A Second *c-myb* Protein Is Translated from an Alternatively Spliced mRNA Expressed from Normal and 5'-Disrupted *myb* Loci

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The major protein encoded by the *c-myb* oncogene in many species has been identified as an unstable, nuclear DNA-binding protein with an apparent molecular mass of 75 to 80 kilodaltons (p75^{*c-myb*}). Recently, an alternatively spliced form of *c-myb*-encoded mRNA has been identified in murine cells containing either normal or rearranged *c-myb* genes. This mRNA includes a new exon, termed E6A, formed through use of cryptic splice sites located in the large intron between *c-myb* exons vE6 and vE7. E6A is predicted to contribute an internal 121-residue in-frame insertion into a region C terminal of the DNA-binding domain the *c-myb*-encoded protein. Here we report the identification of an 85-kilodalton (p85^{*c-myb-E6A*}) protein as the translation product of the alternatively spliced E6A *c-myb* mRNA. This protein as well as p75^{*c-myb*} were precipitated with anti-Myb antibodies raised against the conserved DNA-binding region of c-Myb. Proteolytic mapping studies showed that the two proteins are highly related but not identical. However, only the p85 protein reacted with an antiserum prepared against the E6A region expressed in bacteria, demonstrating that p85 but not p75 contains E6A sequences. In addition, the mobilities of both p85 and p75 were increased in myeloid tumor cell lines containing proviral integrations upstream of the 5' coding exons of v-*myb*, indicating that both proteins are truncated forms of c-Myb expressed from the same disrupted allele. p75^{*c-myb*} and p85^{*c-myb-E6A*} were indistinguishable with respect to nuclear localization and protein half-life. Furthermore, both forms of Myb were synthesized continuously throughout the cell cycle in 70Z pre-B cells. The contribution of the E6A domain to *c-myb* function remains to be elucidated.

The *c-myb* proto-oncogene encodes a predominantly nuclear protein (c-Myb) with an apparent molecular weight of 75 to 80 kilodaltons in avian, murine, and human cells (5, 15, 16, 18). Translation of murine c-Myb is thought to initiate at the first ATG in *c-myb* (located in exon UE3) and to terminate in a 3' exon downstream of vE7 (1, 10; Fig. 1). Expression of *c-myb* appears to be largely, but probably not exclusively, confined to hematopoietic cells (7, 11, 27, 35), and oncogenic activation of *myb* has been associated primarily with myeloid neoplasias (3, 22, 29, 30, 33). It has been suggested that *myb* becomes oncogenically activated through specific structural alterations of Myb. For example, transformation of myelomonocytic cells by avian myeloblastosis virus and E26 leukemia virus is mediated through v-*myb*-encoded proteins that lack similar 5' and 3' coding regions in comparison with c-Myb (8, 26). In addition, murine myeloid tumors induced by murine leukemia virus contain disrupted *c-myb* alleles as a result of proviral insertions in the 5' end of *c-myb*, leading to the loss of the same N-terminal region that is missing in avian v-Myb (30-32).

Myb proteins contain a DNA-binding activity (21) that has been localized to an internal highly conserved 53-residue imperfect threefold repeat in the N-terminal third of c-Myb (17, 24). Two of the three repeats are retained in all activated c-Myb proteins and had been shown to be required for transforming function (14). Whereas the DNA-binding affinity of Myb was initially analyzed on random DNA sequences, more recent experiments have defined a high-affinity interaction with a specific hexanucleotide sequence (2).

Although 5' heterogeneity in *c-myb* transcripts has been detected (1), p75^{*c-myb*} is thought to be translated from the

major *c-myb* mRNA, which contains the *c-myb* coding exons UE3 to UE1 and vE1 to vE7 and 3' exons (Fig. 1). In contrast, tumor-specific Myb proteins expressed from the virus-disrupted allele are predicted to lack either the UE3 to UE1 or the vE1 to vE7 coding exons (9, 30). Recent evidence indicates that an alternatively spliced form of *c-myb* exists. An additional exon, termed E6A, was first identified in cDNA clones isolated from *myb* transcripts derived from one of the tumors that carries a disrupted *c-myb* allele and was proposed to be formed as a consequence of upstream proviral insertion (25). RNA blotting and RNase mapping studies, however, show that the E6A exon is present as a minor form of *c-myb* transcripts in both normal and clonal tumor cells (28). *myb* RNAs containing E6A would be expected to contain 121 additional amino acids inserted in-frame between exons vE6 and vE7.

This study was undertaken to determine whether the alternatively spliced transcript is translated into a Myb-related protein and to investigate the expression of N-terminally truncated Myb proteins from the virus-disrupted *myb* locus. We have identified these proteins and carried out an initial characterization of the various forms of Myb in cells containing normal and rearranged *c-myb* genes.

MATERIALS AND METHODS

Animals and cells. The mouse pre-B-cell line 70Z and the myeloid cell lines MML7, MML9, and ABML4.6 have been described previously (23, 29). Thymocyte suspensions were prepared from thymuses of 4-week-old WC/ReJ mice by using a loosely fitted Dounce tissue homogenizer. Thymocytes were then washed twice in phosphate-buffered saline before being labeled with [³⁵S]methionine.

Generation of the *trpE*-E6A fusion protein. A 274-base-pair *EcoRI*-*AatII* E6A-specific fragment of the mouse *myb* gene

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was isolated from the previously described C3 plasmid (28), blunt ended, ligated with *Bam*HI linkers d(pCGCGGATC-CGCG), and then subcloned into the open reading frame of an inducible *trpE* expression vector, pATH-1 (kindly provided by T. J. Koerner and A. Tzagaloff, Columbia University, New York, N.Y.), by using standard procedures (20). Minilysates of transformed *Escherichia coli* HB101 were screened by restriction enzyme mapping to select the correct clone. The *trpE*-E6A fusion protein product (Bp45-E6A-Myb) was expressed in *E. coli* HB101 and purified by salt extraction.

Antisera. The anti-Myb serum was raised in a rabbit against a fusion protein encoding the 115 N-terminal amino acids of avian myeloblastosis virus p48^{v-myb} (the *myb* expression vector clone Bp52-Myb was kindly provided by J. Lipsick, University of California at San Diego) and has been previously characterized (5, 19).

An antiserum was prepared against a synthetic peptide corresponding to residues 40 to 55 of the predicted E6A-specific insert. The peptide synthesized was *N*-acetyl-HG TRSAGEPSLRVTRRKY (single-letter amino acid code). The high-pressure liquid chromatography-purified peptide was linked through the final tyrosine residue to bovine serum albumin by the bis-diazotized benzidine procedure, and the conjugate was used to prepare a rabbit antiserum. The serum was affinity purified through a bovine serum albumin column to remove anti-bovine serum albumin antibodies and through a column of Sepharose-conjugated E6A peptide to enrich for anti-E6A peptide antibodies (see reference 12 for details).

A second anti-E6A serum was raised in a rabbit against the Bp45-*trpE*-E6A fusion protein by J. Hatgi at the Animal Holding Facility, Frederick Cancer Research Facility, Frederick, Md. It was then characterized by immunoblotting of lysates that express the Bp45-*trpE*-E6A protein.

Metabolic cell labeling, immunoprecipitation, and gel electrophoresis. Labeling of cells, immunoprecipitation of Myb, and gel electrophoresis were performed as described previously (19). For one-dimensional *Staphylococcus aureus* V8 protease digestion, [³⁵S]methionine-labeled protein bands were cut from an unfixed sodium dodecyl sulfate (SDS)-polyacrylamide gel and treated as described by Cleveland et al. (6). For blocking, the antiserum was preincubated for 20 min at room temperature with 20 to 40 µg of cognate fusion protein. For subcellular fractionation, labeled cells were suspended in STM buffer (250 mM sucrose, 50 mM Tris hydrochloride [pH 7.5], 5 mM MgCl₂, 0.5% aprotinin, 10 mM iodoacetamide [IAA]), incubated on ice for 20 min, and broken in a tight-fitting Dounce homogenizer. Nuclei were then pelleted and lysed in Ab buffer (19). The supernatants were adjusted to 0.5% each Nonidet P-40, deoxycholate, and SDS before immunoprecipitation.

Elutriation. 70Z cells from an exponentially growing culture were separated by counterflow centrifugation in Tris-buffered saline containing 0.5 mM EDTA and 5% calf serum at 4°C into fractions 1 to 7 (in order of increasing cell size, measured with an electronic cell counter-sizer). The average DNA content of cells from each fraction was determined by fluorescence-activated cell-sorting analysis after staining of the nuclei with propidium iodide (13).

RESULTS

Identification of multiple forms of Myb. For our initial experiments, we used an antiserum raised against a bacterially expressed fragment of the N-terminal region of p45^{v-myb} (anti-Bp52-Myb-specific antibodies [5]) to identify Myb pro-

FIG. 2. Comparison of c-Myb and truncated c-Myb proteins. (A) The pre-B lymphoma cell line 70Z, with an apparently normal *c-myb* locus, and the myeloid cell lines MML7 and MML9, with proviral integration upstream of vE1 and UE1, respectively, were labeled for 20 min with [³⁵S]methionine. Samples containing equal amounts of radioactivity were subjected to immunoprecipitation with anti-Bp52-Myb expression vector serum. A fluorograph of a 10% SDS-polyacrylamide gel is shown. (B) The [³⁵S]methionine-labeled polypeptides shown in panel A were excised from a parallel unfixed gel and digested with 10 ng (lanes 1 to 6) or 100 ng (lanes 7 to 12) of *S. aureus* V8 protease. Peptides were analyzed on a 20% SDS-polyacrylamide gel. The arrows point to three peptides that are common to the six proteins analyzed.

teins from extracts of 70Z mouse pre-B cells. These cells contain an apparently unrearranged *c-myb* locus. The 70Z cells were labeled with [³⁵S]methionine, lysed in a mixture of detergents, and treated with anti-Bp52-Myb antibodies as described in Materials and Methods. The anti-Myb antibodies precipitated two major proteins with apparent molecular masses of 75 and 85 kDa (Fig. 2A, lane 1). Bands of 48, 110, and 190 kDa as well as several minor proteins were also detected. Precipitation of the 75-, 85-, 110-, and 190-kDa proteins was competitively blocked by addition of the bacterially expressed segment of Myb used to prepare the anti-Bp52-Myb antiserum, suggesting that these proteins were specifically recognized by the antiserum (also see Fig. 4A, lanes 1 and 2). The 75-kDa protein was the size of murine c-Myb previously identified by using antibodies raised against the same immunogen (5), whereas the 85-kDa band was of the size and quantity expected for the c-Myb translated from the alternatively spliced E6A-containing *myb* RNA that is detected in low abundance in these cells (unpublished observation for 70Z cells). The 110- and 190-kDa bands could be Myb-associated or Myb-related proteins, but their low levels and the variability of their precipitation precluded further analysis.

If the 85-kDa protein precipitated with our anti-Bp52-Myb antiserum is actually a form of p75^{c-myb} with additional amino acid sequences derived from E6A, then cells harboring *c-myb* genes with amino-terminal truncations should still

synthesize both proteins but with lower apparent molecular masses. We therefore examined two murine myeloid tumor cell lines that contain different Moloney murine leukemia virus insertions at the 5' side of *c-myb*. MML7 cells contain an integrated Moloney murine leukemia provirus in the intron between UE1 and UE1, whereas MML9 cells possess a proviral insertion in the intron between UE2 and UE1 (Fig. 1; 32). Previous cDNA cloning and sequencing analyses showed that these insertions would result in the production of N-terminally truncated c-Myb proteins lacking approximately 3 kDa (coding exons UE3 and UE2) in MML9 and 5 kDa (coding exons UE3 to UE1) in MML7 (9, 32). When detergent lysates from labeled MML7 and MML9 cells were immunoprecipitated with anti-Bp52-Myb antibodies, we noted that both of the major *myb*-specific proteins were expressed at levels comparable to those in 70Z cells (with an apparently normal *c-myb* locus) but appeared to have shifted to the predicted lower apparent molecular masses of 70 kDa, for MML7 cells and 72 kDa for MML9 cells (Fig. 2A; compare lanes 1 to 3). Precipitation of all six proteins was blocked by the bacterially expressed Bp52-Myb fragment (see Fig. 4A). Further evidence that the anti-Bp52-Myb-reactive proteins from these cell lines are related was derived from partial proteolytic mapping with V8 protease. All of the immunoprecipitated proteins contained multiple comigrating V8 digestion fragments (Fig. 2B). There were clear differences in the V8-generated fragment patterns as

FIG. 3. Expression of E6A sequences in bacteria. Total protein from bacteria that carried the E6A-Myb plasmid was fractionated in SDS-polyacrylamide gels and stained with Coomassie brilliant blue (A) or blotted onto nitrocellulose and stained with anti-E6A peptide serum (B). The bacteria were grown in the absence (lanes 1) or presence (lanes 2) of IAA. Sizes (in kilodaltons) are indicated on the right.

well as similarities, as would be expected from the N-terminal truncations and from the presence of additional sequences or modifications.

Expression of E6A sequences in *E. coli*. The larger form Myb observed in Fig. 2 could have arisen from posttranslational modification, alternative translational initiation, or translation of the alternatively spliced *c-myb* mRNA containing the E6A sequences. To distinguish among these possibilities, we prepared antiserum specifically reactive with the amino acid sequence predicted to be encoded by E6A. To do this, we first expressed 274 base pairs of the E6A sequences in bacteria by inserting the *Eco*RI (nucleotide 32)-*Aat*II (nucleotide 305) fragment (where nucleotide 1 is the first nucleotide in E6A) into the open reading frame of the *E. coli trpE* gene of plasmid pATH-1.

Figure 3A shows a Coomassie blue-stained gel of the proteins present in crude extracts of bacteria transformed with the *trpE*-E6A vector after growth without (lane 1) or with (lane 2) IAA, an inducer of *trpE* expression. IAA clearly induced expression of a protein of 45 kDa, the size expected for the *trpE*-E6A fusion protein, termed Bp45-E6A. That this protein actually contained sequences derived from E6A was demonstrated by immunoblotting of the bacterial proteins, using antisera raised against a synthetic peptide corresponding to residues 40 to 55 of the E6A reading frame (see Materials and Methods). The IAA-induced 45-kDa protein was highly reactive with the anti-E6A peptide serum (Fig. 3B). The Bp45-E6A protein was purified by salt extraction and used to prepare rabbit antiserum (anti-Bp45-E6AMyb).

Proteins encoded by *c-myb* and virus-disrupted *c-myb* contain the E6A domain. We first used the anti-Bp45-E6AMyb serum to analyze proteins in cells expressing normal *c-myb*. 70Z cells and normal thymocytes were labeled with [³⁵S]methionine, lysed, and immunoprecipitated with either anti-Bp52-Myb or anti-Bp45-E6AMyb. The 85-kDa Myb, but not the previously identified 75-kDa Myb, was precipitated by the antiserum against the E6A region (Fig. 4A, lanes 1 to 4; Fig. 4B). This immunoprecipitation was competitively inhibited by addition of the bacterial Bp45-E6A protein. In contrast, the anti-Bp52-Myb serum, raised against the Myb DNA-binding region, specifically reacted with both the 75- and 85-kDa proteins. We also detected low amounts of a 45-kDa protein specifically precipitated by anti-Bp45-

E6AMyb but not anti-Bp52-Myb antibodies. We do not yet understand the relationship, if any, of this protein to Myb.

Analysis of immunoprecipitates from MML7 and MML9 cells, which contain truncated *c-myb* alleles, showed that the larger forms of the truncated c-Myb proteins contained E6A sequences and that the smaller forms did not (Fig. 4A, lanes 5 to 12). An anomaly was found in MML9, in which the anti-Bp45-E6AMyb serum precipitated not only the larger (p82) form, as expected, but apparently also one of the proteins present in the highly heterogeneous p72 band (Fig. 4A, lanes 10 to 12). It is unclear whether this anti-E6A-reactive protein was derived from another alternatively spliced mRNA in these cells or was simply a breakdown product of p82. Our data indicate that alternate forms of Myb that contain E6A coding sequences are present in significant amounts in cells with either normal or 5'-truncated *c-myb* loci. For simplicity, we shall refer to these as Myb-E6A proteins.

Comparison of in vitro and in vivo Myb and Myb-E6A translation products. To further substantiate the conclusion that the various forms of Myb immunoprecipitated by anti-Bp52-Myb and anti-Bp45-E6AMyb sera are encoded by the *c-myb* transcripts previously analyzed in these cells, we subcloned fragments from four different cDNA clones that contain the open reading frames for c-Myb (obtained from 70Z cells [1]), Myb-E6A, and the N-terminally truncated c-Myb proteins (obtained from ABML2, which carries a virus-disrupted *myb* allele analogous to that carried by the MML7 [30]) into pGEM expression vectors. RNAs obtained from linearized vectors by using the appropriate bacteriophage RNA polymerase were translated in vitro by using a rabbit reticulocyte lysate. The [³⁵S]methionine-labeled in vitro translation products were compared with the immunoprecipitated in vivo Myb proteins by SDS-polyacrylamide gel electrophoresis. Cell-free translation of RNA from Myb and Myb-E6A cDNAs produced proteins that differed in apparent molecular mass by approximately 10 kDa (Fig. 5, lanes 1 and 2). Myb and Myb-E6A cDNAs in which the first three 5' exons (UE1 to UE3; Fig. 1) had been removed produced smaller forms of both proteins (Fig. 5, lanes 7 and 8). Immunoprecipitation of the in vitro translation products showed that the anti-Bp52-Myb serum recognized all four forms, whereas the anti-Bp45-E6AMyb serum recognized only the forms of Myb that contained E6A sequences (data not shown). We therefore conclude that our anti-Bp45-E6AMyb serum is specific for the E6A sequences.

When the in vitro-produced proteins were compared with Myb proteins immunoprecipitated from 70Z and MML7 cells, we found that both the in vivo Myb and Myb-E6A proteins migrated slightly slower (by 1 kDa) than did the in vitro products. Treatment with alkaline phosphatase increased the mobility of the in vivo proteins but still did not produce exact comigration with the in vitro forms (Fig. 5; compare lanes 1 and 2 with lanes 3 and 4 and lanes 5 and 6 with lanes 7 and 8). This result suggests that at least part of the difference in electrophoretic mobility between the in vivo- and in vitro-produced proteins was due to phosphorylation. The remaining difference may have been due to phosphorylation that was resistant to alkaline phosphatase treatment or to some other forms of posttranslational modification.

Myb and Myb-E6A exhibit similar short half-lives, are synthesized throughout the cell cycle, and are localized to the nucleus. To determine whether the E6A sequences present in Myb-E6A altered its metabolism with respect to the smaller form of Myb, we carried out experiments to compare the

FIG. 4. Analysis of Myb proteins immunoprecipitated with an E6A-specific serum. Cell lines 70Z, MML7, and MML9 (A) and mouse thymocytes (B) were labeled for 20 min with [35 S]methionine. Samples of the lysates were normalized for equal amounts of incorporated radioactivity and were immunoprecipitated with anti-Bp52-Myb serum preincubated with its cognate *trpE*-Myb fusion protein (block lanes 1, 5, and 9 in panel A; lane 1 in panel B), with anti-Bp52-Myb serum (lanes 2, 6, and 10 in panel A; lane 2 in panel B), with anti-Bp45-E6A serum (lanes 3, 7, and 11 in panel A; lane 3 in panel B), or with anti-Bp45-E6A serum preincubated with its cognate *trpE*-E6A fusion protein (block lanes 4, 8, 12, and 16 in panel A; lane 4 in panel B). i, Samples precipitated with immune sera; b, samples precipitated with block sera. Samples were analyzed on 10% SDS-polyacrylamide gels. Each bracket indicates a set of four small polypeptides and the large arrow marks a protein of 110 kDa, all of unknown origin, immunoprecipitated with the anti-Myb serum. The small arrow points to a protein found in all three cell lines as well as in thymocytes specifically recognized by the anti-E6A serum. The arrowhead identifies an anti-E6A serum-specific protein present only in MML9 cells (see Discussion).

degradation and synthesis of the two forms. We measured the turnover of Myb and Myb-E6A in 70Z cells in a series of pulse-chase experiments. The two protein forms had essentially identical half-lives of approximately 40 min (data not shown), a turnover rate in agreement with previous determinations (15, 19). N-terminal truncation of the proteins, as in MML7 cells, did not alter the rate of turnover (data not shown).

It has been shown that *c-myb* mRNA is constitutively expressed in chicken thymus but varies 5- to 10-fold through the cell cycles of various other cell types, with the highest levels occurring in subpopulations enriched for S-phase cells (34). We measured the synthesis of the two protein forms as a function of the cell cycle. An exponentially growing population of 70Z cells was subjected to centrifugal elutriation to separate cells in different cell cycle phases on the basis of volume. The DNA content of the separated fractions was determined by fluorescence-activated cell-sorting analysis after staining with propidium iodide. The seven elutriated subpopulations represented cells enriched in G1, S, and G2/M phases of the cycle (Fig. 6A). The different populations normalized for cell number were labeled with [35 S]methionine, lysed, and immunoprecipitated with anti-Bp52-Myb. Interestingly, synthesis of both Myb and Myb-E6A appeared to be the same during each of the cell cycle phases (Fig. 6B). Thus, both the E6A-containing and the

c-Myb proteins are not preferentially expressed as a function of the cell cycle of 70Z pre-B cells.

The protein products of the *v-myb* gene and its normal cellular counterpart have been shown to be located in the cell nucleus (4, 18), where Myb is thought to function as a DNA-binding protein. The nuclear transport of Myb appears to require sequences within the highly conserved amino-terminal region of the *v-myb* gene (14). Although this domain is also present in the various forms of Myb identified in this study, it is not clear whether the additional sequences contributed by E6A might affect localization. We therefore examined localization of Myb in 70Z cells and in the myeloid tumor ABML4.6, in which Myb is N-terminally truncated in the same region as MML7 and contains an additional 28 N-terminal amino acids derived from the viral *gag* gene sequences (30). The cells were lysed by Dounce homogenization and separated by centrifugation into nuclear and cytoplasmic compartments. Each fraction was then solubilized with detergents and subjected to immunoprecipitation with anti-Bp52-Myb antibodies. We observed a predominant nuclear localization for both normal and truncated forms of Myb and Myb-E6A (Fig. 7). Little or no Myb was detectable in the cytoplasmic fraction, although in control experiments we were able to detect the cytoplasmic enzyme ornithine decarboxylase by immunoblotting (data not shown). Our

FIG. 5. Comparison of in vivo- and in vitro-synthesized Myb proteins. Myb proteins were immunoprecipitated with anti-Bp52-Myb serum from [35 S]methionine-labeled 70Z (lane 4) and MML7 (lane 5) cells and treated with alkaline phosphatase (lanes 3 and 6). cDNAs coding for c-Myb (lane 2), c-Myb containing E6A sequences (lane 1), UE1- to UE3-truncated c-Myb (lane 7), and UE1- to UE3-truncated c-Myb containing E6A sequences (lane 8) were in vitro transcribed, and the cRNAs were translated in rabbit reticulocyte lysates. Arrowheads point to the in vivo proteins; arrows point to the in vitro-synthesized proteins. The samples were analyzed on a 10% SDS-polyacrylamide gel. For lanes 1, 5, and 6, the gel was exposed three times longer than for the other lanes.

results indicate that all of the Myb protein forms identified thus far are localized in the cell nucleus.

DISCUSSION

Previous studies have identified the protein encoded by the *c-myb* proto-oncogene in avian, murine, and human cells as a 75- to 80-kDa nuclear protein (p75^{c-myb}) that is probably initiated in UE3 and terminated in a 3' exon downstream of vE7 (Fig. 1). The p75^{c-myb} protein contains a highly conserved DNA-binding domain that has been localized to the sequences within vE1 to vE3 (14, 17). We report here the identification of a second, larger *c-myb*-encoded protein (p85^{c-myb-E6A}) in murine cells that is highly related to p75^{c-myb} but contains an additional peptide region. The p85^{c-myb-E6A} must arise from translation of a recently identified alternatively spliced *c-myb* mRNA. This mRNA includes a new in-frame exon termed E6A. We also observed the expression of smaller forms of Myb in tumor cells, presumably as a result of N-terminal truncations caused by provirus insertions.

Several lines of evidence support our contention that p85 is translated from the E6A-containing *c-myb* mRNA. First, both p75 and p85 are precipitated with antibodies raised against the Myb DNA-binding domain and possess comigrating as well as unique partial V8 cleavage products (Fig. 2). This finding indicates that p85 is highly related to p75 but contains unique sequences. Second, tumor cell lines that

FIG. 6. Synthesis of Myb proteins during the cell cycle. 70Z cells from an exponentially growing culture were separated according to volume on a centrifugal elutriator. (A) DNA profile of the fractions as determined by fluorescence-activated cell-sorting analysis. Fractions: 1 to 3, primarily G1-phase cells; 4 and 5, S-phase cells; 6 and 7, G2/M-phase cells. (B) The same number of cells per sample were labeled for 20 min, Myb proteins were immunoprecipitated with anti-Bp52-Myb serum, and the samples were analyzed on a 10% SDS-polyacrylamide gel. U, Unfractionated exponentially growing cells; numbers refer to the fraction numbers in panel A.

contain 5'-truncated *c-myb* genes due to retroviral insertion produce both proteins but each with a lower apparent molecular mass approximately corresponding to the predicted size of the truncation (Fig. 2, 4, and 7). This result suggests that both proteins are encoded by the same disrupted *c-myb* allele and also demonstrates directly that the retroviral insertions alter the structure of c-Myb. It is also worth noting that the total amount of truncated Myb synthesized is not greater than in thymus and 70Z cells, indicating that structural alterations rather than increased levels of Myb are crucial for transformation. Third, RNAs derived from cDNAs with and without the E6A region can be translated in vitro to produce proteins with the same size difference as the in vivo proteins, although the in vivo and in vitro proteins do not comigrate exactly (Fig. 5; see below). Fourth, antisera raised against a bacterially produced fusion protein containing the E6A region, but no other c-Myb sequences, precipitates p85 (and its N-terminally truncated derivatives from the tumors as well as the protein generated by in vitro translations of an E6A-containing cDNA) but not p75. Taken together, our data indicate that the difference between p85 and p75 is due to the E6A-derived insert of 121 amino acids in p85.

Several other properties of p85 are consistent with previous reports on the alternatively spliced E6A mRNA. The levels of E6A-containing mRNA were estimated to be about 10 to 20% of the amount of total *c-myb* mRNA, in agreement with the relative amounts of p75 and p85 observed (Fig. 2, 4, 6, and 7). In addition, although the E6A mRNA was initially thought to be an aberration in tumors containing *c-myb* rearrangements (25), it was subsequently demonstrated to be

FIG. 7. Subcellular localization of Myb. 70Z cells and ABML4.6 cells were labeled for 20 min with [35 S]methionine, Dounce homogenized in STM buffer, and separated into nuclear (N) and cytoplasmic (C) fractions. Myb proteins were immunoprecipitated with anti-Bp52-Myb serum and analyzed on a 10% SDS-polyacrylamide gel.

present in cells containing unrearranged *c-myb* genes (28). Our data, demonstrating the presence of p85^{c-myb-E6A} in 70Z pre-B cells and in normal mouse thymocytes (Fig. 4), are clearly in accord with the idea that the products of alternate splicing are present in both normal and tumor mouse cell types that express *c-myb*. The same alternate splicing event is also found in human *myb* RNAs, suggesting the importance of Myb-E6A product in terms of Myb function (G. L. C. Shen-Ong, unpublished observation). We have detected low amounts of a higher-molecular-weight Myb-related protein in avian cells but have not yet determined whether it arises from avian *c-myb* or a *myb*-related gene (B. Lüscher, unpublished observations). Further studies are required to identify the Myb-E6A product in other species and to determine its importance with respect to Myb function.

Our data on the two Myb forms raise several questions. An unexpected finding was that the *in vitro* translation products of the *c-myb* RNAs did not precisely comigrate with the Myb and Myb-E6A immunoprecipitated from cells even after treatment with phosphatase. It is possible that the *in vivo* forms are posttranslationally modified by phosphorylation that are insensitive to alkaline phosphatase treatment or that other types of modifications have occurred. We have, however, ruled out O-linked glycosylation (unpublished data). Alternatively, there may be some differences between the structures of these cDNAs and the mRNAs transcribed in the 70Z cells. Another point concerns the several minor proteins that appear to be specifically immunoprecipitated by the anti-E6A serum (Fig. 4, arrows and asterisk). Particularly intriguing is a protein close to the size of p72 that is precipitated only in the MML9 tumor cell line.

Further study will be required to determine whether these proteins are generated as breakdown products containing the E6A region, by translation of aberrantly spliced mRNAs in the tumor cells, or by translation from other E6A-related mRNAs. Another unexpected finding was that both Myb and Myb-E6A are constitutively expressed in the 70Z pre-B cells throughout the cell cycle. This observation is in contrast to the previous finding that *c-myb* mRNA accumulation peaks at late G1/S phase of several cell types but is in accord with the finding that *c-myb* mRNA is constitutively expressed in chicken thymus (34). Interestingly, *myb* mRNA has been shown to be constitutively expressed in the 70Z cells, indicating that *myb* expression is regulated at the transcriptional level in these cells. (T. Bender, personal communication). Whether Myb is constitutively expressed throughout the cell cycle in other pre-B cells remains to be determined.

The most important question concerning the two Myb proteins is whether they have distinct functions in the cell. Since the molecular function of Myb is still unknown, we can only test for a few of its known biochemical properties. Thus far, we have been unable to find any obvious differences between Myb and Myb-E6A in nuclear localization, rates of degradation, or synthesis throughout the cell cycle (Fig. 6 and 7). These features, however, probably represent the rather gross properties one might expect to be common to all Myb proteins. It remains to be determined whether the two proteins differ in affinity for the hexameric DNA sequence recently identified as a v-Myb-binding sequence (2) or in their putative interactions with other cellular proteins. It is conceivable that the E6A region constitutes a domain that influences the functional interactions of Myb. Tests of the biological effects of independent expression of Myb and Myb-E6A are under way.

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LITERATURE CITED

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