THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 15, Issue of April 14, pp. 8540–8545, 1995
Printed in U.S.A.

Requirement of B-*myb* Function for Survival and Differentiative Potential of Human Neuroblastoma Cells*

(Received for publication, November 5, 1994, and in revised form, January 17, 1995)

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The B-*myb* gene belongs to a family of transcription factors that also includes A-*myb* and c-*myb*. B-*myb* is expressed in many cell types including human neuroblastoma cells. Here we demonstrate that B-*myb* expression is down-regulated during retinoic acid-induced neural and glial differentiation of neuroblastoma cells. This modulation is an early event, is maintained at late times of induction, and is in part regulated at the transcriptional level. Constitutive expression of B-*myb* prevents retinoic acid-induced neural differentiation as reflected by morphological features and the expression of (or lack of) biochemical markers associated with the undifferentiated phenotype. Furthermore, the expression of antisense B-*myb* transcripts does not allow the rescue of viable cells, suggesting an important role for B-*myb* in the survival of neuroblastoma cells. These results indicate that B-*myb* plays a functional role in the differentiative potential of neuroblastoma cells, raising the possibility that this gene is one of the nuclear regulators in the cascade of events leading to cellular differentiation.

Many transcription factors that normally play a physiological role during development or in adult life can become activated oncogenically upon overexpression or mutation. Several such genes have been characterized with respect to structure, function, and mechanism of action (1).

The Myb family of transcription factors includes A-*myb*, B-*myb*, and c-*myb* (2), which are each located on a different chromosome (3, 4). Each member of the Myb family is characterized by the presence of DNA-binding and transactivation domains. B-*myb*, located on the long arm of chromosome X (Xq13) (3), has a DNA-binding domain consisting of three tandemly repeated segments of 51–52 amino acids located near the NH_2 terminus (5). This region is highly homologous to the corresponding domains of A-*myb* and c-*myb* (2). Deletion of the second and third repeats completely abolishes the DNA binding-dependent transactivating ability of B-*myb* (5). A transcriptional activation domain containing a cluster of acidic amino acids and different from the homologous domain of c-*myb* (6) is located downstream of the DNA-binding domain of B-*myb* (5). Unlike c-*myb*, B-*myb* does not have a negative regulatory domain, and it does not show negative autoregulatory activity; however, like c-*myb*, it has multiple nuclear localization signals located near the COOH terminus (5).

c-*myb* is expressed at high levels in hematopoietic cells, where it plays a major role in the regulation of both normal and malignant hemopoiesis *in vitro* (7, 8) and *in vivo* (9). Several leukemic cell lines express c-*myb* at high levels that decline during terminal differentiation (10). In addition, constitutive expression of c-*myb* blocks Me_2SO-induced differentiation of Friend murine erythroleukemia cells (11). c-*myb* also appears to play an important role in the proliferation of arterial smooth cells *in vivo* (12). Detectable amounts of c-*myb* are also found in many tumors of different embryonic origin such as small cell lung carcinoma (13), colon carcinoma (14, 15), and neuroblastoma (16).

The tissue distribution of B-*myb* expression is more widespread than that of c-*myb* and is detectable in stomach, lung, colon, and thyroid carcinoma and neuroblastoma cell lines (2). Overexpression of B-*myb* reduces the growth factor requirements of Balb/c 3T3 fibroblasts (17) and bypasses, in part, the p53 growth-suppressive effects in human glioblastoma T98G cells (18). In addition, antisense B-*myb* oligonucleotides inhibit the proliferation of human leukemic cell lines (19). Although B-*myb* expression, like that of c-*myb*, correlates with proliferation (17, 20,), transcription regulation by B-*myb* appears to be distinct from that of c-*myb* (21, 22). Furthermore, c-*myb* and B-*myb* appear to recognize the same DNA sequence, but with different affinities (23). Although B-*myb* appears to be involved in cellular proliferation, recent work of Foos *et al.* (24) demonstrating that chicken B-*myb* inhibits c-*myb* transactivation of the *MIM*-1 gene promoter (25) raises the possibility that B-*myb* can act, at least in some instances, as a competitor of c-*myb*.

Despite the wealth of data on B-*myb* structure and B-*myb* function as relates to cell proliferation, no evidence exists for its possible involvement in differentiative processes. In this paper, we have investigated the expression and regulation of the B-*myb* gene during neural and glial differentiative pathways in a neuroblastoma differentiation model.

Neuroblastoma is a malignant childhood tumor with a very undifferentiated appearance and is thought to arise from embryonal neural crest tissue that may be arrested at an early stage of differentiation (26). The mechanisms contributing to the onset and progression of neuroblastomas are largely unknown, but nonrandom chromosomal changes suggest the involvement of genetic alterations (27). The N-*myc* oncogene is frequently expressed in advanced neuroblastomas and cell lines (28) and undergoes early transcriptional down-regulation when cells are induced to differentiate with retinoic acid (RA)[1] (29).

* This work was supported in part by a grant from the Associazione Italiana per la Ricerca sul Cancro and by the American Cancer Society. 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.

¶ Supported by a fellowship from the Associazione Italiana per la Ricerca sul Cancro.

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[1] The abbreviations used are: RA, retinoic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

Like N-*myc*, c-*myb* is down-modulated in neuroblastoma upon induction of differentiation (30), and we recently showed that down-regulation of c-*myb* expression is associated with inhibition of neuroblastoma and neuroepithelioma cell proliferation (31). Thus, to determine the role, if any, of B-*myb* in the growth and differentiation of neuroblastoma cells, we examined the effects of ablating B-*myb* expression on the survival of neuroblastoma cell lines and the effects of constitutive expression of B-*myb* on neural differentiation of neuroblastoma cells.

MATERIALS AND METHODS

Cloning of Antisense and Sense B-myb Vectors—The *Eco*RI-*Sca*I region (714 base pairs) spanning nucleotides 941-1655 of the human B-*myb* cDNA (12) was cloned into plasmid pRc/CMV (Invitrogen, San Diego, CA) in the antisense or sense orientation as confirmed by restriction analysis. The full-length cDNA sequence was subcloned in the sense orientation from plasmid pSV-B-myb (17) into vector pRc/CMV.

Cell Culture and Transfection—The neuroblastoma cell line LAN-5 (32) was grown in RPMI 1640 medium (Sigma) supplemented with 15% fetal bovine serum (Sigma). The neuroblastoma cell line SK-N-SH (33) was grown in minimal essential medium (Life Technologies, Inc.) with 10% fetal bovine serum.

For cell differentiation experiments, cells were seeded at an initial density of 5×10^3 cells/cm^2 and grown for 16 h before the medium was replaced with fresh medium containing 5 μM all-*trans*-retinoic acid (Sigma). Medium with RA was then replaced every 3 days.

LAN-5 cells were transfected by the calcium phosphate precipitation technique as described (31) or by Lipofectin according to the manufacturer's instructions (Boehringer Mannheim). Briefly, cells were seeded at a density of 1.5×10^6 cells/dish and 18 h later exposed to a mixture of 10 μg of plasmid DNA and Lipofectin reagent for an additional 18 h. Cells were washed and grown in normal medium for 24 h. G418 (Sigma) was then added at a concentration of 400 μg/ml to the medium, and transfectant clones were isolated 3 weeks later.

RNA Analysis—Total RNA was prepared by acid guanidinium/thiocyanate/phenol/chloroform extraction (34), separated on a formaldehyde-agarose gel using standard procedures (35), and transferred to nylon membranes (Stratagene, La Jolla, CA). Filters were hybridized with specific probes using the high stringency protocol suggested by the manufacturer. The *Eco*RI-*Sca*I fragment derived from plasmid pSV-B-myb containing the full-length cDNA for human B-*myb* (17) and the 2.1-kilobase *Xho*I insert of clone HFβA-1 containing the cDNA for human β-actin (36) were used as molecular probes in Northern blot analyses.

Nuclei for run-on transcription were prepared as described (37), with at least 2×10^7 cells as starting material. Run-on transcription was carried out with 1×10^7 nuclei in the presence of 100 μCi of [α-^{32}P]UTP (DuPont NEN, Bad Homburg, Germany). Five μg of each linearized plasmid was denatured and blocked on a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a slot blot apparatus. Plasmids used in this experiment were as follows: pSV-B-myb (17), a Bluescript plasmid containing the complete cDNA for human c-*myb* (subcloned in our laboratory); pNB-19–21 containing a fragment spanning the second exon of the human N-*myc* gene (kindly obtained from Dr. F. Alt, Columbia University); β-actin plasmid HFβA-1 (36); and the pVC vector used as negative control. Filters were hybridized for 36 h at 65 °C in 10 mM TES, pH 7.4, 0.2% SDS, 10 mM EDTA, 300 mM NaCl, and 5×10^6 cpm/ml nuclear RNA from the run-on transcriptions. After hybridization, filters were washed with several changes of 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate) for 2 h at 65 °C, incubated at 37 °C in 2 × SSC with 10 μg/ml RNase A (Sigma) for 30 min, washed again with 2 × SSC for 1 h at 37 °C, air-dried, and exposed to x-ray film.

Polymerase Chain Reaction—Primers used to confirm integration of the sense and antisense B-*myb* inserts in transfected cells were as follows: CMV-1, 5′-AATGGGAGTTTGTTTTGGCACCAA-3′, corresponding to nucleotides 699–722 of the pRc/CMV vector; SP6–1, 5′-GCACAGTCGAGGCTGATCAGCGAG-3′, complementary to nucleotides 1020–1043 of the pRc/CMV vector; and B-myb-1, 5′-TGGCATTGCTGGTCAGTGCGGTTA-3′, complementary to nucleotides 313–336 of the human B-*myb* cDNA sequence (2). The oligonucleotide T7-1 (5′-AATACGACTCACTATAGGGAGACC-3′, corresponding to nucleotides 865–888 in the pRc/CMV vector) was used as a specific probe for hybridizations.

Polymerase chain reaction analyses were carried out as described (38). Amplification products were separated on standard agarose gels and transferred to nylon membranes. Filters were prehybridized in 5 × SSC, 1% SDS for 2 h at 47 °C. Hybridizations were carried out in 5 × SSC, 1% SDS, 100 mg/ml sonicated salmon sperm DNA, and 10^7 cpm of 5′-end-labeled probe (T7-1) for 16 h at 47 °C. After washing in 2 × SSC, 0.5% SDS for 15 min at room temperature and in the same buffer for 15 min at 50 °C, filters were exposed in an x-ray cassette with an intensifier screen for 1–4 h.

In Vitro Translation—Plasmid pRc/CMV-B-myb containing the full-length sequence of the human B-*myb* cDNA was linearized with *Xba*I restriction endonuclease (Promega). *In vitro* transcription using T7 RNA polymerase (Promega) was carried out according to the manufacturer's instructions, and 1 μg of the B-*myb* mRNA produced *in vitro* was translated in a rabbit reticulocyte system (Boehringer Mannheim) in the presence of [^{35}S]methionine (DuPont NEN). Translation products were separated by electrophoresis on a 10% SDS-polyacrylamide gel. The gel was dried and exposed in an x-ray cassette for 2 h.

Mobility Shift Assays—Nuclear extracts from B-*myb* transfectants, pRc/CMV transfectants, and LAN-5 cells treated for 6 days with RA (5 μM) were prepared as described (39). The protein content of each extract was determined using the protein assay kit II (Bio-Rad). One strand of the *myb*-specific oligonucleotide MBS-1 (5′-AGAATGTGTGTCAGTTAGGGTGTAGAG-3′) was labeled with [γ-^{32}P]ATP and polynucleotide kinase before annealing to the complementary strand. Mobility shift assays were performed with 6 μg of each nuclear extract or 4 μl of programmed reticulocyte lysate in 40 mM KCl, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 1 mM $MgCl_2$, and 0.2 μg/μl poly[d(I-C)] (Boehringer Mannheim) in the presence of 0.01 pmol of double-stranded ^{32}P-labeled MBS-1 at room temperature for 20 min. Where appropriate, a 100-fold molar excess of the unlabeled double-stranded MBS-1 or YY1 (5′-CCGAGCCCGCTTCAAAATGGAGACCCTC-3′) oligomer was added to the mixtures as a specific or nonspecific competitor, respectively. Reactions were loaded on a native polyacrylamide gel (5%) run on 0.5 × Tris borate/EDTA (1 × Tris borate/EDTA = 0.090 M Tris borate, 0.002 M EDTA) for 4 h at 140 V at 4 °C. The gel was dried and exposed to x-ray film.

Immunoprecipitation—LAN-5, B-*myb* transfectants, and pRc/CMV transfectants were metabolically labeled with 100 μCi/ml [^{35}S]methionine (1000 Ci/mmol) for 3 h. Extracts were prepared from 1×10^6 labeled cells in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin on ice. Immunoprecipitations were carried out essentially as described (40) using a specific anti-B-Myb polyclonal antibody (gift of Dr. Robert E. Lewis, University of Nebraska, Omaha, NB) raised in rabbits by immunization with a glutathione *S*-transferase fusion protein containing amino acids 553–611 of the human B-Myb protein.

Immunocytochemistry—Cells were seeded at a density of 5×10^3 cells/cm^2 in Labtek chamber slides (NUNC, Naperville, IL) and grown under normal conditions or treated with RA for the appropriate time. After washing in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4), cells were fixed in methanol/acetone (25:75) for 20 min at −20 °C and incubated with the appropriately diluted primary monoclonal antibody for 1 h at room temperature. Cells were then extensively washed with phosphate-buffered saline and incubated for 1 h at room temperature with fluoresceinated anti-mouse IgG (Sigma) diluted 1:40. The monoclonal antibodies and relative dilutions used were as follows: anti-vimentin (Sigma), 1:40; anti-glial fibrillary acidic protein (Sigma), 1:400; anti-laminin (Sigma), 1:2000; anti-neurofilament (160 kDa; Sigma), 1:40; anti-collagen type IV (Sigma), 1:500; and anti-S-100 (Boehringer Mannheim), 1:500.

RESULTS

B-myb Is Transcriptionally Down-regulated during Neural Differentiation of Neuroblastoma Cells—B-*myb* mRNA levels in several neuroblastoma cell lines were determined by Northern blot analysis. Transcripts were detected in all cases (Fig. 1, *A* and *C*; data not shown). Since neuroblastoma cells can differentiate toward a neural, Schwann, or melanocytic phenotype depending on the cell line and the inducer (41), we investigated whether B-*myb* mRNA levels might be modulated by differentiative processes. LAN-5 and SK-N-SH cells were treated with RA, which induces predominant neural (42) and Schwann-like (41) phenotypes, respectively. Total RNA was extracted at different times after treatment, separated on a formaldehyde-agarose gel, and transferred to a nylon filter. Filters were hybridized with a B-*myb*-specific probe, washed, and rehybrid-

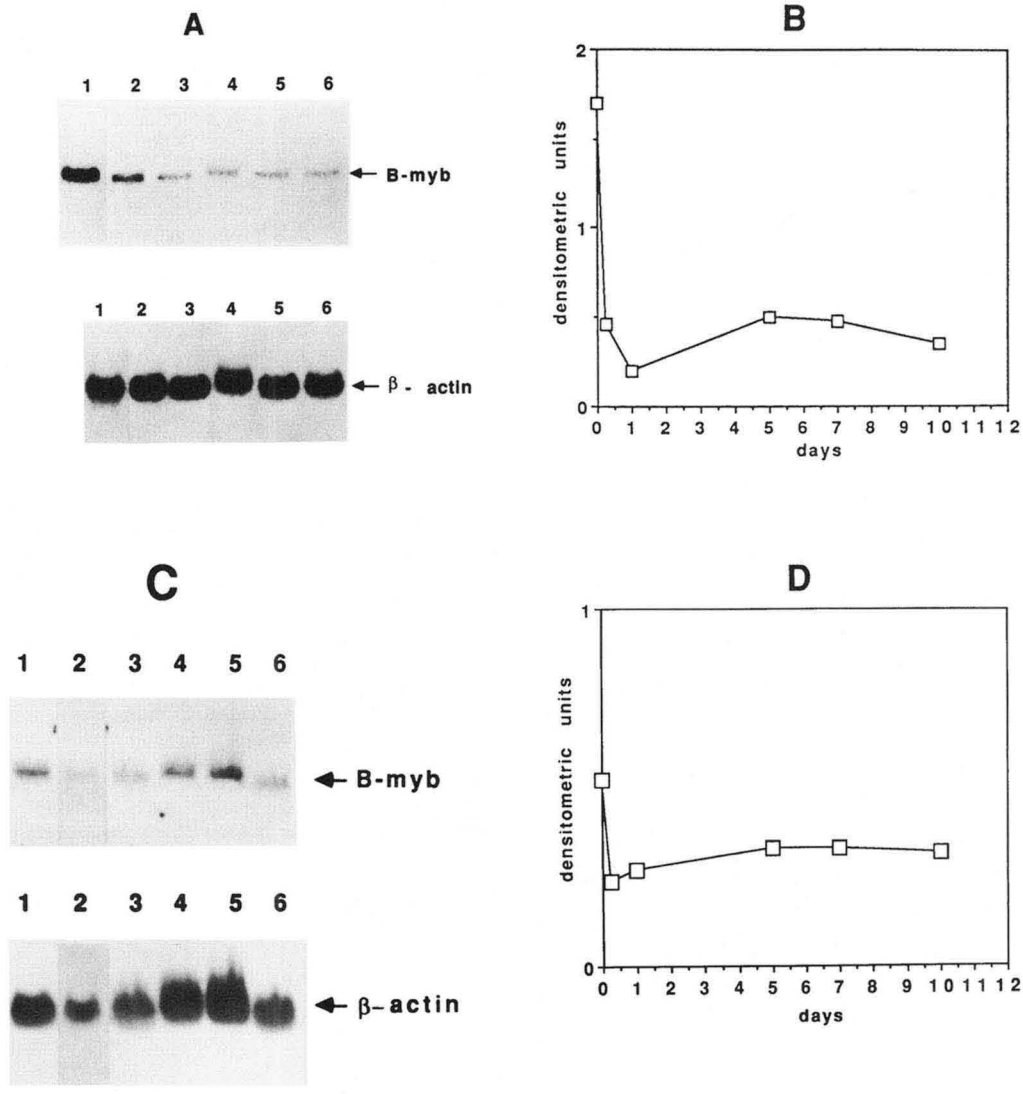


FIG. 1. **Detection of B-*myb* mRNA in neuroblastoma cell lines at different times after RA induction.** LAN-5 (*A*) and SK-N-SH (*C*) cells were uninduced (*lane 1*) or were induced with RA for 6 h (*lane 2*), 1 day (*lane 3*), 5 days (*lane 4*), 7 days (*lane 5*), or 10 days (*lane 6*). Filters were also hybridized with a β-actin probe. Also shown is the densitometric analysis of B-*myb* RNA levels in LAN-5 (*B*) and SK-N-SH (*D*) cells during RA treatment (mean of two experiments in each panel).

ized with a probe for β-actin to control the integrity and the amount of each RNA loaded on the gel. After densitometric reading of the autoradiograms and normalization for β-actin mRNA levels, the amount of B-*myb* mRNA, in arbitrary units, was plotted against the time of RA induction. After RA treatment, B-*myb* mRNA levels decreased sharply in LAN-5 cells (Fig. 1, *A* and *B*), and a moderate, but reproducible decrease was detected in SK-N-SH cells (*C* and *D*); B-*myb* down-regulation occurred as early as 6 h after the beginning of the RA treatment and was maintained at later times in both cell lines (Fig. 1, *A–D*). To determine whether the down-regulation of B-*myb* mRNA during RA treatment was due to a transcriptional mechanism, nuclei from untreated and RA-treated (for 6 days) LAN-5 cells were isolated, and *in vitro* transcription in the presence of [^{32}P]UTP was carried out. The signal intensity for B-*myb* in filters hybridized with the labeled RNA from untreated LAN-5 cells was >3-fold greater than that detected in filters hybridized with RNA from RA-treated cells (data not shown), demonstrating that the rate of B-*myb* transcription decreases during RA-induced differentiation. As expected from previous studies (29, 30), transcription of N-*myc* and c-*myb* was also down-regulated (data not shown).

B-myb Expression Is Required for Neuroblastoma Cell Survival—A B-*myb* fragment (714 base pairs) spanning a region of minimum homology to the other members of the Myb family was cloned in the polylinker region of the pRc/CMV plasmid in the sense or antisense orientation under the control of the cytomegalovirus promoter. Parallel transfections using sense and antisense B-*myb* plasmids were carried out in LAN-5 cells. After 21 days of selection in the presence of the antibiotic G418, plates were fixed and stained, and the number of resistant clones was counted. In four independent experiments (Table I), the number of resistant clones obtained in the antisense B-*myb* transfections was significantly lower than that obtained with the sense B-*myb* transfections, with an average reduction of 79% in the four experiments. Southern blot and polymerase chain reaction analyses of the genomic DNA of the residual antisense B-*myb* transfectants after antibiotic selection demonstrated rearrangements in the antisense B-*myb* insert that prevented expression of the antisense B-*myb* transcript (data not shown), suggesting a counterselection of cells expressing antisense B-*myb* RNA. The marked reduction in the colony-forming ability of antisense B-*myb*-transfected cells is in good agreement with the results of similar experiments demonstrating that antisense B-*myb* RNA or DNA inhibits the proliferation of mouse fibroblasts (17) and human hematopoi-

TABLE I
Clonogenic assay using an antisense B-myb vector in LAN-5 cells

Exp.	Clones		Decrease
	S[a]	AS	
			%
I	87	44	49.50
II	78	8	89.75
III	77	7	90.90
IV	225	26	88.45

[a] S, sense B-*myb* vector (control); AS, antisense B-*myb* vector.

etic cell lines (19). Thus, our findings indicate that B-*myb* is required for the survival of neuroblastoma cells.

Constitutive Expression of B-myb Affects the Differentiative Potential of Neuroblastoma Cells—A full-length B-*myb* cDNA was cloned in the polylinker region of vector pRc/CMV downstream of the cytomegalovirus promoter. The molecular weight of the protein product from the cloned cDNA was verified by *in vitro* translation (Fig. 2*A*). B-*myb*-transfected LAN-5 cells were grown for 21 days in antibiotic selection medium, and a pool of 60–70 resistant clones was expanded. Pooled transfectants were used in order to maintain the heterogeneity of the parental cell line. Integration of the B-*myb* construct was monitored by polymerase chain reaction and Southern blot analyses (data not shown). Overexpression of the B-*myb* transcript in the transfectant clones was analyzed by Northern blot analysis before and after RA treatment for 6 days (Fig. 2*B*, *lanes 1* and *2*). In comparison with transfectants obtained with the pRc/CMV vector (Fig. 2*B*, *lanes 3* and *4*) and with the LAN-5 parental cells (*lanes 5* and *6*), B-*myb* mRNA levels were readily down-regulated in control cells, whereas B-*myb* transfectants expressed B-*myb* mRNA at high levels, which were even increased after RA induction.

Mobility shift assays performed on nuclear extracts from B-*myb* and pRc/CMV transfectants before and after 6-day RA treatment (5 μM) revealed a specific band in B-*myb* transfectants (Fig. 2*C*, *lanes 1* and *2*), but not in RA-treated pRc/CMV transfectants (*lanes 4* and *5*). The B-*myb*-specific band had a shift pattern similar to that obtained with a B-*myb*-programmed reticulocyte lysate (Fig. 2*C*, *lane 7*). This result suggests that the protein product of the transfected B-*myb* construct retains the DNA binding activity, which is still detectable after 6 days of RA induction when endogenous B-*myb* is down-regulated. Finally, B-Myb protein was immunoprecipitated in B-*myb* transfectants and controls using a specific rabbit polyclonal antibody to the human B-Myb protein (data not shown). Consistent with the RNA levels, B-Myb protein in B-*myb* transfectants was more abundant after RA treatment (data not shown).

Rates of growth under normal serum conditions were comparable in B-*myb* transfectants, pRc/CMV controls, and LAN-5 parental cells (data not shown), suggesting that B-*myb* overexpression does not confer a proliferative advantage to neuroblastoma cells. Nevertheless, it should be pointed out that LAN-5 cells express the endogenous B-*myb* mRNA at high levels.

Morphological analysis of B-*myb* transfectants and controls induced to differentiate by 6-day treatment with RA revealed the expected differentiation of LAN-5 cells toward a prevalently neural phenotype, with most cells showing long neuritic processes and few cells with a flat morphology (Fig. 3*F*). The pRc/CMV transfectants behaved similarly (Fig. 3*E*). By contrast, most of the B-*myb* transfectants showed cell body enlargement with a flat morphology without neuritic processes (Fig. 3*D*). To determine whether the morphology of the RA-treated B-*myb* transfectants correlated with a different biochemical phenotype, these cells were tested for the presence of biochemical markers of differentiation (Fig. 4). Vimentin and neurofilaments, two components of the cytoskeleton that are typical markers of differentiation in neuroblastoma cells (43, 44), were initially examined. Vimentin was highly expressed in controls and uninduced B-*myb* transfectants and remained at high levels in RA-treated B-*myb* transfectants, but was dramatically reduced in RA-treated pRc/CMV transfectants and LAN-5 parental cells (Fig. 4, *top panel*). Neurofilaments were absent in all uninduced cells under normal growth conditions (data not shown) and were expressed in pRc/CMV controls and LAN-5 cells after RA induction, but not in the B-*myb* transfectants (Fig. 4, *bottom panel*). This pattern of intermediate filament expression, together with the morphological data, indicates that RA-treated pRc/CMV transfectants and LAN-5 cells undergo the expected pattern of neural differentiation. By contrast, the high level of vimentin expression and the lack of neurofilaments suggest that neural differentiation is inhibited in RA-treated B-*myb* transfectants.

To determine whether RA-treated B-*myb* transfectants were induced to enter other differentiative pathways, several components of the extracellular matrix were also investigated: laminin, fibronectin, and collagen type IV, which are produced by neuroblastoma cells and can be modulated during differentiation processes (45); and glial fibrillary acidic protein, which has been described in human brain tumors (46). Finally, the S-100 protein, which is expressed in ~50% of glial tumors, was also tested (47). Most of the markers analyzed did not change after RA induction in B-*myb* transfectants and in controls (Table II). The only exception was collagen type IV, a marker of Schwann differentiation, which was increased in SK-N-SH cells (Table II). This marker was moderately increased in B-*myb* transfectants after RA treatment, but not in pRc/CMV transfectants or in LAN-5 cells. Overall, the phenotypic data indicate the maintenance of many undifferentiated features in B-*myb* transfectants treated with RA, although they do not rule out the onset of a different, yet not completely defined, differentiative pathway.

DISCUSSION

The induction of differentiation and the commitment to enter a particular pathway are associated with well defined changes in the regulation of gene expression (10). c-*myb*, the most extensively studied member of the Myb family, is transcriptionally down-regulated during differentiation of hematopoietic (48) and neuroblastoma (30) cells. The functional significance of this modulation is suggested by the reported inhibition of Me_2SO-induced differentiation in mouse erythroleukemia cells constitutively expressing c-*myb* (11).

In neuroblastoma, c-*myb* down-regulation is an early event (30). We have previously demonstrated that c-*myb* down-regulation inhibits the proliferation of neuroblastoma and neuroepithelioma cells (31). We now find that B-*myb* is expressed in several neuroblastoma cell lines and that B-*myb* mRNA is down-regulated long before any detectable morphological change when these cells are induced to differentiate along neural and Schwann pathways. Such down-regulation appears to be regulated primarily at the transcriptional level. These findings might be explained in at least three ways. (i) B-*myb* is involved in the control of proliferative mechanisms that must be down-regulated before differentiation can occur; (ii) B-*myb* regulates the expression of genes that inhibit differentiation; or (iii) B-*myb* modulation is a passive event affecting a bystander gene. The last possibility seems unlikely in light of the finding that transfections carried out using an expression vector in which a region of the B-*myb* gene was cloned in the antisense orientation resulted in a substantially lower number of transfected clones as compared with control transfections (Table I). These data are in agreement with previous studies indicating

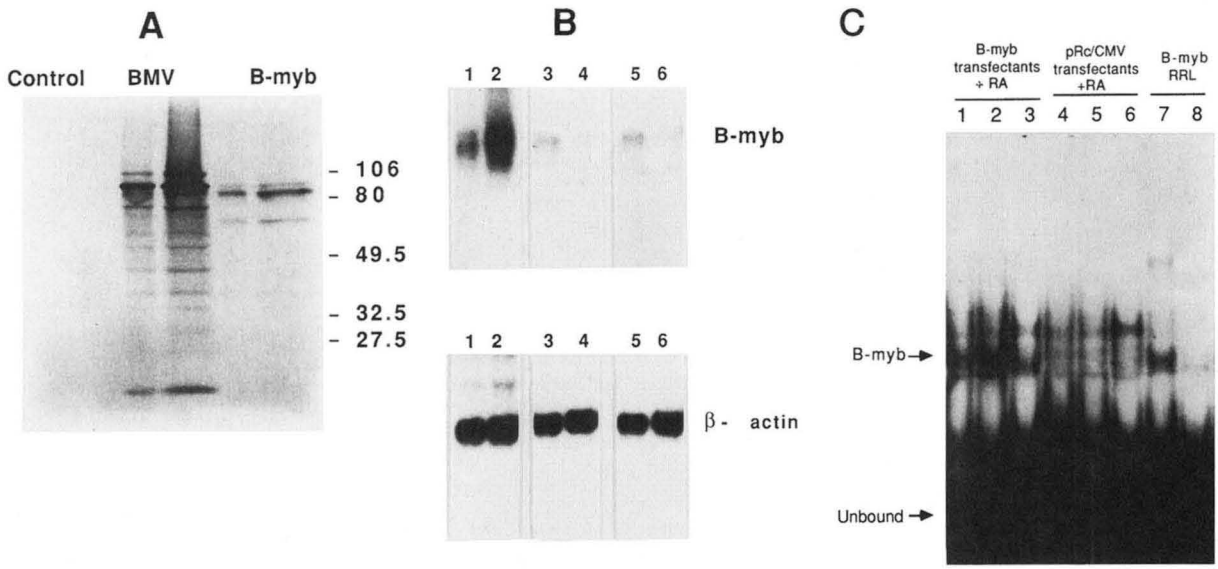


FIG. 2. *A*, *in vitro* translation of RNA obtained from the transcription of plasmid pRc/CMV-B-myb. *Control* and *BMV* are reticulocyte lysate without RNA and stimulated with brome mosaic virus RNA, respectively. Numbers on the right indicate the molecular mass markers in kilodaltons. *B*, B-*myb* mRNA detection in B-*myb* transfectants (*lanes 1* and *2*), pRc/CMV transfectants (*lanes 3* and *4*), and LAN-5 cells (*lanes 5* and *6*) under normal growth conditions (*lanes 1*, *3*, and *5*) or after 6 days of RA treatment (*lanes 2*, *4*, and *6*). Filters were stripped of the previous probe and hybridized with β-actin. *C*, mobility shift assays performed with nuclear extracts from B-*myb* transfectants RA-treated for 6 days (*lanes 1–3*), pRc/CMV transfectants RA-treated for 6 days (*lanes 4–6*), and B-*myb* synthesized in rabbit reticulocyte lysate (*RRL*; *lanes 7* and *8*). The *myb*-specific ^{32}P-labeled oligonucleotide used in this experiment was MBS-1. *Lanes 2* and *5* contain unlabeled nonspecific competitor YY1. *Lanes 3*, *6*, and *8* contain unlabeled specific competitor MBS-1.

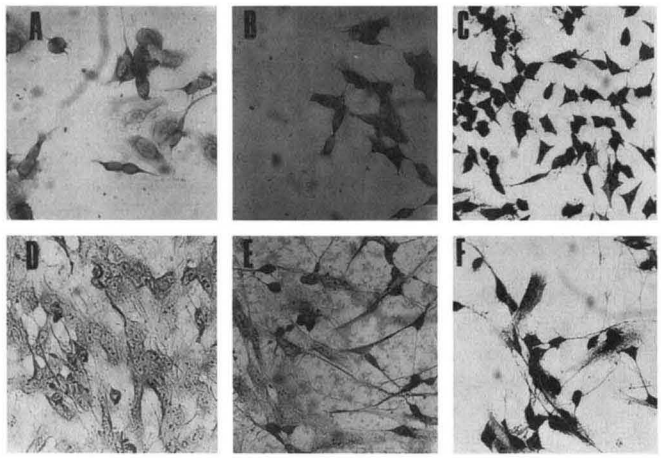
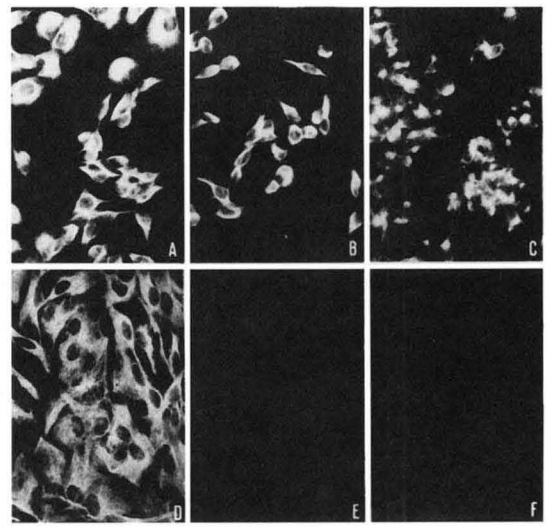


FIG. 3. **Morphology of B-*myb* transfectants (*A* and *D*), pRc/CMV transfectants (*B* and *E*), and LAN-5 cells (*C* and *F*) under normal growth conditions (*A–C*) and after 6 days of treatment with RA (*D–F*).** Original magnification was ×200.



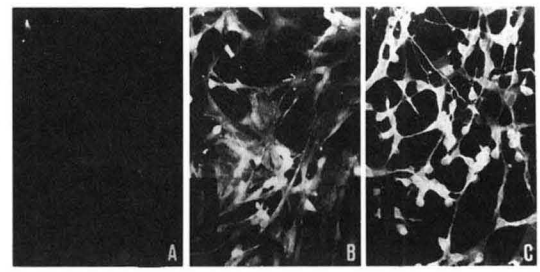


FIG. 4. *Top panel*, indirect immunofluorescence detection of vimentin. Shown are B-*myb* transfectants (*A* and *D*), pRc/CMV transfectants (*B* and *E*), and LAN-5 cells (*C* and *F*) under normal growth conditions (*A–C*) and after 6 days of treatment with RA (*D–F*). *Bottom panel*, indirect immunofluorescence detection of neurofilament (160 kDa). Shown are B-*myb* transfectants (*A*), pRc/CMV transfectants (*B*), and LAN-5 cells (*C*) after 6 days of treatment with RA.

that B-*myb* down-regulation inhibits the proliferation of fibroblasts (17) and hematopoietic (19) cells. Thus, it appears that B-*myb* expression is important for the survival of neuroblastoma cells, although the mechanism by which B-*myb* affects this process remains unclear.

Neuroblastoma cells can differentiate *in vivo* and *in vitro* toward neural, Schwann, and melanocytic phenotypes, recapitulating the multipotential of neural crest cells from which neuroblastomas derive (41). Although many neuroblastoma cell lines show a certain degree of heterogeneity in terms of neurotransmitter expression (33) and differentiative potential (49), each cell line has a prevalent behavior in response to differentiation inducers (41). LAN-5 cells differentiate to a prevalent neural phenotype by induction with RA. When transfected with a B-*myb* cDNA, LAN-5 cells overexpressed B-*myb*. Unlike the parental cell line and control transfectants, B-*myb* transfectants did not show decreased B-*myb* expression after treatment with RA. The continuous B-*myb* expression is due to

TABLE II
Differentiation markers detected by immunofluorescence in B-myb transfectants and controls

	VM[a]		NF 160		C IV		FN		S-100		GFAP	
	UN	RA	UN	RA	UN	RA	UN	RA	UN	RA	UN	RA
LAN-5	++	+/−	−	++	+/−	+/−	+/−	+/−	−	−	−	−
LAN-5-pRc/CMV	++	+/−	−	++	+/−	+/−	+/−	+/−	−	−	−	−
LAN-5-B-myb	++	++	−	−	+/−	+	+/−	+/−	−	−	−	−
SK-N-SH	++	++	−	−	+	++	++	ND	+/−	+/−	+/−	+/−

[a] VM, vimentin; NF 160, neurofilament (160 kDa); C IV, collagen type IV; FN, fibronectin; GFAP, glial fibrillary acidic protein; UN, untreated cells; RA, retinoic acid-treated cells; ND, not done.

the expression of the exogenous gene driven by the cytomegalovirus promoter that is not affected by the RA treatment. By contrast, RA appeared to enhance B-*myb* expression in the B-*myb* transfectants both at the messenger and protein levels (Fig. 2*B*; data not shown). The product of the B-*myb* construct appears to be functional as nuclear extracts from B-*myb* transfectants retain the ability to interact with a B-*myb*-specific binding site even after RA induction (Fig. 2*C*).

The constitutive expression of B-*myb* results in the inhibition of neural differentiation as reflected by the enlargement of B-*myb* transfectant cells, the absence of outgrowing neurites, the maintenance of high level vimentin expression, the lack of neurofilament production, and the moderate increase in collagen type IV expression after 6 days of RA treatment. Such an increase in collagen type IV, which is typical of Schwann-like differentiation, is the only marker of other known differentiative pathways found in RA-treated B-*myb* transfectants. Thus, it is possible that B-*myb*-transfected neuroblastoma cells begin a differentiative pathway that is distinct from neural differentiation, but resembles a Schwann-like differentiation. Consistent with this hypothesis, we found only a slight down-modulation of B-*myb* in SK-N-SH cells differentiating toward a Schwann-like phenotype. Nevertheless, a decrease in B-*myb* expression under a certain threshold might be required for complete schwannian differentiation.

Recently, the mechanism of action of RA on the differentiation of neuroblastoma cells has been analyzed in detail (50). RA reportedly induces expression of the trkB receptor, which, in turn, renders neuroblastoma cells sensitive to the differentiative action of brain-derived nerve growth factor (50). However, the nuclear effectors in the signal transduction cascade promoted by the activation of the trkB receptor remain unknown. Based on the results described here, it is tempting to suggest that B-*myb* is one of the nuclear targets of the differentiation-promoting signal transduced by the trkB receptor. A complete understanding of the process by which a differentiative signal is transmitted to the nucleus is particularly important for the development of effective therapies that may take advantage of the differentiative potential of neuroblastoma cells (51).

Acknowledgments—We thank Dr. Robert E. Lewis for the gift of the antibody to B-Myb and Adelma Di Stefano for technical assistance.

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