THE HUMAN C-MYC GENE:
ITS EXPRESSION AND REGULATION
DURING LEUKEMIC AND NORMAL GRANULOCYTE
DIFFERENTIATION IN VITRO

by

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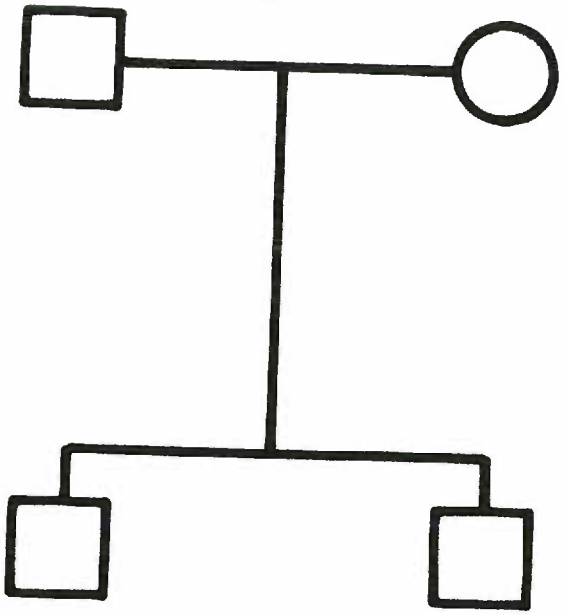

To My Family

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LIST OF ABBREVIATIONS

ALVs.....Avian leukosis virus

CFU-GM.....Colony forming unit-granulocyte/monocyte

C-onc.....Cellular oncogene

CSA.....Colony stimulating activity

CSF.....Colony stimulating factor

DEP/water.....Diethyl pyrocarbonate treated water

DNA.....Deoxyribonucleic acid

DPM.....Disintegrations per minute

Ep.....Erythropoietin

FACS.....Fluorescent activated cell sorter

GTP.....Guanosine triphosphate

HIM.....Hematopoietic inductive microenvironment

HL-60.....Human promyelocytic leukemia cell line

HPCM.....Human placental conditioned medium

HSR.....Homogeneously staining region

K562.....Human erythroleukemia cell line

KG-1.....Human myeloblast leukemia cell line

LDBMC.....Low density bone marrow cells

LTR.....Long terminal repeat

MRA.....Monocyte-derived recruiting activity

NP-40.....Nonidet P-40

PBS.....Phosphate-buffered saline

PDGF.....Platelet-derived growth factor

RNA.....Ribonucleic acid

RSV.....Rous sarcoma virus

SRBC.....Sheep red blood cells

SSV.....Simian sarcoma virus

V-onc.....Viral oncogene

mRNA.....Messenger ribonucleic acid

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ABSTRACT

ABSTRACT

C-*myc* is the cellular homolog of the transforming gene of the avian myelocytomatosis virus; located on the human chromosome 8, band q24, it is highly conserved during evolution. The c-*myc* gene is transcriptionally active in almost all reported acute and chronic myeloid leukemias; myeloid leukemia cell lines HL-60 and KG-1 also contain high levels of c-*myc* transcripts; the transcriptional activity markedly decreases when these cells are induced to undergo maturation by a group of compounds called the "inducers of differentiation". These and other observations have led to the presumption that the c-*myc* gene might play an important role in the maintenance of leukemic growth and differentiation; and that it is the abnormal expression of the c-*myc* gene that contributes to the initiation and/or maintenance of these leukemias. To determine the degree to which the expression of c-*myc* in leukemic cells is inappropriate requires knowledge about the expression and regulation of this gene during the various stages of growth and differentiation of normal myeloid cells.

The objectives of my project were to establish in vitro liquid culture conditions which would permit studies on c-*myc* expression as a function of granulocyte differentiation and second to use these conditions to study the regulation of c-*myc* gene expression in both normal and leukemic granulopoiesis.

A) The cellular characteristics of in vitro granulopoiesis.

Whereas cells of normal bone marrow are predominantly mature hematopoietic elements, leukemic cells are homogeneously immature. Consequently, comparative studies on c-myc expression in unfractionated normal marrow cells and leukemic cells would be unable to distinguish differences that reflect phenomena relating to the neoplastic process and those which merely reflect phenomena which are unique to particular stages of differentiation. I used therefore, in these studies normal marrow cells sequentially depleted of mature blood cells in order to enrich for granulopoietic precursor cells. These progenitor-enriched bone marrow cells were induced to undergo proliferation and differentiation by a myeloid lineage-specific humoral stimulator of growth and differentiation known as colony stimulating activity (CSA). The stimulated cells showed a progressive increase in tritiated thymidine uptake reaching a peak after three days and then declined. Myeloid and T-lymphocyte cell specific monoclonal antibodies and histochemical staining methods were used to examine the pattern of marrow cell differentiation. The CSA-induced differentiation response was largely granulocytic. The differential cell count analysis on Wright stained cyto-spin slides revealed a wave of maturation ranging from predominantly lymphoid appearing immunoglobulin negative cells on day 0 to predominantly non-proliferative meta myelocytes and bands on day 4 with

distinct peaks of myeloblasts after 12 hours, progranulocytes after 24 hours, and myelocytes after 72 hours.

B) C-myc expression in granulopoietic cells.

This in vitro liquid culture system was used in the study of the expression and regulation of c-myc gene during growth and differentiation of normal and leukemic granulocyte progenitor cells. The granulopoietic progenitor cells cultured in the presence or absence of CSA were harvested at various time intervals and the RNA was analyzed for the levels of c-myc and γ-actin and ß-globin mRNA content. The c-myc transcript content was very low on day 0 and increased after 12 hours reaching a peak around 24 hours. The levels reached basal levels consistently by about 36 hours. In contrast to c-myc transcripts, the γ-actin transcript levels remained constant throughout the course of the experiment. ß-globin mRNA was not detected at any time indicating that the differentiating cells in this system are not erythroid cells at least within the limits of detection. An interesting correlation between the peak of c-myc transcripts and predominance of early progranulocytes and their precursors was seen after 24 hours in culture. However, attempts to examine individual cells for c-myc transcripts using in situ hybridization techniques were unsuccessful.

Nonetheless, our findings clearly indicate that some or all of the CSA exposed cells from normal donors express c-myc at a

predictably early stage in culture. Moreover, the number of c-myc transcripts in normal cellsat 24 hours is similar to that found in HL-60 cells. There were three possible explanations for these results. First, that gene amplification is a normal, transient event in granulocyte differentiation. Accordingly, the progenitor enriched human bone marrow cells harvested at various time intervals were analyzed for c-myc gene copy number using a quantitative DNA dot blot method and were compared to that of HL-60 cells. These studies revealed that normal bone marrow cells consistently had 14-16 fold lower signals than HL-60 cells. Therefore, it was concluded that while HL-60 cells used in this study had 14-16 fold higher gene copies (as expected), normal cells do not amplify c-myc gene copy number during myeloid cellular growth and differentiation.

A second explanation of our findings of similar transcript numbers in HL-60 and normal cells was the possibility that HL-60 cells did transcribe c-myc genes 16-fold more than normal cells but that mRNA degradation was substantially (at least 6-8 fold) greater in HL-60 cells. We analyzed this by examining the stability of c-myc mRNA in normal bone marrow and HL-60 cells. These studies revealed that c-myc transcripts in normal bone marrow cells at 24 hours are extremely unstable; the estimated half-life of c-myc mRNA was approximately 15 minutes. Interestingly, however, the c-myc transcripts in HL-60 cells used in these experiments had a slightly longer half-life (20

minutes). Therefore, c-myc transcripts in HL-60 cells are as stable as in normal bone marrow cells. This observation questions the linkage between c-myc gene amplification and transcriptional activity in HL-60 cells.

The third potential explanation of our finding of transcript equivalence, the one we currently favor, is that high transcriptional activity of c-myc is a normal event in CSA-stimulated early granulocyte progenitors and that it is not different from the activity in HL-60 cells in which the gene is amplified 16-fold. A corollary of this explanation is that c-myc transcriptional activity in HL-60 cells, copy-for-copy, is substantially less than that in normal cells. Nuclear run-off experiments may be helpful in this regard and are being carried out at this time.

C) The regulation of the c-myc gene expression.

Because we documented that c-myc transcription is consistently suppressed in normal cells but not in HL-60 cells, we wondered whether freshly obtained human acute leukemia cells also express c-myc gene. Therefore, the differences in the c-myc transcript profiles in normal and leukemic marrow progenitor cells were analyzed by comparing transcript contents at various time intervals. These comparisons revealed that normal marrow cells consistently repress c-myc transcript levels after an initial burst; the leukemic cells on the other hand,

had high c-myc mRNA content; and the levels did not change throughout the culture period. Therefore, the most consistent abnormality in leukemic cells is their failure to suppress c-myc transcription.

In summary, the c-myc gene transcripts increase transiently in normal granulocyte differentiation while leukemic cells show consistently high levels of c-myc transcripts. It is perfectly normal for leukemic cells "arrested" at a primitive stage of granulocyte differentiation to express c-myc gene. The most consistent abnormality in acute myeloid leukemia cells is their failure to suppress c-myc transcription. Therefore, the c-myc gene expression is a normal event in granulopoiesis possibly linked not only to proliferative activity but to the primitive developmental stage as well. The c-myc transcription per se is insufficient to account for the neoplastic phenotype in myeloid leukemia cells. Studies addressing the mechanisms by which normal myeloid cells repress c-myc transcription should provide important insights on the regulatory abnormalities of gene expression which underlie the behavior of myeloid leukemia cells.

CHAPTER 1

CHAPTER 1

THE CELLULAR ONCOGENES

1.1 INTRODUCTION

CONCEPT OF "CANCER GENES"

The genetic origin of cancer has been an article of faith for over half a century (Bishop, 1983). Formal Mendelian analyses revealed that explicit genetic determinants of carcinogenesis were involved in several experimental systems, including fish, insects, and plants (Anders et al., 1984). The examination of human pedigrees engendered the concept of "cancer genes" whose inherited abnormalities predispose to specific forms of neoplasia (Knudson, 1979; Comings, 1973). The discovery of vertically transmitted leukemia in inbred mice brought retroviruses on stage, leading eventually to the proposal that retroviral oncogenes reside in the germ lines of all species (Huebner and Todaro, 1969; Todaro and Huebner, 1972). The proposal proved to be only slightly wide of the mark. Specifically, it is now known that vertebrate and some invertebrate species do indeed harbor genetic loci homologous to retrovirus oncogenes, but these loci are cellular, not viral genes.

UNVEILING CELLULAR ONCOGENES: EXPERIMENTAL STRATEGIES

Three separate experimental strategies have been used to

identify the cellular oncogenes and to implicate these genes in carcinogenesis.

Viral transduction: Many retroviruses bear genetic loci (oncogenes) whose activities are responsible for both the initiation and maintenance of neoplastic transformation induced by viral infection. At the time of discovery, retroviral oncogenes were viewed as biological anomalies akin to the oncogenic determinants of other tumor viruses. However, the discovery that both avian (Stehelin et al., 1976) and mammalian (Spector et al., 1978) DNAs contain nucleotide sequences closely related to the oncogene src of Rous sarcoma virus (RSV) led to the identification of 34 more oncogenes in vertebrate species (Tables 1.1 and 1.2).

Insertional mutagenesis: Integration of viral DNA into the host genome to give a provirus is an inevitable consequence of infection by retroviruses (Bishop, 1983). Integration is potentially mutagenic on two counts: it may disrupt a vital region of the host genome, and it can bring powerful regulatory functions of the virus to bear on the expression of host genes. Almost all of the lymphomas induced by ALVs which do not possess oncogenes, contain viral DNA integrated in a common domain of the host genome (Hayward et al., 1981; Payne et al., 1982). Within this domain lies the cellular gene whose activity is apparently augmented as

a consequence of the insertion of viral DNA (Bishop, 1983; Shibuya, 1982). If other retroviruses without oncogenes act by similar means, the sites at which their proviruses integrate into cellular DNA may reveal previously unrecognized cellular genes whose activation can be implicated in tumorigenesis.

DNA-mediated gene transfer (Transfection): The unveiling of potential cancer genes by transduction and insertional mutagenesis with retroviruses was largely serendipitous. A more deliberate assault on the problem was mounted by the use of transfection, in a quest for evidence that DNA of tumor cells is abnormal (Cooper, 1982). The quest has met with stunning success: DNA from a large variety of solid and hematopoietic tumors "transforms" certain lines of cultured cells to a neoplastic phenotype (Cooper, 1982). Among the first harvests of this endeavor were genes previously known to retrovirologists as the cellular homologs (and presumed progenitors) of two retroviral oncogenes, the Harvey and Kirsten forms of v-ras (Der et al., 1982; Parada et al., 1982; Pulcini et al., 1982). In addition, this approach has yielded five distinct oncogenes that are not found in any of the retroviruses (Table 1.2).

DEFINITIONS

Retrovirologists have adopted a nomenclature in which viral

oncogenes are known as v-onc's, the cellular progenitors of v-onc's as cellular oncogenes (c-onc's), and each viral and cellular gene by terms derived from the names of the virus in question, e.g. v-src, c-src, v-ras, c-ras (Bishop, 1983; Bishop and Varmus, 1982).

1.2 STRUCTURAL AND PHYLOGENETIC FEATURES OF CELLULAR ONCOGENES

Not all c-onc genes have yet been described in full. The ones that have been studied, possess all the cardinal features of an eukaryotic gene, including the regions where transcription of the gene starts and stops, introns, polyadenylation sites etc. Cellular oncogenes behave as classical Mendelian loci. They occupy constant positions within the genomes of particular species (see Table 1.1 and 1.2). They are apparently present in all members of a given species (Bishop, 1983; Hughes et al., 1979a, 1979b). All the cellular oncogenes segregate in a predictable fashion when breedings are analyzed with the assistance of occasional structural polymorphisms that have been identified by restriction mapping (Bishop, 1983).

Evolutionary conservation is a hallmark of all cellular oncogenes. The extent of evolutionary conservation varies from one cellular oncogene to another. Some are readily detectable only in closely related species; as a consequence, their evolutionary age is difficult to judge. Others appear to have taken recognizable form well before the emergence of vertebrates

(Shilo and Weinberg, 1981; Anders et al., 1984). For example, certain cellular oncogenes have been identified in the insect Drosophila and the worm Caenorhabditis elegans (Shilo and Weinberg, 1981). This is of special interest because we can now place the origin of some cellular oncogenes much deeper in the recesses of time than previously anticipated (Gateff, 1978, 1983; Anders et al., 1984).

1.3 EXPRESSION OF CELLULAR ONCOGENES

The possibility that c-onc's might be expressed in phenotypically normal cells first came into view with the discovery of RNA transcribed from c-src in uninfected fibroblasts and tissues of several avian species (Spector et al., 1978a, 1978b). Virtually all c-onc's are transcribed into RNA in normal and/or tumor cells.

Transcription from c-onc's follows a route already familiar from the study of other cellular genes (Breathnach and Chambon, 1981). The initial transcript contains introns as well as exons and is then spliced into a series of smaller intermediates that are found only in the nucleus (Gonda et al., 1982). Introns have been eliminated entirely from cytoplasmic RNAs derived from c-onc's (Gonda et al., 1982).

As the study of transcription from c-onc's broadened over the past few years, several general principles have emerged:

1. Transcription from c-onc's occurs in a variety of tissues and in every vertebrate and invertebrate species that has been examined.

2. The amounts of RNA produced from c-onc's in most cells are extremely small (about 1-10 copies per cell). Occasional exceptions have been found in both normal tissues (Chen, 1980; Gonda et al., 1982; Muller et al., 1982; Shibuya et al., 1982) and tumor cells (Westin et al., 1982; Eva et al., 1982), but the significance of these variations and of the low constitutive levels of expression is not known.

3. C-onc's are not coordinately expressed as a group, nor with the genes of endogenous retroviruses, and the function of each gene may be required only in certain tissues (Bishop, 1983).

4. Each c-onc gives rise to distinctive RNAs whose sizes are generally conserved in various types of cells and in different species (Gonda et al., 1982). The constancy of these RNAs among divergent species of vertebrates is further testimony to the selective pressures that have preserved the structure and presumed function of c-onc's.

5. Many of the mRNAs for c-onc's are appreciably larger (three-fold or greater) than would be required to encode the presumed gene products (Gonda et al., 1982; Bishop, 1983), as if the mRNAs had uncommonly large untranslated regions. Indeed, the c-myc gene transcripts have an 550 base long untranslated region (Battey et al., 1983; Watt et al., 1983).

6. Most of the c-onc's appear to produce a single cytoplasmic RNA, in accord with the expectation that each locus represents one gene (Gonda et al., 1982). Two exceptions identified to date are c-myc which has two promotors producing two transcripts from a single gene locus (Leder et al., 1983; Watt et al., 1983) and avian embryos which contain at least two mRNAs derived from c-erb-A and at least two derived from c-erb-B (Bishop, 1983; Gonda et al., 1982).

7. Many of the oncogenes are transcriptionally active at various stages of pre- and postnatal development of mouse (Muller and Verma, 1984; Muller, 1983a; Muller et al.,1982, 1983b, 1983c, 1983d; Slamon and Cline, 1984).

1.4 CELLULAR ONCOGENE PROTEINS AND THEIR FUNCTIONS

The biochemical functions of cellular oncogenes have not

been easy to discern, principally because they have been available only in small quantities. Currently, oncogene proteins fall into four functional categories:

Tyrosine-specific protein-kinases: Much of the what is known about these proteins stems from the work of Collett and Erickson (1978). They showed that p60^{src} from RSV-infected cells catalyzed the addition of a phosphate molecule to other proteins. Later, it was shown that p60^{src} is unusual in that it phosphorylates tyrosine (Hampe et al., 1984). Since then, the proteins of the known oncogenes have all been tested for tyrosine-specific protein-kinase activity. Six of them, in addition to p60^{src}, were shown to have this enzymatic property (the products of the yes, fgr, abl, fps, fes and ros oncogenes), whereas the others did not display any detectable tyrosine-specific protein-kinase activity (Hampe et al., 1984; Hunter, 1984; Wang and Baltimore, 1985). Initial studies of p60^{src} sequences revealed a stretch of 250 amino acids near one end which is known to be the protein-kinase domain responsible for catalyzing the transfer of phosphates (Hunter, 1984). A recognizably similar domain of about the same length is found in each of the other six oncogenic proteins shown to have tyrosine-specific protein-kinase activity (Hunter, 1984).

The striking similarity of the catalytic domains in the various enzymes suggest that they may have had a common evolutionary progenitor. The normal physiological role of these proteins is not fully understood.

Growth factors and growth factor receptors: One of the most striking properties of tumor cells is their abnormally controlled proliferation. They somehow evade a number of control systems that keep normal cells from dividing too often or continuing to divide indefinitely. Evidence that growth factors might be involved in malignant transformation has recently accumulated. It has become evident that cells stimulated by growth factors and cells transformed by viruses are strikingly similar, having increased solute transport, DNA synthesis, tyrosine kinase activity, failure of contact inhibition, and continued cell division, when contrasted with nonstimulated cells (Deuel and Huang, 1983a, 1983b). A definitive relationship between growth factors and oncogenes was established by the demonstration that the amino acid sequence of a portion of human platelet derived growth factor (PDGF) had striking homology to the predicted amino acid sequence of $p28^{v\text{-}sis}$ (Doolittle et al., 1983; Waterfield et al., 1983). This structural relationship suggested that a PDGF-like growth factor is the product of the transforming gene of simian

sarcoma virus (SSV) and that expression of the growth factor activity might mediate the unregulated growth characteristic of SSV-transformed cells (Devare et al., 1983). Such a growth factor activity has now been identified in SSV-transformed cells but not in nontransformed control cells and these results provide direct evidence for the expression of a PDGF-like molecule encoded by the viral oncogene v-sis, which may in turn be responsible for the abnormal growth regulation of SSV-transformed cells (Deuel et al., 1983). SSV-transformed fibroblasts not only synthesize but also secrete a PDGF-like growth-promoting activity which appears to serve as an autocrine stimulator of growth of SSV-transformed cells (Deuel and Huang, 1983b).

The binding of growth factors is mediated through specific cell surface receptors which, in turn, initiate a cascade of biochemical events leading to DNA synthesis and cellular proliferation (Deuel and Huang, 1983a). A close relationship between growth factor receptors and the transforming proteins of oncogenes has been provided recently by the finding of marked homology between the EGF receptor and the protein encoded by v-erb-B (Downward et al., 1984). It is now known that v-erb-B protein, like EGF receptor possesses tyrosine-specific protein kinase activity (Gilmore et al., 1985; Kris et al., 1985). These

observations suggest an additional mechanism whereby mutations in specific cellular genes might alter the protein products and thereby lead to unregulated growth characteristic of cancer cells.

GTP-binding proteins: The protein products of all three members of the ras gene family (Ha-ras, Ki-ras and N-ras) are immunologicaly related, GTP-binding proteins (McGrath et al., 1984). It is now known that normal ras proteins in addition to GTP-binding activity, possess GTPase activity (McGrath et al., 1984). It has been hypothesized that these proteins on binding GTP function as "coupling factors" in systems relaying signals from the exterior of the cell to the interior (Newbold, 1984). Recently it has been shown that RAS genes in yeast (analogous to mammalian ras genes) are involved in controlling GTP-dependent adenylate cyclase (Toda et al., 1985). Because there has been conservation of immediate biochemical function of ras during evolution, it has been suggested that ras proteins may have analogous functions in humans (Toda et al., 1985).

Nuclear proteins: The oncogene products that fall into this group are perhaps the most intensely studied and at the same time are the most obscure of all known oncogene proteins. This category includes products of oncogenes

myc, myb fos and ski. A number of human cancers show evidence for abnormal expression of the c-myc gene as a result of amplification or of inappropriate regulation. In Burkitt's lymphoma, the c-myc gene is consistently involved in rearrangements with immunoglobulin genes. Therefore, it has been presumed that these events might be involved in the initiation and/or maintenance of these neoplasms. However, without knowing their actual biochemical function, there is no way of telling how this gene is involved in these neoplasms. For example, the expression of the c-myc gene in Burkitt's lymphoma cells may be appropriate for cells at that stage of B-lymphocyte differentiation. Indeed, Croce and his collaborators have shown that the normal and translocated c-myc genes are differentially expressed in cell hybrids with different cellular background representing various stages of B-lymphocyte differentiation (Croce, et al., 1984, 1985).

1.5 CELLULAR ONCOGENES AND NEOPLASTIC TRANSFORMATION

The broad spectrum of neoplasias affecting a wide variety of cell types may involve multiple mechanisms of transformation. Three widely held views on possible mechanisms of neoplastic conversion are quantitative changes, qualitative alteration, and cell type specificity of the products of oncogenes.

Quantitative changes: When the product of a viral or cellular oncogene is synthesized at elevated levels, normal cellular metabolic processes may be altered in a manner that leads to cellular transformation. Experimental support for this notion can be derived from studies on c-mos (mouse) or c-H-ras-1 (human) genes which, when linked to an appropriate regulatory unit (LTR) can lead to cellular transformation (Oskarsson et al., 1980; Blair et al., 1981).

Avian leukosis virus (ALV) induces B-cell lymphomas which is thought to result at least in part from activation of the cellular myc gene (Hayward et al., 1981; Payne et al., 1982). Over 80% of all ALV-induced B-cell neoplasms were found to contain ALV-LTR sequences integrated in the vicinity of the c-myc gene (Hayward et al., 1981). Integration of a provirus next to the c-myc gene has also been observed in 90% of B-cell lymphomas induced by nondefective reticuloendotheliosis virus (Noori-Daloii et al., 1981). In the case of ALV-induced erythroblastosis in chickens, the ALV provirus is integrated next to the c-erb-B gene (Fung et al., 1983). The data strongly implicate transcriptional activation of c-myc and c-erb-B genes in the induction of B-cell lymphomas and erythroblastosis, respectively.

One other mode of quantitative change is brought about

by a process called gene amplification. In the HL-60 cell line, a 16-32 fold amplification of the human c-myc gene is reported (Collins and Groudine, 1982; Dalla-Favera et al., 1982). This amplification appears to be accompanied by a significant increase in the levels of c-myc transcripts (Westin et al., 1982). The amplification of the c-myc gene is also seen in cell lines derived from human colon carcinoma (Alitalo et al., 1983). This mode of quantitative modulation of gene expression is not restricted to the c-myc gene. For example, c-Ki-ras is amplified 30- to 60-fold in mouse adrenocortical tumor (Schwab et al., 1983), c-abl is amplified 4- to 8-fold in human chronic myelogenous leukemia cell line K562 (Collins and Groudine, 1983), and the N-myc gene is amplified 16- to 32-fold in neuroblastoma cell lines (Schwab et al., 1984).

Qualitative alterations: When an oncogene product undergoes a mutation, the altered cellular protein may acquire oncogenic potential. A number of transforming genes have been isolated from mouse and human tumor cells by transforming assays using NIH/3T3 recipient cells (Duesberg, 1983). Molecular analysis of the oncogene of the human bladder carcinoma cell line EJ or T24 revealed that a single amino acid substitution near the N-terminus (position 12) is responsible for the activation of its

transforming potential (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982). As a consequence of this point mutation, the c-Ha-ras-encoded P21 has an altered GTPase function (Tabin et al., 1982). Similar findings have been reported for p21 in human colon and lung tumor cells containing activated c-Ki-ras genes (Der and Cooper, 1982). N-ras has been reported to be activated in human sarcoma, leukemia and neuroblastoma (Hall et al., 1983).

In several instances it has been shown that v-onc genes and their homologs have structurally different c-termini. For example, nucleotide sequence analyses of v-myc and c-myb genes have indicated that the predicted onc gene products differ at their c-termini (Klempnauer et al., 1982). Similarly, the major difference between the deduced amino acid sequences of $p60^{c\text{-}src}$ and $p60^{v\text{-}src}$ is that 19 c-terminal amino acids of $p60^{c\text{-}src}$ are replaced by a new sequence of 12 amino acids in $p60^{v\text{-}src}$ (Muller and Verma, 1984).

Cell type specificity: Expression of a cellular or viral oncogene at abnormal levels or in a structurally aberrant form may not be sufficient to induce malignant transformation. In addition, the induction of neoplasia may require expression of the oncogene in a cell type that is susceptible to malignant conversion by the respective

oncogene product. For example, the level of c-fos transcripts in the late-gestation amnion is nearly equal to the concentration of v-fos mRNA in FBJ-MSV-transformed cells (Muller et al., 1983b, 1983c). C-fos protein can be detected in day 18 mouse amnion by immunofluorescence (Curren et al., 1984). However, such high levels of c-fos expression in the amnion does not lead to malignant transformation although the c-fos gene product can induce neoplastic transformation of other cell types (Miller et al., 1984). Infection of avian macrophages with RSV does not induce any changes in the morphology, growth behavior, or expression of macrophage-specific proteins. This failure may be due to a restriction in the cellular response to a functional src protein, perhaps due to the absence of cellular products which are essential for mediating $p60^{src}$ induced transformation (Lipsich et al., 1984). These results suggest that only certain types of cells can be targets for c-onc transformation. Such target cells could be those cell types where the respective c-onc gene has a physiologic function. These cells could provide intracellular target molecules with which the c-onc protein needs to interact. If the c-onc gene product is expressed constitutively at abnormal levels or has an aberrant (mutated) structure, it may escape the cellular regulatory mechanism and as a consequence, initiate the process of neoplastic transformation.

Table 1.1

Table 1.1. Retroviral oncogenes and their corresponding human proto-oncogenes

Retroviral oncogene	Name and origin of virus	Species of origin	Tumor	Known chromosomal location of human proto-oncogene	Intracellular Location
v-*src*	Rous sarcoma	Chicken	Sarcoma	20	cytoplasm, Plasma membrane
v-*fps*	Fuginami sarcoma	Chicken	Sarcoma	?	Cytoplasm, Plasma membrane
v-*fes*	Snyder-Theilen Feline sarcoma	Cat	Sarcoma	15	Cytoplasm, Plasma membrane
v-*yes*	Yamaguchi sarcoma	Chicken	Sarcoma	?	Cytoplasm, Plasma membrane
v-*ros*	Rochester sarcoma	Chicken	Sarcoma	?	?
v-*myc*	Myelocytomatosis	Chicken	Sarcoma, Carcinoma, Myelocytoma	8	Nuclear matrix
v-*erb-A*	Erythroblastosis	Chicken	Erythroleukemia	17	cytopalasm
v-*erb-B*	Erythroblastosis	Chicken	Sarcoma	7	Plasma membrane
v-*myb*	Myeloblastosis	Chicken	Myeloblastic Leukemia	6	Nucleus
v-*rel*	Reticulo-endotheliosis	Turkey	lymphatic leukemia	?	?
v-*mos*	Molony Sarcoma	Mouse	Sarcoma	8	Cytoplasm
v-*abl*	Abelson leukemia	Mouse/cat	B-cell Lymphoma	9	Inner surface, Plasma membrane
v-*fos*	FBJ murine Osteosarcoma	Mouse	Sarcoma	14	Nucleus
v-*raf*	3611 Murine Sarcoma	Mouse	Fibrosarcoma	3	Cytoplasm
v-Ha-*ras*/ v-*bas*	Harvey Rat Sarcoma	Rat/Mouse	Sarcoma and erythroleukemia	11	Inner surface, Plasma membrane
v-Ki-*ras*	Kirsten Rat Sarcoma	Rat	Sarcoma and erythroleukemia	12	Inner surface, Plasma membrane
v-*fms*	SM feline Sarcoma	Cat	Sarcoma	5	Plasma membrane
v-*sis*	Simian Sarcoma	Woolly Monkey/Cat	Sarcoma	22	Growth factor
v-*ets*	E26 myeloblastisis	Chicken	myeloid, erythtoid leukemia	11	?
v-*ski*	Chicken sarcoma	Chicken	Sarcoma	1	Nucleus
v-*fgr*	Gardner-Rasheed feline sarcoma	Cat	Sarcoma	?	?

Table 1.2

Table 1.2. Proto-oncogenes identified by transfection and other means.

Oncogene	Species of origin	Tumor	Known chromosomal location of human proto-oncogene	Intracellular Location	Method of detection
N-*ras*	Human	Neuroblastoma Promyelocyte leukemia	1	Inner surface, Plasma membrane	Transfection
B-*lym*	Human	Burkitt's lymphoma	1	?	Transfection
N-*myc*	Human	Neuroblastoma	2	?	Homology to c-myc
Int-1	Human	Carcinoma	12	?	Insertion mutagenesis
Int-2	Human	Carcinoma	11	?	Insertion mutagenesis
Neu	Human	Neuroblastoma	17	Plasma membrane	Transfection
Met	Human	Osteosarcoma	7	?	Transfection
Mel	Human	Melanoma	19	?	Transfection
Bcl-1	Human	Burkitt' lymphoma	11	?	Chromosome walking
Bcl-2	Human	Burkitt's lymphoma	18	?	Chromosome walking
Tcl-1	Human	T-cell leukemia	14	?	Chromosome walking
Tcl-2	Human	T-cell leukemia	11	?	Chromosome walking
R-*myc*	Human	?	1	?	Homology to c-myc

CHAPTER 2

CHAPTER 2

THE HUMAN C-MYC GENE

2.1 INTRODUCTION

The viral oncogene, v-myc, which is harbored by avian myelocytomatosis viruses, is derived from a cellular gene (c-myc) found in all vertebrates (Robins et al., 1982; Roussel, 1979; Sheiness and Bishop, 1979; Vennstrom et al., 1982). While little is known about the function of the c-myc protein, the structure of this gene is known in great detail (Battey et al., 1983; Watt et al., 1983). The gene is encoded in three discontinuous exons separated by two large intervening sequences as shown diagramatically in Figure 2.1. The first exon has several interesting features, including the fact that it forms the major portion of a segment of DNA that is tightly conserved between man and mouse (Battey et al., 1983). Given that this evolutionary conservation suggests functional selection, it is surprising that the 550-base-long segment has no translational initiation codons (eight would have been expected in a random sequence of this length). Furthermore, this portion of the c-myc gene contains multiple termination codons in all three reading frames and is, therefore, an untranslated mRNA leader sequence. In addition, this region contains at least two active promotors with transcription initiation sites located about 150 base pairs from one another within the leader sequences (Figure 2.1).

The remaining portion of the c-myc gene contains two coding exons that direct the synthesis of two phosphoproteins of apparent molecular weights of 64 and 66/67 (Hann et al., 1984; Ramsey et al., 1984) encoded either by two mRNA's (Hann et al., 1984) or they could represent differently modified versions of the same gene product (Ramsey et al., 1984). Subcellularly, the c-myc proteins have been localized to the nucleus (Hann et al., 1984; Eisenman et al., 1985) presumably with three types of associations: a major fraction (60 to 90%) is retained in the nuclear matrix, a small fraction (1%) is released during nuclease digestion of the DNA in intact nuclei in the presence of low-salt buffer, and a fraction (10%) is extractable with salt or detergents (Hann et al., 1984; Eisenman et al., 1985).

2.2 REGULATION OF C-MYC GENE EXPRESSION

The c-myc gene encodes two mRNA molecules from two transcription initiation sites located about 150 base pairs from one another within the leader sequence (Battey et al., 1983; Watt et al., 1983). Both mRNA molecules are extremely unstable with a half-life of approximately 15 minutes (Dani et al., 1984). The c-myc proteins also have a very short half-life of about 20-30 minutes (Hann et al., 1985). Mechanisms of c-myc regulation have been very intensely studied in recent years. Two different models, though not mutually exclusive, have been

proposed to explain the regulation of the c-myc gene.

Trans-acting protein repression model: The c-myc gene is translocated consistently into the region of the immunoglobulin heavy chain or of the light-chain loci in a variety of human Burkitt lymphomas and murine plasmacytomas (Adams et al., 1982; Adams et al., 1983; Ar-Rushdi et al., 1983; Battey et al., 1983; Leder et al., 1983; Stanton et al. 1983; Taub et al., 1982). This translocation event creates a situation in which the expression of the two alleles can be compared. Such studies have shown that only the translocated allele is significantly expressed, while that on the normal homologous chromosome seems inactive (Taub et al., 1984). Furthermore, c-myc gene expression could be enhanced when the cells were exposed to cycloheximide, a potent inhibitor of protein synthesis (Kelly et al., 1983; Makino et al., 1984). These data are compatible with the notion that the c-myc gene might be under the control of a labile trans-acting protein (Leder et al., 1983). In order to explain these results, Leder et al. (1983) have proposed that the c-myc gene regulation may be repressed in one or all of three ways: (i) direct autorepression by the c-myc protein. (ii) indirect autoregulation by a repressor that responds to the levels of c-myc protein, or (iii) regulation by a repressor unrelated to the c-myc protein. The translocation events

in Burkitt lymphomas and plasmacytomas could disrupt these regulatory processess leading to the development of neoplasia (Leder et al., 1983). Lymphoblastoid-like hybrids between Daudi and human lymphoblastoid cells co-express both the translocated and normal c-myc genes; this seems to rule out direct c-myc autoregulation (Croce et al., 1984).

Stem-loop model: Saito et al., (1983) have proposed that mRNA from the untranslocated c-myc gene, as opposed to that of the translocated c-myc gene, could form a stable stem-loop structure where the initiator AUG codon would be located within the loop. This secondary structure could severely hinder the translation and the translocated chromosome could escape this translational suppression because of decapitation (separation of the 5' c-myc control elements + exon 1 and exons 2 + 3) of the c-myc gene during translocation.

Even though these models seem attractive, they are not without pitfalls. First of all, both models fail to consider that in many Burkitt lymphomas with the t(8;14) translocation the translocated c-myc gene is not rearranged (Adams et al., 1982, 1983; Ar-Rushdi et al., 1983; Croce et al., 1983; Dalla-Favera et al., 1983; Erickson et al.,1983; Leder et al., 1983; Stanton et al., 1983; Taub et al., 1982) and that in the variant lymphomas with the t(2;8) and t(8;22) translocations,

the rearrangement does not involve sequences 5' of the coding exons but occurs distally (3') to the c-myc gene (Croce et al., 1983).

The repressor model does not consider post-transcriptional regulation. As suggested by Taub et al (1984), the trans-acting element(s) may not be a transcriptional repressor but a factor that acts by accelarating c-myc mRNA degradation. Indeed, Dani et al., (1984) have shown that the c-myc mRNA has an extremely short half-life and that the treatment of cells with emitine, a potent protein synthesis inhibitor has a stabilizing effect on c-myc mRNA. Furthermore, ß-interferon sensitive Daudi cells when exposed to ß-interferon show a significant decrease in cytoplasmic mRNA while the transcription rate does not change (Einat et al., 1985). However, the cells insensitive to ß-interferon do not show any change in the cytoplasmic c-myc mRNA nor in the transcriptional rate (Einat et al., 1985). These observations indicate that post-transcriptional events may be important mechanisms in c-myc gene regulation. I suspect that fine tuning of this gene occurs at several levels.

2.3 C-MYC AND CELLULAR IMMORTALIZATION

When are liberated all the desires that lodge in one's heart,
Then a mortal becomes immortal! Therein he reaches Brahman!
When are cut all the knots of the heart here on earth,
Then a mortal becomes immortal!

[From Katha, II, III, and VI, selected verses, in Hume (1954)]

Use of the 3T3 cell assay to measure transforming function of DNA from a human bladder carcinoma cell line has identified DNA homologous to the ras gene of Harvey rat sarcoma virus (Tabin et al., 1982; Der, et al., 1982). Later it was found that the mutated human proto-Ha-ras, which transforms 3T3 cells, does not transform primary rat embryo cells (Land et al., 1983a; Ruley, 1983) and more significantly, does not transform human embryo cells (Sager et al., 1983). In an effort to explain why mutated proto-Ha-ras transforms cells of the "preneoplastic" 3T3 cells, but not primary rat or human embryo cells, the proposal was made that mutated proto-Ha-ras is only one of at least two activated genes that are necessary to induce transformation (Land et al., 1983a, 1983b; Ruley, 1983). This hypothesis was tested when primary rat cells were transfected with a mixture of the mutated human proto-Ha-ras and either MC29 provirus or activated proto-myc from mouse plasmacytoma (Land et al., 1983a), or with the EIA gene of adenovirus (Ruley, 1983) as helper genes. None of these genes was able to transform rat embryo cells by themselves, but some cells were transformed by the artificially coupled genes. Therefore, it was proposed that "myc-related" genes convert rat embryo cells to cells that are capable of dividing indefinitely, like 3T3 cells, a function termed "immortalization" (Land et al., 1983a, 1983b). However, the immortalization effect of MC29 or of activated proto-myc was not demonstrated independently. Moreover, the proposal did not

explain why an immortalization gene was necessary. Of course, immortalization is necessary to maintain cells in culture. However, immortalization is not necessary for focus formation (Duesberg, 1985) and probably not for tumor formation since embryo cells are capable of sufficient rounds of mitoses (up to 50) in cell culture (and in vivo) to develop tumors (Holliday, 1983). The failure of attempts to maintain cells from many human tumor cells in culture, and our own unsuccessful attempts to maintain myeloid leukemic cells which expressed high levels of c-myc, suggests that immortality may not be an essential criterion of a tumor cell (Duesberg, 1985) and c-myc gene products may not have any role in a proposed immortalizing effect.

2.4 C-MYC AND THE CELL CYCLE

Very little is known about the actual physiological role the c-myc protein plays in the biology of a cell. That the c-myc gene has been highly conserved during vertebrate evolution suggests that it may serve a critical function in a normal cell. The apparent potential of the v-myc gene to interfere with normal cellular proliferation and differentiation suggests that the c-myc gene product may play a role in the regulation of proliferation and differentiation of normal cells (Bishop, 1983). This expectation is borne out by recent observations directly implicating c-myc in the control of normal and abnormal

cell growth.

The progression from quiescence to DNA synthesis (that is, through G_0-G_1 in the cell cycle) involves a phase known as "priming" for growth competence probably triggered by tissue-specific growth factors (Robertson, 1984); and it is in this phase that c-myc is thought to act. Kelly et al., (1983) have been able to show that a fibroblast mitogen PDGF, and two lymphocyte mitogens, concanavalin A and lipopolysaccharide, stimulate the expression of c-myc in those cells that normally respond to them. All three mitogens are believed to act early in the cell cycle to induce competence; and all three stimulate transient expression of c-myc within 1-2 hours, with a return to baseline well before the onset of DNA synthesis. Studies on the expression of the c-myc gene in quiescent A31 and chemically transformed A31 cells, Campisi et al., (1984) found that after a normal cell has been transformed into a tumor cell, the c-myc expression was constitutive. This suggests that the regulation of the c-myc gene may be disrupted in such a way that its activation is independent of growth factors. α-interferon mediated reduction of c-myc mRNA in Daudi cells further implicates its relation to the G_0/G_1 phase of the cell cycle because, α-interferon mediates G_0/G_1 arrest (Einat et al., 1985).

Even though these studies have been interpreted by some investigators to indicate that the expression of c-myc may be

specific to the G_1 portion of the cell cycle, the alternative point of view is that the transient increase in c-myc mRNA following the stimulation of quiescent cells could be a serum dependent phenomenon and c-myc expression may reflect the competency of the cell to enter and progress through the cell cycle. That this is likely the correct view is suggested by work of Hann et al., (1985) and Thompson et al., (1985) in which non-synchronized cells were separated on the basis of their volume into subpopulations representing progressive stages of the cell cycle. They were able to clearly show that c-myc mRNA and protein are constant throughout the cell cycle regardless of the cell cycle stage. The implication of these data is that the rate of synthesis, turnover and modification of c-myc protein (Hann et al., 1985) and mRNA (Thompson et al., 1985) is independent of the cell-cycle stage. In the presence of the appropriate growth factors, both proliferative competency and c-myc expression can be maintained irrespective of the position in the cell cycle (Hann et al., 1985; Thompson et al., 1985). Therefore, it appears that the characteristic mode of c-myc expression is continuous synthesis and rapid turnover throughout the cell cycle in undifferentiated cells and tumor cells which are inherently competent to divide.

2.5 C-MYC AND CELLULAR DIFFERENTIATION

A number of cell lines can be induced to undergo

differentiation by a class of compounds called "inducers of differentiation" (Bustros et al., 1985; Gonda and Metcalf, 1984; Lachman et al., 1984; Reitsma et al., 1983). These cells lose their competency to divide as they terminally differentiate. Upon induction, these cells show a dramatic decrease in the steady-levels of c-myc mRNA (Bustros et al., 1985; Gonda and Metcalf, 1984; Lachman et al., 1984; Reitsma et al., 1983). It is conceivable that c-myc expression may be a differentiation stage specific phenomenon and that when the cells reach the stage where its product is no longer needed, the cells repress the expression of c-myc. Croce et al., (1984) studied the expression of a translocated c-myc gene at various stages of B cell differentiation by creating somatic cell hybrids between human Burkitt lymphoma cells with human lymphoblastoid cells and with mouse plasmacytoma cells. They were able to show that the translocated c-myc gene is transcribed in the plasma cell environment but repressed in the lymphoblastoid cell environment. Interestingly, the non-translocated c-myc gene was completely active in lymphoblastoid cell background and was totally repressed in plasma cell back ground (Croce et al., 1984). The results imply that c-myc expression may be a differentiation stage specific phenomenon, at least in lymphoid cells.

It remains to be seen how these three events can be dissected to describe the role of c-myc gene products in the

biology of the cell. The relationship of c-myc gene products to stages in cell-cycle or to competency of the cells to go through division can easily be studied by using reversible inhibitors of cellular growth or using counterflow centrifugation (Hann et al., 1985; Thompson, et al., 1985). However, the separation of proliferative competency and processes of differentiation is difficult because normal cells progressively lose the former as they exhibit the latter.

2.6 C-MYC AND NEOPLASIA

Alterations in the growth characteristics that accompany the cellular transition from a normal to a neoplastic state could result from changes in a relatively small number of proto-oncogenes (Bishop, 1983a, 1983b; Cairns, 1981; Land et al., 1983). In the case of the c-myc gene, two major notions regarding its action as an oncogene have emerged. The first of these is that c-myc is often activated through an aberrancy of gene regulation rather than a template malfunction. Regulatory disturbances can develop in one of three ways.

Gene Amplifications: In the human promyelocytic cell line HL-60, a 16- to 32-fold amplification of the human c-myc gene has been reported (Collins and Groudine, 1982; Dalla-Favera et al., 1982). The c-myc amplification appears to be localized to a homogeneously staining region

(HSR) and to double minutes of chromosome 8, which has previously been shown to be the chromosomal locus of the c-myc gene (Neel et al., 1982). The c-myc amplification in HL-60 cells is accompanied by a significant increase in the levels of c-myc transcripts (Westin et al., 1982). Since the same c-myc amplification was detected in fresh leukemic cells of the same individual from whom the HL-60 cell line originated, it has been suggested that c-myc amplification may have been involved in the induction of leukemia in this case (Dalla-Favera et al., 1982). This hypothesis will, however, be difficult to prove since nonhematopoietic cells from the same patient are not available for analysis. Even if the amplification had been found only in hematopoietic cells, the possibility would also remain that c-myc amplification might represent a normal event in the differentiation of myelocytic cells (Collins and Groudine, 1982; Dalla-Favera et al., 1982). Some of the studies herein were specifically designed to determine the degree to which the level of c-myc expression in HL-60 cells is linked to gene amplification. Amplification of the c-myc gene was also observed in cell lines derived from human colon carcinoma, where the amplified c-myc sequences were found on double minute chromosomes or within the HSR on the distorted X chromosome (Alitalo et al., 1983).

Insertional Mutagenesis: A second mechanism of c-myc deregulation would be by insertion of a powerful promotor which would activate the c-myc gene. ALVs induce B-cell lymphomas which are thought to result at least in part from activation of the cellular myc gene (Hayward et al., 1981; Payne et al., 1982). Over 80% of all ALV-induced B-cell neoplasias were found to contain ALV-LTR sequences integrated in the vicinity of and apparently resulting in the activation of the c-myc gene (Hayward et al., 1981).

Translocations: The close association between specific chromosomal translocations and certain human neoplasms is well established (Yunis, 1983; Rowley, 1983, 1984). Such translocations are of two types: constitutional, that is, those carried by each of the individual's cells; and somatic, those that arise in a particular cell and are carried by its neoplastic progeny. It is generally held that such constitutional translocations (and other chromosomal abnormalities) predispose an organism to the development of a malignancy, but require a second event, presumably another mutation, to consummate the malignant transformation (Knudson, 1978).

A substantial body of evidence indicates that chromosomal translocations involving the c-myc oncogene and one of the immunoglobulin loci are quite frequent in B-cell

tumors of mouse or human origin (Adams et al., 1982, 1983; Ar-Rushdi et al., 1983; Leder et al., 1983; Stanton et al., 1983; Taub et al., 1982). In over 80% of the mouse plasmacytomas at least one c-myc allele has undergone a recombination within the 11-kb Eco RI fragment containing the c-myc gene (Adams et al., 1982; Harris et al., 1982). The breakpoints generally occur within a 1.0-kb region that spans exon 1 of the c-myc gene and the intron separating it from exon 2 (Adams et al., 1983; Stanton et al., 1983). In Burkitt's lymphoma, heterogeneity of the breakpoint is also observed since some breakpoints occur within or near exon 1 whereas others occur 8 kb or more upstream (Adams et al., 1983; Taub et al., 1982). In approximately 90% of Burkitt lymphomas, the malignant cells show a reciprocal translocation between chromosomes 8 and 14 [t(8;14)], whereas in the remaining 10% the cells show either a t(2;8) or a t(8;22) chromosome translocation, the breakpoint on chromosome 8 consistently affecting band q24 (Ar-Rushdi et al., 1983).

These c-myc translocations result in two types of change: (a) elevated levels of c-myc-related transcripts, and (b) aberrant c-myc transcripts in mouse plasmacytomas. Levels of c-myc expression are usually increased in mouse plasmacytomas and Burkitt's lymphomas, but this transcriptional activation varies from two- to ten-fold

(Leder et al., 1983). It is known that the normal c-myc gene in lymphoblastoid cell line, IARC 100, uses both promotor regions with the steady-state levels of transcripts favoring the promotor producing the shorter transcript (see Fig. 2.1). However, in some Burkitt lines the longer transcript increases relative to the shorter (Leder et al., 1983). This relation between c-myc promotors may not prevail in every Burkitt cell line. In many Burkitt cell lines and in most plasmacytomas, the c-myc genes have lost their promotor-leader exons (Adams et al., 1982, 1983; Ar-Rushdi et al., 1983; Crews et al., 1982; Marcu et al., 1983; Shen-Ong et al., 1982; Stanton et al., 1983; Taub et al., 1982). In view of the conserved nature of the 5' c-myc sequences, the presence of dual promotors, the inversion of transcript ratios, and frequent loss of the promotor-leader sequence in oncogenic translocations, Leder and his collaborators have suggested that these sequences may be important in regulating the expression of the c-myc gene (Leder et al., 1983).

2.7 C-MYC GENE AND HEMOPOIESIS

Hemopoiesis: The formation of mature, usually short-lived, blood cells (hemopoiesis) occurs continuously during fetal and adult life. This cellular proliferation results in the production of

seven major types of blood cell, each with distinctive morphology and highly specialized functional activity. The hemopoietic system itself is a cell renewal system in which balanced cellular proliferation and differentiation are occurring constantly at rates that are sensitive to the demands for new fully differentiated cells. The entire cellular complement of the blood originates from a pluripotent stem cell (Fig 2.2). In normal steady-state human hemopoiesis, the great majority of stem cells are quiescent (Becker et al., 1965); they serve as a reserve from which the system may be replenished if it is depleted. Usually only a few stem cells are active; two cardinal events occur in pluripotent stem cells: "self renewal" and "commitment". "Self-renewal" permits the stem cells to maintain its own pool. "Commitment" leads to the generation of progenitor cells of a given lineage, with the capacity to proliferate extensively but proliferation is not strictly self-renewal in these committed progenitors. Rather, they often lead to daughter cells of increased maturity and the generation of the final class of cells, functionally effective but incapable of division.

The transition from pluripotent stem cells to progenitors capable only of maturation is usually called "commitment" or "determination". There are several models proposed for the mechanisms of stem cell self-renewal and commitment.

The stochastic model developed by Till et al., proposes that the decision of a stem cell to renew itself or to yield cells that are committed to differentiation is governed by definite probabilities (Till et al., 1964). The model as modified by Korn et al., states that the stem cell commitment is governed by progressive and stochastic restriction in the differentiation potentials of hemopoietic stem cells (Korn et al., 1973).

The hematopoietic inductive microenvironment (HIM) model proposed by Trentin et al., argues that the commitment of pluripotent hemopoietic stem cells to monopotent progenitors is determined by a specific inductive microenvironment surrounding individual stem cells (Curry and Trentin, 1967; Trentin, 1970).

The stem cell competition model proposed by Van Zant and Goldwasser argues that the lineage-specific humoral factors such as erythropoietin (Ep) and colony stimulating factor (CSF) play an active role in the patterns of stem cell commitment (VanZant and Goldwasser, 1977, 1979).

Granulopoiesis: Much of the myelopoietic proliferation takes place in progenitors committed to one of the three lineages: erythropoiesis, granulopoiesis and megakaryopoiesis. These "committed progenitors" acquire sensitivity to lineage-specific

regulators. Granulocytes and mononuclear phagocytes are descendents of colonu forming unit-granulocyte/monocyte (CFU-GM) that form granulocyte and macrophage colonies in semisolid media. The clonal growth of these progenitor cells depends on a family of regulatory glycoproteins known as CSA (Burgess and Metcalf, 1980; Das et al., 1981; Stanley, 1981) whose production by T lymphocytes, fibroblasts, and vascular endothelial cells is regulated by another soluble monocyte-derived recruiting activity (MRA) (Bagby et al., 1983).

The committed CFU-GM in the presence of CSA, undergoes a tightly controlled differentiation process coupled with a progressive loss of proliferative capacity. Granulocyte development in the bone marrow from morphologically recognizable precursors can be classically divided into two phases. The first phase which is called the "mitotic phase" includes only cells with proliferative capacity. These cells fall into three morphologically recognizable differentiation stages (myeloblast [the most primitive], promyelocyte, and myelocyte). The second phase called the "postmitotic" phase includes cells which have lost completely the proliferative capacity and can only undergo morphological maturation. These cells fall into three stages (metamyelocyte, band, and mature segmented neutrophil).

The granulocyte of the blood is a highly specialized, nondividing "end" cell with a short life-span (see Fig. 2.3, for

review see Bainton et al., 1971). The bulk of its life cycle is spent in the marrow, where it proliferates, differentiates, and is stored for a few days. The mature cell is then released into the tissues where it functions as a mobile phagocyte.

Myeloblast: The earliest cell of the granulocytic series is the myeloblast. This is a relatively small undifferentiated cell with a high nuclear:cytoplasmic ratio. The cells in this stage have prominent nucleoli. The cytoplasm is scant and devoid of granules.

Promyelocyte: The cells in this stage are largest in size. They have a round nucleus. The cytoplasm is full of large peroxidase-positive azurophilic granules. The cells have prominent golgi filled with azurophilic granules. The cells have nucleoli and the nucleus:cytoplasmic ratio is relatively high.

Myelocyte: The myelocyte is smaller than the promyelocyte. These cells can be recognized by indented nuclei without any nucleoli. The nucleus:cytoplasmic ratio is low. The golgi is still prominent. The granules are mixed and are predominantly peroxidase-negative. The granules continue to accumulate and eventually outnumber the azurophilic granules.

Metamyelocyte: The cells of this developmental stage are non-proliferative and are also called "juveniles". The cytoplasm is filled with prominent neutrophilic granules. There are no nucleoli. The nucleus is indented or kidney-shaped.

The final stages in the maturation of granulocytes are the 'band' stage and finally the mature granulocyte or polymorphonuclear leukocyte. The process of maturation from the metamyelocyte stage includes nuclear elongation and segmentation. The band has a horse-shoe-shaped nucleus. The mature neutrophil has small neutrophilic granules, and the nucleus is segmented into two to five lobes connected by thin chromatin strands.

FIGURE 2.1

Structural features of the human c-myc gene.

The diagram shows a 8.00 kb Hind III-Eco RI fragment encoding the c-myc gene. The boxes within the segment represent the three c-myc exons. The first exon (dotted) encodes two transcription initiation points, indicated as arrows with the "cAp..."symbols. The position of the translational initiation (ATG) and termination (TAA) codons are shown, as are the promotors (TATAA) and the polyadenylate addition (AATAAA) sites. H = Hind III; C = Cla I; P = Pst I; X = Xba I; E = Eco RI.

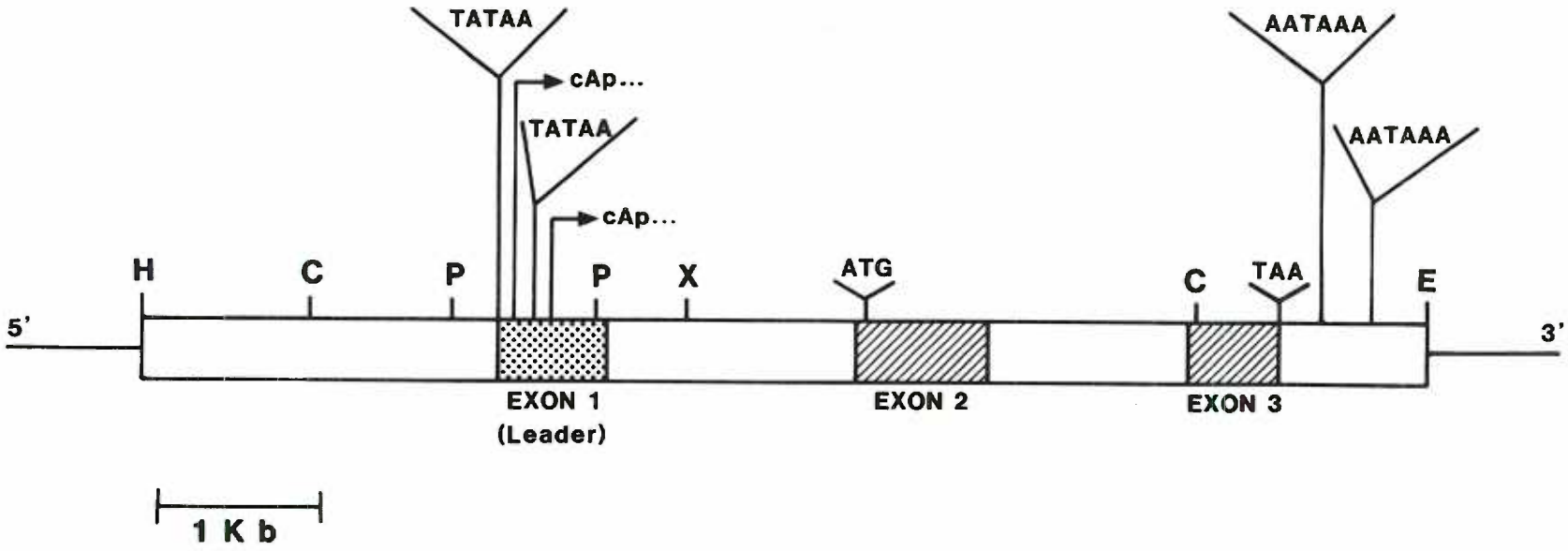

TATAA
cAp...
TATAA
cAp...
AATAAA
AATAAA
H
C
P
P
X
ATG
C
TAA
E
5'
3'
EXON 1
(Leader)
EXON 2
EXON 3
1 K b

FIGURE 2.2

The cell types of hematopoietic system.

CFU-S = colony-forming unit-Spleen; CFU-GM = colony-forming unit granulocyte-monocyte (also called CFU-C); CFU-MEG = colony-forming unit-megakaryocyte; BFU-E = burst-forming unit-erythroid; CFU-E = colony-forming unit-erythroid.

HEMATOPOIESIS

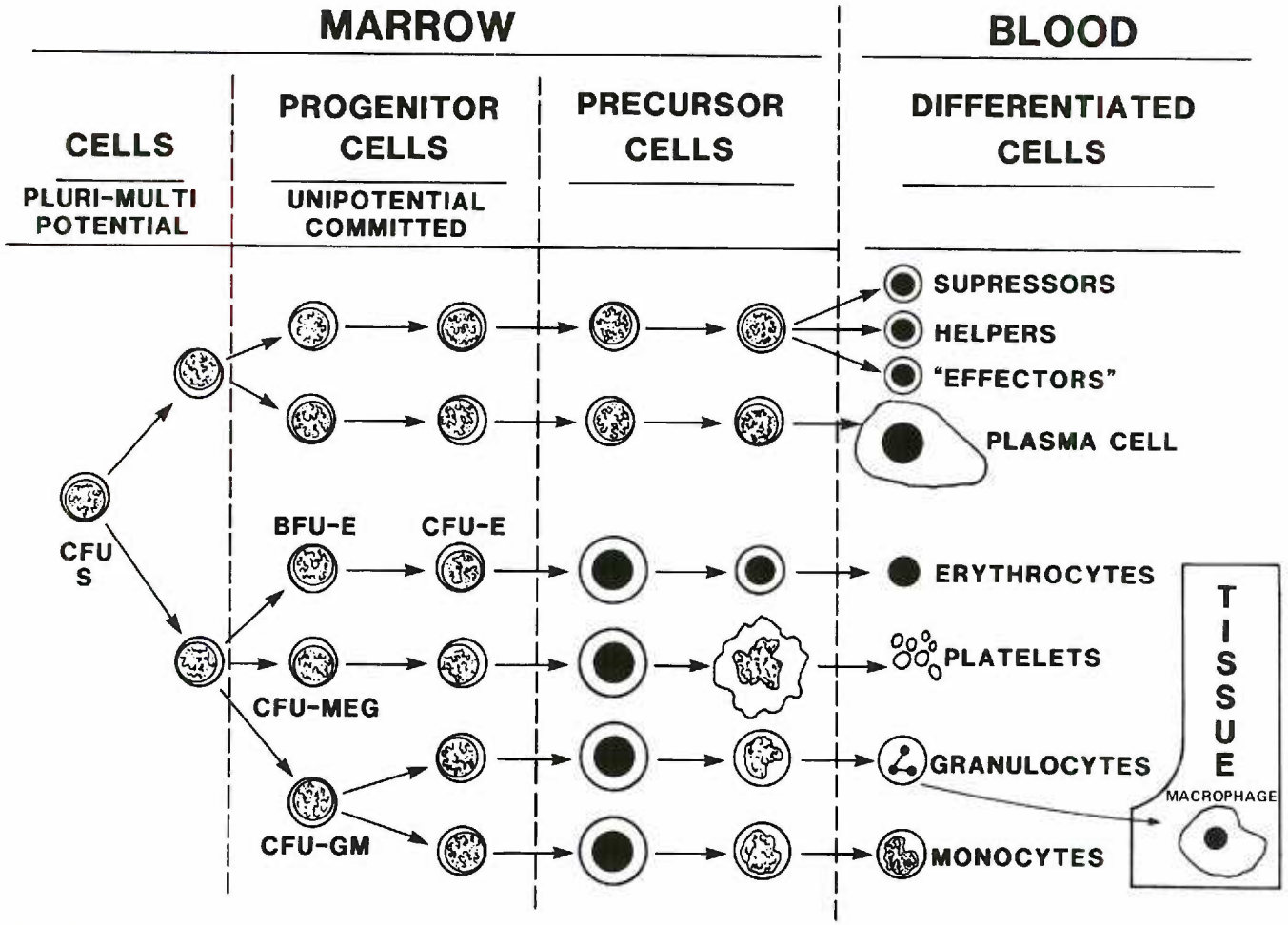
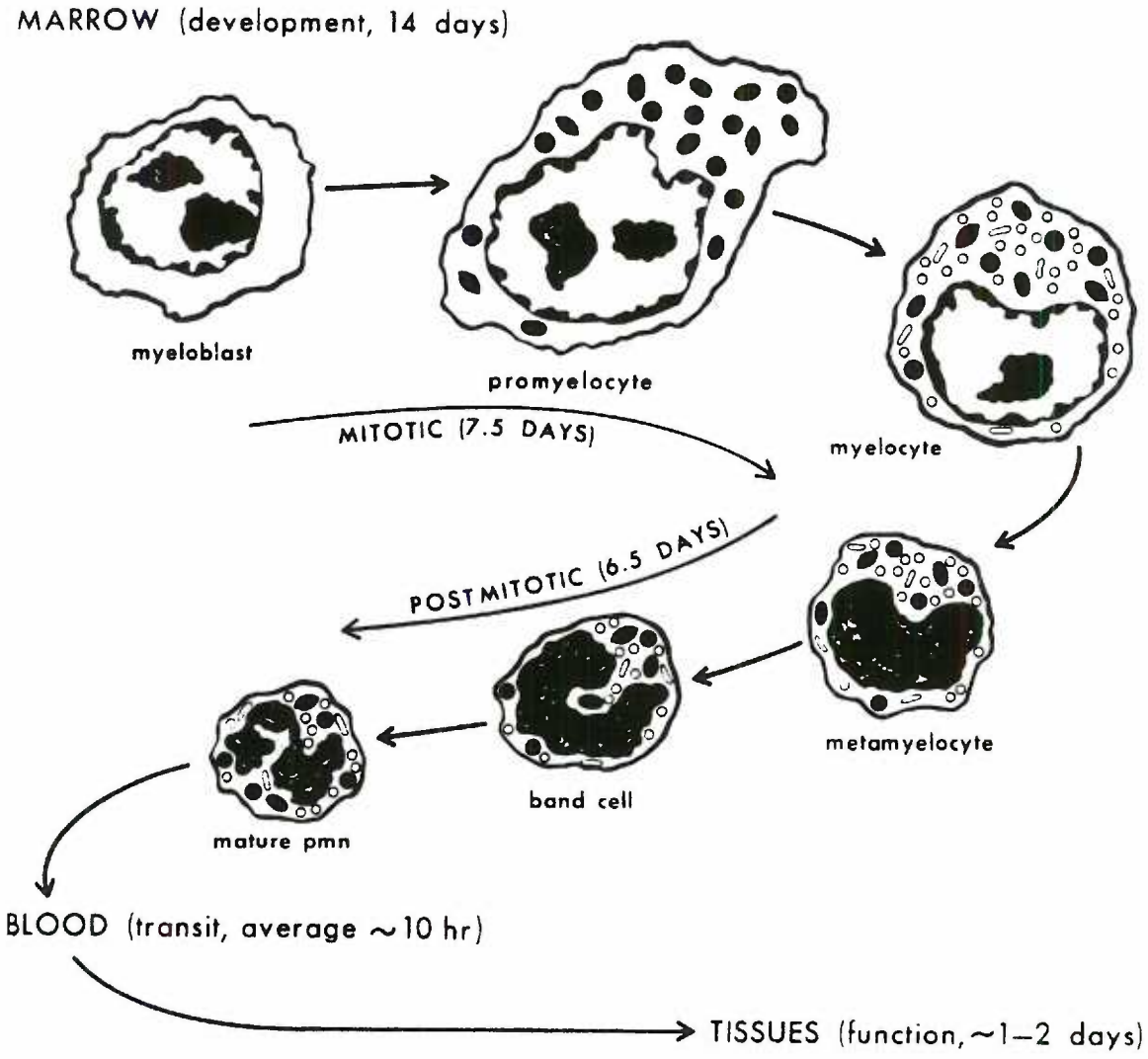


FIGURE 2.3

The life cycle of a mature polymorphonuclear leukocyte.

The myeloblast is a relatively undifferentiated cell with a large oval nucleus, large nucleoli, and cytoplasm lacking granules. It originates from a precursor pool of progenitor cells and is followed by two distinct stages: the promyelocyte and the myelocyte. During each of these stages a distinct type of secretory granule is produced: azurophils (solid black) formed only during the promyelocyte stage, and specific granules (light granules) produced during the myelocyte stage. The metamyelocyte and band forms are nonproliferating stages which develop into the mature polymorphonuclear leukocyte. The latter is characterized by a multilobulated nucleus and cytoplasm containing primarily glycogen and granules.


MARROW (development, 14 days)
myeloblast
promyelocyte
MITOTIC (7.5 DAYS)
myelocyte
POSTMITOTIC (6.5 DAYS)
metamyelocyte
band cell
mature pmn
BLOOD (transit, average ~10 hr)
TISSUES (function, ~1–2 days)

CHAPTER 3

CHAPTER 3

C-MYC GENE EXPRESSION DURING NORMAL AND LEUKEMIC GRANULOPOIESIS

3.1 INTRODUCTION

Acute leukemia has been defined as the clonal proliferation of abnormal hemopoietic stem cells which exhibit abnormalities in cellular differentiation. At the cellular level, leukemia appears to represent an "uncoupling" between proliferation and differentiation (Sachs, 1985). The cardinal feature of acute myeloid leukemia cells is "maturation arrest"; these cells are incapable of undergoing the normal differentiation process (McCullouch, 1984; Metcalf and Nicola, 1985; Reich, 1984). The maturation arrest invariably occurs at stages earlier than myelocyte (stem cell, myeloblast and promyelocyte) (Reich, 1984). In these maturationally arrested primitive cells in acute leukemia, the c-myc transcripts are high (Blick et al., 1984; Reitsma et al., 1983; Westin et al., 1982).

The c-myc transcripts are high in almost all reported acute and chronic myeloid leukemias (Blick et al., 1984; Westin et al., 1982; Koeffler, 1983). This gene is also transcribed in the human myeloid leukemia cell lines, KG-1 and HL-60 (Reitsma et al., 1983; Westin et al.,1982; Koeffler, 1983). The transcription of c-myc is markedly decreased when HL-60 cells differentiate to mature granulocytes and moderately decreases when KG-1 cells differentiate to macrophages (Reitsma et al.,

1983; Koeffler, 1983). The c-myc gene is amplified approximately 16-32-fold in HL-60 cells, as well as in the original leukemic leukocytes obtained from peripheral blood of the patient from whom the HL-60 cell line was established (Collins and Groudine, 1982; Dalla-Favera et al., 1982). These observations have led to the hypothesis that it is the inappropriate and/or untimely expression of c-myc that is involved in the initiation and/or maintenance of these leukemias.

To determine the degree to which the c-myc expression is inappropriate in these abnormal populations of granulopoietic cells requires knowledge of its expression and regulation during growth and differentiation of normal granulopoietic cells. As described above, granulopoietic cells undergo a concatenation of discrete morphological changes in vitro and in vivo. For each successive morphological stage of cellular differentiation, it must be the case that previously untranscribed genes are activated and some genes that had been transcribed in the progenitor cells become inactive. It is conceivable that c-myc may be one of many genes that are transcriptionally active at the early cellular stages of granulopoietic differentiation and, as such, c-myc expression may be perfectly normal for cells arrested at one stage of maturation in acute myeloid leukemias and in chronic myeloid leukemias with an aberrant granulopoietic pool to show high levels of c-myc transcripts. Therefore, it is essential to know the expression profile during growth and

differentiation of normal human myeloid progenitor cells.

To this end, we have characterized an in vitro liquid culture system in which progenitor cell enriched human myeloid cells grow and differentiate under the influence of CSA. Using this system, I have studied the expression and regulation of the c-myc gene during various stages of myeloid differentiation.

The specific objectives of this work were:

EXPERIMENT 1

a) To define the cellular characteristics of the growth and differentiation of progenitor enriched normal human bone marrow cells.

b) To determine whether the c-myc gene is expressed during in vitro growth and differentiation of normal human myeloid cells.

c) To define temporally, the regulation of c-myc mRNA content.

EXPERIMENT 2

a) To determine whether the c-myc gene is amplified during in vitro growth and differentiation of normal myeloid cells and to compare the relative gene copy number to that in HL-60 cells.

EXPERIMENT 3

a) To compare the relative amounts of c-myc mRNA content in normal bone marrow cells to that in HL-60 cells.

b) To compare the c-myc mRNA content profile in normal and acute myeloid leukemia cells during in vitro growth and differentiation.

3.2 MATERIALS AND METHODS

The experimental details for bone marrow progenitor cell enrichment, proliferation assay, surface phenotype analysis, RNA isolation, dot and northern analysis and sources and preparation of the probes are described in the appendix 3.

3.3 RESULTS

Human placental conditioned medium (HPCM) specifically stimulates the expansion of myeloid lineage.

Low density bone marrow (LDBM) cells depleted of T and B lymphocytes, monocytes, and mononuclear phagocytes contained lymphoid appearing cells and granulocyte precursors no more differentiated than myeloblasts. As shown in Figure 3.1a, the majority were lymphoid in appearance but were surface immunoglobulin, OKT3 (which recognizes T-lymphocytes) and OKMI (which recognizes monocytes and granulocytes) negative. These cells were cultured with 10% HPCM, a potent source of CSA (Burgess, et al., 1977), for a period of 96 hours. The cells undergo differentiation to progranulocytes by 24 hours (Fig. 3.1b) and reach non-proliferative stages namely metamyelocytes and bands by 96 hours (Fig. 3.1c). Cells were regularly harvested during the period of culture for morphological studies, analysis of ^{3}H-thymidine incorporation, surface phenotype analysis with monoclonal antibodies and studies of

c-myc expression and gene copy number using a labeled c-myc probe.

As shown in Figure 3.2, the cells cultured in the presence of HPCM revealed a progressive increase in tritiated thymidine uptake up to 72 hours and a decline thereafter. Cells not exposed to HPCM, did not show a significant change in uptake. This 24-fold increase by 72 hours was observed consistently when the LDBM cells were cultured in the presence of HPCM but did not occur in the absence of HPCM. A cytofluorographic phenotype analysis showing the temporal increase in myeloid cells following HPCM stimulation of LDBM cells is seen in Figure 3.3. OKM1 positive cells increased 7-fold by 96 hours. OKT3 positive cells did not increase during the culture period. OKM1 positive cells reflect largely the cells of neutrophilic lineage because cytocentrifuge slides indicated that the majority of cells were chloroacetate esterase positive. Specifically, there was a 7-fold increase in chloroacetate esterase positive cells by 96 hours; while monocyte specific esterase positive cells declined (data not shown). Therefore, the response of LDBM cells to HPCM was largely granulocytic.

In order to define the differentiation stages in vitro, differential cell counts were performed on Wright's stained cytocentrifuged slides. As shown in Figure 3.4a, there was a wave of maturation from primarily lymphoid appearing cells to metamyelocytes and band forms after 96 hours. Peaks of

myeloblasts were noted at 12 hours, progranulocytes at 24 hours and myelocytes after 72 hours. The cells cultured in the absence of HPCM (Fig. 3.4b) showed a similar pattern of maturation (there were more mononuclear phagocytes) without detectable proliferation. We cannot state that this pattern of maturation was wholly independent of CSA however; it could be due to the production of small amounts of CSA by residual monocytes and T-cells (Bagby et al., 1981, 1983).

C-myc transcripts during HPCM stimulated myeloid cell differentiation

Northern blot hybridization analysis with a cDNA probe of the human c-myc mRNA revealed a transcript of approximately 2.4 kb in cells exposed to HPCM for 24 hours (Fig. 3.5a). This transcript was comparable in size to c-myc transcripts found in HL-60 cells (Fig. 3.5a) and probably represents a normally spliced c-myc mRNA species (Adams, et al., 1983; Erickson, et al., 1983; Maguire, et al., 1983) transcribed from the 5.2 kb c-myc gene. Using these techniques it was not possible to detect which of the two promotors (Watt, et al., 1983) was utilized in these cells. The number of c-myc transcripts returned to background level, after 48 hours. The northern blot results which indicate a peak of c-myc transcripts after about 24 hours of culture in the presence of HPCM, were confirmed in five separate dot blot analyses as well (Fig. 3.5c).

To define more exactly the time at which transcripts begin to increase, the cells were harvested at 12 hour intervals for up to 48 hours and mRNA was analyzed by dot blot technique. As shown in Figure 3.5c c-myc transcripts increase between 12 and 24 hours after exposure to CSA and decline to background levels before 36 hours. To further show that the differentiated granulocytes do not express the c-myc gene, we obtained pure population of granulocytes from peripheral blood and analyzed for the c-myc transcripts. As predicted, we did not detect any c-myc mRNA transcripts (data not shown). Cells cultured in the absence of HPCM were also examined. Even though we observed cell maturation, as shown in Figure 3.4b, we did not see any change in the expression of the c-myc gene. However, our assays may not be sensitive enough to detect small changes in c-myc mRNA content.

To control for the relative amount of mRNA in each sample, aliquots of mRNA were analyzed for γ-actin mRNA by dot blot assay. A pBR322 clone containing a 700 bp HindIII-BamHl insert of the 3' untranslated portion of human γ-actin (Ponte, et al., 1983) was used. As shown in Figure 3.5b, γ-actin mRNA remains nearly constant relative to total RNA throughout the course of HPCM stimulated myeloid differentiation. This was confirmed by densitometric scanning of the autoradiogram (data not shown). As a negative control, we used a pBR322 clone containing a 4.4 kb Pst I insert of the human ß-globin probe. We did not detect

any hybridization at any harvest time-point even after prolonged autoradiography (data not shown).

Since some of our target populations had as many as 11% T-lymphocytes and 16% monocytes at the time of exposure to HPCM, it was necessary to examine c-myc gene expression in T-lymphocytes and monocytes from normal marrow after exposure to HPCM. Accordingly, we recovered T-lymphocytes from the sheep red-cell rosettes by hypotonic lysis and recovered monocytes by adherence to serum coated dishes (≃ 92% monocytes) (Bagby, et al., 1981, 1983). These cells were exposed to HPCM for 24 hours and probed for c-myc transcripts. With neither cell type did HPCM induce c-myc gene expression (Fig. 3.5c), nor did a mixture of monocytes and T-cells (data not shown). In view of these results and the findings that T-cells and mononuclear phagocytes declined in number in HPCM stimulated cultures we conclude that c-myc expression seen after 24 hours of exposure to HPCM was in actively proliferating granulocyte precursors.

C-myc gene is not amplified during normal myeloid cell differentiation.

In the progranulocytic cell line HL-60, the c-myc gene is amplified 16-32 fold (Collins and Groudine, 1982; Dalla-Favera et al., 1982). It has been suggested that c-myc amplification may occur in other non-malignant myeloid cells, and thus may represent a normal event in myeloid differentiation (Collins and

Groudine, 1982; Dalla-Favera et al., 1982). We sought, therefore, to measure the abundance of c-myc gene copies in normal cells as a function of differentiation stage. C-myc gene sequences in normal granulopoietic cells were detected in the unfractionated DNA isolated from the nuclei of cells harvested over a 96 hour period at 12 hour intervals by the dot blot technique. A representative experiment is shown in Figure 3.6a. Autoradiograms were scanned for the changes in the intensity of the dots which would indicate changes in the abundance of c-myc sequences. The intensity of the spots was approximately 14-16 times lower than for the DNA from the same number of HL-60 cells (Fig. 3.6b). The results indicate that gene copy number is stable at each developmental stage.

Levels of c-myc transcripts in normal cells and HL-60 cells.

We have compared the levels of c-myc expression in LDBM cells-after 24 hour HPCM stimulation-with the level of c-myc expression in HL-60 cells. As shown in Table 3.1 we noted that for a given amount of mRNA loaded on the blot, c-myc RNA derived from HL-60 cells represented 150% of that derived from normal LDBM cells harvested at 24 hours. In addition mRNA yield from HL-60 cells was 166% that of normal LDBM cells after 24 hour culture in HPCM. Therefore on a cell for cell basis c-myc RNA content of HL-60 cells is 2.5 fold higher than in LDBM cells. We do not yet know the cell type(s) transcribing c-myc in our

system. We can surmise however that cells more differentiated than progranulocytes do not because c-myc is fully repressed in cultures in which such cells predominate. Therefore, we suspect that cell type(s) including early progranulocytes or their progenitors are those which transcribe c-myc.

C-myc transcripts in primary cultures of leukemic cells.

In HPCM-stimulated normal LDBM cells, the number of c-myc transcripts increase approximately 3 fold by day 1 and is followed by a consistent decline to undetectable levels by day 5 (Table 3.2). Unlike normal LDBM cells in which c-myc transcripts decline over time, acute leukemic cells, which failed to differentiate to mature neutrophils in vitro, constitutively exhibited high levels of c-myc transcripts throughout the culture period (Table 3.2).

3.4 DISCUSSION

Although c-myc expression has been found in certain neoplastic cell lines (Erickson, et al., 1983; Maguire, et al., 1983) and in most freshly obtained malignant tissues studied to date (Slamon et al., 1984) expression of this cellular oncogene has been clearly demonstrated in certain proliferating normal cells as well (Bering et al., 1984; Goyette et al., 1984; Keath et al., 1984; Kelly et al., 1983; Muller and Verma, 1984; Pfeifer-Ohlsson et al., 1984). Thus, it is generally

acknowledged that c-myc expression per se is inadequate to account for the assumption of the neoplastic phenotype (Land et al., 1983; Stewart et al., 1984). Certain other observations have been interpreted as suggesting that it may be the inappropriateness of c-myc activation that plays a role in the development or maintenance of the neoplastic phenotype. For example, the integration of avian leukosis virus occurs close to and on the 5' side of c-myc in avian B cell lymphomas (Hayward et al., 1981). In addition, c-myc is regularly translocated close to either the heavy or light chain immunoglobulin genes, and in these cells c-myc is actively expressed (Leder et al., 1983). Finally, amplification of the c-myc gene has been documented in both non-hematopoietic (Little et al., 1983) and hematopoietic (Collins and Groudine, 1982; Dalla-Favera et al., 1982) tumor cell lines. The HL-60 progranulocytic leukemia cell line is one in which the c-myc gene is actively expressed and regularly amplified 16-32 fold. Our studies were designed, in part, to provide contextual information relevant to c-myc amplification and expression in HL-60 cells.

Conceptually, linkage of c-myc expression, the neoplastic phenotype, and gene amplification in malignant cells requires, at the very least, the knowledge that normal cells at the same developmental stage exhibit neither gene amplification nor active c-myc expression. Accordingly, in this study, we sought to determine whether primitive granulopoietic cells from human

bone marrow normally express c-myc. Our results indicate that they do after 24 hours of culture in the presence of CSA (Fig. 3.5). In addition, c-myc gene copy number is not increased at 24 hours or at any other time before or after 24 hours (Fig. 3.6). Moreover, c-myc transcript numbers in normal cells at 24 hours approximates those found in HL-60 cells (Table 3.1).

Although it is of interest that the predominant cell after 24 hours of culture is the progranulocyte, we cannot yet directly compare these progranulocytes to the HL-60 cells because we do not yet know which cell types transcribe c-myc. We do believe, however, that cells more differentiated than progranulocytes do not transcribe c-myc because when such cells predominated in vitro, transcription was fully repressed. Whether early progranucytes or cells more primitive than progranulocytes transcribe c-myc is not yet known. It is conceivable that a CSA-sensitive clonogenic progenitor cell, the CFU-GM, most actively transcribes c-myc. Nor do we yet know whether the activation and repression of c-myc derives from transcriptional activation or post-transcriptional phenomena. Indeed, recent evidence suggests that c-myc mRNA inactivation may be a mechanism by which gene expression is regulated in certain human cell lines, including HL-60 cells (Dani et al., 1984).

Despite unanswered questions, our observations are relevant to our understanding of the role of c-myc in causing or

maintaining the leukemic phenotype. Clearly, active expression of c-myc by primitive non-malignant myeloid precursor cells with only one diploid gene locus is not only legitimate, but quantitatively approximates the expression of c-myc in a cell line in which the c-myc gene is amplified 14-16 fold. Such findings are not entirely without precedent. Recently, normal placental cytotrophoblasts were found to contain as much c-myc RNA as did cells of a malignant cell line (COLO 320 HSR) harboring 16-32 fold amplified c-myc genes (Pfeifer-Ohlsson et al., 1984). Based on these observations one might argue that c-myc amplification in HL-60 cells may not account for c-myc transcriptional activity seen in those cells and that many of the amplified copies may be relatively silent.

Prior work in HL-60 cells in which c-myc transcription was found to decrease markedly when the cells were induced to differentiate (Reitsma et al., 1983) is compatible with the notion that c-myc activity is linked somehow to the maintenance of the neoplastic phenotype. An alternative point of view, one with which our observations are compatible, is that c-myc is simply transcribed in these cells because it is normally transcribed in early progranulocytes or their progenitors and normally becomes quiescent when the cells differentiate further (Collins and Groudine, 1982; Dalla-Favera et al., 1982; Reitsma et al., 1983). Only studies on c-myc expression in single cells can resolve this issue further.

Despite the similarities between them, there are two clear differences between normal and leukemic cells vis-a-vis c-myc expression. First, HL-60 cells proliferate autonomously and express c-myc constitutively, that is without requiring exposure to CSA, whereas c-myc expression can only be induced in normal cells by exposure to CSA. Second, and, because the abnormality is common to both HL-60 cells and primary cultures of leukemic cells, most importantly, normal cells exposed to CSA invariably repress c-myc after 36 hours in culture whereas leukemic cells fail to do so even after three days in culture. Therefore, even if c-myc ultimately proves not to be causally linked with the leukemic phenotype, its expression may prove to be a valuable marker of a primitive developmental stage and failure to suppress transcription in-vitro may prove to be a reliable marker of a leukemic phenotype. Studies addressing the mechanisms by which normal myeloid cells repress c-myc should provide important insights on the regulatory abnormalities of gene expression which underlie the behavior of myeloid leukemic cells.

Table 3.1. Relative levels of c-myc in HL-60 and normal myeloid progenitor cells

Cells	Total RNA yield ($ug/10^7$ cells)	Relative c-myc RNA level* (constant RNA)	Relative c-myc RNA (per cell)
HL-60	7.25 (± 1.5)	1.5	2.5
24 hr HPCM-stimulated LDBM cells	4.37 (± 0.9)	1	1

*The dot blot autoradiograms were examined densitometrically and the linear range intensities were used to calculate the relative levels.

Table 3.2. Comparison of temporal c-*myc* expression in normal and leukemic myeloid cells.

Cells	c-*myc* transcripts*					
Days	0	1	2	3	4	5
LDBM cells (n = 5)	±	+++	+	±	±	-
Acute myeloblastic leukemia (n = 3)	++	+++	+++	+++	NT**	NT
Acute promyelocytic leukemia*** (n = 1)	++	++	++	++	NT	NT

*The dot blot autoradiograms were scanned densitometrically an the lowest reading was assigned '-' and the highest '+++++'.

**Not tested

***The patient was on retinoic acid therapy when the sample was taken.

FIGURE 3.1

Differentiation of LDBM cells cultured for 96 hours in HPCM.

Photomicrographs (magnification, x1250) of: a) Lymphoid appearing surface immunoglobulin negative cells at the start of the culture. b) Progranulocytes predominate after 24 hours of culture. Note the characteristic progranulocytic granules. c) Non-mitotic metamyelocyte and band stage of differentiation which predominated after 96 hours of culture.

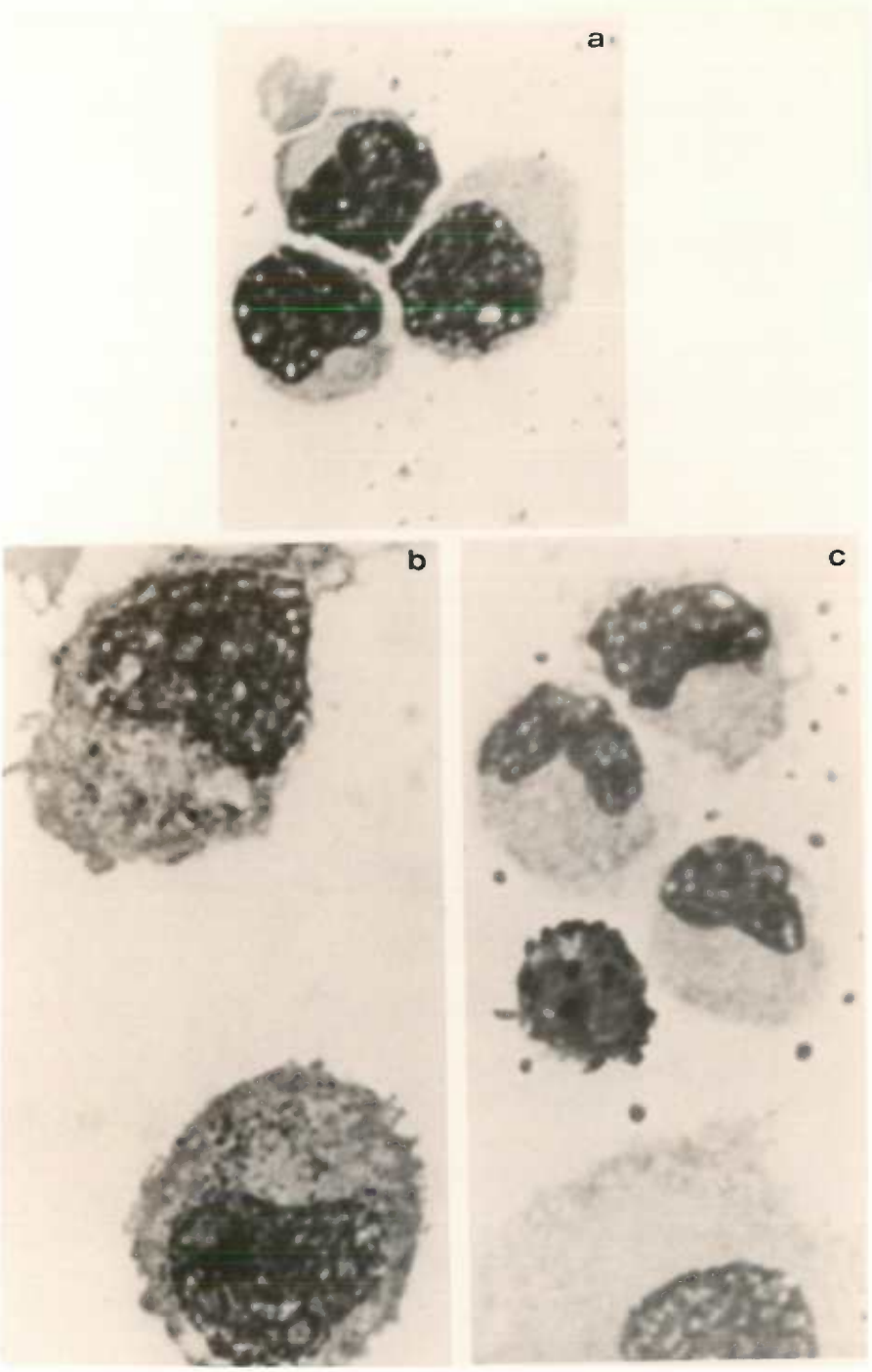

a
b
c

FIGURE 3.2

Proliferative response of HPCM-stimulated LDBM cells.

The incorporation of ^{3}H-thymidine at various times after the stimulation of LDBM cells with HPCM. Triplicate cultures were assayed for each time point. A representative experiment is shown. A similar pattern of incorporation has been seen in all experiments.

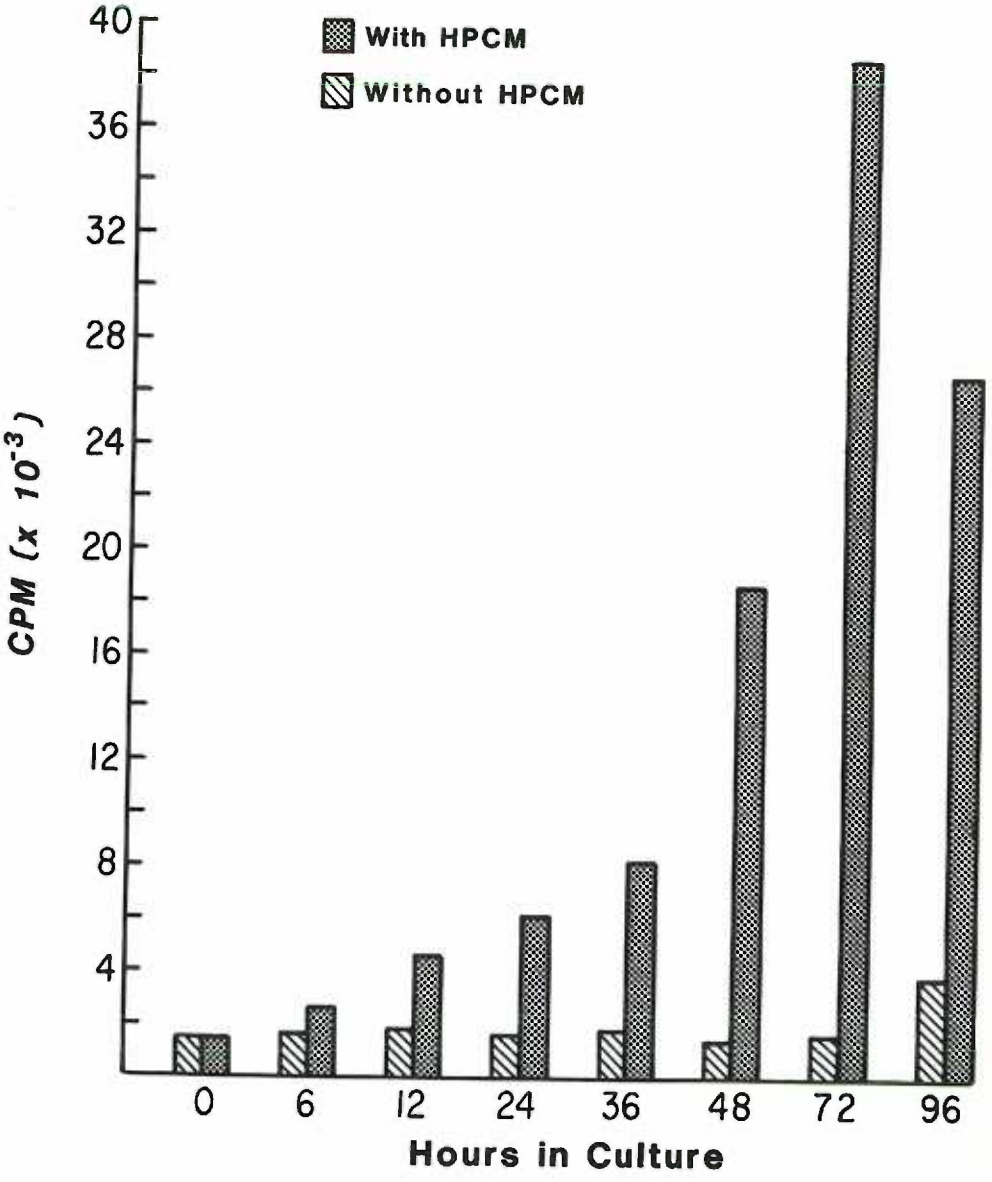

With HPCM
Without HPCM
CPM (x 10⁻³)
40
36
32
28
24
20
16
12
8
4
0
6
12
24
36
48
72
96
Hours in Culture

FIGURE 3.3

Surface phenotype analysis of HPCM-stimulated LDBM cells

Specific expansion of myeloid lineage during HPCM-stimulated differentiation of LDBM cells. LDBM cells were stained with either OKM1 or OKT3 monoclonal antibody and the fraction of cells positive for each antibody was determined by FACS analysis. The results are expressed as number of cells per ml, a value obtained by multiplying the marker positive fraction times the total cell count. A representative experiment is shown. Similar results have been obtained in additional experiments.

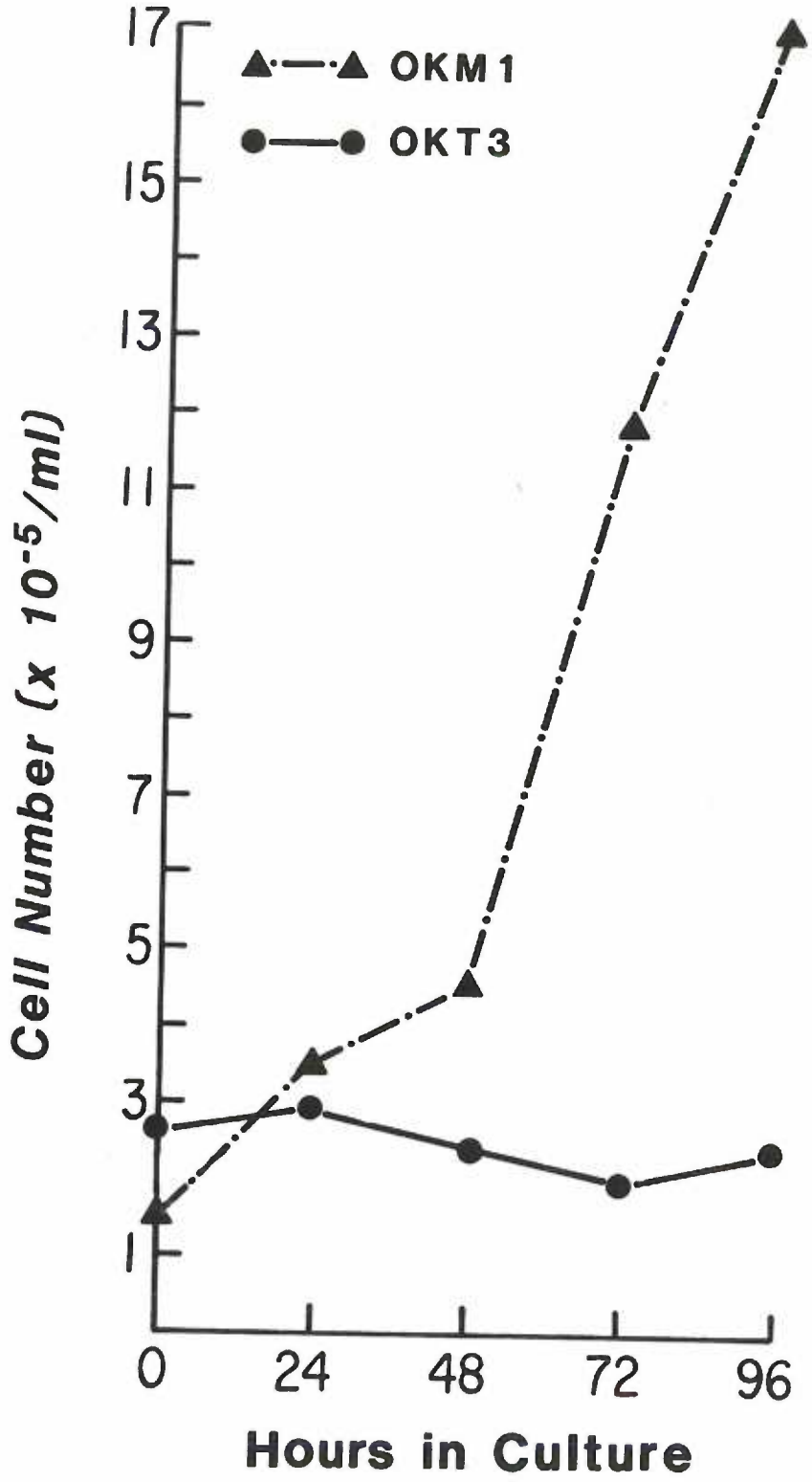

OKM1
OKT3
Cell Number (x 10⁻⁵/ml)
17
15
13
11
9
7
5
3
1
0
24
48
72
96
Hours in Culture

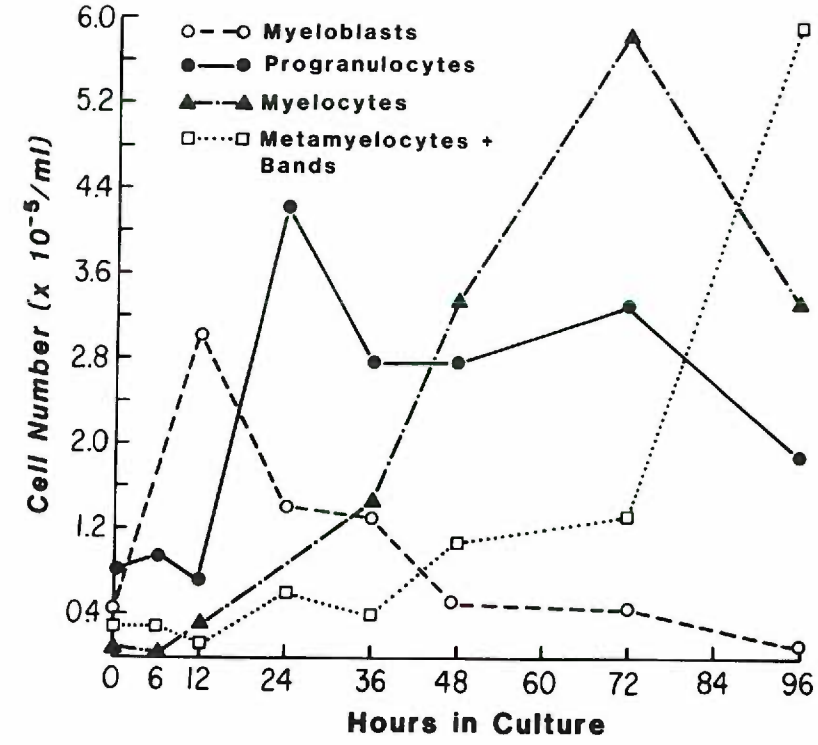
FIGURE 3.4

Differentiation of HPCM-stimulated LDBM cells.

a)Bone marrow cells exposed to HPCM were cyto-centrifuged on slides at 0, 6, 12, 24, 36, 48, 72 and 96 hours, for Wright stained differential counts. A wave of maturation was observed from initially lymphoid appearing and blast cells to metamyelocytes and bands after 96 hours. Peaks of myeloblasts at 12 hours, progranulocytes at 24 hours and myelocytes after 72 hours can be noted. A representative experiment is shown. Similar results have been obtained in additional experiments.

b) Same as in a, but the cells were not exposed to HPCM.

a


Myeloblasts
Progranulocytes
Myelocytes
Metamyelocytes + Bands
Cell Number (x 10⁻⁵/ml)
6.0
5.2
4.4
3.6
2.8
2.0
1.2
0.4
0 6 12 24 36 48 60 72 84 96
Hours in Culture

b

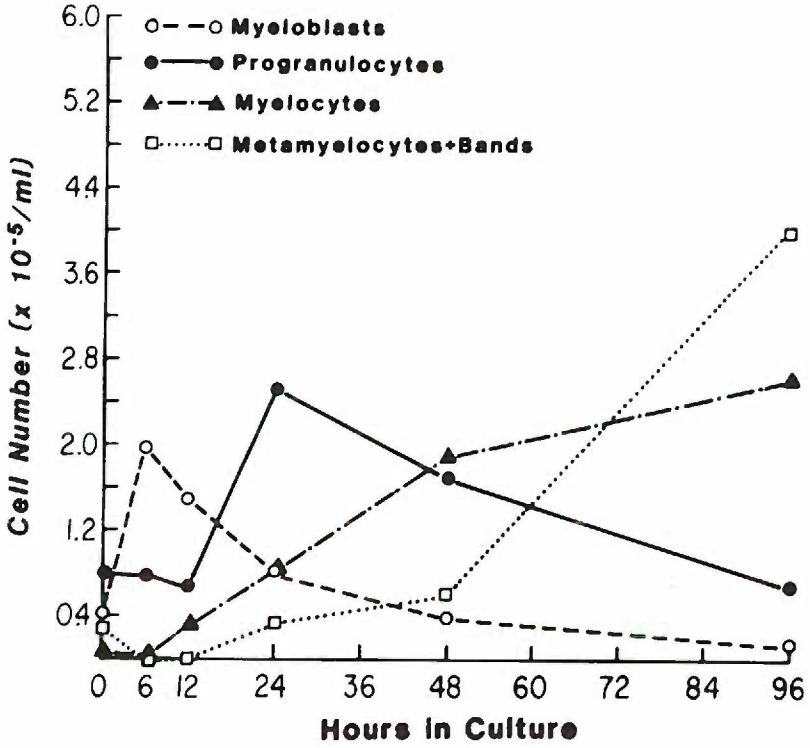

Myeloblasts
Progranulocytes
Myelocytes
Metamyelocytes+Bands
Cell Number (x 10⁻⁵/ml)
6.0
5.2
4.4
3.6
2.8
2.0
1.2
0.4
0 6 12 24 36 48 60 72 84 96
Hours in Culture

FIGURE 3.5

C-myc gene expression in HPCM-stimulated differentiation of LDBM cells.

a)Northern blot analysis of c-myc RNA transcripts in poly (A)-containing RNA from cells harvested at various times of culture with HPCM. Poly (A)-containing RNA was fractionated on a 1% agarose gel, transferred to Genescreen membranes, and hybridized with a ^{32}P labeled cDNA probe of the human c-myc gene. The spot on top of the autoradiogram is a hybridization artifact.

b)Poly (A)-containing RNA from HPCM-stimulated LDBM cells, and HL-60 cells were dot blotted onto Genescreen membranes and hybridized with a probe specific for 3' untranslated region of γ-actin gene.

c)Poly (A)-containing RNA from HPCM-stimulated LDBM cells, and from adherent cells and T-lymphocytes with or without 24 hour-exposure to HPCM, were dot blotted onto Genescreen membranes and hybridized with a probe specific for the second and third exons of c-myc. The dot blot shown was a representative of 5 separate experiments.

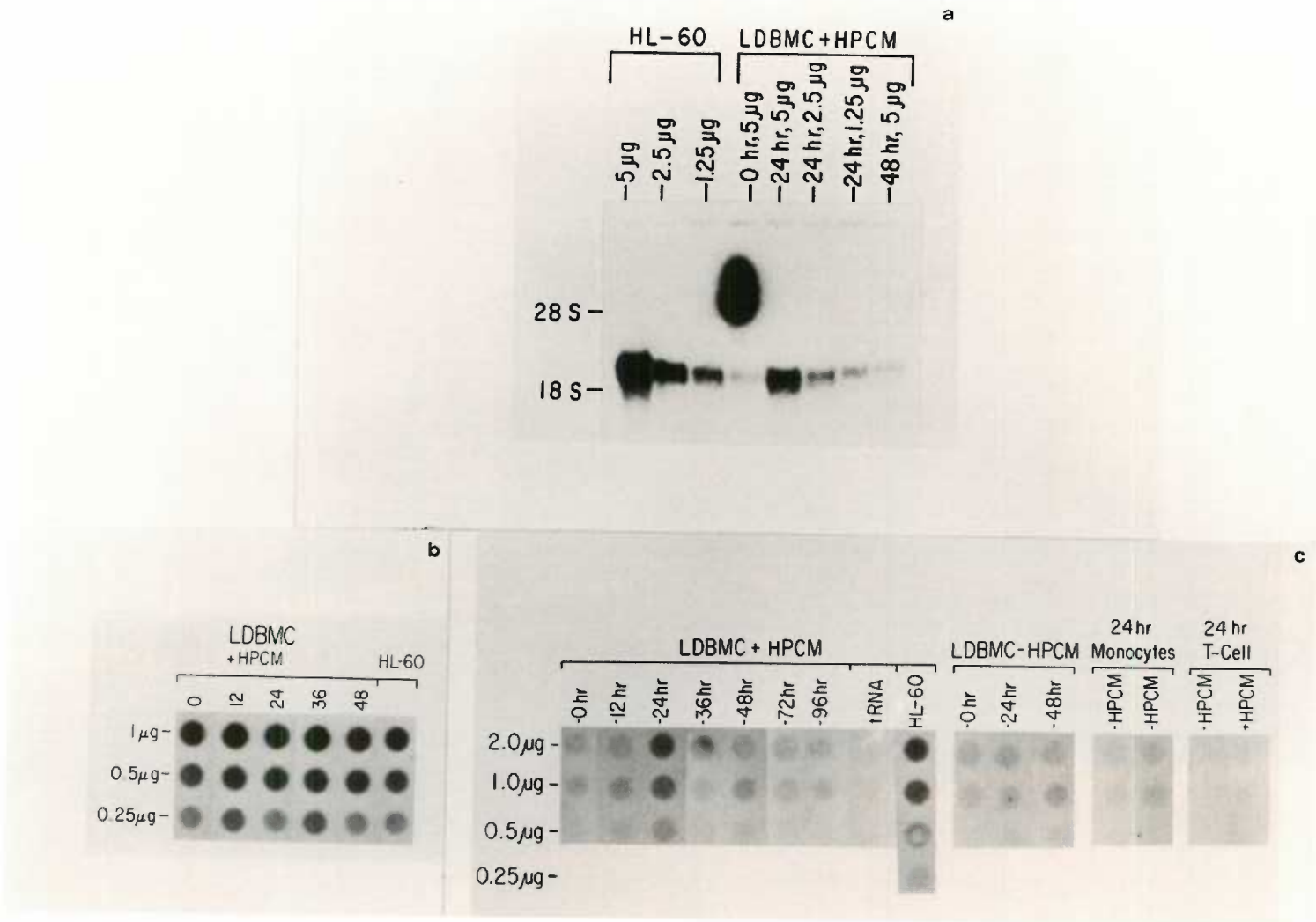
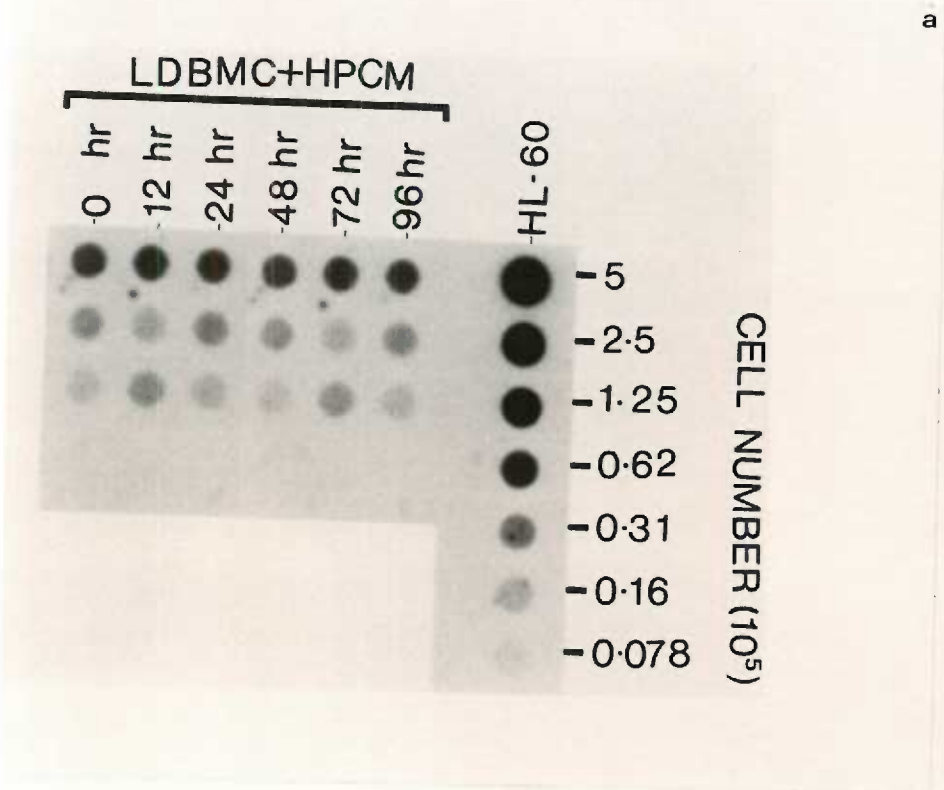

a
HL-60
LDBMC+HPCM
5 µg
2.5 µg
1.25 µg
0 hr, 5 µg
24 hr, 5 µg
24 hr, 2.5 µg
24 hr, 1.25 µg
48 hr, 5 µg
28 S
18 S
b
LDBMC
+HPCM
HL-60
0
12
24
36
48
1 µg
0.5 µg
0.25 µg
c
LDBMC + HPCM
LDBMC-HPCM
24 hr Monocytes
24 hr T-Cell
0 hr
12 hr
24 hr
36 hr
48 hr
72 hr
96 hr
tRNA
HL-60
0 hr
24 hr
48 hr
-HPCM
-HPCM
-HPCM
+HPCM
2.0 µg
1.0 µg
0.5 µg
0.25 µg

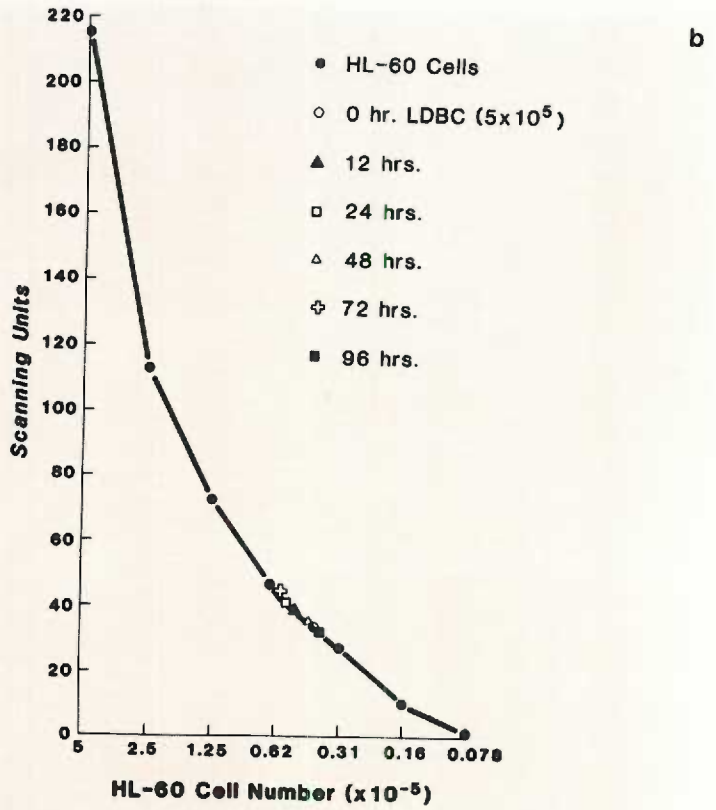
FIGURE 3.6

DNA dot blot of HPCM-stimulated LDBM cells.

a) Cellular DNA isolated from nuclei of HPCM-stimulated LDBM cells at 0, 12, 24, 48, 72 and 96 hours as outlined in Experimental procedures, were dot blotted on Genescreen plus membranes and hybridized with a probe specific for second and third exons. HL-60 DNA was also analyzed similarly. We found that HL-60 cells had 14-16 fold higher gene copies per cell than did normal cells and that gene amplification did not occur in the normal cells.

b) The autoradiogram (the one shown in Fig. 6a) was scanned and the scanning units were plotted against the number of HL-60 cells used per time point. The readings obtained for LDBM cells (5×10^5) were plotted on the curve obtained for HL-60. The relative increase of c-myc gene copies in HL-60 cells was calculated as the ratio of the number of LDBM cells used to the number of HL-60 cells giving similar densitometric reading.


a
LDBMC+HPCM
0 hr
12 hr
24 hr
48 hr
72 hr
96 hr
HL-60
5
2·5
1·25
0·62
0·31
0·16
0·078
CELL NUMBER (10⁵)


b
HL-60 Cells
0 hr. LDBC (5x10⁵)
12 hrs.
24 hrs.
48 hrs.
72 hrs.
96 hrs.
Scanning Units
220
200
180
160
140
120
100
80
60
40
20
0
5
2.5
1.25
0.62
0.31
0.16
0.078
HL-60 Cell Number (x10⁻⁵)

CHAPTER 4

CHAPTER 4

C-MYC EXPRESSION IN LEUKEMIC AND NORMAL HUMAN BONE MARROW CELLS IN VITRO: INSTABILITY OF C-MYC mRNA

4.1 INTRODUCTION

My studies on the temporal expression of c-myc during in vitro differentiation of CSA-stimulated progenitor enriched normal human bone marrow cells revealed that 1) these cells transcribe c-myc transiently; 2) the quantity of c-myc transcripts reach maximum level around 24 hours in culture with HPCM; and 3) the expression was consistently repressed by about 36 hours. In contrast, when progenitor cell preparations from acute myeloid leukemic patients were exposed to HPCM, the level of c-myc expression remained constant throughout the incubation period; and unlike normal cells, leukemic cells did not undergo maturation. In addition, I noted that although HL-60 cells have 16-fold more c-myc gene copies, the relative c-myc transcript number was quantitatively similar to that in appropriate normal myeloid cells. Two interpretations of these observations are that HL-60 cells do transcribe at a rate equal to 16 times that of normal cells but that mRNA degradation is greater than in normal cells. Another explanation is that most c-myc gene copies in HL-60 cells are quiescent. Accordingly, I sought to compare c-myc mRNA turnover in HL-60 cells and normal progenitor-enriched cells expecting at the very least to more

completely understand some of the mechanisms operative in normal bone marrow cells for the regulation of c-myc expression, an understanding of which might provide insights into the regulatory abnormalities of gene expression in myeloid leukemia cells.

4.2 MATERIALS AND METHODS

Cells: Bone marrow cells prepared as described in appendix 3 were cultured in the presence of 10% HPCM for 24 hours. HL-60 cells obtained from ATCC, Maryland were grown in suspension in RPMI 1640 medium containing 15% fetal calf serum. These cells were exposed to actinomycin D (5-10ug/ml) and the cells were harvested as indicated in the figure legends.

The details of RNA isolation, dot and northern blot hybridization, and the sources and the preparation of the c-myc and γ-actin gene probes are presented in appendix 3.

Estimation of the half-life: The dot blot autoradiograms were evaluated by quick scan densitometer. The linear range intensities obtained for matched control and actinomycin D treated samples were used to calculate the proportion of c-myc or γ-actin mRNA remaining. We calculated these proportions as the ratio of scanning units obtained for cells treated with actinomycin D divided by the scanning units for the cells incubated for the same time without actinomycin D. The ratios were plotted against the time of actinomycin D chase; and the

time required for 50% reduction of the ratio was taken as the half-life.

S1 Nuclease mapping: S1 nuclease analysis was performed according to the procedure described be Weaver and Weissmann (1979). 20 ug of cytoplasmic RNA extracted from LDBM cells exposed to HPCM for 24 hours was lyophilized with 20 ug of tRNA and dissolved in 15 ul of 80% formamide hybridization buffer (80% formamide, 1.6M NaCl, 0.16M PIPES [pH 6.4], 4mM EDTA) containing approximately 5 ng of ^{32}P end-labeled 880 base pair 5' Pvu II c-myc gene probe covering the transcriptional initiation sites. The hybridization cocktail was covered with mineral oil, heated to 70°C for 15 minutes and incubated overnight at 44 and 48°C. After the incubation period, the contents were mixed with 300 ul of ice-cold S1 nuclease buffer (0.25M NaCl, 0.03M sodium acetate [pH 4.6], 0.001M $ZnSO_4$, 20 ug/ml denatured salmon sperm DNA [SIGMA]) containing S1 nuclease (final concentration 200U/ml). The contents were cooled on ice immediately for few seconds, vortexed, spun for 15 seconds and incubated for 90 minutes at 25°C. The contents were transferred to a new tube containing 300 ul of saturated phenol/chloroform (1:1), vortexed and spun for 5 minutes in a microcentrifuge. The nucleic acids were ethanol precipitated and dissolved in 5 ul of loading solution (0.05% bromophenol blue, 0.05% xylene cyanol, 1mM EDTA, 90% formamide). The contents were heated for 2 minutes in boiling water and electrophoresed on a 6%

polyacrylamide/7M urea gel in tris-borate buffer. The gel was autoradiographed with X-omat AR (Kodak) X-ray film and an intensifying screen at -70°C.

Preparation of 5'-^{32}P-labeled probes: pHSR-1 human c-myc DNA was digested with Pvu II and dephosphorylated with bacterial alkaline phosphatase (BRL). The 5' c-myc Pvu II fragment was electroeluted and 5'-^{32}P-labeled using T4 polynucleotide kinase (BRL) as described by Maniatis, et al., (1982).

4.3 RESULTS

Levels of c-myc transcripts in CSA-stimulated normal bone marrow cells.

Cytoplasmic RNA was extracted from normal human bone marrow cells cultured in the presence of HPCM for 24 hours. The samples were dot blotted as described in materials and methods, and analyzed for content of c-myc as well as γ-actin mRNA, the latter one serving as a constitutively expressed gene. As shown in Fig. 4.1, the c-myc gene probe used in the study recognizes a 2.3 kb c-myc transcript(s); we could not discern by this technique which of the two c-myc promotors (Battey et al., 1983; Watt et al., 1983) were being used (Fig. 4.1). The γ-actin specific gene probe (Ponte et al., 1983) recognizes a 2.1 kb transcript (Fig. 4.1).

The results of RNA dot blot study for the determination of turnover rates of c-myc mRNA in normal and HL-60 cells are

shown in figures 4.2 and 4.3. In the first experiment (Fig. 4.2), we found that c-myc mRNA quantity fluctuated around 24 hours (Fig. 4.2a), while γ-actin mRNA levels remained steady (Fig. 4.2c) in cells not exposed to actinomycin D. In the second experiment (Fig. 4.3), the variation between the three control samples harvested at different time intervals was not evident (compare lanes 4, 5 and 6 in Fig. 4.3). Most importantly, in both cases, the cells exposed to actinomycin D, revealed a progressive decrease in c-myc mRNA content if these experimental valves are normalized to their matched controls as described in materials and methods (Figs. 4.2 and 4.3). HL-60 cells used in this study had high quantities of c-myc mRNA (Figs. 4.1 and 4.2b). HL-60 cells also contained levels of γ-actin mRNA comparable to that seen in LDBM cells (compare figs. 4.2c and 4.2d).

S1 nuclease mapping experiments were performed to test the specificity of hybridization. The samples were hybridized at two different temperatures. In this experiment, only the samples incubated at 48°C showed interpretable results. Therefore, samples incubated at 44°C will not be discussed further. As shown in Fig. 4.4, the expected 350 base pair c-myc fragment was observed at all time intervals in cells not exposed to actinomycin D (see lanes, 4, 8, and 12 in Fig. 4.4) and interestingly variation in c-myc mRNA content over time was clearly evident in cells not exposed to actinomycin D (Fig. 4.4,

compare lanes 4, 8 and 12). There was a gradual decline in c-myc mRNA content in the cells exposed to actinomycin D (Fig. 4.4, see lanes, 6, 10 and 14).

Stability of c-myc transcripts in CSA-stimulated normal bone marrow and HL-60 cells.

In view of the variations in c-myc RNA content, matched controls were used in all studies. Cytoplasmic RNA was extracted from LDBM cells and HL-60 cells at various times after treatment with actinomycin D (5-10 ug/ml) to block essentially all transcriptional activity (Dani et al., 1985). Each sample was either dot and/or Northern blotted onto a Genescreen membrane as described in materials and methods and analyzed for its c-myc and γ-actin mRNA content. As shown in Figs. 4.2a, 4.3, and 4.4, upon exposure of cells to actinomycin D for 30 minutes, c-myc mRNA content was dramatically reduced while γ-actin remained constant (Fig. 4.2c) in LDBM cells. C-myc content in HL-60 cells also decreased but to a lesser extent than that of normal cells in the presence of actinomycin D (Compare Figs. 4.2a and 4.2b); γ-actin on the other hand, remained unchanged and was quantitatively similar to that in normal cells (Compare Figs. 4.2c and 4.2d).

Densitometry of RNA dot blots showed that the half-life of c-myc in normal and HL60 cells are slightly different (Table 4.1, Figs 4.5a and 4.5b). We estimate that the half-life of c-myc mRNA is 15 minutes in normal human bone marrow cells and

20 minutes in HL-60 cells. γ-actin mRNA was quite stable (Figs. 4.5c and 4.5d). Our estimate of c-myc mRNA half-life is in agreement with that published by Dani et al., (1985).

6.4 DISCUSSION

We have previously observed that CSA-stimulated progenitor enriched normal human bone marrow cells not only transcribe the c-myc gene but also contain c-myc transcripts similar in quantity to that in HL-60 cells (a cell line which has 16-fold higher c-myc gene copies). These results are compatible with two notions. One, HL-60 cells do transcribe c-myc gene at a rate equal to 16 times that of normal cells but mRNA degradation is greater than in normal cells. Two, most c-myc gene copies in HL-60 cells are quiescent. The purpose of the present section was to further characterize regulation of c-myc expression in HL-60 cells and in normal bone marrow cells by determining comparative rates of c-myc mRNA degradation. Our results clearly confirm observations of Dani et al., (1985) that c-myc mRNA is extremely unstable in normal and many transformed cell lines. These results along with the observations of Hann et al., (1985) that c-myc proteins also have a very short half-life (20 minutes), suggest that fine tuning of c-myc expression can occur at multiple levels of gene expression.

Interestingly, however, we find that c-myc mRNA is slightly more stable in HL-60 cells than in normal bone marrow cells

(Table 4.1). This difference could be due to the qualitative differences in the mRNA pool of the two cell types. For example, it is well established that the c-myc gene has two transcriptional initiation sites resulting in two mRNA species differing in length by about 180 nucleotides (Battey et al., 1983; Watt et al., 1983). It is conceivable that the two messages might have a different turnover rate and that in a cell where both initiation sites are being used, the proportion of two mRNA molecules determines the overall turnover rate. This view is supported by two facts. First of all, the data presented in Figures 4.5a and 4.5b for normal bone marrow cells and HL-60 cells are not linear and therefore, the turnover mechanism may not be a first-order reaction. This suggests that the mRNA pool analyzed may contain more than one species of c-myc mRNA and hence the proportion of the two molecules determines the over all turnover time. It should be noted here that in HL-60 cells used by some investigators the shorter transcript predominates (Watt et al., 1983; Nishikura et al., 1985). Should this difference in mRNA pool be confirmed by S1 nuclease mapping, it would strongly suggest that the 5' noncoding region of these mRNA molecules might be an important regulatory target for their degradation. Secondly, cycloheximide is shown to have a differential stabilizing effect on 2.2- and 2.4-kilobase c-myc mRNA species (Dani et al., 1985). This also suggests that the two c-myc mRNA species might

have different turnover rates. However, preliminary results have suggested that the LDBM cells use predominantly, the promotor that transcribes the shorter message (see Fig. 4.4).

We infer from these studies that the c-myc mRNA is extremely unstable in LDBM and HL-60 cells consistent with the earlier studies by Dani, et al., (1985). However, we have made additional observations that are relevant to the quantity of c-myc mRNA in LDBM cells. In particular, the quantity of c-myc mRNA in the control cells is not always constant (see Figs. 4.2a and 4.4). Although, we have not systematically studied these perturbations, the fluctuations could represent a true biological phenomenon related to the differential gene expression during normal granulocyte differentiation or could merely be a technical artifact.

Regardless of the mechanisms involved in differential mRNA degradation, the findings herein confirm that normal marrow cells consistently express c-myc after 24 hours in culture with CSA, that the transcript numbers in these cells 'approximate' the transcripts in HL-60 cells, even though our HL-60 clone has 16-fold amplification of c-myc gene. More importantly, we find that mRNA levels are slightly more stable in HL-60 cells than in normal bone marrow cells. Therefore, we conclude that the most of the amplified genes in HL-60 cells are quiescent relative to c-myc in normal cells. Our findings call into question the long presumed linkage in HL-60 cells between gene copy number and gene activity.

Table 4.1. Half-life of c-myc mRNA in LDBM and HL-60 cells

	Half-life in minutes	
	LDBM	HL-60
	n=2	n=4
C-myc	15	20

FIGURE 4.1

RNA blot analysis of HL-60 c-*myc* and γ-actin mRNAs during an actinomycin D chase.

HL-60 cells were grown in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 5 ug/ml actinomycin D for 10 (lanes 1 and 3) and 30 (lanes 2 and 4) minutes, respectively. Total RNA (15 ug) was electrophoresed under denaturing conditions, transferred to Genescreen membrane and hybridized with nick-translated c-*myc* exons 2 and 3 (top) and γ-actin (bottom) probes.

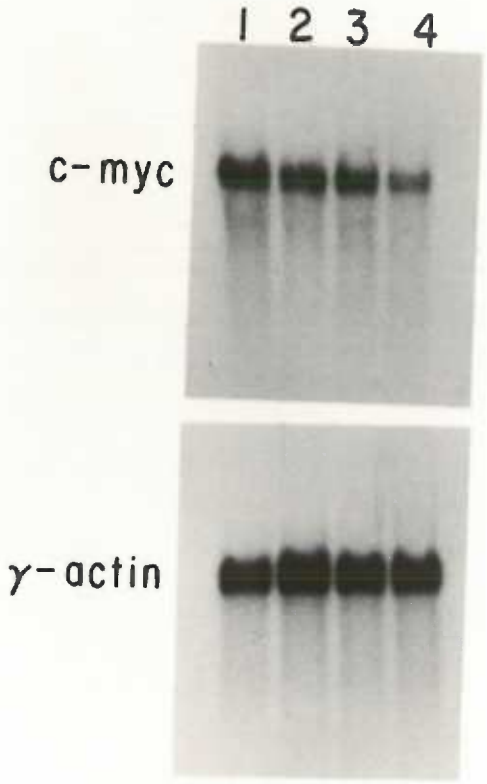

1 2 3 4
c-myc
γ-actin

FIGURE 4.2

RNA dot blot analysis of LDBM and HL-60 c-*myc* and γ-actin mRNAs during an actinomycin D chase.

LDBM (a and c) and HL-60 (b and d) cells were grown in the absence (lanes 1, 2 and 3) or presence (lanes 4 and 5) of 5 ug/ml actinomycin D for 0 (lane 1), 10 (lanes 2 and 4) and 30 (lanes 3 and 5) minutes, respectively. Total RNA was dot blotted onto a Genescreen membrane and hybridized with c-*myc* exons 2 and 3 (a and b) and γ-actin (c and d) probes.

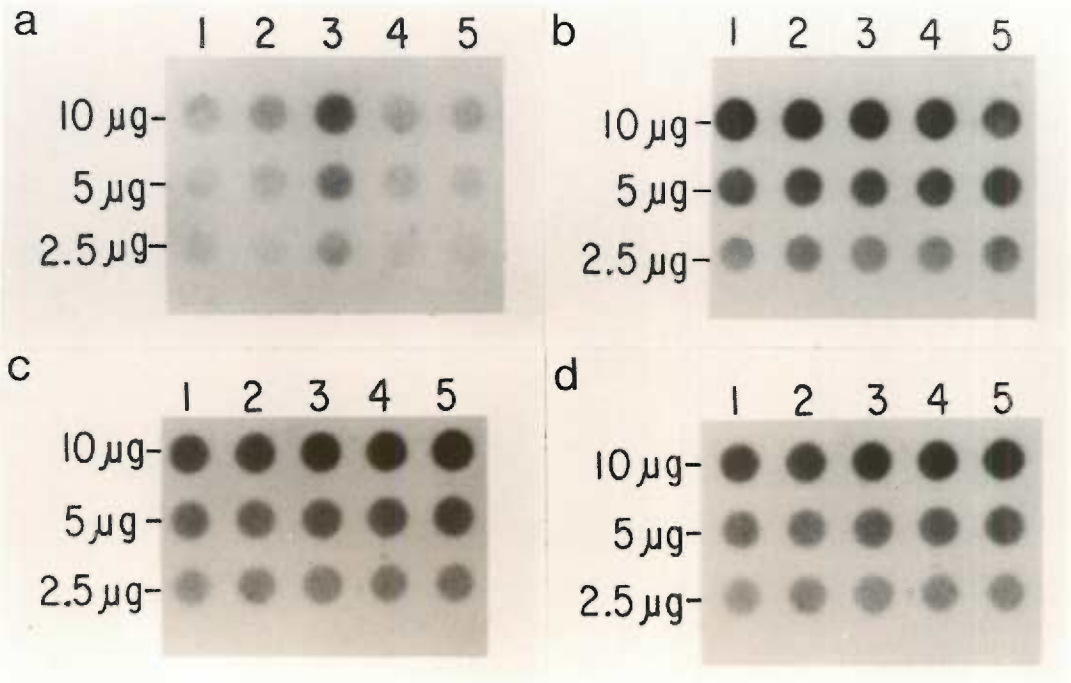

a
1 2 3 4 5
10 µg-
5 µg-
2.5 µg-
b
1 2 3 4 5
10 µg-
5 µg-
2.5 µg-
c
1 2 3 4 5
10 µg-
5 µg-
2.5 µg-
d
1 2 3 4 5
10 µg-
5 µg-
2.5 µg-

FIGURE 4.3

RNA dot blot analysis of LDBM c-myc mRNA during an actinomycin D chase.

LDBM cells were grown in the absence (lanes 4, 5 and 6) or presence (lanes 1, 2 and 3) of 10 ug/ml actinomycin D for 5 (lanes 1 and 4), 10 (lanes 2 and 5) and 30 (lanes 3 and 6) minutes, respectively. Total RNA was dot blotted onto a Genescreen membrane and hybridized with c-myc exons 2 and 3 gene probe.

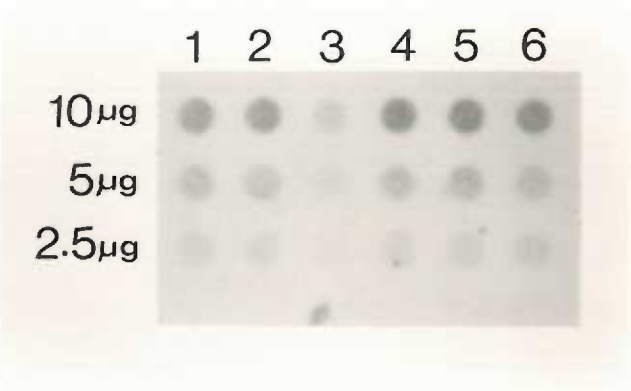

1 2 3 4 5 6
10μg
5μg
2.5μg

FIGURE 4.4

S1 nuclease analysis of LDBM c-myc mRNA during an actinomycin D chase.

LDBM cells were grown in the absence (lanes 3, 4, 7, 8, 11 and 12) or presence (lanes 5, 6, 9, 10, 13 and 14) of 10 ug/ml actinomycin D for 5 (lanes 3, 4, 5 and 6), 10 (lanes 7, 8, 9 and 10) and 30 (lanes 11, 12, 13 and 14) minutes, respectively. The Cytoplasmic RNA was hybridized overnight at 44°C (lanes 3, 5, 7, 9, 11 and 13) and 48°C (lanes 4, 6, 8, 10, 12 and 14) with ^{32}p-labeled 5' c-myc gene probe, digested with S1 nuclease, and the protected nucleic acids were analyzed by polyacrylamide/urea gel electrophoresis and autoradiography. M represents the size markers in base pairs. Lane 1 contains no added RNA and lane 2 represents the probe size.

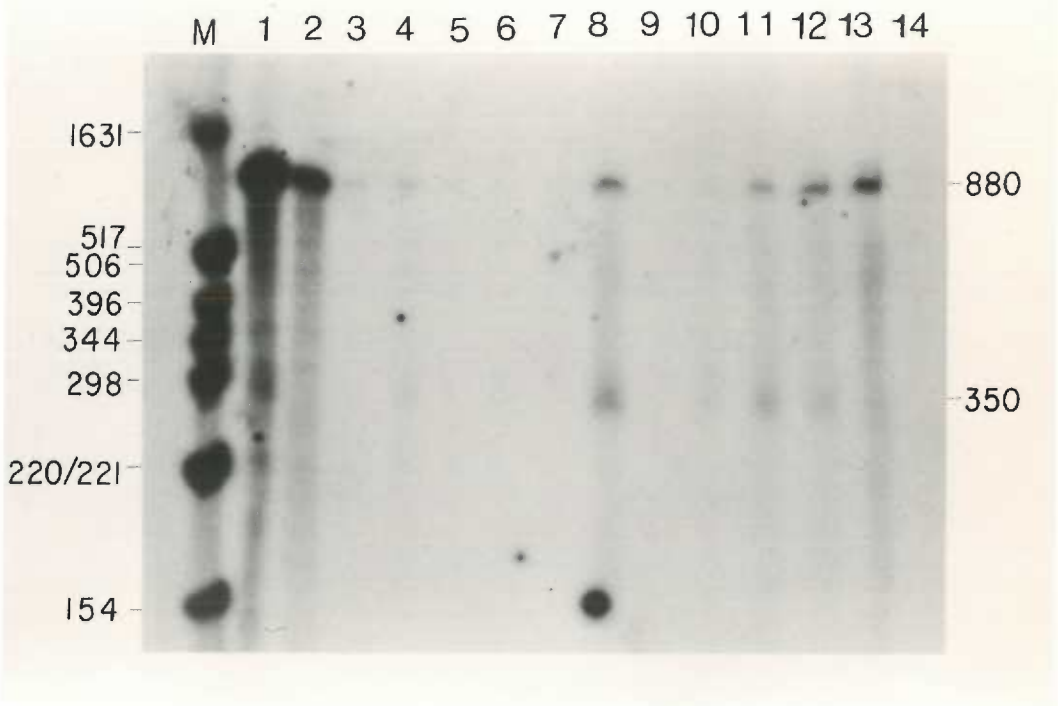
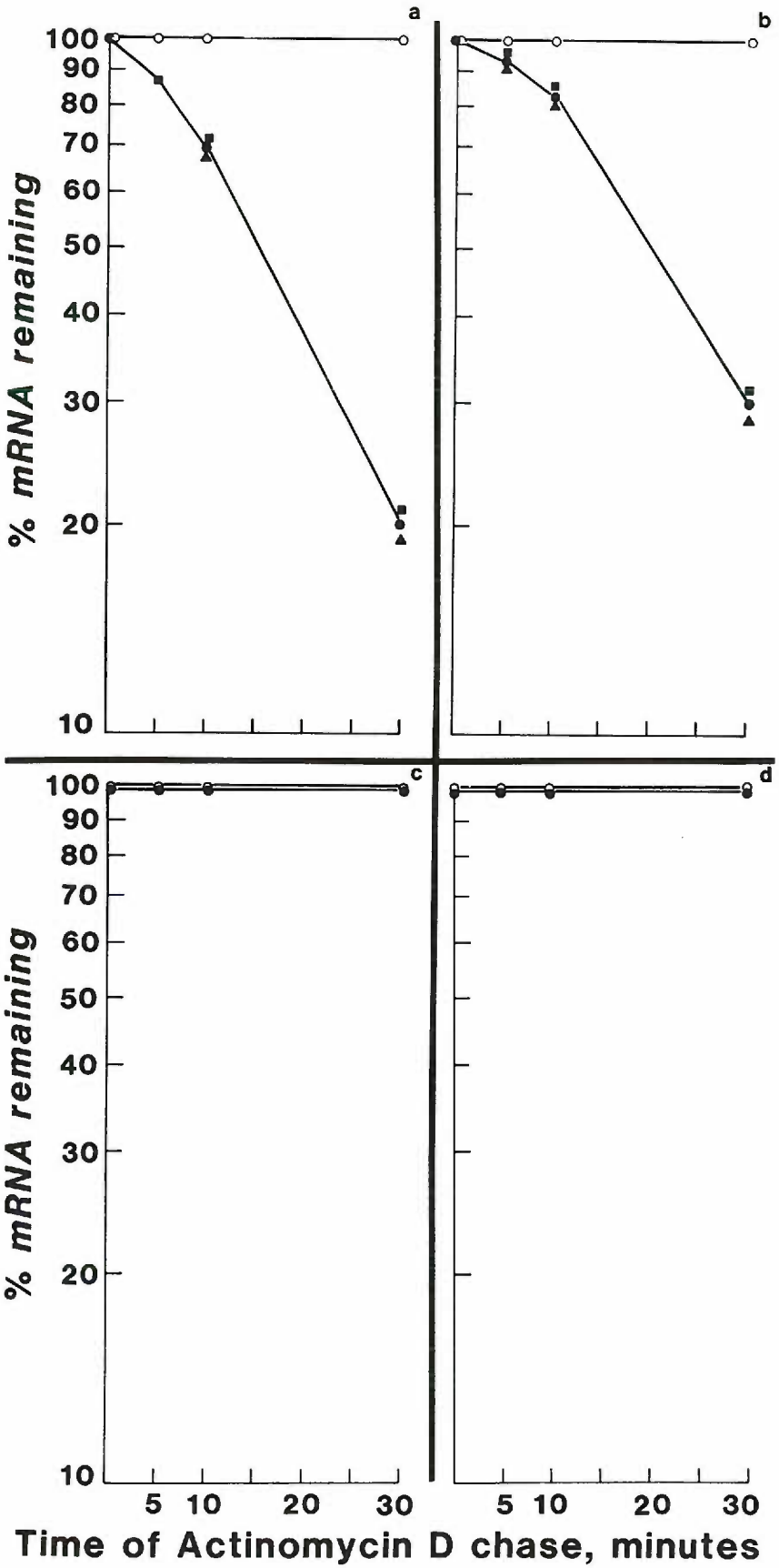

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
1631
517
506
396
344
298
220/221
154
880
350

FIGURE 4.5

Half-lives of LDBM and HL-60 c-myc and γ-actin mRNAs during an actinomycin D chase.

mRNA was quantitated by densitometry scanning of LDBM (a and c) and HL-60 (b and d) autoradiograms for c-myc (a and b) and γ-actin (c and d) genes. (o without actinomycin D; ● , ▲ , ■ with actinomycin D; ■ , ▲ represent two independent experiments).


a
b
c
d
100
90
80
70
60
50
40
30
20
10
% mRNA remaining
5 10 20 30
Time of Actinomycin D chase, minutes

SUMMARY AND CONCLUSIONS

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The present study describes 1) the procedures for the growth and differentiation of human myeloid cells in suspension, 2) the characteristics of myeloid cellular differentiation in vitro, and 3) the expression and regulation of c-myc gene during normal and leukemic granulopoiesis.

Upon induction of proliferation and differentiation by myeloid lineage-specific humoral stimulator, progenitor enriched bone marrow cells showed a progressive increase in tritiated thymidine uptake reaching a peak after three days and then declined; the response was largely granulocytic; while myeloid-lineage specific monoclonal antibody showed a 7-fold increase by day 4, the T-lymphocyte specific monoclonal antibody did not show any change over this period; the chloroacetate esterase (which recognizes only granulocytes) positive cells increased 7-fold by day 4; the non-specific (monocyte-specific) esterase positive cells declined by day 4; the differential cell count analysis on Wright stained cyto-spin slides revealed a wave of maturation ranging from predominantly lymphoid appearing immunoglobulin negative cells on day 0 to a predominantly non-proliferative metamyelocytes and bands on day 4 with distinct peaks of myeloblasts after 12 hours, progranulocytes after 24 hours, and myelocytes after 72 hours.

The granulopoietic progenitor cells undergoing growth and

differentiation were harvested at various time intervals and the RNA was analyzed for the levels of c-myc and γ-actin and ß-globin mRNA content. The c-myc transcript content was very low on day 0 and increased after 12 hours reaching a peak around 24 hours; the levels reached basal levels consistently by about 36 hours. In contrast to c-myc transcripts, the γ-actin transcript levels remained constant; ß-globin mRNA was not detected at any time. An interesting correlation between the peak of c-myc transcripts and predominance of early progranulocytes and their precursors was seen after 24 hours in culture. However, my attempts to examine individual cells for c-myc transcripts using in situ hybridizations were unsuccessful.

The amplification of the c-myc gene in HL-60 cells has led to the hypothesis that the c-myc gene amplification might be a normal event during growth and differentiation of normal myeloid cells. The progenitor enriched human bone marrow cells harvested at various time intervals were analyzed for c-myc gene copy number using a quantitative DNA dot blot method and were compared to that in HL-60 cells. These studies revealed that normal bone marrow cells consistently had 14-16 fold lower signals than HL-60 cells. Therefore, it was concluded that while HL-60 cells used in this study had 10-16 fold higher gene copies, normal cells do not amplify c-myc gene copy number during myeloid cellular growth and differentiation.

The comparisons of the c-myc transcript profiles in normal

and leukemic marrow progenitor cells revealed that normal marrow cells consistently repress c-myc transcript levels after an initial burst; the leukemic cells on the other hand, had high c-myc mRNA content; and the levels did not change through out the culture period.

The examination of the stability of c-myc mRNA in normal bone marrow cells revealed that c-myc transcripts are extremely unstable; the estimated half-life of c-myc mRNA was approximately 15 minutes. Interestingly, however, the c-myc transcripts in HL-60 cells used in these experiments had a slightly longer half-life (20 minutes). Even though the reasons for this discrepancy is not known, it might be related to the relative abundance of the two known c-myc mRNA molecules. Although the RNA T1/2 is slightly longer in HL-60 cells than in normal cells, we conclude that most of the amplified genes in HL-60 cells are quiescent relative to the c-myc gene in normal cells. Our results call into question the long presumed linkage in HL-60 cells between gene amplification and gene activity. Alternatively, the expression of HL-60 c-myc genes at same low levels could account for the similarities in the transcript numbers between normal bone marrow and HL-60 cells.

In conclusion, c-myc expression is a normal event in granulopoiesis possibly linked not only to proliferative activity but to the primitive developmental stage as well. The c-myc transcription per se is insufficient to account for the

neoplastic phenotype in myeloid leukemia cells and the most consistent abnormality in such cells is their failure to suppress c-myc transcription. Studies addressing the mechanisms by which normal myeloid cells repress c-myc transcripts, chromatin structure analysis and changes in methylation profile should provide important insights on the regulatory abnormalities of gene expression which underlie the behavior of myeloid leukemia cells.

APPENDIX 1

APPENDIX 1

IN SITU HYBRIDIZATION

INTRODUCTION

Studies on the expression of the c-myc gene during the growth and differentiation of normal cells revealed an interesting correlation between predominance of early progranulocytes and cells more primitive than progranulocytes and the transient peak of c-myc mRNA content around 24 hours. Cells more differentiated than progranulocytes predominated in the cultures during later hours in culture, when the c-myc mRNA was at its lowest. Moreover, all the acute myeloid leukemic cells are "maturationally arrested" either at the progranulocyte stage, or at stages more primitive than the progranulocyte; the cells obtained from four acute leukemia patients in this study and in almost all the cases reported had a high c-myc mRNA content.

The technique of in situ hybridization has become a powerful tool for mapping specific DNA sequences on chromosomes. Recently, this approach has been applied to the detection of RNA transcripts in individual cells (Angerer and Angerer, 1981; Cox et al., 1984; Brahic and Haase, 1978). It offers the potential for comparing quantitatively the relative concentrations of specific RNA transcripts in different cells of a heterogeneous population. Such an approach is particularly

valuable for systems in which quantities of material are limited and/or in which cell fractionation techniques are not feasible. Therefore, I undertook experiments to identify cell type(s) expressing the c-myc gene during growth and differentiation using in situ hybridization.

MATERIALS AND METHODS

Preparation and pretreatment of the slides: The LDBM cells prepared as described in the appendix or HL-60 cells grown in suspension were cyto-centrifuged onto a clean glass slide using a cyto centrifuge. The slides were then fixed in 1% glutaraldehyde (SIGMA, MO), 3% NaCl, 50 mM sodium phosphate, pH 7.4, at 0° C for 60 minutes. They were washed in two changes of buffer, each 30 minutes at 0° C. The slides were either dehydrated by sequential 30 minute changes through increasing ethanol concentrations (30%, 50%, 70%, 85%, 95%, 99%, and 99%) for storage or processed immediately for hybridization.

The hydrated slides were incubated with 1 ug proteinase K/ml in 0.1M Tris-HCl, 50 mM EDTA, pH 8.0, for 30 minutes at 37° C. Following digestion, the slides were washed briefly in distilled water, and treated with acetic anhydride as described by Hayashi et al., (1978). Acetylated slides were washed briefly in 2X SSC, water and dehydrated by sequential 30 minute changes through increasing ethanol concentrations (30%, 50%, 70%, 85%, 95%, 99%, and 99%).

Preparation of the probe: In order to obtain single-stranded probes, the 5' c-myc Hind III-Xba I fragment was subcloned in reverse orientation (Fig. i) into Xba I-Hind III cut pSP65 plasmid (Promega-Biotec). The plasmid was linearized at the Hind III site and was transcribed using SP6 RNA polymerase as described by Melton et al., (1984) in the presence of tritiated ATP (Amersham). The fragment length of RNA probes was adjusted to a mass average of approximately 150 bases by limited alkaline hydrolysis in 40 mM $NaHCO_3$/60 mM Na_2CO_3, pH 10.2, at 60° C. The sample were neutralized by addition of sodium acetate, pH 6.0, and glacial acetic acid to 0.1M and 0.5% (v/v), respectively, and ethanol precipitated (Cox et al., 1984).

Hybridization: RNA probes and carrier (500 ug yeast tRNA/ml) were boiled for 3 minutes in 10 mM Tris-HCl, pH 8.00, 1 mM EDTA, quick-cooled in ice water, and the other components were added to give final concentrations of 50% Formamide (deionized), 0.3M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2% each of bovine serum albumin (SIGMA), 0.02% Ficoll (Pharmacia), 0.02% polyvinylpyrollidone (Pharmacia), and 10% dextran sulfate (Pharmacia). Hybridization mixtures (5 ul/slide) were applied to slides and covered with silicon-treated, baked coverslips. The edges of the coverslips were sealed with mineral oil to prevent evaporation and incubated at 25° C or 30° C for nick translated probes or at 45° C or at 50° C for single-stranded RNA probes in a humidified incubator for 16-18 hours. After

hybridization, oil was removed from each slide by three 50-ml washes in chloroform, and the slides were air dried. Each slide was then washed in three 50-ml portions of 4X SSC (1X SSC is 0.15M NaCl, 0.015M trisodium citrate, pH 7.0) during which the cover slips were carefully removed. The slides were then treated with RNAase A (20 ug/ml) in 0.5M NaCl, 10 mM Tris-HCl, pH 8.00, at 37° C for 30 minutes, washed in RNAase A buffer at 37° C for 30 minutes, and then in 4 liters of 2X SSC for 30 minutes and 4 liters of 0.1X SSC for 15 minutes, both at room temperature. The slides were dehydrated by sequential 30 minute changes through increasing ethanol concentrations (30%, 50%, 70%, 85%, 95%, 99%, and 99%). Each ethanol solution, except 99%, contained 300 mM ammonium acetate as suggested by Brahic and Haase (1978).

Autoradiography: Kodak NTB-3 nuclear track emulsion was melted at 45° C and diluted 1:1 with 600 mM ammonium acetate. The slides were dipped in melted emulsion for 1-2 seconds and allowed to dry in an upright position for 15-20 minutes at room temperature. They were then transferred to light-proof boxes containing silica gel desiccant and exposed at 4° C for 10, 20, or 40 days. The slides were developed in Kodak D-19 developer for 3 minutes at room temperature followed by a brief wash in 1% acetic acid and fixation for 3 minutes in Kodak fixer.

RESULTS AND INTERPRETATIONS

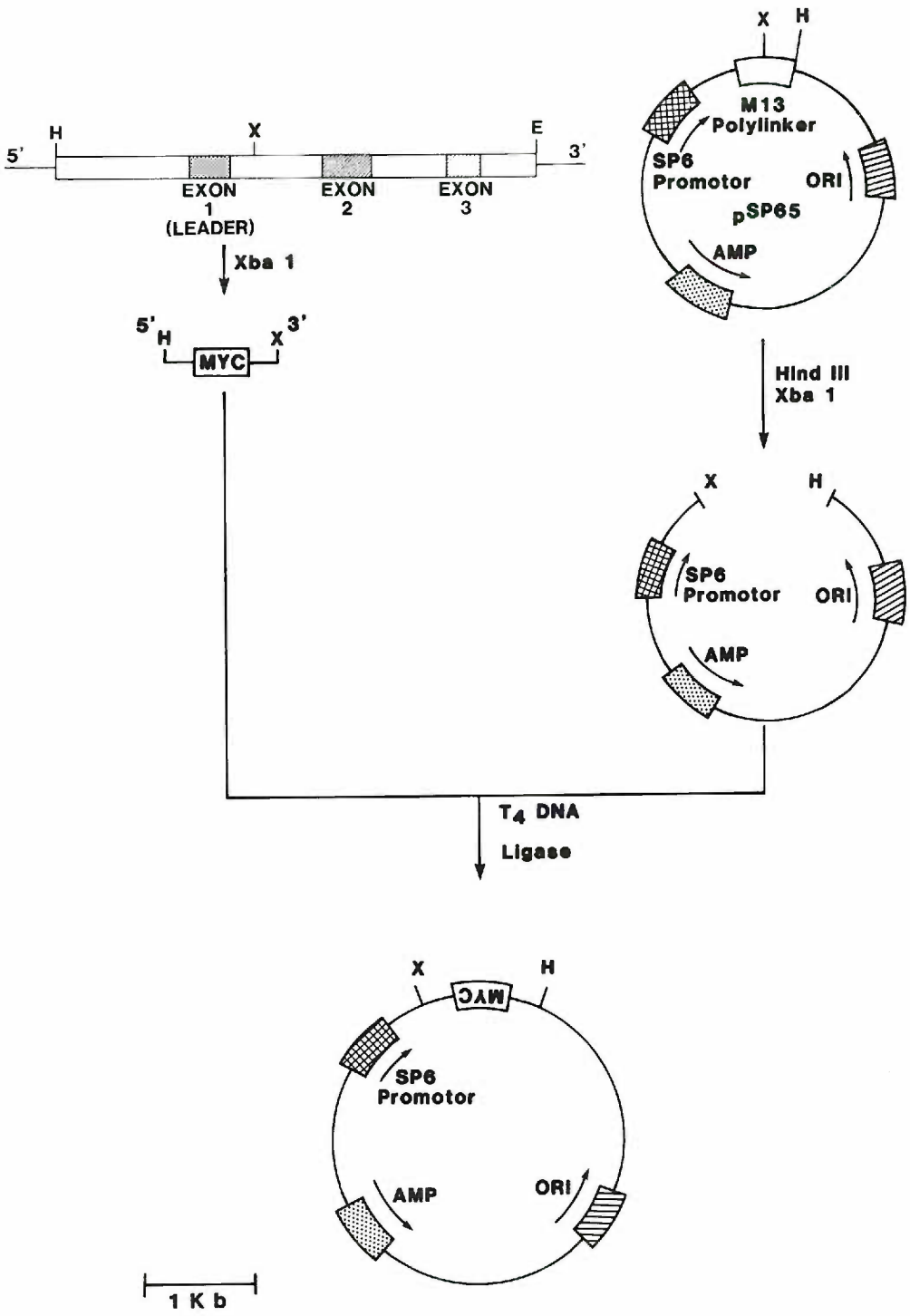
For the initial studies, nick translated c-myc gene probes (method is described in appendix) were used. The examination of the slides revealed that the grain distribution on both control and test slides was comparable; the grains were uniformly distributed without any signs of cell specific accumulation of grains (even HL-60 cells which have high c-myc RNA failed to show cell specific accumulation of grains). One reason for the failure was thought to be related to the effective number of hybridizable c-myc fragments in the nick-translated probe mixture. Therefore, single-stranded probes were prepared as described above and the experiments were repeated. Unfortunately, even these modifications failed to provide any positive signs of cell-specific hybridizations.

Taking into account the facts of uninterpretable hybridizations and the loss of cellular features that enable us to distinguish the myeloid cellular stages during in situ hybridization, these experiments were not pursued further.

Figure i

Construction of pSP65 c-myc riboprobe vector.

The human c-myc gene was digested with Hind III and Xba 1, electroeluted (Maniatis et al., 1982) and inserted in Xba 1-Hind III cut pSP65 (Promaga, Bio-tec) plasmid in reverse orientation. The construct was linearized at Hind III site and transcribed using SP6 RNA polymerase (Melton and Kreig, 1985) to obtain single-stranded RNA probes complementary to c-myc mRNA for in situ hybridization. H = Hind III; X = Xba I; AMP = Ampicillin; ORI = Origin of replication.


5'
H
X
E
3'
EXON 1 (LEADER)
EXON 2
EXON 3
Xba 1
5'
H
MYC
X
3'
X
H
M13 Polylinker
SP6 Promotor
ORI
pSP65
AMP
Hind III
Xba 1
X
H
SP6 Promotor
ORI
AMP
T4 DNA Ligase
X
H
MYC
SP6 Promotor
AMP
ORI
1 K b

APPENDIX 2

APPENDIX 2

DETECTION OF C-MYC REPRESSOR PROTEIN(S) BY FILTER BINDING ASSAY

INTRODUCTION

My studies on c-myc mRNA profiles in normal and leukemic cells suggested that one of the fundamental defects in the leukemic cells might be their inability to repress c-myc expression. It is known that the c-myc gene expression can be enhanced by protein synthesis inhibition (Kelly et al., 1983). Moreover, the lymphoblastoid cell nuclei contain protein(s) that binds to a site near the 5' end of the c-myc gene (Siebenlist et al, 1984). These studies suggest that the c-myc gene might be regulated by a labile trans-acting factor acting as a transcription repressor or as a factor accelerating c-myc mRNA degradation (Taub et al., 1984).

The mechanisms for the control of gene expression in many cases involves either the binding of proteins to DNA at specific sites known as operator sites (Riggs et al., 1970a, 1970b) or modulation of chromatin structure leading to either activation or inactivation of gene(s) (Wu, 1980) or both. In the case of c-myc, Siebenlist et al., (1984) have used the technique popularized by Riggs et al., (1970a) to identify the protein(s) that specifically bind to the 5' end of c-myc gene. They have found that nuclear extracts prepared from lymphoblastoid cell nuclei contain protein(s) that binds to a site near the 5' end

of the c-myc gene. Furthermore, it appears that the sensitivity to DNAase I near site I is modulated by the binding of repressor protein(s) implying that site I may be involved in the process of deregulation of the c-myc gene (Siebenlist et al., 1984). Therefore, my goal in this experiment was to use the filter binding assay 1) to analyze the involvement of DNA-binding protein(s) in the regulation of the c-myc gene in K562 cells; 2) to use this assay to detect, map and quantitate the nuclear protein(s) that bind specifically to the c-myc gene during in vitro differentiation of normal granulocytes. The human erythroleukemia cell line, K562 cells were used to set up the filter binding assay because these cells have low c-myc mRNA content (my studies not included in this thesis) and therefore, I assumed that these cells might have high putative repressor protein(s).

MATERIALS AND METHODS

Preparation of extracts from K562 cells: The procedure to isolate nuclear extracts is essentially as described (Siebenlist et al., 1984). About 10^8 K562 cells were pelleted by low speed centrifugation and resuspended in 10 ml of ice-cold buffer A (10 mM HEPES [pH 8.0], 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA, 0.25 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% (v/v) Triton X-100, 1 mM PMSF, 7 mM 2-mercaptoethanol). All subsequent steps were carried out on ice or 4° C. Cells were

homogenized using a Dounce homogenizer and the nuclei were recovered by centrifugation (1000 x g). The pellet was washed two times with buffer A. After the washing steps, the nuclear pellet was resuspended in 3 ml buffer B (10 mM HEPES [pH 8.0], 100 mM NaCl, 25% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 7 mM 2-mercaptoethanol), stirred for 15 minutes and pelleted (1000g). The supernatant was discarded and the nuclear pellet was then resuspended in 3 ml buffer B containing 300 mM NaCl. After stirring for 30 minutes, the nuclei were pelleted at 2000 g and the supernatant was brought to 45% saturation using solid $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation, redissolved in 0.2-0.5 ml buffer B and stored at -70° C. The Protein concentration as determined by using Protein assay kit (Bio-Rad) was 0.2-0.3 mg/ml.

Restriction of c-myc gene probe: pHSR-1 c-myc (ATCC, Maryland) was prepared as described in the appendix and restricted with Eco RI, Hind III, Xba I, Cla I either individually or in combinations essentially described in Maniatis et al., (1982). The enzymes were from BRL, Maryland.

End-labeling of DNA: 3' recessed ends of the restriction fragments were labeled by replacement synthesis using labeled nucleotides and T4 DNA polymerase enzyme.(Maniatis et al., 1982). Exonuclease reaction was carried out by incubating DNA with T4 DNA polymerase buffer (33 mM Tris-acetate [pH 7.9], 66

mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 100 ug/ml of BSA)and 2 units of T4 DNA polymerase (BRL, Maryland) at 37° for 5 minutes. Resynthesis reaction was carried out by adding 2 mM each of dATP, dGTP, dTTP and 50 uCi [α-^{32}p]dCTP and incubating for 30 minutes at 37° C. The reaction mixtures were incubated for another 30 minutes with all four unlabeled nucleotides at 37°C. Nonincorporated mononucleotides were separated by ethanol precipitation.

Nitrocellulose filter binding assay: Binding reactions (100 ul) were performed in 10 mM HEPES [pH 8.0], 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 5 ug BSA, 5 or 20 ng ^{32}p-DNA and varying amounts (20-200 ng) of nuclear protein. After incubation at 25° C for 30 minutes the reaction mixtures were filtered through nitrocellulose filter (Schleicher and Schuel, 0.45 um) as described by Riggs et al., (1970a). The presoaked (in binding buffer) filters were placed on top of a large (13-cm diameter) porous plastic disc to which a vacuum is applied. The samples were applied and filtered at a rate of 0.06 ml/cm^2/min or slower. After the sample had passed through, the filters were washed twice with 1 ml of binding buffer. DNA was extracted from filters overnight at 37° C with 500 ul 0.25% SDS containing 40 ug of predigested pronase (BRL, Maryland). After adding 0.05 volume 5M NaCl and 2 ug of E. coli tRNA, polynucleotides were ethanol-precipitated, redissolved in 0.2%

SDS, and subjected to 1.0-1.5% agarose gel electrophoresis. Labeled DNA incubated with nuclear extract was used as marker for the identification of the fragment(s) that were retained by the filter due to the binding of the protein. At the end of electrophoresis, the gels were dried using a gel drier (Bio-Rad), and exposed to X-ray films (KODAK XAR-5) using intensifying screen (Dupont, Hi-plus). Labeled DNA filtered without exposure to nuclear extract and replacement of nuclear extract with bovine serum albumin were used as controls for non-specific retention and for nonspecific binding of unrelated proteins to c-myc DNA, respectively.

RESULTS AND INTERPRETATIONS

Filter binding assays have been used to show that the protein extracts prepared from nuclei contain specific protein(s) that bind to gene sequences. The filter binding assay together with the utilization of various restriction enzymes can also be useful in identification and mapping the gene sequences important in protein binding.

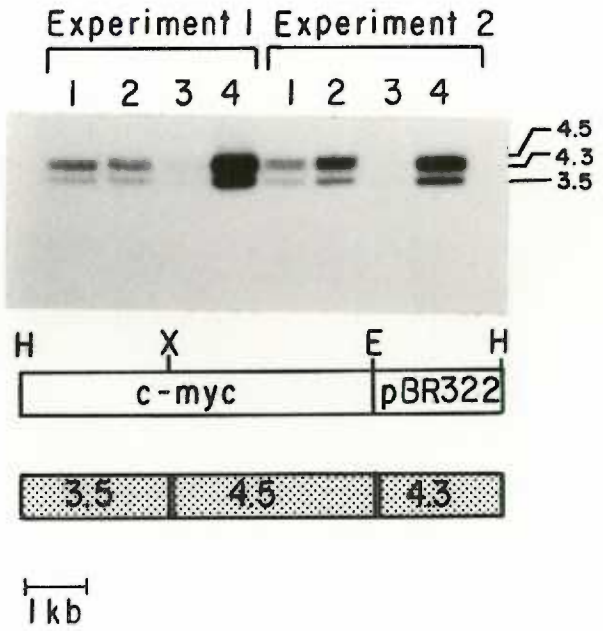
The results obtained were highly variable and uninterpretable. As shown in figure ii, the retention of the fragments was dependent on the concentration of nuclear extract (compare lanes 2 and 4) added; when the nuclear extract was replaced by bovine serum albumin, there was no retention of the fragments (Fig. ii, lane 3). Therefore, the nuclear extract

contained protein(s) that bind to the DNA. However, in contrast to the results of Siebenlist et al., (1984), the retention of the fragments was non-specific. In addition to the expected 5' c-myc fragment, both the 3' c-myc and the pBR 322 vector DNA were retained (Fig. ii). The results obtained from two independent nuclear extract preparations were essentially the same. These results suggested that nuclear preparations might contain non-specific DNA binding proteins. Therefore, competition experiments were used to unmask any specific retention and to clear the nuclear extracts of any non-specific DNA binding proteins. The use of unlabeled linear pBR322, lamda-Hind III, 5' Hind III-Xba I c-myc, 3' Xba I-Eco RI c-myc individually or in any combinations competed equally well with all the fragments. Moreover, the competition experiments were highly variable. Due to this uninterpretable, variable, and non-specific binding of nuclear proteins, the experiments were discontinued and were not performed on normal bone marrow cells.

Figure ii

Filter binding assays

Recombinant plasmid covering the human c-myc gene (see the open line drawing) were cut with restriction enzymes (Hind III, Xba 1, Eco RI) and the resulting fragments were end-labeled (see Materials and Methods). The labeled DNA was incubated with an extract from K562 cell nuclei (NE) for 30 minutes at 25°C and passed through nitrocellulose filters. Protein-bound DNA, retained on the filters, was extracted and analyzed on agarose gels (Lanes 2 and 4). The pattern of input DNA was assayed by ommitting the nitrocellulose filtration step (Lane 1). Background of labeled fragments on nitrocellulose filters was analyzed by substituting an equal amount of BSA for nuclear proteins (Lane 3). Lane 1, +200 ng NE, 5ng DNA, -filtration; Lane 2, +200ng NE, 20ng DNA, +filtration; Lane 3, +200ng BSA, 20ng DNA, +filtration; Lane 4, +400ng NE, 20ng DNA, +filtration. The stippled bars represent the fragments retained on the filter.


Experiment 1
Experiment 2
1 2 3 4 1 2 3 4
4.5
4.3
3.5
H
X
E
H
c-myc
pBR322
3.5
4.5
4.3
1kb

APPENDIX 3

APPENDIX 3

EXPERIMENTAL PROCEDURES

Bone marrow enrichment procedure

Bone marrow obtained from informed consenting normal volunteers was serially passed through 20, 23, and 25 gauge needles. The marrow cells were diluted 1:1 with RPMI containing 10% fetal calf serum. Ten ml. of marrow was layered over 3 mls. of Ficoll-Hypaque. The tubes were spun at 2200 rpm at 25° C for 25 minutes in a Beckman J-6B centrifuge (Boyum, 1976). The low density layer was carefully aspirated and washed twice in RPMI (Fig. i).

Low density mononuclear cells were depleted of T-lymphocytes by incubating cells (10×10^6/ml) with equal volume of 2% washed sheep red blood cells (SRBC) (Prepared media laboratories, Tualatin, OR) at 37° C in a fully humidified incubator for 15 minutes and spun for 5 minutes at 800g in a desk top centrifuge. The tubes were refrigerated for 30 minutes and the cells were resuspended and layered over 3 mls. of Ficoll-Hypaque and centrifuged at 2200 rpm for 25 minutes at 25° C in a Beckman J-6B centrifuge. The low density layer was carefully recovered and washed twice in RPMI (Bagby et al., 1981, 1983; Boyum, 1976). If T-lymphocytes are needed, the cellular pellet after density centrifugation was incubated with hypotonic solution (9 mls. of 0.83% Ammonium Chloride and 1 ml. of

0.05M Tris. buffer [pH 7.5]) at 37° C for 10 minutes and the T-lymphocytes were recovered by centrifugation. The cells were washed once with RPMI.

T-lymphocyte depleted low density marrow cells were subjected to a double adherence step. First, the cells were passed serially through three nylon fiber columns (Julius et al., 1973). The Pasteur pipets were packed compactly with nylon wool fibers and autoclaved. These columns were first wet by passing RPMI and the cell suspension (10 x 10^6/ml) was passed through the columns. The columns were washed with RPMI and the non-adherent cells in the eluted fraction was recovered by centrifugation. This non-adherent fraction of cells was subjected to second adherent step by incubating cells in serum coated plastic dishes (Kumagai et al., 1979). The flasks (T-75 or T-50) were incubated over night with heat-inactivated fetal calf serum at 4° C. The serum was removed just before use and the cell suspension (10 x 10^6/ml) was added to the flasks and incubated at 37° C for 60 minutes in a fully humidified incubator. After the incubation, the cell suspension was removed and the flasks were washed twice with fresh RPMI. The cells were recovered by centrifugation and resuspended in a known volume of RPMI containing 15% heat-inactivated fetal calf serum.

b. Cell culture

The cells were cultured at a density of 5 x 10^5/ml in RPMI containing 15% heat-inactivated fetal calf serum with or without human placental conditioned medium prepared according to Schlunk and Schleyer (Schlunk and Schleyer, 1980) at final concentration of 10%. The cells were incubated in a fully humidified incubator at 37° C. The cells were harvested at various time intervals, counted in a coulter counter.

c. Proliferation assay

The proliferative response to HPCM of LDBM cells was assayed by exposure of sequentially harvested cells to 5 uCi of ^{3}H-thymidine for 30 minutes. The cells were harvested onto to the filters using an automatic cell harvester and air dried. The incorporation of radioactivity was determined by liquid scintillation counting.

d. FACS analysis

1 x 10^6 cells in 200 ul of RPMI (GIBCO) were incubated with 5 ul of OKM1 or OKT3 monoclonal antibodies separately for 30 min. on ice. At the end of the incubation period, cells were washed twice and resuspended in 200 ul of RPMI. The cells were then incubated for 30 min. on ice with 50 ul of 1:30 dilution of fluorescein conjugated goat anti mouse immunoglobulin (Cooper Bio

medicals, Malvern, PA). The cells were washed twice and resuspended in RPMI containing 10% heat inactivated fetal calf serum and 2% formaldehyde. The cells incubated without the 1st antibody were used as controls. Fluorescence sensitivity was assessed using an Ortho 50H cytofluorometric sorting device (Ortho Diagnostics Inc.).

e. Histochemical staining

The cells were stained for naphthol as-D chloroacetate esterase and α-naphthyl acetate esterase enzymes using the procedure of Yam et al., (1971; 1974) using SIGMA kits (SIGMA, MO).

f. RNA isolation

Total RNA was isolated from cells by the procedure described in Maniatis et al., (1982) utilizing either the hot saturated phenol modification or the NP-40 method.

Hot saturated phenol method: The cells were washed once with phosphate-buffered saline and the cells were dissolved in 4M guanidine thiocyanate solution prepared according to Chargwin et al., (1979). The cell suspension was passed through an 18 gauge hypodermic needle and was heated to 60° C to shear the DNA. An equal volume of hot phenol saturated with 0.1M Tris.Cl [pH8.0] and 1mM ß-mercaptoethanol was added and the suspension was passed through the needle. Then 0.5 volume of solution II (0.1M sodium acetate [pH 5.2], 10 mM Tris.Cl [pH7.4], and 1 mM

EDTA) was added to the cell suspension. An equal volume of a 24:1 solution of chloroform and isoamyl alcohol was added, mixed vigorously and maintained at 60° C for 10-15 minutes with periodic mixing. The solution was cooled on ice and centrifuged at 4° C for 10 minutes at 2000g. The aqueous phase was recovered and extracted once with phenol/chloroform at 60° C and then twice with chloroform at room temperature. The aqueous phase was recovered and 2.5 volumes of ethanol was added and stored at -20° C for more than two hours. The nucleic acids were recovered by centrifugation at 12,000g for 20 minutes at 4° C.

The pellet was dissolved in solution III (0.1M Tris.Cl [pH 7.4], 50mM NaCl, 10mM EDTA, and 0.2% sodium dodecyl sulfate) and proteinase K was added to a final concentration of 200 ug/ml. and incubated at 37° C for 1-2 hours. The solution was heated to 60° C and 0.5 volume of saturated phenol preheated to 60° C was added. After adding 0.5 volume of chloroform, the solution was mixed vigorously and held at 60° C for 10 minutes. The solution was cooled on ice and centrifuged at 2000g for 10 minutes at 4° C. The aqueous phase was reextracted once in phenol/chloroform (1:1) to 60° C and twice with chloroform. The nucleic acids were precipitated with 3 volumes of ethanol at -20° C and recovered by centrifugation at 12,000g for 20 minutes at 4° C. The

pellet was washed once with 70% ethanol and resuspended in 0.5 ml. of diethyl pyrocarbonate treated water (DEP/water).

NP-40 method: Total RNA was isolated from cells by the modified procedure of Favalaro et al., (1980). Briefly, the cells were chilled on ice and washed once with ice-cold PBS. The cells were then lysed in ice-cold lysis buffer containing 0.14M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris.Cl (pH 8.6), 0.5% NP-40 and 10 mM vanadyl-ribonucleoside complexes. The cell suspension was overlayed on an equal volume of sucrose solution (24% w/v) containing 1% NP-40. After incubating on ice for 5 minutes, the tubes were centrifuged at 10,000g for 20 minutes at 4° C in a swing-out rotor. The upper turbid layer was recovered and diluted with an equal volume of 2x PK buffer (0.2M Tris.Cl [pH 7.5], 25mM EDTA, 0.3M NaCl, 2% w/v SDS) and Proteinase K was added to a final concentration of 200 ug/ml. The RNA solution was incubated at 37° C for 30 minutes, extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated by 3 volumes of ethanol at -70° C for at least 3 hours. The precipitate was recovered by centrifugation and the pellet was washed once in 75% ethanol containing 0.1M sodium acetate (pH 5.2).

g. Oligo d(T)-Chromatography

Poly (A)-containing RNA was selected by oligo dT-cellulose chromatography (Aviv and Leder, 1972). RNA

solution was mixed with an equal volume of 2X salt solution (40mM Tris.Cl [pH 7.5], 1M NaCl, 2mM EDTA) and 0.1g (dry weight) of oligo(dT)-cellulose was added and mixed well for 10 minutes at room temperature. The tubes were centrifuged in microcentrifuge for 4 minutes. The oligo(dT)-cellulose was washed once with 1X salt and the poly(A)$^+$ RNA was eluted with four 0.2 ml. washes with DEP/water. The concentration of poly(A)$^+$ RNA was determined by optical density (OD) at 260 nm where 1 OD_{260} was taken as 40 ug of RNA.

h. Northern Blot

Glyoxal method: poly(A)$^+$ RNA was first brought to 10% salt with 1X salt (1X salt = 100%) and precipitated with ethanol using tRNA as carrier. The precipitated poly(A)$^+$ RNA was adjusted to 0.5M glyoxal (deionized), 50% DMSO, 15mM sodium phosphate buffer (pH 6.5), heated to 50° C for 1 hour and subjected to electrophoresis in 1% agarose, phosphate buffer (pH 6.5) slab gels. The running buffer was 15mM phosphate buffer (pH6.5); electrophoresis was at 1.6V/cm for 9 hours with constant buffer recirculation. The RNA was transferred to Genescreen membrane (New England Nuclear) by the capillary blot procedure using 25mM phosphate buffer (pH 6.5). The residual agarose was removed by washing the membrane in 25mM phosphate buffer (pH 6.5) for 30 minutes at room temperature with constant

shaking.

Formamide method: Total cellular RNA (15 ug) was adjusted to 6% formaldehyde, 50% formamide (deionized), 1x MOPS buffer (10X MOPS: 0.2M morpholinopropanesulfonic acid sodium salt, 0.05M sodium acetate, 0.01M EDTA adjusted to pH 7.0 with acetic acid), heated to 50° C for 15 minutes and subjected to electrophoresis in 1.2% agarose containing 6% formaldehyde, and 1x MOPS buffer. The running buffer was 1X MOPS; electrophoresis was at 3.0V/cm for 4 hours. The RNA was transferred to Genescreen membrane (New England Nuclear) by the capillary blot procedure using 25mM phosphate buffer (pH 6.5). After the transfer, the membranes were washed in 25mM phosphate buffer (pH 6.5) with shaking for 30 minutes at room temperature and baked at 80° C for 3-4 hours.

i. Dot Blot

For RNA dot blotting, poly (A)-containing RNA or total cellular RNA was diluted to desired concentrations in DEP/water, boiled, quick-cooled on ice and applied with gentle suction to a 4mm diameter spot on Genescreen membrane which previously had been equilibrated with 2X SSC (1.0X SSC is 0.15M Sodium chloride, 0.015M sodium citrate) supported on a #470 paper employing a 96-hole minifold apparatus.

For DNA dot blotting, the DNA was isolated from

HPCM-stimulated LDBM cells and HL-60 cells according to the method described by Meinkoth and Wahl, (1984). The DNA was then dotted onto Genescreen plus membrane (New England Nuclear) as described above.

j. Hybridization, washing and autoradiography

After baking the membranes for 4 hours at 80° C, the blots were prehybridized over night at 42-45° C in a buffer containing 50% Formamide (deionized), 0.2% polyvinyl pyrrolidone (M.W. 40,000), 0.2% Bovine serum albumin, 0.2% Ficoll (M.W. 400,000), 0.05M Tris-HCl (pH 7.5), 1.0M NaCl, 0.1% Sodium pyrophosphate, 1.0% SDS, 10% Dextran Sulphate (M.W. 500,000) and denatured salmon sperm DNA (150 ug/ml). Subsequently, the blots were hybridized for 24 hours at 42-45° C with 7.5×10^5 DPM of nick translated probe per ml of hybridization buffer (same composition as prehybridization buffer). After hybridization, the blots were washed twice with 2X SSC at room temp. (5 mins. each wash), twice with 2X SSC and 1.0% SDS at 65° C (30 mins. each wash), and finally two times with 0.1X SSC at room temp. (30 mins. each). The membrane was dried at 22° C and exposed to X-ray films (X-omat AR5) with an intensifying screen at -70° C for 96 hours. The dot blot autoradiogram shown was evaluated by quick scan densitometer (Helena Laboratories, TX).

k. Preparation of the cloned probes

a. Gene probes and sources

The recombinant plasmids pRyc7.4 (kindly provided by Dr. G. Rovera), pHF$_\gamma$A-3'UT (kindly provided by Dr. L. Kedes), and pHB-1S (kindly provided by Dr. T. Maniatis) were used as c-myc, γ-actin and ß-globin hybridization probes respectively. The presence of the appropriate fragments by restriction enzyme analysis.

b. Bacterial cell culture

The culturing of bacterial cells were carried out by first inoculating cloned, single colonies of bacteria into a 10 ml. aliquot of Luria-Broth medium (per liter: Bacto-Tryptone 10 gms; Bacto-yeast extract 5 gms; Sodium Chloride 10 gms; adjust the pH to 7.5 with sodium hydroxide and autoclave) containing appropriate antibiotic. The cells were allowed to grow overnight in a gyratory water bath shaker at 37°C. This overnight culture was then used to inoculate 1000 ml. of minimal media 9 (MM9)(add 1 gm NH_4Cl, 6 gm Na_2HPO_4, 3 gm KH_2PO_4, 0.5 gm NaCl to 964 ml. water, autoclave and add filter sterilized 20 ml. of 20% casamino acids, 2 ml of 1M $MgSO_4$, 0.1 ml. of 1M $CaCl_2$, 4 ml. of 1% thiamine hydrochloride, and 10 ml. of 20% glucose). After 30

minutes, uridine (1 mg/ml) was added to the culture and incubation was continued until the culture reached an optical density of 0.7-0.8 at 550 nm. Chloramphenicol (final concentration 170 ug/ml) was added to amplify the number of plasmid copies/cell and the culture was further incubated overnight.

c. Plasmid isolation

The bacterial culture was transferred to 250 ml. polypropylene bottles and cooled on ice for 5 minutes. The bottles were then centrifuged at 3000 rpm for 15 minutes in Sorvall GSA rotor at 4° C. The pellet was resuspended in 10 ml. wash buffer (2.5 ml. 1M Tris.Cl [pH 8.0] (25 mM), 2 ml. 0.5M EDTA (10 mM) in 100 mls. water), transferred to four 40 ml. polyallomer tubes and centrifuged at 2000 rpm for 10 minutes at 4° C in a SS-34 rotor. Each pellet was resuspended in 10 ml. resuspension buffer (5 ml. 1M Tris.Cl [pH 8.0] (50 mM), 2 ml. 0.5 M EDTA (10 mM) in 100 ml. water) and incubated on ice for 5 minutes with 25 ul of diethyl pyrocarbonate. Cell suspension was further incubated in ice for 20 minutes with lysozyme (final concentration 1 mg/ml). An equal volume of lysis buffer (2 gm deoxy cholic acid, 5 gm Brig 58, 58.44 gm NaCl to 500 mls. water) was added, mixed gently and held at room temp. for 30 minutes. The

cell debris and chromosomal DNA was pelletted by centrifugation at 20,000 rpm for 60-90 minutes at 0° C in a Sorvall SS-34 rotor. The supernatant was phenol/chloroform/isoamyl alcohol (25:24:1) extracted twice and the nucleic acids were precipitated with 3 volumes of absolute ethanol at -20° C for 2 hours.

The closed circular DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients. The nucleic acids were dissolved in 8 mls. of TE(10mM Tris.Cl [pH 8.0], and 1mM EDTA) and 8 gms. of cesium chloride (BRL, Maryland) was added. 0.8 mls. of ethidium bromide (10 mg/ml) was added so that the final density of solution is about 1.55 gm/ml and ethidium bromide concentration of 600 ug/ml. The solution was transferred to ultra clear Beckman tubes and sealed and centrifuged in a Beckman VTi65 vertical rotor at 45,000 rpm for 30-35 hours at 20° C. The lower band consisting of closed circular DNA was recovered using a #18 hypodermic needle and extracted with cesium chloride-water saturated isopropanol until the pink color disappears. The solution was then diluted with 3 volumes of water and the DNA was precipitated with 3 volumes of ethanol at 4° C. The DNA was recovered by centrifugation and dissolved in an appropriate volume

of TE. The concentration of DNA was determined by optical density (OD) at 260 nm where 1 OD_{260} was taken as 50 ug of DNA.

1. Radiolabeling of probes.

The radiolabeling of DNA was carried out as described by Maniatis et al., (1982). The reaction mixture contained the following:

DNA	1 ug
10X nick-translation buffer	5 ul
unlabeled dATP,dGTP, dTTP	33 nM
[α-^{32}p]dCTP (800 Ci/mmole)	100 uCi
DNAase I (0.1 ng/ul)	2 ul
DNA polymerase (5 units/ul)	1 ul
sterile water to	50 ul

Mix the contents gently and spin in microcentrifuge for 15 seconds and incubate for 70 minutes at 16° C. The reaction was stopped by adding 2 ul of 0.5M EDTA and 1 ul of sonicated salmon sperm DNA was then added. The polynucleotides were separated from mononucleotides by spermine tetrahydrochloride precipitation. The reaction mixture was brought to 10mM spermine tetrahydrochloride and incubated on ice for 15 minutes and spun in a microcentrifuge for 15 minutes at 4° C. The pellet was washed for 60-90 minutes with spermine extraction buffer (70% ethanol, 300mM sodium acetate [pH 4.0], 10mM magnesium

acetate) on ice and recentrifuged for 15 minutes at 4° C. The pellet was dissolved in 10 ul of 0.5M sodium hydroxide and held at room temperature for 5 minutes and 80 ul of TE was added on ice. The sodium hydroxide was neutralized by adding 10 ul of 1.0M sodium acetate [pH 4.0] and stored at -20° C until use.

Figure iii

Bone marrow enrichment procedure.

A flow diagram describing the sequence of methods used to enrich for hematopoietic precursor cells.

Granulopoietic Progenitor Cell Enrichment Procedure

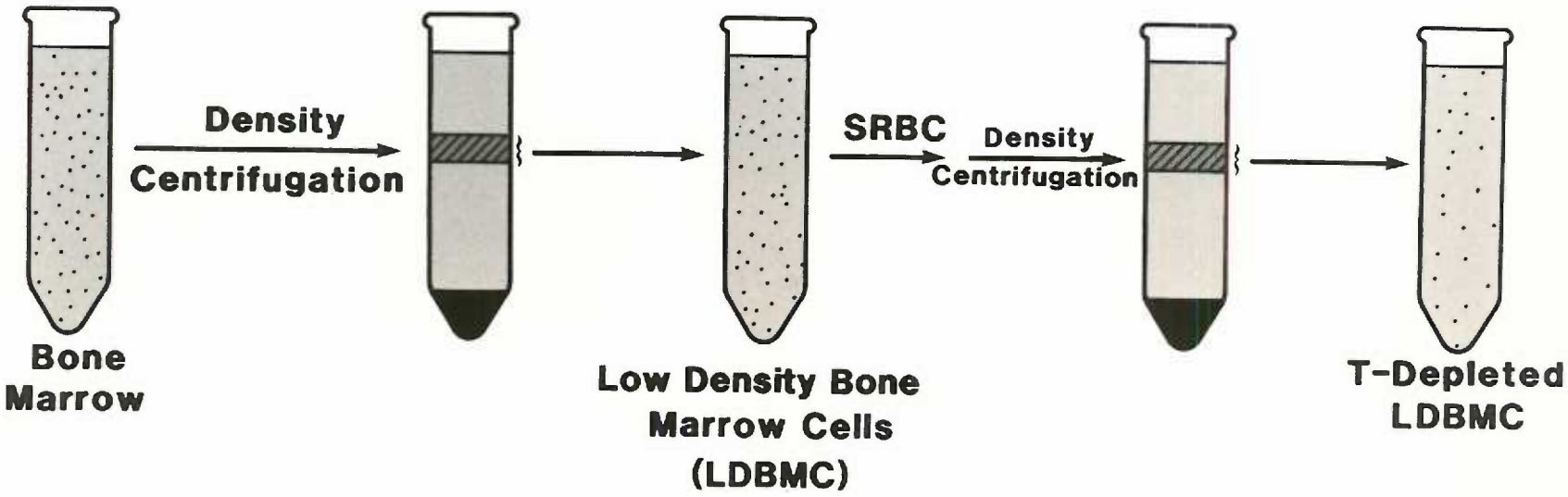
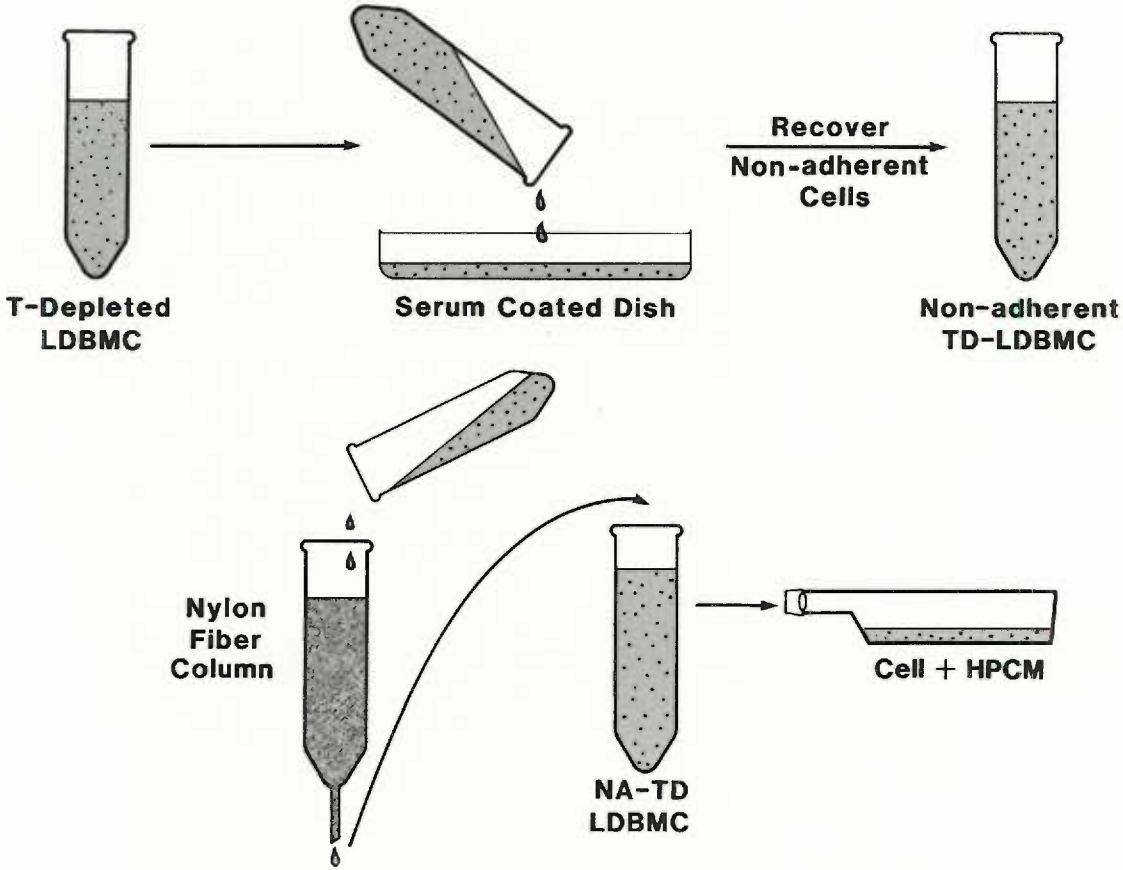


Figure iii (continued)

Granulopoietic Progenitor Cell Enrichment Procedure (Continued)



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