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Purification of the Human NF-E2 Complex: cDNA Cloning of the Hematopoietic Cell-Specific Subunit and Evidence for an Associated Partner

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The human globin locus control region-binding protein, NF-E2, was purified by DNA affinity chromatography. Its tissue-specific component, p45 NF-E2, was cloned by use of a low-stringency library screen with murine p45 NF-E2 cDNA (N. C. Andrews, H. Erdjument-Bromage, M. B. Davidson, P. Tempst, and S. H. Orkin, Nature [London] 362:722–728, 1993). The human p45 NF-E2 gene was localized to chromosome 12q13 by fluorescent in situ hybridization. Human p45 NF-E2 and murine p45 NF-E2 are highly homologous basic region-leucine zipper (bZIP) proteins with identical DNA-binding domains. Immunoprecipitation experiments demonstrated that p45 NF-E2 is associated in vivo with an 18-kDa protein (p18). Because bZIP proteins bind DNA as dimers, we infer that native NF-E2 must be a heterodimer of 45- and 18-kDa subunits. Although AP-1 and CREB copurified with NF-E2, no evidence was found for heterodimer formation between p45 NF-E2 and proteins other than p18. Thus, p18 appears to be the sole specific partner of p45 NF-E2 in erythroid cells. Cloning of human p45 NF-E2 should permit studies of the role of NF-E2 in globin gene regulation and erythroid differentiation.

Sequences within the locus control region (LCR) have been shown to establish an open chromatin domain over the human β-globin cluster and to confer high-level, regulated expression on individual globin genes (10, 11, 13). These sequences span 20 kb of DNA upstream of the β-globin cluster and include four erythroid cell-specific, DNase I-hypersensitive sites (5′HS1 to 5′HS4). There has been considerable interest in reducing the LCR to its minimal, active components. Core enhancer fragments (200 to 400 bp) containing 5′HS2, 5′HS3, or 5′HS4 have been shown to retain significant LCR activity and to have a high concentration of DNA-binding sites for ubiquitous and erythroid cell-specific proteins (38, 39, 51).

While 5′HS2 to 5′HS4 have LCR activity in transgenic mice, only 5′HS2 is able to confer high-level, inducible expression on a transfected γ-globin gene in K562 cells (47). This ability, in turn, depends on the presence within 5′HS2 of tandem binding sites for transcription factors AP-1 and NF-E2 (34, 47). In transgenic mice, 5′HS2-driven β-globin gene expression also requires the tandem AP-1/NF-E2-binding sites (4, 23). These findings suggest that the tandem AP-1/NF-E2-binding sites in 5′HS2 are important for high-level γ- and β-globin gene expression. Consistent with this suggestion is the fact that the region encompassing the AP-1/NF-E2-binding sites has been highly conserved during evolution (57 of 59 and 54 of 57 nucleotides between humans and goats and between humans and mice, respectively) (20, 32).

The tandem AP-1/NF-E2-binding sites in 5′HS2 bind members of the ubiquitous AP-1 family in vitro, as well as the erythroid (and megakaryocytic) cell-specific nuclear factor NF-E2 (33, 41, 51). NF-E2 was originally described as an erythroid cell-specific DNA-binding activity that recognized an AP-1 site in the porphobilinogen deaminase (PBGD) erythroid promoter (30). Several findings suggest that NF-E2 is not related to AP-1 but is a novel transcription factor. NF-E2 contacts nucleotides outside the dyad symmetric AP-1 core (30). Through UV cross-linking, NF-E2 has a higher molecular weight than Jun (29), and it is antigenically unrelated to the Jun or Fos families (33, 51). We used a point mutation in the AP-1/NF-E2-binding site to show that high-level, inducible γ-globin expression in K562 cells depends on NF-E2 (33). In addition to those in the β-globin LCR, AP-1/NF-E2-binding sites have been identified in the α-globin LCR and in the promoters of enzymes of the heme synthetic pathway (6, 17, 30, 50). This result suggests a broader role for NF-E2 in erythroid differentiation.

To study the role of NF-E2 in globin gene transcription and erythroid differentiation, we undertook the purification and cloning of human NF-E2. We found that the native NF-E2 complex is a heterodimer of 45- and 18-kDa subunits. Cloning of the hematopoietic cell-specific 45-kDa subunit (human p45 NF-E2) was facilitated by the recent availability of murine p45 NF-E2 cDNA (2).

MATERIALS AND METHODS

DNA-protein interactions. Gel mobility shift assays were performed as previously described (33) with the following oligonucleotide probes (binding sites are in boldface type): wild-type NF-E2, ATGCTGAGTCATGATGAGTCATG (33); mutant NF-E2 (mNF-E2), ATTCTGAGTCATTATGAGTC ATG (33); and CREB, AAATGACGTAACGG (42).

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For methylation interference, a uniquely end-labeled probe was lightly methylated with dimethyl sulfate. Following the resolution of free and bound probe by gel mobility shift assay, the probe was electrophoretically transferred to an NA45 membrane (Schleicher & Schuell, Inc.) in 0.5× Tris-borate-EDTA buffer (75 V, 1 h). Transferred free and bound probe was eluted, cleaved at dG and dA residues, and resolved on a 20% sequencing gel (27). The probe was derived from the NF-E2 site in the PBGD promoter: CTGGGGAACCTGTGCTGAGTCACTGGAGG (30).

Preparation of DNA affinity columns. Complementary strands of the oligonucleotides to be coupled to the column were synthesized (4 mg each) with 4-bp AGCT 5′ ends, desalted over Sephadex G-25, and ethanol precipitated. Oligonucleotides were resuspended in 200 μl of Tris-EDTA buffer, mixed together, and phosphorylated for 2 h at 37°C in 50 mM Tris (pH 7.5)–10 mM $MgCl_2$–0.1 mM spermidine–0.1 mM EDTA–5 mM dithiothreitol (DTT)–3 mM ATP–1 U of polynucleotide kinase per μl in 2 ml. After phosphorylation, oligonucleotides were precipitated with ammonium acetate-ethanol, extracted with phenol-chloroform, and reprecipitated with sodium acetate-ethanol. Phosphorylated oligonucleotides were annealed and ligated overnight at 18°C in 50 mM Tris (pH 7.5)–10 mM $MgCl_2$–4 mM ATP–20 mM DTT–50 μg of bovine serum albumin per ml–30 U of ligase per μl in 1 ml. Ligated oligonucleotides were extracted with phenol-chloroform, precipitated with ammonium acetate-isopropanol, and reprecipitated with sodium acetate-ethanol. Oligonucleotides were resuspended in 500 μl of high-pressure liquid chromatography-grade water and coupled to 10 ml of cyanogen bromide-activated Sepharose (Pharmacia) in accordance with the manufacturer's instructions. The coupling efficiency was 30 to 50%. The sequences of the oligonucleotides were as follows: NF-E2, (AGCT)**ATGCT GAGTCATGATGAGTCA**TG; and CREB, (AGCT)**TAAAT GACGTAA**CGG.

Purification of human NF-E2. K562 cells (25) were grown in 4-liter spinner flasks containing improved minimal essential medium (Paragon Biotech Inc.) with 5% calf serum and 50 μg of gentamicin per ml. Crude K562 cell extracts were prepared in 40-liter batches by the method of Dignam et al. (7). Extracts were frozen in Dignam buffer B with protease inhibitors [20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.8), 0.42 M KCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM DTT, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and stored above liquid nitrogen. All column chromatography was performed at 4°C with the aid of a fast protein liquid chromatograph (Pharmacia) and a UV monitor.

In a typical study, extracts from 250 g of K562 cells (500 ml; 7.7 mg of protein per ml) were dialyzed against Dignam buffer D (20 mM HEPES [pH 7.8], 0.1 M KCl, 20% glycerol, 0.2 mM EDTA, 1 mM DTT, 0.05% Nonidet P-40, 0.5 mM PMSF) and clarified by centrifugation in 40-ml Oakridge tubes (40,000 × *g*, 15 min, 4°C). Extracts were fractionated by use of an 80-ml heparin-Sepharose column (radius, 2.5 cm; flow rate, 4 ml/min). The 0.28 to 0.6 M KCl fraction containing NF-E2 activity was dialyzed (in Dignam buffer D with 0.2 M KCl), incubated with poly(dI-dC) (Pharmacia; 10 μg/ml; 15 min), and clarified (as described above). The supernatant (125 ml; 2.2 mg/ml) was fractioned by use of a 10-ml DNA affinity column (radius, 1.25 cm; flow rate, 1 ml/min) with CREB-binding sites and a linear gradient of 0.2 to 1.0 M KCl. Fractions with peak NF-E2 activity were pooled, diluted to 0.4 M KCl, and incubated with poly(dI-dC) (10 μg/ml). This pool was fractionated by use of a 3-ml DNA affinity column with NF-E2-binding sites and a linear gradient of 0.4 to 1.5 M KCl. Fractions with peak NF-E2 activity were pooled again and fractionated over a 1-ml NF-E2 DNA affinity column. These fractions were assayed for NF-E2 activity, precipitated in 20% trichloroacetic acid, washed in 100% ice-cold acetone, and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide). The SDS-polyacrylamide gel was transferred to a polyvinylidene difluoride membrane by Western blotting (immunoblotting) (45) and stained for total protein with silver-enhanced gold strain (Bio-Rad Laboratories).

Cloning of human p45 NF-E2. A human genomic library constructed in bacteriophage vector EMBL3 was screened with a full-length murine cDNA insert corresponding to p45 NF-E2 at a reduced stringency (hybridization: 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 55°C; wash: 0.2× SSC at 55°C). From one phage clone, a 1.5-kb *Hin*dIII fragment spanning the third exon of the gene was subcloned and used as a probe to screen a λgt11 cDNA library prepared from human K562 cells. *Eco*RI inserts of positive phage were subcloned and subjected to DNA sequencing.

Genomic localization of the human p45 NF-E2 gene. A 17-kb human p45 NF-E2 genomic probe constructed in lambda (0.5 μg) was labeled with biotin-11-dUTP (Bionick labeling system; GIBCO-BRL). The p45 NF-E2 probe was coprecipitated with Cot-1 DNA (7.5 μg) and resuspended in 1× Tris-EDTA (TE) at 25 μg/ml. Metaphase chromosome preparations from human male peripheral blood lymphocytes were hybridized with the p45 NF-E2 probe in Hybrisol VI (5 μg/ml) in accordance with the manufacturer's instructions (chromosome in situ hybridization kit; Oncor), except for the omission of the RNase treatment. Posthybridization washes included (i) 2× SSC in 50% formamide, 42°C, 15 min; (ii) 2× SSC, 42°C, 5 min (twice); and (iii) 1× phosphate-buffered detergent (PBD) (Oncor), room temperature, 5 min. The biotin-labeled probe was detected and its signal was amplified in accordance with the manufacturer's instructions. Metaphase chromosomes were counterstained simultaneously with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) and propidium iodide (whole-chromosome painting system; GIBCO-BRL). The map position of the NF-E2 probe was determined as the range of the fractional lengths of the total chromosome relative to the telomere of the short arm, designated the FLpter values (22). Fourteen chromosomes 12 were measured to define the range of the FLpter values.

Human p45 NF-E2 expression. With the exception of adult bone marrow and peripheral blood mononuclear cells, human tissue total RNA was obtained from Clontech. Bone marrow and peripheral blood RNA and RNA from cell lines were prepared by the method of Chomczynski and Sacchi (5). Mononuclear cells from adult bone marrow and peripheral blood were isolated by centrifugation over a cushion of lymphocyte separation medium (Organon Teknika). The cell lines for this study were obtained directly from the American Type Culture Collection. Northern (RNA) blotting was performed as described by Sambrook et al. (45) with the following probes: human NF-E2, entire cDNA; human β-actin, entire cDNA; and human $^{A}\gamma$-globin, fragment from positions −408 to +473 (relative to the cap site).

Anti–p45 NF-E2 Western blots and immunoprecipitates.

The glutathione *S*-transferase–murine NF-E2 antibody has been described elsewhere (2). Western-blotted, affinity-purified K562 fractions were probed with preimmune and immune sera and then with alkaline phosphatase-conjugated rabbit anti-immunoglobulin G (ProtoBlot; Promega) in accordance with the manufacturer's instructions. Immunoprecipitation was performed on nuclear extracts from 5×10^8 metabolically labeled K562 and induced MEL cells (1.5% dimethyl sulfoxide; 2 days). Nondialyzed extracts in Dignam buffer B were partially purified by use of an NF-E2 DNA affinity column (0.3-ml column volume). Bound NF-E2 was eluted with 0.9 ml of Dignam buffer D with 1.5 M KCl, divided into two aliquots, and diluted 1:2 with IP buffer (20 mM HEPES [pH 7.8], 1% Triton X-100, 0.5 mM PMSF). Two microliters of anti-murine p45 NF-E2 antibody or preimmune serum was added, and the samples were incubated with rocking on ice for 1 h. One-tenth milliliter of 5% (vol/vol) protein A–Sepharose (PAS) beads was added, and the samples were incubated for another 30 min. The beads were washed three times with IP buffer containing 0.5 M KCl, incubated for 10 min at 85°C in SDS loading buffer, and run on an SDS-polyacrylamide gel. The gel was dried and exposed with a PhosphorImager (Molecular Dynamics). To denature the NF-E2 complex, the NF-E2 eluate was diluted 1:2 in RIPA buffer (IP buffer plus 0.1% SDS and 0.5% deoxycholate), incubated at 95°C for 5 min, and allowed to return to room temperature. Denatured NF-E2 was incubated with antibody and PAS beads as described above, except that the PAS beads were washed with RIPA buffer containing 0.5 M KCl.

Nucleotide sequence accession number. The GenBank accession number for the human p45 NF-E2 gene is L13974.

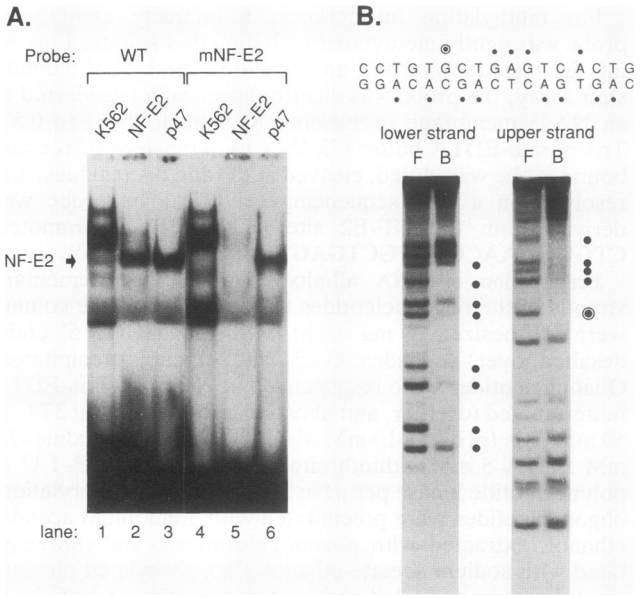

FIG. 1. NF-E2-binding activity in purified K562 fractions. (A) Crude and affinity-purified K562 extracts (1 μl) were studied in gel mobility shift assays with wild-type NF-E2 (WT) and mutant NF-E2 (mNF-E2) probes derived from the HS2 enhancer sequence. The NF-E2–probe complex is indicated by the arrow. Bound to the WT probe, another protein (p47) comigrated with NF-E2. (B) DNA-protein contacts made by affinity-purified NF-E2 were studied by methylation interference. Free (F) and bound (B) probe for both strands is shown. Contacts are indicated by solid circles. A G residue that makes contact with NF-E2 but not AP-1 is indicated by a double circle.

RESULTS

Purification of human NF-E2. NF-E2 is identifiable in nuclear extracts from human erythroleukemia K562 cells as a minor binding activity that migrates below AP-1 complexes in gel shift assays (Fig. 1A, lane 1). This binding activity was purified from crude K562 extracts by conventional and DNA affinity chromatography (18). Purified NF-E2 was defined as fulfilling the following criteria: (i) comigration with NF-E2 in crude K562 extracts in gel shift assays, (ii) ability to discriminate between wild-type and mutant probes, (iii) failure to supershift with anti-Jun or anti-Fos antibodies, and (iv) DNA-protein contacts identical to those of NF-E2 in crude K562 extract, as determined by methylation interference.

Purified NF-E2 was found to elute from an NF-E2 DNA affinity column at between 0.8 and 1.2 M KCl. It comigrated with NF-E2 in crude extracts (Fig. 1A, lanes 1 and 2) and failed to supershift with anti-Jun or anti-Fos antibodies. It also failed to bind to an NF-E2-binding site probe when that probe contained point mutations that selectively ablate NF-E2 binding (Fig. 1A, lanes 1, 2, 4, and 5) (29). In methylation interference assays with a probe derived from the PBGD promoter, purified NF-E2 was found to make the same DNA-protein contacts as previously reported for NF-E2 in crude extracts (Fig. 1B) (30). In particular, it made strong contact with an upstream G residue that discriminates NF-E2 binding from that of members of the AP-1 family (Fig. 1B, double circle). To this extent, affinity-purified NF-E2 exhibited all of the binding properties of NF-E2 in crude K562 extracts.

Purified NF-E2 needed to be distinguished from a 47-kDa protein (p47) present in K562 extracts and having similar binding properties. When a wild-type probe was used, this protein comigrated with NF-E2 in crude K562 extracts (Fig. 1A, lanes 1 and 3). It also recognized AP-1/NF-E2-binding sites in methylation interference assays (data not shown). However, p47 only weakly discriminated between wild-type and mutant probes (lanes 3 and 6) and made weak contact with the discriminatory G residue in NF-E2-binding sites. p47 eluted from the NF-E2 DNA affinity column at between 0.2 and 0.4 M KCl and was readily separated from NF-E2.

Affinity-purified fractions eluting at between 0.8 and 1.2 M KCl were greatly enriched for NF-E2 but still contained AP-1- and CREB-binding activities (Fig. 2A, lanes 1 and 2). For depletion of these fractions of proteins copurifying with NF-E2, a CREB DNA affinity column was used in the purification, permitting the effective separation of NF-E2 from AP-1- and CREB-binding activities (Fig. 2A, lanes 3 and 4). For affinity-purified fractions devoid of AP-1 and CREB, SDS-PAGE revealed a discrete cluster of bands in the 45-kDa region that correlated well with NF-E2 activity (Fig. 2B). These bands contained about 10 ng of purified protein, an amount significantly smaller than that required for sequence analysis.

The murine homolog of these 45-kDa bands (murine p45 NF-E2) recently was purified and cloned from induced murine erythroleukemia (MEL) cells (2). Antibodies raised to a glutathione *S*-transferase–murine p45 NF-E2 fusion protein cross-reacted with human NF-E2 in crude and purified K562 fractions (Fig. 3, lanes 1, 2, 5, and 6). They failed to react with p47 (Fig. 3, lanes 3 and 4). Western blotting of affinity-purified NF-E2 with an anti-p45 NF-E2 antibody confirmed that the 45-kDa bands that we identified are part of the NF-E2 complex (Fig. 2C).

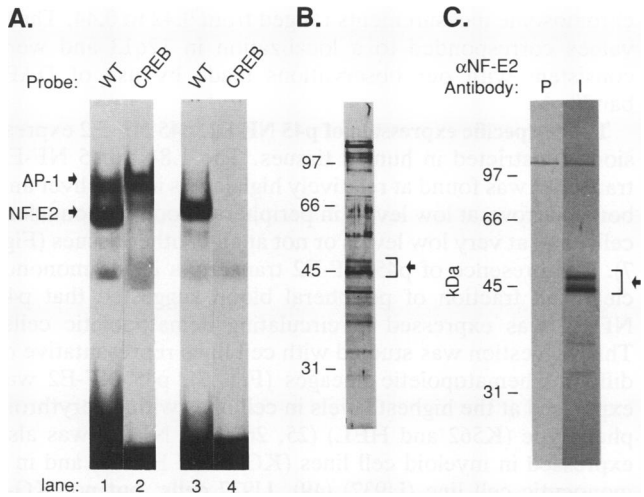

FIG. 2. Human NF-E2 contains a 45-kDa polypeptide. (A) Affinity-purified NF-E2 (1 μl) was studied in gel mobility shift assays with wild-type NF-E2 (WT) and CREB probes. The panel on the left shows AP-1-like-binding activities in an affinity-purified NF-E2 fraction before the introduction of a CREB DNA affinity column. The panel on the right shows an equivalent fraction after the introduction of the CREB column into the purification (see Results). (B) Affinity-purified NF-E2 (1 ml) was precipitated with trichloroacetic acid (TCA) and analyzed by SDS-PAGE (10% polyacrylamide). The bracket marks a cluster of bands at 45 kDa that coeluted with NF-E2 activity. Bands from 54 to 68 kDa represent contamination by skin proteins (35). (C) Western blot of 500 μl of TCA-precipitated, affinity-purified NF-E2. The blot was probed with preimmune serum (P) or an anti-murine p45 NF-E2 antibody (I). The bracket marks immunoreactive bands at 45 kDa. αNF-E2, anti-NF-E2.

Cloning of the human p45 NF-E2 cDNA. For obtaining the human p45 NF-E2 cDNA, a human genomic library was screened with murine p45 NF-E2 cDNA under conditions of reduced stringency. A probe derived from the human p45 NF-E2 genomic clone (exon 3) was then used to screen a K562 cDNA library, ultimately yielding several clones spanning the entire coding region. Human p45 NF-E2 cDNA, like murine cDNA, codes for a predicted protein of 373 amino acids (Fig. 4). Human p45 NF-E2 and murine p45 NF-E2 are highly homologous at the amino acid level (88%), especially in the DNA-binding domain (100%) (Fig. 5). At the C terminus, p45 NF-E2 contains a basic region-leucine zipper (bZIP) element, like members of the Jun, Fos, and CREB families; however, it is not related to these proteins. Within the basic domain, p45 NF-E2 contains a cysteine residue that is highly conserved among members of the AP-1 family. This residue has been shown to be a target of redox regulation of transcription factor binding (1). The N terminus of p45 NF-E2 is rich in acidic and proline residues (29% proline over 100 amino acids). Such domains are characteristic of transcriptional activators (28).

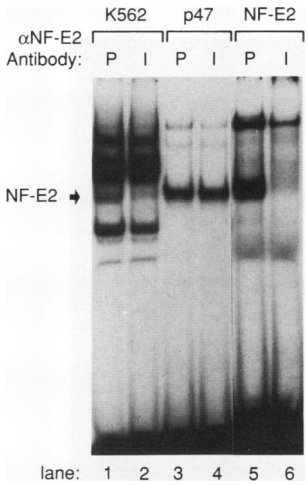

FIG. 3. Recognition of human NF-E2 by an anti-murine p45 NF-E2 antibody. Crude and affinity-purified K562 fractions (1 μl) were studied in gel mobility shift assays with a wild-type NF-E2 probe in the presence of preimmune serum (P) or an anti-murine p45 NF-E2 antibody (I) (1 μl).

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TGCTTGGGGCTCCTGTGCTCAGCTCAGCCTGAGCTTCCACACTCAGCGCTCAGCAATGGC      60
CCGGGGGCGGGGCGCGGTCCTCGCAGATTCTCAAAGGTAGCCGGGATCCTCGTCCAGCAG     120
TGTCAGCTCAGGCTCAGCCTCCCCAGAGACAACACCGGGAGCCTCATCTCTCTCCTCACC     180
CTGCTGTGACTCCACCACAGGTTTCTAGAGCCATCTGGGCTTTCCGGGAACCTGGACCAG     240
ACTCTGGCCCAGTAGGATGTCCCCGTGTCCTCCCCAGCAGAGCAGGAACAGGGTGATACA     300
                M  S  P  C  P  P  Q  Q  S  R  N  R  V  I  Q
GCTGTCCACTTCAGAGCTAGGAGAGATGGAACTGACTTGGCAGGAGATCATGTCCATCAC     360
 L  S  T  S  E  L  G  E  M  E  L  T  W  Q  E  I  M  S  I  T
CGAGCTGCAGGGTCTGAATGCTCCAAGTGAGCCATCATTTGAGCCCCAAGCCCCAGCTCC     420
 E  L  Q  G  L  N  A  P  S  E  P  S  F  E  P  Q  A  P  A  P
ATACCTTGGACCTCCACCACCCACAACTTACTGCCCCTGCTCAATCCACCCAGATTCTGG     480
 Y  L  G  P  P  P  P  T  T  Y  C  P  C  S  I  H  P  D  S  G
CTTCCCACTTCCTCCACCACCTTATGAGCTCCCAGCATCCACATCCCATGTCCCAGATCC     540
 F  P  L  P  P  P  P  Y  E  L  P  A  S  T  S  H  V  P  D  P
CCCATACTCCTATGGCAACATGGCCATACCAGTCTCCAAGCCACTGAGCCTCTCAGCGCT     600
 P  Y  S  Y  G  N  M  A  I  P  V  S  K  P  L  S  L  S  A  L
GCTCAGTGAGCCGCTCCAAGACCCCTTAGCCCTCCTGGACATTGGGCTGCCAGCAGGGCC     660
 L  S  E  P  L  Q  D  P  L  A  L  L  D  I  G  L  P  A  G  P
ACCTAAGCCCCAAGAAGACCCAGAATCCGACTCAGGATTATCCCTCAACTATAGCGATGC     720
 P  K  P  Q  E  D  P  E  S  D  S  G  L  S  L  N  Y  S  D  A
TGAATCTCTTGAGCTGGAGGGGACAGAGGCTGGTCGGCGGCGCAGCGAATATGTAGAGAT     780
 E  S  L  E  L  E  G  T  E  A  G  R  R  R  S  E  Y  V  E  M
GTACCCAGTGGAGTACCCCTACTCACTCATGCCCAACTCCTTGGCCCACTCCAACTATAC     840
 Y  P  V  E  Y  P  Y  S  L  M  P  N  S  L  A  H  S  N  Y  T
CTTGCCAGCTGCTGAGACCCCCTTGGCCTTAGAGCCCTCCTCAGGCCCTGTGCGGCGTAA     900
 L  P  A  A  E  T  P  L  A  L  E  P  S  S  G  P  V  R  R  K
GCCCACTGCACGGGGGGAGGCAGGGAGTCGGGATGAACGTCGGGCCTTGGCCATGAAGAT     960
 P  T  A  R  G  E  A  G  S  R  D  E  R  R  A  L  A  M  K  I
TCCTTTTCCTACGGACAAGATTGTCAACTTGCCGGTAGATGACTTTAATGAGCTATTGGC    1020
 P  F  P  T  D  K  I  V  N  L  P  V  D  D  F  N  E  L  L  A
AAGGTACCCGCTGACAGAGAGCCAGCTAGCGCTAGTCCGGGACATCCGACGACGGGGCAA    1080
 R  Y  P  L  T  E  S  Q  L  A  L  V  R  D  I  R  R  R  G  K
AAACAAGGTGGCAGCCCAGAACTGCCGCAAGAGGAAGCTGGAAACCATTGTGCAGCTGGA    1140
 N  K  V  A  A  Q  N  C  R  K  R  K  L  E  T  I  V  Q  L  E
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 R  E  L  E  R  L  T  N  E  R  E  R  L  L  R  A  R  G  E  A
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 D  R  T  L  E  V  M  R  Q  Q  L  T  E  L  Y  R  D  I  L  E
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 H  L  R  D  E  S  G  N  S  Y  S  P  E  E  Y  A  L  Q  Q  A
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 A  D  G  T  I  F  L  V  P  R  G  T  K  M  E  A  T  D
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CCCCAACCCTGAGTCCCAGAAGGAGCTGAGTTCTCTAGACCAGAAGAGGATGACAA        1496
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FIG. 4. Nucleotide and predicted amino acid sequences of human p45 NF-E2 cDNA.

Localization of the human p45 NF-E2 gene to 12q13. Precise chromosomal assignment of the human p45 NF-E2 gene was accomplished by fluorescent in situ hybridization (Fig. 6). Map positions were determined by use of FLpter values (22). FLpter values obtained from 14 metaphase

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HNFE2  MSPCPPQQSRNRVIQLSTSELGEMELTWQEIMSITELQGLNAPSEPSFEPQAPAPYLGPP  60
MNFE2  MPPCPPQQNRNRLSQLPVGELGEMELTWQEIMSITELQGLNVPSETSFEPQAPTPYPGPL

       PPTTYCPCSIHPDSGFPLPPPPYELPASTSHVPDPPYSYGNMAIPVSKPLSLSALLSEPL  120
       PPPTYCPCSIHPDAGFSLPPPSYELPASTPHVPELPYSYGNVAIPVSKPLTLSGLLNEPL

       QDPLALLDIGLPAGPPKPQEDPESDSGLSLNYSDAESLELEGTEAGRRRSEYVEMYPVEY  180
       PDHLALLDIGLPVGQPKPQEDPESDSGLSLNYSDAESLELEGMEAGRRRSEYVDMYPVEY

       PYSLMPNSLAHSNYTLPAAETPLALEPSSGPVRRKPTARGEAGSRDERRALAMKIPFPTD  240
       PYSLMPNSLAHPNYTLPPTETPLALESSSGPVRAKPAVRGEAGSRDERRALAMKIPFPTD

       KIVNLPVDDFNELLARYPLTESQLALVRDIRRRGKNKVAAQNCRKRKLETIVQEERELER  300
       KIVNLPVDDFNELLAQYPLTESQLALVRDIRRRGKNKVAAQNCRKRKLETIVQEERELER

       KTNERERELRARGEKDRTLEVKRQQLTELYRDILEHLRDESGNSYSPEEYALQQAADGTI  360
       KSSERERELRARGEKDRTLEVKRQQLAELYHDIFQHLRDESGNSYSPEEYVLQQAADGAI

       FLVPRGTKMEATD  373
       FLVPRGTKMEATD
```

FIG. 5. Comparison between human and murine p45 NF-E2 amino acid sequences (HNFE2 and MNFE2, respectively). The basic DNA-binding domain (open box) and hydrophobic residues of the leucine zipper (shaded boxes) are indicated.

chromosome measurements ranged from 0.42 to 0.44. These values corresponded to a localization in 12q13 and were consistent with our observations made by use of DAPI banding.

Tissue-specific expression of p45 NF-E2. p45 NF-E2 expression is restricted in human tissues. The 1.8-kb p45 NF-E2 transcript was found at relatively high levels in fetal liver and bone marrow, at low levels in peripheral blood mononuclear cells, and at very low levels or not at all in other tissues (Fig. 7). The presence of p45 NF-E2 transcripts in the mononuclear cell fraction of peripheral blood suggested that p45 NF-E2 was expressed in circulating hematopoietic cells. This suggestion was studied with cell lines representative of different hematopoietic lineages (Fig. 7). p45 NF-E2 was expressed at the highest levels in cell lines with an erythroid phenotype (K562 and HEL) (25, 26). p45 NF-E2 was also expressed in myeloid cell lines (KG-1 and HL-60) and in a monocytic cell line (U937) (49). U937 cells, but not KG-1 cells, were found to express γ-globin as well. p45 NF-E2 was expressed at very low levels in a lymphoid T-cell line (CEM)

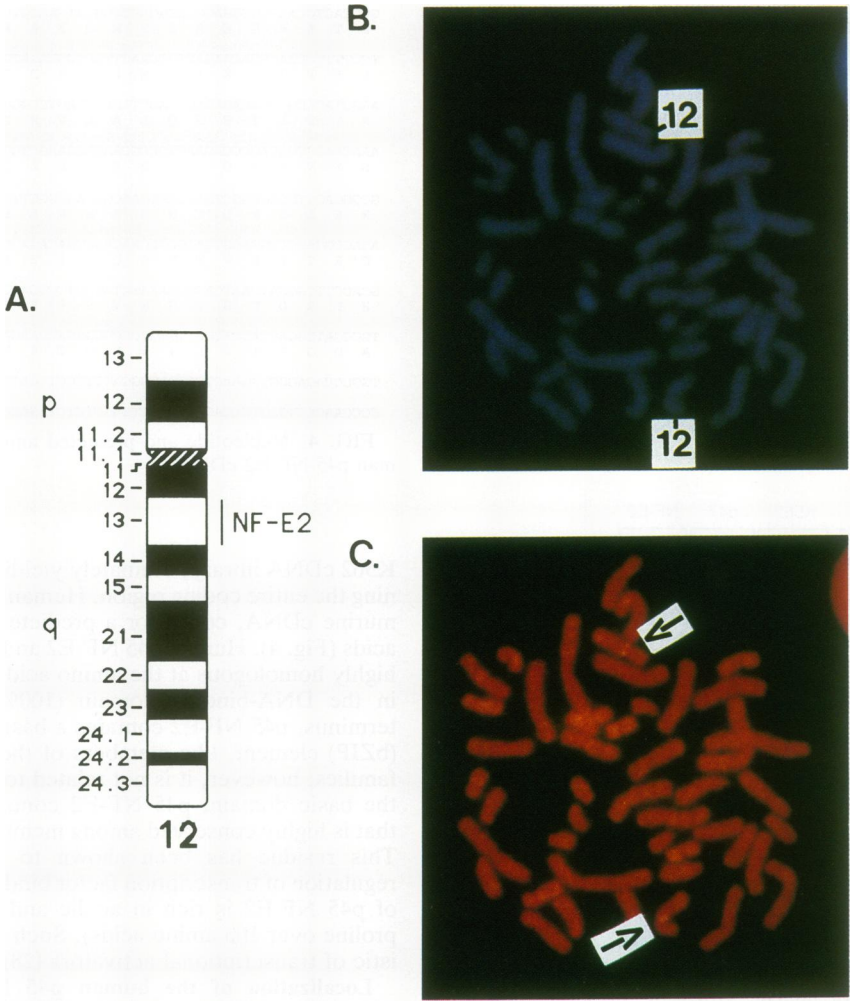

FIG. 6. (A) Ideogram of human chromosome 12 showing the map location of the human NF-E2 gene at 12q13. (B) Photograph of human metaphase chromosomes counterstained with DAPI. The two chromosomes 12 are indicated by numbers. (C) Photograph of human metaphase chromosomes counterstained with propidium iodide following fluorescent in situ hybridization with a probe for the human NF-E2 gene. Arrows point to the site of hybridization on both chromosomes 12 in band q13.

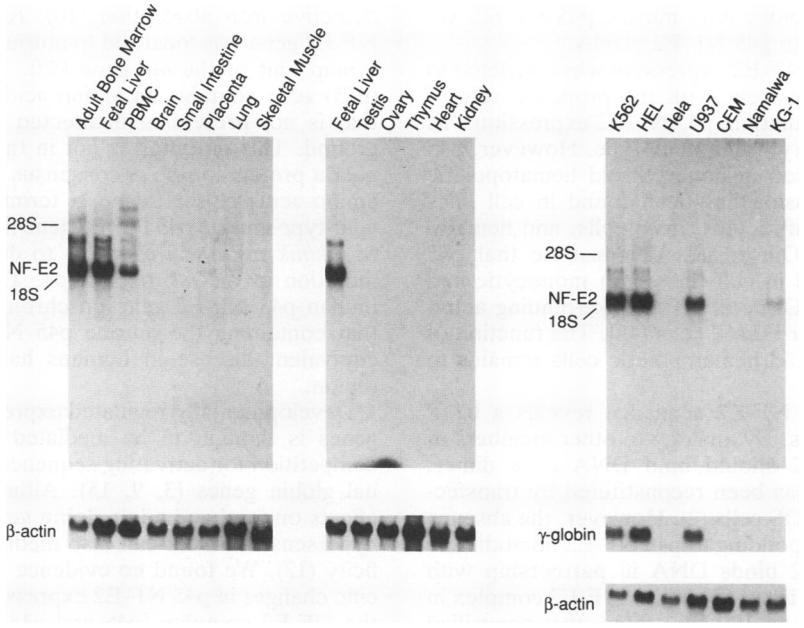

FIG. 7. Tissue-specific expression of human p45 NF-E2. Human tissue RNA (20 μg) and tissue culture cell RNA (10 μg) were studied for p45 NF-E2 and β-actin expression by Northern blotting. Tissue culture cells were also studied for γ-globin expression. The positions of 28S and 18S bands are shown. PBMC, peripheral blood mononuclear cells.

and not at all in an Epstein-Barr virus-transformed B-cell line (Namalwa).

Identification of a 18-kDa partner for p45 NF-E2. Members of the bZIP family that bind DNA bind as homodimers or heterodimers (19). For determination of whether p45 NF-E2 was sufficient for DNA-binding activity, the 45-kDa bands were eluted from SDS-polyacrylamide gels and reconstituted by the Hager-Burgess method (14). NF-E2-binding activity could not be reconstituted, suggesting that NF-E2 binds DNA as a heterodimer. In affinity-purified K562 fractions, an 18-kDa protein that correlated well with NF-E2 activity was identified (data not shown). The native NF-E2 complex was studied further with K562 and induced MEL cell extracts following immunoprecipitation. In metabolically labeled extracts, the anti-p45 NF-E2 antibody coprecipitated p45 and an 18-kDa protein (p18) (Fig. 8, lanes 1 to 4). By immunoprecipitating extracts under nondenaturing and denaturing conditions, we were able to demonstrate that p45 NF-E2 and p18 are associated rather than antigenically related proteins. Under nondenaturing conditions, p45 NF-E2 and p18 were coprecipitated from induced MEL cell extracts (lane 5). Under denaturing conditions, p45 NF-E2 was precipitated equally well by the antibody, but p18 was no longer precipitated (lane 6). In Western blotting, both bands in the 45-kDa doublet but not the 18-kDa protein were recognized by the antibody. Despite the presence of AP-1 and CREB in affinity-purified fractions, no evidence was found for dimerization between p45 NF-E2 and proteins other than p18.

DISCUSSION

We have purified human NF-E2 from K562 cells by DNA affinity chromatography. The study of purified fractions has shown that human NF-E2, like murine NF-E2, contains a doublet of proteins at 45 kDa. Tryptic digestion of the proteins in this doublet yields peptides with the same sequence, suggesting that they are encoded by the same gene (2). Purification of NF-E2 was complicated by the presence of several copurifying proteins. This problem was effectively dealt with through careful identification of NF-E2-binding activity and incorporation of a CREB DNA affinity column into the purification. The limited amount of NF-E2 in K562 extracts ultimately prevented sequence analysis of peptides from human p45 NF-E2. However, because of the high

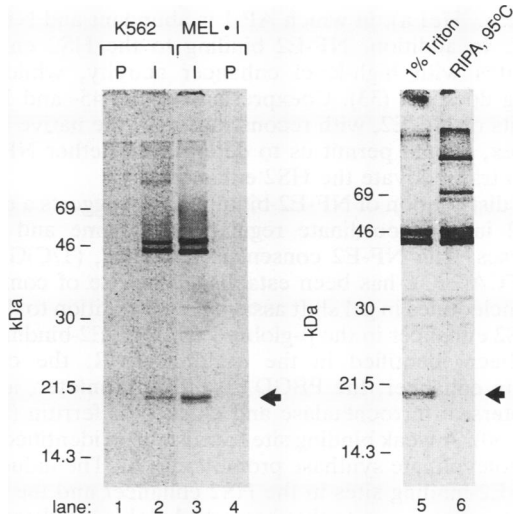

FIG. 8. p45 NF-E2 is associated with an 18-kDa protein. Extracts from metabolically labeled K562 and induced MEL (MEL · I) cells were partially purified by use of an NF-E2 affinity column and precipitated with preimmune serum (P) or an anti-p45 NF-E2 antibody (I). Immunoprecipitates were resolved on an SDS–14% polyacrylamide gel. MEL · I cell extracts were studied under nondenaturing (1% Triton) and denaturing (RIPA, 95°C) conditions. The arrows indicate the position of p18.

degree of sequence homology with murine p45 NF-E2, we were able to obtain human p45 NF-E2 cDNA.

In human tissues, p45 NF-E2 expression was restricted to hematopoietic cells. Consistent with the proposed role of NF-E2 in globin gene regulation, p45 NF-E2 expression was highest in cells with an erythroid phenotype. However, p45 NF-E2 also was expressed in nonerythroid hematopoietic cells. p45 NF-E2 expression has been found in cell lines representative of megakaryocytes, mast cells, and hematopoietic progenitors (2). Our results demonstrate that p45 NF-E2 also is expressed in cell lines with monocytic and myeloid characteristics. Of note, NF-E2-like-binding activity has been described for HL-60 cells (46). The function of p45 NF-E2 in nonerythroid hematopoietic cells remains to be determined.

Inspection of the p45 NF-E2 sequence reveals a bZIP element at the C terminus. By analogy to other members of the bZIP family, NF-E2 should bind DNA as a dimer. NF-E2-binding activity has been reconstituted by transfection of p45 NF-E2 into COS cells (2). However, the absence of binding activity corresponding to p45 NF-E2 homodimers suggests that p45 NF-E2 binds DNA in partnership with another protein. Our studies of the native NF-E2 complex in K562 cells demonstrated an 18-kDa protein that copurified stoichiometrically with p45 NF-E2. This protein also coimmunoprecipitated with p45 NF-E2 but was antigenically unrelated. These findings suggest that p18 is intimately associated with p45 NF-E2. As no members of the AP-1 or CREB families are known to have this molecular mass, we predict that p18 will be found to be a novel member of the bZIP family. Furthermore, the absence of other associated proteins in immunoprecipitates suggests that p18 is the sole specific partner of p45 in vivo.

Like many members of the bZIP family, NF-E2 is likely to function as a transcriptional activator. In HS2 of the β-globin LCR, tandem NF-E2-binding sites have been shown to function as a powerful enhancer. These sites are active (150-fold) in erythroid cells, in which NF-E2-binding activity is present, but are relatively inactive (5-fold) in nonerythroid cells (e.g., HeLa), in which AP-1 is abundant and NF-E2 is absent. In addition, NF-E2 binding to the HS2 enhancer correlates with high-level enhancer activity, while AP-1 binding does not (33). Coexpression of the 45- and 18-kDa subunits of NF-E2, with reconstitution of the native NF-E2 complex, should permit us to determine whether NF-E2 is able to transactivate the HS2 enhancer.

The distribution of NF-E2-binding sites suggests a role for NF-E2 in the coordinate regulation of heme and globin syntheses. The NF-E2 consensus sequence, (T/C)GCTGA (C/G)TCA(T/C), has been established by use of competing oligonucleotides in gel shift assays (2). In addition to those in the HS2 enhancer in the β-globin LCR, NF-E2-binding sites have been identified in the α-globin LCR, the chicken β-globin enhancer, the PBGD erythroid promoter, and the promoters of ferrochetalase and chicken H ferritin (17, 30, 40, 48, 50). A weak binding site has also been identified in the 5-aminolevulinate synthase promoter (2, 6). The inducibility of NF-E2-binding sites in the HS2 enhancer and the PBGD promoter also suggests that heme and globin syntheses may be modulated by NF-E2 during erythroid differentiation.

There is evidence in vivo that mutation of p45 NF-E2 interferes with hemoglobin synthesis. Mice homozygous for the *mk* gene have microcytic anemia characterized by a low mean cellular hemoglobin concentration and a balanced deficit of α- and β-globin chains (43, 44). The underlying defect resides partly in the bone marrow and is partly due to defective iron absorption (16). Recently, the murine p45 NF-E2 gene was localized to murine chromosome 15, within 1 map unit of the *mk* gene (37). Furthermore, the NF-E2 (p45) gene contains an amino acid substitution (V-173→A) that is not present in unaffected mice of the same background. This mutation is not in the bZIP element but falls near a protein kinase A consensus sequence (36), about 100 amino acids closer to the N terminus. Introduction of the wild-type murine p45 NF-E2 gene into the bone marrow cells of *mk/mk* mice will allow us to determine the role of this mutation in the *mk* phenotype. The region containing the human p45 NF-E2 gene on chromosome 12 is syntenic to that containing the murine p45 NF-E2 gene. To date, no equivalent disease in humans has been localized to this region.

Developmentally regulated expression of the β-globin-like genes is thought to be mediated by distance effects and competition for activating sequences in the LCR by individual globin genes (3, 9, 15). Although they differ in their effects on fetal and adult globin gene expression, individual hypersensitive sites can also mediate developmental specificity (12). We found no evidence for developmentally specific changes in p45 NF-E2 expression or the composition of the NF-E2 complex (p45 and p18). Furthermore, the high degree of sequence homology between murine p45 NF-E2 and human p45 NF-E2 implies a conservation of NF-E2 function. However, differences exist between human and murine NF-E2 sequences, mostly in the N-terminal half of the molecule. These differences may permit a stage-specific modification of NF-E2 or an interaction with local stage-specific factors.

The LCR is thought to have a dual role in globin gene expression, as a transcriptional enhancer and as a modifier of chromatin structure. As a transcriptional activator, NF-E2 may exert its effect through a direct interaction with individual globin gene promoters. Such an interaction between distinct sites for transcriptional activators has been shown to occur by DNA loop formation (21). NF-E2 may also participate in hypersensitive site formation. An AP-1/NF-E2-binding site in HS4 contributes to changes in chromatin structure (24). However, NF-E2 does not appear to be sufficient for full LCR function. In stably integrated fragments of HS2, the enhancing properties of the tandem NF-E2-binding sites depend on the presence of additional sequences, which remain to be fully characterized (8, 31, 47, 51). Identification of these sequences and proteins that complement NF-E2 activity will contribute to our understanding of LCR function.

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