

Acceleration of Apoptosis in Transforming Growth Factor β 1-treated M1 Cells Ectopically Expressing B-myb

Juraj Bies¹ and Linda Wolff²

Laboratory of Genetics, National Cancer Institute, NIH, Bethesda, Maryland 20892

Abstract

Inappropriate expression of genes involved in cell proliferation can result in altered regulation of apoptosis, a process of programmed cell death. Since B-myb has recently been implicated in the cell cycle progression we wanted to examine its role in the apoptotic process. For this purpose we used transforming growth factor β 1 (TGF- β 1)-treated M1 myeloid leukemia cell lines that continuously express murine B-myb. It was found that in cells overexpressing B-myb, TGF- β 1-induced apoptosis was accelerated as assessed by cell viability and DNA fragmentation into nucleosomal fragments. A DNA ladder was detected after 24 h of TGF- β 1 treatment in these cells, whereas it was not detected until after 36 h in the parental M1 cells. It was further determined by Northern blot analysis that this higher sensitivity of B-myb overexpressing clones was not due to a change in the expression of TGF- β receptor type I or in the kinetics of the regulation of *c-myc*, *c-myb*, *bcl-2*, and/or *bax*.

Introduction

B-myb is a member of the myb family of transcription factors and has a DNA-binding domain highly homologous to c-myb (1, 2). It is cell cycle regulated with the peak expression at the transition from G₁-S of the cell cycle (3, 4). Interestingly, the overexpression of human B-myb was shown to reduce growth factor requirements and induce a transformed phenotype in BALB/c 3T3 fibroblasts (5). Treatment with an antisense oligomer complementary to the B-myb gene significantly inhibits the proliferation of myeloid or lymphoid cell lines (6). All these results indicate that, like c-myb, B-myb is a growth-promoting gene. Since there have been reports that other growth-promoting genes, when expressed concurrently with growth arrest signals, can cause cells to undergo apoptosis, we wanted to determine if B-myb could also affect this process. It is important to identify and characterize genes that regulate this active program that controls cell numbers since therapeutic modulation of apoptosis may ultimately become a major approach in controlling malignancy. We chose the TGF- β 1³-treated murine myeloid leukemia cell line M1 to evaluate B-myb effects on programmed cell death because it had been shown that treatment of M1 cells with TGF- β 1 induces rapid growth arrest and apoptosis (7, 8) and that c-myb and c-myc oncogenes can accelerate this process (7). Our results show that overexpression of B-myb accelerates programmed cell death in TGF- β 1-treated M1 cells without affecting the regulation of expression of c-myb and c-myc.

Materials and Methods

Cells and Viruses. The murine myeloid leukemia cell line M1 has been described previously (7). The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated horse serum. Murine amphotropic GP+envAm12 (9) and ecotropic GP+E-86 (10) packaging cell lines were maintained in DMEM containing 10% fetal bovine serum. The M1 cells were seeded at a concentration 1.5×10^5 /ml with or without human recombinant TGF- β 1 (10 ng/ml; R&D Systems, Inc., Minneapolis, MN). Viable cell numbers were determined using trypan blue dye exclusion and counting in a hemocytometer.

Recombinant retroviruses were prepared by transfecting plasmids pLXSN (11) or pLB-mybSN into amphotropic packaging cell line GP+envAm12 by the calcium phosphate method. After 48 h, conditioned medium-containing virus was used to infect the ecotropic packaging cell line GP+E-86 in the presence of 4 μ g/ml of Polybrene (Sigma Chemical Co., St. Louis, MO). Infected cells were selected by culturing in medium containing 400 μ g/ml of G418 (GIBCO, Gaithersburg, MD). Selected cells producing recombinant retroviral particles were treated with mitomycin C (10 ng/ml; Sigma) and used to infect M1 cells by cocultivation. The infected M1 cells were subsequently selected for resistance to G418 (400 μ g/ml).

Construction of a Retroviral Vector Expressing B-myb. A retroviral vector for expression of murine B-myb was prepared by digestion of pMB21 (1) with HindIII, followed by conversion of HindIII ends to the BamHI sites and digestion with BamHI to obtain a 2.6-kilobase fragment of B-myb cDNA. This was subsequently subcloned into the BamHI cloning site of retroviral vector LXSN (11).

Analysis for DNA Fragmentation. Briefly, 2×10^6 cells, treated with TGF- β 1 for indicated times, were lysed [0.5% SDS-0.1 M NaCl-1 mM EDTA-50 mM-Tris-HCl (pH 8.0)] and incubated for 4 h at 50°C in the presence of 0.1 mg/ml proteinase K (GIBCO). The samples were then extracted with chloroform-isoamyl alcohol, precipitated with ethanol, treated with RNase for 1 h at 37°C, reextracted, reprecipitated, and finally dissolved in 10 mM Tris-1 mM EDTA (pH 7.4). Equal amounts of DNA were electrophoresed on a 2% agarose gel containing 1 mg/ml ethidium bromide and visualized by UV fluorescence.

Northern (RNA) Analysis. Total RNA was prepared from $3-5 \times 10^7$ cells using TRIzol reagent (GIBCO) as described in the instructions from the manufacturer. Ten- μ g samples of total RNA were electrophoresed on a 1.2% agarose gel containing formaldehyde (0.7%), blotted onto nylon membrane, UV cross-linked using Stratilinker 1800 (Stratagene, La Jolla, CA), and hybridized. The following hybridization probes were labeled using a random priming kit (GIBCO): 2.0-kilobase NcoI fragment of c-myb (12); 2.6-kilobase BamHI-HindIII fragment of B-myb cDNA (1); 1.4-kilobase SstI-HindIII fragment of c-myc (13); cDNA for TGR-I (14); EcoRI fragment of murine bcl-2 (15); murine bax cDNA (16) and rat glyceraldehyde 3-phosphate dehydrogenase cDNA (17).

In Vitro Translation. A 2.6-kilobase BamHI-HindIII fragment of B-myb cDNA was subcloned into pBlueII SK(+) (Stratagene) yielding pBS-B-myb, which was translated *in vitro* using TNT T3 Coupled Reticulocyte Lysate System (Promega, Madison, WI) in the presence of [³⁵S]cysteine according to the manufacturer's protocol. Five μ l of reaction mixture were mixed with an equal volume of 2 \times SDS gel loading buffer and subjected to SDS-PAGE (7%) electrophoresis. The gel was dried and exposed to X-ray film.

Received 11/15/94; accepted 12/19/94.

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.

¹ On leave from the Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovakia.

² To whom requests for reprints should be addressed, at Laboratory of Genetics, Building 37, Room 2B04, National Cancer Institute, NIH, 37 Convent Drive, MSC 4255, Bethesda, MD 20892-4255.

³ The abbreviations used are: TGF, transforming growth factor; TGR-I, TGF- β receptor type I.

Results and Discussion

To analyze the effect of B-myb gene expression on the TGF-β1-induced programmed cell death in M1 cells we constructed a retroviral vector LB-mybSN, where a full length B-myb cDNA was placed under the control of Moloney murine leukemia virus LTR. The protein coding capacity of the B-myb cDNA was checked by carrying out *in vitro* transcription and translation on the plasmid, pBluescriptII SK(+), which contained B-myb cDNA sequence downstream of the T3 promoter (BS-B-myb). As shown in Fig. 1A, we detected the protein of predicted size. Viruses, transiently expressed and packaged in amphotropic cell line GP+envAm12, were used to infect GP+E86 cells, an ecotropic cell line. These cells were subsequently subjected to G418 selection. Infection of the M1 cell line was carried out by cocultivation with B-myb-virus-producing GP+E86 cells, followed by selection of infected clones in medium containing G418 (400 μg/ml). Southern blot analysis of selected M1/B-myb clones confirmed that each clone contained 1–2 integrated retroviral constructs carrying B-myb cDNA of expected size (data not shown). Although the data that follow were largely obtained using three representative clones having a single virus integration, similar results were obtained with all selected M1/B-myb clones. As a negative control, M1 cells were also infected with retroviruses containing the vector LXSXN lacking the B-myb insert (M1/LXSXN). Northern blot analysis using the murine B-myb probe revealed that all infected cell lines expressed high levels of B-myb mRNA transcribed from the retroviral vector and that endogenous levels of B-myb were comparable in all cell lines including parental M1 cells (Fig. 1B). The ectopic expression of B-myb did not affect the doubling time and the morphology of the untreated parental M1 cell line (data not shown), but TGF-β1 treatment resulted in a much more rapid loss of viability in all B-myb-expressing clones comparing to the parental M1 cell line and the cell line infected with the vector LXSXN (Fig. 2A). The acceleration of cell death in B-myb clones was confirmed by the appearance of DNA fragmentation into specific nucleosomal fragments characteristic of apoptosis. Apoptotic DNA ladder was detected in B-myb-expressing clones after 24 h, whereas in M1 and M1/LXSXN cells it was not detected until after 36 h (Fig. 2B).

Considering the previous results of Selvakumaran *et al.* (7) that

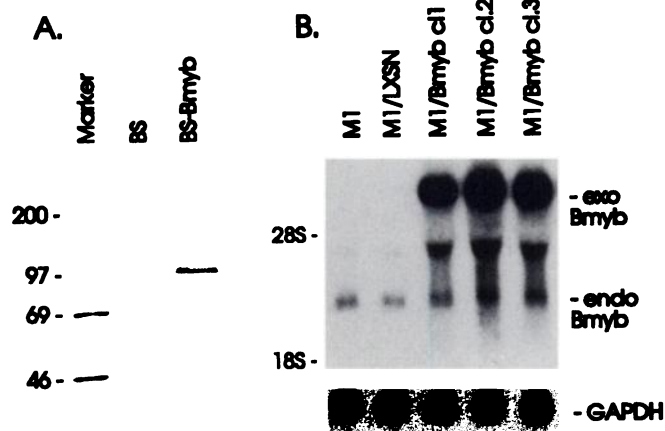


Fig. 1. A, *In vitro* translation product of B-myb cDNA which was subcloned into pBluescriptII SK(+) under the control of T3 promoter (BS-Bmyb), pBluescriptII SK(+) without insert (BS) was used as a negative control. Ordinate, size of protein markers in kilodaltons. B, Northern blot analysis of total RNAs from parental M1 cells and cell lines infected with retroviruses carrying LXSXN or LXSXN expressing B-myb gene. Ten μg of total RNA from each cell line were analyzed by Northern blot hybridization using the probes for murine B-myb and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for integrity and loading consistency.

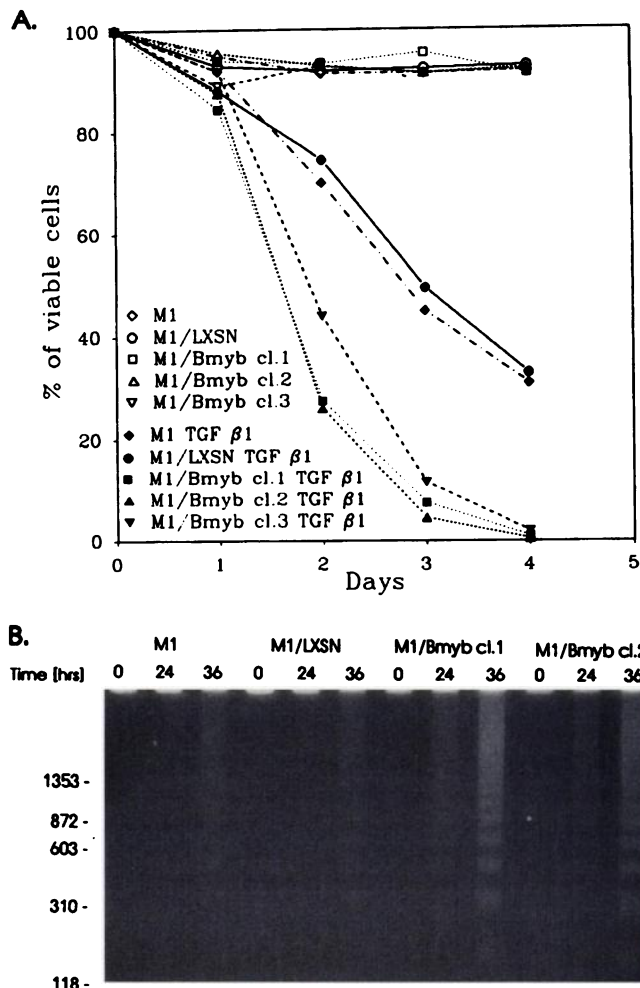


Fig. 2. Effect of deregulated expression of B-myb on TGF-β1-induced loss of viability and apoptosis. A, percentage of viable cells at the indicated times following TGF-β1 treatment of M1, M1/LXSXN, and M1/B-myb clones. The cells seeded at 1.5×10^5 /ml with TGF-β1 (10 ng/ml) were harvested and viability was assessed at the indicated time points by trypan blue dye exclusion. Points, mean from three independent experiments with SDs up to 7% of each value. In B, the cells treated with TGF-β1 for indicated time were harvested for DNA extraction and analyzed as described in "Materials and Methods." Ordinate, migration of the size markers in base pairs.

showed that both *c-myb* and *c-myc* could positively modulate apoptosis, we wanted to see if the acceleration of programmed cell death by B-myb was a consequence of direct or indirect effects on regulation *c-myb* or *c-myc* expression. As shown in Fig. 3, the kinetics of down-regulation of both *c-myb* and *c-myc* mRNAs in B-myb expressing clones was similar to that observed in the parental M1 cell line. It was interesting that although *c-myb* and B-myb accelerate TGF-β1-induced apoptosis similarly, the kinetics of mRNA expression of the two related endogenous genes differs in the cytokine-treated M1 cells; although the level of *c-myb* mRNA is suppressed dramatically after 1 h in TGF-β1, the level of endogenous B-myb mRNA is down-regulated gradually and later (Fig. 3).

Since TGF-β1 exerts its biological effects on cell growth through its cell surface receptors, type I (TGR-I) and type II (TGR-II) (18, 19), we wished to determine if the increased sensitivity of M1/B-myb clones to the TGF-β1 treatment was due to increased expression of mRNA encoding one of the receptors, type I (TGR-I). As shown in the Northern blot in Fig. 4A, however, we did not observe any differences in the expression of message for TGR-I in the cells ectopically expressing B-myb compared to the parental cell line M1 or M1/LXSXN.

Since recent studies demonstrated that *bcl-2* and *bax* genes are the

major players in the control of programmed cell death (16), we wanted to determine how the regulation of these genes is affected by deregulated expression of B-myb in M1 cells. For this purpose we analyzed (by Northern blot hybridization) total RNAs isolated from control M1/LXSN cells as well as cell lines overexpressing B-myb treated with TGF-β1 for indicated times. As shown in Fig. 4B, the regulation of transcription of both genes *bcl-2* and *bax* was similar in both cell lines.

p53 is also an important central molecule in the regulation of programmed cell death as well as the cell cycle but is not expressed in parental M1 cells (20). To resolve the question of whether deregulated expression of B-myb in M1 cells may relieve the transcriptional block imposed on p53 transcription, we analyzed total RNAs isolated from parental M1 cells and from M1/B-myb cells treated for different times with TGF-β1. However, we did not detect p53 mRNA by Northern blot hybridization in any of the cultures suggesting that the pathway leading to programmed cell death in this case does not involve p53 (data not shown).

The present study demonstrates for the first time the positive role of B-myb gene in the induction of apoptosis, and it provides another example whereby a gene involved in cell proliferation can trigger the alternative apoptotic pathway of cell self-destruction in the presence of growth arrest signals. The most likely mechanism by which B-myb may accelerate the programmed cell death would be through the positive or negative regulation of the transcription of genes directly involved in the apoptotic process. This is supported by studies of B-myb from different species that demonstrated its DNA-binding capacity and nuclear localization of protein product (21–23). In an attempt to explain the increased sensitivity of M1 cells overexpressing murine B-myb gene we analyzed the expression of genes such as *c-myc*, *c-myb*, *bcl-2*, *bax*, and *p53* genes, the protein products of which can modulate apoptotic cell death because they could be potential cellular targets for B-myb. Our results showed that none of the studied genes is involved in B-myb-accelerated programmed cell death. Perhaps future experiments aimed at identifying new physiological targets for B-myb will clarify the exact mechanism through which B-myb can modulate the apoptotic process.

Acknowledgments

We thank B. Hoffman for providing murine myeloid cell line M1 and V. Nazarov for critical review of the manuscript. We are also grateful to

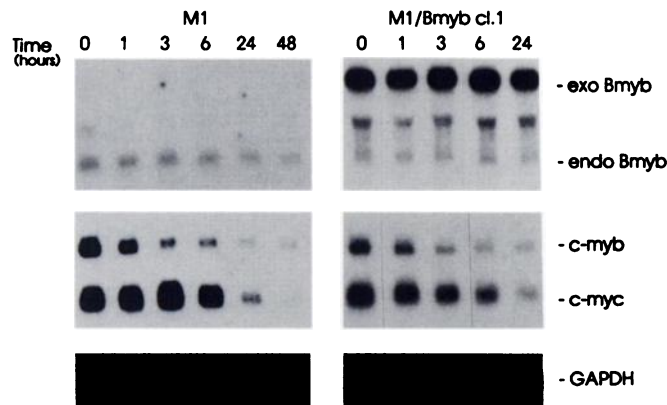


Fig. 3. Northern blot analysis of expression of B-myb, c-myb, and c-myc in parental M1 and M1/B-myb cl.1 cells during culturing in TGF-β1. Total RNAs were isolated from the cells, harvested at the indicated time, and analyzed by Northern blot hybridization with indicated probes. Data from cells overexpressing B-myb at the 48-h time point are not shown because rapid loss of viability caused massive degradation of RNA. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to verify the equivalency in loading and RNA integrity.

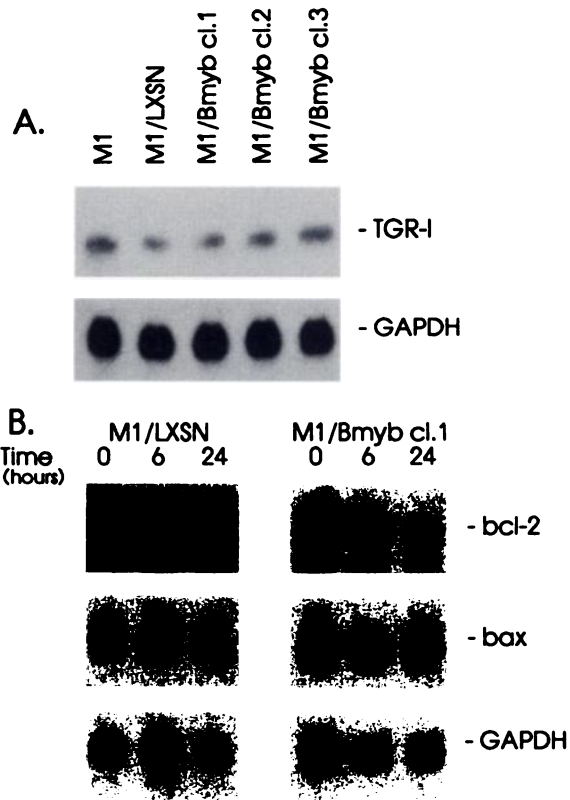


Fig. 4. A, levels of expression of receptor type I for TGF-β1 mRNA in M1 and M1/LXSN cells and M1 clones expressing deregulated B-myb. B, regulation of expression *bcl-2* and *bax* mRNAs in M1/LXSN and M1/B-myb cl.1 cells during TGF-β1 treatment. The total RNAs isolated from the cells cultured in the presence of TGF-β1 for the indicated time were analyzed by Northern blot hybridization. Autoradiograms are all from the same blot, which was stripped and hybridized with different probes. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

R. Watson for providing the murine B-myb cDNA and T. Bender for providing c-myc cDNA.

References

- Lam, E. W-F., Robinson, C., and Watson, R. J. Characterization and cell cycle-regulated expression of mouse B-myb. *Oncogene*, 7: 1885–1890, 1992.
- Nomura, N., Takahashi, M., Matsui, M., Ishii, S., Date, T., Sasamoto, S., and Ishizaki, R. Isolation of human cDNA clones of myb-related genes, A-myb, B-myb. *Nucleic Acids Res.*, 16: 11075–11089, 1988.
- Golay, J., Capucci, A., Arsur, M., Castellano, M., Rizzo, V., and Introna, M. Expression of c-myb and B-myb, but not A-myb, correlates with proliferation in human hematopoietic cells. *Blood*, 77: 149–158, 1991.
- Lam, E. W-F., and Watson, R. J. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *EMBO J.*, 7: 2705–2713, 1993.
- Sala, A., and Calabretta, B. Regulation of BALB/c 3T3 fibroblast proliferation by B-myb is accompanied by selective activation of *cdc2* and cyclin D1 expression. *Proc. Natl. Acad. Sci. USA*, 89: 10415–10419, 1992.
- Arsura, M., Introna, M., Passerini, F., Mantovani, A., and Golay, J. B-myb antisense oligonucleotides inhibit proliferation of human hematopoietic cell lines. *Blood*, 79: 2708–2716, 1992.
- Selvakumar, M., Lin, H-K., Sjin, R. T., Reed, J. C., Lieberman, D. A., and Hoffman, B. The novel primary response gene *MyD118* and the proto-oncogenes *myb*, *myc*, and *bcl-2* modulate transforming growth factor β1-induced apoptosis of myeloid leukemia cells. *Mol. Cell. Biol.*, 14: 2352–2360, 1994.
- Lotem, J., and Sachs, L. Selective regulation of the activity of different regulatory proteins by transforming growth factor-β1 in normal and leukemic myeloid cells. *Blood*, 76: 1315–1321, 1990.
- Markowitz, D., Goff, S., and Bank, A. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology*, 167: 400–406, 1988.
- Markowitz, D., Goff, S., and Bank, A. A safe packaging cell line for gene transfer: separating viral genes on two different plasmids. *J. Virol.*, 62: 1120–1124, 1988.
- Miller, A. D., and Rosman, G. J. Improved retroviral vectors for gene transfer and expression. *Biotechniques*, 9: 980–986, 1989.
- Bender, T. P., and Kuehl, M. W. Murine myb protooncogene mRNA: cDNA sequence and evidence for 5' heterogeneity. *Proc. Natl. Acad. Sci. USA*, 83: 3204–3208, 1986.

13. Stanton, L. W., Watt, R., and Marcu, K. B. Translocation, breakage and truncated transcripts of *c-myc* oncogene in murine plasmacytomas. *Nature (Lond.)*, 303: 401–406, 1983.
14. Tomoda, T., Kudoh, T., Noma, T., Nakazawa, A., Muramatsu, M. A., and Arai, K. I. Molecular cloning of mouse counterpart for human TGF- β type I receptor. *Biochem. Biophys. Res. Commun.*, 198: 1054–1062, 1994.
15. Givol, D. B-CLL, follicular lymphomas, hairy cell leukemia. In: F. Melchers and M. Potter (eds.), *Mechanisms of B-cell Neoplasia*, pp. 257–261. Basel, Switzerland: F. Hoffman-La Roche & Co. Limited Company, 1987.
16. Oltvai, Z. N., Millman, C. L., and Korsmeyer, S. J. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, 74: 609–619, 1993.
17. Fort, Ph., Piechaczyk, M., Sabrouy, S., Dani, Ch., Jeanteur, Ph., and Blanchard, J. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.*, 13: 1431–1437, 1985.
18. Sporn, M. B., and Roberts, A. B. Transforming growth factor- β : recent progress and new challenges. *J. Cell. Biol.*, 119: 1017–1021, 1992.
19. Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Andres, J. L., Laiho, M., Wang, X-F., and Massague, J. TGF β signals through a heteromeric protein kinase receptor complex. *Cell*, 71: 1003–1014, 1992.
20. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature (Lond.)*, 352: 345–347, 1991.
21. Nakagoshi, H., Takemoto, Y., and Ishii, S. Functional domains of the human B-*myb* gene product. *J. Biol. Chem.*, 268: 14161–14167, 1993.
22. Watson, R. J., Robinson, C., and Lam, E. W-F. Transcriptional regulation by murine B-*myb* is distinct from that by *c-myb*. *Nucleic Acids Res.*, 21: 267–272, 1993.
23. Foos, G., Grimm, S., and Klempnauer, K-H. Functional antagonism between members of the *myb* family: B-*myb* inhibits v-*myb*-induced gene activation. *EMBO J.*, 11: 4619–4629, 1992.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Acceleration of Apoptosis in Transforming Growth Factor β 1-treated M1 Cells Ectopically Expressing B-*myb*

Juraj Bies and Linda Wolff

Cancer Res 1995;55:501-504.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/55/3/501>

- E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.
- Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
- Permissions** To request permission to re-use all or part of this article, use this link <http://cancerres.aacrjournals.org/content/55/3/501>. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.