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A Study Of Differentiation Using HL60 Cells As A Model.

Lynn Shona Mitchell

Thesis submitted for the degree of Doctorate of Philosophy in the University of Glasgow, being an account of research conducted at the Beatson Institute for Cancer Research, Glasgow.

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ABBREVIATIONS

A	Adenine
ALL	Acute lymphoid leukaemia
ANL	Acute-non lymphoblastic leukaemia
ATP	Adenosine triphosphate
bp	base pairs
C	Centigrade
cDNA	complementary DNA
CFU-GM	Granulocyte macrophage colony forming unit
CIP	Calf intestinal phosphatase
CLL	Chronic lymphoid leukaemia
CML	Chronic myeloid leukaemia
CsCl	Caesium chloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEP	Diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
ds-	double stranded
dTTP	deoxythymidine triphosphate
EDTA	Ethylene-diamine-tetra-acetic acid
FACS	Fluorescence activated cell sorting
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
hnRNA	heterogeneous ribonucleic acid
HMG	High mobility group
HSTF	Heat-shock transcription factor
HTF	Hpa II tiny fragment
IL-3	Interleukin -3
IPTG	Isopropyl-B-D-thio-galactopyranoside
KCl	Potassium chloride
LiCl	Lithium chloride
M-CSF	Macrophage colony stimulating factor
MgCl	Magnesium chloride
MOPS	Sodium Morpholinopropane sulphonic acid
mRNA	messenger RNA
NaCl	Sodium chloride
NBT	Nitroblue tetrazolium

NSE	Non-specific esterase
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RA	Retinoic acid
RNA	ribonucleic acid
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulphate
ss-	single stranded
SSC	Sodium chloride and sodium citrate
TPA	12-O-tetradecanoyl 13-phorbol acetate
tRNA	transfer RNA
UPE	upstream promoter element
X-Gal	5-Bromo-4-chloro-3-indolyl-B-D-galactocide

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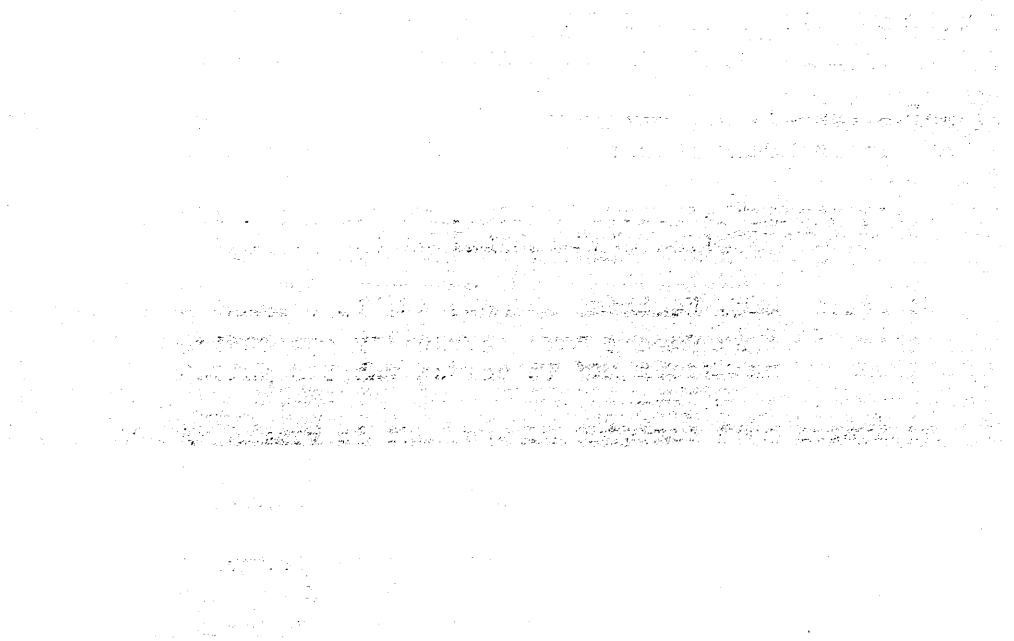

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ABSTRACT

HL60 is a human promyelocytic, leukaemic cell line. The cells are bipotent in culture; treatment with retinoic acid (RA) or dimethyl sulphoxide (DMSO) induces HL60 cells to differentiate to granulocytes whereas treatment with 12-O-tetradecanoyl 13-phorbol acetate (TPA) induces these cells to differentiate to monocytes / macrophages.

Differential screening of a cDNA library representing poly(A)$^+$ RNA from RA induced HL60 cells led to the selection of three different probes for sequences which change in abundance following differentiation of HL60 cells (F6, F10, C6). F6 RNA is highly abundant in uninduced cells but not in induced HL60 cells. Sequencing of F6 cDNA revealed that it consisted of an Alu repetitive element. Both F10 and C6 RNAs increase in abundance during differentiation of HL60 cells to granulocytes. In situ hybridisation indicated that F10 RNA represents a differentiation stage-specific transcript of the myeloid lineage. A probe for C6 RNA detected two transcripts. Analysis of the distribution of these RNAs between nuclear and total RNA isolated from RA induced HL60 cells suggested that the larger transcript is an unprocessed precursor of the smaller C6 RNA.

The timing of changes in expression of these three genes, together with c-myc and lysozyme, has been determined during the induced differentiation of HL60 cells. This has been compared to the timing of the commitment event, as determined by examination of parameters which assess the cell's potential to self-renew and their differentiation status. It appears that c-myc expression bears a direct relationship to proliferation and differentiation of TPA induced HL60 cells. However, no such relationship was observed during DMSO and RA induction. A direct relationship was also observed between C6 and F10 expression and differentiation of HL60 cells to granulocytes.

CHAPTER I : INTRODUCTION

1 CELLULAR DIFFERENTIATION

A) Stem Cells and Commitment to Differentiation

The process of differentiation generates heterogeneous cell populations, crucial to the survival of the multicellular organism. These cell populations are characterised by their unique protein content or phenotype which is essential to the specific functions they perform. For example, in muscle cells the presence of actin, myosin and other proteins contributing to the cytoskeletal and muscle fibres give the cells contractile properties. Similarly, haemoglobin in mature erythrocytes enables these cells to implement oxygen / carbon dioxide gaseous exchange throughout every tissue.

All cells from an individual organism contain the same genetically encoded information. The unique phenotypes displayed by mature, differentiated cells are therefore brought about by differential gene expression. For this to occur in the highly organised manner observed during embryonic development and throughout adult life, strict, intracellular control of gene expression must operate. Determination of the mechanisms for the regulation of differentiation programmes is therefore of vital importance to our understanding of both normal and aberrant development, as it is believed that neoplastic transformation is the result of disruption of normal regulatory processes of differentiation (Sachs, 1980).

i) Stem Cells

The life span of an individual cell is almost always shorter than that of the multicellular organism. Some tissues such as epithelium and blood consist of short-lived cells and therefore to maintain a normal tissue architecture, the loss of cells through natural wastage or damage must be compensated. Most mature cells are incapable of proliferation, therefore to meet this demand a relatively small population of primitive stem cells exist. Other cell types are much longer lived such as brain cells. These cells exist throughout the life-span of the multicellular organism. It is believed that these cells also arise from stem cells during the process of development. Stem cells were originally demonstrated in the bone marrow. In 1961,

Till and McCulloch observed that following total body irradiation of mice, causing the destruction of bone marrow cells, an inoculum of marrow cells from a donor could rescue the animal from death. From analysis of the cells that formed colonies in the spleens of the recipient mice it was proposed that the initial inoculum contained primitive cells capable of self-renewal and differentiation processes which enabled the full restoration of the haematopoietic system (Till and McCulloch, 1961).

Recently, stem cell existence has also been demonstrated in other tissues. Ponder et al (1985) used chromosomal markers to show that adult mouse intestinal epithelium originates from a single stem cell at the base of each intestinal crypt. Stem cells have also been demonstrated in muscle cell populations (Smith Quinn et al, 1985). Difficulties in culturing early progenitor cells and the problem of identifying them has made the search for stem cells from other tissues more complex.

Stem cells are pluripotent; they possess the ability to self renew but also produce progeny capable of development into specialised, differentiated cells. It is believed that early in embryonic development embryonic cells become determined or committed and are laid down as lineages of pluripotent stem cells from which all tissues of the organism are derived. These stem cells proliferate and their progeny differentiate, gradually activating genes that encode the specific proteins of that cell type. Stem cells are differentiated in the sense that the developmental options open to their progeny are restricted to specific lineages of development. Hence a haematopoietic stem cell is committed to produce progeny which differentiate down the haematopoietic differentiation pathway only. Little is known about the process resulting in the conversion of embryonic cells to stem cells. However, all stem cells address the same problem during embryonic development; each must migrate to its final destination in the embryo, and once there it must proliferate and its progeny differentiate into terminally differentiated cells (Hall, 1983). From very small numbers of stem cells the tens of thousands of cells found in the adult are produced through proliferation and differentiation. For example stem cells are believed to represent only 1% of the total population of haematopoietic cells (Till and McCulloch, 1980).

In the embryo, pluripotent cells originate great distances from where they function in the adult. For example, haematopoiesis begins extraembryonically in the blood islands in the yolk sac of the embryo but by birth the major source of haematopoietic stem cells is the bone marrow (Hall, 1983). The stem cells must therefore migrate to the final mature destinations found in the adult. Stem cells from different lineages migrate synchronously during early embryonic development indicative of common differentiation mechanisms operating at early developmental stages (Hall, 1983; Greaves, 1982). With migration the stem cells simultaneously undergo extensive and rapid proliferation. However, it has been shown that stem cell differentiation will not occur unless the cell reaches the appropriate microenvironment. For example, haematopoiesis is believed to be instructed by cells in the bone marrow stroma (Dexter and Allen, 1983). Therefore it can be surmised that the microenvironment must provide support, either structurally or instructively, to initiate appropriate differentiation.

ii) Commitment to Differentiation

Mature cells appear to develop from a stem cell by way of a lineage of intermediate stages of differentiation. These lineages initiate at division of the stem cell producing progeny which either become committed to differentiate or remain in the stem cell pool and retain the potential to self-renew (Johnson and Metcalf, 1977). It is believed that a single cellular event occurs when a stimulus initiates the co-ordinated differentiation programme of a cell. This event is called commitment. The stimulus to initiate differentiation could be a factor(s) from the immediate cell microenvironment or the addition of a differentiation inducing agent <u>in vitro</u> (Dexter, 1984). Commitment is an irreversible stage, once a cell becomes committed it has only one option and that is to progress down the pathway leading to the next stage of differentiation (Levenson and Housman, 1981).

Several models have been proposed for the mechanism determining self renewal and commitment. These models have been formulated from studies of the kinetics of the commitment process to terminal differentiation during haematopoiesis. Two of the most widely accepted models are the stochastic or probabilistic model and the instructive model.

a) The Stochastic Model

This model was first proposed by Till et al (1964) to explain data on proliferation of haematopoietic stem cells following spleen colony formation in mice (Till et al, 1964; Till and McCulloch, 1980). They observed that by transferring the cells from spleen colonies to a second irradiated recipient, the distribution of uncommitted stem cells in individual colonies was not uniform; some colonies had very few uncommitted cells whilst others contained a large number of uncommitted cells. They postulated that the stem cell decision to self renew or to become committed was a random or probabilistic event. A transition probability P was assigned to the chance that during any given generation, an uncommitted cell would become committed. The probability of cell self renewal or commitment must however be sensitive to external signals if differentiation is determined in a stochastic manner. For example, differentiation of haematopoietic colonies grown in semi-solid medium can be determined by external influences such as the addition of colony-stimulating factors and it has been proposed that differentiation in vivo may be influenced by interactions with bone marrow stromal cells (Dexter, 1982; Metcalf and Burgess, 1982). This would then indicate that an over lap exists between the stochastic and the instructive models for commitment to differentiation.

The stochastic model has also been favoured by Bennett (1983). Studies of the differentiation of mouse melanoma cells have supported a modified stochastic model of commitment, the on-off stochastic model. It is postulated that stem cell choice between self-renewal and commitment may be probabilistic but the commitment process is reversible at early times. However, later in the differentiation process, a threshold is reached, when an increase in the concentration of regulatory molecules occurs. Cells at this stage become irreversibly committed to a specific terminal differentiation pathway. Others have also argued that progressive factors must also operate during commitment to differentiation down specific cell lineages, which allow the progression of multipotent progenitors to unipotent, terminally differentiated cells (Ogawa et al, 1983).

b) The Instructive Model

This model was proposed by Trentin et al (1967), again formulated on data based on spleen colony formation. It is proposed that progenitor cells are intrinsically flexible and that commitment of stem cells to unipotent terminally differentiated cells is determined by a specific microenvironment and specific regulatory signals. This has been supported by others studying the interactions between pluripotent haematopoietic cells and bone marrow stromal cells (Dexter and Allen, 1983). However, contradictory evidence arises from the ability of haematopoiesis to occur in semi-solid media, in the absense of a structural, supportive microenvironment. In this system one single colony can still give rise to multi-lineage differentiation (Ogawa et al, 1983).

iii) Terminal Differentiation

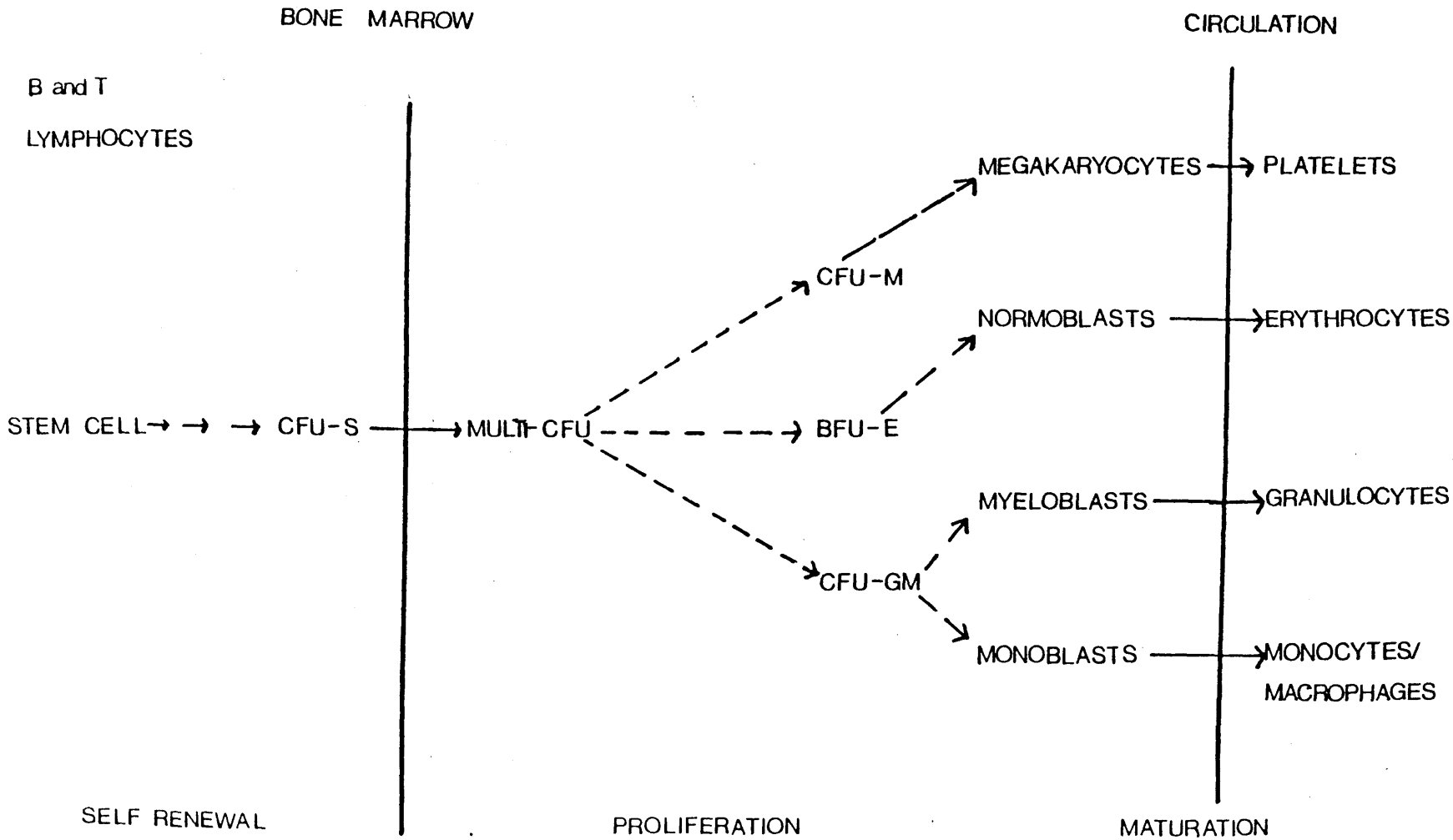
Following the commitment event a specific pattern of differentiation is initiated which results in a terminally differentiated cell, expressing a specific phenotype. However, progression down cell lineages is not totally restrictive. At specific stages the committed cell is faced with further commitment choices, at branch points where the differentiation pathway diverges into sublineages. Figure 1 illustrates the highly divergent nature of the pathway leading to mature haematopoietic cells. At each step of the differentiation lineage there is a progressive narrowing of differentiation potential. Advance towards a terminally differentiated end-point is accompanied by co-ordinated repression and activation of specific gene patterns until the final stages of differentiation are reached. During this progression there must be stages at which crucial molecular and genetic changes occur before the cell progresses to a terminally differentiated end-point. This must involve complex co-ordinated control mechanisms which are regulated by specific elements. With the onset of terminal differentiation the cell loses its proliferative capacity and eventually senesces and dies.

B) Haematopoiesis as an Example of a Differentiation System

Haematopoiesis, the differentiation system leading to the formation

Figure 1 Schematic representation of a model for haematopoietic cell differentiation.

Abbreviations:- CFU-S, spleen colony forming unit; Multi-CFU, multipotential colony forming unit; CFU-M, megakaryocyte colony forming unit; BFU-E, erythroid burst forming unit; CFU-GM, granulocyte macrophage colony forming unit.


BONE MARROW
CIRCULATION
B and T
LYMPHOCYTES
MEGAKARYOCYTES
PLATELETS
CFU-M
NORMOBLASTS
ERYTHROCYTES
STEM CELL
CFU-S
MULTI-CFU
BFU-E
MYELOBLASTS
GRANULOCYTES
CFU-GM
MONOBLASTS
MONOCYTES/
MACROPHAGES
SELF RENEWAL
PROLIFERATION
MATURATION

of mature blood cells, has been studied in depth because of the many advantages this system possesses when compared to other tissues. One main advantage is the availability of blood. It is also a liquid tissue composed of unicellular components which can be identified. Haematopoiesis occurs throughout adult life therefore stem cells and early progenitors are always present. Coupled to this the development of cell culture systems for the clonal growth of haematopoetic progenitor cells in semi-solid media has enabled differentiation to be critically analysed.

During embryogenesis, haematopoiesis occurs in the yolk sac. The cells then migrate to the foetal liver, and by birth the major source of stem cells has shifted from the liver to the bone marrow. It is here and in the lymphoid organs that haematopoietic stem cell differentiation occurs throughout adult life (Hall, 1983).

The haematopoietic system can be divided into two main compartments, lymphoid and myeloid. However, evidence suggests that a pluripotent stem cell gives rise to both lineages (Till and McCulloch, 1980). Following haematopoiesis in the bone marrow, spleen and lymphoid organs the differentiated cells produced, mature granulocytes, monocytes, erythrocytes, platelets and lymphocytes are released into the peripheral blood where each has a specific function.

i) Haematopoietic Stem Cells

The existence of a common haematopoietic stem cell was first proposed by Till and McCulloch (1961). They observed that bone marrow cells, injected into irradiated mice, migrated to the spleen and formed colonies which were able to self-renew and produce cells that revealed terminal differentiation in the erythroid, granulocyte, monocyte and megakaryocyte lineages. Although haematopoietic pluripotent stem cells only comprise 1% of all haematopoietic cells, they are capable of extensive proliferation and the production of committed progenitors capable of the repopulation of the entire haematopoietic system of an irradiated animal, rescuing it from death (Till and McCulloch, 1980). They also showed that cells from a single spleen colony, when injected into a second irradiated mouse, were also capable of rescuing the animal from death. The spleen colonies produced in the second animal contained all the cells necessary for

reconstitution of the haematopoietic system and all had arisen from the original cell inoculum. This was demonstrated by chromosome marker studies and the examination of different isoenzymic forms of glucose-6-phosphate dehydrogenase (Till and McCulloch, 1980).

Treatment of some forms of leukaemia has put the ability of bone marrow stem cells to repopulate the entire haematopoietic system to practical use. The elimination of the "abnormal" bone marrow is accomplished by radiation treatment. This is followed by transplantation of "normal" bone marrow from a donor. Out growth of "normal" mature haematopoietic cells can then occur rescuing the patient from death. The entire haematopoietic cell system is established following bone marrow transplantation indicative of the haematopoietic stem cell's pluripotency (McCulloch, 1983).

ii) Haematopoietic Cell Lineages

With the development of semi-solid clonal cell culture techniques the study of in vitro haematopoiesis was expanded and elaborated. Initial studies of colonies revealed cells possessing bipotent differentiation. These were termed granulocyte-macrophage colony forming units (CFU-GM) and under appropriate conditions gave rise to blast cells expressing granulocyte or macrophage characteristics (Pluznick et al, 1965). Other, monopotent colony forming units were subsequently identified, for example erythroid colony forming units and megakaryocyte forming units (Stephenson et al, 1971 ; Metcalf and MacDonald, 1975). These cells were postulated to be at an almost terminal position in their respective haematopoietic lineages. More recently, it has been demonstrated that cells in haematopoietic cell colonies are capable of differentiation in more than 3 different lineages, for example GEMM-colonies, revealing differentiation in granulocytic, erythrocytic, monocytic and megakaryocytic lineages (Johnson and Metcalf, 1977). These cells are postulated to be early progenitor cells.

Hence, there exists a hierarchy of multipotent haematopoietic stem cells, the earliest of which have the highest self-renewal capacity. However all these cells are committed to differentiate down specific lineages. As cells move down through the different stages of haematopoietic differentiation a progressive loss of self-renewal

potential concommitant with a narrowing in differentiation potential is observed, until a stage is reached when only one cell type is produced. Figure 1 represents a schematic diagram of the human haematopoietic lineages.

Such experimental observations, have led to the conclusion that the haematopoietic differentiation system can be divided into 3 main compartments (Ogawa et al, 1983).

a) The first, most primitive compartment consists of multipotent haematopoietic stem cells that possess self-renewal capabilities and the ability to generate primitive progenitors that are programmed to differentiate.

b) The second compartment consists of committed cells which proliferate and are responsible for maintaining the level of mature blood cells. These cells are responsive to environmental stimuli, as will be discussed later, and show progressive loss of differentiation potential.

c) The third compartment consists of terminally differentiated cells that do not proliferate.

iii) The Role of the Microenvironment in Haematopoiesis

From studies involving haematopoietic differentiation in long term bone marrow cultures it has been postulated that the stromal cells of bone marrow may play a role in haematopoiesis (Dexter and Lajtha, 1976). In these cultures early and late progenitor cells have been identified which is indicative of the capability of the system to support proliferation and differentiation. Bone marrow stromal cells provide mechanical and functional support, in the form of an adherent multilayer. Stroma consists of many cell types of which adipocytes, fibroblasts and macrophages are examples (Dexter and Allen, 1983). It has been observed that cell-cell specific interactions appear to take place between developing haematopoietic cells and stromal cells in vivo and in vitro. In fact it is postulated that cells that undergo differentiation down specific lineages are found associated with a particular stromal cell type (Dexter and Allen, 1983). Synchrony in development of haematopoietic

cells associated with a single stromal cell has also been reported, indicating that differentiation could be the result of locally administered differentiation stimuli to the surrounding haematopoietic cells. This stimulation could be in the form of various molecular compounds found to stimulate proliferation and differentiation of haematopoietic stem cells, the colony-stimulating factors (Clark and Kamen, 1987).

The importance of the microenvironment in the successful differentiation of haematopoietic stem cells has been highlighted by the condition Steel anaemia, a congenital hypoplastic anaemia of mice. Steel (Sl) mice can not be rescued by the inoculation of normal bone marrow, no spleen colonies are formed. However, by grafting of normal spleen into these animals they recover and develop normal haematopoiesis (Dexter and Moore, 1977). This is indicative of the abnormality being present in the microenvironment in which haematopoiesis takes place and not in the haematopoietic stem cells themselves. Further evidence to support this comes from long-term bone marrow culture assays. When stromal cells from Sl mice are used to form the supportive, adherent multilayering no development occurs in the cultures and the cells die. Nevertheless if normal bone marrow cells are mixed with the bone marrow from Sl mice the culture becomes viable and cell proliferation and differentiation is observed (Dexter and Allen, 1983). However the exact nature of the interactions between differentiating cells and the stroma still remains unclear.

Studies of haematopoietic cell colonies, grown from pluripotent progenitor cells, in semi-solid media, have suggested that the restriction in differentiation potentials of stem cells is intrinsically determined in a stochastic manner (Till, 1964; Ogawa et al, 1983). Others suggest that microenvironmental factors and / or interactions with stromal cells instruct the pathway of differentiation (Trentin et al, 1967; Dexter, 1982) (also see Chapter I, 1, A (ii)). The stochastic model has been favoured as a mechanism for determining commitment to differentiation. However evidence gathered from analyses of stromal associations and interactions with differentiating haematopoietic cells would tend to favour the instructive model. Most of the evidence supporting a stochastic model of commitment to differentiation has come from studies of pluripotent progenitor cells grown as colonies in semi-solid media. Therefore

this raises the question of whether the environmental conditions and the availabilty of factors in these in vitro systems is comparable to that found in vivo (Brown et al, 1987). Alternatively, the influences exerted by stromal cells and the microenvironment encountered by differentiating cells in the bone marrow, may influence the probability of commitment to differentiate hence supporting the stochastic model.

iv) Regulatory Factors of Haematopoiesis

Analysis of events in haematopoietic clonal cell culture led to the recognition that haematopoietic cells are intrinsically incapable of unstimulated cell division. Growth of bone marrow progenitors required the addition of factors to sustain proliferation and differentiation. The sources of these factors were very diverse, for example, placenta, peripheral blood cells and tumour cell lines. The characteristics of many regulatory factors have been determined.

a) Haematopoietic growth factors

In the murine system 4 major growth factors have been identified. Two of these are lineage-specific, granulocyte-colony stimulating factor (G-CSF) and macrophage-colony stimulating factor (M-CSF). Colonies grown in the presence of the former consist largely of neutrophilic granulocytes and their precursors, whereas colonies grown in the presence of the latter mainly consist of macrophages (Stanley and Heard, 1977; Metcalf and Nicola, 1983). G-CSF and M-CSF are postulated to support growth and proliferation of only relatively late progenitors, already committed to their respective lineages. In contrast, the remaining well characterised factors are effectors of growth and differentiation in more than one haematopoietic lineage. Granulocyte-macrophage-CSF (GM-CSF) is found to stimulate colonies to give rise to granulocytes, macrophages and eosinophils, and Interleukin-3 (IL-3), or multi-CSF exposed colonies contain many different cell lineages (Metcalf, 1986). It is proposed that GM-CSF and IL-3 interact with early progenitor cells that are pluripotent and therefore capable of producing mature cells from many lineages. In the human system, a set of 4 analogous factors have also been characterised (Nicola et al, 1985; Yang et al, 1986). This evidence supports the theory of the existence of a hierarchy of progenitor

cells along the various haematopoietic cell lineages. Table 1 summarises the cells that produce colony-stimulating factors and the differentiation end-products produced following colony exposure to these factors.

Other regulatory factors have also been identified, for example erythropoietin is a regulatory growth factor for terminal erythrocyte development and IL-2 and IL-4 regulate B and T lymphocyte growth and proliferation (Arai et al, 1986).

b) The Structure and Expression of Colony Stimulating Factors

The regulatory factors or CSFs are glycoproteins. They are produced by a number of different cell sources, as is described in Table 1. It is believed that the degree of glycosylation can vary greatly producing extensive heterogeneity in the sizes of these factors (Clark and Kamen, 1987). The function of the carbohydrate modifications are unknown. Receptors for CSFs have been identified on responsive cells (Metcalf, 1984).

With the availability of DNA probes for CSFs expression of the CSF genes has been examined. Many different cell types have been shown to express CSFs but only when stimulated, for example, monocytes stimulated by phorbol ester or endotoxin and activated T-cells (Wong et al, 1987). However no CSF gene expression has been detected in normal, unstimulated primary cells. This suggests that factors such as GM-CSF, IL-3 and G-CSF do not play a major role in constitutive haematopoiesis but could act to enhance the production of mature haematopoietic cells when the system is under stress, for example during infection. Nevertheless, it cannot be ruled out that extremely small amounts of these factors are produced locally by bone marrow stromal cells, but have not yet been detected (Clark and Kamen, 1987).

The one exception is M-CSF. M-CSF gene expression is detectable in a variety of primary tissues and cells (Kawasaki et al, 1985; Wong et al, 1987). However this may reflect one of the roles of this molecule in vivo, a survival factor for monocytes and macrophages. These cells leave the blood circulation as monocytes and migrate into tissues where they develop into macrophages. It would therefore be

Colony Stimulating Factor	Cellular Source	Haemopoietic Lineages found in Colonies
G-CSF	Monocytes Fibroblasts	Neutrophils
GM-CSF	T-cells Endothelial cells Fibroblasts	Neutrophils Monocytes / Macrophages Eosinophils Megakaryocytes Erythrocytes
IL-3	T-cells	Neutrophils Monocytes/Macrophages Eosinophils Basophils Erythrocytes Megakaryocytes
M-CSF	Monocytes Fibroblasts Endothelial cells	Monocytes/Macrophages

Table 1 Table of the cellular sources of the human colony stimulating factors.

Abbreviations:- G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; IL-3, interleukin 3; M-CSF, macrophage colony stimulating factor.

important for a variety of cells to produce M-CSF for long-term survival of macrophages (Wong et al, 1987).

v) Malignancy due to Abnormality of the Haematopoietic Differentiation System

The process of haematopoiesis can malfunction giving rise to a number of serious disorders from anaemia to leukaemia. Leukaemias can be lymphoid or myeloid, depending on the cell type the malignancy occurs in, and can be either acute or chronic. Leukaemias are therefore classified into 4 main categories, acute non-lymphoblastic leukaemia (ANLL), acute lymphoid leukaemia (ALL), chronic myeloid or granulocytic leukaemia (CML or CGL) and chronic lymphoid leukaemia (CLL). Leukaemic patient characteristically have poorly differentiated haematopoietic cells in their peripheral blood replacing mature, fully functional cells.

It is widely believed that leukaemias arise as a result of transformation event(s) in pluripotent cells. Transformation of different target cells leads to different types of leukaemia, even within the same haematopoietic lineage. Characteristically, the progeny of these transformed cells accumulate at some point analogous to a recognisable stage in the normal pathway of haematopoiesis (Greaves, 1982). Much of the evidence for this theory comes from studies of lineage-specific and differentiation stage-specific proteins as detected by monoclonal antibodies and cytochemical markers (Fialkow, 1982). It is also widely held that different leukaemias represent the clonal proliferation of a single pluripotent stem cell. Fialkow et al (1977) demonstrated this for ANLL by a study of female patients heterozygous for the X-linked isoenzyme glucose-6-phosphate dehydrogenase. Haematopoietic cell lineages from each patient expressed a single, common glucose-6-phosphate dehydrogenase allele. This has also been shown through studies of the karyotypic abnormality, the Philadelphia chromosome (Ph') (t:22,9), found in CGL. This abnormality was found in basophils, erythroid cells, platelet precursors and macrophages, supporting evidence that a pluripotent stem cell is the transformation target (Greaves, 1982; Zalcberg et al, 1896).

It has been postulated that the transformation of normal cells to

malignancy involves changes in the regulation of gene expression. By examination of the requirement for GM-CSF for proliferation and differentiation of normal and malignant myeloid cells (CGL), it was found that the malignant cells could be induced to differentiate by GM-CSF but had lost the requirement for this factor for cell viability and proliferation (Sachs, 1980). In the presence of GM-CSF proliferation of normal cells would be followed by initiation of differentiation and termination of the differentiation programme. These 3 processes would be controlled co-ordinately in the normal system. However it is postulated that uncoupling of the controls for proliferation and differentiation would result in a malignant phenotype. These cells would have an advantage over normal cellular components because of their unrestrained proliferative capacity. This model therefore proposes that defective or incomplete regulation of proliferation and differentiation is the origin of malignancy (Sachs, 1980).

In samples from leukaemic patients, haematopoietic cells are found "maturation arrested". These cells display cytochemical markers of immature cell types from different early stages of the normal differentiation process. The stage of "maturation arrest" varies between the different classes of leukaemia, and is used as a classification criteria. It is thought that "maturation arrest" occurs because of uncoupling of the proliferation and differentiation pathways. The variations between the stages of "maturation arrest" in different types of leukaemia is thought to be the result of the degree of uncoupling of proliferation and differentiation. If the co-ordinated control of these processes was virtually non-existent it would result in highly proliferative, poorly differentiated cells. Alternatively, if the uncoupling was only partial, more mature cell types would result but would still have proliferative capabilities (McCulloch et al, 1982). Phenotypic characterisation of lymphoid malignancies has provided support for this model for malignant transformation. Several groups have observed that lymphoid tumours correspond phenotypically to various stages in lymphocyte maturation (Greaves et al, 1982; Reinherz et al, 1980).

Aberrant gene expression has also been proposed as a model for transformation of haematopoietic stem cells. This might occur through introduction of genes from an exogenous source, by viral infection,

or spontaneously, by mutation. The evidence for this type of model was derived from the discovery of tumour-specific antigens found on malignant cells but not on normal cells (Fioritoni et al, 1980). These were later discovered to be viral antigens and not tumour-specific markers. Lineage infidelity is also thought to support such a model. For example, K-562 cells, a cell line derived from the peripheral blood cells of a patient suffering from CML, were found to express markers for both erythroid and granulocytic cells. However, this could simply reflect the differentiation stage these cells arrested at due to the original transformation event, at an early stage in haematopoiesis before the branch point which causes the divergence of the erythrocytic and myeloid lineages (Till and McCulloch, 1980).

Many cell lines have been established from the haematopoietic cells of patients suffering from leukaemic disorders. These cells have a high proliferative capacity and invariably show some phenotypic characteristics of immature cells from normal haematopoietic lineages. For example, HL60 cells, established from a patient suffering from acute promyelocytic leukaemia, are classified as promyelocytic, both morphologically and cytochemically (Collins et al, 1977). Similarly, Friend erythroleukaemia cells are of murine erythroblastic origin (Friend et al, 1971). These cell lines possess the ability to differentiate to mature cell types similar to the normal cell, when treated with different agents. For example, HL60 cells can be induced to terminally differentiate to granulocytes or monocytes depending on the inducing agent used (Breitman et al, 1980; McCarthy et al, 1983). Due to these properties these cell lines are frequently used as models for haematopoietic differentiation.

vi) Summary

The process of differentiation leads to variety and specialisation of cells in the multicellular organism. For example, haematopoiesis yields a variety of different cell types, erythrocytes, granulocytes, macrophages, monocytes, megakaryocytes, T and B lymphocytes which can all be derived from the same differentiating clone (Till and McCulloch, 1980). Each cell type is highly specialised to undertake a particular function in the multicellular organism. It can therefore be surmised that stem cells play a pivotal role in differentiation.

Differentiation of haematopoietic cells has been studied in detail, not only as a result of technical convenience but also because of the importance in understanding the mechanisms which operate to create the different haematopoietic cell types; these cells maintain the immune system and gaseous exchange processes, amongst many other features which are crucial to survival. It is also hoped that determination of the control mechanisms which operate during haematopoiesis will aid in the elucidation of the origins of abnormal clones characteristic of leukaemia.

During the differentiation process multi-potent stem cells give rise to progenitors which either retain the parental cell's capacity to self-renew or become committed to differentiate down a particular pathway leading to terminal differentiation. Progress along the differentiation pathway results in changes in gene expression and a decrease in the differentiation potential of the cell. Co-ordinated control mechanisms must operate to regulate patterns of gene expression which are specific to any one stage of differentiation. The elucidation of these control mechanisms is therefore of vital importance to our understanding of normal and aberrant gene expression. To enable the study of these mechanisms model systems of differentiation, such as cell lines which are capable of induced differentiation and *in vitro* culture systems for the long term maintenance of bone marrow cells, have been and will be of vital importance.

2 CONTROL MECHANISMS OF CELLULAR DIFFERENTIATION

In the early 1960s it was established that differentiation is implemented by controls operating on the cell genome. Gurdon demonstrated that nuclei transplanted, from cells of early stages of Xenopus laevis embryos, to enucleated oocytes, allowed those oocytes to develop into tadpoles with relatively high efficiency. Hence, it was postulated that the change in nuclear environment exposed the genes in the transplanted nucleus to different control pressures (Gurdon, 1962). These results also demonstrated that controls operating to determine a particular differentiated phenotype are not permanent but under the appropriate conditions can be altered.

Changes in gene expression during differentiation are reflected in the changes in the mRNA and protein content of the cell. This has frequently been reported during the induced differentiation of haematopoietic cell lines (Colbert et al, 1983; Reyland et al, 1986). These changes must follow co-ordinated relaxation and tightening of control mechanisms throughout the entire genome.

There are many stages on the pathway from gene to protein at which control mechanisms could operate to regulate the final functional protein complement of the cell and hence determine the cells ultimate differentiated phenotype. Figure 2 shows the flow of information from DNA to protein and indicates the stages at which potential control of gene expression could operate.

A) Structural Features of Chromatin Which may Affect Gene Transcription

i) Chromatin Structure

Chromatin, the term given to nuclear DNA and its complement of associated protein and RNA molecules, exists in two forms as demonstrated by electron microscopy studies: transcriptionally poised or active chromatin and transcriptionally repressed or inactive chromatin. Active chromatin, believed to occur at sites of frequent transcriptional activity, appears to have a more "open" structure than that of condensed inactive chromatin (Weisbrod, 1982).

Chromatin appears as a "beaded fibre" when examined by electron microscopy. Enzymic studies reveal that the chromatin exists in the form of repeated units of DNA 146-240 base pairs long, wrapped twice around a nucleosome, the unit which gives the chromatin fibre its beaded appearence. The nucleosome is derived from a histone core; histones are a family of basic proteins found exclusively associated with nuclear DNA. The nucleosome core is constructed from 2 X 4 inner histones, H2A, H2B, H3 and H4. A further histone H1 is associated with linker regions of DNA between nucleosomes. Nucleosomes have been demonstrated in both active and inactive chromatin. However nucleosome formation in vitro has been shown to block initiation of transcription. Therefore mechanisms must operate to allow the cell to

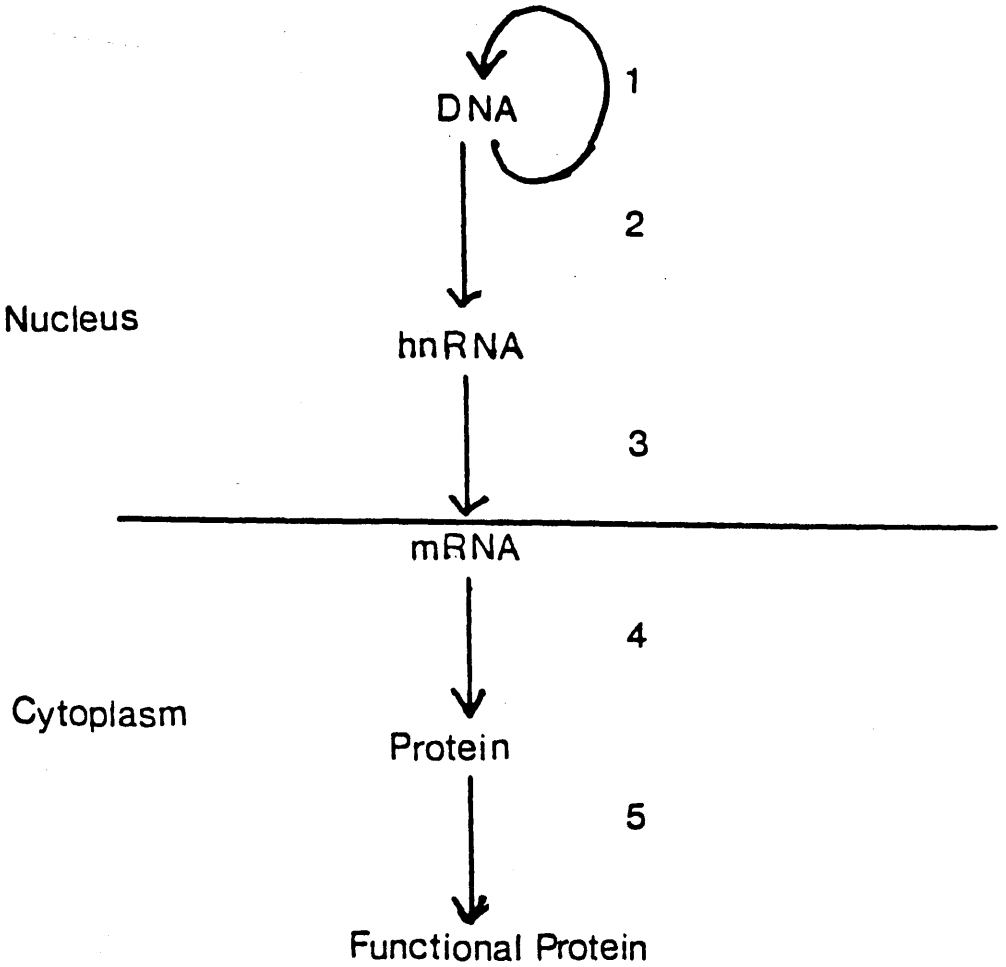


Figure 2 Schematic representation of the stages from the gene to the protein at which potential control of gene expression could operate.

1, Primary and secondary DNA structure; 2, transcription; 3, hnRNA processing; 4, transport/translation; 5, post-translational changes.

transcribe through a nucleosome template (Weintraub, 1985).

It is believed that folding of the chromatin fibre and cross-linking events allow the chromatin to be compacted sufficiently to enable its packaging in the nucleus. Non-histone proteins also aid in chromatin pakaging. These proteins form the scaffold on which the chromosome is constructed. The chromatin fibres are looped around this scaffold and attached to it, allowing further condensation (Weisbrod, 1982).

ii) DNase I Sensitivity

Evidence that chromatin from actively transcribed genes exhibits a structure differing from that of inactive genes developed from the use of endonucleases. Weintraub and Groudine (1976) demonstrated this with DNase I and micrococcal nucleases by examination of their respective capabilities in digesting chick erythrocyte DNA. By determining fragment sizes from the globin gene they discovered that globin DNA was resistant to micrococcal nuclease digestion, a feature of DNA packaged into nucleosomes, but sensitive to DNase I. This led to the suggestion that the globin gene was packaged into nucleosomes but its sensitivity to DNase I indicated that these nucleosomes were conformationally different from most nucleosomes in the chick erythrocyte (Weintraub and Groudine, 1976).

DNase I sensitivity appears to be a tissue specific phenomenon as is demonstrated by the sensitivity of the globin gene to endonuclease activity in erythrocytes but not in brain tissue. Conversely the ovalbumin gene is preferentially sensitive in oviduct and not in the erythrocyte (Garel and Axel, 1976). Stalder et al (1980) also demonstrated that DNase I sensitivity of embryonic and adult β-globin genes changed during development. In mature chick erythrocytes the embryonic β-globin gene is resistant to DNase I digestion but the adult β-globin gene is sensitive. However, in embryonic cells both genes are digested by the endonuclease but only the embryonic gene is expressed. The fact that the adult form of β-globin is sensitive in embryonic cells is indicative of a pre-activation event prior to the stage at which gene expression is required.

DNase I sensitivity appears to be a general phenomenon of actively transcribed genes, but apparently only reflects transcription

potential and not the process of transcription. This has been demonstrated in the chick ovalbumin gene in oviduct. The actively transcribed ovalbumin gene is DNase I sensitive when transcription is induced by the action of steroid hormones. However removal of the hormone stimulus, which results in loss of ovalbumin gene expression, does not affect the DNase I sensitivity of the gene (Palmiter et al, 1977). This is also demonstrated in the Friend erythroleukaemia cell system. These cells are capable of induced differentiation to mature erythrocytes when treated with a variety of agents (Friend et al, 1971). The uninduced cells possess an inactive globin gene which is activated following differentiation. However DNase I sensitivity of the globin DNA does not differ between that observed in the uninduced cell and in the fully differentiated erythrocyte (Miller et al, 1979).

iii) Proteins Associated with Active Chromatin

From endonuclease analyses of chromatin, a structural difference can be demonstrated between those genes capable of transcription and those that remain silent. By examination of the protein moieties released during DNase I digestion of DNA it has been proposed that modified histones, or the presence of non-histone proteins associated with the DNA, results in endonuclease sensitivity.

a) Non-histone Proteins

A family of non-histone proteins, the high mobility group (HMG) are found frequently associated with sites of active transcription. This was first demonstrated by fluorescent antibody detection of HMG proteins associated specifically with the transcriptionally active "puff" regions in salivary gland polytene chromosomes of Drosophila (Mayfield et al, 1978).

HMG proteins are highly conserved throughout evolution, and represent approximately 5% of nuclear proteins. They are slightly acidic and can be easily eluted from chromatin by low salt concentrations. HMGs 14 and 17 can be eluted from chick erythrocyte DNA without any detectable change observed in the gross structure of individual nucleosomes. However the depleted chromatin is no longer DNase I sensitive. Reconstitution of the chromatin by readdition of HMGs 14

and 17 results in the return of endonuclease sensitivity (Weisbrod and Weintraub, 1979; Weisbrod et al, 1980). This is indicative of these proteins playing an active role in the altered structure of active chromatin which could be implemented by protein interaction with the DNA.

From reconstitution studies between HMGs 14 and 17 from brain tissue and depleted chromatin from erythrocytes β globin gene DNase I sensitivity is recovered. This suggests that HMGs are ubiquitous DNA binding factors and not a specific mechanism for gene activation. In fact either HMG 14 or 17 from most cell types can restore DNase I sensitivity to most actively transcribed, HMG-depleted genes (Weisbrod et al, 1980). It is therefore unlikely that such a mechanism would operate to instruct tissue or development-specific transcription. However, the presence of HMGs 14 and 17 at DNase I sensitive sites indicates structural changes in these regions.

Many more non-histone proteins have also been discovered associated with regions of DNA which appear to be important in determining transcriptional activation. Some of these are discussed in Chapter I, 2, B(iii).

b) Histone Proteins

Histone proteins are tightly associated with DNA. The conformation of DNA could therefore be fundamentally altered by modification or variation of the associated histone proteins. Variant forms of all 5 histones have been identified electrophoretically, as have tissue-specific and species-specific subfractions (Insberg, 1978). Changes in histones have also been identified during chick and murine development and during Friend erythroleukaemia cell differentiation (Newrock et al, 1976; Cohen et al, 1979; Grove and Zweilder, 1984). These proteins may play an important part during development and differentiation but their role in these processes is still unclear.

iv) Postsynthetic Modifications of Histone Proteins

Postsynthetic modifications of histones, for example acetylation, methylation and phosphorylation have been described, and a correlation between acetylation of histones and transcriptionally

active chromatin has been reported. For example, exposure of HeLa cells to sodium butyrate produces multiacetlyated forms of histones 3 and 4. This correlates with an increase in detectable DNase I sensitive sites in the cellular DNA (Vidali et al, 1978; Sealy and Chalkley, 1978). It has been postulated that histone acetylation disrupts the overall positive charge of the protein therefore weakening its attraction to DNA. This would make the DNA more accessable to RNA polymerases or nucleases. Although histone acetylation is a characteristic of active genes, its function, if any, in transcriptional activation is still uncertain (Weisbrod, 1982).

v) DNA Modifications

2-7% of the total number of cytosine residues of genomic DNA are in the form 5-methylcytosine. Methylation occurs enzymically, following DNA synthesis, by methyl transfer from S-adenosyl methionine (SAM) to position 5 of cytosine. There appears to be an inverse relationship between methylation of cytosine and gene activation (Razin and Riggs, 1980). The study of this relationship has been aided by the use of specific endonucleases, which recognise the sequence CCGG: Msp I cuts regardless of the methylation status of the internal cytosine residue of this sequence but Hpa II will only cut at unmethylated sites.

Treatment of cells with 5-azacytidine prevents DNA methylation after replication. This treatment is found to activate genes in otherwise non-expressed systems, for example the metallothionien-1 gene in mouse lymphoid cells. Activation of this gene was coincident with demethylation at specific Hpa II sites (Compere and Palmiter, 1981). Genetic inactivation of one X-chromosome occurs in female mammals to ensure that the XX female has an equivalent X complement to the XY male. It has been postulated that methylation acts as an inhibitor of gene activation of the X chromosome and a maintenance mechanism of that state (Monk, 1985).

Sites of undermethylated CpG are frequently demonstrated clustered in G+C rich "islands" which are several hundred base pairs long. These "islands" contain CpG at more than 10 fold its density in bulk DNA (Bird et al, 1985). They have been termed Hpa II Tiny Fragments (HTFs) because of their sensitivity to the Hpa II endonuclease. HTFs

are commonly found associated with the 5' regions of genes in the vertebrate genome. To date most of the associated genes represent those in the "house-keeping" class. It has been proposed that lack of methylation at these sites facilitates transcription of the associated gene but methylation is detrimental to transcription (Gardiner-Garden and Frommer, 1987). For example, the gene for hamster adenine phosphoribosyl transferase, when methylated at the 5' regions where HTFs are situated, is silent. However methylation in the body of the gene does not affect transcription (Stein et al, 1982; Keshet et al, 1985). This suggests that G+C rich "islands" are specifically situated and have a role to play in the regulation of gene activation.

The large majority of HTFs are present upstream from the transcription start site of many genes. However in some "house-keeping" genes, for example glucose-6-phosphate dehydrogenase, HTF clusters are found downstream from the coding region (Bird, 1986). The possibility that some of these 3' islands are actually 5' islands relative to as yet unidentified transcripts can not be excluded or indeed transcription from both DNA strands could account for the apparent presence of 3' G+C rich islands. It can therefore be speculated that the position of CpG islands is critical in "house-keeping" genes.

Others have reported that tissue-specific gene expression can be determined by the degree of methylation of the DNA. Busslinger et al (1983) demonstrated that the globin gene, when methylated at its 5' region, was not expressed in transformed non-erythroid cells. However, when removing a tissue-specific gene from the appropriate cellular environment and placing it in a system in which it is not normally expressed, the sequence is deprived of tissue-specific transcription factors. This would inhibit transcription irrespective of the methylation status of the DNA. Therefore it remains unclear if methylation has a role in tissue-specific gene expression (Jackson and Felsenfeld, 1985).

It has been postulated that hypomethylation is a ubiquitous control mechanism for gene regulation. Evidence to support this has come from studies of the methylation status of CpG islands. To date a large proportion of G+C rich islands appear to be unmethylated, except for

those in the inactive X-chromosome (Gardiner-Garden and Frommer, 1987). It has also been calculated that approximately 30 000 HTFs exist in the mammalian genome which correlates with the predicted number of genes, 20 000 - 50 000 . This would allow a 1:1 relationship between every gene and an HTF (Bird, 1986). However this is unlikely as HTFs appear to occur as clusters and therefore are unlikely to be associated with every individual gene.

A model has been proposed for the role of HTFs in gene control. These sites are postulated to be binding sites for ubiquitous transcription factors (Bird, 1986). A mechanism must function to maintain hypomethylation of HTFs. These regions are not intrinsically unmethylated therefore it has been proposed that the maintenance mechanism would operate in the germ line. "House-keeping" genes must be transcriptionally active in germ line cells hence the binding of transcription factors could obstruct methylation. Once bound these factors would exclude methylases and therefore inhibite methylation. This would enable the progression of active transcription, possibly by causing conformational changes in the DNA, allowing access of RNA polymerases to the genes. Inactivation of the gene would occur with displacement of the factor and methylation of the exposed site (Bird, 1986). Many tissue-specific genes also have HTFs however it appears unlikely that an apparent ubiquitous mechanism for transcriptional activation would function in a tissue-specific manner. It has therefore been proposed that tissue-specific transcription may require both the binding of ubiquitous factors to HTFs and the binding of tissue-specific factors to other elements (Gardiner-Garden and Frommer, 1987). Indeed such tissue-specific factors have been described (Chapter I, 2, B (iii)). Alternatively, CpG islands may remain hypomethylated because they bind an "anti-methylase" protein that is not involved in transcription (Gardiner-Garden and Frommer, 1987).

Uncertainty also exists about the involvement of methylation in events which initiate transcriptional inactivation. Little is known of the molecular events leading to <u>de novo</u> methylation of DNA, but the data implies that most cell types are capable of methylating accessible CpG dinucleotides. Interestingly, teratocarcinoma cell lines and embryonic cells are particularly active at methylation which may be a consequence of the requirement to inactivate gene

expression during development and differentiation (Jaenisch and Jahner, 1984). However, evidence suggests that methylation may maintain the inactive state of DNA but is not the initiating event. For example, the integrated Moloney leukaemia provirus genes are inactive in embryonic cells prior to the detection of de novo methylation (Gautsch and Wilson, 1983). The evidence supporting the inactivation of the X-chromosome is also not conclusive. Comparison of accessibility of DNA sequences on the inactivated X-chromosome to the endonucleases Msp I and Hpa II show variation although all the genes are inactive. It therefore appears that methylation is not sufficient for transcriptional inactivation (Monk, 1985).

vi) Z- Form DNA

Transcriptional activation requires the decondensation of a chromosomal domain which contains the gene to be transcribed. It has been proposed that sequence specific topoisomerases may play an active part in uncoiling the DNA allowing relaxation of torsional stress and accessibility of RNA polymerases to the DNA (Cockerill and Ganard, 1986). Alternatively, decondensation / condensation may be associated with the formation of left-handed or Z-form DNA. In physiological salt concentrations Z-DNA is not stable and readily converts to the B-conformation. However, it can be stabilised by binding of specific basic proteins such as spermine or spermidine, or by the presence of 5-methylcytosine (Behe and Felsenfeld, 1981). It has been proposed that selective demethylation of Z-DNA, thereby destabilising it, would result in torsional stress. This stress would be overcome by positive supercoiling, resulting in unwinding of the double helix at a position removed from the initial event. This would yield nuclease-sensitive, single stranded regions capable of interaction with trans-acting transcriptional factors (Weisbrod 1982).

vii) Summary

Detection of DNase I sensitive sites, sites of methylation, regions containing modified DNA associated proteins and Z-form DNA all appear to coincide. However, it appears unlikely that these events are the initiating mechanisms involved in gene inactivation or activation. Nevertheless, they demonstrate that conformational changes do occur

at the sites of active gene transcription and provide an insight into the possible maintenance mechanisms of these changes. It can also be postulated that these changes in DNA reflect the relaxation of the DNA helix at active transcription sites required to enable trans-acting factors access to the DNA sequence hence stimulating transcription.

B) PRIMARY STRUCTURAL FEATURES OF DNA AFFECTING TRANSCRIPTION RATES

Transcriptionally active genes which encode protein are transcribed by RNA polymerase II (Chambon, 1975). It is believed that recognition of specific DNA sequences by the transcription machinery dictates both the site of initiation of transcription and also the rate at which the gene is transcribed (Maniatis et al, 1987). Initiation of transcription is widely held to be the primary controlling step in regulation of gene expression.

The gene is transcribed in the 5′ to 3′ direction by RNA polymerase II as it moves along the DNA chain adding the correct nucleotides to the growing hnRNA molecule. The hnRNA separates from the DNA as it is assembled but does not become completely separated until the polymerase terminates transcription. Termination of transcription occurs downstream from the 3′ end of the mRNA and is also believed to be determined by specific sequence motifs (Platt, 1986). This is followed by cleavage of the hnRNA molecule near the polyadenylation site and addition of adenosine residues to the 3′ end of the transcript (Nevins, 1983). It remains unclear if the polyadenylation signal has a role in termination of transcription.

i) Cis-Acting DNA Elements

At least two DNA sequence elements are required for transcriptional regulation of gene expression in higher eukaryotes: promoters and enhancers. Promoters are located immediately upstream from the start site of transcription and are typically about 100 base pairs in length whereas enhancers can be found over 1 kb from the affected promoter (Dynan and Tjian, 1985; Serfling et al, 1985). The promoter is required for accurate and efficient initiation of transcription whereas enhancers increase the rate of transcription from promoters.

These sites are thought to be protein binding sites as they are protected from DNase I attack in DNase footprinting experiments (Dynan and Tjian, 1985; Parker and Topol, 1984).

a) Promoters

Promoter regions, required for initiation of transcription, appear to be organised according to a common plan. Many promoters have an element called a TATA box, with a consensus sequence 'TATAAA', located 25-30 base pairs upstream from the transcription start site. It is believed that this sequence determines the efficiency and precise location of the transcription start site (Breathnach and Chambon, 1981). However, it is unlikely that the TATA box determines transcriptional control. This has been postulated following the discovery that not all genes possess a TATA box but are still transcribed (Breathnach and Chambon, 1981). Mutation in this region also does not significantly alter RNA transcription but may generate heterogeneity in the region of initiation of transcription (Benoist and Chambon, 1981).

Typical promoters also include upstream promoter elements (UPEs); these elements increase the rate of transcription and therefore could have an important role to play as a mechanism to stimulate specific gene transcription. UPEs normally occur 70-200 base pairs from the transcriptional start site and contain one or more elements (McKnight and Tjian, 1986). Some upstream promoter elements such as CCAAT and the GC box homologies have been found in many different promoters, whereas others, such as the heat shock response element appears to have a more specialised role (Pelham, 1982; Pelham, 1985).

From mutagenesis studies it has been suggested that the strength of a promoter is determined by the number and the types of UPEs associated with it (Maniatis et al, 1987). These cis-acting elements act regardless of orientation with respect to the TATA box. However insertion of nucleotides between the UPEs and the TATA box can decrease the level of transcription (McKnight, 1982). It has been demonstrated that insertion of odd multiples of half a DNA turn is more detrimental to transcription than insertion of even multiples. This observation has led to the suggestion that one or more proteins bound to the UPEs interact with protein bound to the TATA box to

stimulate transcription. This interaction must require alignment of the proteins on the DNA helix as insertion of DNA between these elements demonstrates (Takahashi et al, 1986).

Studies of the effects of mutagenesis has also revealed that the sequence of the UPEs is functionally important. Mutagenesis studies of the β-globin promoter have revealed the precise location of the TATA box and two UPEs. Single base mutations within any of these three cis-acting elements resulted in a 5-10 fold decrease in transcription in HeLa cells. However, base substitutions elsewhere within the promoter region had no effect (Myers et al, 1986). These results indicate that protein recognition of specific DNA sequences must operate during initiation of transcription. By controlling the availability of the transcription factor(s) and / or the accessibility of the UPEs the cell could therefore control gene expression.

b) Enhancers

A second class of cis-acting DNA elements, the enhancers, have also been identified. These regions are found to dramatically potentiate transcription from RNA polymerase II promoters. They are functionally different from other cis-acting DNA elements in that they function to enhance transcription from distances greater than 1 kb away from the promoter region. Enhancers also functon in an orientation independent manner and in a position either upstream or downstream from the associated promoter (Rogers and Saunders, 1985).

It is believed that enhancer activity requires the interaction of cis-acting elements and trans-acting factors. Mutagenesis experiments on the SV40 enhancer have revealed that the enhancer consists of two functional domains, mutation in either domain led to decrease in transcription which indicated the requirement for both regions for enhancer activity. When one or other of the domains was tested it was found that, singly, they only possessed weak enhancing activity. However, duplication of one element resulted in high enhancing power. Therefore enhancing power appears to be dependent on the number of functional domains present in the enhancer sequence, in a manner analogous to that of UPEs (Herr and Gluzman, 1985; Herr and Clarke, 1986).

In vitro foot printing experiments have demonstrated that enhancer and promoter elements are recognised by cellular protein factors (Dynan and Tjian, 1985; Parker and Topol, 1984). They also show remarkable similarity in function and structure. Therefore it has been postulated that enhancers and promoters are related (Maniatis et al, 1987). The only difference is the ability of enhancers to affect transcription at great distances and in an orientation-independent manner from the promoter. It has been surmised that these differences may simply be a consequence of the arrangement and the number of transcription factor recognition elements in each case rather than differences in the mechanisms by which these elements operate. This conclusion is supported by experiments involving a heat-shock regulatory element. This element can not act at a distance to promote transcription but duplication of this sequence creates an element that functions in a similar manner to an enhancer. (Bienz and Pelham, 1986). Enhancing power can also be used as a mechanism for regulating gene expression. By regulation of the availablity of transcription factors and / or the accessibility of the enhancer regions gene expression could be controlled.

Several models have been proposed to explain how transcription factors, bound to enhancer elements, great distances upstream from a transcription start site, can influence the rate of transcription. The most favoured model is that of DNA looping. It is proposed that proteins bound to the enhancer contact proteins bound to promoter regions by causing the intervening DNA to loop out. This results in the formation of a transcriptional complex the components of which ultimately determine the rate of transcription. The mechanism by which this is accomplished is still unclear (Ptashne, 1986).

ii) Regulated and Inducible Cis-Acting Regulatory Elements

It has been discovered that the activity of some enhancers and UPEs are tissue-specific or development-specific. For example, Gillies et al (1983) described an immunoglobulin enhancer which was functional in murine B-lymphocytes but not in murine fibroblasts. Others have reported 5′ flanking regions of the insulin and chymotrypsin genes which specifically control gene expression in pancreatic cells (Walker et al, 1983). Interestingly, a region 400 nucleotides 3′ to the polyadenylation signal of the β-globin DNA

sequence has also been demonstrated to determine erythrocyte cell specificity. This 3′ enhancer sequence was also shown to be developmentally activated following induced erythrocytic differentiation (Choi and Engel, 1986). The role of enhancer elements in the control of gene expression in differentiation is therefore of fundamental importance. To determine activation it is highly likely that tissue-specific and development-specific trans-acting factors are involved.

Cis-acting regulatory elements may also function to control co-ordinated gene expression in development. By possessing homologous UPEs adjacent to the genes required to act co-ordinately, gene expression would be activated simultaneously, when the appropriate factor bound to each of the elements. This has been demonstrated with inducible enhancer elements. For example, induction of gene expression by the action of steroid hormones is mediated by a number of high affinity binding sites which recognise the hormone bound receptor (Payvar et al, 1981). Regulation of the hormonally induced expression of egg white proteins is thought to be initiated by binding of trans-acting factors to the upstream regions of these genes. Sequence homologies have been observed within the 5′ regions of ovalbumin, conalbumin and ovamucoid genes (Mulvihill et al, 1982).

A protein factor has also been demonstrated to bind to both SV40 and c-fos enhancer regions, PEA 1. However, although the same factor is involved, the binding site is not found in exactly the same context in each case but adjacent to different elements. The fact that enhancers from different sources interact with the same cellular protein may explain the analogous behaviour of these elements in certain physiological situations. For example, both SV40 and c-fos enhancers are weakly active in F9 embryonal carcinoma cells but strongly activated following TPA treatment (Piette and Yaniv, 1987). Indeed it has been demonstrated that a large number of TPA inducible genes share a conserved 9 base pair motif which confers TPA inducibility upon heterologous promoters. It is postulated that the TPA-responsive element is recognised by a common cellular protein. Interaction of this protein with genes containing the appropriate motif would result in co-ordinated gene expression (Angel et al, 1987).

iii) Trans-Acting Factors

RNA polymerase II appears to lack any inherent ability to recognise promoters in an in vitro reaction. Recently, it has become clear that crude extracts from eukaryotic cells contain factors that impart promoter specificity to RNA polymerase II. Some of these factors, when purified, seem to be ubiquitous, required for the activity of most promoters, whereas others function in a specific manner. These trans-acting factors could therefore be used as a mechanism for control of gene expression. Many of the cis-acting DNA elements associated with promoter and enhancer regions, could interact with one or more of these soluble, cellular protein factors resulting in increased gene transcription (Dyan and Tjian, 1985).

Many trans-acting factors have been isolated. One such protein is Sp1. This protein is obtained from cultured human cells and is required for SV40 transcription (Dyan and Tjian, 1983). Sp1 recognises and binds specifically to a GC-rich decanucleotide region (GGGCGG) which is found in multiple copies upstream of the affected promoter. This region appears to represent a HTF discussed in Chapter I, 2, A(v). The consequence of Sp1 binding is an increase in transcription of 10-25 fold depending on the gene. However, Sp1 does not appear to be involved in tissue-specific gene expression as Sp1 binding sites are found associated with a wide range of genes and found in the nuclear extracts from a number of cell types (Short, 1987). Interestingly, the GC box motif, recognised by Sp1, is found associated with many "house-keeping" genes which are constitutively expressed in all cells and would therefore require a ubiquitous mechanism for control of gene expression. However such sites are not unique to 'house-keeping' genes (Short, 1987).

The CCAAT sequence, also frequently found associated with a number of promoters from very diverse genes, appears to be recognised by trans-acting factors resembling Sp1. From nuclear extract of HeLa cells a factor termed CTF has recently been isolated which recognises the CCAAT sequence. On binding to this region CTF increases transcription of the affected promoter up to 10 fold. This has led to the postulation that a number of trans-acting factors exist which share a common, as yet unknown, mode of action. This action however does not appear to be development or tissue-specific (Short, 1987).

A factor which appears to facilitate the transcription of a specific group of genes is the Heat Shock Transcription Factor (HSTF). In most cell types, when exposed to abnormally high temperatures, several heat-shock mRNAs are induced. The induction of transcription of these genes is apparently mediated by a conserved heat-shock regulatory sequence found in the upstream region of the promoters for heat-shock genes (Pelham, 1985). The trans-acting HSTF interacts specifically with those genes possessing the heat-shock regulatory sequence. It is therefore highly probable that a similar mode of control will operate to co-ordinate tissue-specific and development-specific gene expression (Dyan and Tjian, 1985). Indeed this is demonstrated by steroid hormone regulated genes and genes activated by TPA treatment. In the case of TPA induced gene expression the factor(s) involved have still to be identified (Chapter I, 2, B(iii))

From the evidence presented it is apparent that promoter and enhancer interactions with trans-acting factors play a crucial role in determining gene expression. It is interesting to note that most genes possess multiple upstream promoter elements capable of binding a number of different factors. It has therefore been proposed that some factors will prove crucial for initiation of transcription whilst other factors will be involved in the "fine tuning" of the system (Short, 1987).

iv) Promoter Usage

Many genes have been described which possess more than one putative promoter region in the immediate upstream non-coding region of the gene. In many instances the reason why multiple promoters exist is unknown. For example, the gene encoding c-myc contains three promoter regions. Transcription can be detected from all three but the functions of the different transcripts are unknown (Battey et al, 1983; Bentley and Groudine, 1986). However, other genes have been described which possess more than one promoter which function in a tissue-specific or development-specific manner. For example, the isozymic form aldolase A of fructose 1,6-diphosphate aldolase, is expressed as three different sizes of mRNA from the same gene. The three mRNA species are distributed in a tissue-specific manner; one species of mRNA is found in skeletal muscle, one in heart, spleen and

brain and another in liver. On examination of the mRNAs and the aldolase A DNA sequence, it has been postulated that the aldolase A gene has multiple promoters to facilitate the tissue-specific generation of three mRNAs (Joh et al, 1986). A similar system has been proposed to explain the tissue-specific distribution of murine ∝-amylase. This gene possesses two promoters of different transcriptional strengths. The stronger promoter is exclusively active in liver whereas the weaker promoter is active in liver and parotid gland (Schibler et al, 1983). Choice of promoter can therefore be utilised to control gene expression in a tissue-specific manner.

Activation of cryptic promoters has been postulated as a potential mechanism leading to aberrant gene expression, which could result in neoplastic transformation. For example, activated cryptic promoters in the first intron of the c-myc gene have been described following the t(8:14) translocation involved in Burkitt's lymphoma (Nishikura, 1987). This emphasises the importance of control of transcription in normal development and differentiation.

v) Termination of Transcription

Little is known about transcriptional termination but evidence is now gathering to suggest that termination may function as a means of regulating gene expression. It has been postulated that termination sites function constitutively or can be regulated in a manner analogous to promoters (Platt, 1986). However, the study of the potential sequence motifs and the trans-acting factors which could operate to regulate termination is still in its infancy.

It was postulated that termination may be a control mechanism of gene expression following the discovery that not all RNA polymerase II complexes that begin transcription, transcribe the entire sequence. Prematurely terminated transcripts have been described from both viral and cellular genes (Weisbrod et al, 1980; Weintraub, 1979). It is still unclear however where transcription terminates and how specific the termination signals are in eukaryotic cells. Most polyadenylated RNA transcripts terminate at distances far removed from the 3' terminus of the mature mRNA, up to 1 kb - 4 kb, with no apparent similarity in the region of termination. Hence the analysis

of termination signals is complex (Platt, 1986).

From the analysis of 3′ ends of many genes the consensus sequence AATAAA has been identified as the signal for polyadenylation. Deletion of the polyadenylation signal results in apparent "read through" of the gene in many instances, however no evidence has been found to support its direct role in termination (Birnstiel et al, 1985). Endonuclease hypersensitive regions have been mapped to the 3′ termini of many genes. This has led to the postulation that other sequences and trans-acting factors must be involved in transcriptional termination (Fritton et al, 1983; Groudine et al, 1983).

Examination of the 3′ termini of many yeast genes has revealed a sequence which on deletion reduces transcription from the affected gene. If mRNA is detected the transcripts appear larger than normal. Interestingly, there appears to be homology between the 3′ sequence in yeast and a 3′ sequence in the ADH3 gene of Drosophila (Falco et al, 1985; Henikaff et al, 1983). Others have described hair-pin loops at the 3′ end of histone genes. Hair-pin loops have been postulated to act as simple termination signals in prokaryotes. It has been proposed that transcriptional complex pausing at hair-pin loops may facilitate termination (Platt, 1986). The hair-pin loops found in association with the histone genes are associated with a conserved sequence. However, deletion of these elements did not stop termination at the correct region. It has therefore been proposed that these elements exist to aid in the modification and maturation of non-polyadenylated RNA (Birchmeier et al, 1983; Birchmeier et al, 1984).

Premature termination of transcription has been observed in both viral and cellular systems. It is believed that this event may play a role in determining gene expression (Platt, 1986). Termination is thought to be brought about by changes in the secondary structure of DNA. For example, after the initiation of late transcription in SV40, RNA polymerase II terminates prematurely at a hair-pin loop in the VP1 gene, producing short aborted RNA (Hay et al, 1982). The VP1 gene is negatively regulated at the level of transcription by a protein (the agnoprotein) which causes this premature termination (Hay and

Aloni, 1985). It is thought that by alteration of 5′ end of the VP1 gene transcriptional read-through or termination is determined. These changes are in turn determined by the presence or absence of the agnoprotein and have been associated with the formation of stem-loop structures. (Hay and Aloni, 1985). Interestingly, it appears that down regulation of c-myc gene expression following induction of HL60 cells by DMSO may occur by an analogous mechanism to that described for VP1 (Eick and Bornkamm, 1986).

From the evidence available it would appear that termination does play a role in the regulation of gene expression. However, it has been proposed that a number of cis-acting elements possibly act co-ordinately to determine the strength of the termination signal in a manner analogous to the 5′ promoter and enhancer elements (Platt, 1986). To implement termination trans-acting factors must also be involved. One such factor has been described in the prokaryotic system, the rho protein of E. coli. Rho functions as a terminator of transcription by specific sequence recognition of regions in the nascent RNA chain (Platt, 1986). Binding of the Rho factor to the RNA then facilitates the release of the transcript from the transcription machinery (Platt, 1986). It is tempting to speculate that factors involved in the termination of eukaryotic gene expression may function in a tissue-specific or a development-specific fashion. Evidence for development-specific termination has been described by Mather et al, (1984). They have reported that termination of transcription between the μ and the σ immunoglobulin genes only occurs upon maturation of B-lymphocytes.

An example of gene expression determined by termination is demonstrated by adenovirus. The lytic cycle of adenovirus infection is divided into two functionally distinct phases, the early phase preceding, and the late phase beginning at the onset of viral DNA replication. It had been assumed that the major late transcription unit of adenovirus was activated at the shift from early to late phase. However, a low level of transcription was detected from the late unit early in the infectious cycle. During early times of infection the late promoter is active but transcription terminates within the L2 region of the late unit hence only L1 is transcribed. The shift from early to late phase appears to result in loss of a termination signal, allowing transcriptional read-through and the

expression of the other genes on the major late transcription unit (Akusjarvi and Persson, 1981).

vi) Gene Dosage

One potential mechanism for increasing gene expression is to increase the number of transcription units. Quantitative changes in gene copy number are found in many systems. For example, during amphibian development ribosomal RNA genes are amplified 1000 - 2000 fold (Brown and Dawid, 1968). The chorion genes in the ovaries of Drosophila are also amplified (Spradling and Mahowald, 1980). Specific gene amplification has also been detected when bacteria or mammalian somatic cells are cultured under selective conditions (Anderson and Roth, 1977; Schimke, 1984).

However, amplification as a mechanism for increasing gene expression does not appear to be a common event in eukaryotes. Even in cell types almost totally dedicated to the production of one protein only one gene copy exists; for example, erythroid cells are almost solely concerned with the production of globins but only possess one copy of each of the globin genes (Harrison et al, 1977). The amplification of certain genes has been implicated in processes leading to neoplastic transformation. For example, amplification of the c-myc gene has been observed in the promyelocytic cell line HL60, derived from a patient suffering from acute promyelocytic leukaemia (Dalla-Favera et al, 1982). Similarly, L-myc sequences have also been reported to be amplified only in small cell lung carcinoma cells (Little et al, 1983) and amplification of N-myc sequences have been reported in neuroblastomas, retinoblastomas and small cell lung carcinomas (Schwab et al, 1983). It has been speculated that amplification of myc sequences results in over production of myc products which could play a role in the events leading to transformation. Indeed a correlation between the degree of c-myc amplification and the relative abundance of c-myc transcripts in HL60 cells has been reported (Graham et al, 1985). However, it is unknown if amplification is causally related to cancer.

C) Post-Transcriptional Control

RNA processing is the term given to the sequence of events that convert a primary RNA transcript to a mature mRNA. These events begin immediately after RNA polymerase II initiation of transcription, possibly before the polymerase moves past the initiation site. The first known event in RNA processing is the addition of 7-methyl-guanosine triphosphate and methylation of the 5′ end of the nascent RNA chain, the cap structure (Salditt-Georgieff et al, 1980). Few, if any, RNA polymerase II products escape this 5′ modification; this has been demonstrated not only in vivo but also on prematurely terminated RNA chains and RNA transcripts synthesised in vitro by cell extract supplemented RNA polymerase II activity (Salditt-Georgieff et al, 1981). Therefore capping appears to be a ubiquitous event in mRNA processing.

As the RNA chain grows it is thought to become organised into a ribonucleoprotein (RNP) complex by association with basic proteins. This may protect the nascent RNA chain from degradation by endogenous cellular nucleases and maintain the RNA chain in a conformation optimum for further processing steps (Padgett et al, 1986). Transcription then proceeds beyond the 3′ end of the mature mRNA before termination. A processing event follows, at the 3′ end of the RNA chain, resulting in cleavage at the polyadenylation site followed by polyadenylation of the RNA (Nevins, 1983).

RNA, at this stage, is termed heterogeneous (hn) RNA. It has long been recognised that not every part of every hnRNA molecule contributes to cytoplasmic mRNA. This was demonstrated by kinetic labelling studies in cultured cells which showed labelling of hnRNA to be as great as 20 fold that of cytoplasmic RNA (Jelinek et al, 1973; Lengyel and Penman, 1975). In addition complexity studies, based on nucleic acid reassociation kinetics, established that hnRNA has a complexity approximately ten fold greater than cytoplasmic polyadenylated RNA (Getz et al, 1975). This led to the discovery of intron sequences in eukaryotic genes, regions which are specifically excised out of the hnRNA with subsequent splicing of adjacent exons to produce a mature mRNA. This process occurs in the cell nucleus before transportation of the transcript to the cytoplasm but following polyadenylation (Padgett et al, 1986).

Obviously control of gene expression in differentiation could be implemented at any point in the succession of steps leading to the production of a mature mRNA transcript. The evidence to support such controls will be discussed.

i) The Cap Structure

The cap structure is a ubiquitous feature of all RNA polymerase II transcripts and therefore this modification would appear unlikely to play a significant role in the control of gene expression. However, it has been proposed that the cap structure has a role in pre-mRNA splicing. This has been demonstrated using in vitro RNA processing systems. It has been reported that capped pre-mRNAs are spliced more efficiently and that they produce less aberrantly spliced molecules than uncapped pre-mRNAs. Furthermore, cap analogues reduce the efficiency of splicing in HeLa cell extracts (Konarska et al, 1984). Others have reported that the formation of pre-mRNA splicing complexes, a complex structure consisting of a number of nuclear proteins and RNA elements required for efficient splicing, is inhibited by cap analogues and is not correctly formed when uncapped pre-mRNAs are used in in vitro assays of splicing. This has led to the proposal that the splicing complex interacts with the cap structure in some way, which is crucial to the assembly of the active splicing complex (Patzelt et al, 1987). However, it is still unclear if the cap structure has a role in regulation of splicing.

ii) Polyadenylation

Polyadenylation occurs after transcription is completed but prior to splicing events. The signal for polyadenylation is believed to be the consensus sequence AATAAA, found down stream from the coding region of most RNA polymerase II transcription units. Transcription continues past the polyadenylation signal before terminating. This is followed by cleavage at the polyadenylation site and the addition of poly(A) to the 3′ end of the hnRNA (Nevin, 1983). Many genes have been reported which apparently possess multiple polyadenylation consensus sequences, for example the dihydrofolate reductase (DHFR) genes and the gene encoding the cap recognition protein (CRP), involved in initiation of the translation process, possess three. It

has therefore been proposed that selection of the appropriate polyadenylation site may act as a control mechanism in the regulation of gene expression (Rychlick et al, 1987; Kaufman and Sharp, 1983).

Polyadenylation has also been implicated in determining the stability of mRNAs. Stabilisation could act as a potential mechanism of gene control: increase in transcript stability would effectively increase the translation potential of the affected mRNA; instability would obviously have the opposite effect, leading to reduced translation potential or lack of availability altogether due to rapid degradation. Initial experiments demonstrating the effects of polyadenylation on transcript stability utilised in vivo translation systems; injection of non-polyadenylated or polyadenylated transcripts into HeLa cells or Xenopus eggs was carried out followed by comparison of the half-lifes of the transcripts. From this work it was concluded that polyadenylation was a prerequisite for ensuring stability. Non-polyadenylated transcripts were not detectable at short times after injection and were not translated whereas polyadenylated RNA translation products were observed (Huez et al, 1981). Interestingly, a hair-pin loop structure found at the 3' terminal region of histone mRNAs, which are not polyadenylated, has also been shown to confer stability to non-polyadenylated β-globin mRNA when it is exchanged with the globin polyadenylated tail. This highlights the importance of 3' RNA sequences in determining transcript stability (Whitelaw et al, 1986). Transcript stability is discussed in Chapter I, 2, C (v).

iii) Splicing Events

Splicing is a crucial event in determining the correct coding sequence of mature mRNA and therefore the correct cellular protein complement. Regions of RNA sequence, introns, that do not contain coding sequence required for the particular mRNA being processed, are removed from hnRNA with the concomitant splicing of two adjacent exons, to form a mature mRNA transcript. This transcript can then be transported from the nucleus for translation in the cytoplasm (Breathnach and Chambon, 1981). Control of the splicing event in RNA processing can therefore be used as a mechanism of regulation of gene expression. The splicing event is initiated by a splicing complex or splicesome which consists of many components including proteins and

small ribonucleoproteins (SnRNPs) (Grabowski et al, 1985). Splicing results in the production of the spliced exons relating to the mature mRNA and the excised intron RNA which is termed lariat RNA; this is rapidly degraded on release from the splicing complex (Padgett et al, 1986).

Specificity of splicing is determined by recognition of consensus sequences surrounding the intron / exon boundaries. However, some splicing consensus sequences are selected over others. This has led to the postulation that other sequences and trans-acting factors must also be involved in determining the splicing event. Tight control must also operate to eliminate trans-splicing events, that is splicing between two exons from heterologous genes (Padgett et al, 1986). Many genes contain intron sequences of varying size and number. It is therefore feasible that by manipulation of the splicing event and by use of alternative splicing patterns one gene could effectively code for a number of different but related proteins. Examples of this have been described; various mRNAs are transcribed from the major late transcription unit of adenovirus late in infection. These transcripts are produced from one region of DNA by the interaction of alternative splicing events and selection of different termination sites (Akusjarvi and Persson 1981). Alternative splicing has also been reported in the production of two Ia antigen associated invariant chain proteins encoded by a gene located in the HLA-D region of the major histocompatibility complex (MHC) in man (Strubin et al, 1986). Other examples of alternative splicing have been reported for the immunoglobulin genes and the mouse major urinary protein genes (Alt et al, 1980; Clark et al, 1984).

Splicing has also been implicated as one of the requirements for the efficient transport of mRNA to the cytoplasm. However, RNAs which do not possess introns are efficiently transported from the nucleus to the cytoplasm without any splicing event which contradicts this proposal (Padgett et al, 1986). Nevertheless others have demonstrated a requirement for splicing prior to transport. When a cDNA copy of the 16S transcription unit of SV40, replaces itself in a replicating SV40 vector, the inserted cDNA did not produce cytoplasmic mRNA products. Only after reinsertion of an intron into the 16S transcription unit was the production of cytoplasmic mRNA restored (Gruss et al, 1979). Therefore there are contradictory views on the

necessity of splicing for transport to the cytoplasm. However, it seems that these two processes may be linked in some way to determine that only mature mRNA is transported from the nucleus.

It therefore appears that splicing can act as a mechanism for the regulation of gene expression. Alternative splicing patterns enable a number of products to be produced from the one gene. Control of splicing could therefore determine which product is produced. This could be determined by tissue-specific or development-specific factors which could alter the specificity of the splicing reaction. However, the existence of factors which specifically regulate transcription has still to be established. The rate at which the splicing reaction occurs could also act as a mechanism to regulate the production of mature mRNA. It has already been established that the half-life of introns within precursor RNAs can vary from a few seconds to 10-20 minutes (Padgett et al, 1986). However the factors which determine the rate of removal of introns are not known. A model has been proposed by Pikielny and Roshbach (1985) which indicates how the rate of splicing can affect the levels of mRNA in the cytoplasm. It is proposed that if the rate of the splicing reaction is faster than the turnover of hnRNA the level of mRNA will be maximal, almost all the hnRNA is processed into mRNA. However, if the rate of the splicing reaction is less than that of the turnover of the precursor most of the hnRNA will be degraded before it can be spliced. Therefore, by altering the rate of splicing regulation of gene expression could be accomplished. However there have been no reports to support this type of regulation of gene expression.

iv) Nucleo-cytoplasmic Transport

Before a mRNA can be translated into protein and therefore contribute to the cell phenotype the transcript has to be transported from the nucleus to the the cytoplasm and hence to the translation machinery. The mechanisms involved in this process are unknown and examples of control at the level of nucleo-cytoplasmic transport are difficult to determine as other factors, such as increased nuclear stability or decreased cytoplasmic stability could generate data consistent with transport regulation. However, an example of regulation of gene expression at the stage of transcript transportation has been described for the transcripts from the α_{2u}-globulin gene.

α_{2u}-globulin is a glucocorticoid regulated liver gene. However, following adrenalectomy of rats, it was found that in the absence of glucocorticoid stimulation α_{2u}-globulin RNA was depleted in polysomal RNA but increased in nuclear RNA of liver cells. It was proposed that loss of glucocorticoid stimulation led to the inhibition of the nucleo-cytoplasmic transport of α_{2u}-globulin RNA. Injection of Dexamethasone (a synthetic glucocorticoid hormone) resulted in the reduction of nuclear α_{2u}-globulin RNA and a concomitant increase in polysomal transcripts supporting the proposal of control of expression operating at the stage of nucleo-cytoplasmic transport (Fulton et al, 1985).

Evidence that specific transport mechanisms must exist have come from many sources. Injected histone mRNAs are matured and transported from Xenopus nuclei within 30 minutes. This can not simply be due to unrestricted diffusion as unprocessed histone transcripts would also appear in the cytoplasm (Georgiev et al, 1984). Others have studied the regulation of transport of mRNAs from isolated nuclei (Schumm and Webb, 1983). They reported hormonal regulation of gene expression at the level of nucleo-cytoplasmic transport as it appeared that only specific mRNAs were transported from the nucleus in response to a hormonal stimulus. However, these experiments are prone to artifacts therefore specificity of the response of the isolated nuclei is difficult to assess. Chan (1976) has also reported an example of possible control of gene expression at the level of nucleo-cytoplasmic transport. Globin mRNA was found to be synthesised and retained in the nucleus of embryonic erythroid cells during the first 24 hours of culture. Within the next 24 hours there was an almost complete transfer of globin mRNA from the nucleus to the cytoplasm. These results indicate that sequence-specific regulation of transport from the nucleus to the cytoplasm exists. However the mechanisms which function to determine transport are not known.

v) mRNA Stability

It has been demonstrated that different mRNAs have different rates of decay or half-lives. Many transcripts are short lived while others have half-lives of many hours. β-globin mRNA is relatively stable with a half-life greater than 17 hours (Volloch and Housman, 1981). In contrast, c-fos transcripts have a half-life of 15 minutes or less

(Greenberg and Ziff, 1984). Clearly, some factor(s) must operate to determine transcript stability. By changing their stability mRNAs would be available for long periods of time increasing their translation potential, or conversely, could be made so unstable as to effectively eliminate any possibility of translation. Modulation of transcript stability could therefore regulate gene expression.

Many have reported increased transcription as a mechanism of increasing the relative abundance of specific mRNAs. For example, steroid hormones are thought to initiate the transcription of genes in a highly specific manner; oestrogen selectively activates the vitellogenin gene in liver (Brock and Shapiro, 1983a) and the peptide hormone prolactin specifically activates casein gene expression in mammary gland tissue (Guyette et al, 1979). However, it has subsequently been demonstrated that the effects of hormone-activated transcription are amplified by a specific stabilisation of the affected mRNAs. The half-life of the casein message was increased seventeen fold in the presence of prolactin; vitellogenin mRNA half-life increased over thirty fold in the presence of oestrogen (Guyette et al, 1979; Brock and Shapiro, 1983b).

Others have reported the change from stable to unstable transcripts as a means of regulating gene expression. For example, the dihydrofolate reductase (DHFR) genes are active during exponential phase of growth in methotrexate-resistant mouse cell lines but the transcripts from these genes are undetectable in resting cells. Leys et al (1984) have demonstrated that DHFR mRNA is more stable in growing than resting cells and have postulated that most DHFR transcripts are processed into mRNA in growing cells but in resting cells the transcripts are rapidly degraded in the nucleus.

Relatively little is known about the mechanisms controlling mRNA stability. However, evidence is now gathering that indicates that specific sequence recognition could be involved. For example, truncated c-myc genes, found in certain B lymphoid tumour cells, transcribe a more stable mRNA than the full length c-myc gene. Truncation results in the loss of the c-myc gene first 5′ exon which indicates that interaction between this sequence and as yet unknown factors could determine c-myc transcript instability (Rabbits et al, 1985). Shaw and Kamen (1986), recently demonstrated that an A and T

rich sequence in the 3' untranslated region of many short lived transcripts confers instability. The sequence was initially identified within the 3' untranslated region of the lymphokine granulocyte-macrophage colony stimulating factor (GM-CSF) transcript, but has subsequently been identified in many more transcripts including c-fos and c-myc. Shaw and Kamen (1986) demonstrated the effects of this sequence on transcript stability by inserting the 3' untranslated sequence of GM-CSF into the 3' untranslated region of the β-globin gene. In transfection studies the inserted sequence conferred instability on the β-globin transcripts resulting in B-globin mRNA with a half-life of less than thirty minutes compared to the normal seventeen hours (Shaw and Kamen, 1986).

Shaw and Kamen (1986) also demonstrated that cycloheximide treatment, a protein synthesis inhibitor, increased the half-life of the mRNA transcribed from the β-globin construct. This has also been demonstrated for other short lived mRNA species, for example c-fos and c-myc (Greenberg et al, 1986). This has led to the proposal that the conserved sequence at the 3' untranslated region of short lived transcripts may be a recognition site for a labile RNA binding protein involved in the specific degradation of transiently expressed mRNAs (Rahmsdorf et al, 1987). In the light of these discoveries it seems highly probable that other cis-acting elements and trans-acting factors will be involved in the determination of transcript stability.

It has been postulated that polyadenylation of mRNA has a role in determining stability of mature transcripts (see Chapter I, 2, C (ii)). Stability of both polyadenylated and deadenylated transcripts has been determined by the efficiency of translation of these RNAs when injected into Xenopus oocytes. Conflicting results have been reported; deadenylated mRNAs have been observed to be more labile than adenylated species but conversely some data indicate that deadenylated transcripts are as stable as adenylated mRNA in this system (Heuy et al, 1974; Sehgal et al, 1978). Recently it has been reported that c-myc transcripts which lack long poly(A) tails exist in HL60 cells and that these transcripts are more stable than c-myc mRNA which possess a long poly(A) tail. This contradicts the proposal that polyadenylation of RNA is required for stability (Swartwout et al, 1987). Therefore, it remains unclear if polyadenylation is

important in determining RNA stability and hence plays a role in the regulation of gene expression.

vi) Control of Translation

Once transported from the nucleus to the cytoplasm the mRNA transcripts are translated by the translation machinery to protein. Translation requires the interaction of the ribosomal complex with specific sequences at the 5′ end of the transcript. Once this is accomplished the ribosomal complexes move along the RNA chain facilitating the synthesis of protein as directed by the coding region of the mRNA. Gene expression could therefore be determined by the efficiency of translation. This could be implemented by regulation of the interaction between mRNA and the translation machinery.

Examples of differential translation of mature cytoplasmic mRNAs have been described in embryonic development. Rosenthal et al (1980) demonstrated that striking changes occurred in the pattern of protein synthesis shortly after the fertilisation of clam eggs. The synthesis of specific oocyte proteins was reduced whereas the synthesis of many proteins found during early embryogenesis of the clam were increased. However, the mRNAs for both sets of proteins were present at all times but differentially expressed. These results suggest that selection of mRNAs for translation can act as a mechanism for the regulation of gene expression. Others have reported an apparent increase in translation efficiency as a mechanism operating to increase the concentration of translation products. Following fertilisation, sea-urchin oocytes become at least 50 fold more active in protein synthesis, from the same number of mRNAs as is detected at pre-fertilisation times (Brandhorst, (1976). Variation in protein synthesis has also been detected in non-embryonic cells. For example, following heat-shock of mammalian cells, decrease in total protein synthesis occurs despite the presence of a normal mRNA complement (Kelley and Schlessinger, 1978).

To control gene expression at the level of translation mechanisms could operate at the initiation step of mRNA recognition by the translation machinery. Indeed interaction between the 5′ cap structure and cap-binding protein appears to be an important step in

the initiation of translation (Shatkin, 1985). The cap binding protein is thought to interact with the translation complex to facilitate the start of translation (Lax et al, 1985). Therefore, by controlling this interaction the mRNA would effectively be removed from the translation pool.

RNA / RNA interactions have also been proposed as a mechanism for regulation of gene expression at the translation stage. It is postulated that anti-sense RNA, produced from naturally occurring regulatory genes, can directly control gene expression by specific sequence interaction.

Anti-sense RNA was initially discovered in prokaryotes where it has been implicated in the regulation of diverse processes in bacteria such as plasmid replication and bacterial and phage gene expression (Rosen et al, 1981; Mizuno et al, 1984). For example, in E.coli the micF anti-sense RNA represses the synthesis of the outer membrane porin OmpF. The region encoding the micF RNA was found to contain a stretch of approximately 85 base pairs which was homologous to DNA encoding the 5′ end of the OmpF mRNA, including the ribosomal binding site and the OmpF initiation codon. From S1 mapping studies, it was found that the homologous DNA was transcribed in the opposite direction from the Omp F gene. It has been proposed that the complementary micF RNA inhibits OmpF expression by forming a hybrid with the OmpF transcript hence inhibiting translation of the OmpF mRNA (Mizuno et al, 1984).

It has been postulated that a similar mechanism could operate in eukaryotic cells. Potential regions of anti-sense transcription have been demonstrated in vitro, using nuclear run-on assays. For example, regions of anti-sense transcription have been reported in the regions upstream of the human c-myc exon 1 gene. However, no anti-sense transcripts could be detected by Northern blot analysis (Nepvue and Marcu, 1986). Therefore the potential for anti-sense transcription must exist in eukaryote cells. However, to date, no naturally occurring anti-sense genes have been discovered. This implies that the regions of anti-sense transcription detected by nuclear run-on may be artifactual. It is unlikely that all the controlling factors found in the cell will be present in in vitro systems for analysis of transcription therefore this could allow transcription events to

occur in these analyses which would normally be restricted in the cell.

In vitro assays have also revealed that artificially synthesised anti-sense RNA can be used to regulate the expression of a selected gene in eukaryotes. For example, experiments have been carried out involving co-transfection of plasmids containing the sense and anti-sense forms of a specific gene. This resulted in a significantly lower level of expression of the assayed cDNA than was detected when the cDNA was transfected in the sense orientation only. This has now been observed for a number of genes including actin, the thymidine kinase gene and others (Izant and Weintraub, 1984; Izant and Weintraub, 1985). This demonstrates the wide-spread sensitivity to anti-sense inhibition in the eukaryotic genome. However, to successfully regulate gene expression by this method antisense transcripts have to be co-transfected at concentrations 50-100 fold greater than the sense transcripts. It can therefore be surmised that regulation of gene expression by anti-sense transcription is not efficient. Alternatively, anti-sense transcripts may be rapidly degraded hence the need for such a high ratio of anti-sense to sense RNA in transfection analyses.

Repression is thought to be accomplished by the formation of anti-sense RNA : mRNA hybrid complexes. In the prokaryotic system the most effective anti-sense RNAs are complementary to the 5′ end of the target mRNA which generally includes the translation initiation site. However, in eukaryotes, anti-sense RNA complementary to 5′ non-coding regions and 3′ coding regions have been equally associated with efficient inhibition of expression. This may be due to the need for several parameters operating co-ordinately in the eukaryotic genome to facilitate the inhibitory effect (Green et al, 1986).

D) Post-Translational Control

Following translation, a protein may require a number of modifications to become fully functional. This may entail the addition of prosthetic groups, glycosylation, methylation or phosphorylation. Proteolysis of the primary translation product may also be required. Therefore, by controlling the availability of the

effectors of these processes a cell can effectively regulate protein activity.

For example, it has been postulated that proteolytic cleavage occurs in the posterior pituitary, where cells can produce either adrenocorticotropin or β-lipotropin from the same primary translation product (Roberts and Herbert, 1977). Changes in protein glycosylation have been observed during differentiation. The human colon cell line HT-29 differentiates to mature enterocytes when cultured in glucose-free medium. This is accompanied by expression of microvillar hydrolases and other enterocytic proteins. In the case of sucrase-isomaltase, the enzyme activity is absent in undifferentiated cells but present following induced differentiation. The mRNA encoding sucrase-isomaltase is present and the protein is synthesised in undifferentiated cells. However the enzyme is rapidly degraded and appears to have a glycosylation pattern which is altered from that of differentiated cells. Therefore it has been proposed that the enzyme would normally be processed, transported and integrated into the plasma membrane of differentiated enterocytic cells but aberrant glycosylation impairs this process (Trugnan et al, 1987).

The addition of prosthetic groups has also been recognised as occupying a prominent position in the pathways that govern the phenotypes of vertebrate cells. Phosphorylation of specific amino acid residues has been demonstrated as crucial to the function of many proteins (Hunter and Cooper, 1985). Therefore by controlling the addition of prosthetic groups the cell can regulate gene expression. It is interesting to note that some oncogenes have been identified which possess the ability to modify proteins and hence activate them. For example, the retroviral oncogene v-src and its cellular progenitor c-src are protein-tyrosine kinases (Collett and Erikson, 1978; Collett et al, 1978). It is believed that one of the functions of tyrosine kinases is to act as transmembrane receptors for polypeptide hormones, such as insulin and epidermal growth factor. Interaction with the hormone initiates a chain of events from cell division to specific differentiation hence these proteins play a crucial role in gene expression and regulation (Staros et al, 1985; Taylor, 1987).

E) Summary

i) Regulatory Mechanisms for Individual Genes

Many potential mechanisms for the regulation of gene expression have been identified. The primary control step would appear to be at the initiation of transcription but it is becoming increasingly clear that regulation at both transcriptional and post-transcriptional levels contribute to differential gene expression. Studies have revealed that a single gene can possess many potential control elements, indicating that control of gene expression is a complex process probably involving the function of a number of different regulatory mechanisms. For example, the c-myc gene contains many elements upstream from the coding region which could be used as mechanisms for controlling gene expression; these include both cryptic and active promoter sites and regions which have been implicated in anti-sense transcription and transcriptional pausing (Battey et al, 1983; Bentley and Groudine, 1986; Nishikura, 1987; Nepveu and Marcu, 1986 and Eick and Bornkamm, 1986). At the 3' end of the c-myc gene two polyadenylation sites have been identified and also in this region there exists a sequence motif that is believed to be involved in determining transcript stability (Shaw and Kamen, 1986). However, it is unclear if all these elements are functional in determining the expression of c-myc.

It has been proposed that the interaction of transcriptional and post-transcriptional control mechanisms for the regulation of gene expression operate in the form of a cascade to mediate rapid response to changing conditions and act as fine tuning devices (Short, 1987). Interaction of different control mechanisms would also amplify the effects of stimulation on gene transcription. This would result in increasing the abundance of the final protein product by many factors more than would be achieved by stimulation of transcription alone (Tobin, 1979). For example, steroid hormones have been demonstrated to increase the rate of transcription of hormone-responsive genes by 5-10 fold. However, this increase was not sufficient to account for the enormous accumulation of specific mRNA from these genes. It has been demonstrated that this increase is accomplished by interaction of mechanisms for regulation of transcription and also for regulation of mRNA stability (Guyette et al,1979; Brock and Shapiro, 1983b).

Hence, by employing two different mechanisms for regulation of gene expression the stimulatory effect of the hormone is amplified.

ii) Co-ordination of Control

In addition to the interaction of individual control mechanisms operating on single genes, there are wider ranging control mechanisms determining the repressed or active state of sets of genes which are co-ordinately expressed or repressed during differentiation or are required in a co-ordinated manner, for example in an enzyme cascade. For these processes to occur some form of primary control must exist which allows sets of genes to be switched on and off in a controlled manner. From cell hybrid studies between unrelated cell types which under go "phenotypic switching" under different culturing conditions, it has been proposed that "master" regulatory factors must play a role in establishing cell phenotype (Harrison, 1984). However, such factors have yet to be identified.

For sets of genes, present in the genome in tandem, such as the histone genes of sea-urchins, control could conceivably be implemented at one end of the gene array and be sufficient to control all the related genes (Hentschel and Birnsteil, 1981). However, it would be more difficult to co-ordinately control genes separated by large distances or even on different chromosomes. Many different proposals have been made to explain how co-ordinated control of gene expression could be implemented. For example, actively transcribing genes have been shown to be associated with the nuclear matrix with looping out of intervening non-transcribed regions (Ciejek et al, 1983). This would allow genes, which are spatially far apart, to be brought very close to one another and concentrate the action of regulatory molecules in specific regions hence facilitating co-ordinate gene expression.

Other models have proposed the interaction of trans-acting factors with cis-acting sites as a mechanism for co-ordinated control. An initial model, proposed by Davidson and Britten (1979) stated that repetitive sequence transcripts could act in trans to regulate the expression of a set of genes containing homology with the repetitive sequence transcripts. These homologous regions may represent cis-acting regulatory elements for the differentially regulated

genes. Co-ordination of gene control in this model ultimately depends on regulation of transcription of the repetitive elements. Murphy et al (1983) have supported this model. They discovered sets of genes which are activated in all SV40 transformed cell lines and are regulated during embryonic development. All these genes share a repetitive element and it is proposed that this element may act as a co-ordinate regulatory sequence.

Analysis of the 5′ flanking regions of co-ordinately expressed genes have revealed regions of homology and synchronous appearance of nuclease sensitive sites during gene activation. This has been proposed to indicate common control mechanisms operating for each of the co-ordinately expressed genes. For example, Affara et al (1985) demonstrated that genes which are co-ordinately expressed in erythropoiesis are controlled by trans-acting factors which modify the chromatin structure around these genes. Fowlkes et al (1984) have also demonstrated that the three genes encoding the fibrinogen proteins, although separated by large intervening DNA sequences, have conserved homology in their 5′ flanking regions.

A model has also been proposed which states that formation of stable transcription complexes may repress or activate eukaryotic genes. Brown (1984) proposes that transcription complexes are composed of more than one molecule. Formation of these complexes may be involved in commitment to a particular differentiation pathway but binding of trans-acting factors would actually modulate the effect of the stable complex on gene transcription during differentiation. If a set of co-ordinately expressed genes all require the same molecules to construct the transcription complex this would provide a suitable mechanism for co-ordination of gene expression. Evidence to support this model has come from the discovery of tissue-specific and development-specific promoter and enhancer elements which may compete for trans-acting factors (see Chapter I, 2, B). Therefore activation or repression of such trans-acting factors would result in the co-ordinated control of all those genes containing the cis-acting element responsive to the trans-acting factor.

3 THE HL60 PROMYELOCYTIC CELL LINE

The human promyelocytic cell line HL60 was established from the peripheral blood leukocytes from a patient with acute promyelocytic leukaemia (Collins et al, 1977). HL60 cells maintain continuous growth in suspension culture, with a doubling time of 24 hours when the cells are in exponential phase of growth. The cells have been shown to be tumorigenic when inoculated into athymic nude mice (Collins et al, 1978). The majority of HL60 cells are promyelocytic in morphology and histochemistry, but 4-15% can display morphological characteristics of more mature myeloid cells, such as myelocytes, metamyelocytes and polymorphonuclear leukocytes (Collins et al, 1977). Promyelocytes are thought to be an early cell found on the cell lineage leading to mature granulocytic differentiation. Figure 3 shows a schematic representation of the myeloid differentiation lineages and gives the proposed position of the HL60 cell line on the differentiation pathway.

A) The HL60 Genome

HL60 cells are aneuploid; abnormalities have been described in the genetic complement of these cells. Amplification of DNA sequences homologous to c-myc, the oncogene of the transforming avian retrovirus MC29, have been found in the cell line and have also been demonstrated in the primary leukaemic cells from which the cell line was established. The abundance of c-myc RNA, in HL60 cells was also higher than in cells which did not contain amplified c-myc DNA sequences (Collins and Groudine, 1982; Dalla Favera et al, 1982a). However, they did not compare the abundance of c-myc transcripts in HL60 cells and normal promyelocytes therefore it is unclear if the abundance of c-myc RNA in HL60 cells is abnormal for haematopoietic cells at the promyelocytic stage.

The cellular homologs of the oncogenes of transforming retroviruses are highly conserved in evolution and all but one or two are found expressed in normal cells. This has led to the suggestion that these sequences have an important role to play during development and differentiation (Duesberg, 1983). Therefore increased or aberrant expression of these sequences might lead to the uncoupling of the co-ordinated control of proliferation and differentiation leading to

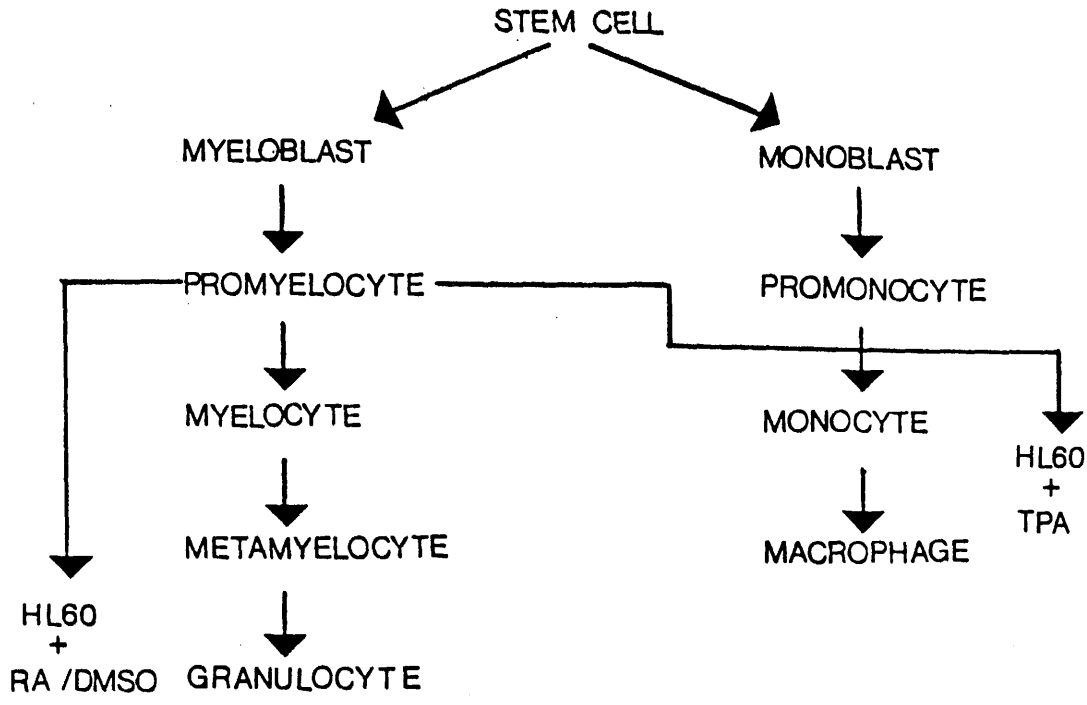


Figure 3 Schematic representation of the myeloid cell lineages.

The proposed position of HL60 cells on this differentiation pathway is indicated. The effects of commonly used chemical agents on the differentiation of HL60 cells is also shown.

neoplastic transformation. Consequently, c-myc amplification may have been involved in the process of leukaemogenesis which gave rise to HL60 cells (Dalla-Favera et al, 1982a). Myc sequences have been frequently postulated to play a role in many different types of cancer, the details of this role being ,as yet undiscovered. For example, in Burkitt's lymphoma translocation occurs between chromosome 8, on which the c-myc gene is found, and chromosomes 2, 14 and 22, carrying the immunoglobulin genes. A similar event has also been described in murine plasmacytomas (Erikson et al, 1983). Amplified c-myc sequences have also been reported in COLO320, a human colon cancer cell line, and in human lung cancer cell lines (Alitalo et al, 1983; Little et al, 1983).

Interestingly, the transforming sequence in HL60 DNA, detected by the 3T3 transformation assay, was shown not to be c-myc but N-ras (Murray et al, 1983). It may be significant that ras, the cellular homolog of the oncogene of the Harvey and Kristen sarcoma virus, and myc oncogenes have been shown to cooperate and be essential for the transformation of primary rat fibroblasts (Land et al, 1983).

Many different sublines of the HL60 cell line exist. Histochemical, karyotypic and immunological analyses have confirmed that they are all HL60. However, it has been demonstrated that c-myc homologous DNA sequence amplification, in many of these sublines, varies from 4-32 fold. This was also reflected in the abundance of c-myc transcripts detected in these cells. Due to this variation between sublines, it has been postulated that while high levels of the c-myc gene, and its transcripts, may have had a role in the initial establishment of HL60 cells, these are not required for the maintenance of the HL60 phenotype (Graham et al, 1985).

p53 is a tumour antigen found at elevated levels in tumour cells of many different tissues and species. However, it has been determined that HL60 cells do not express p53 mRNA due to major deletion in the p53 DNA sequence of these cells. p53 has also been demonstrated to transform primary rat fibroblasts following co-transfection of the p53 gene and the ras oncogene, in an analogous manner to myc and ras (Parada et al, 1984). It has therefore been speculated that deficiency of p53 synthesis in HL60 cells was overcome by using an alternative element which apparently can function in a similar

fashion to the product of p53, this could have been c-myc (Wolf and Rotter, 1985).

B) Induction of Differentiation

HL60 cells are capable of induced differentiation when treated with a number of different agents. A number of other established, haematopoietic cell lines also possess this capacity; for example, U937 cells, a human monoblastic cell line can be induced to differentiate to monocytes by various agents and the Friend murine erythroleukaemia cell line can differentiate to mature erythrocytes under the appropriate stimulatory conditions (Olsson and Breitman, 1982; Rifkind et al, 1984). However, HL60 cells are of particular interest because there is evidence that they are bipotent (Fontana et al, 1981). These cells can differentiate to granulocytes or macrophages depending on the inducing agent used. It has been proposed that uncoupling of the controls which regulate the processes of proliferation and differentiation result in neoplastic transformation (Sachs, 1980). It can be speculated that uncoupling of these processes played a part in the leukaemogenic event from which HL60 cells are derived. Therefore HL60 cells may be "maturation arrested" at a stage similar to that which occurs in the course of normal haematopoietic differentiation. Chemical agents which induce HL60 cells to differentiate may enable the cells to overcome the block in differentiation with the restoration of the differentiation programme.

The most commonly used granulocytic inducing agents are retinoic acid (RA) or dimethyl sulphoxide (DMSO). HL60 cells, when cultured for 5 days in the presence of RA or 5-7 days in the presence of DMSO, differentiate to mature myeloid cells with many of the characteristics attributed to mature granulocytes (Collins et al, 1978; Breitman et al, 1980). The HL60 cell line can also be induced to differentiate to monocyte / macrophages on exposure to many agents, the most commonly used being phorbol ester or 1,25-dihydroxyvitamin D_3. Following the period of induction, 3 days for phorbol ester or 5 days for 1,25-dihydroxyvitamin D_3 treatment, the terminally differentiated cells possess many of the characteristics attributed to mature macrophages (Rovera et al, 1979;

McCarthy et al, 1982).

The bipotent differentiation ability of HL60 cells is unlikely to be due to two cell types present in an HL60 culture. It has been demonstrated that cultures derived from a single cell clone still retain the potential to differentiate to either granulocytes or macrophages depending on the inducing agent used (Fibach et al, 1982). It has also been demonstrated that the presence of inducing agent throughout the whole period required for terminal differentiation characteristics to appear is not necessary; up to 12 hours exposure of HL60 cells to phorbol ester or 48 hours exposure to DMSO is all that is required for the irreversible commitment of the majority of cells to a particular course of differentiation. Removal of the inducer before these times resulted in reversal of the inducer-mediated changes in the cell. However these cells were still bipotent. Hence, cells in which differentiation was initiated by exposure to DMSO were able to develop into macrophages by replacing DMSO with phorbol ester and cells initially exposed to phorbol ester were capable of differentiation to granulocytes with removal of the phorbol ester and addition of DMSO (Fibach et al, 1982). From these results it has been suggested that HL60 cells are derived from an early cell in haematopoiesis common to both myeloid and macrophage cells.

C) Changes Observed During Induction of Differentiation of HL60 Cells

i) Morphological Changes

Uninduced HL60 cells possess large round nuclei, each containing 2-4 nucleoli. The cell cytoplasm is basophilic with prominent azurophilic granules and the nuclear / cytoplasmic ratio is high (Collins et al, 1978). The addition of an agent, which promotes granulocytic differentiation, to the growth medium of an HL60 cell culture, induces striking morphological changes in the majority of cells. These morphological changes have been described by Collins et al (1977) and are characteristic of terminally differentiated myeloid cells. RA or DMSO induced HL60 cells are smaller in size and have a decreased nuclear / cytoplasmic ratio. The cell nucleus is convoluted and lobed with reduced or complete disappearance of nucleoli and the

cytoplasm is diffuse with less prominent cytoplasmic granules. These morphological characteristics are representative of normal metamyelocytes and banded and segmented neutrophils, the terminally differentiated cells of the granulocytic lineage of differentiation. The progress of induction can be followed by analysis of the changes in cell morphology. It has been demonstrated that 24 hours of induction is required before the first morphological changes are found but by 4 days of treatment with either RA or DMSO cells displaying terminally differentiated cell morphology are present in the culture (Breitman et al, 1980).

Following treatment with agents which induce HL60 cells to differentiate to monocyte / macrophages, the cells settle out of suspension and clump together on the surface of the tissue culture flask and attach to the plastic. The adherent HL60 cells have a spindle-shaped morphology. Ruffling of the cell cytoplasm is evident and the cells possess blunt pseudopods which are characteristic features of macrophages (Fibach et al, 1982).

ii) Changes in Cell Growth During HL60 Differentiation

During the induced differentiation of HL60 cells to granulocytes or to monocyte / macrophages, Breitman et al (1980) demonstrated that terminally differentiated cells accumulated in G0/G1 phase of the cell cycle. The loss of cell cycling potential is reflected in the reduced ability of induced HL60 cells to form colonies in semi-solid medium (Daniel et al, 1987). Terminal differentiation of normal cells leads to senescence and death therefore HL60 cells appear to reflect a normal response to a differentiation stimulus by arresting in G0/G1 phase of the cell cycle with the resultant loss of the potential to self-renew.

iii) Functional Changes During HL60 Differentiation

Functional changes can also be detected in differentiating HL60 cells which reflect the functions of normal granulocytes and macrophages. For example, nitroblue tetrazolium (NBT), a water soluble dye, is reduced to insoluble, intracellular blue-black formazan by mature myeloid cells. The reduction process requires the production of superoxide. All terminally differentiated HL60 cells possess this

ability (Breitman et al, 1980). HL60 cells induced to differentiate to granulocytes also phagocytose Candida albicans and opsonised particles (Collins et al, 1978). Increased chemotactic response to formylated peptides has been reported and expression of myeloid surface antigens detected (Tsiftsoglou and Robinson, 1985). All these are characteristic functions of mature granulocytes.

Similarly, HL60 cells induced to differentiate to monocyte / macrophages also show mature cell characteristics. Following HL60 induction by phorbol ester and other monocyte inducing agents an induction of α-napthyl acetate esterase, a histochemical marker of mature macrophages, is detected (Daniel et al, 1987). These cells also display phagocytic properties, increase in lysozyme protein production and expression of monocytic surface antigens which indicate that they are monocytic cells (Munroe et al, 1984; Polansky et al, 1985).

iv) Changes in Gene Expression Following HL60 Differentiation

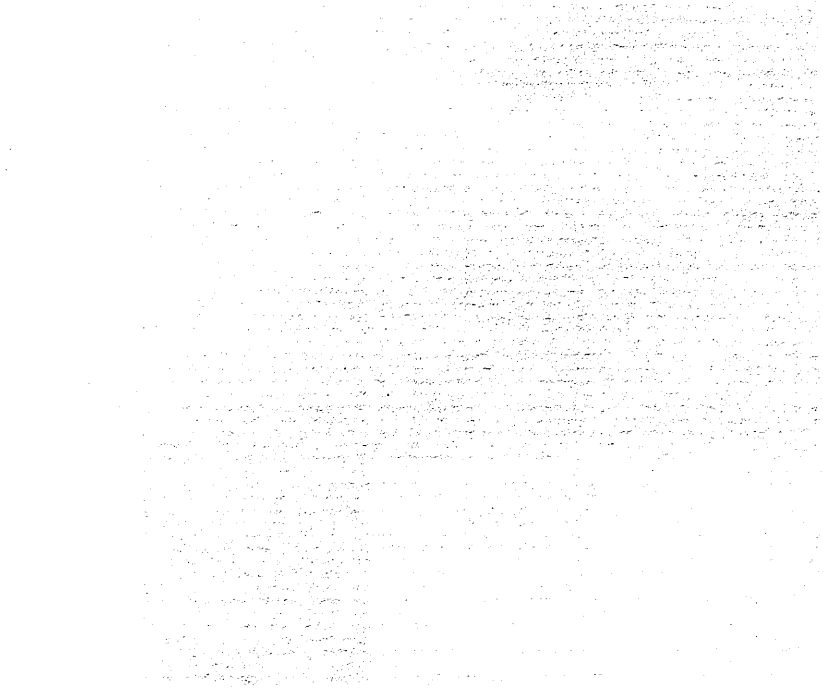
It has been demonstrated that differentiation-associated increase and decrease in the relative abundance of specific mRNAs occurs during induced terminal differentiation of HL60 cells. This was indicated from results of experiments comparing the translation products detected following in vitro translation of polysomal poly(A)$^+$ RNA extracted from uninduced and induced HL60 cells. No gross change in the amount of poly(A)$^+$ RNA was detected following induction but different sets of proteins were translated (Colbert et al, 1983; Reyland et al, 1986). This is indicative of change in patterns of gene expression occuring during the induced differentiation of HL60 cells. The differentiation process is believed to be implemented by changes in the pattern of gene expression therefore HL60 induced differentiation apparently reflects the normal process.

The regulation of specific gene expression has also been reported following HL60 induction. Most notable is the loss of detectable c-myc transcripts in terminally induced HL60 cells (Reitsma et al, 1983; Westin et al, 1982). However more has been determined about the expression of other genes during HL60 induced differentiation with increased availability of DNA hybridisation probes. Analysis of

individual gene expression is useful in determining control mechanisms but in the context of HL60 it is hoped that eventually whole sets of genes will be identified whose abundances change in a co-ordinated manner during differentiation. Identification of these genes will aid in the elucidation of the mechanisms operating to determine co-ordination of gene expression.

D) HL60 as a Model of Differentiation

I have used the HL60 cell line as a means of isolating mRNAs whose abundance varies during differentiation. From analysis of these sequences during the course of HL60 differentiation I wanted to determine the potential control mechanisms operating, on the genes they represented . I also used HL60 cells to determine the order of events which occur as a cell becomes committed to a terminal differentiation pathway. In the future it is hoped that the sequences I have isolated and those isolated by other people will aid in the discovery of the control mechanisms for co-ordinated gene expression.



CHAPTER II : MATERIALS AND METHODS

MATERIALS AND METHODS

1) MATERIALS

All chemicals used were generally Analar Grade, British Drug Houses. Some of the more specialised chemicals, including TPA, RA, sodium lauryl sarcosine, MOPS, antibiotics, ethidium bromide, 2-mercaptoethanol and all the reagents required for hybridisation solutions, except where stated, were purchased from Sigma. Yeast extract, bacto-tryptone and agar for bacterial culture, were purchased from Difco Laboratories. Ultra-pure CsCl, sucrose, urea and agarose were obtained from Bethesda Research Laboratories, Gibco Limited. All tissue culture media were obtained from Gibco Limited or Flow Laboratories. All radioisotopes were supplied by Amersham International.

All glassware, for handling RNA or DNA, was sterilised by treatment with Baysilon-Olemulsion-H and baked at 80°C overnight. All plasticware, tubing and glassware was further sterilised by soaking overnight in 0.05% diethyl pyrocarbonate in water then drying in an oven at 80°C. Exogenous nucleases were removed from all buffers used during the preparation of nucleic acids, by addition of diethyl pyrocarbonate at 0.05% and then destruction of the chemical by autoclaving at 15 lbs/in^2 for approximately 15 min. Disposable plastic pipette tips and tubing were sterilised by autoclaving. Other solutions were either sterilised by autoclaving or by passing through a Millex 0.45 μm filter (Millipore (UK) Limited).

Normal leukocytes were kindly supplied by the West of Scotland Blood Transfusion Service, Law Hospital. Normal mucosa samples were supplied by Dr. P. Elvin, Beatson Institute. Normal human kidney samples were provided by K. Duncan, Western Infirmary, Glasgow and normal human liver samples were supplied by G. Moffat, also of the Western Infirmary, Glasgow.

The vector/host system used, pUC8 / JM83, was described by Viera and Messing (1982).

The cloned probes used were as follows:-

a) pMC41-3RC, containing DNA representing the third exon and 3' flanking region of the human c-myc gene in the Pst I site of pBR322;

originally supplied by Dr. R.C. Gallo (Dalla Favera et al, 1982 b).

b) B_2-microglobulin, containing DNA representing 77% coding region plus 3' flanking sequence of the human B_2-microglobulin gene in the Pst I site of pBR322; supplied by Dr. S. Suggs. (Suggs et al, 1981).

c) pHR28-1, a 7.2kb genomic DNA fragment from the 28S RNA gene, in the Eco RI site of pAT, a gift from A. Sproul.

d) Chick lysozyme cDNA; a full length cDNA cloned into the Pst I site of pBR322; (Land et al, 1981); supplied by Dr. G. Schutz, Heidelberg.

DNA size markers used were λC 857 digested with Hind III or Hind III and Eco RI, and ϕX174 (replicative form) digested with Hae III (Bethesda Research Laboratories, Gibco Limited). RNA size markers were derived from bacteriophage T7, yeast 2 μ, and bacteriophage σ DNA and were purchased as an RNA Ladder from Bethesda Research Laboratories.

2) METHODS

A) Cell Culture

The original HL60 cells used during experimental work in this project were obtained from Dr. R. C. Gallo. However, following mycoplasm contamination the original HL60 subline had to be abandoned. It was replaced with cells received from Dr. G. Brown (Birmingham). These cells were from an early passage of HL60 cells which had originally been obtained from Dr. R. C. Gallo. These HL60 cells were therefore derived from the same source as our initial HL60 subline. HL60 cells were cultured in RPMI-1640 (Flow) plus 0.2% sodium bicarbonate, 2 mM sodium pyruvate, 10% foetal calf serum and 4 mM glutamine. Cells were passaged every 3 days at 2×10^5 cells/ml in Nunclon tissue culture grade flasks and kept at 37°C in 5% CO_2, 95% O_2. The average doubling time for HL60 was 24 h.

U937 cells were cultured in RPMI-1640 (Flow) as described for HL60.

B) Mycoplasma Testing

Cells were tested periodically for the absence of mycoplasma. The cells to be tested were pelleted by centrifugation at 1500 rev/min for 5 min in an MSE bench centrifuge. The cell free supernatant was then placed in a sterile tissue culture grade petri dish (Nunclon), 2 ml of fresh medium were added and 2 x 10^5 NRK49 fibroblasts were seeded into the dish. These cells are known to be mycoplasma free. The dish was incubated at 37°C in a humidified incubator at 5% CO_2, 95% O_2 for 3 days. At the end of the incubation period the cells were fixed by addition of an equal volume of fixative (1 volume of glacial acetic acid : 3 volumes of methanol) for 5 min. The fixative was then removed and the cells air dried. Hoechst 33258 stain was prepared from a 1 mg/ml stock by diluting 1:20 000 in phosphate buffered saline. This solution was poured onto the fixed cells and incubated at room temperature for 10 min. The stain was removed and the cells were washed two times in water. The cells were viewed by fluorescent microscopy using a water immersion lens. Hoechst 33258 is a fluorescent stain for DNA. If the cells were mycoplasma free only the cell nucleus fluoresced, however if the original cells had been mycoplasma infected fluorescence was also detected in the cytoplasm of the NRK49 cells.

C) Induction of Differentiation

i) Phorbol Ester

Stock TPA solution, prepared from Sigma 12-O-tetradecanoyl 13-phorbol acetate, was dissolved in acetone at a concentration of 1.6 x 10^{-4} M and stored at -20°C. HL60 were induced at a concentration of 5 x 10^5 cells/ml at a final concentration of 1.6 x 10^{-7} M TPA. The induction lasted over a 3 day period with only one addition of the inducing agent.

ii) Dimethyl Sulphoxide

HL60 were induced at a concentration of 2 x 10^5 cells/ml with Merck dimethyl sulphoxide giving a final inducer concentration of 1.5% (v/v) DMSO. Induction was over a 5 day period with only one addition of the inducing agent.

iii) Retinoic Acid

Retinoic acid (Sigma) was kept in solution, in ethanol, at a concentration of 10^{-2} M. Stock solution was kept for up to 2 weeks only and stored in a light-tight container at -20°C. HL60 cells were induced at 2 x 10^5 cells/ml giving a final RA concentration of 10^{-6} M. RA was added every 24 h throughout the 5 day induction period. The induced cultures were incubated at 37°C as normal but in the dark to limit degradation of the inducing agent throughout the period of treatment.

All cells used in the inductions were taken from exponentially growing cultures. The cells were passaged 24 h before addition of inducing agent to reduce any potential serum stimulation from the fresh medium.

D) Staining Procedures

i) May-Grunwald and Giemsa Staining

Cells were spun on to clean glass slides using a Shannon cytocentrifuge at 50 rev/min for 5 min and air dried. The cells were then fixed in 70% (v/v) methanol. May-Grunwald stain was prepared by diluting 2 volumes of stain in 3 volumes Sorensens Buffer pH 6.85 (0.066 M disodium hydrogen orthophosphate, 0.066 M potassium dihydrogen orthophosphate) and filtering through Whatman Filter paper. Giemsa stain was also diluted in Sorensens Buffer, 1 volume stain : 9 volumes buffer and filtered. The slides were stained for 5 min in May-Grunwald stain, followed by 10 min in Giemsa stain. They were then washed twice in double distilled water, air dried and mounted in DPX mountant, and examined by light microscopy.

ii) Non-specific Esterase - a Monocyte Specific Stain

Cells were spun on to clean glass slides using a Shannon cytocentrifuge at 50 rev/min for 5 min and air dried. The slides were then incubated at 37°C for 2 h with 0.05 M α-napthyl acetate, 0.1 M hexazonium pararosanilin-HCl in 0.2 M disodium hydrogen orthophosphate solution at pH 7.4. 1% (w/v) methyl green pH 4.0 was used to counterstain prior to washing in double distilled water and dehydration through 50%, 70%, 90% and 100% (v/v) ethanol. The slides were air-dried, mounted in DPX mountant and examined by light microscopy.

iii) Nitroblue Tetrazolium - a Myeloid Cell Specific Stain

1 X 10^6 cells were harvested by centrifugation in an MSE bench centrifuge at 1500 rev/min for 5 min at room temperature. The supernatant was discarded and the cell pellet was resuspended in 10 mM tetra decanoyl phorbol acetate (Sigma) containing 0.2% (w/v) nitroblue tetrazolium (NBT) (Sigma). The cell suspension was incubated at 37°C for 25 min. Following incubation 0.5 ml of the cell suspension was removed and the cells centrifuged on to a clean glass slide using a Shannon cytocentrifuge. The slide was then air dried before staining for 5 min in May-Grunwald stain followed by 10 min in Giemsa stain. The stains were prepared as described in Chapter II, 2, D (i). After removing excess stain by washing in double distilled water the slide was air dried and mounted in DPX mountant before viewing by light microscopy.

E) Lysozyme Protein Assay

i) Secreted Lysozyme Protein

The medium from HL60 cell cultures was decanted and spun at 1500 rev/min for 5 min in an MSE bench centrifuge to remove cells. This cell-free medium was then taken in 1 ml samples, and added to 0.2 mg/ml micrococcus lysodeikticus in 5 ml of 0.1 M PBS pH 6.3. The optical density was measured at 450 nm. Following a 30 min incubation at 37°C the optical density was again determined and expressed as OD_{450nm}. Dilutions of human lysozyme (Sigma) were made in fresh

culture medium and assayed as above to serve as controls and to determine the quantity of lysozyme present in the cell culture medium by comparison.

ii) Intracellular Lysozyme Protein

To measure intracellular lysozyme protein in HL60 cells, 1 x 10^6 cells were collected by centrifugation at 1500 rev/min for 5 min in an MSE bench centrifuge. The supernatant was discarded and the cells were resuspended in 3 ml 0.1% (v/v) triton X-100. The cells were incubated in this solution for 5 min at room temperature to ensure efficient cell lysis. The lysate was then taken in 1 ml aliquots and added to 0.2 mg/ml micrococcus lysodeikticus in 5 ml of 0.1 M PBS pH 6.3. The optical density was measured at 450 nm. Following a 30 min incubation at 37°C the optical density was again determined and expressed as OD_{450nm}. Dilutions of human lysozyme (Sigma) were made in RPMI 1640 medium (Flow) and assayed as above to serve as controls and to determine the quantity of lysozyme present in the cell lysate by comparison.

F) Fluorescence Activated Cell Sorting

Samples containing 1 x 10^6 cells were removed from cultures of HL60 cells. These samples were then centrifuged at 1500 rev/min for 5 min in an MSE bench centrifuge, the medium decanted, and the cells washed 2 times in ice cold PBS before resuspension in 70% (v/v) methanol with continual vortexing. Cells could be kept in this fixed state at 4°C indefinitely. Before analysis, the cells were removed from the methanol and resuspended in 1ml 15 mM $MgCl_2$, (0.002%) (w/v) Chromomycin A3 stain (Sigma). To allow efficient staining the cells were kept on ice, in the dark, for up to 2 h prior to FACS analysis. Samples were examined using a Fluorescence Activated Cell Sorter (FACS II) as described by Young et al (1981). Chromomycin A3 is a fluorescent stain for DNA. The FACS II detects fluorescence emitted from bound stain following exposure to a laser ray. The cells are then sorted according to the intensity of fluorescence detected which is directly proportional to the DNA content of the cell.

G) Measurement of Cell Cloning Potential

HL60 cells were induced as described previously in Chapter II, 2, C. Following induction, cells were removed at specific intervals, counted on a Coulter counter and washed in ice cold PBS. They were then diluted in Flow RPMI 1640 medium to give a final concentration of 5-10 cells/ml. 200 μl volumes of this dilution were plated out into 96 well, flat bottomed Nunclon tissue culture grade plates. These were incubated at 37°C in 5% CO_2 / 95% O_2 in a humidified cell incubator. To analyse the cloning ability of the cells each plate was examined every 24 hours. The number of cells/well were counted and recorded for each plate over a 7 day period.

H) Isolation of RNA

i) Cell Harvesting

1 x 10^8 - 1 x 10^9 cells were harvested from exponentially growing cultures by centrifugation in an MSE bench centrifuge at 1500 rev/min for 5 min, if the volume was less than 100 ml. For larger volumes cells were spun in a GS-3 rotor at 2000 rev/min for 10 min in a Sorvall RC-5B centrifuge at 0°C. The medium was decanted and the cell pellet washed twice in ice-cold PBS.

ii) Cell Fractionation

2 x 10^9 cells were harvested as described in Chapter II, 2, H (i), the cell pellet was resuspended in hypotonic buffer I (0.14 M NaCl, 1.5 mM $MgCl_2$, 100 mM tris-HCl pH 7.4) at a concentration of 5 X 10^7 cells/ml. NP40 was added to a final concentration of 0.5% (v/v) to lyse the cells. The cells were allowed to lyse on ice for 15 min then homogenised gently by hand (10 strokes) with a teflon ball pestle in a tight fitting, pre-chilled glass Potter homogeniser, on ice. Cell lysis was checked by phase contrast microscopy. Nuclei were pelleted from the lysate by centrifugation in an M.S.E. 4L centrifuge (8 x 50 ml rotor) at 2000 rev/min for 15 min at 4°C. The post-nuclear supernatant was retained for preparation of polysomes.

iii) Preparation of Nuclei and Nuclear RNA

Following cell fractionation the pelleted nuclei were resuspended in ice-cold 5% citric acid at a concentration of 1 x 10^8 cells/ml. Nuclei were further purified by homogenisation in this solution, on ice, as before. The homogenate was centrifuged at 4°C for 5 min at 1000 rev/min in the 8 x 50 ml rotor of an M.S.E. 4L centrifuge. The supernatant was removed and the loose pellet was resuspended in ice-cold 0.25 M sucrose in 1.5% (w/v) citric acid. The suspension was then homogenised with 5 strokes of the homogeniser, as described above. The homogenate was transferred to a 50 ml centrifuge tube (20 ml/tube), underlayered with 20 ml of ice-cold 0.88 M sucrose in 1.5% citric acid and centrifuged at 4°C for 5 min at 2000 rev/min in the 8 x 50 ml rotor of an M.S.E. 4L centrifuge. The supernatant was removed by aspiration to just below the level of the interphase then the remaining solution was decanted. The tube was drained and the sides wiped with sterile gauze. The pellet was resuspended, homogenised and centrifuged through sucrose at least twice more to remove any remaining cytoplasmic debris. Purity of the preparation was checked by phase contrast microscopy. The nuclei were washed free of citric acid by resuspending the pellet in 0.25 mM KCl, 3 mM $MgCl_2$, 50 mM tris-HCl pH 7.4 and centrifuging at 4°C for 5 min at 1000 rev/min in the 8 x 50 ml rotor of an MSE 4L centrifuge. The supernatant was discarded and the sides of the tube were wiped with sterile gauze. Total nuclear RNA was prepared by lysing the purified nuclear pellet in 5 M guanidinium thiocyanate, 50 mM tris-HCl pH 7.0, 50 mM EDTA, 5% (v/v) 2- mercaptoethanol at 1-2 x 10^7 nuclei/ml. Preparation of RNA from the nuclear lysate was carried out exactly as described for the preparation of total RNA from whole cells (Chapter II, 2, H (v)).

iv) Preparation of Polysomes and Total Polysomal RNA

Polysomal RNA was prepared essentially as described by Young (1976) and Affara and Young (1976). Following lysis, homogeninisation and isolation of nuclei as described in Chapter II, 2, H (ii), the post-nuclear supernatant was retained for preparation of polysomes. 2 M sucrose in Buffer I (0.14 M NaCl, 1.5 mM $MgCl_2$, 100 mM tris-HCl pH 7.4) was added to the post-nuclear supernatant to a final

concentration of 0.25 M. To remove mitochondria, the post-nuclear supernatant was centrifuged at 8000 rev/min for 10 min at 4°C in the HB-4 rotor of a Sorvall RC-5B centrifuge. The supernatant was collected and triton-X-100 was added to a final concentration of 1%, to release membrane bound polysomes; 15 ml of this solution was transferred to 25 ml polycarbonate MSE ultracentrifuge tubes and underlayered with 5 ml 2 M sucrose in Buffer I. Polysomes were purified and pelleted by centrifugation through the sucrose pad at 55 000 rev/min for 3.5 h at 4°C in an 8 x 25 ml titanium fixed angle rotor in an MSE SS65 ultracentrifuge. Following centrifugation the supernatant was removed by aspiration down to the interphase of the two sucrose solutions then the remaining solution was decanted and the tubes were drained well. After wiping the sides of the tubes with sterile gauze, the pellets were resuspended gently in 1 ml of NETS buffer (100 mM NaCl, 10 mM tris-HCL ph 7.4, 10 mM EDTA, 0.5% SDS). An equal volume of 8 M urea and two volumes of 4 M LiCl were added and the polysomal RNA was precipitated by incubation at 4°C overnight. The polysomal RNA was recovered by centrifugation of the solution at 10 000 rev/min for 15 min at 4°C in the HB-4 rotor of the Sorvall RC-5B centrifuge. The pellet was resuspended in 5 ml NETS buffer and proteins were removed by shaking the solution with an equal volume of a 1:1 mixture of phenol and chloroform saturated with NETS buffer. The phenol and aqueous layers were separated by centrifugation of the mixture at room temperature at 5000 rev/min for 5 min in the Sorvall HB-4 rotor. The aqueous layer was removed and extracted with an equal volume of chloroform to remove residual phenol. The aqueous layer was retained and sodium acetate was added to a final concentration of 0.3 M. RNA was precipitated from solution by addition of 2.5 volumes of ethanol and overnight incubation at -20°C. This step was repeated once more and each time precipitated RNA was collected by centrifugation at 10 000 rev/min for 10 min at -15°C in the HB-4 rotor of a Sorvall RC-5B centrifuge. Pelleted RNA was washed by resuspension in 70% (v/v) aqueous ethanol, then 95% ethanol, collected each time by centrifugation as before. The RNA pellet was then dried and resuspended in DEP treated double distilled water to a final concentration of 1 μg/μl.

v) Preparation of Whole Cell RNA

The method used was adapted from that of Chirgwin et al (1979). Following cell harvesting, cells were lysed by addition of 5 M guanidinium isothiocyanate (Bethesda Research Laboratories), 0.05 M tris pH 8.0, 0.5 M EDTA, 0.7 M 2-mercaptoethanol, at pH 7.0. to give a final cell concentration of 1-2 x 10^8 cells/ml. The preparation could be stored frozen at this stage before the isolation of total whole cell RNA. The solution was then sonicated, in a Bronson sonicator at 20 Hz, by 3 x 30 second pulses, to shear the DNA. The preparation was kept on ice at all times. Lauryl-sarcosine was then added to a final concentration of 2% and the solution heated for 2 min at 68°C to dissolve the sarcosine. This was then loaded on to a 2.5 ml CsCl cushion (5.7 M CsCl, 50 mM EDTA pH 7.0, at a refractive index of 1.3995) in a B60 14 ml polypropylene tube and spun for 36 h at 15°C, 35 000 rev/min in a B60 Damon IEC ultracentrifuge. Following centrifugation the guanidinium isothiocyanate was unloaded leaving the RNA clearly visible as a gelatinous, clear pellet. The tubes were inverted and allowed to drain well. The RNA pellet was then resuspended in DEP treated double distilled water and precipitated from solution at -20°C, by the addition of 3 M sodium acetate to 0.3 M and 2.5 volumes of ethanol. RNA was retrieved by centrifugation at 10 000 rev/min for 10 min at -15°C in a Sorval HB-4 swing out rotor. The pellet was resuspended in DEP treated double distilled water to which was added an equal volume of 4 M LiCl and 8 M Urea. The RNA was precipitated from this solution by incubation overnight at 4°C. This precipitation step removed any residual DNA from the RNA as DNA does not precipitate under these conditions. The RNA was again collected as before by centrifugation at 10 000 rev/min in a Sorval HB-4 swing out rotor. Finally the RNA was precipitated once more by addition of sodium acetate to 0.3 M and 2.5 volumes of 95% ethanol, collected by centrifugation and washed in 70% (v/v) ethanol and then 95% ethanol. The pellet was then dried briefly and resuspended at 1 μg/μl in DEP treated double distilled water.

vi) Preparation of Poly(A)$^+$ and Poly(A)$^-$ RNA Fractions

This method was modified from that of Aviv and Leder (1972). A 1.5 ml column of oligo(dT) cellulose (0.3 g) was prepared as described by the suppliers (Bethesda Research Laboratories). The column was then

well rinsed in approximately 20 bed volumes of 1x Binding Buffer (0.5 M NaCl, 10 mM tris-HCl pH 7.5, 1 mM EDTA). Total whole cell RNA was dissolved in DEP treated double distilled water to give a maximum concentration of 200 µg/ml and then heated at 68°C for 2 min to denature any secondary structure. The solution was then chilled rapidly on ice. An equal volume of 2x Binding Buffer was added to the solution to give a final RNA concentration of 100 µg/ml. This was slowly passed through the oligo(dT) cellulose column. When the RNA solution had passed through, the column was washed with 1x Binding Buffer (approx. 20 bed volumes) until the OD_{260nm} was less than 0.1. The elutate was collected,on ice, as it contained unbound poly(A)$^-$ RNA. The bound poly(A)$^+$ RNA was eluted with sterile water. The RNA was collected in 1 ml fractions and kept on ice. The fractions were then measured by OD_{260nm} and those containing the poly(A)$^+$ RNA pooled. These pooled fractions were again denatured at 68°C for 2 min. 2x Binding buffer was added to adjust salt conditions to that of 1x Binding buffer. The solution was then reapplied to the column and eluted with 1x Binding buffer. The poly(A)$^+$ RNA, bound to the column, was again eluted with sterile water and 1 ml fractions collected. The second application of RNA to the oligo(dT) cellulose column increases the fractionation of poly(A)$^+$ RNA from poly(A)$^-$ RNA. The RNA was then precipitated from solution overnight by addition of 3 M sodium acetate to 0.3 M and 2.5 volumes ethanol at -20°C. The poly(A)$^+$ RNA was collected by centrifugation at 10 000 rev/min at -15°C in a Sorvall RC-5B using an HB-4 rotor, washed in 70% (v/v) ethanol and then 95% ethanol. The pellet was then dried and resuspended in DEP treated double distilled water at a concentration of 1 µg/µl.

The poly(A)$^-$ RNA fractions were retained from the column and the RNA precipitated by addition of 3 M sodium acetate to 0.3 M and two volumes of ethanol. The RNA was precipitated at -20°C overnight and the RNA then collected by centrifugation at 10 000 rev/min at -15°C in a Sorvall RC-5B centrifuge using an HB-4 rotor. The RNA was then washed in 70% (v/v) ethanol followed by 95% ethanol (being collected each time by centrifugation as before), dried and resuspended in DEP treated double distilled water to a final concentration of 1 µg/µl.

I) Analysis of RNA

i) Quantitative RNA Dot Blot Analysis

A stock solution of RNA in DEP treated water (20 µl) was prepared (500 µg/ml for total whole cell RNA); 10 µl of RNA solution was transferred to a tube containing 10 µl of DEP treated double distilled water to give a 1:2 dilution. 10 µl from this tube was then transferred to a third tube containing 10 µl of DEP treated double distilled water and so on until 12 doubling dilutions had been prepared. These samples were then heated at 65°C for 15 min to denature any secondary RNA structure and chilled on ice. The tubes were then spun briefly in an Eppendorf microfuge to collect condensation from the sides of the tube. 4 µl of RNA were spotted on to nitrocellulose membrane (Sartorius 0.10 µm) in duplicate. The nitrocellulose was prepared by soaking in water, then in 20x SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0) for 30 min and air drying. After dotting the membrane was allowed to air dry and was then baked at 80°C for 2 h to immobilise the RNA.

ii) RNA Fractionation on Denaturing Agarose Gels

This method was taken from Boedtker (1971). 10-30 µg of total whole cell RNA or 1-5 µg poly(A)$^+$ RNA were freeze-dried then resuspended in 9 µl formamide denaturation buffer (50% formamide, 2.2 M formaldehyde, 1x MOPS buffer (40 mM sodium MOPS, 10 mM sodium acetate, 1 mM EDTA pH 7.0)). The samples were then denatured by heating for 15 min at 68°C and chilled immediately. Prior to loading on the gel 1 µl of loading buffer (50% glycerol, 0.1% bromophenol blue, 10 mM sodium phosphate pH 7.0) were added. The RNA was electrophoresed through 50 ml horizontal, denaturing, 1% agarose gels containing 2.2 M formaldehyde. Gels were buffered in MOPS buffer (40 mM sodium MOPS, 10 mM sodium acetate, 1 mM EDTA pH 7.0) and subjected to electrophoresis at 90 volts for 1 h with buffer recirculation.

iii) Northern Blot Analysis of RNA

The electrophoresed RNA was transferred directly on to nitrocellulose membrane (Sartorius 0.10 µm) by blotting in 20x SSC (3 M NaCl, 0.3 M

sodium citrate pH 7.0) as described by Thomas (1980). The blotting apparatus was set up as described by Southern (1975). The nitrocellulose was first soaked in DEP treated double distilled water then in 20x SSC for 30 min prior to blotting overnight. At the completion of transfer the nitrocellulose was air dried and then baked at 80°C for 2 h to immobilise the RNA.

J) Preparation of Genomic DNA

Genomic DNA was prepared using a modification of the method of Gross-Bellard et al (1973). 1 x 10^8 cells were harvested as described in Chapter II, 2, H (i). The cells were then lysed by resuspension in 10 mM EDTA, 10 mM tris- HCl pH 8, 10 mM NaCl containing 4% sarcosine (w/v) and proteinase K (20 units/mg) (Boehringer Corporation Limited) at a final concentration of 100 µg/ml. This mix was incubated overnight at 37°C. An equal volume of phenol and chloroform with 0.5 M tris-HCl pH 8.0, 10 mM NaCl, 10 mM EDTA, 0.5% SDS, was added and mixed gently for 10-30 min. This was followed by centrifugation in an MSE 4L centrifuge at room temperature and at 1000 rev/min for 10 min. The aqueous phase was then removed and sodium acetate added to a final concentration of 0.3 M. Following the addition of 2.5 volumes of ethanol the DNA was spooled out from the solution using a sterile glass rod. This was washed twice in 70% (v/v) ethanol, twice in 95% ethanol and then once in chloroform. The DNA was washed, while still wound onto the glass rod, by immersion in each wash for 1 min, followed by expulsion of as much fluid from the DNA as possible before moving on to the next wash. Finally the DNA was air dried and the rod placed in 10 ml of 0.1x SSC overnight to allow the DNA to resuspend. Following resuspension, the solution was RNase treated for 3 h by addition of boiled RNase A (50 µg/ml) and RNase T (1 µg/ml). 0.4 M EDTA was added to a final concentration of 10 mM, sarcosine to 4% and proteinase K (20 units/mg) to a final concentration of 50 µg/ml. This solution was incubated for 3 h at 37°C. At the end of incubation the DNA solution was again phenol / chloroform extracted as before and the DNA from the aqueous phase spooled out of solution containing 0.3 M sodium acetate and 2.5 volumes ethanol. The DNA was washed and dried as described above and then dissolved in TE buffer (10 mM tris pH 8.0, 1 mM EDTA) to give a final concentration of 500 µg/ml.

K) Preparation of Plasmid DNA

i) Large Scale Preparation

This method was a modification of that described by Birnboim and Doly (1979). From 10 ml overnight cultures of the bacteria containing the plasmid to be prepared 1 ml was inoculated into 500 ml of L-broth (1% (w/v) bacto-tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract). 50 µg/ml ampicillin was added if the plasmid contained the appropriate antibiotic resistent gene. The culture was incubated overnight at 37°C in a shaking incubator. Following incubation the cells were pelleted by centrifugation at 7000 rev/min for 5 min at 4°C in a GS-3 rotor of a Sorvall RC-5B centrifuge. The supernatant was decanted and the pellet drained. The cells were then lysed by resuspension of the pellet in 18 ml of cold lysis buffer (50 mM glucose, 25 mM tris-HCl pH 6.8, 10 mM EDTA, 2 mg/ml lysozyme). The resuspended pellet was left on ice for 30 min. To this was added 40 ml of a solution containing 0.2 M NaOH, 1% SDS and the mixture further incubated on ice for 5 min. This solution denatures the bacterial chromosomal DNA. Finally 20 ml of ice cold 5 M potassium acetate pH 4.8 was added to neutralise the lysate. Addition of this causes the bacterial chromosomal DNA to aggregate and become insoluble. After 1 h incubation on ice this insoluble material was pelleted by centrifugation at 8000 rev/min, at 4°C in a GS-3 rotor of a RC-5B Sorvall centrifuge. The supernatant was then removed and filtered through gauze to prevent any contamination by aggregated bacterial DNA. The plasmid DNA was precipitated by addition of 0.6 volumes of ice cold isopropanol and incubated at room temperature for 15 min. DNA was pelleted by centrifugation at 8000 rev/min for 10 min at 4°C as before. The supernatant was removed and the pellet drained then resuspended in 5 ml 50 mM tris-HCl pH 8.0, 10 mM EDTA , 0.5 ml ethidium bromide (10 mg/ml) plus CsCl (Bethesda Research Laboratories) to give a final refractive index of 1.3890. The solution was then transferred to 10 ml polycarbonate tubes for an MSE 10 x 10 ml titanium, fixed angled rotor. The tubes were filled to three quarters with the plasmid DNA solution, the remainder of the volume was filled with paraffin oil. The tubes were centrifuged at 40 000 rev/min for 40-60 h at room temperature. Following centrifugation two bands were usually observed on U.V. exposure. The upper band was comprised of residual bacterial chromosomal DNA, the

lower band contained the plasmid DNA. This band was removed by pipetting using a pasteur pipette. To remove the ethidium bromide from solution the mixture containing the plasmid DNA was extracted 5 times with an equal volume of isopropanol saturated with CsCl. The plasmid solution was then dialysed for 1 day against several changes of TE buffer (10 mM tris-HCl pH 8.0, 1 mM EDTA) to remove the CsCl. Following dialysis the plasmid DNA was precipitated from solution by incubation at -20°C overnight following the addition of 3 M sodium acetate to a final concentration of 0.3 M and 2.5 volumes of ethanol. The plasmid DNA was then pelleted by centrifugation at -15°C, at 10 000 rev/min in an HB-4 rotor on a Sorvall RC-5B centrifuge. The DNA was washed once in 70% (v/v) ethanol and once in 95% ethanol then repelleted by centrifugation.

ii) Further Purification of Plasmid DNA

To further purify the plasmid DNA from contaminating bacterial chromosomal DNA, the DNA was resuspended in 1 M NaCl, 10 mM tris-HCl pH 7.5, 1 mM EDTA at a concentration of 2 mg/ml and sedimented rate zonally by centrifugation through a sucrose gradient. 100 µl samples of DNA were layered on top of 14 mls 5% - 20% neutral sucrose gradients in the same buffer. These samples were then centrifuged in a 6 x 14 ml rotor of a B60 Damon IEC ultracentrifuge for 4 h at 40 000 rev/min at 20°C. Following centrifugation the gradients were unloaded by upward displacement by fluorochemical FC43 (3M Chemical Company Limited) and 1 ml fractions collected. The OD_{260nm} was read for these fractions and those containing plasmid DNA were pooled. The plasmid DNA was precipitated by addition of 3 M sodium acetate to a final concentration of 0.3 M and 2.5 volumes of ethanol followed by overnight incubation at -20°C. The plasmid DNA was then pelleted by centrifugation at -15°C, at 10 000 rev/min in an HB-4 rotor on a Sorvall RC-5B centrifuge. The DNA was washed once in 70% (v/v) ethanol and once in 95% ethanol then collected by centrifugation as before. The DNA pellet was dried and resuspended in 1x TE buffer at 0.5 µg/µl.

iii) Small Scale Preparation

For quick analysis of plasmid DNA small scale preparations were used. To 2 ml of L-broth, containing the appropriate antibiotic if

required, a single bacterial colony was inoculated and incubated overnight at 37°C with shaking. Cells were then pelleted from 1.5 ml of culture medium in screw capped Sarstedt tubes by centrifugation at 10 000 rev/min, at room temperature, in an Eppendorf microfuge. The supernatant was discarded and the cell pellet resuspended in 100 µl of lysis buffer which contained 0.9% (w/v) glucose, 25 mM tris-HCl pH 8.0, 10 mM EDTA pH 7.5, and 25 ul of a fresh solution of lysozyme (10 mg/ml) in the same solution. This mixture was incubated on ice for 15 min. To this was added 200 µl of 0.2 M NaOH, 1% SDS and the mixture incubated on ice for 5 min. 150 µl of ice cold 5 M potassium acetate pH 4.8 was then added and the mixture again incubated on ice for 5 min. This precipitates the bacterial DNA and protein. To remove this the tubes were centrifuged for 1 min in an Eppendorf microfuge and the supernatant containing the plasmid DNA then transferred to a fresh tube. Boiled RNase A (50 µg/ml) was then added to a final concentration of 10 ug/ml and incubated at 37°C for 30 min to remove contaminating RNA. The plasmid DNA was then precipitated by addition of 3 M sodium acetate to 0.3 M and 2 volumes of ethanol and incubated at -20°C for 15 minutes. The DNA was collected by centrifugation in an Eppendorf microfuge for 5 min, the pellet dried and then resuspended in 50 µl TE buffer which gave approximately 1 µg/µl DNA.

L) Restriction Endonuclease Digestion

The digestions were carried out as recommended by the manufacturers. For plasmid DNA restriction enzyme digestions were routinely carried out with the appropriate restriction enzyme added to a final concentration of 5 units/µg of DNA. The mix was then incubated for 1 h at 37°C in the appropriate buffer. Restriction digestion of genomic DNA was carried out using the appropriate restriction enzyme added to a final concentration of 10 units/µg of DNA. Incubation of the mix was 6 h at 37°C; genomic DNA was at a concentration of 50 µg/ml. At the completion of the incubation period the enzymic reaction was stopped by addition of EDTA pH 8.0 to 20 mM. To determine the concentration of genomic DNA present accurately, after digestion, the digested samples were dialysed against 0.1x TE buffer (10 mM tris-HCl pH 8.0, 1 mM EDTA) and then the OD_{260nm} of each sample read on a Cecil spectrophotometer.

M) Analysis of DNA

i) DNA Fractionation by Gel Electrophoresis

Digested genomic DNA samples were fractionated by horizontal electrophoresis on a 1% agarose gel. The gel was made up by boiling the appropriate weight of agarose with 200 ml of 1x TBE electrophoresis buffer (0.089 M tris-base , 0.089 M boric acid, 0.002 M EDTA pH 8.3). This was cast in a 14 cm by 20 cm mould. Separation of plasmid DNA fragments of greater than 1 Kb was also accomplished by the method described above. However DNA fragments of less than 1 Kb were fractionated on 1.5% agarose TBE buffered gels. 20-30 µg of genomic DNA in 30 µl TE buffer , or 0.5-2.0 µg plasmid DNA were routinely electrophoresed. Prior to loading of the DNA samples a sixth volume of sample loading buffer was added to each tube (30% sucrose, 10 mM tris-HCl pH 8.0, 1 mM EDTA, 5% SDS, 0.1% bromophenol blue). Electrophoresis was carried out overnight at 30 volts. Markers were run simultaneously, these were normally λ phage digested with Eco RI / Hind III for sizing of large DNA fragments, or ϕ X174 DNA (replicative form) digested with Hae III for sizing of small fragments. Following electrophoresis the gel was stained for 15 min in ethidium bromide at a final concentration of 0.5 µg/ml, in water. The DNA was visualised by U.V. fluorescence on a Chromato-Vue transilluminator, then photographed through a red No 9 Kodak Wratten gelatin filter.

ii) DNA Fractionation Under Denaturing Conditions

0.5-1.0 µg of restriction digested plasmid DNA was fractionated by electrophoresis on a 1.4% agarose gel under denaturing conditions (55 mM NaOH). Fractionation under these conditions eliminates artifactual results caused by DNA secondary structure. 1.4 g of agarose was added to 100 ml of 50 mM NaCl and 1 mM EDTA then boiled until the agarose dissolved. Following this the gel was poured and allowed to set. Sufficient 1x alkaline electrophoresis buffer (10x alkaline electrophoresis buffer is 300 mM NaOH, 10 mM EDTA) was added to cover the gel and allowed to soak for 30 min. Addition of NaOH to hot agarose solutions can cause hydrolysis of the polymer; hence gels are prepared in neutral unbuffered solution and then equilibriated in

alkaline electrophoresis buffer. Prior to loading of the samples the DNA was dissolved in alkaline loading buffer which is 50 mM NaOH, 1 mM EDTA, 2.5% (w/v) Ficoll and 0.025% (w/v) bromocresyl green. The samples were then loaded and electrophoresed at 80 volts for 1 h. The gel was then removed from the gel apparatus and stained in ethidium bromide (0.5 μg/ml in water). The gel was then washed in water and the DNA visualised by UV fluorescence on a Chromato-Vue transilluminator, then photographed through a red No 9 Kodak Wratten gelatin filter.

iii) Isolation of cDNA Fragments from Recombinant Plasmid DNA

Plasmid DNA was restriction digested as described in Chapter II, 2, L. The reaction mix was electrophoresed on a 1.5% low melting point agarose gel (Bethesda Research Laboratories) at 70 volts for 30 min at 4°C in TBE buffer. The gel was then stained in ethidium bromide at 0.5 μg/ml in water for 10 min. The DNA fragments could then be visualised by UV fluorescence on a "Chromato Vue" transilluminator. The cDNA fragment was cut from the gel and then heated at 70°C for 10 min to melt the agarose. To this was added 100 μl of phenol saturated with TE (10 mM tris-HCl pH 8.0, 10 mM EDTA). The tube was vortexed vigorously then centrifuged at 10 000 rev/min in an Eppendorf microfuge for 5 min. The aqueous phase was removed and this was further phenol extracted until no precipitate was visible between the aqueous and phenol phases. These steps removed the agarose from the DNA. The DNA from the aqueous phase was then precipitated by addition of 3 M sodium acetate to 0.3 M and 2.5 volumes of ethanol and overnight incubation at -20°C. The DNA was collected by centrifugation at 10 000 rev/min for 10 min in an Eppendorf microfuge then resuspended in TE buffer at a concentration of 10 ng/μl.

iv) Southern Blot Analysis of DNA

Following fractionation of endonuclease restriction digested DNA as described in Chapter II, 2, M (i), DNA was transferred to nitrocellulose membrane as described by Southern (1975). Prior to blotting the marker lanes were removed and the gel subjected to a number of washes. The gel was washed twice, for 15 min, in 0.25 M HCl to depurinate the DNA. The gel was then washed twice for 20 min in 1.5 M NaCl, 0.5 M NaOH to shear and denature the DNA. Finally the gel

was neutralised by washing 3 times for 30 min in 3 M NaCl, 0.5 M tris pH 7.4. Each wash was carried out with gentle shaking. The DNA was then transferred from the gel to nitrocellulose membrane by blotting in 20x SSC (3 M NaCl, 0.3 M sodium citrate). The nitrocellulose membrane was first wetted in DEP treated double distilled water then soaked in 20x SSC for 30 min before assembly of the blotting apparatus. Blotting transfer was carried out overnight. The nitrocellulose membrane was then air dried and baked for 2 h at 80°C to immobilise the DNA.

v) Preparation of Plasmid DNA Dot Blots

Plasmid DNA was restriction digested with the endonuclease Eco RI, as described in Chapter II, 2, L. This linearised the supercoiled DNA. The DNA was then denatured by heating at 90°C for 10 min. 5 ul of this mixture was dotted in duplicate on to a nitrocellulose filter (Sartorius, 0.10 μm) which had been soaked in double distilled water then 20x SSC for 30 min. The filter was air dried prior to use. The prepared filters were then air dried and baked at 80°C for 2 h to immobilise the DNA.

N) Preparation of Hybridisation Probes

i) Nick Translation of cDNA

DNA labelling was carried out using the Amersham nick translation kit and following the protocol described in Amersham (1980) which is a modification of the procedure described by Rigby et al (1977). 1 μg of DNA was used in a 50 μl reaction mix which contained 50 μCi ^{32}PdCTP (410 Ci/mmole), 2.5 mM dATP, 2.5 mM dTTP, 2.5 mM dGTP, 100 mM tris-HCl pH 7.8, 10 mM $MgCl_2$, 1% (v/v) 2-mercaptoethanol, 100pg DNase I and 5 units DNA polymerase I. Incubation of the reaction was for 1.5 h at 14°C. The reaction was then terminated by addition of EDTA to 20 mM. Labelled cDNA was separated from unincorporated nucleotides by gel filtration through Biogel A-1.5M agarose (BioRAD) columns. Columns were prepared by plugging a siliconised pasteur pipette with a small plug of sterile glass wool. The column was then filled with A-1.5M Biogel (100-200 mesh) (BioRad) and allowed to settle. Presaturation of the column was carried out by

elution with 10 µl of solution containing 500 µg/ml sonicated salmon sperm DNA. This was eluted with 0.1x SSC solution before the nick translation mix was loaded on to the column. The eluted cDNA peak fraction was collected from the column and used as a hybridisation probe. Prior to use the cDNA was boiled for 10 min to denature any secondary structure, then cooled rapidly on ice.

ii) Reverse Transcription of Total Poly(A)$^+$ RNA

Total poly(A)$^+$RNA was isolated as described in Chapter II, 2, H (vi). Reverse transcription was carried out as described by Getz et al (1975). Using 25 units/µg RNA, Avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories, 5 units/µl), was added to a reaction mix containing 100 mM tris-HCL pH 8.2, 10 mM magnesium acetate, 5 mM dithiothreitol, 50 mM NaCl, 10-20 µg/ml poly(A)$^+$ RNA, oligo(dT)$_{17}$ (Boehringer Corporation Limited) to a final concentration 10 times that of the final RNA concentration, 100 µg/ml actinomycin D (Boehringer Corporation Limited), dATP, dGTP, dTTP (Boehringer Corporation Limited) at 2 mM, 50 µCi ^{32}PdCTP (410 Ci/mmole). The reaction mix was incubated for 90 min at 37°C then terminated by addition of EDTA to 20 mM. Labelled cDNA was isolated from unincorporated nucleotides by gel filtration as described in Chapter II, 2, N (i). Prior to use the cDNA was boiled for 10 min to denature any secondary structure, then cooled rapidly on ice.

iii) Preparation of cDNA Hybridisation Probes by Random Priming

DNA labelling was carried out using the Boehringer Mannheim random priming kit and following the protocol described by the supplier. This in turn is based on the random priming technique of Feinberg and Vogelstein (1984). 25 ng of denatured DNA was added to a reaction mix containing 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dGTP, 50 µCi ^{32}P dCTP (3000 Ci/mmole), and 2 ul of the kit reaction mix which contains 1 M tris pH 7.4, C.T. hexanucleotide (150 OD/ml) 2 M Hepes, 1 M $MgCl_2$, 0.004% (v/v) 2-mercaptoethanol. 1 unit of Klenow enzyme (1 unit/µl) (Boehringer Manneim) was added and the total volume of the reaction mix made up to 20 µl with double distilled water. The mixture was incubated for 30 min at 37°C, following this the reaction was stopped by addition of EDTA to 20 mM. The labelled cDNA was separated

from the unincorporated nucleotides as described previously (Chapter II, 2, N (i)). Prior to use the labelled cDNA was boiled for 10 min to denature any secondary structure, then rapidly cooled on ice.

O) Hybridisation Procedure

The method used to prehybridise and hybridise nitrocellulose membranes produced from the previously described procedures was taken from Jeffreys and Flavell (1977). Prehybridisation was carried out at 42°C in sealed polythene bags. Prehybridisation buffer contained 50% formamide (Fluka), 5x Denharts solution (1x Denharts solution is 0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin), 50 mM sodium phosphate pH 6.8, 0.1% SDS, 100 μg/ml sonicated salmon sperm DNA, 10 μg/ml poly(A) and 10 μg/ml poly(C). Approximately 1.5 ml of prehybridisation buffer was added per 5 cm^2 of filter. Incubation was carried out overnight in a shaking water bath. Following prehybridisation the buffer was removed from the bag and replaced with hybridisation buffer (approximately 1 ml per 5 cm^2 filter). The hybridisation buffer contained radioactively labelled probe at a concentration of 1-2 x 10^6 cpm/ml. The buffer contained 50% formamide (Fluka), 5x SSC, 1x Denharts solution, 20 mM sodium phosphate pH 6.8, 0.1% SDS, 10% dextran sulphate, 100 μg/ml sonicated salmon sperm DNA, 10 μg/ml poly(A) and 10 μg/ml poly(C). The polythene bags were then resealed, ensuring all air bubbles were expelled, and incubated overnight at 42°C in a shaking water bath. Washing of hybridised filters was carried out with buffers preheated to 65°C. Prior to washing the hybridisation solution was removed from the polythene bags, the filters were then put into approximately 250 ml of 3x SSC, 0.1% SDS and washed at room temperature. Five washes in this solution for 5 min each, ensured that all unbound radioactive probe was removed. Filters were then washed two times, for 30 min each wash, in 0.5x SSC, 0.1% SDS at 65°C in a shaking water bath. Finally filters were washed twice in a solution of 0.1x SSC, 0.1% SDS, 1 h for each wash, in a shaking water bath at 65°C. At the completion of washing filters were placed in polythene bags and autoradiographed with Kodak " XAR " or " XRP " X-ray film using Cronex " lightning plus " intensifying screens in Harmer X-ray cassettes. After exposure at

-70°C the films were developed in a Kodak M7A automatic X-ray processor.

P) Stripping of Hybridised Nitrocellulose Filters

Removal of hybridised radioactively labelled probe from nitrocellulose filters was accomplished by incubation of the filters at 55°C for 30 min in a solution containing 70% formamide (Fluka), 0.1 mM EDTA pH 8.0, 10 mM tris-HCl pH 7.4, 0.2% SDS. Approximately 50 ml of strip buffer was added per filter. Following incubation the strip buffer was then removed and replaced with a further 50 ml of fresh buffer and the filters incubated for a further 30 min at 55°C. The second wash ensured the efficient removal of all bound probe. Following incubation the filters were autoradiographed to check the completeness of probe removal. The filters could then be prehybridised and hybridised again, as described in Chapter II, 2, O.

Q) Screening of a cDNA Library by Colony Hybridisation

i) Preparation of Nylon Membranes

This method was adapted from that of Grunstein and Hogness (1975). Biodyne A nylon membranes (Pall) were sterilised by autoclaving. The filters were then placed on to agar plates (0.1% (w/v) bacto tryptone, 0.1% (w/v) NaCl, 0.05% (w/v) yeast extract, 1.5% (w/v) agar) containing ampicillin (5 μg/ml). Using a transfer plate (Dynatech) recombinants were transferred from a 96 well microtitre plate (Titertek) on to the nylon filter. The agar plates, with the filters, were then incubated overnight at 37 C. Following incubation the bacterial colonies should have grown to approximately 1 cm diameter on the filter. Trays were lined with Whatman filter paper soaked in lysis buffer (0.5 M NaOH, 0.2% (w/v) triton-X-100). The nylon membranes were removed from the agar plates and placed on the trays allowing the buffer to soak into them. Following 5 min of lysis the membranes were then transferred to another tray to denature the plasmid DNA. This tray was soaked in denaturation buffer (2.5 M NaCl,

0.5 M NaOH). Membranes were soaked in this buffer for 10 min then transferred into neutralisation buffer (3 M sodium acetate pH 5.5). Neutralisation was for 10 min. The membranes were air dried and then baked at 80°C to immobilise the DNA.

ii) Hybridisation of Nylon Membranes

Biodyne nylon membranes were prehybridised in buffer containing 5x Denharts buffer, 5x SSPE, 0.2% (w/v) SDS, 500 ug/ml denatured salmon sperm DNA (20x SSPE contains 3.6 M NaCl, 0.2 M sodium phosphate pH 7.5). Approximately 1.5 ml of prehybridisation buffer was used per 5 cm^2 of membrane. The filters were then incubated at 65°C overnight in a sealed plastic box in a shaking water bath. Prior to hybridisation the filters were removed from the prehybridisation buffer and placed into polythene bags. Each bag contained 1.5 ml hybridisation buffer per 5 cm^2 of membrane. Hybridisation buffer was made exactly as described for prehybridisation buffer but in addition contained 0.5 x 10^6 cpm ^{32}P dCTP (440 Ci/mmole) labelled, reverse transcribed cDNA. Hybridisation was carried out in a shaking water bath at 65°C overnight. After this time the membranes were washed briefly in wash buffer (5 mM sodium phosphate pH 7.0, 1 mM EDTA, 0.2% (w/v) SDS) to remove unbound cDNA probe. The filters were then washed twice at room temperature in wash buffer, 30 min for each wash, with vigorous shaking. Autoradiography was carried out in Harmer X-ray cassettes using Cornex "lightning plus" intensifying screens using Kodak "XAR" or "XRP" X-ray film. After exposure at -70°C the autoradiographs were developed in a Kodak M7A automatic X-ray processor.

iii) Removal of Hybridised Probe from Nylon Membranes

Membranes were placed in 250-300 ml of strip buffer (10 mM sodium phosphate pH 6.5, 50% formamide) and incubated for 1 h at 65°C with shaking. Following this, to ensure total removal of probe, the filters were washed in wash buffer (2x SSC, 0.1% SDS) for 15 min at room temperature, with vigorous shaking. The membranes were then ready for further prehybridisation and hybridisation.

R) In Situ Hybridisation

i) Fixation of Cells

Glass slides were washed in concentrated nitric acid for 2 h then well rinsed in double distilled water. They were then stored in 70% (v/v) ethanol until required. Prior to use the slides were air dried. Approximately 2 x 10^5 cells were spun onto the glass slides using a Shannon cytocentrifuge at 450 rev/min for 5 min. The slides were air dried and fixed immediately. Fixation entailed incubation of slides in 70% (v/v) methanol, at room temperature, for 10 min. The slides were then transferred through 3 changes of ice cold 10% (w/v) trichloroacetic acid before being dehydrated through 50%, 70%, 90% and 100% ethanol. They were then air dried and stored at -20°C with silica gel.

ii) Preparation of Radioactively Labelled cDNA

1 µg of plasmid DNA was nick translated, as described in Chapter II, 2, N (i). However the labelled nucleotide used was ^{35}S dCTP (600 Ci/mmole). The hybridisation probe was stored at -20°C for up to 2 weeks before use. The labelled cDNA was then separated from unincorporated nucleotides by passing through a Biogel A-1.5 (BioRad) column (Chapter II, 2, N (i)) .The radioactively labelled cDNA was eluted by 0.1x SSC. The DNA was dialysed against 0.1x TE buffer at 4°C then freeze dried. Radioactively labelled cDNA was resuspended in hybridisation buffer (Chapter II, 2, R (iii)) to give 2 x 10^5 cpm/µl.

iii) Procedure for Hybridisation

Before hybridisation the slides were rehydrated through 100%, 90%, 70% and 50% ethanol then rinsed in PBS. They were then treated with proteinase K (1 µg/ml in 2x SSC) for 10 min at 37°C then rinsed briefly in PBS containing 1% (w/v) glycine. Following a further wash in PBS the slides were prehybridised for 2 h at 42°C in coplin jars. The prehybridisation buffer contained 4x SSC, 10 mM tris pH 7.5, 1x Denharts buffer, 1 mM EDTA, 50% formamide (Fluka), 50 mM sodium phosphate pH 7.5, 0.05% (w/v) yeast tRNA and 100 ug/ml denatured salmon sperm DNA. Following prehybridisation the buffer was poured

from the slides and the excess buffer carefully wiped from the glass surface. 5 µl of hybridisation buffer (prehybridisation buffer plus 10% (w/v) dextran sulphate, 0.1 mg/ml poly(A), 0.1 mg/ml poly(C), 2 mM vanadyl ribonucleoside complexes) containing 1 x 10^6 cpm labelled cDNA was spotted over the cells. Coverslips were then placed over the buffer and sealed around the edge with cowgum. The slides were placed in a moist chamber and incubated at 80°C for 10 min and then transferred to a shaking water bath. Hybridisation was carried out at 42°C, overnight. Following hybridisation the cowgum was carefully peeled off and the coverslips removed in a beaker of 2x SSC. Slides were washed twice in 1x SSC for 30 min and rinsed in PBS. They were then dehydrated through 50%, 70%, 90%, and 100% ethanol and air dried .

iv) Autoradiography

Slides were coated in Ilford K2 Nuclear Research emulsion, in the dark. This comes in gel form. To prepare the emulsion an equal volume of gel was added to double distilled water containing 2% (v/v) glycerol. This mixture was heated for 3 min at 50°C until an emulsion was formed. The slides were dipped into the emulsion then left to dry at a 45° angle for 1 h at room temperature. This ensured that excess emulsion drained from the glass surface. The slides were then stored, with silica gel, in a light tight box at 4°C. Following exposure the slides were developed in D19 Ilford developer for 5 min then rinsed in water. They were then fixed in Hypam fixer for 3 min then washed for 1 h under running water and air dried. To visualise the cells the slides were stained using May-Grunwald and Giemsa stains as described in Chapter II, 2, D (i). The slides were mounted in DPX mountant before viewing by light microscopy.

S) Sequencing Procedure

i) Recloning of cDNA into M13

cDNAs were prepared by Eco RI + Bam HI digestion of pUC8 recombinants then isolated by gel fractionation of the restriction digested plasmid DNAs as described in Chapter II, 2, L . M13 bacteriophage DNA was similarly digested and purified. To reduce

self-ligation the vector DNA was then phosphatased by incubation of 2 μg M13 DNA in 2x CIP dilution buffer (10 mM tris-HCl pH 9.2, 1 mM magnesium acetate, 1 mM $ZnSO_4$, 100 μg/ml bovine serum albumin, 50% (v/v) glycerol) and 1 μl calf intestinal phosphatase (1 unit/μl) (Bethesda Research Laboratories) at 45°C for 30 min. A further 1 μl of calf intestinal phosphatase was then added and the reaction re-incubated for a further 30 min at 45°C to ensure the efficiency of the phosphatase reaction. The reaction was stopped by extracting the phosphatased vector DNA by addition of an equal volume of phenol. The mixture was then vortexed and centrifuged for 5 min at 10 000 rev/min in an Eppendorf microfuge. The aqueous phase was removed, containing the DNA. The DNA was then diluted to 1 ng/μl with TE buffer. To ligate the vector and cDNAs 100 ng of vector DNA and 20 ng of cDNA were incubated in 10 mM ATP, 50 mM dithiothreitol, 1x ligase buffer (10x ligase buffer is 0.66 M tris-HCl pH 7.5, 50 mM $MgCl_2$, 50 mM dithiothreitol) plus 1 unit T4 DNA ligase (1 unit/μl) (Bethesda Research Laboratories). Ligation reactions were carried out overnight at room temperature.

ii) Transformation of E.coli JM83 Cells with M13 Recombinants

1 ml of an overnight JM83 E.coli cell culture was inoculated on to 100 mls of 2x TY medium (0.16% (w/v) bacto-tryphone. 0.1% (w/v) yeast extract, 0.05% (w/v) NaCl) and the culture incubated at 37°C for 2 h or until the OD_{600nm} was 0.3. The cells were then pelleted by centrifugation in an MSE 4L centrifuge at 2000 rev/min for 5 min at 0°C. The pellet was resuspended in 10 ml of ice cold 10 mM magnesium sulphate and incubated on ice for 30 min. Following incubation the cells were repelleted by centrifugation in an MSE 4L centrifuge at 2000 rev/min as before. The supernatant was discarded and the cells resuspended in 5ml of ice cold 50 mM $CaCl_2$. These competent cells were then stored on ice for up to 2 h before use. To transform the JM83 cells with M13 phage DNA 0.3 ml of the competent cell mix was added to 0.5 volume of ligation mix (Chapter II, 2, S (i)). The cells were then heat shocked by incubation for 3 min at 42°C then returned to ice. Heat shock facilitates the uptake of exogenous DNA. To each tube of heat shocked cells isopropyl-B-D-thio-galactopyranoside (IPTG) (Sigma) was added to 7 mM, 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-Gal) (Sigma) in dimethyl formamide to 0.13% and 3 ml of molten H top agar (42°C)

(H top agar is 1% (w/v) bacto-tryptone, 0.8% (w/v) NaCl, 0.8% (w/v) agar) was added. This mixture was then poured out on to freshly prepared, prewarmed (37°C) H-plates (1% (w/v) bacto tryptone, 0.8% (w/v) NaCl, 1.2% (w/v) agar). The agar was allowed to set then the plates were inverted and incubated overnight at 37°C.

iii) Preparation of Single Stranded Template

This method was taken from Amersham (1984). 100 ml of 2x TY medium (0.016% (w/v) bacto tryptone, 0.01% (w/v) yeast extract, 0.005% (w/v) NaCl) was inoculated with 1 ml of an overnight E.coli (JM83) culture. 1.5 ml samples of this were aliquoted into sterile bijou tubes (Sterilin). From the H-plates set up previously (Chapter II, 2, S (ii)) single, colourless plaques were removed using sterile, wooden cocktail sticks and inoculated into the bijou tubes. The tubes were then incubated, with shaking, for 5 h at 37°C. At the completion of the incubation period the medium was transferred into Sarstedt microcentrifuge tubes and centrifuged for 5 min at 10 000 rev/min in an Eppendorf microfuge, to collect the cells. The supernatant, containing the viral particles, was transferred to fresh tubes and 0.12 volumes of PEG/NaCl (20% polyethelene glycol 6000, 2.5 M NaCl) added. The tubes were vortexed briefly then left to stand for 15 min at room temperature. To collect the viral DNA the tubes were centrifuged at 10 000 rev/min for 5 min in an Eppendorf microfuge and the supernatant discarded. To remove contaminating protein, the viral DNA pellet was resuspended in TE buffer and 0.5 volume of phenol saturated with TE buffer was added. The tubes were vortexed for 30 sec then centrifuged for 3 min. The aqueous phase, containing the DNA, was removed and transferred to fresh tubes. To precipitate the single stranded viral DNA, 3 M sodium acetate was added to 0.3 M and 2.5 volume of ethanol. The tubes were incubated overnight at -20°C. The viral DNA was collected by centrifugation in an Eppendorf microfuge and then washed once in 1 ml of cold (-20°C) ethanol. The pellet was drained and dried before resuspension in 30 µl of TE buffer.

iv) Sanger " Dideoxy Chain Termination " Sequencing Method

a) Sequencing Reaction

This method is exactly as described by Amersham (1984). The method was previously described by Sanger (1981) and more recently Biggins et al (1983). The Amersham M13 sequencing kit was used exactly as described by the manufacturers (Amersham International). Briefly, annealing of the M13 primer to the single stranded viral template was carried out in a reaction mix containing 0.5 volume of single stranded template, 0.1 volume M13 primer, 0.15 volume 1x Klenow reaction buffer (10x Klenow reaction buffer is 0.5M tris-HCl pH 7.2, 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 ug/ml bovine serum albumin). These components were well mixed then incubated for 2 h at 60°C. To the annealed template / primer hybrid 15 uCi of ^{35}S dATP (600 Ci/mmole) and 1 unit of Klenow fragment (1 unit/µl) were added. 2.5 µl of this mix were added to tubes marked A,C,G,T (4 tubes / clone). To each of the 4 tubes was added the appropriate dideoxy mix (the mixes contained 0.5 mM of each appropriate deoxynucleotide plus the appropriate molarity of dideoxynucleotide). The reaction was incubated for 15 min at room temperature following which 0.5 volume of chase mix was added. The chase mix is 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dTTP and 0.5 mM dGTP. The tubes were again incubated for a further 15 min at room temperature. At the completion of incubation an equal volume of formamide dye mix (20% formamide, 0.002% (w/v) xylene cyanol FF, 0.002% (w/v) bromophenol blue, 500 mM sodium EDTA) was added to each tube to stop the reaction. The tubes were then incubated for 3 min at 90°C, to denature the DNA, before loading on to a polyacrylamide gel.

b) Gel Electrophoresis

To prepare the gel 1.1 M urea, 15% (w/v) acrylamide, and 10x TBE buffer diluted to 0.5 was dissolved in water. 60 ml of this solution was required. To this was added 0.001% (w/v) TEMED, (NNN'N'-tetramethylethylenediamine) and freshly prepared 0.001% (w/v) ammonium persulphate solution. This solution was carefully poured between two glass plates (40 x 20 x 0.04 cm) which had previously been siliconised using 2% dimethyl-dichlorosilane in 1,1,1-trichloroethane (BDH-Replicote). The plates were taped together

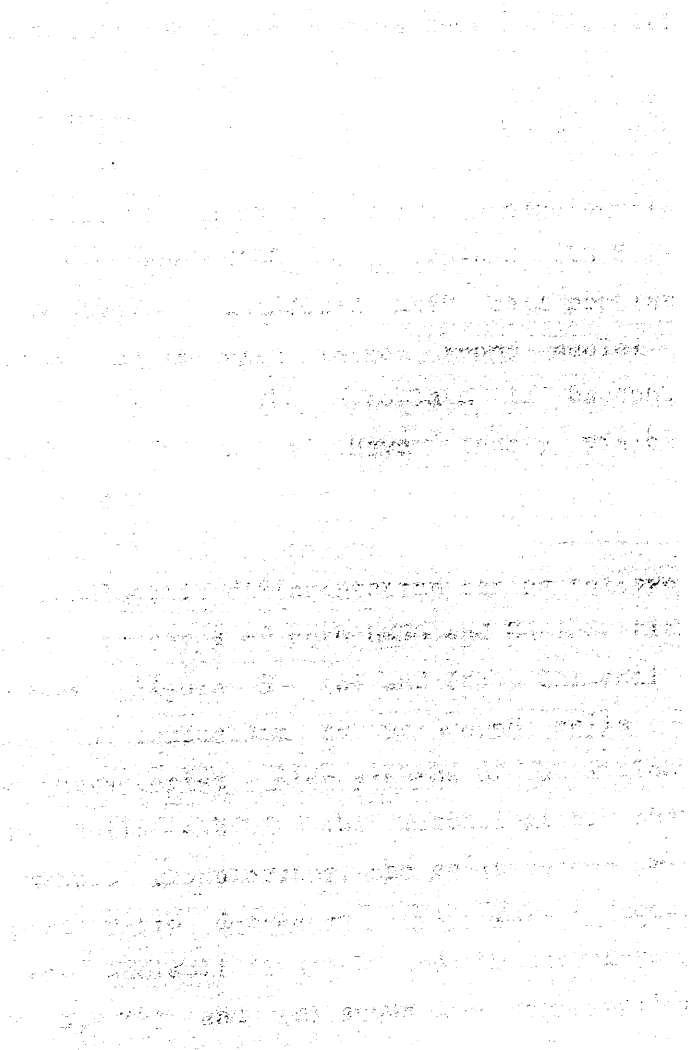
with spacers in place prior to pouring of the gel. The gel was poured by slowly letting the solution flow between the glass plates ensuring that no air bubbles were trapped. The gel was left to set for 1 h at room temperature. Samples were loaded on to the gel and then electrophoresis was carried out at 45 mA, 1.5 kV for 2 h. The gel was buffered in 1x TBE buffer. Following electrophoresis the gel apparatus was dismantled and the glass plates separated. The gel, still resting on one glass plate, was immersed in Fix buffer (10% (v/v) methanol, 10 % (v/v) glacial acetic acid) for 15 min then drained. It was then transferred on to Whatman filter paper, covered in saran wrap, and dried on a Shandon Gel drier for 1 h. The saran wrap was removed and the dried gel autoradiographed using Kodak "XAR" X-ray film in Harmer X-ray cassettes with Cronex "lightning plus" intensifying screens. After exposure the films were developed in a Kodak M7A automatic X-ray processor.

T) End Labelling of Restriction Digested Plasmid DNA

This method is exactly as described by Maniatis et al (1982). 10 µg of restriction digested, linearised plasmid DNA was added to 1x Klenow reaction buffer (10x buffer is 0.5 M tris-HCl pH 7.2, 0.1 M $MgSO_4$, 1 mM dithiothreitol, 500 µg/ml bovine serum albumin), 2 nM dATP, 2 nM dGTP, and 2 nM dTTP, 2 pM ^{32}P dCTP (440 Ci/mmole) and 1 unit of Klenow fragment (1 unit/µl) (Bethesda Research Laboratories). This mixture was incubated at room temperature for 30 min. The reaction was terminated by addition of EDTA to 20 mM. To remove unincorporated nucleotides the labelled DNA was precipitated by adding 3 M sodium acetate to 0.3 M and 2.5 volume of ethanol and incubation at -20°C for 20 min. Labelled plasmid DNA was collected by centrifugation in an Eppendorf microfuge for 10 min at 10 000 rev/min. To determine the size of the labelled fragments the DNA was electrophoresed on a 5% polyacrylamide gel buffered in 1x TBE buffer. The polyacrylamide gel was prepared exactly as described in Chapter II, 2, S (iv). markers digested with Eco RI and Hind III restriction enzymes were used as standards. Following electrophoresis the gel was removed and stained in ethidium bromide (0.5 µg/ml in water). The marker bands were visualised by UV fluorescence on a Chromato Vue transilluminator. Prior to autoradiography the gel was covered in saran wrap then exposed to "XRP" X-ray film at -70°C. X-ray films were developed in a Kodak M7A automatic X-ray processor.

CHAPTER III : RESULTS

RESULTS



1 INDUCED DIFFERENTIATION OF THE HL60 CELL LINE

A) The HL60 Cell Line

The HL60 cell line was initially isolated from the peripheral blood leukocytes of a patient suffering from acute promyelocytic leukaemia by Collins *et al* (1977). HL60 cells are grown in suspension culture and in exponential phase of growth have a doubling time of 24 h. These cells possess the potential to differentiate down the granulocytic differentiation pathway by treatment with DMSO or RA, but can also terminally differentiate to monocyte / macrophage cells by treatment with TPA (Collins *et al*, 1978; Rovera *et al*, 1979; Breitman *et al*, 1980).

B) Changes Observed During the Course of HL60 Differentiation

i) Morphological Changes

Uninduced HL60 cells have a characteristic promyelocytic morphology. Figure 4 shows uninduced HL60 cells stained with May-Grunwald and Giemsa stains. A typical uninduced HL60 cell population contains large, blast-like cells with large, round nuclei and a high nuclear-cytoplasmic ratio. The cytoplasm is basophilic with a granular appearance. The nuclei have clearly visible nucleoli, normally 2-4 per nucleus.

The course of granulocytic differentation can be followed by changes in HL60 morphology. At day 3 of both DMSO and RA induction HL60 cells resemble myelocytes (Figure 5, (a) and (c)). The cell cytoplasm is less basophilic, in comparison to uninduced cells, and the cell nucleus has the characteristic kidney-shape of the myelocyte. By days 4 and 5, the cells exhibit the morphological characteristics attributed to mature granulocytes; the cells become smaller and the nuclear-cytoplasmic ratio decreases, the nucleus acquires a lobed configuration, the nucleoli disappear and the cytoplasm becomes more diffuse. Figure 5, (b) and (d) shows HL60 cells following a 5 day DMSO and RA induction. These cells are classified morphologicaly as banded and segmented neutrophils.

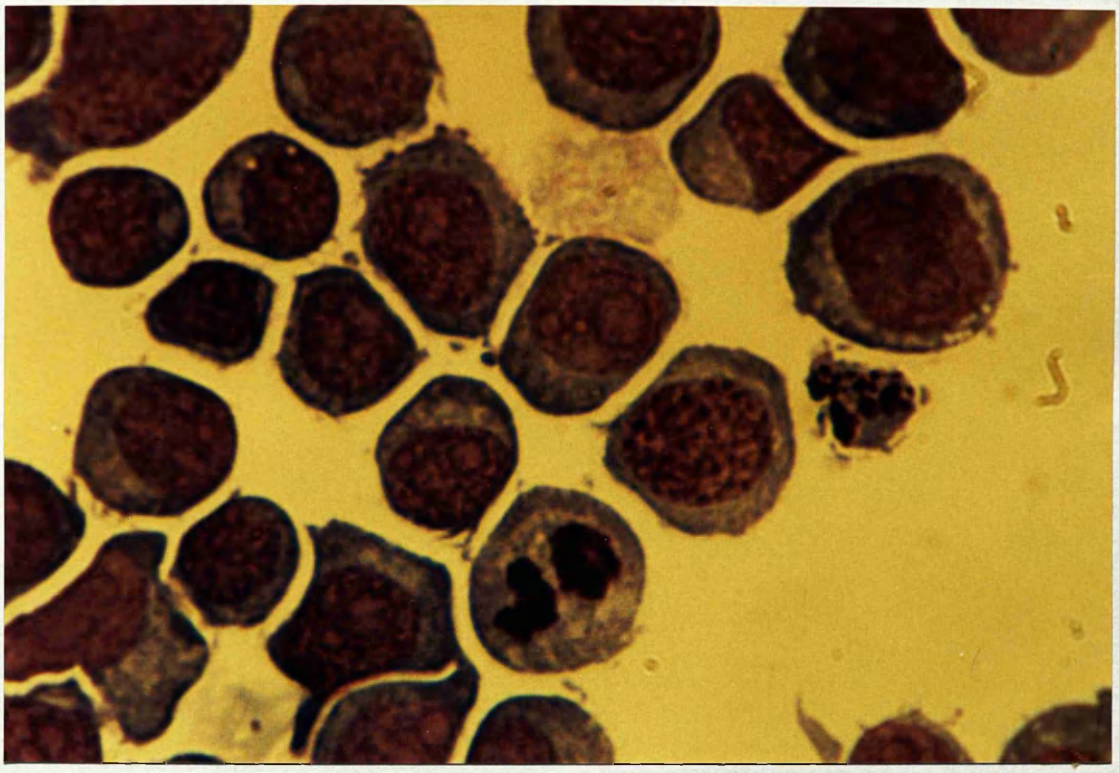


Figure 4 Uninduced HL60 cells.

A 250 µl sample of uninduced HL60 cells was cytocentrifuged on to a clean, glass slide. The cells were fixed in methanol and then stained with May-Grunwald and Giemsa stains.

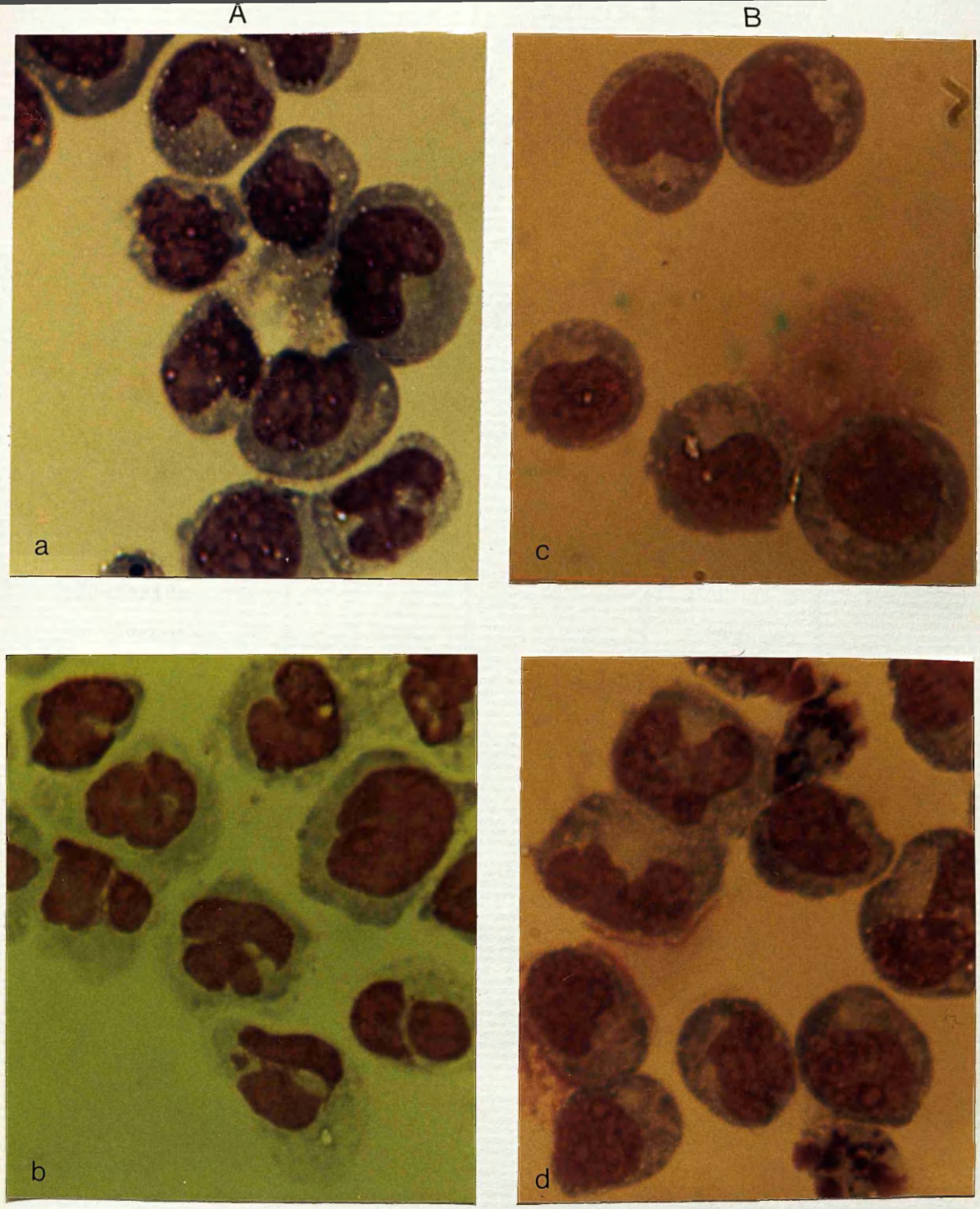


Figure 5 (A) 3 day and 5 day DMSO induced HL60 cells.

250 µl samples of HL60 cells (2 x 10^5 cells) induced for 3 days and 5 days with DMSO (1.5% (v/v)) were cytocentrifuged on to clean, glass slides. The cells were then fixed in methanol and stained with May-Grunwald and Giemsa stains. (a), HL60 cells induced for 3 days with DMSO; (b), HL60 cells induced for 5 days with DMSO.

(B) 3 day and 5 day RA induced HL60 cells.

250 µl samples of HL60 cells (2 x 10^5 cells) induced for 3 days and 5 days with RA (1 x 10^{-6} M) were cytocentrifuged on to clean, glass slides. The cells were then fixed in methanol and stained with May-Grunwald and Giemsa stains. (c), HL60 cells induced for 3 days with RA; (d), HL60 cells induced for 5 days with RA.

HL60 cells induced to differentiate to monocyte/macrophage cells by TPA become adherent after only 8-16 h of treatment. The cells clump together on the tissue culture flask surface, adhere, then spread out with a spindle-shaped morphology. TPA induced HL60 cells stained with May-Grunwald and Giemsa stains can be seen in Figure 6.

ii) Appearance of Histochemical Markers of Differentiation

a) Myeloid cell marker

During HL60 induction, the appearance of histochemical markers of differentation occur concomitant with changes in cell morphology. Mature cells from the myeloid lineage possess the ability to produce superoxide, hence are capable of reducing nitroblue tetrazolium (NBT) to formazan. When HL60 cells, induced by DMSO or RA, are incubated at 37°C for 25 min with 0.2% (w/v) NBT and 10 mM tetradecanoyl phorbol acetate, reduction of NBT can be assessed by the appearance of blue/black granular deposits in the cell cytoplasm. Figure 7 shows NBT positive RA induced cells and uninduced HL60 cells which did not stain and therefore do not produce superoxide. To determine when HL60 cells develop the ability to reduce NBT, and hence become terminally differentiated, cells were sampled at time points throughout the course of a DMSO and RA induction and subjected to the treatment described above. The appearance of NBT positively staining cells during DMSO induction was a late event occurring between 48-72 h of treatment and reaching a maximum at 96-120 h when almost 90% of the cells were capable of reducing NBT. However, only 54% of a RA induced HL60 cell culture were NBT positive at day 5 of treatment. These results are summarised in Figures 16 and 17.

b) Monocyte cell marker

Non-specific esterase (NSE) is a routine histochemical marker for monocytes. Following the incubation of cells with α-napthyl acetate, NSE positive cells can be detected by the presence of black/brown granular deposits throughout the cell cytoplasm. This is due to the hydrolysis of α-napthyl acetate. TPA induced HL60 cells were positive for NSE activity, which was detected as early as 8-16 h following TPA treatment. Figure 8 (a) shows 3 day TPA induced HL60 cells with brown cytoplasmic staining signifying the presence of NSE. The percentage

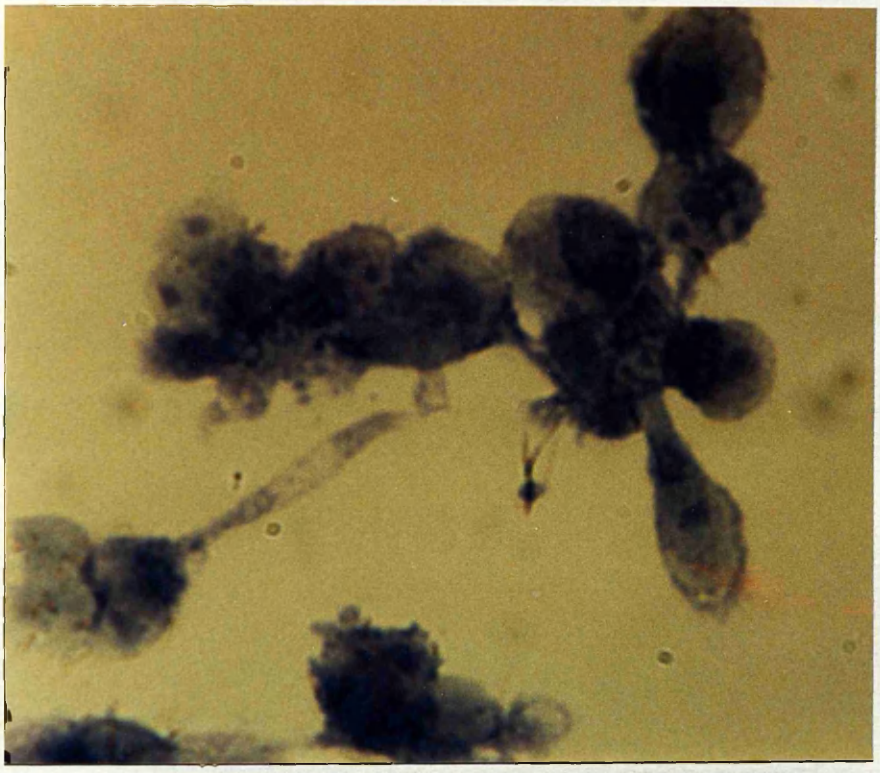
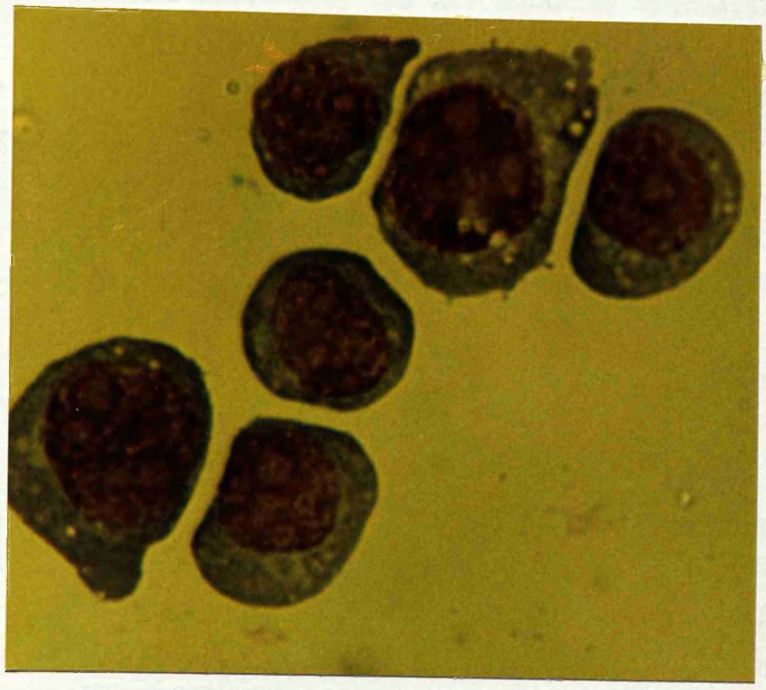


Figure 6 HL60 cells induced to differentiate for 3 days with TPA.

2×10^5 cells were cultured on clean, glass slides for 3 days in the presence of TPA (1.6×10^{-7} M). The medium was then removed, the cells fixed in methanol and then stained with May-Grunwald and Giemsa stains.

a



b

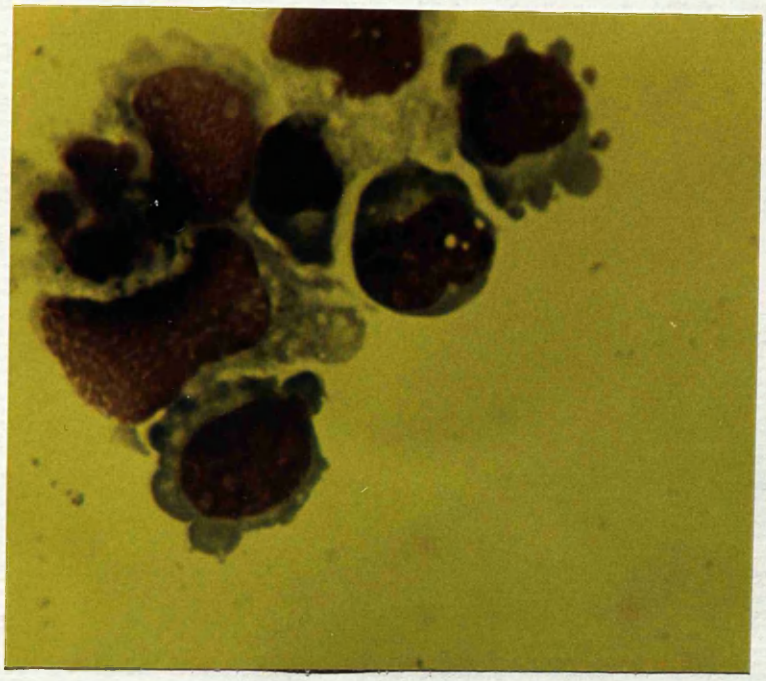


Figure 7 NBT positively stained 5 day RA induced HL60 cells and NBT negatively stained HL60 cells.

250 µl samples (2×10^5 cells) of uninduced HL60 cells and 5 day RA induced HL60 cells were removed from an assay to determine the cells ability to reduce NBT to formazan, a marker for mature myeloid cells. Reduction leads to the appearance of blue/black cytoplasmic granules. The cells were cytocentrifuged on to clean, glass slides, fixed in methanol and then stained with May-Grunwald and Giemsa stains. (a), uninduced HL60 cells; (b), 5 day RA induced HL60 cells.

of cells which stained positively for NSE activity increased rapidly during induction until almost 100% were capable of hydrolysing α-napthyl acetate by 24 h of TPA treatment. Uninduced HL60 cells, however, showed no black granulation and had to be counter-stained with methyl green to visualise the cells by light microscopy (Figure 8 (b)). These results are summarised in Figure 15. RA and DMSO induced HL60 cells also stained negatively when assayed for NSE.

iii) Examination of the Cell Cycling Potential of HL60 Cells During Induced Differentiation

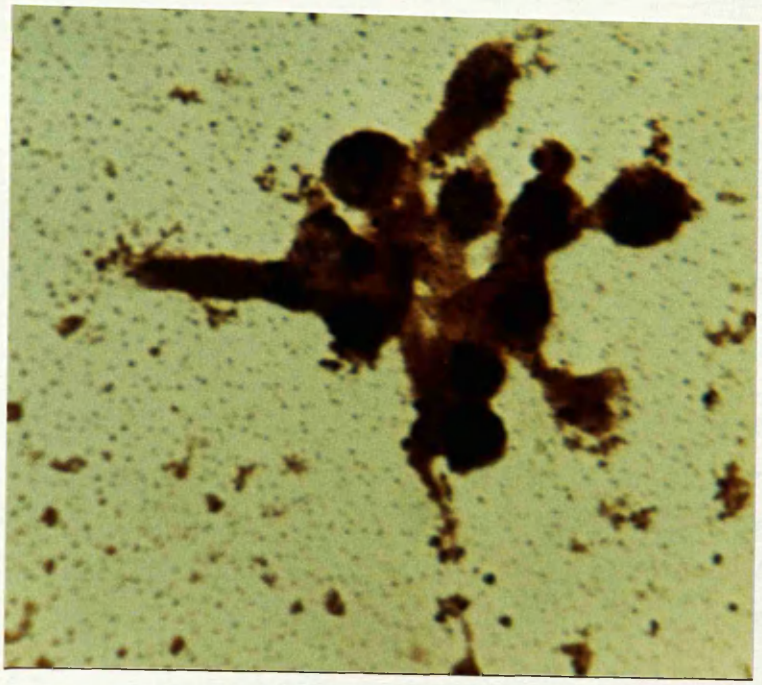
With the onset of commitment to terminal differentation, cells lose their potential to self-renew and cease to proliferate. To investigate the changes occurring in cell cycling potential of HL60 cells undergoing induction, and to determine if and when, during the induction time course, the cells ceased to proliferate, cells were sampled at time points throughout the course of DMSO, RA and TPA treatment, and analysed by Fluorescence Activated Cell Sorting (FACS). The cell profiles generated are shown in Figure 9.

To standardise the profiles, and to eliminate any discrepancies which could have arisen from drift of peaks during analysis on the FACS II, an aliquot of chick red blood cells was added to each sample prior to analysis. No variation occurs in the DNA content of these cells, which enables them to be used as a control.

Routinely, in samples taken from an exponentially growing uninduced HL60 cell culture, the FACS analysis profile showed cells scattered throughout the cell cycle, in G1, G2, M and S phases. However, TPA treatment rapidly reduced the ability of HL60 cells to divide; following only 24 h of induction, approximately 95% of the cells were observed to cease proliferation and arrest at G0/G1. If the doubling time of HL60 cells is 24 h this would only allow cells to traverse the cell cycle once. In the course of DMSO induction the cells divided 2-3 times during the treatment period. Only after 72 h exposure to the inducing agent were the cells also observed to arrest at G0/G1 (Figure 9 (a) and (b)).

RA induced HL60 cells were unusual in their cell cycle behaviour when compared to the induced cells described previously. At completion of

a



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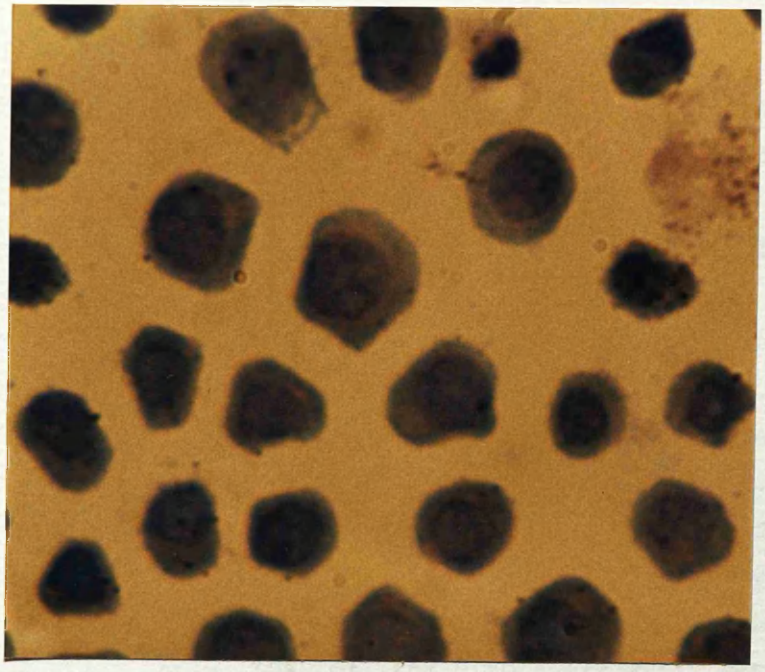


Figure 8 3 day TPA induced HL60 cells and uninduced HL60 cells stained to detect the presence of NSE.

250 µl samples (2 x 10^5 cells) of uninduced HL60 cells and 3 day TPA induced HL60 cells were cytocentrifuged on to clean, glass slides and assayed for the presence of NSE by determining the cells ability to hydrolyse α-napthyl acetate. (a), 3 day TPA induced HL60 cells which stained brown indicating the presence of NSE; (b), uninduced HL60 cells which did not contain NSE and therefore had to be counterstained with methyl green.

the 5 day RA induction period, a proportion of the cells were still present in G2, S and M phases of the cell cycle. The fact that these cells were still cycling indicated that not all the cells had undergone terminal differentiation (Figure 9 (c)).

iv) Changes in Potential to Self-Renew During HL60 Differentiation

FACS analyses give results generated from a population of cells. As was shown, prior to addition of inducing agent to an HL60 culture, the cells are not synchronous but are dispersed throughout the cell cycle. Hence, it is unlikely that these cells will respond to an induction stimulus simultaneously. Therefore, to examine individual cell response to treatment by inducing agents, cell clonogenicity was assayed.

To assess cell clonogenicity throughout the course of TPA, DMSO and RA treatment, cells were sampled at different time points throughout the course of induction, washed, and then plated out into microtitre dishes at 1-2 cells per well. These cells were cultured for up to 7 days in the absense of inducer; the cells were counted every 24 h.

It was found that approximately 15% of an uninduced cell population was incapable of self-renewal before induction. However, this was increased rapidly by pretreatment of HL60 cells with TPA for only 8-24 h when almost 85% of the cell culture had lost the potential to divide. Clonogenicity was also lost by cells treated with DMSO. This occurred gradually throughout the course of induction but with the majority of cells arresting between 24-96 h of DMSO treatment. By 96 h of induction only 10% of the HL60 cells still retained the potential to divide.

When similar analyses were carried out on RA induced HL60 cells, it was found that a proportion of the cells never lost the potential to self-renew, this percentage was as much as 40-50% of the RA treated cell culture, and is in agreement with the results obtained from FACS analysis and examination of histochemical markers of differentiation. The results from the clonogenicity studies are summarised in Figures 15, 16 and 17.

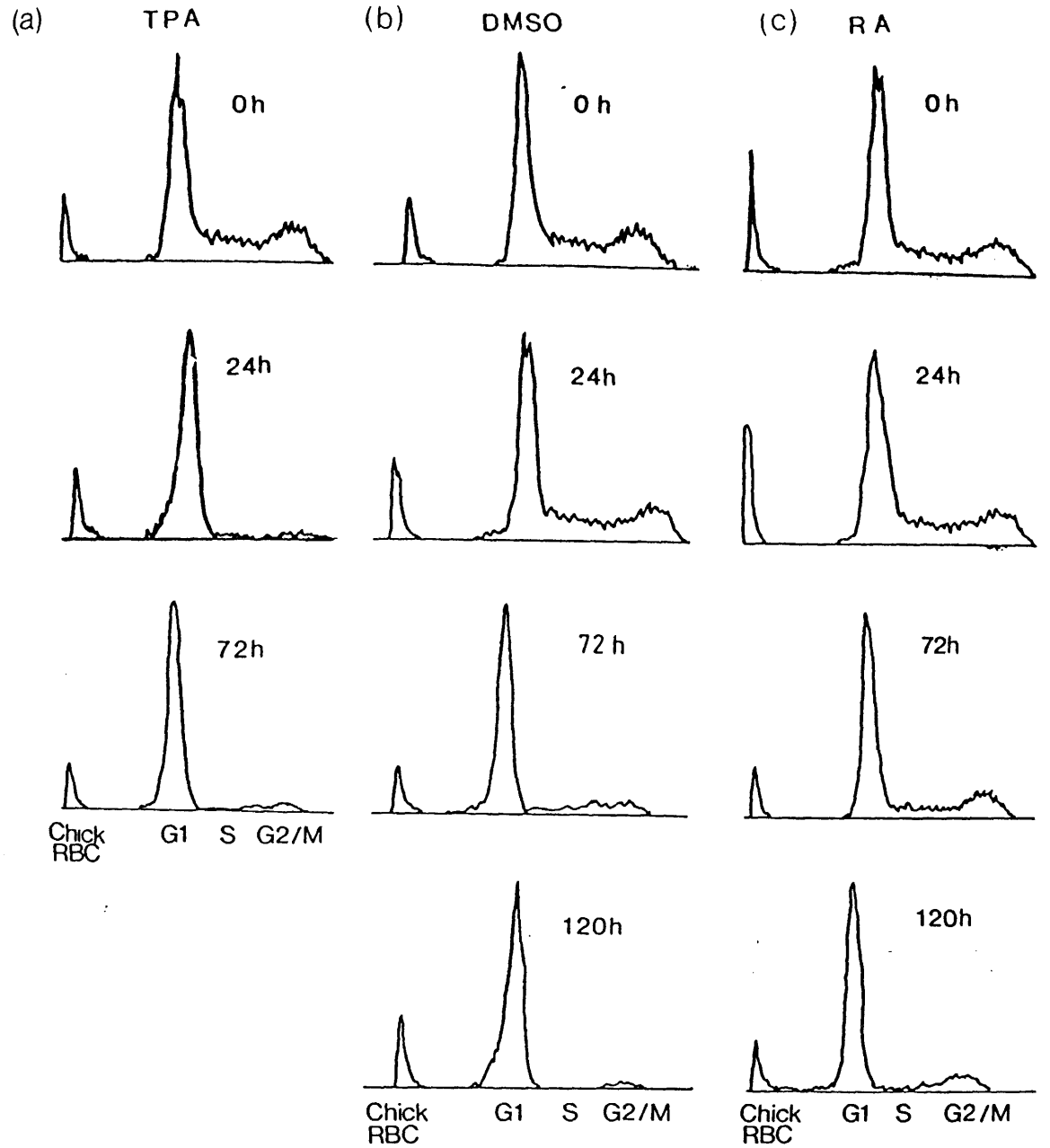


Figure 9 Examination of the cell cycle during HL60 differentiation.

1 x 10^6 cells were sampled at times throughout the course of TPA, DMSO and RA induction of HL60 cells. The cells were fixed in methanol and then stained with a fluorescent stain for DNA. These cells were sorted by fluorescence activated cell sorting according to cell DNA content which was directly proportional to the degree of fluorescent DNA stain bound to each cell. Chick red blood cells were used as standards as these cells have a constant amount of DNA. The profiles displayed are for (a), TPA induced HL60 cells; (b), DMSO induced HL60 cells; (c), RA induced HL60 cells.

From these analyses, the length of induction time required to allow maximum commitment to differentiation could be deduced. 8-24 h exposure to TPA or 48-96 h of DMSO treatment was required to produce almost 90% commitment in an induced HL60 cell culture. However, because RA does not induce a large proportion of HL60 cells to differentiate within the 5 day induction time course, it is unclear what length of exposure to the induction stimulus is required to produce the maximum number of committed cells.

v) Changes in Expression of Specific Genes as Markers of HL60 Differentiation

Changes in morphology, enzymic changes and cell cycle changes, which occur during the course of HL60 differentiation, must all have an initiating event, the induction stimulus. This, in turn, must eventually operate on the cell nucleus, resulting in changes in gene expression. Thus, the expression of two genes, c-myc and lysozyme, where chosen for study during induction of HL60 differentiation.

a) c-Myc

In total cellular RNA of uninduced HL60 cells, c-myc RNA is detectable, by Northern blotting analysis, as a doublet of 2.4 kb and 2.2 kb, when hybridised to ^{32}P-labelled pMC41-3RC cDNA, which contains the exon 3 sequence of the human c-myc gene. However, terminally differentiated HL60 cells, from both granulocytic and monocytic inductions, do not express c-myc RNA (Westin et al, 1982, Reitsma et al, 1983). Loss of detectable c-myc transcripts was used as a marker of terminal differentiation, however, the time at which c-myc expression ceased during HL60 induction was not known.

To investigate the relative abundance of c-myc RNA in HL60 cells during differentiation, total RNA was isolated at time points throughout the course of TPA, RA and DMSO inductions. These RNAs were used to produce Northern blots, which were then hybridised with ^{32}P-labelled pMC41-3RC cDNA. During the course of monocytic differentiation, c-myc RNA could not be detected following 2-4 h of TPA treatment (Figure 10). The transcripts remained undetectable throughout the rest of the induction period. DMSO induced HL60 cells also lost c-myc RNA very rapidly, within 1 h of treatment (Figure 11)

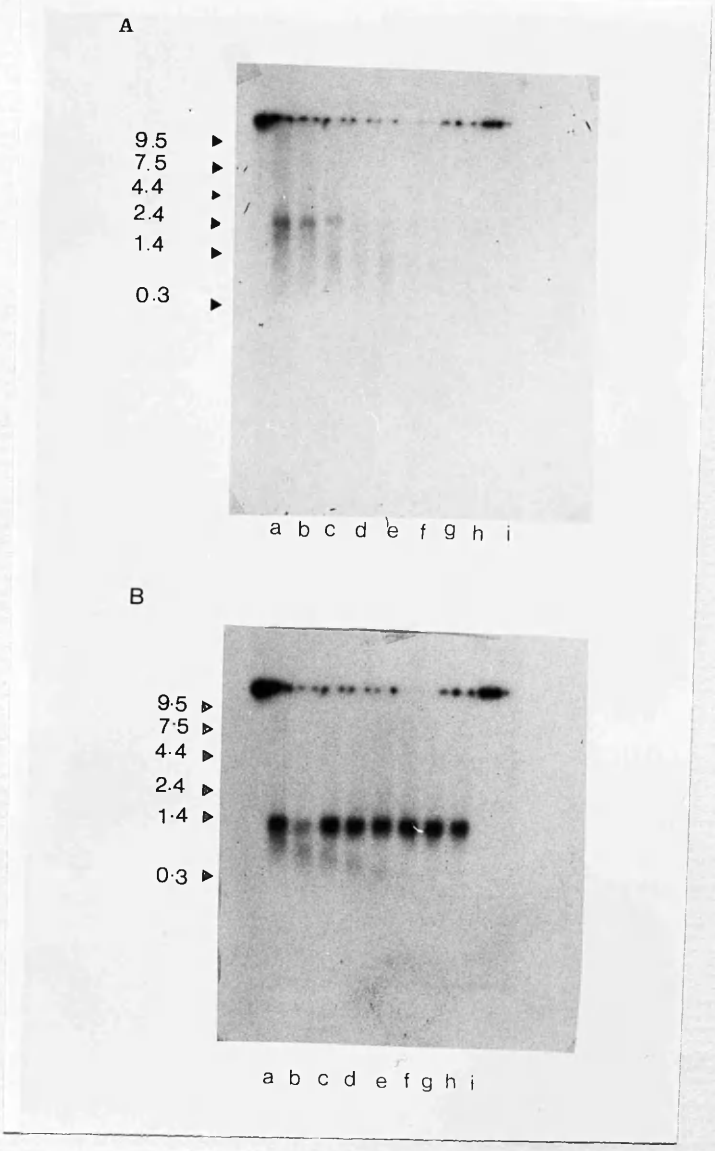


Figure 10 Northern blot analysis of c-myc transcripts during TPA induced differentiation of HL60 cells.

10 µg samples of total cellular RNA from time points throughout the 3 day course of TPA induction of HL60 cells were fractionated by electrophoresis on a denaturing, agarose gel. The RNA was then blotted from the gel on to nitrocellulose membrane. The nitrocellulose membrane was hybridised with ^{32}P-labelled probes for (A), human c-myc (pMC41-3RC); (B), B_2-microglobulin, each at a concentration of 2 x 10^6 cpm/ml. a, 0 h; b, 1 h; c, 2 h; d, 4 h; e, 8 h; f, 16 h; g, 24 h; h, 48 h; i, 72 h of TPA treatment.

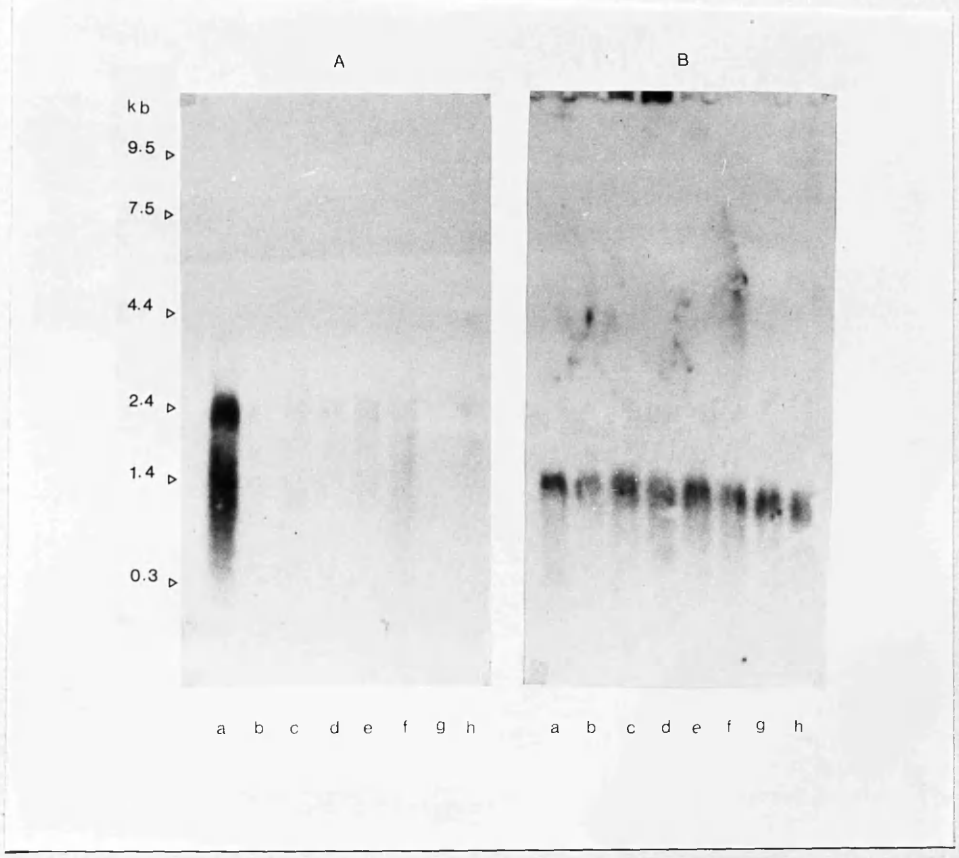


Figure 11 Northern blot analysis of c-myc transcripts during DMSO induced differentiation of HL60 cells.

10 μg samples of total cellular RNA from time points throughout the 5 day course of DMSO induction of HL60 cells were fractionated by electrophoresis on a denaturing agarose gel. The RNA was then blotted from the gel on to nitrocellulose membrane. The nitrocellulose membrane was hybridised with ^{32}P-labelled probes for (A), human c-myc (pMC41-3RC); (B), B_2-microglobulin, each at a concentration of 2 x 10^6 cpm/ml. a, 0 h; b, 1 h; c, 2 h; d, 4 h; e, 8 h; f, 16 h; g, 24 h; h, 72 h; i, 120 h of DMSO treatment.

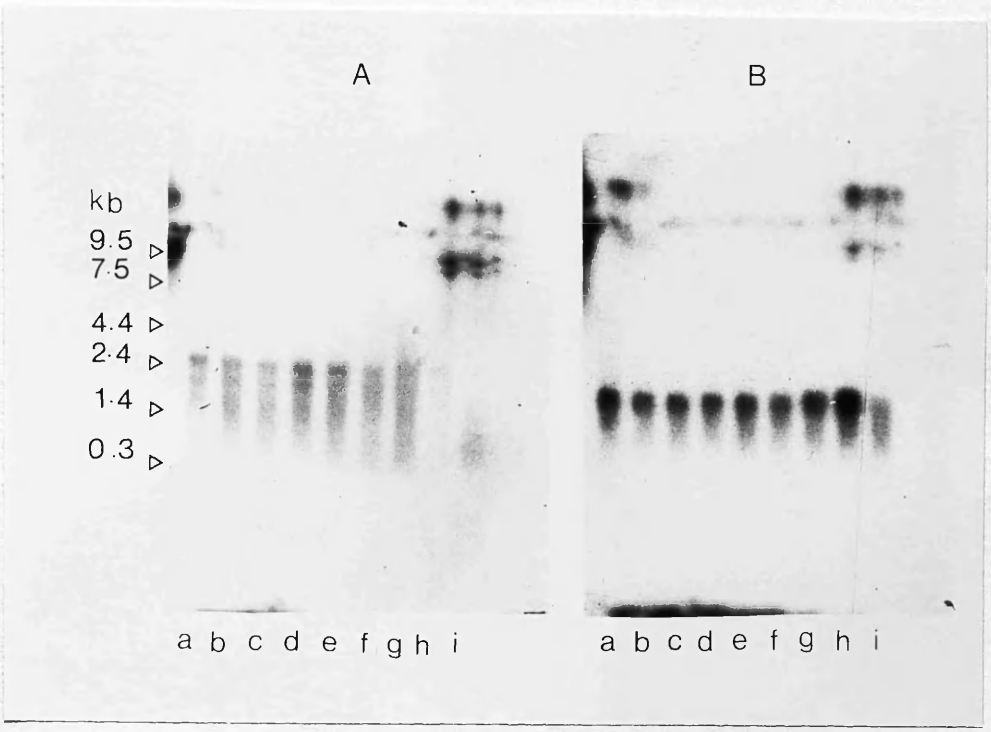


Figure 12 Northern blot analysis of c-myc transcripts during RA induced differentiation of HL60 cells.

10 μg samples of total cellular RNA from time points throughout the 5 day course of RA inducion of HL60 cells were fractionated by electrophoresis on a denaturing agarose gel. RNA was then blotted from the gel on to nitrocellulose membrane. The nitrocellulose membrane was hybridised with ^{32}P-labelled probes for (A), human c-myc (pMC41-3RC); (B), B_2-microglobulin at a concentration of 2 x 10^6 cpm/ml. a, 0 h; b, 1 h; c, 2 h; d, 4 h; e, 8 h; f, 16 h; g, 24 h; h, 72 h; i, 120 h of RA treatment.

However, HL60 cells, induced by RA, did not lose c-myc RNA at early times during induction. It was only after 3 days exposure to RA that these cells showed a drop in the level of c-myc RNA. c-Myc transcripts eventually disappeared at the completion of the 5 day induction period (Figure 12). It is an interesting observation that two agents (DMSO and RA) which both induce granulocytic differentiation in HL60 cells, exhibit such different patterns of c-myc expression. Similar analyses were repeated using total RNA isolated from HL60 cells from a number of different RA, TPA and DMSO inductions, and from cells from different HL60 clones. In each instance it was found that the results were similar to those described above.

The Northern blots were stripped and rehybridised to radioactively labelled B_2-microglobulin cDNA. This hybridises to a 1.1 kb transcript which is present at a constant level during the course of HL60 differentiation (Figures 10, 11, 12). From these results it is apparent that the concentrations of RNA used throughout these analyses were comparable.

b) Lysozyme

b1) Lysozyme protein secretion

The production of lysozyme protein is associated with terminally differentiated macrophages, requiring this protein to accomplish their defensive and scavenging roles in vivo. Uninduced HL60 cells, when assayed for the secretion of lysozyme by the method described in Chapter II, 2, E, (i), do not produce any of this protein. However, during the course of TPA induction, lysozyme protein secretion could be detected as early as 2 h following the addition of inducer to the culture medium. The level of secreted lysozyme protein increased rapidly throughout the first 24 h of induction, reaching a peak between 24-48 h (Figure 13). Interestingly, DMSO and RA induced HL60 cells were found to secrete some lysozyme protein but at a concentration of approximately 5-10 fold less than that of TPA induced cells.

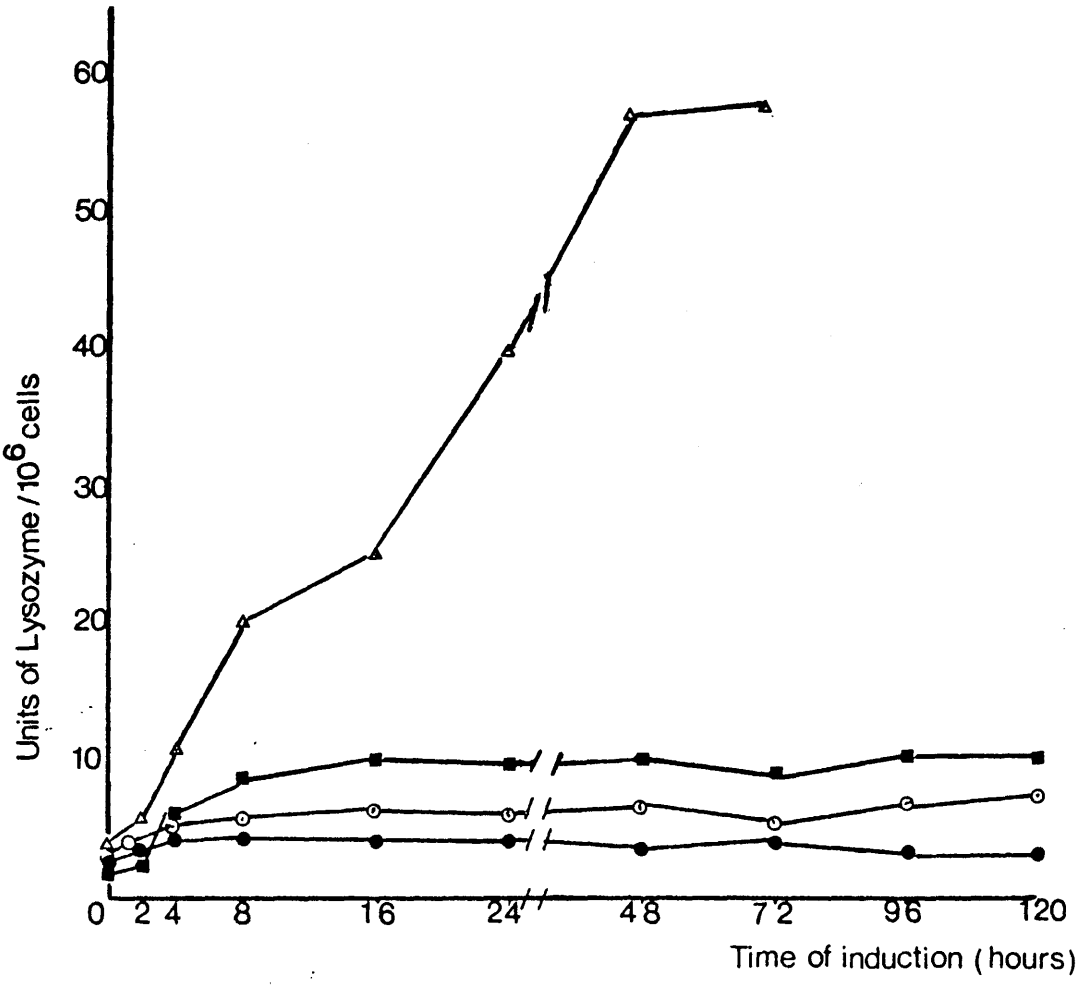


Figure 13 Change in the level of lysozyme protein secretion during induced differentiation of HL60 cells.

Samples of medium were removed from uninduced HL60 cell cultures and from HL60 cell cultures treated with TPA, RA and DMSO at time points throughout the period of induction. The amount of lysozyme protein secreted in to the growth medium was measured and expressed as units/10^6 cells. (△), TPA induced HL60 cells; (○), RA induced HL60 cells; (■), DMSO induced HL60 cells; (●), uninduced HL60 cells.

b2) Intracellular lysozyme protein

To determine if lack of secretion of lysozyme from uninduced and RA or DMSO induced HL60 cells was due to a block in the secretion process, the level of intracellular lysozyme protein present in these cells was examined. HL60 cells were harvested and washed thoroughly to remove all traces of medium. The cells were then lysed in a small volume of 0.2% (v/v) triton X-100 and the lysozyme protein content measured as described previously. The results from this analysis are shown in Table 2. No lysozyme protein was found internally in RA induced, DMSO induced or uninduced HL60 cells; however a small amount was present in TPA induced cells. Therefore, it appeared that the stimulation of lysozyme protein during TPA treatment of HL60 cells, was the result of a change in gene expression initiated by the induction stimulus and not the release of protein already present in the uninduced cell prior to treatment.

b3) Lysozyme mRNA

To examine lysozyme gene expression, total RNA from uninduced and induced HL60 cells were fractionated by electrophoresis on a denaturing agarose gel and then used to prepare a Northern blot. This was hybridised to ^{32}P-labelled lysozyme cDNA. The lysozyme probe, available in our laboratory, is a full-length cDNA of chick lysozyme and therefore only bears approximately 40% homology to the human lysozyme sequence (Land *et al*, 1981). This low homology determined that care had to be taken when washing the blot; it was washed at 42°C and in low stringency wash buffers. The results of this analysis are given in Figure 14. This probe should detect a 0.8 kb RNA in the human system.

At the region around 0.8 kb, the size of the lysozyme mRNA, hybridisation was detected in total RNA from 24 h and 3 day TPA treated HL60 cells and in RNA isolated from normal human monocytes. After 3 days of TPA treatment a larger transcript of 3.8 kb was also apparent. It is interesting to note that this transcript was also present in normal monocyte RNA. A very strong hybridisation signal was detected in uninduced HL60 cells but these transcripts were longer than 0.8 kb. Interestingly, RA induced HL60 cells also appeared to contain high levels of homologous RNA to lysozyme,

HL60 Induction	Units of Lysozyme/10^6 cells	
	Intracellular Protein	Secreted Protein
Uninduced	0	2
3 day DMSO	0	5
5 day DMSO	0	8
3 day RA	0	8
5 day RA	0	9
3 day TPA	6	57

Table 2 Intracellular lysozyme protein compared to secreted lysozyme protein in uninduced and induced HL60 cells.

1 x 10^6 cell samples of uninduced and induced HL60 cells were lysed and the amount of intracellular lysozyme protein determined. Secreted lysozyme protein was determined by analysis of the amount of lysozyme protein present in the growth medium (see Figure 13).

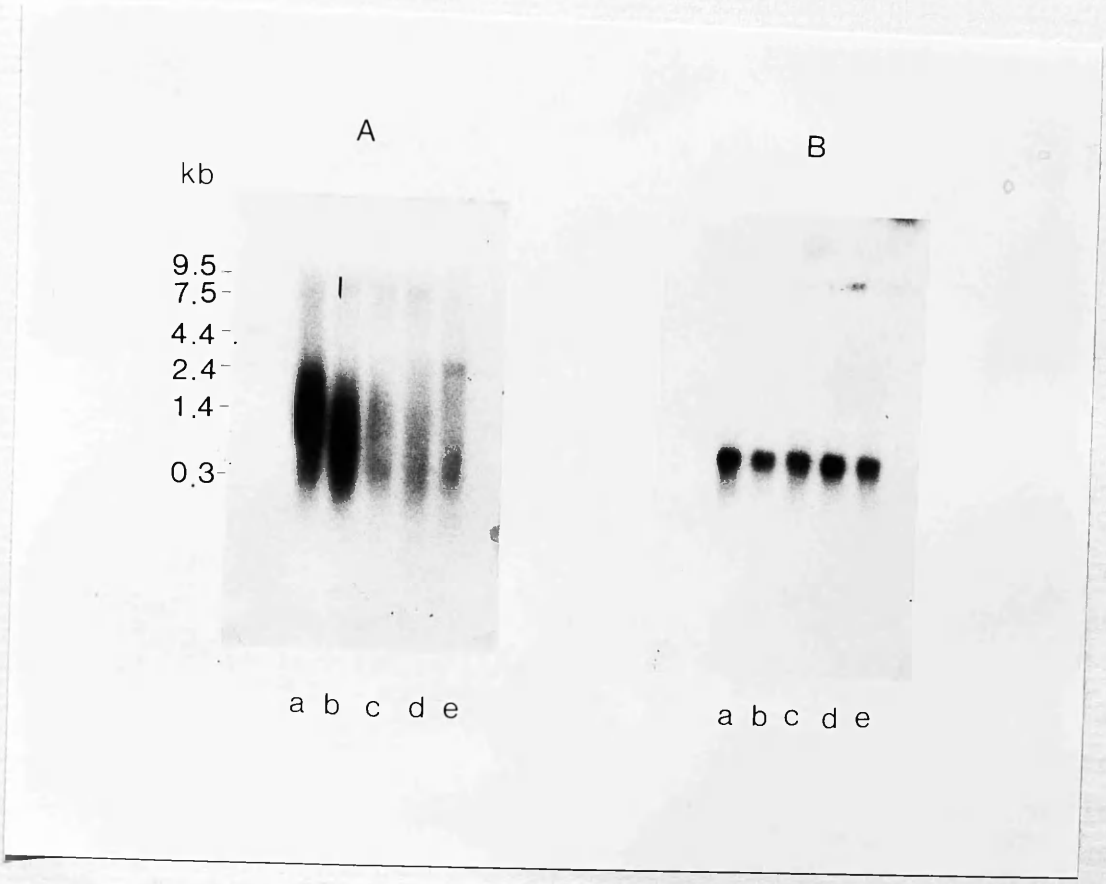


Figure 14 Northern blot analysis of lysozyme transcripts in uninduced and in induced HL60 cells.

30 µg of total cellular RNA from uninduced and induced HL60 cells were subjected to electrophoresis on a denaturing agarose gel. The RNA was then blotted on to nitrocellulose membrane. The nitrocellulose membrane was hybridised with ^{32}P-labelled probes for (A), lysozyme; (B), B_2-microglobulin. a, uninduced HL60; b, 5 day RA induced HL60; c, 16 h TPA induced HL60, d, 24 h TPA induced HL60; e, normal macrophage.

however, the hybridisation signal covered an area relating to 1.0-2.0 kb therefore no exact size could be calculated for these transcripts. From these results it could be concluded that uninduced and RA induced HL60 cells, although not possessing lysozyme protein, did appear to contain RNA species homologous to the lysozyme cDNA sequence; this is discussed in Chapter IV, 3, B.

To determine that a uniform RNA concentration was used in each sample the blot was stripped and rehybridised to radioactively labelled B_2-microglobulin cDNA (Figure 14).

C) Comparison of the Timing of Events Leading to Terminal Differentiation of HL60 Cells

i) TPA induction

Comparisons, made between self-renewal parameters and commitment or differentiation characteristics, suggest that HL60 cells cease to divide at approximately the same time as the appearance of overt differentiation features. Almost 90% of TPA induced cells ceased to proliferate after 16-24 h of treatment. This is concomitant with an increase in the percentage of cells which exhibited differentiated characteristics, such as the appearance of NSE positive cells, maximum production of lysozyme protein and the occurence of almost 100% adherent cells. The timing of the decline in c-myc RNA preceded these events by a number of hours. However, there does appear to be a direct relationship between cell proliferation and loss of detectable c-myc RNA. These results are summarised in Figure 15.

ii) DMSO induction

Decline of self-renewal in HL60 cells, treated with DMSO, also occurred concomitant to the appearance of committed, terminally differentiated cells. In a similar fashion to TPA induction, the loss of c-myc RNA occurred very rapidly after addition of DMSO to the cell culture medium, within 1 h of treatment. This event preceded any other differentiation related response, in DMSO induced cells, by 16-24 h. It is interesting to note that although c-myc transcripts were not detectable after 1 h of induction, DMSO induced cells still

proliferated, infact these cells cycled 2-3 times before arresting in G0/G1 at approximately 72-96 h of treatment. These results are summarised in Figure 16.

iii) RA induction

When used to treat HL60 cells, RA did not result in an efficient differentiation response. c-Myc RNA was detected up until 72 h of induction, FACS analyses (Figure 9) and cloning potential assays showed that 40-50% of HL60 cells were still capable of proliferation at this time. In addition, histochemical analysis revealed that a similar percentage of cells did not stain positively in an NBT test, a positive result revealing the cells ability to produce superoxide which is a characteristic of mature cells of the myeloid lineage. This is summarised in Figure 17. Although c-myc transcripts were not detected following 5 days of RA treatment, this might be due to the accumulation of quiescent cells, as the culture medium was not replenished throughout the induction period.

Chapters 2 and 3 give results obtained from the screening of cDNA libraries, constructed from total poly(A)$^+$ RNA isolated from RA induced HL60 cells. The results described above have considerable bearing on the origin of the cDNAs used to construct this library as it appears that the poly(A)$^+$ RNA used as a template for cDNA synthesis was isolated from a mixed population of cells, containing both RA induced and uninduced HL60 cells.

D) c-Myc Expression and HL60 Differentiation

A general trend throughout induced differentiation of HL60 cells is the loss of self-renewal capacity followed by the appearance of overt differentiation characteristics. c-myc RNA levels decrease prior to this, suggesting that the loss of this message may determine when HL60 cells become committed to a specific differentiation pathway, the loss of c-myc transcripts being a prerequisite in preparing HL60 for terminal differentiation. Evidence is now gathering to support this and is discussed in Chapter IV, 2, B.

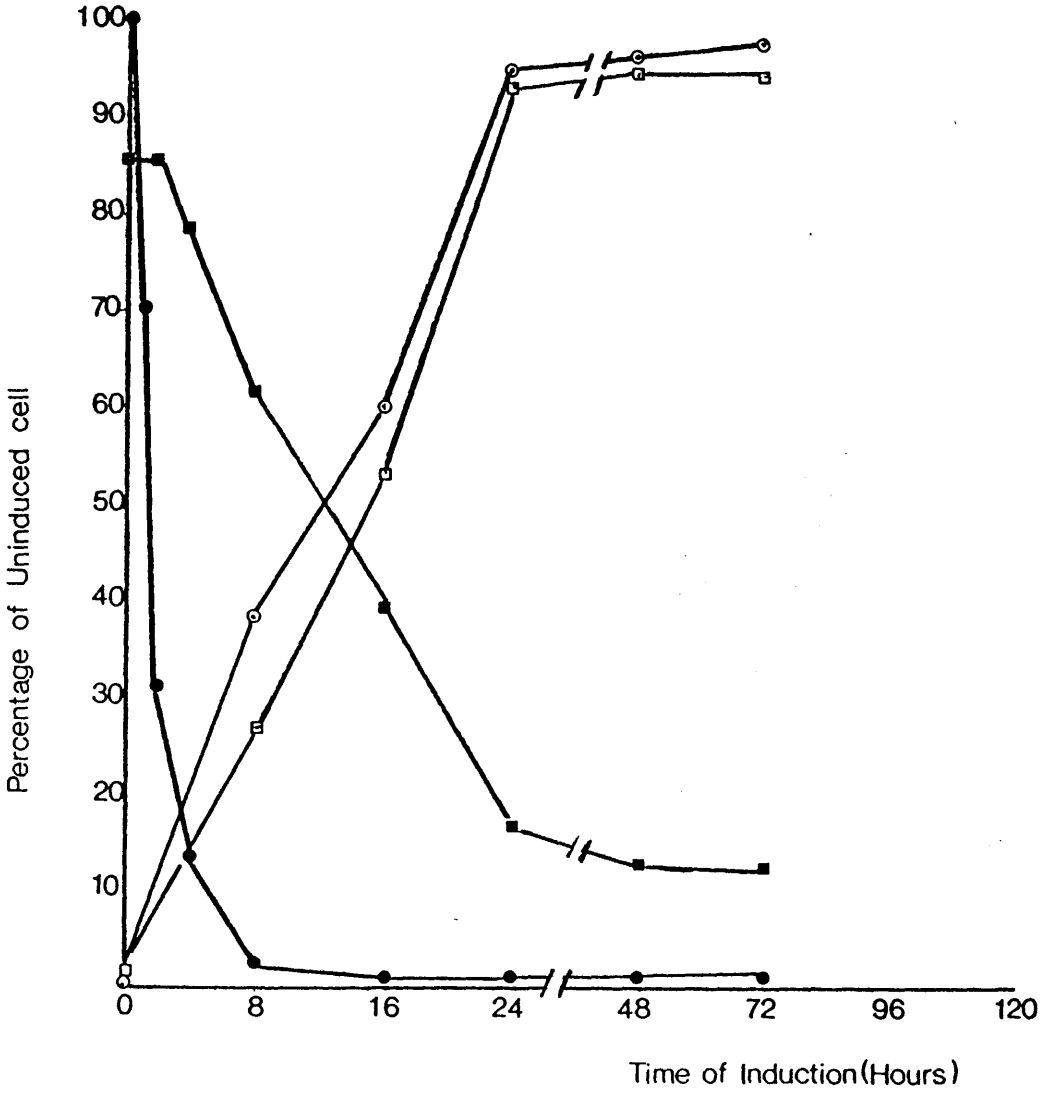


Figure 15 A summary of the results obtained from assays to assess the differentiation status of HL60 cells during a 3 day period of TPA treatment.

The results displayed measure (■), cloning potential of TPA induced HL60 cells; (□), NSE activity in TPA induced HL60 cells; (○), cell adherence; (●), c-myc expression. All percentages were calculated by taking the value obtained for the uninduced cell (cloning potential and c-myc) or the fully induced cell (NSE and adherence) as 100%. The results for c-myc expression were obtained from densitometric scanning of the Northern blot in Figure 10.

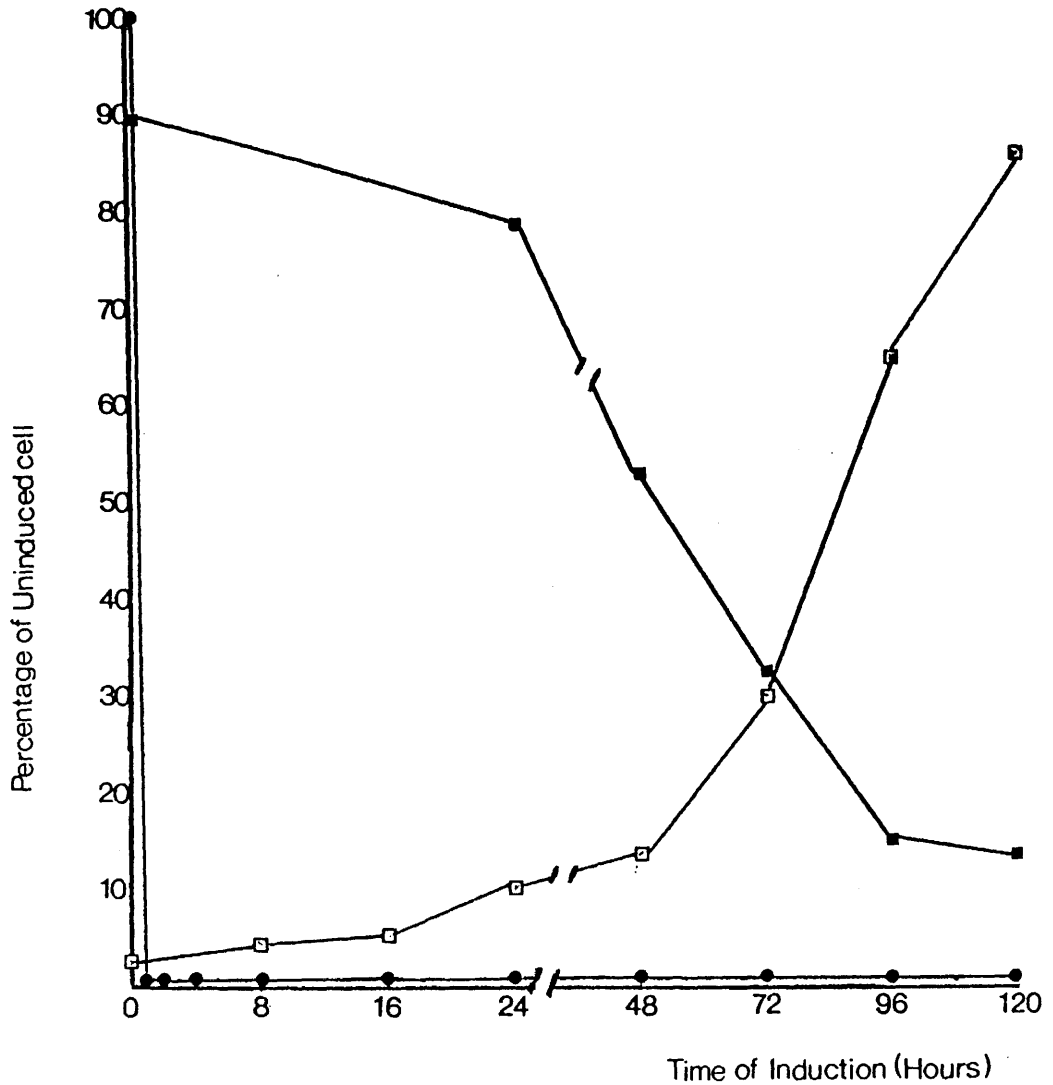


Figure 16 A summary of the results obtained from assays to assess the differentiation status of HL60 cells during a 5 day period of DMSO treatment.

The results displayed measure (■), cloning potential of DMSO induced HL60 cells; (□), the occurence of NBT positive cells during DMSO induction and (●), c-myc expression. All percentages were calculated by taking the value obtained for the uninduced cell (cloning potential, c-myc) or the fully induced cell (NBT) as 100%. The results for c-myc expression were obtained from densitometric scanning of the Northern blot in Figure 11.

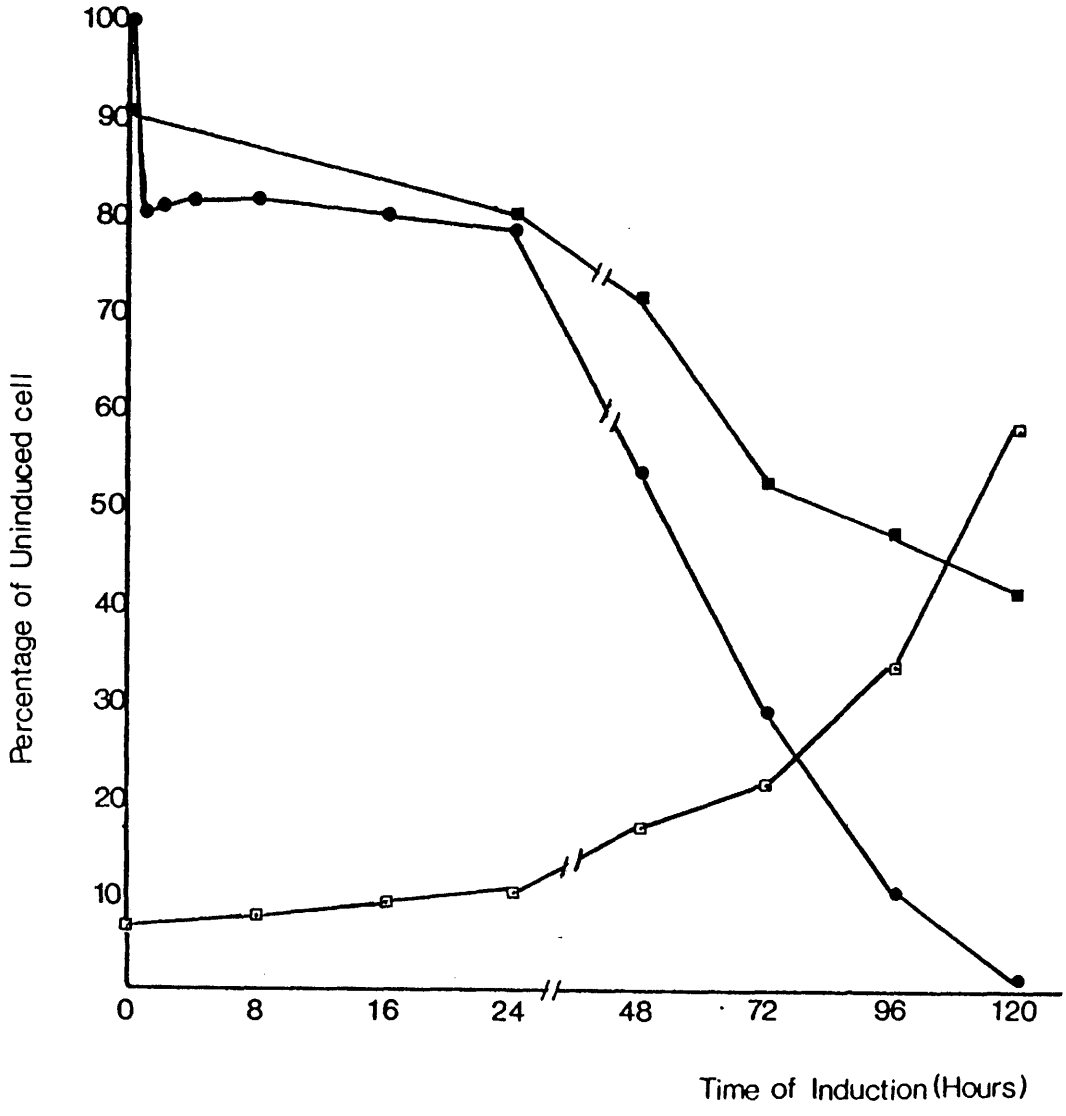


Figure 17 A summary of the results obtained from assays to assess the differentiation status of HL60 cells during a 5 day period of RA induction.

The results displayed measure (■), cloning potential of RA induced HL60 cells; (□), the occurence of NBT positive cells during RA induction and (●), c-myc expression. All percentages were calculated by taking the value obtained from the uninduced cell (cloning potential, c-myc) or the fully induced cell (NBT) to be 100%. The results for c-myc expression were obtained from densitometric scanning of the Northern blot in Figure 12.

2 IDENTIFICATION OF SEQUENCES WHICH CHANGE IN ABUNDANCE FOLLOWING THE INDUCED DIFFERENTIATION OF HL60 CELLS

A) Library Construction

Prior to my arrival at this laboratory, HL60 cDNA libraries had been constructed from uninduced and induced HL60 cellular RNA, by the method of Wickens et al (1978). Briefly, total RNA was isolated from HL60 cells (in the case of the library I screened, the RNA was prepared from HL60 cells following 5 days exposure to RA and was therefore called the RA5 library). Poly(A)$^+$ RNA was isolated from the total RNA by passing through an oligo d(T) cellulose column (Aviv and Leder (1972)). The poly(A)$^+$ RNA was used as a template for the oligo d(T) primed synthesis of a single stranded cDNA in the 5'-3' direction, catalysed by reverse transcriptase. Following this, the mRNA-cDNA first strand hybrids were denatured by boiling. The second cDNA strand was then synthesised in a 5'-3' direction by DNA polymerase I, utilising the hair-pin loop, formed at the 3' end of the first strand cDNA, as a primer for this reaction. This resulted in the formation of a double-stranded cDNA, joined by a single-stranded hair-pin loop, which is variable in length and location. To remove this the cDNA was digested by S1 nuclease and the ends then repaired by DNA polymerase I. The cDNAs were blunt-end ligated into the Sma I site of pUC8. No size selection of cDNAs was carried out before ligation. The resulting recombinant plasmids were used to transform E.coli JM83 cell. Figure 18 depicts the library construction procedure in a schematic form.

B) Library Screening

The RA5 library had initially been screened to isolate those clones which contained sequences present in high abundance in RA induced HL60 cells. Grunstein-Hogness Colony Hybridisation (Grunstein and Hogness (1975)) and DNA dot blot screening had been employed. This had reduced the number of recombinants to be analysed from 10 000 to 190. These 190 positively scoring clones were inoculated into 2 "composite" 96 well culture dishes.

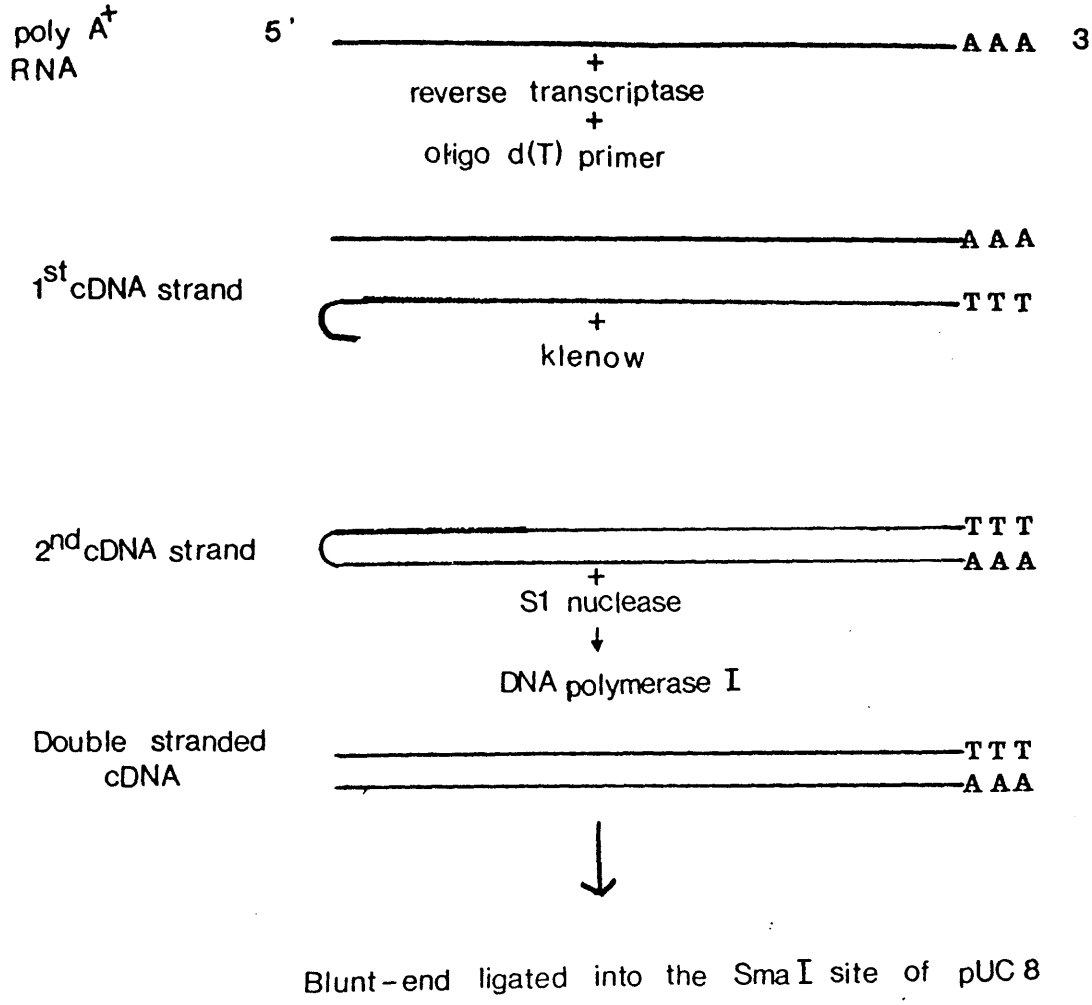


Figure 18 Schematic representation of the construction of the cDNA library representing poly(A)$^+$ RNA from 5 day RA induced HL60 cells.

Briefly, double-stranded DNA was synthesised from a poly(A)$^+$ RNA template, using an oligo(dT) primer, this was blunt end ligated in to the Sma I site of pUC8.

Firstly, I had to confirm that the 190 recombinants were represented in RA induced HL60 cells and, from the results obtained, select those recombinant plasmids which appeared to represent sequences showing changes in abundance during differentiation. To accomplish this the Grunstein-Hogness colony screening method was used. Filters were prepared in triplicate from each "composite" plate. These were then hybridised to ^{32}P-labelled cDNAs, reverse transcribed from poly(A)$^{+}$ RNA which had been isolated from the total cellular RNA of uninduced, TPA induced and RA induced HL60 cells. Recombinant plasmids were selected by comparison of the intensity of hybridisation signal gained from each of the probes, the signal was estimated from a number of different autoradiographic exposure times. To confirm the results obtained from the analyses described above, the experiment was then repeated using freshly prepared filters. The results from both rounds of screening were then compared and only those clones which gave consistant results were chosen to enter the next round of selection assays.

Selection criteria were :-

a) signal increase :- increase in abundance following induction of differentiation. An example of this can be seen in Figure 19A, A9. This recombinant plasmid appeared to represent a sequence which was highly abundant in TPA induced HL60 cells but not present in uninduced or RA induced cells.

b) signal decrease :- decrease in abundance following induction of differentiation. An example of this can be seen in Figure 19B, A6. This recombinant plasmid appeared to represent a sequence which was abundant in uninduced HL60 cells but not present in either RA or TPA induced cells.

c) a hybridisation signal was detected with each probe but the degree of hybridisation varied, being high with one probe but low with one or both the others. An example of this can be seen in Figure 19A, A6. This recombinant plasmid appeared to represent a sequence which was highly abundant in RA induced HL60 cells, present in uninduced cells, but barely detectable in TPA induced cells.

d) the same degree of hybridisation signal intensity was gained with

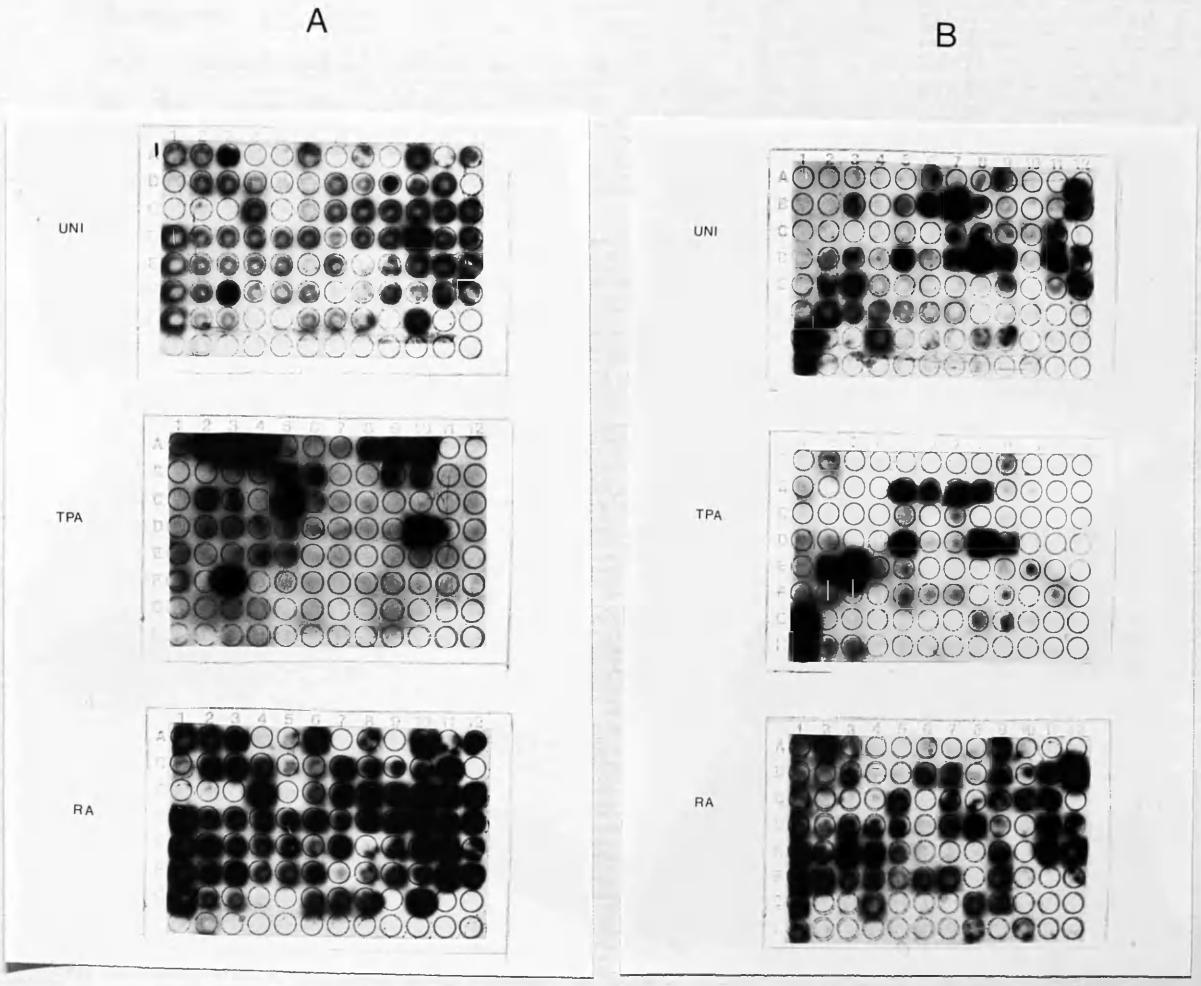


Figure 19 Grunstein-Hogness screening of the cDNA library representing RNA from 5 day RA induced HL60 cells.

Impressions were taken of the colonies containing recombinant plasmids. These were cultured, in triplicate, on nylon membranes. The membranes were then hybridised to ^{32}P-labelled cDNA reverse transcribed from poly(A)$^+$ RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells. (A) represents recombinants from "composite plate 1; (B) represents recombinants from "composite" plate 2.

each probe. An example of this can be seen in Figure 19A, A3. This recombinant plasmid appeared to represent a sequence that was highly abundant in uninduced, RA induced and TPA induced HL60 cells.

Of the 190 clones analysed in this round of screening most were in categories a), c) and d). The results from this experiment are summarised in Table 3. It appeared that the majority (55%) of the clones represented sequences which were found in RA induced or in both RA induced and uninduced HL60 cells. This would be expected as the recombinant plasmids were constructed from cDNA produced from poly(A)$^+$ RNA of RA induced HL60 cells. It can therefore be surmised that, during the induced differentiation of HL60 by RA, a large proportion of the cellular RNA is specific to that differentiation pathway. The large proportion of clones which hybridised to probes produced from poly(A)$^+$ RNA from uninduced HL60 cells is also not surprising as results from Chapter III, 1, B revealed that following 5 days of RA treatment 40-50% of an HL60 cell culture still retain uninduced cell characteristics.

To ensure that a similar plasmid copy number existed in each transfectant, the filters from the analyses described above, were then stripped of hybridised probe and rehybridised to ^{32}P-labelled, nick-translated pUC8 cDNA.

From this first analysis 25 recombinant plasmids were selected which appeared to represent sequences which were highly abundant in RA induced HL60 cells when hybridised to the reverse transcribed probes from HL60 poly(A)$^+$ RNAs. These clones were again screened by the Grunstein-Hogness colony hybridisation method. This third round of screening showed little variation in results from the first or second. At this stage glycerol stocks were made of each of the 25 recombinant plasmids. Each glycerol stock was prepared from a single colony to ensure that it represented only 1 recombinant plasmid. These stocks also ensured that each time a fresh batch of plasmid DNA was prepared, it originated from the initial isolate.

The colony hybridisation technique has two main disadvantages. Firstly, results are dependent on growth of the cells once transferred on to the nylon filter, which consequently affects plasmid copy number and poses problems when comparing individual

Signal with Probes from:-	Number from 190 Clones	Percentage
RA induced cells only	56	29.5 %
Uninduced cells only	20	10.6 %
TPA induced cells only	2	1.0 %
All cells	28	14.8 %
TPA+Uninduced cells	26	13.7 %
RA+ Uninduced .cells	48	25.2 %
TPA+RA induced cells	4	2.0 %
No Signal	6	3.2 %

Table 3 Summary of initial sreening results from the cDNA library.

The percentage of recombinant plasmids which appeared to contai sequences found in uninduced, 3 day TPA induced and 5 day RA induce HL60 cells were calculated from screening data.

colonies. Secondly, the efficiency of the lysis and denaturing processes affect the degree of hybridisation between the plasmid DNA and the labelled probe. In order to overcome these problems, DNA from the 25 recombinant plasmids, selected previously, were prepared using the small-scale plasmid DNA preparation method described in Chapter II, 2, K (iii). The DNA was dot-blotted on to nitrocellulose membrane and hybridised to ^{32}P-labelled probes reverse transcribed from poly(A)$^+$ RNA isolated from total cellular RNA of uninduced, TPA induced and RA induced HL60 cells. Weak hybridisation signals were obtained during this experiment which was probably due to low DNA concentrations used in each DNA dot. However, the intensity of signal improved with longer autoradiographic exposure enabling comparisons to be made between filters hybridised to the three different probes. Very few recombinant plasmid DNAs hybridised to probes from uninduced or TPA induced HL60 poly(A)$^+$ RNA (Figure 20). Filters hybridised with labelled cDNA from RA induced HL60 poly(A)$^+$ RNA also gave a poor signal, however with very long exposure times of upto 2 weeks, almost all of the recombinant plasmid DNAs gave a weak hybridisation signal with this probe (Figure 20). Figures 20 (d) and (e) show the control filter for this analysis, which was hybridised to ^{32}P-labelled pUC8 cDNA, and the filter plan describing the position of each clone.

The recombinant plasmids have been named after the vector, pUC8, plus the position that the plasmid occupied in the initial "composite" plate. Therefore pUC8C3 is a recombinant pUC8 plasmid found at position C3. The cDNA of each clone is named after the position only, dropping the pUC8 prefix, therefore the pUC8C3 cDNA insert would be called C3. The RNA homologous to the C3 sequence would be termed C3 RNA.

When the results from both screening methods were compared, 10 clones were chosen. These appeared to represent sequences which changed in abundance following HL60 differentiation. The results from the screening analyses, for the 10 recombinants are summarised in Table 4 (A). Almost all of the clones appeared to represent RNA sequences that were highly abundant in RA induced HL60 cells, with the exception of pUC8C2, which appeared to be homologous to an RNA abundant in TPA induced HL60 cells. pUC8A3 was selected to act as a control in further analyses, as the cDNA insert present in this

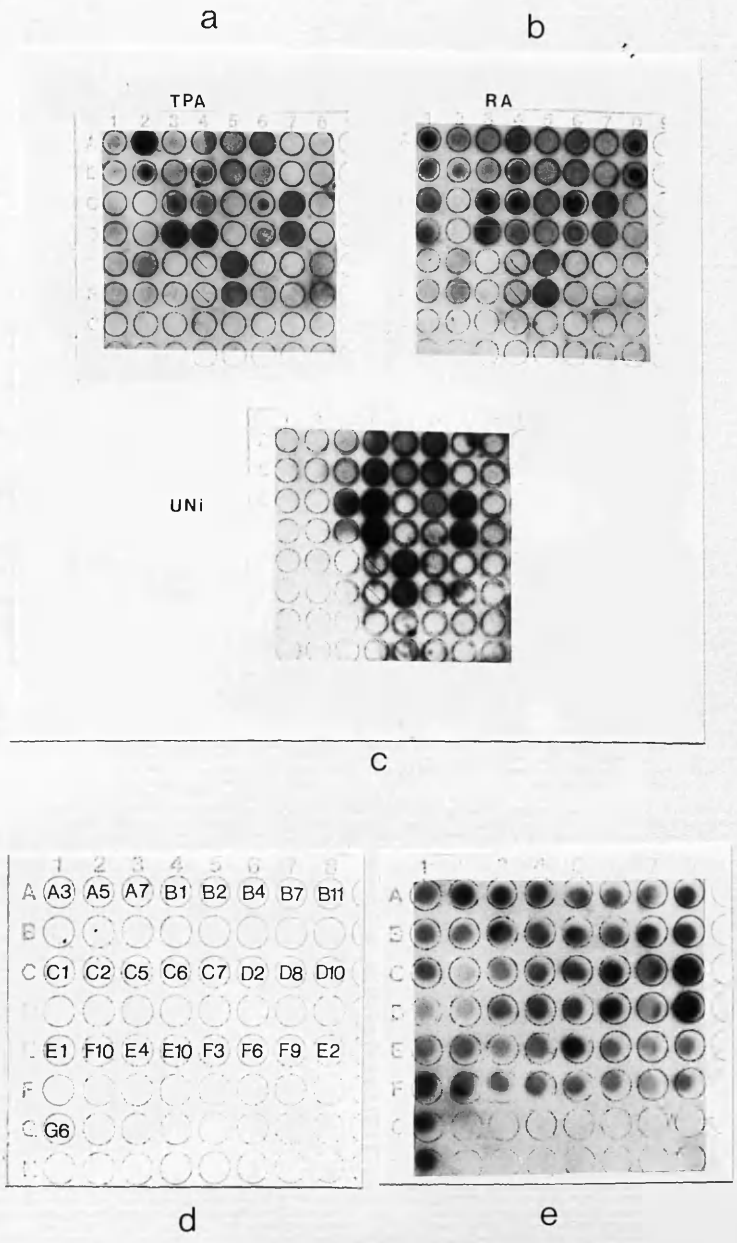


Figure 20 Identification of recombinants homologous to sequences which change in abundance during HL60 differentiation.

Small scale preparations of plasmid DNA were made from 25 colonies, which were selected from the cDNA library representing poly(A)$^+$ RNA from 5 day RA induced HL60 cells. These preparations were used to prepare three nylon membranes. The membranes were hybridised with ^{32}P-labelled cDNA transcribed from poly(A)$^+$ RNA from uninduced HL60 cells, 5 day RA induced HL60 cells and 3 day TPA induced HL60 cells. This figure shows the results of autoradiographs of these membranes. The filters were then stripped of hybridised probe and rehybridised to radioactively labelled pUC8 cDNA to ensure that an equivalent amount of plasmid DNA was present in each dot. A plan of the membranes, giving the position and name of the 25 recombinants is also displayed. a),TPA probed; b),RA probed; c),Uni probed; d),Plan of plate; e),pUC8 probed plasmid DNA.

Table 4 Summary of the data obtained for the 10 recombinants selected following screening of the cDNA library containing sequences present in 5 day RA induced HL60 cells.

(A) The abundance of the sequences represented by each recombinant was established from a number of autoradiographic exposures from the membranes in Figures 19 and 20. (++), very abundant; (+), moderately abundant; (-), not detected.
(B) The sizes of the cDNAs from each of the recombinants was determined by Eco RI + Bam HI restriction digestion of the plasmid DNAs followed by fractionation on a 1.5% (w/v) agarose gel. The sizes were calculated by comparison to DNA size markers which were run simultaneously on the same gel. These were θX174 DNA digested with Hae III.

(A)

Plasmid	UNI	RA	TPA
pUC8 A3	++	++	++
pUC8 B1	-	++	-
pUC8 B4	+	++	-
pUC8 C2	+	+	++
pUC8 C6	+	++	+
pUC8 E1	++	++	+
pUC8E4	+	++	+
pUC8F6	+	++	-
pUC8F10	+	++	-
pUC8G6	+	++	-

(B)

Plasmid	Size of cDNA
pUC8A3	1250
pUC8B1	600,120
pUC8B4	180
pUC8C2	180
pUC8C6	220,190,160
pUC8E1	-
pUC8E4	230
pUC8F6	550
pUC8F10	210
pUC8G6	310, 250

plasmid hybridised almost uniformly to probes from induced and uninduced HL60 cells.

C) Calculation of the Sizes of the cDNA Inserts from the 10 Clones Selected Following the Screening Process of the RA5 cDNA Library

During construction of the cDNA library, derived from poly(A)$^+$ RNA from 5 day RA induced cells, the Sma I restriction digestion site within the pUC8 multicloning site was utilised as the cloning site for the blunt end ligation of the cDNAs into pUC8. To isolate the cDNAs from the recombinant plasmid required restriction digestion with Eco RI and Bam HI. This is shown diagramatically in Figure 21. Therefore, to estimate the size of the cDNAs, from the 10 recombinants selected following screening of the RA5 library, 1 ug of each plasmid DNA was restriction digested with Eco RI + Bam HI, then fractionated by electrophoresis on a 1.5% agarose gel (Figure 22). The sizes of the cDNAs (Table 4 (B)) were calculated by comparison to Hae III restriction digested ϕX174 DNA markers, run simultaneously on the same gel. At this stage, one recombinant plasmid, pUC8E1, did not appear to contain any cDNA and was therefore discarded.

Generally, the cDNAs were small, the largest being A3 of 1250 bp in length. A3, B1, C6, F6, and G6 cDNAs gave multiple fragments following Eco RI + Bam HI digestion of the recombinants. This was indicative of an internal restriction site in the cDNA sequence. Another possibility was that these recombinant plasmids were conjugate, consisting of two plasmids ligated end to end with either the same or different cDNA inserts. Eco RI + Bam HI restriction digestion of such a plasmid would also reveal two cDNAs. From previous work in our laboratory conjugate plasmids had been discovered.

The smaller of the potential cDNAs detected in lanes 1 and 8 of Figure 22, corresponding to pUC8A3 and pUC8F6, were not clearly defined bands on the gel which was indicative of some form of contaminant in the DNA preparation. This could have been caused by contaminating RNA co-purified in the initial preparation of the plasmid DNA. Therefore the plasmid DNAs were further purified by centrifugation at high speed through a sucrose gradient. Re-analysis

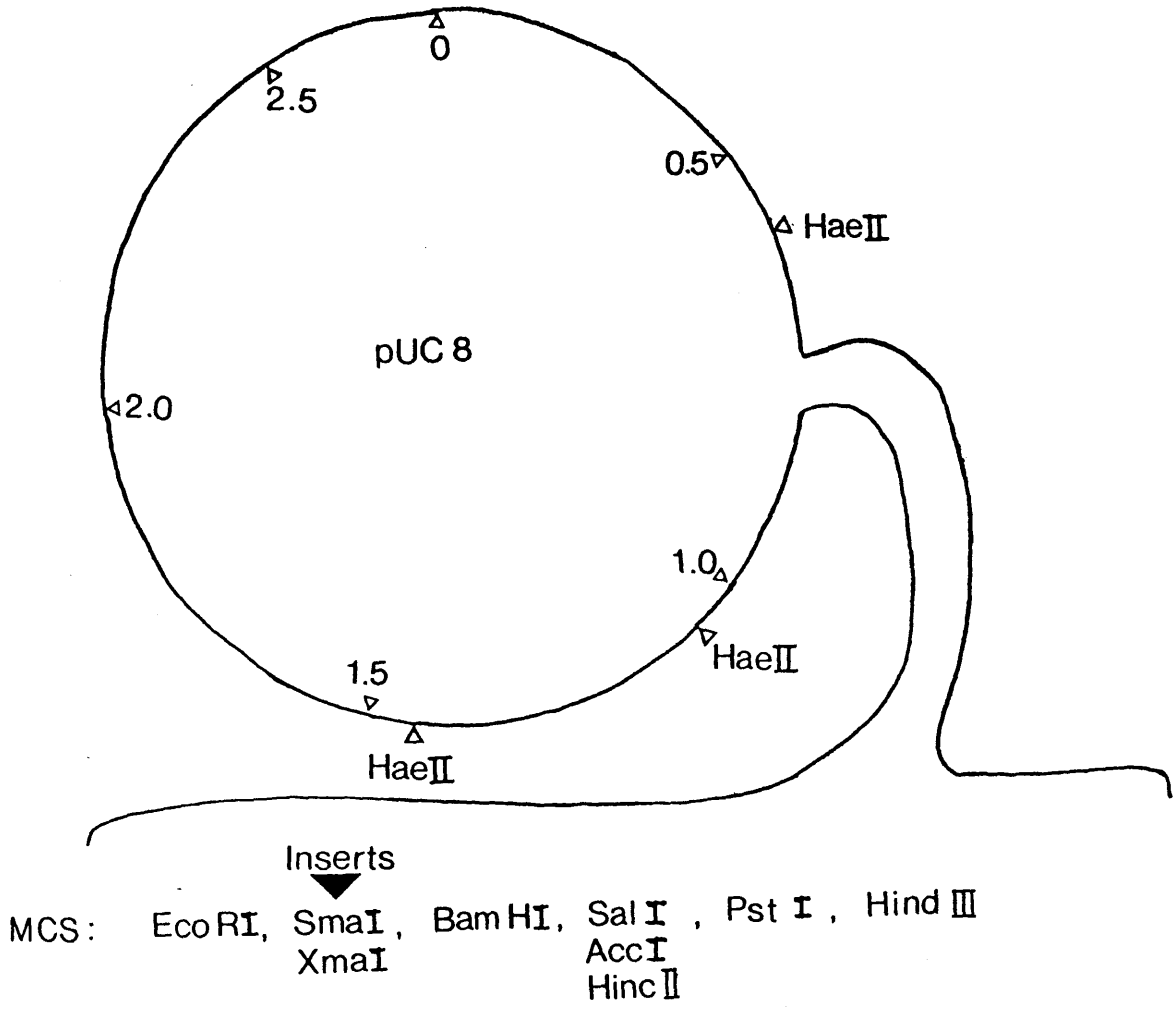


Figure 21 Diagram of the plasmid cloning vector pUC8.

This diagram shows the position of the Hae II restriction digest sites and also the sites in the multicloning site of pUC8. cDNAs were cloned in to the Sma I site therefore their removal from the plasmid vector could be accomplished by Eco RI + Bam HI restriction digestion of the recombinant plasmid DNA.

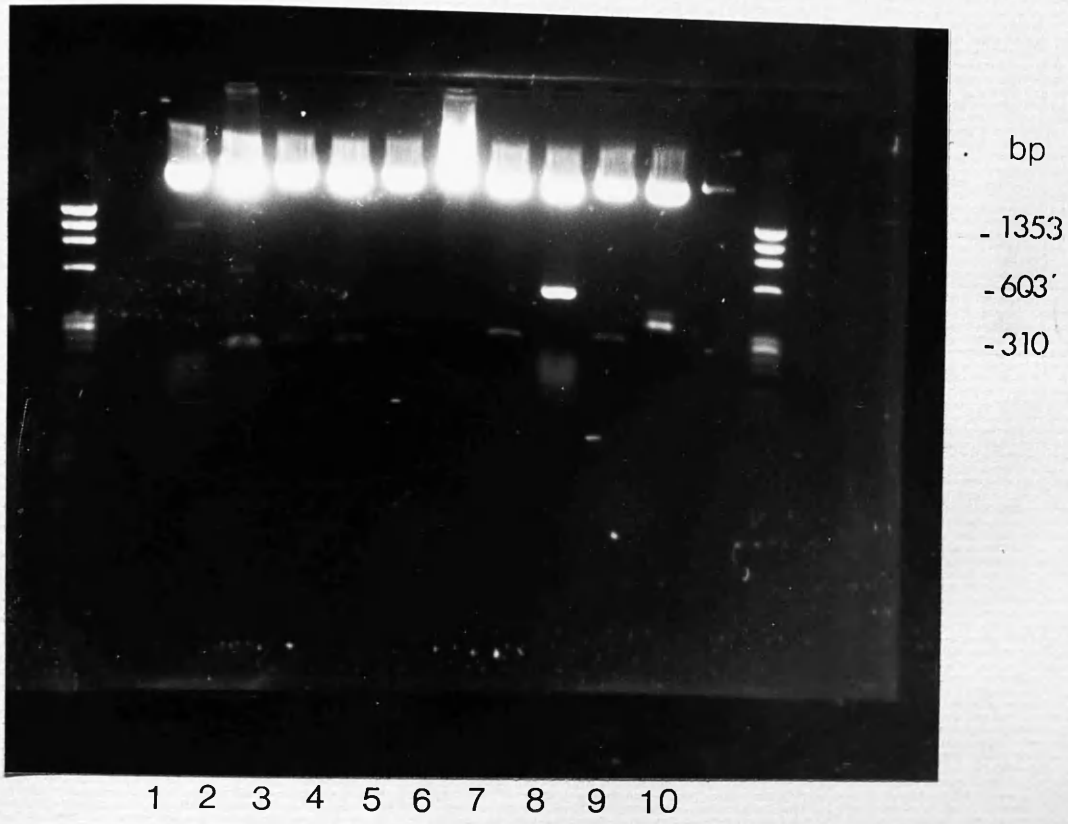


Figure 22 Sizing the cDNAs from the 10 selected recombinants.

Each recombinant was digested with the restriction enzymes Eco RI + Bam HI. The digested plasmid DNAs were electrophoresed in a 1.5% agarose gel. Hae II digested θX174 DNA was also loaded alongside, providing size marker DNA bands. The figure shows the resulting ethidium bromide stained gel visualised by UV light. Lane 1, pUC8A3; Lane 2, pUC8B1; Lane 3, pUC8B4; Lane 4, pUC8C2; Lane 5, pUC8C6; Lane 6, pUC8E1; Lane 7, pUC8E4; Lane 8, pUC8F6; Lane 9, pUC8F10; Lane 10, pUC8G6.

Figure 23 Restriction enzyme digestion of pUC8B1, pUC8C6, and pUC8G6 DNAs.

1 µg of plasmid DNA was digested with Eco RI, Bam HI or Eco RI + Bam HI. The digested DNA samples were then fractionated by electrophoresis on a 1.5% agarose gel. The figure shows the resulting ethidium bromide stained gel visualised by UV light. Hae II digested θX174 DNA was also loaded alongside to provide DNA size markers. Lane 1, pUC8 DNA; Lane 2, pUC8 DNA / Eco RI; Lane 3, pUC8C6 DNA; Lane 4, pUC8C6 / Eco RI; Lane 5, pUC8C6 DNA / Bam HI; Lane 6, pUC8C6 DNA / Eco RI + Bam HI; Lane 7, pUC8B1 DNA; Lane 8, pUC8B / Eco RI; Lane 9, pUC8B1 DNA / Bam HI; Lane 10, pUC8B1 / Eco RI + Bam HI; Lane 11, pUC8G6 DNA; Lane 12, pUC8G6 DNA / Eco RI; Lane 13, pUC8G6 DNA / Bam HI; Lane 14, pUC8G6 DNA / Eco RI + Bam HI.

of pUC8A3 and pUC8F6 DNAs following this revealed only one cDNA fragment for each plasmid.

To determine if pUC8B1, pUC8C6 and pUC8G6 were conjugate plasmids, the plasmid DNAs were digested with Eco RI, Bam HI or Eco RI + Bam HI and fractionated on an agarose gel. The results showed that these plasmids did not appear to be conjugate (Figure 23). When linearised each plasmid appeared as a single band of slightly larger size than linearised pUC8. If indeed they had been conjugate plasmids, it might have been expected that the linearised DNA would run as a doublet. The extra fragments were also unlikely to be the result of a contaminating plasmid. Purification by colony-selection, followed by Eco RI + Bam HI digestion, still revealed an identical restriction pattern. Thus, it appeared that B1, C6 and G6 contained internal Bam HI or Eco RI digestion sites; the fragments generated from these sites, however, were not detected, even when a large concentration of plasmid DNA was analysed (Figure 23).

3 EXAMINATION OF RNAS HOMOLOGOUS TO THE cDNAs OF THREE RECOMBINANTS SELECTED FOLLOWING SCREENING OF THE cDNA LIBRARY

Following the screening of the RA5 library, 10 cDNAs had been selected which appeared to represent sequences that were expressed in high abundance during HL60 induced differentiation by RA. 3 of these cDNAs, F6, F10 and C6, and the RNAs homologous to the sequences the cDNAs represented were then studied in detail.

A) Characterisation and Identification of the F6 cDNA Sequence

Initial results obtained from screening analyis indicated that the F6 cDNA was homologous to RNA which was abundant in both RA and uninduced HL60 cells. To confirm this further analyses were carried out to determine the size and relative abundance of F6 RNA. Results from these analyses, when compared to those obtained from other cDNAs would also determine if the F6, F10 and C6 cDNAs represented different sequences.

i) Southern Blot Analysis

To determine the restriction fragment lengths homologous to F6 in genomic DNA, total cellular DNA from HL60 cells was digested with the restriction endonucleases Eco RI, Bam HI and Hind III. These enzymes were chosen because they cleave DNA relatively infrequently and so should yield simple restriction patterns. A Southern blot was prepared following electrophoresis of the digested DNA which was then hybridised to radioactively labelled F6. The results from this analysis are shown in Figure 24. F6 cDNA was unusual in that it hybridised in a non-specific manner to the DNA. The hybridisation signal was not resolved by increasing the washing time of F6 hybridised filters at high temperatures and in high stringency wash buffers. This pattern is representative of those corresponding to highly repetitive sequences, which occur frequently throughout the genome.

ii) Analysis to Determine the Relative Abundance of F6 RNA in HL60 Cells

To determine the relative abundance of F6 transcripts in HL60 cells, quantitative analyses were carried out. RNA doubling dilution dot blots were prepared from total RNA from uninduced, 3 day TPA induced and 5 day RA induced HL60 cells, then hybridised with radioactively labelled F6 cDNA. The results are shown in Figure 25. F6 hybridised strongly to uninduced HL60 cellular RNA. By measuring the end-point of titration for each RNA sample, it was calculated that RNA homologous to F6 was of 4-fold greater abundance in uninduced HL60 cells than in RA induced cells, and 32-fold more abundant than in TPA induced HL60 cells.

To confirm that the same amount of RNA was present in each series of RNA dots, the dot blot was stripped and rehybridised to ^{32}P-labelled pHR28-1, a probe for human 28S ribosomal RNA sequences. The results, shown in Figure 25, confirm that a uniform concentration of RNA was present in corresponding dots from each series.

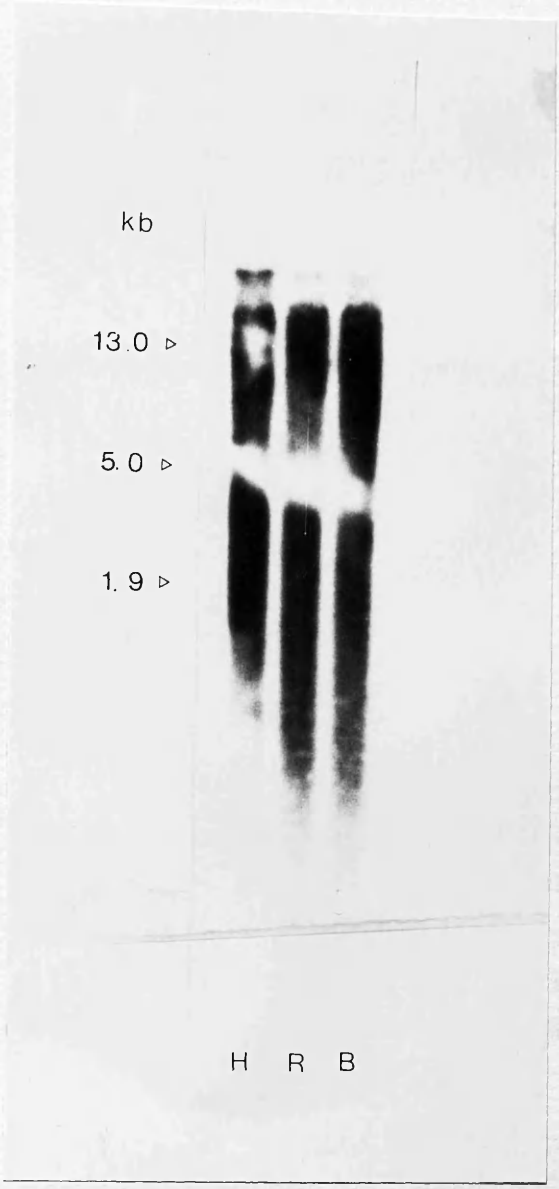


Figure 24 Southern analysis of HL60 DNA hybridised to ^{32}P-labelled F6 cDNA.

30 µg of HL60 total cellular DNA were restriction digested with Eco RI, Bam HI or Hind III then fractionated by electrophoresis on a 1% (w/v) agarose gel. The DNA was transferred to nitrocellulose membrane by blotting. The nitrocellulose membrane was hybridised with ^{32}P-labelled F6 cDNA. (B), Bam HI; (R), Eco RI; (H), Hind III.

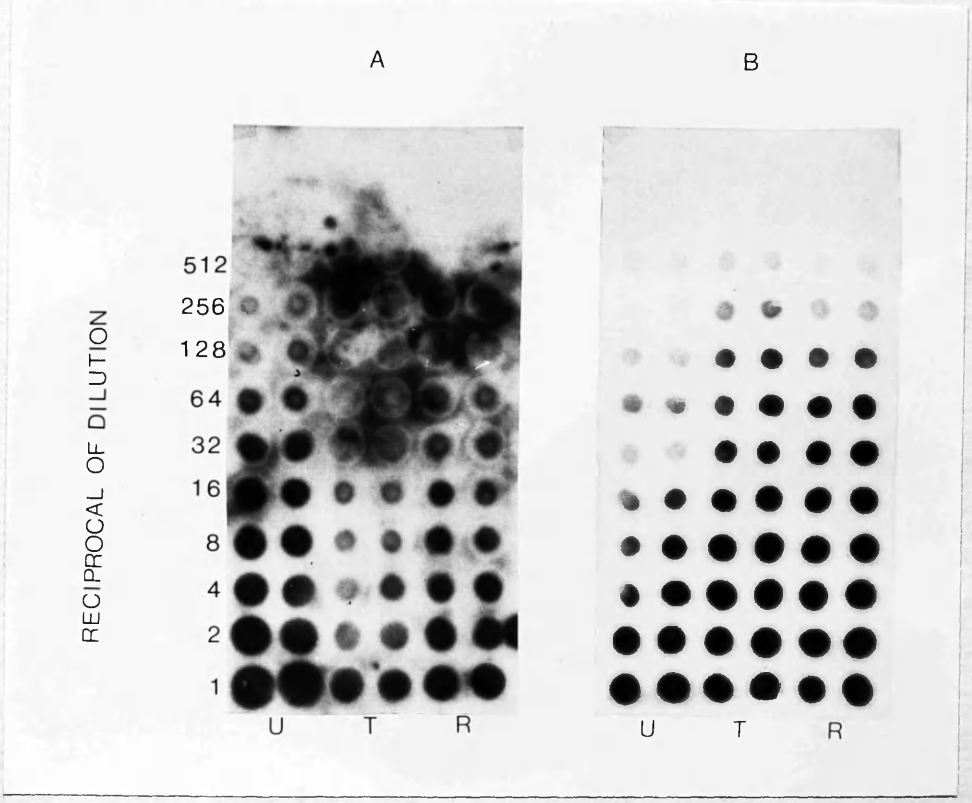


Figure 25 Determination of the abundance of F6 RNA in HL60 cells.

20 µg of total RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were dotted in a doubling dilution fashion on to nitrocellulose membrane. The nitrocellulose membrane was then hybridised with radioactively labelled probes for (A), F6 sequences and (B), human 28S ribosomal RNA sequences. (R), 5 day RA induced HL60 RNA; (T), 3 day TPA induced HL60 RNA; (U), uninduced HL60 RNA.

iii) Northern Blot Analysis

To discover the size of the RNA transcript(s) homologous to F6, a Northern blot was prepared from total cellular RNA from uninduced, 3 day TPA induced and 5 day RA induced HL60 cells. The blot was then hybridised to ^{32}P-labelled F6. The results from this analysis are shown in Figure 26. The transcript sizes were calculated by comparison to RNA size standards, run simultaneously, ranging from 9.5 kb - 0.3 kb.

The F6 cDNA hybridised to an area covering 4.0 kb - 1.0 kb. Although no specific RNA species could be determined, the hybridisation signal was strongest in total RNA from uninduced and RA induced HL60 cells but did not appear in high abundance in TPA induced cells. The results from this analysis suggested that the F6 cDNA contained a repetitive element, in agreement with those obtained from Southern analysis.

To ensure that the RNA analysed by Northern blotting was not degraded and that the concentration of RNA analysed from each sample was uniform the blot was stripped and reprobed with radioactively labelled B_2-microglobulin cDNA. The results, displayed in Figure 26 confirm this.

The results obtained for the size of RNA homologous to F6, from Northern blot analysis and the relative abundance of the F6 transcripts following HL60 differentiation, are summarised in Table 5.

iv) Cell Compartments Containing F6 RNA

a) Poly(A)$^+$ RNA

During the initial characterisation of F6 cDNA, total cellular RNA had been analysed. To determine if F6 transcripts were present in poly(A)$^+$ RNA in HL60 cells, and hence representing mRNA in these cells, poly(A)$^+$ RNA was isolated from uninduced, TPA induced and RA induced HL60 cells by the method of Aviv and Leder (1972). The poly(A)$^+$ RNAs were then used to prepare Northern blots which were hybridised to ^{32}P-labelled F6. The results are given in Figure 27.

cDNA F6		
Size of DNA Fragments (kb)	Hind III	smear
	Eco RI	smear
	Bam HI	smear
	RI / BI	smear
Size of RNA Transcripts (kb)		1.0 - 4.0
Relative Abundance of Transcripts	Uni	1
	RA	0.25
	TPA	0.0625
	DMSO	ND

Table 5 Summary of the results of Northern and Southern blotting analyses of F6 sequences.

These results were gathered from a number of analyses. The values calculated for the relative abundance of F6 RNA are averages of results from a number of different inductions.

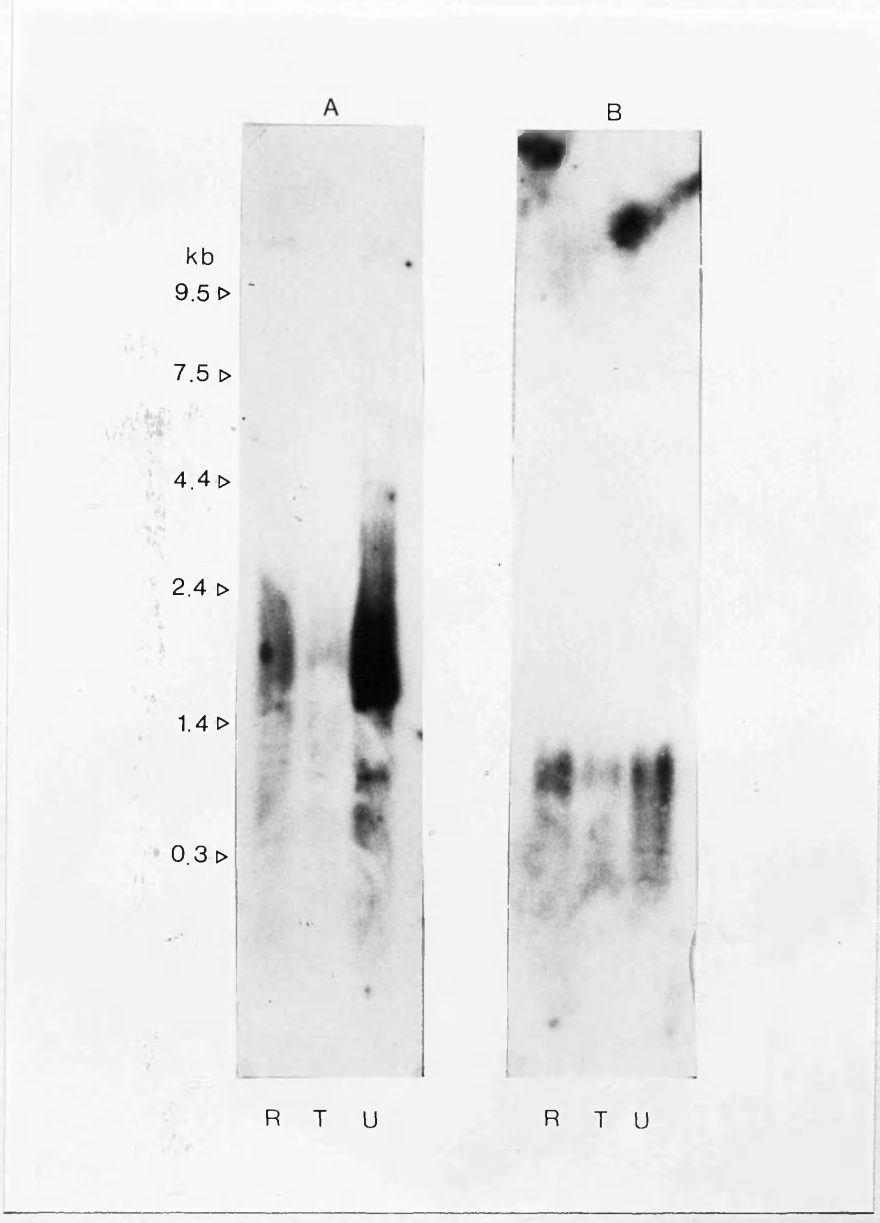


Figure 26 Determination of the size of RNA homologous to F6 by Northern blotting.

20 μg of total RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were fractionated by electrophoresis on a denaturing, agarose gel. The RNA was then transferred on to nitrocellulose membrane by blotting and the membrane hybridised to radioactively labelled probes for (A), F6 sequences and (B), B_2-microglobulin. (R), 5 day RA induced HL60 RNA; (T), 3 day TPA induced HL60 RNA; (U), uninduced HL60 RNA.

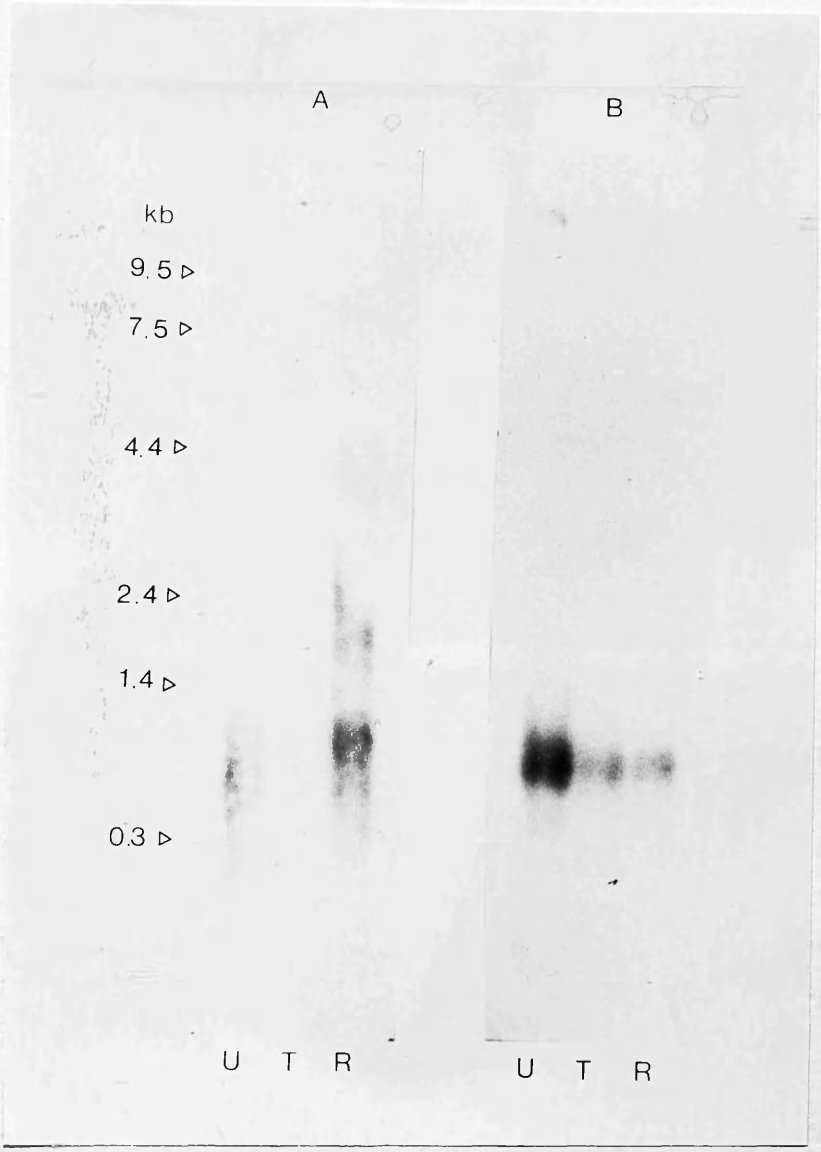


Figure 27 Determination of the presence of F6 RNA in poly(A)$^+$ RNA isolated from HL60 cells.

5 µg of poly(A)$^+$ RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were fractionated by electrophoresis on a denaturing, agarose gel. The poly(A)$^+$ RNA was then transferred on to nitrocellulose membrane by blotting. The nitrocellulose membrane was then hybridised with ^{32}P-labelled probes for (A), F6 sequences and (B), B_2-microglobulin. (U), uninduced HL60 poly(A)$^+$ RNA; (T), 3 day TPA induced HL60 poly(A)$^+$ RNA; (R), 5 day RA induced HL60 poly(A)$^+$ RNA.

Although much of the smearing, which was present when analysing total RNA, was lost, F6 did not hybridise to any specific mRNA. The intensity of hybridisation was again strongest in poly(A)$^+$ RNA from uninduced and RA induced HL60 cells. The smeared results observed when Northern blots were hybridised with F6 suggested that the cDNA contained a non-coding element, supporting the conclusion reached following Southern blotting analysis which resulted in the non-specific hybridisation of ^{32}P-labelled F6 cDNA to all sizes of DNA.

To determine that the poly(A)$^+$ RNA used in this analysis was not degraded and that a similar concentration had been assayed from each sample, the Northern blot was stripped and rehybridised to ^{32}P-labelled B_2-microglobulin cDNA (Figure 27).

b) The abundance of F6 transcripts in Nuclear and Cytoplasmic RNA of HL60 cells

None of the analyses involving the characterisation of F6 RNA had revealed a distinct size of transcript. This led to the conclusion that F6 did not represent a coding sequence but might be a component of hnRNA. To discover if this were true the distribution of RNA homologous to F6 was examined in the different cellular compartments. The cellular compartment to which F6 RNA was localised, in HL60 cells, was discovered by determining the relative abundance of these transcripts, by Northern blotting and RNA dot blot analysis, in total cytoplasmic and nuclear RNA isolated from uninduced HL60 cells. The results from these experiments are given in Figure 28. From the RNA dot blot analysis, cytoplasmic RNA did not hybridise efficiently to F6 but a strong signal was obtained from nuclear RNA. Therefore it appeared that RNA homologous to F6 was 8-10 fold more abundant in nuclear RNA isolated from uninduced HL60 cells than in cytoplasmic RNA. A similar conclusion could be drawn from Northern blot analysis (Figure 28 (ii)). Again considerable smearing occurred but clearly F6 transcripts were more abundant in nuclear RNA. These results suggested that F6 RNA did not contribute to the cytoplasmic RNA pool, was unlikely to represent mRNA and therefore did not contribute to the protein complement of HL60 cells.

The northern blot was stripped and rehybridised with radioactively

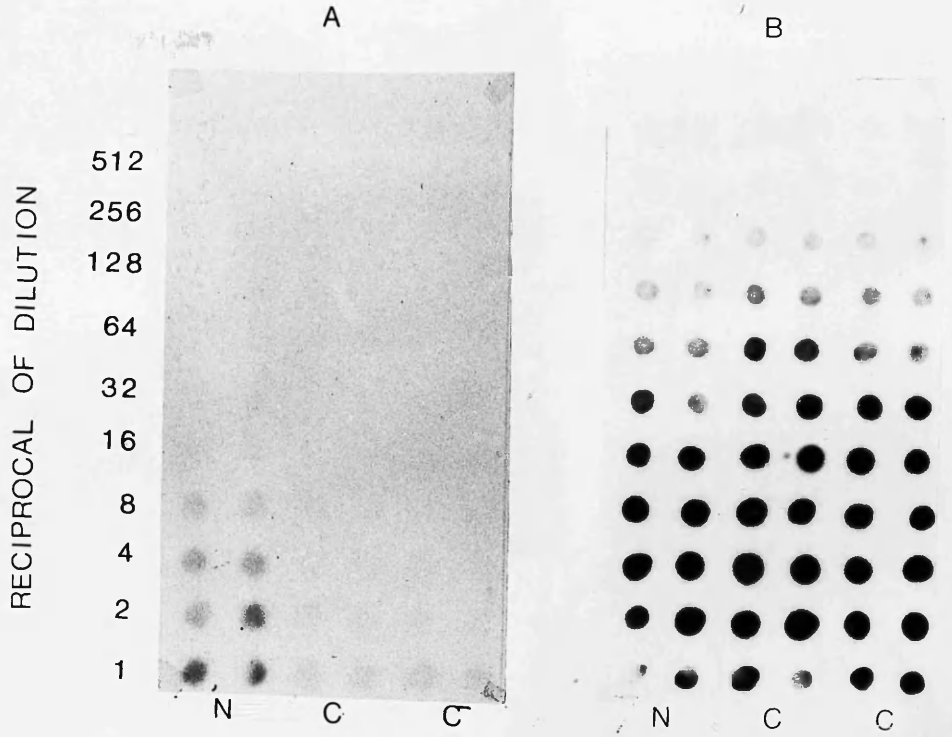
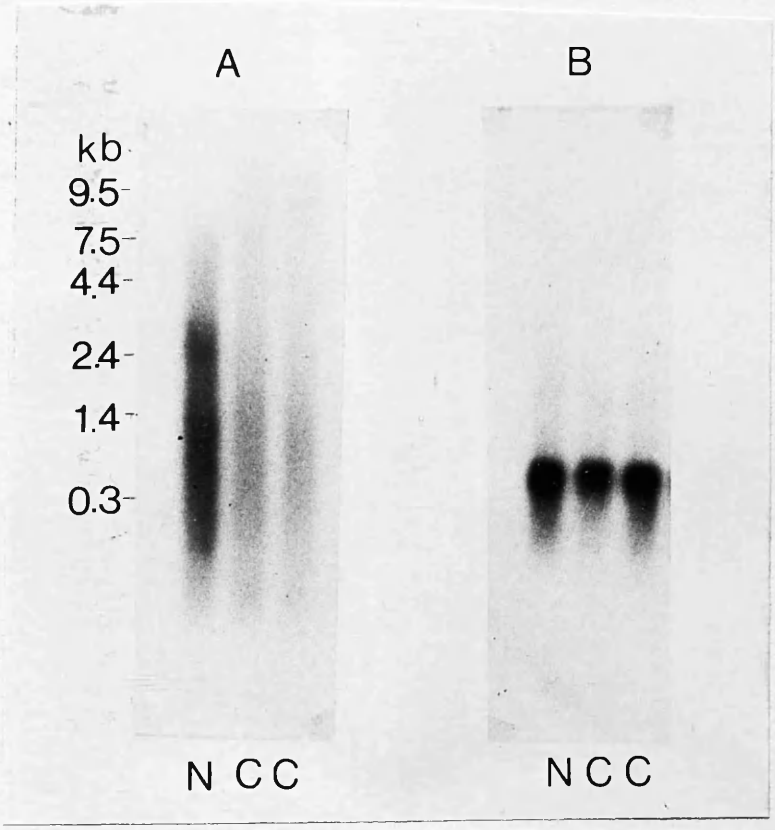


Figure 28 (i) Determination of the abundance of F6 transcripts in total nuclear RNA and total polysomal RNA isolated from uninduced HL60 cells.

5 µg of total cytoplasmic RNA and 5 µg of total nuclear RNA isolated from uninduced HL60 cells were dotted on to nitrocellulose membrane in a doubling dilution fashion. The nitrocellulose membrane was then hybridised with ^{32}P-labelled probes for (A), F6 sequences and (B), human 28S ribosomal RNA sequences. (N), total nuclear RNA; (C), total cytoplasmic RNA.



(ii) Northern blotting analysis of F6 RNA in nuclear and cytoplasmic RNA isolated from uninduced HL60 cells.

5 µg of total cytoplasmic RNA and total nuclear RNA isolated from uninduced HL60 cells were fractionated by electrophoresis on a denaturing, agarose gel. The RNA was then transferred to nitrocellulose membrane by blotting and subsequently hybridised to radioactively labelled probes for (A), F6 sequences and (B), B_2-microglobulin. (N), nuclear RNA; (C), cytoplasmic RNA.

labelled probe for B_2-microglobulin to ensure that the RNA from each sample was at a uniform concentration and not degraded. (Figure 28,ii) confirms this. The RNA dot blot was stripped and rehybridised to a probe for human 28S ribosomal sequences to confirm that a similar concentration of RNA was used in comparable dots from different series (Figure 28,i).

c) Examination of the Translation Potential of RNA Transcripts Homologous to F6

From examination of polysomal fractions obtained from the cytoplasmic extract of fractionated uninduced and induced HL60 cells, it became clear that RNAs homologous to F6 were not translated. The cytoplasmic extract, of uninduced, TPA induced and RA induced HL60 cells was centrifuged through a sucrose gradient which separated the polysomes, plus associated RNA, from free ribosomes. The gradient was then unloaded and collected in 1ml aliquots from which an RNA dot blot was prepared. The RNA dot blots were hybridised with radioactively labelled F6, the results are shown in Figure 29. A greater intensity of hybridisation was detected in those fractions relating to free ribosomes than to those representing polysomes. This was found to be the case in the samples isolated from uninduced HL60 cells and from that of RA induced and TPA induced cells.

v) Sequence and Identification of the F6 cDNA

Southern blot and Northern blot analyses revealed that F6 hybridised in a non-specific manner to total cellular DNA and to total cellular RNA and poly(A)$^+$ RNA isolated from HL60 cells. Analysis of the location of F6 homologous transcripts in HL60 cells determined that these transcripts were abundant in the cell nuclear compartment. It was also shown that F6 RNA is unlikely to be translated as most of the F6 homologous RNA transcripts did not appear to be associated with polysomes. These results led to the conclusion that F6 contained a non-coding, repetitive element. To clarify the situation, the F6 cDNA was sequenced by the Sanger "dideoxy" sequencing method described in Chapter II, 2 (S). The F6 sequence is displayed in Figure 30. The sequence obtained was then compared to recorded sequences in the Genebank Sequence Data Bank.

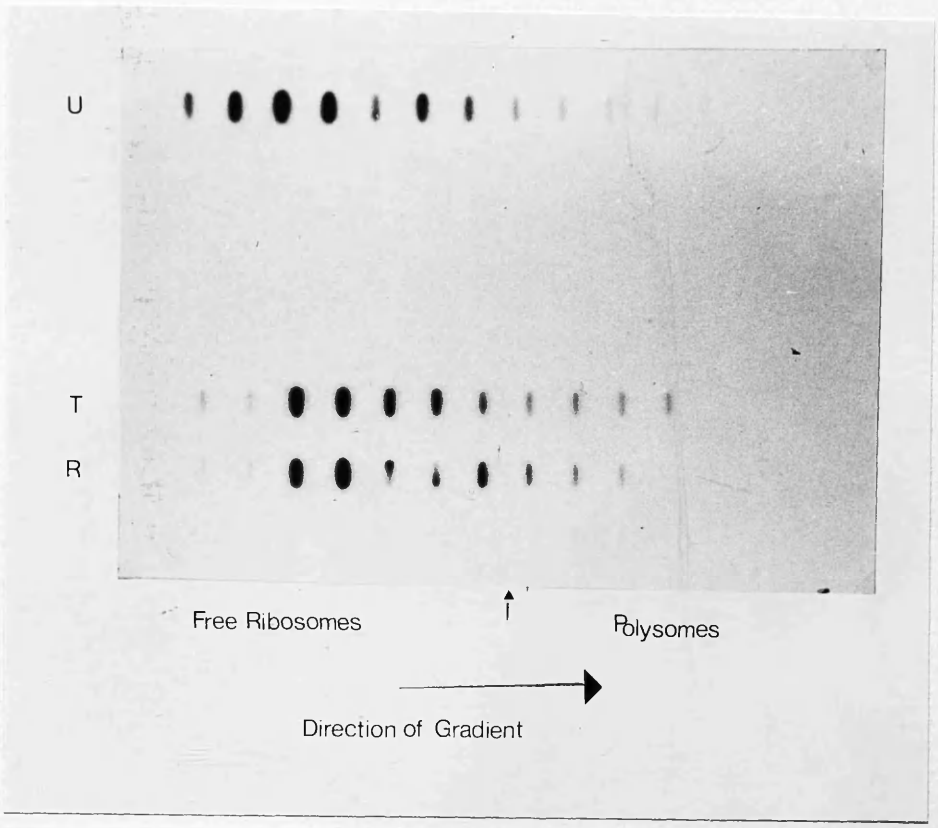


Figure 29 RNA dot blot of polysomal RNA isolated from HL60 cells hybridised to F6.

Polysomal fractions from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were centrifuged through neutral sucrose gradients to separate polysomes plus associated RNA from free ribosomes. 1 ml fractions were removed sequentially from the gradients and RNA was prepared from the samples. 5 μg of RNA from each sample were then dotted on to nitrocellulose membrane and then hybridised with radioactively labelled F6 cDNA. (U), uninduced HL60 RNA; (T), 3 day TPA induced HL60 RNA; (R), 5 day RA induced HL60 RNA.

5'

```
F6  GT   CCAGCTCCTA   GGGTAAGAAC   GTCAAAAGTC   TACTTAGGGT

B13

F6       ACGTTTAATT   TCCCAAACTC   TGTCTCAGGC   CGAGAACAAC
                      ******       ** ****     **********
B13                   AAACTC       TGCCTCAAAG   CGAGAACAAC

F6       CAACGGCACC   TCACGTCACC   GTGTCAGAGC   CGAGTGACGT
              ***     ****** ***       *****    **********
B13      GGGTCCGACC   TCACGTTACC   ACACTAGAGC   CGAGTGACGT

F6       TTGGAGACGA   ACGATCCAAG   CTCACTAAGA   GGGCTGG
         *       *     *  * *         *        *    **
B13      TGTAGACGGA   GGGCCCAAGT   TCGCTAAGAG   GACGAGGTCG

F6

B13      GAGTCGGAGT   CCTCATCGAC   CCCGATGTCC   GTACGCGGTG

F6                                    ATCATG   CTCTACCCCA
                                      *****         *** *
B13      GTGCGGGCCG   ATTAAAACAC   AAAAATCATC   TCAACCCCAA

F6       AAGCGGATCA   CCGCTCCGAC   CAGAGCTTGA   GGACTGGAGT
         **  * *  *   *** ******   **** *****   ****** * *
B13      AACGGTACAA   CCGGTCCGAC   CAGAACTTGA   GGACTGAATT

F6       CCACTACACC   GGTGGAGCTA   TAGGGTTTCA   CGGCCCTAAT
          *****         ****       * ***** *   ** *******
B13      TCACTAGGTG   AACGGAGTCG   GAAGGTTTGA   CGACCCTAAT

F6       GTAAGCAGTT   TAATTTCCAA   AACTAACACT   ACTTTTTGTG
         *
B13      G

F6       TGTATTTTAG   CTAGTCGAAG   TAGTAAAATT   CACATATAAA

B13

F6       GTTCATCACA   ATTTAAAAAA   GTACGATAAC   ACTTTGTGTA

B13

F6       GAGGCCTTGG   AAAAGTAGAA
```

3'

Figure 30 Diagram representing the F6 cDNA sequence compared to that of Blur 13, a human Alu repetitive element.

The sequence of F6 cDNA was found to share approximately 80% homology to Alu repetitive elements. The Blur cDNAs are sequences which represent members of the Alu family of short, interspersed repetitive elements. This diagram indicates the regions of homology between F6 and Blur 13 by (*).

Of the 550 bp of F6 cDNA, the sequence of 400 bp was obtained. From this it became apparent that F6 consisted of almost 300-400 bp of Alu sequence. These are short, interspersed repetitive DNA elements found in eukaryotes and present in the human genome in approximately 500 000 copies per haploid genome (Jelinek et al, 1980). In fact almost all of the F6 sequence shared 80-90% homology to known human Alu repetitive elements. However, 130 bp, at the 3' end of the F6 cDNA did not share any homology to Alu sequence and also bore no similarity to any other recorded sequence in the Genebank Sequence Data files. Attempts to find a suitable restriction digest site which would allow the isolation of this 130 bp unique region, and therefore allow its separation from the Alu element, were not succesful.

Figure 30 also illustrates the regions of homology between F6 and another Alu family member Blur13. The region for which no sequence was determined in F6 cDNA lies between two stretches of Alu homologous regions, which, when compared to other Alu clones, can be mapped 70-80 bp apart. It therefore seems reasonable to assume that this sequence would also be Alu. Although F6 cDNA did not represent a mRNA species, it is interesting to note that expression of Alu repetitive element RNA varied following induced differentiation of HL60 cells.

B) CHARACTERISATION OF F10 cDNA AND ITS HOMOLOGOUS RNA

From initial screening analyses of the RA5 cDNA library, the RNA transcripts homologous to F10 appeared to be highly abundant in 5 day RA induced HL60 cells. To establish this and to determine the size of F10 RNA a number of analyses were carried out.

i) Southern Blot Analysis

To determine the restriction fragment lengths homologous to F10 in DNA, total cellular DNA, from HL60 cells, was digested with the restriction endonucleases Eco RI, Bam HI and Hind III. The DNA was then used to prepare a Southern blot which was subsequently hybridised to radioactively labelled F10 cDNA. The results of this analysis are shown in Figure 31. F10 hybridised to discreet DNA

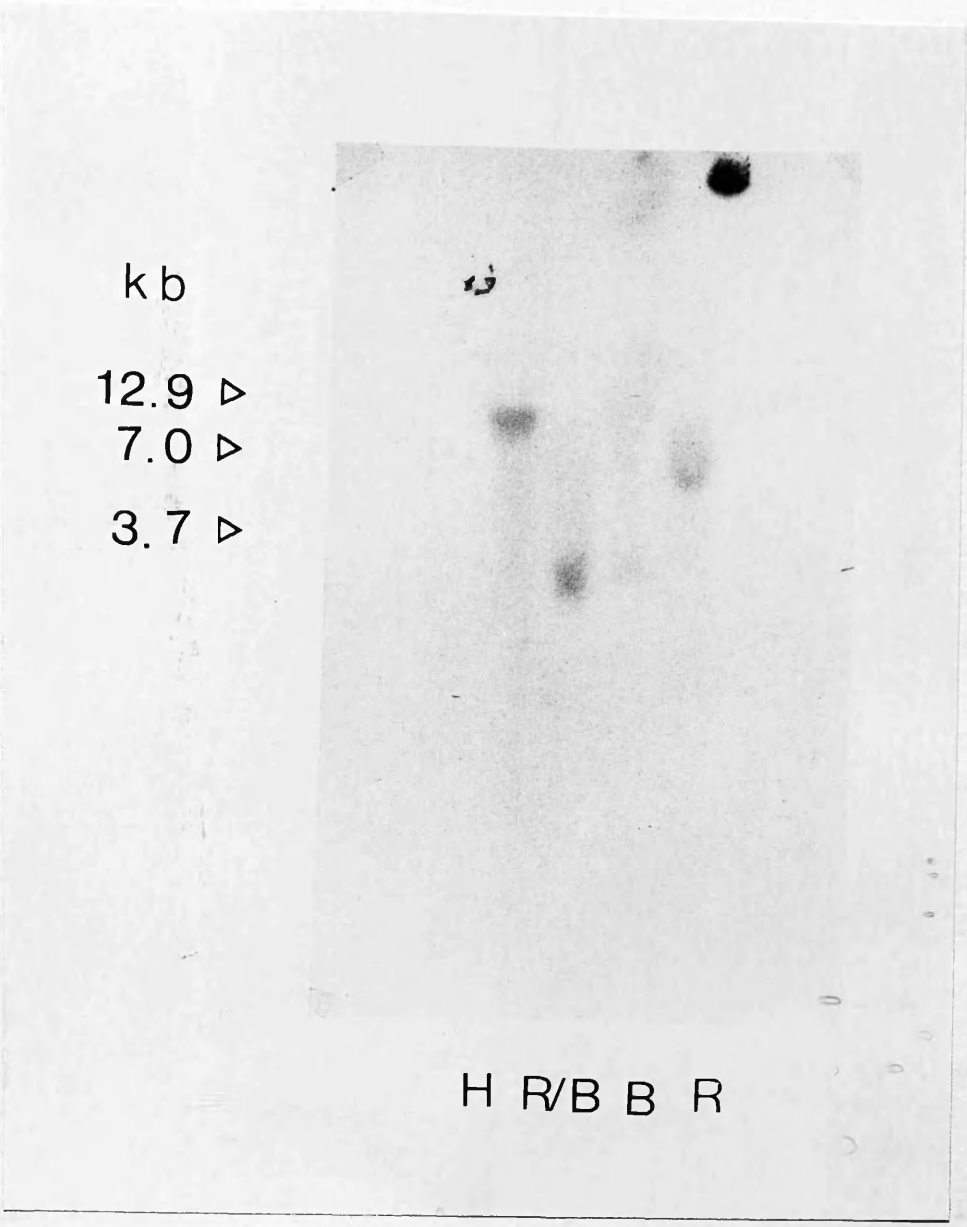


Figure 31 Southern analysis of HL60 DNA hybridised with ^{32}P-labelled F10 cDNA.

30 μg of HL60 total cellular DNA were restriction digested then fractionated by electrophoresis on a 1% (w/v) agarose gel. The DNA was then transferred on to nitrocellulose membrane by blotting. The nitrocellulose membrane was hybridised with radioactively labelled probe for F6 sequences. (R), Eco RI digested DNA; (H), Hind III digested DNA, (B), Bam HI digested DNA; (R/B), Eco RI + Bam HI digested DNA.

fragments. Comparison of the sizes of the restriction fragment lengths obtained from F10 and those of F6 and C6 indicated that F10 represented a unique sequence. The sizes of the restriction fragments homologous to F10 are summarised in Table 6.

ii) Quantitative Analysis of the abundance of F10 RNA Following HL60 Differentiation

To analyse quantitatively the abundance of transcripts related to F10, RNA doubling dot blot dilution assays were carried out.
Using total cellular RNA, isolated from uninduced, RA induced and TPA induced HL60 cells, blots were prepared and then hybridised to radioactively labelled F10 cDNA. The results from this assay are shown in Figure 32. Results from the doubling dot blot displayed in Figure 32 indicated that F10 RNA was 16 fold more abundant in RA induced HL60 cells than in uninduced and TPA induced HL60 cells. However, a number of analyses were carried out using RNA prepared from cells from different inductions. From these results, and those obtained from densitometric scanning of autoradiographs of F10 probed Northern blots, it was concluded that the F10 RNA is 6-10 fold more abundant in 5 day RA induced cells and 3 fold more abundant in 5 day DMSO induced cells, than in the uninduced HL60 cellular counterpart. Table 6 summarises the relative abundance of F10 RNA following HL60 induction.

Rehybridisation of the RNA doubling dilution dot blot with a ^{32}P-labelled 28S ribosomal RNA probe, pHR28-1, confirmed that a similar amount of RNA was present in each of the RNA dot series (Figure 32).

iii) Northern Blot Analysis

To discover if the sequence represented by F10 hybridised to a single RNA species and to determine the size of the transcripts homologous to F10, total RNA isolated from uninduced, 3 day TPA induced, 5 day RA induced, and 5 day DMSO induced HL60 cells was used to prepare a Northern blot. This was subsequently hybridised to ^{32}P-labelled F10 cDNA. The results obtained are shown in Figure 33. F10 cDNA hybridised to a single RNA transcript of 2.3 kb. Results from this initial analysis suggested that the F10 RNA was highly abundant in

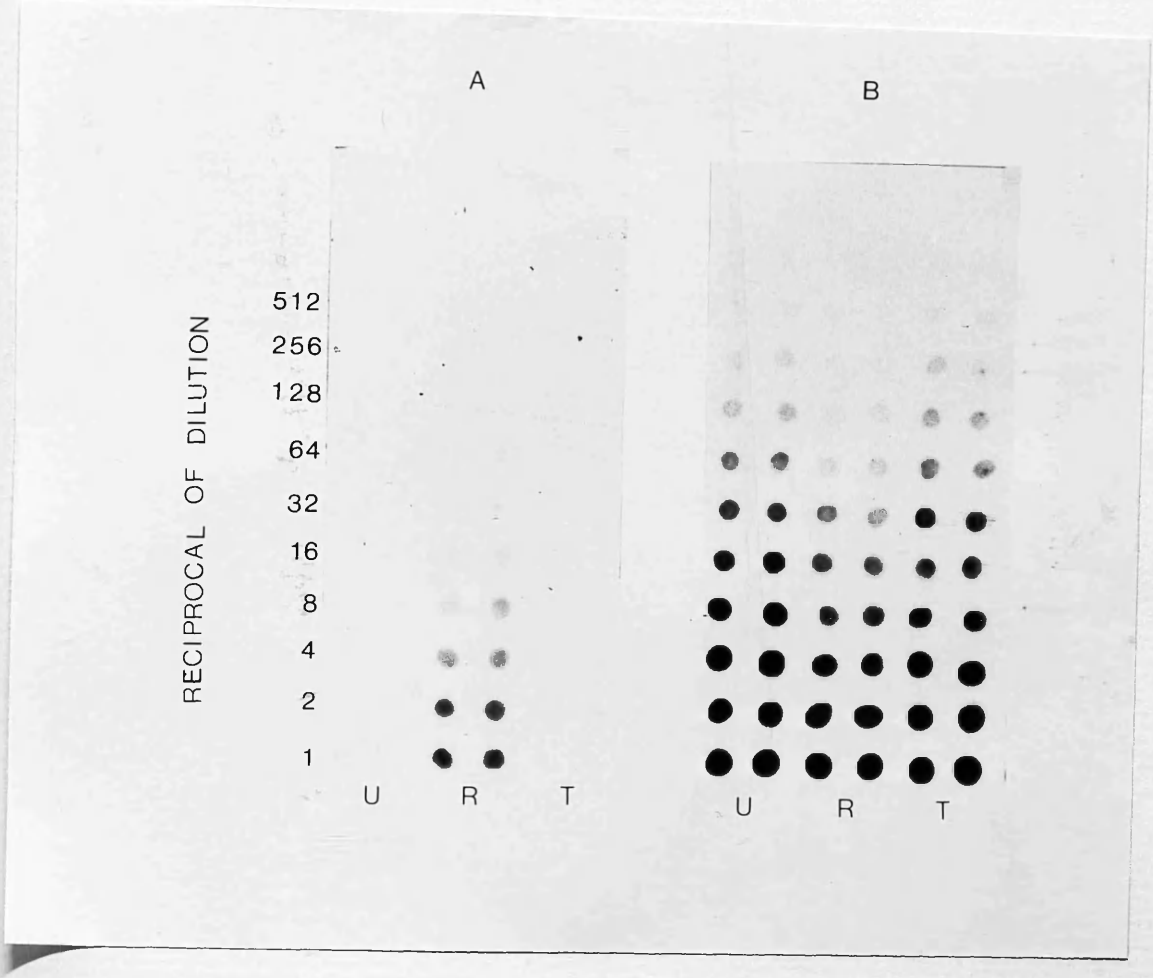


Figure 32 Determination of the abundance of F10 RNA in uninduced, 5 day RA induced and 3 day TPA induced HL60 cells.

20 μg of total RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were dotted on to nitrocellulose membrane in a doubling dilution fashion. The nitrocellulose membrane was then hybridised with radioactively labelled probes for (A), F10 sequences and (B), human 28S ribosomal RNA sequences. (T), 3 day TPA induced HL60 RNA; (R), 5 day RA induced HL60 RNA; (U), uninduced HL60 RNA.

		cDNA F10
Size of DNA Fragments (kb)	Hind III	12.9
	Eco RI	7.0
	Bam HI	3.7
	RI / BI	3.2
Size of RNA Transcripts (kb)		2.3
Relative Abundance of Transcripts	Uni	1
	RA	6
	TPA	2
	DMSO	3

Table 6 Summary of the results of Northern and Southern blot analyses of F10 sequences in HL60 cells.

The values calculated for the relative abundance of F10 RNA are average values calculated from a number of different inductions. Densitometric scanning of Northern blots of total HL60 cellular RNA hybridised with radioactively labelled F10 cDNA were also used to determine the relative abundance of F10 RNA following induction of HL60 cells.

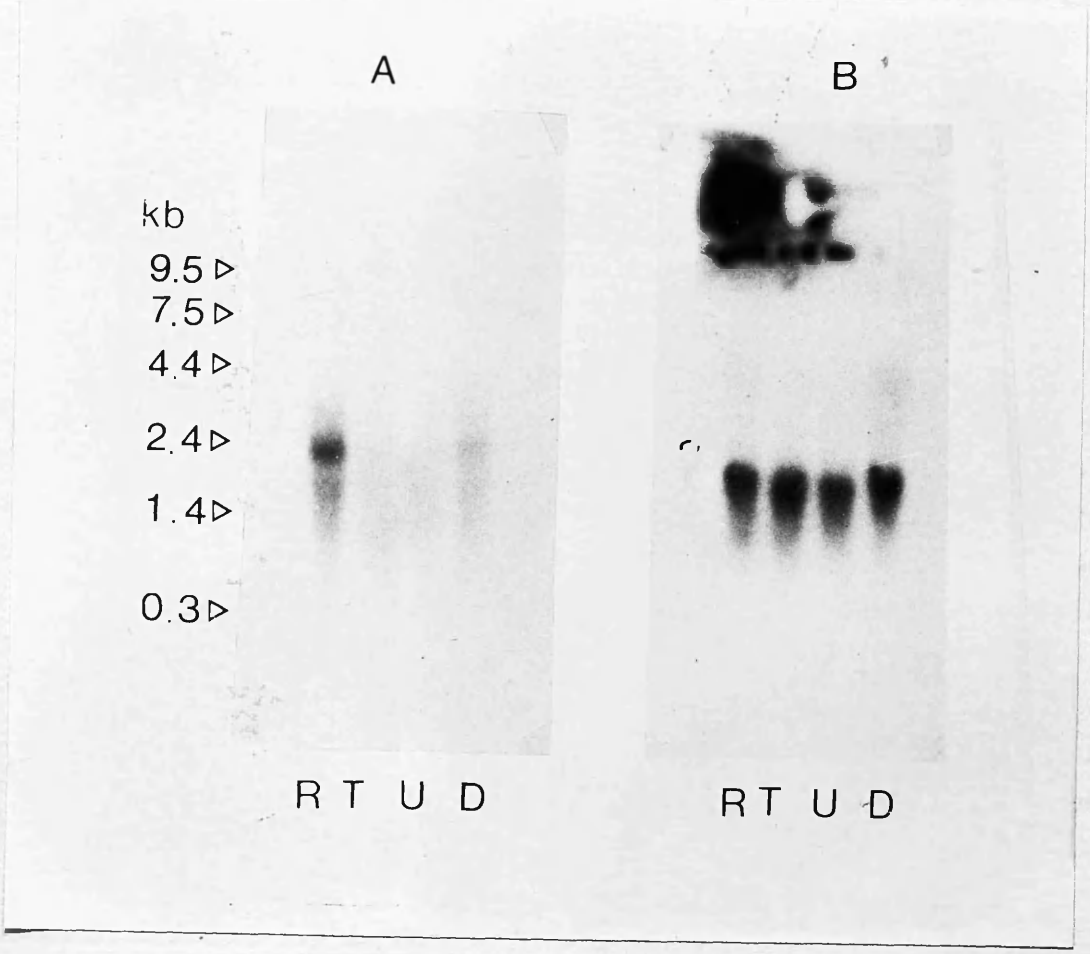


Figure 33 Determination of the size of RNA homologous to F10 cDNA by Northern blotting.

30 μg of total whole cell RNA isolated from 3 day TPA induced, 5 day RA induced, 5 day DMSO induced and uninduced HL60 cells were fractionated in a denaturing, agarose gel. The RNA was transferred on to nitrocellulose membrane by blotting and then hybridised with ^{32}P-labelled probes for (A), F10 sequences and (B), B_2-microglobulin. (R), 5 day RA induced HL60 RNA; (T), 3 day TPA induced HL60 RNA; (U), uninduced HL60 RNA; (D), 5 day DMSO induced HL60 RNA.

HL60 cells induced to differentiate down the granulocytic pathway.

The smearing detected below F10 RNA was not due to degradation of the RNA samples. This was shown by stripping and rehybridising the filters with radioactively labelled B_2-microglobulin cDNA which hybridises to a distinct 1.1 kb RNA (Figure 33).

iv) Analysis of HL60 Poly(A)$^+$ RNA

To determine if the 2.3 kb F10 RNA was present in the poly(A)$^+$ RNA fraction of HL60 cells, poly(A)$^+$ RNA, isolated from total cellular RNA of uninduced, 3 day TPA induced and 5 day RA induced HL60 cells, was used to prepare a Northern blot which was subsequently hybridised to ^{32}P-labelled F10 cDNA. The results from this experiment are displayed in Figure 34. F10 poly(A)$^+$ RNA was found to be abundant in RA induced HL60 cells, however very little hybridisation was detected in the TPA induced or the uninduced poly(A)$^+$ RNA samples. The blot was stripped of hybridised probe and rehybridised to radioactively labelled B_2-microglobulin cDNA to ensure that a similar concentration of RNA had been used in each sample during the analysis (Figure 34).

v) Examination of F10 Expression Throughout the Induced Differentiation of HL60 Cells to Granulocytes

To discover when the increase in the relative abundance of F10 RNA occurred during the course of HL60 granulocytic differentiation, total RNA was prepared from HL60 cells following periods of time throughout the 5 days required for RA and DMSO induction. These RNAs were fractionated by electrophoresis then used to prepare Northern blots which were subsequently hybridised to radioactively labelled F10 cDNA. The results from these analyses are displayed in Figure 35 (a). In both RA and DMSO induced differentiation of HL60, the increase in the relative abundance of F10 RNA was a late event. During DMSO treatment, this increase occurred between days 1-5 of induction, a similar pattern was observed during RA treatment; the level of F10 RNA increased steadily from 24 h of induction to reach a peak between 3-5 days. Densitometric scanning analysis of the autoradiographs from these experiments, showed that the level of F10 RNA did not rise steadily throughout the induction period of both RA

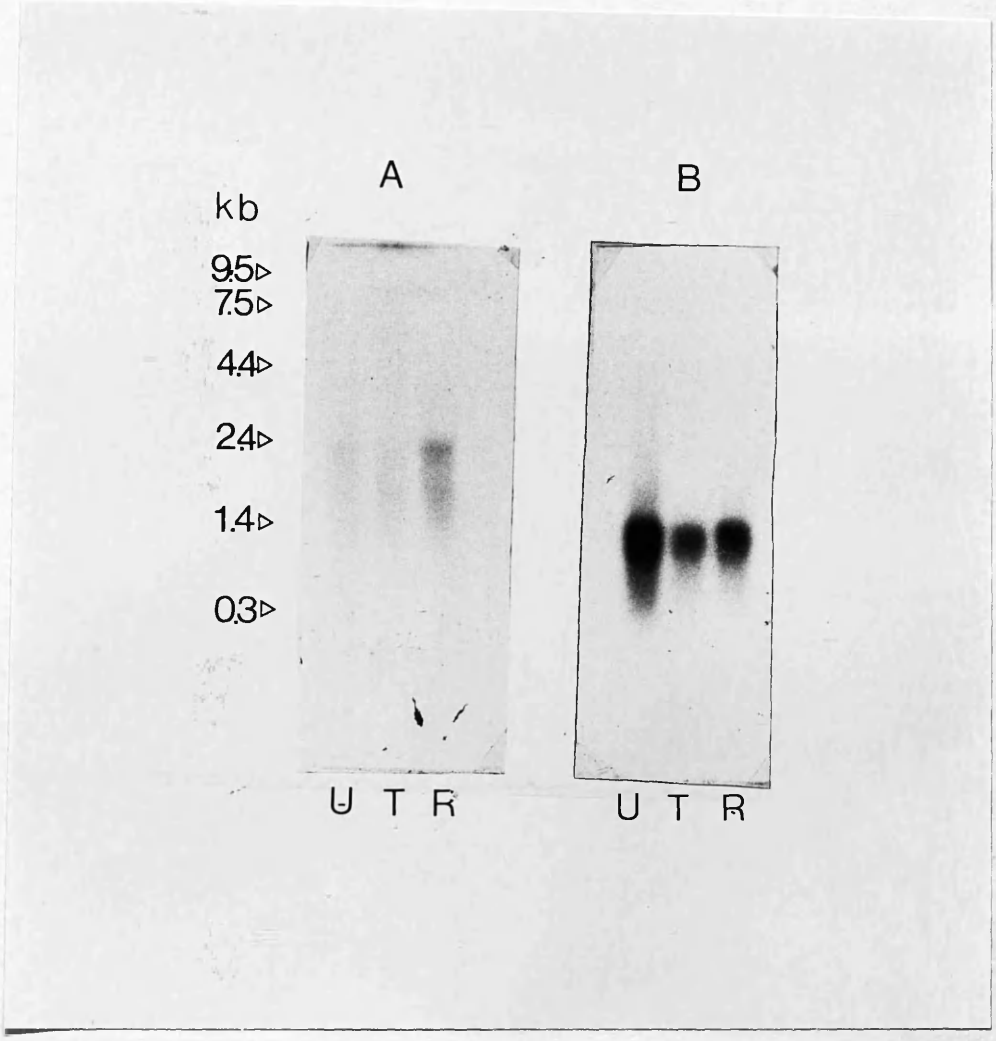


Figure 34 Determination of the presence of F10 RNA in poly(A)$^+$ RNA isolated from uninduced and induced HL60 cells.

5 μg of poly(A)$^+$ RNA isolated from uninduced and induced HL60 cells were fractionated on a denaturing, agarose gel and the RNA then transferred on to nitrocellulose membrane by blotting. The membrane was then hybridised to ^{32}P-labelled probes for (A), F10 sequences and (B), B_2-microglobulin. (U), uninduced HL60 poly(A)$^+$ RNA; (T), 3 day TPA induced HL60 poly(A)$^+$ RNA; (R), 5 day RA induced HL60 poly(A)$^+$ RNA.

and DMSO, but dropped at early times of treatment. The results from the densitometric scan are displayed graphically in Figure 35 (b).

The relative abundance of the 2.3 kb transcript reached a minimum between 16-24 h in DMSO and RA induced HL60 cells. However, after this time the level of F10 RNA increased to a value 3-6 fold of that obtained in uninduced cells, to reach a maximum between days 3-5 of induction. It is interesting to note that, as described in Chapter III, 1, b the appearance of HL60 cells displaying myeloid cell-specific differentiation markers, occurs after 24 h of treatment by inducer, and the number of terminally differentiated cells is maximal at day 5 of both RA and DMSO treatment. The timing of these events correlates exactly with the increase in the level of F10 RNA during HL60 granulocytic induction.

The pattern of F10 RNA relative abundance, observed during granulocytic induction, was not due to variation in the RNA concentrations of the different samples used throughout these experiments. The filters were stripped and rehybridised with ^{32}P-labelled B_2-microglobulin cDNA (Figure 35 a). The levels of this transcript were constant for each sample. This indicated that no variation in concentration arose in the RNAs isolated from HL60.

vi) Examination of the Range of Human Tissues that Express F10 RNA

From the results of previous analyses it appeared that F10 RNA was highly abundant in mature myeloid cells produced by induced differentiation of HL60 cells. The range of distribution of F10 RNA expression was examined by preparing RNA dot blots from total cellular RNA isolated from a number of human tissues; bone marrow, peripheral white blood cells, normal kidney, normal liver and normal mucosa. The blots were hybridised with ^{32}P labelled F10 cDNA, the results are given in Figure 36. A very weak hybridisation signal was detected in the series of RNA dots corresponding to peripheral white blood cell total RNA, however even after a long period of exposure this signal was not improved. No hybridisation was apparent to any of the other samples assayed. It could be surmised from these results that the F10 RNA is restricted to expression in mature haemopoetic cells, but is found in greatest abundance in 5 day RA induced HL60 cells.

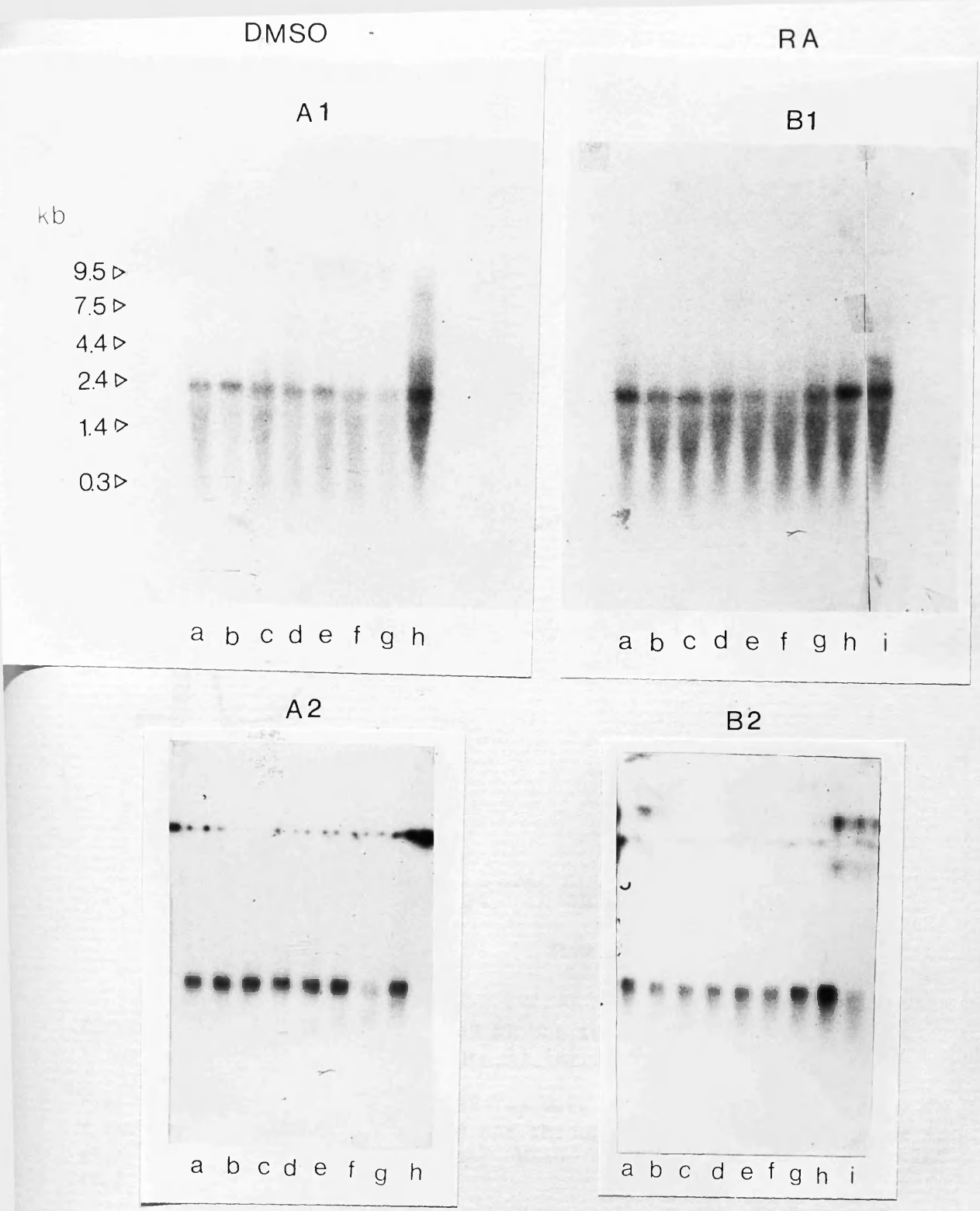


Figure 35 (a) Northern blot analysis of F10 transcripts during granulocytic differentiation of HL60 cells.

30 μg of total cellular RNA isolated from cells harvested at time points throughout the 5 day course of DMSO and RA induction of HL60 cells were electrophoretically fractionated on a denaturing, agarose gel. RNA was then blotted from the gel on to nitrocellulose membrane. The nitrocellulose membrane was hybridised with radioactively labelled probes for (A(1) and B(1)), F10 sequences and (A(2) and B(2)), B_2-microglobulin. a) 0 h; b) 1 h; c) 2 h; d) 4 h; e) 8 h; f) 16 h; g) 24 h; h) 72 h; i) 120 h treatment with inducing agent.

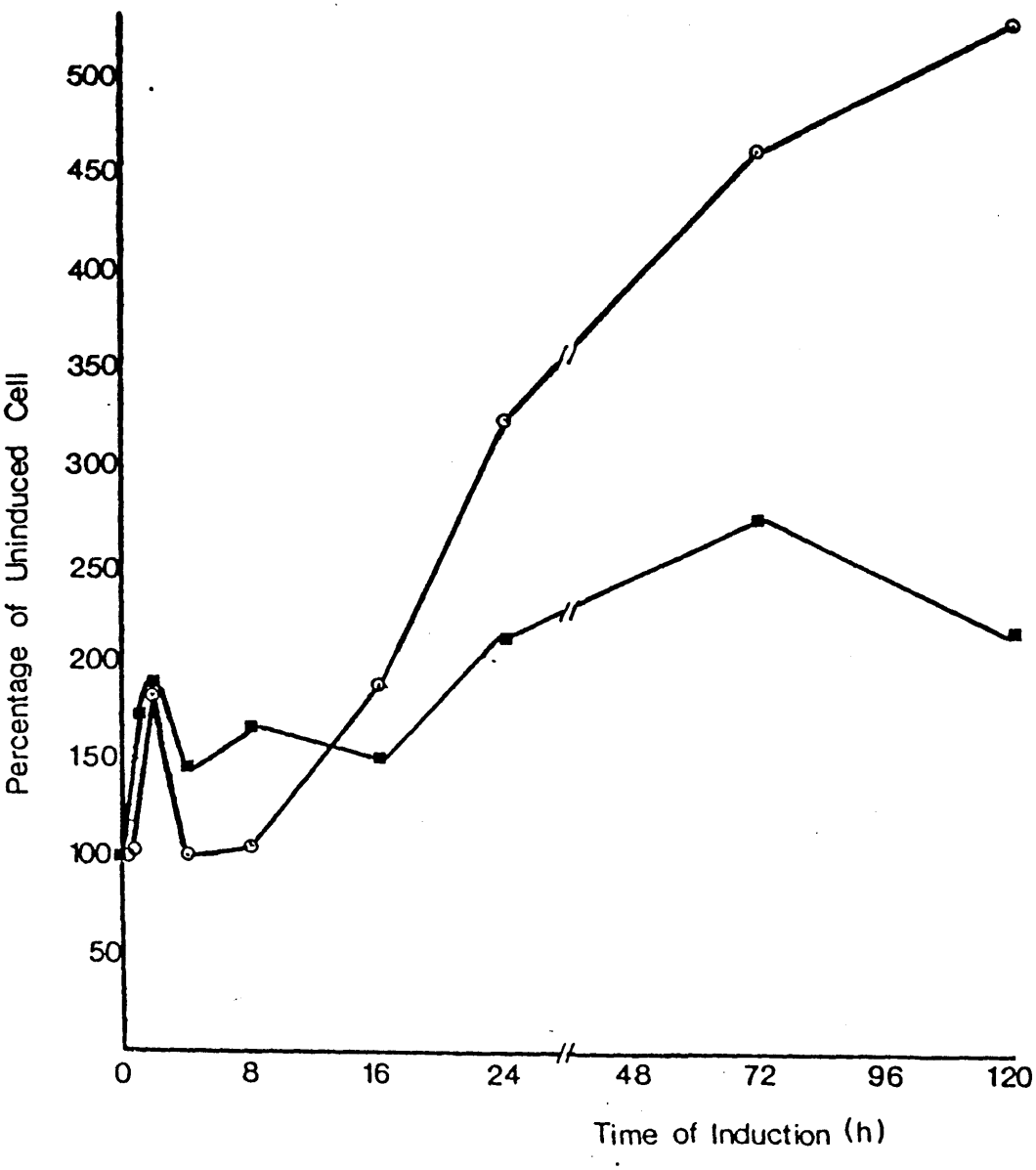


Figure 35 (b) Graphic display of the results from the Northern analyses in Figure 35 (a).

The Northern blots in Figure 35 (a) were densitometrically scanned to quantify the abundance of F10 RNA throughout HL60 differentiation to granulocytes. (○), RA induced HL60 cells; (■), DMSO induced HL60 cells.

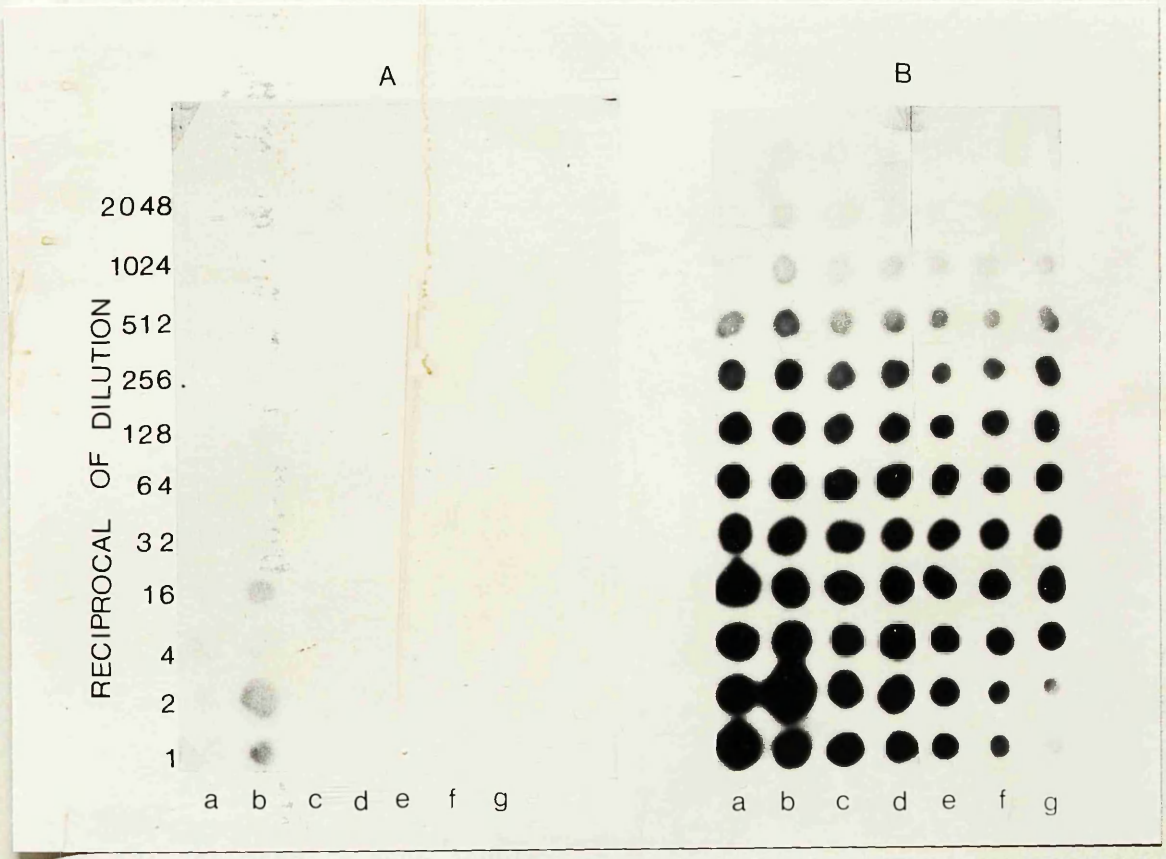


Figure 36 Determination of the range of human tissues that contain F10 RNA.

20 µg of total cellular RNA isolated from a number of different human tissues were dotted on to nitrocellulose membrane in a doubling dilution fashion. The nitrocellulose membrane was then hybridised with ^{32}P-labelled probes for (A), F10 sequences and (B), human 28S ribosomal RNA sequences. (a), uninduced HL60; (b), 5 day RA induced HL60; (C), normal human bone marrow; (d), normal human peripheral white blood cells; (e), normal human kidney; (f), normal human liver; (g), normal human mucosa.

The blot was stripped and rehybridised to radioactively labelled pHR28-1 cDNA (Figure 36). This confirmed that a similar concentration of RNA was present in each series of RNA dots.

vii) Cell Compartments Containing F10 RNA

To discover in which cellular compartment F10 RNA was found in HL60 cells, a comparison was made between the relative abundance of this transcript in nuclear RNA and total cellular RNA prepared from uninduced and induced HL60 cells. Total RNA was prepared from whole cells and from nuclei isolated from uninduced and induced HL60 cells. The nuclear and total RNAs were prepared from cells from the same culture. These RNAs were then used to prepare Northern blots, which were hybridised to ^{32}P-labelled F10 cDNA. Results from this analysis are given in Figure 37. It was found that RNA homologous to F10 was very abundant in the nuclear RNA from uninduced HL60 cells, but almost undetectable in the total RNA isolated from uninduced whole cells. In the case of RA induced HL60 cells, very little hybridisation occurred to the nuclear RNA but 2.3 kb transcripts were detected in the total, whole cell RNA. From these data it could be concluded that F10 RNA was present in the cytoplasmic RNA fraction of RA induced HL60 cells, but not in uninduced HL60 cells where it was detected in the nuclear RNA fraction. The hybridisation signal from the Northern blot, prepared from total RNA was weak because only 3 ug of RNA was used in each sample.

The F10 cDNA probe did not hybridise to a single RNA species in the uninduced cellular nuclear RNA but to an area covering 2.3 kb to very small sizes. This was not due to non-specific degradation of the RNA as is shown in Figure 37. The blots were stripped and rehybridised with radioactively labelled B_2-microglobulin cDNA. This hybridised to a single RNA species of 1.1 kb.

viii) Analysis of the Half-Life of the F10 RNA

Throughout Northern analysis of F10 transcripts, the smearing effect below the 2.3 kb RNA was apparent. From work carried out in this laboratory, examining c-myc RNA, smearing beneath the 2.4 kb - 2.2 kb c-myc transcripts has been explained by the rapid turnover of these RNAs. It has been reported by others that c-myc RNA has a

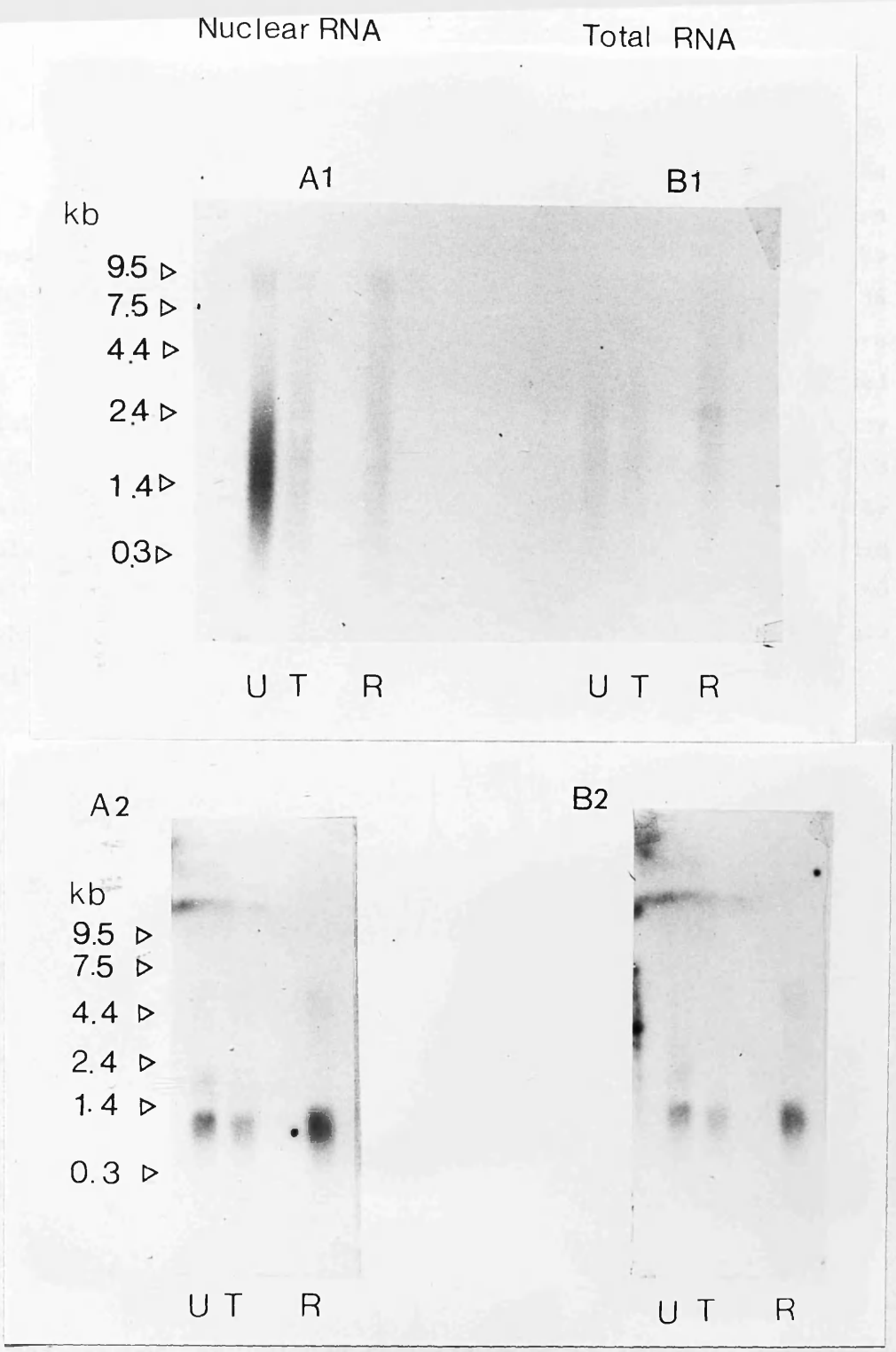


Figure 37 Determination of the cell compartment(s) containing F10 RNA.

3 μg of total cellular RNA and 3 μg of total nuclear RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were fractionated by electrophoresis in a denaturing, agarose gel. The RNA was then transferred on to nitrocellulose membrane by blotting. The nitrocellulose membrane was then hybridised with radioactively labelled probes for (A(1) and B(1)), F10 transcripts and (A(2) and B(2)), B_2-microglobulin. (R), 5 day RA induced HL60; (T), 3 day TPA induced HL60; (U), uninduced HL60.

half-life of 10-15 min (Dani et al, 1984). To examine the half-life of F10 RNA, HL60 cells were cultured in the presence of actinomycin D, an RNA polymerase II transcription inhibitor. The cells were then harvested, total RNA was prepared, which was then analysed by Northern blotting, hybridised to ^{32}P-labelled F10 cDNA (Figure 38 (a)). The autoradiographic results obtained from this experiment were scanned on a densitometer, to give results which could be analysed quantitatively (Figure 38(b)). F10 RNA did not appear to have a very short half-life, when compared to c-myc. After 120 min treatment with actinomycin D, the level of F10 RNA had only decreased by 40%. By extrapolation from the graph in Figure 38 (b), if can be concluded that F10 RNA would have a half-life of 2.5-3 h in the uninduced HL60 cell whereas c-myc transcripts appeared to have a half-life of only 15-20 min.

Figure 38,(a) shows the results from the controls from this analysis. To ensure that actinomycin D did inhibit transcription, the filter was stripped and rehybridised to a ^{32}P-labelled probe for c-myc RNA (pMC41-3RC). The level of c-myc transcripts rapidly decreased , to become undetectable after only 30-60 min of actinomycin D treatment. The filter was then stripped and rehybridised a second time with radioactively labelled B_2-microglobulin, to check the uniformity of the RNA concentrations for each sample.

ix) Analysis of the Proportion of Cells in HL60 Cell Cultures That Express F10 RNA

Only 50-60% of an HL60 cell culture terminally differentiate with RA treatment, however, these cells appeared to contain approximately twice the amount of F10 RNA that was detected in DMSO treated HL60 cells. DMSO induction results in almost 90% of the cells displaying terminally differentiated cell characteristics. Therefore, it was of interest to determine what proportion of the HL60 cells undergoing RA induction actually expressed F10 RNA. To discover if the increase in F10 RNA occurred in all the cells exposed to the RA induction stimulus, the technique of in situ hybridisation was used. The results from this analysis are shown in Figure 39. When uninduced HL60 cells were hybridised to ^{35}S labelled F10 cDNA, virtually no hybridisation signal was detected. (Figure 39 (A)). However, when 5 day RA induced cells were examined, the F10 probe hybridised strongly

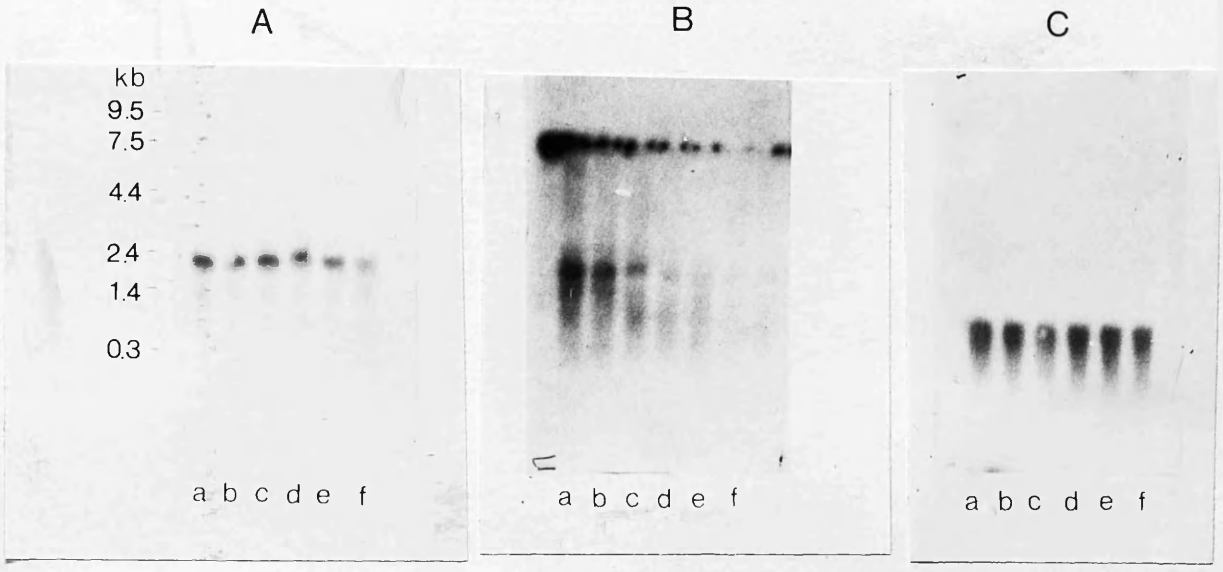


Figure 38 (a) Determination of the half-life of F10 RNA.

30 μg of total cellular RNA from uninduced HL60 cells treated with actinomycin D for periods of time were fractionated by electrophoresis on a denaturing, agarose gel. The RNA was then transferred on to nitrocellulose membrane by blotting. The nitrocellulose membrane was subsequently hybridised with ^{32}P-labelled probes for (A), F10 sequences; (B), human c-myc (pmc41-3RC); (C), B_2-microglobulin. a, 0 min; b, 15 min; c, 30 min; d, 60 min; e, 90 min; f, 120 min of actinomycin D treatment.

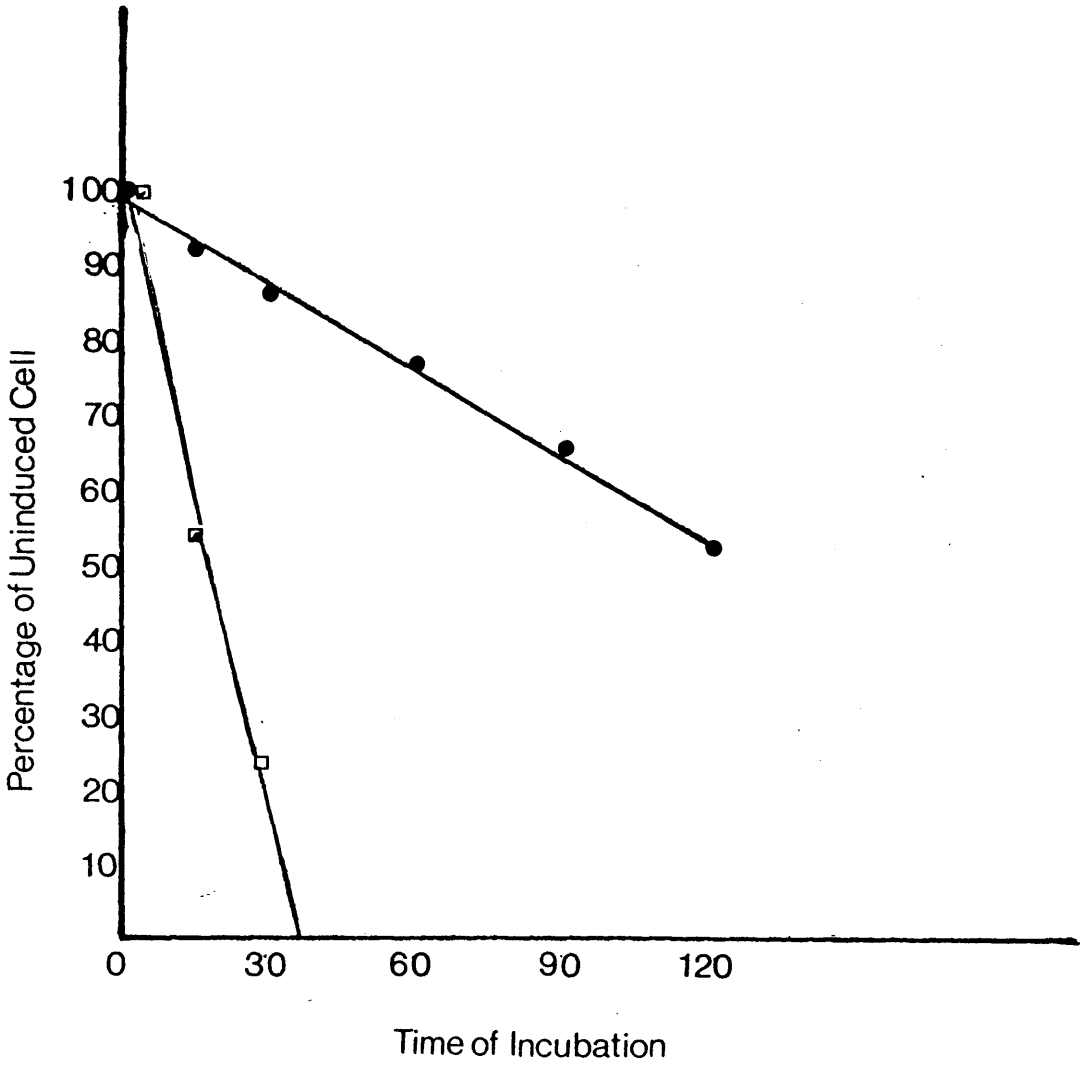


Figure 38 (b) Graph to determine the half-life of F10 RNA.

Results from the autoradiographs in Figure 38 (a) were scanned on a densitometer to quantitate the abundance of F10 transcripts during the period of treatment with actinomycin D. (●), F10 RNA; (□), c-myc RNA.

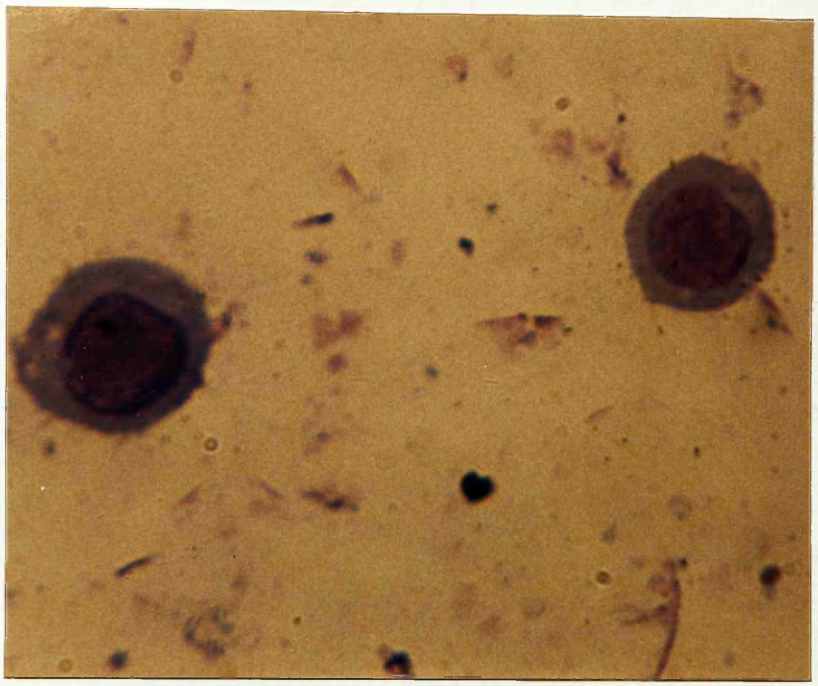
to a large proportion of the cells (Figure 39 (B)). Therefore, it appeared that F10 RNA transcription was increased in almost all RA induced HL60 cells. Following in situ hybridisation, the cells had been stained with May-Grunwald and Giemsa stains to enable viewing by light microscopy. This also enabled the cell morphology to be determined. When a comparison was made between mature cell morphology and hybridisation of ^{35}S-labelled F10 cDNA to individual cells it appeared that those cells displaying a morphology attributed to banded and segmented neutrophils, terminally differentiated myeloid cells, did not hybridise efficiently to F10 and indeed in a few examples no hybridisation was detected at all in these cells. Interestingly, only a small proportion (35%) of 5 day DMSO induced HL60 cells hybridised to ^{35}S-labelled F10 cDNA despite the fact that almost 90% of the DMSO induced HL60 cells appeared terminally differentiated. These results are summarised in Table 7.

x) Investigation of the Effects of RA Treatment on F10 RNA Expression in HL60 Cells

It has been reported that retinoids exert a hormone-like control on cell proliferation or cell differentiation (Roberts and Sporn, 1984). This could have had considerable bearing on the results obtained from analysis of F10 RNA levels throughout HL60 differentiation and from those obtained from in situ hybridisation analyses of uninduced, RA induced and DMSO induced HL60 cells. From examination of a number of granulocytic differentiation markers, it was found that by day 5 of an HL60 RA induction only 50-60% of the cell population had appeared to terminally differentiate to mature myeloid cells. However, at a comparable time during DMSO induction, over 90% of the cells were found to express mature granulocytic characteristics (Figures 16 and 17). Results from previous Northern analyses (Figure 35), suggested that F10 RNA was a mature myeloid cell-specific transcript. However, the fact that RA treatment, leading to the highest levels of this transcript, also resulted in the smallest number of terminally differentiated cells, contradicted this conclusion. Therefore, the F10 sequence in the HL60 genome, may not have responded to RA stimulation in a "differentiation" capacity but could have responded to a "vitamin / hormonal " effect of the retinoid on the cells.

To try and determine which effect resulted in the increase in F10

A



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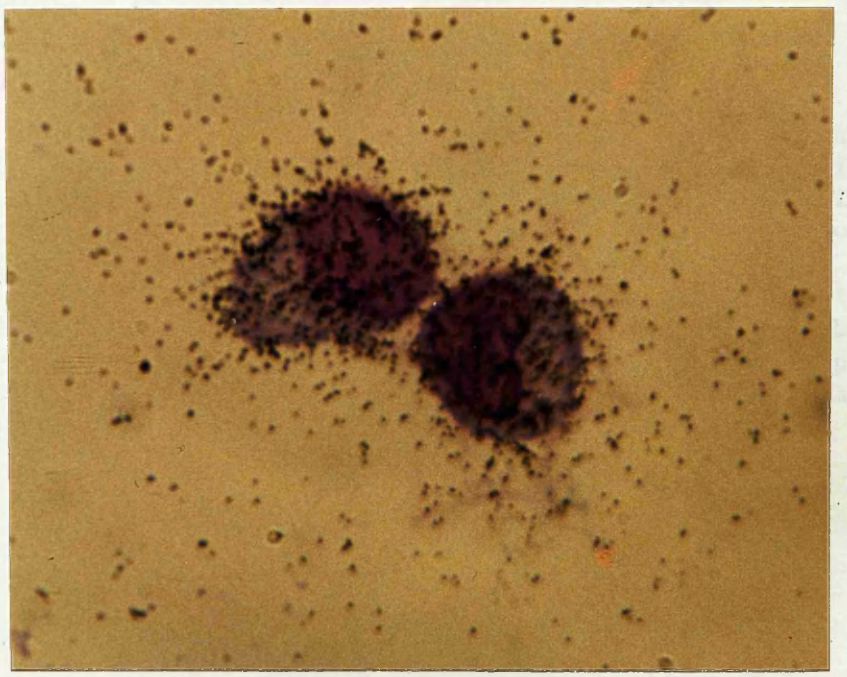


Figure 39 Uninduced and 5 day RA induced HL60 cells following in situ hybridisation with ^{35}S-labelled F10 cDNA.

2 x 10^5 cells were sampled from uninduced and 5 day RA induced HL60 cell cultures. The cells were cytocentrifuged on to glass slides and hybridised to ^{35}S-labelled probe for F10 RNA. Following hybridisation the slides were coated in emulsion; after 5-7 days exposure the slides were fixed and the cells stained with May-Grunwald and Giemsa stains so that the cells could be viewed by light microscopy. (A) Represents uninduced HL60 cells and (B) 5 day RA induced HL60 cells hybridised to ^{35}S-labelled F10 cDNA.

HL60 INDUCTION

TIME OF INDUCTION	UNINDUCED	DMSO	RA	TPA
3 DAYS	0%	70%	40%	0%
5 DAYS	0%	35%	90%	0%

Table 7 Tabulation of the percentage of HL60 cells which hybridised to radioactively labelled F10 cDNA during in situ hybridisation.

2 x 10^5 cells were sampled from HL60 cell cultures and cytocentrifuged on to glass slides. These cells were then hybridised to ^{35}S-labelled F10 cDNA. The slides were then covered in emulsion. Following exposure the cells were fixed and stained with May-Grunwald and Giemsa stains so that the cells could be viewed by light microscopy. Hybridisation of F10 probe to the cells could be detected by black dots on the cell indicating exposure of the emulsion by the isotope. 200 cells were scored for hybridisation. This was repeated three times and the average of the three counts taken as the percentage of HL60 cells containing F10 RNA.

RNA, in the HL60 cell system, another cell line capable of induced differentiation was used, U937. U937 is a human, histiocytic lymphoma cell line with monoblast-like characteristics (Sunstrom and Nilsson, 1976). These cells differentiate into morphologically mature macrophage-like cells after treatment with a number of chemicals, including RA (Olsson and Breitman, 1982). U937 cells were chosen because RA treatment induces differentiation to monocytic cells, not granulocytes as is the case in the HL60 model system. F10 RNA was almost undetectable in monocytic cells produced following 3 days of TPA treatment of HL60 cells. Therefore, if the increase in F10 RNA, in RA induced HL60 cells, was due to RA acting in a "hormonal" fashion only, these transcripts would also increase in the U937 cells incubated with RA, irrespective of the differentiation end point of the cells.

Total RNA was prepared from uninduced U937 cells and cells following 3 days and 5 days treatment with RA. The RNA was used to prepare Northern blots which were then hybridised to ^{32}P-labelled F10 cDNA. The results of this analysis are shown in Figure 40. The level of F10 RNA did not increase with increasing time of exposure of U937 cells to RA. Densitometric scanning of the autoradiographs revealed that the abundance of the F10 transcripts actually decreased 3 fold during 5 days of RA treatment. At this time the U937 cells had begun to show some of the morphological characteristics attributed to monocytes; the cells had begun to clump together but did not adhere to the tissue culture flask surface, as reported by others, (Olsson and Breitman, 1982). It can therefore be concluded that the increase in F10 RNA levels observed during RA induction of HL60 cells was a consequence of the differentiation stimulation of this agent and not due to a "hormonal" effect.

The Northern blot of U937 total cellular RNA was stripped and rehybridised to ^{32}P-labelled B_2-microglobulin cDNA to determine that the concentration of the RNAs used during this analysis was uniform. The results of this analysis are shown in Figure 40.

xi) The F10 cDNA Sequence

The sequence of F10 was determined using the Sanger "dideoxy" sequencing method (Chapter II, 2, (S)). The sequence data is given in

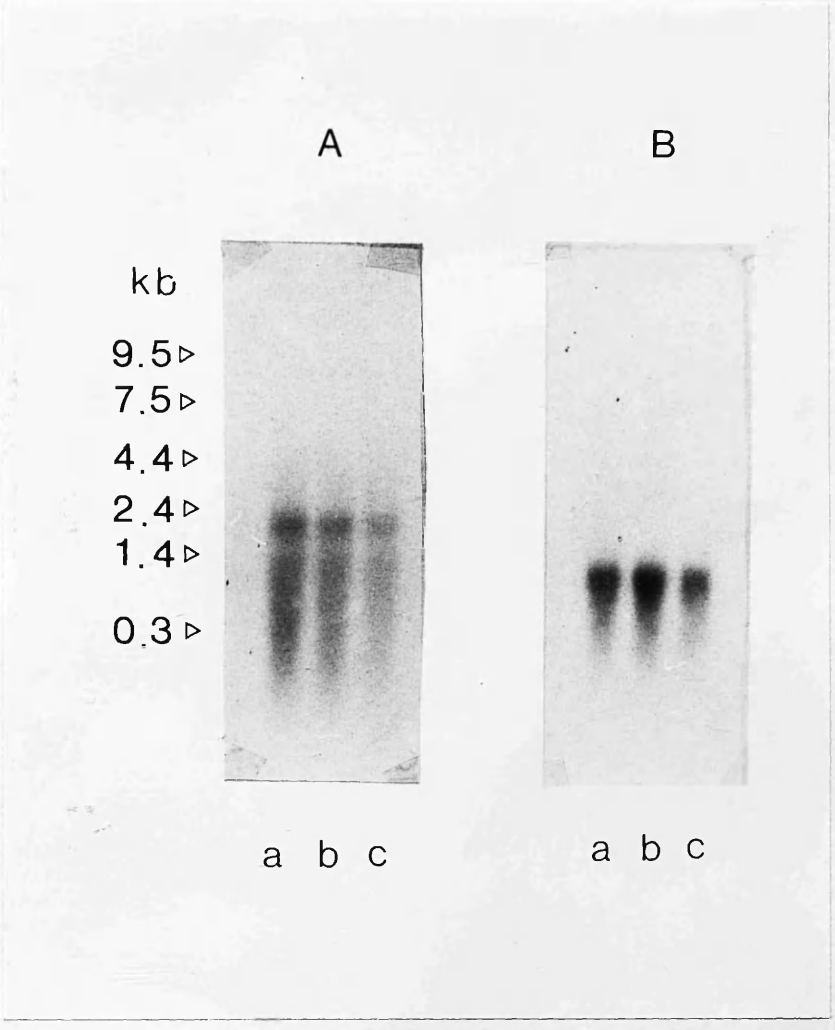


Figure 40 Determination of the presence of F10 RNA in U937 cells by Northern blotting.

30 μg of total cellular RNA isolated from uninduced, 3day TPA induced and 5 day RA induced U937 cells were fractionated by electrophoresis in a denaturing, agarose gel. The RNA was transferred on to nitrocellulose membrane by blotting and then hybridised with radioactively labelled probes for (A), F10 RNA and (B), B_2-microglobulin. a) uninduced U937; b) 3 day RA induced U937; c) 5 day induced U937.

F10

5'

GGACCTAGGC CCAGCCCCTA GCCTGATTGG AGCCCCTGAA GACAGTCCCA
End End End

CCCACTTTAT GGCCTATTGA CATGATCGAT CCTGCTGTGT GTCCTGTTCA
End

TGTTCGGTTC ATCAAATAAT TTGGAAAAAA.....
End

3'

Figure 41 Diagram of the F10 cDNA sequence.

The sequence of F10 cDNA was determined and compared to sequences stored in the Genbank Data System. The underlined sequences represent stop codons.

Figure 41. F10 did not share any significant homology to any of the sequences in the Genebank Data System. The cDNA therefore represents an as yet unknown gene. The non-polyadenylated region has a considerable number of stop codons which indicates that the sequence represented by F10 may be a 3' non-coding region.

C) INVESTIGATION AND CHARACTERISATION OF C6 cDNA

From initial results obtained from the screening of the RA5 library, C6 appeared to represent a sequence which was expressed in high abundance in 5 day RA induced HL60 cells. To confirm this and to investigate the RNA homologous to C6 cDNA further analyses were carried out.

i) Southern Blot Analysis

To determine the sizes of restriction fragments homologous to C6 in HL60 DNA, total cellular DNA from HL60 cells was restriction digested with Eco RI, Bam HI and Hind III. The DNAs were fractionated on a 1% agarose gel which was used to prepare a Southern blot. This was subsequently hybridised to radioactively labelled C6 cDNA. The results from this analysis are shown in Figure 42. During the early stages of analysis of C6 cDNA, all 3 fragments generated from an Eco RI + Bam HI digest of the recombinant plasmid were pooled and used as a template for a single hybridisation probe. Figure 44 (A) depicts a restriction digest of pUC8 which clearly shows these multiple fragments. From the restriction digest pattern obtained from Southern blot analysis it was apparent that C6 represented a unique sequence. The sizes of the restriction fragments homologous to C6 are summarised in Table 8.

ii) Northern Blot Analysis

To discover the size of RNA homologous to C6 a Northern blot was prepared from total cellular RNA isolated from uninduced, 3 day TPA induced, 5 day DMSO induced and RA induced HL60 cells. The blot was then hybridised to ^{32}P-labelled C6 cDNA. Figure 43 shows the results obtained from this analysis. Two clearly distinguishable RNA transcripts hybridised to C6 of 1.3 kb and 2.4 kb respectively. From

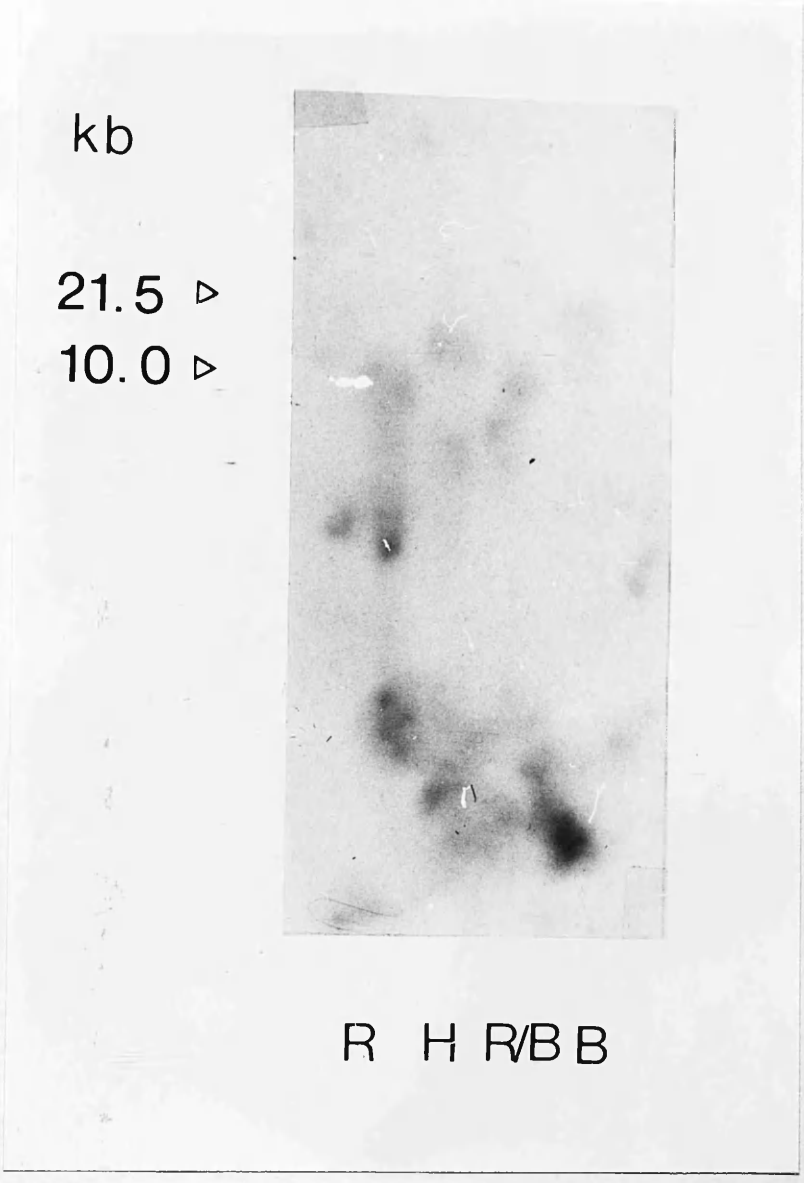


Figure 42 Southern analysis of HL60 DNA hybridised to ^{32}P-labelled C6 cDNA.

30 µg of total cellular DNA isolated from HL60 cells were digested with Eco RI, Bam HI, Hind III and Eco RI + Bam HI. The DNAs were fractionated by electrophoresis, transferred to nitrocellulose membrane and this was hybridised to radioactively labelled probe for C6 sequences. (B), Bam HI, (H), Hind III; (R), Eco RI; (R/B), Eco RI + Bam HI.

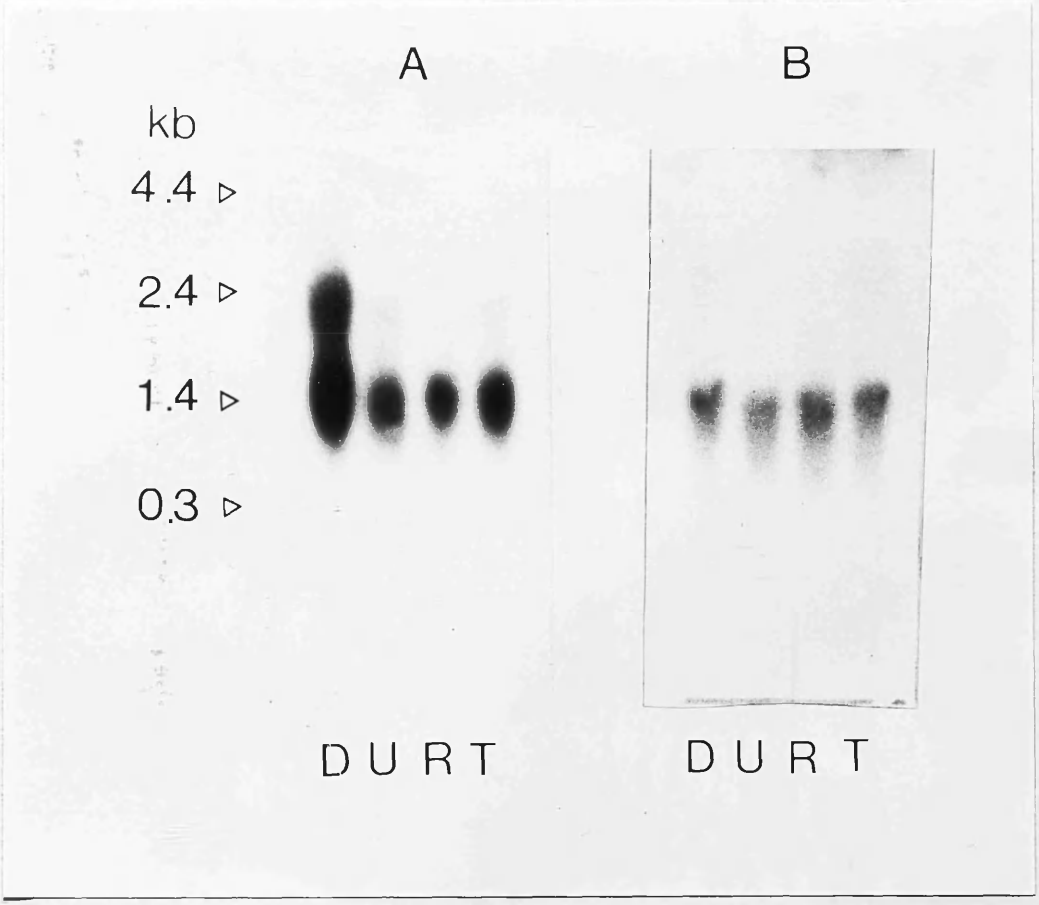


Figure 43 Determination of the size of RNA homologous to C6 by Northern blotting.

30 ug of total RNA isolated from uninduced, 5 day RA induced, 5 day DMSO induced and 3 day TPA induced HL60 cells were fractionated by electrophoresis on a denaturing, agarose gel. The RNA was then transferred on to nitrocellulose membrane and subsequently hybridised to ^{32}P-labelled probes for (A), C6 sequences and (B), B_2-microglobulin. (D), DMSO induced HL60; (U), uninduced HL60; (R), RA induced HL60; (T), TPA induced HL60.

this initial analysis it appeared that both transcripts were highly abundant in HL60 cells induced down the granulocytic pathway.

To ensure that a similar concentration of total RNA was assayed from each sample the blot was stripped and rehybridised to radioactively labelled B_2-microglobulin. The result of this experiment, displayed in Figure 43 confirmed this.

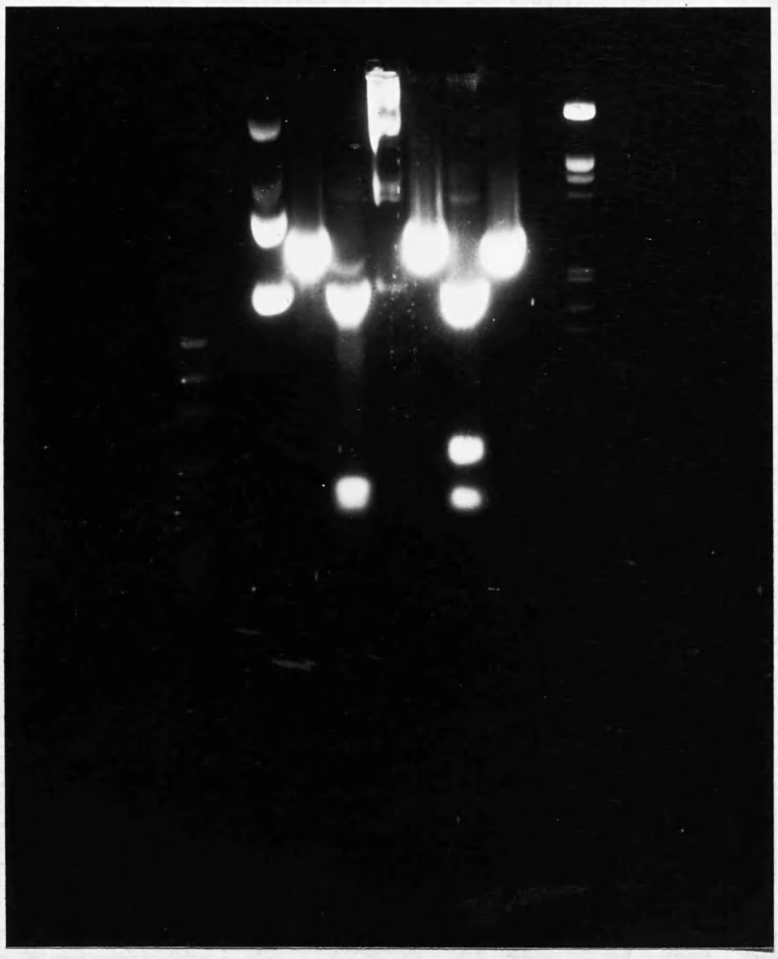
iii) Further Investigation of the Recombinant Plasmid pUC8C6

The initial Northern blot analysis of total HL60 RNA, hybridised to ^{32}P-labelled C6 cDNA, resulted in the resolution of two RNAs of distinct sizes (Figure 43). However, the template for the hybridisation probe used in the initial experiments had consisted of 3 fragments, generated from Eco RI + Bam HI digestion of pUC8C6. This restriction digest should have resulted in the isolation of the C6 cDNA, however, fragments of 220 bp, 190 bp and 160 bp were produced. The initial digestion analysis of pUC8C6 had not revealed any evidence to suggest that it was a conjugate plasmid. However the results from Northern blotting contradicted this conclusion. Therefore further restriction digestion analyses were carried out on the recombinant plasmid pUC8C6 DNA to try and clarify the situation.

a) Analysis of pUC8C6 by Hae II digestion

pUC8 contains three Hae II restriction sites (Figure 21) which give rise to three fragments of 1870 bp, 370 bp and 430 bp, on digestion of plasmid DNA with Hae II. However, on ligation of a cDNA into the multi-cloning site, Hae II digestion of the recombinant pUC8 plasmid gives a 1870 bp and a 370 bp fragment, as previously, but the 430 bp fragment, which contains the multi-cloning site, increases in size in direct proportion to the ligated cDNA. To investigate the construct of pUC8C6 further, plasmid DNA was restriction digested with Hae II and the DNA fractionated on a 1.5% agarose gel. The results from this experiment are displayed in Figure 44(B). Hae II digestion of pUC8C6 revealed the 1870 bp and the 370 bp (plasmid) fragments, however the 430 bp fragment was replaced by what appeared to be a doublet of 800 - 740 bp. This was indicative of :-

B



A

Figure 44 (A) pUC8C6 DNA digested with Eco RI + Bam HI.

3 µg of pUC8C6 DNA were restriction digested with Eco RI + Bam HI and fractionated on a 1.5% (w/v) agarose gel. Ø X174 DNA digested with Hae III was used as DNA size markers. The gel was then stained with ethidium bromide and the DNA visualised by exposure to UV light.

(B) Hae II digestion of pUC8C6 DNA.

3 µg of pUC8 DNA or pUC8C6 DNA were digested with Hae II or Eco RI and fractionated on a 1.0% (w/v) agarose gel. The gel was then stained with ethidium bromide and the DNA visualised by exposure to UV light. Ø X174 DNA digested with Hae II was used as DNA size markers. Lane 1, pUC8; Lane 2, pUC8 digested with Eco RI; Lane 3, pUC8 digested with Hae II; Lane 4, pUC8C6; Lane 5, pUC8C6 digested with Eco RI; Lane 6, pUC8C6 digested with Hae II; Lane 7, pUC8C6 digested with Eco RI + Bam HI.

1) two cDNA inserts, and therefore a conjugate plasmid, or

2) a large cDNA with internal Hae II restriction sites.

It was unlikely that the multiple fragments obtained following Eco RI + Bam HI digestion of pUC8C6 were the result of a contaminating plasmid. pUC8C6 had been subjected to two colony purification procedures to ensure that only one recombinant plasmid was isolated prior to preparation of plasmid DNA.

b) Examination of pUC8C6 by electron microscopy

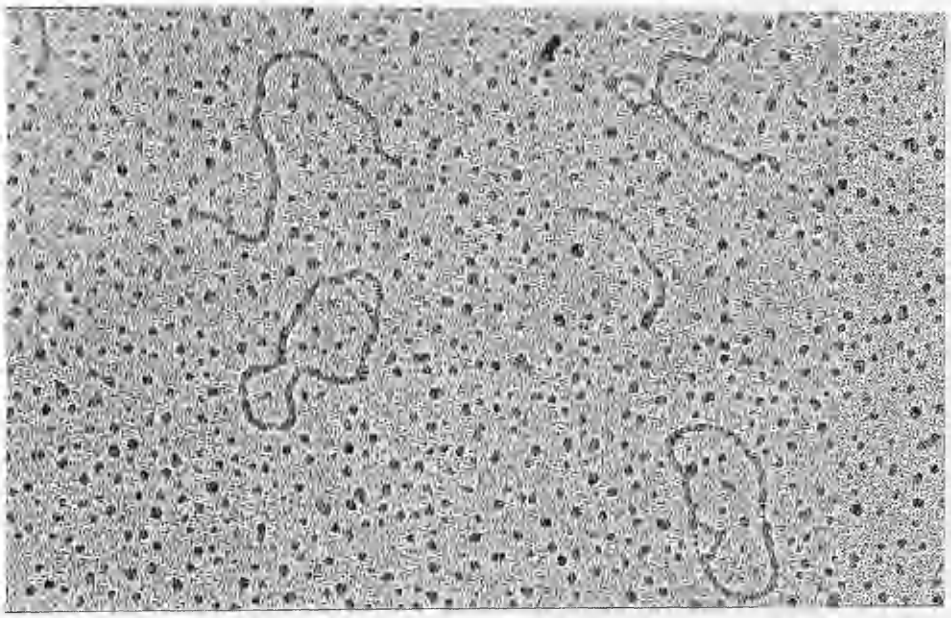
From the results of the previous experiment no conclusion could be reached about the construction of the recombinant plasmid pUC8C6. To examine the size(s) of the C6 cDNA it was decided to view the plasmid DNA directly by electron microscopy. Initial analysis of the plasmid DNA, in closed-circle form, did not reveal any unusually sized plasmid, as might have been expected if pUC8C6 had been conjugate. However, it was difficult to calculate sizes because of the supercoiled state of the DNA. Therefore all further analysis was carried out on pUC8C6 which had been linearised by Sac I digestion. Homoduplex analysis, of Sac I digested pUC8C6 hybridised to self, did not reveal any unusual plasmid fragments. However, heteroduplex analysis, pUC8 hybridised to pUC8C6, revealed small loops or eyes, regions of non-homology between the plasmids. These were measured and the size calculated to be approximately 200 bp. No evidence was discovered to indicate that pUC8C6 was conjugate. The results of this experiment therefore suggested that C6 cDNA was of approximately 200 bp long. Figure 45 gives the results of the analysis of pUC8 DNA by electron microscopy.

iv) Analysis of the Individual cDNA Fragments Generated Following Digestion of pUC8C6 by Eco RI and Bam HI

a) Northern Blot Analysis

The experiments carried out to examine the construction of pUC8C6 had been inconclusive so it was decided to isolate each of the fragments generated from the Eco RI + Bam HI digestion of pUC8C6 and use each one as a hybridisation probe. It was hoped that this would result in

A



B

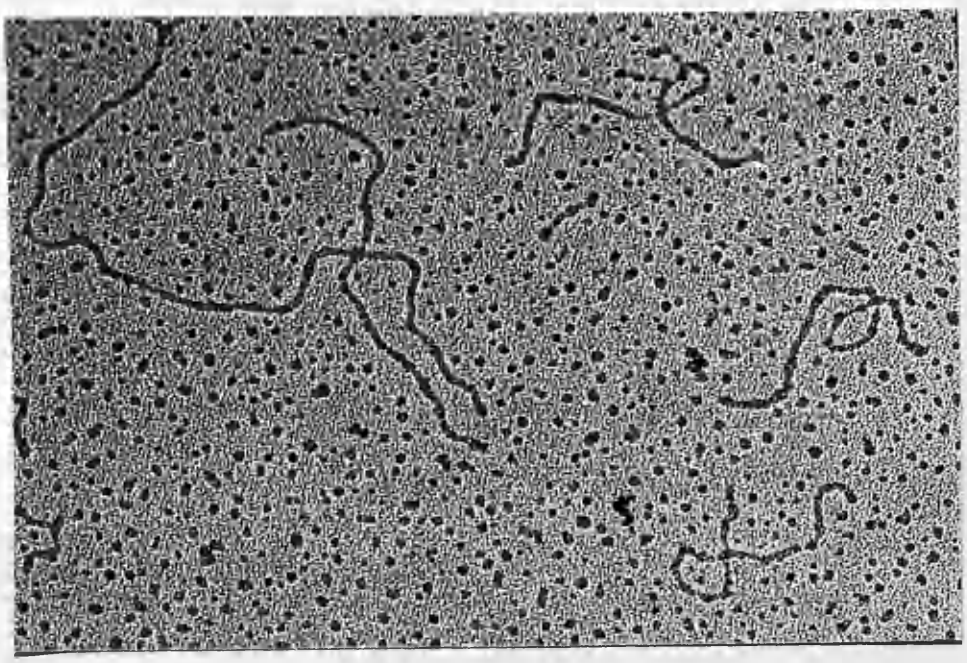


Figure 45 Analysis of pUC8C6 DNA by electron microscopy.

pUC8 DNA and pUC8C6 DNA were restriction digested with Sac I then hybridised together to determine the regions of homology between the two plasmids. (A) represents pUC8 / pUC8C6 heteroduplexes.
(B) represents pUC8C6 / pUC8C6 homoduplexes.

the identification of which cDNA fragment was homologous to which RNA (either the 2.4 kb or the 1.3 kb species). The expression of each of these RNAs could then be analysed separately. This, of course, assumed that pUC8C6 was conjugate, containing at least two different cDNAs of almost identical size.

The fragments were isolated by fractionation of restriction digested pUC8C6 DNA on a 2% low-melting point agarose gel. The cDNAs, once purified, were used as probes to hybridise to Northern blots prepared from total cellular RNA from HL60 cells. The results from this experiment are given in Figure 46. All 3 fragments hybridised to two RNAs of 2.4 kb and 1.3 kb. The RNAs also appeared more abundant in RA and DMSO treated cells, in an identical fashion to initial analyses using the pooled fragments as a single hybridisation probe. Therefore, it appeared that the C6 cDNA fragments corresponded to exactly the same sequence, or regions of the same sequence.

b) Examination of the Role of DNA Secondary Structure in Determining the Size of C6 CDNA

If the assumption that all three C6 cDNA fragments are similar, was true, then the difference in size, as seen during fractionation by electrophoresis, could have been the result of some form of secondary structure which was present in the cDNA. This may have resulted in some C6 fragments maintaining a constrained configuration, following digestion, whilst others would take on a more relaxed, open structure. This would lead to the DNA running at apparently different sizes during electrophoresis.

To determine if this were true, two experiments were carried out. Firstly, prior to electrophoresis, Eco RI + Bam HI restriction digested pUC8C6 DNA was heated to 90°C for 10 min which should denature the DNA (Figure 47 (A)). However, when the DNA was electrophoresed on a 2% agarose gel, then stained with ethidium bromide to visualise the DNA fragments, the same pattern of C6 cDNA fragments was found in both heated and unheated DNA samples. Secondly, digested pUC8C6 DNA was electrophoresed on a denaturing, agarose gel, (55mM NaOH). Following staining of this gel with ethidium bromide, it was found that the pattern of digested C6 fragments had not altered (Figure 47 (B)). These results dismissed

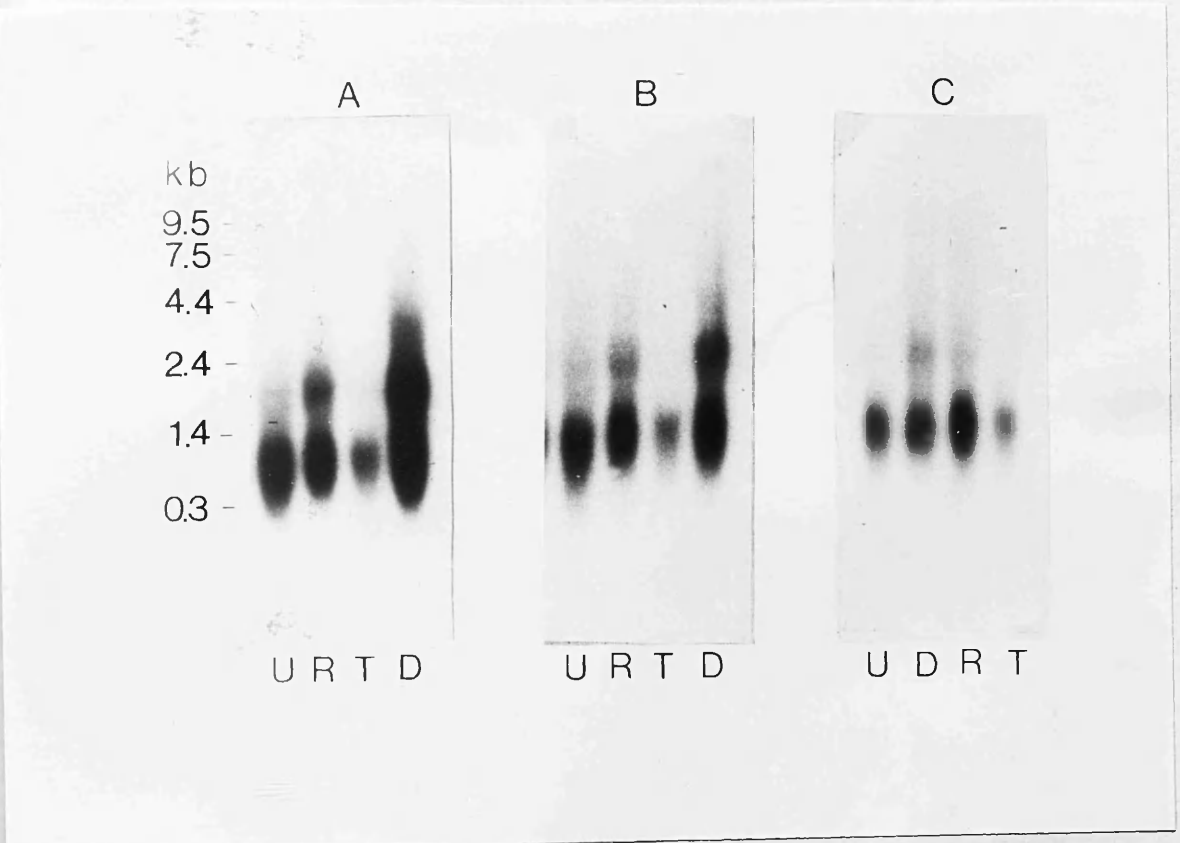


Figure 46 Northern blot analysis of C6 transcripts following differentiation of HL60 cells, using each of the C6 cDNAs as a hybridisation probe.

30 µg of total RNA isolated from uninduced, 5 day RA induced, 5 day DMSO induced and 3 day TPA induced HL60 cells were fractionated by electrophoresis in a denaturing, agarose gel. The RNA was then transferred by Northern blotting on to a nitrocellulose filter which was subsequently hybridised with radioactively labelled probes produced from (A) 220 bp C6 cDNA fragment; (B) 190 bp C6 cDNA fragment; (C), 160 bp C6 cDNA fragment. (U), uninduced HL60; (R) 5 day RA induced HL60; (D), 5 day DMSO induced HL60; (T), 3 day TPA induced HL60.

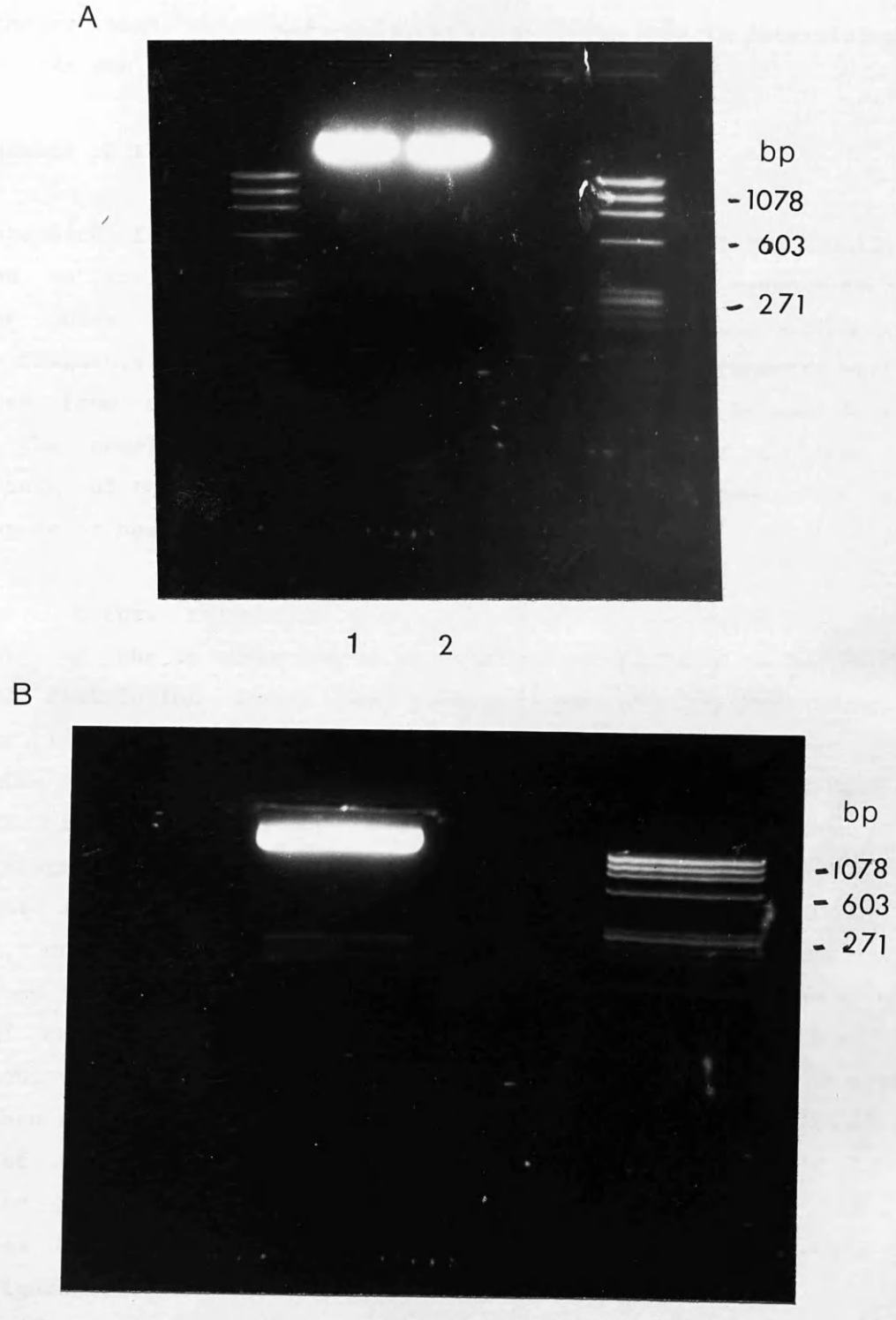


Figure 47 Examination of secondary structure in C6 cDNA.

(A) 3 μg of pUC8C6 DNA were digested with Eco RI + Bam HI. Prior to fractionation in a 1.5% (w/v) agarose gel the plasmid DNA sample was heated at 90°C for 10 min to destroy any DNA secondary structure. Following electrophoresis the gel was stained with ethidium bromide and the DNA visualised by exposure to UV light. Lane 1, pUC8C6 digested DNA; Lane 2, boiled pUC8C6 digested DNA.

(B) 3 μg of Eco RI + Bam HI restriction digested pUC8C6 DNA was fractionated by electrophoresis in a denaturing (55mM NaOH) 1.5% (w/v) agarose gel. This should destroy any DNA secondary structure. Following electrophoresis the gel was stained with ethidium bromide and the DNA visualised by UV light.

the theory that secondary structure played any role in determining how C6 cDNA ran during electrophoresis.

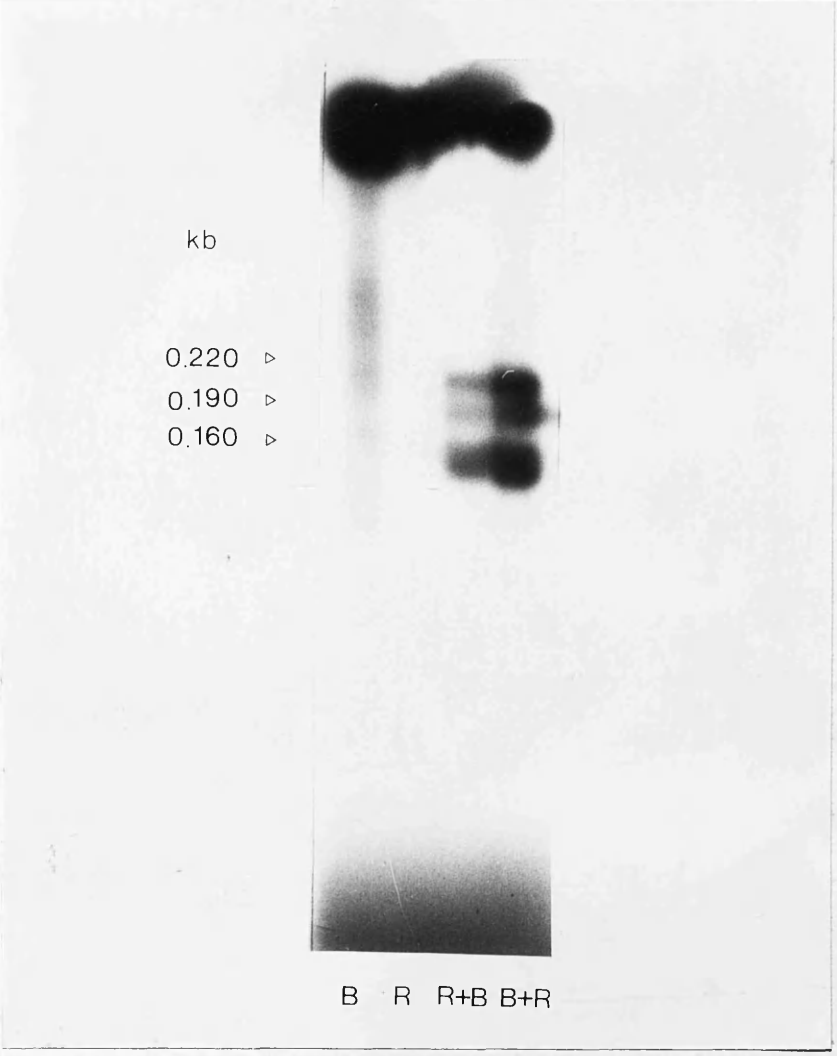
v) Sequence of the Three C6 cDNA Fragments

To determine if the 3 C6 cDNA fragments were similar, it was finally decided to reclone each one into bacteriophage M13 and sequence each of the cDNAs. This procedure would eliminate any contamination by other fragments (this could have happened when the fragments were isolated from agarose) as only one cell clone would be used from which the template would be prepared for the sequencing reaction. A comparison of the sequences obtained would give a clear indication of the degree of homology between the cDNAs.

However, before religation into M13 could be accomplished the termini of the C6 cDNAs had to be established. Although an Eco RI + Bam HI restriction digest had produced the C6 fragments it was unclear if they possessed an Eco RI-end and a Bam HI-end. To determine this, the technique of end-labelling was used. Following an Eco RI or a Bam HI restriction digest of pUC8C6 DNA, the terminii of each fragment produced was labelled with ^{32}P-dCTP, using the Klenow fragment of E.coli DNA polymerase I. The reaction mixes were then halved, one half was retained while the DNA in the remainder was subjected to a further digestion, which would result in a Eco RI + Bam HI cut fragment. Following this, all the DNA samples were fractionated by electrophoresis on a 3.5% polyacrylamide gel. The gel was then autoradiographed for short periods of time. By comparing the size of the fragments generated by Eco RI + Bam HI and the single digested DNA samples it was determined that all 3 C6 cDNAs had Eco RI and Bam HI termini and could therefore be successfully ligated into M13 (Figure 48).

Using the Sanger 'dideoxy' sequencing method , all 3 C6 cDNA fragments were sequenced. The results are given in Figure 48. Only 85 bp of unique sequence existed, this sequence was found in all of the C6 fragments. The remainder of each cDNA consisted of thymidine residues. Interestingly the only difference determined between each of the cDNAs was the number of thymidine residues, this varied by 30-40 nucleotides between the different fragments. It was therefore concluded that the C6 cDNAs represented the same sequence. No

A



B C6

5′

TTCCTGACAC AGTGAAACAC CTTCCTCAAC CCAGGCGGGT GGACAGGGTC

CCCTGTGGTC CAGCAGTAAA AACCATGGTC CCCCCAAAAA.....

3′

Figure 48 Sequences of the C6 cDNA fragments.

(A) Autoradiograph from end-labelling analysis of pUC8C6 DNA Eco RI + Bam HI restriction digested the labelled with ^{32}P-dCTP to determine the terminii of the separate fragments. Lane 1, Bam HI digested pUC8C6 DNA; Lane 2, Eco RI digested pUC8C6 DNA; Lane 3, Eco RI followed by Bam HI digested pUC8C6 DNA; Lane 4, Bam HI followed by Eco RI digested pUC8C6 DNA.

(B) The 85 bp sequence common to all 3 C6 cDNA fragments is illustrated. The Poly(A) sequence has been omitted for convenience.

homology was found between the 85 bp region of sequence common to all the cDNAs and those stored in the Genebank Data system, available in this laboratory. The C6 cDNAs therefore appear to represent the 3' non-coding region of an as yet unidentified sequence.

vi) Quantitative Analysis of C6 RNA Abundance Following HL60 Differentiation

Having established that all 3 C6 cDNA fragments were identical the analysis of the RNA species homologous to this sequence could be continued. The relative abundance of C6 RNA in HL60 cells was calculated by an RNA doubling dilution dot blot prepared from total cellular RNA from uninduced, 3 day TPA induced and 5 day RA induced HL60 cells. The hybridisation probe was synthesised using the 220 bp C6 cDNA fragment as a template. However it must be noted that the figures calculated from this analysis were the sum of both the 2.4 kb and the 1.3 kb transcripts. The results from this experiment are shown in Figure 49, and summarised in Table 8.

To determine that a similar concentration of RNA was present in each series of RNA dots, the dot blot was stripped and rehybridised to ^{32}P-labelled pHR28-1 (Figure 49).

Results from the RNA dot blot in Figure 49 indicated that C6 RNA was 8 fold more abundant in RA induced cells than in uninduced HL60 cells. RA induced HL60 cells also contained 4 times the amount of C6 RNA detected in TPA induced cells. However a number of different analyses were carried out on RNAs isolated from different inductions. A value was calculated for the abundance of C6 RNA in uninduced and induced cells by taking the average of the results from the different experiments. These are shown in Table 8.

vii) Analysis of HL60 Poly(A)$^+$ RNA

To determine if both the 2.4 kb and the 1.3 kb C6 RNAs were present in the poly(A)$^+$ RNA fraction of HL60 cells, poly(A)$^+$ RNA, isolated from uninduced, 3 day TPA induced and 5 day RA induced HL60 cells, were used to prepare a Northern blot which was subsequently hybridised to ^{32}P-labelled C6 cDNA (220 bp fragment). The results from this experiment are displayed in Figure 50. The 1.3 kb C6 RNA

cDNA C6		
Size of DNA Fragments (kb)	Hind III	15.0
	Eco RI	11.0
	Bam HI	> 21.5
	RI / BI	7.0
Size of RNA Transcript(s)	(kb)	2.4 and 1.3
Relative Abundance of Transcripts	Uni	1
	RA	8
	TPA	2
	DMSO	10

Table 8 Summary of the results of Northern and Southern blot analyses of C6 sequences in HL60 cells.

The values calculated for the relative abundance of C6 RNA are average values calculated from a number of different inductions. Densitometric scanning of Northern blots of total HL60 cellular RNA hybridised with radioactively labelled C6 cDNA were also used to determine the relative abundance of C6 RNA following induction of HL60 cells.

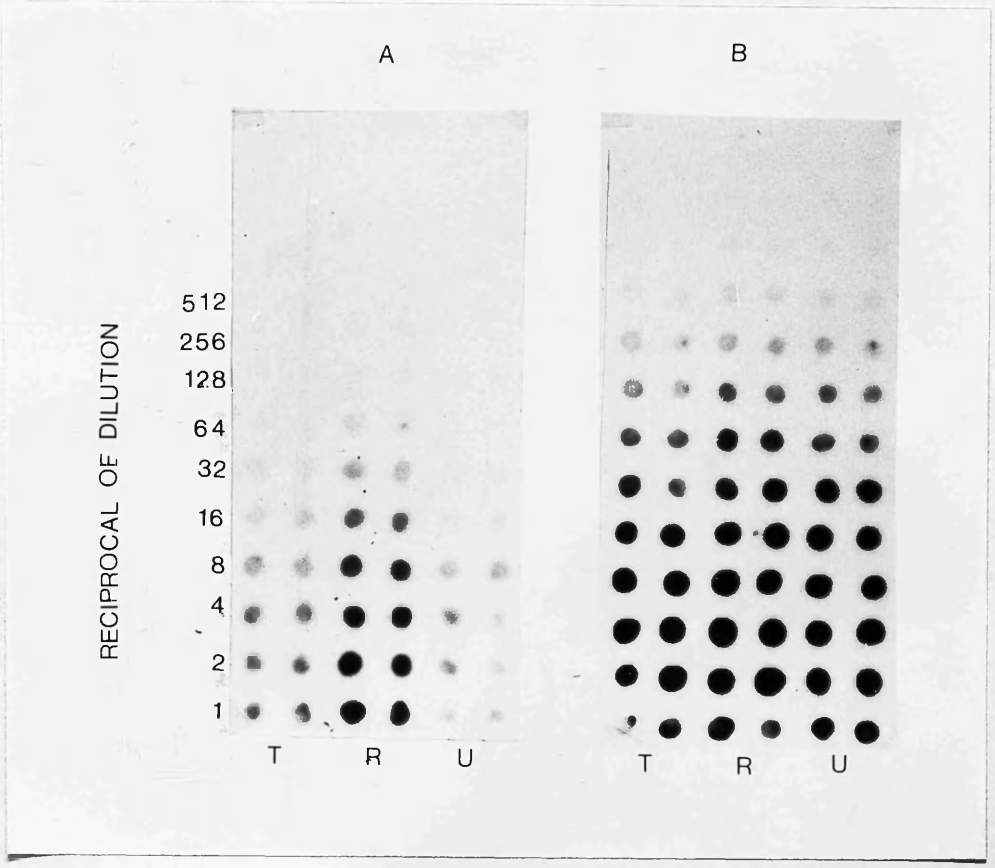


Figure 49 Determination of the abundance of C6 RNA in HL60 cells.

20 ug of total RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were dotted in a doubling dilution fashion on to nitrocellulose membrane. The nitrocellulose membrane was then hybridised with ^{32}P-labelled probes for (A), C6 sequences and (B), human 28S ribosomal RNA sequences. (U), uninduced HL60; (T), TPA induced HL60; (R), RA induced HL60.

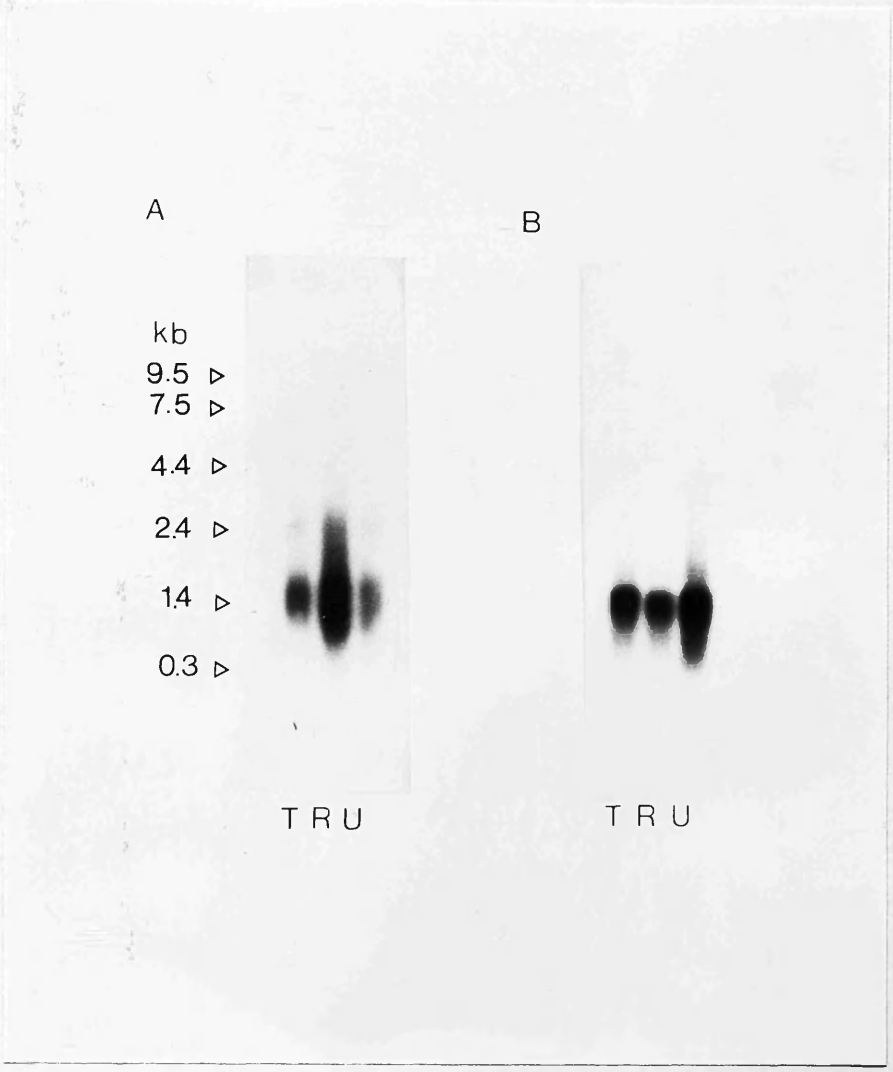


Figure 50 Determination of the presence of C6 transcripts in poly(A)$^+$ RNA isolated from HL60 cells.

5 µg of poly(A)$^+$ RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were fractionated by electrophoresis in a denaturing, agarose gel. The RNA was then transferred by blotting on to a nitrocellulose filter which was subsequently hybridised with radioactively labelled probes for (A), C6 sequences and (B), B_2-microglobulin. The lanes of the gel were (T), TPA induced HL60; (R), RA induced HL60; (U), uninduced HL60.

was found to be abundant in poly(A)$^+$ RNA isolated from 5 day RA induced HL60 cells, however very little hybridisation was detected in the TPA induced or the uninduced poly(A)$^+$ RNA samples. It is interesting to note that the 2.4 kb C6 transcript appeared less abundant in poly(A)$^+$ RNA isolated from HL60 cells induced to differentiate to granulocytes than was observed during analysis of total RNA.

To ensure that a similar concentration of poly(A)$^+$ RNA had been analysed from each sample the blot was stripped and rehybridised to radioactively labelled B_2-microglobulin. The results shown in Figure 50 confirm this.

viii) Examination of C6 Expression Throughout the Induced Differentiation of HL60 Cells to Granulocytes

The previous analyses of C6 RNA had established that following 5 days of RA or DMSO treatment of HL60 cells these transcripts had increased in abundance. To determine when this increase occurred during differentiation, Northern blots were prepared from total cellular RNA isolated from HL60 cells at time points throughout DMSO and RA induction. The Northern blots were then hybridised to ^{32}P-labelled C6 (220 bp) cDNA, however, similar results were obtained using the 190 bp and 160 bp C6 fragments as hybridisation probes. Figure 51 shows the results from these analyses.

The Northern blots from the experiments described above were stripped and rehybridised to ^{32}P-labelled B_2-microglobulin cDNA to confirm that each sample analysed was of a uniform RNA concentration (Figure 51).

To analyse the results quantitatively, the autoradiographs were scanned on a densitometer. The 1.3 kb and the 2.4 kb transcripts were scanned separately as they appeared to display different patterns of expression during HL60 differentiation. The results from the scanning analyses are displayed graphically in Figure 52.

a) DMSO Induction

The level of the 1.3 kb transcript in uninduced HL60 cells was low,

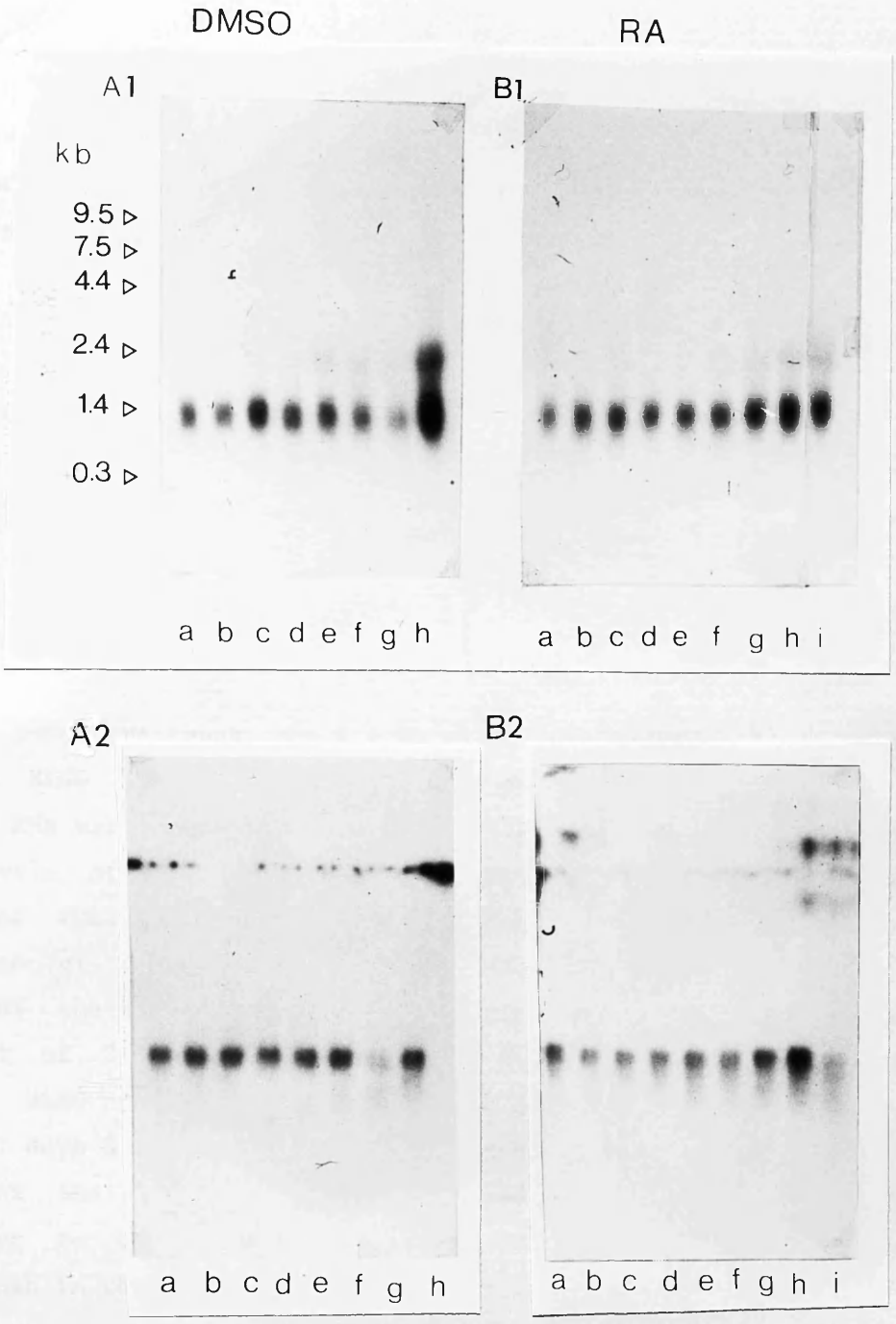


Figure 51 Northern blotting analysis of C6 transcripts during granulocytic differentiation of HL60 cells.

30 µg of total cellular RNA isolated from cells harvested at time points throughout the course of DMSO and RA induction of HL60 cells were fractionated by electrophoresis in a denaturing, agarose gel. The RNA was then blotted from the gel on to nitrocellulose membrane. The nitrocellulose membrane was hybridised with radioactively labelled probes for (A(1) and B(1)), C6 sequences and (A(2) and B(2)), B_2-microglobulin sequences. a) 0 h; b) 1 h; c) 2 h; d) 4 h; e) 8 h; f) 16 h; g) 24 h; h) 72 h; i) 120 h of treatment with inducer.

however the 2.4 kb RNA was almost undetectable. During the first 24 h of DMSO treatment both C6 RNA levels fluctuated, however the sharp decrease observed at 24 h appeared to be artifactual as was shown in the control blot probed with B_2-microglobulin cDNA (Figure 51 (b)). It appeared that the 24 h DMSO sample had less RNA present than the others that were analysed. Nevertheless, the general trend with both the 2.4 kb and the 1.3 kb transcripts was a dramatic increase in abundance following 24 h of DMSO treatment. A 3-fold increase was estimated for the level of the 1.3 kb transcript in 5 day DMSO induced HL60 cells compared to their uninduced counterparts. However the level of the 2.4 kb RNA increased 18-fold during DMSO induction of HL60 cells.

b) RA Induction

As in DMSO treatment, the 2.4 kb C6 transcript was undectable in RA induced HL60 cells till almost 16-24 h of induction, however the 1.3 kb RNA was present in uninduced cells. Again, as described above, the levels of the C6 transcripts were highly variable during early times of treatment but the general trend was a dramatic increase in abundance of C6 RNA by day 3 of RA induction. This increase was 2-3 fold for the 1.3 kb transcript, comparing uninduced HL60 cell levels to that of 3 day RA induced cells. However, unlike DMSO, 5 day RA induced HL60 cells displayed a decrease in the level of 1.3 kb C6 RNA between days 3-5. This was not mimicked by the larger transcript; the level of the 2.4 kb C6 RNA increased dramatically from 4 h of RA treatment to become 6 fold more abundant in the 5 day RA induced cells than in the uninduced HL60 cell (Figure 52). (See Note on page facing 19

From these experiments it could be concluded that the increase observed in the level of the 2.4 kb C6 RNA, during HL60 differentiation, was of a much greater magnitude than that of the 1.3 kb RNA. The increase in the levels of both transcripts, was also a relatively early event during induction, initiating between 4-24 h of treatment. Unlike the cDNA (F10) discussed previously, C6 cDNA hybridised to RNA species which were more abundant in DMSO induced HL60 cells. It should be noted that the abundance of C6 transcripts showed some degree of variation between inductions carried out at different times throughout this project. The discrepancies were small, probably due to slight variations in cell clones which were

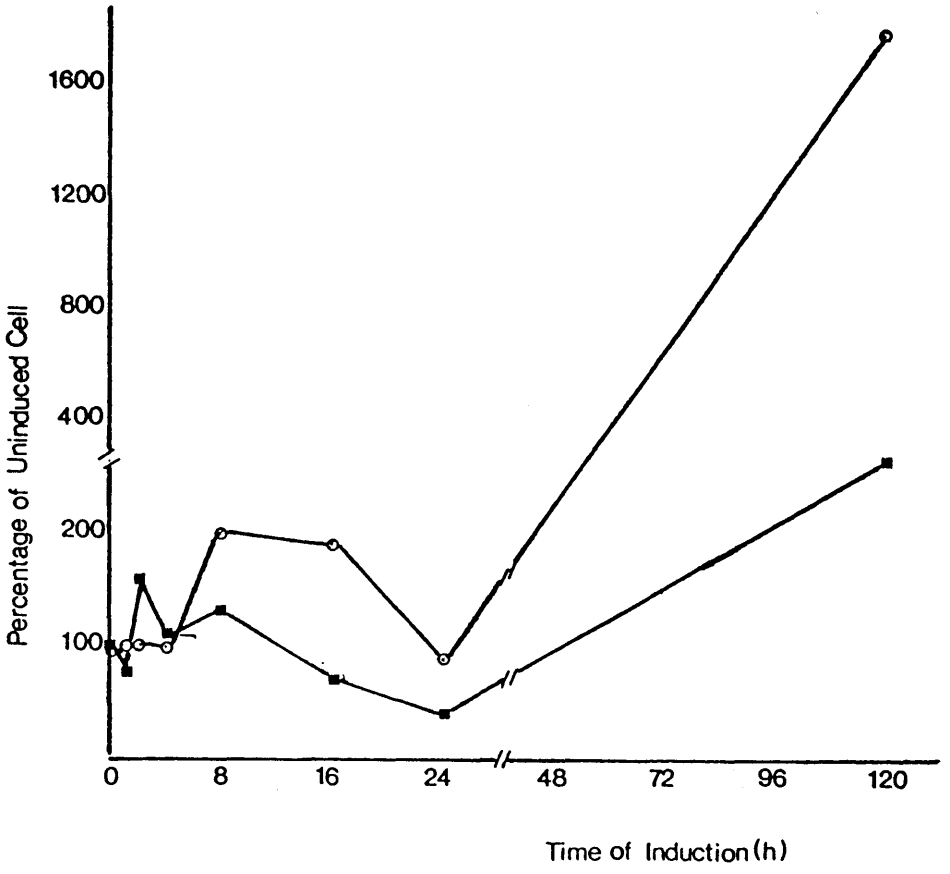
Figure 52 Graphical display of results obtained from densitometric scanning of the autoradiographs from the Northern blots in Figure 51.

(A) Graph of results obtained from densitometric scanning of autoradiographs from Northern blots of HL60 total RNA isolated from times throughout a 5 day DMSO induction period. (O), represents the 2.4 kb C6 RNA; (■), represents the 1.3 kb C6 RNA.

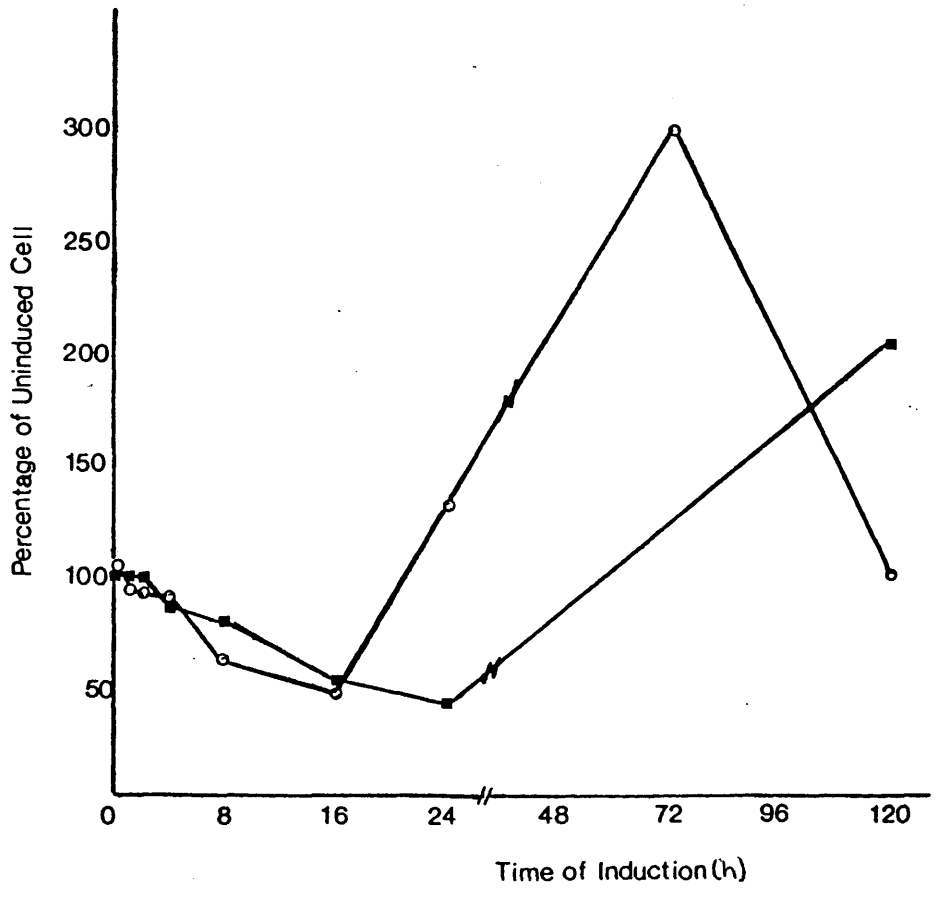
(B) Graph of results obtained from densitometric scanning of autoradiographs from Northern blots of HL60 total RNA isolated from times throughout a 5 day RA induction period. (O), represents the 2.4 kb C6 RNA; (■), represents the 1.3 kb C6 RNA.

Note : The results plotted on graphs (A) and (B) are average figures obtained from densitometric scanning of Northern blots of HL60 total RNA. The RNAs used during these analyses were prepared from different clones of cells and from different RA and DMSO inductions.

A


1600
1200
800
400
200
100
Percentage of Uninduced Cell
0
8
16
24
48
72
96
120
Time of Induction (h)

B


300
250
200
150
100
50
Percentage of Uninduced Cell
0
8
16
24
48
72
96
120
Time of Induction (h)

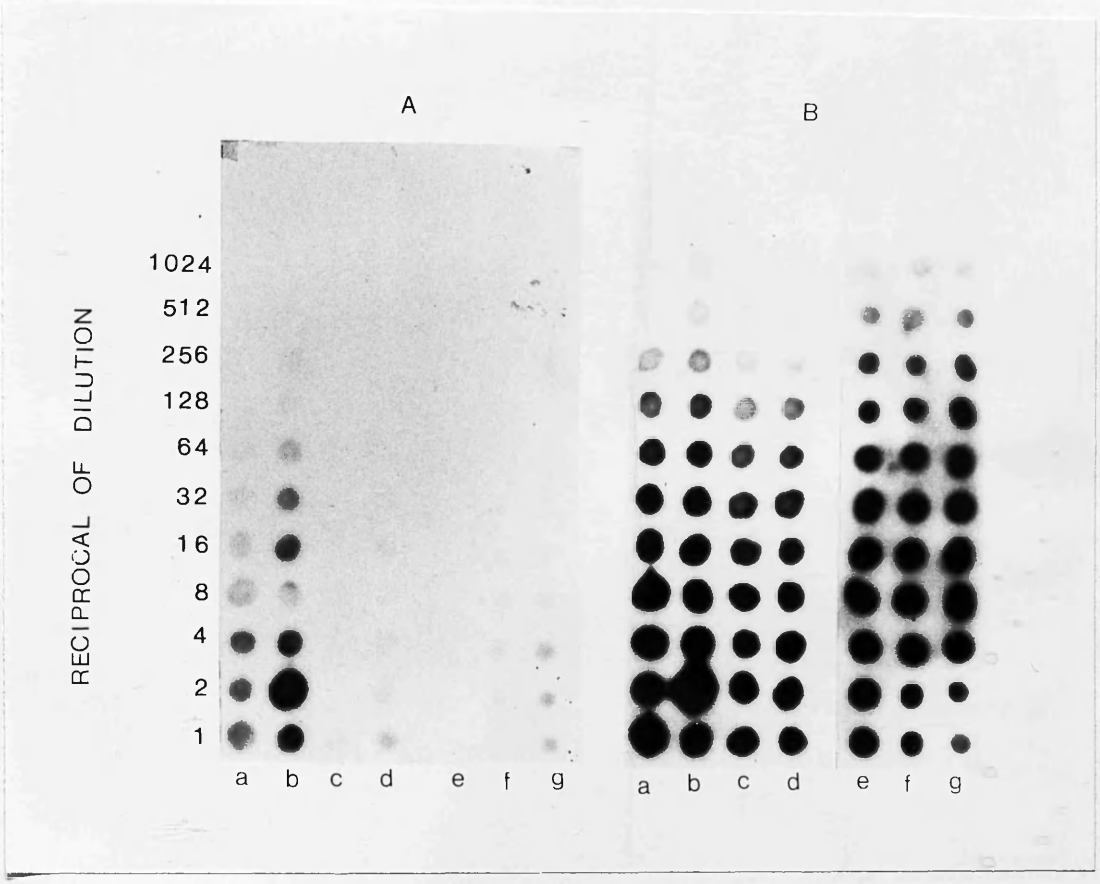


Figure 53 Determination of the range of human tissues that contain C6 RNA.

20 μg of total cellular RNA, isolated from a number of different human tissues, were dotted on to nitrocellulose membrane in a doubling dilution fashion. The nitrocellulose membrane was then hybridised with radioactively labelled probes for (A), C6 sequences and (B), human 28S ribosomal RNA sequences. (a), uninduced HL60; (b), 5 day RA induced HL60; (c), normal human bone marrow; (d), normal human peripheral blood leukocytes; (e), normal human kidney; (f), normal human liver; (g), normal human mucosa.

nuclear and total RNA, isolated from uninduced, 3 day TPA induced and 5 day RA induced HL60 cells were used to prepare Northern blots which were then hybridised to ^{32}P-labelled C6 cDNA (220 bp). As discussed previously, the total RNA and the nuclear RNA were isolated from cells of the same culture. The results of this experiment are shown in Figure 54.

Both C6 transcripts were clearly detectable in total RNA from RA induced HL60 cells, however, only the 2.4 kb RNA could be detected in the nuclear RNA from these cells. Very little hybridisation was found to the uninduced and 3 day TPA induced HL60 RNAs. From these results it could be deduced that the C6 2.4 kb RNA was highly abundant in the nuclear RNA of RA induced HL60 cells. Although total RNA was analysed, it can be assumed that the 1.3 kb C6 RNA if not present in the nuclear fraction must have been in the cytoplasmic RNA pool. These results provided evidence to support the conclusion that the 2.4 kb C6 RNA is a precursor of the 1.3 kb mRNA.

The Northern blots were stripped and rehybridised to radioactively labelled B_2-microglobulin cDNA to determine that a similar RNA concentration was present in each of the RNA samples assayed (Figure 54).

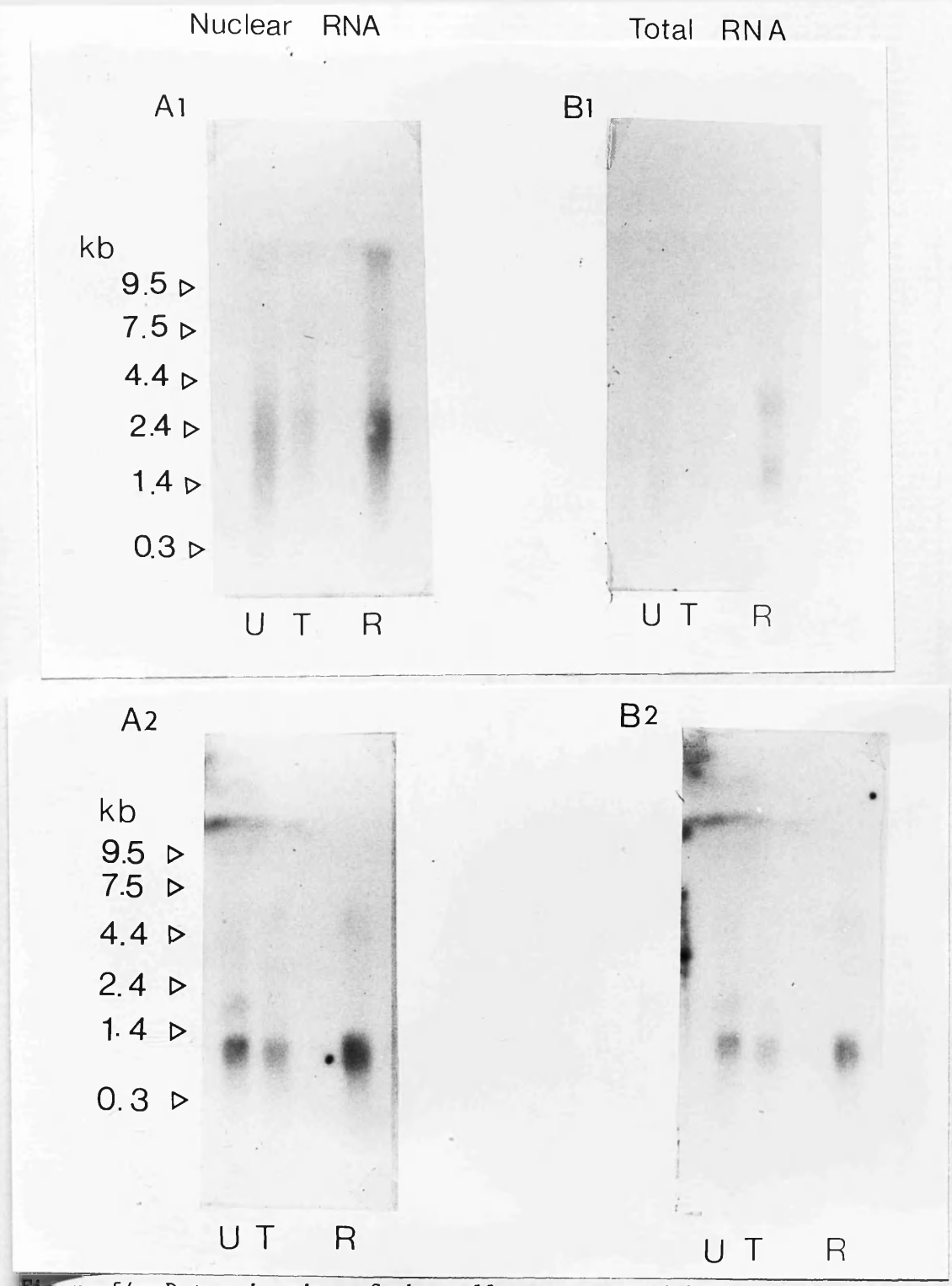


Figure 54 Determination of the cell compartment(s) containing C6 RNA.

3 µg of total cellular RNA and 3 µg of total nuclear RNA isolated from uninduced, 5 day RA induced and 3 day TPA induced HL60 cells were fractionated by electrophoresis in a denaturing, agarose gel. The RNA was then transferred on to nitrocellulose membrane by blotting. The nitrocellulose membrane was hybridised with radioactively labelled probes for (A(1) and B(1)), C6 sequences and (A(2) and B(2)), B_2-microglobulin. (R), 5 day RA induced HL60; (T), 3 day TPA induced HL60; (U), uninduced HL60 cells.

CHAPTER IV : DISCUSSION

DISCUSSION

1 MYELOID CELL DIFFERENTIATION

A) Use of Cell Lines as Model Systems

Many cell lines exist which can be induced to differentiate in culture and which have been used as model systems for differentiation. These include myogenic cell lines, embryonic cell lines and haematopoietic cell lines (Collins *et al*, 1977; Marks and Rifkind; 1978; Strickland and Mahdavi, 1978; Blau and Epstein, 1979). Study of the processes which occur during induced differentiation of these cell lines not only sheds light on the events leading up to terminal differentiation in the individual systems but can also be used to give a general over-view of differentiation in all systems.

There are, however, certain advantages and disadvantages in the use of cell lines to study differentiation. One main advantage is that cell lines allow the isolation of relatively homogeneous, clonal cell populations of differentiated cells, not available *in vivo*. As a result, these systems also enable the differentiation process to be monitored easily as the differentiating cells are not surrounded by a background of other cell types or cells at vastly different differentiation stages. Cell lines which can be induced to differentiate also offer the opportunity to study commitment to differentiation since variant cell lines are available which are resistant to differentiation (Toksoz *et al*, 1982). Furthermore, large amounts of material for analysis, for example RNA and DNA, are easily obtainable. It would prove difficult to obtain similar quantities from animal donors; therefore cell lines are attractive for laboratory use. An added advantage of using a haematopoietic cell line to study differentiation is that the haematopoietic system has been well studied *in vivo* and the cell biology of the differentiation processes well characterised.

Haematopoietic cell lines, and almost all other cell lines, are however derived from tumour cells and therefore are transformed and aberrant. Aberrant gene expression is known to occur in transformed cells therefore it is uncertain if control of gene expression is equivalent in these cells and their normal cellular counterparts (Cairns, 1981). Cell lines are also capable of culture *in vitro*, and

are established hence adaption to tissue culture may also result in aberrant gene expression. The culture conditions may not reflect that of the natural in vivo environment of the cell which may be required for normal growth.

The inducing agents used to initiate differentiation of cell lines are often either non-physiological chemicals, such as DMSO, or are used at concentrations far greater than would commonly be found in vivo. These agents are therefore unlikely to reflect a normal differentiation signal. Furthermore, the mechanisms of action of these agents have not been determined in many cases. Therefore, it is unknown if induction of differentiation by chemical agents occurs by similar patterns of gene expression as are found in vivo. Cell lines are also immortal, with unlimited proliferative capacity, unlike normal cells. During induction of differentiation, cell lines become committed to a specific course of differentiation. This is followed by a change from immortality to mortality. Therefore, many of the changes in gene expression observed during induction of differentiation may result from a shift from immortality to mortality and need not necessarily represent changes important in the differentiation process. Changes in gene expression during induced differentiation may also be due to cessation of proliferation and therefore could be considered secondary to the differentiation process.

B) HL60 as a Model for Differentiation

i) Advantages of Studying Differentiation of HL60 Cells

The HL60 cell line (Collins et al, 1977) has been used as a model system in which to study the biochemical and molecular events necessary for the processes leading to terminal differentiation. The use of haematopoietic, established cell lines has provided a continuous supply of apparently "maturation arrested" cells which can be cultured easily and indefinitely. These cells can also be induced to differentiate by readily available agents, and the progress of differentiation can be monitored by readily assayable characteristics.

In choosing a system in which to study control of changes in gene expression during differentiation it is important to select a system which allows not only the opportunity to study the mechanisms behind gene regulation, but, also one which affords the chance to observe the co-ordination of events which must occur during the differentiation process. HL60 is therefore an important model for differentiation. Comparison of gene expression between uninduced cells and induced cells will allow the determination of changes in gene expression due to differentiation, as in other cell systems. However, there is also good evidence that HL60 is bipotent, therefore these cells also provide the opportunity to investigate the mechanisms that govern the differentiation of progenitor cells down different cellular pathways (Fontana et al, 1981; Fischkoff et al, 1984). This provides interesting possibilities for the study of commitment to differentiation and also to determine gene expression patterns specific to one lineage which could lead to the determination of control mechanisms which operate to co-ordinate gene expression.

The HL60 cell line is also a useful tool for the study of "maturation arrest" in leukaemic disorders. These cells provide an insight into the nature of the leukaemic lesion and are therefore useful as a means of analysing the changes between normal and transformed cells. It is interesting to note that the drugs which are most effective in the treatment of patients with acute myelogenous leukaemia, such as anthracyclines, are also inducers of HL60 differentiation (Schwartz and Sartorelli, 1981). It can be speculated that the effectiveness of these drugs may be due in part to their ability to promote leukaemic cell differentiation and partly to their cytocidal effect on malignant cells.

ii) A Comparison Between Uninduced HL60 Cells and Normal Promyelocytes

HL60 cells were established from the peripheral blood leukocytes of a patient with acute promyelocytic leukaemia (Collins et al, 1977). Hence, these cells represent leukaemic cells and are abnormal. Nevertheless, studies have shown that despite an abnormal karyotype, HL60 cells exhibit many of the functional characteristics of normal promyelocytes (Collins et al, 1977; Gallagher et al, 1979). Studies

involving antisera and monoclonal antibodies directed against normal myeloid cell surface antigens have also demonstrated that HL60 cells possess surface markers equivalent to those found in normal human promyelocytes. For example, HL60 cells react with antiserum directed against cells further down the granulocytic pathway than myeloblasts but not with neutrophil-specific antiserum, hence it has been proposed that HL60 cells are relatively mature myeloid cells (Mulder et al, 1981). Uchanska-Zeigler (1982) has also shown, using a wide range of monoclonal antibodies directed against myeloid cells, that HL60 cells exhibit many of the cell surface antigens associated with cells of the myeloid lineage. However, variation between different HL60 sublines from different laboratories have also been reported. These variations are both karyotypic and biochemical (Gallagher et al, 1985; Graham et al, 1985; Peyman and Sullivan, 1987). The biochemical variations observed between different HL60 sublines from different laboratories may reflect differences in cell culturing techniques. The fact that HL60 cells are established, and therefore can be maintained for long periods of time in culture, could also result in selection and outgrowth of clones of cells which may vary from the initial cell isolate. Genotypes of transformed cells must be less stable than those of normal cells to allow for progression of tumorigenesis in vivo, hence, the longer these cells are maintained in culture the more likely that genotypic variations could arise. This highlights the problems of using cell lines as care has to be taken when comparing results from different laboratories and between in vitro systems and that which occurs in vivo.

iii) Heterogeneity of HL60 Cell Populations During Induction

Uninduced HL60 cell cultures are relatively heterogeneous, consisting of 90% promyelocytes with 10-15% more mature cells of both the granulocytic and the monocyte / macrophage lineages (Chapter III, 1) (Collins et al, 1977). Following addition of the inducing agent to the growth medium and during the differentiation process, the cell population becomes more heterogeneous. The whole HL60 cell population undergoes a programmed differentiation. However, during the course of treatment with inducing agents, cells can be found distributed throughout the many stages of the myeloid cell lineages leading to mature granulocytes or macrophages (Boyd and Metcalf, 1984). I have also detected intermediates between promyelocytes and granulocytes

during induction with DMSO or RA and monocytic cell intermediates during TPA induction. The cell morphologies closely reflect the morphologies observed during *in vivo* differentiation of myeloid cells. At the completion of differentiation however, the majority of cells resemble mature, terminally differentiated myeloid cells (see Chapter III, 1 of this thesis; Breitman *et al*, 1980; Boyd and Metcalf, 1984).

Heterogeneity of an HL60 cell population, during the course of differentiation, may be the result of lack of synchrony between cells both in the position of individual cells in the cell cycle and also in the stage of differentiation of each cell at the time the induction stimulus was added. This may result in variation in the timing of the appearance of overt differentiation between individual cells. Therefore, when interpreting data pertaining to differentiation of HL60 cells the proportion of fully differentiated cells has to be determined and the fact that a proportion of cells will always be relatively undifferentiated has to be noted.

iv) Comparison between Induced Differentiation and Normal Differentiation

Following differentiation of HL60 cells, the cells acquire some of the morphological and functional characteristics of normal granulocytes or macrophages (see Chapter III, 1 of this thesis; Tsiftsoglou and Robinson, 1985). These changes are accompanied by changes in gene expression which is reflected by changes in the protein content of the induced cell (Reyland *et al*, 1986). Differentiating HL60 cells have been shown to undergo many of the phenotypic changes known to occur during normal myeloid differentiation from promyelocytes to granulocytes (Collins *et al*, 1978; Breitman *et al*, 1980). Hence, many of the normal programmed changes in gene expression during myelopoiesis, which give rise to the fully differentiated phenotype, must also take place during HL60 differentiation despite the fact that they are transformed cells. It can therefore be surmised that HL60 differentiation may be very similar to that of the normal myeloid differentiation system.

Nevertheless, results from Skubitz and August (1983), who have studied changes in ^{125}I-labelled surface antigens after induction

of HL60 cells, have reported that although surface antigen characteristics of mature granulocytes could be detected on HL60 cells, they represented only a small proportion of the total population of cell-surface antigens found on these cells. However, the composition of RA induced cell populations reported by Shubitz and August differed from that of others (Breitman et al, 1980) and they did not demonstrate that these cells had undergone full induction. They also did not take into account the heterogeneity of the HL60 cell population. Ferrero et al (1983) also reported that HL60 differentiation is defective. Although they report that over 90% of the HL60 cells appeared morphologically differentiated they did not present data giving the percentage, in cell types, of the final population. Others have demonstrated alterations in polypeptide synthesis down the granulocytic differentiation pathway (Yokota et al, 1984). They showed that, although some of the changes observed were consistent with the normal differentiation process, induction of differentiation of HL60 cells may yield partial or incomplete differentiation since the protein pattern observed from induced HL60 cells was not identical to the normal cell counterpart. From much earlier studies, Gallagher et al (1979) reported that DMSO induced HL60 cells do not synthesise lactoferrin and alkaline phosphatase, markers of mature granulocytes, which also suggests that HL60 differentiation may not be complete. However, different inducing agents were used in these experiments which may contribute to some of the conflicting results. It has been demonstrated that some inducing agents are more effective than others in HL60 cell induction (Breitman et al, 1980). I have also demonstrated this when comparing the proportion of cells which appear terminally differentiated following RA or DMSO induction (Chapter III, 1 of this thesis).

Conflicting results have also been reported about the extent of monocyte / macrophage differentiation observed following TPA induction of HL60 cells. TPA induced HL60 cells are phenotypically very different from uninduced cells or HL60 cells induced to differentiate to granulocytes. I have also shown this (Chapter III, 1). However, Ferrero et al (1983) have reported that, although TPA induced HL60 cells did possess some myelomonocytic cell markers, others could not be detected. These cells were also found to retain some antigens known to be characteristic of granulocytes. Others have shown that TPA induced HL60 cells do express a number of

membrane-bound proteins predominately synthesised by human macrophages (Todd et al, 1981; Feuerstein and Cooper, 1984; Yokota et al, 1984). Therefore, although induced HL60 cells may not be identical to their normal cellular counterparts, the evidence available suggests that DMSO or RA induced HL60 cells are granulocytic and TPA induced HL60 cells are monocyte / macrophage-like.

Classification of haematopoietic cells at specific stages of differentiation is commonly carried out by morphological similarities. However, sub-groups could exist which, although very similar morphologically, could differ phenotypically. This could lead to slight variations in cell-surface antigen expression and functional changes. However, HL60 cells are derived from a cloned cell population, hence comparison of antisera reactivity of HL60 cells with that of normal, haematopoietic cells, an uncloned cell population, may be misleading. Furthermore, HL60 cells are grown in culture which means that they are not influenced by microenvironmental stimuli encountered by normal, differentiating, haematopoietic cells in the bone marrow. Bentley (1982) has proposed that contact between differentiating haematopoietic cells and bone marrow cells may influence the direct administration of appropriate regulatory factors to the differentiating cells. It is not clear how the different inducing agents effect differentiation of HL60 cells or how closely they may mimic in vivo factors. Therefore, it is possible that these agents are not capable of eliciting a full differentiation response from HL60 cells. This would result in phenotypic differences between HL60 in vitro differentiation and normal haematopoietic differentiation in vivo.

It has been reported that leukaemogenesis involves the uncoupling of the mechanisms regulating differentiation and proliferation (Sachs, 1980). Since HL60 cells were derived from a leukaemic source they must therefore be aberrant in this respect. McCulloch (1983) has proposed that a leukaemic clone consists of cells which are "maturation arrested" at a stage similar to a differentiation stage in normal haematopoiesis. Addition of inducing agent to cultures of HL60 cells may allow the cells to overcome the block in maturation and allow progress towards terminal differentiation. It is possible

that the original ancestors of the leukaemic clone which gave rise to HL60 progressed along a differentiation pathway which may have only mimicked the normal course of differentiation. This suggests that HL60 differentiation may only closely resemble the normal, myeloid differentiation pathway, but may actually proceed down a parallel leukaemic differentiation pathway. This could explain the inconsistencies arising from comparison of the characteristics of normal haematopoietic cell and HL60 cells.

v) Classification of HL60 Cells

Studies involving the in vitro growth of normal bone marrow cells have suggested that the myeloid and monocytic lineages share a common ancestor in haematopoietic differentiation. This proposal was based on studies which indicated that colonies grown in semi-solid media, consisting of both granulocytes and macrophages, developed from a normal haematopoietic colony-forming unit (Pluznick et al, 1965). It has also been supported by the ability of myeloid leukaemia cells to differentiate to granulocytes and macrophages following an induction stimulus (Fibach et al, 1973). HL60 cells are bipotent, which has led to the proposal that they represent an early cell which is common to both monocyte and macrophage differentiation pathways. If this is correct, and HL60 cells are early progenitor cells, this raises doubts about the classification of this cell line as promyelocytic. Figure 3 represents the proposed lineages leading to mature granuloytic and monocytic differentiation. Promyelocytes are positioned past the branch point at which these lineages diverge. This would indicate that promyelocytes are committed to differentiate to granulocytes. However, HL60 cells are apparently not committed to one particular lineage as they can differentiate to granulocytes or macrophages. It is unlikely that HL60 cells reverse commitment to granulocytes then become re-committed to monocytic differentiation. Therefore, it can be speculated that the position of the branch point on the differentiation pathway to mature granylocytic and monocytic differentiation may need to be reviewed. However, HL60 cells are derived from leukaemic cells therefore they are not "normal". Although they possess promyelocytic features they may represent a cell type which is neither on the granulocytic nor the monocytic pathway but somewhere in between, an example of lineage infidelity. This would then suggest that HL60 differentiation parallels normal

myeloid differentiation but progresses along a separate "leukaemic lineage" which shares both granulocytic and monocytic features. Alternatively, HL60 bipotency may reflect transdifferentiation of these cells in response to an induction stimulus. Transdifferentiation is the term given to conversion from one distinct cell type to another developmentally related type. This results in expression of proteins in cells which would normally not contain these products (Wyllie et al, 1982). Transdifferentiation of pigmented and neural epithelium to lens tissue has been reported (Clayton, 1982). It would appear that certain conditions, both in cell culture and in cell-cell contact, have to be met before transdifferentiation occurs in this system. Therefore, it could be suggested that the induction stimulus allows HL60 cells to transdifferentiate to monocytic cells with the expression of certain characteristics specific to normal monocytes.

2 INDUCTION OF DIFFERENTIATION AND COMMITMENT

A) Comparison of Inducing Agents

The most commonly used inducing agents for stimulating granulocytic differentiation in HL60 cells are RA and DMSO. TPA and 1,25-dihydroxyvitamin D_3 are commonly used to induce monocyte / macrophage differentiation. Many different parameters have been used to determine the differentiation status of induced HL60 cells, including morphological changes, histochemical changes, functional changes and changes in gene expression (Tsiftsoglou and Robinson, 1985). From such analyses it has become apparent that differences can be observed not only between HL60 cells induced to macrophages and cells induced to granulocytes, but also between cells induced to the same differentiation end point with different chemical agents. This may indicate that not all agents are efficient inducers of differentiation in HL60 cells. Alternatively, different inducing agents may overlap in the patterns of gene expression they induce but may also act individually on other sets of genes. For example, it has been reported that TPA-responsive genes contain a 5' region, a TPA-responsive element, which recognises a specific protein which is expressed following TPA treatment of cells. Interaction of the

protein and the TPA responsive element is thought to induce gene expression (Angel et al, 1987). Therefore, TPA may induce expression of genes in HL60 cells required for monocytic differentiation but may also stimulate expression of "TPA responsive genes" which may or may not be involved in the differentiation process.

Tsiftsoglou and Robinson (1985) have reported that RA is apparently more effective at inducing HL60 cells to terminally differentiate to granulocytes than DMSO. However, Skubitz et al (1982) have observed that DMSO induced HL60 cells have a greater receptor activity for formylated peptides than RA induced HL60 cells. Similarly, a report from Breitman and Keene (1982) demonstrated calcium ionophore activated production of leukotrienes was greater in DMSO induced cells, indicating that DMSO induced HL60 cells were more mature than RA induced HL60 cells. However, Hemmi et al (1982) demonstrated RA induced HL60 cells appeared morphologically more mature at an earlier stage during induction than DMSO induced HL60 cells. I have found that variation exists between RA and DMSO induction of HL60 cells. During analysis of differentiation parameters during RA induction, it became very clear that only 50%-60% of the cell culture expressed characteristics of mature myeloid cells at the completion of the 5 day RA induction period (see Chapter III, 1).

There appears to be great variation in the extent of differentiation of HL60 cells with different inducing agents as reported from different laboratories. The concentrations of the inducing agents used are all very similar therefore it seems reasonable to assume that these results arise through differences in the HL60 sublines from the various laboratories and differences in cell culturing technique (see Section 1 of this chapter). Many different HL60 sublines have been isolated which display varying degrees of resistance to induced differentiation (Gallagher et al, 1985). However the mechanism(s) responsible for resistance is unknown. It is therefore possible that the HL60 cell line used in this laboratory contains a population of cells incapable of responding to RA. Alternatively, RA may only induce HL60 partly along the granulocytic differentiation pathway. At the initiation of induced differentiation the HL60 cells are not synchronous in relation to phase of cell cycle and possibly stage of differentiation, hence this could result in some cells responding quickly to the induction stimulus whilst others

have a delayed response. Therefore, at the end of a 5 day induction period those cells which respond early in the treatment period would display mature cell characteristics but those cells which were later in responding would still be at a very early stage on the granulocytic differentiation pathway and would retain uninduced cell characteristics. I observed that following 5 days of treatment with RA, 50-60% of the cells resembled granulocytes both morphologically and biochemically. However, 30-40% of the cells still resembled uninduced HL60 cells or myelocytes. Cell proliferation could still be observed in the HL60 cell culture, supporting the evidence that a proportion of cells still remained undifferentiated. Interestingly, 5 day RA induced HL60 cells invariably appeared less mature, morphologically, than 5 day DMSO induced HL60 cells (see Chapter III, 1).

Others have also reported differences in the abilities of TPA and 1,25-dihydroxyvitamin D_3 to induce monocyte / macrophage differentiation in HL60 cells. Most reports on HL60 cells induced by TPA state that the majority of the cells terminally differentiate and express morphological, functional and biochemical changes associated with mature macrophages (Rovera et al, 1979). I also found that approximately 90% of HL60 cells terminally differentiated when induced by TPA (see Chapter III, 1). However, others have reported differences in the proportion of terminally differentiated HL60 cells found in cultures treated with 1,25-dihydroxyvitamin D_3. For example, it has been reported that almost 80%-90% of 1,25-dihydroxyvitamin D_3 induced HL60 cells become terminally differentiated (Daniel et al, 1987). However, others report that as few as 30% of the cells showed differentiated characteristics (Studinski and Brelvi, 1987).

B) The Commitment event

The nature of the cellular signal(s), which effects a cellular programme whereby cells yield terminally differentiated progeny, is an outstanding question in leukaemogenesis as well as regulatory biology. Due to its differentiation properties the HL60 cell line has been used to determine the order of events before the appearance of overt differentiation and the timing of the commitment event during

HL60 monocytic and granulocytic differentiation.

From my analyses of terminally differentiated cell characteristics, it appears that the commitment event occurs between 8-24 hours of TPA treatment, and during 48-96 hours of DMSO treatment. At these times the majority of cells display reduced cell cloning ability, are arrested in G0/G1 phase of the cell cycle and display both morphological and histochemical characteristics of mature myeloid cells. Removal of the cells from the induction stimulus before this time resulted in the cells maintaining their immature cell characteristics.

These results are in agreement with those reported by Fibach et al (1982) and Fontana et al (1981). They proposed that 48-96 hours were required to irreversibly commit HL60 cells induced by DMSO to granulocytic differentiation and that 8-12 hours were required for commitment to monocytic differentiation. Data from these workers was derived from analysis of mature cell morphology and histochemical markers of differentiation displayed by HL60 cells exposed for periods of time to inducing agents then cultured in the absense of any induction stimulus. The proportion of cells which became terminally differentiated throughout the period of culture, without any differentiation stimulus, were counted as committed. This was identical to the method I applied to assess cell cloning potential during HL60 differentiation. They determined that after the critical period the presence of inducer in the growth medium was no longer required for completion of the differentiation programme. However, cells removed from the differentiation stimulus before this time showed reversal of mature cell characteristics and the reappearance of those characteristics associated with uninduced HL60 cells. This was also demonstrated during clonal analysis; before the commitment event cells continued to grow indefinately with only a small proportion unable to self renew. During and after the critical time the number of cells incapable of self renewal increased with a concomitant increase in those cells displaying mature cell characteristics.

During these analyses HL60 cells were not synchronous with respect to phases of the cell cycle. It is unlikely that all cells will respond simultaneously to the induction stimulus, therefore, the time of

exposure required for individual cells to become irreversibly committed would vary. This would explain why the estimated time of the commitment event in HL60 cells, after induction, spans a number of hours.

Two models have been proposed for the mechanism of commitment to terminal differentiation, the stochastic model proposed by Till and McCulloch (1964) and the instructive model by Trentin (1967) (see Chapter I, 1, A(ii)). It has been reported that HL60 cells become committed to terminal differentiation in a manner consistent with a stochastic model (Tarella et al, 1982). According to this model commitment events occur randomly in the population with a particular probability. A similar model is also proposed for induced differentiation of erythroleukaemia cells (Guesella et al, 1976). However, it would appear difficult to separate entirely the stochastic model from the instructive model. It has been reported that HL60 cells do not respond to inducing agents in a synchronous manner therefore these cells do not respond to chemical agents instructively (Tarella et al, 1982). However, others have reported that cells respond to induction stimuli at specific stages of the cell cycle (Yen and Albright, 1984). Therefore HL60 cells would have to reach that particular stage before becoming committed and expressing terminally differentiated cell characteristics. This would mask the fact that these cells were responding in a manner consistent with the instructive model. I have found that TPA induction of HL60 cells results in the majority of the cells expressing terminally differentiated characteristics after only 24 hours of treatment. This indicates that these cells responded to the induction stimulus within the time required for the cells to undergo one round of cell division. This would suggest that these cells responded to TPA in a manner consistent to the instructive model. However, DMSO induction of HL60 cells required 3-4 days before the majority of cells were found to express terminally differentiated cell characteristics (see Chapter III, 1). This indicates that the cells had the opportunity to divide 2-3 times before becoming terminally differentiated and could be consistent with a stochastic model for commitment to differentiation.

C) Commitment and the Cell Cycle

Using fluorescence-activated cell sorting, to analyse the distribution of HL60 cells around the cell cycle, I found that TPA and DMSO treatment caused a progressive accumulation of cells in G0 / G1 phase of the cell cycle and a concomitant decrease in the proportion of cells found in S and M phases. The subsequent inhibition of growth demonstrated by decrease in cell cloning ability may be attributed to a reduction in the percentage of cells capable of DNA synthesis. Arrest in G0/G1 occurred early in TPA induction, after 24 hours almost 95% of the cells were localised at this stage of the cell cycle. Others have also reported this (Rovera et al, 1980). Treatment with DMSO resulted in G0/G1 arrest following 72-96 hours of treatment. Similar results have also been reported by Yen et al (1985) but using RA as the induction stimulus. Many other mammalian cell types in advanced stages of differentiation have a DNA content corresponding to the G0/G1 phase of the cell cycle and are no longer capable of proliferation (Prescott, 1976). Therefore this appears to be a normal event in the differentiation process.

It has been proposed that inducing agents transiently block cells in one particular phase of the cell cycle where the probability of spontaneous differentiation is high. This block has been proposed from observations that analogues of purines and pyrimidines, which inhibit DNA synthesis, can induce the differentiation of HL60 cells (Munroe et al, 1984; Griffin et al, 1982). Analysis of induced differentiation in synchronised HL60 cell populations has suggested that HL60 cells become responsive to RA induction during S phase (Yen et al, 1984). Indeed evidence suggests that pretreatment with agents which disrupt DNA synthesis accelerates the onset of terminal differentiation. For example, Yen et al (1987) demonstrated that pretreatment of HL60 cells with hydroxyurea followed by exposure to RA resulted in accelerated HL60 cell differentiation when compared to RA alone. They propose that hydroxyurea induces a precommitment state in HL60 cells. It is proposed that precommitment is a labile, early stage which may be activated specifically in S phase of the cell cycle. Occurence of precommitment is also associated with nuclear structural changes (Yen et al, 1985). Others have reported that early changes in cell cycle kinetics, during differentiation of murine erythroleukaemia cells, resulted in prolongation and finally arrest

at G0/G1 phase of the cell cycle (Terada et al, 1977). My results indicate a progressive decrease in the proportion of HL60 cells found in G2/S phase during early times of TPA induction which could indicate prolongation of the G1 phase.

If induction was facilitated by the cells being in a specific phase of the cell cycle at the time of addition of the inducer, then it could be suggested that cell cycling is a necessary element in the lead up to terminal differentiation. However, it has been demonstrated that commitment to differentiation and indeed the onset of HL60 terminal differentiation does not require DNA synthesis or cell division (Ferrero et al, 1982; Rovera et al, 1980). Hence there is confusion about the importance of the cell cycle in relation to HL60 differentiation.

During commitment of differentiation in normal "stem cells" only one daughter cell becomes committed, the other must retain its ability to produce multipotent daughters to maintain the stem cell pool. In this respect HL60 commitment to differentiation is different. Quite clearly the majority of cells become committed to terminal differentiation. HL60 cells are classified as promyelocytic therefore these cells are committed to differentiation down the myeloid differentiation lineages (Collins et al, 1977). They therefore represent late stages in haematopoiesis and are far removed from early progenitor stem cells. HL60 cells can proliferate but on receiving an induction stimulus it appears that all cells respond unlike stem cells.

D) The Role of c-myc During HL60 Differentiation

It has been suggested that cellular oncogene sequences may encode products that control growth and differentiation. This has been supported in part by study of cellular oncogene expression during the induced differentiation of tumour cell lines in vitro. For example, HL60 cells possess amplified c-myc sequences which has led to the proposal that c-myc may have had a role during the leukaemogenic event from which the cells arose (Dalla Favera et al, 1982a). It has previously been demonstrated that c-myc RNA is undetectable in terminally differentiated HL60 cells (Westin et al, 1982; Reitsma

et al, 1983). To determine when c-myc transcripts became undetectable I examined the presence of c-myc RNA at time points throughout HL60 differentiation.

During the course of induced differentiation of HL60 cells, c-myc RNA was undetectable after very short periods of treatment with either TPA or DMSO: following 4 hours of TPA treatment and after only 1 hour of DMSO treatment. c-Myc transcripts then remained undetected throughout the period of induction. A similar decrease in detectable c-myc transcripts, following DMSO induction of HL60 cells, has been reported by Eick and Bornkamm (1986) and has also been observed during Friend erythroleukaemia cell differentiation (Lachman and Skoultchi, 1984).

From the parameters I measured during HL60 differentiation, decrease in c-myc RNA was the earliest change detected during granulocytic and monocytic differentiation. The loss of c-myc RNA appeared to precede the commitment event, the loss of the potential to self renewal, arrest at G0/G1 and the appearance of overt differentiation. These events occurred a number of hours after c-myc transcripts became undetectable. This is in agreement with results reported by Filmus and Buick (1985). They observed a similar pattern of events during DMSO induction of HL60 cells. I observed that HL60 cells also appeared to stop proliferating before expressing differentiated cell characteristics. It has been postulated that c-myc has a role in cell proliferation and differentiation; however, is decrease in c-myc expression in HL60 cells due to the cessation of growth or due to the differentiation event?

i) c-Myc, a Role in Cell Proliferation

Evidence that myc sequences may be involved in cell proliferation was initially proposed from observations that c-myc expression was induced following serum or growth factor stimulation of quiescent fibroblasts and mitogenic stimulation of lymphocytes (Kelly et al, 1983; Reed et al, 1985). DNA synthesis was also induced when 3T3 cells were transfected with c-myc DNA under the control of the mouse mammary tumour virus promoter and the cells then cultured in the presence of glucocorticoids (Armelin et al, 1984). c-Myc sequences have also been demonstrated to co-operate with other sequences in the

transformation of primary cells (Land et al, 1983). It has been suggested that c-myc protein is a competence factor, it is required for transition from G0 to G1 of the cell cycle but other progression factors are needed for entry into S phase (Bravo et al,1985; Lee et al, 1985; Kaczmarek et al, 1985). Therefore, it has been proposed that c-myc has a role in establishment of cells which enables them to grow continuously in culture.

It was initially believed that c-myc expression was restricted to G1 phase of the cell cycle. This followed reports that serum stimulation of quiescent fibroblasts or activation of lymphocytes by phytohaemagglutinin resulted in proliferation of the cells and induction of c-myc RNA (Kelly et al (1983); Campisi et al, 1984). It was reported that the level of c-myc transcripts increased 20-fold shortly after cells were stimulated to proliferate and then declined before the onset of DNA synthesis. It was inferred from these data that c-myc expression may be specific to G1 phase of the cell cycle. However, it became apparent that the interpretation of these results had been misleading. Shortly after these data had been published others reported that c-myc is rapidly induced upon the cell's transition from a resting to a growing state; however, observation of the cells following stimulation, as they progressed round the cell cycle, indicated that c-myc RNA was present in cells at all phases of the cell cycle (Thompson et al, 1985). It has also been demonstrated that c-myc protein is present in nuclei of cells isolated from phases throughout the cell cycle (Hann et al, 1985). Therefore, in the light of this evidence, it would appear difficult to assign a role for c-myc in cell proliferation. However, it has been proposed that c-myc expression may reflect a cell's competence to enter and progress through the cell cycle but the c-myc gene is not expressed at any particular phase (Thompson et al, 1985).

During my analyses of levels of c-myc transcripts throughout TPA induction of HL60 cells, c-myc expression was undetectable after only 4-8 hours of treatment. Following this event the majority of cells (>90%) arrested at G0 / G1 of the cell cycle within the time estimated for one cell division (24 hours). This indicates that with addition of TPA to the growth medium, those cells which had embarked on the cell cycle completed it but did not enter a second. It can therefore be proposed that loss of c-myc during monocytic

differentiation of HL60 cells appears to reflect the cell's lack of ability to continue cycling. This would support the proposal that c-myc expression reflects a cell's ability to proliferate. However, loss of c-myc RNA during TPA induction of HL60 cells also occurred concomitant to the appearance of mature cell characteristics which indicates a direct link between levels of c-myc RNA and the differentiation process.

From data generated from the analysis of c-myc expression during HL60 induced differentiation by DMSO, I reported a loss of detectable c-myc transcripts within only one hour of treatment. However, from fluorescence activated cell sorting analysis and from observation of cell cloning potential during DMSO induction, it appears that these cells still divide 2-3 times following addition of inducer to the growth medium, before arrest at G0 / G1 and the appearance of overt differentiation characteristics. This suggests that in DMSO induced HL60 cultures the cells are capable of proliferation without the presence of detectable c-myc RNA. Loss of c-myc transcipts also occurs approximately 24-48 hours before the appearance of mature cell characteristics which indicates that the commitment event occurs at a time when the level of c-myc RNA has been negligable for many hours.

Therefore, unlike the TPA induction, it is difficult to determine a direct link between c-myc expression and the loss of cell cycling potential and the appearance of overt differentiation in DMSO induced HL60 cells.

ii) c-Myc, a Role in Differentiation Processes

I demonstrated that the steady-state levels of c-myc RNA dramatically diminish during differentiation of HL60 cells down both the granulocytic and the monocytic differentiation pathways. c-Myc expression has also been reported to decrease during differentiation of many other cell lines. During induction of differentiation of the teratocarcinoma cell line, F9, to parietal and visceral endoderm, c-myc RNA becomes undetectable following only 9-12 hours of treatment with RA (Griep and DeLuca, 1986). c-Myc RNA is also diminished at an early stage of induced differentiation of WEHI-3B cells (a murine myeloid leukaemia cell line) to monocyte / macrophages and a very early biphasic response is observed of c-myc transcripts when murine

erythroleukaemia cells are induced to differentiate to mature erythroid cells (Gonda and Metcalf, 1984; Lachman and Skoultchi, 1984). During the induced differentiation of erythroid cells a 5-15 fold drop in c-myc RNA levels occurred within 1-2 hours. The c-myc RNA then recovered to pretreatment levels by 12-18 hours and then continued to fall off gradually as terminally differentiated cells accumulated. Therefore, decrease in c-myc RNA levels is observed during differentiation of many different cell lines which could indicate that this is a common event during the differentiation process.

Evidence to suggest that c-myc expression is an important factor in differentiation processes has come from a number of different sources. It has been demonstrated that the continuous presence of c-myc RNA prevents differentiation in many systems. Transformation of embryonic quail myogenic cells with avian v-myc myelocytomatosis virus MC-29 resulted in irreversible suppression of their competence for terminal differentiation (Falcone et al, 1985). Furthermore, the constitutive expression of c-myc sequences, as a result of transformation of murine erythroleukaemia cells with v-myc sequences, under the control of the SV40 promoter, resulted in inhibition of erythroid differentiation (Coppola and Cole, 1986). Similar results have been reported by Prochounik et al (1986) and Dmitrovsky et al (1986). Lachman et al, (1986) reported a similar experiment where alterations in the timing of the reappearance of c-myc RNA, during the biphasic response observed during induced differentiation of murine erythroid cells, was demonstrated to change the timing of commitment. For example, introduction of exogenous c-myc sequences reduced the time of reappearance of c-myc transcripts during murine erythroleukaemia cell induction. This resulted in the appearance of committed cell characteristics at earlier times than observed in the parent line. However, transformation of the erythroid cells with anti-sense c-myc sequences resulted in delayed reappearance of c-myc transcripts and a delay in the accumulation of committed cells. From these results it has been proposed that c-myc may have a regulatory role during commitment to differentiation.

However, contradictory evidence has also been reported which suggests that in certain systems the loss of c-myc RNA is not an important event during differentiation. For example, induction of the human

monocytic leukaemia cell line THP-1 showed no decrease in the level of c-myc transcripts when induced to differentiation to functional, adherent macrophages (Lee et al, 1987). Similarly, BC3H1 cells, a myogenic cell line which can be induced to differentiate, showed only partial inhibition of terminal differentiation when transfected with c-myc sequences under the control of the SV40 promoter. This maintains the abundance of c-myc RNA at levels comparable to that of undifferentiated myoblasts (Schneider et al, 1987). Therefore contradictory opinions exist about the role c-myc plays during differentiation. However, it is widely believed that c-myc does have a role to play in both differentiation and proliferation. The fact that some cell lines demonstrate a requirement for regulation of c-myc sequences while others do not, may reflect the nature of the original transforming event which initiated the establishment of the various cell lines. It has been proposed that transformation is the result of de-regulation of the processes of differentiation and proliferation hence some established cell lines may not reflect the events which occur during normal proliferation and differentiation (Sachs, 1980).

I observed that differences existed between DMSO and TPA induction of HL60 cells in respect to c-myc expression. This may reflect differences in the mode of operation of DMSO and TPA in inducing the differentiation process. TPA treatment alters lipid metabolism thereby altering membrane-mediated processes. For example, Cabot et al (1980) have reported enhanced stimulation of phospholipid metabolism during treatment of HL60 cells with TPA. This has been proposed to affect lipid microviscosity, alter lipid components present in the membrane and ion transport across the membrane, all of which could have substantial effects on intracellular processes (Sussman et al, 1985) Protein kinase C has been reported to be the membrane receptor for phorbol esters. Diglyceride, formed by a phospholipase C-catalysed hydrolysis of inositol phospholipids, is the physiological activator of protein kinase C; phorbol esters can substitute for diglycerides in this role. (Castagne et al, 1982). Protein kinases have been implicated in transformation processes but are also believed to play a central role in normal growth regulation (Taylor, 1987). It is thought that they act as means of transmitting signals by phosphorylation events from the cell membrane to the cell nucleus (Taylor, 1987). It has recently been reported that the

co-ordinate expression of TPA-responsive genes is implemented by a specific TPA-response motif in the 5' sequences of these genes. This motif appears to recognise a protein(s) which is abundant following TPA treatment (Angel et al, 1987). Thus, some changes which do or do not take place in response to treatment with TPA may be due to a direct effect of TPA on promoters of genes rather than a response to a differentiation programme.

DMSO is a bipolar molecule, with a high dielectric constant, which has an enormous effect on permeability of membranes; this is indicated by changes in ion movements across the membrane (Friend and Freedman, 1978). Phase transition temperatures of phospholipids are increased by DMSO treatment, causing reduced fluidity and increased membrane stability (Lyman et al, 1976). The importance of these membrane changes on the induction of differentiation by DMSO has been highlighted by treatment of murine erythroleukaemia cells with agents known to increase the fluidity of artificial and biological membranes. This treatment inhibits the induction of erythroleukaemia cells by DMSO (Papahadjopoulos et al, 1975; Poste et al, 1975). However, the exact mechanisms through which DMSO operates are unclear. Changes in membrane permeability could enhance differentiation by altering or enhancing transport or binding of certain "factors" which normally initiate differentiation.

Therefore, both TPA and DMSO appear to have a major effect on the membrane of cells; however the exact nature of the mechanism(s) through which they elicit their induction effects is unclear. To determine if different induction agents affect transcription of the c-myc gene by different mechanisms more would need to be determined about the intracellular processes leading to differentiation and the role c-myc plays in these processes.

Alternatively, c-myc transcription may still occur during DMSO induction but expression may be down-regulated to such an extent that it is below the levels of detectability by Northern blotting analysis. c-Myc sequences are greatly amplified in HL60 cells (Dalla-Favera et al, 1982a). The degree of c-myc transcription is directly proportional to the extent of amplification (Graham et al, 1985). Hence, it could be postulated that transcription is reduced to levels reflecting that from the normal allele which may be difficult

to determine by the method I used. However, the abundance of c-myc protein has not been determined during HL60 differentiation. Therefore, despite the fact that c-myc RNA is undetectable after only one hour of treatment with DMSO, the protein could still be functional for some time. For this to occur in this situation, the c-myc protein would need to be stabilised as it has a short half life of only 15-30 minutes in normally cycling cells (Hann and Eisenman, 1984).

3 CHANGES IN GENE EXPRESSION DURING HL60 DIFFERENTIATION

During induced differentiation of HL60 cells to granulocytes or macrophages, changes in morphological and functional characteristics are observed (see Chapter III, A of this thesis; Tsiftsoglou and Robinson, 1985). Changes in the expression of specific genes have also been reported, in particular c-myc (Westin *et al*, 1982; Reitsma *et al*, 1983). Complex patterns of gene expression are thought to occur during differentiation; this is likely to involve regulation at many levels from the gene to the protein. Some aspects of this regulatory programme have been characterised by looking at changes in the population of mRNA or protein molecules synthesised by cells. From analysis of the availability of specific mRNA transcripts for translation *in vitro*, the extent of regulation of gene expression during HL60 differentiation has been evaluated. Reyland *et al* (1986) reported that 17% of a mRNA population appeared to be differentially regulated during DMSO induction of HL60 cells. Colbert et al (1983) have reported that almost 50% of the mRNA population are differentially regulated and have also observed differences in the translation products produced by HL60 cells induced to granulocytes compared to cells induced to monocytes / macrophages. In other systems, during development of Dictyostelium for example, 25%-27% of proteins synthesised *in vivo* have been shown to be regulated (Alton and Lodish, 1977). These results suggest that extensive changes in gene expression must occur during development and differentiation. From my data, gathered from screening analyses of a cDNA library constructed from poly(A)$^+$ RNA isolated from 5 day RA induced HL60 cells (see Chapter III, B), it appears that almost 30% of the cDNAs represent sequences which are highly abundant in induced cells

compared to uninduced HL60 cells and therefore appear to be differentially regulated. This is in agreement with the results of others from in vitro translation experiments.

I wanted to select sequences which changed in abundance during HL60 differentiation, and to examine the expression of the genes these sequences represented during the induction period, particularly with respect to commitment. To identify the sequences I employed two methods. The first approach was to examine the expression of genes which had been demonstrated to change during HL60 induction. This included c-myc and lysozyme, a macrophage-specific protein which is frequently used as a marker of monocytic differentiation in HL60 cells. The second approach employed the use of cDNA libraries constructed from poly(A)$^+$ RNA isolated from 5 day RA induced HL60 cells. The library was screened to determine sequences which changed in abundance during differentiation. The cDNAs, representing these sequences could then be used as hybridisation probes to investigate the differential characteristics of the homologous RNA. Three mRNAs were selected for analysis by this method. This method has been used successfully by others to determine sequences which change in abundance due to differentiation or hormone stimulation (Goodfellow, 1984; Fulton, 1985).

A) Potential Control Mechanisms for c-Myc Expression During HL60 Differentiation

C-Myc RNA has been shown to be undetectable following very short times of RA or DMSO treatment of HL60 cells (Bentley and Groudine, 1986; Eick and Bornkamm, 1986). I have also observed a rapid loss of c-myc RNA following only 1 hour of treatment with DMSO. Similarly, I observed that only 2-4 hours of TPA treatment was all that was required to result in the loss of c-myc transcripts from HL60 cells (see Chapter III, A of this thesis).

Both transcriptional and post-transcriptional mechanisms appear responsible for alteration of c-myc expression in many systems. Recent studies of c-myc expression, following the addition of serum and defined growth factors to growth-arrested fibroblasts, have indicated that transient increases in c-myc RNA levels occur as a

consequence of both increased transcription and enhanced transcript stability (Greenberg and Ziff, 1984; Blanchard et al, 1985). Inhibition of protein synthesis during mitogenic stimulation leads to "superinduction" of c-myc RNA, suggesting that c-myc mRNA levels may be regulated by a labile protein , a negative regulator (Kelly et al, 1983). Lachman and Skoultchi (1984) also reported data to support this; early decline of c-myc RNA, during DMSO induced Friend erythroleukaemia cell differentiation, was delayed by protein synthesis inhibitors. The initial decrease in c-myc RNA levels during Friend erythroleukaemia cell differentiation is followed by re-expression of c-myc. From these data Lachman and Skoultchi (1984) also postulate that c-myc transcription is controlled by positive regulators which facilitate the biphasic response of c-myc to the induction stimulus.

Evidence is now gathering which indicates that control mechanisms may operate at the 5′ regions of the c-myc locus. The repression of normal c-myc expression in transformed cells harboring c-myc chromosome translocations or c-myc retroviruses, has been proposed to be an auto-regulatory phenomena. This may be mediated, by a repressor, at the level of the first exon of the c-myc gene (Leder et al, 1983; Rabbitts et al, 1984; Rapp et al, 1985). Indeed, control at the first exon of c-myc has been supported by the discovery of a cis-acting negative control element, with the opposite properties of a transcriptional enhancer, which has been localised upstream of the murine c-myc locus (Remmers et al, 1986). The first exon of c-myc has also been reported as a site of anti-sense transcription which may act as a mechanism for control of gene expression but has yet to be demonstrated in vivo (Nepveu and Marcu, 1986).

Control of gene expression during HL60 differentiation also appears to operate at the level of the 5′ regions of the c-myc locus. It has been reported that decrease in the levels of c-myc RNA during DMSO induction of HL60 cells is caused by a block in transcription, which may start immediately after addition of DMSO. Eick and BornKamm (1986) used nuclear run-on assays to determine when and where along the c-myc locus the block occurred. They demonstrated that the block in c-myc transcription was complete after only 30 minutes of DMSO treatment. From my results, using Northern blotting analysis to determine the steady state levels of c-myc RNA during HL60 induction

by DMSO, transcripts were undetectable following only 1 hour of treatment. If the half-life of c-myc RNA is only 10-15 minutes in HL60 cells, as reported by Dani et al (1985), and transcription of the c-myc gene was abolished after only 30 minutes of treatment by DMSO, this would account for the rapid loss of c-myc RNA during DMSO induction. I have also found that the half-life of c-myc RNA in uninduced HL60 cells from this laboratory is only 15 minutes (see Chapter III, A).

The block in c-myc transcription has been localised to regions in the exon 1 / intron 1 boundaries. Interestingly, the first exon remains transcriptionally active when the rest of the gene is silent (Eick and Bornkamm, 1986). In an attempt to detect the exon 1 transcripts, identified by nuclear run-on analysis, I prepared a Northern blot from total RNA isolated from HL60 cells harvested at different times following addition of DMSO to the growth medium. This was hybridised to a c-myc exon 1 probe, however no small RNAs homologous to this sequence could be detected. This has also been reported by others (Eick and BornKamm, 1986). They postulate that the c-myc transcripts, from exon 1, may be so unstable that they are rapidly degraded and therefore are undetectable by Northern blotting analysis.

Bentley and Groudine (1986) have reported that a similar blockage operates to control c-myc expression during RA induction of HL60 cells. They report that the blockage is localised to an area similar to that defined by Eick and Bornkamm, at the boundary of c-myc exon 1 / intron 1. Eick and Bornkamm (1986) suggest that blockage of transcription is caused by DNA secondary structure at the exon 1 / intron 1 regions of the c-myc locus which results in transcriptional termination. They postulate that stem loop structures may facilitate the blockage in response to the induction stimulus. It is unknown if the same mechanisms operate to control c-myc expression during TPA induction of HL60 cells. However, it can be postulated that a different mechanism may operate. Both Eick and Bornkamm (1986) and Bentley and Groudine (1986) have reported that c-myc transcription is abolished in HL60 cell at very short times after the addition of DMSO or RA to the growth medium (see Chapter III, A). However, I found that TPA induced HL60 cells appeared to show a decrease in c-myc RNA at later times than HL60 cells induced to granulocytes, after 2-4 hours instead of 1 hour. The difference in the timing of loss of

c-myc transcripts may therefore indicate the operation of a different mechanism for control of c-myc expression during TPA induction of HL60 cells. Unfortunately RA did not induce a large proportion of my HL60 cells. This made it impossible to determine if and when c-myc transcripts became undetectable in the terminally differentiated cells. c-Myc RNA was detected throughout the first 3 days of RA induction. In the light of data from others this was probably produced from those cells which remained uninduced.

B) Lysozyme Expression in HL60 Cells

Apart from their phagocytic role, mononuclear phagocytes synthesis and secrete a wide variety of biologically important products, some of which are constitutively expressed, some inducible (Gordon, 1980). Lysozyme is a constitutively secreted protein, unaffected by the activation status of monocyte / macrophage cells (Lappin et al, 1986). Therefore this protein is a marker of mature macrophage differentiation and has been used to determine when HL60 cells become terminally differentiated to monocyte / macrophage cells during the course of induction (Reitsma et al, 1983; see Chapter III,A).

From analysis of lysozyme protein secretion during the three days required for TPA induced macrophage differentiation of HL60 cells, it appears that lysozyme protein is highly abundant in TPA induced HL60 cells even at very early times following the addition of inducing agent to the growth medium. An increase in lysozyme protein secretion was detected after only 2 hours of TPA treatment; the amount of secreted lysozyme protein increased dramatically throughout the first 48 hours of treatment but no further increase was observed during the final 24 hours of TPA induction. These results are supported by Reitsma et al (1983). They also reported very early increases in secreted lysozyme protein, between 6-12 hours, during HL60 induction to monocyte / macrophage differentiation by 1,25-dihydroxyvitamin D_3 Polansky et al (1985) also report a similar pattern of lysozyme protein secretion following phorbol ester induction and Krystosek and Sachs (1976) have demonstrated that myeloid leukaemic cells also secrete lysozyme when induced to differentiate to monocyte / macrophages.

The increase in abundance of extracellular lysozyme protein does not appear to be through stimulated release of intracellular protein by the inducing agent. The intracellular lysozyme protein content of uninduced and RA or DMSO induced HL60 cells was almost negligible. This is in agreement with data reported by Polansky et al (1985). Therefore, it can be deduced that the increase in lysozyme secretion observed by TPA induced HL60 cells is a consequence of changes in lysozyme gene expression (Chapter, III, A).

To determine the abundance of lysozyme RNA in HL60 cells, total RNA from uninduced and terminally induced HL60 cells were analysed by Northern blotting for the presence of lysozyme mRNA. Lysozyme transcripts were not clearly resolved but hybridisation was observed in both TPA induced and normal macrophage RNA samples. Interestingly, a very strong hybridisation signal was detected from total RNA isolated from RA induced and uninduced HL60 cells. This has also been reported by Polasky et al (1985). They postulated that some post-transcriptional event may control lysozyme expression in HL60 cells. Indeed Northern analysis reveals that transcripts homologous to the lysozyme probe, in uninduced and RA induced HL60 cells, are not of a specific size and appear larger than the normal lysozyme mRNA. This may indicate that control of lysozyme gene expression operates at the level of RNA processing.

Alternatively, the results from the Northern blotting analysis could be artifactual. The lysozyme probe was of chick origin and is thought to share only 40% homology with the human lysozyme sequence. Due to this the filters were washed in low stringency wash buffers. This could have resulted in non-specific binding of the hybridisation probe. Clearly investigation into the mechanism(s) controlling lysozyme expression in HL60 cells will require production of a human lysozyme probe.

C) F6 RNA Expression During HL60 Differentiation

i) Abundance of F6 RNA in HL60 Cells

F6 cDNA represents RNA which is highly abundant in uninduced HL60 cells, moderately abundant in RA induced cells but barely detectable

following TPA induction. Analyses of HL60 RNA by Northern blotting, revealed that F6 cDNA shared homology with a large range of RNA transcripts in both total whole cell RNA and in poly(A)$^+$ RNA. Probe for F6 also hybridised in a non-specific manner to total genomic DNA. This pattern of hybridisation has been reported by others during the analysis of repetitive DNA elements (Kramerov et al, 1985).

By examination of the distribution of F6 RNA, in nuclear and cytoplasmic RNA fractions isolated from uninduced HL60 cells, it was found that F6 transcripts were highly abundant in nuclear RNA but barely detectable in cytoplasmic fractions. Repetitive sequences have frequently been associated with hnRNA but found at very low levels in cytoplasmic RNA. This has been demonstrated in HeLa cells, in cells from Chinese hamster and also in murine cells (Ryskov et al, 1973; Jelinek, 1978 and Elder et al, 1981).

F6 cDNA was conclusively proved to contain sequences homologous to repetitive elements following sequencing of the cDNA. It was found that approximately 400 nucleotides of F6 bore 80% homology to the Alu family consensus sequence. Alu sequences make up 3-6% of the human genome, with approximately 500 000 copies per haploid genome. The Alu elements are dispersed throughout the DNA with no apparent clustering (Schmid and Jelinek, 1982).

ii) The Alu Repetitive Elements Compared to F6

Alu family members have highly conserved sequences with individual members differing by an average of only 10% from other members. Most of the variations are point mutations which appear randomly distributed throughout the length of the repeat unit (Deininger et al, 1981). Figure 30 represents the sequence of F6 cDNA and that of another Alu family member Blur 13. Approximately 80% homology exists between the two sequences and the variations consist of short sequences, as reported by others.

A stretch of unique sequence of approximately 130 nucleotides in length is associated with the Alu repeat element in F6. This stretch shared no homology with known sequences when compared with those in the Microgene Databank System. Alu repeats, as mentioned previously, are highly represented in hnRNA (Elder et al, 1981). They have been

described in the intervening sequences of many genes, either in introns which are spliced out or in 3' non-coding regions. The 12 Kb c-sis gene contains three Alu family members (Dalla Favera et al, 1981). Similarly, the 56 Kb stretch of human DNA encoding the epsilon, A-gamma, G-gamma, delta and beta globin genes have also been shown to contain seven Alu family members (Coggins et al, 1980). Others have reported the occurrence of Alu sequences in coding regions. Zabarovsky et al (1984) described Alu repeat insertions in the coding region of the human c-mos pseudogene and Quattrochi et al (1986) have demonstrated Alu sequences in the mRNA transcripts of human cytochrome P-450. However, the Alu element present in F6 cDNA hybridises to a number of different sizes of transcripts making it impossible to distinguish the sequence specific to F6. In the light of the data described above it would appear that F6 originated from non-coding sequences. Procedures used to isolate the poly(A)$^+$ RNA transcripts prior to preparation of the cDNA library relied on oligo(dT) recognition of the polyadenylated tail; F6 has a high adenosine content which may have led to the isolation of this sequence during oligo(dT) separation of poly(A)$^+$ RNA. Alternatively, the F6 RNA could have been aggregated with poly(A)$^+$ hnRNA and therefore was co-purified with poly(A)$^+$ RNA.

iii) Repetitive Elements and Differentiation

The abundance of transcripts homologous to F6 cDNA were found to decrease dramatically following three days of TPA treatment. Others have also reported inactivation of transcription of repeat sequences during terminal differentiation (Chou et al, 1984). This could indicate a general decrease in transcription in these cells. However, Colbert et al (1983) reported no overall decrease in poly(A)$^+$ RNA isolated from TPA induced HL60 cells. This indicates that TPA induced HL60 cells do not contain reduced amounts of RNA. Therefore the loss of transcripts which share homology to F6 does not appear to be the result of a general mechanism operating on transcription but may indicate a function for these transcripts during the differentiation process.

It has been postulated that repetitive sequences may have a function in the regulation of gene expression (Davidson and Britten, 1979). The observation that short period interspersed repeats are found in

close proximity to protein coding sequences in DNA and that their transcripts are found associated with nuclear RNA has been used to argue that they function either to regulate transcription or to regulate the processing of hnRNA to mRNA (Jelinek et al, 1980).

Others have described stimulation of transcripts containing sequences sharing homology to repetitive elements when quiescent mouse fibroblasts are stimulated with serum or epithelial growth factor and insulin (Hodgson et al, 1983; Edwards et al, 1985). Scott et al (1983) have discovered a B2 sequence (the murine equivalent of the human Alu sequence) in the 3' non-coding region of a class 1 major histocompatibility antigen which is induced in cells transformed by both viral and chemical agents. Murphy et al (1983) have analysed total foetal RNA at different stages of mouse embryogenesis and shown that transcripts homologous to B2 repeat sequences peak sharply at the 9-10 day stage indicating that these transcripts might have a role during development. They also observed that a B2 probe detected a broad range of transcripts whose abundance decreased upon induction of differentiation of F9 teratocarcinoma cells by RA. These results imply that the transcription of repetitive elements is regulated during development and differentiation.

It is interesting to draw a comparison between the transcripts described in F9 teratocarcinoma cells by Murphy et al (1983) and those detected by F6 cDNA probes in HL60 cells. In both systems a decrease in transcripts homologous to repetitive sequences has been demonstrated following differentiation. HL60 cells are believed to be representative of early progenitor cells on the pathway leading to granulocytic differentiation. Therefore transcripts containing repetitive elements may be specific to immature cell types. Aberrant expression of developmentally significant genes has been proposed as an important factor in cancer and cellular transformation (Pierce et al, 1978). It is therefore interesting that the transcripts homologous to repetitive sequences appear abundant in cell lines derived from cancer cells, and also in normal murine development.

The fact that RA induced HL60 cells did not show total loss of transcripts homologous to F6, may be due to the high proportion of cells which are apparently not terminally differentiated by this agent (40%-50%). Uninduced HL60 cells were found to contain 4 times

as much F6 RNA than RA induced HL60 cells and 16 times more F6 RNA than TPA induced HL60 cells. However, the correlation between decrease in transcripts which show homology to the Alu repetitive elements and increase in terminally differentiated cells during HL60 induction by TPA may be casual rather than causal. It has been thought that Alu sequences are parasitic and that their occurence throughout the human genome has been by chance and not by design. It is proposed that Alu sequences have the ability to self-prime reverse transcription of their RNA transcripts (Jagadeeswaran et al, 1981). It has also been proposed that these sequences may share many of the characteristics attributed to bacterial insertion sequences, as they contain regions of homology to elements which appear to be required for transposition (Calos and Miller, 1980). Therefore, it has been postulated that, following reverse transcription, newly synthesised Alu cDNAs are capable of insertion into the genome. Insertion at some chromosomal loci could have severely disrupted the normal cellular physiological functions hence only those sequences which would not affect the cell phenotype would be tolerated by the cell. Alu repetitive elements have been preserved during recent primate evolution with little change to their sequences (Sawada and Schmid, 1981). This has been postulated to indicate an important cellular function for these sequences. However, it has been argued that without any selective pressure put on Alu sequences the repetitive element would remain relatively stable. It is therefore unclear if Alu repetitive sequences have any function during differentiation and development.

D) C6 RNA Expression During HL60 Cell Differentiation

i) The C6 cDNA

The C6 cDNA represents transcripts which are highly abundant following granulocytic differentiation of HL60 cells. However, DMSO induced cells contain levels of C6 homologous RNA that are approximately 5 fold that detected in RA induced HL60 cells. Interestingly, C6 cDNA hybridises to two transcripts of 2.4 kb and 1.3 kb respectively, in total whole cell RNA from DMSO induced HL60 cells. Only the smaller of the two transcripts can be clearly detected in uninduced cells.

From initial analysis of the recombinant plasmid pUC8C6, following an Eco RI + Bam HI restriction digestion of the plasmid DNA, C6 cDNA was demonstrated to yield three fragments of 220 base pairs, 190 base pairs and 160 base pairs respectively. These fragments had not arisen from internal restriction digestion sites within the C6 cDNA sequence, or from a conjugate plasmid construct. Examination of the recombinant plasmid DNA hybridised to pUC8, by electron microscopy, revealed only one region of non-homology between the two DNAs. This region was estimated to be 200 - 300 base pairs in length. This supported the evidence suggesting that pUC8C6 was not a conjugate of recombinant plasmids and also suggested that the C6 cDNA was only approximately 200 base pairs long. This indicated that the three fragments were not produced from restriction digestion of internal Eco RI or Bam HI sites within a larger cDNA fragment.

From sequencing data, generated from the C6 fragments, it was concluded that all the fragments contained a stretch of 85 nucleotides in common. This was a unique sequence which bore no homology to sequences recorded in the Gene Databank system available in this laboratory. The only apparent difference between the fragments was the length of thymidine residues, the 190 base pair fragment was 30 nucleotides shorter and the 160 base pair fragment 60 nucleotides shorter than the 220 base pair fragment. When used individually, as hybridisation probes, each fragment hybridised to the 2.4 kb and the 1.3 kb transcripts in RA and DMSO induced HL60 total cellular RNA indicating the similarity between the fragments.

It is highly unlikely that the C6 fragments arose because of contamination by another plasmid. During the preparation of the plasmid DNA the transfected cells containing pUC8C6 had been colony-purified twice by selection of distinct cell colonies, which were well separated from other colonies. This eliminated any chance of cross-contamination. The fact that the cDNA fragments are identical, except for the length of thymidine residues, may therefore indicate that the fragments were initially derived from an ancestral plasmid which may have undergone some recombination event during early stages following the construction of the cDNA library. Plasmid DNA was amplified by transfection and subsequent growth in E. coli JM83 cells as recommended by Viera and Messing (1982). These cells are $recA^{+}$ hence recombination events can occur (Messing, 1979).

However, it is interesting to note that if these fragments were generated by recombination events, the sites of recombination would appear very consistent as a similar fractionation pattern has been detected from a number of different pUC8C6 DNA preparations.

Alternatively, the fragments may have been generated by loss of stretches of thymidine residues due to regions of instability along the length of the thymidine sequence. Certain combinations of nucleotides are thought to be intrinsically unstable, for example GC rich regions, but this has not been reported for stretches of A or T. A role for secondary structure in the generation of these fragments also seems unlikely. Fractionation of the fragments on denaturing agarose gels did not alter the pattern observed when C6 cDNA was fractionated normally which suggested that secondary structure was not a factor in producing the C6 cDNAs

C6 cDNA is highly unusual, not only because of the multiple fragments generated on Eco RI + Bam HI digestion of pUC8C6, but also because of the length of thymidine nucleotides detected following sequencing of these fragments. The initial synthesis of the cDNAs during the construction of the cDNA library, followed the method of Wickens et al, (1980). The synthesis of the single stranded cDNA from the poly(A)$^+$ RNA template by Klenow fragment was oligo(dT) primed using a 17mer. The primer was added to a concentration which would maximise binding along the length of the polyadenylated tails of the isolated mRNA molecules and which would, in turn, minimise the synthesis of long stretches of thymidine nucleotides. However, the lengths of thymidine nucleotides detected in the C6 cDNA fragments are very long, approximately 135 nucleotides in the 220 base pair fragment to 75 nucleotides in the 160 base pair fragment.

ii) A Relationship between the 1.3 kb and the 2.4 kb C6 RNAs

Both transcripts detected by C6 cDNA must share the 85 base pair sequence found in all three C6 cDNA fragments in common. This suggested that these transcripts are related in some way. Southern blotting analysis of total cellular HL60 DNA hybridised to C6 probe indicated that the transcripts were derived from a unique DNA sequence. Experiments to determine the location of the transcripts in the different cellular compartments revealed that only the 2.4 kb

transcript could be detected in nuclear RNA isolated from RA induced HL60 cells. Therefore, from this data, it can be tentatively proposed that the 2.4 kb transcript may be an unprocessed form of the 1.3 kb transcript. Since both transcripts can be detected in total whole cell RNA and the 2.4 kb transcript is detected in nuclear RNA it can be assumed that the 1.3 kb RNA must be located in the cytoplasmic cell fraction.

During the processing events leading from hnRNA to mRNA, evidence suggests that following transcription of the hnRNA these molecules are polyadenylated (Breathnach and Chambon, 1981). This event appears to precede splicing out of intron sequences, however the two processes of polyadenylation and splicing are not mechanistically coupled (Zeevi et al, 1981). It is therefore interesting to note that both the 2.4 kb and the 1.3 kb C6 RNAs are present in poly(A)$^+$ RNA isolated from RA induced HL60 cells. The removal of introns from hnRNA containing multiple intron sequences does not appear to occur in a strict order. The order of removal is thought to be kinetically determined as excision of some introns is intrinsically faster than others. This would result in partially spliced molecules with different intron patterns. This has been demonstrated by others. For example, Roop et al (1978) demonstrated that multiple species of ovalbumin RNA could be detected in chick oviduct nuclei only. These RNAs ranged from 1.5 - 5 times larger than the ovalbumin mRNA. Only the mRNA was detected in the cytoplasmic RNA fraction. Nordstrom et al (1979) reported similar results when analysing high molecular weight nuclear RNA for ovamucoid RNA. Hence it could be speculated that the 2.4 kb C6 RNA is the polyadenylated, unspliced precursor of the 1.3 kb transcript.

The half-life of introns within precursor RNAs can vary from only a few seconds to 10 - 20 minutes (Keohavong et al, 1982). Therefore, the fact that the 2.4 kb C6 RNA can be detected by Northern blotting analysis indicates that, if indeed a splicing event is involved in producing the 1.3 kb transcripts, the intron(s) must have a relatively long half-life.

Both C6 transcripts increased in abundance during granulocytic differentiation in HL60 cells, however the 2.4 kb RNA levels increased by a much greater proportion than that of the smaller

transcript. For example, the level of the 2.4 kb RNA increased 5 fold during the 5 day RA induction period whereas the 1.3 kb RNA levels only doubled. During DMSO induction however the levels of the 2.4 kb RNA increased 18 fold during the 5 day induction period whereas the 1.3 kb transcripts doubled in abundance. It is interesting to note that although the level of the smaller RNA increased by a similar degree, during both DMSO and RA inductions, no similarity was found in the extent of increase of the 2.4 kb transcripts.

If the larger transcript is a hnRNA precursor of the 1.3 kb RNA, it is possible to explain the pattern of expression of both RNAs by slight modification of the simple kinetic model of mRNA splicing proposed by Pikielny and Robash (1985). They assumed that all reactions, during splicing, followed first order kinetics and that both precursor molecules and mature RNAs were at steady state. Briefly, they propose that if the rate of the splicing reaction is slower than the turn over of precursor molecules, most precursor RNAs will be degraded and the level of precursor RNA will be maximal and essentially independent of the rate of splicing. The level of mRNA would however be dependent on the rate constant of the splicing reaction. Conversely, when splicing is faster than the turn over of precursor RNA most precursor RNA would be spliced and the level of mRNA would be maximal. The amount of precursor RNA would then be inversely proportional to the rate of the splicing reaction. This model was based on data generated from observation of the abundance of precursor and mRNA molecules after specific mutations were introduced into the introns of yeast genes. It was observed that these mutations either increased or decreased the efficiency of the splicing reaction.

This model also indicates that threshold levels occur in splicing reactions and that the system can become saturated. The situation in the uninduced HL60 cell, in regard to splicing of the 2.4 kb C6 RNA, could be viewed hypothetically as a splicing reaction occurring under non-saturating circumstances. The production of 2.4 kb RNA would occur at a rate comparable to that of the splicing reaction producing the 1.3 kb transcripts. This would result in very low levels of the 2.4 kb transcripts but detectable levels of the 1.3 kb RNA. In effect, the rate of the splicing reaction would be greater than that of the transcription rate of the C6 gene. However, following

induction of HL60 cells by RA or DMSO, it could be proposed that the transcription rate of the C6 gene is increased. As a result, the level of 2.4 kb RNA increases dramatically but the rate of the splicing reaction remains the same. A bottleneck would form at the splicing stage as the reaction reaches saturation. This would result in high levels of precursor RNA with a concomitant increase in mRNA levels which would depend on cytoplasmic stability and other factors which have been proposed to influence the concentration of mature cytoplasmic RNAs.

Pikielny and Rosbash (1985) have also demonstrated that mutation in the sequence motifs in intron sequences in yeast genes could produce the effects described above. In many cases, however, such mutations did not result in reduction of cytoplasmic RNA but the sole effect was an increase in the level of nuclear precursor RNA. Therefore, the pattern of expression of C6 RNA observed during HL60 cellular differentiation may not reflect a normal situation but may be the result of a mutation in HL60 cells which affects the splicing efficiency of the C6 RNA. The study of the defects in the alpha and beta-globin genes present in several forms of thalassemia has demonstrated many examples of natural splicing mutants (Treisman et al, 1983). Alternatively, the abundance of the C6 RNAs may depend on a number of other characteristics which control gene expression, such as the stability of the precursor and mature RNA molecules and the rate of nucleo-cytoplasmic transport.

C6 RNA was detected in whole cell RNA isolated from a number of different human tissues. Interestingly. all the tissues which expressed these transcripts did so at levels below those detected in HL60 cells. It would be interesting to see if both the 2.4 kb and the 1.3 kb C6 transcripts can be distinguished by Northern blotting analyses of these RNAs or is only one species detected. This would indicate if the pattern of expression of C6 RNAs observed in HL60 cells reflected a normal rate limiting step in the splicing processes of C6 transcripts or if it is peculiar to HL60 cells.

RA induced HL60 cells did not contain such high levels of the 2.4 kb C6 RNA as their DMSO induced counterparts. However, the level of the smaller transcripts increased to a similar degree during treatment with both inducing agents. This probably reflects the differentiation

status of RA induced HL60 cells. RA is apparently less efficient at inducing granulocytic differentiation of HL60 cells during the 5 day period of treatment (see Chapter III, A). However, assuming the two C6 transcripts are related and that the relationship is that of unspliced precursor and mature transcript, it can be surmised that the rate of the splicing reaction must be similar in both RA and DMSO induced HL60 cells, hence the similar increase in 1.3 kb RNA abundance during the induction period. This would then indicate that the differences in the levels of the 2.4 kb transcripts at the completion of the induction process may be the result of differences in the rate of transcription of the C6 gene between RA and DMSO induced HL60 cells.

Obviously it will be of vital importance to prove categorically that the 1.3 kb C6 transcript is in the cytoplasmic fraction of DMSO and RA induced HL60 cells. It would also be interesting to determine the transcription rate of the C6 gene during HL60 granulocytic differentiation to see if it is increased. It might also be worth while returning to the RA5 cDNA library to try and identify other cDNAs representing C6 sequence. This would aid in the determination of the full length sequence of C6 and may shed light on the relationship between the 2.4 kb and the 1.3 kb transcripts.

E) F10 Expression During HL60 Cell Differentiation

i) The Effect of RA on the Levels of F10 RNA in HL60 and U937 Cells

F10 cDNA represents transcripts which become highly abundant during granulocytic differentiation of HL60 cells. From Northern analysis of total RNA isolated from HL60 cells harvested at different times throughout the 5 day treatment period required for RA or DMSO induction, F10 RNA was highly abundant during the final days of the induction period. This indicated that these transcripts may represent a mRNA specific to mature granulocytes.

However, when a direct comparison was made between the levels of F10 mRNA in 5 day RA induced HL60 cells and 5 day DMSO induced cells, the F10 transcripts appeared approximately 3 fold more abundant in RA induced cells. This was surprising since previous work, on the timing

of commitment and the appearance of mature cell characteristics during HL60 differentiation, had demonstrated that only 60% of the cells responded to RA induction stimulus (see Chapter III, 1).

Retinoids are vitamin A derivatives. It has been demonstrated that this vitamin is required for normal vision, reproduction and maintenance of differentiated epithelium and mucous secretion in the whole animal (Goodman, 1984). It is also generally believed that retinoids play a basic role in control of differentiation and that these agents suppress the malignant phenotype (Lotan, 1980). Therefore it is suggested that retinoids exert a hormone-like control of either cell proliferation or cell differentiation (Sporn and Roberts, 1984). Assuming that the increase in F10 RNA was associated with the differentiation process, the inconsistency in the extent of terminal differentiation observed following RA induction and the level of F10 RNA, could be explained if the expression of the F10 gene was influenced not only by the differentiation stimulus of RA but also by a vitamin / hormone effect exerted by this agent. To examine this possibility the human histiocytic lymphoma cell line U937 was used. These cells express monoblast-like characteristics, possess receptors for RA and can be induced by this agent into morphologically mature macrophage cells (Sundstrom and Nillsson, 1976; Olsson and Breitman, 1982). If the increase in abundance of F10 RNA, as seen during HL60 differentiation, was due in part to hormomal stimulation, F10 RNA levels in U937 cells would also increase in abundance on exposure of these cells to RA irrespective of the differentiation end-point (macrophages during U937 induction and granulocytes in HL60 cell cultures following RA treatment).

U937 cells were found to express F10 RNA but the levels of this transcript decreased with the length of time the cells were exposed to RA. This decrease also occurred concomitant with the appearance of monocytic differentiation characteristics. Therefore, it appears that F10 RNA expression is decreased as a response to a differentiation stimulus to develop to monocyte / macrophage cells in U937 cells. Interestingly, the levels of F10 RNA are also decreased following induction of monocyte / macrophage differentiation in HL60 cells but increased during granulocytic differentiation. This indicates that increase in abundance of F10 RNA, in HL60 cells induced to

granulocytes by RA treatment, was a differentiation response and not a response to hormone / vitamin effect of RA.

ii) F10 RNA, a Possible Differentiation Stage-Specific Transcript

In Chapter IV, (1), RA induction of HL60 cells was discussed. It was suggested that 5 days of treatment with this agent may not be sufficient to induce the majority of cells to terminally differentiate. When inducing agents are added to the growth medium the cells are not synchronous hence they do not respond to the differentiation stimulus simultaneously. Therefore, at early times following addition of the agent, some cells will have embarked on the pathway to terminal differentiation whilst others will not yet be committed. As the time of exposure to the induction stimulus increases more and more cells will terminally differentiate and ideally at the end of the induction period almost all the cells will express overt differentiation characteristics. However, if the induction is stopped at earlier times cells will be found distributed at different stages of differentiation, some will be terminally differentiated but others will not have completed the developmental process. This model for cell differentiation in a population of non-synchronous cells is displayed diagrammatically in Figure 55.

If it is assumed that 5 days of RA treatment is not sufficient to elicit a differentiation response from the majority of HL60 cells, the pattern of expression of F10 RNA can be explained if this transcript represents a stage-specific RNA of the granulocytic differentiation pathway. If F10 RNA was expressed by cells at late stages of granulocytic differentiation, but before terminal differentiation, this would suggest that at the completion of 5 days of treatment a large proportion of DMSO induced cells had passed this stage and had terminally differentiated ; hence lower levels of F10 RNA would reflect fewer cells at the hypothetical stage of differentiation. However, in RA induced cells a large proportion of cells may have just entered this stage and therefore would contain large amounts of F10 transcripts. If this model is correct increasing the treatment time of HL60 cells with RA should lead to a decrease in F10 RNA.

Others have reported results which fit such a model for

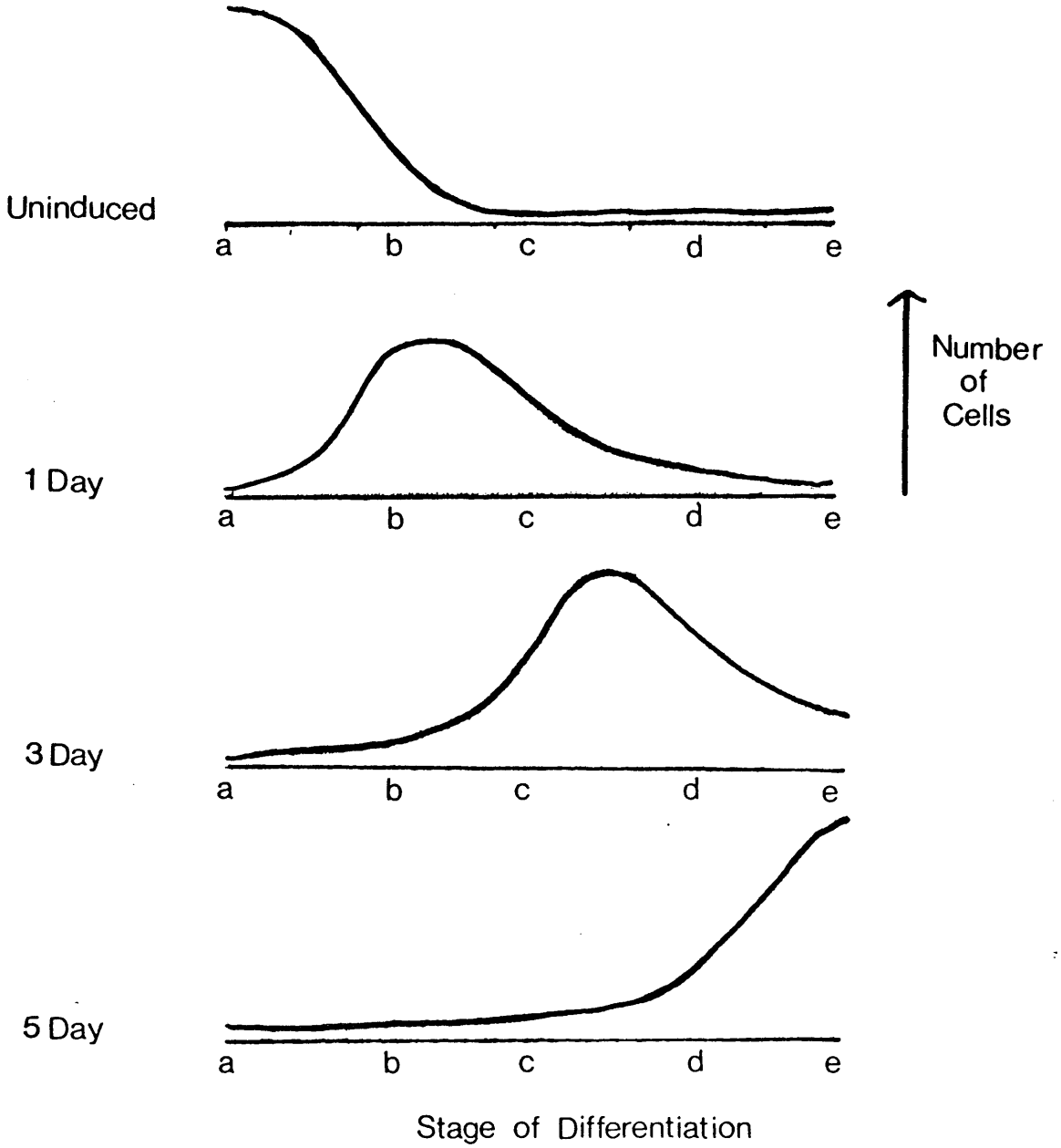


Figure 55 Schematic representation of a model for differentiation of non-synchronous HL60 cell populations.

The solid horizontal line represents a differentiation lineage; a, b, c, d, and e represent stages on the differentiation pathway. (a), uninduced or immature cells; (e), terminally differentiated cells, (b, c, d), are intermediate stages. Each diagram indicates the proportion of HL60 cells at each stage of differentiation after a period of treatment with a stimulus which induces the cells to differentiate, for example RA or DMSO. The majority of uninduced cells are found at very early stages of the differentiation pathway with very few cells at later stages, an almost homogeneous cell population. With induction to differentiate the proportion of cells at (a) diminishes with increasing time of treatment and the population of cells become more heterogeneous and are found spread throughout the intermediate stages of differentiation (b, c and d). After 5 days of treatment with RA or DMSO the majority of cells should have terminally differentiated but a proportion will still remain at early stages.

non-synchronous cell progression down a differentiation pathway. Boyd and Metcalf (1984) determined the progression of sodium butyrate induced HL60 cells along the monocyte / macrophage differentiation pathway during 7 days of treatment. Progression was determined by morphology, the expression of stage-specific antigens and changes in cell function. They demonstrated that at any one time following addition of the inducing agent to the growth medium cells were found at all stages of monocytic differentiation. With longer exposure to sodium butyrate more and more cells acquired mature cell characteristics but in a non-synchronous manner. For example, after 1 day of treatment approximately 19% of the HL60 cells resembled monoblasts, 4% monocytes and the remainder appeared promyelocytic. However, by day 3, 41% of the cells were monoblastic, 56% monocytes and only 3% promyelocytic. By day 7 100% of the cells were classed as monocytic.

From in situ hybridisation analysis of HL60 cells it appeared that uninduced cells did not produce F10 RNA. However, when treated for 5 days with RA, almost 90% of the cells hybridised to a radioactively labelled probe for F10 RNA. It was also found that approximately 30% of a 5 day DMSO induced HL60 cell culture hybridised to F10 cDNA but the remainder of the cells did not. A comparison was made between the degree of hybridisation of cells to F10 cDNA and the morphology of 5 day RA induced cells to see if any correlation could be found. It appeared that virtually all the cells hybridised irrespective of morphology. However, 5-10% of those cells which displayed terminally differentiated morphology of banded and segmented neutrophils did not hybridise indicating that they did not express F10 transcripts. This could suggest that F10 is indeed a stage-specific transcript during haematopoiesis. Analysis of the distribution of F10 RNA in a number of different human tissues also revealed that this transcript was only detected in abundance in HL60 cells and in particular RA induced cells. The transcript was not observed in normal peripheral blood leukocytes or in normal bone marrow. F10 RNA was also barely detectable in normal granulocytes. These results support the proposal that F10 RNA represents a marker for a late stage during granulopoiesis. The identification of stage-specific transcripts is important. Not only do they allow cell differentiation to be monitored in vitro, for example, during HL60 differentiation, but

they can also be used in conjunction with other methods to classify neoplastic tumours (Mills et al, 1987).

Many stage-specific markers have been determined for various differentiation systems. For example, Birnie et al (1983a) have reported a species of RNA, pCG14, which is localised to bone marrow cells and in particular to myelocytes during normal haematopoiesis. However cells in the peripheral blood of patients suffering from CGL were also found to express this transcript (Birnie et al, (1983b). This reflects the stage at which the leukeaemic cells in CGL patients were maturation arrested. Similarly, Warnock et al (1985) have reported an RNA which may be an early monocyte marker.

Others have reported the appearance of proteins during myogenesis which are found specifically during or just prior to the onset of myotube formation. These proteins include myosin heavy chain, myosin light chain, B-actin and tropomyosin (Moss et al, 1981; Shani et al, 1981; Garfinkel et al, 1982). Earlier stages in muscle cell differentiation do not produce these proteins but are found to produce non-muscle actin and other proteins which are not muscle specific (Schwartz and Rothblum, (1981).

During HL60 differentiation the classification of the terminally differentiated cells can be accomplished by the use of monoclonal antibodies which recognise membrane proteins which are specific to the terminal differentiated cell. For example, the monocyte specific monoclonal antibody FMC 17 occurs specifically after 3 days of 1,25-dihydroxyvitamin D_3 treatment of HL60 cells. At this time the cells show many of the characteristics of monocytes (Daniel et al, 1986). Similarly, it has been reported that the pattern of expression of the major histocompatibility complex (MHC) class II antigens is tissue-specific and varies during differentiation (Amatruda et al, 1987). For example, the HLA-DR MHC antigen is expressed by approximately 80% of cells from cell lines with very immature phenotypes such as KG-1 which is believed to be myeloblastic in origin (Koeffler and Golde, 1978). However HLA-DR expression is weak or absent from relatively mature cell lines such as HL60 or U937. Hence these antigens appear to be specific to very immature cell types during haematopoiesis.

To determine which stage F10 RNA expression is related to during haematopoiesis it will be necessary to analyse F10 RNA levels in populations of normal bone marrow cells. In situ hybridisation should enable a comparison to be made between cell morphology and hybridisation to F10 probe which will enable classification of the cell type(s) which express this transcript. It would also be necessary to determine the manner in which F10 RNA expression is regulated in HL60 cells. Initial analysis of the distribution of F10 RNA transcripts in the different cellular compartments of HL60 cells revealed that F10 transcripts were found in the nuclear RNA from uninduced HL60 cells but not in nuclear RNA isolated from RA induced HL60. In these cells the transcripts were present in the total whole cell RNA only. Therefore, it can be speculated that the F10 RNA is present in the cytoplasmic RNA of these cells but has to be proved categorically. If this is found to be the case F10 RNA may represent a transcript whose expression is regulated at post-transcriptional levels, possibly at the stage of nucleo-cytoplasmic transportation.

4 GENE EXPRESSION IN RELATION TO TIMING OF COMMITMENT IN HL60 CELLS

A number of different parameters were used to measure the proportion of committed or terminally differentiated cells during myeloid differentiation of HL60 cells (Chapter III, 1). From these results it is was deduced that 24-96 hours were required for the maximum number of cells to become committed to granulocytic differentiation in HL60 cultures exposed to DMSO or 8-24 hours for commitment to macrophage differentiation. I have analysed the level of abundance of a number of different transcripts throughout induction of HL60 cells by TPA, DMSO and RA. Figure 56 and 57 represent graphs depicting the increase or decrease in the levels of these transcripts compared to the timing of commitment during HL60 differentiation. Only data generated from DMSO and TPA induced HL60 cells were used because of the difficulty in interpreting results from RA treatment.

A) TPA induction

The most striking feature during TPA induction of HL60 cells is the

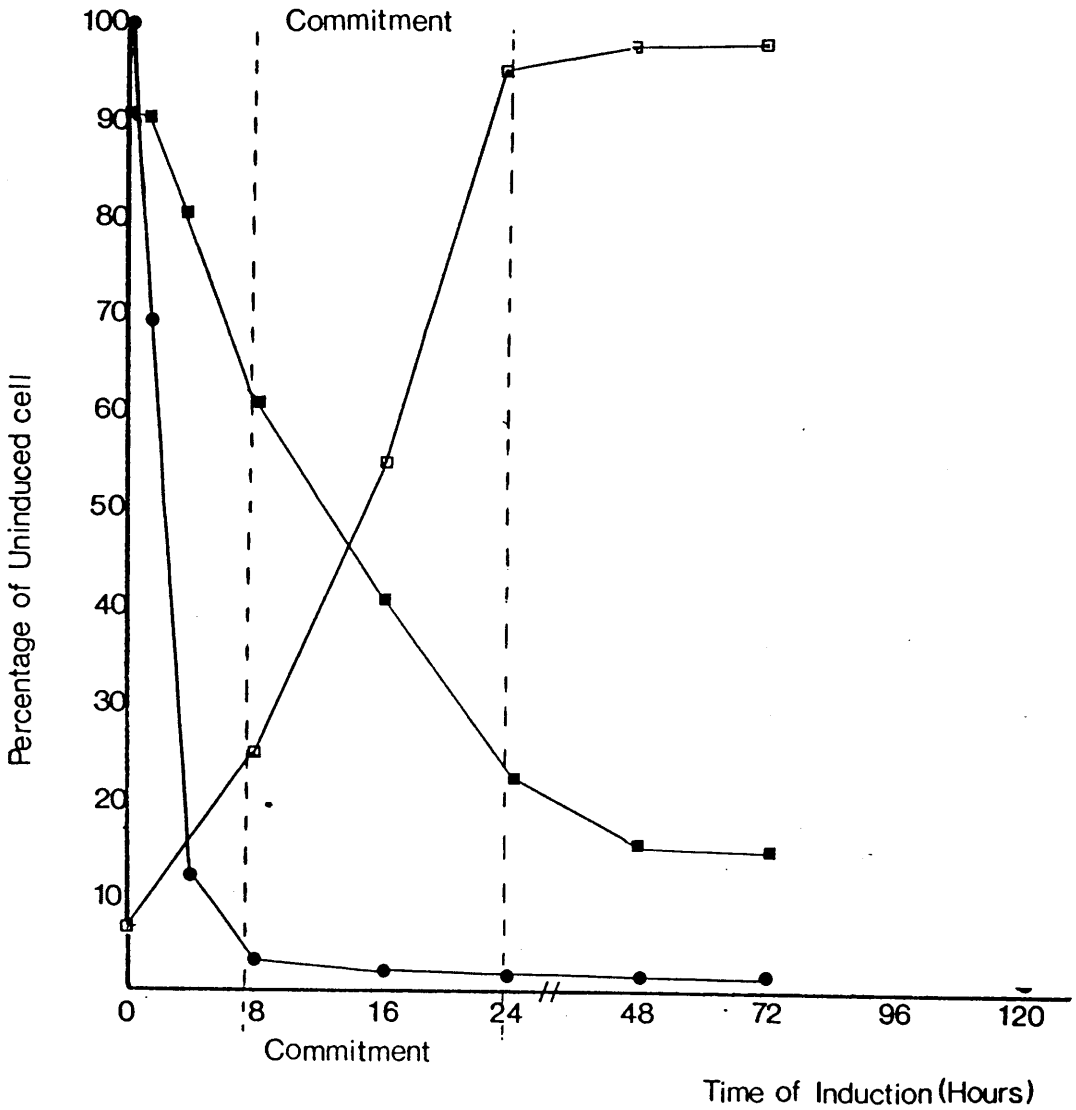


Figure 56 Comparison of the timing of commitment and the timing of changes in c-myc and lysozyme gene expression in TPA induced HL60 cells.

The timing of the changes in abundance of c-myc RNA and lysozyme protein have been compared to the timing of commitment in HL60 cells induced by TPA. This was 8-24 h, which is indicated by the dotted lines on the graph. The results are expressed as percentages calculated by taking the value for uninduced HL60 cells as 100%. (●), c-myc RNA; (□), lysozyme protein; (■), cell cloning potential.

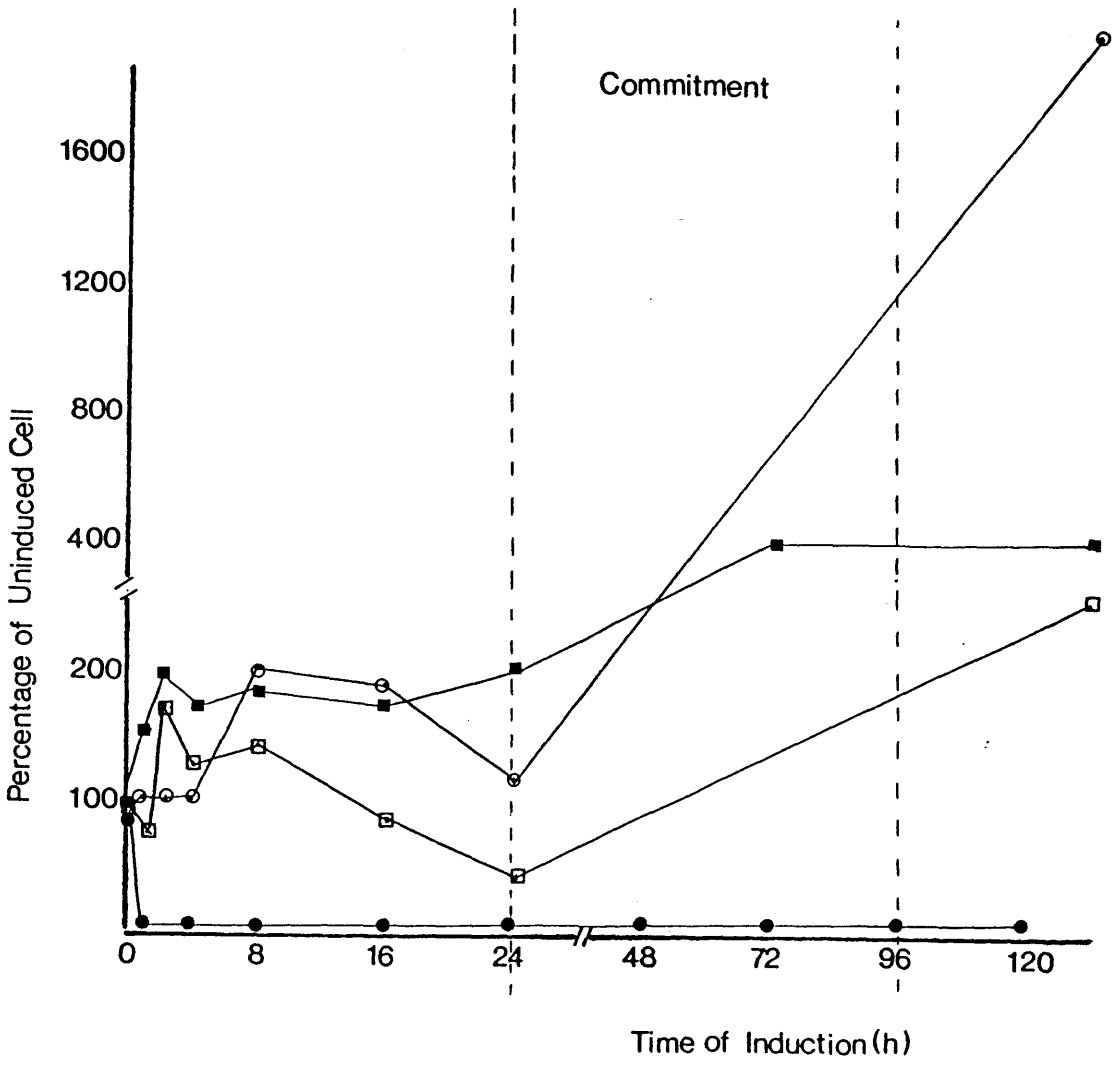


Figure 57 Comparison of the timing of commitment and the timing of changes in c-myc, F10 and C6 gene expression during DMSO induction of HL60 cells.

The timing of the changes in abundance of c-myc RNA, F10 RNA and C6 RNA have been compared to the timing of commitment in HL60 cells induced by DMSO. This was 24-96 h, which is indicated by the dotted line on the graph. The results are expressed as percentages calculated by taking the value for uninduced HL60 cells as 100%.
(●), c-myc RNA; (■), F10 RNA; (○), 2.4kb C6 RNA; (□), 1.3kb C6 RNA.

concomitant increase in lysozyme protein production with that of decrease in cell cloning potential (Figure 56). Hence there appears to be an inverse relationship between growth and differentiation in HL60 cells induced to differentiate by TPA. Daniel et al (1987) have also demonstrated a correlation between growth and differentiation of HL60 cells induced to monocyte / macrophage differentiation. However, they used the chemical agent 1,25-dihydroxyvitamin D_3.

c-Myc RNA becomes undetectable after only 4-8 hours of TPA induction of HL60 cells. It appears that loss of c-myc RNA bears an inverse relationship to differentiation but a direct relationship to proliferation in HL60 cells induced by TPA. This indicates that c-myc may therefore be closely involved in both the processes controlling growth and differentiation in HL60 cells treated with TPA (see Chapter IV, 2).

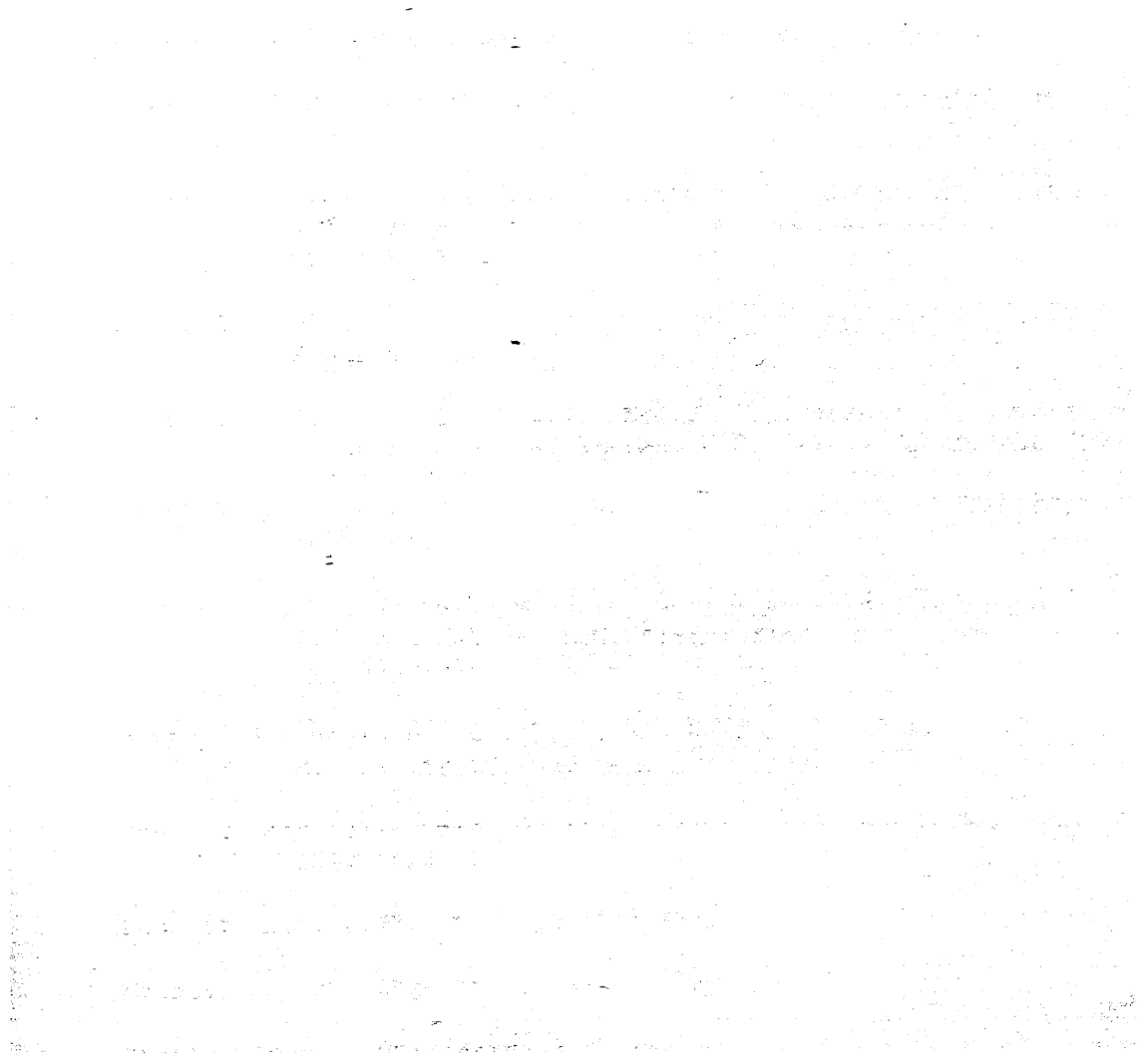
B) DMSO induction

Figure 57 represents a graph summarising the timing of changes in the levels of c-myc RNA, C6 RNA and F10 RNA compared to the timing of the commitment event in HL60 cells induced by DMSO. It is interesting to note that the increase in the levels of F10 RNA and C6 RNA occurred at approximately 24 hours following addition of DMSO to the growth medium. This correlates exactly with the timing of the commitment event estimated for HL60 cells undergoing granulocytic differentiation. As the levels of C6 and F10 RNAs increased the cloning potential of the HL60 cells decreased concomitantly. Similarly, increase in NBT positively stained cells (a marker for mature myeloid cells) bore a direct relationship with increase in F10 and C6 transcripts.

These results indicate that increase in F10 and C6 RNA levels bears an inverse relationship to parameters used to determine the proliferative capacity of HL60 cells but a direct relationship to the timing of appearance of terminally differentiated cells. Therefore it can be proposed that both F10 and C6 gene expression is directly linked to differentiation.

However, c-myc RNA becomes undetectable after only 1 hour of DMSO

treatment of HL60 cells. This event precedes both changes in growth and in differentiation of the cells. It would therefore appear that c-myc is not immediately and directly involved in the processes leading to induced differentiation of HL60 cells by DMSO. This is contradictory to the results obtained from TPA induced HL60 cells.



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