

PEBP2/CBF, the Murine Homolog of the Human Myeloid AML1 and PEBP2 β /CBF β Proto-oncoproteins, Regulates the Murine Myeloperoxidase and Neutrophil Elastase Genes in Immature Myeloid Cells

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The myeloperoxidase (MPO) and neutrophil elastase genes are expressed specifically in immature myeloid cells. The integrity of a polyomavirus enhancer core sequence, 5'-AACCA-3', is critical to the activity of the murine MPO proximal enhancer. This element binds two species, myeloid nuclear factors 1 α and 1 β (MyNF1 α and - β), present in 32D cl3 myeloid cell nuclear extracts. The levels of the MyNF1s increase during early 32D cl3 cell granulocytic differentiation. Both MyNF1 α and - β supershift with an antiserum raised by using a peptide derived from the N terminus of polyomavirus enhancer-binding protein 2/core-binding factor (PEBP2/CBF) α subunit. The specific peptide inhibits these supershifts. In vitro-translated PEBP2/CBF DNA-binding domain binds the murine MPO PEBP2/CBF site. An alternate PEBP2/CBF consensus site, 5'-GACCGCA-3', but not a simian virus 40 enhancer core sequence, 5'-TTCCACA-3', binds the MyNF1s in vitro and activates a minimal murine MPO-thymidine kinase promoter in vivo. The murine neutrophil elastase gene 100-bp 5'-flanking sequences contain several functional elements, including potential binding sites for PU.1, C/EBP, c-Myb, and PEBP2/CBF. The functional element 5'-GGCCACA-3' located at positions -66 to 72 differs from the PEBP2/CBF consensus (5'-PuACCPuCA-3') only by an A-to-G transition at position 2. This DNA element binds MyNF1 α and - β weakly. The N terminus of two PEBP2/CBF α subunit family members, PEBP2 α A and PEBP2 α B (murine AML1), are nearly identical, and 32D cl3 cells contain both corresponding mRNAs. Since t(8;21), t(3;21), and inv(16), associated with myeloid leukemias, disrupt subunits of PEBP2/CBF, we speculate that the resulting oncoproteins, AML1-ETO, AML1-EAP, AML1-Evi1, and CBF β -MYH11, inhibit early myeloid differentiation.

Myeloid cells, which consist of granulocytes, monocytes, and their precursors, account for approximately half of the hematopoietic cells in human and murine marrow. To identify the transcriptional events which initiate myeloid differentiation, we are investigating the regulation of genes expressed in immature myeloid cells. Myeloperoxidase (MPO) and neutrophil elastase (NE) are microbicidal proteins present in the primary granules of myeloid cells (32, 48). These proteins, and their cognate mRNAs, are not found in other cell lineages (48, 51), and transcriptional regulation is in part responsible for their lineage-specific expression (15, 39, 62). MPO and NE are early markers of both the granulocytic and monocytic lineages (14, 38). Human MPO protein has been detected even in myeloid progenitor cells (46).

The murine and human MPO genes have been cloned and sequenced (17, 55). They both lack a TATAA homology. The murine MPO gene initiates transcription from two major sites separated by approximately 400 bp (15). The sequences between, and those 1,200 bp upstream of, these sites are 60% homologous to the region of the human MPO gene located

upstream of its major initiation site (17, 55). Functional analysis of the murine MPO 5'-flanking region delimited a 414-bp myeloid enhancer located just upstream of the murine MPO initiation sites (47). The homologous region of the human MPO gene contains a DNase I-hypersensitive site (19, 25). This enhancer contains several functional elements, including a potential Myb-binding site (47). Mutation of a centrally located enhancer core motif, 5'-AACCA-3', markedly diminished the activity of the murine MPO enhancer, and this DNA element was found to bind a set of myeloid-restricted nuclear factors termed MyNF1s (47).

We have now found that the MyNF1s are members of the recently described polyomavirus enhancer-binding factor 2/core-binding factor (PEBP2/CBF) family of transcriptional activators. We also demonstrate that a PEBP2/CBF will bind and regulate the murine NE gene and that the murine NE enhancer contains additional functional elements, including potential binding sites for a C/EBP, PU.1 or another Ets family member, and Myb.

PEBP2/CBFs were first purified on the basis of their ability to bind the core sites of polyomavirus or Moloney murine leukemia virus (20, 56). Two core-binding species, PEBP2A and PEBP2B, were detected by mobility shift assays (42). These two factors contain a common β subunit and different α subunits. The cDNAs encoding the murine α subunits, PEBP2 α A and PEBP2 α B, have been cloned (4, 36), as has the cDNA encoding the murine β subunit, PEBP2 β /CBF β (35, 56). PEBP2 β /CBF β does not bind DNA directly but strength-

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ens the affinity of α subunits for DNA (35, 57). Alternatively spliced forms of each of the α subunits and of the β subunit have been detected (4, 35, 36, 57).

The PEBP2 α subunits each contain a region homologous to the *Drosophila* Runt protein (21). This 127-amino-acid Runt homology domain is required for DNA binding and heterodimerization (26, 36). The consensus DNA-binding site for PEBP2 α A is 5'-PuACCPuCA-3' (20), and the consensus DNA-binding site for PEBP2 α B is 5'-ACCPuCA-3' (26). The α subunits will not bind the related DNA element 5'-TTC CACA-3', which is found at the core of the simian virus 40 enhancer and interacts with C/EBPs, AP-3, and TEF-2 (9, 18, 30).

The human PEBP2 α B gene, also termed the AML1 gene, is located at the breakpoint of t(8;21)(q22;q22), which is associated with some cases of FAB M2 acute myeloid leukemia (29). These leukemic cells produce a fusion transcript, AML1-ETO (12, 28). The AML1 gene is also involved in t(3;21)(q26;q22), which is associated with some cases of therapy-associated myeloid leukemia and chronic myeloid leukemia blast crisis, and produces an AML1-EAP (34) or AML1-Evi1 (27) fusion gene. Finally, the human PEBP2 β /CBF β gene is fused to a smooth muscle myosin gene as a result of inv(16)(p13q22), which is associated with FAB M4eo cases of acute myeloid leukemia (24). Our findings suggest that some or all of the oncoproteins expressed as a result of these chromosomal abnormalities could impair myeloid differentiation through inhibition of PEBP2/CBF function in leukemic cells.

MATERIALS AND METHODS

Cells and transfection. 32D cl3 cells (53) were maintained at 37°C in a 5% CO₂-Iscove modified Dulbecco medium supplemented with 10% heat-inactivated fetal bovine serum and 5% WEHI-3B supernatant as a source of interleukin 3 (58). For induction of granulocytic differentiation, cells were washed twice with phosphate-buffered saline (PBS) and placed in Iscove modified Dulbecco medium-10% heat-inactivated fetal bovine serum supplemented with 1,000 U of granulocyte colony-stimulating factor (G-CSF) (Amgen) per ml. Mouse L cells (22) were maintained in Dulbecco modified Eagle medium-10% fetal bovine serum.

Transient transfection of 32D cl3 cells and L cells, luciferase assay, and chloramphenicol acetyltransferase (CAT) assay were accomplished as described previously (47).

Nuclear extracts with DFP. Nuclear extracts were prepared by the method of Dignam et al. (11) as modified by Lee et al. (23). Cells (2×10^8) were washed twice with PBS and incubated on ice in 5 ml of buffer A containing 5 mM diisopropylfluorophosphate (DFP) (Sigma) for 15 min. The cells were then resuspended in 2 ml of ml buffer A-0.5 mM DFP-0.75% nonfat dried milk (Carnation)-0.01% Nonidet P-40-0.5 mM dithiothreitol-0.4 mM phenylmethylsulfonyl fluoride-2 mM benzamidine-0.5 mM spermidine-10 μ g of leupeptin per ml-1 μ g of pepstatin A per ml-1 μ g of antipain per ml-1 μ g of chymostatin per ml-2 μ g of soybean trypsin inhibitor per ml-10 μ g of aprotinin per ml. Cells were then lysed by 10 passes of a Dounce homogenizer. Nuclei were pelleted at 850 \times g for 5 min, washed similarly with the same solution lacking DFP, resuspended in 1 ml of this solution, and microcentrifuged at 16,000 \times g for 5 min. Nuclei were then lysed by addition of 0.6 ml of buffer C containing dithiothreitol, phenylmethylsulfonyl fluoride, benzamidine, spermidine, and the peptide protease inhibitors listed above. After being rocked at 4°C for 30 min, the extracts were microcentrifuged, and the supernatants were aliquoted, quickly frozen in liquid

nitrogen, and stored at -70°C. Analysis by the Bradford assay (Bio-Rad) showed that these extracts contained 2 to 2.5 mg of protein per ml.

EMSA. Nuclear extracts (6 to 8 μ g) were preincubated at 4°C for 5 min in a volume of 20 μ l with 2 μ g of dIdC-0.1 mg of bovine serum albumin per ml-50 mM KCl-70 mM NaCl-10 mM Tris (pH 7.5)-1 mM dithiothreitol-0.5 mM EDTA-1 mM phenylmethylsulfonyl fluoride-10% glycerol. When desired, unlabelled competitor oligonucleotides (10 to 200 ng) were included in this 5-min preincubation. For supershift assays, 1 to 2 μ l of either anti-PEBP2/CBF α subunit antiserum (26) or normal rabbit serum was then added, in the presence or absence either of 4 μ g of PEBP2/CBF α subunit N-terminal peptide or of an internal PU.1 peptide, and incubation was continued for 30 min on ice. One nanogram of oligonucleotide, radiolabelled and blunted by Klenow fragment fill-in, was then added, and incubation was continued on ice for 30 min. In vitro transcription and translation in reticulocyte lysates of the PEBP2 α B Runt homology domain was carried out as described previously (26); 1 μ l was used similarly for electrophoretic mobility shift assay (EMSA). The mixtures were then resolved on a 5% acrylamide gel run at 20 mA at 4°C for 3 h in 0.33 \times Tris-borate-EDTA. The resulting gel was then dried and exposed to Kodak XAR film.

Isolation of the murine NE promoter. The human NE cDNA (48) was used to screen a murine BALB/c genomic library (provided by T. Lanahan) at high stringency (40). A single lambda plaque was rescreened twice and contained a 19-kb insert. This insert was restriction mapped and subcloned. Subclones containing exons were identified by Southern blotting with human NE probe. These exons and adjacent DNA segments were sequenced by the dideoxy chain termination method (41), and the first exon and upstream promoter sequences were identified by homology with the human NE gene (49).

Plasmid construction and oligonucleotides. A modified pBS (Stratagene) was created in which a *Bgl*III and *Nco*I site had been placed between the polylinker *Hind*III and *Sal*I sites by using annealed oligonucleotides. A 1.8-kb *Bam*HI-*Nco*I murine NE genomic subclone was ligated into this plasmid after it was digested with *Bgl*III-*Nco*I. The *Nco*I site encodes the first translated ATG in murine NE. The *Nco*I site was then eliminated by using mung bean nuclease, and then a 1.8-kb *Hind*III/*Sal*I fragment containing 1,800 bp of murine NE 5'-flanking sequences, including the 5'-untranslated sequences, was ligated into similarly digested p19LUC (54) to create pNELUC. Clustered point mutations were introduced into pNELUC by oligonucleotide-mediated mutagenesis (45), replacing bases -47 to -42 with 5'-CTCGAG-3', -61 to -55 with 5'-CTCGAGG-3', -69 to -66 with 5'-CTCG-3', -85 to -79 with 5'-CTCGAGC-3', -95 to -91 with 5'-TCGAG-3', and -107 to -103 with 5'-TCGAG-3', thereby creating *Xho*I sites at each location. TATAA is numbered -31 to -27, as in human NE. A 5' deletion series was created by treating plasmids containing clustered point mutations with *Hind*III, *Xho*I, and Klenow enzyme, after which the resulting larger fragments were religated.

Oligonucleotides containing the wild-type murine MPO MyNF1 site (5'-AACCACA-3') and mutant MyNF1 site (5'-TAGCACA-3') have been described previously (47). An oligonucleotide in which the wild-type MyNF1 site is replaced with 5'-GACCGCA-3', which fits the PEBP2/CBF consensus, was obtained by annealing 5'-CTAGACTGACCATTGAC CGCAACCAGTTG-3' with 5'-CTAGCAACTGGTTGCG GTCAATGGTCACT-3'. An oligonucleotide in which the wild-type MyNF1 site is replaced with 5'-TTCCACA-3', the

simian virus 40 enhancer core site, was obtained by annealing 5'-CTAGACTGACCATTTTCCACAACCAGTTG-3' with 5'-CTAGCAACTGGTTGTGGAAAATGGTCAGT-3'. These oligonucleotides were each ligated into *NheI*-digested pM-POH(Δ -552/-241)TKLUC, and the single copy and forward orientation of inserted oligonucleotides were confirmed by *Hin*-dIII-*NheI* digestion of the resulting plasmids.

An oligonucleotide containing the wild-type murine NE MyNF1 site was obtained by annealing 5'-TCGACAGT AGGGCTGTGGCCAGGATGGGG-3' with 5'-TCGACCC CATCCTGGCCACAGCCCTACTG-3'. A similar oligonucleotide containing a mutant MyNF1 site was obtained by annealing 5'-TCGACAGTAGGGCCGAGACCAGGATGGGG-3' with 5'-TCGACCCCATCTGGTCTCGGCCCTACTG-3' or by annealing 5'-TCGACAGTAGGGCTGTCTCGAGGAT GGGG-3' with 5'-TCGACCCCATCCTCGAGACAGCCC TACTG-3'. A similar oligonucleotide containing a murine MPO-type MyNF1 site in place of the murine NE site was obtained by annealing 5'-TCGACAGTAGGGCTGTGGTTA GGATGGGG-3' with 5'-TCGACCCCATCTAACCACAG CCCTACTG-3'. The USF oligonucleotide has been described previously (47).

The PEBP2 α B Runt homology domain (amino acids 50 to 177) was transferred to pBS (Stratagene) by PCR.

RNA preparation and Northern (RNA) blotting. Total cellular RNA was prepared by the acid phenol-guanidinium isothiocyanate procedure (8). Poly(A)⁺ mRNA was obtained by single selection on an oligo(dT)-cellulose (Bethesda Research Laboratories) column as described previously (40). For Northern blotting, 20 μ g of total mRNA or 5 μ g of poly(A)⁺ mRNA was resolved on a 1% agarose-formaldehyde gel and transferred (40) to a nylon membrane (GeneScreen; New England Nuclear). Filters were prehybridized and hybridized at 42°C in 50% formamide-6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-2 \times Denhardt's solution-0.1% sodium dodecyl sulfate (SDS)-100 μ g of sonicated, boiled salmon testis DNA per ml. Probes were radiolabelled with ³²P by random priming (13). Filters were washed to a stringency of 65°C in 0.1 \times SSC-0.05% SDS for 30 min and exposed to autoradiography film at -70°C. The murine β 5-tubulin probe has been described previously (15). Murine NE mRNA was detected by using a 400-bp genomic probe containing murine NE exon 4. A 1.8-kb *EcoRI* fragment containing the murine PEBP2 α B cDNA (4) and a 2.5-kb *EcoRI* fragment containing most of the murine PEBP2 α A cDNA (36) were used as probes as well.

Nucleotide sequence accession number. The GenBank accession number for murine NE is U04962.

RESULTS

The MyNF1s are intact in DFP nuclear extracts. We had previously found that the ubiquitous transcription factor USF was intact in uninduced 32D cl3 or L-cell extracts prepared without DFP. However, USF was completely degraded in 4-day-induced 32D cl3 extracts prepared similarly (47), likely because of abundant proteases in their primary granules. To improve protein integrity, we introduced three modifications into our nuclear extraction procedure. First, intact cells were incubated with DFP, a very strong covalent serine protease inhibitor which can traverse cell membranes (60). DFP was also included in the cell lysis buffer. Second, nonfat dried milk was included in the cell lysis buffer as a protease "sink" (10). Third, extracts were frozen without dialysis. Extracts from uninduced 32D cl3 cells and from 32D cl3 induced to the promyelocytic stage of granulocyte differentiation by exposure

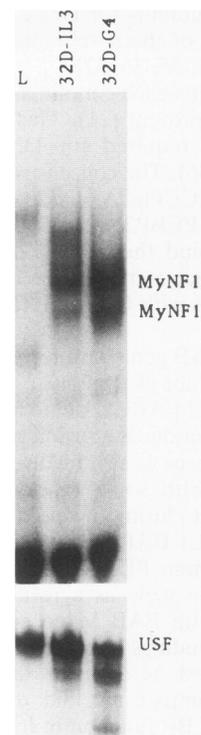


FIG. 1. Integrity of MyNF1s and USF in 32D cl3 cell DFP nuclear extracts. Nuclear extracts were prepared from uninduced (32D-IL3) and 4-day-induced (32D-G4) 32D cl3 cells as described previously (47) with the following modifications: cells were treated with 5 mM DFP prior to lysis, 0.5 mM DFP and 0.75% nonfat dried milk were included in the cell lysis buffer, and extracts were not dialyzed. The integrity of the MyNF1s and of USF in these extracts and in an L-cell extract produced as described previously (47) was assessed by EMSA.

to G-CSF for 4 days (53) were prepared in this manner. The integrity of USF and of the MyNF1s in these extracts and in an L-cell nuclear extract prepared without these modifications was determined by EMSA (Fig. 1). USF was intact in the L-cell and uninduced 32D cl3 cell extracts and was approximately 60% intact in the induced 32D cl3 cell extract. The MyNF1 γ species previously noted in induced 32D cl3 extracts (47) was no longer observed. Instead, now both MyNF1 α and MyNF1 β levels increased during early 32D cl3 cell differentiation, whereas previously only MyNF1 β had been noted to increase (47). Thus, MyNF1 α was not degraded to MyNF1 γ in DFP nuclear extracts.

The MyNF1-binding site fits the PEBP2/CBF consensus. The MyNF1-binding site fits the viral enhancer core motif 5'-(T/A)(T/A)(T/A)CCACA-3' present in a variety of mammalian viruses (59). One group of enhancer core-binding proteins, typified by the C/EBPs, prefers the site 5'-TTC CACA-3' (1, 18). However, a C/EBP oligonucleotide did not compete for MyNF1 binding (47). Also, the murine MPO MyNF1-binding site is 5'-AACCACA-3', which better fits the consensus for the PEBP2/CBF family, 5'-PuACCPuCA-3'. To further test the hypothesis that the MyNF1s are PEBP2/CBFs, we prepared a murine MPO-derived oligonucleotide in which the centrally located MyNF1-binding site had been changed to 5'-GACCGCA-3', which still conforms to the PEBP2/CBF consensus. We also prepared a similar oligonucleotide in which the MyNF1-binding site had been changed to 5'-TTCCACA-

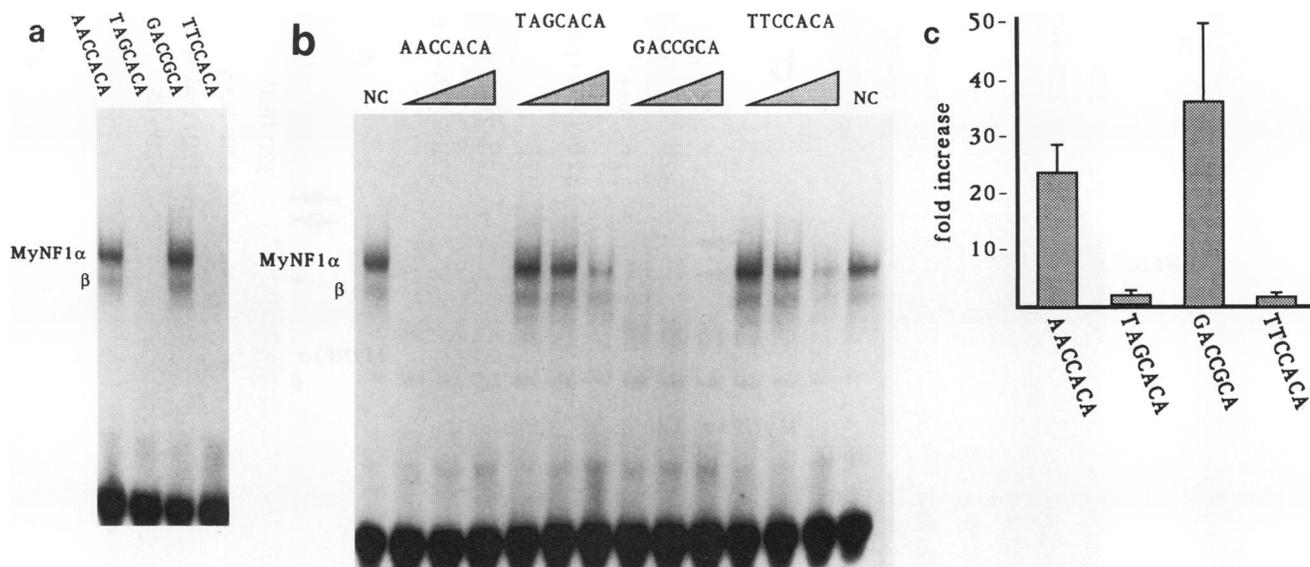


FIG. 2. Characterization of the murine MPO MyNF1-binding site in vitro and in vivo. (a) Four murine MPO-derived, 33-bp oligonucleotides were radiolabelled to similar specific activities and used in an EMSA with 6 μ g of 4-day-induced 32D cl3 cell DFP nuclear extracts. The centrally located MyNF1-binding site either was wild type (AACCACA), was a previously described down-mutant (47) (TAGCACA), fit the PEBP2/CBF consensus (20) (GACCGCA), or matched the simian virus 40 enhancer core (62) (TTCCACA). The positions of MyNF1 α and MyNF1 β are indicated. (b) The wild-type murine MPO oligonucleotide was radiolabelled and incubated with similar extracts either in the absence of competitor (NC) or in the presence of either a 10-, 50-, or 200-fold excess of each of the four, unlabelled, oligonucleotides described for panel a. (c) A single copy of each of these four oligonucleotides was ligated upstream of base -241 in pMPOH(Δ -552/-241)TKLUC. The activities of the resulting four MPO-TK derivatives and of the initial construct in induced 32D cl3 cells were determined by transient transfection. The average fold increase in activity from the insertion of each oligonucleotide, from four experiments, is shown.

3', fitting the C/EBP consensus. These two oligonucleotides, the wild-type oligonucleotide, and a mutant oligonucleotide with site 5'-TAGCACA-3' were labelled to similar specific activities and used in an EMSA with extracts from 4-day-induced 32D cl3 cells (Fig. 2a). Only the oligonucleotides containing enhancer core sites fitting the PEBP2/CBF consensus bound MyNF1 α and MyNF1 β . To confirm these differences in affinity, an EMSA competition experiment was performed (Fig. 2b). The ability of a 10-, 50-, or 200-fold excess of each unlabelled oligonucleotide to compete for MyNF1 binding to 1 ng of radiolabelled wild-type murine MPO oligonucleotide was determined. A 10-fold excess of the two oligonucleotides with DNA elements which fit the PEBP2/CBF consensus prevented binding almost completely, whereas even a 50-fold excess of the other two oligonucleotides only perturbed binding mildly.

These assays show that the MyNF1s bind in vitro to sites which fit the PEBP2/CBF consensus. To determine whether the MyNF1s bind and function through the same sites in vivo, each of the four oligonucleotides employed for Fig. 2 was assessed for its ability to stimulate a minimal MPO-thymidine kinase (TK) promoter in induced 32D cl3 cells. A single copy of each oligonucleotide was inserted in the forward orientation upstream of base -241 in pMPOH(Δ -552/-241)TKLUC. This plasmid lacks the MyNF1-binding site and several surrounding elements that are active only in myeloid cells. It retains an element located between positions -71 and 0 which was equally active in 32D cl3 cells and L cells (47) and also contains a TATAA homology in the TK segment. We employed this method because the wild-type murine MPO oligonucleotide, containing an MyNF1-binding site, stimulated pMPOH(Δ -552/-241)TKLUC 30-fold but stimulated pTKLUC only 4-fold (47). Apparently, the MyNF1s are opti-

mally active in cooperation with additional factors. Also, in 32D cl3 cells the activity of pTKLUC was often near the limits of detection, whereas pMPOH(Δ -552/-241)TKLUC was ninefold more active (47). We found that the two oligonucleotides containing sites which fit the PEBP2/CBF consensus consistently increased the activity of pMPOH(Δ -552/-241)TKLUC 20- to 40-fold, whereas the other two oligonucleotides were not stimulatory (Fig. 2c). Of note, the 5'-GACCGCA-3' site was about twice as active in vivo as the 5'-AACCACA-3' site and bound the MyNF1s with about twice the affinity in vitro as well (Fig. 2a).

To verify that PEBP2/CBF can bind the wild-type murine MPO-derived oligonucleotide, the ability of an in vitro-transcribed and -translated Runt homology domain derived from PEBP2 α B to bind this oligonucleotide was assessed (Fig. 3a). Specific binding at the PEBP2/CBF site was observed.

The MyNF1s supershift with PEBP2/CBF α subunit antiserum. The oligonucleotide containing a centrally located murine MPO MyNF1-binding site was radiolabelled and incubated with extracts from 4-day-induced 32D cl3 cells. For supershift assay, the extracts were preincubated for 30 min with 1 or 2 μ l of a rabbit polyclonal antiserum raised against a 17-amino-acid peptide derived from the N terminus of PEBP2 α B coupled to keyhole limpet hemocyanin (26). To block the antiserum, 4 μ g of the specific peptide was added to the extract prior to antiserum addition (Fig. 3b). Inclusion of the PEBP2 α B antiserum disrupted or shifted the MyNF1 β species completely, and 60 to 80% of the MyNF1 α species were disrupted or shifted as well. A doublet, likely containing supershifted MyNF1 α and MyNF1 β , was evident near the top of the lanes in which antiserum, but no peptide, was added. This doublet is better seen in Fig. 3c and in Fig. 7c, lane 5. A specific peptide, but not a nonspecific peptide derived from

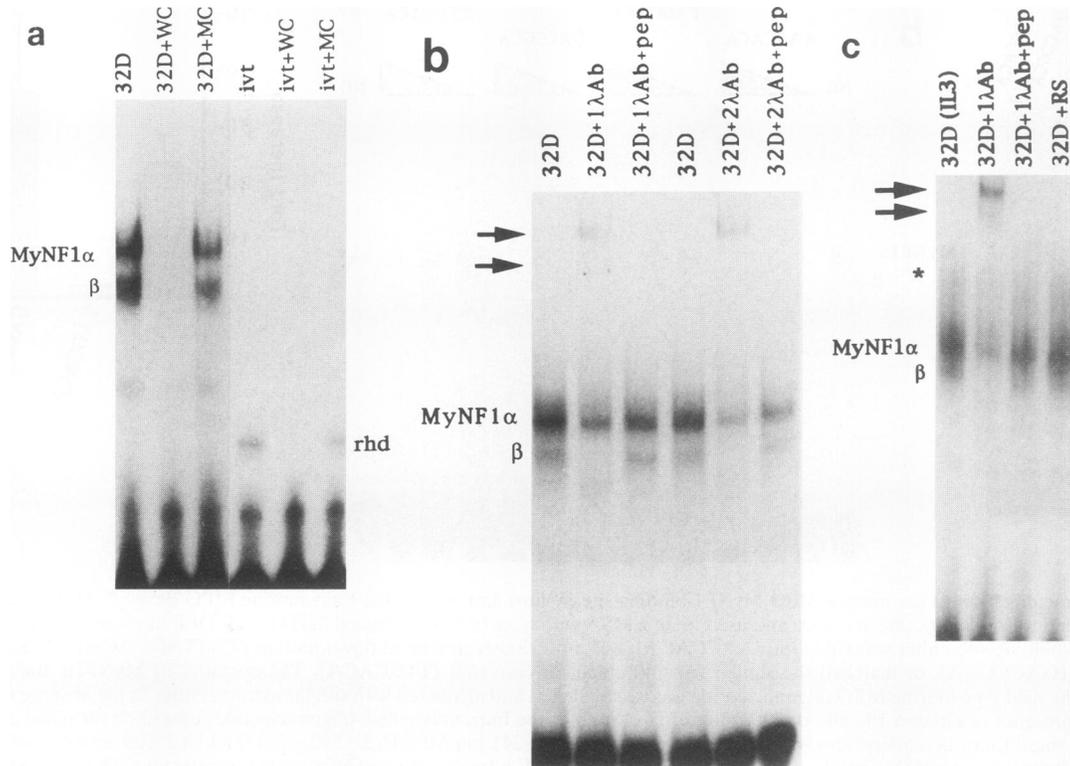


FIG. 3. The murine MPO MyNF1-binding site binds PEBP2/CBF. (a) Binding by in vitro-transcribed and -translated PEBP2/CBF. The DNA-binding Runt homology domain (rhd) of PEBP2 α B was transcribed and translated as described previously (26). The ability of this protein to bind the murine MPO PEBP2/CBF site was compared with that of an induced 32D c3 cell extract in the absence of specific competitor (32D and ivt), in the presence of a 50-fold excess of wild-type competitor (32D+WC and ivt+WC), or in the presence of a 50-fold excess of mutant competitor (32D+MC and ivt+MC). (b) Supershift assay using the murine MPO MyNF1 site and PEBP2/CBF antiserum. The murine MPO oligonucleotide was radiolabelled, incubated with 6 μ g of 4-day-induced 32D c3 cell DFP nuclear extracts, and analyzed by EMSA (32D). The ability of an antiserum raised against the N terminus of the α subunit of PEBP2/CBF to disrupt or supershift the MyNF1 species was assessed by adding 1 or 2 μ l of anti-PEBP2/CBF antiserum to the nuclear extracts prior to probe addition (32D+Ab). The specificity of the antiserum-MyNF1 interaction was assessed by addition of 4 μ g of the specific peptide to the nuclear extracts prior to antiserum addition (32D+Ab+pep). The positions of supershifted species are indicated by arrows. (c) Supershift assay using extracts from uninduced 32D c3 cells. Six micrograms of nuclear extract was incubated with the oligonucleotide alone [32D (IL3)], with the addition of 1 μ l of PEBP2/CBF antiserum (32D+Ab), with the addition of antiserum and specific peptide (32D+Ab+pep), or with the addition of 1 μ l of normal rabbit serum (32D+RS)*, slowly migrating doublet.

PU.1 (not shown), completely prevented the supershift of the MyNF1 species. Addition of specific antiserum to the probe alone, without nuclear extract, did not produce these supershift species (not shown). Since the N terminus of PEBP2 α A is nearly identical to that of PEBP2 α B, with 15 (of 17) identities and two conservative changes (4, 36), we conclude from these experiments only that the MyNF1s are members of the PEBP2/CBF family. Perhaps the unshifted MyNF1 α contains a modified N terminus.

A similar supershift assay was also carried out with extracts from uninduced 32D c3 cells (Fig. 3c). Again the MyNF1 α and MyNF1 β species were supershifted by the PEBP2 α B antiserum, the specific peptide blocked this reaction, and normal rabbit serum did not produce a supershift of the MyNF1s. Of note, the more slowly migrating doublet observed with uninduced, but not induced, extracts (Fig. 1) did not supershift with this antiserum, and neither did the minor species migrating just above MyNF1 α . We previously found that nuclear extracts from SP2 lymphoid cells and MEL erythroid cells did not contain the MyNF1s but did contain species which migrate more slowly in a gel shift reaction with a PEBP2/CBF oligo-

nucleotide (47). These species also did not supershift with the PEBP2 α B N-terminus antiserum (not shown).

Cloning the murine NE promoter. To obtain the gene encoding a second murine myeloid primary granule protein, we screened a BALB/c lambda genomic library with the human NE cDNA and obtained a clone with a 19-kb insert. The insert was subcloned, and fragments containing exons were identified by Southern blotting with the human NE cDNA as probe (not shown). The five exons were sequenced in their entirety, along with some neighboring DNA segments. The predicted amino acid sequence of the mature murine NE protein is 81% identical and 90% homologous to that of human NE. A search of the GenBank and EMBL databases revealed that the next-most-related protein-coding segment is that of human azurocidin, another serine protease (2), which has 46% identity and 56% homology.

To further verify the identity of the murine NE genomic clone, mRNA was prepared from uninduced 32D c3 cells and from cells induced with G-CSF for 4, 7, or 10 days. These mRNAs were subjected to Northern blotting, and the filter was probed sequentially for murine NE and for murine β 5-tubulin

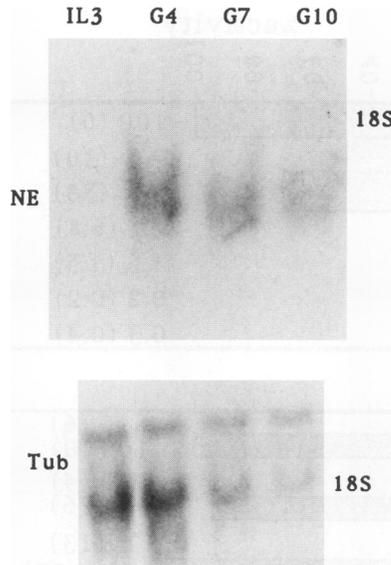


FIG. 4. Northern blot analysis of murine NE and murine β 5-tubulin (Tub) expression in differentiating 32D cl3 cells. Twenty micrograms of total RNA from uninduced (IL3) or 4-, 7-, or 10-day-induced (G4, G7, and G10, respectively) 32D cl3 cells were subjected to Northern blotting and probed sequentially for these two mRNAs. The position of 18S rRNA is indicated for each blot.

(Fig. 4). A 1.3-kb murine NE mRNA was observed in induced, but not uninduced, 32D cl3 cells. The human NE mRNA is also 1.3 kb (49). The expression of murine NE mRNA decreases as 32D cl3 cells mature from the promyelocyte stage (day 4) to the neutrophil stage (day 10), as does the expression of the two β 5-tubulin mRNAs.

Finally, significant homologies in the human and murine NE 5'-flanking regions were also noted (Fig. 5). Within the first 100 bp, both the human and murine genes contain TATAA homologies, as well as potential binding sites for members of the Ets and C/EBP families and also for c-Myb. The murine NE gene also contains an element, 5'-GGCCACA-3', which differs from the PEBP2/CBF consensus, 5'-PuACCPuCA-3', only by an A-to-G transition at the second position. The element 5'-AGCCGCA-3', present in the enhancer core of

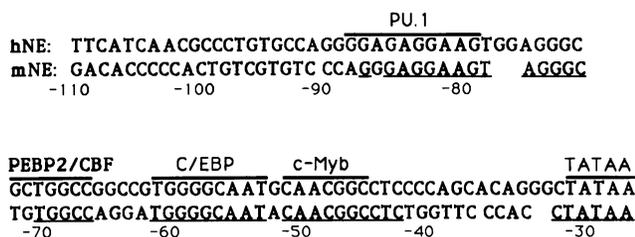


FIG. 5. Comparison of the murine NE and human NE 5'-flanking regions. Both strands of the murine NE promoter were sequenced. The sequences of the promoters of murine NE (mNE) and human NE (hNE), upstream of the TATAA homologies, have been aligned. Identities between these segments are underlined in the murine sequence. Potential binding sites for PU.1, PEBP2/CBF, C/EBP, c-Myb, and TATAA are indicated as well. Note that each of these binding sites is conserved between murine NE and human NE, except for the PEBP2/CBF site, which is disrupted in the human NE promoter.

simian immunodeficiency virus, contains the same A-to-G transition and has been shown to bind PEBP2/CBF (56).

The murine NE promoter contains several functional elements. A DNA fragment containing 1,800 bp of murine NE 5'-flanking sequences and the entire 5'-untranslated region was linked upstream of the luciferase cDNA to create pNELUC. In initial experiments we found that deletion of pNELUC to position -1,000, -180, or -103 was of minimal functional consequence (not shown). Clustered point mutations were therefore introduced at six locations between positions -107 and -42 in pNELUC. The activities of pNELUC and of a 5'-deletion series of pNELUC with endpoints -103, -91, -79, -66, -55, and -42 were assessed in induced 32D cl3 cells by transient transfection (Fig. 6a). The activities of the six clustered point mutations in uninduced and induced 32D cl3 cells were evaluated (Fig. 6b).

Deletion to position -91 was of minimal consequence, and mutation of bases -107 to -103 or -95 to -91 was of no consequence, in induced 32D cl3 cells. Additional deletion to position -79, which removed a potential PU.1-binding site, diminished NE promoter activity 10-fold (range, 9- to 23-fold), and mutation of this site, in pNE(m-85/-79)LUC, decreased activity 28-fold in induced 32D cl3 cells. Further deletion to position -66, which removed a potential PEBP2/CBF-binding site, reduced NE promoter activity fivefold (range, four- to sevenfold), and mutation of this site by altering bases -69 to -66 resulted in threefold-lower activity. To verify that introduction of an *Xho*I site at positions -69 to -66 had not inadvertently created a repressor-binding site, we used mung bean nuclease to remove the central 4 bp of the *Xho*I site from pNE(m-69/-66)LUC. The activity of this plasmid was also threefold less than that of pNELUC in induced 32D cl3 cells, in each of three repetitions.

Deletion to position -55 removed a potential C/EBP-binding element and decreased NE promoter activity an additional sevenfold, although this finding, and the result of further deletion to position -42, is difficult to evaluate because the resulting luciferase activities approached background levels. Disruption of the C/EBP-binding site with clustered mutations at positions -61 to -55 decreased NE promoter activity more than 30-fold, and disruption of the c-Myb site, in pNE(-47/-42)LUC, decreased activity 5-fold. Thus, the conserved segment of the murine NE 5'-flanking region contains four functional elements.

Mutation of the PEBP2/CBF site increased pNELUC activity in L cells (one-, three-, or fivefold in three experiments). Mutation of the murine MPO PEBP2/CBF site had no effect on murine MPO proximal enhancer activity in L cells, and MyNF1s were not detected in L-cell nuclear extracts (47).

Of note, pNELUC was 7-fold more active in induced, compared with uninduced, 32D cl3 cells, pNE(m-107/-103)LUC was 11-fold induced, and pNE(m-95/-91)LUC was 5-fold induced. However, the activity of pNE(m-61/-55)LUC, which lacks a C/EBP site, was not induced by G-CSF. The activities of pNE(m-85/-79)LUC, pNE(m-69/-66)LUC, and pNE(m-47/-42)LUC increased two- to threefold. These data suggest that integrity of the C/EBP site is critical for pNELUC induction and that integrity of the PU.1, PEBP2/CBF, and c-Myb DNA elements is required for optimal induction as well. Perhaps these four factors form a complex on the promoter which stimulates binding of the basal transcription machinery at the TATAA box. This model may account for our observation that the contribution of each DNA element to overall promoter function varied somewhat between experiments, increasing the standard errors for the activities of some of the constructs.

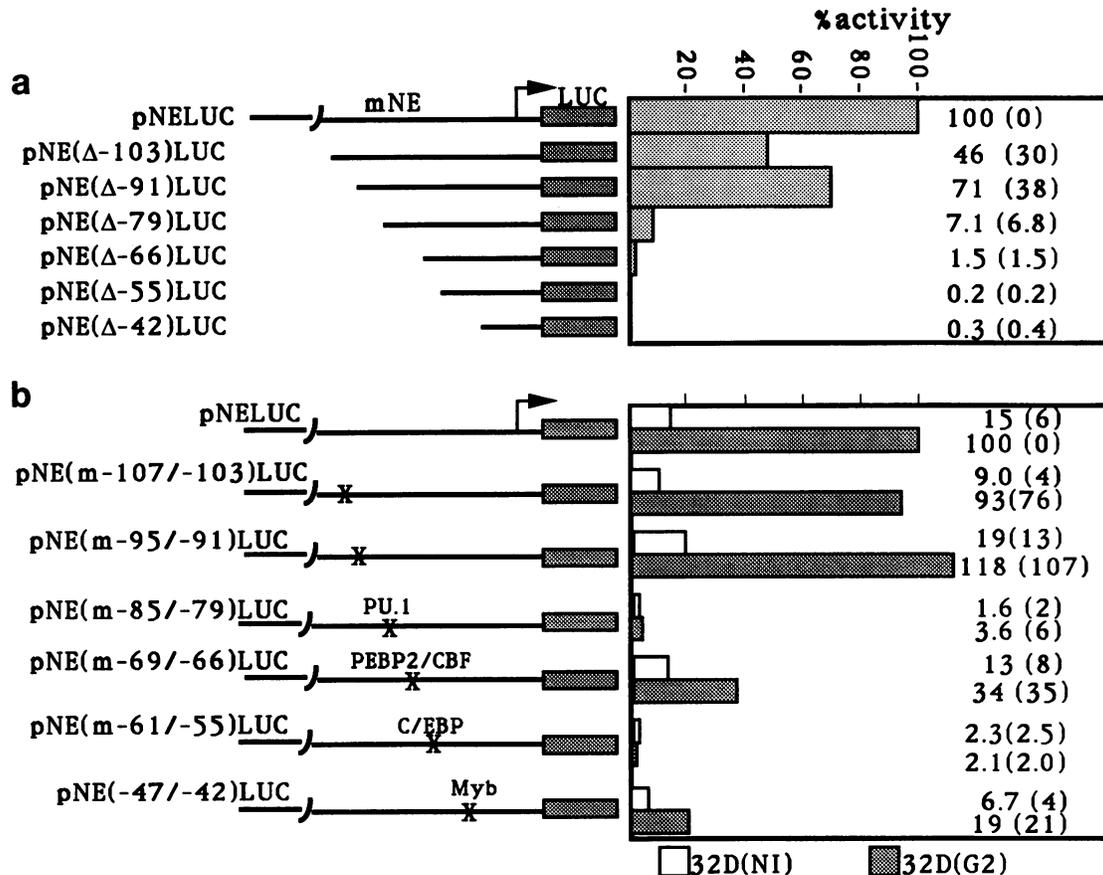


FIG. 6. Activity of murine NE (mNE) 5'-deletion and clustered point mutation constructs in 32D c3 cells. (a) Ten micrograms of each diagrammed plasmid, containing 5' murine NE promoter deletions, was cotransfected with 0.5 μ g of pMSVCAT into 32D c3 cells, which were then cultured in the presence of G-CSF. Cell extracts were assayed for luciferase and CAT activities 2 days later. The ratio of luciferase to CAT activities was set at 100% for pNELUC in each experiment. The average activity of each construct, in five experiments, is shown. Each average is also shown numerically above each bar, along with the standard error in parentheses. (b) Fifteen micrograms of each diagrammed plasmid, containing clustered point mutations introduced into the murine NE promoter segment in pNELUC, was cotransfected with 0.5 μ g of pMSVCAT into 32D c3 cells. These cultures were then split between interleukin 3- and G-CSF-containing media. Two days later, extracts were assayed for luciferase and CAT activities. The activity of pNELUC in induced 32D c3 cells was set at 100% in each experiment. The average activity of each construct, in at least four experiments, is shown.

The murine NE promoter element at positions -72 to -66 binds PEBP2/CBF. An oligonucleotide corresponding to bp -81 to -57 of the murine NE promoter was prepared. This oligonucleotide contains a centrally located, near-consensus PEBP2/CBF site and includes a portion, but not all, of the adjacent PU.1 and C/EBP sites. Similar oligonucleotides, containing either a disrupted PEBP2/CBF element or a consensus PEBP2/CBF site, were also prepared. These oligonucleotides and a murine MPO-derived oligonucleotide containing a consensus PEBP2/CBF element were labelled to similar specific activities and used in an EMSA with nuclear extracts from 4-day-induced 32D c3 cells (Fig. 7a). The murine NE oligonucleotide, but not the variant with a disrupted PEBP2/CBF site, weakly bound both MyNF1 α and MyNF1 β , and no other bands were evident. An additional oligonucleotide in which the murine NE PEBP2/CBF site was disrupted by introduction of an *Xho*I site, as was done for the functional studies, also did not bind the MyNF1s (not shown). Conversion of the near-consensus PEBP2/CBF site to a consensus site, in the context of the murine NE oligonucleotide, increased binding of these factors to a level similar to that obtained with

the murine MPO oligonucleotide. Flanking sequences appear not to influence the affinity of the MyNF1s for the 7-bp PEBP2/CBF site. To confirm these differences in affinity, an EMSA competition experiment was performed (Fig. 7b). The ability of a 10- or 50-fold excess of each unlabelled oligonucleotide to compete for MyNF1 binding to 1 ng of radiolabelled murine NE oligonucleotide was determined. A 10-fold excess of each of the three oligonucleotides with DNA elements which fit, or nearly fit, the PEBP2/CBF consensus prevented binding almost completely, whereas even a 50-fold excess of the mutant murine NE oligonucleotide only perturbed binding mildly.

The wild-type murine NE and MPO oligonucleotides were employed in a supershift assay in the same experiment (Fig. 7c). Binding of MyNF1 β to either oligonucleotide was disrupted or shifted completely, whereas binding of MyNF1 α was disrupted or shifted only partially, by inclusion of anti-PEBP2/CBF α subunit antiserum. A doublet containing these supershifted species was noted near the top of the gel. Finally, the specific peptide again prevented interaction between the antiserum and the MyNF1s.

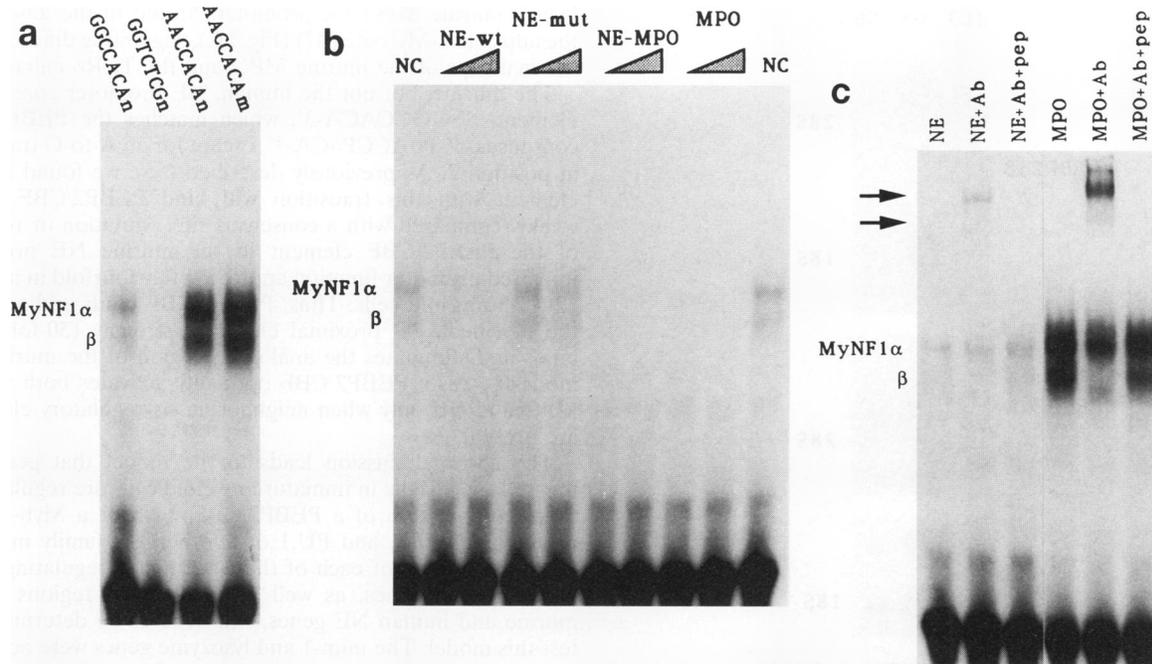


FIG. 7. PEBP2/CBF binds the murine NE promoter. (a) Three murine NE-derived, 33-bp oligonucleotides were radiolabelled to similar specific activities and used in an EMSA with 6 μ g of 4-day-induced 32D cl3 cell DFP nuclear extracts. The centrally located MyNF1-binding site either was wild type (GGCCACAn), was mutant (GGTCTCGn), or matched the MyNF1-binding site in the murine MPO gene (AACCACAn). The n in each of these designations indicates that the surrounding base pairs derive from the murine NE gene. A fourth oligonucleotide, containing the murine MPO MyNF1-binding site and its neighboring sequences (AACCACAm), was assayed similarly. The positions of MyNF1 α and MyNF1 β are indicated. (b) The wild-type murine NE oligonucleotide was radiolabelled and incubated with similar extracts either in the absence of competitor (NC) or in the presence of either a 10- or 50-fold excess of each of the four unlabelled oligonucleotides described for panel a, here designated NE-wt, NE-mut, NE-MPO, or MPO, respectively. (c) The wild-type murine NE or MPO oligonucleotides were radiolabelled, incubated with 6 μ g of 4-day-induced 32D cl3 cell DFP nuclear extracts, and analyzed by EMSA (NE and MPO). The ability of an anti-PEBP2/CBF α subunit antiserum to disrupt or supershift the MyNF1 species was assessed by adding 1 μ l of antiserum to the nuclear extracts prior to probe addition (NE+Ab or MPO+Ab). The specificity of the antiserum-MyNF1 interaction was assessed by addition of 4 μ g of the specific peptide to the nuclear extracts prior to antiserum addition (NE+Ab+pep or MPO+Ab+pep). The positions of supershifted species are indicated by arrows.

32D cl3 cells contain both PEBP2 α B and PEBP2 α A mRNAs. As discussed, the antiserum employed for the supershift assay would likely recognize both PEBP2 α B and PEBP2 α A. We and others are attempting to obtain specific, high-affinity antisera that will recognize these polypeptides. To determine whether 32D cl3 cells contain mRNAs encoding one or both PEBP2 α , we prepared poly(A)⁺ mRNAs from uninduced, 3-day-induced, and 6-day-induced 32D cl3 cells and subjected these mRNAs to Northern blotting (Fig. 8). PEBP2 α B mRNAs of 6.2 and 2.1 kb were detected, as were 7.4- and 6.3-kb PEBP2 α A mRNAs. These mRNAs correspond in size to analogous mRNAs detected in other cell types (4, 36).

DISCUSSION

In a report of an initial investigation of the transcriptional regulation of the murine MPO gene, we described a 414-bp proximal enhancer (47). This enhancer contains several positively acting functional DNA elements. Mutation of one element, 5'-AACCACA-3' was found to markedly diminish enhancer function, and the element was shown to bind a set of myeloid-restricted factors, MyNF1 α and MyNF1 β . This DNA element is also present at the analogous location in the human MPO gene. Optimum function of the factor interacting with this element depended on the presence of neighboring DNA-binding elements (47). We have now demonstrated that the

MyNF1s belong to the PEBP2/CBF family of transcription factors. Mutation of this element to a site which still fit the PEBP2/CBF consensus did not interfere with binding in vitro or function in vivo, whereas mutation to a site which disrupted the PEBP2/CBF consensus, but still fit the consensus for other known viral enhancer core-binding proteins, abolished binding in vitro and function in vivo. In vitro-translated PEBP2/CBF DNA-binding domain bound this element. Also, PEBP2/CBF α subunit-specific antiserum supershifted the MyNF1 complexes. Thus, the MPO gene is the first identified myeloid gene regulated by PEBP2/CBF.

To begin to determine whether PEBP2/CBF plays a general role in regulating early myeloid maturation, we examined the transcriptional regulation of the murine NE gene. The human MPO and NE mRNAs are coordinately expressed in human marrow (14). Striking conservation was noted within the proximal 100 bp of the human and murine NE 5'-flanking regions. Four DNA elements functional in induced 32D cl3 cells were described. One of the elements is predicted to bind a member of the Ets family. The Ets family member PU.1 has been shown to regulate the CD11b and macrophage colony-stimulating factor receptor genes in monocytes (37, 63), and it may regulate the CD18 and CD11a genes as well (6, 44). The murine NE PU.1 site fits the consensus sequence 5'-RRR GAGGAAG-3' noted to be present in several genes expressed in myeloid cells (44), although the active CD11b and macro-

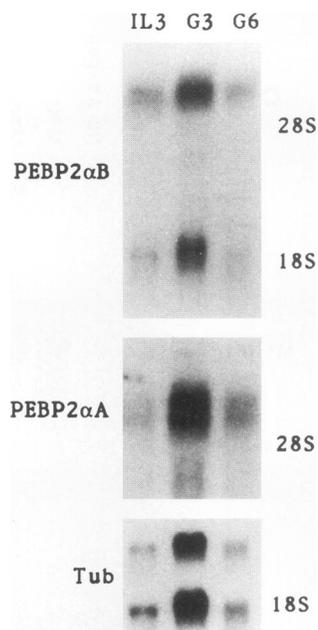


FIG. 8. Northern blot analysis of PEBP2 α B, PEBP2 α A, and tubulin (Tub) expression in differentiating 32D cl3 cells. Five micrograms of poly(A)⁺ RNA prepared from uninduced (IL3) or 3- or 6-day-induced (G3 and G6, respectively) 32D cl3 cells was subjected to Northern blotting and probed sequentially for each of these mRNAs. Note that each probe detected two isoforms of the corresponding mRNAs. The positions of 28S and 18S rRNAs are indicated.

phage colony-stimulating factor receptor PU.1 sites differ from this consensus (37, 63). The murine MPO proximal enhancer contains this element as well, although its function has not been evaluated by point mutation. The Ets family member Ets-1 was recently shown to cooperatively bind DNA with PEBP2/CBF, and there are functional Ets and PEBP2/CBF sites in several viral enhancers (reference 61 and references therein).

The functional murine NE C/EBP site fits the consensus 5'-T(T/G)NNGAA(T/G)-3' (1). Interestingly, conservation between the human and murine NE C/EBP sites extends precisely over this 9-bp site. Of note, this site differs from the CCAAT site known to bind ubiquitously expressed factors (7). A C/EBP has been shown to cooperate with c-Myb to activate the *mim-1* and lysozyme genes in immature avian myeloid cells (33), and a C/EBP activates the *G-CSF* gene in monocytes as well (31). Interestingly, integrity of the C/EBP site was most critical for increased pNELUC activity during 32D cl3 cell differentiation, and the levels of C/EBP α , C/EBP β , and C/EBP γ increase during early 32D cl3 granulopoiesis (43).

Integrity of the segment 5'-CAACGG-3', which is present in both the human and murine NE promoters and fits the c-Myb consensus, 5'-(T/C)AAC(G/T)G-3' (5), is required for optimal murine NE promoter function as well. The murine MPO gene has an element 5'-CAACTG-3' just upstream of the PEBP2/CBF-binding site, and mutation of this element decreased murine MPO proximal enhancer function fivefold (47). The corresponding segment of the human MPO gene has the sequence 5'-TAACTG-3', which also fits the Myb consensus. Integrity of adjacent c-Myb- and PEBP2/CBF-binding sites is critical for activity of the TCR δ enhancer (16). Interestingly, the murine MPO PEBP2/CBF site was able to stimulate a

hybrid murine MPO-TK promoter 20-fold in the absence of the adjacent c-Myb site (47) (Fig. 2C), suggesting differences in the makeups of the murine MPO and the TCR δ enhancers.

The murine, but not the human, NE promoter contains an element, 5'-GGCCACA-3', which matches the PEBP2/CBF consensus, 5'-PuACCPuCA-3', except for an A-to-G transition at position 2. As previously described (56), we found that an element with this transition will bind PEBP2/CBF, albeit weakly compared with a consensus site. Mutation or removal of the PEBP2/CBF element in the murine NE promoter impaired promoter function approximately fourfold in induced 32D cl3 myeloid cells. Thus, PEBP2/CBF binds and activates the murine MPO proximal enhancer strongly (30-fold) and binds and stimulates the analogous region of the murine NE modestly. Also, PEBP2/CBF optimally activates both murine MPO and NE only when neighboring *cis*-regulatory elements are present.

The above discussion leads to the model that genes expressed specifically in immature myeloid cells are regulated by some combination of a PEBP2/CBF, Myb or a Myb-related protein, a C/EBP, and PU.1 or another Ets family member. The contribution of each of these factors to regulating other early myeloid genes, as well as more distal regions of the murine and human NE genes, will need to be determined to test this model. The *mim-1* and lysozyme genes were activated in nonmyeloid cells transfected with Myb and C/EBP expression vectors (33), and the macrophage colony-stimulating factor receptor was activated by a PU.1 expression vector (63) in nonmyeloid cells as well. We cotransfected pMPOHTKLUC and pNELUC with expression vectors for PEBP2 α B and PEBP2 β /CBF β in 32D cl3 cells and in L cells. No consistent *trans*-activation was found in either cell type. Presumably, L cells require expression of additional myeloid activators to allow MPO or NE enhancer function, and 32D cl3 cells already have abundant endogenous PEBP2/CBFs, preventing additional *trans*-activation. Experiments are in progress to identify the additional factors which cooperate with PEBP2/CBFs to regulate the murine MPO and NE proximal regulatory regions. Perhaps we will then be able to identify a combination of these regulators which is sufficient to activate both the MPO and NE genes in nonmyeloid cells and which allows cooperative binding to enhancer fragments *in vitro*. Of note, both PEBP2 α A and PEBP2 α B, the α subunits of two PEBP2/CBF family members, were recently shown to *trans*-activate a T-cell receptor enhancer in two heterologous cell types containing reduced levels of endogenous PEBP2/CBFs (3, 26a).

The distribution of PEBP2/CBF proteins in tissue has not been established. PEBP2/CBF gel shift activities were detected in extracts from myeloid and lymphoid cells but not in liver, kidney, lung, or heart extracts (50, 56), although multiple cell lines contain these activities (26, 47). We demonstrated that 32D cl3 cells contain mRNAs corresponding to two PEBP2/CBF α subunit family members, PEBP2 α A and PEBP2 α B. Development of additional antiserum reagents will be necessary to determine whether the two MyNF1 gel shift bands contain one or both of these PEBP2/CBFs.

Finally, it is provocative that PEBP2/CBF regulates the murine MPO and NE genes and that PEBP2/CBF subunit genes are involved in translocations associated with myeloid leukemias. A portion of the human PEBP2 α B gene, the AML1 gene, is fused to the ETO gene by t(8;21), which is associated with FAB M2 acute myeloid leukemia (12, 29). The marrows of patients with this form of leukemia contain leukemic myeloblasts, as well as more differentiated, MPO-positive, leukemic cells. Thus, if AML1-ETO blocks the differentiation of some leukemic myeloblasts, this effect must be leaky. The

PEBP2 β /CBF β gene, encoding the common β subunit of PEBP2A and PEBP2B, is fused to the tail, coiled-coil region of a smooth muscle myosin gene by inv(16), which is associated with FAB M4eo cases of acute myeloid leukemia (24). The marrows of these patients contain leukemic myeloblasts and monoblasts, as well as leukemic eosinophils. It has been proposed that the CBF β -MYH11 fusion protein produced by inv(16) acts in a dominant-negative fashion with respect to endogenous PEBP2/CBFs (24). The CBF β portion could bind endogenous α subunits, and the myosin portion could then induce the formation of an inactive tetramer. Lack of PEBP2/CBF functions might then prevent differentiation beyond the blast stage, much as interference of RAR α function by the PML-RAR α oncogene, associated with FAB M3 cases of acute myeloid leukemia, is thought to prevent differentiation of myeloid cells beyond the promyelocyte stage of differentiation (52). It will be of interest to determine the effect of AML1-ETO and CBF β -MYH11 on the endogenous MPO and NE genes and on the MPO and NE gene reporter constructs we have characterized. Since arrested differentiation might not be sufficient to transform a normal myeloblast into a leukemic cell, these oncogenes, and also PEBP2/CBF, might also affect the expression of genes which regulate the proliferation of myeloblasts.

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ADDENDUM IN PROOF

The MyNF1s also supershifted with a CBF β antiserum provided by N. Speck.

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