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Mechanisms of Regulation of the Human c-myb Proto-oncogene During Myelomonocytic Differentiation

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Mechanisms of Regulation of the Human c-*myb* Proto-oncogene During Myelomonocytic Differentiation.

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

by

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Bachelor of Science, Philadelphia College of Pharmacy and Science, 1986

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DEDICATION

To my mother for comprimising her lifestyle to benefit that of her three children.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Mechanisms of Regulation of the Human c-*myb* Proto-oncogene During Myelomonocytic Differentiation.

ABSTRACT

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

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Control of hematopoiesis is a complex set of events that is currently being dissected at the molecular level. To determine factors that may be crucial for commitment to terminal differentiation of myelomonocytic cells, a mutant of the HL-60 cell line was characterized at the cellular and molecular level. This clone, termed DMSOr, was shown to differentiate in a similar fashion as parental HL-60 in response to 1.3% DMSO at the morphologic and functional level. The anti-proliferative aspects of differentiation were also present in DMSOr as evidenced by decreased ^{3}H-thymidine incorporation and an increased percentage of cells in the G_0/G_1 phase of the cell cycle. All of these phenotypic changes induced in DMSOr would revert if the DMSO was removed at any

point during the differentiation process, thus DMSOr, despite its ability to functional differentiate, could not commit to terminal differentiation.

Associated with the altered phenotype of DMSOr was the altered expression of the proto-oncogene c-*myb*. Expression of c-*myb* remained detectable at 144 hrs of DMSO treatment in DMSOr but not HL-60. Similar findings were shown for the cell cycle other related genes. This altered gene expression did not extend to the c-*myb* related gene B-*myb* . The possibility that altered transcriptional regulation of c-*myb* was eliminated by nuclear run-on analysis and by the fact that a splice variant of c-*myb* with an altered 3' untranslated region showed no altered regulation. Thus the genetic defect in DMSOr may be in a global control factor for cell cycle related genes such as c-*myb*. This factor may regulate these genes at the post-transcriptional level.

To determine the mechanisms of regulation of c-*myb* during hematopoietic cell differentiation, transcriptional and post-transcriptional studies of c-*myb* following treatment of HL-60 cells with various differentiation inducers were undertaken. Retinoic acid and vitamin D3 regulated c-*myb* at the transcriptional level via an attenuator, while DMSO and phorbol dibutyrate activated multiple mechanisms of regulation. These included attenuation and a post-transcriptional regulation that was dependent on continuous transcription, but not translation in the case of DMSO. Phorbol ester regulation of c-*myb* occurred at the level of an attenuator and possibly a promoter at the transcriptional level. In addition there was post-transcriptional control of c-*myb* by phorbol dibutyrate

that differed from the regulation by DMSO through the lack of transcriptional dependency. Thus c-*myb* is regulated at the transcriptional and the post-transcriptional level in an agent specific fashion during HL-60 differentiation.

A 2.4 kb message is present in the Northern blots probed for c-*myb* expression. This lower molecular weight message is regulated in an abberrant fashion compared to normal message during HL-60 differentiation. Probing of blots with different regions of a full length c-*myb* cDNA and primer extension analysis suggest that the 2.4 kb message may start in the exon of the c-*myb* locus.

INTRODUCTION

The process of hematopoietic cell differentiation is a complex set of events whereby a multipotent progenitor cell of the bone marrow is capable of differentiation to produce the cells required for a functional immune system, oxygen transport and hemostasis. Associated with these changes in function of differentiated cells is the loss of self renewal capacity due to withdrawal from the cell cycle. The differentiation process has "two arms," an antiproliferative arm and the functional/morphological arm. The mechanisms that control these arms of differentiation are extracellular and intracellular signals which are only now being delineated. The extracellular signals include the family of Colony Stimulating Factors (CSF), IL-3 and Stem Cell Factor (SCF). These and other factors convey their differentiation signals to the nucleus via receptor-coupled signal transduction mechanisms which cause changes in the expression of the genes required for defining the phenotypic and proliferative state of the cell. The control of the genes which are important for the phenotypic and proliferative state of the cell have been suggested to be under multiple controlling mechanisms. This is due to the fact that in certain leukemias, such as plasmacytomas, the cells maintain their mature phenotypic features, yet retain the ability to proliferate. The genes that control the proliferative or functional state of the cell are

regulated by specific transcription factors, such as the products of the nuclear, DNA binding class of proto-oncogenes. Many forms of leukemia are the product of the deregulation proto-oncogenes. It is important to understand how these proto-oncogenes are regulated to gain further insight into the control of hematopoiesis and the etiology of leukemia.

To study the processes of gene regulation during human hematopoietic cell differentiation, *in vitro* models of differentiation must be utilized. The HL-60 human promyelocytic leukemia cell line provides an excellent model of differentiation because of its ability to differentiate to a variety of end-stage cells including cells of myeloid and monocytic lineage. The differentiation of HL-60 is induced by a variety of agents, many which are hematopoietic growth factors or activate the signal transduction systems which these factors utilize. Thus gene regulation during differentiation to myeloid or monocytic differentiated cells through the activation of signal transduction systems can be studied.

HL-60 also offers the unique of advantage of *in vitro* models, in that differentiation resistant variants of the parental cell line can be obtained. The procedure for obtaining differentiation resistant mutants is to grow the cells on low levels of the differentiation agent, and to slowly increase the concentration to near maximal differentiation levels. This process will select for cells which are resistant to the induction of differentiation as evidenced by the continued ability to proliferate. These lines can then be characterized at the molecular level to determine what defect is present that may be associated with the altered phenotype.

A prime candidate for a controlling factor in determining the differentiation state of the cell during hematopoiesis is the gene product(s) of the c-*myb* proto-oncogene. This proto-oncogene encodes a transcription factor which, when altered via deletions present in the viral form of the gene, can transform avian hematopoietic cells. A unique feature of v-*myb* is that this truncated form of the gene cannot transform NIH3T3 cells, therefore its action has been considered somewhat specific to the hematopoietic cell system. If c-*myb* encodes a transcription factor, which when deregulated can cause leukemia, then it is important to understand mechanisms that regulate the c-*myb* gene during hematopoietic cell differentiation. To this point all studies that have been published on the regulation of c-*myb* during hematopoietic cell differentiation have been in chicken and murine models. Studies of the human c-*myb* gene have been limited to measurements of changes in steady state levels of mRNA.

The objective of this thesis project were three-fold: i) to characterize a variant of the DMSOr subclone of HL-60 at the cellular level and to determine if altered regulation of c-*myb* in DMSOr may play a role in the unique phenotype of this subclone, ii) to determine the mechanisms of control of the human c-*myb* oncogene during hematopoietic cell differentiation and iii) to utilize HL-60 and DMSOr to further characterize a lower molecular weight c-*myb* related mRNA that is present and which exhibits altered expression patterns from the prototypical c-*myb* message.

LITERATURE REVIEW

Hematopoiesis

Hematopoiesis is the process of supplying and replenishing the body with cells that will carry out the functions of the innate and acquired immune systems, oxygen transport and hemostasis. The majority of the cells required for these functions have lifespans that range from hours to weeks, thus the hematopoietic system must be constantly resupplying the blood with functional cells. This is accomplished through the ability of the plouripotent stem cell of the bone marrow. This cell is unique in that it is capable of self-renewal and can be stimulated to differentiate via multiple pathways to provide all the functional cells of the above mentioned systems. These pathways are initially marked by a committed progenitor cell for that branch of hematopoietic differentiation. The committed progenitor can differentiate through various intermediates to the end-stage cells of that arm of differentiation (Fig. 1). These branches of differentiation are the erythroid branch, the megakaryocytic branch, the lymphoid branch, and the myeloid branch.

Erythroid differentiation provides the erythrocytes which are required for oxygen transport. The erthyroid cells, along with the megakaryocytes which ultimately provide platelets, are derived

from the same precursor cell as the cells of myeloid origin. This progenitor cell (CFU-GEMM) will differentiate to a cell which is committed to becoming either a platelet-providing megakaryocyte, erythrocyte or myeloid cell. The myeloid committed precursor may differentiate to multiple end-stage cells. These cells fall into two classes, monocytic cells and granulocytic cells. The monocytic cells are the blood monocytes and tissue macrophage cells which function in both the innate and acquired immune systems. The granulocytic cells can be further subdivided into three cell types. The most common of these cells is the neutrophil, with differentiation occurring to a lesser extent to basophil and eosinophil forms. These are the majority of cells that make up the cellular portion of innate immunity.

The lymphoid committed progenitor cell is provided directly from the pluripotent stem cell. The differentiation of these committed precursor cells provide the immature B and T lymphocytes which must migrate to the mammalian Bursa equivalent or to the thymus respectively for further development. Mature B lymphocytes, upon stimulation will differentiate to antibody secreting plasma cells to drive the humoral arm of acquired immunity, while mature T lymphocytes are divided into regulatory cells, such as helper or supressor cells, or they may become cytotoxic cells that are the active cells of the cell-mediated arm of acquired immunity.

Hematopoietic Cell Growth Factors

Figure 1. Schematic diagram of Hematopoiesis. The diagram illustrates the ability of the pluripotent stem cell of the bone marrow to differentiate through myeloid and lymphoid precursors to the functional cells of oxygen transport, hemostasis and of the innate and acquired immune systems. Differentiation to end stage cells is through a series of committed progenitor cells such as the CFU-GEMM, the blast cells and the non-mitotic cells such as the megakaryocyte, the proerythrocyte and the B cell.

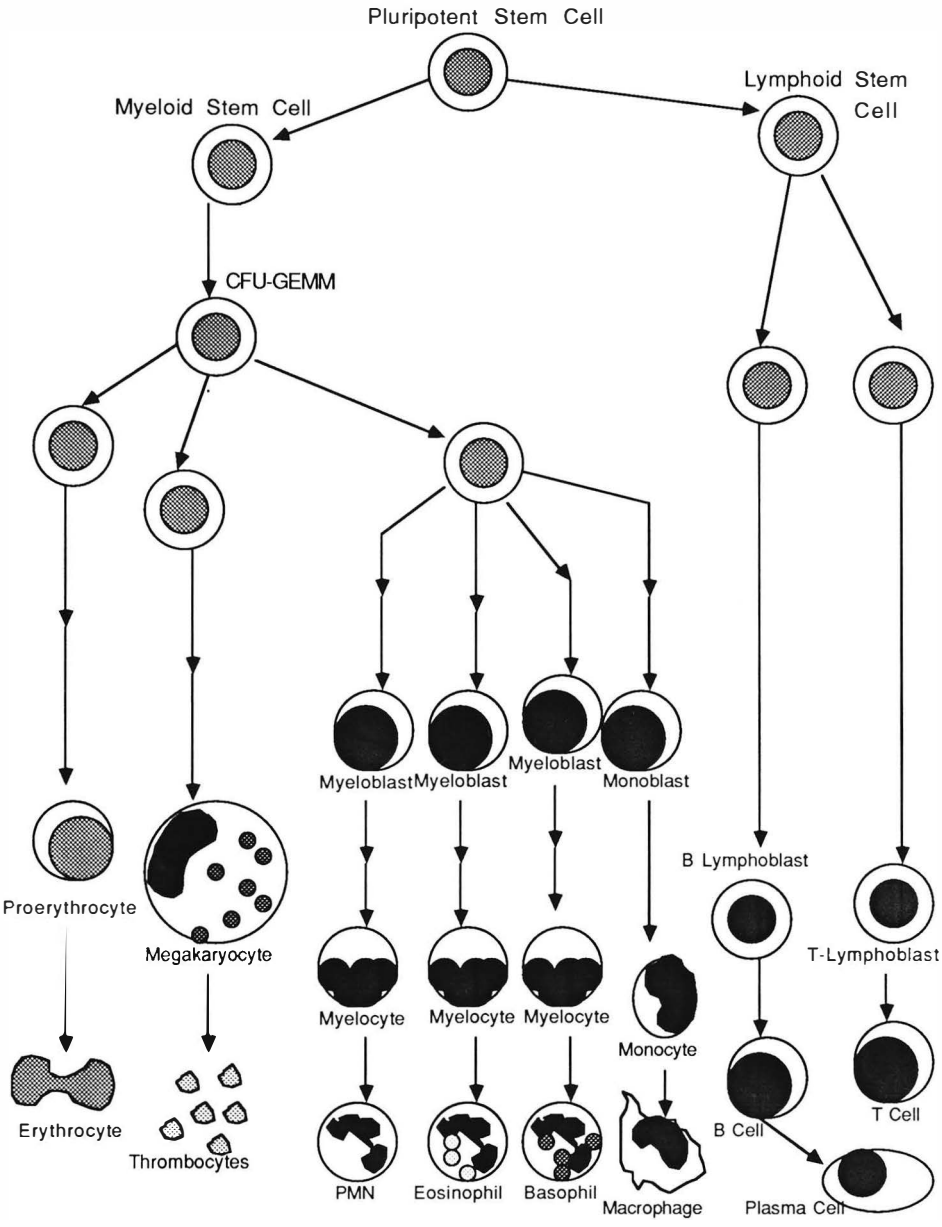
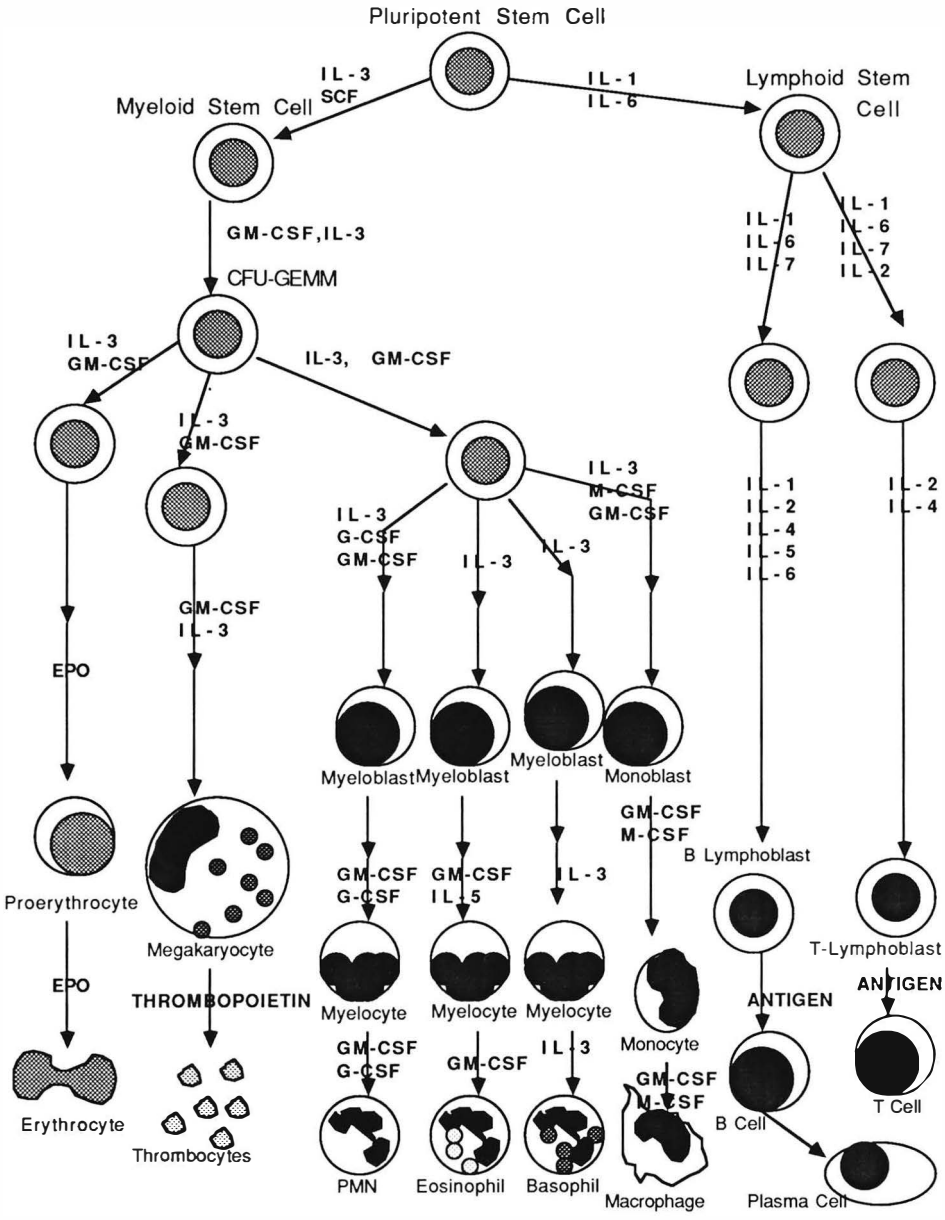

Pluripotent Stem Cell
Myeloid Stem Cell
Lymphoid Stem Cell
CFU-GEMM
Myeloblast
Myeloblast
Myeloblast
Monoblast
Proerythrocyte
Megakaryocyte
B Lymphoblast
T-Lymphoblast
Myelocyte
Myelocyte
Myelocyte
Monocyte
Erythrocyte
Thrombocytes
B Cell
T Cell
PMN
Eosinophil
Basophil
Macrophage
Plasma Cell

Figure 2. Colony-Stimulating Factors (CSFs) and other growth factors involved in hematopoiesis. Differentiation of stem cells, committed progenitors and blast cells requires the external stimuli of growth factors(or antigen in the lymphoid branch). The activity of many of these growth factors is depicted in this schematic representation of hematopoiesis. The general factors such as Stem Cell Factor (SCF), Interleukin 3 (IL-3), Interleukin 6 (IL-6) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) are active in the early stages of differentiation. Specific factors like Granulocyte Colony-Stimulating Factor (G-CSF), Macrophage Colony-Stimulating Factor (M-CSF), erythropoietin (EPO) and thrombopoietin in addition to many of the general factors are active in the latent stages of hematopoieis.


Pluripotent Stem Cell
IL-3
SCF
IL-1
IL-6
Myeloid Stem Cell
Lymphoid Stem Cell
GM-CSF,IL-3
CFU-GEMM
IL-1
IL-6
IL-7
IL-1
IL-6
IL-7
IL-2
IL-3
GM-CSF
IL-3, GM-CSF
IL-3
GM-CSF
IL-3
M-CSF
GM-CSF
IL-3
G-CSF
GM-CSF
IL-3
IL-3
IL-1
IL-2
IL-4
IL-5
IL-6
IL-2
IL-4
GM-CSF
IL-3
EPO
Myeloblast
Myeloblast
Myeloblast
Monoblast
GM-CSF
M-CSF
B Lymphoblast
T-Lymphoblast
Proerythrocyte
Megakaryocyte
GM-CSF
G-CSF
GM-CSF
IL-5
IL-3
EPO
THROMBOPOIETIN
Myelocyte
Myelocyte
Myelocyte
Monocyte
ANTIGEN
ANTIGEN
GM-CSF
G-CSF
GM-CSF
IL-3
GM-CSF
M-CSF
Erythrocyte
Thrombocytes
PMN
Eosinophil
Basophil
Macrophage
B Cell
T Cell
Plasma Cell

The growth and differentiation of the stem cell and lineage specific progenitors is stimulated by soluble and membrane associated growth factors. Many of these factors are produced by cells in the bone marrow, providing a microenvironment consisting of predominantly paracrine and some autocrine stimulation while others, such as the red cell stimulating erythropoietin, are synthesized in the kidney(Sherwood and Goldwasser, 1978). The autocrine activity was initially recognized in *in vitro* clonigenic assays of normal bone marrow cells. Different soluble factors could stimulate the formation of colonies of cells from single precursors(Clark and Kamen, 1987). The colonies would contain cells which had acquired characteristics of a mature cell type. Thus, the soluble factors were stimulating growth and differentiation of the progenitor cells. The factors were named by their ability to form colonies of cells with specific phenotypic features. These Colony Stimulating Factors (CSF) as well as other hematopoietic growth and differentiation factor activities are shown in Fig. 2.

Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF is an 18-24 kd glycoprotein that is produced by a variety of cells including activated peripheral blood lymphocytes (PBL)(Welte et al., 1985), mature T cell lines(Welte et al., 1985) and non-immune tissues(Clark and Kamen, 1987). GM-CSF is capable of stimulating progenitor cells to differentiate to either mature granulocytes or macrophage cells(Metcalf, 1986). In clonigenic assays, GM-CSF stimulated clones will yield colonies that contain both macrophages and neutrophils. The percentage of macrophage

cells in the colony can be influenced through the addition of other CSFs(Metcalf, 1986).

Granulocyte Colony Stimulating Factor (G-CSF).

G-CSF is also a glycoprotein (19 kd) that is produced by cells in and outside of the bone marrow(Wong et al.1986,). G-CSF is named for its ability to stimulate formation of colonies of cells of granulocytic character(Metcalf and Nicola, 1983), although G-CSF is also capable of stimulating the formation of erythroid, macrophage and megakaryocytic colonies to a lesser extent(Wong et al.1986,).

Macrophage Colony Stimulating Factor (M-CSF).

M-CSF (also known as CSF-1) is a heavily glycosylated 45 kd protein dimer that specifically stimulates macrophage committed progenitor cells(Das et al., 1981). The receptor for M-CSF is a tyrosine kinase that is related to the product of the c-*fms* proto-oncogene(Sherr et al., 1985).

Multi-Colony Stimulating Factor (Multi-CSF).

Multi-CSF (also known as IL-3) interacts with early progenitors based on its ability to support the proliferation and differentiation of all the classes of committed progenitors(Yang et al., 1986). Multi-CSF is a 23-28kd glycoprotein that is produced by T cells(Yang et al., 1986).

Stem Cell Factor (SCF).

SCF (also known as Mast Cell Factor) is the ligand for the receptor encoded by the c-*kit* proto-oncogene(Witte, 1990). The factor is encoded in the steel locus of the mouse and is produced by hematopoietic and non-hematopoietic tissues(Zsebo et al., 1990b; Williams et al., 1990; Huang et al., 1990). SCF is capable of stimulating the growth of many hematopoietic cell types including mast cells(Williams et al., 1990; Huang et al., 1990). SCF has moderate effects on myeloid, erythroid and lymphoid cell growth and differentiation by itself, but can also synergize with lineage specific factors(Zsebo et al., 1990a). SCF is expressed as a membrane associated protein or as a soluble molecule, of each which can stimulate cell growth and differentiation(Huang et al., 1990).

Myeloid Differentiation: Morphology and Function.

Differentiation of myeloblasts to end-stage cells such as neutrophils occurs in the bone marrow over a 1 week period. The early or mitotic phase of development consists of differentiation through myeloblasts, promyelocytes and myelocytes. As the cells progress through the pathway a loss of proliferative potential occurs until they reach the non-mitotic phase of differentiation. The metamyelocytes, band cells and mature PMNs make up the late or non-mitotic phase of myeloid differentiation (Fig. 3).

Myeloblast

The myeloblast is the most immature white cell that can be distinguished at the morphological level. These cells have large

round nuclei that are located in the center of the cell. The Wright-Giemsa stain of these cells also reveals prominent nucleoli and a blue cytoplasm that contains no visible granules.

Promyelocyte

The promyelocyte is similar to the myeloblast in its morphology, except for the appearance of dark staining primary granules, which contain the product of the *mim*1 gene(Ness et al., 1989). These granules also contain degradative enzymes such as lipases, proteases, mannodases and glucosidases. The function of these granules is not known.

Myelocyte

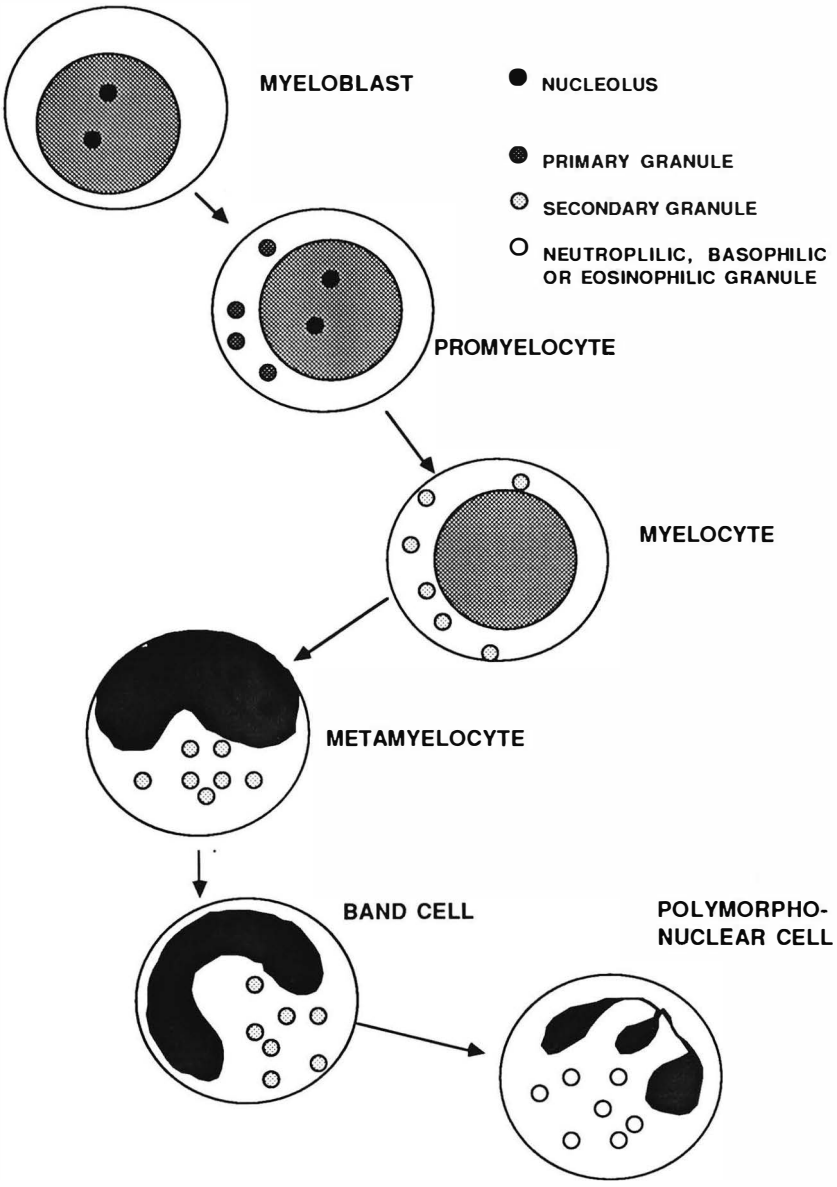
The nucleus of myelocytic cells is round but the nucleoli are no longer present. The primary granules that were present in the promyelocyte are also no longer present. These granules are replaced by secondary granules. These granules contain lysozyme and lactoferrin.

Metamyelocyte

The nucleus of the metamyelocyte is condensed into a "kidney bean" shape that is offset to one side of the cell. The secondary granules remain present in these cells. The Wright-Giemsa staining of the cytoplasm is grey instead of the blue of the mitotic cells.

Band Cell

Figure 3. Myeloid differentiation. This schematic details the differentiation from a myeloblast to a polymorphonuclear cell. The myeloblast is a mitotic cell with prominent nucleoli (black dots) and no cytoplasmic granules. The promyelocyte is morphologically similar to the myeloblast although primary granules are now visible in the cytoplasm. The promyelocyte is also a proliferative cell which contains detectable nucleoli. The myelocyte is a proliferative cell, but not to the same extent as its precursor cells. The nucleus is similar to that of a promyelocyte, with the exception of the nucleoli, which are no longer present. The primary cytoplasmic granules are replaced by secondary granules. The metamyelocyte and band cell are non-mitotic cells with condensed nuclei. These cells also have secondary granules. The polymorphonuclear cell has a segmented nucleus and granules that are specific for the cell type; either neutrophilic, basophilic or eosinophilic.


MYELOBLAST
NUCLEOLUS
PRIMARY GRANULE
SECONDARY GRANULE
NEUTROPLILIC, BASOPHILIC OR EOSINOPHILIC GRANULE
PROMYELOCYTE
MYELOCYTE
METAMYELOCYTE
BAND CELL
POLYMORPHO-NUCLEAR CELL

The band cell represents a continued maturation of the metamyelocyte, with continued condensation of the nucleus into a "crescent moon" shape. The cytoplasm and its granules are similar to the metamyelocyte.

Polymorphonuclear Leukocyte

This fully mature cell contains a lobular nucleus, and many granules in the cytoplasm. Neutrophils, eosinphils and basophils contain granules that are colorless, red or blue respectively when the cells are stained with Wright's stain. These cells function in the innate immune system by producing enzymes and chemicals which are bactericidal. These chemicals such as superoxide anion are produced in response to bacterially elements such as formylpeptides. The enzymes are located in the granules which also contain enzymes found in the secondary granules previously mentioned. PMNs also possess phagocytic capabilities.

Monocytic Differentiation: Morphology and Function.

The blood monocytes and tissue macrophages also differentiate from myeloblast cells in the bone marrow. This differentiation is through an intermediate stage cell, the promonocyte and takes 3-4 days for maturation to occur. Monocytic cells are phagocytes which function in both innate and acquired immunity. The macrophage is capable of processing phagocytized antigen so it can be presented to lymphocytes for activation of a humoral immune response. Monocytes and macrophages also have a gray, granule containing, cytoplasm and

indented nucleus when visualized with Wright's stain. These cells are also characterized by their *in vitro* characteristic of adherence to glass or plastic.

In Vitro Models of Hematopoietic Cell Differentiation.

To perform experiments designed to investigate how hematopoiesis is controlled at the molecular level, *in vitro* models of hematopoietic cell differentiation must be utilized. These models are usually cell lines derived from various forms of leukemia. Many of these tumor cell lines will exhibit the phenotypic characteristics of the normal immature cells prior to transformation. Along with maintaining some of the normal cell's phenotype, the tumor cell lines also retain the normal cell's responsivness to differentiation inducing stimuli.

Some examples of commonly utilized models of differentiation include: HEL(Martin and Papayannopoulo, 1982) and Friend Murine Erythroleukemia (FMEL)(Friend et al., 1971) lines which are human and murine erythroblast cell lines which are capable of differentiating via the erythroid lineage to nucleated red cells; K562 is derived from a human Chronic Myelogenous Leukemia, but is capable of differentiation through erythroid pathways(Anderson et al., 1979); KG-1(Koeffler and Golde, 1978) and ML-1(Takeda et al., 1981) behave in a fashion similar to human myeloblastic cells in culture and are capable of differentiation via myelomonocytic pathways; and HL-60(Collins et al., 1977) is derived from a human promyelocytic leukemia and can be stimulated to differentiate to myeloid or monocytic end-stage cells.

HL-60.

HL-60 was derived from a female patient with acute promyelocytic leukemia(Collins, 1987). A unique feature of HL-60 was its ability to grow in suspension culture in RPMI 1640 medium that was supplemented with only insulin and transferrin(Breitman et al., 1980a). In culture, the cells resemble the original patient sample, which appeared histochemically as promyelocytes. Karyotype analysis revealed chromosomal deletions (5 and 8) and aberrancies of the E chromosome group.

As previously mentioned, the cells resemble promyelocytes in culture and grow as if they are frozen at this stage in development. This is evidenced by the doubling time of 24 hours in liquid culture and the high cloning efficiency in soft agar. Histochemically, HL-60 is positive for primary granules which contain esterase and myeloperoxidase(Collins et al., 1977). HL-60 is not considered to be a normal promyelocyte since it does lack some of the features of normal cells (see Table 1), but is capable of spontaneous or stimulated differentiation via myeloid or monocytic pathways.

Differentiation of HL-60.

Approximately ten percent of HL-60 cells will spontaneously differentiate to mature myeloid or monocytic cells (see Table 1). This percentage can be enhanced dramatically through the use of differentiation inducing agents. These agents include solvents(Collins et al., 1978), cancer chemotherapeutics(Griffin et al., 1982), protein kinase activators(Rovera et al., 1979) and physiologic

Table 1. Characteristics of HL-60

Characteristic	Uninduced	Granulocyte	Monocyte
Myeloperoxidase	positive	decreased	decreased
Nonspecific esterase	negative	negative	positive
Acid phosphatase	positive	increased	increased
Plastic adherence	negative	negative	positive
Chemotaxis	negative	positive	positive
Complement receptors	positive	increased	increased
Fc receptors	positive	increased	increased
Lysozyme	positive	increased	increased
NBT reduction	negative	positive	positive
Phagocytosis	negative	positive	positive
Monocyte markers	+/-	negative	positive
Granulocyte markers	+/-	positive	negative

(derived from Collins, 1987)

Table 2. Inducers of HL-60 differentiation.

	Induced Cell Type	
	Granulocyte	Monocyte/Macrophage
Inducing agents	DMSO	Phorbol esters
	Retinoic acid	Vitamin D3
	Actinomycin D	Sodium Butyrate
	Hypoxanthine	Tumor necrosis factor
	6-thioguanine	Ara-C

(derived from Collins, 1987)

regulators of cell differentiation(Elias et al., 1980; Olsson et al., 1981; Olsson et al., 1984). A partial list of differentiation inducing agents is given in Table 2.

Dimethyl Sulfoxide (DMSO) Induced Differentiation of HL-60.

DMSO stimulates the induction of myeloid differentiation to neutrophilic band cells(Collins et al., 1978). Associated with these morphologic changes induced by DMSO are changes in the cell's functional capabilities. DMSO differentiated HL-60 cells acquire functional chemotactic receptors, including formylpeptide receptors(Niedel et al., 1980), increased production of lysozyme(Rovera et al., 1979), superoxide anion(Collins et al., 1979) and complement receptors(Collins et al., 1979) (see Table 1). These changes allow the cells to respond to bacteria in the same fashion as normal granulocytes(Shakarjian and Carchman, 1990).

Differentiation of HL-60 cells with DMSO occurs when the DMSO is at a concentration of approximately 1.25%. The cells reach maturity six days after the addition of DMSO. The DMSO must be present for at least the first 72 hours of differentiation for the cells to terminally differentiate. This is the point during the time course of DMSO treatment that the cells begin the post-mitotic phase of differentiation.

Phorbol Ester Induced Differentiation of HL-60.

The phorbol esters are a class of tumor promoter derived from the active portion of the oil from *Crotun tiglium*. This class of tumor promoters can stimulate cell growth or differentiation

through the pharmacologic stimulation of Protein Kinase C(Nishizuka, 1986). Differentiation of immature leukemia cell lines including HL-60 has been previously demonstrated(Rovera et al., 1979). Differentiation of HL-60 by phorbol esters will result in cells that resemble monocytes or macrophages in culture. This is evidenced by the adherence of phorbol treated cells to plastic or glass(Rovera et al., 1979), the ability of these cells to phagocytize antigens(Rovera et al., 1979) as well as increased esterase production(Rovera et al., 1979) (see Table 1).

Phorbol esters are potent inducers of HL-60 differentiation, requiring only 1 day for the complete differentiation process to occur. The concentration of phorbol ester needed for stimulation of HL-60 differentiation is in the micromolar range.

Retinoic Acid Induced Differentiation of HL-60.

Retinoic acid (RA; also known as vitamin A) is a lipophilic vitamin that interacts with a receptor of the steroid/thyroid class. Stimulation of the retinoic acid receptor results in a wide variety of cellular changes including differentiation of keratinocytes(Regnier and Darmon, 1989) neuroblastoma derived cells(Matsumoto et al., 1989) and leukemia derived cells(Douer and Koeffler, 1982; Thiele et al., 1988). For this reason, retinoic acid is currently used therapeutically for dermal lesions and in the treatment of Acute Promyelocytic Leukemia.

Retinoic acid is capable of stimulating the differentiation of HL-60 cells to neutrophilic band cells following treatment for six days with a 1 μM concentration of drug(Breitman et al., 1980b).

This makes retinoic acid the most potent of the natural differentiation inducers, although it is not as efficacious as DMSO for granulocytic induction of HL-60. It has recently been demonstrated that RA induced differentiation of HL-60 is via stimulation of the alpha form of the RA receptor(Collins et al., 1991). This may not be the only mechanism of action of RA, as it had been previously reported that RA that was covalently linked to beads could also stimulate HL-60 differentiation(Yen et al., 1984). This suggests that RA may also create membrane perturbations that could effect HL-60 differentiation.

Vitamin D3 Induced Differentiation of HL-60.

The activated form of Vitamin D3, 1-alpha, 25-dihydroxycholicalcerferol (1,25(OH)D3 or D3), is also an oil soluble vitamin that activates a receptor of the steriod/thyroid class. Once activated in the kidney, D3 has multiple actions throughout the body. These activities include stimulation of osteoclast differentiation and activity, differentiation of intestinal cells and effects on calcium and phosphorous transport and homeostasis(Minghetti and Norman, 1988).

Differentiation of HL-60 by D3 results in monocytic cells after six days of treatment with nanomolar concentrations of drug(Miyaura et al., 1981). Although this is as potent as phorbol ester, the differentiation is not as complete. Since nanomolar concentrations of D3 can be achieved in the blood, this compound is currently in clinical trials for treatment of leukemia.

Differentiation Resistant Forms of HL-60.

One method for studying mechanisms important in HL-60 differentiation that has been successful is the use of differentiation resistant mutants of HL-60. These mutants are generated by treating normal cells with low but increasing concentrations of a specific inducing agent. This method will select for spontaneously resistant cells by differentiating the sensitive ones. The remaining population of cells can be assayed for its ability to differentiate and can be recloned to homogenieity. These lines are often maintained on selective pressure, although if the resistance is stable there should be no need for continued selection.

Subclones of HL-60 have been isolated that are resistant to; phorbol ester(Fisher and Grant, 1985; Ely et al., 1987); 5-Azacytidine(Fisher and Grant, 1985); Retinoic acid(Fisher and Grant, 1985); and DMSO(Fisher and Grant, 1985). Reversibly differentiating HL-60 cells have been isolated that will differentiate in the presence of 1,25(OH2)D3, but will revert if the drug is removed(Brelvi and Studzinski, 1987). Many of these resistant and reversible cell lines exhibit altered morphologic and genetic features. These include growth of cells in small clusters, increased number of vacuoles, and altered karyotypes(Ely et al., 1987). Associated with these altered karyotypes has been a change in the expression of oncogenes like c-*myc* and c-*myb*(Ely et al., 1987; Brelvi and Studzinski, 1987).

Oncogenes

The term "oncogene" includes a growing number of cellular genes that are important in the control and regulation of cell growth a differentiation. This eclectic set of genes has been grouped under the name oncogene because of the effects of altered expression resulting in cell transformation, immortalization and tumor formation. The altered expression of proto-oncogenes is the result of; (i) transduction of mRNA of the cellular gene into a retrovirus; (ii) retroviral insertion into the genomic locus of the gene; (iii) amplification of the genomic locus; (iv) chromosomal rearrangement resulting relocation of the gene to a site of active transcription, e.g. an enhancer; (v) genes that contain mutational "hot spots" or, in the case of the "anti-oncogenes," the inactivation of the gene(Bishop, 1988). A list of specific examples of proto-oncogenes which are (de)activated by any of these mechanisms is presented in Table 3.

Genes that have important roles in regulation of cell growth can be oncogenic when normal control mechanisms are altered . It is conceivable that any class of growth regulating protein could be an oncogene if altered by one of the above mentioned mechanisms. This is exemplified by fact that there are proto-oncogenes which encode proteins that are localized to membrane, cytoplasm and nucleus.

Membrane associated proto-oncogenes include proteins normally involved in the first steps of signal transduction. These genes encode receptors, such as the c-*erb*-B gene which encodes the Epidermal Growth Factor receptor(Downward et al., 1984); the c-*fms*

gene which encodes the CSF-1 receptor(Sherr et al., 1985); the c-*trk* gene which encodes the Nerve Growth Factor receptor(Klein et al., 1991); and the c-*kit* gene which encodes the SCF receptor(Witte, 1990). Growth factors like the beta-chain of the Platelet Derived Growth Factor (PDGF) are also encoded by proto-oncogenes, in this case the c-*sis* proto-oncogene and the v-*sis* gene of Simian Sarcoma Virus(Robbins et al., 1983). In the case of receptors, many of the oncogenic mutations lead to production of a truncated protein that lacks the regulatory ligand binding region, therefore the receptors can become constitutively active.

Associated with many receptors are GTP-binding proteins (G-proteins). Gilman initially noted that there was significant sequence homology between the Gs protein of the Beta adrenergic receptor and the proteins encoded by the *ras* proto-oncogenes(Hurley et al., 1984). The *ras* family of proto-oncogenes encode proteins with the ability to bind GTP and GDP, as well as exhibiting intrinsic GTPase activity, but it is not known specifically which "G-protein" is encoded by any of the *ras* family members. The activation of any of the *ras* family members is via a mutation in codons 12 or 61 which inactivates the GTPase activity and therefore the ability of *ras* to return to an inactive state(Reddy et al., 1982; Tabin et al., 1982; Taparowski et al., 1982). A cellular protein called the GTPase Activating Protein (GAP) plays role in the GTPase activity of *ras*(McCormick et al., 1988). The GAP protein can interact with normal *ras* family members but not the oncogenic forms of the protein, and therefore may be an important regulator of cell growth(Trahey and McCormick, 1987).

Table 3. Activitation of proto-oncogenes.

Proto-oncogene	Neoplasm(species)	Lesion
abl	Chronic myelogenous leukemia (human)	Translocation
gip	Carcinoma of ovary and adrenal gland (human)	Point mutations
neu	Adenocarcinoma of breast, ovary and stomach (human)	Amplification
myc	Burkitt's lymphoma (human)	Translocation
	myeloid leukemia (chicken)	Transduction
L-*myc*	Carcinoma of lung (human)	Amplification
N-*myc*	Neuroblastoma; Small cell lung carcinoma (human)	Amplification
sis	Sarcoma (simian, feline)	Transduction
src	Sarcoma (chicken)	Transduction
myb	Leukemia (murine)	Insertion
	Leukemia (chicken)	Transduction

The third type of membrane associated protein that is encoded by proto-oncogenes are the non-receptor tyrosine kinases. The prototype for this family of genes is the c-*src* gene. C-*src* is the cellular homolog of the transforming gene of the Rous Sarcoma Virus, v-*src*(Parker et al., 1981). Many functions have been assigned to c-*src*, including roles in secretion(Ely et al., 1990), cell communication via desmasomes(Loewenstein and Azarnia, 1988), and possibly a role in insulin signalling(Luttrell et al., 1989). There are many *src* related genes that have specific regions of homology with c-*src*. Many of the *src*-related proteins have well defined roles in the signal transduction schemes of receptors of lymphocytes. These include; *lck*, which interacts with CD4 and CD8 receptors(Veillette et al., 1989; Viellette et al., 1988); *fyn*, which is the tyrosine kinase associated with the T cell receptor(Cooke et al., 1991) and *lyn*, which is a *src* related gene encoding the protein that interacts with immunoglobulin receptors on B cells(Yamanashi et al., 1991). One of the regions of homology between all of these proteins is called the SRC Homology 2 or SH2 region, which is important for interactions between proteins(Cantley et al., 1991). SH2 domains allow the tyrosine kinases to interact with non-tyrosine kinase containing proteins such as GAP(McCormick, 1989) and phospholipase C (∂)(Stahl et al., 1988).

The cytoplasmic localized proto-oncogenes are predominantly serine/threonine kinases which may be important in the movement of signals from the membrane to the nucleus. This is suggested by the fact that many of these cytoplasmic proteins have substrates in the nucleus. Examples of such proteins include the mitogen

activated protein kinase (MAP) which is encoded by the *erk* gene(Ely et al., 1990); the *raf* gene product(Rapp et al., 1988) and the *mos* gene product which is the cytostatic factor that is essential for maturation of the oocyte(Sagata et al., 1989).

The nuclear class of proto-oncogenes represent a family of DNA binding proteins that appear to be important in the regulation of gene expression. This would be the final step in signal transduction, and should therefore be under strict control. It is not difficult to perceive that the effects of uncontrolled transcription of these genes involved in cell growth could have oncogenic consequences. The control of gene expression by the nuclear proto-oncogenes can occur at two levels. The majority of the nuclear proto-oncogenes encode genes that have direct effects on gene transcription, while the anti-oncogenes may be important in the regulation of proteins that can activate gene transcription.

Proto-oncogenes that encode transcription factors include the c-*jun* and c-*fos* genes whose products make up the AP-1 transcription factor(Curran and Franza, 1988). The two proteins are capable of interacting through coiled leucine rich domains termed a leucine zipper(Gentz et al., 1989). The leucine zipper allows many forms of *fos* and *jun* homodimers and heterodimers to be formed. These various combinations have different DNA binding affinities and activities(Chiu et al., 1989; Schütte et al., 1989) which provides for a variety of responses to external stimuli. The protein encoded by c-*myc* is also a sequence specific DNA binding protein(Blackwell et al., 1990; Prendergast and Ziff, 1991) that requires an auxiliary protein, *max*, for activity(Blackwood and Eisenman, 1991). MYC

protein binds DNA through a basic-helix-loop-helix (bHLH) domain(Blackwell et al., 1990; Prendergast and Ziff, 1991). The *ets* family of proto-oncogenes encode transcription factors that are important in the regulation of the T cell receptor(Ho et al., 1990), immunoglobulin receptor(Klemsz et al., 1990), and may interact with many other transcription factors(Wasylyk et al., 1990; Ho and Leiden, 1990). The *ets* protein product does not contain the bHLH or leucine zipper domains; it appears to interact with DNA through a tryptophan cluster. The tryptophan cluster is also the mechanism by which the c-*myb* gene product(s) interact with DNA(Kanei-Ishii et al., 1990). Unlike many of the other nuclear proto-oncogenes, c-*myb* appears specific for hematopoietic cell transcriptional regulation. The c-*rel* proto-oncogene encodes the p50 subunit of the lymphocyte specific transcriptional activator NF-kB(Ghosh et al., 1990).

The anti-oncogenes, such as the retinoblastoma susceptibility gene (Rb) and p53, have indirect effects on gene expression by interacting with factors that can regulate gene expression. These factors include the E1A protein of adenovirus(Egan et al., 1989) and the T antigen of the SV40 virus(Wang et al., 1989). The activity of Rb can be regulated by cell cycle specific kinases such as cdc2 kinase(Buchkovich et al., 1989), suggesting an important role for this protein in cell growth.

The *myb* Oncogene.

The c-*myb* proto-oncogene is the cellular homolog of v-*myb*, the transforming gene of the Avian Myeloblastosis Virus

(AMV)(Klempnauer et al., 1982; Souza et al., 1980b; Souza et al., 1980a; Papas et al., 1982) and the E26 avian retrovirus(Nunn et al., 1984; Klempnauer and Bishop, 1984; Klempnauer et al., 1982). These viruses are capable of transforming chicken myeloid cells to leukemic cells in a process that resembles the dedifferentiation of the cell(Beug et al., 1987; Ness et al., 1987). This, along with the fact that v-*myb* does not transform NIH3T3 cells, was the first evidence that *myb* could be important in controlling differentiation, specifically that of hematopoietic cells.

V-*myb*.

The v-*myb* oncogene was isolated from two separate replication defective strains of avian leukemia retroviruses, AMV and E26. Both of these viruses are capable of causing hematopoietic malignancies, but E26 infection predominantly results in erythroid tumors. The presence of a second oncogene, v-*ets* is necessary for the erythroid transformation(Nunn and Hunter, 1989). The protein encoded by the v-*myb* of AMV is 45 kd, due to truncations of the amino and carboxy terminus of c-*myb* encoded proteins(Anderson and Chen, 1981). These truncations are replaced by a fusion of 6 *gag* and 11 *env* amino acids(Klempnauer et al., 1982). The protein encoded by E26 is a fusion protein of 135 kd that includes the virally derived *gag* protein fused to a truncated form of *myb* which is then fused to a portion of the *ets* proto-oncogene(Nunn et al., 1984; Leprince et al., 1983). The truncations in the v-*myb* protein remove regulatory regions of the protein, allowing for the expression of an uncontrolled DNA binding protein. The amino

terminus deletions would remove a casein kinase II phosphorylation site that has been suggested to be important in the regulation of c-*myb* DNA binding activity(Lüscher et al., 1990), while the carboxy terminus deletions remove other negative regulatory domains that are important in controlling the c-*myb* trans-activation domains(Sakura et al., 1989).

The v-*myb* oncogene has been an important tool in determining the function of c-*myb*, due to the lack of negative regulatory domains. Bacterially expressed v-*myb* was utilized to determine the specific DNA binding site of the *myb* proteins, which is pyAAC(G/T)G(Biedenkapp et al., 1988). Both v-*myb* and c-*myb* can trans-activate gene expression from reporter plasmid constructs that contain this sequence(Nishina et al., 1989; Weston and Bishop, 1989; Sakura et al., 1989). Thus the apparent mechanism of action of v-*myb* transforming potential, is by the uncontrolled activation of *myb* responsive genes. One such gene, *mim*1 was cloned by subtractive hybridization of a cDNA library from a temperature sensitive (ts) v-*myb* transformed cell line at the non-permissive temperature from a cDNA library constructed from the same cell line at the permissive temperature(Ness et al., 1989). *Mim*1 encodes a protein that is found in primary granules and is not important in v-*myb* induced transformation. The ability of v-*myb* to activate genes that are not essential for growth (or transformation) must be due to activation of c-*myb* inducible genes that are not involved in growth(Ness et al., 1989). This was confirmed by the presence of 3 *myb* binding sites upstream of the *mim*1 promoter which can be activated by c-*myb*(Ness et al., 1989).

Transformation by the v-*myb* gene is due to a block in the ability of the myeloid cell to complete a differentiation program. This block is evidenced by the immature phenotype of AMV or E26 transformed cells. Many of these cells would normally differentiate to macrophages but are blocked by the dominant effects of v-*myb*. This was demonstrated experimentally with the use of ts mutants of v-*myb*. At the permissive temperature the E26 transformed cells would resemble immature myeloid cells, typical of v-*myb* transformation, but when the cells were shifted to the non-permissive temperature, the cells would differentiate into cells that exhibited the mature markers of a macrophage(Beug et al., 1987). The mature cells would dedifferentiate if shifted back to the permissive temperature. This effect on differentiation differs from the effect of v-*myc* in MC29 transformed avian myeloid cells, which will increase the proliferative capacity of cells without affecting the phenotypic characteristics. Populations of v-*myc* transformed cells may all resemble mature macrophages that have acquired the ability to divide. When cells are transformed with v-*myb* and v-*myc* the cells will exhibit immature characteristics suggesting that the v-*myb* transformation is dominant to v-*myc*(Ness et al., 1987). These studies also suggest the importance of proper myb control in hematopoietic cells, due to its effects on cell differentiation.

C-*myb*.

The c-*myb* gene is expressed predominantly in immature hematopoietic cells(Westin et al., 1982) and mitogenically active T lymphocytes(Sheiness and Gardinier, 1984; Pauza, 1987), but has

also been shown to be present in other tissues such as colon tumor cell lines(Alitalo et al., 1984), neuroblastoma cells(Thiele et al., 1988) and chicken fibroblasts(Thompson et al., 1986). The c-*myb* gene is an important gene from an evolutionary perspective, since there is significant conservation from chicken to man at the protein level(Shen-Ong, 1990) and related genes have been cloned from drosphila(Boyle et al., 1986), *Zea mays*(Paz-Ares et al., 1987) and yeast(Ju et al., 1990; Tice-Baldwin et al., 1989). The region which demonstrates the greatest conservation is the DNA binding domain.

Activation of the c-*myb* gene's transforming potential has been due to retroviral insertion within the gene in both avian B-cell lymphomas and murine myeloid leukemias. Both EU-8 and RAV-1 avian helper viruses can activate c-*myb* by insertion into the 5' end of the gene(Kanter et al., 1988; Pizer and Humphries, 1989). Myeloid transformation is mediated by the insertion of Moloney-Murine Leukemia Virus in the 5' end of the gene, just upstream of the first exon(Shen-Ong and Wolff, 1987; Shen-Ong et al., 1984). The transcriptional orientation of the provirus is the same as the sense transcription of c-*myb*, thus the c-*myb* gene comes under control of the viral promoter. The protein produced by the provirus promoter is a chimera between a viral protein and c-*myb*, with the truncation in the amino terminus of c-*myb*, truncated in a similar fashion as the v-*myb* protein(Shen-Ong et al., 1987). There have also been reports of viral insertions into the 3' end of the *myb* locus detected in myeloid leukemias(Shen-Ong, 1990). These insertions result in the production of proteins that have similar carboxy truncations as v-*myb*, but other insertions within the genome, in

particular into the *evi*-1 locus have made it difficult to prove whether the alterations in c-*myb* are necessary or sufficient for transformation(Mucenski et al., 1988).

Structure of the C-*myb* Gene and Protein.

The c-*myb* locus is approximately 36 kb in length and encodes a transcript that has an average length of 3.5 kb(Westin et al., 1982). The gene has at least 15 exons, not including multiple alternative exons(Westin, 1991). The 5' untranslated region contains a GC rich region with no CAT or TATA boxes in the case of the mouse(Bender and Kuehl, 1986) and the human(Westin, 1991) genes and no TATA box in the chicken gene(Hahn et al., 1989). These features are characteristic of constitutive "housekeeping" promoters.

The primary protein encoded from the locus is 640 amino acids (75 kd)(Lüscher and Eisenman, 1990). The protein has 3 major functional domains, the DNA binding domain, the trans-activation domain, and the negative regulatory domain. There are also sites for protein phosphorylation throughout the protein, although only the casein kinase II site in the first 20 residues has been characterized(Lüscher et al., 1990).

The DNA binding domain consists of three imperfect repeats of 51 to 52 amino acids in length near the amino terminus of the protein (Fig.4). The second and third repeats are essential for DNA binding activity, and are virtually identical from chicken to human(Lüscher and Eisenman, 1990). The DNA binding domain does not resemble that of other transcription factors, such as the

leucine zipper, zinc finger, or basic helix loop helix domains. The repeats do contain a unique feature in that there are three tryptophan residues that are evenly spaced every 18 to 19 amino acids within each of the repeats(Kanei-Ishii et al., 1990). This structure has been demonstrated to be important in the DNA binding activity of c-*myb* and has been termed the tryptophan cluster(Kanei-Ishii et al., 1990).

The trans-activation domain of the c-*myb* protein is located in the center of the protein (Fig. 4) and is approximately 50 amino acid residues in length(Sakura et al., 1989; Weston and Bishop, 1989; Ibanez and Lipsick, 1990). The amino acids in the region are acidic and hydrophillic, which is analogous to other trans-activation domains(Ptashne, 1988). Little is known about how the trans-activation domain works or how it interacts with other proteins, except that it is regulated by a negative regulatory domain in the carboxyl portion of the protein (Fig. 4). The negative regulatory domain has been defined by the ten-fold increase in trans-activation activity when the carboxy-terminus is removed(Sakura et al., 1989; Ibanez and Lipsick, 1990). This domain has a motif that resembles a leucine zipper although this has not yet been proven to be essential for activity of the negative regulatory domain by site-directed mutagenesis.

C-*myb* Protein: Function.

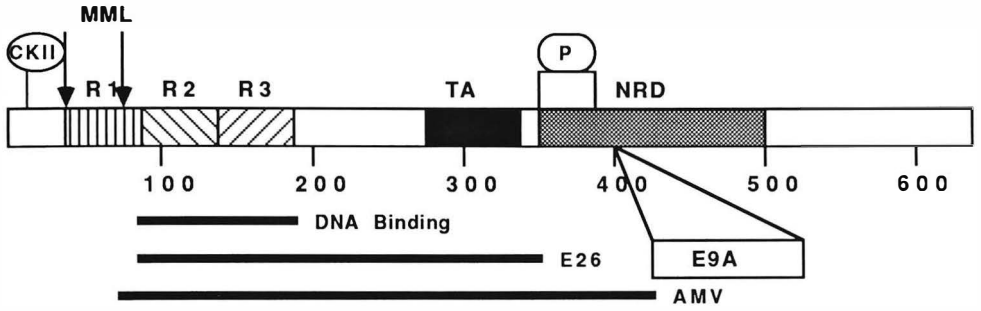
The function of the c-*myb* protein is not entirely known, although it can be presumed to be related to its ability to regulate gene expression. The only gene that is known to be regulated by

the c-*myb* protein is the *mim*1 gene(Ness et al., 1989). *Mim*1 encodes a protein that is located in the primary granules of immature myeloid cells. The promoter for this gene contains three *myb* binding sites, 2 high affinity sites and one low affinity site(Ness et al., 1989). The definition of the high affinity binding and low affinity binding is by the ability of bacterially expressed, full length protein to bind these sites(Nakagoshi et al., 1989). When bound to the high affinity site, *myb* protein is capable of transcriptional activation(Nakagoshi et al., 1989; Sakura et al., 1989; Ibanez and Lipsick, 1990; Weston and Bishop, 1989). A low affinity binding site was initially observed in the SV-40 enhancer. *Myb* can repress transcription, when occupying this site(Nakagoshi et al., 1989). This ability to trans-activate and trans-repress gene expression, taken into context with the expression of c-*myb* itself, suggests that the function of *myb* is probably as a "switch" that is important in controlling the initial differentiation process during myelopoiesis and T-lymphocyte development.

C-*myb* Gene Expression.

The majority of the studies of the expression and regulation of the c-*myb* oncogene have been carried out in normal and leukemic cells from chicken, mouse and to a lesser extent man. As mentioned in a previous section, the c-*myb* gene has a promoter that is characteristic of housekeeping genes. This G/C rich, TATAless promoter has multiple start sites that lead to the 5' heterogeneity in c-*myb* mRNAs that have been found in both chicken(Hahn et al., 1989) and mouse(Bender and Kuehl, 1986; Watson et al., 1991).

Figure 4. Structure of the c-*myb* protein. The structural and functional domains of the c-*myb* protein are presented in this diagram. The three imperfect repeats that form the DNA binding domain are represented by R1, R2 and R3. The region that is essential for DNA binding activity is depicted below the protein diagram. The trans-activation (TA) and negative regulatory (NRD) domains are shown in black and gray respectively. The inclusion site of exon 9A in the negative regulatory domain is depicted by the white box labeled E9A. The arrows below the MML, represent the proviral insertion sites of the Moloney Murine Leukemia virus. Post-translational modifications of *myb* protein such as phosphorylation by Casein Kinase II (CK II) or other potential phosphorylation sites (P) are also shown. The black lines below the c-*myb* protein that are labelled E26 and AMV represent the portions of the c-*myb* protein that are present in the transforming proteins of the two retroviruses respectively. This figure was adapted from a recent review on *myb*(Lüscher and Eisenman, 1990).


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

The c-*myb* promoter is also constitutively active in most cells that have been tested(Bender et al., 1987), thus regulation at this level is probably not the primary mechanism of regulation of c-*myb* expression. An attenuator that is located in the first intron of the murine gene, is active in mature lymphoid, erythroid, and myeloid cells(Bender et al., 1987; Watson, 1988b; Watson, 1988a). This premature termination of transcription is the major mechanism of regulation at the transcriptional level of the murine c-*myb* gene. There is no evidence of attenuation in the chicken form of the gene. Post-transcriptional regulation of both the chicken and murine gene has also been investigated. In the case of the chicken, the post-transcriptional modification was a stabilization during chick embryo fibroblast cell proliferation(Thompson et al., 1986), while during FMEL cell differentiation, destabilization of the murine c-*myb* message has been noted(Watson, 1988b).

A second phenomena that leads to the heterogeneity of the c-*myb* mRNA is the process of alternative splicing of the unprocessed c-*myb* hnRNA. Alternative splicing of c-*myb* has been characterized in both the mouse(Shen-Ong, 1987) and the human(Dasgupta and Reddy, 1989; Shen-Ong et al., 1990; Westin et al., 1990). The alternative splicing involved in the c-*myb* gene, includes alternative use of splice donor sites that result in putative proteins with three amino acids deleted from the transcriptional activation domain(Westin et al., 1990), addition of exons that result in larger proteins(Dasgupta and Reddy, 1989; Dudek and Reddy, 1989b; Dudek and Reddy, 1989a), or potentially smaller proteins(Westin et al., 1990) and altered usage of transcriptional

termination and polyadenylation sites(Westin, 1991). The effect of many of these changes in the c-*myb* message on the ability of the altered proteins to bind DNA has not been determined to date.

Artificial Expression of C-*myb*.

One technique that has been utilized in determining the role of c-*myb* during hematopoietic cell differentiation, has been to study the effects of transfected c-*myb* constructs on leukemic cell lines. This method allows one to determine the outcome of aberrantly expressed c-*myb* on the induced differentiation process. One clone of c-*myb*, pMbm1(Westin et al., 1990), which is similar to the prototypic c-*myb* message except for the use of an alternative splice donor in exon 8 which leads to the deletion of three amino acids, was transfected into FMEL cells and resulted in an inability of these cells to respond to DMSO induced differentiation(Clarke et al., 1988). Another cloned alternative splice form, pMbm2(Westin et al., 1990) was also transfected into FMEL cells, but the outcome of expression of this clone on FMEL differentiation was opposite to that of pMbm1(Weber et al., 1990). When introduced into FMEL cells pMbm2 could induce the differentiation of these cells in response to subthreshold levels of DMSO. The ability of different forms of the c-*myb* message (and presumably protein) to have opposite effects on differentiation is another piece of circumstantial evidence suggesting that c-*myb* is a switch involved in control of hematopoietic differentiation.

Inhibition of C-*myb* Expression.

A second approach to determining the function of c-*myb* by altering its expression, has been by inhibiting the expression of the gene. A series of studies have been performed utilizing antisense oligodeoxynucleotides to block the expression of c-*myb* under cell culture conditions. These reports have implicated c-*myb* as being an essential component during cell cycle progression in stimulated peripheral blood lymphocytes(Gewirtz et al., 1989) and in leukemic cell lines that express c-*myb*(Anfossi et al., 1989). One of the leukemic cell lines that was tested, HL-60, did not differentiate in response to the antisense oligodeoxynucleotides, but in another report it was shown that HL-60 cells that were treated with antisense c-*myb* and DMSO differentiated toward a monocytic cell, as opposed to a myeloid cell(Ferrari et al., 1990).

The inhibition of c-*myb* has also been carried out in an *in vivo* study, where the *myb* locus was altered by homologous recombination in murine embryonic stem cells(Mucenski et al., 1991). The resultant transgenic fetuses were normal through the first 13 days of gestation, but by day 15 the fetuses were anemic. This suggests that c-*myb* is not essential for early fetal development, but is necessary for normal adult hematopoiesis (in particular erythropoiesis). This was determined by the fact that embryonic hematopoiesis, which occurs in the yolk sac was not affected by the "knockout," just the initial adult hematopoiesis, which is initiated in the fetal liver.

C-*myb* related genes.

Diversity in c-*myb* is not only generated through alternative splicing of the mRNA from the c-*myb* locus, but also through proteins that are expressed from related genes. Two of these genes A-*myb* and B-*myb* were cloned from a lymphocyte library(Nomura et al., 1988). Both of these genes have a more diverse spectrum of expression than c-*myb*. Some of the tissues that express A-*myb* and B-*myb* include, heart, intestine and lung(Nomura et al., 1988). B-*myb* is expressed in myeloid cells in a similar fashion to c-*myb*, while A-*myb* expression has not been detected in myeloid cells(Golay et al., 1991). The gene product for B-*myb* is a DNA binding protein that is capable of trans-activating gene expression from c-*myb* response elements(Mizuguchi et al., 1990).

MATERIALS AND METHODS

Characterization of the Phenotype of DMSOr and the Expression and Regulation of c-*myb* During Dimethyl Sulfoxide Induced Differentiation.

This set of experiments was designed to determine the phenotype of the Dimethyl Sulfoxide (DMSO) resistant clone, DMSOr after removal of the constant selective pressure of 0.7% (v/v) DMSO. Phenotypic effects were assayed by morphological changes, proliferation assays, cell cycle analysis and functional analysis. Regulation of c-*myb* was determined by Northern blot analysis and Nuclear Run-on analysis at time points during the DMSO induced differentiation. The effect of antisense oligonucleotides to the c-*myb* gene was also examined. The figures are derived from representative experiments of no less than two assays.

Cell Culture.

HL-60 and DMSOr cells were maintained in RPMI 1640 media supplemented with 10% defined supplemented bovine calf serum (Hyclone) and gentamicin (40 μg/ml) at 37°C in a moist 5% CO_2 atmosphere. Dimethyl Sulfoxide (DMSO) was purchased from Mallinckrodt (Paris, KY) and was added to cells as a 10x (13%) stock solution in RPMI 1640 growth media. Cells were treated with 1.3% DMSO over the entire 6 days of differentiation unless otherwise

specified. Cells were removed at appropriate time points for the subsequent experiments. Washout studies were performed by removing the cells from the DMSO containing growth media washing the cells with fresh serum containing growth media and reculturing the cells for 3 days.

Morphology Studies.

$5x10^4$ cells were harvested at the designated time points and applied to slides by cytospin (600 rpm for 2 min). Slides were then Wright-Giemsa stained. Micrographs were taken at 400x magnification.

^{3}H-Thymidine Incorporation.

10^4 cells were harvested 2 hours prior to each time point and plated in triplicate in 96 well cluster plates in a volume of 100 µl. ^{3}H-Thymidine (0.5 µCi in 100 µl of growth media with or without DMSO) was then added for 2 hrs. Cells were harvested onto filters using a Titer-Tek cell harvester. Filters were quantitated by liquid scintillation in Budget Solve cocktail (Research Products International Corp., Mount Prospect, IL).

Cell Cycle Analysis.

$5x10^6$ cells were harvested and fixed in phosphate buffered saline (Ca^{+2} and Mg^{+2} free):ethanol (1:2) and stored at 4°C until staining. Cells were stained with a propidium iodide staining solution containing $3.8x10^{-3}$M sodium citrate, RNaseA (0.5 mg/ml) and propidium iodide (0.01 mg/ml) for 1 hr on ice. The cells were then

pelleted and resuspended in phosphate buffered saline at $1x10^6$ cells/ml and analyzed on the Becton-Dickenson FACSCAN flow cytometer utilizing CELLFIT software.

Superoxide Production.

Superoxide production was measured using a cytochrome C assay(Shakarjian and Carchman, 1990). $1x10^6$ cells were resuspended in 1 ml of Gey's Balanced Salt Solution. Superoxide release was induced with 10^{-7} M Phorbol-12-myrisitate,13-acetate (PMA) at 37°C/5%CO_2 for 40 min. PMA was a provided by the laboratory of Dr. Richard Carchman. The reaction was stopped by refrigerated centrifugation and the supernatant measured spectrophotometrically at 550 nm. Nonspecific reduction was determined by addition of superoxide dismutase to duplicate tubes.

Northern Blot Analysis.

RNA was isolated from cells as previously described(Chirgwin et al., 1984). Briefly, cells were harvested and lysed in 4 M guanidine isothiocyanate. RNA was pelleted by ultracentrifugation through a 5.7 M cesium chloride cushion at 41,000 rpm in a Beckman 70.1Ti rotor for 20 hours at 20°C. RNA pellets were then washed with 2 volumes of 100% ethanol, resuspended in sterile water and quantitated spectrophotometrically.

Ten µg of total cellular RNA was denatured in 0.02 M MOPS, pH 7.0, 5 mM NaAc, 1 mM EDTA, 2.2 M formaldehyde and 50% formamide. The samples were heated to 65°C for 5 min and then separated on a 6.6% formaldehyde, 1% agarose gel. Equal loading of

RNA in each lane was determined by ethidium bromide staining and/or ß-actin normalization. Blotting was carried out using the method of Thomas(Thomas, 1980) onto BAS-NC transfer membranes (Scheleicher & Schuell). Filters were hybridized to probes, radiolabled by nick translation or 5' end-labelling (Bethesda Research Laboratories kits, Bethesda, MD), in the presence of 50 mM sodium phosphate pH 6.5, 5x Denhardt's (0.1% BSA, 0.1% ficoll, 0.1% polyvinyl pyrollidine), 5x SSC (1x equals 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, yeast RNA (250 µg/ml), 50% formamide and 10% dextran sulfate (not used for oligonucleotide hybridizations). Hybridizations were for 16-20 hours at 42°C. Filters were washed 5 times at 42°C for 5 minutes in 2x SSC and 0.2% SDS followed by one wash in 2x SSC and 0.2% SDS at 60°C for 40 minutes and finally one wash at 60°C in 0.5x SSC and 0.2% SDS for 40 minutes before autoradiography (hybridization and wash conditions that were not carried out under these standard conditions will be mentioned in the figure legends). Autoradiography was performed by exposing filters to Kodak XAR-5 film at -80°C for the indicated times. Sizes of RNA species were estimated by comparison to the 18S and 28S rRNA markers.

Isolation of Specific Probes.

The probes used in these studies are given in Table 4. The plasmids were isolated by the alkaline lysis procedure. Bacterial pellets were resuspended in 10 ml of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and 5.0 mg/ml lysozyme and incubated at room temperature for 5 minutes. Twenty ml of 0.2 M NaOH and 1%

SDS was added to the mixture and incubated for 10 minutes on ice. Fifteen ml of ice cold potassium acetate (5 M, pH 4.8) was then added to the mixture and the solution was thoroughly mixed and incubated for 20 more minutes on ice. This mixture was then centrifuged at 12,000 rpm in an HB-4 rotor (Sorvall) for 35 minutes at 4°C. Nineteen ml of the supernatent was then decanted from the tube and 0.6 volumes of isopropanol (11.4 ml) was added to this solution. DNA was allowed to precipitate from the solution at room temperature for at least 15 minutes. The DNA was pelleted by centrifugation at 12,000 rpm in the HB-4 rotor for 30 minutes at 4°C. The pellets were dried and resuspended in 1:1 cesium chloride:TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with ethidium bromide (100 µg/ml) and centrifuged at 48,000 rpm in a Ti 70.1 rotor (Beckman) for 20 hours at 20°C or for 4 hours at 65,000 rpm at 20°C in a VTi 65.2 rotor (Beckman). Plasmid bands were butanol extracted and ethanol precipitated. The DNA was quantitated at OD_{260}. The specific inserts were isolated by digestion of the plasmid DNA with the appropriate restriction endonuclease and separated on a 0.8% agarose gel. The insert band was then isolated from the gel and subjected to electroelution using an Elutrap electro-eluter (Scheleicher & Schuell). Inserts were quantitated spectrophotometrically at OD_{260} or by approximation based on ethidium bromide staining of a 10 ml agarose gel.

Oligonucleotide probes were created 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 resuspended in water and purified by thin layer chromatography by the method of Alvarado-Urbina(Alvarado-Urbina et al., 1981). Briefly the solid phase was a TLC plate and the mobile phase was 1-propanol:ammonium hydroxide:water (55:35:10). The product was detected by UV shadowing and scraped from the plate. The oligonucleotide was extracted from the sand with water, quantitated spectrophotometrically at OD_{260} and stored at -80°C. For B-*myb*, a 30 base oligonucleotide was used for probing this c-*myb* related gene. The sequence of the B-*myb*.1 oligonucleotide was 5'CTCAGAACGCAGCACCTCCTTCAAGTCCTC3'. An oligonucleotide designed to hybridize specifically to A-*myb* was also utilized. The sequence for the probe A-*myb*.1 was 5'ATGATGGGCATACTGAAGGTCATCATCCTC3'. The sequences for B-*myb* and A-*myb* were obtained from the original publication(Nomura et al., 1988). The probes that were synthesized for cyclin B (cycB.1) and for cdc2 kinase (cdc2.1) had the following sequences 5'GGATCAGCTCCATCTTCTGCATCCACATCA3' and 5'GCTAGTTCAGCAAATATGGTGCCTATACTC3' respectively. The sequences for cyclin B and cdc2 kinase were obtained from the original publications (Pines and Hunter, 1989; Lee and Nurse, 1987).

Antisense Oligonucleotide Experiments.

Cells (10^4) were resuspended in 100 µl of fresh complete media and allowed to acclimate overnight in 96 well culture plates. The antisense or control oligonucleotides were then added to a final concentration of 10 µg/ml in the presence or absence of 1.3% DMSO.

Table 4. DNA probes utilized in Northern blot analysis.

Gene	Plasmid	Citation
c-*myb*	pMbm1	(Westin et al., 1990)
	pMbm28	(Westin, 1991)
c-*myc*	pMC413RC	(Dalla-Favera et al., 1983)
Histone H4	pMUSH4	(Seiler-Tuyns and Birnsteil, 1981)
Ornithine decarboxylase	pODC10/2H	(Hickok et al., 1987)
ß-actin	pLK221	(Gunning et al., 1983)

The cells were then incubated for 24 or 72 hours. During the last 4 hours of the incubation, ^{3}H-thymidine (same as above) was added to the cultures. The cultures were then harvested using a Skatron cell harvester and the radioactivity retained on the filter was quantitated by scintillation counting (same as above). The oligonucleotides used for these experiments were the antisense (Ambm1) (5'CCGGGGTCTTCGGGC3'), the sense control (Smbm1) (5'GCCCGAAGACCCCGG3') and the random sequence control (Rmbm1) (5'GCGTGCGGCTGGCTC3') based on the antisense oligonucleotide sequence. The oligonucleotides were generated and deblocked by the methods described above, but were then washed 3 times with ice cold 75% ethanol and resuspended directly in TE buffer without further purification. The oligonucleotides were then stored at -80oC.

Nuclear Run-on Analysis.

The procedure used for the nuclear run-on analysis of *c-myb* transcription in HL-60 and DMSOr was a modification of the procedure of Groudine(Linial et al., 1985). Nuclei were isolated from cells at the indicated time points of DMSO induced differentiation. The cells ($5x10^7$/set of nuclei) were centrifuged at 1,000 rpm in a swinging bucket rotor at room temperature for 10 minutes. The cell pellet was then washed two times with PBS (Ca^{++}/Mg^{++} free) and pelleted again as above. The pellet was then resuspended in 1 ml of lysis buffer (10 mM Tris-HCl pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$) for $5x10^7$ cells. An equal amount of lysis buffer containing 1% NP-40 (Sigma) was added to the solution and the mixture was then vortexed on setting 6 for 15 seconds. The nuclei were then pelleted

by centrifugation at 1,500 rpm in a swinging bucket rotor for the IEC PR-6000 centrifuge for 10 minutes at 4°C. The supernatant was then aspirated to leave a dry nuclear pellet. The pellet of nuclei was then resuspended in 200 µl of nuclear freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0) per $5x10^7$ nuclei (final volume of nuclei was 210 µl). The nuclei were snap frozen on dry ice and stored at -80°C until needed.

The transcription assay was performed by adding 60 µl of 5x transcription buffer (25 mM Tris-HCl, pH 8.0, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM GTP, pH 7.0, 1.25 mM ATP, pH 7.0, 1.25 mM CTP, pH 7.0) and 30 µl of (alpha ^{32}P)-UTP (3,000 Ci/mmole, 10 µCi/µl, DuPont, Boston, MA) to the 210 µl of nuclei thawed on wet ice. Nucleotides were purchased from Boehringer-Mannheim (Indianapolis, IN) and 100 mM stock solutions were made up in water (final pH, 7.0). The reaction was vortexed at setting 6 for 10 seconds and incubated at 30°C for 30 minutes. The tubes were inverted once during the incubation period. The remaining steps of the procedure were then performed to isolate the radiolabelled RNA from the nucleus.

After completion of the transcription reaction, 50 µl (1 unit/µl) of RQ1 DNase (Promega, Madison, WI) and 5 µl of 100 mM $CaCl_2$ was added to the mixture and incubated for 15 minutes at 30°C. Protein digestion was then carried out by adding 42 µl of 10x SET buffer (1x; 1.0% SDS, 5 mM EDTA, and 10 mM Tris-HCl, pH 7.4) and 15 µl of Proteinase K (25 mg/ml in water) (Boehringer-Mannheim) to the reaction and heating the tubes to 60°C for 2 minutes. This step dissolves the SDS precipitate. The solution was then incubated at

42°C for at least 2 hrs. To remove the digested protein from the samples, a phenol/chloroform extraction was performed. A 1:1 solution of Salt Saturated Phenol(for 50 g of phenol, add 11 ml of 2 M Tris, pH 7.4, 14.3 ml of water; remove aqueous phase and add 11 ml 2 M Tris, pH 7.4, 110 µl 2-mercaptoethanol, 2.75 ml m-cresol, 55 mg 8-hydroxyquinalone)(Davis et al., 1986) to chloroform was made and 360 µl of the mixture was added to the transcription reactions. The tubes were then vortexed and centrifuged in a Fisher microfuge for 5 min and the aqueous phase transferred to a fresh tube. The interface of the phenol/chloroform extraction was re-extracted with 100 µl of 1x SET buffer and the second aqueous combined with the first. The nucleic acids and unincorporated nucleotides were then precipitated by adding 150 µl of 10 M Ammonium Acetate and 650 µl of isopropanol and incubating on dry ice for at least 15 min. The precipitate was pelleted by centrifugation for 10 min in a Fisher microfuge and the pellet resuspended in 100 µl of TE buffer.

To remove small protein fragments and unincorporated nucleotides (which increase background) from the *in vitro* extended RNA, a Sephadex G-50 (fine) (Sigma) spin column was made from a 1 cc syringe. The column was gravity packed with swelled sephadex and then spun at 1,000 rpm in a clinical centrifuge for 3 min. The 100 µl of sample was then loaded on to the spin column and centrifuged at 1,000 rpm for 3 min. The column was then washed with successive washes of 100 µl, 50 µl and 50 µl of TE.

The column eluate (250-300 µl) contains long, extended RNA molecules that must be cleaved to the length of the *in vitro* extension (100-200 bases) for accurate measurement of the "polymerase

density" of a given region of a gene. RNA cleavage was accomplished with the addition of 1/9 volume of 2 M NaOH for 5-10 min on ice. The reaction was stopped by adding 1/3 volume of 1 M HEPES pH 7.0 buffer and the RNA precipitated overnight with 2.5 volumes of ethanol at -20°C.

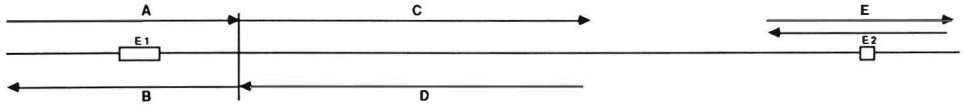
The RNA is then pelleted by centrifugation for 10 min in a Fisher microfuge and the pellet resuspended in 100 µl of TE. To determine the quality of the labelling, 1 µl of the reaction is added to scintillation cocktail and counted. This also provides a method for even loading of multiple samples. The reactions are then hybridized to nitrocellulose filters that have the single and denatured double stranded targets fixed by slot blotting and baking at 80°C for 2 hrs under vacuum (details below). The hybridization buffer and conditions are the same as those described under Northern blot analysis. Following hybridization, the filters were washed 4 times with 2x SSC, 0.2% SDS at 60°C with each wash for 15 min. Two additional washes with 0.1x SSC, 0.1% SDS were then carried at 60°C for 30 min each. The filters were then autoradiographed as described.

Preparation of Targets for Nuclear Run-on Assays.

A. Double Stranded Targets

The double stranded DNAs used as targets for hybridization were the plasmids pLK221(Gunning et al., 1983) for ß-actin, p1XE7 (see Fig 5)(provided by Miss Sarah Jacobs), pMC413RC(Dalla-Favera

Figure 5. Key to nuclear run-on probes. The map represents a genomic map of the 5' untranslated region through exon 1 (E1 box), intron 1, exon 2 (E2 box) and the 5' end of intron 2 of c-*myb*. The arrows represent the size and orientation of the targets termed A through E. The *myb* "read-through" targets (B2 and B2R), and the other targets are described in the lower part of the figure.



200 BASES

SYMBOL	CLONE
A	p13
B	p13r
C	p12
D	p12r
E	p1XE7
B2	pMbm1b2-cDNA containing exons 9-12
B2R	pMbm1b2r-opposite strand of pMbm1b2
RC	pMC413RC-contains 3rd exon of c-myc
MP	pMP1-contains cDNA of myeloperoxidase gene
LK	pLK221- contains cDNA of actin gene
SK+	Bluescript vector
SK-	Bluescript vector

et al., 1983) for c-*myc* and pB-*myb*(Nomura et al., 1988) for B-*myb*. The plasmids were isolated as described.

B. Single Stranded Targets

The procedure for generating single stranded DNAs as targets for hybridization were as described by Stratagene(Stratagene, 1990). An overnight culture of XL1-Blue cells transformed with the plasmid containing the DNA of interest was grown in Superbroth (0.09 M NaCl, 20 g/l yeast extract, 35 g/l bactotryptone; pH to 7.5). An innoculum of 5 ml was added to 50 ml of Superbroth and grown to an OD_{600} of 0.3 ($2.5x10^8$ bacteria/ml). R408 helper phage were then added at an MOI of 20:1 and bacteria were incubated for 8 hrs at 37°C. Cultures were then heated to 65°C for 15 min and the bacterial debris removed by centrifugation at 17,000g for 10 min in a HB-4 rotor at 4°C. The supernatant was then stored for up to 1 month before DNA purification.

For purification of a large culture, the Stratagene procedure was scaled up for a 30 ml culture. The supernatant (30 ml) was added to a 50 ml fresh polypropylene tube and 7.5 ml of a 3.5 M ammonium acetate (NH_4Ac), pH 7.5; 20% polyethylene glycol (PEG) solution was added. The tube was mixed by inversion and set at room temperature for 15 min. The phage were then pelleted by centrifugation at 17,000g for 20 min at 4°C in a HB-4 rotor. The pellet was then resuspended in 7.5 ml of TE buffer and extracted twice with PCI9 (100 g phenol, 100 ml chloroform, 10 ml 50 mM Tris, pH 9, 1 ml isoamylalcohol and 10 ml water) and once with chloroform. The single stranded DNA was then precipitated from the aqueous phase by adding 5 ml of 7.5 M NH_4Ac, pH 7.5 and 20 ml of

100% ethanol and incubating on ice for at least 15 min. The DNA was pelleted by centrifugation at 17,000g for 20 min at 4°C in a HB-4 rotor and resuspended in 0.5 ml of TE.

The single stranded targets utilized for the nuclear run-on assays were generated from the *myb* containing plasmids p13, p13R, p12, p12R, pBSmbm1b2 (pB2), pBSmbm1b2r (pB2R) and the control pBluescript vectors. Fig. 5 shows a map indicating the location and the orientation of the targets noted above. The plasmids p13, p13R, p12 and p12R were provided by Ms. Karen Gorse. pB2 and pB2R were constructed by subcloning the 1.1 kb BamHI fragment of the c-*myb* cDNA pMbm1 (exon 9 to exon 12) into the pBluescript vector pKSII+ in both orientations.

Slot Blot Procedure.

The target DNAs described above were added to nitrocellulose filters by means of a slot blotter (Hoeffer Scientific Instruments). The DNA (1 µg) was brought up to 10 µl in TE buffer and heated for 5 min at 95°C. The samples were then put on ice and 90 µl of 20x SSC was added. The samples were slotted onto a nitrocellulose filter that had been presoaked in distilled water followed by 20x SSC. After the samples were slotted, slots were washed with 20x SSC and the filters removed to dry prior to baking. A map of the c-*myb* targets and a legend of the other targets can be found in fig 4.

Characterization of the Level of Regulation of the Human c-*myb* Proto-oncogene During HL-60 differentiation.

The following methods were used to determine the level of regulation of the c-*myb* gene during differentiation of HL-60. The experiments were designed to determine if regulation occurred at the transcriptional level by down regulation of a promoter or activation of an attenuator. Post-transcriptional events were also examined. The level of regulation was investigated for both the myeloid inducers DMSO and retinoic acid (RA) and the monocytic inducers Phorbol-12,13-dibutyrate (PDbu) and 1,25-dihydroxycholicalciferol (Vitamin D3). To determine the role of *de novo* protein synthesis in c-*myb* regulation, cyclohexamide was also used in some experiments.

Cell Culture.

Cells were treated as above with the following additions. PDbu was purchased from Sigma Chemical Company, St. Louis, MO, and stored as a 2 mM stock solution in DMSO at -80°C. Differentiation with PDbu (250 nM) was for a 24 hr period. Retinoic acid was purchased from Sigma Chemical Company and was stored at -20°C in a desiccator. One mM stock solutions of retinoic acid were made up in 100% ethanol and stored at -20°C. Retinoic acid induced differentiation was also a 6 day induction program following treatment with drug at 1 μM. Vitamin D3 (Sigma) was prepared as a 2.4 mM stock solution in 100% ethanol and was stored at -20°C. Vitamin D3 (10 nM) induces monocytic differentiation over a 6 day program. Cyclohexamide was purchased from Sigma Chemical Company, St. Louis, MO. A 100 mg/ml stock of cyclohexamide was

made up fresh for each experiment in 100% ethanol and added to the cells at a concentration of 10 μg/ml.

Northern Blot Analysis.

Same as above.

Nuclear Run-on Assays.

Same as above.

Post-transcriptional Studies.

To determine if post-transcriptional regulation of c-*myb* occurs during differentiation of HL-60, cells were treated with Actinomycin D (5 μg/ml) in the presence or absence of DMSO or PDbu for the indicated time points. Actinomycin D was purchased from Sigma Chemical Company (St. Louis, MO) and was dissolved in 100% ethanol and stored at 4°C as a 1 mg/ml stock solution. At the indicated time points, cells were removed from culture for isolation of RNA to be used in Northern blot analysis (see above).

Electrophoretic Mobility Shift Assay.

The Gel Shift protocol was based on the procedure of Dignam(Dignam et al., 1983) and kindly provided by Dr. Timothy Bender. Nuclear extracts were prepared from HL-60 at indicated time points of DMSO differentiation and/or cyclohexamide treatment using an NP-40 lysis procedure. Cells were harvested and washed once in PBS. The cells ($1x10^8$) were then resuspended in 250 μl of lysis buffer (150 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0 and

0.5% NP-40), transferred to a microfuge tube and incubated on ice for 10 min. The nuclei were then pelleted by centrifugation in a Sorvall microfuge at 60% speed for 2 min. The supernatant was then removed, the pellet resuspended in 100 µl of Dignam Buffer C (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 20% glycerol, 0.5 mM Dithiothreitol (DTT) and 0.5 mM PMSF) and incubated on ice for 1 hr. The samples were then spun again as above and the supernatant was removed, alliquoted in 25-30 µl fractions, frozen on dry ice and stored at -80°C.

The gel shift reactions were performed using nuclear extracts that were isolated as described above with target DNA from intron 1 of the c-*myb* gene. The targets were isolated from the plasmids p13 and p12. The mapping of the targets can be seen in Fig 27. Targets were radiolabelled by Klenow fill-in or end-labelling. A double stranded oligonucleotide target which contains the high affinity *myb* binding site(Nakagoshi et al., 1989) was used as a positive control. The sequence of the plus strand of MBS-I is 5'TGTGTGTCAGTTAGGGTGTCTCG3', MBS-I was end-labelled, vacuum dried and resuspended in water before use in the gel shift assay.

The binding reactions were carried out by adding water, non-specific competitor (polydI:dC, 0.2-2 µg) and 10x binding buffer (100 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM DTT, 10 mM EDTA, 50% glycerol and 1% BPB) to a microfuge tube. The extracts were then added to each tube at 4 µg of protein per reaction. Extracts were quantitated by Bradford assay(Bradford, 1976) using the Bio-Rad reagent. If dilutions of the protein were necessary, they were done in Dignam Buffer D (20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM

EDTA. 0.5 mM DTT, 0.5 mM PMSF and 20% glycerol). Following addition of the labelled probe ($5x10^4$-$1x10^5$ cpm/reaction), the samples were allowed to incubate at room temperature for 20 min and then loaded onto the gel. The gel was a 4% acrylamide/bis (29/1), 1X TBE (1X TBE-0.089 M Tris-HCl, 0.089 M Boric acid and 0.002 M EDTA) gel that contained 0.2 mM DTT and was polymerized with TEMED and Ammonium Persulfate. The gel was pre-run for 30 min at 170 V prior to sample loading. The samples were loaded, while the gel was running, and electrophoresed at 170 V (210 V for the oligonucleotide probe) until the loading dye reached the bottom of the gel. The gel was then dried onto Whatman 3MM paper and autoradiographed.

Characterization of the 2.4 kb c-*myb* mRNA.

This series of experiments was designed to further characterize the 2.4 kb mRNA that hybridizes to the c-*myb* probe used in the preceding experiments. Standard cloning procedures unfortunately have proven unsuccessful for isolating this message.

Northern Blotting.

Same as above.

Primer Extension Analysis.

The primer LHB.1 (5'GGAGGATCATGCACCTTGCT3') was end-labelled and $2x10^6$ cpm (counts per minute) were annealed to 25 µg of RNA isolated from HL-60 and DMSOr cells treated with DMSO for 0, 72 and 144 hrs. The annealing temperature (Tm) was determined to

be 70°C by the formula, Tm=81.5oC + 16.6log(M NaCl) + (mole fraction of G+C)(41) - 500/length of the primer. Primers were annealed for 2 hrs in 0.4 M NaCl and 40 mM PIPES pH 7.0. Following the annealing reaction, primers were extended for 1 hr at 37°C with 200 units of Moloney Murine Leukemia Virus derived reverse transcriptase (Bethesda Research Laboratories) in reverse transcription buffer (Bethesda Research Laboratories) along with 1 mM DTT and 1 mM each of dATP, dTTP, dCTP and dGTP. The cDNAs were then separated from the RNA by heating the reaction to 95°C for 3 min and the RNAs digested with RNase A (boiled 15 min, 10 µg) at 37°C for 30 min. The cDNA was then extracted with PCI9 and SEVAG and precipitated with 0.3 M sodium acetate, 20µg of carrier tRNA and 2.5 volumes of ice cold ethanol. The cDNA was then pelleted in a Fisher microfuge for 15 min, washed with 80% ethanol, pelleted and dried. The pellet was resuspended in 1 volume of water and an equal volume of sequencing loading buffer. The samples were separated on a 7% acrylamide/8 M urea gel, which was dried and autoradiographed.

RESULTS

Characterization of an HL-60 Mutant That Exhibits Reversible Differentiation in Response to DMSO.

The laboratory received a line of HL-60 that had been reported to be resistant to the induction of differentiation by DMSO(Fisher and Grant, 1985). This line of HL-60, known as DMSOr had been established by growing HL-60 cells on low levels of DMSO (0.4%) initially, then increasing the levels to near maximal differentiation concentration (1.1%). The cells were then maintained on DMSO for continuous selection purposes. Upon arrival into Dr. Westin's laboratory, DMSOr was removed from the maintenance concentration of DMSO, because it was felt that this type of selection did not allow for study of a stable, uninducible phenotype. Following removal of DMSOr from the selective pressure, the line had to be recharacterized.

Morphologic changes induced by DMSO in HL-60 and DMSOr.

A gross comparison of the phenotypic state of HL-60 and DMSOr during DMSO induced differentiation was performed. Cells were stained at the indicated time points of DMSO treatment and representative photomicrographs are presented in Fig. 6. Both control HL-60 and DMSOr cells exhibited the characteristics of an

immature cell with a large, round nucleus and clearly defined nucleoli. After 144 hours of treatment with DMSO, both cell lines have acquired similar levels of morphologic differentiation including the presence of metamyelocytes and band cells. This is exemplified by the condensed, bean shaped nucleus, the loss of nucleoli and the decreased nuclear to cytoplasmic ratio. Washout experiments were performed to determine whether acquisition of this mature cytologic phenotype was associated with terminal differentiation. Following the 3 day reculture period 6 days post-DMSO treatment, the parental cells retained their mature morphology while the DMSOr cells reverted to the immature morphology (Fig. 6).

Examination of the Effect of Induction of Differentiation on Cell Proliferation.

To determine if the DMSOr response to DMSO included the antiproliferative portion of differentiation, ^{3}H-thymidine incorporations were assayed over the treatment time course (Fig. 7A and 7B.). DMSO caused a transient increase in ^{3}H-thymidine incorporation over the first 24 hours of induction in both cell lines. This was followed by decrease in incorporation to 25% and 50% of control in HL-60 and DMSOr respectively by 72 hours. ^{3}H-thymidine incorporation continued to decline at 144 hours to 10% of control in HL-60 and 25% of control in DMSOr. Washout experiments were again performed to determine if the antiproliferative effect was reversible (Figure 7A and 7B, dashed lines). Washout performed at the precommitment time point of 24

Figure 6. Morphologic survey of HL-60 and DMSOr during DMSO induced differentiation. Magnification is 400x. A. HL-60 control. B. DMSOr control. C. HL-60 144 hours post-treatment. D. DMSOr. 144 hours post-treatment. E. HL-60 72 hour treatment with DMSO followed by a 3 day reculture period (3 day washout). F. DMSOr 3 day washout. G. HL-60 144 hour treatment with DMSO followed by a 3 day reculture period (6 day washout). H. DMSOr 6 day washout.

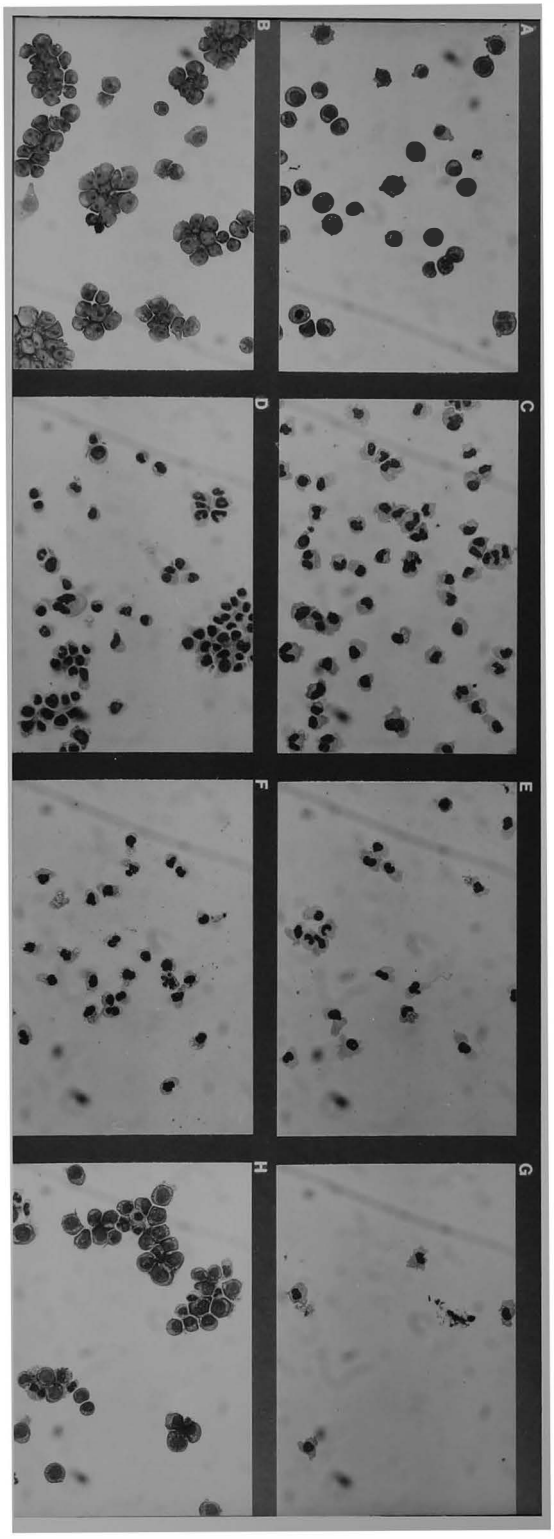
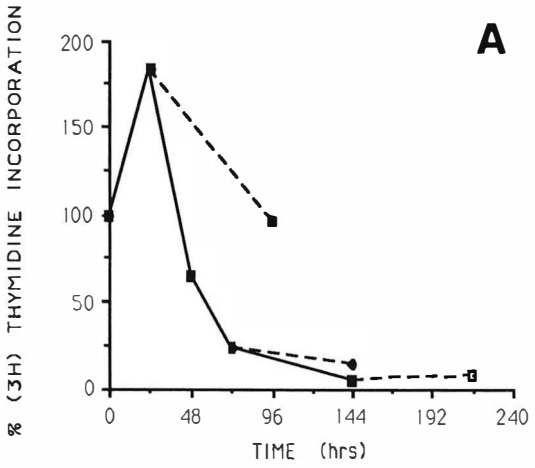
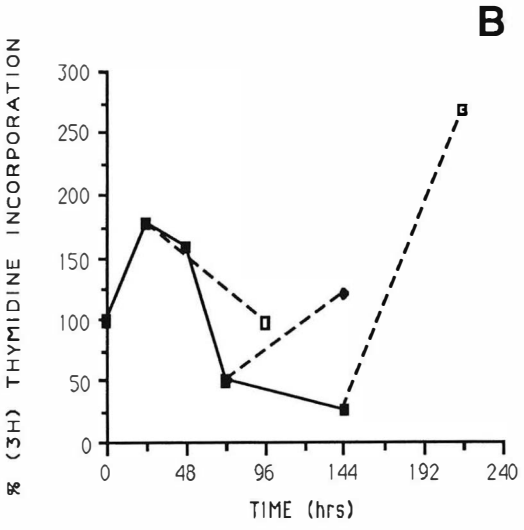

A
B
C
D
E
F
G
H

Figure 7. ^{3}H-thymidine incorporation in HL-60 (A) and DMSOr (B) following DMSO treatment. The results are presented as mean percentage of control (time 0) incorporation (cpm). The standard deviation for each point is no greater than 15% of the mean. Solid lines represent treatment with DMSO while the dashed lines represent washout experiments. The time of treatment with DMSO or reculture period is shown along the X axis.


A
% (3H) THYMIDINE INCORPORATION
200
150
100
50
0
0
48
96
144
192
240
TIME (hrs)


B
% (3H) THYMIDINE INCORPORATION
300
250
200
150
100
50
0
0
48
96
144
192
240
TIME (hrs)

hours showed a return to control levels of incorporation in both cell lines. If the cells had committed to the induction of differentiation, ^{3}H-thymidine incorporation levels would have been closer to that predicted by the graphs for continuous DMSO induction for 96 hours. Washout at 72 and 144 hours demonstrated the reversibility of the antiproliferative effects of DMSO in DMSOr. Following the reculture period, ^{3}H-thymidine incorporation in HL-60 remained less than 20% of baseline, while DMSOr incorporation was elevated to levels greater than control (104% and 260%).

To assure that the reversibility of differentiation seen in the morphology studies and the ^{3}H-thymidine incorporation studies was not simply the selection of a DMSO resistant subpopulation within DMSOr, cells from the washout studies were kept in culture and retreated with DMSO. ^{3}H-thymidine incorporation of cells from the 144 hour washout point retreated with DMSO is shown in Fig. 8. These cells (D6) exhibited an antiproliferative response to DMSO treatment similar to DMSOr or HL-60. A similar response was obtained with cells from the 3 day washout point (data not shown) indicating that the observed phenomena do not represent clonal selection of a DMSO differentiation resistant cell population from within DMSOr.

The ability of cells to maintain the washout phenotype is depicted in Fig. 9, where cells were taken through two cycles of differentiation and reproliferation. DMSOr cells maintained reversible differentiation for two continuous cycles as evidenced by the thymidine incorporations and by morphology (data not shown). If the DMSOr cells were subjected to a third round of sequential

Figure 8. ^{3}H-thymidine incorporation in D6 following treatment with DMSO. The results are presented as mean percentage of control incorporation. The standard deviation for each point is no greater than 10% of the mean. The time of treatment with DMSO is shown along the X axis.

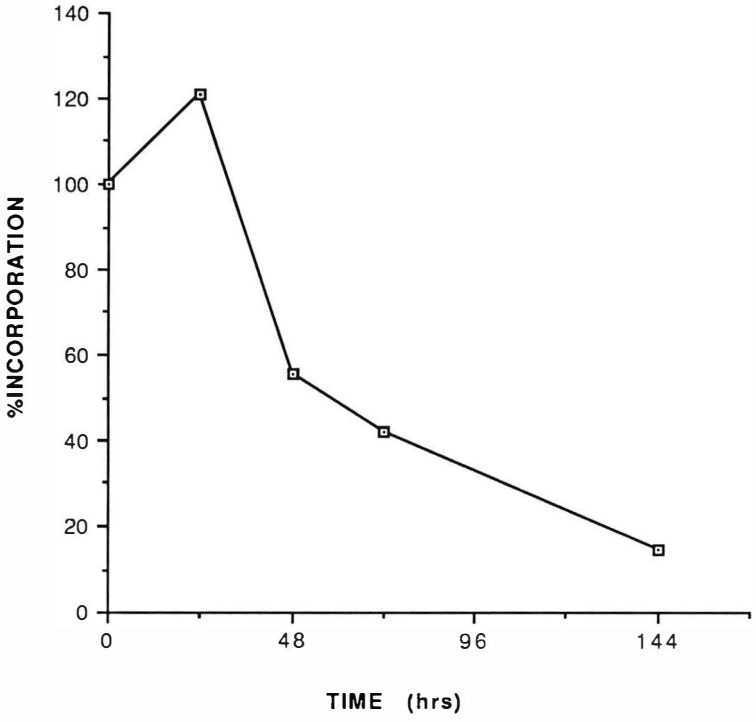
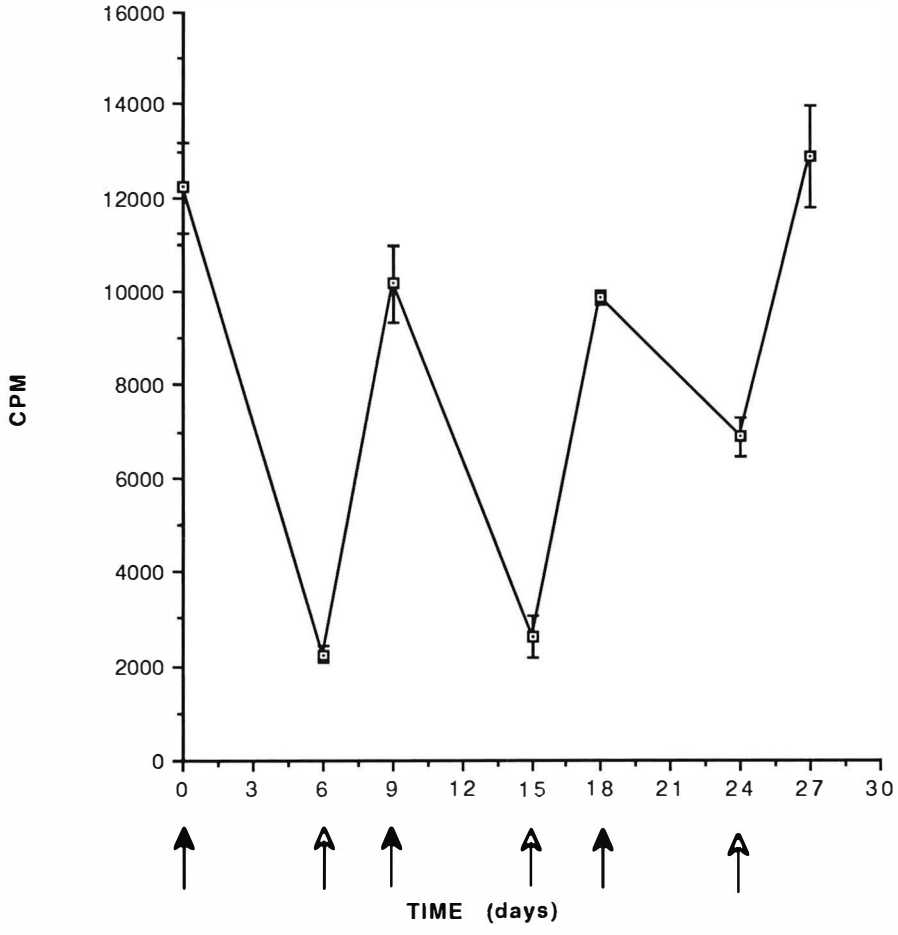

140
120
100
80
60
40
20
0
%INCORPORATION
0
48
96
144
TIME (hrs)

Figure 9. ^{3}H-Thymidine incorporation in DMSOr following three successive DMSO treatment and washouts. The initiation of each six day DMSO treatment is marked with a solid arrow, while the start of each washout period is marked with a open arrow. The results are presented as average counts per minute (cpm) with error bars representing standard deviation. The time of treatment with DMSO or reculture period is shown along the X axis.

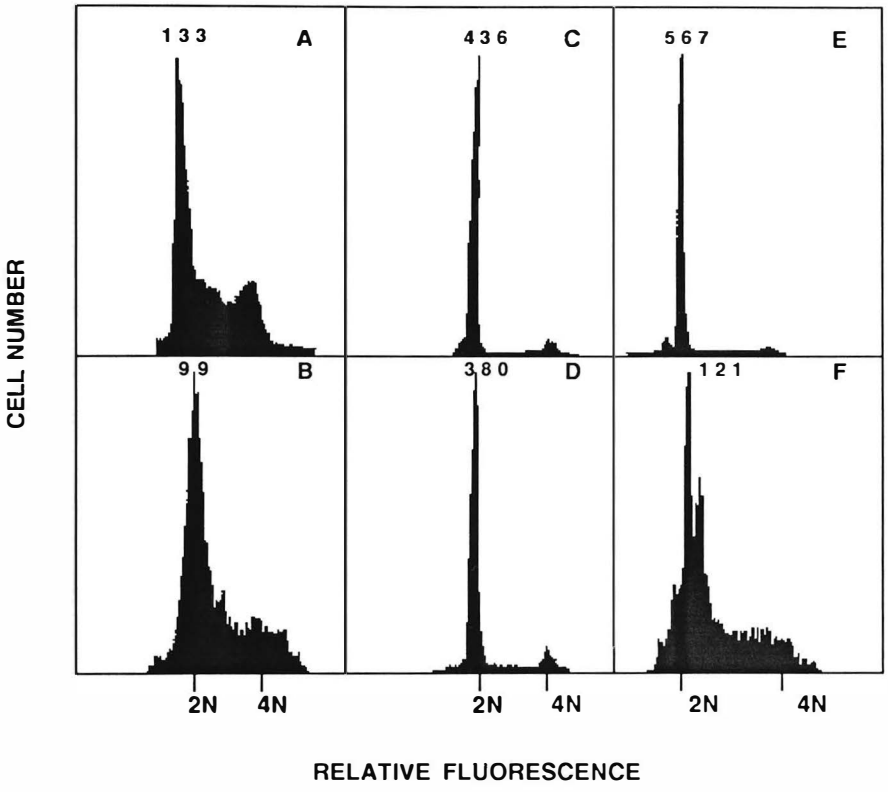

16000
14000
12000
10000
8000
6000
4000
2000
0
CPM
0
3
6
9
12
15
18
21
24
27
30
TIME (days)

DMSO treatment, the cells would begin to exhibit resistant characteristics (Fig. 9). This is probably due to the near continuous exposure of the cells to DMSO for greater than three weeks, which may be enough to select for cells that have reverted to the phenotype they expressed when the line was originally established.

^{3}H-thymidine incorporations represent measures of both the number of cells in S phase of the cell cycle at a given time as well as the rate of DNA synthesis or repair. To obtain a more detailed analysis of the antiproliferative effects following the induction of differentiation and reversibility of this process in DMSOr, cell cycle analysis by flow cytometry was performed (Fig. 10). CELLFIT analysis of DMSO treated HL-60 and DMSOr cells demonstrate that withdrawal from cell cycle occurs by 72 hours following treatment (data not shown). This withdrawal is established to a greater extent in HL-60, with 84.2% of the cells in the G_0/G_1 peak, while DMSOr has 67% of the population in the resting stage at this point. At 144 hours each cell line has greater than 85% of the cells in the G_0/G_1 peak and less than 8% of the cells in S phase (Fig. 10c&10d). Washout studies confirmed the reversibility of these cell cycle events in DMSOr with a return of cells to the cell cycle characterized by an increase in the number of cells in S phase (Fig. 10f). This was not seen to any appreciable extent in the parent HL-60 cell line (Fig. 10e).

Superoxide Anion Production in HL-60 and DMSOr.

Figure 10. Cell cycle analysis of HL-60 and DMSOr. Cell cycle analysis was performed on a Becton-Dickenson FACSCAN and the data was analyzed using the CELLFIT software. The graphs represent the histograms generated by the analysis for the following treatments: 2N represents the G_0/G_1 peak and 4N the G_2/M peak. The number above the G_0/G_1 peak is the height of that peak. A. HL-60 untreated. B. DMSOr untreated. C. HL-60 144 hour treatment with DMSO. D. DMSOr 144 treatment with DMSO. E. HL-60 144 hour treatment with DMSO followed by a 3 day reculture period. F. DMSOr 144 hour treatment with DMSO followed by a 3 day reculture period. For actual percentages of cells in each phase of the cell cycle refer to the text.


133
A
436
C
567
E
99
B
380
D
121
F
2N
4N
2N
4N
2N
4N
CELL NUMBER
RELATIVE FLUORESCENCE

SUPEROXIDE PRODUCTION IN HL-60 AND DMSOr

	HL-60 U	HL-60 D	DMSOr U	DMSOr D
CONTROL	1.50[1] ± 0.77	4.14 ± 0.40	1.07 ± 0.70	0.76 ± 0.61
PMA 10^{-7} M	38.89 ± 1.08	56.14 ± 0.30	9.19 ± 0.54	56.67 ± 2.12

[1] nmoles superoxide/million cells

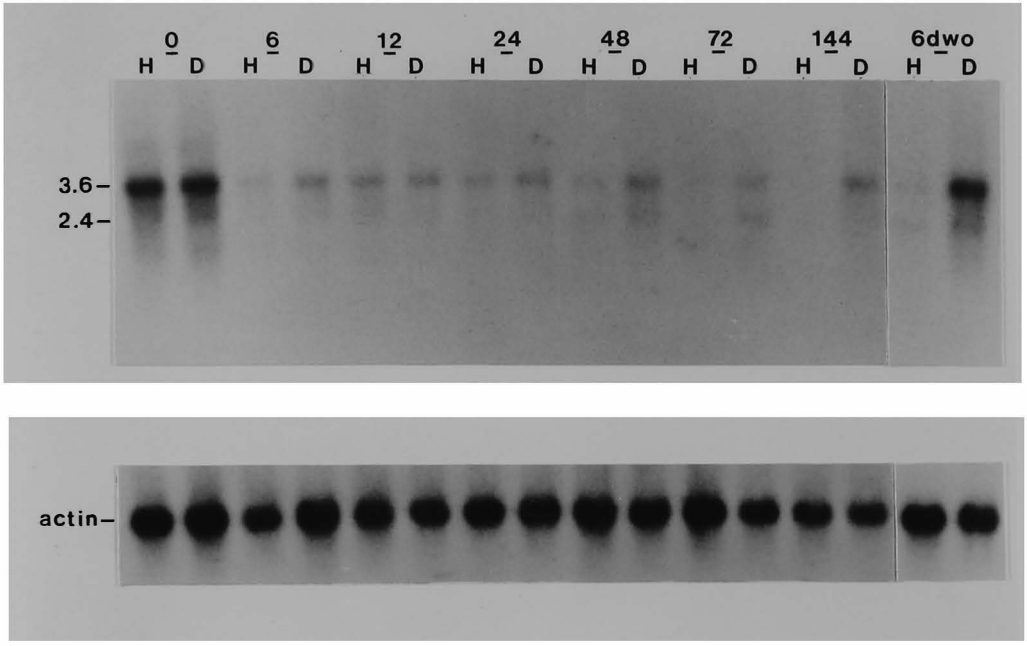
Table 5. Superoxide production in HL-60 and DMSOr. The data is presented as the mean plus or minus the standard deviation. U, Undifferentiated. D, 6 day differentiated with DMSO.

In order to determine if the antiproliferative and morphological effects seen in DMSO treated DMSOr were simply a toxic response as opposed to a differentiation response, a functional assay of differentiation was employed. The production of superoxide anion is a response that is enhanced upon the differentiation of HL-60 toward granulocytes. Superoxide anion is released into the media when the cells are treated with the phorbol ester, phorbol myristate acetate (PMA). This is due to the activation of the NADPH oxidase enzyme system which is present in the mature cells. Some superoxide release was induced from the undifferentiated HL-60 and DMSOr cells. The amount of release from the differentiated cells however was greatly increased and virtually equal between the two cell lines (Table 5).

Expression of c-*myb* During Differentiation of HL-60 and DMSOr.

A comparison of c-*myb* expression during DMSO induced differentiation was performed by isolating total cellular RNA at selected time points and subjecting them to Northern blot analysis. Fig. 11 shows a Northern blot probed with the 1.1 kb BamHI fragment of pMbm1. This probe spans a portion of the coding region of c-*myb* (exon 9 to exon 12), and is therefore a useful probe for measuring the expression of most of the alternative splice forms of the message. The steady state level of expression in HL-60 and DMSOr is equal in untreated cells (time 0 lanes). Each cell line shows a sharp decrease in c-*myb* steady state levels 6 hrs into the DMSO induction with a partial recovery by 12 hrs. In HL-60, c-*myb* expression begins to decline again at the 48 hr time point, with little

Figure 11. Northern blot analysis of c-*myb* expression during DMSO induced differentiation of HL-60 and DMSOr. The probes used in this experiment were pMbm1 (c-*myb*)(Westin et al., 1990)and pLK221 (ß-actin)(Gunning et al., 1983). H represents HL-60 while D represents DMSOr. The time of treatment is given above the sample in hours, with 6dwo a 144 day treatment with DMSO followed by a 3 day reculture period.


0
6
12
24
48
72
144
6dwo
H D H D H D H D H D H D H D H D
3.6–
2.4–
actin–

expression at 72 hrs and no expression of c-*myb* by 144 hrs. In contrast, DMSOr expression is diminished but can still be detected at the 144 hr time point. Following removal of the DMSO at the 144 hr time point followed by a 3 day reculture period, slight c-*myb* re-expression can be detected in HL-60, while control levels are present in DMSOr. Similar data was obtained with cells treated for only 72 hrs with DMSO before washout. (data not shown). A 2.4 kb. message is also detected with this probe. This yet undefined mRNA appears to be up-regulated with respect to the c-*myb* mRNA represented by the 3.6 kb. band at the later time points of differentiation. The up-regulation occurs in each cell line at the 48 hr time point to the same extent.

This difference in expression of c-*myb* between parental HL-60 and DMSOr was not seen with all the splice forms of c-*myb*, during DMSO induced differentiation. In Fig. 12, a Northern blot of a differentiation time course was probed with a splice variant of c-*myb* termed pMbm28. This cDNA clone was isolated from an HL-60 library and is altered by a truncated 3' end of the mRNA. Expression of pMbm28 is discordant with other c-*myb* clones in that it does not change significantly during the entire differentiation process. It should also be noted that the size (2.0 kb) and the pattern of expression of pMbm28 does not correspond to the 2.4 kb message present in Fig 11. The patterns of expression of pMbm28 are similar between HL-60 and DMSOr, suggesting that the protein encoded by this splice form of c-*myb* may not play a significant role in determining the phenotype of DMSOr.

Figure 12. Northern blot analysis of the expression of the pMbm28 clone of c-*myb* (Westin, 1991)during DMSO induced differentiation of HL-60 and DMSOr. H represents HL-60 while D represents DMSOr. The time of treatment is given above each sample in hours.

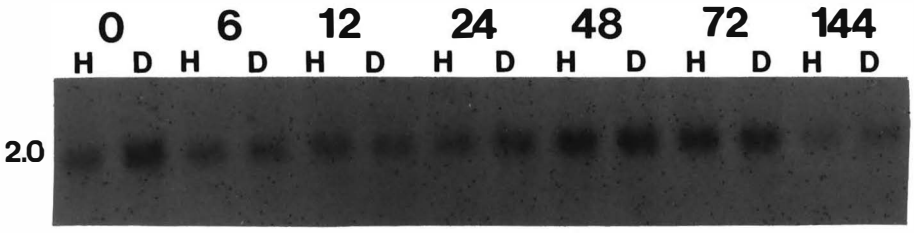
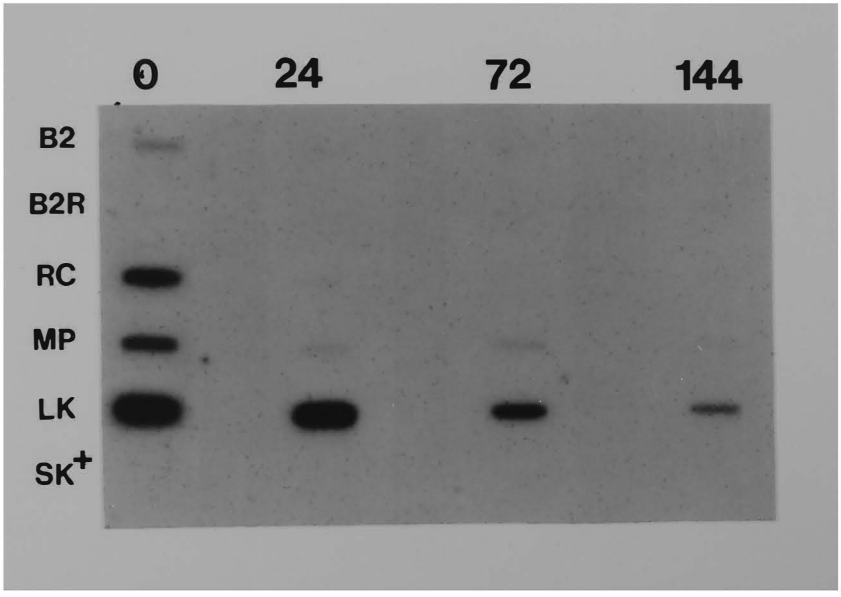

0
6
12
24
48
72
144
H D H D H D H D H D H D H D
2.0

Figure 13. Nuclear run-on analysis of DMSOr. Nuclei were isolated from DMSOr cells at the given time points (in hours), and transcription of selected genes was determined. For a description of the target DNAs please refer to figure 5.


0
24
72
144
B2
B2R
RC
MP
LK
SK+

Transcriptional Regulation of c-*myb* in DMSOr.

To determine if the aberrant expression of c-*myb* in DMSOr is at the transcriptional level, nuclear run-on analysis was performed. Nuclei were isolated from cells at the time points indicated in Fig. 13, and ^{32}P-UTP was incorporated into the nascent transcripts *in vitro*. The radiolabelled RNA was isolated and hybridized to DNA targets (defined in Fig. 5) that are fixed to the filter by a slot blotter. The results indicate that c-*myb* (B2 probe) is regulated at the transcriptional level, as well as c-*myc* (RC probe) and myeloperoxidase (MP probe). The kinetics of the transcriptional regulation of c-*myb* are similar to that of the parental HL-60 line (Fig. 22). It is therefore unlikely that transcriptional regulation is the mechanism of altered c-*myb* expression DMSOr.

Expression of B-*myb* During HL-60 and DMSOr Differentiation.

To examine B-*myb* regulation during HL-60 differentiation and to determine if it is the 2.4 kb message, a Northern blot was probed with an oligonucleotide designed to hybridize to B-*myb* based on the published sequence(Nomura et al., 1988) (Fig. 14). The hybridizing message is 2.8 kb in size and showed an expression pattern that did not correspond to the 2.4 kb band seen in Fig. 10. The pattern of expression of B-*myb* in both HL-60 and DMSOr was one of down regulation at 48 hours. This is consistent with the pattern of expression reported in other leukemic cell lines and normal hematopoietic cells(Golay et al., 1991). Unlike c-*myb* expression, there was also no difference in the regulation of B-*myb*

by DMSO between HL-60 and DMSOr. A-*myb* expression was examined but not detected in HL-60 or DMSOr (data not shown).

Expression of Genes Associated With the Cell Cycle.

The previous results demonstrated an altered expression of c-*myb* but not B-*myb* in DMSOr cells in response to DMSO. To determine if this altered expression was unique to c-*myb*, additional genes were also tested. The genes tested were either involved in cell cycle control, or were cell cycle regulated, since c-*myb* is cell cycle regulated and there were slight differences in the thymidine incorporation, and cell cycle experiments between HL-60 and DMSOr. A delay in the down regulation of expression of cdc2 kinase, cyclin B, and histone H4, was similar to that of c-*myb* in DMSOr, while ornithine decarboxylase gene expression was not significantly affected (Fig. 15). The parental cell line showed a significant decline in the expression of all these genes by 72 hrs, with completely ablated expression by 144 hrs. The 72 hr time point represents the beginning of the period of non-mitotic differentiation, so down regulation of these genes at this point would be expected. These data suggest that the genetic alteration in DMSOr may affect the control of genes that are important in cell cycle regulation.

Effect of Antisense Oligodeoxynucleotides on HL-60 and DMSOr.

To determine if continued c-*myb* expression was essential for the phenotype of DMSOr, antisense oligodeoxynucleotides against c-

Figure 14. Northern blot analysis of B-*myb* expression during DMSO induced differentiation of HL-60 and DMSOr. The probe used in this experiment is the 30 base oligonucleotide B*myb*.1. H, HL-60 and D, DMSOr. Each time point appears centered above the two lanes.

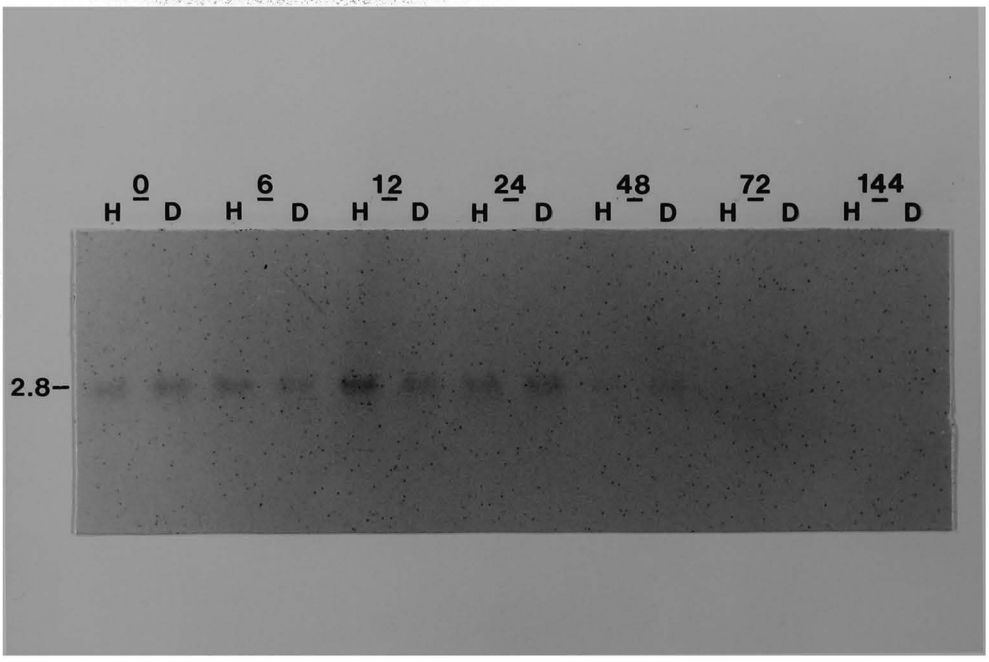
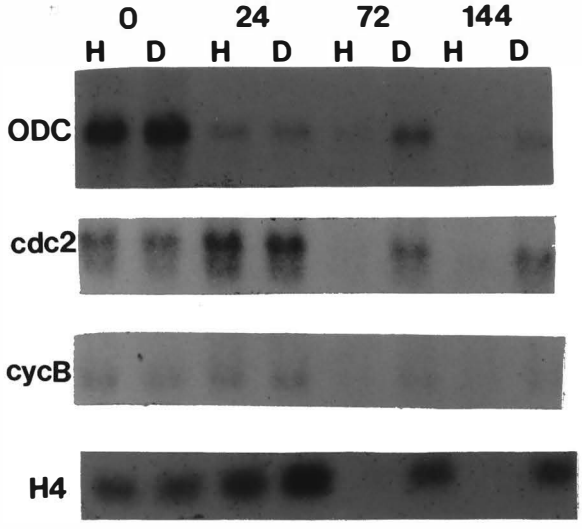

0
H D
6
H D
12
H D
24
H D
48
H D
72
H D
144
H D
2.8–

Figure 15. Northern blot analysis of cell cycle related genes during DMSO treatment of HL-60(H) and DMSOr(D). The probes used for these studies were the 2.0 Eco RI fragment of pODC10/2H(ornithine decarboxylase-ODC)(Hickok et al., 1987), the 30 nucleotide oligomers cdc2.1 and cycB.1 that hybridize to the mRNA for cdc2 kinase and cyclin B respectively and the 1.8 kb fragment of pMUSH4 (histone H4)(Seiler-Tuyns and Birnsteil, 1981). The time of treatment (in hours) with DMSO is centered above the two lanes.


0
24
72
144
H D H D H D H D
ODC
cdc2
cycB
H4

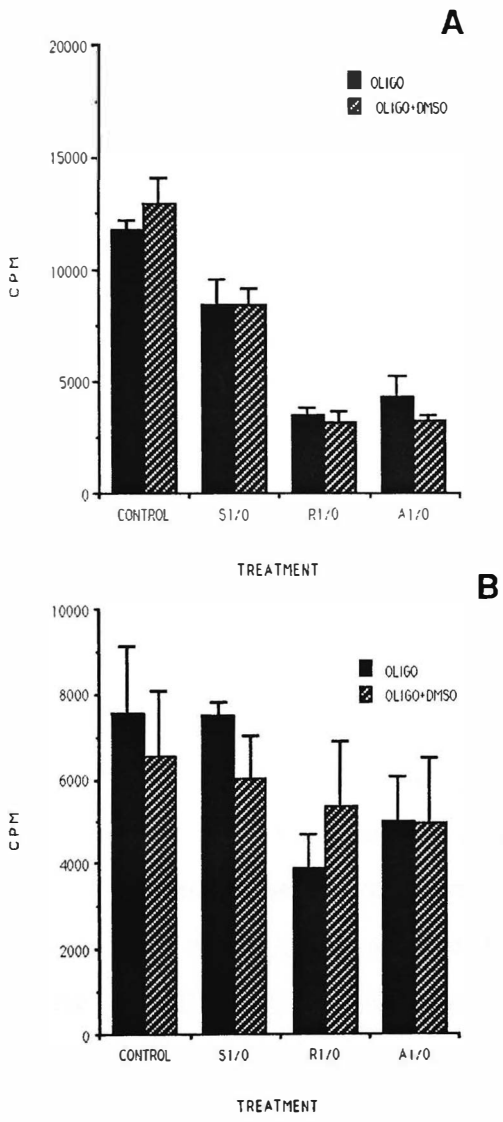
myb were synthesized and used in the presence or absence of DMSO. Both HL-60 and DMSOr cells were treated for 24 or 72 hrs (72 hr data not shown) with vehicle (control), Smbm1/0 (sense control), Rmbm1/0 (randomized antisense sequence control), and Ambm1/0 (antisense oligo that can bind at the translation start site of most forms of the protein). None of the oligonucleotides had any significant effect on the DMSOr cells as measured by tritiated thymidine incorporation (Fig. 16). The antisense oligodeoxynucleotide did not alter the effect of DMSO either, but the significance of the data must be questioned because of the effect of Rmbm1/0 on the parental HL-60. This data suggests that the oligodeoxynucleotides may have non-specific effects on cell proliferation unrelated to the effects of antisense oligonucleotide on c-*myb*.

Determination of the Level of Regulation of the Human c-*myb* Proto-oncogene during Hematopoietic Cell Differentiation.

Strategy and Definition of Probes.

To determine the level of regulation of c-*myb* during differentiation of HL-60, the changes in mRNA levels as determined by Northern blot analysis was compared to Nuclear Run-on analysis. The first four probes depicted in Fig. 5 are single stranded targets for the 5' untranslated region through the first portion of intron 1 that can detect both sense and antisense transcription. The region covered by probe A and B contains promoter activity and the majority of the mRNA start sites(Westin, 1991) The murine attenuator would map

Figure 16. The effect of antisense oligonucleotides on ^{3}H-thymidine incorporation in HL-60(A) and DMSOr(B). All treatments were for 24 hours. Ambm1/0 and Smbm1/0 are 15mers derived from sequence 5 bases downstream from the translational start site of the prototypic *myb* message. Ambm1/0(A1/0) would bind to sense strand message and Smbm1/0(S1/0) to a complementary message. Rmbm1/0(R1/0) was generated by randomizing the Ambm1/0 sequence. The data is presented as counts per minute (CPM) on the y-axis and treatment on the x-axis. Cells were treated with an oligonucleotide or vehicle (TE, Control) in the presence (striped bars) or absence (solid bars) of 1.25% DMSO.


A
20000
15000
10000
5000
0
CPM
OLIGO
OLIGO+DMSO
CONTROL
S1/0
R1/0
A1/0
TREATMENT
B
10000
8000
6000
4000
2000
0
CPM
OLIGO
OLIGO+DMSO
CONTROL
S1/0
R1/0
A1/0
TREATMENT

at the end of probe C. The "post-attenuator" probes are the single stranded targets B2 and B2R. This probe is derived from the cDNA pMbm1 and spans a 1.1 kb region from the end of exon 9 into exon 12. This cDNA fragment was also used for probing the Northern blots. Probe E from Fig. 5 is present in one experiment. This probe could also serve as a post-attenuator probe, but it does not hybridize well and its use was discontinued in later experiments. The other targets used in the Run-on analysis are for the third exon of c-myc (RC), a full length cDNA of ß-actin (LK) and single stranded vector (pBluescript) controls for non-specific binding (+/-).

Transcriptional Regulation of c-*myb* by Retinoic Acid and Vitamin D3.

Retinoic acid induces myeloid differentiation of HL-60 cells over a 6 day period while vitamin D3 induces monocytic differentiation over the same time course. Both of these agents induce similar changes in c-*myb* message over this time (Figure 17a and 18a). The message levels decline in a time dependent fashion with a near complete loss of message at the 144 hr time point for both agents. Nuclear run-on transcription was analyzed at the same time points for each of these agents (Figure 17b and 18b) and these experiments yielded three observations. First the transcription at the promoter and early region of c-*myb* was unchanged during the time course of differentiation with respect to ß-actin transcription (probes A and C), suggesting a constitutive promoter. Second, antisense transcription is present in the 5' region of the gene

Figure 17. Regulation of c-*myb* by retinoic acid. A) Northern blot analysis of c-*myb* expression during retinoic acid induced differentiation of HL-60. This blot was probed with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990). The time of treatment is given above the lane. B) Nuclear run-on analysis of c-*myb* transcription during retinoic acid induced differentiation of HL-60. The key to the slot blot targets is presented in Fig. 5. The time of treatment (in hrs) is presented at the top of each blot.

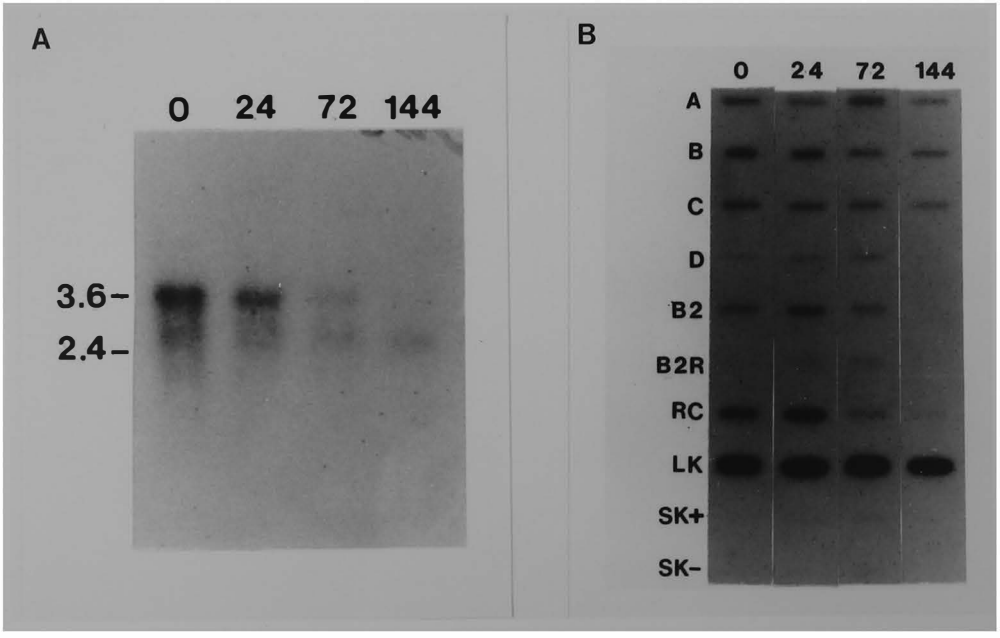
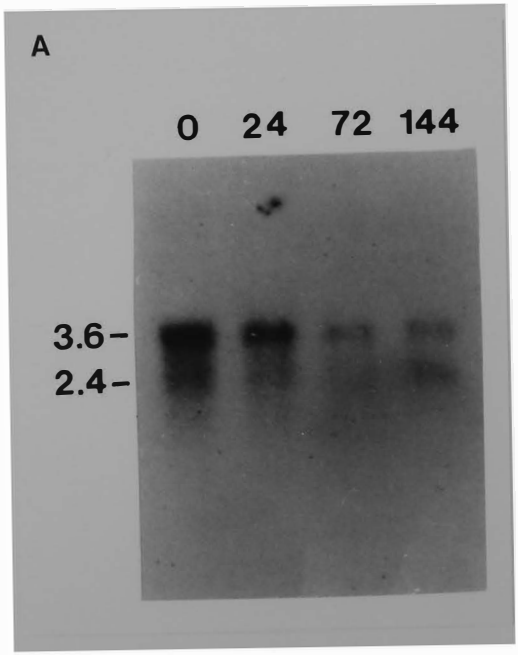
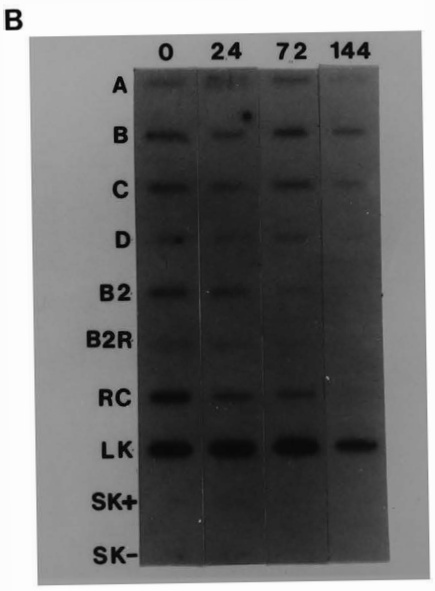

A
0
24
72
144
3.6–
2.4–
B
0
24
72
144
A
B
C
D
B2
B2R
RC
LK
SK+
SK–

Figure 18. Regulation of c-*myb* by vitamin D3. A) Northern blot analysis of c-*myb* expression during vitamin D3 induced differentiation of HL-60. This blot was probed with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990). The time of treatment is given above the lane. B) Nuclear run-on analysis of c-*myb* transcription during vitamin D3 induced differentiation of HL-60. For a description of the slot blot targets, refer to Fig. 5. The time of treatment (in hrs) is presented at the top of each blot.


A
0
24
72
144
3.6-
2.4-


B
0
24
72
144
A
B
C
D
B2
B2R
RC
LK
SK+
SK-

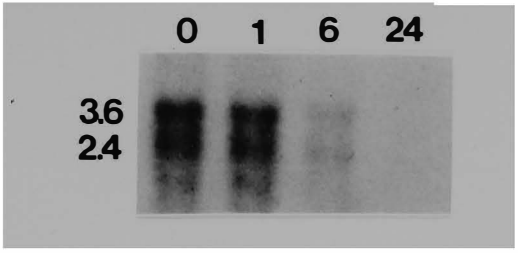
(probes B and D) but not the 3' end (probe B2R). Finally, the change in transcription due to differentiation with both retinoic acid and vitamin D3 is due to an attenuation of transcription. This is evident in the change in transcription in the 3' end of the gene (probe B2). When the Northern blots and nuclear run-ons were compared by densitometry, the down-regulation of steady-state message correlated with the decrease in transcription in the 3' region of the gene (Fig. 21a and b). It can be concluded that attenuation is the prevailing mechanism of c-*myb* down regulation by retinoic acid and vitamin D3.

Phorbol Ester Regulation of c-*myb* During HL-60 Differentiation.

PDbu induces monocytic differentiation of HL-60 cells over a 24 hr time course, with changes in steady c-*myb* message detectable in 1 hr and significantly reduced by 6 hrs (Fig. 19a). Nuclear run-on analysis revealed that the levels of read-through transcription at 6 hr was greater than would be expected from the change in steady state message at this time point (Fig. 19b). This suggests that attenuation is not the only mechanism of regulation activated by PDbu. One possibility is that c-*myb* is regulated by PDbu at the post-transcriptional level. To investigate post-transcriptional regulation as a potential mechanism of PDbu regulation, the Northern blot in Fig. 20 shows the effect of Actinomycin D on changes in c-*myb* steady message induced by PDbu. This blot compares the effect of PDbu (P), Actinomycin D (A), or the combination (PA) on c-*myb* expression. The decline in c-*myb*

Figure 19. Regulation of c-*myb* by phorbol dibutyrate. A) Northern blot analysis of c-*myb* expression during phorbol dibutyrate induced differentiation of HL-60. This blot was probed with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990). The time of treatment is given above the lane. B) Nuclear run-on analysis of c-*myb* transcription during phorbol dibutyrate induced differentiation of HL-60. The key to the slot blot targets is presented in Fig. 5. The time of treatment (in hrs) is presented at the top of each blot.

A



B

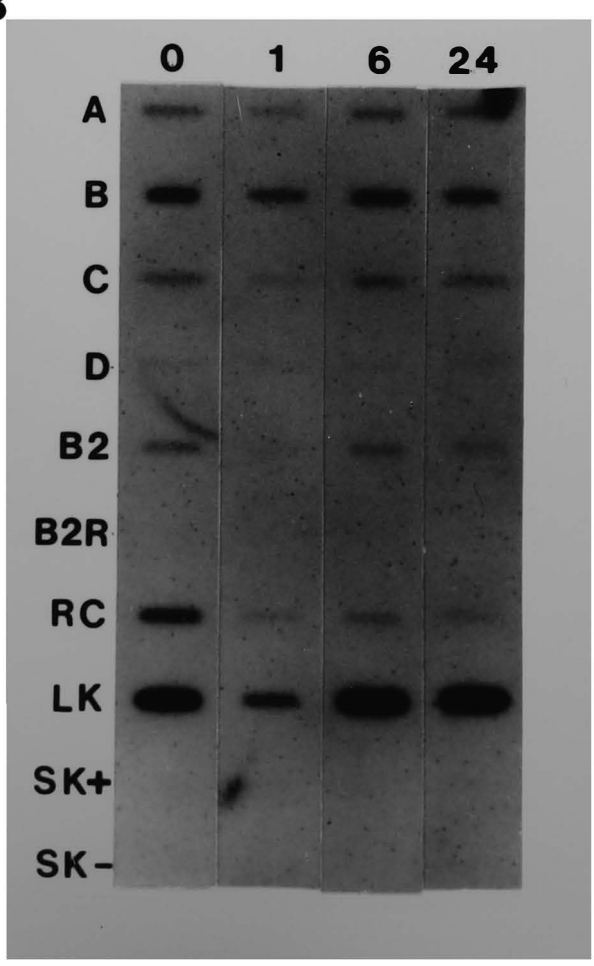


Figure 20. Post-transcriptional regulation of c-*myb* by phorbol dibutyrate. HL-60 cells were treated with phorbol ester (P), actinomycin D (A) or the combination of the two (P+A), for up to 3 hrs. The Northern blot was probed with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990).

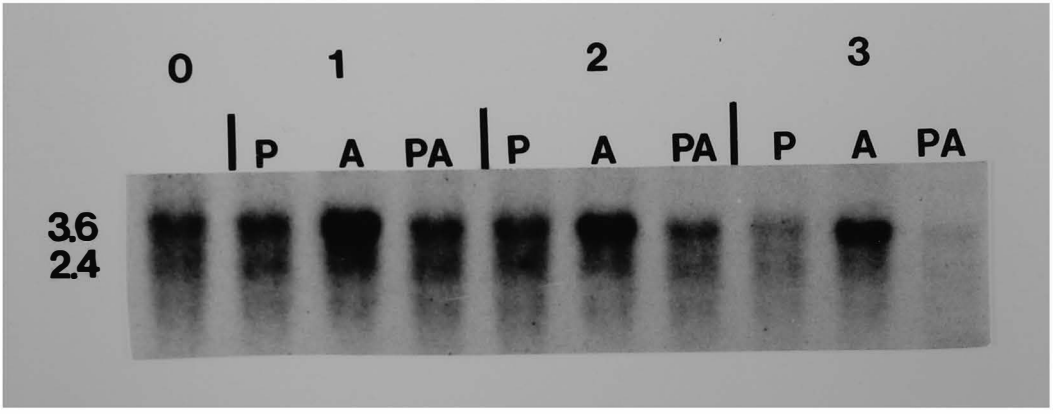
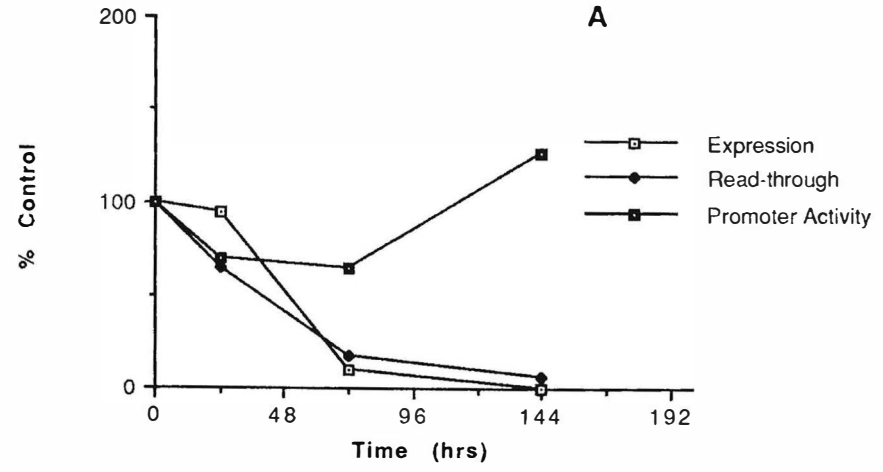
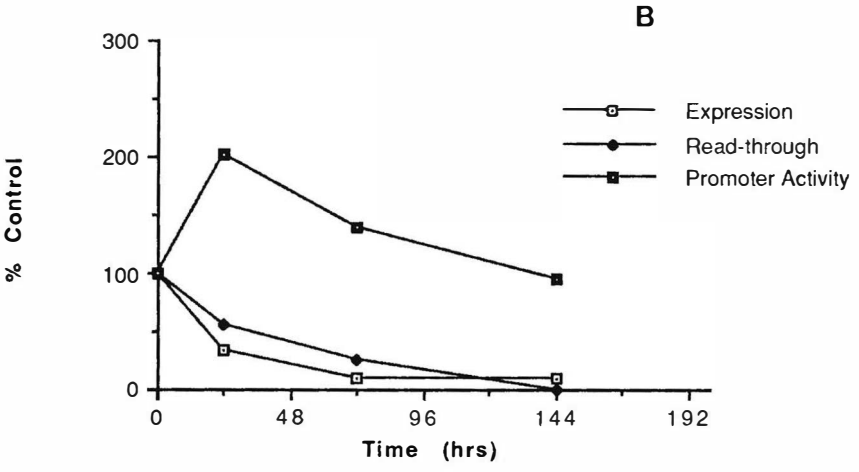
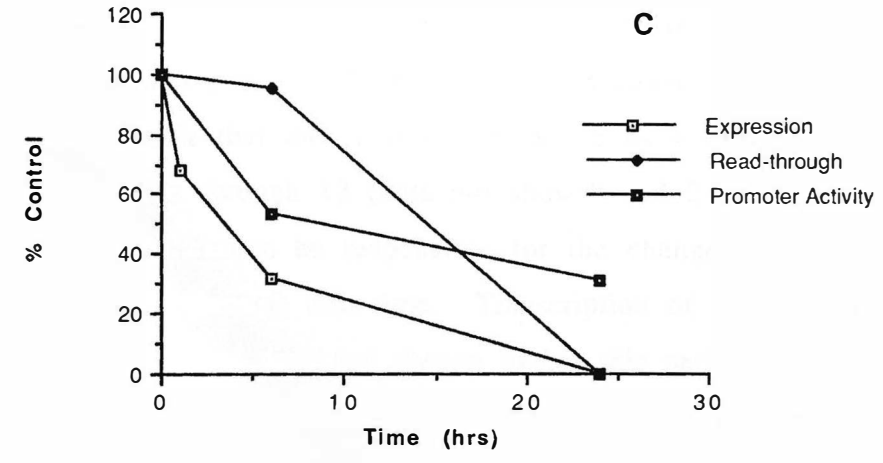

0
1
2
3
P
A
PA
P
A
PA
P
A
PA
3.6
2.4

Figure 21. Densitometric analysis of Northern blots and nuclear run-ons. All blots were scanned and normalized to the actin control for comparisons. The data is presented as percent of control promoter activity ([(A/LK)/(A_0/LK_O)]x100)(closed boxes),percent of control read-through transcription ([(B2/A)/($B2_0$/A_0)]x100)(diamonds) and percent of control steady state expression ((RNA/RNA_0)x100)(open boxes). A) Retinoic acid induced changes. B) Vitamin D3 induced changes. C) Phorbol dibutyrate induced changes.


A
200
100
0
% Control
0
48
96
144
192
Time (hrs)
Expression
Read-through
Promoter Activity


B
300
200
100
0
% Control
0
48
96
144
192
Time (hrs)
Expression
Read-through
Promoter Activity


C
120
100
80
60
40
20
0
% Control
0
10
20
30
Time (hrs)
Expression
Read-through
Promoter Activity

message due to PA is greater than that of A alone suggesting that c-*myb* is controlled at the post-transcriptional level by phorbol esters.

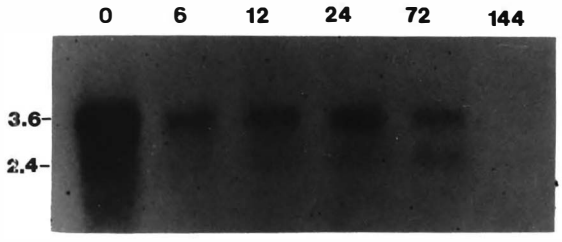
A second potential mechanism of PDbu induced regulation was revealed by comparing the densitometric analysis of changes in steady state mRNA to changes in transcription of the region of initiation. In Fig. 21c, the changes in message levels are compared with changes in transcription of the 5' untranslated region of c-*myb*. When these transcriptional changes are normalized to the constitutively transcribed actin gene, there is a good correlation with changes in steady state message from 0 to 6 hrs. This correlation suggests that PDbu can induce a decrease in the initiation of transcription as well, presumably by regulation of the c-*myb* promoter.

The Biphasic Regulation of c-*myb* by DMSO at the Transcriptional and Post-transcriptional Level.

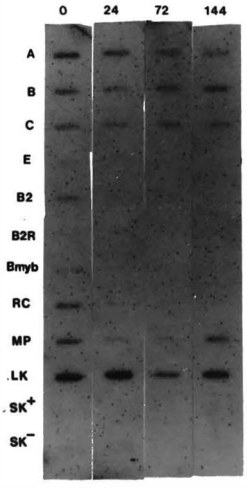
DMSO induced differentiation of HL-60 cells produces a biphasic pattern of regulation of c-*myb* expression. This is similar to the pattern observed in FMEL differentiation, with a sharp decrease in c-*myb* expression at 6 hr followed by an increase in expression that peaks at 24 hr (Fig. 22a)(Watson, 1988b). Run-on analysis reveals that the attenuator is active by 6 hr (Fig. 26b) and remains active through 12 (data not shown) and 24 hr (Fig. 22b) and therefore cannot be responsible for the changes in steady state mRNA levels through this time. Transcription of the 5' untranslated region of the gene does not change during this period, thus it is possible that post-transcriptional mechanisms may be present. To

Figure 22. Regulation of c-*myb* by DMSO. A) Northern blot analysis of c-*myb* expression during DMSO induced differentiation of HL-60. This blot was probed with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990). The time of treatment is given above the lane. B) Nuclear run-on analysis of c-*myb* transcription during DMSO induced differentiation of HL-60. For a description of the slot blot targets, refer to Fig. 5. The time of treatment (in hrs) is presented at the top of each blot.

A



B



investigate the possibility of post-transcriptional mechanisms of c-*myb* regulation, Actinomycin D was employed as an inhibitor of transcription to determine if a change in mRNA stability was induced by DMSO. The pattern of c-*myb* expression suggested that if a change in mRNA stability were to occur it would be in the first 6 hours of DMSO treatment. Depicted in Fig. 23a is a Northern blot of RNA isolated from cells treated with DMSO (D), Actinomycin D (A) or the combination (AD) for a 3 hour period. When D and A are compared over this time course, DMSO alone leads to a decline in c-*myb* mRNA that was greater than that of inhibition of transcription. This suggests that a message destabilization occurred during the DMSO treatment. The DMSO induced destabilization is inhibited when cells are treated with Actinomycin D and DMSO, thus the post-transcriptional regulation of c-*myb* by DMSO is dependent on ongoing transcription. This pattern of regulation was detected for c-*myc* under similar circumstances, but not characteristic of the regulation of ornithine decarboxylase (Fig. 23b and c).

One possibility for the transcriptional dependency of c-*myb* destabilization is the induction of a protein necessary to direct mRNA degradation. To explore this, it was determined if protein synthesis was essential for post-transcriptional control by DMSO. If DMSO induces a protein that is essential for message turnover, then the protein synthesis inhibitor cyclohexamide should be able to impede the change in steady state mRNA that is due to post-transcriptional mechanisms. Cyclohexamide was not capable of

Figure 23. Post-transcriptional regulation of gene expression by DMSO. HL-60 cells were treated with DMSO (D), actinomycin D (A) or the combination of the two (DA), and samples were taken for up to 3 hrs. A) The Northern blot was probed for c-*myb* with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990) and for ß-actin (pLK221)(Gunning et al., 1983). B) Samples treated under similar conditions to part A were probed for c-*myc* expression with the Eco RI/Cla I fragment of pMC413RC(Dalla-Favera et al., 1983). C) Samples were probed with the Eco RI fragment of pODC10/2H(Hickok et al., 1987) for detection of ornithine decarboxylase.

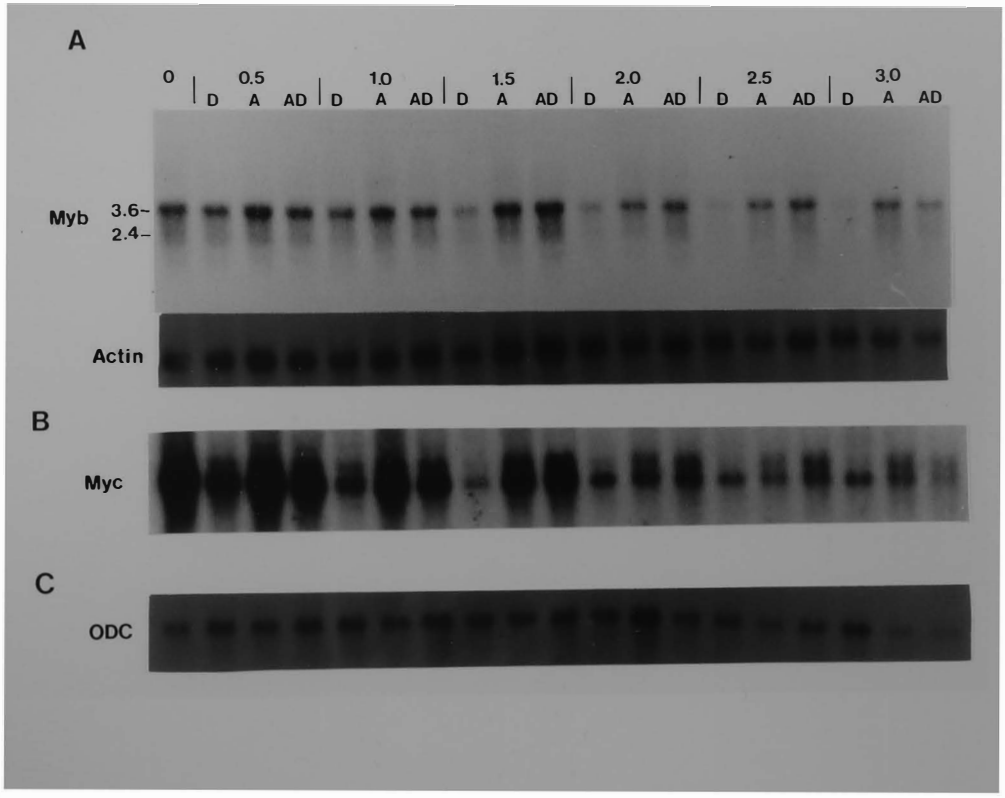
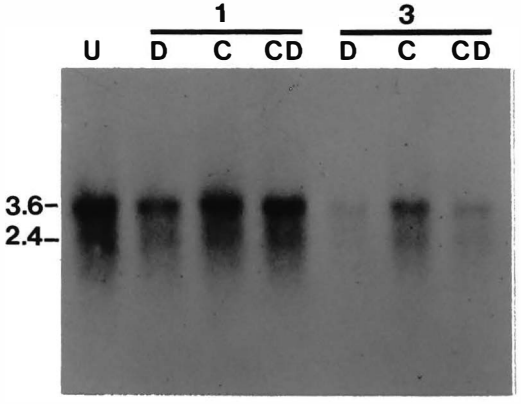

A
0
0.5
1.0
1.5
2.0
2.5
3.0
D
A
AD
Myb
3.6–
2.4–
Actin
B
Myc
C
ODC

Figure 24. The effect of cyclohexamide on c-*myb* expression and DMSO induced changes in c-*myb* expression in HL-60. Expression of c-*myb* was detected in HL-60 cells that were untreated (U), treated with DMSO (D), treated with cyclohexamide (C) or treated with DMSO and cyclohexamide (DC). The time of treatment is centered above the sample. C-*myb* detection was with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990).

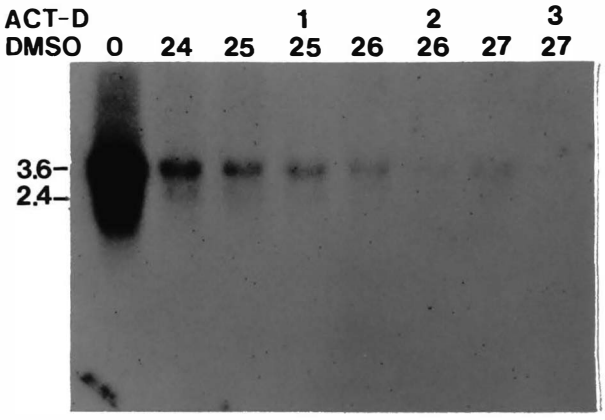

1
3
U
D
C
CD
D
C
CD
3.6–
2.4–

superinducing c-*myb* nor could it significantly affect DMSO induced changes at 1 or 3 hrs (Fig. 24). Similar results were obtained at 6 hrs (see Fig. 26a), thus the post-transcriptional regulation of c-*myb* by DMSO does not require protein synthesis.

If a message destabilization is responsible for a portion of the regulation of c-*myb* during the first six hours of DMSO induced differentiation of HL-60, and there is no change in the transcriptional regulation during the first 24 hours of DMSO treatment then the increase in steady state *myb* mRNA from 6 to 24 hours may reflect a restabilization of message. This is impossible to determine because an accurate half-life of c-*myb* message during the first 3 hrs of DMSO treatment cannot be obtained due to the transcriptional dependency of the regulation. If the transcriptional dependency is a cross talk mechanism between transcriptional and post-transcriptional regulation, then transcriptional dependency would not be necessary following the initial period of message destabilization. This was tested by adding Actinomycin D to HL-60 that had treated with DMSO for 24 hours (Fig. 25). This Northern blot demonstrates the loss of transcriptional dependency and presumably post-transcriptional regulation at the 24 hour time point. If the transcriptional dependency remained active, then the addition of Actinomycin D would not enhance changes in steady state c-*myb* mRNA as in Fig. 23a.

Effect of Cyclohexamide on Transcriptional Control of c-*myb*.

Figure 25. Post-transcriptional regulation of c-*myb* by DMSO following 24 hrs of DMSO induced differentiation. HL-60 cells were pretreated with DMSO for 24 hrs, then either (D), actinomycin D (A) or the combination of the two (DA), for up to 3 additional hrs. The Northern blot was probed with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990).


ACT-D
DMSO 0 24 25 1 25 26 2 26 27 3 27
3.6–
2.4–

Proteins have been implicated in the control of the process of attenuation(Reddy and Reddy, 1989). In an attempt to make a gross observation on the need for protein synthesis on the attenuator that regulates c-*myb*, cells were treated with cyclohexamide (CHX) in the presence or absence of DMSO. The cells were treated for 6 hrs when total cellular RNA and nuclei were isolated and the Northern blot and nuclear run-on analyses are presented in Fig. 26. The Northern blot (Fig. 26a) demonstrates a similar pattern of steady state c-*myb* levels following the 6 hr treatment as that seen in Fig. 24, where DMSO caused a decline in c-*myb* message that is not affected by CHX. CHX alone also has minimal effect on c-*myb* expression following a 6 hr treatment. The nuclear run-on experiments yielded a different result (Fig. 26b), where CHX caused the activation of the attenuator in a similar fashion as DMSO. This result suggests that ongoing translation may be important for read-through transcription of the attenuator of c-*myb*.

One possibility for this requirement is the presence of an anti-terminator protein. Such proteins have been postulated to exist and would assist the RNA polymerase to pass premature termination sites(Adamkiewicz et al., 1990). To determine if an anti-terminator protein may bind to the first intron of the c-*myb* gene, and if this binding was inhibited by CHX, electrophoretic mobility shift assays (EMSA) were performed. The targets for the EMSA were selected by evolutionary conservation between the murine and human intron 1 sequences (the human sequence and the comparison was

Figure 26. Regulation of c-*myb* by DMSO and/or cyclohexamide. A) Northern blot analysis of c-*myb* expression during treatment with DMSO, cyclohexamide (CHX) or the combination (DMSO+CHX). This blot was probed with the 1.1 kb Bam HI fragment of pMbm1(Westin et al., 1990). The time of each treatment is 6 hrs. B) Nuclear run-on analysis of c-*myb* transcription during treatment with DMSO, cyclohexamide (CHX) or both (DMSO+CHX). For a description of the slot blot targets, refer to Fig. 5. The time of treatment is 6 hrs.

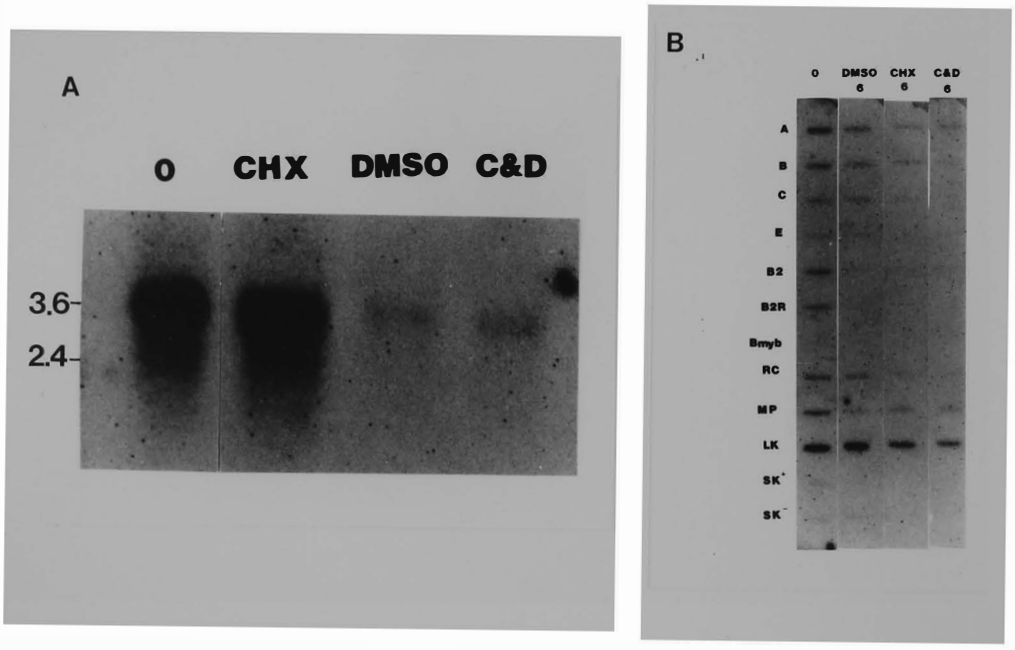

A
0
CHX
DMSO
C&D
3.6
2.4
B
0
DMSO 6
CHX 6
C&D 6
A
B
C
E
B2
B2R
Bmyb
RC
MP
LK
SK+
SK-

Figure 27. Identity map of intron 1 of c-*myb*. The map illustrates regions of identity between the murine and human forms of the c-*myb* gene. The areas of significant identity are presented as hatches regions. Three regions were shown to have significant identity and were termed regions 1, 2 and 3(Westin, 1991). The restriction sites utilized in the EMSA analysis are shown; Eco RI (Eco), Bam HI (Bam), Pvu II (Pvu), Hinc II (Hinc), Bgl I (Bgl) and Sph I (Sph).

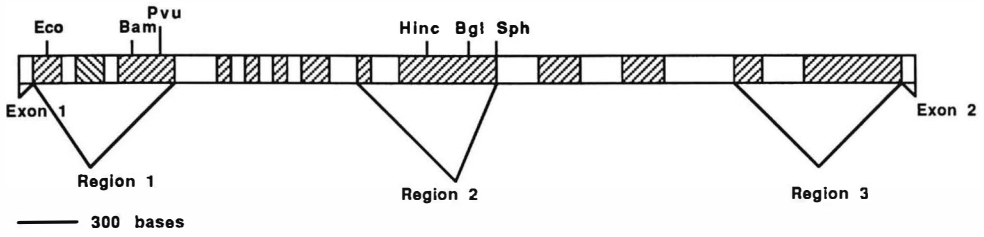
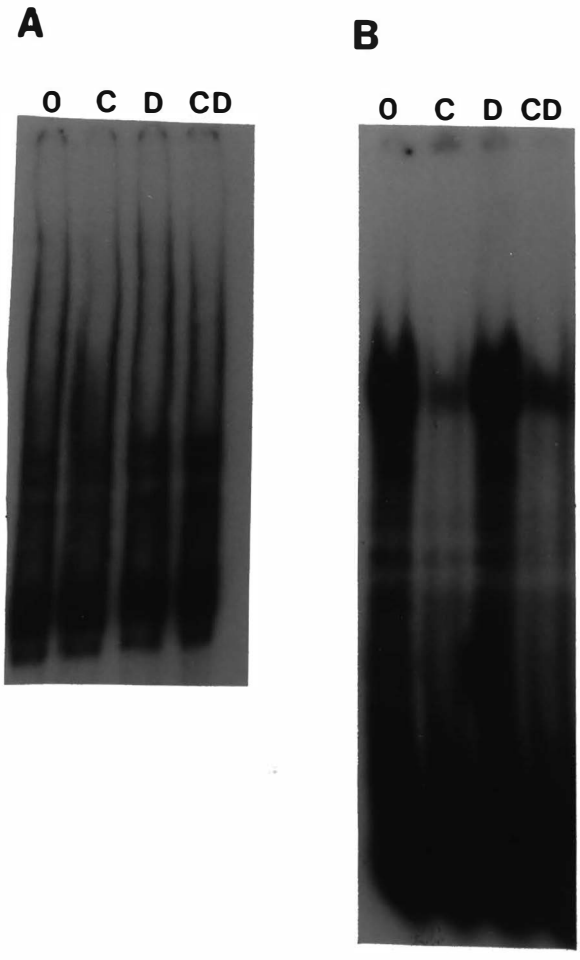

Eco
Bam
Pvu
Hinc
Bgl
Sph
Exon 1
Exon 2
Region 1
Region 2
Region 3
300 bases

Figure 28. EMSA analyses of nuclear extracts from HL-60 cells. The cells were untreated (0), treated with DMSO (D), cyclohexamide (C) or the combination (CD). HL-60 cells were treated for 6 hrs prior to isolation of nuclear extracts. A) EMSA of the 156 bp Bam HI/Pvu II fragment of intron 1 of c-*myb*. B) EMSA of the MBS-I site.


A
O C D CD
B
O C D CD

completed by Dr. Eric Westin and Miss Sarah Jacobs). A restriction map of the human intron 1 that showing the three regions of homology is depicted in Fig. 27. From the map it was decided that the two regions that might contain a protein binding site that could be important for allowing read-through transcription would be region 1 or region 2. In Fig. 28a an EMSA of the Bam HI/Pvu II (156 bp) fragment of region 1 is shown. The nuclear extracts are from cells which were treated with DMSO, CHX, or the combination for 6 hrs. None of these treatments has any effect on DNA binding of this or any of the other regions tested (the Eco RI/Bam HI fragment of region 1, the Hinc II/Bgl II and Bgl II/Sph I of region 2, data not shown). To test if the extracts were capable of supporting an EMSA, the same extracts were tested with a double stranded oligodeoxynucleotide that contains the high affinity *myb* binding site (MBS-I). The extracts were capable of shifting this fragment and the CHX inhibited such shifting, presumably by inhibiting the synthesis of c-*myb* protein (Fig. 28b).

Characterization of a Low Molecular Weight c-*myb* mRNA.

In many of the Northern blots previously presented, a 2.4 kb molecular weight message is present. This mRNA appears to be regulated in an alternative fashion compared to the prototypical c-*myb* message, and is easily detected in the DMSOr cell line following treatment with DMSO (see Fig. 11). This lower molecular weight message, if it is a splice form of c-*myb* would be of great interest

Figure 29. Bam HI map of pMbm1. This schematic representation of the c-*myb* cDNA pMbm1(Westin et al., 1990), maps the Bam HI fragments with respect to the translational start site (ATG) and the spice acceptors that marker the beginning of exon 10 and exon 12. The BAM 1 fragment is used as a probe in Fig. 30, while the BAM 2 fragment is used as a probe on all of the previous Northern blots.

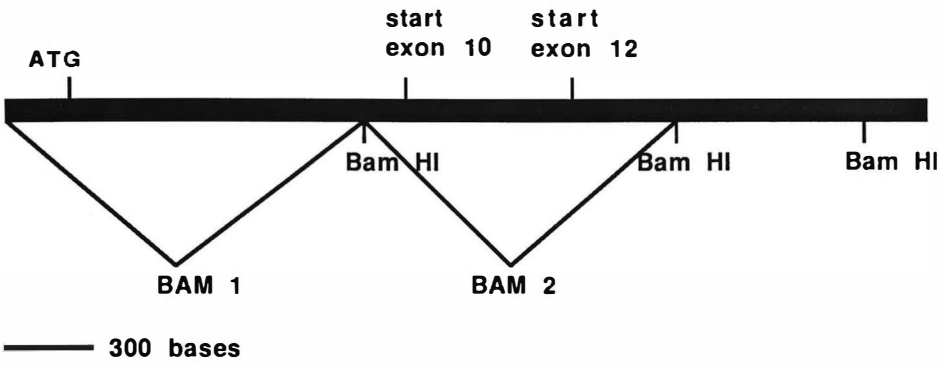
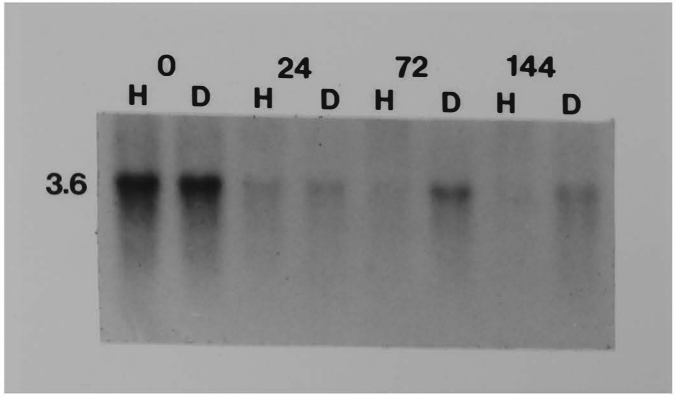

start
exon 10
start
exon 12
ATG
Bam HI
Bam HI
Bam HI
BAM 1
BAM 2
300 bases

Figure 30. Northern blot of HL-60 and DMSOr cells probed with BAM 1. The Northern blot contains RNA from HL-60 (H) and DMSOr (D) cells that have been treated with DMSO for the given times (in hours). The blot was probed with the 1.4 kb Bam HI fragment termed BAM 1 in Fig. 29.


0
24
72
144
H
D
H
D
H
D
H
D
3.6

because of its apparent increase in expression during differentiation. Such a message could be important in the regulation of events during the latter stages of differentiation or may also exhibit the differentiation enhancing qualities of the splice clone pMbm2.

Initial studies had already ruled out the possibility that the 2.4 kb message is pMbm28 (Fig. 12) or the c-*myb* related gene B-*myb* (Fig. 14). Other alternative splice clones were also tested but none showed a similar pattern of expression to the 2.4 kb message. The following studies were designed to characterize this message by attempting to find unique start sites in the c-*myb* locus, based on observations made during the screening of Northern blots with c-*myb* probes.

Differential Screening of Northern Blots with c-*myb* Probes.

To determine if there are potential start sites within the interior of the c-*myb* locus, two Bam HI fragments of pMbm 1 were used to screen identical Northern blots. The first fragment, BAM 1 contains the 5' half of the cDNA, while the second probe, BAM 2 contains the next three exons (Fig. 29). When these two probes were hybridized to Northern blots with RNA isolated from HL-60 and DMSOr cells taken through a time course of DMSO differentiation, only the BAM 2 probe could detect the 2.4 kb message (Fig. 11 and 30).

Primer Extension Analysis of the BAM 2 Fragment.

The Northern blots in Fig. 12 and 30 suggested that the 2.4 kb message may initiate in the 3' end of the gene, since only the probe containing exons 9 through 12 could detect its expression. To test for initiation sites in this region of the c-*myb* locus, a primer extension analysis was performed. The strategy for the primer extension is presented in Fig. 31, where a primer was selected which could be annealed to mRNA within the 5' Bam HI site of the BAM 2 probe. The primer extension analysis revealed a potential start site within this region of the gene 170 bases from the primer annealing site (Fig. 32). The region where this start site maps is the beginning of exon 9 (Fig. 33). The pattern of expression of the 2.4 kb message and the results of differential probing with the two Bam HI fragments suggest that the exon 9 start site may be the start point for the 2.4 kb message.

Figure 31. Primer extension strategy. The genomic sequence provided maps the start of exon 9 (*EXON 9), the Start of BAM 2 (BamHI) and the sequence and location utilized for the primer extension primer LHB.1.

PRIMER EXTENSION STRATEGY

Figure 32. Primer extension analysis of c-*myb* from the LHB.1 probe. RNA was isolated from HL-60 (H) and DMSOr (D) cells at the given time points (in hours) and subjected to primer extension analysis. The size of the primer extension product was estimated by comparison with size standards.

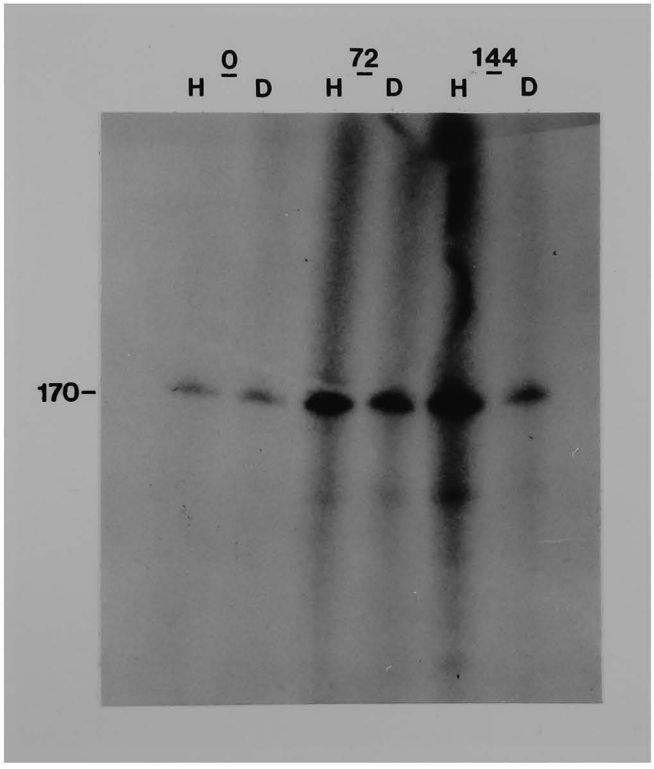

0
72
144
H
D
H
D
H
D
170–

Figure 33. Primer extension summary. The start site mapped by the primer extension in Fig. 32 is shown as arrows. This putative start site is within the borders of exon 9 (*EXON 9) and 120 bases from the beginning of the BAM 2 fragment.

EXON 9 PRIMER EXTENSION SUMMARY

DISCUSSION

Characterization of DMSOr.

Terminal differentiation is the process whereby a cell commits to a program of changes which usually result in the loss of the cell's proliferative capacity and the acquisition of a specific function. During hematopoiesis, terminal differentiation gives rise to cells which have functions such as phagocytosis, bactericidal activity and oxygen transport. However these macrophage, neutrophil and erythrocyte cell types all lose the ability to proliferate. To examine the events that are important or essential to the commitment to terminal differentiation, the HL-60 *in vitro* model of differentiation and a subclone that was selected for a differentiation resistant phenotype were utilized. By removing the selective pressure from DMSOr, a new stable phenotype developed. The current studies defined this phenotype as a reversible differentiation response to DMSO. The cells are capable of differentiating morphologically and functionally while undergoing withdrawal from the cell cycle as evidenced by ^{3}H-thymidine incorporation and cell cycle analyses. The magnitude of these changes is similar to that of the parental clone. However, all of these changes are reversible in DMSOr by the removal of the DMSO at any point during the differentiation process. This is not the case for the parental clone where treatment with

DMSO for at least 72 hours results in the commitment of the cells to the induction of myeloid differentiation(Siebenlist et al., 1988). These studies suggest that many of the changes in morphology, function and proliferative state of the cell are not necessarily linked to the process of commitment to terminal differentiation. Thus the DMSOr cell line may prove to be an excellent model for examining the events that are required for commitment to a terminal differentiation program.

Earlier studies with differentiation resistant subclones of HL-60 have demonstrated altered regulation of oncogenes(Ely et al., 1987; Studzinski and Brelvi, 1987; Fisher and Grant, 1985; Collins et al., 1991). The c-*myb* oncogene has been shown to be expressed and regulated during hematopoietic cell differentiation in both normal and leukemic cells(Gonda and Metcalf, 1984; Blick et al., 1984; Westin et al., 1982). This regulation includes the expression of multiple transcripts generated by alternative splicing and 5' heterogeneity(Westin et al., 1990; Bender and Kuehl, 1986; Dasgupta and Reddy, 1989; Shen-Ong, 1987; Shen-Ong et al., 1990). At least two of these alternative transcripts have opposing effects on the differentiation of FMEL cells(Clarke et al., 1988; Weber et al., 1990). Taken in context with the fact that c-*myb* has trans-activation(Ibanez and Lipsick, 1990; Nishina et al., 1989; Sakura et al., 1989; Weston and Bishop, 1989)and trans-repression(Nakagoshi et al., 1989) capabilities, this gene product is a candidate for being a controlling factor in the differentiation of hematopoietic cells. In the DMSOr cell line, c-*myb* is aberrantly expressed at the later time points of DMSO induced differentiation. This could link c-*myb*

regulation to terminal differentiation of HL-60. Although no causal relationship was demonstrated (in part due to the difficulty in interpreting the antisense experiments), the expression of c-*myb* at the late time points of differentiation and the subsequent dedifferentiation following removal of the differentiation inducing agents associated with re-expression of c-*myb*, parallels the results of Beug et al.(Beug et al., 1987) where a temperature sensitive v-*myb* mutant could cause a dedifferentiation of chicken macrophages at the permissive temperature. The current studies also suggest that the early down regulation of c-*myb* during HL-60 differentiation is not sufficient to permit the cells to commit to terminal differentiation.

Other molecular events in DMSOr, which may occur in an abnormal fashion, could play a role in its reversible phenotype. One other gene that was investigated was the c-*myb* related gene B-*myb*. B-*myb* encodes a transcription factor that can activate transcription from the c-*myb* DNA binding site(Mizuguchi et al., 1990). B-*myb* is expressed in many more tissues than c-*myb*(Nomura et al., 1988) and has been shown to be expressed in immature but not mature hematopoietic cells(Ibanez and Lipsick, 1990). This pattern of expression in hematopoietic cells is similar to the c-*myb* oncogene but B-*myb* expression in DMSOr was the same as the pattern observed in HL-60. This indicates that altered regulation of c-*myb* in DMSOr following induction of differentiation is specific. The down regulation at 48 hours is the first demonstration of B-*myb* expression kinetics during HL-60 differentiation. A-*myb* gene expression was not observed in either

HL-60 or DMSOr. This is consistent with previous findings in hematopoietic cells(Ibanez and Lipsick, 1990).

It is also worthy to note that B-*myb* is not the 2.4 kb. mRNA that is recognized by the c-*myb* probe. This lower molecular weight message carries a different pattern of expression than the normal c-*myb* or B-*myb* mRNA. The expression of this message is up regulated at the later time points of differentiation relative to the c-*myb* mRNA. This pattern is present in both HL-60 and DMSOr and therefore may not play a specific role in commitment to terminal differentiation. The kinetics of expression of this mRNA suggest a role in regulation of expression of genes late in the differentiation process. It is not clear at this time if this mRNA is an alternatively spliced message of c-*myb* or a message from an as yet uncharacterized *myb* related gene. There is evidence for each possibility. A truncated form of the c-*myb* mRNA; HMYB.1 has been previously described(Slamon et al., 1986) and additional *myb* related genes may exist. The splice form pMbm 28, which also encodes a truncated form of the c-*myb* message, does not encode the 2.6 kb message, but does show discordant regulation when compared to the prototypic c-*myb* message, and is not regulated in an altered fashion during the differentiation of DMSOr.

It has been previously demonstrated that c-*myb* expression is important for proper transition from G_1 phase to S phase of the cell cycle in lymphocytes(Gewirtz et al., 1989). Northern blots were hybridized to probes for genes encoding cdc2 kinase, cyclin B, histone H4, and ODC to determine if other genes that are important in cell cycle control or are cell cycle regulated are appropriately

expressed during DMSOr differentiation. The results from these experiments revealed that all of these genes showed altered patterns of regulation during DMSOr differentiation. The pattern of expression for these genes was similar to that of c-*myb* with the delay in down regulation witnessed at the later time points. Altered regulation of this many cell cycle related genes suggests that the alteration in DMSOr is probably a mutation in the cell cycle control pathways. Since the nuclear run-on analyses of c-*myb* and c-*myc* regulation during DMSOr differentiation is similar to HL-60 differentiation, it is unlikely that the genetic alteration in DMSOr is in a transcriptional controlling element. This alteration could be in a gene that is active in controlling the expression of cell cycle genes at the post-transcriptional level, because the cells do respond to the differentiation induction by DMSO and the changes in the cell cycle analyses (FACSCAN) were similar to the parental cell line. If a gene that is important in post-transcriptional regulation of any of these genes was effected, then mRNA levels could be elevated at later points during differentiation, and following removal of the DMSOr would be available to express proteins that are normally repressed at this point. The fact that the DMSOr cells are capable of responding to DMSO induced differentiation at all may be due to normal control of gene expression at the transcriptional level for c-*myb* and possibly the other cell cycle related genes. Proper translational control of these genes during differentiation may also play a role in DMSOr differentiation. If the mechanisms that control c-*myb* and the other genes at the transcriptional and translational level are under a kinetic control then these mechanisms may no

longer be active at the post-commitment time points and thus re-expression can occur as the cells begin to cycle.

It is also interesting to note that pMbm 28 does not show the altered pattern of expression in DMSOr that pMbm 1 displays. This form of the c-*myb* message is truncated at the 3' end, through the utilization of an alternative polyadenylation site. The 3' untranslated regions of these messages are different, and this is a region of mRNA that is important in post-transcriptional regulation(Brawerman, 1989). A motif that is of particular importance in the post-transcriptional regulation of gene expression is the AUUUA box. There have been at least two proteins isolated that effect the stability of mRNA by binding to this sequence, one that destabilizes lymphokine and oncogene mRNA(Malter, 1989) and one that is inducible that may stabilize lymphokine mRNA(Bohjanen et al., 1991). The prototypic c-*myb* message contains 6 AUUUA sites in the 3' untranslated region, while pMbm28 has no AUUUA sites in its alternative 3' untranslated domain(Westin, 1991). This may reflect a difference in the mechanisms of regulation between c-*myb* splice clones, with ones like pMbm28 not susceptible to the post-transcriptional regulation that clones with the normal 3' end are. If a post-transcriptional mechanism of regulation of c-*myb* is affected in DMSOr, this could explain the lack of correlation between expression patterns witnessed for the prototypic c-*myb* compared to pMbm 28. Thus, the mechanism important for regulating pMbm1 expression during myeloid differentiation is not a normal mechanism of regulation for pMbm 28 and therefore might not be altered during DMSOr

differentiation. This lends support to the theory that the mutation in DMSOr may be involved in a gene that is important in the post-transcriptional regulation of cell cycle related genes like c-*myb*. It should be noted that it is unclear why the pMbm 28 probe does not hybridize to the 3.6 kb message. Further characterization of this form of the c-*myb* message should be undertaken.

The current studies confirm the importance of control of the c-*myb* oncogene for proper differentiation of hematopoietic cells. These studies also characterize a cell line, DMSOr, which may be valuable for examination of factors that are essential for terminal differentiation. These studies present evidence for the uncoupling of withdrawal from cell cycle and functional changes from commitment to terminal differentiation. DMSOr will therefore be useful in determining what changes are necessary for commitment to a terminal event as opposed to acquisition of a differentiated phenotype. The reversible phenotype of DMSOr also correlates well with the altered, late expression of c-*myb* and other cell cycle related genes during differentiation. Thus this cell line will be an important tool in dissecting the role and regulation of c-*myb* during the differentiation process. Future studies that should be performed would include determining if there is a difference in the post-transcriptional regulation of c-*myb* in DMSOr compared to parental HL-60. If differences in regulation at the post-transcriptional level are evident it should also be determined if these differences are present for the other cell cycle regulated genes. Another potential area of pursuit would be to determine if the 3' untranslated regions, in particular the AUUUA sites are

important in the regulation of c-*myb* mRNA. These results could be achieved experimentally *in vitro* by exposing c-*myb* messages with different 3' ends to cellular extracts from HL-60 and DMSOr cells at various stages of DMSO induced differentiation. By crosslinking the RNA/protein complexes it could be determined if proteins bind to these messages in a pattern that correlates with DMSO induced differentiation, and if these binding patterns are altered in DMSOr. By comparing the pMbm 1 3' end to the pMbm 28 3' end , it could be determined if the differences in the regulation of these two processing variants during HL-60 differentiation is due to the differences in the 3' untranslated region of these messages. Another important question that should be addressed is if there are differences in c-*myb* protein levels in HL-60 and DMSOr. All of the interpretation that is presented above is based on differences that are at the mRNA level and make the assumption that these differences are also present at the protein level.

Transcriptional and Post-transcriptional Regulation of c-*myb*.

The regulation of the c-*myb* gene during hematopoietic cell differentiation must be under precise control since altered expression of the gene may have varied effects on the differentiation process as has been discussed above. Studies of the murine clone of the gene have indicated that c-*myb* is regulated at the transcriptional level by an attenuator located in the first intron of the gene(Bender et al., 1987; Watson, 1988b; Watson, 1988a). Murine and chicken studies have also suggested that post-transcriptional regulation may also be important in controlling the

expression of c-*myb*(Watson, 1988b). The current studies were designed to determine if the human c-*myb* gene shared regulatory schemes with the mouse and chicken genes or if novel mechanisms of regulation were present. The HL-60 cell line provided an *in vitro* model of human hematopoietic cell differentiation, whereby differences in c-*myb* regulation due to myeloid or monocytic differentiation or differentiation agent specificity could be determined. The results from these experiments indicate that the human c-*myb* gene is regulated at the transcriptional level primarily via an attenuator, although the data suggests that one differentiation agent, PDbu may also regulate the promoter of the gene. Differentiation of HL-60 cells through myeloid or monocytic lineages showed no qualitative differences in the regulation of c-*myb*, as demonstrated by the similarities in regulation induced by the myeloid inducer retinoic acid and the monocytic inducer vitamin D3. The similarities in the regulation of c-*myb* induced by these agents may involve the mechanism of action by which these agents act. Both retinoic acid and the activated form of vitamin D3 bind to the cytosolic, steriod/thyroid class of receptors and it is possible that upon entering the nucleus and binding to the appropriate response elements, these agents can activate expression of genes necessary for attenuation to occur. These similarities in transcriptional regulation were shared by the other two differentiation agents, DMSO and PDbu, but these two agents also demonstrated unique mechanisms of c-*myb* regulation. These unique events included post-transcriptional control and possibly

promoter control, thus during HL-60 differentiation, c-*myb* is regulated in an agent specific manner.

Read-through of the c-*myb* attenuator appears to be dependent on the *de novo* translation of protein, as evidenced in the nuclear run-on assays where cyclohexamide could induce the attenuation of c-*myb* in HL-60 cells. The possibility that cyclohexamide was inhibiting the translation of anti-terminator proteins was investigated by testing the effects of this protein synthesis inhibitor on DNA-protein interactions in the first intron of c-*myb*. The gel shift analyses of regions of evolutionary conservation did not yield any consistent data that suggested that a protein that could allow read-through transcription of the attenuator was inhibited from binding by cyclohexamide.

Other possibilities for anti-terminator proteins that could be affected by cyclohexamide would be proteins that are bound to RNA polymerase, that allow read-through of attenuators but not termination sites that are associated with polyadenylation sites. Logan and colleagues postulated that this process would explain how genes like c-*myc* could be elongated past the attenuator(Adamkiewicz et al., 1990; Logan et al., 1987). The anti-terminator would associate with the elongating polymerase and allow read-through of premature termination sites. When the polyadenylation signal was reached the anti-terminator protein would be released from the polymerase and soon after the polymerase would terminate the nascent transcript.

Future experiments that would be necessary in the characterization of the transcriptional regulation of the human c-

myb gene, include fine mapping of the attenuator region. The current studies have not mapped the region of termination within the first intron of the human gene. Mapping of this region would require the use of short (300-500 bp), contiguous targets within intron 1 in the nuclear run-on assay. Fine mapping of the region could provide further insight for locating possible DNA-protein interactions that are important for attenuation. RNA-protein interactions should also be studied in this region of the c-*myb* gene. One possible method for obtaining such data would be to incubate radiolabelled *in vitro* transcribed RNA with nuclear extracts from HL-60 cells at different points of differentiation. This procedure may yield unique RNA-protein interactions that correlate with attenuation or with read-through transcription. If any of these experiments showed a differential binding that correlated with read-through transcription, it should then be determined if cyclohexamide could block the DNA or RNA binding. If any protein binding site correlates with attenuation it should be tested to see if cell treatment with cyclohexamide could mimic the protein binding induced by the differentiation agent. This experiment is proposed because many transcriptional activators like AP-1(Auwerx and Sassone-Coral, 1991) and NF-kB(Baeurele and Baltimore, 1988) are regulated by cellular inhibitors. These transcription factors are DNA binding proteins, so it is not difficult to imagine that other DNA binding proteins could be regulated in a similar fashion. If such a protein had a shorter half-life than the attenuator protein, then cyclohexamide could be causing attenuation by effectively releasing the attenuator protein from the regulatory protein. To test this

possibility, control cellular extracts could be added to the cyclohexamide binding reaction to see if binding could be inhibited.

Post-transcriptional regulation of c-*myb* appears to have multiple mechanisms of action. In the DMSO treated HL-60 cells, c-*myb* steady state mRNA levels are expressed in a biphasic pattern. During the first 6 hrs of treatment there is a sharp decline in c-*myb* message levels. This is followed by an increase in levels that peaks at 24 hrs. During the first 24 hrs of DMSO treatment the c-*myb* attenuator is activated, but does not change during the period where message levels rise (6-24 hrs). This suggested the presence of post-transcriptional control of c-*myb*, particularly in the first 6 hrs of DMSO treatment. A change in the message stability was shown by the fact that DMSO induced changes in c-*myb* steady state levels during the first three hours were greater than Actinomycin D changes during the same time period. Interestingly, the changes induced by DMSO were blocked by Actinomycin D when the two drugs were used in concert. This suggested a transcriptional dependency to the post-transcriptional regulation of c-*myb* during the early portions of DMSO induced differentiation. If the large decrease in c-*myb* message during the first 6 hrs of DMSO treatment is due to post-transcriptional changes and the attenuator activity does not change during the period which the message levels rise again, then it is possible that the message destabilization is transient and once the attenuator is operational, the post-transcriptional regulation becomes secondary. This hypothesis is suggested by the fact that the transcriptional dependency of the post-transcriptional regulation of c-*myb* is no longer present at 24

hrs. This experiment does not prove that all post-transcriptional changes in c-*myb* are inactive at this point since an accurate determination of c-*myb* half-life in unstimulated cells cannot be obtained. Similar findings were shown for c-*myc* expression during the first three hrs of DMSO treatment of HL-60, but not for ODC. The post-transcriptional regulation that is transcriptional dependent may be a global regulatory axis that is important in controlling genes that are considered "early-response" types. It should also be noted that of all the cell cycle related genes tested in the first section of this thesis, ODC showed the least difference in alterations in DMSOr expression. These differences between ODC and the oncogenes (& histone) may be due to a difference in the magnitude of post-transcriptional regulation of ODC compared to other genes.

One possible explanation for the transcriptional dependency of the post-transcriptional regulation of c-*myb* by DMSO is that the expression of an inducible protein that is necessary for post-transcriptional regulation is being inhibited. If this were the case then cyclohexamide treatment should be able to, an extent, block the changes in c-*myb* message induced by DMSO. The results from this experiment revealed that protein synthesis was not necessary for c-*myb* regulation during the first 6 hrs of DMSO treatment since cyclohexamide could not block the effect of DMSO. Cyclohexamide had no effect on c-*myb* expression in HL-60 as had been previously shown(Golay et al., 1991). When this data is considered in context with the effect cyclohexamide has on the c-*myb* attenuator, it provides further evidence that c-*myb* is regulated at the post-transcriptional level.

A possible explanation for the transcriptional dependency, without the need for translation on the early post-transcriptional regulation of c-*myb* by DMSO is a role for the antisense transcription that occurs in the 5' end of the gene. Antisense transcripts have been isolated and RNA:RNA duplexes found *in vivo* for the N-*myc* gene(Krystal et al., 1990). These duplexes have been hypothesized to play a role in the regulation of post-transcriptional events, such as splicing or metabolism. In the case of c-*myb*, the antisense transcription could play a role in DMSO induced changes. There are no obvious changes in the antisense transcription rate during HL-60 differentiation, but it is not known if any message is produced from this transcription. If a message is produced from the antisense transcription, the half-life may be much shorter than the sense transcription. The Actinomycin D could then effectively remove any such transcript, thus removing this regulatory feature and leading to the apparent transcriptional dependency. Future experiments in this area of research should include a characterization of the antisense transcription in the 5' portion of c-*myb*.

The monocytic inducer, PDbu, also induced a change in the message stability that was different from that induced by DMSO. The post-transcriptional changes induced by PDbu were not dependent on *de novo* transcription. One possible explanation for the differences between the post-transcriptional changes induced by DMSO and those induced by PDbu, may be differences in the kinetics of the differentiation induced by the two agents. PDbu requires only 24 hrs to differentiate HL-60 cells, and it is possible

that changes that occur late in DMSO induced differentiation occur in the first few hours of PDbu induced differentiation. Such changes could include post-transcriptional changes postulated in the first part of the discussion (changes that occur after 72 hrs), and possibly after the first 24 hours of DMSO induced differentiation, where the transcriptional dependency is absent. The possibility that two levels of post-transcriptional regulation could be active can be extrapolated from the data of Watson, which showed that c-*myb* is post-transcriptionally regulated in DMSO induced FMEL cell differentiation(Watson, 1988b). The post-transcriptional studies revealed that the half-life of c-*myb* from cells treated for 2 hrs was less than those untreated. The half-life from cells treated for 96 hrs was also less than the control cells but it was greater than the 2 hr treated cells.

The c-*myb* promoter has many of the characteristics of a constitutive promoter. These include the GC rich regions, that contain SP1 binding sites and the lack of TATA and CAT boxes. The current studies confirmed that the promoter of the human form of the c-*myb* gene is also not a site of regulation during myeloid or monocytic differentiation, with one possible exception in the case of PDbu induced changes. Quantitation of the nuclear run-on data suggested that the early drop in steady state levels of c-*myb* message that occurred during PDbu induced differentiation correlated with changes in transcription in the promoter region of the gene as opposed to the post-attenuator region. This data does not take into consideration the simultaneous post-transcriptional

changes and therefore should be an area of pursuit in future studies.

Characterization of the 2.4 kb c-*myb* mRNA.

C-*myb* is a gene that encodes a transcription factor associated with hematopoietic cell differentiation. Transcription factors are capable of activating or repressing the expression of many genes. One possible mechanism for determining whether one gene is affected vs. another at a given time is to generate a family of transcription factors that have different affinities for cis elements that share a core binding sequence. The differences in the proteins within the family may allow regulatory domains to interact with different sets of proteins, thus similar proteins can elicit varied responses. The two known mechanisms for generating such proteins are alternative splicing and "gene families." It has been revealed that the *fos* and *jun* genes utilize both of these mechanisms to create AP-1 transcription factors with different binding affinities and activation or repression functions(Ryder et al., 1989; Nakabeppu and Nathans, 1991). The c-*myb* gene has also been shown undergo extensive alternative splicing(Westin et al., 1990; Shen-Ong et al., 1990; Dasgupta and Reddy, 1989; Shen-Ong, 1987) and to have to at least two family members, A-*myb* and B-*myb*(Nomura et al., 1988). The B-*myb* gene product is the only family member or alternatively spliced product that has been characterized as a transcription factor to date(Mizuguchi et al., 1990).

The expression of a c-*myb* splice product or related gene that is up-regulated during HL-60 differentiation could have interesting implications. If the gene product encodes a transcription factor that is expressed during the later stages of differentiation, such a factor could be important in the expression of genes that define the mature cell phenotype, or repress genes that are essential for self-renewal of immature cells. One splice clone of c-*myb*, pMbm 2 is capable of inducing the differentiation of FMEL cells, possibly through such a mechanism(Weber et al., 1990). The expression of pMbm2 is minimal in HL-60(Westin et al., 1990) and therefore may not be the primary form of c-*myb* involved in a differentiation enhancing process in this cell line.

The pattern and level of expression of the 2.4 kb message seen in the Northern blots presented, suggested that this mRNA species may encode a differentiation enhancing protein and therefore an attempt to further characterize the mRNA was made. The pattern of expression and the size of the c-*myb* message pMbm28 and the *myb* related gene B-*myb* did not correlate with the expression of the 2.4 kb message ruling out these two *myb* forms as being this message. To define which regions of the c-*myb* message were homologous to the 2.4 kb message, two BamHI fragments of pMbm1 were used to screen Northern blots. The first fragment which contained 5' untranslated region into exon 9 did not hybridize well to the 2.4 kb message, while the second BamHI fragment did. This second fragment contained the remainder of exon 9 to 12. This suggested the possibility of a start site within the 3' portion of the gene. To test this possibility, a primer extension

analysis was performed, which demonstrated a well defined potential start site in the beginning of exon 9. The usage of this start site correlated with the expression of the 2.6 kb message during HL-60 differentiation, and therefore this message may use an alternative start site located at the beginning of exon 9.

The current experiments do not prove that the 2.4 kb message is transcribed from a start site located in the 3' portion of the c-*myb* gene, but they may provide some clues for cloning the message, which has proven difficult by standard techniques. By using an anchored PCR method, the 5' end of the clone could be readily obtained, this could then be used as a probe for probing Northern blots and eventually screening cDNA libraries. The putative promoter at the intron 8/exon 9 border could also be characterized. It is interesting to note that the sequence in the 3' portion of the intron 8 of the human c-*myb* gene has significant identity with the same region of the chicken c-*myb* gene(Westin, 1991). The only other intron region that contains such identity is the 3' portion of intron 1, which which functions as a promoter(Westin, 1991).

The protein that would be encoded from a message that started in exon 9 of c-*myb* would not likely be a transcription factor that is similar to the prototypic *myb* protein due to the lack of the *myb* DNA binding domain and a complete *myb* transcriptional activation domain. This does not rule out the possibility that the protein could affect transcription through as yet undefined motifs. Another possibility for this putative c-*myb* protein could further define previous studies where c-*myb* was isolated from the cytoplasm of mature myeloid cells(Bading et al., 1988). The

immunoblots in this report show an undefined band of lower molecular weight than 75 kd in the cytoplasm of the mature cells. This band could be the protein that is encoded by the 2.4 kb message. A role for cytoplasmic c-*myb* is not known.

Concluding Remarks.

The c-*myb* proto-oncogene is an important regulator of hematopoietic cell differentiation. For this reason it is important to gain further insight into the mechanisms that regulate the expression of c-*myb* itself, since aberrant regulation can lead to altered differentiation and leukemia. The studies detailed in this thesis have demonstrated that the human c-*myb* gene is regulated at multiple transcriptional and post-transcriptional levels during myelomonocytic cell differentiation. This regulation may, in part be under global cell cycle control. These studies have also described a previously unknown transcriptional dependence of post-transcriptional regulation, that was specific for the proto-oncogenes tested. These multiple levels of regulation of c-*myb* underscore the importance of proper regulation of the gene.

With a clear understanding of the regulation of genes like c-*myb*, the pathways that are essential for appropriate regulation of oncogenes can be exploited in cancer chemotherapy. It is therefore important to continue the study of the regulation of c-*myb* and other similar genes at all potential levels of regulation.

LITERATURE CITED

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Adamkiewicz, J., Brüller, H.-J., Gausepohl, H., Frank, R., and Müller, R. (1990). Mapping of functional domains in Fos and Jun proteins using epitope-specific antibodies. Oncogene *5*, 525-533.

Alitalo, K., Winqvist, R., Lin, C.C., de la, CA., Schwab, M., and Bishop, J.M. (1984). Aberrant expression of an amplified c-myb oncogene in two cell lines from a colon carcinoma. Proc. Natl. Acad. Sci. U. S. A. *81*, 4534-4538.

Alvarado-Urbina, G., Sathe, G., Liu, W., Gillen, M., Duck, P., Bender, R., and Ogilvie, K. (1981). Automated Synthesis of Gene Fragments. Science *214*, 270-273.

Anderson, L., Jokinen, M., and Gahmberg, C. (1979). Induction of erythroid differentiation in the human leukemia cell line K562. Nature *278*, 364.

Anderson, S.M. and Chen, J.H. (1981). In vitro translation of avian myeloblastosis virus RNA. J. Virol. *40*, 107-117.

Anfossi, G., Gewirtz, A.M., and Calabretta, B. (1989). An oligomer complementary to c-*myb*-encoded mRNA inhibits proliferation of human myeloid leukemia cell lines. Proc. Natl. Acad. Sci. USA *86*, 3379-3383.

Auwerx, J. and Sassone-Coral, P. (1991). IP-1: A dominant inhibitor of fos/jun whose activity is modulated by phosphorylation. Cell *64*, 983-993.

Bading, H., Gerdes, J., Schwarting, R., Stein, H., and Moelling, K. (1988). Nuclear and cytoplasmic distribution of cellular **myb** protein in human haematopoietic cells evidenced by monoclonal antibody. Oncogene *3*, 257-265.

Baeurele, P.A. and Baltimore, D. (1988). Iκ-B: a specific inhibitor of the NF-κB transcription factor. Science *242*, 540-546.

Bender, T.P. and Kuehl, W.M. (1986). Murine **myb** protooncogene mRNA: cDNA sequence and evidence for 5' heterogeneity. Proc. Natl. Acad. Sci. U. S. A. *83*, 3204-3208.

Bender, T.P., Thompson, C.B., and Kuehl, W.M. (1987). Differential expression of c-myb mRNA in murine B lymphomas by a block to transcription elongation. Science *237*, 1473-1476.

Beug, H., Blundell, P.A., and Graf, T. (1987). Reversibility of differentiation and proliferative capacity in avian myelomonocytic cells transformed by tsE26 leukemia virus. Genes. Dev. *1*, 277-286.

Biedenkapp, H., Borgmeyer, U., Sippel, A.E., and Klempnauer, K.H. (1988). Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature *335*, 835-837.

Bishop, J.M. (1988). The molecular genetics of cancer: 1988. Leukemia. *2*, 199-208.

Blackwell, T.K., Kretzner, L., Blackwood, E.M., Eisenman, R.N., and Weintraub, H. (1990). Sequence-specific DNA binding by the c-Myc protein. Science *250*, 1149-1151.

Blackwood, E.M. and Eisenman, R.N. (1991). Max: A helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with myc. Science *251*, 1211-1217.

Blick, M., Westin, E., Gutterman, J., Wong-Staal, F., Gallo, R., McCredie, K., Keating, M., and Murphy, E. (1984). Oncogene expression in human leukemia. Blood *64*, 1234-1239.

Bohjanen, P.R., Petryniak, B., June, C.H., Thompson, C.B., and Lindsten, T. (1991). An inducible cytoplasmic factor (AU-B) binds selectively to AUUUA multimers in the 3' untranslated region of lymphokine mRNA. Mol. Cell. Biol. *11*, 3288-3295.

Boyle, W.J., Lipsick, J.S., and Baluda, M.A. (1986). Antibodies to the evolutionarily conserved amino-terminal region of the v-myb-encoded protein detect the c-myb protein in widely divergent metazoan species. Proc. Natl. Acad. Sci. U. S. A. *83*, 4685-4689.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of protein Utilizing the principles of Protein-dye Binding. Analytical Biochemistry *72*, 248-254.

Brawerman, G. (1989). mRNA decay: Finding the right targets. Cell *57*, 9-10.

Breitman, T., Collins, S., and Keene, B. (1980a). Replacement of serum by insulin and transferrin supports growth and differentiation of the human promyelocytic leukemia cell line, HL-60. Exp. Cell Res. *126*, 494.

Breitman, T.R., Selonick, S.E., and Collins, S.J. (1980b). Induction of differentiation of human promyelocytic leukemia cells (HL-60) by retinoic acid. Proc. Natl. Acad. Sci. U. S. A. *77*, 2936-2940.

Brelvi, Z.S. and Studzinski, G.P. (1987). Coordinate expression of c-myc, c-myb, and histone H4 genes in reversibly differentiating HL-60 cells. J. Cell Physiol. *131*, 43-49.

Buchkovich, K., Duffy, L.A., and Harlow, E. (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell *58*, 1097-1105.

Cantley, L., Auger, K., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. Cell *64*, 281-302.

Chirgwin, J.M., Przybyla, H.E., MacDonald, R.J., and Rutter, W.J. (1984). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. *18*, 5294-5299.

Chiu, R., Angel, P., and Karin, M. (1989). Jun-B differs in its biological properties from, and is a negative regulator of, c-Jun. Cell *59*, 979-986.

Clark, S. and Kamen, R. (1987). The Human Hematopoietic Colony-Stimulating Factors. Science *236*, 1229-1245.

Clarke, M.F., Kukowska-Latallo, J.F., Westin, E., Smith, M., and Prochownik, E.V. (1988). Constitutive expression of a c-myb cDNA blocks friend murine erythroleukemia cell differentiation. Mol. Cell. Biol. *8*, 884-892.

Collins, S., Robertson, K., and Mueller, L. (1991). Retinoic Acid-Induced Granulocytic Differentiation of HL-60 Myeloid Leukemia Cells Is Mediated Directly through the Retinoic Acid Receptor (RAR-α). Mol. Cel. Biol. *10*, 2154-2163.

Collins, S., Ruscetti, F., Gallagher, R., and Gallo, R. (1978). Terminal differentiation of human promyelocytic leukemia cell induced by dimethylsulfoxide and other polar compounds. Proc. Natl. Acad. Sci. USA *75*, 2458.

Collins, S., Ruscetti, F., Gallagher, R., and Gallo, R. (1979). Normal functional characteristics of cultured human promyelocytic leukemia cells after induction of differentiation by dimethylsulfoxide. J. Exp. Med. *146*, 969-974.

Collins, S.J. (1987). The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. Blood *70*, 1233-1244.

Collins, S.J., Gallo, R.C., and Gallagher, R.E. (1977). Continuous growth and differentiation of human myeloid leukemia cells in culture. Nature *270*, 347-349.

Cooke, M., Abraham, K., Furbush, K., and Perlmutter, R. (1991). Regulation of T-cell receptor signalling by a SRC-family protein tyrosine kinase (p59*fyn*). Cell *65*, 281-292.

Curran, T. and Franza, B.R.,Jr. (1988). Fos and Jun: the AP-1 connection. Cell *55*, 395-397.

Dalla-Favera, R., Westin, E., Gelmann, E.P., Martinotti, S., Bregni, M., Wong-Staal, F., and Gallo, R.C. (1983). The human onc gene c-myc: structure, expression, and amplification in the human promyelocytic leukemia cell line HL-60. Hamatol. Bluttransfus. *28*, 247-254.

Das, S., Stanley, E., Guilbert, L., and Forman, L. (1981). Human colony-stimulating factor (CSF-1) radioimmunoassay: Resolution of three subclasses of human colony-stimulating factors. Blood *58*, 630-641.

Dasgupta, P. and Reddy, E.P. (1989). Identification of alternatively spliced transcripts for human c- *myb*: Molecular cloning and

sequence analysis of human c-*myb* exon 9A sequences. Oncogene *4*, 1419-1423.

Davis, L., Dibner, M., and Battey, J. (1986). Basic Methods in Molecular Biology (New York: Elsevier Science Publishing, Inc.).

Dignam, J., Lebowitz, R., and Roeder, R. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Research *11*, 1475-1489.

Douer, D. and Koeffler, H. (1982). Retinoic acid inhibition of the clonal growth of human myeloid leukemia cells. J. Clin. Invest. *69*, 277.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schessinger, J., and Waterfield, M. (1984). Close similarity of epidermal growth receptor and v-erbB oncogene protein sequences. Nature *307*, 521-527.

Dudek, H. and Reddy, E.P. (1989a). Identification of two translational products for c-*myb*. Oncogene *4*, 1061-1066.

Dudek, H. and Reddy, E.P. (1989b). Murine myeloid leukemias with aberrant *myb* loci show heterogeneous expression of novel *myb* proteins. Oncogene *4*, 1489-1495.

Egan, C., Bayley, S.T., and Branton, P.E. (1989). Binding of the *Rb1* protein to E1A products is required for adenovirus transformation. Oncogene *4*, 383-388.

Elias, L., Wogenrich, F., Wallace, J., and Longmire, J. (1980). Altered patterns of proliferation and differentiation of HL-60 human promyelocytic leukemia cells in the presence of leukocyte-conditioned medium. Leuk. Res. *4*, 301.

Ely, C., Oddie, K., Litz, J., Rossomando, A., Kanner, S., Sturgill, T., and Parsons, S.J. (1990). A 42-kD tyrosine kinase substrate linked to chromaffin cell secretion exhibits an associated MAP kinase activity and is highly related to a 42-kD mitogen-stimulated protein in fibroblasts. J. Cell Biol. *110*, 731-742.

Ely, C.M., Leftwich, J.A., Chenevix-Trench, G., Hall, R.E., and Westin, E.H. (1987). Altered regulation of c-myc in an HL-60 differentiation resistant subclone, HL-60-1E3. Cancer Res. *47*, 4595-4600.

Ferrari, S., Donnelli, A., Manfredini, R., Sarti, M., Roncaglia, R., Tagliafico, E., Rossi, E., Torelli, G., and Torelli, U. (1990). Differential effects of c-myb and c-fes antisense oligodeoxynucleotides on granulocytic differentiation of human myeloid leukemia HL60 cells. Cell Growth and Differentiation *1*, 543-548.

Fisher, P. and Grant, S. (1985). Effects Of Interferon On Differentiation Of Normal And Tumor Cells. Pharmac. Ther. *27*, 143-166.

Friend, C., Scher, W., and Holland, J. (1971). Hemoglobin synthesis in murine-virus-induced leukemia cells in vitro: Stimulation of erythroid differentiation by dimethylsulfoxide. Proc. Natl. Acad. Sci. USA *68*, 378.

Gentz, R., Rauscher, F.J.,III, Abate, C., and Curran, T. (1989). Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. Science *243*, 1695-1699.

Gewirtz, A.M., Anfossi, G., Venturelli, D., Valpreda, S., Sims, R., and Calabretta, B. (1989). G_1/S transition in normal human T-lymphocytes requires the nuclear protein encoded by c-*myb*. Science *245*, 180-183.

Ghosh, S., Gifford, A., Riviere, L., Tempst, P., Nolan, G., and Baltimore, D. (1990). Cloning of the p50 DNA binding subunit of NF-kappaB: homology to *rel* and *dorsal*. Cell *62*, 1019-1029.

Golay, J., Capucci, A., Arsura, M., Castellano, M., Rizzo, V., and Introna, M. (1991). Expression of c-*myb* and B-*myb* but not A-*myb*, Correlates With Proliferation in Human Hematopoietic Cells. Blood *77*, 149-158.

Gonda, T.J. and Metcalf, D. (1984). Expression of myb, myc and fos proto-oncogenes during the differentiation of a murine myeloid leukaemia. Nature *310*, 249-251.

Griffin, J., Munroe, D., Major, P., and Kufe, D. (1982). Induction of differentiation of human myeloid leukemia cells by inhibitors of DNA synthesis. Exp. Hematol. *10*, 774.

Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H., and Kedes, L. (1983). Isolation and characterization of full-length cDNA clones for the human α-, β-, γ-actin mRNAs: Skeletal but not cytoplasmic actins have an amino-terminal cysteine that is subsequently removed. Mol. Cell. Biol. *3*, 787-795.

Hahn, S., Hahn, M., and Hayward, W. (1989). Structural organization of upstream exons and distribution of transcription start sites in the chicken c-*myb* gene. Mol. Cell. Biol. *9*, 837-843.

Hickok, N.J., Seppänen, P.J., Gunsalus, G.L., and Jänne, O.A. (1987). Complete amino acid sequence of human ornithine decarboxylase deduced from complementary DNA. DNA *6*, 179-187.

Ho, I., Bhat, N.K., Gottschalk, L., Lindsten, T., Thompson, C.B., Papas, T.S., and Leiden, J.M. (1990). Sequence-specific binding of the human ets-1 to the T cell receptor α gene enhancer. Science *250*, 814-818.

Ho, I. and Leiden, J.M. (1990). Regulation of the T-cell receptor a gene enhancer: Multiple ubiquitous and T-cell specific nuclear proteins interact with four hypomethylated enhancer elements. Mol. Cell. Biol. *10*, 4720-4727.

Huang, E., Nocka, K., Beier, D., Chu, T.-Y., Buck, J., Lahm, H.-W., Wellner, D., Leder, P., and Besmer, P. (1990). The hematopoietic growth factor KL is encoded at the *Sl* locus and is the ligand of the c-*kit* receptor, the gene product of the *W* locus. Cell *63*, 225-233.

Hurley, J., Simon, M., Teplow, D., Robishaw, J., and Gilman, A.G. (1984). Homologies between signal transducing G proteins and the *ras* gene products. Science *226*, 860-862.

Ibanez, C.E. and Lipsick, J.S. (1990). Trans Activation of Gene Expression by v-myb. Mol. Cell Biol. *10*, 2285-2293.

Ju, Q., Morrow, B.E., and Warner, J.R. (1990). REB1, a yeast DNA-binding protein with many targets, is essential for cell growth and

bears some resemblance to the oncogene *myb*. Mol. Cell. Biol. *10*, 5226-5234.

Kanei-Ishii, C., Sarai, A., Sawazaki, T., Nakagoshi, H., He, D.-N., Ogata, K., Nishimura, Y., and Ishii, S. (1990). The tryptophan cluster: A hypothetical structure of the DNA- binding domain of the *myb* protooncogene product. J. Biol. Chem. *265*, 19990-19995.

Kanter, M.R., Smith, R.E., and Hayward, W.S. (1988). Rapid induction of B-cell lymphomas: insertional activation of c-myb by avian leukosis virus. J. Virol. *62*, 1423-1432.

Klein, R., Jing, S., Nanduri, V., O'Rourke, E., and Barbacid, M. (1991). The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell *65*, 189-197.

Klempnauer, K.H. and Bishop, J.M. (1984). Neoplastic transformation by E26 leukemia virus is mediated by a single protein containing domains of gag and myb genes. J. Virol. *50*, 280-283.

Klempnauer, K.H., Gonda, T.J., and Bishop, J.M. (1982). Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell *31*, 453-463.

Klemsz, M.J., McKercher, S.R., Celada, A., Van Beveren, C., and Maki, R.A. (1990). The macrophage and B cell-specific transcription factor PU.1 is related to the *ets* oncogene. Cell *61*, 113-124.

Koeffler, H. and Golde, P. (1978). Acute myelogenous leukemia: A human cell line responsive to colony stimulating activity. Science *200*, 1153.

Krystal, G.W., Armstrong, B.C., and Battey, J.F. (1990). N-*myc* mRNA forms an RNA-RNA duplex with endogenous antisense transcripts. Mol. Cell Biol. *10*, 4180-4191.

Lee, M. and Nurse, P. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. Nature *327*, 31-35.

Leprince, D., Gegonne, A., Coll, J., de Taisne, C., Schneeberger, A., Lagrou, C., and Stehelin, D. (1983). A putative second cell-derived

oncogene of the avian leukaemia retrovirus E26. Nature *306*, 395-397.

Linial, M., Gunderson, N., and Groudine, M. (1985). Enhanced transcription of c-*myc* in bursal lymphoma cells requires continuous protein synthesis. Science *230*, 1126.

Loewenstein, W.R. and Azarnia, R. (1988). Regulation of intercellular communication and growth by the cellular *src* gene. Ann. NY Acad. Sci. *551*, 337-346.

Logan, J., Falck-Pedersen, E., Darnell Jr., J.E., and Shenk, T. (1987). A poly(A) addition site and a downstream termination region are required for efficient cessation of transcription by RNA polymerase II in the mouse B^{maj}-globin gene. Proc. Natl. Acad. Sci. USA *84*, 8306-8310.

Luttrell, L.M., Luttrell, D.K., Parsons, S.J., and Rogol, A.D. (1989). Insulin and phorbol ester induce distinct phosphorylations of $pp60^{c\text{-}src}$ in the BC3H-1 murine myocyte cell line. Oncogene *4*, 317-324.

Lüscher, B., Christenson, E., Litchfield, D.W., Krebs, E.G., and Eisenman, R.N. (1990). Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation. Nature *344*, 517-522.

Lüscher, B. and Eisenman, R.N. (1990). New light on myc and myb. Part II. Myb. Genes. Dev. *4*, 2235-2241.

Malter, J. (1989). Identification of an AUUUA-Specific Messenger RNA Binding Protein. Science *246*, 664-666.

Martin, P. and Papayannopoulo, T. (1982). HEL cells: A new human erythroleukemia cell line with spontaneous and induced globin expression. Science *216*, 1233.

Matsumoto, M., Akiyama, T., Miyatake, S., Oda, Y., Kikuchi, H., Hanoaka, M., and Namba, Y. (1989). Expression of proto-oncogene products during drug-induced differentiation of a neuroblastoma cell line SK-JN-DZ. Acta Neuropathol. *79*, 217-221.

McCormick, F. (1989). ras GTPase activating protein: signal transmitter and signal terminator. Cell *56*, 5-8.

McCormick, F., Adari, H., Trahey, M., Halenbeck, R., Koths, K., Martin, G.A., Crosier, W.J., Watt, K., Rubinfeld, B., and Wong, G. (1988). Interaction of *ras* p21 proteins with GTPase activating protein. Cold Spring Harbor Symp. Quant. Biol. *53*, 849-854.

Metcalf, D. (1986). The molecular biology and functions of the granulocyte-macrophage colony-stimulating factors. Blood *67*, 257-267.

Metcalf, D. and Nicola, N. (1983). Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. J. Cell. Physiol. *116*, 198-206.

Minghetti, P.P. and Norman, A.W. (1988). 1,25(OH)2-vitamin D3 receptors: gene regulation and genetic circuitry. FASEB. J. *2*, 3043-3053.

Miyaura, C., Abe, E., Kuribayashi, T., Tanaka, H., Konno, K., Nishii, Y., and Suda, T. (1981). 1 alpha 25 dihydroxyvitamin D3 induces differentiation of human myeloid leukemia cells. Biochem. Biophys. Res. Commun. *102*, 937.

Mizuguchi, G., Nakagoshi, H., Nagase, T., Nomura, N., Date, T., Ueno, Y., and Ishii, S. (1990). DNA binding activity and transcriptional activator function of the human B-*myb* protein compared with c-MYB. J. Biol. Chem. *265*, 9280-9284.

Mucenski, M., McLain, K., Kier, A., Swerdlow, S., Schreiner, C., Miller, T., Pietyga, D., Scott Jr., W., and Potter, S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell *65*, 677-690.

Mucenski, M.L., Bedigian, H.G., Shull, M.M., Copeland, N.G., and Jenkins, N.A. (1988). Comparitive molecular genetic analysis of lymphomas from six inbred mouse strains. J. Virol. *62*, 839-846.

Nakabeppu, Y. and Nathans, D. (1991). A Naturally Occurring Truncated form of FosB That Inhibits Fos/Jun Transcriptional Activity. Cell *64*, 751-759.

Nakagoshi, H., Nagase, T., Ueno, Y., and Ishii, S. (1989). Transcriptional *trans*-repression by the c-*myb* proto-oncogene product. Nucleic Acids Res. *17*, 7315-7324.

Ness, S.A., Beug, H., and Graf, T. (1987). v-myb dominance over v-myc in doubly transformed chick myelomonocytic cells. Cell *51*, 41-50.

Ness, S.A., Marknell, A.,and Graf, T. (1989). The v-*myb* oncogene product binds to and activates the promyelocyte-specific *mim-1* gene. Cell *59*, 1115-1125.

Niedel, J., Kahane, I., Lachman, L., and Cuatrecasas, P. (1980). A subpopulation of cultured human promyelocytic leukemia cells (HL-60) displays the formyl peptide chemotactic receptor. Proc. Natl. Acad. Sci. USA *77*, 3664.

Nishina, Y., Nakagoshi, H., Imamoto, F., Gonda, T.J., and Ishii, S. (1989). Trans-activation by the c-myb proto-oncogene. Nucleic. Acids. Res. *17*, 107-117.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. Science *233*, 305.

Nomura, N., Matsui, M., Ishii, S., Date, T., Sasamoto, S., Ryataro, I., and Takahashi, M. (1988). Isolation of Human cDNA Clones of myb-related Genes, a-myb and b-myb. Nucleic Acids Res. *16*, 11075-11282.

Nunn, M., Weiher, H., Bullock, P., and Duesberg, P. (1984). Avian erythroblastosis virus E26: nucleotide sequence of the tripartite onc gene and of the LTR, and analysis of the cellular prototype of the viral ets sequence. Virology. *139*, 330-339.

Nunn, M.F. and Hunter, T. (1989). The ets sequence is required for induction of erythroblastosis in chickens by avian retrovirus E26. J. Virol. *63*, 398-402.

Olsson, I., Olofsson, T., and Mauritzon, N. (1981). Characterization of mononuclear blood cell derived differentiation inducing factors for the human promyelocytic leukemia cell line HL-60. J. Natl. Cancer Inst. *67*, 1225.

Olsson, I., Sarngadharan, M., Breitman, T., and Gallo, R. (1984). Isolation and characterization of a T-lymphocyte-derived differentiation inducing factor for the myeloid leukemic cell line HL-60. Blood *63*, 510.

Papas, T.S., Rushlow, K.E., Watson, D.K., Lautenberger, J.A., Perbal, B., Baluda, M.E., and Reddy, E.P. (1982). Nucleotide sequence and organization of the transforming region and large terminal redundancies (LTR) of avian myeloblastosis virus (AMV). J. Cell Biochem. *20*, 95-103.

Parker, R., Varmus, H., and Bishop, J.M. (1981). Cellular homologue (c-*src*) of the transforming gene of Rous sarcoma virus: Isolation, mapping, and transcriptional analysis of c-*src* and flanking regions. Proc. Natl. Acad. Sci. USA *78*, 5842-5846.

Pauza, C.D. (1987). Regulation of human T-lymphocyte gene expression by interleukin 2: immediate-response genes include the proto-oncogene c-myb. Mol. Cell Biol. *7*, 342-348.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P.A., and Saedler, H. (1987). The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO. J. *6*, 3553-3558.

Pines, J. and Hunter, T. (1989). Isolation of the human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell *58*, 833-846.

Pizer, E. and Humphries, E.H. (1989). RAV-1 insertional mutagenesis: Disruption of the c-*myb* locus and development of avian B-cell lymphomas. J. Virol. *63*, 1630-1640.

Prendergast, G.C. and Ziff, E.B. (1991). Methylation-sensitive sequence-specific DNA binding by the c-myc basic region. Science *251*, 186-189.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. Nature *335*, 683-689.

Rapp, U.R., Heidecker, G., Huleihel, M., Cleveland, J.L., Choi, W.C., Pawson, T., Ihle, J.N., and Anderson, W.B. (1988). *raf* Family

serine/threonine protein kinases in mitogen signal transduction. Cold Spring Harbor Symp. Quant. Biol. *53*, 173-184.

Reddy, C.D. and Reddy, E.P. (1989). Differential binding of nuclear factors to the intron 1 sequences containing the transcriptional pause site correlates with c-*myb* expression. Proc. Natl. Acad. Sci. USA *86*, 7326-7330.

Reddy, E., Reynolds, R., Santos, E., and Barbacid, M. (1982). A point mutation is responsible for the acquistion of transforming properties of the T24 human bladder carcinoma oncogene. Nature *300*, 149-152.

Regnier, M. and Darmon, M. (1989). Human epidermis reconstructed in vitro: A model to study keratinocyte differentiation and its modulation by retinoic acid. In Vitro Cell. Dev. Biol. *25*, 1000-1008.

Robbins, K., Antoniades, N., Devare, S., Hunkapiller, M., and Aaronson, S. (1983). Structural and immunological similarities between simian sarcoma virus gene products and human platelet derived growth factor. Nature *305*, 605-608.

Rovera, G., Santoli, D., and Damsky, C. (1979). Human promyelocytic leukemia cells in culture differentiate into macrophage like cells when treated with phorbol diesther. Proc. Natl. Acad. Sci. U. S. A. *76*, 2779-2783.

Ryder, K., Lanahan, A., Perez-Albuerne, E., and Nathans, D. (1989). *Jun-D*: A third member of the *Jun* gene family. Proc. Natl. Acad. Sci. USA *86*, 1500-1503.

Sagata, N., Daar, I., Oskarsson, M., Showalter, S.D., and Vande Woude, G.F. (1989). The product of the *mos* proto-oncogene as a candidate "initiator" for oocyte maturation. Science *245*, 643-646.

Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T.J., and Ishii, S. (1989). Delineation of three functional domains of the transcriptional activator encoded by the c-*myb* protooncogene. Proc. Natl. Acad. Sci. USA *86*, 5758-5762.

Schütte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J., and Minna, J. (1989). *jun-B* inhibits and c-*fos* stimulates the transforming and *trans*-activating activities of c-*jun*. Cell *59*, 987-997.

Seiler-Tuyns, A. and Birnsteil, M. (1981). Structure and expression in L-cells of a clone of H4 histone gene of the mouse. J. Mol. Biol. *151*, 607.

Shakarjian, M. and Carchman, R. (1990). Alteration of Human Granulocytic Functional Responses by Menadione. Archives of Biochemistry and Biophysics *283*, 1-11.

Sheiness, D. and Gardinier, M. (1984). Expression of a proto-oncogene (proto-myb) in hemopoietic tissues of mice. Mol. Cell Biol. *4*, 1206-1212.

Shen-Ong, G.L. (1987). Alternative internal splicing in c-myb RNAs occurs commonly in normal and tumor cells. EMBO. J. *6*, 4035-4039.

Shen-Ong, G.L., Holmes, K.L., and Morse 3d, H.C. (1987). Phorbol ester-induced growth arrest of murine myelomonocytic leukemic cells with virus-disrupted myb locus is not accompanied by decreased myc and myb expression. Proc. Natl. Acad. Sci. U. S. A. *84*, 199-203.

Shen-Ong, G.L., Potter, M., Mushinski, J.F., Lavu, S., and Reddy, E.P. (1984). Activation of the c-myb locus by viral insertional mutagenesis in plasmacytoid lymphosarcomas. Science *226*, 1077-1080.

Shen-Ong, G.L. and Wolff, L. (1987). Moloney murine leukemia virus-induced myeloid tumors in adult BALB/c mice: requirement of c-myb activation but lack of v-abl involvement. J. Virol. *61*, 3721-3725.

Shen-Ong, G.L.C. (1990). The *myb* oncogene. Biochim. Biophys. Acta Rev. Cancer *1032*, 39-52.

Shen-Ong, G.L.C., Skurla, R.M.,Jr., Owens, J.D., and Mushinski, J.F. (1990). Alternative splicing of RNAs transcribed from the human c-*myb* gene. Mol. Cell. Biol. *10*, 2715-2722.

Sherr, C., Rettenmier, C., Sacca, R., Roussel, M., Look, A.T., and Stanely, E.R. (1985). The c-*fms* Proto-oncogene Product Is Related to the Receptor for the Mononuclear Phagocyte Growth Factor, CSF-1. Cell *41*, 665-676.

Sherwood, J. and Goldwasser, E. (1978). Extraction of erythropoietin from normal kidneys. Endocrinology *103*, 866-870.

Siebenlist, U., Bressler, P., and Kelly, K. (1988). Two distinct mechanisms of transcriptional control operate on c-myc during differentiation of HL60 cells. Mol. Cell Biol. *8*, 867-874.

Slamon, D.J., Boone, T.C., Murdock, D.C., Keith, D.E., Press, M.F., Larson, R.A., and Souza, L.M. (1986). Studies of the human c-myb gene and its product in human acute leukemias. Science *233*, 347-351.

Souza, L.M., Komaromy, M.C., and Baluda, M.A. (1980a). Identification of a proviral genome associated with avian myeloblastic leukemia. Proc. Natl. Acad. Sci. U. S. A. *77*, 3004-3008.

Souza, L.M., Strommer, J.N., Hillyard, R.L., Komaromy, M.C., and Baluda, M.A. (1980b). Cellular sequences are present in the presumptive avian myeloblastosis virus genome. Proc. Natl. Acad. Sci. U. S. A. *77*, 5177-5181.

Stahl, M., Ferenz, C., Kelleher, K., Kriz, R., and Knopf, J. (1988). Sequence similarity of phospholipase C with the non-catalytic region of src . Nature *332*, 269-272.

Stratagene, (1990). pBluescript II Exo/Mung DNA sequencing system: Instruction Manual (Stratagene Cloning Systems).

Studzinski, G.P. and Brelvi, Z.S. (1987). Changes in proto-oncogene expression associated with reversal of macrophage-like differentiation of HL 60 cells. J. Natl. Cancer Inst. *79*, 67-76.

Tabin, C., Bradley, S., Bargman, C., Weinberg, R., Papageorge, A., Scolnick, E., Dhar, R., Lowy, D., and Chang, E. (1982). Mechanism of activation of a human oncogene. Nature *300*, 143-148.

Takeda, K., Minonada, J., and Bloch, A. (1981). Kinetics of appearance of differentiation associated characteristics in ML-1, a line of human myeloblastic leukemia cells, after treatment with 12-O-tetradeconylphorbol 13-acetate, dimethylsulfoxide, 1-beta-D-arabinofurnosylcytosine. Cancer Res. *42*, 5152.

Taparowski, E., Suard, Y., Fascano, O., Simizu, K., Goldfarb, M., and Wigler, M. (1982). Activation of T24 bladder carcinoma

transforming gene is linked to a single amino acid change. Nature *300*, 762-765.

Thiele, C.J., Cohen, P.S., and Israel, M.A. (1988). Regulation of c-myb expression in human neuroblastoma cells during retinoic acid-induced differentiation. Mol. Cell Biol. *8*, 1677-1683.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U. S. A. *77*, 5201-5205.

Thompson, C.B., Challoner, P.B., Neiman, P.E., and Groudine, M. (1986). Expression of the c-myb proto-oncogene during cellular proliferation. Nature *319*, 374-380.

Tice-Baldwin, K., Fink, G.R., and Arndt, K.T. (1989). BAS1 has a myb motif and activates *HIS4* transcription only in combination with BAS2. Science *246*, 931-935.

Trahey, M. and McCormick, F. (1987). A cytoplasmic protein stimulates normal n-*ras* GTPase, but does not affect oncogenic mutants. Science *238*, 542-545.

Veillette, A., Bookman, M.A., Horak, E.M., Samelson, L.E., and Bolen, J.B. (1989). Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56*lck*. Nature *338*, 257-259.

Viellette, A., Bookman, M., Horak, E., and Bolen, J. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56*lck*. Cell *55*, 301-308.

Wang, E.H., Friedman, P.N., and Prives, C. (1989). The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. Cell *57*, 379-392.

Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Leprince, D., and Stehelin, D. (1990). The c-*ets* proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature *346*, 191-193.

Watson, R., Dyson, P., and McMahon, J. (1991). Multiple c-*myb* transcript cap sites are variosly utlized in cells of mouse hematopoietic origin. EMBO. J. *6*, 1643-1651.

Watson, R.J. (1988a). A Transcriptional Arrest Mechanism Involved in Controlling Constitutive Levels of Mouse c-myb mRNA. Oncogene *2*, 267-272.

Watson, R.J. (1988b). Expression of the c-myb and c-myc Genes is Regulated Independently in Differentiating Mouse Erythroleukemia Cells by Common Processes of Transcription Arrest and Increased mRNA Turnover. Mol. Cell Biol. *8*, 3938-3942.

Weber, B.L., Westin, E.H., and Clarke, M.F. (1990). Differentiation of Mouse Erythroleukemia Cells Enhanced by Alternatively Spliced c-myb mRNA. Science *249*, 1291-1293.

Welte, K., Platzer, E., Lu, L., Gabrilove, J., Levi, E., Mertelsmann, R., and Moore, M. (1985). Purification and biochemical characterization of human pluripotent hematopoetic colony-stimulating factor. Proc. Natl. Acad. Sci. USA *82*, 1526-1530.

Westin, E. (1991). unpublished observation.

Westin, E.H., Gallo, R.C., Arya, S.K., Eva, A., Souza, L.M., Baluda, M.A., Aaronson, S.A., and Wong-Staal, F. (1982). Differential expression of the amv gene in human hematopoietic cells. Proc. Natl. Acad. Sci. U. S. A. *79*, 2194-2198.

Westin, E.H., Gorse, K.M., and Clarke, M.F. (1990). Alternative splicing of the human c-myb gene. Oncogene *5*, 1117-1124.

Weston, K. and Bishop, J.M. (1989). Transcriptional activation by the v-*myb* oncogene and its cellular progenitor, c-*myb*. Cell *58*, 85-93.

Williams, D.E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K., March, C.J., Park, L.S., Martin, U., Mochizuki, D.Y., Boswell, H.S., Burgess, G.S., Cosman, D., and Lyman, S.D. (1990). Identification of a ligand for the c-*kit* proto-oncogene. Cell *63*, 167-174.

Witte, O. (1990). Steel locus defines new multipotent growth factor. Cell *63*, 5-6.

Wong, G., Temple, T., Leary, A., Witek-Giannotti, J., Yang, Y., Ciarletta, A., Chung, M., Murtha, P., Kriz, R., Kaufman, R., Ferenz, C., Sibley, B., Turner, K., Hewick, R., Clark, S., Yanai, N., Yokota, H., Yamada, M., Minoru, S., Motoyoshi, K., and Furimaro, T. (1987). Human CSF-1: Molecular cloning and expression of 4 kb cDNA encoding the human urinary protein. Science *235*, 1504-1508.

Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamoto, T., and Toyoshima, K. (1991). Association of B cell antigen receptor with protein tyrosine kinase Lyn. Science *251*, 192-194.

Yang, Y., Ciarletta, A., Temple, P., Chung, M., Kovacic, S., Witek-Giannotti, J., Leary, A., Kriz, R., Donahue, R., Wong, G., and Clark, S. (1986). Human IL-3 (Multi-CSF): Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell *47*, 3-10.

Yen, A., Reece, S., and Albright, K. (1984). Membrane origin for a signal eliciting a program of cell differentiation. Exp. Cell Res. *152*, 493.

Zsebo, K., Williams, D., Geissler, E., Broudy, V., Martin, F., Atkins, H., Hsu, R., Birkett, K., Okino, K., Murdock, D., Jacobsen, F., Langley, K., Smith, K., Takeishi, T., Cattanach, B., Galli, S., and Suggs, S. (1990a). Stem cell factor is encoded at the *Sl* locus of the mouse and is the ligand for the c-*kit* tyrosine kinase receptor. Cell *63*, 213-224.

Zsebo, K., Wypych, J., McNeice, I., Lu, H., Smith, K., Karkare, S., Sachdev, R., Yuschenkoff, V., Birkett, N., Williams, L., Satyagal, V., Tung, W., Bosselman, E., Mendiaz, E., and Langley, K. (1990b). Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. Cell *63*, 195-201.

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