# Alternative RNA Splicing Produces Multiple Forms of c-Myb with Unique Transcriptional Activities[▽][†]

John P. O'Rourke* and Scott A. Ness

*Department of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131*

The c-Myb transcription factor regulates the proliferation and differentiation of hematopoietic cells, and activated alleles of c-*myb* induce leukemias and lymphomas in animals. Relatively minor changes in the structure of c-Myb protein change the genes that it regulates and can unleash its latent transforming activities. Here, quantitative assays were used to analyze the alternative splicing of human c-*myb* transcripts. We identified an array of variant transcripts, expressed in highly regulated, lineage-specific patterns, that were formed through the use of alternate exons 8A, 9A, 9B, 10A, 13A, and 14A. Expression levels of the different splice variant transcripts were regulated independently of one another during human hematopoietic cell differentiation, and the alternative splicing of c-*myb* mRNAs was increased in primary leukemia samples. The alternatively spliced c-*myb* transcripts were associated with polysomes and encoded a series of c-Myb proteins with identical DNA binding domains but unique C-terminal domains. In several types of assays, the variant c-Myb proteins exhibited quantitative and qualitative differences in transcriptional activities and specificities. The results suggest that the human c-*myb* gene encodes a family of related proteins with different transcriptional activities. Enhanced alternative splicing may be a mechanism for unmasking the transforming activity of c-*myb* in human leukemias.

The c-*myb* gene is the normal, cellular counterpart of v-*myb*, the oncogenic component of avian myeloblastosis virus (AMV) that transforms immature myeloid cells and induces myeloid and lymphoid leukemias in animals (1, 25, 26). Several types of evidence suggest that c-*myb* plays a critical role in regulating hematopoietic cell differentiation, proliferation, and function (25, 26). The c-*myb* gene is duplicated and is a target for chromosomal translocation in a subset of human leukemias, suggesting that c-*myb* is an oncogene in human cancers (4). The c-Myb protein is the founding member of a family of transcription factors that also includes the related proteins A-Myb (MYBL1) and B-Myb (MYBL2). All the Myb proteins are structurally similar and have nearly identical DNA binding domains near their N termini, but they are more divergent in their central transcriptional activation domains and C-terminal negative regulatory domains. Although c-Myb, A-Myb, and B-Myb can bind the same DNA sequences and activate the same promoters in reporter gene assays (31), the three proteins are differentially expressed, and mouse knockout experiments have shown that they have different biological roles (23, 36, 37). In addition, microarray assays showed that each protein activates a unique set of genes in human cells (30), and the differences in the activities of these proteins have been mapped primarily to differences in their unique transcriptional activation and C-terminal domains (18). Thus, posttranslational modifications or other changes that affect the transcriptional activation domains of Myb proteins may have profound effects on their abilities to regulate specific genes (20, 27).

Alternatively spliced c-*myb* transcripts have been detected in human, mouse, and chicken hematopoietic cells (33, 34, 40). The best-characterized of these transcripts includes an alternate exon that encodes a variant of c-Myb with a 121-amino-acid in-frame insertion in the transcriptional activation domain (7, 32, 34, 42). Although biological roles for the variant c-Myb proteins remain unclear, alternatively spliced forms of chicken c-*myb* have increased transcriptional and transforming potential (42). For example, a variant of mouse c-*myb* formed through alternative RNA splicing enhanced cell survival in hematopoietic cell assays in which wild-type c-*myb* accelerated cell death (15). Thus, alternative RNA splicing permits the c-*myb* gene to encode multiple versions of the c-Myb transcription factor with unique biological activities.

Several recent studies have suggested that relatively minor changes in the transcriptional activation domain of c-Myb can dramatically affect its transcriptional activity. Since the alternative splicing of c-*myb* gene products is a mechanism for producing variants of c-Myb with different transcriptional activation domains, we established qualitative and quantitative assays to begin investigating the extent and the significance of alternative RNA splicing for the human c-*myb* gene. We found that normal primary human hematopoietic and leukemia cells produce a large and diverse set of c-*myb* transcripts that show cell type-specific expression patterns and that the alternately spliced c-*myb* transcripts encode proteins with distinct activities. The results have significant implications for understanding the role of Myb proteins in the regulation of hematopoiesis and for determining how wild-type and variant c-Myb proteins contribute to leukemogenesis.

* Corresponding author. Mailing address: Department of Molecular Genetics & Microbiology, MSC08 4660, 1 University of New Mexico, Albuquerque, NM 87131-0001. Phone: (505) 272-3464. Fax: (505) 272-3463. E-mail: jorourkejr@salud.unm.edu.

## MATERIALS AND METHODS

**Cell lines and primary hematopoietic cells.** The human hematopoietic cell lines K562 (ATCC CCL-240), THP-1 (ATCC TIB-202), Jurkat (ATCC TIB-152), and KG-1 (ATCC CCL-246) and the fibroblast cell line 293 (ATCC CRL-1573) were cultured as suggested by the American Type Culture Collection. The avian monocyte cell line HD11 was cultured in Dulbecco's modified Eagle's medium supplemented with 2% chicken serum, 8% fetal bovine serum, and $1\times$ penicillin-streptomycin solution. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

Buffy coats from healthy donors were obtained from United Blood Services (Albuquerque, NM). Peripheral blood mononuclear cells were isolated by Ficoll (Amersham, Piscataway, NJ) density centrifugation, and cell populations were depleted of monocytes by differential adherence. Protein or RNA was isolated from half of the nonadherent peripheral blood leukocytes (PBLs) immediately, while the remaining cells were cultured in Iscove's modified Dulbecco's medium (IMDM) with 10 U each of interleukin-2 (IL-2; PeproTech, Rocky Hill, NJ) and phytohemagglutinin (PHA; Gibco, Carlsbad, CA)/ml for 4 days prior to protein and RNA isolation.

Cryopreserved CD34$^+$ cells derived from cytokine-mobilized peripheral blood were obtained from the Fred Hutchinson Cancer Research Center Large-Scale Cell Processing Core. For the CD34$^+$ cell assays, $10^6$ CD34$^+$ cells per ml were cultured in IMDM supplemented with IL-3 (20 ng/ml), IL-6 (20 ng/ml), and stem cell factor and FLT-3 ligand (100 ng/ml each) (CC 100 cytokine cocktail; Stem Cell Technologies, Vancouver, Canada) for 4 days. Half of the cells were then cultured for 6 days in long-term myeloid medium (Stem Cell Technologies, Vancouver, Canada) supplemented with IL-3 and granulocyte-monocyte colony-stimulating factor (PeproTech, Rocky Hill, NJ) for myeloid differentiation as described previously (38). The remaining cells were cultured for 5 days in IMDM supplemented with IL-6, stem cell factor, and erythropoietin as described previously (14). Total RNA from CD34$^+$ cell experiments was isolated using RNAzol Bee (Tel-Test Incorporated, Friends, TX) at 0, 4, and 10 days postculture. Pediatric T-cell acute lymphoblastic leukemia (T-ALL) samples and adult acute myeloid leukemia samples were obtained from the Children's Oncology Group and the Southwest Oncology Group, respectively.

**RNA isolation and real-time quantitative PCR (qPCR) assays.** The isolation of total and cytoplasmic RNAs was performed using the RNeasy mini kit (Qiagen, Valencia, CA), and poly(A)$^+$ RNA was purified from the cytoplasmic RNA using the poly(A)$^+$ mRNA isolation kit (Miltenyi Biotech, Auburn, CA). cDNA synthesis using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) and SYBR green-based real-time PCR using a Dynamo PCR kit (New England Biotech, Waltham, MA) were done as suggested by the manufacturers. Analyses of PCR efficiency and melting-point curves of PCR products for each SYBR green primer pair were performed to verify that the primers were specific (see Fig. S2 in the supplemental material), and PCR was performed in triplicate using primers described in Table S1 in the supplemental material. The results of relative gene expression assays were normalized to the level of β2-microglobulin gene expression, and the data were analyzed using the comparative threshold cycle method (21). Plasmid standard curves ($10^6$ to $10^2$ copies) were generated for experiments measuring absolute (copy number) expression values.

**Polyribosome analysis.** Transcripts associated with polyribosomes were analyzed as described previously (9). Briefly, K562 cell cytoplasmic fractions were layered onto 40 to 60% Nyzode (Sigma, St. Louis, MO) gradients containing either cycloheximide (for ribosome clamping) or EDTA (for ribosome release). After overnight centrifugation, 24 gradient fractions were recovered and RNA was isolated using RNAzol Bee (Tel-Test, Friends, TX). RNA from each fraction was analyzed using a total RNA Nano Chip kit (Agilent Technologies, San Jose, CA). Virtual gels showing tRNA and 18S and 28S rRNA were prepared using the Agilent software, and transcript levels were measured by qPCR as described above. The percentage of each transcript in each gradient fraction was calculated by dividing the number of transcript copies in the gradient fraction by the sum of the numbers of transcript copies in all the gradient fractions analyzed.

**Expression plasmids, analysis of protein production, and transcription assays.** PCR products containing unique alternative exons were cloned in frame into a human c-Myb expression vector (pCDNA3; Invitrogen, Carlsbad, CA). For transfection assays, different amounts of expression plasmids (250 ng of the 14A variant c-Myb plasmid, the 13A variant c-Myb plasmid, and the 9B variant c-Myb plasmid, 180 ng of the wild-type c-Myb plasmid and the 8A variant c-Myb plasmid, and 90 ng of the 10A variant c-Myb plasmid and the 9A variant c-Myb plasmid), balanced by an empty vector, were used to equalize the levels of the proteins detected by Western blotting. The avian monocyte cell line HD11 was transfected using Superfect as recommended by the manufacturer (Qiagen, Valencia, CA). For endogenous *mim-1* gene assays, HD11 cells were transfected
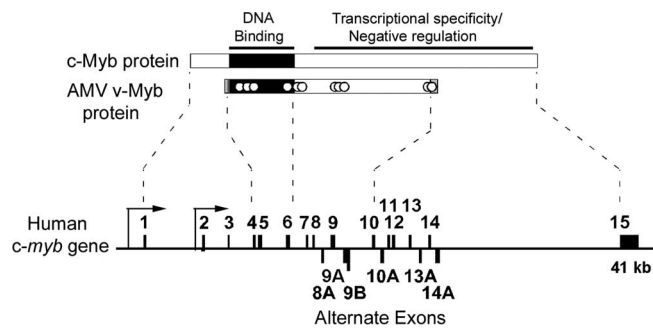


FIG. 1. Structure of the human c-*myb* gene. The 640-amino-acid c-Myb protein (top) contains a highly conserved DNA binding domain (shaded black) near the N terminus and a large C-terminal domain that controls transcriptional specificity and is the target of numerous regulatory pathways. The chicken v-Myb protein is a truncated and mutated version of c-Myb that lacks the N and C termini and that harbors 11 point mutations (indicated by white dots). The human c-*myb* gene (bottom) spans 41,000 nucleotides and contains 15 exons (shaded black, above the line). The arrows represent the 5′ and intronic promoters. The alternative exons are shown below the line and encode proteins that differ in the transcriptional specificity and negative regulation domain of c-Myb (dotted lines).

as described above, total RNA was isolated 48 h later from half the cells, and the remainder of the cells were lysed in boiling 2× sodium dodecyl sulfate buffer for protein analysis. The expression of *mim-1* RNA was analyzed using SYBR green-based real-time PCR, and relative *mim-1* gene expression levels were calculated using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression levels to normalize for equivalent cDNA levels. Primer sequences for *mim-1* and the GAPDH gene are given in Table S1 in the supplemental material. Proteins were analyzed by Western blotting as described previously by using a polyclonal antibody raised against the conserved DNA binding domain of c-Myb (8). For reporter gene activation assays, triplicate samples of cells in 24-well dishes were transfected with 350 ng of the c-Myb-responsive EW5 reporter gene, along with 50 ng of the wild-type c-Myb expression plasmid (or the splice variant expression plasmids indicated in Fig. 6 using the same ratios listed above) and 10 ng of a renilla luciferase plasmid as a control for transfection efficiency. Luciferase levels were analyzed 2 days later with a dual luciferase kit (Promega, Madison, WI).

Hep27, CXC chemokine receptor 4 (CXCR-4), cyclin A1, CFTR/MRP, and keratin-16 gene transcript levels in MCF-7 cells after adenoviral transduction were determined as previously described (30). Briefly, adenoviral vectors expressing the c-Myb splice variants indicated in Fig. 7 and coexpressing green fluorescent protein as a marker were constructed using the Adeasy system (22). More than 80% of MCF-7 cells were transduced, as assessed by green fluorescent protein expression. RNA was isolated from 90% of the cells by using an RNeasy mini kit (Qiagen, Valencia, CA), while the remaining 10% of the cells were boiled in sodium dodecyl sulfate buffer for Western blot analysis. Relative expression levels of the indicated genes were evaluated by quantitative reverse transcription-PCR using an assay-on-demand Taqman kit (Applied Biosystems, CA), and the results were analyzed as described above.

## RESULTS

**Alternative splicing of human c-*myb* RNA is frequent and cell type specific.** The human c-*myb* gene encompasses 15 exons on chromosome 6q22-q23 (Fig. 1). Exons 4 to 6 encode the highly conserved DNA binding domain (25), while exons 7 to 15 encode the C-terminal domains involved in transcriptional activation, specificity, and regulation (17, 18). The region encoded by exons 4 to 10 corresponds to the protein encoded by the v-*myb* oncogene from AMV and includes all the activities required for transformation and leukemogenesis. Several previous studies have investigated an alternatively spliced RNA containing exon 9B, which in its mouse and
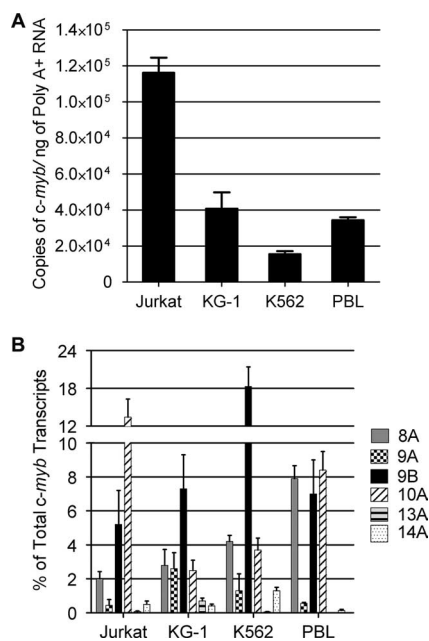
FIG. 2. The c-*myb* splice variants exhibit a cell type-specific expression pattern. (A) Total numbers of c-*myb* mRNA copies from various hematopoietic cells. Absolute levels of c-*myb* RNA were detected by performing reverse transcription and SYBR green-based real-time PCR with primers from exons 14 and 15, starting with 20 ng of cytoplasmic, polyadenylated RNA from the Jurkat T-cell line, the KG-1 myeloid leukemia cell line, or the K562 erythroid leukemia cell line or from PBLs after 4 days of activation. Total numbers of c-*myb* copies were determined using a plasmid standard curve. The bar graph shows the average numbers of c-*myb* mRNA copies detected per nanogram of starting RNA in two independent experiments, each with triplicate assays. Error bars represent standard deviations. (B) Percentages of c-*myb* transcripts containing alternative exons. Real-time PCR was performed as described above using primers to detect individual alternative exons, and the results are plotted as percentages of total c-*myb* transcripts containing the indicated exons. Error bars represent standard deviations.

chicken forms is referred to as 9A (7, 34). However, a search of entries for the human c-*myb* gene in the GenBank database (accession number U22376) suggested the presence of additional alternative exons (8A, 9A, 10A, 13A, and 14A), some of which have also been detected by PCR methods or analyses of expressed sequence tag clones (5).

Initial experiments using conventional reverse transcription-PCR assays with primers specific for the predicted alternative exons showed that each exon could be detected in RNA isolated from CD34$^+$ cells and bone marrow aspirates from leukemia patients, suggesting that hematopoietic cells expressed a wider-than-expected range of c-*myb* gene products (see Fig. S1 in the supplemental material). Therefore, we utilized quantitative reverse transcription and real-time PCR assays with purified cytoplasmic, poly(A)$^+$ mRNA as the template to obtain accurate expression data for various c-*myb* splice products. First, the absolute total levels of c-*myb* mRNAs were measured using primer sets designed to detect all variants of c-*myb*. As shown in Fig. 2A, Jurkat T-cell leukemia cells expressed the highest total levels of c-*myb* transcripts per nanogram of reverse-transcribed poly(A)$^+$ RNA, followed by the myeloid leukemia cell line KG-1 and normal peripheral blood lymphocytes

(PBLs) stimulated with IL-2 and PHA, each of which expressed c-*myb* at about one-third the level of expression in the Jurkat cells. The erythroid leukemia cell line K562 expressed sixfold less c-*myb* mRNA than the Jurkat cells.

For transcripts containing alternative exons, specific primers were used in qPCR assays, and plasmid standard curves were used to measure the absolute amount of each type of transcript as a percentage of total c-*myb* transcripts. In these assays, the percentage of alternately spliced c-*myb* isoforms varied from ~15% of total c-*myb* transcripts in KG-1 cells to ~30% of total c-*myb* transcripts in K562 cells. Approximately 22% of the c-*myb* transcripts in PBLs and Jurkat cells contained alternative exons (Fig. 2B). While the total fraction of alternatively spliced c-*myb* isoforms did not vary dramatically among cell types, each type of cell expressed a unique, qualitatively different pattern of alternatively spliced c-*myb* products (Fig. 2B). For example, exon 9B-containing transcripts were about 5% of the total in Jurkat cells but more than 18% of the c-*myb* transcripts in K562 cells. In contrast, exon 10A was detected in about 12% of the Jurkat cell c-*myb* transcripts but in only 2, 4, and 8% of the transcripts in the KG-1 and K562 cells and the PBLs, respectively. The 8A exon was detected in about 8% of transcripts in PBLs but only 2 to 4% of those in the leukemia cell lines. In all cell types, transcripts containing the 9A, 13A, or 14A exon were present at very low levels. The qPCR results presented here were confirmed using a shotgun cloning and sequencing approach (see Table S2 in the supplemental material), which gave similar results. From these quantitative assays, it appears that approximately 15 to 20% of the c-*myb* transcripts in primary human hematopoietic cells and several common cell lines contain at least one alternative exon, and the relative levels of different transcripts were variable and cell type specific. Thus, the repertoire of c-*myb* transcripts is complex and varies in different hematopoietic cell types.

**Alternative splicing of c-*myb* transcripts during hematopoietic cell differentiation.** The comparisons of different hematopoietic cell lines described above suggested that the alternative splicing of c-*myb* transcripts was regulated in a lineage-specific manner. We next analyzed the expression of various splice variants to determine whether c-*myb* alternative splicing was regulated during normal hematopoiesis. Primary human hematopoietic CD34$^+$ cells were cultured in vitro using a protocol that stimulated proliferation for several days, and then the cells were divided and transferred to conditions designed to stimulate differentiation toward granulocytes or erythrocytes for an additional 11 days (Fig. 3A). This standard CD34$^+$ differentiation protocol was previously used to investigate changes in gene expression and alternative splicing (6, 38). At various time points, the relative total c-*myb* transcript levels or levels of variant transcripts containing alternate exons were analyzed by qPCR. Differentiation-specific cell surface markers were assayed using flow cytometry in order to monitor the effectiveness of the differentiation protocols (data not shown). The results are shown in Fig. 3B, where the relative levels of each type of c-*myb* variant are compared to the changes in total c-*myb* transcript levels and plotted as the amounts of change (*n*-fold) compared to the starting levels in the unstimulated CD34$^+$ cells. (Note that, for illustrative purposes, each panel in Fig. 3B is plotted with a different scale, and the total c-*myb* transcript levels are plotted in all the panels.) As shown in Fig.
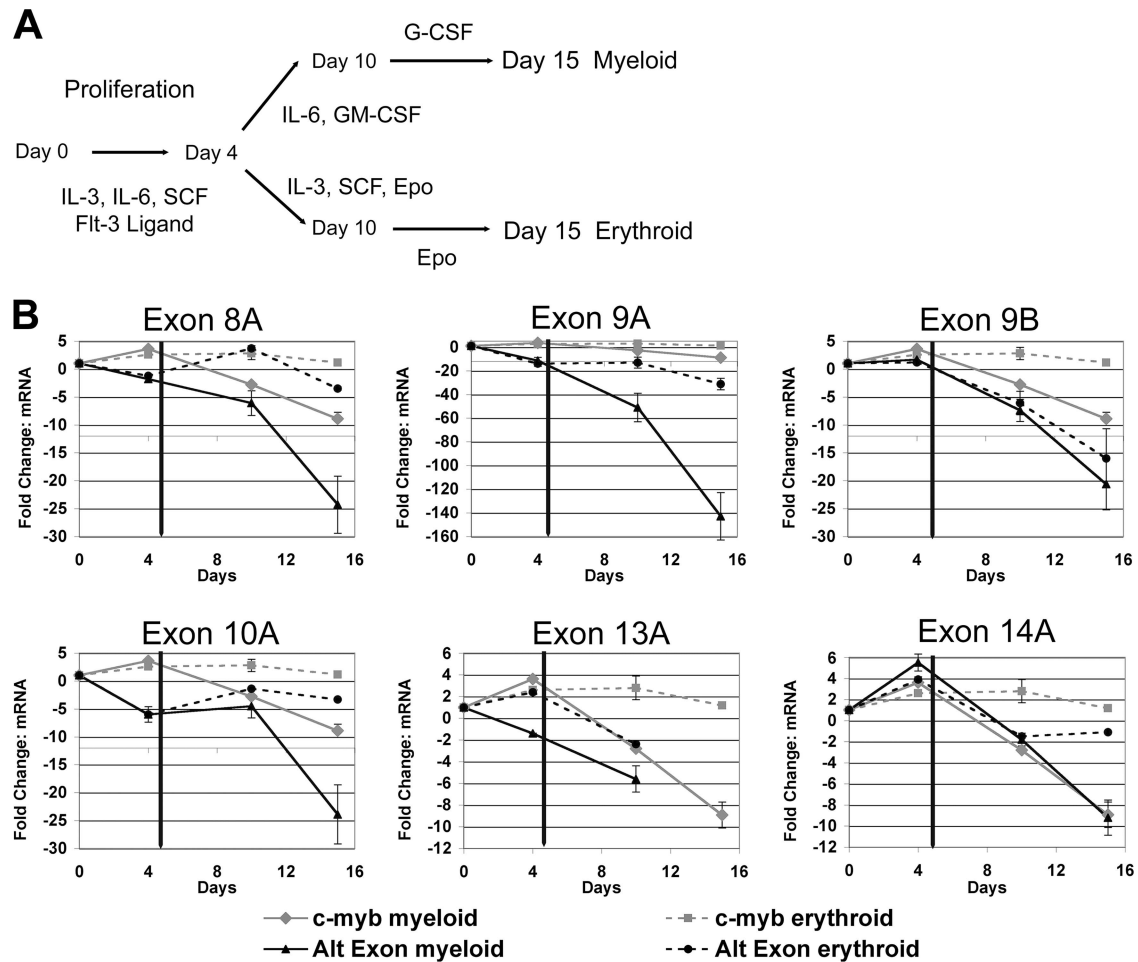
FIG. 3. Independent and lineage-specific expression of c-*myb* transcripts containing alternate exons during in vitro differentiation of CD34$^+$ hematopoietic cells. (A) Differentiation protocol for CD34$^+$ cells. Populations of primary human CD34$^+$ cells were expanded (days 1 to 4) and then differentiated toward the myeloid or erythroid lineage (days 5 to 15) by using the indicated cytokines. SCF, stem cell factor; G-CSF, granulocyte colony-stimulating factor; Epo, erythropoietin. (B) Results from exon-specific qPCR analysis of differentiating CD34$^+$ cells. RNA was isolated at the indicated time points, and qPCR was used to measure the levels of c-*myb* transcripts containing the indicated alternate exons. The graphs show the change (*n*-fold) in mRNA levels for the alternatively spliced c-*myb* variants, normalized by comparison to β2-microglobulin gene expression levels, relative to the mRNA levels in unstimulated CD34$^+$ (day 0) cells. The solid vertical line on day 4 represents the transition of the cells from proliferation toward differentiation. The solid black lines indicate splice variant levels in cells undergoing myeloid differentiation, while the dashed black lines represent those in cells undergoing erythroid differentiation. For comparison purposes, the relative change (*n*-fold) in total c-*myb* mRNA levels was plotted on each graph and is represented by the gray lines. Exon 13A was not detectable after 10 days in culture. Error bars represent standard deviations of triplicate qPCR measurements. Similar results were obtained in two independent experiments. Note that the scale on the y axis is different in each graph to show the large variation in relative mRNA levels for the different c-*myb* splice variants. Alt exon, alternative exon.

3B, the total levels of c-*myb* transcripts increased severalfold during the initial proliferation phase of the experiment (days 1 to 4) and remained fairly constant when the cells were switched to erythroid differentiation medium. However, total c-*myb* levels in the cells that were switched to granulocyte differentiation conditions (days 4 to 15) declined about ninefold, which is consistent with data in previously published reports (2).

The analysis of the alternatively spliced variants showed that each was expressed in a unique pattern during this proliferation and differentiation protocol. For example, the initial proliferation phase led to increases in the levels of transcripts containing exon 9B, 13A, or 14A (twofold, threefold, or sixfold, respectively), similar to the increase in total c-*myb* transcript levels. In contrast, during the same period, levels of transcripts

containing exon 8A, 9A, or 10A decreased 2-, 13-, and 6-fold, respectively, suggesting that the levels of the individual alternative transcripts were regulated independently. Even bigger differences were noted when the cells were stimulated to differentiate. Granulocyte differentiation conditions caused the levels of all the alternative exon-containing transcripts to decrease at least 2-fold (for those containing exon 13A or 14A) and as much as 50-fold (for those containing exon 9A) by day 10, although total c-*myb* transcript levels declined only about 2-fold during that time period. The erythroid differentiation conditions, which led to very little change in total levels of c-*myb* transcripts, caused the most unique changes in the levels of some of the variants. For example, by day 15 of erythroid differentiation, levels of transcripts containing exon 9A or 9B
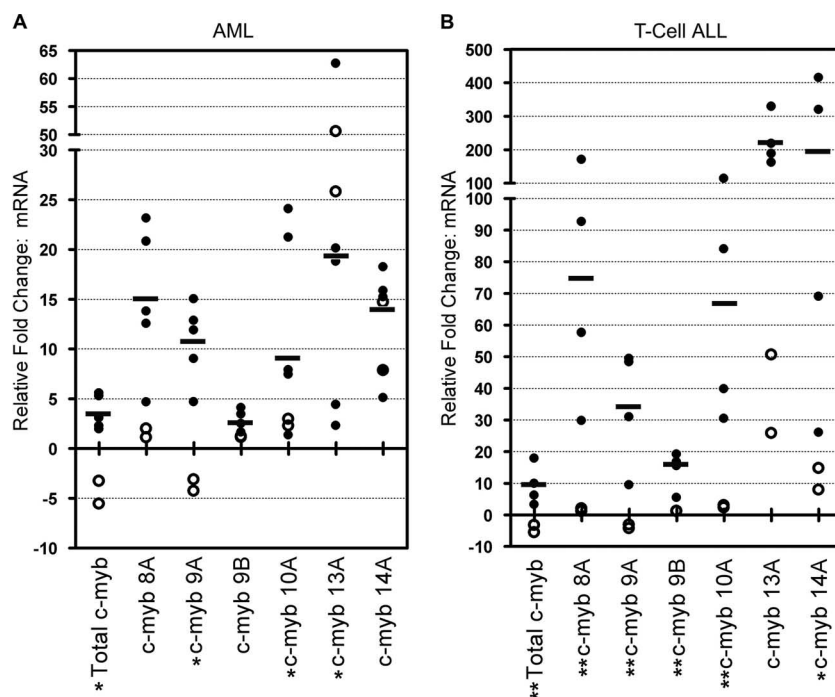
FIG. 4. The expression of the c-*myb* splice variants is altered in primary leukemia bone marrow samples. qPCR assays using primers for the indicated alternative exons were performed on bone marrow aspirates from patients with AML ($n = 5$; filled circles) or pediatric T-ALL ($n = 4$; filled circles) or from normal donors ($n = 2$; empty circles). Each dot represents the average of triplicate measurements from a single sample, and the horizontal bars represent the median values for the leukemia samples. The plots show the differences (*n*-fold) in the levels of each transcript compared to the levels in PHA-activated PBLs. Statistical analysis was performed using the two-sample F-test for variances. The c-*myb* splice variants that gave significantly different results for leukemia samples and samples from normal donors are labeled with * ($P \leq 0.05$), and highly significant differences are indicated with ** ($P \leq 0.01$).

had decreased about 15- to 30-fold while those of transcripts containing exon 8A, 10A, or 14A had decreased only 1.5- to 3-fold. These results suggest that changes in the patterns of c-*myb* alternative RNA splicing occur during normal hematopoietic cell differentiation, at least under these in vitro conditions, and occur in a lineage- and differentiation-specific manner. The results also imply that different types of hematopoietic cells express different combinations of normal and variant forms of c-Myb proteins, which may have unique transcriptional activities.

**Altered levels of c-*myb* transcripts in primary leukemia samples.** Truncated versions of c-*myb* have oncogenic activity in animals. Since the alternatively spliced forms of c-*myb* transcripts had the potential to encode truncated versions of the protein (see below), we tested whether expression levels of the alternative c-*myb* transcripts were altered in leukemia samples. RNA was prepared from normal bone marrow samples or from bone marrow samples collected from patients with pediatric T-ALL or adult acute myelogenous leukemia (AML), and then total levels of c-*myb* transcripts and levels of the splice variants were measured using qPCR assays and compared to the levels observed in PHA-activated PBLs. Consistent with data in previous reports (4, 16), total c-*myb* mRNA was overexpressed 13- and 40-fold in AML and T-ALL, respectively, compared to the level of expression in normal bone marrow samples (Fig. 4). Transcripts containing exon 8A or 10A were overexpressed at levels similar to the total c-*myb* overexpression levels in both AML and T-ALL, whereas relative levels of

transcripts containing exon 9A were significantly increased in T-ALL samples (as much as 180-fold compared to those in normal bone marrow samples). In contrast, levels of transcripts containing exon 9B, 13A, or 14A relative to total c-*myb* transcript levels in leukemia samples were decreased compared to those in normal bone marrow samples. A leukemia-specific pattern of c-*myb* splice variant expression was also observed in a pediatric B-cell ALL sample compared to the expression patterns in CD34^{+} and PHA-activated PBLs by using a shotgun cloning and sequencing approach (see Table S2 in the supplemental material). The data suggest that the c-*myb* gene may be a target for aberrant splicing during leukemogenesis, leading to the expression of a leukemia-specific signature of c-*myb* transcripts.

**Alternately spliced c-*myb* transcripts are associated with polyribosomes.** The expression profiles of the alternatively spliced c-*myb* transcripts suggested that the transcripts were individually regulated in specific patterns in different lineages and during hematopoietic cell differentiation. We next investigated whether the alternatively spliced c-*myb* variants were associated with polyribosomes, which would indicate that they are likely to be translated to produce different forms of c-Myb protein. Nyzode density gradients were used to fractionate the polysome-containing cytoplasmic extracts derived from K562 cells. In order to accurately assess polysome-associated mRNA and non-polysome-associated (free) mRNA density fractions, we compared samples analyzed on gradients containing cycloheximide, which prevents ribosome dissociation from mRNA,
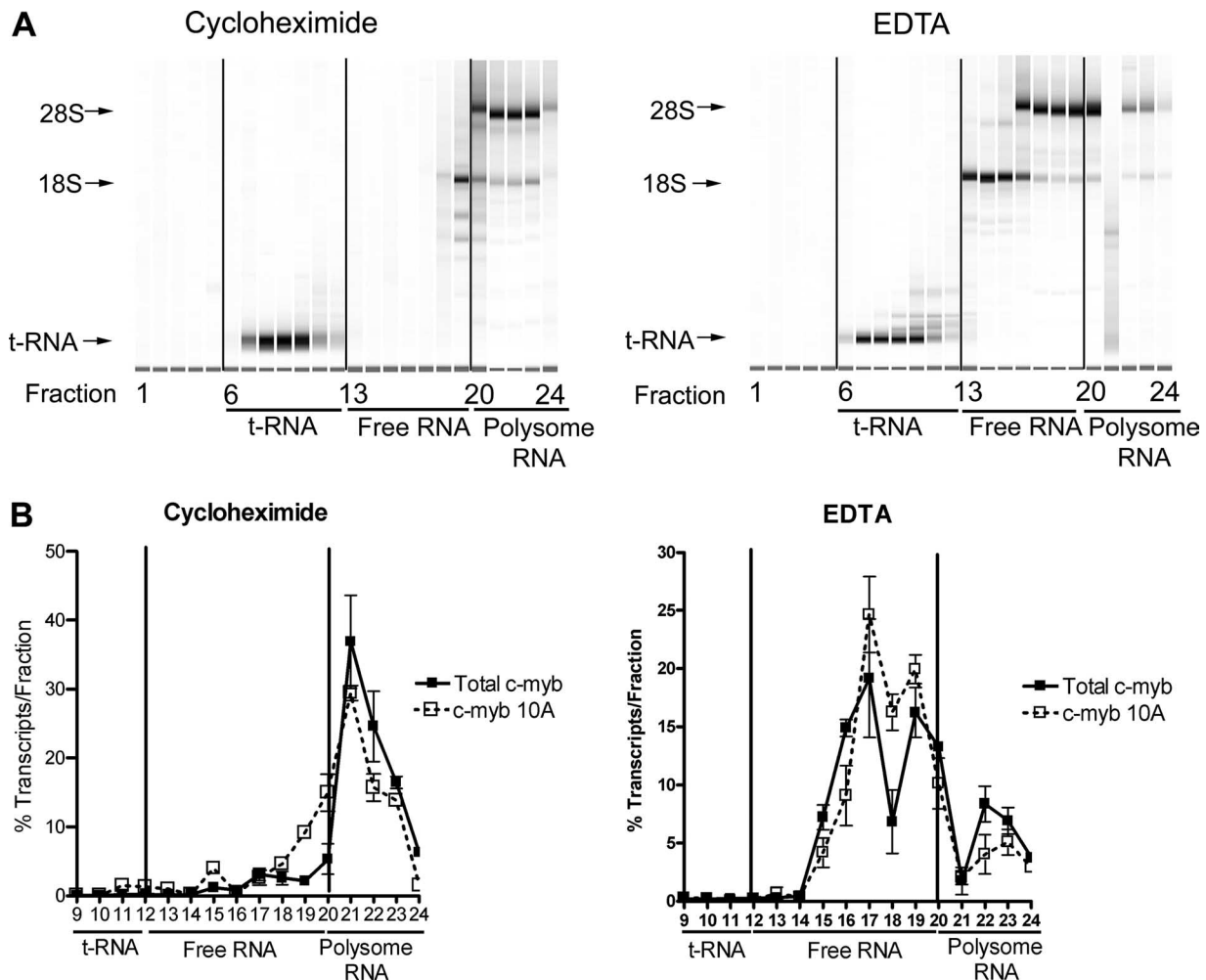
FIG. 5. The c-*myb* splice variants associate with polysomes. Cytoplasmic extracts isolated from K562 cells were fractionated on Nyzode density gradients containing cycloheximide (to inhibit polysome-RNA dissociation) or EDTA (to release polysomes from RNA). The gradients were separated into 24 fractions (fraction 1, top; fraction 24, bottom). (A) RNA profiles. RNA samples isolated from the gradients containing cycloheximide (left panel) or EDTA (right panel) were analyzed as described in Materials and Methods. Fractions 20 to 24 contained polysomes, as indicated by the presence of both the 18S and 28S rRNAs. Non-polysome-associated RNA was present in fractions 13 to 19, between the tRNA in fractions 7 to 12 and the ribosome-containing fractions. Note the shift toward lower-density fractions for the 18S ribosomal subunit in the EDTA gradient (compare fractions 13 to 19 in EDTA and cycloheximide gradients), confirming that the EDTA treatment disrupted the polysomes. (B) Results from qPCR analysis. Relative total c-*myb* transcript levels or levels of transcripts containing exon 10A were detected in the gradient fractions by qPCR. Copy numbers of each transcript were determined using plasmid standard curves. The plots show the percentages of transcripts recovered from each fraction. Note the shift in both total c-*myb* and exon 10A transcripts in the EDTA gradient. Nearly identical results were obtained for transcripts containing exons 8A, 9A, 9B, 13A, and 14A (not shown).

to samples run on gradients containing EDTA, which disrupts the ribosome-RNA complexes. Actively translated mRNAs are expected to shift to lower-density fractions in EDTA gradients than in cycloheximide gradients, since EDTA causes the more rapidly sedimenting polysomes to dissociate into mRNAs and free ribosomes. RNA samples from the gradient fractions were analyzed by electrophoresis and qPCR. As shown in Fig. 5A, RNA fractionated on the cycloheximide gradient yielded three distinct sets: tRNA (fractions 7 to 12), free RNA (fractions 13 to 19), and polysome-associated RNA (fractions 20 to 24). Treatment with EDTA did not change the migration of the tRNA, but it did cause a dramatic shift of the 18S and 28S rRNAs to lower-density fractions (fractions 13 to 16), confirming that the EDTA treatment disrupted the polysomes.

When the cycloheximide gradient fractions were analyzed by qPCR, the majority of total c-*myb* transcripts and the alternative exon-containing transcripts were found in the polysome-associated fractions (Fig. 5B, top panel). However, both types of transcripts shifted to the lower-density fractions containing the free mRNAs in the EDTA gradients. Although Fig. 5B shows the data only for exon 10A-containing transcripts, nearly identical results were obtained with the other alternative exon-containing transcripts (data not shown). Overall, a vast majority of the alternatively spliced transcripts (68 to 94%) were associated with polysomes in human K562 cells. Even the rarest c-*myb* splice variants were predominantly associated with polyribosomes. The results suggest that the c-*myb* splice variants are likely to be translated into proteins and imply that

human hematopoietic cells produce a mixture of different c-Myb proteins, which may have unique activities.

**Alternative splicing produces c-Myb proteins with different activities.** All of the alternative c-*myb* exons are in the region of the gene that encodes the transcriptional activation, specificity, and regulatory functions of c-Myb (18, 30). That part of c-Myb has been shown to be very sensitive to mutations: numerous studies have shown that c-Myb proteins with parts of that domain mutated or truncated have dramatically altered transcriptional activities (18, 20, 30). The c-*myb* splice variants are predicted to encode proteins with altered transcriptional activation and specificity domains, raising the possibility that they may display unique transcriptional activities.

Figure 6A summarizes the differences in the proteins encoded by the normal c-*myb* transcript, by the v-*myb* oncogene from AMV, and by the variants of c-*myb* containing the alternative exons described in this paper. Compared to c-Myb, the AMV v-Myb protein is truncated at both the N and C termini and has numerous point mutations, many of which are important for its transforming activity and which affect its ability to activate specific target genes (18). Each of the alternatively spliced c-*myb* variants described above is predicted to encode a different version of c-Myb, with the same N terminus and DNA binding domain as those of the normal c-Myb but with a unique C terminal domain. The transcript containing exon 8A would encode a protein containing the first 316 amino acids of c-Myb, followed by 34 novel amino acids. The 9A variant-encoded protein includes the first 401 amino acids of c-Myb, plus one additional methionine residue. Exon 9B leads to the insertion of 121 additional amino acids in the middle of the transcriptional activation and specificity domain. The exon 10A version includes 449 amino acids of c-Myb, prematurely truncated without any new amino acids. The addition of exon 13A produces a protein with 566 amino acids from c-Myb fused to a novel 100-amino-acid domain at the C terminus. Finally, adding exon 14A leads to a protein with the first 602 amino acids of c-Myb fused to 10 novel amino acids at the C terminus.

The activities of the normal and variant proteins were tested in several types of transcription assays to determine whether the altered C-terminal domains affected these activities. First, plasmids expressing normal c-Myb and the six variant proteins produced through alternative splicing were constructed. As shown in Fig. 6B, when HD11 monocyte cells were transfected with the plasmid vectors expressing the different splice variants, Myb proteins of the expected sizes, ranging from the 40,000-molecular-weight protein encoded by the 8A variant (lane 3) to the 83,600-molecular-weight protein encoded by the 9B variant (lane 5), were produced.

We first tested the activities of the various Myb proteins by using a well-established cotransfection assay incorporating a Myb-responsive reporter gene. Myb-negative HD11 cells were transfected with plasmids expressing normal and variant c-Myb proteins, along with a Myb-responsive reporter plasmid containing five high-affinity Myb binding sites from the Myb-regulated *mim-1* gene promoter upstream of a minimal promoter and a luciferase reporter gene. As shown in Fig. 6C, the expression of wild-type c-Myb resulted in an approximately 10-fold increase in reporter gene activity compared to the vector (cDNA) control. In contrast, the expression of the v-Myb protein from AMV led to >100-fold activation, illustrating that

the oncogenic mutations in v-Myb, including the changes in the C-terminal domain, lead to greater activity in this assay. Although they were expressed at similar levels, the six variant forms of c-Myb, produced from transcripts incorporating alternative exons, had widely different transcriptional activities. The proteins encoded by the 9B and 14A variants activated the reporter gene sevenfold and fivefold, respectively, and so had activities similar to that of normal c-Myb. The 8A variant protein was less active than normal c-Myb and activated the reporter gene only about 2.5-fold. Weakest of all was the 13A variant protein, which had little detectable activity in this assay. However, two proteins, the 10A and 9A variants, activated much better than normal c-Myb, stimulating the reporter gene about 10-fold and nearly 60-fold better than normal c-Myb and, thus, exhibiting activity similar to that of the oncogenic v-Myb protein. Since protein expression levels in the transfected HD11 cells were nearly equivalent (Fig. 6B), the nearly 600-fold range in transcriptional activities observed is likely due to differences in the C-terminal domains of the c-Myb splice variants.

The *mim-1* gene is a well-characterized Myb-regulated target gene that can be activated by the ectopic expression of c-Myb, but not the oncogenic v-Myb allele, in avian myeloid cells (11, 19, 20, 28, 29). Interestingly, c-Myb, but not v-Myb, is able to activate the endogenous *mim-1* gene, while both c-Myb and v-Myb are able to activate plasmid-based reporter genes containing the *mim-1* promoter, suggesting that the regulation of the endogenous gene is more restrictive, possibly requiring protein-protein interactions that are disrupted by the mutations in v-Myb (11, 18, 19, 20). We next tested the abilities of the variant c-Myb proteins to activate the *mim-1* gene to see if they behaved qualitatively more like c-Myb or v-Myb. Briefly, the HD11 myeloid cell line was transfected with the various Myb expression vectors, and the resulting *mim-1* gene expression was measured by qPCR 2 days later. As shown in Fig. 6D, the endogenous *mim-1* gene was activated about 22-fold in cells transfected with the vector expressing wild-type c-Myb compared to *mim-1* expression in cells transfected with a v-Myb expression vector or a control vector. The c-Myb splice variants had very different activities in this assay, ranging from little or no ability to activate the *mim-1* target gene (8A and 13A variants) to activity much higher than that of c-Myb (the 9A variant gave about 35-fold activation). Several variants, those corresponding to exons 9B, 10A, and 14A, activated the *mim-1* gene between 10- and 18-fold and so had activities that were comparable to that of normal c-Myb.

Recently, microarray assays have identified a number of human genes that are activated or repressed by different Myb proteins in mamma-derived cells (18, 20, 30). To investigate if the unique transcriptional properties observed for the c-*myb* splice variants in hematopoietic cells also occurred in other c-*myb*-expressing cell types, we tested the abilities of the variant proteins to activate several target genes in the MCF-7 mammary cell line. Briefly, adenoviral vectors expressing v-Myb, c-Myb, and the splice variants corresponding to exons 8A, 9A, 10A, and 13A were constructed and used to transduce the MCF-7 mammary cell line. After 18 h, Western blot analysis was used to check protein levels and qPCR was performed to test the expression of genes regulated by c-Myb (Hep27 and keratin-16 genes) and those regulated by v-Myb (cyclin A1 and
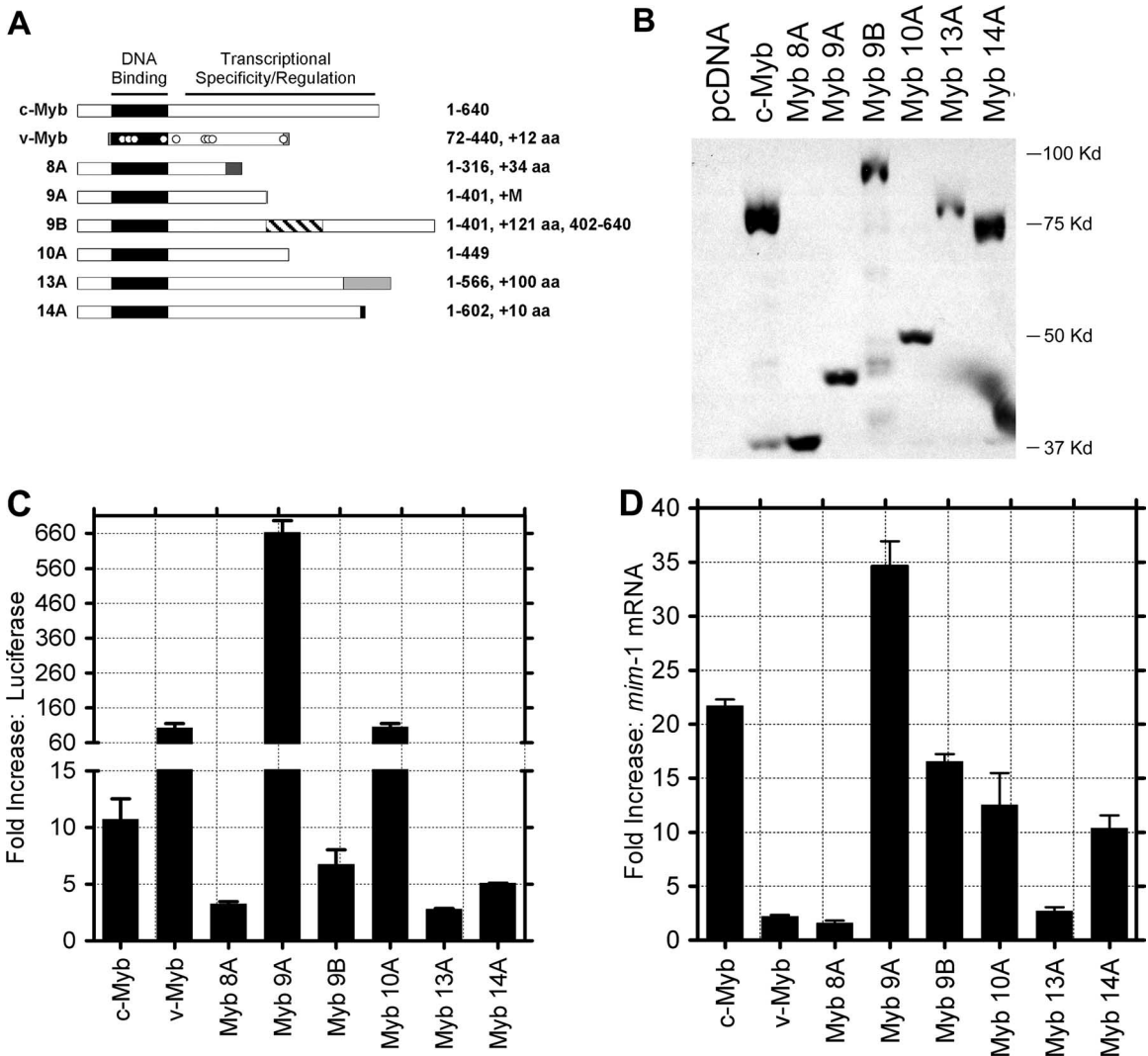
FIG. 6. Variants of c-Myb display unique transcriptional activities. (A) Diagrams of c-Myb proteins encoded by normal c-*myb*, the v-*myb* oncogene from AMV, and the alternatively spliced forms of c-*myb* containing exon 8A, 9A, 9B, 10A, 13A, or 14A. The highly conserved Myb DNA binding domain is labeled and shaded black. The transcriptional specificity and regulation domain of c-Myb (labeled) is affected by the alternative exons, as indicated by shaded boxes. The numbers on the right indicate amino acid (aa) residues from c-Myb plus novel amino acids encoded by the alternative exons. (B) Western blot analysis. HD11 monocyte cells were transfected with plasmids expressing the indicated c-*myb* splice variants. Whole-cell extracts were isolated 2 days later for Western blot analysis using anti-Myb antibodies to detect the expressed proteins. The numbers on the right show the migration of molecular size markers. (C) Activation of a c-*myb*-responsive reporter gene. Cells were cotransfected with plasmids expressing the indicated Myb proteins and a Myb-responsive reporter plasmid, and extracts were prepared and assayed for Myb-dependent luciferase activity after 2 days. Results are plotted as the average normalized luciferase activities from triplicate measurements in a representative experiment performed twice. Error bars represent standard deviations. Note that the scale has been split to show the large variation in the activities of the different c-Myb proteins. (D) Activation of an endogenous target gene. Chicken monocyte HD11 cells were transfected with the indicated c-Myb expression plasmids. After 2 days, RNA was isolated and qPCR was used to measure the levels of the Myb-regulated *mim-1* gene and the GAPDH control gene. The plots show the levels of induction (n-fold) of *mim-1*, normalized by comparison to the GAPDH gene, relative to the activation by an empty vector control. Error bars represent standard deviations of triplicate qPCR measurements. Similar results were obtained in two independent experiments.

CXCR-4 genes) (20, 30). Figure 7A shows that the 9A and 10A variants were expressed at levels comparable to those of wild-type c-Myb and v-Myb and that the 8A and 13A variants were expressed at somewhat lower levels in MCF-7 cells. When the gene expression assays were performed, each of the Myb proteins displayed a unique pattern of target gene activation. For example, the 9A variant activated the keratin-16 and Hep27 genes like c-Myb but also strongly activated the cyclin A1 and

CXCR-4 genes, which c-Myb did not activate but which were activated by v-Myb. Thus, the 9A variant appeared to have some activities that resembled those of normal c-Myb and others like those of v-Myb. The 10A variant had activities similar to those of the 9A variant but had less activity toward the cyclin A1 and CFTR target genes. Although the 13A variant had no detectable activity in the reporter gene or *mim-1* gene activation assays summarized in Fig. 6, it was a strong
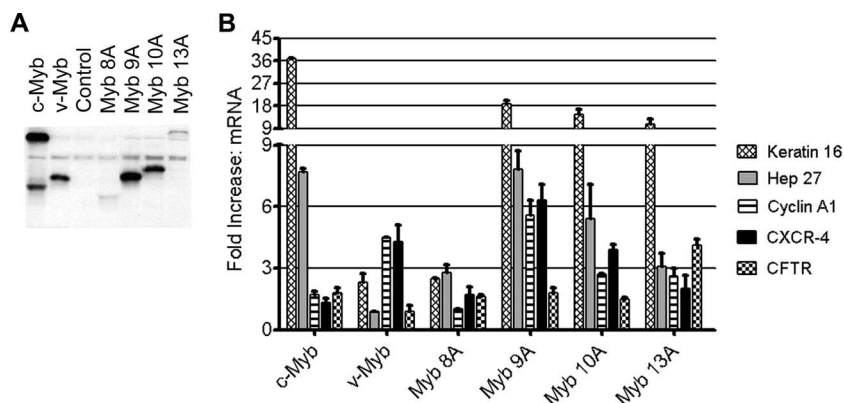
FIG. 7. Variants of c-Myb display qualitatively different transcriptional properties. (A) Western blot analysis. MCF-7 cells were transduced with adenoviral vectors expressing the indicated c-Myb variants. Whole-cell extracts were isolated from 20% of the cells 18 h posttransduction for Western blot analysis using anti-Myb antibodies to detect the expressed proteins. (B) Activation of endogenous target genes. RNA was isolated from the remaining MCF-7 cells, and qPCR was used to measure the levels of expression of a panel of genes regulated by c-Myb (keratin-16 and Hep27 genes) or v-Myb (cyclin A1 and CXCR-4 genes) and the GAPDH control gene. The CFTR gene was activated by c-Myb in primary monocyte cells but not MCF-7 cells. The plot shows the levels of induction (n-fold) of the indicated genes, normalized by comparison to the GAPDH gene, relative to the activation by a control adenoviral vector. The data presented are representative of an experiment performed two times, and error bars represent standard deviations of triplicate qPCR measurements. Note that the scale has been split to show the large variation in activities of the different c-Myb proteins on the various target genes.

activator of the keratin-16 and CFTR target genes in MCF-7 cells. Thus, the additional protein sequences at the C-terminal end of the 13A variant (Fig. 6A) had a dramatic effect on its activities. Taken together, these results suggest that the variant c-Myb proteins encoded as a result of alternative RNA splicing have unique transcriptional activities and regulate distinct sets of target genes.

## DISCUSSION

The expression of the c-*myb* gene is required for normal hematopoiesis (23) and for T-cell development (3), and this gene is expressed in all proliferating hematopoietic cells, where it presumably regulates genes involved in proliferation and differentiation. However, the mechanisms of c-*myb* regulation of multiple cell fate pathways and the cell type-specific genes required for such complex regulation are not yet understood. In this report, we demonstrate that the human c-*myb* gene generates several different transcripts through alternative RNA splicing whose protein products have diverse transcriptional properties. Quantitative assays showed dramatically different expression patterns and relative levels of c-*myb* variants in PBLs, CD34$^+$ cells, and primary leukemia samples, implying that the splicing of c-*myb* transcripts is highly regulated and lineage specific and that the ratios or the relative levels of alternatively spliced c-Myb variants play a role in the lineage-specific functions of hematopoietic cells. Furthermore, the ratios of alternately spliced c-*myb* transcripts changed dramatically in a lineage-specific manner following the in vitro differentiation of CD34 cells (Fig. 3). Thus, the regulation of c-*myb* alternative splicing may play an important role in hematopoietic cell differentiation.

Recent reports suggest that human gene transcripts undergo extensive alternative splicing (12), providing an important mechanism for creating diversity from a limited number of genes. Here, we categorized a variety of human c-*myb* splice variants containing alternative exons. During these studies, we also discovered additional alternatively spliced c-*myb* transcripts from primary hematopoietic cells that were formed by a wide range of complex splicing events, including the combinatorial use of alternative exons, the use of alternative 3′ splice donor sites in some exons, and exon skipping (see Table S2 in the supplemental material). Others have speculated that the variant c-*myb* gene products, many of which contain premature stop codons, are likely to be subject to nonsense-mediated RNA decay (5). However, our results for hematopoietic cell lines indicate that the stabilities of the alternatively spliced c-*myb* transcripts were not significantly affected by cycloheximide treatment and that the variant transcripts had rates of decay similar to that of the wild-type transcript (data not shown). Transcripts subject to nonsense-mediated decay are generally rapidly degraded following cycloheximide treatment, suggesting that most of the c-*myb* transcripts are not significantly affected by that regulatory mechanism. Furthermore, the alternatively spliced c-*myb* transcripts were found to be associated with polyribosomes (Fig. 5), suggesting that most of the variants are stable and are likely to encode functional proteins with altered C termini. Interestingly, Western blot analyses of hematopoietic cell lines with the use of an antibody to the c-Myb DNA binding domain detected multiple immunoreactive bands whose protein sizes were consistent with the predicted sizes of some of the variants (see Fig. S3 in the supplemental material). Antibodies specific for novel peptides in the variant proteins will be required to confirm that these proteins are stably expressed in primary cells.

One of the most interesting conclusions from our studies is that the different c-Myb splice variants display qualitative differences in their transcriptional activities. While all of the splice variants contained identical DNA binding domains, they had different C termini. Recent studies from our laboratory demonstrate that small changes in the C-terminal domains influence target gene specificity (18, 20, 30). In this study, we

showed that each splice variant product can activate a unique set of genes, suggesting that the alternate splicing of c-*myb* produces a family of transcription factors with unique biological activities. Therefore, it may be the ratios of alternatively spliced c-Myb proteins present in a specific cell type that allow c-Myb to regulate hematopoietic cell function in a lineage-specific manner.

In order for c-Myb to be transforming, it must be activated to unmask its transforming potential. For example, the c-*myb* gene is a common target of retroviral insertion leading to leukemia formation in mouse models (24, 41). Recently, two genetic abnormalities in the human c-*myb* gene in T-ALL patients were found, a duplication of the c-*myb* gene and a reciprocal translocation of c-*myb* and T-cell receptor beta loci, with both of these abnormalities leading to the overexpression of c-Myb (4, 16). Our results show that the c-*myb* gene may also be a target for deregulated or aberrant splicing in leukemia. An association between cancer and the deregulated alternative splicing of many different genes has been well documented previously (35, 39). However, recent reports suggest that the alternately spliced forms of genes such as those for the tyrosine kinase receptor RON (10) and the kinase SK61 (13) are themselves oncogenic when expressed inappropriately. Also, the overexpression of splicing factor 2/alternative splicing factor, which changes alternative splicing in a limited cohort of genes, can transform fibroblast cells, further linking aberrant splicing and oncogenesis (13). Our results show that the c-*myb* splicing patterns were different in bone marrow samples from control subjects and leukemia patients and, in particular, that exon 9A-containing transcripts were overexpressed nearly 120-fold in T-ALL samples. The inclusion of the alternate exon 9A produces a protein that is truncated similarly to the AMV v-Myb protein, which transforms immature myeloid cells and induces myeloid leukemias in animals. It is interesting that in our transcriptional assays, the exon 9A variant of c-Myb activated both c- and v-Myb-regulated genes (Fig. 7). These results suggest that aberrant or deregulated alternative splicing of the c-*myb* gene may be an additional mechanism to unmask its transforming potential. Additional experiments will be required to determine how different versions of c-Myb contribute to normal hematopoiesis and leukemogenesis and to identify the signaling pathways that regulate the alternative splicing of c-*myb* transcripts in human cells.

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