THE EXPRESSION AND REGULATION OF THE GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR RECEPTOR (GM-CSFR)

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A thesis submitted to the University of London for the degree of Doctor of Philosophy

1996

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This thesis is dedicated to the memory of my father

Yash Pal Chopra
ABSTRACT

The granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) consists of a specific α chain which binds ligand with low affinity and a β chain which confers high affinity binding. The aim of this thesis was to investigate the expression and regulation of the GM-CSFR.

RNase protection assays were developed in order to detect the low abundance mRNA species. The GM-CSFRα mRNA expression was determined to be in the pg range and the β mRNA expression at least one log lower. GM-CSFRα mRNA expression was demonstrated in a number of haemopoietic cell lines and the expression of GM-CSFRα mRNA was consistently higher than that of GM-CSFRβ mRNA, which is in keeping with the receptor protein expression.

RNase protection assays of GM-CSFRα yielded extra bands in addition to the expected mRNA transcript. Characterisation studies, using RT-PCR, established the existence of at least two additional mRNA transcripts involving alternative splicing of the 5' untranslated region of the GM-CSFRα chain. Sequencing demonstrated that these isoforms were the result of a deletion of 24 nucleotides at the 3' end of exon 2 (exon 2b deleted isoform) and complete deletion of exon 2 (exon 2 deleted isoform). These isoforms were detected in primary haemopoietic cells, blasts from patients with acute myeloid leukaemia and malignant cell lines. Together the isoforms were more highly expressed than the full length sequence. Translation of constructs corresponding to the exon 2 deleted isoforms was assessed using an in-vitro reticulocyte lysate system. Deletion of exon 2 resulted in a significantly lower in vitro translation of the receptor protein compared to the full length sequence, whilst deletion of exon 2b results in higher translation of the α chain protein. These in vitro studies therefore identified putative regulatory sequences that may modulate translation of the GM-CSFRα chain.
In order to investigate the underlying mechanisms by which the receptor expression is regulated, GM-CSFR binding and mRNA expression were investigated using two model systems: TF-1 cells undergoing down and up-regulation of surface receptors; and HL-60 cells undergoing DMSO-induced differentiation. The half-life of the GM-CSFR protein in TF-1 cells growing in steady state conditions in the presence of Epo and the translation inhibitor cycloheximide, was approximately 4 hours, which would indicate a constant non-ligand mediated turnover of cell surface receptor protein. Incubation of the TF-1 cells in the presence of the transcription inhibitor actinomycin-D indicated that the half-life of the GM-CSFRα mRNA was between 1 - 3 hours and that of the GM-CSFRβ mRNA greater than 8 hours. The cell surface expression of the specific GM-CSFRα and β chains and mRNA levels however, did not change in the presence of actinomycin D, implying post-transcriptional regulation of the GM-CSFR expression. In order to further clarify the nature of this post-transcriptional regulation, cell surface receptor expression in cells undergoing up-regulation of their receptor was measured. Up-regulation was inhibited in the presence of cycloheximide, but not by the lysosomotropic agent ammonium chloride, suggesting that new protein synthesis, rather than receptor recycling, is important when GM-CSFR expression is dynamically modulated.

Uninduced HL-60 cells express low numbers of both low and high affinity receptors and on induction with DMSO for 5 days, the receptor numbers increased and both the GM-CSFRα and β mRNA levels showed a co-ordinate increase. This implies transcriptional regulation of the GM-CSFR α and β chains during differentiation. The different mechanisms described for GM-CSFR regulation may explain at least in part, how GM-CSF can act through its receptor to modulate long term differentiation events and at the same time augment mature cell function where the control mechanisms take place over a time course of minutes and hours.
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ACKNOWLEDGEMENTS

The work presented in this thesis would not have been possible without the help and support of family, friends and colleagues.

I am grateful to all the staff at 98 Chenies Mews and in particular would like to thank Steve Devereux, Rosemary Gale, Asim Khwaja, Pam Roberts and Shaun Thomas who not only generously shared their expertise with me, but also taught me most of the laboratory methods used in this study. I would like to offer a special thank you to Rosemary Gale for painstakingly reading my thesis and offering constructive criticism - her trans-world communication via e-mail was particularly appreciated.

Last but not least, I would like to thank Professor DC Linch for giving me the opportunity to work in his department and for his close supervision. His constant encouragement and ability to enthuse was a significant factor in the completion of this work.
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>ALA</td>
<td>5-aminolevulinate synthase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CFU-S</td>
<td>Colony forming units-spleen</td>
</tr>
<tr>
<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAB</td>
<td>French American British</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidyl inositol</td>
</tr>
<tr>
<td>GTC</td>
<td>Guanidine thiocyanate</td>
</tr>
<tr>
<td>HGF</td>
<td>Haemopoietic growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-b-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IRE</td>
<td>Iron responsive element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>STATs</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>JCML</td>
<td>Juvenile chronic myeloid leukaemia</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LMP</td>
<td>Low melting point</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>OSM</td>
<td>Oncostatin M</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonylfluoride</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA Ends</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor,</td>
</tr>
<tr>
<td>SD</td>
<td>Subdomain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>snRNA</td>
<td>small nuclear RNAs</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
</tr>
<tr>
<td>TCA</td>
<td>Tri-chloro acetic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethlenediamine</td>
</tr>
<tr>
<td>TIR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol 13 acetate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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—CHAPTER 1—

INTRODUCTION
1.1 Haemopoiesis

Haemopoiesis is the process by which a minimum of eight distinct populations of mature blood cells are generated from a pool of haemopoietic stem cells that reside in the bone marrow. Mature cells of each of these lineages have finite life spans ranging from several days to months or even years. The wide range of different cell type specific half-lives requires that different cell populations be produced at different rates. Every day $10^{12}$ blood cells, including $2 \times 10^{11}$ erythrocytes and $7 \times 10^6$ neutrophils are produced. This prodigious feat in terms of daily output must be precisely regulated, since circulating levels of mature cells are maintained within narrow limits in the steady state, but can be altered rapidly in response to increased or decreased demand. The ability of this system to respond appropriately in a cell specific manner to pathological deficiency (such as red cells in anaemia), further suggests a sophisticated regulatory mechanism. Such precise regulation is thought to occur through a series of complex interactions between the bone marrow stem cells, haemopoietic growth factors (HGFs) acting through their specific receptors and the bone marrow microenvironment.

1.1.1 Historical perspective

The existence of the haemopoietic stem cell was suspected as early as the turn of the century (Pappenheim, 1900) and subsequently Maximow (1924) postulated the existence of a cell capable of giving rise to all blood cells. The original hypothesis, that the stem cell could produce further stem cells which were pluripotent for myeloid cells and lymphoid systems, and that these latter cells could then produce stem cells of more restricted potential, proved to be remarkably accurate.

Whilst most of these assertions were originally based upon purely morphological study of human bone marrow, subsequent murine reconstitution /
transplantation studies yielded further experimental evidence for the existence of multipotent haemopoietic stem cells. In these studies, syngeneic bone marrow cells were injected into lethally irradiated mice. Macrosopic splenic colonies were present 7 - 14 days later and analysis showed that these colonies were composed of haemopoietic cells of apparently single lineage (i.e. erythroid or granulocytic) at day 8, but by day 12 - 14, there were colonies composed of a number of different haemopoietic cells (Till & McCulloch, 1961). These colonies were termed colony forming units-spleen (CFU-S). Secondary transplantation of the CFU-S colonies and subsequent serial transplantation established that the CFU-S had a self-generating capacity, although this capacity was finite.

The CFU-S assays enabled initial characterisation of haemopoietic progenitor cells and suggested that CFU-S cells could give rise to the full haemopoietic lineage, including B lymphoid cells, but not T lymphocytes which require a specific thymic microenvironment (Wu et al, 1983). The factors that regulated haemopoiesis could not, however, be purified until techniques for in vitro culture of haemopoietic cells were established. Subsequently, Pluznick & Sachs (1965) and Bradley & Metcalf (1966) independently described methods for the maintenance of haemopoietic colonies in semi-solid culture. These cultures were maintained by conditioned medium derived from a variety of organs including the lung, muscle and pregnant mouse uterus (Metcalf, 1984). Conditioned media were capable of 'colony stimulating activity', mostly of granulocyte, erythroid and macrophage lineages (Robinson et al, 1967). The purification of haemopoietic regulatory factors from these conditioned media was a truly heroic task. For example, the purification of murine granulocyte colony stimulating factor (G-CSF) required three litres of mouse lung conditioned media (derived from 800 mice) with a final yield of 2.7 μg pure G-CSF (Nicola et al, 1983).
By the early 1980s, studies had led to the purification and characterisation of four different murine colony stimulating factors, G-CSF, granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF) and interleukin-3 (IL-3, multi-CSF) (Burgess et al, 1977; Stanley et al, 1976; Ihle et al, 1983; Nicola et al, 1983). Subsequently, the corresponding human CSFs were purified, and cDNAs encoding all four murine and human CSFs were cloned. This work rapidly led to the clinical application of HGFs.

Additional haemopoietic regulators have been subsequently identified and include the following: stem cell factor (SCF) (Zsebo et al, 1990; Huang et al, 1990; Williams et al, 1990); IL-6 (Kishimoto, 1995); IL-11 (Paul et al, 1990), IL-12 (Wolf et al, 1991); Flt2/Flk3 ligand (Lyman et al, 1994); and mpl ligand (Kaushansky, 1995). There are, however, undoubtedly other cytokines which play a role in the regulation of haemopoiesis. The remarkable thing about the HGFs isolated, purified and cloned to date, has been their rapid introduction into clinical practice. For example, mpl ligand was introduced into patients within 18 months of its original isolation (CG Begley, personal communication). The isolation of the HGFs has facilitated the characterisation of their corresponding specific cell receptors, which in turn has given further insight into the mode of action of HGFs.

1.1.2 The haemopoietic stem cell
The understanding of how a small number of progenitor cells can give rise to a variety of haemopoietic cells remains a fundamental challenge in haematology. Recent studies have gone some way in the characterisation of so called stem cells.

One of the reported characteristics of the stem cell is its capacity for self renewal and retention of multilineage differentiation (Moore, 1991). This definition is based on transplantation studies in mice (Lemischka, 1992) and in vitro assays (Gordon, 1993). Transplantation studies in mice using retrovirally
marked cells showed that only a few cells are necessary for restoration of haemopoiesis (Jordan & Lemischka, 1990; Harrison, 1980; Keller & Snodgrass, 1990). This data supports the clonal succession model hypothesis in which it was proposed that self renewal is limited, and that a proportion of stem cells are drawn on to be active to replace others that have undergone differentiation, end cell maturation and ultimately cell death (Kay, 1965). Haemopoiesis is therefore initially supported by a succession of short-lived clones. Furthermore, engraftment may be divided into an early phase of a few months during which multiple stem cell clones, with transient and restricted development potential, are active, and a later stage with a stable continuation from one or two pluripotent stem cells with long life spans.

A variety of *in vitro* assays enabled similar conclusions and suggested that stem cells are heterogeneous with a hierarchical organisation of the stem cell and progenitor cell compartment (Figure 1.1). It should be noted, however, that the data supporting a clonal succession model is based on post-transplantation and *in vitro* studies, and may therefore have limited relevance to steady state haemopoiesis. Characterisation studies of early stem cells have yielded the following observations:

(i) The cells have self-renewal potential which reduces with age (Metcalf & Moore, 1971) - the role in steady state haemopoiesis remains controversial (Lord & Dexter, 1995);

(ii) The cells which are capable of supporting long term engraftment are Rhodamine (Rh) dull (Rh is a supravital fluorochrome which fluoresces when activated by mitochondrial enzymes - quiescent stem cells have low mitochondrial activity) (van der Sluijs *et al*, 1990);

(iii) The cells are HLA-DR negative (Moore *et al*, 1980; Keating *et al*, 1984);
Figure 1.1 Diagram illustrating the hierarchical organisation of the stem cell and progenitor cell compartments as identified by currently available bioassays.

The compartments are shown in relationship to proliferative potential and cell-cycle status in steady state haemopoiesis. Stage related phenotypic characteristics used for stem cell purification are also used.
(iv) The cells are Ly6/Scal positive in mice (Spangrude et al, 1988) and CD34 positive in mice and humans (Caux et al, 1989; Andrew et al, 1989);
(v) Most cells are in G0 (Lajtha, 1963 and 1979);
(vi) The cells require HGFs to proliferate and survive, and withdrawal of HGFs leads to cell death by apoptosis (Williams et al, 1990).

1.1.3 Possible mechanisms of haemopoiesis

The progeny of these early stem cells maintain the balance between self renewal and differentiation. The mechanism of such regulation is poorly understood and the subject of some debate.

The inductive hypothesis Studies with cultured haemopoietic cells have shown that the formation of haemopoietic colonies, as well as the proliferation and survival of various haemopoietic cells, typically requires the presence of HGFs. This has led to the inductive hypothesis, which suggests that receptor binding of the HGFs (perhaps in combination with elements of the cellular microenvironment) acts as an inducer or controller of the differentiation and determines the lineage choice for multipotent cells (Curry & Trentin, 1967; Metcalf, 1991).

The stochastic hypothesis The inductive hypothesis has been rejected by the adherents of the stochastic theory of lineage commitment (Till et al, 1964; Ogawa, 1993; Landsdorp, 1995a). Evidence for a stochastic process is derived from the original work of Till et al, (1964) and subsequently Ogawa (1993). Ogawa examined the commitment of micro manipulated single progenitors. After taking a two cell "colony", separating the cells and then comparing the progeny in subsequent colonies, Ogawa was able to demonstrate a variety of lineage combinations. This work lead to the proposal that self-renewal and / or lineage commitment is a random process intrinsic to the cells. Furthermore, the lineage commitment data follows a mathematical distribution, suggesting that this is a
random process (Landsorp, 1995a). According to the mathematical model, intrinsic control of stem cell decision may be described by $p$ (probability of self-renewal) and $f$ (fraction of stem cells that at any time point ($t$) contribute to haemopoiesis. By decreasing this probability from $1 > p > 0.5$ to $p = 0.5$, and decreasing $f$ with time, the pool of stem cells is functionally increased or maintained as required for a particular stage in development. A self-renewal rate probability of $> 0.5$ has been found by a number of investigators (Vogel et al, 1980; Nakahata & Ogawa, 1982). Experimental evidence as to how the $p$ and $f$ values alter during periods of stress, for example as a result of bacterial sepsis, however, is not forthcoming.

**The intrinsic control hypothesis** Lansdorp et al (1995b) have suggested an intrinsic control model (a refinement of the stochastic hypothesis), whereby an inbuilt genetic programme governs the decision to differentiate and / or proliferate. This would have the theoretical advantage of prevention of *in vivo* stem cell expansion, or expansion in response to external stimuli. This group further suggests that the balance between self-replication and differentiation may be controlled by telomerases and telomere length. As cells age, the length of telomere shortens and this is of importance in deciding whether the cell has the ability to divide. Adult haemopoietic stem cells have a reduced length of telomere compared to cord blood or fetal liver (Vaziri et al, 1994) and the latter may have a greater proliferative capacity (Hows et al, 1992).

Other evidence for intrinsically determined differentiation has come from studies after transfection of Bcl2 into a murine FDCP- mix cell line (a multipotent primary cell line able to undergo granulocyte / erythroid differentiation under different conditions, such as culture on stroma or in the presence of exogenous growth factors like IL-3). Bcl2 facilitated survival of cells in the absence of IL-3, and single cell experiments showed that differentiation could occur independently of proliferation (Fairbairn et al, 1993). However, this data does not preclude the
possibility that HGFs may be able to influence stem cell differentiation, since some of these experiments were performed in the presence of horse serum. Indeed, comparison of the differentiation profiles of the FDCP-mix (Bcl2) cells in the presence and absence of horse serum indicated that serum factors other than IL-3 may have been able to modulate lineage choice in these cells.

The critical issue is therefore whether HGFs actually control differentiation or whether they modulate, facilitate, or influence this process (although this may be considered a matter of semantics). Documentation of how HGFs may modulate differentiation commitment is technically demanding, because selective cell survival needs to be carefully eliminated as an alternative explanation for any observed changes. Indirect evidence, however, comes from the fact that growth factor receptors have intracytoplasmic domains that influence differentiation of cells, as opposed to proliferation (Fukunaga et al., 1993; Miyajima et al., 1993). Whilst there is no doubt that maturation of any one lineage requires a multiplicity of carefully regulated and sequential genetic events including transcription factors and / or proto-oncogenes, exactly how these are influenced by HGFs still remains to be elucidated.

1.1.4 Role of haemopoietic growth factors
Having established that HGFs are necessary for the survival, proliferation and, at least in part, differentiation of haemopoietic stem cells, studies of HGFs have been made in an attempt to elucidate their mechanisms of action. Such investigation has identified some common features of HGFs.

**Lineage restriction** It is conventional to show the action of HGFs in a schematic fashion with different HGFs and related molecules acting at different stages of development of various lineages. This however, is a simplified representation, as none of the known regulators have been shown to be restricted. Regulators most closely approaching absolute lineage restriction are possibly
erythropoietin (Epo) or G-CSF. However, Epo has been reported as having actions on megakaryocytic cells (Sakaguchi et al, 1987), and G-CSF on early stem cells (Metcalf & Nicola, 1991).

**Redundancy** The cross lineage actions of haemopoietic regulators has fostered the notion of redundancy. Failure to produce one regulator resulted in continued haemopoiesis as a result of the actions of the other regulators (Kishimoto et al, 1994). Further credence to the notion of redundancy comes from the molecular structure of the cytokine receptors (Section 1.3.1). A number of cytokines consist of specific α chains and shared β chains. The receptors also share common signalling pathways (Section 1.3.3).

Redundancy and the pleiotropic effects of the HGFs have become increasingly apparent in gene knockout studies (using homologous recombination) of HGF genes. Such studies have shown that inactivation of the GM-CSF gene results in normal fetal and post-natal haemopoiesis. The only defect noted in this case was defective function of alveolar macrophages, which resulted in surfactant accumulation and alveolar proteinosis (Stanley et al, 1994). There was no compensatory rise in G-CSF, IL-3 or M-CSF, suggesting that apart from alveolar macrophage function, the GM-CSF can be readily compensated by normal physiological levels of other regulators. The specific functions of GM-CSF and IL-5 have also been highlighted by the generation of mice carrying a null mutation for the shared β chain between GM-CSFR, IL-5R and IL-3R (Nishinakamura et al, 1995; Robb et al, 1995). The IL-3 function was not affected since the mouse IL-3R has an additional β chain (see Section 1.3.1). The mice had alveolar proteinosis and exhibited low basal numbers of eosinophils. Infection of these mice with a parasitic tape worm resulted in the absence of blood and lung eosinophilia, showing the primary importance of IL-5 in generating mature eosinophils, although other factors were capable of compensating to some extent. In an IL-3 unresponsive A/J mouse strain, where a deletion in a branch
point splice site, results in loss of exon 8 which contains a WSXWS motif common to all HGF receptors (Section 1.3.2), the IL-3 receptor is not expressed at the cell surface. Haemopoiesis is normal, suggesting that either IL-3 has a minimal role in regulating basal haemopoiesis, or is easily compensated for by other regulators, at least in the mouse (Hara et al, 1995).

It has been suggested that the redundancy and pleiotropy displayed by the HGFs is a function of the evolutionary persistence of molecules, some of which may have little or no function in normal physiology (Lajtha, 1994). However, in a seminal review, Metcalf has pointed out that the existence of multiple regulatory factors indicates subtlety rather than redundancy (Metcalf, 1993), and knockout experiments are already highlighting the specific effects of the HGFs. In addition to the specific effect of GM-CSF on alveolar macrophage function identified by knockout experiments, M-CSF knockout has been shown to lead to osteopetrosis in the face of normal macrophage function (Lieschka et al, 1994). The term redundancy therefore is misleading as haemopoiesis is regulated by multiple regulators which allow synergistic, or additive, responses, therefore broadening the cellular repertoire.

Polyfunctionality of cytokines It is being increasingly recognised that HGFs not only act to regulate haemopoiesis but have important effects on other organ systems. The leukaemia inhibitory factor (LIF) / IL-6 / IL-11 / Oncostatin M (OSM) family of cytokines (which share a common high affinity gp130 chain (Section 1.3.1) are the paradigm of this pleiotropy in that these regulators modulate haemopoietic cell proliferation and differentiation to some extent, but also modulate osteoclast function and hepatocyte acute phase release of proteins. Furthermore, LIF is important for blastocyst implantation, T cell responses and CNS development (reviewed by Hilton, 1992). Metcalf (1994) has suggested that such polyfunctionality is nature's design to integrate the response of tissue units to a functional or emergency demand.
1.1.5 Haemopoietic growth factor receptors

In order to establish the mechanism of action of haemopoietic growth factors and attempt to understand both their pleiotropy and redundancy, study has focused on the cell surface receptors for these molecules. The purification of many of these ligands enabled investigation of the biochemical interactions between the ligands and their receptors (reviewed in Nicola, 1987 & 1989). Such studies demonstrated the following:

(i) Each haemopoietic growth factor binds to a unique cell surface receptor.

(ii) Receptors are expressed on the cell surface at very low levels (100 - 2000 receptors per cell).

(iii) Receptors for different HGFs may be expressed simultaneously on any given cell.

(iv) The distribution of expression of receptors reflects the known cellular targets for the HGF, both in and out of the haemopoietic system.

(v) Binding of one HGF to its receptor may down-modulate receptors for other HGFs. On murine cells this occurs in a hierarchical fashion, for example IL-3 is capable of modulating the GM-CSF receptor and GM-CSF can down-modulate GM-CSF and M-CSF receptors, but the reciprocal does not hold (Walker et al, 1985). It has been hypothesised that the biological consequence of this may be to activate the down-regulated receptor (Nicola, 1987). This may in part explain why some HGFs, such as IL-3, have a broad action and can down-modulate all HGF receptors, whereas others, such as G-CSF, can only down-modulate a specific receptor.

(vi) In the human system, GM-CSF and IL-3 cross-compete for the same receptor on eosinophils and acute myeloid leukaemia (AML) cells (Lopez et al, 1989; Park et al, 1989a).

(vii) Cross-linking studies suggest that G-CSF receptors are likely to be single chain molecules (Nicola & Peterson, 1986), while GM-CSF and IL-3 receptors
are comprised of at least two sub-units (Nicola & Peterson, 1986; Chiba et al., 1990).

(viii) Scatchard analysis suggests that some HGFs bind their receptors with high affinity (eg. G-CSF, Demetri & Griffin, 1991), while others display both low and high affinity binding (eg. GM-CSF and IL-3, Park et al., 1989b). In the case of GM-CSF and LIF at least, detergent solubilisation of cell membranes converts high affinity binding to low affinity binding, suggesting that the generation of high affinity binding sites depends on the membrane association of two or more proteins (Nicola & Cary, 1992).

The recent cloning of genes encoding these receptors has provided further insight into the actual molecular entities involved in both the ligand-receptor and receptor-receptor interactions, and go some way in explaining the cross-competition and hierarchical down-modulation described for the receptors.

The first of the HGF receptors to be cloned was the receptor for M-CSF which, by serendipity, was equated to the product of the c-fms proto-oncogene (Sherr et al., 1985). It has since transpired that the HGF receptors (HGFR) can be classified into either (i) the tyrosine kinase family of receptors, eg. M-CSFR (c-fms) (Sherr et al., 1985) and SCFR (c-kit, Yarden et al., 1987); or (ii) the recently defined cytokine receptor superfamily (Bazan, 1990). Members of this superfamily can be further divided into class I, the haemopoietin receptor family (mpl, gp130 and receptors for growth hormone, prolactin, IL-2 (β and γ chains), IL-3, 4, 5, 6, 7, 9, 11, 12-p40, 13, 15, GM-CSF (Figure 1.2), G-CSF, ciliary neurotrophic factor (CNTF), OSM and LIF and class II, the interferon receptor family.

The cytokine receptor superfamily have certain characteristics in common and these are specifically described for the GM-CSFR (Section 1.3.1). Dimerization appears to be an important feature of these receptors. Homodimerization is a feature of some cytokines and is a feature of the receptors
for growth hormone, prolactin, Epo and G-CSF (de Vos et al, 1992; Fukunaga et al, 1991; Watowich et al, 1992). Heterodimerization, with sharing of a common component, however, is a characteristic of other members of the haemopoietin receptor superfamily. The receptors for IL-3, IL-5 and the human GM-CSF are heterodimers consisting of specific α chains and a shared common β chain (Section 1.3.1). The receptors for IL-2, IL-4, IL-7, IL-9 and IL-13 contain a common component, isolated initially as the γ chain of the IL-2 receptor (Takeshita et al, 1992; Kondo et al, 1993; Noguchi et al, 1993; Kishimoto et al, 1994). In the case of IL-4, IL-7 and IL-13, the receptors may consist of homodimerized or single specific receptor subunits in association with the γ chain, but for IL-2, in contrast, there is a heterocomplex consisting of α, β and γ subunits (Nikaido et al, 1984; Leonard et al, 1984; Hatakeyama et al, 1989).

The situation for IL-6, IL-11, CNTF, LIF and OSM is more complex. The receptor for each cytokine contains gp130 which, although it does not bind to IL-6, was first identified because of its ability to interact with the complex between IL-6 and its specific, low affinity receptor subunit to yield a high affinity receptor capable of signal transduction (Hibi et al, 1990). The stoichiometry of the IL-6 receptor was initially reported to be two molecules of gp130 to a single α chain (Murakami et al, 1993). However, recently Paonessa et al (1995) have suggested that in vitro at least, the fully assembled IL-6R complex consists of two IL-6, two IL-6Rα and two gp130 molecules. CNTF, LIF and OSM receptors differ in that they contain one molecule of gp130 and one molecule of a LIF receptor polypeptide (Gearing et al, 1991; Gearing & Bruce, 1992; Davis & Yancopoulos, 1993; Davis et al, 1993). CNTF also binds to a specific α subunit that is most similar in primary sequence to the α subunit of the IL-6 receptor, but which, unlike other members of the haemopoietin / interferon receptor family, is tethered to the plasma membrane by a glycoposphatidyl inositol (GPI) anchor rather than a classical transmembrane domain (Davis et al, 1991).
Figure 1.2 GM-CSFR structure
This structure is common to the haemopoietin receptor family
(Features of fibronectin and SD100 domain are described in Section 1.3.2)
The haemopoietin receptor family therefore not only have unique structural features, but some also have complex stoichiometric arrangements. A further level of complexity is the presence of a number of isoforms of the haemopoietin receptor superfamily (Section 1.3.1).

### 1.2 GM-CSF

GM-CSF is produced in a paracrine manner by a number of different cell types, including endothelial cells, fibroblasts (exposed to TNF or IL-1), macrophages (stimulated by lipopolysaccharide) and T cells (stimulated by antigen) (Gasson et al, 1991).

Human GM-CSF is a 127 amino acid glycoprotein which has undergone N- and O-linked glycosylation, giving rise to several molecular weight species, ranging from 18 to 30 kDa. N-linked glycosylation appears to reduce both the specific biological activity and receptor affinity of the human GM-CSF, relative to the non-glycosylated molecule (Cebon et al, 1990). The structure of GM-CSF has been determined by X-ray crystallography and, like several other cytokines, GM-CSF has been shown to assume the conformation of an anti-parallel four α helical bundle with two anti-parallel β strands in the loops between helices A and B and helices C and D (Figure 1.3). A variety of structure-function studies have suggested that the C and D helices are important for binding to the α chain of the GM-CSFR, while residues in the A helix, particularly Glu 21, are important for binding to the β chain of the GM-CSFR (Kaushansky et al, 1989; Shanafelt et al, 1991; Lopez et al, 1992).
Figure 1.3 Crystal structure of GM-CSF
The human GM-CSF gene is localised to chromosome 5q23 - 31, and is close to the IL-3, IL-4 and IL-5 genes. The long arm of chromosome 5q13 -31 also encodes a number of other important oncogenes, HGFs and receptors including Ras p21, IL-9, endothelial growth factor, p40 sub-unit of IL-12. The long arm of chromosome 5 has been the focus of a great deal of study following the demonstration of its deletion in 5q- myelodysplastic syndrome (MDS), a condition which gives rise to specific clinical features in predominantly female patients (Boultwood et al, 1994). Deletion of the long arm of chromosome 5 has been hypothesised to result in loss of a tumour suppressor locus which has been mapped to just beyond 5q31 (Boultwood et al, 1994), which therefore excludes GM-CSF, IL-3, IL-4 and IL-5 as candidate genes for this locus.

1.2.1 Role in haemopoiesis

In vitro assays show that GM-CSF increases the production of neutrophil, eosinophil and monocyte colonies, and under certain conditions, will promote the formation of erythroid and megakaryocytic precursors (Metcalf, 1992). GM-CSF is thought to act at the intermediate progenitor level and shows synergistic activity with early acting factors, eg. SCF (Bernstein et al, 1991), other intermediate acting factors, eg. IL-3 (Sieff et al, 1989) and with late acting factors eg Epo (Sieff et al, 1985). Low concentrations of GM-CSF act on a common bipotential precursor to preferentially produce monocyte colonies, and higher concentrations lead to the development of increased numbers of neutrophil colonies (Metcalf et al, 1985). Like the majority of HGFs, GM-CSF is not normally detectable in the circulation and probably exerts its effects locally in a paracrine fashion. In support of this locally acting hypothesis, it has been shown to bind to stromal elements in long term bone marrow cultures (Gordon et al, 1987).

The clinical administration of GM-CSF leads to striking increases in neutrophil, eosinophil and monocyte numbers, with a lesser effect on lymphocyte
counts (Ganser et al, 1989). The last is likely to be due to an increase in the production of other cytokines by accessory cells, eg macrophages primed by GM-CSF, although there have been reports that GM-CSF can act on lymphoid cells of both T and B lineages (Gasson, 1991). GM-CSF administration also leads to marked increases in circulating myeloid and erythroid progenitor cells, and this property has been utilised to increase the yield from leukopheresis in patients undergoing autologous peripheral blood progenitor cell transplants (Gianni et al, 1989).

1.2.2 Effects on mature cells

The availability of significant amounts of purified material led to the surprising observation that, in addition to its effects on haemopoietic progenitors, GM-CSF could alter the functional capabilities of mature phagocytic white blood cells (Gasson, 1991). Factors which had previously been characterised on the basis of their effects on phagocytic activity, eg. neutrophil migration inhibitory factor (NIF), were shown to be identical with GM-CSF (Gasson, 1991).

GM-CSF may exert its effect directly, eg by increasing cellular adhesion molecule expression (Arnaout et al, 1986), or it may enhance the cell's responses to subsequent stimuli, eg. by priming the neutrophil respiratory burst (Roberts et al, 1990a). However, the distinction between the two types of responses are sometimes unclear.

Although GM-CSF does not show a significant sequence homology with IL-3 or IL-5, all three HGFs exhibit surprisingly similar activities (Table 1.1).
Biological effects of GM-CSF

Blast colony formation*
Mixed cell colony formation*
GM colony formation*
Eosinophil colony formation*®

Up-regulation of CD11b/CD18
Down-regulation of LAM-1
Down-regulation of GM-CSFR
Up-regulation of fMLPR

Inhibition of migration
Priming of the respiratory burst
Enhanced direct and ADCC
Increased microbial phagocytosis/killing*
Secretion of primary and secondary granules in neutrophils
Stimulation of eosinophil degranulation*®
Stimulation of histamine release from basophils*®
Enhanced cytokine secretion
Enhanced antigen presentation

Table 1.1 The effects of GM-CSF on phagocytic cells (Neutrophils, monocyte / macrophages and eosinophils)

* IL-3 activity
® IL-5 activity
1.3 The GM-CSF Receptor

1.3.1 Structure

Early studies with murine bone marrow cells demonstrated the presence of low numbers of specific surface receptors of both low GM-CSF binding affinity ($k_D$ approximately 1 nM, 500 receptors / cell) and high affinity ($k_D$ approximately 50 pM, 50 receptors / cell) (Walker et al., 1985). Initially, human neutrophils and mature haemopoietic cells were reported to express only high affinity GM-CSFR (Gasson et al., 1986), but later investigations confirmed that whereas neutrophils have only high affinity receptors, immature cells and monocytes have both low and high affinity receptors (Park et al., 1989b; Chiba et al. 1990). GM-CSFR binding has been demonstrated in non-haemopoietic cell lines including small cell lung cancer (Baldwin et al., 1989), some COS cells (Baldwin et al., 1989), breast cancer and osteosarcoma (Dedhar et al., 1988) and colon cancer (Bardel et al., 1989). Most of this binding is of low affinity, and high affinity binding has only been demonstrated in haemopoietic cells. Using specific monoclonal antibodies (Mabs) directed against GM-CSFR $\alpha$ and $\beta$ chains, Sato et al. (1993a) showed that both $\alpha$ and $\beta$ chains are expressed in myeloid lineages, as well as early CD34+ progenitors.

The human GM-CSFR has been shown to consist of an $\alpha$ chain which specifically binds specific ligand with low affinity (Gearing et al., 1989). This binding is converted to high affinity binding when the $\alpha$ chain heterodimerizes with a $\beta$ chain (KH97) (Hayashida et al., 1990). The $\beta$ chain is shared with the specific $\alpha$ chain for IL-3R (Kitamura et al., 1991) and IL-5R (Tavernier et al., 1991; Murata et al., 1992). The sharing of a common $\beta$ chain between IL-3R, IL-5R and GM-CSFR explains the earlier mentioned finding of cross-competition between these cytokines in haemopoietic cells.
The murine GM-CSFR has also been characterised. Mouse GM-CSFR consists of a low affinity α chain (Park et al, 1992) with only 35% sequence identity with the human protein. As is the case for the human GM-CSFR, the low affinity binding α chain can be converted to high affinity binding on heterodimerization with the β chain shared with the IL-3R and IL-5R (A1C2B protein) (Devos et al, 1991; Miyajima et al, 1992). The murine IL-3R however, displays a greater degree of complexity than the human counterpart, in that it can bind to an alternative high affinity chain (A1C2A) (Gorman et al, 1992). A1C2A and A1C2B have a 95% homology and there is no clear difference between the two high affinity IL-3 receptors.

The GM-CSFRα gene is located on the pseudoautosomal region of the human X and Y chromosome, about 100 to 1300 kb from the telomeres (Gough et al, 1990). The GM-CSFRβ chain is on human chromosome 22q 12.3-13.1 (Shen et al, 1992). Whilst the genomic organisation of the human GM-CSFRα has been published, that of human GM-CSFRβ has not. The GM-CSFRα gene spans approximately 44 kb and has 13 exons (Nakagawa et al, 1994). The relationship of the gene, exon / intron, protein structure is shown in Figure 1.4.

To date at transcripts of least six isoforms of the GM-CSFRα, produced by alternative splicing at the 3' end, have been described (Figure 1.5). These include the originally isolated low affinity receptor (Gearing et al, 1989), a serine rich insertion of ten amino acids at the cytoplasmic end of the receptor (Crosier et al, 1991), two soluble forms lacking the transmembrane and intracytoplasmic regions (Raines et al, 1991; Hu et al, 1994), an isoform with a novel 62 amino acid C terminus including an alternative membrane anchoring domain (Hu et al, 1994) and finally, an isoform that contains an insertion of 34 amino acids between the WSXWS box and the transmembrane domain (Devereux et al, 1993) (Figure 3 4.
Figure 1.4 Genomic organisation of GM-CSFRα
The relationship between exon / intron boundaries and protein structure
(fibronectin-like and SD100 domains are described in Section 1.3.2)
Figure 1.5 Isoforms of the GM-CSFRα chain
1.5). These isoforms would result in an alteration of the protein, although the physiological consequences have yet to be fully elucidated.

The soluble GM-CSFRα arises from alternative splicing which excludes the exon encoding the transmembrane amino acids that anchor the membrane-bound protein to the cell surface (Raines et al, 1991; Heaney et al, 1995). Similar alternative splicing results in a soluble IL-4R, IL-5R and LIFR (the latter only found in the mouse) (Moseley et al, 1989; Takaki et al, 1990; Layton et al, 1992). Interestingly, no soluble form of the shared β chain has been described to date.

Soluble IL-5Rα and GM-CSFRα inhibit the binding of their respective ligand to isolated membrane in a dose-dependent manner (Devos et al, 1993; Brown et al, 1995). In the case of GM-CSF, this activity is biologically relevant, since the isolated α subunit may signal for increased glucose uptake (Spielholz et al, 1993). Recent evidence suggests that soluble IL-5R and GM-CSFRα may also inhibit functions mediated by their high affinity receptors and points to a mechanism of inhibition that extends beyond single competitive binding of ligand (Tsuruoka et al, 1993; Williams et al, 1994; Brown et al, 1995). Perhaps, the soluble α subunits can associate in some manner with the β chain to prevent ligand signalling.

1.3.2 Extracellular domains and ligand binding

While many haemopoietic receptors include extracellular fibronectin domains, the unifying feature of this family is the presence of one or two domains of 200 amino acids, termed D200. D200 domains may in turn be divided into subunits of 100 amino acids (SD100) which are themselves homologous (Figures 1.2 and 1.4) (Bazan, 1990). There are four well conserved cysteine residues in the N-terminal SD100 subdomains, and a unique Trp-Ser-X-Trp-Ser (WSXWS) sequence (or box) in the C-terminal module. Each of the SD100 domains contains
seven anti-parallel \( \beta \) strands arranged to form a barrel-like structure. Bazan (1991) has predicted that a binding crevice is formed between linked \( \beta \) barrel folds.

Study of the exon organisation and corresponding intron masses of the HGFR genes has allowed the following rules to be established (Nakagawa et al., 1994):

(i) Each SD100 domain is encoded by two exons, and the boundaries of the domain are defined by phase I introns (phase I introns interrupt codons after the first nucleotide).

(ii) Each N-terminal SD100 domain is interrupted by a phase 2 intron, and the C-terminal domain by a phase 0 intron (phase 2 introns interrupt the reading frame after the second nucleotide; phase 0 interrupt between codons).

Comparison of the intronic phases allows construction of an evolutionary model of the haemopoietic superfamily, and suggests that IL-3R\( \alpha \), IL-5R\( \alpha \) and GM-CSFR\( \alpha \) form a sub family. These studies have also revealed a role for exon insertion and exon duplication in the evolution of these receptors.

Other conserved domains described include the following (Doshi & DiPersio, 1994):

(i) a CXN motif comprising the second of the four conserved cysteine residues

(ii) an XW motif in the C-terminal SD100 domain

(iii) an XYFLY motif located 11 amino acids downstream from the second of the four conserved extracellular cysteines.

Mutation analysis of the above motifs and the WSXWS box, suggest that these domains are important for receptor expression (Doshi & DiPersio, 1994). Yoshimura et al. (1990) have shown that mutation of the WSXWS box affects protein folding and endoreticular transport, and therefore interferes with the cell surface receptor expression.

**Ligand binding** The binding of GM-CSF to the \( \alpha \) chain results in low affinity binding and this is converted to high affinity binding with the interaction of the \( \beta \)
chain. A mutant murine GM-CSF, with a substitution of Glu 21 with Ala, abrogates the high affinity binding to the GM-CSFR, but is still capable of transducing a growth signal at high concentrations. These results indicate that binding of the cytokine to its α subunit leads to a tight association with the β subunit, and the association between the α and β subunits seems to be the critical step for signal transduction (Lopez et al, 1992).

This has however been questioned by Ronco et al (1994) who have suggested that the GM-CSFRα and β exist in a preformed heterodimeric protein complex at the surface. This group transfected COS cells with mutant human GM-CSFRα (loss of second conserved extracytoplasmic cysteine) ± wild type GM-CSFRβ. The cells transiently transfected with mutant hGM-CSFRα did not bind ligand but transfection of both mutant α chain and wild type β chain resulted in high affinity binding. Transient expression studies should, however, be treated with caution, as the levels of GM-CSFRα and β in transiently co-transfected COS7 cells were shown to be variable and dependent on the efficacy of the transfection, and studies of GM-CSFRα in a variable background of GM-CSFRβ are difficult to interpret.

1.3.3 Intracytoplasmic domains and signal transduction
The GM-CSFRβ chain is not only responsible for high affinity binding but is essential for signal transduction in most circumstances. Cytoplasmic deletions of the intracytoplasmic tail of GM-CSFRβ identified critical domains within the β chain which are responsible for proliferation (Sakamaki et al, 1992). A membrane proximal domain of 32 amino acids was necessary for human GM-CSF signal transduction; this domain includes a highly conserved amino acid sequence common to the human β chain and the murine A1C2A and A1C2B chains, as well as EpoR (He et al, 1994), gp130 (Murakami et al, 1990) and G-CSFR (Fukunaga et al, 1990). This sequence has been termed box1. A second domain of 26 amino
acids which is important, but not essential, for growth has been termed Box2 (Figure 1.6). Box2 homology sequences have also been shown for gp130, EpoR and G-CSFR (Murakami et al, 1990; Zeigler et al, 1993; Dong et al, 1993).

Recently other homologous sequences have been described, including an extended box2 (Figure 1.6). The function of this sequence is unknown for GM-CSFRβ but in EpoR it is the site of interaction with kit (SCF receptor) (Wu et al, 1995). This leads to tyrosine phosphorylation of the EpoR and explains the synergy between SCF and Epo. Since SCF (a tyrosine kinase receptor) synergises with a number of other cytokines, it would not be surprising if a similar pattern of interaction was observed for the other members of the haemopoietin receptor family.

Whilst the proximal part of the GM-CSFRβ intracytoplasmic domain is therefore important for mitogenic signalling, the distal portion has been implicated in activation of Ras, Raf-1, MAP kinase and p70 S6 kinase (Sato et al, 1993b) (See below). The distal portion of G-CSFR has been shown to be necessary for differentiation and maturation of myeloid cells (Fukunaga et al, 1993; Dong et al 1993). Whether there is such distinct delineation between differentiation and proliferation has not been proven satisfactorily for the GM-CSFRβ.

Although the GM-CSFRα is largely extracellular, it does contain a 54 amino acid intracytoplasmic tail. Removal of this tail appears to inhibit the ability of the subunit to stimulate growth of murine cells (Sakamaki et al, 1992; Polotskaya et al, 1994), which is in contrast to IL-6, where the cytoplasmic domain is dispensable for signal transduction (Taga et al, 1989). The GM-CSFRα chain may therefore be important for cytokine-specific signals and GM-CSFRα isoforms with different intracytoplasmic tails (described earlier) give a potential for even greater complexity.

GM-CSF induces rapid tyrosine phosphorylation of intracellular proteins even though the GM-CSFR (like the rest of the haemopoietin receptor family)
Figure 1.6 Intracytoplasmic domains of GM-CSFRβ chain
lacks intrinsic tyrosine kinase activity. Recent work has attempted to elucidate the mechanism by which GM-CSF induces signals that are specific to its function, and at the same time induce signals that are common to a number of cytokines. Furthermore, it has yet to be clarified whether it is the receptor per se, or downstream signalling molecules, which are responsible for receptor-specific responses. The past two years have seen a dramatic increase in our understanding of receptor-mediated signalling events, but the central question of specificity still remains to be answered.

One common signalling pathway utilised by mitogenic cytokines is activation of Ras followed by activation of the kinase cascade Raf, MAP kinase kinase and MAP kinase, which leads to induction of various genes including c-fos (Miyajima et al, 1993). Growth factor receptors with an intrinsic tyrosine kinase activity activate Ras through the GDP/GTP exchange factor Sos which is linked to the activated receptor by the SH2-containing adaptor molecules Shc and Grb2 (Pellici et al, 1992). A deletion of the C terminal region of GM-CSFRβ (described above) simultaneously abrogates Shc phosphorylation, activation of Ras, Raf and MAP kinase and induction of c-fos/jun (Sato et al, 1993). These results indicate that HGFs activate a pathway similar to that activated by receptors with intrinsic tyrosine kinase activity.

GM-CSFRs ectopically expressed on fibroblasts or T cells (which usually do not express GM-CSFR) stimulate tyrosine kinase phosphorylation of MAP kinase, induce nuclear proto-oncogenes such as c-fos, and stimulates cell proliferation (Eder et al, 1993; Watanabe et al, 1993). This suggests that no myeloid-specific kinase is required for signal transduction by the GM-CSFR, at least for the common pathway, although the kinase that activates Ras is currently unknown.

Another link from the GM-CSFR to the nucleus is triggered by the JAK kinase family (reviewed by Darnell et al, 1994). The JAK kinase family was
initially described in the interferon signalling pathway (closely linked to the
haemopoietin receptor family and called Class II cytokine receptors). Initially
three members of the JAK kinase family were described: JAK1, JAK2 and
TYK2, and more recently JAK3. The JAKs have been of considerable interest in
haemopoiesis because all the cytokines that utilise receptors of the haemopoietin
receptor superfamily have been found to activate one or more JAKs. With the
exception of JAK3, the JAKs are expressed at comparative levels in all tissue and
cell lines. In contrast, JAK3 is predominantly expressed in myeloid and lymphoid
cells.

The JAK family of tyrosine kinases are thought to be activated by ligand-
induced dimerization (hetero or homo) of cytokine receptor chains. This brings
about local aggregation of the JAKs, resulting in their activation by cross-
phosphorylation, a mechanism analogous to the well-established system of cross-
activation of the tyrosine kinase family of receptors (Ulrich & Schlessinger,
1990). In all cases examined, cytokines induce the tyrosine phosphorylation of
JAKs at multiple sites including a tryptic peptide containing the KEYY sequence
which has been predicted to affect kinase activity (Ihle, 1995). The membrane
proximal region (containing Box 1 and Box 2) of the cytokine receptors is also
required for the activation of the JAKs (Figure 1.6). The GM-CSFRβ chain
associates with the membrane proximal region, possibly box1 (Quelle et al, 1994)
and this region is essential for proliferative signals (see above). However, it
should be noted that the activation of JAKs, whilst necessary, is not sufficient for
proliferation. This is illustrated by the ability of interferon to activate JAKs but
failure to induce mitogenic responses in a variety of cells. This has also been
illustrated by studies with EpoR mutants (He et al, 1994) which indicate that
JAK2 can be activated by a mutant that lacks the extended BOX2 region and
cannot induce mitogenic responses. Thus other substrates recruited by the
domains of the receptor may be required.
A key question is how different HGFRs activate different sets of genes through the same JAK kinase. JAK2 for example is activated by a number of different cytokines including IFNγ, IL-3, GM-CSF, Epo, IL-6, LIF, OSM and CNTF. Signal specificity is thought to be achieved via rapid transduction of signals to the nucleus and activation of specific genes. A recently identified set of transcription factors called STATs (Signal Transducer and Activator of Transcription) have been the focus of a great deal of attention, and it is thought that they may provide this specificity (Darnell et al., 1994). STAT factors are rapidly tyrosine phosphorylated by JAKs after stimulation with cytokines, and subsequently dimerize and translocate to the nucleus where they activate transcription, either directly or through other transcription factors (Figure 1.7). To date, six members of the STAT family have been identified and characterised. STATs are widely expressed in different cell types and tissues, with the exception of STAT4 which is expressed predominantly in testes and cells of haemopoietic origin. Table 1.2 summarises the STAT family members involved in different cytokine responses. It should also be noted that there is no pattern between cytokine response and the STAT used, and that more than one cytokine activates a specific STAT factor. The specificity of action may therefore result from: the presence of different STAT isoforms - eg. STAT5a and b; heterodimerization of different STAT molecules; interactions with additional proteins, such as p48, which alter DNA binding specificity (Darnell et al., 1994). It is also possible that the GM-CSF stimulated JAK2 binding to the receptor is of a higher affinity than IL-3 stimulation, and therefore results in a more potent kinase signal to activate specific STAT factors. Such differences have been shown for mpl ligand and IL-3 (Morella et al., 1995).

Although STAT molecules are undoubtedly important mediators of the JAK pathway it should be noted that there is no evidence that they are involved in proliferative responses. Since there is a correlation between JAK2 and
Figure 1.7 Signalling pathways of the GM-CSFR
proliferative responses, there must be other JAK2 substrates apart from the STAT factors. One such substrate may be MAP kinase (David et al, 1995).

There is also evidence that the GM-CSFR stimulates other pathways of signal activation. These include the following:

(i) PI3 kinase (Corey et al, 1993) which on phosphorylation activates Protein kinase B and then p70 S6 kinase, a ribosomal protein activated in response to a number of mitogens (Downward et al, 1994).

(ii) c-fps/fes, a protein tyrosine kinase found in haemopoietic cells which has been shown to be induced by GM-CSF and IL-3 and physically associated with the β chain (Hanozano et al, 1993). c-fps/fes is involved in the signal transduction of myeloid cells undergoing differentiation.

(iii) vav, a proto-oncogene that is specifically expressed in haemopoietic cells (Katzav et al, 1989) and functions both as a transcription factor and also has properties of a signal transduction molecule (Adams et al, 1992). vav has been shown to be phosphorylated by GM-CSF and at the same time regulated by JAK

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Kinase</th>
<th>STAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prolactin</td>
<td>JAK2</td>
<td>STAT5a (MGF)</td>
</tr>
<tr>
<td>Epo</td>
<td>JAK2</td>
<td>STAT5b</td>
</tr>
<tr>
<td>mpl ligand</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>G-CSF</td>
<td>JAK2 / JAK1</td>
<td>STAT3</td>
</tr>
<tr>
<td>IL-2, IL-4, IL-7, IL-9, IL-12</td>
<td>JAK3 / JAK1</td>
<td>STAT5b / STAT3 / STAT6</td>
</tr>
<tr>
<td>IL-6, CNTF, LIF, IL-11</td>
<td>JAK1 / JAK2 / Tyk2</td>
<td>STAT1 / STAT3</td>
</tr>
<tr>
<td>IL-3, IL-5, GM-CSF</td>
<td>JAK2</td>
<td>STAT5a / STAT5b</td>
</tr>
</tbody>
</table>

Table 1.2 JAKS and STATS in cytokine signalling
kinases. Thus this pathway may be as important as the STAT pathway and requires further investigation (Matsuguchi et al, 1995).

In addition to signal activation, inactivators of the GM-CSFR signalling pathway have been identified. JAK kinases have been shown to be inactivated by the phosphatase SH/PTP1 (Ihle, 1995). SH/PTP1 has been shown to attach to the distal portion of the EpoR and inactivate JAK2 (Klingmuller et al, 1995). The consequences of recruitment of SH/PTP1 to the cytokine-receptor complex were initially recognised from the phenotype of moth-eaten mice in which the SH/PTP1 gene is functionally disrupted and results in the proliferation of a variety of haemopoietic cells (Shultz et al, 1993). Consistent with this, deletion of the SH/PTP1 binding sites results in increased sensitivity of the EpoR to Epo and constitutive activation of JAK2 (Klingmuller et al, 1995). The potential site of action of SH/PTP1 (as correlated from the EpoR) in the β chain is shown in Figure 1.7. Recently an adaptor-like molecule analogous to Grb2 has been shown to attach to the proximal domain of the GM-CSFRβ on ligand binding, and results in decreased proliferation (Yoshimura et al, 1995). The exact regulatory role of this CIS protein is not known and further emphasises the complex array of molecules that interact with the GM-CSFR.

1.4 GM-CSFR and Leukaemogenesis

The manner in which HGFs modulate leukaemogenesis and how they may play a role in the pathogenesis of this process has been the focus of a number of studies (Lowenberg & Touw, 1993). Autocrine mechanisms of growth may render neoplasms independent of exogenous growth factor stimulation, thereby removing one normal mechanism of growth control. In experimental models, autocrine growth factor stimulation can be a critical step in myeloid
leukaemogenesis. The main mechanism for autocrine loops in AML is the extracellular secretion of HGFs including GM-CSF, G-CSF, M-CSF, IL-1 and IL-3 (Lowenberg & Touw, 1993). Neutralising antibodies generally inhibit the actions of these cytokines, and therefore abrogate the autocrine loop (Young et al, 1988). However, some AML blasts that show characteristics of autonomous growth are not inhibited by neutralising antibodies but are inhibited by anti-sense oligonucleotides directed against the specific cytokine. These results suggest the existence of an intracellular autocrine loop, the mechanism of which has not been elucidated (Rogers et al, 1994).

Transgenic mice expressing the GM-CSF cDNA, as a result of retroviral transfer, develop myeloproliferative syndromes but not acute leukaemia (Johnson et al, 1989). These findings suggest that autocrine stimulation is not sufficient to induce leukaemia, and must be accompanied by other genetic events that block differentiation. The extent to which autocrine loops are of any significance in AML is unknown. AML blasts generally require HGFs for survival and only a proportion are capable of autonomous growth (Lowenberg & Touw, 1993). The proportion of factor independent AML cells varies considerably from study to study (from as low as 10% to greater than 70%) (Griffin & Lowenberg 1986; Begley et al, 1987; Young et al, 1988; Salem et al, 1989; Lowenberg et al, 1993; Rogers et al, 1994). There are a number of technical considerations which may in part explain these discrepancies. While leukaemic samples usually contain a large proportion of leukaemic cells (>70%) the remaining normal cell component may contribute to the assay by synthesising and releasing HGFs capable of stimulating the leukaemic cells to proliferate, hence giving false positive results. In addition, in order to prove autocrine HGF secretion, 'factor free conditions' should be employed. While serum free media were used in a few of these studies (Lowenberg et al, 1988; Salem et al, 1989), the majority have included fetal calf serum or Chinese hamster ovary (CHO) or COS cell conditioned medium (Griffin
et al, 1986; Begley et al, 1987; Young et al, 1988; Rogers et al, 1994), either of which may contain cross-species reactive factors capable of supporting blast growth in vitro.

If autocrine mechanisms represent a minor mechanism for uncontrolled growth, other potential mechanisms could include increased receptor expression and altered receptor affinities. There is no evidence, however, to suggest that HGF receptor numbers or affinities are altered in AML. In most cases of AML studied, GM-CSFR and G-CSFR numbers are low, with both high and low affinity binding sites present (Lowenberg & Touw, 1993). It should, however, be noted that in some AML patients, the GM-CSFR may be resistant to ligand mediated down-regulation (Cannistra et al, 1990).

Certain viral oncogenes encode for truncated (transforming forms) of HGFrs. Consequently, a possible role for mutations in these receptors in the development of human leukaemia has been widely considered. The cellular counterpart of the cellular oncogene v-fms encodes for M-CSFR. However, although certain c-fms mutations have been shown to be leukaemogenic in mice (Sherr, 1990), there is no evidence of c-fms mutations being pathogenic in AML or MDS in humans. In more than 200 patients investigated, the few mutations identified have either found to be the result of polymorphisms, or are non-transforming in nature (Ridge et al, 1990; Roussel et al, 1990; Tobal et al, 1990). Similarly, mutational analysis of c-kit has not proven fruitful (Wang et al, 1989). More recently, the GM-CSFRα and β chains have been investigated in AML patients. Restriction fragment length polymorphism analysis did not show any gross rearrangements in the α or β genes (Bardy et al, 1992; Brown et al, 1993). Single strand conformation polymorphism (SCCP) analysis of 32 patients identified four point mutations in the GM-CSFRα cDNA, but these were shown to be polymorphisms (Wagner et al, 1994). Similar SCCP analysis of the β chain has failed to detect any significant mutations in AML or Juvenile chronic myeloid
leukaemia (JCML) (Freeburn et al, 1995a and b). Further study and elucidation of the mechanisms for regulation of GM-CSFR are necessary in order to establish whether the receptor is involved in leukaemogenesis.

1.5 Possible modes of regulation of GM-CSFR expression

The expression of the GM-CSFR is generally restricted to myeloid cells and must therefore be regulated in a cell-specific manner. Murine embryonic stem cells express neither the IL-3Rβ or the β common chain under culture conditions that maintain totipotency. Dramatic up-regulation of the two β subunit genes occurs at day 6 or 7 in culture conditions that allow differentiation of the stem cells to various lineages (Schmidt et al, 1991; Keller et al, 1993). Similarly, blastocysts at day 3 or 4 expressed no detectable β subunit mRNA (measured by reverse transcriptase polymerase chain reaction, RT-PCR) and induction of the β subunit occurred after 7 to 9 days culture in vitro, which is consistent with the time for blood island formation in the mouse (McClanahan et al, 1993). Thus the temporal pattern of the β subunit expression may serve as an excellent marker for the commitment to the haemopoietic lineage. Surprisingly, in contrast to the β subunits, expression of GM-CSFRα mRNA, as well as IL-3Rα mRNA, was detectable in embryonic stem cells, as well as day 3 or 4 blastocysts. However, in the model their expression in differentiated cells is restricted to haemopoietic cells, which would suggest the presence of a complicated regulatory mechanism for expression of these subunits.

Like other genes, expression of the GM-CSFR genes may be regulated at different levels. The potential regulatory mechanisms for any gene including haemopoietin receptor genes are summarised in Figure 1.8. Several mechanisms of gene regulation may be utilised and each individual mechanism will be
Figure 1.8 Schematic representation of possible mechanisms for regulation of GM-CSFR
discussed where possible with reference to GM-CSFR. However, there has been limited study of regulation of GM-CSFR gene expression and, where necessary, examples of other haemopoietic receptors or eukaryotic proteins will be used to illustrate a process.

### 1.5.1 Transcriptional regulation

Transcription involves the assembly of an RNA molecule from ribonucleotides, using a DNA template, by RNA polymerase I, II or III. In eukaryotes, the role of RNA polymerase II has been most extensively studied (reviewed in Latchman, 1991). For initiation of transcription, a multi-component stable transcriptional complex is necessary (summarised in Figure 1.9). The transcription complex requires binding of a transcription factor and an assembly factor with RNA polymerase. Constitutive transcription requires a TATA box (an AT-rich consensus sequence TATAA/TAT/T) which is found about 30 bp upstream from the transcriptional start site in most, but not all, genes (Goodwin et al, 1990).

This sequence binds RNA polymerase II and the assembly factor TFII. Other promoter regions found in association with the TATA box include: (i) a CCAAT box found upstream of the TATA box; (ii) an SpI box which has a consensus sequence CGGCGG.

The nucleotide sequence flanking the 5' end of the human GM-CSFRα gene has been published, whereas that for the human β chain has not (Nakagawa et al, 1994). The putative promoter region does not contain a typical TATA motif or an SpI binding site. This 'TATA-less' feature appears to be common for genes encoding other members of the haemopoietin receptor family including the human IL-2Rγ, the human G-CSF and the murine IL-3Rα (Nakagawa et al, 1994). Lack of a TATA box has also been demonstrated in some regulatory genes such as lck (Garvin et al, 1988), and the function of the TATA box can be substituted by a 17
DNA

RNA polymerase

Protein factors

Assembly inhibited by low detergent concentration or competing DNA

Complex not inhibited by low detergent concentration or competing DNA

Assembly factor can dissociate

Repeated rounds of transcription

RNA transcripts

Figure 1.9 Transcription is regulated by a multi-component complex
bp Inr element (initiator element) that contains the necessary information for basal transcription (Weiss & Reinberg, 1992). The human GM-CSFRα has such an Inr sequence and one may speculate as to whether basal transcription of the GM-CSFRα chain under certain circumstances is not a regulated process.

Nakagawa et al (1994) have also shown that there is a striking homology between the IL-2Rγ, G-CSFR and GM-CSFRα at the 5' end of the gene, and it has been suggested that these genes may have similar mechanisms of basal transcription. The upstream region of the GM-CSFRα also contains potential binding sites for NF-IL6, a basic helix-loop-helix transcriptional factor. NF-IL6 was initially described as a nuclear target of gp130 signalling via MAP kinase (Kishimoto et al, 1995), and since GM-CSF also activates MAP kinase, this may be a potential target for the GM-CSF activated MAP kinase pathway. The upstream region also contains a consensus binding sequence for PU.1, an ets family transcription factor principally expressed in myeloid cells. The PU.1 GAGGAA sequence is frequently found in genes expressed in myeloid cells and is thought to be important for myeloid differentiation (Moreau-Gachelin et al, 1994). Studies in the U7 cell line suggest that the transcription factor (GATA-1) modulates EpoR expression. Whether the PU.1 transcription factor will prove to be an important regulator of the GM-CSFR remains to be seen.

1.5.2 RNA processing

After the synthesis of specific mRNA, it is processed in the nucleus (summarised in Figure 1.10). Heteronuclear RNA, or pre mRNA, contains both exon and intron sequences. Before RNA can be transported from the nucleus to the cytoplasm, RNA processing appears to be necessary (reviewed by Lamond, 1991). A 5' terminal cap structure containing a mono-methylated terminal G residue (m7GpppG) apparently facilitates the export of all RNA polymerase II transcripts from the nucleus. 3' addition of poly-A RNA is also important.
Figure 1.10 Mechanisms of RNA processing
most cases, the removal of introns by splicing is also necessary for nuclear export
of RNA.

Since a number of isoforms of the GM-CSFR and other members of the
haemopoietin receptor family have been described, alternative splicing and
regulation of the splicing machinery must play an important role in the post
transcriptional control of the receptor (Figure 1.5).

So far it has been shown that only three sequences are required for
splicing:

(i) The 5' donor sequence with a consensus of (AG/GURAGU) at the exon-
    intron boundary
(ii) 3' recipient sequence AG/G at the exon-intron boundary
(iii) Branch site sequence CACUGAC usually found 30 nucleotides 5' of the
    3' end of the intron (Figure 1.11).

Recognition of the splice sites occurs by a complex of trans-acting RNA
molecules found in the nucleus - small nuclear RNAs (snRNA) and ribonuclear
proteins called snRNPs. Splicing is regulated by a complex of snRNPs U1 to U6
and a large number of additional individual factors which are not fully described.

Just as splicing of RNA is tightly regulated, alternative splicing which involves
the differential use of splicing functions, often in a cell-specific manner, would
also require a similarly highly regulated mechanism. This is discussed further in
Chapter 4.

Once RNA is processed, it is exported from the nucleus to the cytoplasm
and is chaperoned by ribonuclear proteins which protect it from RNase
degradation. How this process is modulated in higher prokaryotes and what
signals control preferential transport of one RNA transcript over another, are not
clearly understood. Recently, viral proteins such as Rev protein of HIV-I have
been shown to modulate preferential export of viral mRNAs as opposed to host
mRNAs (reviewed by Krug et al, 1993).
Figure 1.11 Mechanisms and nucleotide sequences involved in RNA splicing
1.5.3 RNA stability and degradation

Once the RNA has reached the cytoplasm, the rate of production of the protein that it encodes can also be regulated. The mRNA may be sequestered away from the ribosome so that it is not translated. The efficiency with which it is translated can vary, and finally the rate at which the message is degraded can be controlled.

The control of mRNA stability has received a great deal of attention recently. This interest was generated as a result of the original observation by Shaw and Kamen (1986) that AU rich sequences were common in the 3' untranslated regions (UTR) of mRNA encoding certain lymphokines, cytokines and oncogenes. Shaw and Kamen (1986) demonstrated that the 7 AU rich sequences in the 3' UTR of GM-CSF mRNA were capable of decreasing the half-life of normally stable β globin mRNA to less than 30 minutes. Such a role for AU sequences in regulation of mRNA stability, and hence expression, has also been demonstrated in studies of the oncogene c-fos (Decker & Parker, 1994). Similarly, sequences in the 3' UTR of the transferrin receptor have been found to modulate its mRNA stability, in response to cellular iron stores (see below). The AUUA elements and the iron response element formed in the 3' UTR of GM-CSF and transferrin are thought to promote degradation of the poly A tail which in turn leads to rapid endonuclease cleavage of the mRNA (Decker & Parker, 1994).

1.5.4. Translation

The steps involved in translation are catalysed by eukaryotic initiation factors (eIFs), of which eIF 2 and 4 are best characterised. Translation starts with the dissociation of an 80S ribosome into 40S and 60S subunits (Figure 1.12). The 40S subunit attaches to met-tRNA and phosphorylated eIF2 to form a pre-initiation complex. The pre-initiation complex then binds at, or near, the m7GpppG 5' cap structure with another initiation factor eIF4. The complex then scans the 5' untranslated region of the mRNA for an AUG start codon which is in
good context and joins the 60 S at this AUG start site. Translation may now begin. Scanning of the 5' UTR is therefore an important step in translation initiation. Elements in the 5' untranslated leader sequence can therefore modulate translation and may be one of the sites for post-transcriptional regulation of gene expression (Kozak, 1991a). Although the scanning model is the commonly utilised mechanism for eukaryotic translation, internal ribosomal entry initially described in picornaviruses and recently for some eukaryotic proteins is an alternative means of translation (McBratney et al, 1993).

Apart from the elongation and initiation factors shown in Figure 1.12, translation can also be regulated by trans-acting proteins interacting with cis RNA sequences. Perhaps the best characterised are the mRNAs whose protein products are involved in iron metabolism, (Melefors & Hentze, 1993). The mRNAs encoding ferritin heavy and light chains, erythroid 5-aminolevulinate synthase (ALA) and transferrin receptor (TfR) contain stem-loop structures which function as iron responsive elements (IREs), and to which regulatory protein (IRE-BP) binds when cells are depleted of iron. There have been reports of translational control of other eukaryotic proteins involving RNA-protein interaction, in particular p70 S6 kinase (Downward, 1994) which is an important protein involved in mitogenic signalling.

Another level of translational control of the GM-CSFRα may be the short 22 codon open reading frame (ORF) prior to the main ORF. Interestingly, such short ORFs are found in the 5' UTR of other members of the haemopoietin receptor family (Gearing et al, 1989) and might act, if translated, to depress the translation of the main receptor coding regions. Such a mechanism has been described in the yeast GCN4 system (Gabelle & Morris, 1994).
Figure 1.12 Schematic representation of the initiation of eukaryotic translation
1.5.5 Post-translational modification
Both the GM-CSFR α and β chains are glycosylated (Gearing et al, 1989; Hayashida et al, 1991). Traditionally it has been thought that the signal peptide leader sequence allows trafficking through the endoplasmic reticulum (ER) and allows its insertion in to the cell surface. However, recently, lipidation of receptor proteins has been shown to be an important regulatory mechanism in directing proteins to the cell membrane. Such lipidation occurs for the G receptors and Ras proteins (Casey, 1995), but whether it plays a role in the haemopoietin receptor superfamily remains to be elucidated.

1.5.6 Regulation of receptor at the cell surface
On binding ligand, both low and high affinity GM-CSFR binding sites are internalised (Walker & Burgess, 1985; Khwaja et al, 1993). If GM-CSF is then removed, the receptor is re-expressed. Internalisation has been shown to down-regulate the cellular responses to GM-CSF, since inhibition of internalisation results in continued GM-CSF mediated response in neutrophils (Khwaja et al, 1990). Down-regulation of the GM-CSFR can also occur in the presence of IL-3 (Walker et al, 1985; Kuwaki et al, 1989; Khwaja et al, 1993). This down-regulation not only affects high affinity binding, as would be expected from a shared β common chain, but also affects the low affinity α chain. The mechanism of such trans down-modulation of the α chain still remains to be elucidated. Re-expression of the receptor after ligand-induced down-regulation, may be due to new protein synthesis, or receptor recycling. Burgess and Walker (1985) have suggested that both resynthesis and recycling of the GM-CSFR takes place in a murine myelomonocytic cell line. However, there have been no reports of the rate-limiting step involved in the re-expression of the human receptor, and it is not known whether GM-CSFR α and β chains are distinctly regulated.
1.6 Aims

The aim of this study was to investigate the expression and regulation of the GM-CSF receptor α and β chains, both at the mRNA and protein levels, in order to evaluate the critical steps involved. This would give a better insight into the mode of action of GM-CSF and how its receptor may be potentially dysregulated in pathological states such as acute myeloid leukaemia.

Standard radioligand binding assays were used for measurement of protein expression. In order to obtain optimal measurement of mRNA levels, sensitive RNAse protection assays of GM-CSFRα or β mRNA were established (Chapter 3). Using the RNAse protection assay, initial focus was on the 5' UTR of GM-CSFRα. Two alternatively spliced 5' UTR isoforms of the GM-CSFRα chain were isolated and their sequence characterised (Chapter 4). Functional evaluation of these isoforms was performed using in vitro translation assays (Chapter 5).

Regulation of GM-CSFR was investigated in relation to long term haemopoietic differentiation and proliferation, and also more rapid modulation. These aims were approached using two model systems: (i) In HL-60 cells undergoing DMSO-induced differentiation over five days and; (ii) TF-1 cells on whose cells surface GM-CSFR was modulated over a time course of hours, by incubating the cells either in medium containing GM-CSF (resulting in down-modulation) or medium containing Epo (resulting in up-regulation) (Chapter 6).
— CHAPTER 2 —

GENERAL METHODS
2.1 Selection and culture of *Eschericia coli*.

**Reagents** All reagents were from Merck Ltd (Poole, Dorset) unless otherwise stated.

* LB (Luria-Bertani) medium -(2% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract in 20 mM NaCl) (Difco Laboratories, Michigan, USA) - autoclaved.

* Bacto agar (Difco Laboratories).

* Ampicillin (Sigma Chemical Co) - stock: 10 g/l; working concentration: 50 mg/l in agar plates and 25 mg/l in broth.

* Kanamycin (Sigma Chemical Co) - stock: 50 g/l; working concentration: 50 mg/l in agar plates and 25 mg/l in broth).

* 5-Bromo-4-chloro-3-indoyl -B-D-galactoside (X-Gal, Promega, WI, USA) - stock: 50 g/l in dimethylformamide.

* Isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega) - stock: 10 mM.

* JM109 *E. coli* bacterial strain (Laclq+, Promega).

**Methods** For preparation of agar plates, 15 g of agar were added to 1 litre of LB medium in a glass flask and boiled in a microwave oven. The flask was then transferred to a water bath at 55°C and allowed to cool before addition of selection antibiotics (kanamycin or ampicillin). Approximately 20 ml of melted agar were then poured into sterile petri dishes on a level surface. After the plates were set, the surface of the agar was dried by opening the plates and placing them, medium surface down, in a 37°C oven. Twenty µl of the stock X-gal and 10 µl of IPTG were then spread across the agar surface. These were allowed to diffuse into the plates for at least an hour before use. Twenty five to 100 µl of transformed JM109 *E. coli* were then spread on the agar plates and incubated overnight at 37°C.

Small scale broth cultures of *E. coli* were prepared in 5 ml LB medium (containing the appropriate antibiotic selection) in 10 ml polypropylene tubes. The
LB medium was inoculated with a single colony isolated from the agar plates. Large scale broth cultures were carried out in 500 ml of LB medium with appropriate antibiotic selection in 2 litre conical flasks. All broth cultures were incubated at 37°C in a rotary shaker incubator at 225 rpm for at least 12 - 15 hours.
2.2 Cell culture of established cell lines

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

* Recombinant human GM-CSF - Produced in *E. Coli* (Behringwerke, Marburg, Germany).
* Recombinant Epo - Produced in CHO cells (Boehringer Mannheim, UK).
* RPMI 1640 + L glutamine (Gibco-BRL, Uxbridge, Middlesex).
* Fetal Calf Serum (FCS) (ICN, Thame, Oxfordshire).
* Trypsin EDTA solution (Gibco-BRL).
* The following established cell lines were used:

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>TISSUE OF ORIGIN</th>
</tr>
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<tbody>
<tr>
<td>HL-60</td>
<td>Acute promyelocytic leukaemia (Collins et al, 1977)</td>
</tr>
<tr>
<td>U937</td>
<td>Histiocytic lymphoma (Sundstrom et al, 1976)</td>
</tr>
<tr>
<td>TF-1</td>
<td>Erythroleukaemia (Kitamura et al, 1989)</td>
</tr>
<tr>
<td>Daudi</td>
<td>B cell lymphoma (Silverman et al, 1982)</td>
</tr>
<tr>
<td>K562</td>
<td>CML blast transformation (Lozzio et al, 1975)</td>
</tr>
<tr>
<td>JAR</td>
<td>Choriocarcinoma (Patillo et al, 1971)</td>
</tr>
</tbody>
</table>

**Method** All cell culture studies were performed in tissue culture flasks and 96 well flat bottomed culture plates (Becton Dickinson Ltd, Cowley, Oxford). Cell lines were grown in RPMI 1640 containing 10% (v/v) heat inactivated FCS, at 37°C in a 5% CO₂ humidified atmosphere. Antibiotics were not added to the cultured cells. TF-1 cells were routinely grown in 5 ng/ml of GM-CSF unless otherwise stated. HL-60, U937, K562 and Daudi cells were cultured in the absence of growth factors. JAR cells are adherent and cells were passaged after removal with trypsin-EDTA.
2.3 Isolation of haemopoietic cells from whole blood

Reagents  All reagents were from Merck Ltd unless otherwise stated.

* Recombinant human GM-CSF - Produced in E. Coli (Behringwerke, Marburg, Germany).
* Recombinant Epo - Produced in CHO cells (Boehringer Mannheim).
* RPMI 1640 + L glutamine (Gibco-BRL).
* Fetal calf serum (FCS) (ICN).
* Ficoll Histopaque (Sigma Chemical Co, Poole, Dorset).
* Lymphoprep (Nycomed, Oslo Norway).
* Dextran (Pharmacia Biotech, Uppsala, Sweden).
* Nycodenz gradient (Nycodenz ©Monocytes, Nycomed).
* Heparin - (Monoparin, CP Pharmaceutical Ltd, Wrexham, UK).
* Phosphate buffered saline (PBS) - 0.01 M phosphate, 0.145 M NaCl, pH 7.4.
* EDTA - 0.1 M in distilled water.
* Phytohaemagglutinin (Sigma Chemical Co).

Method  Blood samples were obtained from healthy human subjects. Venous blood was collected into heparin (12 IU/ml, final concentration) and processed without delay. The following protocols were used for isolation of the different haemopoietic cell populations from whole blood.

NEUTROPHILS were separated from whole blood using a Ficoll-Histopaque based gradient technique (Khwaja et al, 1990). Dextran was added to whole blood (10% (w/v) final concentration) which was then allowed to stand at room temperature for 15 - 30 minutes, in order to allow the red cells to sediment. Five ml of the leucocyte-rich supernatant was then removed and layered onto 3 ml of a gradient consisting of Histopaque and Lymphoprep (1:1) in a polypropylene centrifuge tube. This was then centrifuged at 700 x g for 30 minutes. After this
time, the neutrophil containing pellet was washed in PBS. Any contaminating red cells were removed by a hypotonic lysis procedure in which 4 mls of 0.25% (v/v) PBS in distilled water were added to the leukocyte pellet and mixed for 30 seconds before addition of 4 mls 1.55% (w/v) NaCl solution. The tube was then topped up to 50 ml with PBS and the cells were washed once more. The cells were finally resuspended in RPMI containing 2% (v/v) FCS. Neutrophils isolated by this method were shown to be >95% pure by Romanowsky staining.

MONOCYTES were prepared using a density gradient separation method (Boyum, 1983). Blood was processed as reported above, but after isolation using Nycodenz gradient, the mononuclear cell layer (containing monocytes, lymphocytes and some contaminating neutrophils) was added to 45 ml RPMI 1640 containing 10% (v/v) FCS in a polypropylene centrifuge tube. The mononuclear cell rich suspension was centrifuged at 600 x g for 10 minutes and the supernatant was then removed and discarded. The cells were washed once again in RPMI/10% FCS and then 2 mls of the washed suspension were layered onto sterile 16 cm petri dishes (Grenier Labortechnik, Dursley, UK). After incubation for 2 hours at 37°C, the supernatant, containing lymphocytes and other non-adherent cells, was decanted off. The adherent monocytes were then gently scraped off with a sterile cell scraper (Grenier Labortechnik). The cells were then washed twice in RPMI containing 10% (v/v) EDTA and 10% (v/v) FCS, using a centrifugation protocol of 50 x g for 10 minutes, in order to remove any contaminating platelets. The monocytes were finally resuspended in this solution.

T-LYMPHOCYTES were isolated using the method described by Cantrell et al (1984). Non-adherent mononuclear leukocytes were isolated using the technique reported above. The cells were then activated with Phytohaemagglutinin (1 g/l, final concentration) for 3 days. After this time, the cells were washed three times with RPMI/10% FCS, and finally resuspended in RPMI/10% FCS containing IL-
2 (20 μg/l, final concentration). The cells were grown for 4 days and passaged twice with fresh RPMI/10% FCS after every 48 hours. The cells remaining after 4 days of incubation were >95% pure T lymphocytes.
2.4 Electrophoresis Techniques

2.4.1. Agarose gel electrophoresis

Reagents  All reagents were from Merck Ltd unless otherwise stated.

* Agarose (Sigma Chemical Co),
* Stock Tris borate EDTA (TBE) buffer, pH 8.0 - 0.45 M Tris, 0.45 M boric acid, 0.01 M disodium EDTA. Diluted 1 in 10 in distilled water for use.
* Ethidium bromide (Biorad Ltd, Hemel Hempstead, Herts.) - 10 g/l in distilled water.
* Stock loading buffer - 0.25% (v/v) bromophenol blue, 40% (w/v) sucrose in water. Diluted 1 in 6 in distilled water for use.
* DNA size standards (Gibco BRL) - 1 Kb marker.

Method  For preparation of 1% (w/v) agarose, 2 g of agarose was added to 200 ml TBE buffer in a glass container and the resultant slurry was boiled in a microwave oven for 2 minutes. After this time, the gel was allowed to cool and then 2.5 μl ethidium bromide were added and mixed thoroughly. Where different agarose gel densities were required the amount of agarose added to buffer was adjusted accordingly.

A sample application comb was appropriately positioned on the gel tray of an electrophoresis unit (Minigel Electrophoresis system, BRL) and the agarose gel was then gently poured onto the gel tray and allowed to set at room temperature. After this had completely set, the sample comb was removed and the buffer reservoirs were filled with enough TBE buffer to cover the agarose gel by approximately 1 mm.

The DNA samples for analysis were mixed with loading buffer and then 10 - 20 μl of the mixture were gently loaded into the sample wells. An appropriate DNA size standard was also prepared in loading buffer and applied to the gel. Electrophoresis was carried out at a constant current of 65 - 80 mA until the
bromophenol blue dye front was within approximately 2 cm of the anodal gel edge.

After electrophoresis, the gel was examined under ultra-violet light and then photographed.

2.4.2 Low Melting Point (LMP) Agarose Gel Electrophoresis and DNA Recovery

Reagents All reagents were from Merck Ltd unless otherwise stated.

* LMP Agarose (Gibco-BRL).
* Stock Tris acetate EDTA (TAE) buffer, pH 8.0 - 2 M Tris, 1 M glacial acetic acid, 0.05 M EDTA. Diluted 1 in 50 in distilled water for use.
* Ethidium bromide (Biorad Ltd) - 10 g/l in distilled water.
* Stock loading buffer - 0.25% (v/v) bromophenol blue, 40% (w/v) sucrose in water. Diluted 1 in 6 in distilled water for use.
* DNA size standards (Gibco-BRL) - 1 Kb marker.
* DNA Purification kit (US Biochemicals, Ohio, USA).

Method LMP agarose gel was added to TAE buffer and prepared in the same way as standard agarose gel (Section 2.4.1). Electrophoresis in TAE buffer was performed using the procedure reported in Section 2.4.1.

After completion of electrophoresis, the band of interest was identified under UV light and excised using a sharp scalpel blade. This agarose was transferred to a microcentrifuge tube and weighed in order to estimate volume (1 ml = 1 g). The DNA was then extracted from the agarose using the DNA purification kit. In this procedure, 3 volumes of the salt solution supplied were added to 1 volume of agarose, and this was then incubated at 55°C for 5 minutes in order to allow the agarose to dissolve. Five volumes of the DNA binding matrix were then added to the agarose / DNA mixture and mixed thoroughly. This was then incubated for 5 minutes while mixing regularly in order to keep the matrix in suspension. After
this time, the mixture was centrifuged at 10,000 x g to pellet the matrix and the supernatant was removed. The pellet was then washed using washing buffer provided and the DNA was then eluted from the matrix by resuspension in TAE buffer and incubation at 55°C for 5 minutes. Finally, the matrix was again pelleted and the supernatant containing the DNA removed.

2.4.3 Polyacrylamide gel electrophoresis (PAGE) for sequencing studies and RNase protection assays

Reagents All reagents were from Merck Ltd unless otherwise stated.
* Stock Tris borate EDTA (TBE) buffer, pH 8.0 - 0.45 M Tris, 0.45 M boric acid, 0.01 M disodium EDTA. Diluted 1 in 10, or 1 in 20, in distilled water for use.
* 40% stock acrylamide - N,N'-methylenebisacrylamide (19:1) - Acrylogel 5 solution.
* Urea.
* Ammonium persulphate (Biorad Ltd) - 10% (w/v) in distilled water (prepared immediately before use).
* N,N,N',N'-Tetramethylethylenediamine (TEMED) (Biorad Ltd).
* Stock loading buffer - 80% (v/v) formamide, 0.1% (v/v) xylene cyanol, 0.1% (v/v) Bromophenol Blue, in 2 mM EDTA.
* DNA size standards (Gibco-BRL) - 1 Kb marker.

Method For preparation of a 6% acrylamide / 8M urea gel, 46 g urea were added to 15 ml of stock acrylamide solution. 20 ml of stock TBE buffer were then added and the volume was made up to 100 ml with distilled water. 100 µl of TEMED were added to this solution and finally, just before pouring, 800 µl ammonium persulphate were added.

Electrophoresis was performed using The BRL Sequencing System (BRL). Prepared gel was poured into the pre-assembled glass plates which were separated
by wedge spacers (0.25 - 1.2 mm), and a sample well comb was immediately positioned in the gel. The gel was then allowed to polymerise at room temperature for approximately 1 hour. After this time, the sample well comb was carefully removed and the wells were washed out with water. The formed wells were then gently filled with TBE buffer and this buffer was also used to fill the buffer reservoirs of the electrophoresis unit.

$^{32}$P labelled nucleic acid samples and size standards were prepared in loading buffer, denatured by boiling for 5 minutes and then carefully applied to the sample wells of the gel using disposable pipette tips. Electrophoresis was carried out using a fixed voltage of 1200 V until the bromophenol blue dye front reached the anodal edge of the gel.

The gel was then vacuum dried for 45 minutes and exposed to X-ray film overnight at -80°C using an intensifying screen.

### 2.4.4 Polyacrylamide gel electrophoresis (PAGE) of in vitro translated protein

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

* Stock 30% acrylamide / bis (37.5:1) (Boehringer Mannheim).
* Sodium dodecyl sulphate (SDS) (ICN).
* Stock separating gel buffer, pH 8.8 - 1.5 M Tris, 0.4% (w/v) SDS.
* Stock stacking gel buffer, pH 6.8 - 0.5 M Tris, 0.4% (w/v) SDS.
* Electrophoresis buffer - 0.025 M Tris, 0.2 M glycine, 0.1% (w/v) SDS.
* Ammonium persulphate (Sigma Chemical Co) -10% (w/v) in distilled water.
* N,N,N',N'-Tetramethylethlenediamine (TEMED) (Sigma Chemical Co).
* Loading buffer - Stacking gel buffer (stock solution) containing 20%(v/v) glycerol, 20% (v/v) SDS, 5% (v/v) β-mercaptoethanol and 2.5% (w/v) Bromophenol Blue.
Method For preparation of 20% acrylamide gel, 3.8 ml stock separating gel buffer and 1.2 ml distilled water were added to 10 ml of stock acrylamide solution. 7.5 µl of TEMED were added to this solution and finally, just before pouring, 150 µl ammonium persulphate were added.

The separating gel was then poured into the pre-assembled cassette of a Biorad Minigel system (Biorad Ltd), leaving sufficient space above for the stacking gel to be added later. An overlay of butanol was immediately pipetted over the gel surface and the gel was left to polymerise at room temperature for approximately 45 minutes. After this time, the butanol was removed and the gel surface was rinsed with distilled water.

For preparation of 5% stacking gel, 750 µl of stock acrylamide solution and 1.2 ml stock stacking gel buffer were added to 2.5 ml distilled water. 5.0 µl of TEMED were added to this solution and finally, just before pouring, 40 µl ammonium persulphate were added.

The stacking gel was then poured onto the surface of the 20% gel and a sample well comb was immediately positioned in the gel. The gel was then left to polymerise at room temperature for approximately 30 minutes. After this time the comb was gently removed and the sample wells rinsed with distilled water. The formed wells were then gently filled with electrophoresis buffer and the gel unit was then transferred into the electrophoresis tank. The remaining buffer was then used to fill the buffer reservoirs of the electrophoresis unit.

Protein samples and molecular weight markers were prepared in loading buffer and boiled for 5 minutes before being carefully applied to the sample wells of the gel using disposable pipette tips. Electrophoresis was carried out, using a constant current of 30 mA, until the bromophenol blue dye front reached the anodal edge of the gel.
The gel was then vacuum dried for 45 minutes and exposed to X-ray film overnight at -80°C using an intensifying screen. The gel was also exposed to a phosphorimaging plate (Fuji Photocompany Ltd, Japan).
2.5 DNA and RNA Extraction

2.5.1 Plasmid DNA - small scale preparation

**Reagents**  All reagents were from Merck Ltd unless otherwise stated.

* Tris buffer with glucose and EDTA, pH 8.0 - 25 mM Tris, 50 mM glucose, 10 mM EDTA (autoclaved)

* Alkali solution - 0.2 N sodium hydroxide containing 10% (w/v) SDS

* Sodium acetate solution, pH 5.2 - 3 M in distilled water

* Tris EDTA (TE) buffer, pH 8.0 - 10 mM Tris, 1 mM EDTA

* 'Phenol-chloroform' - 5 ml of the lower phase of a 10 ml aliquot of phenol and 5 ml 24 : 1 (v/v) chloroform : isoamyl alcohol.

* Isopropanol

* Ethanol - 70% (v/v)

**Method**  Mini-preparations of plasmid DNA were prepared using an alkali lysis method (Sambrook & Maniatis, 1989). 1.5 ml of a bacterial culture, prepared from a single bacterial colony grown up overnight, were placed in a microfuge tube and centrifuged for 30 seconds at 12,000 x g and 4°C. The medium was then pipetted off and the pellet resuspended in 100 μl ice cold Tris buffer with glucose and EDTA, by vigorous vortexing. 200 μl of alkali solution were then added and the tube was mixed by inversion and then stored on ice for 4 minutes. After this time, 150 μl of ice cold acetate buffer were added and mixed by inversion and the tube was then stored on ice for 5 minutes. 100 μl chloroform were added and mixed by inversion and the tube was again centrifuged for 30 seconds at 12,000 x g and 4°C. The supernatant was added to an equal volume of phenol chloroform in a fresh microfuge tube and mixed by vortexing. After a further refrigerated centrifugation step, the supernatant was transferred to a fresh microfuge tube. One volume of isopropanol and a one-tenth volume of sodium acetate solution were added and mixed and the tube was incubated at room temperature for 2 minutes.
The tube was then centrifuged at 12000 x g for 5 minutes and the supernatant was removed. The pellet was washed once with ethanol, dried using a vacuum desiccator and finally resuspended in an appropriate buffer.

2.5.2 Plasmid DNA - large scale preparation

Reagents All reagents were from Merck Ltd unless otherwise stated.

* Tris buffer, pH 8.0 with glucose and EDTA - 25 mM Tris, 50 mM glucose, 10 mM EDTA (autoclaved)
* Alkali solution - 0.2 M sodium hydroxide containing 10% (w/v) SDS
* Sodium acetate solution, pH 5.2 - 3 M in distilled water
* Tris EDTA (TE) buffer, pH 8.0 - 10 mM Tris, 1 mM EDTA
* Isopropanol
* Ethanol
* Tris-HCl, pH 8.0 - 10 mM Tris
* Caesium chloride
* Ethidium bromide - 10 g/l in distilled water
* Butanol - water saturated

Method Large scale preparations of plasmid DNA were prepared using a modification of the alkali lysis method reported in Section 2.5.2, followed by purification by a caesium chloride density centrifugation step. Generally, 0.5 l volumes of culture were processed.

The bacteria were first incubated at 4°C for at least 30 minutes before processing, then centrifuged at 3000 rpm for 30 minutes at 4°C and the supernatant removed. The bacteria were then resuspended in 50 mls Tris buffer with glucose and EDTA, and incubated at room temperature for 10 minutes. After this time, 100 ml of alkali solution were added and the mixture was incubated for a further 10 minutes. 75 ml potassium acetate solution were added and mixed and the flask was stored on ice for 10 minutes. The flask was then centrifuged at 3000
rpm for 20 minutes. The supernatant was removed and filtered through a gauze filter and a 0.6 volume of isopropanol was then added. After mixing and incubation at room temperature for 10 minutes, this was then centrifuged at 6000 rpm for 10 minutes. The pellet was washed once with ethanol and then dried using a vacuum desiccator.

The pellet was dissolved in TE buffer and further TE buffer was added until the total weight of the solution was 9 g. 10 g of caesium chloride were then added and mixed until solution was complete. 1 ml ethidium bromide was added and mixed and the solution was then transferred to a sealable tube (Quickseal Tube, Beckman) which was then heat sealed. The tube was the centrifuged at 55,000 rpm for 24 hours at 20°C in an 80Ti rotor in a L80 7M ultracentrifuge (both Beckman, High Wycombe, Bucks). Of the two bands of DNA located in the centre of the gradient, the lower one represents the plasmid DNA of interest, while the upper, thinner band represents linear DNA and nicked plasmid DNA. The lower band was therefore removed by needle aspiration through the side of the centrifuge tube. The DNA was resuspended in 1.6 g/ml caesium chloride in TE buffer in a fresh sealable tube and ultracentrifuged again, using the same conditions.

The DNA was then resuspended in TE buffer and added to an equal volume of water-saturated butanol and centrifuged at 3000 rpm for 3 minutes. The aqueous phase was then added to a further volume of butanol. This was repeated until all the 'pink colour' was removed from the aqueous phase (usually 6 - 7 extraction cycles). Distilled water was then added to a volume of 9 ml and 1 ml of sodium acetate solution was then added. Twenty ml ethanol were added and the mixture was incubated at room temperature overnight. The tube was then centrifuged at 10,000 rpm for 30 minutes at 4°C and the resultant pellet was washed in ethanol. The final DNA pellet was dried using a vacuum desiccator and resuspended in an appropriate buffer.
2.5.3 Total RNA

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

* Diethylpyrocarbonate (DEPC) treated water - mixed overnight at 37°C and then autoclaved.
* β mercaptoethanol (Sigma Chemical Co)
* Guanidine thiocyanate (GTC) solution, pH 7 - 4 M GTC, 0.025 M sodium citrate, 0.5% (w/v) sodium lauryl sarcosine (SLS), 0.7% (v/v) β-mercaptoethanol.
* EDTA - 0.5 M, pH 7 in DEPC treated water
* Caesium chloride solution - 240 g Caesium chloride and 5 ml EDTA was made to 250 ml with DEPC treated water. This was incubated overnight at 37°C overnight and then autoclaved.
* Sodium acetate - 2M
* Ethanol
* Sodium acetate solution - 0.3 M sodium acetate, pH 4 containing 0.1% (w/v) SDS and 10 mM EDTA
* Precipitation solution - 3 volumes of sodium acetate solution added to 8 volumes of ethanol (obtained from a previously unopened bottle). Prepared immediately before use.

**Method** Isolation of RNA was performed according to the method of Chirgwin *et al* (1979). Care was taken at all times to minimise the risk of RNase contamination. All glassware was DEPC treated overnight and then autoclaved, and all solutions were prepared in DEPC treated water.

Cells previously stored in GTC at 4 - 20°C (6 ml of GTC / 10^8 cells), had their DNA sheared by passing the GTC cell lysate six times through a 23 gauge needle (Terumo, Leuven, Belgium). The GTC lysate was then carefully layered onto 5 ml of caesium chloride solution cushion in ultracentrifuge tubes (14 x 89 mm, Beckman). The ultracentrifuge tubes were then centrifuged at 27 000 rpm for
22 hours at 20°C in a SW41 rotor and L80 7M ultracentrifuge (both Beckman). The resulting supernatant was gently aspirated with a drawn out pasteur pipette, being careful not to disturb the pellet of RNA. The ultracentrifuge tube containing the pellet was inverted and residual GTC allowed to drain. The cylindrical top of the ultracentrifuge tube was cut off with a hot scalpel and the RNA pellet at the base of each tube was precipitated by addition of 200 μl precipitation solution and incubation at room temperature for 1 hour. The resulting opaque RNA pellet was carefully transferred to a 1.5 ml microfuge tube containing 900 μl of the precipitation solution. This was allowed to further precipitate at 4°C for 24 hours and finally pelleted by centrifugation at 10,000 x g for 20 minutes. The pellet was washed twice in 70% ethanol and then dissolved in 50 - 200 μl of DEPC water.

**2.5.4 Isolation of nuclear and cytosolic RNA**

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

* Reagents given in Section 2.4.2.
* HEPES buffer, pH 7.9 - 10 mM HEPES, 10m M potassium chloride, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonylfluoride (PMSF)
* 10% (w/v) Nonidet P40 (Fluka)
* Ammonium acetate - 10 M

**Method** Nuclear and cytoplasmic RNA was prepared from TF-1 cells by a mini extract method modified from Schreiber *et al* (1989). Care was taken at all times to minimise the risk of RNase contamination. All glassware was DEPC treated overnight and then autoclaved; and all solutions were prepared in DEPC treated water.

3 x 10^7 cells were pelleted in a 1.5 ml microfuge tube and resuspended in 400 μl HEPES buffer by gentle pipetting. The cells were allowed to swell on ice for 15 minutes and 25 μl of Nonidet P40 were then added and mixed gently, in order
to disrupt the cytoplasmic membrane, whilst leaving the nuclei intact. The homogenate was spun at 13000 rpm for 1 minute at 4°C and the supernatant, containing the cytoplasm, was removed. The nuclear pellet was washed twice in HEPES buffer and then resuspended in 6 ml GTC. The nuclear RNA was then extracted using the caesium chloride gradient method reported in Section 2.5.2.

The cytoplasmic RNA was extracted with phenol/chloroform (Section 2.5.1) and precipitated with a 0.1 volume of ammonium acetate and 2.5 volume 100% ethanol at -70°C for at least 30 minutes. The tube was then centrifuged at 12000 x g for 10 minutes and the supernatant gently removed. The resulting RNA pellet (often not visible) was dried using a vacuum desiccator and finally resuspended in an appropriate buffer. The relative cross-contamination between nuclear and cytoplasmic RNA was evaluated by agarose gel electrophoresis. Cytoplasmic RNA contained 28, 18 and 4S RNA, whereas the nuclear RNA did not.
2.6 DNA Sequencing

Reagents  All reagents were from Merck Ltd unless otherwise stated.

* Alkali denaturing solution - 1 M sodium hydroxide containing 1 mM EDTA
* Sequenase Version 2.0 kit (US Biochemicals)
* Primers at 20 ng / μl (various sources)
* 32P-dCTP (Amersham)

Method  Sequencing of a plasmid DNA or PCR amplified product was carried out using a modified version of the Sequenase reaction (Keohavong & Tilley, 1989). The annealing and labelling reactions were carried out in a single step using automated PCR equipment (Thermocycler, Hybaid Ltd, Teddington, Middlesex).

PLASMID DNA TEMPLATE PREPARATION  A single stranded DNA template was produced by alkali denaturation of the plasmid (Sambrook & Maniatis, 1989). Four μg of a 3 kb plasmid were prepared in a total volume of 22.5 μl of DDW. Six μl of denaturing solution were added to the plasmid preparation and incubated at room temperature for 5 minutes. Single stranded DNA was then isolated by size fractionation on a Sepharose CL-4B (Sigma Chemical Co) column pre-equilibrated with TE buffer and was then immediately placed on ice. 7 μl of this material (equal to 1 μg of starting plasmid) was then used in each sequencing reaction.

PCR PRODUCT TEMPLATE PREPARATION. The DNA was purified from a LMP agarose gel (see Section 2.4.2). Approximately 500 ng of this material was then used in each sequencing reaction.

SEQUENCING REACTION  The sequencing reaction was performed according to a previously described method (Keohavong & Tilley, 1989). The reaction was set up in a final volume of 13 μl, containing 7 μl (1 μg) of DNA template, 3 μl of 5 x (dCTP) labelling mix, 2.5 μl of annealing primers and 0.5 μl of 32P-CTP.
This was then incubated at 99°C for 5 minutes and after pulsing down, left at room temperature for 30 seconds and transferred to ice. 2 μl of dilute Sequenase enzyme was added (1 volume of Sequenase to 8 volumes of ice cold enzyme dilution buffer) to the sequencing reaction which was then transferred to 30°C and incubated at this temperature for 3 minutes. After this time, the tube was again placed on ice. The reaction was terminated by adding 3.3 μl of the reaction mixture to four tubes, pre-incubated at 37°C, each containing 2.5 μl of dideoxy-termination mix: dGTP, dATP, dTTP or dCTP respectively. The tubes were then incubated at 37°C for exactly 4 minutes, before addition of 4.2 μl of Stop Solution. The samples were then heat denatured at 80°C for 3 minutes and run on a 6% acrylamide/8M urea denaturing PAGE (Section 2.4.3).
2.7 Radioligand Binding Studies

2.7.1 $^{125}$I-GM-CSF Binding assays

**Reagents** All reagents were from Merck Ltd unless otherwise stated.

* Foetal calf serum (FCS) (ICN)
* Binding buffer - RPMI 1640 (Gibco-BRL) containing 25 mM HEPES and 2% (v/v) FCS
* $^{125}$I GM-CSF (Amersham UK)
* Phosphate buffered saline (PBS), pH 7.4 - 0.01 M phosphate, 0.145 M NaCl
* Phosphate buffered saline (PBS), pH 3.0 - 0.01 M phosphate, 0.145 M NaCl
* TF-1 cells (Kitamura et al, 1989)

**Method** Cells were incubated at 4°C for 16 hours or at room temperature for 2 - 4 hours, with $^{125}$I GM-CSF (specific activity of 22.2 - 44.4 TBq/mmol in binding buffer). These conditions have previously been shown to give maximum binding (data not shown). The concentration of $^{125}$I GM-CSF ranged from 1 pM to 2 nM. Non-specific binding was measured by parallel incubation in the presence of >100 fold excess of unlabelled GM-CSF (0.5 - 1 mg).

Cell suspensions were then layered onto a cushion of chilled FCS in 1.5 ml polypropylene tubes, centrifuged at 1200 x g in a desktop microcentrifuge and snap frozen in liquid nitrogen. The cell pellet was cut and counted in a γ counter.
2.7.2 Measurement of GM-CSFR α and β chain expression by antibody binding

Reagents All reagents were from Merck Ltd unless otherwise stated.

* Foetal calf serum (FCS) (ICN)
* Binding buffer - RPMI 1640 (Gibco-BRL) containing 25 mM HEPES and 2% FCS
* Phosphate buffered saline (PBS) pH 7.4 - 0.01 M phosphate, 0.145 M NaCl, 0.02% (w/v) azide
* Bovine serum albumin (BSA) (Sigma Chemical Co)
* TF-1 cells (Kitamura et al, 1989)
* Mouse anti-human GM-CSFRα monoclonal IgG (Santa Cruz Technology, Santa Cruz, CA) - Stored in 3% (w/v) BSA in PBS
* Mouse anti human GM-CSFRβ (Santa Cruz) - Stored in 3% (w/v) BSA in PBS
* 125I labelled sheep anti-mouse IgG (Amersham UK) - Stored in 3% (w/v) BSA in PBS

Method 1-2 x 10^6 TF-1 cells were resuspended in 50 μl of binding buffer and incubated in round bottomed 96 well plates (Beckton-Dickinson Ltd), with mouse anti-human GM-CSFRα (5 mg/ml, final concentration) or GM-CSFRβ (5 mg/ml, final concentration) for 1 hour on ice. After this time, the cells were washed three times in binding buffer and incubated with 125I labelled sheep anti mouse antibody for 30 minutes. Cell suspensions were then layered onto a cushion of chilled FCS in 1.5 ml polypropylene tubes, centrifuged at 1200 x g in a desktop microcentrifuge and snap frozen in liquid nitrogen. The cell pellet was cut and counted in a γ counter.
EXPRESSON OF GM-CSFRα and β mRNA
3.1 Introduction

The cells that express the GM-CSF receptor include myeloid cells and their precursors. GM-CSFR expression has also been demonstrated on leukaemic cell lines and blasts from patients with acute myeloid leukaemia (AML) (Park et al., 1989b; Kelleher et al., 1988; Budel et al., 1993; Khwaja et al., 1993). The number of receptors per cell is low, with AML blasts expressing as few as 50 receptors per cell, and TF-1 cells up to 9000 receptors (8600 low affinity and 2000 high affinity binding sites). One would therefore expect the GM-CSFR mRNA expression to be commensurately low. This has been confirmed by the difficulties encountered when the GM-CSFRα and β cDNA were first cloned (Gearing et al., 1989; Hayashida et al., 1990, respectively).

Gearing et al. (1989) screened large cDNA libraries using a sensitive detection method they had developed, in order to isolate GM-CSFRα. A human placental cDNA library of approximately $5 \times 10^6$ independent recombinants in a COS cell expression vector was subfractionated, and each subfraction transfected into $1.5 \times 10^6$ COS cells by electroporation. The transfected COS cells, grown on glass slides, were then screened for $^{125}$I GM-CSF binding using direct autoradiography. Only 2 pools out of the first 250 pools of cells screened gave rise to one or two positive cells. These positive cells were then isolated by three further rounds of subcloning of the positive cell pools.

The human GM-CSFRβ cDNA has not been fully isolated in a single clone, despite screening $4 \times 10^5$ independent clones in a TF-1 derived cDNA library with a mIL-3 receptor probe (AIC2A). Screening did however isolate a cDNA clone KH 85 homologous to the mIL-3 receptor cDNA (70% homology). This clone lacks about 600 bases from its 5' end compared with the sequence of the mouse cDNA. The KH 85 clone was used to design specific primers to prepare further cDNA libraries and these were further screened for more complete
Twenty six further clones were identified, but all either had an insertion or deletion resulting from alternative splicing. The cDNA was therefore constructed by joining some of the clones to give sequence that encoded for the entire protein. This clone was called KH97 (Hayashida et al, 1990).

In order to overcome potential difficulties with measuring mRNA expression, we elected to use RNase protection assays to detect GM-CSFR expression in preference to more conventional techniques. The RNase protection assay is a powerful tool for detecting and quantifying specific RNAs, in particular low abundance RNA species (Zinn K et al, 1983; Lee & Costlow, 1987).

PRINCIPLE OF RNase PROTECTION ASSAYS

The assay is based upon the hybridisation of a radioactive antisense RNA probe with the RNA pool being tested, in solution (Figure 3.1). The antisense RNA probe is synthesised by inserting the probe fragment into a transcription vector under the control of a bacteriophage promoter (T3, T7 or SP6) and using the corresponding T3, T7 or SP6 RNA polymerase to generate a probe of high specific activity. Complementary portions of the test RNA and radioactive probe RNA form hybrids. RNase A and T1 are used to digest the single-stranded portions of the hybrids, leaving the double stranded 'protected regions' intact. RNase A cleaves 'unprotected' RNA fragments 3' of C and U and RNase T1 cleaves 3' to G. Separation of the digestion products on a denaturing polyacrylamide gel reveals the size of the protected RNA fragment.

Compared to more conventional methods of RNA detection, which rely on RNA bound to a solid support (Northern blotting or slot blotting), low abundance mRNAs (up to 0.1 pg) are more readily detected and quantified using a solution hybridisation procedure. Another advantage is that the single stranded probes hybridise specifically only to their complementary strand. Since the probes used in RNase protection assays are usually significantly shorter than the mRNA species being detected, the target RNA preparation does not need to be completely
Target mRNA

Radiolabelled antisense probe

Hybridisation

Nuclease treatment (usually RNase A and T1)

Radiolabelled probe 'protects' complementary RNA

Unhybridised material is removed by digestion with nuclease.

The 'Protected' Fragment Is Analysed By Denaturing PAGE and Autoradiography.

Figure 3.1 Schematic representation of the RNase protection assay
intact (breaks in the mRNA that occur outside the region that hybridises to the probe will have no effect on the assay, but will result in band smearing on Northern blots). S1 nuclease assays have also been used for RNA detection and to determine the intron-exon structure of genes (Myers et al, 1985). However, as S1 nuclease assays involve RNA : DNA hybrids, there is greater potential for digestion artefacts.

3.2 Methods

3.2.1 Cloning of GM-CSFRα and β cDNA fragments.

*Principle* In order to produce high specific activity probes for the RNase protection assays, GM-CSFRα cDNA fragments were produced by RT-PCR (Gearing et al, 1989). GM-CSFRβ cDNA fragments were produced by PCR amplification of the GM-CSFRβ cDNA clone KH97 (Hayashida et al, 1990) (kindly provided by Dr Miyajima, DNAX, WA, USA).

To clone the blunt ended, double stranded products of PCR into a plasmid vector, it is necessary to perform blunt end ligations which are obviously less efficient than cohesive end ligations. To achieve cohesive end ligation, restriction sites are included at the 5' end of each primer to allow direct cloning. However, low cloning frequencies are sometimes encountered, presumably due to incomplete enzyme digestion or *Taq* polymerase carryover (Lorens, 1991). This problem has been circumvented by a number of commercially available vectors which use the process of TA cloning. This process exploits the fact that when DNA fragments are generated by *Taq* polymerase in a PCR reaction, the enzyme introduces one or two extra deoxynucleotides at the 3' end of blunt double stranded DNA (Clark et al, 1988). Any one of the four nucleotides can be added when they are present in the mixture but for reasons unknown, preference is
given to the incorporation of dATP. To achieve cohesive end ligation for the DNA products with adenine overhangs, vectors with thymidine overhangs have been produced using either *Taq* polymerase and dTTP alone, Xcm I digestion, or terminal deoxynucleotidyl transferase (Hengen *et al*., 1995).

TF-1 cell total RNA was utilised as a template for reverse transcriptase in order to produce DNA for amplification with *Taq* polymerase. Primers used for RT-PCR of the GM-CSFRα probe (A and B) were directed to the 5' end of the cDNA and those for GM-CSFRβ (C and D) were directed to the membrane proximal region of the KH97 cDNA. The primer sequences and their position relative to the published exon arrangement and the transmembrane region, are summarised in Figure 3.2. The β genomic sequence (human) has not been published.

The PCR product (without gel purification) was ligated using a commercial vector containing both ampicillin and kanamycin resistance genes. This vector has the multiple cloning site situated in phase with a *lacZ* reading frame, which allows use of a simple blue to white visual assay to indicate whether a PCR amplified molecule has been cloned. If a DNA fragment is inserted into this indicator gene it will disrupt the normal translation of the β-galactosidase protein, resulting in a white colony phenotype on growth media containing the chromogenic dye X-Gal. The undisturbed gene yields a blue colony phenotype. Transformation or transfection of the bacteria is carried out by electroporation.

**Reagents** All reagents were from Promega (WI, USA) unless stated otherwise.

* RNA from TF-1 cells (Section 2.5.3)
* GM-CSFRα PCR primer A (nucleotides 90 - 110)
* GM-CSFRα PCR primer B (nucleotides 372 - 392)
* GM-CSFRβ PCR primer C (nucleotides 1281 - 1301)
* GM-CSFRβ PCR primer D (nucleotides 1600 - 1620)
* Oligo dT
Figure 3.2 5' Exon positions of GM-CSFRα and primers used in PCR reactions for preparation of GM-CSFRα and β RNase protection probes
* dNTP
* Taq polymerase buffer
* RNase inhibitor
* AMV reverse transcriptase
* Taq polymerase
* Magnesium chloride (Promega)
* PCRII plasmid vector (Invitrogen, CA, USA)

**Method**  One µg of total RNA from TF-1 cells was used as a template in a reverse transcriptase reaction primed with oligo dT (12.5 ng/µl, final concentration) in a total volume of 20 µl containing Taq polymerase buffer, 5.25 mM magnesium chloride, 1 mM dNTP, 20 U RNase inhibitor and 3.75 U AMV reverse transcriptase. All reagents were added to a 0.5 ml microfuge tube stored on ice, and the tube was then transferred to a thermocycler (Hybaid) and incubated at 42°C for 1 hour and 99°C for 5 minutes.

Four µl of the RT reaction was used for PCR in a total volume of 20 µl containing 0.5 U Taq polymerase, 2.25 mM magnesium chloride, 0.2 mM dNTP, and 4 ng/µl primers. PCR was carried out using a hot start at 95°C for 5 minutes prior to addition of the Taq polymerase, followed by 35 cycles of 95°C for 30 seconds, 30 seconds at 60°C for α primers or 64°C for β, and 72°C for 1 minute. The final extension step was 72°C for 5 minutes. In this way, a 302 bp fragment of GM-CSFRα corresponding to nucleotides 90 - 392 of the published cDNA sequence, and a 340 bp fragment of GM-CSFRβ, corresponding to nucleotides 1281 - 1620 of the published sequence, were produced.

Fresh PCR product was ligated with the Invitrogen PCR II vector. The ligation was set up according to the manufacturer's instructions using reagents provided in the kit. Two sets of ligations were carried out at a 1:1 and 1:3 molar ratio of vector to GM-CSFRα/β PCR products. JM109 competent bacteria were used in place of the competent bacteria provided in the cloning kit. Transformation
or transfection of the bacteria was carried out by electroporation (Gene Pulser System, Biorad Ltd). The ligation mixture was diluted 1 in 5 with distilled water, to decrease the salt concentration and 5 μl of this added to 40 μl of JM109 competent bacteria in an electroporation cuvette (Biorad Ltd). The electroporation was carried out at 25 μF and 1.5 kV with a time constant between 19.3 and 20.6 seconds. The cells were then added to LB medium to recover at 37°C for 1 hour, plated on agar and grown up overnight. Bacteria containing cloned gene were isolated using X-gal selection (Section 2.1). The cDNA was then digested using the appropriate restriction enzyme and analysed by agarose gel electrophoresis (Section 2.4.1).

### 3.2.2 Transcription of radioactive RNA probe and non-radioactive sense RNA transcripts

**Principle** A clone containing the insert of the 302 bp fragment of the GM-CSFRα in the sense orientation was selected (Figure 3.2). The clone was digested with Rsa 1 to produce a 379 bp fragment containing the T7 polymerase promoter and the GM-CSFRα sequence from nucleotides 102-392. This was predicted to yield a protected fragment of 291 bases on RNase protections. The linearised template was used to produce the antisense probe for the RNase protection assays. To produce transcripts in the sense direction the plasmid was digested with Dde I within the α insert and transcribed from the SP 6 polymerase promoter. This yields a sense transcript of 377 bases containing the GM-CSFRα from nucleotides 90-369 (Figure 3.3).

Similarly, the 340 bp fragment of the GM-CSFRβ was digested with Rsa1 to produce a 253 base antisense probe. This contains the GM-CSFRβ sequence from 1429-1620, which should give a protected fragment of 191 bases in RNase protection assays. To produce transcripts in the sense direction the plasmid was digested with Dde I within the β insert and transcribed from the SP 6 polymerase
promoter. This results in a sense transcript of 385 bases containing the GM-CSFRβ from nucleotides 1281-1584 (Figure 3.4).

Care was taken to linearise the plasmid containing the probe with an appropriate enzyme yielding a 5' overhang, or at least blunt ended. In this way, formation of hairloops of the transcribed probe, which would result in excessive length of self protected species of the probe, was prevented.

**Reagents** All reagents were from Promega unless otherwise stated.

* Plasmid DNA (Section 2.5.1)
* Restriction enzymes
  - Rsa 1 (Boehringer Mannheim)
  - Dde 1 (Boehringer Mannheim)
  - Hinf I (Boehringer Mannheim)
* Enzyme buffers (Boehringer Mannheim)
* Transcription buffer
* rNTPs (ATP, UTP, and GTP) (Pharmacia Biotech)
* Dithiothreitol (DTT) (Sigma Chemical Co)
* RNasin
* 32p CTP (400 Ci/mmol; 10 mCi/ml)
* T7 RNA polymerase
* SP6 RNA polymerase
* RQ1 DNase - 1000U/ml
* Ammonium acetate (Merck Ltd) - 10 M
* Glycogen (Boehringer Mannheim)
* Tris EDTA buffer pH 7.5 (TE buffer) - 10 mM Tris, 1 mM EDTA
* TE buffer pH 7.5, containing SDS (TES buffer) - TE buffer containing 20% (w/v) SDS
* Tri-chloro acetic acid (TCA) - 10% (v/v)

**Method** The linearised DNA template was produced by digestion of 4 μg of plasmid in a total volume of 50 μl containing 50 U Rsa 1. The reaction mixture
Figure 3.3 Transcription of GM-CSFRα
Figure 3.4 Transcription of GM-CSFRβ
was incubated at 37°C overnight and then 3 µl of reaction mixture were analysed by agarose gel electrophoresis, in order to ensure digestion was complete (Section 2.4.1). After determining that digestion was complete, the digested DNA was extracted (Section 2.5.1) and this template was used to transcribe the radioactive antisense probe.

Four µl of stock transcription buffer were first added to 5 µl DNA template and then 2 µl stock rNTP were added to this mixture. 0.8 µl of 250 mM DTT, 1 µl RNasin, 1µl DEPC treated water, 5 µl ³²P CTP (>400 Ci/mmol) and 1 µl T7 RNA polymerase were then added in the order indicated. This reaction mixture was incubated at 37°C for 90 minutes and then 10 µl of RNase-free DNase were added. The reaction was incubated at 37°C for a further 30 minutes in order to remove the DNA template, as remaining DNA template could result in competition for the probe during hybridisation.

The resulting probe was phenol/chloroform extracted (Section 2.5.1) and then precipitated with 0.3 vol ammonium acetate and 2.5 vol 100% ethanol at -80°C, or on dry ice for at least 1 hour. To facilitate precipitation, and to get optimal recovery of the probe, 1 µl of glycogen was added to the precipitating solution. The probe was recovered by centrifugation at 13,000 rpm in a benchtop microfuge for 10 minutes, in order to pellet the RNA. The probe was then washed with 70% ethanol and resuspended in 100 µl of TES buffer.

The specific activities of the probes were measured using Cerenkov counting (Kobayashi, 1974) in which 1 µl of the probe was analysed using a β scintillation counter (Pharmacia Biotech Ltd) without the use of scintillant. This method does not allow distinction of unincorporated and incorporated ³²P-CTP and therefore overestimates the specific activity of the probe. In order to overcome this difficulty, the amount of ³²P incorporation was determined by TCA precipitation. Briefly, 2 µl of the final probe reaction in TES was removed and added to 198 µl of TE buffer containing 100 µg of carrier RNA. 100 µl of this
mixture were counted directly in the β scintillation counter and the remaining 100 μl of the mixture were subjected to TCA precipitation. In this method, 2 μl of ice cold TCA were added to the 100 μl sample and the mixture was vortexed and incubated on ice for 5 minutes. The precipitate was collected by filtering under a vacuum on GF/C glass fibre filters (Whatman UK). The filter was counted and the counts compared with the non-TCA precipitated reaction to give the proportion of CTP incorporation in the probe. The transcribed probe specific activity was then calculated in each case (Section 3.3).

Non-radioactive sense GM-CSFR α and β transcripts were synthesised exactly as for the radioactive transcription but with the 32P-CTP substituted by 5 mM CTP and SP6 RNA polymerase instead of T7 polymerase. The reaction was carried out in a final volume of 100 μl to give a high yield of RNA, and therefore the relative amount of reagents used were increased accordingly.

Expression of actin RNA was used as an internal control for the RNase protection assays. The human actin probe was synthesised as above from a plasmid which was a kind gift of LJZ Penn and H Land (ICRF, London). The plasmid was linearised using Hinf I and the RNA probe transcribed using the SP6 polymerase promoter. This probe is 140 bases in size and yields a 120 bp protected fragment. Actin shows a much higher expression than the GM-CSFRα or β chain and in order to get similar intensities of the signals, a 1/50 dilution of the actin probe was made in TES. tRNA was added to the required volume of TES to achieve a final concentration of 1 ng/μl (the tRNA acts as a non specific carrier and prevents loss of the probe added subsequently). Sufficient cold actin probe was added to achieve a final concentration of 2 ng/μl and, finally, 'hot' actin probe was added (2% (v/v), final concentration), this ensures the actin probe is always in excess in the reaction mix and should be able to cope with up to 40 μg of total RNA.
3.2.3 RNase Protection Assays

**Reagents** All reagents were from Sigma Chemical Co unless otherwise stated.

* Total RNA (Section 2.5.3)
* Hybridisation buffer - 40 mM PIPES (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl containing 80% (v/v) formamide (prepared immediately before use)
* Probe of interest (Section 3.2.2)
* Actin probe (diluted 1 in 50) (Section 3.2.2)
* RNase A and T1 (Boehringer Mannheim)
* RNase solution - 10 mM Tris pH 7.5, 5 mM EDTA pH 8.0, 300 mM NaCl, containing 40 μg/ml RNase A and 2 μg/ml RNase T1
* SDS - 10% (w/v)
* Proteinase K - 10 mg/ml
* 'Phenol-chloroform' - 5 ml of the lower phase of a 10 ml aliquot of phenol and 5 ml of 24 : 1 (v/v) chloroform : isoamyl alcohol.
* Glycogen (Boehringer)
* Ethanol (Merck Ltd)
* RNA loading buffer - Tris borate EDTA (TBE) buffer, pH 8.0 - 89 mM Tris, 89 mM boric acid, 2 mM disodium EDTA, containing 80% (v/v) Formamide, 0.1% (w/v) Xylene cyanol and 0.1% (w/v) Bromophenol Blue.

**Method** Five - 25 μg of total RNA sample were freeze dried in a 1.5 ml microfuge tube using a vacuum desiccator (Speed-Vac, Savant). Twenty five μl of hybridisation buffer were then added and the tube was mixed well to ensure all material went into solution. The probe of interest together with the actin probe (diluted 1 in 50) were mixed in equal volumes and 2 μl of the probe/actin mix added to the hybridisation sample. The reaction was then thoroughly mixed and boiled at >90° C for 10 minutes and then incubated at 50° C for > 8h (usually overnight). The following control hybridisation reactions were also analysed on each occasion:
(i) Positive control - reaction containing sense GM-CSFRα or β transcripts and probe;
(ii) Negative control (1) reaction containing tRNA, probe and actin (to rule out non specific protection);
(iii) Negative control (2) reaction containing only probe and actin (to assess activity of the nuclease mixture).

Nuclease treatment was carried out using RNase A and T1. 350 µl of freshly made RNase solution were added to each hybridisation sample and the reactions were incubated at 30°C for 1 hour. After this time, the nuclease activity was stopped by first adding 10 µl of SDS and then 10 µl of Proteinase K. The reaction was then thoroughly mixed and incubated at 37°C for 30 minutes. 400 µl of phenol/chloroform were then added and the resulting mixture thoroughly mixed by vortexing. The tube was centrifuged at 13,000 rpm in a benchtop microfuge for 5 minutes and the uppermost 350 µl of the aqueous phase transferred to a 1.5 ml microfuge tube. RNA was precipitated by the addition of 1 µl of glycogen and 650 µl of 100% ethanol, and incubation on dry ice, or at -80°C, for at least 1 hour. An obvious white pellet was seen at the bottom of the tube (due to the high amount of salt in the reaction) and this was washed with 70% ethanol and thoroughly vacuum dried. The RNA containing pellet (which may or may not be visible) was then resuspended in 10 µl of RNA loading dye and the protected probe was separated on a 6% denaturing sequencing gel (Section 2.4.3).

The gel was dried and the RNA detected either by autoradiography on pre-flashed X-OMAT AR (Kodak, NY USA) or MP film (Amersham International) with a single intensifying screen or exposed to phosphorimager plates and evaluated on a Fujimax bas 1000 phosphorimager (Fuji Photo Film Co Ltd). Quantitation of phosphorimager data was performed using Millipore Whole Band Analyzer software (Millipore UK) run on a Sun Sparc workstation (Sun Sparc Corp, CA, USA). The background in each lane was subtracted and the area under
the curve integrated for each band detected by phosphorimager, then corrected for CTP content. Autoradiograph film images were scanned with a flat bed scanner (Howtec scanmaster 3+, Howtec Ltd) and quantified using the Millipore software.

3.3 Results

3.3.1 Cloning of GM-CSFRα and β cDNA fragments

GM-CSFRα. Four colonies grew on the agar plates and of these, 3 (2 white and 1 blue) grew on the 1:3 ligation plate and 1 (blue) grew on the 1:1 ligation plate. There were no colonies present on the self ligation control plate. Plasmid DNA was extracted from all four colonies and digested with Dde I to assess the orientation of the insert. In the absence of an insert, such digestion would yield four fragments of 2817, 540, 409, and 166 bp. In the presence of an insert the 540, 409 and 166 bp fragments would be present plus fragments of 2105 and 1013 bp in the case of an insert in the sense direction, or 2361 and 757 bp in the case of an antisense insert. Clones B, E and F contained the GM-CSFRα in the sense orientation (Figure 3.5). Clone B was used for a large scale plasmid preparation in order to synthesise the GM-CSFRα antisense probe.

GM-CSFRβ. Four colonies grew on the agar plates and of these, 3 (2 white and 1 blue) grew on the 1:3 ligation plate and 1 (blue) grew on the 1:1 ligation plate. There were no colonies present on the self ligation control plate. Plasmid DNA was extracted from all four colonies and digested with EcoR I to confirm the presence of the insert and Dde1 and BssHII to assess the orientation of the insert. The presence of an insert was indicated by a 339 bp fragment in addition to the 3932 bp fragment on digestion with EcoR I. Sense orientation of the insert would yield fragments of 1810 and 2460 bp with BssHII digestion, and 166,
409, 540, 2118, and 1037 bp with Dde1 digestion (Figure 3.6). An insert in the antisense orientation would result in digestion fragments of 1547 and 2723 bp with BssHII and 166, 409, 540, 2385 and 770 bp with Dde1. Clone W1 contained the fragment in the sense orientation and was used for a large scale plasmid preparation in order to synthesise the GM-CSFRβ antisense probe.

3.3.2 Probe characteristics

SEQUENCE The plasmids were sequenced in order to confirm the presence of the cloned inserts. Clone B (GM-CSFRα) demonstrated the complete GM-CSFRα sequence from 90-392 nucleotides. Clone W1 (GM-CSFRβ) demonstrated the GM-CSFRβ sequence from 1281-1620 but included a point mutation at position 1592 with a guanine nucleotide substituted by a thymidine.

SPECIFIC ACTIVITY The activity of each synthesised probe was measured before and after TCA precipitation, and the specific activity was calculated using the method shown in the following example:

The GM-CSFRα probe used for the RNase protections shown in Figure 3.7 (probe 1) was transcribed in a 20 μl reaction mixture containing 5 μl 32P-CTP (400 Ci/mmol; 10 mCi/ml). At the end of the synthesis reaction, 5 μl of DNase was added and the probe was extracted using phenol/chloroform and resuspended in a final volume of 100 μl. A 2 μl aliquot was removed and added to 198 μl of buffer containing 100 μg of carrier tRNA. 100 μl of this reaction mixture was counted in a scintillation counter and was found to contain 2.97 x 10^5 cpm. The remaining 100 μl of this reaction mixture was subjected to TCA precipitation and the precipitate was counted and found to contain 1.48 x 10^5 cpm. The counting efficiency was assumed to be 100% and so the specific activity of the RNA probe was calculated using the method shown below. The specific activities of the GM-CSFRβ chain and actin probes were determined using the same procedure.
Figure 3.5 GM-CSFRα clones - 1% agarose gels stained with ethidium bromide showing plasmid DNA digested with DdeI

Clones B, E and F - Insert in sense orientation; Clone K - No insert

Figure 3.6 GM-CSFRβ clones - 1% agarose gels stained with ethidium bromide showing plasmid DNA digested with DdeI and BssHII

Clone W1 - in sense orientation; Clone W2 - antisense orientation
DETERMINATION OF SPECIFIC ACTIVITY OF PROBE

(i) Proportion of CTP incorporated into the RNA:
\[
\frac{1.48 \times 10^5}{2.97 \times 10^5} = 50\%
\]

(ii) Number of moles of \(^{32}\text{P}-\text{CTP}\) in the reaction (calculated by converting the volume of \(^{32}\text{P}-\text{CTP}\) added to the number of mCi of \(^{32}\text{P}\) added, and then further converting to the molar amount using the known specific activity):

\begin{align*}
(a) \quad & 5 \mu l \times 10 \text{mCi/ml} (5 \times 10^{-3} \times 10) = 0.05 \text{ mCi} \quad ^{32}\text{P}-\text{CTP} \\
(b) \quad & 0.05 \text{ mCi} = 0.125 \text{ nmoles in reaction} \\
& 400 \times 10^3 \text{ mCi}
\end{align*}

(iii) Number of moles of \(^{32}\text{P}-\text{CTP}\) actually incorporated:
\[
\frac{50\% \times 0.125 \text{ nmoles}}{100} = 0.06 \text{ nmoles}
\]

(iv) Amount of RNA synthesised in reaction (assuming that the RNA synthesised contains equi-molar amounts of ATP, CTP, GTP and UTP) using a total molecular mass for all four ribonucleotides of approximately 1320 Da.
\[
1320 \text{ g} \times 0.06 \times 10^{-9} \text{ moles} = 79.2 \text{ ng}
\]

(v) Cpm incorporated into the RNA product: The total reaction volume from which the 2 \(\mu l\) sample was removed to determine label incorporation was 100 \(\mu l\). The TCA precipitated probe contained \(1.48 \times 10^5\) cpm, so the whole probe contained \(1.48 \times 10^7\) TCA precipitable material.

Specific activity of the GM-CSFR\(\alpha\) probe:
\[
\frac{14.8 \times 10^6 \text{ cpm}}{80 \text{ ng}} = 1.78 \text{ cpm} \times 10^8/\mu g
\]

The specific activities of the probes varied considerably (Table 3.1). It was therefore only possible to directly compare and quantify expression of the mRNA transcripts in different experiments if the same probe had been used. In cases where different probes had been employed, it was necessary to make adjustment for the relative intensities of the probes used in each case.

<table>
<thead>
<tr>
<th></th>
<th>GM-CSFR(\alpha)</th>
<th>GM-CSFR(\beta)</th>
<th>Actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe 1</td>
<td>(1.78 \times 10^8)</td>
<td>(2.4 \times 10^8)</td>
<td>(0.88 \times 10^8)</td>
</tr>
<tr>
<td>Probe 2</td>
<td>(3.4 \times 10^8)</td>
<td>(4.6 \times 10^8)</td>
<td>(4.1 \times 10^8)</td>
</tr>
<tr>
<td>Probe 3</td>
<td>(2.1 \times 10^8)</td>
<td>(3.9 \times 10^8)</td>
<td>(1.6 \times 10^8)</td>
</tr>
</tbody>
</table>

*Table 3.1* The specific activities (cpm/\(\mu g\)) of probes synthesised in three separate experiments from a single stock of digested DNA template.
3.3.3 Sensitivity of the GM-CSFRα and β RNase protection assays

In order to establish the sensitivity of this assay and to set up a calibration curve for the quantification of receptor mRNA transcripts, the α and β antisense probes were hybridised to target synthesised RNA sense transcripts. The RNA transcripts were serially diluted to the concentration of GM-CSFR mRNA transcripts expected in target tissue. The target sense transcripts were spiked with a constant amount of TF-1 cell total RNA, to obtain an actin signal and internally control this experiment.

GM-CSFRα: The signals from the serially diluted target sense transcripts (1 pg to 1 ng) and actin are shown in Figure 3.7. The signals from the sense transcripts and actin were expressed as a ratio and a calibration curve was plotted. As the target RNA concentration decreases the calibration curve becomes non-linear (Figure 3.8). The lower limit of detection of the sense transcript in the GM-CSFRα mRNA protection assay was approximately 10 pg per sample, with a linear signal obtained up to 6 ng.

GM-CSFRβ: The signals from the serially diluted target sense transcripts (1.25 pg to 12.5 pg) are shown in Figure 3.9. The signals from the sense transcripts and actin were expressed as a ratio and a calibration curve was plotted (Figure 3.10). As in the case of GM-CSFRα, when the target RNA concentration decreases the calibration curve is non-linear.

The GM-CSFRβ mRNA protection assay was more sensitive than that for GM-CSFRα, as the detection limit was approximately 1 pg per sample. However, the maximum was approximately 12 pg per sample and so the detectable range for GM-CSFRβ was more restricted than GM-CSFRα. Higher amounts could not be accurately quantified above this level as the intensity of the specific GM-CSFRβ mRNA signal was too high relative to the actin signal derived from the total mRNA from either HL-60 or TF-1 cells.
Figure 3.7 RNase protection of the serially diluted target GM-CSFRα sense transcripts containing 15 μg of TF-1 total RNA

Figure 3.8 RNase protection assay calibration curve for GM-CSFRα
Figure 3.9 RNase protection of the serially diluted target GM-CSFRβ sense transcripts containing 15 µg of TF-1 total RNA

Figure 3.10 RNase protection assay calibration curve for GM-CSFRβ
The calibration curves were used to estimate the amount of GM-CSFRα and β mRNA in 15 μg total RNA extracted from TF-1 cells, HL-60 cells and neutrophils. The RNase protection assays were performed using the same probe in each case in order to control for differences in the specific activity. The results are summarised in Table 3.2.

It was not possible to give accurate absolute quantities on a single cell basis as it could not be assumed that the RNA recovery was similar in the different cell types. Furthermore there was considerable variation in the amount of RNA extracted from the same cell type but carried out on different days. The variation of RNA recovery obtained using a single RNA extraction protocol is shown in Table 3.3.

<table>
<thead>
<tr>
<th></th>
<th>HL-60 (uninduced)</th>
<th>TF-1</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSFRα mRNA</td>
<td>1.4</td>
<td>4.0</td>
<td>1.3</td>
</tr>
<tr>
<td>GM-CSFRβ mRNA</td>
<td>0.06</td>
<td>0.08</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*Table 3.2 GM-CSFα and β mRNA (pg) measured by RNase protection assay per μg total RNA extracted from different cell types*

<table>
<thead>
<tr>
<th></th>
<th>HL-60 (uninduced)</th>
<th>TF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>4.5</td>
<td>10</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>70</td>
<td>40</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>12</td>
<td>80</td>
</tr>
</tbody>
</table>

*Table 3.3 RNA yields (μg) from different extractions of 10 x 10^6 cells*
3.3.4 Expression of GM-CSFRα and β mRNA in cell lines and primary haemopoietic cells

**GM-CSFRα:** RNase protection assays were performed on total RNA with a probe spanning bases 102 to 392, and the expected fragment of 291 bases was detected in a number of myeloid haemopoietic cell lines (TF-1, HL-60, and U937) (Figure 3.11). However, this mRNA species was not detected in RNA from the K562 cell line. The lymphoid cell lines (CEM and Daudi cells) did not express the GM-CSFRα mRNA (Figure 3.11). Monocytes and neutrophils (Figure 3.11), as well as blasts from AML patients (Figure 3.12), expressed this mRNA species, whilst T lymphocytes did not.

As well as the expected 291 base fragment, an extra 269 base fragment was consistently present in all the positive cell lines, as well as in the primary haemopoietic cells (Figures 3.11 and 3.12). A 167 bp fragment (seen as a faint band on Figure 3.11) was detected in U937 cells and in mature monocytes (Figure 3.13).

**GM-CSFRβ:** The 253 base antisense probe, spanning nucleotides 1429-1620 gave the expected 191 base protected fragment on this assay and this was present in all myeloid cell lines except K562 cells and in primary myeloid cells (Figure 3.14). There was no expression of the GM-CSFRβ mRNA in Daudi cells or T lymphocytes. In addition to the expected 191 base fragment, two extra bands were observed (163 base and 64 base fragments).

The extra bands seen on the RNase protection assays were not present in the negative control lanes and must therefore be considered to be 'legitimate'. Such extra bands could be the result of hybridisation of the probe with transcripts containing different, but related sequences. It is also possible that the probe may be detecting heterogeneity in initiation, termination or processing of mRNA transcripts (heteronuclear RNA) or mRNA that is of the same species of transcript but represents an alternatively spliced isoform. Alternatively, the probe may...
Figure 3.11 GM-CSFRα RNase protection assays of total RNA extracted from haemopoietic cell lines and primary haemopoietic cells

Figure 3.12 GM-CSFRα RNase protection assays of total RNA extracted from blasts obtained from patients with AML

AML was classified according to The FAB classification system (Bennett et al, 1985)
Figure 3.13 GM-CSFRα RNase protection assays of total RNA extracted from monocytes and U937 cells

Figure 3.14 GM-CSFRβ RNase protection assays of total RNA extracted from haemopoietic cell lines and primary myeloid cells
contain a mutation and hence a region of mismatch with the target mRNA transcripts.

3.3.5 Investigation of the extra bands

GM-CSFRβ

163 Base Fragment The 163 base band could have been due to any of the reasons presented above but the most likely reason would be the mutation at nt 1592 (G to T) in the probe used. This would result in separation on the denaturing gel of the expected 191 bp protected fragment and, as a result of RNase digestion at the point of the mismatch, a 163 base fragment (Figure 3.15). To investigate this possibility, the assay was repeated with 25 μg of TF-1 RNA. Each of the TF-1 RNase protection assays was subjected to different concentrations of RNase treatment. As the concentration of the RNase A and T1 was increased the lower bands increased in intensity (Figure 3.16).

GM-CSFRβ

64 Base Fragment An additional 64 base fragment was observed when the RNase protection assays were performed in the absence of actin. Characterisation studies performed by R Gale (Department of Haematology, UCL) using RT-PCR and direct sequencing of the PCR products, have demonstrated that the 64 base resulted from a truncated form of the β chain. A deletion of a 104 bp exon in the membrane-proximal region of the chain, which creates a frame shift and introduces a premature stop codon has been identified (Figure 3.17). This isoform of the GM-CSFRβ chain has been designated βIT for βINTRACYTOPLASMIC TRUNCATED. Twenty three amino acids of the βIT tail would be identical to the full length β chain followed by a new sequence of 23 amino acids. RNase protection assays show that this isoform comprises approximately 20% of the total β chain message in haemopoietic cells lines and primary myeloid cells
Figure 3.15 Possible origin of 163 bp fragment in GM-CSFRβ
and was increased in blast cells in a number of patients with AML, in some cases up to 90% of the total (Gale et al., 1994).

**GM-CSFRα**

269 *Base Fragment* Full sequencing of the plasmid used to transcribe the probe for the protection assays did not reveal any point mutations. Furthermore, the extra 269 base band did not change in intensity when the conditions for the RNase digestion were altered as they had done for the β probe.

In order to exclude the possibility that the smaller extra band represented an intermediate heteronuclear RNA species, RNase protection assays were carried out on TF-1 cell nuclear and cytoplasmic RNA (Figure 3.18). Both bands were present in nuclear and cytoplasmic RNA.

The relative mRNA expression of the two fragments was investigated in a number of haemopoietic cell lines. In each case, the background for a single lane
Figure 3.17 Schematic representation of alternative splicing leading to truncated isoform of the GM-CSFRβ chain
was subtracted and the area under the curve was integrated for each band detected by the phosphorimagner. The combined signal from the full length (291 base) and 269 base fragment was taken as 100% and the signals of the separate bands expressed as a percentage of the combined signal intensity. The abundance of the 269 base band was consistently higher than the 291 base band in TF-1, HL-60 and U937 cell lines as well as in neutrophils and monocytes from normal individuals (Table 3.4).

![RNAse protection assay of GM-CSFRα mRNA from TF-1 cell nuclear and cytoplasmic RNA](image)

**Figure 3.18** RNAse protection assay of GM-CSFRα mRNA from TF-1 cell nuclear and cytoplasmic RNA

A = nuclear RNA; B = cytoplasmic RNA.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Full length fragment (%)</th>
<th>269 base fragment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF-1</td>
<td>36.0 (2.8)</td>
<td>64.0 (5.5)</td>
</tr>
<tr>
<td>HL-60</td>
<td>31.5 (6.0)</td>
<td>68.5 (6.0)</td>
</tr>
<tr>
<td>U937</td>
<td>37.0 (4.0)</td>
<td>63.0 (5.0)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>37.5 (3.0)</td>
<td>62.5 (4.0)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>35.0 (1.3)</td>
<td>65.0 (1.3)</td>
</tr>
</tbody>
</table>

Table 3.4 Relative mRNA expression of the full length and 269 base fragment

Results indicate % total signal and represent the mean and SEM of four separate experiments.

3.4 Discussion

The low number of mRNA transcripts of the GM-CSFR has been an important obstacle in initially cloning of the receptor chains and subsequently studying its expression. RNase protection assays were therefore used to detect the low abundance mRNA species. The results presented here confirm that the expression of both the GM-CSFRα and β mRNA is low, with the GM-CSFRα mRNA expression being in the pg range and the β mRNA expression at least one log lower. The lower limit of detection of the assays performed was 1 pg for GM-CSFRβ, and 10 pg for GM-CSFRα. The difference in the sensitivities between the GM-CSFRα and β assay is attributable to the differences in the specific activity of their respective probes. The specific activity of the β probe was consistently higher than the α probe (Table 3.1).

Few studies have investigated GM-CSFR mRNA expression and quantitative data has been limited. However, recently Heaney et al (1995) have studied GM-CSFRα levels in haemopoietic cells. This group, using quantitative RT-PCR, reported fg levels of GM-CSFRα mRNA in haemopoietic cells. The
differences between the data presented in this thesis and the data of Heaney et al may be explained by the different methodologies used. The detection limit for RNase protection assays has been reported to be in the 0.1 pg range, but the data presented here suggests that when using a target mRNA species as a standard, the detection limit is in the order of 1 pg.

RT-PCR is technically simpler than RNase protection assays and also has the potential to be a more sensitive method. However, there are a number of disadvantages to the use of RT-PCR for the purposes of quantification. The PCR process is by definition a chain reaction, and therefore an exponential process, and this exponential nature means that small changes in the amplification efficiency of any PCR reaction can yield dramatic differences in the amount of product. Several experimental factors, including the following, may affect the efficiency of amplification: the sequence being amplified; the sequence of the primers; the length of the sequence being amplified; and impurities in the sample. These factors can very easily give rise to-tube-to-tube variations in amplification efficiency. Another disadvantage of PCR is the plateau effect, with the initial exponential amplification levelling off as the rate of production of the product slows down. In order to overcome some of these problems, quantitative PCR using internal standards (Chelly et al 1988; Noonan et al 1990, Wang et al 1989) or competitive PCR (Gilliland et al 1990, Siebert & Fukuda, 1984) have both been employed.

Quantitative PCR using internal standards can either rely on amplification of endogenous sequence known to be present at constant levels (typically house keeping genes such as β-actin or GAPDH), or exogenous sequences added to the amplification reaction. However, the disadvantages of RT-PCR mentioned above still apply to both approaches. The amplification efficiencies may be different, plateaus for target and standards may occur at different time points, and since two sets of primers are utilised, primer interference may be a problem.
Competitive PCR uses homologous competitor fragments, known quantities added to the amplification reaction will compete for the same primers and therefore for amplification. A dilution series is made of either the target or the standard sequence, and a constant amount of the other component is added to each of the reactions. Quantification is performed after competitive amplification of the entire series of reactions, and is achieved by distinguishing the two PCR products from each tube by difference in size, hybridisation properties, or restriction enzyme sites. An important advantage of competitive PCR is that, because the ratio of target to standard remains constant during amplification, it is not necessary to obtain data before the reaction reaches a plateau phase. Whilst competitive PCR does give reliable results compatible with other methods, Pannetier et al (1993) have cautioned that competitive PCR may not provide accurate results when the sequences of the target and standard molecules are completely different (except for the primer sequences) and when the data are collected well after the plateau phase of the reaction. Therefore for each set of reactions, the amplification efficiency should be examined and because a series of dilutions are required for quantitation, this procedure may be as demanding as RNase protection assays. The latter may be less sensitive, but the results are highly reproducible and once optimal conditions are established, the technique is reasonably rapid to perform. Heaney et al (1995) employed a PCR technique that did not include an internal standard and therefore have not satisfactorily addressed the problem of tube to tube variation in amplification efficiency.

The results presented in this chapter demonstrate GM-CSFRα mRNA expression in a number of haemopoietic cell lines (TF-1, HL-60 and U937). This is consistent with the previous demonstration of GM-CSFRα protein on the surface of these cells (Chiba et al, 1990; Khwaja et al, 1993; Roberts et al, 1994). In these cells, the expression of GM-CSFRα mRNA was consistently higher than
that of GM-CSFRβ mRNA, which is in keeping with the receptor protein expression.

There was no detectable GM-CSFRα or β mRNA expression in the lymphoid cell lines nor surprisingly in the erythroleukaemia cell line K562. This finding is consistent with receptor binding studies which have failed to demonstrate detectable membrane protein (Khwaja et al, 1993; Heaney et al, 1995). Although the significance of this finding is unclear, it may be exploited to study downstream signalling events associated with the GM-CSFR. Most studies to date have used murine lymphoid cell lines (BaF 3 and CTLL) for study of signalling events in cells transfected with the GM-CSFRα and β cDNA (Sakamaki et al, 1992; Polotskaya et al, 1994). Arguably studies with a human erythroleukaemia cell line may be a closer reflection of events in myeloid cells.

The extra, unexpected bands resulting from the RNase protection assays presented in this chapter may be attributable to a number of different processes. Of these, point mutations in the probe, especially those initially cloned using RT-PCR, should be considered as prime candidates. RNase protection assays are useful for detection of alternatively spliced transcripts which can give rise to various isoforms. The 64 base band on the GM-CSFRβ assays represents the βTT isoform. This truncated isoform, which has an intracytoplasmic tail of 46 amino acids instead of 432, represents approximately 20% of the total β chain message in haemopoietic cell lines and primary haemopoietic cells, and up to 90% of the GM-CSFRβ mRNA in blasts of patients with AML. This increased level in AML was not reflected in early progenitor CD34+ cells and did not relate to a particular stage of myeloid differentiation, as AML patients with all FAB types were found to have high levels of βTT message (Gale et al, 1994).

The 269 base fragment detected using the α chain protection assays might have been due to the probe hybridising to transcripts which do not encode for GM-CSFRα but have related sequences. This is unlikely since the assay is
sensitive to a single base mismatch between target and probe (as clearly shown by
the data for the GM-CSFRβ mRNA protection assays) and therefore differing but
related target RNA will result in much smaller protected fragments after RNase
treatment. A polymorphism resulting from a single base mismatch could be
excluded as the relative intensity of the 269 band was similar in all cells. Another
possibility is that this fragment could have been formed by alternative splicing in
the 5' end of the GM-CSFRα. At the time these experiments were completed
(August 1993), the organisation of the 5' region of the GM-CSFRα chain had
been defined by analysis of 3 clones from a cosmid library using restriction site
mapping, Southern hybridisation and nucleic acid sequencing (Rappold et al,
1992). These data, together with genomic sequencing data (data not shown),
suggested that exon 2 consisted of 88 bp, exon 3 of 102 bp and exon 4 of 143 bp.
The size of exon 5 was unknown. The group initially describing the organisation
of the 5' region were unable to find the location of exon 1 encoding the first 35
bp. The probe used for the GM-CSFRα RNase protection assay covers part of
exons 2 to exon 5 (Figure 3.2). If alternative splicing was responsible for the
extra 269 base band, it is unlikely to have been due to loss of exons 3 or 4 as this
would result in bands of 167 and 22 bases or 124 and 24 bases, respectively. The
only other explanation was that exon 2 was deleted by alternative splicing. In
order to investigate this possibility a nested RT-PCR approach was used and the
results are presented in Chapter 4.

The 167 bp protected fragment seen on the RNase protection assays may
also represent an alternatively spliced isoform, possibly a result of deletion of
exon 3, which encodes for the signal peptide of the GM-CSFRα chain. This has
recently been characterised (Wagner et al, 1994) and the implications of this will
be discussed in Chapter 4.
— CHAPTER 4 —

ALTERNATIVE SPLICING OF THE 5' UNTRANSLATED REGION OF THE GM-CSFRα mRNA
4.1 Introduction

The aim of this chapter was to further investigate the possibility of an alternative splicing process at the 5' end of the GM-CSFRα. In the results presented in chapter 3, an extra 269 base band fragment was observed consistently in all the cell lines that expressed GM-CSFRα, and indeed represented the main mRNA transcript. One possible explanation is the existence of an mRNA transcript that had undergone deletion of exon 2. In order to investigate this possibility, RT-PCR of haemopoietic cell RNA was carried out using primers that spanned the sequence from nt 1 to 392 - covering the first 5 exons of the 5' end of the GM-CSFRα.

4.2 Methods

RT-PCR ANALYSIS OF 5' ISOFORMS  500 ng-1 µg of total cellular RNA from TF-1 cells was used as a template for the reverse transcriptase PCR reaction. The RT-PCR was performed as described in Section 3.2.1, with the exception that an annealing temperature of 62°C was used. Primers E and B were used to amplify a 392 bp fragment (nucleotides 1-392 of the GM-CSFRα cDNA, Figure 1.4).

PURIFICATION OF DNA FROM LOW MELTING POINT AGAROSE Section 2.4.2
DNA SEQUENCING Section 2.6
Results of the RNase protection assays (Chapter 3) suggested that a deletion of exon 2 of GM-CSFRα may have occurred. If this was in fact the case, the RT-PCR strategy should yield 2 bands: a 392 bp band from the full length GM-CSFRα and a 304 bp band representing the product of splicing of the whole of exon 2 (which consists of 88 bp).

In fact 3 bands were observed: the expected 392 bp fragment; a 368 bp fragment; and a faint 304 bp band, which would have been consistent with the predicted deletion of exon 2. These bands were gel purified and the PCR products sequenced. The 3 bands were not only present in TF-1 cells but also in HL-60 cells, monocytes, neutrophils and in blast cells from 4 patients with AML. Although the RT-PCR assay was not strictly quantitative, the intensity of the 368 bp band on ethidium bromide staining was similar to the larger 392 bp band, and the 304 bp band was in the order of 10% of the total (Figure 4.2).
Figure 4.2 RT-PCR of RNA from cell lines, primary haemopoietic cells and blasts from patients with AML using GM-CSFRα primers

A=TF-1 cells; B=HL-60 cells; C=neutrophils; D=monocytes; E, G, F and H=AML blasts; I=no RNA (negative control)

SEQUENCING The three bands obtained by large scale RT-PCR of total RNA from TF-1 cells were sequenced. The 392 bp band had a sequence corresponding exactly to the published sequence of the full-length GM-CSFRα (Gearing et al, 1989). The sequence of the 304 bp fragment indicated that this band was due to a deletion of the entire second 88 bp exon (nucleotides 36-123). The sequence of the 368 bp fragment indicated that there was a 24 bp deletion (nucleotides 100-123) at the 3' end of exon 2 (Figure 4.3). This results in a new exon-intron boundary as a consequence of the use of an alternative donor site (GT) within exon 2, whilst still retaining the same 3' acceptor site. This alternative splicing at
Figure 4.3 Alternative splicing of GM-CSFRα as indicated by sequencing of RT-PCR products

The alternative splice donor site GT and a polypyrimidine tract at the 3’ end of exon 2 are underlined
the 5' end of the mRNA concurs with the results of the RNAse protection assays. Deletion of either exon 2 or deletion of part of exon 2 could explain the 'extra' 269 base band on the RNAse protection assays (Figure 3.11). Neither of the deletions alter the signal peptide or the mature protein which arises from the second AUG start site which is at nucleotide 150 in exon 3. This is in fact the second open reading frame (ORF) since there is a first AUG start site at nucleotide 25. Although the AUG of the first ORF is in poor context (Kozak, 1984), it can nonetheless potentially give rise to a peptide of 22 amino acids. Deletion of exon 2 would alter the peptide sequence from this first ORF and result in a peptide of 23 amino acids as shown in Figure 4.4. Deletion of part of exon 2 does not alter the first ORF.

Evaluation of the probable effect of the exon 2 deletions on the GM-CSFRα mRNA secondary structure was made using the Vienna RNA programme (Zuker, 1989). The 5' untranslated leader full length GM-CSFRα mRNA has a predicted hairpin loop between nucleotides 70 and 120. Both the partial and complete deletion of exon 2 result in loss of this hairpin loop (Figure 4.5).

![Figure 4.4 Exon structure of the 5' end of GM-CSFRα](image)

This shows the second open reading frame which gives rise to the receptor protein.
The full length GM-CSFRα mRNA contains a hairpin loop between nucleotides 70 and 120 (as predicted by computer modelling). This is absent in both exon 2 and exon 2b deleted isoforms.
4.4 Discussion

The results presented in this chapter indicate that transcripts containing a deletion of a 24 bp fragment at the 3' end of exon 2 and a complete deletion of exon 2 (88 bp) of the GM-CSFRα, are present in primary myeloid haemopoietic cells and cell lines. Part of the exon 2 is deleted by alternative use of the 5' donor site within exon 2 (Figure 4.3). The deleted forms together are more abundant than the full length transcript, as shown by quantification of the RNase protection assays in Chapter 3 (Table 3.4). The exon 2 deleted forms being twice as highly expressed as the originally described full length GM-CSFRα sequence. It should be noted that both of these alternatively spliced forms give rise to the same sized band in this assay and were only distinguishable by means of RT-PCR. These transcripts are expressed in both cytoplasmic and nuclear RNA, suggesting that they do not simply represent intermediate spliced forms of heteronuclear RNA (Figure 3.18). Alternative splicing of primary transcripts can give rise to different proteins, as is the case with 3' end of the GM-CSFRα, but obviously this is not the case where the alternative splicing occurs in the 5' untranslated region. In this case the alternative splicing could affect translational efficiency by the removal of control regions which bind to trans acting RNA binding proteins or by alteration of secondary structure.

The alternative isoforms described here for GM-CSFRα lead to a shorter leader sequence and therefore one may expect more efficient translation of the exon 2 deleted mRNA isoforms if leader length per se regulated translational efficiency. The role of RNA secondary structure in eukaryotes has been extensively evaluated and it has been shown that RNA from regulatory genes such as growth factors, cytokine receptors and oncogenes, have 5' untranslated leader sequences with considerable secondary structure. These 5' sequences have been shown in some circumstances to regulate translation (Kozak, 1991a). The fact that
both exon 2 deleted isoforms result in removal of a hairpin loop at nt 70-120 would also suggest that these isoforms should be more efficiently translated. The relative translational efficiencies of the full length and both exon 2 deleted isoforms were investigated and the results are presented in Chapter 5. The exon 2 and part exon 2 deleted isoforms will henceforth be referred to as exon 2 and 2b deleted isoforms, respectively.

As described in Chapter 1, there are at least 6 isoforms described to date, arising from alternative splicing occurring at the 3' end of the GM-CSFRα. The data presented in this chapter suggests that variable splicing may also occur at the 5' end of the receptor cDNA. Alternative splicing of exon 2 or 2b gives rise to three transcripts with different 5' UTR leader sequences and may potentially regulate translation of the receptor. In addition to deletion of exon 2 or exon 2b, there is evidence to suggest that exon 3 is also spliced out in certain cell lines and in some patients with AML, as RNase protection assays of TF-1 cells, U937 cells and monocytes showed a consistently high expression of a 167 base fragment (Figure 3.13).

The mechanisms controlling of the variable splicing of the GM-CSFRα chain have yet to be elucidated but may involve a network of trans-acting protein factors and cis-acting pre mRNA sequence elements. The mechanisms involved in splicing are described in chapter 1. Alternative splicing which involves the differential use of splicing functions, often in a cell specific manner (Screaton et al, 1995), would also require a highly regulated mechanism, the nature of which is largely unknown. The protein and RNA sequences that interact to yield one RNA splice form rather than another remain undetermined. The few alternative splicing factors which have been isolated so far, include members of the SR protein family. This protein family comprises at least six members (SRp75, SRp55, SRp40, SRp30a, SRp30b and SR20). Interestingly, these factors are conserved from Drosophila to humans (Zahler et al 1992). The SR proteins share
a characteristic N-terminal domain that binds RNA and a C-terminal serine-arginine rich domain. Most of the information about the functions of SR proteins has come from in vitro experiments. In nuclear extracts, SR proteins are able to regulate alternative splicing of a variety of pre-mRNAs, including thalassaemic human β-globin (Mayeda et al 1992) and bovine growth hormone (Sun et al 1993). More recently it has been shown that ASF/SF2, the human SRp30a protein, is able to affect the selection of 5' splice sites in vivo (Caceres et al 1994). Moreover, AS/SF2 has been shown to bind to 5' splice sites and to U1 snRNPs and can mediate interactions between U1 and U2. These interactions may be important for both constitutive splicing and the regulation of alternative splicing. Interestingly, different tissues exhibit variations in the relative amounts of individual SR proteins, and the switching of splice choice may be affected by subtle changes in concentration of the SR proteins. Whether SR proteins regulate GM-CSFR expression is open to speculation, although it is worth noting that one of the targets of the Drosophila SR proteins is a polypyrimidine tract (Heinrichs & Baker, 1995) which is absent in the exon 2b deleted isoform (Figure 4.3).
IN VITRO TRANSLATION OF THE 5' UNTRANSLATED ISOFORMS OF GM-CSFRα
5.1 Introduction

The aim of this chapter was to investigate the functional relevance of the exon 2 deleted isoforms described in chapter 4. These isoforms alter the leader sequence prior to the AUG translation start site and could therefore possibly modulate the translational efficiency, and hence the expression of the GM-CSFRα protein. Studies performed during the seventies and early eighties have led to a greater understanding of the processes involved in the regulation of eukaryotic translation. Although the last few years have seen the discovery of increasing numbers of factors/proteins that participate in this process, the basic elements of the translation pathway follow the principles established in the earlier studies (Melefors & Hentze, 1993). Figure 1.12 shows a simplified schematic representation of the current model for the initiation of eukaryotic translation.

The steps involved in translation are catalysed by eukaryotic initiation factors (eIFs), of which eIF 2 and 4 are best characterised. Translation starts with the dissociation of an 80S ribosome into 40S and 60S subunits. The 40S subunit attaches to met-tRNA and phosphorylated eIF2 to form a pre-initiation complex. The pre-initiation complex binds at, or near, the m7GpppG 5' cap structure on the mRNA with another initiation factor eIF4. The complex then scans the 5' untranslated region of the mRNA for an AUG start codon which is in good context and joins the 60 S subunit at this AUG start site. Translation may now begin. Scanning of the 5' UTR is therefore an important step in translation initiation. Elements in the 5' untranslated leader sequence can therefore modulate translation and may be one of the sites for post-transcriptional regulation of gene expression (Kozak, 1991b).

Cell-free protein synthesis systems have been used in recent years for the translation of eukaryotic mRNAs. Of these, the rabbit reticulocyte lysate and the
wheat germ extract have received the most attention and have been fairly well
characterised (Trachsel et al., 1978).

There are a number of reasons for the preferred use of the rabbit
reticulocyte lysate system: the preparation can be prepared with relative ease; it has
a high translational activity with endogenous or exogenous mRNA; and it is
relatively stable when stored at -80°C. The reticulocytes are purified to remove
contaminating cells, which could otherwise alter the translational properties of the
final extract. After the reticulocytes are lysed, the extract is treated with nuclease
to destroy endogenous mRNA, and thus reduce background translation to a
minimum. The lysate contains the cellular components necessary for protein
synthesis tRNA, ribosomes, amino acids, and initiation, elongation, and
termination factors. Reticulocyte preparations are further optimised for mRNA
translation by addition of the following: (i) an energy generating system
consisting of pre-tested phosphocreatine kinase and phosphocreatine; (ii) a
mixture of tRNAs to expand the range of mRNAs which can be translated; (iii)
hemin to prevent inhibition of initiation; and (iv) potassium acetate and
magnesium acetate to maintain an endogenous concentration of the potassium and
magnesium ions.

In this chapter, the relative translational efficiencies of GM-CSFRα
protein from constructs representing the naturally occurring 5' UTR alternatively
spliced isoforms described in chapter 4 are investigated. RNA binding proteins
have recently been shown to bind 5'UTR sequences and modulate translation,
particularly in iron metabolism (Klausner et al. 1994), and in the regulation of
ribosomal proteins (Kaspar et al., 1990; Standart & Jackson 1994). Changes in the
5' UTR sequences may therefore be of particular interest as there is data to
indicate that the regulation of both the GM-CSFRα and β chains are controlled at
the post-transcriptional level (Chapter 6).
5.2 Methods

RT-PCR products of the full length GM-CSFRα and the exon 2 deleted isoforms were generated using the nested RT-PCR strategy and primers described in Section 4.2. The primers E and B would yield a 392 bp fragment which represents the full length GM-CSFRα sequence, a 368 bp fragment resulting from alternative splicing of exon 2b, and a 304 bp fragment due to alternative splicing of exon 2 (Figure 5.1).

PURIFICATION OF DNA FROM LOW-MELTING POINT AGAROSE Section 2.4.2

CLONING OF THE PCR PRODUCTS Section 3.2.1. The gel purified PCR products were cloned into the pGEM-T plasmid vector (Promega) in the sense direction to the T7 promoter. In order to juxtapose the GM-CSFRα PCR inserts within 4 nucleotides of the T7 promoter, and thus minimise any interference of the plasmid sequence in the translation experiments, a 39 bp plasmid sequence that occurs between the plasmid sequence and the cloning site was digested out using ApaI and Sac II restriction digests, and then subjected to re-ligation (Figure 5.1).

TRANSCRIPTION OF THE mRNA Each construct was transcribed using the protocol reported in Section 3.2.2. Transcription was controlled by monitoring mRNA transcribed from equimolar amounts of each construct using T7 polymerase in the presence of α\(^{32}\)P-CTP (>400Ci/mM). Two μl of the final transcription reaction were removed and added to 198 μl of TE buffer (25 mM Tris, 10 mM EDTA, pH 7.5) containing 100 μg of carrier tRNA. One hundred μl of this diluted reaction was directly counted in a scintillation counter and the other 100 μl was used to determine the amount of \(^{32}\)P incorporated CTP by adding 2 ml of ice cold 10% (v/v) TCA to the sample, vortexing and leaving it on ice. The resulting precipitate was filtered under vacuum on GF/C glass fibre filters.
Figure 5.1  pGEM-T cDNA constructs for translation experiments
(Whatman UK). The filter was washed through under vacuum, once with 2 ml of 5% (v/v) TCA and twice with 2 ml of 100% ethanol. The filter was then dried in an 80°C oven for 1 hour and counted in a scintillation counter. By dividing the counts of the washed and TCA-precipitated filters by non-TCA precipitated reaction, the proportion of incorporated radioactivity was calculated (Section 3.3.2).

**IN VITRO TRANSLATION OF THE 5' ISOFORMS** The Promega TNT™ Coupled Reticulocyte Lysate System was used in these experiments. This system allows transcription and then translation of the first 81 amino acids of the GM-CSFRα from the constructs cloned into the pGEM-T vector. Only 81 amino acids would be translated because the constructs contained the second AUG start site in good context at nucleotide 150 and the second open reading frame up to nucleotides 392. The calculated molecular weight of the resulting translation product is 11 kDa.

The TNT Rabbit Reticulocyte Lysate reaction was carried out according to the manufacturer's instructions. All the reagents for the reaction were stored at -70°C and then thawed on ice immediately before use. The reaction components were added in the following order to a 1.5 ml microfuge tube stored on ice:

- **TNT rabbit Reticulocyte Lysate** 25 μl
- **TNT Reaction Buffer** 2 μl
- **TNT T7 RNA Polymerase** 1 μl
- **Amino acid mixture minus methionine** 1 μl
- **35S-methionine (1000 Ci/mM) at 10 mCi/ml** 4 μl
- **RNasin Ribonuclease Inhibitor 40 u/μl** 1 μl
- **DNA template** 1 μM
- **DEPEC treated H2O** to final volume 50 μl
The reaction was then incubated at 30°C. The optimal plasmid concentrations and incubation times for these reactions were established, such that the yield of the translation product, as calculated by 35S methionine incorporation, was on the linear part of the protein translation curve. According to the manufacturer's instructions, it was not necessary to linearise the plasmid for the coupled transcription and translational experiments, as circular plasmids give the best translation results. A luciferase control DNA template, yielding a monomeric 61 kDa translation product, was used as positive control.

The translation product was separated by electrophoresis through a 20% SDS polyacrylamide gel. The gel was fixed in 15% (v/v) methanol containing 5% (v/v) acetic acid for 30 minutes, dried and exposed to phosphorimaging plates. The radioactive signal was then visualised and quantified using the Fujimax phophorimager as described in Section 3.2.

5.3 Results

TRANSCRIPTION OF THE mRNA FROM THE CLONED CONSTRUCTS
Since the TNT system used required a coupled translation and translation step, it was necessary to ensure that equal amounts of RNA were transcribed from each of the 3 constructs. Equimolar amounts of the cDNA constructs (1 μM) were therefore used to calculate the amount of RNA transcribed by measuring 32P-CTP incorporation using T7 polymerase (Section 3.3.2). The specific activities of the transcribed RNAs were similar: 1.79 x 10^8, 1.68 x 10^8 and 1.72 x 10^8 cpm/μg for the full length construct, exon 2 and exon 2b deleted constructs, respectively. These data demonstrate that equimolar amounts of cDNA could be used to produce equal amounts of RNA.
OPTIMISATION OF THE IN-VITRO TRANSLATION REACTION CONDITIONS

In order to compare the relative translation of the full length GM-CSFRα and both exon 2 deleted isoforms the in vitro translation reaction was optimised such that the yield of the translation product, as calculated by $^{35}$S methionine incorporation, was on the linear part of the protein translation curve. These optimisation experiments were performed using the full length construct.

The effect of DNA template concentration on rate of translation was first investigated. DNA template concentrations between 125 ng and 2 μg were investigated using a reaction time of 120 minutes (time recommended by manufacturer for optimal translation). At 250 ng of template, translation was almost maximal (Figure 5.2) and lower concentrations of the DNA template were therefore used. 125 ng was selected as the concentration for further experiments.

Using 125 ng of DNA template, the effect of incubation time on the reaction was investigated over a time course of 120 minutes. In this case, the plateau phase was reached at 45 minutes (data not shown) and a 30 minute incubation period was selected for further experiments.

COMPARISON OF THE IN-VITRO TRANSLATION OF THE 5' UTR CONSTRUCTS

The full length, exon 2 and exon 2b deleted constructs were translated at the optimal conditions established as described above using 125 ng of DNA in the reaction mixture, which is roughly equivalent to 1 μmol of plasmid DNA.

Using these conditions, all three DNA constructs yielded the expected 11 kDa product (Figure 5.3). The luciferase control gave the expected 61 kDa protein. The radioactive signal from each construct was quantified using a phosphorimager and the results expressed both as arbitrary 'phosphorimaging units' and as a percentage of the full length translation product. Although there
Figure 5.2 The effect of DNA template concentration on rate of translation of the full-length GM-CSFRα construct
was variation in translation between the different batches of reticulocyte preparations, the results from each preparation consistently showed greater translation with the Exon 2b deleted construct, with means of 164, 128 and 305%, as compared with the full length construct in the three reticulocyte preparations. Conversely, there was reduced translation from the Exon 2 deleted construct as compared to the 392 bp construct, with means of 53, 73, 56 %, respectively. The amount of translation was significantly different for the three constructs when analysis of variance (ANOVA) was applied to the results of all 9 experiments (p=0.01). The relative signals for 1 μM template are shown in Figure 5.4.
Figure 5.4 Results of translation experiments using GM-CSFRα constructs (1 μM DNA)

- □ full length construct (392 nt)
- □ exon 2b deleted (368 nt)
- □ Exon 2 deleted (304 nt)

(results represent the mean and SEM of 3 separate experiments for each preparation)
5.4 Discussion

Alternative splicing of primary transcripts can give rise to different proteins. This has been demonstrated for 3' end of the GM-CSFRα (Figure 1.5). However, this cannot occur if alternative splicing occurs in the 5' UTR. The data presented in this chapter, however, demonstrate that the alternatively spliced 5' isoforms of the GM-CSFRα, involving the 5' UTR, have the potential to be translated with different efficiencies. Deletion of exon 2b increases the translation of the receptor protein, whereas splicing out the whole of exon 2 decreases translation. In the \textit{in vitro} system used here, the 5' end of each mRNA was not capped, and we cannot therefore rule out the possibility that cap binding proteins affect the translation of these species. However, these \textit{in vitro} studies have allowed identification of putative regulatory sequences that may modulate translation of the GM-CSFRα chain.

It has previously been shown for a number of genes such as the Hox 5.1 (Cianetti \textit{et al}, 1990), c-sis (Ratner \textit{et al}, 1989), L-myc (Dosaka \textit{et al}, 1991), Ick (Reynolds \textit{et al}, 1990) and TGFβ (Arrick \textit{et al}, 1991), that transcripts containing the short leader sequences are produced in the cell in addition to full length mRNAs. The shorter leader sequences are translated more efficiently and a switch to the shorter mRNA species can be regulated developmentally (Kozak \textit{et al}, 1991b). The alternative isoforms described here for GM-CSFRα lead to a shorter leader sequence, and whilst the exon 2b deleted isoform does indeed translate better than the full length isoform, the exon 2 deleted isoform does not. It should be noted that there is no developmental associated change in the GM-CSFRα isoforms as the same relative amounts are present in blasts and mature neutrophils (Section 3.3.5).

The role of RNA secondary structure in eukaryotes has been extensively evaluated and it has been shown that RNA from regulatory genes such as growth
factors, cytokine receptors and oncogenes have 5' untranslated leader sequences with considerable secondary structure (Kozak, 1991a). These 5' sequences have been shown in some circumstances to regulate translation (Gabelle & Morris, 1994). The fact that both exon 2 deleted isoforms result in removal of a hairpin loop at nt 70 - 120, but translate with different efficiencies, suggests that loss of this particular secondary structure does not explain the translation differences. Furthermore, removal of the hairpin loop does not result in a change in mRNA stability as the half lives of the mRNA species described here were similar (between 1 and 3 hours in three experiments; Figure 6.3a). Sequence elements in exon 2 may therefore be more important in this regulatory function, and these sequences may act as recognition sites for as yet unidentified RNA binding proteins.

One potential regulatory sequence is a polypyrimidine tract at the 3' end of exon 2, that is deleted in both isoforms (sequence underlined in Figure 4.3). Recent studies have suggested that transcripts containing a polypyrimidine tract at their 5' end are preferentially translated when the ribosomal protein S6 is phosphorylated by S6 kinase (Jefferies et al, 1994). S6 kinase is activated by a variety of mitogens, including insulin, at the cell surface, and this process is itself mediated by one of the phosphatidylinositol-3-OH kinase family (Downward, 1995). This represents a mechanism of translational control which would be dependent on the mitogenic signal received by the cell. Removal of the polypyrimidine tract in the GM-CSFRα resulted in decreased translation from the exon 2 deleted construct, but increased translation when only exon 2b was deleted. One explanation for this discrepancy may be that there are as yet other sequences within exon 2 which are involved in modulating translation, or there is complex cis interaction between the 5' and 3' sequences of exon 2. Such complex interactions are often dependent on a cascade of events induced by mitogenic signals and will not be identified by in vitro reticulocyte lysate experiments.
It is also worth noting that the α chains of the IL-3 and IL-5 receptor untranslated sequences contain a polypyrimidine tract. Further studies to evaluate the alternative splicing of these two receptors would be of value in order to determine whether the regulation mechanism for the GM-CSFRα was common to these closely related cytokine receptors.
REGULATION OF THE HUMAN GM-CSFR DURING DYNAMIC CHANGES IN CELL SURFACE EXPRESSION AND DIFFERENTIATION
6.1 Introduction

Complex processes such as growth factor-mediated cell proliferation require multiple levels of molecular regulation for the variety of proteins involved (Pardee, 1994), and there is a body of evidence that the IL-4R is regulated both transcriptionally and at the post transcriptional level (Dokter et al, 1992). For GM-CSF, IL-3 and IL-5 receptors there is limited data as to how cell surface expression is regulated. There is some evidence in the murine mast cell line, MC 9, that the β chain may be post transcriptionally regulated (Hara et al, 1994). An understanding of the mechanisms involved in the regulation of GM-CSFR expression, may be of importance in understanding the processes involved in leukaemogenesis as well as normal haemopoiesis.

Haemopoietic growth factors, including GM-CSF, have been reported to be involved in autocrine or paracrine pathways of growth stimulation in myeloid malignancies such as AML, CML and JCML, resulting in a growth advantage for the malignant clones (Daley et al, 1990; Lowenberg & Touw, 1993). Structural abnormalities in the receptor may alter responses to growth factor, but studies have failed to demonstrate specific point mutations in the GM-CSFRα or β chains in AML or JCML (Wagner et al 1995; Freeburn et al, 1995a; Freeburn et al, 1995b). The mechanism by which the haemopoietic growth factors influence leukaemogenesis may therefore be dependent on alterations in their specific signal transduction pathways, or alterations in the regulation of haemopoietic growth factor receptor expression.

Modulation of the receptor expression can be short-term, such as rapid ligand-induced receptor turnover, or longer-term, due to differentiation-linked regulation. Such modulation has been demonstrated using haemopoietic cells lines. For example, the human erythroleukaemia TF-1 cell line, which can grow
either in GM-CSF or Epo (Kitamura et al, 1989), expresses both low and high affinity GM-CSFR (Table 6.1), and in media containing either GM-CSF or Epo the receptor expression undergoes dynamic changes over a period of hours, with rapid down- or up-regulation respectively (Khwaja et al, 1993). Differentiation-linked changes in GM-CSFR expression have been demonstrated in HL-60 cells, where dimethylsulfoxide (DMSO) induced maturation was accompanied by a two-fold increase in the number of both low and high affinity receptors expressed, although there was no apparent change in receptor affinity (Roberts et al, 1994; Table 6.1). In addition, primary myeloid blast cells are known to express both low and high affinity receptors, whereas mature neutrophils express only a single class of receptor with relatively high affinity (Budel et al, 1993; Khwaja et al, 1993).

In order to investigate the underlying mechanisms by which the receptor expression is regulated, GM-CSFR binding and mRNA expression were investigated in two model systems: in TF-1 cells undergoing down and up-regulation of surface receptors to examine short term dynamic changes in expression; and in HL-60 cells induced to differentiate to longer term changes.

### 6.2 Methods

**Reagents** All reagents were from Merck Ltd unless stated otherwise.

- Dimethyl sulfoxide (DMSO)
- Cycloheximide (Sigma Chemical Co) - 10 μg/ml
- Actinomycin-D (Sigma Chemical Co) - 5 μg/ml in DMSO and ethanol (5:1 v/v)
- Nitroblue tetrazolium (NBT)
<table>
<thead>
<tr>
<th></th>
<th>HL-60*</th>
<th>TF-1**</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 0</strong> (uninduced)</td>
<td><strong>Day 5</strong> (DMSO induced)</td>
<td><strong>Neutrophils</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Low affinity receptors</strong></td>
<td><strong>(receptor number / cell)</strong></td>
<td><strong>(receptor number / cell)</strong></td>
<td><strong>(receptor number / cell)</strong></td>
</tr>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 5</td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td>(uninduced)</td>
<td>(DMSO induced)</td>
<td>(DMSO induced)</td>
</tr>
<tr>
<td><strong>Low affinity receptors</strong></td>
<td><strong>(receptor number / cell)</strong></td>
<td><strong>(receptor number / cell)</strong></td>
<td><strong>(receptor number / cell)</strong></td>
</tr>
<tr>
<td></td>
<td>1231 ± 398</td>
<td>2366 ± 788</td>
<td>2000 ± 450</td>
</tr>
<tr>
<td></td>
<td>(k_d =1.5±0.50 nM)</td>
<td>(k_d =1.6±0.14 nM)</td>
<td>(k_d =1.8±0.3 nM)</td>
</tr>
<tr>
<td><strong>High affinity receptors</strong></td>
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<td><strong>(receptor number / cell)</strong></td>
</tr>
<tr>
<td></td>
<td>299 ±98</td>
<td>647 ±249</td>
<td>1100 ± 200</td>
</tr>
<tr>
<td></td>
<td>(k_d =62±10 pM)</td>
<td>(k_d =36±6 pM)</td>
<td>(k_d =50±15 pM)</td>
</tr>
</tbody>
</table>

* Roberts *et al.*, 1994
** Khwaja *et al.*, 1993

Table 6.1 Expression of GMCSFR protein in HL60 and TF-1 cells, and neutrophils
* 12-O-tetradecanoylphorbol 13 acetate (TPA)
* Tricholoroacetic acid (TCA)
* Phosphate buffered saline (PBS), pH 3.0 - 0.01 M phosphate. 0.15 M sodium chloride
* TF-1 cells (Section 2.2)
* HL-60 cells (Section 2.2)
* Neutrophils (Section 2.3)

Methods

DIFFERENTIATION OF HL-60 CELLS  HL-60 cells were grown in parallel with or without DMSO (1.25% (v/v)) for 5-7 days in order to induce maturation (Roberts et al, 1990a). Cells were initially seeded at 1x 10^4/ml and cell concentrations (determined using a Neubauer counting chamber) were kept below 5 x 10^5/ml in order to prevent overcrowding. Cell viability was assessed by the exclusion of trypan blue, and cell morphology was determined using cytospin preparations stained with Leishman's stain.

FUNCTIONAL MATURATION  Cell maturation was assessed by an adaptation of the NBT test (Roberts et al, 1989). Briefly, cells were incubated at 37°C for 30 minutes in NBT (0.05% (w/v) in RPMI medium containing 1 μg/ml TPA) in order to stimulate the respiratory burst. Control samples were incubated without TPA to measure the resting level of NBT reduction. Positive cells containing black deposits of formazan were identified by light microscopy. The percentage of positive cells was calculated from a total of 300 cells in each sample. Cell viability was assessed by trypan blue exclusion.

125I-GM-CSF BINDING ASSAYS (Section 2.7.1)  In some experiments multiple concentrations of 125I-GM-CSF, ranging from 1 pM to 2 nM, were used with Scatchard analysis to determine receptor numbers and affinities (Khwaja et al, 1993). In other experiments either a single concentration (500 pM) or two concentrations (100 pM and 2 nM) of 125I-GM-CSF were used. Previous investigation of GM-CSFR number and affinities demonstrated that at 100 pM
$^{125}$I-GM-CSF, 80% of cell associated radioactivity was attributable to binding to high affinity receptors and 20% to low affinity receptors; at 500 pM $^{125}$I-GM-CSF, cell associated radioactivity was equally bound to both high and low affinity receptors; and at 2 nM $^{125}$I-GM-CSF, 30% of radioactivity was attributable to binding to high affinity receptors and 70% to binding to the low affinity receptor (Khwaja et al., 1993).

Cells were incubated with ligand in order to maximally down-regulate GM-CSFR (Khwaja et al., 1993). Briefly, cells maintained in Epo (1 IU/ml) for 18 hours were washed and then incubated for 18 hours at 37°C in medium containing either GM-CSF (360 pM) or FCS diluent as control. To assess residual surface GM-CSF receptors, any remaining molecules bound at the cell surface were removed by a low pH wash (Cannistra et al. 1990). Following centrifugation, the cell pellet was resuspended in ice-cold PBS (pH 3) for 2 minutes, topped up with an excess of binding buffer and washed three times in binding buffer. Control cells underwent the identical procedure. In pilot experiments this process did not affect subsequent binding of $^{125}$I-GM-CSF significantly (greater than 95% binding was obtained following a low pH wash compared with 100% binding on unwashed TF-1 cells) and was shown to remove more than 90% of surface-bound GM-CSF. The re-expression of the receptor was examined by removing the GM-CSF by four large volume washes and resuspending the cells in Epo (1 IU/ml) to maintain viability. Re-expressed receptor was measured by $^{125}$I-GM-CSF binding at 4°C for 16 hours, after a further low pH wash.

**MEASUREMENT OF GM-CSFR α AND β CHAIN EXPRESSION BY ANTIBODY BINDING** (Section 2.7.2)

**EVALUATION OF LIGAND DEGRADATION** TF-1 cells (3 x $10^6$ cells/ml), were incubated with $^{125}$I-GM-CSF (500 pM in binding buffer) for 2 hours at 4°C. After this time, cells were washed twice with ice-cold binding buffer,
resuspended at the original cell concentration and transferred to a 37°C water bath. At each stated time point, two aliquots of 3 x 10^6 cells were removed, centrifuged at 180 x g for 5 minutes and the supernatants removed. One cell pellet was resuspended in 200 µl of binding buffer, the other cell pellet was resuspended in PBS (pH 3) for 3 minutes at 4°C, and the radioactivities of the cell pellets were then determined. TCA was added to the supernatants (10% (v/v), final concentration), and these were incubated at 4°C for 30 minutes before centrifugation at 10,000 x g for 8 minutes. The radioactivities of the supernatant containing low molecular weight TCA soluble fragments of 125I-GM-CSF and the precipitate containing intact 125I-GM-CSF were determined.

RNase PROTECTION ASSAY (Section 3.2.3)

6.3 Results

6.3.1 Short-term dynamic changes in receptor expression

EXPRESSION OF GM-CSFR PROTEIN AND mRNA IN TF-1 CELLS IN THE ABSENCE OF SPECIFIC LIGAND.

Protein In the absence of specific ligand, expression of the GM-CSFR is up-regulated (Khwaja et al, 1993). Therefore, in order to investigate the half-life of the surface GM-CSFRs, TF-1 cells cultured in Epo (1 IU/ml) for twenty four hours were split into aliquots and cycloheximide added to one aliquot at a dose that inhibits protein synthesis (10 µg/ml) (Walker & Burgess, 1987). Binding of 125I-GM-CSF (500 pM, final concentration) was then measured at 2 hour intervals for a 6 hour time period, followed by a further time point at 20 hours (Figure 6.1). At 4 hours the receptor binding had decreased to 53% ± 5 of time 0 values, and to 29% ± 3 at 6 hours. These data indicate that both high and low affinity receptors had been lost, as at this concentration of 125I-GM-CSF,
Figure 6.1  Half-life of surface GM-CSFR in TF-1 cells incubated in cycloheximide

(Mean ± SEM 3 separate experiments)
approximately 50% of binding could be attributed to the low affinity receptors, and 50% to the high affinity receptors. The half-life of the GM-CSFR (high and low affinity receptors together) in the absence of specific ligand is approximately 4 hours (Figure 6.1).

Surface receptor expression was also measured in the absence of transcription. Aliquots of TF-1 cells cultured in Epo were incubated with or without the transcription inhibitor actinomycin D (final concentration 5 μg/ml) and 125I-GM-CSF binding (500 pM) was measured after 0, 3 and 6 hours. There was no difference between surface ligand binding of treated and untreated cells during this time period: at 3 hours the 125I-GM-CSF binding was 123% ± 12 of time 0 values for cells treated with actinomycin, and 99% ± 5 for cells without actinomycin (mean ± SEM of 3 experiments). At 6 hours, the values were 125% ± 2 and 117% ± 4, respectively. The switching off of transcription by actinomycin-D thus results in a fall in GM-CSFRA mRNA expression without a concomitant fall in 125I-GM-CSF binding. This could be the result of efficient α chain translation maintaining cell surface receptors, or alternatively, be due to altered GM-CSFR affinity. Such a change in GM-CSFR affinity may occur if the relative numbers of α or β chains is altered by the actinomycin-D treatment, resulting in alternative oligomerisation of the α and β subunits (Budel et al, 1993; Wheadon et al, 1995).

The surface expression of the α and β chain proteins was therefore measured using specific anti-GM-CSFRA and β monoclonal antibodies in cells incubated with Epo and actinomycin-D. There was no significant change in the expression of the α or β chains over a 6 hour period, the α expression being 74 and 98% of time 0 values (mean 86%) and the β expression 89% and 101% of time 0 values (mean 95%) in two separate experiments. Thus the α chain expression was maintained at the cell surface even though the mRNA levels had fallen to 25%. These data suggest that post-transcriptional control of the α chain can occur to
maintain steady state receptor levels, albeit under the artificial circumstances of exposure to actinomycin-D.

**mRNA** Assessment of the half life of the GM-CSFRα and β chain mRNA in TF-1 cells was carried out using experiments parallel to those in which the half-life of surface binding of 125I-GM-CSF was determined. In these experiments, one aliquot of cells was incubated with Epo alone and the other was incubated with Epo and actinomycin-D, at a final concentration of 5 µg/ml, in order to inhibit transcription. The GM-CSFRα and β chain mRNA levels were measured serially using the RNase protection assay including actin as an internal control. The half-life of the GM-CSFRα mRNA was between 1-3 hours (Figure 6.2a), whereas the GM-CSFRβ mRNA half life was greater than 8 hours (Figure 6.2b). The viability of the TF-1 cells fell rapidly after 6 hours in actinomycin D from 82% at 6 hours to 55% at 12 hours, and the experiment was not therefore extended beyond 8 hours.

**EXPRESSION OF GM-CSFR IN THE PRESENCE AND ABSENCE OF SPECIFIC LIGAND**

**Protein** TF-1 cells cultured in Epo, that is with maximal GM-CSFR expression, were washed and incubated with GM-CSF (360 pM) at 37°C. At varying times, surface-bound ligand was removed using a low pH wash and the expression of both high and low affinity GM-CSFR was estimated using radio-ligand binding at two concentrations of 125I-GM-CSF (100 pM and 2 nM). Incubation in GM-CSF induced rapid down-regulation of surface receptors, after 4 hours in unlabelled GM-CSF, the binding of 100 pM 125I-GM-CSF was reduced to 12% ± 4 of time 0 values, and after 24 hours was 6% ± 5 (mean ± SEM, n=3) (Figure 6.3). This indicates that virtually all high affinity receptors were down-regulated.
Figure 6.2 (a) Half-life of GM-CSFRα mRNA and (b) GM-CSFRβ mRNA in TF-1 cells incubated in 5 μg/ml actinomycin D (time after exposure to Actinomycin D)
Figure 6.3 - Expression of high and low affinity GM-CSFR in TF-1 cells during specific ligand induced down-regulation and subsequent up-regulation following removal of ligand.

Ligand was removed by high volume wash as indicated on figure. O high affinity (binding of 100 pM $^{125}$I-GM-CSF); + low affinity (binding of 2 nM $^{125}$I-GM-CSF).
The binding of 2 nM \(^{125}\text{I}-\text{GM-CSF}\) was 27\% ± 3 of time 0 values after 4 hours exposure to GM-CSF (mean ± SEM, n=3), and 9\% ± 2 at 24 hours (Figure 6.3) indicating that there had also been down-regulation of low affinity receptors.

To examine receptor up-regulation, TF-1 cells exposed to unlabelled GM-CSF (360 pM) for 24 hours were thoroughly washed and transferred to medium containing 1 IU/ml of Epo. The GM-CSFRs were rapidly up-regulated with a rise in the binding of 100 pM \(^{125}\text{I}-\text{GM-CSF}\) to 55\% ± 3 of time 0 values after 6 hours, and 128\% ± 6 after 18 hours (Figure 6.3). The binding of 2 nM \(^{125}\text{I}-\text{GM-CSF}\) was 41\% ± 5 at 6 hours and 93\% ± 8 at 18 hours. Therefore both high and low affinity receptors were up-regulated once specific ligand had been removed.

EVALUATION OF THE ROLE OF PROTEIN SYNTHESIS IN GM-CSFR UP-REGULATION

In order to assess the contribution of new protein synthesis during receptor up-regulation, TF-1 cells were cultured overnight with GM-CSF (720 pM) and then incubated either with or without cycloheximide (10 \(\mu\text{g/ml}\)) for 1 hour. The cells were then washed three times in RPMI, ± cycloheximide, to remove GM-CSF and resuspended in medium containing Epo (1 IU/ml), ± cycloheximide. Six hours after the addition of Epo, the cells incubated in cycloheximide were unable to bind \(^{125}\text{I}-\text{GM-CSF}\) (500 pM), whereas binding in the control cells had increased to 36\% ± 8 of binding measured at 18 hours (mean ± SEM, n=3) (Figure 6.4). At 18 hours, the binding of control cells was assumed to represent 100\%, as demonstrated in the studies above. This suggests that synthesis of new receptor chains may be necessary for receptor up-regulation, and that surface receptors are not replenished from an intracellular pool.
Figure 6.4 - Evaluation of the role of protein synthesis in GM-CSFR up-regulation.

- In the presence of 10 \( \mu \text{g/ml} \) cycloheximide (protein synthesis inhibited);
  + Control

Mean ± SEM of 3 separate experiments
EVALUATION OF THE ROLE OF RECEPTOR RECYCLING IN GM-CSFR UP-REGULATION

In certain receptor systems, not all the internalised receptors are degraded, some are recycled to the cells surface (Knutson 1992). To examine this possibility, a series of experiments were performed using the lysosomotropic agent ammonium chloride, which prevents acidification of internalised endosomes and thus interferes with ligand-receptor dissociation and degradation in many receptor systems (Dean et al 1984). However, ammonium chloride has also been shown to inhibit protein synthesis at doses above 25 mM (Jessup et al, 1983), so it was therefore important to first establish the minimum dose that would inhibit lysosomal acidification. This was achieved by determining the effect of ammonium chloride on the fate of internalised $^{125}$I-GM-CSF. TF-1 cells grown for 18 hours in Epo (1 IU/ml) were incubated with $^{125}$I-GM-CSF (500 pM) at 4°C for 2 hours, and then transferred to medium at 37°C for up to 3 hours. Every hour after transfer, samples were removed for determination of both total cell-associated radioactivity and the proportion of radioactivity that was internalised (ie. resistant to a low pH wash). Two separate experiments were performed and Figure 6.5 is representative of one experiment with absolute radioactive counts plotted. After 1 hour, 58% of cell-associated radioactivity was internalised (Figure 6.5). The solubility of the radioactive protein released into the supernatant was then determined by TCA precipitation. Intact GM-CSF is insoluble in 10% TCA, whereas degraded GM-CSF has a lower molecular weight and is soluble (Walker & Burgess, 1987). Internalisation of $^{125}$I-GM-CSF was followed by the appearance of TCA soluble radioactivity (ie degraded ligand) in the supernatant, and after 3 hours, this represented 61% of the initially bound $^{125}$I-GM-CSF. The decline in the total and internalised counts was almost entirely attributable to a rise in the amount of degraded $^{125}$I-GM-CSF. Hence $^{125}$I-GM-CSF accumulated in the TF-1 cell and was rapidly degraded, indicating
that an intact ligand-receptor complex was not recycled. Parallel experiments in
the presence of ammonium chloride (10 mM) showed that 43% of the initial cell-
associated radioactivity was internalised after 1 hour, and by 3 hours 60% had
been internalised The TCA soluble counts, however, were lower than in the
control cells, as at 3 hours they were 37% of initially bound $^{125}$I-GM-CSF,
compared to 61% in control cells. The degradation of the $^{125}$I-GM-CSF was
therefore inhibited in the presence of ammonium chloride and most of the radio
ligand remained internalised (Figure 6.5b). Having ascertained that lysosomal
acidification is required for ligand degradation and that lysosomal acidification is
inhibited by this dose of 10 mM ammonium chloride, the effect of this
concentration of lysosomotropie agent on receptor up-regulation was then
evaluated.

TF-1 cells were cultured overnight with GM-CSF (720 pM), were washed
three times in RPMI, resuspended in medium containing Epo (1 IU/ml) ±
ammonium chloride (10 mM) and then $^{125}$I-GM-CSF binding was measured
every two hours for a period 6 hours. No difference was observed in either the
level or rate of receptor up-regulation between cell incubated with or without
ammonium chloride (Figure 6.6). Furthermore, there was no effect on the re-
expression of individual $\alpha$ or $\beta$ chains as measured using specific monoclonal
antibodies (data not shown). This is compatible with the hypothesis that receptor
up-regulation represents new protein synthesis rather than recycling.

EVALUATION OF THE ROLE OF LIGAND INDEPENDENT RECEPTOR
TURNOVER DURING RECEPTOR UP-REGULATION

Up-regulation of receptor after removal of ligand is likely to be due to increased
production of receptors, but could conceivably be due to suppression of ligand-
independent receptor loss from the cell surface. In order to assess whether
Figure 6.5- Chase of internalised ¹²⁵I GM-CSF

a control; b in the presence of 10 mM ammonium chloride
O Total surface bound; ♦ Internalised (resistant to low pH wash); + Degraded
(TCA soluble); A Un degraded (TCA precipitable)
Figure 6.6- GM-CSFR Re-expression in the presence of ammonium chloride

○ Control; + In the presence of 10 mM ammonium chloride
(Mean ± SEM of 3 separate experiments)
receptor up-regulation could be due to retardation of the latter process rather than new protein synthesis, TF-1 cells were cultured overnight in medium containing GM-CSF (360 pM) to down-regulate the receptor, then transferred to medium containing Epo (1 IU/ml). After 6 hours when the re-expression of the receptor was approximately 50%, as predicted from experiments shown above, the cells were divided into two and incubated ± cycloheximide (10 mg/ml), in order to inhibit protein synthesis. Binding of $^{125}$I-GM-CSF (500 pM) was determined at 0, 30, 60 minutes and 4 hours after exposure to protein inhibitor. In cells not exposed to the protein synthesis inhibitor, $^{125}$I-GM-CSF binding increased from 100% at 0 minutes to 128% ±1 of time 0 values at four hours (n=2), whereas in cells incubated with cycloheximide, there was a decrease in ligand binding to 46% ± of time 0 values (Figure 6.7). This represented a receptor half-life of 4 hours, which is similar to the half-life demonstrated in the steady state in cells that had not been down-regulated by culture in GM-CSF. This indicates that up-regulation of the receptor requires new protein synthesis and does not involve alteration in receptor turnover by retardation of ligand-independent receptor protein loss from the surface.

**EXPRESSION OF GM-CSFR α and β mRNA DURING SPECIFIC LIGAND INDUCED DOWN-REGULATION AND SUBSEQUENT UP-REGULATION FOLLOWING REMOVAL OF LIGAND**

Alterations in the expression of GM-CSFRα and β chain mRNA during receptor down- and up-regulation were evaluated by RNase protection assays. Total RNA was prepared from TF-1 cells at 4 and 24 hours after transfer to 360 pM GM-CSF to induce receptor down-regulation and then at 6 and 18 hours after resuspension in Epo to allow receptor up-regulation. As a control, RNA was also prepared at the same time intervals from cells grown continuously in Epo and
Figure 6.7 Evaluation of the role of ligand independent GM-CSFR turnover during receptor up-regulation

O control; + In the presence of 10 μg/ml cycloheximide - added 6 hours after up-regulation of receptor was approximately 50% (as predicted by experimental data shown in figures 6.3, 6.4 and 6.5). Mean ± SEM of 2 separate experiments.
processed in the same manner. At each time point the levels of GM-CSFRα and β mRNA were quantitated for both sets of cells. There was no difference between the expression of either the GM-CSFRα or β chain mRNA during down and subsequent up-regulation of the surface protein, when compared to the levels in cells with continuously up-regulated receptor (Figure 6.8). This indicates that the protein synthesis required for up-regulation of the receptor is not due to increased mRNA production.

6.3.2 Longer-term changes in receptor expression with cell differentiation

CHANGES IN EXPRESSION OF GM-CSFR IN DIFFERENTIATING CELLS

In order to evaluate changes in GM-CSFR expression, HL-60 cells were incubated in DMSO (1.25% v/v) for 5 to 7 days in order to induce cell maturation. Morphological examination confirmed differentiation of the cell population from 95% ± 2% immature promyelocytes to 84% ± 18% morphologically mature cells (myelocytes and metamyelocytes) by day 5 (mean ± SEM of 3 experiments). Acquisition of the ability to generate superoxide was confirmed using the NBT test which demonstrated that although only 4.5% ± 1.5% were NBT positive at day 0, 56% ± 2.5% were NBT positive at day 5 (mean ± SEM of 3 experiments). Equilibrium binding studies and Scatchard analysis using $^{125}$I-GM-CSF have previously shown that uninduced HL-60 cells expressed both low and high affinity GM-CSFR and exposure to DMSO led to a two-fold increase in receptor numbers, without any change in the receptor affinity or the ratio of high to low affinity receptors (Roberts et al, 1994; Table 6.1).

Total RNA was prepared form the HL-60 cells each day after 5 days in DMSO and levels of GM-CSFR α and β chain mRNA were quantified using RNase protection assays. Both α and β chain GM-CSFR mRNA expression
Figure 6.8 GM-CSFR α/β mRNA expression during specific ligand-induced down-regulation and subsequent up-regulation;
+ α mRNA, O β mRNA

Down-regulation is achieved by culture in the presence of 360 pM GM-CSF and up-regulation by extensive cell washing (cell wash indicated in figure). Results are expressed as % of control (cells cultured in the presence of 1 IU/l erythropoietin throughout experiment). Results represent the mean and SEM of 3 separate experiments.
increased during DMSO induced differentiation of HL-60 cells if the signals were standardised for actin mRNA expression (Figure 6.9). If values at day 0 are considered to be 100%, by day 5, expression of \( \alpha \) chain mRNA was \( 340\% \pm 61\) of day 0 values (mean \( \pm \) SEM of 3 experiments) (Figure 6.9a). \( \beta \) chain mRNA expression increased to \( 207\% \pm 69\) over the same time period (Figure 6.9b). There is thus a co-ordinate rise in \( \alpha \) and \( \beta \) chain mRNAs which correlates with an increase in cell surface protein levels as HL-60 cells differentiate. The ratio of the GM-CSFR \( \alpha:\beta \) mRNA levels (standardised to actin or 18 S ribosomal RNA levels) was 22:1 in uninduced HL-60 cells and 35:1 in day 5 DMSO-induced cells. An actin probe together with a probe for a highly conserved region of the 18S ribosomal RNA gene were used as internal controls, as it has previously been suggested that the actin content of the HL-60 cells increases as they differentiate (Meyer et al, 1983). However, the present data do not confirm a concomitant increase in actin mRNA levels during HL-60 differentiation (Figure 6.9), and therefore actin alone could be satisfactorily used as an internal control. The excess of \( \alpha \) chain mRNA observed is in accord with the presence of dual affinity receptors on these cells.

In TF-1 cells, which also express dual affinity receptors, the ratio of the \( \alpha:\beta \) chain mRNAs was similar to that of HL-60 cells. However, in mature neutrophils the ratio was considerably lower at 9:1 (Table 6.2). It is not possible to give accurate absolute quantities on a single cell basis as it cannot be assumed that the mRNA recovery was similar in the different cell types (Section 3.3.3), nevertheless, these data do show that neutrophils have less GM-CSFR\( \alpha \) mRNA, relative to GM-CSFR\( \beta \) mRNA, than HL-60 cells which is in accord with the absence of low affinity GM-CSFRs on neutrophils.
Figure 6.9 (a) GM-CSFRα mRNA and (b) GM-CSFRβ mRNA expression in HL-60 cells undergoing DMSO induced differentiation for 5 days.
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<th>Neutrophils</th>
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<td></td>
<td>Day 0 (uninduced)</td>
<td>Day 5-DMSO induced (% day 0 uninduced)</td>
<td>NA</td>
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<tr>
<td>GM-CSFRα mRNA</td>
<td>100%</td>
<td>340% ± 61</td>
<td>NA</td>
</tr>
<tr>
<td>GM-CSFRβ mRNA</td>
<td>100%</td>
<td>207% ± 69</td>
<td>NA</td>
</tr>
<tr>
<td>Ratio of GM-CSFR α : β mRNA</td>
<td>22 : 1</td>
<td>35 : 1</td>
<td>50 : 1</td>
</tr>
</tbody>
</table>

*Table 6.2* Expression of GM-CSFR mRNA in HL-60 and TF-1 cells, and neutrophils (NA = not applicable)
6.4 Discussion

The expression of GM-CSFRs has been extensively studied in a range of haematological cell types (Budel et al, 1993; Khwaja et al, 1993; Roberts et al, 1994) but there is little information about the molecular regulation of these receptors. The work presented in this chapter was performed in order to define the changes in GM-CSFRα and β chain mRNAs that occur during differentiation and in response to dynamic changes in cell surface receptor expression.

The half life of the GM-CSFR protein in TF-1 cells grown in steady state conditions in the presence of Epo and the translation inhibitor cycloheximide, was approximately 4 hours. This is considerably shorter than has been reported for other receptors, for example, up-regulated insulin receptors in dexamethasone treated mouse fibroblasts have a receptor half-life of 19 hours (Knutson, 1992). The rapid down-regulation of the receptors under these conditions was unlikely to be due to ligand induced internalisation by contaminating GM-CSF in the extracellular medium for several reasons. The cells had been washed extensively prior to transfer to Epo and the estimated GM-CSF contamination in the Epo culture phase is in the low femtomolar range. In addition, experiments utilising TCA precipitation demonstrated that internalised GM-CSF is degraded, and would not therefore be released into the supernatant. In accord with this, if the washed cells were cultured without Epo there was cell death by apoptosis detectable by 36 hours (data not shown). This suggests that loss of receptors from the cell surface could either be by enzymatic cleavage as demonstrated for the M-CSFR (Downing et al, 1989) or by internalisation occurring even in the absence of specific ligand as in the insulin receptor (Knutson, 1992). This latter process appears to be metabolically controlled and is the rate-limiting step in the expression of the insulin receptor, whereas receptor recycling to the cell surface does not appear to be a regulated process. Similarly, internalisation of the
epidermal growth factor receptor (EGFR) in the absence of ligand has been demonstrated in the epidermoid carcinoma cell A431 and skin fibroblasts to have a receptor half life of 9 - 16 hours (Kruppet al 1982). Despite this apparent GM-CSFR turnover, surface expression of the receptor remained constant over 6 hours, even in the absence of transcription as shown by GM-CSF binding in the presence of actinomycin D. As the half-life of the GM-CSFRα chain mRNA was found to be between 1 and 3 hours (Figure 6.2a), this indicated that there was a fall in α chain mRNA without a concomitant decrease in 125I-GM-CSF binding. This could either be due to more efficient a chain translation, or to a change in GM-CSFR affinity, possibly as a result of a change in relative numbers of α and β chains leading to alternative oligomerisation of these chains (Budel et al, 1993; Wheadon et al, 1995). However, there was no change in the surface expression of either chain during this period, as measured by surface binding of GM-CSFRα and β chains with monoclonal antibodies. Therefore the level of α chain protein was maintained at the cell surface, even when the α chain mRNA had fallen to 25%. This suggests that translational control of the GM-CSFRα chain production can occur to maintain steady-state receptor levels.

The surface expression of the GM-CSFR can be rapidly modulated by the presence of specific ligand, with nearly all high affinity receptors and approximately 70% of low affinity receptors being down-regulated by 4 hours. If the GM-CSF was then removed by extensive washing, the surface ligand binding ability is up-regulated to the pre-GM-CSF exposure levels after 18 hours. For ligand-binding to increase to steady state levels, one or more of four conditions must be operative: receptors internalised in the presence of GM-CSF must be recycled to the cell surface; the steady state turnover of the receptors must be markedly reduced; the affinity of receptors present must increase; or the rate of receptor chain synthesis must be increased relative to the steady state level. The present data demonstrates no evidence for significant receptor recycling, Firstly,
receptor up-regulation was inhibited in the presence of cycloheximide suggesting that this process requires new protein synthesis and that a pool of intracellular receptors is not available for surface expression. Secondly, once internalised, the receptor bound ligand was degraded, as demonstrated by an increase in radioactivity in the TCA-soluble fraction containing low molecular weight fragments of $^{125}$I-GM-CSF. Inhibition of lysosomal acidification by incubation with 10 mM ammonium chloride prevents ligand dissociation and degradation of the ligand, but did not lead to an alteration either in GM-CSFR re-expression after ligand-induced down-regulation (as measured by binding of radioligand) or re-expression of $\alpha$ and $\beta$ chains (as measured by specific monoclonal antibodies). This suggests that recycling of the receptor from the dissociated receptor-ligand complex does not make a major contribution to its up-regulation. In addition, although Knutson (1992) demonstrated that changes in the internalisation rate constant of the insulin receptor can lead to changes in the relative number of recycled to degraded receptors, we could find no alteration in the rate of ligand-independent receptor loss from the cell surface which might explain an increase in receptor expression (Figure 6.7). Using the murine myelomonocytic line WEHI-3BD+, Walker & Burgess (1987) have reported that a considerable proportion of the murine GM-CSFR is recycled to the surface, and although new synthesis does contribute to the re-expression of receptors, this occurs in the first hour after down-regulation. In their model cycloheximide alone did not inhibit receptor re-expression after ligand-induced down-regulation and only a combination of 50 mM ammonium chloride and cycloheximide resulted in inhibition of receptor re-expression. The differences between the results presented here may be due to differences between species or between the different cell models used.

The internalisation and intracellular degradation of GM-CSF and its receptor would therefore be a mechanism by which the effects of the ligand are down-
regulated and are in agreement with the findings of Khwaja et al, 1990. This has also been well described for other growth factor receptors EGFR (Felder et al, 1990) and M-CSFR (Downing et al, 1989). Both these receptors homo-dimerize on interaction with their cognate ligands, whereas interaction of GM-CSF with its receptor results in a heterodimer or complex of α and β chains. The data presented here would suggest that both the α and β chains are degraded on internalisation of the receptor complex. Inhibition of receptor up-regulation by cycloheximide indicates that the major mechanism for up-regulation is by increased protein synthesis. However, throughout the period of receptor up-regulation, α and β mRNA levels remained constant (Figure 6.8) suggesting that the increase in receptor synthesis is due to increased translational efficiency. Translational control has been described for a number of proteins including ferritin, vertebrate ribosomal proteins, Xenopus oocyte maturation and the yeast GCN4 system (Melefors & Hentze, 1993).

During DMSO-induced maturation of HL-60 cells, up-regulation of both high and low affinity receptors was observed, indicating an increase in both GM-CSFRα and β chain production. This was associated with a commensurate rise in α and β mRNA levels, suggesting either increased transcriptional activity, or an increase in mRNA stability associated with differentiation. One way of confirming transcriptional regulation would be to perform nuclear run-off assays, using nuclear extract of induced HL-60 cells. Nuclear run-off assays are, however, notoriously unreliable. When these assays were performed using the murine IL-3 receptor AIC2A, nascent transcripts were barely detectable in the MC9 cell line (Hara & Miyajima, 1994). In mature neutrophils only a single class of the high/intermediate affinity receptors was seen, indicating a relative increase in the surface β chains compared to α chains. The fact that induced HL-60 cells do not have this expression pattern is in line with previous reports that HL-60 cells do not acquire all the characteristics of mature neutrophils, in that they lack
specific granules (Newburger et al, 1979) and have monocyte DNase (Roberts et al, 1990b). Analysis of the relative amounts of α and β chain mRNAs in neutrophils indicated a lower GM-CSFRα to β mRNA ratio compared to HL-60 cells, which is in accord with the expression of α and β chain proteins.

In conclusion, the expression of the GM-CSFRs is highly regulated following both ligand-induced down-regulation of receptors and during differentiation but the mechanism of regulation appears to differ. Whereas changes GM-CSFR protein during differentiation are associated with co-ordinate changes in mRNA levels, rapid changes in receptor expression induced by transient exposure to GM-CSF do not involve any change in mRNA levels and are at the level of translation.
— CHAPTER 7 —

SUMMARY
Summary

Early studies with murine and human bone marrow demonstrated the presence of a relatively low number of GM-CSF specific surface receptors. The cloning of the GM-CSFRα chain and the common β chain has enabled the study of the receptor at the level of mRNA, as well as cell surface protein expression. The problems associated with the quantification of biological systems in general, and molecular cell events in particular, have resulted in the majority of studies to be qualitative in nature. The data presented in this thesis attempts a quantitative approach of molecular events utilising low substrate concentration.

Chapter 3 highlights the problem of attempting to measure mRNA transcripts which are present in the femtomolar range. Although RNase protection assays were able to detect both GM-CSFRα and β mRNA transcripts, it is clear from the calibration curves that the mRNA transcripts are at the lower end of the detection limit for some cells, e.g., DMSO induced HL-60 cells (Figure 3.2). The wide variation in the yields of mRNA extracted from the cells, reflecting the vulnerability of RNA to rapid degradation, makes it difficult to quantify mRNA transcripts on a per cell basis. Although some groups have attempted this task, the results are always reported on the basis of average RNA extraction, and little is mentioned of the variation. Whatever the problems associated with quantification of GM-CSFRα and β mRNA transcripts, it is clear that they are present in the 0.1 to 1.0 pg, or 0.1 to 1 femtomolar range.

The RNase protection assays of GM-CSFR yielded extra, unexpected bands. These results thus highlight the sensitivity of the method for detection of a single base pair mismatch. Further investigation of the two extra band demonstrated by RNase protection assays of GM-CSFRβ demonstrated that one of these bands was attributable to a point mutation in the probe used for assay. The second extra band, however, was the result of alternative splicing in the
intracytoplasmic region of the β chain. This isoform has the potential to form a truncated β chain which lacks the distal sequence involved in differentiation signals from the β chain.

Chapter 4 includes the results of experiments undertaken in order to characterise the three extra bands demonstrated in RNase protection assays of GM-CSFRα. Using RT-PCR and direct sequencing techniques, these bands were shown to be a result of variable alternative splicing at the 5' end of GM-CSFRα. Characterisation of these extra fragments identified deletions of exon 2 and 2b in the 5' UTR, and deletion of exon 3, resulting in a cDNA sequence with the potential to form a receptor without a signal peptide sequence.

The exon 2 deleted isoforms do not result in the formation of a new receptor protein, but give rise to alternative 5' UTR leader sequences (Chapter 5). The results of in vitro translation experiments indicated that the alternative splicing of exon 2b resulted in increased translation of the receptor protein compared to the originally described full length isoform. Conversely, splicing out of exon 2 results in reduced translation efficiency. This study is unique in attempting to elucidate the significance of the 5' UTR leader sequences of the GM-CSFRα. Recent unpublished data using the RACE (rapid amplification of cDNA ends) technique to investigate the 5' leader sequences of the IL-11 receptor α chain, shows the existence of similar alternatively spliced isoforms (Dr L Robb, WEHI, Australia, personal communication). It would be interesting to know whether the translation of the IL-11 receptor is similarly affected.

In chapter 1, a minimum model that includes the major processes involved in gene expression was presented. The aim of chapter 6 was to investigate the mechanism by which the GM-CSFR expression was regulated. Bioassay of the receptor protein expression (radioligand binding studies) and RNase protection assay of GM-CSFRα and β mRNA were employed. TF-1 cells undergoing dynamic changes of cell surface receptor protein, and HL-60 cells, induced to
undergo differentiation by DMSO, were studied. The results in chapter 6 go some way towards elucidating the mechanisms for regulation of GM-CSF expression (Figure 7.1).

In HL-60 cells, DMSO induced differentiation resulted in an increase in GM-CSFα and β mRNA, and a co-ordinate increase in both low and high affinity receptor numbers. This increase occurred over a time period of days, which would suggest transcriptional regulation of the receptor. However, in TF-1 cells undergoing significant change in receptor expression during a period of hours, the regulation of receptor protein expression was shown to be at the post-transcriptional level, in particular at the level of translation. Furthermore, re-expression of cell surface receptor was demonstrated to be the result of de novo protein synthesis, and was not attributable to changes in cell surface receptor protein half-life, or receptor recycling. The differences observed between regulation of receptor expression in induced HL-60 cells and TF-1 cells undergoing dynamic changes, may in part explain how GM-CSF modulates differentiation of immature progenitors, and yet is able to facilitate mature cell function. Translational regulation would of course be advantageous in mature cell function, since this would allow rapid up-regulation of the receptor. Internalisation and receptor degradation rather than recycling would also prevent haemopoietic cells being 'continually' activated by ligand.

It is tempting to speculate that the alternative mRNA transcripts presented in this thesis play a role in the translational regulation of the GM-CSFRβ chain. In this way, the exon 2b deleted isoform would be used if rapid new receptor expression was required, and the exon 2 deleted isoform would be translated if reduced receptor levels were desirable. In vivo translation models, utilising polysomal RNA, could be used to evaluate which of the transcripts are translated in any given situation. Although laborious, these experiments would be worth undertaking in order to confirm the 'default transcript' hypothesis presented here.
Transcriptional regulation of GM-CSFR important in differentiating HL-60 cells

Figure 7.1 Schematic representation of the regulation of GMCSFR
Demonstration of the regulatory processes in human primary haemopoietic cells would, however, be necessary before extrapolation of these observations to the *in vivo* situation can be made. CD34+ cells undergoing differentiation and neutrophil or monocytes undergoing dynamic modulation, would be suitable models for study. Such investigation would however require large numbers of cells to enable binding studies and RNA extraction at different time points.
References

Adams JM, Houston H, Allen J, Lints T and Harvey R. (1992) The haematopoietically expressed vav proto-oncogene shares homology with the dbl GDP-GTP exchange factor, the bcr gene and a yeast gene (CD24) involved in cytoskeletal organization. Oncogene, 7, 611.

Andrew RG, Singer JW, and Bernstein ID. (1989) Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. Journal of Experimental Medicine, 169, 1721.


Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, 18, 5294.


Downing JR, Roussel MF and Sherr C (1989) Ligand and Protein kinase c downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. Molecular and Cell Biology, 9, 2890


Freeburn RW, Linch DC (1995b). The beta subunit common to the GM-CSF, IL-3 and IL-5 receptors is highly polymorphic but pathogenic point mutations in patients with acute myeloid leukaemia (AML) are rare. Leukaemia (In press).


Hara T and Miyajima A. (1994). Regulation of IL-3 receptor expression: evidence for a post-transcriptional mechanism that dominantly suppresses the expression of beta subunits. *International Immunology*, 6, 1525.


Maximow AA. (1924) Relation of blood cells to connective tissues and endothelium. *Physiology Reviews*, 4, 533.


Metcalf D, Begley CG, and Nicola NA. (1985) the proliferative effects of human GM-CSFα and β and murine G-CSF in microwell cultures of fractionated human marrow cells. Leukaemia Research, 9, 198, 521.


Roussel MF, Downing JR and Sherr CJ. (1990) Transforming activities of human CSF-1 receptors with different point mutations at codon 301 in their extracellular domains. *Oncogene*, 5, 25.


Tavernier J, Devos, R, Cornelis S, Tuytens T, van der Heyden J, Fiers W and Plaetinck G. (1991) A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. Cell, 66, 1175.


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