Characterization of the Hematopoietic Transcription Factor NF-E2 in Primary Murine Megakaryocytes*

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Biochemical analysis of megakaryocytes, the precursors of blood platelets, is limited by their rarity *in vivo*, and studies on lineage-specific gene expression have been conducted exclusively in cell lines with limited megakaryocytic potential. Mice lacking the transcription factor NF-E2 display arrested megakaryocyte differentiation and profound thrombocytopenia. To study the heterodimeric NF-E2 protein in primary cells, we cultured mouse fetal livers with the c-Mpl ligand, obtained highly enriched megakaryocyte populations, and readily detected NF-E2 activity in nuclear extracts. As in erythroid cells, p45 NF-E2 is the only large subunit in primary megakaryocytes that dimerizes with distinct small Maf proteins to constitute a heterogeneous NF-E2 complex. Whereas p18/MafK is the predominant small Maf protein in erythroid cells, the related polypeptides MafG and/or MafF predominate in megakaryocytes. Although this represents the first example of differential small Maf protein expression among closely related blood lineages, the DNA-binding specificity of NF-E2 is similar in both cell types. Although the megakaryocyte protein preferentially binds an asymmetric AP-1-related motif, it also recognizes cAMP-responsive element-related sequences, albeit with lower affinity, and nucleotides outside the core sequence influence the DNA-protein interaction. These results demonstrate the feasibility of biochemical studies on primary murine megakaryocytes and provide a basis to dissect the critical functions of NF-E2 in megakaryocyte differentiation.

Megakaryocytes, which give rise to blood platelets, constitute an extremely rare subpopulation of cells *in vivo*. Therefore, despite the importance attached to the process by which megakaryocytes form and release platelets, molecular aspects of their development are poorly understood and have largely been studied in a few multipotential hematopoietic cell lines with limited megakaryocyte differentiation. The c-Mpl ligand (also known as thrombopoietin or the megakaryocyte growth and development factor) is the major cytokine that regulates megakaryocyte proliferation and differentiation and raises platelet counts substantially *in vivo* (reviewed in Ref. 1). Recombinant c-Mpl ligand has facilitated *in vitro* culture of sufficient numbers of primary megakaryocytes to permit biochemical studies (2),

partially relieving the dependence on transformed cell lines. However, studies on gene expression and transcription factors have not been reported in primary cells.

Studies in immortalized cell lines suggest that several genes expressed specifically within megakaryocytes are regulated in part by a combination of transcription factors belonging to the GATA and Ets protein families (3–5). Indeed, mice with megakaryocyte-selective loss of GATA-1 activity are severely thrombocytopenic as a result of arrested megakaryocyte differentiation (6). Analysis of megakaryocyte promoters *per se* has not consistently pointed to other proteins that may mediate lineage-specific gene expression and participate in platelet biogenesis.

Like GATA-1, the transcription factor NF-E2 was originally identified through its interaction with critical *cis*-elements within the β-globin locus control region (7, 8). NF-E2 is an obligate heterodimer between a 45-kDa hematopoietic restricted polypeptide (p45 NF-E2) and widely expressed ~18-kDa proteins related to the avian oncogene v-*maf*; both subunits belong to the bZip (basic leucine zipper) family of transcriptional regulators (9–11). Although several lines of evidence point to NF-E2 as the major enhancer protein acting at the β-globin gene locus in developing erythroid cells, mice lacking p45 NF-E2 display only mild, albeit consistent, red blood cell abnormalities (12). The most notable feature of these knockout mice is the virtual absence of circulating blood platelets, associated with arrested megakaryocyte maturation (13). Thus, two distinct "erythroid" transcription factors play critical roles in proper megakaryocyte development and are required to generate normal platelets *in vivo*.

In contrast to the biochemical basis of interactions between the monomeric GATA factors and their cognate DNA-binding sites (14, 15), our knowledge of NF-E2 interactions with DNA is more limited and is based exclusively on studies in erythroid cells (7–9) or with purified recombinant proteins (16, 17). Several aspects of the NF-E2 protein lend complexity to this question. First, at least in erythroid cells, NF-E2 recognizes the extended DNA sequence **GCTGA(G/C)TCA**, which lacks dyad symmetry but includes the core symmetric AP-1 motif (underlined) known to bind dimers between the Jun and Fos subfamilies of bZip proteins. Second, there is the potential for variable dimerization between p45 NF-E2 and a number of small Maf proteins, which appear to dictate the binding site preference on DNA (10, 16, 18). Finally, a number of p45 NF-E2-related polypeptides have been identified through molecular cloning (19–23), although their interactions with specific DNA sequences have not been defined completely. These considerations raise the possibility that NF-E2·DNA complexes distinct from the one characterized in erythroleukemia cells may function within megakaryocytes *in vivo*. As attention turns to the mechanisms responsible for thrombocytopenia and arrested megakaryocyte differentiation in the absence of NF-E2, it is particularly important to establish the nature of this transcription factor in

bona fide megakaryocytes. Here we report on our studies characterizing NF-E2 in primary murine megakaryocytes.

EXPERIMENTAL PROCEDURES

Cell Culture—Whole livers were recovered from mouse fetuses between embryonic days 13 and 15, and single cell suspensions were prepared by successive passage through 22- and 25-gauge needles. Fetal liver and mouse erythroleukemia (MEL) cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.1 mM nonessential amino acids. Fetal liver cultures were further supplemented with 0.1 μg/ml polyethylene glycol-conjugated recombinant human c-Mpl ligand (Amgen Inc., Thousand Oaks, CA) or 1% tissue culture supernatant from a murine c-Mpl ligand producer cell line (generously provided by Dr. J.-L. Villeval) (24). 75% of the cultured megakaryocytes were harvested on the fifth day of culture; with further expansion of the remaining cells, almost the same number of cells were harvested on the eighth day, after which the culture was terminated.

Purification of Primary Megakaryocytes—For most experiments, the entire culture of fetal liver cells was used without additional purification of megakaryocytes. To further enrich for megakaryocytes (see Fig. 2), fetal liver cultures containing 50–60% acetylcholinesterase-positive cells were depleted of non-megakaryocytic cells by incubation for 30 min at 4 °C with the monoclonal antibodies Mac-1, GR-1, and TER-119 (Pharmingen, Los Angeles, CA), followed by addition of magnetic beads coated with sheep anti-rat IgG (Dynal A. S., Oslo, Norway) for 30 min at 4 °C. The immunodepleted cell fraction contained ≥90% large acetylcholinesterase-positive cells. Acetylcholinesterase activity was detected as described previously (25).

Electrophoretic Mobility Shift Assay (EMSA)[1]—Nuclear extracts from native or cultured fetal liver and MEL cells were prepared according to a modification of previously described techniques (26). Cells were lysed in 10 mM HEPES, pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol for 10 min at 4 °C, and nuclei were pelleted by centrifugation at 13,500 $\times$ g for 10 s at 4 °C. Nuclear proteins were extracted over 20 min in 20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, and 0.5 mM dithiothreitol at 4 °C. Both incubations included protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 mM antipain; all from Sigma). The nuclear residue was eliminated by centrifugation at 13,500 $\times$ g for 2 min at 4 °C, and nuclear extracts were stored at −80 °C.

To reveal NF-E2·DNA complexes, nuclear extracts (2.5–5.0 μg) were incubated in a binding buffer containing 20 mM HEPES, pH 7.9, 60 mM KCl, 6 mM MgCl$_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mg/ml poly(dI-dC), and 10% glycerol for 5 min in a volume of 14 μl at ambient temperature. One μl of 5′-end-radiolabeled NF-E2 probe (25,000–50,000 cpm) was added before further incubation for 15 min at ambient temperature. Complexes were resolved on 5% nondenaturing polyacrylamide gels in 0.5$\times$ Tris borate/EDTA at room temperature and detected by autoradiography. GATA-1·DNA complexes were studied as described previously (15), with incubations and electrophoresis at 4 °C.

For competition experiments, a variable (see Fig. 7) or 100-fold (all other experiments) molar excess of unlabeled self- or modified oligonucleotide was incubated with the nuclear extracts for 15 min prior to addition of the labeled probe. Besides the competitors used in Fig. 6, which are specified therein and also described elsewhere (9), the following oligonucleotides were used (modified nucleotides are in boldface).

<div align="center">

NF-E2   site
AP-1 core

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NF-E2:	TGGGGAACCTGT	GCTGAGTCA	CTGGAG
NF-E2 M1:	TGGGGAACCTGT	**T**CTGAGTCA	CTGGAG
NF-E2 M2:	TGGGGAACCTGT	GCT**T**AGTCA	CTGGAG
NF-E2 M3:	TGGGGAACCTGT	**A**CTGAGTCA	CTGGAG
NF-E2 M4:	TGGGGAACCTGT	GCTGAGT**AA**	CTGGAG
HS2NFE2X2:	AGCACAGCAAT	GCTGAGTCATCATGAGTCA	TGCTGAGCC
NA.CRE:	TGGGGAACCTGT	GCTGA**CG**TCACTGGAG	
MAF:	TGGGGAACCTGT	GCTGA**CG**TCAGCAGAG	

<div align="center">

OLIGONUCLEOTIDES FOR FIGS. 2, 3, 5, AND 7

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For antibody super shift assays, extracts were incubated with antisera for 20 min at ambient temperature before addition of the radiolabeled probe. The anti-p45NF-E2 rabbit antisera are directed against either full-length murine protein (p45A) (10) or a peptide corresponding to amino acids 40–56 (p45N; kindly provided by Paul Ney). Rabbit anti-MafG antiserum was raised against a recombinant glutathione *S*-transferase fusion protein containing amino acids 1–162 of human MafG (27) and cross-reacts weakly with p18/MafK and possibly with MafF as well. Anti-GATA-1 monoclonal antibody N6 and anti-p18/MafK, anti-c-Jun family, and anti-c-Fos family antisera were purchased from Santa Cruz Laboratories (Santa Cruz, CA).

Immunoblot Analysis—Immunoblots were performed according to standard protocols (28). 20–40 μg of nuclear extract from MEL cells or primary cultured megakaryocytes was resolved by SDS-polyacrylamide gel electrophoresis; transferred to nitrocellulose; and incubated with a 1:1000 dilution of anti-p45 NF-E2, anti-MafG, or anti-p18/MafK antiserum for 1 h at room temperature. After five washes, incubation with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham International, Buckinghamshire, United Kingdom) for 1 h, and five additional washes, the bound antibodies were detected using an enzymatic chemiluminescence kit (Amersham International) and exposure to Reflections autoradiography film (NEN Life Science Products). The anti-MafK blot was exposed approximately five times longer than the anti-MafG and anti-p45 NF-E2 blots.

RESULTS

Detection of Transcription Factor DNA-binding Activity in Primary Murine Megakaryocytes—To date, knowledge about gene regulation in megakaryocytes has derived almost exclusively from experiments conducted in immortalized cell lines. To initiate studies on transcription factor structure and function in primary murine megakaryocytes, we cultivated fetal liver cells in the presence of fetal calf serum and recombinant c-Mpl ligand for several days. Within 3–4 days into the culture, megakaryocytes, identified by their large size and acetylcholinesterase activity, were the predominant differentiated cell; although at 5 days, they constituted 50–60% of all viable nonadherent cells (Fig. 1, *A* and *B*), their substantially increased DNA content suggested that nuclear extracts from the mixed culture probably contained >90% megakaryocyte-derived proteins. Nuclear extracts prepared from nonadherent cells at 5 or 8 days included an abundant quantity of the erythroid-megakaryocytic transcription factor GATA-1, as detected by binding of this monomeric zinc finger protein to a radiolabeled specific probe in EMSAs (Fig. 1*C*). Hence, megakaryocytes cultured directly from mouse fetal livers constitute a useful source of nuclear proteins for biochemical and functional studies.

The bZip transcription factor NF-E2 plays an essential role in late megakaryocyte maturation and thrombocytopoiesis (13); however, this heterodimeric protein has only been characterized in erythroid cells, where it fulfills distinct functions *in vivo*. As with nuclear extracts from MEL cells, from which the p45·p18 NF-E2 complex was originally isolated (9, 10), extracts prepared from cultured primary megakaryocytes readily revealed DNA-binding activity for oligonucleotides derived either from the promoter of the human porphobilinogen deaminase gene (Fig. 2*A*) or from DNase I-hypersensitive site 2 of the β-globin locus control region (oligonucleotide HS2NFE2X2) (data not shown). In contrast to MEL cells, the DNA-protein complexes formed by megakaryocyte nuclear extracts on these probes were virtually limited to the doublet previously identified as the NF-E2 heterodimer. The slower migrating complex that includes c-Jun, c-Fos, and possibly other related bZip proteins in MEL cells (AP-1) (Fig. 2*A*) was barely detected in megakaryocytes, although it was clearly present in whole fetal liver extracts prior to culture with the c-Mpl ligand, when erythroid cells predominated (for example, see Fig. 6 below). This difference underscores the lack of significant contamination of these nuclear extracts by non-megakaryocyte proteins. Indeed, the results from EMSAs performed with nuclear extracts prepared from cultures in which contaminating cells

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[1] The abbreviations used are: EMSA, electrophoretic mobility shift assay; CRE, cyclic AMP-responsive element.
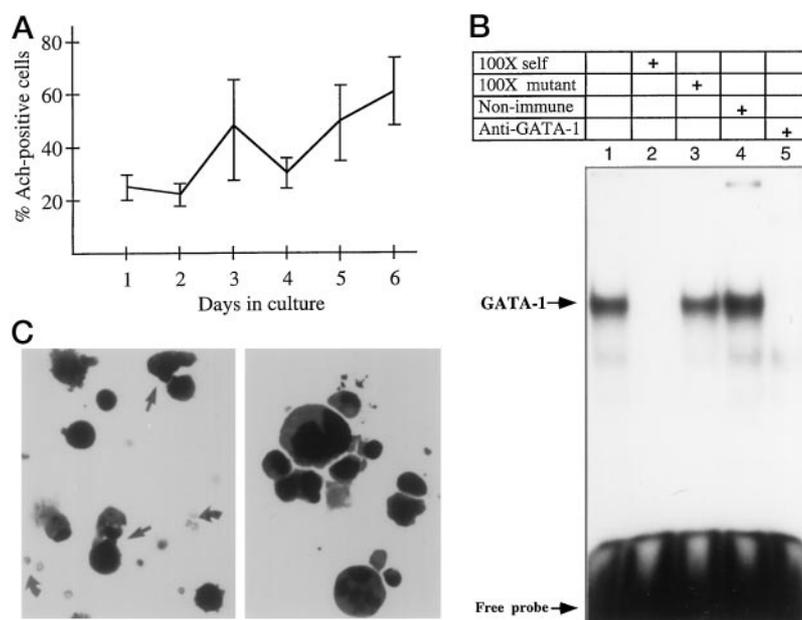
FIG. 1. **Characterization of megakaryocytes cultured from mouse fetal livers.** *A*, percentage of acetylcholinesterase-positive cells obtained at the indicated day of culture in the presence of recombinant c-Mpl ligand. These values represent *bona fide* megakaryocytes in a dynamic culture in which the latter cells expand at the expense of other lineages. *Ach*, acetylcholinesterase. *B*, low-power (*left panel*; original magnification × 100) and high-power (*right panel*; original magnification × 400) views of acetylcholinesterase-stained cytospin preparations of mouse fetal liver cells on the fourth day of culture with the c-Mpl ligand, demonstrating advanced megakaryocyte differentiation and emphasizing the high ratio of megakaryocyte nuclear mass (*straight arrows*) relative to other hematopoietic lineages (*curved arrows*). *C*, gel retardation assay performed by incubating the ³²P-labeled GATA probe from the erythropoietin receptor gene promoter with nuclear extracts from primary megakaryocytes. Addition of a 100-fold molar excess of unlabeled wild-type (*lane 2*) or mutant (*lane 3*) oligonucleotides and abrogation of the relevant complex (*arrow*) upon preincubation with anti-GATA-1 antibody (*lanes 4* and *5*) demonstrated specificity of the DNA-protein complex. *Lane 1*, control.
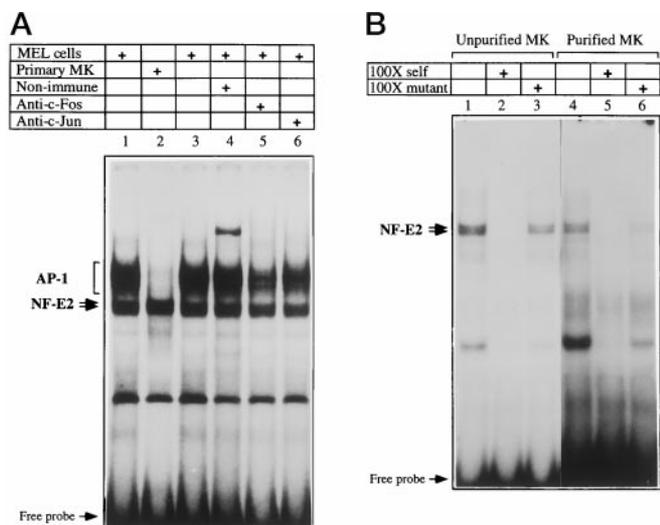

FIG. 2. **DNA-binding activity of NF-E2 in primary megakaryocytes and MEL cells.** *A*, the ³²P-labeled NF-E2 probe from the porphobilinogen deaminase promoter was incubated with nuclear extracts from MEL cells (*lanes 1* and *3–6*) or primary megakaryocytes (*MK; lane 2*). The doublet corresponding to the NF-E2·DNA complex is indicated by the *double arrow*. Nuclear extracts from MEL cells revealed an additional, slower migrating complex, designated AP-1, that diminished when antibodies against the c-Fos or c-Jun protein family (*lanes 5* and *6*) were included in the reaction. *B*, EMSA was carried out with nuclear extracts from fetal liver cells cultured with the c-Mpl ligand for 5 days and used directly (*lanes 1–3*) or after further enrichment for megakaryocytes by immunodepletion (*lanes 4–6*).

were depleted to achieve ≥90% purity of mature megakaryocytes were indistinguishable from the results obtained when no additional purification was performed (Fig. 2*B*). To avoid the substantially lower cell yield associated with immunopurification, this step was therefore omitted for most of the experiments described here.

Although hematopoietic cells derived from the fetal liver, including megakaryocytes, typically manifest a greater proliferation potential *in vitro* than cells from adult tissues, bone marrow-derived megakaryocytes can also be cultured to high purity in the presence of the c-Mpl ligand (2). Nuclear extracts from wild-type cultured bone marrow-derived megakaryocytes also display NF-E2 DNA-binding activity; as expected, this activity was missing in megakaryocytes cultured from p45 NF-E2 knockout mice (Fig. 3*A*). Furthermore, the latter extracts did not contain a detectable amount of any alternate NF-E2 DNA binding activity, whereas GATA-1 protein levels were normal (Fig. 3*B*). Thus, megakaryocytes cultured from various tissue sources and from single adult mice harbor a specific DNA-binding activity on NF-E2 oligonucleotides that is absent in cells cultured from p45 NF-E2 null mice.

Nature of the NF-E2 Complex in Primary Megakaryocytes—In erythroid cells, the NF-E2 heterodimer is composed of p45 and p18 subunits. Three distinct genes that are homologous to p45 NF-E2, *Nrf1* (also known as LCR-F1), *Nrf2* (for N̲F-E2-r̲elated f̲actors 1 and 2) and *Ech*, have been isolated (19–23), but their functions in hematopoiesis remain uncertain (29, 30). Furthermore, the Maf family encompasses at least three small proteins that can heterodimerize with p45 NF-E2 and related proteins *in vitro* and *in vivo*, including MafK (the avian homologue of p18 NF-E2), MafG, and MafF (31, 32). Thus, a heterogeneity of DNA-protein complexes is possible at relevant *cis*-elements. To investigate the composition of the NF-E2 complex in primary megakaryocytes, we included a set of antibodies directed against p45 NF-E2 and the different Maf proteins in the EMSA reactions. Addition of two distinct anti-p45 antisera abolished the NF-E2 DNA-protein complex in both MEL cells and primary megakaryocytes (Fig. 4*A*, *lanes 3, 4, 10,* and *11*). Together with the finding obtained with p45 NF-E2⁻/⁻ megakaryocytes (Fig. 3), these results establish that
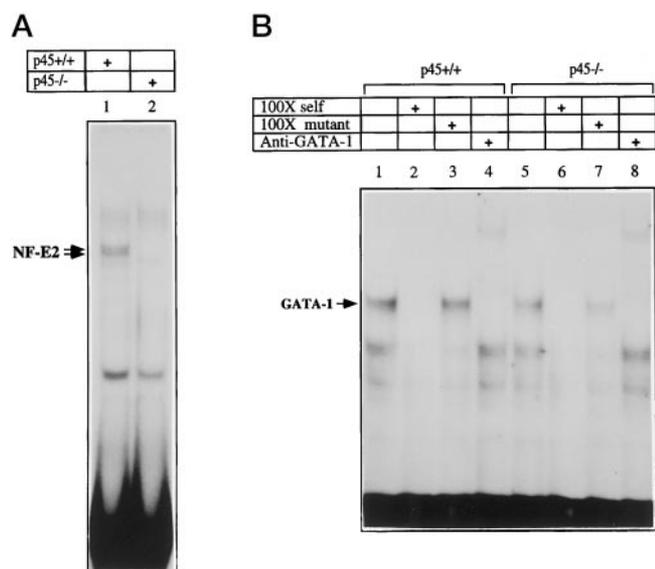
FIG. 3. **Bone marrow megakaryocytes from p45$^{-/-}$ mice lack NF-E2-like DNA-binding activity.** *A*, EMSA was carried out with nuclear extracts from p45$^{+/+}$ (*lane 1*) or p45$^{-/-}$ (*lane 2*) adult bone marrow cells treated for 4 days with the c-Mpl ligand. Neither NF-E2 nor alternate binding activity was detected in primary megakaryocytes from p45$^{-/-}$ mice. *B*, to establish specificity, the same nuclear extracts from either p45$^{+/+}$ (*lanes 1–4*) or p45$^{-/-}$ (*lanes 5–8*) adult bone marrow cells were incubated with the GATA probe. A specific GATA-1·DNA complex (*arrow*), recognized by its competition profile and response to anti-GATA-1 antibody, was readily seen in both p45$^{+/+}$ and p45$^{-/-}$ samples.

FIG. 4. **Nature of the NF-E2 complex in primary megakaryocytes and MEL cells.** *A*, antibodies directed against p45 NF-E2 (*lanes 3, 4, 10,* and *11*), different small Maf proteins (*lanes 5–7* and *12–14*), or nonimmune serum (*lanes 2* and *9*) were incubated with the EMSA reaction mixture for 20 min before addition of the radiolabeled probe. Some degradation products were present in the case of primary megakaryocytes (*MK*) in this experiment (*lanes 8–14*). *B*, immunoblot analysis of nuclear extracts from MEL cells or primary megakaryocytes with anti-p18/MafK, anti-MafG, and anti-p45 NF-E2 (control) antisera. Either 40 µg (small Maf antisera) or 20 µg (anti-p45 antiserum) of nuclear extracts was resolved by 12.5 or 10% SDS-polyacrylamide gel electrophoresis, respectively. Relative molecular masses are indicated to the right in kilodaltons.

p45 NF-E2 is the only large subunit contributing to the dominant complex in primary megakaryocytes.

A specific anti-p18/MafK antibody and an anti-MafG antiserum (27), each, inhibited formation of the NF-E2·DNA complex, whereas the combination of these reagents abrogated the NF-E2·DNA complex completely (Fig. 4*A*, *lanes 5–7* and *12–14*). This indicates that the polypeptides recognized by these antibodies together account for all the small subunits constituting the NF-E2 protein complex in primary megakaryocytes as well as MEL cells. Notably, the anti-MafG antiserum consistently displayed a greater effect on the NF-E2 complex in primary megakaryocytes compared with that in MEL cells (Fig. 4*A*, *lanes 5* and *12*). In contrast, the anti-p18/MafK antibody had a reproducibly lesser effect on the complex in primary megakaryocytes compared with that in MEL cells (Fig. 4*A*, *lanes 6* and *13*). Taken together, these findings suggest that the NF-E2 protein complex in primary megakaryocytes has a lesser contribution from the p18/MafK subunit compared with the complex in MEL cells. Immunoblot analysis further revealed that this observation directly reflects differences in the relative amounts of small Maf proteins between the two cell types. The ratio of p18/MafK to other small Maf proteins is high in MEL cells, whereas the converse is true for primary megakaryocytes (Fig. 4*B*). The level of p45 NF-E2 was equivalent in the two cell types. These data represent the first example of differential expression of small Maf proteins among closely related hematopoietic lineages and raise the possibility that p45 NF-E2 function in diverse cell types may be mediated in part through distinct complexes with small Maf proteins and *cis*-regulatory DNA elements.

DNA-binding Specificity of NF-E2 in Primary Megakaryocytes—The specificity of DNA binding by the NF-E2 heterodimer depends not only on p45, which recognizes the (C/G)**TCA** half-site, but also on the small Maf subunit, which binds to the larger **GCTGA**(C/G) half-site (10, 16, 18). To establish the binding specificity of the NF-E2 complex in primary
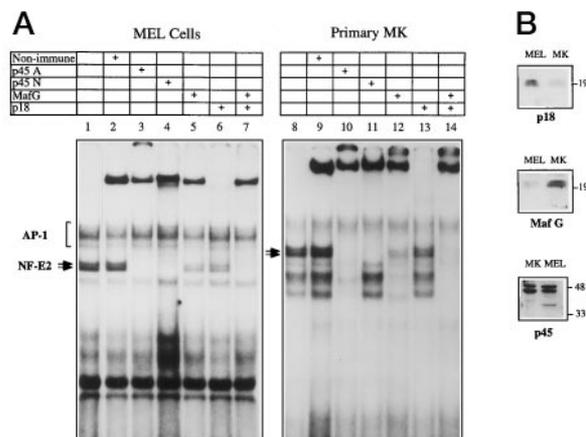
megakaryocytes, we tested the ability of various modified oligonucleotides to compete for binding between the standard probe and nuclear extracts from these cells. With four different oligonucleotides modified either within the AP-1 core sequence (M2 and M4) or in the extended half-site that distinguishes NF-E2 binding from AP-1 (M1 and M3), the pattern of competition was similar between MEL cells and primary megakaryocytes (Fig. 5). Notably, however, oligonucleotide M3, which competes effectively for binding to the p45·p18 complex purified from MEL cells (9), consistently competed less well when crude nuclear extracts from either MEL cells or primary megakaryocytes were used in the EMSA (Fig. 5, *lanes 5* and *11*). To identify any potential differences in DNA-binding specificity between erythroid cells and megakaryocytes, we further used an extended panel of 22 oligonucleotides. Again, no significant differences in NF-E2 binding were seen in nuclear extracts from MEL cells, uncultured fetal liver (*FL*, representing primary erythroid cells), and primary megakaryocytes (Fig. 6). Hence, the observed difference in small Maf subunit abundance between MEL cells and primary megakaryocytes (Fig. 4) does not translate into discernible differences in DNA-binding specificity between erythroid cells and megakaryocytes. The present resolution of the NF-E2·DNA complex therefore suggests that the various Maf homologues impart overlapping rather than exclusive DNA-binding specificities. Finally, oligonucleotides 6, 8, and 17 (Fig. 6), which have an intact NF-E2 core recognition sequence, competed only partially for binding to the NF-E2 protein, indicating that residues outside the previously established consensus sequence (9, 27) can also influence the DNA-protein interaction.

Interaction of NF-E2 with Non-consensus DNA Sequences—The demonstrated importance of NF-E2 in megakaryocyte maturation has spurred interest in identifying its direct transcriptional targets. Because one criterion that candidate target genes must fulfill is the presence of an NF-E2-responsive site in a *cis*-regulatory element, it is important to define the spectrum of DNA sequences that can bind the NF-E2 protein complex found in primary megakaryocytes. AP-1-related bZip proteins can recognize both AP-1 sites and the cyclic AMP-responsive
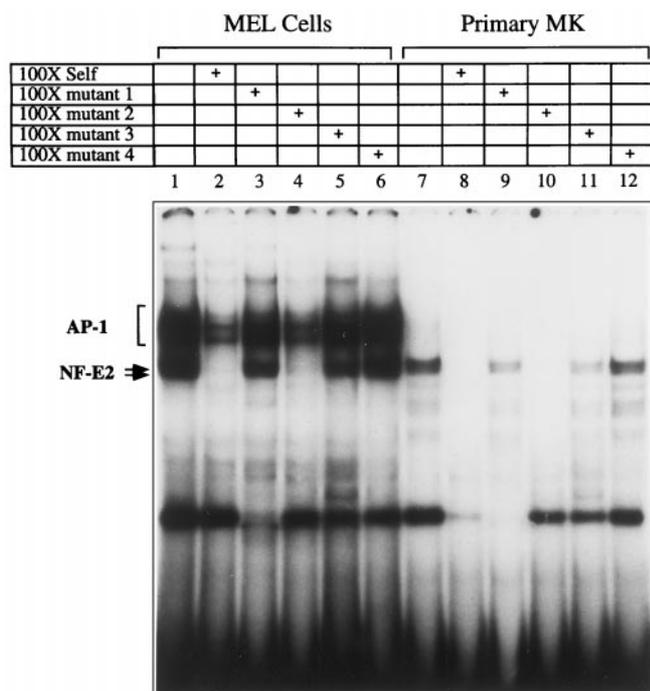
	MEL Cells						Primary MK					
100X Self		+					+					
100X mutant 1			+					+				
100X mutant 2				+					+			
100X mutant 3					+						+	
100X mutant 4						+						+
	1	2	3	4	5	6	7	8	9	10	11	12

FIG. 5. **Effects of mutations in AP-1 or NF-E2 core sequences on DNA-binding activity of NF-E2 in primary megakaryocytes.** Specificity of the DNA-binding activity of NF-E2 was tested by competition with oligonucleotides bearing mutations either within the AP-1 core sequence (*lanes 4, 6, 10,* and *12*) or outside it (*lanes 3, 5, 9,* and *11*); the oligonucleotides are specified under "Experimental Procedures." Nuclear extracts of MEL cells (*lanes 1–6*) or primary megakaryocytes (*MK; lanes 7–12*) were incubated for 15 min with a 100-fold molar excess of competitor oligonucleotides before addition of the ^{32}P-labeled NF-E2 probe. The migration positions of the NF-E2·DNA and AP-1·DNA complexes are indicated.

element (CRE), and NF-E2 heterodimers assembled from bacterially expressed recombinant proteins *in vitro* can bind a CRE sequence (GCTGAGNTCA) distinct from the NF-E2 consensus site (17). Indeed, a CRE-like DNA sequence represents the only potential NF-E2-responsive *cis*-element in the human thromboxane synthase gene, a candidate transcriptional target of NF-E2 in megakaryocytes (33). Additionally, symmetric sequences that preferentially bind dimers of small Maf proteins could potentially interact with the heterodimeric NF-E2 complex and mediate lineage-specific gene expression. We addressed each of these possibilities.

Although a CRE oligonucleotide (oligonucleotide 14 in Fig. 6) competed partially for binding of the NF-E2 protein to the porphobilinogen deaminase promoter probe, we did not detect direct binding of the NF-E2 protein in megakaryocyte nuclear extracts to a radiolabeled CRE probe (data not shown); other nuclear proteins dominated binding to this sequence *in vitro*. Furthermore, the relative affinity of the megakaryocyte NF-E2 protein for this selected CRE sequence was lower than that for the porphobilinogen deaminase promoter sequence; complete inhibition by unlabeled oligonucleotide required a ≤25-fold molar excess of the porphobilinogen deaminase promoter probe (Fig. 7, *lanes 1–5, 11,* and *12*), but a ≥100-fold excess of the NA.CRE oligonucleotide (*lanes 11–17*). In contrast, oligonucleotide MAF, which includes two symmetric Maf half-sites, competed poorly for binding to the megakaryocyte NF-E2 complex (Fig. 7, *lanes 6–10*). While these data do not address the extent to which other factors might modify DNA-protein interactions *in vivo*, they do indicate that a subset of AP-1-related sequences constitute preferred NF-E2-binding sites, whereas CRE-like sequences can also bind the protein, but with lower

affinity. Notably, the two oligonucleotides examined here differ only outside the core sequence, again emphasizing the influence of residues outside this core on the DNA-protein interaction. The relative importance of these residues and of AP-1 *versus* CRE motifs in megakaryocyte *cis*-elements *in vivo* will only become clear with the identification of additional transcriptional targets of NF-E2.

DISCUSSION

Transcription factors regulate many aspects of cell differentiation. Our current appreciation of this process is based on the premise that lineage-restricted and ubiquitous transcription factors integrate the various signals that impinge upon immature cells and cooperate to establish or maintain lineage-specific programs of gene expression. The study of transcription factor functions within individual cell lineages thus greatly improves our understanding of differentiation.

Study of the transcriptional regulation of megakaryocyte maturation and platelet production has been particularly hampered by the rarity of this cell type *in vivo* and by the limited megakaryocyte differentiation potential of established cell lines. Targeted disruption of the p45 NF-E2 gene provided the first *in vivo* demonstration of the critical role of a transcription factor in megakaryocyte development: absence of p45 NF-E2 leads to arrested megakaryocyte maturation, a uniformly profound platelet deficit, and frequent death due to hemorrhage (13). As with other key lineage-restricted transcription factors, however, the mechanism of NF-E2 action in megakaryocytes is not known, making it important to study its functions within the natural context of primary cells. To this end, we have used recombinant c-Mpl ligand to culture adequate numbers of mature megakaryocytes and thus obtain materials from a physiologic source. Although the c-Mpl ligand promotes proliferation of multiple immature blood cell lineages (34), its most potent effects are exerted through all stages of megakaryocyte maturation, including platelet differentiation *in vivo* (35–37) and *in vitro* (38, 39). This relative specificity of the c-Mpl ligand on murine hematopoietic cells makes it possible to achieve significant expansion of mature megakaryocytes at the expense of other lineages (2); in our experience, the fetal liver is a superior source of megakaryocytes relative to the bone marrow, probably because of considerably greater proliferation potential of its megakaryocyte progenitors. In both cases, a large number of primary megakaryocytes serve as an excellent source of proteins and nucleic acids for biochemical analysis of NF-E2 and other transcription factors.

At least four potential large subunits (p45 NF-E2, Nrf1/LCR-F1/TCF11, Nrf2, and Ech) and three potential small subunits (p18/MafK, MafG, and MafF) of an NF-E2 or NF-E2-like protein have been identified by molecular cloning in chicken, mouse, and man (9–11, 19–23, 27, 31, 32). In addition, heterodimers of Jun- and Fos-related bZip proteins also bind the same DNA sites *in vitro*. While the enormous potential for heterodimerization inherent in this system suggests a complex circuitry regulating cell differentiation, such models are difficult to test *in vivo*. To date, only p45 NF-E2 has a clear and nonredundant role in megakaryocyte development; for example, mice lacking p18/MafK or Nrf2 show no hematologic or other detectable abnormalities (29, 40), and embryonic stem cells lacking Nrf1/LCR-F1 contribute normally to blood cell lineages in chimeric mice (30). In erythroid cells, binding to an NF-E2 site is represented by an AP-1-related complex as well as a mixture of heterodimers between p45 and two or three of the known small Maf proteins (10, 11, 40). In contrast, the nuclei of highly differentiated normal megakaryocytes harbor little AP-1 activity, and p45-containing heterodimers account for almost all of the binding to an NF-E2 site probe. This AP-1

FIG. 6. **Comparison of NF-E2 DNA-binding specificity in primary megakaryocytes and erythroid cells.** Nuclear extracts from uncultured fetal liver cells, primary megakaryocytes (*MK*), or MEL cells were preincubated for 15 min with each oligonucleotide indicated before addition of the ^{32}P-labeled NF-E2 probe. The previously determined consensus binding site for NF-E2 is *boxed*, and altered nucleotides are *underlined*. Oligonucleotides 2–6 and 9 were derived from promoter sequences (9); all others represent mutations of the consensus sequence, except oligonucleotide 1, which is the consensus sequence found in the porphobilinogen deaminase promoter. *Double arrows* and *brackets* indicate the NF-E2·DNA and AP-1·DNA complexes, respectively. Only the relevant portion of the autoradiogram, with 90° clockwise rotation relative to all other figures, is shown.
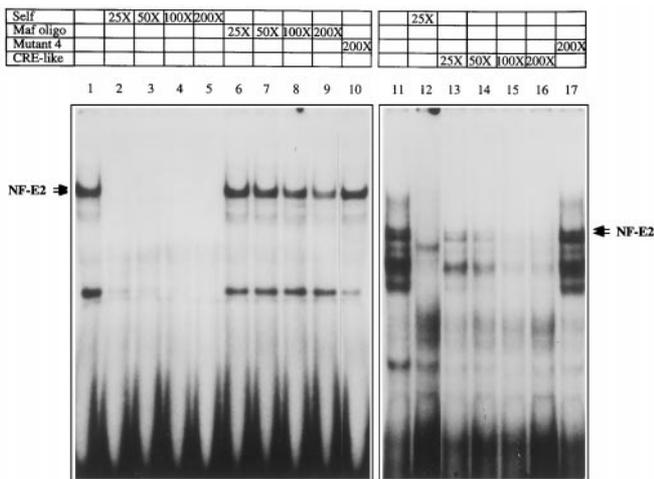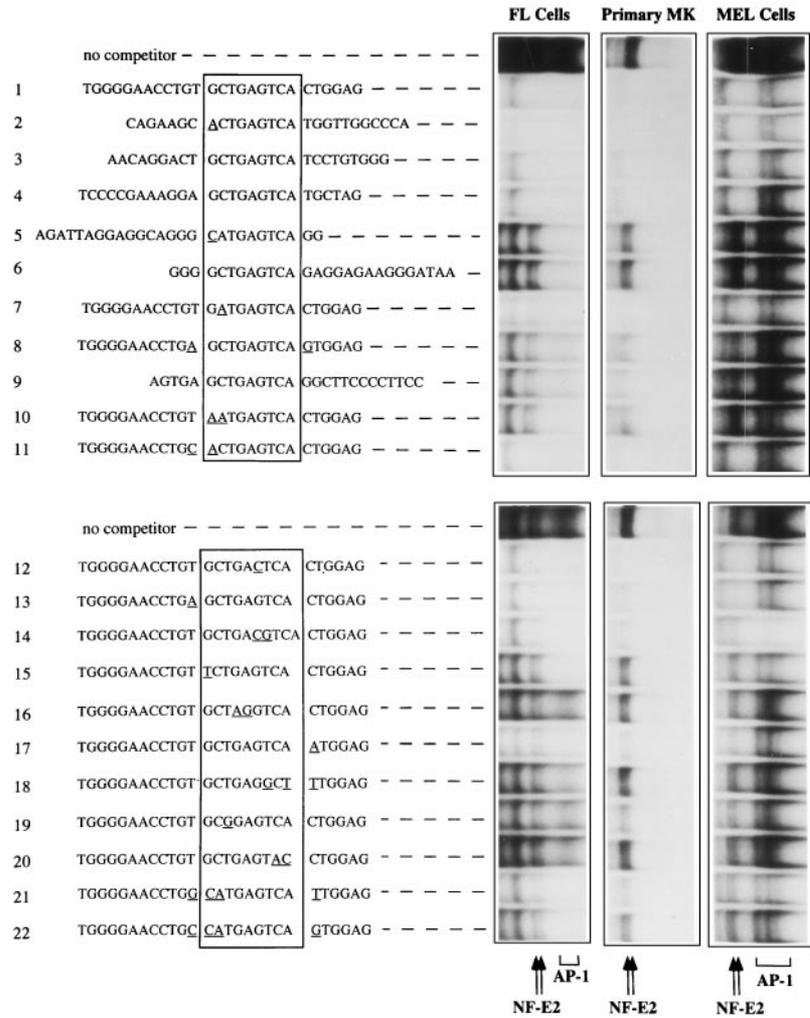

FIG. 7. **Binding of NF-E2 to alternate DNA sequences.** Nuclear extracts from primary megakaryocytes were incubated with the ^{32}P-labeled NF-E2 consensus probe, and competitions were performed with increasing molar excesses of the unlabeled probe (*lanes 2–5*), the Maf consensus sequence oligonucleotide (*lanes 6–9*), or a CRE-like oligonucleotide (*lanes 13–16*). *Lanes 1* and *11*, no competitor; *lanes 10* and *17*, 200-fold molar excess of oligonucleotide M4. *Double arrows* indicate the position of the NF-E2·DNA complex.

deficit may simply reflect the lower proliferation of differentiated megakaryocytes relative to maturing erythrocytes; alternately, this might account, at least in part, for the megakaryocyte-restricted severity in the phenotype of p45 NF-E2 knockout mice (12, 13).

Many conclusions regarding structural and functional interactions between p45 NF-E2 or related proteins and the small Maf proteins are based on *in vitro* studies using modified recombinant proteins (16, 18). For example, MafG and MafF bind an NF-E2 probe as heterodimers with p45, Ech, c-Fos, or Nrf1/LCR-F1/TCF11, with each other, or as homodimers, with different affinities. Primary megakaryocytes do not reveal significant binding of Maf homodimers to an NF-E2 probe. However, as in erythroid cells, at least two (and possibly all three) of the known small Maf proteins are present in the dominant p45-containing complex. More interesting is the clear heterogeneity of small Maf protein expression between primary or transformed erythroid cells and primary megakaryocytes (Fig. 4): p18/MafK is under-represented in the latter relative to either whole fetal livers or MEL cells, from which the p45·p18 complex was originally isolated (9, 10). Previous studies have suggested that the expression patterns of the small Maf proteins, although wide, overlap significantly (31, 32). Our demonstration of heterogeneity in small Maf protein expression in two closely related hematopoietic lineages in which NF-E2 fulfills distinct functions emphasizes the potential significance of these differences *in vivo*. Again, the possibility that this disparity is partially responsible for the lack of megakaryocyte abnormalities in p18 knockout mice (40) suggests itself, but remains unproven.

Indeed, the extent to which the small Maf proteins, which lack canonical transactivation domains, might compensate for

each other functionally is presently unclear. Experimental evidence indicates that these subunits recognize the extended portion of the NF-E2 consensus sequence that distinguishes it from an AP-1 site and thus establish the DNA-binding specificity of the heterodimer (10). Although this raises the possibility that the various small Maf proteins manifest distinct DNA-binding preferences, our data rather suggest that the DNA sequence preferences overlap. Despite the differences between erythroid and megakaryocytic cells in the contribution of individual small Maf proteins to the NF-E2 complex (Fig. 4), the profile of competition by unlabeled oligonucleotides with either source of nuclear extract is substantially similar (Fig. 6). Nevertheless, the heterogeneity in small Maf proteins among closely related hematopoietic lineages leaves open the possibility that these proteins, which appear to lack a transactivation domain, fulfill important functions other than determining DNA-binding site preferences, such as interacting with other transcriptional regulators. This hypothesis may be tested directly once megakaryocyte promoters with functional NF-E2-binding sites are identified and characterized.

In conclusion, our studies provide the first example of detailed characterization of a transcription factor in primary megakaryocytes rather than immortalized multipotential cell lines. Further analysis of NF-E2 and other important lineage-restricted transcription factors in primary megakaryocytes should continue to shed light on the transcriptional control of platelet biogenesis.

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