Differentiation of Mouse Erythroleukemia Cells Enhanced by Alternatively Spliced c-*myb* mRNA

BARBARA L. WEBER, ERIC H. WESTIN, MICHAEL F. CLARKE*

C-*myb*, the normal cellular homolog of the retroviral transforming gene v-*myb*, encodes a nuclear, transcriptional regulatory protein ($p75^{c\text{-}myb}$). C-*myb* is involved in regulating normal human hematopoiesis, and inhibits dimethyl sulfoxide–induced differentiation of Friend murine erythroleukemia (F-MEL) cells. An alternately spliced c-*myb* mRNA encodes a truncated version of $p75^{c\text{-}myb}$ (*mbm2*) that includes the DNA binding region and nuclear localization signal present in the c-*myb* protein, but does not contain the transcriptional regulatory regions. Constitutive expression of *mbm2*, in contrast to c-*myb*, here resulted in enhanced differentiation of F-MEL cells. These data suggest that the c-*myb* protooncogene encodes alternately spliced mRNA species with opposing effects on differentiation.

C-*myb* ENCODES A DNA-BINDING protein with transcriptional activating activity in vitro (*1–9*). Recent studies provide evidence that c-*myb* plays a role in growth and differentiation of normal hematopoietic cells (*10*). In addition, constitutive expression of c-*myb* inhibits dimethyl sulfoxide (DMSO)–induced differentiation of Friend murine erythroleukemia (F-MEL) cells (*11–13*). C-*myb* encodes several mRNA species ranging from 3.4 to 4.5 kb (*14, 15*), which are thought to originate from variable initiation sites or alternate splicing events. The predominant transcript is 3.8 kb. While characterizing a full-length c-*myb* cDNA (*11*), we identified an alternately spliced c-*myb* mRNA which appears to promote DMSO-induced differentiation of F-MEL cells.

Approximately 50 clones were isolated by homology to a human genomic c-*myb* probe (*16*) from a cDNA library constructed from the T-lymphoblastic leukemia cell line CCRF-CEM. One 3.4-kb clone, *mbm1*, was sequenced and found to be homologous to known human c-*myb* sequences. A second clone, *mbm2*, of similar size and with a restriction site pattern similar to that of *mbm1*, was also sequenced. In *mbm2*, an alternate splice donor-acceptor site is used at the junction of exons five and six, which results in the insertion of a 122-bp cryptic sixth exon. This exon contains an in-frame stop codon (UAA) 102 bp beyond the alternate splice junction. Southern (DNA) blot and sequence analysis demonstrates that the sixth exon of *mbm2* is derived from the fifth intron of the genomic c-*myb* clone (*15*).

Mbm1 and *mbm2* also diverge at their 5′ end, where an alternate initiation site results in an *mbm2* protein product beginning 20 amino acids downstream of the *mbm1* initiation codon. Primer extension analysis of RNA from CCRF-CEM cells verifies the existence of an mRNA species predicted by the 5′ *mbm2* sequence (*15, 17*). The 5′ region of c-*myb*, which is deleted in *mbm2* and v-*myb*, contains a phosphorylation-sensitive element that inhibits sequence-specific DNA binding when phosphorylated (*18*). The functional significance of the 5′ alteration of *mbm2* is unknown.

Mbm2 mRNA was shown to be present in vivo by Northern (RNA) blot analysis of both uninduced HL-60 and CCRF-CEM cells, representing 5 to 10% of the predominant c-*myb* transcript (*15*). No signal was detected in differentiated HL-60 cells (Fig. 1).

After exposure to DMSO, F-MEL cells differentiate along the erythroid pathway and produce hemoglobin (*19*). Optimal differentiation, typified by the presence of hemoglobin in 60 to 80% of cells, occurs after exposure to 1.8% DMSO for 5 days. In order to examine the function of the truncated protein, *mbm2* was inserted into a eukaryotic expression vector under the control of the SV40 promoter (Fig. 2). The recombinant plasmid pMbm2 was linearized with Kpn I and transfected into F-MEL cells (*20*). Three separate transfections with pMbm2 were performed in parallel with the following control transfections (Fig. 2): (i) pMbm2 was linearized with Eco RI, which cleaves twice within the protein coding sequence, such that no full-length message was expressed. (ii) A frame shift mutant of pMbm2 ($pSst^-$) was constructed by deletion of 4 bp at the Sst I restriction site located 206 bp downstream from the *mbm2* initiation codon (*21*). This deletion altered the reading frame and allowed expression of the *mbm2* mRNA but not the *mbm2* protein product. (iii) A construct similar to pMbm2 but with a full-length c-*myb* cDNA, pMbm1, was linearized with Kpn I.

Stable transfectants were isolated from each experiment and treated with 0.5 or 1.5% DMSO for 3 days. Differentiation was determined by analysis of intact cells with benzidine staining before (spontaneous) and after (induced) DMSO exposure. In all three transfections, the pMbm2-transfected cells showed significantly enhanced differentiation ($P < 0.01$) after 3 days in DMSO, as compared to control cells (Table 1 and Fig. 3). Cells transfected with $pSst^-$ (Fig. 3) or

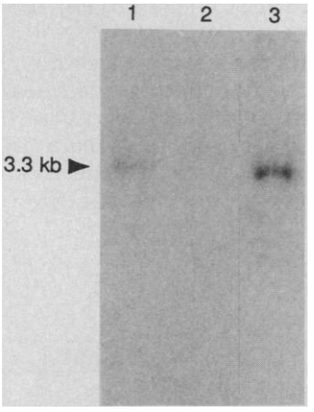

Fig. 1. Northern blot analysis of CCRF-CEM and HL-60 mRNA. Five micrograms of oligo(dT)-selected RNA from uninduced HL-60 cells (lane 1), HL-60 cells induced to differentiate with 1.3% DMSO (lane 2), and CCRF-CEM cells (lane 3) was probed with a 30-base oligonucleotide homologous to the unique region of the *mbm2* exon 5/6 splice junction (*28*).

Table 1. Benzidine staining of pMbm2-transfected clones. Percentage (±1 SD) of cells stained with benzidine after 3 days exposure to DMSO at the concentrations indicated. Statistical significance was determined from the Behrens-Fisher *t* test. Control ($n = 21$), wild-type F-MEL cells or cells transfected with Kpn I–linearized $pSst^-$ or Eco RI–linearized pMbm2; pMbm2 ($n = 18$), cells transfected with pMbm2. Each clone was analyzed at least three times. Differences between control subsets were not statistically significant ($P > 0.2$).

DMSO (%)	Benzidine-positive (%)		
	Control	pMbm2	
0	2.4 ± 2.7	10.5 ± 8.1	$P < 0.001$
0.5	12.2 ± 6.8	29.9 ± 17.5	$P < 0.001$
1.5	17.4 ± 7.7	43.8 ± 20.8	$P < 0.001$

B. L. Weber and M. F. Clarke, Department of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, MI 48109.
E. H. Westin, Departments of Medicine, Microbiology and Immunology, Medical College of Virginia, Richmond, VA 23298.

*To whom correspondence should be addressed.

Eco RI–linearized pMbm2 (*22*) were indistinguishable from wild-type F-MEL cells at both concentrations of DMSO tested. As has been reported previously, pMbm1 transfectants were resistant to induction with DMSO (Fig. 3) (*11*).

Ten random uninduced pMbm2–Kpn I clones were assayed for presence of *mbm2* mRNA by S1 nuclease protection. A 1.1-kb probe (Fig. 2) was used that was specific to *mbm2* and included upstream 5′ nontranslated regions and the first 494 bp of the coding sequence, to detect transfected human mRNA sequences but not endogenous mouse c-*myb* mRNA sequences. Transcripts of *mbm2* were detected in all clones. As expected, wild-type F-MEL cells did not contain detectable levels of *mbm2* transcripts. Analysis of the clones demonstrated that a tendency toward spontaneous differentiation and the extent of enhanced DMSO-induced differentiation generally correlated with higher expression of *mbm2* (Fig. 4). Clone 2, which did not show evidence of enhanced spontaneous differentiation, appeared to express a form of *mbm2* with a deletion in the 5′ portion of the cDNA. We postulate that this may have resulted in the production of a nonfunctional *mbm2* protein.

Several oncogenes are known to encode more than one protein product, sometimes by means of alternately spliced mRNA species. The clone *mbm2* represents an alternately spliced c-*myb* mRNA. Other structurally distinct alternately spliced c-*myb* mRNAs have been identified (*14, 23*), but the function of these transcripts is not known. The presence of *mbm2* mRNA appears to have an effect directly opposing that of c-*myb* mRNA. The mechanism for this effect remains unknown. The predicted *mbm2* protein product encodes the DNA binding and nuclear localization signals present in c-*myb* but not the distinct, highly conserved domain shown to have transcriptional activating and suppressing activity (*2, 3*). We speculate that the *mbm2* protein may be functioning as a competitive inhibitor of c-*myb* protein by occupying the same DNA binding sites without regulatory activity, a type of dominant negative function (*24*). This hypothesis remains to be tested directly. Such a competition has been described for alternately spliced translation products of the protooncogene c-*erb*A (*25*). Both proteins recognize the same DNA sequence, but only one protein also binds thyroid hormone and functions as a transcriptional activator. The alternate protein acts as an inhibitor, presumably through competitive binding. The experimentally produced yeast GAL4-LacZ fusion protein may also function in that manner (*26*). GAL4 protein binding to a specific site upstream of both the GAL1 and GAL10 loci is required for efficient transcription of these genes. The GAL4-LacZ fusion protein, containing only the DNA binding regions of GAL4, does not activate transcription and functions as a repressor when properly positioned in front of the genes.

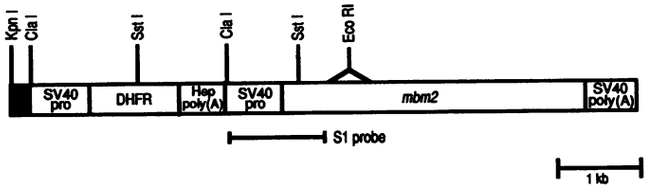

Fig. 2. Structure of pMbm2. The Eco RI–Cla I restriction fragment used as a probe for the S1 nuclease protection assay is indicated. The plasmid was linearized with Kpn I for pMbm2 transfections and with Eco RI for control transfections. The Sst I site in the *mbm2* coding region was deleted from pSst$^-$.

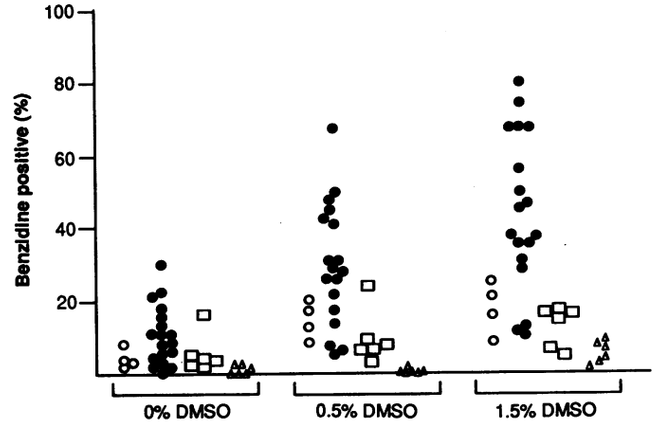

Fig. 3. Differentiation patterns of F-MEL cells transfected with pMbm2 and control cells. Individual clones of F-MEL cells transfected with pMbm2 (●), pSst$^-$ (□), or pMbm1 (△), and wild-type F-MEL cells (○) were exposed to DMSO in the concentrations indicated for 3 days. Cells were stained with benzidine as previously described (*11*) and counted in a hemocytometer. At least 100 cells per sample were counted.

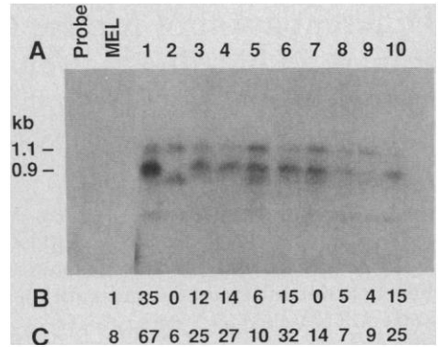

Fig. 4. S1 nuclease analysis of mRNA from ten representative *mbm2*-transfected cloned cell lines. (**A**) The Cla I–Eco RI fragment (Fig. 2) of pMbm2 was isolated, end-labeled with [γ-^{32}P] ATP, and hybridized overnight at 51°C to 10 μg of total cellular RNA from each clone. Samples were digested for 1 hour with S1 nuclease and analyzed by gel electrophoresis. The gel was fixed and dried under vacuum before autoradiography. Probe, hybridization without added RNA; MEL, RNA from uninduced F-MEL cells; lanes 1 to 10, individual clones of F-MEL cells transfected with Kpn I–linearized pMbm2. The 1.1-kb band represents undigested probe and the 0.9-kb band represents digested probe protected by hybridization to *mbm2* mRNA. (**B**) Percentage of cells in each clone that differentiate spontaneously (benzidine-positive) during exponential growth. (**C**) Percentage of cells that differentiate (benzidine-positive) after treatment for 3 days with 0.5% DMSO.

The role of *mbm2* in normal differentiation is unknown. The absence of *mbm2* transcripts in differentiated HL-60 cells suggests that *mbm2* may be required only briefly in the differentiation process, but this remains speculative. It is possible that deletion or inactivation of splice sites required to generate the *mbm2* mRNA would result in cells with decreased potential for normal differentiation. This could explain the unexpectedly high incidence of homozygosity at the c-*myb* locus found in a panel of 35 solid tumors (*27*). In order to elucidate the role of *mbm2* in differentiation, further studies will be needed to identify *mbm2* mRNA in differentiating cells and to determine the cellular location and DNA binding activity of the *mbm2* protein.

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20. Complementary DNA from *mbm2* mRNA was cloned into the plasmid pCD as previously described (*11*). For selection purposes, the dihydrofolate reductase (DHFR) transcription unit, derived from pFR400 (*28*), was also inserted into this vector. This construct was cotransfected with pSV2neo into F-MEL cells by electroporation, and transformants were selected by sequential growth in media containing geneticin and methotrexate. Individual transfected clones were obtained by limiting dilution.
21. pSst$^-$ was generated by partial digestion of pMbm2 with Sst I, removal of the protruding ends with T4 polymerase, and subsequent blunt-end re-ligation. Absence of the Sst I restriction site was verified by digestion with Sst I, Eco RI, and Cla I. The plasmid was transfected into F-MEL cells as described (*20*).
22. Mean values for percent of differentiated cells (benzidine-positive) after transfection with Eco RI–linearized pMbm2. Individual clones were treated with DMSO and compared to wild-type F-MEL cells; 0% DMSO, 3% benzidine-positive ($P = 0.55$); 0.5% DMSO, 21% benzidine-positive ($P = 0.79$); 1.5% DMSO, 35% benzidine-positive ($P = 0.78$).
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28. Total cellular RNA was isolated from CCRF-CEM cells, uninduced HL-60 cells, and HL-60 cells induced to differentiate with 1.3% DMSO. Messenger RNA was selected by passage over an oligo(dT) column. A 30-base oligonucleotide specific for the pMbm2 exon 5/6 junction was synthesized and hybridized as previously described (*15*).
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Induction of Donor-Specific Unresponsiveness by Intrathymic Islet Transplantation

ANDREW M. POSSELT, CLYDE F. BARKER, JOHN E. TOMASZEWSKI, JAMES F. MARKMANN, MICHAEL A. CHOTI, ALI NAJI*

The application of isolated pancreatic islet transplantation for treatment of diabetes mellitus has been hampered by the vulnerability of islet allografts to immunologic rejection. Rat islet allografts that were transplanted into the thymus of recipients treated with a single injection of anti-lymphocyte serum survived indefinitely. A state of donor-specific unresponsiveness was achieved that permitted survival of a second donor strain islet allograft transplanted to an extrathymic site. Maturation of T cell precursors in a thymic microenvironment that is harboring foreign alloantigen may induce the selective unresponsiveness. This model provides an approach for pancreatic islet transplantation and a potential strategy for specific modification of the peripheral immune repertoire.

TRANSPLANTATION OF ISOLATED pancreatic islets is the most specific therapy for insulin-dependent diabetes mellitus (*1*). However, despite immunosuppression, no human islet allografts have escaped rejection, possibly because cellular allografts such as bone marrow or islets are more vulnerable to rejection than vascularized organ allografts such as kidney, liver, or whole pancreas (*2*). Greater success has been achieved in experimental animals by chronic administration of potent immunosuppressants or by reducing the immunogenicity of the graft before transplantation (*3, 4*). The latter can be achieved by prolonged tissue culture, ultraviolet irradiation, or treatment of islets with specific antibodies to delete or inactivate intra-islet antigen presenting cells (APCs) (*1, 3, 5*). An alternative strategy would be transplantation to an immunologically privileged site. Thus far only the brain and the testicle have shown promise in this regard (*6, 7*). Our data support the thymus as a new immunologically privileged site for islet transplantation. The prolonged residence of allogeneic islets in the thymus also induced unresponsiveness to donor islets transplanted extrathymically.

A. M. Posselt, C. F. Barker, J. F. Markmann, M. A. Choti, A. Naji, Department of Surgery, Hospital of the University of Pennsylvania, Philadelphia, PA 19104.
J. E. Tomaszewski, Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

*To whom correspondence should be addressed.

Islets were isolated from Lewis (RT1^l) or DA(RT1^a) donors and transplanted to major histocompatibility complex (MHC) incompatible Wistar Furth (WF,RT1^u) recipients (8 to 10 weeks old) in which diabetes (blood glucose > 300 mg/dl) had been induced 2 to 3 weeks earlier with intravenous streptozotocin (65 mg per kilogram of body weight). Islets (1000 to 1200) were inoculated into conventional islet transplant sites (beneath the renal capsule or into the liver, via the portal vein) (*8*), into a known privileged site (the testicle), or into the thymus by direct injection of 500 to 600 islets into each lobe. In some instances freshly isolated islets were transplanted, whereas in others islets were maintained in tissue culture for 14 days before transplantation (to delete intra-islet APCs). Where noted, recipients received a single intraperitoneal injection (1 cm^3) of rabbit antiserum to rat lymphocytes (ALS) at the time of islet transplantation. Islet grafts were considered technically successful if the nonfasting blood glucose returned to normal (<200 mg/dl) within 2 to 3 days. Rejection was diagnosed by subsequent recurrence of hyperglycemia (>200 mg/dl) and confirmed by histological examination of the graft.

All transplants of freshly isolated (non-APC–depleted) Lewis islets were rejected promptly by nonimmunosuppressed WF rats if transplanted to the liver or renal subcapsule [median survival times (MST), 8 and 9.5 days respectively; Table 1]. Survival of freshly isolated islets in the thymus was prolonged (MST, 17 days), and one of seven such islet allografts survived permanently.

Administration of ALS (1 cm^3) at the time of transplantation delayed rejection of freshly isolated islets transplanted to the liver (MST, 29 days) or renal subcapsule (MST, 47 days). Although rejection was further postponed by transplantation to a clas-

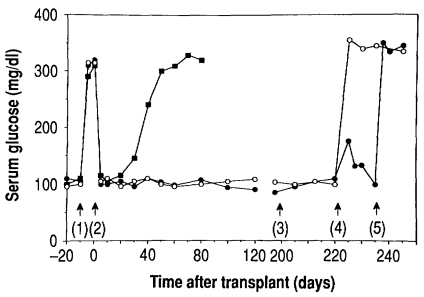

Fig. 1. Representative blood glucose profiles of WF rats transplanted with freshly isolated pancreatic islets in various sites. (1) Streptozotocin administration. (2) Transplantation of fresh Lewis islets (■–■, intraportal; ●–●, intrathymic, animal #1; ○–○, intrathymic, animal #2). All animals received ALS (1 cm^3) at time of transplantation. (3) Second renal subcapsular transplants of either Lewis (●–●) or DA (○–○) islets. (4) Thymectomy. (5) Removal of islet bearing kidneys.