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 megakaryocytic differentiation. The c-Mpl ligand (also known as thrombopoietin or the megakaryocyte growth and differentiation 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/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

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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 20-gauge needles. Fetal liver and mouse erythroleukemia (MEL) cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 1% 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 mM 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-megakaryocyte cells by incubation for 30 min at 4 °C with the monoclonal antibodies Mac-1, GR-1, and TER-119 (Pharmering, 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. At this incubation time, the doublet previously isolated (9, 10), extracts were virtually limited to the doublet previously identified (data not shown). In contrast to MEL cells, from which the NF-E2 in Primary 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), the 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-megakaryocyte transcription factor GATA-1, as detected by binding of this monomeric zinc finger protein to a radiolabeled specific probe in EMSAs (Fig. 1C). 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 thrombocytopenia (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 p45NF-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. 2A) 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. 2A) was barely detected in megakaryocytes, although it was clearly present in 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

b. The abbreviations used are: EMSA, electrophoretic mobility shift assay; CRE, cyclic AMP-responsive element.
were depleted to achieve ≥90% purity of mature megakaryocytes were indistinguishable from the results obtained when no additional purification was performed (Fig. 2B). 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. 3A). 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. 3B). 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 NF-E2-related factors 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 primary megakaryocytes, whereas GATA-1 protein levels were normal (Fig. 3B). This demonstrated specificity of the DNA-protein complex.

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![Figure 1](http://www.jbc.org/)

**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. 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 (curved arrows) relative to other hematopoietic lineages (straight 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.

**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).

![Figure 2](http://www.jbc.org/)

**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).
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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. 4A, 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. 4A, 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. 4A, 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. 4B). 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 GGTTGA/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 to 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 from p45/MafK, anti-MafG, and anti-p45 NF-E2 (control) antisera. Either 40 μg (small Maf antisera) or 20 μg (anti-p45 antisera) 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.

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, 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 antisera) 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.

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 DNA elements.

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 GGTTGA/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 to 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 from p45/MafK, anti-MafG, and anti-p45 NF-E2 (control) antisera. Either 40 μg (small Maf antisera) or 20 μg (anti-p45 antisera) 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.
It's unclear what specific question or task the image represents. However, based on the content, we can infer that it is related to DNA-binding activity and the effects of mutations on this activity in primary megakaryocytes. The table and graph likely show results of experiments comparing the binding of NF-E2 and AP-1 to various DNA sequences.

For the transcription factors, the discussion mentions the important role of NF-E2 in megakaryocyte development and maturation, highlighting the significance of specifiers and regulators of cell differentiation. The study of transcription factors and their interactions with DNA is crucial for understanding cell biology and disease development.
deficit may simply reflect the lower proliferation of differentiated megakaryocytes relative to maturing erythrocytes; alternatively, 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 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...
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each other functionally is presently unclear. Experimental evidence indicates that these subunits utilize 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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