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Regulation of c-*myb* Expression in Human Neuroblastoma Cells during Retinoic Acid-Induced Differentiation

CAROL J. THIELE,* PAMELA S. COHEN, AND MARK A. ISRAEL

Molecular Genetics Section, Pediatric Branch, Clinical Oncology Program, National Cancer Institute, Building 10, Room 13N240, Bethesda, Maryland 20892

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We detected expression of the c-*myb* proto-oncogene, which was initially thought to be expressed in a tissue-specific manner in cells of hematopoietic lineage, in human tissues of neuronal origin. Since the level of c-*myb* expression declined during fetal development, we studied the regulation of its expression in human neuroblastoma cell lines induced to differentiate by retinoic acid. The expression of c-*myb* declined during the maturation of neuroblastoma cells, and this change was mediated by a decrease in c-*myb* transcription.

The c-*myb* gene is the normal cellular homolog of the retroviral transforming gene v-*myb*, which causes malignant transformation of cells of granulocyte-monocyte lineage in chickens (2). In normal tissues, c-*myb* expression is most readily detected in hematopoietic cell precursors (6, 7, 36), suggesting that this gene may play a specialized role in the growth and development of these cell types. The expression of c-*myb* in nonhematopoietic tissues, such as fetal kidney, liver, and brain (14), is controversial and difficult to assess because of tissue vascularization. Recently, c-*myb* expression has been detected in chicken embryo fibroblast cell lines (34) and several human transformed cell lines from tumors of neuroectodermal origin (15, 31), as well as from colon and breast carcinomas (1, 29). These findings suggest that c-*myb* may also play a more generalized role in cell growth and differentiation.

We reported detection of c-*myb* expression in several human tumors of neuroectodermal origin, including neuroblastoma (NB) and neuroepithelioma (31). Human NB is a tumor of the peripheral nervous system and is thought to arise in migratory cells of the embryonal neural crest. The development of this tumor may be due to alterations in normal cellular maturation, since there are documented cases of spontaneous (11) as well as therapy-associated maturation of NB to benign ganglioneuroma (12). Additionally, a variety of chemicals and biologic-response modifiers are known to induce morphological, biochemical, ultrastructural, and electrophysiological differentiation of NB cell lines (23, 26).

Previously, we reported that the steady state levels of the N-*myc* proto-oncogene, which is amplified in most NB cell lines (19, 25) and in 38% of stage III and IV NBs (5), are dramatically decreased when NB cell lines are induced to differentiate by retinoic acid (RA) or dibutryl cyclic AMP (32). The decrease in N-*myc* mRNA did not appear to be related to RA-induced growth arrest, since the steady state levels of N-*myc* mRNA were not significantly altered in growth-arrested cells (32). Since both c-*myb* and N-*myc* encode nuclear proteins (18, 28) and have been postulated to play a role in the regulation of gene expression, we were interested in studying the regulation of c-*myb* expression in neuronal tissue. In this report, we examine the molecular mechanisms of the regulation of c-*myb* expression during RA-induced maturation of NB cell lines.

* Corresponding author.

MATERIALS AND METHODS

Materials. Actinomycin D, *trans*-RA, and cycloheximide (Chx) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Aphidicolin was kindly provided by M. Suffness of the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. [^{3}H]thymidine (800 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [α-^{32}P]dUTP (3,000 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, Mass.). NTB-2 emulsion was purchased from Eastman Kodak Co. (Rochester, N.Y.). Age-estimated human fetal tissue was obtained from Thomas Shepherd of the Embryology Laboratory, University of Washington, Seattle, Wash.

Cell culture. Human NB cell lines SMS-KCNR (32) and LA-N-5 (35) were cultured in RPMI 1640 medium containing 15% fetal calf serum, glutamine, penicillin, and streptomycin. NB cells (3×10^6) were plated into 150-mm tissue culture plates and incubated for 2 days at 37°C and 5% CO_2. The medium was removed from the cells, adjusted to 5 μM RA, and then returned to the cells, which were then incubated for different periods of time. Subsequently, cells were fed every 2 days with medium containing 5 μM RA or equivalent amounts of solvent control and harvested at the indicated times for the isolation of nucleic acids. Cells were growth arrested by nutrient deprivation as previously described (32). Actinomycin D (5 μg/ml) or Chx (5 μg/ml) was added to cell cultures for different periods of time, as indicated. In other experiments, 5 μg of aphidicolin per ml was added to cultures for 24 h, after which time the cultures were washed three times in medium without drug and incubated for an additional 6 h. To determine the percentage of mitotic cells, cultures were labeled with 1 μC of [^{3}H]-thymidine for 6 h, and cytocentrifuge slide preparations were then made from cell suspensions and processed for autoradiography as previously described (30).

Isolation and analysis of RNA. Total RNA was isolated from tissue culture cells as previously described (33) and from fetal tissues by the method of Chirgwin et al. (8). RNA was studied by Northern (RNA) blot analysis as previously described (32), although Nytran filters (Schleicher & Schuell, Inc., Keene, N.H.) were used in place of nitrocellulose. A 2.2-kilobase-pair *Hae*III DNA fragment probe of pODAmyb (a gift from J. Frederic Mushinski, National Cancer Institute), which encodes exons 3 to 7 of human c-*myb*, was radiolabeled by nick translation (24) and used in hybridizations, as previously described (32). The filters were

reused for sequential hybridizations after they were treated for 1 h in 50% formamide–1× SSC (0.15 M sodium chloride plus 15 mM sodium citrate [pH 7.0]) at 75°C. Relative amounts of RNA were determined by quantitative densitometric scanning of appropriately exposed autoradiograms.

In vitro nuclear transcription assay. Nuclei were isolated from cells by the method of Bitter and Roeder (4) and frozen at −70°C. Nuclei from different time points were thawed simultaneously, and RNA synthesis was assayed by the method of Groudine et al. (16). Equal counts per minute of the ^{32}P-labeled RNA transcription products were diluted in 50% formamide–3× SSC–0.5% Denhardt solution–10% dextran sulfate–0.1% sodium dodecyl sulfate and hybridized to filters containing 5 μg of the 2.2-kilobase-pair *Hae*III DNA fragment of c-*myb* that encodes exons 3 to 7 and an actin-containing plasmid (22) for 48 h at 42°C.

RESULTS

Expression of c-*myb* in developing neural tissue. The c-*myb* gene is expressed during the physiologic maturation of hematopoietic cells and in tumors of hematopoietic origin (13, 36). Since we found that the c-*myb* proto-oncogene is expressed in several pediatric malignancies of neuroectodermal origin, including NB, peripheral neuroepithelioma (31), and Ewing's sarcoma (C. McKeon, C. Thiele, and M. Israel, unpublished data), as well as several glial tumor cell lines (R. La Rocca and M. Israel, unpublished data), we were interested in determining whether the expression of c-*myb* could be documented in normal developing neural tissue. Equal amounts of embryonic, fetal, and adult brain, spinal cord, and lung mRNAs were examined by Northern blot analysis for c-*myb* expression. Expression of c-*myb* was detected in each of the neural tissues examined, with higher levels detected in embryonic and fetal tissues than in adult. The highest level of c-*myb* mRNA expression was found in embryonic brain cells (Fig. 1A). To quantitate the level of c-*myb* RNA found at these different times during development, we normalized the intensity of the c-*myb* signal detected (Fig. 1A) to the level of actin mRNA in these same specimens (data not shown). A graphic representation of these data (Fig. 1B) reveals a decrease in c-*myb* expression during nervous system development. Similar results were obtained when data were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GADPH) mRNA (data not shown).

To study the regulation of c-*myb* mRNA expression during neuronal development, we examined human NB cell lines which were induced to differentiate in vitro by RA. NB cell lines SMS-KCNR and LA-N-5 were treated with RA, and after different periods of time, the extent of morphological differentiation was assessed and total RNA was isolated and examined by Northern blot analysis. The morphologic changes that occurred during RA-induced differentiation of LA-N-5 cells are depicted in Fig. 2. Within the first 2 days after RA treatment, there were no dramatic morphologic changes, although RA-treated cells appeared to be more substrate adherent. After 4 days of RA treatment, the majority of cells had begun to extend processes (32). By day 6, the migration of cells into ganglionlike clusters was associated with an extensive network of neuritic processes. Evaluation of these changes by electron microscopy has shown that the extensive network of processes and bundles mimics, untrastructurally, normal neuronal development (27). Growth inhibition of RA-treated cells was apparent by days 2 to 4 and, previously, we have shown that the RA-treated cells become arrested at G_1 in the cell cycle (32).

Expression of c-*myb* RNA during RA-induced differentiation of two NB cell lines, SMS-KCNR and LA-N-5, is shown in Fig. 3A and B. In both cell lines, the level of c-*myb* expression increased slightly within the first 4 h of treatment with RA; however, by day 4 of RA treatment, there was a sharp decrease in c-*myb* expression in both cell lines. Decreased c-*myb* expression continued throughout the course of treatment of SMS-KCNR cells (panel A), while in LA-N-5 cells (panel B), expression of c-*myb* increased slightly above nadir levels at 10 days of RA treatment. Analysis of these Northern blots for the expression of actin and GADPH mRNA confirmed that equivalent amounts of RNA had been examined at each time point (data not shown).

To examine the kinetics of growth arrest which occurs in NB cell lines induced to differentiate with RA, we simultaneously analyzed the number of mitotic cells in these cell cultures by [^{3}H]thymidine labeling and autoradiography. For each cell line studied, the percentage of cells containing radiolabeled nuclei at different times after the initiation of RA treatment is shown in Fig. 3C and D. These results documented the arrested growth of these cell cultures and indicated that the data are compatible with c-*myb* expression being regulated in association with either the antiproliferative or the differentiating effects of RA.

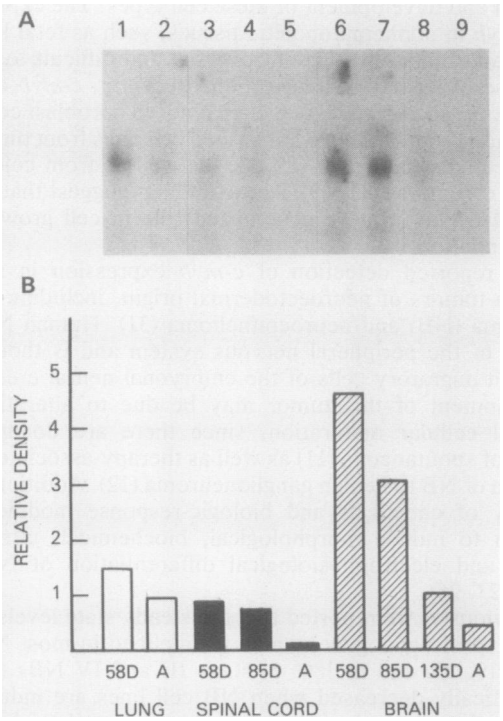

FIG. 1. c-*myb* expression in fetal and adult tissues. Total RNA (25 μg) isolated from age-estimated embryonic, fetal, and adult tissues was electrophoresed, blotted onto Nytran filters, and hybridized to ^{32}P-labeled c-*myb* as described in Materials and Methods. (A) Northern blot containing 58-day embryonic lung (lane 1), adult lung (lane 2), 58-day embryonic spinal cord (lane 3), 85-day fetal spinal cord (lane 4), adult spinal cord (lane 5), 58-day embryonic brain (lane 6), 85-day fetal brain (lane 7), 135-day fetal brain (lane 8), and adult brain (lane 9). (B) Graph of relative intensity of c-*myb* hybridization signal obtained from densitometric scanning of autoradiograph pictured in panel A. The intensity was normalized to the intensity of the actin hybridization signal (data not shown). Abbreviations: 58D, 58-day embryo; A, adult; 85D, 85-day fetus; 135D, 135-day fetus.

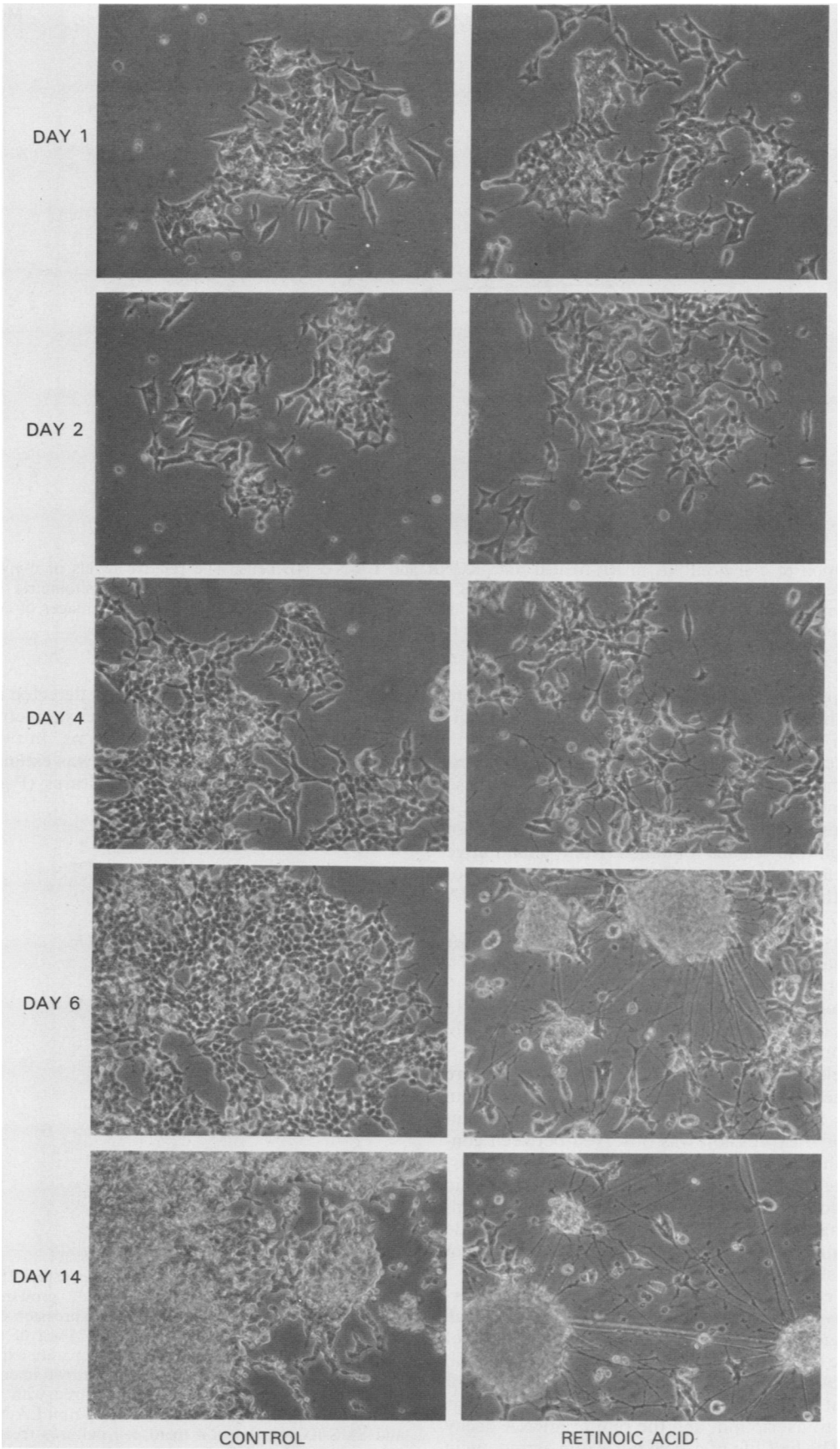

FIG. 2. Morphological differentiation of RA-treated LA-N-5 NB cells. Replicate cultures of 3×10^6 LA-N-5 NB cells were treated with 5 μM RA or control solvent, as described in Materials and Methods, and photographed. Control cells (left panels) and cells treated with RA (right panels) are shown after various periods of RA treatment.

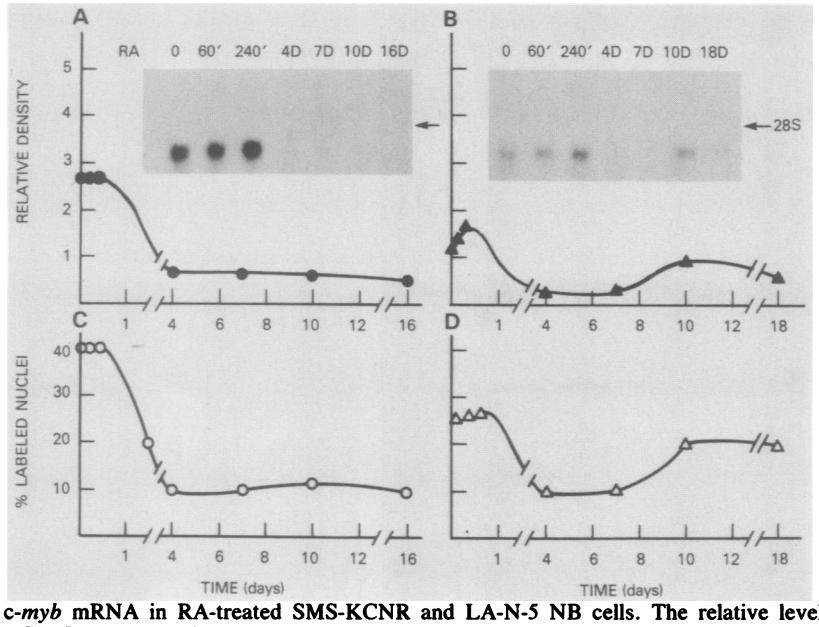

FIG. 3. Expression of c-*myb* mRNA in RA-treated SMS-KCNR and LA-N-5 NB cells. The relative levels of c-*myb* expression in RA-treated cultures of SMS-KCNR (A) and LA-N-5 (B) NB cells were determined by quantitative densitometric scanning of the autoradiograms pictured in the corresponding insets. The arrow indicates the position of 28S RNA. The percentages of cells with labeled nuclei in cultures of SMS-KCNR (C) and LA-N-5 (D) cells were determined at different times after RA treatment.

c-*myb* mRNA expression and growth arrest. To evaluate more critically the relationship between growth arrest, differentiation, and c-*myb* expression, we arrested the growth of SMS-KCNR cells by serum starvation under conditions which do not cause morphologic differentiation of these cells. After serum starvation of NB tumor cells for 5 days, a 75% decrease in radiolabeled nuclei was obtained (data not shown), although the values obtained from quantitative densitometric scanning of appropriately exposed autoradiograms revealed that the steady state levels of c-*myb* mRNA decreased only 7 to 18% (Fig. 4A).

Aphidicolon is a drug which reversibly inhibits DNA polymerase alpha, arresting cells at the G_1-S interface (17). We incubated replicate cultures of SMS-KCNR and LA-N-5 cells in control medium or medium containing aphidicolin (5 μg/ml) for 24 h and harvested them for RNA. To evaluate c-*myb* expression after cells were released from the G_1-S aphidicolin block, a duplicate aphidicolin-treated culture was washed three times, cultured in complete medium for an additional 6 h, and harvested for RNA. No significant difference in c-*myb* expression was observed between control NB cell cultures containing 30 to 40% mitotic cells, aphidicolin-treated cultures containing less than 1% mitotic cells, and aphidicolin-treated and washed cultures containing 40 to 50% mitotic cells (Fig. 4B). These data provide additional evidence that growth arrest alone is not sufficient to decrease the steady state levels of c-*myb* mRNA.

Regulation of c-*myb* expression during RA-induced differentiation. To investigate the mechanism by which c-*myb* expression is regulated during RA-induced differentiation of NB cell lines, we determined the half-life of c-*myb* mRNA in untreated and RA-treated LA-N-5 cells (Fig. 5). After 2- or 10-day treatment with RA, actinomycin D (5 μg/ml) was added to inhibit transcription, and the rate of mRNA decay was examined. The half-life of c-*myb* mRNA in actinomycin D-treated LA-N-5 cells was estimated by linear regression analysis of the rate of c-*myb* mRNA decay as a function of time after treatment with actinomycin D. Values for the steady state levels of c-*myb* mRNA detected in each culture were obtained from quantitative densitometric scanning of appropriately exposed autoradiograms. In two separate experiments, a 25- to 30-min half-life was estimated for c-*myb* mRNA in control LA-N-5 cell cultures (Fig. 5A). As ex-

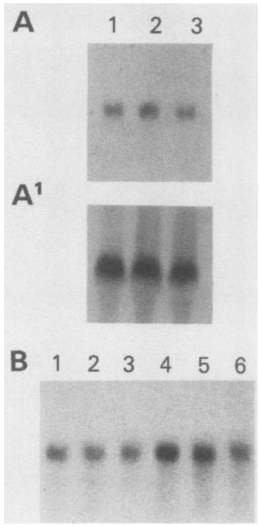

FIG. 4. c-*myb* expression in growth-arrested NB cells. (A) Total RNA, isolated from nutrient-deprived, growth-arrested SMS-KCNR cells, was analyzed for c-*myb* expression by Northern blot hybridization. Lanes 1 and 3 contain 25 μg of total RNA from serum- starved cultures of cells in two separate experiments; lane 2 contains 25 μg of total RNA from cells grown in complete medium. (A^1) Northern blot in panel A rehybridized with ^{32}P-labeled actin plasmid DNA. (B) Total RNA, isolated from LA-N-5 (lanes 1 to 3) and SMS-KCNR (lanes 4 to 6) cell cultures treated with control solvent (lanes 1 and 4), 5 μg of aphidicolin per ml for 24 h (lanes 2 and 5), or 5 μg of aphidicolin per ml for 24 h, washed 3 times with complete medium, and cultured for an additional 6 h (lanes 3 and 6), was analyzed for c-*myb* expression by Northern blot analysis.

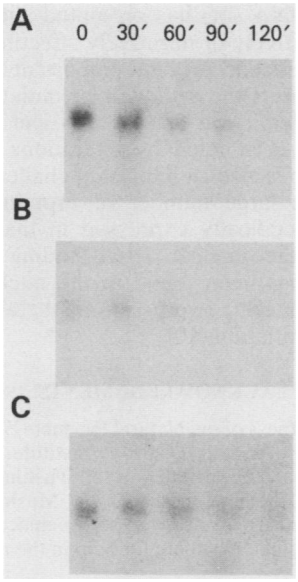

FIG. 5. Estimation of c-*myb* mRNA stability in actinomycin D-treated NB cells during RA treatment. Cellular RA was isolated from solvent control- and RA-treated cultures of SMS-KCNR NB cells incubated with 5 μg of actinomycin D per ml at the indicated times (minutes after initiation of actinomycin D treatment). (A) Northern blot of 30 μg of total RNA from solvent control-treated SMS-KCNR cultures; (B) similar Northern blot of SMS-KCNR cultures treated for 2 days with RA; (C) similar Northern blot treated for 10 days with RA.

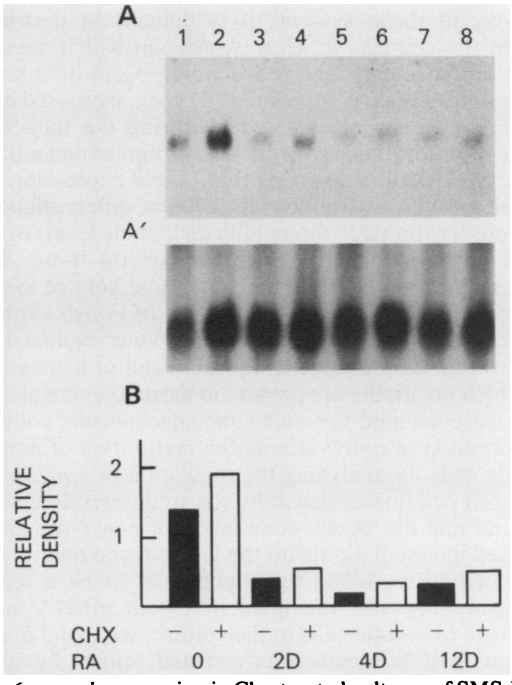

FIG. 6. c-*myb* expression in Chx-treated cultures of SMS-KCNR cells during RA treatment. RA-treated cultures were incubated with 5 μg of Chx per ml for 3 h, after which total RNA was isolated and evaluated by Northern blot hybridization for c-*myb* expression. (A) Northern blot of 30 μg of total RNA from control SMS-KCNR cultures without (lane 1) and with (lane 2) Chx and from experimental cultures 2 days after RA treatment, without (lane 3) and with (lane 4) Chx; 4 days after RA treatment, without (lane 5) and with (lane 6) Chx; and 12 days after RA treatment, without (lane 7) and with (lane 8) Chx. The RNA was hybridized to ^{32}P-labeled c-*myb* DNA. (A′) The same blot hybridized to ^{32}P-labeled actin plasmid DNA. (B) Graph of the relative intensity of the c-*myb* hybridization signal normalized to the actin signal, which was obtained from densitometric scanning of the autoradiogram pictured above.

pected, the 2- and 10-day RA treatments resulted in decreased c-*myb* mRNA levels. Interestingly, there was a slight increase (45 to 50 min) in the half-life of the c-*myb* mRNA in these cultures of differentiated cells (Fig. 5B and C). The half-life of the c-*myb* mRNA expressed in SMS-KCNR cells was unchanged after RA treatment (data not shown). These results indicated that the decrease in the steady state levels of c-*myb* mRNA was not due to an increased rate of mRNA degradation.

To ascertain whether proteins may be affecting the stability of c-*myb* mRNA, we incubated control and RA-treated cultures with Chx. At a concentration of Chx which we determined inhibits protein synthesis by 99% (5 μg/ml), cultures treated with Chx had a slight but consistent 1.5- to 2-fold increase in the steady state levels of c-*myb* mRNA compared with those of controls (Fig. 6). A graphic representation of these data, normalized to actin mRNA levels, was obtained from quantitative densitometric scanning of appropriately exposed autoradiograms and is depicted in Fig. 6B. RA treatment of cells for different periods of time did not significantly alter the relative increase in c-*myb* mRNA levels seen in Chx-treated control cultures. Thus, labile proteins do not appear to play a major role in the regulation of c-*myb* mRNA levels in RA-treated NB cells.

Since decreased mRNA stability could not account for the observed decrease in levels of c-*myb* mRNA in RA-treated cultures, it appeared likely that the decrease was due to a change in the transcriptional activity of this gene. We isolated nuclei from control cells and cells treated for 2 or 14 days with RA and assayed for changes in the transcriptional activity of the c-*myb* gene. After 2 and 14 days of RA treatment, there was a marked decrease in the transcriptional activity of the c-*myb* gene, while transcription of an actin gene was not altered (Fig. 7). Since the c-*myb* cDNA fragment used in the transcription analysis contains exons 3 to 7, we are unable to evaluate whether the decrease in transcription was due to a block in initiation or elongation of c-*myb* mRNA.

DISCUSSION

A tissue-specific role in the maturation of hematopoietic cells has been postulated for c-*myb* because the steady state levels of mRNA decrease in hematopoietic tumor cell lines induced to differentiate by dimethyl sulfoxide (36), granulocyte colony-stimulating factors (13), and phorbol esters (9).

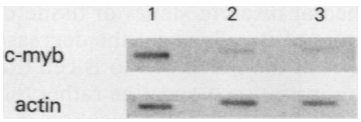

FIG. 7. Transcription of c-*myb* during RA treatment of SMS-KCNR cells. A 2.2-kilobase-pair *Hae*III cDNA fragment of c-*myb* (encoding exons 3 to 7) (5 μg) and an actin-containing plasmid DNA (5 μg) were immobilized on Nytran filters according to the recommendations of the manufacturer. ^{32}P-labeled nuclear RNA transcripts were prepared and isolated from solvent control-treated SMS-KCNR nuclei (lane 1) and 2-day (lane 2) and 14-day (lane 3) RA-treated SMS-KCNR nuclei as described in Materials and Methods.

However, in these systems it is difficult to discriminate between the changes in gene expression which are due to growth arrest of cells and those which occur as a result of cellular differentiation. In some cell types, increased expression of c-*myb* has been detected during the induction of cellular proliferation as well as during exponential cell growth (9, 34, 20), suggesting that c-*myb* expression varies with cell growth, as well as with cellular differentiation.

Our observation that the readily detectable levels of c-*myb* mRNA found in normal developing neural tissue decline during maturation extends the functional role of c-*myb* to nonhematopoietic cells. The detection of c-*myb* expression in several cell lines of peripheral nervous system tumors indicates that its presence is not the result of hematopoietic cells which invariably are present in tissue specimens. In this study, we evaluated the molecular mechanisms controlling the expression of c-*myb* during the maturation of nonhematopoietic cells by analyzing the regulation of c-*myb* expression in NB cell lines induced by RA to differentiate in vitro. We found that the steady state levels of c-*myb* dramatically decreased in association with the RA-induced differentiation of NB cell lines. While there appeared to be a temporal relationship between the level of c-*myb* mRNA and the percentage of mitotic cells in the culture, we found that after the growth of NB cells was arrested, either by nutrient deprivation or aphidicolin treatment, the steady state levels of c-*myb* did not decrease to the level detected during RA-induced differentiation. Thus, under these cell culture conditions, the expression of c-*myb* did not correlate with cellular proliferation or the arrest of cell growth.

Our data indicate that the decreased levels of c-*myb* expression during RA-induced differentiation of NB are largely due to a decreased transcription rate of the c-*myb* gene rather than a decrease in the stability of c-*myb* mRNA. Chx treatment of NB cells at various times after differentiation did not seem to significantly alter the steady state levels of c-*myb* mRNA. Thompson et al. (34) have shown that in cell types in which c-*myb* expression is associated with cellular proliferation, the steady state levels of mRNA are regulated posttranscriptionally and are dramatically altered by Chx treatment of cells. In contrast, they found that the high levels of c-*myb* expression detected in immature (but not mature) thymocytes are transcriptionally regulated and not significantly altered by Chx treatment. These investigators postulate that c-*myb* serves two roles, a specific function in thymic differentiation and a more generalized function in cell proliferation. The transcriptional regulation of c-*myb* in cell lines of NB, a tumor of nonhematopoietic origin, suggests that c-*myb* expression may be of significance in the maturation of multiple cell lineages.

To understand the importance of the c-*myb* gene during differentiation, it will be important to determine whether regulatory factors specifically affecting c-*myb* expression can be identified at discrete stages of tissue differentiation. In murine tumors of B-cell origin, the decreased expression of c-*myb* mRNA at the pre-B cell to B cell transition is due to a block in transcription elongation rather than a change in transcription initiation. Also, the location of the transcriptional block at a DNase I hypersensitivity region in the c-*myb* gene suggests the presence of factors blocking elongation of c-*myb* transcripts in more mature B-cell tumors (3). If such factors can be identified, RA-induced differentiation of NB cells provides an interesting model for evaluating the specificities of these factors and their effects on regulatory elements in the c-*myb* gene.

The nuclear localization of the c-*myb* protein (18) and its ability to bind DNA (21) has prompted speculation that it functions by positively or negatively affecting the regulation of genes which encode proteins important for determining whether a cell enters the cell cycle or initiates a differentiation program. Identifying these genes and evaluating how their expression is affected by alterations in the level of c-*myb* expression is also an important challenge. Such genes may include the large number of proteins and peptides known to be specifically expressed in maturing neuronal tissue, since another nuclear, DNA-binding proto-oncogene product, c-*fos*, has been found in the nucleoprotein complexes that regulate the expression of the aP-2 gene during adipocyte differentiation (10).

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