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Direct Transactivation of the Anti-apoptotic Gene Apolipoprotein J (Clusterin) by B-MYB*

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Maria Cervellera‡, Giuseppe Raschella§, Giorgia Santilli‡, Barbara Tanno§¶, Andrea Ventura‡, Camillo Mancini§, Cinzia Sevignani‖, Bruno Calabretta‖, and Arturo Sala‡**

From the ‡Laboratory of Molecular Pharmacology and Pathology, Consorzio Mario Negri Sud, 66030 S. Maria Imbaro, Italy, the §Section of Toxicology and Biomedical Sciences, ENEA 00060 Rome, Italy, and the ‖Department of Microbiology/Immunology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

B-MYB is a ubiquitously expressed transcription factor involved in the regulation of cell survival, proliferation, and differentiation. In an attempt to isolate B-MYB-regulated genes that may explain the role of B-MYB in cellular processes, representational difference analysis was performed in neuroblastoma cell lines with different levels of B-*MYB* expression. One of the genes, the mRNA levels of which were enhanced in B-*MYB* expressing cells, was ApoJ/Clusterin$^{SGP-2/TRMP-2}$ (ApoJ/Clusterin), previously implicated in regulation of apoptosis and tumor progression. Here we show that the human ApoJ/Clusterin gene contains a Myb binding site in its 5′ flanking region, which interacts with bacterially synthesized B-MYB protein and mediates B-MYB-dependent transactivation of the ApoJ/Clusterin promoter in transient transfection assays. Endogenous ApoJ/Clusterin expression is induced in mammalian cell lines following transient transfection of a B-*MYB* cDNA. Blockage of secreted clusterin by a monoclonal antibody results in increased apoptosis of neuroblastoma cells exposed to the chemotherapeutic drug doxorubicin. Thus, activation of ApoJ/Clusterin by B-MYB may be an important step in the regulation of apoptosis in normal and diseased cells.

MYB family members (A-Myb, B-Myb, c-Myb, and v-Myb) are nuclear transcription factors that recognize the Myb consensus sequence (C/T)AACNG in viral and cellular promoters (1). Whereas c-MYB and A-MYB play important roles in many cellular processes and in development in a tissue specific manner, B-MYB is thought to have a general role in cellular proliferation and differentiation (2, 3). However, whereas c-MYB and A-MYB transactivate promoters containing the Myb consensus sequence in different cell systems, the activity of B-MYB appears to be regulated by cell-type specific factors (4). Endogenous B-MYB protein is phosphorylated at the onset of DNA synthesis by cyclin A/cyclin-dependent kinase 2 kinase (5). Consistent with this finding, several laboratories have demonstrated that B-MYB transactivating potential correlates with phosphorylation induced by the cyclin A/cyclin-dependent kinase 2 kinase complex (6–9). Accordingly, B-MYB function parallels endogenous levels of cyclin A in different cell types (8, 10). B-*MYB* expression has been detected in all cell lines analyzed so far and is regulated at the G_1/S border of the cell cycle (11–13). *In vivo*, B-*MYB* expression is detected only in proliferating tissues of the developing mouse (14), consistent with studies showing that transcription of the B-*MYB* gene is growth-regulated through an E2F site located in its promoter (15). The retinoblastoma family members p107 and p130, which are associated with E2F4, are involved in B-*MYB* promoter silencing during the G_0/G_1 phase of the cell cycle (16, 17). De-repression of the E2F site leading to B-*MYB* gene transcription at the G_1/S border of the cell cycle is linked to the disappearance of an Rb/E2F complex from the B-*MYB* promoter (17). These results are consistent with a model in which regulation of B-*MYB* expression is achieved through repression of transcription during the G_0/G_1 phase of the cell cycle, coupled to cyclin-dependent phosphorylation of B-MYB protein during S phase. This concerted regulation suggests that B-MYB activity is restricted to the phase of DNA synthesis, in accord with experiments showing that ectopic expression of a B-*MYB* transgene in T98G glioblastoma cells results in S phase accumulation of cells, but not in a shortened G_1 phase (18). Ablation of endogenous levels of B-MYB protein by means of the antisense techniques causes block of cellular proliferation and/or survival of normal and transformed cell lines, consistent with a function in cell cycle progression (19–22).

Overall, the existing experimental evidence suggests that B-*MYB* is a gene required for cell cycle progression that may be involved in tumorigenesis. In this regard, serial analysis of gene expression in non-small cell lung cancer biopsies has shown that B-*MYB* is overexpressed in primary tumors, as compared with normal tissue (23). Exogenous B-*MYB* expression blocks differentiation and prevents the arrest of cell proliferation imposed by differentiative agents in the M1 and LAN-5 murine myeloid and human neuroblastoma cell lines, respectively (22, 24). This may be particularly important in the light of recent data showing that expression of B-*MYB*, but not of A-MYB or c-MYB, in tumor biopsies of children affected by neuroblastoma correlates with poor survival (25). Like other factors promoting cell cycle progression, B-MYB is subjected to negative control by growth suppressors. p107, a member of the retinoblastoma family, inhibits B-MYB transactivating activity and p107/B-MYB complexes are co-immunoprecipitated from human leukemic cell lines (26). Also, B-MYB can rescue the G_1 arrest imposed by the tumor suppressor gene p53 (21).

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** To whom correspondence should be addressed. Tel.: 39-0872-570341; Fax: 39-0872-578240; E-mail: asala@cmns.mnegri.it.

Because B-MYB is a transcriptional activator, we reasoned that its function in normal and transformed cells might be mediated by downstream effector genes. Myb target genes, such as *MIM*-1 and *TOM*-1, are involved in hematopoietic cell differentiation (27, 28) but are not likely to be the mediators of B-MYB effects in nonhematopoietic cells. To identify genes that may be directly transactivated by B-MYB, we used a LAN-5 cell line stably transfected with B-*MYB* (LAN-5 B-*MYB*), previously shown to be refractory to the differentiation-inducing effects of retinoic acid (22). Representational difference analysis (RDA)[1] of the LAN-5 B-*MYB* cell line revealed that several genes were differentially expressed in comparison to the parental cell line. We focused our interest on the ApoJ/Clusterin gene, which encodes a sulfated glycoprotein initially thought to be associated with apoptotic cell death but later shown to be expressed by apoptosis-resistant cells (reviewed in Ref. 29) and to be required for survival of cells treated with tumor necrosis factor α or subjected to oxidative stress (30–33). Expression of ApoJ/Clusterin is also activated during carcinogenesis induced by *N*-nitroso-*N*-methylurea and dysplastic growth of rat prostate or during progression of prostate cancer in humans (34–36). The results presented in this study provide evidence that the ApoJ/Clusterin gene is a direct downstream target of the transcription factor B-MYB and that its expression is required for resistance to apoptotic cell death induced by the chemotherapeutic drug doxorubicin.

MATERIALS AND METHODS

Cell Lines and Cell Death Assay—The neuroblastoma cell line LAN-5 and the B-*MYB*-transfected LAN-5 clone (LAN-5-B-*MYB*) (22) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. The SAOS2 and COS-7 cell lines were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium containing 15 and 10% fetal calf serum, respectively. For cell death assay, LAN-5 or LAN-B-*MYB* cells were cultured with or without 0.5 μg/ml of doxorubicin in 96-well plates in the presence of 40 μg/ml ApoJ monoclonal antibody (Upstate Biotechnology, Inc.) or control antibody (c-myb monoclonal IgG; Upstate Biotechnology, Inc.). After 24 h of culture, cell death was assessed by trypan blue dye exclusion assay.

Plasmids—The pGL-ApoJ/Clusterin promoter plasmid was obtained as follows: a fragment of the human ApoJ/Clusterin promoter from nucleotide −4 to nucleotide −300, starting from the origin of transcription, was obtained by PCR. Restriction sites for *Xho*I and *Hin*dIII were introduced in the 5′ and 3′ primers, respectively, to facilitate cloning. After digestion with *Xho*I and *Hin*dIII, the PCR fragment was ligated to the PGL-basic vector (Promega) that had been digested with *Xho*I and *Hin*dIII. The pGL-ApoJ/Clusterin(mutant) promoter plasmid was obtained by introducing a 3-nucleotide mutation in the putative Myb sequence in the forward primer (wild-type, GGGCTAACCG; mutant, GGGTTTTCCG). Correct orientation and sequence of the ApoJ/Clusterin promoter plasmids were confirmed by sequencing. CMV-B-MYB is described in Ref. 21. To obtain a construct producing a GST-B-MYB fusion protein, an *Eco*RI-*Bam*HI fragment containing the B-*MYB* DNA-binding domain was released from the SK-B-MYB vector (20) and ligated to the *Eco*RI/*Bam*HI-digested pGEX2T plasmid (Amersham Pharmacia Biotech).

RDA—For the analysis, we followed an improved RDA procedure (a detailed protocol was kindly provided by Michael O'Neill, Royal Children's Hospital, Melbourne, Victoria, Australia), which introduces a high performance liquid chromatography purification step of primers/adaptors and requires reduced amounts of starting RNA (37). This method allows the obtainment of well isolated bands in difference product 2 (DP2), so that a third round of subtraction/amplification is generally not required. In brief, 10 μg of total cellular RNA was extracted from LAN-5 or LAN-5 B-*MYB* cells with Trizol reagent (Life Technologies, Inc.) following the manufacturer's instructions. Poly(A)$^+$ mRNA was obtained by flow of total RNA through an oligo-dT push-column (Stratagene). 200 ng of poly(A)$^+$ RNAs was transcribed into cDNA with the aid of a cDNA-construction kit, following the manufacturer's instructions (Roche Molecular Biochemicals). 300 ng of TESTER amplicon (LAN-5 B-*MYB*) and 30 μg of DRIVER amplicon (LAN-5) were subjected to one round of hybridization/PCR to obtain DP1. A 1:400 ratio of TESTER/DRIVER amplicons (63 ng/25 μg) was used in the second hybridization/PCR step to obtain DP2. DP2 products digested with *Dpn*II were separated by agarose gel electrophoresis, eluted from the gel, subcloned into the *Bam*HI-linearized pBluescript plasmid, and sequenced.

Production of GST Fusion Proteins and Gel Shift Assays—GST-B-MYB protein was produced *in vitro* from pGEXB-MYB-transformed bacterial cultures grown for 2 h in LB medium containing 0.1 mM IPTG. GST or GST-B-MYB protein was mixed to the glutathione-Sepharose resin and washed with phosphate-buffered saline, and the purified proteins were eluted from the resin with free glutathione.

Double-stranded oligonucleotides from the MIM-1 (TCGACACATTATAACGGTTTTTTAGC) or the ApoJ/Clusterin (AGACAGCCGGGCTAACCGCGTGAGAGG) promoters and including the underlined Myb consensus sequence were labeled with polynucleotide kinase and [γ-^{32}P]ATP and used as probes for gel shift experiments. In brief, 300 ng of fusion proteins was mixed at room temperature for 20 min with the different probes in a binding buffer containing 10 mM Tris-HCl, 80 mM KCl, 1 mM dithiothreitol, 6% glycerol, and 3 μg of poly(dI-dC). At the end of the incubation period, the samples were loaded onto a 4% polyacrylamide gel containing 0.5× TBE buffer and run in 0.5× TBE for 2 h at 200 mV.

Reverse Transcription (RT)-PCR—Total RNA (500 ng) from LAN-5 and LAN-5-B-myb cell lines was reverse transcribed for 45 min at 40 °C in the presence of 2 μM random hexamers (Amersham Pharmacia Biotech), 0.8 mM dNTPs in 1× reverse transcriptase buffer (Amersham Pharmacia Biotech), and 200 units of Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech) in a total volume of 50 μl. PCR mixtures were prepared using 5 μl of the reverse transcriptase mixture in 10 mM Tris HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.4 μM upstream primer, 0.4 μM downstream primer, 0.2 mM dNTPs and 2 units of Ex *Taq* polymerase (TaKaRa Shuzo Co., Ltd., Otsu, Shiga, Japan) in a total volume of 50 μl. PCR conditions were as follows: 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C for 20, 25, 30, or 35 cycles to set the appropriate conditions for linearity. 30 PCR cycles were then used for detection of ApoJ-1 transcripts, whereas 25 cycles were utilized to detect β-actin. A final extension step of 7 min at 72 °C was carried out in each case. 15 μl of each PCR product was run on a 1% agarose gel in 1× TBE and transferred onto a nylon membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The filter was hybridized to a ApoJ-1-specific or to a β-actin-specific 5′ end-labeled oligonucleotide probe (specific activity, 5 × 10^8 cpm/μg; concentration in the hybridization mixture, 1 × 10^6 cpm/ml), representing an internal portion of the amplified product at 45 °C in 5× SSC, 100 μg/ml sonicated salmon sperm DNA for 16 h. Washings were carried out in 2× SSC, 0.1% SDS for 15 min at room temperature and for 30 min in the same buffer at 50 °C. The filter was exposed to an x-ray film (Eastman Kodak Co.) in the presence of an intensifier screen for 3 h at −80 °C. Primers and probes were as follows. ApoJ-1: upstream primer, 5′GTGCAATGAGACCATGATGG3′; downstream primer, 5′CAGGTAGTGGTAGGTATCCT3′; probe, 5′AGCATCATAGACGAGCTCTT3′. β-Actin: upstream primer, 5′TCATCACCATTGGCAATGAG3′; downstream primer, 5′CACTGTGTTGGCGTACAGGT3′; probe, 5′ATGGAGTCCTGTGGCATCCACGAA3′.

Transient Transfections and Luciferase Assay—SAOS2 or LAN-5 cells were transfected according to the calcium phosphate precipitation method. 1 μg of each plasmid was used in 30-mm wells; after 36 h, the cells were harvested, and luciferase activity was monitored with a luciferase assay kit following the manufacturer's instructions (Promega). Because B-MYB can significantly modulate the activity of viral promoters driving β-galactosidase plasmids,[2] rendering control of transfection efficiency difficult, the assays were performed in triplicate, and each experiment was repeated at least three times with different plasmid preparations. Light emission was evaluated with the aid of a scintillation counter or luminometer and expressed as cpm.

RESULTS

RDA of Gene Expression in Neuroblastoma Cells Overexpressing B-MYB—The neuroblastoma cell line stably expressing B-*MYB* (LAN-5 B-*MYB*) was subjected to RDA to identify

[1] The abbreviations used are: RDA, representational difference analysis; CMV, cytomegalovirus; DP, difference product; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; RT, reverse transcription.

[2] M. Cervellera, G. Raschella, G. Santilli, B. Tanno, A. Ventura, C. Mancini, C. Sevignani, B. Calabretta, and A. Sala, unpublished observations.

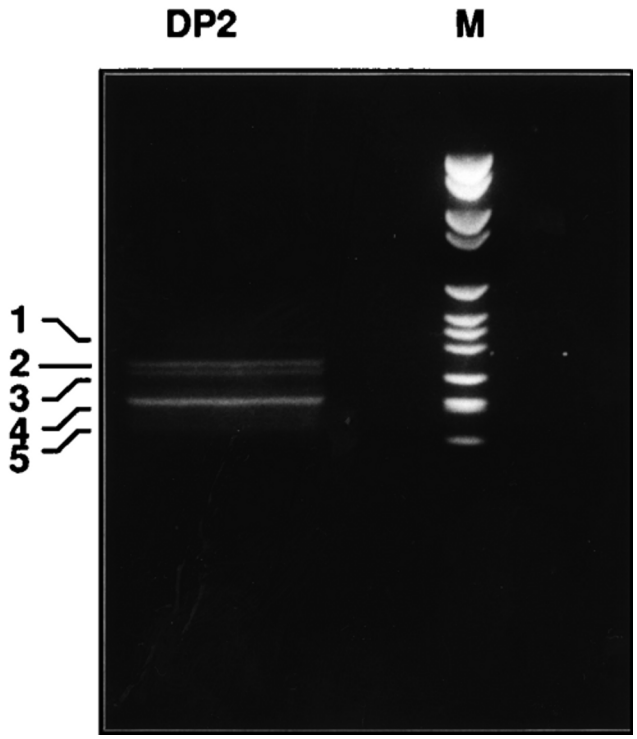


FIG. 1. **Representational difference analysis of gene expression between parental and LAN-5** B-*MYB* **cells.** DP2 was run on a 2% agarose gel and stained with ethidium bromide. Visible bands (*bands 1–5*) were excised from the gel and subcloned into the pBluescript plasmid. *M,* DNA marker.

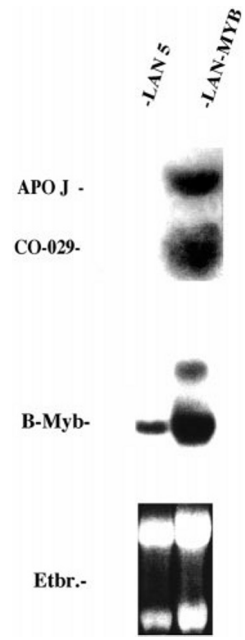


FIG. 2. **ApoJ/Clusterin and CO-029 expression in LAN-5** B-*MYB* **cells.** 15 μg of total RNA from parental or B-*MYB*-expressing cells was loaded onto a 5% agarose gel, transferred to nitrocellulose, and hybridized with the ApoJ/Clusterin and CO-029 (*top panel*) or the B-*MYB* (*middle panel*) probes. Ethidium bromide (*Etbr*) staining of RNA (*bottom panel*) confirmed equal loading of the lanes.

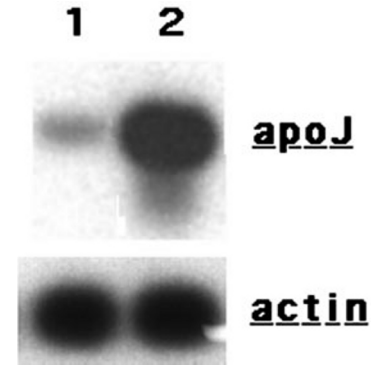


FIG. 3. **RT-PCR phenotyping of LAN-5 and LAN-5** B-*MYB* **cells.** Expression of ApoJ/Clusterin was analyzed in parental (*lane 1*) or in B-*MYB*-transfected (*lane 2*) LAN-5 cells. RT-PCR and southern hybridization were performed as described under "Materials and Methods." β-Actin levels were monitored to normalize the RT-PCR reaction.

genes preferentially expressed, compared with the parental cell line. After two rounds of hybridization/PCR, the DP2 products, consisting of five distinct bands (Fig. 1), were subcloned into the pBluescript (SK+) and sequenced. The cDNA clones from band 1 corresponded to a ribosomal protein and collagen type III; the clones from bands 2 and 3 were identified as the sulfated glycoprotein Apolipoprotein J/Clusterin; those from band 4 contained tropomyosin III; and those from band 5 contained fibrillin and the tumor-associated antigen CO-029.

As initial validation of the RDA procedure, Southern blot hybridization of the Driver (LAN-5) and Tester (LAN-5 B-*MYB*) amplicons with ^{32}P-labeled probes representative of the various RDA fragments, revealed selective hybridization with the TESTER amplicon, suggesting that the DP2 products were preferentially expressed in the LAN-5 B-*MYB* cDNA population (not shown).

Apolipoprotein J/Clusterin Is Overexpressed in LAN-5 B-MYB Neuroblastoma Cells—To further confirm the result obtained by RDA, ApoJ/Clusterin expression was assessed by Northern blot in total cellular RNA from LAN-5 and LAN-5 B-*MYB* cells. Compared with parental cells, B-*MYB*-expressing cells showed markedly increased levels of ApoJ/Clusterin and of the tumor-associated antigen CO-029 (Fig. 2). This result was confirmed by a semiquantitative RT-PCR analysis with ApoJ/Clusterin and CO-029-specific primers (Fig. 3). To exclude the possibility that overexpression of ApoJ/Clusterin in the LAN-5 B-*MYB* cell line was the consequence of clonal selection, SAOS2 and COS-7, two highly transfectable cell lines, were transiently transfected with the CMV-B-*MYB* expression plasmid; 36 h later, they were assessed for ApoJ/Clusterin mRNA levels. Northern blot analysis confirmed that cells transfected with the B-*MYB* cDNA, but not mock-transfected cells, overexpressed the ApoJ/Clusterin mRNA (Fig. 4). As a control of RNA loading, expression of β-actin remained unchanged among the different transfectants (Fig. 4).

B-MYB Binds to the 5′-Flanking Sequence of the ApoJ/Clusterin Gene—Inspection of the 5′ flanking region of the human ApoJ/Clusterin gene revealed a putative Myb site, not described previously, starting at nucleotide –292 of the 5′ flanking sequence (38). Notably, the Myb site is conserved in the rat promoter, although in the opposite orientation, in a region that presents no homology between the human and rat genes, suggesting that it may be important for ApoJ/Clusterin gene regulation in different species (Fig. 5). Gel shift experiments were carried out to assess whether B-MYB interacts with this putative Myb binding site. A ^{32}P-labeled double-stranded oligonucleotide probe containing the putative Myb binding site of the human ApoJ/Clusterin promoter bound to GST/B-*MYB*, but not to GST alone (Fig. 6, *lanes 1* and *2*). Competition with an excess of wild-type unlabeled probe abolished the binding, whereas the interaction was not abolished in the presence of an oligonucleotide carrying a mutated Myb binding site (Fig. 6, *lanes 3* and *4*).

B-MYB Transactivates the ApoJ/Clusterin Promoter through a B-MYB Binding Site—To further demonstrate that the ApoJ/Clusterin promoter contains a functional Myb binding site involved in B-MYB-dependent promoter regulation, an ApoJ/Clusterin promoter plasmid containing a wild-type or a mutated Myb binding site was co-transfected with the empty vector or with CMV-B-MYB in LAN-5 or SAOS2 cells. B-MYB significantly activated the wild-type plasmid in both cell lines, whereas transactivation of the mutant plasmid was markedly diminished, especially in SAOS2 cells (Fig. 7, *A* and *B*). Interestingly, the basal activity of the ApoJ/Clusterin promoter carrying a mutated B-*MYB* binding site is lower than that of the wild-type promoter, suggesting that endogenous levels of MYB proteins are important for ApoJ/Clusterin promoter activity.

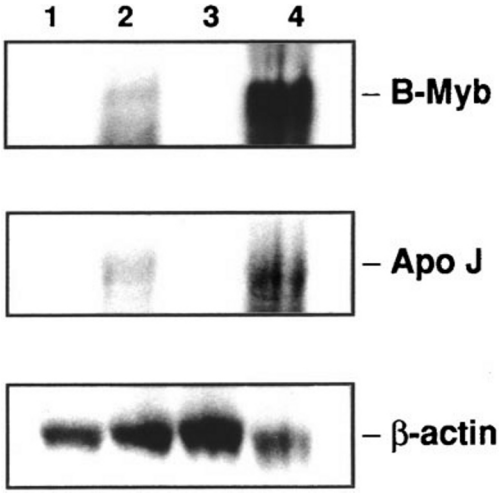


FIG. 4. **Enhanced expression of ApoJ/Clusterin mRNA in cells transiently transfects with** B-*MYB*. COS-7 (*lanes 1* and *2*) or SAOS2 (*lanes 3* and *4*) were transfected with CMV-B-MYB (*lanes 2* and *4*) or the CMV empty vector (*lanes 1* and *3*). After 36 h, cells were harvested and 20 μg of total RNA was hybridized to the indicated probes.

SAOS2 cells contain almost undetectable levels of endogenous cyclin A protein and are especially useful to study the cooperative effects of B-*MYB* expression and cyclin-dependent kinase 2/cyclin A kinase activity (8). Accordingly, co-expression of B-*MYB* and cyclin A in SAOS2 cells resulted in a dramatic increase in promoter transactivation, suggesting that augmented expression of cyclins also may contribute to abnormal expression of ApoJ/Clusterin (Fig. 7*C*).

ApoJ/Clusterin Blockage Accelerates Cell Death Induced by the Chemotherapeutic Drug Doxorubicin—Neuroblastoma tumors expressing high levels of B-*MYB* and ApoJ/Clusterin might exhibit growth advantage through increased resistance to stress or apoptotic stimuli delivered by the host environment. To test this hypothesis, LAN-5 or LAN-5 B-*MYB* cells were exposed to the chemotherapeutic drug doxorubicin, which causes apoptotic death of neuroblastoma cells (39), in cultures supplemented with an anti-ApoJ monoclonal antibody. B-*MYB*-overexpressing cells showed a significant resistance to cell death induced by doxorubicin, as compared with parental cells (14.5 ± 3 *versus* 32 ± 5% of cell death, respectively) (Fig. 8). Interestingly, the anti-ApoJ antibody, but not a IgG-matched control antibody, accelerated cell death induced by doxorubicin of both cell lines (Fig. 8). These results suggest that secreted ApoJ/Clusterin is important in protecting neuroblastoma cells from cell death induced by apoptotic agents, such as doxorubicin, and that B-*MYB* exerts its protective effects on neuroblastoma cells through activation of ApoJ/Clusterin expression.

DISCUSSION

In this report, we provide evidence that B-MYB, the ubiquitous member of the Myb family, positively regulates expression of the anti-apoptotic gene ApoJ/Clusterin, conferring on B-*MYB*-expressing cells resistance to death induced by the chemotherapeutic drug doxorubicin. A growing body of evidence implicates B-MYB in the control of cell proliferation and differentiation, and it is becoming apparent that B-*MYB* expression in tumor cells is associated with disease progression (26).

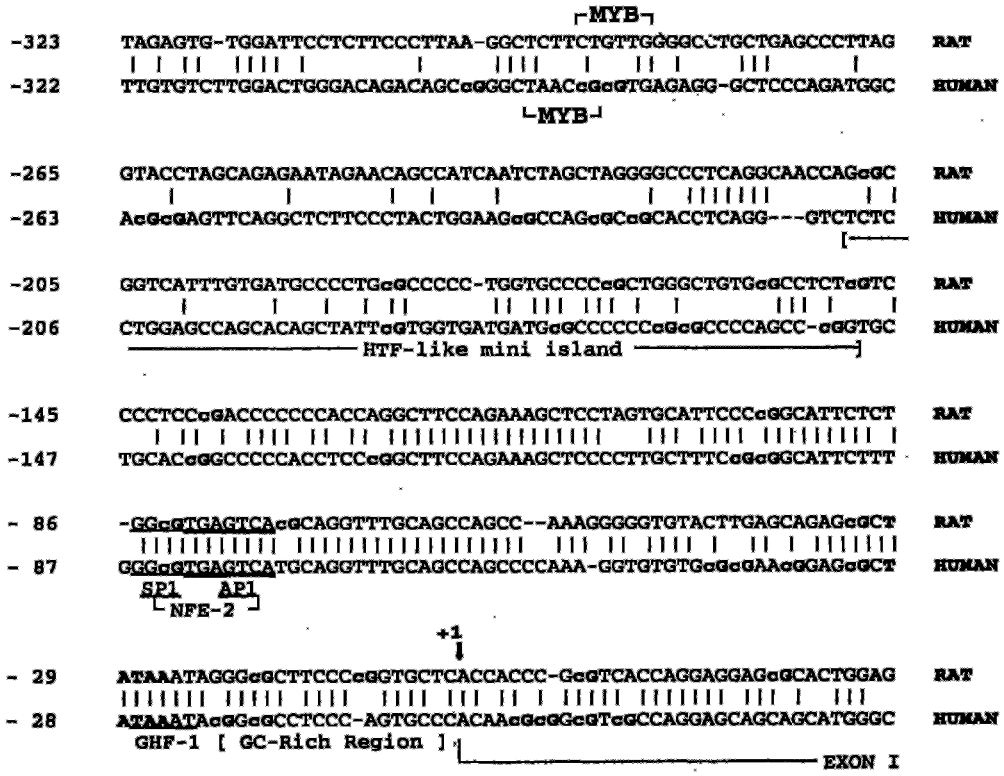


FIG. 5. **Promoter structure of the ApoJ/Clusterin gene.** Putative MYB binding sites in the human and rat promoters are indicated.

FIG. 6. **Interaction of B-MYB with the 5′ flanking region of the ApoJ/Clusterin gene.** Electrophoretic mobility shift assay of GST B-MYB with the ^{32}P-labeled ApoJ/Clusterin Myb binding site. The double-stranded oligonucleotide including the putative Myb binding site of the human ApoJ/Clusterin promoter was labeled with ^{32}P and mixed with the GST/B-MYB fusion protein or GST alone for a gel shift assay. Electrophoretic mobility shift assay was also performed with a 300-fold excess of an unlabeled wild-type oligonucleotide (*WT*) or an oligonucleotide carrying a 3-nucleotide substitution in the putative Myb binding site (*MUT*) to assess specificity.

An important step in the understanding of B-MYB function is to identify target genes that may be responsible for the phenotypic changes imposed by B-*MYB* expression. In a recent report (25), we noted a correlation between high levels of B-*MYB* expression in neuroblastoma and decreased patient survival. We hypothesized that several B-MYB target genes may be up-regulated in response to augmented levels of B-*MYB*, resulting in more aggressive tumors. To directly identify genes activated by B-MYB, we compared gene expression patterns of parental and B-*MYB*-expressing neuroblastoma LAN-5 cells (22). LAN-5 cells, similar to the majority of tumor biopsies from neuroblastoma patients, express average levels of B-*MYB* mRNA (Fig. 2). In contrast, B-*MYB* transfected LAN-5 cells express high levels of B-*MYB* mRNA (Fig. 2). Thus, comparing gene expression in these two cell lines may lead to the identification of B-MYB target genes involved in normal and pathological processes. By RDA, we identified several genes that were differentially expressed in B-*MYB*-transfected cells. Notably, at least two genes overexpressed in the LAN-5 B-*MYB* cell line are implicated in tumor progression and/or tumor survival. The CO-029 antigen is a tetraspanin molecule the expression of which is observed in human carcinoma but not in normal tissues (40). The rat homologue of human CO-029 imposes a metastatic phenotype to transfected cells, suggesting that this molecule is involved in tumor spreading (41). Apolipoprotein J/Clusterin is a ubiquitously expressed sulfated glycoprotein that appears to promote cell survival based on experiments in which its expression was ablated by antisense strategies or neutralizing antibodies (30, 32). Its expression seems to be directly regulated by B-MYB as indicated by the transactivation assays with the wild-type or the mutant ApoJ

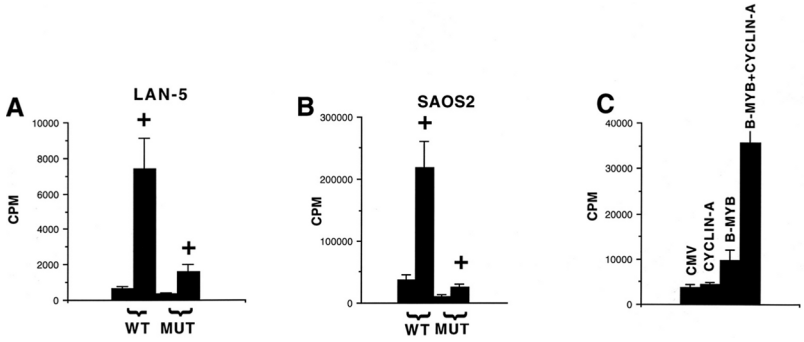
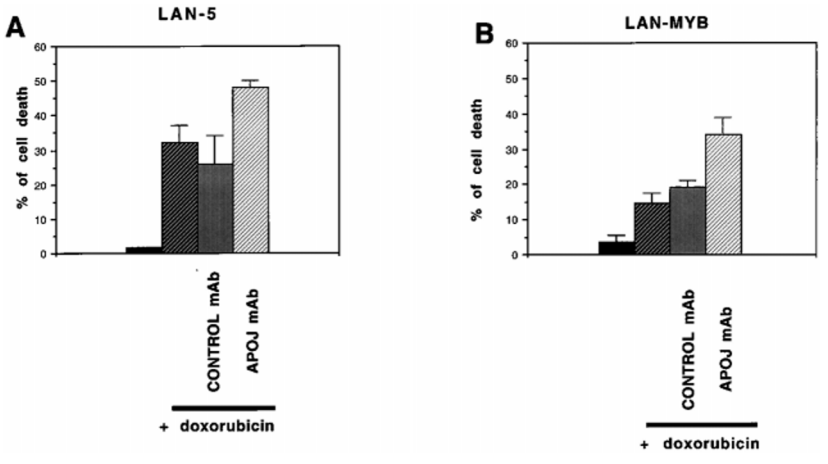


FIG. 7. **B-MYB-dependent transactivation of the ApoJ/Clusterin promoter cloned upstream of a luciferase reporter gene.** Transient transfections and luciferase assay were performed as described under "Materials and Methods." Four independent transfections, each performed in triplicate, gave similar results, and representative experiments are shown. Standard deviation is indicated by *error bars*. *A,* LAN-5 cells transfected with the wild-type (*WT*) or mutant (*MUT*) pGL-APOJ promoter plasmid with or without CMV-B-MYB (indicated by +). *B,* SAOS2 cells transfected with the pGL-ApoJ promoter (wild-type or mutant) with or without CMV-B-MYB (indicated by +). *C,* SAOS2 cells transfected with the WT pGL-ApoJ promoter plasmid with CMV-B-MYB alone or in combination with a cyclin A expression plasmid, as indicated.

FIG. 8. **Cell death induced by doxorubicin is accelerated in the presence of an ApoJ/Clusterin monoclonal antibody.** LAN-5 cells (*A*) or LAN-5 cells stably transfected with B-*MYB* (*B*) were cultured with 0.5 μg/ml of doxorubicin (indicated by the + sign) for 24 h in the presence of 40 μg/ml of anti-ApoJ/Clusterin or control monoclonal antibody. Cell viability was assessed by trypan blue exclusion dye. The *column* referring to untreated cells is indicated in the *Black* color. Each *column* shows the average of triple replicates, and the *error bars* denote S.D. A typical experiment (out of three independent assays) is shown.



promoter (Fig. 7). ApoJ/Clusterin expression appears to increase resistance of neuroblastoma cells to death induced by environmental factors or chemotherapeutic drugs, at least *in vitro* (Fig. 8). This hypothesis is consistent with data showing that ApoJ/Clusterin expression is associated with advanced stages of neuroblastoma (data not shown). The mechanisms by which ApoJ/Clusterin exerts its protective effects during cell injury are presently unknown. ApoJ/Clusterin synthesis and posttranslational modification is a complex phenomenon, involving glycosylation and proteolytic cleavage of the precursor protein in two chains (α and β) that associate through disulfide bridges to form the secreted heterodimeric protein (reviewed in Ref. 42). Given the amphipathic nature of the protein, it has been proposed that ApoJ/Clusterin acts as a detergent, binding to hydrophobic complexes and denatured proteins and aiding in their uptake and clearance (42). The unprocessed form of ApoJ/Clusterin can remain intracellular, and it has been shown to associate with the Ku70 DNA-protein kinase, a protein involved in DNA repair, resulting in impaired Ku70 binding to DNA ends (43). Thus, it is possible that increased ApoJ/Clusterin expression may affect tumor progression by increasing cell survival and accumulation of detrimental mutations (through Ku70 inactivation), which may be consistent with the establishment of a fully malignant phenotype.

Consistent with previous observations (8), we have shown that concomitant overexpression of cyclin A and B-*MYB* results in the synergistic activation of the ApoJ/Clusterin promoter (Fig. 7*C*). In light of this result, it will be important to evaluate the activity of cyclin/cyclin-dependent kinase in tumors. One could hypothesize that overexpression of B-*MYB* and cyclins may result in higher levels of ApoJ/Clusterin and increased survival of tumor cells. Our finding that ApoJ/Clusterin is a direct target of B-MYB further suggests that the spectrum of genes of which the expression is controlled by MYB proteins extends beyond those involved in cell proliferation and differentiation and includes genes associated with apoptosis resistance and cell survival, as previously reported (44–47).

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