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Alternative internal splicing in c-*myb* RNAs occurs commonly in normal and tumor cells

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Activation of the c-*myb* gene by viral transduction or proviral insertional mutagenesis that is likely to result in the production of structurally altered *myb* proteins has been shown to be predominantly associated with myelomonocytic tumors. An alternative splicing event in which a portion of the intron bounded by the vE6 and vE7 exons with v-*myb* homology is included as an additional 363-nucleotide coding exon has recently been identified in a mouse tumor that carries a provirus-activated *myb* gene. This alternative splicing was hypothesized to be a tumor-specific aberrant form of 3′-*myb* RNA processing as a consequence of the disruption of upstream 5′-sequences by proviral insertion. However, RNA blot analyses and RNase mapping studies presented here show that a significant portion (~10%) of all *myb* transcripts examined, whether in normal or in clonal tumor cells, contains the additional exon. Hence the alternative splicing is a hitherto unrecognized common normal event that potentially increases the diversity of the *myb* proteins expressed in normal tissues including thymus and spleen, as well as in tumor cells with either normal or 5′-rearranged *myb* alleles. The lack of change in the ratio of the two spliced products expressed from either the normal or the 5′-rearranged *myb* further indicates that the insertion of the unique 121 amino acids in the larger *myb* transcripts is not a consequence of tumor-specific activation of the mouse *myb* oncogene.

Key words: ABLSs/ABMLs/NFS60/thymus/non-rearranged and provirus-disrupted c-*myb*

Introduction

Previous studies on each of the two independently derived viruses that contain a virus-transduced *myb* (v-*myb*) indicate that the *myb* oncogene is closely associated with the induction of myeloid tumors (reviewed in Bishop and Varmus, 1985). Comparison of nucleotide sequences shows that both v-*myb* genes lack the same 5′ and similar portions of the 3′ cellular *myb* (c-*myb*) coding exons (Klempnauer *et al.*, 1982; Rushlow *et al.*, 1982; Leprince *et al.*, 1983; Nunn *et al.*, 1983; Gonda *et al.*, 1985), suggesting that the tumorigenic capacity of *myb* may be due to the structural alteration of the *myb* protein.

A unique group of tumors termed Abelson virus-induced plasmacytoid lymphosarcomas (ABPLs, Potter *et al.*, 1973; also named as ABMLs due to their myeloid phenotype, Shen-Ong *et al.*, 1987) has been shown to carry a disrupted c-*myb* allele (Mushinski *et al.*, 1983) resulting from the clonal insertion of the Moloney murine leukemia virus (M-MuLV) helper component of the Abelson virus complex (Shen-Ong *et al.*, 1984). M-MuLV alone can induce ABML-like tumors, and are termed Moloney virus-induced myelongenous leukemias (MMLs); each of these tumors also carries a disrupted c-*myb* allele as a result of proviral insertion (G.L.C.Shen-Ong and L.Wolff), in preparation). In order to elucidate the sequence content of the tumor-specific *myb* RNAs, *myb* hybridizing clones have been obtained previously from an oligo(dT)-primed cDNA library prepared from poly(A) RNA derived from ABML2 (Shen-Ong *et al.*, 1986). One of three clones isolated was shown by cDNA sequence analysis to be derived from a *gag-myb* chimeric transcript, and S1 mapping studies of the *myb* mRNAs isolated from six ABMLs and seven MMLs demonstrated that proviral insertion had, in each tumor, resulted in the absence of the same 5′ c-*myb* coding exons that are lacking in the v-*myb* gene. It was therefore speculated that truncation of the 5′ amino terminus of the c-*myb* protein alone might play an important role in the development of these tumors (Shen-Ong *et al.*, 1986; G.L.C.Shen-Ong and L.Wolff, in preparation).

Further detailed studies, as reported here, have shown that another ABML2-derived cDNA clone contains ~400 bp of additional sequences between the vE6 and vE7 exons with v-*myb* homology. The presence of the additional sequences (designated as exon E6A in this study) in a region of the coding sequences where other virus-transduced or proviral-inserted forms of the *myb* gene have 3′ deletions (Leprince *et al.*, 1983; Nunn *et al.*, 1983; Shen-Ong *et al.*, 1986; Weinstein *et al.*, 1987) raises the possibility that the presence of this exon may also be important in the c-*myb* activation in these tumors. Recently, the sequence of a group of ABPL2-derived *gag-myb* clones which contains a stretch of alternatively spliced sequences that encode 121 extra amino acids has been reported (Rosson *et al.*, 1987). It was concluded that the presence of the novel exon has resulted from a tumor-specific aberrant 3′ splicing event that had been induced by upstream proviral integration. Restriction enzyme mapping and nucleotide sequence analyses indicate that the ABPL2-derived cDNA clones obtained from the two independent studies contain the same additional sequences.

The present study was undertaken to determine if the presence of E6A sequences is indeed an obligatory splicing event induced by the upstream provirus, or if it represents a hitherto unrecognized normal alternative internal splicing event in c-*myb*. To this end, RNA blot analyses and RNase mapping studies were carried out to detect the presence of the typical and alternate *myb* transcripts in normal and tumor cells with or without virus-disrupted *myb*.

Results

Structure of two different forms of chimeric gag-myb transcripts in ABMLs

It has previously been shown that the major tumor-specific *myb* transcripts in ABMLs and MMLs, which have clonal rearrangements in the *myb* locus as a result of provirus integration, are initiated in the upstream 5′ long terminal repeat (LTR) of the integrated provirus, and processed via a cryptic splice donor sequence in the *gag* (p30) region to the splice acceptor site in

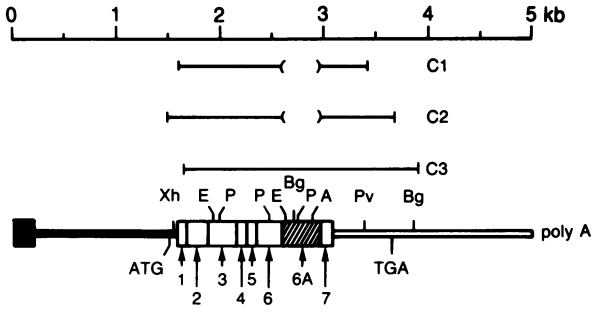

Fig. 1. A composite restriction map of an ABML chimeric *gag-myb* transcript with the E6A sequences. The three ABML2-derived cDNA inserts of plasmids C1, C2 and C3 are shown above the restriction map. Brackets in C1 and C2 indicate the absence of E6A sequences in these two clones. The 5′ LTR is identified by a solid box, the M-MuLV *gag* sequences by a solid bar, the vE1−vE7 exons that correspond to avian myeloblastosis virus (AMV)-transduced *myb* exons by seven open boxes as marked, the E6A sequences by a shaded box and the remaining 3′ c-*myb* sequences by an open bar. The positions of the putative tumor-specific *myb* translation start ATG codon and the termination TGA codon are as shown. Restriction sites are: A, *Aat*II, Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; Xh, *Xho*I.

vE1 of the c-*myb* gene (Shen-Ong *et al.*, 1986; G.L.C.Shen-Ong and L.Wolff, in preparation). In addition, some of the chimeric *gag-myb* transcripts have been found to contain E6A sequences, and have been shown by Rosson *et al.* (1987) to correspond to an exon that begins ~850 bases from the 3′ end of the vE6 exon. A composite restriction map of a tumor-specific chimeric *gag-myb* transcript which includes the E6A sequences (shaded box) is shown in Figure 1. Probes used in the RNA blot analysis and RNase mapping studies described below were isolated from the ABML2-derived C3 clone that contains the E6A sequences.

E6A sequences are found in normal and tumor-specific myb transcripts

To determine if the alternative 3′ *myb* RNA processing is a result of upstream provirus insertion or a common event in normal c-*myb* expression, RNA blot analyses were first performed to determine if the E6A sequences are found in transcripts from either normal or tumor cells. A E6A-specific probe was first prepared by subcloning a 0.28-kbp *Eco*RI−*Aat*II fragment isolated from the C3 clone into a pGEM3Z vector (see Figure 1). The insert was verified by DNA sequencing, and is found to be void of repetitive sequences since it hybridizes to a 16.5-kbp mouse genomic *Eco*RI fragment and a 7.7-kbp human genomic *Eco*RI fragment (data not shown). Northern blot analyses of mRNAs from mouse thymus and four different ABMLs using the E6A-specific probe showed high levels of E6A-hybridizing transcripts in the ABMLs and a much lower level (~1/10) in mouse thymus (Figure 2, probe i). An apparently similar hybridization pattern, though with slightly faster migrating bands, was obtained when the same blot was stripped, rehybridized to a c-*myb* probe that contains the vE2 sequences, and autoradiographed for 16 h (Figure 2, probe ii for ABML1 and ABML2). When the blot was autoradiographed for 10 days, less abundant, larger *myb*-hybridizing bands that co-migrate with the E6A hybridizing bands were observed for the thymus, ABML3 and ABLS 109 (Figure 2). Hence RNA blot analyses indicate that the additional E6A sequences, which presumably account for the increase in size of each of the larger *myb* hybridizing bands, are present in mRNAs from normal and ABML cells.

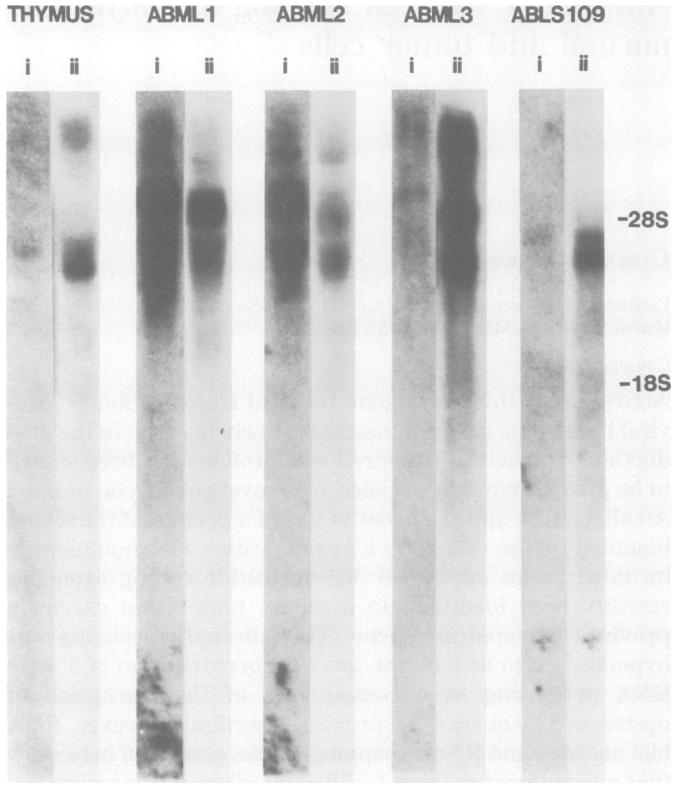

Fig. 2. RNA blot analyses of mRNAs derived from thymus and four different ABMLs using: (i) E6A-specific probe (see text) and (ii) *myb*-specific probe that contains the vE2 sequences. Positions of the 28S (4.7-kb) and 18S (2.0-kb) rRNAs are as shown. The exposure time for probe i is 15 days while the time for probe ii hybridization with ABML1 and ABML2 is 1 day (since the intense 10-day exposure obliterated all bands) and with thymus, ABML3 and ABLS109 is 10 days.

The alternative splicing occurs in myb transcripts expressed from either the normal or provirus-activated myb gene in normal and tumor cells

RNase protection experiments were performed using a 332-nucleotide probe that spans 131 bp of the vE6 exon and 136 bp of E6A sequences which lie immediately downstream (top of Figure 3). In two separate experiments, the presence of a fully protected 267-bp band (denoted as 'a') that corresponds to *myb* transcripts with both vE6 and E6A sequences was detected in poly(A) RNAs isolated from the mouse thymus (Figure 3). This indicates that the alternative splicing occurs in *myb* transcripts expressed by normal cells as already suggested by RNA blot analyses described above. A more abundant 131-bp band (denoted as 'b') which corresponds to the typically spliced *myb* transcripts (top of Figure 3) is also present in thymus mRNAs.

The same fully protected 267-bp species band a that represents alternative splicing, and the 131-bp band b that represents typical splicing are also found in *myb* RNAs isolated from normal spleen and various cell lines (Figure 3). These lines include three different Abelson virus-induced lymphosarcomas (ABLSs) that contain the normal c-*myb* gene, as well as six different ABMLs (ABLS109 and ABLS122 are considered as ABMLs since they also carry a provirus-disrupted *myb* allele; Shen-Ong *et al.*, 1986) and three different MMLs that have undergone rearrangements in the *myb* locus as a result of proviral insertion upstream of the 5′-most exon with v-*myb* homology (vE1). Hence the RNase

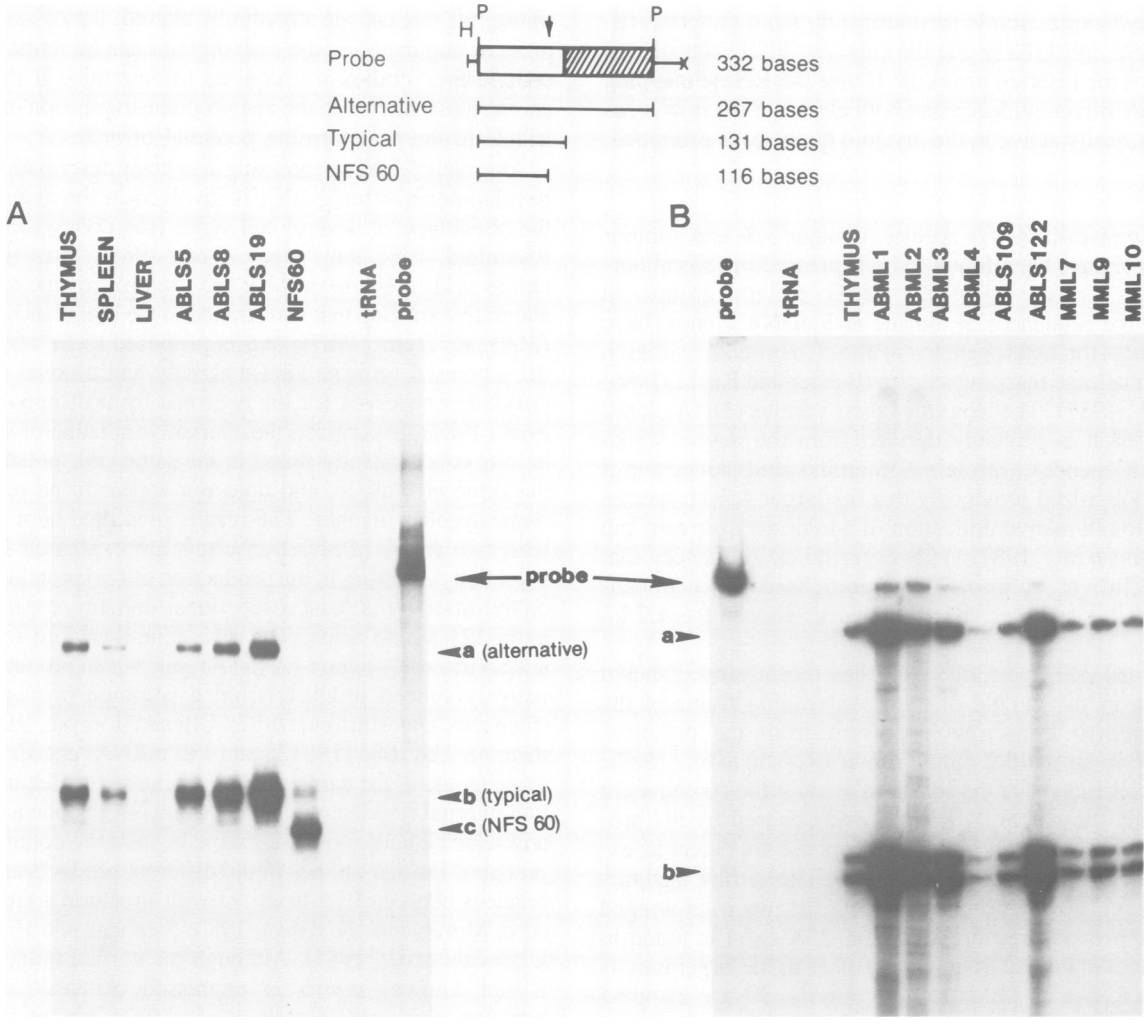

Fig. 3. RNase protection studies of *myb* ABML3 transcripts from normal and tumor cells. (Note: ABLS109 is considered as an ABML since it carries a *myb* allele that has undergone 5′ rearrangement due to the insertion of a single viral LTR; Shen-Ong *et al.*, 1986.) The ^{32}P-labeled antisense transcript probe [332 bases that are originated and promoted by T7 polymerase (start marked by X) and contain portions of vE6 (open box) and E6A (shaded box) sequences] is as shown (top of figure). The site of CasBr-M provirus insertion found in NFS60 cells as determined previously (Shen-Ong *et al.*, 1986), is marked by the arrow shown above the probe. The various fragments which would be protected by the alternative internal splicing, the typical splicing and the NFS60 disrupted *myb* transcripts are illustrated below the probe. Restriction sites are: H, *Hin*dIII and P, *Pst*I. The ^{32}P-labeled antisense probe (332 bases) was hybridized to 10 μg poly(A) RNAs: (**A**) from normal cells and tumor cells with unaltered *myb* alleles; and (**B**) from thymus with normal *myb* alleles, and ABMLs, ABLSs and MMLs with a provirus-activated *myb* allele. 40 μg of total RNA derived from MMLs were used. Hybrids were then digested with RNase A and RNase T1 and then electrophoresed under non-denaturing condition as in panel A and under denaturing condition as in panel B. The intact probes which are larger than the fully protected band a due to the presence of polylinker sequences are as indicated. The absence of any protected bands in the presence of 40 μg of tRNA shows the complete digestion of the probes by RNase in the absence of hybridizing RNAs. The bands are marked a, representing the fully protected alternative 3′ spliced band; b, representing the typically spliced bands; and c, representing the NFS60-specific band. A major 131-bp protected band b is found when the RNase-treated sample was electrophoresed under non-denaturing condition as in panel A. Under denaturing conditions, this 131-bp band resolves into two closely migrating bands (± a few nucleotides) as in panel B, presumably due to 'end-nibbling' or 'underdigestion' of the 131-bp fragment by the RNase A (C and U specific) and RNase T1 (G specific). End-labeled mol. wt standards (BRL) were used to verify to sizes of the probe and the protected fragments.

mapping studies show that the E6A sequences in *myb* mRNAs as revealed by cDNA cloning and nucleotide sequence analyses can easily be detected. More importantly, the E6A sequences represent an additional exon in normal *myb* transcripts, rather than arising as the consequence of a tumor-specific aberrant splicing event due to the upstream proviral insertion.

Expression from both myb alleles in NFS60 tumor cell line

Previous studies have shown that one of the *myb* alleles in the NFS60 myeloid tumor cell line carries a 3′ rearrangement in the *myb* locus as a result of proviral insertion within the vE6 exon, thus giving rise to a novel 2.3-kb *myb* transcript which utilizes the polyadenylation site provided by the 5′ LTR of the provirus that is inserted downstream (Shen-Ong *et al.*, 1986). As illustrated in Figure 1B, only a 116-bp band (designated as 'c') would be protected by transcripts that are expressed from the provirus-disrupted allele of the *myb* locus. Figure 3A shows the presence of the fully protected 267-bp species (band a) and the 131-bp species (band b) at levels comparable with that found in spleen cells, as well as a novel 116-bp species (band c) in *myb* mRNAs isolated from the NFS60 cells. This result clearly indicates that in this cell line *myb* is expressed from the provirus-disrupted *myb* allele and from the non-rearranged c-*myb* allele via typical and alternative splicing (Figure 3A). The more abundant 116-bp band

shows that *myb* expression is predominantly from the provirus-disrupted allele, most likely due the enhancer effect contributed by the LTR of the inserted provirus. Hence the RNase mapping results provide direct evidence that both alleles of the *myb* locus are transcriptionally active in the myeloid tumor cells examined.

Discussion

At least two *myb* mRNA size species, a major 3.8- and a minor 4.2-kb species, have been found to be expressed in several normal cell types (Mushinski *et al.*, 1983). Several groups, in reporting on the sequences of murine *myb* cDNA clones, have found evidence for heterogeneity in the 5′ non-coding region based on S1 nuclease mapping studies (Bender and Kuehl, 1986; Watson *et al.*, 1987). The lengths of their various cDNA clones suggest that each is probably derived from the major 3.8-kb species which encodes a protein 636 amino acids long, and it has been hypothesized previously that the larger 4.2-kb species may be due to alternative utilization of additional upstream cap sites (Watson *et al.*, 1987). However, no 5′ sequences that hybridize exclusively to the 4.2-kb species have yet been identified. Hence it is not clear what sequences encode the 4.2-kb species.

An additional 363-nucleotide exon has recently been shown to be present in the middle of the open reading frame of the chimeric *gag-myb* transcripts expressed from the provirus-activated *myb* in an ABPL2 mouse tumor (Rosson *et al.*, 1987). That report concluded that the additional exon is the consequence of an aberrant alternative 3′ splicing event caused by the provirus inserted upstream since these authors did not detect its expression in *myb* RNA from normal cells. RNA blot analyses presented here, however, demonstrate that upon prolonged autoradiograph exposure, the additional E6A exon is found in a 4.2-kb species in normal thymus, as well as in the larger tumor-specific *myb* RNAs in ABMLs. Furthermore, RNase mapping studies clearly show that the alternative splicing that includes the E6A exon in the transcripts occurs in normal and tumor cells whether or not c-*myb* is disrupted in its 5′ end by proviral insertion.

A major 75-kd nuclear protein product, presumably encoded by the major 3.8-kb mRNA species, has been shown to be immunoprecipitated from mouse cells by c-*myb*-specific antiserum (Boyle *et al.*, 1986). Since the additional 121 codons generated by the splice are in the same reading frame as c-*myb*, one would expect the production of an additional c-*myb* that is >75 kd. Preliminary immunoprecitation experiments using c-*myb*-specific antiserum have shown the presence of a minor 85-kd *myb*-related protein and the previously characterized $p75^{myb}$ in a pre-B tumor cell line which does not have any alteration within the c-*myb* locus (in collaboration with R.N.Eisenman, unpublished observation). We are currently attempting to determine whether the 85-kd species is encoded by the *myb* transcripts that contain the E6A sequences, and whether the major 75-kd species is encoded by the typically spliced 3.8-kb *myb* mRNA species.

Another interesting finding comes from RNA analysis of NFS60 cells, which shows that *myb* is expressed from both the normal and the provirus-disrupted alleles. This result differs from that observed with another frequently rearranged nuclear proto-oncogene, c-*myc*. In every tumor in which transcripts from the rearranged and normal alleles can be distinguished, only the rearranged c-*myc* allele is expressed (reviewed in Cole, 1986). This observation has led to the proposal that the main functional consequence of c-*myc* activation may be the escape from negative regulation in cells that would otherwise not express c-*myc*. Since transcripts that encode structurally altered, tumor-associated *myb* proteins and the normal c-*myb* proteins can be found in the same cell, further studies are required to determine if the structural alternations rather than the inappropriate expression of c-*myb* contribute to the transforming potential of *myb*.

Alternative RNA processing has been described as a normal developmental strategy that is involved in the determination of the phenotype of several cell types (for reviews, see Leff and Rosenfeld, 1986 and Padgett *et al.*, 1986). Most recently, six-amino acid insertion in the c-*src* proto-oncogene as a result of alterantive splicing that is restricted to the brain has been reported (Martinez *et al.*, 1987), and is proposed to be responsible for the increased tyrosine kinase activity and a novel serine phosporylation observed for the neuronal form of $pp60^{c\text{-}src}$ (Brugge *et al.*, 1987). In contrast, the alternative splicing of *myb* reported here is concomitantly found in the same cell, presumably from the same *myb* allele, and does not appear to be regulated in a tissue-specific manner. The results presented here show that at least two forms of *myb* transcripts are expressed from c-*myb*, potentially resulting in the production of two proteins which probably differ by at least a functional domain constituted by the extra 121 amino acids. The *myb* gene therefore resembles the adenovirus early region 1A (E1A) gene which produces five alternatively spliced products from a common primary transcript (Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987 and refs therein). The 10S, 11S, 12S and 13S mRNAs encode related E1A proteins using the same reading frame but differ in one and/or two internal regions, while the 9S mRNA encode a smaller protein which changes reading frame beyond the common 3′ splice junction. Further studies of the different products allow the correlations between E1A functions with structural domains (reviewed in Moran and Matthews, 1987; Stephens and Harlow, 1987; Ulfendahl *et al.*, 1987). Although the normal function of c-*myb* is not known, c-*myb* is expressed primarly in immature hematopoeitic cells (Gonda *et al.*, 1982; Westin *et al.*, 1982), and its disruption, resulting in the deletion of the same amino-terminal region, is predominantly associated with myeloid tumors. It is possible that *myb* may contain different functional domains that are required for different biological activities, especially during hematopoeisis. Consequently, the E6A exon found in a minor population of normal c-*myb* transcripts as reported here may constitute an additional domain that is required for a particular biologic function. Southern blot analyses showed the presence of E6A sequences in human and chicken DNAs (data not shown), again suggesting the importance of these conserved sequences. Further investigation of possible cell-type specificity and the developmental profile of these two species of c-*myb* mRNA, and the detailed characterization of the two *myb* 'isoforms' might shed some insights into the possible function of *myb*.

Materials and methods

Tissues, tumors and cell lines

Thymus, spleen and liver tissues were obtained from normal BALB/c mice. Solid tumors were excised from subcutaneous or mesenteric sites after transplantation of ascites tumor cells into syngenic mice. The origin of the BALB/c tumors (ABLS5, ABLS8, ABLS19, ABML1, ABML2, ABML3, ABML4, ABLS109, ABLS122) used in this study has been described previously (Mushinski *et al.*, 1983). MML7, MML9 and MML10 cells were maintained as tissue culture lines established from transplanted M-MuLV-induced myelomonocytic tumors. NFS60 cells were maintained as a tissue culture line as previously described (Holmes *et al.*, 1985).

RNA extraction and Northern blot analysis

Total RNA was extracted from tissues and cells using the guanidium/cesium chloride method (Maniatis *et al.*, 1982). Polyadenylated RNA was selected by

the messenger affinity paper (Hybond-mAP, Amersham). Five micrograms of polyadenylated RNA were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde as described by Maniatis *et al.* (1982). RNAs were blotted onto hybridization transfer membranes (Hybond-N, Amersham). Hybridization with the appropriate probes was carried out under stringent conditions in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0.5% SDS at 42°C for 16 h. Washing was performed in 0.1 × SSC, 0.5% SDS at 65°C for 1 h. Filters were autoradiographed at −80°C with Kodak XAR-5 films for various times.

RNase protection analysis

A uniformly labeled antisense RNA transcript was generated from the c-*myb* probe (contains sequences from the vE6 and E6A exons cloned into the *Pst*I site of a pGEM-3 vector and linearized with the *Hin*dIII restriction enzyme that cut once in the linker region on the distal end of the T7 promoter sequences), using T7 polymerase and [^{32}P]UTP as recommended by Promega Biotec (see Figure 1B). Labeled transcripts were hybridized to 10 μg of poly(A) RNA or 40 μg of total cellular RNA in 30 μl of hybridization solution (80% formamide, 40 mM PIPES, pH 6.7, 0.4 M NaCl, 1 mM EDTA) at 49°C for 16 h. RNase solution (300 μl) containing RNase A (8 U/ml, Cooper Biomedical) and RNase T_1 (1400 U/ml, BRL) in 10 mM Tris−HCl (pH 7.5), 5 mM EDTA and 300 mM NaCl was then added. RNase digestion was allowed to proceed at 37°C for 1 h prior to termination by the addition of 10 μl of 20% SDS and 50 μg of proteinase K. The mixture was incubated at 37°C for 15 min. After phenol−chloroform extraction and ethanol precipitation, samples were either resuspended in 10 mM Tris−HCl (pH 8.0), 1 mM EDTA, 0.01% xylene cyanol and 0.005% bromophenol blue and the duplex RNAs were electrophoresed in a 5% polyacrylamide non-denaturing gel, or in 97% formamide, 0.1% SDS, 10 mM Tris−HCl, 0.01% xylene cyanol and 0.005% bromophenol blue, denatured at 90°C for 3 min and electrophoresed in a denaturing 6% acrylamide−8 M urea gel. Autoradiography was performed on dried gels using intensifying screens.

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