Regulation of Proliferation and Cytokine Expression of Bone Marrow Fibroblasts: Role of c-*myb*

By Cezary Szczylik,* Tomasz Skorski,* De-Hui Ku,* Nicholas C. Nicolaides,* Shau-Ching Wen,* Lidia Rudnicka,‡ Antonio Bonati,* Lucia Malaguarnera,* and Bruno Calabretta*

*From the *Department of Microbiology and Immunology, and Jefferson Cancer Institute; and the ‡Department of Dermatology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107*

Summary

The c-*myb* protooncogene plays a major role in regulating the process of in vitro and in vivo hematopoiesis via its activity as transcriptional regulator in hematopoietic progenitor cells. Since the bone marrow microenvironment appears to regulate in vivo hematopoiesis by maintaining the growth of multipotent progenitors via secretion of specific cytokines, we asked whether c-*myb* is also required for the proliferation of and/or cytokine production by stromal cells that generate fibroblast-like colonies (fibroblast colony-forming units [CFU-F]). Using the reverse transcriptase polymerase chain reaction technique, we detected low levels of c-*myb* mRNA transcripts in human normal bone marrow fibroblasts. Treatment of these cells with c-*myb* antisense oligodeoxynucleotides caused downregulation of c-*myb* expression, decrease in the number of marrow CFU-F colonies (~54% inhibition) and in the cell number within residual colonies (~80%), and downregulation of granulocyte/macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) mRNA expression. Transfection of T98G glioblastoma cells, in which expression of c-*myb*, GM-CSF, and SCF mRNAs is undetectable or barely detectable, with a plasmid containing a full-length c-*myb* cDNA under the control of the SV40 promoter induced the expression of biologically active SCF and GM-CSF in these cells. Regulation of GM-CSF expression by c-*myb* was due in part to transactivation of the GM-CSF promoter. These results indicate that, in addition to regulating hematopoietic cell proliferation, c-*myb* is also required for proliferation of and cytokines synthesis by bone marrow fibroblasts.

Hematopoietic tissue is constantly renewed through the proliferation and differentiation of stem cells residing in the bone marrow in close contact with multiple adherent (stromal) cells that comprise the hematopoietic microenvironment (HM) (1–4). In vitro stromal cells form fibroblast colonies (CFU-F) that, under defined conditions support long-term bone marrow growth of primitive hematopoietic stem cells (long-term bone marrow cultures [LTBMC][1] or Dexter-type cultures) (1–4). The interactions between the hematopoietic cells and the microenvironment are still poorly understood, due to the cellular heterogeneity of this microenvironment and to the difficulties in isolating homogeneous populations of its components for genetic and functional studies. The development of the PCR technology has enabled detection of growth factor transcripts in stromal marrow fibroblasts, suggesting that, via these cytokines, these cells play an important role in hematopoiesis in mammals in vivo (5, 6).

Different experimental approaches have revealed that the protooncogene c-*myb* plays an important role in regulating not only hematopoietic cell growth (7–10), but also proliferation of nonhematopoietic cells (11–13). Treatment with synthetic c-*myb* antisense oligodeoxynucleotides inhibits formation of colonies derived from normal hematopoietic progenitors (7–9). Inactivation of the endogenous c-*myb* gene by homologous recombination in mouse embryonic stem cells drastically impairs liver hematopoiesis (10). To address the possibility that c-*myb* also regulates stromal cell function, we measured c-*myb* mRNA levels in bone marrow fibroblasts and assessed whether downregulation of c-*myb* expression affects proliferation and cytokine production of these cells.

Materials and Methods

Cells and Cell Cultures. Bone marrow aspirates were obtained from the iliac crest of healthy individuals after informed consent.

[1] *Abbreviations used in this paper:* LTBMC, long-term bone marrow culture; NBM, normal bone marrow; SCF, stem cell factor.

The cell suspensions were diluted 1:4 with IMDM, layered on Ficoll/Histopaque density gradient, and centrifuged for 30 min at 1,500 rpm. Light-density mononuclear cells were washed in the same medium, and plated (5×10^5 cells/dish) into 35-mm petri dishes (Nunc, Inc., Naperville, IL) in IMDM supplemented with 15% FCS. After 90 min, supernatant containing nonadherent cells was discarded and the remaining adherent cells were cultured. Culture medium was changed every 3 d. After a 14-d culture, the number of colonies was counted under light microscope after staining with 5% crystal violet for 8 min. Cells to be used for RNA extraction were grown as monolayers, washed twice with HBSS, passaged four times, washed with IMDM, and lysed with lysing solution directly in the plates. The cell composition of this population of stromal cells was analyzed by surface phenotyping as described below. T98G human glioblastoma cells and TK-ts13 hamster fibroblasts (kind gifts of Drs. E. Mercer and R. Baserga, respectively) were maintained in culture as described (14, 15).

Indirect Immunofluorescence. Cells were grown on chamber slides (Nunc, Inc.) to subconfluence. The slides were rinsed with PBS and fixed with ice-cold 100% ethanol. After a 30-min incubation with 1% BSA in PBS the samples were exposed to either factor VIII (Dako, Denmark), CD14, CDw32, or type I collagen (ICN, Costa Mesa, CA) mAbs for 45 min at room temperature. The slides were then rinsed with PBS and exposed to rhodamine- or fluoresceine-conjungated anti-mouse IgG mAbs (Cappel Laboratories, West Chester, PA) for 45 min. Nonadherent secondary antibodies were then washed off with PBS. Nonspecific binding of the antibodies was excluded by performing controls with secondary antibodies only. In additional controls, microvascular endothelial cells were stained with antibodies to factor VIII.

Oligomers and Primers. These were synthesized on a DNA synthesizer (308B; Applied Biosystems, Inc., Foster City, CA) by means of β-cyanoethyl-phosphorymidite chemistry as described (7). The sequences of the c-*myb* sense and antisense oligodeoxynucleotides used were: 5′-GCC CGA ACA CCC CGG CAC-3′ and 5′-GTG CCG GGG TCT TGG GGC-3′, respectively. c-*myb* mRNA was detected by reverse transcriptase (RT)-PCR amplification of a segment of c-*myb* mRNA in the 3′ untranslated region with a 5′ synthetic primer, 5′-ATT AGG TAA TGA ATT GTA GCC AG-3′, and a 3′ synthetic primer, 5′-ACT TAG AGT AAT GCT TTT ACT GA-3′. The amplification product was detected by hybridization to the synthetic oligomer included in the amplified fragment (5′-ATT TTT TTA AAA AAA AAC ATA AAA TGA TTT ATC TGG TAT TTT AAA GGA TCC-3′) encompassing nucleotides 2351–2400 of the human c-*myb* cDNA (16). β-Actin mRNA levels were also analyzed by RT-PCR. The 5′ primer corresponds to nucleotides 224–244; the 3′ primer corresponds to nucleotides 411–433 of β-actin cDNA. A 39-base probe used to detect the amplification product corresponds to nucleotides 258–296 (17). GM-CSF mRNA was detected by RT-PCR technique with a pair of synthetic primers: 5′ primer, 5′-ATG TGA ATG CCA TCC AGG AG-3′; 3′ primer, 5′-CTT GTA GTG GCT GGC CAT CA-3′ and detected with a synthetic probe, 5′-TAG AGA CAC TGC TGC TGA GA-3′ (18). Stem cell factor (SCF) mRNA was detected using the following set of primers: 5′ primer, 5′-ATG AAG AAG ACA CAA ACT TGG-3′; 3′ primer, 5′-GTC CAG AAG ATC AGT CAA GCT-3′, and detected by synthetic oligomer probe, 5′-GCC GAG CTG GAC AGC ACC GTG CTC CTG ACC CGC TCT CTC-3′ (19). IL-6 mRNA was detected using the following set of primers: 5′ primer, 5′-AAG ATT CAT ACC TCA GAG CG-3′; 3′ primer, 5′-ATG AGA TCA CCT AGT CCA CC-3′; and detected by synthetic oligomer probe, 5′-AGC CCA GAC TCG AAT TCT GGT TCT GCC AAA-3′ (20). IL-11 mRNA was detected using the following set of primers: 5′ primer, 5′-ATG AAC TGT GTT TGC CGC-3′; 3′ primer, 5′-CCC CTG AGC TGG GAA TTT-3′; and detected by synthetic oligomer probe, 5′-GCC GAG CTG GAC AGC ACC GTG CTC CTG ACC CGC TCT CTC-3′ (21).

Oligomer Treatment of the Cells. Normal bone marrow (NBM) cells were plated into 35-mm dishes (Nunc, Inc.) in 1 ml IMDM 1640 supplemented with 15% FCS. c-*myb* sense and antisense oligodeoxynucleotides were added three times during a 14-d culture: 40–120 μg/ml, as indicated, was added during the first 18–24 h of incubation (37°C, 5% CO_2), half of the initial dose was added on day 3, and a third dose (50% of initial dose) was added on day 6 after changing the medium. On day 14, the medium was discarded, the cells were stained with 5% crystal violet for 8 min, and the number of colonies per plate and cell number per colony were determined.

Transfection. T98G human glioblastoma cells were transfected using the calcium-phosphate precipitation method (22). Briefly, 2×10^5 cells were either cotransfected with 10 μg of the plasmid pMbmI, which contains the human c-*myb* cDNA under control of the SV40 early promoter and enhancer, and 1 μg of the plasmid pLHL4, which contains the gene encoding hygromycin resistance or transfected with the plasmid pLHL4 only. After 12-d selection in culture medium containing 0.5 mg/ml of hygromycin B (Calbiochem-Behring Corp., San Diego, CA), colonies were pooled and cultured in medium containing 0.3 mg/ml of hygromycin B.

Detection of c-myb, β-Actin, GM-CSF, and SCF mRNA Transcripts in Marrow Fibroblasts and in T98G Glioblastoma Cells. To analyze the effect of c-*myb* sense and antisense oligodeoxynucleotide treatment on c-*myb* expression, 10^5 marrow fibroblasts obtained after the fourth passage were plated into 35-mm plastic dishes (Nunc, Inc.), and were left untreated or exposed to c-*myb* sense or antisense oligodeoxynucleotides (40 μg/ml at time 0; 20 μg/ml at 24 h); 24 h later, cells were collected separately from each experimental group, before extracting total RNA as described (23, 24). RNA from each group was divided into four identical portions, and reverse transcribed using 400 U of Moloney murine leukemia virus RT (Bethesda Research Laboratories, Gaithersburg, MD), and 0.1 μg of 3′ primers of c-*myb*, β-actin, GM-CSF, and SCF for 1 h at 37°C. Resulting cDNA fragments were amplified with 5 U of Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT) in the presence of 3′ and 5′ primers generating c-*myb*, β-actin, GM-CSF, and SCF fragments during 50 cycles of PCR. Amplified DNA was subjected to electrophoresis, transferred to Zetabind nylon filters (Cuno, Inc., Meriden, CT), and detected by Southern hybridization with [^{32}P]ATP end-labeled c-*myb*, β-actin, GM-CSF, or SCF synthetic probes, as described (21, 22). Densitometric analysis of hybridization bands was performed using an Ultro Scan XL (Pharmacia LKB) apparatus.

GM-CSF and SCF mRNA levels were determined in 1 μg of total RNA derived from parental and SVmyb-transfected T98G cells. As positive control for the expression of GM-CSF and SCF, RNA derived from PHA-stimulated PBMC and the bladder carcinoma HTB9 line, respectively, was used. RNA from each sample was divided into four portions, and c-*myb*, GM-CSF, SCF, and β-actin expression was determined by RT-PCR technique. As negative control, RT-PCR amplifications were performed in the absence of RNA.

Detection of GM-CSF and SCF in Cell-free Culture Supernatants of SV-myb-transfected T98G Cells. Cell-free culture supernatants were collected from exponentially growing T98G transfected with plasmid pLHL4 encoding hygromycin resistance and SV-*myb*-transfected T98G cells cultured in the presence of 10% FCS. Supernatants were sterilized by filtration through 0.22-μm filters (Mil-

lipore). Concentration of GM-CSF and SCF in the supernatants was measured using commercial enzyme immunoassays (R & D Systems, Minneapolis, MN), with sensitivity of 1.5 and 3.0 pg/ml, respectively.

Isolation of the 5' Flanking Region of Human GM-CSF Gene and Plasmid Construction. A 600-bp fragment of the 5' flanking region of the human GM-CSF gene (18) was isolated by PCR amplification of placenta genomic DNA using the following primers: 5' primer, 5'-AAG CTT GCT GAG AGT GGC TGC-3'; 3' primer, 5'-CAG AGA ACT TTA GCC TTT CTC-3'. The amplified fragment was then subcloned into the SmaI site of the Bluescript vector (Strategene, La Jolla, CA), 5' of the T7 promoter, and subjected to sequence analysis to confirm its identity with the 5' flanking segment of the human GM-CSF gene. This plasmid was called GMCSF17. A CAT construct was prepared after digestion of GMCSF17 with EcoRV and HindIII to isolate the 600-bp fragment and clone it into pucCAT linearized using HindIII and SalI restriction enzymes to obtain the GMCSF fragment in sense orientation with respect to the CAT gene. This construct was named GM-CSF CAT2.

Transient CAT Analysis. CAT assays were performed as described (25). Briefly, 2 μg of CAT reporter plasmid was transfected with or without 8 μg of effector plasmid plus 1 μg of pSV-β-gal, which contains the bacterial β-galactosidase gene driven by the SV40 promoter as an internal control of transfection efficiency, into wild-type or SV-*myb*-transfected Tk-ts13 Syrian hamster fibroblasts using the calcium-phosphate precipitation method (22). 48 h after transfection, cells were harvested and proteins were extracted by freeze/thawing and normalized for transfection efficiency by β-galactosidase assay as described by the manufacturer (Promega Biotec, Madison, WI). For each assay, cellular lysates were incubated with [^{14}C]chloramphenicol and acetyl-CoA for 1 h at 37°C. Transactivation of reporter constructs was assayed measuring the amount of acetylated [^{14}C]chloramphenicol by thin-layer chromatography followed by autoradiography and scintillation counting.

Results

Detection of c-myb mRNA in Bone Marrow Stromal Fibroblasts. c-*myb* mRNA levels are relatively abundant in undifferentiated and proliferating hematopoietic cells but are undetectable or present in few copies in other normal cell types. To determine whether c-*myb* mRNA is present in marrow stromal fibroblasts and to compare its expression to that found in hematopoietic cells, RNA was extracted from an equal number of cells from different sources and mRNA levels were measured by RT-PCR analysis (Fig. 1). High levels of c-*myb* mRNA were detected in the myeloid leukemia cell line HL 60 (Fig. 1, lane *a*), in the Philadelphia[1] lymphoid leukemia line BV173 (lane *b*), in normal marrow mononuclear cells enriched in early progenitors (CD34+ cells) (lane *e*), and in the glioblastoma T98G line transfected with a human c-*myb* cDNA plasmid (SV-*myb* T98G) (lane *d*); c-*myb* mRNA levels were barely detectable in WI38 human fibroblasts (lane *f*) and in the parental T98G cells (lane *c*); intermediate c-*myb* mRNA levels were detected in marrow stromal fibroblasts (lane *g*).

Effect of c-myb Oligomers on CFU-F Colony Growth. To determine whether cloning efficiency of stromal fibroblasts derived from human normal bone marrow depends on c-*myb* expression, the effect of c-*myb* sense or antisense oligodeoxynucleotides (80 μg/ml at time 0; 40 μg/ml after 24 h), on stromal fibroblast colony formation was analyzed in 14-d cultures. In four separate experiments, untreated and sense-treated cells formed a similar number of colonies, each containing a similar number of cells. In contrast, cultures treated with c-*myb* antisense oligodeoxynucleotides showed an ~54% decrease in colony number and an ~80% decrease in cell content per colony (Table 1). Fig. 2 illustrates these effects in a representative experiment.

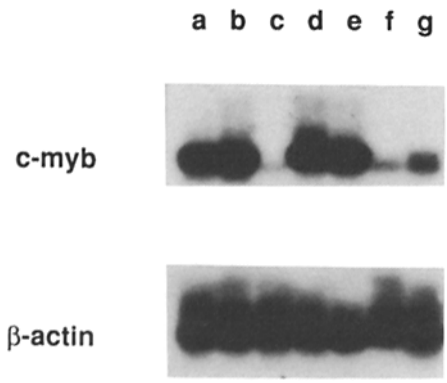

Figure 1. C-myb mRNA levels in human hematopoietic and non-hematopoietic cells. Total RNA extracted from 5 × 10^4 HL-60 (lane *a*), BV-173 (lane *b*), T98G (lane *c*), SV-*myb* T98G (lane *d*) marrow mononuclear cells enriched in CD34+ cells (lane *e*), WI-38 (lane *f*), and human marrow stromal fibroblasts (lane *g*), as described (35), was analyzed by RT-PCR for c-*myb* and β-actin expression.

Effect of c-myb Antisense Oligonucleotides on c-myb, GM-CSF, and SCF mRNA Levels in Stromal Cells. To determine whether the inhibition of stromal fibroblasts colony formation was associated with downregulation of c-*myb* expression, c-*myb* mRNA levels were determined by RT-PCR after exposure of stromal cells collected after the fourth passage, to c-*myb* sense or antisense oligodeoxynucleotides. These cells showed an intense staining for type I collagen, and were negative for the expression of factor VIII, CD14, and CDw32 normally expressed by endothelial cells and megakaryocytes, and by monocyte/macrophages respectively. These results and the characteristic morphology of the cells indicate that the culture consisted of fibroblasts only, in agreement with a published study (6). c-*myb* mRNA expression was easily detected at similar levels in untreated (Fig. 3 *a*) and sense-treated stromal cells (Fig. 3 *b*), whereas significantly lower c-*myb* mRNA levels were present in cells treated with c-*myb* antisense oligonucleotides (Fig. 3 *c*). Densitometric measurement of the c-*myb* hybridizing bands in sense-vs.-antisense oligodeoxynucleotide-treated samples indicated that the signal from the antisense-treated samples was <10% of that from the sense-treated sample.

To determine whether inhibition of c-*myb* expression affected stromal fibroblast cell functions, we investigated GM-CSF, SCF, IL-6, and IL-11 mRNA levels in c-*myb* sense- or antisense-treated cultures in four different experiments. High levels of GM-CSF and SCF mRNAs were detected in untreated and c-*myb* sense-treated cultures (Fig. 3, lanes *a* and

Table 1. *Inhibition of CFU-F Colony Formation by c-myb Sense or Antisense 18-mer Oligodeoxynucleotides*

	Exp.			
	1	2	3	4
Control				
Colony No.	14.4 ± 2*	34.5 ± 3.5	16.7 ± 2.3	26.8 ± 6.8
Cells/colony	1718.5 ± 127.3	500 ± 50.9	1781 ± 88.1	110.3 ± 41.7
Sense				
Colony no.	17.5 ± 0.7	47.08 ± 2.8	13.5 ± 0.7	31 ± 4.2
Cells/colony	1843 ± 391.2	578.5 ± 44.5	1408.3 ± 218.2	65 ± 19.3
Antisense				
Colony no.	7.5 ± 2.1	17.7 ± 1.5	7.01 ± 1.4	16.25 ± 3.4
Cells/colony	327.3 ± 40.2	90.3 ± 32	309.2 ± 47.3	20.2 ± 5.9
Percent inhibition				
Colony growth‡	57.2	62.4	48.7	47.5
(significance)	p <0.001	p <0.002	p <0.005	p <0.03
Percent inhibition				
Cells/colony§	82.2	84.3	78.8	68.9
(significance)	p <0.001	p <0.005	p <0.005	p <0.015

* Data are mean ± SD from quadruplicate plates.
‡ Calculated from the ratio between colony number in sense- and antisense-treated cultures.
§ Calculated from the ratio of number of cells/colony (10 for each group) in sense- and antisense-treated cultures.

b), whereas these were reduced by >90% in cultures exposed to c-*myb* antisense oligodeoxynucleotides (Fig. 3, lanes *c*). In contrast, IL-6 and IL-11 mRNA levels were not affected by the treatment with c-*myb* antisense oligodeoxynucleotides (not shown). IL-3 mRNA levels, barely detectable in stromal cells, were not modified in cultures treated with c-*myb* antisense oligodeoxynucleotides (not shown). Levels of β-actin mRNA, used as control, were constant.

Expression of GM-CSF and SCF in T98G Cells Constitutively Expressing c-myb. To further investigate whether c-*myb* expression is linked to that of GM-CSF and SCF, T98G glioblastoma cells, which express undetectable or low levels of endogenous c-*myb*, were transfected with a human c-*myb* cDNA driven by the SV40 promoter in the presence of the plasmid carrying the gene encoding hygromycin resistance. After selection, a mixed cell population of T98G cells transfected with a human c-*myb* cDNA driven by the early SV40 promoter (SV-*myb* T98G) (Fig. 4, lane *b*) and expressing the exogenous c-*myb* at high levels appeared to express higher levels of GM-CSF or SCF mRNA compared with T98G cells transfected only with the plasmid pLHL4 encoding hygromycin resistance (Fig. 4, lane *a*). In SV-*myb* T98G cells the level of expression of GM-CSF and SCF mRNAs increased linearly with increase in the number of PCR cycles from 30 to 50 (not shown). Consistently, we were unable to detect SCF mRNA in control T98G cells, whereas GM-CSF mRNA could be detected only after as many as 50 cycles of PCR amplification (not shown). In SV-*myb* T98G cells, SCF mRNA levels appeared essentially identical to those found in HTB9 cells, whereas GM-CSF mRNA levels were less abundant than in PHA-stimulated PBMC. GM-CSF and SCF levels were measured in the cell-free culture supernatant from the control and the SV-*myb*-transfected T98G cells. A 3–4- and a 10–14-fold increase in secreted GM-CSF and SCF proteins, respectively, was detected in SV-*myb* T98G cells, compared with T98G cells transfected only with plasmid pLHL4 (Fig. 5). Biological activity of the secreted cytokines was analyzed using the acute myelogenous leukemia MO7 cell line, whose proliferation is dependent on exogenously added IL-3, GM-CSF, or SCF (26–28). Either 10^4 or 10^5 MO7 cells were seeded on a feeder layer of exponentially growing control or SV-*myb*-transfected T98G cells and the number of cells in suspension was counted at different days. MO7 cells seeded on the feeder layer of SV-*myb* T98G cells continued to proliferate over a 5-d period. In contrast, T98G cells transfected only with plasmid pLHL4 encoding hygromycin resistance were not able to support the growth of MO7 cells (Fig. 6). T98G cells are growth arrested when they reach confluence (14, 29, and our own observations); to exclude the possible presence of contaminating T98G cells among MO7-growing cells, expression of CD45, present on MO7 cells and absent on SV-*myb* T98G cells, was analyzed by flow cytometry in the cells growing in suspension. More than 90% of the cells were $CD45^+$ (data not shown), thus confirming

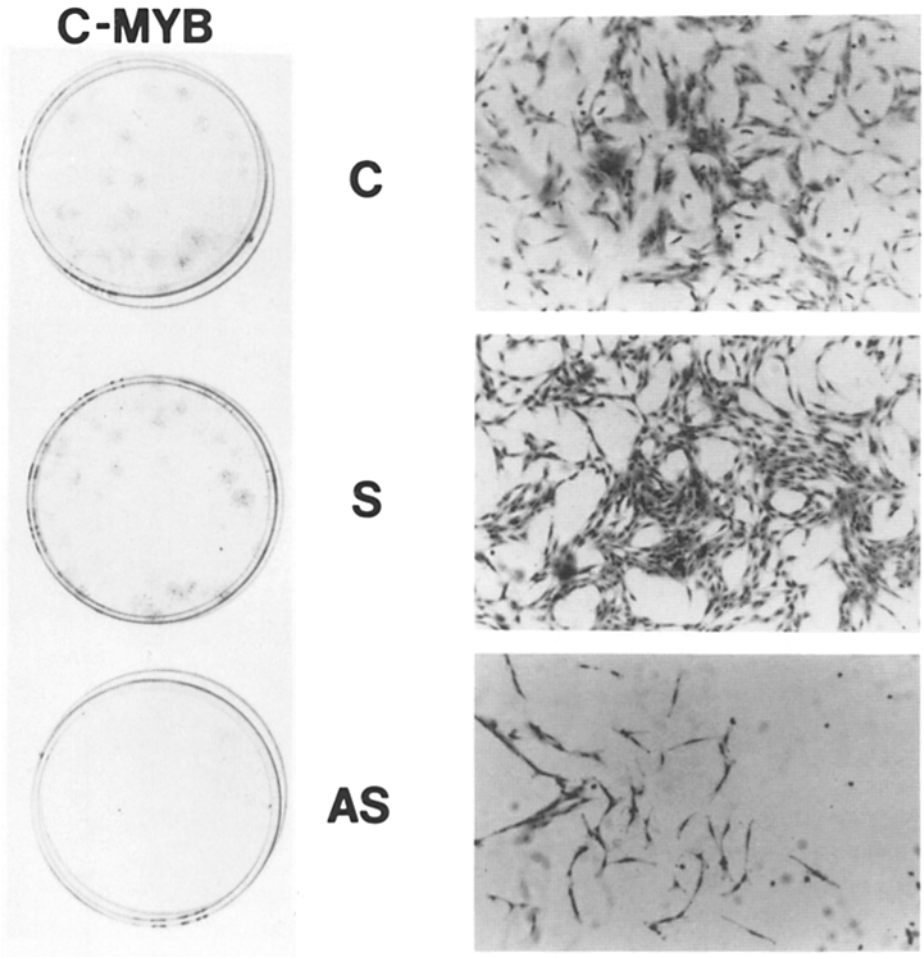

Figure 2. Effect of c-*myb* antisense oligodeoxynucleotides on human CFU-F NBM colony formation. (*Left*) Effect of c-*myb* oligomers on the number of CFU-F colonies from NBM cells. The corresponding higher magnification pictures (*right*) show the effect of c-*myb* oligomers on CFU-F colonies cellularity. (*A*) Untreated cultures; (*S*) c-*myb* sense-treated cultures; (*AS*) c-*myb* antisense-treated cultures.

the proliferation of MO7 cells on the feeder layer of SV-*myb* T98G cells.

c-myb Transactivation of CAT Gene Expression Driven by the Human GM-CSF 5' Flanking Region Containing Putative Myb Binding Sites. To assess the ability of c-*myb* to transactivate GM-CSF, transient expression assays were performed using a CAT reporter construct containing a 600-bp fragment of the human GM-CSF 5' flanking region found to contain several putative Myb binding sites. In Tk-ts13 hamster cells transfected at a 5:1 E/T ratio and assayed 48 h later, the SV40 c-*myb* effector plasmid induced a fivefold increase in CAT expression driven by the 600-bp GM-CSF 5' flanking segment (Fig. 7 *A*); a similar level of transactivation of the GM-CSF 5' flanking sequence was found in SV-*myb*-Tk-ts13 cells constitutively expressing a human c-*myb* cDNA (Fig. 7 *B*, lane *2*).

These latter cells were also transfected with the GM-CSFCAT2 construct in the presence of an excess (100:1, molar ratio) of a 22-base synthetic oligomer containing two canon-

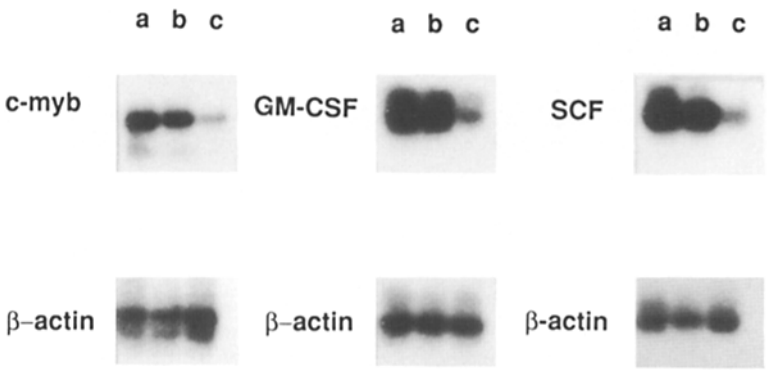

Figure 3. Effect of c-*myb* antisense oligodeoxynucleotides on c-*myb* GM-CSF and SCF mRNA levels in human marrow stromal fibroblasts. (Lanes *a*) Untreated cells; (lanes *b*) c-*myb* sense-treated cells; and (lanes *c*) c-*myb* antisense-treated cells. As control, β-actin mRNA expression was evaluated in each sample. Identical qualitative results were obtained with 30 or 40 RT-PCR amplification cycles.

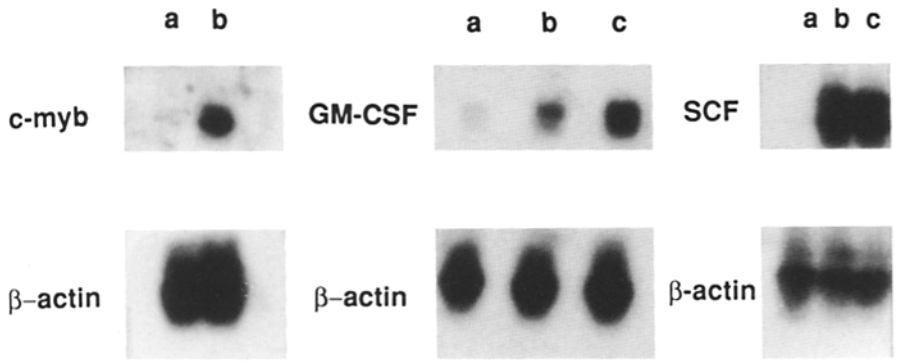

Figure 4. Expression of c-*myb*, GM-CSF, and SCF in T98G and SV-*myb* T98G cells. RNA extracted from: (lanes *a*) T98G glioblastoma cells, (lanes *b*) SV-myb T98G cells, (lanes *c*) PHA-stimulated PBMC (*GM-CSF*), and HTB cells (*SCF*) was analyzed by RT-PCR for c-myb, GM-CSF, and SCF expression. As control, β-actin mRNA expression was analyzed in all samples.

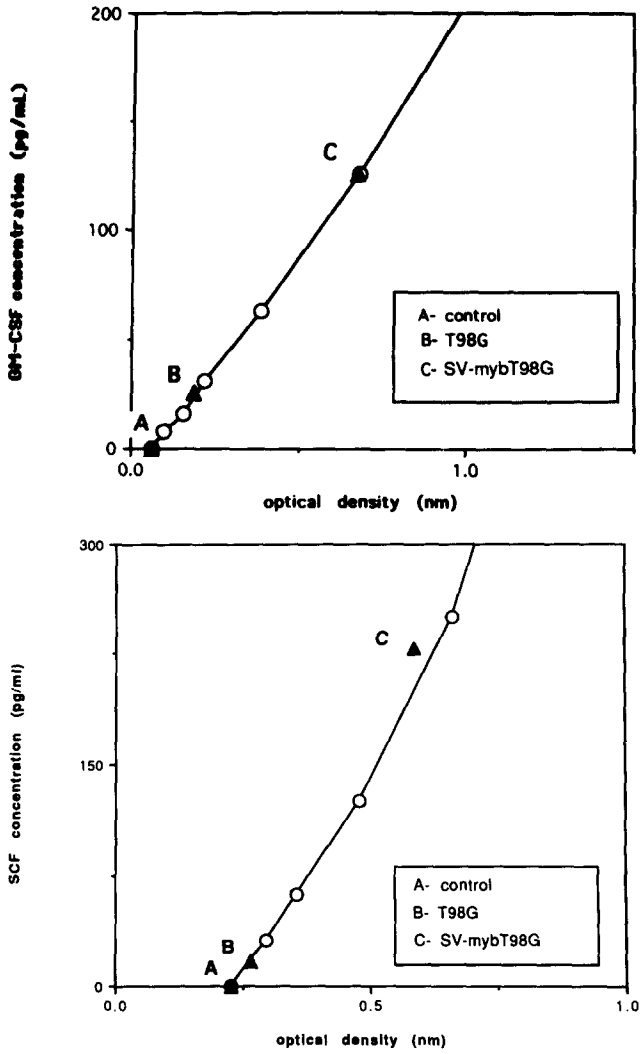

Figure 5. Detection of GM-CSF (*top*) and SCF (*bottom*) in cell-free supernatants from cultures of control and SV-*myb*-transfected T98G cells. Levels of GM-CSF and SCF were measured by ELISA in RPMI, 10% FCS medium (*A*), and in the cell-free supernatants from control T98G cells (*B*) and SV-*myb* T98G cells (*C*). Each point represents data collected from two independent experiments. (○) GM-CSF or SCF standard (pg/ml); (▲) GM-CSF or SCF detected in supernatant (pg/ml).

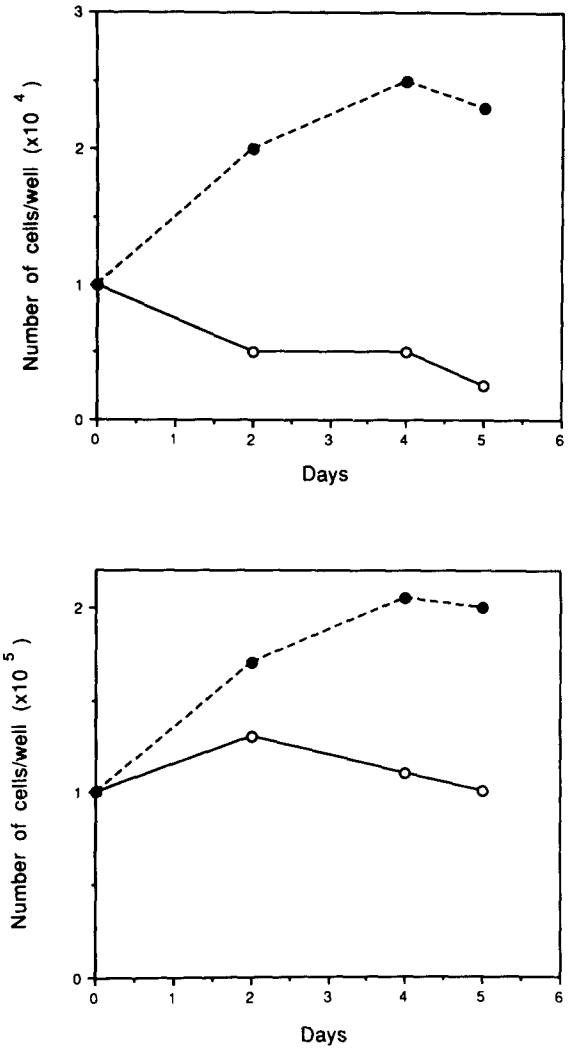

Figure 6. Proliferation of MO7 cells upon coculture with c-*myb*-transfected T98G cells. MO7 cells (10^4 or 10^5, as indicated) were added to 80% confluent monolayers of adherent T98G cells transfected with pLHL4 (hygromycin resistance gene) (○) or cotransfected with pLHL4 and pMbml (c-*myb* driven by the SV-40 promoter) (●). The number of cells growing in suspension was counted at the indicated times.

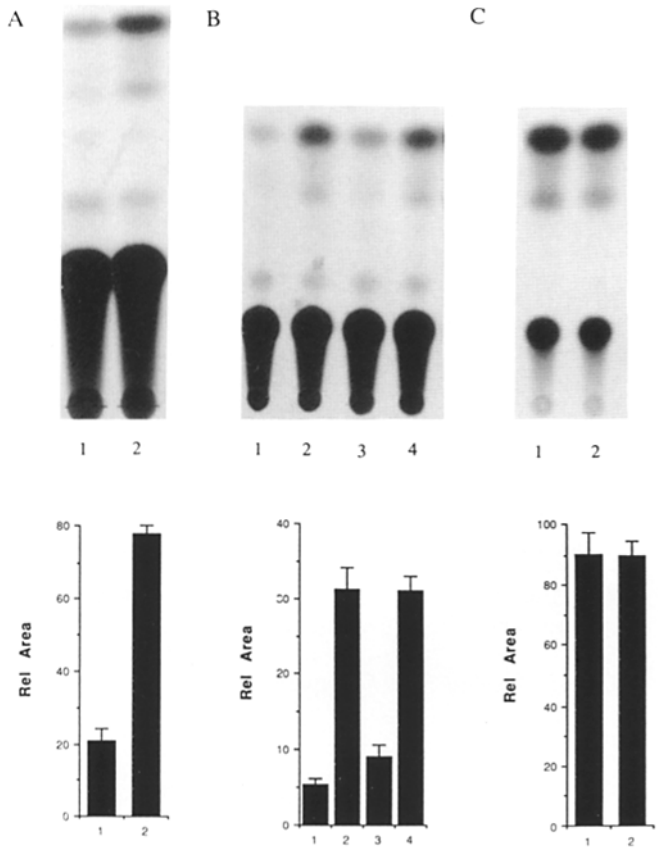

Figure 7. *c-myb* transactivation of the human GM-CSF promoter in Tk-ts 13 hamster fibroblasts. (*A*) Autoradiogram shows CAT activity in lysate of Tk-ts13 cells transfected with: (lane *1*) GM-CSF CAT 2 only; (lane *2*) GM-CSF CAT 2 and pMb ml (human *c-myb* cDNA driven by the SV 40 promoter). (*B*) Autoradiogram shows CAT activity in lysate of: (lane *1*) Tk-ts13 cells transfected with GM-CSF CAT 2; (lane *2*) SV-*myb* Tk-ts13 cells transfected with GM-CSF CAT 2; (lane *3*) SV-*myb* Tk-ts13 cells transfected with GM-CSF CAT 2 plus a 22-base synthetic oligomer containing two Myb binding sites; (lane *4*) SV-*myb* Tk-ts13 cells transfected with GM-CSF CAT 2 plus a 22-base synthetic oligomer containing mutated (three nucleotide substitutions) Myb binding sites. (*C*) Autoradiogram shows CAT activity in lysate of: (lane *1*) Tk-ts13 cells transfected with pSV-CAT (5 mg); (lane *2*) Tk-ts13 cells transfected with pSV-CAT plus a 22-base synthetic oligomer containing two Myb binding sites. (*Bottom*) Scintillation counting of acetylated [^{14}C]chloramphenicol.

ical Myb binding sites or a 22-base synthetic oligomer with mutations at both sites. Transactivation of the GM-CSF CAT2 construct in the transfected cells was abolished by the wild type 22-mer competitor, but was unaffected by the mutated competitor (Fig. 7 *B*, lanes *3* and *4*), suggesting that the transactivation of the GM-CSF promoter directly depended on *c-myb* expression and interaction with Myb binding sites.

To demonstrate that the 22-base oligomer containing two canonical Myb binding sites was nontoxic, CAT activity was analyzed in Tk-tk13 cells transfected with pSV-CAT in the presence (Fig. 7 *C*, lane *2*) or in the absence (Fig. 7 *C*, lane *1*) of excess amount (100:1, molar ratio) of the synthetic oligomer. Levels of CAT activity were identical, confirming the specificity of the effects observed.

Discussion

We have recently shown that the product of *c-myb* plays an important role in normal and leukemic hematopoiesis, perhaps by directly regulating the proliferation of normal early hematopoietic progenitors and leukemic cells. The role of *c-myb* does not appear to be restricted to hematopoietic cells, since other normal and neoplastic nonhematopoietic cells expressing *c-myb* require this gene for proliferation (11–13).

Because normal bone marrow contains nonhematopoietic adherent progenitor cells (CFU-F) capable of forming fibroblast colonies that support long-term growth of hemopoietic stem cells in vitro, we investigated whether *c-myb* is expressed in these cells and whether it is important for their proliferation and function. Downregulation of *c-myb* expression in marrow stromal cells was associated with inhibition of colony formation derived from CFU-F progenitors (~54%, inhibition of colony formation) and with a reduced number of cells in each residual colony (~80% reduction), suggesting that the relatively low levels of *c-myb* expression in these cells were nevertheless important for their proliferation. Marrow fibroblasts appear to express *c-myb* mRNA at levels lower than those of normal or leukemic hematopoietic cells but significantly higher than those found in the WI-38 human fibroblasts, cells considered to be negative for *c-myb* expression. In agreement with our findings, low-level expression of *c-myb* in murine fibroblasts and requirement of *c-myb* for G_1/S transition in these cells has recently been shown (30). The limited effect of *c-myb* antisense oligodeoxynucleotide on marrow fibroblast proliferation may reflect the requirement by these cells of the function of other members of the *myb* gene family, such as B-*myb*, which we have recently shown to behave as a *c-myb* functional equivalent in fibroblasts (31). Nevertheless, the findings reported here provide additional evidence that the role of *c-myb* is more general than previously thought and are consistent with similar findings in other nonhematopoietic systems, such as that of colon carcinoma and neuroblastoma cell proliferation and normal smooth muscle cell proliferation (11–13).

To further investigate the relevance of *c-myb* expression for stromal fibroblast cell function, we assessed whether cytokine production was regulated by *c-myb*. Several lines of evidence support this hypothesis: (*a*) downregulation of *c-myb* expression in stromal fibroblasts was associated with a specific decrease in GM-CSF and SCF mRNA levels; (*b*) constitutive expression of *c-myb* in a glioblastoma cell line with low or undetectable levels of endogenous *c-myb* was associated with upregulation of GM-CSF and SCF mRNAs and proteins; and (*c*) *c-myb* transactivated the expression of a reporter gene driven by a segment of the 5′ flanking region of the human GM-CSF gene. Although we cannot exclude that downregulation of GM-CSF and SCF expression in marrow fibroblasts exposed to *c-myb* antisense oligodeoxynucleotides is, at least in part, a consequence of the growth inhibition of these cells, the observation that GM-CSF and SCF expression is upregulated in T98G cells whose growth is independent of *c-myb* expression, and that *c-myb* has a direct effect on the GM-CSF promoter, makes it unlikely that GM-CSF and SCF

production are solely related to proliferative effects. The possible involvement of c-*myb* in regulating the expression of hematopoietic growth factors is not completely surprising in light of the recent observation of selective upregulation of insulin-like growth factor 1 (IGF-1) expression in murine fibroblasts constitutively expressing c-*myb* (32). The significance of the functional link between c-*myb* and hematopoietic growth factors may not be restricted to marrow fibroblasts; a subset of primary leukemic cells expresses GM-CSF (33, 34). Perhaps the overexpression of c-*myb* often observed in leukemic cells leads to autocrine cytokine expression that, in turn, contributes to the growth advantage of leukemic cells.

In summary, c-*myb* appears to play a role in regulating both proliferation and cytokine production in marrow fibroblasts. Although the mechanisms involved in this function remain unknown, our findings underscore the importance of the role of c-*myb* in the regulation of cell physiology and suggest potential consequences of c-*myb* activation in hematological malignancies.

We thank B. Perussia for critical reading of the manuscript and for many useful suggestions.

This work was supported, in part, by National Institutes of Health and American Cancer Society grants. D.-H. Ku is supported by training grant 1 T32 CA-09670. T. Skorski is supported by a fellowship of the Cancer Research Foundation of America. B. Calabretta is a scholar of the Leukemia Society of America.

Address correspondence to Bruno Calabretta, Department of Microbiology and Immunology, Jefferson Cancer Institute, Room 630, Bluemle Life Sciences Building, 1020 Locust Street, Philadelphia, PA 19107.

Received for publication 25 February 1993 and in revised form 5 May 1993.

References

1. Zipori, D., and M. Tamir. 1989. Stromal cells of hemopoietic origin. *Int. J. Cell. Cloning.* 7:281.
2. Dexter, T.M., T.D. Allen, and L.G.J. Laitha. 1977. Conditions controlling the proliferation of stem cells in vitro. *J. Cell. Physiol.* 91:335.
3. Allen, T.D., and T.M. Dexter. 1984. The essential cells of the hematopoietic microenvironment. *Exp. Hematol.* 12:517.
4. Allen, T.D., and T.M. Dexter. 1990. Marrow biology and stem cells. *In* Colony Stimulating Factors. Marcel Dekker, Inc., New York. 1–38.
5. Kittler, E.L., H. McGrath, D. Temeles, R.B. Crittenden, V.K. Kister, and P.J. Quesenberry. 1992. Biologic significance of constitutive and subliminal growth factor production by bone marrow stroma. *Blood.* 79:3168.
6. Guba, S.C., C.I. Sartor, L.R. Gottschalk, J.H. Ying-Hu, T. Mulligan, and S.G. Emerson. 1992. Bone marrow stromal fibroblasts secrete interleukin-6 and granulocyte-macrophage colony-stimulating factor in the absence of inflammatory stimulation: demonstration by serum-free bioassay, enzyme-linked immunoabsorbent assay, and reverse transcriptase-polymerase chain reaction. *Blood.* 80:1190.
7. Gewirtz, A.M., and B. Calabretta. 1988. A c-myb antisense oligodeoxynucleotide inhibits human hematopoiesis in vitro. *Science (Wash. DC).* 242:1303.
8. Caracciolo, D., D. Venturelli, M. Valtieri, C. Peschle, A.M. Gewirtz, and B.J. Calabretta. 1990. Stage-related proliferative activity determines c-myb functional requirements during normal human hematopoiesis. *J. Clin. Invest.* 85:55.
9. Valtieri, M., D. Venturelli, E. Pelosi, C. Labbaye, G. Mattia, C. Fossati, A.M. Gewirtz, B. Calabretta, and C. Peschle. 1991. Antisense myb inhibition of purified erythroid progenitors in development and differentiation is linked to cycling activity and expression of DNA polymerase-α. *Blood.* 77:1181.
10. Mucenski, M.L., K. McLain, A.B. Kier, S.H. Swerdlow, C.M. Schreiner, T.A. Miller, D.W. Pietryga, J.W. Scott, and S.S. Potter. 1991. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell.* 65:677.
11. Melani, C., L. Rivoltini, B. Parmiani, B. Calabretta, and M. Colombo. 1991. Inhibition of proliferation by c-myb antisense oligodeoxynucleotides in colon adenocarcinoma cell lines that express c-myb. *Cancer Res.* 51:2897.
12. Raschella, G., A. Negroni, T. Skorski, S. Pucci, M. Nieborowska-Skorska, A. Romeo, and B. Calabretta. 1992. Inhibition of proliferation by c-myb antisense RNA and oligodeoxynucleotides in transformed neuroectodermal cell lines. *Cancer Res.* 52:4221.
13. Simons, M., E.R. Edelman, J.-L. Dekeyser, R. Langer, and R.D. Rosenberg. 1992. Antisense c-myb oligonucleotides inhibit intimal arterial smooth muscle cell accumulation in vivo. *Nature (Lond.).* 359:67.
14. Mercer, W.E., M.T. Shields, M. Amin, G.J. Sauvé, E. Appella, J.W. Romano, and S.J. Ullrich. 1990. Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA.* 87:6166.
15. Reiss, K., S. Travali, B. Calabretta, and R. Baserga. 1991. Growth-regulated expression of B-myb in fibroblasts and in hematopoietic cells. *J. Cell. Physiol.* 148:338.
16. Majello, B., L.C. Kenyon, and R. Dalla-Favera. 1986. Human c-myb proto-oncogene: nucleotide sequence of cDNA and organization of the genomic locus. *Proc. Natl. Acad. Sci. USA.* 83:9616.
17. Tukunaga, K., H. Taniguchi, K. Yoda, M. Shimizu, and S. Sakiyama. 1986. Nucleotide sequence for a full-length cDNA for mouse cytoskeletal β-actin mRNA. *Nucleic Acids Res.* 14:2829.

18. Kaushansky, K., P.J. O'Hara, K. Berkner, G.M. Segal, F.S. Hagen, and J.W. Adamson. 1986. Genomic cloning, characterization and multilineage growth-promoting activity for human granulocyte macrophage colony stimulatory factor. *Proc. Natl. Acad. Sci. USA.* 83:3101.
19. Martin, F.H., S.V. Suggs, K.E. Langley, H.S. Lu, J. Ting, I.K. McNiece, F.W. Jacobsen, E.A. Mendiaz, N.C. Birkett, K.A. Smith, et al. 1990. Primary structure and functional expression of rat and human stem cell factor cDNA. *Cell.* 63:203.
20. Yasakawa, K., T. Hirano, Y. Watanabe, K. Muratani, T. Matsuda, S. Nakai, and T. Kishimoto. 1987. Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2939.
21. Paul, S.R., F. Bennett, J.A. Calvett, K. Kelleher, C.R. Wood, R.M. O'Hara, A.C. Leary, B. Sibley, S.C. Clark, D.A. Williams, and Y.-C. Yang. 1990. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphohemopoietic and hematopoietic cytokine. *Proc. Natl. Acad. Sci. USA.* 87:7512.
22. Huttner, K.M., J.A. Barbosa, G.A. Scangos, D.D. Pratcheva, and F.H. Ruddle. 1981. DNA-mediated gene transfer without carrier DNA. *J. Cell Biol.* 91:153.
23. Szczylik, C., T. Skorski, L. Manzella, L. Malaguarnera, D. Venturelli, A.M. Gewirtz, and B. Calabretta. 1991. Selective inhibition of leukemia cell proliferation by bcr/abl antisense oligodeoxynuleotides. *Science (Wash. DC).* 253:562.
24. Skorski, T., C. Szczylik, L. Malaguarnera, M. Ratajczak, A. Gewirtz, and B. Calabretta. 1992. Growth-factor dependent inhibition of normal hematopoiesis by N-*ras* antisense oligodeoxynucleotides. *J. Exp. Med.* 175:743.
25. Ku, D.-H., S.-C. Wen, A. Englehard, N.C. Nicolaides, K.E. Lipson, T.A. Marino, and B. Calabretta. 1993. c-myb transactivation of cdc2 expression via Myb binding sites in the 5′ flanking region of the human cd2 gene. *J. Biol. Chem.* 268:2255.
26. Avanzi, G.C., P. Lista, B. Giovinazzo, R. Miniero, G. Saglio, G. Benetton, R. Coda, G. Cattoretti, and L. Pegoraro. 1988. Selective growth response to IL-3 of a human leukemic cell line with megakaryoblastic features. *Brit. J. Haematol.* 69:359.
27. Kanakura, Y., B. Druker, S.A. Cannistra, Y. Furukawa, Y. Torimoto, and J.D. Griffin. 1990. Signal transduction of the human granulocyte-macrophage colony-stimulating factor and interleukin-3 receptors involves tyrosine phosphorylation of a common set of cytoplasmic proteins. *Blood.* 76:706.
28. Hendrie, P.C., K. Miyazawa, Y. Yang, C.D. Langefield, and H.E. Broxmeyer. 1991. Mast cell growth factor (c-kit ligand) enhances cytokine stimulation of proliferation of human factor dependent cell line MO7e. *Exp. Hematol.* 19:1031.
29. Stein, G.H. 1979. T98G: An anchorage-independent human tumor cell line that exhibits stationary phase G_1 arrest in vitro. *J. Cell. Physiol.* 99:43.
30. Olson, J.E., J.T. Winston, J.A. Whitlock, and W.J. Pledger. 1993. Cell cycle-dependent gene expression in V point-arrested Balb/c3T3 cells. *J. Cell. Physiol.* 154:333.
31. Sala, A., and B. Calabretta. 1992. Regulation of Balb/c3T3 fibroblast proliferation by B-myb is accompanied by selective activation of cdc2 and cyclin D1 expression. *Proc. Natl. Acad. Sci. USA.* 89:10415.
32. Travali, S., K. Reiss, A. Ferber, S. Petralia, W.E. Mercer, B. Calabretta, and R. Baserga. 1991. Constitutively expressed c-myb abrogates the requirement for IGF-1 in 3T3 fibroblasts. *Mol. Cell. Biol.* 11:731.
33. Young, D.C., and J.D. Griffin. 1986. Autocrine secretion of GM-CSF in acute myeloblastic leukemia. *Blood.* 68:1178.
34. Young, D.C., K. Wagner, and J.D. Griffin. 1987. Constitutive expression of the granulocyte-macrophage colony stimulating factor gene in acute myeloblastic leukemia. *J. Clin. Invest.* 79:100.
35. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.