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# CHARACTERIZATION OF A PROMOTER REARRANGEMENT AND A SECOND PROMOTER IN THE HUMAN C-MYB PROTO-ONCOGENE

Sarah Margaret Jacobs

Virginia Commonwealth University
School of Basic Health Sciences

This is to certify that the dissertation prepared by Sarah M. Jacobs entitled "Characterization of a Promoter Rearrangement and a Second Promoter in the Human C-*myb* Proto-oncogene" has been approved by her committee as satisfactory completion of the dissertation required for the degree of Doctor of Philosophy.

[redacted]
Eric H. Westin, M.D. Director of Dissertation

[redacted]
Gail E.Christie, Ph.D., School of Basic Health Sciences

[redacted]
Joy L. Ware, Ph.D., School of Medicine

[redacted]
Zendra E. Zehner, Ph.D. School of Basic Health Sciences

[redacted]
Francis L. Macrina, Ph.D., School of Basic Health Sciences

[redacted]
John G. Tew, Ph.D. Acting Department Chairman

[redacted]
William Dewey, Ph.D. Dean, School of Basic Health Sciences
Chairman, MCV Graduate Committee

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Date

# CHARACTERIZATION OF A PROMOTER REARRANGEMENT AND A SECOND PROMOTER IN THE HUMAN C-*MYB* PROTO-ONCOGENE

A dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia, Virginia Commonwealth University

BY

Sarah Margaret Jacobs
B.S., Bucknell University, 1986

Director: Eric H. Westin, M.D.
Associate Professor, Department of Medicine

Virginia Commonwealth University
Richmond, Virginia

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# TABLE OF CONTENTS

# LIST OF TABLES

## LIST OF FIGURES

CHARACTERIZATION OF A PROMOTER REARRANGEMENT AND A SECOND PROMOTER IN THE HUMAN C-*MYB* PROTO-ONCOGENE

## ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Medical College of Virginia, Virginia Commonwealth University.

Sarah M. Jacobs

Eric H. Westin, M.D.

Medical College of Virginia/Virginia Commonwealth University

The human cellular proto-oncogene c-*myb* has been implicated as important in the regulation of hematopoietic cell growth and differentiation. Aberrant expression of this gene and chromosomal aberrations near the c-*myb* locus have been associated with a number of carcinogenic processes. An alternatively spliced cDNA clone of c-*myb*, pMbm-2, contains unique 5' sequences which replace exon 1. The existence of this 5' divergent cDNA clone led us into a study of the promoter activity of the c-*myb* gene.

Intron 1 of c-*myb* is highly conserved between human and mouse throughout the intron, while only those sequences directly adjacent to exons 1 and 2 are conserved between human and chicken. The unique sequence of pMbm-2 was located directly adjacent to exon 2, suggesting that it arose as a

product of alternative transcription initiation within intron 1. A cluster of transcription start sites was detected at the 5' end of exon 2. Levels of messages utilizing these start sites are expressed proportionally to those arising from the primary promoter. Functional characterization of this region revealed that this region can function as a promoter. Deletion studies have revealed the presence of negative and positive regulatory elements within this region which are utilized with different efficiencies in different cell lines. These studies suggest that *cis* or *trans* factors acting in this region may serve a dual function in both attenuation and transcription initiation.

Studies of the c-*myb* promoter utilizing the acute lymphoblastic cell line CCRF-CEM revealed that a portion of the c-*myb* promoter is lost in this cell line. The rearranged locus, which we have designated MRR (**m**yb **r**earranged **r**egion), has been cloned and mapped to chromosome 6. The MRR sequence is linked to the c-*myb* locus, suggesting that the rearrangement is due to a submicroscopic deletion. The rearrangement appears to have no effect on c-*myb* promoter activity as analyzed in CCRF-CEM cells. The normal locus of the MRR sequence shows a high degree of homology to a member of the *myc* family of oncogenes. Therefore, although attenuation may be the primary mechanism of c-*myb* regulation, the existence of a second promoter in the c-*myb* gene and a rearrangement of the primary c-*myb* promoter in a leukemia cell line suggests that other regions at the 5' end of this gene are important in the regulation of c-*myb* transcription.

# INTRODUCTION

Hematopoiesis is a complex process by which organisms generate the cells necessary for the various functions of the immune system as well as oxygen transport and coagulation. These cells are derived from a single pluripotent stem cell which may differentiate into multiple cell types. When the normal process of hematopoiesis is disrupted, the immature cells fail to differentiate into their mature forms. The result is acute leukemia, a disorder which is characterized by uncontrolled growth of immature cell types of each of the major hematopoietic lineages. An understanding of how the genes that control hematopoiesis are regulated may lead to an understanding of what goes awry during leukemogenesis.

The human cellular proto-oncogene c-*myb* has been implicated as important in the regulation of hematopoietic cell growth and differentiation. This gene has been identified in evolutionarily divergent organisms including plant and insect systems as well as mammalian species. C-*myb* is highly expressed in a number of leukemias and in immature hematopoietic cells. Furthermore, c-*myb* expression is regulated during the process of hematopoietic

differentiation suggesting that the regulation of this gene is critical for the correct development of the hematopoietic system. The c-*myb* gene is located on chromosome 6q22-23; chromosomal translocations and deletions in this region have been associated with a number of hematopoietic and non-hematopoietic malignancies. Antisense oligonucleotides to c-*myb* mRNA inhibit hematopoietic cell proliferation and c-*myb* expression is necessary for the maintenance of adult-type but not embryonic hematopoiesis. Taken together, these facts suggest that the regulation of c-*myb* expression is important in the proper control of hematopoiesis and that aberrations near the c-*myb* locus may be important in the altered regulation of hematopoietic cell growth and differentiation that occurs in leukemia.

The primary mechanism of regulation of c-*myb* is transcriptional attenuation; this has been localized to the center of intron 1 in both the human and murine c-*myb* genes. In both chicken and mouse acute myeloid and some lymphoid leukemias, c-*myb* is activated by retroviral insertions into sequences at the 5' end of the c-*myb* locus which are not associated with the region of transcriptional attenuation. This work suggests that additional regions in the 5' end of c-*myb* may be important in the regulation of c-*myb* either by effecting the function of the attenuator or by other mechanisms.

The acute lymphoblastic leukemia cell line CCRF-CEM has proved to be an excellent model for studies of the regulation of c-*myb* expression. C-*myb* is expressed at high levels in this cell line; it is therefore useful in the study of the

promoter activity of c-*myb* and in identification of alternatively spliced forms of c-*myb*. Recently, alternatively spliced c-*myb* messages have been cloned from a CCRF-CEM cDNA library. One of these messages contains unique 5' sequences in place of the prototypic c-*myb* exon 1 sequences. The presence of these unique sequences suggests the possible existence of a second promoter within intron 1 of the c-*myb* locus. This promoter would be located at the 3' end of intron 1 in a region which is altered in a number of retroviral induced leukemias in mice and chickens. The objective of this project was to further study regions of the 5' end of the c-*myb* proto-oncogene in order to determine more precisely its role in the regulation of c-*myb* transcription. This analysis was carried out in two ways: i) characterization of a potential second promoter in intron 1 of the c-*myb* proto-oncogene; ii) structural and functional analysis of a rearranged primary c-*myb* promoter in CCRF-CEM.

# LITERATURE REVIEW

Eukaryotic transcriptional regulation.

In eukaryotes, regulation of mRNA synthesis is a highly complex process requiring specific *cis* and *trans* factors for correct transcriptional initiation, elongation, and termination. Transcription may be regulated at any or all of these steps in different genes.

**Regulation of transcriptional initiation.**

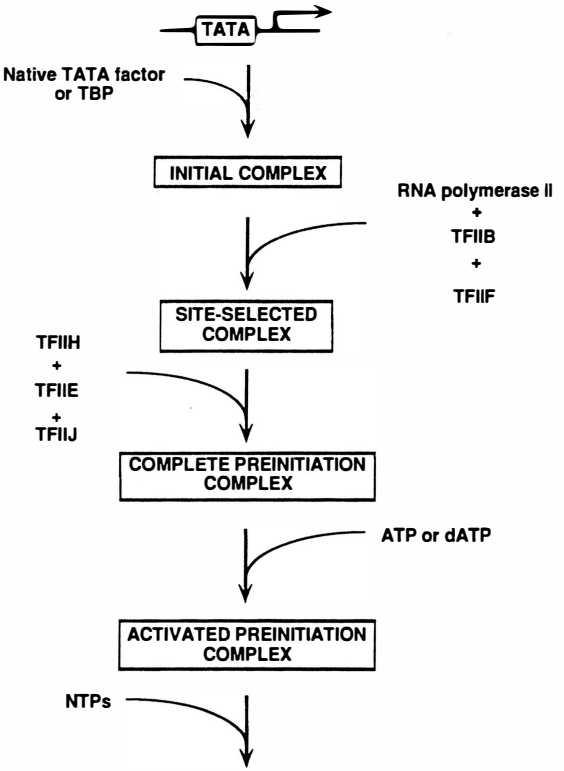
The correct initiation of mRNA synthesis requires specific DNA sequence elements which fall into two classes: a core promoter element and upstream promoter elements. The core promoter element, a TATA box, is recognized by general initiation factors which are required for all RNA polymerase II transcription. Included in the core promoter element may be initiator upstream promoter elements, such as the CAAT box. However, these are recognized by regulatory factors which are not required for initiation, but modulate the initiation event. Some eukaryotic promoters do not contain any consensus TATA or CAAT sequences; these promoters, commonly called housekeeping promoters, are more difficult to define; they may be characterized

by the presence of GC-rich regions, or GpC "islands." Transcription initiates from within these islands in many housekeeping promoters, such as that for c-*myb* (Dvorák et al., 1989).

To initiate transcription, an initiation complex is formed at the TATA box by binding of the TATA binding factor TFIID (O'Shea, Greenfield and Smale, 1992) (Figure 1); this binding is promoted by the initiation factor TFIIA (Hahn et al., 1989). Other initiation factors must then bind the initiation complex: TFIIB, which may play a role in correct positioning of the polymerase (Hahn et al., 1989), and TFIIF and TFIIE, which do not bind DNA but associate with RNA polymerase II (Hahn et al., 1989; Killeen and Greenblatt, 1992). RNA polymerase II binds to form the complete preinitiation complex, and transcription is initiated in an ATP-dependent manner. The mechanism of initiation from eukaryotic promoters which do not contain TATA boxes is not clear; it has been hypothesized that in these systems the TATA binding protein (TBP) enters the initiation complex via binding to RNA polymerase II (Carcamo et al., 1991).

The initiation of transcription by RNA polymerase II is regulated primarily by phosphorylation of its carboxyl terminal domain (CTD); polymerases with hyperphosphorylated CTD's are associated with elongating messages, whereas hypophosphorylated forms of RNA polymerase II are capable of interacting with initiation complexes (Kim and Dahmus, 1988). The mechanism by which phosphorylation regulates RNA polymerase II

Figure 1. Initiation of RNA polymerase II transcription. For specific initiation of RNA polymerase II transcription, a TATA binding factor (TBP) must bind to the TATA sequence in the eukaryotic promoter to form an initiation complex. RNA polymerase II may then bind to the initial complex; the correct positioning of the polymerase is aided by the binding of other transcription factors to RNA polymerase II. Transcription then initiates in an ATP dependent manner.


TATA
Native TATA factor or TBP
INITIAL COMPLEX
RNA polymerase II
+
TFIIB
+
TFIIF
SITE-SELECTED COMPLEX
TFIIH
+
TFIIE
+
TFIIJ
COMPLETE PREINITIATION COMPLEX
ATP or dATP
ACTIVATED PREINITIATION COMPLEX
NTPs

transcription is currently unknown.

Some RNA polymerase II transcripts are constitutively expressed; other genes may need to be initiated in a cell-type or temporally specific manner. In order to regulate the transcriptional initiation of cell-type or developmentally specific genes, other transcription factors must bind to regions near the initiation complex. These transcription factors may be activated or repressed by extracellular signals which are transmitted through signal transduction pathways to the transcription factors. Modification of transcription factors by differential phosphorylation or dephosphorylation effects the DNA binding or protein binding activity of the factor. These modifications may lead to an enhancement or repression of transcription initiation of a gene depending on its role in the growth or development of the organism.

**Transcriptional attenuation.**

In a number of prokaryotic and eukaryotic systems, the site of regulation of transcription is not at the site of initiation but further downstream of this event. In these systems, levels of transcription initiation are steady state, but the transcription elongation complex is paused or terminated downstream of the site of initiation (Bentley and Groudine, 1986; Reddy and Reddy, 1989; Bender et al., 1987). In prokaryotes, the mechanism of attenuation occurs primarily by the formation of a "termination structure" through changes in the secondary structure of the nascent RNA transcript; attenuation can also occur by modulation of rho dependent termination by a *trans*-acting factor (Landick and

Turnbough, 1992). In eukaryotes, the c-*myc*, L-*myc*, N-*myc*, c-*myb*, c-*erbB*, c-*fos*, and c-*mos* proto-oncogenes utilize attenuation as their major mechanism of transcriptional regulation (Landick and Turnbough, 1992). In these systems, the attenuator is regulated according to the proliferative state of the cell; proliferating hematopoietic cells do not demonstrate c-*myc* and c-*myb* attenuation. Upon induction of differentiation, however, the level of transcripts from exon 1 are approximately tenfold higher than exons 2 and 3 for these two genes (Bentley and Groudine, 1986; Reddy and Reddy, 1989; Bender et al., 1987). Because efficient regulation of these genes is crucial during growth and development, it is not surprising that the region of attenuation is altered in some cancers. The site of c-*myc* attenuation is the target of multiple somatic mutations in Burkitt's lymphoma; these mutations alleviate the transcriptional block and allow for high levels of c-*myc* expression (Spencer et al., 1990). The factors which cause this pausing are not well defined; there is evidence that proteins bound to the DNA may block the ability of the elongation complex to read through (Reddy and Reddy, 1989). These factors may bind at the site of attenuation (Reddy and Reddy, 1989) or in the promoter region of the gene (Dufort et al., 1993).

**Alternative splicing of pre-mRNA.**

Following the transcription of full length mRNA, the introns of the mRNA must be spliced out to form fully matured messages. An important mechanism for the generation of diversity in eukaryotic mRNA is alternative

usage of splice donor and acceptor sites. This alternative splicing may exclude exons from a gene or incorporate new exons into its message. In this manner, multiple mature mRNAs may be generated by the same pre-mRNA, yielding structurally diverse proteins. These alternative proteins may be tissue specific or developmentally regulated. Despite all that is known in general about the splicing event, little is known about how the splice sites themselves are specifically and efficiently chosen. There is mounting evidence that the secondary structure of the mRNA may be important in splice site selection (Balvay et al., 1993). A role for factors acting in *trans* has been implicated in cell-specific alternative splicing of pre-RNA (Saga et al., 1990; Barone et al., 1989; Streuli and Saito, 1989); in addition, *cis* elements in both exon (Weil et al., 1989; Streuli and Saito, 1989) and/or intron (Shen-Ong et al., 1984a) sequences may also function to mediate the splicing events. Further studies suggest that base substitutions in exons may activate cryptic splice sites (Nelson and Green, 1990; Weil et al., 1989) or effect splicing of other introns (Talerico and Berget, 1990). Transcription factors which use alternative splicing to generate multiple transcripts include *fos*-B, a member of the *fos* family of transcription factors (Nakabeppu and Nathans, 1991), *ets*-1 (Jorcyk et al., 1991), and c-*myb* (Westin et al., 1990; Shen-Ong, 1987; Dasgupta and Reddy, 1989; Dudek and Reddy, 1989a). In the case of the *fos*-B gene, alternative splicing yields a protein product which can inhibit *Fos*/*Jun* activity, suggesting that alternatively spliced protein products can aid in both positive and negative

control of cellular processes (Nakabeppu and Nathans, 1991).

**Alternative promoter usage.**

In prokaryotes, many genes are under the control of a single promoter for coordinate expression of these genes. Eukaryotic mRNAs, by contrast, are monocistronic. Both systems, however, use a similar mechanism for differential expression of single genes: the use of alternative promoters. In prokaryotes, a second promoter may be used to differentially regulate individual genes that are part of a multicistronic operon. This is evidenced in bacteriophage P1, which utilizes a second promoter inside the ParA/ParB operon (these genes are necessary for the correct partitioning of DNA during cell division) (Friedman and Austin, 1988); the second promoter in front of *ParB* is immune to the normal feedback regulation of the operon and therefore allows for less stringent control of *ParB*. In the Shiga toxin operon (*stx*) of *Shigella* dysenteriae, which contains genes for the A and B subunits of the holotoxin, a second promoter in front of the B subunit gene is not as sensitive to iron repression as the primary promoter (Habib and Jackson, 1992). The subsequent overproduction of this gene may contribute to the stoichiometry of the 1A:5B complex of the holotoxin.

In eukaryotes, alternative promoter usage is widespread in a number of gene systems. Many systems use two promoters for differential regulation of a single gene. The regulation may be temporal; the vaccinia virus *hemagglutinin* (HA) gene is regulated by an early/late and a distinct late promoter (Brown et

al., 1991). In *Drosophila*, the antennapedia gene (*Antp*) also has two promoters; the second promoter is tightly repressed during the initial stages of zygotic cell division and is then activated by specific zygotically-active repressors and activators (Riley et al., 1991). The temporal usage of these alternative promoters, therefore, is important to the correct development and life cycle of these systems.

Alternative promoters may also be used in a tissue specific manner. In *Drosophila*, the dual promoters in the tropomyosin II gene are regulated in a tissue specific manner: one promoter is muscle specific while the other resembles a housekeeping promoter and expresses cytoskeletal forms of the protein (Hanke and Storti, 1988). In the rat, the gamma-glutamyl transpeptidase (*ggt*) gene is encoded by three promoters which may be expressed in a tissue specific manner; promoter II was found to be expressed only in the epididymis and kidney (Lahuna et al., 1992). In the mouse, the alkaline phosphatase gene is expressed in a number of tissues (Studer et al., 1991). This alternatively spliced message is controlled by two promoters; transcripts from the first promoter are expressed in a variety of tissues and in embryo-derived cells while the second promoter is silent in embryonic tissue and expressed specifically at high levels in the heart (Studer et al., 1991). Therefore, this single gene uses two forms of regulation to express different messages in different tissues.

In some genes, the use of alternative promoters may lead to structurally and functionally distinct proteins. The human progesterone receptor has two

protein forms, Form A and Form B. Form A is transcribed from a distinct promoter 537 bases downstream of the Form B promoter; this excludes the Form B translation start site (Kastner et al., 1990). Form A translation, therefore, creates a protein with an unique N terminus. The two forms of the receptor differentially induce target genes, indicating that this dual promoter system creates functionally distinct proteins from the same gene.

There are many genes in which alternative promoters are regulated in a similar fashion and encode the same message (Shull, 1991; Kim et al., 1989b; Kim et al., 1989a; Shull, 1991). The well characterized dual promoter system in the c-*myc* gene demonstrates how a promoter may work in coordination with another form of transcriptional regulation to control the expression of a gene. The c-*myc* gene has two constitutively expressed promoters which would not alter the encoded protein and lie upstream of the region of attenuation in the gene (Miller et al., 1989). Transcripts from the first promoter, P1, are always elongated while transcripts from P2 are elongated or terminated at the site of attenuation. Deletions and point mutations in P2 abolish the transcriptional block (Miller et al., 1989). Recently it was shown that nuclear factors binding between the promoters near P2 contribute to the transcriptional block (Dufort et al., 1993). Therefore, while the second promoter does not encode a novel translated protein product, its usage is important in the regulation of the gene during differentiation.

Signal transduction and oncogenes.

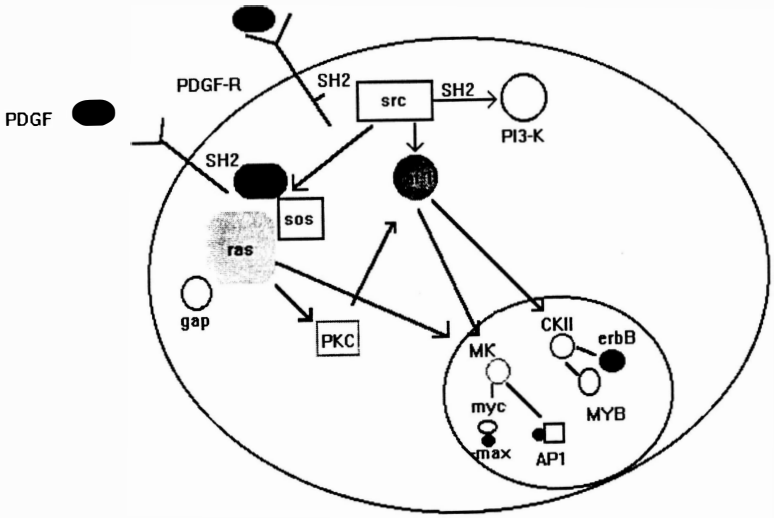
In order to control the specific expression of genes during cell growth and differentiation, it is important for the ubiquitous transcription machinery to interact with cell-specific transcription regulatory factors. The cell- or developmentally regulated transcription factors are activated by external signals which may be hormones or growth factors. These external signals are transmitted through the cell by a complex system know as the signal transduction pathway (Figure 2).

Oncogenes resemble their cellular counterparts, proto-oncogenes (c-onc), but they are functionally locked in their biochemically activated form. Since cancer results from the loss of control of cellular growth and differentiation, it is not surprising that proto-oncogenes function in normal signal transduction and frequently interact with one another at the molecular level. There are four broad classes of proto-oncogenes involved in signal transduction: i) growth factors, which carry the signals received from the extracellular environment; ii) growth factor receptors, which are modified and activated upon ligand binding; iii) cytosolic factors, which associate with and are activated by the receptors; iv) transcription factors, which are altered by the cytoplasmic signals and act in coordination with the transcription machinery to specifically activate genes involved in cell growth and differentiation.

**Growth factors.**

Growth factors are a class of extracellular signalling substances which effect the growth and differentiation of developing cells. Growth factors such as

Figure 2. A signal transduction pathway. In this representation of how a signal is transmitted through the cell, the extracellular signal, platelet derived growth factor (PDGF) is recognized by its receptor (PDGF-R) on the cell surface. The receptor then propagates the signal by subsequent phosphorylation of cytosolic proteins, which may increase the GTPase activity of the protein (in the case of ras/GAP) or the protein tyrosine kinase activity of the protein (in the case of *Src*). These proteins then activate other protein kinases such as *Raf*-1 and protein kinase C (PKC) which modify the activity of transcription factors via MAP kinase (MK) and casein kinase II (CKII). These transcription factors (*Myc*, the AP1 complex, *erbB*, and *Myb*) may then activate or repress genes in response to the extracellular signal. This figure adapted from the textbook *Immunology*, by Roitt et.al. (Roitt et al., 1985)


PDGF
PDGF-R
SH2
src
SH2
PI3-K
SH2
sos
ras
gap
PKC
CKII
erbB
MK
myc
MYB
max
AP1

nerve growth factor, epidermal growth factor, and platelet derived growth factor recognize specific receptors on the cell surface and transmit the signal across the cell membrane via this receptor. The best characterized oncogene which encodes a growth factor is the v-*sis* oncogene, which was isolated from the Simian sarcoma virus (Doolittle et al., 1983); this protein is a homolog of the $\beta$-chain of platelet-derived growth factor (PDGF) (Waterfield et al., 1983).

**Growth factor receptors.**

The majority of growth factor receptors which are implicated in human cancers belong to a family of protein tyrosine kinase receptors (Bishop, 1991). The members of this family of receptors have a highly glycosylated extracellular ligand binding domain, a single membrane spanning region, and a cytosolic portion containing the tyrosine kinase domain (Ullrich and Schlessinger, 1990). Many members of this family, including platelet derived growth factor (PDGF) receptor, have an interruption in their kinase domain known as the kinase insert; this insert has been shown to be important in determining specific signal transduction properties of the receptor (Williams, 1989).

The PDGF receptor is activated by ligand binding (Ullrich and Schlessinger, 1990), which causes dimerization and *trans* phosphorylation of adjacent monomers (Williams, 1989). This phosphorylation increases the intrinsic tyrosine kinase activity of the receptor and creates binding sites which allow cytosolic proteins to associate with the receptor via specific *src*

homology-2 (SH2) domains. These SH2 domains function as binding sites for phosphotyrosine (Koch et al., 1991) and are important for substrate recognition by a number of proteins in the signal transduction cascade (Matsuda et al., 1990). These cytosolic proteins may then become targets for phosphorylation by the receptor. Alternatively, the receptor may simply recruit cytosolic factors to the membrane so that they may interact with other proteins.

Tyrosine kinase receptors are regulated by ligand binding in the extracellular domain (Yarden and Ullrich, 1988). Oncogenic activation of receptor tyrosine kinases occurs due to mutations of the receptor which allow for constitutive protein-tyrosine kinase activity in the absence of bound ligand (Yarden and Ullrich, 1988). Alternatively, the simultaneous expression of the receptor and its ligand may lead to autocrine stimulation of a receptor, as is seen with the v-*sis* oncogene which transforms fibroblasts in an autocrine manner by activating the endogenously expressed PDGF receptor (Waterfield et al., 1983).

**Membrane associated cytosolic factors.**

Following activation, protein tyrosine kinase receptors associate with a number of different cytosolic proteins which, in turn, activate a number of different signal transduction pathways. In the case of the PDGF receptor, ligand activation causes the receptor to associate with at least seven other proteins: $pp60^{c\text{-}src}$, $pp60^{c\text{-}yes}$, $pp60^{c\text{-}fyn}$ (all members of the src-related family of protein tyrosine kinases), phospholipase (PLC-$\gamma$1), ras/GTPase activating protein

(ras/GAP), phosphatidylinositol-3 kinase (PtdIns 3-kinase, a cytosolic tyrosine kinase which phosphorylates the D-3 position of the inositol ring of phosphatidylinositol and appears to act in a new signalling pathway (Auger et al., 1989)), and *Raf*-1, a serine/threonine kinase. In each case, the intracellular protein can only associate with a ligand-activated receptor; mutant PDGF receptors which lack tyrosine kinase activity fail to associate with cytosolic proteins (Coughlin et al., 1989). Two pathways of PDGF activation will be discussed: the src related tyrosine kinase signal transduction pathway, and the ras/GTPase activating protein (ras/GAP) pathway. It is well worth noting that in these systems, as with many other signalling pathways, there is a great deal of redundancy in cellular substrates and secondary messengers utilized.

The members of the family of non-receptor tyrosine kinases share a number of functional domains. Like receptor tyrosine kinases, all have a region responsible for tyrosine kinase activity (Hanks et al., 1988). The src subfamily, which includes $pp60^{c\text{-}src}$, $pp60^{c\text{-}yes}$, and $pp60^{c\text{-}fyn}$ as well as the T-cell receptor associated kinases $p56^{lck}$ and $p59^{fynT}$, also share a myristylation site at their N termini which is required for membrane localization (Cross et al., 1985). Two additional conserved domains include the src homology-2 domain (SH2), described above, and the src homology 3-domain (SH3). The SH3 domain binds proline-rich stretches of nine or ten amino acids in target proteins (Cicchetti et al., 1992).

Upon PDGF receptor activation, it is believed that $pp60^{c\text{-}src}$ associates

with phosphorylated tyrosine on the receptor via its SH2 domain (Cantley et al., 1991). $pp60^{c\text{-}src}$ is negatively regulated by tyrosine phosphorylation at its carboxyl-terminus (Cartwright et al., 1987); the P-Tyr associates with the SH2 domain on the same molecule to maintain the protein in an inactive form. Association of $pp60^{c\text{-}src}$ with the PDGF receptor via its SH2 domain leads to activation of *src* kinase activity. Substrates for $pp60^{c\text{-}src}$ include PtdIns 3-kinase, ras/GAP, and c-Raf-1 (Morrison et al., 1989). $pp60^{c\text{-}src}$ interacts with PtdIns 3-kinase and ras/GAP via its SH2 domain (O'Brien et al., 1990; McCormick et al., 1991).

Activation of tyrosine kinase receptors may also lead to the activation of GTP binding proteins. $p21^{ras}$ is a GTP/GDP binding protein which has a strong GTPase activity (Field et al., 1987). $p21^{ras}$ is oncogenically activated by point mutations in the guanine binding domain (Parada et al., 1982). The biological activity of the protein appears to by determined by the bound state of the nucleotide; $p21^{ras}$ is inactive when bound by GDP and active when bound by GTP. Therefore, $p21^{ras}$ is believed to activate its targets by exchange of GDP for GTP (Marshall et al., 1989). $p21^{ras}$ is negatively regulated by the GTPase activating protein (GAP), which accelerates the GTPase activity of $p21^{ras}$ (Zhang et al., 1990), and positively regulated by the *Drosophila son of sevenless* (dSOS) protein product, which stimulates the exchange of bound GDP for GTP (Rogge et al., 1991).

Recent evidence suggests that $p21^{ras}$ regulation by tyrosine kinases is

mediated by growth factor receptor bound protein 2 (GRB2). This protein contains an SH2 and two SH3 domains which allows it to associate with the insulin receptor (Baltensperger et al., 1993; Skolnik et al., 1993a) via its SH2 domain, and the *sos* gene product via its SH3 domain. Activation of these receptors leads to an increased association of GRB2 and sos with the receptors, thereby increasing the Ras GTP-GDP exchange (Skolnik et al., 1993b). Ras has been shown to activate a number of mitogenic signalling molecules, including serine/threonine kinases such as protein kinase C (PKC) (Morris et al., 1989), mitogen activated protein (MAP) kinases, and the ribosomal protein S6 kinase (*rsk* kinase).

**Non-membrane associated cytosolic factors.**

Activated membrane associated receptors and cytoplasmic factors interact with other cytosolic factors which either directly or indirectly effect transcription of specific genes required for cell growth and differentiation. Recent studies have linked the membrane associated proteins with the transcriptional machinery by selective phosphorylation of transcription factors.

The serine/threonine kinase $pp74^{c\text{-}raf}$ is activated following interaction with activated $pp60^{c\text{-}src}$ and *ras*/GAP, as well as a number of tyrosine kinase receptors (Morrison et al., 1989). *Raf*-1 is phosphorylated on tyrosine (Morrison et al., 1989), but the major sites of phosphorylation are on serine and threonine. *Raf*-1 is phosphorylated by protein kinase C (PKC) (Sozeri et al., 1992), a serine/threonine kinase which is activated both by ras/GAP

(Morris et al., 1989) and diacylglycerol (DAG), a by-product of $pp60^{c\text{-}src}$ activation of PLC-$\gamma$1.

*Raf*-1 has provided the link between transcription factor modifications and the signal transduction pathway. *Raf*-1 activates mitogen activated protein kinase (MAP kinase), a serine/threonine kinase which phosphorylates transcription factors (Howe et al., 1992). The mechanism of activation appears to be phosphorylation of MAP kinase kinase, which, in turn, phosphorylates and activates MAP kinase. Because MAP kinase is a central factor in a number of signal transduction pathways, it is now possible to determine the effects of different signal transduction pathways on the activity of transcription factors.

**Transcription factor modifications.**

In the final step of the signal transduction pathway, transcription factors are modified to either activate or repress genes involved in cell growth and regulation. Two serine/threonine kinases have been implicated in the regulation of sequence specific DNA binding by transcription factors: MAP kinase and casein kinase II.

MAP kinase directly phosphorylates a number of transcription factors. The c-*myc* proto-oncogene is a widely expressed sequence specific DNA-binding protein (Blackwell et al., 1990) which requires a cofactor, MAX, for efficient DNA binding (Blackwood et al., 1992b; Blackwood et al., 1992a). *Myc* is directly phosphorylated by MAP kinase on its N terminus, which is the transactivation domain of the protein. Phosphorylation of this region stimulated

the transactivation ability of c-myc (Seth et al., 1991). Therefore, the *Myc* protein is able to respond to extracellular signals from a variety of receptors to transactivate genes in a signal specific manner.

MAP kinase also effects the AP1 transcription factor complex comprised of the c-*fos* and c-*jun* proto-oncogene products (Curran and Franza, Jr., 1988). These two proteins interact through a series of leucine containing alpha helical domains, termed leucine zippers (Gentz et al., 1989). As with *Myc*, *Jun* is phosphorylated by MAP kinase in its transactivation domain which positively regulates the transactivation activity of *Jun* (Gille et al., 1992). The transcription factor p62TCF, which is necessary for activation of the c-*fos* promoter, is also phosphorylated by MAP kinase; phosphorylation of this factor leads to transactivation of the c-*fos* promoter. Therefore, MAP kinase is able to activate the AP1 complex by both direct phosphorylation of AP1 proteins and by stimulation of transcription of one of its products. Recently, it has been determined that MAP kinase also phosphorylates the *Myb* protein (Aziz et al., 1993).

Casein kinase II (CKII) is another serine/threonine kinase which may play a major role in the regulation of both mitosis and transcription. This enzyme phosphorylates serines within a negatively charged amino acid region containing the consensus sequence $\beta$-turn-S-X-X-E (Figge et al., 1988). CKII is activated by the cell-cycle specific kinase *cdc2*. Activated CKII has been shown to phosphorylate a number of cell-cycle related proteins, such as p53, a tumor

suppressor gene which regulates cell-cycle progression from G1 to S phase (Patschinsky and Deppert, 1990), and cAMP response element binding factor (CREB), which binds cAMP responsive elements and mediates the induction of these genes by cAMP (Roesler et al., 1988).

CKII phosphorylation sites are deleted in a number of oncogenically activated proteins. Examples of these include the v-*erbA* oncogene product; this protein contains an amino terminal truncation which deletes a CKII-sensitive phosphorylation site (Glineur et al., 1989). V-*Fos*, which is derived from the Finkel-Biskis-Jinkins murine osteosarcoma virus, is missing CKII-sensitive phosphorylation sites which are present in the carboxyl terminus of c-*Fos* (Van Beveren et al., 1983).

In both of these cases, the consequences of the deletion of these CKII sites is unknown. CKII phosphorylation of *Myb* has been implicated in the regulation of this nuclear protein. *Myb* contains two CKII sites at its amino-terminus which are deleted in the v-*myb* protein. Phosphorylation of these two CKII sites has been shown to inhibit the DNA binding ability of c-*Myb*, indicating that these phosphorylation events may be important in the regulation of c-*Myb* activity (Lüscher et al., 1990; Lüscher and Eisenman, 1992).

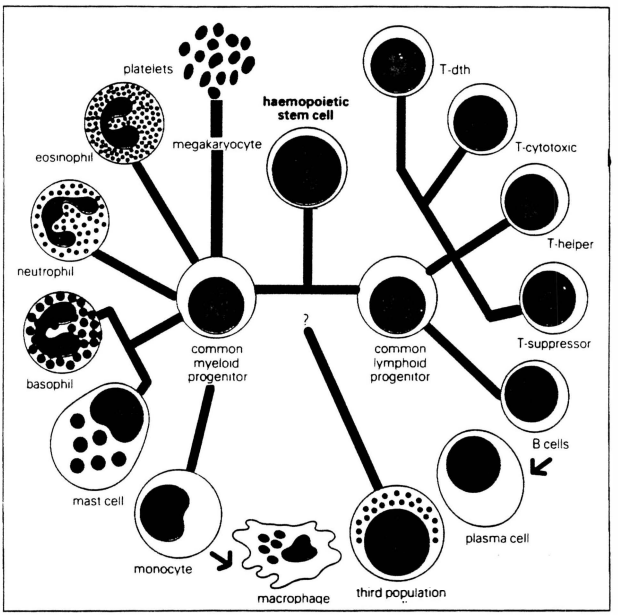
Hematopoiesis and leukemogenesis.

Hematopoiesis is the process by which organisms supply themselves with multiple types of cells required for blood formation. All cells of the hematopoietic system are derived from a common hematopoietic stem cell

which is found in the bone marrow of adult humans (Figure 3). This stem cell may be induced to develop into any of the cells which comprise the hematopoietic spectrum. Depending on the inducing agent, the stem cell develops into a committed progenitor cell for each of the hematopoietic differentiation pathways. The committed progenitor cells are then further induced to mature into their end stage forms through various intermediates.

The branches of the hematopoietic system are the erythroid, myeloid, megakaryocyte and lymphoid branches. The myeloid, erythroid and megakaryocytic cells are all derived from a single committed progenitor cell (Abramson et al., 1977). This cell is induced to differentiate into cells which are committed to be either myeloid (monocytes or granulocytes), erythroid (red blood cells) or megakaryocytic (platelets) lineages. These committed cells are then induced to differentiate via various intermediate forms to their mature phenotype. Myeloid-committed cells develop into granulocytic cells, which may be neutrophils, basophils, eosinophils, or monocytic cells which develop into blood monocytes and tissue macrophages. The lymphoid branch develops from the lymphoid committed progenitor cell (Wu et al., 1968). These committed cells develop into immature B- and T- cells, which must then migrate to the thymus for further maturation. These cells are induced to terminally differentiate by antigens; B- cells are induced into antibody-secreting plasma cells, while T- cells are induced into regulatory cells, T-helper and T-suppressor cells, or cytotoxic T-cells (McCulloch et al., 1974).

Figure 3. Hematopoiesis. All blood cells are derived from a pluripotent hematopoietic stem cell. This cell may develop into a common myeloid or lymphoid progenitor. The development of these progenitors into mature cells is stimulated by cell-type specific growth factors (in myeloid and T- cell development) or by antigens (in B-cell development).


platelets
megakaryocyte
haemopoietic stem cell
T-dth
T-cytotoxic
T-helper
T-suppressor
B cells
plasma cell
third population
macrophage
monocyte
mast cell
basophil
neutrophil
eosinophil
common myeloid progenitor
common lymphoid progenitor
?

Neoplastic transformation of hematopoietic cells reflects an arrest of these cells at different stages along the normal differentiation pathway. Leukemic cells are unable to terminally differentiate and are trapped in a proliferative state. Leukemias are generally classed according to the lymphoid and myeloid markers expressed by the neoplastic cells and by their arrested stage in differentiation. Leukemias are grouped into two broad categories; chronic and acute leukemias. Cells involved in chronic leukemia reflect a more differentiated state of the involved lineage type. Chronic leukemias include chronic myelogenous leukemia (CML), which is characterized by overproduction of granulocytic cells, hairy cell leukemia, a rare form of leukemia similar to CML, and chronic lymphocytic leukemia, which is characterized by an accumulation of monoclonal mature B or occasionally T-lymphocytes (Keating et al., 1992).

Acute leukemias reflect malignant transformation of early hematopoietic precursors (Keating et al., 1992). These cells, therefore, give rise to cells which cannot be induced to differentiate and instead proliferate in an uncontrolled manner. These immature "blast" cells accumulate and replace the normal bone marrow cells leading to anemia, bleeding and an inability to fight infection. Transformation of an immature myeloid cell leads to acute myelogenous leukemia (AML), while transformation of an immature lymphoid cell leads to acute lymphoblastic leukemia (ALL)(Keating et al., 1992).

Like leukemia, lymphomas may be related to specific stages of normal

B- and T- cell development. There are two classes of lymphomas: Hodgkin's disease, and non-Hodgkin's lymphoma (NHL). Hodgkin's disease is an unique neoplasm which arises in the lymph nodes and is defined by the presence of specific Reed-Steinberg giant cells. Non-Hodgkin's lymphomas (NHL) are the most common immune system neoplasms and reflect a heterogeneous group of neoplasms which are T-and B-cell in origin (Keating et al., 1992).

Chromosomal abnormalities in leukemias and lymphomas.

Chromosomal translocations and deletions have been implicated in a number of lymphoid and myeloid malignancies. Chronic myelogenous leukemia may be diagnosed by a frequent reciprocal translocation between chromosomes 9 and 22 (the Philadelphia chromosome, Daley et al., 1990). In addition, translocations may affect two important areas of T-and B- cell regulation: the correct expression of antigen receptor genes, and aberrant expression of oncogenes. Both heavy and light immunoglobulin (Ig) chain genes are abnormally rearranged in lymphomas and chronic leukemias of B-cell lineages (Korsmeyer et al., 1983), while the T cell receptor (TCR) may be abnormally rearranged in mature T-cell or B-cell lineages (Aisenberg et al., 1987). In these neoplasms, the rearrangements have been ascribed to errors arising from the recombinase system used in the normal rearrangement of Ig and TCR genes during the generation of receptor diversity. These rearrangements can result in lineage inappropriate expression of these receptors; in other cases, the rearrangement may bring a proto-oncogene under the control of

Table 1. Chromosomal translocations implicated in the tumorogenic process. Shown are the tumor type, the chromosomal abnormality associated with the tumor, and the gene or genes which may be involved in the formation of the tumor, and the gene function, if known (Perkins and Vande Woude, 1993).

| Tumor | Abnormality | Gene(s) Involved | Gene Feature or Function |
|---|---|---|---|
| Chronic myelogenous leukemia | t(9;22))(q34;q11) | BCR-ABL | Fusion gene involving nonreceptor tyrosine kinase (ABL) |
| T-cell acute lympho-blastic leukemia (T-ALL) | t(9;22))(q34;q11) | BCR-ABL | p180 gene product |
| Follicular center cell lymphoma | t(14:18)(q32;q21) | BCL2 activation | Prevents apoptosis |
| Burkitt lymphoma | t(8:14)(q24;q32) | c-MYC IG locus | Transcription factor |
| Acute promyelocytic leukemia | t(15;17) | APL-RAR fusion | Fusion gene involving the retinoic acid receptor X |
| T-ALL | t(11;14)(p15;q11) | TCR locus | Zinc-finger gene |
| T-ALL | t(17;19)(q34;p13) | LYL1 ($\beta$TCR) | Nuclear, helix-loop-helix |
| Pre-B-ALL | t(1;19)(q23;p13.3) | E2A-PR1 fusion | Fusion gene containing homeobox from PRL |
| T-ALL | t(1;14)(p32;q11) | SCL(tal;TCL5) | Helix-loop-helix gene |
| T-ALL | del(1), p33 | SCL/SIL (SCL interrupter locus) | SIL-SCL fusion gene |
| T-ALL | t(10;14) | HOX11 are TCL3 | Homeobox gene |
| T-ALL | t(7;9) | TAN1 | Homolog of *Drosophila* notch gene |
| B-cell lymphoma | t(11;14)(q13;l32) | PRAD1, BCL1 | Cyclin D |
| Amplifications in breast and squamous cell carcinomas | inv(11) | $G_1$ cyclin gene | |
| Parathyroid adenoma | inv(11) | PRAD1 | Cyclin D |

Table 2. Chromosome 6q abnormalities in cancer.

| Aberration | Localization | Tumor | Reference* |
|---|---|---|---|
| Translocation | t(X:6q15);t(X:6q16) | ALL | (1) |
| | t(6:11)(q27:q23) | AML | (3) |
| | t(6:9) | AML | (4) |
| Deletion | del(6)(q13q27) | CLL | (2) |
| | del(6)(q22q27) | melanoma | (5) |
| | del(6)(q12q14) | AML | (6) |
| | del(6q-) | AML | (7) |
| | del(6)(q21q23) | ALL | (8) |
| | del(6)(q21q23) | NHL | (9) |
| | del(6)(q21q23) | AML,CML | (10) |
| | del(6)(q22) | ALL,AML | (11) |
| | | NHL | (12) |

* (1)- (Carney et al., 1992); (2)- (Philip et al., 1991); (3)- (Hatfill et al., 1991); (4)- (von Lindern et al., 1992); (5)- (Millikin et al., 1991); 6-(Hirata et al., 1992); 7-(Fagioli et al., 1990); 8-(Oshimura and Sandberg, 1976) (9)-(Bloomfield et al., 1983); (10)-(Mitelman, 1983); (11)-(Barletta et al., 1987); (12)-(Park and Reddy, 1992).

immunoglobulin gene enhancers, resulting in the deregulation of the proto-oncogene and the creation of an oncogene (Table 1). This phenomenon is best demonstrated in the neoplasm known as Burkitt's lymphoma; in 80% of these neoplasms, a translocation has occurred between chromosomes 8 and 14 [t(8:14)]. This translocation juxtaposes the IgM heavy chain gene to the c-*myc* proto-oncogene (Taub et al., 1982); the deregulation of the c-*myc* gene contributes to unrestricted B-cell proliferation and thus to the pathogenesis of this B-cell malignancy. Other immunoglobulin translocations implicating specific oncogenes in leukemia pathogenesis include *bcl*-2, a gene shown to be important in programmed cell death (McDonnell et al., 1989) and rearranged in follicular lymphomas (Rowley, 1988) and *bcl*-1, which was identified at a t(11:14) translocation (Raffeld and Jaffe, 1991) and is important in cell cycle regulation (Motokura et al., 1991).

6q abnormalities in leukemias and lymphomas.

Deletions and translocations of the long arm of chromosome 6 have been implicated in a number of leukemias and lymphomas (Table 2). These deletion and translocation breakpoints occur at a number of points in the 6q locus, ranging from 6q13-6q27 (Hirata et al., 1992; Carney et al., 1992; Philip et al., 1991; Millikin et al., 1991). These studies suggest that genes in this region of 6q, which include c-*myb*, play an important role in the neoplastic process.

Recent studies have suggested the possibility of tumor suppressor genes which may be located on chromosome 6. In a study of childhood ALLs with

6q- deletions, all of the deletions encompassed the 6q21 region (Hayashi et al., 1990). Hubbard-Smith et al. immortalized human fibroblasts with an origin-defective SV40 virus and analyzed the karyotypes of the immortalized fibroblasts. All of the immortalized cell lines had alterations in the 6q21 region, which does not correlate to the locus of any known oncogenes or tumor suppressor genes (Hubbard Smith et al., 1992). These results suggest that genetic loss of regions at or distal to 6q21 may be important in the immortalization process and suggests the presence of a tumor suppressor gene.

The *myb* oncogene.

The cellular proto-oncogene c-*myb* was first identified as the transforming agent of the avian myeloblastosis virus (AMV). AMV specifically transforms cells of the myelomonocytic lineage in chickens, producing AMV-transformed myeloblasts with an unrestricted growth potential (Symonds et al., 1984). The transforming phenotype of this replication-defective virus and the E26 virus, which induces erythroid tumors (Nunn and Hunter, 1989), was shown to be associated with the presence of normal cellular sequences within the viral genome (Souza et al., 1980).

The cellular sequences of the AMV virus (then known as v-*amv*) were used to determine the cell-specific expression of this gene in human leukemia cell lines. Westin, et al (Westin et al., 1982), determined that v-*amv* was expressed in immature precursor cells of lymphoid, myeloid and erythroid lineages, but not in immature B-cells nor mature T-cell and myeloid cell

lineages. Cellular sequences related to v-*amv* were not expressed in any solid tumors screened; therefore, the expression of this gene correlated with hematopoietic cells of immature phenotypes (Westin et al., 1982).

The cellular homologue to the v-*amv* sequences, designated cellular *myb* (c-*myb*) was transduced from the chicken cellular *myb* gene in both AMV (Klempnauer et al., 1982) and E26 leukemia virus (Radke et al., 1982). The c-*myb* locus is approximately 36 kb in length and encodes a transcript of approximately 3.5 kb (Westin et al., 1982). The gene has 15 exons not including alternatively spliced exons recently identified (Prochownik et al., 1990; Dasgupta and Reddy, 1989; Shen-Ong et al., 1990) (Figure 4).

V-*myb* is a spliced version of its cellular homologue, c-*myb*, containing seven exons of c-*myb* with sequences deleted at the 5' and 3' ends of the gene (Klempnauer et al., 1983). The v-*myb* protein encoded by AMV is 45 kilodaltons (kDa) (Klempnauer et al., 1982), designated $p45^{v\text{-}myb}$; the protein is smaller than the cellular c-*myb* gene due to amino- and carboxyl- terminal truncations in the protein (Figure 5). The full length *Myb* protein is 75 kDa and is extensively conserved at the protein level in multiple functional domains between Drosophila (Katzen et al., 1985), chicken, mouse, and human species (Cole, 1990). *Myb*-related genes have also been cloned from *zea mays*, and Dictyostelium (Cole, 1990). Two human c-*myb* related genes, A-*myb* and B-*myb*, have also been identified (Nomura et al., 1988). Unlike c-*myb*, A-*myb* and B-*myb* are expressed in tissue types beyond the hematopoietic system; some

of the tissues that express A-*myb* and B-*myb* include heart, intestine, and lung (Nomura et al., 1988). Little is known about their function, although B-myb is down-regulated during hematopoiesis (Reiss et al., 1991b).

***Myb* structure and function.**

The exact role of c-*myb* in normal cellular processes is not known. However, c-*myb* has been implicated as an important factor in hematopoietic growth and differentiation. Myeloid cells infected with a temperature sensitive mutant of the v-*myb* gene proliferated at the permissive temperature, but exhibited a more differentiated phenotype at the non-permissive temperature (Ness et al., 1989). Therefore, the mechanism of transformation by v-*myb* appears to be its ability to block the differentiation of hematopoietic cells.

Both v-*Myb* and *Myb* proteins have been localized to the nucleus and are associated with chromatin, suggesting these may be DNA binding proteins (Boyle et al., 1985; Ibanez and Lipsick, 1988). Bacterially expressed v-*Myb* was used to localize the DNA binding domain for this protein to the N-terminus (Klempnauer and Sippel, 1987) and to determine that the protein binds the specific 6 base pair motif pyAACG/TG (Biedenkapp et al., 1988).

The DNA binding domain of c-*Myb* is comprised of approximately 150 amino acids in three imperfect repeats of 51 to 53 amino acids in length (Figure 5). These repeats contain a large proportion of basic residues, a motif associated with other DNA binding domains (Paz-Ares et al., 1987). The three imperfect repeats in the DNA binding domain also contains a series of regularly

Figure 4. The c-*myb* locus. The c-*myb* locus encompassed approximately 30,000 bp and is comprised of 19 exons: four of these exons are alternatively spliced in a number of forms of c-*myb*. The primary c-*myb* promoter (P1) and coding ATG translation start sites (ATG) are indicated. Some of the alternatively spliced forms utilize different splice donor (SD) sites (such as pMbm-1 and pMbm-2) or utilize an internal polyadenylation site within intron 8 (pMbm-28). Two alternative translation termination sites are also present in exon 8A and exon 13A; usage of these sites would result in a C-terminally truncated protein.

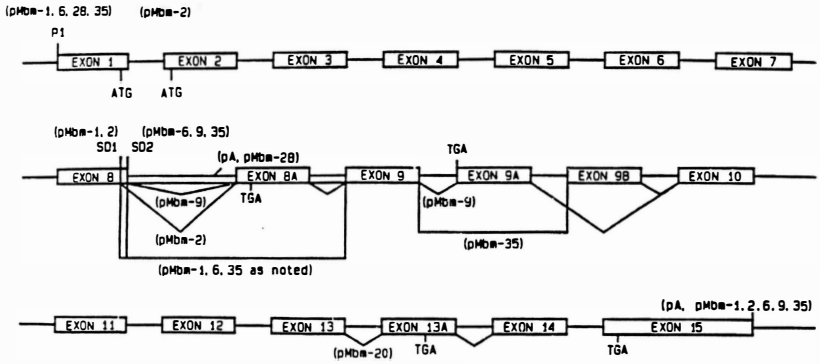
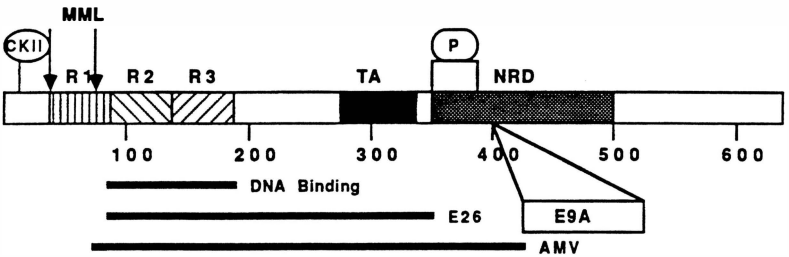

(pMbm-1, 6, 28, 35)
(pMbm-2)
P1
EXON 1
EXON 2
EXON 3
EXON 4
EXON 5
EXON 6
EXON 7
ATG
ATG
(pMbm-1, 2)
(pMbm-6, 9, 35)
SD1
SD2
(pA, pMbm-28)
TGA
EXON 8
EXON 8A
EXON 9
EXON 9A
EXON 9B
EXON 10
(pMbm-9)
TGA
(pMbm-9)
(pMbm-2)
(pMbm-35)
(pMbm-1, 6, 35 as noted)
(pA, pMbm-1, 2, 6, 9, 35)
EXON 11
EXON 12
EXON 13
EXON 13A
EXON 14
EXON 15
(pMbm-20)
TGA
TGA

Figure 5. The c-*myb* protein. The prototypic c-*myb* protein is 640 amino acids in length. The DNA binding domain (R1, R2, and R3), transactivation domain (TA) and negative regulatory domain (NRD) are indicated by the shaded regions. Downward arrows indicate sites of retroviral insertion by moloney murine leukemia virus (MML)(Shen-Ong et al., 1984b). The casein kinase II (CKII) -sensitive phosphorylation site and MAP kinase (P) sensitive phosphorylation sites are also indicated. The minimal DNA binding domain and the *Myb* protein sizes of the E26 and AMV viruses are indicated below. This figure adapted from a review by Thomas Graf (Graf, 1992).


MML
CKII
R1
R2
R3
TA
P
NRD
100
200
300
400
500
600
DNA Binding
E26
E9A
AMV

spaced tryptophans; recent nuclear magnetic resonance (NMR) studies have indicated that a novel helix-turn-helix motif is formed around these tryptophans in the third repeat of the protein (Ogata et al., 1992). This unique structure therefore represents a new DNA binding motif.

The DNA binding affinity of *Myb* appears to depend both on the specific sequence of the *Myb*-responsive element (MRE) and the structure of the protein. The protein binds as a monomer but the interaction appears to be enhanced when tandem copies of the MRE are placed close together (Howe et al., 1990). Carboxyl-truncated *Myb* proteins bind to the MRE with a seven-fold higher affinity than full length *Myb* protein, which may contribute to the transforming activity of the viral forms of c-*myb* (Ramsay et al., 1992).

Both c-*myb* and v-*myb* can transactivate a reporter gene via the MRE (Nishina et al., 1989; Weston and Bishop, 1989). C-*myb* contains a transactivation domain of approximately fifty amino acids in the center of the protein (Sakura et al., 1989; Weston and Bishop, 1989; Lane et al., 1990; Ibanez and Lipsick, 1990). The region is comprised of hydrophilic and acidic amino acids, consistent with other transactivation domains (Saha et al., 1993). Little is know about the exact mechanism of transactivation by the *Myb* protein except that it is regulated by a negative regulatory domain located at the carboxyl-terminus of the protein; deletion of this negative regulatory domain leads to a ten-fold increase in the transactivation ability of *Myb* (Sakura et al., 1989; Lane et al., 1990). It has recently been determined that the negative

regulation of transactivation is mediated by a leucine zipper motif in the negative regulatory domain (Kanei-Ishii et al., 1992); this motif is absent in oncogenically activated *Myb* proteins suggesting that one of the mechanisms of transformation is removal of this leucine zipper.

*Myb* also contains a region of transrepression, located downstream of the putative transcriptional activation domain (Ibanez and Lipsick, 1990). This region is missing in a number of transformation associated *Myb* proteins (Rosson et al., 1987), suggesting that transformation by c-*myb* may also be mediated by increased expression of other genes. The activation and repression activities of *Myb* appear to depend on the affinity of the protein for different MRE's (Ibanez and Lipsick, 1990). When *Myb* is bound to a high affinity site, the protein is capable of transactivation (Ibanez and Lipsick, 1990; Sakura et al., 1989; Lane et al., 1990; Weston and Bishop, 1989); when the protein is bound to a low affinity site, transrepression activity is observed (Ibanez and Lipsick, 1990).

The first gene identified as a target for v- or c-*myb* transactivation was the *mim*-1 gene. This gene is expressed in the granules of promyelocytes but its function is currently unknown. The *mim*-1 promoter contains three myb responsive elements (MRE) and is activated by both the v-*myb* (Ness et al., 1989) and c-*myb* (Dudek et al., 1992) proteins, although the transactivation ability of c-*myb* is much lower than v-*myb*.

*Myb* has also be found to activate other genes important in cell growth

and differentiation. The *Myb* protein can regulate c-*myc* expression in a cell-type specific manner (Cogswell et al., 1993) and can interact with MRE's in the c-*myc* promoter (Zobel et al., 1991). A murine variant of the E26 virus can activate the erythroid transcription factor GATA-1 (Aurigemma et al., 1992), and c-*myb* can increase the expression of insulin-like growth factor 1 and insulin-like growth factor 1 receptor (Reiss et al., 1991a). Finally, there is evidence that the c-*myb* gene is autoregulatory; MRE's in the c-*myb* promoter can activate the thymidine kinase (TK) reporter gene when the reporter gene is co-transfected with the full length c-*myb* gene (Nicolaides et al., 1991).

Further information on the function of *Myb* may be garnered from its expression during the cell cycle and during hematopoietic differentiation. Expression of c-*myb* is cell cycle regulated; *Myb* levels increase during the cell cycle and peak during S phase (Thompson et al., 1986). Other data linking c-*myb* expression to hematopoietic cell growth include evidence that c-*myb* is necessary for G1/S transition in human T lymphocytes (Gewirtz et al., 1989). C-*myb* expression is highest during cellular proliferation and decreases as the cells leave the cell cycle. Induction of differentiation in both Friend murine erythroleukemia (FMEL) (Watson, 1988b) and human HL-60 promyelocytic leukemia cells (Ibanez and Lipsick, 1988) correlates with a decrease in c-*myb* expression during differentiation.

In FMEL cells, c-*myb* expression during differentiation appears to be biphasic with levels decreasing during the early period of commitment, rising

again, and then decreasing following loss of cellular proliferation (Watson, 1988b). Constitutive expression of c-*myb* has been shown to block differentiation in two erythroleukemic cell lines (Watson, 1988b; Kirsch et al., 1986). Inhibition of $p75^{c\text{-}myb}$ expression using antisense oligonucleotides inhibits the growth of hematopoietic cells *in vitro* (Anfossi et al., 1989; Ratajczak et al., 1992). Embryonic "knockout" experiments revealed that c-*myb* was necessary for adult-type but not embryonic hematopoiesis (Mucenski et al., 1991). Together, these results suggest that the function of c-*myb* is to maintain hematopoietic cells in an immature state and act as a "switch" during early stages of differentiation (Lüscher et al., 1990).

**6q abnormalities and the c-*myb* gene.**

Three genes have been localized to the long arm of chromosome 6: c-*myb*, *ros*1, and *mas*1. Of these three, translocations and deletions associated with leukemias and lymphomas have been localized to regions surrounding the c-*myb* locus which has been sublocalized to 6q22-23 (Janssen et al., 1986). Because c-*myb* has been demonstrated to be important in the regulation of hematopoiesis, a number of studies have attempted to make a correlation between deletion or rearrangement of the c-*myb* gene and myeloid and lymphoid malignancies. In a series of in situ hybridizations of 6q- hematopoietic neoplasms with human c-*myb* probes, two different studies (Barletta et al., 1987; Okada et al., 1990) demonstrated that while the 6q- interstitial and distal deletions correlated with an increase in c-*myb* expression,

the gene itself was neither rearranged nor deleted (Barletta et al., 1987). The breakpoints for these deletions mapped to 6q22, cytogenetically close to the c-*myb* locus, but no rearrangements could be found within 12 kilobases (kb) of the 5' end of this gene.

In a recent series of studies using infrequently cutting restriction enzymes and the clamped homogeneous electric field system (CHEF) to resolve high molecular weight DNA fragments, the c-*myb* genomic locus was extensively mapped (Park and Reddy, 1992). This map, created from genomic DNA isolated from normal peripheral blood lymphocytes, was compared to genomic maps of the c-*myb* loci from 7 6q- leukemia cell lines with high levels of c-*myb* expression. Of the 7 myeloid and lymphoid leukemia cell lines tested, no large scale deletions were detected within 1 megabase (1000 kb) of the c-*myb* locus.

Other studies involving 6q abnormalities have also failed to implicate rearrangements of the c-*myb* locus in this abnormality (Barletta et al., 1987; Okada et al., 1990; Park and Reddy, 1992; Philip et al., 1991; Hirata et al., 1992), although amplification of the c-*myb* locus has been reported in occasional cases of AML (Pelicci et al., 1984; Barletta et al., 1987; Castaneda et al., 1991).The results of these studies suggests that while c-*myb* expression may be activated in 6q- abnormalities and regions at or near the c-*myb* locus are important in the development of leukemias and lymphomas, specific rearrangements of the c-*myb* locus have not been detected in 6q- cytogenetic abnormalities. It has been speculated that a growth suppressive and/or a c-*myb*

suppressive gene may be deleted in these leukemias (Park and Reddy, 1992; Hubbard Smith et al., 1992); deletion of this gene may then deregulate c-*myb* expression and, possibly, the expression of other genes important in the regulation of hematopoiesis.

**Regulation of c-*myb* expression.**

The importance of the proper temporal and quantitative levels of c-*myb* expression in the growth and differentiation of hematopoietic cells indicates that it must be tightly regulated at the transcriptional level. The majority of the mechanisms that govern c-*myb* expression are located at the 5' end of the gene, spanning the 5' untranslated region through the first intron.

The c-*myb* promoter is a GC-rich promoter which does not contain any consensus TATA or CAAT sequences in the mouse (Bender and Kuehl, 1986), or the human (Westin et al., 1990), and no TATA sequences in the chicken (Hayward et al., 1989). This structure is indicative of a housekeeping promoter. Because there are no specific transcription initiation factor binding sites in the c-*myb* promoters, mRNAs initiated from these promoters exhibit extensive 5' heterogeneity. In the chicken, the transcription start sites initiate within a GpC island (Dvorák et al., 1989), a region rich in guanine and cytosine. Although the transcripts are heterogenous at the 5' end, there is cell-type specificity to the transcription start sites; in the chicken, T-cell and B-cell precursors each exhibited patterns of 5' heterogeneity specific for that cell type (Kim and Baluda, 1989). Similar patterns of cell-type specific 5' heterogeneity have been

detected in the mouse (Watson et al., 1987).

Little is known about what transcription factors are responsible for the activation of c-*myb*. As stated in the previous section, c-*myb* may be autoregulated (Nicolaides et al., 1991). There is also evidence that members of the *Jun* family of transcription factors, which are important in the early stage of the cell cycle, can transactivate the c-*myb* gene (Nicolaides et al., 1992). However, because the c-*myb* promoter is constitutively active in most cells that have been tested (Biunno et al., 1988), promoter regulation does not appear to be the primary mechanism of regulation of c-*myb* expression.

In the murine and human systems, c-*myb* expression is regulated by a transcriptional pause; nuclear runoff assays have shown a steady rate of c-*myb* transcription despite varying levels of c-*myb* mRNA during cellular proliferation (Thompson et al., 1986). A number of recent studies have demonstrated that, in the mouse, c-*myb* is regulated primarily by a constitutive premature arrest within the first intron of the gene (Watson, 1988a; Bender et al., 1987; Sobieszczuk et al., 1989). Recently, this region of transcriptional arrest or pausing has been mapped in the murine system to a 1.0 kb region of intron 1. DNAse hypersensitivity and gel mobility assays have correlated the binding of nuclear factors to this 1.0 kb region of intron 1 with a decrease in expression of c-*myb* mRNA (Reddy and Reddy, 1989). A similar region of transcriptional pausing was also detected in the human c-*myb* intron 1 (Godbout et al., 1992). No attenuator has ever been detected in the chicken c-*myb* gene. Although the

actual regions of transcriptional pausing have been mapped in the human and mouse systems, it is not known what factors are necessary for attenuation nor whether sequences outside the pausing region are necessary for pausing to occur.

It was once believed that c-*myb* encoded a single mRNA and protein in the absence of viral integration, rearrangements, or deletions (Bender and Kuehl, 1986). Recent evidence indicates, however, that this is not the case. Alternative c-*myb* splicing occurs in both normal (Shen-Ong, 1987) and tumor (Westin et al., 1990; Shen-Ong, 1987; Dasgupta and Reddy, 1989; Dudek and Reddy, 1989a) cell lines. The alternative splicing may be due to alternative use of splice donor/acceptor sites which may delete 3 amino acids (Westin et al., 1990), the inclusion of novel exons which increase the size of the protein (Dudek and Reddy, 1989b; Dasgupta and Reddy, 1989; Dudek and Reddy, 1989a; Shen-Ong et al., 1990), or the inclusion of an exon which may lead to premature termination of translation (Weber et al., 1990) (Figure 4). Most of this alternative mRNA processing occurs between exons 8 and 9 indicating that changes in this region, which encodes a portion of the transactivation domain, may effect the transactivation ability of c-*myb*. The function of these alternatively spliced c-*myb* products in the regulation of hematopoiesis is currently unknown.

**Activation of c-*myb*.**

The viral form of $p75^{c\text{-}myb}$, $p45^{v\text{-}myb}$, contains amino- and carboxyl-

terminal truncations in addition to fusion to viral protein sequences and amino acid substitutions. *In vitro* studies have been conducted to determine which alterations in the c-*myb* gene enhance the transforming activity of the v-*myb* protein. Neither the virally-encoded residues nor the amino acid substitutions are required for transformation by v-*myb* (Ibanez and Lipsick, 1988). Rather, it is the amino- and carboxyl- terminal truncations which confer the transforming phenotype of the v-*myb* protein. The deletion of either the amino- or carboxyl-terminus of the protein is sufficient to cause the transforming phenotype (Grässer et al., 1991; Gonda et al., 1989). The carboxyl terminal truncations appear to cause transformation by disrupting the negative regulatory domain at the carboxyl- terminus of the protein (Hu et al., 1991); also, these carboxyl-terminally deleted proteins appear to have an increased DNA binding affinity for target sites (Olins and Rangwala, 1990).

The mechanism of increased transforming capacity of amino-terminally deleted *Myb* protein is not as clear; N-terminal truncation removes a putative regulatory region of the protein which may regulate the DNA binding activity of *Myb*; the amino terminal truncation removes a casein kinase II (CKII) sensitive site which has been shown to inhibit DNA binding when phosphorylated (Lüscher et al., 1990; Lüscher and Eisenman, 1992). Also, an amino-terminally truncated c-*myb* protein was shown to transactivate with a four-fold greater efficiency than full length c-*myb* (Punyammalee et al., 1991). These results suggest that the amino-terminus of the protein is also important in

the regulation of c-*myb* protein activity.

Another mechanism by which c-*myb* is activated in leukemias is by retroviral insertion into the 5' end of the gene. Many of these studies were conducted in the mouse systems using Moloney murine leukemia virus (MML),which induces myeloid leukemia (Shen-Ong and Wolff, 1987; Mukhopadhyaya and Wolff, 1992; Wolff et al., 1991) or plasmacytoid lymphosarcomas (ABPL's) (Mukhopadhyaya and Wolff, 1992; Wolff et al., 1991; Shen-Ong and Wolff, 1987; Lavu and Reddy, 1986; Rosson et al., 1987; Shen-Ong et al., 1984a; Shen-Ong et al., 1984b; Lavu et al., 1984; Mushinski et al., 1983). In some cases, viral integrations at the 3' end of the c-*myb* gene led to an activation of the gene (Mukhopadhyaya and Wolff, 1992). In most cases, however, activation of c-*myb* expression occurred by retroviral integrations into the 5' end of the c-*myb* gene, specifically into intron 1 (Dudek and Reddy, 1989a; Lavu and Reddy, 1986; Kanner et al., 1991; Rosson and Reddy, 1987; Rosson et al., 1987). In some cases, the retroviral integrations caused aberrant splicing of c-*myb* products (Dudek and Reddy, 1989a; Rosson and Reddy, 1987; Rosson et al., 1987); in others, the retrovirally activated genes encoded amino-terminally truncated proteins similar to those encoded by the v-*myb* protein (Kanner et al., 1991; Lavu and Reddy, 1986; Shen-Ong et al., 1984a).

Similar studies in the avian system using the avian retrovirus RAV-1 have further characterized retroviral insertions in this region (Pizer et al., 1992;

Pizer and Humphries, 1989). Insertion of RAV-1 into the c-*myb* gene induces the rapid onset of avian B-cell lymphomas. Assessment of the altered c-*myb* genes in these lymphomas revealed that the majority of retroviral insertions occurred at the 3' end of intron 1 (Figure 6). The altered gene utilized an ATG translational start codon in exon 2 and thus encoded amino-terminally truncated proteins. These studies establish that not only are amino-terminally truncated c-*myb* proteins implicated in transformation, but that intron 1, especially the 3' end of intron 1, is a "hot spot" for alterations which may lead to malignant transformation. By analogy, the 3' end of intron 1 may be also important in the regulation of the normal c-*myb* gene.

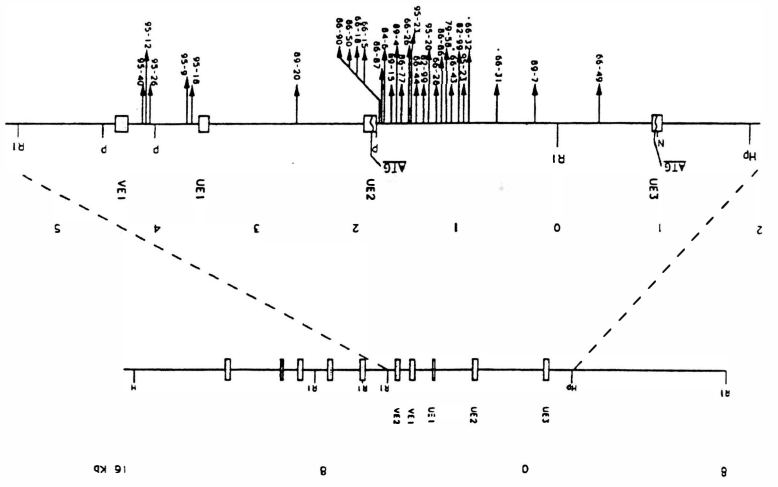
**CCRF-CEM.**

Non-transformed cells have a limited proliferative ability and pure populations of specific immune cells are difficult to isolate. Therefore, immortal transformed cell lines have been useful in the study of c-*myb* expression and its role in the regulation of hematopoiesis. Many of these cell lines are derived from various forms of leukemia. *In vitro* models of hematopoietic differentiation include the promyelocytic HL-60 cell line and the Friend murine erythroleukemia (FMEL) cell lines which can be induced to differentiate with a variety of agents. Immature lymphocytic leukemia cell lines, on the other hand, express high levels of c-*myb* (Ibanez and Lipsick, 1988) but cannot be induced to differentiate. These cell lines are therefore useful to study the promoter activity of c-*myb* and to identify alternatively spliced forms of c-*myb*.

The cell line CCRF-CEM was isolated in 1965 from a 3 year old female with acute lymphoblastic leukemia (Foley, 1965). These cells have proved to be useful in the study of acute leukemia because they will grow in suspension culture in the absence of lymphoid growth factors and do not require blastogenic agents such as phytohemagglutinin (PHA) for induction of mitosis. These clonally-derived cells exhibit the characteristic morphology of lymphoblastic cells: i) the nuclei are densely stained and of unusual morphology, ii) there is little cytoplasm present in each cell. Recent karyotypic analysis of CCRF-CEM revealed that the cell line is near tetraploid (Pittman et al., 1993). Numerous chromosomal aberrations are observed; however, the cell line is not 6q-, and no other aberrations were associated with chromosome 6 (Pittman et al., 1993).

Two alternatively spliced c-*myb* DNA clones were isolated from a cDNA library constructed from CCRF-CEM. These two clones, pMbm-1 and pMbm-2, represent two different alternatively spliced products of c-*myb* when compared to the c-*myb* prototype cDNA LMC-8 (Majello et al., 1986). Both clones have been sequenced and analyzed with regard to predicted protein structure and mechanism of alternative splicing. Both pMbm-1 and pMbm-2 are expressed in a number of lymphoid and myelomonocytic cell lines including CCRF-CEM, HL-60, and MOLT-4 (Westin et al., 1982). The prototypic c-*myb* message is present in the greatest abundance, followed by pMbm-1, while

Figure 6. RAV-1 retroviral insertion into intron 1 of the c-*myb* gene. (Top) Schematic representation of the chicken c-*myb* gene. Boxed indicate chicken c-*myb* exons from exon 1 (UE3) to exon 10. (Bottom) Sites of retroviral insertion into the 5' end of the chicken c-*myb* gene. Downward arrows indicate the positions of the viral integrations. The alternative ATG translation start site used to translate the interrupted c-*myb* messages in exon 2 (UE2) is also indicated. RI-*Eco*RI; Hp- *Hpa*I; H-*Hind*III; N-*Nco*I; P-*Pst*I (Pizer et al., 1992).


8
0
8
16 kb
uE3
uE2
uE1
V1
V2
ATG
ATG
ATG
uE3
uE2
uE1
V1
0
1
2
3
4
5

pMbm-2 is expressed at low levels. As with the prototypic c-*myb* gene, both pMbm-1 and pMbm-2 are down-regulated during DMSO induced HL-60 myeloid differentiation (Weber et al., 1990).

Functional studies of pMbm-1 and pMbm-2 were conducted using the Friend murine erythroleukemia (FMEL) cell model system in collaboration with Dr. Michael Clarke. Transfection and overexpression of these two clones in FMEL cells revealed opposing roles for the two clones in the differentiation process. Expression of high levels of pMbm-1 leads to a block in the ability of FMEL cells to withdraw from the cell cycle and differentiate when induced with dimethyl sulfoxide (DMSO). By contrast, high levels of pMbm-2 expression were shown to promote DMSO induced differentiation of FMEL cells. These results indicate the human c-*myb* proto-oncogene encodes alternatively spliced mRNA species with opposing roles in the differentiation process. The precise mechanism of this effect is unknown, but it is postulated that the predicted protein for pMbm-2 is functioning as a competitive inhibitor of *Myb* DNA binding sites.

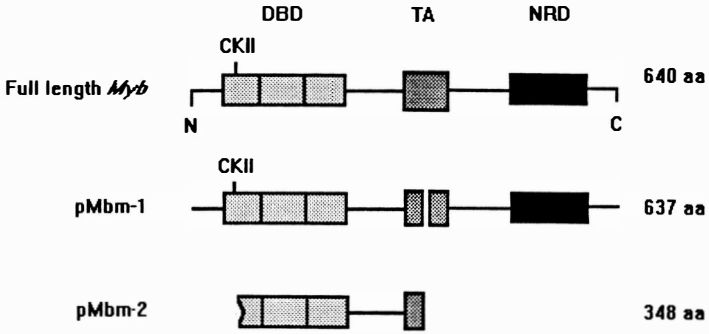
A comparison of pMbm-1 and pMbm-2 reveals a great deal of heterogeneity between the two clones and the prototypic c-*myb* gene at the protein level (Figure 7). The pMbm-1 message is most similar to the full length *Myb* protein, containing only a 9 base pair (bp) deletion in the message which arose from an alternate splice site within vE5 (exon 8 of c-*myb*). This splice deletes 3 amino acids from the protein, allowing for a near full length c-

*myb* protein to be translated. The pMbm-2 message, by contrast, contains an additional 122 bp exon between exons 8 and 9 designated exon 8A. The predicted protein from this message would encode the DNA binding and nuclear localization regions of c-*myb*, but would terminate within this alternative exon. This would lead to deletion of the carboxyl- terminus of the protein containing the negative regulatory domain.

In addition to these differences, the two clones also diverge at their N-terminus. The pMbm-1 5' sequences are similar to the prototypic c-*myb* gene, containing a normal 5' untranslated region and a correct splice between UE3 (exon 1) and exon 2. The pMbm-2 message, on the other hand, possesses an unique 26 bp 5' sequence in the place of exon 1 resulting in the loss of 20 amino acids at the N terminus of the protein. This loss deletes the casein kinase II phosphorylation sensitive element which inhibits sequence-specific DNA binding of *Myb* when phosphorylated (Lüscher et al., 1990; Lüscher and Eisenman, 1992). The same region is also deleted in the v-*myb* oncogene product (Perbal et al., 1986). Southern blot hybridization of an oligonucleotide probe to genomic clones of c-*myb* mapped the unique 26 bp sequence of pMbm-2 to intron 1 of the c-*myb* locus (Weber et al., 1990).

The presence of a cDNA clone which potentially encodes an amino-terminally truncated form of c-*myb* indicates that, while attenuation may be the major form of regulation of c-*myb* expression, other mechanisms in the 5' end of the gene may also be important in the regulation of c-*myb*.

Figure 7. Predicted structure of alternatively spliced c-*myb* clones. (Top) The full length *Myb* protein, containing an intact DNA binding domain (DBD), transactivation (TA), negative regulatory domain (NRD), and casein kinase II sensitive phosphorylation site. (Middle) Predicted structure of the pMbm-1 protein. This protein would possess a 3 amino acid deletion in the transactivation domain due to usage of an alternative splice donor site in exon 8; it would have an intact N- and C- terminus. (Bottom) The predicted structure of the pMbm-2 protein. This protein would have a 20 amino acid deletion at the N- terminus and would terminate prematurely in the alternatively spliced exon 8A, deleting part of the transactivation domain and all of the negative regulatory domain.


DBD
TA
NRD
CKII
Full length Myb
640 aa
N
C
CKII
pMbm-1
637 aa
pMbm-2
348 aa

# METHODS

Characterization of intron 1 of the human c-*myb* proto-oncogene and a second promoter within the intron.

The purpose of these studies was to identify and characterize the origin of the pMbm-2 specific transcript by analysis of the structure and function of critical regions of the human c-*myb* intron 1. The intron was sequenced and compared to previously identified mouse and chicken intron 1 sequences. Transcripts originating from the intron were identified by primer extension and RNAse protection analysis. Intron 1 sequence was assayed for promoter activity in chloramphenicol acetyl transferase (CAT) transient expression assays. The ability of the intron 1 sequence to bind specifically to nuclear proteins was assessed by gel mobility shift assays.

**Phage DNA preparation.**

*Escherichia* coli LE392 host cells ($6 \times 10^{10}$) were infected with $3 \times 10^{8}$ lambda ($\lambda$) phage particles with adsorption for 15 minutes at 37°C in 1 ml of Luria broth (LB:1% bacto-tryptone (Difco)/ 0.75% yeast extract (Difco)/ 86 mM NaCl (pH 7.0)). One-half liter of LB/ 10 mM $MgCl_2$ was inoculated with

the infected cells and incubated overnight at 37°C with shaking. The culture was centrifuged to remove all cellular debris. Sodium chloride (NaCl) and polyethylene glycol-8000 (PEG-8000) were added to the supernatant to a final concentration of 1 M and 10%, respectively, and incubated two hours at 4°. Phage particles were collected by centrifugation for 15 minutes at 6,000 rpm in an GSA rotor (Sorvall) and resuspended in a solution of 6 g CsCl/ 8 ml SM buffer (50 mM Tris-HCl (pH 7.5)/ 0.1 M NaCl/ 10 mM $MgSO_4$/ 0.1% gelatin). This mixture was centrifuged for 4 hours at 65,000 rpm in a VTi65.1 rotor (Beckman), and the phage band was recovered and re-banded. The phage suspension was diluted with eight volumes of TE (10 mM Tris-HCl (pH 8.0)/ 1 mM EDTA) to dilute the cesium chloride. Phage DNA was purified by treatment of the phage suspension with 10 mM RNAse and 10 mM DNAse for 15 minutes at 37°C and 2.5 mM proteinase K for 30 minutes at room temperature, followed by two extractions of the suspension with PCI9 (1:1:0.1:0.1 w/v phenol/ chloroform/ isoamyl alcohol/ 0.09 M Tris-HCl (pH 8.0)) and one extraction with SEVAG (1:0.1 chloroform:isoamyl alcohol). The DNA was precipitated by the addition of 0.1 volume 3 M NaOAc (pH 5.5) and two volumes of 95% EtOH, followed by mixing, incubation at -80°C for 10 minutes (or -20°C for 30 minutes), and centrifugation for 15 minutes at 13,000 rpm in a microcentrifuge (Fisher, DuPont or Sorvall). Unless otherwise indicated, this method of was used to precipitate DNA or RNA. The DNA was redissolved in water and quantified spectrophotometrically at $OD_{260}$.

**Plasmid DNA preparation.**

Five hundred ml of LB/ 25 μg/ml tetracycline/ 25 μg/ml ampicillin was inoculated with a 5 ml overnight culture of XL1-Blue bacterial host cells (Stratagene) containing the appropriate plasmid and incubated 5 hours at 37°C with shaking. Chloramphenicol was added to the culture to a final concentration of 62.5 μg/ml and the culture was incubated overnight at 37°C with shaking. Bacteria were collected by centrifugation in a GSA rotor for 10 minutes at 6000 rpm. Bacterial pellets were resuspended in 10 ml of 50 mM glucose/ 25 mM Tris-HCl (pH 8.0)/ 10 mM EDTA/ 5.0 mg/ml lysozyme and incubated at room temperature for 5 minutes. Twenty ml of 0.2 N NaOH/ 1% sodium laurel sulfate (SDS) was added, and the mixture was inverted several times to allow the solutions to mix and then incubated for 15 minutes on ice. Fifteen ml of 5 M potassium acetate (pH 4.8) was then added to the mixture, and the sample was inverted again and incubated 15 minutes on ice. The cellular debris was pelleted by centrifugation for 15 minutes at 12,000 rpm in an HB-4 rotor (Sorvall). The supernatant was combined with 0.6 volumes of isopropyl alcohol and incubated 15 minutes at room temperature. The DNA was pelleted by centrifugation for 15 minutes at 12,000 rpm in an HB-4 rotor. The pellet was dried and redissolved in a 1:1 weight/ volume solution of CsCl/ TE with ethidium bromide (100 μg/ml) and centrifuged for 4 hours at 65,000 rpm in a VTi65.2 rotor (Beckman). The plasmid band was isolated and re-banded. The plasmid band was extracted with 1-butanol three times and then diluted 1:4 in

$dH_2O$. Two volumes of 95% EtOH was added to the DNA, and the precipitated DNA was collected by centrifugation in an HB-4 rotor for 15 minutes at 12,000 rpm. The DNA was redissolved in 0.5-1 ml of sterile water and quantitated spectrophotometrically at $OD_{260}$.

**Isolation of specific probes.**

The DNA fragments used in the characterization of intron 1 sequence are shown in Figure 8. All intron 1 clones were derived from the genomic lambda clone Lambda 1, a gift from G. Francini (Harper et al., 1983). The p13, p12, and p1BG3 subclones were obtained from Ms. Karen Gorse. The clones were obtained by digestion of Lambda 1 with the appropriate enzymes: the insert for p1XE7 was derived by digestion with *Xba*I and *Eco*RI, and pINT1CAT by digestion with *Pst*I. For the transient transfection studies, the gene for chloramphenicol acetyl transferase (CAT) from the pCAT-PROMOTER construct (Promega) (Gorman et al., 1982) was isolated by digestion with *Hind*III. The digested DNAs were separated on an 0.8% agarose gel. The insert bands were then isolated from the gel and subjected to electro-elution using an Elutrap electro-eluter (Schleicher and Schuell). The insert was then checked for integrity and approximate quantity on a 0.8% agarose/ 1X TAE (40 mM Tris-acetate (pH 8.0)/ 1 mM EDTA) gel. Unless otherwise indicated, the agarose used in the electrophoresis studies was Ultrapure agarose (BRL).

**Fragment subcloning.**

For subcloning of pINT1 and p1XE7, pBluescript II KS+ (for pINT1

and pBSCAT(R)) or pBluescript II SK+ (for p1XE7) vectors (Stratagene) were digested with the appropriate enzymes and purified by two extractions with PCI9 and one extraction with SEVAG, and ethanol precipitated. The insert was ligated to the vector in 1X ligation buffer (50 mM Tris-HCl (pH 7.8)/ 10 mM $MgCl_2$/ 10 mM DTT/ 1 mM ATP/ 25 $\mu$g/ml BSA)/ 200 units T4 DNA ligase (New England Biolabs) overnight at 15°C. The ligated plasmids were transformed into XL1-BLUE competent cells (Stratagene) by incubation of 1 x $10^6$ competent cells with 40 ng of the ligated plasmid on ice for 30 minutes, heat shocking the cells for 90 seconds at 42°C, addition of the cells to 1 ml LB, and incubation of the cells 1 hour at 37°C with shaking. Positive clones were detected by differential $\beta$-galactosidase expression of bacterial colonies on 100 mm LB/ 0.35% bacto-agar (Difco)/ 10 mM $MgSO_4$ plates supplemented with 25 $\mu$g/ml tetracycline and 25 $\mu$g/ml ampicillin using 0.1 M isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) and 0.004 M 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (X-GAL) (Bethesda Research Laboratories) and verified by restriction digest of the recombinant plasmid and resolution on a 0.8% agarose/ 1X TAE gel (40 mM Tris-acetate (pH 8.0)/ 1 mM EDTA). Unless otherwise indicated, the agarose used in the electrophoresis studies is Ultrapure agarose (Sigma).

Plasmids used in transfection studies consisted of portions of intron 1 fused to the gene for chloramphenicol acetyl transferase (see Figure 9). These were created as follows: the gene for chloramphenicol acetyl transferase (CAT)

was subcloned on a 1.6 kb *Hind*III fragment into pBluescript II KS+ to create pBSCAT(R). The 1.6 kb *Pst*I fragment encompassing the 3' end of intron 1, ending at the intron 1/exon 2 junction, was isolated from the Lambda 1 clone and subcloned into pBSCAT(R) in the forward and reverse orientation to create pINT1CAT and pINT1CAT(R), respectively. Deletion constructs of pINT1CAT were created using the Kilo-sequencing kit (Takara Industries, INC) (Henikoff, 1984) (to create pINT1CAT.40 and pINT1CAT.12) and by restriction of pINT1CAT with *Bst*XI enzyme, incubation with Klenow fragment of DNA polymerase I to generate blunt ends, and religation (to create pBXPCAT).

**Oligonucleotide synthesis.**

Oligonucleotides for primer extension and DNA sequencing were synthesized by the Nucleic Acid Core Facility at the Medical College of Virginia. The synthesis products were deblocked in concentrated ammonium hydroxide at 55°C for 15 hours. The solution was then evaporated in a Speedvac centrifuge connected to a vacuum trap (Savant). The synthesis product was then redissolved in water and purified by thin layer chromatography. Briefly, the solid phase was a fluorescent TLC plate and the mobile phase was 1-propanol:ammonium hydroxide:water (55:35:10). The oligonucleotide was detected by UV light shadowing and the product was scraped from the TLC plate, eluted in water, and quantitated spectrophotometrically at $OD_{260}$.

**DNA sequencing.**

All constructs were sequenced using the dideoxy method described by Sanger et al (Sanger et al., 1977) using Sequenase version 2.0 (United States Biochemical). Briefly, templates were denatured by boiling the DNA in 0.2 N NaOH for two minutes followed by chilling for 2 minutes on ice and ethanol precipitation. The DNA was redissolved in 6 $\mu$l sterile $dH_2O$ and annealed to 2 $\mu$l (40 ng/$\mu$l) of the appropriate primer and 2 $\mu$l of 5X sequencing buffer (1X is 20 mM Tris-HCl (pH 7.5)/ 10 mM $MgCl_2$/ 25 mM NaCl) (USB) by heating to 65°C and cooling slowly to 37°C. The template/primer mixture was then incubated with 2 $\mu$l of a 1:4 dilution of Sequenase version 2.0, 2 $\mu$l of 1.5 $\mu$M dGTP, dCTP, dTTP and 0.5 $\mu$l (6 $\mu$Ci) $\alpha$-$^{35}$S-dATP for 3 minutes at room temperature. The reaction mix was then aliquotted to each of four deoxy/ dideoxy termination mixes containing G,A,T, and C termination mixes and incubated for 5 minutes at 37°C. The reactions were stopped by addition of sequencing loading dye (95% formamide/ 20 mM EDTA/ 0.05% bromophenol blue/ 0.05% xylene cyanol). Sequencing reactions were resolved on a 7% acrylamide/ 8 M urea/ 1X TBE (0.09 M Tris-HCl (pH 8.0)/ 0.9 M boric acid/ 2 mM EDTA) gel. The gel was adhered to one of the plates by treatment of the plate with two coats of methacrylsaure-3-trimethoxysilylpropylester. The gel was pre-heated before sample loading by subjecting the gel to electrophoresis at 50 watts for 30 minutes in 1X TBE . The samples were loaded and electrophoresed at 50 watts until the bromophenol blue ran to the bottom of the gel. The bottom chamber was drained and filled with a 1:1 solution of 1X TBE/

3M NaOAc. The remainder of the sample was loaded onto the gel and run at 50 watts for two hours. The gel was then removed form the gel rig, and the urea was eluted from the gel by washing it three times in 10% glacial acetic acid/ 10% MeOH in $dH_2O$. The gel was dried overnight in a fume hood, and the DNA was visualized by autoradiography.

Sequencing data was assembled and analyzed using the sequence analysis software package (version 7.1) of the Genetics Computer Group mounted on the Virginia Commonwealth University VAX cluster (Lipman et al., 1989). Sequence conservation between species was analyzed using alignment algorithms and the FASTA program (Lipman et al., 1989). Homologous regions were defined as those regions of intron 1 which showed significant homology to murine or chicken c-*myb* sequences when searched against the entire sequence database.

**Cell culture and differentiation studies.**

K562 (Andersson et al., 1979), CB23 (EBV transformed cord blood B-cell line, a gift of Ian McGrath), CCRF-CEM (Foley, 1965), U937 (Larsson et al., 1988), HL60 (Collins et al., 1977), and MOLT-4 (Ohyashiki et al., 1988), REH (Greaves and Janossy, 1978), Jurkat-E61 (Weiss et al., 1984), and HSB-2 (Adams et al., 1968) cell lines were grown in RPMI 1640 (GIBCO) media supplemented with 10% defined calf serum (Hyclone), 40 $\mu$g/ml gentamicin at 37° C in a moist, 5% $CO_2$ environment. U937 cells were differentiated by aliquotting 5 x $10^7$ cells to four 100 mm petri dishes containing 50 ml RPMI

1640 (Gibco/Brl)/ 10% defined calf serum/ 40 $\mu$g/ml gentamicin/ 250 nm phorbol ester dibutyrate (PDbu) (Rovera et al., 1979). Cells were harvested at 0, 6, 12, and 24 hours post induction with PDbu by scraping the cells from each petri dish at a time point, followed by centrifugation at 1000 rpm for ten minutes in a clinical centrifuge. HL60 cells were differentiated in 1.3% dimethyl sulfoxide (DMSO) (Collins et al., 1977); cell were harvested at 0, 1, 3, 6, 12, 24, and 96 hours post-induction with DMSO by removal of an aliquot of cells at the appropriate time point and centrifugation as indicated for the U937 cells. The differentiated phenotype was monitored by adherence of the cells to plastic (for U937 cells) and differential staining using Wright/ Giemsa stain (for U937 and HL60 cells).

**Northern blot analysis.**

Total RNA was isolated by the method of Chirgwin et al (Chirgwin et al., 1979). Log-phase cells (between 6 x $10^5$ and 1 x $10^6$ cells/ml) were harvested and lysed in 4 M Guanidine hydrochloride/ 7% $\beta$-mercaptoethanol/ 0.5% sarcosyl. RNA was pelleted by ultracentrifugation through a 5.7 M CsCl cushion in a 70.1Ti rotor (Beckman) for 20 hr at 20°C. The RNA pellets were washed with 100% ethanol, air dried, and redissolved in sterile $dH_2O$. The RNA was quantitated spectrophotometrically at $OD_{260}$.

Poly A+ selection was performed using the Poly A Tract System (Promega). In brief, total RNA was incubated with oligo d(T) conjugated to magnetic particles. Poly A+ RNA annealed to the oligo d(T) was separated

from poly A- RNA by capturing the magnetic particles using a magnetic stand. The poly A- RNA was removed, and the poly A+ RNA was washed four times in 0.1X SSC (1X is 0.14 M NaCl/ 0.015 M sodium citrate) and then eluted from the magnetic beads in $dH_2O$.

Ten $\mu$g of total RNA or 5 $\mu$g poly A+ RNA were denatured in 1X MOPS buffer (0.02 M MOPS (pH 7.0) / 5 mM NaOAc/ 1 mM EDTA)/ 2.2 M formaldehyde/ 50% formamide. The samples were heated for 5 minutes at 65°C and then separated on a 6.6% formaldehyde, 1% agarose gel in 1X MOPS buffer. Equal loading of samples was determined by staining the gel in 1 $\mu$g/ml ethidium bromide and visualization with UV light. The RNA was transferred to NYTRAN blotting membrane (Schleicher and Schuell) using the method described by Thomas (Thomas, 1980). For Northern blot analysis, a 1.1 kb *Bam*HI fragment (BAM2) containing exons 9-14 of the normal c-*myb* message was nick translated (Bethesda Research Laboratory kit). Filters were hybridized to the probe in 1X prehybridization buffer (50 mM sodium phosphate (pH 6.5)/ 0.1% BSA/ 0.1% ficoll/ 0.1% polyvinyl pyrollidine/ 5X SSC/ 0.1% SDS/ 250 $\mu$g/ml yeast RNA)/ 50% formamide/ 10% dextran sulfate). The blots were hybridized for 16-20 hours at 42°C. The filters were washed 3 times in 2X SSC/ 0.2% SDS at 42°C, twice in 0.5X SSC/ 0.2% SDS at 60°C, and twice in 0.1X SSC/ 0.1% SDS for 10 minutes at 60°C. The blots were exposed to Kodak XAR-5 film for the times indicated in the figure legends at -80°C with Quanta III intensifying screens.

Following exposure to determine levels of c-*myb*, the blots were stripped by incubating the filters at 90°C in 0.01X SSC/ 0.01% SDS for 10 minutes and re-probed with a 1.9 kb nick-translated *Bam*HI fragment of β-actin, derived from the LK221 plasmid (Gunning et al., 1983). The blots were hybridized, washed and exposed to XAR-5 film as described above.

To determine relative levels of c-*myb* expression between cell lines in the poly A+ selected blot, the radioactive blot was scanned using the Betascope 603 Blot Analyzer (Betagen) provided by the Nucleic Acids Core Facility at the Medical College of Virginia. Counts per minute per lane were determined for c-*myb* and for the corresponding β-actin hybridization, and each c-*myb* lane was standardized for β-actin levels.

**Southern blot analysis.**

For hybridization of DNA fragments to radioactive probes, genomic and plasmid DNA (digested with the appropriate enzymes) or cDNA (from the RT-PCR assay described below) was subjected to electrophoresis in a 300 ml 1% agarose/ 1X TAE gel in 1X TAE buffer overnight at 15 volts. The DNA was transferred to NYTRAN blotting membrane (Schleicher and Schuell) using the method described by Southern (Southern, 1975). Briefly, the gel was incubated for 15 minutes in denaturing solution (1.5 M NaCl/ 0.5 N NaOH), then neutralized for 15 minutes in neutralizing solution (1.5 M NaCl/ 1 M Tris-HCl (pH 8.0)) and equilibrated 15 minutes in 10X SSC. Following capillary transfer of the DNA to NYTRAN membranes in 10X SSC, the blot was baked

2 hours *in vacuo* at 80°C.

For hybridization, 1 x $10^6$ cpm/ ml of either $\gamma^{32}$P-ATP labeled oligonucleotide (for RT-PCR assay) or nick-translated probe (for genomic DNA, phage DNA, and plasmid DNA blots) was hybridized to the filters for 18 hr at 42°C using 1X prehybridization buffer/ 40% formamide (for oligonucleotide probes) or 1X prehybridization buffer/ 50% formamide/ 10% dextran sulfate (for nick-translated probes). The blots were wash as indicated in the Northern blot analysis section (for nick translated probes) or in 2X SSC/ 0.2% SDS for 30 minutes at 42° C (for oligonucleotide probes). The blots were exposed to Kodak XAR-5 film for the time indicated in each specific section.

**Primer extension analysis.**

A 30 base oligonucleotide, PE1, (5'-CCAAGTCCTCATCATCCTCGTCACTGCTAT-3') complimentary to c-*myb* exon 2 sequence was synthesized and end labeled with $\gamma^{32}$P-ATP using the 5' termini labeling kit (Bethesda Research Laboratories) (Thomas, 1980) and purified using NENSORB columns (Dupont/NEN). For primer extension (Thomas, 1980), 1 x $10^6$ cpm (counts per minute) of the end-labeled primer were annealed to 10 $\mu$g of total CCRF-CEM, K562, HL-60 RNA and tRNA (as a negative control). The melting temperature (Tm) of the primer was determined according to the formula Tm = 81.5°C + 16.6 log (M NaCl) + (mole fraction of G+C) - 500/length of primer. The primer was annealed at Tm-10° for 2 hours in 0.4 M NaCl and 400 mM PIPES (pH 7.0). Following

annealing, the primer was extended for 1 hour at 37°C in 50 mM Tris-HCl (pH 8.3)/ 40 mM KCl/ 6 mM $MgCl_2$/ 1 mM DTT/ 1 mM each dATP, dCTP, dGTP, and dTTP/ 200 units reverse transcriptase (Mo-MuLV-RT, Bethesda Research Laboratories). The cDNA was purified by denaturing the cDNA/RNA complex at 95°C for 5 minutes, followed by incubation of the reaction mix with 10 $\mu$g RNAse A (DNAse free). The cDNA was then extracted with PCI9 and SEVAG (once each). Twenty $\mu$g of carrier tRNA was added to the cDNA, and the cDNA was ethanol precipitated as indicated in the sequence analysis section. The cDNA was pelleted by centrifugation for 15 minutes at 12,000 rpm in a microcentrifuge (Fisher), washed once in 70% EtOH (-20°C) and dried *in vacuo*. The pellet was redissolved in one volume of water and one volume of sequencing loading buffer (Maniatis et al., 1989). The cDNA extension products were separated by loading the entire sample on a 7% polyacrylamide/ 8 M urea gel/ 1X TBE (preheated as in the sequence analysis section) and running the gel at 50 watts for two hours. The gel was transferred to 3M paper (Whatman), dried *in vacuo* for 1 hour at 60°C and exposed to Kodak XAR5 film for three days at -80°C with an intensifying screen. The size of the extended fragments were mapped against a standard curve of [$\gamma^{32}$P]ATP labeled $\phi$X174 (*Hae*III digested) marker (Bethesda Research Laboratories) with a $\pm$2bp error.

***In vitro* transcription.**

The construct p1XE7, containing the last 500 bp of intron 1, exon 2 and

363 bp of intron 2, was shortened by deleting the intron 2 sequence using a *Sac*I site at the end of exon 2 and a convenient *Hinc*II restriction site in the polylinker of pBluescript II SK+, followed by restriction site fill-in with T4 DNA polymerase and blunt end ligation to create p1XE7SH (Figure 14).

For *in vitro* transcription, p1XE7SH was linearized at a *Bst*XI site 220 bp upstream of the intron 1/ exon 2 junction to detect transcripts originating from the 3' end of intron 1. The linearized template was incubated with transcription buffer (40 mM Tris-HCl (pH 8.0)/ 25 mM NaCl/ 8 mM $MgCl_2$/ 2 mM spermidine-$(HCL)_3$)/ 5 mM DTT/ 2.5 mM each ATP, CTP, and GTP/ 100 $\mu$M UTP/ 1 unit RNAsin (RNAse inhibitor, Promega)/ 50 $\mu$Ci $^{32}$P-UTP/ 5 units T7 RNA polymerase (Bethesda Research Laboratories). The reaction mix was incubated 30 minutes at 37°C. Ten units of RNAse free DNAse (Promega Laboratories) was added to the reaction mix, and the mix was incubated 5 minutes at 37°C. The RNA template was purified by PCI9 and SEVAG extraction followed by ethanol precipitation. The RNA was redissolved in DEPC (diethyl pyrocarbonate) treated $dH_2O$.

**RNAse protection analysis.**

RNAse protection was carried out with the RPAII kit (Ambion, INC). Four $\mu$g of poly A+ RNA from the indicated cell lines was annealed to 6 x $10^5$ cpm of the RNA probe in 20 $\mu$l hybridization buffer (80% deionized formamide/ 100 mM sodium citrate (pH 6.4)/ 300 mM sodium acetate (pH 6.4)/ 1 mM EDTA) for 14-16 hours at 20°C. The annealed RNA:RNA

complex was digested in 25 units RNAse A/ 1000 units RNAse T1 in 200 $\mu$l of RNAse digestion buffer (solution Bx, patent pending, Ambion) for 30 minutes at 15°C. The digested RNAs were precipitated with the addition of 1.5 volumes ethanol/ guanidine thiocyanate solution (solution Dx, patent pending, Ambion), storage of the samples for 30 minutes at -20°C, and centrifugation in a microcentrifuge (Fisher) for 15 minutes at 12,000 rpm. The pellets were washed once in 70% EtOH (-20°C) and dried *in vacuo*. The RNA was redissolved in 6 $\mu$l loading buffer (80% formamide/ 2 mM EDTA/ 0.1% bromophenol blue/ 0.1% xylene cyanol) and resolved on a 4% polyacrylamide/ 8 M urea/ 1X TBE gel as indicated in the primer extension analysis section. The gel was dried *in vacuo* and exposed to Kodak XAR5 film for three days at -80°C with an intensifying screen. The transcription start sites were mapped as indicated in the primer extension analysis section.

**Transient transfection assays.**

For transient expression assays, CCRF-CEM and K562 cells were grown to log phase (8 X $10^5$ to 1 x $10^6$ cells/ml). The cells were harvested, and CCRF-CEM cells were resuspended in serum-free RPMI 1640 media to a final concentration of 2 x $10^7$ cells/ml. One ml aliquots of cells were cotransfected with 70 $\mu$g of covalently closed circular test plasmids and 35 $\mu$g of pCMV-$\beta$ (MacGregor and Caskey, 1989), which contains the gene for $\beta$-galactosidase under the control of the CMV promoter. K562 cells were resuspended in serum free media to a final concentration of 1 x $10^7$ cells/ml. Aliquots of 0.7

ml were transfected with 50 $\mu$g of covalently closed circular test plasmid and 25 $\mu$g of pCMV-$\beta$ plasmid. All cell lines were transfected using a BTX electroporater and a 0.4 mm electrode at 216 V and 1000 microfarads. Following electroporation, the cells were incubated in 9.5 ml RPMI 1640/ 10% defined calf serum/ 40 $\mu$g/ml gentamicin for 48 hrs. at 37°C in a 5% $CO_2$ environment, harvested by centrifugation for 10 minutes at 1000 rpm in a clinical centrifuge, washed once in 1X PBS (140 mM NaCl/ 10 mM $Na_2HPO_4$/ 2 mM KCl/ 1.5 mM $KHPO_4$), and resuspended in 0.1 ml 0.2 M Tris-HCl (pH 7.4).

Cell free lysates were prepared from transfected cells by three cycles of freezing on dry ice and thawing at 37°C, followed by sonication for two seconds using a fine tip sonicator (Microson Ultrasonic Cell Disruptor, Heat Systems) on setting 2. Supernatants were clarified by centrifugation for 15 minutes at 12,000 rpm in a microfuge at 4°C. The lysates were standardized for protein content by incubating 5 $\mu$l of the lysate in an 80% solution of Bradford protein reagent (Bradford, 1976) (Biorad) and monitoring the absorbance of the mixture spectrophotometrically at $OD_{595}$. Known quantities of bovine serum albumin (BSA) were used to derive a standard curve of absorbance vs quantity of protein, and the lysates were quantitated against the standard curve.

Transfection efficiency was measured by assessing the level of $\beta$-galactosidase activity of the cell free lysates. Equal quantities of protein were incubated in 1 mM $MgCl_2$/ 45 mM $\beta$-mercaptoethanol/ 0.1 M sodium

phosphate (pH 7.5)/ 0.8 M o-nitrophenyl-β-D-galactopyranoside (ONPG) for 1 hour at 37°C. The reactions were stopped by the addition of 2 volumes of 1 M $NaCO_2$, and the cleavage of ONPG was monitored spectrophotometrically at $OD_{420}$.

For the CAT assays, extracts containing equal β-galactosidase units for each set of constructs were incubated with 10 μl 4 mM Acetyl CoA and 10 μl fluorescent chloramphenicol (Molecular Probes, INC) (Hruby and Brinkley, 1990). The reactions were incubated for 5 hours at 37°C. Acetylated chloramphenicol was isolated by extraction with ethyl acetate and desiccation of the ethyl acetate *in vacuo*. The samples were redissolved in 10 μl ethyl acetate and spotted onto a non-fluorescent thin layer chromatography plate. The plate was subjected to thin layer chromatography in a chloroform:methanol (9:1) mobile phase for 1 hour.

Conversion of chloramphenicol to the acetylated forms was measured by obtaining a photographic image of the thin layer chromatography plate using the Star 1 Camera Controller System (Star 1 Image Processing Software, Photometrics), kindly provided by Dr. Sarah Rutan. Histograms were derived for each assay by converting the amount of UV absorption in each fluorescent spot to a numerical value using the Star 1 data reduction software.

Percent conversion of chloramphenicol was determined by conversion of the histograms to bar graphs using the Quattro Pro spreadsheet program (Borland). Briefly, the numerical values of the peaks corresponding to the

acetylated chloramphenicol in each lane were added and compared to the value of all spots in the lane. Percent conversion for each sample was determined as the value of the acetylated peaks/total value. The percent conversion of each construct for each set of transfections was then plotted as a bar graph.

**RT-PCR assay.**

CCRF-CEM cells were transfected with pI2NT1CAT.12 and incubated as indicated above. The cells were harvested, and total RNA was isolated from the transfected cells and 1 x $10^7$ untransfected CCRF-CEM cells using a guanidine-HCL lysis procedure (AutoGen). Following isolation, the RNA samples were incubated in 5 units RQ1 DNAse (Promega) for 30 minutes at 37° C to remove any contaminating plasmid DNA. The samples were extracted twice with an equal volume of PCI9, once with chloroform, and ethanol precipitated in the presence of 20 $\mu$g carrier tRNA. CDNA was synthesized from each sample using the Superscript Preamplification System (BRL).

For reverse transcription, 39 $\mu$l of untransfected CCRF-CEM and pINT1CAT.12-transfected CCRF-CEM total RNA was incubated with 3 $\mu$l of oligo d(T) (0.5 mg/ml), mixed gently, heated to 70°C for 10 minutes, then quick chilled. The contents were collected by brief centrifugation. The annealed RNA/ oligo d(T) sample was incubated in 20 mM Tris-HCl (pH 8.4)/ 50 mM KCl/ 25 mM $MgCl_2$/ 1 mg/ml bovine serum albumin (BSA)/ 10 mM dNTP mix/ 0.1 M DTT/ 2 units Superscript RT. The samples were incubated at room temperature for 10 minutes, then at 42°C for 50 minutes. The samples

Table 3. Oligonucleotides utilized in the RT-PCR studies. The two intron 1 primers, MBM2-5' and INT1.5', were each used with a primer specific for the gene for chloramphenicol acetyl transferase (CAT.3'). If the promoter activity observed in the transient transfection assays corresponds to start site utilization observed in the RNAse protection analysis (Figures 15, 16, and 17). amplification of RNA should only be observed with the MBM2-5'/CAT.3' oligos. To verify that the RT-PCR reactions were working, oligonucleotides to the gene for human $\beta$-actin (ACTIN.5' & ACTIN.3') were used as an internal control.

| Primer | Sequence |
|---|---|
| MBM2-5' | 5'-GTCTACCCATTCTTATTTCTG-3' |
| INT1.5' | 5'-CCAGTAGTAGTCTAAATCCTC-3' |
| CAT.3' | 5'-TGCCACTCATCGCAGTACTGT-3' |
| ACTIN.5' | 5'-CCACGAAACTACCTTCAACTCC-3' |
| ACTIN.3' | 5'- TCATACTCCTGCTTGCTGATCC-3' |
| CAT.PROBE | 5'-ACGTGGCCAATATGGACAACTTCTTCGCCC-3' |
| ACTIN.PROBE | 5'-GCACCCAGCACAATGAAGATCAAGATCATT-3' |

were denatured 5 minutes at 95°C, chilled 10 minutes on ice, and then incubated 20 minutes in the presence of 2 units RNAse H.

Following reverse transcription, each reaction was divided into three aliquots. One gem of Ampliwax (Perkin-Elmer Cetus) was added to each tube with a sterile needle and heated for 1 minute at 80°C. The samples were cooled to room temperature.

For the PCR reactions, a number of oligonucleotides were generated (Table 3). Two intron 1 primers were created as 5' PCR primers: MBM2-5',a 21 base oligonucleotide beginning 26 bp upstream of exon 2 , and INT1.5', a 21 base oligonucleotide beginning 61 bases upstream of exon 2 (to detect non-specific start sites within the intron). A 21 base 3' PCR primer, CAT.3', in the CAT gene of pINT1CAT.12 was also created to generate a 759 and 724 bp PCR fragment with the INT1.5' and MBM2.5' primers, respectively. Primers were created to the human $\beta$-actin gene, ACTIN.5' and ACTIN.3' to generate a 350 bp PCR fragment as an internal control for the reverse transcriptase reaction.

The cDNAs were amplified with the three sets of primers using the GeneAmp Kit (Perkin-Elmer Cetus) (Silver and Keerikatte, 1989; Mole et al., 1989). The cDNAs were combined with 1X Synthesis Buffer (10 mM Tris-HCl (pH 8.3)/ 50 mM KCl), 0.5 units Amplitaq DNA polymerase (Perkin-Elmer Cetus), and 4 $\mu$M of each of the appropriate primers. The samples were heated to 95°C for 4 minutes and then subjected to thirty cycles of the following

reactions: denaturing at 94°C for 1.5 minutes, annealing at 55°C for 2 minutes, and extension at 72°C for 2 minutes. The PCR reactions were separated on a 1% agarose gel and transferred to NYTRAN using the Southern blot procedure. For hybridization, CAT.PROBE, a 30 base oligonucleotide internal to the PCR fragments generated by the intron 1/CAT.3' PCR reactions was labeled with [$\gamma^{32}$P]ATP as indicated in the primer extension analysis section. The blot was hybridized and washed using the procedure described in the Southern blot analysis section and exposed 48 hours at -80°C with an intensifying screen.

Following exposure to determine the presence of CAT- derived PCR fragments, the blot was stripped as described in the Northern blot analysis section and re-probed with a $\gamma^{32}$P-ATP labeled 30 base oligonucleotide probe specific to the $\beta$-actin PCR fragment, ACTIN.PROBE. The blots were hybridized, washed and exposed to film as with the CAT.PROBE oligonucleotide.

**Electrophoretic mobility shift assay (EMSA).**

For the EMSA, nuclear extracts were prepared from CCRF-CEM and K562 cells grown to log phase using the procedure described by Dignam (Dignam et al., 1983). Cells were harvested and washed once in 1X PBS. The volume of the cell pellet was measured, and the cell membranes were lysed by incubation of the cells in two volumes of Solution A (10 mM HEPES (pH 7.9)/ 1.5 mM $MgCl_2$/ 10 mM KCl/ 0.5 mM phenylmethyl sulfonyl fluoride (PMSF)/ 0.5 mM DTT) with douncing of the cells ten times in a B pestle homogenizer.

The nuclei were pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C. The cytoplasmic supernatant was then removed. The nuclei were resuspended in 1 volume of Solution C (20 mM HEPES (pH 7.9)/ 1.5 mM $MgCl_2$/ 0.2 mM EDTA/ 0.5 mM PMSF/ 0.5 mM DTT/ 20% glycerol). While stirring, 5 M NaCl was added to the nuclei to a final concentration of 420 mM. The nuclei were incubated on ice 30 minutes, and the extract was cleared by centrifugation at 13,000 rpm for 30 minutes at 4°C. The cleared extract was then dialyzed for two hours against Solution D (20mM HEPES (pH 7.9)/ 1.5 mM $MgCl_2$/ 80 mM KCl/ 0.2 mM EDTA/ 0.5 mM PMSF/ 0.5 mM DTT/ 15% glycerol).

The DNA fragments used in the gel mobility shift assay are shown in Figure 22. Fragments I, II, and III were generated by the polymerase chain reaction (PCR). The six primers were use to make the three fragments are indicated in Table 4. Fragment IV was generated by digestion of the pINT1 plasmid, containing a 1.6 kb PstI fragment from the 3' end of intron 1 (see Figure 9), with XbaI to generate a 166 bp fragment.

The PCR products were generated using the GeneAmp kit (Perkin-Elmer Cetus). The appropriate primers were combined with 100 ng of the pINT1 plasmid and amplified using the procedure described in the RT-PCR assay section. The integrity of the PCR products were verified by electrophoresis of 1/10 of the PCR reaction on a 0.8% agarose/ 1X TAE gel. The remainder of the PCR products was extracted twice with PCI9, once with SEVAG, and ethanol precipitated as described previously.

Table 4. Oligonucleotides used to generate PCR fragments for the electrophoretic mobility shift assay (EMSA).

| Fragment | Oligo | Sequence |
|---|---|---|
| I | SMJ24 | 5'-GGATTACATCATGTCGCCATTCA-3' |
| | SMJ21 | 5'-TCATTGCAATGTTGGGA-3' |
| II | SMJ25 | 5'-GTTTCCCAACATTGCAATGAC-3' |
| | SMJ26 | 5'-GCCATGGATTTAAAGAACCC-3' |
| III | SMJ14 | 5'-GGCCACTTGTTAGTCAG-3 |
| | SMJ22 | 5'-ACAAACAGTAAGCATAT-3' |

For the EMS assay, the four intron 1 fragments were end labeled with [$\gamma^{32}$P]ATP as indicated in the primer extension analysis section. Five $\mu$g of nuclear extract was incubated with 5 x $10^4$ cpm of each fragment with varying amounts of non-specific or specific competitor, as indicated in the figure legends. The samples were incubated at room temperature for 20 minutes, and fragments which bound nuclear proteins were resolved on a 4% non-denaturing polyacrylamide gel (29:1 polyacrylamide: bisacrylamide)/ 1X TBE using a vertical gel electrophoresis system (BRL) for 1.5 hours at 150 volts. The gel was dried *in vacuo* and exposed to Kodak XAR5 film overnight at -80°C.

Characterization of a rearrangement in the c-*myb* promoter in the acute lymphoblastic leukemia cell line CCRF-CEM.

The purpose of these studies was to determine the cause of a restriction fragment length polymorphism (RFLP) detected by Southern blot analysis in the promoter region of c-*myb* in the acute lymphoblastic leukemia cell line CCRF-CEM. A CCRF-CEM genomic DNA library was created and screened with a DNA probe specific for the c-*myb* promoter. Sequence analysis was used to characterize the alteration in the c-*myb* promoter. The unique sequence found adjacent to the promoter (designated the myb rearranged region (MRR) region) was mapped to chromosome 6 using Southern blot analysis of somatic cell hybrids. The MRR sequence was linked in its normal state to the c-*myb* locus using field inversion gel electrophoresis (FIGE) mapping. Both the c-*myb* promoter probe and MRR probes were used to screen a panel of AMLs and

ALLs to look for similar rearrangements. The effect of the rearrangement on c-*myb* promoter activity was assessed in chloramphenicol acetyl transferase (CAT) transient expression assays. The normal locus of the MRR sequence was identified and characterized by screening a human placental genomic library with a portion of the MRR sequence.

**Phage DNA preparation.**

Same as above.

**Plasmid DNA preparation.**

Same as above.

**Sequence analysis.**

Same as above.

**Genomic DNA isolation.**

Leukemia cell samples (ranging from 1 x $10^7$ to 2 x $10^7$ cells/ sample) were obtained through the Massey Cancer Center Bone Marrow Transplant Laboratory Shared Resource. Genomic DNA was isolated from the leukemic samples and cell lines using standard methods (Maniatis et al., 1989). Briefly, the cells were collected by centrifugation for 10 minutes in a clinical centrifuge and washed once with 1X PBS. The cells were resuspended in 9.5 ml of 1X SSCE (1x SSC/ 1 mM EDTA)/ 0.5 ml 20% SDS/ 1 mg/ml proteinase K (Sigma)/ 10 $\mu$g/ml RNAse A (Sigma). The cells were incubated at 50°C for four hours and then at 42°C overnight (12-16 hr). The protein was separated from the DNA by two extractions with PCI9 and one extraction with SEVAG

(equal volume for each extraction). The DNA was then precipitated by the addition of 0.5 ml 10 M $NH_4OAc$ and 20 ml 95% EtOH (-20°C). The precipitated DNA was air dried, redissolved in sterile $dH_2O$ and quantitated spectrophotometrically.

**Isolation of specific probes.**

The probes used in the Southern blot analysis and library screenings are shown in Figure 25. The 1E2 c-*myb* promoter fragment (Figure 25) used to screen the CCRF-CEM genomic library and in Southern blot analysis was derived from the genomic human c-*myb* lambda clone Lambda 1 (Harper et al., 1983). The *Kpn*I-*Sph*I fragment (MRR-ks) used to clone the normal locus of the MRR1 unique sequence and in Southern blot analysis was derived from the MRR1 clone obtained from the CCRF-CEM genomic library, described below. Insert isolation was the same as that described previously. Both fragments were subcloned from the lambda clones into pBluescript II KS+ (Stratagene) using the indicated restriction sites described.

**Southern blot analysis.**

Same as above.

**Localization of the MRR sequence to chromosome 6.**

DNA from two human/hamster somatic cell hybrids GM10629 (10629) and GM03700 (3700) were obtained from the National Institute of General Medial Sciences Repository (NIGMS), along with human parental cell culture DNA (NAIMR90) and hamster DNA (NA10658). Ten $\mu$g of each DNA were

digested with *Pst*I and subjected to electrophoresis, blotted, hybridized, washed and exposed to X-ray film as indicated in the Southern blot procedure. The blot was hybridized to the nick-translated MRR-ks probe indicated in Figure 25B.

**Field inversion gel electrophoresis (FIGE) analysis.**

Agarose plugs of normal peripheral blood lymphocytes (PBLs) were created for genomic mapping of the MRR and c-*myb* promoter sequences in the following manner: one hundred milliliters (ml) of whole blood was obtained from a volunteer and mixed with 2 ml of heparin (10,000 u/ml) to prevent clotting. The blood was diluted 1:1 in serum-free RPMI-1640. The diluted blood was transferred to 50 ml conical tubes in 40 ml aliquots. Ten ml of Ficoll-paque (Pharmacia) was layered underneath the diluted blood, and the tubes were centrifuged for 20 minutes at 1000 rpm in a clinical centrifuge. The peripheral blood lymphocytes were removed from the Ficoll gradient by aspiration of the serum layer and removal of the PBLs with a pipet. The PBLs were diluted 1:4 in 1X PBS, and cell number and viability was determined by diluting 0.2 ml of cells in 0.2 ml Trypan Blue and counting the cells in a hemocytometer. The cells were centrifuged for 10 minutes at 1000 rpm in a clinical centrifuge, and 1.5 x $10^7$ cells were resuspended in 0.5 ml of 1X PBS. An equal volume of 2% inCERT agarose (FMC Bioproducts) in 0.5X TBE (boiled 30 seconds and cooled to 50°C) was added to the PBLs, and this mixture was aliquotted into an agarose plug mold in 100 $\mu$l aliquots. The plugs were chilled for 15 minutes at 4°C, removed from the gel mold and incubated

for 2 hours at 50°C in 0.5 M EDTA (pH 8.0)/ 1% sarcosyl/ 100 μg/ml proteinase K. An additional 100 μg/ml of proteinase K was then added to the plugs, and the plugs were incubated overnight at 50°C. Following overnight incubation, the plugs were washed twice in TE/ 1 mM PMSF for two hours at room temperature and three times in TE. The plugs were stored in TE at 4°C.

For field inversion electrophoresis, the agarose plugs were cut in half with a cover slip, and half plugs were digested with 40 units of *Eag*I enzyme (New England Biolabs) overnight at 37°C. The following day, a 300 ml 1% Ultrapure agarose (BRL)/ 0.5X TBE gel was cast with a 50 mm comb arranged with the teeth touching the bottom of the casting mold. The digested agarose plug was loaded into a well, one half plugs containing lambda high molecular weight markers (FMC Bioproducts) were loaded on both sides of the *Eag*I digest, and all the wells were filled with liquid 1% inCERT agarose/ 0.5X TBE (50°C). The gel was chilled at 4°C for 15 minutes. The gel was then subjected to field inversion electrophoresis in 0.5X TBE using the Switchback Pulse Controller (Hoefer Scientific Instruments) for 28 hours at 10°C with buffer recirculation (200 volts, with a ramping pulse time ranging from 1 to 50 seconds, and a forward:reverse ratio of 3:1). Following electrophoresis, the gel was stained for 30 minutes in 1 μg/ml ethidium bromide. The high molecular weight DNA was nicked prior to transfer by exposure to a UV light source (Hoefer Transilluminator, 254 nm) for one minute. The DNA was transferred to NYTRAN nylon membrane using the Southern blot procedure. The blot was

baked 2 hours *in vacuo* at 80°C. The blots were hybridized to the nick-translated 1E2 probe, washed, and exposed to Kodak XAR-5 film for 48 hours at -80°C as indicated in the Southern blot analysis section.

Following exposure to determine hybridization to the 1E2 probe, the blot was stripped as indicated in the Northern blot analysis section and re-hybridized to the nick-translated MRR-ks probe. The blot was hybridized, washed and exposed to XAR-5 film as with the 1E2 probe.

**Screening a CCRF-CEM genomic library.**

A CCRF-CEM genomic library was created in the lambda vector λDASHII (Stratagene) in the following manner: Two hundred μg of CCRF-CEM genomic DNA were partially digested with *Mbo*I at a concentration of 0.3 units enzyme/ 0.9 μg DNA at 37°C for 1 hour. The DNA was extracted twice with PCI9 and once with SEVAG and ethanol precipitated as previously described. The DNA was loaded onto a 10%-40% sucrose gradient. The gradient was centrifuged 22 hours at 22,000 rpm in an SW-28.5 rotor (Beckman). One ml fractions of the gradient were collected, and 5 μl of each fraction was subjected to electrophoresis on a 0.8% agarose/ 1X TBE gel. The gel was stained for 15 minutes in 1 μg/ml ethidium bromide, and the DNA was visualized using ultraviolet light to size the DNA fragments in each fraction. One fraction, containing DNA fragments between 17 and 23 kb, was precipitated as described previously and ligated to *BamH*I-digested λDASHII arms as indicated in the fragment subcloning section. The ligated arms were

packaged into phage heads and tails using the Gigapack Gold II packaging system (Stratagene).

To plate the library, 0.5 ml of an overnight culture of *Escherichia coli* host SRB cells were incubated in 50 ml NYZ media (86 mM NaCl/ 8 mM $MgSO_4$/ 0.5% yeast extract/ 1% casamino acids) for 6 hours at 37°C. The cells were harvested and resuspended in 25 ml ice cold 10 mM $MgSO_4$. The SRB cells were infected with 1.2 x $10^6$ plaque forming units (pfu) and aliquotted to 13 ml tubes at 700 $\mu$l/tube. The phage were allowed to adsorb to the bacteria for 15 minutes at 37°C. Seven ml of NYZ media/ 0.75% agarose (boiled to dissolve the agarose and cooled to 50°C) were added to each tube, and the mixture was plated on a 150 mm NYZ/ 0.35% bacto-agar/ 10 mM $MgSO_4$ plate. The plates were incubated overnight at 37°C.

Following overnight incubation, duplicate plaque lifts were performed on each plate using nitrocellulose filters (Schleicher and Schuell). After air drying, the filters were denatured 1.5 minutes in denaturing solution, neutralized for 1.5 minutes in neutralizing solution, and the DNA was fixed for 1.5 minutes in 3X SSC. The filters were baked 2 hours at 80°C *in vacuo*. The filters were hybridized to the nick-translated 1E2 probe as described in the Southern blot analysis section. The blots were exposed to Kodak XAR-5 film for three days at -80°C. Plaques corresponding to duplicate signals were cored out of the plate using the large end of a Pasteur pipette and placed in 1 ml of SM buffer (1 mM NaCl/ 8 mM $MgSO_4$/ 50 mM Tris-HCl (pH 7.5)/ 0.1%

gelatin) with a drop of $CHCl_3$ added. The phage were eluted for 2 hours at room temperature, and 50 $\mu$l of an overnight culture of LE392 cells was infected with 200-400 pfu of phage/sample. The infected bacteria were plated on a 100 mm LB/ 0.35% bacto-agar/ 10 mM $MgSO_4$ plate. Duplicate filter lifts were performed as before, and the filters were denatured, neutralized, baked and hybridized as for the primary library screen. Positive plaques were identified in this secondary screen, cored out, and re-plated as described in the primary library screen to obtain plaque pure samples. DNA was isolated from each sample as described in the phage DNA preparation section with the following exceptions: 1,000 pfu of each phage sample was incubated with 50 $\mu$l of an overnight culture of LE392 cells, and 5 ml of LB/ 10 mM $MgCl_2$ was inoculated with the phage/ bacteria mixture and incubated overnight at 37°C with shaking. Following centrifugation to remove cellular debris, the supernatant was incubated 30 minutes at 37°C in the presence of 10 $\mu$g RNAse A (Sigma) and 10 $\mu$g DNAse (Sigma) and then PEG precipitated as described previously. The precipitated phage were resuspended in 1 ml TE/ 25 $\mu$g/ml proteinase K/ 10 $\mu$g/ml RNAse A (Sigma) and incubated 30°C for 30 minutes. The phage DNA was isolated by two phenol extractions and one SEVAG extraction, followed by ethanol precipitation as described previously. The lambda DNA was digested with *EcoR*I, and the digested DNA was separated on a 0.8% agarose/ 1X TAE gel. The DNA was transferred to NYTRAN nylon membrane using the Southern blot procedure, hybridized to the nick-translated

1E2 probe and washed as described previously. The blot was exposed to XAR-5 film for 30 minutes at room temperature to identify a clone which contained the rearranged c-*myb* promoter. A high titer lysate was prepared from this clone by plating 1000 plaques on a 100 mm LB/ 0.35% bacto-agar/ 10 mM $MgSO_4$ plate. Following overnight incubation of the plate at 37°C, 5 ml of SM buffer was layered on top of the plate, and the phage were allowed to elute from the plate by incubation for two hours at room temperature with shaking. The SM buffer was removed from the plate, 250 $\mu$l of $CHCl_3$ was added to the eluted phage, and a mass culture of phage was grown using the titered phage stock. Purified lambda DNA was isolated as described above.

To clone the normal locus of the MRR unique sequence, the MRR-ks fragment (Figure 25) was nick translated and used as a probe to screen 1 x $10^6$ pfu of a normal human placental genomic DNA library in the λFIXII vector (Stratagene). Primary and secondary screens, plaque purification and phage DNA isolation were performed as described for the CCRF-CEM genomic library screen.

**Transient transfection studies.**

The plasmids used in transfection studies consisted of portions of the c-*myb* promoter and MRR1 clone fused to the gene for chloramphenicol acetyl transferase (Figure 30). These were created as follows: a 1.6 kb *Xba*I-*Eco*RI fragment encompassing the c-*myb* promoter was subcloned into pBluescript II KS+ to create p1XB. This construct was digested with *Nco*I (in the first exon

of c-*myb*) and *Eco*RI (in the polylinker) followed by digestion with mung bean nuclease to generate blunt ends and religation to create p1XBd. The gene for chloramphenicol acetyl transferase (CAT) from the pCAT-Promoter construct (Promega Inc.) (Gorman et al., 1982) was subcloned on a 1.6 kb *Hind*III fragment into p1XBd to create PRO-1. This construct was deleted by digestion with *Bam*HI and *Sph*I, followed by T4 DNA polymerase fill in and ligation, to create PRO-2 and PRO-3, respectively. To recreate the rearranged promoter found in CCRF-CEM, an *Xba*I-*Bam*HI fragment from the MRR1 clone (see Figure 30) was subcloned into the PRO-2 construct to create MRR. The CAT gene was cloned in the reverse orientation to create MRR(R).

CCRF-CEM cells were co-transfected with the indicated CAT constructs and the pCMV-$\beta$ plasmid as described previously with the following exceptions: 1.5 x $10^7$ cells/ sample were transfected at 300 V and 1000 $\mu$farads; the cells were incubated in 14.5 ml RPMI 1640/ 20% defined calf serum/ 40 $\mu$g/ml gentamicin for 48 hrs.

Cell free lysate preparation, $\beta$-galactosidase assays protein determinations and CAT assays were conducted as indicated above.

# RESULTS

Characterization of intron 1 of the human c-*myb* proto-oncogene and a second promoter within the intron.

**Sequence analysis of the human c-*myb* intron 1.**

Three overlapping genomic subclones were sequenced to derive the complete sequence for intron 1 of the human c-*myb* gene (Figure 8). A number of potential transcription factor binding elements are indicated; the intron contains Sp1 binding sites throughout the 5' end and center of the intron (Briggs et al., 1986). Three *Myb* responsive elements (MREs) are also seen (Prendergast and Ziff, 1989; Howe et al., 1990). Approximately 2.0 kb from the 5' end of the intron, an Oct 1 enhancer binding site is found in the opposite orientation of c-*myb* transcription (Stern et al., 1989). Pyrimidine-rich regions similar to those seen in the murine c-*myb* gene are seen; however, these regions are located farther downstream than in the murine gene. Two potential mRNA cap sites are present 17 and 32 bases upstream of exon 2 (Bucher and Trifonev, 1986). The 26 bp 5' unique sequence of pMbm-2 is located directly adjacent to exon 2.

Figure 8. Sequence of the 5' end of human c-*myb*. The two shaded regions indicate the coding region of c-*myb*. Brackets (⌐¬) delineate intron 1 sequence. Potential transcription factor binding sites for SP1(___), Oct 1(●●●) and *Myb* (↑↑↑↑↑↑), are shown. Downward arrows (↓) indicate intron 1 transcriptional start sites as mapped by RNAse protection (Figure 15). Single ovals (●) represent potential mRNA cap sites. Boxes (■■■) underscore the pMbm-2 5' unique sequence. Underlined sequences indicate the region of identity between human and chicken c-*myb* intron 1. Diamonds (◆ ◆ ◆) indicate potential ATG translation start site within exon 2. This sequence may be found the the GenBank database under accession number M95584.

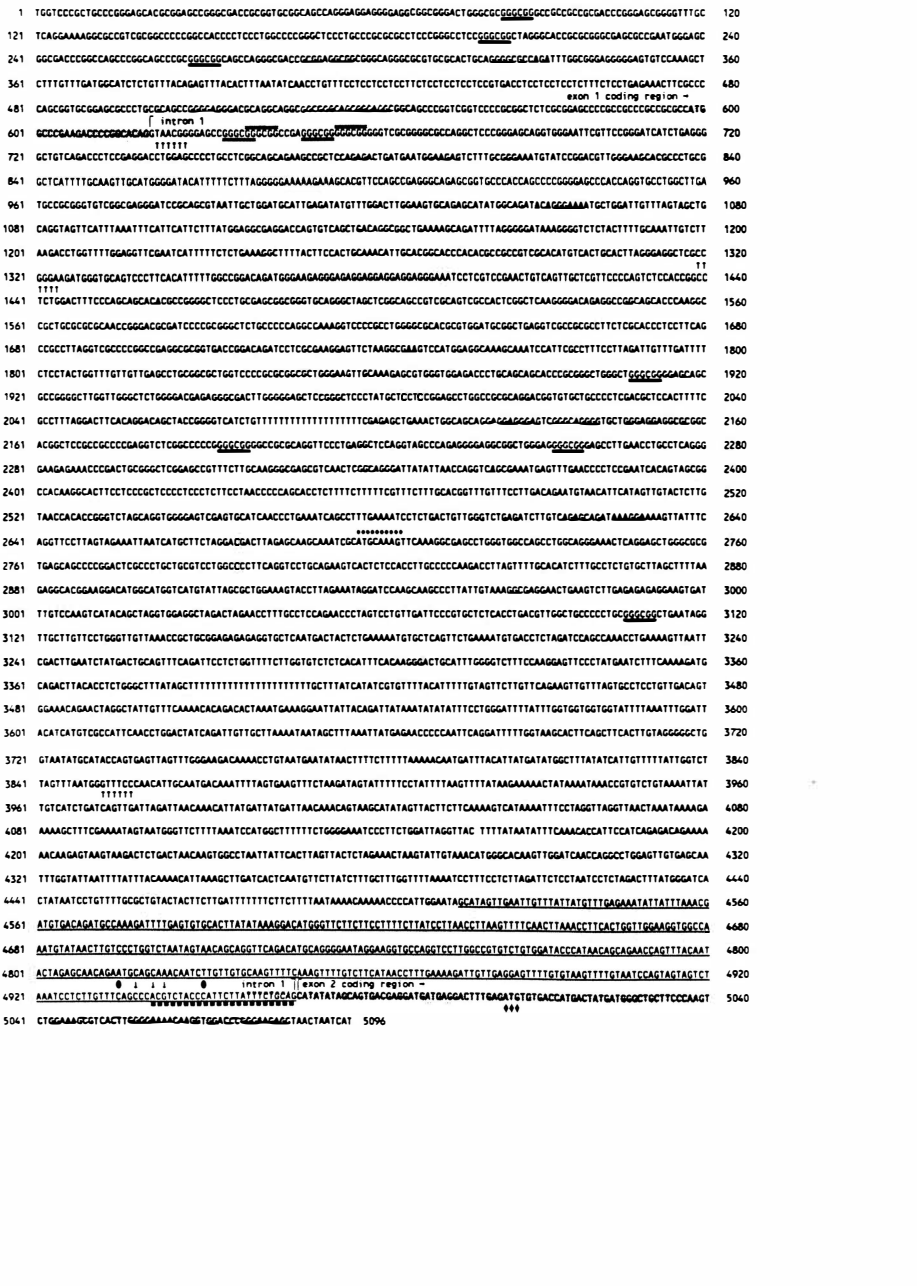
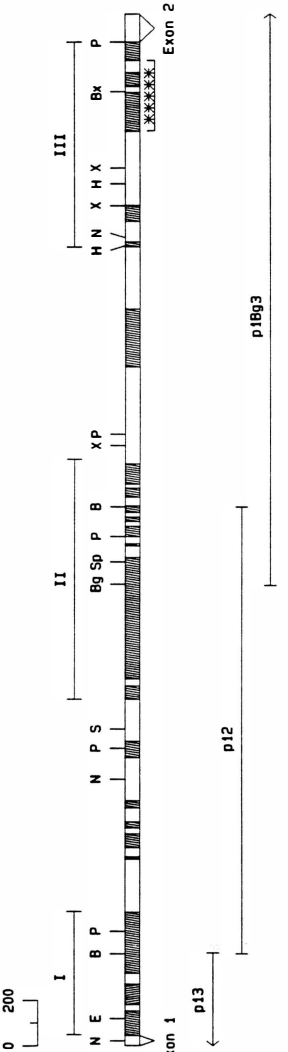

exon 1 coding region →
intron 1
intron 1
exon 2 coding region →

Figure 9. Schematic representation of sequence conservation between murine, chicken and human c-*myb* intron 1 sequences and subclones used to obtain intron 1 sequence. Conservation between human and mouse is indicated by hatched regions. Regions I, II, and III which constitute the major regions of identity between the human and mouse intron 1 sequence are indicated by bracketed regions. Conservation between human and chicken is indicated by asterisks(***) . Restriction sites are abbreviated as follows: N-*Nco*I; E-*Eco*RI; B-*Bam*HI; P-*Pst*I; S-*Sma*I; Bg-*Bgl*II; Sp-*Sph*I; X-*Xba*I; H-*Hind*III; Bx-*Bst*XI. (Below) Genomic fragments which were used to derive the sequence of intron 1.


0
200
I
N E
B P
N P S
II
Bg Sp P B
X P
III
H N X H X
Bx
P
Exon 1
Exon 2
p13
p12
p18g3

Comparison of human intron 1 to previously published mouse sequences revealed many regions of significant identity between the two species (Figure 9). Both species contain a GC-rich region containing Sp1 binding sites directly adjacent to exon 1. In addition, three larger regions of identity exist; region I consists of two clusters showing 72% and 83% identity between human and mouse, respectively. This region begins 153 bases from the 5' end of the intron and spans 440 bases. Region II is 613 bp long and begins 1489 bases from the 5' end of the intron. At 889 bases in length, region III contains the longest stretch of identity and encompasses the 3' end of the intron, including the pMbm-2 unique sequence. When human c-*myb* intron 1 sequence was compared with analogous sequences in the chicken only two regions showed significant identity: i) both species have the GC-rich region containing Sp1 binding sites near the 5' end of the intron; ii) sequences at the 3' end, directly adjacent to exon 2, show significant identity. In the latter region, 69% of intron 1 sequence is conserved between human and chicken, which is comparable to conservation seen between coding sequences (Li et al., 1985).

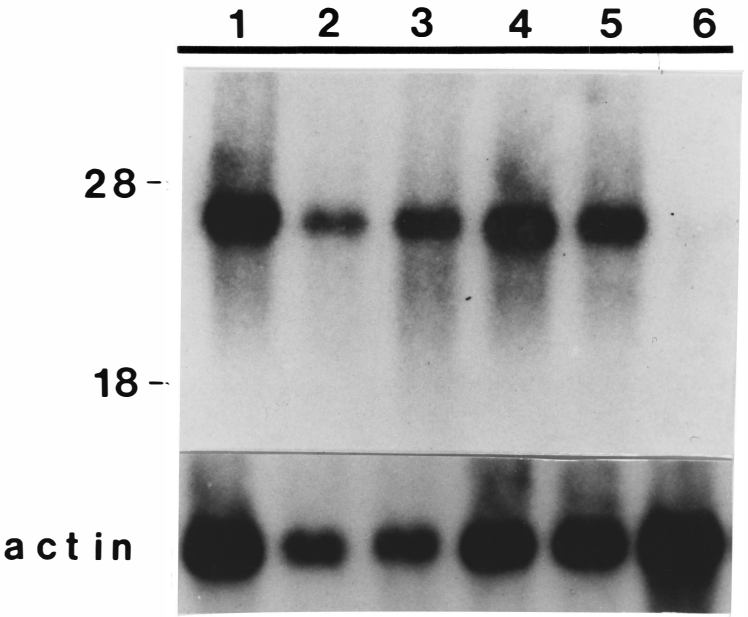
**Northern blot analysis.**

Northern blot analysis of six cell lines was performed to determine the expression of c-*myb* in these cell lines (Figure 10). Due to different efficiencies of poly A+ selection in each sample, relative expression of c-*myb* was determined by comparison to $\beta$-actin. Although levels of $\beta$-actin may vary slightly between cell lines, we have used it previously to compare c-*myb* levels

between cell lines and during differentiation (Boise et al., 1992b). C-*myb* expression is highest in immature lymphocytic leukemia cell lines such as CCRF-CEM (Figure 10, lane 1) and MOLT-4 (Figure 10, lane 3); c-*myb* levels are lower in myeloid leukemia cell lines such as HL-60 (Figure 10, lane 5) U937 (Figure 10, lane 4) and K562 (Figure 10, lane 2). The EBV transformed cord blood B- cell line CB23 expresses the lowest levels of c-*myb*. These results correspond to the expression levels of c-*myb* previously characterized (Westin et al., 1982) and indicate that the cell lines have not changed their levels of c-*myb* expression during passage in culture. C-*myb* expression during hematopoietic differentiation was analyzed by Northern blot analysis of two different myeloid leukemia cell lines induced to differentiate with different agents. U937 cells were induced to differentiate into cells with a monocytic phenotype with phorbolester dibutyrate (PDbu) (Figure 11), and HL-60 cells were induced to differentiate into cells with a myeloid phenotype with dimethyl sulfoxide (DMSO) (Figure 12). During PDbu-induced differentiation of U937 cells, c-*myb* mRNA levels drop (Figure 11). At the end of the time course, c-*myb* levels rise slightly but remain below undifferentiated levels. As is seen with FMEL differentiation (Clarke et al., 1988), c-*myb* exhibits biphasic regulation during DMSO induced HL-60 differentiation (Figure 12); c-*myb* levels drop during the first six hours of differentiation, rise again at twelve hours, and drop to nominal levels after twenty-four hours.

Attempts were made to detect c-*myb* sequences containing the 26 bp 5'

Figure 10. Northern blot of c-*myb* expression in leukemia cell lines. The blot was probed with a 1.1 kb cDNA fragment of exons 9-14 of the normal c-*myb*. The blot was hybridized as indicated in the methods section and exposed to X-Ray film overnight at -80°C. Lane 1- CCRF-CEM; lane 2-K562; lane 3-MOLT-4; lane 4- U937; lane 5- HL60; lane 6-CB23. Shown below is the levels of $\beta$-actin expression in each cell line. Numbers below reflect c-*myb* expression relative to $\beta$-actin. To determine relative levels of c-*myb* expression, the radioactive blot was scanned using the Betascope 603 Blot Analyzer (Betagen). Counts per minute per lane were determined for c-*myb* and for the corresponding $\beta$-actin hybridization, and each c-*myb* lane was standardized for $\beta$-actin levels.




| | | | | | | |
|---|---|---|---|---|---|---|
| MYB | 0.81 | 0.64 | 1.00 | 0.87 | 0.63 | 0.06 |

unique sequence of pMbm-2 on poly A+ selected Northern blots (data not shown). However, levels of this message were so low as to be undetectable by Northern blot analysis using an end labelled oligonucleotide probe. As a result, primer extension and RNAse protection analysis were undertaken.

**Primer extension analysis.**

Primer extension studies were performed to determine if transcription initiation sites within intron 1 could be detected. For this preliminary study, RNA from one lymphoid cell line (CCRF-CEM) and one myeloid cell line (K562) were utilized to detect transcription start sites from both upstream of exon 1 and within intron 1 (Figure 13). Extension of the pMbm-2 message would yield a 63 bp fragment, while transcripts originating from the primary promoter would undergo a normal splice between exons 1 and 2 and would yield a larger extended fragment. If this promoter shows 5' heterogeneity as is seen in the mouse and chicken c-*myb* promoters (Dvorák et al., 1989; Bender and Kuehl, 1986), multiple transcription start sites between 100 and 230 bp would be detected.

An autoradiogram of the extended products (Figure 13) reveal that a 230 bp fragment corresponding to transcripts initiating from the 5' end of exon 1, previously identified in cDNA clones, is detected in both cell lines (upper arrow). The primary human c-*myb* promoter exhibits extensive 5' heterogeneity in both of these cell lines (middle arrows). Some of the start sites are similar for each cell line, and some are unique to each cell line. In addition, a cluster

Figure 11. C-*myb* expression in U937 cells at 0, 6, 12 and 24 hours post induction with PDbu. The blot was hybridized as indicated in the methods section and exposed to X-Ray film overnight at -80°C. Shown below is the levels of β-actin expression in each cell line.

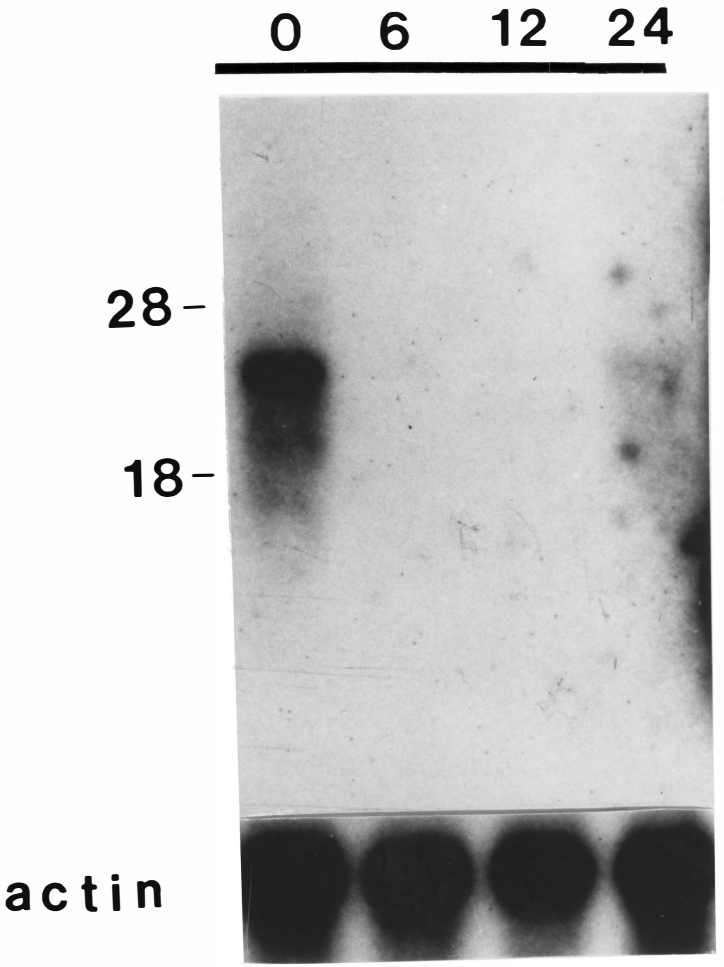
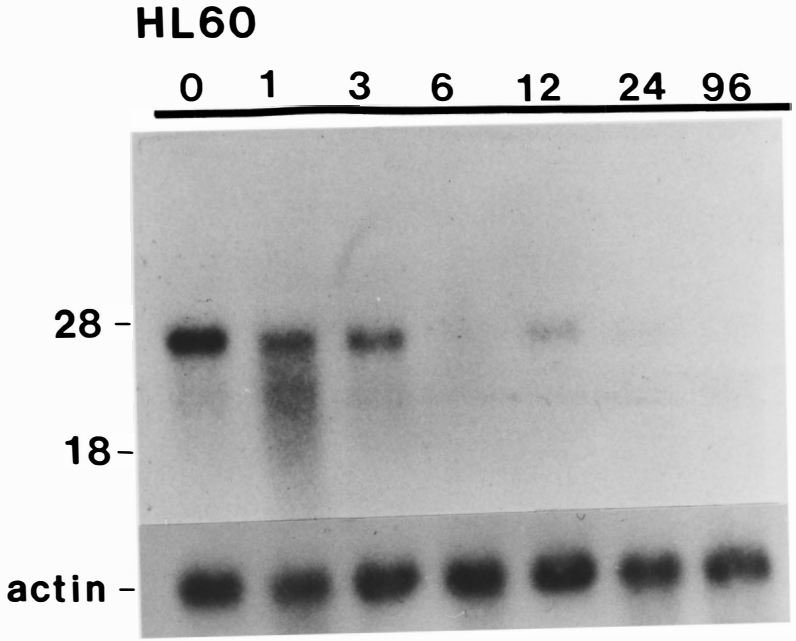

0
6
12
24
28
18
actin

Figure 12. C-*myb* expression of HL60 cells at 0, 1, 3, 6, 12, 24, and 96 hours post-induction with DMSO. The blot was hybridized as indicated in the methods section and exposed to X-Ray film overnight at -80°C. Shown below is the levels of $\beta$-actin expression in each cell line.

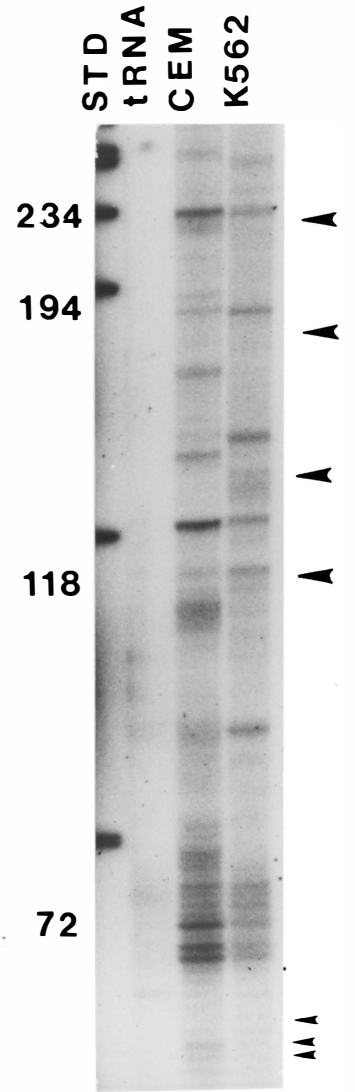

HL60
0 1 3 6 12 24 96
28
18
actin

of extended products between 60 and 70 bases in length is apparent (lower 3 arrows). The three fragments in this cluster are approximately 61, 63, and 66 bp in length. The size of these fragments corresponds to the 5' end of the pMbm-2 cDNA clone, and are in proximity of the potential cap sites identified by sequence analysis (Figure 8).

**RNAse protection analysis.**

Identification of the three potential sites in the primer extension analysis as specific to a promoter within intron 1 was obscured by the multiple transcriptional start sites originating from exon 1. Therefore, RNAse protection analysis was used to verify the presence of transcriptional start sites within intron 1. The single stranded probe derived from the construct p1XE7SH, shown in Figure 14, contains 200 bases of intron 1 sequence and 113 bases of exon 2 sequence; therefore, any c-*myb* messages containing a normal exon 1/ exon 2 splice junction will protect 113 bases of the probe. Any protected fragments larger than 113 bases would originate within the intron. Figure 15 shows RNAse protection analysis of poly A+ RNA from CCRF-CEM, K562, MOLT-4, and U937 cell lines. All cell lines show a 113 bp protected fragment corresponding to the exon 1/ exon 2 splice observed with the prototypic c-*myb* gene (bottom arrow). In all cell lines, a cluster of three protected fragments 131, 136 and 139 bases in length is also observed. These fragments correspond to transcription initiation sites 23, 26 and 29 bases upstream of the intron 1/ exon 2 junction. The position of these sites is consistent with the position of the

Figure 13. Primer extension analysis of CCRF-CEM and K562 total RNA. Extension from the primer complimentary to exon 2 sequences (see Methods) yielded a number of larger products corresponding to normal c-*myb* transcripts (larger arrows) as well as three smaller transcripts (smaller arrows). Extended products were resolved on a 7% polyacrylamide/8 M urea gel and exposed to X-ray film for 18 hr at -80°C.


STD
tRNA
CEM
K562
234
194
118
72

extended fragments in the primer extension analysis (Figure 13) as well as the presence of the pMbm-2 message cloned from CCRF-CEM. RNAse protection analysis using a probe linearized at the *Pst*I site at the intron 1/ exon 2 junction eliminates these three protected fragments (data not shown). The level of stable transcripts arising from this region varies from cell line to cell line; levels of transcript initiating within this region are proportional, however, to that seen from the upstream promoter.

Analysis of start site utilization during the differentiation process was assessed using poly A+ RNA from U937 cells differentiated along the monocytic pathway with PDbu (Figure 16). Messages from transcripts initiating from the intron 1 promoter and the upstream promoter decrease during differentiation. Thus, the primary and intron 1 derived messages are expressed concurrently during PDbu- induced differentiation of U937 cells. Transcriptional start site usage during DMSO-induced HL-60 differentiation was also assessed (Figure 17). RNAse protection of poly A+ RNA shows the biphasic expression of c-*myb* described previously (Boise et al., 1992b) and observed in the Northern blot analysis (Figure 12). As is seen during U937 differentiation, transcripts originating from intron 1 increase and decrease concurrently with c-*myb* messages originating in exon 1.

**Functional characterization of the intron 1 promoter.**

A series of constructs containing regions of intron 1 upstream of the chloramphenicol acetyl transferase gene (Figure 18) was assayed for promoter

Figure 14. Schematic representation of the probe used in RNAse protection analysis. 500 bp of the 3' end of intron 1 are shown above and the 328 bp single stranded probe generated by *in vitro* transcription is shown below. This probe will protect 113 bases of exon 2, which would represent c-*myb* message spliced normally between exons 1 and 2. Larger protected fragments would then represent messages originating from intron 1.

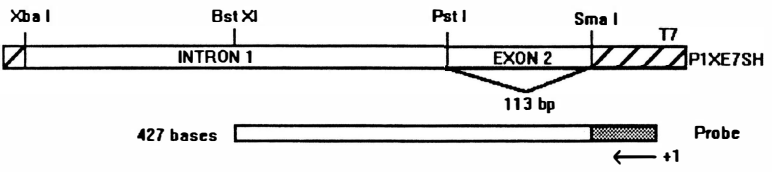

Xba I
BstXI
Pst I
Sma I
T7
INTRON 1
EXON 2
P1XE7SH
113 bp
427 bases
Probe
+1

Figure 15. RNAse protection analysis of leukemia cell lines. Five $\mu$g of poly A+ RNA from the indicated cell lines was annealed to the single stranded probe shown in Figure 14. The top arrow indicates nuclear RNA protected by the probe. C-*myb* message originating from exon 1 (lowest arrow) and intron 1 (three smaller arrows) are indicated. t ,10 $\mu$g tRNA; C, CCRF-CEM; K, K562; M4, MOLT-4; U, U937. M, molecular weight markers ($\gamma^{32}$[P]-ATP labeled $\phi$X174 DNA digested with HaeIII); P, 5 x $10^6$ cpm of undigested probe. The gel was exposed to film for three days at -80°C with an intensifying screen.

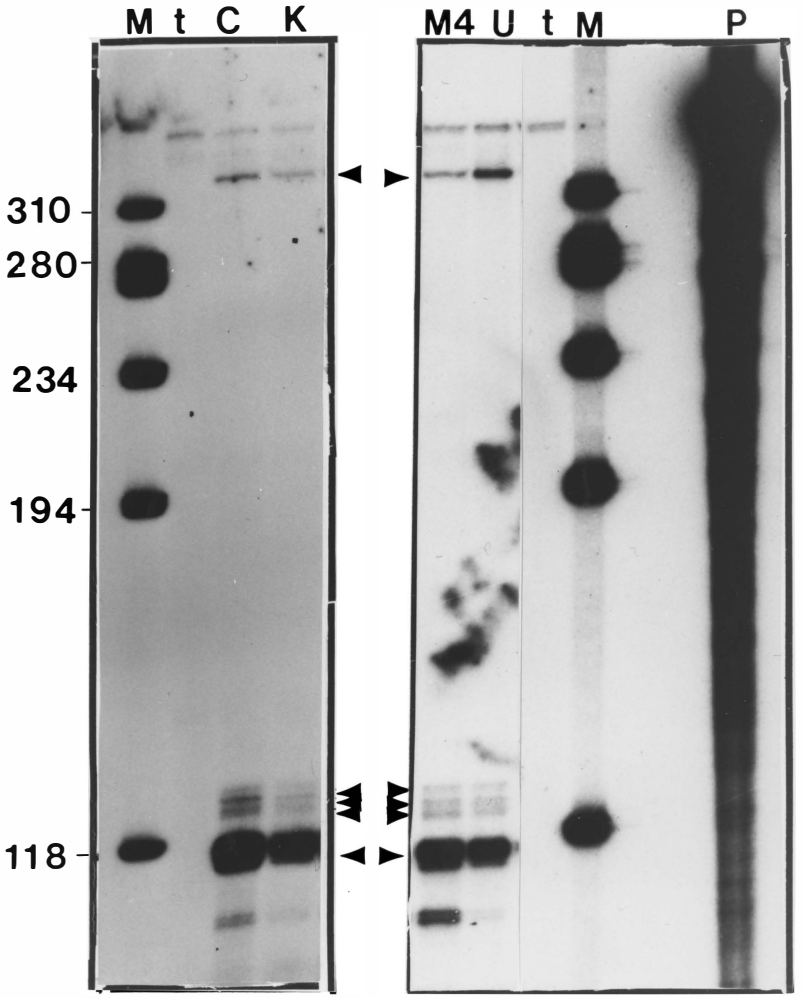

M t C K
M4 U t M P
310
280
234
194
118

Figure 16. RNAse protection analysis of exon 1 and intron 1 initiated c-*myb* transcripts following PDbu-induced differentiation of U937 cells. RNA was harvested at 0, 12, and 24 hours post induction of PDbu. The top arrow indicates nuclear RNA protected by the probe. C-*myb* message originating from exon 1 (lowest arrow) and intron 1 (three smaller arrows) are indicated. t,10 $\mu$g tRNA; M, molecular weight markers ($\gamma^{32}$[P]-ATP labeled $\phi$X174 DNA digested with HaeIII); P, 5 x $10^6$ cpm of undigested probe. The gel was exposed to film for three days at -80°C with an intensifying screen.

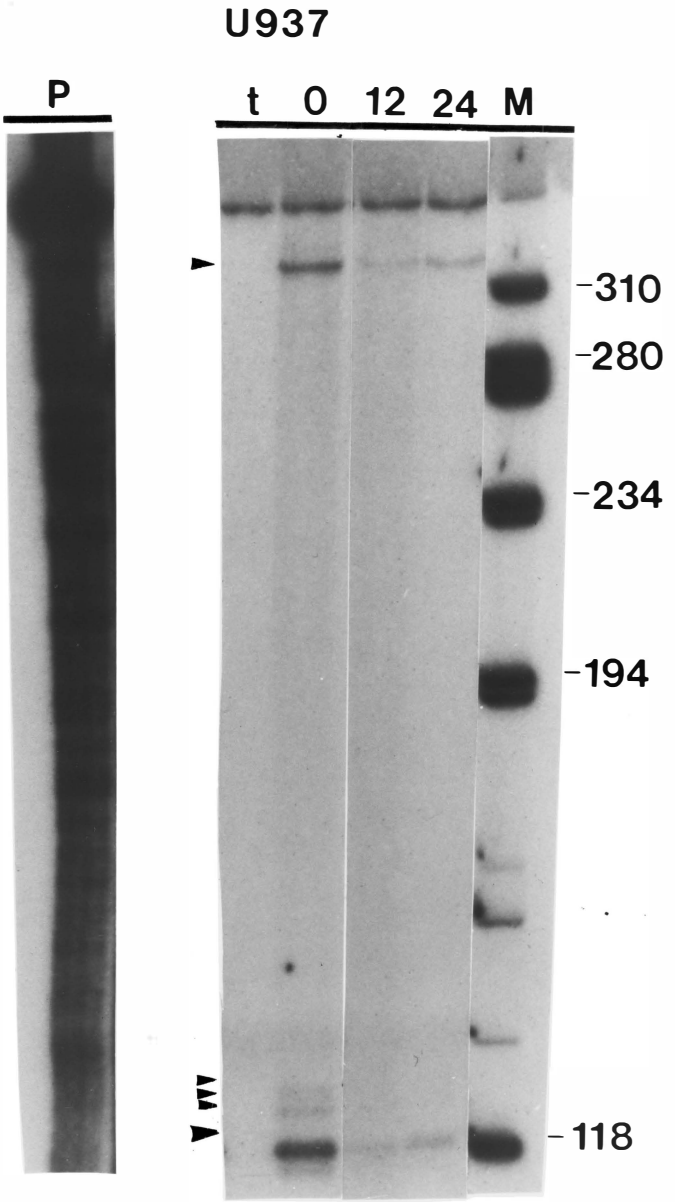
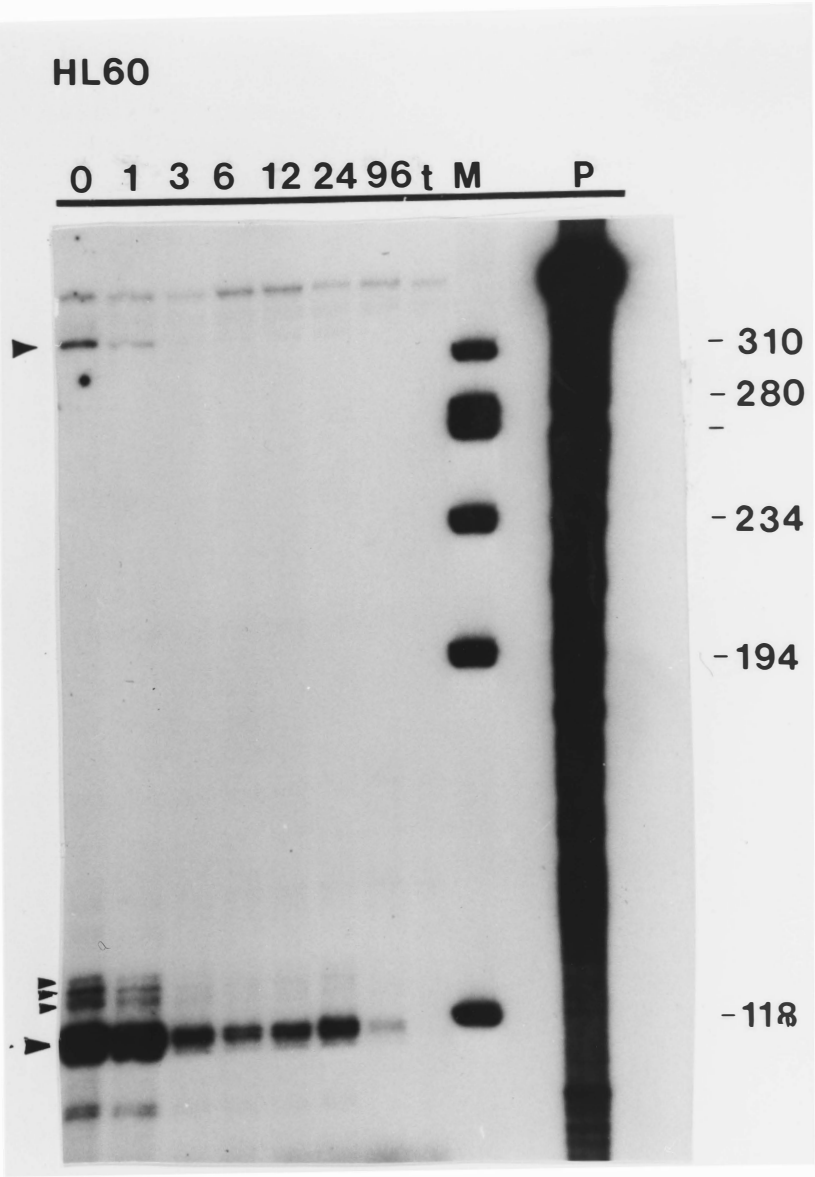

U937
P
t
0
12
24
M
310
280
234
194
118

Figure 17. RNAse protection of exon 1 and intron 1 initiated c-*myb* transcripts in HL-60 cells at 0, 1, 3, 6, 12, 24, and 96 hours following induction of differentiation with DMSO. The top arrow indicates nuclear RNA protected by the probe. C-*myb* message originating from exon 1 (lowest arrow) and intron 1 (three smaller arrows) are indicated. t ,10 $\mu$g tRNA; M, molecular weight markers ($\gamma^{32}$[P]-ATP labeled $\phi$X174 DNA digested with HaeIII); P, 5 x $10^6$ cpm of undigested probe. The gel was exposed to film for three days at -80°C with an intensifying screen.


HL60
0 1 3 6 12 24 96 t M P
310
280
234
194
118

activity in the CCRF-CEM and K562 cell lines. A 1.6 kb *Pst*I fragment containing the 3' end of intron 1 was tested to determine if potential promoter activity could be detected from this region of intron 1 (pINT1CAT). Deletions of this fragment were created to assess if any *cis* elements in intron 1 could affect promoter activity and to determine the size of the minimal promoter (pINT1CAT.12, pINT1CAT.40, pXPCAT, pBxPCAT). The activity of each of these promoter constructs is shown in Figures 19 and 20.

In CCRF-CEM (Figure 19), the promoter exhibits changes in activity dependent on *cis* elements in the intron; deletion of 653 bp of the intron (seen in construct pINT1CAT.12) leads to a 6-fold increase in promoter activity. Further deletion causes a decrease in promoter activity (pINT1CAT.40), suggesting that an element necessary for the increase in promoter activity is removed. Intron 1 can be deleted up to 213 bases 5' of exon 2 without abolishing promoter activity (pBxPCAT). By contrast, in K562, intron promoter activity does not vary significantly as intron 1 sequence is deleted (Figure 20). It appears, therefore, that the promoter activity in this cell line is not regulated in the same manner as in CCRF-CEM.

**RT-PCR analysis of CCRF-CEM reporter assay transfections.**

To verify that the increased CAT activity seen in pINT1CAT.12-transfected CCRF-CEM cells originated from the transcription start sites mapped in the RNAse protection studies (Figure 15) and was not due to non-specific initiation events, RT-PCR analysis was performed (Figure 21).

Figure 18. Regions of intron 1 used in reporter constructs for chloramphenicol acetyl transferase transient assays. Shown in this figure is the last 200 bp of intron 1. A portion of the region of transcriptional attenuation found in the center of intron 1 (Attenuator) is indicated. The regions of intron 1 indicated were fused to the chloramphenicol acetyl transferase gene as indicated in the methods section. Conservation between human and mouse is indicated by hatched regions. Conservation between human and chicken is indicated by asterisks(***). Restriction sites are abbreviated as follows: N-*Nco*I; E-*Eco*RI; B-*Bam*HI; P-*Pst*I; S-*Sma*I; Bg-*Bgl*II; Sp-*Sph*I; X-*Xba*I; H-*Hind*III; Bx-*Bst*XI.

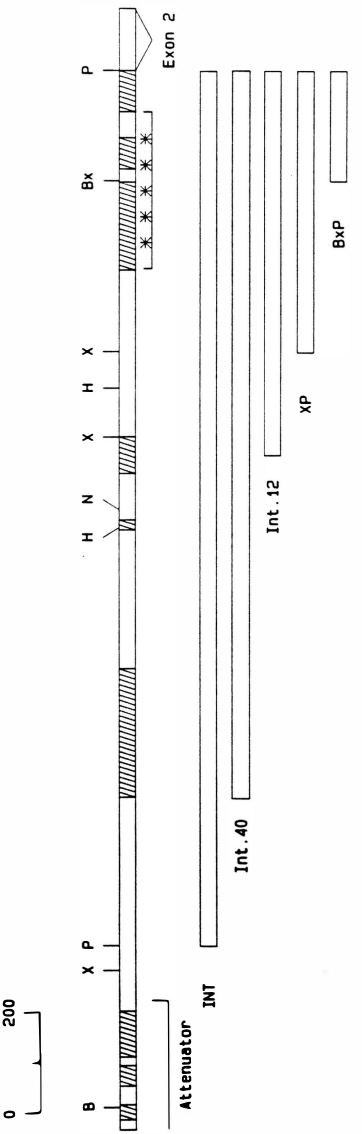

0
200
B
X
P
H
N
X
H
X
Bx
P
Exon 2
Attenuator
INT
Int.40
Int.12
XP
BxP

Figure 19. Promoter activity of human c-*myb* intron 1 sequence in CCRF-CEM cells. Representative results are shown from three independent transfections of SV40 and intron 1 constructs into CCRF-CEM. Constructs utilized for this figure are indicated in the figures and refer to fragments shown in Figure 18. A plasmid containing the gene for $\beta$-galactosidase under the control of the CMV promoter was co-transfected with the CAT constructs as an internal control. The plasmids tested are indicated on the X-axis. Percent conversion of chloramphenicol is shown on the y-axis as normalized to $\beta$-galactosidase activity of each cell free extract. SV40 - CAT gene under the control of the SV40 promoter and enhancer. REV - INT fragment cloned in reverse orientation to the CAT gene.

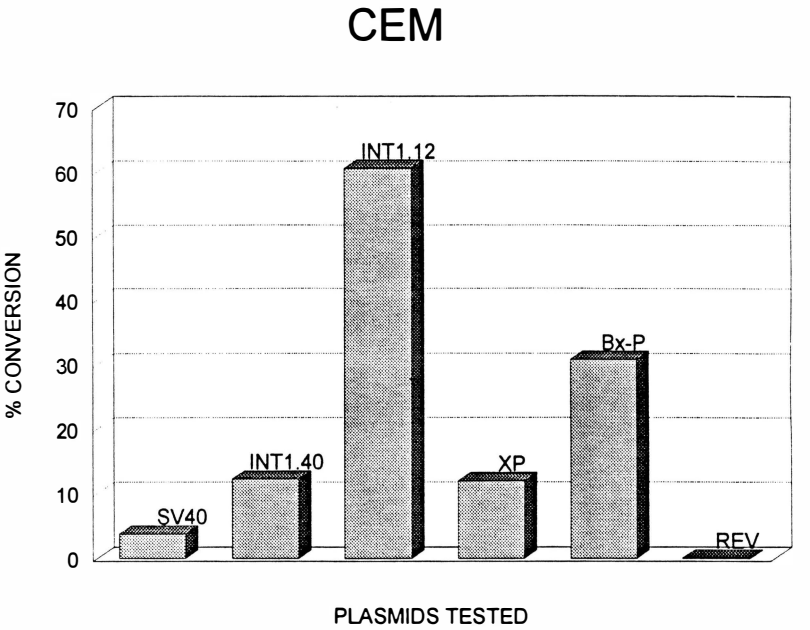
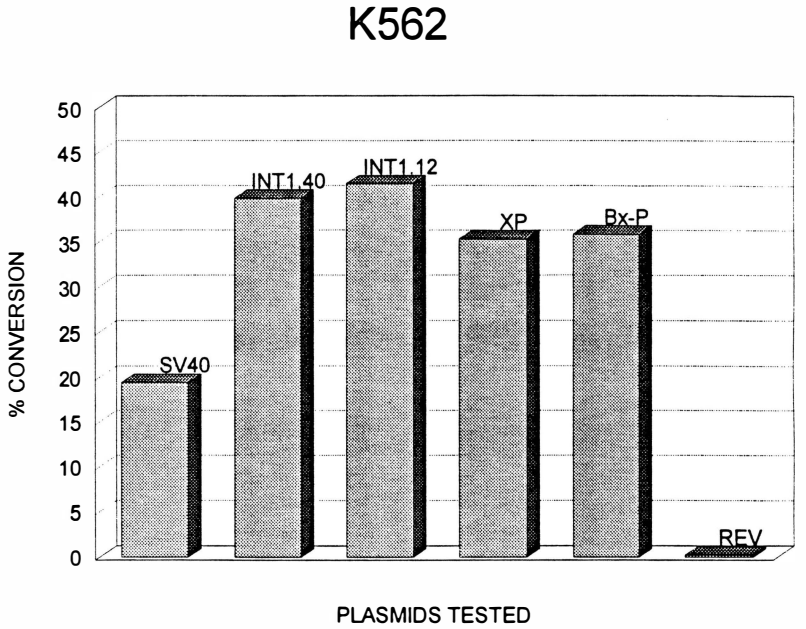

CEM
70
60
50
40
30
20
10
0
% CONVERSION
SV40
INT1.40
INT1.12
XP
Bx-P
REV
PLASMIDS TESTED

Figure 20. Promoter activity of human c-*myb* intron 1 sequence in K562 cells. Constructs utilized for this figure are indicated in the figures and refer to fragments shown in Figure 18. A plasmid containing the gene for β-galactosidase under the control of the CMV promoter was co-transfected with the CAT constructs as an internal control. The plasmids tested are indicated on the X-axis. Percent conversion of chloramphenicol is shown on the y-axis as normalized to β-galactosidase activity of each cell free extract. SV40 - CAT gene under the control of the SV40 promoter and enhancer. REV - INT fragment cloned in reverse orientation to CAT gene.


K562
50
45
40
35
30
25
20
15
10
5
0
% CONVERSION
SV40
INT1.40
INT1.12
XP
Bx-P
REV
PLASMIDS TESTED

Southern blot analysis using a probe specific for the chloramphenicol acetyl transferase gene (CAT) (Figure 21A) reveals that hybridization is seen to a 724 bp band specific for initiation at the expected sites.

**Electrophoretic mobility shift assay (EMSA).**

Because *cis* elements within intron 1 appear to affect the activity of the intron 1 promoter differentially in CCRF-CEM and K562, the regions of intron 1 which correspond to these changes in CAT activity were tested for their ability to bind nuclear proteins in an electrophoretic mobility shift assay (EMSA). The four double stranded DNA fragments (Figure 22) were radioactively labeled and incubated with nuclear extracts from CCRF-CEM and K562 cell lines. Resolution of these samples on a polyacrylamide gel revealed that while fragment I (Figure 23, I) and fragment II (data not shown) could not bind nuclear extracts from either cell line, fragments III and IV could bind nuclear proteins from both CCRF-CEM and K562 nuclear extracts (Figure 23, III and IV). The binding to both fragments III and IV is not competed off by increasing amounts of non-specific competitor (n.s. comp). The difference in binding to fragment III between CCRF-CEM and K562 appears to be qualitative; the fragments shift to different regions of the gel, indicating that different proteins may be bound. The difference in binding to fragment IV, by contrast, appears to be quantitative; the fragment shifts to the same position using nuclear protein from either cell line, but more protein is bound in CCRF-CEM extracts compared with K562 extracts.

Figure 21. RT-PCR analysis of CCRF-CEM cells transfected with pINT1CAT.12. A. Southern blot hybridization using the CAT.PROBE oligonucleotide to PCR amplified RT reactions of cells either transfected with pINT1CAT.12 (construct 12, lanes 1,2,4,8, and 9) or untransfected (construct (-), lanes 5-7). Primer sets for the PCR reactions are as follows: 5': INT1.5'/CAT.3' primers; M: MBM2.5'/CAT.3' primers; A: actin 5' and 3' primers. Lane 1 and 2: PCR reactions of pINT1CAT.12-transfected cells prior to DNAse treatment. Lane 3: RT-PCR reaction using $H_2O$ instead of RNA. Lane 4: No reverse transcription PCR reaction of DNAse-treated RNA from pINT1CAT.12-transfected cells. Lanes 5-7: RT-PCR reactions of untransfected cells with the 5', M and A sets of PCR primers, respectively. Lanes 8 and 9: RT-PCR reaction of cells transfected with the pINT1CAT.12 construct and amplified with the 5' and M sets of PCR primers, respectively. B. Southern blot hybridization using the human $\beta$-actin probe to RT-PCR reactions from untransfected (lane 1) and pINT1CAT.12 transfected (lane 2) cells using the actin PCR primers. Markers indicate 1.0 kb and 0.5 kb from top to bottom.

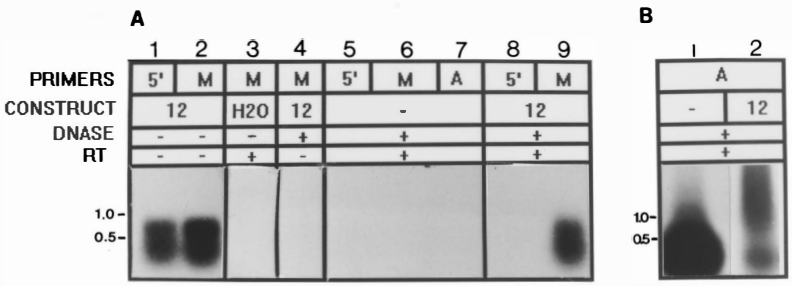

A
1 2 3 4 5 6 7 8 9
PRIMERS
5' M M M 5' M A 5' M
CONSTRUCT
12 H2O 12 - 12
DNASE
- - - + + + +
RT
- - + - + + +
1.0
0.5
B
1 2
A
- 12
+
+
1.0
0.5

Figure 22. Schematic representation of the fragments used in the electrophoretic mobility shift assay (EMSA). Shown is the last 2000 bp of intron 1. The region of transcriptional attenuation in the center of intron 1 (Attenuator) is indicated. Regions corresponding to positive and negative regulation of promoter activity in CCRF-CEM transient transfections are also indicated. Conservation between human and mouse is indicated by hatched regions. Conservation between human and chicken is indicated by asterisks(***). Start of pINT1CAT.40 (Int.40) and pINT1CAT.12 (Int.12) are indicated. Restriction sites are abbreviated as follows: N-*Nco*I; E-*Eco*RI; B-*Bam*HI; P-*Pst*I; X-*Xba*I; H-*Hind*III; Bx-*Bst*XI.

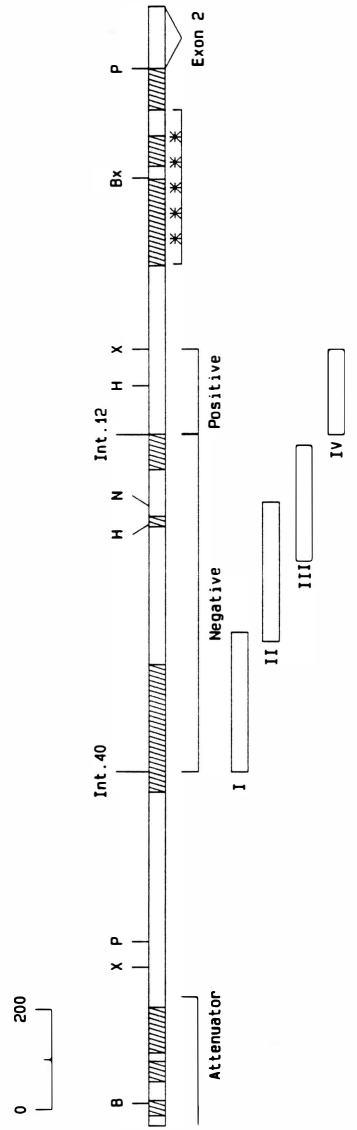

0
200
B
Attenuator
X
P
Int.40
H
N
Int.12
H
X
Bx
P
Exon 2
Negative
Positive
I
II
III
IV

Figure 23. EMSA of fragments I, III, and IV incubated with CCRF-CEM (C) and K562 (K) nuclear extracts with increasing amounts of poly dIdC non-specific competitor (n.s. comp.). The concentration of the non-specific competitor is indicated on the figure.

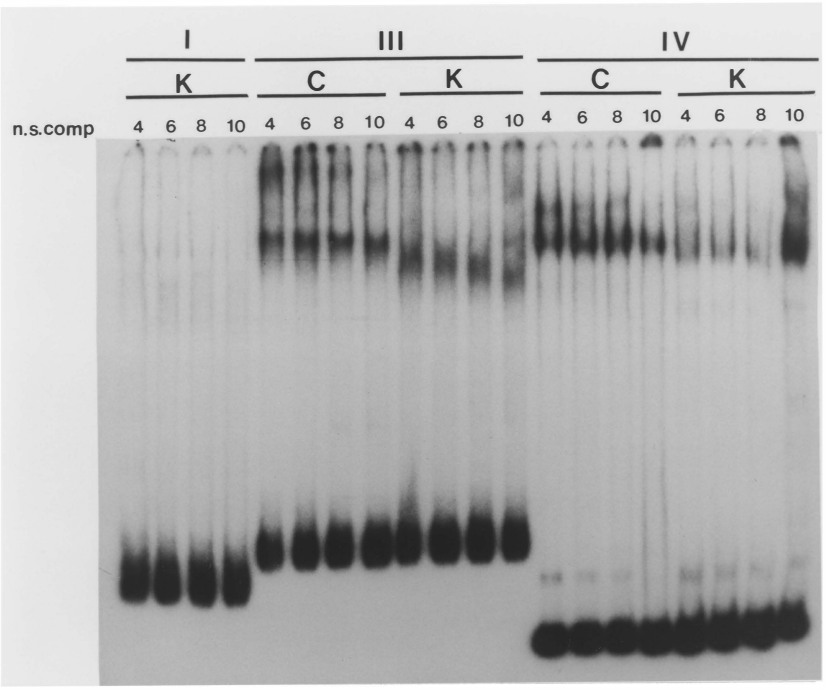
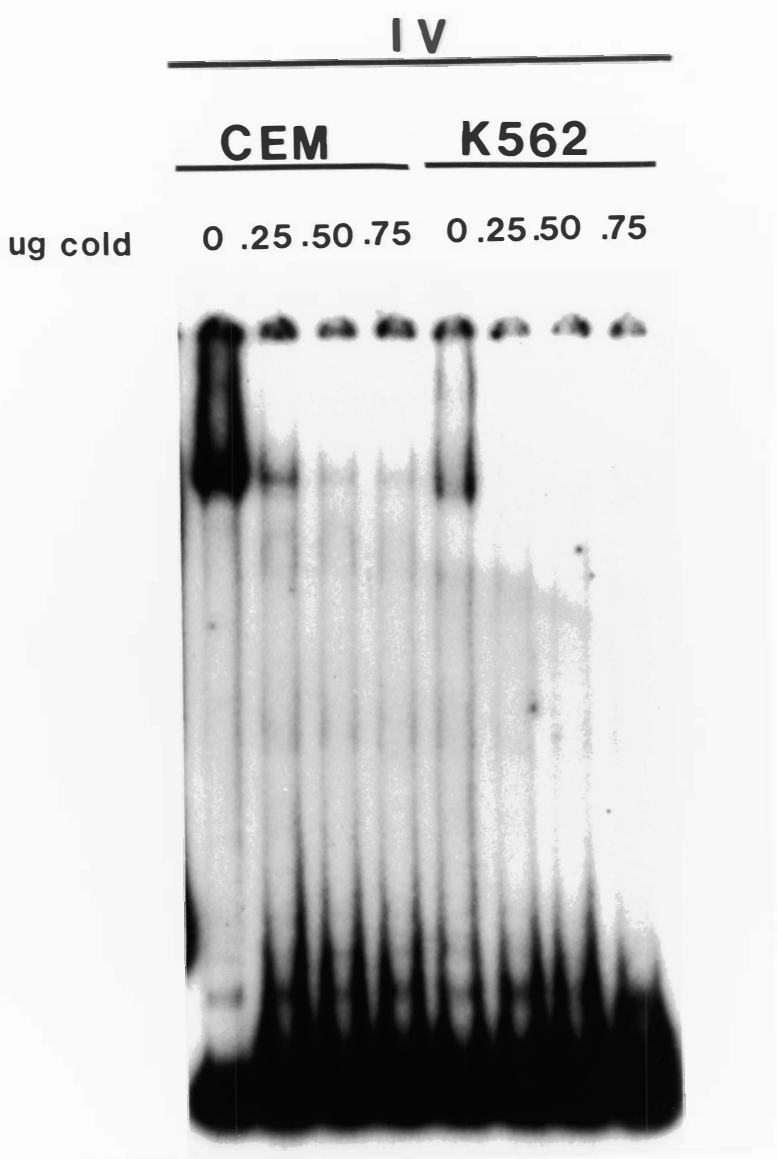

I
III
IV
K
C
K
C
K
n.s.comp
4 6 8 10 4 6 8 10 4 6 8 10 4 6 8 10 4 6 8 10

Figure 24. EMSA of fragment IV incubated with CCRF-CEM (CEM) and K562 nuclear extract with increasing amounts of unlabeled fragment IV ($\mu$g cold). The concentration of the specific competitor is indicated on the figure.


IV
CEM
K562
ug cold
0 .25 .50 .75 0 .25 .50 .75

In order to determine if the binding of nuclear extracts by fragment IV was sequence specific, the radioactively labeled DNA fragment and nuclear extracts were incubated with increasing amounts of unlabeled fragment IV. An EMSA of this experiment (Figure 24) revealed that the binding of both CCRF-CEM and K562 nuclear proteins to radioactive fragment IV was competed by increasing amounts of unlabeled fragment IV ("cold comp"). As was seen in Figure 23, the binding of the nuclear proteins appears to be quantitative; more protein was bound in CCRF-CEM extracts than K562 extracts. This is confirmed from the competition assays where ten-fold less cold competitor is needed to compete for binding to K562 extracts.

Characterization of a rearrangement in the c-*myb* promoter in the acute lymphoblastic leukemia cell line CCRF-CEM.

**Identification of a rearrangement in the c-*myb* promoter in CCRF-CEM.**

A schematic representation of the c-*myb* promoter is shown in Figure 25. Southern blot analysis of a panel of *Eco*RI digested leukemic cell line genomic DNAs using a probe which encompasses the c-*myb* promoter reveals the presence of a restriction fragment length polymorphism (RFLP) in the CCRF-CEM cell line which is not present in any other cell line tested (Figure 26). The CCRF-CEM sample shown in Figure 26 is an ATCC stock sample, indicating that the RFLP did not occur due to continuous subsequent passage of the cell line in culture. Further analysis with other restriction enzymes (data not shown) showed that the RFLP was not due to a single point mutation, but

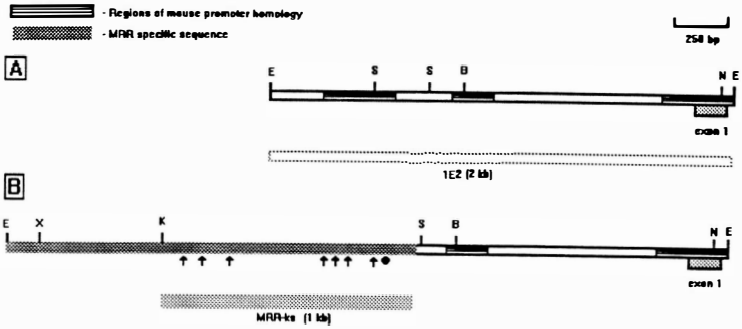
represented a rearrangement in the promoter region upstream of a *Sph*I site in the normal c-*myb* promoter region (see Figure 25B).

**Cloning the rearranged CCRF-CEM c-*myb* promoter.**

Using the 1E2 probe shown in Figure 25A, a CCRF-CEM genomic lambda library was screened to isolate and characterize the promoter rearrangement. Sequence analysis of the clone, which we have termed MRR1 (**m**yb **r**earranged **r**egion 1), revealed the presence of unique DNA juxtaposed to the normal c-*myb* promoter (Figure 25C). This unique sequence is also associated with the RFLP seen in CCRF-CEM (Figure 27). In addition, a portion of the promoter sequence which is conserved between the human and murine c-*myb* promoters is lost; no *myb* specific sequence upstream of the *Sph*I site indicated in Figure B was associated with any RFLP seen in CCRF-CEM (Figure 26 and data not shown), indicating a loss of this promoter sequence material from the CCRF-CEM cell line. The MRR unique sequence has been mapped by Southern blot analysis of somatic cell hybrids to chromosome 6 (Figure 28). Together, these data suggest that the rearrangement seen in CCRF-CEM is a deletion and not a translocation or insertion.

Using the probes generated from this cloning, a panel of fresh leukemic DNAs was screened to identify similar RFLP's in other leukemias. Both the 1E2 probe and the MRR-ks probe (Figure 25) were used to detect either c-*myb* specific or MRR specific aberrations. Southern blot analysis of 23 acute myelogenous leukemias, 16 acute lymphoblastic leukemias, 3 chronic

Figure 25. Schematic representation of the human c-*myb* promoter (A) and the myb rearranged region (MRR)/c-*myb* rearrangement cloned from a CCRF-CEM genomic library (B). Probes used to detect *myb* specific (1E2) and MRR specific (MRR-ks) sequence are indicated below. Lined boxes indicate regions of promoter sequence conserved between the mouse and human c-*myb* promoters. Shaded region indicates unique sequence found upstream of the c-*myb* promoter in the CCRF-CEM rearrangement. Arrows indicate potential AP1 transcription factor binding sites. Filled circle indicates a potential TATA box. E-*Eco*RI; X-*Xba*I; S-*Sph*I; B-*Bam*HI; K-*Kpn*I; N-*Nco*I. (C) Sequence of the MRR/c-*myb* junction region. Shaded sequence indicates the MRR specific sequence; underlined sequence indicates normal c-*myb* promoter sequence. Arrow indicates the rearrangement junction.




C

```
  1  TAAATAAACAGATCAACAGAATAGTATAGA   30
 31  GTGCCCAGGGTGTAAATCACAAACTGTTTT   60
                     ↑
 61  ACTTCTAAAACCATGCATGCAAACGTGGGG   90
 91  TTTCCTGAGT  100
```

Figure 26. Southern blot analysis of *Eco*RI digested genomic DNA from the leukemia cell lines indicated using the 1E2 c-*myb* promoter specific probe. A single altered band in the human acute lymphoblastic leukemia cell line CCRF-CEM is detected (arrows). The probe used is illustrated in Figure 25. The blot was exposed to X-ray film one week at -80°C with an intensifying screen.

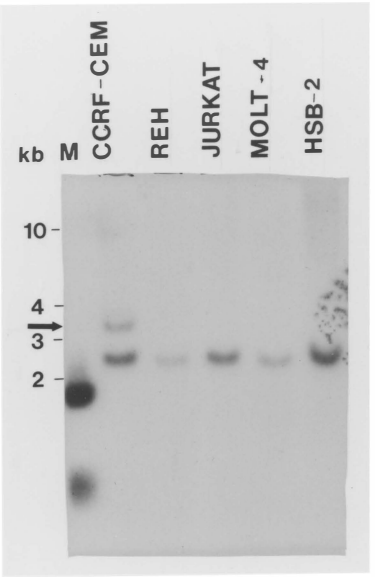
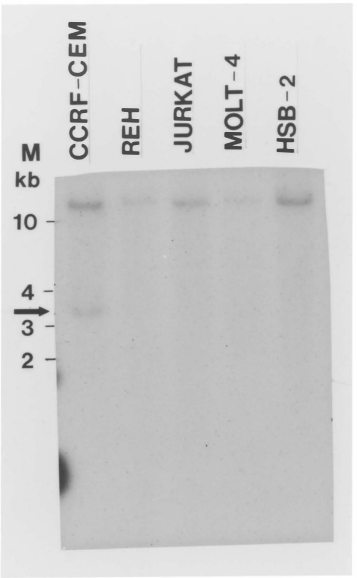

kb
M
CCRF-CEM
REH
JURKAT
MOLT-4
HSB-2
10
4
3
2

Figure 27. Southern blot analysis of *Eco*RI digested genomic DNA from the leukemia cell lines indicated using the MRR-ks probe specific for the CCRF-CEM rearrangement. A single altered band in the human acute lymphoblastic leukemia cell line CCRF-CEM is detected (arrows). The probes used are those illustrated in Figure 25. The blot was exposed to X-ray film one week at -80°C with an intensifying screen.


M
kb
10
4
3
2
CCRF-CEM
REH
JURKAT
MOLT-4
HSB-2

myelogenous leukemias and one chronic lymphocytic leukemia using restriction with *Eco*RI, *Bam*HI, and *Pst*I (data not shown) revealed a single rearrangement associated with the c-*myb* promoter: a T-cell acute lymphoblastic leukemia (ALL) was amplified and rearranged upstream of the *Bam*HI site in the c-*myb* promoter (Figure 25A). Due to limited sample quantities, further analysis of this rearrangement was not possible. In this leukemia, the MRR specific sequence did not reveal a rearrangement at the MRR locus.

**Field inversion gel electrophoretic studies of the MRR/c-*myb* region.**

Field inversion gel electrophoresis (FIGE) of DNA isolated from peripheral blood lymphocytes (PBLs) was used to determine the approximate size of the deletion identified in CCRF-CEM. Southern blot hybridization of *Eag*I digested agarose plugs of PBLs (Figure 29) showed that both the 1E2 c-*myb* promoter fragment (Figure 29, left) and the MRR *Kpn*I-*Sph*I (MRR-ks) fragment (Figure 29, right) hybridize to a 450 kilobase (kb) fragment (Figure 29, upper arrows).

**Promoter activity of the CCRF-CEM MRR/c-*myb* promoter.**

Sequence analysis of the 1.6 kb *Xba*I-*Bam*HI fragment shown in Figure 25 revealed the presence of potential transcriptional activator binding sites in the MRR sequence juxtaposed to the altered c-*myb* promoter. Therefore, a series of constructs containing regions of the normal c-*myb* promoter and the MRR/c-*myb* sequence placed upstream of the chloramphenicol acetyl transferase

Figure 28. Southern blot hybridization of the MRR-ks probe to Pst I digested somatic cell hybrids. The somatic cell hybrids (10629 & 7300), human (donor) and hamster DNAs (hamster) are indicated. The probe hybridizes to a single band in the human donor and the somatic cell hybrids DNAs containing human chromosome 6.

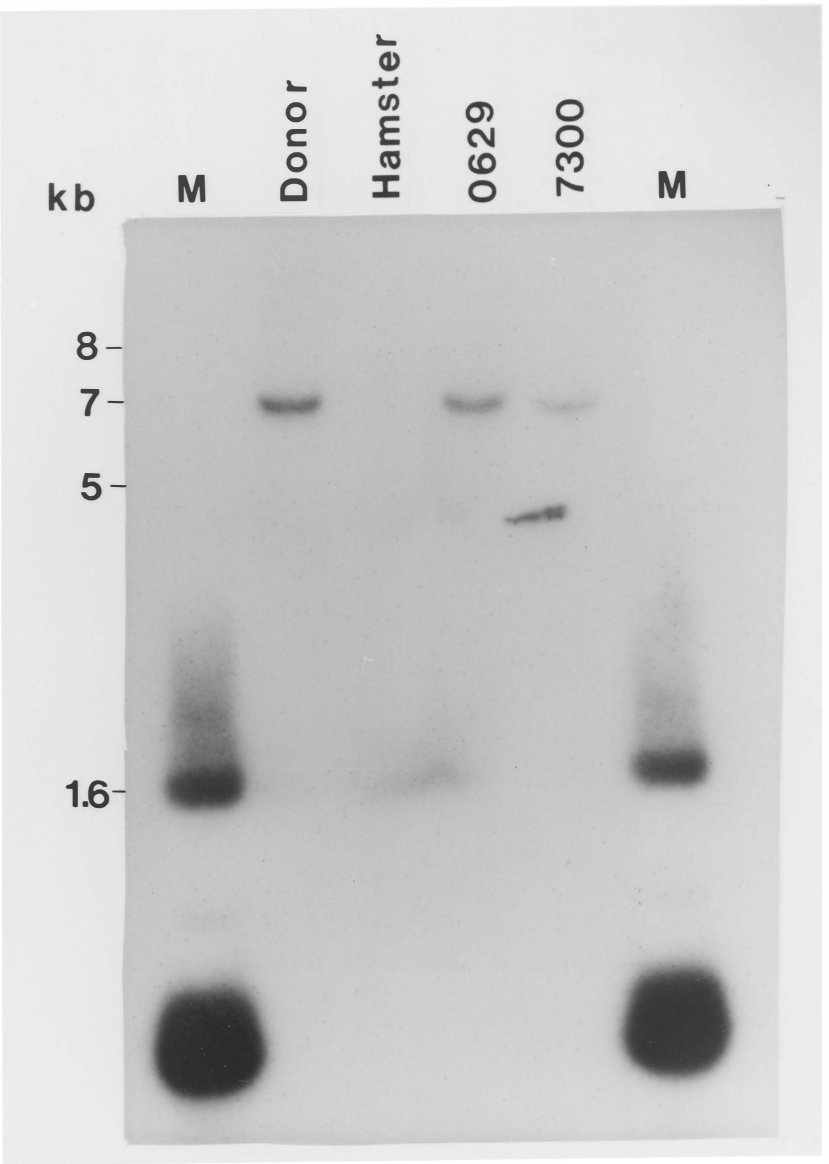
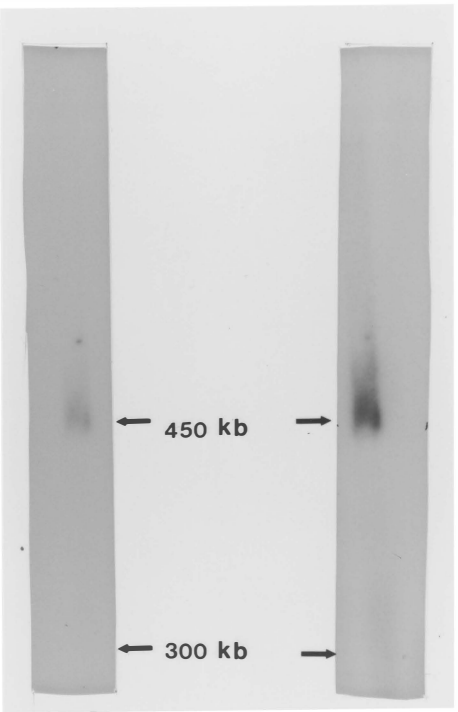

kb
M
Donor
Hamster
0629
7300
M
8
7
5
1.6

Figure 29. Southern hybridization of *Eag*I-digested human peripheral blood lymphocyte (PBL) DNA separated by field inversion gel electrophoresis. Hybridization is detected with both the 1E2 (left) and MRR-ks (right) probes. A specific 450 kb band is detected by both probes (upper arrows).


450 kb
300 kb

gene (Figure 30) were assayed for promoter activity in proliferating CCRF-CEM cells (Figure 31). The presence of the unique MRR sequence (Figure 31, lane MRR) does not appear to substantially effect c-*myb* promoter activity when compared to the full length promoter (Figure 31, lane PRO1), although deletion of the c-*myb* promoter does have a nominal effect on promoter activity (Figure 31, lane PRO2).

**Characterization of the sequence deleted in CCRF-CEM.**

The MRR-ks fragment (Figure 25B) was used to screen a normal human placental genomic DNA library to identify the normal locus containing the MRR sequence. From this library, a 17 kb clone was isolated which contains the MRR sequence identified in the CCRF-CEM rearrangement (Figure 32). The upstream deletion junction from CCRF-CEM was sequenced (Figure 33) and compared to known sequences to determine if any nearby potential genes may be deleted. Sequence comparison revealed the presence of a 133 bp sequence with 66% identity to the *myc*L2 gene, a member of the *myc* oncogene family (Morton et al., 1989) (Figure 33). Directly flanking this region of identity are potential splice donor and acceptor sites (Figure 33, double diamonds). This sequence also shows significant identity to a region of the p-*myc* gene, a *myc*-family pseudo-oncogene (DePinho et al., 1987), but no homology to any other known member of the *myc* family. An oligonucleotide probe to this *myc*L2-like sequence, however, failed to hybridize to specific mRNA in a number of leukemia cell lines tested (CCRF-CEM, MOLT-4,

Figure 30. Schematic representation of constructs used in transient transfections. Top map represents the normal c-*myb* promoter region with shaded regions indicating sequences conserved between the mouse and human. PRO-1 to PRO-3 and MRR represent reporter constructs as described in the Methods section. Shaded region in the MRR construct indicates MRR specific sequence. E-*Eco*RI; X-*Xba*I; S-*Sph*I; B-*Bam*HI; K-*Kpn*I; N-*Nco*I.

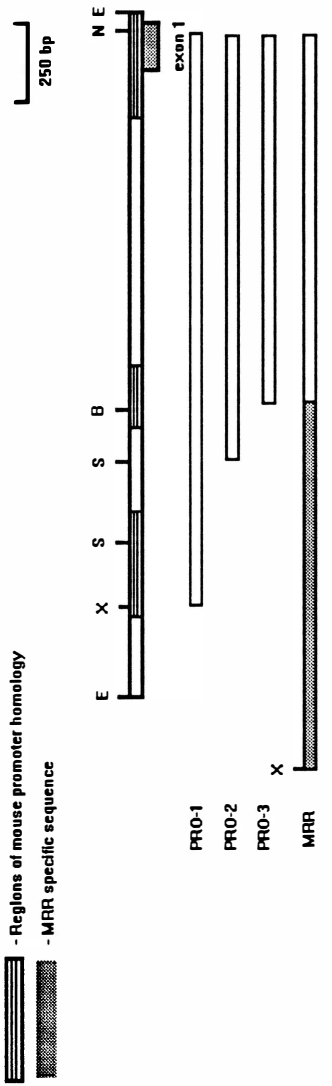

- Regions of mouse promoter homology
- MAR specific sequence
250 bp
E
X
S
S
S
B
N
E
exon 1
PRO-1
PRO-2
PRO-3
X
MAR

Figure 31. Promoter activity of c-*myb* and MRR/c-*myb* promoter constructs in CCRF-CEM cells. The PRO1, PRO2, PRO3 and MRR constructs, indicated in Figure 30, were co-transfected with the gene for β-galactosidase under the control of the CMV promoter. Shown is a thin layer chromatography plate of fluorescent chloramphenicol after incubation with cell-free extracts of each construct normalized by relative β-galactosidase activity. The unacetylated form is the slowest migrating form of chloramphenicol and is seen at the bottom of the photograph. The acetylated forms migrate faster and are seen above. SV40-activity of the CAT gene placed under control of the SV40 promoter and enhancer. REV -activity of MRR(R) (CAT in reverse orientation). STD-acetylated chloramphenicol internal standard.

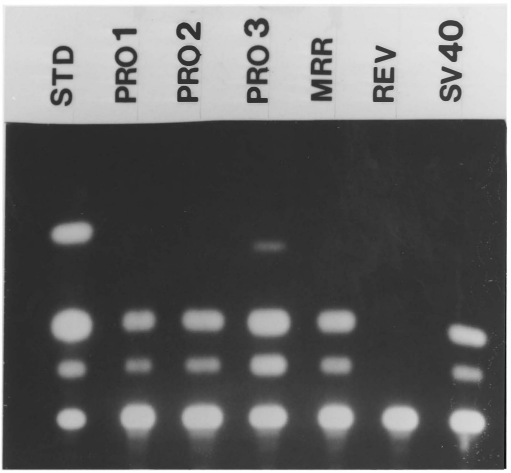
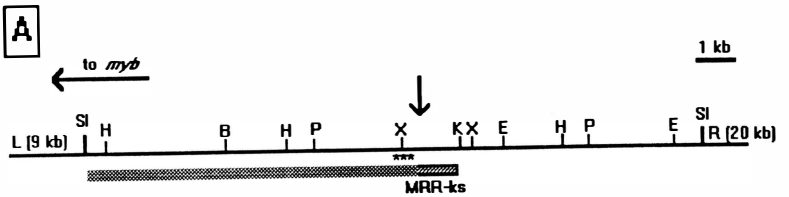

STD
PRO 1
PRO 2
PRO 3
MRR
REV
SV 40

Figure 32. (A) Schematic representation of the normal locus of the MRR sequence. L and R indicate left and right arms of the λFIXII vector, respectively. Downward arrow indicates breakpoint in the MRR locus of CCRF-CEM. Hatched region indicates the MRR-ks specific sequence. Shaded region indicates sequence lost in the CCRF-CEM deletion. Asterisks indicate location of sequence with identity to the *myc*L2 gene. Sl-*Sal*I (in multiple cloning site of vector); H-*Hind*III; B-*Bam*HI; P-*Pst*I; X-*Xba*I; K-*Kpn*I; E-*Eco*RI. (B) Sequence flanking the normal locus of the MRR DNA. Shaded region indicates DNA found 5' to c-*myb* in the rearrangement. Double underlined sequence indicates region with identity to the *myc*L2 gene. Double diamonds indicate potential consensus splice donor and acceptor sites.




B

```
  1  AATAAACAGATCAACAGAATAGTATAGAGT  30
 31  GCCCAGGGTGTATAGACAAAGTATAAAGGC  60
 61  CATTCAGTGGAGAAAAGACAGTTTTTTCCA  90
 91  ACAAATGGTGTTAGAATAGTTGGATATTCA 120
121  TATGTGAAAAAAATGAACTTTGATCCATAC 150
151  TTTACAACATGTACAAAAGTAACTCAAAAT 180
181  GAACCACAGATCTAATGTAAACCCAAGCTG 210
```

K562, HSB-2, HUT-78, REH, Jurkat-E61; data not shown).

## DISCUSSION

Intron 1 of the human c-*myb* gene was sequenced to determine the origin of the unique pMbm-2 5' sequence (Weber et al., 1990) and to identify regions of the intron with potential functional significance. Three regions of significant identity were revealed through sequence analysis and comparison to previously published mouse sequence. The first encompasses 600 bp of DNA located at the 5' end of the intron adjacent to exon 1. The first 153 bases of identity fall in a region which corresponds to a GC rich region containing Sp1 binding sites found in both the human and the mouse, suggesting that this region may function to regulate transcription of the upstream promoter. This would fit with data from the characterization of the primary c-*myb* promoter as lacking TATA- and CAAT boxes and utilizing multiple transcription start sites. The function of the remaining 447 bases of identity in this region is unknown; electrophoretic mobility shift assays using this region of murine intron 1 failed to correlate with changes in c-*myb* expression (Reddy and Reddy, 1989). This region may play a role in regulation of either the primary c-*myb* promoter or the constitutive antisense transcription which has been detected in this region (Boise et al.,

1992a).

The second region of identity is located in the center of the intron. The position of this sequence corresponds to DNAse I hypersensitive sites identified in the murine c-*myb* intron 1 which correlate with pausing activity. Electrophoretic mobility shift studies using DNA fragments in this region showed a correlation between nuclear factor binding and changes in c-*myb* expression (Reddy and Reddy, 1989). The region of transcriptional attenuation in the human has also been roughly mapped to this region (Boise et al., 1992a). These results indicate that this region of identity corresponds to the region of transcriptional attenuation for the human and murine c-*myb* genes and may be involved functionally in this process.

When human, murine and chicken c-*myb* intron 1 sequences are compared, different patterns of identity emerge. Except for the first 153 bases of the intron, which correlate to the GC rich region seen in human and murine intron 1, there is no significant identity between the human and chicken sequences in the first 3200 bp of the intron. The presence of an attenuator in the chicken c-*myb* gene has not yet been demonstrated; the lack of identity throughout the center of the intron suggests that 1) attenuation may not be the primary means of c-*myb* regulation in the chicken; or 2) while attenuation may occur in the center of the intron, the signals for pausing may be located elsewhere in the gene. The only other region of intron 1 which showed significant identity to chicken sequences was located at the 3' end of the intron.

This region also showed significant identity to murine sequences. The conservation of this noncoding region of sequence throughout evolution suggests that this region may also have functional significance.

As previously noted, the 5' unique sequence of the alternatively spliced clone pMbm-2 was mapped via Southern hybridization to intron 1. Sequence analysis revealed that this sequence is located directly adjacent to exon 2. The position of this unique sequence indicates that this message did not arise from alternative splicing but instead arose from a transcriptional initiation site within intron 1. No TATA consensus sequences for eukaryotic promoters are found in this region; therefore, as with the primary promoter, multiple start sites would be expected. Primer extension analysis using a primer common to both the pMbm-1 and pMbm-2 cDNA clones revealed that, as has been detected in the mouse and chicken c-*myb* genes, primary human c-*myb* messages exhibit extensive 5' heterogeneity (Figure 13). Some of the start sites are common to both the CCRF-CEM and K562 cell lines; other sites are unique for each cell line. This result is consistent with the cell-type specificity seen in the 5' heterogeneity of the murine and chicken c-*myb* gene (Kim and Baluda, 1989; Watson et al., 1987).

In addition to the start sites which map to the primary promoter, a cluster of three transcriptional start sites which map close to the 5' end of the pMbm-2 message was detected (Figure 13, lower three arrows). However, identification of these sites as specific to a promoter within intron 1 was

obscured by the multiple transcriptional start sites originating from exon 1. Therefore, RNAse protection analysis was used to verify the presence of transcriptional start sites within intron 1. RNAse protection analysis confirmed the existence of a cluster of three transcriptional start sites within thirty bases of exon 2 in cell lines expressing c-*myb*. Based on this evidence, there is a TATA-less promoter at the 3' end of c-*myb* intron 1.

The regulation of stable transcripts arising within intron 1 was studied by comparing transcriptional start site usage within intron 1 with primary c-*myb* expression in various myeloid and lymphoid leukemia cell lines. In all cell lines tested, the levels of c-*myb* messages which are initiated within intron 1 are proportional to those initiated from exon 1. Due to the biphasic nature of DMSO-induced HL-60 differentiation, we were curious as to the level of intron 1 transcripts during the early stages of differentiation; if levels of intron 1 transcripts remained constant during the initial stages of differentiation, a role for intron 1-originating transcripts in differentiation could be surmised. However, we observed that, as seen in PDbu-induced U937 differentiation, stable message levels arising from within the intron varied proportionally with normal c-*myb* message levels.

Functional studies using the 3' portion of intron 1 fused to the reporter gene chloramphenicol acetyl transferase (CAT) demonstrates the ability of this region to function as a promoter. Deletion studies reveal that the promoter activity is influenced both by positive and negative regulatory elements in a cell

type specific manner. A transcription factor database scan of the putative regulatory region revealed a *Myb* binding site. Given the recent evidence that c-*myb* may be autoregulatory (Nicolaides et al., 1991), it is interesting to speculate that the intron 1 promoter may be regulated by a related mechanism.

A number of facts supports the proposition that the promoter activity derived from the intron 1 DNA in transient transfection assays represents an *in vivo* promoter. RT-PCR analysis of transfected CCRF-CEM cells verified that the mRNA start site utilized by the promoter constructs corresponds to the pMbm-2 message and do not map upstream of this site; this result suggests that the CAT constructs utilize the *in vivo* mRNA start site which corresponds to the pMbm-2 message and RNAse protection studies, this correlating promoter activity in this region with *in vivo* mRNA start sites. Furthermore, tumor derived mRNAs which contain 5' deletions utilize the ATG start site in exon 2 to produce an in-frame, amino-terminally deleted protein in the same manner predicted for the pMbm-2 message (Pizer et al., 1992).

To determine if the positive and negative regulatory *cis* elements could be correlated with the binding of cell-type specific nuclear factor, portions of intron 1 were tested in the electrophoretic mobility shift assay (EMSA). Two regions of intron 1 were found to bind to nuclear extracts of CCRF-CEM and K562 and were not competed for by non-specific competitors (Figure 23). One region, encompassing fragment III, contains a *Myb* responsive element (MRE). However, because binding of this fragment to nuclear factor could not be

competed by specific competitor, its role in the regulation of the intron 1 promoter is not clear. The second region, encompassing fragment IV, bound specifically to both K562 and CCRF-CEM nuclear extracts (Figure 24). A scan of this region reveals no known potential transcription factor binding sites. However, the specific binding of this fragment corresponds to the region of positive regulation detected in the CCRF-CEM transient transfection studies (Figure 19). Suggesting that nuclear extract binding in this region may effect the intron 1 promoter activity.

An important question remains, therefore, as to how proportional levels of primary promoter versus intron 1 initiating transcripts are maintained. In transient CAT assays, the intron 1 promoter is influenced differentially by positive and negative regulatory elements in different cell lines. Transcription from the upstream promoter is regulated primarily by attenuation. There has been no evidence for the presence of a second attenuator downstream from exon 2. Therefore, although the relative levels of transcripts from the two promoters is the same, the exact mechanism of regulation of transcript levels must be different for transcripts arising from the two promoters. It is possible that *cis* or *trans* factors which are necessary for attenuation are also necessary to repress the intron 1 promoter. This possibility may explain the quantitative differences seen between the binding of fragment IV to the two lymphoid and myeloid nuclear extracts in the EMSA. Less fragment IV is bound to K562 nuclear extracts; the lack of binding may prevent transcription through this

region. Likewise, the greater amount of probe bound to CCRF-CEM nuclear extracts may allow for the greater transcriptional read through in this cell line. Furthermore, a second promoter identified in the c-*myc* gene has been shown to be important in the regulation of attenuation in that gene; binding of nuclear factors and deletions or mutations at this second promoter effect the attenuation of transcription in this gene (Miller et al., 1989; Dufort et al., 1993). Thus, the binding (or release) of these factors to intron 1 could serve a dual role in c-*myb* regulation: attenuation factors and promoter repressors. Alternatively, post-transcriptional mechanisms may function to maintain the proportional transcript balance observed between different cells lines and in models of myelo-monocytic differentiation such as HL-60 or U937.

A recent study in which proviral insertions into the avian c-*myb* gene were found to induce B-cell lymphomas revealed that the majority of proviral insertions which caused the disease occurred within the last 1 kb of intron 1 of the chicken c-*myb* gene (Pizer et al., 1992). The region of integration corresponds to both fragments III and IV in the EMSA (Figure 22). Given that this region of the intron was conserved between chicken and human, it is possible that sequences near the 3' end of the intron may be necessary for regulation of c-*myb* expression in both chickens and humans. Alternatively, integrations within this area may disrupt normal regulation of the downstream promoter.

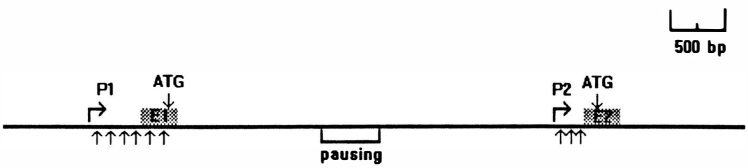
The presence of a promoter downstream from the site of translation

initiation in exon 1 of the human c-*myb* proto-oncogene (Figure 33) raises speculation as to the function and role of N- terminal truncated proteins whose messages originate from this region. Transcription of the pMbm-2 message, which also contains an additional exon leading to C- terminal protein truncation, was apparently initiated from the intron 1 promoter. The opposing role of this alternatively spliced form of c-*myb* in FMEL cell differentiation (Weber et al., 1990) suggests an opposing role for this form of c-*myb* in the differentiation process. The relative contributions of the N- and C- terminal truncations to promotion of FMEL cell differentiation is currently unknown. Recent studies have shown that both N- and C- terminal deletions of *Myb* can affect the transactivation ability of this protein (Dubendorff et al., 1992). Furthermore, the unique 26 bp 5' sequence of the pMbm-2 message results in the loss of 20 amino acids at the N terminus of the protein, assuming that translation starts at the first coding ATG of the message (see Figure 8). (This ATG was used as the translational start sit for *Myb* proteins in the RAV-1 insertional mutagenesis studies mentioned above.) This deletion occurs in a region that has been shown to contain a casein kinase II phosphorylation sensitive element which inhibits sequence-specific DNA binding of the c-*myb* protein when phosphorylated (Lüscher et al., 1990; Lüscher and Eisenman, 1992). The phosphorylation status of *Myb* during differentiation is unknown. If phosphorylation is a mechanism used to inhibit *Myb* binding during differentiation, it is possible that by deleting this phosphorylation site, the

pMbm-2 protein and/or N-terminal related proteins are able to bind DNA preferentially as differentiation occurs. Alternatively, this amino-terminally truncated c-*myb* protein may have a greater transactivation efficiency than full length c-*myb*, as has been demonstrated with yeast *Myb* proteins (Punyammalee et al., 1991). Therefore, further studies of the *in vivo* expression and post-translational modifications of these and other alternatively spliced c-*myb* proteins may give additional insight as to their role in the regulation of hematopoiesis.

Although chromosome 6q abnormalities have been associated with a number of malignant processes, and many of these abnormalities map within proximity of the c-*myb* locus, a direct role of c-*myb* rearrangements or deletions in these cancers has not previously been demonstrated. We have identified a rearrangement in the c-*myb* promoter of the acute lymphoblastic leukemia cell line CCRF-CEM. This is the first report of such an event in human leukemia despite a number of studies in which attempts were made to detect c-*myb* rearrangements in 6q- leukemias and lymphomas (Park and Reddy, 1992; Barletta et al., 1987). Hybridization of the MRR sequence to somatic cell hybrids containing chromosome six and the loss of c-*myb* promoter sequence upstream of the rearrangement indicates that this is a 6q interstitial deletion; the linkage of the MRR and c-*myb* sequences on a 450 kb fragment indicates that the deletion is small by cytogenetic standards. A similar rearrangement of c-*myb* was also detected in another T-cell acute lymphocytic leukemia. Since

Figure 33. Schematic representation of the two promoter regions in the human c-*myb* gene. Upward arrows are a representation of the multiple transcription start sites for the primary (P1) (Bender and Kuehl, 1986) and intron 1 (P2) promoters. Downward arrows indicate potential ATG translation start sites within exon 1 (E1) and exon 2 (E2). The 0.5 kb region of transcriptional pausing (Reddy and Reddy, 1989) is also indicated.


500 bp
P1
ATG
E1
P2
ATG
E2
pausing

there is evidence of cell-line specificity to the 5' heterogeneity in c-*myb* messages (Kim and Baluda, 1989; Watson et al., 1987) it is possible that the deleted region may be important in the regulation of c-*myb* expression in T-cells. The MRR junction sequence was not detected as altered in any other cell lines or leukemias; the limited size of the probe may have precluded detection of similar rearrangements in this locus. Genomic DNA screenings with probes derived from other regions of the normal MRR locus may yield further rearrangements in the MRR region.

Due to the deletion, a portion of the c-*myb* promoter which is evolutionarily conserved between the human and mouse promoters is removed. However, the deletion has no effect on the intrinsic activity of the promoter in proliferating CCRF-CEM cells, nor does it remove the *myb* responsive elements (MREs) which have been suggested to have an autoregulatory effect on c-*myb* expression (Nicolaides et al., 1991). The effect of this deletion on the regulation of c-*myb* expression is therefore unclear. Given that deletions in promoter sequences have been demonstrated to affect attenuation in the c-*myc* gene (Dufort et al., 1993), it is possible that deletions in the c-*myb* promoter may affect attenuation in this system as well. Alternatively, the conserved region of the c-*myb* promoter that is deleted in CCRF-CEM may play a role yet to be defined in cell type or differentiation state specific promoter control of c-*myb* expression.

We have also identified a portion of the sequence which is lost in the

CCRF-CEM deletion. Analysis of the sequences flanking the 5' junction of the deletion revealed that a portion of the DNA lost in the rearrangement included a potential exon with a high degree of homology to the *myc*L2 gene, a processed, X-linked member of the *myc* oncogene family which shares a high degree of homology to the L-*myc* (*myc*L1) gene (Morton et al., 1989). This gene is expressed almost exclusively in human adult testes (Robertson et al., 1991). The identity seen with the MRR sequence occurs in the 5' untranslated region of both *myc*L2 and the *myc* pseudogene p-*myc*. The consensus splice donor and acceptor sites flanking the region of identity indicate that the sequence may represent an exon. Alternatively, the region may be important in regulation of another currently unidentified gene.

It is unknown whether the MRR region of identity corresponds to a processed message; a preliminary screen of cell lines did not detect expression of this sequence as a processed mRNA. Given that it has been postulated that regions surrounding the c-*myb* locus have been implicated in the development of leukemia and other malignant disorders and may contain a tumor suppressive or c-*myb* suppressive gene (Park and Reddy, 1992; Hubbard Smith et al., 1992), it is possible that the *myc*L2 like sequence identified in these studies may correspond to a tumor suppressive gene. Two members of the *myc* oncogene family have been demonstrated to have tumor suppressor activity. S-*myc*, another processed gene, has been isolated from the rat. Transfection of this gene into rat tumor cells has been found to suppress the tumorigenicity of these

cells in nude mice (Kuchino et al., 1989; Sugiyama et al., 1989). In addition, the B-*myc* protein shares homology with the transcriptional activation domain of c-*myc* but does not share identity in the c-*myc* DNA binding domain; B-*myc* can inhibit neoplastic transformation and transcriptional activation by c-*myc* (Resar et al., 1993). Therefore, the identification of sequences deleted in this acute lymphoblastic leukemia cell line and linked to the c-*myb* gene may provide additional information and probes for the analysis of sequences lost in 6q- abnormalities detected frequently in leukemias and lymphomas.

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