E2 is not required to maintain these hypersensitive sites in vivo.

The tandem NF-E2 sites in β-LCR HS-2 are occupied in vivo in CB3 and dominant-negative p18 NF-E2 MEL cells. The sustained presence of HS-2 led us to examine whether the

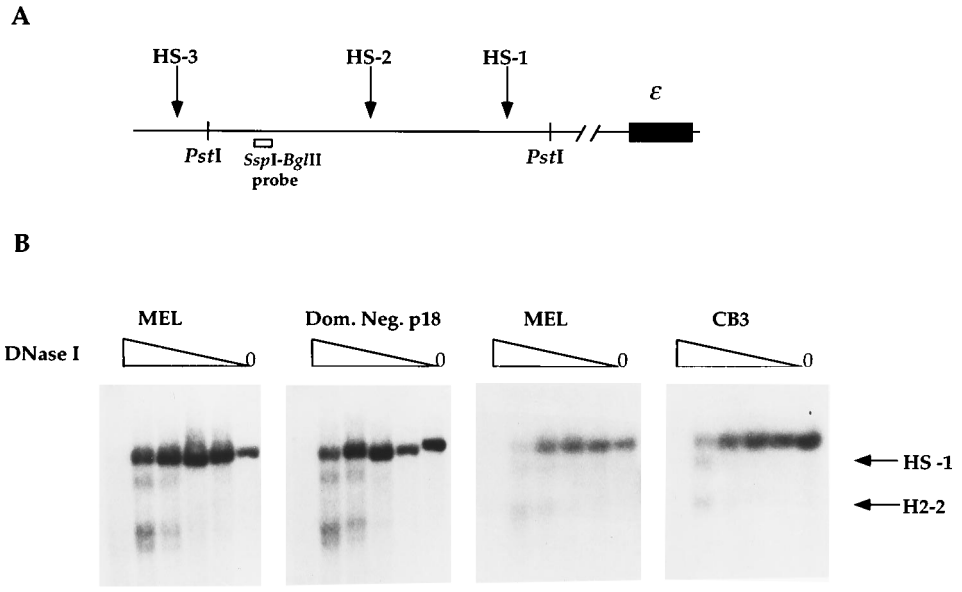

FIG. 3. DNase I hypersensitivity site mapping of the β-LCR in MEL cells, CB3 cells, and dominant-negative p18 MEL cells. (A) Schematic diagram of mapping strategy. The hypersensitive sites (vertical arrows), the probe (open box), and the ε-globin gene (solid box) are indicated. The *Ssp*I-*Bgl*II fragment hybridizes to a 13-kb *Pst*I fragment that contains both HS-1 and HS-2. Digestion with DNase I yields a 6.5-kb band (*Pst*I–HS-2 fragment) and an 11.5-kb band (*Pst*I–HS-1). (B) Results of hypersensitivity site mapping. DNase I concentrations increase from right to left (0, 0.13, 0.25, 0.50, 1.0, and 2.0 U/ml). HS-1 and HS-2 are indicated on the right. Mapping of CB3 and dominant-negative (Dom. Neg.) p18 MEL cells was done on separate occasions; for comparison, MEL cells were included each time.

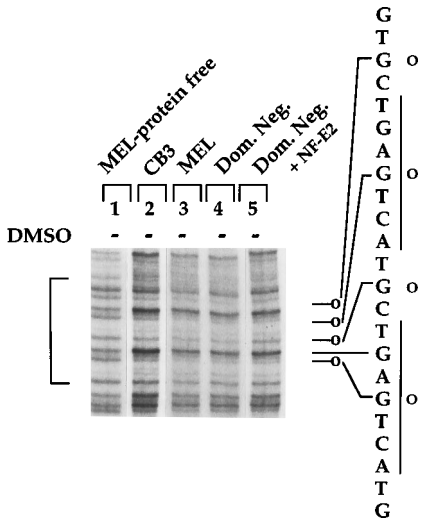

FIG. 4. Ligation-mediated in vivo footprinting of the tandem NF-E2 sites of β-LCR HS-2. Footprinting of the sense strand of HS-2 is shown. Lane 1, in vitro-methylated protein-free MEL DNA; lane 2, in vivo-methylated CB3 DNA; lane 3, in vivo-methylated MEL DNA; lane 4, in vivo-methylated dominant-negative (Dom. Neg.) p18 MEL DNA; lane 5, in vivo-methylated NF-E2-rescued dominant-negative MEL DNA. The area of the footprint encompassing the tandem NF-E2 sites (bracket on the left), protections (open circles), and residues which constitute the AP-1 sites (vertical lines) are indicated.

tandem NF-E2 sites were occupied in vivo. Ligation-mediated PCR (29, 41) was performed on in vivo-methylated genomic DNA isolated from MEL, CB3, dominant-negative p18 NF-E2 MEL, and rescued dominant-negative MEL cells expressing tethered NF-E2 by using primers specific for the sense strand of β-LCR HS-2. As shown in Fig. 4, protection was detected at both guanine residues at the −2 position (relative to the AP-1 site), as well as at both +4 guanine residues within the AP-1 site in all cell lines. From these data, we conclude that the tandem NF-E2 sites of β-LCR HS-2 are occupied in vivo at all times in these cell lines. The nature of the protein(s) occupying these sites in the absence of NF-E2 is unknown. Our results are consistent with previous data in which no alteration in the in vivo footprint after treatment of MEL cells with DMSO was observed (42a).

The amino terminus of p45 NF-E2 is required for rescue of globin expression. To better understand the function of the NF-E2 complex, we sought to delineate regions of erythroid p45 NF-E2 required for rescue of globin expression. We generated a deletion mutant lacking the amino-terminal 206 residues of the protein and tested its ability to rescue globin expression in stably transfected CB3 cells. Despite the appearance of abundant gel shift activity in transfected clones, we observed no detectable rescue of either α- or β-globin RNA expression (Fig. 5). Thus, the truncated p45 NF-E2 species efficiently dimerizes with endogenous p18 NF-E2 to form a heterodimer, but this complex is inactive with respect to rescue of globin expression. We conclude, therefore, that an essential function of the NF-E2 complex relies on the integrity of the amino terminus of the p45 subunit.

A tethered LCRF1-p18 heterodimer fails to rescue globin expression. At least two widely expressed proteins, Nrf1/LCRF1 and Nrf2, that are closely related to p45 NF-E2 in the basic CNC domain have been described (8, 9, 26). Homology in their leucine zippers is less apparent. Defined partners for these NF-E2-related proteins are unknown. Nrf1 and LCRF1

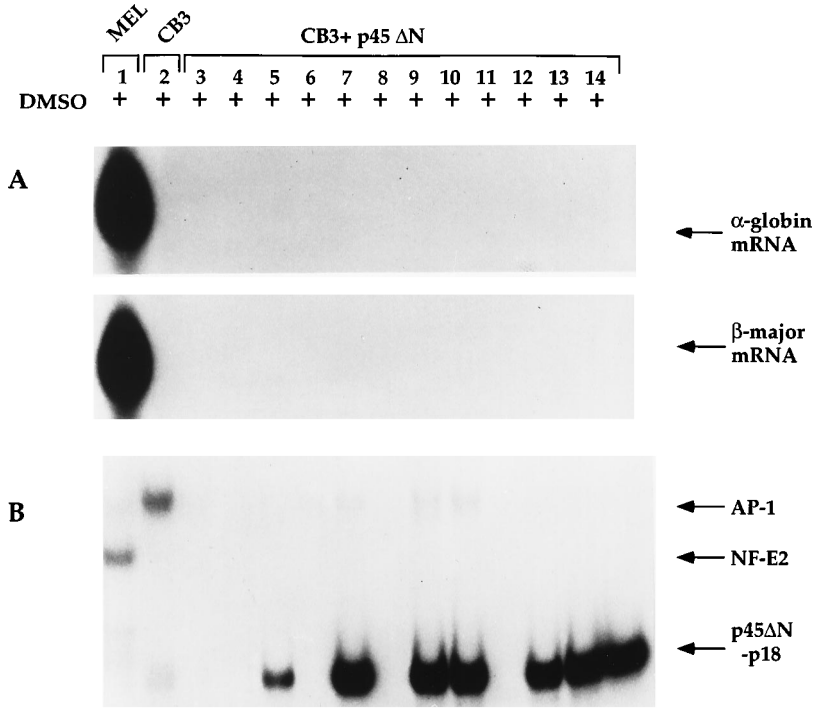

FIG. 5. A p45 mutant lacking amino acids 1 to 206 fails to rescue globin gene expression in CB3 cells. (A) Northern analysis of cytoplasmic RNAs from MEL cells, CB3 cells, and CB3 clones expressing various concentrations of the truncated p45 mutant (p45ΔN). All cell lines were induced with DMSO. A 15-μg sample of RNA was loaded in each lane. The blot was hybridized first with an α-globin cDNA probe and then with a β-major globin cDNA probe. (B) Gel shift analysis of NF-E2 binding activity in nuclear extracts prepared from MEL cells, CB3 cells, and CB3 clones expressing various concentrations of the truncated p45 mutant. Oligonucleotide-bound AP-1, NF-E2, and p45ΔN-p18 complexes are indicated on the right. The p45ΔN-p18 heterodimer-DNA complex migrates significantly faster than the NF-E2–DNA complex.

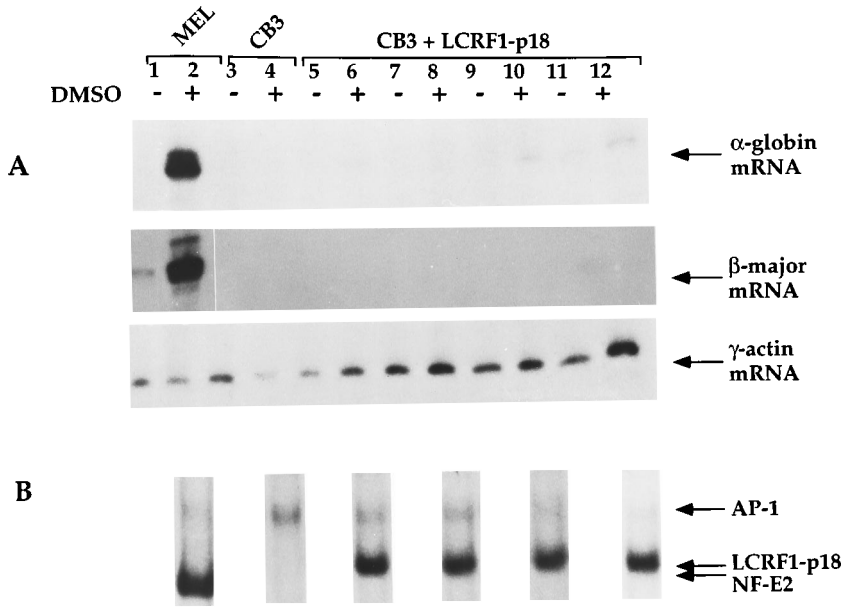

FIG. 6. A tethered LCRF1-p18 heterodimer fails to rescue globin gene expression in CB3 cells. (A) RNase protection assays for α-globin and β-major mRNAs from MEL cells, CB3 cells, and CB3 clones expressing various concentrations of tethered LCRF1-p18 heterodimer. In all samples, γ-actin mRNA was assayed in parallel. (B) Gel shift analysis of NF-E2 binding activity in nuclear extracts prepared from MEL cells, CB3 cells, and CB3 clones expressing various concentrations of tethered LCRF1-p18. Oligonucleotide-bound AP-1, NF-E2, and LCRF1-p18 complexes are indicated on the right. The tethered LCRF1-p18 heterodimer-DNA complex migrates slightly more slowly than the NF-E2–DNA complex.

are the products of the same gene but differ in their amino-terminal extents. Nrf1 has been reported to activate transcription in *Saccharomyces cerevisiae* (9), while LCRF1 has been proposed to function as an erythroid-cell-specific activator (8). To examine the potential specificity of NF-E2 action in vivo, we questioned whether rescue of globin expression might be achieved with one of these p45 NF-E2-related molecules. Initial attempts to express stable Nrf1 or LCRF1 protein in MEL cells were unsuccessful (unpublished data). Therefore, we tethered LCRF1 to p18 NF-E2. The binding specificity of the heterodimer in monkey kidney COS cells was assessed and shown to be indistinguishable from that of the native NF-E2 complex (not shown). We proceeded to examine the ability of the tethered LCRF1-p18 heterodimer to restore globin expression in CB3 cells. Despite the appearance of abundant NF-E2-like gel shift activity approximating that of endogenous NF-E2 in wild-type MEL cells, we observed no significant rescue of either α- or β-globin RNA expression (Fig. 6). We conclude that the NF-E2-related factor LCRF1 is unable to replace the function of p45 NF-E2 in the rescue of globin expression in erythroleukemia cells.

DISCUSSION

In this report, we provide new insights into the requirement for NF-E2 in globin gene expression within intact erythroid cells. First, we demonstrated that inhibition of NF-E2 activity by a dominant-negative form of the widely expressed smaller p18 NF-E2 subunit leads to impaired globin gene expression that can be overcome by a tethered p45-p18 NF-E2 heterodimer. Second, we determined the levels of NF-E2 required to rescue globin gene expression in a MEL cell line completely lacking NF-E2 because of retroviral insertion. Third, using DNase I hypersensitivity mapping and in vivo footprinting assays, we observed that no detectable chromatin alterations due to loss of NF-E2 occurred in β-LCR HS-2. Fourth, using a truncation mutant, we showed that the amino terminus of p45 NF-E2 is required for rescue of globin gene expression. Finally, we demonstrated that a p45 NF-E2-related molecule, LCRF1, is unable to rescue globin gene expression in CB3 cells.

The inhibition of globin gene expression by the dominant-negative p18 NF-E2 mutant and the virtual absence of globin gene expression in CB3 cells argue persuasively for an important role for NF-E2 in erythroid cells, formally consistent with that predicted from prior studies of transgenic mice and cell lines (23, 31, 32, 44). The functional effect of the dominant-negative mutant provides strong evidence that dimers containing p18 NF-E2 constitute the active NF-E2 species in vivo (3). Alternatively, it could be argued that the dominant-negative p18 mutant could inactivate other, related leucine zipper proteins present in these cells. However, the complete rescue of globin gene expression in the dominant-negative MEL cells by a tethered p45-p18 heterodimer argues that p45 NF-E2, rather than another, related factor, is primarily responsible for globin gene expression in MEL cells. These conclusions are in agreement with biochemical characterization of the NF-E2 complex which showed that the major DNA-binding activity in MEL cells is a heterodimer of p45 and p18 (1, 3).

Several aspects of the role of NF-E2 in globin gene expression are raised by these findings. The differing sensitivity of α- and β-globin gene expression to loss of NF-E2, as revealed in the dominant-negative mutant experiments (Fig. 1), suggests that different concentrations are needed for full activation of α- versus β-globin gene expression. Even at relatively low levels of functional NF-E2, considerable α-globin gene expression persists. Similarly, expression of low levels of tethered heterodimer is sufficient to allow for some rescue of α-globin gene expression in CB3 cells (Fig. 2). Whether the differing requirement for NF-E2 in the α- and β-loci reflects the distribution of binding sites in the respective clusters or other properties is unclear. We have also observed a consistent difference be-

tween CB3 cells and dominant-negative MEL cells rescued with the tethered heterodimer. Although β-globin gene expression can be fully restored in both cells, the maximal level of α-globin gene expression in rescued CB3 cells is low relative to those in wild-type or rescued dominant-negative cells. This result suggests the possibility that there are other genetic lesions in CB3 cells, apart from inactivation of the p45 NF-E2 gene, that may limit α-globin gene expression. We note, for example, that the gene adjacent to p45 NF-E2, heterogeneous nuclear ribonucleoprotein A1, is also inactive in CB3 cells (5). Lastly, from the correlation of globin rescue with the tethered heterodimer in either cell line, we conclude that high concentrations of NF-E2 are required for normal globin gene expression.

Our finding that the chromatin architecture of hypersensitivity sites HS-1 and HS-2 is unaltered in cells lacking NF-E2 is unexpected, as detailed analysis of the core elements required to form the DNase I-hypersensitive sites has implicated an NF-E2 site and two GATA sites (40). However, it is probable that other proteins that recognize NF-E2 binding sites in the absence of NF-E2, such as the AP-1 complex, assist in maintaining the chromatin structure of LCRs. Indeed, our in vivo footprinting studies have demonstrated binding of another factor to the tandem NF-E2 sites of HS-2 in the absence of NF-E2. This is also consistent with the observation that in the human β-LCR HS-1 an AP-1 site, not an NF-E2 site, and two GATA sites are present. Alternatively, these observations may indicate that NF-E2 does not function at the level of the LCR but instead influences globin expression indirectly.

The demonstration that the amino-terminal portion of p45 NF-E2 is required for rescue of globin gene expression in CB3 cells assigns an important function to this proline-rich region. Presumably, residues within this area provide the transcriptional activation functions of the NF-E2 heterodimer. The inability of a tethered LCRF1-p18 heterodimer to substitute for NF-E2 in activating globin gene expression in CB3 cells points to specificity of NF-E2 action in vivo. Among the proteins capable of binding the NF-E2 elements in LCR cores or HS-40, only the NF-E2 heterodimer has been shown to function in globin gene expression. The inability of LCRF1 to rescue globin gene expression is perhaps to be anticipated, as LCRF1 is expressed at similar levels in wild-type and CB3 MEL cells (unpublished data). However, it would not be predicted from the findings of Caterina et al., who described transfection experiments with LCRF1-Gal4 fusion proteins and artificial β-globin gene reporter constructs that suggested an erythroid-cell-specific activation function for this p45 NF-E2-related molecule (8). We are left to conclude that an intact-cell rescue assay provides a more stringent test of transcriptional function.

Of possible relevance to our findings are recent studies of Bieker and Southwood (6) that demonstrate activation of β-globin gene reporter constructs by the erythroid Krüppel-like factor (EKLF) and not by the related, ubiquitous factor Sp1. Thus, globin gene transcription appears to be facilitated selectively by two factors, EKLF and NF-E2. It is of interest that the putative activation domains of both EKLF and p45 NF-E2 are proline rich, in contrast to the glutamine-rich and acidic domains of Sp1 and LCRF1, respectively. Perhaps these proline-rich domains establish erythroid-cell-specific protein-protein interactions essential for directing globin gene expression.

Finally, in vivo findings with MEL cells need to be compared with the results of disruption of the p45 NF-E2 gene in mice (39). Surprisingly, NF-E2-null mice exhibit only a subtle deficit in hemoglobin accumulation in mature erythrocytes. Although the precise basis for the phenotype of NF-E2-null mice is unknown, several possibilities may account for this discrepancy. MEL cells represent only one stage of erythroid development, that analogous to an adult, committed erythroblast. Therefore, they may be frozen at a point at which globin gene expression is particularly dependent on NF-E2. It is probable that the repertoire of compensatory mechanisms available to erythroid progenitor cells in mice is broader than that operative within MEL cells. Although we might expect compensating proteins to be capable of activating globin gene expression in MEL cells, it is unlikely that these factors are present constitutively, since we have shown that the p45-p18 heterodimer constitutes the major DNA-binding activity in MEL cells. Moreover, it is uncertain if compensating proteins act as heterodimers with p18 NF-E2 or in another manner. Notwithstanding the striking differences between the phenotype of NF-E2 loss in MEL cells and that in animals, the experiments reported here provide strong evidence for a specific role of NF-E2 within erythroid cells and implicate the amino-terminal region of p45 NF-E2 in erythroid-cell-specific transcription.

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REFERENCES

1. **Andrews, N. C., H. Erdjument-Bromage, M. Davidson, P. Tempst, and S. H. Orkin.** 1993. Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. Nature (London) **362:**722–728.
2. **Andrews, N. C., and D. V. Faller.** 1991. A rapid micropreparation technique for extraction of DNA binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res. **19:**2499.
3. **Andrews, N. C., K. J. Kotkow, P. A. Ney, H. Erdjument-Bromage, P. Tempst, and S. H. Orkin.** 1993. The ubiquitous subunit of erythroid transcription factor NF-E2 is a small basic-leucine zipper protein related to the v-*maf* oncogene. Proc. Natl. Acad. Sci. USA **90:**11488–11492.
4. **Baron, M. H., and T. Maniatis.** 1986. Rapid reprogramming of globin gene expression in transient heterokaryons. Cell **46:**591–602.
5. **Ben-David, Y., M. R. Bani, B. Chabot, A. de Koven, and A. Bernstein.** 1992. Retroviral insertions downstream of the heterogeneous nuclear ribonucleoprotein A1 gene in erythroleukemia cells: evidence that A1 is not essential for cell growth. Mol. Cell. Biol. **12:**4449–4455.
6. **Bieker, J. J., and C. M. Southwood.** 1995. The erythroid Krüppel-like factor transactivation domain is a critical component for cell-specific inducibility of a β-globin promoter. Mol. Cell. Biol. **15:**852–860.
7. **Bowerman, B., B. A. Eaton, and J. R. Priess.** 1992. *skn*, a maternally expressed gene required to specify the fate of ventral blastomeres in the early C. elegans embryo. Cell **68:**1061–1075.
8. **Caterina, J. J., D. Donze, C.-W. Sun, D. J. Ciavatta, and T. M. Townes.** 1994. Cloning and functional characterization of LCR-F1: a bZIP transcription factor that activates erythroid-specific, human globin gene expression. Nucleic Acids Res. **22:**2383–2391.
9. **Chan, J. Y., X.-L. Han, and Y. W. Kan.** 1993. Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. Proc. Natl. Acad. Sci. USA **90:**11371–11375.
10. **Cordes, S. P., and G. S. Barsh.** 1994. The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. Cell **79:**1025–1034.
11. **Crossley, M., and S. H. Orkin.** 1993. Regulation of the β-globin locus. Curr. Opin. Genet. Dev. **3:**232–237.
12. **Forrester, W. C., S. Takegawa, T. Papayannopoulou, G. Stamatoyannopoulos, and M. Groudine.** 1987. Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin-expressing hybrids. Nucleic Acids Res. **15:**10159–10177.
13. **Glover, J. N. M., and S. C. Harrison.** 1995. Crystal structure of the heterodimeric bZIP transcription factor c-Fos-c-Jun bound to DNA. Nature (London) **373:**257–261.
14. **Grosveld, F., G. B. van Assendelft, D. R. Greaves, and G. Kollias.** 1987. Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell **51:**975–985.
15. **Higgs, D. R., W. G. Wood, A. P. Jarman, J. Sharpe, J. Lida, I.-M. Pretorius,**

and H. Ayyub. 1990. A major positive regulatory region located far upstream of the human α-globin gene locus. Genes Dev. **4:**1588–1601.
16. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
17. **Hug, B. A., A. M. Moon, and T. J. Ley.** 1992. Structure and function of the murine β-globin locus control region 5′ HS-3. Nucleic Acids Res. **20:**5771–5778.
18. **Igarashi, K., K. Kataoka, K. Itoh, N. Hayashi, M. Nishizawa, and M. Yamamoto.** 1994. Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. Nature (London) **367:**568–572.
19. **Jarman, A. P., W. G. Wood, J. A. Sharpe, G. Gourdon, H. Ayyub, and D. R. Higgs.** 1991. Characterization of the major regulatory element upstream of the human α-globin gene cluster. Mol. Cell. Biol. **11:**4679–4689.
20. **Kerppola, T., and T. Curran.** 1995. Zen and the art of Fos and Jun. Nature (London) **373:**199–200.
21. **Lowrey, C. H., D. M. Bodine, and A. W. Nienhuis.** 1992. Mechanism of DNase I hypersensitive site formation within the human globin locus control region. Proc. Natl. Acad. Sci. USA **89:**1143–1147.
22. **Lu, S.-J., S. Rowan, M. R. Bani, and Y. Ben-David.** 1994. Retroviral integration within the *Fli-2* locus results in inactivation of the erythroid transcription factor NF-E2 in Friend erythroleukemias: evidence that NF-E2 is essential for globin expression. Proc. Natl. Acad. Sci. USA **91:**8398–8402.
23. **Mignotte, V., L. Wall, E. deBoer, F. Grosveld, and P.-H. Romeo.** 1989. Two tissue-specific factors bind the erythroid promoter of the human porphobilinogen deaminase gene. Nucleic Acids Res. **17:**37–54.
24. **Mizushima, S., and S. Nagata.** 1990. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res. **18:**5322.
25. **Mohler, J., K. Vani, S. Leung, and A. Epstein.** 1991. Segmentally restricted, cephalic expression of a leucine zipper gene during Drosophila embryogenesis. Mech. Dev. **34:**3–10.
26. **Moi, P., K. Chan, I. Asunis, A. Cao, and Y. W. Kan.** 1994. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the β-globin locus control region. Proc. Natl. Acad. Sci. USA **91:**9926–9930.
27. **Moi, P., and Y. W. Kan.** 1990. Synergistic enhancement of globin gene expression by activator protein-1-like proteins. Proc. Natl. Acad. Sci. USA **87:**9000–9004.
28. **Moon, A. M., and T. J. Ley.** 1991. Functional properties of the β-globin locus control region in K562 erythroleukemia cells. Blood **10:**2272–2284.
29. **Mueller, P. R., and B. Wold.** 1989. *In vivo* footprinting of a muscle specific enhancer by ligation mediated PCR. Science **246:**780–786.
30. **Neuhold, L. A., and B. Wold.** 1993. HLH forced dimers: tethering MyoD to E47 generates a dominant positive myogenic factor insulated from negative regulation by Id. Cell **74:**1033–1042.
31. **Ney, P. A., B. P. Sorrentino, C. H. Lowrey, and A. W. Nienhuis.** 1990. Inducibility of the HS II enhancer depends on binding of an erythroid specific nuclear protein. Nucleic Acids Res. **18:**6011–6017.
32. **Ney, P. A., B. P. Sorrentino, K. T. McDonagh, and A. W. Nienhuis.** 1990. Tandem AP-1-binding sites within the human β-globin dominant control region function as an inducible enhancer in erythroid cells. Genes Dev. **4:**993–1006.
33. **Nishizawa, M., K. Kataoka, N. Goto, K. T. Fujiwara, and S. Kawai.** 1989. v-*maf*, a viral oncogene that encodes a "leucine zipper" motif. Proc. Natl. Acad. Sci. USA **86:**7711–7715.
34. **Philipsen, S., D. Talbot, P. Fraser, and F. Grosveld.** 1990. The β-globin dominant control region: hypersensitive site 2. EMBO J. **9:**2159–2167.
35. **Pondel, M. D., M. George, and N. J. Proudfoot.** 1992. The LCR-like α-globin positive regulatory element functions as an enhancer in transiently transfected cells during erythroid differentiation. Nucleic Acids Res. **20:**237–243.
36. **Pruzina, S., O. Hanscombe, D. Whyatt, F. Grosveld, and S. Philipsen.** 1991. Hypersensitive site 4 of the human β globin locus control region. Nucleic Acids Res. **19:**1413–1419.
37. **Reddy, P. M. S., and C.-K. J. Shen.** 1991. Protein-DNA interactions *in vivo* of an erythroid-specific, human β-globin locus enhancer. Proc. Natl. Acad. Sci. USA **88:**8676–8680.
38. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
39. **Shivdasani, R. A., M. F. Rosenblatt, D. Zucker-Franklin, C. W. Jackson, P. Hunt, C. J. M. Saris, and S. H. Orkin.** 1995. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. Cell **81:**695–704.
40. **Stamatoyannopoulos, J. A., A. Goodwin, T. Joyce, and C. H. Lowrey.** 1995. NF-E2 and GATA binding motifs are required for the formation of DNaseI hypersensitive site 4 of the human β-globin locus control region. EMBO J. **14:**106–116.
41. **Strauss, E. C., N. C. Andrew, D. R. Higgs, and S. H. Orkin.** 1992. In vivo footprinting of the human α-globin locus upstream regulatory element by guanine and adenine ligation-mediated polymerase chain reaction. Mol. Cell. Biol. **12:**2135–2142.
42. **Strauss, E. C., and S. H. Orkin.** 1992. *In vivo* protein-DNA interactions at hypersensitive site 3 of the human β-globin locus control region. Proc. Natl. Acad. Sci. USA **89:**5809–5813.

42a. **Strauss, E. C., and S. H. Orkin.** Unpublished data.

43. **Swaroop, A., J. Xu, H. Pawar, A. Jackson, C. Skolnick, and N. Agarwal.** 1992. A conserved retina-specific gene encodes a basic motif/leucine zipper domain. Proc. Natl. Acad. Sci. USA **89:**266–270.
44. **Talbot, D., and F. Grosveld.** 1991. The 5′HS2 of the globin locus control region enhances transcription through the interaction of a multimeric complex binding at two functionally distinct NF-E2 binding sites. EMBO J. **10:**1391–1398.
45. **Talbot, D., S. Philipsen, P. Fraser, and F. Grosveld.** 1990. Detailed analysis of the site 3 region of the human β-globin dominant control region. EMBO J. **9:**2169–2178.
46. **Tuan, D., W. Solomon, Q. Li, and I. M. London.** 1985. The "beta-like globin" gene domain in human erythroid cells. Proc. Natl. Acad. Sci. USA **82:**6384–6388.
47. **Wu, C.** 1980. Analysis of hypersensitive sites in chromatin. Methods Enzymol. **170:**269–289.
48. **Zhen, L., A. A. J. King, Y. Xiao, S. J. Chanock, S. H. Orkin, and M. C. Dinauer.** 1993. Gene targeting of X-linked chronic granulomatous disease locus in a human myeloid leukemia cell line. Proc. Natl. Acad. Sci. USA **90:**9832–9836.