Characterization of a gene expressed during human myeloid cell differentiation

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CONTENTS

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ABBREVIATIONS

A	Adenine
ALL	Acute Lymphocytic Leukaemia
ANLL	Acute Non-Lymphocytic Leukaemia
ATP	Adenosine triphosphate
bp	base pairs
C	Centigrade
cDNA	complementary DNA
CFU	colony-forming unit
CGL	Chronic Granulocytic Leukaemia
CIP	Calf intestinal phosphatase
CLL	Chronic Lymphocytic Leukaemia
CsCl	Caesium chloride
CSF	colony-stimulating factor
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ds-	double-stranded
EDTA	ethylene-diamine-tetra-acetic acid
IL-3	interleukin-3
IPTG	isopropyl-β-D-thio-galactopyranoside
MOPS	sodium morpholinopropane sulphonic acid
mRNA	messenger RNA
NBT	nitroblue tetrazolium
PBS	phosphate-buffered saline

PEG	polyethylene glycol
PMA	phorbol myristal acetate
RA	retinoic acid
RNA	ribonucleic acid
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ss-	single-stranded
SSC	sodium chloride and sodium citrate
TPA	12-O-tetradecanoyl 13-phorbol acetate
Tris	trihydroxymethylaminomethane
tRNA	transfer RNA
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

LIST OF FIGURES

ABSTRACT

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Previous work using differential screening of a cDNA library representing poly A^+ RNA from the peripheral blood leukocytes of a chronic phase CGL patient had led to the isolation of cDNA clones representing mRNAs found at high abundance in this leukaemia compared to ANLL, CLL or normal peripheral blood leukocytes. The gene encoding one clone, pCG14, was also found to be expressed during normal human myelopoiesis in the neutrophilic myelocyte, a bone-marrow restricted neutrophil progenitor.

In this work the pCG14 cDNA was completely sequenced and shown to encode HNP3, one of a group of three closely homologous peptides called defensins. These peptides are found in abundance in the granules of neutrophils and are involved in destruction of micro-organisms. The human defensin peptides HNP1 and HNP3 were shown to be encoded by two mRNAs differing at only one coding position that could be distinguished by PCR, and the regulation of the abundance of these mRNAs during myelopoiesis was investigated using induced differentiation of HL60 cells as a model system.

Four different genes, two encoding HNP1 and two encoding HNP3, were isolated and one gene encoding HNP1 was characterized in detail. It was shown to be comprised of three exons and its 5'-regulatory region shown to contain two functional promoters. Using the presence of a single nucleotide change which distinguishes the coding regions of HNP1 and 3 genes, giving rise to a polymorphic Hae3 restriction enzyme site, individuals were shown to contain variable relative amounts of HNP1 and HNP3 genes.

To explain these results it was hypothesized that individuals contain four defensin genes per diploid cell, one tandem pair on each chromosome 8. This model was tested using DNAs from a family where it was shown that defensin genes were inherited as a pair.

SECTION 1 : INTRODUCTION

1. INTRODUCTION

1. INTRODUCTION

1.1 NORMAL HEMATOPOIESIS

Active hematopoiesis in humans begins extraembryonically early in development in the embryonic yolk sac with cells subsequently migrating to the foetal liver. By birth, however, the major site of hematopoietic stem cell differentiation has shifted to the bone marrow and lymphoid organs (Metcalf, 1971). The regulated production of blood cells is one of the most complex examples of multilineage differentiation in man. Essentially the process consists of a common set of highly proliferative pluripotent stem cells, residing mostly in the bone marrow, giving rise to large numbers of non-dividing red cells, neutrophils, basophils, eosinophils, monocytes, platelets and lymphocytes in the circulation. As many of these blood elements are short lived (for example, a few hours for neutrophils, a few weeks for erythrocytes), they must be continually replenished. Moreover, the levels of mature cells can respond dramatically to environmental stress. For example, in cases of severe infection, granulocyte counts can increase from the normal ~5000/ml to more than 50,000/ml, a variation corresponding to the production of 2 x 10^{11} cells which can occur within a matter of a few days (Williams *et al*, 1983).

1.1.1 Stem cells

It is thought that hematopoiesis originates from a multipotential stem cell, the main source of which by birth is the bone marrow. The stem cells are relatively few in number, but can persist throughout life by undergoing proliferation to produce daughter stem cells. This process of self-renewal is the distinguishing feature of hematopoietic stem cells and is a characteristic shared by stem cells in other regenerating systems (Potten, 1983). The assay *in vivo* for this type of cell was first developed by Till and McCulloch (1961) after it was recognised in the murine system that animals given lethal doses of irradiation (and thereby suffering bone marrow failure) could be saved by injection of unirradiated bone marrow cells (Ford *et al*, 1956) and that these animals were restored in all hemato-lymphoid cell types by cells of bone marrow donor origin. Till and McCulloch (1961) performed the first quantitative experiments on bone marrow restoration of lethally irradiated mice, showing that limiting numbers of bone marrow cells called CFU-S (colony forming units - spleen) gave rise to clonal colonies of myeloid-erythroid cells in the spleen and bone marrow of the irradiated hosts. Subsequent studies using chromosomal markers showed that each spleen colony was unlike any other and that certain distinguishing chromosomal markers in reconstitution experiments could be shared by cells of lymphoid as well as the myeloid lineages (Wu *et al*, 1967;

Abramson et al, 1977). Recently, Lemiska et al, (1986) confirmed and extended this finding using retroviruses as insertion markers to follow the progeny of single stem cells.

The spleen colonies in the irradiated mice also contained stem cells, moreover, since injection of cells from the spleen colonies into another lethally irradiated mouse also led to secondary spleen colonies in the recipient, shown by chromosomal markers to have been derived from the injected cells (Till and McCulloch, 1980). The vast self-renewal capacity of these stem cells can in fact allow repopulation of the entire hematopoietic system of one animal from a single stem cell (Till and McCulloch, 1980). In many respects then, spleen colony forming cells (CFU-S) posses the charateristics of stem cells. However, more recent work by Magli et al, (1982) has shown that CFU-S are heterogeneous; they include not only multipotent stem cells, but also more mature cells that have lost their capacity to fully re-establish hematopoiesis, although they often retain a multipotential nature.

Circulating blood itself has a low number of stem cells, although it characteristically lacks proliferative cells between the blast and mature cells in the developmental lineages (Metcalf, 1971). One implication of this observation is that committed cells must mature elsewhere, and indeed there is impressive evidence that only in the

appropriate hematopoietic inducing microenvironment (HIM) in hematopoietic tissue will stem cells proliferate and differentiate.

The question as to what proportion of bone marrow cells are stem cells has been answered using the irradiated mouse system. Boggs et al, (1982) have shown that infusion of as few as 10^4-10^5 marrow cells into an irradiated mouse is sufficient to restore hematopoiesis. Since a normal mouse has 3×10^8 marrow cells, the infused amount represents only 0.01% of this total. Since these transplant recipients have a normal lifespan and their hematopoietic system shows no evidence of decline with age (Schofield et al, 1986), this implies that there must be a vast proliferative reserve built into the hematopoietic system that would be sufficient to last several times the normal lifespan.

1.1.2 Committed cells

Like stem cells, the committed progenitor cells are distinguished by the progeny they produce. This distinction is generally made on the basis of an in vitro analysis in clonogenic soft-gel culture systems (Metcalf, 1977). For example, certain cells undergo proliferation and differentiation in vitro to produce colonies containing cells of several different lineages: neutrophils, megakaryocytes, macrophages, basophils and erythroid cells (Johnson, 1984). Such colonies can be obtained from all mammalian species so

far examined (mouse, rat, cat, dog, sheep, cows) including man. Since the colonies are clonal, the mature cells must be derived from a multipotent cell called colony-forming cell (mixed) or CFC-MIX. Moreover, some of the colonies contain cells that can be plated in soft gels to produce more mixed colonies, which means that some of the CFC-MIX can themselves undergo self-renewal. In fact, some of the colonies derived from CFC-MIX may contain CFU-S, which would indicate a considerable overlap between these two populations (Metcalf and Burgess, 1982). Not all colonies undergo self-renewal, however, perhaps indicating the likelihood of heterogeneity in self-renewal capacity of CFC-MIX.

Lineage - restricted progenitor cells are also classified on the basis of the progeny they produce in clonogenic soft-gel systems. Granulocyte-macrophage colony-forming cells, GM-CFC, undergo proliferation and development to produce netrophils and/or macrophages, depending on the growth stimulus used. The most primitive erythroid progenitor cells, BFU-E (burst-forming units - erythroid) can also undergo proliferation in vitro. The cells thus produced, CFU-E (colony-forming units - erythroid) migrate through the soft-gel system where they begin to synthesize hemoglobin thereby generating typical erythroid "bursts". Megakaryocyte, eosinophil and basophil colony-forming cells (Meg-CFC, Eos-CFC and Bas-CFC, respectively) can also be recognized by their ability to undergo proliferation and differentiation in vitro to produce mature progeny (Metcalf,

1977). All of these lineage-restricted progenitor cells are continuously generated from the multipotent stem cells and, in this way, a constant supply of mature cells is ensured and a balance is maintained throughout the different cell compartments. The central question of hematopoiesis, i.e. how this controlled process of self-renewal, commitment, proliferation and maturation is regulated, has been investigated in two main *in vitro* assay systems. These systems are the *in vitro* soft-gel culture system of Metcalf (Bradley and Metcalf, 1966) and the long-term bone marrow culture system of Dexter *et al* (1977). The first system has strongly underscored the importance of colony-stimulating factors (CSFs) in the regulation of hematopoiesis and will now be discussed.

1.1.3 Growth factors in hematopoiesis

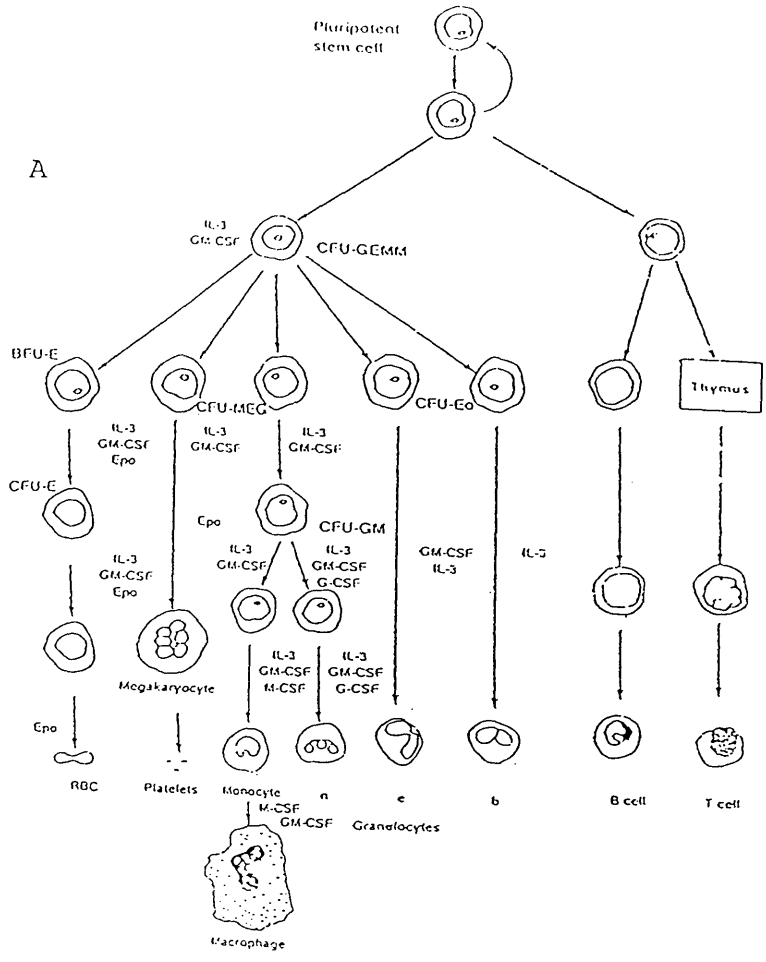
The development of semisolid culture systems supporting the clonal growth of hematopoietic cultures independently by Metcalf (Bradley and Metcalf, 1966) and Sachs (Ichikawa *et al*, 1966) and the recognition that hematopoietic precursor cells are unable to survive or proliferate *in vitro* unless specifically stimulated (Metcalf, 1984), led to the discovery of a group of specific regulatory glycoproteins, the colony-stimulating factors, that stimulate cell proliferation and at least some aspects of the functional activity of various hematopoietic sub-populations (Metcalf, 1984). These colony-stimulating factors were initially discovered to be

Fig.1 Schematic views of hematopoiesis.

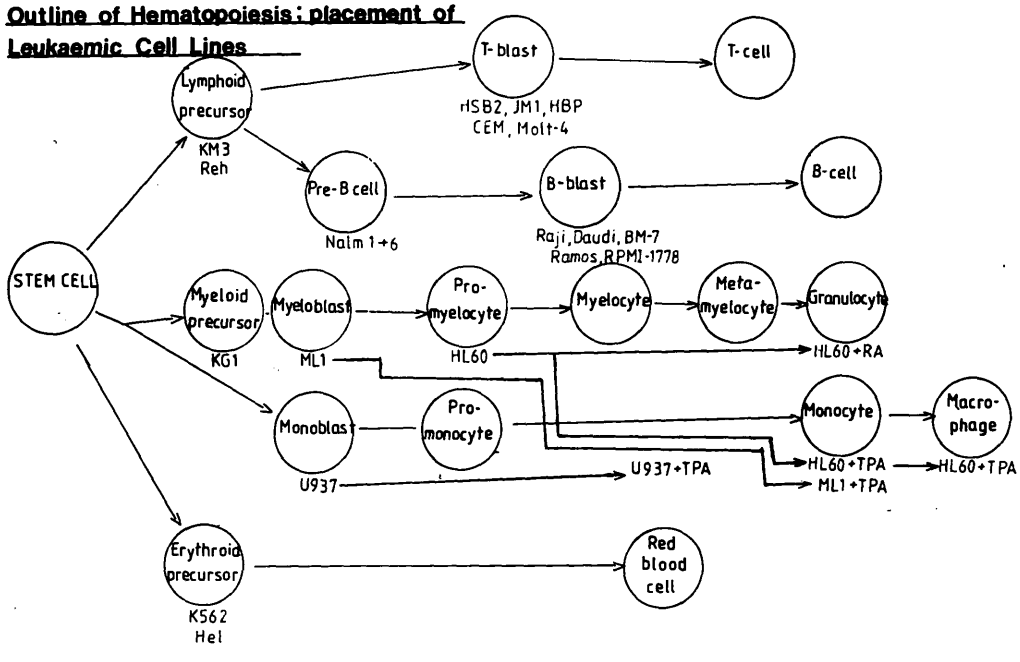
A: Interaction of colony stimulating factors (CSFs) with hematopoietic cells. The different progenitor cells shown are those identified by in vitro culture systems (see Section 1.1.3). CFU-GEMM (colony-forming-unit granulocyte-erythrocyte-monocyte-megakaryocyte), CFU-Meg (CFU-megakaryocyte), CFU-Eo (CFU-eosinophil), CFU-GM (CFU-granulocyte/monocyte), CFU-E (CFU-erythroid), and BFU-E (burst-forming unit-erythroid). The abbreviations for the hematopoietic lineages are : n, neutrophil; b, basophil; m, monocyte/macrophage; E, erythrocyte; e, eosinophil; M, megakaryocyte. The interactions of the different CSFs with the various lineages are as indicated. These interactions are based on analysis of mature cells found in colonies grown in the presence of CSFs. The sites of action are intended to indicate that at least some, but not all, progenitors of that lineage are responsive to the indicated CSF (Figure from Clark and Kamen, 1987).

B: This is a simplified model of hematopoiesis in which various stages of normal development are represented by leukaemic cell lines (where available) which resemble the normal cell stage, using a number of criteria such as reactivity with a panel of monoclonal antibodies, morphology, ultrastructure and histochemistry (see Section 1.2). Considering the myeloid cell lineage, HL60 cells resemble immature myeloid cells (promyelocytes) although they are bipotent in vitro (ie they can be induced to differentiate along both the granulocytic and the monocytic lineages) unlike normal promyelocytes present in bone marrow.

A


Pluripotent stem cell
IL-3 GM-CSF
CFU-GEMM
BFU-E
CFU-MEG
CFU-Eo
Thymus
IL-3 GM-CSF Epo
IL-3 GM-CSF
IL-3 GM-CSF
CFU-E
Epo
CFU-GM
IL-3 GM-CSF Epo
IL-3 GM-CSF
IL-3 GM-CSF G-CSF
GM-CSF IL-3
IL-5
Megakaryocyte
IL-3 GM-CSF M-CSF
IL-3 GM-CSF G-CSF
Epo
RBC
Platelets
Monocyte
n
e
b
B cell
T cell
M-CSF GM-CSF
Granulocytes
Macrophage

B


Outline of Hematopoiesis: placement of Leukaemic Cell Lines
Lymphoid precursor
KM3
Reh
T-blast
HSB2, JM1, HBP
CEM, Molt-4
T-cell
Pre-B cell
Nalm 1+6
B-blast
Raji, Daudi, BM-7
Ramos, RPMI-1778
B-cell
STEM CELL
Myeloid precursor
KG1
Myeloblast
ML1
Pro-myelocyte
HL60
Myelocyte
Meta-myelocyte
Granulocyte
HL60+RA
Monoblast
U937
Pro-monocyte
U937+TPA
Monocyte
HL60+TPA
ML1+TPA
Macro-phage
HL60+TPA
Erythroid precursor
K562
Hel
Red blood cell

present in the conditioned media from a variety of normal and leukaemic cell lines, or from various tissues or activated T-lymphocyte cell clones (Metcalf, 1984). Several different colony-stimulating factors have been distinguished through careful analysis of the cell types found in hematopoietic colonies grown with various sources of growth factor activity (Bradley and Metcalf, 1966; Metcalf, 1984; Pluznik and Sachs, 1965). In the best characterized murine system, four major types have been identified. Two of these have proven to be relatively lineage-specific; that is, colonies grown in the presence of granulocyte-CSF (G-CSF) consist largely of neutrophilic granulocytes and their precursor cells (Metcalf and Nicola, 1983), whereas those grown in the presence of macrophage-CSF (M-CSF, CSF-1) consist largely of macrophages (Stanley and Heard, 1977). In contrast, the colonies grown in the presence of multi-CSF (IL3) are generally found to contain many different lineages (Shrader, 1986; Ihle et al, 1983), whereas those found in cultures grown in the presence of granulocyte-macrophage-CSF (GM-CSF) are found to contain neutrophilic granulocytes, macrophages, eosinophils and other cell types. These results, in a model proposed by Metcalf and Nicola (1983), define a hierarchy of progenitor cells along the various lineages. In this model, 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, GM-CSF is presumed to interact additionally with somewhat earlier progenitor cells that are still capable of differentiating into neutrophils,

eosinophils and monocytes. The multiplicity of activities attributable to IL3 is believed to be a consequence of its ability to support the growth of cells from relatively early pluripotent progenitors to mature cells of multiple lineages. In humans a set of four analogous factors have been described each having properties similar to those of the corresponding murine G-, M-, GM- or Multi-CSFs (Metcalf, 1984, 1985; Yang et al, 1986).

Before considering the role of these polypetides in vitro and in vivo in more detail, it is worthwhile mentioning that analogous regulatory proteins exist for hematopoietic cells in other lineages. For example erythropoietin has the general properites of a CSF (Miyake et al, 1977; Jacobs et al, 1985). This molecule is thought to act in a classical hormonal fashion to promote proliferation and hemoglobinization of fairly mature erythroid progenitor cells (CFU-E). Production of this hormone occurs in the kidney and is regulated by the number of mature erythrocytes in the circulating blood and their oxygen-carrying capacity (Adamson et al, 1978) i.e. via a classical feedback loop control system. Other less well characterised polypeptides with the general properties of CSFs include T-cell regulator (IL2) (Taniguchi et al, 1983), B-cell growth factor (Yoshizaki et al, 1983) and a factor stimulating eosinophil differentiation in vitro (Sanderson et al, 1985).

The most extensively characterised growth factors, G-CSF, M-CSF, GM-CSF and IL3 not only commit progenitors to differentiate, they are also required for cell survival *in vitro* in the absence of bone marrow stromal cells. This is not, however, a withdrawal into a quiescent state as can be seen in other growth factor systems such as PDGF and EGF stimulation of cultured fibroblast cell lines; hematopoietic cells cultured in the absence of growth factors die. Thus, *in vitro* these growth factors subserve several functions; survival, proliferation and differentiation, which are perhaps not mutally exclusive events. How then do these factors exert their pleiotropic effects *in vitro*? cDNAs encoding all the above factors from mice and humans have been cloned and have been found to be unrelated in nucleotide or amino acid sequence. By analogy with other growth factor systems, one would expect there to be specific receptors for the colony-stimulating factors and, since each factor is unrelated to any of the others, one prediction would be that there would be at least four such receptors. This prediction is indeed borne out. A single class of specific membrane receptor for M-CSF exists of mol.wt. 165KD (Morgan and Stanley, 1984; Guilbert and Stanley, 1980) with receptor numbers highest on cells of the moncyte-macrophage lineage (Byrne *et al*, 1981; Chen *et al*, 1984). This receptor is structurally related, and possibly identical to, the c-*fms* proto-oncogene product that is expressed at high levels in mature macrophages (Sherr *et al*, 1985). M-CSF can sometimes support the proliferation of granulocyte clones (Metcalf and Burgess, 1982) and in

accord with this is the finding that some cells of the granulocyte series also contain the 165KD M-CSF receptor (Shaddock et al, 1983). Interestingly, receptor numbers are relatively high (up to 50,000/cell) on some macrophage tumour cell lines, whilst lower numbers (3,000-15,000/cell) are found on mature monocyte-macrophages from various normal sources (Byrne et al, 1981; Stanley and Guilbert, 1981). The reason for this is unknown, but may represent an adaptation to cell culture.

A similar situation occurs with G-CSF. There exists a single receptor for this growth factor on the membranes of all murine granulocytic cells, receptor density increasing as the cells mature to postmitotic polymorphs (Nicola and Metcalf, 1985). Receptors are however demonstrable on some cells of the monocytic lineage, but not on eosinophil, erythroid or lymphoid cells. Average receptor numbers for this growth factor are low (5-500/cell), with an apparent dissociation constant for binding of 60-80 pmol/L. These two observations may be significant, since they imply that G-CSF is able to exert half-maximal proliferative effects on responding cells at low receptor occupancy.

For GM-CSF a slightly different situation exists. It appears that normal marrow cells have both high and low-affinity receptors (average number 70/cell and 350/cell respectively) (Walker and Burgess, 1985). All granulocytes, monocytes and eosinophils have receptors for GM-CSF, the

numbers decreasing with increasing maturation and with mature eosinophils exhibiting half the receptor numbers of neutrophils. Significantly, no receptors are found on lymphoid or erythroid cells. Despite the high and low affinity types, it appears that there exists a single receptor of mol. wt. 51KD (Metcalf, 1986).

For IL3, high numbers of receptors of a single class have been observed on continuous hematopoietic cells lines dependent on this growth factor (Palaszynski and Ihle, 1984). Lower receptor numbers are observed on normal marrow cells (receptor numbers being ~50-1,000/cell) with all granulocytic, monocytic and eosinophilic cells containing the receptor. Similar to the situation with the GM-CSF receptor, receptor numbers decrease with increasing maturation although in this case eosinophils exhibit twice the number as mature neutrophils. Lymphoid or nucleated erythroid cells, moreover, do not contain the receptor.

It appears then that most murine granulocyte-macrophage cells simulatenously exhibit receptors for three or four CSFs. The function of this redundancy is not known, but clearly has some bearing on notions of lineage commitment. Perhaps, for example, the binding of one factor to its cognate receptor somehow commits the cell to a particular lineage and also ensures that other lineage choices are obviated. There is some evidence from Walker et al (1985) that there exists a hierarchical decrease in abundance of growth factor receptors

in response to factor binding. They have found that although there is no direct competition for receptor binding between the CSFs, binding of IL3 to its receptor can lead to down-modulation of receptors for all other CSFs. Binding of GM-CSF to its receptor, furthermore, does not influence IL3 receptor binding but can down-modulate receptors for G-CSF and M-CSF. Finally, high concentrations of M-CSF down-modulates GM-CSF receptors and high concentrations of G-CSF down-modulate receptors for M-CSF. The mechanism of this down-modulation is unknown, but could clearly operate at a number of levels of control, transcriptional or post-transcriptional. Elucidating exactly what controls are operating in such an *in vitro* system is of paramount importance in understanding the molecular basis of commitment, but will clearly be a difficult task simply due to the small numbers of pluripotent stem cells that can be isolated and characterised biochemically. Model systems consisting of one cell type which can grow in cell culture in sufficient numbers for molecular analysis may provide some insight as to genetic changes underlying commitment and development and will be discussed later.

It is apparent, then, that IL-3, GM-CSF, G-CSF and M-CSF can support the survival, proliferation and development of the appropriate target cell *in vitro*. This does not, however, prove that they exert the same influence *in vivo*. Both M-CSF and GM-CSF have been detected in the serum and urine of animals and man (Metcalf, 1986). In terms of roles for these

molecules *in vivo*, it may be significant that substantial increases in the circulating concentrations of both GM-CSF and M-CSF are observed in animals treated with bacterial lipopolysaccharides, and this correlates with GM progenitor cell proliferations (Whetton and Dexter, 1986). Additionally, the CSFs can stimulate a number of functional aspects of mature granulocytes, eosinophils and macrophages. These effects include survival, cell mobility, shape, phagocytic activity, synthesis of biologically active molecules, antibody-dependent cytotoxicity, autofluorescence and expression of various membrane markers (Metcalf, 1984).

The accumulated evidence suggests that *in vivo* the role of the colony-stimulating factors is to protect the body from infective organisms (helminths, protozoa and bacteria) and to stimulate accessory cells in the immune response. Thus high local or circulating levels of these growth factors may represent the outcome of a peripheral immune response rather than having a bearing on marrow myeloporesis.

1.1.4 Growth factors and commitment

It is clear that hematopoietic differentiation in Metcalf's *in vitro* system requires the presence of growth factors that are progressively restricted in their biological activities and target cells. However, as previously mentioned, one of the fundamental unanswered questions concerns the process by which cells acquire the capacity to

respond to growth factors and just how the commitment process operates. For example, multipotential stem cells can respond to IL3, but not to M- or G-CSF. Presumably this means that stem cells hve receptors that respond to IL3 but not to M- or G-CSF. It is possible, then, that commitment involves activation for the gene coding for the M- or G-CSF receptor, or it may be that control operates at the translational/post-translational level.

Whichever is the case, the expectation would be that IL3 could then initiate the production of all the subsequent lineages. In the ideal situation, one would wish to isolate such IL3-responsive pluripotent stem cells and determine whether they are indeed expressing each of the receptor genes at the mRNA and protein levels and also whether they are expressing each receptor in a correct membrane orientation suitable for interaction with growth factor. If such a system were available, one would be able to test directly whether simple non-expresion of growth factor receptor genes was involved in commitment. Such systems, however, are as yet not available.

Some recent studies on a particular effector of hematopoiesis, a molecule known as hematopoietin-1 (H1) may, however, be shedding light on the nature of commitment in vitro. This factor, first described by Bradley and Hodgson (1979) acts synergistically with other known growth factors to recruit more colony-forming cells although by itself it has

no growth stimulatory effect. Using fluorenscence-activated cell sorting (FACS) to obtain marrow cells enriched for CFU-S, Lord and Spooncer (1986) have shown that in the absence of Hl, 4-40% of cells produce multilineage colonies in vitro when cultured in the presence of IL3, i.e. this proportion has the receptor for, and can respond to IL3. When this stem cell-enriched fraction is cultured in vitro in the presence of only Hl they die, but when IL3 and Hl are combined, however, 50-100% more colonies are produced than in the presence of IL3 alone. Therefore, it seems that Hl acts to recruit more stem cells to an IL3-responsive state. Most interesting, however, is the interaction between M-CSF and Hl. With M-CSF alone no colonies are produced from the CFU-S-enriched fraction and the stem cells die as would be expected if they did not express receptors for M-CSF. However, in the presence of M-CSF and Hl, as many colonies develop as in the presence of IL-3 and Hl. One interpretation of this is that Hl facilitates the development of multipotent stem cells to a stage at which they can respond to M-CSF, i.e. Hl commits the cells to an M-CSF-responsive state. An immediate possibility then would be that Hl and possibly other as yet unidentified hematopoietic regulators may act by inducing the expression of particular growth factor receptors on pluripotent stem cells. Perhaps by allowing these cells to express the receptors for all the growth factors, the particular lineage finally chosen could be decided by which growth factor is present and can actively bind to its receptor. This hypothesis cannot be totally correct, however, since Lord and

Spooncer (1986) have also shown that preincubation of enriched stem cells with H1 followed by its removal and addition of M-CSF alone leads to stem cell death; both must be present simulatenously to show the enhanced colony development effect. This would seem to imply that M-CSF receptor expression per se is not sufficient to maintain a response to M-CSF. If, then, the role of Hl is indeed to induce expression of growth factor receptors, as this work implies, the question of how this is accomplished is a fundamental, but as yet unresolved, one.

1.1.5 Role of the hematopoietic microenvironemnt

The extreme complexity of hematopoiesis is further exemplified by the results from another in vitro sytem, the long-term bone marrow culture system of Dexter et al (1977). The development of this system, in which hematopoiesis can be maintained, not just for a few days as in the soft-gel in vitro system, but for several months, arose from experiments and observations on the importance of the hematopoietic microenvironment in hematopoietic development.

Initial experiments indicating the role of the hematopoietic microenvironment were on mice with congenital genetically determined macrocytic anaemias, termed Steel (SL) and W, and used the irradiation/spleen colony-forming assay previously described. CFU-S are greatly reduced or absent in W anaemic mice, but are present in normal numbers in SL mice (Russel and Bernstein, 1966), consistent with the view that

lesions in W mice are due to intrinsic defects in hematopoietic stem cells, whereas in SL mice the defect resides in the hematopoietic microenvironment into which these cells migrate (Russel and Bernstein, 1966; Bernstein et al, 1968; Bennett et al, 1968; Sutherland et al, 1970). Thus, W mice, both irradiated or unirradiated, support spleen-colony formation after inoculation of bone marrow from non-anaemic litter-mates or from SL mice, whereas spleen-colony formation does not occur in SL mice after normal marrow inoculation.

The hematological defects in W mice can, thereore, be cured by an inoculum of marrow cells from SL mice, and the macrocytic anaemia of SL mice can be reversed after grafting of neonatal spleen or whole bone marrow from W anaemic mice as a source of non-defective hematopoietic microenvironment.

In Dexter's in vitro system, stem cells and committed progenitor cells can be shown to be present in culture, indicating that the system supports both self-renewal and commitment to differentiation. Bone marrow stromal cells, in the form of an adherent multilayer, are the crucial feature of this in vitro system, forming a mechanical and functional support in a similar manner to that occurring in vivo (Dexter and Allen, 1983). Moreover, specific hematopoietic cell/stromal cell interactions seem to be reproduced in this system. Thus when adherent layers of stromal cells prepared from the bone marrow of SL mice are used to support hematopoiesis in these cultures the hematopoietic stem cells

do not survive. However, when marrow cells from SL mice are added to normal adherent layer bone marrow stromal cells, survival of stem cells and hematopoiesis can take place (Dexter and Moore, 1977). This result proves that the hematopoietic microenvironment is located in the bone marrow and also that the hematopoietic defect in the SL mice is in the stromal cells of the bone marrow.

A second feature of these long-term cultures is that hematopoiesis is sustained in the absence of added growth factors. Since it is known that isolated hematopoietic cells (grown in soft-gel clonogenic systems) die in the absence of growth factor, this would imply that the marrow stromal cells provide the necessary extracellular matrix and growth factors essential for establishment of hematopoiesis. Furthermore, the production of these regulatory molecules is obviously stringently controlled in these long-term cultures, since homeostasis is maintained for many months, i.e. a balance is maintained between stem cells self-renewal/differentiation and growth and development of the committed progenitor cells. How then is this exquisite regulation acheived? Several points have emerged concerning the interactions of stromal cells (which comprise a heterogeneous collection of various cell types such as endothelial cells, fibroblasts, adipocytes and macrophages) with hematopoietic cells (Dexter et al, 1977 and 1984). Firstly, the hematopoietic cells must be in direct contact with the stromal cells to permit their survival, growth and development: if they are prevented from

attaching to the stroma, they die. Secondly, discrete stromal elements show characteristic interactions with the different maturing myeloid cells and with each other. Dexter has suggested (Dexter and Spooncer, 1987) that this might be indicative of different environmental 'niches' existing that could specifically modulate lineage development. In this regard, it may be significant that extracellular proteoglycans produced by stromal cells have been found to bind GM-CSF and in this way possibly mediate stromal cell/hematopoietic cell interactions (Gordon et al, 1987; Roberts et al, 1987). How might such niches (better described as hematopoietic inducing microenvironments (HIMs)) ensure that cells of only one lineage type are retained? An immediate possibility is that cells of a particular lineage might somehow bind to HIMs through specific adhesion molecules in the extracellular matrix (ECM). A possible candidate for such a molecule, haemonectin, has been described by Campbell et al (1987). This protein, of approximately 60KD relative molecular mass, is a lineage-specific attachment molecule for granulocyte lineage cells and is found only in bone marrow ECM. Lastly, stromal cells from drug-treated animals or congenitally anaemic mice may be compromised in their ability to support hematopoiesis, which indicates that some myeloproliferative disorders may in fact represent a defect in the stromal cell environment rather than the hematopoietic target cells.

1.1.6 Role of growth factors in long-term marrow cultures and in vivo

The expectation that marrow stromal cells will themselves produce growth factors is a strong one. However, if they do produce growth factors, these are not normally released as diffusible regulatory molecules since both Dexter *et al* (1977) and Quesenberry *et al* (1984) have shown that, not only is direct contact of the hematopoietic cells with the stroma required to stimulate hematopoiesis, but that media conditioned by the growth of marrow stromal cells contains little or no colony-stimulating factor activity. The implication of this is that growth factors, presumably produced by stromal cells, represent surface-bound molecules that exert their effects through direct cell-to-cell contact. In this respect, it is worthwhile to note that Gough *et al* (1986) have suggested that a variant cDNA clone of IL3 with an upstream exon 14kb from the main body of the gene may encode a larger IL3 present on the cell surface. This could conceivably be produced by differential splicing of the IL3 mRNA in stromal cells.

One immediate question is whether the growth factor stimuli operating in long term cultures are the same factors defined in the soft-gel clonogenic assays. Initial (unpublished) *in situ* hybridisation studies by Dexter have failed to detect mRNAs for IL3, GM-CSF or G-CSF in marrow stromal cells of hematopoietically active cultures (Dexter and

Spooncer, 1987). Thus, either these particular factors are not synthesized by stromal cells (and as a corollary, they are not essential for stromal cell-associated hematopoiesis) or they are produced at levels below the limit of detection by present molecular techniques but in biologically active amounts. This latter possibility is not as unlikely as it appears when it is borne in mind that receptors for hematopoietic cell growth factors on target cells are few in number (100-1000) and that relatively few receptors (5-10%) have to bind a growth factor to elicit a maximal response (Nicola and Metcalf, 1985; Park et al, 1986). Dexter has also found, perhaps significantly, that one of the factors secreted in appreciable amounts by stromal cells is hematopoietin-1 (H1). This factor may act in helping progenitor cells to be growth factor-responsive in situations where growth factor alone initiates no response.

These results, showing that stromal cells by themselves are capable of stimulating hematopoiesis in the absence of detectable growth factors, could be interpreted as implying that some of the growth factors previously described (IL3, GM-CSF, G-CSF, M-CSF) may in fact represent in vitro artifacts rather than in vivo biological response-inducers or modifiers of hematopoiesis. Recent results using recombinant factors, however, are not in agreement with this interpretation. Injection of IL3 into the mouse can cause a rapid recruitment of quiescent stem cells (CFU-S) into the cell cycle (Lord et al, 1986). Continued administration of IL3, moreover, leads

to a major disturbance in the kinetic behaviour of stem and progenitor cells in the bone marrow; it also causes the mouse spleen (normally a lymphoid organ) to increase in size and cellularity, become actively myelopoietic, and show a dramatic increase in the numbers of CFU-S and committed progenitor cells (Metcalf et al, 1986; Kinder et al, 1986). The response to G-CSF is also dramatic in the mouse, inducing a 10-20 fold increase in circulating blood leukocyte levels and increasing the total number of multipotent stem cells. Studies in primates treated with GM-CSF have reported effects similar to those in mice. Donahue et al (1986) have shown, for example, that administration of GM-CSF to normal macaque monkeys leads to a tenfold rise in circulating leukocyte levels. The evidence is clear, therefore, that growth factors can have some role in hematopoiesis, although whether they do under normal circumstances is unclear.

1.2 NORMAL MYELOPOIESIS

Three major compartments of differentation during normal myelopoiesis have been distinguished. As previously described (Section 1.1.1) the most primitive compartment is characterized by pluripotent stem cells. These stem cells have a large proliferative potential including the potential for self-renewal, and serve as the cellular basis for a self-maintaining hematopoietic clone containing multiple lineages. The second major compartment of differentiation in the marrow consists of progenitor cells committed to a single hematopoietic lineage. Although these cells have great proliferative potential and have acquired sensitivity to the regulatory mechanisms of their lineage, they are not self-renewing. When triggered by specific stimuli, they undergo terminal differentiation and become effector cells with various functions. The third and most familiar stage of myelopoiesis consists in cells identifiable by morphologic features e.g. a characteristic nuclear configuration and the presence of obvious cytoplasmic granules. These cells are either fully mature PMN or those undergoing the last few divisions leading to maturity.

1.2.1 Cell biology of myelopoiesis

Myeloid cells are located in humans in three main locations: bone marrow, peripheral blood and tissues. Bone marrow is the site of the important processes of proliferation

and terminal maturation of neutrophilic granulocytes (from committed cells, myeloblasts, to mature PMN). Proliferation, consisting in approximately 5 divisions (Bainton, 1977), takes place only through the first three stages of neutrophil maturation (myeloblast, promyelocyte and myelocyte). At the myelocyte stage, the cells become 'end cells' (cells no longer capable of mitosis) and enter a large storage pool. About 5 days later they are released into the blood where they generally circulate for about 10 hours. Their fate after they have migrated to tissues is generally senescence and they generally live for only 1-2 days (Bainton, 1977).

Prior to the development of clonogenic soft-gel culture systems and the culturing of primitive hematopoietic cells and their differentiation in response to colony-stimulating factors (Section 1.1.3), the main descriptions of the cell biology of myelopoiesis came from examination of suitably stained smears of bone marrow material by microscopy. This approach has been instructive and has led to the identification of distinctive stages of myeloid development during which immature committed cells (myeloblasts) differentiate to mature PMN.

The immediate precursor of mature granulocytes is a cell called the metamyelocyte. Morphologically this cell contains an indented or horseshoe-shaped nucleus with extensive clumping of chromatin. The metamyelocyte does not divide and it not normally found in peripheral blood.

Preceding the metamyelocyte is the myelocyte. The myelocyte stage begins with the formation of the first specific granules (the sites of strorage of a number of proteins involved in PMN function including lysozyme, collagenase, lactoferrin and alkaline phosphatase) and ends when the cell has a full complement. Late myelocytes can be firmly classified as neutrophilic, eosinophilic or basophilic because of the characteristic staining of the granules, classification is more difficult in earlier stages. All myelocytes can be readily distinguished from precursors of monocytes, lymphocytes and megakaryocytes. The myelocyte is a fairly large cell, 16-25 um in diameter with a rounded nucleus. The chromatin is mostly loose, though some clumping appears late in this stage, and one or two nucleoli are usually present. Myelocytes do divide and 1-2% of cells are typically in mitosis. Early myelocytes have deeply basophilic cytoplasm, but this is lost during the stage and late myelocytes have faintly pink cytoplasm. The specific granules synthesized during this stage of myeloid development are formed by the Golgi complex. They vary in size and shape and are typically spherical ($\sim$ 200 nm) or rod-shaped (130 x 1000 nm). Kinetic studies on human cells indicate that about three divisions occur at this stage of maturation (Cronkite and Vincent, 1969).

The earliest cell that can be identified with certainty as a neutrophil precursor is the promyelocyte. This stage of maturation is characterized by the production and accumulation

of a large population of peroxidase-positive granules that vary in contour and size; most are spherical (~ 500 nm), but there are also ellipsoid, crystalline forms as well as small granules connected by filaments. Morphologically, promyelocytes are of rather variable size (15-20 um) with a large, rounded central nucleus, usually containing one or two nucleoli, and a small amount of deeply basophilic cytoplasm.

Overall, granulocyte precursors constitute about 60% of all the cells in bone marrow. Of this about 5% are promyelocytes, 12% myelocytes, 22% metamyelocytes and 20% mature PMN. These figures are, in fact, averages with a very considerable variance from individual to individual. They also vary within an individual in response to infection.

1.2.2 Neutrophils: cellular and functional aspects

Neutrophils, the terminally differentiated end-cells of myelopoiesis are the most abundant human white blood cell (typically 50-60%) in peripheral blood. These cells are involved in a number of body processes including inflammation and destruction of invading microorganisms. These cells show a variety of behaviours, the most important of which, chemotaxis, allows the cells to cross two cell boundaries (to leave bone marrow and enter tissues) to reach sites of tissue damage and infection. The total amount of neutrophils can be regulated such that their number in peripheral blood can vary more than ten-fold. Infection, inflammation, and stress have been shown then to increase the

rate of production of neutrophils from precursors. Additionally, such conditions can shorten the time usually required for each stage of neutrophil maturation, decrease the time mature neutrophils reside in the bone marrow and result in the release of immature neutrophil precursors into the circulation.

Neutrophils are equipped with a variety of specific proteins and reactive compounds which allow their specific responses to infection. The most prominent cytoplasmic constituents of neutrophils are the granules, which are visible by light microscopy and have given the cell its alternative name of 'granulocyte'. There is considerable variation in granule morphology among species, but at least in the rabbit and in man there are two main types.

The azurophil granules constitute 10-20% of the total population. In electron micrographs these granules are dense, with a diameter of about 0.5 um and are surrounded by a unit membrane. The specific granules, the other main granule type, are smaller (0.2 um), stain faintly pink with Wright's stain, and are also surrounded by a unit membrane. The two granule types can be separated by ultracentrifugation and their constituents have been analyzed. Many of the constituents of azurophil (primary) and specific (secondary) granules have been identified. Primary granules contain two of the cell's most abundant proteins, defensins and myeloperoxidase, involved in oxygen-independent and -dependent

microbial killing, respectively. The secondary granules also contain antimicrobial proteins including lactoferrin (involved in sequestration of iron, required for bacterial growth) and various collagenases.

Both primary and secondary granules are enveloped, thus protecting the cell from intracellular damage. The envelope surrounding granules is also vital to the processes of phagocytosis and degranulation whereby micro-organisms are ingested by neutrophils into phagocytic vescicles which fuse with granule membranes allowing direct contact of granule constituents with microorganisms. The molecular nature of this phenomenon is complex and only partially understood. The initial recognition process involves a number of specific proteins covering the microorganism and receptors on the neutrophil surface. Wright and Douglas (1903) first showed that human serum contains both heat-stable and heat-labile factors that dramatically enhance the ability of blood neutrophils to ingest Staphylococci. These factors, which include IgG and complement C3, were later shown to act by binding to bacteria. Neutrophils recognize the coated bacteria via specific receptors for IgG and C3, and phagocytosis follows. Phenomenologically, phagocytosis is a process whereby, once a microorganism is attached to the PMN, pseudopods pass around each side of it, meet, and then fuse together. The microorganism is enclosed in a pouch of cell membrane, the inner layer of which was originally the outer layer of the cell membrane. The phagocytic vesicle is

originally connected to the cell surface by two opposed layers of cell membrane but these soon disappear freeing the vescicle with its contained microorganism.

It was first observed by Hirsch and Cohn (1960) that, following phagocytosis *in vitro*, there occured a loss of neutrophil granules and that the extent of loss varied with the number of microorganisms phagocytosed. Careful electron microscopy studies have shown that engulfment of particles is accompanied by the assembly of contractile elements around the developing vescicle which are sequentially removed starting at the apex of the vescicle as it moves towards the apex of the cell (Stossel, 1988). Granules then fuse with the vescicle and discharge their contents (Zucker-Franklin and Hirsch, 1964; Weissmann *et al*, 1971).

There is evidence that degranulation is an ordered process. The neutrophil contains at least two and probably three morphologically distinct populations of granules and there is evidence that secretion from them may be under separate control (Lew *et al*, 1986). Specific granules, then, seem to interact with the phagosome earlier than do the azurophil granules and perhaps contribute the first events of microbial killing such as removal of iron (by lactoferrin) and breakdown of the outer envelope of bacteria (by lysozyme).

1.2.3 Neutrophil antimicrobial mechanisms

One of the main functions of neutrophils is their capacity to ingest and destroy organisms, both bacterial and fungal (Klebanoff and Clarke, 1978; Spitznagel, 1977). Neutrophils destroy invading microorganisms by two principal mechanisms. One of these depends on production of reactive oxygen intermediates (ROI) by stimulated phagocytes. These ROI such as H_2O_2 (hydrogen peroxide) and $OH^{\cdot}$ (hydroxyl radical) can act directly or in concert with other granulocyte components, such as myeloperoxidose, to damage or kill ingested microbes (Klebanoff and Clarke, 1978; Root and Cohen, 1981). In addition to such oxidative and peroxidative microbicidal mechanisms, neutrophils are equipped with antimicrobial mechanisms that can operate independently of ROI (Spitznagel, 1984; Elsbach and Weiss, 1983). Thus neutrophils obtained from patients with chronic granulomatous disease (defective in production of ROI), or neutrophils tested under anaerobic conditions, retain substantial efficacy against certain bacteria and fungi (Spitznagel and Okamura, 1983; Vel et al, 1984).

Studies of oxygen-independent microbicidal mechanisms, have shown that broken cell preparations from neutrophils can be fractionated, and that certain cell fractions are capable of killing a range of both Gram-positive and Gram-negative bacterial species. Hirsch (1956) was the first to show that a protein-rich fraction from acid-extracted neutrophils named

'phagocytin' had potent bacteriocidal activity. The discovery of the degranulation phenomenon whereby membranes of the granules and the phagocytic vacuole of neutrophils fuse covering phagocytosed bacteria with granular contents (Hirsch and Cohn, 1960; Section 1.2.3) further implicated granular substances in microbicidal activity. A more detailed dissection of neutrophil granule constituents with bactericidal activity by Zeya and Spitznagel (1968) led to the partial resolution of a phagocytin-like fraction into a series of apparently low molecular weight (3-8 KD) proteins with overlapping microbicidal activity towards a spectrum of both Gram-negative and Gram-positive bacterial species. Mechanistically, these O_2-independent activities appeared to be relatively fast-acting, with killing of microorganisms accompanied by little structural disorganisation of the organism (Elsbach and Weiss, 1988). Thus E.coli treated with crude neutrophil extracts, for example, have been shown to incur subtle envelope alterations involving the outer membrane (in less than one minute) after treatment, despite continued protein and nucleic acid metabolism for at least one hour (Elsbach et al, 1973).

A number of distinct microbicidal proteins have now been identified and some partially characterised. These proteins are found in both primary (azurophilic) and secondary (specific) granules. Some are typical lysosomal hydrolases

capable of degrading microbial macromolecules, such as elastase, collagenases, lysozyme, chymotrypsin-like protease (Cathepsin G), lipases, sulphatases and phosphatases. Others, amongst which include bacteri cidal permeability increasing protein (BPI), defensins (see Section 1.2.4) lactoferrin, B_{12}-binding proteins and major basic protein have no known catalytic activity.

Two of the best-studied microbicidal proteins involved in O_2-independent killing are defensins and BPI. BPI is present in primary granules of both human and rabbit neutrophils (Weiss et al, 1978; Elsbach et al, 1979) and is a possible candidate for the previously described activity that kills E.coli with minimal apparent initial damage to bacterial structure.

Human and rabbit BPI are similar in their biological effects and show identical target specificity. The protein is potently bactericidal toward a broad range of enteric Gram-negative bacterial species and strains. However, even at concentrations of up to 100-fold higher it is non-toxic to all of the Gram-positive bacteria and eukaryotic cells tested (Elsbach and Weiss, 1988).

Studies on BPI have addressed the question of how the molecule acts in such a way as to increase permeability within one minute despite continued metabolic activity of the microorganism for at least one hour. Mechanistically, four

elements have been identified in the interaction of BPI with susceptible bacteria; (i), insertion into the outer membrane of the Gram-negative bacterial envelope through electrostatic and hydrophobic forces; (ii), irreversible loss of colony-forming ability of the cells within 30 seconds of exposure to BPI; (iii), a reversible increase in the permeability of the outer membrane for normally impermeable hydrophobic substances; and (iv), a reversible and highly selective activation of enzymes degrading bacterial phospholipids and peptidoglycans (Elsbach and Weiss, 1988).

The specificity of BPI for Gram-negative bacteria and the highly discrete envelope alterations that are associated with its actions appears to be explained by the unique composition and organisation of the Gram-negative bacterial outer membrane with its negatively-charged lipopolysaccharides (LPS) which allow homing-in of the highly-basic BPI (Weiss et al, 1983). Although binding is necessary for the effects of BPI on bacterial multiplication and outer envelope it is not sufficient. Post-binding steps apparently involving hydrophobic interactions must follow (Weiss et al, 1983). This dependence on post-binding steps can be demonstrated by creating conditions that impede hydrophobic interactions without preventing protein binding. Under such conditions, the bacteria can be rescued from the bactericidal effect (Weiss et al, 1983). However, the primary irreversible event that leads to bacterial death is unknown.

1.2.4 Isolation and characterisation of defensins

Defensins, small peptides or Mr. < 4000 present in secondary granules of PMN from several species, are one of the main effectors of oxygen-independant bactericidal activity of neutrophils. These peptides were first described by Zeya and Spitznagel (1968) as bactericidal components of an arginine- and cysteine-rich cationic protein fraction from granules of rabbit PMN. Acid extraction of granule-rich cell fractions solubilizes large quantities of these cysteine-rich peptides that can be separated by preparative electrophoresis and HPLC into several closely homologous single peptides (Selsted et al, 1984; 1985a). Two have been purified to homogeneity from rabbit alveolar macrophages (MCP1 and MCP2, Selsted et al, 1983), six from rabbit peritoneal neutrophils (MCP1, MCP2, NP3a, NP3b, NP4, NP5, Selsted et al, 1985a) and three from human neutrophils (HNP1-3, Ganz et al, 1985; Selsted et al, 1985b). The primary structures of all eleven of these defensins have been determined and both the rabbit NP2 and the human HNP1 have been crystallized (Westbrook et al, 1984; Stanfield et al, 1988). All defensin peptides isolated thus far contain between 28 and 32 amino acids and share at least 11 residues, including six cysteines without apparent free -SH groups.

A number of roles have now been ascribed to these peptides including tumour cell cytolysis (Lichtenstein et al, 1986 and 1988) and macrophage chemotaxis (Territo et al, 1989). The most studied role of these peptides however is that of inactivation of bacteria, fungi and enveloped viruses. A large body of evidence now exists that these peptides can inactivate bacteria (Selsted et al, 1985b; Ganz et al, 1985; Viljanen et al, 1988; Lehrer et al, 1988a) certain fungi (Selsted et al, 1985c; Lehrer et al, 1985; Lehrer et al, 1988b) and enveloped viruses (Lehrer et al, 1985b, Daher et al, 1986).

1.2.5 Activities of human defensins against microorganisms

Three human defensin peptides have been isolated and characterized. As shown in Figure 12, HNP1 and HNP3 are identical in amino acid sequence apart from their N-terminal amino acid (alanine in HNP1, aspartic acid in HNP3, whilst HNP2 lacks this N-terminal residue (Selsted et al, 1985b). All three peptides can be isolated from PMN where they are present in large amounts (30-40% of total granule protein, Ganz et al, 1988). Ultrastructural immunochemistry has localized these peptides to the azurophilic granules (Ganz et al, 1985). Ganz et al (1985) have purified defensins HNP1, HNP2 and HNP3 and investigated their relative capacity to inactivate various bacteria. Interestingly, they found that a mixture of HNP1 and HNP2 were just as active as a mixture of

all three peptides. HNP3 was less microbicidal against four out of five of the bacterial strains tested, although against one strain it had similar activity to HNPsl and 2.

Daher et al (1986) have also addressed the question of the relative activities of HNP1-3. Since it was already well-established that neutrophils could inhibit viral replication (Rouse, 1981; Allison, 1974) they tested the three defensins' capacity to inactivate the enveloped human virus, HSV-1. In an in vito plaque-reduction assay, all 3 defensins had equal activity. Additionally, HNP1 was found to inactivate several other enveloped viruses such as cytomegalovirus, vesicular stomatitis virus and influenza virus. However two non-enveloped viruses, echovirus and reovirus were resistant to inactivation. Interestingly, a similar study has been performed with rabbit defensins (Lehrer et al, 1985b) and a functional homology shown between the human defensins HNP1, 2 and 3 and MCP1 and 2, two rabbit defensins expressed in both alveolar macrophages and neutrophils. MCP1 and 2 are identical in sequence except for an amino acid substitution (arginine in MCP1 for leucine in MCP2) (Selsted et al, 1983). In this respect they are similar to HNP1 and 3 which are also identical in primary sequence except for one residue (alanine in HNP1, aspartic acid in HNP3; Selsted et al, 1985b). Like HNP1 and 3, MCP1 and 2 are potent in activating HSV-1. Four other rabbit defensins NP3a, NP3b, NP4 and NP5 which are rather more divergent in sequence than MCP1 and 2 have, in contrast, no HSV-1-inactivating capacity (Lehrer et al, 1985b).

The three human defensins have also been tested *in vitro* for their ability to inactivate certain fungi (e.g. *C.albicans;* Lehrer *et al*, 1988b). The relative activities of HNP1, 2 and 3 when the peptides were tested against *C. albicans* were similar to that obtained with bacteria. Thus, both HNP1 and HNP2 had significant activity against fungi, whilst HNP3 had no detectable activity. Thus the single amino acid difference between HNP1 and HNP3 results in a marked difference in the capacity of the two peptides to inactivate both bacteria and fungi, though interestingly not enveloped viruses.

1.2.6 Other activities of defensin peptides

As well as their capacity for inactivating phagocytosed microorganisms, there is evidence that human defensins secreted from neutrophils might contribute to inflammatory lesions. Thus, *in vitro* HNP1-3 have been shown to be secreted when purified PMN are stimulated with PMA or opsonized (antibody coated) zymosan (Ganz, 1987). The secretion of these peptides *in vitro* indicates that they may be present *in vivo* at effective concentrations at sites of infection/ inflammation, though this has not yet been directly shown. What role might these peptides have there? One possibility is that they may contribute to cytotoxicity. Lichtenstein *et al* (1986), using a chromium-release assay to measure cell lysis, have shown that *in vitro* all three human defensins have the capacity to lyse a number of eukaryotic cell lines (eg.

WIL-2, IM-4, Raji, K562), target lysis occuring after a lag period of to 3-4 hours. They further showed (Lichtenstein et al, 1988) that binding of defensins to target membranes was a necessary precondition for lysis. However, subsequent internalization was then required before lysis could occur. Such internalization was mediated by the target cell and could be antagonized by agents that interfered with target cell energy metabolism, the cytoskeletal apparatus, lysosomal function or calmodulin-mediated activities. Okrent et al (1990) have addressed the possibility that such cell lysis by defensins might be involved in neutrophil-mediated cytotoxicity of the lung by showing that a crude fraction from neutrophils containing mainly defensins was cytotoxic to three lung-derived cell lines. They showed, moreover, that the cytotoxicity of this extract could be completely accounted for by its defensin content. Thus there is a strong possibility that the defensins play a role in tissue damage in the extracellular millieu. What defensins might be doing once bound to lipid bilayers is unknown. However, Kagan et al, (1990) have shown that, at least in artificial lipid bilayers, MCP1, a rabbit defensin similar in many respects to the human defensins, can integrate into the membrane and form voltage-dependent, weakly anion-selective channels. This MCP1-conductance was found to be moderately selective for monovalent anions such as Cl^- compared to Na^+, K^+, Ca^{2+} and SO_4^{2-}. This may be one way in which defensins cause membrane damage during lysis, but precisely how this is effected is unknown.

Lastly, another role in the extracellular millieu has recently been ascribed to defensins. Territo et al (1989) have shown that in vitro the defensins show significant chemotactic activity for monocytes. Purified preparations of each of the three human defensins have all been tested and HNP1 shown to have significant chemotactic activity (approximately 50% of that elicited by chemotactic agent, FMLP). HNP2, on the other hand, showed somewhat less activity (20% of FMLP), whilst HNP3 showed no activity. Again then, in common with their activities against bacteria and fungi, HNP3 has least activity; HNP2 more and HNP1 highest. Since this chemotactic effect is shown at very low defensin concentrations (10^{-4}M), the possibility is clear that defensins secreted by neutrophils may play a role in the recruitment of monocytes by neutrophils into sites of infection/inflammation.

1.3 THE HL60 CELL LINE AS A MODEL SYSTEM FOR MYELOPOIESIS

The human promyelocytic cell line HL60 was established from the peripheral blood leukocytes of a patient with acute promyelocytic leukaemia (FAB class M2) (Collins et al, 1977). The ability of these growth-factor independent cells to be induced to differentiate in vitro make this cell line a useful model for human myeloid differentiation. 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 PMN (Collins et al, 1977). Promyelocytes are normally found in bone marrow of humans and comprise immature granulocyte-committed cells (Fig. 1B). Fig. 1B indicates the position of HL60 cells and some other myeloid and non-myeloid cell lines in hematopoietic lineages used in this study.

1.3.1 HL60 cells are karyotypically abnormal transformed cells

HL60 cells are a suspension cell line and grow with a doubling time of 20-45 hours, depending on the subline. Morphologically, the cells are large, with characteristic large rounded nuclei containing typically 2-4 nucleoli and a basophilic cytoplasm with azurophilic granules. Most cells in a culture carry a variety of cell surface antigens characteristic of immature myeloid cells as shown by their reaction with an extensive panel of monoclonal antibodies (Graham et al, 1985). Amongst these markers include myeloperoxidase and acid phosphatase key proteins in

neutrophil antimicrobial function as well as receptors for insulin, transferrin and complement (Tsiftsoglou and Robinson, 1985; Collins, 1987).

Cytc genetically, HL60 cells show many karyotypic abnormalities. A variety of such abnormalities have been described, including monosomy, polyploidy and a variety of chromosomal translocations (Wolman et al, 1985; Donti et al, 1988). Moreover, a number of specific genetic lesions have been described. For example, the p53 gene on chromosome 17p13 has been largely deleted (Wolf and Rotter, 1985) and one allele of the GM-CSF gene on chromosome 5q21-q23 is rearranged and partly deleted (Huebner et al, 1985). Additionally, N-ras contains an activating codon 61 mutation in HL60 cells (Bos et al, 1984) responsible for the transforming activity of HL60 DNA in an NIH3T3 transformation assay (Murray et al, 1983).

Amplification of the c-myc oncogene has also been found in HL60 cells (Collins and Groudine, 1982) as well as in the primary leukaemic cells from which the cell line was established. The extent of amplification of c-myc varies from 4-30 fold in different HL60 sublines (Graham et al, 1985; Donti et al, 1988), with a concomitant variation in c-myc mRNA abundance (Graham et al, 1985). It seems possible that this overexpression of c-myc may have acted in cooperation with a mutated N-ras gene in the establishment of the HL60 line.

1.3.2 Induction of differentiation

From the viewpoint of understanding how myeloid genes such as those encoding defensins are controlled during differentiation, the most important property of HL60 cells is their capacity to differentiate *in vitro* to mature cells of the granulocytic or monocytic lineages. HL60 cells are capable of induced differentiation when treated with a number of different agents. They are not unique in this respect. A number of other established hematopoietic cell lines also posess this capacity. U937 cells, for example, a human monoblastic cell line, can be induced to monocytes by various agents; Friend murine erythroleukaemia cells can be induced to mature erythrocytes under appropriate stimulatory conditions (Olsson and Breitman, 1982; Rifkind *et al*, 1984). However, HL60 cells can be induced to differentiate along either of two distinct lineages, depending on the inducing agent used. If, as seems to be the case, the neoplastic transformation of HL60 cells is due to an uncoupling of the processes of proliferation and differentiation resulting in a 'maturation arrest' at a stage similar to that which occurs in the course of normal hematopoietic differentiation, the effect of agents which induce HL60 cells to differentiate may be to enable the cells to overcome this block in differentiation with the restoration of the differentiation programme.

HL60 cells can be induced to differentiate to both granulocytic and monocytic cell types. The most commonly used granulocytic inducing agents are retinoic acid (RA) and dimethyl sulphoxide (DMSO). HL60 cells, when cultured for 5 days in the presence of retinoic acid or 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/macrophage-like cells by exposure to other agents such as phorbol ester (TPA) or 1,25-dihydroxyvitamin D_3. Following the period of induction, the terminally differentiated cells possess many of the characteristics attributed to monocyte/macrophages (Rovera et al, 1979; McCarthy et al, 1982).

1.3.3 Morphological and functional changes during HL60 cell differentiation

The course of terminal differentiation of HL60 cells is accompanied by striking morphological, histochemical and immunological changes. These changes have been described by Collins et al (1977) and are characteristic of terminally differentiated myeloid cells. Thus incubation with DMSO or retinoic acid leads to a progressive decrease in the size of HL60 cells, a condensation of nuclear material and the appearance of kidney-shaped nuclei often with a reduction or disappearance of nucleoli characteristic of banded and segmented neutrophils. The nuclear/cytoplasmic ratio

decreases and the cytoplasm becomes more diffuse. The process of differentiation can be followed by analysis of these changes in cell morphology. It has been demonstrated that 24 hours of induction is required before the first morphological changes are observed, but by 4 days of treatment with either retinoic acid or DMSO cells displaying terminally differentiated cell morphology are present in culture (Breitman et al, 1980).

Concomitant with these morphological changes, there occur marked changes in histochemistry, including decreased myeloperoxidase activity and the appearance in culture of cells capable of reducing nitroblue tetrazolium, a marker for functionally mature granulocytes. 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. These adherent cells have a spindle-like morphology. Ruffling of the cell membrane is apparent and the cells possess blunt pseudopods characteristic of macrophages (Fibach et al, 1982).

A number of functional changes can also be detected in differentiating HL60 cells which reflect the functions of normal PMN and macrophages. For example, HL60 cells terminally differentiated to granulocytes have the ability to phagocytose fungi (C.albicans) and opsonized particles (Collins et al, 1978), as well as the ability to respond

chemotactically to FMLP in a manner similar to mature granulocytes (Tsiftsoglou and Robinson, 1985). Similarly, HL60 cells induced to differentiate to monocyte/macrophages also show characteristics of mature cells of this lineage. Thus, following HL60 induction by phorbol ester, an induction of α- napthol esterase, a histochemical marker for mature macrophages is detected (Daniel et al, 1987). These cells are also capable of phagocytosis, show increased synthesis of lysozyme and express a number of monocyte surface antigens (Monroe et al, 1984; Polansky et al, 1985).

1.3.4 Changes in HL60 gene expression during differentiation

The large number of morphological, functional and biochemical changes seen when HL60 cells are induced to differentiate are accompanied by large qualitative and quantitative changes in gene expression. This was first indicated from results of experiments comparing the translation products detected following in vitro translation of polysomal polyA$^+$ RNA extracted from uninduced and induced HL60 cells. No gross change in the total amount of polyA$^+$ RNA was detected following induction of differentiation, but different sets of proteins were translated (Colbert et al, 1983; Reyland et al, 1986). This relatively insensitive method still detected large differences in mRNA populations between induced and uninduced cells and between HL60 granulocytes and HL60 macrophages.

Another approach to detecting changes in gene expression occuring during HL60 differentiation is that of isolation of genes whose abundance is related to differentiation of the cells by screening cDNA libraries derived from induced and uninduced HL60 cells. This approach has identified a number of recombinant clones homologous to a large number of mRNAs that change in abundance during differentiation (Davis et al, 1987; Mitchell, 1987). Interestingly, a large number of these clones contained highly repeated sequences (including Alu sequences) or belonged to members of extensive gene families (Davis et al, 1987)

For genes for which cloned probes are available, the HL60 differentiation system has proven useful for a number of reasons. It provides a system in which the relationship between gene expression and both proliferation and differentiation can be investigated. One much-studied example is the c-myc proto-oncogene, the cellular homologue of the transforming avian retrovirus MC29. There is much evidence that c-myc proteins are involved in both proliferation and differentiation in many diverse cell types. Briefly, evidence for an involvement of c-myc proteins in proliferation was initially proposed from the observation that c-myc expression was induced following serum or growth factor stimulation of quiescent fibroblasts or following mitogenic stimulation of lymphocytes (Kelly et al, 1983, Reed et al, 1985). Armelin et al (1984) showed, moreover, that DNA synthesis could be induced when 3T3 mouse

fibroblasts were transfected with a construct containing c-myc under the control of an MMTV promoter and the cells stimulated with glucocorticoids to induce expression.

A link between c-myc expression and differentiation has also been inferred from work on a number of inducible cell lines including HL60, Friend erythroleukaemia and F9 cells (Lachman and Skoultchi, 1984; Dean et al, 1986; Collins, 1987). Thus a marked decrease in c-myc RNA and protein levels occur in HL60 cells in response to initiation of differentiation. This decrease has been shown to be due to a two-phase down-regulation of transcription. In uninduced HL60 cells there is a partial block to transcript elongation at the end of exon 1 of the c-myc gene that is rapidly and greatly enhanced by induction of differentiation with DMSO (Bentley and Groudine, 1986; Eick and Bornkamm, 1986; Siebenlist et al, 1988). A later event which serves to consolidate this down-regulation is a decrease in the rate of initiation of transcription (Siebenlist et al, 1988). This is correlated with changes in chromatin structure 5' of exon 1 as shown by changes in DNAase I hypersensitive sites.

For a number of other genes expressed in the myeloid lineages (granulocytic and monocytic) the HL60 cell line has been of value in understanding if and how the expression of particular genes is related to differentiation. This system is especially useful for proteins expressed during normal granulopoiesis, since almost all synthesis and processing of

individual mRNAs and proteins begins and is completed in relatively immature myeloid cells such as the promyelocyte and myelocyte which are normally confined to the bone marrow and therefore physically difficult to study. Thus the HL60 cell line can help serve as a model for the synthesis and processing of normal myeloid proteins since it is active in transcription and translation during its terminal differentiation.

One such protein which has been investigated is myeloperoxidase. This protein is a major constituent of azurophilic granules and contributes to oxygen-dependent bactericidal functions. Myeloperoxidase mRNA is confined to cells of the granulocytic lineage and is found at highest abundance in immature myeloid cells (promyelocytes and myelocytes) (Koeffler et al, 1985; Jaffe et al, 1988) but not in mature granulocytes. Induction of HL60 cells to differentiate to granulocytes, moreover, leads to a similar down-regulation of myeloperoxidase gene expression implying that the types of controls operating during normal myelopoiesis on myeloperoxidase mRNA levels may be conserved during HL60 cell differentiation. (Yamada and Kurahashi, 1984; Weil et al, 1984; Tobler et al, 1988).

The mRNAs encoding defensins HNP1 and HNP3 are similar in their range of expression in some respects to myeloperoxidase. They too are expressed only in the myeloid lineage although in a smaller window of differentiation (the

neutrophilic myelocyte) than myeloperoxidase (Weidemann et al, 1989). In common with myeloperoxidase mRNA, defensin mRNAs are not detected in mature PMN, although their corresponding proteins are found at high abundance. For an analysis of the fine details of how defensin gene expression might be controlled during myelopoiesis, induced differentiation of HL60 cells would seem to provide a useful model system.

1.4 LEUKAEMIA: ABNORMAL REGULATION OF HEMATOPOIESIS

1.4.1 Maturation arrest

The normal process of multi-lineage hematopoiesis, involving as it does intricate control of both highly proliferative pluripotent cells and very long-lived self-renewing stem cells, is an obvious target for neoplastic transformation. Most evidence supports the notion of a unicellular (monoclonal) origin of most leukaemias and lymphomas with the original transforming event(s) in leukaemogenesis taking place in the hematopoietic stem cell compartment in the bone marrow (and thus giving rise to a number of transformed stem cells) and an apparent uncoupling of proliferation and differentiation known as maturation arrest (Greaves and Janossy, 1978). It has been possible in many cases, from a consideration of the composite phenotype of leukaemic cells in comparison with normal populations, to designate the predominant lineage and cell type involved, and at least to speculate as to the possible target cell for clonal expansion (Greaves et al, 1981; Stein et al, 1984; Foon and Todd, 1986). Thus proliferating leukaemic cells are maturation-arrested at a stage similar, but not identical, to one occuring during normal hematopoiesis. It is this loss by the cells of the ability to differentiate terminally together with a capacity for continued cell division that leads to overt leukaemia. Several conceptual models have been formulated to explain this phenomenon. Sachs (1982) and

Greaves (1982) have both suggested that chromosomal changes occuring concomitantly with progression in leukaemia affect genes whose expression is intimately associated with the coupling of proliferation with differentiation (for example, the growth factor or growth factor receptor genes previously discussed). The more stringent the uncoupling of these two processes in a leukaemic clone due to these chromosomal changes, these two authors suggest, the less mature the phenotype of cells in the transformed clone. Similarly Nowell (1977) has argued that a small shift uncoupling proliferation with differentiation in the stem cell compartment due to tumorigenesis may result in a gradual clonal expansion allowing cells to differentiate almost to termination. Alternatively, a large shift in the balance between proliferation and differentiation would result in a very rapid clonal proliferation and a reduction in the capacity of these cells for differentiation. Would such a model explain different leukaemia types? For the myeloid leukaemias the former situation could possibly explain the predominance of more mature relatively normal myeloid cell types in the peripheral blood of a typical case of CGL, while the latter situation would be reflected in the high proportion of rapidly dividing early blast cells of the myeloid lineage seen in acute non-lymphocytic leukaemia (ANLL).

Leukaemias are highly heterogeneous, but can be divided into two main categories, lymphoid and myeloid. Each category contains both acute (rapid development) and chronic

(relatively slow development) types of disease. The four main classifications of human leukaemia are therefore: acute lymphocytic leukaemia (ALL), acute non-lymphocytic leukaemia (ANLL), chronic lymphocytic leukaemia (CLL) and chronic myeloid (granulocytic) leukaemia (CML, CGL). A more subtle classification of leukaemias based on reactivity with panels of monoclonal antibodies to cell surface markers as well as enzyme markers will be discussed in Section 1.4.3.

1.4.2 Growth factors and leukaemia

With our present knowledge of the role of growth factors in the control of normal hematopoiesis, the possibility presents itself that one mechanism whereby a pluripotent stem cell could uncouple proliferation from differentiation would be simply by proliferating independently of an exogenous supply of growth factor. Such growth factor autonomy could subsequently lead to maturation arrest or might possibly be concomitant with it. In this respect it is logical to ask two main questions; are the genes for the CSFs or CSF receptors oncogenes and does autocrine production of CSFs lead to leukaemia?

In answer to the first question no sequence homology between the CSFs and known oncogenes has been found. However, it should be borne in mind that the list of oncogenes (dominantly-acting genes implicated in a wide variety of

neoplasias) is probably incomplete - particularly in respect to hematopoietic neoplasias, because of the use of a fibroblast (NIH 3T3) detection system.

The possible role of CSFs as autocrine stimulators of proliferation in myeloid leukaemia cells is a complex question for which evidence is quite conflicting. Certainly, for primary myeloid leukaemia in the murine system and in man, the leukaemic cells remain absolutely dependent on exogenous CSF for survival and continued proliferation in vitro. This situation persists throughout the clinical course of the disease. Thus, it seems unlikely that myeloid leukaemias exhibit autocrine growth, assuming that the in vitro data reliably reflects the situation in vivo. Furthermore, there are multiple normal tissue sources of CSF, probably producing CSF levels greatly in excess of those conceivably able to be produced by the first emerging leukaemic cell. Nonetheless, it is conceivable that independence from normal growth inducer could explain the survival and growth of metastasizing cells in places in the body where growth inducer required for the viability of normal cells is absent. Might leukaemic cells simply produce more CSFs or abnormal forms of these molecules to which they themselves are abnormally or selectively responsive? Most evidence suggests that this is not the case: where leukaemia cell-derived CSFs have been biochemically characterized, they are demonstrable as being similar to the normal equivalent molecule and moreover, are produced in amounts similar to those produced by normal hematopoietic cells.

Despite this evidence against an involvement of growth factors in leukaemia, certain other observations have linked acquisition of constitutive CSF production with leukaemic transformation. For example, some murine nonleukaemic but immotransformation.opoietic cells dependent on extrinsic IL3 for survival and proliferation show the interesting property of generating extrinsic IL3-independent subclones in vitro. These subclones are capable of autonomous proliferation and IL3 production and, most importantly, thereby acquire the capacity to produce transplanted leukaemias in syngeneic recipients (Hapel et al, 1981; Schrader and Crapper, 1983). One could argue, however, that perhaps some other changes occuring during cell culture in this system were responsible for autonomous proliferation and leukaemic capacity and that autocrine CSF production was an advantageous byproduct of these changes. However, a more causal relationship has been established by Lang et al (1985) who showed that infection of cells of one such IL3-or GM-CSF-dependent line, FDC-P1, with a retroviral construct containing the GM-CSF cDNA leads to the emergence of autonomous GM-CSF-producing sublines that are uniformly leukaemogenic on transplantation to syngeneic recipients. Several other experimental systems utilizing retroviruses as transduction vehicles have also demonstrated that expression of some known oncogenes or growth factor genes can induce factor production and thus lead to growth autonomy in certain hematopoietic cells. However, it appears that prior immortalization of the target cells (whatever that means

in molecular terms) is a prerequisite for malignant transformation. When the same retroviruses are introduced into normal, freshly isolated hematopoietic progenitor cells, these cells also acquire the ability to grow in the absence of added growth factor, but in this case the growth is accompanied by terminal maturation; the cells do not become malignant (Metcalf, 1986). Thus it seems that, as in other systems, at least two events are required for acquisition of a malignant phenotype. Moreover, some studies using similar systems have demonstrated, in contrast to the above findings, that a loss of factor dependency and acquisition of the leukaemic phenotype can occur without detectable factor production after introduction of oncogenes. For example, Cook et al, (1985) and Pierce et al (1985) have shown that Abelson virus infection of immortalized CSF-dependent murine hematopoietic cell lines can transform the cells to leukaemic cells under conditions where autocrine production of either IL3 or GM-CSF could be excluded and in which membrane receptors for CSF appeared to be unaltered in the transformed cells.

Thus, although CSF production by preleukaemic cells may be able to lead to the transformation of such cells to fully leukemic cells, leukaemic transformation can also occur by other mechanisms. Recently, Stocking et al (1988) using a factor-dependent myeloid precursor cell line D35 derived from in vitro infection of Dexter's long-term murine marrow cultures, have shown that growth factor-independent sublines

do indeed show a mechanistic heterogeneity in transformation to growth autonomy. Essentially, all mutant sublines in their study were tumorigenic in nude mice and, furthermore, could be grouped into two classes depending on whether they secreted a diffusible stimulating factor. Of the mutants releasing a factor (10/11), approximately two-thirds secreted GM-CSF, one-third secreting IL3 with one mutant releasing a possible new hematopoietic growth factor. Activation in most cases was due to rearrangement of the activated allele associated with juxtaposition of enhancer and other transcriptional regulatory signals contained in a virus or virus-like LTR either 5' or 3' of the factor coding region. In some other cases of mutants releasing a factor, no gross genotypic alterations were observed and small rearrangements not detectable by conventional Southern analysis of genomic DNA within the immediate 5'-regulator region or within distant upstream promoter sequences were postulated by the authors to account for aberrant expression of the growth factor. Most interesting in this study, was the finding that one of the eleven growth-autonomous mutants tested did not produce a diffusible factor as assayed by stimulation of the parental D35 line or other factor-dependent hematopoietic cell lines. It seems a distinct possibility that this type of rare mutant, able to grow autonomously by a mechanism not involving autocrine stimulation by growth factors, may represent an initial stage in leukaemogenesis hitherto uncharacterised. The authors themselves suggest that, by the constitutive expression of a receptor or transducer in an active

configuration, these types of mutants are able to short-circuit and thus activate the normal signal transduction pathway as proposed for several oncogenes (Weinberg, 1985).

Although much work has concentrated on growth factors and the role of growth factor autonomy in leukaemogenesis, there is some evidence that inhibitory factors may have some role. In normal hematopoiesis, Lord et al, (1976), for example, have identified a factor that specifically and reversibly inhibits the entry of multipotent stem cells into S-phase of the cell cycle and is found in medium conditioned by bone marrow stromal cells. Moreover, they have shown also that it can prevent entry into the S-phase of CFU-S stimulated by cell cycle activators (Lord et al, 1977) and that its inhibitory effects are apparently restricted to multipotent stem cells with no cell-cycle inhibitory effects on lineage-restricted progenitor cells. Clearly, since the basic mechanism underlying leukaemogenesis is perturbation of the growth control system that operates in normal hematopoiesis, it is possible to envisage that one such possible perturbation may well be unresponsiveness to the normal growth inhibition system. In fact, there is some evidence that multipotent stem cells from CGL patients do not respond to the CFU-S inhibitory factor produced in vitro in long-term marrow cultures. It is a possibility that this may be the reason for the selective advantage seen for CML stem cells over normal stem cells in CGL patients (Eaves et al, 1986).

The phenomenon of maturation arrest, involving as it does a defect in differentiation, has been approached from another angle by asking the question of exactly how colony-stimulating factors might induce differentiation of committed precursors. It appears unlikely that a growth factor that induces cell multiplication would also induce differentiation which stops cell division in mature cells. In fact, proteins that induce myeloid cell differentiation but not growth have been identified (Sachs and Lotem, 1984; Sachs, 1980; Olsson et al, 1984) termed macrophage and granulocyte inducers-type 2 (MGI-2). Sachs (1987) has suggested that induction of these differentiation factors by growth factors might serve as an effective mechanism to couple growth and differentiation in vivo. Differences in the time of switch-on of the differentiation inducer, he suggests, might be responsible for the observed differences in the amount of cell division before differentiation. Furthermore, different growth inducers could switch on different differentiation inducers which might in turn determine the differentiated cell type. Since it has been found that a differentiation inducer can in turn switch on growth factor production in myeloid cells, this could also presumably ensure cell viability and enhance the function of mature cells. It should be stressed, however, that evidence for the importance of these differentiation factors has mainly come from studies on their role in inducing primary myeloid leukaemia to differentiate in vitro. Can maturation arrest be overcome by these factors and, as a corollary, is maturation arrest perhaps caused by a defect in the synthesis of, or response to, these factors? There are no data on the

second question, but Sachs has provided evidence that at least some myeloid leukaemias can be induced to differentiate in response to these factors (Sachs and Lotem, 1984; Ichikawa et al, 1976). Mature cells from these differentiation-positive (D^+) clones, moreover, stop multiplying and are no longer malignant in vivo (Lotem and Sachs 1981 and 1984). Furthermore, studies in animals and man have shown that normal differentiation of D^+ myeloid leukaemia cells, to mature non-dividing cells can be induced not only in culture but also in vivo (Lotem and Sachs (1978), (1979); Fearton et al, 1986). Sachs has suggested (1987) from this work that these D^+ leukaemias may grow progressively only when there are too many leukaemic cells for the normal amount of differentiation inducer in the body. In support of this idea, he has found that development of leukaemia can be inhibited in mice with these D^+ leukaemia cells by increasing the amount of differentiation inducer protein, either by injecting it or by injecting a compound that increases its production by cells in the body (Lotem and Sachs, 1981 and 1984). Interestingly, Sachs has also described differentiation-defective (D^-) clones. Some D^- clones are induced by differentiation inducing factors to an intermediate stage of differentiation that then slows down the growth of the cells; others, though, cannot be induced to differentiate even to this intermediate stage. The immediate significance of this work is not clear, but Sachs has proposed (Sachs, 1987) that D^+ clones may be the early stages of leukaemia and that possibly the formation of different types of D^- clones may be later stages in the progression to malignancy.

1.4.3 Using molecular markers to classify leukaemias

We have seen that the concept of maturation arrest has arisen from a consideration of the composite phenotype of leukaemic cells in comparison with normal populations, allowing in most cases a designation to be made of the predominant lineage and cell type involved in a given leukaemia. Such considerations increasingly do not rely simply on morphological/ultrastructural considerations, but on the expression of cell-surface antigens on the leukaemic cells and their detection by monoclonal antibodies (Foon and Todd, 1986). Furthermore, the use of molecular probes that identify immunoglobulin and T-cell receptor genes (the protein products of which in normal hematopoiesis are expressed in B- and T- cells respectively) and the use of enzymatic markers such as myeloperoxidase (expressed in immature cells of the myeloid lineage), have strengthened the idea that leukaemic cells resemble their normal counterparts in hematopoiesis in many details (Greaves, 1986). However, it must be kept in mind that leukaemic phenotypes are not perfect replicas of normal ones. In addition to specific chromosomal changes and alteration in the structure and/or control of particular genes, leukaemic cells may show some asynchrony of phenotypic expression in comparison to their equivalent maturation compartment in normal tissue. An example of this occurs in T-cell leukaemia. In one study of the composite immunophenotype in 82 cases of T-cell leukaemia, 26 showed

some asynchrony or abnormal combination of T-cell markers with respect to intralineage maturation status (Greaves et al, 1981). Additionally, all cases of thymic or T-acute lymphoblastic leukaemia (T-ALL) tested in this study expressed high density HLA (A;B) combined with nuclear terminal deoxynucleotidyl transferase (TdT) in individual cells (Greaves et al, 1981; Bradstock et al, 1980a) whereas in the normal thymus TdT-positive cells have very low density HLA (A;B), the density of this glycoprotein increasing with further T-cell maturation and loss of TdT activity (Bradstock et al, (1980b). Similarly in leukaemia of B-cell precursors, a substantial proportion of cases are cytoplasmic μ chain/TdT-positive (Vogler et al, 1978; Greaves et al, 1979), whereas in normal populations this is an extremely rare (though detectable) cellular phenotype (Janossy et al, 1979), pre-B cells presumably losing TdT prior to detectable μ chain synthesis. Although a case can be made for this extremely small sub-population being the target for clonal expansion, the finding that these distortions in phenotype are much more pronounced in cell lines established from ALL patients compared with uncultured cells studied at diagnosis, suggests that outwith normal homeostatic control asynchrony of phenotypic expression manifests itself. Although the significance of these findings is unclear, Greaves has suggested that they may still be consistent with the concept of maturation arrest if the arrest is not an absolute bar to maturation, but rather a regulatory uncoupling of variable stringency (Greaves et al, 1986).

The concept of maturation arrest/clonal expansion arises principally from the rather faithfully conserved lineage specificity observed in leukaemia and lymphoma. An example of this is the expression of the gp100/common acute lymphoblastic antigen (CALLA, CD10) structure. This structure itself is non-lineage specific (Metzgar et al, 1981) but is selectively expressed on certain cell types in concert with maturation status. Thus, although granulocytes express CALLA weakly, myeloid progenitors are unreactive (Clavell et al, 1981; Braun et al, 1983), in contrast to lymphoid precursors in the bone marrow and thymus which are CALLA-positive (Greaves et al, 1981; Greaves et al, 1983a). Significantly then, in a survey of over 2000 cases of leukaemia with the anti-CALLA monoclonal antibody J5, almost 75% of ALLs were reactive compared with only 2.4% cases of ANLL. Furthermore, of these 'positive' ANLL cases, on further examination, all turned out to be either dual lymphoid-myeloid, had no unequivocal evidence for myeloid cytochemistry, or subsequently relapsed as ALL. Thus, mirroring the situation in normal hematopoiesis, it is clearly exceedingly rare for myeloblasts to express illegitimately the CALLA marker. Similarly, glycophorin, an erythroid cell specific membrane glycoprotein expressed on most but not all erythroleukaemias (Greaves et al, 1983b) shows a similar lineage-restrictedness. When a monoclonal antibody LICR.LON.R10 (Edwards, 1980) was used to screen 329 cases of ALL (Greaves et al, 1983b), only two had positive blasts. Subsequent review of these two cases, moreover, revealed that

although both had lymphoblast morphology, they did not express an ALL immunophenotype and were almost certainly cryptic erythroleukaemias.

1.4.4 Lineage infidelity in leukaemia

From the assembled data then, the idea that leukaemic cells are phenotypically similar in many details to normal hematopoietic progenitors or subsets seems compelling. Further insight on how the choice of lineage is established in normal hematopoiesis has also come from the study of molecular markers on leukaemic cells. Several models for self-renewal and differentiation of pluripotent stem cells have been proposed (Ogawa et al, 1983). Two major competing models which make quite different predictions exists. The model first suggested by Till et al (1964) and later developed by others (Johnston, 1981; Eaves et al, 1982; Kurnit et al, 1985), predicts that adoption of lineage commitment at a genetic level is indifferent to external directive influences, but rather follows an intrinsic program that is either stochastic (probabilistic) or deterministic (ordered) in nature. An alternative model, first proposed by Trentin (1970), suggests that, in contrast to lineage commitment existing as a mechanistically 'hard-wired' system indifferent to external influences, choice of commitment to a particular lineage may be determined or perhaps imposed by external microenvironmental influence, e.g. growth factors (van Zant et al, 1978; Metcalf and Burgess, 1982) and/or stromal elements

(Dexter, 1982) or other positional information (Wolpert, 1969). In considering these models, it is worthwhile to ask whether strict lineage fidelity of markers is always observed in leukaemia, as the concept of maturation arrest would suggest, and, if not, what this may imply about normal hematopoietic development. In fact, a number of instances of so-called 'lineage infidelity' (McCulloch, 1983) have been reported in leukaemia. As argued by Greaves, however (Greaves et al, 1986) some such claims can be discounted for purely technical reasons (for example monoconal antibody cross-reactivity) as well as for reasons of interpretation (for example where a given marker is mistakenly thought to be lineage-specific in normal hematopoiesis but is, in fact, expressed at different developmental stages in more than one lineage). However, some bona fide examples of lineage infidelity exist and should be considered. One example is the finding of immunoglobulin gene rearrangements and/or expression in T-cell leukaemia (Korsmeyer et al, 1981; Ford et al, 1983; Ha et al, 1984a) and some myeloid leukaemia (Rovigatti et al, 1984; Ha et al, 1984b; Palumbo et al, 1984) and T-cell receptor gene rearrangements and/or expression in some non-T-cell leukaemias. Since the immunoglobulins and T-cell receptor proteins are highly cell-type specific in normal development, are the above findings indicative of a type of aberrant programming of gene expression in these leukaemias (i.e. lineage infidelity) or do they indicate a type of loose plasticity of genetic events occuring in early progenitor cells? The question is, then,

whether the molecular rearrangements underlying the assembly of these genes occurs in a lineage-restricted way. In fact, in the murine system it has been demonstrated that, contrary to expectations, normal mouse thymocytes (T-cell precursors) as well as some cytotoxic T-cell clones, leukaemic T-cells and myeloid cells all can have immunoglobulin heavy chain gene rearrangements (Kemp et al, 1980; Cory et al, 1980; Forster et al, 1980; Kurosawa et al, 1981). Immunoglobulin transcripts of various sizes have also been reported and, in one case, shown to result from joining of the D (diversity) to J (joining) segments in the absence of V (variable) region (Kurosawa et al, 1981). Furthermore, studies on Abelson virus-transformed B-cell precursors in fetal liver (Alt et al, 1984; Reth and Alt, 1984) have revealed that DJ joining with transcription of a truncated mRNA probably actually precedes recombination involving V— region genes in normal B-cell ontogeny. Moreover, transcripton of unrearranged V—gene segments is predominant in cells underoing V_H to DJ rearrangement and is B-cell specific (Yancopoulos and Alt, 1985). Significantly, later events than these in B-cell ontogeny, rearrangement of K or λ light chain genes, have not been reported in non-B cell leukaemias.

The situation with respect to the T-cell receptor β-chain gene is similar (Moller, 1984). This gene has an immunoglobulin—like VDJC suprastructure and also undergoes rearrangement. In both the murine system and in man both normal and leukaemic immature thymocytes transcribe a 1Kb mRNA

product of the DJC β-gene lacking a V-region. Very immature T-ALL also have these β-gene rearrangements and express this transcript. Some, significantly also have a rearrangement of the immunoglobulin μ chain gene. Finally, β-gene rearrangement and/or expression of the β-gene mRNA has also been detected in a few immature myeloid and pre-B leukaemias.

It would seem then that the early events in programming gene expression in normal hematopoiesis of the immunoglobulin and T-cell receptor genes are not lineage-restricted. Thus this 'lineage infidelity' seen in leukaemia would seem to present a molecular 'snap-shot' of early lineage markers being coexpressed on cells of different lineages. Greaves (Greaves et al, 1986) has termed this phenomenon 'lineage-promiscuity' and his conception of this phenomenon is that some markers that are exclusively expressed in a lineage-restricted fashion on precursor cells committed to a single lineage are also transiently coexpressed on some or all multipotential cells, and perhaps briefly on the progeny of these cells following the initiation of commitment, but prior to full or irreversible determination. Thus, if some limited degree of lineage promiscuity of gene expression preceded lineage fidelity in the normal course of events, then if a leukaemia arose in such a precommitment phase of hematopoiesis and was subject to a relatively stringent maturation arrest, appropriate marker analysis might reveal a mixed lineage phenotype on individual cells and be interpreted as lineage infidelity. Since several lineage- and maturation-associated

markers (e.g. CALLA, glycophorin, T3) are not found on multipotent progenitors, a more realistic version of this model would be that limited or selective promiscuity may occur with respect to genes that are among the first to be expressed in a lineage and whose activity is perhaps functionally involved in lineage commitment. The case of immunoglobulin heavy chain rearrangement may be an example of just such a phenomenon. Speculating, perhaps successful joining of VDJ is some signal for cessation of β-gene T-cell receptor rearrangement and thus commits a progenitor cell to B-cell ontogeny. Unsuccessful joining might in turn lead to the choices of successful joining of the β-gene segments (and thus T-cell ontogeny) or even unsuccessful joining here and the choice of other lineages.

The lineage promiscuity model implies that if lineage-associated genes are indeed required as part of the initiation process of commitment (and are thus coexpressed in some multipotent cells) we would expect to see 'lineage infidelity' more often with markers expressed early in a lineage than for markers that arise during maturation. One marker which shows this pattern is TdT.

TdT is a gene expressed in leukaemic and normal cells of the pre-B and pre-T phenotype, is absent from mature lymphocytes (Bollum, 1979; Bertazzoni and Bollum, 1982) and is postulated to play a role in increasing the diversity of

immunoglobulin and T-cell receptor genes by the random addition of nucleotides in the N region (Desiderio et al, 1984; Siu et al, 1984). Since it is expressed in pre-B lymphocytes, it is a useful marker for ALL and lymphoid blast crisis of CGL. However, its expression is not restricted to ALL; it is also expressed in some ANLLs. In two extensive surveys of ANLLs, 5-10% of cases were found to be TdT positive (Paietta et al, 1985; Jani et al, 1983). Furthermore, in several instances it has been unambiguously demonstrated that TdT and a myeloid marker such as myeloperoxidase coexist in the same cells. Although this may well be a case of lineage infidelity/abnormal expression, lineage promiscuity cannot be ruled out. As Greaves suggests (Greaves et al, 1986), it is quite conceivable that TdT could be induced very early in hematopoiesis in preparation for the modification of immunoglobulin and T-cell receptor rearrangements but be carried over and transiently expressed in some cells that adopt myeloid commitment. The latter might, especially if arrested in their maturation, continue to express TdT as a relic of their prior promiscuity. If maturation were to continue, then, expression of such inappropriate genes would be expected to be switched off. This indeed seems to be the case for TdT, since, in contrast to TdT-positive ANLL, no TdT-positive CGL has been recorded in chronic phase (relatively mature granuloycyte lineage cells) despite the origin of this leukaemia in pluripotent stem cells. Blast crisis of CGL (early myeloid cells) that arises in the progenitor compartment with associated maturation arrest can, however, be TdT positive (McCafferty et al, 1975; Hoffbrand et al, 1977).

With these observations of possible lineage promiscuity in mind, Greaves et al, (1986) have suggested a model incorporating features of the previously alluded to models of Till et al, (1964) and Trentin (1970) in sequence. The model proposed that if certain early lineage-associated markers are promiscuously coexpressed on bi- or multipotent cells (and their corresponding leukaemias) then they may function to advertise the accessibility of particular lineage options. Activation of particular early genes could then arise intrinsically in a programmed or randomly generated fashion as suggested by Till and thereby indicate spontaneous initiation of the commitment process in one or more lineages. The actual lineage option endorsed in individual cells might subsequently be determined both by the particular pattern of markers expressed (and hence lineages actually available) and by the balance of external influences according to the model of Trentin. What kind of genes would one expect then to be prime candidates for promiscuous expression? The previously described colony-stimulating factor receptors and as yet undiscovered receptors for regulators of early lymphoid development are obvious candidates. This suggestion, that uncommitted progenitors might be expected to express concomitantly or sequentially receptors for growth factors that regulate growth and maturation in a lineage-restricted fashion, has previously been made by Till et al (1976) and Van Zant and Goldwasser (1978).

1.5 CHRONIC GRANULOCYTIC LEUKAEMIA

1.5.1 Cellular aspects of CGL

Chronic granulocytic leukaemia (CGL) is a hematologic malignancy characterized by the proliferation and subsequent accumulation of myeloid cells and their precursors (Koeffler and Golde, 1981). It is probably the best characterized naturally-occuring human leukaemia with the primary leukaemogenic event identified at the molecular level (Groffen et al, 1984; Shtivelman et al, 1985). The disease can be clinically divided into two phases. The initial chronic phase lasts for a median 3-4 years and is characterized by a marked increase in the stem-cell compartment committed to myelopoiesis. The acute phase (blastic crisis) of 3-6 months duration almost invariably follows the chronic phase of the disease and is characterized by a nonregulated outgrowth of immature myeloid or lymphoid blast-like cells and a loss of differentiation.

CGL was the first human neoplasia to be associated with a consistent chromosome abnormality, the Philadelphia chromosome (Rowley, 1973), which is present in 90-95% of cases (Galton, 1981; Clarkson, 1985). The target for leukaemic transformation in CGL appears to be at the level of the pluripotent stem cell, since the Philadelphia chromosome is present in all hematopoietic elements of patients with CGL, including B- and T- cells (Tough et al, 1963; Whang et al,

1963; Golde et al, 1977; Martin et al, 1980; Fauser et al, 1985). The clonal origin of CGL was indicated further by the analysis of patterns of expression of glucose-6-phosphate dehydrogenase and adenylate kinase (Fialkow et al, 1977 and 1978).

Although multiple cell lineages are involved in CGL, the clinical features of the disease are usually limited to excessive granulocytosis alone or with thrombosis (Spiers, 1977). The increased myelopoiesis observed, however, is not due to an accelerated proliferative rate as measured by labeling index or doubling time of myeloid cells (Chervenick and Boggs, 1968). Rather, the disease is characterized by massive expansion of pools of committed progenitors (Galbraith and Abu-Zahra, 1972; Moore et al, 1973; Goldman et al, 1980). The peripheral blood of a person presenting with CGL in chronic phase contains a large number of circulating leukocytes, many of which resemble normal mature neutrophil polymorphonuclear cells, although progenitor cells such as promyelocytes, myelocytes and metamyelocytes are also present (Koeffler and Golde, 1981). The presence of these immature cells (especially myelocytes) was relevant to the isolation of the recombinant cDNA pCG14 (the starting point for the work described in Section 3) and will be discussed in Section 4.1.

Like most primary leukaemia cells, CGL cells require colony-stimulating activity for growth in vitro i.e. their growth is not autonomous (Chervenick et al, 1971; Metcalf et

al, 1974). Similarly, erythropoeisis in CGL is generally erythropoietin-dependent (Goldman et al, 1980; Greenberg et al, 1980). Interestingly, CGL stem cells seem to be compromised in their ability to interact with (adhere to) stromal cells in Dexter's long term bone marrow culture system (Coulombel et al, 1983; Gordon et al, 1987). However, whether this has any relevance to their growth in vivo remains to be established. What is known is that during the chronic phase of CGL the proportion of Philadelphia chromosome-positive cells progressively increases over time to typically involve 99% of dividing bone marrow cells. This growth advantage may be due to suppression of growth of persisting Philadelphia chromosome-negative cells by the malignant clone (Gupta et al, 1984; Dube et al, 1984), although convincing evidence for an inhibitory activity is lacking.

In general, during the chronic phase of CGL, although there are subtle abnormalities of granulocyte (Broxmeyer et al, 1977; Olofsson et al, 1976) and platelet (Shafer, 1984) function, myeloid cells mature normally. CGL cells are minimally invasive in chronic phase with malignant cells generally remaining restricted to hematopoietic tissues such as the marrow, spleen and cords of the liver.

As mentioned previously the chronic phase of CGL is unstable and is followed by an acute phase (blast crisis) characterized by loss of cell maturation and frequently death

within 6-12 months. Since acute phase CGL is often refractory to common anti-leukaemic regimens, the duration of chronic phase is the major determinant of survival. Although most patients with CGL enter acute phase within 7 years, approximately 20% have a longer chronic phase. There appears to be a link between the length of chronic phase of the disease and the position of the breakpoint in bcr, the site involved in the reciprocal translocation giving rise to the Philadelphia chromosome (Birnie et al, 1989) although why this might be so in molecular terms in unclear.

1.5.2 Molecular nature of the Philadelphia translocation

CGL is one of the few leukaemias for which the primary leukaemogenic event has been identified. The Philadelphia chromosome, first described by Nowell and Hungerford (1960) is the cytogenetic hallmark of CGL. Using a combination of staining procedures, Rowley (1973) showed that this small chromosome 22 resulted from a specific reciprocal translocation t (9;22) q34.1; q11.21. In a small percentage of CGL cases the Philadelphia chromosome arises from anomalous complex translocations, but nonetheless studies using in situ hybridization have demonstrated that chromosomes 9 and 22 are usually involved.

The localization of the cellular oncogenes c-abl and c-sis close to or at the breakpoints of chromosomes 9 and 22 respectively and the finding that these oncogenes are included

in the exchanged segments of these chromosomes (de Kline et al, 1982), first suggested that one or both were involved in the development of CGL. It appears, however, that the c-sis oncogene is not transcribed in CGL leukocytes at a detectable level indicating that this oncogene does not play a direct role in the disease (Gale and Cananni, 1984). In contast, the c-abl gene is indeed one partner in the translocation.

The t(9;22) translocation in CGL leads to the fusion of c-abl sequences from chromosome 9 to sequences from a gene termed either phl or BCR in a small region of this gene termed bcr (break-point cluster region) (Groffen et al, 1984). The c-abl gene is transcribed in a variety of human cell lines (Gale et al, 1984; Wang and Baltimore, 1983) giving rise to two normal RNA species of 6kb and 7kb. However, in CGL leukocytes, both in chronic and acute phases a novel 8kb c-abl transcript is detected. Direct cloning of this novel c-abl transcript from K562 cells, a CGL-derived cell line, and isolation of the genomic sequences of bcr, c-abl and bcr-abl fusions (Shtivelman et al, 1985; Grosveld et al, 1986; Groffen et al, 1984) led to the following molecular picture. The breakpoints on chromosome 22 q11 occur in a small region of 5.8kb (bcr). The bcr is itself part of a large gene (BCR) and comprises 5 exons of the gene. In contrast, breakpoints on chromosome 9 are scattered over a distance of at least 200kb and are all located 5' of the tyrosine kinase domain of the c-abl proto-oncogene (Heisterkamp et al, 1985; Shtivelman et al, 1985).

As a result of the Philadelphia translocation, the c-abl gene is transferred from its normal position on chromosome 9 q34 to the Philadelphia chromosome. This event creates a novel head-to-tail bcr-abl fusion on the Philadelphia chromosome, with the BCR gene closer to the centromere (Heisterkamp et al, 1983). The fusion gene is thus transcribed using the BCR promoter into a chimeric bcr-abl mRNA consisting at its 5'-end of approximately 3.2kb of bcr sequences linked to 5.3kb of c-abl sequences, lacking the first exon of c-abl, lost during splicing. The result of the transcription of the chimeric bcr-abl mRNA then is the production of an in-frame fusion protein of 210kD which exhibits an in vitro tyrosine kinase activity distinguishable from the enzymatic activity of the wild-type 145kD c-abl protein (Konopka and Witte, 1985; Konopka et al, 1984; Ben-Neriah et al, 1986).

The very high correlation of the clinical pattern of CGL with the presence of the Philadelphia chromosome and expression of the chimeric protein argues strongly for a central role of the bcr-abl gene in the pathogenesis of the disease. One can envisage at least two possible mechanisms whereby fusion of bcr to c-abl could lead to neoplasia. Firstly, since the t(9;22) translocation subjects c-abl to the control of the BCR promoter elements, it is possible that bcr-abl is expressed in a cell type that does not normally

express c-abl. This possibility seems unlikely, however, since c-abl is expressed in diverse tissues including the hemolymphoid system and CGL cells (Gale et al, 1984; Westin et al, 1982). It is possible though that the neoplastic effect of bcr-abl expression may be largely confined to a stem cell compartment within a CGL cell population which would not normally express c-abl protein; whether that minor sub-population of cells expresses c-abl is not known. An extension of this idea is the possibility that increased transcription of the bcr-abl and/or increased stability of the hybrid bcr-abl mRNA or fusion protein in such a stem cell compartment may contribute to neoplasia simply by unregulated expression of the c-abl tyrosine kinase activity. Relevant to this idea is the finding that although the normal BCR and c-abl genes are expressed at a low level (around 10-20 copies/cell) in a relatively tissue non-specific fashion, the chimeric bcr-abl gene can be expressed at a 10-20-fold higher level (Collins and Groudine, 1983; Heisterkamp et al, 1985; Shtivelman et al, 1985).

A second possibility as to how bcr-abl protein might transform comes from in vitro studies on the tyrosine kinase activity of it in relation to the proteins encoded by c-abl and v-abl. Protein tyrosine kinases themselves have been implicated in both neoplastic transformation and in the control of normal cell growth (reviewed in Foukes and Rich-Rosner, 1985; Hunter and Cooper, 1985). In in vitro assays the bcr-abl p210 polypeptide has similar tyrosine

kinase activity to that of v-abl, the p160 transforming protein of Abelson murine leukaemia virus in which the N-terminal portion of the viral gag gene has replaced that of c-abl (Konopka and Witte, 1985). Moreover, in contrast to the normal c-abl gene product (p145), both p210 (bcr-abl) and p160 (v-abl) proteins are phosphorylated on tyrosine in vivo (Konopka and Witte, 1985). The possibility arises then that the fusion of bcr to c-abl sequences may either increase the tyrosine kinase activity or alter the substrate specificity of the c-abl protein either or both of which could potentiate transformation. However, despite the in vitro similarity of p210 (bcr abl) and p160 (v-abl) their properties in vivo show major differences. The Abelson virus can transform bone marrow cells to yield tumorigenic cell lines (Whitlock and Witte, 1985) and can render factor-dependent cell lines independent of exogenous factor (Pierce et al, 1985; Cook et al, 1985). In contrast, the development of CGL appears to be a slow multi-stage process and CGL cells synthesizing the bcr-abl protein still require exogenous factor for growth (Metcalf, 1985), and only rarely form permanent cell lines. Furthermore, for most of the tyrosine kinases so far isolated, association with the plasma membrane appears to be crucial to their transforming ability (Hunter and Cooper, 1985). In both v-abl and v-src, myristylation of the N-terminal glycine residue leads to association with the inner surface of the cell membrane (Kamps et al, 1985; Mathey-Prevot and Baltimore, 1985); disruption of myristylation removes this association and prevents cellular transformation. The

sequence of bcr however, (Adams and Hariharan, 1987) provides no indication that bcr-abl would associate with the membrane. Direct proof that bcr-abl is not membrane bound, however, is lacking.

An intriguing finding which might relate to any mechanism whereby bcr-abl expression might lead to neoplasia is that the BCR gene itself is expressed in a variety of cell types, including fibroblasts, diverse lymphoid and myeloid cell lines and HeLa cells (Ben-Neriah et al, 1986). It follows then that the bcr-abl gene would most probably be transcribed in many cell types should a t(9;22) translocation occur. However, t(9;22) translocation and bcr-abl fusion is absolutely restricted to CGL and some cases of ALL. If, as seems likely, the translocation in CGL is a random event that could occur in any cell type, the marked specificity of t(9;22) for CGL and ALL requires an explanation. Evidence from Epstein-Barr virus immortalized B-lymphoid cell lines derived from patients with CGL (Konopka et al, 1986) and from interspecies human CGL cell-mouse fibroblast hybrids (Kozbor et al, 1986) demonstrates, respectively, that p210 is expressed from the Philadelphia chromosome even in hematopoietic cells which do not have a frankly malignant phenotype and, in a non-hematopoietic cell background (mouse fibroblasts). This pattern suggests that the simple expression of p210 is not in itself, at least in these cell types, sufficient to elicit a full malignant phenotype. The specificity of t(9;22) for CGL then could be due to the

restriction of crucial substrates of the p210 kinase (substrates which could in turn mediate transformation) to the pluripotent stem cell from which CGL originates.

Additionally, the long life-span of such pluripotent CGL stem cells with the Philadelphia chromosome could increase the likelihood of further carcinogenic events occuring stochastically. For example, it is known that blast crisis phase CGL cells are characterized by additional non-random chromosome abnormalities (Rowley, 1980; Champlin and Golde, 1985) such as trisomy 8 or 19 or isochromosome 17q. The precise role of these further genetic changes in the progress of CGL from its chronic to its acute phase has yet to be established.

1.6 GENE EXPRESSION IN NORMAL AND LEUKAEMIC LEUKOCYTES

A priori it would seem that classification of leukaemias by surface marker expression alone is not ideal. For example, any differences observed in marker expression may represent phenotypic changes not directly relevant to actual gene expression in leukaemic cells. Lack of correct display on the membrane, post-translational modification/processing, in addition to any translational controls, might all serve to complicate a classificiation based simply on surface antigen expression. A more direct objective way of classifying leukaemias (or indeed normal tissue sources) should be by directly detecting the abundance of individual mRNAs, since the most important single factor in determining the protein composition of a cell is the concentrations of these individual mRNAs. It has been known from some years that while many mRNAs are common to a variety of types of cell the abundance of some mRNAs can vary through several orders of magnitude in cells of different lineages (Hastie and Bishop, 1976; Young et al, 1976), as well as for cells at different stages of differentiation (Harrison, 1976; Minty et al, 1978). Consequently, a comparison of the mRNAs of two populations of cells might be expected to show whether these populations are composed of cells of the same or different types. How might one investigate leukaemic gene expression using differences in mRNA abundance between different leukaemias and between leukaemic and normal tissue? One way

chosen by many workers is simply to isolate cells from leukaemic individuals peripheral blood or bone marrow and measure the abundances of mRNAs for which cloned probes are available, for example the mRNAs for the proto-oncogenes implicated in a wide range of neoplasias. Many studies of this type exist and have been instructive. For example, in CGL this approach demonstrated that there is an abnormal transcript of c-abl expressed in the bone marrow and peripheral blood cells of leukaemic individuals. This mRNA was subsequently shown to be a chimeric mRNA containing both sequences from c-abl and sequences from a gene termed BCR (breakpoint cluster region) (See Section 1.5.2). In most cases, however, no abnormal transcripts of oncogenes are seen in leukaemia. In general, though, oncogene expression is higher in leukaemias which represent maturation arrest of more immature hematopoietic cells. For example, the c-myc mRNA is elevated in the peripheral blood and marrow cells of ANLL patients, as compared to CGL (Birnie et al, 1984) or normal hematopoietic cells (Westin et al, 1981; McLain, 1984; Rothberg et al, 1984). This type of finding for c-myc is in accord with its role in cell differentiation and proliferation and the findings from other systems such as HL60 differentiation that c-myc tends to be switched off before or concomitant with differentiation (Eick and Bornkamm, 1986; Bentley and Groudine, 1986; Siebenlist et al, 1988).

1.6.1 Differential cDNA libary screening

An alternative approach (and perhaps a more instructive one) in characterising leukaemias by gene expression is to isolate genes either expressed in one leukaemia and not in others, or expressed at higher abundance in one type than in others. This method was first used for human leukaemias by Wiedemann et al (1983). By the process of differential screening of cDNA libraries derived from the mRNA populations of leukaemic patients peripheral blood leucocytes, it was possible in this instance to isolate clones for mRNAs found at higher abundance in CGL than CLL leukocytes. Furthermore, using a panel of such recombinants it was possible to distinguish other patients by the patterns of hybridization since the patterns were significantly different between all CGLs and other populations of normal and leukaemic leukocytes. One clone isolated in this study, pCG14, will be discussed in detail shortly. A similar approach was later used by Mars et al (1985) with a similar outcome (Mars et al, 1988).

This approach was also taken by Warnock et al (1985) by making a cDNA libary from an ANLL leukaemia (acute myelomonocytic) and screening with CGL, CLL and ALL cDNA. One clone in this study (designated pAM6) appeared to be characteristic of cells of early monocyte stage in differentiation, although its expression in individual ANLL cases was variable. Thus pAM6 expression was high in blast

crisis of CGL where early myelomonocytic blast cells predominate. Moreoever, pAM6 was also found to be expressed in normal bone marrow (i.e. was not leukaemia-specific) and localized to NSE-positive (monocyte) cells by density gradient separation and Northern blotting of RNA prepared from normal bone marrow cells.

For all of the clones so far isolated in these two studies, where it has been checked, none have been found to be leukaemia-specific, i.e. they were also expressed at some stage in normal hematopoiesis. This is to be expected, however, since most of the programme of gene expression in a maturation-arrested leukaemic clone might be expected to be very similar if slightly asynchronous compared to the normal cell equivalent (see Section 1.4.3).

1.6.2 Isolation of cDNAs for mRNAs found at high abundance in CGL

The lead-in to the work described in Section 3 was the isolation of cDNA clones representing mRNAs found at high abundance in CGL leukocytes. Using differential screening of cDNA libraries (a forerunner of subtractive hybridization) Wiedemann et al (1983) isolated several cDNA clones representing mRNAs found at high abundance in the peripheral blood leukocytes of chronic phase CGL patients. One of the recombinants, termed pCGL4, was found to be expressed at high abundance in the leukocytes investigated. In fact out of 26

recombinants chosen at random, 4 of them were shown to be homologous to pCG14 (Wiedemann et al, 1983). One of these, pCG32, was also chosen for investigation.

1.6.3 Expression of the pCG14 gene

Several studies on the expression of the gene encoding pCG14 were carried out prior to the work described in Section 3. The first finding was that the gene encoding pCG14 seemed only to be expressed in chronic phase CGL cells, and not in the leukocytes of other leukaemias or normal peripheral blood leukocytes (Wiedemann et al, 1983; Warnock et al, 1985; Birnie et al, 1983 and 1984). Birnie et al (1983) first showed that pCG14 hybridized to an approximately 750-base mRNA species. Interestingly, this mRNA was undetectable in leukocytes from CLL, ANLL and ALL patients. In contrast 7 out of 7 chronic phase CGL patients' leukocytes had moderate to high levels of this mRNA.

The immediate possibility from these results was that expression of this gene might be diagnostic of CGL leukaemia. However, expression of the gene was also found at moderate to high levels in normal bone marrow (Mills et al, 1987). This was the first indication that expression in CGL leukocytes might be a result of an increased amount of immature myeloid cells spilling out into the peripheral blood. Expression of the gene in the myeloid lineage seemed most likely because of the well-known increase in myelopoiesis

found in chronic phase CGL (see Section 1.5.1) and this was shown directly by Wiedemann et al (1989). Using a combination of density gradient fractionation of normal bone marrow cells (and then Northern blotting and in vitro clonogenic growth of fractions) and in situ hybridization, expression of the pCG14 gene was shown to be restricted to a particular differentiation stage of myelopoiesis, that of the neutrophilic myelocyte. This cell is committed to neutrophilic differentiation and forms pure neutrophil colonies in vitro (Wiedemann et al, 1989). In situ hybridization studies on CGL peripheral blood leukocytes, moreover, localized expression to a cell type morphologically indistinguishable from that seen in normal bone marrow (Birnie et al, 1984; Wiedemann et al, 1989), confirming the hypothesis for expression of the gene in CGL leukocytes.

The above results were of obvious interest since although the gene encoding pCG14 was not expressed in a leukaemia-specific way, its normal expression was highly differentiation stage-specific. However, both the nature of the protein encoded by the gene and precisely how its expression was controlled during differentiation was unknown. The work in Section 3 clarified the first point and also shed some light on the second. Thus Section 3.1 describes the characterization of the pCG14 cDNA (and the homologous clone pCG32 shown to encode the same protein) whilst Section 3.2 describes how the gene is expressed during

myelopoiesis *in vitro*, using the HL60 cell line differentiation system. Lastly, Section 3.3 describes the characterization of the genes encoding both pCG14 and *mrs*, a closely homologous gene.

SECTION 2 : MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents and glassware

All chemicals used were generally Analar Grade, British Drug Houses. Some of the more specialised chemicals, including sodium lauryl sarcosine, MOPS, antibiotics, ethidium bromide, 2-mercaptoethanol and all the reagents required for hybridization 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° overnight. All plasticware, tubing and glassware was further sterilised by soaking overnight in 0.05% diethyl pryocarbonate 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 used in Western blotting were kindly supplied by the West of Scotland Blood Transfusion Service, Law Hospital.

Both normal and leukaemic leukocytes used to provide material for Southern blotting, PCR amplification and Northern analysis were obtained by informed consent from patients attending hospitals mainly in the Glasgow area.

A rabbit anti-peptide antiserum against human defensins was raised by Pat Barber and Neville Husskins at the AFRC in Cambridge using an oligopeptide representing sequences from mature defensin (see Fig. 12).

2.1.2 E.coli strains and cloning vectors

Several E.coli strains were utilized, the particular strain used depending on the experiment.

For plasmid and M13 cloning, both JM83 and JM101 (Viera and Messing, 1983) were used. For plating of genomic DNA libraries in lambda vectors both LE392 (lambda L47.1 vector) and NM538 (EMBL3 vector) were used.

Vectors used were pUC8 (Viera and Messing, 1982), pAT153 (Twigg and Sherratt, 1980) and pUC 18/19 (Yanish-Perron et al, 1985), all for plasmid cloning. For M13 cloning, both M13 mp 10 and 11 (Messing and Viera, 1982) and M13 mp 18 and 19 (Yanish-Perron et al, 1985) were used.

For genomic library construction in lambda vectors, both L47.1 (Loenen and Brammar, 1980) and EMBL3 (Frischauf et al, 1983) were used.

2.1.3 Cloned probes used

β_2-microglobulin, containing DNA representing 77% coding region plus 3'-flanking sequence of the human β_2-microglobulin gene in the PstI site of pBR322; supplied by Dr. S. Suggs (Suggs et al, 1981).

C-myc exon 1, contains exon 1 sequences (AvaI-AvaI, 340bp fragment) of human c-myc gene cloned into the AvaI site of pBR322.

C-myc exon 2, contains exon 2 sequences (PstI - PstI 415bp fragment) of the human c-myc gene cloned into the PstI site of pBR322.

2.1.4 DNA, RNA and protein size markers

DNA size markers used were lambda CI857 digested with Hind 3 or Hind 3 and EcoR1, and ϕ X174 (replicative form) digested with Hae3 (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.

Protein molecular weight size markers were obtained from BRL and contained pre-stained proteins at a concentration of 1mg/ml varying in size from 3kD (insulin A and B chains) to 44kD (ovalbumin).

2.2 METHODS

2.2.1 Cell culture

HL60 cells were cultured in RPMI-1640 (Gibco) plus 0.2% sodium bicarbonate, 2 mM sodium pyruvate, 10% foetal calf serum and 4 mM glutamine. Cells were passaged every 3 days at 5 x 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 HL60P25 cells was 36 hours.

2.2.2 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 was 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 fluorescence 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.

2.2.3 Induction of differentiation

(i) Dimethyl Sulphoxide

HL60 cells were induced at a concentration of 2×10^5 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.

(ii) 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 5×10^5 cells/ml giving a final RA concentration of 10^{-6} M. RA was added every 24 hours 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 hours before addition of inducing agent to reduce any potential serum stimulation from the fresh medium.

2.2.4 Staining procedures

(i) May-Grunwald and Giemsa Staining

Cells were spun onto 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 Sorensen's Buffer pH 6.85 (0.066M disodium hydrogen orthophosphate, 0.066M potassium dihydrogen orthophosphate) and filtering through Whatman Filter paper. Giemsa stain was also diluted in Sorensen's buffet (1 volume stain: 9 volumes buffer) and filtered. The slides were stained for 5 mins in May-Grunwald stain, followed by 10 mins in Giemsa stain. They were then washed twice in double-distilled water, air dried and mounted in DPX mountant (BDH), and examined by light microscopy.

(ii) 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 tetradecanoyl phorbol acetate (Sigma) containing 0.2% (w/v) nitroblue tetrazolium (NBT) (Sigma) in PBS. 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 Section 2.2.4.

After removing excess stain by washing in double distilled water, the slide was air dried and mounted in DPX (BDH) mountant before viewing by light microscopy.

2.3 ANALYSIS OF RNA

2.3.1 Cell harvesting

$1 \times 10^7 - 1 \times 10^9$ cells were harvested from exponentially growing cultures by centrifugation in an MSE bench centrifuge at 1500 rev/min for 5 mins, if the volume was less than 100ml. For larger volumes, cells were spun in a GS-3 rotor at 2000 rev/min for 10 mins in a Sorvall RC-5B centrifuge at 0°C.

2.3.2 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 $1-2 \times 10^8$ 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 (5.7M 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 isolthiocyanate 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 DEPC-treated double distilled water and precipitated from solution at $-20^{0}C$, 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 $-15^{0}C$ in a Sorval HB-4 swing out rotor. The pellet was resuspended in DEPC-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 $4^{o}C$. 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, colleted by centrifugation and washed in 70% (v/v) ethanol and then 95% ethanol. The pellet was then dried briefly and resuspended at 1 µg/ul in DEPC-treated double distilled water.

2.3.3 Preparation of poly(A)$^{+}$ RNA

Poly A^{+} mRNA was separated from whole cell RNA using Hybond-mAP (Amersham), a diazonium-activated paper substituted with polyuridylic acid according to a method described in the

manufacturer's manual. Briefly, approximately 1 mg of whole cell RNA as a 1 mg/ml solution in DEPC-treated water was denatured by incubation at 65°C for 5 minutes, chilled on ice and 4M NaCl added to give a final concentration of 0.5M. To this solution was added Hybond m-AP ($7cm^2$), prewetted by washing in 0.5M NaCl for 5 mins at room temperature. The mixture was then incubated for 2 hours at room temperature with agitation. The mAP paper was then removed from the RNA solution and washed three times in 30ml 0.5M NaCl, 5 mins per wash, to remove unbound RNA. Salt was removed by washing the paper in 70% ethanol for 2 mins with continuous shaking. Poly A^+ RNA was eluted from the mAP paper by the addition of 500 µl DEPC-treated water and incubation for 5 mins at 70°C. The paper was then removed from the poly A^+ RNA solution and the yield of poly A^+ RNA obtained determined by spectophotometric means.

2.3.4 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 was 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) was 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. Prior to transfer, gels were stained in a solution of ethidium bromide (5 ug/ml) in DEPC-treated water (20 mins, room temperature), visualized under UV to check integrity and amount of RNA, and photographed.

2.3.5 Northern blot analysis of RNA

The elecrophoresed RNA was transferred directly onto Hybond-N (Amersham) by blotting in 20 x SSC (3M NaCl, 0.3M sodium citrate pH 7.0) as described by Thomas (1980). The blotting apparatus was set up as described by Southern (1975). The membrane was first soaked in DEPC-treated double distilled water then in 20x SSC for 30 min prior to blotting overnight. At the completion of transfer the membrane was air dried and then irradiated on a UV transilluminator for 5 mins to immobilize the RNA.

2.3.6 Slot blotting of RNA

2 µg aliquots of total cellular RNA in DEPC-treated water (10 ul) were heated at 65°C for 15 mins and chilled on ice. Samples were then slot-blotted onto Hybond-N (Amersham) using

a Hybri-Slot Manifold slot-blotter (BRL) following manufacturers instructions. The Hybond-N membrane was prepared by soaking in DEPC-treated water, then in 20x SCC (3M NaCl, 0.3M sodium citrate pH 7.0) for 30 mins and air dried. After slot-blotting, the membrane was allowed to air dry and then irradiated on a UV transilluminator for 5 mins to immobilize the RNA.

2.3.7 Primer extension analysis of RNA

Primer extension reactions were performed by a modification of the method of Jones et al (1985). Lyophilized RNA (typically 10 µg poly A^+ or 50 µg total RNA) was dissolved in 8 µl of 10 mM Tris-HCl, pH 7.9, containing 25 ng of ^{32}P-5'-end-labelled synthetic oligodeoxynucleotide and heated at 85°C for 5 mins. After addition of 2 µl of a solution containing 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1.25 M KCl, the mixture was annealed at 65°C for 60 min, then diluted with 25 µl of 20 mM Tris-HCl, pH 8.7, containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 µM deoxynucleoside triphosphates (dNTPs), 10 µg/ml actinomycin D, and 20 units of avian myeloblastosis virus (AMV) reverse transcriptase (Boeringer Corporation Ltd, London). The mixture was incubated at 48°C for 60 mins, chilled and mixed with 300 µl of 95% ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol, and dried. The precipitate was resuspended in 5 µl of water, mixed with 5 µl of formamide dye mixture (United States Biochemicals

Sequenase Kit), heated at 90°C for 5 mins, and fractionated on an 8% polyacrylamide sequencing gel. Dried gels were exposed to Kodak X-OMAT AR film with intensifying screens at -70°C for varying times.

2.3.8 Primer extension sequencing

The 5'-end of pCG14-homologous mRNA was sequenced directly by the primer extension/dideoxynucleotide method of Geliebter et al (1986). Briefly, lyophilized RNA (10 µg poly A^+ from CGL leukocytes containing highly abundant pCG14-homologous RNA (see Fig. 22A, lane 9) was dissolved in 8 µl of 10 mM Tris-HCl, pH 7.9, containing 25 ng of ^{32}P-5'-end-labelled sythetic oligodeoxynucleotide A (see Fig. 3) and heated at 85°C for 5 mins. After addition of 2 µl of a solution containing 10 mM Tris-HCl pH 7.9, 1 mM EDTA, 1.25 M KCl the mixture was annealed at 65°C for 60 mins and split into 5 2 µl aliquots. Four sequencing mixes (A, C, G and T) containing 10 mM $MgCl_2$, 5 mM dithiothreitol, 50 µM deoxynucleoside triphosphates (dNTPs), 10 µg/ml actinomycin D and 2 Units AMV reverse transcriptase, in addition to 0.5 mM dideoxy-ATP (A), 0.25 mM dideoxy-CTP (C), 0.5 mM dideoxy-GTP (G) or 1 mM dideoxy-TTP (T) in 3.3 µl aliquots was then added to each of the four 2 µl aliquots of oligo A-annealed RNA. A parallel reaction lacking dideoxynucleoside triphosphates was also set up to rule out premature terminations obtained during primer extension. The mixtures were then extended at 48°C for 60 mins, chilled on ice, and 5 µl formamide dye mixture

(United States Biochemicals Sequenase Kit) added. The mixtures were then heated at 90°C for 5 mins and fractionated on an 8% polyacrylamide sequencing gel. Dried gels were exposed to Kodak X-OMAT AR film with intensifying screens at -70°C for 7 days.

2.3.9 S1 nuclease protection analysis

(i) Probe Preparation

To map the junction in pCG14 cDNA of pCG14 mRNA-derived and foreign sequences, a single-stranded uniformly-labelled probe containing sequences spanning the hypothesized junction was prepared by subcloning a Bgl 2-PstI probe (see Fig. 2) of pCG14 into M13 mp18. Cloning into M13, identification of recombinants and preparation of single-stranded M13 DNA are as described in Section 2.7. A uniformly-labelled probe was prepared essentially using the method of Bentley (1984). Approximately 1 µg of M13 recombinant (containing the coding strand of pCG14) was annealed with 200 ng M13 universal primer (BRL) in a volume of 7 µl buffer containing 15 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$ by placing the mixture (in a 1.5 ml eppendorf) in a large beaker of water at 70°C and allowing it to cool to 30°C. An antisense uniformly-labelled probe was synthesized by the addition of 7 µl of a buffer containing 0.7 mM dATP, 0.7 mM dGTP, 0.7 mM dTTP, 0.35 mM dCTP, 30 µCi (^{32}P)dCTP (400 Ci/mM, Amersham), 2 units Klenow E.coli DNA polymerase (Boeringer Corporation Ltd, London) and incubating

for 15 mins at room temperature. DNA synthesis was terminated by incubation at 60°C for 10 mins. A secondary restriction enzyme cut was performed on the DNA using EcoR1 by the addition 5 µl of 5x EcoRI buffer (0.5M NaCl, 0.1M Tris-HCl, pH 7.5, 0.05M $MgCl_2$) and 2 µl EcoRI (10U/µl, Boeringer Corporation Ltd, London) incubating for 4 hours at 37°C.

The mixture was then extracted twice with phenol/chloroform, the DNA ethanol precipitated, washed in 70% ethanol, dried and resuspended in 5 µl TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA) prior to the addition of 5 µl formamide dye mixture (United States Biochemicals Sequenase Kit). The DNA was denatured by heating at 90°C for 5 minutes and the 255-base probe purified by elecrophoresis on a 6% polyacrylamide sequencing gel. The probe was localized by autoradiography and extracted from the gel slice by incubation at 37°C for 12 hours in 200 µl of a buffer containing 0.5 M ammonium acetate, 1 mM EDTA. Acrylamide fragments were removed by passing the solution through glass wool and the DNA ethanol precipitated, washed in 70% ethanol, dried and resuspended in 50 µl of TE buffer. The probe was frozen at -20°C until use within 7 days.

(ii) S1 protection

60,000 cpm of probe and either 10 µg poly A^+ RNA or 50 µg total RNA or tRNA were lyophilized together and resuspended in 8 µl formamide prior to the addition of 2 µl of a buffer containing 2M NaCl, 0.2M PIPES, pH 6.4, 0.005M EDTA. The mixtures, in 500 µl eppendorfs, were overlaid with 20 µl mineral oil to prevent evaporation, denatured by incubation at 90°C for 10 mins, and allowed to hybridize at 50°C for 18 hours. To each mixture 235 µl of a buffer containing 0.25M NaCl, 0.03M sodium acetate, pH 4.6, 0.001M $ZnSO_4$, 200 µg/ml salmon sperm DNA and 500 Units/ml S1 nuclease (Boeringer Corporation Ltd, London) was added and S1 digestion allowed to proceed for 1 hour at 37°C. The mixtures were then extracted twice with phenol/chloroform, nucleic acids ethanol-precipitated, washed with 70% ethanol, dried and resuspended in 5 µl TE. After addition of 5 µl formamide dye mixture (United States Biochemicals Sequenase Kit), the samples were heated at 90°C for 5 mins and fractionated on an 8% polyacrylamide sequencing gel. Dried gels were exposed to Kodak X-OMAT AR film with intensifying screens at -70°C for 7 days.

2.3.10 PCR amplification of RNA

(i) cDNA Reaction

10 µg total or 0.2 µg poly A^+ RNA was freeze-dried and lyophilized under vacuum prior to the addition of 18 µl annealing buffer (250 mM KCl, 10 mM Tris-HCl, pH 8.3, 1mM EDTA) and 1 µl of a 500 ng/µl solution of oligo D (see Fig. 3) in H_2O. The samples were then denatured for 3 mins at 80°C before being transfered to a large beaker of water at 70°C for 25 mins to allow annealing. Samples were then allowed to cool to room temperature and 30 µl cDNA buffer (24 mM Tris-HCl, pH 8.3, 16 mM $MgCl_2$, 8mM DTT, 0.4 mM (each) dATP, dCTP, dGTP, dTTP, 10 µg/ml actinomycin D and 20U AMV reverse transcriptase (Boeringer Corporation Ltd, London) added before incubating at 45°C for 60 mins.

(ii) PCR Protocol

To amplify by the polymerase chain reaction (Saiki et al 1985), 25 µl of the cDNA reaction was added to 10 µl Taq polymerase buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin). 20 µl 2.5 mM dNTPs, 1 µl oligo B' (500 ng/µl) (see Fig. 3) and 3 µl Taq polymerase (1U/µl, Cetus) were then added and the reactions made up with water to 100 µl. The mixtures were then heated to 90°C for 5 mins, overlaid with 100 µl mineral oil to reduce evapor ation and subjected to 30 cycles of the polymerase chain reaction on a

Perkin Elmer Cetus DNA thermal cycler. Each cycle consisted of 1 min denaturation, 3 mins annealing and 2 mins extension. Following PCR, the mixtures were extracted with phenol/chloroform, DNA ethanol-precipitated, washed with 70% ethanol, dried and resuspended in 20 μl water. Following gel electrophoresis on a 1.8% TBE-agarose gel, the 270bp amplified products were purified and $^1/_3$ of the amount digested with Hae 3 (20U) or Hind 3 (20U, to ensure complete digestion with another restriction enzyme) for 12 hours at 37°C in buffers recommended by the manufacturer (Boeringer Corporation Ltd, London), including 5 mM spermidine. Both undigested amplified DNAs Hae 3-digested and Hind 3-digested DNAs were then electrophoresed on a 1.8% TBE agarose gel and the gel Southern blotted onto a Hybond-N membrane as described in Sections 2.4.5 and 6. The membrane was then hybridized with 5'-end labelled oligo C as described in Section 2.6.3.

2.3.11 Nuclear run-on analysis

(i) Preparation of nuclei

Cells at a density of 5 x 10^5/ml were harvested from exponentially growing cultures of HL60P25 (see Section 2.3.1) and washed with PBS three times. Nuclei were prepared by resuspension of the cell pellet (containing approximately 10^8 cells) in 5 ml of NP40 lysis buffer (10 mM Tris-HCl,

pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) NP40) and incubating on ice for 5 mins. Nuclei were pelleted by centrifugation at 500g (2,400 rev/min on an MSE centrifuge) for 5 mins prior to resuspension in 1 ml of nuclei storage buffer (50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA). Nuclei were stored at -70°C for a period of up to six months before nuclear run-on assay.

(ii) Nuclear run-on assay

Nuclei (200 µl, approximately 2 x 10^7 nuclei) were added to 200 µl nuclear run-on reaction buffer (2 mM dithiothreitol, 10 mM Tris-Hcl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 0.5 mM each of ATP, CTP, GTP and 200 µCi (^{32}P)-UTP) and incubated for 30 mins at 30°C. Reactions were stopped by the addition of 30 µl RNase-free DNaseI (Bethesda Research Laboratories) incubating for 5 mins at 30°C. The reaction mix was made 1 X SET (5 X SET is 5% SDS, 25 mM EDTA, 50 mM Tris-HCl, pH 7.4) prior to the addition of proteinase K (BRL) to a final concentration of 200 µg/ml. Reaction mixes were incubated at 37°C for 45 mins then extracted with an equal volume of phenol/chloroform. The interphase was also extracted with 1 X SET, both aqueous phases pooled prior to the addition of ammonium acetate to a final concentration of 2.3M, and an equal volume of isopropyl alcohol. Labelled RNA was precipitated by incubation on dry ice for 15 mins, and the precipitate collected by centrifugation at 10,000 rev/min for

10 mins. The precipitate was then washed with 70% (v/v) ethanol, washed with 95% ethanol, dried and resuspended in 100 µl TE.

(iii) Hybridization

Nitrocellulose filters containing 500 ng/slot denatured double-stranded cDNA probes were prepared on a Hybri-Slot Manifold slot-blotter (BRL) under conditions suggested by the manufacturer. Filters were prehybridized in hybridization solution (10 mM TES, pH 7.4, 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 1X Denhardts and 250 µg/ml E.coli RNA) for 12 hours at 65°C. Following prehybridization, the filters were hybridized to the run-on RNAs in hybridization solution for 36 hours at 65°C. A typical reaction contained 2 ml of hybridization solution at 1X 10^7 cpm/ml. Following hybridization, the filters were washed twice for 15 mins in 0.1X SDS, 2X SSC at room temperature and then washed at 60°C (0.1% SDS, 0.1X SSC) for 30 mins. Filters were then exposed to Kodak XAR film in cassettes containing intensifying screens at -70°C for various times prior to autoradiography.

2.4 ANALYSIS OF DNA

2.4.1 Preparation of genomic DNA

Genomic DNA was prepared using a modificaiton of the method of Gross-Bellard et al (1973). 1 x 10^8 cells were harvested as described in Section 2.3.1. The cells were then lysed by resuspension in 10 mM EDTA, 10 mM tris-HCl pH 8, 10 mM NaCl contaiing 5% sarcosine (w/v) and proteinase K (20 units/mg) (Boehringer Corporation Ltd) 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 then 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, 1mM EDTA) to give a final concentration of 500 μg/ml.

2.4.2 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 resistance 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. The solution denatures the bacterial chromosomal DNA. Finally, 20 ml of ice-cold 5 M potassium acetate pH 4.8 was added to neutralize 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 then centrifuged at 40,000 rev/min for 40-60 h at room temperature. Following centrifugation, two bands were

usually observed on UV 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 centrifigation 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 ml 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 additon 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 µl 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 ul 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 5 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 µg/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/ul DNA.

2.4.3 Purification of lambda DNA

Defensin-positive plaques were carefully picked with a sterile Pasteur pipette and transferred to 10 ml L-broth medium supplemented with 10 mM $MgSO_4$ and 0.4% (w/v) maltose. A 50 µl aliquot of an overnight culture of E. coli LE392 cells (for lambda L47.1 recombinants) or NM538 (for EMBL3 recombinants) was added to each tube, which were then incubated at 37°C on an orbital shaker for 12 hours. 100 µl of chloroform was then added and the tubes shaken for 2 mins at 37°C. Baterial debris was then removed by

centrifugation at 2000 rev/min (560g) for 10 mins in a IEC Centra-8R centrifuge. The aqueous phase containing bacteriophage virions was then transferred to a fresh 50 ml sterile Falcon tube and 100 µl of 1 M $MgSO_4$ added. To 10 ml of lysate, 10 ml of TM buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$) and 320 µl of DNase 1 (100 µg/ml, BRL) were then added, gently mixed, and incubated for 15 mins at room temperature. Bacteriophage virions were precipitated by the addition of 2 ml 5 M NaCl and 2.2 g of PEG-6000, incubating for 15 mins on ice and centrifuged at 12,000 rev/min at 4°C on a Sorval HB-4 swing-out rotor. All traces of PEG were carefully removed and the phage pellet resuspended in 300 µl of TM buffer. The mixture was then extracted twice with an equal volume of chloroform. Following the addition of 15 µl of 0.5 M EDTA, pH 8.0 and 30 µl of 5M NaCl, the mixtures were extracted with an equal volume of phenol. After two more chloroform extractions, phage DNA was precipitated by the addition of two volumes of ethanol and incubation on ice for 15 mins. Precipitates were collected by centrifugation (10,000 rev/min, 10 mins, 4°C), washed with 70% ethanol and dried under vacuum. DNAs were resuspended in 100 µl TE and stored at 4°C. This procedure typically yielded 50-100 µg of phage DNA. For restriction endonuclease digestion of phage DNA, approximately 1 µg was digested with the relevant enzyme according to manufacturers instructions. At the completion of digestion (typically in 10 µl volumes), 1 µl of 40 mg/ml boiled RNase A (BRL) was added to digest bacterial RNA.

2.4.4 Restriction endonuclease digestion

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 12 h at 37°C; genomic DNA was at a concentration of 50 µg/ml. At the completion of the incubation period the 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.

2.4.5 DNA fractionation by gel electrophoresis

Digested genomic DNA samples were fractionated by horizontal electrophoresis on a 0.8% agarose gel. The gel was made up by boiling the appropriate weight of agarose with 200 ml of 1X TBE electrophoresis buffer (0.089M Tris base, 0.089M 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% agarose TBE buffered gels. 10-20 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 lambda phage digested with EcoR1/Hind3 for sizing of large DNA fragments, or ϕ X174 DNA (replicative form) digested with Hae3 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 UV fluorescence on a Chromato-Vue transilluminator, then photographed through a red No. 9 Kodak Wratten gelatin filter.

2.4.6 Southern blot analysis of DNA

Following fractionation of endonuclease restriction digested DNA as described in Section 2.4.5, DNA was transferred to nylon membranes 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 20 min in 1.5 M NaCl, 0.5 M NaOH to shear and denature the DNA. The gel was then 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 Hybond-N membrane by blotting in 20X SSC (3 M NaCl, 0.3 M sodium citrate). The nylon membrane was first wetted in DEPC-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 nylon membrane was then air dried and irradiated on a UV transilluminator for 5 mins to immobilize the DNA.

2.4.7 Synthetic oligodeoxynucleotides

All oligonucleotides (17-20 mers) were synthesized on an Applied Biosystems Model 381 DNA sythesiser. Oligonucleotides were synthesized trityl-on and purified on OPC purification cartridges (Applied Biosystems) following manufacturers instructions. All oligonucleotides used for primer extension and PCR amplification were additionally purified by gel electrophoresis prior to use.

2.4.8 Restriction mapping of defensin genomic clones

For each of the five defensin genomic clones isolated from human genomic libraries (Section 3.3) the 8kb BamH1 fragment containing the entire coding region of the genes (see Figs. 26 and 32C) was isolated by digestion of the relevant plasmid or lambda clone and purification of the 8kb BamH1 fragment from a 0.8% LMP-agarose (BRL) gel after visualization by U.V-staining. The 5'-3' order of restriction fragments generated by digestion of these 8kb BamH1 fragments with EcoR1 or Hinc 2 was established by oligonucleotide hybridization to Southern blots (Sections 2.4.6, 2.6.3). To establish a complete restriction map of all 5 clones with respect to the enzymes EcoR1, Hinc 2 and Hind 3 the 8kb BamH1 fragment for all 5 clones was digested with each of the three enzymes and fragments purified from ethidium bromide stained LMP-agarose gels. Each of the fragments were then digested with either of the other restriction enzymes either singly or as double digests. For mapping of the 3' Hae 3 sites of the 5 clones, the order of sites was established using a singly end-labelled 2.6kb Hinc2-BamH1 fragment (see Figs. 27 and 32) and employed partial digestion with Hae 3 (Smith and Bernstiel, 1976).

2.5 RADIOLABELLED PROBES

2.5.1 Preparation of cDNA hybridization 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 µl of the kit reaction mix which contains 1 M tris pH 7.4, C.T. hexanucleotide (150 OD/ml) 2 M Hepes, 1 M $MgCl_2$ and 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 unincorporated nucleotides by gel filtration through Biogel A-1.5M agarose (BioRAD) columns. Columns were prepared by plugging a siliconized pasteur pipette with a small plug of sterile glass wool. The column was then filled with A-1.5M Biogel (100-200 mesh) and allowed to settle. Presaturation of the column was carried out by elution with 10 µl of a solution containing 500 µg/ml sonicated salmon sperm DNA. This was eluted with 0.1X SSC solution before the reaction mix was loaded on to the column. The eluted DNA peak fraction was collected from the column and used as a

hybridization probe. Prior to use, DNA probes were boiled for 10 mins to denature any secondary structure, then cooled rapidly on ice.

2.5.2 5'-end-Labelling of DNA oligonucleotides

100-500 ng of oligonucleotides were 5'-end-labelled in a final volume of 20 µl in a buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM spermidine, 0.01 mM EDTA, 0.1 mM $ZnSO_4$, 30 µCi (γ-^{32}P) dATP and 10 U T4-polynucleotide kinase (BCL) by incubation at 37°C for 30 mins. The end-labelling reaction was terminated by the addition of 1 µl 0.25M EDTA (pH 8.0) and DNA precipitated by addition of 10 µl 7.5M ammonium acetate and 90 µl ethanol followed by storage at -70°C for 15 mins. The precipitate was then collected by centrifugation at 12,000 rev/min for 10 mins, at 4°C, washed twice with 70% ethanol to remove unincorporated nucleotides, dried under vacuum and resuspended in 100 µl H_2O.

2.6 HYBRIDIZATION PROCEDURES

2.6.1 Hybridization procedure for Southern blots (cDNA probes)

The method used to prehybridise and hybridize nylon membranes produced from the previously described procedures was taken from Jeffreys and Flavell (1977). Prehybridization was carried out at 42°C in sealed polythene bags. Prehybridization buffer contained 50% formamide (Fluka), 5X Denhardts solution (1X Denhardts solution is 0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpryrrolidone, 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 prehybridization buffer was added per 5 cm^2 of filter. Incubation was carried out overnight in a shaking water bath. Following prehybridization the buffer was removed from the bag and replaced with hybridization buffer (approximately 1 ml per 5 cm^2 filter). The hybridization 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 Denhardts 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 hybridized filters was carried out with buffers preheated to 65°C. Prior to washing the

hybridization 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.

2.6.2 Hybridization procedure for Northern blots (cDNA probes)

Prehybridization and hybridization of Northern blots of Hybond-N (Amersham) membranes were essentially as recommended by the manufacturers. Prehybridization was carried out at 42°C in sealed polythene bags in a buffer containing 5X SSPE (20X is 3.6M NaCl, 0.2 M sodium citrate, pH 7.7, 0.002M EDTA), 50% (v/v) formimide, 5X Denhardt's solution (1X is 0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin), 0.5% (w/v) SDS and 100 µg/ml sonicated salmon sperm DNA. Approximately 1.5 ml of prehybridization buffer was added per 5 cm^2 of filter. Incubation was carried out overnight in a shaking water bath. Following

prehybridization the buffer was removed from the bag and replaced with hybridization buffer. The hybridization buffer contained radioactively labelled probe at a concentration of 1-2 X 10^6 cpm/ml and was otherwise the same as prehybridization buffer. 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 hybridized filters was carried out as detailed in Section 2.6.1. At the completion of washing filters were placed in polythene bags and autoradiographed with Kodak XAR film in cassettes with intensifying screens. After exposure at -70°C the films were developed in a Kodak M7A automatic X-ray processor.

2.6.3 Oligonucleotide hybridization

20-mer digonucleotides 5'-end labelled as described in Section 2.5.2 were hybridized to nylon filters in a buffer containing 6X SSC (0.9 M NaCl, 0.09M sodium citrate), 10X Denhardts (0.2% (w/v) ficoll 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) bovine serum albumin) and 100 µg/ml denatured salmon sperm DNA. Filters were first prehybridized at 65°C for 4 hours in this solution, prehybridization buffer removed and hybridization fluid containing 1X 10^6 cpm/ml of labelled oligonucleotide added. Oligonucleotide hybridization temperature was calculated from an empirical formulae T_i = 4X (G or C) + 2X (A or T) - 5°C and hybridization was

allowed to proceed for 12-18 hours. Filters were then washed at T_i for 60 mins in a buffer containing 6X SSC, 0.1% SDS and for 5 mins in the same buffer at T_i + 5°C. At the completion of washing, filters were placed in polythene bags and autoradiographed with Kodak XAR film in cassettes with intensifying screens.

2.6.4 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 prehybridization and hybridization.

2.7 MOLECULAR CLONING

2.7.1 Preparation of recombinant DNA molecules

Vector DNA (plasmid or M13) was prepared in the following manner. The vector was linearized with the appropriate restriction endonuclease (in certain cases the vector was digested with two restriction endonucleases), run on an agarose gel and then purified. The linearized vector DNA was then phosphatased by treatment with 1 unit of calf intestinal phosphatase (ClP, Boeringer Corporation Ltd) in phophatase buffer (0.05M Tris-HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine) for 30 mins at 37°C. Following this, another 1 unit of CIP was added to the reaction mix which was incubated at 37°C for a further 30 mins. After phosphatase treatment, the reaction mix was incubated at 65°C for 15 mins to destroy the ClP. The reaction mix was then run on a 1% agarose gel and the vector DNA isolated. Insert DNA was prepared by digesting the appropriate recombinant plasmid or phage with restriction endonuclease(s). The digestion mixture was then run on an agarose gel and the relevant DNA fragment purified. In both cases all restriction endonuclease digestions were carried out according to the manufacturer's instructions.

Following the preparation of the vector and insert DNA, recombinant DNA molecules were made by ligation reactions. In these reactions approximately 50 ng of the linearized

vector DNA was incubated with approximately 200 ng (1 μg in the case of blunt-ended fragments) of insert DNA with 1 unit of T4 DNA ligase (BCL) in ligation buffer (0.07M Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT and 1 mM ATP). This provided a high ratio of insert DNA ends to linearized vector DNA which favours the insertion of the insert DNA fragment into the linearized vector DNA. The ligation reaction was incubated for 12-18 hours at 13°C.

2.7.2 Preparation of competent JM83 cells

A glycerol stock of E.coli JM83 cells (see Section 2.1.2) was used to inoculate 10 ml of L-broth (1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% NaCl). This was incubated at 37°C overnight in an orbital shaking incubator. The following morning 1 ml was taken from this overnight culture and added to 100 ml of L-broth which was incubated at 37°C in an orbital shaking incubator. When the OD_{600} of the culture was approximately 0.6 it was split between two 50 ml Falcon tubes. The bacterial cells were pelleted by centrifugation of the culture at 2000 rev/min (560 g) for 5 mins at 4°C in an IEC Centra-8R centrifuge. The cells were then resuspended in 10 mM $MgSO_4$ (10 ml in each 50 ml Falcon tube) and incubated on ice for 30 minutes. Following this incubation, the bacterial cells were pelleted once more by centrifugation at 2000 rev/min for 5 mins at 4°C in a IEC Centra-8R centrifuge. The pelleted cells were then resuspended in 50 mM $CaCl_2$ (10 ml total for both 50 ml

Falcon tubes) and incubated on ice for at least one hour. Following this procedure, the cells were competent i.e. they were capable of being transformed by circular recombinant molecules.

2.7.3 Transformation of competent JM83 by recombinant plasmids

One half of the ligation reaction (10 µl) was placed in a fresh, sterile 1.5 ml eppendorf and 100 µl of competent JM83 cells added. This mixture was incubated on ice for 20-60 mins, heat-shocked at 42°C for two minutes and then allowed to stand at room temperature for 10 minutes. The mixture was then mixed with 800 µl of L-broth and incubated at 37°C for 90 minutes. Following this incubation, an appropriate aliquot (100-250 µl) of each culture was spread onto L-broth agar plates containing ampicillin (L-broth, 1.5% bactoagar 50 µg/ml ampicillin). The culture was allowed to dry onto the agar plate which was then incubated overnight at 37°C. In the case of pUC plasmids, plates also contained 0.5 mM IPTG and 0.05% (w/v) X-gal.

2.7.4 Screening of bacterial colonies containing plasmids

Any bacterial colonies that form after the overnight incubation at 37°C contained circular plasmid DNA molecules that conferred ampicillin resistance to the bacterial cells. To determine which of the colonies contained recombinant

plasmids, small scale plasmid preparations were carried out to isolate plasmid DNA for restriction endonuclease analysis. This procedure was also carried out with pUC transformations with the additional selection that white (recombinant) colonies were chosen. Bacterial colonies were picked from the agar plates and used to inoculate 2 ml of L-broth containing 50 µg/ml ampicillin and processed as described in Section 2.4.2 (iii). Plasmid DNA was finally resuspended in 30 ul of TE. For each restriction endonuclease reaction 5 µl of the plasmid preparation was used.

2.7.5 Transformation of competent JM83 by M13 recombinants

To transfer JM83 cells with M13 phage DNA, 0.3 ml of competent cells were added to 10 µl of ligation mix and the mixture incubated on ice for 2 hours. The cells were then heat-shocked by incubation for 2 mins at 42°C then returned to ice. To each tube of heat shocked cells IPTG and X-gal were added to 0.5 mM and 0.05% (w/v) respectively and 3 ml of molten H top agar (at 48°C) (H top agar is 1% (w/v) bactotryptone, 0.8% (w/v) NaCl, 0.8% (w/v) bactoagar) 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) bactoagar). The top agar was allowed to set then the plates inverted and incubated overnight at 37°C. Recombinants (white plaques) were then picked and the desired clone identified by sequencing.

2.7.6 Preparation of single-stranded template

This method was taken from Amersham (1984). 100 ml of 2X TY medium (0.016% (w/v) bactotryptone, 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 (Section 2.7.3) single, white 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 particles, 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 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.

2.7.7 M13 dideoxy chain-termination sequencing

(i) Using Klenow enzyme

Initial sequencing (for example of the clone pCG14) was performed with a commercial Klenow sequencing kit (Amersham). The method used is exactly as described by Amersham (1984). 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 µg/ml bovine serum albumin). These components were well mixed then incubated for 2 h at 60°C. To the annealed template/primer hybrid 15 µCi 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, 0.0002% (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.

(ii) Using Sequenase

All later sequencing of both M13 and plasmid DNA was performed using a modified T7 DNA polymerase Sequenase (United States Biochemical Corporation) using protocols described by the manufacturers. Annealing of primer (5-10 ng) to single stranded M13 templates (1-2 µg) was performed in a 1X Sequenase buffer (40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) by warming to 65°C and allowing to cool to 30°C in an eppendorf placed in a breaker of water. Following annealing, the reaction mix was labelled by the sequential addition of 1 µl 0.1 M DTT, 2 µl diluted labelling mix (1.5 µM each of dGTP, dCTP and dTTP) 10 µCi (^{35}S)- dATP and 0.5 units Sequenase (2 µl) and incubation for 3 mins at room temperature. Chain-terminating mixes were set up (4 tubes, 2.5 µl/tube, of mixes containing 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 50 mM NaCl and 8 µM of the relevant dideoxynucleoside triphosphate ddATP (A), ddCTP (C), ddGTP (G) or ddTTP (T) and to each 3.5 µl of the reaction mix added and

incubated at 37°C for 5 minutes. At the completion of incubation, 4 µl of formamide dye mix (95% formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% xylene cyanol FF was added to terminate the reactions and the tubes incubated for 3 mins at 90°C to denature before loading onto a polyacrylamide sequencing gel.

2.7.8 Sequencing of plasmid DNA

All plasmid DNA sequenced was prepared using the CsCl method described in Section 2.4.2(i). The DNA (3-5 µg) was denatured by treating with 0.2M NaOH for 5 minutes (a total volume of 20 µl). After this incubation, 8 µl of 5M ammonium acetate was added along with 100 µl of 100% ethanol. This mixture was incubated at -70°C for 10 minutes and then centrifuged for 15 minutes at 4°C in an Eppendorf centrifuge 5415. The pellet of denatured DNA was resuspended in 9 µl of 1X Sequenase buffer (USB Sequenase Kit) and 1 µl (5-10 ng) of sequencing primer added. This mixture was heated to 65°C and then allowed to cool very slowly to below 30°C (1-2 hours). This solution was then ready for sequencing as described in Section 2.7.5(ii).

2.7.9 Gel electrophoresis

Sequencing gels consisted of 6-8% (w/v) acrylamide, 7M urea, 1X TBE (filter sterilized, 0.45 um (Millipore)). 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.

2.8 ISOLATION OF GENOMIC DEFENSIN CLONES

A total of three libraries of human genomic sequences were screened. Two were construcrted from BamHl-digested size-selected DNA cloned into lambda L47.1 (Loenen and Brammar, 1980), the other was constructed from human DNA partially digested with Sau3A cloned into lambda EMBL3 (Frischauf et al, 1983) and was purchased from Clontech Laboratories (Palo Alto, USA).

2.8.1 Construction of human genomic libraries in lambda L47.1

High molecular weight DNA from both white blood cell DNA (WBC) and normal granulocyte DNA (G) was prepared and 300 µg of each DNA cut to completion with BamHl and size-fractionated on 30 ml neutral 10-40% sucrose gradients. Fractions containing pCG14-hydridizable sequences (8-12 Kb in size) were pooled for both WBC and G DNAs. Lambda vector L47.1 was digested to completion (50 µg) with BamHl and size fractionated twice on neutral 10-40% sucrose gradients to remove the stuffer fragment. Vector DNA and both WBC and G human DNAs were ligated and packaged using extracts made for E.coli BHB2688 and BHB2690 and the titre of plaques obtained assessed by titering on E.coli DB102. For the WBC and G libraries the titres obtained were 2.4 $X10^6$ pfu/µg and 1.4 $X10^6$ pfu/µg respectively. (7.5 $X10^6$ plaques and 4.3 $X10^6$ plaques from 3.1 µg genomic DNA ligated).

2.8.2 Screening of L47.1 and EMBL3 libraries

Screening of both L47.1 libraries and the EMBL3 library was performed using similar methodologies. In the case of the WBC and G libraries approximately 5 X10^5 plaques were screened in both cases as follows. 1 X10^5 pfu of each library was added to 1 ml of an overnight culture of LE392 (in 10 mM $MgSO_4$) and phage allowed to absorb by incubation at 37°C for 15 mins. To this was added 50 ml of top agarose (at 45°C) which was then plated onto a large plate (23 x 23 cm, Nunc) containing LB agar preheated to 37°C. After the top agar had set, the plates were incubated at 37°C for 12 hours until plaques were visible. 5 plates, each containing approximately 1 X10^5 plaques were prepared for both WBC and G libraries. For the EMBL3 library 10 plates each containing approximately 1 X10^5 plaques were similarly prepared. The plating cells used for EMBL3 were E.coli NM538 (see Section 2.1.2). Plaque lifts of all plates were performed with Biodyne nylon membrane (Gallenkamp) using conditions recommended by the manufacturer. The method used was essentially that of Benton and Davis (1977). Filters were cut (23 x 23 cm) and placed onto the surface of the plate allowing surface moisture to absorb the filter throughout its surface. After 5 minutes the filter was carefully lifted and placed, plaque side up, on filter paper (Whatman) saturated in 1.5 M NaCl 0.5 M NaOH, left for 5 minutes and then placed, again plaque side up, on filter paper (Whatman)

saturated in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0. Filters were then removed, air dried and baked for 60 minutes at 80°C.

For screening of the WBC and G libraries, filters were prehybridized and hybridized with a radiolabelled full-length HNP3 cDNA probe as described in Section 2.6.1. Positive areas (one per library) containing 10-20 plaques were picked and placed in 200 µl of SM buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM $MgSO_4$, 2% gelatin) vortexed, and serial dilutions made in order to perform a secondary screen, each dilution was used to infect 200 ul of E.coli LE392 cells (in 10 mM $MgSO_4$) at 37°C for 15 mins and 3 ml molten top agar (supplemented with 10 mM $MgSO_4$, 0.4% (w/v) maltose) at 45°C added and poured onto 90 mm L-agar plates preheated to 37°C. Plates were incubated at 37°C until plaques became visible (12-18 hours). One plate from each library was then chosen containing 50-100 plaques and rescreened with a radiolabelled full-length HNP3 cDNA probe. From each plate defensin-positive plaques were identified and grown up as described in Section 2.4.3. One clone (HNPIA) as derived from the G library, the other (HNPIB) from the WBC library.

A similar methodology was used to isolate defensin-related clones from the library cloned into EMBL3. In this case, however, a radiolabelled E2-E3 HNP3 cDNA probe was used to screen a total of 1 $X10^6$ recombinant plaques.

Three were isolated in a primary screen and individual plaques picked following a secondary screen. For EMBL3, E.coli NM538 cells were substituted for LE392.

Cytoplasmic extracts from normal white cells or HL60 cells were prepared by NP40 lysis. Approximately 1 $X10^8$ cells were resuspended in 2 ml of NP40 buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) NP40 plus protease inhibitors 0.5 mM PMSF, 0.5 mM benzamidine, 1 ug/ml pepstatin A, 1 ug/ml aprotinin, 1 ug/ml leupeptin). Nuclei were sedimented by centrifugation on an MSE bench centrifuge at 500 g (2400 rev/min) and cytoplasmic extracts stored at -70°C until use. The protein concentration of extracts was determined using a Biorad Protein Assay Kit. Approximately 10-20 µg of protein in a volume of 15 µl was added to an equal volume of sample buffer (0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% (v/v) glycerol, 0.02% bromophenol blue), heated to 90°C for two minutes to denature proteins and electrophoresed on an 17.5% SDS PAGE gel. This gel contained 17.5% acrylamide, 0.073% bisacrylamide, 0.375 M Tris-HCl pH 8.7, 0.4% SDS, 0.033% TEMED in a discontinuous buffer system as described by Laemmli (1970). The gel solution, before addition of TEMED, was degassed by placing under a vacuum for 15 mins to remove oxygen. TEMED was added to catalyze polymerization of the gel and the gel was cast in a vertical gel mould (150 mm X 165 mm X 0.75 mm) to within 20 mm of the top. The surface was overlayered with a solution containing 0.375 M Tris-HCl, pH 8.7 and allowed to polymerize. After polymerization was complete, the buffer on the gel surface was removed and a stacking gel containing 5% acrylamide, 0.13%

bisacrylamide, 0.125 M Tris-HCl, pH 6.8, 0.2% SDS, 0.05% TEMED, 0.05% ammonium persulphate was formed in the remainder of the gel mould with slots. Heated samples were then loaded. Electrophoresis was carried out at 75 volts using a 0.192 M glycine, 0.025 M Tris-HCl, pH 8.7, 0.1% SDS buffer at 4°C for 12-18 hours. If gels were to be stained, they were first fixed in a solution of 10% acetic acid, 50% methanol, 40% water for 2 hours then in the same solution containing 0.2% Coomassie brilliant blue R (Sigma) for 4 hours. Gels were destained by washing for 6-8 hours in the same solution lacking Coomassie stain. For Western blotting, gels were placed on a sandwich-type appratus. Filter paper (Whatman) was cut to the size of the gel, 6 pieces soaked in dry blot buffer (50 mM Tris-HCl, pH 9.2, 0.3% (w/v) glycine, 0.04% SDS, 20% methanol) and the gel placed on top. Nitrocellulose (Schleicher and Schull BA85) was placed carefully onto the gel surface and the sandwich completed by six more pieces of filter paper placed on top. Bubbles and excess buffer were carefully removed and the gel blotted for 45 mins in a Sartoblot Electroblotter (Sartorius) at 250 mA. Transfer was monitored by visualization of pre-stained low size range molecular weight standards (BRL). Nitrocellulose was then carefully removed from the gel surface and placed in TBST solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (Promega)). To detect defensin peptides, an alkaline

phosphatase-conjugated secondary antibody system was used (Protoblot Western Blot AP system, Promega) using a protocol suggested by the manufacturer. Filters were first blocked in blocking solution (1% (w/v) BSA, 2% Marvel in TBST) for 1 hour at room temperature. This solution was then removed and the blot incubated with the primary rabbit anti-defensin antiserum. The third bleed (3 months after the first injection of peptide) rabbit antiserum was used and found to be effective at a 1:500 dilution in TBST. Incubation was for 2 hours at room temperature. Filters were then removed and washed in fresh TBST (3 X 5 min/wash). The secondary antibody incubation utilized goat anti-rabbit IgG alkaline phosphatase conjugated antibody (at a dilution of 1:7500 of that supplied in the kit) incubating for 2 hours at room temperature. Filters were washed in TBST as before and then placed in 10 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). To this was added 66 µl nitroblue tetrazolium solution (50 mg/ml in 70% dimethylformamide) and 33 µl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in dimethylformamide) and colour development allowed to proceed for 10-15 mins. Filters were then washed in double-distilled water and stored in the dark prior to photography. In peptide blocking experiments, 1 µl of 1 mg/ml peptide (used to elicit antibody response) was incubated with 10 µl antiserum at room temperature for 15 mins. This was found to abolish the defensin signal detectable on Western blots.

2.10 IMMUNOPRECIPITATION

Exponentially growing HL60 cells were pulse-labelled by addition of 500 µCi (^{35}S)- cysteine to cultures of 2 X10^7 cells. Labelling of protein was carried out for 8 hours at 37°C, cells harvested, washed three times in PBS and cytoplasmic extracts made. Cells were lysed by the addition of 1 ml lysis buffer (Na_2HPO_4 pH 8.0, 140 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5% (v/v) NP40 containing protease inhibitors 0.5 mM PMSF, 0.5 mM benzamidine, 1 µg/ml pepstain A, 1 µg/ml aprotinin and 1 µg/ml leupeptin) and nuclei sedimented by centrifugation at 500 g (2400 rev/min on an MSE bench centrifuge). To 200 µl of extract was added 40 µl of correction mix (5% (w/v) sodium deoxycholate, 2.5% (v/v) NP40, 0.5% (w/v) SDS) and 0.5 µl anti-defensin antiserum (1:500 final dilution) or 0.5 µl preimmune serum. Immunoprecipitations were carried out at 4°C for 12 hours on a Spiramix agitator. Immunoprecipitates were complexed by the addition of 40 µl Protein-A-Sepharose (Pharmacia) and another 4°C incubation for 2 hours. Immunoprecipitates were collected by centrifugation and washed six times in wash buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl 1% (v/v) NP40, 1% (w/v) sodium deoxycholate) and once in 10 mM Tris-HCl, pH 7.5. In later experiments sodium deoxycholate was left out and NP40 reduced to 0.05% (v/v) to reduce washing stringency. After the final wash, immunoprecipitates were resuspended in 30 µl sample buffer (125 mM Tris-HCl, pH 6.8, 20 mM DTT, 3.3% (w/v) SDS, 0.2% bromophenol blue) the samples heated for 5

mins and centrifuged for 5 mins to remove Protein-A-Sepharose. Supernatants were then run for 12 hours at 4°C on an 17.5% SDS PAGE gel (see Section 2.10). Following electrophoresis, gels were fixed for 30 mins in fixative solution (50% isopropanol, 10% acetic acid), this solution removed and fluorographic agent added (Amplify, Amersham). The gels were washed for 20 mins in this solution and then dried. Fluorography was carried out at -70°C in cassettes with intensifying screens with Kodak XAR film for 7-10 days.

SECTION 3 : RESULTS

3.1 IDENTIFICATION AND CHARACTERISATION OF A CGL cDNA CLONE

Two cDNA clones were isolated by differential screening of a CGL library on the basis of increased expression of their complementary mRNA in leukocytes from a CGL patient, as compared to leukocytes from a patient with CLL (Wiedemann *et al*, 1983).

Initial experiments indicated that both these cDNA clones hybridized to a mRNA of approximately 750 nucleotides in size abundant in leukocytes of most patients in chronic phase of CGL, but absent from the leukocytes of normal individuals and non-CGL leukaemias (Birnie *et al*, 1983; Birnie *et al* 1984). Moreover, this mRNA was also expressed in normal bone marrow cells, although not to an appreciable extent in normal peripheral blood leukocytes (Wiedemann *et al*, 1989).

In situ hybridization to normal bone marrow cells of one of these cDNAs termed pCG14, further indicated that expression of this mRNA had an almost unique differentiation-stage-specific specificity of expression in an immature cell of the myeloid lineage, the neutrophilic myelocyte (Birnie *et al*, 1984; Wiedemann *et al*, 1989).

3.1.1 Characterisation of cDNA clones pCG14 and pCG32

Initial restriction mapping of the two clones pCG14 and pCG32 showed that both clones were homologous where they overlapped at their 3'-ends, but contained non-homologous sequences at their 5'-ends (Fig. 2). Most work subsequently concentrated on clone pCG14; this cDNA was completely sequenced on both strands in bacteriophage vectors M13 mp10 and mp11 (see Section 2.1.2). The sequencing strategy is shown in Fig. 2 and the complete sequence shown in Fig. 3.

To determine the coding strand of pCG14 cDNA, two 20-mer oligonucleotides, oligos B and B' (see Fig. 3) representing antisense and sense mRNA sequences, were synthesized and each used to probe Northern blots of RNA from CGL leukocytes previously shown to contain a high abundance of the 750-nucleotide pCG14 mRNA. Oligo B' failed to detect any RNA species (data not shown) whilst oligo B detected a single 750-nucleotide species (Fig. 7C). This established the coding strand of the pCG14 cDNA as that shown in Fig. 3, presented translated in all three reading frames.

At the time this work was done, no sequences similar to pCG14 had been entered into EMBL nucleic acid and protein databases. Thus all three potential reading frames shown in Fig. 3 seemed equally likely to encode the pCG14 polypeptide. Reading frame one (RF1) contained the longest ORF, with its coding potential apparently stretching from outwith the clone

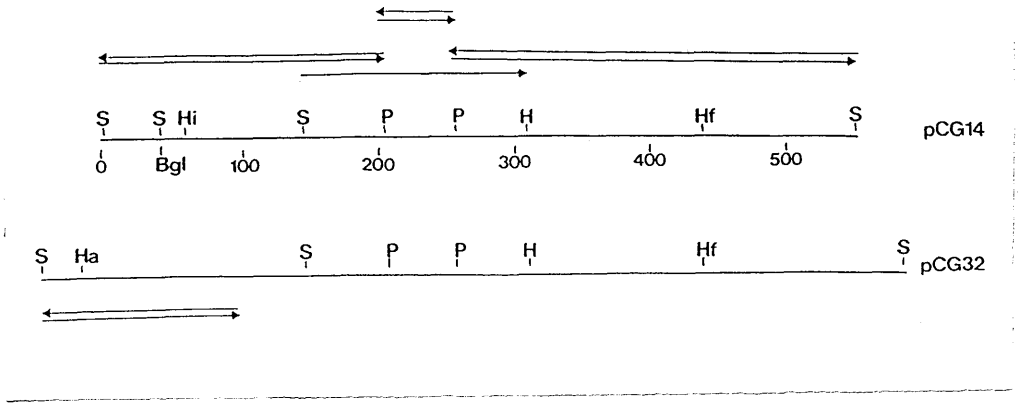


Fig.2 Restriction map of clones pCG14 and pCG32.

Both cDNA clones isolated from the CGL library had been cloned into the BamH1 site of pAT153 (Twigg and Sherratt, 1980) Arrows above clone pCG14 represent restriction fragments cloned into bacteriophage M13 vectors mp10 and 11 (Messing, 1982) with the direction of the arrow indicating the direction of sequencing. Sequencing was performed with a commercial Klenow dideoxy sequencing kit. Arrows below clone pCG32 represent the extent of plasmid sequencing of this clone: top arrow, primed with oligo A (Fig.3); bottom arrow, primed with pAT153 oligo
(5′ GCCGGCCACGATGCGTC 3′).
Sau3A sites (S) at ends of cDNA clones are those constructed during cloning into the BamH1 site of pAT153.
Abbreviations: S, Sau3A; H, Hind3; Ha, Hha1; Hi, Hinc2; Hf, HinF1; P, Pst1; Bgl, Bgl2.

Fig.3 Sequence of coding strand of pCG14.

The sequence is shown translated in all 3 reading frames RF1, RF2, and RF3. Open reading frames are shown in bold typeface. The position of oligonucleotides X, A, B, C and D used in primer extension, oligonucleotide hybridization and sequencing experiments is indicated in the figure. Overlining of the sequence indicates that the oligonucleotide is complementary to that sequence; underlining indicates that the oligonucleotide is that sequence. Thus oligos X, A, B, C and D are all complementary to the sequences underlined, whilst oligos A′, B′, C′ and D′ are the sequences underlined. The mapped end-points of primer extension at nucleotides 77 and 78 (p-ex) are indicated with points above them. The junction with non-pCG14 sequence revealed by S1 nuclease protection is indicated with a line above the sequence (S1, nucleotides 75-78). The methionine residue most likely to initiate protein synthesis is shown underlined (nucleotides 166-168). At either end of the sequence the Sau3A site constructed during cloning is shown underlined. Also shown is the position of Hae3 restriction enzyme sites (see Section 3.3) and the positions of the Pst1 and Rsa1 sites used to bind the E2-E3 HNP3 cDNA probe used in Section 3.3.


oligoX
oligos A/A'
oligos B/B'
oligos C/C'
oligos D/D'
Hae3
Pst1
Rsa1
RF1
RF2
RF3

to nucleotide 447, giving rise to a nascent polypeptide in excess of 147 amino acids. RF2 had a somewhat smaller coding potential stretching from an AUG at nucleotide 245 to nucleotide 469 and would have given rise to a 100 amino acid polypeptide. Lastly, RF3 stretched from nucleotide 309 to outwith the clone at its 3'-end and would have coded for a polypeptide in excess of 97 amino acids.

Since for most eukaryotic mRNAs the 5'-most AUG is used to initiate protein synthesis (Kozak, 1983), of the three possible ORFs, the ORF in RF1 seemed the most likely to encode the pCG14 polypeptide (see Fig. 3). However, since the coding potential of this ORF apparently stretched from outwith the cloned sequence, it seemed likely that the 5'-end of pCG14 mRNA had not been cloned during construction of the CGL cDNA library.

3.1.2 Primer extension analysis of pCG14 mRNA

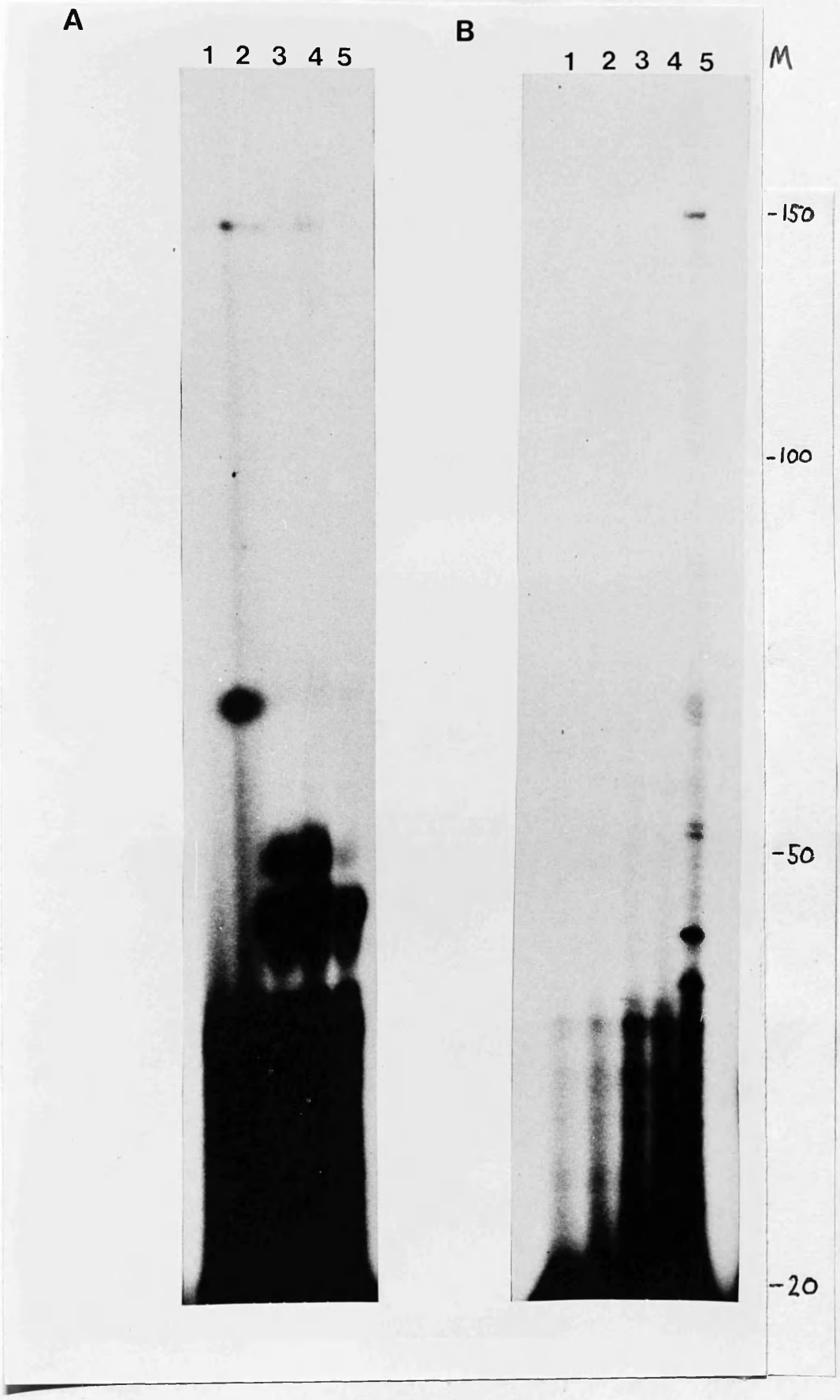
To determine therefore how much 5'-sequence was absent from the pCG14 cDNA clone, a 20-mer oligonucleotide, oligo X, was synthesized in order to carry out primer extension with CGL RNA, shown previously to contain abundant pCG14-homologous mRNA. However, after several attempts using this oligonucleotide, no specific primer extension could be demonstrated (Fig. 4A) i.e. no unambiguous primer extension product was present with CGL RNA that was not also present in HL60B RNA (see Section 3.2.7) or yeast tRNA, RNA samples known

Fig.4 Primer extension with oligos X and B.

A: Primer extension with oligo X (see Fig.3). Lane 1, oligo X alone (no RNA); lane 2, 50µg yeast tRNA; lane 3, 50µg HL60B total cellular RNA; lane 4, 50µg HL60P25 total cellular RNA; lane 5, 10µg CGL polyA+ RNA. Note lack of CGL-specific primer extension (lane 5).

B: Primer extension with oligo B. Lane 1, oligo B alone (no RNA); lane 2, 50µg yeast tRNA; lane 3, 50µg HL60B total cellular RNA; lane 4, 50µg HL60P25 total cellular RNA; lane 5, 10µg CGL polyA+ RNA. Note CGL-specific primer extension (lane 5). This extension is approximately 150 nucleotides relative to a sequencing gel run in parallel as size markers (data not shown).

Note: the lack of defensin-specific primer extension seen in Fig.4B lane 4 reflects the relatively small amount of polyA$^+$ mRNA present in this <u>total</u> RNA sample (approximately 1-5% of total RNA is mRNA). Thus the detection of defensin-specific primer extension in Fig.4B lane 5 but not in lane 4 reflects, in part, an approximately 20-fold difference in the amount of mRNA present in these two samples.


A
1 2 3 4 5
B
1 2 3 4 5
M
-150
-100
-50
-20

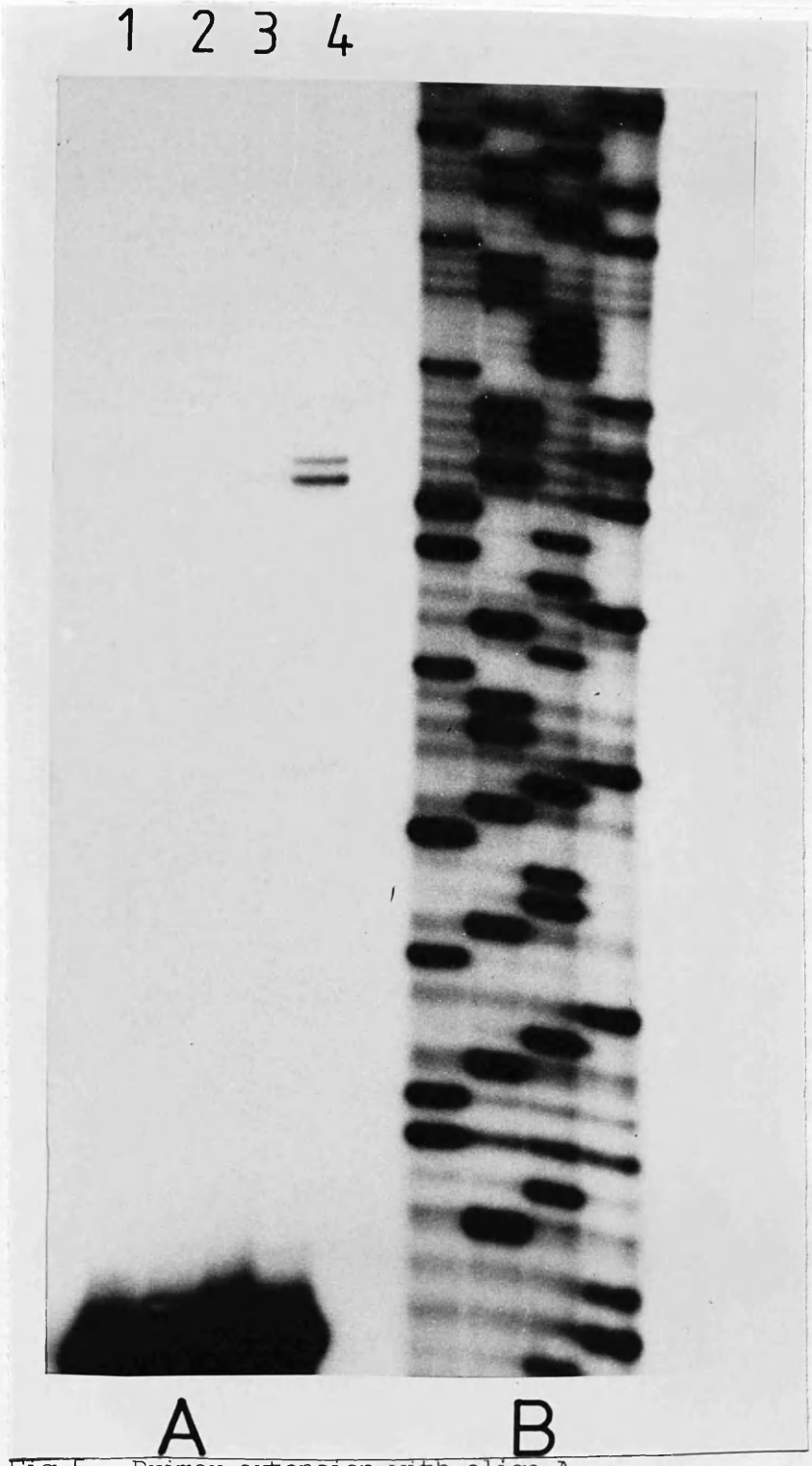


Fig.5 Primer extension with oligo A.

A: Primer extension with oligo A (Fig.3). Lane 1, oligo A alone (no RNA); lane 2, 50µg yeast tRNA; lane3, 50µg HL60P25 total cellular RNA; lane 4, 10µg CGL polyA+ RNA. Note two main primer extension products, one nucleotide apart (lane 4).

B: DNA sequence ladders generated from M13 mp18 which were used for size estimation of the primer extended material.

to contain no pCG14-homologous mRNA. In contrast, with two other 20-mer oligonucleotides 3' to oligo X, oligos A and B (see Fig. 3), CGL - specific primer extension could be demonstrated (Figs. 4B, 5).

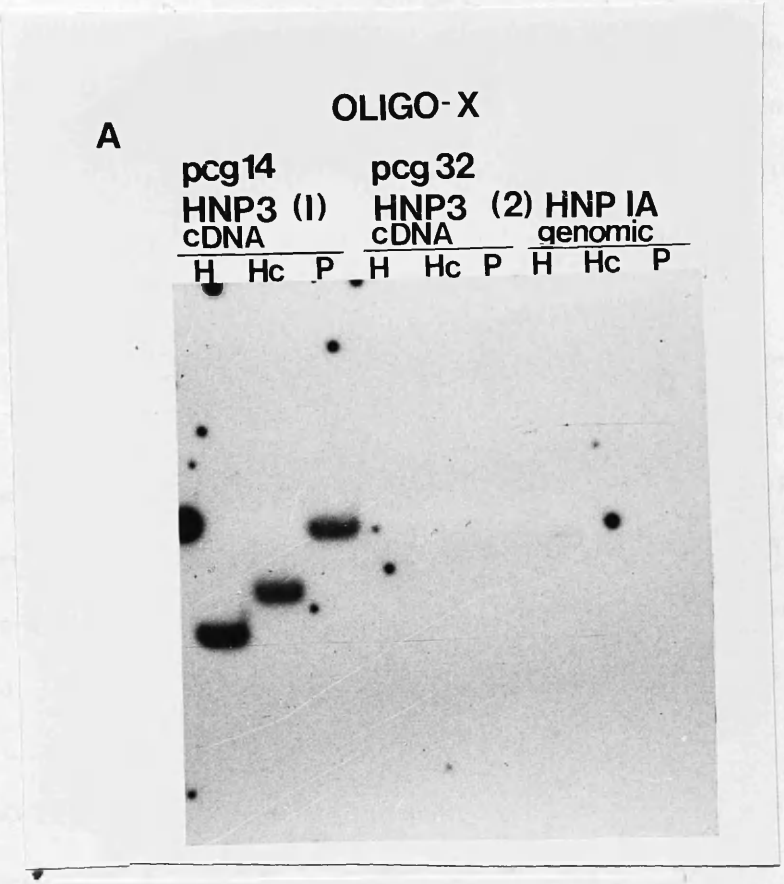
With oligo A this analysis established that the mRNA(s) to which this oligonucleotide hybridized contained a further 29 and 30 nucleotides of 5'-sequence, with two main extension products, one base apart. The same conclusion could also be drawn from the same experiment performed using oligo B (Fig. 4B): CGL-specific primer extension in this case extending 150± 2 nucleotides.

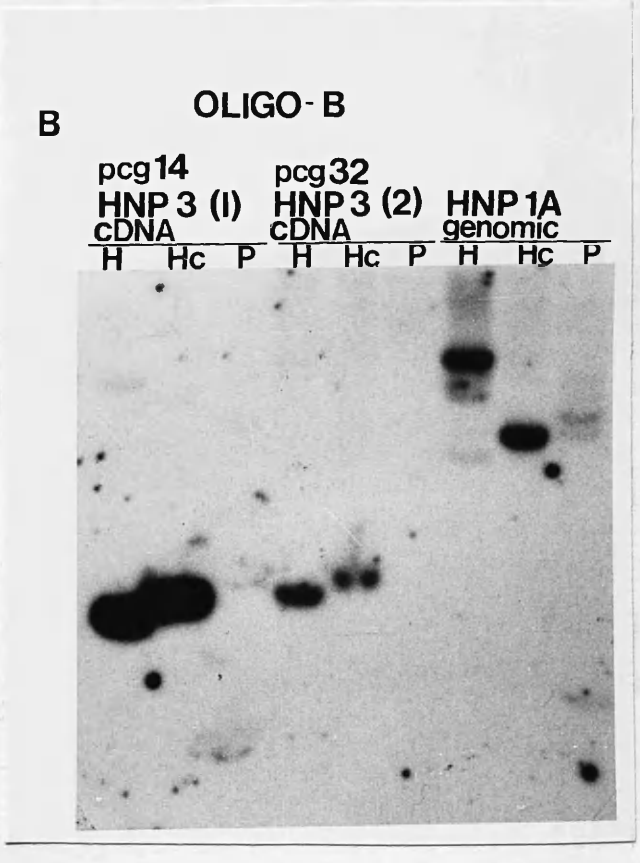
3.1.3 Oligonucleotide hybridization

The most obvious possibility from these results was that most, if not all, sequence in pCG14 5' to oligo A was not derived from pCG14 mRNA and had been fused to pCG14-homologous sequences during construction of the CGL cDNA library from which the clone was isolated (Wiedemann et al, 1983). This idea was tested in two ways. Firstly, oligos X and B were used to probe a Southern blot of pCG14 and pCG32 plasmid DNAs in addition to a 8Kb genomic clone for HNP1A (see Section 3.3) digested with a number of restriction enzymes. Probing with oligo X indicated that oligo X sequences were only present in the pCG14 clone and absent from both pCG32 and the HNP1A genomic clone (Fig. 6A). In contrast, the same blot stripped and probed with oligo B showed that both pCG14 and pCG32 as

Fig.6 Oligonucleotide hybridization to plasmid Southern blots.

Restriction enzyme digests of approximately 1µg each of plasmids pCG14, pCG32 (inserts cloned into the filled-in BamH1 site of pAT153) and pHNP1A (an 8kb defensin genomic clone recloned from lambda L47.1 into pUC8, see Section 3.3) were performed and digestion products electrophoresed on a 1% agarose gel. The gel was Southern blotted onto a Hybond N membrane. Approximately 150ng of each oligonucleotide was 5'-end labelled and each hybridized to the filter in turn. A: blot hybridized to oligo X ; B: stripped blot hybridized to oligo B. The absence of signal with oligo B in the Pst1 lanes of pCG14, pCG32 and pHNP1A is due to the position of this oligo. lying on a Pst1 fragment of 50bp which runs off the gel. Faint bands seen in the Pst1 lane of pHNP1A (B) are partial digestion products.Abbreviations: H, Hind3; Hc, Hinc2; P, Pst1.


A
OLIGO-X
pcg14
HNP3 (1)
cDNA
H Hc P
pcg 32
HNP3 (2)
cDNA
H Hc P
HNP 1A
genomic
H Hc P


B
OLIGO-B
pcg14
HNP 3 (1)
cDNA
H Hc P
pcg32
HNP 3 (2)
cDNA
H Hc P
HNP 1A
genomic
H Hc P

well as the HNP1A clone contained oligo B-homologous sequences (Fig. 6B). Thus pCG14 and pCG32 were shown to have distinct 5'-sequences, a result later confirmed by sequencing of pCG32 at its 5'-end (Fig. 10).

Since the possibility existed that clone pCG14 was derived from an alternatively spliced mRNA differing from the predominant form(s) revealed by primer extension by the presence of sequences 5' to oligo A, oligos X, A and B were used to probe Northern blots of CGL and HL60P25 RNAs (known to contain the 750-nucleotide pCG14-homologous mRNA) as well as HL60B and human mucosal RNAs (known to contain no pCG14-homologous mRNA). This experiment revealed that oligo X detected a single 1500-nucleotide mRNA present at very low abundance in human colonic mucosal, HL60P25 and HL60B RNAs and undetectable in CGL RNA (Fig. 7A) but did not detect the 750-nucleotide pCG14 mRNA. In contrast, oligos A and B only detected this 750-nucleotide mRNA (Figs. 7B, C).

3.1.4 S1 nuclease protection of pCG14 cDNA

Both these experiments supported the idea that the pCG14 cDNA clone was a chimaera, with sequences at its 5'-end derived from a 1500-nucleotide mRNA distinct from the 750-nucleotide mRNA from which the main body of the clone was derived. To confirm this hypothesis, and to map the position of the junction between the two sequences, an S1 nuclease experiment using a single-stranded uniformly-labelled pCG14

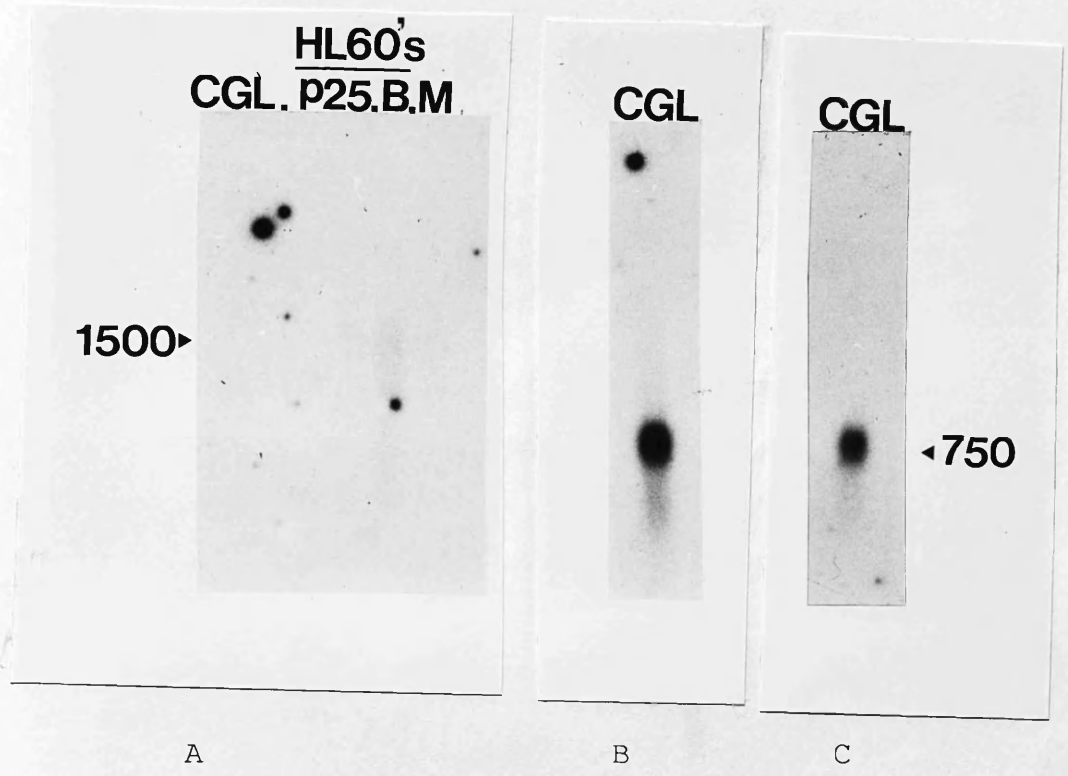


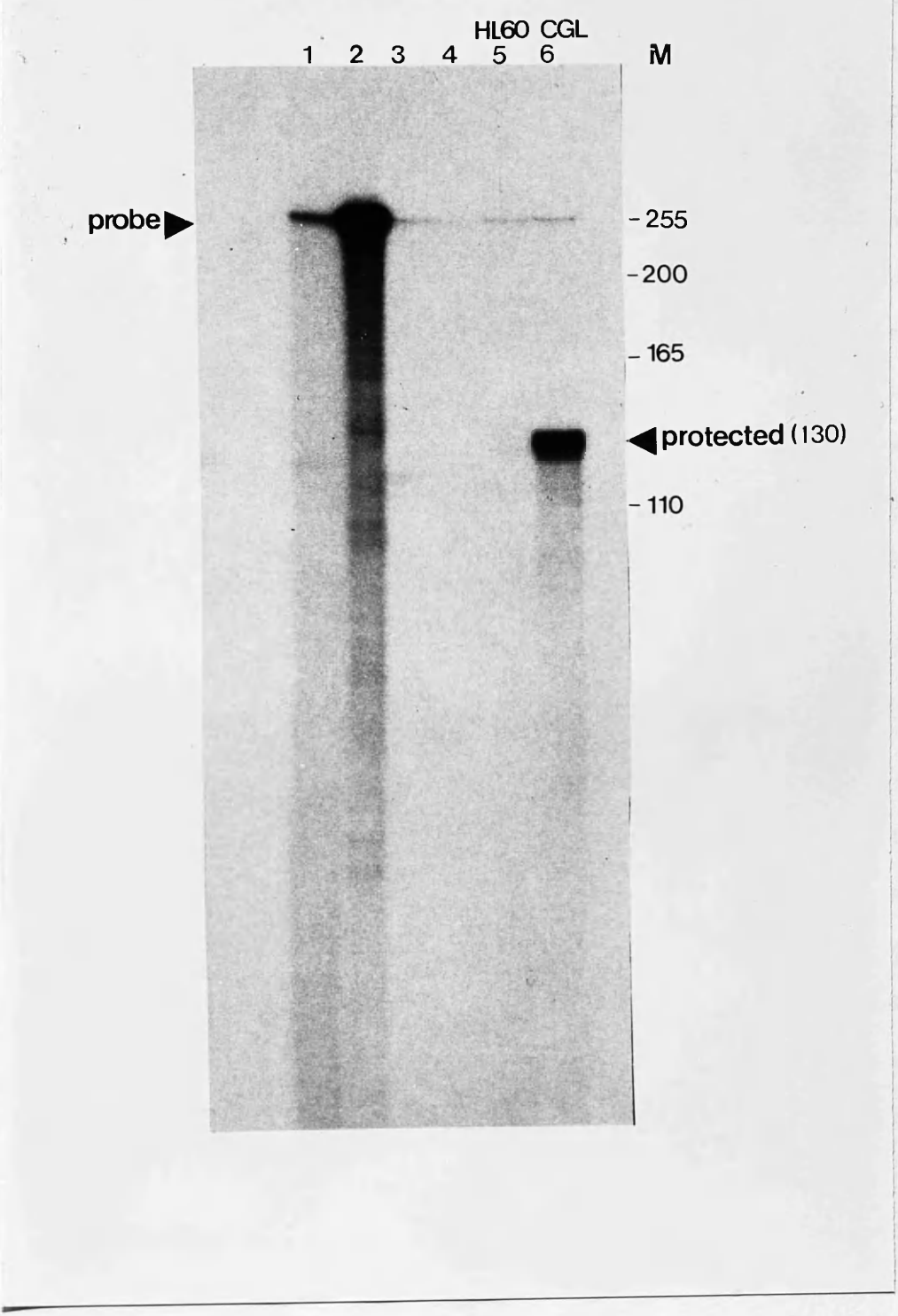
Fig.7 Oligonucleotide hybridization to Northern blots.

Approximately 150ng of each oligonucleotide was 5'-end labelled and hybridized to Northern blots of various RNAs. A: Oligo X; CGL, 0.2µg polyA+ RNA; P25, 6µg total cellular RNA from HL60P25 cells; B, 6µg total cellular RNA from HL60B cells; M, 6µg human mucosal total cellular RNA (a kind gift from Dr. Paul Elvin). B: Oligo A; CGL, 0.2µg polyA+ RNA; C: Oligo B; CGL, 0.2µg polyA+ RNA.

Fig.8 S1 nuclease protection experiment.

A Bgl2-Pst1 fragment of pCG14 was cloned into M13mp18. A single-stranded uniformly-labelled probe complementary to pCG14 mRNA was prepared using an M13 universal primer to prime DNA synthesis using the Klenow fragment of E.coli DNA polymerase. A 255-nucleotide probe was gel-purified and hybridized to various RNAs, prior to S1 nuclease digestion. Lane 1, S1 probe digested; lane 2, S1 probe undigested; lane 3, 50µg yeast tRNA; lane 4, 50 µg HL60B total cellular RNA; lane 5, 50µg HL60P25 total cellular RNA; lane 6, 10µg CGL polyA+ RNA. An identically sized S1-protected fragment is detected with both HL60P25 (faint) and CGL (strong) RNAs.* The size of the protected fragment was estimated relative to a sequence ladder generated from the same M13mp18/Bgl2-Pst1 pCG14 clone run in parallel (data not shown) and was 130(± 2) nucleotides.

* see Fig.4 (Note) regarding the 20-fold difference in starting mRNA between the samples in lanes 5 and 6 which accounts in part for the difference in signal of the protected fragment in these two samples.


HL60 CGL
1 2 3 4 5 6 M
probe
255
200
165
protected (130)
110

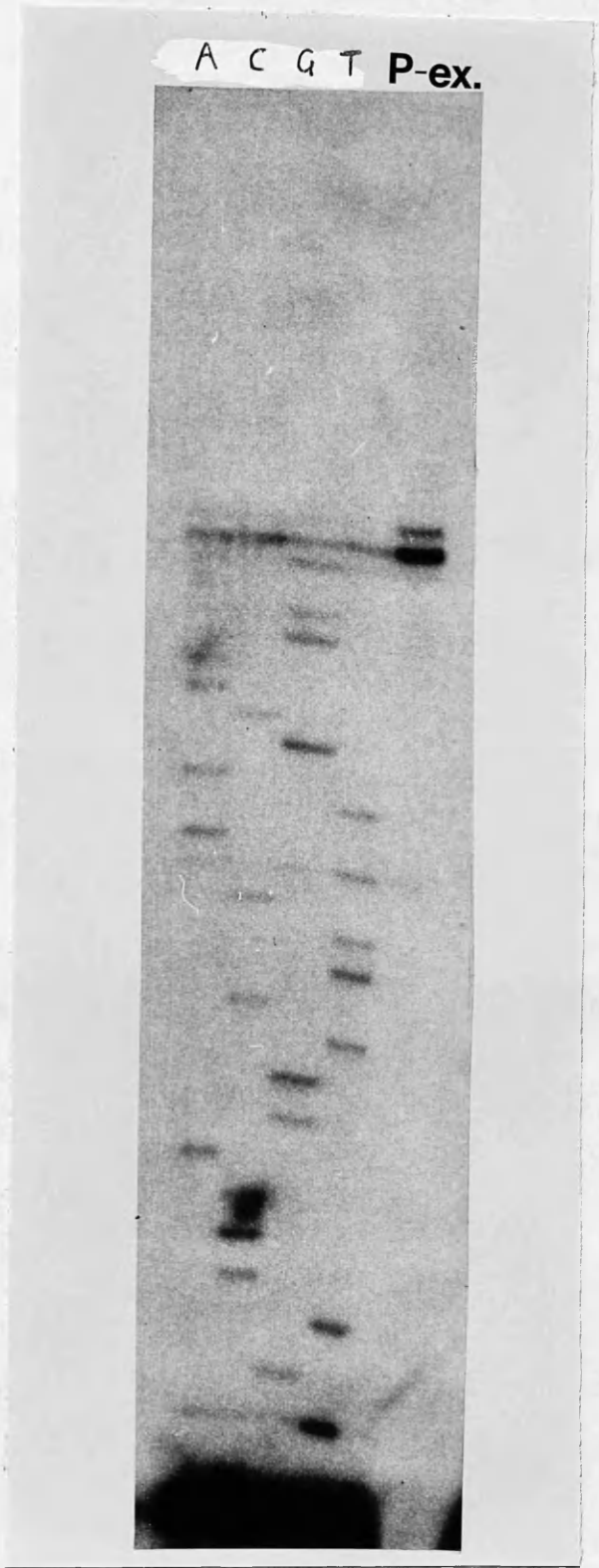
cDNA probe prepared by subcloning a Bgl2 - Pstl fragment (see Fig. 2) into M13 mp18 was undertaken. The S1 probe spanned the hypothesized junction between pCG14 homologous and non-homologous sequences since it contained both oligo X and oligo A sequences. Complete protection from S1 digestion of sequences from Bgl2 to Pstl would have yielded a protected fragment of approximately 180 nucleotides after digestion of M13 probe sequences. However CGL RNA protected a sequence 130 ± 2 nucleotides in length. (Fig. 8, lane 6), indicating that the junction hypothesized in pCG14 lay around nucleotide 78 (see Fig. 3) in the clone. Since this position mapped to within a few nucleotides of the predominant 5'-ends of pCG14-homologous mRNA (Fig. 5), it seemed likely that almost the complete 5'-end sequence of pCG14 mRNA was present in the pCG14 clone and was juxtaposed to non-pCG14-derived sequences which thereby appeared to lengthen RF1.

3.1.5 Direct primer extension sequencing of pCG14 mRNA

To establish unequivocally the identity of sequences in the pCG14 cDNA clone 5' to oligo A, up to the junction with non-pCG14-homologous sequences with those in pCG14 mRNA, oligo A was used to primer extend sequence pCG14 mRNA present in CGL RNA. The sequence obtained (Figs. 9 and 11B) indicated that only the last 3 or 4 nucleotides were missing from the 5'-end of the pCG14 clone. Thus, although the fusion of non-pCG14 homologous sequences had appeared to lengthen RF1, the 5'-most AUG in the clone in a good sequence context (Kozak, 1986) most

Fig.9 Sequence of 5′-end of pCG14 mRNA.

The sequence was determined by primer extension sequencing using reverse transcriptase. Each lane T, G, C, and A represent primer extension reactions (2µg CGL polyA+ RNA, 25ng 5′-end labelled oligo A) carried out in the presence of the corresponding dideoxynucleotide triphosphate. P-ex is the same reaction run in the absence of dideoxynucleotide triphosphates and is the same result (2 main primer extension products representing extensions of 28 and 29 nucleotides) as that shown in Fig.5. The intense autoradiographic signal at the bottom of the gel represents unextended oligo A.


A C G T
P-ex.

likely to initiate protein synthesis occured at nucleotide 166 (Fig. 3) such that RF1 would encode a 94-amino acid polypeptide (Fig. 11A).

Sequencing of the 5'-end of pCG32 (Fig. 10) indicated that this clone was homologous to pCG14 3' to nucleotide 89 in pCG14 (Fig. 3), but diverged in sequence 5' of this position. A computer search in an EMBL nucleic acid sequence database indicated that the non-pCG14 mRNA sequence in pCG32 was identical to part of human mitochondrial URF1 sequence. However, the 1500-nucleotide mRNA from which the foreign sequence in pCG14 is derived remains unidentified.

Thus both cDNA clones pCG14 and pCG32 were shown to be chimaeric at their 5'-ends. Further experiments (data not shown) using oligonucleotides C and D (see Fig. 3) as hybridization probes to Northern blots of CGL RNA indicated that the pCG14 cDNA clone at its 3'-end contained no foreign sequences introduced during cloning.

3.1.6 Clone pCG14 encodes defensin HNP3 peptide

Thus the clone pCG14 was shown to have the sequence and coding potential shown in Fig. 11A. At this time Mars et al (1988) published the sequence of a cDNA clone (termed mrs) almost identical (differing at only one nucleotide position) to pCG14 cDNA. Mars et al (1988) had also isolated their cDNA clone from a cDNA library derived from CGL leukocytes and

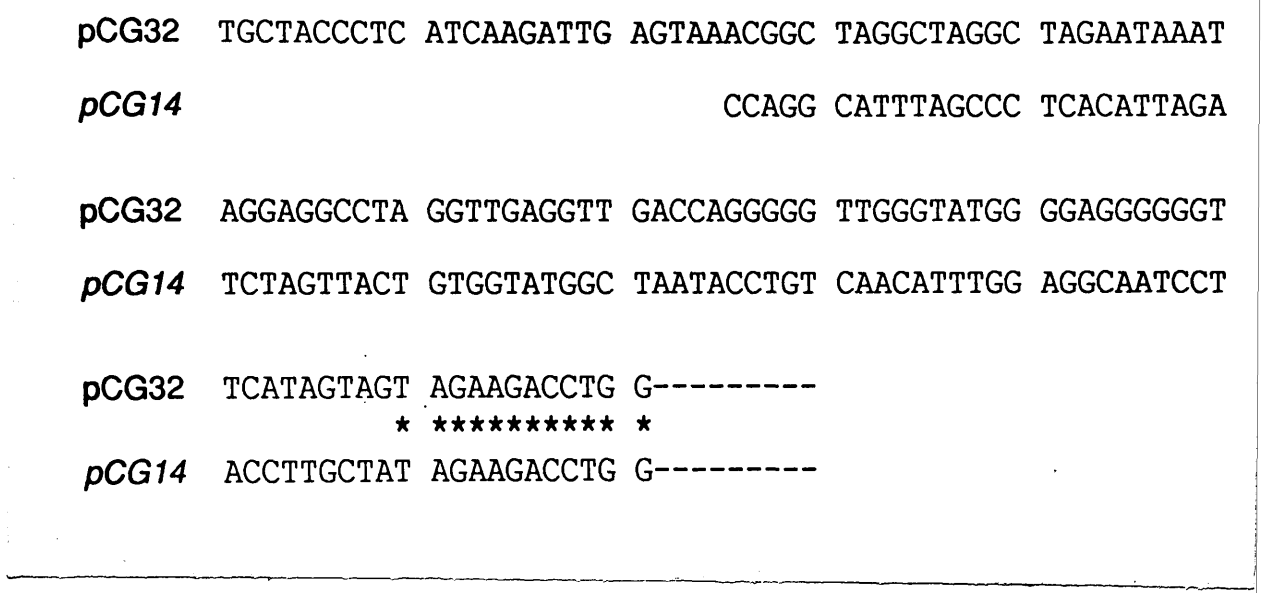


Fig.10 Sequences of 5′-ends of pCG14 and pCG32 cDNAs.

The 5′ sequences of clones pCG14 and pCG32 are shown aligned. Identity of sequences is indicated by stars between the sequences. The sequence of the 5′-end of pCG14 is derived from an as yet unidentified 1.5kb mRNA (see Fig.7). The sequence at the 5′-end of pCG32 is different and shares 100% identity with part of a human mitochondrial transcript URF1. Thus both these cDNA clones are very nearly full-length at their 5′-ends and contain foreign sequences fused to this. The strategy for sequencing these clones at their 5′-ends is shown in Fig.2.

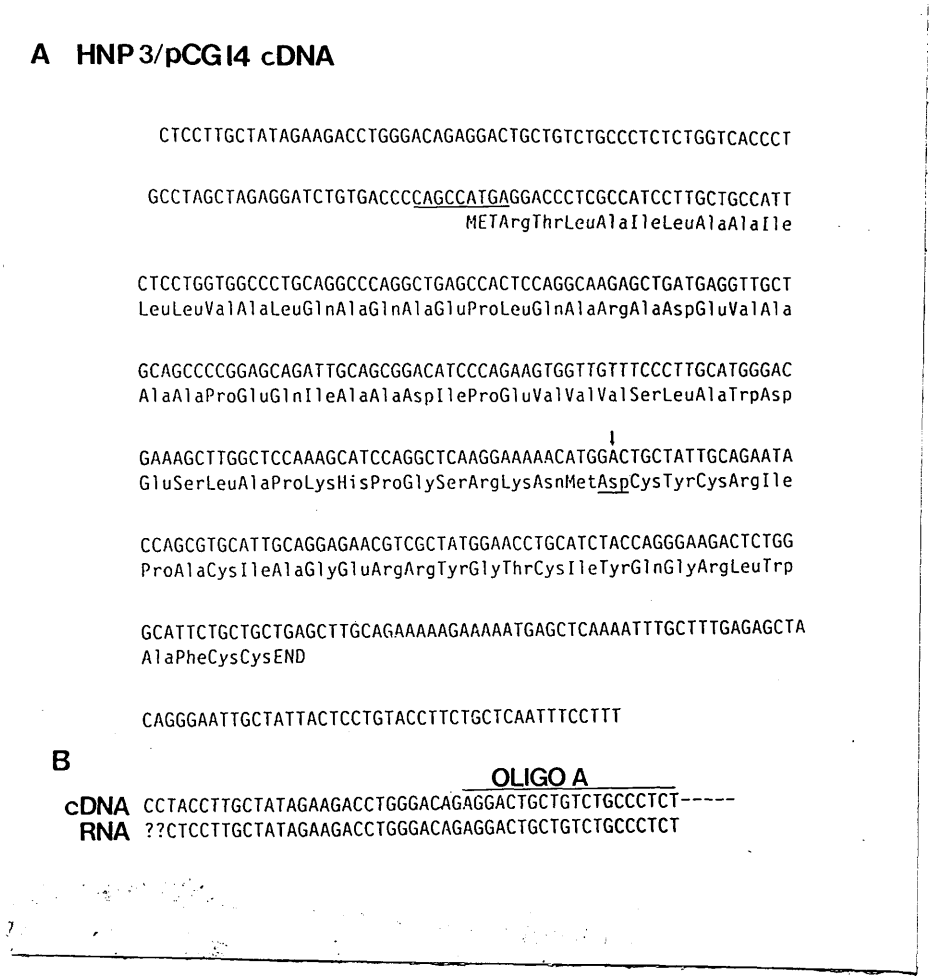


Fig.11 Revised sequence of cDNA clone pCG14.

A: A revised sequence of clone pCG14 is shown together with its predicted primary translation product. The predicted translational initiation region is shown underlined and has a 7/9 match with the initiation consensus (CC[A/G]CCATGG) proposed by Kozak (1986) and predicts a 94-amino acid polypeptide. The arrow indicates the nucleotide which differs from the sequence presented by Mars et al (1988): A in pCG14, C in mrs.
B: Comparison of the sequence of the 5'-end of pCG14 cDNA (top) with that of the 5'-end of pCG14 mRNA (bottom), as determined by primer extension sequencing using the oligonucleotide complementary to bases 106-125 of the sequence shown in Fig.3.

selected it on the basis of its increased expression in CGL leukocytes compared with normal human placental tissue and the leukocytes of an ANLL patient (Mars et al, 1985). The relevance of the one nucleotide difference between the pCG14 and mrs cDNA clones (indicated by an arrow in Fig. 11A) became clear very shortly afterwards by the publication by Selsted et al (1985b) of the isolation from human neutrophils and amino acid sequencing of a group of three peptides called defensins.

Fig. 12 shows the primary amino acid sequence of the three defensin peptides. It was clear that HNP1 defensin is the C-terminal 30 amino acids of the mrs ORF, whilst HNP3 defensin is the C-terminal 30 amino acids of the pCG14 ORF (Fig. 11). Thus the single nucleotide difference between the two cDNAs (C in mrs, A in pCG14) results in two almost identical peptides differing only at their N-terminal residue (alanine in HNP1, aspartic acid in HNP3). HNP2, the other defensin peptide isolated by Selsted et al lacks this N-terminal residue (Fig. 12) and may be a processing product of either or both of defensins HNP1 and HNP3.

3.1.7 Western blotting of defensin peptides

Since in both cases HNP1 and HNP3 represent only the C-terminal 30 amino acids of their predicted 94-amino acid primary translation products (Fig. 11A), proteolytic cleavage would also have seemed to be involved in their derivation. As an attempt to prove this and also to confirm the 3kD (30

HNP1
ALACysTyrCysArgIleProAla**CysIleAlaGlyGluArgArgTyrGlyThrCys**
IleTyrGlnGlyArgLeuTrpAlaPheCysCys

HNP2
CysTyrCysArgIleProAla**CysIleAlaGlyGluArgArgTyrGlyThrCys**
IleTyrGlnGlyArgLeuTrpAlaPheCysCys

HNP3
ASPCysTyrCysArgIleProAla**CysIleAlaGlyGluArgArgTyrGlyThrCys**
IleTyrGlnGlyArgLeuTrpAlaPheCysCys

Fig.12 Primary amino acid sequences of defensin peptides.

The primary amino acid sequences of the three cysteine-rich peptides abundant in human PMN characterized by Selsted et al (1985b) are shown aligned. HNP1 and HNP3 are identical in sequence apart from their N-terminal residue (alanine in HNP1, aspartic acid in HNP3). HNP2 is identical in sequence to both HNP1 and 3, except that it lacks their N-terminal residue. In bold is the sequence of the oligo-peptide used to raise a rabbit antipeptide antiserum against all 3 defensin peptides.

amino acid) size of the mature defensins, it was decided to raise an antipeptide antiserum to a 10-amino acid region common to all three defensins. A rabbit antipeptide antiserum was raised against this peptide (see Section 2.1.1) and used to probe a Western blot of human leukocyte proteins (Fig. 13). The anti-defensin antiserum recognized species migrating as a single band with an apparent molecular weight of 3kD, the molecular weight of the mature defensins HNP1, 2 and 3, described by Selsted et al (1985b). To demonstrate specificity of the antipeptide antiserum for defensins, control experiments were performed in which the antiserum was first preincubated with the 10-mer peptide used to raise it and then used to probe a Western blot of human leukocyte proteins. This resulted in the loss of the 3kD signal shown in Fig. 13 (data not shown). Furthermore, preimmune serum from the same rabbit used to raise the antipeptide immune serum failed to detect any species in the 3kD range (data not shown). The demonstration of no species detectable on Western blots other than the 3kD mature defensins indicated that if indeed HNP1 and HNP3 are derived from larger precursor polypeptides that these precursors are absent or present at undetectable levels in human peripheral blood leukocytes. Experiments described in Section 3.2.4 further addressed the question of the presence of defensin precursors in HL60 cells.

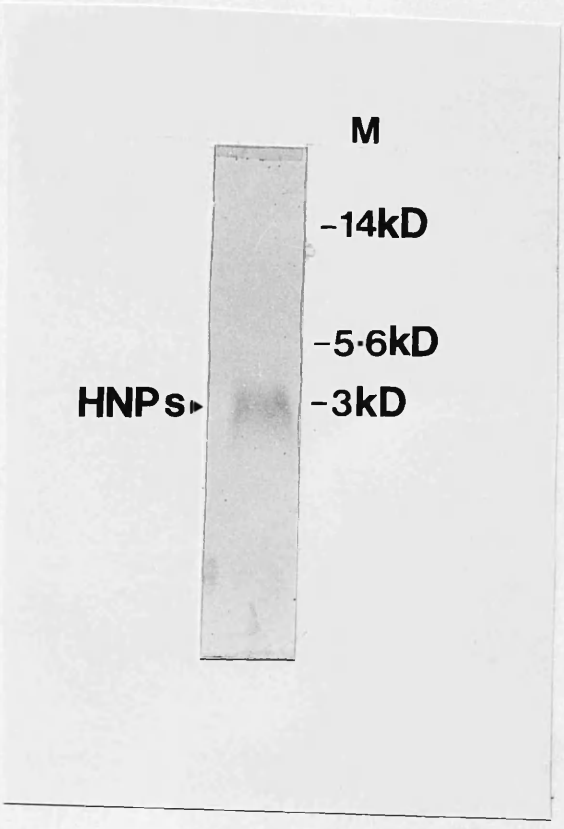


Fig.13 Western blot of mature defensin peptides.

Normal peripheral blood leukocytes were prepared from a buffy coat and a cytoplasmic extract made by NP40 lysis. Approximately 20µg of protein was electrophoresed by SDS-PAGE and the gel electroblotted onto nitrocellulose. Details of the detection of defensins using a secondary alkaline-phosphatase conjugated antibody are given in Section 2.9 . Size markers ran in parallel were pre-stained molecular weight protein standards (3kD is insulin A and B chains, 5.6kD is bovine trypsin inhibitor and 14kD is lysozyme).

3.2 DEFENSIN GENE EXPRESSION DURING MYELOID DIFFERENTIATION

As described in Section 1.3.4 one of the most useful systems to investigate changes in gene expression during myeloid differentiation utilizes HL60 cells. Cultures of these cells can be induced to differentiate to mature granulocyte-like cells by treatment with a variety of chemical inducers, including DMSO and retinoic acid.

Since during normal hematopoiesis defensin gene expression is both lineage-specific (restricted to the myeloid lineage) and stage-specific (restricted to myelocytic cells) (see Section 1.6.3), it was of interest to investigate whether, in a model system, defensin gene expression was also strictly linked to differentiation.

3.2.1 Defensin gene expression in HL60 cells DMSO-induced

One particular subline of HL60 cells (HL60P25) used in the laboratory was very similar to the original cell line established by Collins et al (1977), having, for example, a rather long doubling-time (36-48 hours) in comparison to other laboratories' HL60 cells. This line was used in the majority of experiments described in this section. Of many other hematopoietic cell lines investigated (Fig. 21) only HL60P25 cells expressed defensin mRNAs. Thus in the following experiments we are looking at a system in which the defensin

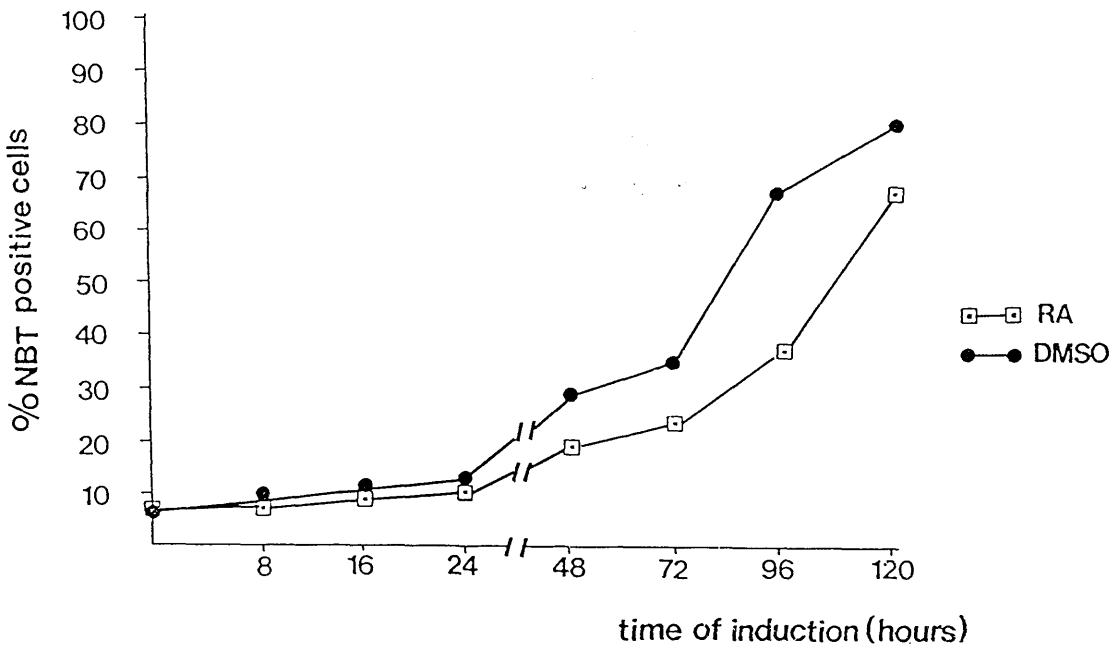


Fig.14 Induction of differentiation of HL60 cells.

Exponentially growing HL60 cells were induced to differentiate by treatment with either DMSO (Section 3.2.1) or retinoic acid (Section 3.2.3) and the proportion of NBT-positive cells in culture measured. Growing cultures of HL60P25 cells typically had 4-10% of NBT-positive cells due to spontaneous differentiation , rising to 80-90% and 60-70% after treatment with DMSO and retinoic acid (RA), respectively.

gene(s) are already synthesizing defensin mRNAs, a situation which is followed during normal myeloid differentiation by down-regulation of expression (Wiedemann et al, 1989).

Inducing HL60P25 cells to differentiate along the myeloid lineage by treatment with DMSO (Fig. 14), led to a down-regulation of defensin mRNA levels. Fig. 15A shows a Northern blot of polyA$^+$ mRNA from HL60 cells, harvested at various times following their treatment with DMSO, and probed with a defensin cDNA (HNP3; pCG14 minus non-defensin sequences). During the first hour after addition of DMSO, the abundance of defensin mRNAs decreases, whereafter their levels are restored to near pre-induction levels 7 hours later. Subsequently, the abundance of defensin mRNAs is again decreased, this time much more slowly. Both ethidium bromide staining of the gel (data not shown) and reprobing of the filter with a cDNA probe for β_2-microglobulin (Fig. 15B), detecting an mRNA whose level is known not to change to any extend during HL60 differentiation (Mitchell, 1987), indicated that each lane in Fig. 15A contained approximately the same amount of mRNA and, therefore, that the observed changes in autoradiographic signal intensity reflected real changes in the abundance of defensin mRNAs.

Changes in gene expression can occur at a variety of levels, both transcriptional and post-transcriptional. The early transient decrease seen with defensin mRNA during treatment of HL60P25 cells with DMSO could, for example, have

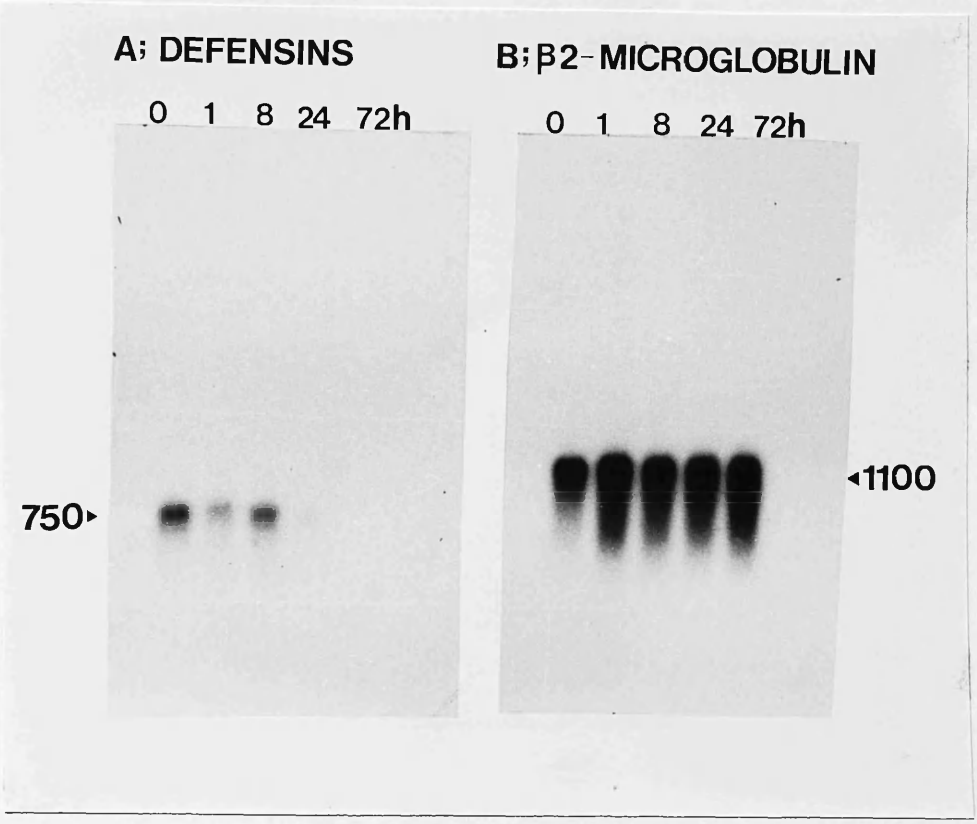


Fig.15 Northern blot analysis of defensin gene expression during granulocytic differentiation of HL60P25 cells induced by DMSO.

Exponentially growing HL60P25 cells were induced to differentiate by treatment with DMSO (1.5% v/v) and total cellular RNA harvested at various time intervals. PolyA+ RNA was prepared, and 1µg from each time fractionated on a denaturing agarose gel. The RNA was then blotted from the gel onto a Hybond-N nylon membrane. A: blot probed with a radiolabelled HNP3 defensin cDNA probe. B: blot stripped and reprobed with a radiolabelled β2-microglobulin cDNA probe (Section 2.1.3), to ensure equal amounts of RNA on each lane of the blot.

occured at the level of mRNA stability, a level of control of gene expression shown for certain other mRNAs including those for GM-CSF and c-myc (Greenberg et al, 1986; Shaw and Kamen, 1986). Alternatively, the same early decrease could have been accounted for by a transient transcriptional block as described (though not in these cases transiently) for the c-myc and c-myb genes (Bentley and Groudine, 1986; Bender et al, 1987).

To attempt to decide which of these levels of control were operating, it was decided to investigate the stability of defensin mRNAs in HL60 cells. The rationale for this experiment is as follows. If defensin mRNAs in uninduced HL60P25 cells were ordinarily very stable, then if a transcriptional block were operating exclusively after treatment of the cells with DMSO, one would expect a comparatively high level of defensin mRNAs after one hour, which is not what is observed (see Fig. 15A). Therefore treatment of the cells with DMSO must also result in a rapid destabilization of defensin mRNAs.

3.2.2 mRNA stability and transcription of defensin genes

One way of measuring mRNA stability is to utilize a potent inhibitor of transcription by RNA polymerase 2, such as actinomycin D, thus allowing a decoupling of the process of transcription from post-transcriptional levels of control of mRNA abundance. Fig. 16A shows a Northern blot of whole cell RNA harvested at various times following treatment of HL60P25 cells with actinomycin D and probed with a defensin cDNA. Two features are noticeable. Firstly, a transient increase in the level of defensin mRNAs 30 min after treatment. This transient increase appears to be a real one and not due to more RNA loaded in this lane, since ethidium bromide staining of the gel prior to transfer onto the nylon filter shows that there was an equal amount of RNA on each lane (data not shown).

The second feature of Fig. 16A is that, even after 3 hours of treatment (no defensin transcription for 3 hours), there is still significant defensin mRNAs present (approximately two-thirds of untreated cells). A slot blot of the same RNAs probed with a c-*myc* exon 2 probe (Fig. 16B) demonstrated that the actinomycin D treatment is indeed inhibiting transcription since c-*myc* RNA is turning over with a characteristic short half-life (Dani *et al*, 1984).

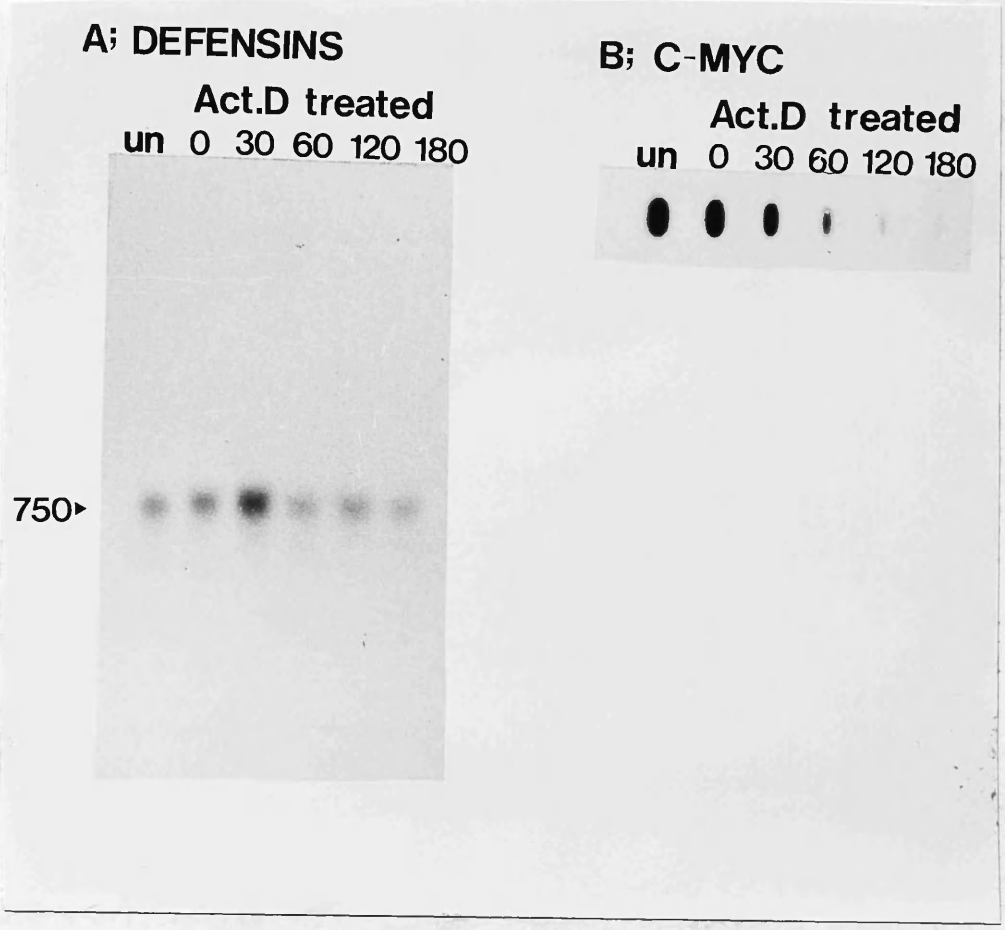


Fig.16 Determination of stability of defensin mRNAs.

Exponentially growing HL60P25 cells were treated with actinomycin D (5µg/ml) and total cellular RNA harvested immediately (0 time-point) and at various time intervals. A: Northern blot analysis of 10µg of total cellular RNA fractionated on a denaturing agarose gel and hybridized with a radiolabelled HNP3 defensin cDNA probe. B: Slot-blot of 2µg of total cellular RNAs from the same experiment hybridized with a radiolabelled human c-myc exon 2 probe (Section 2.1.3).

Thus defensin mRNAs are ordinarily rather stable in HL60P25 cells. This finding implies then that whatever mechanism is involved in the initial decrease in abundance of defensin mRNAs during 1 hour of treatment of the cells with DMSO, a decrease in mRNA stability must also be involved. Although this established that an early decrease in the stability of defensin mRNAs is necessary to bring about the observed decrease in abundance, it does not establish that it is in itself sufficient to bring this about, leaving open the possibility that some transient transcriptional pause could also be involved. For the reasons explained in Section 3.2.5 it was impossible to directly measure the stability of defensin mRNAs after treatment with HL60P25 cells with DMSO.

To investigate the transcription of defensin gene(s) in HL60P25 cells, with a view to answering this question and also the question of whether the later, slower decrease in abundance of defensin mRNAs after treatment of the cells with DMSO (Fig. 15A) is accomplished by a transcriptional switch-off, nuclear run-on transcription experiments, which allow one to investigate how actively a given gene or gene segment is being transcribed (Groudine et al, 1981), were attempted.

In the experiment, nuclei harvested from exponentially growing HL60 cells were used in an in vitro run-on reaction, radiolabelled RNA harvested and used to probe a slot-blot of double-stranded cDNA probes bound to a nitrocellulose filter,

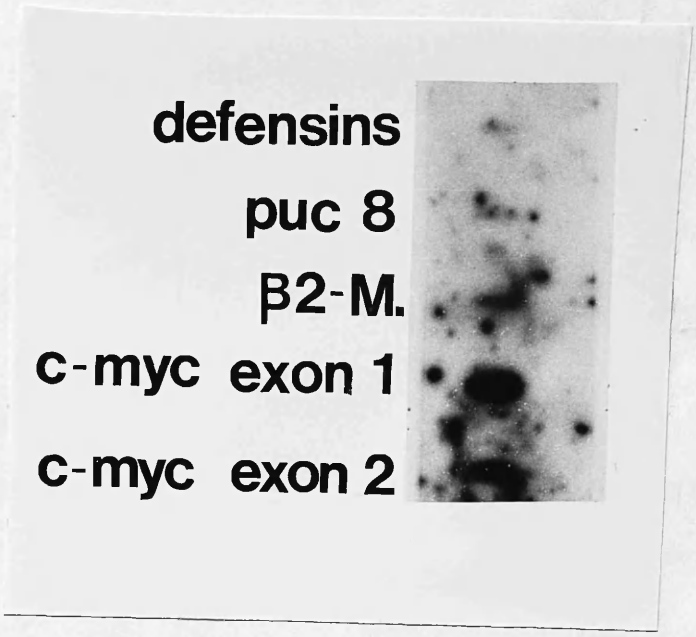


Fig.17 Nuclear run-on transcription assay in HL60P25.

1µg of double-stranded plasmid inserts for HNP3 defensin, β2-microglobulin, c-myc exon 1, c-myc exon2 and EcoR1 cut pUC8 were alkali-denatured and slot-blotted onto nitrocellulose. The filter was hybridized with radiolabelled RNA transcripts generated from a nuclear run-on transcription assay using nuclei prepared from exponentially growing HL60P25 cells.

including a full-length defensin cDNA. Fig. 17 shows the result. Defensin gene transcription in HL60 cells is very low, barely in excess of a negative control (pUC8) and certainly not as high as transcription of the genes for β_2-microglobulin (single copy) or c-myc (amplified some 30-fold in these cells (Graham et al, 1985). The excess of c-myc exon 1 transcription over exon 2 observed is due to the presence of a transcriptional pause site at the end of exon 1 (Bentley and Groudine, 1986). Unfortunately, this low level of transcription observed for defensin genes in HL60P25 cells made it impossible to study the impact of any possible decrease in transcription to the decrease in abundance of defensin mRNAs following treatment of the cells with DMSO, since any decrease would be hard to establish unequivocally.

3.2.3 Defensin gene expression in HL60 cells RA-induced

It was decided at this juncture to change tack and look at the regulation of defensin mRNAs following treatment of HL60P25 cells with another inducer of granulocytic differentiation, retinoic acid, since there were indications of marked differences in the regulation of certain other mRNAs, including c-myc (Mitchell, 1987) when cells were incubated with this granulocytic inducer rather than DMSO. Fig. 18A shows a Northern blot of whole cell RNA harvested at various times following treatment of HL60P25 cells with retinoic acid, probed with a defensin cDNA. It can be clearly seen that treatment with this inducer, in contrast to

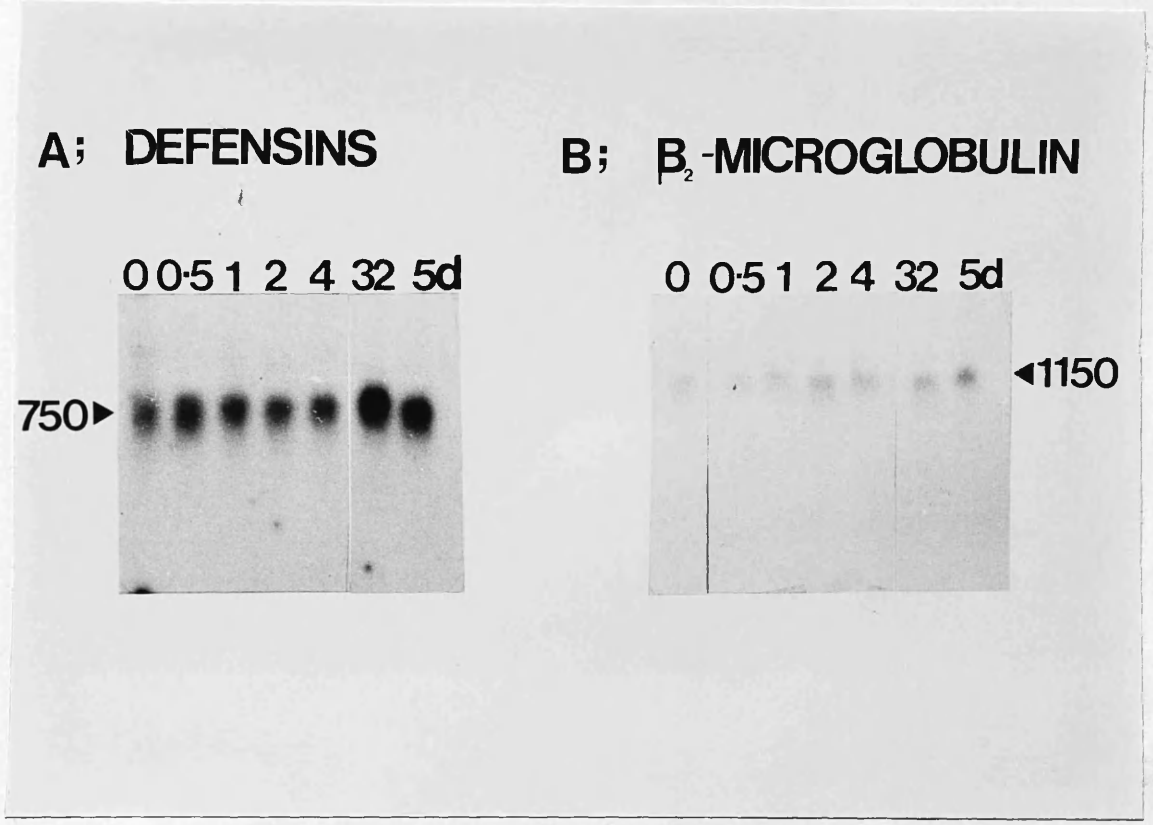


Fig.18 Northern blot analysis of defensin gene expression during granulocytic differentiation of HL60P25 cells induced by retinoic acid.

Exponentially growing HL60P25 cells were induced to differentiate by treatment with retinoic acid (1μM), and total cellular RNA harvested at various time intervals. 10μg of RNA from each time-point was fractionated by electrophoresis on a denaturing agarose gel. The RNA was then blotted from the gel onto a Hybond-N nylon membrane. A: blot hybridized with a radiolabelled defensin cDNA probe. B: blot stripped and reprobed with a β2-microglobulin cDNA probe.

treatment with DMSO, does not lead to a decrease in abundance of defensin mRNAs. Levels of defensin mRNAs are constant up to 32 hours, whereupon an increase (2-4 fold) is seen. At day 5 of treatment defensin mRNAs are still detected at a level similar to uninduced cells. Fig. 18B shows the same composite blot reprobed for β_2-microglobulin mRNA, indicating that approximately the same amount of RNA is present in each lane.

Thus, although retinoic acid is effective at inducing HL60P25 cells to differentiate (as monitored by the ability of the mature cells to reduce the dye NBT (see Fig. 14), the changes observed in the steady-state levels of defensin mRNAs are quite different to those observed after treatment of the cells with DMSO (compare Figs. 15A and 18A). Possible reasons for this will be discussed in Section 4.2.

3.2.4 Investigation of defensin proteins in HL60 cells

At the end of these experiments all our batches of frozen HL60P25 cells had been used. All subsequent experiments with this subline are therefore with cells of the original batch grown up and frozen in new batches and then grown from frozen (now termed HL60P25(1)). All media, serum etc are, however, identical to the previous set of experiments and are as described in Section 2.2.1.

As previously detailed, the mature defensin peptides are 30 amino acids (3kD) as found in mature PMN (Fig. 13). However, the nascent polypeptides encoded by both the pCG14 and mrs cDNAs (encoding HNP3 and HNP1 defensins, respectively) have a predicted size of 94 amino acids (approximately 9kD). To try to show a product-precursor relationship between these two forms of defensin, it was decided to label the protein in vivo by briefly pulse-labelling HL60 cells with (^{35}S)-cysteine and immunoprecipitate defensins and defensin-precursors using the anti-defensin antiserum used to detect mature defensins on Western blots (Figs. 12 and 13). Fig. 19A shows the result of this experiment. As can be seen, some proteins are weakly immunoprecipitated. However, these proteins are immunoprecipitated with both immune and preimmune antisera and cannot, therefore, be defensin-specific (Fig. 19A, comparing PRE and IMM).

Despite three separate attempts at different conditions for immunoprecipitation (altering the stringency of washing after the first immunoprecipitation), this result remained the same. There seemed to be two possible explanations for this: first, the anti-defensin antiserum, although able to recognize epitopes on denatured defensins presented on Western blots, might not be able to recognize defensins or their precursors in their native non-reduced forms in cytoplasmic extracts; second, HL60P25(1) cells might not be sythesizing defensin protein at all. To test this latter hypothesis, a Western blot of a cytoplasmic extract from HL60P25(1) cells was probed

with anti-defensin antiserum. As a control, the same amount of total protein (estimated by staining of the gel with Coomassie Blue) from human PMN was run and transferred to nitrocellulose on the same blot. The result (data not shown) was a failure to detect any defensin-related species in the HL60P25(1) extract despite ready detection of the mature defensin peptides in the extract from human PMN.

3.2.5 Loss of defensin gene expression in HL60P25(1)

To determine whether HL60P25(1) cells were still expressing defensin mRNAs, whole cell RNA was harvested, and a Northern blot run with both HL60P25(1) and HL60P25 RNAs (harvested previously) and probed for defensin mRNAs. The result (Fig. 19Bi) shows that the HL60P25(1) cells used in both the immunoprecipitation and Western blotting experiments were not expressing defensin genes (Fig. 19Bi, lane 2). Reprobing of the blot for β_2-microglobulin mRNA (Fig. 19Bii) showed that there was approximately the same amount of RNA in both samples.

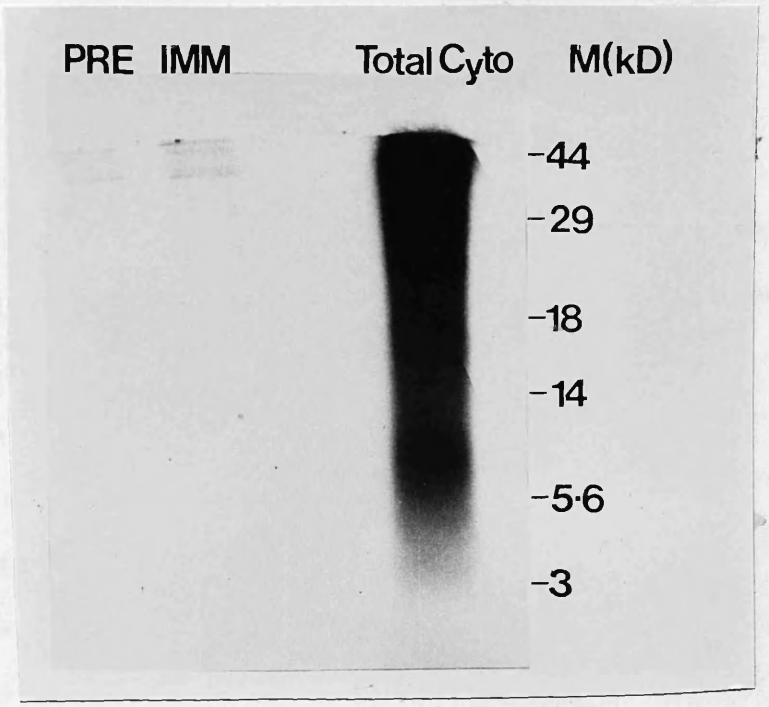
Thus it seemed that although this newly grown batch of HL60P25 cells (HL60P25(1)) were similar to the previous batch of cells (with respect to, for example, ability to differentiate to granulocyte-like cells upon treatment with DMSO, doubling time and morphology (all data not shown)) they differed with respect to expression of defensin genes.

Fig.19 Loss of defensin gene expression in HL60P25(1).

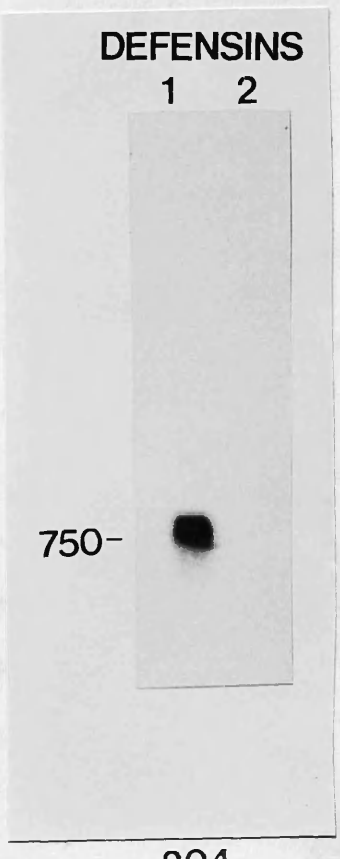
A: Immunoprecipitation of [35S]-cysteine labelled proteins. Exponentially growing HL60P25(1) cells were pulse-labelled with [35S]-cysteine for 8 hours, a cytoplasmic extract made by NP40 lysis and 1/5th volume immunoprecipitated using either preimmune (PRE) or immune (IMM) anti-defensin antisera. Immunoprecipitates collected by protein-A sepharose were denatured, electrophoresed by SDSPAGE and fluorographed prior to autoradiography. Also run on the gel was 5µl of cytoplasmic extract (out of a total volume of 1ml) included to check labelling (TOTAL CYTO), and prestained protein molecular weight size markers (M).

B: Northern blot analysis of defensin gene expression in HL60P25(1). (lane 1) (lane 2)
Total cellular RNA from HL60P25^ and HL60P25(1)^ was harvested from exponentially growing cultures and 10µg each fractionated by electrophoresis on a denaturing agarose gel. The RNA was then blotted from the gel onto a Hybond-N nylon membrane. Bi: blot hybridized to a radiolabelled HNP3 defensin cDNA probe. Bii: stripped blot reprobed with a radiolabelled β2-microglobulin cDNA probe,

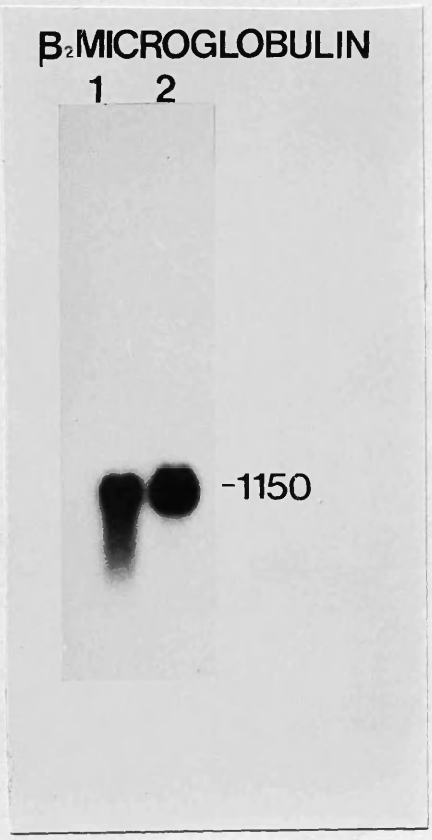
A


PRE IMM
Total Cyto
M(kD)
-44
-29
-18
-14
-5·6
-3

B (i)


DEFENSINS
1 2
750-

(ii)


β2MICROGLOBULIN
1 2
-1150

204

3.2.6 Defensin gene expression in other cell lines

The mechanism(s) of this loss of defensin gene expression is unknown and was not investigated further. However, it would appear to be a phenomenon not restricted to the P25 subline of HL60 cells. Fig. 20 shows a Northern blot of RNA harvested from four different sublines of HL60 cells and probed for defensin mRNAs. Of these 4 sublines (and subsequently another one was tested, data not shown) only the initial P25 subline expressed defensin mRNAs. In fact out of a total of 16 hematopoietic cell lines tested (including 6 different HL60 lines) only the P25 subline of HL60 cells expressed defensin mRNAs (Fig. 21). The loss of defensin expression in HL60P25(1) has resulted in there being no cell line available currently in our hands in which to investigate defensin gene expression.

3.2.7 Differentiation of defensin expression-negative HL60 cells does not induce expression

Inducing another HL60 subline, HL60B, to differentiate along the granulocyte lineage by treatment with DMSO did not induce the cells to synthesize defensin mRNAs. Fig. 22A shows a Northern blot probed for defensin mRNAs of whole cell RNA harvested at various times following treatment of these cells with DMSO. Lane 9 is a positive control of a CGL peripheral blood leukocyte RNA sample containing abundant defensin mRNAs. As a control for equal RNA on each lane of the HL60B RNA

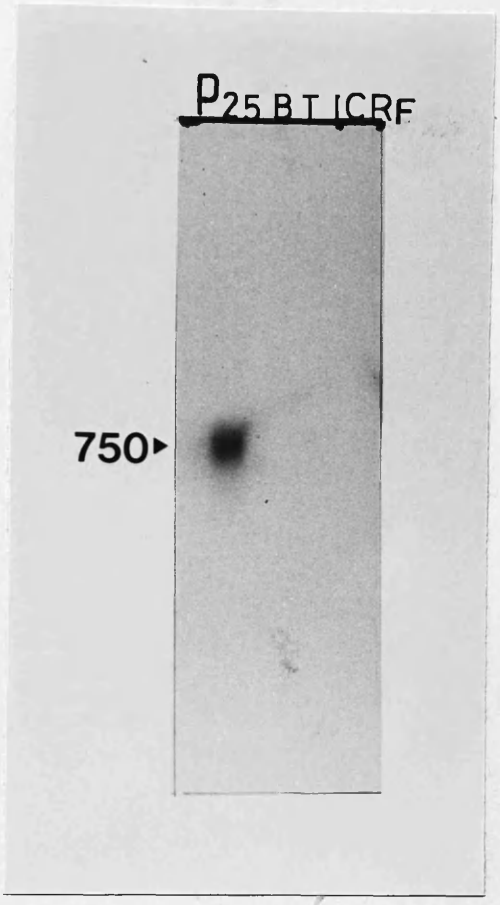


Fig.20 Northern blot analysis of defensin gene expression in 4 HL60 sublines.

Exponentially growing HL60 cells from 4 different laboratories (P25, B, T, and ICRF) growing in RPMI 1640/ 10%FCS were harvested and total cellular RNA prepared. 10µg of each RNA was fractionated by gel electrophoresis, stained with ethidium bromide to ensure equal amounts were present in each lane, and blotted onto a Hybond-N nylon membrane. The blot was then hybridized with a radiolabelled HNP3 defensin cDNA probe.

		EXPRESSION OF DEFENSIN mRNAs (+/−)
HL60 lines	P25	+
	P25(1)	−
	B	−
	T	−
	ICRF	−
	N	−
	K562	−
	ML1	−
	KG1	−
	KG1A	−
	Daudi	−
	Raji	−
	CEM	−
	MOLT-4	−
	Km3	−
	U937	−

Fig.21 Summary of defensin gene expression in hematopoietic cell lines.

Defensin mRNA levels were determined in a variety of HL60 sublines and other hematopoietic cell lines either by Northern blot analysis or by quantitative dot-blot analysis. Only the original HL60P25 line obtained from the laboratory of Dr.Robert Gallo [NIH, USA] expressed detectable defensin RNAs.

samples, the blot was stripped and reprobed for β_2-microglobulin mRNA (Fig. 22B). From this experiment it can be deduced that whatever mechanism is responsible for the loss of expression of defensin mRNAs in this subline of HL60 cells, it cannot be reversed by inducing the cells to differentiate. Whether this is also the case for other HL60 sublines, including HL60P25(1) remains to be established.

. In summary then, using the HL60 differentiation system to study defensin gene expression during myeloid development has shown that DMSO-induced differentiation of these cells more closely resembles in its gross characteristic of down-regulation of expression the in vivo regulation of these genes than RA-induced differentiation.

A seemingly more fruitful avenue for investigation, in the absence of an in vitro system more closely resembling in vivo myelopoiesis, was to investigate the defensin gene(s) themselves, with the ultimate view of understanding the mechanism(s) of control of differentiation-stage-specific gene transcription. It was therefore decided to isolate and investigate the human defensin genes.

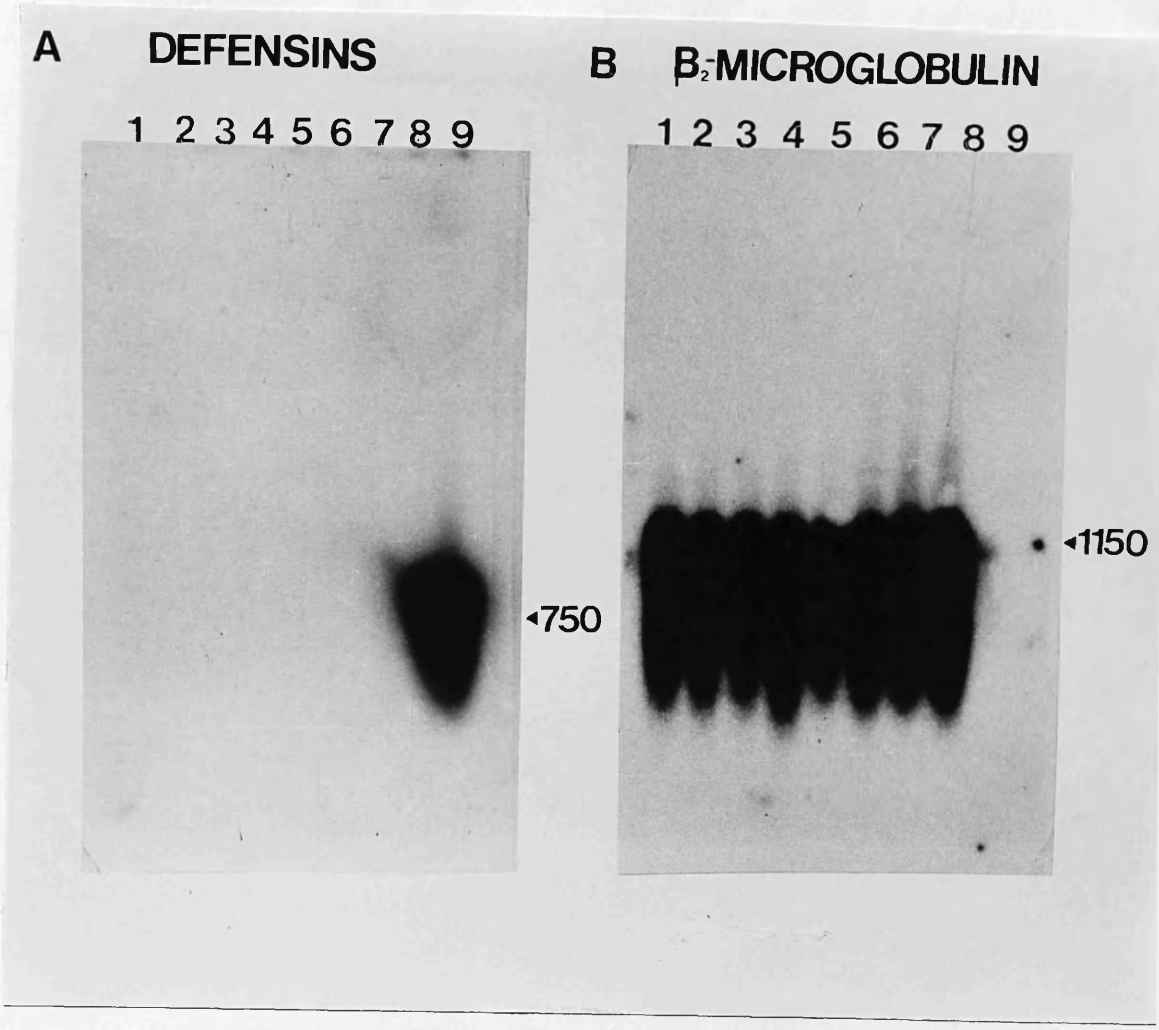


Fig.22 Northern blot analysis of defensin gene expression during granulocytic differentiation of HL60B induced by DMSO.

Exponentially growing HL60B cells were induced to differentiate by treatment with DMSO (1.5% v/v), total cellular RNA harvested at various time-points and analysed by Northern blotting of 15µg of each sample fractionated by electrophoresis on a denaturing agarose gel. A: blot hybridized with a radiolabelled HNP3 defensin cDNA probe. Lane 1, uninduced; lane 2, 1 hour; lane 3, 2 hours; lane 4, 4 hours; lane 5, 8 hours; lane 6, 24 hours; lane 7, 2 days; lane 8, 4 days; lane 9, RNA from peripheral blood leukocytes from a CGL patient, 0.2µg polyA+. B: blot stripped and probed with a radiolabelled β2-microglobulin cDNA probe.

3.3 ISOLATION AND CHARACTERIZATION OF HUMAN DEFENSIN GENES

The realization that human defensin peptides HNP1 and HNP3 were encoded by distinct mRNAs (this work and Daher et al, 1988) immediately implied that there ought to be at least two distinct defensin genes, one encoding HNP1, the other encoding HNP3. The cloning of both HNP1 and HNP3 cDNAs from HL60 cells (Daher et al, 1988) added support to the notion that both genes co-exist in the same cells.

There were, however, other possibilities to there being two distinct defensin genes. One possibility was that both HNP1 and HNP3 mRNAs were encoded by a single gene, but that this gene encoded two mRNAs with alternatively spliced exons, one exon containing a GCC triplet (alanine) and thus encoding HNP1, the other containing a GAC triplet (aspartic acid) and thus encoding HNP3. Another possibility was that again there was only a single gene, in this instance encoding either HNP1 or HNP3, such that both mRNAs were derived by modification of the single gene-encoded mRNA by a post-transcriptional mRNA editing mechanism (nucleotide substitution) as has been described in the case of apolipoprotein B mRNAs (Powell et al, 1987).

Prior to the isolation of defensin-encoding genomic clones, it was decided to ask the question as to whether the single nucleotide change between HNP1 and HNP3 mRNAs previously described might be useful in distinguishing whether

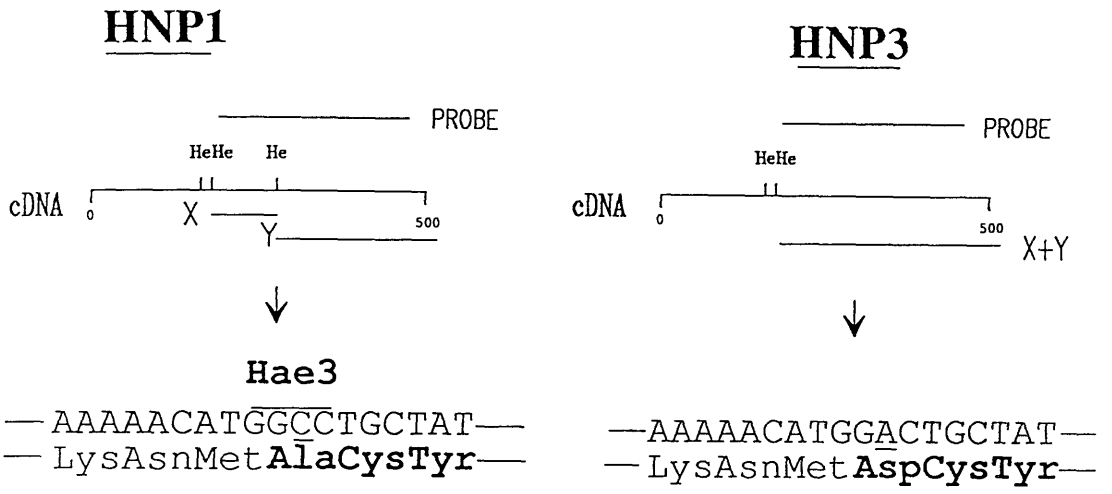


Fig.23 Schematic view of the coding difference between HNP1 and HNP3 cDNAs.

The Hae3 restriction maps of the cDNAs encoding defensins HNP1 and HNP3 are shown together with the single base coding change (C in HNP1, A in HNP3) which distinguishes them (Wiedemann et al, 1989; Mars et al, 1988). Also shown is the cDNA probe (PROBE) used to distinguish the genomic sequences of HNP1 and HNP3. This probe is a 3' Pst1-Rsa1 fragment derived from pCG14 (see Fig.3) and spans the polymorphic Hae3 site. It was later shown to include sequences from exons 2 and 3 of the HNP1A gene (see Fig.27B) and was therefore termed E2-E3 cDNA probe.

subsequently isolated genomic clones encoded HNP1 or HNP3. Since the C nucleotide in the GCC triplet of the HNP1 cDNA gave rise to a GGCC tetranucleotide sequence, a cutting site for the restriction enzyme Hae3, whilst the A nucleotide in the GAC triplet of HNP3 gave rise to a GGAC tetranucleotide sequence not recognized by Hae3, a possible means of distinguishing HNP1 from HNP3 on the basis of Hae3 digestion was clear (shown in Fig. 23). The simple model that HNP1 gene sequences around the polymorphic Hae3 site, upon Hae3 digestion should be detected as two fragments, whilst HNP3 gene sequences should be detected as one larger fragment was tested by Southern blotting of a number of Hae3-digested human DNAs, probing with a HNP3 cDNA probe spanning the Hae3 polymorphism (Fig. 23).

3.3.1 Human DNAs have variable relative amounts of genomic sequences encoding HNP1 and HNP3

An example of this Southern blot analysis is shown in Fig. 24. Out of 13 DNAs (and a subsequent 19 have since been analyzed, data not shown) four simple patterns involving three fragment sizes of 350, 650 and 1150 bp have been observed. Of these four patterns the most striking is represented in Fig. 24, lanes 4 and 5 where only the 350 and 650 bp fragments were detected.

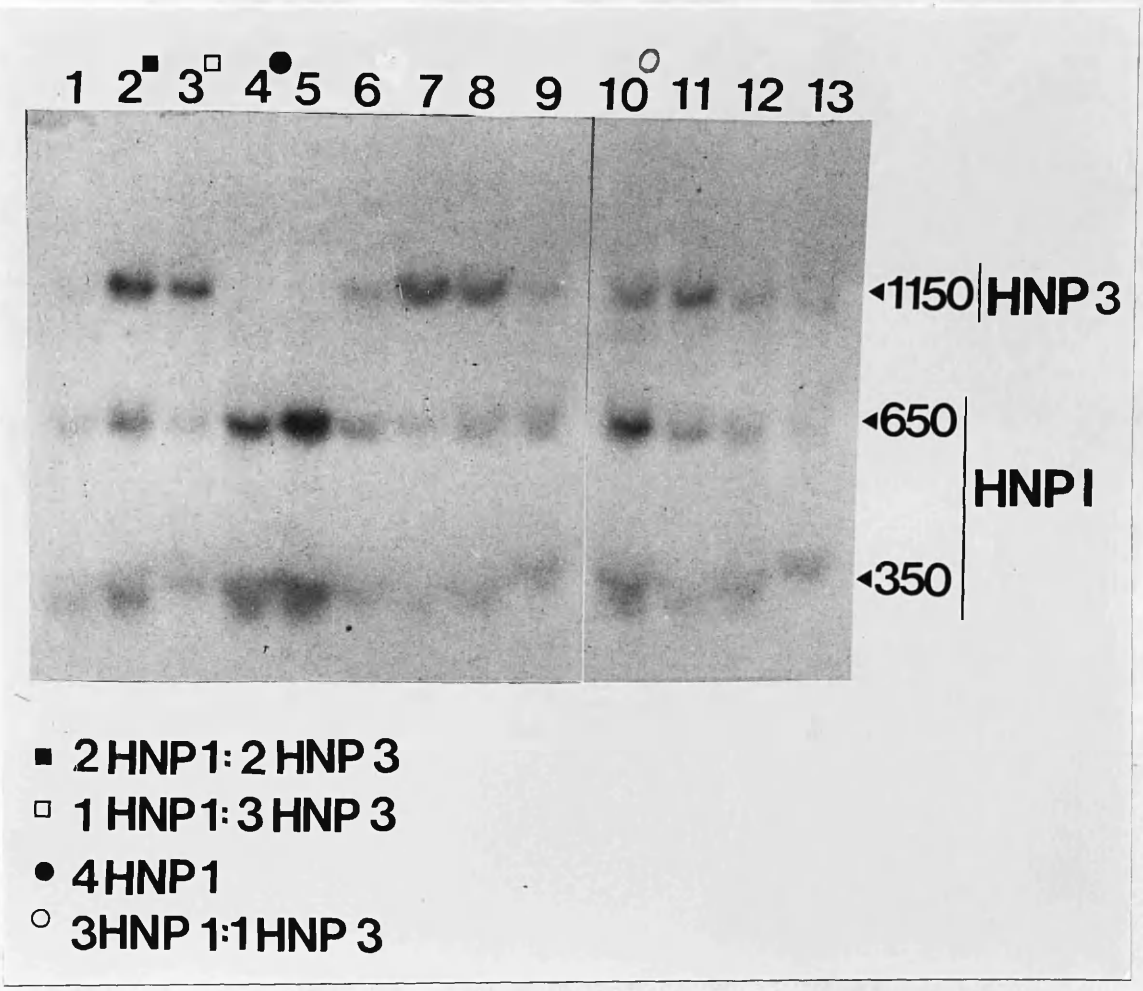


Fig.24 Southern blot analysis of Hae3 digested human leukocyte DNAs.

15µg of DNA from the peripheral blood leukocytes of 13 individuals were digested with Hae3 and Southern blotted onto a Hybond-N membrane. The blot was hybridized with a radiolabelled Pst1-Rsa1 HNP3 cDNA probe (shown in Fig.23). The 1150bp fragment and the 350/650bp fragments are those fragments derived from HNP3 and HNP1 genes, respectively. This was shown directly by the isolation of genomic clones for HNP1 and HNP3 (Sections 3.3.2, 3.3.4). Four distinct patterns, shown most clearly in lanes 2, 3, 4 and 10, have been found in a survey of 32 DNAs (see Fig.25).

In the absence of any detailed information on the structure of defensin-encoding genes, the interpretation of this result was that the 1150 bp fragment detected in the majority of the DNAs represented HNP3 genes (lacking the polymorphic Hae3 site) which, in the case of HNP1 gene sequences (containing the polymorphic Hae3 site) was digested into two fragments of 350 and 650 bp (the discordance between 350 + 650 = 1000 bp and the observed 1150 bp fragment size remained at this stage unexplained, see Fig. 31 for explanation).

This interpretation, which proved to be essentially correct (see Fig. 31), implied that individuals had variable copy number of HNP1- and HNP3-encoding sequences. Some individuals (for example lanes 4 and 5, Fig. 24) seemed to have only HNP1-encoding gene sequences, whilst others seemed to have either equal amounts of HNP1 and HNP3, relatively more HNP3- or relatively more HNP1- encoding sequences (compare lanes 2, 3 and 10 respectively, Fig. 24). The relative autoradiographic intensity of the HNP3 (1150 bp fragment) and HNP1 (350/650bp) fragment signals in Southern blots of human DNAs (as evaluated by densitometric scanning of autoradiographs, data not shown) fitted with a model in which individual human DNAs had a total of four defensin-encoding genes. According to this model, the individuals of Fig. 24 lanes 4 and 5 contained four HNP1-encoding gene sequences, whilst the individual in lane 2 had two HNP1- and two HNP3-encoding gene sequences. Similarly, the individual

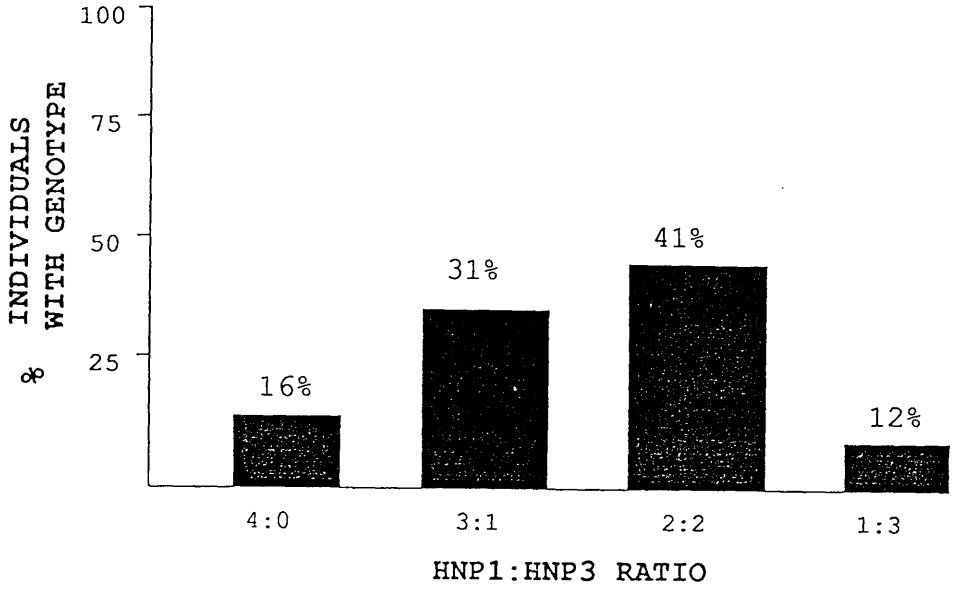


Fig.25 HNP genotype survey.

DNAs from a total of 32 individuals (mainly UK nationals) were digested with Hae3 and probed with a probe which distinguishes HNP1 from HNP3 genes (see Figs.23 and 24). Densitometric scanning of autoradiographs allowed all individuals tested to be placed into one of 4 patterns (see Fig.24 lanes 2,3,4 and 6). A simple model, in which all individuals tested had a total of 4 defensin genes per diploid genome, was invoked to explain these 4 patterns. According to this model, individuals can have either 4 HNP1-encoding genes (4:0) or ratios of HNP1:HNP3 genes (3:1, 2:2, 1:3, 0:4). The proportions of the total number of individuals tested having each of these 4 patterns is expressed here in percentage terms. Out of a total of 32 DNAs no individuals containing only HNP3 genes (0:4 ratio) have been observed.

represented by Fig. 24 lane 3 contained one HNP1- and three HNP3- encoding sequences, whilst the individual of Fig. 24 lane 10 had the converse. The proportions of individuals out of a total of 32 tested having each of these four patterns is shown in Fig. 25. This model is discussed in more detail in Section 4.3. and its validity addressed in the experiment described in Section 3.3.7.

If there were indeed four defensin-encoding genes, one should be able to isolate them from individuals, with the caveat that the genomes of these individuals should actually contain both HNP1 and HNP3 genes.

3.3.2 Isolation of genomic clones for defensin HNP1

To ensure that the entire coding region of defensin genes were isolated on putative defensin genomic clones, a number of restriction enzymes were tested by digesting genomic DNAs from a number of individuals, probing Southern blots with a full-length defensin HNP3 cDNA probe. Thus, in all individuals tested, two EcoR1 fragments of 1.7 and 4.5 Kb were detected (data not shown). In contrast, a single 8-9 Kb fragment size was detected in a number of individuals DNAs' digested with BamH1, indicating that all defensin-encoding sequences were present on a 8-9 Kb BamH1 fragment (Fig. 26).

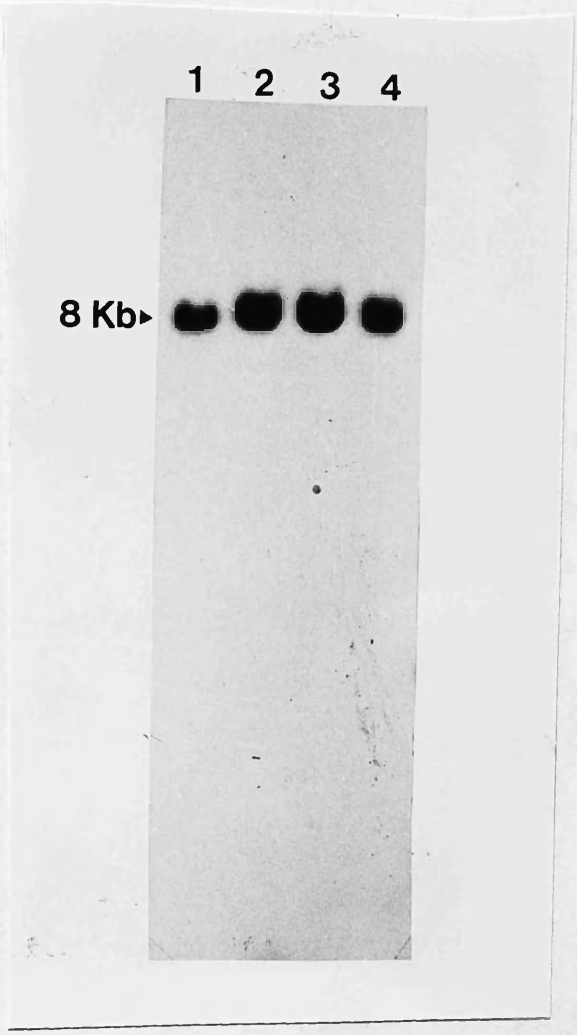


Fig.26 Southern blot of leukocyte DNAs digested with BamH1.

15µg of DNA from the peripheral blood leukocytes of 4 individuals was digested with BamH1 and Southern blotted onto Hybond-N. The blot was hybridized with a radiolabelled full-length defensin HNP3 cDNA probe. DNAs from both normal and leukaemic individuals have been similarly analyzed and no gross deletions or insertions of defensin coding sequences noted using this probe (data not shown).

With this knowledge, it was decided to construct a BamH1 library of human leukocyte DNA and screen with a defensin cDNA in order to isolate defensin genomic clones. Two libraries were constructed (from two individuals) in lambda L47.1 and two independent defensin-encoding clones isolated, one from each library. Both genomic glones were approximately 8-9 Kb in size and had similar restriction maps (Fig. 27A).

It was decided to investigate one of these genomic clones (termed HNP1A in Fig. 27A) in detail by a combination of hybridization with defensin cDNA oligonucleotides (see Fig. 3) to Southern blots of restriction enzyme digests of the clone (data not shown), and by sequence analysis (Fig. 28).

3.3.3 Characterization of HNP1A genomic clone

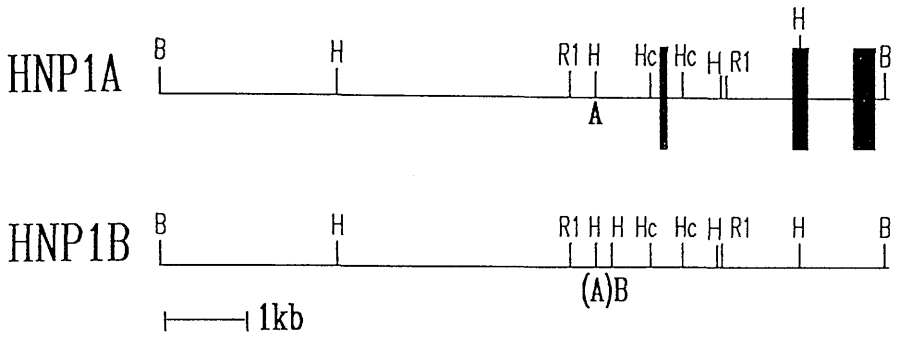
Since oligonucleotide hybridization using oligo A (Fig. 3) had revealed that the 5'-coding region of this defensin clone was contained on a 500bp Hinc2-Hinc2 fragment whilst more 3'-coding regions (as represented by oligos B, C and D) were present on a 2.6 Kb Hinc 2 - BamH1 fragment (data not shown, and Figs. 27A and B), these portions of HNP1A genomic clone were subcloned into M13mp18 and 19 vectors and sequenced as shown in Fig. 27B.

Fig. 28 shows the result of this sequence analysis, the sequence shown representing the coding strand and the translation product shown underneath.

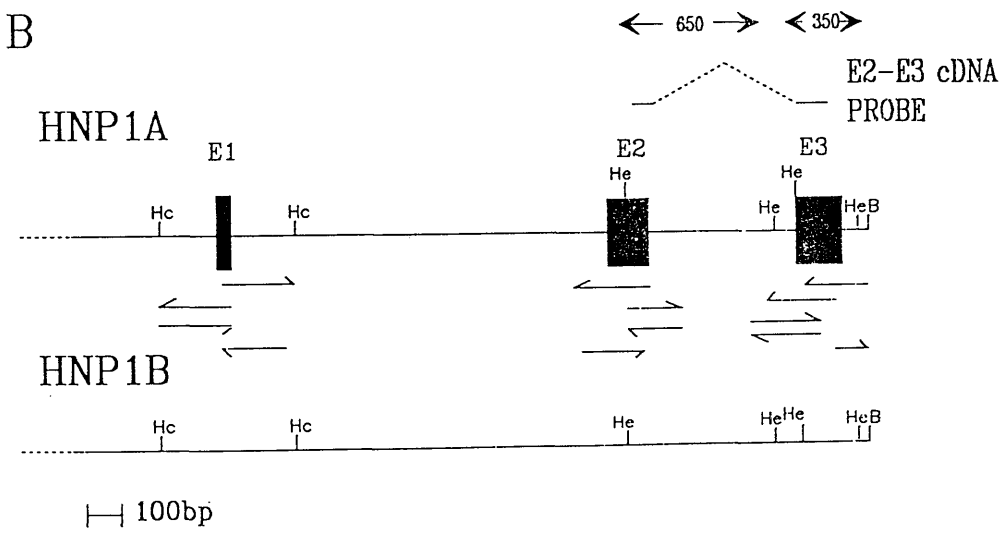
Fig.27 Characterization of two HNP1-encoding defensin genes.

A: Restriction Maps.
Clones HNP1A and HNP1B were isolated as 8-9kb BamH1 genomic fragments cloned into bacteriophage vector lambda L47.1 (Loenen and Brammer, 1980). The library from which HNP1A was derived was constructed from DNA from the peripheral blood granulocytes of a normal donor. Likewise, the library from which HNP1B was derived was constructed from the peripheral blood leukocytes (total wbc) of another normal donor. In both cases the 8-9kb genomic fragment was then recloned into the plasmid vector pUC8 (Viera and Messing, 1982) and restriction mapped with the enzymes Hinc2 (Hc), EcoR1 (R1) and Hind3 (H).
The subscript A or B refers to the position of the first Hind3 site upstream of exon 1 used as a convention to distinguish the clones. Bold rectangles represent exons.
B: Coding regions of HNP1-encoding defensin genes.
The 3'-regions of the two HNP1 defensin clones isolated are shown. The region was restriction mapped from the 3'-most Hae3 site in E2 to the BamH1 site at the 3'-end of the clone with the restriction enzyme Hae3. The map predicts that an E2-E3 cDNA probe, which spans the second intron, should detect two Hae3 fragments (650/350bp) which is what is observed (Fig.31).
HNP1A, the most extensively characterized HNP1 gene, consists of 3 exons (E1, E2, and E3) and two introns (I1 and I2) and was sequenced on both strands as shown in the Figure by subcloning into bacteriophage M13 vectors mp18 and 19 (Yanish-Perron et al, 1985), the direction of the arrow indicating the direction of sequencing (performed at least twice per direction). Sequencing was performed with a commercial T7 dideoxy chain terminating sequencing kit.
The following defensin cDNA oligonucleotides were used, in addition to the M13 universal primer, as sequencing primers:
First exon region: oligo A; oligo A'.
Second exon region: oligo B; oligo B'.
Third exon region: oligo D; oligo D'.
Two other oligonucleotides derived from HNP1A intron sequences were also used as sequencing primers:
Oligo I1 (Intron 1 oligo. used to sequence into E2 5'-3');
5' CCACACAGCTGCTCCTGCTC 3'.
Oligo I2 (Intron 2 oligo.used to sequence into E3 5'-3');
5' TGACGATTGAGGTATGAGTT 3'.

A


HNP1A
B
H
R1 H
Hc
Hc
H R1
H
B
A
HNP1B
B
H
R1 H H Hc Hc H R1
H
B
(A)B
1kb

B


650
350
E2-E3 cDNA
PROBE
HNP1A
E1
E2
E3
He
He
Hc
Hc
He
HeB
HNP1B
Hc
Hc
He
HeHe
HeB
100bp

(i) Structure of coding regions of HNP1A

The coding regions of this gene correspond exactly in sequence to the cDNA for HNP1 defensin (Mars et al, 1988; Daher et al, 1988; and to my sequence for pCG14 (HNP3) shown in Fig. 11 apart from one base: A (HNP3); C (HNP1), the relevant GCC triplet is in exon 3, Fig. 29) and thus this gene encodes HNP1. The gene is organized into three exons and two introns as revealed by sequence analysis. Exon 1 (E1) is small (76 bp) and non-coding, and is separated from exon (E2) by an intron (I1) of approximately 1600 bp. The sequence of the 5'-end of E1 corresponds exactly to that found by direct sequencing of defensin mRNAs in the experiment shown in Fig. 9. E2 is larger than E1 (187 bp) and encodes the first methione and 58 residues of the defensin precursor. E3 (219bp) encodes the mature HNP1 defensin peptide and is separated from E2 by an intron (I2) of approximately 650 bp. As shown in Fig. 29, the splice junctions E1-E2 and E2-E3 correspond well to the consensus sequences proposed by Mount (1982).

(ii) HNP1A promoter

239 bp of sequence upstream of E1 was sequenced and revealed to contain two AT-rich elements (shown underlined in Fig. 28), regions typically 20-30 bases upstream of most eukaryotic promoters required for normal initiation at the cap site (Breathnach and Chambon, 1981) 60 bases apart. The

Fig.28 Sequence of HNP1A.

The sequence of the entire coding region of HNP1A is shown. Also shown are the sequences of the 5′-regulatory region (239bp determined), some intronic and 3′ non-coding regions. Exon sequences (E1-E3) are shown in bold and correspond exactly with the published sequence of HNP1 defensin (mrs) (Mars et al, 1988). The transcriptional initiation points on the gene revealed by primer extension analysis are shown arrowed and occur 20-30 bases downstream of two AT-rich sequence elements (underlined). Also shown at the 3′-end of the gene is a polyadenylation signal AATAAA (underlined). The sequence shown was determined using the sequencing strategy shown in Fig. 27B.

-239

AACTGTGTTAGGAGCCATTGAGAATCCATAGTTGGTTGCTGCCTGGGCCTGGCCAGGGCT

-179

GACCAAGGTAGATGAGAGGTTCCTCTGTGGAGTTCTACTTTAACCTCACCTTCCCACCAA

-119

ATTTCTCAACTGTCCTTGCCACCACCATTATTTAATGGACCCAACAGAAAGTAACCCCGG

-59

AAATTAGGACACCTCATCCCAAAAGACCTTTAAATAGGGGAAGTCCACTTGTGCACGGCT

GCTCCTTGCTATAGAAGACCTGGGACAGAGGACTGCTGTCTGCCCTCTCTGGTCACCCTG

E1

CCTAGCTAGAGGATCTGTAAGTACTACAAAACTTAAACTTTACACTGAGTTTTCATCATT

GAAGCTATGCCTCCAATCTGACCTCTGACTGTGGGGCCGCCCCAGAGGGACCCAGCGGGT

GAATCCCTGCTAGGAACGTCTGTCCGGACCTCTGGTGACTGCTGGGGACGATGGCTTCCA

GCTAACTTAATAGAGAAACTCAAGCAGTTTCCTTCTAAATACACATGTCACATGTCCTGG

TTCCGGATCCTCTAGAGTCGACCTGCA----------1350 bp----------C

CACACAGCTGCTCCTGCTCTCCCTCCTCCAGGTGACCCCAGCCATGAGGACCCTCGCCAT

MetArgThrLeuAlaIl

CCTTGCTGCCATTCTCCTGGTGGCCCTGCAGGCCCAGGCTGAGCCACTCCAGGCAAGAGC

eLeuAlaAlaIleLeuLeuValAlaLeuGlnAlaGlnAlaGluProLeuGlnAlaArgAl

E2

TGATGAGGTTGCTGCAGCCCCGGAGCAGATTGCAGCGGACATCCCAGAAGTGGTTGTTTC

aAspGluValAlaAlaAlaProGluGlnIleAlaAlaAspIleProGluValValValSe

CCTTGCATGGGACGAAAGCTTGGCTCCAAAGCATCCAGGTGAGAGA-----500 bp---

rLeuAlaTrpAspGluSerLeuAlaProLysHisProG

---------GAGGTTGTTCGTGCTACCGGCTGCAATGCAGCTGCAAGCTACACCTGTCAG

CTAGCAGTGACTTCCCCGAGATTCTTTTTCTTACCCACTGCTAACTCCATACTCAATTTC

TCATGCTCTCCCTGTCCCAGGCTCAAGGAAAAACATGGCCTGCTATTGCAGAATACCAGC

lySerArgLysAsnMetAlaCysTyrCysArgIleProAl

GTGCATTGCAGGAGAACGTCGCTATGGAACCTGCATCTACCAGGGAAGACTCTGGGCATT

aCysIleAlaGlyGluArgArgTyrGlyThrCysIleTyrGlnGlyArgLeuTrpAlaPh

E3

CTGCTGCTGAGCTTGCAGAAAAAGAAAAATGAGCTCAAAATTTGCTTTGAGAGCTACAGG

eCysCys

GAATTGCTATTACTCCTGTACCTTCTGCTCAATTTCCTTTCCTCATCCCAAATAAATGCC

TTGTTACAAGATTTCTGTGTTTCCACCTCTTTAATGTGTGATATGTGTCTGTGTCAAGAC

ACTTGGGATACACGTACCAAAACGCAAAATCAAATTTTTGAACAATATAAAATTCCAAAT

TCTAGGAATTTCAAGCAGGAGTTTGGGCTTCAGATCCAAATTGAAAAGAAGGCCCATATG

ACACCACTGATTTCCCCACCCACTGCTCTGCCTTTTCACCCTGCCTCATTTTCTCTGGAT

CC

SPLICE JUNCTION	DONOR	ACCEPTOR
E1–E2	GATCT/ GTAAGT	CCTCCAG/GTGACC
E2–E3	TCCAG/ GTGAGA	GTCCCAG/GCTCA
CONSENSUS	$^{C}_{A}$AG/ GT$^{A}_{G}$AGT	$^{T\ T\ T\ T}_{C\ C\ C\ C}$NAG/G

Fig.29 Sequences of HNP1A splice junctions.

The sequences of HNP1A splice junctions between exon 1 and exon 2 (E1-E2) and between exon 2 and exon 3 (E2-E3) are shown compared to consensus eukaryotic splice donor and acceptor sites proposed by Mount (1982).

proximal TATA-like box lies 30 bp upstream from the major start sites of transcription. Fine mapping primer extension using oligo A (Fig. 30) and mRNA from CGL peripheral blood leukocytes and HL60P25 revealed that both TATA-like elements are used to initiate transcription 30 bases downstream, albeit at very different levels.

Initiation from the proximal promoter (P1) (heterogeneous, with two main sites of initiation one nucleotide apart, arrowed in Fig. 30) is at least 20-fold greater than transcription initiating from the distal promoter P2, as judged by densitometric scanning (data not shown). P2 transcription was only detected in CGL leukocytes and not HL60P25 under the experimental conditions detailed in Fig. 30. The question as to whether both P1 and P2 transcripts originate solely from the HNP1A gene remains to be established, however, since the oligonucleotide (oligo A) used in this primer extension experiment, being common to both HNP1 and HNP3 mRNAs, should extend on both.

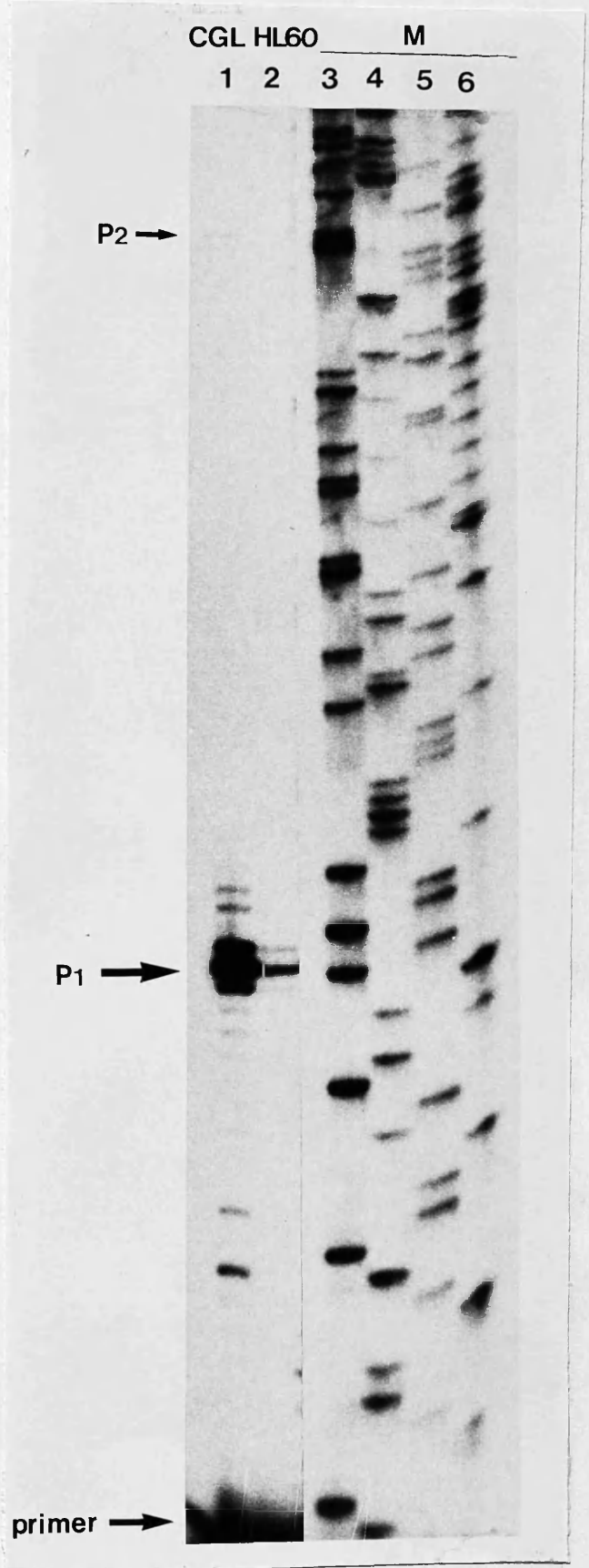
(iii) 3' Hae3 sites in HNP1A

Hae3 mapping of HNP1A and the other clone isolated during library screening HNP1B (shown in Fig. 27B) revealed that they had identical 3' Hae3 maps, with both containing a Hae3 site in the 3rd exon and thus encoding HNP1 defensins. This site results in the detection of the 350/650 bp fragment pairing (Figs. 27B and 31) previously seen with Hae3-digested human

Fig.30 Both promoters of HNP1A are functional.

Primer extension with oligo A. This experiment was performed in a manner identical to that used in Fig.5, with the following modifications: 1) 100ng of 5'-end labelled oligo A (PRIMER) was used in each extension reaction. 2) 5µg of polyA+ RNA from CGL leukocytes (lane 1) or HL60P25 (lane 2) was substituted for the previous amounts used (10µg polyA+ and 50µg total cellular RNA, respectively). The use of an increased amount of primer resulted in an increase in sensitivity such that a minor start-site of transcription in CGL leukocytes could clearly be detected (P2, arrowed) not apparent in the previous experiment (Fig.5). This species corresponds to initiation at a region 20-30 bases downstream of the distal TATA-like element shown in Fig.28. The main start-sites of transcription (P1) are rather heterogeneous and initiate 20-30 bases downstream of the proximal TATA-like element shown in Fig.28. DNA sequence ladders generated from M13 mp18 were used for size estimation of the primer-extended material.

Note: these start-points of transcription revealed by primer extension would ideally be confirmed by S1-protection experiments using cloned defensin genomic sequences.


CGL HL60
M
1 2 3 4 5 6
P2
P1
primer

DNAs. Although both HNP1A and HNP1B apparently both encode HNP1, the clones are distinct as indicated in Fig. 27A. This will be discussed in Section 3.3.5.

3.3.4 Isolation of genomic clones for defensin HNP3

Since library screening had thus far isolated only HNP1-encoding genes, it remained to isolate genes encoding HNP3 defensin. The knowledge that genes encoding HNP3 ought to have a characteristic 1150bp Hae3 fragment at their 3'- coding regions due to their lacking a Hae3 site in their 3rd exons (Figs. 23, 24) aided rapid screening of clones from a new library constructed from human leukocyte DNA. A partial Sau3A library in EMBL3 of DNA from human leukocytes was constructed and screened (see Fig. 33) using the E2-E3 HNP3 cDNA probe used previously (Fig. 27B).

Southern blots of three clones isolated from this library indicated that two of them encoded HNP3 (Figs. 34 and 35, lane 6). One of these clones, HNP3B, was sequenced in the region of the 3rd exon and in this way shown directly to encode HNP3 defensin (Figs. 32B, 37). Both genomic clones contain a characteristic 1150 bp Hae3 fragment seen in human genomic DNAs (Fig. 31) and have their entire coding regions present on a 8-9 Kb BamHl fragment (Figs. 32A and B) as predicted from the result of the experiment shown in Fig. 26.

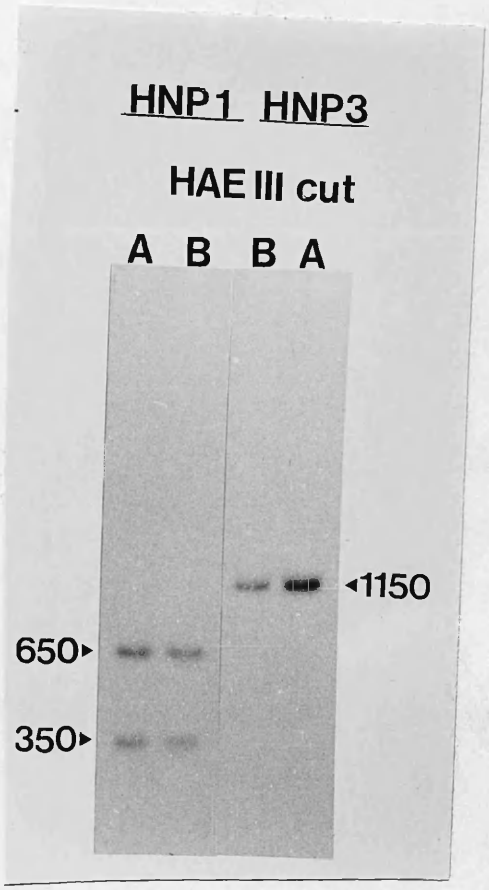


Fig.31 HNP1 and HNP3 genes distinguished by Hae3 digestion and Southern blotting.

Approximately 0.5µg (rather more in the case of HNP3A) of the 8-9kb BamH1 fragment of clones HNP1A, HNP1B, HNP3A and HNP3B were digested with Hae3, the products run on a 1.2% agarose gel and the gel blotted onto a Hybond-N membrane. The blot was then hybridized with a radiolabelled E2-E3 defensin cDNA probe. Both HNP1 clones have a characteristic 350/650bp fragment pairing detected with this probe, whilst the HNP3 clones have a characteristic 1150bp fragment (see Figs.27B & 32B). In the cases of HNP1A and HNP3B this has been shown directly to be due to a single base change in the GGCC site (HNP1) giving a GGAC site (HNP3). HNP3 clones also lack a GGCC site present in the second intron of the HNP1 clones as determined by restriction mapping (see Fig.32B).

3.3.5 Two distinct genes encode HNP3 mRNAs

Although both HNP3 clones were similar, they were not identical. Fig. 32A shows the restriction maps of these two clones. Identity of sites existed for a number of restriction enzymes (Hind 3, Hinc 2 and EcoR1 shown) mainly in and around the coding regions of the clones, with differences occuring outwith (5' to) this region. On the basis of one easily detected difference (Fig. 32D) in the Hind3 map upstream of the presumptive first exon, these clones were termed HNP3A (1450 bp E1 Hind3 fragment) and HNP3B (1300 bp E1 Hind3 fragment) as shown in Fig. 32A. Subsequent reinvestigation of the two HNP1 clones isolated during the first library screening revealed that they too differed in their 5'-regions and could also be distinguished by Hind3 digestion due to a change analogous to that observed with the two HNP3 clones (Fig. 27A and 32D).

Returning to the HNP3 clones, using HNP3 cDNA oligonucleotides A, B, C and D (see Fig. 3) as hybridization probes to Southern blots of restriction enzyme digests of the HNP3A and HNP3B clones, their intron-exon structures were shown to be similar to that of the HNP1A gene. Thus both clones contained three non-contiguous coding regions with a small upstream exon (E1) present on a 500bp Hinc2-Hinc2 fragment (Fig. 32A; B) separated from E2 by an intron ($<$1.75kb in size) which in turn was separated from E3 sequences by a smaller intron ($<$1kb in size) (data not shown).

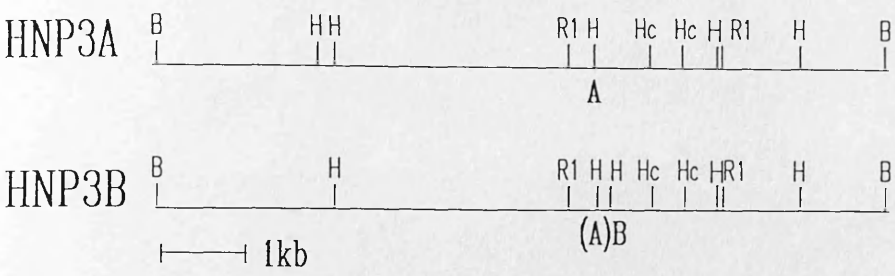
Fig.32 Characterization of HNP3 defensin genes.

A: Restriction maps.
Genomic clones HNP3A and HNP3B were isolated, along with a larger clone for HNP1A (Fig.36), as partial Sau3A genomic fragments (>12kb in size) cloned into bacteriophage lambda vector EMBL3 (Frischauf et al, 1983). For each of these 3 clones, a central 8-9kb BamH1 fragment was isolated and restriction mapped with Hinc2, EcoR1 and Hind3. The subscript A or B refers to the position of the first Hind3 site upstream of exon 1 described in the text, used as a convention to distinguish the clones.

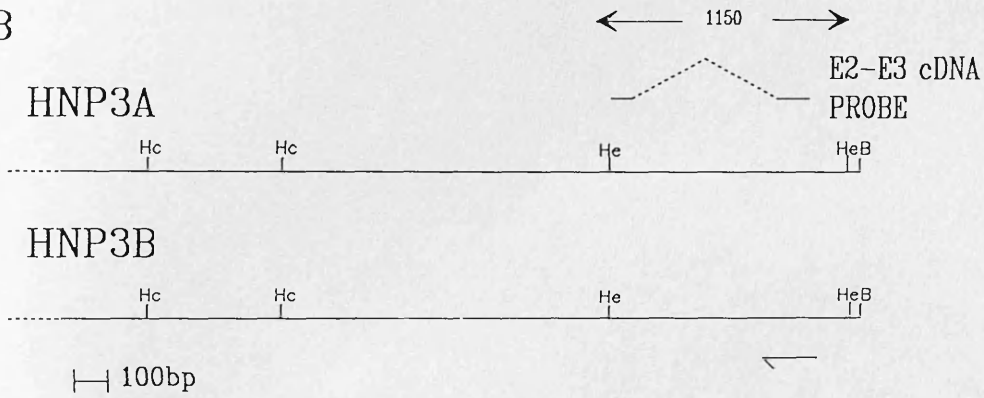
B: Coding regions of HNP3-encoding defensin genes.
The 3'-regions of the two HNP3 clones isolated are shown. Both clones were mapped at their 3'-ends (from the 3'-most Hae3 site in the E2 position of the HNP1A gene to the BamH1 site at the 3'-end the 8-9kb fragment on which the HNP3 genes reside) with Hae3. The map predicts a 1150bp Hae3 fragment detectable with the E2-E3 cDNA probe, which is observed (see Fig.31). The HNP3B gene was partially sequenced by subcloning a 3'-portion (a 2.7kb Hind3 fragment shown in Fig.35, lane 4 containing exon 3 and 1.5kb of 3'-sequence downstream of the BamH1 site) into M13mp18; sequencing primed with oligo D.

C and D: Southern blotting of defensin genomic clones.
C: BamH1; 1µg of plasmids HNP1A/pUC8 and HNP1B/pUC8 and approximately 5µg of HNP3A/EMBL3 and HNP3B/EMBL3 DNA were digested with BamH1, the products run on a 0.8% agarose gel, and the gel blotted onto a Hybond-N membrane. The blot was then hybridized with a radiolabelled full-length HNP3 defensin cDNA probe.
D: Hind3; 0.5µg of the 8-9kb BamH1 fragments of HNP1A, HNP1B, HNP3A and HNP3B were digested with Hind3, the products run on a 0.8% agarose gel, and the gel blotted onto a Hybond-N membrane. The blot was then hybridized with a radiolabelled HNP3 defensin E1 cDNA probe.

A


HNP3A
B
H H
R1 H
Hc
Hc
H R1
H
B
A
HNP3B
B
H
R1 H H
Hc
Hc
H R1
H
B
(A)B
1kb

B


1150
E2-E3 cDNA
PROBE
HNP3A
Hc
Hc
He
HeB
HNP3B
Hc
Hc
He
HeB
100bp

C


HNP I HNP 3
BAM HI cut
A B B A
8kb

D

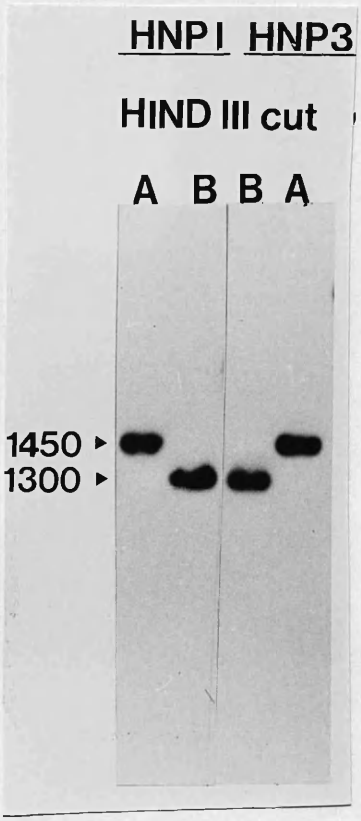
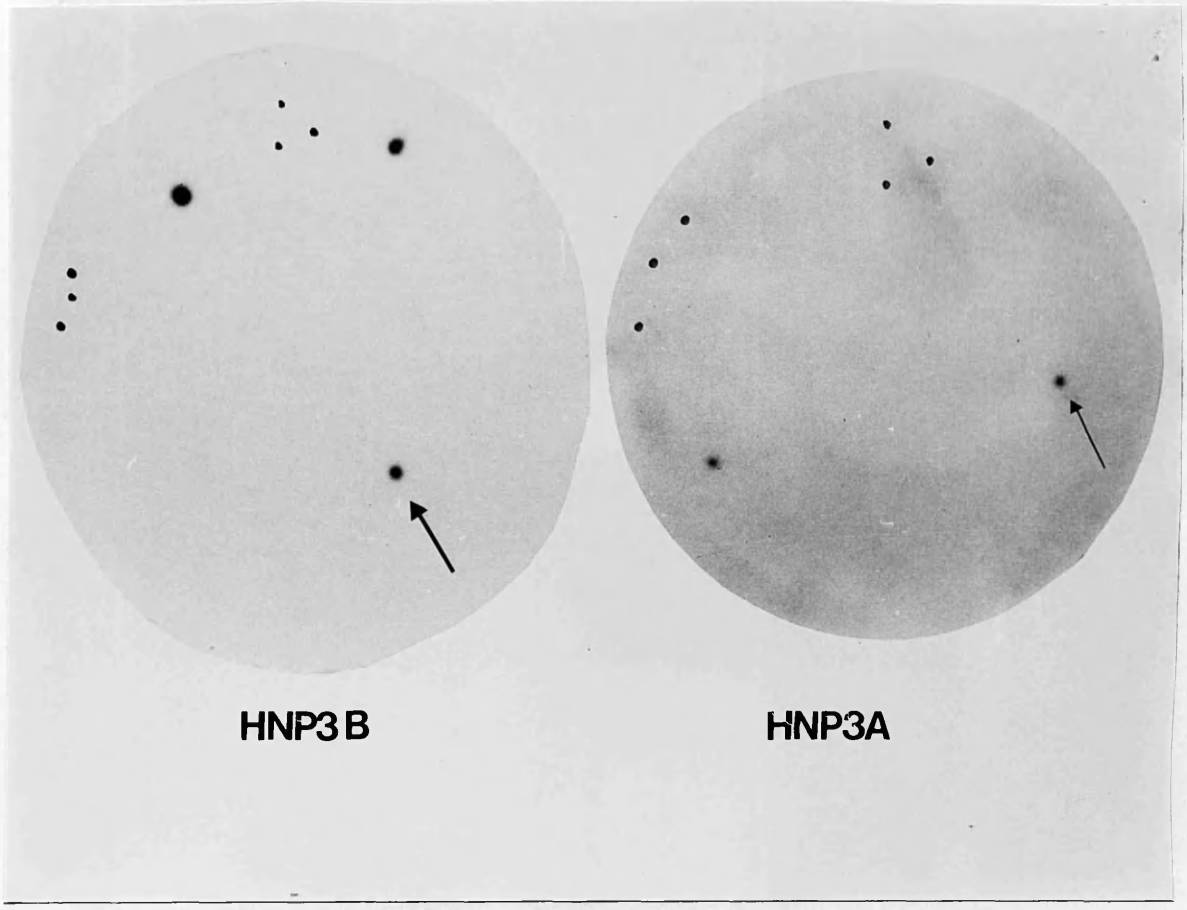

HNP I HNP3
HIND III cut
A B B A
1450
1300

Fig.33 Secondary plaque screening of HNP3 clones.

As described in Section 2.8.2 positive areas of hybridization from primary lambda EMBL3 screenings were replated at various titres and plates containing 50-100 lambda plaques screened using an E2-E3 HNP3 cDNA radiolabelled probe. A: secondary screen isolating HNP3A/EMBL3 clone (a single hybridizing plaque isolated and grown indicated by an arrow). B: secondary screen isolating HNP3B/EMBL3 clone (a single hybridizing plaque isolated from this plate indicated by an arrow). The other clone isolated from the primary EMBL3 library screen was similarly isolated through replating and rescreening (data not shown).


HNP3 B
HNP3A

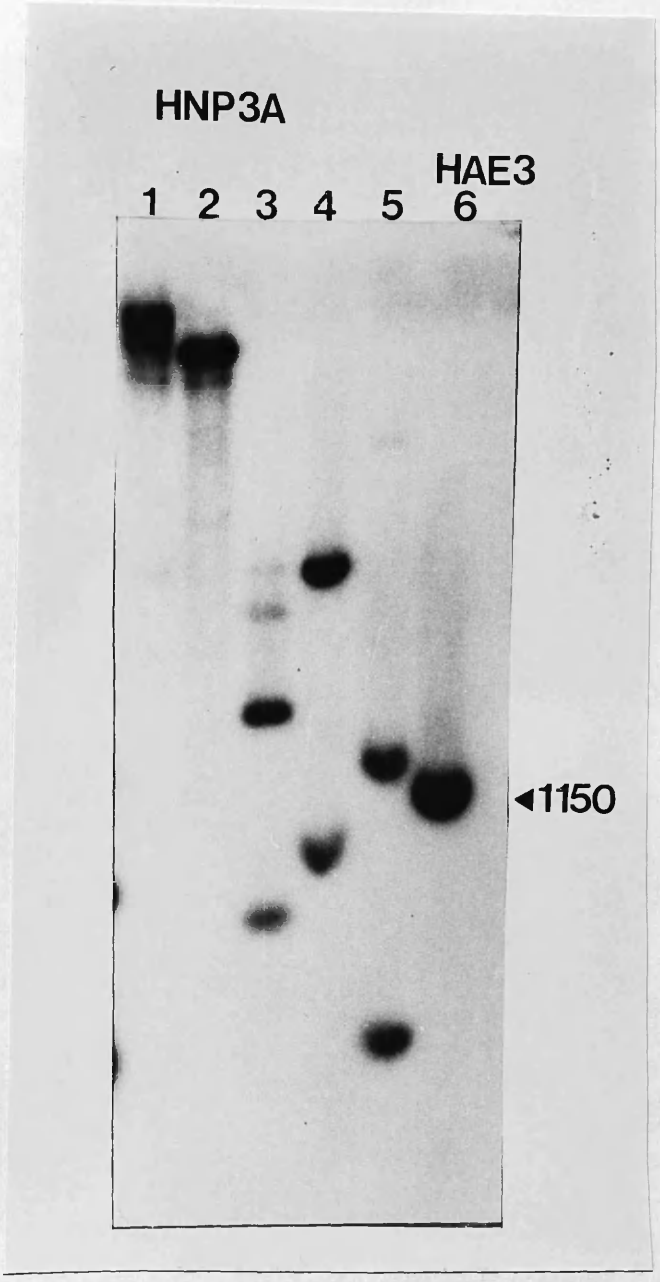


Fig.34 Southern blot of HNP3A/EMBL3 clone.

Lambda clone HNP3A/EMBL3 DNA was prepared as described in Section 2.4.3 and 1µg was digested with various restriction enzymes. The digestion products were run on an agarose gel and the gel blotted onto a Hybond-N membrane. The blot was then hybridized with a radio-labelled E2-E3 HNP3 defensin cDNA probe. Lane 1, Sal1; lane 2, BamH1/Sal1; lane 3, EcoR1/Sal1 (star activity of EcoR1 due to wrong buffer strength used, later shown to be a single 4.5kb fragment detected with this probe); lane 4, Hind3; lane 5, Pst1; lane 6, Hae3. This partial Sau3A genomic clone contains at least another 5.5kb of sequence (Lane 1; genomic insert plus data not shown) more than the 8-9kb shown in Fig.32A.

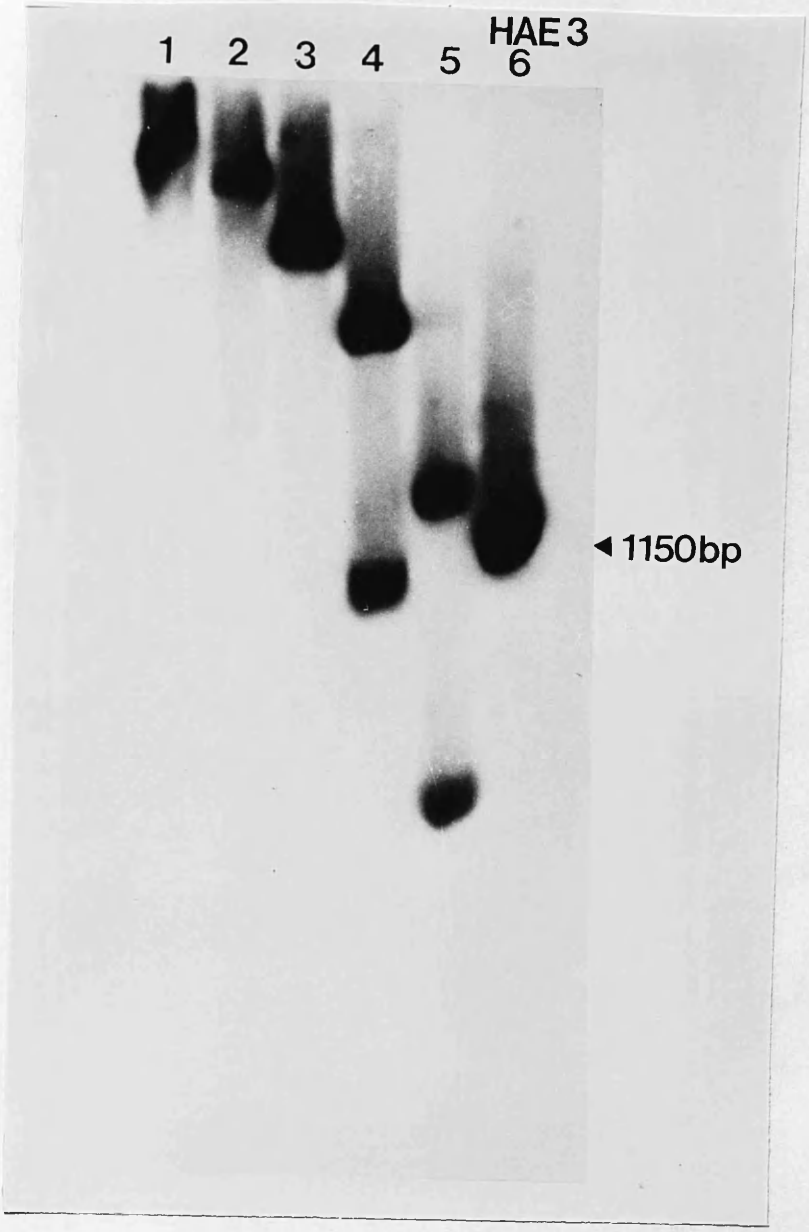


Fig.35 Southern blot of HNP3B/EMBL3 clone.

Lambda clone HNP3A/EMBL3 DNA was prepared as described in Section 2.4.3 and 1μg was digested with various restriction enzymes. The digestion products were run on an agarose gel and the gel blotted onto a Hybond-N membrane. The blot was then hybridized with a radiolabelled E2-E3 HNP3 defensin cDNA probe. Lane 1, Sal1; lane 2, BamH1/Sal1; lane 3, EcoR1/Sal1; lane 4, Hind3; lane 5, Pst1; lane 6; Hae3. This partial Sau3A genomic clone contains at least another 7.3kb of sequence (lane 1; genomic insert plus data not shown) more than the 8-9kb BamH1 fragment shown in Fig.32A.

3.3.6 Four distinct defensin-encoding genes

Screening for HNP3-encoding genes also isolated another defensin-encoding clone (Fig. 36). On the basis of its characteristic 350/650 bp Hae3 fragment pairing (Fig. 36, lane 6) this clone encoded HNP1. Restriction mapping of the clone revealed that, in common with the other defensin-encoding genes, it too had its entire coding region contained on a 8-9Kb BamH1 fragment (data not shown and Fig. 36, lane 2). Furthermore, the restriction map of this fragment (with the restriction enzymes Hind 3, Hinc2 and EcoR1) indicated that it was indistinguishable from the previously isolated HNP1A clone (Fig. 27A).

Thus the screening of this partial Sau3A EMBL3 library, constructed from the DNA of one individual, resulted in the isolation of three distinct defensin-encoding genes, two enc oding HNP3, one encoding HNP1. This indicated that there could be at least three different defensin genes present in the genome of any one individual. Furthermore, a total of four different defensin genes had been isolated in total (see Figs. 27A; 32A). These four genes HNP1A, HNP1B, HNP3A and HNP3B, although similar, are clearly distinct by restriction enzyme analysis of their 5'- non-coding regions (compare Hind 3 maps Figs. 27B and 32A) and by the presence, in the cases of HNP1A and B, of two Hae3 sites absent in HNP3A and B (compare Fig. 27B and Fig. 32B).

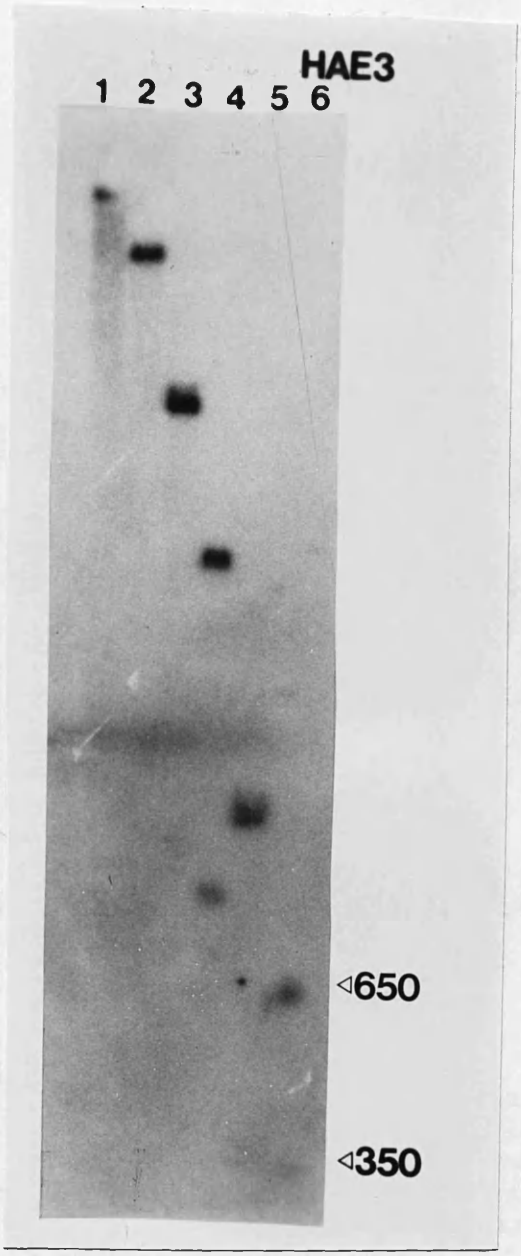


Fig.36 Southern blot of HNP1A/EMBL3 clone.

Lambda clone HNP1A/EMBL3 DNA was isolated as described in Section 2.4.3 and 1µg digested with various restriction enzymes. The digestion products were run on an agarose gel and the gel blotted onto a Hybond-N membrane. The blot was then hybridized with a radiolabelled E2-E3 HNP3 defensin cDNA probe. Lane 1, Sal1; lane 2, BamH1/Sal1; lane 3, EcoR1/Sal1; lane 4, Hind3; lane 5, Pst1; lane 6, Hae3. This clone was isolated from the same library as HNP3A and HNP3B indicating that all 3 genes are present in the same individual. It was isolated as a partial Sau3A fragment and contains at least another 5kb of sequence (lane 1: genomic insert plus data not shown) more than the 6-9kb shown in Fig.27A.

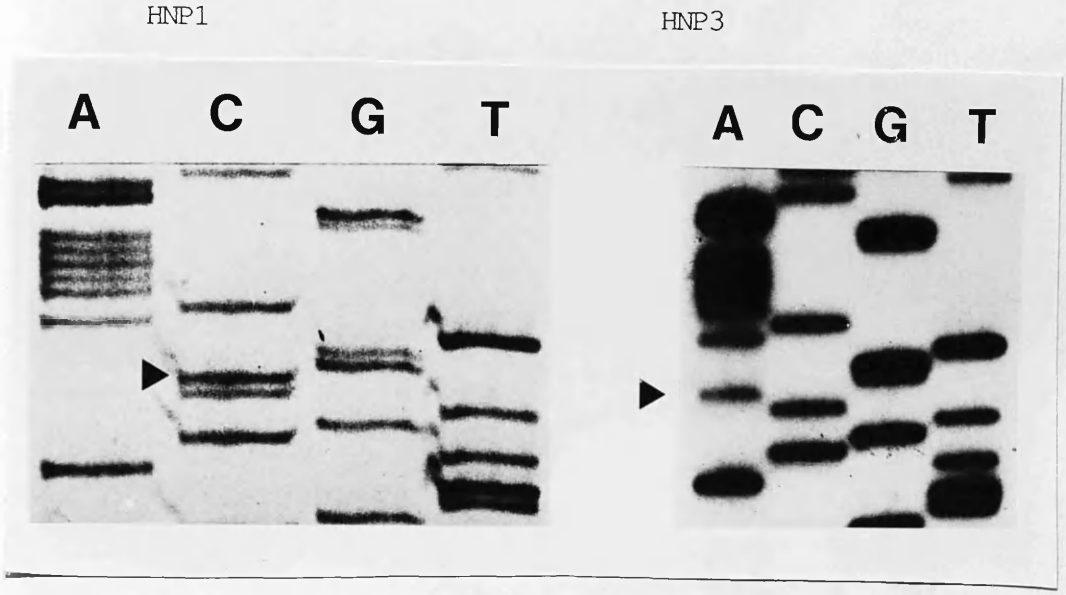


Fig.37 Sequences of HNP1 and HNP3 genes at Hae3 polymorphic site.

A 2.6 Hinc2-BamH1 fragment of clone HNP1A and a 2.7kb Hind3-Hind3 fragment (see Fig.35, lane 4) of clone HNP3B cloned into appropriately cut M13 mp18 were sequenced through their E3 regions (see Fig.28) using oligo D (Fig.3) as a sequencing primer. The sequence obtained for HNP1A was identical to that of HNP3B apart from one base change (C in HNP1A, A in HNP3B) indicated by an arrowhead at the relevant nucleotide. This change results in the presence of a Hae3 restriction enzyme site present in HNP1A that is absent in HNP3B and the encoding of distinct defensin peptides.

The question as to whether all these genes are functional remains unanswered. However, as revealed by the PCR experiment shown in Fig. 40, at least two of the defensin-encoding genes must be actively transcribed.

3.3.7 Inheritance of defensin-encoding genes

Both the isolation of four distinct defensin-encoding genes (Section 3.3.6) and the analysis of the relative amounts of HNP1 and HNP3 defensin-encoding genes by Hae3 digestion of human DNAs (Section 3.3.1) implied that there were at least four defensin-encoding genes per diploid genome. Since two studies on the chromosomal localization of defensin genes (Wiedemann et al, 1989; Sparkes et al, 1989), had previously indicated that defensin genes were present only on chromosome 8 (8p23 in the study of Sparkes et al, 1989) it seemed reasonable to suppose that the genes were clustered. This is in fact known to be the case for the closely homologous rabbit defensin genes MCP1 and MCP2 which share with HNP1, for example, the same intron-exon organization and 86% homology in their 5'-untranslated regions (Ganz et al, 1989, discussed in Section 4.3). The rabbit genes are tandemly duplicated and are within 10 Kb of each other (Ganz et al, 1989).

A simple model for the organization of human defensin genes is that they too are tandemly duplicated (perhaps with the same separation between genes as with MCP1 and MCP2) and that a number of alleles are present in the population, each

allele consisting of a tandem pair of defensin-encoding genes (for example HNP1-HNP1, HNP1-HNP3, HNP3-HNP3, see Fig. 38A). Accordingly, individuals containing, for example, only HNP1-encoding genes (eg. Fig. 24, lanes 4 and 5), since they have two copies of chromosome 8, would have two alleles each consisting of a pair of HNP1-encoding genes (Fig. 38A). A prediction from this model is that since the recombination frequency between two defensin genes would be vanishingly small due to the small separation between two genes of a pair, inheritance of two copies of chromosome 8 should result in the inheritance of two pairs of defensin-encoding genes, one pair derived from the mother, one pair from the father.

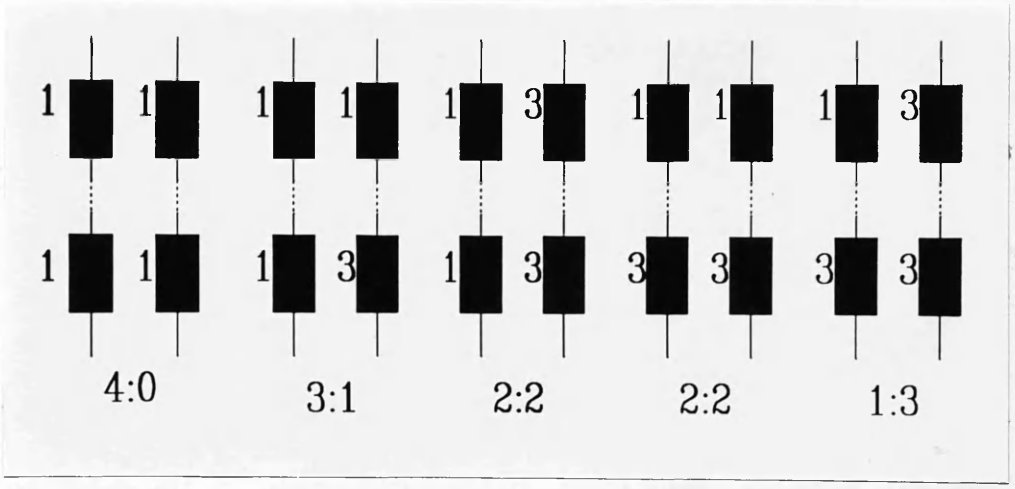
Fig. 38B shows that this is indeed the case. DNAs from a mother, father and two children were digested with Hae3 and a Southern blot probed with a E2-E3 cDNA probe which distinguishes HNP1 from HNP3 sequences. One parent (the mother) was homozygous for HNP1 i.e. contained two alleles each consisting of a pair of HNP1-encoding genes. The other parent had a 2:2 ratio of HNP1:HNP3 genes. However, both children had a different pattern from either parent and contained a 3:1 ratio of HNP1:HNP3 genes. By simple Mendelian inheritance then they must have received a tandem pair of HNP1 genes (HNP1-HNP1 allele) from their mother and a mixed tandem pair (HNP1-HNP3 allele) from their father. Furthermore, if no recombination or gene conversion events occured in the father, this result also implies that his genotype consists of 2 copies of a HNP1-HNP3 allele rather

Fig.38 Inheritance of defensin-encoding genes.

A: Model for arrangement of defensin-encoding genes. In this model there are 4 defensin-encoding genes per diploid cell. From the analysis of Hae3-cut human DNAs (see Fig.24) four distinct genotypes could be distinguished each having different relative amounts of HNP1-:HNP3- encoding seguences. Thus some individuals contained 4 HNP1- encoding sequences (4:0), some had 3 HNP1:1 HNP3 (3:1), some 2 HNP1: 2 HNP3 (2:2) and some 1 HNP1:3 HNP3 (1:3). The model assumes that defensin genes are tandemly duplicated, as shown for the closely homologous rabbit defensins MCP1 and MCP2 (Ganz et al, 1989) with two copies contained on each chromosome 8, the sole location of defensin-encoding genes (Wiedemann et al, 1989; Sparkes et al, 1989). For 2:2 HNP1:HNP3 ratios, two alternative configurations for the arrangement of defensin-encoding genes are possible and are shown. The model predicts that defensin genes should be inherited as a linked pair due to their proximity (< 10kb between MCP1 and MCP2).

B: Inheritance of tandem pairs of defensin genes. 15µg of genomic DNA from a mother, father and two children was digested with Hae3 and Southern blotted onto a Hybond-N membrane. The blot was then hybridized with a radiolabelled E2-E3 cDNA probe (see Fig. 23). This probe distinguishes HNP1- from HNP3- encoding sequences. The mother contained 4:0 HNP1:HNP3 encoding sequences, the father 2:2 HNP1:HNP3 and the two children 3:1 HNP1:HNP3 ratios. According to the model both children received a HNP1-HNP1 tandem pair allele from their mother and a HNP1-HNP3 tandem pair allele from their father. This establishes their father as having a two copies of an allele with a HNP1-HNP3 tandem pair as his 2:2 HNP1:HNP3 configuration.

A


1 1 1 1
1 1 1 3
4:0
1 1 1 1
1 3 1 3
3:1
1 3 1 1
3 3 3 3
2:2
1 3
3 3
2:2
1 3
3 3
1:3

B


4:0
2:2
3:1
3:1

than one copy of an HNP1-HNP1 and one of a HNP3-HNP3, since neither of the children could have inherited either of these two alleles from their father (Fig. 38B).

This experiment then is confirmatory evidence of the model shown in Fig. 38A. However, it is not direct proof, which requires a knowledge of the long-range organization of the defensin genes by the cloning of a tandem pair of genes on lambda or cosmid clones. If the human defensin genes are in fact homologous to the genes for MCP1 and MCP2 isolated from rabbits and 10 Kb apart then with the genomic clones thus far isolated (HNP1A; two clones of 8Kb and approx 12Kb; HNP1B, 8Kb; HNP3A, approx 13.5 Kb; HNP3B approx 15.3 Kb) it may be the case that two clones might overlap at their 3'- or 5'-ends. Whether this is the case is currently being addressed by restriction mapping of the larger genomic clones outwith the 8-9 Kb BamHl fragment previously mapped.

3.3.9 HNP1 and HNP3 defensin mRNAs can be distinguished utilizing PCR

Having established that individuals have different relative amounts of HNP1- and HNP3- encoding sequences it was decided to test whether this results in the expression of different relative amounts of HNP1 and HNP3 mRNAs. As shown in Fig. 39, HNP1 and HNP3 mRNAs can be distinguished by virtue of the single base difference between them resulting in an additional Hae3 site in HNP1 absent in HNP3. Both HNP1 and

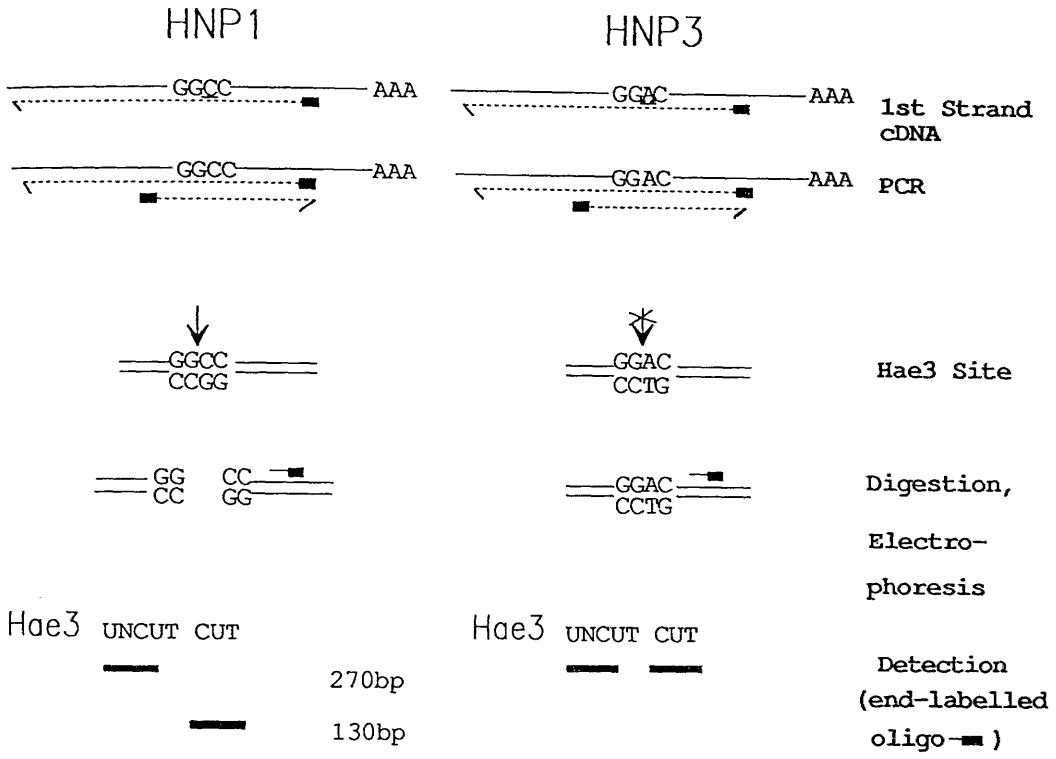


Fig.39 Schematic representation of PCR experiment.

Defensin mRNAs present in RNA from HL60 cells or peripheral blood leukocytes of individuals can be amplified by PCR (Saiki et al, 1985) by first synthesizing cDNA primed by oligo D (1st Strand cDNA) and then amplifying using a combination of oligos B' and D (PCR). The 270bp amplified fragments derived from both HNP1 and HNP3 mRNAs can then be distinguished from each other by digestion with Hae3 (Hae3 Site), since the HNP1-derived fragment contains a cutting site for this restriction enzyme and is digested giving two fragments of 90 and 130bp whereas the HNP3-derived fragment remains undigested since it lacks this site. Both the 130bp HNP1-derived fragment and the 270bp HNP3-derived fragment can be resolved by gel electrophoresis, Southern blotted and detected with an oligonucleotide not used to amplify (oligo C).

HNP3 mRNAs can be amplified by PCR giving identically-sized double-stranded DNA fragments which, upon digestion with Hae3, can be distinguished on the basis of size. These fragments are easily detected by oligonucleotide hybridization using an end-labelled oligonucleotide (oligo C) not used to amplify.

3.3.10 Expression of defension mRNAs in individuals with different HNP genotypes

From three individuals (Fig. 40, lanes 2, 3 and 4, the same individuals as Fig. 24, lanes 5, 6 and 10) and from HL60P25 cells (defensin-expression-positive), RNA and DNA were prepared. Fig. 40B shows a Southern blot of these DNAs digested with Hae3 and probed with an E2-E3 cDNA probe. Lane 1 is an individual (HL60P25 cells) with equal representation of HNP1- and HNP3- encoding sequences (2:2 ratio), whilst the individuals of lanes 3 and 4 both have 3:1 ratio of HNP1:HNP3. Lane 2 is an individual containing only HNP1-encoding sequences (see Section 3.3.1 for an explanation of this interpretation). Fig. 40A shows the result of amplifying defensin mRNAs present in the leukocytes of these 4 cases. Three out of four samples amplified successfully each giving the expected 270 bp fragment (Fig. 40A UNCUT). Digestion of this fragment with Hae3 established how much of the total amount of this fragment is derived from each of the two defensin mRNAs (Fig. 40A, Hae3 CUT).

Fig.40 Variable expression of HNP1 and HNP3.

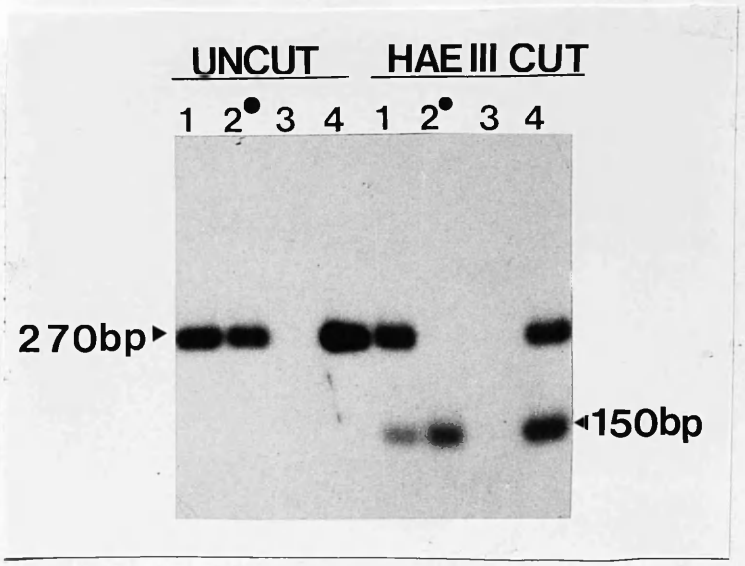
A: PCR Experiment.

0.2µg polyA+ RNA from exponentially growing HL60P25 cells (lane 1) or 10µg total cellular RNA from the peripheral blood leukocytes of 3 leukaemia patients (lane 2, myelofibrosis; lane 3, ANNL; lane 4, CGL) were amplified by PCR using oligos B' and D as described in Section 2.3.10. Amplified products were run on an agarose gel and a discrete 270bp band detected for the samples of lanes 1, and 4 purified and digested with Hae3 (Hae3 cut) or Hind3 (to ensure complete digestion with another enzyme, data not shown). Digestion products were run on a 1.5% agarose gel and the gel blotted onto Hybond-N. The blot was then hybridized with a 5'-end labelled oligo C. Lane 3 failed to amplify in this experiment, probably due to the extremely low level of defensin mRNAs present in ANNL leukocytes (Birnie et al, 1983). In a subsequent experiment (data not shown) defensin mRNAs were amplified successfully from this individual's leukocyte RNA, the result showing a pattern of expression of defensins identical to Fig.34A, lane 4 (Hae3 cut).

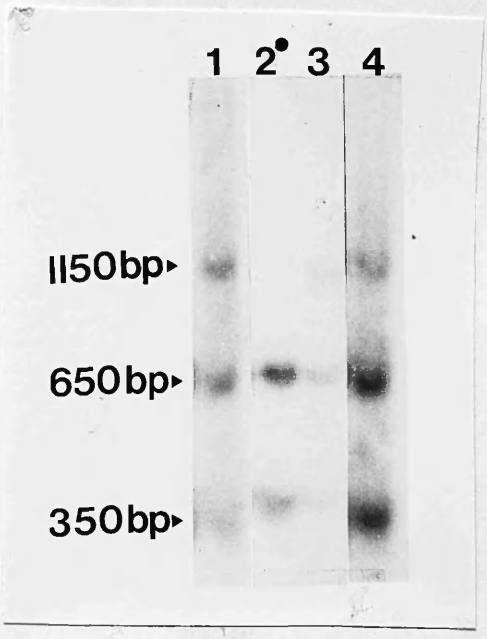
B: Southern Blotting of Genomic DNAs used in PCR Experiment.

DNA was extracted from exponentially growing HL60P25 cells (lane 1) or from the peripheral blood leukocytes of the 3 individuals described in A (lanes 2, 3 and 4) as described in Section 2.4.1. 10ug of DNAs digested with Hae3 were Southern blotted onto Hybond-N and the blot hybridized with a radiolabelled E2-E3 HNP3 defensin cDNA probe.

A


UNCUT
HAE III CUT
1 2 3 4 1 2 3 4
270bp
150bp

B


1 2 3 4
1150bp
650bp
350bp

The main conclusion from this experiment was that the individual hypothesized to contain only HNP1-encoding genes (Fig. 40B, Lane 2) only synthesized HNP1 mRNAs (Fig. 40A, lane 2 UNCUT), lane 2 (Hae3 CUT). In contrast HL60P25 cells, which contained both HNP1- and HNP3- encoding sequences (Fig. 40B, lane 1) synthesized both HNP1 and HNP3 mRNAs (approximately 4-fold more HNP3 than HNP1). Interestingly, an individual containing relatively more HNP1- than HNP3-encoding sequences (Fig. 40B, lane 4) had a higher abundance of HNP1 mRNAs than an individual containing equal proportions of HNP1- and HNP3- encoding sequences (compare Fig. 40B, lanes 1 and 4 and Fig. 40A, lanes 1 and 4 (Hae3 CUT)). This general result, that of increased abundance of a particular HNP mRNA in individuals containing more of that homologous HNP-encoding sequence, holds true for a number of other individuals analyzed (data not shown). However explanations other than their being a direct relationship between gene dosage and mRNA abundance exist which might explain this phenomenon and these will be discussed in Section 4.3.

SECTION 4 : DISCUSSION

4. DISCUSSION

4.1 CHARACTERIZATION OF pCG14 cDNA

4.1.1 Isolation of pCG14 cDNA

The isolation of pCG14 (Wiedemann et al, 1983) and its subsequent characterization (this work, Section 3.1) was of interest from several stand-points. Firstly, the gene encoding pCG14 was expressed in the leukocytes of CGL patients. As described in Section 1.5, CGL is a stem cell disease characterized by two phases: (i) a long chronic phase in which unregulated myelopoiesis occurs due to a rapid expansion in the pool size of committed myeloid precursors (myeloblasts, promyelocytes and myelocytes primarily; Galbraith and Abu-Zahra, 1972; Moore et al, 1973; Goldman et al, 1980); and (ii) a shorter acute phase characterized by an arrest of leukocyte differentiation in which immature myeloid or lymphoid blast-like cells predominate both in bone marrow and in the circulation (Koeffler and Golde, 1981). Initially then, it was hoped that expression of the gene encoding pCG14 might be a useful molecular marker in the diagnosis of CGL. However, it was soon realized that pCG14 gene expression was neither leukaemia-specific nor indeed absolutely CGL-specific (Daher et al, 1988).

The finding that the gene encoding pCG14 was not expressed specifically as a cause or consequence of transformation in CGL, but was in fact expressed during normal bone-marrow-confined myelopoiesis (Birnie et al, 1984) first indicated that pCG14 might encode a myeloid gene and that its isolation from CGL was a consequence of the large numbers of immature leukaemic myeloid cells in the peripheral blood leukocytes of CGL patients. This was confirmed by Birnie et al (1984) and Wiedemann et al (1989) who showed that the gene encoding pCG14 was expressed in a differentiation stage-specific manner during myelopoiesis. Thus expression of the gene was shown to occur only in the neutrophilic myelocyte in normal bone marrow. During this stage of myelopoiesis, neutrophil-committed cells synthesize and assemble many of the key proteins found in granules which contribute to the functions of mature neutrophils. In the chronic phase of CGL, large numbers of these cells are produced and many end up in the peripheral blood. By using in situ hybridization, the cell type expressing the pCG14 gene in CGL was shown to be morphologically indistinguishable from that expressing the gene during normal myelopoiesis i.e. the neutrophilic myelocyte (Wiedemann et al, 1989).

One of the first tasks undertaken (Section 3.1) was the complete sequencing of a pCG14 cDNA. Another cDNA clone, pCG32, apparently identical to pCG14 where the clones overlapped (and subsequently also shown to encode HNP3 defensin by Hae3 restriction enzyme analysis (data not

shown)), was also partially sequenced. Using a combination of primer extension, S1 analysis and RNA sequencing, both cDNAs were shown to contain distinct foreign sequences at their 5'-ends, but were otherwise almost full-length. These foreign sequences were probably introduced during blunt-end ligation of cDNA fragments into the vector pAT153 (Wiedemann et al, 1983; Affara et al, 1981). The sequence of pCG14 did not match any sequence then present in databases and seemed to encode a cysteine-rich 94-amino acid protein. However, what this protein was and how it functioned in myeloid cells was unknown until the reports by Mars et al (1988) and Selsted et al (1985b) of the isolation and sequencing of (respectively) mrs and human defensins.

4.1.2 Isolation of mrs

Concurrent with the work described in Section 3.1, Mars et al (1988) reported the sequence of a cDNA they termed mrs shown to be homologous to pCG14. In the isolation of mrs, Mars et al (1985) used a similar approach to that of Wiedemann et al (1983) to isolate preferentially expressed genes in CGL. One clone (first termed C-A3, then mrs) was isolated and characterized which was found to represent an mRNA found at high abundance in chronic phase CGL leukocytes. In common with pCG14, no mrs gene expression was detectable in normal leukocytes or leukocytes from ANLL, ALL or CLL patients. In situ hybridization analysis indicated that this gene was also

expressed at high levels in immature myeloid cells (promyelocytes and myelocytes) (Mars et al, 1987). The sequence of mrs (Mars et al, 1988) indicated that it was identical to pCG14 where the clones overlapped apart from one base (C in mrs, A in pCG14) and also encoded a cysteine-rich 94-amino acid protein.

4.1.3 pCG14 and mrs encode defensins

The nascent polypeptides of pCG14 and mrs encode precursors of defensins HNP3 and HNP1, respectively, peptides involved in neutrophil antimicrobial killing (this work, Section 3.1.6; Selsted et al, 1985b; Wiedemann et al, 1989; Daher et al, 1988). These peptides are found at high abundance in mature PMN and are discussed in Section 1.2.4. Both pCG14 and mrs cDNAs were also isolated by Daher et al (1988) using an oligonucleotide probe representing defensin peptide sequences to probe an HL60 cDNA library. They showed that their cDNAs for HNP1 and HNP3 differed by a further base change in their 3'-untranslated regions (C in HNP1, T in HNP3) in a stretch of 3' sequence not cloned by Wiedemann et al (1989) or Mars et al (1988), in addition to the coding change previously described. They too found that expression of defensins was high in CGL leukocytes, but they also detected expression in some CLL leukaemias. However, using an anti-defensin antibody this expression was localized to immature myeloid and not lymphoid cells (Daher et al, 1988).

4.1.4 Chromosomal location of mrs and pCG14 (HNP1 and 3) genes

The gene encoding mrs (HNP1 defensin) was first localized by Mars et al (1988) to the long arm of chromosome 8 (8q21.1-23) using a combination of somatic cell hybrid analysis and in situ hybridization to metaphase chromosomes. Using a similar approach, Wiedemann et al (1989) also localized the gene encoding pCG14 to chromosome 8, but to the short arm rather than the long. Due to the almost 100% identity of the probes used in these two studies (mrs and pCG14 cDNAs differ by only one base) the different localization of the two genes is hard to explain since each probe should hybridize to both genes and therefore give two peaks of hybridization, one on each arm of chromosome 8, if the genes were indeed present on either arm of the chromosome. It seems more likely that in one of the studies the defensin gene(s) have been assigned to the wrong arm of chromosome 8. In support of the assignment of defensin gene(s) to the short arm of chromosome 8, Sparkes et al (1989) have also localized mrs (HNP1) to the short arm of chromosome 8 (8p23) using the twin methods of a mouse/human somatic cell hybrid panel and in situ hybridization to normal human metaphase chromosomes. In this study, only one strong peak of in situ hybridization on chromosome 8p23 was detected, suggesting that all defensin genes must be located in this region.

4.1.5 Expression of pCG14 and mrs in vivo

The finding that both pCG14 and mrs are expressed in both normal and leukaemic (CGL) myelocytes (Birnie et al, 1984; Mars et al, 1985) is in accord with other work comparing normal and leukaemic gene expression (see Section 1.4.3). Thus, in the majority of cases, the phenotypes and gene expression of leukaemic cells are similar in many characteristics to normal hematopoietic progenitors (Greaves et al, 1986). In this regard, it is interesting to note that, in contrast to the expression of pCG14 (HNP3 defensin) during the chronic phase of CGL, expression during the acute phase of the disease (blast crisis) is low or variable. Thus in the study of Birnie et al (1984) all (14/14) CGL patients in chronic phase contained defensin mRNAs in their peripheral blood leukocytes whilst only 1/3 patients in blast crisis contained detectable defensin mRNAs. Similarly, Mars et al (1985) in a survey of CGL patients showed that 17/18 contained defensin mRNAs in their peripheral blood leukocytes and that this expression correlated with the presence of myelocytes in peripheral blood as evaluated by differential counts. Significantly, the one CGL patient in this study not containing detectable defensin mRNAs did not contain myelocytes in his peripheral blood leukocytes. This study also investigated the presence of defensin mRNAs during blast crisis of CGL and produced results similar to that of Birnie et al (1984). Thus defensin gene expression during blast crisis was variable with 2/4 individuals containing detectable

defensin mRNAs in their peripheral blood leukocytes (Mars et al, 1985). Interestingly, the two positive patients peripheral blood contained myelocytes, whilst the two negative patients did not.

These results with blast crisis CGL are consistent with what is known about the differentiation stages of the leukaemic cells during this phase of CGL. The blast cells seen during the acute phase of CGL usually resemble myeloblasts seen in ANLL (Rosenthal et al, 1977); however in a number of cases they may have morphological and/or cytochemical features of lymphoblasts (Peterson et al, 1976). Since the normal window of differentiation of defensin gene expression occurs in more differentiated myeloid cells than myeloblasts, and expression is not found at all in the lymphoid lineage (Mars et al, 1987; Wiedemann et al, 1989), if one assumes that during blast crisis the majority of cells are one of these two types, the lack of/variable defensin gene expression observed can be understood. In the cases of CGL blast crisis where defensin gene expression is detected, the presence of a small residual population of more mature myeloid cells around the myelocyte stage of differentiation accounts for the observed expression (Mars et al, 1985).

4.2 DEFENSIN GENE EXPRESSION

4.2.1 Use of cell lines as model systems

Understanding the myriad changes in gene expression occuring during lineage commitment and terminal maturation occuring during normal hematopoiesis is made difficult by both the physical inaccessibility of the site of maturation (bone marrow for non-lymphoid lineages in humans) and the complex number and organization of both hematopoietic and bone marrow cell types as well as the underlying extracellular matrix. At best, for example in the long-term bone marrow culture system of Dexter (see Section 1.1.5), if appropriate maturation stages of a particular lineage can be identified, expression of particular markers can be evaluated using specific monoclonal antibodies and the techniques of immunocytochemistry. However, the underlying causes of changes in the expression of such markers remain obscure using such culture systems.

Many investigators have turned to clonal cell lines capable of differentiation *in vitro* to answer questions relating to how individual or sets of genes are regulated during hematopoietic (or non-hematopoietic) differentiation. Amongst these are myogenic cell lines, embryonic lines, neuronal cell lines and hematopoietic cell lines (Blau and Epstein, 1979; Strickland and Mahdavi, 1978; Marks and Rifkind, 1978; Temple and Raff, 1985; Patterson, 1978;

Collins et al, 1977). An analysis of the changes which occur during induced differentiation of these cell lines in vitro 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 overview of differentiation in all systems.

The main advantage of cell lines is that they allow the isolation of relatively pure clonal cell populations of differentiated cells not available either in vivo or in Dexter-type culture systems. As a result, these systems also enable maturation to be monitored easily as the differentiating cells are not surrounded by a background of other cell types or cells at markedly different differentiation stages. Cell lines which can be differentiated also allow the process of commitment to differentiate to be studied since variant cell lines resistant to differentiation are available (Toksoz et al, 1982). Moreover, large amounts of material for analysis, for example DNA, RNA and protein, are easily obtainable. Another advantage of using hematopoietic cell lines to study differentiation in this system is that hematopoiesis in man has been well-studied and the cell biology of the different lineages well-characterized (Metcalf, 1971).

However, with few exceptions (see, for example, Dexter et al, 1980; Greenberger et al, 1983; Hapel et al, 1981), hematopoietic cell lines are derived from tumour cells and are therefore transformed and aberrant. Since aberrant gene

expression is known to occur in transformed cells, it remains problematic whether, as a rule, control of gene expression may also be aberrant due to adaptation of the cells to tissue culture, and might therefore be expected to vary depending on how close culture conditions are to those encountered by the cells *in vivo*. Almost certainly for most cell lines, culture conditions do not reflect that of the natural *in vivo* microenvironment of the cell which may be required for normal growth and gene expression.

The inducing agents used to initiate differentiation of cell lines are often either highly non-physiological, such as DMSO, or are physiological agents used at concentrations far greater than that likely to be encountered by cells *in vivo*. These agents (with the probable exception of the CSFs sometimes used to effect hematopoietic differentiation *in vivo* (Koeffler, 1983)) are unlikely to reflect a normal differentiation signal. Furthermore, the mechanisms of action of these agents remain, for the most part, obscure. Therefore it is unknown if induction of differentiation by chemical agents occurs by similar patterns of gene expression as those 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 loss of immortality. 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 cell's differentiation programme.

4.2.2 Differentiation of HL60 cells as a model of myelopoiesis

The HL60 cell line affords several advantages as a model system in which to study differentiation. This cell line provides a continuous supply of apparently "maturation arrested" cells which can be cultured easily and indefinitely (Collins et al, 1977). The cells can be induced to differentiate by readily available agents, and the progress of differentiation can be monitored by easily measurable characteristics. The HL60 cell line is also a useful model for the study of maturation arrest itself in leukaemic disorders. These cells provide an insight into the nature of leukaemic lesion(s) and are therefore useful as a means of analyzing 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 ANLL, such as anthracyclines, are also potent inducers of HL60 cell 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 as well as direct cytotoxic effect on rapidly proliferating malignant cells.

Uninduced HL60 cell cultures are somewhat heterogeneous, consisting of 90% promyelocytic cells with 10-15% more mature cells of both the granulocyte and monocyte/macrophage lineages (Collins et al, 1977). Following addition of the inducing agent to the growth medium, during the differentiation process, the cell population becomes more heterogeneous. The whole HL60 cell population appears to undergo a programmed differentiation, such that 3-5 days following the addition of inducing agent most cells resemble mature cells of either the granulocyte or monocyte/macrophage lineage depending on the inducing agent used. However, during the course of treatment with inducing agents, cells can be found distributed throughout the stages of the myeloid cell lineages leading to mature granulocytes or monocytes (Boyd and Metcalf, 1984). Thus intermediates between promyelocytes and granulocytes during induction with DMSO or retinoic acid and monocytic intermediates during TPA induction can be detected in culture (Mitchell, 1987). 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 (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 inducing agent 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 (see Section 4.2.5).

4.2.3 Mechanisms of regulation of gene expression

The control of defensin gene expression, in common with other non-housekeeping eukaryotic genes, might be expected to be a complex affair both *in vivo* and during *in vitro* differentiation of HL60 cells. Currently accepted models to explain the regulation of gene expression in both eukaryotes and prokaryotes are essentially similar (Ptashne, 1986) with the overriding control of gene expression in both types of organism exerted at the transciptional level. Thus, the first level of control of gene expression is generally exerted at the transcriptional level.

For both prokaryotes and eukaryotes the basic mechanisms that lead to gene activation or inactivation involve interaction of specific cis-acting regulatory elements in the DNA with trans-acting factors, which include the enzymatic machinery of transcription, although in eukaryotes these steps are inherently more elaborate than in prokaryotes, reflecting the relatively greater size and complexity of the eukaryotic genome.

So far as eukaryotic gene expression is concerned, the pathway of mRNA biogenesis involves several steps, all of which potentially offer the cell points at which gene expression may be regulated. These steps include transcriptional initiation, elongation and termination, RNA capping, 3' processing of the primary transcript, polyadenylation, splicing, nucleo-cytoplasmic transport and cytoplasmic stability. There is considerable evidence that most, if not all, of these options are used in eukaryotic cells. Genes for which transcriptional initiation, elongation or termination is exploited for regulation of gene expression are said to be under transcriptional control, while those genes for which other steps are used for control of gene expression are under post-transcriptional control.

Transcriptional control of eukaryotic genes has received the most attention regarding possible levels of control of gene expression. Many of the cis-acting sequences responsible for normal and inducible transcription as well as

the trans-acting factors both general and specific, responsible for gene activation have been isolated and partially characterized (Maniatis et al, 1987). Although eukaryotic cells possess three classes (1, 2 and 3) of RNA polymerase, it is almost exclusively pol2 which trancribes genes encoding proteins. Evidence from in vitro transcription of cloned protein-coding genes indicates that RNA polymerase 2 initiates transcription at the mRNA cap site (Breathnach and Chambon, 1981). A conserved AT-rich element (TATA box), generally located 25-30 bases from this site (Darnell, 1982), was first shown to be involved in regulating gene expression by directing the transcriptional machinery to the correct site for initiation by RNA polymerase 2 (Nevins, 1983). Two similar elements are present upstream of the HNP1A defensin gene at appropriate positions to mediate transcriptional initiation from two sites approximately 60 nucleotides apart (Section 3.3.3).

Many other sequences have since been characterized which serve to modulate transcription of certain genes. Enhancer elements (Khoury and Gruss, 1983), themselves constructed from smaller sequence elements termed enhansons (Ondek et al, 1988), steroid hormone receptor binding sites (Beato, 1989; Evans, 1988), metal responsive regulatory elements (Karin et al, 1985) and heat-shock transcriptional regulatory elements (Pelham, 1984) are examples of such cis-acting transcriptional control sequences all under the influence of various trans-acting proteins some of which themselves are tissue or differentiation-stage specific (Scheidereit et al, 1988).

A more recently described control of transcription occurs during transcriptional elongation itself. For both the c-myc (Bentley and Groudine, 1986; Eick and Bornkamm, 1986) and the c-myb (Bender et al, 1987) proto-oncogenes, this novel mode of intragenic control has been shown to operate. For the c-myc gene, a specific sequence at the end of exon 1 has been shown to be responsible for premature termination or pausing of elongation (Bentley and Groudine, 1988). This mechanism of control may be most appropriate for genes whose control must be tightly coupled to cellular processes such as proliferation and differentiation.

Termination of transcription has been found to occur at a defined site in genes transcribed by RNA polymerase 2 (Manderious and Chen-Kiang, 1984). The 3'-end of most primary transcripts is generated by 3' processing of the nascent transcript (Birnstiel et al 1985). This process potentially affords a point of control of gene expression since differential termination of transcription can generate a number of different mRNAs. This sort of mechanism has been described for transcripts of adenovirus genes (Darnell, 1982) and for regulation of expression of the secreted and membrane-bound forms of the immunoglobulin μ-membrane protein (Early et al, 1980).

The process of mRNA polyadenylation accompanies cleavage of the nascent transcript to form the 3'-end of the mRNA (Birnstiel et al, 1985). A conserved sequence AAUAAA has

been identified 10-30 nucleotides upstream of the polyA addition site at the 3'-end of polyA - containing transcripts, and this sequence may direct or regulate mRNA cleavage and polyadenylation (Birnstiel et al, 1985). This sequence is also found at the 3'-end of the HNP1A gene (see Fig. 28) as might be expected since HNP1 mRNAs are polyadenylated (Daher et al, 1988). Experiments showing a decrease in stability on removing the polyA tail from globin mRNA after injection into HeLa cells (Huez et al, 1981), have highlighted the importance of a polyA tail to mRNA stability.

Although the initial events of mRNA biogenesis occur rapidly, further processing of the precursor RNA is a slower event (Nevins, 1979). Most higher eurkaryotic transcripts undergo splicing to remove intron sequences to yield mature mRNAs. Splicing reactions are directed by splice site signals at the intron/exon junctions (Breathnach and Chambon, 1981) and may be facilitated by formation of lariat RNA intermediates (Keller, 1984). The HNP1A gene characterized in Section 3.3.3 contains three exons and two introns both of which must be spliced out to produce a functional HNP1 mRNA. For the HNP1A gene, the splicing signals at the intron/exon junction correspond well to consensus splicing signal sequences proposed by Mount (1982). Splicing control is common in eukaryotic genes with more than one mature transcript often being derived from the one gene in different tissues (Lewin, 1985a).

Transcript export from the nucleus to polysomes represents another possible level of control of gene expression. How such a control might operate is unknown, nor is it known whether such a process is active or passive. Results to date, however, suggest that only mature mRNAs enter the cytoplasm (Nevins, 1979). Since it seems that there is sequence-specific selection of transcripts for transport (Jacobs and Birnie, 1982; Babich et al, 1983; Fulton et al, 1985), this step in the pathway of gene expression may afford an important control point.

Lastly, the rates of cytoplasmic mRNA turnover in eukaryotes vary widely and this is often a factor in the control of expression of particular genes. The half-lifes of some mRNAs span several hours or even days (Singer and Penman, 1973; Volloch and Houseman, 1981). Some mRNAs, however, such as those encoding interferon (Raj and Pitha, 1981), GM-CSF (Shaw and Kamen, 1986), c-myc and c-fos (Greenberg et al, 1986) are relatively unstable, with a half-life in the range of 30 min or less. Moreover, in response to physiological and pharmacological stimuli, preferential stabilization (Guyett et al, 1979; Brock and Shapiro, 1983; Raghow et al, 1987) or destabilization (Hamalainen et al, 1985; Dani et al, 1985; Raghow et al, 1986) of specific mRNAs has been demonstrated.

4.2.4 Changes in defensin gene expression during granulopoiesis

Induction of HL60 cells along the granulocytic lineage by treatment with DMSO leads to a biphasic down-regulation of defensin gene expression (Section 3.2.1). Thus uninduced HL60P25 cells used in the experiments described in that section synthesized defensin mRNAs (both HNP1 and HNP3, as evaluated by PCR amplification and Hae3 digestion), whilst cells which had been induced to differentiate for 72 hours had ceased to synthesize detectable defensin mRNAs. This down-regulation of defensin gene expression was not gradual as might perhaps be expected if defensin gene expression was strictly linked to the process of differentiation, since defensin mRNA abundance did not correlate linearly with the proportion of mature granulocytes appearing in culture (Section 3.2.1). Rather, defensin gene expression was rapidly down-regulated, then re-elevated before a second, slower down-regulation which seemed to correlate with the differentiation process. The kinetics of this change in expression is similar to that seen for the change in abundance of c-*myc* mRNAs when murine erythroleukaemia cells are induced to differentiate to mature erythroid cells by treatment with HMBA or DMSO (Lachman and Skoultchi, 1984). Thus, during the induced differentiation of these cells a 5 to 15 -fold drop in c-*myc* mRNA levels occurs within 1 to 2 hours of treatment. The c-*myc* mRNA level then recovers to pretreatment levels by 12 to 18 hours and then continues to fall off gradually as terminally differentiated cells

accumulate. The striking similarity between this regulation and the regulation of defensin gene expression suggests that similar mechanisms of control of gene expression may be operating for both genes.

In contrast to treatment of HL60 cells with DMSO, treatment with retinoic acid does not lead to a down-regulation of defensin gene expression. Rather a 2-4 fold up-regulation 32 hours after treatment is observed which, after 5 days of treatment, has returned to pre-induction levels. Thus, although the cells apparently differentiate normally to mature granulocyte-like cells (65% NBT-positive by day 5 of treatment), albeit to a lesser extent than with DMSO (85% NBT-positive by day 5 of treatment), defensin gene expression is maintained and indeed transiently increased during the course of induced differentiation.

However, for DMSO at least two distinct events would seem to be occuring. The initial down-regulation and subsequent re-elevation of expression may well be simply a result of DMSO treatment and not linked in any direct way to differentiation of the cells. DMSO is a bipolar molecule with a high dielectric constant which has an enormous effect on permeability of membranes. Phase transition temperatures of phospholipids are increased by DMSO treatment causing reduced fluidity and increased membrane stability. However, the mechanism(s) through which DMSO operates to induce differentiation are obscure. One possibility is that changes

in membrane permeability could enhance differentiation by altering the binding of certain factors (perhaps those described by Sachs (1987)) which normally initiate differentiation.

The striking similarity in the kinetics of c-myc regulation during Friend cell differentiation and defensin gene expression during HL60 cell differentiation induced by DMSO may be instructive and shed some light on how DMSO alters gene expression. Like c-myc in Friend cells, defensin gene expression is rapidly down-regulated following the addition of DMSO to HL60 cells in a manner not directly linked to differentiation. Moreover, it is known that for other cell lines DMSO can lead to a rapid mRNA destabilization of c-myc in the absence of differentiation. For example, Darling et al (1989) have shown that this occurs in several diverse cell lines (K562 (myeloid;) Daudi and Raji (Burkitt lymphoma lines); CEM (T-cell lymphoblastoid cells) and L1210 (mouse lymphoma line)). If, as seems to be the case, the mechanism of this decrease in abundance of c-myc mRNA is post-transcriptional, then for c-myc (and also defensin) it seems also to be reversible, such that mRNA levels are re-elevated 4-12 after treatment (this work, Section 3.2.1 Lachman and Skoultchi, 1984; Darling et al, 1989). This re-elevation of c-myc mRNA levels seems to be absent or greatly attentuated in HL60 cells induced to differentiate by treatment with DMSO (Siebenlist et al, 1988) where the rapid decrease of c-myc mRNA abundance is not reversible and

probably occurs via an increase in transcriptional pausing at the end of exon 1 of the c-myc gene (Bentley and Groudine, 1986; Eick and Bornkamm, 1986; Siebenlist et al, 1988).

The third facet of defensin gene expression during DMSO-induced HL60 differentiation is a down-regulation of gene expression which seems to parallel the differentiation process. If retinoic acid and DMSO induce differentiation of HL60 cells in different ways, then despite a population shift of HL60 cells through the myelocyte stage of differentiation (where the defensin genes are normally expressed) then, in contrast with retinoic acid-induced differentiation, gene expression would not seem to be up-regulated after DMSO treatment of HL60 cells.

In summary then, the differences in defensin gene regulation between retinoic acid- and DMSO-induced differentiation may be as a result of two factors: (i) DMSO inducing a higher proportion of HL60 cells to mature non-expressing granulocyte-like cells; (ii) both agents effecting differentiation and co-ordinate gene expression in different ways. For retinoic acid it is possible that additionally it may act directly to increase defensin gene expression and thereby overcome the normal down-regulation of gene expression occuring with differentiation. Retinoids are derivatives of vitamin A, a hormone demonstrated to be required for normal vision, reproduction as well as maintenance of differentiated epithelium and mucous secretion

in the whole animal (Goodman, 1984). It is also thought that retinoids play a basic role in control of differentiation and can suppress the malignant phenotype (Lotman, 1980) and may exert a hormone-like control of either or both of proliferation or differentiation (Sporn and Roberts, 1984). Retinoic acid can increase gene expression acting via specific nuclear receptors which bind the hormone and increase the rate of transcriptional initiation of retinoic acid-responsive genes (de Thé et al, 1987; Petkovich et al, 1987; Brand et al, 1988). It is possible, then, that defensin genes may be directly responsive to this hormonal effect of retinoic acid independently of the indirect effect of induced HL60 differentiation promoted by the agent.

4.2.7 Comparison of inducing agents

The reason for this difference in regulation of defensin gene expression in response to different inducing agents is not known. At first sight it would appear to be unlikely that the small difference in the induction capacity of retinoic acid and DMSO could result in such entirely different regulations of defensin gene expression. Some workers have directly compared the efficacies of retinoic acid and DMSO at inducing HL60 cells to differentiate with conflicting results. Tsifsoglou and Robinson (1985) have reported that retinoic acid 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 (a granulocyte marker) than retinoic acid-induced HL60 cells. Similarly, a report by Breitman and Keene (1982), demonstrated that calcium ionophore—activated production of leukotrienes (another marker of mature granulocytes) is greater in DMSO- than retinoic acid-induced cells. In contrast, Hemmi et al (1982) demonstrated that retinoic acid-induced HL60 cells appeared morphologically more mature at an earlier stage during induction than DMSO-induced HL60 cells. However, the HL60 cells used in this work (HL60P25) reproducibly could be induced to differentiate to a greater extent with DMSO than with retinoic acid, using the ability of the cells to reduce NBT (Section 2.2.4) as an assay for terminally differentiated cells. In conclusion, there appears to be great variation in the extent of differentiation of HL60 cells with different inducing agents used in different laboratories. Since the concentrations of the inducing agents used are all very similar, it seems reasonable to suppose that these inconsistencies result from differences in the HL60 sublines from various laboratories and/or differences in cell culture technique.

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 such resistance remain obscure. In this regard it is possible that the maintenance of defensin gene expression during retinoic acid-induced differentiation

may be due to the presence in the HL60P25 line of a population of cells incapable of responding to retinoic acid. A second possibility is that retinoic acid may only induce HL60 cells partly along the granulocytic lineage. It is known that during initiation of retinoic acid-induced differentiation HL60P25 cells are not synchronous in relation to phase of the cell cycle (Mitchell, 1987) and possibly stage of differentiation, either of which could result in some cells responding more quickly to the induction stimulus than others. Therefore, at the end of a 5-day retinoic acid induction period those cells which responded early in the treatment period would display mature cell characteristics whilst those cells which were later in responding would still be at an early stage on the granulocytic differentiation pathway and hence would retain uninduced cell characteristics. Interestingly, with the HL60P25 line, Mitchell (1987) also observed following 5 days of incubation with retinoic acid 50- 60% of cells resembling granulocytes both morphologically and biochemically. However, 30-40% of the cells still resembled uninduced HL60 cells or myelocytes. In this study, cell proliferation could still be observed in the HL60 cell culture after 5 days of retinoic acid treatment, supporting the notion that a significant proportion of the cells remained undifferentiated. Moreover, 5 day retinoic acid-induced HL60 cells invariably appeared less mature morphologically than 5 day DMSO-induced HL60 cells.

One hypothesis, then, for the difference between DMSO and retinoic acid on the regulation of defensin gene expression in HL60 cells might be that if indeed retinoic acid leads to a culture of cells containing 30-40% of defensin-expression-positive immature cells then significant overall expression of defensin might still be seen by day 5 of treatment. Furthermore, the increase in defensin gene expression observed by 32 hours of incubation of retinoic acid may be real and reflect increased expression of defensin genes as a significant proportion of HL60 cells become differentiated to myelocyte-like cells, the cell type of highest defensin gene expression *in vivo* (Wiedemann *et al*, 1989; Mars *et al*, 1987). *In vivo* the average transit time from promyelocyte to myelocyte in man is 40-50 hours (Bainton, 1977) which agrees well with the timing seen of highest defensin gene expression during retinoic acid-induced HL60 differentiation. The slower decrease in abundance of defensin mRNAs observed by day 5 of treatment with retinoic acid may then represent a further shift in the population of cells to metamyelocyte and band-like cells for the majority of cells in culture with a concomitant down-regulation of defensin gene expression similar to that observed *in vivo*.

4.2.6 Control of defensin gene expression

Of a number of hematopoietic cell lines investigated (Section 3.2.6) only one HL60 cell line expressed human defensins. Since both HNP1 and HNP3 mRNAs were found in

HL60P25 cells (Section 3.3.9), both types of genes must therefore have been transcribed. Defensin genes are normally located on chromosome 8, only one copy of which is found in HL60 cells (Gallagher et al, 1979). Interestingly in Southern blots of HL60 cells DNA digested with Hae3, direct comparison with identical quantities of DNA suggest that although an equal ratio of HNP1- to HNP3 - sequences is present there is about 1/2 the autoradiographic signal intensity of a normal 2:2 pattern DNA (data not shown). This result should be treated with caution until a more quantitative test is carried out (for example DNA dot-blot hybridization comparing defensin genes to a control gene present at two copies/diploid cell in HL60 cells and another cell type). However, if correct it is of some interest since it implies that HL60 cells contain one HNP1 gene and one HNP3 gene, both of which seem to be actively transcribed. Interestingly, the relative abundance of the two types of defensin mRNA in HL60 cells was not equal; HNP3 mRNAs seemed to be 3-4 fold more abundant than HNP1 mRNAs. The reason for this is not clear. One possibility is that HNP3 and HNP1 mRNAs may have inherently different stabilities. How might this occur? Two possibilities present themselves. One possibility is that either or both of the two base changes between the two mRNAs (one in the 3'-coding region, one in the 3'-non-coding region (Section 4.1.3)) may serve to stabilize HNP3 relative to HNP1 mRNA. Alternatively, a significant increase in the length of polyA tail of HNP3 mRNA may enhance its stability relative to HNP1 mRNA as has been described for tubulin mRNA abundance during the cell cycle (Green and Dove, 1988).

A more likely explanation, though, is that the genes encoding HNP1 and HNP3 were transcribed at different rates in HL60 cells. How this might have been effected will require a direct comparison of the expression of HNP1 and HNP3 genes. This could be investigated using the isolated genomic clones for HNP1 and 3 described in Section 3.3. Certainly, the limited restriction analysis of the 4 isolated defensin genes (Section 3.3) indicated that differences exist between the two isolated HNP1 or HNP3 genes as well as between HNP1 and HNP3 genes in their 5'-regulatory regions which may be responsible for different levels of expression.

During induced differentiation of HL60 cells only the overall regulation of defensin gene expression was investigated. Since both HNP1 and HNP3 genes would seem to be independently regulated in uninduced HL60P25 cells as indicated by the different mRNA abundances of HNP1 and 3, it is possible that they were also independently regulated during differentiation. This question could be addressed, should a defensin-expressing HL60 cell line become available, using a PCR-based approach rather than Northern blotting to distinguish HNP1 from HNP3 mRNAs during differentiation. An analysis of the results of Section 3.2 must therefore be viewed as results of expression of both HNP1 and HNP3 genes.

As revealed by actinomycin D treatment of HL60 cells, defensin mRNAs seem to be relatively stable. Interestingly, a rapid transient increase in the level of defensin mRNAs

occurred shortly (30 min) after actinomycin D treatment (Section 3.2.2). The reason for this induction is unknown since actinomycin D is best known as a potent inhibitor of transcription. A comparison of the effect of the protein synthesis inhibitor cycloheximide on c-myc RNA levels may be instructive. In this case, cycloheximide treatment of cells leads to a super-induction of c-myc gene expression during mitogenic stimulation of lymphocytes (Kelly et al, 1983). In this case a labile negative regulator protein was invoked to explain the effect of a protein synthesis inhibitor on c-myc gene expression. However, both the extreme rapidity and transience of the effect on defensin gene expression would argue against such a protein regulator being involved. How actinomycin D might rapidly and transiently increase defensin gene expression as seems to be the case may merit future studies.

Nuclear run-on experiments with uninduced HL60P25 cells indicated that defensin genes are transcribed at a low level in these cells (Section 3.2.2). Again with this technique, transcription of the HNP1 gene was not distinguished from the HNP3 gene. However such an analysis, even if technically possible, would have been difficult to perform due to the low level of transcription seen in HL60P25 cells. Most probably the down-regulation of defensin gene expression seen during the latter part of DMSO-induced HL60 differentiation reflects a decrease in the transcription of defensin genes, but this awaits direct proof.

4.2.7 Loss of defensin gene expression

The loss of defensin gene expression on reculture at a later date of a new batch of frozen and rethawed HL60P25 cells, HL60P25(1) (Section 3.2.5), is one of the more puzzling results in the work described in Section 3.2. It seems that the defensin genes are still retained in HL60P25(1) (data not shown) and therefore the loss of defensin gene expression is not simply due to a gross deletion of defensin sequences. There are, however, other possible reasons for the observed loss of defensin gene expression. The first possibility is that in the intervening period between experiments with HL60P25 and HL60P25(1) some aspect of cell culture was changed. For both medium and serum used to culture the cells this is unlikely since the same manufacturer (Gibco) was used for both medium and serum in both sets of experiments. However, differences between batches of either of these two constituents cannot be ruled out as a variation between culture conditions. Alternatively, growth of a culture of HL60P25(1) may have selected for a clone of cells not expressing defensin genes. This is a distinct possibility bearing in mind that there is no positive selection for HL60 cells expressing defensins since the peptides have no documented role in cell viability or proliferation (see Section 1.2.4-6).

Any mechanism pertaining to lack of expressing must, however, account for the loss of expression of both (or possibly 4) defensin genes in HL60 cells, since the different levels of gene expression of HNP1 and 3 indicate that they are independently regulated. Investigation of the methylation status of the defensin genes in HL60P25(1) may help to rule out gene inactivation via methylation as the mechanism of loss of defensin gene expression (Jackson and Felsenfeld, 1985).

4.3 CHARACTERIZATION OF DEFENSIN GENES

4.3.1 Hae3 restriction analysis of human DNAs indicates the presence of at least 4 defensin genes/diploid cell

The occurence of a single coding change between defensin cDNAs encoding HNP1 and HNP3 (this work, Section 3.3.1; Daher et al 1988) and the realization that this was a possible way to distinguish HNP1 from HNP3 genes (this work, Section 3.3.1), by virtue of the presence or absence of a cutting site for the Hae3 restriction enzyme, provided the first insight into the structure and copy number of human defensin genes (Section 3.3.1). Thus prior to any knowledge of the fine structure of defensin genes, individuals could be shown to contain variable relative amounts of HNP1 and HNP3 genes and a hypothesis proposed in which individuals contained a total of 4 defensin genes, two per chromosome 8.

In a total of 32 human DNAs analyzed, individuals could be placed into one of four groups, depending on their relative amounts of HNP1 and HNP3 genes. Thus some individuals (16%) contained only HNP1 genes, about twice this number (31%) contained three HNP1 genes and one HNP3 gene, most individuals (41%) contained two HNP1 and two HNP3 genes whilst a small number (12%) contained one HNP1 gene and three HNP3 genes. Interestingly, no individuals were found out of a total of 32 analyzed that contained only HNP3 genes.

The strength of an analysis of defensin genotypes using the Hae3 restriction enzyme to distinguish HNP1 from HNP3 genes was firmly underscored by the demonstration (Section 3.3.7) that defensin genes are inherited in pairs. Thus it could be shown that a parent containing only HNP1 genes passed on one pair of these to each of two offspring in a manner consistent with the hypothesis that human defensin genes are closely linked as a tandem pair and therefore co-inherited.

A more extensive analysis using DNAs from a large family tree or several families, however, is required to prove the generality of this inheritance.

4.3.2 Characterization of an HNP1 defensin gene

The first defensin genes isolated from human DNA libraries encoded HNP1 defensin. The two genes were isolated from two individuals and shown by restriction analysis to have distinct 5'-regulatory sequences but great similarity in their coding regions (Section 3.3.2). On the basis of one easily detected Hind3 polymorphism the genes were termed A or B and the HNP1A gene investigated in detail.

The HNP1A was found to be split into three exons separated by two introns. Exon 1 of the HNP1 gene was found to be non-coding, with exons 2 and 3 encoding the nascent HNP1 defensin precursor. Both by Hae3 restriction enzyme analysis and by sequencing, the gene was shown to encode HNP1.

Sequencing of 239 bp of the upstream region of the HNP1A gene revealed the presence of two promoters containing AT-rich sequences, both of which seem to be functional since they serve to initiate transcription 30 bases downstream (P1 and P2 transcripts). The proximal promoter P1 is the stronger of the two and serves to initiate transcription over a 2 or 3 nucleotide region (Section 3.3.3). The reason for the presence of two promoters is unknown but it is also a characteristic of the rabbit defensin genes MCP1 and MCP2 (Ganz et al, 1989). At least for MCP2, both TATA boxes may also be functional as suggested by the existence of two classes of cDNA clones with 5'-termini positioned appropriately by the two promoters (Ganz et al, 1989).

Continuing the analogy with rabbit MCP defensin genes, HNP1 shares the same intron-exon organization as the rabbit genes, although the sizes of the rabbit MCP introns are somewhat smaller (500 bp and 600 bp) than the equivalent HNP1A introns (1500 bp and 650 bp). Like HNP1A, the first exon of the rabbit MCP genes is non-coding, with exons 2 and 3 encoding the MCP precursor protein. Interestingly, a comparison of MCP1 or MCP2 cDNAs with those encoding HNP1 or 3 (Ganz et al, 1989), shows a 61%-64% overall homology between the sequences. Moreover, a 110 nucleotide region of 86% homology occurs which includes the 5'-untranslated region (exon 1 of HNP1A or MCP1 and 2) and 73 nucleotides of HNP1A exon 2 (encoding 20-residues of the defensin precursor). The hydrophobicity of this 20-residue peptide has prompted some

authors to suggest that it represents a signal sequence which is cleaved during proteolytic processing to produce mature defensin peptides (Daher et al, 1988; Ganz et al, 1989). If this is correct it implies that at least two processing events must occur during the maturation of defensin peptides: (i) removal of a signal peptide and; (ii) removal of a residual 44 residues to give the mature HNP1 or HNP3 peptide. If a myeloid cell line expressing defensins could be found, the occurence of such processing steps could be tested by both pulse-labelling and pulse-chase experiments utilizing the anti-defensin antiserum used in Section 3.1.7 to immunoprecipitate defensin precursors. Whatever the function of the first 20 residues of the defensin precursor, its sequence conservation between HNP and MCP precursors suggests that it is required for the production of both human and rabbit defensins.

4.3.3 Isolation of 4 different defensin genes

Subsequent to the isolation of the two different HNP1 genes, three more defensin genes were isolated by screening a single genomic library (Sections 3.3.4 and 3.3.5). One of these was shown by restriction mapping to be indistinguishable from the previously isolated HNP1A gene, the other two by Hae3 restriction enzyme analysis shown to encode HNP3.

The two HNP3 genes were restriction mapped and shown to have distinct 5'-regulatory regions but great similarity in their coding regions. Interestingly, the two genes had the same upstream Hind3 polymorphism as the two HNP1 genes and were thus also termed A or B. As yet the two genes encoding HNP3 have not been analyzed in as much detail as the HNP1A gene. However, both genes lack a second 3' Hae3 site present in their putative second introns compared to HNP1 genes as well as the Hae3 site which results from their encoding HNP3. Of special interest will be the promoters of HNP3 genes where a comparison with HNP1 genes will shed light on how the two genes are expressed at different levels (Section 4.2.6).

If, as seems to be the case by analogy with rabbit MCP1 and 2 genes, human defensin genes are tandemly duplicated, it is of interest how they might be organized. For the four defensin genes isolated it is presently unknown which genes are allelic (i.e. present at equivalent positions on different copies of the same chromosome) and which non-allelic (i.e. present at different positions on the same chromosome). Stated this way allelic genes would be expected to be more alike than non-allelic genes. To satisfactorily answer this question, a more complete sequence analysis of the four defensin genes would be required.

4.3.4 HNP genotype influences relative abundance of HNP1 and 3 mRNAs

A number of important issues are raised by the finding of different HNP genotypes amongst individuals (Section 3.3.1). The first issue is how variable relative amounts of HNP1 and HNP3 genes affect the relative abundances of HNP1 and 3 mRNAs. This issue was addressed in Section 3.3.9 using a PCR-based approach and Hae3 digestion to distinguish HNP1 from HNP3 mRNAs. Interestingly, it was found that an individual containing only HNP1 genes expressed only HNP1 mRNAS. Thus a small percentage (16%) of individuals might be expected to only express HNP1 peptides. Furthermore, it was found that individuals containing relatively more HNP1 genes expressed relatively more HNP1 mRNAs. However, a measure of caution should be expressed about this latter interpretation. In the experiment described in Section 3.3.9, RNA for determination of the relative abundance of HNP1 and HNP3 mRNAs, with the exception of that derived from HL60 cells, was derived from mixed populations of leukocytes for which data on the relative expression of HNP1 and HNP3 genes is not available. Thus it can be imagined that increased expression of the HNP1 genes, for example, may be a consequence of the particular leukocyte population chosen and not be directly related to the relative amount of HNP1 compared to HNP3 genes if the HNP1 gene was expressed at a higher level in such a leukocyte population.

A second issue arising from this result and the results of Section 3.3.1 concerns the biological consequences, if any, of expressing only one defensin peptide (in the case of HNP1 homozygotes) or relatively more HNP3 than HNP1 peptide (in the case of individuals containing 1:3 ratios of HNP1:HNP3 genes). As detailed in Section 1.2.5, in several different assays investigating the relative activities of defensin peptides, HNP1 has been shown to have greatest activity, HNP2 less and HNP3 least or none (Ganz et al, 1985; Lehrer et al, 1988b; Territo et al, 1989). Simplistically, one might expect individuals expressing only HNP1 peptides not to be disadvantaged whilst individuals expressing only HNP3 peptides may be. However this analysis is simplistic and does not take into account the HNP2 peptide which is almost as active as HNP1 and may be derived from either or both of HNP1 or 3. If HNP2 defensin can be derived from HNP3, then the presence of relatively more HNP3 genes might not be expected to be disadvantageous. In this regard, it is of interest to know why HNP3 genes occur at all since HNP1 genes would seem to provide adequate protection in the form of HNP1 (and HNP2?) peptides. If a coding change converting an HNP1 to an HNP3 gene was genetically neutral one might expect several other changes in the coding and non-coding structural regions of defensin genes, which is not found. It would seem therefore that the HNP3 genotype has been fixed and must be of some purpose. Just what functions the HNP3 peptide can perform, however, remain to be established.

4.4.5 Hypothesis for the evolution of HNP1 and HNP3 genes

The above argument presupposes that HNP3 genes are derived from HNP1 genes. Is there any evidence for this? The occurence of homozygotes containing only HNP1 genes and the relative infrequency of individuals containing 1:3 ratios of HNP1:HNP3 genes is consistent with the ancestral defensin gene being of the HNP1 type. Fig. 41 shows a model of how the present HNP genotypes may have arisen. The first event in such a scheme is that of gene duplication. Such an event is common in eukaryotes with many genes, for example, the interferons and globins, occuring in clusters deriving initially from gene duplication (Lewin, 1985b). Following duplication, at least one of the genes is free to collect mutations some of which may become fixed. In the case of defensin genes, one such mutation which may have become fixed was that converting an HNP1 to an HNP3 gene. Such a change can create chromosomes containing an HNP1-HNP3 tandem pair. How might one then create a chromosome with an HNP3-HNP3 tandem pair, seen in a small number of individuals? For genes occuring in clusters some interesting molecular mechanisms operate both to spread mutations and to homogenize gene sequences, the result being a process called concerted evolution (Lewin, 1985b; Hartl and Clarke, 1989). A good comparison is with the human globin genes (Maniatis, 1980). For example, humans have duplicated α-globin genes encoding identical proteins and there is only a single amino acid difference between the two human δ-globin proteins. How do

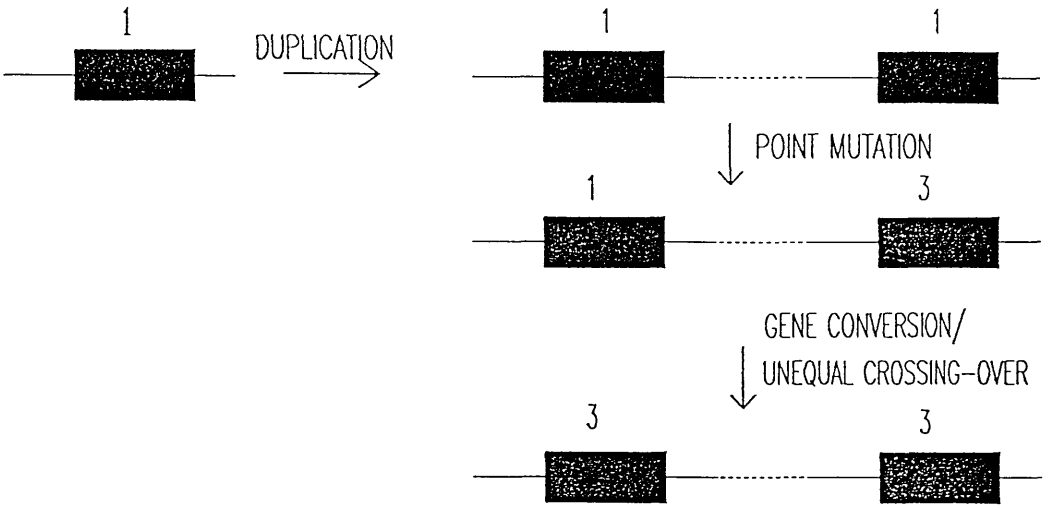


Fig.41 Hypothesized evolution of defensin genes.

There are two varieties of human defensin genes (HNP1 and HNP3). On the basis of the present allele frequencies of tandem pairs of defensin genes (i.e. HNP1-HNP1, HNP1-HNP3, HNP3- HNP3), it is hypothesized that the ancestral defensin gene was of the HNP1 type. Subsequent duplication of this gene led to the presence of tandem pairs of HNP1 genes (DUPLICATION). One gene of the tandem pair was free to collect mutations, one of which (the nucleotide substitution C→A converting HNP1 to HNP3) became fixed (POINT MUTATION). To bring about the relatively rare HNP3-HNP3 tandem pair allele it is hypothesized that either gene conversion or two or more rounds of unequal crossing-over converted a HNP1-HNP3 allele into a HNP3-HNP3 allele (GENE CONVERSION/ UNEQUAL CROSSING-OVER).

these sequences remain so similar if one accepts that both are not under selective pressure in order to produce enough functional protein? Two general types of mechanism which maintain identity of gene sequences are gene conversion and unequal crossing-over (Hartl and Clarke, 1989). Gene conversion is a process whereby nucleotide pairing between two sufficiently homologous genes is accompanied by the loss of all or part of the nucleotide sequence in one gene and its replacement by a replica of the nucleotide sequence of the other gene. Formally, the result is that the sequence in one gene converts the sequence in the other gene to be exactly like itself. An example of the frequency of such conversion may be the presence of a particular β-thalassemia nonsense mutation (β-globin gene $\beta^{39(CAG-TAG)}$)which is present on nine different β-thalassemia chromosomes in a Sardinian population (Pirastu et al, 1987).

Concerted evolution can also occur by means of unequal crossing-over. In tandem gene families, misaligned pairing of the genes during meiosis can occur and crossing-over results in gametes that can have either an increase or a decrease in copy number. Thus, a subsequent crossing-over can either correct the copy number or increase it further. Formally, the final result is genetically equivalent to gene conversion because the nucleotide sequences of some members of the multigene family have been replaced with the nucleotide sequences of other members. At the same time that unequal crossing-over increases the variance in copy number, it acts to homogenize the members of the family.

By comparing the sequences of defensin genes one should be able to judge whether they are indeed subject to concerted evolution. If they are, one should not see the accumulation of silent site substitutions between them because the homogenization process applies to these as well as to the replacement sites. However, apparent spreading of the HNP3 base change to both copies of the defensin gene present on one chromosome suggests that such mechanisms of concerted evolution may have acted on defensin genes in the past.

With the rather small number of individuals (12%) containing an allele with an HNP3-HNP3 tandem pair the absence of individuals (out of 32 tested) with an HNP3 homozygous genotype becomes easier to understand. Thus if this proportion of individuals is representative of the frequency of the HNP3-HNP3 allele, one would expect the frequency of HNP3 homozygotes to be in the region of 1/270 (i.e. 1/4 X 0.12 X 0.12) and thus be unlikely to be present in the small sample size (32) of individuals analyzed.

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