A COMPARISON OF INDUCER MEDIATED DIFFERENTIATION OF HL60 CELLS AND A TPA RESISTANT DERIVATIVE OF THE HL60 CELL LINE

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A COMPARISON OF INDUCER MEDIATED DIFFERENTIATION OF HL60 CELLS AND A TPA RESISTANT DERIVATIVE OF THE HL60 CELL LINE

by

Tina M. Calderon

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

A COMPARISON OF INDUCER MEDIATED DIFFERENTIATION
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OF THE HL60 CELL LINE

by

Tina M. Calderon

Adviser: Professor Judith K. Christman

The HL60 cell line can be induced to differentiate along the myelocytic and monocytic developmental pathways and thus provides a model invitro system for the study of cellular differentiation. The HL60 T cell line, a derivative of the HL60 cell line, was isolated for its resistance to TPA and the effects of various inducing agents on both cell lines was studied in order to try to identify the biochemical events critical to the process of cellular differentiation.

The response of HL60 and HL60 T cells to inducing agents was found to be markedly different. HL60 T cells did not differentiate when exposed to a variety of known inducers. However, 13-Cis-retinoic acid promoted the granulocytic maturation of these cells which revealed that the HL60 T cell line maintained the capacity to

terminally differentiate.

The correlation between the ability of inducing agents to promote cellular differentiation and their effects on biochemical events that have been postulated to play an important role in the regulation of cellular differentiation were then examined. Experiments utilizing a variety of weak acids and bases failed to support the theory that transient cytoplasmic ion fluxes are a critical regulator of HL60 cell differentiation. The use of monoclonal antibodies that recognized lineage specific cell surface antigens confirmed the induction of granulocytic differentiation of HL60 cells by specific inducing agents and also identified a protein whose appearance was indicative of the responsiveness of HL60 cells to TPA.

Inducing agents were also shown to promote a decrease in S-Adenosylmethionine levels in both cell lines without affecting the pattern of 5-methylcytosine residues in DNA sequences coding for specific cellular oncogenes. Subsequent studies on the effects of inducing agents on the level of poly A+ RNA revealed the one major difference between the two cell lines. The HL60 T cell line maintains a much higher level of c-myb poly A+ RNA, implicating a major role for the expression of this cellular oncogene in the control of cellular differentiation.

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LIST OF ABBREVIATIONS

ammonium sulfate	$AmSO_4$
cycloheximide	ch
deoxyribonuclease	DNAase
1.25 dihydroxyvitamin D_3	$1,25(OH)_2D_3$
5,5 dimethyloxazolidine-2,4-dione	DMO
dimethylsulfoxide	DMSO
dithiothreitol	DTT
ethylenediamine tetraacetic acid	EDTA
fetal bovine serum	FBS
guanidine hydrochloride	GnHCL
guanidinium thiocyanate	GuSCN
hexamethylenebisacetamide	HMBA
hexose monophosphate shunt activity	HMSA
high pressure liquid chromatography	HPLC
murine erythroleukemia cells	MELC
nitroblue tetrazolium	NBT
2[N-Morpholino]ethane sulfonic acid	MES
2[N-Morpholino]propane sulfonic acid	MOPS
12-O-tetradecanoyl-phorbol-13-acetate	TPA
phenylmethanesulfonylfluoride	PMSF
phosphate buffered saline	PBS
picomoles	pmol
ribonuclease	RNAase
s-adenosylmethionine	SAM
sodium dodecyl sulfate	SDS

INTRODUCTION

Human neoplasms result from a breakdown in the regulation of cellular proliferation. Eukaryotic cells have evolved a complex system to maintain controlled responses to their environment and carry out the instructions of their genetic program. Various components of this system are under intensive study in order to understand how normal growth and differentiation is regulated and the mechanisms by which this regulation is subverted resulting in tumorigenesis.

The HL60 cell line was derived from a patient with acute promyelocytic leukemia and provides a good model system for the study of cellular differentiation. These cells exhibit a transformed phenotype, as evidenced by their continuous proliferation in suspension and their tumorigenicity in nude mice (Collins, et al., 1977). HL60 cells can mature morphologically and functionally along the granulocytic and monocytic developmental pathways after treatment with a variety of inducing agents (Rovera, et al., 1979, Collins, et al., 1978). HL60 T cells, a differentiation defective derivative of HL60 cells, fail to respond to most of these agents (Mendelsohn, et al., 1983). Together these cell lines provide an invitro model system for the study of the hierarchy of factors which regulate cellular differentiation.

The functional state of eukaryotic cells depends on a constant interplay between plasma membrane, cytoplasm and nucleus and inducers of differentiation alter biochemical pathways at all three

levels with subsequent changes in the differentiated state of cells. Thus, this study was designed to compare a number of events which have been proposed to be critical to differentiation in cells that undergo terminal differentiation (HL60) and cells that fail to provide this ultimate response (HL60 T).

BACKGROUND

HL60 cells are a human leukemic cell line derived from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia (Collins, et al., 1977). They have retained a typical promyelocytic character even after prolonged passage invitro. Promyelocytes are the precursors of the mature granulocytes which function as an important part of the immune system in the defense against foreign invaders.

The granulocytic series encompasses three morphologically distinct cell types: the neutrophil, the eosinophil, and the basophil. Immature promyelocytes contain large, round nuclei, each containing two to four nucleoli and dispersed nuclear chromatin. The cytoplasm is basophilic and the nuclear to cytoplasmic ratio is relatively high. Differentiation along the myelocytic pathway entails marked morphological changes including a decrease in cell size and nuclear to cytoplasmic ratio culminating in cells containing very elongated and segmented nuclei.

Mature granulocytes also exhibit many characteristic functional traits. These include the presence of Fc and complement receptors in the outer membrane and the ability to phagocytize. The Fc receptor will bind to an immunoglobulin molecule when it is complexed with an antigen. In this way antibody coated microorganisms are attached to the cell surface of the granulocyte so that the phagocytic process can begin. Complement receptors are also present on the cell surface. Complement consists of at least twenty

serum proteins which interact with each other, with antibody, and with cell membranes in a complex system that leads to various biological activities including lysis of bacteria and induction of phagocytosis (Cline, 1975).

The phagocytic process involves the activation of a series of oxidative metabolic reactions known as respiratory burst activity. These include activation of the hexose monophosphate shunt pathway and rapid generation of superoxide anion and hydrogen peroxide. The interaction of these reactive chemical species and the granulocytic enzymes lysozyme, peroxidases, and acid hydrolases, results in the killing of the ingested microorganism (Cline, 1975).

HL60 cells can be induced to differentiate to morphologically mature granulocytes by incubation with dimethylsulfoxide (DMSO) (Collins, et al., 1978), a small polar compound which was a known inducer of Friend erythroleukemia cells (Friend, et al., 1971). The treated HL60 cells also possessed many of the functional characteristics of mature granulocytes. Complement receptors increased two fold upon induction of HL60 cells with DMSO while the number of Fc receptors increased more than twenty fold (Fleit, et al., 1984). Phagocytosis of the yeast Candida Albicans increased more than ten fold with DMSO treatment (Collins, et al., 1978).

Mature granulocytes and HL60 cells treated with DMSO immediately exhibit a number of biochemical reactions normally associated with the phagocytic process upon exposure to the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA). These reactions include the production of superoxide anion and oxidation of

glucose via the hexose monophosphate shunt pathway (Estensen, et al., 1974, Repine, et al., 1974). The ability of granulocytic cells to respond to TPA with increased oxidative metabolism depends on the extent to which they have matured and this responsiveness is associated with the presence of a membrane bound reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. TPA activates this enzyme resulting in an increased production of NADP+ and superoxide anion (Mendelsohn, et al., 1980). The increase in NADP+ in turn activates the hexose monophosphate shunt with subsequent replenishment of NADPH pools (Babior, 1978). The measurement of these TPA induced biochemical reactions is a useful quantitative indicator of the differentiated state of HL60 cells. Hexose monophosphate shunt activity (HMSA) is measured by the release of labeled carbon dioxide from [1-^{14}C] glucose when HL60 cells are incubated with TPA (Zabos, et al., 1978). HL60 cells incubated with DMSO for five days exhibit an approximately four fold higher HMSA in response to TPA than do non-induced cells (Mendelsohn, et al., 1980). The number of cells producing superoxide anion is measured by the reduction of nitroblue tetrazolium (NBT). NBT is a yellow, water soluble dye that is converted to insoluble, intracellular blue particles upon reduction by superoxide anion (Segal, et al., 1974). The percentage of cells reducing NBT dye in the presence of TPA is five to fifteen fold greater for HL60 cells exposed to DMSO for five days than for untreated cells (Collins, et al., 1979, Mendelsohn, et al., 1980).

In addition to its ability to rapidly activate (1-5 minutes)

the membrane bound NADPH oxidase of mature granulocytes, TPA also induces monocytic differentiation of immature HL60 cells. Incubation with TPA for twenty four hours causes HL60 cells to adhere to the surface of tissue culture vessels, assume the appearance of macrophages, produce monocyte specific enzymes, and acquire macrophage-associated surface markers (Rovera, et al., 1979). Thus, HL60 cells are bi-potent with the ability to mature along the myelocytic and monocytic developmental pathways. They provide a model system for studying the process that generates several classes of hematopoietic cells from a common precursor cell, as well as the process that mediates the maturation of these cells to their terminally differentiated state.

A variant of the HL60 cell line was isolated in our laboratory from an HL60 culture that had been exposed to 400pm TPA for seven days. The inducing effects of TPA are concentration dependent and treatment with this very low concentration of TPA did not generate any observable changes. However, subsequent karyotypic analysis revealed that greater than 90% of the diploid HL60 cells had been converted to a tetraploid state. These cells were designated HL60 T and have been maintained in culture for over four years (Mendelsohn, et al., 1983).

Many variants of the HL60 cell line have been isolated in other laboratories. Cell lines resistant to a single inducer like DMSO have been produced by continuous exposure of HL60 cells to the appropriate inducer over prolonged periods of time. These cell lines gradually lose their resistance if they are cultured

without inducing agent (Grosso and Pitot, 1984, Perella, et al., 1986). Estensen et al. isolated TPA resistant cell lines by exposing HL60 cells to a frameshift mutagen with subsequent exposure to TPA. Over 90% of the cells in the most resistant cell line were found to be tetraploid and their resistance to TPA was a stable characteristic. (Mascioli and Estensen, 1984).

The response of HL60 and HL60 T cells to inducing agents is markedly different. HL60 T cells are refractive to inducers of both monocytoid (TPA) and myeloid (DMSO) differentiation and these properties have been stable with continuous culture. However, they can be induced along the granulocytic pathway by the vitamin A analog, 13-Cis-retinoic acid and the monocytoid pathway by 13-Cis-retinoic acid and TPA. The genetic information essential for differentiation is present and can be activated leading to the expression of the normal phenotype. In addition, HL60 T cells secrete a factor into their culture medium that inhibits the induction of HL60 cells with DMSO. This inhibitory factor is not able to block the inducing effects of 13-Cis-retinoic acid (Mendelsohn, et al., 1983).

HL60 and HL60 T cells possess characteristics very well suited to studies on the mechanisms by which cells proliferate and differentiate. Gene activity in these cells has been altered, resulting in neoplastic transformation. The inducing effects of various agents reverse or overcome these changes leading to the expression of the normal, mature phenotype. The number of agents that can trigger this maturation process is fairly extensive and is increas-

ing daily. The properties of these inducing agents are extremely varied and it is not known how they activate signals involved in the control of cellular differentiation. A vast array of biochemical pathways must control this essential function and researchers have focused on discrete regulatory sites in order to gain a better understanding of this process.

Recent evidence has indicated that changes in the intracellular levels of certain ions may play a role in signalling the onset of cellular differentiation. The growth and differentiation of eukaryotic cells seems to be affected by changes in the flux of protons and/or cations across the plasma membrane leading to altered cytoplasmic pH, Na+, K+, or Ca++ levels (Harold, 1977, Levinson, et al., 1983). Ca++ ionophores stimulate development of unfertilized echinoderm and fish eggs and stimulates terminal differentiation of several mammalian cell lines (Steinhardt and Epel, 1974, Ridgeway, et al., 1977, Winkler, et al., 1980, Hennings, et al., 1980, Roufa, et al., 1981). Changes in Na+ and K+ fluxes have been implicated as early signals in serum stimulation of cell growth (Smith and Rozengurt, 1978). Very early changes in transmembrane potassium fluxes occur in HL60 cells following DMSO treatment and the fact that these changes occur so rapidly after exposure to inducer indicates that they may trigger cellular differentiation rather than being a consequence of this process (Gargus, et al., 1984). An increase in intracellular calcium was also found to be essential for the terminal differentiation of Friend erythroleukemia cells (Levenson, et al., 1980).

Alterations in internal concentration of calcium and other ions is closely connected with biochemical events occurring at the cell membrane. Many biologically active substances interact with their specific cell surface receptor and elicit a wide variety of intracellular responses. The tumor promoter, TPA, induces monocytic differentiation of HL60 cells and was shown to directly activate protein kinase C (Castagna, et al., 1982, Kikkawa, et al., 1983). This enzyme is normally activated by an increase in its affinity for calcium after interaction with diacylglycerol. Diacylglycerol is produced by the turnover of inositol phospholipids in the membrane following receptor mediated signalling by biologically active compounds (Takai, et al., 1979, Kishimoto, et al., 1980, Nishizuka, 1980). This subsequently leads to an activation of protein kinase C which is associated with a wide range of biological events including changes in ionic transport, the release of bioactive substances, and the phosphorylation of various proteins within the cell. The activation of protein kinase C is also closely associated with an increase in intracellular calcium and both events act synergistically in influencing cellular function (Nishizuka, 1984). The importance of changes in the intracellular concentration of specific ions like calcium are likely to play an important role in cellular proliferation and differentiation as membrane events are manifested within a cell.

HL60 T cells are refractive to the inducing effects of TPA and provide an interesting contrast to HL60 cells in assessing the role of ion fluxes in cellular differentiation. In order to deter-

mine whether alternative methods of inducing ion fluxes would mimic the events that have been postulated to initiate cellular differentiation, we tested 5,5 dimethyloxazolidine-2,4-dione (DMO) as an inducer of HL60 and HL60 T cells. DMO is a nonmetabolizable, weak acid used in the measurement of differences between intracellular and extracellular pH. In the unionized form, DMO can freely cross cell membranes and once inside of a cell can dissociate to an extent dependent on the intracellular pH. The ionized form of DMO is then trapped inside of the cell and quantification of the amount of DMO that has dissociated is a measure of internal pH (Gillies and Deamer, 1979). However, it should also act to cause a transient decrease in intracellular pH and the effect of this change on the differentiated state of HL60 and HL60 T cells was studied as well as the effects of other weak acids and bases.

Any comprehensive study of the molecular basis of cellular differentiation has to also focus on events occurring in the nucleus. With minor exceptions, the genetic information in all of our cells is the same. The process of differentiation entails a selective activation and repression of myriad combinations of genes which gives rise to a pattern of gene activity that defines each kind of differentiated cell. What we need to know is how this complicated process is controlled.

One regulatory mechanism which may play an important role in cellular proliferation and differentiation is the expression of cellular oncogenes. These genes are the cellular progenitors of the genetic information responsible for transformation by acutely

transforming retroviruses and changes in the structure or expression of these genes has been implicated in the process of tumorigenesis.

Cellular oncogenes have been highly conserved throughout evolution and are expressed in various cells at specific times during the course of growth and development. The prevalence of these genes and their origins very early in evolution suggests that they play a fundamental role in the control of cellular proliferation and differentiation. During normal development they are not tumorigenic and generally require either expression at inappropriate times in development, activation to abnormal levels of expression, or mutations resulting in the production of an abnormal gene product in order to elicit neoplastic transformation (Bishop, 1983).

More than twenty viral oncogenes with known cellular homologues have been described and several of them are thought to play important roles in human cancers. Activated oncogenes have been detected in cancers of the bladder, ovary, breast, lung, and colon as well as in several types of leukemia. The normal role of cellular oncogenes in the regulation of cellular proliferation and differentiation is under intensive study. Many oncogenes are thought to code for products that are involved in the normal growth of cells such as peptide hormones, hormone receptors, DNA binding proteins, protein kinases, and G proteins or GTPases (Bishop, 1983).

The cellular homologue of the transforming gene of Simian

Sarcoma Virus (c-sis) and Avian Erythroblastosis Virus (c-erb B) code for a polypeptide chain of the platelet derived growth factor and a truncated form of the epidermal growth factor receptor respectively (Doolittle, et al., 1983, Downward, et al., 1984). The viral oncogene of the McDonough strain of Feline Sarcoma Virus (v-fms) codes for a transmembrane glycoprotein closely related to the receptor for the macrophage growth factor, CSF 1, which is necessary for the growth and differentiation of mononuclear phagocytes in mice (Sherr, et al., 1985). The viral oncogenes of the Harvey and Kirsten murine sarcoma viruses (v-Ha-ras and v-Ki-ras) code for proteins that have structural and functional similarities to a class of proteins (known as G or N proteins) that control the activity of adenylate cyclase in vertebrate cells (Uno, et al., 1985, Defeo, et al., 1983, Powers, et al., 1984). Mutations in the ras oncogene have been associated with a high number of human cancers (Bishop, 1983). The gene product of c-myc and c-myb, the cellular homologues of the transforming genes of the Avian Myelocytomatosis Virus, MC29, and the Avian Myeloblastosis Virus respectively, are nuclear proteins which may regulate cellular proliferation (Favera, et al., 1982, Watt, et al., 1985, Eisenman, et al., 1985, Molling, et al., 1985, Klempnauer, et al., 1986). The gene product of the cellular homologue of the FBJ Murine Osteosarcoma Virus (c-fos) has been implicated in the onset of monocytic differentiation of human hematopoietic cells, in particular HL60 cells (Muller, et al., 1984, Sariban, et al., 1985).

HL60 cells have an elevated level of c-myc expression when

compared with other human hematopoeitic cell lines. This has been attributed to an amplified, structurally normal c-myc gene. Expression of the cellular oncogenes c-myb and N-ras can also be detected in this cell line (Westin, et al., 1982). Incubation of HL60 cells with various inducers of differentiation results in the modulation of oncogene expression in accordance with the differentiated state of the cells. The expression of c-myc is diminished when HL60 cells are treated with TPA, DMSO, and other inducing agents (Westin, et al., 1982). Conversely, TPA induced monocytic differentiation results in the rapid, transient induction of the c-fos and c-fms oncogenes (Muller, et al., 1984, Sariban, et al., 1985). These results suggest a correlation between oncogene expression and the neoplastic transformation of these cells.

A complete quantitative study of cellular oncogene expression in HL60 and HL60 T cells will determine whether cellular proliferation and differentiation can be correlated to levels of oncogene expression and whether alterations in the expression of these genes contribute to the tumorigenic state of these cells. The regulation of expression of specific DNA coding sequences like cellular oncogenes is not fully understood but enhancer elements, trans-acting protein factors, and the methylation of DNA sequences are just a few components of this complicated regulatory system that are under study today.

It has been shown that Friend erythroleukemia cells synthesize hypomethylated DNA when induced to terminally differentiate (Christman, et al., 1977) and numerous experiments have revealed

that the tissue specific expression of various genes can be directly correlated to the degree they are undermethylated (Razin and Riggs, 1980). Also, agents known to inhibit trans-methylation reactions, such as L-ethionine and 5-azacytidine, were found to act as inducers of granulocytic differentiation in HL60 cells (Mendelsohn, et al., 1980, Creusot, et al., 1982). The DNA of all higher eukaryotes examined thus far contains the minor base 5-methylcytosine and the level and pattern of methylation of mammalian DNA has been implicated as an important regulatory mechanism in gene expression (Holliday and Pugh, 1975, Mandel and Chambon, 1979, McGhee and Ginder, 1979, Shen and Maniatis, 1980).

DNA methylation occurs by an enzymatic transfer of methyl groups from S-Adenosylmethionine (SAM) to cytosine (Srinivasan and Borek, 1964). Ultimately anything that affects SAM levels has the potential to affect the efficiency of DNA methylation, therefore we examined the levels of SAM present in HL60 and HL60 T cells in addition to the level and pattern of 5-methylcytosine residues in DNA sequences coding for specific cellular oncogenes. In this way it could be determined whether changes in the level of expression of cellular oncogenes could be linked to changes in DNA methylation before and after treatment with different inducing agents.

In summary, the process of cellular proliferation and differentiation will certainly entail a very complicated system of interconnecting biochemical pathways which will be very difficult to fully understand. However, studies utilizing HL60 and other cell lines as invitro model systems can concentrate on various,

discrete aspects of this process and help provide the information necessary to help piece this puzzle together.

MATERIAL AND METHODS

Cell Cultures

HL60 and HL60 T cells are maintained in suspension culture in RPMI 1640 (Grand Island Biological Company) supplemented with 10% heat inactivated fetal bovine serum (FBS-Grand Island Biological Company) and grown at 37^0C in a moist 5% CO_2 atmosphere. Cells initially suspended at 2.5×10^5 cells/ml are subcultured every five days.

HL60 T conditioned medium is obtained during four to five days growth of HL60 T cells initially suspended at 2.5×10^5 cells/ml. The cells are then removed by centrifugation and the medium is sterilized by filtration through a 0.2 μm nitrocellulose filter.

Induction of granulocytic differentiation of HL60 cells is achieved by suspending cells at 2.5×10^5 cells/ml in sterile medium containing the appropriate inducing agent. The cells are grown in this medium for five days.

Induction of monocytic differentiation of HL60 cells is achieved by suspending cells at 4.5×10^5 cells/ml in sterile medium containing 17nm TPA for twenty four hours or 5×10^{-7}M 1,25 dihydroxyvitamin D_3 for two days.

Measurement of Granulocytic Differentiation

NBT Reduction Assay 2×10^6 cells are suspended in 0.5 ml RPMI 1640 + 10% FBS. The cells are incubated at 37^0C for 20 minutes with an equal volume of 0.2% NBT dissolved in phosphate buff-

ered saline (137mM NaCl, 2.7mM KCl, 8mM Na_2HPO_4, 1.5mM KH_2PO_4). TPA is also added to a concentration of 17nM. After twenty minutes the cells are checked under a microscope to determine the percentage of cells containing blue, intracellular material (Collins, et al., 1979).

Phagocytosis of Latex Beads Latex beads (1.0 μm, Dow Diagnostics) are washed three times in phosphate buffered saline (PBS). They are then suspended in RPMI 1640 + 10% FBS. 400 μl of latex bead suspension is added giving a 40:1 ratio of beads to cells. After a one hour incubation at 37^0C, the cells are washed with RPMI 1640 + 10% FBS and checked under a microscope for the percentage of cells that have ingested more than five beads (Mendelsohn, et al., 1980).

Measurement of Hexose Monophosphate Shunt Activity 5×10^5 cells/ml are washed in 0.9% NaCl and resuspended in glucose-free Earle's balanced salt solution (Grand Island Biological Company) buffered at pH 7.2 with 20mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, supplemented with 10% FBS and 0.2 μCi of [1-^{14}C] glucose (specific activity 5.7 mCi/mmole, Amersham) in the presence of 17nM TPA. The CO_2 released during a one hour incubation at 37^0C is trapped in a hyamine soaked filter suspended above the reaction mixture. The reaction is terminated by the addition of 100 μl of concentrated sulfuric acid and the amount of radioactivity trapped in the filter paper is counted (Zabos, et al., 1978).

Measurement of Monocytic Differentiation

Adherence HL60 cells, at 4 x 10^5 cells/ml, are incubated with 17nM TPA for twenty four hours. The medium is removed from the cell culture and after several washings with PBS the adherent cells are removed by treatment with trypsin/EDTA (Grand Island Biological Company) and counted.

Analysis of Cell Surface Antigens by Immunofluorescence

HL60 cells are washed twice with PBS. 5 x 10^5 cells in 100 μl of PBS is added to coverslips which have been soaked in poly L-lysine (20 μg/ml in H_2O, Sigma Chemical Company) for thirty minutes at room temperature. The cells are incubated at 37^0C for thirty minutes and then fixed overnight in 1% formaldehyde in PBS at 4^0C.

The coverslips are washed several times in PBS and then are soaked in PBS + 1% FBS for thirty minutes in order to block non-specific binding of antibody. Primary antibody is added (25 μl of a proper dilution in PBS + 1% FBS) and the cells are incubated on ice for thirty to sixty minutes. After thorough washing in PBS and PBS + 1% FBS, 50 μl of fluorescein conjugated secondary antibody (diluted 1:40 in PBS + 1% FBS) is added and the coverslips are incubated on ice for thirty minutes. They are then washed in PBS + 1% FBS and are mounted in glycerol and examined with a fluorescent microscope.

Isolation and Assay of the Inhibitory Factor Secreted by HL60 T Cells

100 ml of HL60 T conditioned medium is brought to various percentages of saturation with ammonium sulfate ($AmSO_4$). The solution is centrifuged and the precipitated material is dissolved in 10 ml PBS to give a 10 fold concentrated solution. The solution is then dialyzed overnight at 4^0C in PBS. One ml is added to nine ml of HL60 cells (2.5×10^5 cells/ml) in medium containing 1.25% DMSO after filter sterilization. The cells are checked five days later for the percentage of cells reducing NBT dye. Inhibitory activity results in an approximately five fold decrease in the percentage of cells reducing NBT dye.

HPLC Analysis of S-AdenosylMethionine Levels

Cells are initially suspended at 2.5×10^5 cells/ml in sterile medium containing the appropriate inducing agent. After various incubation periods, 10^6 cells are centrifuged and the cell pellet is dissolved in 100 μl of 5% perchloric acid. Cell samples were then frozen at -70^0C until they were shipped to the University of Louisville to be analyzed by high pressure liquid chromatography (HPLC) by Dr. Jerald Hoffman.

Western Blotting of Cell Surface Proteins

Cells with and without treatment of inducing agents are washed twice with PBS and then incubated at room temperature for thirty minutes in 7mM octyl-β-D-thioglucopyranoside (Calbiochem) in PBS,

1mM PMSF, 10mM iodoacetamide at 4 x 10^6 cells/ml. Octyl-β-D-thio-glucopyranoside is a non-ionic, non-denaturing detergent that is used for the solubilization and reconstitution of membrane proteins. The cells are then centrifuged and the supernatant is concentrated five to ten fold in Centricon 30 micro-concentrator units (Amicon Company).

Cell samples are run on 12% polyacrylamide-SDS gels with Tris-glycine buffer at 15mA for approximately four hours with continuous cooling. The proteins were then transferred electrophoretically onto nitrocellulose paper. The gel and nitrocellulose paper are soaked in transfer buffer (120mM glycine, 24.8mM Tris and 20% methanol) for a few minutes before the proteins are transferred at 25 volts for four to five hours.

Non-specific binding of antibody is eliminated by incubating the nitrocellulose twice in 5% Carnation Dry Milk in PBS and 0.05% sodium azide at room temperature for thirty minutes. The nitrocellulose paper is then incubated at 4^0C overnight with ^{125}I labeled primary antibody or unlabeled antibody diluted in 5% Carnation Dry Milk in PBS. Four washes with PBS at room temperature removes unbound antibody. If radioactive primary antibody was used, the nitrocellulose can be exposed directly to x-ray film. If not, the nitrocellulose must be incubated with an enzyme linked secondary antibody with subsequent incubation with a solution of substrates that will produce color development at sites of bound primary antibody.

Southern Blotting

Isolation of Cellular DNA 10^8 cells are washed twice with PBS and the cell pellet is dissolved in three to four volumes of RSB Nonidet (10mM NaCl, 10mM Tris, 3mM $MgCl_2$, and 0.5% Nonidet P-40). This suspension is homogenized until only cell nuclei remain. After centrifugation the supernatant is removed and the pellet is dissolved in five volumes of 150mM NaCl, 100mM EDTA, pH 8.0. SDS is added to 1% and 1/10 volume of Proteinase K (4 mg/ml in H_2O) is added. This mixture is incubated at 37^0C until the pellet is entirely dissolved. The solution is brought to 1M with sodium perchlorate and then extracted twice with chloroform-isoamyl alcohol (24:1). The supernatant is precipitated with 0.1 volume 2M sodium acetate pH 5.6 and 1 volume cold isopropanol and the DNA is spooled out of this solution. The DNA is washed with 70% ethanol and dissolved in 0.1 x TNE (50mM Tris, 100mM NaCl, 5mM EDTA). Ribonuclease (20 μg/ml) is added to the DNA solution and incubated at room temperature for one hour with subsequent addition of 100 μg/ml Proteinase K (4 mg/ml in H_2O) and incubation at 37^0C for two hours. The solution is then extracted with phenol-chloroform and the supernatant is precipitated with 0.1 volume 2M sodium acetate, pH 5.6 and 0.54 volume cold isopropanol and the DNA is spooled out and redissolved in 0.5M Tris, pH 7.5, 0.1mM EDTA.

Restriction and Electrophoresis of Cellular DNA

Five to ten units of restriction enzyme (Boehringer Mannheim) was incubated with each μg of cellular DNA in the appropriate

buffer overnight at 37^{0}C. The enzymes used were either Msp I, Hpa II, or Hha I and a typical reaction mixture contained 20 μg of DNA which was run on 1% agarose (Seakem ME) horizontal slab gels at 120 volts for 4 hours in 40mM Tris, pH 8.0, 5mM sodium acetate, 1mM EDTA. The gel is then stained with ethidium bromide (1 mg/ml) and the DNA visualized under UV light.

DNA Transfer

The agarose gel is soaked in 300ml of 0.25M HCl for five to fifteen minutes at room temperature. The gel is briefly washed with distilled water to remove any residual acid and then soaked in 300 ml of 0.4M NaOH for fifteen minutes. The DNA is transferred to Zeta Probe Membrane (Bio-Rad) in 0.4M NaOH for 20 hours by placing a weight on top of the Zeta Probe Membrane and gel with the buffer flowing through the gel and membrane by capillary action. The membrane is then washed with 300 ml 2 x SSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7.0) for 10 minutes and air dried.

Preparation of Oncogene Hybridization Probes

All of the probes used in my studies originated from plasmids obtained from transformed bacteria that were purchased from American Type Culture Collection except for c-fos, c-myc and B actin. The bacteria containing the c-fos plasmid was a kind gift of Dr. Tom Curran.

Plasmid Preparation

A 10 ml culture of transformed bacteria is grown overnight at 37^0C in L-broth containing the appropriate antibiotic. The bacteria are then added to one liter of sterile M9 medium (0.4% glucose, 0.1mM $CaCl_2$, 1mM $MgSO_4$, 0.4% casamino acids, and essential salts and amino acids). The culture is shaken at 37^0C for approximately three hours when 200 mg of chloroamphenicol is added with an additional 12 to 20 hour incubation time for amplification of the plasmid. The solution is centrifuged and the pellet frozen at -20^0C overnight. After thawing, the pellet is suspended in 15 ml cold SET buffer (20% sucrose, 50mM Tris, pH 7.6, 50mM EDTA) with the addition of 3 ml lysozyme (5 mg/ml in 10mM Tris, pH 7.6, 1mM EDTA, 10mM NaCl). Incubation on ice for fifteen to thirty minutes is followed by the addition of 15 ml triton lysis mix (0.3% triton X-100, 0.19M EDTA, 0.15M Tris, pH 8.0). After gentle mixing on ice for fifteen to thirty minutes, the solution is centrifuged and an equal volume of H_2O and 10 μl RNAase (10 mg/ml) is added. A thirty to sixty minute incubation at room temperature is followed by extraction with an equal volume of phenol and chloroform. The DNA is precipitated by adding NaCl to 0.2M, 2 volumes cold 95% ethanol and storage at -20^0C overnight. The solution is centrifuged and the DNA is dissolved in TEN buffer (10mM Tris, pH 8.0, 1mM EDTA, 10mM NaCl) and run on an A-50 column (Bio-Rad) to remove contaminating RNA. The DNA is reprecipitated and run on a Cesium Chloride gradient to isolate pure plasmid DNA.

v-myb Probe pVM2 plasmid is isolated from transformed bacteria. The DNA is digested with Kpn I and Xba I restriction enzymes and then extracted with phenol-chloroform and reprecipitated with 1/4 volume 5M ammonium acetate and 2 volumes 95% ethanol. The DNA is resuspended in TE buffer (10mM Tris, pH 8.0, 1mM EDTA) and run on a preparative 1% agarose gel at 120 volts for approximately four hours. The gel was stained with ethidium bromide (1 mg/ml) and visualized under long wave UV light. The band corresponding to the approximately 1.5 kb fragment containing the entire v-myb coding sequence was cut out of the gel and the DNA was isolated using an IBI electroeluter where DNA is run from agarose fragments into 7.5M ammonium acetate by electrophoresis. The DNA is precipitated by addition of 2 volumes 95% ethanol and storage at -20^{0}C overnight. The solution is spun and the DNA is dried and resuspended in TE buffer.

c-myc Probe pMC415PP plasmid is isolated from transformed bacteria. The DNA is digested with Pst I restriction enzyme and the 3 kb fragment corresponding to the first exon and part of the second exon of c-myc is isolated as described previously.

c-Ha-ras Probe pUCEJ6.6 plasmid is isolated from transformed bacteria and digested with Sac I restriction enzyme yielding a 3 kb fragment corresponding to the entire c-Ha-ras coding sequence that was isolated as described previously.

c-fos Probe pc-fos(human)-1 plasmid is isolated from transformed bacteria and digested with Eco RI and Bam HI restriction enzymes yielding an approximately 3.5 kb fragment that was isolated as described previously and corresponds to the first three and part of the fourth exon of c-fos.

β-Actin Probe pHFBA-1 is isolated from transformed bacteria and digested with Bam HI restriction enzyme yielding an approximately 2 kb fragment that was isolated as described previously.

Nick Translation

DNAase I (Sigma Chemical Company) is stored at 1 mg/ml in 50mM Tris, pH 7.5, 10mM $MgSO_4$, 1mM dithiothreitol (DTT), and 50% glycerol at -20^0C. An aliquot of 0.5 μl is diluted to 100 μl in 1X buffer (50mM Tris, pH 7.5, 10mM $MgSO_4$, 1mM DTT, and 50 μg/ml bovine serum albumin) at 0^0C. A subsequent 0.5 μl aliquot is then further diluted to 100 μl in the same buffer. The reaction mixture consists of 5 μl 10X buffer (0.5M Tris, pH 7.5, 0.1M $MgSO_4$, 10mM DTT, 500 μg/ml bovine serum albumin), 7.5 μl dNTP mix (0.07mM dATP, dGTP, dTTP in H_2O), 0.25 μg DNA, 100 to 150 μCi ^{32}P-CTP (Amersham, 2000 Ci/mmole), 0.5 μl diluted DNAase I, 1.5 μl DNA polymerase I (10,000 U/ml), and H_2O to bring volume to 50 μl. This solution is incubated for three hours at 14^0C. The reaction mixture is then run on a Sephadex G-50 (Bio-Rad) column to separate unincorporated labeled deoxynucleotide triphosphates from the radioactively labeled DNA which has approximately 10^8 cpm/μg DNA.

Pre-hybridization and Hybridization

Zeta Probe membrane is placed in a plastic pouch with 10 ml pre-hybridization solution containing 5 ml deionized formamide, 2 ml 20 x SSPE (3M NaCl, 0.2M $NaH_2PO_4.H_2O$, 25mM EDTA, pH 7.4), 1 ml Denhardt's solution (0.02% wt./vol. each of bovine serum albumin, polyvinyl pyrrolidone and Ficoll), 1 ml denatured salmon sperm DNA (5 mg/ml), 0.5 ml 20% SDS, and 0.5 ml H_2O. This is incubated at 42^0C overnight after which the pre-hybridization solution is removed.

Hybridization solution is then added which consists of 5 ml deionized formamide, 2 ml 20 x SSPE, 0.2 ml Denhardt's solution, 1.0 ml denatured salmon sperm DNA (5 mg/ml), 2 ml 50% sodium dextran 500, 0.5 ml 20% SDS, and the denatured, radioactively labelled probe. This is incubated at 42^0C for 16 to 18 hours.

The Zeta Probe membrane is washed once with 250 ml 2 x SSC, 0.1% SDS for fifteen minutes at room temperature. The next wash is for fifteen minutes in 0.1 x SSC, 0.1% SDS at room temperature with a final wash at 50^0C in 0.1 x SSC, 1% SDS for thirty minutes. The Zeta Probe membrane was air dried and exposed to x-ray film (Kodak XR-5) at -70^0C using Kodak intensifying screens.

RNA Isolation

Solutions - Guanidinium thiocyanate stock (4M) is prepared by mixing 50 grams guanidinium thiocyanate (GuSCN-Fluka) with 0.5 grams sodium N-lauroylsarcosine, 2.5 ml 1M sodium citrate, pH 7.0, 0.7 ml 2-mercaptoethanol, and 0.33 ml of Antifoam A (Sigma Chemical

Company). Deionized water is added with warming and stirring until the volume equaled 100 ml. The solution is filtered and the pH adjusted to 7.0 with a small amount of 1N NaOH.

Guanidine hydrochloride stock (GnHCl, 7.5M-Fluka) is filtered, neutralized to pH 7.0, buffered with 2.5 ml 1M sodium citrate, pH 7.0, and DTT is added to bring the solution to 5mM.

All glassware and solutions, whenever possible, are treated with 0.1% diethylpyrocarbonate overnight and then autoclaved to prevent RNAase contamination.

GuSCN Extraction Procedure

10^8 cells are washed three to four times with cold PBS and centrifuged. The cell pellet is dissolved in 10 ml GuSCN stock solution and homogenization is used if necessary. After the addition of 0.25 ml 1M acetic acid, 7.5 ml 95% ethanol is added very slowly while the solution is vortexed with subsequent storage at -20^0C overnight. After centrifugation, the pellet is dissolved in 5 ml GnHCl with incubation of the sample at 68^0C for a few minutes if the pellet is not completely dissolved. Precipitation with the addition of 0.125 ml 1M acetic acid and 2.5 ml 95% ethanol and storage at -20^0C overnight follows. After centrifugation, the pellet is dissolved in 2.5 ml GnHCl, and 0.06 ml 1M acetic acid and 1.25 ml 95% ethanol is added with overnight storage at -20^0C. The last centrifugation yields an RNA pellet that is washed once with 75% ethanol, dried, and redissolved in 1.5 ml H_2O with centrifugation to discard any undissolved material. At this point only use sterile plasticware or diethylpyrocarbonate treated glassware

and solutions. RNA samples are routinely stored as 70% ethanol suspensions at pH 5.0 and -20^{0}C.

Total cellular poly A+ RNA is isolated by affinity chromatography on oligo dT Cellulose (Collaborative Research Company). A 0.5 ml column is washed with 10 column-volumes each of 1) 10mM Tris, pH 7.5, 1mM EDTA, and 0.5% SDS, 2) 10mM Tris, pH 7.5, 1mM EDTA, 0.5% SDS, and 0.1N NaOH, and 3) 10mM Tris, pH 7.5, 1mM EDTA, 0.5% SDS, and 0.5M NaCl. The RNA sample is dissolved at 0.65 mg/ml in 10mM Tris, pH 7.5, 1mM EDTA, and 0.5% SDS and denatured at 85^{0}C for five minutes and cooled to room temperature. Sodium chloride is added to 0.5M and the solution is then applied to the column. The column is washed with 15 column-volumns of 10mM Tris, pH 7.5, 1mM EDTA, 0.5% SDS, and 0.5M NaCl. The poly A+ RNA is eluted with 4 column-volumns of 10mM Tris, pH 7.5, 1mM EDTA, and 0.05% SDS. Sodium chloride is added to 0.5M to the original flow through solution and recycled through the column with subsequent isolation of any residual poly A+ RNA. The combined poly A+ RNA solutions are brought to 0.5M with NaCl and recycled through the column to insure a pure poly A+ RNA solution. The RNA is precipitated by bringing the solution to 1M with NaCl and adding two to three volumes 95% ethanol with storage at -20^{0}C overnight. The pellet is washed with 70% ethanol, dried, and dissolved in H_2O.

RNA Dot Blotting Procedure

Poly A+ RNA samples are prepared by adding an equal volume of 29% formaldehyde, 20 x SSC with subsequent incubation at 55^{0}C for

ten minutes in order to denature the RNA.

Nitrocellulose membrane (12cm x 14cm) is soaked in 1 x SSC for a few minutes. The nitrocellulose is then placed over two sheets of pre-wet filter paper in a Schleicher and Schuell Manifold Dot Blot Apparatus. Samples containing 2 μg of poly A+ RNA as determined by A_{260} readings are applied to each well under vacuum.

After air drying the nitrocellulose is baked at 80^0C for two hours and placed in a sealed plastic pouch with 10 ml pre-hybridization solution containing 5 ml deionized formamide, 2.5 ml 20 x SSPE, 200 μl Denhardt's solution, 0.5 ml denatured salmon sperm DNA (5 mg/ml) and 1.8 ml H_2O. This is incubated at 42^0C overnight after which the pre-hybridization solution is removed. Hybridization solution is then added which consists of 5 ml de-ionized formamide, 2.5 ml 20 x SSPE, 1 ml 5% Denhardt's solution, 0.5 ml denatured salmon sperm DNA (5 mg/ml), 1 ml 50% sodium dextran 500, and the denatured, radioactively labelled probe. This is incubated at 42^0C for 16 to 18 hours.

The nitrocellulose is washed once with 250 ml of 2 x SSPE, 0.2% SDS for fifteen minutes at room temperature followed by two washes with the same solution at 50^0C for thirty minutes and then twice with 0.1 x SSPE, 0.1% SDS for thirty minutes at 50^0C. The nitrocellulose was air dried and exposed to x-ray film (Kodak XR-5) at -70^0C using Kodak intensifying screens.

RESULTS

I. Similarities and Differences in the Response of HL60 and HL60 T Cells to Inducers

Initial studies centered on characterizing the HL60 T cell line in its response to inducing agents. These cells were isolated for their resistance to TPA (Mendelsohn, et al., 1983) and the effects of other inducers were unknown. Tables 1 and 2 illustrate the differentiation inducing abilities of various compounds on both the HL60 and HL60 T cell lines. The first compound tested, DMSO, was well known for its inducing ability (Friend, et al., 1971, Collins, et al., 1978) and it immediately revealed a basic difference between the two cell lines. HL60 T cells did not differentiate as evidenced by NBT reduction, phagocytosis of latex beads, and HMSA activity. Other known inducers of HL60 cells like 1,25 dihydroxyvitamin D_3 (Bar-Shavit, et.al., 1983, Tanaka, et.al., 1983) were also tested in order to try to determine whether HL60 T cells retained the capacity to differentiate under appropriate conditions. This was shown to be the case as evidenced by the positive response to the granulocytic inducer, 13-Cis-retinoic acid (Breitman, et.al., 1981). HL60 T cells are also sensitive to simultaneous exposure to TPA and 13-Cis-retinoic acid resulting in the appearance of adherent, monocytic-like cells.

II. Effect of Inducers on Cytoplasmic Ion Fluxes

Many reports have suggested that transient ion fluxes may trigger a variety of biochemical responses such as activation of

Legend to Table 1.

Cells were suspended at 2.5 x 10^5 cells/ml in medium containing the indicated concentration of inducing agent and incubated for five days except for TPA and 1,25 dihydroxyvitamin D_3 (1,25 $(OH)_2D_3$) treatment where cells were suspended at 5 x 10^5 cells/ml and incubated for one day and three days respectively. NBT reduction and phagocytosis of latex beads were measured as described in Materials and Methods. The appearance of cells adhering to the bottom of tissue culture flasks are indicated with a plus or minus.

N.D., not determined

Table 1

Response of HL60 and HL60 T Cells to Inducers

Treatment	% of cells reducing NBT dye	% of cells phagocyti-zing 5 beads or more	Adherence
HL60 cells			
None	3 ± 0.6	9	-
1.25% DMSO	40 ± 10	31	-
1.5μM Cis-retinoic Acid	35 ± 12	N.D.	-
$5x10^{-7}$M $1,25(OH)_2D_3$	81 ± 10	55	+
17nM TPA	N.D.	85	+
17nM TPA + 1.5μM Cis-retinoic Acid	N.D.	40	+
HL60 T Cells			
None	2 ± 1	8	-
1.25% DMSO	4 ± 2	10	-
1.5μM Cis-retinoic Acid	20 ± 6	20	-
$5x10^{-7}$M $1,25(OH)_2D_3$	5	22	-
17nM TPA	N.D.	10	-
17nM TPA + 1.5μM Cis-retinoic Acid	N.D.	17	+

Legend to Table 2

Cells were suspended at 2.5 x 10^5 cells/ml in medium containing the indicated concentration of inducing agent and incubated for five days. Hexose monophosphate shunt activity was measured as described in Materials and Methods and is expressed in cpm/10^6 cells/hour.

Table 2

TPA-Stimulated Hexose Monophosphate Shunt Activity

Cells	Treatment	$^{14}CO_2$ Release
HL60	None	1500 ± 350
HL60	1.25% DMSO	16,500 ± 2300
HL60 T	None	1690 ± 620
HL60 T	1.25% DMSO	1780 ± 450

the Na+/H+ antiporter and the Na+/K+ ATPase that in turn signal the onset of cellular differentiation (Levenson, et.al., 1983). Various investigators have hypothesized that inducing agents like DMSO immediately trigger the transient ion fluxes that are thought to be crucial to cellular differentiation (Levenson, et al., 1980, Smith, et.al., 1982). In order to test this hypothesis the effects of DMO on HL60 and HL60 T cells were studied. DMO is a non-metabolizable, weak acid which is used to measure the differences between intracellular and extracellular pH. In the un-ionized form, DMO can freely cross cell membranes and once inside the cytoplasm can dissociate to a degree dependent on the intracellular pH and become trapped inside the cell (Gillies and Deamer, 1979).

A starting assumption was that the influx of DMO into HL60 cells would lead to a transient increase in intracellular H+ ions which would then duplicate events postulated to play a role in initiating cellular differentiation. As shown in Table 3 DMO is a strong inducer of granulocytic differentiation of HL60 cells. The effects of other weak acids and bases were studied to determine whether changes in intracellular pH were essential for promoting cellular differentiation. Benzoic acid was a moderate inducer of HL60 cells but other weak acids and bases tested had no effect on cellular differentiation as measured by the NBT reduction assay and phagocytosis of latex beads (Table 3).

The optimal concentration of each inducing agent was determined by testing a wide range of concentrations of each compound and identifying the amount that elicited the greatest response or

Legend to Table 3.

Cells were suspended at 2.5 x 10^5 cells/ml in medium containing the indicated concentration of inducing agent and incubated for five days. NBT reduction and phagocytosis of latex beads were measured as described in Materials and Methods.

N.D., not determined

MES - 2[N-Morpholino]Ethane Sulfonic Acid

MOPS - 3[N-Morpholino]Propane Sulfonic Acid

Table 3

Response of HL60 and HL60 T Cells to Inducers

Treatment	% of cells reducing NBT dye	% of cells phagocyti-zing 5 beads or more
HL60 Cells		
None	3 ± 0.6	9
40mM DMO	54 ± 11	30
15mM Benzoic Acid	30 ± 15	34
80mM MES	10 ± 2	N.D.
90mM MOPS	6 ± 4	N.D.
5mM Benzamide	10 ± 2	N.D.
10mM 3-Aminobenzamide	15 ± 3	N.D.
HL60 T Cells		
None	2 ± 1	8
40mM DMO	5 ± 2	15
15mM Benzoic Acid	5 ± 3	N.D.

was just below the level that proved toxic to the cells. Although the pH of the culture medium was adjusted to 7.2 after the addition of each agent so as to duplicate the proper conditions for the growth of HL60 and HL60 T cells, the possibility arose that the addition of these various acids and bases may have resulted in changes in the buffering capacity of the culture medium. However, a correlation between the inducing abilities and the pKa of these various compounds was not observed (Table 4). The lower pKa of benzoic acid did not make it a better inducer of HL60 cells than DMO and 2(N-Morpholino)Ethane Sulfonic Acid (MES), which has approximately the same pKa as DMO, did not induce HL60 cells at all. Benzamide and 3-aminobenzamide inhibit poly (ADP-ribose) polymerase (Purnell, et.al., 1980) and have been shown to induce murine erythroleukemia cells (Terada, et.al., 1979). Although 3-aminobenzamide has been reported to induce granuloctyic differentiation of HL60 cells (Damji, et.al., 1986), I found it to have very little effect on functional maturation as measured by the NBT reduction assay. It is of interest that benzoic acid, which is used as a negative control in studies that focus on the effect of agents on poly ADP ribosylation, was an inducer of HL60 cells.

These experiments suggested the inducing abilities of DMO were not based solely on its ability to affect ion concentrations within cells or to buffer the pH of the growth medium. Dilution experiments also demonstrated that the continuous presence of DMO is needed for extended periods of time in order to induce differentiation. Table 5 illustrates that DMO was like other inducers of

Table 4

Weak Acids and Bases Utilized in This Study

	pKa
Benzoic Acid	4.19
MES	6.1
DMO	6.2
MOPS	7.2
3-Aminobenzamide	9.4
Benzamide	13-14

Legend to Table 5.

HL60 cells were suspended at 2.5×10^5 cells/ml in medium containing the initial concentration of inducing agent. At the indicated time, warm medium was added to bring the inducing agent to the final concentration indicated. Five days after the cells were initially suspended, NBT reduction was measured as described in Materials and Methods.

Table 5

Continuous Presence of Inducing Agent Essential for Granulocytic Maturation of HL60 Cells

Treatment	Initial Concentration	Final Concentration	Time of Dilution (hours)	% of cells reducing NBT dye
DMSO	1.25%	1.25%	-	37
DMSO	1.25%	0.62%	24	15
DMO	40mM	40mM	-	58
DMO	40mM	40mM	24	51
DMO	0	40mM	24	41
DMO	40mM	4mM	4	6
DMO	40mM	4mM	16	4
DMO	4mM	4mM	-	7
DMO	40mM	8mM	24	19
DMO	8mM	8mM	-	11
Benzoic Acid	12mM	12mM	-	21
Benzoic Acid	12mM	12mM	24	20
Benzoic Acid	0	12mM	24	10
Benzoic Acid	12mM	2.4mM	24	3
Benzoic Acid	2.4mM	2.4mM	-	8

granulocytic differentiation in that the effects of these agents are mediated over several days. If DMO was acting solely via transient changes in ion fluxes, cellular differentiation would be triggered after a short incubation period. However, DMO exposure for twenty four hours with subsequent dilution of the DMO concentration to non-inducing levels did not result in the differentiation of HL60 cells. This type of response is typical for other granulocytic inducers and indicates that transient ion fluxes are not sufficient although they may be necessary for cellular differentiation. In order for DMO to mediate its full effect it must be incubated with HL60 cells for an extended period of time. In this way DMO can affect cellular processes that occur subsequent to the initiation of transient ion fluxes, resulting in cellular differentiation. The similarities between the inducing abilities of DMO and other granulocytic inducers is also evident in Table 6 which reveals a similar time course of differentiation with DMO, DMSO, and 13-Cis-retinoic acid.

The biochemical signals elicited by exposure to DMO and other granulocytic inducers are clearly deficient in HL60 T cells as illustrated in Tables 1 and 2. These cells are also resistant to TPA and are partially resistant to 1,25 dihydroxy-vitamin D_3, another inducer of monocytic differentiation of HL60 cells (Bar-Shavit, et.al., 1983, Tanaka, et.al., 1983). However, HL60 T cells are sensitive to the inducing effects of 13-Cis-retinoic acid. Thus, these cells have maintained the capability to differentiate but are blocked from maturing along their normal devel

Legend to Table 6.

Cells were suspended at 2.5 x 10^5 cells/ml in medium containing the indicated concentration of inducing agent and incubated for five days. At the times indicated, an aliquot was removed from the cell cultures and the cells assayed for NBT reduction as described in Materials and Methods.

Table 6

Time Course of Differentiation of HL60 Cells

Treatment	Days in Culture 2	3	4	5
	(% of cells reducing NBT dye)			
None	2	2	2	2
1.25% DMSO	8	19	21	35
1.5μM Cis-retinoic Acid	5	14	20	33
40mM DMO	17	34	58	74

opmental pathway at a point different from that of HL60 cells.

A factor secreted by HL60 T cells into their culture medium was found to inhibit the induction of HL60 cells upon incubation with DMSO for five days as measured by the NBT reduction assay (Mendelsohn et.al., 1983). Conditioned medium from HL60 T cell cultures was able to block the inducing effects of DMSO. The identity of this unknown factor was of great interest because it could help explain the molecular events contributing to the increased neoplastic state of the HL60 T cell line and the biochemical pathways utilized by inducing agents in promoting differentiation.

Initial steps to isolate this factor involved ammonium sulfate ($AmSO_4$) fractionation of HL60 T conditioned medium. The inhibitory activity was found to be non-dialyzable and present in the 50-90% fraction of $AmSO_4$ precipitated material (Table 7). The data in this table also shows that the factor was active after incubation at 37^0C for twenty four hours while incubation at 60^0C for one hour abolished the inhibitory activity. The heat lability of the factor suggested it may be a protein. Subsequent purification steps proved not to be feasible as the biological assay system for detecting the inhibitory factor was very time consuming and too insensitive for use in isolation procedures involving column chromatography.

III. Effect of Inducers on the Cell Membrane

The ability of the HL60 cell line to differentiate along the

Legend to Table 7.

HL60 cells were suspended at 2.5 x 10^5 cells/ml in medium containing the indicated inducer and HL60 T conditioned medium which comprised 80% of the total volume of the cell culture. $AmSO_4$ fractions of HL60 T conditioned medium were obtained as described in Materials and Methods. The fractions were dialyzed and concentrated so that they could be added in an amount that would be equivalent to 100% of the total volume of the cell culture. After five days the percentage of cells reducing NBT dye was assayed as described in Materials and Methods.

Table 7

Effect of HL60 T Inhibitory Factor on the Differentiation of HL60 Cells

Additions to medium	% of cells reducing NBT dye	% inhibition
None	3	-
1.25% DMSO	50	0
1.25% DMSO + conditioned medium	12	76
1.25% DMSO + 0-30% $AmSO_4$ fraction	34	32
1.25% DMSO + 0-50% $AmSO_4$ fraction	31	38
1.25% DMSO + 50-90% $AmSO_4$ fraction	10	80
1.25% DMSO + 50-90% $AmSO_4$ fraction incubated at 37^0C for 1 hour	15	70
1.25% DMSO + 50-90% $AmSO_4$ fraction incubated at 37^0C overnight	14	72
1.25% DMSO + 50-90% $AmSO_4$ fraction incubated at 60^0C for 1 hour	33	34

granulocytic and monocytic developmental pathways led to a collaborative effort with Dr. Josef Michl to study hematopoietic cell specific surface antigens. Monoclonal antibodies have been used for the identification of cell surface antigens that are unique to granulocytes or monocytes. The isolation of two monoclonal antibodies, BH2-C6 and IE8, specific for neutrophils and eosinophils respectively (Pytowski, et.al., in press, Calderon, et.al., 1986), provided a unique means of characterizing the appearance of lineage-specific plasma membrane antigens in HL60 and HL60 T cells. The maturation of HL60 cells along the neutrophil lineage after treatment with inducers like 13-Cis-retenoic acid is well documented (Breitman, et.al., 1981) and the eosinophilic differentiation of HL60 cells cultured in alkaline medium has also been reported (Fischkoff, et.al., 1984). These two monoclonal antibodies would thus prove very useful in documenting the inducer mediated appearance of cell surface markers of terminal differentiation in HL60 cells and whether similar results are obtained with the differentiation defective HL60 T cell line.

The BH2-C6 monoclonal antibody was isolated from a mouse immunized with human neutrophils and its reactivity is confined to this lineage of hematopoietic cells. Immunofluorescent staining of HL60 cells with this antibody revealed a very small number (<4%) of positive cells. By contrast, 82% of cells induced with DMO and 90% of cells induced with 13-Cis-retinoic acid expressed the BH2-C6 cell surface antigen (Pytowski, et.al., in press). These results show that these two inducers of granulocytic dif-

ferentiation stimulate the expression of the BH2-C6 antigen which correlates well with other biochemical markers of cellular differentiation. HL60 T cells incubated with 13-Cis-retinoic acid also expressed the BH2-C6 cell surface antigen (75% of cells were positive by immunofluorescent staining) while incubation with DMO had little effect (<5% of cells were positive). These results show a direct correlation between the ability of these compounds to act as inducers of granulocytic differentiation and the expression of this cell surface marker.

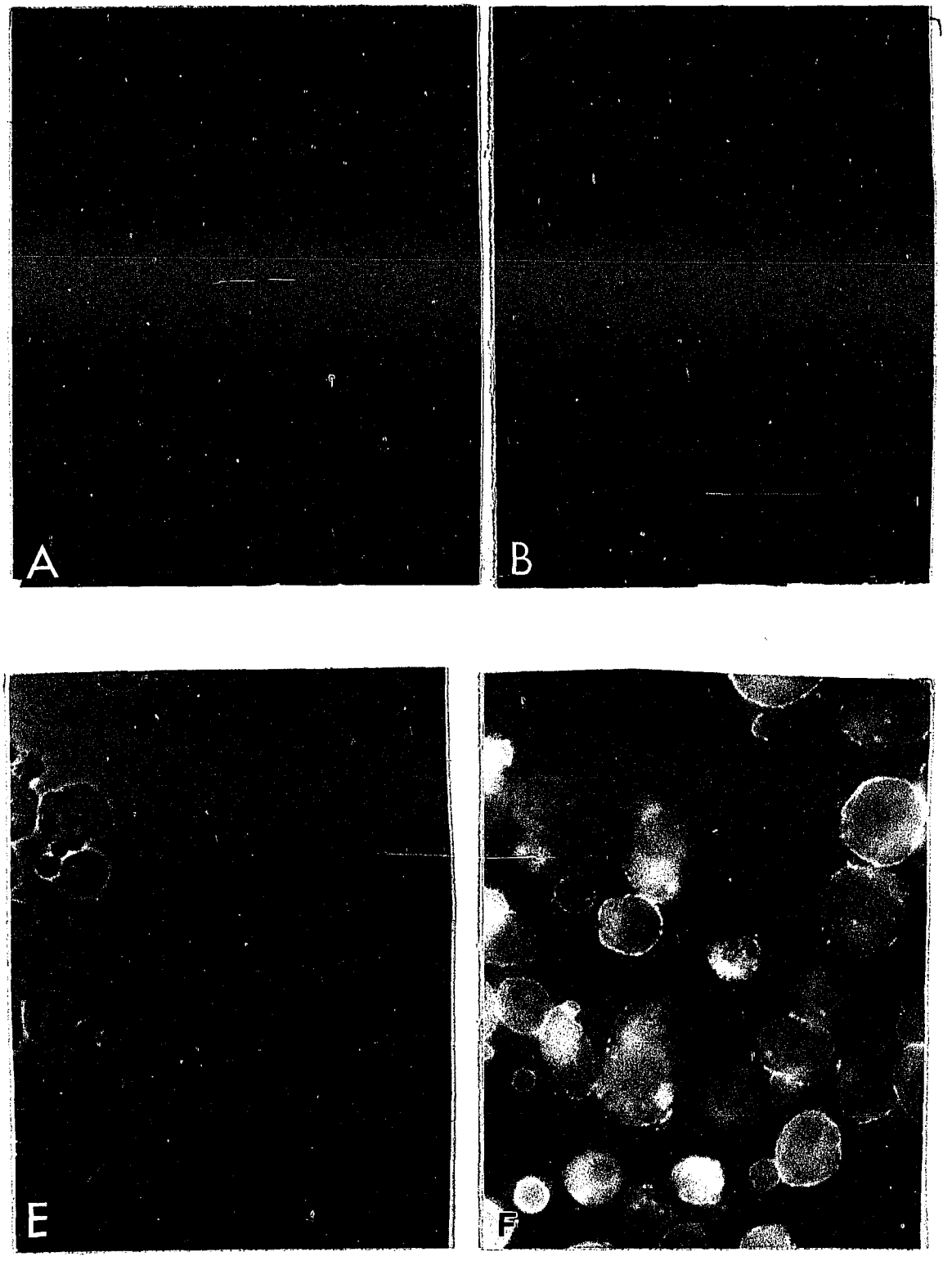
The monoclonal antibody, IE8, reacts specifically with a cell surface antigen restricted to human eosinophils, megakaryocytes, and platelets. Less than 5% of HL60 cells were positive and treatment with various inducers of granulocytic differentiation failed to promote the appearance of this antigen in the cell membrane as revealed by immunofluorescent staining. Unexpectedly, exposure to TPA resulted in greater than 90% of the cells staining positively with the IE8 antibody within twelve hours (Figure 1) (Calderon, et.al., 1986).

The appearance of this antigen could be detected within six hours of TPA exposure (Table 8) and this effect was blocked by pretreatment of HL60 cells with cycloheximide (20 μg/ml) for one hour before the addition of TPA. These results suggested a rapid activation of protein synthesis to produce this cell surface antigen as a direct result of TPA treatment. Pretreatment of HL60 cells with tunicamycin, an inhibitor of protein N-glycosylation, failed to block the induction of this antigen with exposure to TPA

Figure 1. Reactivity of HL60 Cells Treated with TPA to IE8 Antibody.

HL60 cells (A) photographed with a light microscope and HL60 cells (B) incubated with the IE8 monoclonal antibody, a fluorescent secondary antibody and photographed with a fluorescent microscope (400X magnification).

HL60 cells (E) incubated with TPA and photographed with a light microscope and HL60 cells (F) incubated with TPA, the IE8 monoclonal antibody and a fluorescent secondary antibody and photographed with a fluorescent microscope (400X magnification) as described in Materials and Methods.


A
B
E
F

Legend to Table 8.

Cells were incubated with 17nM TPA for the times indicated and then assayed for membrane fluorescence as described in Materials and Methods. More than 200 cells on each of three coverslips were counted to obtain the percentage of the population exhibiting membrane fluorescence.

Table 8

Time Course of Induction of IE8 Reactivity in TPA Treated HL60 Cells

Time	% Population with intense membrane fluorescence	Total fluorescent cells (% Population)
0 hours	4	6
6 hours	11	15
12 hours	41	51
18 hours	48	65
22 hours	58	88

which suggested that the IE8 antibody was not recognizing an N-glycosylated protein. Subsequent experiments utilizing Elisa assays showed that trypsin digestion failed to inhibit IE8 reactivity while proteinase K was found to destroy the recognizability of this antigen by the IE8 antibody.

To determine whether this antigen was associated with monocytic differentiation of HL60 cells, the effect of phorbol ester analogues as well as other monocytic inducers were tested. Elisa assays indicated that incubation of HL60 cells with phorbol ester analogues that lack tumor promoting activity (phorbol, 4-phorbol-didecanoate) failed to promote the appearance of this antigen while the tumor promoter, phorbol-didecanoate, was found to induce IE8 reactivity. Immunofluorescent staining of HL60 cells incubated with 1,25 dihydroxyvitamin D_3 revealed a negligible number of positive cells (Table 9). Thus, the presence of this surface antigen does not correlate with development of either myeloid or monocytic differentiation of HL60 cells but seems to be an early marker for exposure to TPA and other tumor promoters.

The IE8 antibody also served as a marker for TPA resistance. HL60 T cells were negative for IE8 reactivity even after treatment with TPA for twenty four hours (Table 9). Other inducers of granulocytic and monocytic differentiation also failed to promote the appearance of this membrane antigen. In addition, the inhibitory factor produced by HL60 T cells blocked TPA induction of this antigen in HL60 cells. The number of positive cells, as seen by immunofluorescent staining, decreased five fold which provides a

Legend to Table 9.

Cells were suspended at 5 x 10^5 cells/ml in medium containing the indicated concentration of inducing agent and incubated with TPA for twenty four hours and 1,25 dihydroxyvitamin D_3 for seventy two hours. NBT reduction and phagocytosis of latex beads were measured as described in Materials and Methods as well as the method of immunofluorescent staining.

Table 9

Effects of TPA and 1,25 Dihydroxyvitamin D_3 on Induction of Reactivity with IE8 Antibody

Addition	Relative staining intensity (% of population)			% of cells reducing NBT dye	% of cells phagocyti-5 beads or more
	neg.	weak	mod./strong		
HL60					
None	9	5	0	7	8
17nM TPA	10	64	26	-	80
$5x10^{-7}M$ $1,25(OH)_2D_3$	97	3	0	81	50
HL60 T					
None	95	5	0	5	8
17nM TPA	88	10	2	-	10
$5x10^{-7}M$ $1,25(OH)_2D_3$	98	2	0	5	2

rapid and quantitative assay system for the inhibitory factor. This may prove very useful in future attempts to purify this factor.

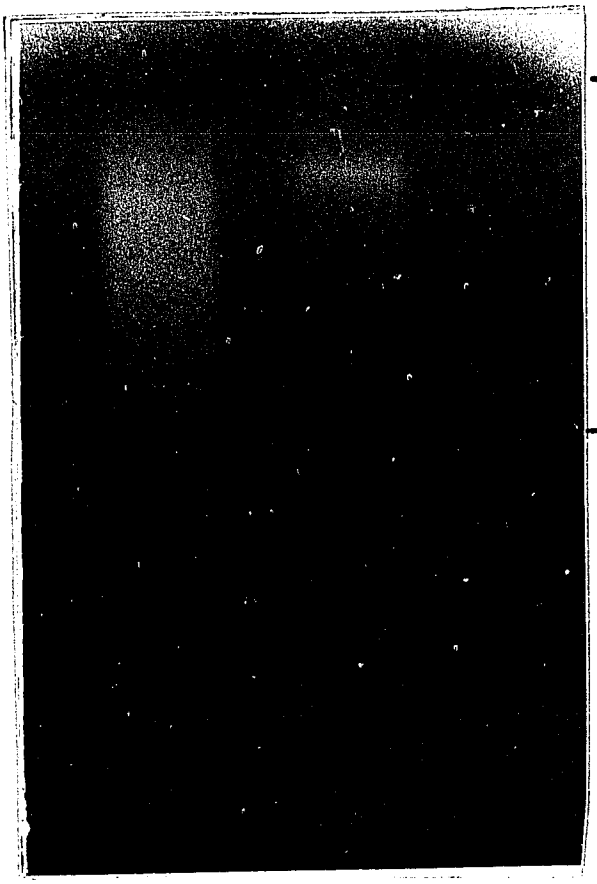
As mentioned previously, the specific cell surface binding protein for TPA is protein kinase C (Castagna, et.al., 1982, Kikkawa, et.al., 1983) and activation of this enzyme is associated with a wide range of biological functions. Many investigators have tried to identify the various proteins phosphorylated by protein kinase C and to elucidate the exact mechanism by which protein phosphorylation exerts control over cellular proliferation and differentiation. The IE8 antibody recognizes an antigen that seems to be indicative of the responsiveness of HL60 cells to TPA treatment and may prove helpful in elucidating cellular responses to protein kinase C activation.

Initial efforts at identifying and characterizing the membrane antigen recognized by the IE8 monoclonal antibody involved Western Blotting of detergent cell extracts. Octyl-β-D-thio-glucopyranoside extracts of human platelets contained a high concentration of cell surface antigen and were used primarily for these experiments. Samples were run on 12% polyacrylamide gels without the addition of DTT and boiling as this was found to destroy the epitope recognized by the IE8 antibody.

Figure 2 is an autoradiogram of a Western blot incubated with ^{125}I labelled IE8 antibody. There is diffuse staining but a prominent band can be seen which identifies a protein with a molecular weight of approximately 51,000. This establishes the identity of

Figure 2. Western Blot of Octyl-β-D-thio-glucopyranoside Extract of Human Platelets.

Ten ml of a human platelet pack was spun and washed twice with PBS. The pellet was then extracted in 7mM octyl-glucoside in PBS, 1mM PMSF and 10mM iodoacetamide. The solution was centrifuged after thirty minutes and the supernatant was concentrated approximately ten fold in Centricon 30 microconcentrator units. 50 μl of two different preparations were run on 12% polyacrylamide gels and transferred to nitrocellulose as described in Materials and Methods.


x10^3
68
45

the cell surface antigen as a protein and future experiments will center on its characterization using gene cloning techniques. The cloning of the regulatory regions of the gene coding for this protein would prove very interesting in regard to TPA regulation of expression.

IV. Effect of Inducers on DNA Methylation and Oncogene Expression

The relationship between the expression of specific cellular oncogenes and neoplastic transformation is under intensive study. Cellular oncogenes are thought to participate directly in the control of cellular proliferation and differentiation and changes in the normal activity of these genes most likely play an important role in transformation.

The expression of the cellular oncogenes Ha-ras, myc, myb, and fos was studied in HL60 and HL60 T cells incubated with different inducing agents for varying periods of time. The level of messenger RNA expression was ascertained using RNA dot blots and the relationship of this expression to the degree to which the DNA coding sequences were methylated was determined by Southern blot analysis. Since inhibitors of DNA methylation can act directly as inducers of HL60 cell differentiation, I examined the possibilities that: 1) other inducers affected DNA methylation indirectly through effects on the S-Adenosylmethionine pools and; 2) the differences in the response of HL60 and HL60 T cells to inducers was related to differences in the methylation pattern of specific cellular oncogenes.

Table 10 reveals the concentration of S-Adenosylmethionine (SAM) in HL60 and HL60 T cells after incubation with various inducing agents for forty eight hours. The concentration of SAM was determined by HPLC as described in Materials and Methods. SAM levels drop significantly in HL60 cells in an amount proportional to the potency of the compound used as an inducer of differentiation. However, treatment with these compounds also decreases SAM levels in HL60 T cells. All of these compounds have affected both cell lines in a manner that has led to the reduction of SAM levels. This treatment has resulted in cellular differentiation of the HL60 cell line but the HL60 T cell line is only sensitive to the inducing effects of 13-Cis-retinoic acid. This indicates that the reduction of SAM levels is necessary but not sufficient to trigger cellular differentiation.

The extent of methylation of specific coding sequences in genomic DNA can be determined using specific restriction enzymes. Approximately 90% of the 5-methylcytosine residues in eukaryotic DNA are located in the dinucleotide CpG (Doskocil and Sorm, 1962). Several restriction endonucleases have recognition sequences that contain CpG (Table 11) (Roberts, 1978). These restriction enzymes are unable to cleave at its recognition site if one of the C residues in that site is methylated. This allows a determination of the degree to which sequences in specific regions of DNA are methylated.

Genomic DNA is digested with the restriction enzymes already mentioned and the resulting fragments are then separated according

Legend to Table 10.

HL60 and HL60 T cells were suspended at 2.5 x 10^5 cells/ml in medium containing the indicated concentration of inducing agent and incubated for forty eight hours. Aliquots were removed to be analyzed for S-Adenosylmethionine levels as described in Materials and Methods. The cell cultures were then incubated for an additional three days and then assayed for NBT reduction as described in Materials and Methods.

Table 10

Effect of Inducers on S-Adenosylmethionine Levels

Treatment	pmol SAM/10^6 cells (48 hours)	% of cells reducing NBT dye
HL60		
None	83.9 ± 4.1	5
1.25% DMSO	35.7 ± 3.5	43
40mM DMO	23.8 ± 4.3	57
1.5μM Cis-retinoic Acid	47.7 ± 3.4	25
15mM Benzoic Acid	59.1 ± 2.9	15
HL60 T		
None	82.9 ± 5.5	2
1.25% DMSO	30.8 ± 2.9	5
40mM DMO	34.6 ± 5.6	8
1.5μM Cis-retinoic Acid	46.6 ± 1.7	29
15mM Benzoic Acid	57.5 ± 1.9	2

Table 11

Restriction Endonucleases Sensitive to 5-MethylCytosine

Endonuclease	Recognition sequence cleaved	Recognition sequence not cleaved
Hpa II	CCGG, MeCCGG	CMeCGG
Msp I	CCGG, CMeCGG	MeCCGG
Hha I	GCGC	GMeCGC

to size by agarose gel electrophoresis. The DNA is then blotted and hybridized with a radioactively labelled DNA probe specific for each cellular oncogene as described in Materials and Methods. Exposure of the blot to x-ray film reveals a band pattern that is representative of the extent of methylation of the DNA sequences in question. Sequences containing a high degree of 5-methylcytosine residues will reside in large DNA fragments while unmethylated sequences will give rise to smaller fragments.

Figures 3 and 4 are Southern blots of DNA from HL60 (Blot A) and HL60 T (Blot B) cells treated with various inducers, digested with Hpa II or Msp I, and hybridized with a labelled c-myc probe. The c-myc gene is unmethylated at CCGG sites as evidenced by the pattern of small DNA fragments which is identical in both Hpa II and Msp I digests. This low level of methylation correlates well with the high level of c-myc expression in HL60 cells which decreases after inducer mediated cellular differentiation (Westin, et.al., 1982, Reitsma, et.al., 1983). Since the gene is highly unmethylated no further loss of methyl groups can occur but no increase was seen after treatment with inducing agents in both HL60 and HL60 T DNA.

Southern blots of HL60 (Blot A) and HL60 T (Blot B) DNA digested with Hpa II or Msp I and probed with c-fos are shown in Figures 5 and 6. HL60 and HL60 T DNA produce very similar banding patterns when digested with Hpa II or Msp I which is not changed after incubation with the various inducers tested. However, Hpa II digestion reveals a somewhat higher degree of methylation of the

Figure 3. Hybridization of Hpa II Digested HL60 and HL60 T DNA With c-myc.

20 μg of HL60 (A) and HL60 T (B) DNA digested with Hpa II was electrophoresed in each lane of a 1% agarose gel, blotted onto Zeta Probe and hybridized with ^{32}P-labelled c-myc DNA as described in Materials and Methods. (Lane a) DNA from untreated cells; (lane b) DNA from cells treated with 1.25% DMSO for four days; (lane c) DNA from cells treated with 17nM TPA for twenty four hours. The DNA of HL60 and HL60 T cells treated with 40mM DMO and 1.5μM 13-Cis-retinoic Acid for four days gave identical results (not shown).

It was assumed that each lane of every gel was loaded with 20 μg of DNA as measured by A_{260} readings but subsequent staining of the gels revealed that some lanes contained less DNA than others.

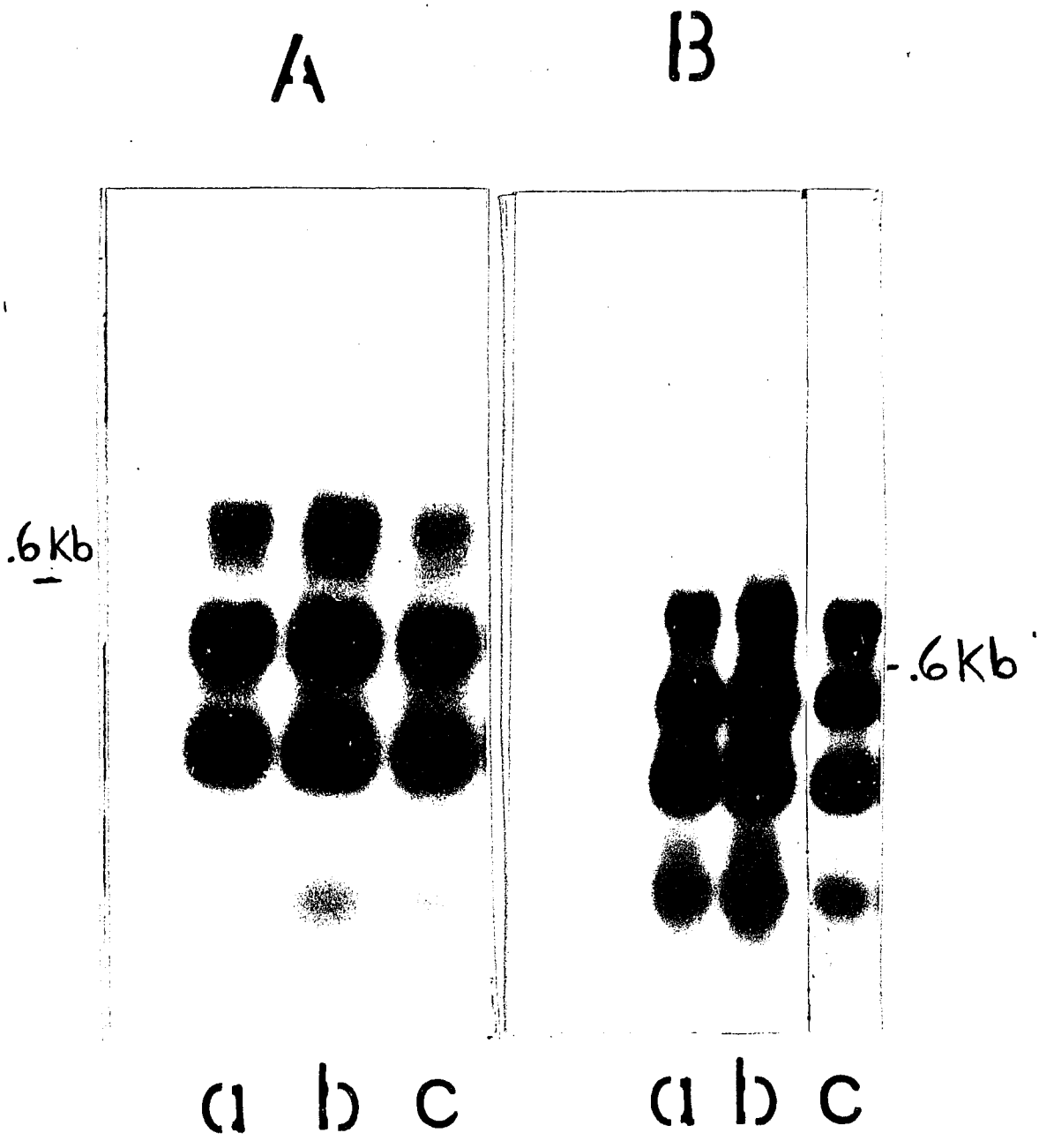

A
B
.6Kb
.6Kb
a b c
a b c

Figure 4. Hybridization of Msp I Digested HL60 and HL60 T DNA With c-myc.

20 μg of HL60 (A) and HL60 T (B) DNA digested with Msp I was electrophoresed in each lane of a 1% agarose gel, blotted onto Zeta Probe and hybridized with ^{32}P-labelled c-myc DNA as described in Materials and Methods. (Lane a) DNA from untreated cells; (lane b) DNA from cells treated with 1.25% DMSO for four days; (lane c) DNA from cells treated with 17 nM TPA for twenty four hours. The DNA of HL60 and HL60 T cells treated with 40mM DMO and 1.5μM 13-Cis-retinoic acid for four days gave identical results (not shown).

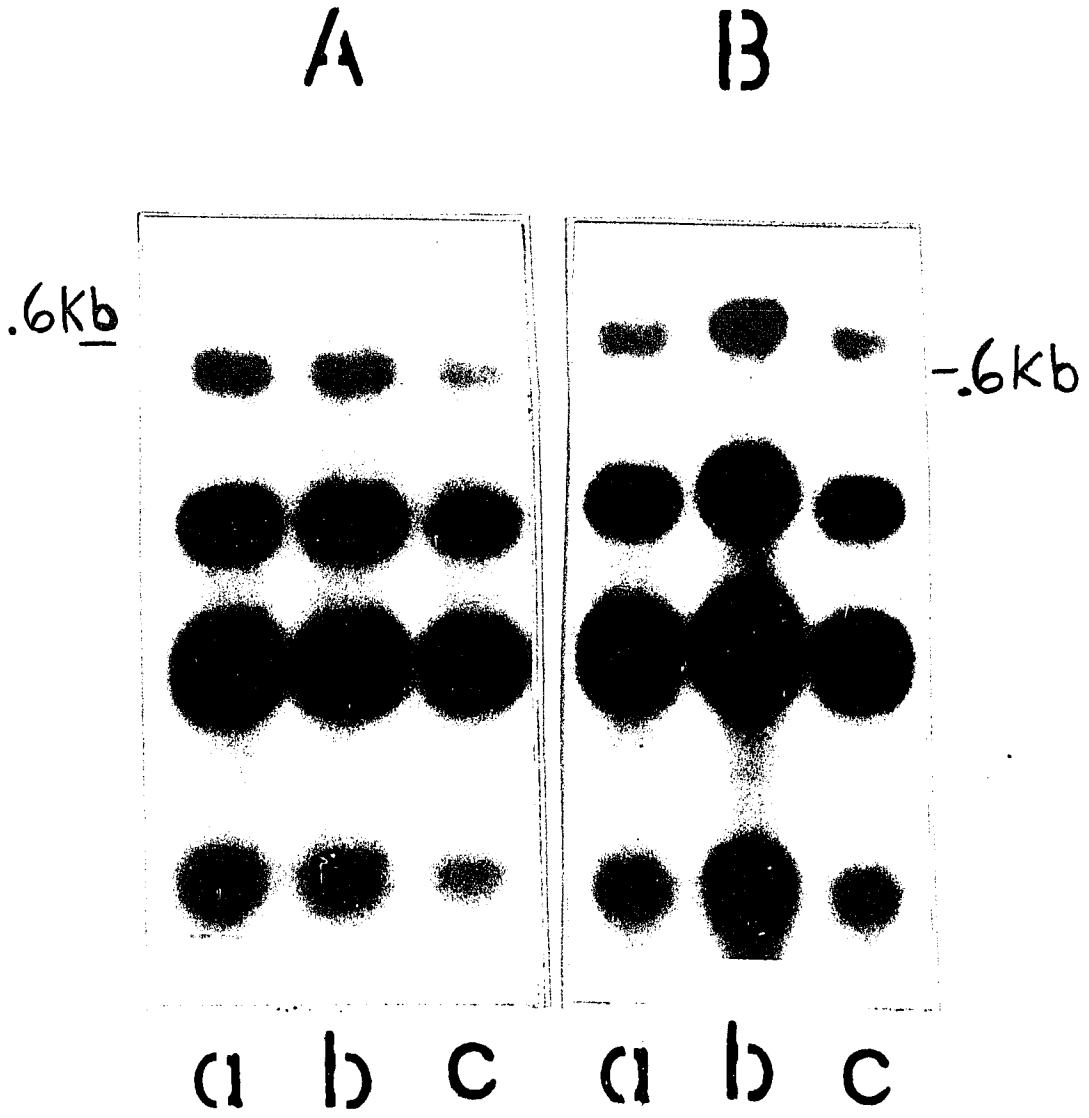

A
B
.6Kb
-.6Kb
a b c a b c

Figure 5. Hybridization of Hpa II Digested HL60 and HL60 T DNA With c-fos.

20 μg of HL60 (A) and HL60 T (B) DNA digested with Hpa II was electrophoresed in each lane of a 1% agarose gel, blotted onto Zeta Probe and hybridized with ^{32}P-labelled c-fos DNA as described in Materials and Methods. (Lane a) DNA from untreated cells; (lane b) DNA from cells treated with 1.25% DMSO for four days; (lane c) DNA from cells treated with 17nM TPA for twenty four hours. The DNA from HL60 and HL60 T cells treated with 40mM DMO and 1.5μM 13-Cis-retinoic Acid for four days gave identical results (not shown).

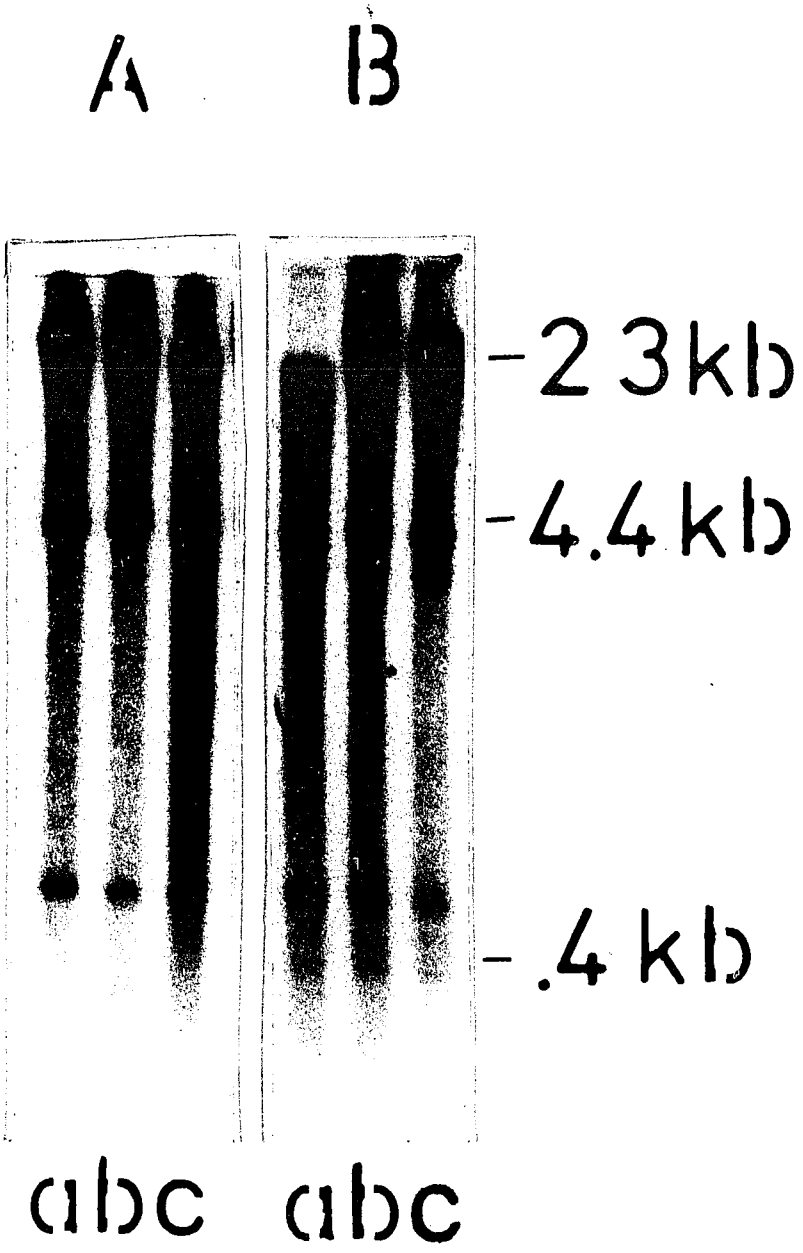
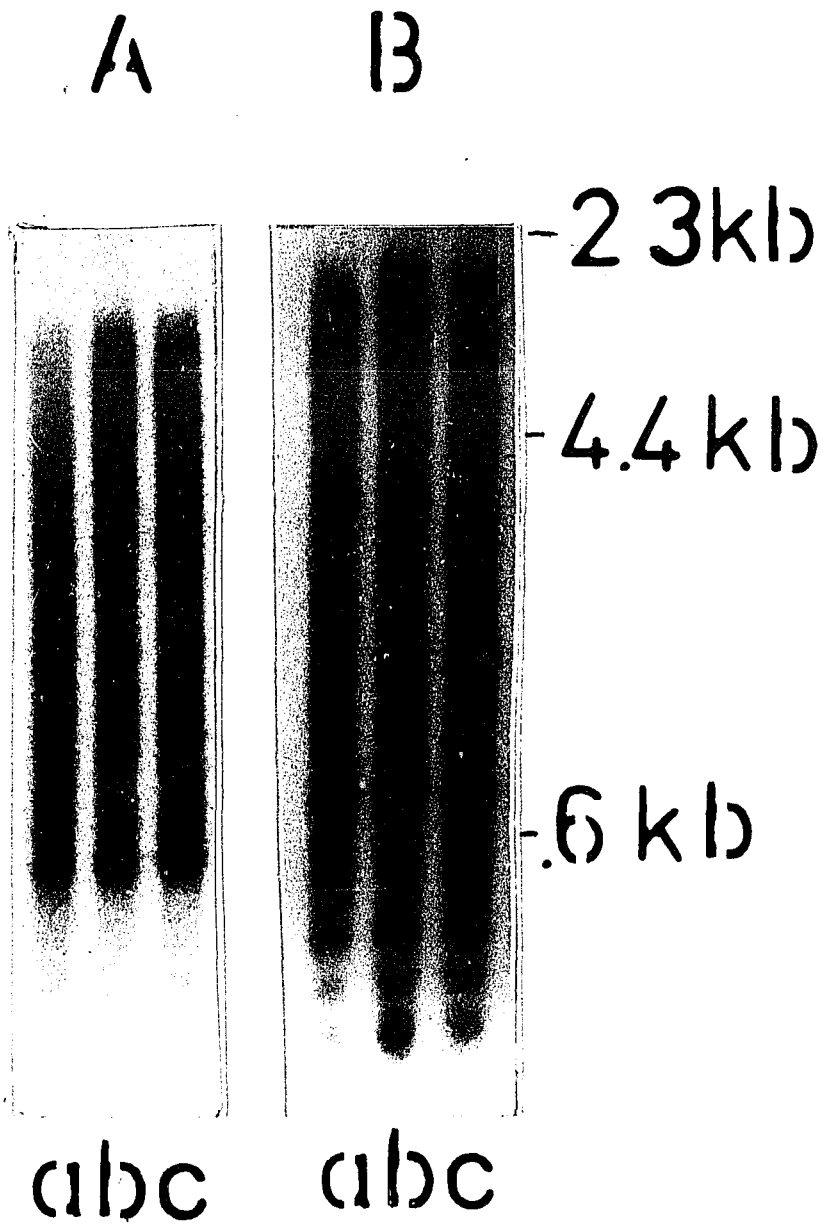

A
B
-2.3kb
-4.4kb
-.4kb
abc
abc

Figure 6. Hybridization of Msp I digested HL60 and HL60 T DNA With c-fos.

20 μg of HL60 (A) and HL60 T (B) DNA digested with Msp I was electrophoresed in each lane of a 1% agarose gel, blotted onto Zeta Probe and hybridized with ^{32}P-labelled c-fos DNA as described in Materials and Methods. (Lane a) DNA from untreated cells; (lane b) DNA from cells treated with 1.25% DMSO for four days; (lane c) DNA from cells treated with 17nM TPA for twenty four hours. The DNA from HL60 and HL60 T cells treated with 40mM DMO and 1.5μM 13-Cis-retinoic Acid for four days gave identical results (not shown).


A
B
2.3kb
4.4kb
6kb
abc
abc

DNA sequence coding for the c-fos gene. The amount of smearing in these gels is probably due to non-specific hybridization.

HL60 (Blot A) and HL60 T (Blot B) DNA digested with Hpa II and probed with c-Ha-ras is shown in Figure 7. The treatment with various inducers does not alter the pattern of DNA fragments but there is a significant increase in the methylation of the c-Ha-ras gene at CCGG sites in HL60 T DNA. Figure 8 reveals a high degree of methylation of the c-myb oncogene in both HL60 (Blot A) and HL60 T (Blot B) DNA digested with Hha I and the inducers tested had no effect on the pattern of DNA fragments.

In summary, I found no changes in the methylation of these oncogene sequences with the induction of differentiation although the c-Ha-ras gene was methylated to a greater extent in HL60 T cells. To determine how these results correlated with gene activity, the expression of these various oncogenes was studied using dot blots of messenger RNA isolated from HL60 and HL60 T cells treated with inducing agents for various periods of time. The aim was to determine whether inducer mediated maturation of HL60 cells would lead to changes in oncogene expression which were absent or altered in HL60 T cells and thus might contribute to the unresponsiveness of this cell line to specific inducing agents.

HL60 and HL60 T cells were treated with the inducers DMSO, DMO, 13-Cis-retinoic acid, and TPA for time periods ranging from 3 hours to 4 days. In addition, the effect of cycloheximide treatment on messenger RNA expression was also studied (Table 12) to determine whether changes in the level of messenger RNA were

Figure 7. Hybridization of Hpa II Digested HL60 and HL60 T DNA With c-Ha-ras.

20 μg of HL60 (A) and HL60 T (B) DNA digested with Hpa II was electrophoresed in each lane of a 1% agarose gel, blotted onto Zeta Probe and hybridized with ^{32}P-labelled c-Ha-ras DNA as described in Materials and Methods. (Lane a) DNA from untreated cells; (lane b) DNA from cells treated with 1.25% DMSO for four days; (lane c) DNA from cells treated with 17nM TPA for twenty four hours. The DNA from HL60 cells treated with 40 mM DMO and 1.5μM 13-Cis-retinoic Acid for four days gave results comparable to blot A and the DNA from HL60 T cells treated under the same conditions gave results comparable to blot B (not shown).

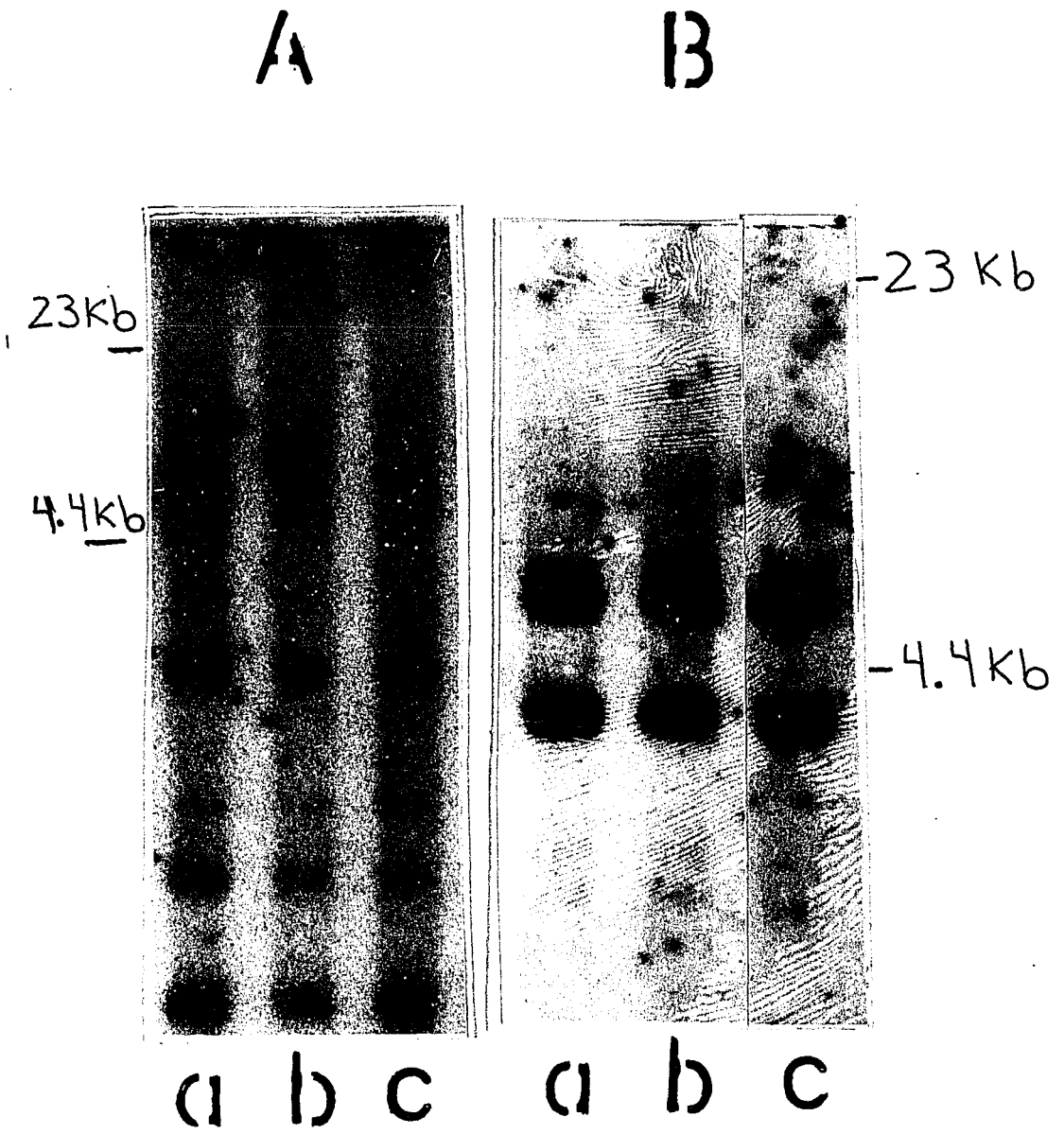

A
B
23Kb
4.4Kb
-23 Kb
-4.4Kb
a b c
a b c

Figure 8. Hybridization of Hha II Digested HL60 and HL60 T DNA With v-myb.

20 μg of HL60 (A) and HL60 T (B) DNA digested with Hha I was electrophoresed in each lane of a 1% agarose gel, blotted onto Zeta Probe and hybridized with ^{32}P-labelled v-myb DNA as described in Materials and Methods. (Lane a) DNA from untreated cells; (lane b) DNA from cells treated with 1.25% DMSO for four days; (lane c) DNA from cells treated with 17nM TPA for twenty four hours. The DNA from HL60 and HL60 T cells treated with 40mM DMO and 1.5μM 13-Cis-retinoic Acid for four days gave identical results (not shown).


A
B
-23 Kb
a b c
a b c

Legend to Table 12.

HL60 and HL60 T cells were incubated with inducers for the time periods indicated. Pre-treatment with 20 μg/ml cycloheximide for 15 minutes with subsequent addition of inducers for a 3 hour incubation was also included. Poly A+ RNA was isolated from these cultures as described in Materials and Methods.

Table 12

Incubation of HL60 and HL60 T Cells With Inducers Before Poly A+ RNA Isolation

Treatment	Time of Incubation	
	No pre-treatment	Pre-treatment with 20 ug/ml cyclo-heximide
1.25% DMSO	3,6,12,24,96 hours	3 hours
40mM DMO	3,6,12,24,96 hours	3 hours
1.5μM Cis-retinoic Acid	3,6,12,24,96 hours	3 hours
17 nM TPA	3,6,12,24,96 hours	3 hours
1.5μM Cis-retinoic Acid + 17nM TPA	3,6,12,24,96 hours	3 hours

dependent on protein synthesis or whether messenger RNA's were stabilized or destabilized by newly synthesized proteins. The most striking changes in the expression of each oncogene will be discussed.

Figure 9 is a poly A+ RNA dot blot probed with c-myc as described in Materials and Methods. Cycloheximide pre-treatment of HL60 and HL60 T cells increases the level of c-myc poly A+ RNA, especially in HL60 T cells. Incubation with the various inducing agents generally leads to a decrease in the level of c-myc poly A+ RNA except for DMO treatment where the decrease is not as great as with the other inducers. The time course of this decrease differs depending on the inducer. A marked decrease can be detected by 12 hours in HL60 cells and 24 hours in HL60 T cells with TPA treatment. DMSO, DMO, and 13-Cis-retinoic acid significantly decreased the level of poly A+ RNA in both cell lines after incubation for four days but the level of c-myc poly A+ RNA in HL60 T cells did not seem to be affected by incubation with DMO.

Figure 10 reveals a slightly lower level of c-Ha-ras poly A+ RNA in HL60 T cells and in general the level of c-Ha-ras poly A+ RNA in both cell lines is lower than that of c-myc. Treatment with inducing agents lowers the level of c-Ha-ras poly A+ RNA in HL60 cells in a manner similar to c-myc except that the decrease is much less pronounced. HL60 T cells generally maintain a constant level of c-Ha-ras poly A+ RNA that is comparable to HL60 cells after incubation with all the inducing agents tested. These results can be compared to the level of methylation of the c-Ha-

Figure 9. Dot Blot of HL60 and HL60 T Poly A+ RNA Hybridized With c-myc.

2 μg of Poly A+ RNA isolated from cell cultures described in Table 12 was blotted onto nitrocellulose and hybridized with ^{32}P-labelled c-myc DNA as described in Materials and Methods.

Blot A - HL60 RNA Blot B - HL60 T RNA

Lane a - Cells treated with 17nM TPA

Lane b - Cells treated with 17nM TPA and 1.5μM 13-Cis-retinoic acid

Lane c - Cells treated with 1.5μM 13-Cis-retinoic acid

Lane d - Cells treated with 1.25% DMSO

Lane e - Cells treated with 40mM DMO

The times indicated represent the period of incubation with each inducing agent.

ch - pre-treatment with cycloheximide before incubation with inducer for three hours

This blot was exposed to x-ray film for 20 hours.

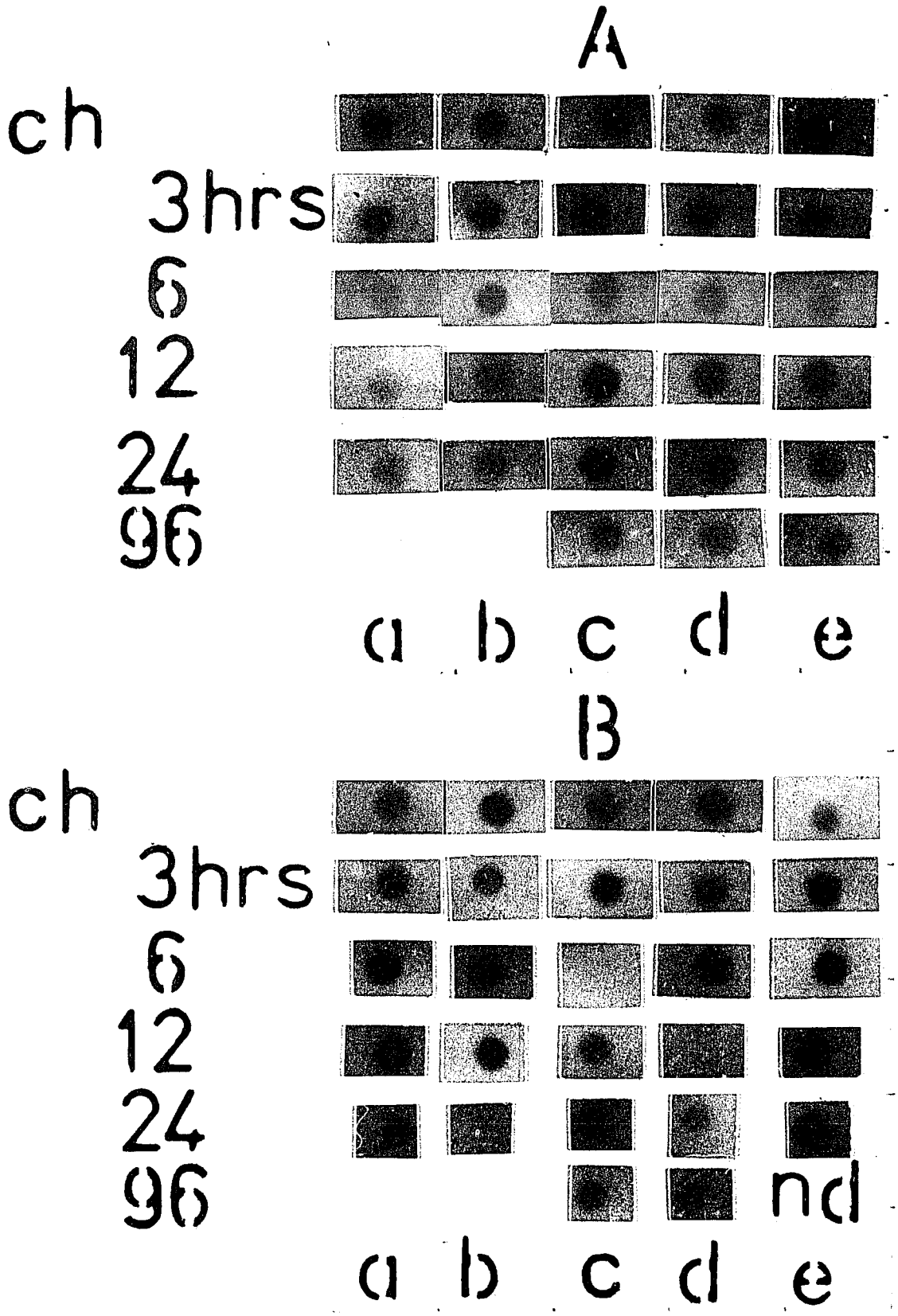

A
ch
3hrs
6
12
24
96
a b c d e
B
ch
3hrs
6
12
24
96
nd
a b c d e

Figure 10. Dot Blot of HL60 and HL60 T Poly A+ RNA Hybridized With c-Ha-ras.

2 μg of Poly A+ RNA isolated from cell cultures described in Table 12 was blotted onto nitrocellulose and hybridized with ^{32}P-labelled C-Ha-ras DNA as described in Materials and Methods.

Blot A - HL60 RNA Blot B - HL60 T RNA

Lane a - Cells treated with 17nM TPA

Lane b - Cells treated with 17nM TPA and 1.5μM 13-Cis-retinoic acid

Lane c - Cells treated with 1.5μM 13-Cis-retinoic acid

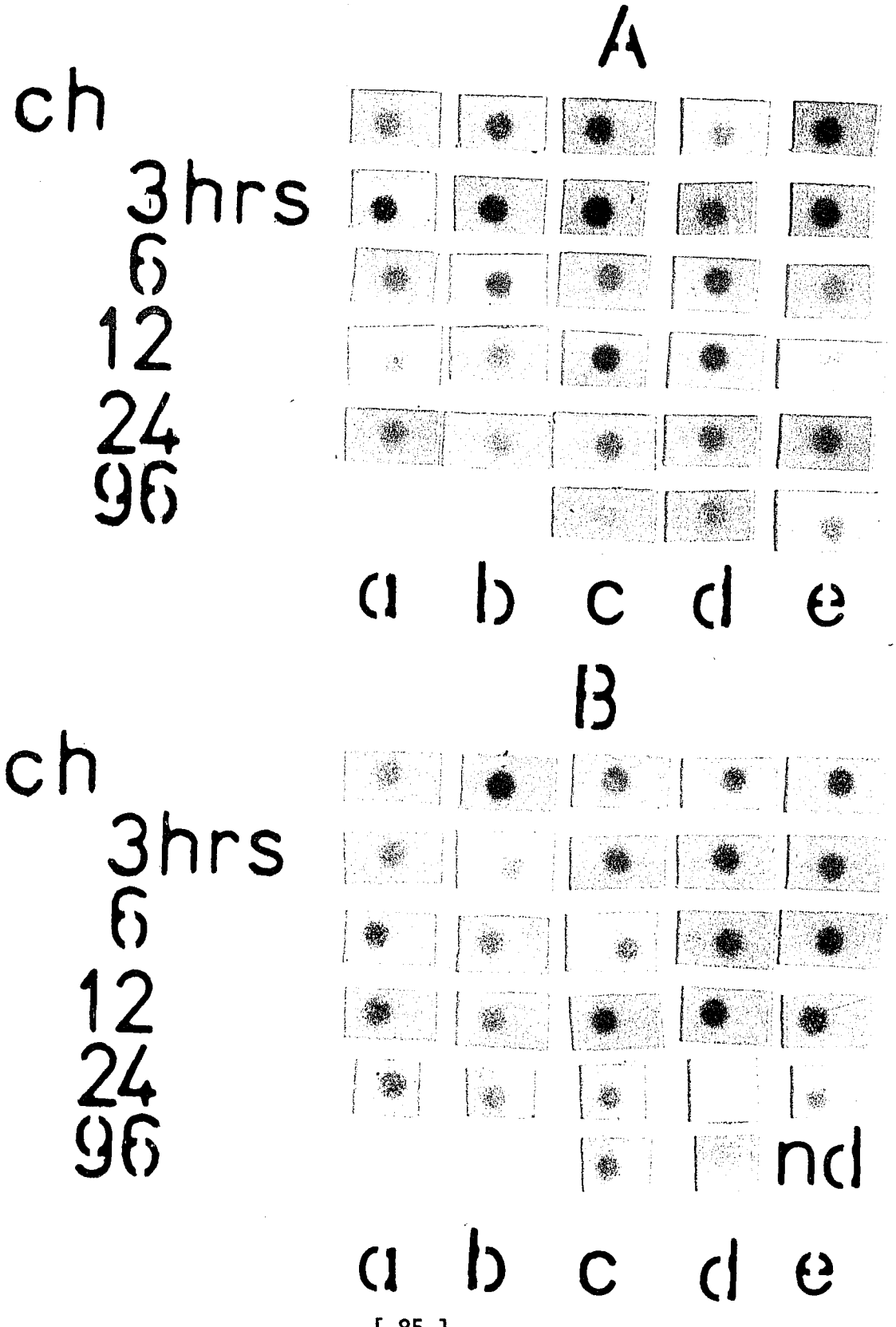
Lane d - Cells treated with 1.25% DMSO

Lane e - Cells treated with 40mM DMO

The times indicated represent the period of incubation with each inducing agent.

ch - pre-treatment with cycloheximide before incubation with inducer for three hours

This blot was exposed to x-ray film for 20 hours.


A
ch
3hrs
6
12
24
96
a b c d e
B
ch
3hrs
6
12
24
96
nd
a b c d e

ras gene. Even though this gene is methylated to a greater extent in HL60 T cells this has not manifested itself in a large reduction of the level of c-Ha-ras poly A+ RNA as compared to HL60 cells.

The level of c-fos poly A+ RNA is shown in Figure 11. Previous investigators have established the induction of c-fos expression in HL60 cells after incubation with TPA, which has been implicated as playing a role in triggering monocytic differentiation (Muller, et.al., 1984). The expression of c-fos has also been shown to be further augumented by cycloheximide treatment of HL60 cells before and during TPA treatment (Mitchell, et.al., 1985). The results in Figure 11 agree with these previous findings and also show that the other inducers tested had little effect on the level of c-fos poly A+ RNA. HL60 T cells behaved in a similar manner to HL60 cells except that the level of c-fos poly A+ RNA after incubation with cycloheximide and TPA, and TPA alone was much greater. In addition, incubation with DMSO seems to increase expression after six hours of exposure. Even though HL60 T cells are unresponsive to the inducing effects of TPA, there is no difference in the modulation of the level of c-fos poly A+ RNA upon TPA exposure between HL60 and HL60 T cells.

Figure 12 reveals substantially higher levels of c-myb poly A+ RNA in HL60 T cells when compared to HL60 cells as well as the levels of the other oncogenes already mentioned. This blot was exposed to x-ray film for only five hours so that the levels of poly A+ RNA are much higher when compared to the previous blots which were exposed for twenty hours. The level of poly A+ RNA is

Figure 11. Dot Blot of HL60 and HL60 T Poly A+ RNA Hybridized With c-fos.

2 μg of Poly A+ RNA isolated from cell cultures described in Table 12 was blotted onto nitrocellulose and hybridized with ^{32}P-labelled c-fos DNA as described in Materials and Methods.

Blot A - HL60 RNA Blot B - HL60 T RNA

Lane a - Cells treated with 17nM TPA

Lane b - Cells treated with 17nM TPA and 1.5μM 13-Cis-retinoic acid

Lane c - Cells treated with 1.5μM 13-Cis-retinoic acid

Lane d - Cells treated with 1.25% DMSO

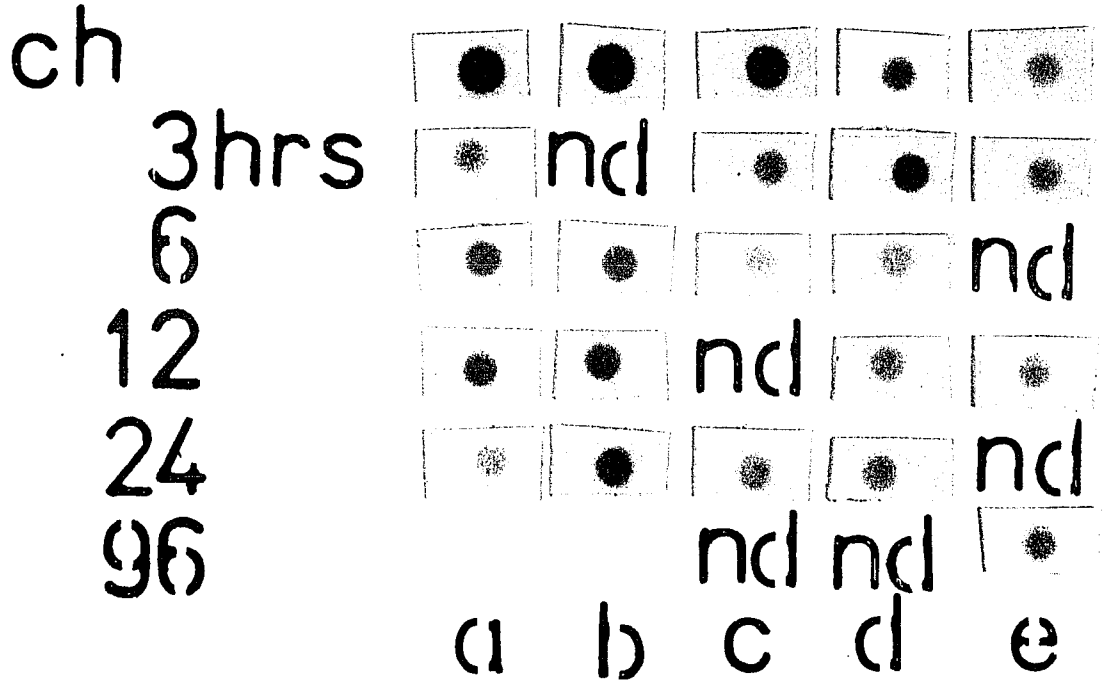
Lane e - Cells treated with 40mM DMO

The times indicated represent the period of incubation with each inducing agent.

ch - pre-treatment with cycloheximide before incubation with inducer for three hours

This blot was exposed to x-ray film for 20 hours.

A


ch
3hrs
6
12
24
96
ncl
ncl
ncl
ncl
ncl
ncl
a b c d e

B

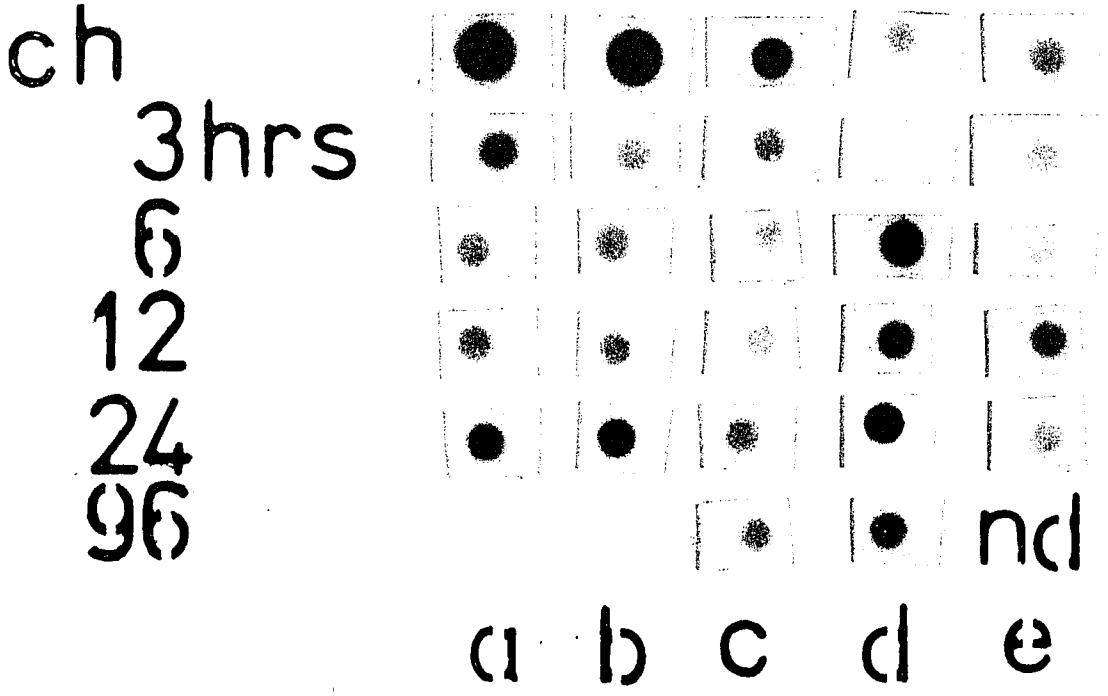

ch
3hrs
6
12
24
96
ncl
a b c d e

Figure 12. Dot Blot of HL60 and HL60 T Poly A+ RNA Hybridized With v-myb.

2 μg of Poly A+ RNA isolated from cell cultures described in Table 12 was blotted onto nitrocellulose and hybridized with ^{32}P-labelled v-myb DNA as described in Materials and Methods.

Blot A - HL60 RNA Blot B - HL60 T RNA

Lane a - Cells treated with 17nM TPA

Lane b - Cells treated with 17nM TPA and 1.5 μM 13-Cis-retinoic acid

Lane c - Cells treated with 1.5μM 13-Cis-retinoic acid

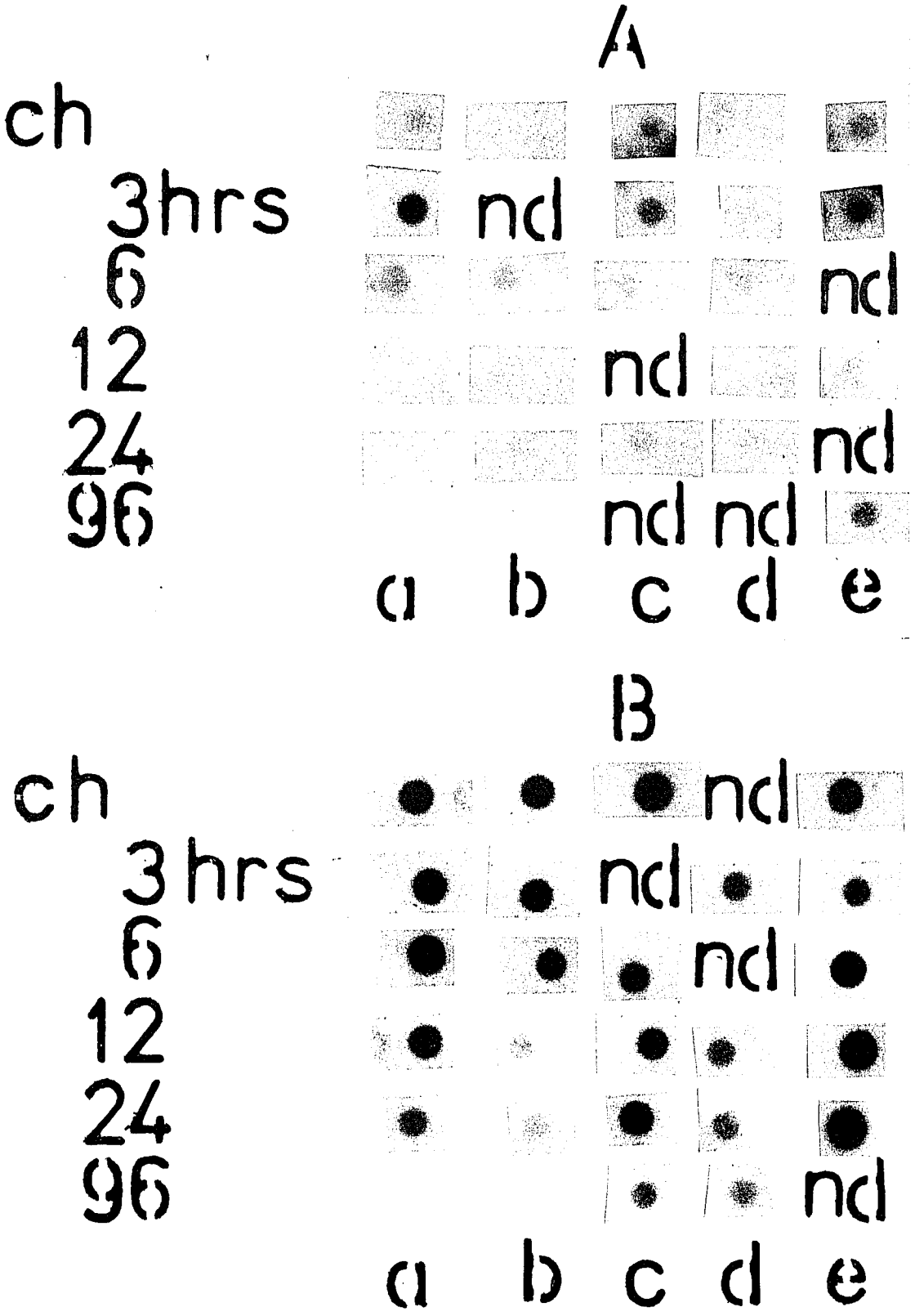
Lane d - Cells treated with 1.25% DMSO

Lane e - Cells treated with 40mM DMO

The times indicated represent the period of incubation with each inducing agent.

ch - pre-treatment with cycloheximide before incubation with inducer for three hours

This blot was exposed to x-ray film for five hours.

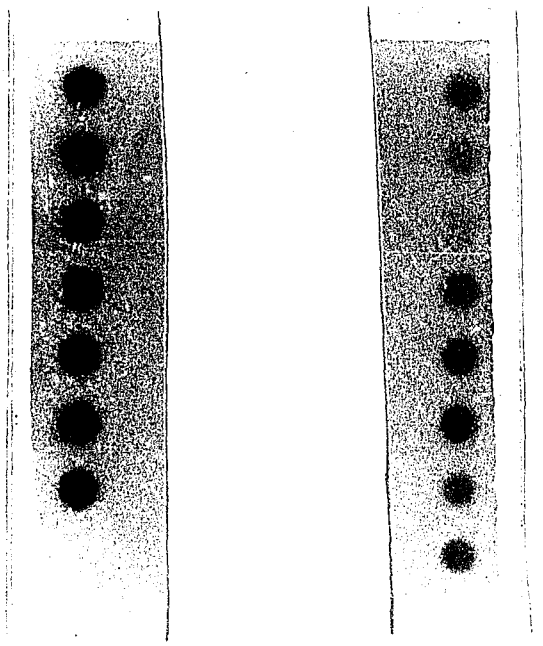

A
ch
3hrs
6
12
24
96
ncl
a b c d e
B
ch
3hrs
6
12
24
96
ncl
a b c d e

very high in HL60 T cells under all treatments while HL60 cells have a much lower level of c-myb poly A+ RNA that is decreased with extended incubation with inducing agents. However, if one looks exclusively at the HL60 T cell line, the level of c-myb poly A+ RNA is slightly decreased after incubation with DMSO for twenty four hours and 13-Cis-retinoic acid for four days. In addition, incubation with 13-Cis-retinoic acid and TPA leads to a slight decrease beginning at six hours. The difference in the levels of c-myb poly A+ RNA between the HL60 and HL60 T cell lines is very significant and implicates the suppression of c-myb expression after incubation with inducing agents as a critical event in the terminal differentiation of HL60 cells. The fact that HL60 T cells maintain a high level of c-myb poly A+ RNA that does not seem to be responsive to the effects of inducing agents may play a significant role in the inability of these agents to induce terminal differentiation.

A poly A+ RNA dot blot probed with human β-actin was used as a control for RNA concentration (Figure 13). Equal amounts of poly A+ RNA as determined by absorbance at 260 nm was applied to each dot. Densitometric quantification of the dots are in progress but it appears the amount of poly A+ RNA blotted from HL60 T cell cultures was half the amount blotted from HL60 cells. However, the concentration of my RNA samples were confirmed by a method utilizing the annealing of tritiated labelled poly U to poly A+ RNA (Bishop, et.al., 1974). Concentrations determined by A_{260} readings and this method were equivalent for the most part. The

Figure 13. Dot Blot of HL60 and HL60 T Poly A+ RNA Hybridized With β-Actin.

2 μg of Poly A+ RNA isolated from cell cultures described in Table 12 was blotted onto nitrocellulose and hybridized with ^{32}P-labelled β-actin DNA as described in Materials and Methods.
Lane a - HL60 RNA Lane b - HL60 T RNA.
These two lanes are representative of the RNA dots from cell cultures treated with all the inducers previously mentioned.
This blot was exposed to x-ray film for five hours.



a b

concentration of a few of the RNA samples were different by 20% at most. Thus, the β-actin blot reveals that HL60 and HL60 T cells are producing different levels of β-actin.

DISCUSSION

The biochemical pathways involved in the normal regulation of cellular proliferation and differentiation are poorly understood resulting in a lack of guidance in devising ways to reverse the neoplastic transformation of eukaryotic cells. The HL60 and HL60 T cell lines provide an invitro model system for the study of parameters critical to the initiation of cellular differentiation. The studies presented here have centered on three specific aspects of the induction of HL60 and HL60 T cells: 1) the effect of inducers on cytoplasmic ion fluxes; 2) the effect of inducers on the cell membrane; and 3) the effect of inducers on DNA methylation and cellular oncogene expression.

The study of the role of cytoplasmic ion fluxes in cellular maturation led to the identification of DMO as a new inducer of HL60 cells. Levenson, et.al.(1980) have suggested that early changes in ion fluxes are a major contributor to the regulation of murine erythroleukemia cell differentiation. The use of DMO and other weak acids and bases in my studies disproves this contention as it relates to HL60 cells or else suggests that the initiation of cellular differentiation involves a complicated process that entails other biochemical events in addition to changes in ion fluxes. DMO, like other inducers of granulocytic differentiation, initiates a number of early biochemical changes in HL60 cells but the continuous presence of these agents for extended periods of time is necessary for the full commitment to terminal differentiation. If the initiation of transient ion fluxes were the integral

event in the induction of cellular differentiation, a short period of exposure to DMO should be sufficient to achieve these results which was found not to be the case.

The biochemical responses elicited by exposure to DMO and other inducers of granulocytic and monocytic differentiation are lacking in HL60 T cells or else are not sufficient to induce differentiation of this cell line. HL60 T cells are blocked from maturing along their normal developmental pathway at a point different from that of the HL60 cell line yet the biochemical events precipitated by exposure to 13-Cis-retenoic acid promote cellular differentiation in both cell lines.

The effect of inducers on the cell membrane centered on the appearance of two cell surface antigens specific for mature human neutrophils and eosinophils in induced HL60 and HL60 T cells. The granulocytic differentiation of HL60 cells after treatment with DMO was confirmed by the appearance of the neutrophil specific cell surface antigen recognized by the BH2-C6 monoclonal antibody (Pytowski, et.al., in press). In addition, the unresponsiveness of the HL60 T cell line to the inducing effects of DMO was also verified.

The IE8 monoclonal antibody was found to recognize a plasma membrane antigen that was indicative of the responsiveness of HL60 cells to TPA (Calderon, et.al., 1987). This antibody had originally been intended to study the eosinophilic differentiation of HL60 cells but instead has led to an ongoing investigation of this cell surface antigen as a marker for the responsiveness of immature or

transformed hematopoietic cells to TPA and other tumor promoters. TPA induces monocytic differentiation of HL60 cells but the appearance of the IE8 antigen did not correlate to this maturation process as evidenced by the absence of surface antigen after incubation with 1,25 dihydroxyvitamin D_3. Incubation with other phorbol ester analogues revealed that the tumor promoting activity of TPA was essential for promoting the appearance of the IE8 antigen. Tumor promoting activity is a consequence of the binding of phorbol esters to protein kinase C which implicates the activation of this enzyme as a critical event in the production of this cell surface antigen.

HL60 T cells, which are resistant to the inducing effects of TPA, did not exhibit reactivity with the IE8 monoclonal antibody after treatment with TPA. In addition, the inhibitory factor produced by the HL60 T cell line can block the TPA promoted appearance of this cell surface antigen in HL60 cells. The amount of protein kinase C and the binding of TPA to this protein is the same in both the HL60 and the HL60 T cell lines (R. Estensen, personal communication). This suggests that a part of the cascade of biochemical events that occurs in HL60 T cells after the binding of TPA to protein kinase C has been altered in some way so that TPA exposure fails to promote the appearance of this cell surface antigen. Another interesting question is whether this defect also contributes to the unresponsiveness of the HL60 T cell line to the inducing effects of TPA.

Future experiments will concentrate on the purification of

the IE8 antigen and the study of whether this protein is indicative of the responsiveness of HL60 and other eukaryotic cells to TPA and the activation of protein kinase C. In addition, the gene coding for this protein will be cloned in order to study the direct effect of TPA exposure on the regulation of expression of this gene.

The results obtained from the study of inducer related effects on oncogene expression in HL60 and HL60 T cells have implicated the suppression of c-myb messenger RNA levels as a critical event in the process of commitment to terminal differentiation. Koeffer (1986) concluded that certain myeloid cell lines display invariant expression of c-Ha-ras, c-Ki-ras and c-abl, a decline in c-myc and c-myb with induction and a biphasic response with c-fos and c-fms, which initially rise and then fall with inducers of monocytic differentiation. The levels of c-fos and c-fms messenger RNA have been shown to transiently increase in HL60 cells induced to differentiate along the monocytic developmental pathway while c-myc and c-myb gene expression declines with granulocytic and monocytic differentiation (Westin, et.al., 1982, Muller, et.al., 1984, Mitchell, et.al., 1985, Sariban, et.al., 1985, Koeffler, 1986).

Recent evidence indicates that v-myb encodes a trans-acting DNA binding protein (Molling, et.al., 1985, Klempnauer, et.al., 1986) and expression of the cellular homologue of this gene occurs predominately in cells of all the hematopoietic lineages. However, in each lineage c-myb messenger RNA levels are much greater in immature cells than in more differentiated cells (Westin, et.al.,

1986, Westin, et.al., 1982). Studies also indicate that v-myc encodes a nuclear protein which localizes with ribonucleoprotein particles (Spector, et.al., 1987) and the expression of the cellular homologue of this gene is associated with the proliferative state of a variety of different cell lines including those of hematopoietic origin (Gonda, et.al., 1982, Hann, et.al., 1983).

Ramsay, et.al., (1986) have reported that the continued suppression of c-myb messenger RNA levels is critical for the commitment of murine erythroleukemia cells (MELC) to terminally differentiate after induction with hexamethylenebisacetamide (HMBA). MELC exhibit a decrease in c-myc and c-myb messenger RNA in the period immediately after exposure to HMBA when the cells are not yet committed to differentiation. The level of c-myc returns to control levels after cell commitment but c-myb messenger RNA levels remain suppressed throughout this period. Dexamethasone inhibits HMBA induced differentiation but does not block the initial decrease in c-myc or c-myb levels. However, with prolonged exposure, c-myb messenger RNA levels reaccumulates to control levels. Thus the continued suppression of c-myb levels may be an important factor in the irreversible commitment of MELC to terminal differentiation.

Symonds, et.al., (1986) have studied the effect of transformation of a chick myelomonocytic cell line with v-myb and v-myc and have reported that doubly transformed cells attain an intermediate phenotype that is characteristic of neither v-myb nor v-myc transformation. Cells transformed by v-myc resemble macrophages, where-

as cells transformed by v-myb appear similar to myeloblasts which can be induced by TPA to differentiate to mature macrophages. Cells transformed by both c-myc and c-myb are resistant to the inducing effects of TPA.

These results are contradicted by the work of Ness, et.al., (1987) who also studied the transformation of chick myelomonocytic cells with v-myc and v-myb. Their results indicated that v-myb is dominant over v-myc and that, while v-myc only induces cell proliferation without influencing differentiation, the v-myb oncogene affects both growth and differentiation in these cells. Transformation with v-myc leads to the appearance of macrophage-like cells while v-myb transformation results in less mature myeloblastic cells. The activity of v-myb is dominant to that of v-myc and is able to alter the differentiation program of these cells as evidenced by the appearance of myeloblastic cells when v-myc transformed cells are superinfected with v-myb. Different cell systems are cited as a possible explanation for the different results obtained by Symonds, et.al., who used a cell line that was growth factor-independent and which continuosly generated more differentiated, adherent cells.

The results obtained in the study of oncogene expression in HL60 and HL60 T cells also suggests that c-myb expression plays a critical role in the differentiation of HL60 cells. The levels of c-myc, c-Ha-ras and c-fos are modulated in a manner that has been reported by other investigators in a cell line that undergoes cellular differentiation (HL60) and a cell line that does not

(HL60 T). This suggests that these changes in messenger RNA levels are not sufficient to induce terminal differentiation. However, the significantly increased levels of c-myb messenger RNA levels in HL60 T cells indicate a critical role for the level of c-myb messenger RNA in the control of cellular differentiation as already postulated by Ramsay, et.al. and Ness, et.al. This high level of expression is not due to an amplified c-myb gene and incubation with standard inducers of differentiation apparently does not result in a decrease in the level of messenger RNA that is sufficient to promote the commitment to terminal differentiation.

The possible role of DNA methylation and S-Adenosylmethionine levels in altering gene activity was also studied in HL60 and HL60 T cells during inducer mediated differentiation. The methylation of CCGG sites of oncogene sequences in HL60 and HL60 T DNA did not seem to be altered by incubation with inducing agents. However, the c-Ha-ras gene did exhibit a higher degree of methylation in HL60 T cells which did not correlate to significant differences in the levels of c-Ha-ras poly A+ RNA when compared to HL60 cells.

The results obtained from my study of S-Adenosylmethionine levels showed a decrease in the level of this compound in both HL60 and HL60 T cells after incubation with all the inducers tested. This result is very significant because it reveals that all of these inducers are affecting both cell lines and influencing an important biochemical pathway. However, this change is not sufficient to induce cellular differentiation. Even though SAM levels decrease in HL60 T cells after exposure to DMSO, DMO, and benzoic

acid, this does not promote cellular maturation.

In summary, these results reveal that the initial steps in the response to inducing agents seems to be intact in both cell lines. The modulation of the expression of the c-myc, c-fos, and c-Ha-ras oncogenes and the decrease in S-Adenosylmethionine levels after incubation with inducers of differentiation is the same. A step in the program of cellular differentiation beyond these initial biochemical changes is blocked in the HL60 T cell line which can be compensated for by incubation with 13-Cis-retinoic acid and once the block is overcome differentiation is normal. The high levels of c-myb messenger RNA may be responsible for this block in the normal regulation of cellular differentiation.

REFERENCES

Babior, B.M., N. Engl. J. Med., 298: 659-668, 1978

Bar-Shavit, Z., Teitelbaum, S.L., Reitsma, P., Hall, A., Pegg, L.E., Trial, J. and Kahn, A.J., Proc. Natl. Acad. Sci., USA, 80: 5907-5911, 1983

Bishop, J.M., Rosbash, M. and Evans, D.J., J. Mol. Biol., 85: 75-86, 1974

Bishop, J.M., Ann. Rev. Biochem., 52: 310-354, 1983

Breitman, T.R., Collins, S.J. and Keene, B.R., Blood, 57: 1000-1004, 1981

Calderon, T., Kirtane, Y., Easton, T., Michl, J. and Christman, J.K., J. Cell Biol., 103: 510a, 1986

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y., J. Biol. Chem., 257: 7847-7851, 1982

Christman, J.K., Price, P., Pedrinan, L. and Acs, G., Eur. J. Biochem., 8: 53-61, 1977

Cline, M.J., The White Cell, Cambridge, Ma., Harvard, 1975

Collins, S.J., Gallo, R.C. and Gallagher, R.E., Nature, 270: 347-349, 1977

Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C., Proc. Natl. Acad. Sci., USA, 75: 2458-2468, 1978

Collins, S.J., Ruscetti, F.W., Gallagher, R.E. and Gallo, R.C., J. Exp. Med., 149: 969-974, 1979

Creusot, F., Acs, G. and Christman, J.K., J. Biol. Chem., 257: 2041-2048, 1982

Damji, N., Khoo, K.E., Booker, L. and Browman, G.P., Am J. Hematol., 21: 67-78, 1986

Defeo-Jones, D., Scolnick, E., Koller, R. and Dhor, R., Nature, 306: 707-709, 1983

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devane, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.W., Science, 221: 275-277, 1983

Doskocil, J. and Sorm, F., Biochim. Biophys. Acta, 55: 953-959, 1962

Downward, J., Yarden, Y., Mayes, E., Scraie, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D., Nature, 307: 521-527, 1984

Eisenman, R.N., Tachibana, C.Y., Abrams, H.D. and Hann, S.R., Mol. Cell. Biol., 5: 114-126, 1985

Estensen, R.D., White, J.G. and Holmes, B., Nature, 248: 347-348, 1974

Favera, R.D., Wong-Staal, F. and Gallo, R.C., Nature, 299: 61-63, 1982

Fischkoff, S.A., Pollak, A., Gleich, G.J., Testa, J.R., Misawa, S. and Rebor, T.J., J. Exp. Med., 160: 179-196, 1984

Fleit, H.B., Wright, S.D., Durie, C.J., Valinsky, J.E. and Unkeless, J.C., J. Clin. Invest., 73: 516-525, 1984

Friend, C., Scher, W., Holland, J.G. and Sato, T., Proc. Natl. Acad. Sci., USA, 68: 378-382, 1971

Gargus, J.J., Adelberg, E.A. and Slayman, C.W., J. Cell. Physiol., 120: 83-90, 1984

Gillies, R.J. and Deamer, D.W., Curr. Topics Bioenergetics, 9: 63-87, 1979

Gonda, T.J., Sheiness, D.K. and Bishop, J.M., Mol. Cell. Biol., 2: 617-624, 1982

Grosso, L.E. and Pitot, H.C., Cancer Letters, 22: 55-63, 1984

Hann, S.R., Abrams, H.D., Rohrschneider, L.R. and Eisenman, R.N., Cell, 34: 789-798, 1983

Harold, F.M., Ann. Rev. Microbiol., 31: 181-203, 1977

Hennings, H., Michael, D., Chang, C., Steinhert, P., Holbrook, K. and Yuspa, S.H., Cell, 19: 248-254, 1980

Holliday, R. and Pugh, J.E., Science, 187: 226-232, 1975

Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. and Nishizuka, Y., J. Biol. Chem., 255: 2273-2276, 1980

Kishimoto, A., Takai, Y., Moto, T., Kikkawa, U. and Nishizuka, Y., J. Biol. Chem., 255: 2273-2276, 1980

Klempnauer, K.H. and Sippel, A.E., Mol. Cell. Biol., 6: 62-69, 1986

Koeffler, H.P., Blood, 23: 223-236, 1986

Levenson, R., Housman, D. and Cantley, L., Proc. Natl. Acad. Sci., USA, 77: 5948-5952, 1980

Levenson, R., Macara, I., Cantley, L. and Housman, D., J. Cell. Biochem., 21: 1-8, 1983

Mandel, J.L. and Chambon, P., Nucl. Acids Res., 7: 2081-2103, 1979

Mascioli, D.W. and Estensen, R.D., Cancer Res., 44: 3280-3285, 1984

McGhee, J.D. and Ginder, G.P., Nature, 280: 419-420, 1979

Mendelsohn, N., Gilbert, H.S., Christman, J.K. and Acs, G., Cancer Res., 40: 1469-1474, 1980

Mendelsohn, N., Michl, J., Gilbert, H.S., Acs, G. and Christman, J.K., Cancer Res., 40: 3206-3210, 1980

Mendelsohn, N., Calderon, T., Acs, G. and Christman, J.K., Exp. Cell Res., 148: 514-519, 1983

Mitchell, R.L., Zokas, L., Schreiber, R.D. and Verma, I.M., Cell, 40: 209-217, 1985

Molling, K., Pfaff, E., Beug, H., Beimling, P., Bunte, T., Schaller, H.E. and Graf, T., Cell, 40: 983-990, 1985

Muller, R., Muller, D. and Guilbert, L., Embo J., 3: 1887-1890, 1984

Ness, S.A., Beug, H. and Graf, T., Cell, 51: 41-50, 1987

Nishizuka, Y., Mol. Biol. Biochem. Biophys., 32: 113-135, 1980

Nishizuka, Y., Nature, 308: 693-698, 1984

Nishizuka, Y., Science, 225: 1365-1370, 1984

Perella, F.W., Hellmig, B.D. and Diamond, L., Cancer Res., 46: 567-572, 1986

Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J. and Wigler, M., Cell, 36: 607-612, 1984

Purnell, M.R. and Whish, W.J.D., Biochem. J., 185: 775-777, 1980

Pytowski, B., Easton, T.G., Valinsky, J.E., Calderon, T., Sun, T., Christman, J.K., Wright, S.D. and Michl, J., J. Exp.Med., in press

Ramsay, R.G., Ikeda, K., Rifkind, R. and Marks, P.A., Proc. Natl. Acad. Sci., USA, 83: 6849-6853, 1986

Razin, A. and Riggs, A.D., Science, 210: 604-610, 1980

Reitsma, P.H., Rothberg, P.G., Astrin, S.M., Trial, J., Bar-Shavit, Z., Hall, A., Teitelbaum, S.L. and Kahn, A.J., Nature, 306: 492-494, 1983

Repine, J.E., White, J.G., Clawson, C.C. and Holmes, B.M., J. Lab. Clin. Med., 83, 911-920, 1974

Ridgeway, E.B., Coilkey, J.C. and Jaffe, L.F., Proc. Natl. Acad. Sci., USA, 74: 623-627, 1977

Roberts, R.J., Gene, 4: 183-193, 1978

Roufa, D., Wu, F.S. and Martonosi, A.N., Biochim. Biophys. Acta, 674: 225-237, 1981

Rovera, G., Santoli, D. and Damsky, C., Proc. Natl. Acad. Sci., USA, 76: 2779-2783, 1979

Rovera, G., O'Brien, T.G. and Diamond, L., Science, 204: 868-870, 1979

Sariban, E., Mitchell, T. and Kufe, D., Nature, 316: 64-66, 1985

Segal, A.W., Lancet, 2: 1248-1252, 1974

Shen, C.J. and Maniatis, T., Proc. Natl. Acad. Sci., USA, 77: 6634-6638, 1980

Sherr, C.J., Rettenmeir, C.W., Sacca, R., Roussel, M.F., Look, A.T. and Stanley, E.R., Cell, 41: 665-676, 1985

Smith, J.B. and Rozengurt, E., J. Cell. Physiol., 97: 441-450, 1978

Smith, R.L., Macara, I.G., Levenson, R., Housman, D. and Cantley, L., J. Biol. Chem., 257: 773-780, 1982

Spector, D.L., Watt, A.R. and Sullivan, N.R., Oncogene, 1: 5-12, 1987

Srinivasan, P.R. and Borek, K.E., Science, 145: 548-553, 1964

Steinhardt, R.A. and Epel, D., Proc. Natl. Acad. Sci., USA, 7: 1915-1919, 1974

Symonds, G., Klempnauer, K.H., Snyder, M., Moscovici, G., Moscovici, C. and Bishop, J.M., Mol. Cell. Biol., 6: 1796-1802, 1986

Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y., Biochem. Biophys. Res. Commun., 91: 1218-1224, 1979

Tanaka, H., Abe, E., Miyaura, C., Shiina, Y. and Suda, T., Biochem. Biophys. Res. Commun., 117: 86-92, 1983

Terada, M., Fujiki, H., Marks, P.A. and Sugimura, T., Proc. Natl. Acad. Sci., USA, 76: 6411-6414, 1979

Uno, I., Mitsuzawa, H., Matsumoto, K., Tanaka, K., Oshima, T. and Ishikawa, T., Proc. Natl. Acad. Sci., USA, 82: 7855-7859, 1985

Watt, R.A., Shatzman, A.R. and Rosenberg, M., Mol. Cell. Biol., 5: 448-456, 1985

Westin, E.H., Gallo, R.C., Arya, S.K., Eva, A., Souza, L.M., Baluda, M.A., Aaronson, S.A. and Wong-Staal, F., Proc. Natl. Acad. Sci., USA, 79: 2194-2198, 1982

Westin, E.H., Wong-Staal, F., Gelman, E.P., Dalla Favera, R., Papas, T.S., Lautenberger, J.A., Eva, A., Reddy, E.P., Tronick, S.R., Aaronson, S.A. and Gallo, R.C., Proc. Natl. Acad. Sci., USA, 79: 2490-2494, 1982

Winkler, M.M., Steinhardt, R.A., Grainger, L.F. and Minning, L., Nature, 287: 558-560, 1980

Zabos, P., Kyner, D., Mendersohn, N., Schreiber, C., Waxman, S., Christman, J.K. and Acs, G., Proc. Natl. Acad. Sci., USA, 1978