MOLECULAR AND CELLULAR BIOLOGY, Feb. 1993, p. 841–851
0270-7306/93/020841-11$02.00/0

Vol. 13, No. 2

Proto-Oncogenes of the *fos/jun* Family of Transcription Factors Are Positive Regulators of Myeloid Differentiation

KENNETH A. LORD, ABBAS ABDOLLAHI, BARBARA HOFFMAN-LIEBERMANN,* AND DAN A. LIEBERMANN*

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

Received 10 August 1992/Returned for modification 25 September 1992/Accepted 2 November 1992

The proto-oncogenes c-*jun*, *junB*, *junD*, and c-*fos* recently have been shown to encode for transcription factors with a leucine zipper that mediates dimerization to constitute active transcription factors; *juns* were shown to dimerize with each other and with c-*fos*, whereas *fos* was shown to dimerize only with *juns*. After birth, hematopoietic cells of the myeloid lineage, and some other terminally differentiated cell types, express high levels of c-*fos*. Still, the role of *fos/jun* transcription factors in normal myelopoiesis or in leukemogenesis has not been established. Recently, c-*jun*, *junB*, and *junD* were identified as myeloid differentiation primary response genes stably expressed following induction of terminal differentiation of myeloblastic leukemia M1 cells. Intriguingly, c-*fos*, though induced during normal myelopoiesis, was not induced upon M1 differentiation. To gain further insights into the role of *fos/jun* in normal myelopoiesis and leukemogenicity, M1*fos* and M1*junB* cell lines, which constitutively express c-*fos* and *junB*, respectively, were established. It was shown that enforced expression of c-*fos*, and to a lesser extent *junB*, in M1 cells results in both an increased propensity to differentiate and a reduction in the aggressiveness of the M1 leukemic phenotype. M1*fos* cells constitutively expressed immediate-early and late genetic markers of differentiated M1 cells. The in vitro differentiation of normal myeloblasts into mature macrophages and granulocytes, as well as the increased propensity of M1*fos* leukemic myeloblasts to be induced for terminal differentiation, was dramatically impaired with use of c-*fos* antisense oligomers in the culture media. Taken together, these observations show that the proto-oncogenes which encode for *fos/jun* transcription factors play important roles in promoting myeloid differentiation. The ability of the M1 leukemic myeloblasts to be induced for terminal differentiation in the absence of apparent *fos* expression indicates that there is some redundancy among the *fos/jun* family of transcription factors in promoting myeloid differentiation; however, *juns* alone cannot completely compensate for the lack of *fos*. Thus, genetic lesions affecting *fos/jun* expression may play a role in the development of "preleukemic" myelodysplastic syndromes and their further progression to leukemias.

Growth and differentiation of animal cells is a well-controlled and highly conserved process involving multiple changes in gene expression that are developmentally regulated and result in the conversion of proliferating, undifferentiated cells into nonproliferating, highly specialized cells. A profound example of this process, which continues throughout life, is the complex blood cell formation, whereby a hierarchy of hematopoietic progenitor cells in the bone marrow proliferate and differentiate along multiple, distinct cell lineages, including the proliferation and differentiation of myeloid precursor cells into mature granulocytes and macrophages (57). The establishment of in vitro culture systems for the clonal development of bone marrow cells (6, 43) and the availability of M1 myeloblastic leukemia cells, which proliferate autonomously and can be induced for differentiation and loss of leukemogenicity by physiological myelopoietic factors (15, 47, 49), provide an excellent biological system with which to study, side by side, the molecular biology of normal blood cell development and lesions that afflict it in leukemia and upon its progression (20, 23, 29, 30).

To enhance our understanding of the regulation of normal terminal differentiation and alterations in these regulatory processes that block differentiation, leading to leukemogenicity and its progression, recently we isolated and characterized cDNA clones of myeloid differentiation primary response (*MyD*) genes (1, 32–36). *MyD* genes are activated in the absence of de novo protein synthesis in M1 cells following induction for terminal differentiation and growth arrest by conditioned medium of mouse lungs (LUCM), a potent physiological source of hemopoietic differentiation inducers (29, 50). These studies have led to the conclusion that the immediate-early genetic response of terminal myeloid differentiation is complex. Both known genes, previously identified in the context of other biological systems, and novel genes, with yet unknown functions, are induced (1, 2, 32–36). In the course of this work, the proto-oncogenes c-*jun*, *junB*, and *junD* were identified as *MyD* genes, which are stably induced, suggesting that they may play key roles in the initiation, progression, and maintenance of the myelopoietic differentiation program.

The proto-oncogenes c-*jun*, *junB*, *junD*, and c-*fos* recently have been shown to encode proteins with a leucine zipper that mediates dimerization to constitute active transcription factors; *jun*s were shown to dimerize with each other and with c-*fos*, whereas *fos* was shown to dimerize only with *jun*s (44, 56). After birth, hematopoietic cells of the myeloid lineage (reference 13 and references; 29) and some other terminally differentiated cell types (51) express high levels of c-*fos*. However, the role of *fos/jun* transcription factors in normal myelopoiesis or in leukemogenesis has not been established. Intriguingly, c-*fos*, though stably induced during normal myelopoiesis, was not induced upon M1 differentia-

* Corresponding author.

tion (29, 32). To gain further insights into the role of *fos/jun* in normal myelopoiesis and leukemogenicity, M1 cells were stably transfected with a c-*fos* or *junB* transgene (*junB* was chosen as a paradigm for a *fos/jun* gene similarly induced in both M1 and normal myeloblasts), to obtain M1*fos* and M1*junB* cell lines that constitutively express c-*fos* or *junB*, and their growth and differentiation properties were analyzed. In addition, the effect of constitutive expression of c-*fos* on leukemogenicity of M1 cells in vivo was analyzed. The results of these experiments indicate that the proto-oncogenes of the *fos/jun* family of transcription factors are positive regulators of myeloid differentiation.

MATERIALS AND METHODS

Cells, cell culture, and mice. The murine M1 myeloid leukemic cell line, obtained from E. R. Stanley (Albert Einstein College of Medicine), was recloned in soft agar; clones were tested for differentiation-associated properties as described previously (29, 32, 34). M1 differentiation-competent clone 9 (M1D+) was used in this study. Myeloblast-enriched bone marrow cells were obtained from femurs of CD-1 mice (Charles River Laboratories) injected intraperitoneally 3 days earlier with 3 ml of 10% sodium caseinate (Difco) in phosphate-buffered saline (PBS) (29). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) and 10% horse serum (M1) or 10% fetal calf serum (bone marrow) at 37°C in a humidified atmosphere with 10% CO_2. Cells were seeded at densities as indicated (0.1×10^6 to 0.2×10^6 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 $>0.25 \times 10^6$ when harvested. Viable cell numbers were determined by trypan blue dye exclusion, with counting in a hemocytometer. CD-1 *nu/nu* mice, 4 to 6 weeks old, were obtained from Charles River Laboratories. Cloning of M1 cells in soft agar was done as described previously (29).

Cytokines, biologicals, and other compounds. Serum-free LUCM was prepared with LiCl (24) and used at a concentration of 10%. Purified human recombinant interleukin-6 (IL-6) (0.5 mg/ml) was a gift from L. Souza, Amgen, Inc., Thousand Oaks, Calif., and used at a concentration of 100 ng/ml. Purified murine leukemia-inhibitory factor (10^6 U/ml), obtained from AMRAD Corp., Victoria, Australia, was used at a concentration of 200 U/ml. Purified recombinant granulocyte colony-stimulating factor (G-CSF) was a gift from Amgen and was used at a concentration of 160 ng/ml. Macrophage colony-stimulating factor (M-CSF) from serum-free conditioned medium of L929 fibroblasts, concentrated 100-fold by using high-molecular-weight polyethylene glycol (Serva), or purified M-CSF was used at 100 U/ml. The cytokines were titrated for differentiation-inducing and growth-inhibitory activities as described previously (22, 29, 34). Experiments were conducted by using concentrations which represent the optimum of the linear differentiation and/or growth inhibition dose-response curves.

The phosphorothioate-capped antisense oligonucleotide (5′-AsAsACCCGAGAACATCsAsT-3′), targeted against the first five codons plus two additional bases of c-*fos* (55), was used. Control sense (5′-AsTsGATGTTCTCGGGTsTsT-3′) oligodeoxynucleotides are the complementary sense strand of the antisense oligomers similarly modified. Phosphorothioate-capped oligodeoxynucleotides were synthesized and obtained from the Regional DNA Synthesis Laboratory Calgary, Alberta, Canada. Lyophilized oligomers were resuspended in PBS without Ca^{2+} or Mg^{2+} at 2.5 mM. c-*fos* antisense oligomers were added to cells at concentrations which represent the optimum of dose-response curves, and cytokines were added 3 h following incubation with oligomers only. All batches of oligomers were tested at the same concentration with uninduced M1 cells and found to have no effect on cell growth and viability, determined by trypan blue dye exclusion and counting in a hemocytometer. Results shown are representative of at least three independent experiments, each done in duplicate, using three different batches of antisense and sense oligomers. Rabbit anti-c-*fos* antibodies (689/1) (25) were a kind gift from R. Bravo.

Assays for differentiation-associated properties. Fc and C3 receptors were assayed as previously described (32, 37). Cell attachment was determined as previously described (32). 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 or granulocyte stages of differentiation, and mature macrophages or granulocytes (21, 32, 34).

General recombinant DNA techniques and DNA probes. Plasmid preparations, restriction enzyme digestions, DNA fragment preparations, and agarose gel electrophoresis were carried out as described before (21, 29). The probes for *junB* (*MyD21*), c-*jun* (*MyD42*), *junD* (*MyD63*), *IRF1* (*MyD32*), and *MyD88* were cDNAs cloned in this laboratory (1, 32–35). Also, murine ferritin light chain and lysozyme were cloned in this laboratory from a cDNA library of myeloid-enriched bone marrow, sequenced by S. Suggs (Amgen), and found to match the known sequence (5, 21). Probes for c-*fos*, β-actin, and IL-6 were the same as those used previously (29, 33). The probe for *fos*B was the 2-kb *Eco*RI insert of cDNA clone AC113-1, kindly provided by Rodrigo Bravo (58), whereas the probe for *fra*1 was the 1.5-kb insert of cDNA clone pSP65-*fra*1 (11), a kind gift of Donna Cohen. DNA for probes was labeled by random priming to a specific activity equal to or greater than 10^9 cpm/μg (14).

c-*fos* and *junB* expression vectors. pCMV-fos, in which rat c-*fos* is under control of the cytomegalovirus (CMV) immediate-early promoter (52), was obtained from Tom Curran. The *junB* expression vector (pAc.junB), in which *junB* is under control of the β-actin promoter, was constructed in this laboratory. Briefly, the 1.2-kb *Sma*I-*Xho*I restriction fragment, containing the entire coding region of a mouse *junB* (*MyD21*) cDNA (34), was cloned into the *Hin*dIII site of pHb APr-1-neo (19), and the sticky ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase (Promega).

Stable and transient DNA transfections. M1 cells were stably transfected by electroporation (Bio-Rad Gene Pulsor). To obtain M1*fos* cell lines, M1 cells were transfected with both pCMV-fos and pSV2neo (American Type Culture Collection) at a 10:1 ratio. pCMV-fos was linearized at the *Pvu*I site. pSV2neo, linearized by digestion with *Eco*RI, was necessary to select for transfectants by resistance to geneticin (G418). To obtain M1*junB* cell lines, M1 cells were transfected with linearized (*Pvu*I) plasmid pAc.junB, harboring the Neo^r gene. In either case, a pulse was delivered to a 0.7-ml suspension containing 1.5×10^7 cells and 50 to 55 μg of linearized plasmid DNA. After 48 h, the cells were subjected to selection in growth medium containing 400 μg of geneticin (G418 sulfate; GIBCO) per ml. Within 4 weeks, surviving cells were detected. As judged from the frequency of positive wells, each well contained a clonal population of transfectants which was maintained in 200 μg of drug per ml. Transient transfection of plasmid AP-1 col/TK-CAT (kindly

provided by M. Karin (3) into M1 cell lines was performed by using DEAE-dextran as described elsewhere (4).

RNA extraction, Northern (RNA) blotting, and hybridization. RNA was extracted by the method of Chomczynski and Sacchi (9), using guanidinium thiocyanate. Total RNA (5 μg 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 in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 1 M NaCl, 1% sodium dodecyl sulfate [SDS]) at 42°C for 2 to 3 h and, following removal of the buffer, hybridized in additional buffer containing probe (10^6 cpm/ml) and 100 μg of sheared salmon sperm DNA per ml at 42°C for 12 to 16 h. Northern blots were washed twice (10 min each time) at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and twice (30 min each time) at 60°C in 0.1× SSC–1% SDS and were exposed to X-ray film at −80°C. Stripping blots of probe to rehybridize was done as described previously (32). 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 Beckman DU7 spectrophotometer. To compare *fos* RNA levels in M1 with those in normal myeloid cells, autoradiograms were obtained from blots with the same amount of RNA per lane, hybridized with the same batch of ^{32}P-labeled *fos* probe.

RT-PCR. To increase the sensitivity of detection of IL-6 transcripts in M1 cells. Polymerase chain reaction (PCR) was used on aliquots of RNA as described previously (1). Briefly, 1 μg of total RNA was reverse transcribed with 200 U of murine leukemia virus reverse transcriptase (RT) (Bethesda Research Laboratories) in 20 μl containing 1 mM deoxynucleosides (dNTPs), 4 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.001% gelatin, and 0.2 μg of oligo(dT) as the primer. Samples were diluted to 100 μl with buffer, yielding 0.2 mM dNTPs–2 mM $MgCl_2$–10 mM Tris (pH 8.3)–50 mM KCl–0.001% gelatin; 50 pmol of each primer and 1.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) were added, and samples were covered with mineral oil, heated at 95°C for 5 min, and subjected to 15 or 21 cycles of PCR in a Perkin-Elmer Thermal Cycler, using 2 min of denaturation at 95°C, 1 min of annealing at 55°C, and 4 min of polymerization at 72°C. According to the numbering of Chiu et al. (7), the 21-mer primers used spanned positions 388 to 408 and 617 to 597. After extraction with $CHCl_3$, 20 μl of products was electrophoresed, blotted, and hybridized with the IL-6 probe excised from pSP6mifB (32). λ DNA with appropriate primers (Perkin-Elmer Cetus) was used as an internal marker to monitor for efficiency and reproducibility of PCR amplification, and control samples not reverse transcribed were used to monitor for possible contamination with genomic DNA. RT-PCR for quantitation of c-*fos* with a low number of cells ($<10^6$; as in the case of antisense experiments) was performed essentially as indicated above except that RNA was extracted in the presence of 20 μg of *E. coli* rRNA (Boehringer Mannheim) per ml and subjected to 14 cycles of amplification. The 24-mer primers used correspond to nucleotides 205 to 228 and 645 to 622 for c-*fos* (rat/mouse) RNAs (12, 55). In all cases, PCR analysis, as determined empirically, was within the linear range of PCR cycles for IL-6 or *fos* mRNA. For quantitation of PCR products, 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 Beckman DU7 spectrophotometer.

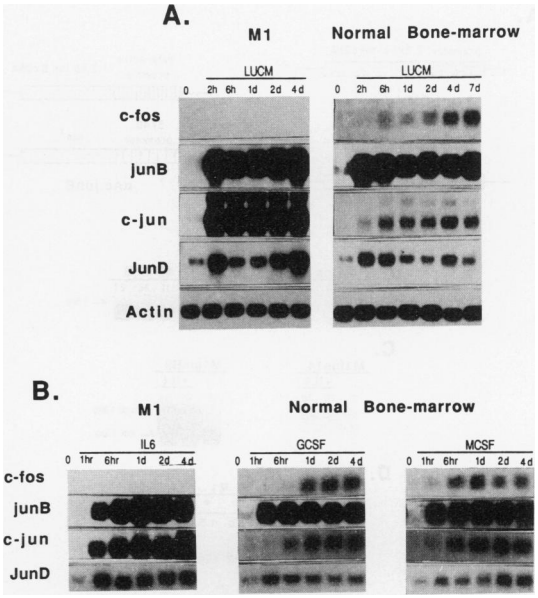

FIG. 1. Expression of c-*fos* and *jun* mRNAs during differentiation of normal and M1 leukemic myeloblasts. (A) Analysis of the expression of c-*fos* and *jun* during differentiation of M1 leukemic myeloblasts compared with differentiation induced in normal myeloblast-enriched bone marrow cells, using 10% LUCM. (B) c-*fos* and *jun* expression during IL-6 (100 ng/ml)-induced differentiation of M1 cells compared with G-CSF (160 ng/ml)-induced granulocytic and M-CSF (100 U/ml)-induced macrophage differentiation of myeloblast-enriched bone marrow cells. Expression was analyzed by hybridization to Northern blots, using total RNA (5 μg per lane) extracted from cells at the indicated times. All autoradiograms gave similar hybridization signals, comparable to what is shown in panel A, following hybridization to a β-actin probe.

RESULTS

***fos/jun* expression upon induction of differentiation of M1 leukemic myeloblasts compared with normal myeloblasts.** We have shown that LUCM is a potent physiological source of myelopoietic differentiation inducers, including IL-6 (33, 50), which can be used to induce differentiation of M1 leukemic myeloblasts as well as normal myeloblast-enriched bone marrow cells (29). LUCM induces primarily macrophage differentiation in M1 cells and both macrophage and granulocyte differentiation in normal myeloblasts (29). As shown in Fig. 1A, both *jun* (c-*jun*, *junB*, and *junD*) and *fos* mRNAs were stably expressed during LUCM-induced differentiation of normal myeloblasts. Further analysis of normal myeloid cells showed that *fos/jun* mRNAs were stably expressed upon induction of either granulocyte or macrophage differentiation, using G-CSF and M-CSF, respectively (Fig. 1B). In contrast, only *jun* (c-*jun*, *junB*, and *junD*) mRNAs were expressed during M1 differentiation induced by either LUCM or purified IL-6 (Fig. 1). G-CSF and M-CSF, which do not induce M1 differentiation (29), also did not induce *fos/jun* in M1 cells (data not shown).

Establishment and analysis of M1*fos* and M1*junB* cells constitutively expressing c-*fos* and *junB* transgenes. M1*fos* and M1*junB* cell lines were established via electroporation of M1 cells with the expression vectors pCMV-fos and

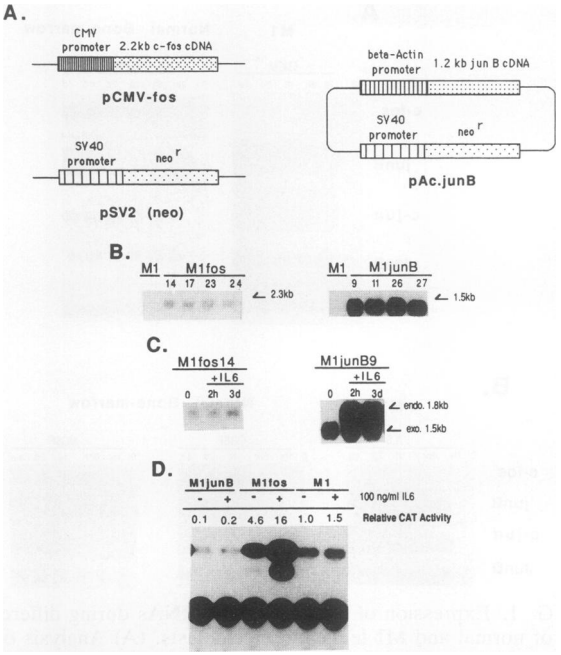

FIG. 2. Establishment of M1*fos* and M1*junB* cell lines. (A) Vectors used for transfection. M1*fos* cell lines were established by coelectroporation of M1 cells with pCMV-fos plus pSV2neo. M1*junB* cell lines were established by electroporation of M1 cells with pAc.junB. (B) Analysis of the expression of exogenous c-*fos* and *junB* mRNAs in different M1*fos* and M1*junB* cell lines, respectively. (C) Analysis of c-*fos* and *junB* expression in M1*fos*14 and M1*junB*9 cell lines, respectively, before and after stimulation for differentiation with IL-6 (100 ng/ml). The two *junB* hybridization bands observed with RNA obtained from M1*junB* cells following stimulation with IL-6 represent endogenous (1.8-kb) and exogenous (1.5-kb) *junB* transcripts. Expression was analyzed by hybridization to Northern blots, using total RNA (5 μg per lane) extracted from cells at the indicated times. (D) Analysis of transactivation of the AP-1 col/TK promoter in M1 versus M1*fos*9 and M1*jun*B14 cell lines. Transactivation was determined by measuring CAT activity in cell extracts obtained from the indicated cell lines following transfection with plasmid TRE col/TK-CAT, either untreated (−) or treated for 24 h with IL-6 (100 ng/ml) (+). TRE col/TK-CAT plasmid (5 μg/5 × 10^6 cells per 5 ml) was cotransfected with plasmid pMLV-β-gal (2 μg) to correct for DNA uptake. Relative CAT activity values represent CAT/β-galactosidase enzymatic activity ratios relative to that of TRE col/TK-CAT in M1 cells (=1) and are averages of at least three independent experiments.

pAc.junB, respectively (Fig. 2A), as described in Materials and Methods. As shown in Fig. 2B, M1*fos* and M1*junB* cell lines expressed c-*fos* and *junB* mRNAs, respectively, whereas no expression was observed in parental M1 cells prior to stimulation with IL-6. Expression of c-*fos* or *junB* was not detected in M1 transfectants obtained by using a vector carrying the selectable marker only (M1*neo*). DNAs obtained from the transfectants contained c-*fos*- or *junB*-hybridizing fragments characteristic of the transfecting vectors (data not shown). Following induction of differentiation with IL-6, expression of c-*fos* in M1*fos*14 was maintained at similar levels at early as well as at late times, whereas expression of exogenous *junB* RNA (under control of the β-actin promoter) in M1*junB*9 increased slightly (Fig. 2C). Similar results were obtained with other M1*fos* and M1*junB* cell lines. It should be pointed out that only M1*fos* cell lines expressing lower levels of *fos* than did normal cells could be established, even when an expression vector containing the strong β-actin promoter was used; 8- to 12-fold-lower levels of *fos* RNA (quantitated by densitometry) and much lower levels of *fos* protein (assessed by indirect immunofluorescence [42]) were observed in M1*fos* cells than in terminally differentiated normal myeloid cells (e.g., granulocytes and macrophages).

It was demonstrated that c-*fos* greatly enhances binding of the different *jun* proteins to the tetradecanoyl phorbol acetate-responsive element (TRE) (AP-1) consensus sequences (41, 46) and enhances transactivation compared with *jun* only (40). Also, it was shown that TRE-containing promoters are largely unresponsive to *junB* and can be activated by the other *juns* and that *junB* inhibits transactivation of TRE promoters (8, 40). Thus, transactivation of the TRE col/TK promoter (with the thymidine kinase [TK] promoter fused to a synthetic consensus TRE sequence [3]) was used to ascertain the functionality of the c-*fos* and *junB* transgenes. This was done by measuring chloramphenicol acetyltransferase (CAT) activity following transient transfection of plasmid TRE col/TK-CAT into M1, M1*junB* and M1*fos* cell lines. As shown in Fig. 2D, transactivation of the TRE col/TK promoter was relatively low in untreated M1 cells and slightly higher following stimulation with optimal concentrations (100 ng/ml) of IL-6. Transactivation of the TRE col/TK promoter was lower in M1*junB* cells than in M1 cells, both untreated and following stimulation with IL-6, which is consistent with a functional *junB* transgene. Apparently, in unstimulated M1*junB* cells, the protein product of the *junB* transgene can compete with proteins from endogenous *jun* genes, which are expressed at low basal levels (notably *junD*; Fig. 1) for binding to the TRE site, resulting in repression of TRE transactivation relative to M1; similarly, following stimulation of M1*junB* cells with IL-6, the protein product of the *junB* transgene, in addition to endogenous *junB*, can bind to and titrate out TRE sites, accounting for the failure to significantly increase TRE transactivation. In contrast, the TRE col/TK promoter was highly transactivated in untreated M1*fos* cells, indicative of the presence of a functional c-*fos* transgene, and further increased following stimulation of the cells with IL-6. The differential activation of the TRE col/TK promoter in M1*junB*9 and M1*fos*14 cells compared with M1 cells, as shown in Fig. 2D, also was observed with two other M1*junB* (11, 27) and M1*fos* (22, 24) cell lines and not with three different M1*neo* control cell lines. Taken together, the results of these experiments unequivocally demonstrate the presence of functional protein products of the *junB* and *fos* transgenes in M1*junB* and M1*fos* cells lines, respectively.

Effects of constitutive expression of c-*fos* or *junB* on growth and differentiation characteristics of M1 leukemic myeloblasts. Having the M1*fos* and M1*junB* cell lines allowed us to ascertain the effects that constitutive expression of c-*fos* or *junB* have on growth and differentiation of M1 myeloid leukemia cells. Detailed results are presented for M1*fos*14 and M1*junB*9 and compared with results for the parental M1 cell line. Three control M1 transfectants (M1*neo*) displayed growth and differentiation characteristics that were essentially indistinguishable from those obtained with M1, whereas the growth and differentiation characteristics of two other M1*fos* (22, 24) and M1*junB* (11, 27) cell lines (Fig. 2B) were essentially the same as described below for M1*fos*14 and M1*junB*9 cell lines, respectively.

As shown in Fig. 3A, constitutive expression of c-*fos* and to a lesser extent *junB* had a dramatic effect on growth arrest associated with M1 terminal differentiation induced by IL-6.

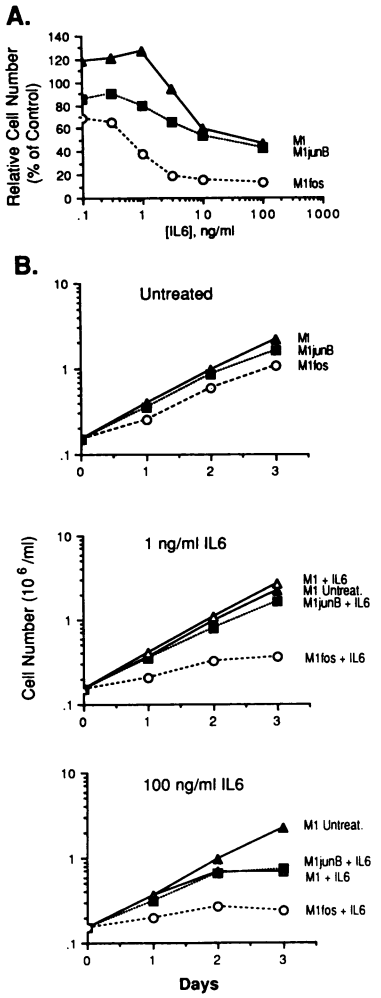

FIG. 3. () IL-6 dose response of the growth inhibition of M1*fos*14 and M1*junB*9 cell lines compared with M1 cells. Cells were seeded at 0.15 × 10^6/ml, and cell number was determined after 3 days. Results are presented as percentage of untreated M1 cells (% control). Note that low IL-6 concentrations (≤1 ng/ml) were observed to stimulate proliferation of M1 cells. (B) Growth kinetics of M1*fos*14 and M1*junB*9 cell lines compared with M1 cells in the absence (untreated) or presence of a low (1 ng/ml) or optimal (100 ng/ml) concentration of IL-6.

The growth of M1*fos* cells was markedly inhibited with concentrations of IL-6 as low as 1 ng/ml. In contrast, this concentration of IL-6 did not inhibit, and even enhanced, proliferation of M1 cells and marginally decreased proliferation of M1*junB*. Growth kinetics of M1, M1*junB*, and M1*fos* cell lines in the absence and presence of low or high concentrations of IL-6 are shown in Fig. 3B. It can be seen that untreated M1*junB* cells proliferated at a rate similar to that of parental M1 cells, whereas proliferation of M1*fos* cells was somewhat slower. The growth kinetics corroborate the results of the dose-response curves of Fig. 3A and demonstrate that IL-6 at 1 ng/ml enhanced proliferation of M1 cells, marginally affected proliferation of M1*junB* cells, and completely inhibited proliferation of M1*fos* cells (Fig. 3B). Proliferation of all of these cell lines was inhibited, however, by IL-6 at 100 ng/ml (Fig. 3A and B), the optimal concentration for M1-induced differentiation. Consistent with the proliferative capabilities of the cells in mass culture,

TABLE 1. Growth and differentiation characteristics of M1, M1*junB*9, and M1*fos*14 cells

Cells and concn (ng/ml) of IL-6 added	Colonies in agar[a]	Fc receptors[b] (%)	C3 receptors[b] (%)	Cell type (%)[b]		
				Blast	Intermediate	Mature
M1D+	347	2.1	1.3	>99	<1	0
1	423	23	38	97	3	0
100	6	82	75	10	58	32
M1*junB*	320	5.1	3.3	>99	<1	0
1	298	29	41	93	7	<1
100	0	78	77	6	48	46
M1*fos*	234	14	16	98	2	0
1	11	63	68	3	45	52
100	0	87	80	1	21	78

[a] Colonies in soft agar were counted 14 days after seeding of 500 cells in 5-cm-diameter dishes. Numbers represent the mean of three independent determinations with standard deviations of up to ±20%.

[b] Determined 4 days after the cells were seeded in liquid culture (0.1 × 10^6/ml). Values represent the mean of three independent determinations with standard deviations of up to ±15% (e.g., 2.1% = 2.1% ± 0.3%).

at 1 ng of IL-6 per ml, the relative cloning efficiency of M1 cells in soft agar was enhanced, whereas the cloning efficiency of M1*fos* cells decreased dramatically; colony formation of all of these M1 cells was inhibited by 100 ng of IL-6 per ml (Table 1 and Fig. 4A).

Furthermore, it was observed that in the absence of IL-6, a significant number (~25%) of the colonies formed by M1*fos* cells displayed a diffuse morphology at 7 days (Fig. 4B), characteristic of colonies with differentiated myeloid cells (31); the majority of these colonies degenerated by 14 days. No diffuse colonies were observed with M1 or M1*junB* cells. Also, the majority of the M1*fos* colonies formed after 7 days in the presence of 1 ng of IL-6 per ml displayed a diffuse morphology, whereas no such colonies were observed with M1 or M1*junB* cells. These observation indicate that a proportion of the M1*fos* cells have the propensity to spontaneously undergo terminal differentiation, unlike M1 and M1*junB* cells, and that M1*fos* cells are more susceptible than M1 and M1*junB* cells to be induced by IL-6 for terminal differentiation.

Analysis of the morphological characteristics of M1*junB* and M1*fos* cells in mass culture showed that in the absence of IL-6, the majority of the M1*junB* and M1*fos* cells grew in aggregates, in contrast to M1 cells, which grew as single cells in suspension (Fig. 5A). In the case of M1*fos*, the majority of these cell aggregates attached to the surface of the tissue culture plate. It should be pointed out that aggregation is an early differentiation-associated characteristic of M1 cells (34). It also was observed that M1*fos* cells were induced for macrophage differentiation associated with attachment of the cells to the surface of the tissue culture plate with 1 ng of IL-6 per ml, which did not induce macrophage differentiation and cell attachment of M1 or M1*junB* cells (Table 1 and Fig. 5A and B). This result is consistent with the colony morphology seen in Fig. 4B.

Fc and C3 receptors are early myeloid differentiation markers that start to appear at about 6 h, following induction of differentiation of M1 cells and continue to increase (Fig. 6). As shown in Table 1 and Fig. 6, only very few (<2.1%) M1 cells display Fc and C3 receptors prior to stimulation for differentiation with IL-6. The number of M1*junB* cells which displayed Fc and C3 receptors was only slightly higher than what was observed for M1 cells, whereas a significant

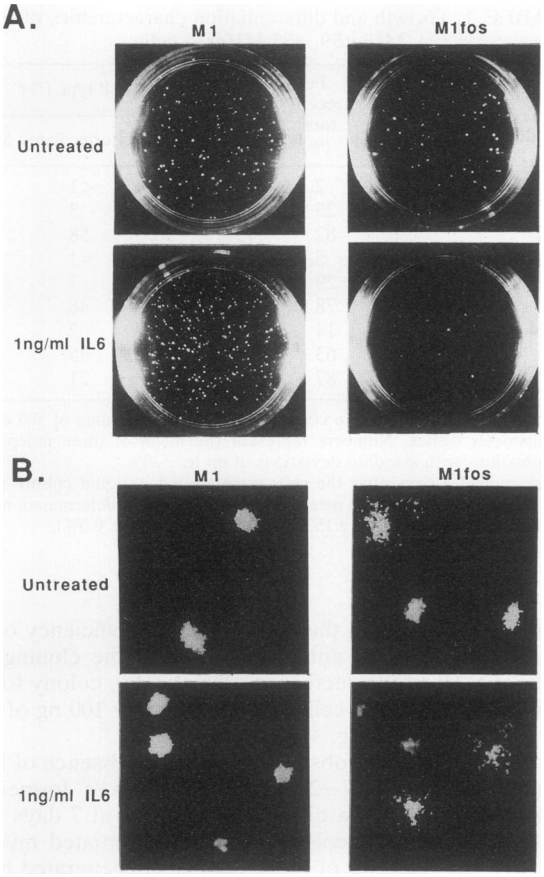

FIG. 4. Photographs and photomicrographs of M1 and M1*fos*14 colonies in soft agar. (A) Cells were seeded (500 cells per 5-cm-diameter dish) without or with IL-6 (1 ng/ml), and photographs were taken following 14 days. (B) Photomicrographs (magnification, ×80) of M1 and M1*fos* colonies 7 days after the cells were seeded in soft agar without or with IL-6.

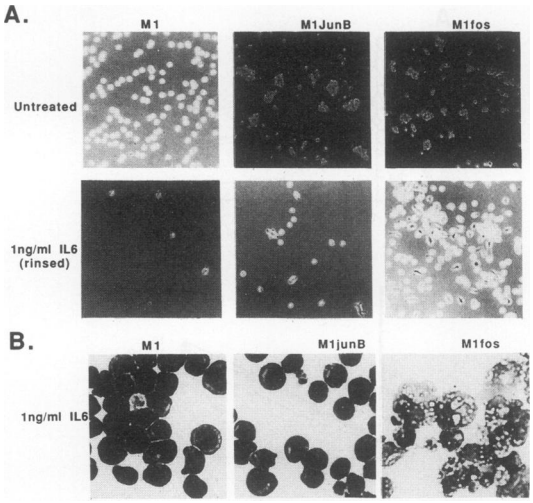

FIG. 5. Morphological characteristics of M1*junB*9 and M1*fos*14 cells compared with M1 cells in mass culture. (A) Photomicrographs (magnification, ×90) of M1, M1*junB*, and M1*fos* cells in culture without (untreated) or with IL-6 for 4 days. Photomicrographs of cells with IL-6 were taken after the tissue culture plates were rinsed three times with Dulbecco's modified Eagle's medium; thus, only cells which remained attached to the surface of the tissue culture plate are shown. (B) Photomicrographs (magnification, ×300) of May-Grünwald-Giemsa-stained cytospin smears of M1, M1*junB*, and M1*fos* cells following 4 days of treatment with IL-6 (1 ng/ml). Blasts are characterized by scant cytoplasm and round or oval nuclei; mature cells have large amounts of cytoplasm, irregularly shaped nuclei, and vacuoles.

increase was observed with M1*fos* cells (≥16%). It was also observed that at low concentrations of IL-6 (1 ng/ml), the number of M1*fos* cells which displayed Fc and C3 receptors was significantly higher than the number of M1 and M1*junB* cells (Table 1). The optimal concentration of IL-6 (100 ng/ml) induced similar high numbers of cells displaying Fc and C3 receptors in all three cell lines following 3 days (Table 1); however, the kinetics of induction of Fc and C3 receptors was accelerated in M1*junB* and M1*fos* cells compared with M1 cells (Fig. 6).

Taken together, these observations indicate that M1*fos* cells and to a lesser extent M1*junB* cells spontaneously display certain myeloid differentiation-associated characteristics and that M1*fos* cells are much more susceptible than M1 cells to induction for terminal differentiation.

Effects of constitutive expression of c-*fos* or *junB* on immediate-early and late genetic markers of M1 myeloid differentiation. To further characterize the effects that constitutive expression of c-*fos* and *junB* have on the myeloid differentiation program, we examined the expression of several myeloid differentiation immediate-early response (*MyD*) genes, which are induced within 30 min in the absence of de novo protein synthesis following stimulation of M1 cells for terminal differentiation (34). Five genes were selected: *junB* (*MyD21* [34]), c-*jun* (*MyD42* [32]), *junD* (*MyD63* [33]), *IRF1* (*MyD32* [1]), and *MyD88*, a novel *MyD* gene whose sequence has been reported recently (35). We have also looked at the effects of constitutive expression of c-*fos* or *junB* on two late genetic markers associated with M1 myeloid differentiation, namely, the ferritin light chain and lysozyme (21, 26).

As shown in Fig. 7A, stimulation of M1 cells with a low concentration of IL-6 (1 ng/ml) stably induced c-*jun* and *junD* mRNAs, although induction of c-*jun* mRNA was lower than what was observed with the optimal concentration (100 ng/ml) of IL-6. However, *junB*, *IRF1*, and *MyD88* mRNAs were only transiently induced, unlike the stable induction observed following stimulation with 100 ng of IL-6 per ml. In contrast, stimulation of M1*fos* cells with 1 ng of IL-6 per ml was sufficient to muster stable induction of all *MyD* gene

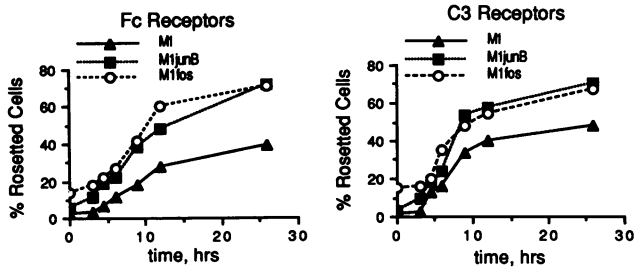

FIG. 6. Kinetics of induction of Fc and C3 receptors in M1, M1*junB*9, and M1*fos*14 cell lines following stimulation with IL-6. Cells were seeded at 0.15×10^6/ml, and the percentage of cells with Fc and C3 receptors was determined at the indicated times following stimulation with IL-6 (100ng/ml).

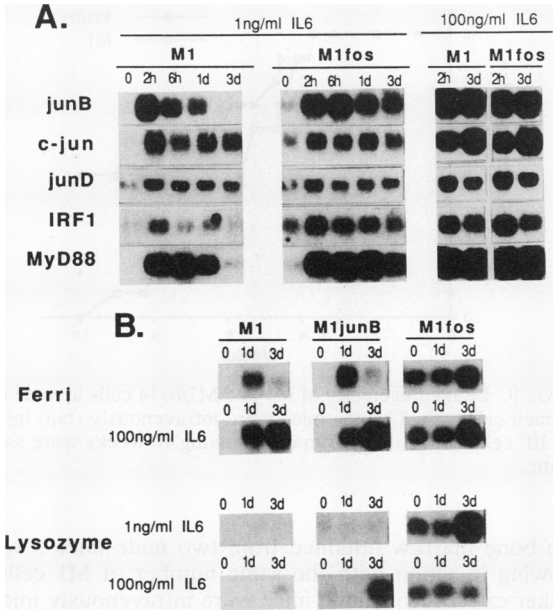

FIG. 7. Analysis of expression of immediate-early (A) and late (B) genetic markers before and after stimulation of M1, M1*fos*14, and M1*jun*B9 cells with IL-6. Expression was analyzed by hybridization to Northern blots, using total RNA (5 μg per lane) extracted from cells at the indicated times. All autoradiograns gave similar hybridization signals, comparable to what was shown in Fig. 1A, following hybridization to a β-actin probe.

mRNAs, including *junB*, *IRF1*, and *MyD88*. In addition, unlike in M1 cells, in M1*fos* cells, *junB*, c-*jun*, and *IRF1* mRNAs were expressed at low basal levels. No difference in the expression of *MyD* genes was observed in M1*junB* cells compared with M1 cells before or after stimulation with a low concentration of IL-6 (data not shown). At 100 ng of IL-6 per ml, no difference in *MyD* gene expression was detectable between M1 and M1*fos*.

As shown in Fig. 7B, in M1 cells, ferritin light-chain mRNA was induced following 1 day of stimulation with either 1 or 100 ng of IL-6 per ml; however, stable expression was observed only at the optimal IL-6 concentration (100 ng/ml). Induction of lyzozyme mRNA in M1 cells was observed 3 days following stimulation of the cells with 100 ng of IL-6 per ml, and no induction was detected with 1 ng/ml. In contrast, M1*fos* cells constitutively expressed high levels of both ferritin light-chain and lysozyme mRNAs, and ferritin transcript levels were further increased following stimulation with IL-6. Interestingly, in the case of lysozyme expression, it was observed reproducibly that in M1*fos* cells, the level of mRNA increased following stimulation with 1 but not 100 ng of IL-6 per ml (Fig. 7B); an analogous observation may be that the level of lysozyme mRNA was much lower following stimulation of M1 cells with supraoptimal concentrations (≥500 ng/ml) of IL-6 compared with stimulation with the optimal concentration (100 ng/ml) (data not shown). Again, in M1*junB* cells, expression of these late genetic markers did not vary significantly compared with what was observed in M1 cells, except that induction of lysozyme mRNA was higher in M1*junB* cells than in M1 cells following treatment with IL-6 at 100 ng/ml (Fig. 7B).

Effects of antisense c-*fos* oligomers on the differentiation of normal myeloblasts and M1*fos* leukemic myeloblasts. Expression of c-*fos* was observed to be induced upon induction of macrophage or granulocyte differentiation in normal bone marrow-derived myeloblasts (Fig. 1B). It was also observed that constitutive expression of c-*fos* in M1 leukemic myeloblasts dramatically increased the propensity of the cells to be induced for terminal differentiation by IL-6.

To further assess the role of c-*fos* in myeloid cell development, we examined the effects of c-*fos* antisense oligodeoxynucleotides (oligomers) in the culture medium on the differentiation of normal and the M1*fos* leukemic myeloblasts.

As shown in Fig. 8A and C, stimulation of myeloblast-enriched bone marrow cells with M-CSF or G-CSF, in the presence of control sense oligomers, resulted in differentiation of the majority of the cells into macrophages or granulocytes, respectively, similar to what was observed in the absence of oligomers (not shown). In contrast, in the presence of c-*fos* antisense oligomers, the number of mature macrophages or granulocytes was significantly reduced, and the number of myeloblasts was significantly increased.

Similar experiments with M1*fos* cells have shown (Fig. 8A) that in the absence of IL-6, neither sense nor antisense c-*fos* oligomers had a marked effect on the growth and differentiation properties of the cells, except that proliferation of M1*fos* cells treated with antisense oligomers appeared, reproducibly, to be slightly enhanced compared with their proliferation in the presence (or absence) of c-*fos* sense oligomers (Fig. 8A). However, the ability of the M1*fos* cells to be induced for differentiation with a low concentration of IL-6 was observed to be markedly impaired in the presence of c-*fos* antisense oligomers compared with sense oligomers, as evident from the lower number of mature macrophage-like cells and concomitant increase in the number of myeloblasts (Fig. 8A). Neither c-*fos* sense nor antisense oligomers had any effect on the proliferation or differentiation of M1 cells (data not shown). As shown in Fig. 8B, treatment of bone marrow or M1*fos* myeloblasts with c-*fos* antisense, but not sense, oligomers (at the same concentration that was used in the experiments described above) also reduced c-*fos* RNA.

Taken together, these observations provide further evidence for the important role that proto-oncogenes of the *fos/jun* family play in myeloid cell development (keeping in mind that *fos* must dimerize with *jun* to constitute functional transcription factors [44, 56]) and further substantiate the notion of the role that enforced expression of a c-*fos* transgene in M1 cells plays in increasing the propensity of these leukemic myeloblasts to be induced for differentiation by IL-6.

Constitutive expression of c-*fos* decreases the leukemogenicity of M1 cells. M1 cells are leukemogenic when injected into syngeneic (SL) or nude mice, and their leukemogenicity is lost following induction of differentiation in vitro or in vivo (47, 49). Thus, it is important to understand the relationship between an increase in the propensity of M1*fos* cells to be induced for differentiation in vitro and their leukemogenicity in vivo.

As shown in Fig. 9, all nude mice that were intravenously injected with 10^4 M1 cells died within 10 weeks, whereas only half of the nude mice injected with the same number of M1*fos* cells died within this time period. The surviving mice showed no sign of leukemogenicity, characterized by massive weight loss and lethargic behavior. Also, no myeloid leukemic cells were recovered from bone marrow obtained from two mice of this group, as determined by growth autonomy and growth and differentiation characteristics (49). In contrast, myeloid leukemic cells were recovered

A.

Cells & Treatment	Cell number 10^6/ml	Cell Type (%) Blast	Intermediate	Mature
Bone Marrow				
M-CSF + sense	0.31	4	33	63
+ AS	0.39	38	30	32
G-CSF + sense	0.22	2	23	75
+AS	0.24	17	35	48
M1fos				
Control + sense	1.5	90	10	0
+ AS	1.9	96	4	0
3 ng/ml IL6 + sense	0.33	3	41	56
+ AS	0.56	28	47	25

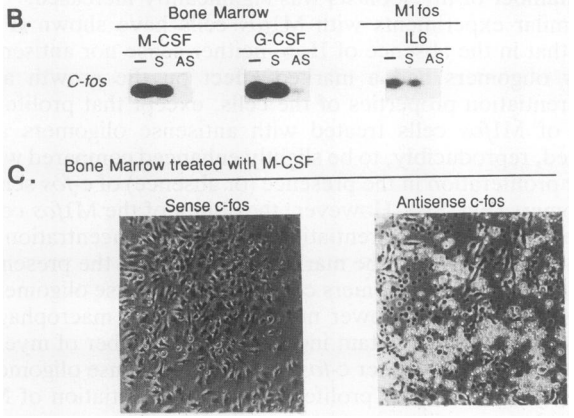

FIG. 8. Effect of c-*fos* antisense oligodeoxynucleotides in the culture medium on differentiation of normal and M1*fos* myeloblasts. (A) Growth and differentiation characteristics of myeloblast-enriched bone marrow cells and M1*fos*14 cells seeded in the presence of sense or antisense c-*fos* phosphorothioate-capped oligomers (20 bases in length) corresponding to the first five codons plus two additional bases of murine c-*fos* mRNA. (B) Effects of antisense c-*fos* oligomers on c-*fos* RNA in normal and M1*fos* myeloblasts (C) Photomicrographs (magnification, ×200) of myeloblast-enriched bone marrow cells 3 days following stimulation with M-CSF in the presence of sense or antisense c-*fos* phosphorothioate-capped oligomers. Myeloblast-enriched bone marrow cells (2.5×10^5/ml) were seeded in the presence of 30 μM sense or antisense c-*fos* oligomers, and M-CSF (100 U) or G-CSF (160 ng/ml was added to the culture medium following 3 h of incubation with oligomers only. Cell number and cells type were determined after 3 days. M1*fos* cells (1.5×10^5/ml) were seeded in the presence of 70 μM oligomers, without or with IL-6, as indicated above, and cell number and type were determined after 4 days. Values represent means of three independent determinations, with standard deviations of up to ±15% (e.g., for values expressed in percentage, 63% = 63% + 9%). Randomized oligomers, used at the same concentration as sense oligomers, gave results similar to those shown with c-*fos* sense oligomers. Neither sense nor antisense c-*fos* oligomers had an effect on the differentiation of M1 cells. To analyze for c-*fos* RNA levels, RNA was extracted from 0.5×10^6 myeloblast-enriched bone marrow or M1*fos* cells following 24 h of incubation in the presence of M-CSF, G-CSF, or IL-6, in medium without oligomers (−) or supplemented with sense (S) or antisense (AS) c-*fos* oligomers, at concentrations as indicated above. RNA was extracted in the presence of *E. coli* rRNA, one-fifth of the RNA was used for RT-PCR with c-*fos* primers as described in Materials and Methods, and one-fifth of the products were electrophoresed. Control samples not reverse transcribed gave no evidence of PCR products, and PCR with mouse β-actin amplimers (Clontech) has shown similar PCR amplification for the different RNA samples.

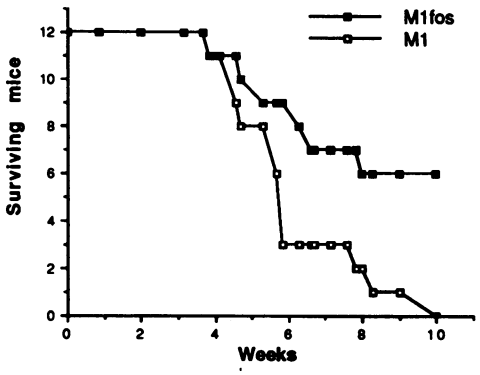

FIG. 9. Leukemogenicity of M1 and M1*fos*14 cells in nude mice. For each cell type, 12 nude mice were intravenously (tail) injected with 10^4 cells. Mice that survived following 10 weeks were asymptomatic.

from bone marrow obtained from two nude mice 5 weeks following injection with the same number of M1 cells. In another experiment, nude mice were intravenously injected with 2×10^6 M1 or M1*fos* cells that were treated for 5 days with 3 ng of IL-6 per ml; all 10 nude mice injected with IL-6-treated M1 cells died within 6 weeks, whereas none of the 10 mice injected with IL-6-treated M1*fos* cells died, or showed signs of disease, within this time period. Thus, constitutive expression of c-*fos*, which was observed to increase the propensity of M1 cells to be induced for terminal differentiation in vitro, also decreased leukemogenicity of the cells in vivo.

Effect of constitutive expression of c-*fos* on IL-6 expression in M1 cells. Multiple cytokine- and second-messenger-responsive elements have been located within the 5′ regulatory region of the IL-6 gene, including AP-1 binding sites that are recognized by AP-1 transcription factor complexes encoded by proto-oncogenes of the *fos/jun* family (45, 53). These observations have raised the possibility that some of the distinct characteristics of M1*fos* cells may reflect endogenous synthesis of IL-6 due to expression of the c-*fos* transgene. To test this possibility, we have used quantitative PCR (1) to determine endogenous IL-6 mRNA levels in M1*fos* compared with M1 cells before and after stimulation for differentiation.

As shown in Fig. 10, endogenous IL-6 mRNA was detected in untreated M1*fos* cells but not in M1 cells. Following stimulation with exogenous IL-6, endogenous IL-6 mRNA increased to a higher level in M1*fos* cells than in M1

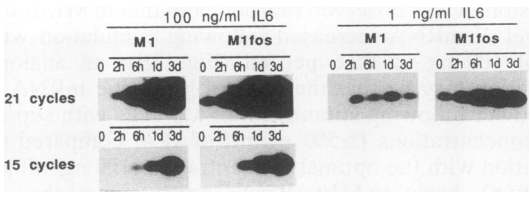

FIG. 10. Quantitation of endogenous IL-6 transcripts in M1 and M1*fos*14 cells by RT-PCR. RT-PCR was used with 1-μg aliquots of total RNA as described in Materials and Methods, and one-fifth of the products were electrophoresed. Control samples not reverse transcribed gave no evidence of PCR product, and PCR with mouse β-actin amplimers (Clontech) has shown that PCR amplification was the same for the different RNA aliquots. PCR analysis was within the linear range of PCR cycles for IL-6 mRNA.

cells, most notably following stimulation with a low concentration of IL-6 (1 ng/ml). Additional experiments, conducted to further test the relationship between endogenous IL-6 synthesis and the growth and differentiation characteristics of M1*fos* cells, have shown, however, that although endogenous IL-6 levels in M1*fos* cells stimulated with 1 ng of IL-6 per ml were lower than in M1 cells stimulated with 10 ng of IL-6 per ml, growth inhibition and differentiation of the M1*fos* cells was still more pronounced than that observed with the M1 cells (data not shown). No difference in IL-6 synthesis, before or after stimulation for differentiation, was observed between M1*jun*B and M1 cells.

Thus, constitutive endogenous synthesis of IL-6 and higher inducibility of the IL-6 gene in M1*fos* cells, presumably due to constitutive expression of the c-*fos* transgene, may account for some of the distinct characteristics of the M1*fos* cell lines.

DISCUSSION

As shown in this work, the expression of *fos* and *jun*s was induced during differentiation of normal myeloid precursor cells, whereas only *jun*s were induced during differentiation of M1 leukemic myeloblasts. The stable expression of *fos* and *jun*s during myeloid differentiation (this work; 18, 29) is in contrast to their transient expression following serum stimulation of quiescent fibroblasts (27, 28), thereby suggesting that they may play a role in the initiation, progression, and maintenance of the differentiation program. We would like to point out that expression of the RNA of neither *fosB* (58) nor *fra*1 (11), two *fos*-related genes, was detectable in the M1 cells used in this study or in myeloid-enriched bone marrow cells. More recently, the isolation of a third distinct *fos*-related gene, termed *fra*2 (38), has been documented. Whether the product of this gene plays a role in myeloid differentiation remains to be determined.

M1*fos* cells, which constitutively express a c-*fos* transgene, were shown to have a dramatically greater propensity to be induced for terminal differentiation by IL-6 than did M1 cells, which do not express *fos* during differentiation. Low levels of IL-6 were sufficient to muster stable induction of a set of immediate-early *MyD* genes in M1*fos* but not in M1 cells, including *MyD* genes regulated at the transcriptional or posttranscriptional levels (33). Also, M1*fos* cells constitutively express genetic markers of M1-induced differentiation, including the immediate-early genetic markers c-*jun*, *junB*, and *IRF1*, the early marker IL-6, and the late genetic markers ferritin and lysozyme. The differentiation of normal myeloblasts in vitro into mature macrophages or granulocytes, as well as the increased propensity of M1*fos* leukemic myeloblasts to be induced for terminal differentiation, was dramatically impaired via the use of c-*fos* antisense oligomers in the culture media. Finally, M1*fos* cells were shown to display a less aggressive leukemic phenotype than did the parental M1 cells when injected into nude mice.

Taken together, these observations clearly provide the first evidence of an important role for proto-oncogenes of the *fos/jun* family of transcription factors, notably c-*fos*, in the control of hematopoietic cell differentiation. To what extent all of these characteristics of the M1*fos* cells reflect either the direct function of *fos/jun* transcription factors or an indirect function, further down the ladder of the *fos/jun* regulatory cascade, remains to be determined.

The ability of the M1 leukemic myeloblasts to be induced for terminal differentiation in the absence of apparent *fos* expression indicates that there is some redundancy among the *fos/jun* family of transcription factors in promoting myeloid differentiation; however, the *jun*s alone cannot completely compensate for the lack of *fos*. Thus, genetic lesions affecting *fos/jun* expression may play a role in the development of "preleukemic" myelodysplastic syndromes and their further progression to leukemias.

Recently, by using a transgenic mouse line containing a *fos-lacZ* fusion gene, high constitutive levels of *fos-lacZ* were observed in skin, hair follicle, and bone, suggesting that constitutive expression of c-*fos* is associated with terminal differentiation of other cell types as well (51). Involvement of other leucine zipper transcription factors, e.g., transcription factors of the C/EBP family, in terminal differentiation of adipocytes has been demonstrated recently (54). By using this cellular system, it also was shown that constitutive expression of c-*myc* prohibited the induction of C/EBPα and prevented adipogenesis and that enforced expression of C/EBPα overcame the *myc*-induced block of adipocyte differentiation (17).

Clearly, further genetic manipulation of M1 cells as well as M1*myc* and M1*myb* cells, recently established in our laboratory, in which the genetic program of myeloid maturation has been disrupted at distinct developmental stages (21, 49), will be carried out. This analysis will be instrumental in determining the molecular nature and functions of different hetero- and homodimer transcription factor complexes of the *fos/jun* family in the initiation, progression, and maintenance of the normal myeloid differentiation program and the type of lesions that may afflict the function of these genes, thereby playing a role in leukemogenesis and its progression.

ACKNOWLEDGMENTS

This work was supported by NIH grant 1RO1CA43618-01 (D.L.) and American Cancer Society grant IM-544 (B.H.-L.). We also acknowledge Amgen's support for this work.

REFERENCES

1. **Abdollahi, A., K. A. Lord, B. Hoffman-Liebermann, and D. Liebermann.** 1991. Interferon regulatory factor-1 (IRF1) is a primary response gene to IL6 and leukemia inhibitory factor (LIF) induced terminal differentiation: role in growth inhibition. Cell Growth Differ. **2**:401–407.
2. **Abdollahi, A., K. A. Lord, B. Hoffman-Liebermann, and D. Liebermann.** 1991. Sequence and expression of a cDNA encoding MyD118: a novel myeloid differentiation primary response gene induced by multiple cytokines. Oncogene **6**:165–167.
3. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Ramhsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49**:729–739.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1992. Current protocols in molecular biology. Greene & Wiley-Interscience, New York.
5. **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.
6. **Bradely, T. R., and D. Metcalf.** 1966. The growth of mouse bone marrow cells in-vitro. Aust. J. Exp. Biol. Med. Sci. **44**:287–300.
7. **Chiu, C. P., C. Moulds, R. L. Coffman, D. Rennick, and F. Lee.** 1988. Multiple biological activities are expressed by mouse interleukin-6 cDNA clone isolated from mouse bone marrow stromal cells. Proc. Natl. Acad. Sci. USA **85**:7099–7103.
8. **Chiu, R., P. Angel, and M. Karin.** 1989. *Jun*-B differs in its biological properties from, and is a negative regulator of, *c-jun*. Cell **59**:979–986.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloro-

form extraction. Anal. Biochem. **162:**156–159.
10. **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.
11. **Cohen, D. R., and T. Curran.** 1988. *fra*-1: a serum-inducible cellular immediate-early gene that encodes for a *fos*-related antigen. Mol. Cell. Biol. **8:**2063–2069.
12. **Curran, T., M. B. Gordon, K. L. Rubino, and L. C. Sambucetti.** 1987. Isolation and characterization of the c-fos (rat) cDNA and analysis of post-translational modification in vitro. Oncogene **2:**79–84.
13. **Distel, R. J., and B. M. Spiegelman.** 1990. Protooncogene c-*fos* as a transcription factor. Adv. Cancer Res. **55:**37–55.
14. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
15. **Fibach, E., and L. Sachs.** 1975. Control of normal differentiation of myeloid leukemic cells. VIII. Induction of differentiation to mature cells in mass culture. J. Cell. Physiol. **86:**221–230.
16. **Fornace, A. J., J. Jackman, M. C. Hollander, B. Hoffman-Liebermann, and D. A. Liebermann.** Genotoxic-stress-response genes and growth arrest genes: the gadd, MyD, and other genes induced by treatments eliciting growth arrest. Ann. N.Y. Acad. Sci., in press.
17. **Freytag, S. O., and T. J. Geddes.** 1992. Reciprocal regulation of adipogenesis by *myc* and C/EBPa. Science **256:**379–382.
18. **Gonda, T. J., and D. Metcalf.** 1984. Expression of myb, myc, and fos during differentiation of a murine myeloid leukemia. Nature (London) **310:**249–254.
19. **Gunning, P., J. Leavitt, G. Muscat, S. Y. Ng, and L. Kedes.** 1987. A human β-actin expression vector system directs high-level accumulation of antisense transcripts. Proc. Natl. Acad. Sci. USA **84:**4831–4835.
20. **Hoffman-Liebermann, B., D. Liebermann, and L. Sachs.** 1981. Control mechanisms regulating gene expression during normal differentiations of myeloid leukemic cells: differentiation defective mutants blocked in mRNA production and mRNA translation. Dev. Biol. **81:**255–265.
21. **Hoffman-Liebermann, B., and D. Liebermann.** 1991. IL6 and leukemia inhibitory factor terminal differentiation of myeloid leukemia cells is blocked at an intermediate stage by constitutive c-*myc*. Mol. Cell. Biol. **11:**2375–2381.
22. **Hoffman-Liebermann, B., and D. 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.
23. **Hoffman-Liebermann, B., and L. Sachs.** 1978. Regulation of actin and other proteins in the differentiation of myeloid leukemic cells. Cells **14:**825–834.
24. **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.
25. **Kovary, K., and R. Bravo.** 1991. Expression of different jun proteins during the G0-to G1 transition in mouse fibroblasts: in vitro and in vivo associations. Mol. Cell. Biol. **11:**2451–2459.
26. **Krystosek, A., and L. Sachs.** 1976. Control of lysozyme induction in the differentiation of myeloid leukemic cells. Cell **9:**675–684.
27. **Lau, L. F., and D. Nathans.** 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-*fos* or c-*myc*. Proc. Natl. Acad. Sci. USA **84:**1182–1186.
28. **Lau, L. F., and D. Nathans.** 1991. Genes induced by serum growth factors. *In* P. Cohen and J. G. Foulkes (ed.), Molecular aspects of cellular regulation, vol. 6. Elsevier, Amsterdam.
29. **Liebermann, D., and B. Hoffman-Liebermann.** 1989. Proto-oncogene expression and dissection of the myeloid growth to differentiation developmental cascade. Oncogene **4:**583–592.
30. **Liebermann, D., B. Hoffman-Liebermann, and L. Sachs.** 1980. Molecular dissection of differentiation in normal and leukemic myeloblasts: separately programmed pathways of gene expression. Dev. Biol. **79:**46–63.
31. **Liebermann, D., and L. Sachs.** 1979. Increase of normal myeloblast viability and multiplication without blocking differentiation by type C RNA virus from myeloid leukemic cells. Proc. Natl. Acad. Sci. USA **76:**3353–3357.
32. **Lord, K. A., A. Abdollahi, B. Hoffman-Liebermann, and D. Liebermann.** 1990. Dissection of the immediate early response of myeloid cells to terminal differentiation and growth inhibitory stimuli. Cell Growth Differ. **1:**637–645.
33. **Lord, K. A., A. Abdollahi, S. M. Thomas, M. DeMarco, J. S. Brugge, B. Hoffman-Liebermann, and D. Liebermann.** 1991. Leukemia inhibitory factor (LIF) and IL6 trigger the same immediate early response including tyrosine phosphorylation upon induction of myeloid leukemia differentiation. Mol. Cell. Biol. **11:**4371–4379.
34. **Lord, K. A., B. Hoffman-Liebermann, and D. Liebermann.** 1990. Complexity of the immediate early response of myeloid cells to terminal differentiation and growth arrest includes ICAM-1, *Jun*-B and histone variants. Oncogene **5:**387–396.
35. **Lord, K. A., B. Hoffman-Liebermann, and D. 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.
36. **Lord, K. A., B. Hoffman-Liebermann, and D. Liebermann.** 1990. Sequence of MyD116 cDNA: a novel myeloid differentiation primary response gene induced by IL6. Nucleic Acids Res. **18:**2823.
37. **Lotem, J., and L. Sachs.** 1976. Control of Fc and C3 receptors on myeloid leukemic cells. J. Immunol. **117:**580–586.
38. **Matsui, M., M. Tokuhara, Y. Konuma, N. Nomura, and R. Ishizaki.** 1990. Isolation of human fos-related genes and their expression during monocyte-macrophage differentiation. Oncogene **5:**249–255.
39. **Morgan, J. I., and T. Curran.** 1991. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. Annu. Rev. Neurosci. **14:**421–51.
40. **Nakabeppu, Y., and D. Nathans.** 1991. A naturally occurring truncated form of *fos*B that inhibits *fos/jun* transcriptional activity. Cell **64:**751–759.
41. **Nakabeppu, Y., K. Ryder, and D. Nathans.** 1991. DNA binding activities of three murine jun proteins: stimulation by *fos*. Cell **55:**907–915.
42. **Nguyen, H., B. Hoffman-Liebermann, and D. Liebermann.** Unpublished data.
43. **Pluznik, D. H., and L. Sachs.** 1965. The cloning of normal "mast" cells in tissue culture. J. Cell. Comp. Physiol. **66:**319–324.
44. **Ransone, L. J., and I. M. Verma.** 1990. Nuclear proto-oncogenes *fos* and *jun*. Annu. Rev. Cell Biol. **6:**539–577.
45. **Ray, A., P. Sassone-Corsi, and P. B. Sehgal.** 1989. A multiple cytokine-and second messenger-responsive element in the enhancer of the human interleukin-6 gene: similarities with c-*fos* gene regulation. Mol. Cell. Biol. **9:**5537–5547.
46. **Ryseck, R.-P., and R. Bravo.** 1991. c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. Oncogene **6:**533–542.
47. **Sachs, L.** 1987. Cell differentiation and bypassing of genetic defects in the suppression of malignancy. Cancer Res. **47:**1981–1986.
48. **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.
49. **Selvankumaran, M., D. Liebermann, and B. Hoffman-Liebermann.** Deregulated c-*myb* disrupts Interleukin-6- or Leukemia Inhibitory Factor-induced myeloid differentiation prior to c-*myb*: role in leukemogenesis. Mol. Cell. Biol. **12:**2493–2500.
50. **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.
51. **Smeyne, R. J., K. Schilling, L. Robertson, D. Luk, J. Oberdick, T. Curran, and J. I. Morgan.** 1992. Fos-lacZ transgenic mice: mapping of sites of gene induction in the central nervous

system. Neuron **8:**13–23.
52. **Sonnenberg, J. L., F. J. Rauscher III. J. I. Morgan, and T. Curran.** 1989. Regulation of proenkephalin by fos and jun. Science **246:**1622–1265.
53. **Tanabe, O., S. Akira, T. Kamiya, G. G. Wong, T. Hirano, and T. Kishimito.** 1988. Genomic structure of the murine IL6 gene. J. Immunol. **141:**3875–3881.
54. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT-enhancer binding protein: a component of a differentiation switch. Science **251:**288–292.
55. **Van Beveren, C., F. Van Straaten, T. Curran, R. Muller, and I. M. Verma.** 1983. Analysis of FBJ-MuSV provirus and c-*fos* mouse gene reveals that viral and cellular *fos* gene products have different carboxy termini. Cell **32:**1241–1245.
56. **Vogt, P. K., and T. J. Bos.** *Jun*: Oncogene and transcription factor. Adv. Cancer Res. **55:**1–35.
57. **Wintrobe, M. M., G. R. Lee, D. R. Boggs, T. C. Bithell, J. Foerster, J. W. Athens, and J. N. Lukens (ed.).** 1981. Clinical hematology, p. 35–74. Lea & Febiger, Philadelphia.
58. **Zerial, M., L. Toschi, R. P. Ryseck, M. Schuermann, R. Muller, and R. Bravo.** 1989. The product of a novel growth factor activated gene, *fos* B, interacts with JUN proteins enhancing their DNA binding activity. EMBO J. **8:**805–813.