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# An oligomer complementary to c-*myb*-encoded mRNA inhibits proliferation of human myeloid leukemia cell lines

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ABSTRACT **To study the role of the protooncogene c-*myb* in regulating myeloid leukemia cell proliferation and differentiation, we exposed cells of the human leukemia lines HL-60, ML-3, KG-1, and KG-1a to an oligodeoxynucleotide complementary to an 18-base-pair (bp) sequence of c-*myb*-encoded mRNA. This treatment resulted in a significant decrease in cell proliferation in all of the lines, which was most marked in HL-60 cells. After 5 days in culture, in several separate experiments with different oligomer preparations, 75% growth inhibition was observed in c-*myb* antisense treated cells in comparison to untreated HL-60 cells. Two c-*myb* antisense oligomers of identical length with either 2- or 4-bp mismatches had no effect on cell growth nor did an 18-bp c-*myb* sense or myeloperoxidase antisense oligomer. The effect of a c-*myc* antisense oligomer (18 bp) on the growth of HL-60, KG-1, and KG-1a cells was also studied. This oligomer had much less inhibitory effect on cell proliferation than did the c-*myb* antisense sequence. Interestingly, although c-*myc* antisense treatment induced maturation of HL-60 cells while it inhibited cell proliferation, such an effect was not noted in c-*myb* antisense treated cells. These studies indicate that the nuclear protein encoded by the c-*myb* protooncogene is required for maintenance of proliferation in certain leukemia cell lines. In addition, the stringent requirements for c-*myb* protein as compared to c-*myc* protein suggest that, at least in HL-60 cells, c-*myc* amplification or N-*ras* activation may not be sufficient to maintain the leukemic growth in the absence of c-*myb* protein. These findings support the hypothesis that development and maintenance of a malignant phenotype requires a multiplicity of interrelated genetic events.**

Experimental evidence suggests that the protooncogene c-*myb* plays a major role in the regulation of hemopoiesis *in vitro* (1, 2). For instance, a number of leukemia cell lines have high c-*myb*-encoded mRNA levels that decline during the process of terminal differentiation (3–5). It has also been shown that constitutive expression of an exogenously introduced c-*myb* construct inhibits the erythroid differentiation of a murine erythroleukemia cell line (MEL) in response to known inducing agents (6). Most recently we have demonstrated that exposure of partially purified human hematopoietic progenitor cells to a synthetic oligodeoxynucleotide complementary to c-*myb* mRNA inhibits the formation of erythroid, myeloid, and megakaryocytic colonies in semisolid culture medium (7). In this regard, we found that when normal human hematopoietic progenitor cells were cloned in the presence of the oligomer complementary to c-*myb*, the resulting colonies had fewer cells than normal colonies. The reduction in the number of cell colonies was noted very early (4 days after plating) during the process of colony formation. These findings suggested that the effect of the c-*myb* antisense oligodeoxynucleotide was to inhibit the proliferation of early hematopoietic progenitor cells. To determine if a c-*myb* antisense oligomer preferentially inhibits proliferation at a specific stage of development, four human myeloid leukemia cell lines (HL-60, ML-3, KG-1, and KG-1a), each blocked at different stages of differentiation, were exposed to a synthetic oligodeoxynucleotide complementary to c-*myb* mRNA. We found that proliferation of all lines was partially dependent on the protooncogene *myb* gene product. Interestingly, HL-60 cells, which are blocked at the promyelocytic stage, were the most sensitive to the inhibition of c-*myb* product; KG-1a cells, which are the most immature and do not differentiate in response to common inducers, appeared to be the least dependent on the product of c-*myb* gene.

## MATERIALS AND METHODS

**Cell Lines.** The leukemia cell lines employed in this study—HL-60 (8), ML-3 (9), KG-1 (10), and KG-1a (11)—have been described previously. The majority of HL-60 cells are promyelocytes and are capable of granulocytic or monocyte/macrophage differentiation (12). ML-3 and KG-1 are arrested at the differentiation stage of myelomonoblast and myeloblast, respectively, and can be induced to differentiate into monocyte/macrophage by phorbol 12-myristate 13-acetate (PMA) treatment (12). KG-1a is blocked at a more immature stage of myeloid differentiation than the parental line KG-1 and does not mature into monocyte/macrophage elements in response to PMA (12). Cell lines were maintained in RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 2 mM L-glutamine, and antibiotics.

**Synthesis and Purification of Oligomers.** Unmodified, 18-base deoxynucleotides were made on an Applied Biosystems 380B DNA synthesizer by means of β-cyanoethyl phosphoramidite chemistry. Oligodeoxynucleotides were purified by ethanol precipitation and multiple washes in 70% ethanol. They were lyophilized to dryness and redissolved in culture medium at a concentration of 1 μg/μl.

**Measurement of c-*myb* and Myeloperoxidase Protein in HL-60 Cells.** Cells exposed for appropriate time to antisense *myb* or antisense myeloperoxidase were cytocentrifuged (Shandon II, 500 rpm × 8 min) and fixed with methanol/acetone (1:9) for 15 min at room temperature. Incubation with sheep anti-myb serum (Cambridge Research Biochemicals, Valley Stream, NY; 1:40 dilution), rabbit anti-myeloperoxidase serum (1:250 dilution), or normal sheep or rabbit serum (at the same dilutions) was carried out for 45 min at 37°C. After extensive washes with phosphate-buffered saline, the slides were stained with fluorescein-conjugated donkey anti-sheep and sheep anti-rabbit IgG antibodies, respectively.

**Determination of Growth Rate and DNA Content Analysis.** Cells were cultured in 24-well Costar plates at an initial

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; NBT, nitroblue tetrazolium.

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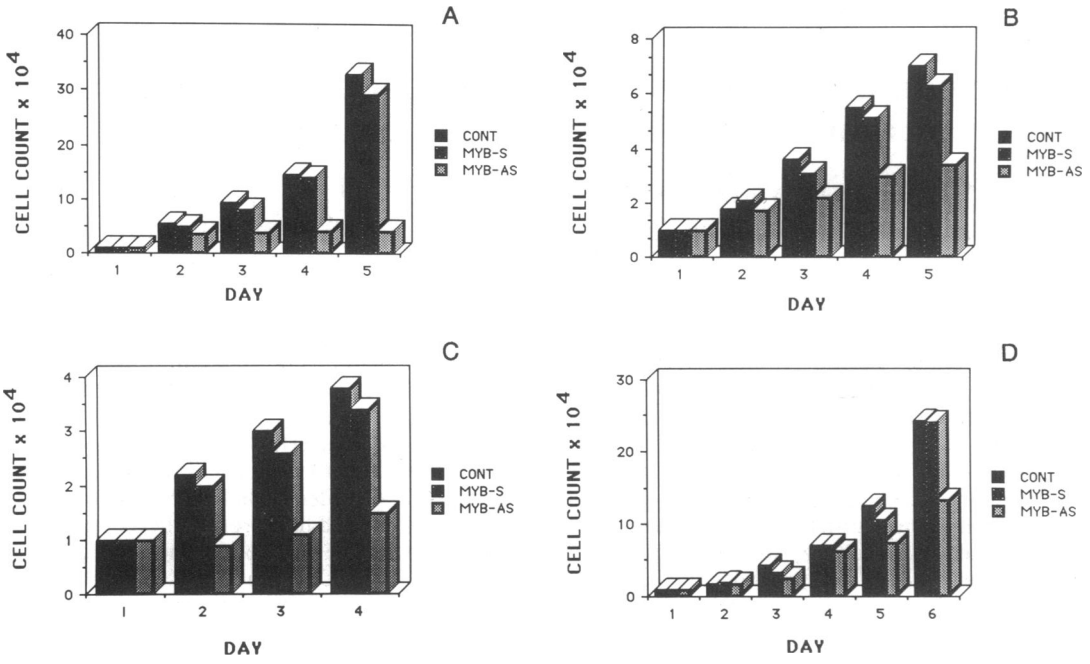


FIG. 1. Effect of c-*myb* sense and antisense oligodeoxynucleotides on the proliferation of HL-60, ML-3, KG-1, and KG-1a cells. Leukemia cells ($1 \times 10^4$) growing in RPMI 1640 medium in the presence of 15% fetal calf serum at an initial concentration of $2 \times 10^4$ per ml were exposed to a sense (-S) and antisense (-AS) oligomer complementary to c-*myb* mRNA. Each oligonucleotide was added at the beginning of the experiment at a concentration of 40 $\mu$g/ml (except in KG-1a, in which 80 $\mu$g/ml was used); this was followed by two additions at 24 hr and 48 hr of 10 $\mu$g/ml each. The final oligonucleotide concentrations were 10.5 $\mu$M for HL-60, ML-3, KG-1 and 17.5 $\mu$M for KG-1a. (*A*) HL-60. (*B*) ML-3. (*C*) KG-1. (*D*) KG-1a. The sequence of the c-*myb* (15) sense oligomer is 18 base pairs (bp) in length starting from the second codon (5'-GCCCGAAGA CCCCGG CAC-3'); the sequence of c-*myb* antisense oligomer is 5'-GTG CCG GGGTCTTCGGGC-3'. CONT, control.

concentration of $1 \times 10^4$ per well ($2 \times 10^4$ per ml). For cell lines HL-60, ML-3, and KG-1, a starting oligomer dose of 20 $\mu$g was followed by two additional doses of 5 $\mu$g each at 24 and 48 hr. For KG-1a, double the amount of oligomers was used. Cell counts and viability (trypan blue exclusion) were determined daily until the fifth day. The doubling time was determined with the following formula: $(t_2 - t_1)\log 2/\log(N_2/N_1)$, where $N_2$ and $N_1$ are the number of cells determined at the times $t_2$ and $t_1$, respectively. The DNA content of HL-60 cells incubated with sense or antisense c-*myb* oligomer was determined with the aid of a computer-operated microspectrofluorimeter as described (7, 13).

**Measurement of Differentiation Markers.** Morphology was assessed by cytocentrifugation followed by methanol fixation and Wright/Giemsa staining. Nitroblue tetrazolium (NBT) reduction was assayed by the method of Collins *et al.* (14). Briefly, 200 $\mu$l of cell suspension ($0.5 \times 10^6$ per ml) in RPMI 1640 medium with 15% fetal calf serum was incubated with an equal volume of NBT solution (200 mg in 100 ml of Dulbecco's phosphate-buffered saline containing 200 ng of PMA per ml at 37°C for 30 min). The cells recovered by cytocentrifugation into slides were subsequently stained by the method of Wright and Giemsa.

## RESULTS

**Effect of c-*myb* 18-mer Oligodeoxynucleotides on the Proliferation of Human Myeloid Leukemia Cell Lines.** The effect of *myb* sense and *myb* antisense oligomers on the growth rate of four myeloid leukemia lines is shown in Fig. 1. For each cell line the experiments have been repeated at least three times with different preparations of oligodeoxynucleotides. It is evident that c-*myb* antisense oligodeoxynucleotide inhibits the proliferation of each leukemia cell line, although the effect is most pronounced in HL-60 cells (Fig. 1*A*). The effect of *myb*-AS (perfectly matched antisense), *myb*-5 (2-bp mismatch), and *myb*-6 (4-bp mismatch) on the growth rate of HL-60 cells is shown in Fig. 2.

The growth of HL-60 cells treated with *myb*-6 (4-bp mismatch) is undistinguishable from that of untreated cells; *myb*-5 (2-bp mismatch) has a very modest effect (≈10% inhibition) only at the highest dose (total dose, 30 $\mu$g), whereas *myb*-AS (no mismatch) has the expected substantial effect at the highest dose. At a dose of 12.5 $\mu$g, *myb*-2 antisense still inhibited cell proliferation, although the effect was not as pronounced as at the higher dose. To determine whether the treatment with c-*myb* antisense oligomer mod-

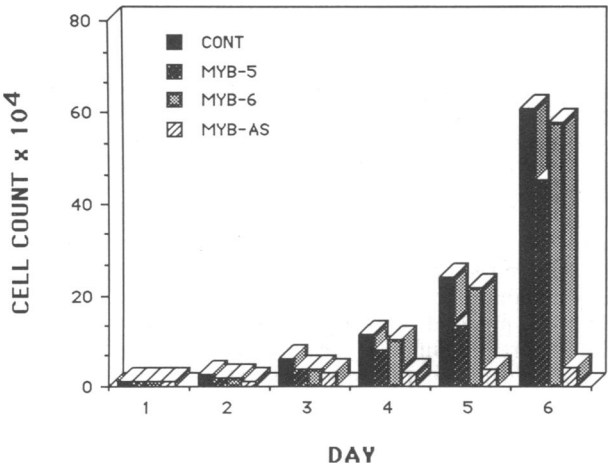


FIG. 2. Effect of c-*myb* antisense oligomers with 2- and 4-bp mismatches on the proliferation of HL-60 cells. Growth conditions of HL-60 cells and oligodeoxynucleotide concentrations are as described in the legend to Fig. 1. The sequence of the c-*myb* antisense oligomer with two mismatches (*myb*-5) is 5'-GTGTCGGGGTCTC-CGGGC-3'; the sequence of the c-*myb* antisense oligomer with four mismatches is 5'-GTGTCGAGGCCTCCGGGC-3'. CONT, control.

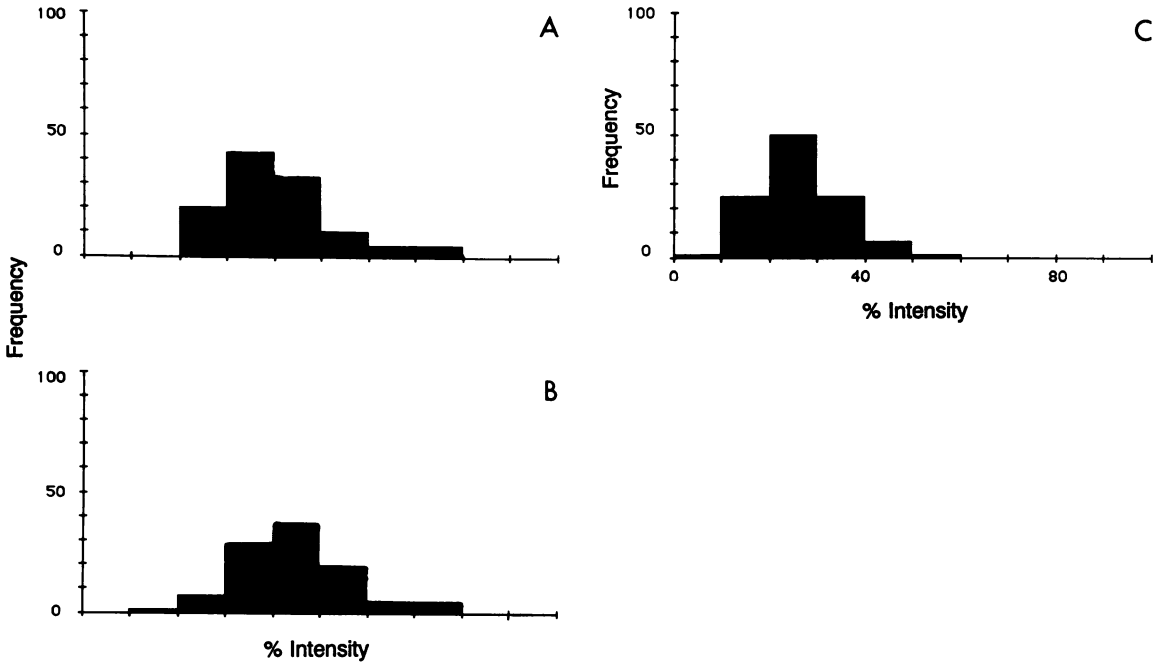


FIG. 3. DNA content histogram of HL-60 cells exposed to c-*myb* sense and antisense oligomers. DNA content of individual cells was determined by measuring the green fluorescence of acridine orange-stained cells with a computer-operated microspectrofluorimeter. Frequency indicates the number of measured cells in a given intensity class. Intensity is the relative fluorescence per nucleus in arbitrary units (0–100) of green fluorescence (520–540 nm). Green fluorescence is proportional to the relative amount of DNA (13). (*A*) Exponentially growing HL-60 cells. (*B*) Exponentially growing HL-60 cells exposed to c-*myb* sense oligomer. (*C*) Exponentially growing HL-60 cells exposed to c-*myb* antisense oligomer. Growth conditions of HL-60 cells and oligomer concentrations are as described in the legend to Fig. 1.

ified the cell cycle distribution of HL-60 cells, we measured the DNA content in exponentially growing HL-60 cells exposed to either sense or antisense *myb* oligomers (11). Control cells or cells treated with c-*myb* sense oligomer gave very close values of DNA content; in cells treated with c-*myb* antisense oligomer, $G_2$ cells completely disappeared and most cells were shifted in the $G_1$ compartment (Fig. 3). These data suggest that HL-60 cells exposed to c-*myb* antisense oligomer are blocked in $G_1$ phase or at the $G_1$/S boundary.

To demonstrate that c-*myb* antisense oligomer is taken up by HL-60 cells and inhibits the expression of c-*myb* protein, we determined, by indirect immunofluorescence, the level of c-*myb* protein in untreated cells and in cells treated for 18 hr with either *myb* antisense or *myb* sense oligomers. Immunofluorescence and subcellular fractionation studies indicate that viral and cellular *myb* proteins are localized in the nucleus (16, 17). Fig. 4 shows that treatment of HL-60 cells with *myb* antisense oligomer resulted in a clear decrement in the amount of c-*myb* nuclear protein in comparison to *myb* sense treated cells.

**Comparison of the Effect of c-*myc* and c-*myb* Antisense Oligodeoxynucleotides on the Growth of Leukemia Cell Lines.** It has been reported that an oligomer complementary to the first five codons of c-*myc* mRNA inhibits proliferation of HL-60 cells and induces differentiation (19, 20). Accordingly, we deemed it of interest to compare the effects of two oligomers of identical size (18 bp) complementary to the same region of c-*myc* and c-*myb* mRNAs (from codon 2 to codon

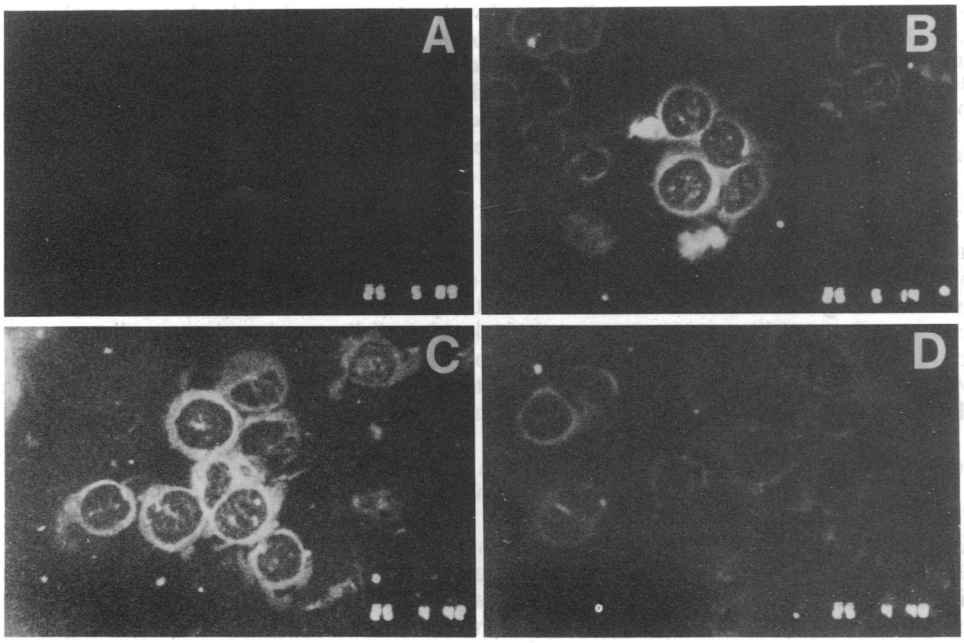


FIG. 4. c-*myb* protein expression in HL-60 cells exposed to c-*myb*. Protein expression was assessed by indirect immunofluorescence (18) in exponentially growing HL-60 cells exposed for 18 hr to a c-*myb* sense oligomer (*C*) and c-*myb* antisense oligomer (*D*) at a final concentration of 10.5 $\mu$M. (*B*) Untreated exponentially growing HL-60 cells. (*A*) HL-60 cells that reacted with normal sheep serum.

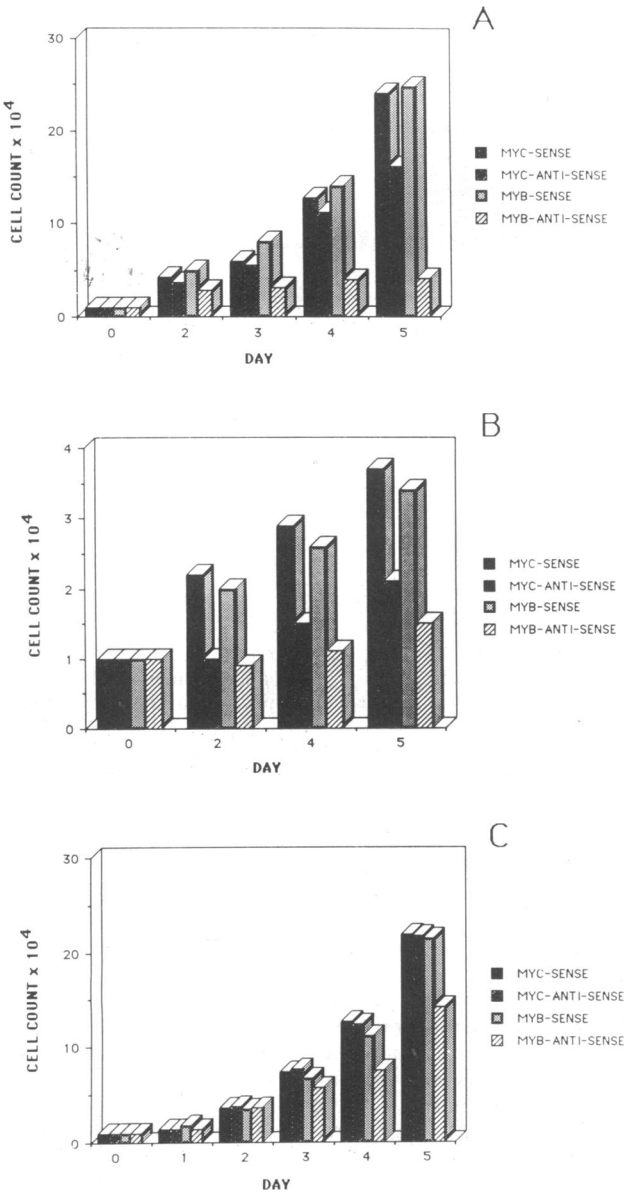


FIG. 5. Comparison of the effect of c-*myc* and c-*myb* antisense oligomers on the proliferation of HL-60, KG-1, and KG-1a cells. Growth conditions of leukemia cell lines HL-60, KG-1, and KG-1a and oligodeoxynucleotide concentrations are as described in the legend to Fig. 1. The sequence of c-*myc* (21) sense oligomer is 18 bp in length starting from the second codon (5′-CCCCTCAACGT-TAGCTTC-3′); the sequence of c-*myc* antisense oligomer is 5′-GAAGCTAACGTTGAGGGG-3′.

7) in HL-60, KG-1, and KG-1a cells. Fig. 5*A* shows that c-*myb* antisense oligomer has a much more pronounced effect than c-*myc* antisense oligomer on the growth rate of HL-60 cells; Fig. 5*B* shows that c-*myb* antisense oligomer inhibits KG-1 cells slightly more than c-*myc* antisense oligomer. Lastly, c-*myb* antisense oligomer still retains an inhibitory effect (37% inhibition) on the growth of KG-1a cells, whereas these cells are insensitive to the treatment with c-*myc* antisense oligomer (Fig. 5*C*).

In the experiment of Fig. 5 the doubling time of untreated HL-60 cells was 20–21 hr, the doubling time of c-*myc* antisense treated HL-60 cells was 30–31 hr, and the doubling time of c-*myb* antisense treated HL-60 cells was 50–52 hr. We note that the effect of c-*myc* antisense oligomer on the proliferation of HL-60 cells was, in our studies, of the same order reported in a previous investigation (19).

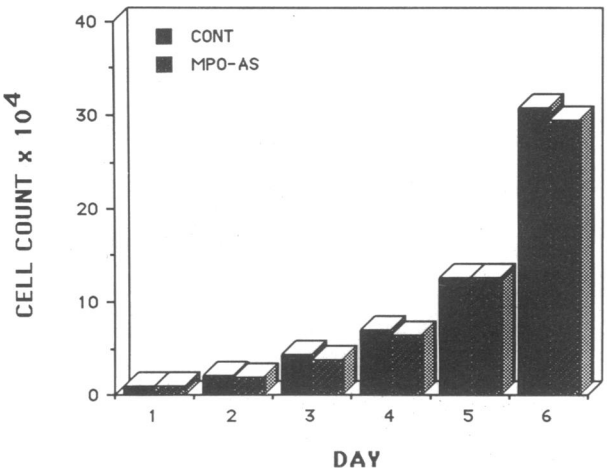


FIG. 6. Effect of a myeloperoxidase antisense oligomer on the proliferation of HL-60 cells. Growth conditions of HL-60 cells and the concentration of the anti-myeloperoxidase (MPO) oligomer are as described in the legend to Fig. 1. The sequence of the myeloperoxidase (23) antisense oligomer is 5′-AGAGAAGAAGGGACCCC-3′. CONT, control.

We next asked whether treatment of HL-60 cells with c-*myb* antisense oligomer would induce differentiation along the granulocytic pathway as observed when these cells were exposed to c-*myc* antisense oligomer (19). Incubation of HL-60 cells with a c-*myc* antisense oligomer for 5 days was followed by the appearance of 15–20% of the cells showing NBT reduction and morphological changes associated with a more differentiated phenotype than that of untreated cells (not shown). On the contrary, <5% of HL-60 cells exposed to c-*myb* antisense oligomer were NBT positive (not shown).

**Effect of a Myeloperoxidase Antisense Oligodeoxynucleotide on the Growth Rate of HL-60 Cells.** We next asked if inhibition of a gene product that is not related to the control of cell proliferation might perturb the growth rate of HL-60 cells. For this purpose we selected the myeloperoxidase gene since its protein product is an abundant and specific marker of myeloid differentiation (22). Fig. 6 shows that the growth rate of HL-60 cells incubated in the presence of myeloperoxidase antisense oligodeoxynucleotide is indistinguishable from that of untreated cells. To demonstrate uptake of the oligomer, the level of myeloperoxidase in untreated HL-60 cells and in cells treated with antisense *myb* and antisense myeloperoxidase was determined by indirect immunofluorescence.

The treatment of HL-60 cells with antisense myeloperoxidase resulted in a decrement in the amount of protein in comparison to untreated cells; in HL-60 cells treated with *myb* antisense, myeloperoxidase protein level was unaffected (not shown).

## DISCUSSION

The findings described in this report demonstrate that the reduction of the protein level of c-*myb* protooncogene that occurs in leukemia cell lines exposed to an oligomer complementary to c-*myb* mRNA is associated with a substantial inhibition of their proliferative rate. This phenomenon was best documented in HL-60 cells, where the number of cells were 75–80% lower than control after 5 days of growth in the presence of a c-*myb* antisense oligomer. In HL-60 cells, in addition to the activation of N-*ras* by point mutation (24), c-*myc* gene is amplified 10- to 30-fold and is highly expressed (25, 26). It has been previously shown that exposure of HL-60 cells to an oligomer against c-*myc* caused inhibition of proliferation and induction of differentiation (19). Therefore it is significant that the decrement of c-*myb* protein level is

sufficient to abrogate the growth advantage presumably provided to HL-60 cells by N-*ras* activation and amplification of c-*myc* gene.

It is likely that c-*myc* and c-*myb* regulate cell proliferation in the myeloid lineage by independent mechanisms; alternatively, the inhibition of c-*myb* may cause down-regulation of c-*myc* expression, which, in turn, inhibits the proliferation of myeloid cell lines. We favor the hypothesis that separate mechanisms operate in the control of cell proliferation because (*i*) the effect of c-*myb* antisense oligomer on the proliferation of HL-60 cells is more dramatic than that of c-*myc* antisense oligomer and (*ii*) differentiated cells are not detected following the exposure of HL-60 cells to c-*myb* antisense oligomer. The detection of differentiated cells after exposure of HL-60 cells to c-*myb* antisense oligomer could argue in favor of down-regulation of c-*myc* gene, since differentiated cells are observed after exposure of HL-60 cells to c-*myc* antisense oligomer.

Induction of terminal differentiation is likely to contribute to the overall growth inhibition caused by c-*myc* antisense oligomer because as HL-60 cells differentiate they exit from the pool of proliferative cells; in contrast, the growth inhibition observed in HL-60 cells exposed to c-*myb* antisense oligomer is entirely due to an antiproliferative effect.

Our findings indicate that there is a preferential growth inhibition of HL-60 cells in comparison to the other less differentiated cell lines; this suggests that stage of differentiation arrest and proliferative potential depend on the presence of distinct genetic defects in each cell line. Accordingly, the effects of such abnormal gene functions appear to be mitigated to a different extent by down-regulation of c-*myb* protein.

Finally, our observations that a c-*myb* antisense oligomer inhibits proliferation of myeloid leukemia lines validate previous studies demonstrating the inhibition of specific gene functions by the antisense approach (27–30). The use of the antisense approach to inhibit the function of proliferation-associated genes in mammalian cells may appear of less practical utility than that aimed to the inhibition of viral replication (31–33). However, the findings that the inhibition of two nuclear protooncogene functions, c-*myc* and c-*myb*, affects the growth of HL-60 with distinct mechanisms set the stage for the delineation of a pattern of gene functions required for the growth of myeloid leukemia lines *in vitro*. These observations and the fact that c-*myb* protooncogene is preferentially expressed in hemopoietic tissues (3, 34) may provide the rationale for an antisense based therapy of leukemia patients.

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