

Stage-related Proliferative Activity Determines *c-myb* Functional Requirements during Normal Human Hematopoiesis

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Abstract

To determine if MYB protein is preferentially required during specific stages of normal human hematopoiesis we incubated normal marrow mononuclear cells (MNC) with *c-myb* antisense oligodeoxynucleotides. Treated cells were cultured in semisolid medium under conditions designed to favor the growth of specific progenitor cell types. Compared with untreated controls, granulocyte-macrophage (GM) CFU-derived colonies decreased 77% when driven by recombinant human (rH) IL-3, and 85% when stimulated by rH GM colony-stimulating factor (CSF); erythroid burst-forming unit (BFU-E)- and CFU-E-derived colonies decreased 48 and 78%, respectively. In contrast, numbers of G-CSF-stimulated granulocyte colonies derived from antisense treated MNC were unchanged from controls, though the numbers of cells composing these colonies decreased ~ 90%. Similar results were obtained when MY10⁺ cells were exposed to *c-myb* antisense oligomers. When compared with untreated controls, numbers of CFU-GM and BFU-E colonies derived from MY10⁺ cells were unchanged, but the numbers of cells composing these colonies were reduced ~ 75 and > 90%, respectively, in comparison with controls. *c-myc* sense and antisense oligomers were without significant effect in these assays. Using the reverse transcription-polymerase chain reaction, *c-myb* mRNA was detected in developing hematopoietic cells on days 0–8. At day 14 *c-myb* expression was no longer detectable using this technique. These results suggest that *c-myb* is required for proliferation of intermediate-late myeloid and erythroid progenitors, but is less important for lineage commitment and early progenitor cell amplification. (*J. Clin. Invest.* 1990. 85:55–61.)
antisense • *c-myb* • hematopoiesis

Introduction

Normal circulating myeloid and erythroid cells are derived from a small complement of bone marrow progenitors that give rise to these morphologically recognizable elements after a myriad of cell proliferation and maturation events (1, 2). The molecular processes that regulate these events, especially in humans, remain largely unknown because of difficulties in

isolating sufficient cellular material with which to conduct such investigations. Based on studies demonstrating that the generation of hematopoietic colonies closely mimics *in vivo* hematopoiesis (3–5); that a variety of recombinant hematopoietic growth factors can be used for the growth of specific progenitor cell types (6, 7); and that antisense oligodeoxynucleotides can block the activity of specific genes in hematopoietic cells (8–10), we have developed a strategy that allows direct assessment of the role of specific genes in regulating hematopoiesis. With this approach we recently showed that exposure of partially purified human hematopoietic progenitor cells to a synthetic oligodeoxynucleotide complementary to *c-myb* mRNA inhibits human hematopoietic colony formation *in vitro* (11). Although those studies provided direct evidence that the *c-myb* gene product plays an important role in regulating hematopoiesis, the stage of hematopoietic development during which *c-myb* gene function is most important remained unknown. In particular, it remained unclear whether *c-myb* function was most relevant for the proliferation of progenitor cells or whether it was needed at an earlier (lineage commitment) or later (precursor differentiation) stage of hematopoietic development. To address these questions, we analyzed the growth of late and early progenitors *in vitro* after exposure to a *c-myb* antisense oligomer. Our findings suggest that *c-myb* gene function is preferentially required for the proliferation of intermediate-late hematopoietic progenitors; is less critical for the proliferation of early progenitors; and may not be required for lineage commitment.

Methods

Cells. Human bone marrow cells were obtained from normal healthy volunteers after informed consent. Light density mononuclear cells (MNC)¹ were obtained by Ficoll-Hypaque density gradient centrifugation and enriched for hematopoietic progenitors by first removing adherent and phagocytic elements and then T lymphocytes as previously described (12). MNC depleted of adherent/phagocytic and T cells (A[–]T-MNC) were then plated in semisolid medium or further enriched for immature progenitors by immunorsetting with anti-HPCA-1 MAb (Becton Dickinson, Mountain View, CA), which recognizes the MY10 or CD34 antigen (13). Briefly, packed sheep red blood cells (SRBC) were incubated with an equal volume of 0.1% CrCl₃ and F(ab)₂ goat anti-mouse affinity-purified F(ab)₂ (1 mg/ml; Cappel Labo-

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1. **Abbreviations used in this paper:** A[–]T-MNC, MNC depleted of adherent/phagocytic and T cells; BFU-E, burst-forming unit-erythrocyte; CFU-E, CFU erythrocyte; CFU-G, CFU granulocyte; CFU-GM, CFU granulocyte/macrophage; FBS, fetal bovine serum; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; IMDM, Iscove's modified Dulbecco's medium; MNC, mononuclear cells; RT-PCR, reverse transcriptase-polymerase chain reaction; SRBC, sheep red blood cells.

ratories, Cochranville, PA) for 10 min at room temperature. SRBC were then extensively washed in normal saline and resuspended at a concentration of 2% in normal saline (200 μ l of packed SRBC in a final volume of 10 ml). Marrow cells were incubated for 60 min at 4°C (6 \times 10⁶ cells/ml) in the presence of 1:50 anti-HPCA-1 MAb. After extensive washing, antibody-treated SRBC (0.4 ml/10⁶ cells) were added to cells, pelleted, and incubated for 1 h at 4°C. Rosetted cells were then separated on a Ficoll gradient and SRBC were subsequently eliminated with a lysing reagent (Ortho Pharmaceutical, Raritan, NJ).

Colony assays. 2.5 \times 10⁴ A⁺T⁺MNC or 4 \times 10³ MY10⁺ MNC were plated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum (FBS) and 0.3% agar (Difco Laboratories Inc., Detroit, MI) or in IMDM, 30% FBS, 10⁻⁴ M, β -mercaptoethanol (Sigma Chemical Co., St. Louis, MO), and 0.9% methylcellulose (Dow Chemical Co., Indianapolis, IN) in 35-mm petri dishes (Corning Glass Works, Corning, NY) at 1 ml/dish. For myeloid colony stimulation, granulocyte/macrophage colony-stimulating factor (GM-CSF; 5 ng/ml), IL-3 (20 U/ml), or (granulocyte colony-stimulating factor (G-CSF; 10% of transfected Chinese hamster ovary cell supernatant) were used. CFU erythroid (CFU-E) colonies were obtained in the presence recombinant human erythropoietin alone (3 U/ml; Amgen Biologicals, Thousand Oaks, CA). Burst-forming units-erythroid (BFU-E) colonies were obtained in the presence of a combination of IL-3 (10 U/ml), GM-CSF (5 ng/ml), and erythropoietin (3 U/ml). Colonies were scored after 14–16 d of culture in a humidified 5% CO₂ incubator. CFU-E and G-CSF-stimulated granulocytic colonies were scored at 7–9 d of culture. To determine colony size, 30 consecutive individual colonies from each plate were plucked from methylcellulose, dispersed in tissue culture medium, and then counted in a hemocytometer. Cells in smaller colonies were counted directly in the plates.

Oligodeoxynucleotides. Unmodified, 18-base oligodeoxynucleotides were made on a DNA synthesizer (model 380B; Applied Biosystems Inc., Foster City, CA) by means of β -cyanoethyl-phosphorymidite chemistry. Oligodeoxynucleotides were purified by ethanol precipitation and multiple washes in 70% ethanol. They were subsequently lyophilized to dryness and redissolved in culture medium at a concentration of 1 mg/ml. The sequence of the *c-myb* antisense oligomer is 5'-GTGCCGGGTCTTCGGGC-3' and is complementary to 18 nucleotides starting from the second codon of *c-myb* mRNA (14); the *c-myb* sense oligomer has the sequence 5'-GCCCCAAGACCCCGG-CAC-3'. The sequence of the *c-myc* antisense oligomer is 5'-GAAGC-TAACGTTGAGGGG-3' and is complementary to 18 nucleotides starting from the second codon of *c-myc* mRNA (15). The *c-myc* sense oligomer has the sequence 5'-CCCCTCAACGTTAGCTTC-3'.

Oligomer treatment of cells. 2.5 \times 10⁴ A⁺T⁺MNC or 4 \times 10³ MY10⁺ MNC from each sample were incubated in 0.2 ml of IMDM, 20% FBS in polypropylene tubes (Falcon Plastics, Cockeysville, MD) in the presence of optimal concentrations of the relevant hematopoietic growth factor. Some cultures were also supplemented with 100 μ g/ml of either sense or antisense oligomers for 18 h at a final concentration of 17.5 μ M. Cells were not washed before plating. Control cultures were left untreated.

Detection of *c-myb* mRNA in bone marrow cells by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Expression of *c-myb* mRNA in A⁺T⁺MNC and MY10⁺ MNC was analyzed as follows: cells seeded at 4 \times 10⁴ were collected at the end of the required time of culture and total RNA was extracted in the presence of 20 μ g of *Escherichia coli* ribosomal RNA as described (16). RNA was then reverse-transcribed using 500 U of Moloney murine leukemia virus reverse transcriptase and 0.2 μ g of oligo(dT) as primer for 1 h at 37°C. The resulting cDNA fragments were amplified with 7.5 U of *Thermus aquaticus* (Taq) polymerase in the presence of *c-myb* mRNA sequence-specific synthetic primers; the 5' oligonucleotide corresponds to the *c-myb* mRNA sequence from nucleotides 2,258–2,279 and the 3' oligonucleotide corresponds to nucleotides 2,466–2,487. Therefore, a 230-bp fragment corresponding to a portion of the 3' untranslated region of *c-myb* (14) was generated during 60 cycles of polymerase

chain reaction (17). 10 μ l of the 50- μ l polymerase chain reaction was separated in a 4% Nusieve agarose gel and transferred to a nitrocellulose filter. The resulting blot was hybridized with a synthetic 50-base *c-myb* oligomer complementary to the amplified *c-myb* cDNA from nucleotides 2,351–2,400 (14). The synthetic *c-myb* oligomer was end-labeled with [³²P]-ATP and polynucleotide kinase as described (18).

Detection of *myb* protein. 5 \times 10⁴ MY10⁺ MNC, separated as described above, were plated in 96-well plates (Costar, Cambridge, MA) in 200 μ l/IMDM, 20% FBS per well in the presence of GM-CSF (5 ng/ml) plus IL-3 (20 U/ml). At days 0 and 21 of culture in a humidified incubator at 37°C cells were placed on polylysine-treated slides, cytocentrifuged at 400 rpm for 5 min (Shandon II), and fixed with methanol/acetone (1:9) for 15 min at room temperature. Expression of *myb* antigen was assessed by indirect immunofluorescence with a sheep anti-human *myb* serum (Cambridge Research Biochemicals, Valley Stream, NY) at a 1:40 dilution (19, 20).

Results

Effect of sense and antisense *myb* and *myc* oligomers on human bone marrow (A⁺T⁺MNC). A⁺T⁺MNC were exposed to oligomer preparations for 18 h and then cloned in cultures optimized for the growth of specific progenitor cell subsets (see methods). Data relative to erythroid progenitor cell growth are shown in Fig. 1. Bars express percent of growth of antisense- vs. sense-treated cells (assumed as 100%) and are mean \pm SD of three different experiments. Mean colony yield was 54 \pm 2 (2.5 \times 10⁴ cells plated) for BFU-E and 298 \pm 20 for CFU-E in three different experiments. Sense oligomers gave 2–20% inhibition compared with untreated controls. Treatment with the *c-myb* antisense oligomer resulted in a 48% decrement in the number of BFU-E colonies, whereas the decrease of CFU-E colonies was 78%, a value consistent with our previous findings (11). Colony size was also analyzed (Table I). In the case of BFU-E, median colony size was 12,000 for untreated controls. Sense oligomers gave only a small reduction in the number of cells per colony (10,000 = 16.6% inhibition), whereas antisense oligomers effected a drastic reduction in colony size (1,500 cells per colony = 87.5% inhibition). When erythroid colonies formed in the presence of a *c-myc* antisense oligomer, no decrement in either colony size or number was noted as compared with control colonies. An analysis of myeloid colony formation in the presence of *c-myb* and *c-myc* oligomers is shown in Fig. 2. Data relative to CFU-granulocyte/macrophage (CFU-GM) growth in the presence of IL-3 or GM-CSF, and G-CSF-stimulated CFU-granulocyte (CFU-G) growth are presented. Mean colony yield (per 2.5 \times 10⁴ cells plated) was 83 \pm 6 and

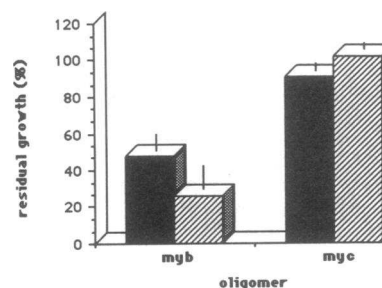


Figure 1. Effect of antisense vs. sense *c-myb* and *c-myc* oligomers on in vitro colony formation of human erythroid progenitors. Bars express percent of colony number of antisense- vs. sense-treated A⁺T⁺MNC. Values are mean \pm SD of three and four different experiments for BFU-E (■) and CFU-E (▨), respectively, from duplicate plates. CFU-E were scored after 7 d of culture. BFU-E were scored after 16 d of culture.

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Table I. Effect of Sense and Antisense Oligodeoxynucleotides on the Size of Colonies Obtained from A⁺T⁺MNC

	Erythroid progenitors		
	BFU-E	CFU-E	
Control	12,000 (10,500–14,000)	36 (6–40)	
<i>c-myb</i> sense	10,000 (9,000–10,500)	38 (6–40)	
<i>c-myb</i> antisense	1,500 (1,000–2,000)	36 (6–40)	
<i>c-myc</i> antisense	12,500 (11,000–14,000)	37 (6–40)	
	Myeloid progenitors		
	CFU-GM (GM-CSF)	CFU-GM (IL-3)	CFU-G (G-CSF)
Control	650 (80–800)	500 (80–600)	850 (100–1,000)
<i>c-myb</i> sense	500 (50–600)	350 (50–400)	650 (100–800)
<i>c-myb</i> antisense	55 (40–80)	45 (40–60)	60 (40–70)
<i>c-myc</i> antisense	700 (80–800)	550 (80–600)	800 (100–1,000)

Partially purified bone marrow cells (A⁺T⁺MNC) were cultured in semisolid medium under optimal conditions for the different subsets tested as described in Methods. After appropriate incubation time (14 d for BFU-E and CFU-GM, and 7–9 d for CFU-G and CFU-E, respectively) the colony size was evaluated. From each culture plate 30 consecutive individual colonies were examined for their size. For smaller colonies a cell count was made directly on the culture plate; bigger colonies were picked up with a drawn Pasteur pipette and dispersed in culture medium, and cells were counted in a hemocytometer. Numbers express the median value observed. The variation range is given in parentheses.

163±9 in GM-CSF- and IL-3-stimulated cultures, respectively. Treatment with sense oligomers resulted in a 6–20% inhibition compared with growth in untreated control cultures. Regardless of the hemopoietin used, colony growth was inhibited in the presence of *c-myb* antisense oligomers (77% inhibition of colonies formed in presence of IL-3 and 85% inhibition of colonies formed in presence of GM-CSF). Most of the residual colonies were macrophage and eosinophilic in type. CFU-GM colony growth was unaffected when progenitor cells were exposed to *c-myc* antisense oligomers (94–112% growth vs. controls).

Day 7 granulocyte progenitors (CFU-G) are developmentally more mature than day 14 CFU-GM and give rise to single lineage colonies in response to G-CSF after 7 d of culture. In

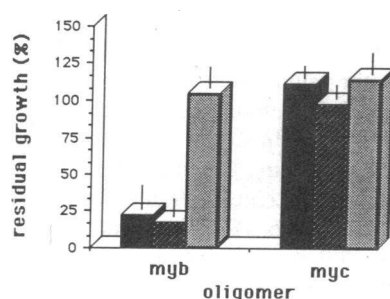


Figure 2. Effect of antisense vs. sense *c-myb* and *c-myc* oligomers on in vitro colony formation of human myeloid progenitors. Bars express percent of colony number of antisense- vs. sense-treated A⁺T⁺MNC. Values are mean±SD of four different experiments from

duplicate plates. Colonies were scored after 14 d of culture (7–9 d for G-CSF-stimulated cultures). ■, CFU-GM (IL-3); □, CFU-GM (GM-CSF); ▨, CFU-G (G-CSF).

our culture system, control CFU-G cultures contained 327±28 colonies per 2.5×10^4 cells plated. Treatment with *c-myb* sense oligomer inhibited the number of colonies in 9–20% of controls, and no further decrease in colony number was observed when cells were exposed to *c-myb* antisense or *c-myc* sense or antisense oligomers (Fig. 2). However, analysis of CFU-G colony size (Table I and Fig. 3) revealed a ~90% reduction in the numbers of cells/colony formed in the presence of *c-myb* antisense oligomer (median size, 60 cells/colony) as compared with control colonies (median size, 850 cells/colony) or those arising in the presence of the *c-myb* sense oligomer (median size, 650 cells/colony) or the *c-myc* antisense oligomer (median size, 800 cells/colony).

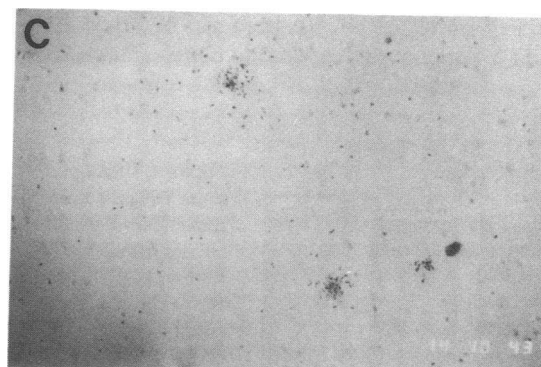
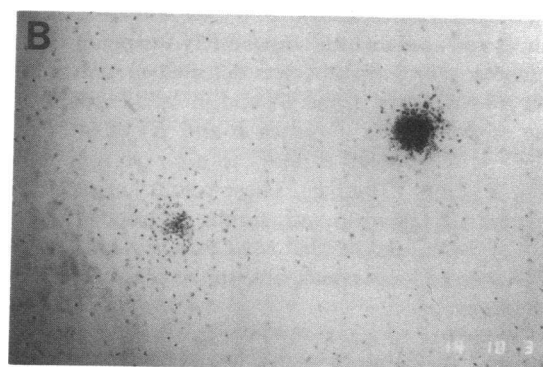
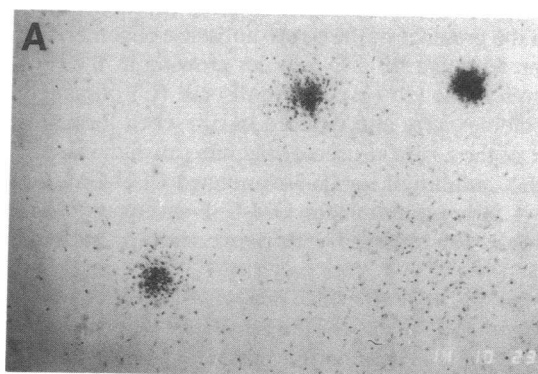


Figure 3. Effect of *c-myb* sense and antisense oligomers on neutrophilic granulocytic colony formation. CFU-G colonies grown in the presence of recombinant human G-CSF from A⁺T⁺MNC after 7 d of culture. A, control; B, colonies after treatment with a sense oligomer; C, colonies after treatment with an antisense oligomer.

Effect of sense and antisense *myb* and *myc* oligomers on marrow MY10⁺ MNC. To analyze the effect of antisense oligomers on the growth of a more homogeneous, less mature population of progenitors the same set of experiments were performed on marrow cells enriched for MY10⁺ MNC. Because neither CFU-E nor CFU-G are contained within this cell population (21), only day 14 CFU-GM and BFU-E derived colonies were studied. Mean colony yield was 56±3 for BFU-E and 63±6 or 86±4 for CFU-GM, stimulated with IL-3 or GM-CSF, respectively. Little inhibition in the numbers of colonies formed was observed in either the sense- or antisense-treated cultures (Fig. 4). However, colony size was consistently affected (Table II). Median colony size for BFU-E formed in presence of *c-myb* sense oligomer was 15,500 cells/colony. This value was reduced to 4,000 cells/colony (average inhibition 75%) in the presence of the *c-myb* antisense oligomer. Fig. 5 shows representative BFU-E colonies growing in the presence of *c-myb* sense (B) or *c-myb* antisense (C) oligomers. CFU-GM colonies were also reduced in size when formed in the presence of the *c-myb* antisense oligomer (median value 55 vs. 700 = 93% inhibition for IL-3-stimulated CFU-GM, and 55 vs. 550 = 90% inhibition for GM-CSF-stimulated CFU-GM). No effect was observed with *c-myc* sense or antisense oligomers.

Expression of *c-myb* mRNA in bone marrow cells exposed to *c-myb* oligomers. To address the possibility that the differential effect of the *c-myb* antisense oligodeoxynucleotides on the various progenitor subsets was due to differential cellular uptake of oligomers, equal numbers of MY10⁺ MNC and A-T⁺MNC (4×10^4) were exposed to *c-myb* sense and antisense sequences and analyzed for *c-myb* mRNA expression by RT-PCR analysis. *c-myb* mRNA was detected in untreated marrow cells (Fig. 6, lanes A and D) and in cells exposed to *c-myb* sense oligomer (Fig. 6, lanes B and E) but not in MY10⁺ MNC or A-T⁺MNC exposed to a *c-myb* antisense oligomer (Fig. 6, lanes C and F). These results suggest that *c-myb* oligomers are taken up with similar efficiency by the majority of A-T⁺MNC and MY10⁺ MNC, since in both cases *c-myb* mRNA was no longer detectable in the presence of the antisense oligomer.

The disappearance of *c-myb* mRNA in antisense-treated cultures probably results from *c-myb* mRNA degradation after the formation of specific DNA-RNA duplexes that may provide a substrate for cellular RNase-H activity (22); a specific disappearance of *c-myb* mRNA has been also observed in peripheral blood T lymphocytes exposed to *c-myb* antisense oli-

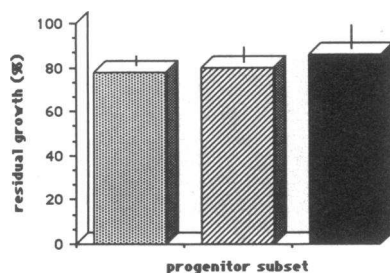


Figure 4. Effect of antisense vs. sense *c-myb* oligomers on in vitro colony formation of human MY10⁺ MNC. Bars express percent of colony number of antisense- vs. sense-treated cultures. Values are mean±SD of three different experiments in

duplicate plates. 4×10^3 cells/ml were plated. Colonies were scored after 16 d of culture. □, BFU-E; ▨, CFU-GM (IL-3); ■, CFU-GM (GM-CSF).

godeoxynucleotides (20), and disappearance of *c-abl* transcripts has been observed in K562 cells exposed to *c-abl* antisense oligodeoxynucleotides (22a).

Detection of *c-myb* mRNA and *c-myb* protein during in vitro differentiation of MY10⁺ MNC. To follow the expression of *c-myb* mRNA during the differentiation of marrow MY10⁺ MNC, these cells were cultured for different time periods in the presence of IL-3 and GM-CSF, and *c-myb* mRNA levels were measured by RT-PCR analysis using 1.6×10^5 or 1×10^5 cells. In each experiment $7-8 \times 10^7$ marrow cells were used for purification with a final yield of $1-3 \times 10^5$ MY10⁺ MNC enriched elements. Fig. 7 shows a Southern blot of the products of cDNA amplification using a 5' and a 3' *c-myb* sequence-specific primers hybridized to an end-labeled diagnostic probe 50 bases long corresponding to the amplified *c-myb* fragment from nucleotides 2,351-2,400 (14). A 230-bp *c-myb* amplified fragment is detected in MY10⁺ MNC at day 0 (Fig. 7, lane A) and after growth in culture in the presence of GM-CSF and IL-3 for 4 and 8 d when *c-myb* mRNA levels were slightly increased (Fig. 7, lanes B and C), but not after 14 d in culture (Fig. 7, lane D); in a second experiment *c-myb* mRNA was detected in MY10⁺ MNC at day 0 (Fig. 7, lane E) but was no longer detectable at day 14 (Fig. 7, lane F). This kinetics suggests that *c-myb* mRNA expression is maximal in actively cycling cultured MY10⁺ MNC, whereas *c-myb* mRNA is undetectable when MY10⁺ MNC are well advanced in their process of differentiation (not shown). We also analyzed the expression of *c-myb* protein by immunofluorescence and in agreement with the observations of Kastan et al. (23) detected *c-myb* protein in marrow MY10⁺ on day 0, but not after 21 d in culture in the presence of GM-CSF and IL-3 (not shown).

Discussion

Under homeostatic conditions hematopoiesis is maintained by a small subset of pluripotent stem cells and unipotent progenitor cells that reside in the G₀ phase of the cell cycle. When needed, these cells enter into the cell cycle, synthesize DNA, and divide to generate an amplified population of functionally mature elements. The cloning of the genes for several hematopoietic growth factors and the resulting production of the encoded recombinant proteins have led to major conceptual advances in understanding the regulation of normal hematopoiesis (6). However, the intermediary events that occur distal to the interaction between growth factors and their own receptors and the nuclear processes that specifically regulate proliferation and differentiation during hematopoietic development are still poorly understood (24). In theory, the activation of nuclear protooncogenes could provide a functional link between events occurring at the cellular membrane and the ensuing proliferative and maturative responses of the hematopoietic and other cellular systems (25). In fact, several studies have provided indirect evidence suggesting the importance of the *c-myb* nuclear protooncogene in regulating hematopoietic proliferation and differentiation (26-28). We recently took a direct approach and reported that a *c-myb* antisense oligomer inhibits in vitro growth of CFU-E, CFU-GM, and CFU-megakaryocyte colonies from normal bone marrow progenitors depleted of adherent cells and T lymphocytes (11). In the present study we analyzed different subsets of human hematopoietic progenitors to ascertain whether inhibition of colony forma-

Table II. Effect of Sense and Antisense Oligodeoxynucleotides on the Size of Colonies Obtained from Marrow MY10 MNC

	BFU-E	CFU-GM (IL-3)	CFU-GM (GM-CSF)
Control	17,500 (16,000–20,000)	1,000 (100–1,200)	700 (100–800)
<i>c-myb</i> sense	15,500 (13,500–18,000)	700 (80–800)	550 (80–700)
<i>c-myb</i> antisense	4,000 (3,500–5,000)	55 (40–80)	55 (40–60)

MY10 MNC were cultured in semisolid medium under optimal culture conditions for the different subsets tested as described in Methods. After appropriate incubation time (16 d) the colony size was evaluated. For each culture plate the size of 30 consecutive individual colonies was examined as described in Table I. Numbers express the median value observed. The variation range is given in parentheses.

tion by a *c-myb* antisense oligomer is restricted to a specific level of maturation. Inhibition of the proliferation of erythroid progenitors was observed when the target cells analyzed were

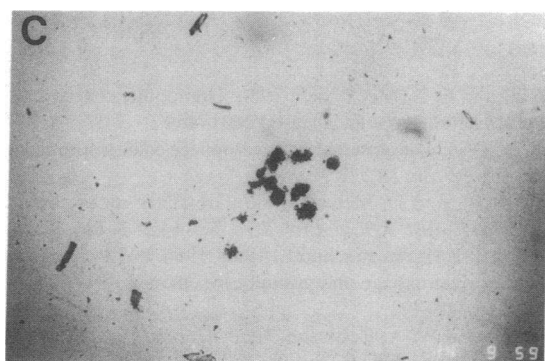
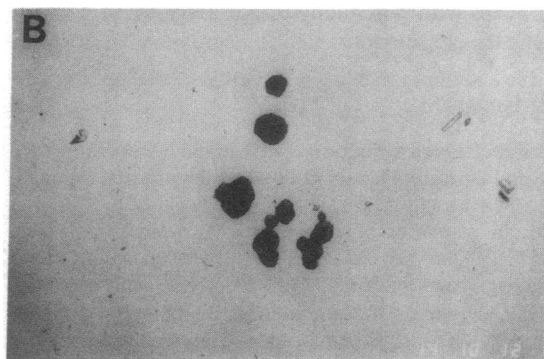
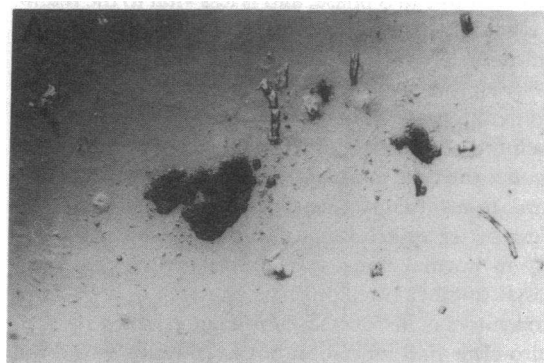


Figure 5. Erythroid bursts from MY10⁺ MNC. A, control; B, colonies after treatment with a *c-myb* sense oligomer; C, colonies after treatment with a *c-myb* antisense oligomer.

CFU-E and BFU-E derived from bone marrow cells depleted of adherent cells and T lymphocytes. Colony inhibition was most pronounced in CFU-E (22% of that observed in *c-myb* sense-containing dishes) but was also observed in the growth of BFU-E colonies (52% of *c-myb* sense-treated dishes). No inhibition of colony number was observed with a subset of more primitive BFU-E derived from the population enriched in the more immature MY10⁺ progenitors (21), but colony size was much smaller (Table II, Fig. 5). Analogous results were obtained from BFU-E grown from peripheral blood (not shown), which is known to contain only the more immature progenitor subsets (29, 30).

Interestingly, the inhibitory effect of the *c-myb* antisense oligomer is most pronounced in the CFU-E compartment in which 60–80% of the cells are in S phase and virtually all CFU-E are actively progressing through the cell cycle (31). In contrast, < 30% of the primitive BFU-E are in S phase and at least half of the cells in the BFU-E compartment are not cycling (31). Since the exposure of BFU-E progenitors to the *c-myb* antisense oligomer reduced the size of the BFU-E colonies without affecting the total colony number, it is likely that the antisense *c-myb* oligomer maximally inhibits colony formation once the primitive BFU-E enter into the pool of actively cycling progenitors.

A similar line of reasoning might explain the effects of the *c-myb* antisense oligomer on the CFU-GM and CFU-G progenitors. The most pronounced effect is on CFU-GM progenitors derived from partially purified marrow cells (~ 80% growth inhibition), while the effect on the more primitive

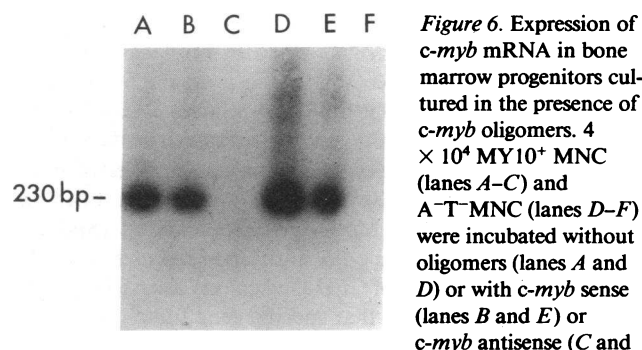


Figure 6. Expression of *c-myb* mRNA in bone marrow progenitors cultured in the presence of *c-myb* oligomers. 4×10^4 MY10⁺ MNC (lanes A–C) and A-T MNC (lanes D–F) were incubated without oligomers (lanes A and D) or with *c-myb* sense (lanes B and E) or *c-myb* antisense (C and F) oligomers in the presence of IL-3 and GM-CSF. Cells were exposed to oligomers (final concentration 14 M) for 48 h, collected, and total RNA extracted as described (16). *c-myb* mRNA was amplified by the RT-PCR technique (17) and detected by hybridizing the products of cDNA amplification with a diagnostic ³²P-end-labeled 50-base oligomer corresponding to a region within the amplified *c-myb* mRNA from nucleotides 2,351–2,400 (14).

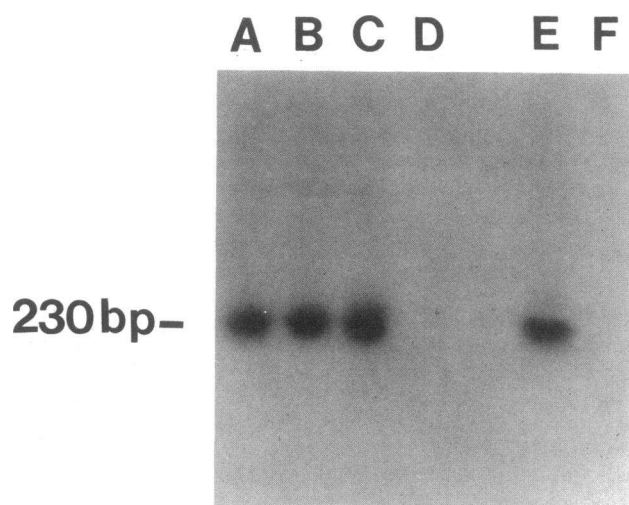


Figure 7. Kinetics of expression of *c-myb* mRNA in MY10⁺ MNC cultured in the presence of GM-CSF and IL-3. Detection of *c-myb* mRNA was as described in Fig. 6. Experiment 1: (A, MY10⁺ MNC RNA at day 0; B, MY10⁺ MNC at day 4; C, MY10 MNC at day 8; D, MY10⁺ MNC at day 14); experiment 2: (E, MY10⁺ MNC at day 0; F, MY10⁺ MNC at day 14).

CFU-GM progenitors (MY10⁺ MNC) is manifested as a reduction in colony size (Table II). Analogous results were obtained from CFU-GM growth from peripheral blood (not shown), which contains only the more immature progenitors (30, 32). Exposure of the more mature CFU-G to the *c-myb* antisense oligomer resulted in a significant decrement in colony size (Table I). CFU-G have very high proliferative activity (50% of the cells in S phase; 29), but the number of cells per colony is much higher than that of the analogous late progenitors in the erythroid compartment (CFU-E; Table I). This may explain why the antisense *myb* oligomer reduces CFU-E derived colony formation while the effect on CFU-G is primarily manifested as reduction in colony size.

The differential effect the *c-myb* antisense oligomers on early and late progenitors could have occurred as consequence of differences in cellular uptake of these oligomers that affects their ability to inhibit *c-myb* function equally in both progenitor types. The experiment in Fig. 6 suggests that *c-myb* oligomers are taken up with similar efficiency by the majority of A-T⁺MNC and MY10⁺ MNC since in both cases *c-myb* mRNA was no longer detectable in the presence of the antisense oligomer. However, the data do not prove unequivocally that *c-myb* antisense oligodeoxynucleotides equally inhibit *c-myb* function in the colony-forming progenitors of each compartment, since these cells are a minority both in A-T⁺MNC and in MY10⁺ MNC. On the other hand, exposure of MY10⁺ MNC to several *c-abl* antisense oligomers resulted in a significant inhibition of CFU-GM colony formation (22a), validating the assumption that specific gene functions are inhibited in MY10⁺ progenitors exposed to synthetic oligomers.

We have also noted that exposure of early and late progenitors to *c-myc* antisense oligodeoxynucleotides does not have measurable effects on in vitro hematopoietic colony growth. It has been shown that the expression of a *c-myc* antisense construct introduced by transfection in Friend murine erythro-

leukemia cells accelerates erythroid differentiation in response to a DMSO (33) and that exposure of HL-60 cells to a *c-myc* antisense oligomer inhibits proliferation and induces terminal differentiation (10). In contrast, exposure of HL-60 cells to a *c-myb* antisense oligomer does not induce differentiation (19). These findings suggest that *c-myb* and *c-myc* play different roles in regulating hematopoietic growth and differentiation: downregulation of *c-myb* expression might directly affect hematopoietic proliferation, whereas low levels of *c-myc* protein might be necessary to allow hematopoietic cells to enter the differentiation pathway (10, 33). If downregulation of *c-myc* expression also accelerates the differentiation of normal marrow progenitors it would be difficult to observe such effect under the standard conditions of colony formation we used.

In summary, our findings suggest that *c-myb* function is most needed in the compartments containing the highest number of progenitors in S phase, and is less vital to the maintenance of compartments containing earlier progenitors with low proliferative activity. Nevertheless, while these results suggest that *c-myb* protein is most important for cells that are actively proliferating, we cannot exclude the possibility that actively cycling progenitors undergo maturational changes that may render the cells more dependent on *c-myb* function. In this regard, it has been suggested that *c-myb* protein levels may correlate better with cell maturation than cell proliferative activity in normal bone marrow progenitor cells (23). Since the total number of colonies of any type derived from MY10⁺ progenitors is not decreased in comparison with controls it is also clear that inhibition of *c-myb* function did not adversely affect lineage commitment. Of more general importance, these studies should enable more rigorous analyses of many other genes with potentially important roles in normal hematopoietic development.

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References

1. Till, J. E., and E. A. McCulloch. 1980. Hemopoietic stem cell differentiation. *Biochim. Biophys. Acta.* 605:431-459.
2. Metcalf, D. 1985. The granulocyte-macrophage colony-stimulating factors. *Science (Wash. DC)*. 229:16-22.
3. Bradley, T. R., and D. Metcalf. 1966. Growth of mouse bone marrow cells in vitro. *Aust. J. Exp. Biol. Med. Sci.* 44:287-296.
4. Ichikawa, Y., D. H. Pluznik, and L. Sachs. 1966. In vitro control of development of macrophage and granulocyte colonies. *Proc. Natl. Acad. Sci. USA*. 56:488-492.
5. Pike, B. L., and W. A. Robinson. 1970. Human bone marrow colony growth in agar gel. *J. Cell Physiol.* 76:77-85.
6. Clark, S. C., and R. Kamen. 1987. The human hematopoietic colony-stimulating factors. *Science (Wash. DC)*. 236:1229-1237.

7. Seiff, C. A. 1987. Hematopoietic growth factors. *J. Clin. Invest.* 79:1549-1557.
8. Zamecnik, P. C., J. Goodchild, Y. Taguchi, and P. S. Sarin. 1986. Inhibition of replication and expression of human T-cell lymphotropic virus type III in culture cells by exogenous synthetic oligonucleotides complementary to viral RNA. *Proc. Natl. Acad. Sci. USA.* 83:4143-4146.
9. Heikkila, R., G. Schwab, E. Wickstron, S. L. Loke, D. H. Pluznik, R. Watt, and L. M. Neckers. 1987. A c-myc antisense oligodeoxynucleotides inhibit entry into S phase but not progress from G₀ to G₁. *Nature (Lond.)*. 328:445-449.
10. Holt, J. T., R. L. Redner, and A. W. Nienhuis. 1988. An oligomer complementary to c-myc messenger-RNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol. Cell. Biol.* 8:963-973.
11. Gewirtz, A. M., and B. Calabretta. 1988. A c-myc antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. *Science (Wash. DC)*. 242:1303-1306.
12. Caracciolo, D., N. Shirsat, G. G. Wong, B. Lange, S. Clark, and G. Rovera. 1987. Recombinant human M-CSF requires subliminal concentration of GM-CSF for optimal stimulation of human macrophage colony formation in vitro. *J. Exp. Med.* 166:1851-1860.
13. Civin, C. I., L. C. Strauss, C. Brovall, M. J. Fackler, J. F. Schwartz, and J. H. Shaper. 1984. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-la cells. *J. Immunol.* 133:157-165.
14. Majello, B., L. C. Kenyon, and R. Dalla-Favera. 1986. Human c-myc proto-oncogene: nucleotide sequence of cDNA and organization of the genomic locus. *Proc. Natl. Acad. Sci. USA.* 83:9636-9640.
15. Watt, R., L. W. Stanton, K. B. Marcu, R. C. Gallo, C. M. Croce, and G. Rovera. 1983. Nucleotides sequence of cloned cDNA of the human c-myc oncogene. *Nature (Lond.)*. 303:725-728.
16. Rappolee, D. A., D. Mark, M. J. Benda, and Z. Werb. 1988. Wound macrophages express TGF- and other growth factors in vitro: analysis by mRNA phenotyping. *Science (Wash. DC)*. 241:708-712.
17. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Wash. DC)*. 239:487-491.
18. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
19. Anfossi, G., A. M. Gewirtz, and B. Calabretta. 1989. An oligomer complementary to c-myc encoded mRNA inhibits proliferation of human myeloid leukemia cell lines. *Proc. Natl. Acad. Sci. USA.* 86:3379-3383.
20. Gewirtz, A. M., G. Anfossi, D. Venturelli, S. Valpreda, R. Sims, and B. Calabretta. 1989. G₁/S transition in normal human T-lymphocytes requires the nuclear protein encoded by c-myc. *Science (Wash. DC)*. 245:180-183.
21. Brandt, J., N. Baird, L. Lu, E. Srouf, and R. Hoffman. 1988. Characterization of a human hematopoietic progenitor cell capable of forming blast cell containing colonies in vitro. *J. Clin. Invest.* 82:1017-1027.
22. Dash, P., I. Lotan, M. Knapp, E. R. Kandel, and P. Goelet. 1987. Selective elimination of mRNA in vivo: complementary oligodeoxynucleotides promote RNA degradation by an RNase H-like activity. *Proc. Natl. Acad. Sci. USA.* 84:7896-7900.
- 22a. Caracciolo, D., M. Valtieri, D. Venturelli, C. Peschle, A. M. Gewirtz, and B. Calabretta. 1989. Lineage specific requirement of c-abl function in normal hematopoiesis. *Science (Wash. DC)*. 245:1107-1109.
23. Kastan, M. B., D. J. Slamon, and C. I. Civin. 1989. Expression of proto-oncogene c-myc in normal human hematopoietic cells. *Blood.* 73:1444-1451.
24. Sachs, L. 1987. The molecular control of blood cell development. *Science (Wash. DC)*. 238:1374-1379.
25. Weinberg, R. A. 1985. The action of oncogenes in the cytoplasm and nucleus. *Science (Wash. DC)*. 230:770-776.
26. Westin, E. H., R. E. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal. 1982. Differential expression of the amv-gene in human hematopoietic cells. *Proc. Natl. Acad. Sci. USA.* 79:2194-2198.
27. Gonda, T. J., and D. Metcalf. 1984. Expression of myb, myc, and fos protooncogenes during the differentiation of a murine myeloid leukemia. *Nature (Lond.)*. 310:249-251.
28. Clarke, M. D., J. F. Kukowska-Latallo, E. Westin, M. Smith, and E. V. Prochownik. 1988. Constitutive expression of a c-myc cDNA blocks Friend murine erythroleukemia cells differentiation. *Mol. Cell. Biol.* 8:884-882.
29. Lipton, J. M., M. Kudish, and D. G. Nathan. 1981. Response of three clones of human erythroid progenitors to the absence of erythropoietin in vitro as a measure of progenitor maturity. *Exp. Hematol. (NY)*. 9:1035-1041.
30. Ferrero, D., H. E. Broxmeyer, G. L. Pagliardi, S. Venuta, B. Lang, S. Pessano, and G. Rovera. 1983. Antigenically distinct subpopulations of myeloid progenitor cells (CFU-GM) in human peripheral blood and marrow. *Proc. Natl. Acad. Sci. USA.* 80:4114-4118.
31. Iscove, N. N. 1977. Role of erythropoietin in regulation of population size and cell cycle of early and late erythroid precursors in mouse marrow. *Cell Tissue Kinet.* 10:323-350.
32. Tebbi, K., L. Rubin, D. H. Cowan, and E. A. McCulloch. 1978. Granulopoiesis in culture from blood and marrow cells of nonleukemic individuals and patients with acute leukemia. *Blood.* 48:235-243.
33. Prochownik, E. V., J. Kukowska, and C. Rodgers. 1988. c-myc antisense transcripts accelerate differentiation and inhibit G₁ progression in murine erythroleukemia cells. *Mol. Cell. Biol.* 8:3683-3695.