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The C-Terminal Domain of B-Myb Acts As a Positive Regulator of Transcription and Modulates Its Biological Functions

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The *myb* gene family consists of three members, named A-, B-, and c-*myb*. All three members of this family encode nuclear proteins that bind DNA in a sequence-specific manner and function as regulators of transcription. In this report, we have examined the biochemical and biological activities of murine B-*myb* and compared these properties with those of murine c-*myb*. In transient transactivation assays, murine B-*myb* exhibited transactivation potential comparable to that of c-*myb*. An analysis of deletion mutants of B-*myb* and c-*myb* showed that while the C-terminal domain of c-Myb acts as a negative regulator of transcriptional transactivation, the C-terminal domain of B-Myb functions as a positive enhancer of transactivation. To compare the biological activities of c-*myb* and B-*myb*, the two genes were overexpressed in 32Dcl3 cells, which are known to undergo terminal differentiation into granulocytes in the presence of granulocyte colony-stimulating factor (G-CSF). We observed that c-*myb* blocked the G-CSF-induced terminal differentiation of 32Dcl3 cells, resulting in their continued proliferation in the presence of G-CSF. In contrast, ectopic overexpression of B-*myb* blocked the ability of 32D cells to proliferate in the presence of G-CSF and accelerated the G-CSF-induced granulocytic differentiation of these cells. Similar studies with B-*myb*–c-*myb* chimeras showed that only chimeras that contained the C-terminal domain of B-Myb were able to accelerate the G-CSF-induced terminal differentiation of 32Dcl3 cells. These studies show that c-*myb* and B-*myb* do not exhibit identical biological activities and that the carboxyl-terminal regulatory domain of B-Myb plays a critical role in its biological function.

The *myb* gene family currently consists of three members, named A-, B-, and c-*myb* (28). All three members of this family encode nuclear proteins that share extensive sequence homology and bind DNA in a sequence-specific manner and function as regulators of transcription (4, 11, 16, 24, 34, 42). The first one-third of each protein consists of an unusual structure of three tandem 50- to 52-amino-acid direct repeats which appear to mediate binding of the Myb proteins to DNA (4, 33, 34, 42). The central portion of the molecule contains a region which mediates the transactivating function of the proteins (34, 42). The C-terminal ends of these proteins contain a third conserved region whose function is at present unclear. A negative regulatory function has been proposed for this domain because deletion in this region appears to enhance the transcriptional transactivation potential of the c-*myb* gene product (10, 34, 42).

The sequence-specific DNA binding activity and ability to activate transcription of reporter genes linked to certain promoter-enhancer sequences suggest that Myb proteins act as nuclear transcription factors (11, 34, 42). Studies with antisense oligonucleotides demonstrate that expression of the c-*myb* gene product is essential for the proliferative potential of several myeloid and T-cell lines (15). In addition, studies with hematopoietic and neuronal cell lines suggest that terminal differentiation of these cells is accompanied by downregulation of c-*myb* gene expression (31, 37, 41) and that constitutive expression of c-*myb* blocks terminal differentiation of these cells (5, 7, 29, 30, 35, 38). Elimination of c-*myb* function in vivo by gene knockout techniques, has indicated that homozygous null c-*myb* mutant mice fail to show effective fetal hepatic hematopoiesis, resulting in their death in utero (25).

While c-*myb* and A-*myb* are known to be transactivators of transcription, there appears to be some controversy regarding the transactivation function of B-*myb*. In the chicken system, Foos et al. (13) have reported that B-*myb* lacks transactivation function and indeed functions as an antagonist to c-*myb*. However, Mizuguchi et al. (24), using human B-*myb* clones, reported the transcriptional transactivation of reporter genes containing Myb recognition sequences. Furthermore, deletion of the carboxyl terminus of B-Myb has been found to downregulate the transactivation potential of B-*myb* (26), suggesting that the C-terminal domains might have distinctive functions in c-*myb* and B-*myb*. However, like c-*myb*, the expression of B-*myb* is cell cycle dependent, and antisense inhibition of either c-*myb* or B-*myb* was found to independently inhibit cell proliferation, suggesting that both c-*myb* and B-*myb* regulate cell growth (1, 14, 15, 20).

In this report, we analyzed transcriptional transactivation properties of murine B-*myb* and the contribution of the three functional domains to this biochemical function. In addition, we studied the effect of ectopic overexpression of c-*myb* and B-*myb* on granulocyte colony-stimulating factor (G-CSF)-induced granulocytic differentiation of 32Dcl3 cells to determine if the two genes can exhibit similar biological effects in this cell system. These studies show that c-*myb* and B-*myb* genes perform distinctive biological functions, and these distinctions are determined by their C-terminal sequences.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum and 0.5% penicillin-streptomycin (Gibco). COS cells were cultured in the same medium, except that it was

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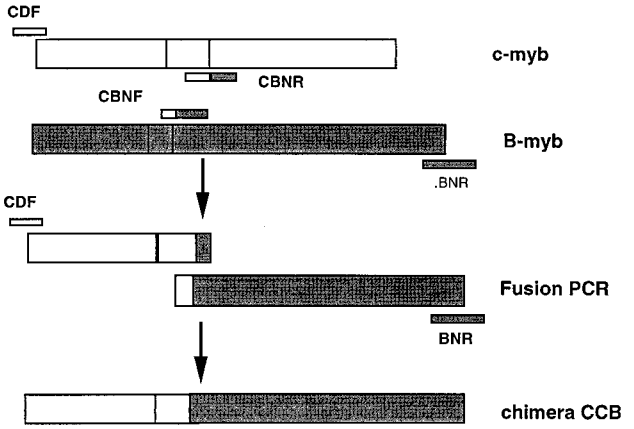

FIG. 1. Schematic representation of the fusion PCR protocol used for the generation B-*myb*–c-*myb* chimeras. Construction of chimera CCB is illustrated. Outer primer CDF is derived from the c-*myb* coding sequence, while BNR is derived from the B-*myb* coding sequence. Primers CBNR and CBNF contain sequences derived from c-*myb* and B-*myb* and determine the point of fusion between the two genes.

supplemented with 10% fetal bovine serum (FBS). WEHI3B cells were cultured in RPMI 1640 with 10% FBS. 32Dcl3 (17, 39) was maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 10% WEHI 3B cell-conditioned medium as a source of interleukin 3 (IL-3). HD11 cells were cultured in Iscove's modified DMEM supplemented with 2% chicken serum and 8% FBS.

Plasmids. Construction of the reporter plasmids pMIL-luc and pTA3-luc has been previously described (11, 27). For expression of c-*myb*, B-*myb*, and B-*myb*–c-*myb* chimeras in COS cells or NIH 3T3 cells, each construct was inserted into the expression vector pRC/CMV (In-vitrogen), which places the inserts under control of the cytomegalovirus (CMV) immediate-early promoter. Rous sarcoma virus (RSV)–β-galactosidase (β-Gal) plasmid (Invitrogen), which expresses the *lacZ* gene, was used as an internal standard to determine transfection efficiencies.

The inducible vector pMT-neo, which has the human metallothionein promoter containing metal-responsive elements (19) and which was modified from parental plasmid, was kindly provided by Dan Libermann.

Construction of chimeric genes and truncation mutants. The C-terminal truncated mutants of c-*myb* and B-*myb* were constructed by PCR utilizing primers tagged with *Not*I and *Xba*I sites, and the PCR product was ligated to pRC/CMV (Invitrogen). The inserts were completely sequenced to ensure that the PCRs did not introduce any mutations in the two genes. Chimeric constructs between B-*myb* and c-*myb* were generated by the gene fusion technique described earlier (12). For example, chimera CCB, which has a 5′ end encoding the DNA binding and transactivation domains of c-*myb*, was fused to the 3′ end of B-*myb*, following the protocol described below, which is schematically illustrated in Fig. 1. First, a PCR fragment was generated by using a pair of primers. These included a forward primer (CDF), 5′-ATA GCGGCCGC ATG GCC CGG AGA CCC CGA CAC-3′, which spans the 5′ end of c-*myb* c-DNA with a *Not*I site (underlined), and a reverse primer (CBNR), 5′-GCG CAC TTC TCC CAG ATC AGC GGG GTA GCT GCA AGT GTG GTT-3′, which has the antisense sequence of c-*myb* up to the end of the transactivation domain (amino acid 325) fused to the antisense sequence of B-*myb* (underlined), corresponding to the starting region of the B-*myb* C terminus which encodes amino acids 260 to 704. A second PCR was performed to amplify the 3′ end of B-*myb* encoding the amino acid sequence 260 to 704 with overlapping c-*myb* sequences in its 5′ terminus. For this, the forwarding primer, CBNF (5′-AAC CAC ACT TGC AGC TAC CCC GCT GAT CTG GGA GAA GTG CGC-3′), which contains the fusion sequences of c-*myb* and B-*myb* coding for amino acids 317 to 325 (underlined) of c-*myb* and amino acids 261 to 267 of B-*myb*, was used along with the reverse primer, BNR (5′-ATA TCT AGA TCA GGA CAG AAT GAG GGT CCG AGA-3′), which has an *Xba*I restriction site (underlined) and the 3′ terminus of the B-*myb* c-DNA. Both PCR products were combined, and a third PCR was carried out with primer CDF and primer BNR in order to fuse the two fragments by virtue of their shared overlapping regions; this reaction generated a full-length chimeric gene, CCB, with a 5′ *Not*I site and a 3′ *Xba*I site. In similar manner, a series of domain swapped chimeric genes between B-*myb* and c-*myb* were generated, and each construct was subcloned into the pRC/CMV expression vector with the corresponding *Not*I/*Xba*I sites in the vector. All of the constructs which were generated in this manner were completely sequenced to ascertain that no mutations were introduced into the coding sequences during PCR.

For the insertion of a hemagglutinin (HA) tag into *myb* coding sequences, the c-*myb* cDNA cloned into the pRC/CMV vector was digested with *Bsm*BI (which cuts the cDNA approximately 500 bp upstream of the terminator codon) and *Xba*I (which is in the polylinker site of the vector), and this fragment was replaced by a PCR product of the corresponding coding region, into which the sequence 5′-TAC CCA TAC GAC GTC CCA GAC TAC GCT-3′ encoding the sequence YPYDVPDYA was inserted immediately upstream of the terminator codon TGA.

Antisera. To generate anti–c-Myb antiserum, the 5′ end of the c-*myb* c-DNA encoding amino acids 1 to 325 was subcloned into the pDS5-6His vector (Quiaexpress), and the resulting fusion protein was purified by nickel chelate affinity chromatography (Quiaexpress). The purified protein was injected into rabbits for the generation of polyclonal antibodies. To generate B-Myb-specific antibodies, a region of the B-*myb* c-DNA which shows minimal homology to c-*myb* encoding the amino acid sequence 321 to 446 was subcloned into the pDS5-6His vector. Purified B-Myb fusion protein was used for injection into rabbits to raise B-Myb-specific antibodies. To detect HA-tagged proteins, a rabbit polyclonal antibody, Y11, raised against the HA epitope (purchased from SantaCruz Biotechnology, Santa Cruz, Calif.) was used.

Transient transfection assays. For transfection into NIH 3T3 cells, cells were seeded into 100-mm petri dishes at a density of 1.5×10^5 to 2×10^5 cells per plate. After 18 to 24 h, DNA was transfected by the calcium phosphate precipitation method (2). In each transfection, 5 μg of reporter and 5 μg of effector plasmids were transfected along with 1 μg of RSV-β-Gal DNA as an internal standard. Following incubation for 60 to 70 h, the cells were harvested in 900 μl of reporter lysis buffer (Promega). Luciferase activity was assayed with a luciferin substrate (Promega) according to the manufacturer's protocol. Luciferase activities were normalized against β-galactosidase activity to determine relative luciferase activities.

Establishment of stable cell lines expressing transgenes. Exponentially growing 32Dcl3 cells were electroporated with DNA with a Gene-Pulser (Bio-Rad) at a pulse of 230 V, 960 μF. The transfected cells were selected in 500 μg of G418 (Gibco-BRL) per ml for 2 to 3 weeks. To isolate single-cell clones, transfected cells were serially diluted in 96-well plates in the presence of G418 and selected for clonal expansion.

Northern blot analysis. Total RNAs from individual 32Dcl3 cell lines were purified with the Ultra-spec RNA (Biotecx) purification reagent. To purify RNA from Zn^{2+}-induced cells, the cells were incubated with 100 μM $ZnCl_2$ for 30 h prior to RNA isolation. Northern blot analysis was performed as previously described (2). To detect c-*myb* transcripts, a 2.3-kb c-DNA fragment that contained the entire coding region was isolated following digestion with *Bam*HI and *Hin*dIII and was used as a probe. To detect B-*myb*-specific transcripts, a 1.72-kb B-*myb*-specific probe was prepared by digestion with *Sal*I and *Xba*I.

Western blot analysis. To analyze the protein products of the transfected genes, normalized amounts of protein from each cell lysate were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore) in transfer buffer (10 mM CAPS, 10% methanol [pH 11.0]). The filter was blocked with 5% nonfat milk in TTS solution (0.05% Tween 20, 25 mM Tris-HCl [pH 7.4], 150 mM NaCl) for 4 h, incubated with primary antibody in the same buffer for 1 h, and washed three times in TTS solution. The filters were then incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham) and washed in the same manner. The Amersham ECL detection system was used for visualization as specified by the manufacturer.

Growth and differentiation assays. 32Dcl3 cells were maintained in IMDM containing 10% FBS and IL-3. Prior to the induction of differentiation, the cells were washed twice in IL-3-free medium and plated at a density of 10^5 cells per ml in medium containing 10% FBS and GCSF as described earlier (29, 39). The viability and proliferation rate of the cell cultures were monitored at regular intervals. Morphological analysis of G-CSF-treated cells was performed with an aliquot of cells that were cytospun and stained with May-Grunwald-Giemsa stain. To determine the proliferation rate in IL-3, cells were plated at a density of 10^5 cells per ml in medium containing IL-3 and 10% FBS, and cell numbers were determined at daily intervals.

RESULTS

Comparison of the transactivation potentials of murine c-*myb* and B-*myb*. Figure 2A shows a structural comparison between c-Myb and B-Myb proteins. The two proteins share highest structural homology in the DNA binding domain (87%), while they are least homologous (48%) in the transactivation domain. In the C-terminal regulatory domain, they share 51% sequence identity (21). As can be expected from the high degree of homology in the DNA binding domain, it has been shown that both c-Myb and B-Myb bind to the sequence motif PyAACT/GG (24).

To gain an understanding of the relative transactivation potential of murine B-*myb* and c-*myb*, we carried out transcriptional transactivation studies. Figure 2B shows the two re-

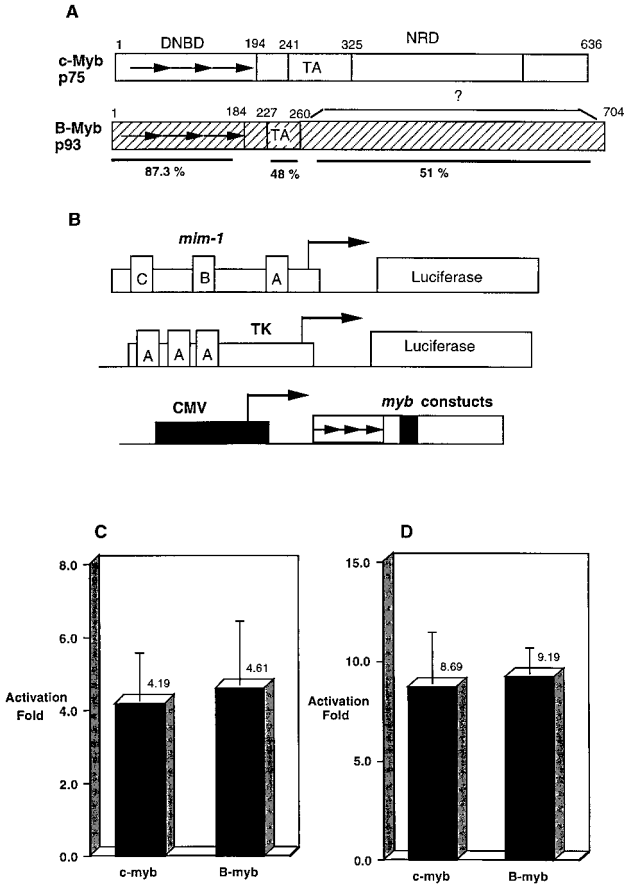

FIG. 2. (A) Structural comparison of c-Myb and B-Myb. Schematic structures of B-Myb and c-Myb proteins are presented. The numbers above each diagram are the positions of amino acid residues in each corresponding region. Thick bar, region of homology; percent homology values to c-Myb in each domain are given. Horizontal arrows, the three 51-to-52-amino-acid repeats that constitute the DNA-binding domain; DNBD, DNA-binding domain; TA, transactivation domain; NRD, negative regulatory domain. (B) Schematic representation of reporter and effector plasmids used in transient transactivation assays. Dotted box, promoters; arrows in promoters, starting sites of transcription; arrows in Myb, the three 51-to-52-amino-acid repeats of the DNA-binding domain; black box, transactivation domain; boxed A, B, and C in pMIL-luciferase, the three Myb binding sites in the *mim-1* promoter; TK, herpes simplex virus thymidine kinase (TK) promoter; CMV, immediate-early promoter for CMV. (C and D) Transcriptional activation by B-Myb and c-Myb. Each *myb* expression plasmid was transfected into NIH 3T3 cells along with either reporter plasmid pMIL-luc (C) or pTA3-luc (D) and the RSV–β-Gal plasmid as described in Materials and Methods. After 60 to 70 h of transfection, the cells were harvested and assayed for β-Gal activity. The luciferase activities were normalized to β-Gal activities, and the degrees of activation were obtained by setting the value of empty vector as 1.0. Shown are the means of activation obtained from at least three independent experiments. Vertical bars, standard deviations of the values.

porter plasmids pMIL-luc and pTA3-luc. The reporter plasmid pMIL was generated by cloning the naturally occurring Myb-inducible promoter of the *mim-1* gene into a luciferase vector (11, 27). The reporter plasmid pTA3-luc contained three copies of Myb binding sites, cloned in tandem upstream of a truncated thymidine kinase promoter (27). The expression vectors for c-*myb* and B-*myb* were generated by cloning the two murine cDNAs into pRC/CMV (Invitrogen), which places the two genes under the control of the CMV early promoter. Following transfection of the reporter and *myb* expression plasmids into a murine cell line, NIH 3T3, we analyzed the relative transcriptional transactivation potentials of the two genes. The results presented in Fig. 2D show that both c-Myb and B-Myb transactivated pTA3-luc reporter approximately 9-fold. Similar results were obtained with the pMIL-luc reporter, in which c-*myb* and B-*myb* exhibited somewhat lower but comparable transactivation potentials of 4.2- and 4.6-fold, respectively (Fig. 2C). These results demonstrate that murine B-Myb can transactivate promoters containing Myb-binding sites with the same efficiency as that of c-Myb.

The C terminus of B-Myb is a positive regulatory domain. Since deletion of the C-terminal domain of c-Myb results in increased transactivation potential, we carried out transcriptional transactivation experiments with a mutant of B-*myb*, from which the C-terminal sequences were similarly deleted (Fig. 3A). In addition, since the transactivation domain of B-Myb has been found to lack the acidic amino acid residues that are considered critical for the transactivation function of many transcription factors, we examined the transactivation potential of a mutant form of B-*myb*, in which the transactivation domain of the protein had been deleted and the DNA-binding domain was fused to the C-terminal domain maintaining the reading frame of the gene (Fig. 3A). The integrity of each construct was verified by sequence analysis and in vitro translation experiments to ascertain that different deletion mutants produced translational products of expected sizes (data not shown). Each expression construct was then transfected into NIH 3T3 cells along with the pTA3-luc reporter plasmid, since both c-*myb* and B-*myb* showed higher transactivation levels with this reporter (Fig. 2D). The results presented in Fig. 3B show that deletion of the 3′ end of c-*myb* cDNA which encodes the negative regulatory domain results in a two- to threefold increase in the transactivation potential of this mutant. On the other hand, deletion of the 3′ end of B-*myb*, which encodes the corresponding domain, resulted in a substantial loss of its transcriptional transactivation potential. Deletion of the transactivation domain of the B-*myb* gene resulted in a complete loss of its transactivation potential.

To rule out the possibility that the observed increase and decrease of the transactivation potential of the truncated forms of c-*myb* and B-*myb* are not due to differences in the expression levels of effector molecules, we carried out experiments to determine the intracellular levels of effector molecules in NIH 3T3 cells transfected with the effector and reporter DNAs. Since the antibodies that are currently available do not recognize C-Myb and B-Myb proteins with the same affinity, we constructed c-*myb* and B-*myb* expression vectors, which are tagged with the HA epitope, thus allowing detection of these proteins with a single monoclonal antibody. The transactivation potentials of the various HA epitope-tagged molecules were found to be identical to those of their nontagged counterparts (data not shown). Analysis of the NIH 3T3 cell extracts (normalized to units of β-Gal activity) transiently transfected with normal and truncated c- and B-*myb* expression vectors revealed that the full-length c-Myb protein was expressed at the highest levels in these cells, which was approximately twofold higher than that seen with the truncated c-Myb. If one were to normalize the transactivation activities of the two constructs to the intracellular effector protein levels, one could conclude that the truncated form of c-Myb exhibits four- to sixfold higher transactivation potential than that seen with the full-length c-*myb* expression vector. This is in agreement with previously published data (24, 42). When a similar analysis was carried out with NIH 3T3 cells transfected with full-length and truncated forms of B-Myb, it was found that the truncated form of B-Myb was expressed at a level similar to or slightly higher (1.5- to 2-fold) than that of the full-length counterpart. These results allow us to conclude that the C-terminal domain of c-Myb acts as a negative regulator of its transacti-

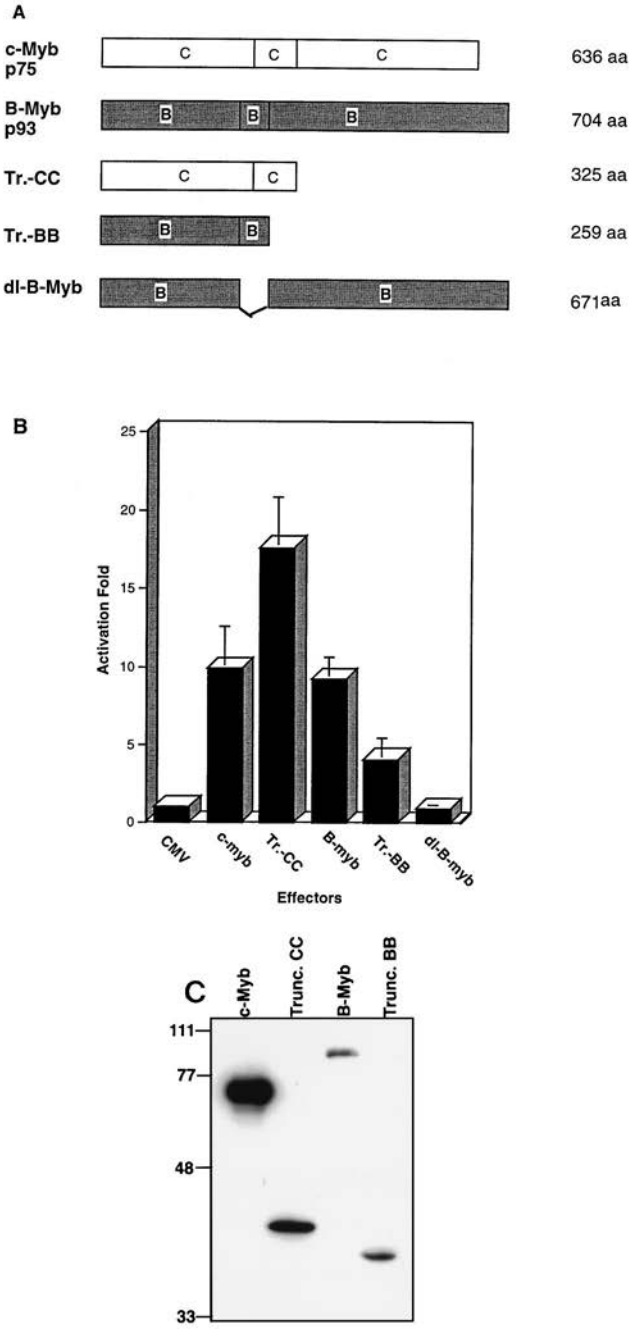

FIG. 3. (A) Structures of deleted and truncated mutants of B-*myb*. The schematic structures of B-*myb* mutants in which the transactivation domain (dl-B-Myb) or the C-terminal domain (Tr.-BB) have been deleted are presented. Also shown are the structures of wild-type c-*myb* and the C-terminus-truncated mutant of c-*myb* (Tr.-CC). aa, amino acid. (B) Transcriptional activation by the wild type and deletion mutants of B-*myb*. NIH 3T3 cells were transfected with expression plasmids containing the deletion mutants of B-*myb* or c-*myb*. In each transfection, 5 μg of effector plasmid and 5 μg of pTA3-luc were transfected along with 0.5 μg of RSV–β-Gal DNA. Shown are the mean values of activation obtained from at least three independent experiments with standard deviations. Other abbreviations are as defined in the legend to Fig. 2. (C) Transient expression of effector molecules. NIH 3T3 cell lysates transfected with expression plasmids and expressing equal amounts of β-Gal activity were subjected to Western blot analysis and probed with an antibody raised against the HA epitope. Trunc., truncated.

vation potential in NIH 3T3 cells, while under identical conditions, the C-terminal domain of B-Myb functions as a positive enhancer of this transactivation function. This is in agreement with the results reported for the human B-*myb* gene (26).

Construction of chimeric genes between B-*myb* and c-*myb*. To further demonstrate the distinctive roles played by the C-terminal domains of c-Myb and B-Myb, we constructed a series of chimeric molecules where the three functional domains of the c-Myb protein were replaced by the homologous domains of B-Myb. The structures of these chimeras are shown in Fig. 4A. Each DNA construct was subcloned into pRC/CMV (Invitrogen), and the integrity of each construct was verified by sequence analysis and in vitro transcription-translation assays, which showed that all of the constructs produced translation products of expected sizes (data not shown). In addition, the constructs were transfected into NIH 3T3 cells or COS-1 cells, and the cell lysates were examined by Western blot analysis, which showed that all of the chimeric genes produced proteins of appropriate sizes and appropriate immune reactivities.

Each expression construct was then transfected into NIH 3T3 cells along with the pTA3-luc reporter plasmid to study their transactivation potentials (Fig. 4B). Replacement of the transactivation domain of c-Myb with that of B-Myb in chimera CBC resulted in a reduction of its transcriptional activity by twofold, an activity identical to that seen with the deletion mutant of B-*myb*, BB, which lacks the C-terminal end. This suggests that the transactivation domain of B-Myb loses its ability to transactivate transcription in the absence of the C-terminal domain of B-Myb. Replacement of the C-terminal domain of B-Myb with that of c-Myb (chimera BBC) failed to restore the full transactivation potential of B-Myb, further indicating that the two domains do not perform the same function. These results suggest that the transactivation and the C-terminal domains of B-Myb act in concert in the transcriptional transactivation of target genes. Replacement of both the transactivation domain and the C-terminal domain of B-Myb with that of c-Myb (chimera BCC) restored the transactivation potential of the chimera to that seen with wild-type c-Myb, suggesting that the DNA-binding domains of the two genes are interchangeable.

Interestingly, replacement of the C-terminal negative regulatory domain of c-Myb with the C-terminal domain of B-Myb, as seen in chimera CCB, resulted in a twofold enhancement of the transactivation potential compared to that of full-length c-Myb. Similarly, chimera CBB, which contains the DNA-binding domain of c-Myb and the transactivation and C-terminal domains of B-Myb, showed a transactivation potential comparable to that of chimera CCB. While the transactivation potentials of chimeras CCB and CBB are higher than that of wild-type c-Myb, it should be noted that there is little or no difference among the activities of CCB, CBB, and truncated c-Myb (CC) in this assay. These results suggest that the B-Myb carboxyl terminus functions as a positive enhancer of transcription in conjunction with its own transactivation domain and fails to exhibit a negative regulatory activity when fused to the DNA-binding and transactivation domains of c-Myb. Analysis of the intracellular levels of the effector molecules suggests that these chimeric proteins are expressed at similar levels in transfected cells (Fig. 4C).

Since the inhibitory activities of the c-Myb C-terminal region vary considerably among cell lines, we studied the transactivation potentials of wild-type and various truncated and chimeric forms of c- and B-*myb* genes in another cell line, HD-11, which is of hematopoietic origin and which has been used extensively

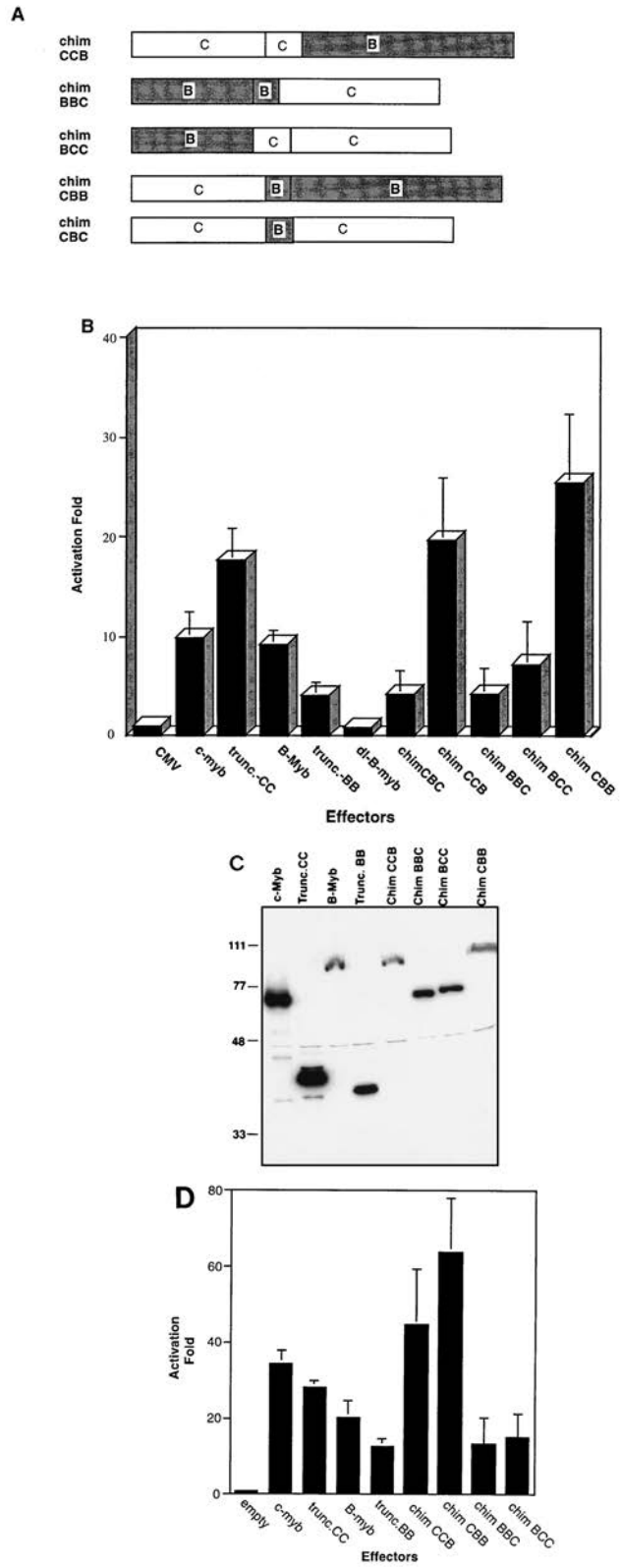

FIG. 4. (A) Schematic structures of chimeric genes between B-*myb* and c-*myb*. Schematic structures of different chimeric constructs between B-*myb* and c-*myb* are presented along with those of wild-type c-*myb* and B-*myb*. Crossed boxes, domains derived from B-*myb* (B); blank boxes, domains derived from c-*myb* (C). (B) Transactivational activities of chimeric genes between B-*myb* and c-*myb*. NIH 3T3 cells were transfected with 5 μg of each expression plasmid DNA along with 5 μg of pTA3-luc DNA and 0.5 μg of RSV–β-Gal DNA. Activation values were obtained as described above. Shown are the mean values of activation with standard deviations obtained from at least three independent experiments. (C) Transient expression of effector molecules. Cell lysates transfected with expression plasmids and expressing equal amounts of β-Gal activities were subjected to Western blot analysis and probed with an antibody raised against the HA epitope. (D) Transactivational activities of chimeric genes between B-*myb* and c-*myb* in HD-11 cells. HD-11 cells were transfected with 5 μg of each expression plasmid DNA along with 5 μg of pTA3-luc DNA and 0.5 μg of RSV–β-Gal DNA. Activation values were obtained as described above. Shown are the mean values of activation with standard deviations obtained from at least three independent experiments. chim, chimera; trunc., truncated.

by other investigators for studies with the c-*myb* gene. For this set of experiments, we used the reporter plasmid pTA3-luc, since this construct gives the highest levels of transactivation. Following transfection of the reporter and *myb* expression plasmids into HD-11 cells, we analyzed the relative transcriptional transactivation potentials of the various constructs, and the results of this experiment are presented in Fig. 4D. These results show that deletion of the C-terminal domain of c-Myb had little or no effect on its transactivation potential. However, deletion of the C-terminal domain of B-myb resulted in two-fold decrease of its transactivation potential, a result similar to that seen with NIH 3T3 cells. These results suggest that while the negative regulatory effect of the C-terminal domain of c-Myb is dependent on the cell type, the C-terminal domain of B-Myb exhibits its positive influence in both cell types. In the HD-11 cell background, chimeras CCB and CBB exhibited transactivation potentials 1.5- to 2-fold higher than that of c-Myb as well as that of the truncated form of c-Myb (CC), suggesting a positive regulatory effect of this domain. As can be expected, chimeras BBC and BCC exhibited levels of transactivation similar to those seen with B-Myb, again suggesting that the C-terminal domain of c-Myb does not exert its negative regulatory effect in HD-11 cells.

Effect of ectopic overexpression of B-*myb* and c-*myb* on myeloid cell proliferation. Based on the observation that both c-*myb* and B-*myb* are expressed during the late G_1 phase of the cell cycle and that antisense oligonucleotides directed against c-*myb* and B-*myb* inhibit cell proliferation, it has been proposed that these two proteins might have an analogous function in cells in which the two genes are expressed (1). To determine whether c-*myb* and B-*myb* can function in an analogous manner, we studied the effect of ectopic overexpression of these two genes on myeloid cell differentiation. For this we utilized the 32Dcl3 cell line, which is a myeloid precursor cell line derived from normal mouse bone marrow. This cell line was found to be strictly dependent on IL-3 for growth and, when cultured in a medium containing GCSF, was found to undergo terminal differentiation to granulocytes in a period of 10 to 12 days (17, 29, 32). We had earlier demonstrated that v-*myb* and c-*myb* genes, when ectopically overexpressed in this cell line, blocked its ability to undergo terminal differentiation in the presence of G-CSF and enabled this cell line to continuously proliferate in G-CSF (29, 30). This cell line provides a convenient assay system to test whether c-*myb* and B-*myb* can exert similar biological effects in a cell system. For these experiments, the complete coding regions of both c-*myb* and B-*myb* cDNAs were subcloned into the expression vector containing the human metallothionein promoter (pMT), the DNAs were electroporated into 32Dcl3 cells and selected in G418, and both mass cultures and single cell clones were established. Deletion of noncoding sequences from the c-*myb* cDNA allowed detection of transgene mRNA as a smaller transcript. As negative controls, the cells were transfected with an empty pMT vector that carried the G418 resistance gene.

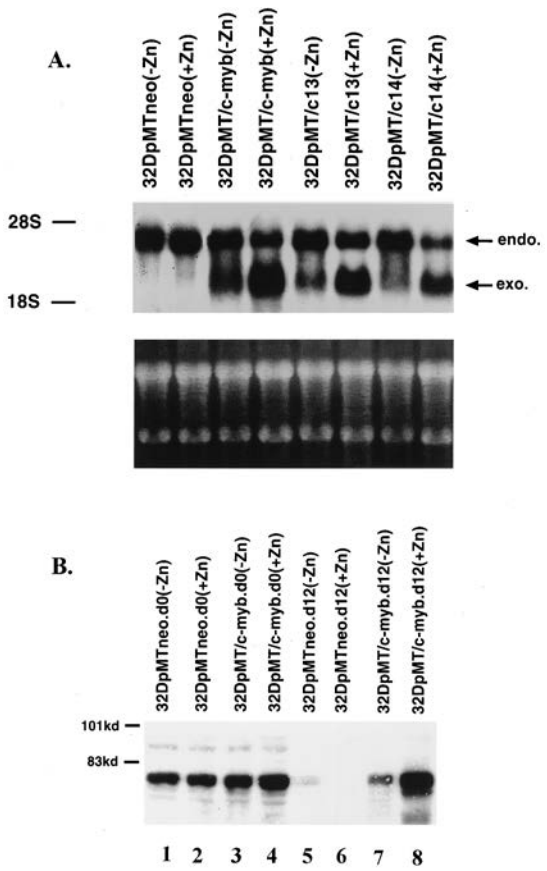

FIG. 5. Inducible expression of c-*myb* in 32Dcl3 cell lines. c-*myb* c-DNA in pMT-neo vector was transfected into 32Dcl3 cells, and mass cultures (32DpMT/c-*myb*) as well as single-cell clones (32DpMT/c13 and -14) were established as described in Materials and Methods. (A) Northern blot analysis of total RNA extracted from different cell lines with a full-length c-*myb* c-DNA probe. Endogenous (end.) c-*myb* transcript (upper band) and c-*myb* transcript encoded by the transgene (exo; lower bands) are marked. Ethidium bromide staining of RNA after completion of RNA transfer onto the nitrocellulose filter is shown in the lower panel. (B) Expression of c-Myb protein in empty vector-transfected cells (lanes 1, 2, 5, and 6) and c-*myb* expression in vector-transfected cells (lanes 3, 4, 7, and 8). Lanes: 1 and 2, cell lysates from empty vector-transfected cells grown in the presence of IL-3; 3 and 4, lysates from cells transfected with c-*myb* expression vector grown in the presence of IL-3; 5 and 6, lysates from cells transfected with empty vector but grown in the presence of G-CSF for 12 days (note the absence of c-Myb protein); 7 and 8, lysates from cells transfected with c-*myb* expression vector, also grown in the presence of G-CSF for 12 days. Note the low levels of c-Myb protein in cell lysates in the absence of Zn^{2+} (lane 7) and induction of high levels of transgenic Myb protein in the presence of Zn^{2+} (lane 8).

Following the establishment of permanent cell lines, we examined the effect of Zn^{2+} on the expression of the two transgenes.

Figure 5A and B shows the expression of c-*myb* in transfected 32Dcl3 cell lines. 32D cells transfected with empty vector DNA (32DpMT neo) showed the presence of a 3.8-kb endogenous c-*myb* band in the presence or absence of Zn^{2+}. In mass cultures (32DpMT/c-*myb*) as well as single-cell clones (32DpMT/c13 and -14) that were transfected with c-*myb* expression vectors, transgene expression was seen as a 2.3-kb transcript which could be detected at low levels in the absence of Zn^{2+}. In the presence of Zn^{2+}, the levels of this transcript were elevated by approximately 5- to 10-fold (Fig. 5A). Interestingly, the endogenous levels of c-*myb* transcripts were found to be downregulated concomitant to transgene expression, suggesting that an autoregulatory mechanism operates in these cells to maintain a constant amount of c-*myb* (18). This does not appear to be due to the addition of Zn^{2+}, since such a downregulation was not observed in cells transfected with empty vector. When the same cultures were examined for induction of c-Myb protein synthesis, we did not observe a significant increase in the levels of the Myb protein (Fig. 5B). This appears to be due to the downregulation of endogenous c-*myb* transcript levels, which results in the maintenance of a constant amount of c-Myb protein. In order to demonstrate that cells transfected with c-*myb* expression vectors do express transgenic c-Myb protein, we compared the c-Myb protein levels in cells that were cultured for 12 days in the presence of G-CSF. It had been previously shown that in 32Dcl3 cells grown in the presence of G-CSF, the endogenous levels of c-*myb* RNA and protein are downregulated and become undetectable by the 10th day of G-CSF treatment (29). Taking advantage of this observation, we analyzed the levels of c-Myb protein in control and c-*myb* expression vector-transfected cells following their incubation in G-CSF for 12 days. As shown in Fig. 5B, c-Myb protein could be readily detected in control and vector-transfected cells prior to their treatment with G-CSF. However, incubation of the empty vector-transfected cells in the presence of G-CSF for 12 days resulted in a complete downregulation of Myb protein levels, which became undetectable. In sharp contrast, in the c-*myb* expression vector-transfected cells, a small amount of c-Myb protein was observed in the absence of Zn^{2+} and these levels were markedly increased by Zn^{2+} treatment.

Figure 6A and B shows expression of transgenic B-*myb* RNA and protein in the mass cultures as well as single-cell clones (pMT/B-10, -11, and -12) by Northern and Western blot analyses. 32Dcl3 cells transfected with empty vector were found to express little or no endogenous B-*myb* RNA in the presence or absence of Zn^{2+}. However, cell lines transfected with B-*myb* expression vectors showed a high level of B-*myb* RNA expression in the presence or absence of Zn^{2+}. To analyze the expression of the B-*myb* protein in these cells, we carried out Western blot analyses using a B-Myb-specific polyclonal rabbit antiserum. As shown in Fig. 6B, there was little or no B-Myb protein expressed in cells transfected with empty vector in the presence or absence of Zn^{2+}. However, in cells that were transfected with B-*myb* expression vector, a high level of B-Myb protein could readily be detected in the absence or presence of Zn^{2+}. These results show that the expression of B-*myb* driven by the metallothionein promoter showed a significant level of leakiness such that there was no discernible difference in the expression levels between induced and uninduced cells at both RNA and protein levels. Despite the apparent lack of inducible expression, the absence of B-*myb* expression in 32Dcl3 cells and a high-level expression of B-*myb* in cells transfected with B-*myb* expression vectors allowed us to study the effects of ectopic overexpression of B-*myb* on the growth and differentiation properties of these cells.

The effects of overexpression of c-*myb* and B-*myb* on G-CSF-induced terminal differentiation of 32Dcl3 cells are shown in Fig. 7 and 8 and Table 1. As shown in Fig. 7A, upon incubation in a medium containing G-CSF, 32D cells transfected with the empty vector underwent several rounds of cell division followed by growth arrest around day 10 in the absence or presence of Zn^{2+}. On the other hand, cells transfected with the c-*myb* expression vector continued to proliferate without showing any growth arrest. In addition, the proliferation rates of these cells were higher in Zn^{2+}-treated cells compared to uninduced cells (Fig. 7B). These results suggest that even low-level c-*myb* expression (which is seen in cells in the absence of Zn^{2+}) delays G-CSF-induced growth arrest and that induction of high levels of c-Myb expression

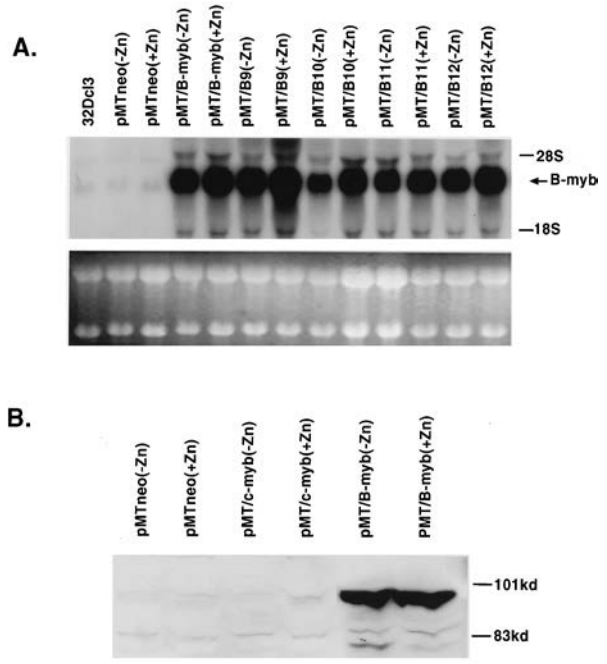

FIG. 6. Overexpression of B-*myb* in 32Dcl3 cell lines. B-*myb* c-DNA was subcloned into pMT-neo and transfected into 32Dcl3 cells by electroporation. After selection in G418, both mass cultures (pMT/B-*myb*) and single-cell lines (pMT/B9, -10, -11, and -12) were established. (A) Northern blot analysis of RNAs extracted from different cell lines grown in the presence (+Zn) or absence (−Zn) or Zn^{2+} was performed with a B-*myb*-specific probe as described in Materials and Methods. A picture of the nitrocellulose filter after ethidium bromide staining is shown below the Northern blot. (B) Analysis of B-Myb protein in 32Dcl3 cells transfected with B-*myb* (pMT/B-myb), c-*myb* (pMT/c-myb), and empty vector (pMTneo) in the presence (+Zn) or absence (−Zn) of Zn^{2+} in the medium. Each 32D cell line overexpressing B- or c-*myb* expression vector was lysed, and the cell lysates were subjected to Western blot analysis as described above. For detection of B-*myb*-specific translational product, antibodies specific for B-Myb protein (as described in Materials and Methods) were used.

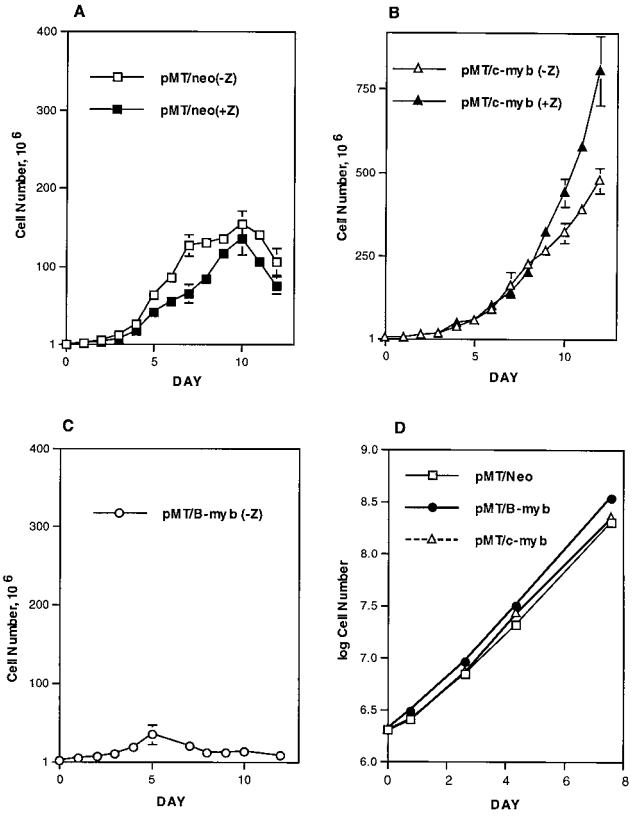

FIG. 7. Effect of B-*myb* overexpression on the growth of 32Dcl3 cells in the presence of G-CSF or IL-3. Cultures of 32Dcl3 cells transfected with empty vector (pMT/neo) (A), c-*myb* (pMT/c-*myb*) (B), or B-*myb* (pMT/B-*myb*) (C) were analyzed for growth in the presence of G-CSF. The cells were washed twice in IL-3-free medium and plated at a density of 10^5 per ml in medium containing 10% G-CSF. On each indicated day, the numbers of viable cells were determined by trypan blue exclusion. (D) Cell lines described for panels A to C were plated in IL-3-containing medium at a density of 10^5 per ml, and the cell numbers were determined at the indicated time points. Results are the means ± standard deviations of triplicate determinations from one representative experiment.

results in a higher rate of cell proliferation in the presence of G-CSF. In contrast, when 32D cells transfected with B-*myb* expression vector were placed in a medium containing G-CSF, they underwent growth arrest and failed to proliferate in the presence of G-CSF (Fig. 7C). Identical growth profiles were obtained with mass cultures as well as all of the single-cell clones. These results show that while c-*myb* expression results in increased proliferation of 32D cells in the presence of G-CSF, expression of B-*myb* results in a block to their ability to proliferate in the presence of G-CSF. Interestingly, this growth-inhibitory effect of B-*myb* was not seen in cells that were cultured in the presence of IL-3 (Fig. 7D), suggesting that the growth-inhibitory effect of B-*myb* is dependent on factors induced by G-CSF.

Effect of ectopic overexpression of B-*myb* and c-*myb* on myeloid cell differentiation. To determine the effects of ectopic overexpression of B-*myb* on G-CSF-induced 32D cell differentiation, we carried out a morphological analysis of these cells cultured in the presence of G-CSF, which is shown in Fig. 8. As was expected, cells transfected with empty vector underwent terminal differentiation to granulocytes by day 10 of G-CSF treatment (Fig. 8A), in the presence or absence of Zn^{2+}. However, 32Dcl3 cells transfected with the c-*myb* expression vector were slow to undergo terminal differentiation in the absence of Zn^{2+}, even though they all differentiated into mature granulocytes following culturing of cells in the presence of G-CSF for 15 days. On the other hand, in the presence of Zn^{2+}, these cells failed to undergo terminal granulocytic differentiation and were blocked in the promyelocytic stage and proliferated indefinitely as promyelocytes (Fig. 8A). These results confirm earlier observations that ectopic overexpression of c-*myb* in 32Dcl3 cells results in their continued proliferation in the presence of G-CSF as promyelocytes (29, 30). In sharp contrast, 32D cells overexpressing B-*myb* showed an accelerated pattern of differentiation, and within 4 days following the addition of G-CSF, approximately 50% of the cells were found to have differentiated into metamyelocytes and mature granulocytes (Fig. 8B and Table 1). By day 7, the entire culture consisted of granulocytes and the onset of apoptotic death could be seen in cells that had differentiated into granulocytes at an earlier time point. These results suggest that ectopic overexpression of B-*myb* results in growth arrest of cells in the presence of G-CSF and an acceleration of the granulocytic differentiation program in these cells.

Localization of the B-Myb-specific domain which accelerates myeloid cell differentiation. To determine the molecular basis for the observed differences between the biological activities of c-*myb* and B-*myb*, we examined the effects of ectopic overexpression of various chimeras that contained portions of c-*myb* and B-*myb* on G-CSF-induced 32D cell differentiation.

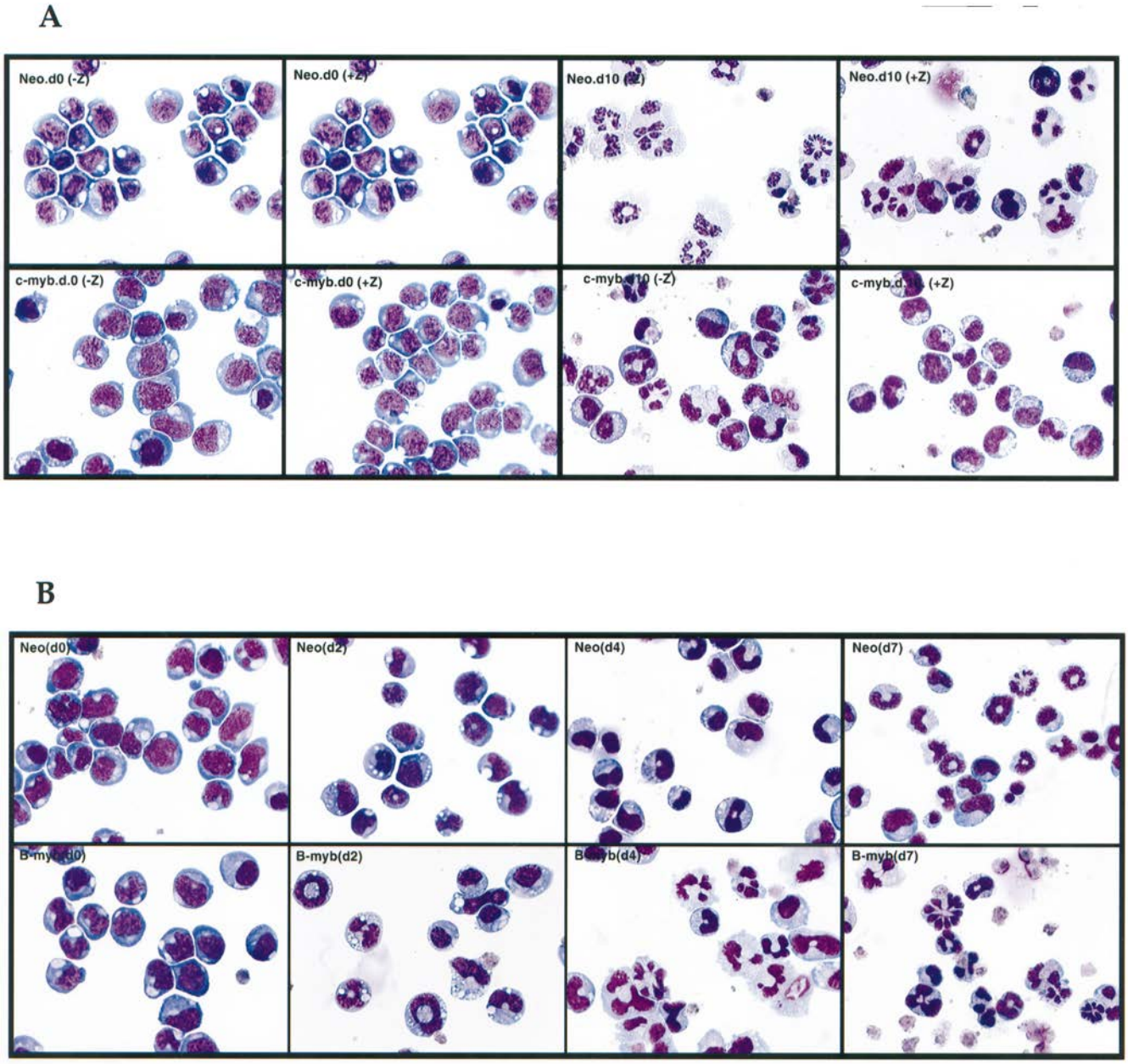

FIG. 8. (A) Morphological analysis of 32Dcl3 cells overexpressing c-*myb* following incubation in G-CSF-containing medium. 32D cells overexpressing c-*myb* were compared with mock-transfected 32Dcl3 cells for their responses to G-CSF addition. Aliquots of the cells were cytospun, stained with May-Grunwald-Giemsa stain, and analyzed for morphological changes during incubation in the presence (+Z) or absence (−Z) of Zn^{2+} on day 0 (d0) and day 10 (d10). (B) Morphological analysis of 32Dcl3 cells overexpressing B-*myb* following incubation in G-CSF. 32D cells overexpressing B-*myb* (B-*myb*) were compared with mock-transfected cells (Neo) for their responses to the addition of G-CSF. Aliquots of cells cultured in the absence of Zn^{2+} for 0, 4, and 7 days in the presence of G-CSF were analyzed by May-Grunwald-Giemsa staining as described in Materials and Methods.

These chimeras were subcloned into the pMT-neo plasmid vector and stably transfected into 32Dcl3 cell line as previously described. As can be seen in Fig. 9A, all three cell lines transfected with the expression vectors expressed the transgene, which, in all cases is smaller than the endogenous c-*myb* transcript. Since the C-terminal domain of B-Myb was larger than that of c-Myb, chimeras CCB and CBB expressed transcripts slightly larger than that of chimera CBC. In Western blot analysis, an antiserum directed against the N terminus of c-Myb was used. All three chimeric gene products migrated on SDS-polyacrylamide gels with the expected mobilities and exhibited immune reactivity to the N-terminal Myb antibody (Fig. 9B). Again, as was described for wild-type B-*myb*, the inducible promoter was leaky. With the exception of chimera CBC, in which slight induction of B-*myb* RNA and protein synthesis was observed in the presence of Zn^{2+}, no significant difference was seen with the other constructs in the levels of RNA and protein in the presence and absence of Zn^{2+}.

The cell lines overexpressing these chimeric genes were then examined for their responses to the addition of G-CSF. The results of this experiment are presented in Fig. 10 and 11 and Table 2. Upon incubation in a medium containing G-CSF, cells

TABLE 1. Differentiation profile of 32D cells overexpressing c-*myb* and B-*myb*[a]

Cell line[b]	Days after G-CSF addition	% of following cell types		
		Myeloblast	Promyelocyte/myelocyte	Metamyelocyte/granulocyte
32D/PMT-Neo (−Zn)	0	97.7 ± 1.5	2.3 ± 1.5	
	4	7.0 ± 1	85.7 ± 1.5	7.3 ± 0.6
	7	0.3 ± 0.6	76.0 ± 4.6	23.3 ± 2.5
	10		1.3 ± 1.5	98.7 ± 5.3
32D/PMT-Neo (+Zn)	0	98.3 ± 0.6	1.7 ± 0.6	
	4	12.0 ± 3	77.7 ± 6.8	10.3 ± 4
	7	0.7 ± 0.6	80.3 ± 2.5	19.0 ± 2.6
	10		3.3 ± 3.5	96.7 ± 10.1
32D/c-myb (−Zn)	0	97.7 ± 0.7	2.3 ± 0.6	
	4	10.7 ± 1.2	86.3 ± 1.5	3.0 ± 1
	7	1.0 ± 1.7	83.0 ± 1.5	15.4 ± 2.5
	10		66.7 ± 5.7	33.3 ± 10.8
32D/c-myb (+Zn)	0	99.0 ± 1	1.0 ± 1	
	4	23.0 ± 8.7	75.0 ± 7.8	2.0 ± 1
	7	2.7 ± 2.5	95.0 ± 3	2.3 ± 0.6
	10	10.0 ± 2	87.3 ± 3.2	2.7 ± 1.5
32D/B-myb (−Zn)	0	95.7 ± 2.5	4.3 ± 2.5	
	4	1.7 ± 1.2	50.0 ± 1	48.3 ± 5.6
	7		6.7 ± 3.2	93.3 ± 8.5

[a] 32D cells overexpressing each transgene were induced to differentiate by the addition of G-CSF. On the indicated days, aliquots of the cells were cytospun and stained with May-Grunwald-Giemsa. The values are means of percentages of the indicated cells from three independent studies ± standard deviations.
[b] −Zn, lacking zinc; +Zn, with zinc.

which overexpress the chimera CBB and CCB exhibited almost complete growth arrest, a phenotype which is very similar to that seen with the overexpression of wild-type B-*myb*. On the other hand, cells which overexpress the chimera CBC displayed a slightly suppressed growth rate compared to that of the empty vector-transfected cells (Fig. 10). As shown in Fig. 11, morphological analysis of these cells showed that cells overexpressing the chimera CBB and CCB terminally differentiated between days 4 and 6, while cells expressing chimera CBC followed the same differentiation course as the empty vector-transfected cells, which terminally differentiated at day 12. These results are also schematically presented in Fig. 12. Addition of Zn^{2+} to these cell cultures did not make a significant difference in the observed differentiation rate. Taken together, these results demonstrate that chimeras CCB and CBB, but not chimera CBC, mimic B-Myb in their biological activities. Since all of the chimeras that contain the C-terminal domain of B-Myb induce growth arrest and accelerate the terminal differentiation of 32Dcl3 cells, we conclude that the B-Myb carboxyl-terminal domain which appears to function as a positive regulator of transcription confers a unique set of properties to the B-Myb protein, which distinguishes it from that of c-Myb. In addition, our results suggest that the observed B-Myb-specific effects are not due to differential recognition of target promoters by the B-*myb* gene, because both chimeras CBB and CCB are expected to recognize the same sets of target promoters as c-Myb since they contain the DNA-binding domain of c-Myb.

DISCUSSION

The *myb* gene family consists of three members, named A-, B-, and c-*myb*, all of which bind DNA in a sequence-specific manner and function as regulators of transcription. While A-*myb* and c-*myb* have been described as potent activators of transcription, there appears to be a controversy regarding the ability of B-*myb* to transactivate transcription and its role in mediating cell proliferation and differentiation. Thus, some of the earlier reports had provided evidence for transcriptional activation of promoters containing Myb-responsive elements by B-*myb* (24, 26), while others showed that B-*myb* is incapable of transactivating transcription of promoters containing Myb-binding elements (13, 40). It should be noted that these conflicting results were obtained with B-*myb* clones derived from different species, and their transactivation potentials were often tested in cell lines derived from exogenous species. In addition, different investigators used widely different reporter plasmids which could have contributed to these experimental variations. A recent report provides support to the argument that the transcriptional transactivating activity of the human B-*myb* gene is highly dependent on the cell type used (36). In addition, most of the studies conducted so far were limited to transcriptional transactivation assays, since a simple biological assay was not available for *myb* genes until recently.

In this communication, we have examined two aspects of B-*myb* function. In the first set of experiments, we have studied the comparative abilities of murine c-*myb* and B-*myb* genes to activate transcription of reporter genes that are driven by promoters containing Myb-binding sites. To avoid problems associated with species specific variations that may be associated with these transcription factors, we used murine c-DNA clones to transactivate transcription in a mouse cell line. Our results show that both c-*myb* and B-*myb* c-DNA clones exhibit comparable transactivation potentials with two different sets of reporters. Both proteins exhibit a modular structure, with an N-terminal DNA-binding domain, a central transactivation domain, and a C-terminal regulatory domain. It had earlier been shown that the C-terminal domain of c-*myb* acts as a negative regulator of transcriptional transactivation function, since deletion of this region was found to considerably enhance this activity. Our results presented here show that unlike the case with c-Myb, deletion of the C-terminal domain of B-Myb was found to considerably reduce the ability of this protein to transactivate transcription of target genes, suggesting that this domain might act as an enhancer of transactivational function of this protein.

To further delineate the function of the C-terminal regulatory domain of B-Myb, we constructed several chimeric mole-

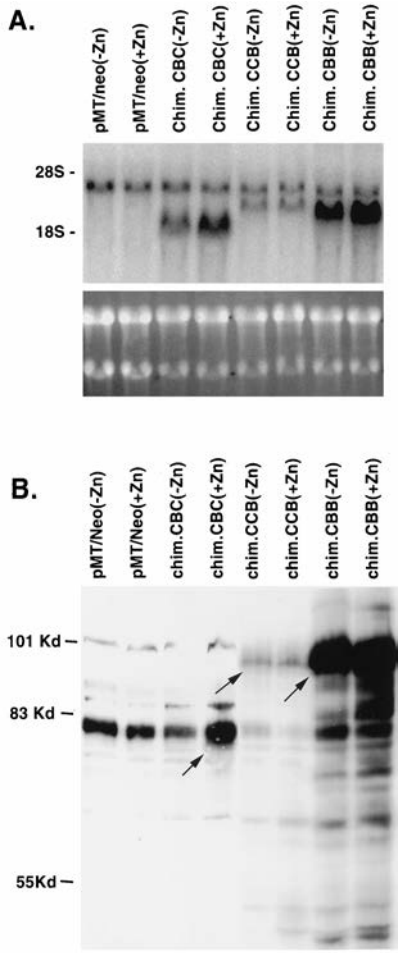

FIG. 9. Establishment of 32Dcl3 cell lines overexpressing chimeric genes between c-*myb* and B-*myb*. Each chimeric gene was subcloned into pMT-neo and transfected into 32Dcl3 cells. Following G418 selection, the established cell lines were analyzed for expression of each transgene in the presence (+Zn) or absence (−Zn) of Zn^{2+}. (A) Northern blot analysis of total RNA extracted from each transfectant was performed with labeled c-DNA of chimera CBC as a probe. The endogenous c-*myb* (upper bands) transcript and transcripts from transfected genes (lower bands) could be distinguished due to differences in the sizes of the transcripts. The staining of RNA with ethidium bromide after completion of transfer to nitrocellulose paper is shown below the Northern blot. (B) Western blot analysis of cell lysates from control and expression vector-transfected cells was performed with antiserum raised against the N terminus of c-Myb. Arrows, each gene product of the expected size. Chim, chimera.

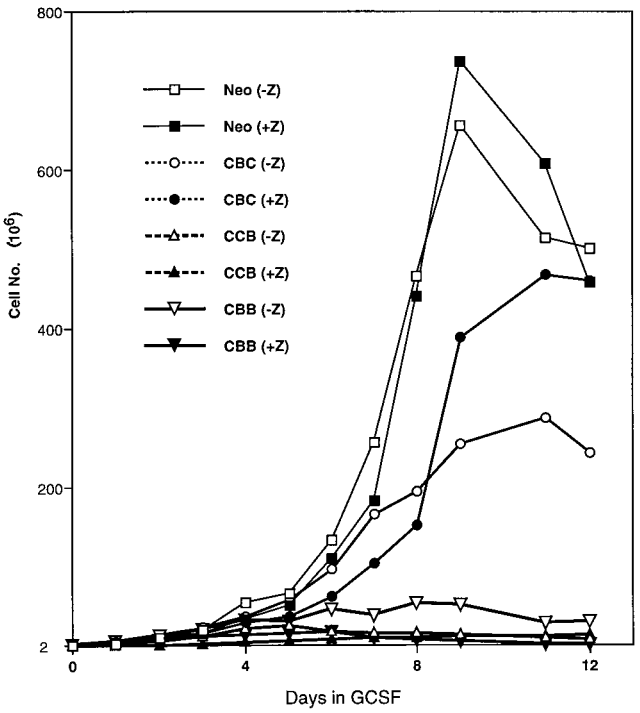

FIG. 10. The growth of 32Dcl3 cells overexpressing chimeras between c-*myb* and B-*myb* in the presence of G-CSF. Each 32Dcl3 cell line overexpressing chimeras CBC, CCB, and CBB and mock-transfected cells were analyzed for their growth in the presence of G-CSF as described above, except that the cells were plated at a density of 2×10^5 per ml of G-CSF-containing medium.

cules in which we systematically replaced the three functional domains of c-Myb with those of B-Myb. An analysis of the transactivation potentials of these chimeric molecules suggests that the C-terminal domain of B-Myb functions as an enhancer of transactivation and that its deletion results in a substantial decrease of this activity of B-Myb. The molecular basis for this difference between c-Myb and B-Myb is at present unclear. It has been recently shown that c-Myb protein contains a Ser motif at position 528, which is phosphorylated by p42MAPK, resulting in an inhibition of the transactivation potential of c-Myb (3). While the Ser moiety is conserved in B-Myb, the A/EVES motif (9) adjacent to this Ser is not well conserved in B-myb, and at present it is unclear that this Ser can be phosphorylated by MAP kinase. Additional mutagenesis studies with the C-terminal domain of B-Myb, including creation of c-Myb–B-Myb chimeras in which small regions of the C-terminal domain are interchanged between the two molecules, might shed additional light on the mechanisms by which the carboxyl terminus of B-Myb exerts this enhancer effect.

To test whether ectopic overexpression of B-*myb* and c-*myb* can produce similar phenotypic effects in a biological assay system, we studied the effect of overexpression of these two genes in 32Dcl3 cells, which provided us with an unexpected insight into the function of B-*myb*. As shown previously, ectopic overexpression of c-*myb* in these cells resulted in a block to G-CSF-induced differentiation, resulting in continued proliferation of cells in G-CSF. In contrast, when the 32D/B-*myb* cells were cultured in a medium containing G-CSF, they underwent growth arrest and completed the differentiation process in 4 to 6 days, which otherwise takes 10 to 12 days for normal 32Dcl3 cells. Most strikingly, unlike c-*myb*, B-*myb* appears to be incapable of inducing a block to G-CSF-induced terminal differentiation of 32Dcl3 cells. In addition, while transgenic expression of c-*myb* allowed the cells to proliferate indefinitely in G-CSF, transgenic expression of B-*myb* inhibited proliferation of cells in G-CSF. These results provide strong evidence for the argument that c-*myb* and B-*myb* function very differently in a biological context.

The mechanisms by which B-*myb* brings about this biological effect on 32Dcl3 cells could be due to activation of a new set of genes, which could be dictated by subtle differences that the two proteins might exhibit with respect to their target sequence binding (24). A second mechanism could be due to promoter occupancy by B-*myb*, which blocks the ability of c-*myb* to transactivate transcription of proliferation-associated genes. A third possibility is that the DNA-binding domains of c-Myb and B-Myb have similar binding specificities but that the two proteins perform different biological functions due to the unique role played by the C-terminal domains, which could mediate

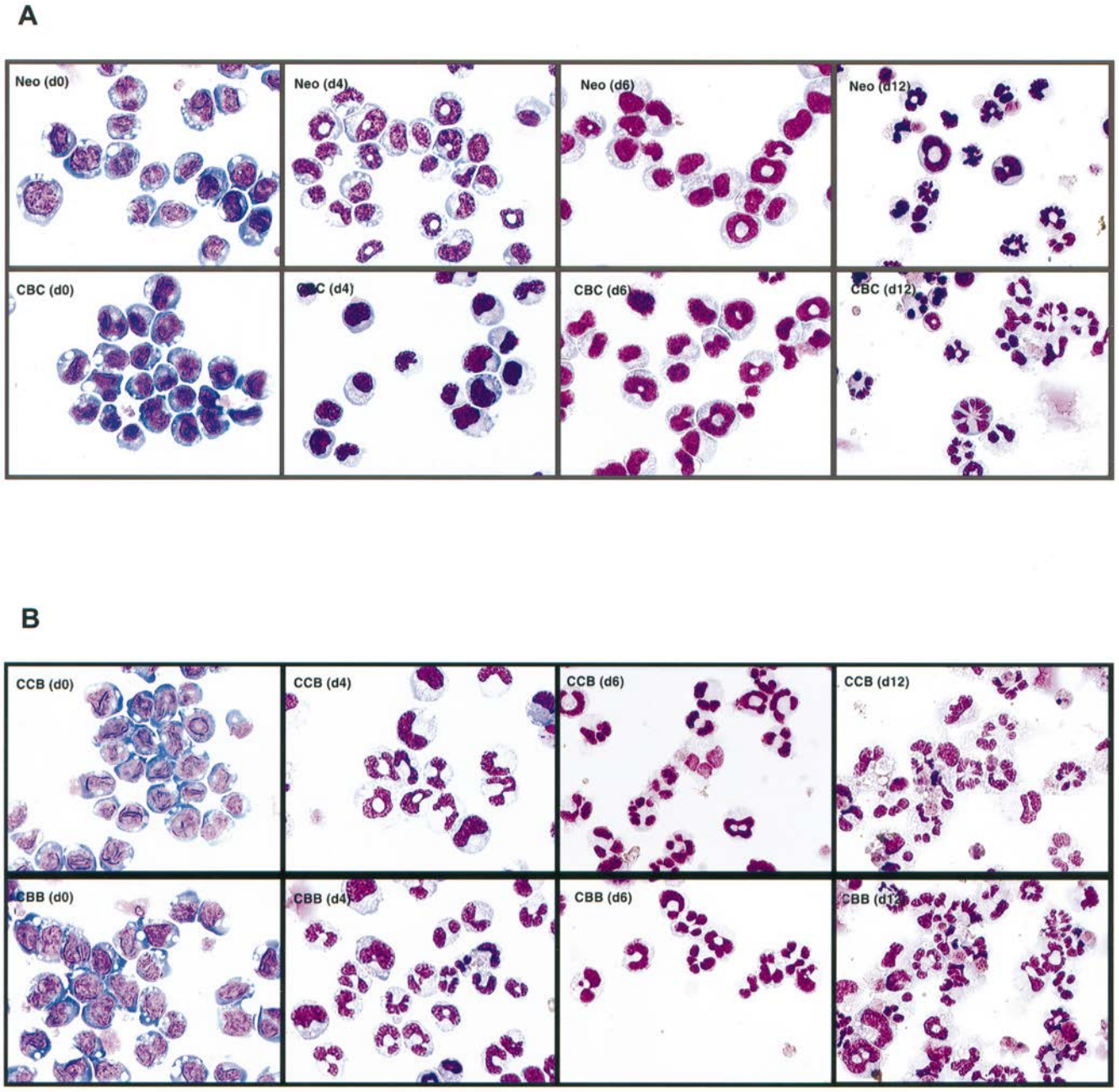

FIG. 11. Morphological analysis of 32Dcl3 cells overexpressing chimeras between c-*myb* and B-*myb* following the addition of G-CSF. 32D cell lines overexpressing each chimeric gene were analyzed for morphological changes during G-CSF-induced differentiation in the absence of Zn^{2+}. On days 0, 4, 6, and 12 (d0, d4, d6, and d12, respectively) of G-CSF treatment, aliquots of the cells were cytospun and stained with May-Grunwald-Giemsa stain as described above. (A) Morphology of 32D cells overexpressing chimera CBC and mock-transfected cells following G-CSF-induced differentiation; (B) analysis of cells overexpressing chimeras CBB and CCB following incubation in G-CSF-containing medium.

the assembly of specific transcriptional complexes. To discern among these possibilities, we transfected different B-*myb*–c-*myb* chimeras into 32Dcl3 cells and examined the abilities of these chimeras to induce growth arrest and accelerate terminal differentiation of 32Dcl3 cells. Our results show that only chimeras that contain the C-terminal domain of B-Myb are capable of bringing about an effect similar to that seen with B-*myb*. Substitution of the DNA-binding domain of B-Myb with that of c-Myb or substitution of the DNA-binding and transactivation domains of B-Myb with that of c-Myb seemed to produce no alteration of this biological phenotype produced by B-*myb*. It is interesting to note that chimeras CCB and CBB exhibited the highest levels of transcriptional transactivation in transient transfection assays and that both contained the DNA-binding domain of c-Myb. These results strongly suggest that the unique biological effects of B-*myb* are exclusively dictated by its C-terminal portion of the molecule and not by its DNA-binding or transactivation domains.

It is now established that c-Myb and B-Myb function effectively in the presence of appropriate cooperating factors, which

TABLE 2. Differentiation profiles of 32D cells overexpressing chimeric genes between c-*myb* and B-*myb*[a]

Cell line	Days after GCSF addition	% of following cell type (mean ± SD)		
		Myeloblast	Promyelocyte/myelocyte	Metamyelocyte/granulocyte
32D/PMT-Neo (−Zn)	0	97.7 ± 1.5	2.3 ± 1.5	
	4	7.0 ± 1	85.7 ± 1.5	7.3 ± 0.6
	7	0.3 ± 0.6	76.0 ± 4.6	23.3 ± 2.5
32D/PMT-Neo (+Zn)	0	98.3 ± 0.6	1.7 ± 0.6	
	4	12.0 ± 3	77.7 ± 6.8	10.3 ± 4
	7	0.7 ± 0.6	80.3 ± 2.5	19.0 ± 2.6
Chimera CBC (−Zn)	0	99.0 ± 1	1.0 ± 1	
	4	3.7 ± 1.2	85.0 ± 3.6	11.3 ± 2.5
	7	0.7 ± 1.1	77.7 ± 4.6	21.3 ± 3.8
Chimera CBC (+Zn)	0	97.7 ± 1.5	2.3 ± 1.5	
	4	2.7 ± 2	84.0 ± 1	13.3 ± 2.3
	7		74.0 ± 7.8	26.0 ± 6.5
Chimera CCB (−Zn)	0	98.0 ± 1	2.0 ± 1	
	4		47.3 ± 2.5	52.7 ± 2.9
	7		2.7 ± 2.5	97.3 ± 8.7
Chimera CCB (+Zn)	0	97 ± 1	3.0 ± 1	
	4		44.0 ± 3.6	56.0 ± 6
	7		2.7 ± 2.5	97.3 ± 7.5
Chimera CBB (−Zn)	0	97 ± 1	3.0 ± 1	
	4		34.7 ± 2.5	65.3 ± 4.9
	7		1.0 ± 1	99.0 ± 3.4
Chimera CBB (+Zn)	0	97.7 ± 1.5	2.3 ± 1.5	
	4		10.3 ± 2.3	89.7 ± 6.5
	7		3.0 ± 1	97.0 ± 3.6

[a] 32D cells overexpressing each transgene were induced to differentiate by G-CSF. On the indicated days, aliquots of the cells were cytospun and stained with May-Grunwald-Giemsa. The values are means from three studies ± standard deviations.
[b] −Zn, lacking zinc; +Zn, with zinc.

interact with the two proteins and form an enhancer complex (6, 8, 9, 11, 23). We propose that the nature of transcription factors that interact with B-Myb or c-Myb is determined by their C-terminal domains, which dictate the nature of the enhancer complex formed between c-Myb and B-Myb proteins and other cooperating factors (Fig. 9). In the absence of any cooperating factors, it is expected that c-Myb assumes an inactive state via an intramolecular conformation such as the one suggested recently (9). However, following posttranslational modifications, which seem to activate the transactivating potentials of these proteins (3, 22), c-Myb appears to assume a conformation that allows its interaction with discrete sets of transcription factors which specify the nature of target genes that are transactivated by c-Myb. It is possible that the B-Myb protein is not so very stringently regulated, since it does not seem to exist in an inactive state under normal conditions. We propose that the C-terminal domains of the two proteins play an active role in dictating the nature of factors that interact with each other. It is likely that some of these factors might be common to both c-Myb and B-Myb, while others might be unique to the individual Myb proteins. This combination of interacting factors is likely to dictate the nature of target genes that are transactivated by individual members of the Myb family of proteins. It is conceivable that the C-terminal domain of c-Myb allows the interaction of c-Myb with a defined set of nuclear factors that activate transcription of a group of target genes that promote proliferation and block terminal differentiation of myeloid precursor cells. On the other hand, B-Myb might transactivate transcription of a different set of genes by virtue of its ability to interact with a distinctive set of nuclear factors, and this transcription complex transactivates genes that are associated with terminal differentiation of myeloid cells. This model predicts that c-*myb* and B-*myb* cannot compensate for the biological function of each other. It is at present unclear whether the C-terminal domains of B-Myb and c-Myb regulate the nature of the enhancer complex formed by participating in protein-protein interactions or solely through steric effect. Studies aimed at the identification of nuclear factors that interact with B-Myb and c-Myb and identification of the domains of c-Myb and B-Myb that participate in these interactions are likely to shed further insight into the mechanism of action of this gene family.

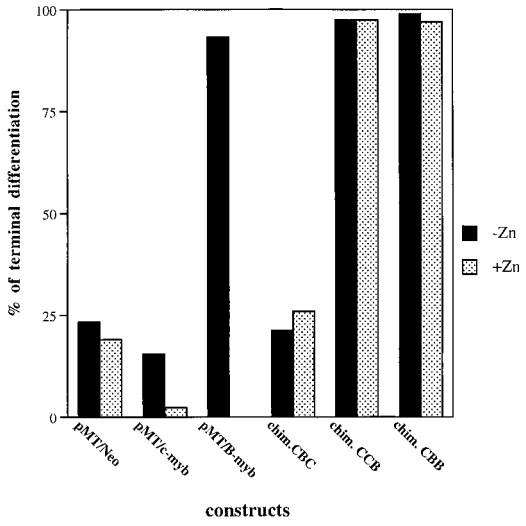

FIG. 12. Schematic representation of differentiation profiles of 32D cells overexpressing c-*myb*, B-*myb*, and B-*myb*–C-*myb* chimeras (chim). The percentages of terminally differentiated cells (metamyelocytes plus granulocytes) were determined on the seventh day of G-CSF-induced differentiation in the presence (+Zn) or absence (−Zn) of Zn^{2+}.

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REFERENCES

1. **Arsura, M., M. Introna, F. Passerini, A. Mantovani, and J. Golay.** 1992. B-myb antisense oligonucleotides inhibit proliferation of human hematopoietic cell lines. Blood **79:**2708–2716.
2. **Ausubel, F. M., R. Brent, R. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
3. **Aziz, N., M. R. Miglarese, R. C. Hendrickson, J. Shabanowitz, T. W. Sturgill, D. F. Hunt, and T. P. Bender.** 1995. Modulation of c-Myb-induced transcription activation by a phosphorylation site near the negative regulatory domain. Proc. Natl. Acad. Sci. USA **92:**6429–6433.
4. **Biedenkapp, H., U. Borgmeyer, A. E. Sippel, and K. H. Klempnauer.** 1988. Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature **335:**835–837.
5. **Bies, J., R. Mukhopadhyaya, J. Pierce, and L. Wolff.** 1995. Only late, nonmitotic stages of granulocyte differentiation in 32Dcl3 cells are blocked by ectopic expression of murine c-myb and its truncated forms. Cell Growth Differ. **6:**59–68.
6. **Burk, O., S. Mink, M. Ringwald, and K. H. Klempnauer.** 1993. Synergistic activation of the chicken *mim*-1 gene by v-*myb* and C.EBP transcription factors. EMBO J. **12:**2027–2038.
7. **Clarke, M. F., J. F. Kukowska-Latallo, E. Westin, M. Smith, and E. V. Prochownik.** 1988. Constitutive expression of a c-myb cDNA blocks Friend murine erythroleukemia cell differentiation. Mol. Cell. Biol. **8:**884–892.
8. **Dai, P., H. Akimaru, Y. Tanaka, D. X. Hou, T. Yasukawa, C. Kanei-Ishii, T. Takahashi, and S. Ishii.** 1996. CBP as a transcriptional coactivator of c-Myb. Genes Dev. **10:**528–540.
9. **Dash, A. B., F. C. Orrico, and S. A. Ness.** 1996. The EVES motif mediates both intermolecular and intramolecular regulation of c-Myb. Genes Dev. **10:**1858–1869.
10. **Dubendorff, J. W., L. J. Whittaker, J. T. Eltman, and J. S. Lipsick.** 1992. Carboxy-terminal elements of c-Myb negatively regulate transcriptional activation in cis and in trans. Genes Dev. **6:**2524–2535.
11. **Dudek, H., R. V. Tantravahi, V. N. Rao, E. S. Reddy, and E. P. Reddy.** 1992. Myb and Ets proteins cooperate in transcriptional activation of the mim-1 promoter. Proc. Natl. Acad. Sci. USA **89:**1291–1295.
12. **Erlich, H. A. (ed.).** 1989. PCR technology: principles and applications for DNA amplification. Stockton Press, London, United Kingdom.
13. **Foos, G., S. Grimm, and K. H. Klempnauer.** 1992. Functional antagonism between members of the myb family: B-myb inhibits v-myb-induced gene activation. EMBO J. **11:**4619–4629.
14. **Gewirtz, A. M., G. Anfossi, D. Venturelli, S. Valpreda, R. Sims, and B. Calabretta.** 1989. G1/S transition in normal human T-lymphocytes requires the nuclear protein encoded by c-myb. Science **245:**180–183.
15. **Gewirtz, A. M., and B. Calabretta.** 1988. A c-myb antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. Science **242:**1303–1306.
16. **Golay, J., L. Loffarelli, M. Luppi, M. Castellano, and M. Introna.** 1994. The human A-myb protein is a strong activator of transcription. Oncogene **9:** 2469–2479.
17. **Greenberger, J. S., M. A. Sakakeeny, R. K. Humphries, C. J. Eaves, and R. J. Eckner.** 1983. Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. USA **80:**2931–2935.
18. **Guerra, J., D. A. Withers, and L. M. Boser.** 1995. Myb binding sites mediate negative regulation of c-*myb* expression in T-cell lines. Blood **86:**1873–1880.
19. **Karin, M., A. Haslinger, A. Heguy, T. Dietlin, and T. Cooke.** 1987. Metal-responsive elements act as positive modulators of human metallothionein-lla enhancer activity. Mol. Cell. Biol. **7:**606–613.
20. **Lyon, J., C. Robinson, and R. Watson.** 1994. The role of Myb proteins in normal and neoplastic cell proliferation. Crit. Rev. Oncogen. **5:**373–388. (Review.)
21. **Mettus, R. V., J. Litvin, A. Wali, A. Toscani, K. Latham, K. Hatton, and E. P. Reddy.** 1994. Murine A-myb: evidence for differential splicing and tissue-specific expression. Oncogene **9:**3077–3086.
22. **Miglarese, M. R., A. F. Richardson, N. Aziz, and T. P. Bender.** 1996. Differential regulation of c-Myb-induced transcription activation by a phosphorylation site in the negative regulatory domain. J. Biol. Chem. **271:**22697–22705.
23. **Mink, S., U. Kerber, and K.-H. Klempnauer.** 1996. Interaction of C/EBPβ and v-Myb is required for synergistic activation of the *mim*-1 gene. Mol. Cell. Biol. **16:**1316–1325.
24. **Mizuguchi, G., H. Nakagoshi, T. Nagase, N. Nomura, T. Date, Y. Ueno, and S. Ishii.** 1990. DNA binding activity and transcriptional activator function of the human B-myb protein compared with c-MYB. J. Biol. Chem. **265:**9280–9284.
25. **Mucenski, M. L., K. McLain, A. B. Kier, S. H. Swerdlow, C. M. Schreiner, T. A. Miller, D. W. Pietryga, W. J. Scott, Jr., and S. S. Potter.** 1991. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell **65:**677–689.
26. **Nakagoshi, H., Y. Takemoto, and S. Ishii.** 1993. Functional domains of the human B-myb gene product. J. Biol. Chem. **268:**14161–14167.
27. **Ness, S. A., A. Marknell, and T. Graf.** 1989. The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell **59:**1115–1125.
28. **Nomura, N., M. Takahashi, M. Matsui, S. Ishii, T. Date, S. Sasamoto, and R. Ishizaki.** 1988. Isolation of human cDNA clones of myb-related genes, A-myb and B-myb. Nucleic Acids Res. **16:**11075–11089. (Erratum, **17:**1282, 1989.)
29. **Patel, G., B. Kreider, G. Rovera, and E. P. Reddy.** 1993. v-myb blocks granulocyte colony-stimulating factor-induced myeloid cell differentiation but not proliferation. Mol. Cell. Biol. **13:**2269–2276.
30. **Patel, G., R. Tantravahi, I. H. Oh, and E. P. Reddy.** 1996. Transcriptional activation potential of normal and tumor-associated *myb* isoforms does not correlate with their ability to block GCSF-induced terminal differentiation of murine myeloid precursor cells. Oncogene **13:**1197–1208.
31. **Ramsay, R. G., K. Ikeda, R. A. Rifkind, and P. A. Marks.** 1986. Changes in gene expression associated with induced differentiation of erythroleukemia: protooncogenes, globin genes, and cell division. Proc. Natl. Acad. Sci. USA **83:**6849–6853.
32. **Rovera, G., B. Kreider, N. Shirsat, D. Venturelli, G. Naso, and F. Mavilio.** 1989. Alteration of the program of terminal differentiation caused by oncogenes in the hemopoietic progenitor cell line 32D C13 (G). Ann. N.Y. Acad. Sci. **567:**154–164. (Review.)
33. **Saikumar, P., R. Murali, and E. P. Reddy.** 1990. Role of tryptophan repeats and flanking amino acids in Myb-DNA interactions. Proc. Natl. Acad. Sci. USA **87:**8452–8456.
34. **Sakura, H., C. Kanei-Ishii, T. Nagase, H. Nakagoshi, T. J. Gonda, and S. Ishii.** 1989. Delineation of three functional domains of the transcriptional activator encoded by the c-myb protooncogene. Proc. Natl. Acad. Sci. USA **86:**5758–5762.
35. **Selvakumaran, M., D. A. Liebermann, and B. Hoffman-Liebermann.** 1992. Deregulated c-myb disrupts interleukin-6- or leukemia inhibitory factor-induced myeloid differentiation prior to c-myc: role in leukemogenesis. Mol. Cell. Biol. **12:**2493–2500.
36. **Tashiro, S., Y. Takemoto, H. Handa, and S. Ishii.** 1995. Cell type-specific trans-activation by the B-myb gene product: requirement of the putative cofactor binding to the C-terminal conserved domain. Oncogene **10:**1699–1707.
37. **Thiele, C. J., P. S. Cohen, and M. A. Israel.** 1988. Regulation of c-myb expression in human neuroblastoma cells during retinoic acid-induced differentiation. Mol. Cell. Biol. **8:**1677–1683.
38. **Todokoro, K., R. J. Watson, H. Higo, H. Amanuma, S. Kuramochi, H. Yanagisawa, and Y. Ikawa.** 1988. Down-regulation of c-myb gene expression is a prerequisite for erythropoietin-induced erythroid differentiation. Proc. Natl. Acad. Sci. USA **85:**8900–8904.
39. **Valtieri, M., D. J. Tweardy, D. Caracciolo, K. Johnson, F. Mavilio, S. Altmann, D. Santoli, and G. Rovera.** 1987. Cytokine-dependent granulocytic differentiation. Regulation of proliferative and differentiative responses in a murine progenitor cell line. J. Immunol. **138:**3829–3835.
40. **Watson, R. J., C. Robinson, and E. W. Lam.** 1993. Transcription regulation by murine B-myb is distinct from that by c-myb. Nucleic Acids Res. **21:**267–272.
41. **Westin, E. H., R. C. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal.** 1982. Differential expression of the amv gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79:**2194–2198.
42. **Weston, K., and J. M. Bishop.** 1989. Transcriptional activation by the v-myb oncogene and its cellular progenitor, c-myb. Cell **58:**85–93.