

# Murine A-*myb* Gene Encodes a Transcription Factor, Which Cooperates with Ets-2 and Exhibits Distinctive Biochemical and Biological Activities from c-*myb*\*

(Received for publication, March 12, 1997, and in revised form, June 5, 1997)

Il-Hoan Oh and E. Premkumar Reddy‡

From the Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

The *myb* gene family consists of three members, named A-, B-, and c-*myb*, which encode nuclear proteins that bind to DNA and function as regulators of transcription. Our results show that murine A-*myb* is a poor transactivator of transcription compared with murine c-*myb*. Deletion of the COOH-terminal domain of A-Myb, or co-expression with Ets-2 resulted in increased transactivation potential. While ectopic overexpression of c-*myb* in 32Dcl3 cells results in a block to the ability of these cells to undergo terminal differentiation resulting in indefinite growth in granulocyte-colony-stimulating factor (G-CSF), similar overexpression of A-*myb* results in growth arrest and concomitant terminal differentiation of 32D cells into granulocytes. Co-expression of A-*myb* and *ets-2* in these cells results in the restoration of the proliferative activity of the cells in G-CSF, but fails to induce a block to G-CSF-induced terminal differentiation. However, overexpression of the COOH-terminal deletion mutant of A-*myb* results in a block to G-CSF-induced differentiation of 32D cells, suggesting that the distinctive biological phenotypes produced by A-*myb* and c-*myb* genes are mediated by their COOH-terminal domains.

The *myb* gene family currently consists of three members, named A-, B-, and c-*myb* (1). All the three members of this family code for nuclear proteins that bind DNA in a sequence-specific manner and function as regulators of transcription (2–9). The first one-third of each protein consists of the DNA-binding domain followed by the central portion of the molecule, which seems to mediate the transactivating function of the protein (3, 4). The COOH-terminal end of these proteins contains a third domain, which appears to regulate the transactivating function of these three proteins (3, 4).

c-*myb* is predominantly expressed in hematopoietic cells and is readily induced upon treatment of these cells with interleukins or mitogens (10, 11). 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 (12). In addition, studies with hematopoietic cell lines suggest that terminal differentiation of these cells is accompanied by down-regulation of *myb* gene expression and constitutive expression of c-*myb* blocks their terminal differentiation (13–22). Homozygous null c-*myb* mutant mice die *in utero* due to defects in fetal hepatic hematopoiesis, confirming

an essential role for c-*myb* in hematopoiesis (23).

In contrast to c-*myb*, little is known about the role of the A-*myb* and B-*myb* genes in development. While B-*myb* seems to be expressed ubiquitously, A-*myb* is expressed at high levels in testis, germinal centers of spleen, and immature neuronal cells (9, 24). Homozygous null A-*myb* mutant mice show defects in spermatogenesis and breast development, suggesting that this gene may play an essential role in the development of tissues where it is expressed (25). In addition, A-*myb* was shown to be expressed in Burkitt's lymphoma cell lines, suggesting that A-*myb* might be also involved in pre-B-cell development (26). To date, at least two reports indicate that human and chicken A-*myb* genes are potent transactivators of transcription (7, 8) and based on structural similarities to c-*myb*, and its apparent transactivating potential, it has been proposed that A-*myb* might function in an identical manner to that of c-*myb* *in vivo* (7, 8). To test this hypothesis, we have examined the relative transactivating potential of murine A-Myb and c-Myb. It was earlier demonstrated that c-Myb co-operates with Ets-2 in transcriptional transactivation of Myb target genes (6). In this report, we examined whether A-*myb* can similarly co-operate with Ets-2 in transcriptional activation of promoters containing Myb-binding sites. In addition, we have examined the effects of overexpression of A-*myb* and c-*myb* on myeloid cell differentiation using the murine myeloid precursor cell line, 32Dcl3. Our results show that c-*myb* and A-*myb* differ in their ability to transactivate transcription of reporter genes linked to Myb-responsive promoters but resemble each other in their ability to co-operate with Ets-2. Overexpression of c-*myb* in 32D cells was found to result in a block to G-CSF<sup>1</sup>-induced terminal differentiation and accelerated proliferation of these cells, while similar overexpression of A-*myb* was found to result in their growth arrest. These results suggest that A-*myb* and c-*myb* do not perform identical functions *in vivo*. Our results also suggest that the COOH-terminal domain of A-Myb might confer these distinctive properties to A-Myb.

## MATERIALS AND METHODS

**Cell Culture**—NIH3T3 cells were cultured in Dulbecco's Modified Eagle medium supplemented with 10% calf serum and 0.5% penicillin/streptomycin (Life Technologies, Inc.) in a humidified incubator maintained at 37 °C circulated with 5% CO<sub>2</sub>. The murine myeloid progenitor cell line, 32Dcl3 (27–29) was maintained as described earlier supplemented with 10% WEHI3B cell-conditioned medium as a source of IL-3 (30).

**Plasmids**—Construction of the reporter plasmid pMIL-luc, pΔE-luc-AM, pTA3-luc, and pT81-luc has been described previously (6, 31–33). For expression in NIH3T3 cells, each construct was inserted into the expression vector pRC/CMV (Invitrogen), which places the inserts un-

\* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Tel.: 215-707-4307; Fax: 215-707-1454.

<sup>1</sup> The abbreviations used are: G-CSF, granulocyte-colony-stimulating factor; IL, interleukin; kb, kilobase(s); kbp, kilobase pair(s); CAPS, 3-(cyclohexylamino)propanesulfonic acid.

der the control of cytomegalovirus immediate early promoter. RSV- $\beta$ -Gal plasmid (Invitrogen), which expresses *lacZ* gene, was used as an internal standard for transient transfection assays. The inducible vector pMT-neo, which has the human metallothionein promoter containing metal-responsive elements (34) and modified from parental plasmid LK-444 (35), was kindly provided by Dr. Dan Libermann. The wild-type A-myb and c-myb cDNAs were subcloned into the pMT-neo vector to express these genes in 32Dcl3 cells in a metal ion-inducible manner. *ets-1* and *ets-2* expression vectors were prepared by the insertion of the two cDNAs (6) into pSG5 vector for SV40-driven expression.

**Construction of Truncation Mutants**—The COOH-terminal truncated mutants of the c-myb and A-myb were constructed using the polymerase chain reaction utilizing primers tagged with *NotI* and *XbaI* sites and the polymerase chain reaction product was ligated to pRC/CMV (Invitrogen) and pMT-neo. The inserts were completely sequenced to assure that the polymerase chain reaction reactions did not introduce any mutations in the two genes.

**Antisera**—To generate anti-c-Myb antiserum, the part of the c-myb cDNA corresponding to the DNA-binding and transactivation domains (amino acid 1–325) was subcloned into the pDS5–6His vector (Quiaexpress) to produce a fusion protein with 6 histidine residues at the NH<sub>2</sub> terminus. The resulting fusion protein was purified by nickel chelate affinity chromatography (Quiaexpress) and used to produce polyclonal antibodies in rabbits.

**Transient Transfections and Luciferase Assays**—For transfection into NIH3T3 cells, cells were seeded into 100-mm dishes at a density of  $1.5 \times 10^5$  cells/plate. The following day, DNA was transfected by the calcium phosphate precipitation method (36). In each transfection, 5  $\mu$ g of reporter and 5  $\mu$ g of effector plasmids were transfected along with 0.5  $\mu$ g of RSV- $\beta$ -Gal plasmid as an internal standard. Following incubation for 60–70 h, cells were harvested in 900  $\mu$ l of reporter lysis buffer (Promega). Luciferase activity was assayed using a luciferin substrate (Promega) according to the manufacturer's protocol. Luciferase activities were normalized against the  $\beta$ -galactosidase activity to determine relative luciferase activity, and the activation fold was obtained by setting the value of the empty vector control as 1.0.

**Establishment of Stable Cell Lines Expressing Transgenes**—For stable transfection of 32Dcl3 cells, exponentially growing 32Dcl3 cells were electroporated with various plasmid DNAs using a Gene-Pulser (Bio-Rad) at a pulse of 230 V, 960 microfarads. The surviving cells were selected in 500  $\mu$ g/ml of G418 (Life Technologies, Inc.) for 2–3 weeks. To isolate single cell clones, mass cell cultures were serially diluted in 96-well plates in the presence of G418 and selected for clonal expansion.

**Northern Blot Analysis**—Total RNA from each cell line was purified using the Ultra-spec RNA (Biotex) purification reagent. To purify RNA from zinc-induced cells, the cells were incubated in the presence of 100  $\mu$ M ZnCl<sub>2</sub> for 30 h prior to RNA isolation. Northern blot analysis was performed as described previously (36). To detect the A-myb transcript, an A-myb-specific probe was generated from the 1.35-kbp *HindIII*-*SacI* fragment (9). To detect truncated A-myb transcripts, full-length A-myb cDNA (9) was used. To detect c-myb transcripts, a 2.3-kbp cDNA fragment containing the entire stretch of coding sequences was isolated by digestion with *Bam*HI and *Hind*III and used as a probe.

**Western Blot Analysis**—To analyze protein products of the transfected genes, normalized amounts of protein from each cell lysate were separated by SDS-polyacrylamide gel electrophoresis (36), 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% non-fat milk in TTS solution (0.05% Tween 20, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 4 h and incubated with primary antibody in the same buffer for 1 h and washed three times in TTS solution. The secondary antibody reaction was performed by incubating the filters with horseradish peroxidase-conjugated anti-rabbit Ig (Amersham Corp.) and washed in a similar manner as was described for the primary antibody reaction. The Amersham ECL detection system was used for visualization as specified by the manufacturer.

**Growth and Differentiation Assays**—To induce granulocytic differentiation, parental or transfected 32D cells were washed twice in IL-3-free medium and plated at a density of  $1 \times 10^5$  cells/ml in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum and 10% G-CSF (27). The viability and proliferation of the cell cultures were monitored for the indicated days. Morphological analysis of G-CSF-treated cells was performed using an aliquot of cells that was cytospun and stained with May-Grunwald-Giemsa stain. The differentiation state of cells was scored for 12 days at 2-day intervals following the addition of G-CSF to the culture medium. In experiments where metallothionein promoter was used, transcriptional induction of the transgenes was achieved by

the addition of 100  $\mu$ M (final concentration) of ZnCl<sub>2</sub> 30 h before the addition of G-CSF.

## RESULTS

**Comparison of the Transcriptional Transactivation Potential of Murine A-myb and c-myb Genes**—Fig. 1A provides a comparison of the structure of murine A-Myb and c-Myb proteins. Both these proteins contain an NH<sub>2</sub>-terminal DNA-binding domain, a central transactivation domain, and a carboxyl-terminal negative regulatory domain. The two proteins exhibit 93, 63, and 62% sequence identities in the DNA-binding, transactivation, and negative regulatory domains, respectively (9). As is expected, both proteins have been shown to bind to the same target sequence (PyrAACGTPur, where Pyr indicates a pyrimidine and Pur indicates a purine) in DNA (7). Based on the structural homology and DNA binding properties, several investigators have suggested that the two genes might possess overlapping biochemical and biological functions (7, 8, 24, 37). To test this hypothesis, we carried out transcriptional transactivation studies using eukaryotic expression vectors that express full-length murine A-Myb and c-Myb proteins. To avoid problems associated with interspecies differences, all experiments were carried out using the mouse cell lines and mouse cDNAs. Fig. 1B shows the two reporter 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. *mim-1* promoter was previously shown to contain three Myb-binding sites (A, B, and C), of which site A was found to be the high affinity site (33), and mutations in this site (p $\Delta$ E-luc-AM) were found to drastically reduce Myb-mediated transcriptional transactivation. The reporter plasmid, pTA3-luc, contains three copies of Myb-binding sites upstream of a truncated thymidine kinase promoter (31). and pT81-luc, which lacks Myb-binding sites, was used as a negative control for this reporter. Fig. 1C illustrates the levels of transactivation seen with full-length c-myb and A-myb genes when used with *mim-1* promoter. When the reporter gene was placed under the control of *mim-1* promoter, low levels of transactivation (approximately 3.5 fold) was seen with c-myb, while no transactivation of the promoter was observed with the A-myb expression vector. p $\Delta$ E-luc-AM, which contains a mutation in the high-affinity Myb-binding site of the *mim-1* promoter (A-box) showed very little or no transactivation, when co-transfected with c-myb or A-myb expression vectors.

To test whether higher transactivation levels can be observed with the synthetic target promoter, we tested the ability of c-myb and A-myb genes to transactivate transcription from the reporter plasmid pTA3. Results shown in Fig. 1D show that while c-myb could transactivate transcription of this reporter by about 10-fold, the A-myb expression vector showed considerably lower transactivation potential (approximately 3-fold), suggesting that murine A-Myb is a weak transactivator, when compared with murine c-Myb. Little or no transactivation was seen with the control vector (pT81-luc) with c-myb and A-myb.

It has been reported previously that the transactivation potential of A-Myb is dose-dependent with a bell-shaped distribution (7). To rule out the possibility that the lower transactivating potential of A-Myb is due to inadequate amounts of DNA used in the transfection studies, we examined the effects of varying amounts of DNA on the transactivation activity of A-Myb. When we varied the amounts of DNA used for transfection in the range of 0.1–10  $\mu$ g, we observed a dose-dependent increase of activity up to 7  $\mu$ g of input DNA, which was followed by a drop with increasing concentration of input DNA. A similar dose-response curve was seen with c-myb, which, however, was consistently higher than that of A-myb, suggesting that murine A-myb is indeed a weak transactivator of transcription

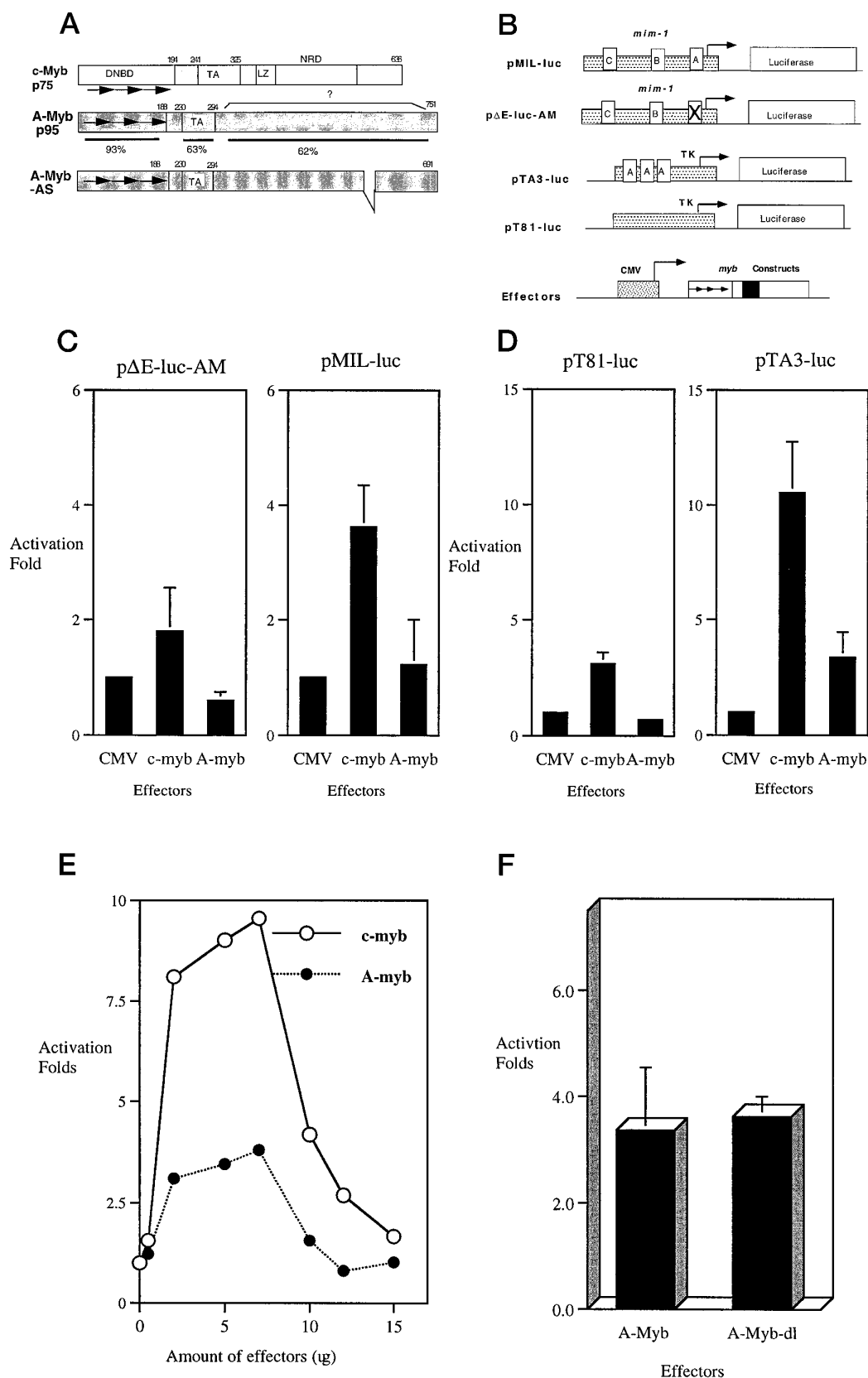


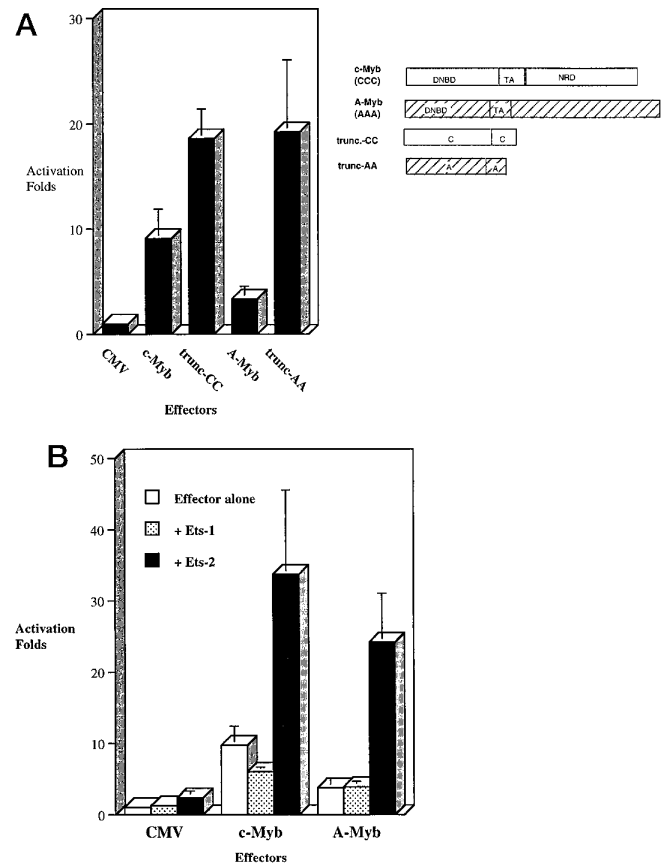
FIG. 1. Transcriptional transactivation of reporter genes by murine A-myb and c-myb. A, structural comparison of murine c-Myb and A-Myb. Schematic structures of wild-type A-Myb and c-Myb proteins are presented. The numbers above each diagram are the positions of amino acid residues in each corresponding region. The thick bar represents the region of homology, and the numbers below are percent identity to c-Myb in each domain. Horizontal arrows represent the three 51–52-amino acid repeats that constitute DNA binding domain. A-Myb-AS, alternatively spliced form of A-Myb; DNBD, DNA-binding domain; NRD, negative regulatory domain; TA, transactivation domain; LZ, leucine zipper. The interruption in A-Myb-AS represents the region deleted in this molecule due to alternative splicing. B, schematic representation of reporter and effector plasmids used in transient transactivation assays. The dotted box represents promoters, and the arrows in the promoters represent starting sites of transcription. The arrows in Myb represent the three 51–52 amino acid repeats of DNA-binding domain, and the black box

compared with *c-myb* (Fig. 1E)

We had previously isolated an alternatively spliced form of A-myb (see A-Myb-AS in Fig. 1A), which codes for a protein that has an internal deletion of 60 amino acids in COOH terminus (9). Because this deletion occurs in the negative regulatory domain, it is conceivable that the protein encoded by this alternatively spliced mRNA exhibits higher transactivation potential than that of full-length A-myb. To test this possibility, we carried out transcriptional transactivation studies using the cDNA that codes for this alternatively spliced form. The results presented in Fig. 1F show that the alternatively spliced form of A-Myb does not significantly differ from the wild-type form, suggesting that this internal deletion does not affect the transactivation potential of the A-Myb protein.

**Deletion of the COOH-terminal Negative Regulatory Domain Enhances the Transactivation Potential of Murine A-myb**—It has been shown previously that deletion of COOH-terminal sequences within c-Myb results in a substantial increase in the transactivating potential of the truncated protein (3, 4, 6, 38). To determine whether a similar phenomenon is observed with murine A-myb, we carried out transfection studies with the truncated forms of A-myb and c-myb, where the 3' ends of the two genes which encode the negative regulatory domains have been deleted. The results of these studies show that deletion of COOH-terminal sequences from both A-Myb and c-Myb markedly enhance their transactivation potential (Fig. 2A). These results suggest that like with c-Myb, the COOH-terminal domain of A-Myb acts as a negative regulatory element and that A-myb can function as a strong transactivator under conditions that relieve the negative regulatory effect of the COOH-terminal domain. This is in agreement with the observations made with human A-myb (37).

**A-myb Co-operates with *ets-2* in Transcriptional Transactivation of Target Promoters**—We had earlier demonstrated that c-Myb and Ets-2 co-operate with each other in transcriptional transactivation of promoters containing Myb-binding sites (6). To test whether the A-myb gene can similarly cooperate with *ets-2*, the expression plasmid containing A-myb was co-transfected with expression plasmids encoding *ets-1* or *ets-2* into NIH3T3 cells. As positive controls, identical experiments were carried out with c-myb expression vectors. The two myb genes were expressed under the control of the CMV promoter, while *ets-1* or *ets-2* genes were driven by the SV40 promoter. This experiment was designed to minimize any promoter interference that might occur when two different constructs are expressed under the control of the same promoter. pTA3-luc was used in this assay, since both c-myb and A-myb genes showed higher transactivation activity with this reporter. As shown in Fig. 2B, co-transfection of the c-myb and *ets-2* expression plasmids resulted in approximately 3-fold increase in transactivation as compared with the transactivation levels seen with c-myb alone. However, this increase in transactivation was not observed when the c-myb expression vector was co-transfected with *ets-1* expression plasmid, confirming our earlier observa-



**FIG. 2. Carboxyl-terminal deletions or co-operation with Ets-2 enhance the transactivation potential of A-Myb.** A, carboxyl terminus of A-Myb functions as a strong negative regulatory domain. Expression plasmids containing wild-type A-myb and c-myb and carboxyl terminus-truncated A-myb (AA) and c-myb (CC) were transfected into NIH3T3 cells along with pTA3-luc and RSV- $\beta$ -gal DNA as described earlier. Shown are the mean activation folds from three experiments with their standard deviations. B, transcriptional cooperation of Ets-2 with A-Myb and c-Myb. 5  $\mu$ g of each expression plasmid was co-transfected with 5  $\mu$ g of *ets-1* or *ets-2* expression plasmid into NIH3T3 cells along with 5  $\mu$ g of pTA3-luc and 0.5  $\mu$ g of RSV- $\beta$ -gal. In each set of transfection, the total amount of DNA transfected was kept constant by the addition of 11Zf plasmid DNA, where necessary. Activation folds were obtained as described earlier, and mean value of activation folds from at least three experiments are shown with standard deviation.

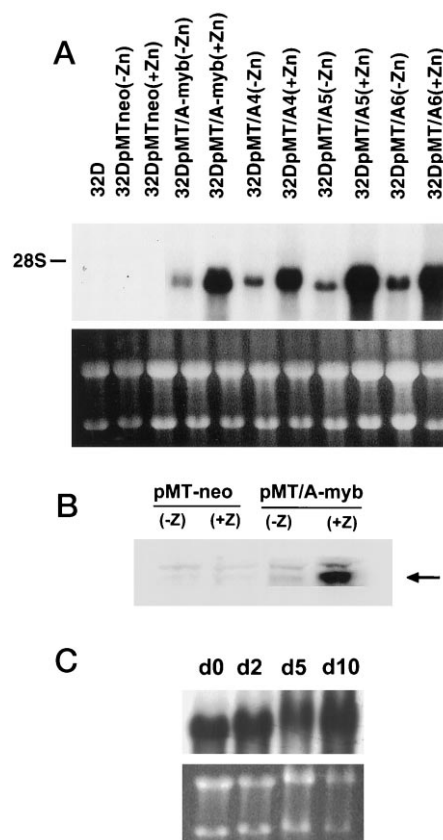
tion that only *ets-2* can mediate this co-operativity (6). We next tested the ability of A-myb to cooperate with *ets-1* or *ets-2* using the same methods. As presented in Fig. 2B, the transactivation potential of full-length A-Myb was markedly increased (8-fold) when co-transfected with *ets-2*. The level of co-operativity was considerably higher than that seen with c-Myb, suggesting that A-Myb has a greater ability to co-operate with Ets-2 compared with c-Myb. On the other hand, co-transfection of A-myb and

represents the transactivation domain. The boxes (A, B, and C) in pMIL-luc represent the three Myb-binding sites in *mim-1* promoter, and the cross in the A-box of pAE-AM represents mutations in the Myb-binding site A. TK, HSV-thymidine kinase promoter; CMV, immediate early promoter for cytomegalovirus. C and D, transcriptional activation by A-Myb and c-Myb. Each myb expression plasmid was transfected into NIH3T3 cells with reporter plasmids pTA3-luc or pT81-luc (C) or pMIL-luc or pAE-AM-luc (D) and the RSV- $\beta$ -gal plasmid as described under "Materials and Methods." After 60–70 h of transfection, the cells were harvested and assayed for  $\beta$ -galactosidase activity. The luciferase activities were normalized to  $\beta$ -galactosidase activities, and the activation fold was obtained by setting the value of empty vector as 1.0. Shown are the means of activation folds obtained from at least three independent experiments, and the vertical bars represent standard deviations of the values. E, comparison of transactivation potential between wild-type A-Myb and the alternatively spliced form of A-Myb. 5  $\mu$ g of each expression plasmid for wild-type A-myb or alternatively spliced form of A-myb (A-Myb-AS) was transfected along with 5  $\mu$ g of pTA3-luc and 1  $\mu$ g of RSV- $\beta$ -gal. Shown are the means of activation folds from three independent experiments, and the vertical bars represent standard deviation. F, effect of DNA dose on A-Myb- and c-Myb-mediated transactivation. Mixtures of 7  $\mu$ g of the reporter plasmid and varying amounts of the effector plasmid DNA mixed with 1  $\mu$ g of RSV- $\beta$ -Gal plasmid DNA were transfected into NIH3T3 cells. After 60–70 h following transfection, cells were harvested, and luciferase assays were performed as described under "Materials and Methods."

*ets-1* did not increase the transactivation potential of A-Myb, suggesting that A-Myb, like c-Myb, cannot cooperate with Ets-1.

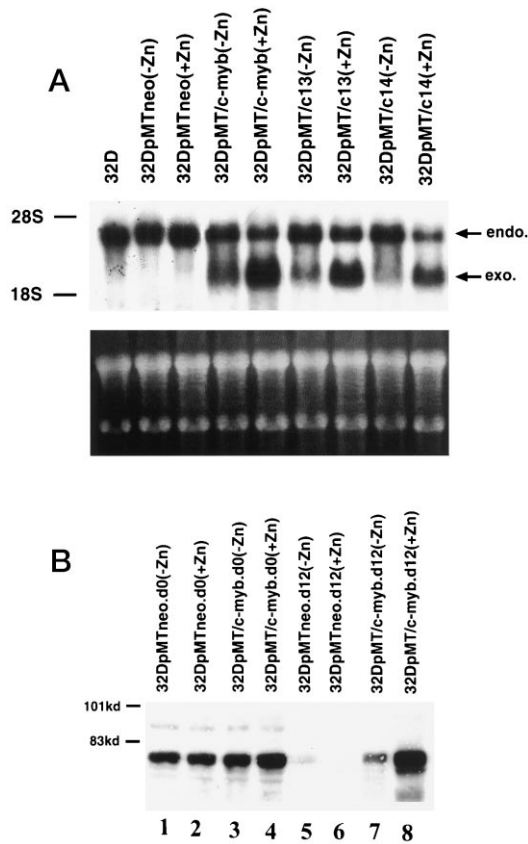
**A-myb and c-myb Exert Distinctive Biological Effects when Constitutively Expressed in Myeloid Precursor Cells**—Since A-Myb and c-Myb proteins share extensive sequence homology and bind to identical DNA sequence elements, it had been proposed that the two genes might exert a similar, if not identical, overlapping biological functions *in vivo* (7, 8). We had reported earlier a biological assay for v-myb and c-myb using the myeloid cell line 32Dcl3 (16, 22). These cells, derived from normal mouse bone marrow, have been found to be strictly dependent on IL-3 for growth and undergo terminal differentiation when placed in an IL-3-free medium containing G-CSF. We had shown previously that overexpression of v-myb or c-myb in 32Dcl3 cells results in a block to their ability to terminally differentiate into granulocytes in the presence of G-CSF (16, 21, 22). To determine whether A-myb can exert a similar influence during granulocytic differentiation of 32Dcl3 cells, we constructed inducible expression vectors where the A-myb and c-myb genes were placed under the control of the human metallothionein promoter. Following the construction of these vectors, the plasmid DNAs encoding the A-myb and c-myb genes were transfected into 32Dcl3 cells by electroporation. As a negative control, empty vector DNA was similarly introduced into 32Dcl3 cells. Following selection in G418, mass cultures as well as single cell clones were established from each stable transfection. The derived cell lines were tested for the expression of each transgene in the presence and absence of  $Zn^{2+}$ , which acts as an inducer of transcription from the metallothionein promoter. The profiles of RNA induction in these cell lines are shown in Figs. 3A and 4A. As can be seen in Fig. 3A, 32D cells transfected with the empty vector showed no hybridization with A-myb probe, either in the presence or absence of  $Zn^{2+}$ . On the other hand, mass cultures (32DpMT/A-myb) as well as three single cell clones (32DpMT/A4, -5, and -6) transfected with the A-myb expression vector showed low levels of expression of A-myb transcripts in the absence of  $Zn^{2+}$ , with approximately 10-fold elevation in the transcription of A-myb transgene in the presence of  $Zn^{2+}$ . When we analyzed the same cell lines for A-Myb protein expression (Fig. 3B), we did not detect any A-Myb protein in empty vector-transfected cells, while very low levels of A-Myb protein was seen in cells transfected with pMT/A-myb but grown in the absence of  $Zn^{2+}$ . However, considerable elevation of A-Myb protein levels was seen in these cells upon treatment with  $Zn^{2+}$ , which is in agreement with RNA expression data. Incubation of 32DpMT/A-myb cell line in G-CSF did not result in a decrease of A-myb transcripts, suggesting that G-CSF does not down-regulate the expression of the transgene (Fig. 3C).

A similar result was obtained with cells transfected with the c-myb expression vector, the results of which are shown in Fig. 4. 32Dcl3 cells transfected with empty vector DNA (32DpMT neo) showed the presence of a 3.4 kb endogenous c-myb band in the presence and absence of  $Zn^{2+}$ . In mass cultures (32DpMT/c-myb) as well as single cell clones (32DpMT/cl3 and -4) transfected with c-myb expression vectors, low levels of transgene expression were found to occur, which is seen as a 2.3-kb transcript in the absence of  $Zn^{2+}$ . The smaller size of the transgenic transcript is due to the absence of 3'-untranslated sequence, which was deleted during the construction of the expression plasmid. In the presence of  $Zn^{2+}$ , the levels of the 2.3-kb transcript were elevated by approximately 5–10-fold (Fig. 4A). Interestingly, the endogenous levels of c-myb RNA were found to be down-regulated upon the addition of  $Zn^{2+}$ , showing an inverse correlation between transgene expression



**FIG. 3. Inducible expression of A-myb RNA and protein in 32Dcl3 cells transfected with A-myb expression vectors.** 32Dcl3 cells were electroporated with either pMT-neo or pMT-A-myb expression vectors and selected in G418 for establishment of mass cultures (32DpMT/A-myb) and single cell clones (32DpMT/A4, -5, and -6). The established cell lines were treated with 100  $\mu M$  of  $ZnCl_2$  for the induction of transgene and analysis for A-myb RNA and protein. **A**, 32D cells transfected with A-myb expression vector were cultured in the absence and presence of  $Zn^{2+}$ , and total RNA was extracted. 20  $\mu g$  of RNA from each cell line was subjected to Northern blot analysis and hybridized to an A-myb-specific probe. Before hybridization, the blot was stained with ethidium bromide to determine the levels of total RNA transferred onto the filter. **B**, Western blot analysis of cell lysates transfected with A-myb expression vectors. Total cell lysates were prepared as described under "Materials and Methods," and 100  $\mu g$  of the lysate from each cell line was subjected to Western blot analysis using rabbit anti-Myb antibody as described under "Materials and Methods." **C**, Northern blot analysis of RNAs extracted from 32DpMT/A-myb following incubation in G-CSF containing medium. 32D cells transfected with A-myb expression vectors were washed in IL-3-free medium and incubated for 10 days in the presence of G-CSF and 100  $\mu M$   $Zn^{2+}$ . On indicated days, total RNAs were extracted from these cells and subjected to Northern blot analysis.

and endogenous c-myb RNA expression. Such an alteration of endogenous myb RNA level was not observed in empty vector transfected cell lines, suggesting that this is not an effect of addition of  $ZnCl_2$  to the culture medium and that autoregulatory mechanisms function to maintain a constant amount of c-myb transcript in the cell (39). In agreement with the RNA expression results, we did not detect a significant increase in the levels of c-Myb protein upon the addition of  $Zn^{2+}$ . To definitively demonstrate expression of c-Myb protein from the transgene, we compared the c-Myb protein levels following incubation of 32D cells in the presence of G-CSF for 12 days. It was shown previously that, in 32Dcl3 cells, the endogenous levels of c-myb transcript and protein remain relatively high until the 8th day of G-CSF treatment followed by a down-regulation of RNA and protein, such that they become undetectable by day 10 of G-CSF treatment (16, 21). Taking advan-



**FIG. 4. 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 4) were established as described under "Materials and Methods." A, Northern blot analysis of total RNA extracted from different cell lines using a full-length c-myb c-DNA probe. Endogenous c-myb transcript (upper band) and c-myb transcript encoded by the transgene (lower bands) are marked. Ethidium bromide staining of RNA after completion of RNA transfer onto the nitrocellulose filter is presented below. B, expression of c-Myb protein in empty vector-transfected cells and c-myb expression vector transfected cells. Lanes 1 and 2 contain cell lysates from empty vector-transfected cells grown in the presence of IL-3; lanes 3 and 4 contain lysates from cells transfected with c-myb expression vector grown in the presence of IL-3; lanes 5 and 6 contain 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; lanes 7 and 8 contain 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).

tage of this observation, we analyzed the level of c-Myb protein on the 12th day of G-CSF treatment in 32DpMT/c-myb-transfected cells to determine the levels of c-Myb transgenic protein expressed in the absence of the endogenous gene product. As shown in Fig. 4B, on the 12th day of G-CSF treatment, the empty vector-transfected cells were found to express very little or no c-Myb protein (Fig. 4B, lane 5 and 6). On the other hand, in the pMT/c-myb-transfected cells, high levels of c-Myb protein could be induced by  $Zn^{2+}$  (Fig. 4B, lane 7 and 8).

Following the verification of the induction of the transgene, we tested the effects of overexpression of A-myb and c-myb on G-CSF-induced terminal differentiation of 32D cells. The results of these experiments are shown in Fig. 5 and Table I. 32Dcl3 cells transfected with the empty vector undergo several rounds of cell division followed by growth arrest around day 8 of G-CSF treatment in the absence or presence of  $Zn^{2+}$  (Fig. 5A). On the other hand, cells transfected with the c-myb expression vector showed a higher proliferative potential, even in the absence of  $Zn^{2+}$  induction (Fig. 5B). This appears to be due

to the "leaky" expression of low levels of c-myb from the pMT vector used. However, the proliferation rate of these cells was dramatically higher in the presence of  $Zn^{2+}$  than in the absence, suggesting that a higher level of c-myb expression was responsible for the higher rate of cell proliferation in the presence of G-CSF (Fig. 5B). In contrast to cells transfected with c-myb, 32D cells overexpressing A-myb did not show any increase in the rate of proliferation. In the absence of  $Zn^{2+}$ , the cells transfected with A-myb expression vectors behaved in a manner similar to mock-transfected cells, where the cells underwent several rounds of division followed by growth arrest. On the other hand, the induction of high levels of A-myb expression in these cells by the addition of  $Zn^{2+}$  resulted in profound growth suppression (Fig. 5C), with a decrease in the number of viable cells during the 10-day course of G-CSF treatment (data not shown). Levels of A-myb expression remained constant through the entire period of incubation in G-CSF (Fig. 3C), suggesting that the observed growth arrest is not a result of shut off of A-myb transgene expression.

Morphological analysis of the cells treated with G-CSF is presented in Fig. 6. Cells transfected with empty vector plasmid, when treated with G-CSF, undergo terminal differentiation into granulocytes by day 10. Similarly, in the absence of  $Zn^{2+}$ , a portion of the cells transfected with c-myb terminally differentiated into granulocytes by day 10. However, the number of granulocytes were less than that observed with the mock-transfected cells, presumably due to low level expression of c-myb from the metallothionein promoter. These cells were found to undergo growth arrest in an unsynchronized manner and gradually differentiate into granulocytes by day 15–16 (data not shown). In the presence of  $Zn^{2+}$ , where high levels of transgene expression were achieved, these cells failed to undergo terminal differentiation and continued to proliferate indefinitely in G-CSF containing media as myelocytes/promyelocytes. In contrast to c-myb transfected cells, the cells transfected with A-myb expression vector terminally differentiated into granulocytes in the absence or presence of  $Zn^{2+}$ , suggesting that unlike c-Myb, A-Myb is incapable of blocking G-CSF-induced terminal differentiation of 32D cells. From these results, we conclude that ectopic overexpression of c-myb in 32Dcl3 cells results in a block to G-CSF-induced terminal differentiation with a concomitant induction of proliferation, while similar overexpression of A-myb results in an opposite effect leading to a block to proliferation without affecting G-CSF-induced terminal differentiation.

**Effect of A-Myb and Ets-2 Co-expression on 32D Cell Differentiation**—The observation that the transactivating function of A-Myb is highly enhanced in the presence of Ets-2 raised the possibility that A-Myb might mimic the biological activity of c-Myb in the presence of Ets-2, if the weak transactivating activity of A-myb alone is responsible for its inability to induce proliferation of 32Dcl3 cells in the presence of G-CSF. Since the results presented above show that the transactivation potential of A-myb can be considerably enhanced when co-expressed with ets-2, it was of interest to see whether co-expression of ets-2 along with A-myb in 32Dcl3 cells would result in a phenotype that resembles the one produced by c-myb overexpression.

To overexpress Ets-2, an expression plasmid was prepared where the ets-2 gene was placed under the control of the SV40 promoter (pSG5) along with the puromycin resistance gene, which was placed under the control of the PGK-1 promoter. This expression vector was transfected into 32Dcl3 cells, which overexpress A-myb under the control of metallothionein promoter (pMT-neo). As negative controls, empty vectors pSG5 and pMT-neo were similarly electroporated into cells. Following selection of cells in the presence of neomycin and puromy-

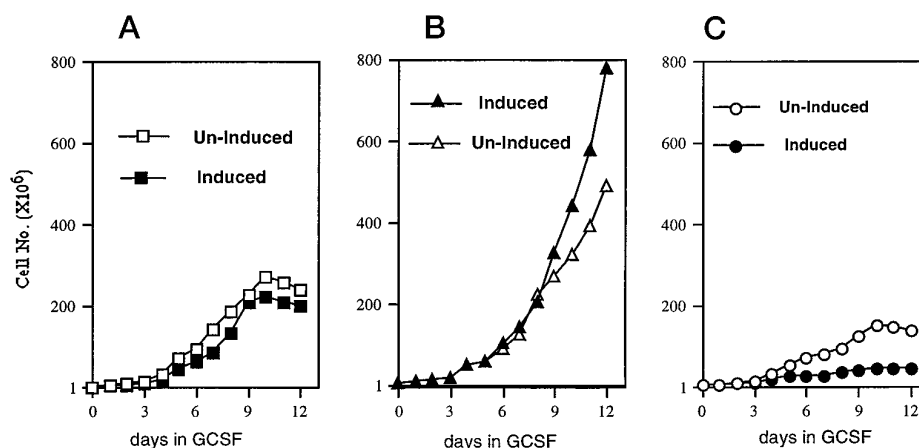


FIG. 5. **Growth and differentiation of 32Dcl3 cells overexpressing A-myb or c-myb in the presence of G-CSF.** 32Dcl3 cells transfected with empty vector (32D/pMT-neo), *c-myb* (32D/pMT-c-myb), or *A-myb* (32D/pMT-A-myb) expression vector were analyzed for their growth in the presence of G-CSF. Cells were washed two times in IL-3-free medium and cultured in the medium containing G-CSF and were split 1:4 with complete medium containing G-CSF, when the cell density reached to  $5-7 \times 10^5$  per ml. For induction of transfected genes, the cells were treated with  $100 \mu\text{M}$  of  $\text{ZnCl}_2$  approximately 30 h before G-CSF treatment. On each indicated day, the numbers of the cells were determined by trypan blue exclusion. A, growth curve of 32D cells transfected with empty vector. B, growth curve of 32D cells transfected with *c-myb* expression vector. C, growth curve of 32D cells transfected with *A-myb* expression vector.

TABLE I

The effect of A-myb and c-myb overexpression on G-CSF-induced granulocytic differentiation of 32Dcl3 cells

Each cell line was plated in IMDM containing 10% FBS, 1% penicillin/streptomycin, and 10% G-CSF at a density of  $2 \times 10^5$  cells/ml. On indicated days smears were prepared from an aliquot of cell culture and stained with May-Grunwald stain for morphological analysis.

Cell lines	Zinc treatment	Days after G-CSF	Myeloblasts	Promyelo/myelocytes	Metamyelo/granulocytes
32D/pMT-neo	—	0	93	7	0
		5	0	54	46
		10	0	13	87
	+	0	94	6	0
		5	1	54	45
		10	0	12	88
32D/pMT A-Myb	—	0	90	10	0
		5	1	26	73
		10	0	10	90
	+	0	91	9	0
		5	4	34	62
		10	0	7	93
32D/pMT C-Myb	—	0	90	10	0
		5	0	61	39
		10	2	39	59
	+	0	92	8	0
		5	6	83	10
		10	6	88	6

cin, the expression of *A-myb* and *ets-2* in these cells was determined by Northern blot analysis. As shown in Fig. 7A (lane 1), *ets-2* transcripts were undetectable in the 32Dcl3 cell line transfected with empty vector but were readily detectable in cell lines that were transfected with pSG5-ets/puro vector (Fig. 7A, lanes 2 and 4). Each cell line was then induced to differentiate by the addition of G-CSF, and their proliferation rate and differentiation potential were monitored by cell counting and morphological analysis. As shown in Fig. 7B, 32Dcl3 cells transfected with *ets-2* expression vector alone in the absence of *A-myb* did not show an appreciable increase in the growth rate compared with control cells that neither expressed *A-myb* or *ets-2*. On the other hand, growth of the 32Dcl3 cell line overexpressing *A-myb* alone was almost completely blocked in the presence of G-CSF. However, when the cell lines expressing both *A-myb* and *ets-2* were treated with G-CSF, a remarkable increase in the growth of the cells was observed in the presence of G-CSF (Fig. 7C). In addition, cell viability was also maintained at a higher level in *ets-2* co-expressing cell line (data not

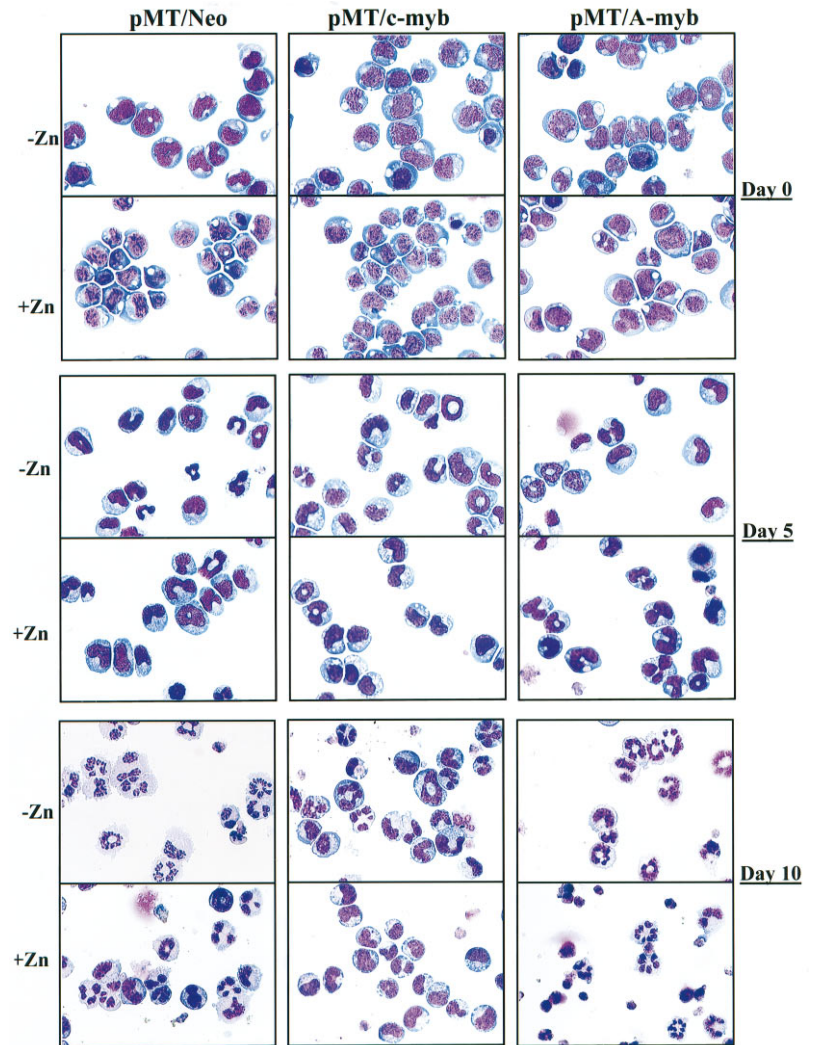
shown). These results show that *A-myb*, when co-expressed along with *ets-2*, can support the growth of 32Dcl3 cells in the presence of G-CSF. To determine whether co-expression of *A-myb* along with *ets-2* resulted in a block to the ability of 32Dcl3 cells to terminally differentiate into granulocytes, morphological analysis of cell lines transfected with *ets-2* alone, *A-myb* alone, as well as *A-myb* along with *ets-2* was carried out. The results of this experiment are shown in Fig. 7D. These results show that despite their increased growth rate, the differentiation of 32Dcl3 cells co-expressing *A-myb* and *ets-2* was not affected. These results show that co-expression of *A-myb* along with *Ets-2*, while resulting in increased transactivation of the A-Myb protein, fails to mimic the phenotypic effect produced by c-Myb.

**Effect of COOH-terminal Truncation of A-Myb on 32D Cell Differentiation**—Since the transactivation potential of A-Myb is dramatically enhanced by COOH-terminal truncations, we examined the effects of overexpression of this mutant in 32Dcl3 cells (Fig. 8). For this, we constructed inducible expression vectors where the truncated *A-myb* (tA-myb) gene was placed under the control of the human metallothionein promoter. Following selection in G418, the derived cell lines were tested for the expression of the transgene in the presence and absence of  $\text{Zn}^{2+}$ . Fig. 8A shows the RNA and protein expression profiles of truncated *A-myb* in these transfectants. Interestingly, the expression levels of tA-Myb were very high even in the absence of  $\text{Zn}^{2+}$ , which appears to be due to leaky expression of the transgene from the metallothionein expression vector.

We next tested the effects of overexpression of tA-myb on G-CSF-induced terminal differentiation of 32D cells. 32Dcl3 cells transfected with the tA-myb expression vector showed a high proliferation potential (Fig. 8B), which is in sharp contrast to cells transfected with full-length *A-myb* expression vectors. Because of the leaky expression of tA-Myb, addition of  $\text{Zn}^{2+}$  did not make a difference to the phenotype observed. Morphological analysis of the cells treated with G-CSF (Fig. 8C) showed that differentiation of these cells is arrested at the myelocytic stage, a phenotype that is similar to that seen with *c-myb*. From these results, we conclude that the DNA-binding and transactivation domains of A-Myb, in the absence of its COOH-terminal negative regulatory domain can mimic the biological effects of c-Myb. However, full-length A-Myb cannot mimic the biological effects of c-Myb, suggesting that the COOH-terminal domain of A-Myb dictates the biological phenotype produced by this gene.



**FIG. 6. Effect of ectopic overexpression of A-myb and c-myb on G-CSF induced granulocytic differentiation of 32Dcl3 cells.** 32Dcl3 cells overexpressing *c-myb* (C), A-myb (A) and mock-transfected cells (E) were induced to differentiate by the addition of G-CSF in the presence (+Z) or absence (–Z) of  $Zn^{2+}$  as described under “Materials and Methods.” Following incubation for 0, 5, and 10 days in the presence of G-CSF (Day 0, Day 5, or Day 10), aliquots of cells were cyto-spun and stained with May-Grunwald-Giemsa. A, morphology of mock-transfected cells following incubation in the presence of G-CSF. B, morphology of *c-myb* overexpressing 32D cells following incubation in the presence of G-CSF. C, morphology of 32Dcl3 cells overexpressing A-myb following incubation in the presence of G-CSF.



#### DISCUSSION

In this paper, we have described the transactivating potential of murine A-Myb and compared this biochemical activity with that of c-Myb. Our results show that murine A-Myb is a very weak transactivator of transcription compared with that of c-Myb. However, the deletion of the COOH-terminal regulatory domain of A-Myb seemed to restore the full transactivating potential of A-Myb, suggesting that the COOH-terminal domain of this protein exerts a strong negative regulatory effect on this protein. Our results also show that this negative regulatory effect of the COOH-terminal domain could be compensated by Ets-2, which appears to co-operate with A-Myb in transactivation of reporter genes containing Myb-binding sites. Earlier reports with human A-myb (7, 37) had indicated that both A-myb and c-myb gene possess comparable transactivating activity. A similar observation was made with chicken A-myb (8). The reason for this difference is unclear. It is possible that the A-myb gene derived from different species exhibits different transactivational activities. Alternatively, these transactivation assays for human and chicken A-myb were conducted in hematopoietic cell lines, which often express *ets-2*. Since our results show that co-expression of *ets-2* with A-myb results in high transactivational activities, the differences seen by different investigators could be due to the nature of co-operating factors that are expressed in different cell lines.

It is now well established that *c-myb* is essential for the proliferative potential of several myeloid and T-cell lines (12, 40, 41). In addition, several lines of evidence suggest that

hematopoietic cell differentiation is accompanied by down-regulation of *c-myb* gene expression (10, 11, 42, 43), and constitutive overexpression of *c-myb* blocks cytokine-induced terminal differentiation of hematopoietic cells (17–22). To test whether A-Myb can function in a manner similar to that of c-Myb in a biological system, we expressed A-myb and c-myb genes in the myeloid precursor cell line 32Dcl3 and examined their effects on the ability of this cell line to undergo terminal differentiation in the presence of G-CSF. Our results clearly show that while expression of *c-myb* in this cell line results in a block to the ability of these cells to undergo terminal differentiation in the presence of G-CSF, the expression of A-myb failed to bring about this phenotypic effect. In addition, expression of *c-myb* appears to result in an increased proliferative potential of this cell line in G-CSF, while expression of A-myb alone results in a complete block to the ability of this cell line to proliferate in the presence of G-CSF. The observed inhibitory effect of A-Myb could be attributed to the possibility that A-Myb, by virtue of a highly related DNA-binding domain, could occupy c-Myb binding sites on DNA and act as a competitor to these sites. The inability of A-Myb to bring about transactivation of these c-Myb target genes could bring about the observed growth arrest.

Our observation that the transactivation potential of A-Myb is considerably elevated in the presence of Ets-2 allowed us to test whether A-Myb can mimic the biological effects of c-Myb under conditions where its transactivation potential is enhanced to levels exhibited by c-Myb. Our results show that A-Myb-Ets-2 cooperation results in the restoration of cell pro-



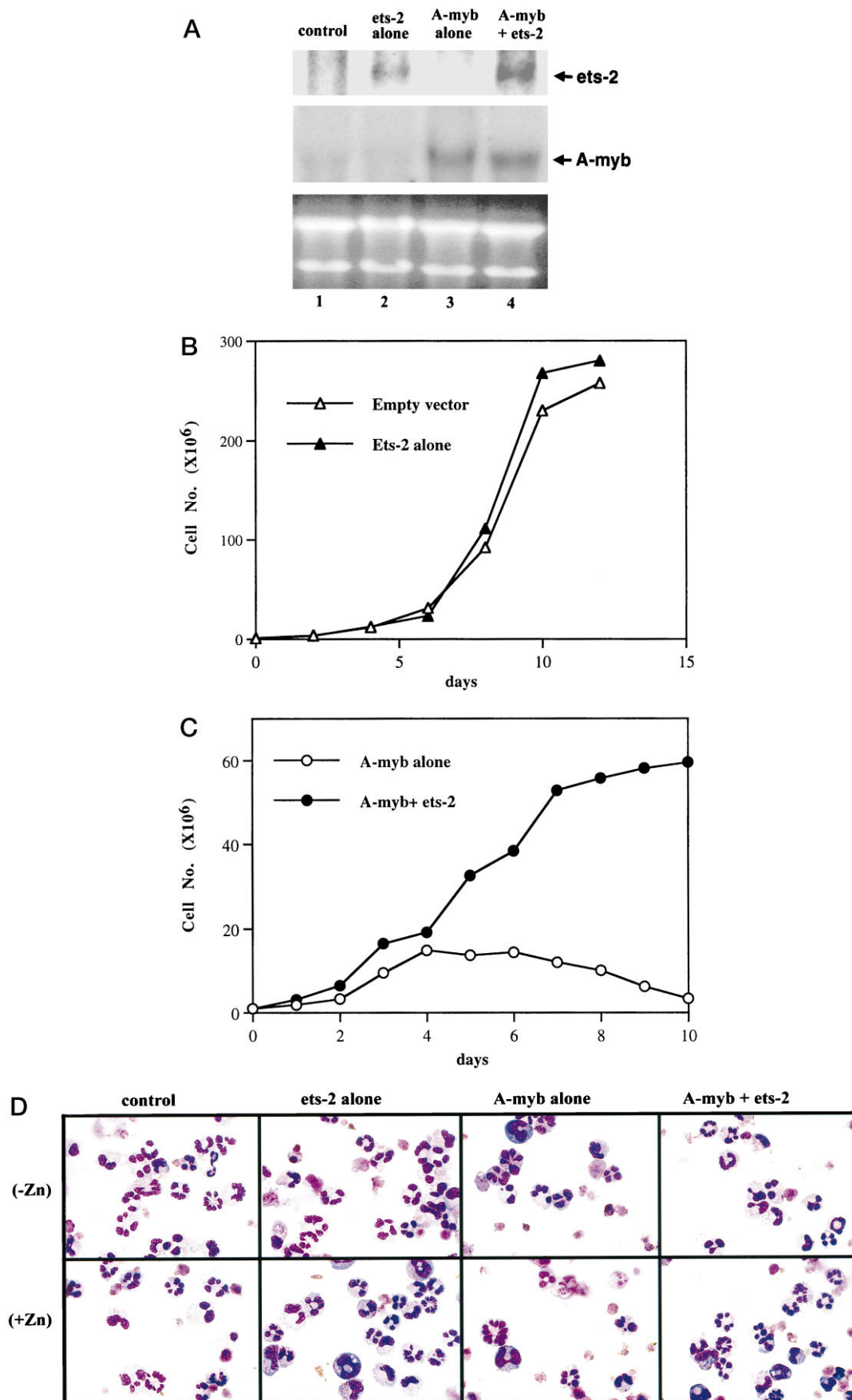
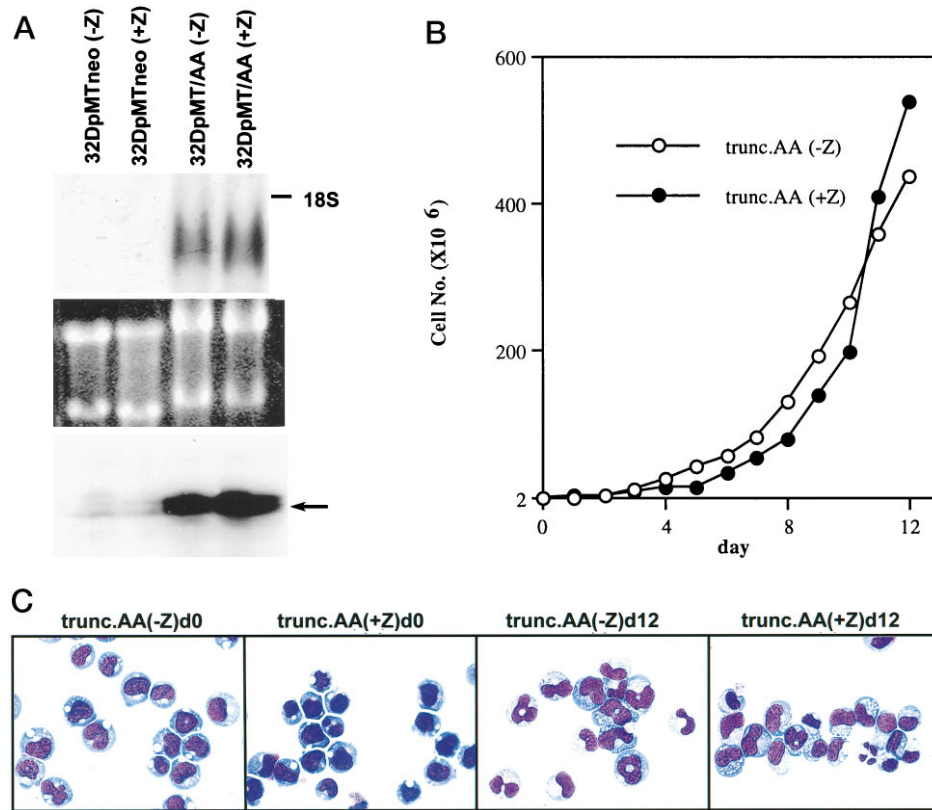


FIG. 7. Growth of 32Dcl3 cells co-expressing A-myb and ets-2 in the presence of G-CSF. A, establishment of 32Dcl3 cell line co-expressing ets-2 and A-myb. 32Dcl3 cells transfected with A-myb expression vector (32DpMT/A-myb) or empty vector (32D/pMT-neo) were re-transfected with either pSG5/puro or pSG5-ets/puro and selected in G418 (500  $\mu$ g/ml) and puromycin (2  $\mu$ g/ml). The established cell lines were analyzed for expression of ets-2 and A-myb. Lane 1, total RNA from the cell line, which was transfected with two empty vectors (pMT-neo and pSG5/puro) and



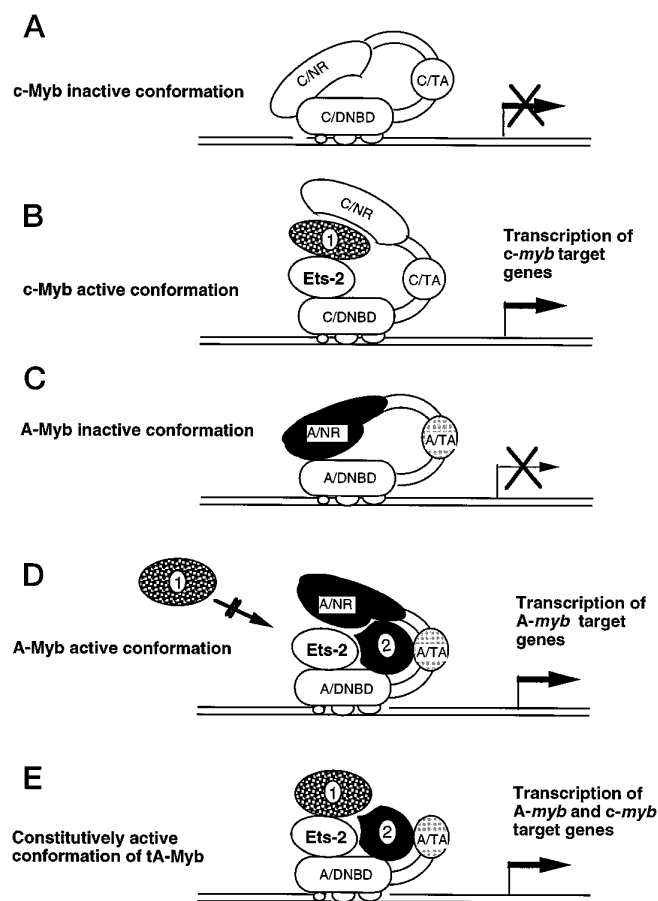
**FIG. 8. The effect of C terminus truncated A-myb on growth and differentiation of 32D cells.** A, establishment of 32D cell line overexpressing truncated A-myb. Top, the 3'-truncated A-myb c-DNA cloned into pMT-neo vector was transfected into 32D cells as described earlier. 20  $\mu$ g of total RNA was analyzed by Northern blot using full-length A-myb c-DNA as a probe. Middle, before hybridization, the blot was stained with ethidium bromide to determine the levels of total RNA transferred onto the filter. Bottom, Western blot analysis of cell lysates transfected with truncated tA-myb expression vector. Total cell lysates were prepared as described under "Materials and Methods," and 150  $\mu$ g of the lysate from each cell line was subjected to Western blot analysis using rabbit anti-Myb antibody as described under "Materials and Methods." The protein product of the gene is marked with an arrow. B, growth of 32D cells transfected with truncated A-myb in the presence of G-CSF. Cells overexpressing truncated A-myb were incubated in G-CSF, and the cell count was determined as described earlier. C, morphology of 32D cells overexpressing tA-myb following incubation in G-CSF. Cells overexpressing tA-myb were incubated in G-CSF and analyzed on indicated days by May-Grunwald-Giemsa staining.

liferation to 32Dcl3 cells incubated in the presence of G-CSF. However, the increase in growth of the cells was not accompanied by an arrest to differentiation process as was observed with c-Myb expression. These results suggest that the cooperation between Ets-2 and A-Myb, while being able to restore its transactivation function, failed to produce the phenotype caused by c-Myb.

Since deletions in the COOH-terminal domain of A-Myb also enhance its ability to transactivate transcription, we studied the effect of ectopic overexpression of this mutant on granulocytic differentiation of 32Dcl3 cells. Our results show that this mutant, tA-myb, was very effective in promoting proliferation of 32Dcl3 cells in G-CSF and like c-myb blocked G-CSF-induced differentiation of 32D cells. These results suggest that the COOH-terminal domain of A-Myb plays a critical role in the biological phenotype produced by A-Myb and its deletion allows

this protein mimic the function of c-Myb. This was an unexpected result because of the belief that A-Myb and c-Myb may both act by similar, if not identical, mechanisms (7, 8). If this were not to be the case, as suggested by our results, one need to find an explanation for the opposing effects of A-Myb and c-Myb on 32Dcl3 cell proliferation and differentiation programs. It is now established that c-Myb and A-Myb function effectively in the presence of appropriate co-operating factors, which interact with the two proteins and form an enhancer complex (6, 44–46). We propose that the nature of transcription factors that interact with A-Myb or c-Myb is determined by their COOH-terminal domains, which dictate the nature of the enhancer complex formed between c-Myb and A-Myb proteins and other co-operating factors (Fig. 9). In the absence of any co-operating factors, it is expected that these two proteins assume an inactive state via an intramolecular conformation

expresses neither *ets-2* or A-myb. Lane 2, total RNA from 32D cell line transfected with pSG5-ets/puro and empty pMT-neo vector. Lane 3, total RNA from cells transfected with pMT-A-myb and empty pSG-5/puro vector. Lane 4, total RNA from cells transfected with pMT-A-myb and pSG5-ets-2/puro such that both of the transgenes are expressed in these cells. Upper panel, Northern blot analysis of cellular RNAs using full-length *ets-2* c-DNA as a probe. Middle panel, analysis of the same filter for A-myb expression with an A-myb-specific probe. Lower panel, ethidium staining of RNA after completion of RNA transfer onto the nitrocellulose filter. B, growth of cells transfected with two empty vectors, pMT-neo and pSG5/puro, was compared with cells transfected with pSG5-ets/puro along with an empty pMT-neo vector. On each indicated day, the numbers of viable cells were determined by trypan blue exclusion, and the total numbers of viable cells per culture were estimated. C, growth of cells transfected with pMT-A-myb alone (along with pSG5/puro empty vector) was compared with cells transfected with pMT-A-myb along with pSG5-ets/puro vector. For induction of A-myb expression, cells were treated with 100 mM ZnCl<sub>2</sub> approximately 30 h before the addition of G-CSF. Cell counts were determined as described in A. D, morphological analysis of 32D cells co-expressing A-myb and *ets-2* following incubation in G-CSF. Cell lines expressing A-myb and *ets-2*, A-myb alone, *ets-2* alone, and control cell line transfected with empty vectors were analyzed for morphological changes after 10 days of G-CSF treatment in the presence of (+Z) or absence (-Z) of Zn<sup>2+</sup> using May-Grunwald-Giemsa stain.



**FIG. 9. A hypothetical model for Myb-mediated transactivation.** A and C, the carboxyl-terminal negative regulatory domain of c- and A-Myb interact with their amino termini, resulting in an inactive conformation of the protein as has been proposed earlier (47). B, transcriptional co-activators such as Ets-2 interact with the amino-terminal region of c-Myb, resulting in a change in conformation, which allows further interaction of c-Myb with other members of transcriptional machinery, which is required for the transactivation of c-Myb-specific target genes. D, a similar interaction of A-Myb with Ets-2 or other co-operating factors provides a relief from negative regulation allowing interaction of specific cellular factors, resulting in transactivation of A-Myb-specific target genes. The C-terminal domain of A-Myb provides a conformation that specifies the nature of other co-operating factors that interact with A-Myb. E, deletion of the C-terminal domain of A-Myb results in a conformation, which allows interaction of this truncated protein with multiple cellular factors, resulting in aberrant transcriptional activation of both c- and A-Myb target genes, which could lead to a neoplastic state. Since the amino-terminal domain of Myb has been shown to interact with multiple proteins (44–47), Ets-2 could be replaced by other co-operating co-activators, depending on the cell type. C, c-Myb; A, A-Myb; DNAB, DNA-binding domain; TA, transactivating domain; NR, negative regulatory domain.

such as the one suggested recently for c-Myb (47). However, following post-translational modifications, which seem to activate the transactivating potential of these proteins (48, 49), c-Myb and A-Myb assume conformations that allow their interaction with discrete sets of transcription factors, which specify the nature of target genes that are transactivated by A-Myb and c-Myb. We would propose that the COOH-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 such as Ets-2 might be common to both c-Myb and A-Myb, while others (depicted as 1 and 2 in Fig. 9) 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 possible that c-Myb and A-Myb

have some common targets and some specific targets, which dictate the final biological effects produced by these individual proteins. It is conceivable that the COOH-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 A-Myb, whose function appears to be restricted primarily to germ cells (9, 24, 25), might transactivate transcription of a different set of genes by virtue of its ability to interact with a distinctive set of nuclear factors. This model predicts that c-myb and A-myb cannot totally compensate for the biological function of each other. In myeloid cells such as 32Dcl3, it is conceivable that co-factors that relieve the negative regulation of c-Myb exist but not for A-Myb. Such factors for A-Myb are likely to be expressed in cell types such as male germ cells, where A-Myb has a well defined function (25). In the presence of Ets-2, which can interact with A-Myb, it is possible that the A-Myb protein undergoes a conformational change, that allows it to form an enhancer complex that can transactivate transcription of a few but not all of the target genes of c-Myb. On the other hand, truncation of A-Myb might relieve the steric inhibitory effects, resulting in the indiscriminate interaction of the truncated A-Myb protein with co-operating factors that interact with both c-Myb or A-Myb. Several lines of study suggest that truncation of c-Myb at the COOH-terminal region is associated with enhanced transforming activity (50–54), and it is conceivable that this may also be true for A-Myb. It is at present unclear whether the COOH-terminal domains of A-Myb and c-Myb regulate the nature of enhancer complex formed by participating in protein-protein interactions or solely through steric effect. Studies aimed at studying the biological effects of A-Myb truncation *in vivo* and identification of nuclear factors that interact with A-Myb and c-Myb and co-operate to bring about the transcriptional transactivation of their target genes are likely to shed further insight into the mechanism of action of this gene family.

#### REFERENCES

- Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Sasamoto, S., and Ishizaki, R. (1988) *Nucleic Acids Res.* **16**, 11075–11089
- Biedenkapp, H., Borgmeyer, U., Sippel, A. E., and Klempnauer, K.-H. (1988) *Nature* **335**, 835–837
- Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T. J., and Ishii, S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5758–5762
- Weston, K., and Bishop, J. M. (1989) *Cell* **58**, 85–93
- Mizuguchi, G., Nakagoshi, H., Nagase, T., Nomura, N., Date, T., Ueno, Y., and Ishii, S. (1990) *J. Biol. Chem.* **265**, 9280–9284
- Dudek, H., Trantravahi, R. V., Rao, V. N., Reddy, E. S., and Reddy, E. P. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1291–1295
- Golay, J., Loffarelli, L., Luppi, M., Castellano, M., and Introna, M. (1994) *Oncogene* **9**, 2469–2479
- Foos, G., Grimm, S., and Klempnauer, K. H. (1994) *Oncogene* **9**, 2481–2488
- Mettus, R. V., Litvin, J., Wali, A., Toscani, A., Latham, K., Hatton, K., and Reddy, E. P. (1994) *Oncogene* **9**, 3077–3086
- Pauza, C. D. (1987) *Mol. Cell. Biol.* **7**, 342–348
- Reed, J. C., Alpers, J. D., Scherle, P. A., Hoover, R. G., Nowell, P. C., and Prystowsky, M. B. (1987) *Oncogene* **1**, 223–228
- Anfossi, G., Gewirtz, A. M., and Calabretta, B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 3379–3383
- Westin, E. H., Gallo, R. C., Arya, S. K., Eva, A., Souza, L. M., Baluda, M. A., Aaronson, S. A., and Wong-Staal, F. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 2194–2198
- Ramsay, R. G., Ikeda, K., Rifkind, R. A., and Marks, P. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6849–6853
- Thiele, C. J., Cohen, P. S., and Israel, M. A. (1988) *Mol. Cell. Biol.* **8**, 1677–1683
- Patel, G., Kreider, B., Rovera, G., and Reddy, E. P. (1993) *Mol. Cell. Biol.* **13**, 2269–2276
- Clarke, M. F., Kukowska-Latallo, J. F., Westin, E., Smith, M., and Prochowik, E. V. (1988) *Mol. Cell. Biol.* **8**, 884–892
- McClinton, D., Stafford, J., Brents, L., Bender, T. P., and Kuehl, W. M. (1990) *Mol. Cell. Biol.* **10**, 705–710
- Todoroki, K., Watson, R. J., Higo, H., Amanuma, H., Kuramochi, S., Yanagisawa, H., and Ikawa, Y. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 8900–8904
- Selvakumaran, M., Liebermann, D. A., and Hoffman-Liebermann, B. (1992) *Mol. Cell. Biol.* **12**, 2493–2500
- Bies, J., Mukhopadhyaya, R., Pierce, J., and Wolff, L. (1995) *Cell Growth*

- Differ.* **6**, 59–68
22. Patel, G., Tantravahi, R., Oh, I. H., and Reddy, E. P. (1996) *Oncogene* **13**, 1197–1208
  23. Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., Jr., and Potter, S. S. (1991) *Cell* **65**, 677–689
  24. Trauth, K., Mutschler, B., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and Klempnauer, K. H. (1994) *EMBO J.* **13**, 5994–6005
  25. Toscani, A., Mettus, R. V., Coupland, R., Simpkins, H., Litvin, J., Orth, J., Hatton, K. S., and Reddy, E. P. (1997) *Nature* **386**, 713–717
  26. Golay, J., Luppi, M., Songia, S., Palvarini, C., Lombardi, L., Aiello, A., Delia, D., Lam, K., Crawford, D. H., Biondi, A., Barbui, T., Rambaldi, A., and Introna, M. (1996) *Blood* **87**, 1900–1911
  27. Rovera, G., Kreider, B., Shirsat, N., Venturelli, D., Naso, G., and Mavilio, F. (1989) *Ann. N. Y. Acad. Sci.* **567**, 154–164
  28. Valtieri, M., Twardy, D. J., Caracciolo, D., Johnson, K., Mavilio, F., Altmann, S., Santoli, D., and Rovera, G. (1987) *J. Immunol.* **138**, 3829–3835
  29. Greenberger, J. S., Sakakeeny, M. A., Humphries, R. K., Eaves, C. J., and Eckner, R. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 2931–2935
  30. Ymer, S., Tucker, W. Q. J., Sanderson, C. J., Hapel, A. J., Campbell, H. D., and Young, I. G. (1985) *Nature* **317**, 255–258
  31. Ness, S. A., Marknell, A., and Graf, T. (1989) *Cell* **59**, 1115–1125
  32. Nordeen, S. K. (1988) *BioTechniques* **6**, 454–458
  33. Ness, S. A., Kowenz-Leutz, E., Casini, T., Graf, T., and Leutz, A. (1993) *Genes Dev.* **7**, 749–759
  34. Karin, M., Haslinger, A., Heguy, A., Dietlin, T., and Cooke, T. (1987) *Mol. Cell. Biol.* **7**, 606–613
  35. Gunning, P., Leavitt, J., Muscat, G., Ng, S. Y., and Kedes, L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 4831–4835
  36. Ausubel, F. M., Brent, R., Kingston, R., Moore, D. D., Seidman, J. G., J. A., S., and Struhl, K. (eds) (1989) *Current Protocols in Molecular Biology*, pp. 1061–1085, John Wiley and Sons, New York
  37. Takahashi, T., Nakagoshi, H., Sarai, A., Nomura, N., Yamamoto, T., and Ishii, S. (1995) *FEBS Lett.* **358**, 89–96
  38. Kalkbrenner, F., Guehmann, S., and Moelling, K. (1990) *Oncogene* **5**, 657–661
  39. Guerra, J., Withers, D. A., and Boxer, L. M. (1995) *Blood* **86**, 1873–1880
  40. Gewirtz, A. M., and Calabretta, B. (1988) *Science* **242**, 1303–1306
  41. Gewirtz, A. M., Anfossi, G., Venturelli, D., Valpreda, S., Sims, R., and Calabretta, B. (1989) *Science* **245**, 180–183
  42. Gonda, T. J., and Metcalf, D. (1984) *Nature* **310**, 249–251
  43. Kuehl, W. M., Bender, T. P., Stafford, J., McClinton, D., Segal, S., and Dmitrovsky, E. (1988) *Curr. Topic. Microbiol. Immunol.* **141**, 318–323
  44. Burk, O., Mink, S., Ringwald, M., and Klempnauer, K. H. (1993) *EMBO J.* **12**, 2027–2038
  45. Mink, S., Kerber, U., and Klempnauer, K. H. (1996) *Mol. Cell. Biol.* **16**, 1316–1325
  46. Dai, P., Akimaru, H., Tanaka, Y., Hou, D. X., Yasukawa, T., Kanei-Ishii, C., Takahashi, T., and Ishii, S. (1996) *Genes Dev.* **10**, 528–540
  47. Dash, A. B., Orrico, F. C., and Ness, S. A. (1996) *Genes Dev.* **10**, 1858–1869
  48. Miglarese, M. R., Richardson, A. F., Aziz, N., and Bender, T. P. (1996) *J. Biol. Chem.* **271**, 22697–22705
  49. Aziz, N., Miglarese, M. R., Hendrickson, R. C., Shabanowitz, J., Sturgill, T. W., Hunt, D. F., and Bender, T. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6429–6433
  50. Rosson, D., and Reddy, E. P. (1986) *Nature* **319**, 604–606
  51. Weinstein, Y., Ihle, J. N., Lavu, S., and Reddy, E. P. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5010–5014
  52. Shen-Ong, G. L. C., Morse, H. C., III, Potter, M., and Mushinski, J. F. (1986) *Mol. Cell. Biol.* **6**, 380–392
  53. Gonda, T. J., Buckmaster, C., and Ramsay, R. G. (1989) *EMBO J.* **8**, 1777–1783
  54. Hu, Y. L., Ramsay, R. G., Kanei-Ishii, C., Ishii, S., and Gonda, T. J. (1991) *Oncogene* **6**, 1549–1553