Deregulated *c-myb* Disrupts Interleukin-6- or Leukemia Inhibitory Factor-Induced Myeloid Differentiation Prior to *c-myc*: Role in Leukemogenesis

MUTHU SELVAKUMARAN, DAN A. LIEBERMANN,* AND BARBARA HOFFMAN-LIEBERMANN*

Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine,
Philadelphia, Pennsylvania 19104-6059

Received 9 January 1992/Accepted 5 March 1992

The *c-myb* proto-oncogene is abundantly expressed in tissues of hematopoietic origin, and changes in endogenous *c-myb* genes have been implicated in both human and murine hematopoietic tumors. *c-myb* encodes a DNA-binding protein capable of *trans*-activating the *c-myc* promoter. Suppression of both of these proto-oncogenes was shown to occur upon induction of terminal differentiation but not upon induction of growth inhibition in myeloid leukemia cells. Myeloblastic leukemia M1 cells that can be induced for terminal differentiation with the physiological hematopoietic inducers interleukin-6 and leukemia inhibitory factor were genetically manipulated to constitutively express a *c-myb* transgene. By using immediate-early to late genetic and morphological markers, it was shown that continuous expression of *c-myb* disrupts the genetic program of myeloid differentiation at a very early stage, which precedes the block previously shown to be exerted by deregulated *c-myc*, thereby indicating that the *c-myb* block is not mediated via deregulation of *c-myc*. Enforced *c-myb* expression also prevents the loss in leukemogenicity of M1 cells normally induced by interleukin-6 or leukemia inhibitory factor. Any changes which have taken place, including induction of myeloid differentiation primary response genes, eventually are reversed. Also, it was shown that suppression of *c-myb*, essential for terminal differentiation, is not intrinsic to growth inhibition. Taken together, these findings show that *c-myb* plays a key regulatory role in myeloid differentiation and substantiate the notion that deregulated expression of *c-myb* can play an important role in leukemogenicity.

Blocks in differentiation appear to be a major step in tumor progression (31). To diagnose and treat different malignancies, including leukemias, it is important to gain a better understanding of molecular mechanisms that promote terminal differentiation and growth arrest of cells and, concomitantly, how blocks in differentiation can arise. An experimental approach toward dissecting the regulation of differentiation is to genetically manipulate cells to constitutively express genes which are normally suppressed and exert pleiotropic effects on gene expression, directly or indirectly (7, 15).

Normal myeloid precursor cells can be induced in vitro for proliferation coupled to terminal differentiation, using a variety of hematopoietic growth and differentiation inducers (20). The availability of the murine myeloid leukemic M1 cell line, which proliferates autonomously and can be induced with the physiological inducer interleukin-6 (IL-6) or leukemia inhibitory factor (LIF) to undergo terminal differentiation and growth arrest, makes it possible to analyze differentiation and its associated growth arrest segregated from other biological processes (15, 22, 23). In addition, M1 cells are no longer leukemic after they are induced to differentiate, so loss of the leukemic phenotype is amenable to genetic analysis (10, 29). We have shown that expression of the nuclear proto-oncogenes *c-myc* and *c-myb* is high in proliferating normal and M1 leukemic myeloid precursor cells and is suppressed following induction of terminal differentiation and growth arrest. Recently, we have shown that enforced expression of *c-myc* blocks M1 terminal differentiation,

leading to intermediate-stage myeloid cells which continue to proliferate in the absence of *c-myb* expression (15).

c-myb proto-oncogene transcripts are most abundant in tissues of hematopoietic origin (4, 13, 19), and changes in endogenous *c-myb* genes have been implicated in both human and murine hematopoietic tumors (27, 33). Yet the consequences of enforced expression of *c-myb* on physiologically induced myeloid differentiation have not been determined. Recently, it has been shown that *c-myb* encodes a DNA-binding protein (2, 11) which can *trans*-activate the *c-myc* promoter (8). It also was shown that suppression of both of these proto-oncogenes occurred upon induction of terminal differentiation by IL-6 or LIF but not upon induction of growth inhibition with antiproliferative compounds which did not induce terminal differentiation of M1 cells (16). Therefore, it was of obvious interest to determine (i) whether deregulated *c-myb* blocks physiologically induced differentiation of myeloid cells in vitro and subsequent loss of leukemogenicity in vivo, (ii) if *c-myb* does block differentiation, then whether the block is mediated via deregulation of *c-myc*, and (iii) whether deregulated expression of *c-myb* interferes with growth inhibition induced by antiproliferative compounds which do not induce terminal differentiation. In this report, we describe the establishment and use of M1 cell lines which constitutively express human *c-myb* to address these issues. Our findings indicate that *c-myb* plays a pivotal role in the regulation of myeloid differentiation and substantiate the notion that genetic lesions which result in continuous, deregulated expression of *c-myb* can provide a major step in the development and progression of leukemias.

* Corresponding authors.

MATERIALS AND METHODS

Cells, cell culture, and mice. The murine M1 myeloid leukemic differentiation-competent clone 6 (M1D+) (20, 21, 23), induced for differentiation by IL-6 or LIF, was used in this study. Cells, cultured as described previously (15, 20, 23), were seeded at densities as indicated (0.1×10^6 to 0.2×10^6 /ml for 3 to 4 days in culture); for RNA extraction using cells treated for less than 3 days, cell concentrations were adjusted so that final densities were $>0.25 \times 10^6$ /ml when harvested. Viable cell numbers were determined by trypan blue dye exclusion, counting in a hemocytometer. CD-1 *nu/nu* mice, 4 to 6 weeks old, were obtained from Charles River Laboratories.

Cytokines and other compounds used. Serum-free conditioned medium from mouse lungs (LUCM) was used at a concentration of 5% (15, 17). Purified human recombinant IL-6 (0.5 mg/ml) was used at a concentration of 100 ng/ml. Murine recombinant LIF in Cos cell conditioned medium was prepared by using the expression vector pcD-LIF (F. Lee, DNAX) and was used at a concentration of 10 U/ml (21). Purified murine recombinant interleukin-1 α (IL-1 α ; 1.2×10^8 U/ml), given to us by P. Lodmico, Hoffmann-La Roche Inc., Nutley, N.J., was used at a concentration of 240 U/ml. Murine alpha-plus-beta interferon (IFN- α + β ; 4.2×10^5 U/ml; Lee Biomolecular Research Inc.) was used at a concentration of 1,000 U/ml, and rat IFN- γ (10^6 U/ml; Amgen, Thousand Oaks, Calif.) was used at a concentration of 400 U/ml. Lipopolysaccharide (LPS) type B from *Salmonella typhimurium* (Difco Laboratories) was used at a concentration of 1 μ g/ml. Experiments were conducted by using concentrations which represent the optimum of the linear differentiation and/or growth inhibition dose-response curves.

Assays for differentiation-associated properties. Fc and C3 receptors (21, 25), lysozyme (18, 21), and cell attachment (21) were assayed as described previously. Morphological differentiation was determined by counting at least 300 cells on May-Grünwald-Giemsa-stained cytospin smears and scoring the proportion of immature blast cells, cells at intermediate monocyte stages of differentiation, and mature macrophages and granulocytes (15, 21, 23). M1 cells differentiate predominantly to macrophages; however, low percentages (2 to 7%) of granulocytes are also detected.

General recombinant DNA techniques and DNA probes. Plasmid preparations, restriction enzyme digestions, DNA fragment preparations, and agarose gel electrophoresis were as described previously (15, 20). The probe for murine *c-myb* was the 1.3-kb *Pst*I-*Sac*I fragment of the murine MM46 cDNA clone (12), a gift from P. Reddy. The human *c-myb* probe was the 1.5-kb *Hind*III fragment from pMbm I, obtained from M. Clarke (7). The 3.4-kb *Xho*I fragment from pMbm I was used for Southern blot analysis of genomic DNA (7).

Probes for *c-myc* and β -actin were used as previously described (15, 20). *junB* (*MyD21*), *MyD88*, and *IRF-1* (*MyD32*) probes were cDNAs cloned in this laboratory (1, 21–24). Murine ferritin light chain was cloned in this laboratory, sequenced by S. Suggs (Amgen), and found to match the known sequence (3, 15). DNA for probes was labeled by random priming to a specific activity equal to or greater than 10^9 cpm/ μ g (9).

DNA transfections. M1 cells were transfected via electroporation (Bio-Rad Gene Pulsor) with pMbm I/DHFR (7) and pSV2neo (American Type Culture Collection) at a 10:1 ratio. Plasmid pMbm I/DHFR, generously provided by Michael F.

Clarke, containing the human *c-myb* gene under control of the simian virus 40 promoter and a transcription unit for dihydrofolate reductase (*dhfr*) cDNA, was linearized at the *Kpn*I site. pSV2neo, linearized by digestion with *Eco*RI, was necessary to select for transfectants by resistance to geneticin (G418; 400 μ g/ml). Control transfections using pSV2dhfr (American Type Culture Collection) and pSV2neo, or pSV2neo only, were done. Based on the frequency of positive wells, each well contained a clonal population of transfectants which was maintained in 200 μ g of drug per ml. Increasing amounts of methotrexate up to 0.50 μ M were added to the culture medium of M1myb transfectants selected by growth in G418. Since the level of exogenous *c-myb* expression was low, the *dhfr* cDNA facilitated selective amplification of plasmid sequences in the presence of high concentrations of methotrexate (28), with a concomitant increase in the expression of the *c-myb* transgene. The M1dhfr transfectants were treated similarly. Seven M1myb clones and three of each control were established as cell lines. When cells were maintained in methotrexate, the drug was removed 3 days prior to analysis of growth and differentiation properties.

Genomic DNA extraction, Southern blotting, and hybridization. DNA was extracted as described previously (30); 20 μ g of restriction enzyme-digested DNA was size fractionated on a 1% agarose gel and blotted onto GeneScreen Plus (NEN). Filters were prehybridized (10% dextran sulfate, 1 M NaCl, 1% sodium dodecyl sulfate [SDS]) at 65°C for 2 to 3 h and hybridized at 65°C for 12 to 16 h in additional buffer containing denatured probe at 2×10^6 cpm/ml and sheared salmon sperm DNA at 100 μ g/ml. Blots were washed under stringent conditions down to $0.2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C (7) so that the 3.4-kb entire human *c-myb* cDNA used as a probe did not cross-hybridize with murine *c-myb* genomic DNA and were exposed to X-ray film at -80°C .

RNA extraction, Northern (RNA) blotting, and hybridization. RNA was extracted by using guanidinium thiocyanate (5). Total RNA (5 μ g per lane) was electrophoresed on 1% agarose formaldehyde gels. Northern blots, using Duralone-UV membranes (Stratagene), were prepared and UV cross-linked (Stratalinker; Stratagene) prior to baking. Filters were prehybridized (50% deionized formamide, 10% dextran sulfate, 1 M NaCl, 1% SDS) at 42°C for 2 to 3 h and hybridized in additional buffer containing probe (10^6 cpm/ml) and sheared salmon sperm DNA (100 μ g/ml) at 42°C for 12 to 16 h. To eliminate cross-hybridization of human and murine *c-myb* sequences, blots were washed twice (10 min each time) at room temperature in $2 \times$ SSC–0.1% SDS and twice (30 min each time) at 65°C in $0.1 \times$ SSC–1% SDS and were exposed to X-ray film at -80°C . The latter wash was done at 60°C for other probes. Stripping blots of probe to rehybridize was done as described previously (21). For quantitation of individual mRNAs, films were exposed for periods during which band intensity was linear with respect to time, and relative intensities of hybridization signals were measured at 560 nm with the gel scan program of a DU7 Beckman spectrophotometer.

Western immunoblots. Extracts of total proteins were prepared as described previously (16). Extracts from equal cell numbers were fractionated on SDS–10% polyacrylamide gels, transferring resolved proteins to an Immobilon-P membrane (Millipore), using the Trans-blot (Bio-Rad) apparatus at 50 V for 18 h. To block nonspecific sites, the filter was incubated in phosphate-buffered saline (PBS; pH 8.3) containing 5% bovine serum albumin (BSA) for 2 h at room

FIG. 1. (a) Southern blot analysis of genomic DNA demonstrating that M1myb cell lines contain the exogenous human *c-myb* gene. Twenty micrograms of genomic DNA and 125 pg (five copies) of the transfecting plasmid DNA were digested with *Bam*HI, resolved on a 1% agarose gel, transferred to GeneScreen Plus, and hybridized with the full-length 3.4-kb *Xho*I cDNA fragment. The copy numbers of the exogenous *myb* transgene for M1myb3, M1myb7, and M1myb11 are estimated to be 10, 3, and 20, respectively; those for the seven cell lines checked, using a dilution series of pMbm I/DHFR, ranged between 2 and 40. Lane C, control; lane PL, plasmid pMbm I/DHFR. (b) Analysis of *c-myb* RNA expression in M1 and M1myb11 cells before and after treatment with IL-6 at the indicated times (d, days). Northern blots were hybridized sequentially to DNA probes specific for murine *c-myb* and human *c-myb*. (c) Western blot analysis of *c-myb* protein in M1 and M1myb11 cells untreated (–) and treated for 3 days (+) with IL-6.

temperature. Blots were probed with sheep anti-*c-myb* antibodies (Cambridge Research Biochemicals) for 1 h at room temperature in PBS (pH 7.2)–0.5% BSA–0.5% Tween 20. After three rinses in PBS containing 0.5% Tween 20, blots were incubated for 20 min at room temperature in antibody binding solution containing rabbit anti-sheep immunoglobu-

lin peroxidase-conjugated antibodies (Cambridge Research Biochemicals). Blots were rinsed six times in PBS containing 0.5% Tween 20. Signals were developed by using the enhanced chemiluminescence Western blotting system (Amersham).

RESULTS

Transfection and analysis of M1myb cells constitutively expressing *c-myb* RNA and protein. M1myb transfectants were established as described in Materials and Methods. Seven M1myb cell lines were analyzed for the presence of exogenous human *myb* sequences by Southern blotting. DNAs from three representative transfectants (Fig. 1a) contained the same three human *myb* hybridizing fragments as did the transfecting pMbm I/DHFR plasmid; similar results were obtained for the four additional cell lines (data not shown). These data demonstrated that the M1myb cell lines contained the human *myb* gene and that no apparent rearrangements had occurred. No endogenous murine *c-myb* genomic sequences were detected by hybridizing to the human *myb* probe because of the highly stringent conditions used for the hybridization and subsequent washes.

Expression of exogenous (human) and endogenous (murine) *c-myb* was analyzed by Northern blots, using probes specific for the human or murine *myb* transcripts, employing hybridization conditions as specified (see Materials and Methods). As seen in Fig. 1b, the steady-state level of murine *myb* transcripts was the same in the M1myb11 transfectant as in the M1 parental cell line. In addition, the steady-state level of human *myb* transcripts was higher in M1myb11 cells than in the human promyelocytic cell line HL60 but was comparable to the endogenous M1 levels. It can be seen that after treatment of M1myb11 with IL-6, expression of endogenous *c-myb* was down-regulated just as for the parental M1 cells. However, the steady-state level of the exogenous human *c-myb* transcripts remained high (Fig. 1b). Therefore, the exogenous *c-myb* gene was constitutively expressed in M1myb11 following induction for differentiation with IL-6. The results were the same for cells treated with LIF (data not shown).

As observed for M1myb11, the other six M1myb cell lines studied showed the same level of murine *myb* transcripts as were detected in the parental M1 cell line. There was variability in the level of exogenous human *myb* transcripts, but it was at least as high as that seen for HL60. Following

TABLE 1. Induction of growth inhibition, differentiation markers, and terminal differentiation in parental M1 compared with M1myb11 cells

Clone	Inducer ^a	Cell no. ^b (10 ⁶)	Cell adherence ^b (%)	Fc receptor ^b (%)	C3 receptor ^b (%)	Lysozyme ^{b,c}	Cell type (%) ^b		
							Blast	Intermediate	Mature
M1	None	2.2	<1	0.6	1.1	0.13	>99	<1	0
	IL-6	0.41	69	38	53	7.5	8	56	36
	LIF	0.39	65	30	51	7.2	9	58	33
	LUCM	0.25	88	48	65	12	2	22	76
M1myb11	None	1.8	<1	0.5	1.3	0.08	>99	<1	0
	IL-6	1.5	<1	0.7	1.0	0.11	96	4	0
	LIF	1.4	<1	0.4	1.2	0.07	93	7	0
	LUCM	0.99	2	2	4	0.10	73	27	0

^a IL-6 was used at 100 ng/ml, LIF was used at 10 U/ml, and LUCM was used at 5% (vol/vol).

^b Cells were seeded at a density of 0.1×10^6 /ml and were analyzed after 4 days. All values represent the means of three independent determinations, with standard deviations up to 15%.

^c Given in microgram equivalents per 5×10^6 cells.

FIG. 2. Comparison of M1 and M1myb11 cells following treatment with IL-6. Shown are M1 and M1myb11 cells in culture without (a and b) and with (c and d) IL-6 for 4 days (magnification, $\times 150$). Photomicrographs of May-Grünwald-Giemsa-stained cytospin smears reveal the morphology of M1 and M1myb11 cells without (e and f) and with (g and h) IL-6 for 4 days (magnification, $\times 300$). Blasts are characterized by scant cytoplasm and round or oval nuclei; mature cells have large amounts of cytoplasm, irregularly shaped nuclei, and vacuoles.

treatment with IL-6 or LIF, all results were comparable to what has been shown for M1myb11 (data not shown).

Western blot analysis of cell extracts showed that no *myb* protein was present in M1 cells induced to differentiate but was found in M1myb11 cells following treatment with the differentiation inducer IL-6 (Fig. 1c). Similar results were observed by using LIF.

Taken together, these results show that *myb* mRNA and protein continue to be synthesized in M1myb cells following treatment with differentiation inducers, whereas following

similar treatment, the parental M1 cells, as well as the control transfectants (data not shown), no longer expressed *c-myb*.

Effect of continuous, elevated expression of *c-myb* on terminal myeloid differentiation and its associated growth arrest. Having the M1myb cell lines allowed us to ascertain whether continuous expression of *myb* had any effect on terminal differentiation and growth arrest of the M1 myeloid leukemic cells induced by IL-6 or LIF. In addition to IL-6 and LIF, LUCM, containing a variety of myelopoietic growth and differentiation inducers, including IL-6 and LIF (14, 15, 20, 32), was used, because its inducing effects are stronger and the intent was to challenge the M1myb cells to differentiate with the most potent inducers available. Detailed results are presented for M1myb11 and compared with the parental M1 cell line results. The control M1 transfectants gave results similar to those for M1; three M1myb cell lines which constitutively expressed levels of exogenous *c-myb* mRNA comparable to the M1myb11 level, which is similar to the level of endogenous *c-myb*, behaved similarly to M1myb11.

In contrast to the results obtained with the parental M1 cell line, there were no mature cells present in the M1myb11 cell line following stimulation with LUCM, IL-6, or LIF, and most of the cells were blasts (Table 1 and Fig. 2). Four days following stimulation with LUCM, IL-6, or LIF, the cell concentration was much higher for M1myb11 than for the parental M1 cell line, which was growth arrested; however, it was lower than for cells not induced to differentiate, most notably in the case of LUCM treatment. To distinguish whether M1myb11 was growth arrested at a later time than M1 or continued to proliferate in the presence of differentiation inducers, growth kinetics were analyzed (Fig. 3). The kinetic studies demonstrated that M1myb11 continued to proliferate for at least 6 days in the presence of LUCM, IL-6, or LIF, when M1 no longer proliferated (Fig. 3), and was observed to continue to proliferate beyond 21 days (data not shown). Enforced expression of *c-myb* reversed the growth arrest normally induced by LUCM, IL-6, or LIF.

Although M1myb11 appeared to maintain the myeloblast morphology and continued to proliferate following stimulation with IL-6, LIF, or LUCM, it was important to ascertain whether expression of other terminal myeloid differentiation markers was inhibited. Fc and C3 receptors start to appear at about 9 and 12 h, respectively, following induction of differentiation of M1 cells and continue to increase. There was no induction of either Fc or C3 receptors following treatment of M1myb11 cells with IL-6 or LIF, and there was very low induction with LUCM (Table 1).

Ferritin light-chain mRNA is induced in the human myeloid leukemic cell line HL60 (6), and we have recently shown that in M1 cells induced for differentiation with IL-6, increased levels of ferritin light-chain transcripts also are induced, with high expression by 3 days (15). No induction of an increase in steady-state levels of ferritin transcripts in IL-6-treated M1myb11 cells was detected (Fig. 4).

Lysozyme is induced in M1 cells, in which it is detected as early as 2 days following stimulation for terminal differentiation and continues to increase (18). No lysozyme was detected in M1myb11 cells treated with IL-6, LIF, or LUCM (Table 1).

Taken together, these results demonstrated that M1myb11 cells, which constitutively expressed *c-myb* transcripts, did not express markers normally associated with terminal myeloid differentiation following treatment with IL-6, LIF, or LUCM.

Effects of continuous *c-myb* expression on suppression of the

FIG. 3. Growth of M1 and M1myb11 cells following treatment with IL-6, LUCM, or LIF. Cells were seeded ($0.2 \times 10^6/\text{ml}$) with or without the appropriate treatment, and viable cell counts were determined on days 1, 2, and 3. On day 3, cells were harvested and reseeded at a final concentration of $0.2 \times 10^6/\text{ml}$ in fresh medium with the indicated treatment, and again viable cell counts were determined at the indicated times. Each time point is the average of three experiments, with a standard deviation up to 15%. C, control.

proto-oncogene *c-myc* and induction of primary response (*MyD*) genes. In autonomously proliferating M1 cells, the proto-oncogene *c-myc* is expressed. Following induction for terminal differentiation and growth arrest with IL-6, LIF, or LUCM, the steady-state transcript levels of *c-myc* increased rapidly and transiently and were no longer detectable by 1 day (15). Given that *c-myb* was reported to play a role in *trans*-activation of *c-myc* (8), it was of interest to ascertain what effect deregulated and continuous expression of *c-myb* would exert on the regulation of *c-myc* expression. As seen in Fig. 4, in M1myb11 cells treated with IL-6, the steady-state level of *c-myc* underwent the early transient increase; however, steady-state *c-myc* levels declined only to the basal level found in untreated cells. This observation is consistent with the report that *c-myb* *trans*-activates the *c-myc* promoter.

Immediate-early response genes (*MyD*), which are induced within 30 min and in the absence of protein synthesis following stimulation of M1 cells with IL-6, LIF, or LUCM, have been isolated in this laboratory (23). We wanted to ascertain whether any *MyD* genes were induced in M1myb11 cells following stimulation for myeloid differentiation. Three genes were selected, *junB* (*MyD21* [21, 23]), *IRF-1* (*MyD32* [1]), and *MyD88*, a novel gene whose sequence has been reported recently (24). Both *junB* and *IRF-1* are transcriptionally activated, whereas *MyD88* is posttranscriptionally regulated, most probably at the level of mRNA stabilization (22). As can be seen in Fig. 4, the three *MyD* genes were induced in both M1 and M1myb11. In contrast to M1 cells, in which both *junB* and *MyD88* continued to be expressed, in M1myb11 cells their expression was not maintained. In the case of *IRF-1*, expression peaked 1 h following stimulation with IL-6 for both M1 and M1myb11 cells and then fell off. However, for M1, there was biphasic kinetics of expression, whereas for M1myb11, the second increase was not detected. Thus, in M1myb11 cells, IL-6 activated the immediate-early response genes; however, expression was subsequently suppressed.

Continuous *myb* expression blocked loss in leukemogenicity. The leukemogenicity of M1 myeloid leukemic cells is lost following induction of differentiation *in vitro* or *in vivo* (10, 29). To understand the relationship between leukemogenicity and blocks in differentiation, it is important to ascertain whether continuous *myb* expression, which blocked the

myeloid differentiation program at an early stage, will prevent M1 cells from losing their leukemic phenotype.

Nude mice (CD-1 *nu/nu*) were intravenously injected with M1 and M1myb11 cells, either untreated or 5 days following treatment with LUCM, IL-6, or LIF. As seen in Table 2, M1 cells induced to differentiate with LUCM were no longer leukemogenic; however, similarly treated M1myb11 cells were as leukemogenic as untreated M1 and M1myb11 cells. In addition, 3 weeks after the mice were injected, one mouse from each group was sacrificed and the bone marrow was analyzed. Myeloid leukemic cells, as determined by growth and differentiation characteristics, were recovered from mice injected with untreated M1 or M1myb11 leukemic cells and M1myb11 LUCM-treated cells. The results obtained for smaller populations of nude mice that had been injected with M1 and M1myb cells treated with IL-6 or LIF were consistent with those obtained with LUCM (data not shown). Thus, continuous, deregulated expression of *c-myb* blocked the loss of leukemogenicity.

***c-myb* down-regulation is not necessary for growth suppression.** Previously, we showed that the proto-oncogenes *c-myc* and *c-myb* were not suppressed when M1 was growth inhibited following treatment with IL-1, LPS, IFN- β , or IFN- γ (16). In addition, M1myc cells, which contained a *myc* transgene under control of the β -actin promoter such that *c-myc* continued to be expressed when these cells were treated with IL-6 or LIF, were as responsive as the parental M1 cells to growth suppression by the different antiproliferative compounds which do not induce terminal differentiation (16).

Since M1myb cell lines, which continuously express *c-myb*, have become available, we took the opportunity to look at the effects of different antiproliferative compounds, including IL-1, IFN- γ , IFN- α + β , and LPS, on these cells. We found that M1myb11 cells were, like M1myc cells, as responsive as the parental M1 cells to growth suppression by the different antiproliferative compounds which do not induce terminal differentiation (Table 3).

FIG. 4. Analysis of expression of ferritin, *c-myc*, *junB*, *MyD88*, and *IRF-1* following IL-6 treatment of M1 and M1myb11 cells. Expression was analyzed by hybridization to Northern blots, using 5 μg of total RNA extracted from cells at the indicated times (d, days).

TABLE 2. Effect of enforced expression of *myb* on leukemogenicity of myeloid leukemic cells in nude mice

Cells ^a	LUCM	Mice dead (%)		Myeloid leukemic cells recovered from bone marrow ^b
		3 wk	6 wk	
None	—	0	0	—
M1	—	40	100	+
M1	+	0	0	—
M1myb11	—	30	100	+
M1myb11	+	20	100	+

^a For each cell type, 10 nude mice were intravenously (tail) injected with 2×10^6 cells. Cells were treated for 5 days with or without 5% LUCM prior to inoculation of the nude mice.

^b Three weeks following inoculation, one mouse injected with each cell type was sacrificed, and the bone marrow was analyzed for the presence of myeloid leukemic cells.

DISCUSSION

In this study, it is shown for the first time that IL-6- or LIF-induced terminal differentiation and growth arrest of myeloid leukemic cells required suppression of *c-myb* expression. Enforced, continuous expression of *c-myc* in M1myb cells disrupted the IL-6- or LIF-induced genetic program of myeloid differentiation at a very early stage (Fig. 5), the cells maintaining the blast morphology and continuing to proliferate. The very early block is consistent with the observation that *c-myc* is normally suppressed to very low levels 3 h following treatment with differentiation inducers. In this regard, it is interesting to point out that enforced expression of *c-myc* previously has been shown to block erythroleukemia cell differentiation, for which its suppression is required at late times following induction with dimethyl sulfoxide (7, 26).

Abnormalities in both growth and differentiation appear to be required for leukemogenesis (31). Consistent with the notion that enforced expression of *c-myc* blocked myeloid differentiation at a very early stage, in this work we also found that continuous expression of *c-myc* prevented the loss in leukemogenicity which takes place when M1 cells are induced to differentiate. Thus, continuous, deregulated expression of *c-myc* may play a role in leukemogenesis.

Deregulated *c-myc* blocks myeloid differentiation at an earlier stage than does deregulated *c-myc*. Previously, it has been shown in this laboratory that deregulated and continuous expression of *c-myc* blocks terminal myeloid differentiation induced by IL-6 or LIF, leading to intermediate-stage myeloid cells which continue to proliferate (15). Recently, it has been reported that *c-myc* trans-activates the *c-myc*

FIG. 5. Schematic representation of induction of M1 differentiation and the blocks in M1myc2 and M1myb11 cells following treatment with IL-6 or LIF.

promoter in T-lymphocytic cell lines (8). This finding is consistent with the observation that *c-myc* continued to be expressed in M1myb11 cells induced to differentiate. However, the block in differentiation caused by enforced expression of *c-myc* was not mediated via *c-myc* expression, since M1myb cells were blocked at an earlier stage than were M1myc cells (Fig. 5). Since both *c-myc* and *c-myc* continued to be expressed in M1myb cells, it is highly probable that M1 cells containing both *myc* and *myb* transgenes regulated by constitutive promoters would exhibit the same growth and differentiation characteristics as do M1myb cells. Finally, if any M1myb cells pass the early block caused by *myb* expression, continuous expression of *c-myc* would block differentiation at the intermediate stage; this may account for the intermediate-stage cells observed following treatment of M1myb11 cells with the potent differentiation inducer LUCM.

Early changes induced by IL-6 or LIF are reversed when *c-myc* fails to be suppressed. The few changes that M1myb cells treated with myeloid differentiation factor underwent, with time, to be reversed. Specifically, *MyD* genes which were activated very early were turned off, with only very low levels detected by 1 day. In addition, endogenous *c-myc*, which was suppressed following treatment with IL-6 or LIF, was expressed again after M1myb11 cells were treated with IL-6 for 25 days (earlier times were not monitored; data not shown). When these cells (M1myb11-25) were removed from IL-6 for a period of several days to several weeks and then challenged with IL-6 again, they responded as did their parental M1myb11 cells, transiently expressing *MyD* genes and suppressing endogenous *c-myc*. Thus, prolonged treatment of M1myb11 cells with IL-6 did not result in selection of IL-6-resistant cells. These observations are consistent with M1myb cells treated with IL-6 undergoing some changes at very early times and then, in response to continuous expression of exogenous *c-myc*, reversing these changes and behaving like untreated M1 or M1myb cells.

The failure to maintain *MyD* gene expression demonstrated that continuous expression of *c-myc* prevented changes required for maintenance of *MyD* gene expression. Since IL-6 or LIF is in the culture medium throughout the differentiation process, these results suggested that there are differences in the regulation of induction and maintenance of expression of *MyD* genes. In cells containing a *c-myc*

TABLE 3. Growth inhibition induced by cytokines or LPS in M1 and M1myb11 cells

Inducer	No. of cells (10^6 /ml) ^a	
	M1	M1myb11
None	2.12	1.83
IL-6	0.39	1.51
LIF	0.38	1.45
IL-1	0.69	0.71
IFN- γ	0.49	0.51
IFN- α + β	0.37	0.43
LPS	0.28	0.24

^a Cells were seeded at a density of 0.1×10^6 /ml and counted after 4 days. All values represent the means of three independent determinations, with standard deviations up to 15%.

transgene, *MyD* genes continue to be expressed similarly to what is found in M1 cells (15). M1myb cell lines provide a vehicle for dissecting the molecular requirements for maintenance of *MyD* gene expression in IL-6- or LIF-stimulated cells. Interestingly, enforced *myb* expression blocked maintenance of expression of a *MyD* gene regulated at the level of transcription (*junB*) as well as of one regulated posttranscriptionally (*MyD88*) (22).

Suppression of c-myb is tightly linked to terminal differentiation and not growth inhibition. M1myb11 cells, in which the exogenous *c-myb* transcript continued to be expressed following treatment with the antiproliferative compounds IL-1, LPS, IFN- α + β , and IFN- γ , were, like M1myc cells, as responsive as the parental M1 cells to growth suppression by the different antiproliferative compounds which do not induce terminal differentiation (Table 3). Taken together with the fact that both M1myb and M1myc cells are blocked in the IL-6- or LIF-induced differentiation program, albeit at different stages, it can be concluded that *c-myc* and *c-myb* down-regulation is not intrinsic to growth suppression but is essential for terminal myeloid differentiation, which is consistent with our previous observations (16).

ACKNOWLEDGMENTS

We thank Michael Clarke (University of Michigan, Ann Arbor) for generously providing plasmids pMbm I and pMbm I/DHFR, Lawrence Souza (Amgen, Thousand Oaks, Calif.) for providing human recombinant IL-6, and Frank Lee (DNAX Research Institute, Palo Alto, Calif.) for providing the murine LIF expression vector.

This work was supported by American Cancer Society grant IM-544 (B.H.L.) and NIH grant 1ROICA43618-01 (D.A.L.).

REFERENCES

- Abdollahi, A., K. A. Lord, B. Hoffman-Liebermann, and D. A. Liebermann. 1991. Interferon regulatory factor-1 is a myeloid differentiation primary response gene induced by interleukin 6 and leukemia inhibitory factor: role in growth inhibition. *Cell Growth Differ.* 2:401-407.
- Bading, H., J. Hansen, and K. Moelling. 1987. Selective DNA binding of the human cellular *myb* protein isolated by immunofluorescence chromatography using a monoclonal antibody. *Oncogene* 1:395-401.
- Beaumont, C., I. Dugat, F. Renaudie, M. Souroujon, and B. Grandchamp. 1989. Transcriptional regulation of ferritin H and L subunits in adult erythroid and liver cells from the mouse. *J. Biol. Chem.* 264:7498-7504.
- Bender, T. P., C. B. Thompson, and W. M. Kuehl. 1987. Differential expression of *c-myc* in RNA in murine B lymphomas by blocked transcription elongation. *Science* 237:1473-1476.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Chou, C., R. A. Gatti, M. L. Fuller, P. Concannon, A. Wong, S. Chada, R. David, and W. A. Salser. 1986. Structure and expression of ferritin genes in a human promyelocytic cell line that differentiated in vitro. *Mol. Cell. Biol.* 6:566-573.
- Clarke, M. F., J. F. Kukowska-Latallo, E. Westin, M. Smith, and E. V. Prochownik. 1988. Constitutive expression of a *c-myc* cDNA blocks Friend murine erythroleukemia cell differentiation. *Mol. Cell. Biol.* 8:884-892.
- Evans, J. L., T. L. Moore, W. M. Kuehl, T. Bender, and J. P.-Y. Ting. 1990. Functional analysis of c-Myb protein in T-lymphocytic cell lines shows that it *trans*-activates the *c-myc* promoter. *Mol. Cell. Biol.* 10:5747-5752.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Fibach, E., and L. Sachs. 1975. Control of normal differentiation of myeloid leukemic cells. VIII. Induction of differentiation to mature granulocytes in mass culture. *J. Cell. Physiol.* 86:221-230.
- Gabrielsen, O. S., A. Sentenac, and P. Fromageot. 1991. Specific DNA binding by *c-myc*; evidence for a double helix-turn-helix-related motif. *Science* 253:1140-1143.
- Gonda, T. J., N. M. Gough, A. R. Dunn, and J. de Blaquiére. 1985. The nucleotide sequence of cDNA clones of the murine *myb* proto-oncogene. *EMBO J.* 6:3995-4002.
- Gonda, T. J., D. K. Sheiness, and J. M. Bishop. 1982. Transcripts from the cellular homologs of retroviral oncogenes: distribution among chicken tissues. *Mol. Cell. Biol.* 2:617-624.
- Gough, N. Personal communication.
- Hoffman-Liebermann, B., and D. A. Liebermann. 1991. Interleukin-6- and leukemia inhibitory factor-induced terminal differentiation of myeloid leukemia cells is blocked at an intermediate stage by constitutive *c-myc*. *Mol. Cell. Biol.* 11:2375-2381.
- Hoffman-Liebermann, B., and D. A. Liebermann. 1991. Suppression of *c-myc* and *c-myb* is tightly linked to terminal differentiation induced by IL6 or LIF and not growth inhibition in myeloid leukemia cells. *Oncogene* 6:903-909.
- Horak, H., A. R. Turner, and O. W. Yau. 1982. Comparison of colony stimulating activities secreted into mouse lung conditioned medium in the presence and absence of lithium chloride. *Exp. Hematol.* 10:123-129.
- Krystosek, A., and L. Sachs. 1976. Control of lysozyme induction in the differentiation of myeloid leukemic cells. *Cell* 9:675-684.
- Kuehl, W. M., T. P. Bender, J. Stafford, D. McClinton, S. Segal, and E. Dmitrovsky. 1988. Expression and function of the *c-myc* oncogene during hematopoietic differentiation. *Curr. Top. Microbiol. Immunol.* 141:318-323.
- Liebermann, D. A., and B. Hoffman-Liebermann. 1989. Proto-oncogene expression and dissection of the myeloid growth to differentiation developmental cascade. *Oncogene* 4:583-592.
- Lord, K. A., A. Abdollahi, B. Hoffman-Liebermann, and D. A. Liebermann. 1990. Dissection of the immediate early response of myeloid leukemic cells to terminal differentiation and growth inhibitory stimuli. *Cell Growth Differ.* 1:637-645.
- Lord, K. A., A. Abdollahi, S. M. Thomas, M. DeMarco, J. S. Brugge, B. Hoffman-Liebermann, and D. A. Liebermann. 1991. Leukemia inhibitory factor and interleukin-6 trigger the same immediate early response, including tyrosine phosphorylation, upon induction of myeloid leukemia differentiation. *Mol. Cell. Biol.* 11:4371-4379.
- Lord, K. A., B. Hoffman-Liebermann, and D. A. Liebermann. 1990. Complexity of the immediate early response of myeloid cells to terminal differentiation and growth arrest includes ICAM-1, JunB and histone variants. *Oncogene* 5:387-396.
- Lord, K. A., B. Hoffman-Liebermann, and D. A. Liebermann. 1990. Nucleotide sequence and expression of a cDNA encoding *MyD88*, a novel myeloid differentiation primary response gene induced by IL6. *Oncogene* 5:1095-1097.
- Lotem, J., and L. Sachs. 1976. Control of Fc and C3 receptors on myeloid leukemic cells. *J. Immunol.* 117:580-586.
- McClinton, D., J. Stafford, L. Brents, T. P. Bender, and W. M. Kuehl. 1990. Differentiation of mouse erythroleukemia cells is blocked by late up-regulation of a *c-myc* transgene. *Mol. Cell. Biol.* 10:705-710.
- Mushinski, J. F., M. Potter, S. R. Bauer, and E. P. Reddy. 1983. DNA rearrangement and altered RNA expression of the *c-myc* oncogene in mouse plasmacytoid lymphosarcomas. *Science* 220:795-798.
- Prochownik, E. V., and J. Kukowska. 1986. Deregulated expression of *c-myc* by murine erythroleukemia cells prevents differentiation. *Nature (London)* 322:848-850.
- Sachs, L. 1987. Cell differentiation and bypassing of genetic defects in the suppression of malignancy. *Cancer Res.* 47:1981-1986.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular

- cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Sawyers, C., C. T. Denny, and O. N. Witte.** 1991. Leukemia and the disruption of normal hematopoiesis. *Cell* **64**:337–350.
 32. **Shabo, Y., J. Lotem, M. Rubinstein, M. Revel, S. C. Clark, S. F. Wolf, R. Kamen, and L. Sachs.** 1988. The myeloid blood cell differentiation-inducing protein MGI-2A is interleukin-6. *Blood* **72**:2070–2073.
 33. **Slamon, D. J., T. C. Boone, D. C. Murdock, D. E. Keith, M. F. Press, R. A. Larson, and L. M. Souza.** 1986. Studies of the human *c-myb* gene and its product in human acute leukemias. *Science* **233**:347–351.