Myeloblastin is a granulocyte colony-stimulating factor-responsive gene conferring factor-independent growth to hematopoietic cells

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Hematopoiesis depends on a pool of quiescent hematopoietic stem/progenitor cells. When exposed to specific cytokines, a portion of these cells enters the cell cycle to generate an amplified progeny. Myeloblastin (MBN) initially was described as involved in proliferation of human leukemia cells. The granulocyte colony-stimulating factor (G-CSF), which stimulates the proliferation of granulocytic precursors, up-regulates MBN expression. Here we show that constitutive overexpression of MBN confers factor-independent growth to murine bone marrow-derived Ba/F3/G-CSFR cells. Our results point to MBN as a G-CSF responsive gene critical to factor-independent growth and indicate that expression of the G-CSF receptor is a prerequisite to this process. A 91-bp MBN promoter region containing PU.1, C/EBP, and c-Myb binding sites is responsive to G-CSF treatment. Although PU.1, C/EBP, and c-Myb transcription factors all were critical for expression of MBN, its up-regulation by G-CSF was associated mainly with PU.1. These findings suggest that MBN is an important target of PU.1 and a key protease for factor-independent growth of hematopoietic cells.

Myeloblastin (MBN), also called proteinase 3, is a myeloid-specific serine protease involved in the control of growth and differentiation of cells derived from the human leukemia cell line HL-60 (1). Down-regulation of MBN expression by antisense oligodeoxynucleotides inhibited proliferation of promyelocytic-like leukemia cells (1). MBN is overexpressed in myeloid leukemia cells (2) and in colony-forming unit–granulocyte/monocyte of patients with chronic myelogenous leukemia (3).

Hematopoiesis is maintained by a limited subset of quiescent hematopoietic stem cells and progenitors. When exposed to specific cytokines, these cells can enter a limited number of cell cycles and divide to generate an amplified progeny (4). The mechanisms underlying these processes remain largely unknown. Granulocyte colony-stimulating factor (G-CSF) is a myeloid-specific cytokine stimulating survival, proliferation, and maturation of granulocytic progenitors (5). The biologic activities of G-CSF are mediated by the G-CSF receptor (G-CSFR) on the surface of responsive cells (6). The G-CSFR is expressed on myeloid progenitor cells, myeloid leukemia cells, mature neutrophils, monocytes, platelets, and some T and B lymphoid cell lines (7). G-CSFR expression is up-regulated in immature myeloid cells in response to G-CSF (8) and in acute promyelocytic leukemia cells induced to differentiation with all-trans retinoic acid (9). Targeted mutation of the G-CSF or its receptor resulted in reduced numbers of hematopoietic progenitors and circulating granulocytes in mice (10, 11).

Despite a pivotal role assigned to G-CSF in growth control of granulocytic progenitors, few G-CSF-responsive genes are known to trigger or facilitate proliferation. Such genes include junB (12) and DNA topoisomerase II (13), which are induced by G-CSF and have been described in myeloid leukemia cells. Promoter regions of genes expressed specifically in immature myeloid cells are regulated by at least four transcription factors: PEPB2/CBF, C/EBP, PU.1, and c-Myb (14). PU.1, which is encoded by the protooncogene *Spi-1* (15), is required for the development of both myeloid and lymphoid lineages (16, 17). In myeloid progenitors, PU.1 is required for proliferation/survival and controls responsiveness to myeloid-specific cytokines (17).

We have investigated the regulation of MBN in normal hematopoietic progenitors induced to differentiate toward the myeloid lineage. Here we demonstrate that MBN expression is up-regulated by G-CSF and that its constitutive overexpression is sufficient to confer factor-independent growth to bone marrow-derived Ba/F3 cells expressing the G-CSFR. Our results show that the proximal part of the MBN promoter, which harbors binding sites for the PU.1, C/EBP, and c-Myb, is responsive to G-CSF. Furthermore, we found that up-regulation of MBN by G-CSF is associated mainly with PU.1.

Materials and Methods

Human-Cell Preparation, Liquid Culture, and Hematopoietic Progenitor-Cell Assays. Bone marrow cells were obtained with informed consent from healthy donors. Low-density mononucleated cells were separated on a Ficoll–Isopaque density gradient (1.077 g/ml; Seromed, Munich), washed, and resuspended at a concentration of 1–5 $\times$ 10^7 cells per ml in RPMI medium 1640 (GIBCO). $CD34^+$ cells were selected by using superparamagnetic microbead selection and minimacs columns (Miltenyi Biotec, Auburn, CA), and the mean $CD34^+$ cell purity was 92 $\pm$ 2%. $CD34^+$ cells (5×10^4 per ml) were suspended in 6-well plates (Falcon) containing serum-free long-term culture medium and IL-3 (0.1 ng/ml; Sandoz Pharmaceutical), granulocyte–macrophage colony-stimulating factor (GM-CSF) (0.1 ng/ml), and G-CSF (50 ng/ml; Shugai Rhone Poulenc) as described (18). Cultures were incubated at 37°C in a 100% humidified, 5% CO_2 atmosphere for 10 days. Every 3 days, an equal volume of fresh long-term culture medium and cytokines was added to each well. Cells were collected every 3 days, washed in Iscove-Modified Dulbecco's Medium (IMDM, Seromed), counted by trypan blue exclusion, and assayed for burst-forming unit-erythroid cells and colony-forming unit–granulocyte/monocyte as described (19). Cell cultures were conducted with 20 ng/ml G-CSF and GM-CSF, 10 ng/ml IL-3, 3 units/ml erythropoietin, and 50 ng/ml stem cell factor (Immunex). Burst-forming unit-erythroid cells and colony-forming unit–granulocyte/monocyte were scored on day 14 of culture. Differentiation was assessed by cell morphology as examined under light microscopy on cytospin slides stained with May–Grünwald–Giemsa.

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Abbreviations: G-CSF, granulocyte colony-stimulating factor; MBN, myeloblastin; G-CSFR, G-CSF receptor.

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Leukemia Cell Lines and Culture Conditions. The mouse IL-3-dependent Ba/F3 cell line (20), which constitutively expresses G-CSFR (Ba/F3/G-CSFR cells), was kindly provided by K. Welte, Medizinische Hochschule Hannover, Germany. This cell line was cultured in RPMI medium 1640 supplemented with 10% FBS, 2 mM L-glutamine, and 0.1 ng/ml recombinant mouse IL-3 (R & D Systems) or 1 ng/ml G-CSF (Amgen). Promyelocytic HL-60 cell line was obtained from the American Type Culture Collection and was cultured in RPMI medium 1640 supplemented with 10% FBS and 2 mM L-glutamine (GIBCO). Cell viability was estimated by using standard trypan blue dye-exclusion assay.

Transformation Assay. Exponentially growing Ba/F3/G-CSFR cells were washed twice in serum-free medium, once in Opti-Mem medium (GIBCO), resuspended in the same medium at 5×10^6 cells per 0.5 ml, and electroporated (Gene Pulser, Bio-Rad) at 300 V, 960 μF in the presence of the plasmids. Transfected Ba/F3/G-CSFR cells were maintained in IL-3-containing medium for 48 hr and then selected with 100 μg/ml zeocin (Invitrogen). Zeocin-resistant populations were washed twice in serum-free medium before plating in medium lacking IL-3 to select for IL-3-independent growth. Immunoblotting were performed as described (1).

RNA Isolation and Northern Blot Analysis. Total RNA extracted by using an RNeasy Mini Kit (Qiagen, Chatsworth, CA) was electrophoresed and transferred onto Hybond-N nylon filters (Amersham Pharmacia). Hybridization and washing conditions were as described (21). The MBN probe was the full-length cDNA (22). The 36B4 probe was an internal *Pst*I fragment of the 36B4 cDNA, corresponding to a ubiquitously expressed gene (23). Probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by using a random primer labeling kit (Amersham Pharmacia).

Recombinant Plasmids. The human MBN cDNA was derived from HL-60 cells by using reverse transcriptase–PCR with Moloney murine leukemia virus reverse transcriptase (Superscript II, GIBCO) and oligo(dT)$_{12–18}$ (Sigma) as recommended by the manufacturer. The sense primer was 5′-CGAAGCTTGCCACCATGGCTCACCG-3′, and the antisense was 5′-ATGCGGCCGCTTCAGGGGCGGCCCT-3′. Denaturation was conducted for 5 min at 95°C followed by 40 PCR cycles (94°C for 30 sec, 62°C for 20 sec, and 72°C for 45 sec), and extension for 10 min at 72°C by using *Taq* polymerase (Perkin–Elmer). The human MBN cDNA was subcloned into the pcDNA3.1/Zeo expression vector (Invitrogen) generating the pcDNA3.1/Zeo/hMBN. Construction of the pcDNA3.1/Zeo/hMBNS203A vector was achieved by using the QuickChange site-directed mutagenesis kit (Stratagene). For this we used a mutated oligonucleotide sequence as indicated in boldface: 5′-GCTTCGGAGAC**TGC**G-GTGGCCCCCTG-3′. pcDNA3.1/Zeo/hMBN and pcDNA3.1/Zeo/hMBNS203A vectors were verified by DNA sequencing. Expression vectors for c-Myb (CMV-c-Myb) and for PU.1 (pECE-PU.1) (17) were obtained from B. Lüscher, Medizinische Hochschule Hannover, Germany (24) and R. Maki, The Burnham Institute, La Jolla, CA (15), respectively.

A 658-bp MBN promoter region was subcloned into *Bam*HI–*Xho*I sites of the pBLCAT6 vector (25) generating the pMBN-658 reporter plasmid. A 91-bp promoter fragment was generated by using PCR (5′ and 3′ primers were: 5′-ATGGATCCAAGGCAAAAGGAGGAAGT-3′ and 5′-ATCTCGAGGATATCGAATTCCTGCAG-3′, respectively) and cloned into *Bam*HI–*Xho*I sites of the pBLCAT6 vector to generate the pMBN-91 reporter plasmid. For constructing the pMBN-658PU.1 mutant, pMBN-658C/EBP mutant, and pMBN-658Myb mutant, mutagenesis of the PU.1, c/EBP, and c-Myb binding sites were achieved by using the QuickChange site-directed mutagenesis kit and the following oligonucleotide sequences (mutated base pair are in boldface): PU.1 site, 5′-CAAGGCAAAAGGA**TT**AAGTGGGGACCCAG-3′; C/EBP site, 5′-CCAGCCTGGGC**G**T**A**GT**G**G**G**ACTCAACGGCC-3′; c-Myb, 5′-GGGCAACTCA**TG**GGCCTCTGGC-3′. Promoter constructs were verified by DNA sequencing.

***In Vivo* Expression Assays.** Exponentially growing Ba/F3/G-CSFR cells were washed twice in serum-free medium, once in Opti-Mem medium, resuspended in the same medium at 5×10^6 cells per 0.5 ml, and electroporated (Gene Pulser, Bio-Rad) at 300 V, 960 μF with 20 μg of reporter plasmids. Transfected cells then were cultured in 10 ml of medium for 40 hr. Plasmid (5 μg) containing the luciferase cDNA driven by the cytomegalovirus promoter was included as internal control for transfection efficiency. All transfections were performed at least in triplicate with independent template preparations. Assays to measure chloramphenicol acetylase (26) and luciferase (27) activities were as described.

Protein Extracts and Electrophoretic Mobility-Shift Assays. Ba/F3/G-CSFR nuclear extracts were prepared essentially as described (28). Leupeptin, pepstatin, aprotinin, antipain, and chymostatin (2.5 ng/ml each; Sigma) were added in lysis and extraction buffers. Electrophoretic mobility-shift assays were carried out by using the following oligonucleotides: PU.1, 5′-AGCTTAGGCAAAAGGAGGAAGTGGGGACG-3′; mut PU.1, 5′-AGCTTAGGCAAAAGGATTAAGTGGGGACG-3′; C/EBP, 5′-AGCTTGGGCATTGGGCAACTCG-3′; mut C/EBP, 5′-AGCTTGGGCGTAGTGGGACTCG-3′; Myb, 5′-ATGGATCCGCAACTCAACGGCCTCTGGCAT-3′; mut Myb, 5′-ATGGATCCGCAACTCATGGGCCTCTGGCAT-3′. ^{32}P 5′-end-labeled, synthetic double-stranded oligonucleotide (0.25 ng, 15,000 cpm) was incubated with 1 μg of Ba/F3/G-CSFR nuclear extracts in the presence of poly(dI–dC) in 10 mM Tris·HCl (pH 7.9), 50 mM KCl, 2 mM EDTA, 0.05% NP40, 0.3 mg/ml BSA. After 15 min at room temperature, DNA–protein complexes were separated on a 5% polyacrylamide gel (acrylamide/bisacrylamide ratio, 37.5:1) in TBE (25 mM Tris base/25 mM boric acid/0.5 mM EDTA) at 4°C. For retardation competition experiments, extracts were preincubated with poly(dI–dC) and unlabeled competitor oligonucleotides for 15 min before addition of the probe and further incubation.

Cell-Surface Expression of the G-CSFR. To assess G-CSFR levels, cells were incubated at room temperature for 60 min with 30 μg/ml biotinylated mouse anti-human G-CSFR mAb LMM741 (PharMingen), and then at 4°C for 60 min with 2 μg/ml phycoerythrin-conjugated streptavidin, with washing between each step. Similar procedures were used to assess the binding capacity of the G-CSFR except that (*i*) 1.5 μg/ml biotinylated G-CSF was used instead of the biotinylated anti-G-CSFR mAb and (*ii*) a 7-fold excess of nonbiotinylated G-CSF was used as a competitor. Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson).

Results

MBN Is a G-CSF-Responsive Gene Conferring Factor-Independent Growth to Bone-Marrow Derived Hematopoietic Cells. Purified bone marrow CD34$^+$ cells expressed low levels of the MBN mRNA (Fig. 1*Aa*, lane 1), which were gradually enhanced in hematopoietic stem/progenitor cells induced toward the myeloid lineage (Fig. 1*A b* and *c*) in the presence of G-CSF (Fig. 1*Aa*, lanes 2 and 3), with a maximum expression at day 6 as cells reached the promyelocytic stage (Fig. 1*Aa*, lane 3, and 1*Ac*). To investigate whether MBN could confer factor-independent growth to murine bone marrow-derived cells, we used Ba/F3/G-CSFR

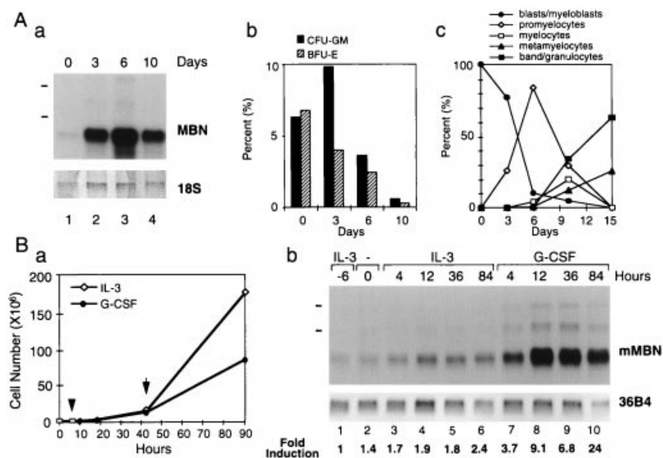

Fig. 1. MBN is a G-CSF-responsive gene. (*A*) Expression of MBN during growth and differentiation of CD34$^+$ cells toward the myeloid lineage. (*a*) Autoradiogram of MBN mRNA expression in CD34$^+$ cells cultured in the presence of G-CSF. Total RNA (1.8 μg) was loaded in each lane. Positions of the 28S and 18S rRNAs are indicated on the left as size markers; *Lower* is methylene blue-stained 18S rRNA on membrane after transfer as assessment of RNA quantities in each lane. Days of culture are as indicated. (*b*) *In vitro* colonies formation. Cells harvested at 0, 3, 6, and 10 days were seeded in semi-solid culture conditions and percent of colony-forming unit–granulocyte/monocyte and burst forming-unit–erythroid cells were assessed at day 12. (*c*) Morphological differentiation of CD34$^+$ toward the myeloid lineage. (*B*) Induction of MBN expression by G-CSF in Ba/F3/G-CSFR cells. (*a*) Growth of Ba/F3/G-CSFR cells with IL-3 or G-CSF. Arrowhead indicates addition of either IL-3 or G-CSF. Arrow indicates that cells were split at 1×10^6 cells per ml. Concentrations of IL-3 and G-CSF were maintained at 0.1 ng/ml and 1 ng/ml, respectively. (*b*) Autoradiogram of murine MBN (mMBN) mRNA expression in Ba/F3/G-CSFR cells cultured in IL-3- or G-CSF-containing medium. Ba/F3/G-CSFR cells maintained in IL-3-containing medium (lane 1) were washed with factor-free medium. After 6 h incubation in factor-free medium (lane 2), cells were cultured with either IL-3 (lanes 3–6) or G-CSF (lanes 7–10) for the indicated times. Total RNA (2 μg) was loaded in each lane. Positions of the 28S and 18S rRNA are indicated on the left as size markers. *Lower* is an autoradiogram of 36B4 mRNA expression as assessment of RNA quantities in each lane. Fold inductions were calculated by the ratio of mMBN expression to 36B4 expression.

cells whose continued growth depends on either IL-3 or G-CSF (Fig. 1*Ba*) and that do not differentiate in response to G-CSF (data not shown). In these cells, MBN mRNA was barely induced when cells, deprived of factors (Fig. 1*Bb*, lane 2) were re-exposed to IL-3 (Fig. 1*Bb*, lanes 1 and 3–6). In contrast, MBN mRNA expression was drastically induced after shifting cells to G-CSF-containing medium (Fig. 1*Bb*, lanes 7–10). Altogether, these results indicated that MBN is a G-CSF-responsive gene. We then assessed whether MBN overexpression could substitute for the requirement of G-CSF for continued growth of G-CSF-dependent Ba/F3/G-CSFR cells. For this, Ba/F3/G-CSFR cells were transfected with the pcDNA3.1/Zeo/hMBN or the pcDNA3.1/Zeo/hMBNS203A vectors expressing the wild-type human MBN and a mutant lacking the serine protease catalytic activity (29), respectively. In these experiments, the pcDNA3.1/Zeo vector was used as a control. Both the Zeo/hMBN- and Zeo/hMBNS203A-transfected cells had similar levels of MBN (Fig. 2*Aa*). In contrast to control (Zeo) cells and cells expressing the MBN mutant (Zeo/hMBNS203A), cells expressing the wild-type MBN (Zeo/hMBN) were able to grow continuously in a factor-independent manner (Fig. 2*Ab*). The fact that conditioned medium from two factor-independent Ba/F3/G-CSFR cell lines expressing the wild-type MBN did not support the proliferation of Ba/F3/G-CSFR cells in factor-free medium (Fig. 2*B*) indicated that neither IL-3 nor G-CSF were secreted

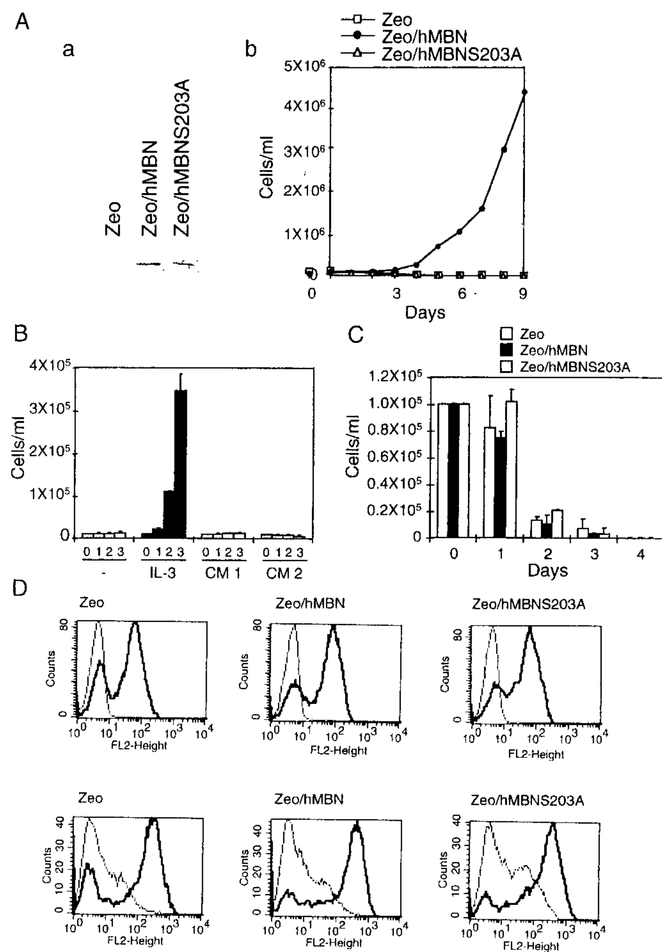

Fig. 2. MBN is critical to transformation to factor-independent growth; requirement of the G-CSFR. (*A*) Semiquantitative immunodetection of MBN in zeocin-resistant cells (*a*) and growth (*b*) of zeocin-resistant Ba/F3/G-CSFR cells after transfection of 5 μg of the pcDNA3.1/Zeo (Zeo), pcDNA3.1/Zeo/hMBN (Zeo/hMBN), or pcDNA3.1/Zeo/hMBNS203A (Zeo/hMBNS203A) in factor-free medium. In *b*, the experiment was repeated three times with similar results with independent zeocin-resistant Ba/F3/G-CSFR cells. (*B*) An autocrine loop is not involved in pcDNA3.1/Zeo/hMBN-transfected Ba/F3/G-CSFR cells expressing the wild-type MBN. Growth capacity of wild-type Ba/F3/G-CSFR cells cultured in conditioned medium from factor-independent Ba/F3/G-CSFR cell lines expressing MBN. Cells were washed three times in factor-free RPMI 1640 medium and 1×10^5 cells per ml were cultured in factor-free medium (−), IL-3 (0.1 ng/ml), or conditioned medium from two factor-independent Ba/F3/G-CSFR cell lines expressing the wild-type MBN (CM1 and CM2). Cell concentrations at days 0, 1, 2, and 3 are indicated. Error bars indicate SDs from the results of three independent experiments. (*C*) Requirement of G-CSFR. Growth of zeocin-resistant Ba/F3 cells after transfection of 5 μg of the pcDNA3.1/Zeo (Zeo), pcDNA3.1/Zeo/hMBN (Zeo/hMBN), or pcDNA3.1/Zeo/hMBNS203A (Zeo/hMBNS203A) in factor-free medium. Cell counts were obtained at days 0, 1, 2, 3, and 4. The experiment was repeated three times with two independent zeocin-resistant Ba/F3 cells giving similar results. Error bars indicate SDs from the results of three independent experiments. (*D*) MBN does not affect cell-surface expression and binding capacity of the G-CSFR in Ba/F3/G-CSFR cells. Flow cytometric analysis of G-CSFR expression (*Upper*) and binding capacity (*Lower*) on pools of Ba/F3/G-CSFR cells expressing the control (Zeo), wild-type (Zeo/hMBN), or mutant (Zeo/hMBNS203A) MBN. Cells were stained with either biotinylated mouse anti-human G-CSFR antibodies or biotinylated G-CSF. This step was followed by phycoerythrin-conjugated streptavidin (bold curve). Left part of bold curves represents negative cells. (*Upper*) Light curves are without the anti-G-CSFR step. (*Lower*) Light curves are with a 7-fold excess nonbiotinylated G-CSF competitor. All samples were analyzed for fluorescence (FL2-Height).

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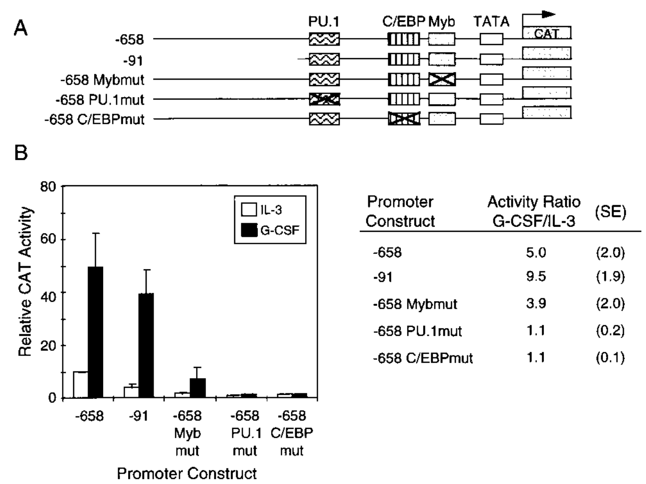
and that an autocrine loop was unlikely to be the cause of factor-independent growth.

Expression of G-CSFR Is Necessary for MBN to Render Ba/F3 Cells Factor-Independent. We examined whether MBN could confer IL-3-independent growth to Ba/F3 cells. As shown in Fig. 2*C*, factor-independent growth was not observed in these cells whether they expressed the wild-type (Zeo/hMBN) or mutant (Zeo/hMBNS203A) MBN. The fact that, in contrast to Ba/F3/G-CSFR cells, the parental Ba/F3 cells expressing MBN remained factor-dependent for growth indicated that expression of the G-CSFR was necessary for MBN to render Ba/F3 cells factor-independent. It should be noted that, although ≈50% of the Ba/F3/G-CSFR cells were alive after 2 days of culture without factor, the parental Ba/F3 cells had no survival capacity (data not shown). This suggested that expression of the G-CSFR was required for providing Ba/F3 cells with enough survival capacity in the absence of factor while MBN-driven transformation to factor-independent growth was taking place. Cell-surface expression and G-CSF binding capacity of the G-CSFR then were determined by using fluorescence-activated cell-sorter analysis. As shown in Fig. 2*D*, expression of the wild-type MBN (Zeo/hMBN) in Ba/F3/G-CSFR cells did not affect G-CSFR expression levels when compared with their control (Zeo)- and mutant (Zeo/hMBNS203A)-expressing counterparts (Fig. 2*D Upper*). Furthermore, under the same conditions, expression of MBN had no effect on the binding capacity of G-CSFR to its ligand (Fig. 2*D Lower*). Altogether these results indicated that MBN was sufficient to confer factor-independent growth to Ba/F3 cells expressing the G-CSFR.

Importance of PU.1, C/EBP, and c-Myb in MBN Regulation by G-CSF. We assessed critical regulatory sequences involved in G-CSF-induced expression of MBN. Both the human and mouse MBN promoters harbored conserved TATA box and binding sites for c-Myb, C/EBP, and PU.1. Constructs of the human MBN promoter (Fig. 3*A*) cloned into pBLCAT6 reporter plasmid were transiently transfected into Ba/F3/G-CSFR cells. We observed a 5-fold induction of the pMBN-658 reporter plasmid in G-CSF-treated cells (Fig. 3*B*). Deletion from position −685 to −91 was still responsive to G-CSF treatment, demonstrating that this part of the promoter retained responsive elements involved in the induction of MBN by G-CSF (Fig. 3*B*). Mutation of either the PU.1, C/EBP, or c-Myb site resulted in a drastic decrease of the promoter activity, indicating that all three sites were critical for the MBN promoter activity in Ba/F3/G-CSFR cells (Fig. 3*B*). Our data indicate that the proximal part of the MBN promoter is responsive to G-CSF and that G-CSF-induced MBN expression depended on the integrity of each PU.1, C/EBP, and c-Myb binding sites.

To investigate the mechanisms by which G-CSF induces MBN expression, EMSA were carried out with $CD34^{+}$ cells differentiating through the granulocytic pathway by using the PU.1 probe. G-CSF-induced differentiation of $CD34^{+}$ cells was accompanied by a progressive increased binding of PU.1 to its probe (Fig. 4*Aa*), which specificity was attested by competition and supershift assays (data not shown). A specific increase also was observed in G-CSF-treated Ba/F3/G-CSFR cells (Fig. 4*Ab*, lanes 3–6 versus lanes 1 and 2). The PU.1 mRNA was increased 2-fold in Ba/F3/G-CSFR cells transferred from IL-3 to G-CSF (Fig. 4*B a* and *b*). No difference was observed in C/EBP binding (Fig. 4*Ac*) and in c-Myb mRNA expression (Fig. 4*B a* and *b*) on G-CSF-treatment. Overexpression of PU.1 in IL-3-treated Ba/F3/G-CSFR cells was sufficient to activate the MBN promoter to a level comparable to that obtained when the cells were cultured with G-CSF (Fig. 4*Ca*). This activation depended on the integrity of the PU.1 binding site (Fig. 4*Cb*). MBN promoter activity was not induced in Ba/F3/G-CSFR transfected with the



Promoter Construct	Activity Ratio G-CSF/IL-3	(SE)
-658	5.0	(2.0)
-91	9.5	(1.9)
-658 Mybmut	3.9	(2.0)
-658 PU.1mut	1.1	(0.2)
-658 C/EBPmut	1.1	(0.1)

Fig. 3. The human MBN proximal promoter region harbors PU.1, C/EBP, and c-Myb binding sites critical for G-CSF-induced MBN expression in Ba/F3/G-CSFR cells. (*A*) Wild-type (−658), 5′-deletion mutant (−91), and point mutation constructs (PU.1 mut, C/EBP mut, and Myb mut) of the MBN promoter were fused to the chloramphenicol acetyltransferase (CAT) gene. (*B*) MBN promoter activity in Ba/F3/G-CSFR cells exposed to G-CSF or IL-3. Indicated MBN promoter constructs (20 μg each) were transfected into Ba/F3/G-CSFR cells, which then were cultured in IL-3- or G-CSF-containing medium. Transfection efficiencies were normalized by cotransfection of a cytomegalovirus–luciferase vector. Cells were harvested 40 h posttransfection, and reporter activity was measured. Open and filled bars represent IL-3- and G-CSF-treated cells, respectively. Error bars indicate the SD from the results of at least three independent experiments. Adjacent Table represents calculated G-CSF/IL-3 activity ratios as well as standard errors (SE) for each construct. CAT activities presented for each MBN promoter construct were calculated relative to the −658 in IL-3 culture conditions.

empty vector (Fig. 4*Cb*). Altogether, these results suggest that, among the other factors, PU.1 played a major role in inducing MBN expression in hematopoietic cells treated with G-CSF.

Discussion

Hematopoiesis strictly depends on cytokines and growth factors for survival and limited growth of hematopoietic progenitor cells, which then can continue toward a specific hematopoietic lineage (30). We found that MBN, which has been described as being involved in proliferation of human leukemia cells (1), is expressed specifically in immature myeloid cells. We have shown that MBN is a G-CSF-responsive gene and that its constitutive overexpression confers factor-independent growth to Ba/F3 cells expressing the G-CSFR. The fact that a MBN mutant lacking the serine protease catalytic activity failed to confer factor-independent growth strongly suggests that the protease activity is required for this transformation. The Ba/F3 cell line is likely to represent a nonleukemic primitive multipotent progenitor. This cell line has been used extensively to demonstrate transforming capacities of a number of fusion proteins (31–34) and start deciphering subsequent transformation pathways (35, 36). However, factor-independent growth is likely to represent only one of the early stages in leukemia development that may result in autonomous growth of primitive leukemia cells. It therefore is expected that normal G-CSF-responsive genes required for limited growth of normal hematopoietic progenitors remain abnormally expressed in primitive leukemia cells providing factor-independent growth. Little is currently known about normal genes whose overexpression can induce factor-independent growth. Our work provides evidence that factor-independent growth can be obtained by overexpressing a normally expressed protease and points to MBN as a critical G-CSF

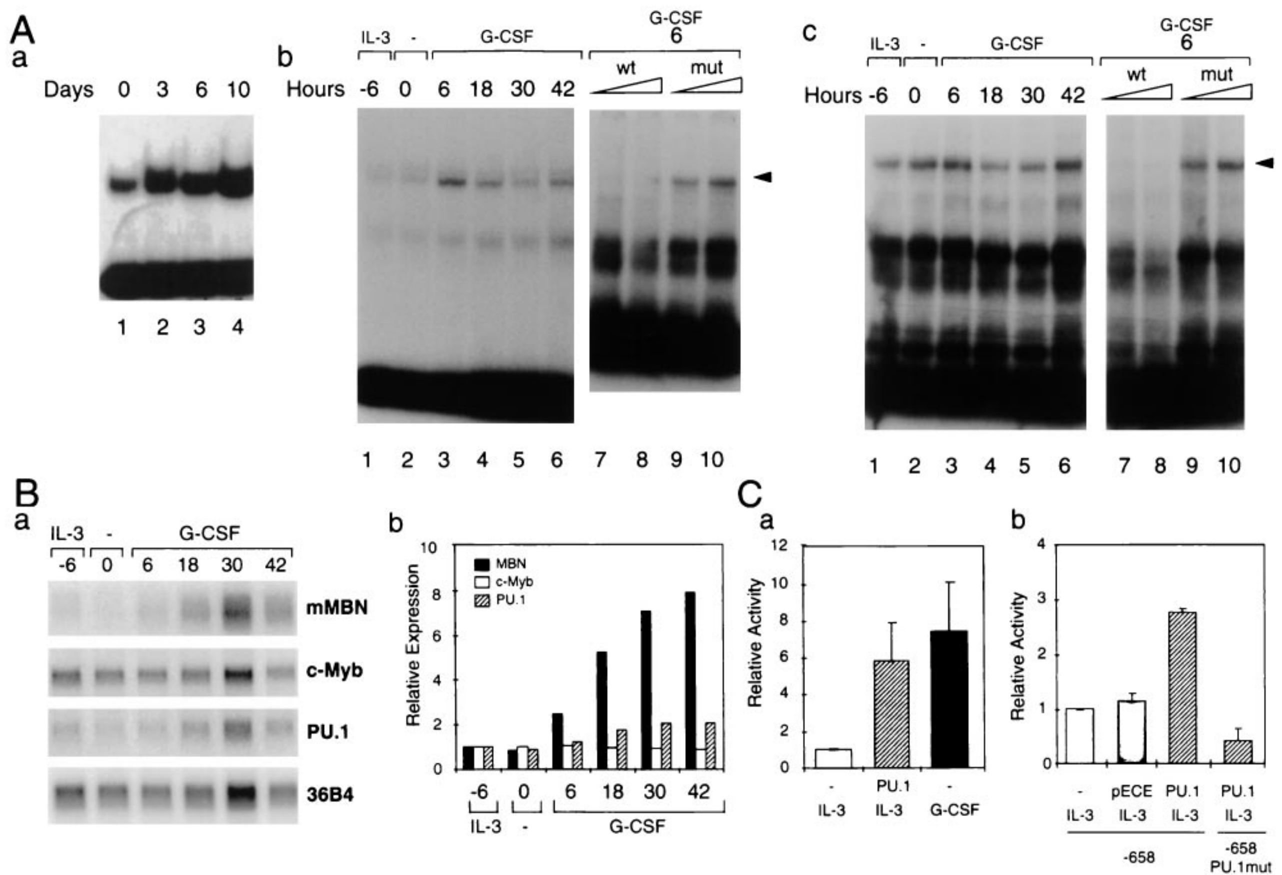


Fig. 4. PU.1 affects MBN expression in G-CSF-treated CD34$^+$ and Ba/F3/G-CSFR cells. (*A*) Nuclear extracts (1 μg) from CD34$^+$ cells induced to differentiate toward the myeloid lineage by G-CSF as described in *Materials and Methods* (*a*) and from Ba/F3/G-CSFR cells cultured with either IL-3 or G-CSF for the indicated times (*b* and *c*) were subjected to EMSA. DNA-binding assays were performed with the PU.1 (*a* and *b*) or the C/EBP probe (*c*) in the presence of 5 μg and 0.2 μg of poly(dI–dC), respectively. Competition assays were carried out with a 40- and 200-fold molar excess of the wild-type (wt) or mutated (mut) oligonucleotides. Arrowheads indicate specific complexes. (*B*) Up-regulation of MBN mRNA by G-CSF correlates with up-regulation of PU.1 mRNA. Autoradiogram (*a*) and relative expression (*b*) of MBN, PU.1, and c-Myb mRNAs. Total RNA (2 μg) was loaded in each lane. In *a*, the lower part is an autoradiogram of 36B4 mRNA expression as assessment of RNA quantities in each lane. In *A* and *B*, cells were cultured with IL-3 (lane 1), incubated for 6 hr in factor-free medium (lane 2), and cultured with G-CSF (lanes 3–6). (*C*) Overexpression of PU.1 in IL-3-cultured Ba/F3/G-CSFR cells is sufficient to induce MBN expression. (*a*) Twenty micrograms of the pMBN-658 construct was either transfected alone (−) or cotransfected with 5 μg of pECE-PU.1 (PU.1) into Ba/F3/G-CSFR cells. Cells then were cultured in IL-3- or G-CSF-containing medium as indicated. (*b*) Twenty micrograms of the pMBN-658 (-658) or pMBN-658 PU.1 mut (-658 PU.1 mut) constructs were individually either transfected (−) or cotransfected with 5 μg of pECE or with 5 μg of pECE-PU.1 (PU.1) into Ba/F3/G-CSFR cells. Cells then were cultured in IL-3-containing medium as indicated. Transfection efficiencies were normalized by cotransfection of a cytomegalovirus–luciferase vector. Cells were harvested 40 hr posttransfection. Chloramphenicol acetyltransferase activities calculated relative to the −658 in IL-3 culture conditions as a reference are presented. Error bars indicate the SDs from the results of three independent experiments.

responsive gene. Furthermore, our results indicate that expression of the G-CSFR was a prerequisite for MBN to render cells factor-independent. In this context, MBN affected neither the level of G-CSFR expression nor its binding capacity. The high levels of G-CSFR found in Ba/F3/G-CSFR cells may provide enough spontaneous G-CSF survival signals in the absence of factor, allowing MBN to confer G-CSF-independent growth. That such signals also may allow MBN to confer factor-independent growth in the absence of IL-3 is consistent with the fact that G-CSF can stimulate the proliferation of mouse IL-3-dependent NFS-60 cells in the absence of IL-3 (37) and the observation that human G-CSF has effects similar to IL-3 in the development of multipotential hematopoietic progenitors in transgenic mice expressing receptors for human G-CSF (38).

We have shown that the MBN promoter activity in G-CSF-treated Ba/F3/G-CSFR cells depended on the integrity of each of the three functional sites: C/EBP, c-Myb, and PU.1. Because treatment of Ba/F3/G-CSFR cells with G-CSF induced growth without differentiation, our results indicate the relevance of C/EBP, c-Myb, and PU.1 in G-CSF-induced regulation of a growth-related gene. That PU.1 mainly mediated G-CSF-induced MBN expression in hematopoietic cells is reminiscent of previous observations showing that *PU.1* −/− myeloid progenitors fail to proliferate in response to G-CSF (17). Whether posttranscriptional modifications such as phosphorylations of c-Myb or C/EBP are important for G-CSF-induced MBN expression remains to be established.

Cytokines and growth factors coordinate cell growth and differentiation. Aberrations in their function play a major role in the transformation mechanism possibly as a result of abnormal clonal expansion of progenitor cells that may become targets for additional genetic events leading to leukemia transformation. Leukemia cells may (*i*) produce a growth factor in an uncontrolled fashion and (*ii*) dysregulate a growth factor receptor downstream target. The finding that the normal IL-3 gene is overexpressed in a subtype of human acute pre-B cell leukemia with a t(5;14) translocation (39) has suggested that this leukemia resulted from an IL-3 autocrine loop. However, IL-3 overex-

pression in mouse models indicated that an autocrine loop was insufficient to produce leukemia on its own (39). Another observation has suggested that, in a mouse model for chronic myelogenous leukemia, leukemic cells expressed excess of IL-3 (40). To date, no other translocation in human leukemia has been identified that disrupts G-CSF or IL-3 activation. Our results indicate that an autocrine loop was unlikely to be involved in factor-independent growth of MBN-expressing Ba/F3/G-CSFR cells. High-affinity G-CSF receptors frequently are expressed on blasts of human acute leukemia of myeloid origin (41–43). This, together with the fact that MBN is a downstream target of G-CSF and is overexpressed in myeloid leukemia cells (44, 45), suggests that it may confer factor-independent growth to emerging myeloid leukemia cells in connection with activated G-CSFR survival signals. Altogether, our results may provide insights into early steps in myeloid leukemia transformation and reinforce the view that MBN may be a candidate of choice for targeted T cell therapy of myeloid leukemia (44, 45).

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