



Regulation of expression of the human interleukin 3 gene.

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To my wife, Sharon Ryan.

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Addendum

1). An additional comment should appear in chapter 5 at the end of the first paragraph on page 126:

The use of shorter oligonucleotides encompassing only the CK1 or CK2 elements alone, or the isolated elements in a nonsense background may assist in defining whether these elements can independently form the expected complexes. However, similar experiments in this laboratory with isolated G-CSF and GM-CSF CK1 regions have been unsuccessful at either clarifying protein binding sites or more importantly defining the function of the isolated elements.

2). In the discussion of chapter 5 on page 133 the noncoding strand base sequence AAGG is written in a 3' to 5' sense. To avoid confusion the sentences containing the sequence should read:

In respect of NFGMa, Shannon *et al.* (1990) and Kuczek *et al.* (1991) showed by EMSA and modification interference assays that the central AAGG (in a 3' to 5' sense) bases in the noncoding strand of the GM-CSF and G-CSF CK1 elements are important for NFGMa binding and also that this motif is required for the TNF- α responsiveness of the G-CSF CK1 element. The 3' AAGG5' motif is also conserved in the IL-3 CK1 element.

3). These additional comments should appear on page 133 immediately after the two sentences shown in 2) above:

This conserved sequence resembles that shared by the Ets family of DNA binding factors as a common core (5'GGAA/T3') to their binding sites. While the ability of Ets family proteins to bind the IL-3 CK-1 element has not been tested, Ets family members do not bind the GM-CSF CK-1 element (M.F. Shannon and I Kola, personal communication). However, since there is evidence of the binding of AP-1 to the IL-3 CK1 element (figure 5.4) and this complex is known to cooperate with Ets family members for transcriptional induction in T-cells (Wasylyk *et al.*, 1990 and Boise *et al.*, 1993), the role of Ets factors binding at the IL-3 CK-1 element should be further investigated.

4). An additional comment should appear in the discussion of chapter 5 at the end of the first paragraph on page 136:

Furthermore, until the requirements for double- or single-stranded DNA binding sites are known for all of the IL-3 CK1/CK2 binding factors, the results of competition EMSA assays should be interpreted with caution. If the binding of any of these factors does require single stranded DNA then the results of the competition assays will be very dependent on the amount of single stranded DNA in the annealed oligos. In future EMSA competition experiments, the double-stranded oligonucleotides should be repurified after annealing to avoid potentially anomalous results.

Declaration

The work reported in this thesis was carried out by the author in the Division of Human Immunology at the South Australian Institute of Medical and Veterinary Science. This thesis contains no material that has been submitted for another degree or diploma in any university or tertiary institution and to the best of my knowledge contains no material previously published by any other person except where due reference has been made in the text. I consent to this thesis being made available for loan and photocopying when deposited in the University library.

Gregory R. Ryan.

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Abstract

Interleukin 3 (IL-3) is an haemopoietic growth factor (HGF) which regulates the production and the functional activity of blood cells. The primary aim of the work presented in this thesis was to investigate the mechanisms involved in regulating the expression of the human IL-3 gene. At the outset of this work the restriction of human IL-3 expression to antigen or mitogen activated cells of the T-lymphoid lineage had been described, however, nothing was known of the mechanisms involved in the tissue specific and inducible expression of IL-3.

This thesis examines the stimulation requirements, kinetics and mechanisms involved in regulation of IL-3 mRNA accumulation. The results of RNase protection analyses of a number of T-cell and non-T-cell cell lines are presented. IL-3 mRNA was detected only after stimulation of the T-cell lines, despite a low level of constitutive transcription. Both induction of transcription and specific modulation in mRNA stability were shown to play a role in IL-3 mRNA accumulation in stimulated cells. The roles of cis-acting transcription regulatory elements, that were identified by sequence comparison between the murine and human IL-3 genes and with other related HGF genes, were examined in both T-cell and fibroblast cell lines using transient transfection assays. For these experiments either a series of restriction fragments from the 5'-promoter region of the IL-3 gene or short synthetic oligonucleotides were attached to the chloramphenicol acetyl transferase reporter gene. Both positive and negative regulatory regions were identified using this approach.

This thesis also presents the identification and partial characterisation a number of nuclear proteins which bind specifically to regulatory elements in the IL-3 promoter. Of particular interest are proteins which bind to promoter sequence elements which are conserved in the promoters of IL-3, Interleukin 5, granulocyte-macrophage colony stimulating factor (GM-CSF) and other related HGF genes. Nuclear protein complexes that bind specifically to the conserved cytokine promoter elements of both the IL-3 and GM-CSF genes or exclusively to IL-3 sequences were identified by electrophoretic mobility band shift assays. The possible role of these complexes and that of other transcription factor complexes in the regulation of IL-3 transcription and their relationship to factors that bind related sequence elements in the promoters of other cytokine genes is also discussed.

Abbreviations

Abbreviations used are as described in The Journal of Biological Chemistry "Instructions to authors" (1992). Additional abbreviations are listed below.

A_n :	Absorbance at wavelength n.
bp:	base pair.
BCIG:	5-bromo 4-chloro 3-indolyl- β -D-galactopyranoside.
con A:	concanavalin A.
cpm:	counts per minute.
dNTP:	deoxyribonucleotide triphosphate.
ddNTP:	di-deoxyribonucleotide triphosphate.
DTT:	dithiothreitol.
FITC:	fluorescein isothiocyanate
g:	gravitational force.
IPTG:	Ethylene glycol-bis (β -amino-ethyl ether) isopropyl-thiogalactoside.
kb:	kilobase (1000 bp).
ONPG:	O-nitrophenyl galactose.
PMSF:	phenylmethanesulphonyl fluoride.
Py:	Pyrimidine nucleotide (C or T)
S:	Svedberg unit of sedimentation coefficient.
TCA:	trichloroacetic acid.



Chapter 1

Introduction

1.1 Introduction

The work presented in this thesis focuses on the regulation of expression of the haemopoietic growth factor, human interleukin 3 (IL-3). This introductory chapter will attempt to put the experimental work presented in this thesis into context by reviewing the three main areas relevant to this work; firstly, haemopoiesis and the haemopoietic functions of IL-3, secondly, the genomic structure and organisation of the IL-3 gene and thirdly, the control of eukaryotic gene expression with particular emphasis on transcriptional regulation.

1.2 Introduction to the Haemopoietic system

Haemopoiesis, the regulated production of blood cells, is a complex example of multilineage differentiation. Progenitor cells, residing mostly in the bone marrow, give rise to the large numbers of red cells, granulocytes, monocytes, platelets and lymphocytes circulating in the blood. In normal health the circulating numbers of mature cells are maintained at a constant level. Many of these blood cell types are short lived and must be continually replenished. The system also allows fluctuations to meet environmental challenges such as blood loss, infection and reduced oxygen tension. The dynamic nature of the haemopoietic system suggests a flexible and complex regulation.

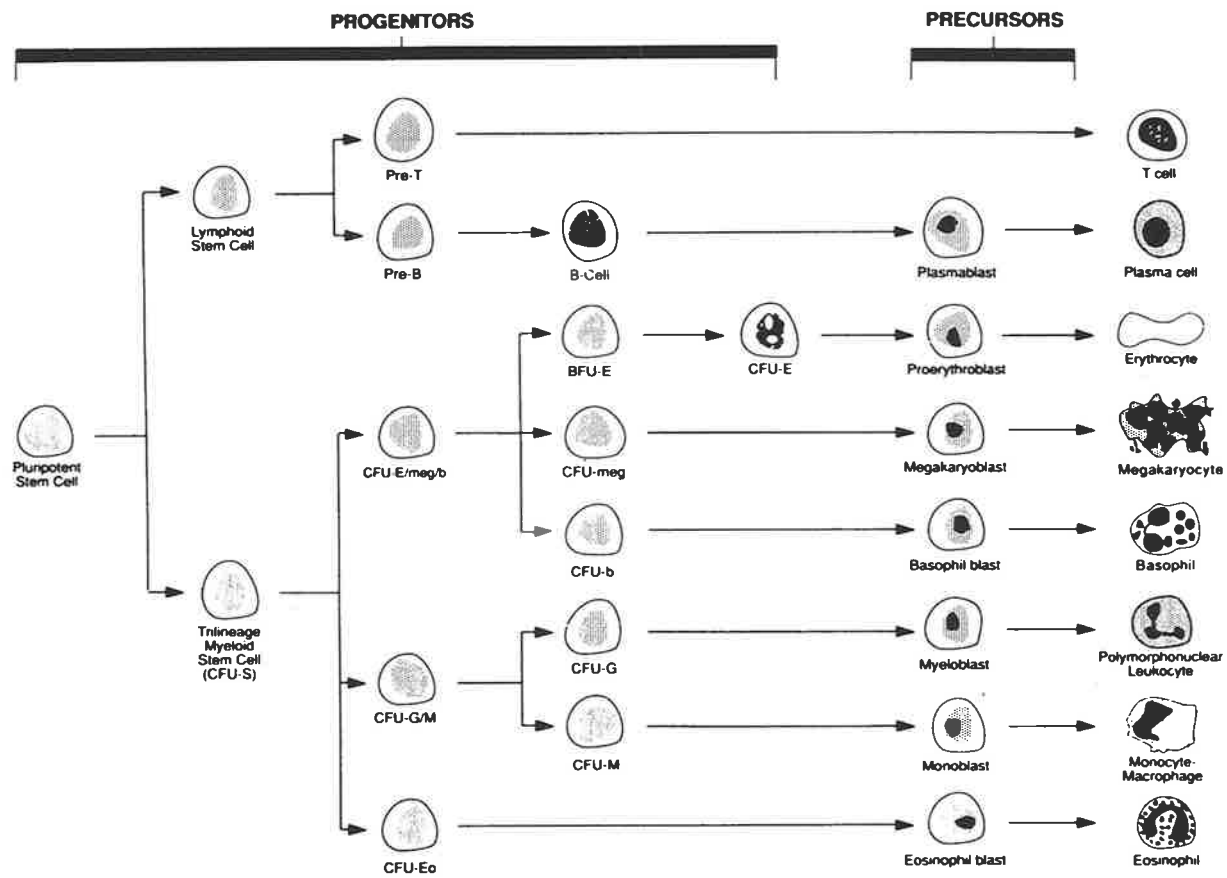
The concept that the circulating blood cells are derived from the proliferation and differentiation of pluripotent stem cells, which are induced to form specific lineages as a result of their interaction with growth factors, began with experiments carried out

in the laboratories of Sachs and Metcalf (Pluznik and Sachs, 1966, Bradley and Metcalf, 1966). The pluripotent stem cell is found in the medullary cavity of the marrow together with stromal cells, fibroblasts and endothelial cells. The restricted location of haemopoiesis implies that the specialised local stromal cells in these tissues must play a key role in controlling or at least permitting haemopoiesis (reviewed by Daniel and Dexter, 1989). This microenvironment provides a vital surface to which progenitors can adhere, and gives rise to many of the growth factors that stimulate specific progenitor differentiation and may also contribute to the renewal rate of earlier stem cells. Stem cells comprise between 0.01% and 0.05% of the total marrow population and possess two features that distinguish them from other haemopoietic cells. First is their ability to proliferate and produce more stem cells, a process called self renewal. Second, is their potential to undergo differentiation to produce at least nine highly specialised mature cell types, erythrocytes, neutrophils, eosinophils, basophils, platelets, monocytes/macrophages, osteoclasts, and T and B lymphocytes as shown diagrammatically in Figure 1.1. In this process the pluripotent stem cells, under the influence of the haemopoietic growth factors (HGFs), produce more developmentally restricted stem cells, for example the tri-lineage myeloid stem cells (CFU-S), that can proliferate, differentiate and develop into mature cells of the various myeloid cell lineages (but not T or B lymphocytes). These multipotential colony forming cells first differentiate to cells called progenitor cells (CFU-E, CFU-GM and CFU-Eo) that are more lineage restricted. These cell types can in turn proliferate and differentiate under the influence of lineage specific HGFs to produce a variety of unipotent blast cells that finally develop into mature cells of only one cell lineage. Although Figure 1.1 is drawn to represent a stepwise differentiation of stem cells, Suda *et al.* (1983) have shown that the various maturation stages may be stochastic and stages may be skipped.

The progressive restriction in developmental potential and the ability of progenitor cells to proliferate under the influence of HGFs ensures a tremendous amplification of specific mature cell types. For example an individual mouse is capable of contributing,

Figure 1.1 The progenitor basis of Haemopoiesis

Diagrammatic representation of the production of blood cells from pluripotent stem which are the ultimate source of the progenitors of the myeloid and lymphoid systems. On differentiation, this cell may randomly enter either the lymphoid or the myeloid pathway. Initially myeloid progenitors have a multipotential capacity (CFU-S), they may differentiate to a cell that will give rise to erythroid, megakaryocytic, basophilic or granulocyte-macrophage lineages or to an eosinophilic progenitor (CFU-E/meg/b, CFU-GM or CFU-Eo). The precursor or blast cells divide and differentiate into the mature blood cells.



through sequential marrow grafts, enough marrow cells to reconstitute the haemopoietic systems of 10,000 siblings. It is clear that the most primitive cells in the bone marrow graft are capable of an extent of self renewal which far exceeds a normal life span. It is also clear that it is the stem cells that are ultimately responsible for regenerating haemopoiesis following bone marrow transplantation or following severe damage to the haemopoietic system by radiation or treatment with chemotherapeutic agents for malignant disease. However, short term survival following such damage to the haemopoietic system may depend more on the pools of committed progenitor and blast cells which can undergo rapid proliferation and maturation.

1.2.1 Haemopoietic growth factors

Over the last several years, much has been learned regarding the HGFs involved in regulating the proliferation and differentiation of committed haemopoietic progenitors. These factors, also termed colony stimulating factors (CSFs), appear to act on various subsets of lineage committed progenitor cells that are derived from the pluripotent stem cells. Using enriched populations of multipotent cells and lineage restricted progenitor cells, the target cells for the individual haemopoietic growth factors were determined in both liquid and soft gel clonogenic assay systems. Eventually, the widespread use of recombinant DNA technology allowed isolation of the genes coding for the HGFs and the production of large amounts of protein using bacterial, yeast, lepidopteran or mammalian cell expression systems. In addition, various cytokines termed Interleukins, that are able to modulate proliferation and/or differentiation of B-or T-lymphocytes and their precursors have also been characterised and molecularly cloned. The nomenclature of the haemopoietic regulators and interleukins is complex, therefore throughout this thesis the term haemopoietic growth factor will be used to define factors, including the CSFs and some interleukins, that regulate cells of the myeloid and erythroid lineages. The more general term, cytokine, will be used to define regulatory factors that are active on lymphoid and other tissues.

The haemopoietic growth factors represent a diverse family of proteins and do not share significant homology at the nucleotide or amino acid level. It has been shown by analysis of crystallised HGFs that some have common structural motifs involving four α -helical bundles, at least two of which contribute to the specific binding site (section 1.3.5, Bazan, 1990 and reviewed by Goodall *et al.*, 1993). Each factor binds to a distinct receptor which is part of the haemopoietin receptor superfamily (see section 1.2.3) present on the target cell surface. So far at least 18 distinct haemopoietic regulators have been identified, their cDNA's cloned and the regulators produced in active recombinant form (Table 1.1). The target cell specificity and biological activities of the HGFs will not be discussed at length and have been recently reviewed by Clarke and Kamen (1987), Metcalf (1989), Mizel (1989), Nicola (1989), Arai *et al.* (1990), Oster (1991), Metcalf (1992) and Hamilton (1993). This introduction will concentrate on the myeloid CSFs and on IL-3.

In the human and murine system several HGFs that stimulate the production of the myeloid lineage have been identified. In general, the myeloid CSFs are characterised by overlapping spectra of biological functions as depicted in Figure 1.2. For example, neutrophil production can be stimulated at least *in vitro* by three colony stimulating factors, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF) and IL-3 (multi-CSF) and also by interleukin 6 (IL-6) and the stem cell factor (kit ligand, steel factor) (SCF). Similarly, eosinophil production can be stimulated by IL-3, GM-CSF or interleukin 5 (IL-5 or Eo-CSF). None of these factors is exclusively neutrophil or eosinophil specific as each has some actions on some other cell types. Macrophage-CSF (M-CSF or CSF-1) is more restricted and acts upon CFU-GM to preferentially stimulate the development of monocytes/macrophages and only occasionally neutrophils. In addition, these growth factors are not simply proliferative stimuli, but according to the type of the responding cell, they can also influence cell maturation (reviewed by Nicola, 1989). Finally, erythropoietin (Epo) together with IL-3, is the primary physiological stimulus for proliferation, induction of

Table 1.1 Haemopoietic regulators

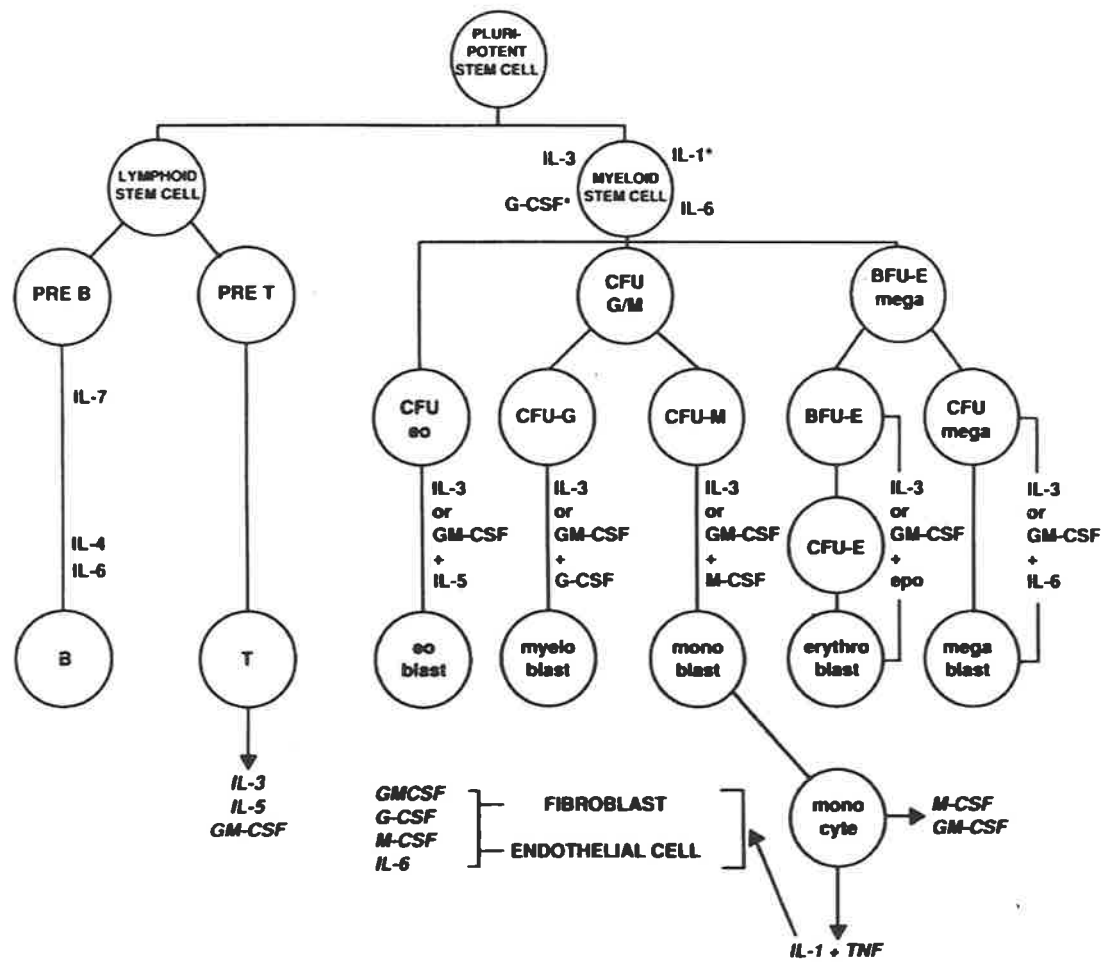
Regulator	Abbreviation	Molecular mass Da	Responding haemopoietic cell
Erythropoietin	Epo	18,400	E, Meg
Granulocyte-macrophage colony stimulating factor	GM-CSF	14,400	G, M, Eo, Meg, E
Granulocyte-colony stimulating factor	G-CSF	19,100	G, M
Macrophage-colony stimulating factor	M-CSF(CSF-1)	21,000(2X) 18,00(2X)	M, G
Interleukin 1	IL-1	17,900	T, Stem
Interleukin 2	IL-2	19,400	T, B
Interleukin 3	IL-3	16,200	G, M, Eo, Meg, Mast, E, Stem
Interleukin 4	IL-4	14,000	B, T, M, G, Mast
Interleukin 5	IL-5	13,300(2X)	Eo, B
Interleukin 6	IL-6	21,700	B, G, Stem, Meg
Interleukin 7	IL-7	14,900	B, T
Interleukin 9	IL-9	14,200	T, Meg, Mast
Interleukin 10	IL-10	18,700	T
Interleukin 11	IL-11	21,000	Meg, B
Interleukin 12	IL-12	-	N.K.
Interleukin 13	IL-13	17,000	M
Stem cell factor (Steel factor/kit ligand)	SCF	18,400	Stem, G, E, Meg, Mast
Leukaemia inhibitory factor	LIF	20,000	Meg

G, granulocytes; M, macrophages; Eo, eosinophils; E, erythroid cells; Meg, megakaryocytes; Stem, stem cells; Mast, mast cells; T, T lymphocytes; B, B lymphocytes; N.K., natural killer cells.

Figure 1.2 The target cells for myeloid haemopoietic growth factors

The growth factors that regulate the proliferation and differentiation of the particular progenitors are shown to the right of lines connecting the progenitors to their more mature progeny. The complexity of growth factor interactions with T-cells are not shown. The lower section of the diagram emphasises the importance of monocytes as a source of IL-1 and TNF which in turn stimulate fibroblasts and endothelial cells to produce four growth factors. T-cells are the predominant source of IL-3, IL-5 and IL-4. (after Williams and Nathan, 1991)

Abbreviations; TNF, Tumour Necrosis Factor- α ; IL-1, Interleukin-1; IL-4, Interleukin 4; IL-6, Interleukin 6; IL-7, Interleukin 7; epo, Erythropoietin.



haemoglobin expression and terminal differentiation of erythroid precursor cells (reviewed by Metcalf, 1989).

Certain growth factors also exhibit strong synergistic activity *in vitro*. For example, M-CSF and G-CSF when used in combination can recruit more primitive multipotent cells that are unresponsive to either factor used alone (Whetton and Dexter, 1989, Dexter, 1989), and allow these to develop into neutrophils and macrophages. Similarly interleukin-1 which is not a growth factor for multipotent cells, can synergise with IL-3, with GM-CSF and with M-CSF (Moore *et al.*, 1987). IL-6 is also a powerful stimulus when used in combination with IL-3. The observed synergistic effects of a wide range of growth factor combinations *in vitro* may be particularly relevant to how regulation of haemopoiesis takes place in the bone marrow. Although IL-3 has not been detected by biological assays in the bone marrow, IL-1 and IL-6 are constitutively produced in the marrow stromal cells (Miossec *et al.*, 1986) and may act in a synergistic manner with low levels of other HGFs in the regulation of blood cell production.

1.2.2 Activation of functional capacity of mature haemopoietic cells by HGFs

The *in vitro* and *in vivo* biological functions of IL-3 are discussed in more detail in sections 1.3.1 and 1.3.2. This section will focus briefly on the other myeloid CSFs and their interactions with mature haemopoietic cells. Myeloid CSFs, with the exception of M-CSF, do not stimulate the proliferation of mature cells in the blood. They do however, prolong their life span and directly enhance their migration and functional activities associated with host defence. One of the best studied examples is GM-CSF, which increases the capacity of neutrophils to phagocytose and kill parasites or bacteria, and to adhere to cell surfaces by inducing the expression of surface adhesion molecules. GM-CSF also primes neutrophils to respond more effectively to bacterial formyl peptides by releasing superoxide anions and participate in antibody dependent cell killing (Lopez *et al.*, 1986). Most of these activities on neutrophils are shared with G-CSF and these factors can enhance the general immunological response by

stimulating the release of other mediators of inflammatory responses, for example, arachadonic acid and IL-1. IL-5 and GM-CSF (but not G-CSF) have a similar activating function on eosinophils and enhance the capacity of eosinophils to participate in antibody dependent cell killing and production of superoxide anion as well as increased phagocytosis of opsonised yeast (Lopez *et al.*, 1991). Macrophages can be induced to proliferate by M-CSF. In addition, GM-CSF is able to synergise with M-CSF and increase the proliferative response of macrophages, their production of interferons, IL-1 and TNF- α and to increase the capacity of macrophages to kill intracellular parasites and yeast (Weiser *et al.*, 1987, Hamilton *et al.*, 1988 and Metcalf, 1989). The release of the myeloid CSFs at the site of infection would be expected to directly enhance the ability of local mature myeloid cells to resolve the infection and to recruit circulating cells to the site of infection or inflammation and immobilise them there. The induced release of other cytokines would recruit further cells and also enhance their functional capacity at the site of infection.

1.2.3 Haemopoietic growth factor receptors

Like other polypeptide hormones the HGFs interact with cells through specific glycoprotein receptors found on the cell surface. Typically the numbers of receptors of any one type are relatively small (several hundred to 1000). However, these can be of high affinity, in the range of 20-100 pM and maximal stimulation of the responding cells can also occur with a relatively low receptor occupancy (Lopez *et al.*, 1991). HGF receptors within the haemopoietin superfamily have been classified into two broad classes (1) transmembrane glycoproteins with a tyrosine kinase domain for signalling following activation by ligand binding, exemplified by M-CSF and SCF receptors. (2) transmembrane glycoproteins lacking a tyrosine kinase domain that combine with at least one other receptor chain (β subunit) to form a high affinity receptor. This class includes the receptors for IL-3, GM-CSF, G-CSF, IL-2, IL-4, IL-6, IL-7, LIF and Oncostatin M (OSM) as well as the receptors for hormones such as human growth factor and prolactin. These receptors exhibit clear structural

homology in their extracellular domains and have a double β barrel configuration similar to the classical growth hormone receptor structure (Bazan, 1990 and De Vos *et al.*, 1992). Molecular cloning of the receptor α and β chains has led to an explanation for the observed receptor cross competition between apparently high affinity specific binding sites of some of the myeloid HGFs. For example, the high affinity receptors of human IL-3, GM-CSF and IL-5 all show some degree of cross competition (Lopez *et al.*, 1991). It has been shown that the α chains of these receptors share and compete for a common β chain subunit, that α - β dimerisation is necessary for high affinity binding of the ligand and that the β subunit initiates signalling from the occupied receptor (reviewed by Nicola and Metcalf, 1991). A similar competitive sharing of β chains has been described for IL-6, LIF and OSM (Gearing *et al.*, 1991).

1.3 Cellular and molecular biology of IL-3

Several reviews are available describing the properties of IL-3 (Schrader, 1986; Clark and Kamen, 1987; Metcalf, 1989; Arai *et al.*, 1990; Ihle, 1990; Morris, Young and Happel, 1990 and Ihle, 1992). This section will focus on the biological and physical properties of IL-3 and the mechanisms by which IL-3 induces haemopoietic cell growth and supports differentiation.

1.3.1 Biological activities of IL3 *in vitro*

IL-3 was first defined in the murine system by its ability to induce the enzyme 20- α -hydroxysteroid dehydrogenase (20- α -HSD), a marker of T-cell maturation, in spleen cells of athymic nude mice (Ihle *et al.*, 1981). Following purification to homogeneity and its eventual molecular cloning (Fung *et al.*, 1984, Cohen *et al.*, 1986, Otsuka *et al.*, 1988, Yang *et al.*, 1989), it became clear that IL-3 has a broad spectrum of activities on haemopoietic cells and is responsible for a range of other biological activities present in conditioned medium, termed multi-colony-stimulating factor (multi-CSF), mast cell growth factor, P cell-stimulating factor, burst-promoting

activity, WEHI-3 growth factor and others (reviewed by Ihle *et al.*, 1983). All these activities stem from the ability of IL-3 to support the proliferation of haemopoietic cells at various stages of differentiation (see Figure 1.2).

The involvement of IL-3 in the regulation of early multipotential stem cells has been studied using *in vitro* blast cell colony assays (Sanderson, Warren and Strath, 1985). IL-3 supports the survival of stem cells capable of reconstituting lethally irradiated mice (Spangrude, Heimfeld and Weissman, 1988, Muller-Sieburg *et al.*, 1988, Bodine, Karlsson and Neinhuis, 1989) and the combination of IL-3 together with IL-6 increases the number of primitive reconstituting cells. While IL-3 does not stimulate these stem cells to enter the G₀ state to begin active proliferation and differentiation, it has been described as providing a "permissive milieu" for the continued proliferation and survival of the early haemopoietic progenitors (Koike, Ihle and Ogawa, 1986 and Koike *et al.*, 1987). In humans, IL-3 has been shown to support the growth and differentiation of primitive multilineage (CD34⁺) bone marrow progenitor cells (Kannourakis and Johnson, 1990). In semisolid *in vitro* cultures IL-3 supports the development of mixed myeloid/erythroid, neutrophil, eosinophil and basophil colonies as well as early stages of growth of stem-cell colonies containing progenitors of the erythroid and megakaryocyte lineages (Quesenberry, 1985; Sanderson *et al.*, 1985 and Burstein, 1986). Furthermore, direct effects of IL-3 on progenitors of mast cells, neutrophils, megakaryocytes and macrophages have also been established by experimentation with single isolated cells (Suda *et al.*, 1986). Comparisons with *in vitro* colony assays of the activities of human recombinant IL-3 to those of GM-CSF show extensive overlap in the haemopoietic functions of these two factors. However, while both factors, at saturating levels, promote the formation of granulocyte, granulocyte-macrophage, macrophage and mixed erythroid colonies, IL-3 is more effective than GM-CSF in promoting growth of the megakaryocyte containing colonies from the progenitor cells of fresh bone marrow (Koike *et al.*, 1987, Kobayashi *et al.*, 1989 and Ottman *et al.*, 1989).

A particularly interesting aspect of IL-3 biology is its synergism with many other growth factors. IL-3 and human recombinant IL-6 or IL-1 act synergistically on primitive bone marrow precursors to support multilineage colony formation (Ikebuchi *et al.*, 1987, Mochizuki *et al.*, 1987 and Dexter, 1987). Studies have also shown that IL-3 synergises with M-CSF when assayed on bone marrow enriched for early progenitor cells. While macrophages do not proliferate in response to IL-3 alone (Chen and Clark, 1986), the combination of IL-3 and M-CSF enhanced M-CSF receptor levels synergistically and generated large macrophage colonies in soft agar cultures (Bartelmez, Sacca and Stanley, 1985, McNiece *et al.*, 1984, Cannistra *et al.*, 1988). Similarly, the optimum production *in vitro*, of erythrocytes and the full development of megakaryocytes to the platelet shedding stage, requires in addition to IL-3, erythropoietin (Goldwasser, 1975) and thrombopoietin (Williams *et al.*, 1984) respectively. These observations suggest that macrophages, megakaryocyte and erythroid progenitors, lose the ability to proliferate in response to IL-3 following commitment and that lineage specific factors such as M-CSF, thrombopoietin and erythropoietin may act on more mature cells that have acquired the receptor as part of their differentiation program supported by IL-3.

Although the majority of studies have focused on the effects of IL-3 on myeloid cells, several studies have reported effects on cells of other cell lineages. In particular, IL-3 has been reported to support the differentiation (Barton and Mayer, 1989 and Chang *et al.*, 1989) of osteoclasts from preparations of bone marrow cells. There is also evidence for a role for IL-3 in pro-B cell and pro-T cell development based largely on the properties of IL-3-dependent cell lines, isolated from bone marrow, foetal liver or spleen (Palacios *et al.*, 1984 and Palacios, 1987). Many of these IL-3-dependent cell lines are able to differentiate along either T or B cell lineages *in vivo* (Palacios and Steinmetz, 1985 and Palacios *et al.*, 1987). Murine long term bone marrow cultures (LTBMC) support the differentiation of early B cells (Whitlock and Witte, 1982) and the addition of IL-3 to LTBMC can enhance the production of B cell precursors (Rennick *et al.*, 1989). Cells from these cultures are unresponsive to IL-3

alone but IL-3 synergises with stromal cell conditioned media to support their growth. The strongest evidence for an effect of IL-3 on differentiation of T lymphocytes has been the identification of IL-3-dependent pro-T cell lines (Palacios and Samaridis, 1987). It has been reported that IL-3 may support the proliferation of early T cells that can initiate delayed-type hypersensitivity responses in an antigen-specific manner (Keever *et al.*, 1989) and in addition, that IL-2-dependent, cytotoxic, large granulated lymphocytes can be obtained from IL-3-induced progenitors (Minato *et al.*, 1988). In contrast, IL-3 has no effects on T-lineage committed early thymocytes (Okazaki *et al.*, 1989 and Palacios and Von Boehmer, 1986). Taken together the data are consistent with the hypothesis that IL-3 alone or in synergy with other factors, supports the proliferation of early pluripotential stem cells prior to commitment to the lymphoid lineages. Once committed to the T or B cell lineage, or shortly after commitment, they lose the ability to respond to IL-3.

Among the haemopoietic growth factors IL-3 is the most evolutionally divergent (Burger *et al.*, 1990) and there is little *in vitro* cross species reactivity even between closely related species. For example, Cohen *et al.* (1986) found very low or undetectable levels of cross reactivity between either rat and mouse IL-3's. Similarly, administration of recombinant human IL-3 (hIL-3) to rhesus (*Macaca mulata*) or cynomologous monkeys (*Macaca fascicularis*) exerted limited and inconsistent effects on the production of peripheral blood cells (Donahue *et al.*, 1988 and Umemura *et al.*, 1989) and recombinant rhesus IL-3 (RhIL-3) was 100 fold more effective in stimulating rhesus monkey haemopoietic progenitor cells *in vitro* than human IL-3 (Wagemaker *et al.*, 1990). Sequencing of the IL-3 genes from these species (Dorssers *et al.*, 1987, Yang *et al.*, 1986, Cohen *et al.*, 1986 and Yang and Clark, 1988) revealed an unusual number of substitutions of bases that change codon meaning, although in many cases the changes are conservative (section 1.3.4) these changes indicate that IL-3 is subject to a high evolution rate. Considering the lack of species cross reactivity with IL-3, it is interesting to speculate that IL-3 is rapidly evolving divergent sequences and perhaps functions. It has been suggested that evolution of the IL-3 gene

is occurring at a mutation rate approaching that of pseudogenes which are not believed to be subject to functional constraint (Burger *et al.*, 1990). Although the potency of IL-3 in stimulating bone marrow haemopoiesis has been demonstrated, it is worth emphasising that all of the target cells for IL-3 can also be recruited using other growth factors, either alone or in combination, suggesting that there may well be no absolute requirement for IL-3. The rapid divergence of the rodent and human and non-human primate IL-3 genes over such a relatively short evolutionary period compared to that of other HGFs, is all the more remarkable considering the restraint of co-evolution of high affinity receptor binding and that the co-evolution of the IL-3 specific α -chain of the receptor is also constrained by interaction with the β -chain which is shared with a number of other CSF specific α -chains (see section 1.2.3)

1.3.2 The role of IL-3 *in vivo*

The first *in vivo* animal experiments using high doses of recombinant murine IL-3 surprisingly showed only modest effects upon circulating leukocyte levels particularly in view of the *in vitro* potency of IL-3 (Lord *et al.*, 1986, Kindler *et al.*, 1986, Metcalf *et al.*, 1986, Kalland, 1987, and Kimoto *et al.*, 1988). The effects most commonly noted were induction of splenomegaly and a shift of haemopoiesis from the bone marrow to the spleen, with increases in eosinophils, neutrophils, mast cells, and macrophages (Molineux, Pojda and Dexter, 1990). Subsequent experiments using mice pretreated with iron-saturated lactoferrin, which suppresses myelopoiesis and the release of some HGFs *in vitro*, were successful in detecting the effects of relatively small doses of IL-3 which were sufficient to enhance the cycling status and absolute numbers of all progenitors in the bone marrow (Broxmeyer *et al.*, 1987a). Even lower doses of IL-3 were effective in combination with GM-CSF or M-CSF (Broxmeyer *et al.*, 1987b) and confirmed the significance of combinations of HGFs in the physiological regulation of blood cell production *in vivo*. In addition, it has been observed that chronic overproduction *in vivo* of murine IL-3 causes a non-malignant myeloproliferative disorder (Chang *et al.*, 1989 and Wong *et al.*, 1989). In man initial

dose finding clinical trials with IL-3 showed small increases in neutrophils, eosinophils, monocytes, lymphocytes and basophils, with increases in bone marrow cellularity in patients with bone marrow failure (Ganser *et al.*, 1989). Administration of IL-3 is well tolerated, the side effects are minor and consist of flu-like symptoms and headache. Recent phase II trials involving long term recombinant IL-3 treatment of patients with myelodysplastic syndromes, have produced increases in platelets in some patients and an increase in leukocyte and also reticulocyte counts, although without an increase in total haemoglobin levels. Morphological analysis of the marrow revealed an expansion of the myeloid compartment in most patients due to stimulation of precursor cells (Ganser, 1993). Recently, Frisch *et al.*, (1993) have shown, in studies where IL-3 was combined with GM-CSF and administered to patients with intact haemopoietic function, an enhanced stimulation of megakaryopoiesis combined with a more pronounced stimulation of granulopoiesis than with IL-3 alone. Data by the same authors on patients with chemotherapy induced myelosuppression showed beyond the known amelioration of myelosuppression by GM-CSF treatment, an additional ability to enhance platelet recovery when GM-CSF and IL-3 are coadministered. As a corollary, chemotherapy postponement due to insufficient bone marrow recovery is less frequent when IL-3 is administered (de Vries, van Gameren and Willemsse, 1993). Recent reports have demonstrated that primitive progenitor cells are also mobilised into the circulation of patients receiving G-CSF and GM-CSF and IL-3 (reviewed by Guillaume, D'Hondt and Symann, 1993 and de Vries, van Gameren and Willemsse, 1993). The potential clinical uses of blood borne stem cells are obvious and has led to the use of blood stem cells for autologous transplantation (Gianni *et al.*, 1989 and de Vries, van Gameren and Willemsse, 1993).

1.3.3 IL-3 gene structure and genomic organisation

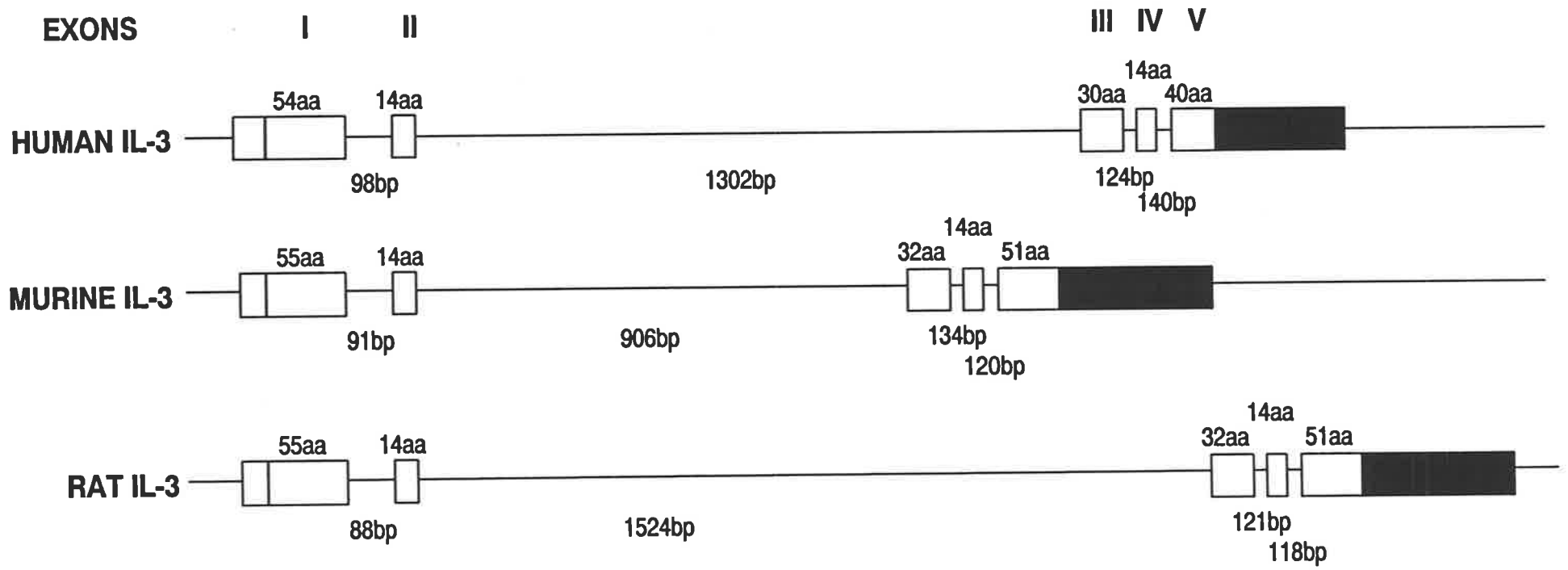
Recombinant cDNA clones encoding murine IL-3 was first isolated by Fung *et al.* (1984) by translation of an mRNA library in *Xenopus laevis* oocytes and cDNA cloning of the active mRNA fraction. The translation products were assayed for

biologically active IL-3 using the IL-3 dependent cell line, 32D cl-23. The source of mRNA was the myelomonocytic cell line WEHI-3B which produces IL-3 constitutively. Murine IL-3 was independently isolated by its mast cell growth factor activity by Yokota *et al.*, (1984). Southern hybridisation showed that there is a single copy of the IL-3 gene in the haploid mouse genome (Miyatake *et al.*, 1985 and Campbell *et al.*, 1985) and hybridisations on genomic DNA from a range of mammalian species including humans showed that only the rat sequence was significantly similar to the murine probe. Rat IL-3 was subsequently cloned and characterised using the mouse sequences as a molecular probe (Cohen, Hapel and Young, 1986). For some time the human IL-3 gene had proved elusive but eventually the gibbon gene was cloned by expression cloning, providing a molecular probe for the cloning of the human gene (Yang *et al.*, 1986). Rhesus monkey IL-3 was cloned to facilitate *in vivo* primate studies by using the gibbon IL-3 as a probe (Burger *et al.*, 1990).

The mammalian IL-3 genes have very similar structures (Clark and Kamen, 1987 and Yang and Clark, 1988). All genes consist of 5 small exons separated by one large intron (intron 2), and three small introns as shown in Figure 1.3. The human and murine cDNAs share a low but significant similarity of 49% at the nucleotide level and 29% at the amino acid level. The regions of highest similarity within the IL-3 genes are found in the promoter region and in the 3' non-coding sequence of the cDNA. For example, the rat and murine genes flanking regions (200 bp both 3' and 5' of the coding region) and the introns share 80% to 90% similarity respectively. Similarly, the human and mouse genes show 59% similarity in the proximal promoter region compared to 45% similarity in the coding regions and the introns (Yang *et al.*, 1988). In the 3' non-coding region of these two genes the similarity is also approximately 59%. The high degree of conservation in these non-coding sequences may be important in the regulation of the expression of these genes. Several sequence elements found in the promoters of the genes are also highly conserved and some of these elements are found in the promoters of other cytokine genes (discussed in chapter 4). There is also a

Figure 1.3 The genomic organisation of the human mouse and rat IL-3 genes.

The diagram represents a comparison of human (Yang and Clark, 1988), murine (Miyatake *et al.*, 1985) and rat (Cohen Hapel and Young 1986) IL-3 gene structures. The coding sequences of the IL-3 genes from the AUG codon to the termination codon, are represented by open boxes. The exons are numbered (roman numerals) and the number of amino acids encoded by each exon is shown above the exon. In addition, the size of each intron is shown in bp under each intron.



tandem repeat ATTTA element in the 3' non-coding region which is present in all of the IL-3 genes. Similar AT rich sequences are present in the untranslated mRNA regions of genes encoding the murine and human GM-CSF and several other cytokine and oncogene mRNAs (Shaw and Kamen, 1986). Experimental evidence suggests that these regions regulate mRNA stability and may therefore play a critical role in the regulation of haemopoietic growth factor and cytokine gene expression (Shaw and Kamen, 1986 and Chapter 3).

The human (Yang *et al.*, 1988) and murine (Ihle, Silver and Kozak, 1987) IL-3 genes have been genetically mapped to chromosomes 5 and 11, respectively. The human IL-3 gene is located on the long arm of chromosome 5 in the region of 5q23.3 to 5q32 (Yang *et al.*, 1988) and in mice it is positioned in a region of synteny or linkage conservation between the mouse and human genomes. In humans the IL-3 gene is located 9 kb 5' of the GM-CSF gene and is oriented in the same transcriptional direction (Yang *et al.*, 1988) and in mice, it is 14 kb 5' of the GM-CSF gene also in the same transcriptional orientation (Lee and Young, 1989). Human chromosome 5 also contains the loci for M-CSF (Kaushansky, 1987), IL-5 (Sutherland *et al.*, 1988), IL-4 (Miyajima *et al.*, 1988), the M-CSF receptor c-fms (Neinhuis *et al.*, 1985) and platelet derived growth factor receptor (PDGFR, Westermark *et al.*, 1990). The close proximity of the IL-3 and GM-CSF genes, along with the similarity in their gene structures and extensive overlap in the biological activities of their protein products, suggest that the two genes may have evolved from a common ancestor through gene duplication and divergence.

Chromosomal rearrangements that affect the IL-3 gene have been identified in both murine and human haemopoietic malignancies or tumours cell lines. Rearrangements of the murine IL-3 gene that result in its constitutive expression have been demonstrated in a variety of murine myeloid cell lines (Ymer *et al.*, 1985 and Isfort, 1990). In all cases that have been examined, the rearrangements involve the insertion of intracisternal A particle elements or retroviruses in the 5' or 3' regions of the gene. The intracisternal A particle acts as a strong enhancer to cause constitutive high level

expression of the IL-3 gene. The ability of the endogenously produced IL-3 to support growth has been demonstrated by transfecting the IL-3 gene into growth factor-dependent cell lines (Wong, Chung and Nienhuis, 1987 and Hapel *et al.*, 1986). Interestingly, a carboxyl modified form of murine IL-3 that is retained intracellularly, can stimulate autocrine growth (Dunbar *et al.*, 1989). In humans, deletion of the IL-3 gene, as well as the GM-CSF gene, occurs in patients with myelodysplastic syndrome or acute nonlymphocytic leukemia secondary to cytotoxic therapy for other diseases (Wisniewski and Hirschhorn, 1983, Sokal *et al.*, 1975). The significance of these deletions to haemopoietic disorders or to the development of malignancies is not known. There are also recent reports demonstrating the rearrangement of the IL-3 gene in cases of pre-B cell ALL with t(5;14)(q31;q32) translocations that are associated with eosinophilia (Grimaldi and Meeker, 1989 and Meeker *et al.*, 1990). In the translocations that have been characterised, the breakpoint in the IL-3 gene is -462 or -934 bp from the transcriptional start site and joins the IL-3 gene to the J region of the IgH locus on chromosome 14 in a head-to-head orientation and results in the constitutive transcription of the IL-3 gene. In one patient, circulating, biologically active IL-3 was also detected. Not all cases of myelodysplastic disorders however, involve the IL-3 gene. In particular, Ihle *et al.*, (1992) have recently identified a case of B cell ALL with t(5;12)(q31;p13) translocation and eosinophilia, in which transcripts were not detected for IL-3, GM-CSF, IL-4 or IL-5.

1.3.4 Regulation of IL-3 production

In general, the expression of all HGFs is highly regulated and reflects the intricate mechanisms that must be involved in production of haemopoietic cells. IL-3 is the only CSF to be produced predominantly in T-lymphocytes (Ihle and Weinstein, 1986 and Niemeyer *et al.*, 1989). The expression of IL-3 in these cells is inducible and is restricted to antigen or mitogen activated cells. More recently it has been reported that human thymic epithelial cells produce IL-3 (Dalloul *et al.*, 1991). The significance of this observation is not known. In addition, mouse but not human mast cells can produce IL-3

in response to the stimulation of IgE receptors (Plaut *et al.*, 1989) and an IL-3 like activity has been detected in neutrophilic and eosinophilic granulocytes (Kita *et al.*, 1991). These observations have considerable importance since it can be envisioned that with antigenic stimulation, the autocrine production of IL-3 could significantly expand the mast cell population and enhance the cellular immune response at sites of infection and inflammation.

The T-cell specificity for the production of IL-3 is particularly striking when compared to GM-CSF which, like IL-3, is expressed by activated T cells, but unlike IL-3, is also expressed by macrophages in response to inflammatory stimuli and by stromal fibroblasts and endothelial cells in response to IL-1 or TNF (Bagby, 1987, Broudy *et al.*, 1986, Thorens, Mermoud and Vassali, 1987 and Wimperis *et al.*, 1989). The production of IL-3 predominantly in activated T-cells has led to the concept that IL-3 may only be involved in immunological regulation of haemopoiesis and specifically does not play a role in normal, constitutive, haemopoiesis (Azoulay, Webb and Sachs, 1987).

A number of studies have examined the ability of sub-populations of murine CD4⁺ T-cells to produce various HGFs. Distinct classes of helper, inducer T-cell populations have been defined by the spectrum of HGFs that they produce (Mosmann *et al.*, 1987 and Cherwinski *et al.*, 1987). One class, Th1, produces IL-2 but not IL-4, while the second class, Th2, produces IL-4 but not IL-2. Both populations of helper T-cells produce IL-3. However, other studies with primary antigen reactive T-cell clones have found less of an indication for the coordinate expression of various HGFs in activated T-cells (Gough and Kelso, 1989).

The production of IL-3, like other HGFs, is specifically inhibited by glucocorticoids (Culpepper and Lee, 1985) or cyclosporin A (Palacios, 1985). These inhibitors do not affect transcription of the gene or secretion of IL-3 in cell lines in which the gene is constitutively transcribed due to retroviral insertions (*i.e.* WEHI-3 cells, Palacios, 1985), suggesting that they function through blocking the specific signal transducing events that are initiated at the T-cell receptor and are responsible for activating gene transcription.

1.3.5 Structural properties of IL-3

Purified, native murine IL-3 is a glycoprotein which exists as a monomer with an average molecular size of 28 kD and contains on average approximately 38% carbohydrate by weight (Ihle *et al.*, 1983). Studies with native, recombinant and synthetic IL-3 indicate that glycosylation is not necessary for biological activity (Clark-Lewis *et al.*, 1986 and Clark-Lewis, Hood and Kent, 1988). It has not been determined whether glycosylation effects the stability or biological half life of IL-3 *in vivo*. Human IL-3 has an apparent molecular size of about 15-30 kD reflecting the heterogeneity in the carbohydrate component.

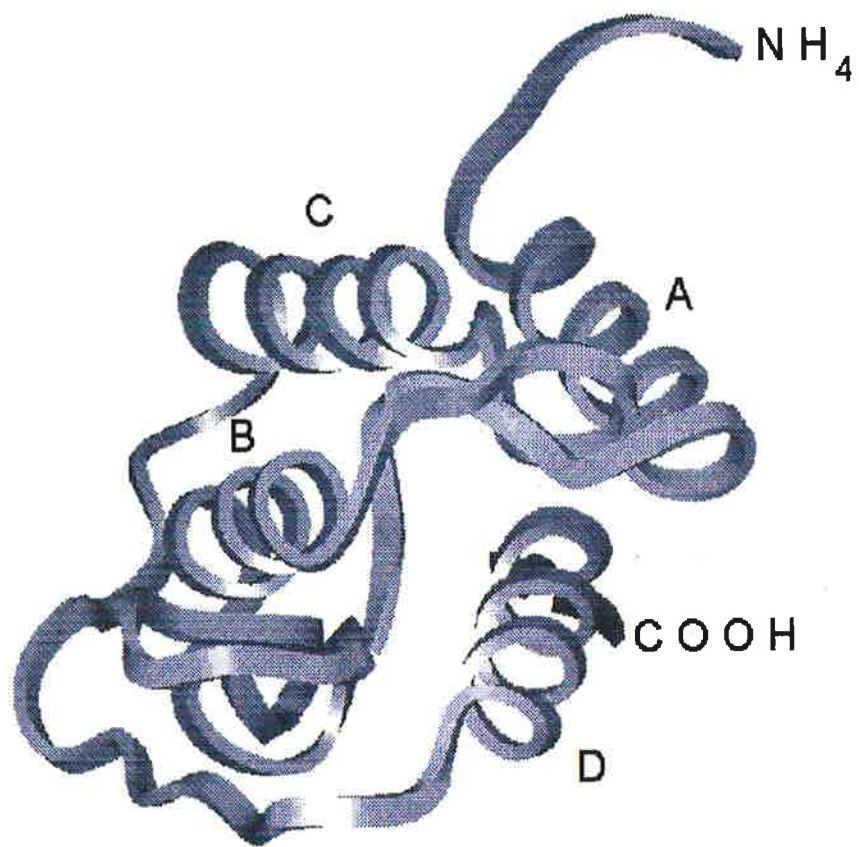
The primary structures for murine (Yokota *et al.*, 1984 and Yang *et al.*, 1986), human (Yang *et al.*, 1986), gibbon ape (Yang *et al.*, 1986), rat (Cohen, Hapel and Young, 1986) and rhesus monkey (Burger *et al.*, 1990) IL-3 have been deduced from the sequence of cDNA clones. The murine and human proteins have been the most intensively studied. The murine IL-3 gene encodes a protein of 166 amino acids and contains four sites of potential N-glycosylation. The first 26 amino acids encode a typical hydrophobic leader sequence required for secretion. The human and gibbon IL-3 genes encode a protein of 152 amino acids including a leader sequence of 19 amino acids and contains two sites for potential N-glycosylation. Consistent with the lack of biological cross-reactivity between murine and human IL-3, there is little sequence homology between the proteins. At the amino acid level, human and murine IL-3 only show a 29% similarity or, if conservative changes are included, a 38% similarity. The degree of amino acid homology between IL-3 proteins from different species is low when compared to other lymphokines. In comparison, IL-2 and GM-CSF have mouse/human similarities of 64% and 54% respectively (Cerretti *et al.*, 1986 and Cantrell *et al.*, 1985). Interestingly, a greater similarity between the mouse and human proteins is evident in the amino terminus of the mature protein (residues 7-21) and in sequence of basic residues near the carboxy terminus (residues 125-135) (Yang and Clark, 1988). The primate IL-3 proteins share significantly greater

homology. Rhesus monkey IL-3 shares 80.5% similarity to the mature human IL-3 although it is nine residues shorter than the human protein (Mr 13,974 non-glycosylated) and gibbon ape IL-3 shares 93% similarity with the human protein. This higher homology is reflected in the greater level of species cross reactivity especially between gibbon and human IL-3 (Lopez *et al.*, 1987 and reviewed by Yang and Clark, 1988). Interestingly, all but one of the nine human:gibbon amino acid differences in gibbon IL-3 are conserved between gibbon and rhesus IL-3, these non-human primate IL-3 proteins have an 86% identity.

It has been suggested by Goodall *et al.* (1993) that IL-3 probably adopts a tertiary structure, as depicted in Figure 1.4, similar to the known structures of GM-CSF (Diederichs *et al.*, 1993), IL-5 (Milburn *et al.*, 1993), IL-4 (Garrett *et al.*, 1992), IL-2 (Bazan, 1992) and growth hormone (GH, de Vos *et al.*, 1992), all of which have similar topologies involving four helical bundles. Studies using synthetically prepared murine IL-3, directed at identification of the domains involved in biological activity have suggested that the receptor-binding function is in the amino-terminal region of the protein (Clark-Lewis *et al.*, 1986). However, recent experiments involving chimeras between gibbon and murine IL-3 (Kaushansky *et al.*, 1992) and site-directed mutagenesis of human IL-3 (Lokker *et al.*, 1991 and Lopez *et al.*, 1992) have identified putative contact points for the IL-3 specific receptor α -chain on IL-3 helix D and the C-terminal of helix A as well as the loop between helices A and B. For the receptor's common β -chain these experiments predict contact points on helix C and the N-terminal region of helix A (Figure 1.4). Elucidation of the mechanism of high affinity receptor binding has important implications for development of new agonists and antagonists of IL-3 function. One agonist with increased binding affinity and biological activity has been described by Lopez *et al.* (1992). The rational design of new IL-3 analogs would obviously be facilitated by a more thorough understanding of the protein structure after the crystallisation of IL-3 or co-crystallisation with the IL-3 receptor complex.

Figure 1.4 Structural model of human IL-3

A model structure of human IL-3 based on predicted similarity and the structural coordinates of human GM-CSF (Dr. C. Bagley, unpublished data). The α -carbon backbone, depicted by a ribbon, is shown with the four predicted α -helices labelled A-D. The amino and N terminal residues are also labelled. The sequences of IL-3 and GM-CSF were aligned both on amino acid similarity and secondary structure prediction using the GOR II algorithm. A Personal Iris computer was used to run the molecular modelling programs Insight II and Homology (Biosym Technologies Inc., San Diego, CA). The proposed coordinates of IL-3 were assigned from the homologous backbone coordinates of GM-CSF and some side-chain coordinates, the remaining side-chain coordinates were assigned from an amino acid library.



1.4 Regulation of eukaryotic gene expression.

The function of human cells requires thousands of genes to be turned on and off in a spatially and temporally regulated manner. Studies of tissue specific expression of genes have revealed that tissue specificity is largely due to differences in the rate of transcription between different tissues. In contrast, induced gene expression is related to virtually every known mechanism of control. Gene expression can be controlled at a multiplicity of points along the way from gene to protein product. The rate of initiation of transcription, premature termination of transcription, RNA processing, transport and stability, translational regulation and post-translational regulation all represent stages at which gene expression can be controlled. Other less common regulatory mechanisms include gene amplification, DNA rearrangement and transposition. It would be impossible to cover all the pertinent areas in the diverse field of eukaryotic gene regulation in this introduction, therefore the rest of this chapter will concentrate on aspects central to the work in this thesis, the organisation and structure of gene regulatory regions, the regulation of basal, inducible and tissue specific gene expression by transcriptional and post-transcriptional means, and the characteristics of transcription regulatory proteins and their mechanisms of action and regulation.

1.4.1 Regulation of Transcription

Transcription in eukaryotes is dependent on three separate RNA polymerase complexes each of which transcribes a different class of genes. RNA polymerase I (RNA Pol I) transcribes genes encoding the large ribosomal RNAs (class I genes), RNA Pol II transcribes genes that encode messenger RNA for protein synthesis and some small stable nuclear RNAs (class II genes) and RNA Pol III transcribes genes encoding the 5S RNA, tRNAs and RNAs involved in mRNA processing and protein transport (class III genes). These classes of genes also differ in their genomic organisation and the proteins that regulate their transcription (reviewed by Sawadogo and Sentenac, 1990). Reviews of class I and III genes and their regulation have

recently been published by Murphy *et al.*, 1989 and Palmer and Folk, 1990 and will not be discussed here.

Transcription of eukaryotic structural genes (class II genes) is influenced by DNA regulatory regions termed promoters, enhancers, locus control regions and silencers. (reviewed by Maniatis *et al.*, 1987, Johnson and McKnight, 1989 and Orkin, 1990). These regions in turn are composed of discrete DNA sequence motifs which constitute binding sites for the sequence specific DNA binding proteins that interact directly or indirectly with the RNA polymerase pre-initiation complex to regulate transcription initiation. (reviewed by Johnson and McKnight, 1989, Ullman *et al.*, 1990 and Green *et al.*, 1992). Promoters are located directly upstream of the start site of transcription and are required for accurate and efficient initiation of transcription. Transcriptional repression regions or silencers are also commonly found in the sequences proximal to the transcription start site of many tissue- or developmental-specific genes (Scholer and Gruss, 1984). These sequences control the activity of a constitutive promoter or positively acting region. Enhancers increase the rate of transcription obtained from the promoter alone. Enhancers have traditionally been defined as cis-acting regulatory elements that function independent of distance, orientation or position with respect to the coding region of the gene. Locus control regions, similar to enhancers, may be located several kilobases from gene promoters and are required for the activation of some multi-gene loci. Recent evidence has shown that the distinction between these regulatory regions appears to be arbitrary, the basic components of these regulatory regions, the DNA sequence elements which constitute binding sites for transcription regulatory proteins, may, in some cases be identical in promoters, enhancers and locus control regions and the mechanisms by which they facilitate transcription from these different regions may be indistinguishable (Schatt Rusconi and Schaffner, 1990)

1.4.1.1 Promoters

(a) Basal elements.

The detailed molecular analysis of many different promoters revealed a common pattern of organisation (Dyana and Tjian, 1985 and McKnight and Tjian, 1986). A typical promoter includes an AT rich region with an asymmetrical consensus sequence TATAAA designated the TATA box and one or more upstream activator elements. The TATA box is located 25-30 bp upstream of the transcription initiation site in higher eukaryotes and from between 40 to 120 bp in yeast (Ogden, 1986 and reviewed by Struhl, 1989) and serves as an assembly point for the polymerase holoenzyme complex. The TATA box functions primarily to ensure that transcripts are accurately initiated. Many gene specific variants of the TATA sequence have been conserved between species suggesting that the variants are required for optimal gene function (reviewed by Nussinov, 1990).

A guanosine and cytosine rich region or G/C box is common to many genes transcribed by RNA Pol II and is often present in multiple copies. This element represents a class of general transcription regulatory elements. In the Herpes simplex virus promoter, recognition of the G/C box by the transcription factor SP1 is required for transcriptional activation (reviewed by Kadonaga *et al.*, 1986). In addition to the G/C box, a sequence recognition motif, GGCCAATCT, often referred to as the CAAT box is also commonly found within 70 to 90 bp of the transcription initiation site (or cap site). This element was originally identified as an important RNA Pol II element in the vertebrate globin genes (Santoro *et al.*, 1988). Activity of the G/C or CAAT box elements depends in part on their orientation and on the distance from the TATA box. Insertions of odd multiples of a half turn of DNA between the upstream elements and the TATA box is more detrimental to transcription than addition of even multiples suggesting that proteins bound to upstream elements require specific alignment and interact directly with proteins bound to the TATA box (Takahashi *et al.*, 1986).

The TATA box is necessary for transcription in many but not all promoters. Substitution of the TATA element by G/C rich regions containing several binding sites for the transcription factor SP1 has been observed in one group of genes transcribed by Pol II (reviewed by Wasylyk, 1988). The genes in this group, found only in vertebrates and some higher plants, usually perform housekeeping functions (Hatton *et al.*, 1988) and are expressed constitutively at a low level in all cell types. Transcription of these genes is characterised by initiation at multiple sites.

A third group of genes is characterised by promoters which lack both the TATA element and obvious G/C rich elements. These genes are typically expressed in a developmental and tissue specific manner. The unique organisation of these promoters may be responsible for the restrictive pattern of expression observed for these genes during development and differentiation. Comparison of promoter structure of Pol II genes lacking a TATA element has revealed a loose consensus sequence $\text{PyPyC}\underline{\text{A}}\text{PyPyPyPyPy}$ that includes the transcription initiation site (underlined). This element, known as Initiator or Inr, has also been found in many TATA box containing promoters and can direct low level transcription in TATA-less genes from a single initiation site (Smale and Baltimore, 1989).

Studies of changed order, of the basal promoter elements, have revealed that it is primarily the relative order of the upstream element/TATA box rather than the sequence asymmetry of the TATA element that determines the polarity of transcription, while the nucleotide sequence of the cap site region affects the precision and efficiency of initiation. When the Inr site is eliminated or moved from its natural position other sequences about 25 bp downstream of the TATA box, are used as new initiation points (Breathnach and Chambon, 1981, Carcamo *et al.*, 1990)

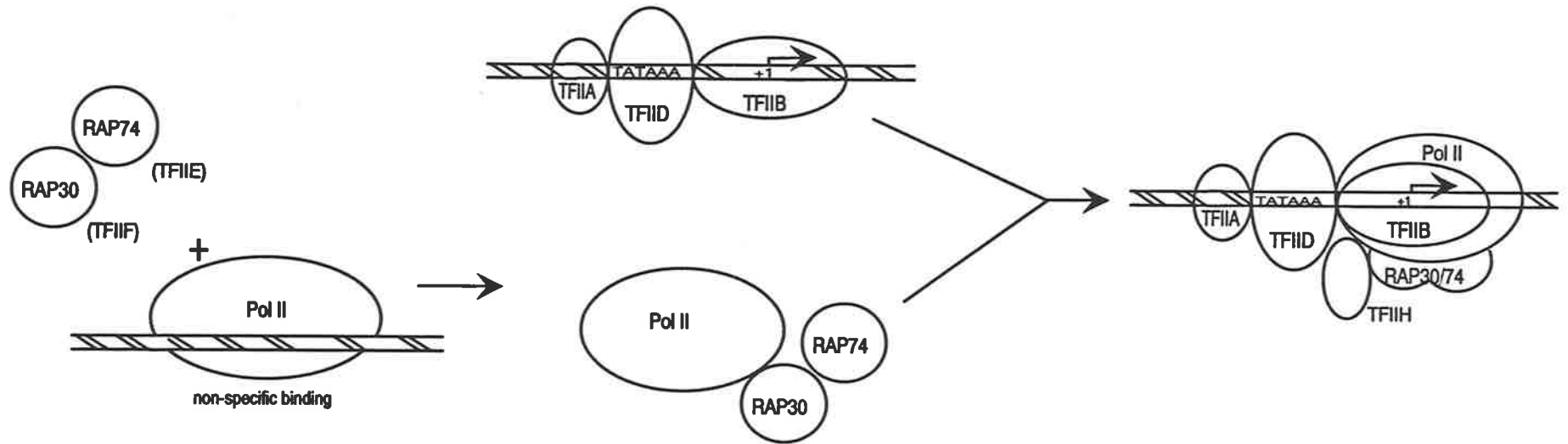
(b) TATA box binding factors and transcription initiation complex assembly

The TATA box binding protein (TBP), is required for transcription by all three nuclear RNA polymerases in eukaryotes and may be regarded as a commitment factor whose binding to the TATA box (and in its absence to Inr, Hernandez, 1993) is the

prerequisite for assembly of the basal transcription apparatus (reviewed by Sharp, 1992 and Hernandez, 1993). Initiation of transcription however, requires other general transcription factors (TFIIA, TFIIB, TFIIE, TFIIIF and TFIIH) and RNA polymerase to bind in a defined order to build the transcription pre-initiation complex as shown in Figure 1.5. In higher eukaryotes the association of TBP with transcription by different polymerases is determined by its interactions with tightly associated peptides called TAFs (for TBP Associated Factors, reviewed by Pugh and Tjian, 1992). The TBP/TAF complex involved in RNA polymerase II transcription has also been called TFIID (Matsui *et al.*, 1980). Purified TBP can function with the other polymerase II general transcription factors in basal level transcription *in vitro* from a TATA box promoter but the TAFs associated with TFIID are required for transcriptional activation by activator proteins (Dynlacht *et al.*, 1991 and Tanese *et al.*, 1991). Assembly of the pre-initiation complex begins with the stable binding of TFIID to the TATA element in a reaction that may be facilitated by another factor TFIIA. The subsequent binding of TFIIB acts as a bridge between the promoter bound TFIID and RNA Pol II. TFIIIF may also be required for the unwinding of the promoter DNA to form an open complex. The binding of RNA Pol II is also facilitated by the RAP (RNA Pol II Associated Proteins, reviewed by Greenblatt, 1991) factor complex, RAP30/RAP74, also known as TFIIIF and TFIIE respectively. The pre-initiation complex cannot initiate transcription without another general initiation factor known as TFIIH. TFIIE is required for the stable association of TFIIH with the pre-initiation complex. TFIIH contains a kinase activity capable of phosphorylating the carboxy-terminal domain of the largest subunit of RNA polymerase II which is believed to control the transition from initiation complex to elongation (Lu *et al.*, 1992). TFIID remains bound at the TATA box following initiation, perhaps blocking nucleosome formation, and may catalyse multiple rounds of transcription (Workman and Roeder, 1987). RAP30/74 remains associated with Pol II during transcription and may influence transcription elongation with other factors such as RAP38 (Conaway *et al.*, 1991).

Figure 1.5 Assembly of Polymerase II transcription pre-initiation complex

Specific binding of TBP and TAFs or the TFIID complex to the TATA box is followed by binding of TFIIA and TFIIB. This complex, termed DAB, adopts an open structure, melting the DNA helix. RAP30/74 guides Pol II to the promoter bound DAB complex because it suppresses the binding of Pol II to non-promoter sequences. Additional factors such as TFIIF and TFIIS (not shown) may be required for transcriptional initiation and elongation.



(c) Tissue and developmental specific promoter elements.

In addition to the constitutive promoter elements, sequence motifs termed upstream promoter elements (UPEs), that confer cell type- or developmental-specific cis-regulation of genes or response to specific environmental stimuli are continually being discovered. A very large and growing number of these elements and their binding factors have been identified. The majority have been compiled and cross-referenced by D. Ghosh into a computer database containing 2155 binding sites and 523 transcription factor entries (TFD release 7.3, Ghosh, 1990 and Ghosh, 1993). An example is the GATA-1 element which contains a central core recognition sequence of 5'-GATA-3' and is bound by an erythroid/megakaryocyte specific protein also termed GATA-1 (Reddy and Shen, 1991). A GATA-1-DNA-protein complex plays an essential role in erythroid specific transcription of the ϵ -globin gene and although transcriptionally inactive by itself, is required by the distant globin locus enhancer to activate transcription (Gong, Stern and Dean, 1991). Recently, GATA-1 sequences were implicated in some rare forms of δ -thalassemia where homozygous mutations in the GATA-1 sequence (at -77bp) in the δ -globin promoter impairs δ -globin synthesis (Matsuda, Sakamoto and Fukumaki, 1992). Many of these sequence elements are found both in proximal promoters regions and in more distant transcriptional enhancers. Another well-studied example is the "octamer" sequence, which was thought initially to be involved in regulating immunoglobulin gene transcription in a tissue-specific manner in lymphoid cells (Forster, Stafford and Queen, 1985). The octamer oligonucleotide alone, coupled to a TATA element, has been found to be sufficient for lymphoid specific promoter activity (Wirth, Staudt and Baltimore, 1987). The octamer element illustrates the complexity of transcriptional regulation since the octamer sequence is recognised by a number of binding factors and is also found in the promoters of genes whose expression is not limited to lymphoid cells, such as the histone H2B (Harvey, Robins and Wells, 1982) and the herpes virus thymidine kinase promoter (Parslow *et al.*, 1987) among others. A large family of octamer binding proteins have been identified, of these OCT-1 an ubiquitous

factor (Sturm *et al.*, 1988), and OCT-2A (Staudt *et al.*, 1986) which is restricted mostly to the lymphoid lineage, are both able to activate transcription from octamer containing Ig heavy chain promoters in vitro (LeBowitz *et al.*, 1988).

(d) The modular nature of promoter regulatory elements.

An important general point to emerge from the study of promoter regulatory elements, is that many genes are regulated by combinations of elements that have a modular nature and these modules can often operate independently of each other (reviewed by Serfling *et al.*, 1985). Each promoter module may contain one or a number of DNA-protein consensus recognition sites specifying a type of regulatory function of the promoter. Elements which play equivalent roles in two promoters can be exchanged without seriously affecting the function of the promoter. An example of the modular nature of promoters is provided by the three distinct sequence elements that have been located for the thymidine kinase gene of the herpes virus (McKnight and Tjian, 1986). The first is the TATA box, located upstream (-20bp) of the transcription initiation site, which is needed for accurate initiation. The second middle region at -50 to -70bp and the third at -80 to -110bp are needed for efficient transcription initiation. The β -globin gene also has three analogous elements. A functional promoter can be constructed by joining the distal components of the TK gene to the middle and TATA components of the β -globin gene (Courey *et al.*, 1986).

1.4.1.2 Enhancers

Enhancers were first identified in the 5' control regions of eukaryotic viruses as elements that are effective in increasing transcription of a gene when placed in either orientation and at considerable distances either 5' or 3' to the promoter. Examples of enhancers positioned upstream or 5' to the initiation site have been found associated with a large number of eukaryotic genes (reviewed by Muller, Gerster and Schaffner, 1988 and Harrison, 1990). In some instances, the promoter control regions described above can also act as enhancers. In other cases, the enhancer lies 3' to the promoter, such as in the heavy and light chain immunoglobulin genes (Banerji, Olson and

Schaffner, 1983) and in the adult β -globin gene (Collias *et al.*, 1987). The pattern of transcriptional regulation by enhancers can be (a) constitutive, that is, active in all cell types of a given organism, an example is the simian virus SV40 enhancer (Jones *et al.*, 1988), (b) inducible, where enhancers respond to changes in the external environment of a cell to activate a gene, for example the heat shock element (Bienz and Pelham, 1986) and (c) temporal and tissue-specific, where enhancers are active only at specific times during development, or only in specific cell types such as the lymphoid specific immunoglobulin enhancer (Gillies *et al.*, 1983). Most enhancers or other control elements can operate as independent units irrespective of the promoter or gene to which they are attached, although in some cases there may be a synergism between the enhancer and its natural promoter. For example, such synergy has been observed in the transcriptional regulation of immunoglobulin heavy-chain gene, possibly due to cooperative interactions between two molecules of the same protein bound to both the promoter and enhancer regulatory regions (Garcia *et al.*, 1986). Mutational analysis and *in vivo* footprinting studies of many enhancers has revealed that in fact they are complex regulatory units, like the SV40 enhancer, containing subelements that bind different proteins (Ondek, Shepard and Herr, 1987 and reviewed by Dynan, 1989). Individual elements of an enhancer can be found in other enhancers and promoters (Bohman, 1987). In many cases the deletion of one element can be compensated by the duplication of another, thus, these elements also appear to be modular and functionally interchangeable (reviewed by Dynan, 1989).

How enhancers and other cis-control elements function to increase transcription from the promoter is not clear. The general model of enhancer function envisages them as entry sites for transcription factors that are then able to interact with RNA polymerase and other factors to set up a functional transcription complex. This might be by scanning the DNA until a promoter is found. Earlier work suggesting that enhancers had greatest effect on the most proximal of two promoters was considered to support the view that enhancers act as bidirectional entry sites for transcription factors, which then scan the DNA for nearby promoters (Wasylyk *et al.*, 1983). However, recent

evidence shows that tandem immunoglobulin promoters are equally active in the presence of the immunoglobulin enhancer suggesting that the enhancer exerts its influence uniformly over large distances and independently of the presence of intervening promoters (Atchison and Perry, 1986). Recent experiments by Muller *et al.* (1990), where the positioning of the SV40 transcriptional terminator between the mouse β -globin enhancer and promoter had no effect on β -globin transcription do not support the scanning hypothesis.

There is considerable evidence in prokaryotes and yeast to support the idea that transcription factors can interact at some distance with the promoter by looping out of the intervening DNA (reviewed by Ptashne, 1986, Amouyal, 1991 and Schleif, 1992). Evidence supporting this DNA bending or looping model is that cooperative binding between, for example, lambda repressors only occurs if the repressors can interact on the same face of the DNA helix and looping out of intervening DNA between two lambda repressor sites *in vitro* has been shown by electron microscopy (Ptashne, 1986). In addition, a single protein binds to both a promoter and enhancer region of the long-terminal repeat (LTR) of the murine sarcoma virus. Interestingly, mutations in the promoter that affect binding of the protein also affect its binding to the enhancer, implying cooperativity over a long distance (Johnson *et al.*, 1987). Also consistent with the looping-out model is the observation that the SV40 or cytomegalovirus enhancers can stimulate transcription of the β -globin promoter *in trans* when attached to the promoter via a protein (streptavidin) bridge (Müller, Sogo and Schaffner, 1989).

Other models for enhancer function suggest that they are involved in organising the surrounding chromatin into an active conformation. These models are supported by the observation that tissue-specific enhancers are almost invariably associated with DNase I hypersensitive sites (DHSS), which are thought to reflect regions of nucleosome-free DNA in the chromosome. The SV40 enhancer, for example, is associated with a stretch of nucleosome-free DNA several hundred base pairs in

length even if transposed to an abnormal position in the viral genome (Jongstra *et al.*, 1984).

A further model of enhancer function that has been proposed is that the enhancers affect distant genes by altering the twist of the DNA in the region, thereby increasing the negative supercoiling of an entire DNA domain and so increasing the transcription of supercoiling-dependent promoters within this domain. The association of topoisomerase II cleavage sites with some enhancers supports this model (Cockerill and Garrard, 1986). Recent experiments correlating DNA topology with transcriptional activity suggests that localised transient domains of supercoiling allow transcription from an otherwise quiescent rRNA promoter in a *Xenopus* oocyte transcription system (Dunaway and Ostrander, 1993). These authors suggest that supercoils created from the disassembly of nucleosomes may participate in the activation of the first transcriptional event. It is likely that more than one of these proposed mechanisms will be used by enhancers to stimulate transcription.

1.4.1.3 Locus control regions

A recently described transcription control element, the locus control region (LCR), has been shown to be involved in the regulation of the β -globin gene family. (Grosveld *et al.*, 1987 and reviewed by Orkin, 1990). This region confers developmentally regulated and erythroid specific expression on the embryonic (ϵ), foetal ($G\gamma$ and $A\gamma$) and adult (δ and β) globin genes. Regions with similar function have also been described for the human CD2 gene (Greaves *et al.*, 1989) and the α -globin gene family (reviewed by Orkin, 1990). In the case of the β -globin gene family, the LCR is located approximately 50 kb upstream, and consists of a number of DHSSs. When this region is linked to the β -globin gene or heterologous genes and introduced into mice, it confers high level, erythroid specific and copy number dependent expression, independent of site of integration in the host genome (Grosveld *et al.*, 1987). In addition, deletion of the LCR upstream of the α -globin gene family occurs in some rare forms of thalassaemia and inactivates the whole gene complex

(Orkin, 1990). Although the mechanisms by which the LCR region functions is unknown, it has been proposed that it organises the β -globin locus into transcriptionally competent DNase I hypersensitive domains and permits regulatory factors to gain access to individual genes in the locus and then additionally serves as an enhancer of β -globin transcription (Orkin, 1990).

1.4.1.4 Negative control of gene transcription

For some time, it was assumed that the tissue-specific or developmental regulatory effects of control regions in increasing transcription meant that they were activated by some positively acting factor(s) which were only present in a particular cell type or developmental situation. However, recent work suggests that in many cases genes are regulated by tissue- or developmental-specific repressors binding to specific sequences that in turn control the activity of a constitutive enhancer or other positively acting element. The evidence supporting this conclusion has come from two approaches. Firstly, finer dissection of control regions by deletion and single base-pair mutation and second, by competition experiments where increasing amounts of the control element to be tested are cotransfected with the complete gene, if the element acts as a negative control element, then the transcription of the test gene will be increased due to removal of negatively-acting factors (Scholer and Gruss, 1984). Examples of negative regulation are seen in the genes encoding β -interferon (Goodbourne 1990), *c-myc* (Chung *et al.*, 1986), *c-fos* (Sassone-Corsi and Verma, 1987), IL-2 (Williams *et al.*, 1991), IL-3 (Mathey-Prevot *et al.*, 1990) and GM-CSF (Coles *et al.*, 1993). The general model to emerge from studies of these and other genes is that tissue-specific and developmentally regulated genes are repressed in most tissues. These genes can be activated by transcription activation factors in certain tissues where the repressor is no longer synthesised or is inactivated by binding to some developmental-specific signal (reviewed by Karin, 1991).

The mechanisms employed in negative transcriptional control have been grouped into three broad classes, (1) inhibition of DNA binding by transcription activation factors,

(2) blocking or masking of activation domains and (3), silencing or direct repression by a DNA binding repressor protein. (reviewed by Renkawitz, 1990). The repression of transcription by exclusion of DNA binding of activator proteins by a site specific repressor has been observed in the β -interferon promoter. In response to environmental signals, such as viral infection, the repressor is inactivated and leaves the DNA, allowing the positively acting factors to bind and transcription to proceed (Goodbourn *et al.*, 1990 and Zinn and Maniatis, 1986). By a similar mechanism, the induction of IL-6 gene expression by a wide range of stimuli (IL-1, TNF- α phorbol esters and forskolin) is efficiently repressed by dexamethasone (Dex). DNase I footprinting experiments have shown that ligand activated glucocorticoid receptor, from Dex treated extracts, can bind specifically over the TATA box, the Inr site and the multiple cytokine- or second-messenger-responsive element (MRE II) in the IL-6 promoter and represses the IL-6 gene by occlusion of these basal promoter elements as well as the inducible IL-6 MRE II site (Ray, LaForge and Sehgal, 1990).

Squelching, the blocking of an activation domain of a transcription factor, occurs in the regulation of c-myc gene expression. The negative factor Myc-PRF binds to a repressor element abutting the binding site of the positively acting factor Myc-CF1 and prevents this factor from activating transcription (Kakkis *et al.*, 1989). Blocking of transcriptional activation does not always require specific DNA binding, for example the yeast factor GAL80 prevents gene activation by the GAL4 protein by binding to and masking the transcription activation domain of GAL4. GAL80 dissociates in the presence of galactose allowing activation of transcription of catabolic genes by GAL4 (Ma and Ptashne, 1988). Another similar mechanism termed quenching has also been described, where intermediary proteins that transmit the transcription activation signal from DNA bound transcription factors to the transcription complex are sequestered from activation domains of the bound transcription factors by repressor proteins (reviewed by Renkawitz, 1990). An example of regulation by this mechanism is repression by adenovirus E1a protein, of viral and cellular enhancers (Lillie *et al.*, 1986). In a similar fashion, repression of

c-Jun activation can be achieved by over expression of the acidic transcription activation domains of JunB, GAL4 or VP16. These domains can competitively bind an essential factor (p52/54) required for transmission of Jun activation signal to the polymerase (Oehler and Angel, 1992).

Factors that bind specifically to negative regulatory regions and have a direct repression/inactivation effect on the transcription initiation complex are termed silencers. An example of transcriptional repression by a silencer is provided by the developmentally regulated human ϵ -globin gene. A silencer element which has been identified in the promoter of the human ϵ -globin gene, (-177 to -392 bp), prevents expression of this gene after the embryonic stage of development (Cao *et al.*, 1989). Silencers may share some of the properties of enhancers in that this negative element can act at a distance and from positions both 5' and 3' to the transcriptional unit. In addition to factors which act solely as silencers, regulatory proteins that can function both as an activator and as a repressor have been identified. For example the homeobox protein, *Ultrabithorax*, has been shown to possess dual function, suppressing the *Antennapedia* promoter activity while activating the *Ultrabithorax* promoter (Krasnow *et al.*, 1989). The mechanism causing the different regulatory patterns in this case is poorly understood. One explanation is that the transcription factors may possess discrete activating and repressing domains and the relative contribution of the domains may depend on protein conformation, slight differences in DNA binding site sequence or interactions with other proteins (reviewed by Levine and Manley, 1989).

1.4.2 Transcription regulatory proteins

The identification and cloning of genes encoding eukaryotic transcription factors in the last decade has led to an avalanche of information on the structure and function of these factors. These factors appear to be modular having several broadly defined classes of transcription activation domains. Several families of DNA-binding domains that recognise specific promoter or enhancer sequences have also been identified by

truncation and mutagenesis experiments using cloned transcription factors. Transcription factors are grouped together according to the type of DNA binding domain that they possess. In some cases these domains can be interchanged between proteins, showing that they are independently folded units. The three-dimensional structures have been determined for members of most of these groups. The DNA binding domains fold in such a way that they present a protruding surface, a flexibly extended structure, or both, in order to contact the DNA, or the pre-initiation complex and its transcription accessory proteins. Contacts with the DNA, in most cases, occur in the major groove and includes hydrogen bonds and non-polar van der Waals interactions (reviewed by Pabo and Sauer, 1992). Interactions with atoms of the sugar-phosphate backbone especially hydrogen bonds to non-esterified phosphate oxygens are critical for positioning the binding domains so that their protruding structures align correctly with the base pairs they contact. They are also important for fixing the DNA in a particular conformation. The final part of this section describes briefly, the classes of DNA-binding domains for which structural data are available (see Table 1.2), the different activation domains and outlines how the activities of transcription factors are regulated.

1.4.2.1 The helix turn helix motif

Members of the most thoroughly studied group of DNA-binding proteins contain domains with a helix-turn-helix (HTH) motif (reviewed by Aggarwal and Harrison, 1990). Most of the prokaryotic transcriptional regulatory proteins fall into this group, as do the eukaryotic homeodomain proteins. The HTH is generally defined as a 20-residue segment, with two α -helices that cross at an angle of about 120° (Figure 1.6a). This motif is found embedded in domains of remarkably varied structure (reviewed by Aggarwal and Harrison, 1990). The second helix in the HTH has been called a 'recognition helix', because it lies in the major groove when HTH-containing domains bind DNA and because experiments that involve changing residues on its outer surface suggest that it is critical for specificity (Wharton, Brown

TABLE 1.2 Examples of DNA-binding domain classes based on structural similarities[†].

Group I	Helix turn helix
	Eukaryotic regulatory proteins
	Homeodomains
	<i>Antennapedia</i>
	<i>Engrailed</i>
	<i>Ultrabithorax</i>
	Hox 11, 2.4, 2.3
	Prokaryotic regulatory proteins
	lac repressor
	λ repressor
	434 repressor
	434 Cro
	λ Cro
	catabolite activator protein (CAP)
	trp repressor
	inversion stimulation factor (PIS)
Group II	Zinc-binding proteins
Class I	Zinc fingers
	Xfin (finger 31)
	SP1
	TFIIIA
	ADR1 (finger ADR1b)
	Zif 268 (fingers 1-3)
Class 2	Double loop zinc helix (LZnH)
	glucocorticoid receptor
	oestrogen receptor
	progesterone receptor
	vitamin D receptor
Class 3	
	GAL4
Class 4	
	GATA-1
Group III	Leucine zipper or coiled coil (bCC or bZip)
	GCN4
	Fos, Jun
	CREB/ATF
Group IV	Helix loop helix motif
	E2A, E12 and E47
	Myo D
	c-myc (N-myc and l-myc)
	<i>daughterless</i>
Group V	β-ribbon motif
Class 1	
	Met J
	Arc
Class 2	
	HU

[†] Harrison, 1991, Nichols and Nimer, 1992 and Pabo and Sauer, 1992.

Figure 1.6 Three dimensional structures of transcription factor DNA binding domains.

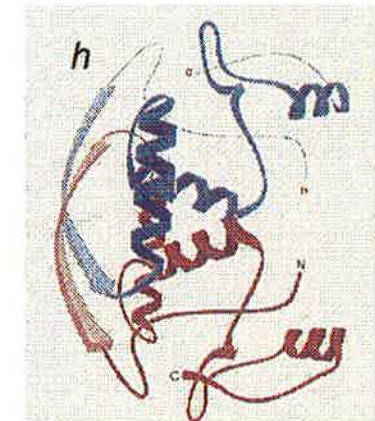
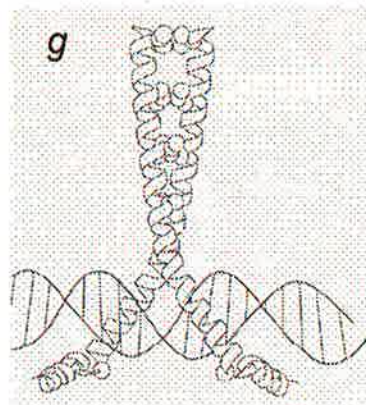
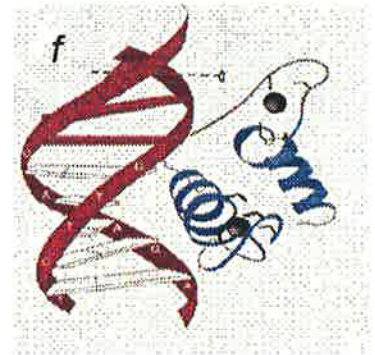
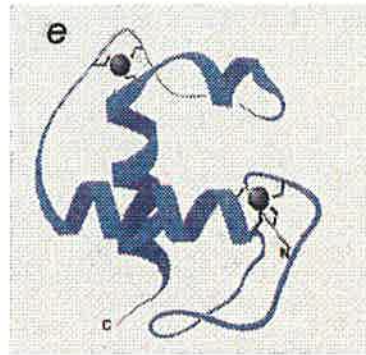
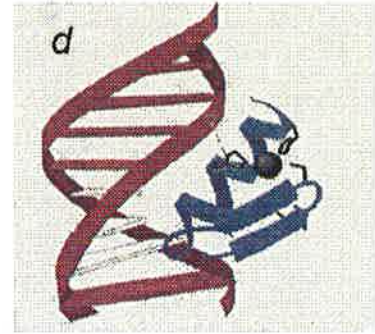
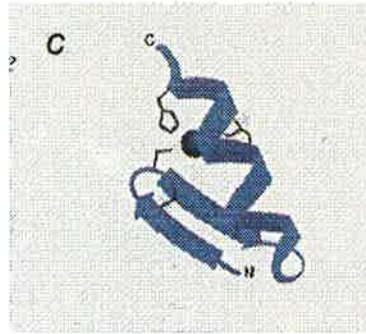
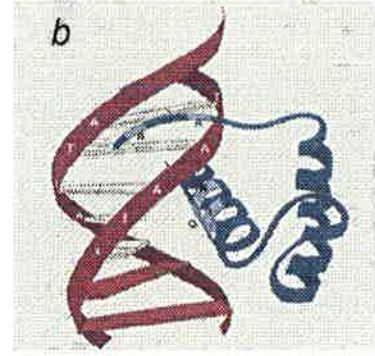
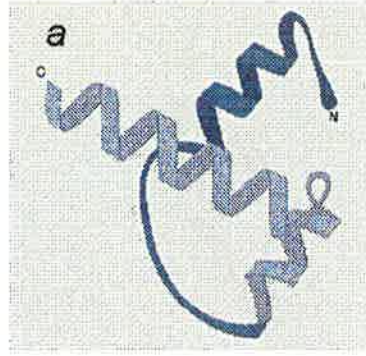
The three dimensional conformation of the α -carbon atoms of 5 families of DNA binding domains are represented by ribbon diagrams. (a) The helix turn helix (HTH) family, defined as a 20 residue segment with 2 α -helices that cross at an angle of 120° . (b) The second or C-terminal helix of the HTH motif lies in the major groove when bound to DNA. Additional N-terminal residues of HTH containing homeodomain proteins make contact with bases in the minor groove and enhance specificity of DNA recognition.

(c) The Class I 'Zn-finger', an approximately 30-residue module with one Zn ion (black sphere) liganded by two cysteines and two histidines. The folded part of the structure is a compact 12-residue helix. In each finger two cysteines flank a turn in the hairpin, and the two Zn-liganding histidines are on the inward-facing side of the helix. (d) Multiple fingers bind DNA each in essentially an identical manner at intervals of three base pairs. Subtle differences in the structure of the fingers and the immediate surrounding sequences defines the binding specificity.

(e) Class II (LZnH) zinc binding structures have an approximately 70-residue domain found in the receptors for the steroids and related hormone-like molecules. They contain two loop-helix elements, each with a Zn ion liganded by two cysteines at the beginning of the loop and two others at the N-terminus of the α -helix instead of the histidine pairs described for the group above. (f) The consensus DNA elements these proteins recognise have a two fold symmetry consistent with dimerisation of the binding factors. The axis of symmetry is shown by the dotted line.

(g) Leucine zipper motif has an approximately 30-residue segment forms the C-terminal half of a binding domain that contains a positively charged 'basic region' at its N terminus. The basic N-terminal region is thought to be responsible for contacting and binding to the DNA and the α helical C-terminus facilitates the dimerisation of two similar leucine containing helices

(h) The β -ribbon recognition motifs interact with DNA in the major groove as dimers through the extended β sheets. All of the proteins in this class are prokaryotic transcription factors.



and Ptashne, 1984). In some cases, such as the N-terminal arms outside of the helices of homeodomains or of the lambda repressor, additional residues make direct base-pair contacts (Figure 1.6b) and other proteins seem to enhance specificity by helping to position, or reposition, the homeodomain itself (Pabo and Sauer, 1992). Recently, another group of transcriptional regulatory proteins has been identified in which the homeobox forms part of a longer conserved domain, referred to as the POU domain. This domain also includes another 75-80 amino acid POU specific region. Proteins included in this group, among others, are the pituitary specific protein Pit-1 (Ingraham *et al.*, 1988) and the octamer binding factors OCT-1 (Sturm *et al.*, 1988) and OCT-2 (Clerc *et al.*, 1988). The relative contributions to DNA binding of the homeodomain and the POU-specific domain vary between the different proteins (Sturm and Herr, 1988 and Theill *et al.*, 1989).

1.4.2.2 Zinc binding domains

One of the first transcription factors to be cloned was TFIIIA a protein required for the transcription of 5S ribosomal RNA genes by RNA Pol III (Ginsberg, King and Roeder, 1984). It was found that TFIIIA was complexed with zinc and that zinc was necessary for DNA binding. Three distinct classes of zinc binding protein have been recognised since this time (Harrison, 1991).

The first is the 'Zn-finger', an approximately 30-residue module with one Zn ion liganded by two cysteines and two histidines. TFIIIA falls into this class (Miller, McLachlan and Klug, 1985 and Brown Sander and Argos, 1985). Most proteins containing these modules have three or more fingers in direct succession. The folded part of the structure is a compact 12-residue helix. In each finger two cysteines flank a turn in the hairpin, and the two Zn-liganding histidines are on the inward-facing side of the helix (Figure 1.6c). The direct concatenation of several fingers in Zn-binding proteins suggests that they form a repeating structure when bound to DNA (Berg, 1988). The fingers bind DNA in the major groove (Figure 1.6d) each in an essentially identical manner at intervals of three base pairs. Subtle differences in the structure of

the fingers and the immediate surrounding sequences defines the binding specificity of this group and interchanging fingers between two proteins that recognise related consensus elements creates interchanged specificities (reviewed by Harrison, 1991).

The second class of zinc binding structures is an approximately 70-residue domain found in the receptors for the steroids and related hormone-like molecules, with two Zn ions, each liganded by four cysteines (Freedman *et al.*, 1988). The structures of several class-2 Zn domains have been determined by NMR (Hard *et al.*, 1990 and Shwabe Neuhaus and Rhodes, 1990) (Figure 1.6e). They contain two loop-helix elements, each with a Zn ion liganded by two cysteines at the beginning of the loop and two others at the N-terminus of the α -helix instead of the histidine pairs described for the group above. The name 'double loop-Zn-helix' (LZnH) has been proposed for this structure (Shwabe Neuhaus and Rhodes, 1990). The consensus DNA elements these proteins recognise have a two fold symmetry consistent with dimerisation of the binding factors (Figure 1.6f) (Mader *et al.*, 1989).

A third class is found in a set of yeast activators, including GAL4, with two closely spaced Zn ions sharing six cysteines (Pan and Coleman, 1990). The motif incorporates a zinc coordination scheme similar to that of the metallothionine group of proteins. GAL4 binds as a dimer to a twofold-symmetric, 17-bp site (Bram and Kornberg, 1985 and Giniger, Varnum and Ptashne, 1985). NMR results show that the two Zn ions bound by each subunit form a binuclear cluster, with each Zn coordinated by four cysteines; two of the six cysteines are therefore shared (Pan and Coleman, 1990).

The structure of the haemopoietic-regulatory factor GATA-1 has recently been solved and in addition to the three previously defined classes of zinc binding domains it may represent a fourth class of Zn domain DNA binding protein (Omichinski *et al.*, 1993 and Orkin, 1990).

1.4.2.3 Leucine zipper and coiled coil motifs

A third type of DNA binding motif was originally identified by its dimerisation motif, an α -helical segment referred to as a 'leucine zipper' (Landschultz, Johnson and McKnight, 1988). Transcription factors in this group include the liver specific transcription factor C/EBP which binds both the CAAT recognition and SV40 enhancer core sequences, the yeast factor GCN4, and the proto-oncogenes c-Jun, c-Fos and c-Myc (reviewed by Bausch and Sassone-Corsi, 1990). This approximately 30-residue segment forms the C-terminal half of a binding domain that contains a positively charged 'basic region' at its N terminus. The basic N-terminal region is thought to be responsible for contacting and binding to the DNA and the α helical C-terminus facilitates the dimerisation of two similar leucine containing helices (Figure 1.6g). Because dimerisation occurs through formation of an α -helical coiled-coil the structure has been called a 'bCC domain' and the name 'bZIP' is also commonly used (O'Shea, Ruthowski and Kim, 1989). Dimerisation may represent a common property of sequence specific DNA binding proteins since it may increase the efficiency and specificity of DNA recognition. The leucine zippers of some of the bZIP proteins do not form stable homodimers, and a suitable heteromeric partner (such as Jun in the case of Fos) is required for dimerisation and DNA binding (reviewed by Kerpolo and Curran, 1991). Dimerisation between multiple related proteins with different abilities to influence transcription allows for more complex regulatory circuits than can be obtained with homodimers alone.

1.4.2.4 The helix-loop-helix motif

Originally identified in leucine zipper containing proteins (Figure 1.6g), the basic helix-loop-helix domain has also been found in several other transcription factors including the immunoglobulin enhancer binding proteins E12 and E47, MyoD a muscle regulatory protein, c-Myc and the *drosophila daughterless* gene (reviewed by Murre, McCaw and Baltimore, 1989). This domain is characterised by a basic region of about 15 residues which lies immediately N-terminal to a 15-residue amphipathic

helix. A region of variable size (9-20 residues) separates this helix from another of similar length. The helix-loop-helix motif mediates dimerisation. In almost all cases, a heterodimer of two such proteins is the active form in binding DNA. Thus the mechanisms of DNA binding of this class of protein may be similar to that of the leucine zipper proteins, but they may dimerise by a different structure.

1.4.2.5 β -ribbon proteins and other structures

In addition to the motifs described above, there are two classes of proteins that interact with DNA through β -ribbon recognition motifs (reviewed by Kim, 1992). All of the proteins in this class are prokaryotic transcription factors. The first class includes the Met J repressor of *E. coli* (Figure 1.6h) and the *arc* and *mnt* repressors of Salmonella phage P22. The second class includes the protein HU, which forms condensed nucleoprotein structures in many prokaryotes, the *E. coli* integration host factor IHF, and the *B. subtilis* phage transcription factor TF1. This class of DNA recognition motifs has recently been reviewed by Kim (1991) and Harrison (1991).

1.4.3 Transcriptional activation domains

It is generally held that transcription regulatory proteins must interact in some way with each other and/or with the RNA polymerase pre-initiation complex in order to influence transcription initiation. Surveys of the known transcriptional regulators have, together with deletion and mutagenesis experiments, defined a number of loosely conserved transcriptional regulatory domains which mediate the activation (or repression) functions of the transcription factor.

1.4.3.1 Acidic domains

While the activation domains of a number of transcription factors do not share amino acid homology, they do contain a high proportion of acidic amino acids. Examples of well studied transcription factors with acidic activation domains are the yeast factors GAL4 and GCN4 (Hope and Struhl, 1986 and Ma and Ptashne, 1987a). Consistent

with the hypothesis that a relatively nonspecific but predominantly acidic domain activates transcription, Ma and Ptashne (1988) have shown that random peptides that were able to activate transcription when fused to the binding domain of GAL4 contained an excess of acidic residues but no obvious sequence homology. The strength of the transcriptional activation by these fragments also correlated with the extent of their negative charge (Gill and Ptashne, 1987). In addition, Giniger and Ptashne (1987) have shown that a peptide which could form an acidic amphipathic helix with all the negative charges displayed along one helix could activate transcription when linked to the DNA binding domain of GAL4, but a random distribution of the same negatively charged amino acids was unable to do so. The ability of the acidic activation domain to activate transcription in the context of heterologous binding proteins indicates that this domain represents an independent functional unit. It has been suggested that the acidic domains activate transcription by interacting directly with the transcriptional pre-initiation complex, specifically TFIID (Stringer *et al.*, 1990) or TFIIB (Lin and Green, 1991). Herpes virus VP16 contains an acidic activation domain but does not bind directly to DNA. It forms complexes with other DNA binding proteins such as OCT-1 and OCT-2 and is able to transmit an activation signal by direct interaction with the transcription pre-initiation complex factors TFIIB and TBP (Brou *et al.*, 1993 and Siepel *et al.*, 1993). Recently, the oncoproteins v-Rel and c-Rel and RelA have also been shown to interact directly via their acidic activation domains with TBP and TFIID.

1.4.3.2 Non-acidic domains

Less common than acidic activation domains, glutamine rich activation domains were first identified in the transcription factor SP1 (Courey and Tjian, 1988). Since that time similar glutamine rich activation domains have been described in many mammalian transcription factors including, among others, OCT-1 (Sturm *et al.*, 1988) and OCT-2 (Arnosti *et al.*, 1993), AP2 (Williams *et al.*, 1988), the serum response factor (SRF) (Norman *et al.*, 1988) and in the *Drosophila Antennapedia*,

Ultrabithorax and *zeste* genes (Hunter and Karin, 1992). A correlation between the glutamine content and transcriptional activation capacity has been demonstrated for the factors OCT2 and SP1 (Müller-Immerglück *et al.*, 1990). A second non-acidic domain, rich in proline, serine and threonine has been described by Mermod *et al.*, (1989) This domain characteristically contains approximately 20-30% proline residues. Transcription factors reported to contain this activation motif are AP2 (Williams *et al.*, 1988), OCT-2 (Tanaka and Herr, 1990) and SRF (Norman *et al.*, 1988). Like the acidic activation motif, both the proline rich and the glutamine rich domains can activate transcription when in various positions in the DNA binding protein with respect to the DNA binding region, and are able to activate when placed in the context of heterologous DNA binding domains (reviewed by Mitchel and Tjian, 1989).

1.4.4 Regulation of transcription factor activity

The regulation of activity of transcription factors has been observed at multiple levels (reviewed by Hunter and Karin, 1992). Post-translational mechanisms are commonly used to modify transcription factor activity. The advantages of this form of regulation is that a transcriptional response to external stimuli, directed by the regulated transcription factor, can be delivered more rapidly and effectively than a response that relies on transcriptional or post-transcriptional mechanisms. Common post-translational mechanisms include modulation of the ability to directly or indirectly activate transcription by masking or modification of an activation (or silencing) domain, alterations in DNA binding affinity, nuclear transport and compartmentalisation by specific inhibitory proteins, dimerisation, and activation by ligand binding, in the case of steroid hormone receptor family. Recent evidence suggests that the convergence of more than one of these regulatory mechanisms on a single transcription factor is increasingly common (Hunter and Karin, 1992).

Over the past 5 years it has become evident that phosphorylation is able to regulate transcription factor activity by many of the mechanisms just described. One well

characterised example of a transcription factor whose trans-activating potential is regulated by phosphorylation is CREB (cyclic-AMP responsive element (CRE) binding protein, Hoeffler *et al.*, 1988). The CREB protein is a weak transcriptional activator in several cell types. However upon stimulation of the adenylyl cyclase pathway, CREB is phosphorylated by protein kinase A (PKA) which leads to a dramatic increase in its trans-activating potential (Wadzinski *et al.*, 1993). It has been suggested that this increase results from a phosphorylation-induced conformational change in the CREB protein leading to exposure of the glutamine-rich activation domain (Gonzales *et al.*, 1991). A similar unmasking of transcriptional domains also occurs in the Ets transcription factor family members Ets-1 and Ets-2 (Wasylyk, Kerckaret and Wasylyk, 1992). In addition, the DNA binding activity of the serum response factor is enhanced after phosphorylation by casein kinase II (Manak and Prywes, 1991).

Activation of the transcription factor NF- κ B occurs following translocation from the cytoplasm to the nucleus. NF- κ B exists in the cytoplasm as a complex with its repressor I κ B. The translocation of NF- κ B to the nucleus is dependent on the phosphorylation of I κ B by protein kinase C (PKC) which allows dissociation of the cytoplasmic complex (Ghosh and Baltimore, 1990). In contrast to PKC's regulation of NF- κ B, the dephosphorylation of NFATp, a component of the T-cell regulatory factor NFAT-1 and a target of the T-cell Ca²⁺ dependent signalling pathway, by the calmodulin dependent phosphatase calcineurin results in translocation of NFATp from the cytoplasm to the nucleus and the formation with AP1 of the NFAT complex (Schreiber and Crabtree, 1992). Nuclear transport is also a mechanism used by ligand-bound steroid receptor transcription factors (reviewed by Hunter and Karin, 1992) and the human heat shock proteins. Activation of the heat shock factor HSF1 is followed by oligomerisation and rapid translocation to the nucleus (Baler, Dhal and Voellemy, 1993).

A second method of post-translational modification, glycosylation, may also be involved in regulation of transcription factor activity. Glycosylation has traditionally been regarded as being restricted to exported proteins, those situated on cell membranes or within intracellular organelles, although glycosylated proteins have been detected in the nucleus (Jackson and Tjian, 1988). Jackson and Tjian (1988) demonstrated that a number of different transcription factors, including SP1 and AP1, bear multiple O-linked N-acetylglucosamine residues. Furthermore, within families of closely related transcription factors, only a subset of the family was glycosylated. These data suggest that differential glycosylation may be an important mechanism regulating the functional differences between closely related transcription factors. Recently, extensive glycosylation was detected on the carboxy terminal regulatory domain (CTD) of the large subunit of RNA Pol II (Kelly, Dahmus and Hart, 1993). The CTD of Pol II is also phosphorylated at multiple sites throughout the domain and the phosphorylation is believed to play an important role in the transition from pre-initiation to elongation (Lu *et al.*, 1992). The carbohydrate, however, is not detected on the phosphorylated form of the enzyme, suggesting that differential modification of the CTD may play an important role in regulation of transcription initiation by RNA Pol II.

A third type of post-translational modification used in the regulation of transcription factor activity, redox regulation, has recently been described (Matthews *et al.*, 1992 and Xanthoudakis *et al.*, 1992). Matthews *et al.* (1992) have shown that over-expression of human thioredoxin results in increased NF- κ B dependent transcription. The same authors suggest that modification of NF- κ B (p50) by thioredoxin, a gene induced in parallel with NF- κ B by stimulation of T-lymphocytes, may contribute to full NF- κ B activation. Another redox factor, Ref-1, regulates the binding activity of oncoproteins Fos and Jun through a conserved cysteine residue located in the DNA binding domain (Xanthoudakis *et al.*, 1992). Ref-1 also stimulated the DNA binding ability of several other transcription factors such as Myb and members of the CREB/ATF family (Xanthoudakis *et al.*, 1992).

An interesting example of a very complex array of post-transcriptional, post-translational and translational regulatory mechanisms, is demonstrated by another member of the cyclic-AMP (c-AMP) response factor family, CREM (CRE modulator, Foulkes *et al.*, 1991). This gene generates both transcriptional activators (CREM τ , τ 1 and τ 2, Laoide *et al.*, 1993) and repressors (CREM α , β and γ , Foulkes *et al.*, 1991) by alternate splicing. CREM τ is also phosphorylated by PKA upon activation of the c-AMP signalling pathway (de Groot *et al.*, 1993). Interestingly, the CREM gene encodes two different bZip DNA binding/dimerisation domains, which are used alternatively in the different isoforms (Foulkes *et al.*, 1991). In addition to these post-translational mechanisms, the CREM gene produces a further product (S-CREM) by alternative translational initiation at an internal AUG codon of CREM τ mRNA. This factor lacks the kinase inducible domain and acts as a repressor of c-AMP induced transcription (Delmas *et al.*, 1992).

In addition to the post-translational mechanisms described above, the alteration of mRNA stability is also used to regulate transcription factor activity. Changes in stability have been linked to the occurrence of AU rich regions in the 3' UTRs of several unstable transcription factor mRNAs such as that of c-Fos (Rahmsdorf *et al.*, 1987 and Wilson and Triesman, 1987) and c-Myc (Jones and Cole, 1987). Highly unstable mRNAs are characteristic of immediate-early-type and transiently acting response factors (Hunter and Karin, 1992).

Finally, evidence also shows that the regulation of transcription factors activity is controlled at the transcriptional level, particularly in the case of tissue-restricted transcription factors such as OCT-2 (Clerc *et al.*, 1988) and C/EBP (Xanthopoulos *et al.*, 1989). Auto-regulation of transcription factor gene expression is also increasingly common. A well studied example is the negative auto-regulation of c-fos transcription (reviewed by Mitchel and Tjian, 1989).

1.4.5 Post-transcriptional control mechanisms

1.4.5.1 Changes in mRNA Stability

In addition to the transcriptional control mechanisms described above, regulation of mRNA stability is an important control mechanism used by cells to achieve a rapid response to changes in the environment or during cell differentiation (reviewed by Brawerman, 1987). Individual mRNAs differ widely with respect to stability, but mRNAs encoding proteins whose levels change rapidly in a cell, such as the mRNA for c-fos (Greenberg and Ziff, 1984), are often unstable whereas others such as β -globin or β -actin appear to be very stable (Goodier and Kay, 1991 and Aharon and Schneider, 1993). The mechanisms involved in regulation of mRNA stability are poorly understood, however, sequences contained within the mRNAs are thought to play a role in cis-regulation of mRNA stability. These sequences are often located in the 3' untranslated region (UTR) of the mRNA and one class includes palindromic sequences with the potential to form stem loop structures that appear to confer resistance to 3' exonuclease attack (reviewed Brawerman, 1987). An example is the human transferrin receptor (hTR), where high iron levels increase the instability of hTR mRNA by inhibiting the binding of a protective factor, which is specific for palindromic sequences in the hTR 3' UTR. The same protein can also bind in the absence of iron to the 5' region of ferritin mRNA, which is responsible for the regulation of translation initiation of ferritin, and achieves coordinate reciprocal regulation of hTR and ferritin expression via a common factor (Mulner, Neupert and Kühn, 1989). Another example is the change in stability of β -tubulin mRNAs during liver regeneration. It has been shown that the levels of unpolymerised β -tubulin subunits govern the stability of the mRNA via a direct interaction with sequences lying within the first 16 translated codons of the mRNA and thereby auto regulate their own synthesis (reviewed by Cleaveland, 1988). An additional class of RNA regulatory signal is comprised of a rather nonspecific stretch of adenosine and uridine residues also in the 3' untranslated region of several HGF and oncogene mRNAs. That

the AU rich regions are present in many unstable mRNAs suggests an important role and a common function. AU rich elements are found in IL-3 mRNA and in GM-CSF mRNA and in the case of GM-CSF have been shown to mediate susceptibility of the mRNA to nucleolytic degradation (Shaw and Kamen, 1986) and to stabilise the mRNA in appropriately stimulated cells (Akashi *et al.*, 1991). Interestingly in the case of GM-CSF several mechanisms may be involved since TNF- α has been shown to stabilise the mRNA through a mechanism independent of the AU sequences (Akashi *et al.*, 1991). Further evidence of the role of these AU rich sequences in mRNA degradation was obtained by Peppel, Vinci and Baglioni (1991) whose results suggest that glucocorticoids that down regulate HGF production may activate a specific ribonuclease that degrades mRNAs containing AU rich sequences. Recently, a cytoplasmic protein termed adenosine-uridine binding factor (AUBF) that binds to the AU rich domains in GM-CSF and a variety of labile RNAs has been described by Malter and Gillis(1991). The binding of this protein may enhance the stability of the otherwise labile mRNAs. The binding of AUBF is regulated by external stimuli. A redox switch and phosphorylation are involved in the post-translational up-regulation of AUBF binding in response to phorbol ester and calcium ionophore (Malter and Hong, 1991).

Other less specific mechanisms such as translation rate may also have an effect on mRNA half life. It has been suggested that rapid translation of the μ immunoglobulin heavy chain mRNA in plasma cells compared to pre-B cells may confer stability in part by ribosomal protection from enzymatic degradation (Jack, Berg and Wabl, 1989).

1.5 Thesis aims

The primary aim of the work presented in this thesis was to investigate the mechanisms regulating the expression of the human IL-3 gene. At the outset of this work the restriction of human IL-3 expression to antigen or mitogen activated cells of the T-lymphoid lineage was apparent, although nothing was known of the mechanisms involved in the tissue specific and inducible nature of human IL-3 expression.

The specific aims were, (a) to obtain a model cell system to examine the contribution of transcriptional, post-transcriptional or translational mechanisms to the production of IL-3, (b) to determine the regulatory elements within the proximal region of the IL-3 promoter by transient transfection of IL-3 promoter/reporter gene hybrids, (c) to establish the role of the conserved cytokine elements CK1 and CK2, that are found also in the promoter of GM-CSF and in the case of CK1, in other HGF genes. It was hoped that an understanding of the function of these and other promoter elements may establish the mechanisms underlying the apparent coordinate expression of the GM-CSF and IL-3 genes in activated T-cells and (d) to identify and characterise the putative transcriptional regulatory proteins that bind to the IL-3 promoter and to establish their relationship to those that bind the closely related sequences in the GM-CSF promoter.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Drugs, chemicals and reagents

The following were obtained from Sigma Chemical Company, St. Louis, MO.

A23187, Acrylamide, Agarose (Type 1), Ampicillin, Bovine Serum Albumin (BSA), Chloramphenicol, Dithiothreitol (DTT), Ethidium bromide, Ethylenediaminetetra-acetic acid (EDTA), IPTG, N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (Hepes). Nonidet P40 (NP40), ONPG, salmon sperm DNA, Poly(dI:dC), sodium dodecyl sulphate (SDS), Tris base, Phorbol-12-myristate acetate (PMA).

Sources of other reagents were as follows:

Actinomycin D: Boehringer Mannheim, Mannheim, FRG.

BCIG: Bethesda Research Laboratories (BRL), Bethesda, MD.;

Ficoll 400, deoxyribonucleotide triphosphates (dNTPs), ribonucleotide triphosphates (rNTPs) and *E. coli* tRNA: Pharmacia-LKB, Uppsala, Sweden;

Human placental ribonuclease inhibitor (RNasinTM), Promega Corporation, Madison, WI.

Phenol, polyethylene glycol 6,000 and trichloroacetic acid (TCA): BDH

Chemicals, Poole, UK,

N,N,N',N'-teramethylethylenediamine (TEMED), bis-acrylamide (N,N'-methylene-bis-acrylamide): BioRad, Richmond, CA;

Phytohaemagglutinin (PHA): Wellcome Pharmaceuticals, Kent, UK;

Kits for random hexamer labelling of DNA and in vitro synthesis of RNA were obtained from Biotechnology Research Enterprises of South Australia (Bresatec). All other chemicals and reagents were of analytical grade.

2.1.2 Radiochemicals

[α -³²P]dATP (3,000 Ci/mmol), [α -³²P]dCTP (3,000 Ci/mmol), [γ -³²P]rATP (4,000 Ci/mmol) and [α -³²P]rUTP (3,000 Ci/mmol) were purchased from Bresatec.

[Dichloroacetyl-1,2-¹⁴C]-Chloramphenicol (60mCi/mmol) was purchased from Amersham, Amersham, UK. [³H]Uridine (Amersham) gift of Dr. T. M^cNaughton, University of Adelaide, South Australia.

2.1.3 Enzymes

All restriction enzymes and other DNA modifying enzymes used during the course of this work were purchased from either Pharmacia-LKB or BRL except where specifically indicated.

Other enzymes were obtained from the following sources:

lysozyme: Sigma Chemical Co.

Calf intestinal phosphatase, Chloramphenicol acetyltransferase (CAT), b-galactosidase, Proteinase K, ribonuclease A (RNase A): Boehringer Mannheim.

AMV reverse transcriptase: Promega Corporation.

T4 DNA polymerase: International Biotechnology Inc. (IBI), New Haven, CT.

2.1.4 Antibodies

Mouse monoclonal antibody (MAb) 9.3 supplied as a sterile ascites fluid: Bristol-Myers Squibb Pharmaceuticals, Seattle, WA.

Mouse MAb OKT3-rhodamine conjugate: Coulter Immunology, Melbourne, Victoria.

Mouse monoclonal OKT3-sepharose bead conjugate was a gift of Dr. L.K. Ashman, University of Adelaide, South Australia.

Sheep anti-mouse-FITC conjugate: Silenius, Sydney, New South Wales.

2.1.5 Buffers

Denhardt's solution: 0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidone, 0.1%(w/v) BSA.

PBS: 136mM NaCl, 2.6mM KCl, 8mM NaHPO₄, 2mM KH₂PO₄ pH 7.3.

NET: 100mM NaCl, 1mM EDTA, 10mM Tris-HCl pH 7.5.

SSC: 150mM NaCl, 15mM sodium citrate.

TAE: 40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA, pH 8.2.

TBE: 90mM Tris-HCl, 90mM boric acid, 2.5mM EDTA, pH 8.3

TBS: 10mM Tris-HCl pH 7.4, 137mM NaCl, 5mM KCl, 0.6mM Na₂PO₄, 0.7mM CaCl₂, 0.5mM MgCl₂.

TE: 10mM Tris-HCl pH 7.5, 0.1mM EDTA

TES: 25mM Tris-HCl pH 8.0, 10mM EDTA, 15% sucrose

All buffers were sterilised by autoclaving or where necessary by filtration through a Sartorius™ Minisart NML 0.2mm filter.

2.1.6 Cloning vectors

Plasmids pUC18, pUC19, pSP64 and pSP65 were purchased from Bresatec.

The bluescript vector pBS (KS-) was purchased from Strategene, La Jolla, CA.

2.1.7 Cloned DNA sequences

The following cloned DNA sequences used throughout this study were generous gifts.

Human Growth Hormone Expression Plasmid, p0GH: Dr. B. May, University of

Adelaide, South Australia. Transient transfection plasmids, pBLCAT3 and pBLCAT2:

Dr. B. Luckow, Institut für Zell-und Tumorbologie, Heidelberg, FRG. (Luckow, 1987).

Gibbon ape IL-3 cDNA clone, pMLA-IL-3, and the human IL-3 genomic DNA clones,

λ J1-16 and λ 66: Dr. S. Clark., Genetics Institute, CA. Human c-fos cDNA plasmid,

pBK28: Dr. I. Verma., Salk Institute, CA. Human β-actin cDNA clone, pHF-βA1: Dr. L.

Matchoss., Stanford University CA. Human GM-CSF cDNA clone, pCH5.2: Dr. J.

Gasson., University of California at Los Angeles, CA. RSV promoter driven

β -galactosidase expression plasmid, pRSV β -Gal: Dr. K. Kaushansky., University of Washington, Seattle, WA.

2.1.8 Synthetic oligonucleotides

Synthetic DNA oligonucleotides were synthesised by Mr. H. Suskin and Ms. J. Phillips on an Applied Biosystems automated DNA synthesiser using β -cyanoethyl phosphoramidites. Oligonucleotides cleaved from the column with the trytyl group attached were purified by reverse phase HPLC (BioRad) by Mr H. Suskin and the author. Oligonucleotides without an attached trytyl group were cleaved from the column and purified by gel electrophoresis (Section 2.2.10.2). The sequences are listed below:

PE-IL-3(1) 5'-dCTGGTCCGCCCCGGACTCCAAGCTCCCATGAC-3'

PE-IL-3(2) 5'-dTGAAGACAAGCTGGGTAACTGCTCTAAC-3'

CK-IL-3(1) 5' -dCTGTGGTTTTCTATGGAGGTTCCATGTCAGATAAAGATCC-3'

CK-IL-3(2) 5' -dGGATCTTTATCTGACATGGAACCTCCATAGAAAACCACAG-3'

CK-GM(1) 5' -dAATTCTGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTG-3'

CK-GM(2) 5' -dAATTCAACTACCTGAACTGTGGAATCTCCTGGCCTTATCAG-3'

M1CK-IL-3(1) 5' -dCTGTGGTTTTCTACTCTGGTTCCATGTCAGATAAAGATCC-3'

M1CK-IL-3(2) 5' -dGGATCTTTATCTGACATGGAACCAGAGTAGAAAACCACAG-3'

M2CK-IL-3(1) 5' -dCTGTGGTTTTCTATGGAGGTACGCTGTCAGATAAAGATCC-3'

M2CK-IL-3(2) 5' -dGGATCTTTATCTGACAGCGTACCTCCATAGAAAACCACAG-3'

M3CK-IL-3(1) 5' -dCTGTGGTTTTCTATGGAGGTTCCATGCTGTATAAAGATCC-3'

M3CK-IL-3(2) 5' -dGGATCTTTATACAGCATGGAACCTCCATAGAAAACCACAG-3'

Unrelated(X) 5' -dTCGATCGCCAATGAGCTCCCGGGTTCGACTGCAGAAGCTTC-3'

Unrelated(Y) 5' -dTCGAGAAGCTTCTGCAGTCGACCCGGGAGCTCATTGGCGA-3'

M13 Universal sequencing 17mer: 5' -dGTAAAACGACGGCCAGT-3'

M13 Reverse sequencing 25mer: 5' -dCACACAGGAAACAGCTATGACCATG-3'

2.1.9 Bacterial strains and bacterial growth media

The following *E. coli* K12 strains were used:

- (1) *E. coli* MC1061: *hsdR514 lacX74*, host for recombinant plasmids: Gift of Dr. J.B. Eagan. University of Adelaide, South Australia.
- (2) *E. coli* JM109: *recA1, endA1, gyrA96, thi1, hsdR17(rk⁻, mk⁻), supE44, Δ(lac-proAB), relA1, [F', traD36, proAB, lacI^q ZΔM15], λ⁻*: Gift of Prof. P. Reeves., University of Sydney, New South Wales.
- (3) *E. coli* JM101: *SupE44, thi1, Δ(lac-proAB), [F, traD36, proAB, lacI^q ZΔM15], λ⁻*: Gift of Prof. P. Reeves., University of Sydney, New South Wales.
- (4) *E. coli* LE392: *supE44 supF58 hsdR17* host for bacteriophage lambda DNA library: Gift of Prof. P. Reeves, University of Sydney, New South Wales.

E. coli Strains JM101 and JM109 were used as a plasmid host strains for blue/white selection of recombinant plasmids in the presence of BCIG and IPTG. Strain JM109 was used to prevent homologous recombination and *recA* dependant deletions of the propagated plasmid DNA. Stock cultures for long term storage of these strains (and plasmid transformed bacteria) were prepared by dilution of an overnight culture with an equal volume of 80% glycerol and stored at either -20°C, or -70°C Single colonies of bacteria, obtained by streaking the glycerol stock onto agar plates of suitable medium, as described below, were used to inoculate liquid growth medium. The bacterial cultures were grown at 37°C with continuous shaking to provide adequate aeration. Growth media was prepared using pure water obtained by ion exchange and filtration through a MilliQ water purification plant and was sterilised by autoclaving. Antibiotics and other labile chemicals were added after the solution had cooled to <50°C.

Luria (L) broth contained 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl. Ampicillin (100µg/ml) was added where appropriate for growth of transformed bacteria, to maintain selective pressure for the presence of the plasmid. 2xYT Medium contained 1.6% (w/v) Bacto-tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl. Ampicillin (100µg/ml) was added where appropriate. Agar plates were prepared by

supplementing the above media with 1.5% (w/v) Bacto-agar, antibiotics were added if appropriate. Soft overlays were 0.7% (w/v) agar in L broth or 2xYT medium.

2.1.10 Tissue Culture Cell Lines

The Human lymphoblastic leukaemia cell line Jurkat, was a gift from Dr. H. Zola, Flinders Medical Centre, South Australia. The gibbon lymphosarcoma cell line, UDC-144-MLA (MLA144) was a gift from Dr. G. Burns, Royal Newcastle Hospital, New South Wales (NSW). The following cell lines were obtained from the American Tissue Culture Collection (ATCC): HUT 78, HEL-Human embryonic lung fibroblasts, HL60, 5637 and LiBr.

2.1.11 Tissue culture media

Tissue culture cell growth medium contained RPMI 1640 medium diluted to the manufacturers specification, supplemented with 28mM NaHCO₃, 19mM glucose, 20mM HEPES pH 7.3, 1mM L-glutamine (Cytosystems, Castle Hill, NSW.), 100 U/ml Penicillin and 100µg/ml Streptomycin (Cytosystems). The medium was sterilised by filtration through Whatman 0.2µm filters. Growth medium for HEL cells contained Dulbecco's minimal essential medium (DMEM) diluted to manufacturer's specification and supplemented with 1mM L-glutamine, 28mM NaHCO₃, 20mM HEPES pH 7.3 and supplemented with 100 U/ml penicillin, 100µg/ml Streptomycin. The medium was filter sterilised as described above. Foetal calf serum (FCS, Cytosystems) was inactivated by heating to 56°C for 1 hour. Trypsin/EDTA solution: 0.1% trypsin (Flow) and 1 x EDTA (Commonwealth Serum Laboratories (CSL) was sterilised by filtration through a 0.2µm filter (Whatman).

2.1.12 Miscellaneous Materials

GF/A glass fibre filter discs and 3MM paper: Whatman Ltd.

Nitrocellulose (BA 85): Schleicher and Schuell.

Nylon membrane (Hybond N), X-ray film: (Hyperfilm MP) Amersham Ltd.

2.2 Recombinant DNA methods

2.2.1 General DNA methods

The following methods were performed as described by Maniatis *et al.*, (1982). Growth, maintenance and preservation of bacterial and viral strains; UV quantitation of DNA and RNA; autoradiography; denaturation and glyoxylation of RNA; agarose and polyacrylamide gel electrophoresis; DNA and RNA precipitations; phenol/chloroform/isoamyl alcohol extractions; end-filling or end-labelling of DNA fragments using the Klenow fragment of *E. coli* DNA polymerase I.

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Institute of Medical and Veterinary Science Biohazards Committee, The Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

2.2.2 Plasmid DNA preparation

The rapid alkaline lysis procedure described by Birboim and Doly (1979), was used for the isolation of plasmid DNA from 10ml overnight cultures for analytical restriction enzyme digestions. This method was also employed for the bulk preparation of plasmid DNAs from 500ml cultures for use as probes either for radiolabelling in Northern hybridisation analysis (Section 2.3.2) or for immobilisation on nitrocellulose filters for nuclear transcription run-on analysis (Section 2.4.2). DNA used for transfection of tissue culture cell lines, cloning vectors or *in-vitro* transcription templates was routinely prepared from 500ml cultures inoculated with a single colony from a freshly streaked agar plate. The plasmid was extracted using the alkaline lysis procedure described above and further purified by equilibrium density gradient centrifugation in a CsCl/ethidium bromide gradient using either Beckman L880:80ti or TL100 benchtop/TL100.3, centrifuge/rotor combinations. Ethidium bromide was removed by repeated (4x) extraction with equal

volumes of water saturated butanol. CsCl was removed by three precipitation steps, each following the addition of 0.1 volume of 3M sodium acetate pH 5.2 and 2.5 volumes of ethanol. Plasmid DNA was resuspended and stored in TE at -20°C .

2.2.3 Restriction enzyme digestions

For analytical digestions, 0.5–1 μg of DNA was incubated with 2–5 units each of the appropriate enzyme(s) for a minimum of 1 hour in the buffer conditions specified by the manufacturer. Reactions were terminated by the addition of a 1/3 volume of dye load buffer and electrophoresed on 1% mini–agarose gels in TAE buffer. In preparative digests, 5 μg of DNA was digested in a reaction volume of 30 μl , and the desired DNA fragments were isolated as detailed in sections 2.2.4 and 2.2.5

2.2.4 Preparation of cloning vectors

Plasmid DNA was linearised with the appropriate restriction enzyme(s). To prevent self–ligation of the vector, 5'–terminal phosphate groups were removed by incubation in 50mM Tris–HCl pH 9.0, 1mM MgCl_2 , 0.1mM ZnCl_2 , with 0.5 units of calf intestinal alkaline phosphatase (CIP) for 1 hour at 37°C . The vector DNA was isolated after electrophoresis on a 1.0% agarose TAE gel using GeneCleanTM (Bio 101, La Jolla, CA.) according to the manufacturers instructions. The DNA was resuspended at a concentration of 20–50ng/ μl , for use in ligation reactions.

2.2.5 Preparation of DNA restriction fragments

The DNA to be digested was incubated with the appropriate restriction enzyme(s) as described above (Section 2.2.3) and all restriction fragments were isolated from either a horizontal 0.8%–2.0% agarose gel or a vertical 10% polyacrylamide gel depending on the size of the DNA restriction fragment(s). Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide and the appropriate fragment(s) excised from the gel. Fragments isolated from polyacrylamide gels were eluted from the gel slice by incubation in 400 μl of water at 37°C for 16 hours. The DNA

was precipitated by the addition of 0.1 volume of sodium acetate and 2.5 volumes of ethanol, washed in 70% ethanol, air dried and resuspended in 10–20µl of Water.

Alternatively the DNA fragments were electrophoresed on 0.8%–2.0% TAE agarose gels and the DNA isolated using the GeneClean™ protocol. If flush DNA ends were required for cloning purposes the DNA fragments were treated prior to electrophoresis with 1–5 units of Sequenase v2.0 (Unites States Biochemicals) in the presence of 50mM dNTP's at 37°C for 30 minutes.

2.2.6 Ligation conditions

Ligation of DNA was carried out in 10µl reactions which contained 20–50ng of vector DNA, the DNA restriction fragment, 50mM Tris–HCl pH 7.4, 10mM MgCl₂, 1mM DTT, 1mM rATP, and 1–2 units of T4 DNA ligase. For cloning into plasmid vectors, a 3–5 molar excess of restriction fragment insert to vector DNA was used. The reactions were incubated for either 1–4 hours at 26°C, or overnight at 16°C. A control ligation, with vector only, was included in the subsequent transformation to determine levels of undigested or recircularised vector DNA.

2.2.7 Transformation of *E. coli* with recombinant plasmids

A single colony of the *E. coli* host strain was inoculated into 5ml of L–broth and the culture incubated overnight at 37°C with continuous shaking. The overnight culture was then diluted 100 fold into 50ml of L–broth and the incubation continued at 37°C, with shaking, until the culture reached an absorbance (A_{600}) of 0.6. The cells were then pelleted by centrifugation at 5,000g for 5 minutes, resuspended in 2.5ml of ice cold 0.1M MgCl₂, centrifuged again at 5,000g for 5 minutes then resuspended in 100µl 0.1M CaCl₂ and left on ice for >60 minutes. 200µl of this cell suspension was mixed with 2–5µl of the DNA ligation reaction (section 2.2.6) and left on ice for 10 minutes. The cells were then heat shocked at 42°C for 1 minute. L–broth supplemented with 20mM glucose was added (1ml if the overlay method of plating was used or 500µl if the cells were spread directly onto the agar plates), and the cells were incubated at 37°C for 20–30 minutes. The

transformed cells were then plated onto L-agar by spreading with a wire spreader. The agar plates were routinely incubated upside down at 37°C overnight.

2.2.8 Dideoxy-chain termination sequencing analysis

Double stranded sequencing was performed using plasmid DNA purified by centrifugation in a CsCl gradient (section 2.2.2). 3–5µg of plasmid DNA was used per reaction. The DNA was denatured in 0.2M NaOH, 2mM EDTA at 37°C for 30 minutes. The mixture was then neutralised by the addition of 0.1 volumes of 3M sodium acetate pH 4.6 and the DNA precipitated with 2.5 volumes of ethanol. The DNA pellet was recovered after centrifugation in an eppendorf centrifuge, washed in 70% ethanol, and resuspended in 7µl of 0.1mM EDTA. The sequencing reactions were carried out using a Sequenase version 2.0 sequencing kit (USB Corp.) in accordance with the protocol accompanying the kit.

2.2.9 Gel electrophoresis of DNA for sequence analysis

Sequencing reactions (2µl) were electrophoresed on 6% polyacrylamide gels containing 7M urea in TBE buffer at 1800 V. After electrophoresis, gels were fixed in 10% (v/v) acetic acid, washed with 20% (v/v) ethanol and dried onto whatman 3MM paper using a BioRad gel dryer at 80°C for 30 minutes. The gels were then autoradiographed for 16–48 hours at room temperature. All sequencing data was compiled and analysed using the Microgenie computer program (section 2.7.5).

2.2.10 Preparation of [³²P]–labelled DNA probes

2.2.10.1 Oligo–labelling of DNA

In all experiments a Bresatec kit was used for the oligolabelling of recombinant plasmids. 0.1–0.5µg of DNA was labelled in a 25µl reaction containing 100mM Tris–HCl pH 7.6, 20mM MgCl₂, 100mM NaCl, 200µg/ml BSA, 4µM each of unlabelled dGTP and dTTP, 50µCi each of [α -³²P]dATP and [α -³²P]dCTP, and 5.0 units of the large Klenow

fragment of *E. coli* DNA polymerase I. The reaction was incubated at 37°C for 60 minutes and then stopped by the addition of 5µl of 0.5M EDTA pH 8.0 and 5µl of stop buffer (10% (w/v) SDS, 10mg/ml tRNA). The DNA was precipitated by addition of 50µl of NET buffer, 125µl of 4M ammonium acetate and 400µl of ethanol. Precipitation was carried out at either -70°C for 30 minutes or at -20°C overnight. The DNA was pelleted by centrifugation for 30 minutes at 13,000g, washed with 1ml of ice-cold 70% ethanol, air dried and resuspended in 300µl of TE buffer.

Determination of TCA-precipitable radioactivity using 1µl aliquots (section 2.7.1) demonstrated that over 80% of total radioactivity in the ethanol precipitate was TCA-precipitable. The specific activity of probes was generally 1-2 x 10⁸cpm/µg. Immediately before addition to the hybridisation mix, the oligolabelled DNA was denatured by incubation at 100°C for 10 minutes and then snap-cooled on ice.

2.2.10.2 5'-labelling of synthetic DNA oligonucleotides

Synthetic DNA oligonucleotides used as probes were labelled at the 5' end using [γ -³²P]rATP and T4 polynucleotide kinase. The reaction mixture contained 10mM MgCl₂, 50mM Tris-HCl pH 7.4, 5mM DTT, 0.1mM EDTA, 50µCi [γ -³²P]rATP and 2 units of T4 polynucleotide kinase in a final volume of 10µl. The reaction was incubated at 37°C for 60 minutes. Following the addition of 10µl formamide/dye loading buffer, the end labelled DNA was electrophoresed on a 10-20% polyacrylamide gel at 30mA for 60 minutes to separate the [³²P]-labelled oligomer from unincorporated nucleotides. The labelled oligomer was localised by autoradiography, excised from the gel and eluted into 400µl of 1 x TE at 37°C for 16 hours. This solution was then used directly in hybridisations or was precipitated following the addition of 0.1 volume sodium acetate and 3 volumes of ethanol for use in primer extension experiments (section 2.3.5).

2.3 Methods for isolation and analysis of RNA

2.3.1 Isolation of RNA from tissue culture cells

Total RNA was extracted from tissue culture cells essentially as described by Chomczynski and Sacchi (1987). Following RNA extraction, absorbance values of each RNA sample were determined at 260nm and 280nm on a Beckman DU-50 spectrophotometer. The A_{260}/A_{280} ratios of the RNA samples were consistently in the range 1.6–2.0. The relationship of one A_{260} unit equal to 40 μ g/ml RNA was used in the calculation of RNA concentrations. RNA samples were stored in 75% (v/v) ethanol at -20°C .

2.3.2 Northern hybridisation analysis of RNA

Northern hybridisation analysis of total RNA was carried out by electrophoresis of glyoxal treated RNA on 1% agarose gels using 0.1M sodium phosphate buffer, followed by capillary transfer onto Hybond-N filters (Amersham UK). Filters were then irradiated for 3–5 minutes on an UV transilluminator (UVP Inc. Illinois) which results in the RNA being covalently cross-linked to the filter (manufacturer's instruction manual). Filters were pre-hybridised for 4–16 hours at 42°C in 50% (v/v) formamide, 5 x SSC, 5 x Denhardt's solution, 0.1% (w/v) SDS, 0.05% (w/v) sodium pyrophosphate (Na_4PP_i), and 200 μ g/ml of heat denatured sonicated salmon sperm DNA. Hybridisations were carried out for 18–24 hours under exactly the same conditions except for the addition of 20ng of radiolabelled probe ($1-5 \times 10^8$ counts/ μ g). Filters were washed twice in 2 x SSC, 0.1% (w/v) SDS at room temperature for five and twenty minutes respectively, followed by one wash in 0.2 x SSC, 0.1% (w/v) SDS at 60°C for 40 minutes.

2.3.3 *In-vitro* synthesis of [^{32}P]-labelled RNA

Plasmid DNA (40 μ g) containing the antisense template for RNA synthesis was linearised completely with the appropriate restriction enzyme. The extent of digestion was checked by electrophoresis and the template was purified by the GeneCleanTM protocol. 2 μ g of this DNA was added to a transcription reaction containing 40mM Tris-HCl pH 7.6, MgCl_2 ,

10mM DTT, 500 μ M each of rATP, rCTP and rGTP, 15 μ M rUTP, 1 μ g BSA, 100 μ Ci [α -³²P]rUTP and 4 units of SP6 RNA polymerase in a total volume of 20 μ l. 50 μ M rUTP was included in reactions for low specific activity probes, the reaction was incubated at 42°C for 60 minutes. Full length RNA probes were purified by electrophoresis on a 14cm long sequencing gel. The band corresponding to the full length probe was excised and the labelled probe was eluted for 1–2 hours in 400 μ l of RNA elution buffer (0.5M ammonium acetate, 1mM EDTA, and 0.1% (w/v) SDS) at 37°C. The buffer containing the probe was then aspirated away from the gel slice and the RNA was stored under 75% (v/v) ethanol at –20°C.

2.3.4 RN'ase protection analysis

10–20 μ g of cytoplasmic RNA and approximately 50,000 cpm each of the single stranded RNA probes were combined in a 1.5ml polypropylene tube with 1ml of ethanol and pelleted by centrifugation at 13,000g. The supernatant was removed and the RNA pellet dissolved in 24 μ l of deionised formamide and 6 μ l of 5 x hybridisation buffer (2M NaCl, 0.2M PIPES pH 6.4, and 0.01M EDTA) The mixture was heated to 85°C for several minutes, allowed to slowly cool to 45°C and incubated overnight at 45°C. Following hybridisation 350 μ l of RNase digestion buffer (0.3M NaCl, 10mM Tris–HCl pH 7.5, 5mM EDTA, 10 μ g/ml RNase A) was added. The reaction was incubated at 30°C for 40 minutes. 5 μ l of Proteinase K (25mg/ml) and 10 μ l of 20% (w/v) SDS were added and the incubation continued at 37°C for a further 40 minutes. The reaction was extracted with an equal volume of phenol/chloroform/isoamyl alcohol(25:24:1) and the undigested RNA recovered by precipitation after addition of 20 μ g of carrier tRNA and 1ml of ethanol. The protected RNA was dissolved in formamide/dye loading buffer, denatured by heating at 85°C for 2 minutes and analysed by electrophoresis on a 14cm, 6% sequencing gel. The gel was dried and autoradiographed using Hyperfilm MP X–ray film at –70°C overnight or for several days.

2.3.5 Primer extension analysis of RNA

Full length oligonucleotide primers were purified by polyacrylamide gel electrophoresis and the terminal phosphate labelled with ^{32}P using *E. coli* polynucleotide kinase (section 2.2.10.2). Cytoplasmic RNA (20–50 μg) isolated from Jurkat cells (section 2.3.1) was precipitated together with 1ng of kinased oligonucleotide primer by the addition of 0.1 volume of sodium acetate and 3 volumes of ethanol. Following centrifugation in an eppendorf centrifuge for 15 minutes the pellet was resuspended in 10 μl of hybridisation buffer, (10mM Tris–HCl pH 8.3, 200mM NaCl, 20U RNasinTM), heated to 80°C for 3 minutes then cooled slowly and allowed to hybridise at 42°C for >90 minutes or overnight. The extension reaction was carried out at 42°C for 60 minutes after the addition of 1 unit of AMV reverse transcriptase and 24 μl of extension buffer to give a final concentration of 10mM Tris–HCl pH 8.3, 100 mM NaCl, 14mM MgCl₂, 14mM DTT and 700 μM of each dNTP. The RNA template was then digested with 1 μg of RNase A at 37°C for 15 minutes. The primer extension reactions were then extracted with phenol/chloroform/isoamyl alcohol. The products of the primer extension reaction were precipitated by addition of 10 μg of *E. coli* tRNA, 0.1 volume of sodium acetate and 3 volumes of ethanol and centrifuged in an eppendorf centrifuge for 15 minutes. The pellet was resuspended in 5 μl of formamide/dye electrophoresis buffer, heated to 85°C for 2 minutes and electrophoresed in a 6% sequencing polyacrylamide gel. A double stranded di–deoxy sequencing reaction using the extension primer and the IL–3 genomic DNA clone (pGRIL3–1) as template, was employed as a size and sequence marker to identify the base corresponding to the IL–3 mRNA initiation site.

2.4 Methods for analysis of gene transcription

2.4.1 Isolation of nuclei from Jurkat cells

To ensure the cells were in the logarithmic growth phase they were routinely divided 1:2 the day before the nuclei were to be isolated. Cells were grown to an initial density of 5×10^5 cells/ml and were then stimulated for various time periods. Cells from one 100ml

flask (approximately 7.5×10^7 – 1×10^8 cells) were harvested by centrifugation in 50ml polypropylene tubes (Falcon) at 1,000g for 3 minutes at 4°C in a Beckman GPP Benchtop centrifuge. A sample of 5×10^6 cells was kept for extraction of cytoplasmic RNA. The supernatant was removed and the cells washed with 10ml of ice-cold PBS, centrifuged for 3 minutes at 1,000g, resuspended in ice-cold PBS and the wash repeated. The supernatant was completely removed and the cell pellet was loosened by vortexing gently prior to the addition of 5ml of NP-40 lysis buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl₂, and 0.1% (v/v) Nonidet P-40). The vortexing was continued as the buffer was added and for 10 seconds afterwards, this allowed the uniform resuspension of the cells and inhibited clumping. The lysed cells were incubated on ice for 5 minutes and then carefully loaded above 20ml of ice-cold sucrose, buffer (20% (w/v) sucrose 10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM MgCl₂). This density gradient was then centrifuged for 5 minutes at 1,500g and 4°C. The supernatant was discarded, and the nuclei were resuspended in 210µl of ice-cold glycerol storage buffer (50mM Tris-HCl pH 8.0, 0.1mM EDTA, 40% (v/v) glycerol, 1mM DTT, and 0.1mM PMSF) by gentle vortexing. The yield of nuclei was approx 3 – 5×10^7 . Nuclei were then transferred to 2ml polypropylene cryotubes (A/S Nunc, Denmark) and stored under liquid nitrogen for up to 1 week.

2.4.2 Nuclear run-on transcription assay

2.4.2.1 [³²P]-labelling of nascent RNA transcripts

Frozen nuclei were used in the transcription run-on experiments immediately after rapid thawing. Each transcription reaction contained 50mM Tris-HCl pH 8.0, 15mM KCl, 2.5mM MgCl₂, 0.6mM EDTA, 0.1mM PMSF, 1mM DTT, 24% (v/v) glycerol, 0.5 mM of each rATP, rCTP, rGTP, 2µM of unlabelled rUTP, 100µCi of [α -³²P]rUTP and 20 units of RNasin™ ribonuclease inhibitor in a final volume of 300µl. Transcription reactions were incubated for 20 minutes at 30°C.

2.4.2.2 Isolation of [³²P]-labelled RNA

The nuclear DNA was removed from run-on transcription reactions by addition of 40 units of DNase 1 and incubation for 30 minutes at 37°C. If still viscous another 40 units of DNase 1 was added and the samples incubated for a further 30 minutes at 37°C. The DNase 1 digestion was terminated by addition of 40µl of 10 x STE buffer (10% (w/v) SDS, 50mM EDTA, 10mM Tris pH 7) and 5µl of proteinase K (20mg/ml). The reaction was, after gentle mixing, incubated for 45 minutes at 37°C. RNA was isolated by extraction with 1.5ml of water saturated acidic phenol/chloroform/isoamyl alcohol (25:24:1). The top phase was collected and the phenol phase was back extracted with 200µl of 1 x STE buffer. 5M ammonium acetate was added to the pooled, phenol extracted, supernatants to a final concentration of 2.5M and the RNA precipitated by the addition of 1.5ml of isopropanol. After precipitation for 1 hour at -70°C or overnight at -20°C the RNA was collected by centrifugation for 30 minutes at 13,000g. The pellet was resuspended in 200µl of water and reprecipitated using the above conditions. Finally the pellet was washed with 75% ethanol and resuspended in 200µl water and TCA precipitable radioactivity was determined (section 2.7.1) in 2µl aliquots diluted into 100µl of stop buffer (10% SDS, 10mM EDTA, 100µg/ml of heat denatured, sonicated salmon sperm DNA).

2.4.2.3 Hybridisation of [³²P]-labelled RNA to immobilised DNA

DNA hybridisation target was applied to a nitrocellulose filter in a grid pattern using a dot-blot apparatus (BioRad). For each target dot, 5µg of DNA in 1 x TE buffer was incubated with 20µl of 3M NaOH at 65°C for 30 minutes, the mixture was then neutralised with 220µl of 4M ammonium acetate and kept on ice. The nitrocellulose filter was soaked, first in water and then in 2M ammonium acetate, for 10 minutes each. The DNA (final volume 440µl) was applied to the nitrocellulose under gentle suction, the dot-blot apparatus wells were then flushed with 1ml of 2M ammonium acetate containing 0.001% (w/v) bromophenol blue. The filter was then irradiated for 3 minutes on an UV

transilluminator (UVP Inc.) and air dried. The array of dots was then cut into strips (80mm x 5mm) containing a row of the target DNA dots. Filter strips were pre-hybridised in 1ml of hybridisation buffer (50% (v/v) formamide, 5 x SSC, 10mM Tris-HCl pH 7.6, 1mM EDTA, 0.1% Na₄PP_i, 0.1% SDS, 100µg/ml *E. coli* tRNA and 0.2% each of Ficoll, polyvinyl-pyrrolidone and BSA) at 45°C overnight. Hybridisations with equal amounts of [³²P]-labelled RNA added to each reaction (section 2.4.2.2) were carried out at 45°C for 72 hours in hybridisation buffer. Filters were washed twice at room temperature for 30 minutes in 2 x SSC, 0.1% (w/v) SDS, 0.1% (w/v) Na₄PP_i, then twice in 0.5 x SSC, 0.1% (w/v) SDS, 0.1% (w/v) Na₄PP_i at 65°C for 60 minutes. The filters were then incubated at 37°C in 1 x SSC, 5µg/ml RNase A for 30 minutes, washed briefly in 1 x SSC, air dried and autoradiographed at -70°C with Hyperfilm MP x-ray film (Amersham) and Cronex Lightening plus intensifying screens (Dupont, Wilmington, DE) for 3-14 days.

2.5 Methods for transient expression of plasmid constructs in tissue culture cell lines

2.5.1 Cell maintenance

All cells were maintained routinely in 150 cm³ or 600 cm³ flasks (Falcon) at 37°C in an atmosphere of 5% CO₂ in air and were subcultured every 3-4 days. To subculture adherent cells the culture media was removed and the cells washed in PBS before the addition of 3ml of trypsin/EDTA solution (Cytosystems). The cells were left at room temperature until they began to detach from the flask, 10ml of culture media was added and the flask washed to remove any remaining cells. The cells were routinely diluted 1:4 into fresh media and replated into fresh flasks or dishes. Non-adherent cells were subcultured by seeding at a dilution of at least 1:2 into pre-warmed growth medium supplemented with 10% FCS. If the cells were to be harvested they were washed once in 10ml of PBS and pelleted by centrifugation at 1,000g for 5 minutes before being resuspended in the appropriate buffer.

2.5.2 Transfection of Jurkat cells by electroporation.

Transfection of the human Jurkat T-cell line by electroporation was performed by a modification of the method of Doffinger *et al.*, (1988). To ensure the cells were in logarithmic growth phase they were routinely split 1:2 the day before electroporation. Jurkat cells were grown to a density of approximately 5×10^5 cells/ml and were harvested by centrifugation for 5 minutes at 1,000g. In initial experiments cells were resuspended in HeBs buffer that contained 20mM Hepes pH 7.0, 137mM NaCl, 5mM KCl, 0.7mM NaHPO₄, and 6mM Glucose. Subsequently this buffer was replaced with RPMI 1640 medium. The cell concentration was adjusted to 1.3×10^7 cells/ml. FCS was added to a final concentration of 20% (v/v). The cells were gently mixed and dispensed into electroporation cuvettes (4.0mm inter-electrode gap, 10mm width) (BioRad), 10–15µg of the DNA construct to be transfected was then added, gently mixed and the cuvettes were allowed to stand at room temperature (24°C) for 10 minutes. The cells were then exposed to a single voltage pulse of 270V from the 960µf capacitor of a BioRad gene pulser electroporation unit. The cells were allowed to recover for 10 minutes after electroporation and were gently plated in 25 cm³ culture flasks containing 8ml of RPMI 1640 growth medium supplemented with 10% FCS. In experiments where stimulation with reagents such as PHA or PMA was used, the cells were routinely left to recover from electroporation and to start to express the reporter gene for approximately 24 hours. After this time the reagents were added and the cells incubated for a further 16 hours before the transfected cells were harvested, cell extracts were made and assayed for protein concentration and chloramphenicol acetyl transferase activity. Where cells were cotransfected with the plasmid RSV-βGal (which has the reporter gene β-galactosidase under the control of the Rous Sarcoma virus promoter) as an internal control, the cells extracts were first assayed for β-galactosidase activity to allow standardisation of cell extracts used in the chloramphenicol acetyl transferase assays, based on the level of expression of the internal control reporter gene. PMA was prepared freshly each time. Stock solutions of PMA, A23187, PHA and ascites were dissolved or diluted in growth

medium supplemented with 10% FCS, at concentrations 100 fold greater than the final concentration in the culture medium. The working titre of the anti-CD28 ascites was determined by titration and staining of CD28 positive Jurkat cells and peripheral T-lymphocytes (1×10^6 cells/ml). The titre was defined as the final dilution of ascites which produced saturation levels of binding. Binding of the MAb was detected by staining with FITC-conjugated sheep anti-mouse-Ig antibody. The T-cells were also directly stained with rhodamine conjugated OKT3 MAb. Control samples were stained with class matched irrelevant antibody. The red/green fluorescence of double stained cells was measured with a coulter FACS star plus analyser (Coulter Electronics).

2.5.3 DEAE-dextran mediated transfection of HEL cells

HEL cells were routinely split and replated on 100mm diameter dishes 1-2 days before transfection to achieve approximately 60-70% confluency at the time of transfection. Each plate (approximately $0.6-1 \times 10^6$ cells) was transfected with 10 μ g of CAT reporter plasmid construct and 5 μ g of internal control plasmid (pRSV- β -galactosidase). The DNA for each transfection was ethanol precipitated, redissolved in 80 μ l of TBS and added dropwise to 180 μ l of DEAE dextran solution (10mg/ml in TBS) just prior to addition to the cells. The cell growth medium was removed and replaced with 4ml of fresh medium. The DNA/DEAE-dextran was added dropwise to the plate and swirled to mix with the medium. The final concentration of DEAE-dextran was 400 μ g/ml. Plates were then incubated at 37°C for 2 hours. After incubation the DNA/DEAE dextran solution was removed and replaced with 5ml of 10% (v/v) Me₂SO in PBS, which was left in contact with the cells for 1 minute. The cells were rinsed with 10ml of PBS. Fresh medium was added and the transfected cells were incubated for 48 hours. In experiments where stimulation with reagents such as TNF- α or PMA were used the cells were left to recover for 24 hours before stimulation.

2.5.4 Preparation of extracts from transfected tissue culture cells

Transfected Jurkat cells were collected in 50ml polypropylene tubes (Falcon) after firmly tapping the tissue culture flasks to resuspend the cells. Each flask was rinsed with 5ml of PBS. The cells were pelleted by centrifugation at 1,000g for 5 minutes and washed with 20ml of PBS. The washed cell pellets were resuspended in 200 μ l of 0.25M Tris-HCl pH 7.5 and transferred to 1.5ml polypropylene tubes. Cells were lysed by rapid freeze/thawing three times, cell debris was removed by centrifugation for 5 minutes at 13,000g. The supernatant was collected and stored at -20°C . Extracts of transfected HEL cells were prepared by removal of the cell culture medium from each plate by suction, the cells were then washed with 20ml of PBS. The cell monolayer was detached by scraping with a plastic policeman (Costar) and washed from the plate with 10ml of PBS. The cells were recovered by centrifugation for 5 minutes at 1,000g and resuspended in 250 μ l of 0.25M Tris-HCl buffer pH 7.5. The cells were lysed by freeze/thawing as described above.

2.5.5 Human Growth Hormone (hGH) Assay

The Pharmacia-LKB hGH immunoradiometric assay system which incorporates two monoclonal antibodies with high affinity and specificity for the hormone was used, according to the manufacturers instructions, for hGH assays performed during this study. In this assay 100 μ l aliquots of transfected cell culture supernatant were tested for the presence of hGH. Radioactivity was measured in a Packard gamma scintillation counter. A dose response curve of radioactivity versus hGH concentration was generated using the results obtained from standard controls included in the assay. The concentration of hGH present in the transfected cell supernatants was determined directly from this curve.

2.5.6 β -galactosidase assay

To measure β -galactosidase (β -Gal) in extracts from transfected cells a microscale β -gal assay was developed for 96 well microtitre trays (Titretek). 30 μ l of cell extract was

incubated in 150 μ l of freshly made β -Gal reaction buffer (0.1M NaPO₄ pH 7.5, 10mM KCl, 1mM MgCl₂, 50mM 2-mercaptoethanol) for 5 minutes before the addition of 50 μ l of the chromogenic substrate, ONPG (4mg/ml). The reaction was stopped by the addition of 50 μ l of 2M NaHCO₃. Multiple sequential absorbance measurements at a wavelength of 415nm and at a reference wavelength of 615nm were taken with a BioRad 3550 microplate spectrophotometer. Serial dilutions of purified β -Gal (Boehringer Mannheim) were used to construct a standard curve for each assay. The assay was linear between absorbance values of 0.2 to 1.0 at 415nm. β -Gal enzyme activity was calculated from the data within the linear range of the assay using BioRad Microplate Manager Software and a Macintosh computer.

2.5.7 Chloramphenicol acetyl transferase (CAT) activity assay

Transfected cell extracts prepared as described above (section 2.5.4) were thawed on ice and mixed by inversion. The CAT assay was carried out essentially as described by Gorman *et al.* (1982). Up to 30 μ g of cell extract was incubated for 2 hours in 180 μ l of CAT assay buffer (0.25M Tris-HCl pH 7.8, 1.0mM acetyl CoA, 1 μ Ci of [¹⁴C]-chloramphenicol). After incubation the [¹⁴C]-chloramphenicol was extracted into 500 μ l of ethyl acetate by vigorous vortexing. The upper organic layer was removed and dried under vacuum. The extracted [¹⁴C]-chloramphenicol was then redissolved in 25 μ l of ethyl acetate. The acetylated and non-acetylated forms of chloramphenicol were separated by thin layer chromatography (TLC) on silica gel plates (Merk) using an ethanol:chloroform (1:9) mobile phase. [¹⁴C]-chloramphenicol was detected by autoradiography or scanning phosphorimage analysis with a Molecular Dynamics phosphorimager. The activity of chloramphenicol acetyl transferase in the cell extracts was calculated as the percentage conversion of chloramphenicol to the mono- and di-acetylated forms. This was quantified by liquid scintillation counting of radioactive bands excised from the TLC plates or by image analysis with Molecular Dynamics phosphorimager software.

2.5.8 Estimation of protein concentration

The protein content of cell extracts was determined by a modification of the method of Bradford (1976). 5µl of cell extract was assayed using the BioRad protein microassay kit (according to the manufacturers instructions). Bovine serum albumin was used as the protein standard.

2.6 Methods for isolation and characterisation of nuclear DNA binding proteins

2.6.1 Extraction of nuclear proteins

Nuclear extracts were prepared by a modification of the method of Dignam *et al.* (1983). Cells (usually $1-5 \times 10^7$) were collected by centrifugation at 1,000g for 5 minutes. The cell pellet was resuspended (1ml/ 10^7 cells) in ice cold lysis buffer (10mM Tris-HCl pH 7.9, 10mM KCl, 1.5 mM MgCl₂ 0.5% (v/v) NP-40, 1mM DTT and 0.1mM PMSF). The cells were kept on ice for 15 minutes and vortexed periodically. Nuclei were then collected by centrifugation at 4°C for 5 minutes at 13,000g. The nuclei were washed in ice-cold lysis buffer without NP-40, centrifuged at 4°C for 5 minutes and resuspended in extraction buffer (50mM Tris-HCl pH 7.5, 10% (w/v) sucrose, 450mM KCl. 0.1mM EDTA, 20% (v/v) glycerol, 2mM DTT and 0.1mM PMSF). Nuclear proteins were extracted at 4°C for 45 minutes on a gently rotating orbital mixer. Nuclear DNA and debris was removed by centrifugation at 4°C in a TL100 benchtop centrifuge with a TL100-3 rotor at 25,000 rpm for 1 hour. The supernatant was collected and dialysed with several changes of TM.1 buffer (50mM Tris-HCl pH 7.9, 12mM MgCl₂, 1mM EDTA 20% (v/v) glycerol, 1mM DTT and 100mM KCl). Insoluble material was removed after dialysis by centrifugation for 2 minutes in an eppendorf centrifuge. PMSF was added to the nuclear protein extract to a final concentration of 0.1mM. Finally the protein concentrations of the extracts were determined (section 2.5.8) and the extracts were stored in small aliquots at -70°C.

2.6.2 Nuclear protein DNA binding assays

2.6.2.1 Purification of oligonucleotides

Stocks of pure full length synthetic oligonucleotides were prepared by electrophoresis of approximately 25µg of crude oligonucleotide preparation through 10% polyacrylamide gels. Bands containing full length oligonucleotides were identified by staining with ethidium bromide and visualised with UV light and excised from the gel. The full length oligonucleotide DNA was eluted from gel slices into 500µl of TE at 37°C for 16 hours. The eluted DNA was aspirated away from the gel slice, its concentration was determined by its absorbance at 260nm (A_{260}) using a Beckman DU-50 spectrophotometer. The relationship of 1 A_{260} unit equal to 33mg/ml of single stranded DNA was used in the calculation of oligonucleotide concentrations. Oligonucleotide stock solutions were kept at -20°C.

2.6.2.2 Labelling and hybridisation of oligonucleotide probes

100ng of each oligonucleotide was labelled with ^{32}P and purified by electrophoresis (section 2.2.10.2). Complementary oligonucleotides were annealed by combining 41µl (approximately 5-10ng) of each labelled and eluted oligonucleotide in oligo hybridisation buffer (25mM Tris-HCl pH 7.6 and 150mM NaCl). The hybridisation reaction was heated to 100°C for 5 minutes, allowed to cool slowly to room temperature (24°C) and stored on ice or at -20°C. Non-isotopically labelled oligonucleotides used as specific binding competitors in band shift assays were prepared and hybridised as above using 500ng of the complementary oligonucleotides in 100µl of oligo hybridisation buffer.

2.6.2.3 Gel mobility shift assays

Gel mobility shift reactions were carried out as described by Shannon *et al.*, (1990). Crude nuclear extract 5µg or the indicated amount of enriched or purified material was mixed with 0.1 to 0.5ng of radiolabelled double stranded (annealed) oligonucleotide (5,000-10,000 cpm) in a final volume of 20µl of binding buffer (25mM Tris-HCl pH 7.6,

6.25mM MgCl₂, 0.5mM EDTA, 0.5mM DTT, 10% glycerol, 0.1mM PMSF and 200mM KCl). Poly(dI:dC) (1–5µg) was used as non-specific competitor in the reactions. For binding competition experiments, specific competitor DNAs were always mixed with the reaction before addition of the radiolabelled probe. The reactions were analysed on 140mm long x 1mm thick 12.5% polyacrylamide gels in 0.5 x TBE buffer (45mM Tris-HCl, 45mM boric acid, 1.25mM EDTA pH 8.3). After electrophoresis the gels were dried at 80°C for 1 hour. The induced electrophoretic mobility shift of the radiolabelled probe DNA was detected by autoradiography for 16–48 hours at –70°C with Hyperfilm MP and intensifying screens.

2.6.3 Column chromatography of nuclear extracts

Nuclear DNA binding proteins in crude Jurkat cell nuclear extracts were enriched and fractionated by heparin sepharose chromatography as described by Shannon *et al.*, (1988). To assess the native size of nuclear DNA binding proteins, crude extracts were fractionated under non-denaturing conditions on a sepharose G100 (Pharmacia-LKB) column (370ml, 20mm diameter x 1200mm long). The column was calibrated with blue dextran 2000, gamma-globulin, ovalbumin, myoglobin and vitamin B12 as molecular weight standards (BioRad). 10mg samples (in 5ml of TM.1 buffer) were loaded and eluted at 4°C with TM.1 buffer. 10ml fractions were collected and 10µl aliquots from these fractions were assayed for DNA binding activity (section 2.6.2.3).

2.6.4 SDS Polyacrylamide gel electrophoresis (SDS-PAGE) of nuclear extracts

Gel electrophoresis in the presence of SDS was used to fractionate the extracted nuclear proteins. 30–50µg samples of nuclear extract were loaded in either reducing sample loading buffer (75mM Tris-HCl pH 6.8, 2% (w/v) SDS, 5% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 2% (v/v) β-mercaptoethanol) or non-reducing load buffer (75mM Tris-HCl pH 7.8, 2% (w/v) SDS, 5% (v/v) glycerol, 0.01% (w/v) bromophenol blue) onto 140mm x 1mm thick, 12.5% polyacrylamide gels and were electrophoresed at 30mA for 4 to 6 hours or at 10mA overnight. Nuclear extracts in reducing load buffer were heated at

95°C for 3 minutes prior to electrophoresis. BioRad low range molecular weight marker proteins were used to construct a standard curve of protein mobility.

2.6.5 Elution and renaturation of SDS–PAGE fractionated nuclear protein extracts

SDS–gel lanes which contained the fractionated nuclear extracts were cut into small (2mm) slices and crushed, then placed into 1.5ml polypropylene tubes with 500µl of elution buffer (50mM Tris–HCl pH 7.9, 0.1% (w/v) SDS, 100µg/ml BSA, 0.2mM EDTA, 1mM DTT, 0.1mM PMSF and 2.5 % (v/v) glycerol). Protein was eluted at 4°C for 16 hours. After elution, 400µl of the elution buffer was aspirated away from the gel fragments and the eluted protein was precipitated at –20°C for 2 hours by addition of 800µl of acetone. The precipitate was collected by centrifugation for 5 minutes in an eppendorf centrifuge, washed once with 500µl of ice–cold methanol and air dried. The dried pellets were dissolved in 2.5µl of a saturated solution of urea by gentle mixing with a gilson pipette tip and then allowed to renature overnight at 4°C in 130µl of renaturation buffer (20mM Tris–HCl pH 7.6, 10mM KCl, 2µM DTT and 0.01mM PMSF). Nuclear DNA binding proteins eluted and renatured in this manner were stable for 1 week and were stored at –70°C. 15µl samples of this renatured material were assayed for their ability to induce gel mobility shifts of ³²P–labelled oligonucleotides (section 2.6.2.3).

2.7 Miscellaneous Methods

2.7.1 Measurement of TCA–precipitable radioactivity

1–15µl of radioactive sample was added to 100µl of Water, with 100µg of carrier nucleic acid (denatured salmon sperm DNA for DNA samples, or E. coli tRNA for RNA samples). 1–5ml of ice–cold 10% (w/v) TCA, 1% (w/v) Na₄PP_i was added and the samples kept on ice for 30 minutes. The precipitate was collected by filtration through a Whatman GF/A glass fibre disc and washed with 25ml of ice cold 5% (w/v) TCA, 1% (w/v) Na₄PP_i. The discs were then washed with 10ml of ethanol, dried and counted in 3ml of scintillation fluid in Packard 2000CA liquid scintillation counter.

2.7.2 Preparation of Peripheral T-lymphocyte cell cultures

Human peripheral T-lymphocytes were isolated from freshly collected, whole blood from anonymous donors (Red Cross Transfusion Service, Adelaide, South Australia).

Mononuclear cells were prepared by centrifugation of whole blood on Ficol-Hypaque gradients and washed twice in PBS. The remaining monocytes and lymphocytes were separated in a Beckman J-6M/E elutriator. T-lymphocytes recovered from the elutriator were washed twice in PBS and resuspended in RPMI medium supplemented with 10% FCS. The peripheral T-lymphocyte preparations were at least 90% pure as judged by morphology and fluorescence activated cell sorter (FACS) analysis of T-cell preparations stained with rhodium conjugated anti-CD3 monoclonal antibody. The major contaminating cell types were B cells (<10%) monocytes and granulocytes (principally basophils)(<2%).

2.7.3 Measurement of IL-3 protein

Human IL-3 protein was quantified by means of a competitive radioimmunoassay (RIA) using [¹²⁵I]-labelled IL-3 and rabbit anti-IL-3 serum. A modified IL-3 protein with an added octapeptide in the amino-terminus containing an extra tyrosine to facilitate iodination, (gift from L. S. Park, Immunex) was radioiodinated using the iodine monochloride method as previously described by Lopez *et al.*, (1990). This IL-3, has been shown by Park *et al.*, (1989) to have the same properties as the unmodified IL-3. The rabbit anti-IL-3 serum (gift from S. C. Clark, Genetics Institute) was found in preliminary experiments to recognise IL-3 but not GM-CSF (A. F. Lopez, Personal communication). Rabbit anti-IL-3 antiserum at a dilution of 1:20,000 was mixed and incubated overnight at 4°C with tissue culture supernatants or, to construct a standard curve, with known concentrations of CHO-derived unlabelled IL-3 (gift from S. C. Clark, Genetics Institute, MA.). 50pM [¹²⁵I]-labelled IL-3 was then added to the mixture for a further 4h at 4°C. At the end of this incubation period 200µl of previously titrated goat anti-rabbit IgG coupled to Sepharose beads was added, the mixtures centrifuged,

washed and the pellets counted in a Packard gamma counter. The amount of IL-3 in the tissue culture supernatants was calculated from the linear part of the curve constructed with known amounts of unlabelled IL-3.

2.7.4 Quantitation of radioactive DNA and RNA

Densitometric quantitation of autoradiography bands was performed on a LKB Bromma ULTRASCAN XL enhanced laser densitometer. Autoradiograph exposure times were adjusted so that the signals were within the linear range of the film used. In experiments where indicated, radioactive material was excised from dried gels or filters after visualisation by autoradiography and the radioactivity was quantified by liquid scintillation counting in a Packard liquid scintillation counter or radioactivity in bands was quantified directly, using a molecular dynamics phosphorimager.

2.7.5 Computer programmes

Microgenie (Beckman), a suite of DNA analysis programmes was used to identify restriction enzyme sites and also to identify putative nuclear DNA binding factor sites with data from the transcription factor database (TFD) described by Ghosh (1990).

Pascal computer programs were written by the author to transfer the relevant sites table data (site_name and seq) from the Ghosh TFD sites tables to Microgenie search file formats and to convert the IUPAC ambiguous base abbreviations used in the Ghosh sites table to the ambiguous base code format used by the Microgenie programmes. DNA sequence acquisition codes and DNA sequence entries were obtained from the Genbank sequence repository using the IRX query facility. Sequence comparisons were performed at Genbank using the FASTA program of Lipman and Pearson.

Chapter 3

Regulation of IL-3 mRNA expression

3.1 Introduction

The cloning of the gene for human IL-3 has enabled the determination of the cellular sources of the human IL-3 protein. In contrast to some of the other human HGFs which are expressed in a wide variety of tissue types, expression of the human IL-3 gene was initially thought to be restricted to the antigen or mitogen activated, CD28⁺ subset, of peripheral blood T-lymphocytes and some T-cell tumour lines (Sieff *et al.*, 1988, Thompson *et al.*, 1989, Oster *et al.*, 1989, Niemeyer *et al.*, 1989 and Guba *et al.*, 1989). Recently, however, human IL-3 has been detected by *in-situ* hybridisation and sensitive bio-assays in thymic epithelial cells (Dalloul *et al.*, 1991) and in both neutrophilic and eosinophilic granulocytes (Kita *et al.*, 1991). In addition, recent reports have shown that the supernatants of cultured human epidermal keratinocytes, which were known to have an IL-3 like activity (Luger *et al.*, 1988), contain IL-3 protein and that these cells express IL-3 mRNA (Dalloul *et al.*, 1992). The synthesis of IL-3 by these peripheral and circulating cells and the absence of IL-3, detectable by enzyme linked immunosorbent assay (ELISA) or reverse transcriptase polymerase chain reaction (RT-PCR) assays, in bone marrow stromal cells and cultured mesenchymal cells (Sieff *et al.*, 1988 and Guba *et al.*, 1992) supports the assertion that the primary role of IL-3 is as a potentiating signal for allergic and immune responses rather than basal level haemopoiesis in the bone marrow. Consistent with this view is the fact that constitutive expression of human IL-3 has not been observed in any normal cell type yet examined. For example, the expression of human IL-3 in thymic epithelial cells and epidermal keratinocytes requires continuous

stimulation of these cells with epidermal growth factor (EGF) and hydrocortisone (Dalloul *et al.*, 1991 and Dalloul *et al.*, 1992) and eosinophils and neutrophils produce IL-3 only after stimulation with calcium ionophore (Kita *et al.*, 1991). T-lymphocytes coordinately express IL-3 and an array of cytokines and HGFs including IL-2, IL-4, IL-5, IL-6, GM-CSF, tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) only when the cells are appropriately activated (Oster *et al.*, 1989, Guba *et al.*, 1989, Lindsten *et al.*, 1989, Bradley *et al.*, 1991 and Fraser and Weiss, 1992).

It has been observed that similar to IL-3, most of the cytokines produced by T-cells have extremely short half lives *in-vivo* and that removal of the stimulus to T-cell activation results in the rapid closing down of the expression of cytokines and HGFs (Crabtree, 1989). Of fundamental interest, therefore, is the relationship of T-cell activation and signal transduction to cytokine transcription and its control. Activation of human T-cells is complicated by the existence of a number of cell surface molecules that can all be involved in transducing signals either singly or in cooperation. These include the T-cell antigen receptor (TcR) which controls the immune specificity of T-cell activation (reviewed by Izquierdo and Cantrell, 1992), adhesion molecules such as CD2 (T11, the sheep E rosette receptor) and CD28 which deliver a costimulatory signal when bound by their ligands LFA-3 and B7 respectively (Meuer *et al.*, 1984, Oster *et al.*, 1989, and reviewed by Springer, 1990, June *et al.*, 1990 and Jenkins *et al.*, 1991) and the CD4 and CD8 molecules which are receptors for class I and class II major histocompatibility complex (MHC) proteins (reviewed by Bierer *et al.*, 1989). Similarly, a wide variety of ligands will deliver stimulatory signals to T-cells. They range from physiological signals delivered by antigens recognised by the TcR when the antigen is presented in association with a MHC molecule on the surface of an antigen presenting cell (APC), to monoclonal antibodies directed against the CD3 component of the TcR (Izquierdo and Cantrell, 1992), to lectins such as PHA, which interact with sugar residues on cell surface glycoproteins, thereby potentially crosslinking of cell surface and antigen receptors (Ng *et al.*, 1987).

The induction of cytokine gene expression and the activation of T-lymphocytes is inhibited by glucocorticoids and other immunosuppressive agents such as cyclosporin A (CSA) or FK506 (Shaw *et al.*, 1987, Vacca *et al.*, 1990). The mechanisms involved in repression of T-cell activation and cytokine expression by glucocorticoids are not well understood, although recent evidence suggests that interference with the activity of transcription activation factors may be involved (Paliogianni *et al.*, 1993). The immunosuppressive activity of CSA and FK506 may also be explained by their ability to coordinately inhibit expression of cytokine genes at the transcriptional level (Tocci *et al.*, 1989).

The coordinate induction and repression of groups of cytokines including IL-3 and GM-CSF in activated T-lymphocytes in both the murine and the human system suggests that antigen or lectin (PHA) stimulation induces expression of the cytokine genes by a common pathway. It has been proposed that the signal generated by interaction of antigen and MHC with the TcR is transmitted to the cytoplasm *via* stimulation of phosphatidyl inositol turnover resulting in an increased concentration of cytoplasmic free calcium (Ca^{2+}) and activation of protein kinase C (Ledbetter *et al.*, 1986). Activation of naive or resting T-cells and maximal cytokine expression requires however, in addition to stimulation of the TcR, a second signal. This signal is supplied physiologically when the T-cells are co-stimulated with the macrophage factor interleukin 1 (IL-1) or, in situations where cell-cell contact is required, when B-cell or APC surface regulatory molecules interact with the T-cell surface activation molecules, for example B7 with CD28 or LFA-3 with CD2 (Granelli-Piperno, Andrus and Steinman 1986, June *et al.*, 1987 and Plunket *et al.*, 1987). Synthetic diacylglycerol analogues such as the phobol ester 12-O-tetradecanoyl-phorbol-13-acetate (PMA) which maximally activate PKC obviate the need for IL-1 co-stimulation in optimal production of T-cell cytokines (Rosentreich and Misel, 1979). The biochemical events that accompany activation by CD2 appear to be identical to activation *via* the CD3-T-cell receptor pathway (Pantaleo *et al.*, 1987).

Stimulation of the CD28 receptor by monoclonal antibodies induces a distinct but as yet unidentified pathway that synergises with the TcR generated signals to increase both T-cell proliferation and cytokine production (Ledbetter *et al.*, 1985, Weiss, Manger and Imboden, 1986 and Fraser and Weiss, 1992)

The targets of signals generated by specific antigen receptors and secondary T-cell activation antigens are among others, the nuclear regulatory proteins controlling gene transcription. The rate of cytokine gene transcription may be largely responsible for tissue specific expression in T-cells. However, the mechanisms used by lymphocytes to regulate induction of gene expression include not only alterations in transcription rate but also termination of transcription and mRNA stability (reviewed by Crabtree 1989 and Eick and Bornkamm, 1986). The signals affecting these modes of regulation have generally not been elucidated.

At the time this work began there was little known of the kinetics or molecular mechanisms involved in IL-3 mRNA expression in activated human T-lymphocytes. This chapter presents an analysis of the regulatory mechanisms involved in human IL-3 mRNA expression in T-lymphocytes. The first aim of the work presented in this chapter was to establish a model cell line in which the mechanisms involved in regulation of IL-3 mRNA expression could be studied. IL-3 transcriptional induction, mRNA accumulation and stability were examined using northern blotting, RNase protection assays and nuclear transcription run-on analysis.

3.2 Results

3.2.1 Kinetics of IL-3 mRNA expression in human T-lymphocytes

To establish a cell line model for IL-3 production, the stimulation requirements and kinetics of IL-3 accumulation in normal freshly isolated peripheral blood lymphocytes (PBLs) were compared to that of a panel of human cell lines. First, the pattern of expression of IL-3 mRNA was examined in freshly isolated PBLs. Cells were

prepared from donated whole blood by Ficoll-Hypaque density centrifugation and elutriation (see section 2.7.2) and stimulated (5×10^7 cells/treatment) for 0, 4, 8, and 24 hours with the lectin PHA ($2\mu\text{g/ml}$) or PHA together with PMA ($2\mu\text{g/ml}$ and 10ng/ml respectively). Whole cell RNA was extracted and equal amounts ($20\mu\text{g}$) of RNA were electrophoresed, transferred to nylon membrane and probed using a ^{32}P labeled gibbon IL-3 cDNA Xho I fragment as a molecular hybridisation probe (sections 2.2.10.1, 2.3.1 and 2.3.2). Cells from three different donors gave only low level or barely detectable signals for IL-3 mRNA in tracks where the cells were treated with both PMA and PHA for 24 hours. No IL-3 mRNA could be detected in freshly isolated or unstimulated cell aliquots or where the cells were stimulated with PHA or PMA alone (data not shown). To improve the sensitivity of IL-3 mRNA detection, the same samples were examined by RNase protection analysis. An IL-3 transcription template was constructed for this purpose by cloning the 301bp Sma I/Pst I fragment of the human IL-3 gene, which contains the first exon and part of the first intron, into the plasmid vector pSP64 (Figure 3.1A and B). Using this more sensitive technique IL-3 mRNA was detected in normal, freshly isolated PBLs that were stimulated with PHA/PMA ($2\mu\text{g/ml}$ and 10ng/ml respectively) for at least 4-6 hours (Figure 3.2). IL-3 mRNA levels had decreased by 8 hours of stimulation. However, a second strong peak of IL-3 mRNA accumulation was detected at 24-36 hours after stimulation in PBLs from two separate donors.

At the time this study was commenced it was unclear which human cell lines would be able to produce IL-3, although investigations in the murine system had shown that IL-3 was often expressed in activated T-cells coordinately with the related cytokine GM-CSF (Mosmann and Coffman, 1989). To establish a human cell line model for IL-3 expression, a panel of cells was chosen for their ability to express human GM-CSF. Initially both T-cell and non-T-cell lines were surveyed. Non T-cell lines which were tested included the bladder carcinoma 5637 which constitutively produces GM-CSF, the human skin melanoma line LiBr, and a promyelocytic line HL-60. All

Figure 3.1 Construction of IL-3 and β -actin RNase protection assay probe templates

Human IL-3 and β -actin RNase protection assay probe templates were constructed in the SP6 phage transcription vectors pSP64 or pSP65 (Melton et al., 1984).

A and B). The human IL-3 probe was constructed by cloning the 301 bp Sma I/Pst I fragment of the human IL-3 gene (obtained from λ J1.16, Gift Dr. S. C. Clark, Genetics Institute, MA) into the Sma I/Pst I sites of plasmid pSP64 to create plasmid pGR42. This region of the IL-3 gene encompasses the entire first exon and small regions of the first intron (30 bp) and the promoter (65 bp). To generate transcription templates the plasmid was linearised by Eco RI digestion 3' to the IL-3 insert (*c.f.* the SP6 Pol start site). Transcription from the SP6 promoter generates a 320 bp RNA probe, 206 bp of this probe are protected from RNase digestion by hybridisation to complimentary sequences of IL-3 mRNA.

C). A β -actin transcription template was generated by cloning the 134 bp Sma I/ Pst I fragment of the β -actin cDNA clone pHF- β A-1 (Gunning et al., 1983) into the plasmid pSP64, generating plasmid pGR62. Transcription templates were obtained from this plasmid by digestion with Pvu II. Transcription from the SP6 promoter of the template generated a 356 bp β -actin probe of which 165 bp are protected by hybridisation to β -actin mRNA.

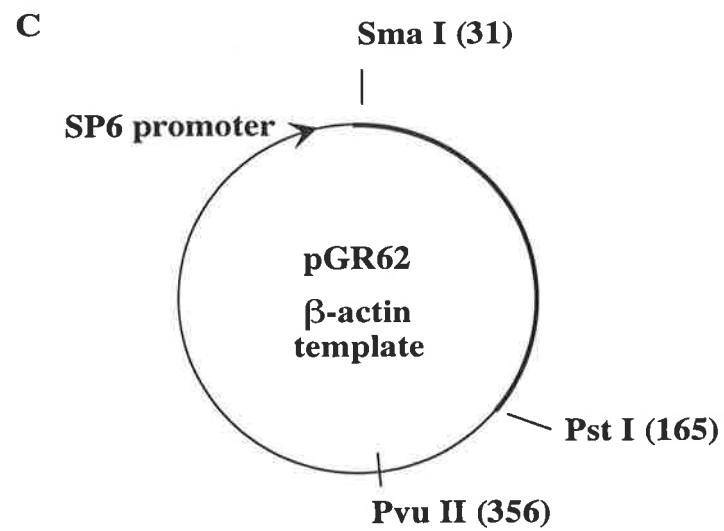
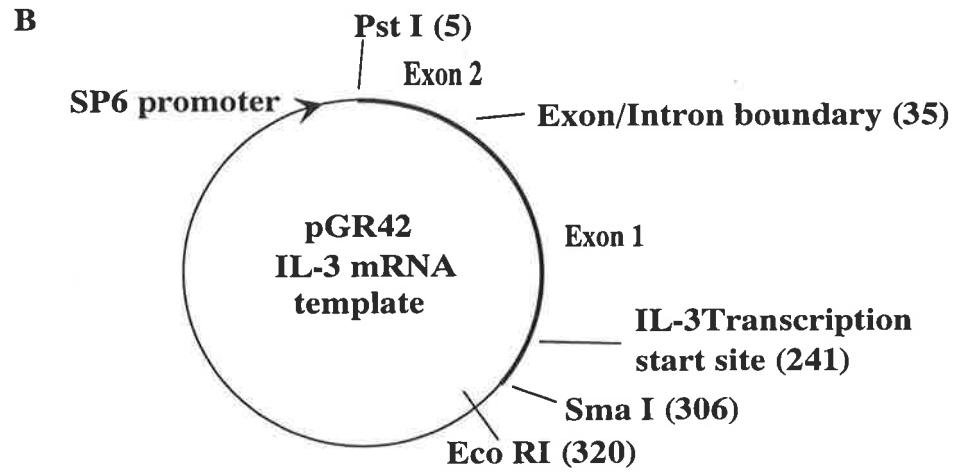
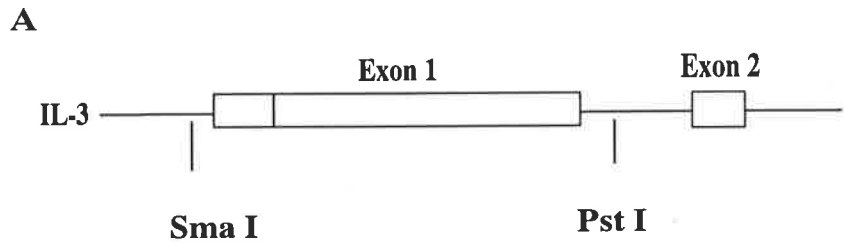


Figure 3.2 IL-3 mRNA expression in normal peripheral blood lymphocytes

Peripheral blood lymphocytes were prepared from donated whole blood by density centrifugation with ficol-hypaque and elutriation (section 2.7.2). The cells were stimulated (5×10^7 /treatment) for the times as indicated, 0, 4, 8, or 24 hours, with PHA and PMA (2 mg/ml and 10 ng/ml respectively). Whole cell RNA was extracted and equal (20 μ g) amounts of RNA were analysed by RNase protection assay for the expression of IL-3 mRNA (section 2.3.4). The autoradiograph shown is representative of three independent experiments with separate donors. Protected bands corresponding to IL-3 mRNA (arrowed) were detected in samples treated with PHA and PMA for 4 and 8 hr. A large accumulation of IL-3 mRNA was detected in these cells following 24 hrs of PHA/PMA stimulation. No IL-3 mRNA was detected in unstimulated or freshly isolated cells. The negative control track contained 20 μ g of *E. coli* tRNA in place of T-cell RNA. Undigested probe was electrophoresed in the first track. Small amounts of the hybridisation probe remain undigested in the experimental tracks (arrowed). Other bands, lower in the experimental tracks than the signal for IL-3 mRNA, represent mRNA breakdown and/or probe background.

**PROBE
NEGATIVE**

0 4 8 24Hr



origin

◀ probe

◀ IL3

were negative for IL-3 mRNA production when either unstimulated or stimulated for 12 hours with PMA (Figure 3.3A). T-cell lines were tested for their ability to express IL-3 mRNA following activation for 12 hours by PHA/PMA or alternatively with PMA and calcium ionophore (A23187). These reagents were used at concentrations shown to be optimal in our laboratory for the expression of T-cell cytokines and activation antigens (PHA 2µg/ml, PMA 10ng/ml and A23187 0.3µM) (Dr. Gordon Burns, personal communication). The human T-lymphocytic leukaemia line HUT78 failed to express detectable levels of IL-3 mRNA following stimulation (Figure 3.3A). In addition to these human cell lines the gibbon lymphosarcoma cell line UDC-144-MLA (MLA-144), from which primate IL-3 was first isolated (Yang *et al.*, 1988) was included as a positive control. This cell line, as reported by Yang *et al.* (1988) expressed large amounts of IL-3 mRNA when stimulated for 12 hours with PMA alone (10ng/ml) but was not induced to express IL-3 with PHA stimulation (Figure 3.3A). IL-3 mRNA expression was easily detected in the human T-cell lines Jurkat and HSB2. IL-3 mRNA was induced within 4 hours in the Jurkat cell line with PHA and PMA (PHA/PMA, 2µg/ml and 10ng/ml respectively) or PHA and A23187 (2µg/ml and 0.3µM respectively) (Figure 3.3B and 3.4). In contrast, induction of IL-3 mRNA in HSB2 cells was detected only with PMA/A23187 stimulation at approximately 4-8 hours after stimulation (Figure 3.3B). Interestingly this cell line may share characteristics of primitive T-cells in that by FACS analysis, T-cell surface markers (including CD2, CD3, CD4 and CD8) are absent, with the exception of a marker of primitive T-cells, CD7. IL-3 mRNA expression was not detected in unstimulated Jurkat or HSB2 cells (figures 3.2 and 3.3) by RNase protection or by using the more sensitive PCR reaction on the same RNA samples (Dr. Peter Bardy, personal communication). These preliminary experiments indicated that expression of IL-3 mRNA in the Jurkat cell line was similar to that of freshly isolated human PBLs and enabled the use of this cell line as a model for IL-3 production in normal T-cells.

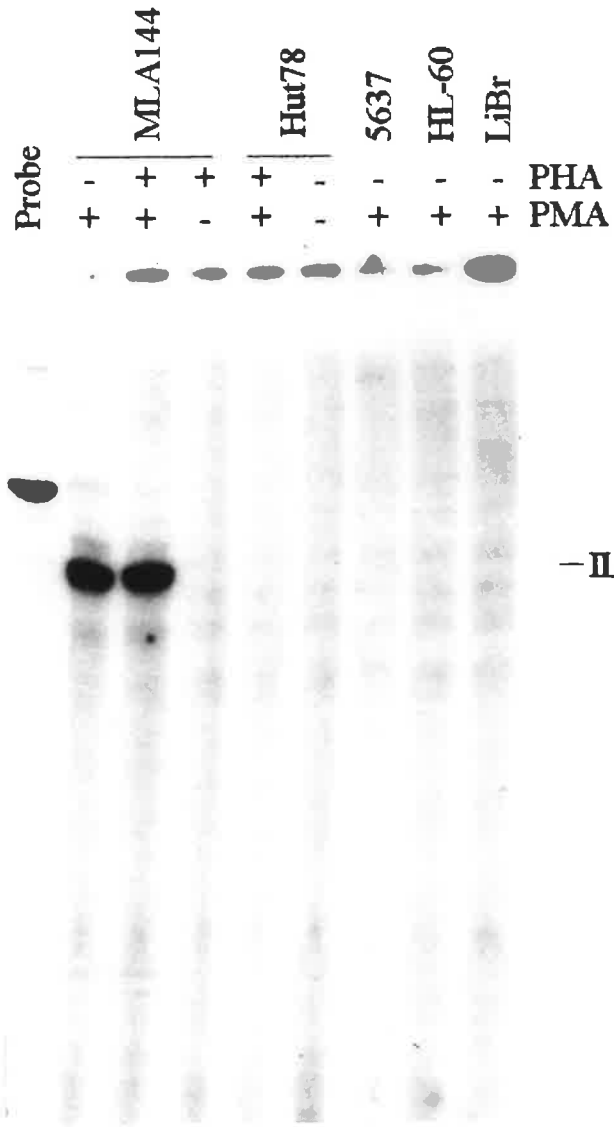
Figure 3.3 Expression of IL-3 mRNA in human cell lines

IL-3 mRNA was detected by RNase protection assays in whole cell RNA extracted from cultured human cell lines. The positions of the protected band representing IL-3 mRNA is indicated to the right of the panels.

(A) Cell cultures were treated with either PMA (20ng/ml), PHA (2 μ g/ml) or PHA/PMA as indicated above the tracks for 12 hours. A control track containing undigested IL-3 probe is shown in the first track.

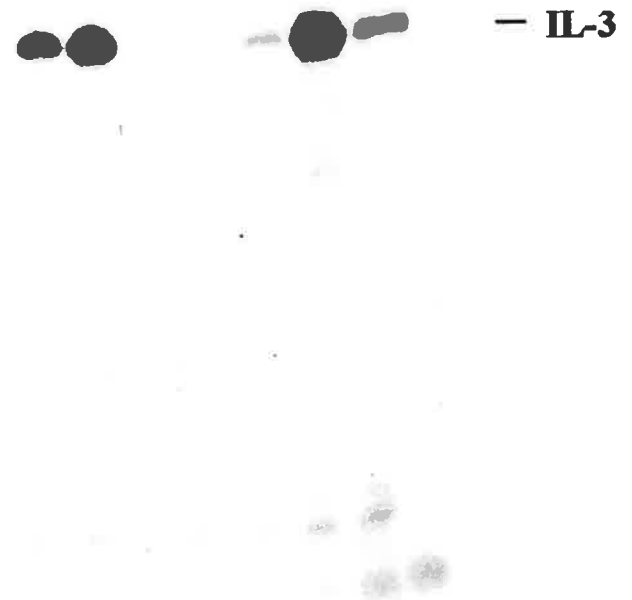
(B) HSB2 and Jurkat cells were treated with PMA/calcium ionophore A23187 (2 μ g/ml and 0.3 μ M) and PHA/PMA (2 μ g/ml/20ng/ml) respectively, for the time periods 1-24 hours as indicated above the tracks. Unstimulated Jurkat cell RNA (central track) was included as a negative control.

A



B

HSB2 PMA/A23187				Jurkat PHA/PMA				
24	8	4	1	NIL	1	4	8	24

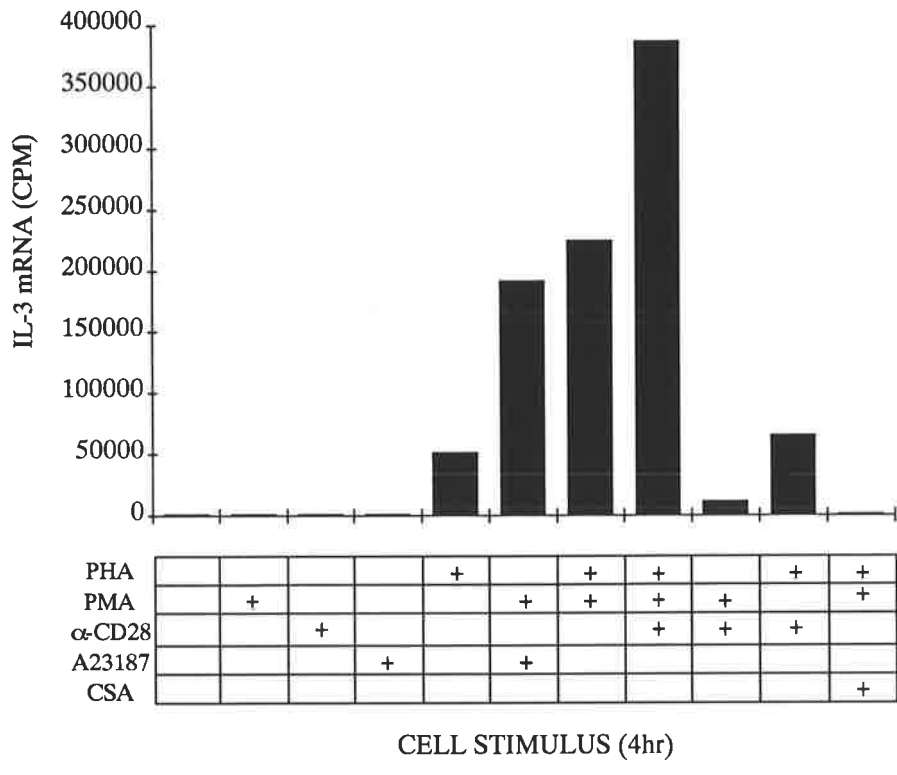


To establish the range of stimulation conditions that elicit IL-3 production in these cells, RNA was extracted from Jurkat cells treated with a panel of reagents and analysed by RNase protection assay for expression of IL-3 mRNA. To allow comparison and quantitation of the IL-3 mRNA content of these samples, both between independent experiments and between samples with different stimuli, mRNA for the housekeeping gene β -actin was measured as an internal control in each RNA sample. The β -actin probe was transcribed from a template constructed from a 130bp fragment of β -actin clone pHF- β A1 (Gunning *et al.*, 1983, Figure 3.1). IL-3 mRNA was detected in Jurkat cells following stimulation of the cells for 4 hours with PHA (2 μ g/ml) (Figure 3.4). Stimulation with PHA together with A23187 or PMA, in three separate experiments resulted in an average of 4-4.5 fold greater IL-3 mRNA accumulation after 4 hours than stimulation with PHA alone. Maximum IL-3 mRNA accumulation was obtained by stimulation of CD28 with the monoclonal antibody 9.3 (see section 2.5.2) in combination with both PHA and PMA (Figure 3.4). Stimulation *via* CD28 appeared to co-operate with PHA/PMA treatment, since stimulation of the cells with α -CD28 alone did not induce the expression of IL-3 mRNA and α -CD28 together with PMA or α -CD28 and PHA produced only slight and inconsistent increases over the levels obtained with PMA or PHA respectively (Figure 3.4). The immunosuppressant CSA (0.1 μ M), when applied to the cells immediately before PHA/PMA treatment, completely abolished the induction of IL-3 mRNA in these cells (Figure 3.4).

To examine more closely the kinetics of induction of IL-3 mRNA in Jurkat cells, the time course of IL-3 mRNA accumulation was investigated. In PHA treated cells IL-3 mRNA expression was detectable at 2 hours and then increased over this initial level by approximately 3.5 fold to reach a maximum at 6 hours returning again to low levels by 24 hours. Stimulation of Jurkat cells with PHA/PMA induced a similar transient increase (Figure 3.5). The more efficient induction of IL-3 mRNA allowed detection of IL-3 mRNA at 1 hour after stimulation and by 6 hours the levels had increased to

Figure 3.4 Induction of IL-3 mRNA in Jurkat cells

IL-3 and β -actin mRNA levels were measured by RNase protection assay in total cell RNA (10 μ g) extracted from stimulated or unstimulated Jurkat cells. The IL-3 mRNA signals were quantitated with a scanning phosphorimager (Molecular Dynamics) and normalised to the levels of the signal from the β -actin internal control. The bars represent the mean of two independent experiments. The cells were treated for 4 hours with the reagents where indicated by a cross under the bar. PHA (2mg/ml), PMA (10ng/ml), anti-CD28 monoclonal antibody 9.3 ascites (α -CD28, titre 1/10,000), Ionophore A23187 (0.3 μ M), Cyclosporin A (0.1 μ M).



400000
350000
300000
250000
200000
150000
100000
50000
0

				+		+	+		+	+
	+				+	+	+	+		+
		+					+	+	+	
			+		+					
										+

CELL STIMULUS (4hr)

Figure 3.5 Kinetics of IL-3 mRNA expression in activated Jurkat cells

The time course of IL-3 and β -actin mRNA accumulation was measured by RNase protection assay. Whole cell RNA was collected over a 24 hour period from Jurkat cells that were stimulated with PHA (upper panel, 2 μ g/ml) or PHA and PMA (lower panel, 2 μ g/ml and 10ng/ml respectively). Protected bands, 213 bp for IL-3 mRNA and 130 bp for β -actin mRNA are labelled and marked by arrows.

approximately 4.5 fold higher levels than those observed after stimulation with PHA alone (Figure 3.5). Stimulation with either PHA, PMA or A23187 alone or in combination for periods less than 24 hours had no consistent effect on the levels of β -actin mRNA in these cells.

To determine if the IL-3 mRNA induced by PHA and PMA stimulation of Jurkat cells resulted in the production of IL-3 protein, serial samples of Jurkat cell culture supernatants (5×10^5 cells/ml) were assayed by the author and Ms. Susan Milton using an IL-3 radioimmunoassay (see section 2.7.3). The production of IL-3 protein in stimulated cells closely followed the accumulation of IL-3 mRNA and was detected as early as 4 hours after PHA stimulation and continued to accumulate in the cell supernatant up to 24 hours (Figure 3.6). Similar to IL-3 mRNA, maximum IL-3 protein levels were obtained after stimulation with PHA/PMA (Figure 3.6) which induced an approximate 3.5 fold greater increase in the amount of IL-3 detectable in the culture supernatant compared to PHA stimulation alone. In these assays no IL-3 was detected in unstimulated cells. As a negative control, Jurkat cells were treated with cycloheximide (CHX) for one hour prior to stimulation with PHA/PMA and as expected IL-3 production was also undetectable in supernatant fractions from these cells, indicating that new protein synthesis is required for the secretion of IL-3 into the cell supernatant (Figure 3.6).

3.2.2 Protein Synthesis is required for optimal induction of IL-3 mRNA

The production of detectable levels of IL-3 mRNA appeared to be delayed by at least 1 hour after Jurkat cells were stimulated (Figure 3.5) suggesting that new protein synthesis may be required for the induction of IL-3. The requirement for new protein synthesis during this time was examined by inhibition of protein synthesis with CHX one hour prior to stimulation. This treatment resulted in a decrease of greater than 3 fold in the steady state accumulation of IL-3 mRNA (measured at 6 hours) after PMA/PHA stimulation (Figure 3.7A), compared to cells where protein synthesis was

Figure 3.6 Production of IL-3 protein by stimulated Jurkat cells.

Jurkat cells were grown to an initial density of 5×10^5 /ml. One ml supernatant aliquots were collected from unstimulated cells (closed circles), cells pretreated with cycloheximide for 1h. prior to PMA/PHA stimulation (closed squares), PHA (2mg/ml) stimulated cells (closed triangles) or PHA/PMA (2mg/ml)/(20ng/ml) stimulated cells (open circles) at various times after stimulation. The level of IL-3 in these aliquots was measured by radioimmunoassay (section 2.7.3). Results (ng/ml) are presented as mean (\pm S.E.) of 3 experiments, each experiment in triplicate

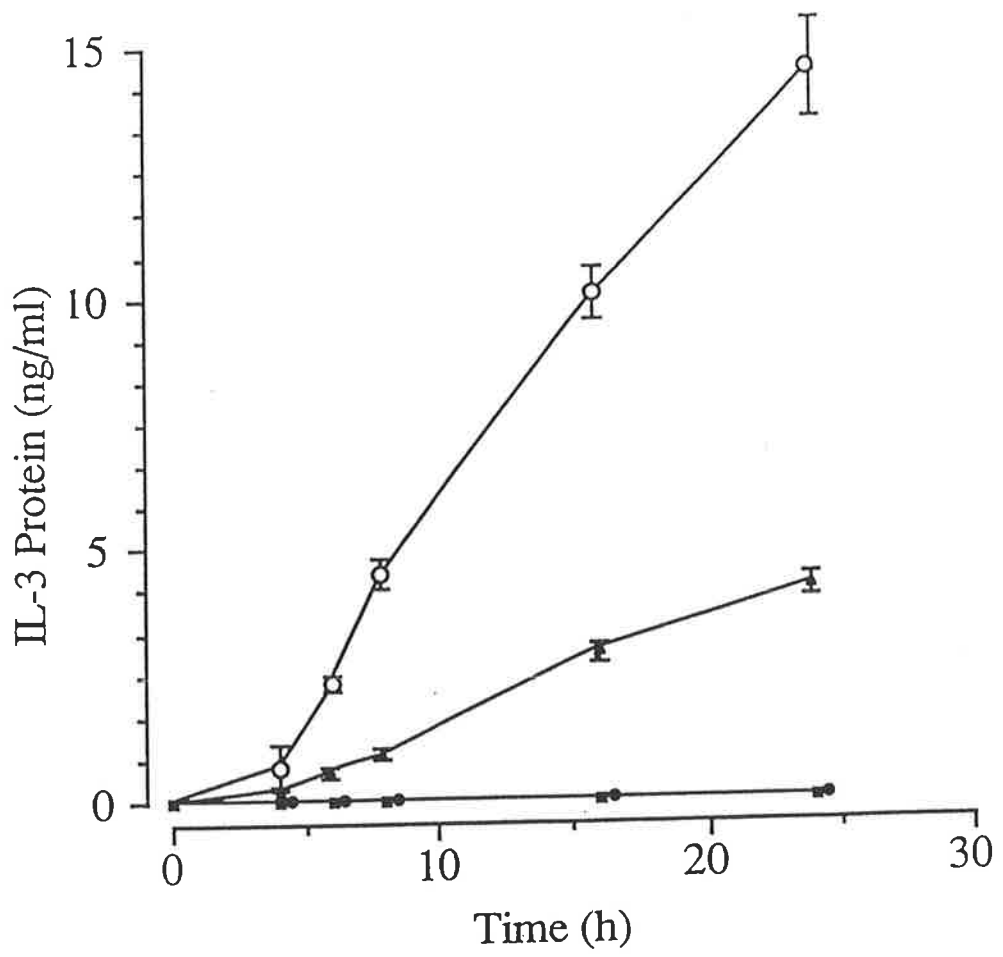
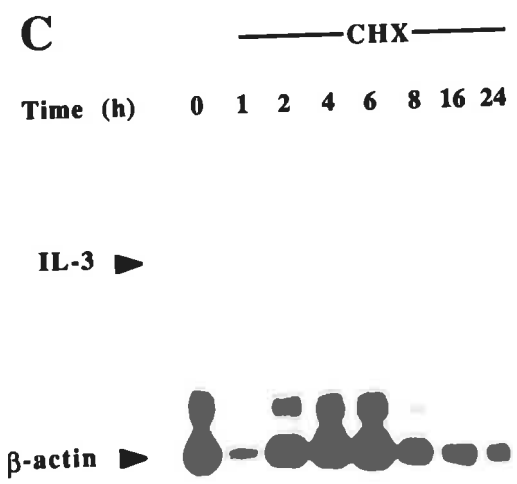
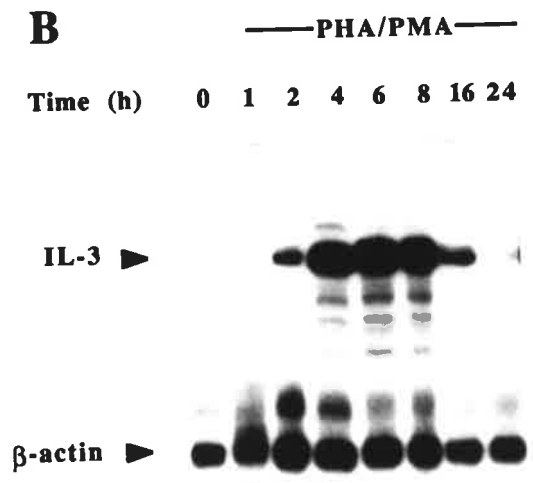
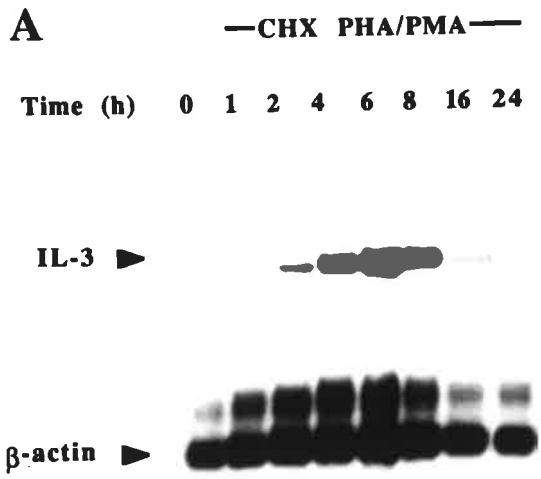


Figure 3.7 Effect of inhibition of protein synthesis on IL-3 mRNA accumulation.

Jurkat cells were treated with (A) CHX (10 μ g/ml) for 1 hour followed by PHA/PMA stimulation (2 μ g/ml)/(20ng/ml), (B) PHA/PMA alone and (C) CHX alone. IL-3 and β -actin mRNA levels were measured by RNase protection assay at the time points shown using equivalent amounts of total cellular RNA (10 μ g).



unimpaired (Figure 3.7B). IL-3 mRNA was not detected in Jurkat cells treated with CHX alone (Figure 3.7C). In contrast, inhibition of protein synthesis by CHX treatment did not deplete the steady state levels of β -actin mRNA (Figure 3.7 panels A and C). However, cell viability measured by trypan blue exclusion and β -actin mRNA levels began to decrease after 24 hours of protein synthesis inhibition. These data indicate that inhibition of protein synthesis does not induce IL-3 mRNA production and that while inhibition of protein synthesis does not completely abrogate accumulation of IL-3 mRNA, protein synthesis is required for maximal induction of IL-3 mRNA.

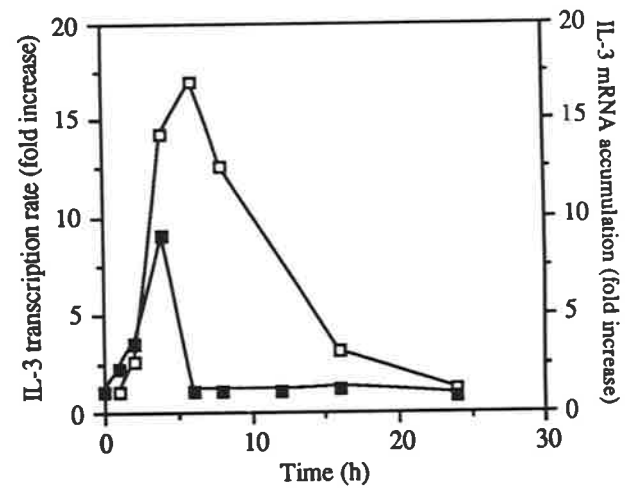
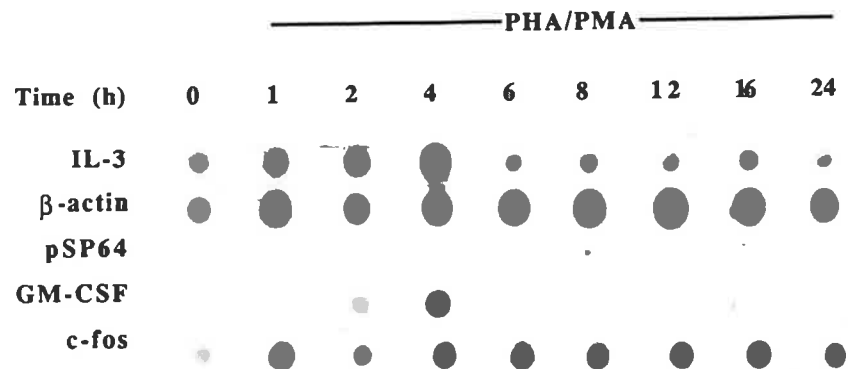
3.2.3 Analysis of IL-3 gene transcription in Jurkat cells

The strong induction of IL-3 mRNA accumulation following activation of T-cells suggests that transcriptional mechanisms may play an important role in IL-3 expression. However, both transcriptional and post-transcriptional mechanisms have been implicated in the regulation of the closely related GM-CSF gene and other cytokine genes in human T-cells (reviewed by Crabtree, 1989). To determine the contribution of transcriptional activation to the observed accumulation of IL-3 mRNA in stimulated cells, nuclear run-on assays were performed in isolated Jurkat cell nuclei. Jurkat cells (8×10^7 cells/ml) were stimulated either with PHA or PHA/PMA for the various times up to 24 hours to induce expression of the IL-3 gene. Immediately before the nuclei were harvested for nuclear run-on assays, small samples (5×10^6 cells) were removed from each culture vessel for extraction of whole cell RNA. RNase protection assays were used to assess the steady state levels of IL-3 and β -actin mRNA in these cells and the nuclei were isolated from the remaining cells and *in-vitro* transcription assays performed in the presence of ^{32}P rUTP (sections 2.4.1 and 2.4.2). The transcription rate of the IL-3 gene was measured by hybridisation of equal amounts of ^{32}P -labeled RNA to filters which held the hybridisation target sequences of the genomic IL-3 gene (section 2.4.2.3). As controls, the transcription rate of two inducible genes, GM-CSF and c-fos were measured in addition to that of a

housekeeping gene β -actin which is not induced under the stimulation conditions employed here. The plasmid vector pSP64 was also included on the filter as a background or negative control. The results were quantitated by direct measurement of radioactivity on the filters or where the signal was very low, by scanning densitometry of autoradiographs. To minimise experimental variation from the hybridisation reactions, the level of transcriptional signal from the IL-3 gene was normalised relative to that of the β -actin signal. A reproducible but low level transcription of the IL-3 gene in unstimulated cells was apparent (Figure 3.8), although IL-3 mRNA was not detected (section 3.2.1). Stimulation with PHA/PMA induced a transient, approximately 9 fold, increase in IL-3 gene transcription, averaged over four experiments (Figure 3.8). The induction of IL-3 transcription was apparent as early as 1 hour after stimulation, reached a maximum at 4 hours after stimulation and then decreased rapidly to pre-stimulation levels by 6 hours. The steady state IL-3 mRNA level in these cells, measured by RNase protection assay, rose by an average of 17 fold over levels detected at one hour and reached maximum levels at 6 hours (graphed in Figure 3.8). Treatment of Jurkat cells with PHA alone induced a lower, approximately 2.5 fold, transient increase in IL-3 gene transcription, averaged over 2 experiments (Figure 3.9). This finding is consistent with the lower steady state level of IL-3 mRNA in these cells (graphed in Figure 3.9). Increased transcription of the GM-CSF gene was also induced by PHA or PHA/PMA treatment of Jurkat cells (figures 3.8 and 3.9) and was accompanied by accumulation of GM-CSF mRNA (data not shown). However, constitutive transcription of the GM-CSF gene was not detected in any of the experiments (see figures 3.8-3.11). The transcription rate of the c-fos gene increased within one hour of PMA/PHA stimulation but remained at high and greatly variable levels over the time course of these experiments (Figure 3.8). The significance of the apparent sustained high level of c-fos transcription in these cells especially after several hours of PHA or PHA/PMA stimulation remains unclear, however, it is possible that cross hybridisation of an actively transcribed mRNA is responsible for the signal. Transcription of the β -actin gene was constitutive and was

Figure 3.8. Nuclear run on analysis of IL-3 transcription in Jurkat cells.

Nuclei and steady state whole cell RNA were isolated from Jurkat cells that were stimulated with PHA/PMA for various times between 1 and 24 hours as shown. The rate of gene transcription in the isolated nuclei was determined by nuclear run-on analysis. Transcriptional activity of the IL-3 gene was measured using the Hind III fragment of genomic human IL-3 clone λ J1.16 (Yang et al., 1989) as the hybridisation target. The transcriptional activity of GM-CSF, β -actin and c-fos was also determined. Hybridisation targets for measurement of the transcription of these genes were pSP64 plasmids containing the following inserts, a 5.2kb genomic fragment of plasmid pCH5.2; a 1.8kb c-fos cDNA fragment from pBK28 and the 2.1kb β -actin fragment from pHF- β A1. (see section 2.1.7) pSP64 plasmid DNA was used as a negative control. The results shown are representative of 4 experiments. Autoradiographs were exposed for 4 days and results were quantified by scanning densitometry and liquid scintillation counting of filter bound RNA. The average fold increase in IL-3 transcriptional rate (closed squares) was normalised with respect to the β -actin signal and is compared in graphic form with the fold increase in accumulation of steady state IL-3 mRNA in the same cells relative to levels which were first detected at one hour after stimulation (open squares).



unaffected by either PMA/PHA or PHA stimulation over the time period used in these experiments.

The finding of constitutive transcription of the IL-3 gene in the absence of IL-3 mRNA accumulation suggested the possibility that the transcriptional signal may be due to cross hybridisation of the genomic IL-3 hybridisation targets. To determine whether the apparently constitutive transcription observed in unstimulated Jurkat cells was specific for IL-3 coding sequences and not flanking or repetitive sequences which may be present in the genomic probe, nuclear run-on experiments were carried out with an IL-3 cDNA hybridisation target. The 865bp Xho I fragment of gibbon IL-3 cDNA, which is approximately 95% homologous to the human gene, was cloned into the plasmid pSP65 for use as a hybridisation target. Although nuclear run-on experiments using the gibbon cDNA target gave an overall lower signal in both stimulated and unstimulated cells, a low level of constitutive transcription of the IL-3 gene was consistently detected (Figure 3.10).

The requirement, at least in part, for nascent protein synthesis in the accumulation of maximal levels of IL-3 mRNA and the delay observed before transcription induction in stimulated cells suggested that protein synthesis may be required before the induction of IL-3 transcription. To examine the role of protein synthesis in the induction of IL-3 gene transcription Jurkat cells were treated with CHX to inhibit protein synthesis for one hour prior to stimulation with PHA/PMA. This CHX pretreatment abolished the increase in the transcription of IL-3 normally seen following stimulation with PHA/PMA (Figure 3.11 (*c.f.* Figure 3.8)). However, as shown by RNase protection analysis, accumulation of steady state IL-3 mRNA in CHX pretreated cells was detected after PHA/PMA stimulation (see Figure 3.7A and Figure 3.11). The induction of GM-CSF transcription was also blocked by inhibition of protein synthesis (Figure 3.11). These data suggest that there may be a low level of constitutive transcription of the IL-3 gene but not GM-CSF in Jurkat cells and that following stimulation, new protein synthesis is required for the increase in both IL-3

Figure 3.9 Nuclear run on analysis of IL-3 transcription in PHA stimulated Jurkat cells.

The rate of gene transcription in nuclei isolated from PHA treated Jurkat cells was determined by nuclear run-on analysis. Nuclei and steady state whole cell RNA were isolated from cells that were stimulated for various times between 1 and 24 hours as shown. Transcription hybridisation target probes used (see section 2.1.7) are described in the legend for figure 3.8. The results shown are representative of 2 experiments. Autoradiographs were exposed for 10 days and results were quantified by scanning densitometry. The average fold increase in IL-3 transcriptional rate (closed squares) was normalised with respect to the β -actin signal and is compared in graphic form with the fold increase in accumulation of steady state IL-3 mRNA in the same cells relative to levels which were first detected at two hours after stimulation (open squares).

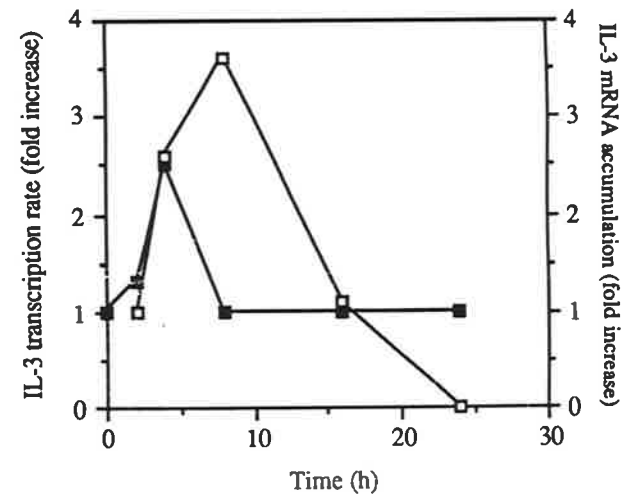
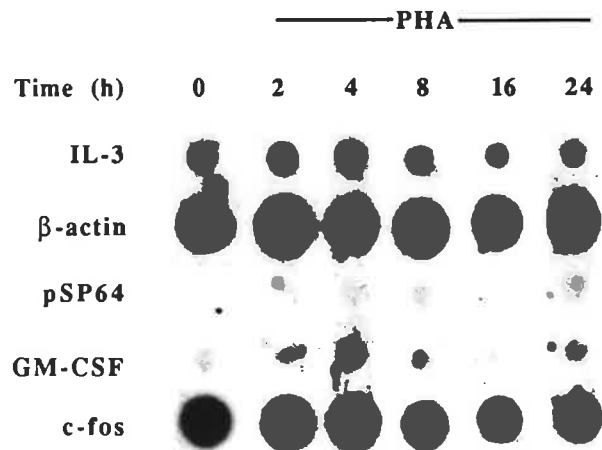


Figure 3.10 Nuclear run on analysis of IL-3 transcription in PHA/PMA stimulated Jurkat cells.

The rate of gene transcription in nuclei isolated from PHA/PMA treated Jurkat cells was determined by nuclear run-on analysis. Nuclei and steady state whole cell RNA were isolated from cells that were either unstimulated (shown in duplicate) or stimulated for four hours as shown. A 865 bp Xho I fragment of gibbon IL-3 c-DNA was used as the hybridisation target probe for IL-3 gene transcription. Transcription hybridisation target probes for β -actin, GM-CSF, c-fos and the background control are described in the legend for figure 3.8 (also see section 2.1.7). The results shown are representative of 4 experiments. Autoradiographs were exposed for 10 days and results were quantified by scanning densitometry. The average fold increase in IL-3 transcriptional rate shown graphically (closed squares) was normalised with respect to the β -actin signal

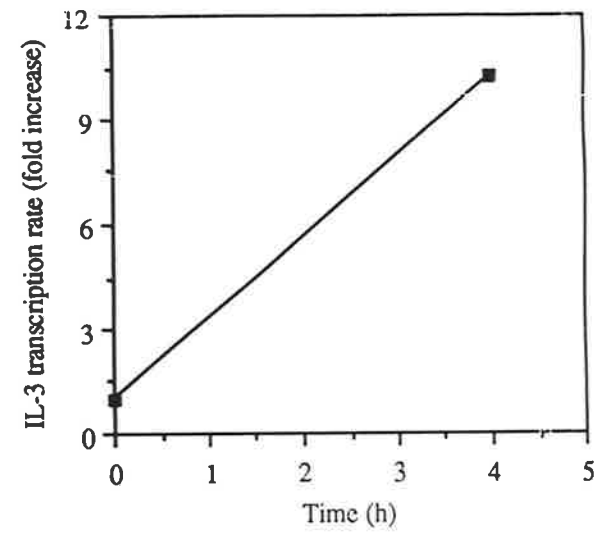
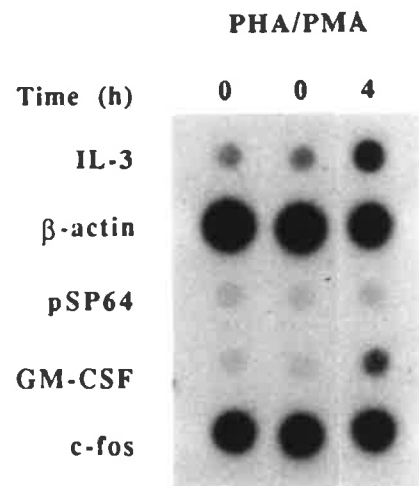
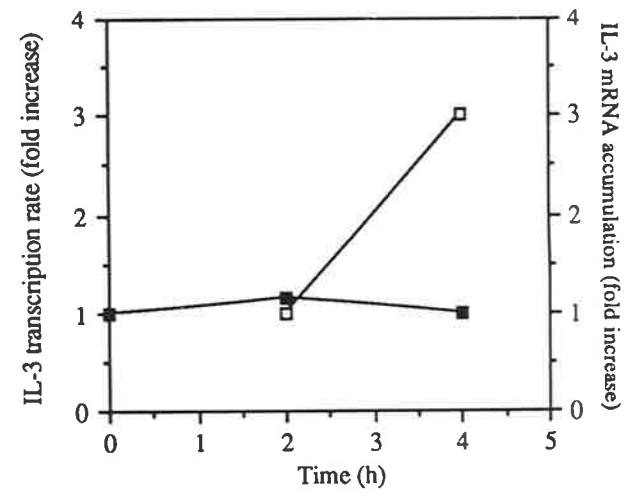
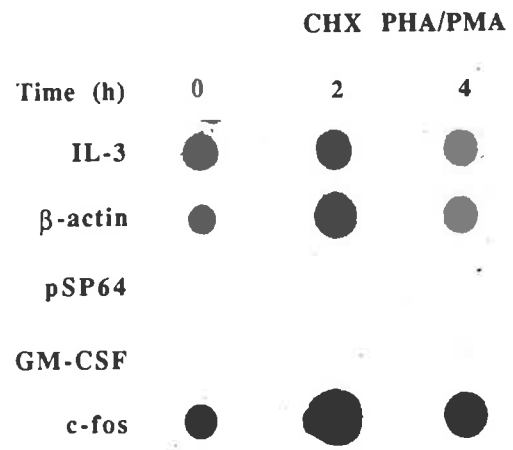


Figure 3.11 Nuclear run on analysis of IL-3 transcription in cycloheximide treated Jurkat cells.

Jurkat cells were treated with CHX to inhibit peptide synthesis for one hour prior to stimulation of the cells with PHA/PMA. The rate of gene transcription in nuclei isolated from these cells was determined by nuclear run-on analysis. Nuclei and steady state whole cell RNA were isolated from cells that were unstimulated or stimulated for two or four hours as shown. Transcription hybridisation target probes for β -actin, GM-CSF, c-fos and the background control are described in the legend for figure 3.8 (also see section 2.1.7). The results shown are representative of two experiments. Autoradiographs were exposed for 10 days and results were quantified by scanning densitometry. The average fold increase in IL-3 transcriptional rate (closed squares) was normalised with respect to the β -actin signal and is compared in graphic form with the fold increase in accumulation of steady state IL-3 mRNA in the same cells relative to levels which were first detected at two hours after stimulation (open squares).



and GM-CSF transcription. Moreover, the increase in the rate of transcription of IL-3 in PHA/PMA stimulated cells appears insufficient to account for the large increase of IL-3 mRNA accumulation in these cells.

3.2.4 Regulation of IL-3 mRNA Stability

The absence of IL-3 mRNA in unstimulated cells despite the apparent constitutive transcription signal coupled with the observation that IL-3 mRNA accumulates following PMA/PHA stimulation in CHX pretreated cells, where transcription is not induced above the constitutive level, suggests that post-transcriptional mechanisms play a role in the induction of high levels of IL-3 mRNA. To examine the role of mRNA stability in induction of IL-3 mRNA, the half lives ($t_{1/2}$) of IL-3 and β -actin mRNAs in PHA or PHA/PMA stimulated Jurkat cells, were analysed by RNase protection assay following inhibition of transcription by actinomycin D (act D) treatment. Preliminary experiments were carried out to determine the concentration of act D that is required for the complete inhibition of Jurkat cell RNA polymerase II (Pol II) dependent transcription. In these experiments, unstimulated Jurkat cell cultures (2×10^6 cells/ml in fresh medium) were treated with act D at final concentrations ranging from 0.1 to $10 \mu\text{g/ml}$ (as indicated in Figure 3.11) and were immediately ($< \text{one minute}$) or after the times indicated, pulsed with [^3H]-Uridine ($1 \mu\text{Ci/ml}$). Total cell RNA was extracted after 60 minutes incubation and the TCA precipitable radioactivity (section 2.7.1) was assessed by liquid scintillation counting. A plateau level of [^3H]-Uridine incorporation that was not sensitive to act D was apparent in these experiments no matter how high the concentration of act D used (data not shown) and is presumably due to non RNA Pol II activity that is not sensitive to interference by act D. Actinomycin D at $10 \mu\text{g/ml}$, was sufficient to inhibit 90% of the Pol II dependent (act D inhibitable) [^3H]-Uridine incorporation within the first 5 minutes (Figure 3.12) and this concentration was used in RNA stability assays.

Figure 3.12 Inhibition of transcription in Jurkat cells by Actinomycin D treatment.

To determine the concentration of the transcription inhibitor Actinomycin D (Act D) required to inhibit transcription of structural or Pol II transcribed genes Jurkat cells were treated with Act D at either 0.1, 1.0 or 10 $\mu\text{g/ml}$. Ongoing transcription was measured by the incorporation of [^3H]-Uridine (1 $\mu\text{Ci/ml}$) into total cell RNA. After the addition of Act D the first time points were immediately (< one minute) pulsed with [^3H]-Uridine, subsequent pulses were at 5,10, 20 and 40 minutes. Total cell RNA was extracted after a 60 minute incubation and ^3H incorporation was determined by liquid scintillation counting.

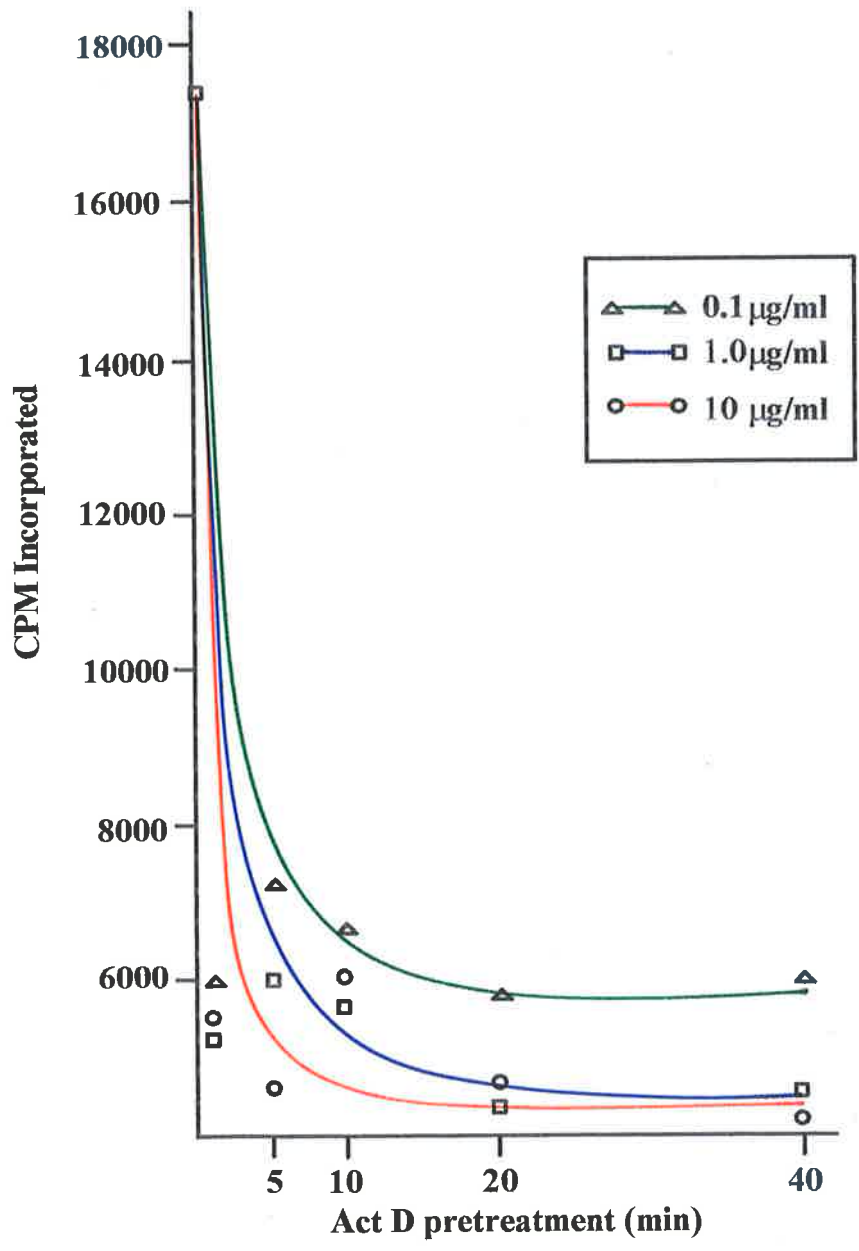


Figure 3.13 Measurement of IL-3 mRNA halflife.

IL-3 mRNA stability was measured by RNase protection assay in Jurkat cells where transcription had been inhibited by Act D. Jurkat cells were stimulated with PHA for 4h. (open circles); PHA/PMA for 4h. (open squares); pretreated with CHX for 1h. followed by PHA/PMA stimulation for 4h. (closed squares) and then treated with actinomycin D (10 μ g/ml) to inhibit transcription. Samples were withdrawn from the cultures at 15, 30 120, 240 and 360 minutes after Act D addition and RNase protection assays were carried out to determine IL-3 mRNA levels remaining at these times. The results shown are the means of three separate experiments for PMA/PHA and CHX-PMA/PHA treated cells and two separate experiments for PHA treated cells. Results were quantified by liquid scintillation counting of labelled RNA bands excised from the gels The results are expressed relative to levels detected at the time of actinomycin D addition.

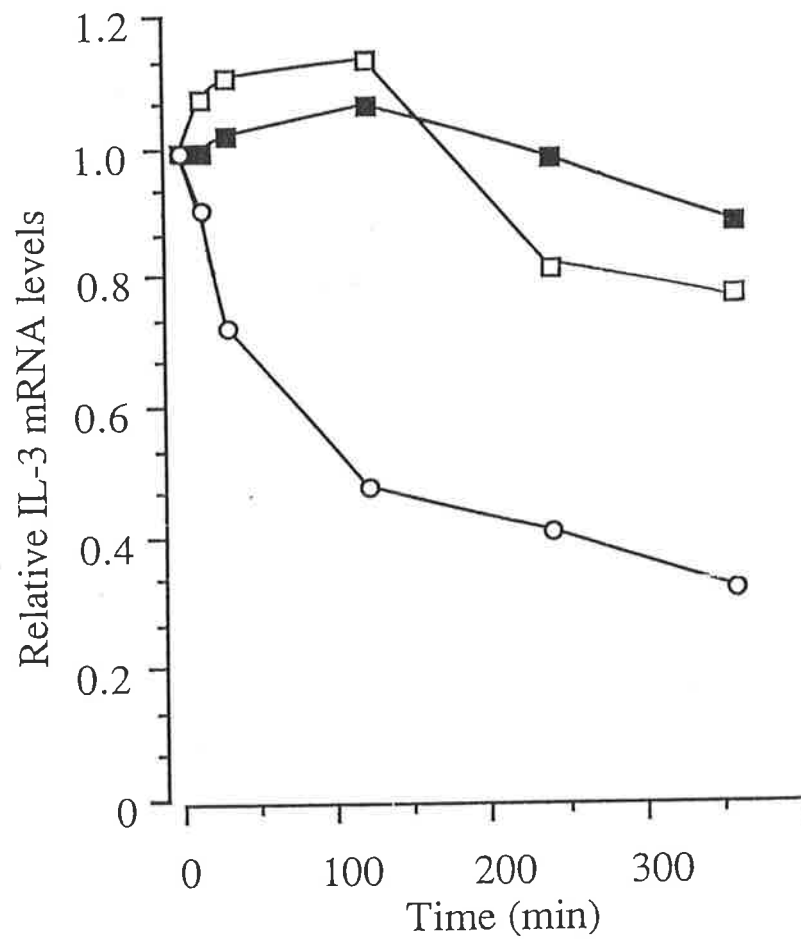
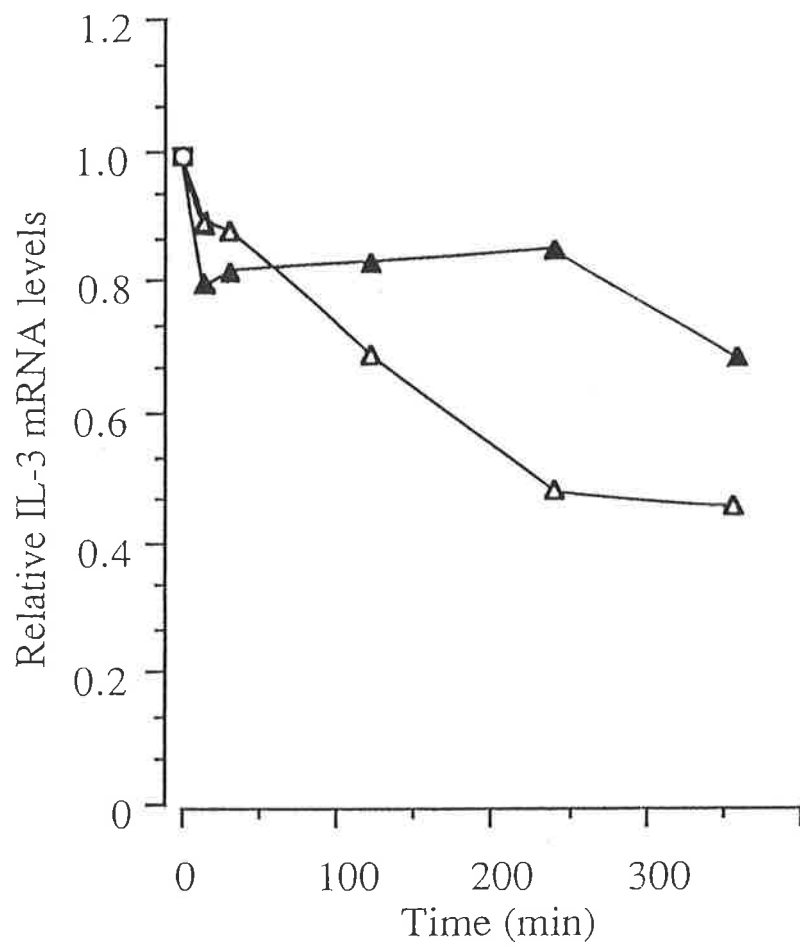


Figure 3.14 Measurement of IL-3 mRNA half life in Jurkat cells at 16 hours after PHA/PMA stimulation

Jurkat cells were stimulated with PHA/PMA for 16h. (open triangles) or pretreated with CHX for one hour followed by PHA/PMA stimulation for 16h. (closed triangles) and then treated with Actinomycin D (10 μ g/ml) to inhibit transcription. Samples were withdrawn from the cultures at 15, 30 120, 240 and 360 minutes after Act D addition and RNase protection assays were carried out to determine IL-3 mRNA levels remaining at these times. The results shown are the means of three separate experiments. Results were quantified by liquid scintillation counting of labelled RNA bands excised from the gels The results are expressed relative to levels detected at the time of Actinomycin D addition.



The $t_{1/2}$ of IL-3 mRNA was measured by removing sequential samples (1×10^7 cells) from a culture flask of Jurkat cells in which transcription had been blocked by act D treatment. Whole cell RNA was extracted from these cells and the IL-3 mRNA was quantitated by RNase protection assay. To minimise experimental variation between replicate assays, the IL-3 mRNA signal was normalised to the internal control signal for β -actin. Preliminary experiments showed that β -actin mRNA was highly stable in Jurkat cells (data not shown). The experimental design used in this study allowed measurements of RNA, following cessation of transcription, for up to 6 hours. This assay therefore is limited to assigning stable mRNAs an apparent $t_{1/2}$ greater than 6 hours. Treatment of Jurkat cells with either PHA or PHA/PMA did not affect the stability of β -actin mRNA (data not shown). As no IL-3 mRNA could be detected in unstimulated cells, IL-3 mRNA $t_{1/2}$ was measured in PHA stimulated cells. Stimulation of Jurkat cells with PHA alone at 4 hours led to the production of IL-3 mRNA which had an $t_{1/2}$ of approximately 1.5 hours (Figure 3.13, open circles). Jurkat cells were also treated with PHA/PMA to determine the additional consequences of PMA treatment to IL-3 mRNA stability. Stimulation with PHA/PMA for 4 hours increased the stability of IL-3 mRNA produced in these cells (Figure 3.12 open boxes). Under these conditions IL-3 mRNA became very stable and had an apparent $t_{1/2}$ of greater than 6 hours (Figure 3.13). A small increase in the IL-3 mRNA level was consistently observed in the first 15 minutes after act D addition in cells that had been stimulated with PHA/PMA. While the bulk of cellular transcription is inhibited almost immediately by act D treatment, the rise may however reflect accumulation of the IL-3 mRNA before the effects of act D treatment are complete.

To determine if labile proteins or nascent protein synthesis plays an active role in stabilisation of IL-3 mRNA in stimulated cells, Jurkat cells were pretreated with CHX for at least 1 hour before stimulation with PHA/PMA. The IL-3 mRNA produced in the absence of nascent protein synthesis after 4 hours of PHA/PMA stimulation was also very stable with a $t_{1/2}$ of greater than 6 hours (Figure 3.13, closed squares).

The long half life of IL-3 mRNA despite the apparent transient nature of steady state levels in stimulated cells suggests that changes in IL-3 mRNA stability may occur during the period following the peak of IL-3 mRNA accumulation. To determine if modulation of mRNA stability plays a role in the transient nature of IL-3 mRNA accumulation, IL-3 mRNA $t_{1/2}$ was measured after 16 hours of PHA/PMA stimulation when the steady state levels were in decline. These experiments showed that the mRNA became more unstable at this time point, having an $t_{1/2}$ of approximately 4 hours (Figure 3.14). Pretreatment with CHX before 16 hours of PHA/PMA stimulation again increased the IL-3 mRNA $t_{1/2}$ to greater than 6 hours.

In summary these data show that IL-3 mRNA produced by stimulation with PHA alone is relatively unstable. Stimulation in addition with PMA serves to increase the stability of the mRNA, in part, accounting for the relatively higher steady state mRNA levels in PHA/PMA stimulated cells. Moreover the decline in steady state levels of IL-3 mRNA at 16 hours after stimulation appears to be associated with decreased stability of IL-3 mRNA and this decline in mRNA stability may be associated with mechanisms requiring protein synthesis.

3.3 Discussion

Human IL-3 gene expression has been detected in only a small number of cell types, primarily in cells which constitute or support the effector arms of the immune system, *i.e.* in activated T-lymphocytes, both neutrophilic and eosinophilic granulocytes and thymic epithelial cells. Moreover, in the mouse although not in the human, IL-3 is produced by mucosal mast cells (Plaut *et al.*, 1989). Since IL-3 rarely reaches detectable levels in the circulation its action is probably restricted to the site of synthesis, regulating activation of local effector cells in situations of infection and in allergic inflammatory responses where the rapid supply of functionally active mature haemopoietic cells is required. The approach taken in this study to investigate the molecular mechanisms involved in the expression and induction of the human IL-3

gene in T-lymphocytes was to use as a model, a tumour cell line that is able to express IL-3 under similar stimulation conditions shown to be required for IL-3 production in normal PBLs. Accurate and sensitive quantitation of IL-3 mRNA in PBLs and cell lines required the optimisation of an RNase protection assay. Using this technique IL-3 mRNA was detected in PBLs and tissue culture cell lines only after lectin and phorbol ester stimulation. Stimulation of PBLs with PHA resulted in only low levels of IL-3 mRNA. Additional stimulation with PMA induced an increase in the accumulation of total IL-3 mRNA after a short lag, with peaks at 8 and 24 hours. Niemeyer *et al.*, (1989) have reported similar diphasic responses for both GM-CSF and IL-3 mRNA. These authors have suggested that the second wave of cytokine mRNA accumulation may be due to stimulation of the PBLs by monokines expressed by PMA activation of the contaminating monocytes in the PBL preparation, since stimulation of the PBLs with IL-1 and α -CD3 antibodies induced only the earlier peak of IL-3 expression.

The lack of constitutive expression and the rapid induction of IL-3 mRNA in activated PBLs resembles that of other cytokines which are collectively expressed on T-cell activation and comprise a set of early phase T-cell activation genes which includes among others GM-CSF, interleukins (IL) 2 through 6 and interferon- γ (IFN- γ) (Mizutani *et al.*, 1987, Ullman *et al.*, 1990). The coordinate expression of cytokine genes in activated T-lymphocytes particularly the tightly linked expression of IL-3 and GM-CSF has led to the suggestion of the existence of a common cytokine regulatory pathway in T-lymphocytes (Nishida *et al.*, 1991). However, several lines of evidence argue against this proposal. In mice the synthesis of some cytokines can be reciprocally regulated, most notably IFN- γ *versus* IL-4, IL-5, IL-6 and IL-10 which are products of T-cell helper subsets Th1 and Th2 respectively. IL-3 and GM-CSF are however, produced in both the Th1 and Th2 subsets (Mosmann and Coffmann, 1989). In addition, Kelso *et al.* (1986) demonstrated the selective production of murine GM-CSF in the absence of parallel generation of IL-3 in T-cells that were stimulated

by exogenous IL-2. Furthermore, Bickel *et al.* (1988) demonstrated a differential susceptibility of these two cytokines to the immunosuppressant CSA. While throughout this work the transcription of the IL-3 gene was associated in all experiments with GM-CSF mRNA accumulation measured by RNase protection assays (results not shown), this study did not address the question of differential control mechanisms of the two cytokines. Other studies in humans have shown subtle differences in the regulation of GM-CSF and IL-3 gene expression in human T-cells. The expression of IL-3 requires, as a prerequisite, an increase in intracellular calcium (Guba *et al.*, 1989) and contrasts with the expression of GM-CSF and IL-2 which can occur in PMA and α -CD28 stimulated cells by a Ca^{2+} independent mechanism that is not regulated by CSA (Ledbetter *et al.*, 1986, Thompson *et al.*, 1989 and June *et al.*, 1987). This difference in stimulation requirements implies the existence of alternate regulatory pathways which allow independent regulation of IL-3, GM-CSF and other cytokines.

Production of IL-3 mRNA was detected only in the T-lymphocytes, other cell lines such as the bladder carcinoma 5637 which constitutively expresses GM-CSF did not express IL-3 mRNA when stimulated with PMA (Kaashoek *et al.*, 1991). Although an IL-3 like activity, and more recently the detection of IL-3 mRNA, has been described in primary human keratinocyte cell cultures (Luger *et al.*, 1988), no IL-3 mRNA was detected in the unstimulated or PMA treated melanoma cell line LiBr. In addition, the promyelocytic leukaemia cell line HL60 did not express IL-3 mRNA. The gibbon lymphosarcoma cell line UDC-MLA-144 (MLA-144) from which the primate IL-3 gene was first isolated, was used as a positive control in the production of IL-3 mRNA. Interestingly, this cell line was unresponsive to PHA stimulation, although MLA144 cells express very large amounts of IL-3 after stimulation with PMA alone (Yang *et al.*, 1988). In this respect, MLA 144 cells may resemble partially activated T-lymphocytes. Niemeyer *et al.*, (1989) observed that MLA 144 may express very low levels or non-functional T-cell receptors, and this may account for the lack of response

of this cell line to PHA stimulation. Moreover, disregulated production of IL-2 has also been observed in MLA 144 cells. Constitutive expression of IL-2 by these cells was shown to result from the integration of two copies of the San Francisco strain of the gibbon ape leukaemia virus (GALV-SF) provirus inserted both in the 5' and 3' vicinity of the IL-2 gene (Durand *et al.*, 1986 and Holbrook *et al.*, 1987). Whether other copies of the provirus play a direct or indirect role in the regulation of IL-3 in these cells is not known. Of the T-cell lines screened for IL-3 production, one, HUT78, did not produce IL-3 mRNA when stimulated with PMA/PHA or with PMA/A23187 (not shown). Although IL-3 is not expressed in these cells GM-CSF expression is constitutive at a low level and is induced by stimulation with PMA alone, suggesting that signaling pathways involving the activation and translocation of PKC to the membrane are intact and that these cells may also display a partially activated phenotype (Niemeyer *et al.*, 1989). The expression of IL-3 mRNA in the T-cell tumour lines Jurkat and HSB2 after stimulation followed similar kinetics to that of the first wave of expression in PBLs. In both of these cell lines the expression of GM-CSF mRNA is coordinated with that of IL-3 (Niemeyer 1989 and Cockerill *et al.*, 1993). Interestingly, the HSB2 cell line expressed IL-3 mRNA only after stimulation with agents that bypass the requirement for stimulation through the TcR, that is, direct activation of PKC and elevation of intracellular Ca^{2+} by PMA and calcium ionophore A23187. It is puzzling however, that Niemeyer *et al.*, (1989) in a similar survey for IL-3 producing cell lines reported the detection of IL-3 mRNA in HSB2 cells stimulated with PHA and PMA. FACS analysis showed that the HSB2 cell line used in this study lacks the CD3/TcR complex and has a surface marker profile similar to that of immature thymocytes. If this cell line is representative of untransformed cells the data suggests that commitment to express IL-3 and GM-CSF may occur during T-cell ontogeny prior to TcR rearrangement. Furthermore, expression of IL-3 in the Peer cell line which is characterised by expression of the primitive T-cell γ/δ -TcR chains has led to the suggestion that immature cells may account for the production of IL-3 and GM-CSF in patients with severe combined immunodeficiency whose

residual T-cells also express the γ/δ TcR (Niemeyer *et al.*, 1989 and Brenner *et al.*, 1986)

Since the production of IL-3 mRNA in Jurkat cells most closely matched the pattern observed in normal PBLs, this cell line was used as a model to elucidate the regulatory mechanisms of IL-3 mRNA expression. The production of IL-3 mRNA and protein by Jurkat cells that were stimulated with PHA was detected after a short lag (<two hours). Stimulation in addition with PMA increased the level of IL-3 mRNA expression and IL-3 mRNA was then detected less than one hour post stimulation. Bypassing the TcR by stimulation with the Ca^{2+} ionophore A23187 and PMA induced a similar accumulation of IL-3 mRNA. Interestingly, CD28 stimulation alone or in combination with either one of PHA or PMA had very little effect on the production of IL-3 mRNA, however, stimulation of CD28 by monoclonal antibody synergised with PHA and PMA treatment. The combination of PHA/PMA and the α -CD28 monoclonal antibody produced the maximum IL-3 mRNA accumulation and suggests that the CD28 receptor stimulates a distinct and complimentary pathway to that of TcR or those induced by PHA and PMA stimulation. Recent reports have linked the stimulation of the CD28 receptor with the induction of IL-3 mRNA transcription as well as an increase in IL-3 mRNA stability (Fraser and Weiss, 1992 and Guba *et al.*, 1989).

To determine the contribution of PHA/PMA stimulation to the induction and regulation of IL-3 gene transcription, the rate of IL-3 gene transcription was examined in nuclei isolated from stimulated or unstimulated Jurkat cells by nuclear run on experiments. The data indicated that transcriptional induction plays a major role in the regulation of steady state levels of IL-3 mRNA in activated T-cells. These experiments also demonstrated that Jurkat cells have a low level of constitutive IL-3 transcription. It was initially considered likely that the signal may be due to cross hybridisation of other labeled nascent RNAs in the run-on assay to either the IL-3 coding regions or to repetitive sequences within the introns or in the sequences

flanking the IL-3 coding region of the genomic fragment used as a probe. However, despite the substitution of the human genomic probe sequences used in these experiments with that of the gibbon IL-3 cDNA, a low level of constitutive transcription was still consistently observed in the unstimulated cells. Recent evidence obtained by Dokter *et al.*, (1993) also shows constitutive transcription of IL-3 in freshly isolated PBLs. It is curious then, that no IL-3 mRNA could be detected in unstimulated cells by RNase protection. Furthermore, analysis of mRNA from unstimulated Jurkat cells and freshly isolated PBLs in this laboratory by PCR and southern hybridisation also failed to detect IL-3 mRNA (Dr. Peter Bardy, personal communication). There are at least three possible explanations. Firstly, it is possible that the nuclear run-on probes contained some less obvious repetitive sequence elements that could cross-hybridise within the heterogenous population of radiolabelled RNA generated by the run-on reactions. This potential artifact has been demonstrated with a number of full length mouse HGF cDNA sequences (Brorson *et al.*, 1991). Removal of small regions having homology to highly expressed repetitive or ribosomal RNA sequences or the 3' untranslated region of the hybridisation target eliminated the cross hybridisation and the transcription signal from resting non-transformed T-lymphocytes in cases where no mRNA could be detected by northern blot. Although searches against the Genbank sequence databank do not detect any obvious homology among the known sequences with the IL-3 cDNA probe they reveal that the c-fos sequences which were used as a probe in the nuclear run-on experiments share limited homology with ribosomal RNA and repetitive sequences in the human genome. Cross hybridisation of these sequences may be responsible for the surprisingly high c-fos transcription observed in resting cells by the nuclear run-on experiments. To establish whether the constitutive IL-3 signal observed in these nuclear run-on experiments results from such cross hybridisation would require the testing of several probes from various regions of the IL-3 gene. A second alternative is that in Jurkat cells, IL-3 transcription may abort before it reaches the end of the IL-3 gene and so result in an unstable transcript. Such a mechanism has

been proposed for regulation of c-myc and IL-2 gene transcription in T-lymphocytes (Lindsten, June and Thompson, 1988, Eick and Bornkamm, 1986 and reviewed by Ullman *et al.*, 1990). There is however no evidence of short IL-3 mRNA products from protection assays which employ a probe directed against the first exon of the IL-3 gene. A series of protection assay probes covering different segments of the IL-3 gene may be useful in determining the likelihood of this possibility. Thirdly, it has been suggested that the transcription of tissue specific genes may not be absolutely shut off in all non-permissive tissues and that a low level of expression of mRNA species which are particularly unstable in "non producing" cells may escape detection even by PCR whereas low level transcription of more stable mRNA species is more easily detected (Gilliland *et al.*, 1990). Such a proposal argues that post-transcriptional mechanisms, that is the rapid degradation of small amounts of constitutively expressed IL-3 mRNA, may be the predominant regulatory mechanisms for IL-3 in unstimulated cells. This proposal predicts that the inhibition of protein synthesis by CHX, a treatment which is known to increase mRNA stability of cytokine genes (Shaw and Kamen, 1986), would reveal accumulation of IL-3 mRNA in unstimulated cells since CHX treatment did not affect the constitutive transcription of IL-3 (see Figure 3.11). On the contrary, in this study no IL-3 mRNA was detected by RNase protection assays in unstimulated cells following CHX treatment. However, RNA samples were tested only at one hour post CHX treatment and it is still possible that low levels of IL-3 mRNA may be detected under these circumstances with the PCR technique.

While inhibition of nascent protein synthesis did not induce the accumulation of IL-3 mRNA, it was found to be necessary for the induction of IL-3 transcription. It is possible then, that IL-3 mRNA detected after PHA/PMA stimulation in CHX treated cells by RNase protection assays (see Figure 3.7A) may be due to stabilisation by PMA/PHA treatment of the mRNA produced by constitutive transcription. The dependence on new protein synthesis for induction of IL-3 transcription is presumably

due to the need for the synthesis of essential nuclear transcriptional activation factors. Similarly, the transcription of other "early" T-cell activation cytokine genes, such as GM-CSF and IL-2, has been shown to be contingent upon protein synthesis and on expression and/or activation of "immediate early" T-cell activation genes, a group which includes the transcription factors c-fos, NF-AT and NF- κ B (reviewed by Ullman *et al.*, 1990). Some of these may play a direct role in the regulation of IL-3 transcription.

The accumulation of IL-3 mRNA observed in the absence of protein synthesis and the undetectable levels of IL-3 mRNA in unstimulated or cycloheximide treated cells, despite constitutive transcription, suggested that IL-3 mRNA expression may be regulated at the post-transcriptional level, perhaps by a mechanism independent of novel protein synthesis. To assess the contribution of PMA and PHA stimulation to regulation of IL-3 mRNA stability, RNase protection assays were used to measure IL-3 mRNA levels in the absence of continuing transcription. Steady state levels of IL-3 mRNA could not be detected in unstimulated cells and therefore, an increase in the stability of IL-3 mRNA in PHA treated cells can only be inferred by indirect means. Additional stimulation by PMA together with PHA produced a marked increase in IL-3 mRNA stability with little appreciable decay over a 6 hour period. Hence the role of PHA/PMA may be mediated through stabilisation of existing mRNA as well as augmentation of the transcriptional response. Modulation of the stability of IL-3 mRNA probably serves to down regulate the expression of IL-3 in resting or unstimulated T-lymphocytes, to augment rapid accumulation of mRNA during cell activation and may also be involved in regulation of the transient nature of the response following the peak of transcription. Inhibition of protein synthesis at this later stage of the IL-3 timecourse prolongs the half-life of IL-3 mRNA suggesting that mechanisms involving translation are predominantly responsible for the removal of IL-3 mRNA after the transcriptional phase of IL-3 mRNA expression. Similar modulations in GM-CSF mRNA stability following PMA or α -CD28 stimulation of

T-lymphocytes have been observed (Shaw and Kamen, 1986 and Lindsten *et al.*, 1989). In the case of GM-CSF mRNA, an AU rich sequence in the 3'-untranslated region (UTR) confers mRNA instability. Related AU rich sequences are found in the UTR of other HGF genes including IL-3, IL-2, IFN- γ and β and also in several oncogene mRNAs including c-myc and c-fos. (Reeves *et al.*, 1987, Caput *et al.*, 1986 and Shaw and Kamen, 1986) When the AU rich region of GM-CSF mRNA was inserted into the normally stable β -globin mRNA the chimeric message was rapidly degraded (Shaw and Kamen, 1986) and when the corresponding region was removed from c-fos the message produced from transfected constructs had a greater half-life as well as the ability to transform cells (Meijlink *et al.*, 1985). AU rich sequences in the murine IL-3 UTR may also have a similar effect. IL-3 over-expression enhanced the tumorigenicity of a mast cell line when transfected with truncated IL-3 constructs missing their AU rich UTR (Wodnar-Filipowicz and Moroni, 1990). These highly conserved AU rich 3' UTR regions may also be involved in other regulatory mechanisms such as translational blockade (Kruys *et al.*, 1989).

The precise mechanism involved in the regulation of mRNA stability through the AU rich domains is presently unknown. Recently, a cytoplasmic phosphoprotein AUBF which complexes *in-vitro* to a variety of labile RNAs containing AU rich sequences has been described (Malter and Gillis, 1991 and Malter and Hong 1991). AUBF is inactive in resting T-cells and can be activated by phosphorylation following PMA or ionophore treatment. It has been suggested that the binding of this factor to the AU rich motif in the cytokine mRNA UTR delays the turnover of otherwise labile message RNA. A similar mechanism has been proposed for the regulation of stability of the human transferrin receptor mRNA (Mullner, Neupert and Kühn, 1989).

Although this study has not examined the effects of CD28 stimulation on IL-3 mRNA stability, treatment with an α -CD28 monoclonal antibody has been shown to result in increased half-life of IL-3 mRNA as well as that of GM-CSF, IL-2 and IFN- γ (Lindsten *et al.*, 1989 and Guba *et al.*, 1989). However, it is unclear whether the stabilisation in

general of cytokine mRNAs induced by CD28 stimulation is mediated by an AU rich UTR sequence. At least some mRNAs that contain AU rich sequences, for example *c-myc* and *c-fos* mRNAs, are not stabilised by activation of the CD28 pathway (Lindsten *et al.*, 1989).

In conclusion, both transcriptional and post-transcriptional mechanisms are involved in the regulation of IL-3 expression in human T cells. These experiments suggest that despite a low constitutive level of transcription in unstimulated cells, steady state levels of IL-3 mRNA are maintained at undetectable levels probably by rapid mRNA destruction. Whether constitutive transcription and post-transcriptional processing of the IL-3 mRNA in Jurkat cells is a reflection of their transformed phenotype or whether these mechanisms operate in other cell types remains to be determined although in one other case constitutive transcription of IL-3 has been observed in freshly isolated PBLs. In situations where high doses of IL-3 may be needed, induction of IL-3 expression is achieved by both increasing gene transcription, presumably by induction of one or several transcription factors, and by stabilising the newly synthesised IL-3 mRNA. In Jurkat cells transcriptional induction of IL-3 is transient and peaks at approximately 4 hours, rapidly returning to basal levels by 6 hours. Finally, changes in the half-life of the accumulated IL-3 mRNA contribute to the accelerated decay of the mRNA at times later in the timecourse of expression and reduce accumulated IL-3 mRNA to background levels 24 hours after stimulation.

Chapter 4

Functional analysis of the human IL-3 gene promoter

4.1 Introduction

While the stimuli required for the expression of IL-3 in T-lymphocytes had been documented, at the time this work began nothing was known at the molecular level about regulation of induction and the tissue specific nature of IL-3 gene transcription. Nuclear run-on experiments described in the previous chapter demonstrated that expression of the human IL-3 gene is regulated, at least in part, at the transcriptional level and that the kinetics of IL-3 transcription are similar to that of a group of other cytokine genes including IFN- γ , GM-CSF, IL-2, IL-4, IL-5 and LD78 α and β , all of which are coordinately expressed early in T-cell activation (Crabtree, 1989, Ullman *et al.*, 1990 and Zipfel *et al.*, 1989). It has been suggested that the early T-cell cytokine genes may be activated through common *trans*-activators that interact with conserved *cis*-acting regulatory elements (Crabtree, 1989 and Shannon *et al.*, 1990) and it was anticipated that the cloning and sequencing of the coordinately regulated T-cell derived cytokine genes would allow the identification of common sequence elements involved in cytokine gene regulation.

Transfection experiments have established that, in general, the 5' flanking regions covering a few hundred base pairs from the transcription initiation start site of many of the early T-cell cytokine genes play a major role in inducible expression (reviewed by Arai *et al.*, 1990). However, these studies do not discount the possibility that additional *cis*-acting elements for the inducible responses could reside outside the 5' flanking regions of the genes, such as in the introns or the exons of the genes, or that enhancer like regions are located at distant sites from the gene. The T-cell

expressed cytokine genes have little obvious DNA sequence similarity within their 5' flanking regulatory regions except for a conserved 10 bp consensus sequence which is present in several of the genes. This cytokine sequence element termed CK1 (Shannon Gamble and Vadas, 1988) and also known as CLE1 (Miyatake *et al.*, 1988) is conserved in the rodent and primate IL-3 genes. The IL-3 gene has two copies of this element within the 5' flanking sequences in both distal and proximal positions (-334 bp and -127 bp in the human). Similar sequences have been found at comparable positions in the promoters of the GM-CSF, IFN- γ , IL-2, IL-4, IL-5 and LD78 α and β genes (Nakao, Nomiyama and Shimada, 1990 and reviewed by Arai *et al.*, 1990). In contrast, the same sequence is found in the promoter of the G-CSF gene which is not expressed in T-cells (Shannon *et al.*, 1990). Recent evidence has shown that although identical in sequence, the CK1 element in the G- and GM-CSF genes have distinct functions in cells that express both genes (reviewed by Shannon *et al.*, 1993). Among the cytokine genes expressed early after activation of T-cells, GM-CSF shares the highest similarity with the 5' flanking region of the IL-3 gene. These two genes share in addition to the general cytokine element CK1, a second conserved element termed CK2 (Shannon, Gamble and Vadas, 1988) which is positioned close to the CK1 element in both genes. A GC rich region is present downstream of the CK2 element, this region contains sequences typical of the CG box (GGGCGG or CCGCCC for IL-3 and CCGCGCCCCC for GM-CSF) that is conserved in the promoters of many Pol II genes. Computer searches of the 5' flanking region of the IL-3 gene using the Ghosh transcription factor database (TFD, Ghosh, 1990) predict that the GC rich element may bind the general transcription factor SP1. This type of search also detects the consensus binding sites for transcription factors of the AP1, AP2, CREB/ATF, octamer and Ets families in both the GM and IL-3 promoters. Besides these elements, one further conserved sequence element, a 13 bp motif, is found in both the IL-3 and GM-CSF promoters (positions -55 to -43 in human IL-3, the sequence is not conserved in the murine IL-3 gene). In the GM-CSF gene this element overlaps two of the CATT(A/T) repeat motifs that may be involved

in inducible GM-CSF expression. Deletion or mutation of this element in the GM-CSF promoter eliminates inducible promoter activity (Nimer *et al.*, 1990).

Despite having in common, consensus binding sites for known transcription factors and a similar arrangement of conserved cytokine elements, differences in the regulation of IL-3 and GM-CSF are apparent. GM-CSF transcription, in contrast to that of IL-3, can be induced in T-cells independently of TcR activation and intracellular Ca²⁺ increase by stimulation with PMA and α -CD28 antibodies (Thompson *et al.*, 1989, June *et al.*, 1987 and Guba *et al.* 1989). In addition, GM-CSF expression is detected readily in macrophages, fibroblasts and endothelial cells which do not express IL-3.

As a first step towards elucidating the mechanisms controlling the coordinate expression of cytokine genes in T-lymphocytes and identifying what distinguishes IL-3 regulation from that of GM-CSF, regulatory sequences controlling IL-3 transcription were characterised in the leukaemic cell line Jurkat. To identify the IL-3 promoter sequences that conferred responsiveness to T-cell mitogenic signals, a series of deletion mutants were created, each mutant containing progressively shorter segments of the IL-3 gene 5' flanking sequences. The transcriptional activity of these constructs was tested with transfection assays using human cell lines. The functions of the highly conserved CK1 and CK2 sequence elements of the IL-3 promoter were also tested in the context of heterologous (thymidine kinase) promoter/reporter constructs.

4.2 Results

4.2.1 Determination of the IL-3 transcription initiation site

To facilitate the construction of IL-3 promoter/reporter plasmids, the transcription initiation or CAP site of IL-3 mRNA was determined by primer extension reactions (section 2.3.5). The human IL-3 transcription initiation site first proposed by Yang

et al. (1988) was identified only by sequence comparison between murine and human IL-3 genes. This site was however one of several likely initiation sites (Y.-C. Yang, personal communication). Oligonucleotide primers used in the primer extension reactions were chosen to be complimentary to IL-3 mRNA sequences within the first exon, approximately 100 bp to 130 bp 3' to the TATA box element. Oligonucleotides were labelled with [³²P]-ATP at the 5' terminal phosphate (section 2.2.10.2) and reverse transcribed products detected by electrophoresis and autoradiography. Double-stranded di-deoxy sequence markers were electrophoresed in tracks adjacent to the primer extension reaction products. The sequencing reactions utilised the relevant primer extension oligonucleotide as sequencing primer and a plasmid, pGR-P25, containing a 926 bp PstI fragment of the IL-3 gene which includes all of the first exon and part of the promoter as the template.

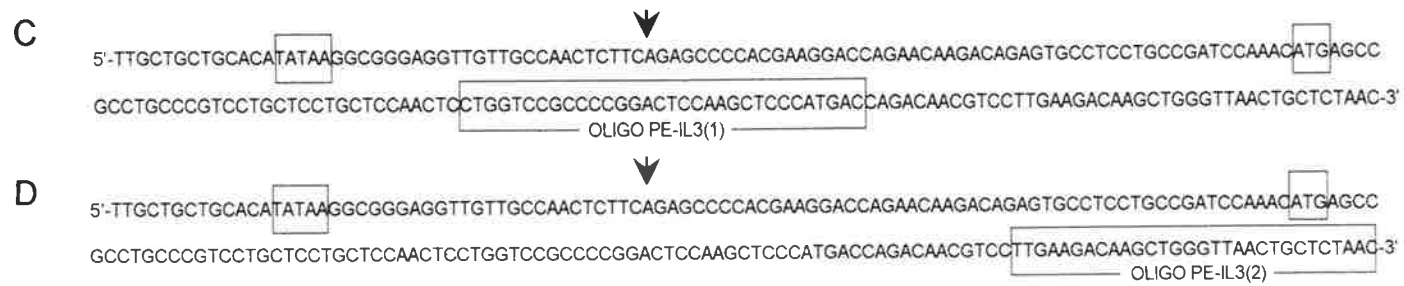
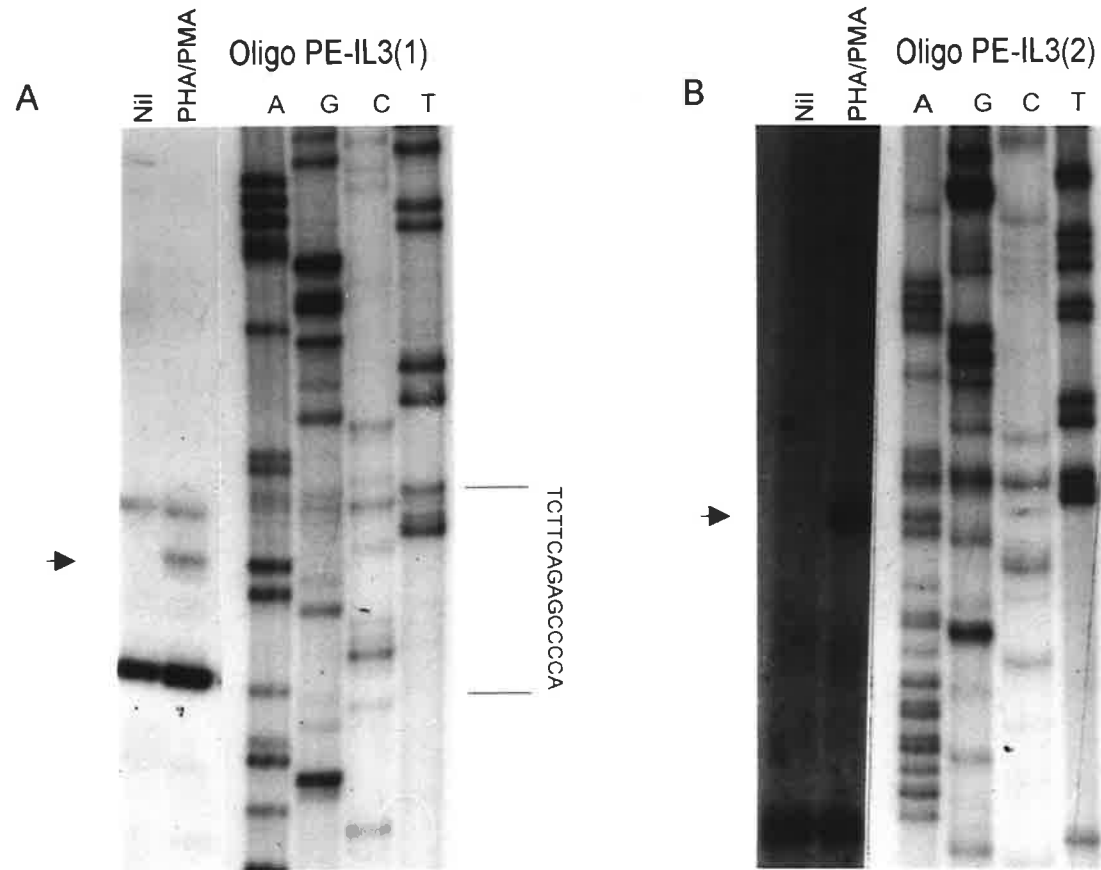
The conditions for hybridisation and primer extension of IL-3 mRNA were optimised using RNA prepared from gibbon MLA-144 cells that had been treated for 16 hours with PMA (20ng/ml). Under these conditions MLA-144 cells express very high levels of IL-3 mRNA. Whole cell RNA was prepared for primer extension experiments from human Jurkat T-cells that were stimulated for 12 hours with PMA (20ng/ml) and PHA (2µg/ml). Primer extension experiments using the primer PE-IL3(1) (section 2.1.8) produced several bands representing extended products in reactions with RNA (50µg/reaction) from both unstimulated and PHA/PMA treated cells (Figure 4.1A). However, only one band, indicated with an arrow, was unique to tracks containing RNA from stimulated cells (Figure 4.1A). Since no IL-3 mRNA was detected in unstimulated Jurkat cells by RNase protection assays or PCR it was assumed that a band present in extension reactions with RNA from both unstimulated and stimulated cells was non-specific and was probably due to primer cross hybridisation. To further distinguish the specific primer extended products from non-specific products and to confirm the position of the CAP site a second primer, PE-IL3(2) (section 2.1.8), was used. This primer produced a single specific extended product (Figure 4.1B), which

Figure 4.1 Determination of the IL-3 transcription initiation site

The transcription initiation site (or CAP site) of the IL-3 gene was determined by extension of oligonucleotide primers by AMV reverse transcriptase (section 2.3.5).

Autoradiographs of primer extension reactions are shown (A) using primer PE-IL3(1) and (B) PE-IL-3(2). Whole cell RNA used in the primer extension reactions was prepared from Jurkat cells that were either untreated (Nil) or stimulated for 12 hours with PMA (20ng/ml) and PHA (2µg/ml) (PHA/PMA). Bands representing primer extension products specific for PHA/PMA treated samples are indicated by arrows. The DNA sequence immediately surrounding the CAP site is noted on the right of each panel. Double-stranded di-deoxy sequence reactions were electrophoresed in tracks adjacent to the primer extension reaction products to serve as size and sequence markers. The sequencing reactions utilised the relevant primer extension oligonucleotide as sequencing primer and the plasmid pGR-P25 as a template. The di-deoxy used for chain termination is marked above each lane.

(C and D) The genomic IL-3 DNA sequence flanking the start of transcription is shown. The sequence and positions of the two oligonucleotide primers used in the primer extension reactions are labelled and indicated by boxes. The position of the TATA box and AUG initiation codon are also boxed. The IL-3 mRNA CAP site is indicated by an arrow.



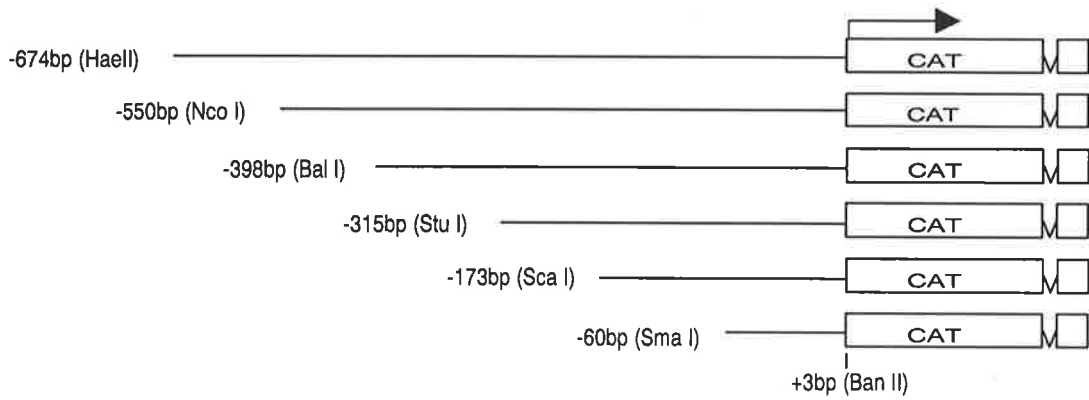
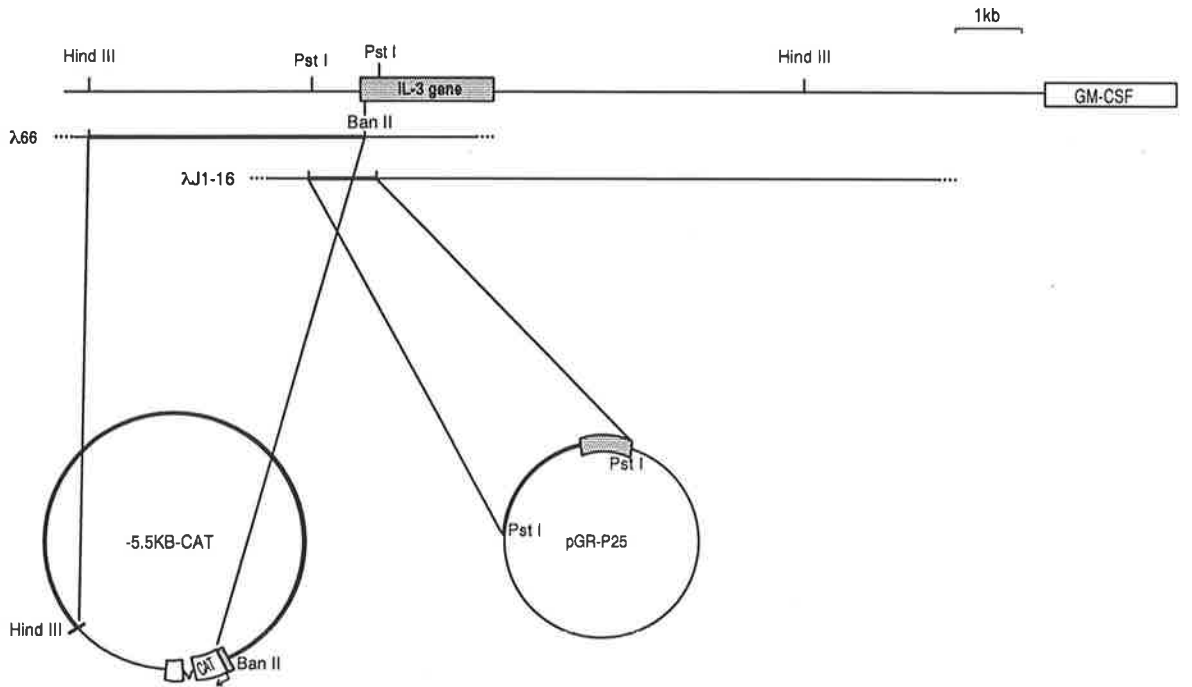
corresponded to transcription initiation at the same adenine residue detected with the PE-IL3(1) primer. In summary, two independent oligonucleotide primers identified a single initiation site for IL-3 gene transcription at an adenine residue 31 bp 3' to the TATA box and 52 bp 5' to the ATG initiation codon. IL-3 transcription initiation occurs in a position which is conserved between the human and murine IL-3 genes (12/13 bases) and corresponds to the position first proposed by Yang *et al.* (1988).

4.2.2 Construction of IL-3 promoter/reporter gene chimeric plasmids

To identify the regulatory regions within the IL-3 5' flanking DNA in transient transfection assays, chimeric plasmids containing the chloramphenicol acetyl transferase (CAT) reporter gene and a series of fragments from the IL-3 promoter were prepared. The promoterless eukaryotic CAT expression plasmid pBLCAT3 (Luckow and Schütz, 1987) was used as the vector for all of the promoter/reporter constructs. This plasmid also contains the SV40 virus small-t-antigen splice site and polyadenylation signal located 3' to the CAT gene for efficient processing and termination of eukaryotic transcription. IL-3 promoter sequences for these constructs were isolated from the genomic λ clones λ 66 and λ J1-16 (gift, Dr. S. Clark). The 3' end of all of the promoter constructs was defined by a BanII restriction site which occurs immediately 3' (+3bp) to the CAP site in the IL-3 mRNA 5' untranslated region. The removal of the 3' protruding end of this restriction site during the cloning procedure leaves the IL-3 transcriptional start site intact. The longest promoter/reporter construct was assembled by cloning the 5.5kb HindIII/BanII fragment of λ clone 66 into the HindIII and blunted SalI sites of pBLCAT3, thereby generating a reporter construct with the complete 5.5kb of 5' flanking sequences from the IL-3 gene including the natural IL-3 transcription initiation site (Figure 4.2). IL-3 promoter sequences were further subcloned by first isolating, from λ J1-16, a 926 bp PstI fragment containing a portion of the first exon, the IL-3 transcription start site and 686 bp of 5' flanking DNA. This fragment was cloned into the PstI site of plasmid pUC18 and designated pGR-P25 (Figure 4.2). Six unique restriction sites within the

Figure 4.2 Construction of IL-3 promoter/reporter plasmids.

The diagram shows the details of construction of reporter plasmids containing the chloramphenicol acetyl transferase (CAT) reporter gene. The top line shows the genomic arrangement of the IL-3 (shaded box) and GM-CSF genes. IL-3 promoter DNA fragments for the reporter constructs (bold lines), were isolated by enzyme digestion from the genomic clones λ 66 or λ J1-16 (gift Dr. S. Clark) as shown in the upper part of the diagram. The longest promoter/reporter construct (-5.5KB-CAT) was assembled by cloning the 5.5kb Hind III/Ban II fragment of λ 66 into the Hind III and blunted Sal I sites of pBLCAT3. IL-3 promoter sequences were further subcloned by first isolating from λ J1-16, a 926bp Pst I fragment containing a portion of the first exon, the IL-3 transcription start site and 686bp of 5' flanking DNA. This fragment was cloned into the Pst I site of plasmid pUC18 and designated pGR-P25. Six unique restriction sites within the first 680bp of the IL-3 5' flanking sequences define the 5' ends of promoter fragments used in these reporter constructs. The extent (bp) of each promoter fragment is indicated together with the restriction site used. All of the promoter fragments used have a common 3' end defined by a Ban II site at +3bp with respect to the IL-3 promoter transcription initiation site.



first 680 bp of the IL-3 5' flanking sequences, were chosen for the construction of the reporter plasmids to define the 5' ends of the promoter fragments at intervals of approximately 100 bp in positions where the previously defined conserved sequence elements were not disrupted. Each promoter construct has a common 3' end defined by the unique BanII restriction site at position +3 bp of the IL-3 mRNA. All of these fragments were made blunt ended by treatment with *E. coli* polymerase and each was cloned into the blunted SalI site, upstream of the CAT gene, in the promoterless expression vector pBLCAT3 (Luckow and Schütz, 1987) (Figure 4.2). The orientation, size and correct insertion of the 3' and 5' ends of the IL-3 promoter fragments into the reporter plasmid constructs was verified by restriction enzyme digestion and double-stranded di-deoxy sequencing reactions (section 2.2.8).

4.2.3 Transfection of Jurkat cells by electroporation

Experiments were carried out to optimise the electroporation parameters for optimal transfection of Jurkat cells. For these experiments Jurkat cells were transfected with a plasmid expression vector derived from the pSV series, pSV2CAT (Subramani and Southern, 1983). This construct contains the SV40 viral early promoter/enhancer linked to the CAT reporter gene, small-t-antigen splice site and early gene poly adenylation signal. Efficient expression of this plasmid in transient expression assays has been reported in a wide variety of cell types (Subramani and Southern, 1983, Sleight and Lockett, 1985). A series of different voltage and capacitance combinations were tested to determine the electroporation conditions that resulted in the maximum expression of the CAT reporter gene. Cell extracts were prepared by repeated freeze/thaw cycles and assayed for CAT activity as described by Gorman *et al.* (1982) (section 2.5.7). Peak CAT activity was consistently measured in extracts from cells electroporated at 270V and 960 μ F and these voltage and capacitance settings were used for all subsequent electroporation experiments (results not shown). To ensure the linearity of CAT assays, increasing amounts of purified *E.coli* CAT enzyme were added to Jurkat cell extracts in a standard two hour CAT assay. The detection of CAT

assay. The detection of CAT activity in 25 μ g aliquots of protein extract was linear up to a level of 60% conversion of chloramphenicol to the acetylated forms (results not shown). The amount of protein extract subsequently used in CAT assays was adjusted, and reassayed if necessary, to achieve CAT activity within this linear range of the assay. To optimise the protein recovery from cell extracts the cell numbers were increased for 1×10^6 up to 4×10^6 cells per electroporation reaction, which increased total protein yield although it had no effect on the specific activity of the CAT enzyme in the cell extracts. In addition to the increased cell numbers, an increase in the FCS concentration of the electroporation buffer from 10% to a final concentration of 20% improved cell survival and increased recovery of protein. The protein yield (50-200 μ g) from electroporation experiments under these conditions was sufficient for the routine measurement of CAT activity in 20 μ g samples of protein extract from transfected cells.

Initial transfection experiments with the pSV2CAT plasmid demonstrated unpredictable experimental variation between transfection experiments. The main differences between transfection experiments were in the levels of induction between independent experiments. The quality of DNA preparation and recent cell culture history appeared to be the most important variables. To reduce variations, due to electroporation efficiency within a series of experiments where treatment with different reagents was planned for cells transfected with the same DNA construct, the transfected cells were pooled before stimulation and incubated overnight. The pooled transfectants were divided evenly, immediately prior to stimulation with reagents such as PHA and PMA.

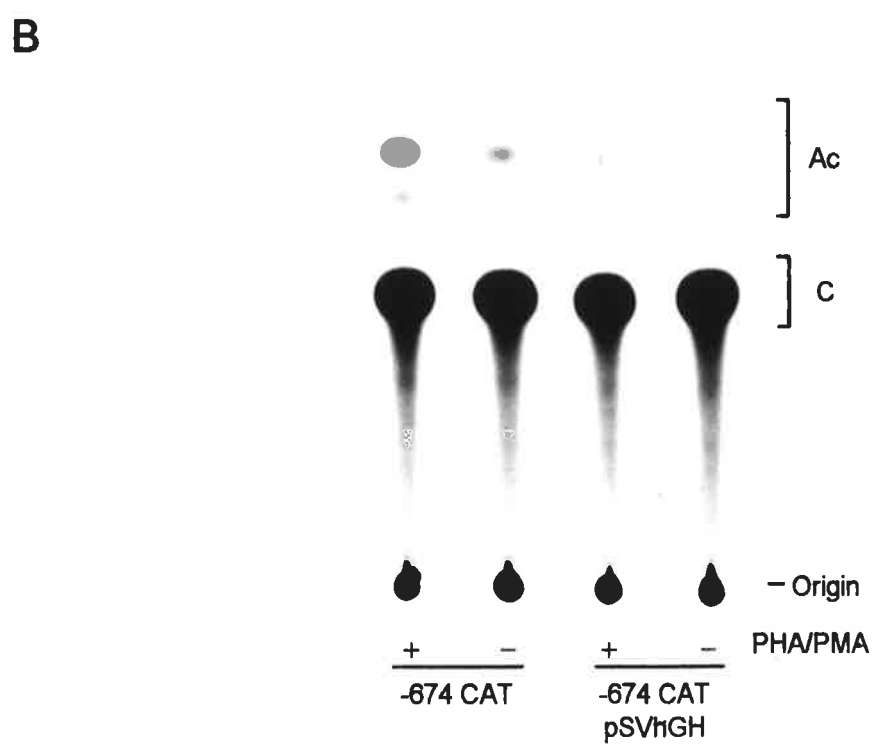
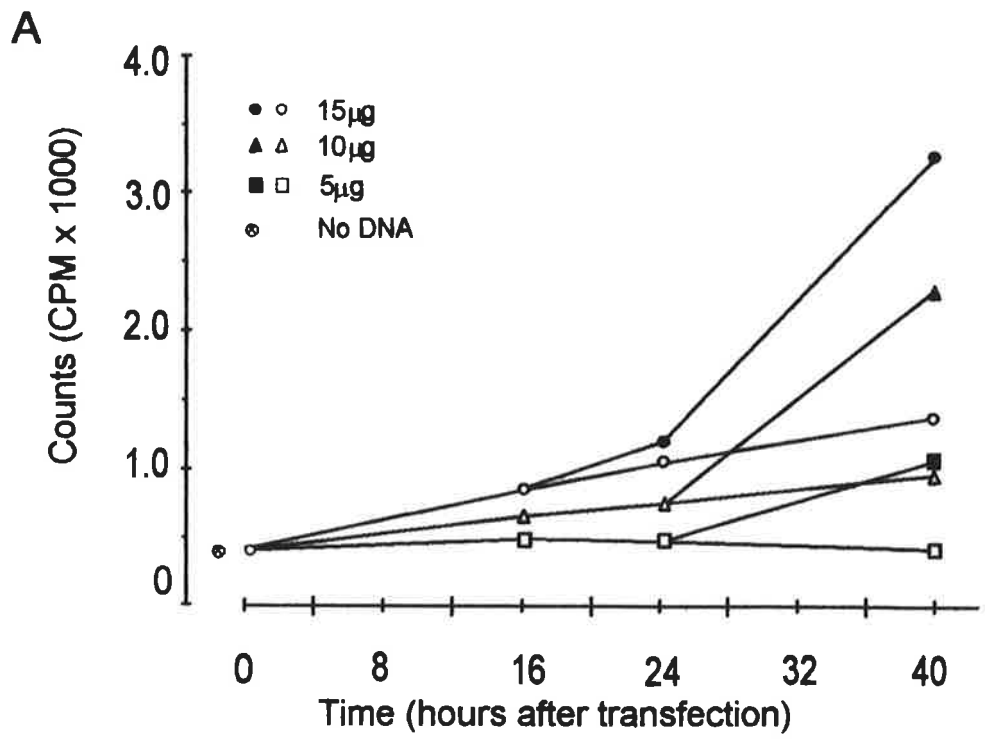
The use of internal control plasmids was also evaluated to enable a more accurate comparison of data both within experiments and between a series of independent transfection experiments. Two reporter genes which could be assayed independently of CAT were tested as internal controls in transfection experiments with the IL-3 promoter constructs. The first reporter gene, human growth hormone (hGH) produces

an extracellular protein and can be detected in transfected cell supernatants by a sensitive immuno-radiometric assay (section 2.5.5). An hGH expression plasmid was assembled by cloning the SV40 viral early promoter upstream of the hGH gene in the promoterless plasmid p0GH (gift of Dr. Brian May, University of Adelaide). The 342 bp HindIII/PvuII fragment of the SV40 promoter was cloned into the HindIII/XbaI sites of p0GH (SVhGH). Transfection of Jurkat cells with the SVhGH construct were carried out to determine both the efficiency of hGH expression in stimulated and unstimulated cells, and the sensitivity of the assay system for hGH detection. In these experiments increasing quantities, either 5, 10 or 15 μ g, of SVhGH construct were transfected into Jurkat cells. The hGH concentration in the transfected cell supernatants was measured with a two site immuno-radiometric assay (IRMA) and standardised with known concentrations of purified hGH (section 2.5.5). Low levels of hGH expression in unstimulated Jurkat cell supernatants were detected 24 hours after transfection and continued to increase until 40 hours. Stimulation of the transfected Jurkat cells induced, at each DNA concentration, an increase in hGH production measured at the 40 hour time point (Figure 4.3A). However the concentration of hGH could not be reliably quantitated as it was below the linear range (1.5-50 mU/l) of the assay in all supernatants except those from stimulated cells transfected with 10 or 15 μ g of SVhGH construct. In addition, co-transfection experiments were also conducted with 15 μ g of IL-3 -674 bp promoter construct and 10 μ g of the SVhGH construct. CAT enzyme activity was detected in cells co-transfected with both plasmids, however CAT activity driven by the IL-3 promoter was markedly decreased when compared to that in cells transfected with the -674 bp CAT construct alone (Figure 4.3B). IRMA assays did not detect the expression of significant levels of hGH in the co-transfected cell supernatants (results not shown). These data suggest that co-transfection of SVhGH may interfere with IL-3 promoter directed expression of the CAT gene and that transfection of lesser amounts of the

Figure 4.3 Expression of pSVhGH internal control plasmid in Jurkat cells

(A) The graph represents RIA measurements of human growth hormone in transfected cell supernatants. The results shown are an average of two independent transfection experiments both measured in triplicate. Jurkat cells were transfected with increasing amounts of pSVhGH construct from 5 to 15 μ g or mock transfected (\otimes , no DNA). Supernatant samples were assayed at 0, 16, 24 and 40 hours post transfection (\circ Δ \square , DNA amounts as indicated in the key). A second set of cells transfected in the same manner was stimulated at 16 hours post transfection with PHA/PMA and samples were taken at 24 and 40 hours (\bullet \blacktriangle \blacksquare).

(B) The panel shows an autoradiogram detecting CAT enzyme activity in extracts of Jurkat cells transfected with the -674-CAT IL-3 promoter construct alone (first two tracks) and of Jurkat cells co-transfected with 15 μ g of IL-3 -674bp promoter construct together with 10 μ g of the SVhGH construct. Transfected cells were either unstimulated (-) or stimulated with PHA/PMA (+). [14 C]-chloramphenicol (C) and acetylated chloramphenicol (Ac) were separated by TLC. The figure is representative of two independent experiments.



hGH construct results in levels of hGH expression insufficient for its use as an internal control plasmid.

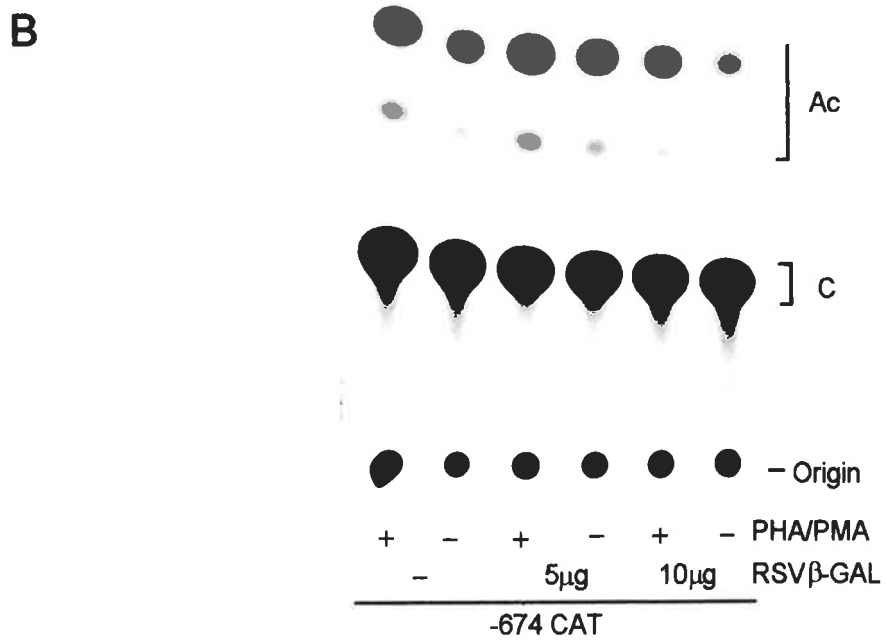
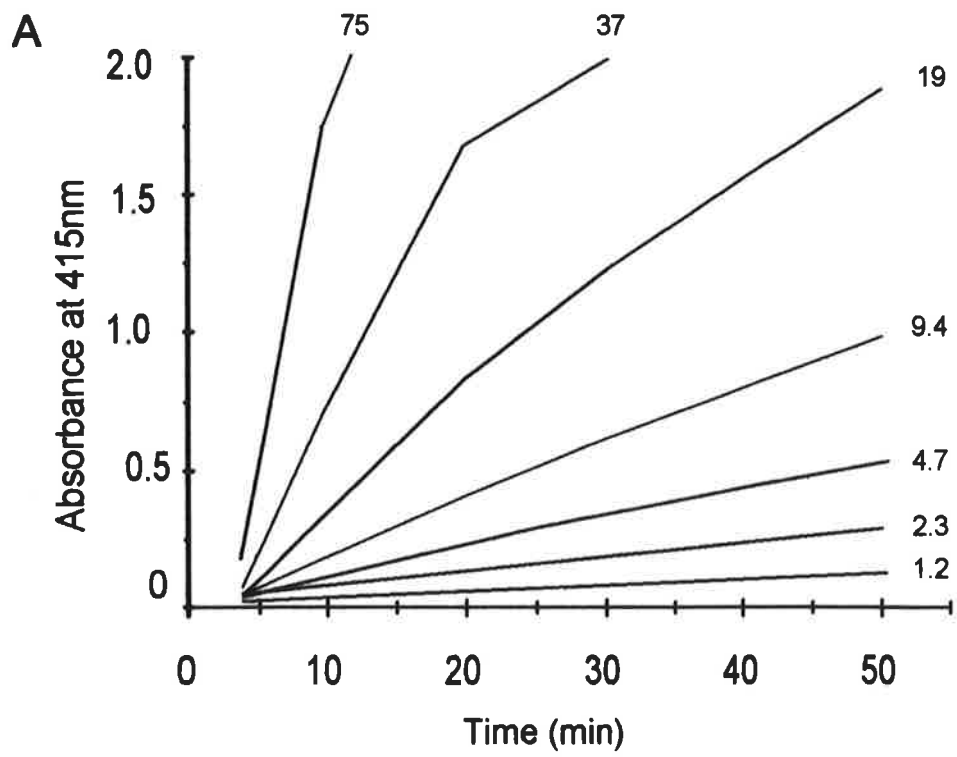
In view of these results a second internal control promoter/reporter plasmid was evaluated. Expression of β -galactosidase has been used widely as a reporter or internal control in co-transfection experiments. The plasmid pRSV β -GAL (gift of Dr. Kenneth Kaushansky, University of Washington WA, MacGregor *et al.*, 1987), which contains the viral LTR/promoter from the Rous sarcoma virus (RSV) linked to the *E.coli* β -galactosidase gene, was used for these experiments. β -galactosidase activity was measured in freeze/thaw cell extracts by a colorimetric assay as described by Sambrook *et al.*, (1989). Transfection of pRSV β -GAL in preliminary experiments established that the RSV LTR was a strong constitutive promoter in Jurkat cells and that β -galactosidase activity of approximately 60 to 100 μ U/ml of cell extract, could be detected in unstimulated Jurkat cells electroporated with 5 μ g of pRSV β -GAL. In addition, expression of pRSV β -GAL is inducible (up to approximately 50 fold) by PHA/PMA treatment of transfected cells. To facilitate the simultaneous measurement and comparison of β -galactosidase from a series of transfected cell extracts, the colorimetric assay was modified to develop a microscale assay suitable for the volume of a 96 well microtitre tray (section 2.5.6). The linearity and sensitivity of this assay was tested over a wide range of enzyme concentrations shown in Figure 4.4A. The results from these experiments show that the assay is linear over the range of 0.1 to 1.0 absorbance units and can accurately detect 1 μ U of β -galactosidase activity in cell extracts at 24°C in a 50 minute assay. Co-transfection of 5 μ g of pRSV β -GAL with the -674 bp IL-3 reporter construct (10 μ g), had no effect on the expression of the CAT gene from the 674 bp IL-3 construct, however, an increase to 10 μ g of pRSV β -GAL reduced the CAT reporter gene levels (Figure 4.4B).

The induction of β -galactosidase levels in stimulated cells complicates the use of β -galactosidase as an internal control in transfection experiments which compare the CAT expression of the IL-3 promoter constructs under several different stimulation

Figure 4.4 Optimisation of β -galactosidase enzyme assay and internal control transfections

(A) The graph represents measurements of linearity and sensitivity of the modified β -galactosidase assay. β -galactosidase was serially diluted over a range of enzyme activities from 75 μ U to 1.2 μ U as shown to the right of each line. The increase in absorbance at 415nm plotted against time for each reaction is shown. The absorbance values were read automatically at 5 minute intervals and β -galactosidase enzyme activity was calculated from the kinetic data by computer (BioRad). The assay is linear between 0.1 and 1.0 absorbance units.

(B) Jurkat cells were co-transfected with either 0 (-), 5 μ g or 10 μ g of pRSV β -GAL and 10 μ g of the -674 bp IL-3 promoter/CAT reporter construct. Each group of transfected cells was split evenly and then either stimulated with PHA/PMA for 24 hours (+) or left untreated (-). CAT assays were performed on freeze/thaw extracts from the cells. Acetylated (Ac) and non-acetylated chloramphenicol (C) were separated by thin layer chromatography (TLC) and detected by autoradiography.



conditions. To overcome this difficulty one CAT construct (the longest) was chosen as a standard, β -galactosidase assays were conducted with extracts from cells transfected with this standard construct under each stimulation condition. The β -galactosidase levels from each stimulation condition of the standard construct were used as the internal standard level for the other similarly stimulated transfectants.

In summary, constitutive and inducible expression of pRSV β -GAL can be detected in Jurkat cells at a level which provides a suitable internal control for transfection experiments with IL-3 reporter constructs. All subsequent co-transfection experiments were carried out with 10 μ g of IL-3 reporter construct DNA and 5 μ g of the internal control plasmid, pRSV β -GAL. β -galactosidase enzyme activity was calculated by computer from kinetic data within the linear range (0.1 and 1.0) of absorbance values. The β -galactosidase values were then used to normalise the results of subsequent CAT assays.

4.2.4 Expression of IL-3 promoter/CAT constructs in transiently transfected Jurkat cells

To identify the regions of the IL-3 promoter required for transcriptional activation, Jurkat cells were co-transfected with the IL-3 promoter constructs together with the pRSV β -GAL internal control plasmid. CAT activity was measured and normalised for transfection efficiency using the β -galactosidase activity as described above. Jurkat cells transfected with 10 μ g of the 5.5kb IL-3 promoter/reporter construct showed a low level of constitutive CAT enzyme expression (approximately 0.5% conversion) in unstimulated cells (Figure 4.5A). Stimulation of the transfected cells with PHA/PMA increased the CAT activity by an average of approximately 1.9 fold in three independent experiments. Similar levels of CAT activity were obtained by stimulation with PMA in combination with the ionophore A23187 (data not shown). The removal of 5' flanking sequences from the upstream Hind III site at -5.2kb to the Hae II site at position -674 increased the fold induction over the basal level of CAT expression in

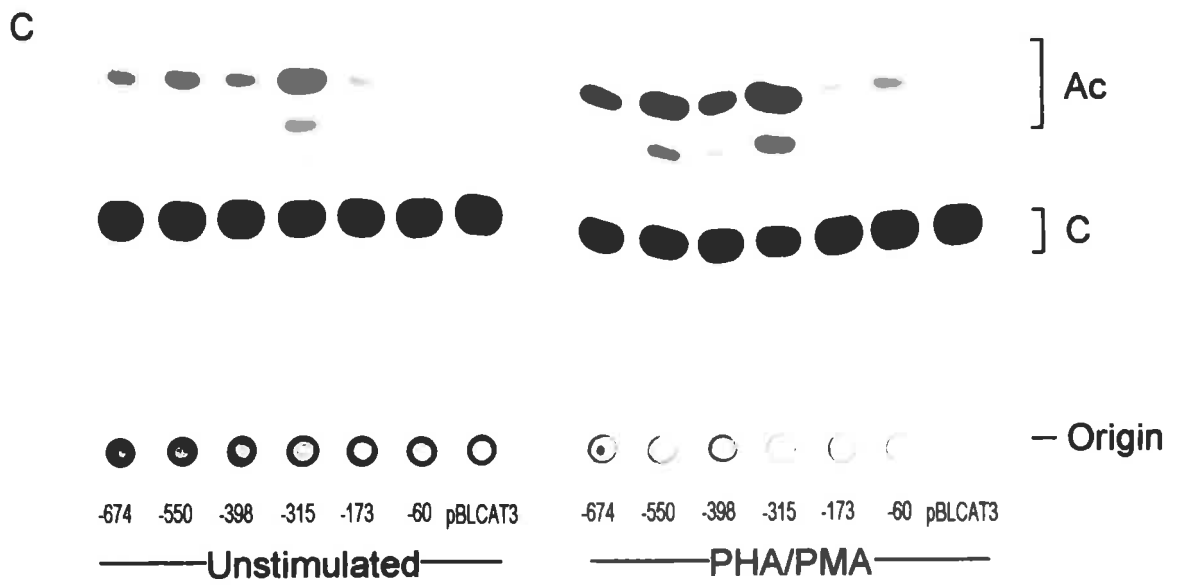
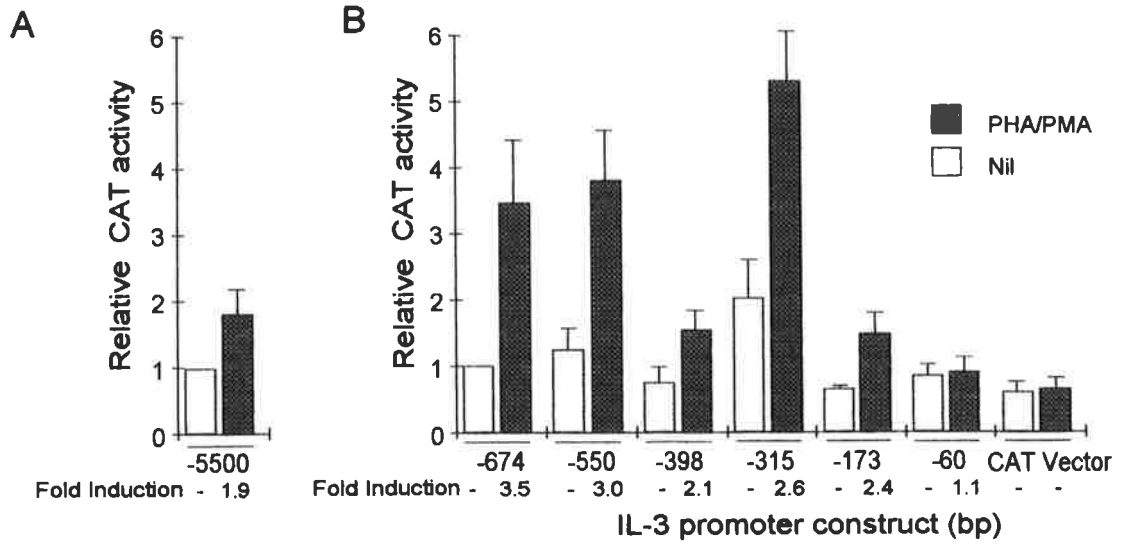
Figure 4.5 Expression of IL-3 promoter constructs in transfected Jurkat cells

Jurkat cells were transfected with deletion constructs of the IL-3 5' flanking region cloned upstream of the CAT reporter gene in promoterless CAT expression plasmid pBLCAT3. (Figure 4.2). The CAT activity was determined as the percentage conversion of chloramphenicol to the acetyl forms and was quantitated by liquid scintillation counting of bands isolated from the TLC plate and by phosphorimage analysis.

(A) The CAT activity detected in PHA/PMA stimulated cells that were transfected with the 5.5kb IL-3 promoter construct (10 μ g) is expressed relative to the level of constitutive expression set at a value 1 (approximately 0.56% CAT conversion) of this promoter/reporter construct. The fold induction of CAT activity over the unstimulated level is shown below the column representing stimulated cells. The values represent the mean and standard error of 3 independent experiments.

(B) Jurkat cells were co-transfected by electroporation with IL-3 promoter constructs (10 μ g) and the internal control expression plasmid pRSV β -GAL (5 μ g). The transfected cells were then either stimulated (PHA/PMA) or left unstimulated (Nil). The constructs used are designated according to the length of upstream promoter sequence. The protein concentrations used in the CAT assays were normalised by the β -galactosidase activity in each sample. CAT activity is expressed relative to the value of the -674 construct which is set at 1 (average CAT conversion for this construct was 0.86%). The values represent the mean and standard errors of 7 independent transfections. The fold induction of CAT activity over the unstimulated level of each construct is shown below the column representing stimulated cells.

(C) An autoradiogram of one representative transfection experiment incorporating the IL-3 promoter/reporter constructs. The transfected cells were either stimulated with PHA/PMA or unstimulated as shown. The constructs used are designated according to the length of upstream promoter. The autoradiogram shows CAT conversion of [¹⁴C]-chloramphenicol (C) to the acetyl forms (Ac) which have been separated by TLC.



PHA/PMA stimulated cells from 1.9 to approximately 3.5 fold (Figure 4.5B and C). This increased CAT induction suggests that essential promoter elements required for the inducible expression of the IL-3 promoter must be contained within this proximal portion of the IL-3 promoter and that negative regulatory elements may be present in the more distal regions of the IL-3 promoter. No significant change was observed in either the basal level CAT expression or the level detected after PHA/PMA stimulation when sequences between -674 and -550 bp were deleted. However, transfection of the -398 bp promoter construct resulted in significantly lower CAT activity levels in unstimulated and stimulated cells than those obtained with the longer constructs, although the ratio of induction for this construct in stimulated cells was approximately 2 fold. These results imply that a constitutive activating element exists between -550 bp and -398 bp in the IL-3 promoter. Further truncation of the promoter sequences to -315 bp increased the expression of both basal and induced levels of CAT activity suggesting that a strong constitutive transcriptional silencer is present between -398 bp and -315 bp. The IL-3 promoter construct deleted to -315 bp expressed the highest absolute levels of both basal and inducible CAT activity, however this construct yielded approximately the same level of induction over its unstimulated level (2.6 fold). Positive regulatory elements within the -315 bp promoter fragment must lie between the positions -315 bp and -173 bp, since truncation of the IL-3 promoter to -173 bp substantially decreased the overall expression of the CAT reporter gene. Although the absolute levels of CAT activity were lower, PHA/PMA stimulation of Jurkat cells transfected with the -173 bp construct induced CAT expression by approximately 2.4 fold suggesting that PHA/PMA responsive element(s) still exist downstream of -173 bp. Removal of these elements by truncation of the IL-3 promoter to position -60 bp deleted all the essential promoter elements required for the induction of IL-3 expression in Jurkat cells. The level of CAT reporter gene activity obtained with this promoter construct was only

slightly greater than that of the promoterless vector alone and CAT activity did not increase on PHA/PMA stimulation.

Stimulation of the Jurkat cells with PHA alone, which mimics activation of the TcR, also induced transcription of the transfected promoter constructs. In a series of three experiments, the IL-3 promoter constructs, stimulated by PHA alone, displayed a similar pattern of CAT gene expression except that the overall levels of CAT enzyme and the fold induction of CAT activity over the constitutive levels was notably reduced (Figure 4.6A) for all but the -550 bp and -173 bp constructs.

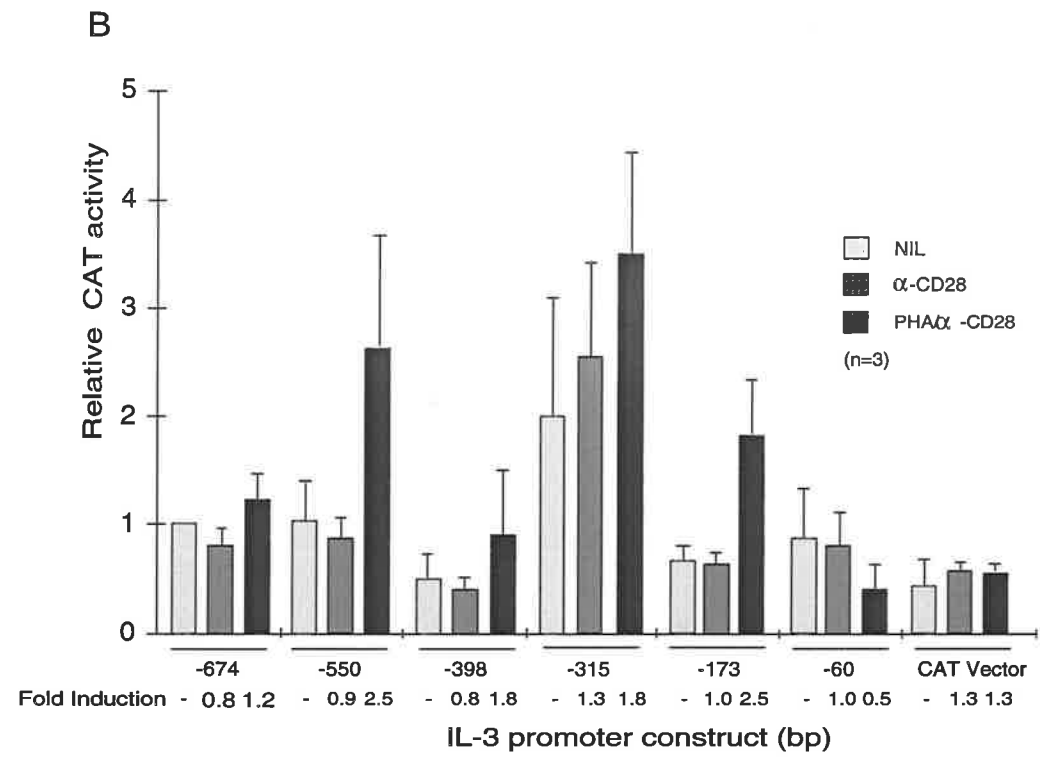
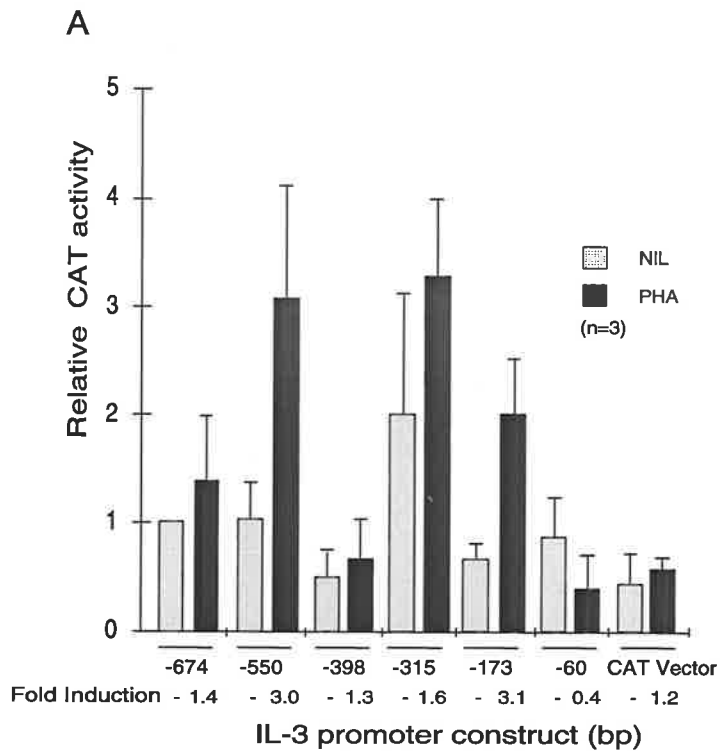
Evidence has also shown that a number of additional T-cell surface receptors serve to regulate the responses of antigen activated T-cells (reviewed by Weaver and Unanue, 1990). One such accessory molecule, CD28, initiates or regulates a signal transduction pathway distinct from those of the TcR pathway (reviewed by June *et al.*, 1990).

During the course of this work a CD28-responsive transcription regulatory element, CD28RE, was identified in the IL-2 promoter (Verweij Geerts and Aarden, 1991, Fraser *et al.*, 1991 and Civil *et al.*, 1992). The sequence of this element is similar to the conserved cytokine (CK1) regions found in IL-3, GM-CSF and in other cytokine gene promoters (Fraser *et al.*, 1991). Moreover, in this study co-stimulation of Jurkat cells with PMA/PHA and the α -CD28 monoclonal antibody 9.3, enhanced the accumulation of IL-3 and other cytokine mRNAs over the levels obtained by stimulation with PHA and PMA (see Figure 3.4). To determine whether CD28 responsive elements are functional in the IL-3 promoter, transfection experiments with the IL-3 reporter constructs were carried out and the transfected cells were stimulated with the α -CD28 monoclonal antibody 9.3, alone or in combination with PHA or PHA/PMA. In three independent transfection experiments, where the cells were co-transfected with the IL-3 promoter constructs and the internal control plasmid pRSV β -GAL, stimulation of the transfected Jurkat cells with α -CD28 antibody alone did not induce an increase in CAT activity, compared to the unstimulated levels, from any of the truncated IL-3 promoter constructs (Figure 4.6B). Similarly, co-stimulation

Figure 4.6 Expression of IL-3 promoter constructs in PHA and α -CD28 treated Jurkat cells

(A) Jurkat cells were co-transfected by electroporation with IL-3 promoter constructs (10 μ g) and the internal control expression plasmid pRSV β -GAL (5 μ g). The transfected cells were then either stimulated (PHA) or left unstimulated (Nil). The constructs used are designated according to the length of upstream promoter sequence. The protein concentrations used in the CAT assays were normalised by the β -galactosidase activity in each sample. CAT activity is expressed relative to the value of the -674 construct which is set at 1. The CAT activity was quantitated by scintillation counting of bands excised from the TLC plates and represent the mean and standard errors of 3 independent transfections. The fold induction of CAT activity over the unstimulated level of each construct is shown below the column representing stimulated cells.

(B) In a series of transfection experiments conducted in parallel with those presented in (A), Jurkat cells were co-transfected by electroporation with IL-3 promoter constructs and the pRSV β -GAL internal control plasmid and stimulated with α -CD28 monoclonal antibody 9.3 alone (α -CD28), α -CD28 monoclonal in combination with PHA (PHA/ α -CD28) or left unstimulated (Nil). CAT activity is expressed relative to the value of the -674 construct which is set at 1. The values represent the mean and standard errors of 3 independent transfections. The fold induction of CAT activity over the unstimulated level for each construct is shown below the column representing stimulated cells.



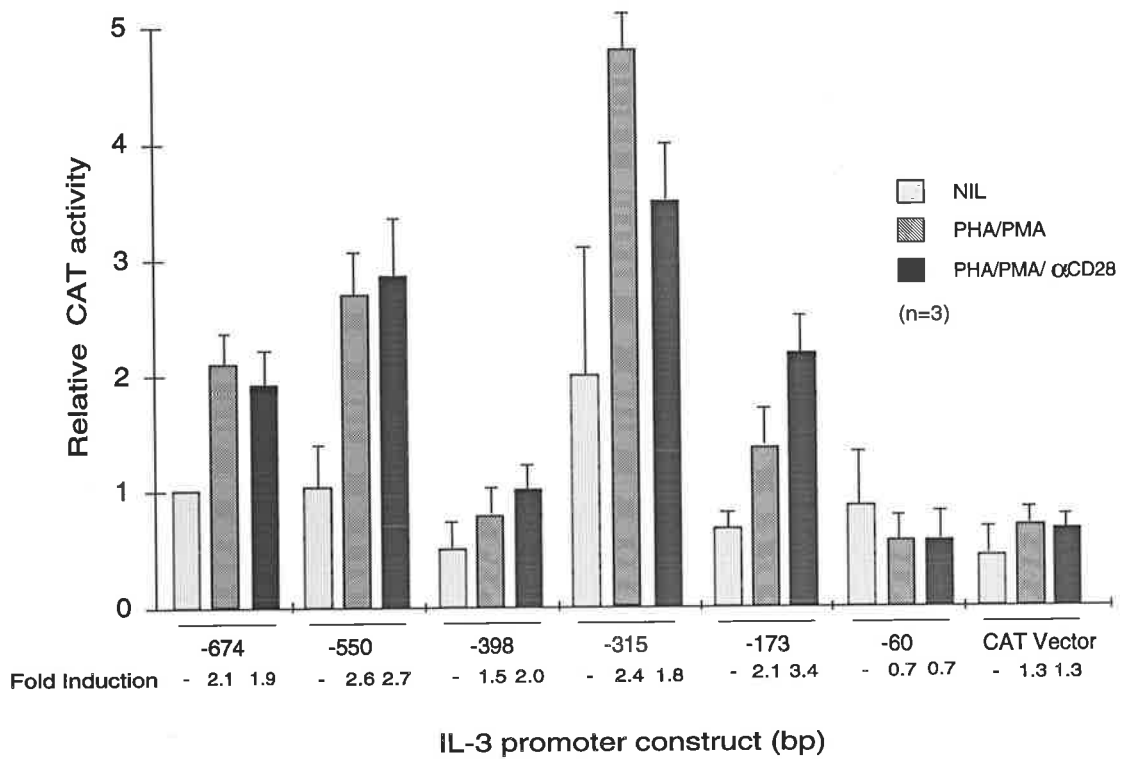
with α -CD28 and PHA did not alter the levels of CAT activity compared to those obtained by stimulation with PHA alone (compare Figures 4.6A and 4.6B). However, co-stimulation of transfected Jurkat cells with PHA/PMA and α -CD28, in three independent experiments, consistently increased (from 2.1 to 3.4 fold) the induction of CAT expression from the -173 bp promoter construct (Figure 4.7). It is intriguing, however, that no such increase was detected in transfection experiments with the longer promoter constructs. These results suggest that CD28 responsive elements may be present within the -173 bp region of the IL-3 promoter but that the response may be decreased by upstream sequences. As the conserved cytokine element CK1, found within the -173 bp fragment of the promoter, resembles the IL-2 CD28RE transfection experiments described below (section 4.2.7) were carried out to determine the ability of the CK1 element from the IL-3 promoter to enhance transcription of a heterologous promoter following α -CD28 treatment of transfected Jurkat cells .

4.2.5 Examination of transcriptional regulation by sequences within the IL-3 coding region

Measurements of the transcriptional induction of the IL-3 gene by nuclear run-on experiments showed that transcription is induced by approximately 10 fold following PHA/PMA stimulation (see Figure 3.8). The lower levels of transcriptional induction obtained with the constructs described above, led to the suggestion that there may be one or more regulatory regions 3' to the IL-3 coding region or within the intervening sequences. Computer analysis of the sequence of the intronic regions of the IL-3 gene reveal consensus recognition sites for several transcription factors (eg AP-1, SRE and PEA3) and conserved regions between the murine and human sequences in the second intron (Yang and Clark, 1988). To test the possibility that elements within the IL-3 coding and intronic sequences contribute to the transcriptional regulation of IL-3, a 1.6 kb fragment of the IL-3 gene from the SacI site at position +378 bp, at the start of the second intron to the Eco RI site in the fifth exon, was cloned downstream of the CAT gene in the -550 bp promoter/reporter construct. The addition of the 1.6kb

Figure 4.7 Expression of IL-3 promoter constructs in PHA/PMA and α -CD28 treated Jurkat cells

Jurkat cells were co-transfected by electroporation with IL-3 promoter constructs (10 μ g) and the internal control expression plasmid pRSV β -GAL (5 μ g). The transfected cells were then stimulated with either PHA/PMA, PHA/PMA and α -CD28 monoclonal antibody 9.3 (PHA/PMA/ α -CD28) or left unstimulated (Nil). The constructs used are designated according to the length of upstream promoter sequence. The protein concentrations used in the CAT assays were normalised by the β -galactosidase activity in each sample. CAT activity is expressed relative to the value of the -674 construct which is set at 1. CAT activity was quantitated by scintillation counting of bands excised from the TLC plates and represents the mean and standard errors of 3 independent transfections. The fold induction of CAT activity over the unstimulated level of each construct is shown below the columns representing stimulated cells.



fragment to the functional -550 bp promoter/reporter construct produced a small but consistent decrease in both the constitutive CAT expression and the level of CAT expression after PHA/PMA stimulation of transfected cells. However, there was no change in the average fold induction of CAT activity in stimulated transfectants (Figure 4.8). These data suggest that although consensus transcription factor recognition sites were identified by computer analysis within the IL-3 intron sequences, the fragment tested contains no functional PHA/PMA responsive transcriptional enhancer or regulatory regions that can influence expression from the IL-3 promoter at least in the transient expression system.

4.2.6 Examination of IL-3 promoter expression in fibroblasts.

The restricted nature of IL-3 expression in activated T-cells contrasts with that of many other cytokine genes. Transcription of the GM-CSF gene for example is found in a wider variety of cell types including T-cells, endothelial cells (Broudy *et al.*, 1986 and Kaushansky, 1989) and is constitutive in human lung fibroblasts and macrophages (Koeffler, Gasson and Tobler, 1988, and Thorens, Mermoud and Vassali, 1987). To determine if the proximal region of the IL-3 promoter is functional in non-T-cells and to attempt to identify regions of the IL-3 promoter that may be involved in general transcriptional regulation or in tissue restricted expression, human embryonic lung (HEL) fibroblasts were transfected with the IL-3 promoter constructs. Adherent HEL fibroblast cells were transfected by a DEAE-dextran method which had previously been optimised for this cell line in this laboratory (Shannon *et al.*, 1990) (section 2.5.3). The IL-3 reporter constructs were co-transfected together with the internal control plasmid pRSV β -GAL. These experiments used, as a positive control for cell stimulation, an Ig κ -TK-CAT clone which is expressed to high levels in TNF- α or PMA treated HEL fibroblasts. This plasmid contains five copies of a 26 bp sequence spanning the NF- κ B site from the murine kappa immunoglobulin gene (Dunn *et al.*, 1990). CAT expression was not detected from HEL cells transfected with any of the IL-3 promoter constructs (Figure 4.9), although the transfection efficiency was

Figure 4.8 Construction and transfection of IL-3 promoter/intron CAT reporter plasmids

(A) The diagram shows the construction of a reporter plasmid containing sequences from the coding region and introns of the IL-3 gene. The organisation of the intron-exon structure of the IL-3 gene is depicted to the right of the panel. Exons are numbered (roman numerals) and the 3' untranslated region is shown as a solid box. To construct this reporter plasmid a 1.6kb fragment of the IL-3 gene from the Sac I site at position +378bp, at the start of the second intron to the Eco RI site in the fifth exon, was cloned downstream of the CAT gene of the -550 bp promoter construct. The construct was designated p-550CAT-I.

(B) Jurkat cells were co-transfected with either the -550CAT, -550CAT-I or the promoterless vector pBLCAT3 and the internal control plasmid pRSV β -GAL. The transfected cells were stimulated with PHA/PMA or untreated (NIL). The protein concentrations used in the CAT assays were normalised by the internal control β -galactosidase activity in each sample. CAT activity is expressed relative to the value of the unstimulated -550CAT construct which is set at 1. CAT activity was quantitated by scintillation counting of bands excised from the TLC plates and represent the mean of two independent transfections. The fold induction of CAT activity over the unstimulated level of each construct is shown below the columns representing stimulated cells.

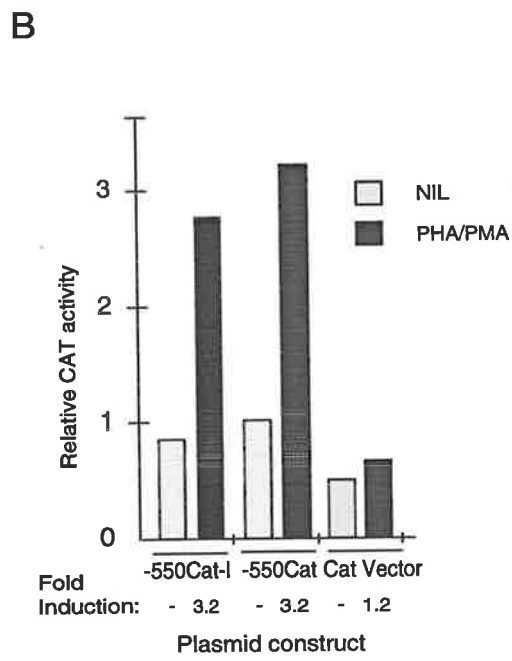
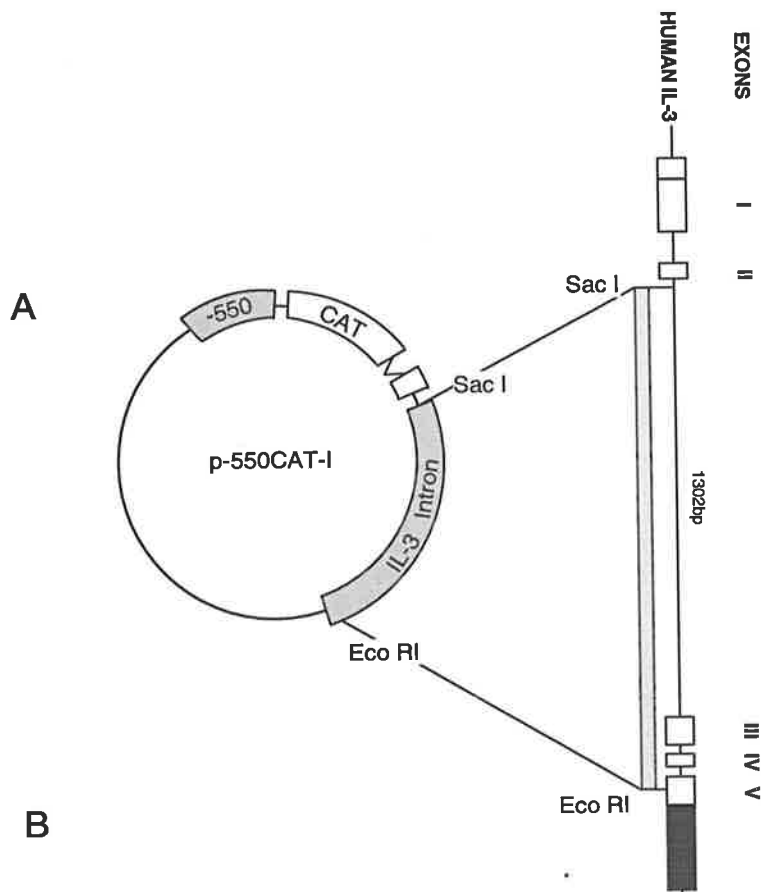
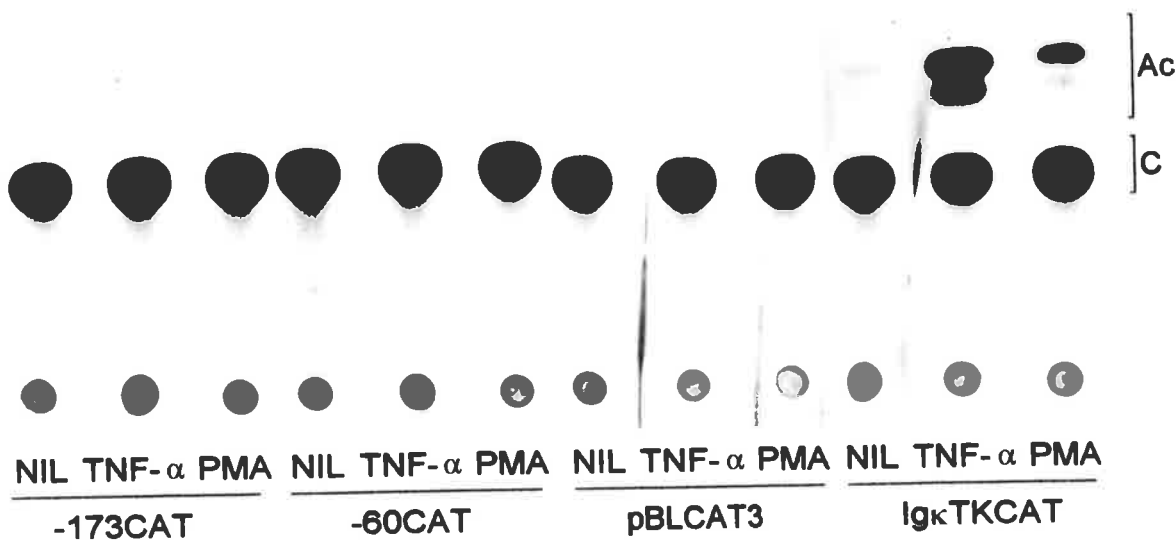
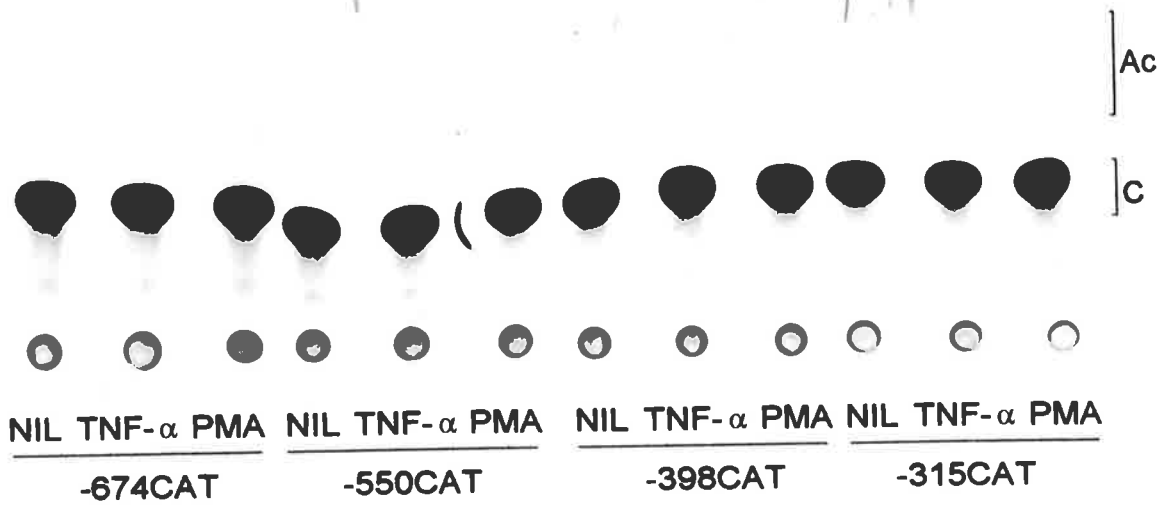


Figure 4.9 Transfection of HEL fibroblasts with IL-3 promoter CAT reporter constructs

Human embryonic lung fibroblasts (HEL) were transfected by the DEAE dextran method with the deletion constructs of the IL-3 5' flanking region and the internal control expression plasmid pRSV β -GAL. An Ig κ -TKCAT clone was included as a positive control for cell stimulation. This plasmid contains five copies of a 26bp sequence spanning the NF- κ B site from the murine kappa immunoglobulin gene (see figure 4.10). The transfected cells were stimulated after 16 hours incubation with PMA (10ng/ml) or TNF- α (100 U/ml) or left unstimulated (Nil). Freeze/thaw protein extracts were recovered at 40 hours after transfection. The constructs used are designated according to the length of upstream promoter sequence. The protein concentrations used in the CAT assays were normalised by the β -galactosidase activity in each sample. The autoradiogram shows CAT conversion of [14 C]-chloramphenicol (C) to the acetyl forms (Ac) which have been separated by TLC.



adequate since internal control β -galactosidase enzyme activity was measured and CAT expression from the Ig κ -TK-CAT positive control was easily detectable in TNF- α and PMA stimulated cells. To eliminate any possible interference in the expression of the IL-3 promoter constructs by the internal control plasmid, the transfection experiments were repeated in HEL cells in the absence of the internal control plasmid and similarly no CAT activity, above the background level of the vector pBLCAT3, was detected in extracts from these cells (data not shown).

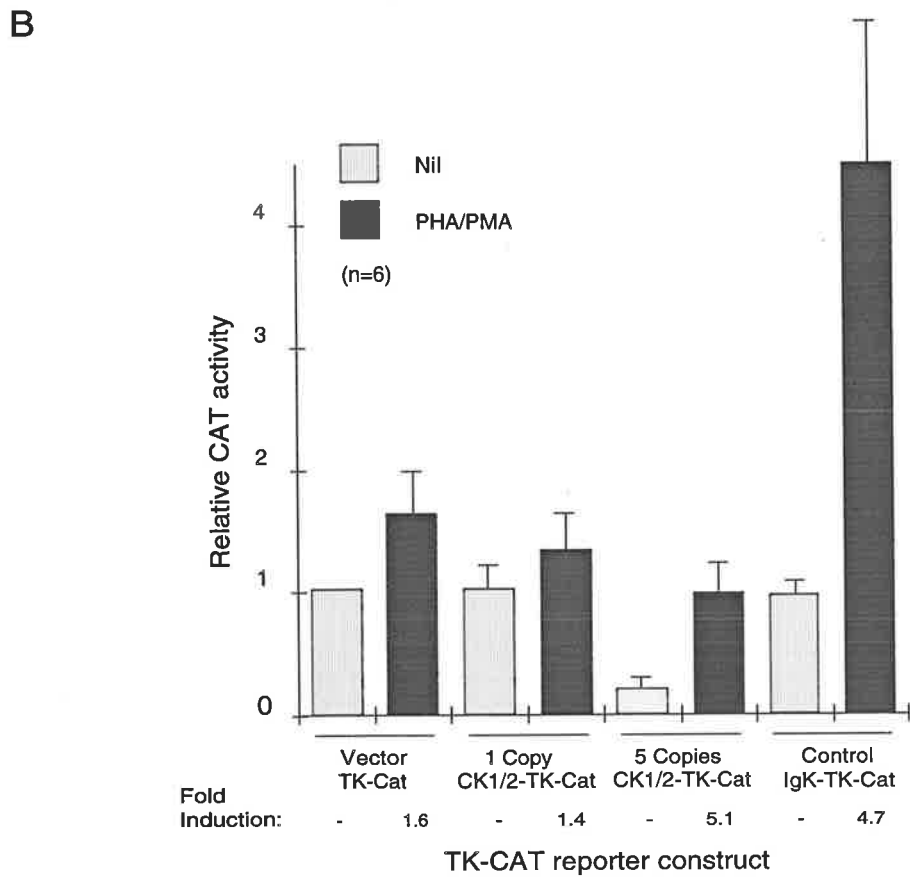
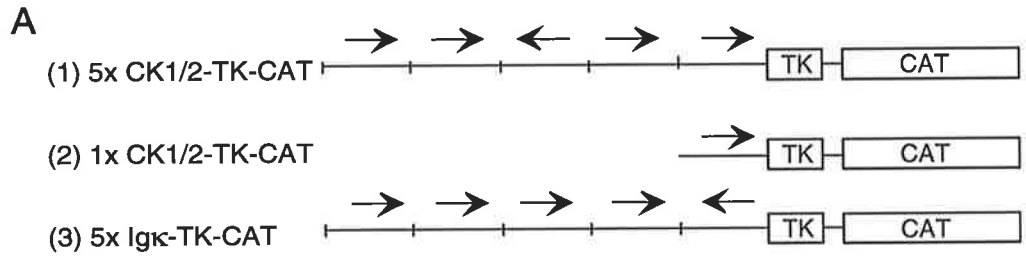
4.2.7 Analysis of the transcriptional function of the IL-3 promoter region containing the cytokine sequences CK1 and CK2 in Jurkat cells and HEL fibroblasts

The presence of the conserved elements, CK-1 and CK-2, in the proximal promoter regions of IL-3 and several other cytokine genes has led to the suggestion that these elements may contribute to the similarity in the pattern of cytokine gene expression (Shannon *et al.* 1990). Although the function of these elements in T-lymphocytes has not yet been defined, Shannon *et al.* (1990) have shown that the CK1 element can function in transfected lung fibroblasts as an IL-1 or TNF- α responsive element in the context of the G-CSF promoter and confer TNF- α and IL-1 responsiveness on an heterologous thymidine kinase (TK) promoter. In addition, Fraser *et al.* (1991) and Verweij *et al.* (1991) showed that an element similar in sequence to CK1 activated IL-2 expression in response to crosslinking of the CD28 T-cell surface receptor. The conserved CK1 and CK2 sequences lie between -173 bp and -60 bp of the IL-3 promoter and could contribute to the function, observed above, for this region. To determine the function of the IL-3 CK1/CK2 sequences, oligonucleotides spanning the region -142 bp to -102 bp (CK-IL-3(1-2), see section 2.1.8) were synthesised and cloned into the blunted Sall cloning site 5' to the TK promoter in the expression vector pBLCAT2 (pTK-CAT, Luckow and Schütz, 1987). TK-CAT clones that contained either one or five tandem copies of the IL-3 CK1/CK2 oligonucleotides (Figure 4.10A) were used in co-transfection experiments with pRSV β -gal in both

Figure 4.10 Expression of IL-3 CK1/CK2 thymidine kinase promoter constructs in Jurkat cells

(A) The diagram shows the orientation and number of cloned CK1/CK2 oligonucleotides inserted 5' to the thymidine kinase promoter of the CAT reporter plasmid pBLCAT2 (TK-CAT) and an I κ B-TKCAT reporter plasmid that was included as a positive control for cell stimulation. This plasmid contains five copies of a 26bp sequence spanning the NF- κ B site from the murine kappa immunoglobulin gene cloned upstream of the TK promoter of pBLCAT2. The number and arrangement of the oligonucleotides was determined by restriction analysis and di-deoxy sequencing reactions (sections 2.1.8 and 4.2.7).

(B) The CK1/CK2 oligomer constructs and controls in (A) were co-transfected into Jurkat T cells by electroporation with the internal control plasmid pRSV β -GAL. The cells were either stimulated (PHA/PMA) or left unstimulated (Nil). Protein concentrations used in the CAT assays were normalised by the β -galactosidase activity in each sample. CAT activity is expressed relative to the value of the unstimulated vector TK-CAT construct which is set at 1. The values represent the mean and standard error of six independent experiments. The fold induction of CAT activity over the unstimulated level of each construct is shown below the columns representing stimulated cells.



Jurkat cells and human embryonic lung fibroblast (HEL) cells. The plasmid pIg κ -TK-CAT which contains five copies of the NF- κ B site from the immunoglobulin kappa gene and is inducible in both these cell types was used as a positive control (2 μ g/transfection).

The results of a series of six Jurkat cell transfection experiments with each TK-CAT construct, either unstimulated or stimulated with PHA/PMA, are shown in Figure 4.10. Both constitutive (approximately 5% conversion) and inducible CAT activity was detected in Jurkat cells transfected with the TK-CAT expression plasmid pBLCAT2 (Figure 410B). A small decrease in the fold induction of CAT expression from the TK-CAT construct containing a single copy of the IL-3 CK1/CK2 region was consistently observed in transfected cells treated with PHA/PMA (Figure 410B). Interestingly, the introduction of five copies of the CK1/CK2 region in front of the TK promoter markedly decreased the constitutive expression from the TK promoter by a factor of five fold (Figure 410B). Stimulation by PHA/PMA of cells transfected with this construct restored the levels of CAT activity to that of the unstimulated TK-CAT construct (an approximately five fold activation) (Figure 410B). High levels of PHA/PMA induced CAT expression from the Ig κ -TK-CAT positive control plasmid were detected as expected (Figure 410B). These data suggest that a constitutive transcriptional silencer is present within the region -142 bp to -102 bp containing the conserved CK1/CK2 elements of the IL-3 promoter. However, activation of the -173 bp fragment of the IL-3 promoter with PHA/PMA overcomes the inhibitory functions of this element. The results also suggest that the inhibitory function of the CK1/CK2 region in the context of an heterologous promoter may require the interaction of more than one copy of the sequence elements. Interestingly, in the IL-3 promoter a second copy of the CK1 sequence is found further upstream, in the negative regulatory region we have defined between -315 bp and -398bp.

To determine if the IL-3 CK-1 element was responsive to CD28 stimulation, Jurkat cells transfected with the TK-CAT constructs were stimulated with α -CD28 antibody

alone or combined with PHA or PHA/PMA (Figure 4.11). Stimulation of transfected cells with α -CD28 alone did not change the level of CAT gene expression obtained from any of the TK-CAT plasmids in unstimulated cells (Figure 4.11A). Similarly, the addition of α -CD28 treatment to cells stimulated with PHA (Figure 4.11A) or PHA/PMA (Figure 4.11B) did not affect the level of CAT activity. PHA treatment by itself was able to only partially overcome the suppression of the TK-CAT reporter by multiple copies of the CK1/CK2 elements. (compare PHA and PHA/PMA stimulated transfectants in Figures 4.11A and B)

In summary, these experiments show that although the IL-3 promoter region containing the conserved cytokine elements bears some sequence similarity to the IL-2 CD28RE, this region did not respond under the conditions used here to binding of CD28 by soluble monoclonal antibody alone or in combination with PHA or PHA/PMA. Suppression of CAT expression from the TK promoter by the IL-3 CK1/CK2 region is relieved, in part by stimulation of the T-cell receptor signalling pathway by PHA and to a greater extent by the additional activation of PKC dependent signalling pathways by PMA treatment.

Given the conservation of the CK1 cytokine element in the IL-3, GM-CSF and G-CSF promoter, it was of interest to compare the function of the IL-3 cytokine region in cell types, such as fibroblasts, that express both GM- and G-CSF but do not express the IL-3 gene. Kuczek *et al.*, (1991) have shown that both GM- and G-CSF are induced by TNF- α and IL-1 treatment of fibroblast cells and that the CK1 sequence in the context of the G-CSF promoter acts as a TNF- α and IL-1 response element but that the CK1 sequence from the GM-CSF gene is not transcriptionally active. To examine the function of the IL-3 cytokine region the IL-3 TK-CAT constructs described above were transfected into HEL fibroblasts by a DEAE-dextran method. The transfected HEL cells were stimulated with either TNF- α or PMA at concentrations (100U/ml or 20ng/ml respectively) that were sufficient to induce the expression of G-CSF and GM-CSF mRNAs in these cells (Shannon *et al.*, 1992). The TK promoter-driven

Figure 4.11 Expression of IL-3 CK1/CK2 TK promoter constructs in Jurkat cells

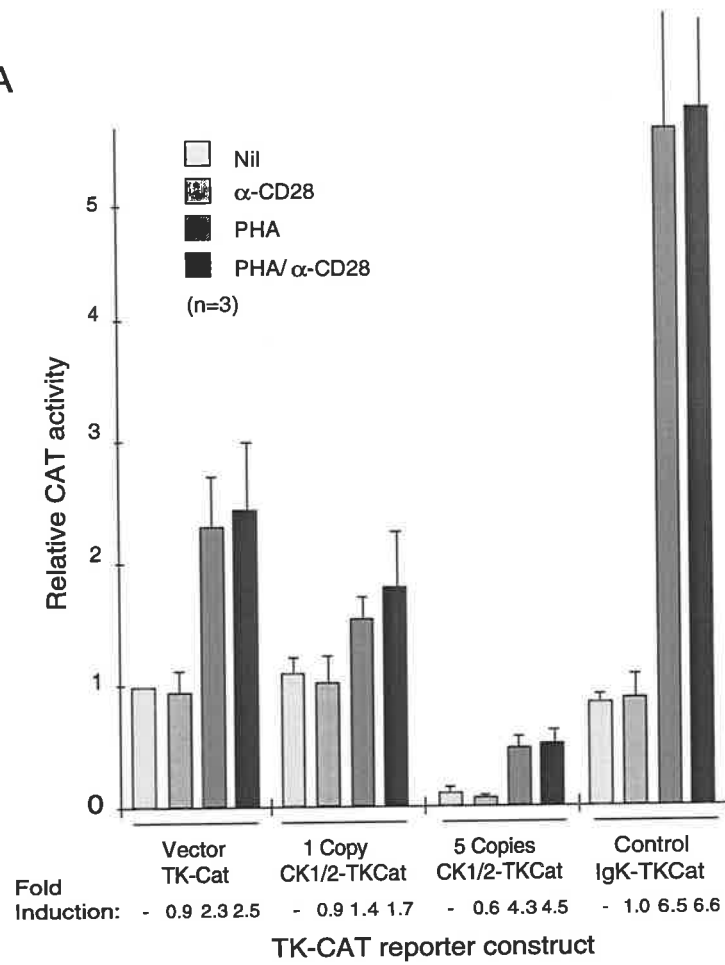
The CK1/CK2 oligomer constructs (10 μ g) (Figure 4.10) were co-transfected into Jurkat T cells by electroporation with the internal control plasmid pRSV β -GAL. An Ig κ -TKCAT reporter plasmid that was included as a positive control for cell stimulation.

(A) Cells were stimulated with an α -CD28 monoclonal antibody, PHA, PHA together with α -CD28 antibody or left unstimulated (Nil).

(B) Cells were stimulated with PHA/PMA, α -CD28 antibody together with PHA/PMA or left unstimulated (Nil).

Protein concentrations used in the CAT assays were normalised by the β -galactosidase activity in each sample. CAT activity is expressed relative to the value of the unstimulated vector TK-CAT construct which is set at 1. In each panel the values represent the mean and standard error of three independent experiments. The fold induction of CAT activity over the unstimulated level of each construct is shown below the columns representing stimulated cells.

A



B

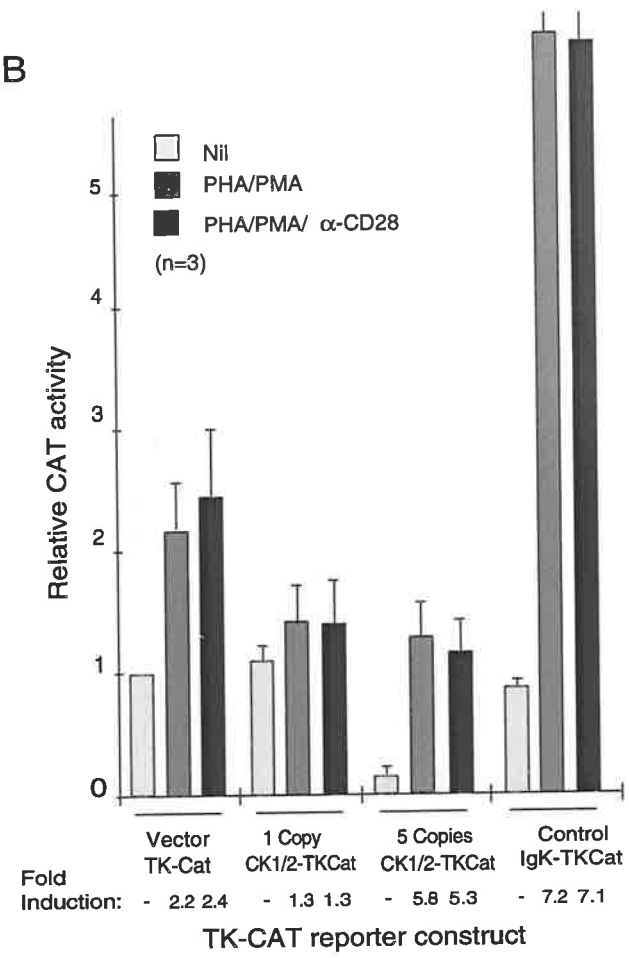
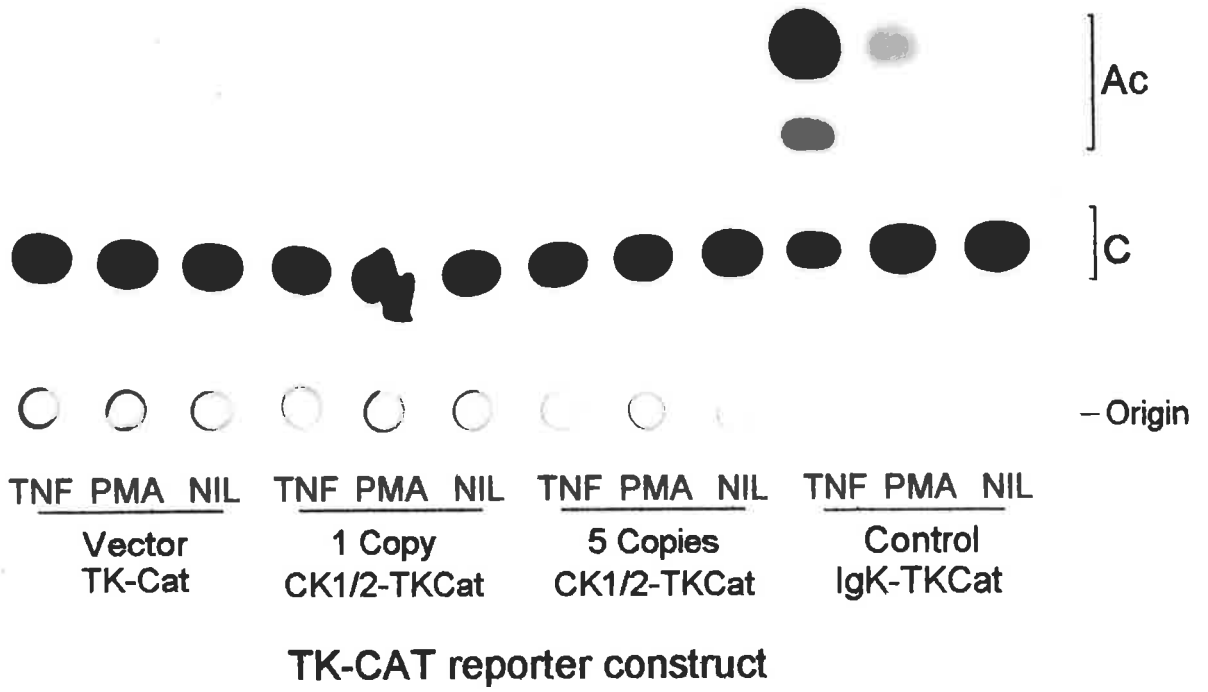


Figure 4.12 Transfection of IL-3 CK1/CK2 TK promoter constructs in HEL fibroblasts

CAT assay autoradiograph of HEL fibroblasts co-transfected by the DEAE/Dextran method with the CK1/CK2 oligomer constructs (10 μ g) (Figure 4.10) and the internal control plasmid pRSV β -GAL. An Ig κ -TKCAT reporter plasmid was included as a positive control for cell stimulation. The cells were stimulated with TNF- α (100 U/ml), PMA (10ng/ml) or unstimulated (NIL). Protein concentrations used in the CAT assays were normalised by the β -galactosidase activity in each sample. The autoradiogram shows CAT conversion of [14 C]-chloramphenicol (C) to the acetyl forms (Ac) which have been separated by TLC.



expression of the CAT gene was undetectable in transfected HEL cells and did not respond to TNF- α or PMA stimulation, and unlike that of the G-CSF gene, no induction of the TK-CAT constructs containing single or multiple copies of the IL-3 CK1/CK2 region was detected in three independent transfection experiments (Figure 4.12). However, the control for the transfection, stimulation of Ig κ -TK-CAT generated a high level of inducible CAT activity in TNF- α and PMA treated cells. Due to the lack of constitutive or inducible expression from the basal TK promoter in HEL cells any inherent inhibitory activity could not be measured with these constructs. These experiments show, however, that the IL-3 CK1/CK2 region is not responsive to TNF- α or PMA stimulation, a situation that resembles the function of the GM-CSF CK1/CK2 region rather than the CK1 element of the G-CSF promoter.

4.3 Discussion

The mechanisms involved in IL-3 transcriptional regulation were investigated using functional assays that employed IL-3 promoter reporter gene constructs transfected into human Jurkat T-cells. Deletion mutants of the IL-3 promoter, which contained the natural IL-3 transcription start site and a variable length of 5' regulatory sequences, were co-transfected with an internal control plasmid to minimise variation due to differences in transfection efficiency. This approach identified both activation and inhibitory regions of the IL-3 promoter including a novel upstream activation region between -398 bp and -550 bp and a negative regulatory region between -398 bp and -315 bp. The work presented in this chapter has also shown that the conserved IL-3 cytokine elements, CK-1/CK-2, found in many other cytokine gene promoters can function in T-cells as a repressor of constitutive expression from a heterologous promoter.

Transfection of the 5.2kb 5' upstream fragment of the IL-3 gene showed surprisingly low levels of transcriptional activity in stimulated T-cells when compared to the induced level of transcription of the endogenous IL-3 gene measured previously by

nuclear run-on analysis (section 3.2.3). This low level of activity was also observed for the shorter deletion constructs. In contrast, other investigators have shown higher levels of IL-3 promoter activation in Jurkat cells and other T-cell lines (Shoemaker *et al.*, 1990 and Gottschalk *et al.*, 1993). There is no obvious explanation for the difference in the levels of activation, although, there were minor differences in the transfection methodologies used in the different studies. For example, Shoemaker *et al.* (1990) used the lectin Concanavalin A not PHA and cells were harvested three days after transfection rather than two. Transfection constructs used in other studies contained additional IL-3 sequences downstream of the IL-3 transcription start site it is possible that these sequences may contribute to IL-3 transcriptional regulation. Transfection experiments did not show any evidence of enhancer elements within the introns and coding region of the IL-3 gene. The low levels of transcriptional induction particularly from long IL-3 promoter constructs has led to the suggestion that IL-3 may be regulated by more distant enhancer elements (Arai *et al.*, 1990). Since the completion of this work, Cockerill *et al.* (1993) identified an inducible DNase I hypersensitive site approximately 7.5 kb downstream of IL-3, between IL-3 and the closely linked GM-CSF gene. This site mediates an 80-95 fold induction of the transcription response from the GM-CSF promoter and an 11 fold enhancement of IL-3 induction (Cockerill *et al.*, 1993). This enhancer, therefore, could account for the induction of the endogenous IL-3 gene observed in the nuclear run on experiments. In addition, Cockerill *et al.* (1993) showed that IL-3 and GM-CSF promoter constructs were insensitive to suppression by the immunosuppressant cyclosporin A and that the intergenic enhancer restores cyclosporin A sensitivity to both IL-3 and GM-CSF promoter constructs. However, it remains to be established whether this element can influence the expression of the IL-3 gene from its natural position 14 kb 3' to the IL-3 gene. More recently a second cyclosporin A sensitive DNase I hypersensitive site has been identified approximately 14 kb 5' of the IL3 gene (Dr. P. Cockerill, personal communication) but the role of this region in the regulation of the IL-3/GM-CSF locus is presently unknown. Although the IL-3 promoter may not be highly inducible *per se*,

it may be required to regulate T-cell-specific expression since a DNase I hypersensitive site observed on the IL-3 proximal promoter is T-cell specific (Dr. P. Cockerill, G. Ryan and M. Vadas, manuscript in preparation). It will be of interest to determine how enhancer/promoter interactions occur.

The transfection experiments with the proximal promoter constructs suggest that the promoter elements required for transcriptional induction are contained within the -550 bp promoter fragment. A negative regulatory region was exposed by further deletion of the promoter to position -398 bp. This promoter construct exhibited a decrease in both the absolute basal and stimulated level of CAT activity, however the induction of CAT activity from this construct remained at approximately two fold. Higher absolute levels of promoter activity were restored by further deletion of sequences between -398 bp and -315 bp, suggesting that a constitutive negative regulatory element is contained within this promoter segment. It is clear, however, that upstream sequences from -398 bp to -550 bp can overcome this repression, therefore identifying a new activating region of the IL-3 promoter. We have termed the negative regulatory region NIP2 with regard to the nomenclature of Mathey-Prevot *et al* (1990). Computer searches against the TFD database (Ghosh, 1990) have revealed no exact matches to known silencer or repression sequences within the region. Promoter deletion studies by Park *et al.* (1993) suggest that the repressor region may be further defined between -356 and -315 bp. Interestingly, the region between -398 bp and 313 bp contains a single copy of the conserved cytokine specific promoter element, CK1 (at -330 bp to -323 bp) (Shannon *et al.*, 1988). The function of this element has not been determined. Also found in the NIP2 region is an imperfect tandem repeated motif with the core sequence of AGCAGG/A (Figure 4.13A). This AGCAGG repeat is similar to the ICK-1 transcriptional repressor element repeat identified by Nomiya *et al.*, (1993) in the promoter of the LD78 α gene and to the previously described imperfect direct repeat in the NIP repressor region of the human IL-3 promoter (Mathey-Prevot *et al.*, 1990) (Figure 4.13A). The

Figure 4.13 Alignment of IL-3 promoter element sequences with related elements of cytokine gene promoters


(A) Alignment of the coding (C) and non-coding (N) strand nucleotide sequences of human IL-3 NIP2 and NIP region with the ICK-1 negative regulatory element of the LD78 α gene and the ICK-1A binding site of the murine GM-CSF gene. Related sequence elements are shaded. Directly repeated sequences are overlined with arrows. Mismatches in the direct repeats are indicated by white boxes. Positions of the nucleotide sequences (bp) with respect to the starts of transcription are also shown.

(B) Alignment of the CK-2 elements of IL-3 and GM-CSF with the Nil2A binding site of the IL-2 promoter (overlined). Conserved nucleotides between the IL-3 and GM-CSF CK-2 core sequences and the IL-2 Nil2A sequences are indicated by asterisks

A

IL-3	C	-380	CAGGACCA		AGCAGGG		CGGGCAGCAGAG	-363
IL-3 NIP	N	-243	AGATGGGA		AGCATGGC		CAGCAGGTGAGA	-267
LD78- α ICK-1	C	-112	GGCAACTT		AGCATGAC		CAGCATCACTAC	-86
mGM-CSF ICK-1	C	-65	TCGGGGTC		ACCATTA		AATCATTTCCTC	-36

B

				Nil2A		
IL-2	C	-115	TTTCAGACAGG		TAAAGTCTT	-96
				* * * * *		
IL-3	C	-121	TCCATGTCAGATAAAGATCC			-102
				* * * * *		
GM-CSF	C	-93	CACAGTTCAGGTAGTTCCCC			-74

function of the IL-3 NIP region (at -263 to -249) is controversial since deletion or mutation of this region in the context of a longer promoter construct failed to increase CAT activity (Gottschalk, Giannola and Emmerson, 1993). However, Nomiyama *et al.* (1993) have shown both IL-3 NIP and ICK-1 bind a nuclear factor, ICK-1A, which may repress the activities of positive factors bound at adjacent sites.

Interestingly, ICK-1A also binds to a region containing two of the three CATT/TA repeats in the proximal region of the GM-CSF promoter. This region has been shown to be critical in GM-CSF transcriptional induction (Nomiyama *et al.*, 1993 and Nimer *et al.*, 1990). Nomiyama *et al.* (1993) suggest that although ICK-1A binds this site with high affinity, the positive factors important for GM-CSF expression may have higher binding affinities and compete for binding with ICK-1A. Thus ICK-1A can also act as a modulator of cytokine gene transcription by regulating the activity of positive factors at the same site.

Finally, a third NIP like element (8/10 match) is found further 5' at position -657 bp to -647 bp in the IL-3 promoter. However, there was no significant change in the basal level or induction of CAT reporter expression when sequences containing this element were deleted. It will be interesting in future experiments to determine whether the related sequence motifs in the NIP (ICK-1) like regions of the IL-3 promoter bind ICK-1A and what role the negative regulatory elements may have in control of the lineage restricted expression of IL-3.

The highest absolute levels of reporter gene activity were measured with the -315 bp promoter fragment. A consensus binding site for the transcriptional activation complex AP-1 has been previously identified by sequence comparison at position -301 bp to -295 bp (Figure 4.13) (Shoemaker *et al.*, 1990 and Mathey-Prevot *et al.*, 1990). This AP-1 site, referred to as activator region 2 (ACT-2, Mathey-Prevot *et al.*, 1990), may account for the activation function of this region of the IL-3 promoter. Direct evidence for *in vitro* binding of the c-jun protein has been obtained using PHA or PHA/PMA stimulated T-lymphoblast nuclear extracts (Park *et al.*, 1993 and

Gottschalk, Giannola and Emmerson, 1993). Gottschalk *et al.* have also shown that a non-consensus, ets family nuclear protooncogene binding site (EBS, -288 bp to -278 bp) can bind the ets family transcription factor Elf-1 and that the combined AP-1/Elf-1 site is important in T-cell specific activation of IL-3 expression. It has been shown that an AP-1/ets family member nuclear protein complex is implicated in the activation of other cytokine genes (Wasylyk *et al.*, 1990 and Wang *et al.*, 1994).

Further truncation of the IL-3 promoter to -173 bp reduced the overall levels of CAT activity but did not eliminate the response to PHA or PHA/PMA. This study has shown by DNase I footprinting that purified AP-1 can also bind to a non-consensus AP-1 element (at -154 to -148, section 5.2.2) in this part of the IL-3 promoter (Mathey-Prevot *et al.*, (1990). This result has been confirmed recently in gel shift experiments by Park *et al.*, (1993) and Davies *et al.*, (1993). The Oct-1 protein appears to bind to an adjacent site and the interaction of these proteins may also be important in mediating T-cell-specific expression (Davies *et al.*, 1993 and Park *et al.*, 1993). All of the IL-3 promoter constructs were tested in human embryonic lung fibroblasts, a cell type that doesn't express IL-3 but expresses the closely related cytokine GM-CSF. No reporter gene activity was detected in these cells implying that the proximal region of the IL-3 promoter acts in a cell specific manner. While the expression of both the AP-1 and Oct-1 proteins is not restricted to T-cells, to explain the T-cell specificity of IL-3 transcription, it has been suggested that these proteins may interact with additional T-cell specific mitogen-inducible factors. Recently Park *et al.* (1993) showed by UV crosslinking that proteins other than AP-1 or Oct-1 associate with the ACT-1 site. Moreover, the functional activity of Oct-1 in Jurkat cells requires the direct interaction of Oct-1 and the T-cell specific Oct-1 associated protein, OAP40 (Ullman *et al.*, 1991). Similar AP-1/Oct interactions contribute to the T-cell specific expression of the IL-2 promoter (Ullman *et al.*, 1991).

In addition to the ACT-1 region, the -173 bp fragment of the IL-3 promoter contains the conserved cytokine elements CK-1 and CK-2. Shannon *et al.* (1992) have

previously shown that the conserved CK-1 region of the G-CSF promoter acts as a TNF- α responsive activation element in fibroblasts. In contrast, the GM-CSF CK-1 region is not TNF- α responsive in fibroblasts (Kuczek *et al.*, 1991) and transfection experiments here demonstrated that the IL-3 CK-1/CK-2 region is also non-functional in fibroblasts. Recently Coles *et al.*, (1994) have demonstrated that the GM-CSF CK-1 region can repress the TNF- α response of downstream GM-CSF promoter sequences. This study has shown that the IL-3 CK-1/CK-2 region acts as a repressor region upstream of a heterologous promoter in T-cells. In addition, the IL-3 CK1/CK2 region has been implicated in the repression of IL-3 transcription in HTLV infected T-cells (Wolin *et al.*, 1993). It is interesting to note that the G-CSF CK-1 element is not linked to a CK-2 element but the GM-CSF CK-1/CK-2 region is very similar to the IL-3 CK-1/CK-2 region (Shannon *et al.*, 1988). Therefore, it appears that the coexistence of the CK-1 and CK-2 elements may generate repressor activity. There are at least two possible explanations for repression by the cytokine elements. Coles *et al.* (1994) have recently identified a 22 kD nuclear protein complex, NF-GMb, that binds to tandem CAGG sequences within the conserved CK-1/CK-2 region of the GM-CSF promoter. The binding of this factor to the GM-CSF CK1/CK-2 region is correlated with repressor activity in fibroblasts. It is possible that a similar protein may function on the IL-3 CK-1/CK-2 region. It is interesting to note also, that a number of repeats of the sequence CAGG occur in the upstream NIP2 region that may be involved in repression of IL-3 transcription. Alternatively, the CK-2 regions of IL-3 and GM-CSF have a 5 and 6 out of 7 match respectively (Figure 4.13B) to the binding site for the transcriptional repressor protein Nil2A which inhibits transcription from the IL-2 promoter (Williams *et al.*, 1991). It is therefore possible that Nil2A may repress the IL-3 promoter. It will be of interest in future experiments to determine if Nil2A does bind to the conserved cytokine elements.

Activation of helper T-lymphocytes and cytokine gene expression is thought to require, in addition to stimulation of the TcR, a second signal, normally delivered by IL-1 or stimulation of T-cell surface accessory molecules including CD2, LFA-1, CD8

and CD28 by the antigen presenting cells (Oster et al 1989, Barber *et al.*, 1989, De Jung *et al.*, 1991 and Maraskovsky, Troutt and Kelso, 1992). Moreover, in the absence of concomitant TcR stimulation, ligation of the CD2 and CD28 accessory molecules is also able to induce T-cell proliferation *in-vitro*. Stimulation of the TcR together with the CD28 receptor enhances the expression of cytokine mRNAs by a mechanism involving increased mRNA stability (Lindsten *et al.*, 1989). More recent reports however, have demonstrated induction of IL-2, IL-3, IFN- γ and GM-CSF promoters and activation of the HIV LTR in T-cells by stimulation of CD28 in the presence of TcR like signals (Verweij, Geerts and Aarden, 1991, Fraser *et al.*, 1991, Fraser et al 1992 and Gruters *et al.*, 1991). Fraser *et al.*, (1992) have demonstrated that a CD28 responsive element (CD28RE) in the IL-2, IL-3 and GM-CSF gene promoters, which overlaps the CK-1 element, is bound in stimulated T-cells by an inducible nuclear complex, CD28RC, and that deletion of the CD28RE in the IL-3 and GM-CSF promoters abrogates the CD28-induced activity without affecting the PHA/PMA induced activity. In this study, α -CD28 monoclonal antibody (Mab 9.3) treatment produced only a small increase in CAT activity from cells that were transfected with the -173 bp proximal promoter constructs and α -CD28 treatment of cells transfected with the CK-1/CK-2 TK constructs also failed to increase CAT reporter gene activity. Ledbetter *et al.* (1990) have shown that transmembrane signalling by the CD28 receptor is dependent on the degree of receptor aggregation and that bivalent binding of the α -CD28 Mab 9.3 acts primarily through a mechanism that results in stabilisation of otherwise labile cytokine mRNAs. A higher degree of crosslinking of the CD28 receptor, which can depend on antibody concentration or aggregation, activated the transcription of IL-2 in PBLs even in the absence of other stimuli. The effectiveness of stimulation then, may be exquisitely sensitive to the ability of the monoclonal antibody to cause extensive crosslinking of the CD28 receptor. In the present work Mab 9.3 was used at saturating levels not expected to cause a high degree of crosslinking of the CD28 receptor. Although Mab 9.3 used in this way induced increased steady state levels of IL-3 mRNA in PHA/PMA treated

Jurkat cells, possibly through a stability related mechanism, it did not increase transcription from the IL-3 promoter. It will be of interest therefore to further examine the function of the CD28 responsive elements with purified and immobilised or crosslinked monoclonal antibodies.

In conclusion, the function of the regions of the IL-3 gene involved in the regulation of transcription have been investigated in transiently transfected T-cells by linking the 5' or intron and coding regions of IL-3 gene to the chloramphenicol acetyltransferase reporter gene. This approach has shown, by serial deletion of the promoter, that there are both positive and negative regulatory regions within the first -647 bp of the IL-3 promoter and that the minimal functional or inducible promoter unit extends to -173 bp. This study has also characterised the transcriptional regulatory function of the conserved CK-1/CK-2 region that exists between positions -142 and -102 bp in the IL-3 promoter in the context of a heterologous thymidine kinase promoter. This region acts as a transcriptional silencer of the constitutive expression of the thymidine kinase promoter in transfected Jurkat cells.

Chapter 5

Characterisation of nuclear proteins that interact with the IL-3 promoter

5.1 Introduction

Our comprehension of the biology of transcription regulatory factors in general is expanding at a rapidly increasing pace, however, the level of characterisation of transcription factors varies greatly. Some have been cloned and their primary sequences are known while others have been identified only by their DNA binding and *in vivo* functional analysis. The detection of rare DNA binding activities in crude cell extracts by sensitive gel mobility shift assays and DNA protection assays is largely responsible for the rapidly increasing amount of information concerning transcription regulatory proteins. Purification to homogeneity and cloning of these proteins has been expedited by refinement of sequence specific DNA affinity chromatography techniques and cell free extracts have been developed that accurately reproduce transcription from RNA polymerase II promoters using cloned DNA templates and the purified regulatory components (Nishida *et al.*, 1991 and reviewed by Johnson and McKnight 1989).

Evidence presented in the preceding chapters of this thesis and that of others (Nishida *et al.*, 1991, Shoemaker *et al.*, 1990, Mathey-Prevot *et al.*, 1990 and Dokter *et al.*, 1993) has established that the tissue restricted expression of IL-3 and the induction of IL-3 mRNA in activated T-lymphocytes is regulated at least in part at the transcriptional level. Furthermore, functional studies of the human IL-3 promoter have led to the identification of regulatory areas of the promoter that contain discrete activation and silencing regions *vis.* ACT-1, ACT-2, NIP (Mathey-Prevot *et al.*, 1990,

Shoemaker *et al.*, 1990 Davies *et al.*, 1993 and Park *et al.*, 1993), NIP2 and the CK1/CK2 elements (this work). Recent studies have implicated AP1 transcription factor binding sites in the ACT-1 and ACT-2 activation regions (Mathey-Prevot *et al.*, 1990) as important to the induction of IL-3 mRNA expression (Shoemaker *et al.*, 1990, Gottschalk *et al.*, 1993 and Park *et al.*, 1993). Other studies have shown that the regulatory regions of several other inducible, T-cell expressed, cytokine genes also contain AP1 binding sites that are important for mRNA induction and that some of these, like the IL-3 AP1 site (Gottschalk *et al.*, 1993), occur in close proximity to an Ets family nuclear protein binding site and may be involved in T-cell specific gene expression (Wasylyk *et al.*, 1990 and Boise *et al.*, 1993).

The frequent appearance of CK1 like sequences in cytokine gene promoters suggests that they may play a pivotal role in the regulation of cytokine gene transcription. The presence of the IL-3 CK1/CK2 elements in the -173 bp minimal functional fragment of the IL-3 promoter further supports this suggestion. Initial deletional analyses failed to provide a role for the CK1/CK2 region in the IL-3 gene in response to T-cell activation signals (Mathey-Prevot *et al.*, 1990 and Shoemaker *et al.*, 1990). However, evidence presented in the preceding chapter has shown that the IL-3 CK1/CK2 region can function as a transcriptional silencer at least in the context of a constitutive heterologous promoter and that this region also responds to stimulatory signals that activate T-cell function. Other groups have shown that the CK-1 elements may mediate the response to CD28 stimulation (Fraser *et al.*, 1991 and Fraser and Weiss, 1992). In addition, the CK 1 element has been shown to be essential for full activation of the mouse and human GM-CSF promoters by the HTLV-I *trans*-activator P40^x or *tax* in Jurkat cells (Miyatake *et al.*, 1988 and Himes *et al.*, 1993) but paradoxically, this region may be involved in the repression of IL-3 in HTLV infected human cells (Wolin *et al.*, 1993). Himes *et al.*, (1993) have also implicated the GM-CSF CK2 and the NF- κ B binding site of human GM-CSF together with a potential CREB/ATF site

overlapping the CK2 element as critical to the response of that gene to the *tax* *trans*-activator.

Our understanding of the role of the conserved cytokine elements in the context of the IL-3 promoter and the molecular mechanisms underlying the regulation of IL-3 transcription will be advanced by the cloning and characterisation the transcriptional regulatory factors involved in binding to these elements. The work presented in this chapter focuses firstly on identifying the nuclear transcriptional regulatory complexes that bind to the highly conserved IL-3 cytokine gene promoter elements CK1 and CK2 and the preliminary characterisation of these complexes using electrophoretic mobility shift assays and secondly, on the development of chromatographic procedures useful for the isolation and purification of the factors to facilitate their eventual molecular cloning.

5.2 Results

5.2.1 Identification of nuclear factors that bind to the conserved IL-3 CK1 and CK2 region

The presence of nuclear factors that could bind to the conserved IL-3 CK1/CK2 region was investigated by electrophoretic mobility shift assays (EMSA, section 2.6.2).

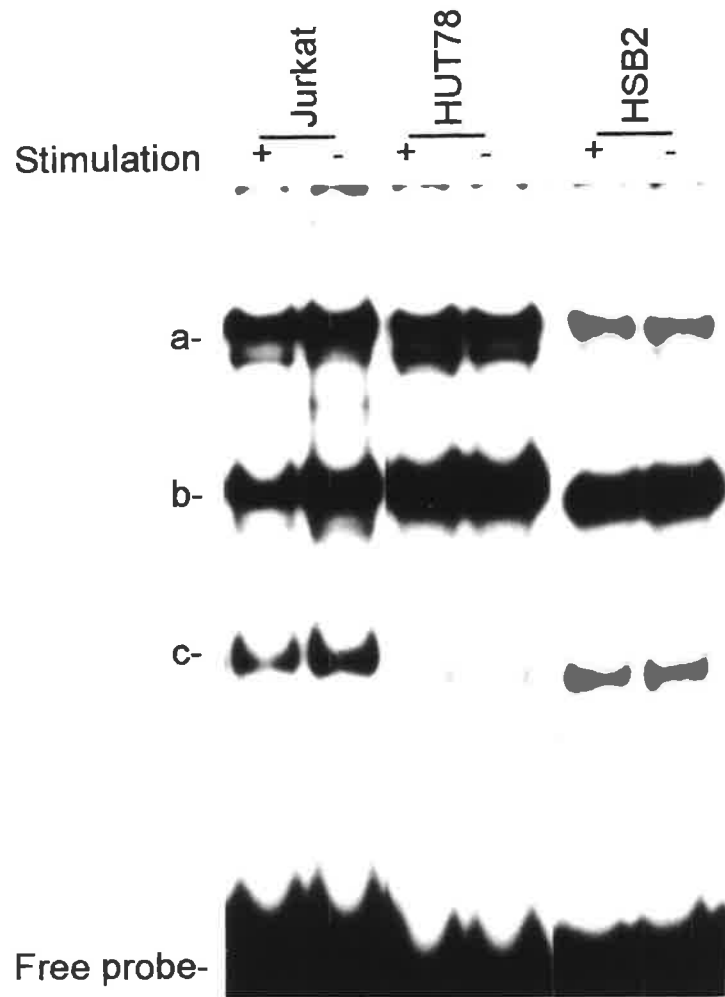
Nuclear protein was extracted from a panel of human cell lines and the Gibbon T-cell line MLA144 (section 2.6.1) (Figure 5.1). At least three prominent bands were observed in EMSA employing these nuclear extracts and the complimentary 40 mer CK1/CK2 oligonucleotides, CK-IL-3(1 and 2) (section 2.1.8). Complexes of slow, medium and fast mobility were identified (designated a, b and c in Figure 5.1).

Interestingly, there was no clear distinction between the pattern of nuclear protein binding to this region in extracts from the T-cell lines Jurkat, HSB2 and MLA 144 which express IL-3 and from HUT78 cells which do not express IL-3, regardless of whether the cells were untreated or had been stimulated with PHA/PMA (or A23187/PMA for HSB2). However, the intensity of the fastest mobility complex, "c",

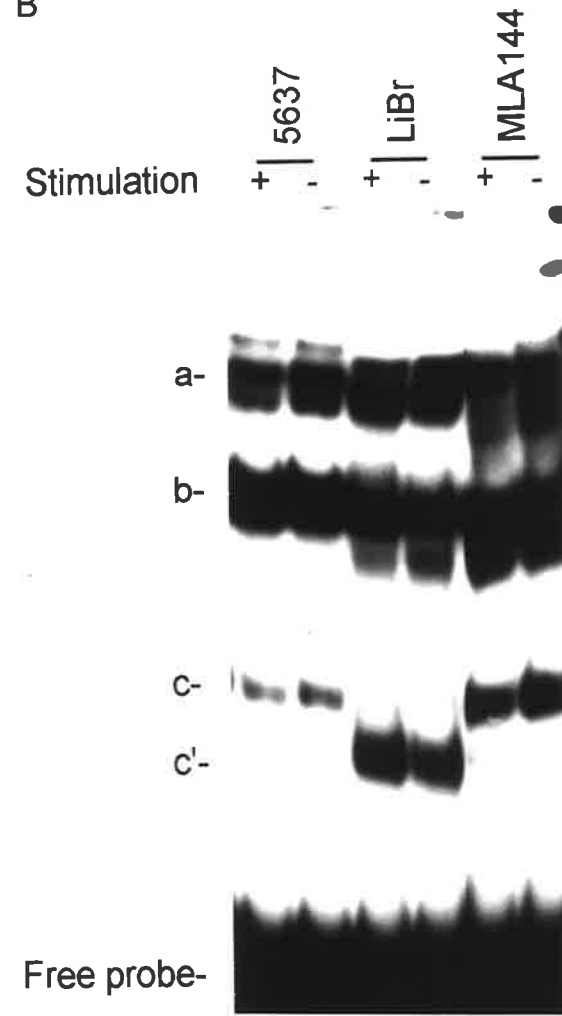
Figure 5.1 Identification of nuclear factors that bind to the conserved IL-3 CK1 and CK2 region

Autoradiographs of EMSA employing extracts from a panel of T-cell and non-T-cell lines. Nuclear protein extracts were obtained from (A) the human T-cells Jurkat, HUT78 and HSB2. (B) The bladder carcinoma cell line 5637, a human melanoma cell line LiBr and the gibbon lymphosarcoma MLA144. Cells were either unstimulated (-) or stimulated (+), Jurkat HUT78 and MLA144 cells with PHA/PMA, HSB2 cells with A23187/PMA, 5637 and LiBr with PMA. Each EMSA reaction included 2 µg of each crude nuclear extract and 0.1 ng of double stranded ³²P-labelled IL-3 CK1/CK2 probe (section 2.1.8). The positions of the probe bands retarded by binding of nuclear proteins and that of unbound or free probe are indicated to the left of the panels.

A



B



was consistently lower in HUT78 cells. A similar pattern of DNA binding was observed in extracts from either unstimulated or PMA stimulated 5637 bladder carcinoma cells (Figure 5.1B). Extracts from the melanoma LiBr contained a fourth binding protein with faster mobility in EMSA (c' in Figure 5.1B) as well as the "a" and "b" complexes. The nature of this complex was not further investigated.

To establish whether the binding of these putative transcriptional regulatory complexes was specific for the IL-3 sequences rather than nonspecific DNA binding, nuclear factor binding was competed in EMSA by the addition of unlabelled oligonucleotides, of identical or related sequences, to the binding reactions for 15 minutes prior to the addition of the labelled oligonucleotides (Figure 5.2).

Competition by oligonucleotide sequences identical to that of the labelled probe, or self-competition together with the absence of competition by an unrelated sequence (Figure 5.3A) indicates the binding of the nuclear proteins is specific for the labelled oligonucleotide probe sequences. Self-competition for the binding of the three major EMSA bands is shown in Figure 5.2A where up to 20 ng, representing a 200 fold excess, of identical unlabelled oligonucleotides was included in the EMSA. These data indicate that the binding of the nuclear proteins involved in these complexes is specific for the IL-3 sequences in the oligonucleotide probes. A band showing very slow mobility in this gel system was not competed by unlabelled IL-3 or GM-CSF CK1/CK2 competitors (CK-GM(1 and 2) section 2.1.8) (Figure 5.2A and B) and appears to be due to non-specific binding of the probe. Moreover, this band was observed inconsistently in EMSA.

Previous studies in this laboratory have shown that binding of the HUT78 nuclear protein extracts to the GM-CSF CK1 and CK2 sequences revealed a similar pattern of specific nuclear protein/DNA complex formation. At least two nuclear protein complexes that bind specifically to GM-CSF CK1 and CK2 elements have been identified in this laboratory and termed NFGMa and NFGMb (Shannon, Gamble and Vadas, 1988, Shannon *et al.*, 1990 and Coles *et al.*, 1994). Given the similarity of

Figure 5.2 Comparison of nuclear complexes binding to IL-3 and GM-CSF CK1/CK2 regions.

Autoradiographs of EMSA employing nuclear extracts from PHA/PMA stimulated Jurkat cells.

(A) EMSA using the ^{32}P -labelled IL-3 CK1/2 binding probe and either none (-) or the indicated amount of unlabelled IL-3CK1/2 oligonucleotide as a specific binding self-competitor. The positions of nuclear DNA binding complexes and unbound or free probe are labelled to the left of the panel. The complexes correspond to the three complexes, a, b and c shown in Figure 5.1.

(B) EMSA using the ^{32}P -labelled IL-3CK1/2 binding probe and either none (-) or the indicated amount of unlabelled GMCK1/2 oligonucleotide as a binding competitor.

(C) EMSA using the ^{32}P -labelled GMCK1/2 binding probe and either none (-) or the indicated amount of identical unlabelled IL-3CK1/2 oligonucleotide as a binding competitor.

The positions of the probe bands retarded by specific binding of nuclear proteins and that of free probe are indicated to the left of the panel.

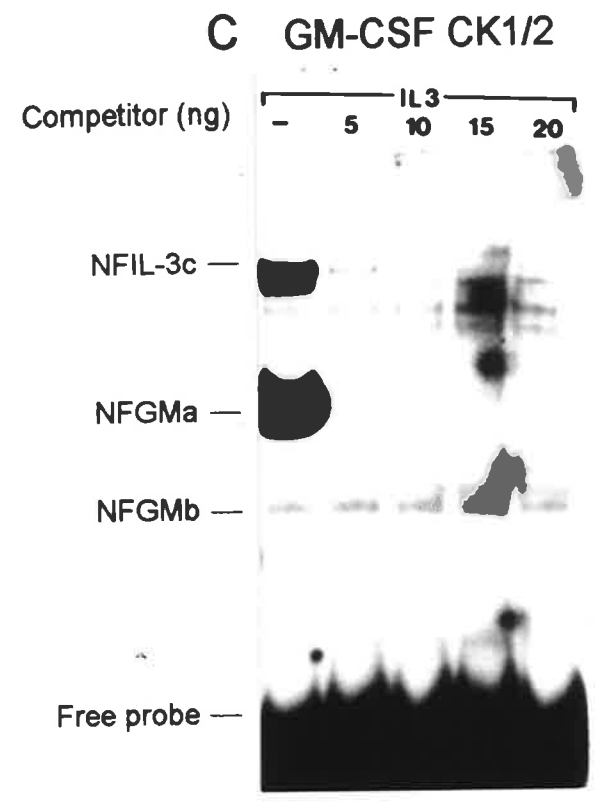
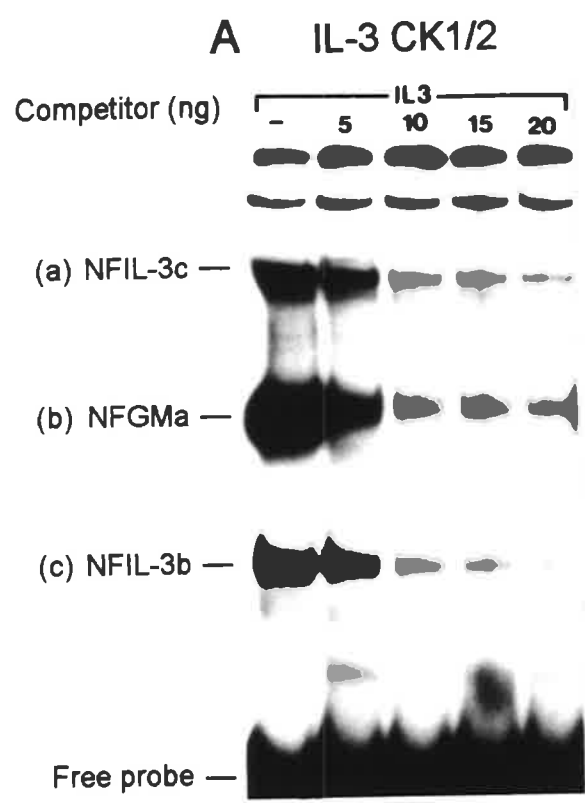


Figure 5.3 Identification of the binding sites of NFGMa, NFIL-3b and NFIL-3c within the IL-3 CK1/CK2 region

(A) Autoradiographs of EMSA employing nuclear extracts from PHA/PMA stimulated Jurkat cells and a ³²P-labelled IL-3CK1/2 binding probe. Before the addition of the labelled probe the nuclear extracts were first incubated with either none (-), 5, 10 or 20ng of unlabelled self-competitor oligonucleotide IL-3CK1/2, or unlabelled mutant oligonucleotides M1, M2 and M3 as shown above each panel. A synthetic random sequence (section 2.1.8) was used as an unrelated competitor in the EMSA reactions shown in the rightmost panel. The positions of specific retarded bands and that of free probe are indicated to the left of the first panel.

(B) The DNA sequence of the oligonucleotides used in (A) are shown. The natural IL-3 CK1/CK2 sequence is shown in double stranded form (wt). The positions of the conserved CK1 and CK2 elements are indicated by horizontal brackets. The coding strands only of the mutant competitor oligonucleotides M1, M2 and M3 are shown. Dots represent sequence identity with the natural sequence, bases that were mutated are shown. Inverted repeat sequences within and between the IL-3 CK1/CK2 elements are indicated by boxes and hollow arrows. A direct repeat structure, within and 3' to the CK2 element, is boxed and indicated by solid arrows.

sequence between the IL-3 and GM-CSF CK1/CK2 elements it was of interest to compare the nature of the nuclear binding complexes which bind to these regions. The mobility in EMSA of one of these, NFGMa, matched that of a complex (initially identified as "b" in Figure 5.1) formed with the IL-3 CK1/CK2 probes (compare band "b" or NFGMa in Figure 5.2 panels A, B and C). Cross competition experiments with the IL-3 and GM-CSF CK1/CK2 EMSA probes showed that the binding of NFGMa to the IL-3 EMSA probes can be competed by the GM-CSF CK1/CK2 probe (Figure 5.2B) albeit at a lower efficiency than self competition with the IL-3 CK1/CK2 probes (compare band "b" or NFGMa in Figure 5.2A and B). Conversely, NFGMa binding to the GM-CSF CK1/CK2 region can be competed, with high efficiency, by the IL3 CK1/CK2 sequences (Figure 5.2C). A 50 fold excess (5ng) of the IL-3 CK1/CK2 probes was completely effective in competition of the NFGMa complex. These results suggest that NFGMa complex can bind specifically to both the IL-3 and GM-CSF CK1/CK2 region. Furthermore, the high efficiency competition of the NFGMa complex by the IL-3 EMSA probes compared to that of the GM-CSF probes suggests that the affinity of the nuclear protein complex is higher for the IL-3 DNA sequences than for related sequences in the GM-CSF CK1/CK2 region. Interestingly the second GM-CSF specific nuclear protein complex NFGMb is not apparent in EMSA utilising the IL-3 CK1/CK2 sequences and cross competition of NFGMb complex with unlabelled IL-3 CK1/CK2 oligonucleotides does not effect the formation of the complex with labelled GM-CSF probe (Figure 5.2C). However, a complex with slightly faster mobility is observed in EMSA with the IL-3 probe (initially identified as "c" in Figure 5.1). Cross-competition EMSA show that GM-CSF sequences do not compete the binding of this complex with the IL-3 sequences(Figure 5.2B). Taken together these data suggest that the EMSA complex "c" is specific for IL-3 CK1/CK2 sequences and does not bind to the GM-CSF CK1/CK2 elements. This complex was therefore designated NFIL-3b. These experiments also show that NFGMb does not bind to the IL-3 CK1/CK2 oligonucleotides. The third and slowest mobility EMSA complex (labelled "a" in

Figure 5.1) is competed by both IL-3 and GM-CSF CK1/CK2 probes (Figure 5.2A and B). The comparative efficiency of cross competition of this complex by IL-3 and GM-CSF EMSA probes suggests that, like NFGMa, it has a higher affinity for IL-3 sequences than to the GM-CSF sequences, therefore the complex was subsequently termed NFIL-3c.

Both the IL-3 CK1 and CK2 sequence elements are contained within the 40 bp oligonucleotides used for the EMSA described above. Thorough inspection of the sequence of these elements and the immediately surrounding region reveals the presence of inversely repeated sequences within the CK1 element. In addition there are two short, four base pair, direct repeats within and 3' to the CK2 element (Figure 5.3B). Such repeat sequences appear to be partially preserved in the GM- but not in G-CSF or IL-5 cytokine elements (see Figure 5.11). Nevertheless, their presence in the conserved region of IL-3 promoter suggests that they may be candidate targets for putative transcription factor binding. To determine if these DNA sequence elements are responsible for the binding of one or more of the three nuclear factor complexes described above, oligonucleotides with mutated sequences in the repeat structures of CK1 and CK2 were used as competitive inhibitors of complex formation in EMSA. Three, double stranded mutant oligonucleotides, M1 to M3 (section 2.1.8), were assembled with base mutations in the repeated structures of CK1 and CK2 as shown in Figure 5.3B. EMSA competition studies with these mutant oligonucleotides and an unrelated, random sequence control oligonucleotide (Figure 5.3A), show that while unrelated sequences have no effect on factor binding with crude Jurkat cell nuclear extracts the M1 mutant competes efficiently all three of the nuclear protein complexes. The M2 mutant does not efficiently compete the binding of NFGMa or the slower mobility complex NFIL-3c, suggesting that sequences altered by the M2 mutation are involved in the binding of the NFGMa and NFIL-3c nuclear complexes. Binding of the NFIL-3b was competed by the M2 in a dose responsive manner, albeit less efficiently than that of the M1 mutant or wild type CK1/CK2 sequences. In

contrast, mutant M3 did not compete the binding of NFIL-3b while it did compete, again inefficiently, for the binding of NFGMa and NFIL-3c. These data suggest that NFIL-3b may bind to sequences within the IL-3 CK2 element. These EMSA competition experiments, however, can indicate only indirectly that the CK1/CK2 elements are responsible for nuclear factor binding and further investigation of the precise bases involved in the binding of NFIL-3b and NFIL-3c will require more extensive EMSA using the mutant oligonucleotides as labelled probes and other methods such as methylation or modification interference assays (Gilman *et al.*, 1986).

5.2.2 DNase I footprinting of the IL-3 promoter with purified transcription factors AP1, AP2 and AP3

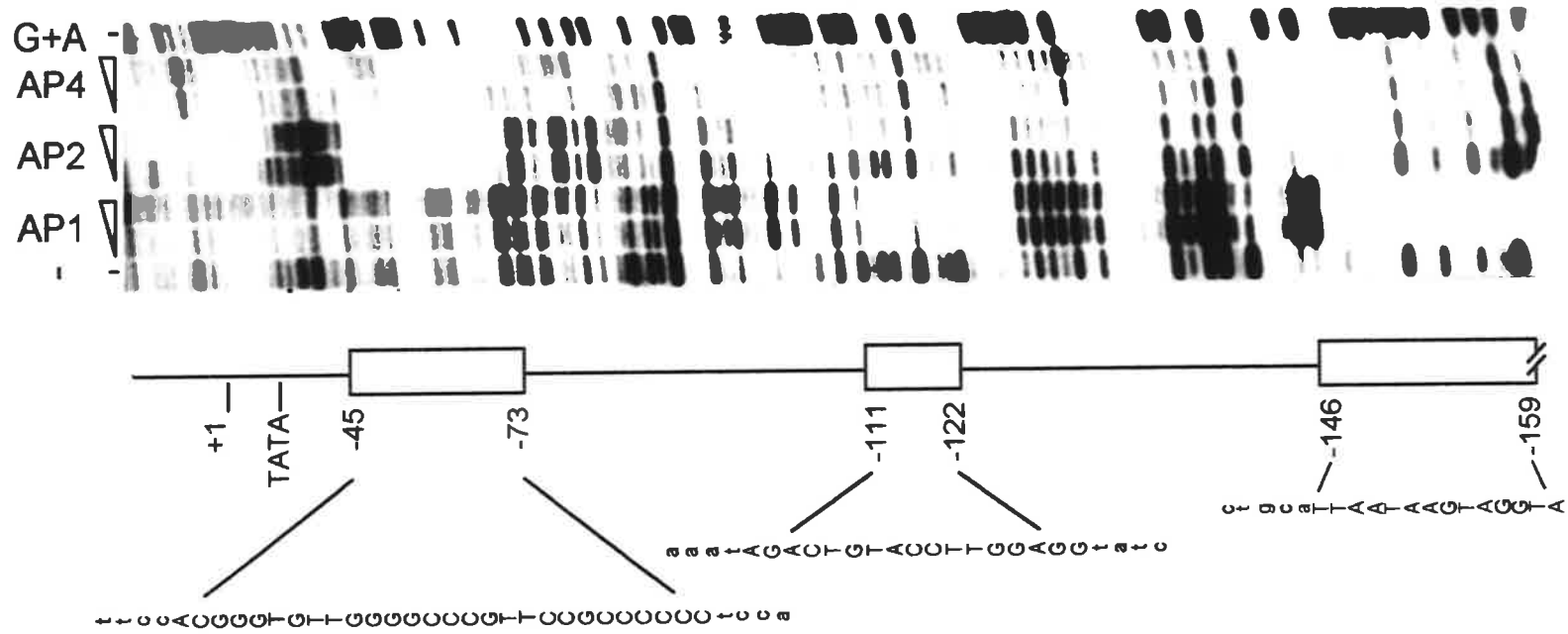
Analysis of the sequence of the IL-3 CK1 and CK2 elements of IL-3 promoter revealed a similarity to the consensus AP1 binding site further upstream in the IL-3 promoter at -305 bp (5/8 bp) and also to the non consensus AP1 binding site in the ACT-1 region at -154 bp (5/6 bp) (Figure 5.4B). To determine if AP1 could bind to the IL-3 CK1 and CK2 elements or surrounding regions of the IL-3 proximal promoter, purified AP1 protein was tested in a DNase I footprinting assay which used a fragment of the IL-3 promoter from -173 bp to +3 bp. In addition, the activator proteins AP-2 (Williams *et al.*, 1988) and AP-4 (Fodor *et al.*, 1991) were tested for their ability to bind to the IL-3 promoter (Figure 5.4A). DNase I footprinting was carried out as described by Galas and Schmitz (1978) in collaboration with Dr. D. Bohmann. The probe used in these reactions was obtained from the -173 bp IL-3 promoter/CAT reporter construct by digestion with Hind III and Bam HI. This 210 bp probe contained the IL-3 promoter sequences from -173 bp to +3 bp and some flanking vector polylinker sequence. The results of the DNase I footprint analysis show that AP1 can bind strongly to sequences immediately upstream of position -146 bp of the IL-3 promoter. A second weaker AP1 DNase I footprint is observed over the CK1 element and is flanked by bands that are characteristically over digested

Figure 5.4 DNase I protection footprint of the IL-3 proximal promoter region by purified transcription activation complexes AP1, AP2 and AP4.

(A) DNase I footprint reactions with the -173 bp IL-3 promoter fragment and purified AP1, AP2 and AP4 protein. The lower track (-) shows the digestion control reaction. The nuclear protein complexes were used in increasing amounts as indicated by the triangles. A G+A Gilbert Maxam sequencing track was included as a sequence and position marker. Three footprints were obtained, the sequences of protected regions are shown in capital letters in the diagram representing the IL-3 promoter shown below the autoradiogram.

(B) Alignment of sequence similarity between the IL-3 CK1/CK2 region at -128 bp to -109 bp (the conserved CK1 and CK2 bases are shown in upper case letters), the AP1 binding site (overlined upper case letters) of the -159 bp to 143 bp IL-3 ACT-1 region and the consensus AP1 binding site in the IL-3 promoter at -305 bp to -290 bp. Base identity is indicated with asterisks.

A



B

hIL-3 ACT1		-159	tcattg	<u>GATGA</u>	ataatta	-143
				*** **		
hIL-3 CK1/CK2	-128		GGAGGTTCCATgt	CAGATAaa		-109
			CCTCCAAGGTACa	GTCTATtt		

hIL-3 AP1		-305	TGAGTCag			-289

by DNase I in the presence of a DNA bound protein (Figure 5.4A). Purified AP-2 also generated a footprint flanked by over digested bands on the IL-3 promoter at the predicted AP-2 binding site from -69 bp to -40 bp (Figure 5.4A). The AP-4 protein did not bind to any sequences from -173 bp to +3 bp. These results provide evidence that AP1 may be involved in the response of the -173 bp, ACT-1 region of the IL-3 promoter in stimulated Jurkat cells and that the conserved IL-3 CK1/CK2 elements can also bind AP1 albeit weakly and so may be involved in regulation of IL-3 transcriptional induction. Moreover, alignment of sequence similarity between the IL-3 CK1/CK2 region at -128 bp to -109 bp, the AP1 binding site of the IL-3 ACT-1 region (-159 bp to 143 bp) and the consensus AP1 binding site in the IL-3 promoter at -305 bp to -290 bp shows that several bases centering on the CK-2 element share a partial match to these AP-1 binding sites (Figure 5.4B).

5.2.3 Separation and enrichment of Jurkat cell nuclear extracts by affinity chromatography.

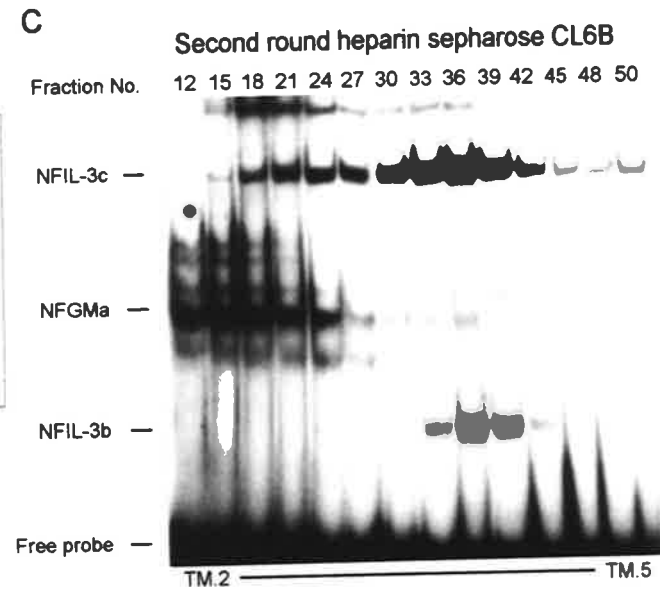
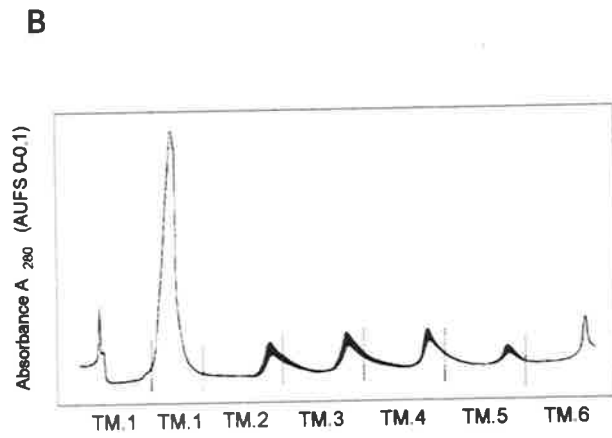
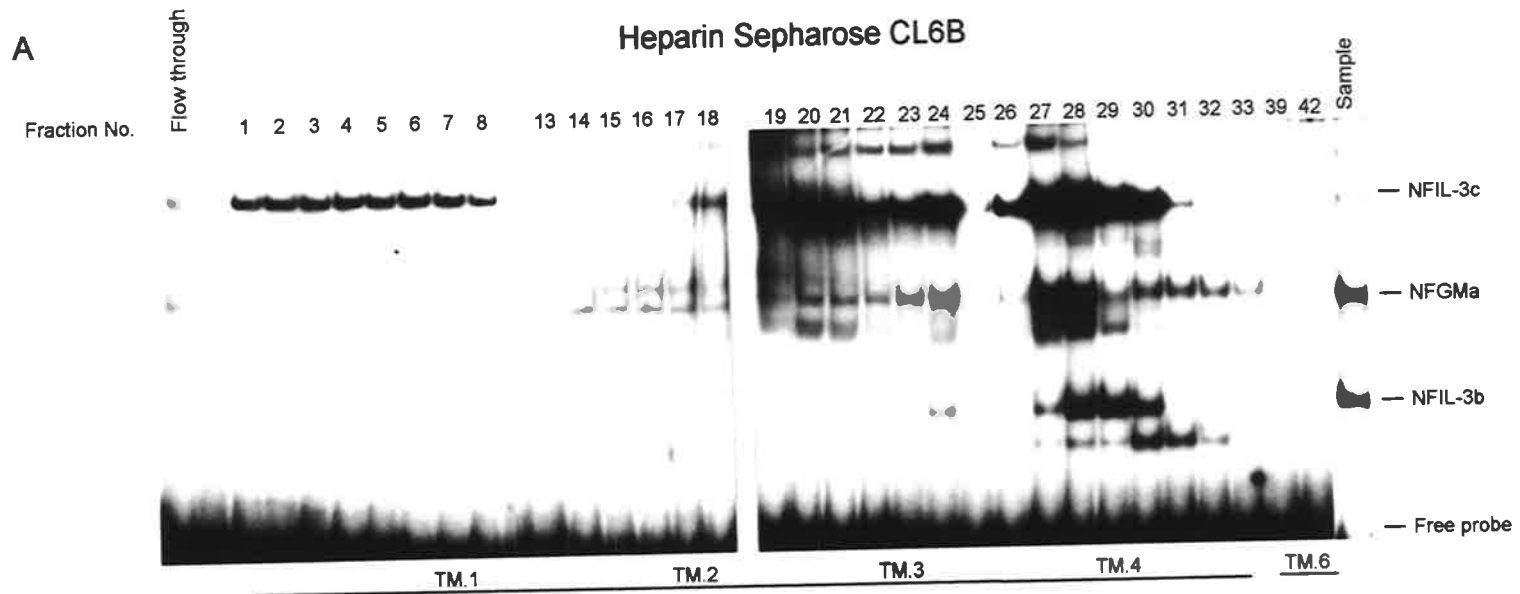
To obtain nuclear extracts that were enriched in the nuclear complexes that bind to the IL-3 CK1/CK2 region and to facilitate the further characterisation of the nuclear binding complexes two different affinity chromatography stationary phases, heparin sepharose and hydroxylapatite, were evaluated for use for the initial enrichment and fractionation of the nuclear extracts. The nuclear extracts were chromatographed on a 100 ml heparin sepharose column (20 mm x 50 mm) equilibrated with TM.1 buffer (section 2.1.5) at 4°C. DNA binding complexes were eluted with a discontinuous salt concentration step gradient from TM.1 to TM.6 buffer. Crude nuclear extract material (20 mg) was loaded onto the column in the TM.1 buffer used to store the nuclear extracts. The column wash fractions and eluted nuclear proteins were collected from several column runs, aliquots of the fractions were diluted if necessary and adjusted to a final concentration of 200 mM salt (TM.2) and assayed immediately by EMSA (Figure 5.5A). Significant amounts of protein (Figure 5.5B) but only small amounts of DNA binding activity (Figure 5.5A) were recovered from the column flow-through

Figure 5.5 Heparin sepharose chromatography of crude Jurkat cell nuclear extracts

(A) Autoradiograph of EMSA of heparin sepharose enriched nuclear extracts bound to the IL-3 CK1/CK2 oligonucleotide probe. DNA binding complexes were eluted with a discontinuous salt concentration step gradient. Equal volume eluat fractions were collected and aliquots of the fractions adjusted to a final concentration of 200 mM salt (TM.2) and assayed by EMSA. The fractions are numbered above each lane. Column flow through during loading is shown in the leftmost lane. The column was washed with 2.6 volumes of the loading buffer (TM.1) fractions 1-8. Fractions 9-12 and 34-37 contained no binding activity and are omitted. Approximate salt concentration of the eluting fractions is shown under the panel. An EMSA reaction containing a sample of material applied to the column is shown in the rightmost lane. The positions of specific IL-3 DNA binding complexes and free probe are indicated to the right of the panel.

(B) The absorbance profile (at 280nm) of material eluting from the heparin sepharose column run shown in (A). The timing of changes in the salt concentration of the TM elution buffer are indicated by vertical lines in the trace and the TM buffer used is shown underneath each concentration step. The eluted nuclear protein was measured by UV detector and chart recorder. Full scale deflection represents 0.1 absorbance units.

(C) Autoradiograph of second round heparin sepharose chromatography assayed by EMSA. Fractions 24-30 in (A) were pooled with similar fractions from two successive column runs, Fractions from the second round of heparin sepharose chromatography were eluted with a shallow continuous salt gradient from TM.2 to TM.5. The fractions are numbered above each lane. The positions of specific IL-3 DNA binding complexes and free probe are indicated to the left of the panel.



and wash fractions (fractions 1-8). Fractions 9 to 12 contained no binding activity. Elution of the nuclear binding complexes was detected within the 200 and 400 mM TM buffer steps (Figure 5.5 fractions 13-33). Approximately 20% of the total material absorbing at 280 nm was contained within these fractions (Figure 5.5B). Bands migrating at the same position as NFGMa and NFIL-3c appeared in the TM.2 buffer step in a wide peak and were completely removed from the matrix at 400 mM salt. A band corresponding to NFIL-3b eluted in 400 mM TM buffer in a narrow peak. Additional prominent EMSA bands not previously observed were present below NFGMa and NFIL-3b in the heparin sepharose fractionated material. It is not clear from these experiments whether the additional bands represent unmasked binding activities present in the crude extracts or degradation products of the specific nuclear complexes. Fractions 24 to 30 were pooled with similar fractions from two successive column runs, stored for less than one week at -70°C and subjected to a second round of heparin sepharose chromatography. Fractions from the second round of heparin sepharose chromatography were eluted with a shallow continuous salt gradient from TM.2 to TM.5 (Figure 5.5C). Fractions from the 200 to 300 mM salt buffer eluate were enriched for NFGMa. A complex corresponding to NFIL-3c eluted in a broad peak from TM.2 to TM.5 buffer and although NFIL-3b was eluted in a sharp peak at 400 mM salt it was not possible to separate NFIL-3c and NFIL-3b with this chromatographic system. Interestingly the additional EMSA band present in the primary separation below NFIL-3b (Figure 5.5A) was absent in the material recovered from the second fractionation and may represent a binding activity that is not stable to storage.

As an alternate separation procedure an hydroxylapatite column, commonly used for the separation of nuclear proteins, was prepared and equilibrated with TM.1 buffer (column dimensions, 20 mm diameter x 60 mm, total volume approximately 140 ml). Fractions of crude nuclear extract were eluted with a discontinuous step gradient between TM.1 and TM.6. Analysis of the fractions of this separation by EMSA

(Figure 5.6A) show that the fractions of the crude extract containing NFGMa, NFIL-3b and NFIL-3c overlapped and were eluted mainly in the TM.2 buffer step, although a peak containing NFGMa tailed throughout the separation and was retained on the column in TM.4 and TM.6 buffer (Figure 5.6A). The samples from fractions 3 to 30 of the first round separation were pooled. These pooled fractions contained approximately 23% of the nuclear protein loaded onto the column. The enriched and pooled nuclear extract fractions were stored at -70°C and re-chromatographed on the same column with a shallow continuous TM buffer salt gradient from 100 mM to 400 mM (Figure 5.6B). This chromatographic method achieved a separation of NFIL-3b and NFIL-3c and may be useful as a second purification step following heparin sepharose chromatography in which NFIL-3b and NFIL-3c co-chromatograph (Figure 5.5A and C). A peak containing NFGMa also eluted at approximately 350 mM salt. In addition, a prominent new binding activity of slightly faster mobility than NFGMa eluted in a broad peak from 100 mM to 300 mM salt in fractions 8 to 36. This binding activity was also present in the heparin sepharose enriched material (Figure 5.5A). It is unclear from these experiments whether this complex represents a new binding or a breakdown product of one of the other prominent nuclear complexes. For reference, the complex was termed NFGMa' although the specificity and binding characteristics of this complex have not yet been investigated.

5.2.4 Estimation of molecular size of nuclear complexes that bind to the IL-3 CK1/CK2 region

To establish the molecular weight of the three complexes NFGMa, NFIL-3b and NFIL-3c, pooled enriched fractions from the second round of heparin sepharose chromatography (fractions 12 to 24 for NFGMa and 33 to 42 for NFIL-3b and NFIL-3c) were subjected to size exclusion chromatography with a sepharose G100 column. The binding activity in eluted fractions was detected by EMSA. The quantity of binding complex in the eluate was estimated by liquid scintillation counting of radioactivity in the EMSA retarded bands (Figure 5.7). The G100 column was

Figure 5.6 Hydroxyl apatite chromatography of crude Jurkat cell nuclear extracts

(A) Autoradiograph of EMSA of hydroxyl apatite enriched nuclear extracts bound to the IL-3 CK1/CK2 oligonucleotide probe. DNA binding complexes were eluted from a hydroxyl apatite column with a discontinuous TM salt concentration gradient using steps of 100mM, 200mM, 400mM and 600mM KCl. Approximate salt concentration of the eluting fractions is shown under the panel. Equal volume eluate fractions were collected and each third fraction assayed by EMSA. The fractions are numbered above each lane. An EMSA reaction containing a sample of material applied to the column is shown in the rightmost lane. The positions of specific IL-3 DNA binding complexes and free probe are indicated to the right of the panel.

(B) Autoradiograph of EMSA of second round hydroxyl apatite chromatography. Fractions 3 to 30 from (A) were pooled and subjected to a second round of hydroxyl apatite chromatography on the same column. Nuclear proteins were eluted from the column with a shallow continuous salt gradient from TM.1 to TM.4. Equal volume fractions were collected and are numbered above each lane. The positions of IL-3 DNA binding complexes and free probe are indicated to the right of the panel.

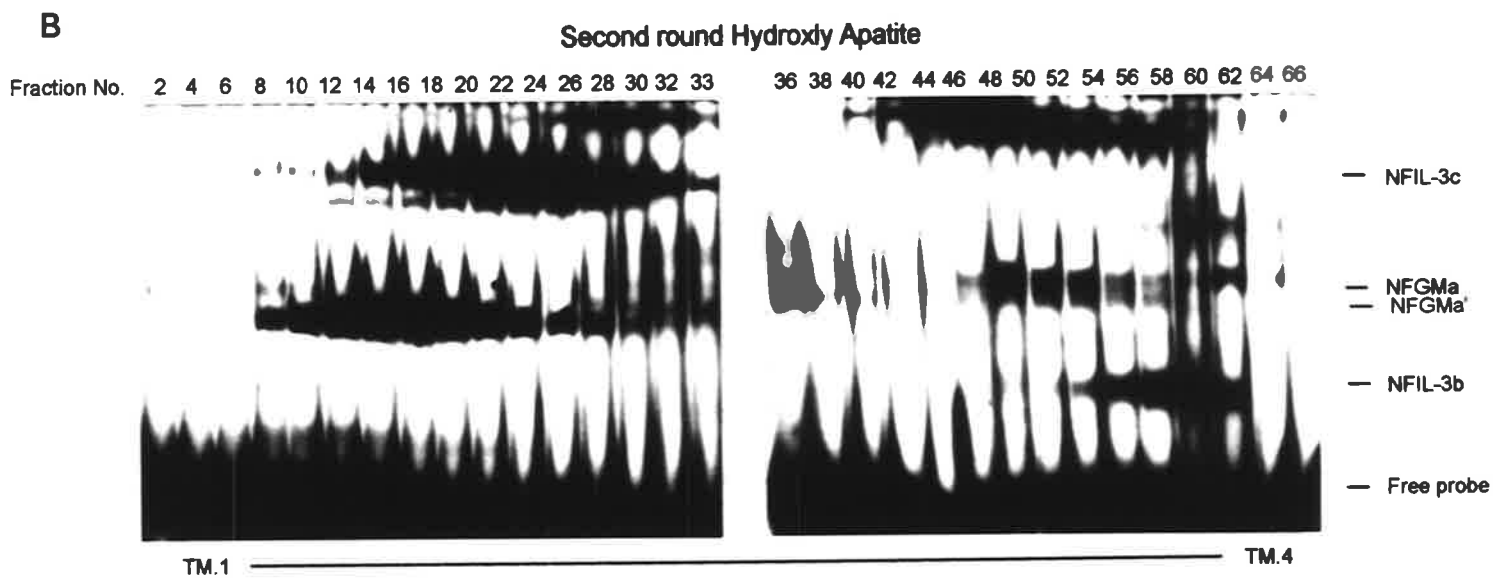
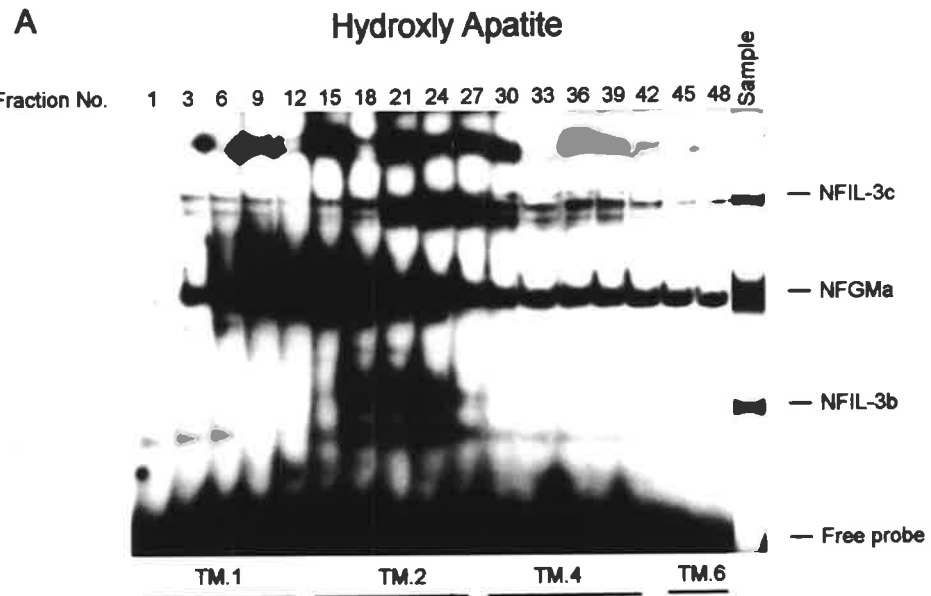
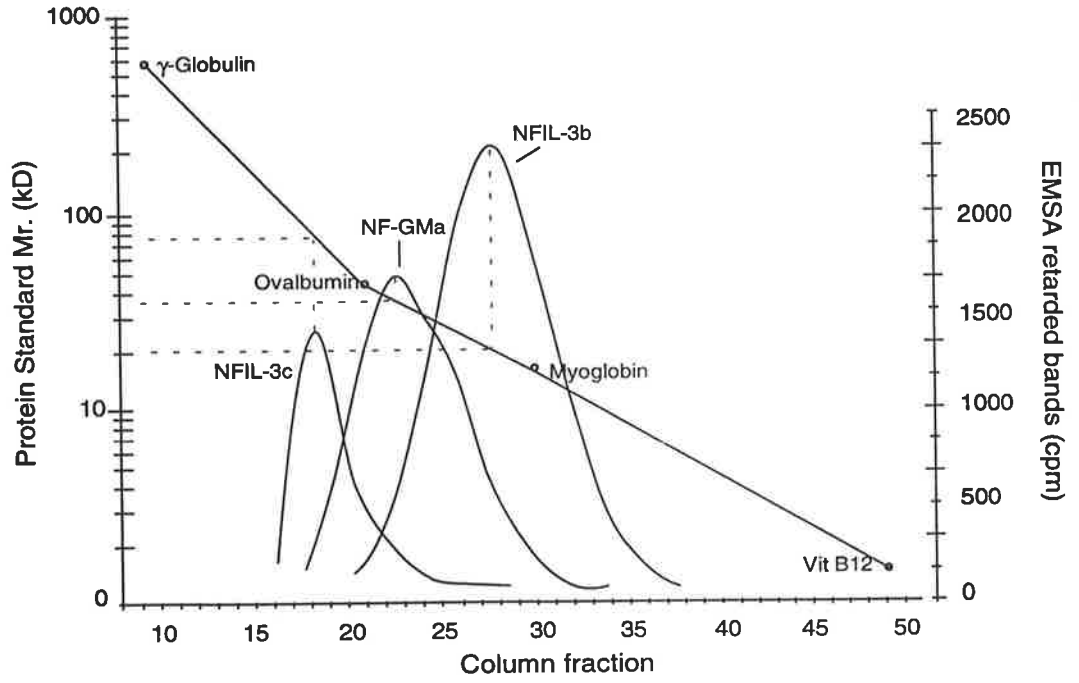


Figure 5.7 Sephadex G100 size exclusion chromatography of heparin sepharose enriched Jurkat cell nuclear extracts

Graph of DNA binding activity recovered from native gel size exclusion chromatography with a sepharose G100 column. The column was loaded with pooled fractions from the second round of heparin sepharose chromatography (Figure 5.5A, fractions 12 to 24 for NFGMa and 33 to 42 for NFIL-3b and NFIL-3c). Equal volume column fractions were collected and EMSA reactions were used to follow elution of IL-3 CK1/CK2 region binding activity. Each curve represents liquid scintillation counting of radioactivity in the EMSA retarded bands of eluted NFIL-3c, NFGMa or IL-3b. The curves for each specific DNA binding complex are labelled. The molecular mass standard curve was constructed with the protein mass standards gamma-globulin, ovalbumin, myoglobin and vitamin B12 (section 2.6.3). Estimations of the molecular mass of the heparin sepharose enriched binding activities were made by interpolation from the standard curve (dotted lines)

Sephadex G100 Chromatography



equilibrated in TM.1 buffer and pre-calibrated with protein standards (section 2.6.3 and Figure 5.7). The results of this separation show that the binding activity of NFIL-3b peaked at 20 kD, NFGMa at 35 kD and that of NFIL-3c at approximately 70 to 75 kD. These data also show that size exclusion chromatography was also effective in separation of NFIL-3b and NFIL-3c. However, the separation of molecular mass standards above 100 kD was non-linear with this matrix suggesting that a more open matrix may further improve the separation of these two complexes.

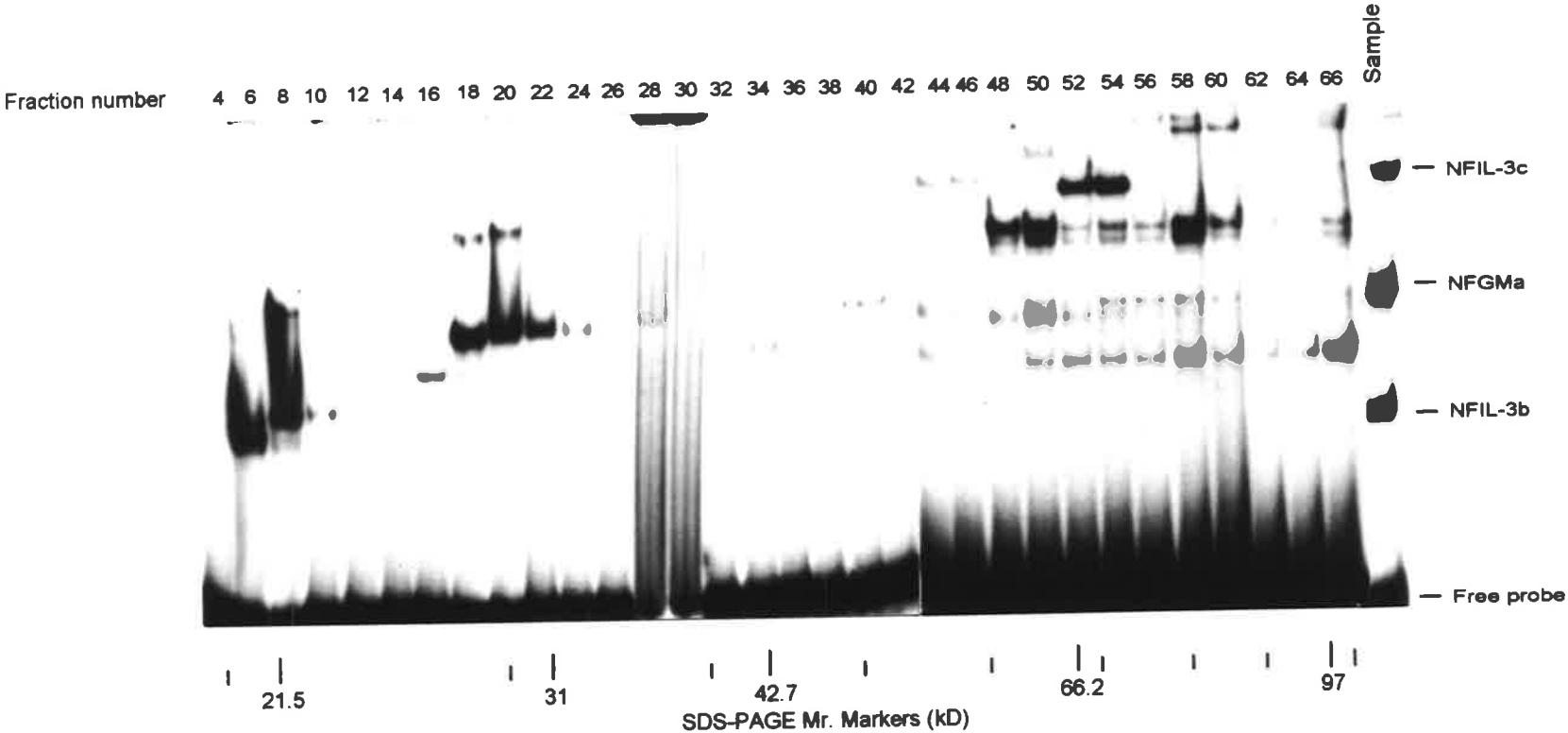
5.2.5 Estimation of subunit composition and size by SDS polyacrylamide gel electrophoresis and renaturation of IL-3 CK1/CK2 nuclear binding complexes

To investigate subunit composition of the IL-3 CK1/CK2 region binding complexes and to more accurately establish their molecular mass under denaturing conditions, crude nuclear extracts were separated by electrophoresis on SDS polyacrylamide gels, protein bands were then recovered in thin horizontal gel slices, eluted, renatured and analysed by EMSA (section 2.6.4). The results of these experiments show that binding activity of NFGMa NFIL-3b and NFIL-3c could be reconstituted after non-reducing SDS-PAGE electrophoresis and detected by EMSA (Figure 5.8). EMSA analysis of the renatured extracts detected binding activities corresponding to that of NFIL-3b at approximately 20 kD and NFIL-3c at 66 to 70 kD and is in close agreement with the size estimates of these factors obtained by native G100 gel chromatography. A prominent peak corresponding to NFGMa binding activity was detected at approximately 30 to 35 kD. The formation of less mobile EMSA complexes above the NFGMa bands is also apparent. It is possible that these bands are formed by multimerisation of the renatured NFGMa subunits. A faint binding activity similar to NFGMa was consistently detected at 45 kD and also in a wide spread peak from 65 to 80 kD. A peak that may correspond to the slightly more mobile complex, NFGMa', was detected at approximately 25 kD and also in a wide peak between 60 to 90 kD. Surprisingly a number of other, as yet uncharacterised, bands were detected by EMSA

Figure 5.8 SDS polyacrylamide gel electrophoresis, renaturation and EMSA of crude Jurkat cell nuclear extracts.

Autoradiograph of EMSA analysis of nuclear protein recovered from non-reducing SDS-PAGE gel slices. Nuclear protein bands were recovered in 66 thin horizontal slices from the top to the bottom of the SDS-PAGE gel, the nuclear proteins were then eluted, renatured and analysed by EMSA. The gel slice fractions are numbered on the top of the panel. The crude PHA/PMA stimulated Jurkat cell nuclear extract applied to the SDS-PAGE gel is shown in the rightmost lane. The positions of specific IL-3 DNA binding complexes and free probe are indicated to the right of the panel. The molecular mass of the proteins in the gel slices was estimated by comparison to migration in SDS-PAGE of Biorad protein molecular mass standards which are marked together with a log scale at the bottom of the autoradiograph (Phosphorylase, 97.4 kD; BSA, 66.2 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD; Soybean trypsin inhibitor, 21.5 kD and lysozyme, 14.4 kD).

SDS-PAGE Renaturation and EMSA



of the renatured nuclear proteins. Many of these bands are not detectable in crude nuclear extracts. Particularly prominent bands which migrate in EMSA to a position between NFIL-3c and NFGMa were detected in the 60 to 70 kD SDS-PAGE fractions. At the present time the binding characteristics and the degree of specificity of these bands for IL-3 promoter sequences are unknown.

Size estimations NFGMa were obtained earlier in this laboratory by Shannon *et al.* (1990) These authors determined the size of NFGMa bound to the GM-CSF CK1 element to be approximately 45 kD by protein/DNA UV-crosslinking studies. Subsequently, the same authors recovered NFGMa binding activity from renatured protein eluted from a 16 kD fraction of an SDS-PAGE gel where the nuclear extract samples were first heated and reduced with 2-mercaptoethanol before electrophoresis (Dr. M.F. Shannon and F. Occhiodoro, personal communication). Similarly, the size of NFGMa in renatured bands detected in EMSA using the IL-3 CK1/CK2 region was smaller in samples of crude nuclear extract that were heated in reducing buffer before SDS-PAGE (compare Figures 5.9A and B). A comparison of the same crude nuclear extract that was electrophoresed in parallel under either reducing or non reducing conditions shows that binding activity corresponding to NFGMa does appear in fractions from 16 to 20 kD of heated and reduced extracts (Figure 5.9B) but the same activity is not present or present very weakly in samples that were not heated or reduced prior to SDS-PAGE (Figure 5.9A). These results suggest that the NFGMa complex may be composed of two or more 16 kD subunits which can be separated in heated or reduced samples by SDS-PAGE. Interestingly, in addition to the renatured NFGMa binding activity, a second more mobile EMSA band migrating close to the free probe was consistently detected in only the 16 kD fractions containing NFGMa from heated and reduced nuclear extracts (Figure 5.9B fraction 6). This band may represent the 16 kD NFGMa subunit monomer. In contrast to the changes observed in the mobility of the NFGMa complex, the electrophoretic positions of either NFGMa',

Figure 5.9 SDS polyacrylamide gel electrophoresis, renaturation and EMSA of crude Jurkat cell nuclear extracts: Comparison of reduced and non-reduced samples.

Autoradiographs of EMSA analysis of renatured SDS-PAGE fractionated nuclear protein. Samples of a crude PHA/PMA stimulated Jurkat cell nuclear extract were separated in parallel by electrophoresis on (A) non-reducing SDS polyacrylamide gel or (B) SDS polyacrylamide gel under reducing conditions (section 2.6.4). The nuclear extract samples that were electrophoresed under reducing conditions were heated in load buffer containing 2-mercaptoethanol to 95°C for 5 minutes prior to loading. Protein bands were recovered in 18 horizontal slices numbered from the bottom to the top of the SDS-PAGE gel. Gel slice fraction numbers are shown on the top of the autoradiograph. The sample applied to the SDS-PAGE gel is shown in the leftmost lanes of both panels (M) where the positions of the specific binding proteins and the free probe are labelled. The molecular mass of the proteins in the gel slices was estimated by comparison to migration of Biorad protein molecular mass standards which are marked with a log scale at the bottom of the autoradiograph (Phosphorylase, 97.4 kD; BSA, 66.2 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD; Soybean trypsin inhibitor, 21.5 kD and lysozyme, 14.4 kD).

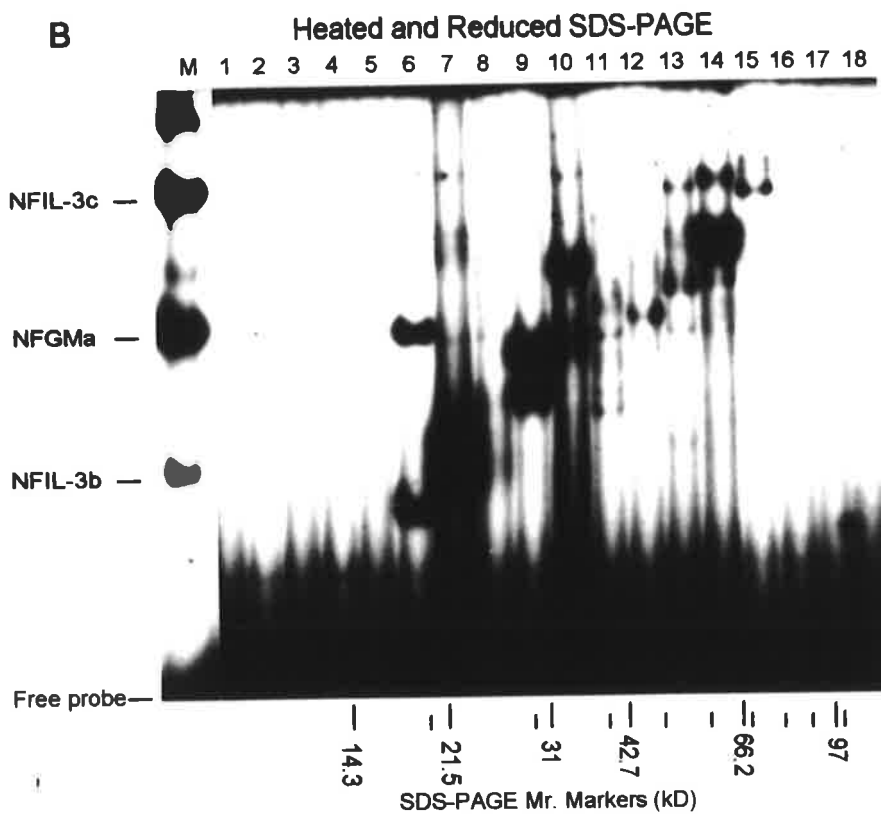
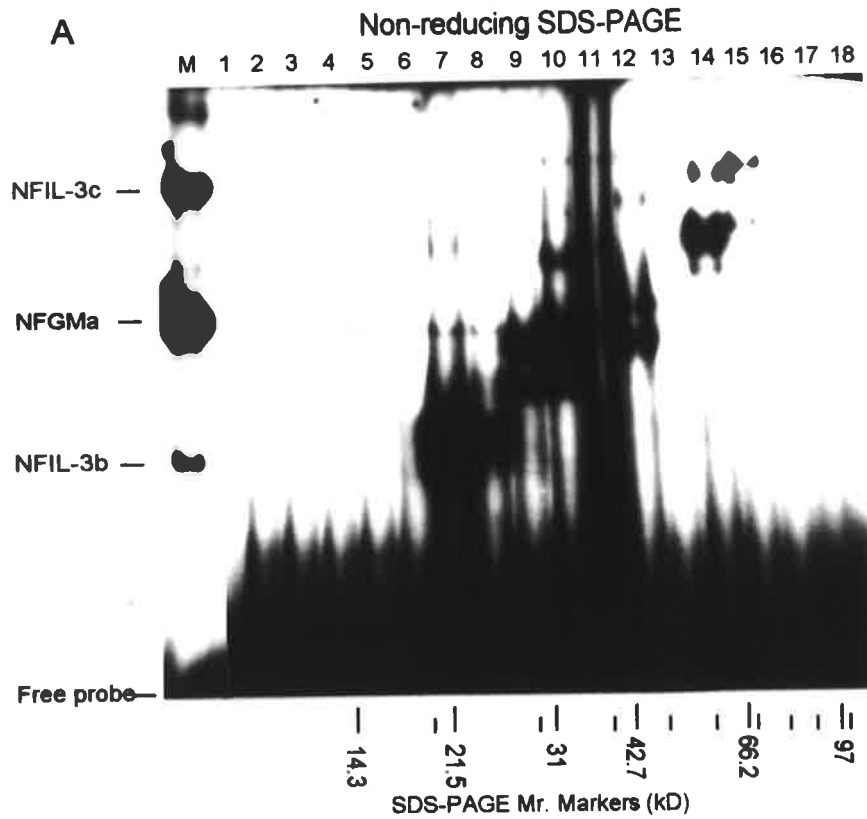
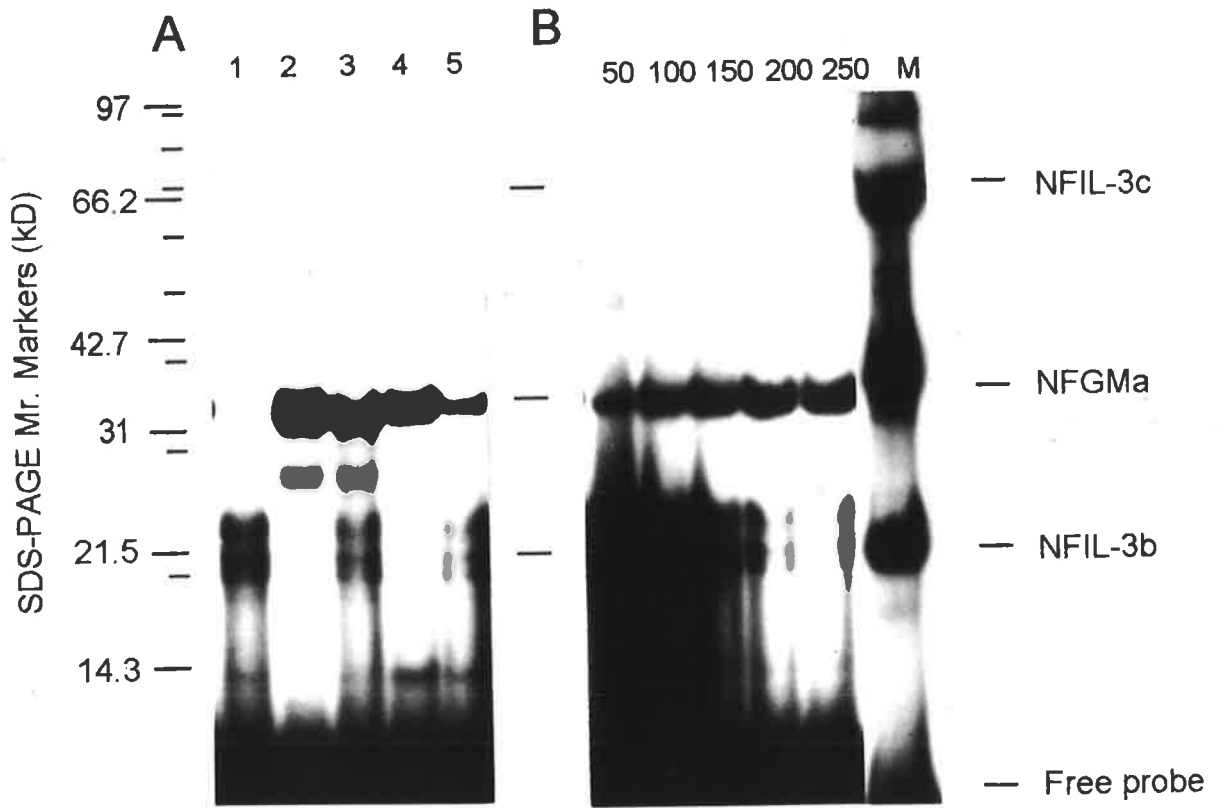


Figure 5.10 Combination and EMSA of SDS-PAGE eluted nuclear protein fractions

(A) Autoradiograph of EMSA where different fractions of renatured protein from SDS-PAGE were combined before addition of the labelled IL-3 CK1/CK2 EMSA probes. The fractions used were taken from the SDS-PAGE separation shown in figure 5.9A and B. Track 1, 21 kD NFIL-3b (fraction 5.9B #7); track 2, 35 kD non-reduced form of NFGMa (fraction 5.9A #12); track 3, mixed NFIL-3b (fraction 5.9B #7) and NFGMa (fraction 5.9A #12); track 4, the 16 kD reduced form of NFGMa (fraction 5.9B #6); track 5, mixed NFGMa (fraction 5.9B #6) and NFIL-3b (fraction 5.9B #7). A log scale showing the position of protein molecular mass standards is shown to the left of the panel and the positions of specific IL-3 DNA binding complexes are indicated to the right of the panel.

(B) Titration of the KCl concentration of the EMSA buffer of the mixed NFGMa and NFIL-3b fractions (NFGMa, reduced form fraction 5.9B #6 and NFIL-3b, fraction 5.9B #7) from 50 mM to 250 mM. The sample applied to the SDS-PAGE gel is shown in the rightmost lane (M). The positions of specific IL-3 DNA binding complexes and free probe are indicated to the right of the panel.



NFIL-3b or NFIL-3c do not vary in samples that were heated and reduced suggesting that these binding factors may consist of only one subunit.

It was of also interest to determine if any of the less mobile bands evident in the EMSA of renatured nuclear proteins, particularly NFIL-3c, could be reconstituted by mixing of fractions containing SDS-PAGE purified and renatured NFGMa or NFIL-3b. Aliquots of the nuclear proteins that were renatured from SDS-PAGE gel slices were mixed prior to addition of labelled IL-3 CK1/CK2 EMSA probes (Figure 5.10A). Although faint less mobile EMSA bands were present above NFGMa, particularly in the track containing the non-reduced form of NFGMa, no evidence of the reconstitution of NFIL-3c or other or less mobile complexes was obtained by EMSA analysis of mixed samples that included either the 35 kD non-reduced form of NFGMa (Figure 5.10A track 3) or the 16 kD reduced form of NFGMa (Figure 5.10A track 5). Titration of the KCl concentration of the EMSA buffer in the mixed samples from 50 mM to 250 mM also had no effect on the formation of higher complexes from mixed fractions containing NFGMa and NFIL-3b although a marked salt dependency in NFIL-3b complex formation was detected with an optimum KCl concentration of 50 mM or less (Figure 5.10B). In contrast, the binding of the 16 kD form of NFGMa was increased by the addition of up to 250 mM KCl.

5.3 Discussion.

The transcriptional regulation of the IL-3 gene is likely to be complex and may involve the interaction of both negative and positive acting nuclear regulatory proteins from binding sites in several regions of the IL-3 promoter. The properties of the cytokine elements CK1 and CK2 (Shannon *et al.*, 1988) are of particular interest because of their appearance in the promoters of other cytokine genes. The experiments detailed in this chapter have identified at least three constitutive nuclear factor complexes, NFGMa, NFIL-3b and NFIL-3c, that bind to the IL-3 CK1/CK2 elements. One of these, NFGMa, has been previously characterised in this laboratory by

Shannon *et al.* (1988) as a factor that binds to the GM-CSF CK1 element. Cross-competition EMSA with GM-CSF and IL-3 probes show that NFIL-3c, also recognises the related cytokine elements of the GM-CSF promoter. Mobility shift assays employing competing binding sites suggest that NFIL-3b binds exclusively to the IL-3 sequences and probably to the CK2 element and that both NFGMa and NFIL-3c may bind to the sequences within the CK1 element. In respect of NFGMa, Shannon *et al.* (1990) and Kuczek *et al.* (1991) showed by EMSA and modification interference assays that the central AAGG bases in the noncoding strand of the GM-CSF and G-CSF CK1 elements are important for NFGMa binding and also that this motif is required for the TNF- α responsiveness of the G-CSF CK1 element. The AAGG motif is also conserved in the IL-3 CK1 element. Shannon *et al.* (1990) have established a hierarchy in the affinity of the binding of NFGMa to the CK1 element with the IL-3 element having the strongest binding followed by GM-CSF, G-CSF and IL-5 in descending order of binding affinity. Interestingly the IL-3 CK1 element contains an indirectly repeated motif overlapping the non-coding strand AAGG sequence, part of this palindrome is preserved in the GM-CSF CK1 element but only one related repeat unit is found in the G-CSF and IL-5 CK1 elements (Figure 5.11). It is tempting to speculate that NFGMa binds to the CK1 element through this palindromic structure perhaps as a dimer of similar or identical subunits. The differences in the NFGMa binding affinity for the different conserved CK1 elements may therefore be explained by the extent of preservation of the symmetrical repeat units within the CK1 elements. Observations of the multiple subunit structure of the NFGMa support such a proposal. Size exclusion chromatography suggests that the NFGMa complex is approximately 35 kD. Although the mobility of protein complexes bound to DNA in EMSA is influenced by protein complex conformation it is largely dependent on size of the protein/DNA complex (Orchard and May, 1993). Alignment of the positions of the 20 kD NFIL-3b and 70 kD NFIL-3c EMSA complexes to a log scale of molecular weight allows a crude approximation of size of the NFGMa complex in EMSA. Estimates of NFGMa size by this method correlate

Figure 5.11 Alignment of palindromic sequences within the conserved cytokine promoter element CK1

The base sequence of the conserved CK1 elements and their flanking bases in the IL-3, GM-CSF, G-CSF and IL-5 genes (Shannon *et al.*, 1990) are aligned to show maximum similarity. The extent of the CK1 element is indicated by an horizontal line. Inverted repeats in the IL-3 CK1 element are indicated by converging arrows. Similar imperfect repeats in the GM-CSF, G-CSF and IL-5 CK1 elements are also indicated by arrows. Bases that do not conform to the IL-3 repeat sequence are indicated by an asterisk.

CK1

IL-3 TATGGAGGTTCCATGTCA
 → ←

GM-CSF CCAGGAGATTCCACAGTTC
 → ←

G-CSF ATCAGAGATTCCACAATT
 ←*

IL-5 CCCAAAGATTCCTTCGTAA
 ←*

approximately to the 35 kD size estimate obtained by native G100 gel chromatography (Figure 5.10). In contrast, SDS-PAGE fractionation of heated and reduced nuclear extracts detected a 16 kD fraction that, after renaturation, could reconstitute the NFGMa binding activity in EMSA (Figure 5.9). These initial observations of the nature of the subunit structure of the NFGMa complex are consistent with the formation of NFGMa from two 16 kD subunits. Furthermore, it is possible that higher order, tetrameric, NFGMa complexes can also be formed. Preliminary evidence was obtained in SDS-PAGE experiments of novel EMSA complexes at approximately 60 to 70 kD (Figure 5.8 tracks 48-60). Complexes with the same EMSA mobility are also present in only the NFGMa containing fractions at 30-35 kD (Figure 5.8 tracks 18 and 20). However, since these novel EMSA bands do not appear in EMSA of crude nuclear extracts, it remains to be determined whether they represent specific IL-3 binding activities, contain NFGMa, or are artefacts of the manipulation of the nuclear extracts. It is possible that the redox state of the NFGMa subunits may regulate their recruitment into the 35 kD multimeric form found in crude extracts since the 16 kD subunit form was detected under reducing conditions in SDS-PAGE/EMSA assays. However, the 16 kD form of NFGMa was also detected in this laboratory by SDS-PAGE/EMSA analysis of HUT78 cell nuclear extract samples that were heated in loading buffer in the absence of reducing agents prior to SDS-PAGE (Dr. M.F. Shannon and F. Occhiodoro personal communication), suggesting that the NFGMa subunits may form a stable dimeric complex that is partially resistant to denaturation by SDS in the loading buffer used in non-reducing SDS-PAGE, but which disassociate after heating in SDS-PAGE loading buffer. The presence of a faint NFGMa like binding activity detected in the 45 kD non-reduced SDS-PAGE fractions matches with the size, previously determined by protein/DNA UV-crosslinking, of HUT78 NFGMa bound to the GM-CSF CK1 element (Shannon *et al.*, 1990). Clearly, further studies are needed to understand the characteristics of NFGMa complex formation and to resolve the exact nature of the IL-3 NFGMa binding site. Initial attempts in this laboratory of modification interference assays with

crude nuclear extracts were not successful and these experiments suggested that such an approach may require cloned, pure or at least enriched fractions of the nuclear factor complexes in question.

The binding of NFIL-3c like that of NFGMa is competed by the addition of both IL-3 and GM-CSF CK1/CK2 sequences. SDS-PAGE/EMSA analysis and the native G100 column chromatography suggests that NFIL-3c is a factor of approximately 70 to 75 kD. Interestingly the affinity and competition behaviour of NFIL-3c closely matches that of NFGMa and it is competed by the same mutant oligonucleotides, however, the possible relationship of these two complexes remains equivocal. No NFIL-3c binding activity was detected however, in EMSA of the separate 16 kD or 35 kD forms of NFGMa obtained from SDS-PAGE fractions (Figure 5.8 and 5.9A and B) and combining the low molecular mass binding factors also failed to reconstitute NFIL-3c (Figure 5.10). Interestingly the addition of one protein fraction to the other in this experiment did not result in an increase or decrease in the binding of the other implying that there is no cooperativity between NFGMa and NFIL-3b in binding to the IL-3 CK1/CK2 elements. The EMSA in this study were carried out under conditions of probe excess and so do not detect the exclusion of binding of one factor by the others. Further EMSA experiments under conditions of limiting probe would be required to determine whether the binding of any of the specific IL-3 CK1/CK2 complexes is mutually exclusive of the others.

In contrast to that of the NFGMa and NFIL-3c complexes, the binding of the 20 kD NFIL-3b complex seems to be specific for the conserved IL-3 CK2 element and is not altered in EMSA by competition with the GM-CSF CK1/CK2 oligonucleotides. Analysis by EMSA of renatured SDS-PAGE fractions and size exclusion chromatography fractions suggests that the NFIL-3b binding complex is a single protein complex approximately 20 kD in size. Recently in this laboratory, Coles *et al.* (1994) have enriched nuclear extracts from HUT78 by heparin sepharose chromatography and characterised a 22 kD protein, NF-GMb, that can bind the

GM-CSF CK1/CK2 elements at repeated 5'CAGG3' sequences which overlap binding sites for positive factors. In addition, a second nuclear binding protein that binds to the same sequence, NF-GMc, was identified. These authors also showed that the 5'CAGG3' sequences are involved in repression of TNF- α inducible expression directed by GM-CSF promoter sequences in fibroblasts. Although NF-GMb is of similar size, co-migrates with NFIL-3b in the same SDS-PAGE gels slice (G. Ryan, Dr. M.F. Shannon, and F. Occhiodoro, data not shown) and neither binding complex changes apparent size when electrophoresed under reducing conditions, the relationship between the two nuclear binding complexes still remains to be determined. Sequence comparison shows that the 5'CAGG3' sequence is not conserved in the IL-3 CK1/CK2 elements (Figure 5.3B) perhaps explaining the inability of the IL-3 and GM-CSF CK1/CK2 sequences to cross compete for the binding of either NFIL-3b or NF-GMb complexes. Surprisingly, NF-GMb and NF-GMc bind only to single stranded DNA and it will therefore be interesting in future experiments to determine if the NFIL-3b also binds exclusively to single stranded DNA.

The NFIL-3b complex, like NFGMa and NFIL-3c, is present in nuclear extracts of a wide variety of cell types, although it appears to be absent or as an altered form (labelled c' in Figure 5.1) in the melanoma cell line LiBr. It is interesting that nuclear extracts from either stimulated or unstimulated HUT78 T-cells consistently contained a lower level of the NFIL-3b complex but comparable levels of NFGMa and NFIL-3c (Figure 5.1A). Neither HUT78 or LiBr cells can be induced to express the endogenous IL-3 gene (G. Ryan and Dr. P. Cockerill, unpublished results). Stimulation of HUT78, LiBr, or of the bladder carcinoma cell line 5637, which produce GM-CSF or of Jurkat, HSB2 and MLA144 T-cells failed to change the level of any of the three specific nuclear protein complexes that bind to the IL-3 CK1/CK2 elements suggesting that the role of these proteins play in the regulation of IL-3 transcription may be regulated

by post-translational modification of the DNA binding complexes or through their interactions with other inducible regulatory proteins.

The involvement of the AP1 complex in the regulation of IL-3 transcription has been examined and both consensus and non-consensus AP1 binding sites have been identified in the IL-3 promoter (Shoemaker *et al.*, 1990, Mathey-Prevot *et al.*, 1990, Park *et al.*, 1993, Davies *et al.*, 1993 and Gottschalk *et al.*, 1993). In this study the low level binding of purified AP1 to the CK1/CK2 elements was detected by DNase I footprinting. It has been well documented that DNA binding of the transcription factor, AP-1 is activated by phorbol ester treatment of cells (Angel and Karin, 1991) and is also induced in T-cells by PHA treatment (Park *et al.*, 1993). Although the CK1/CK2 region is only partially protected from DNase I digestion by AP1 binding it is puzzling that no PHA/PMA inducible binding complexes were detected by EMSA reactions in the crude Jurkat cell nuclear extracts nor in heparin sepharose enriched extracts from stimulated cells. It is possible that the *in-vitro* binding conditions and EMSA gel system used do not allow detection of weak AP1 binding, however under similar conditions, subsequent EMSA analysis in this laboratory of nuclear protein interactions with the IL-3/GM-CSF intergenic enhancer using extracts from the same Jurkat cells detected AP1 binding to the NFAT sites present in the enhancer (Cockerill *et al.*, 1993).

To assist in the characterisation of the specific IL-3 CK1/CK2 binding complexes and to facilitate the eventual sequencing and cloning of the complexes, the suitability of techniques for the separation and purification of the nuclear binding proteins were evaluated. Both heparin sepharose and hydroxylapatite column chromatography enriched the nuclear extracts for the binding proteins by removing a large amount of nuclear protein that did not bind to the column in TM.1 buffer and fractionated the bound nuclear protein based on the salt optimum of binding to the column matrix. A second round of chromatography on either of the columns with a lower protein load and a continuous salt gradient increased the separation efficiency of the columns

particularly in the case of the heparin sepharose matrix. Although neither matrix achieved complete separation of the three IL-3 CK1/CK2 specific binding complexes, chromatography on heparin sepharose was able to separate much of the NFGMa binding activity from NFIL-3b and NFIL-3c and hydroxlyapatite chromatography allowed separation of NFIL-3b and NFIL-3c. Furthermore, molecular size fractionation can also effectively separate the 20 kD NFIL-3b from the 70 kD NFIL-3c therefore the combination of heparin sepharose and higher resolution FPLC molecular sieving matrices may be useful in obtaining enriched binding factors for further analysis or as the starting material for DNA affinity chromatography to obtain quantities of sufficiently pure binding factor preparations required for protein sequencing. In addition, since binding in EMSA was detected after SDS-PAGE and renaturation from urea containing elution buffer, denaturing molecular sieving chromatography may also be an effective method to enrich for the lower molecular weight binding factors NFGMa and NFIL-3b since the bulk of the extracted nuclear protein would be eliminated in the higher molecular weight fractions in one step. A subsequent or second round heparin sepharose affinity chromatography step in such a purification scheme would help to concentrate specific binding factors diluted by molecular sieving chromatography.

In conclusion, EMSA analysis of Jurkat cell nuclear extracts have identified at least three widely expressed and constitutive putative nuclear transcription factor complexes that can bind in a specific manner to the conserved IL-3 CK1/CK2 elements. One of these, NFIL-3b, is appears to be a novel factor that binds only to the IL-3 CK2 element. The others, NFIL-3c and a previously identified factor, NFGMa, bind also to the conserved CK1 element in the GM-CSF promoter. The functions of the NFGMa, NFIL-3b and NFIL-3c binding sites in the IL-3 promoter are at present unknown, however, NFGMa binding to the G-CSF CK1 element is induced in embryonic fibroblasts by TNF- α and the CK1 element has been shown to participate in the response of the G-CSF promoter in TNF- α and IL-1 β stimulated fibroblasts

(Shannon *et al.*, 1992). The molecular size of the IL-3 specific binding factors was investigated by native molecular sieving chromatography which indicated the size of NFGMa to be approximately 35kD, NFIL-3b, 20 kD and NFIL-3c, approximately 70 to 75 kD. Separation of crude Jurkat cell nuclear extracts by reducing SDS-PAGE suggested that NFGMa may composed of at least two 16 kD subunits and that both NFIL-3b and NFIL-3c appear to be single protein complexes. It is to be expected that further elucidation of the function of the conserved IL-3 cytokine elements will be facilitated by the characterisation of the specific CK1/CK2 element binding factors. The role of the transcriptional activator AP1 remains in question. Transient transfection experiments in Jurkat cells have suggested that the conserved cytokine elements can respond to PHA/PMA stimulation at least in the context of an heterologous promoter and DNase I footprinting assays show that the CK1/CK2 elements may also bind the transcriptional activator complex AP1, however, no inducible nuclear complexes were detected by EMSA analysis. Chromatography techniques employing heparin sepharose and hydroxylapatite affinity matrices were tested for the ability to enrich or separate the IL-3 specific binding factors from T-cell nuclear extracts. These preliminary data will be of use in subsequent efforts towards the purification to homogeneity, peptide sequencing and the eventual cloning of these putative IL-3 transcription regulatory factors.

Chapter 6

Final discussion and summary

6.1 Final discussion and future directions

The work presented in this thesis has shown that both induction of transcription and stabilisation of mRNA have important roles in the regulation of IL-3 gene expression in T-lymphocytes. Moreover, the coordinate induction and repression of groups of cytokine genes, including IL-3, in activated human and murine T-lymphocytes suggests that T-cell activation induces expression by common mechanisms. The transcription control elements found in both the IL-3 proximal promoter and the promoters of similarly regulated cytokine genes are likely to be major determinants of the T-cell restricted nature of IL-3 expression. This work, therefore, focused on the conserved cytokine elements CK-1 and CK-2 which are found in the promoters of IL-3 and several other T-cell expressed cytokine genes. Over the past several years and during the course of this work considerable information has accumulated regarding transcriptional and post-transcriptional regulatory mechanisms and the promoter elements and nuclear factors that regulate expression of the IL-3 gene and related cytokines. This final chapter will therefore discuss recent progress and summarise important developments in two areas relevant to the work in this thesis. Firstly, recent findings on the molecular mechanisms involved in regulation of cytokine mRNA stability and secondly, the identification of *cis*-elements that enable the regulation of IL-3 gene transcription and the role of these factors in maintaining

the tissue specificity of IL-3 expression in T-cells. Throughout the discussion I will also provide suggestions for the directions of future research.

6.1.1 Cell restricted expression of IL-3 mRNA

Following the cloning of the human IL-3 gene, initial studies showed that IL-3, although co-expressed in T-cells, is distinguished from other haemopoietic growth factors such as G- and GM-CSF by its restricted cellular sources and more stringent requirements for induction. While no expression of IL-3 mRNA or the reporter constructs was detected in this study in HEL human fibroblasts, human IL-3 has recently been detected in other cell types including neutrophilic and eosinophilic granulocytes (Kita *et al.*, 1991 and Fujisawa *et al.*, 1994), stromal cells of the thymus (Dalloul *et al.*, 1991) and keratinocytes (Dalloul *et al.*, 1992). Thymic epithelial cells for example, produce IL-3 and a range of cytokines, including interleukins -1 α , 1 β , -6 and 8, GM- and M-CSF, IGF and TGF α . Interestingly, the production of thymic epithelial cell-derived cytokines is regulated both by adhesion molecules (LFA-3) and by a range of soluble factors IL-1 α , TGF α , EGF, IL-4 and IFN γ . At least two of these, EGF and TGF α , have been shown to regulate various cytokine mRNAs at a post-transcriptional level by increasing cytokine mRNA stability (reviewed by Le and Singer, 1993). The recent development of culture systems and cell lines from these IL-3 expressing tissues (Frischkoff and Rossi, 1990, Hurlin *et al.*, 1991, Mayumi, 1992 and Ueno *et al.*, 1994) presents an opportunity for future studies to define more clearly the cis-elements and the molecular mechanisms involved in tissue-specific and inducible regulation of IL-3 and other related cytokine genes in a non-T-cell background.

6.1.2 IL-3 mRNA stability

Work described in this thesis and other reports have shown that the production of IL-3 and related cytokines is absent in freshly isolated or resting human peripheral T-cells (reviewed by Ullman *et al.*, 1990 and Dokter *et al.*, 1993) despite the measurable constitutive transcription detected by the nuclear transcription run-on experiments in our hands. These observations suggest that in unstimulated cells, IL-3 mRNA may be actively and rapidly degraded. Furthermore, addition of PMA to cells stimulated with PHA (this work) or concanavalin A (Dokter *et al.*, 1993), produced an increase in both the IL-3 mRNA transcription rate and the stability or half-life of the IL-3 mRNA. The same or a similar mechanism can be triggered through a different pathway by the CD28 receptor (Lindsten *et al.*, 1989, Williams *et al.* 1992, August *et al.*, 1994 and reviewed by Umlauf *et al.*, 1993). Recently, Dokter *et al.* (1993) have shown that the stromal derived growth factor, human IL-7, also modulates the expression of T-cell cytokines including IL-3, by a mechanism involving increased mRNA stability. Furthermore, the inhibitory effects of glucocorticoids (Peppel *et al.*, 1991 and Amano *et al.*, 1993) and cyclosporin A (Nair *et al.*, 1994) on cytokine and pro-inflammatory mediators may also be due, in part, to specific increases in mRNA turnover. The convergence of both stimulatory and inhibitory signals on the regulation of cytokine mRNA stability suggests that this mechanism plays a crucial role in the regulation of induction or suppression of cytokine gene expression in T-cells. Moreover, the importance of modulations in mRNA stability in the regulation of IL-3 expression was illustrated in murine lymphoid cell lines which contain alterations in the IL-3 locus. Aldgate and McCubrey (1993) showed that an intracisternal A particle which disrupts the AUUUA sequence motifs of the 3' mRNA untranslated region of murine IL-3 can

activate IL-3 gene expression, in this case, by altering the IL-3 mRNA half-life without a change in the constitutive transcription rate. The deregulated expression of IL-3 in these cells resulted in their autocrine transformation and their ability to form tumours upon injection into syngeneic mice.

At present, the precise molecular mechanisms involved in the maintenance of the very low level of IL-3 mRNA in unstimulated T-cells and the removal of IL-3 mRNA after the transcriptional phase of expression remains poorly understood. Recent developments indicate that at least 6 cytoplasmic proteins bind to reiterated AUUUA sequences present in the 3'-UTR sequences of cytokine mRNAs (AREs, Hamilton *et al.*, 1993). Two of these were identified by immunoblotting as hnRNP A1 and hnRNP C and it has been suggested that these proteins may play an important role in regulating the stability of ARE containing cytokine mRNAs in addition to their previously characterised roles as pre-mRNA binding proteins involved in nuclear mRNA processing (Hamilton *et al.*, 1993). Furthermore, Gillis and Malter (1991) and Malter and Hong (1991) identified and characterised a cytoplasmic phosphoprotein, AUBF, which can be induced to bind AREs by phosphorylation following PMA or ionophore treatment. Recently, Rajagopalan and Malter (1994) showed that AUBF was associated largely with the polysomal fraction and that its depletion destabilises GM-CSF mRNA. Muller *et al.* (1992) have suggested that AUBF may also be involved in regulating nuclear transport of mRNAs that contain AREs. The absence, however, of a clear understanding of the sequences and structural features of AREs that are required for the destabilising function have hampered further elucidation of their mode of action and the basis of their specificity. Most recently, however, Akashi *et al.* (1994) demonstrated that the number and location of AUUUA elements

correlates with rapidity of turnover but that the AUUUA motifs cannot alone account for the rapid degradation of ARE containing mRNA. Lagnado *et al.* (1994) have suggested that the smallest functional sequence within the AREs may be UUAUUUUA(U/A)(U/A). Their studies also showed that the degree of destabilisation depends on the number of copies of such a sequence and the degree of mismatch in the first and last two positions. In addition, Chen *et al.* (1994), combined extensive mutagenesis of the *c-fos* ARE with *in vivo* analysis of mRNA stability and revealed the presence of two functionally distinct but interdependent domains that constitute the *c-fos* ARE. Domain I contains the AUUUA repeats and forms the essential core unit necessary for mRNA destabilisation and can function as a destabilising element by itself. The second domain is a U-rich sequence which is located 3' to domain I. Although domain II can not act as a destabiliser it serves two critical roles. Firstly, it enhances the destabilisation ability of domain I by accelerating deadenylation and secondly, it may buffer decay impeding effects of mutations introduced in domain I. Similar AU- and U-rich sequences can be found in the IL-3 mRNA UTR but their exact contribution to the regulation of mRNA stability remains to be determined. Transfection of T-cells with synthetic IL-3-ARE constructs that can be expressed either constitutively or in a short pulse may help determine the fate of IL-3 mRNA in resting and stimulated cells of different tissues and would allow the characterisation of sequence motifs and factors responsible for the targeted destruction and/or protection of IL-3 mRNA. Clarification of the molecular mechanism(s) that control IL-3 mRNA stability will provide important information on IL-3 regulation and activation of T-cell cytokine gene expression.

Although AREs have been strongly implicated in the regulation of IL-3 in resting cells, conceivably other mechanisms, such as premature mRNA termination could also be involved. Of interest, Strobl and Eick (1992) have shown that retention of RNA pol II at, or just downstream of, the transcription initiation site is a critically important mechanism in the regulation of c-myc and there is now increasing evidence that a number of genes in *Drosophila* are regulated in a similar manner (O'Brien and Lis, 1991). Such mechanisms may account for the transcriptional signal detected in the absence of accumulating IL-3 mRNA in unstimulated T-cells. Strobl and Eick (1992) also showed that the preparation of nuclei for run-on experiments induces the release of Pol II held at the c-myc P₂ promoter and the generation of a strong run on transcription signal. If such a mechanism is active in the regulation of IL-3 mRNA expression it may account for the constitutive run-on transcription signal observed here and it will therefore be of importance to determine what signals are required to stall and/or release the activated IL-3 RNA Pol II complex.

6.1.3 Transcriptional control of IL-3 expression

Although much is now known about the biological role of IL-3 and its requirements for expression *in vitro*, the promoter regulatory elements and their cognate nuclear binding factors that regulate transcription of the IL-3 gene are less well understood. This thesis has shown by transient expression of a series of CAT reporter constructs that the proximal 600bp of the IL-3 promoter consists of several activating regions with intervening inhibitory regions that contribute to inducible and perhaps the constitutive expression of the IL-3 gene. The complexity of interactions between these positive and negative regions is just beginning to be understood.

It is puzzling that the levels of induction from the CAT reporter constructs used in this study were lower than those obtained by other groups who also used the Jurkat T-cell line in CAT reporter gene transfection experiments (Shoemaker, Hromas and Kaushansky, 1990, Park, Kaushansky and Levitt, 1993 and Gottschalk *et al.*, 1993). In each case reporter constructs used in those studies contained up to 42 bp of mRNA sequence from the 5' untranslated region. The reporter constructs used in this study were truncated immediately downstream of the start of transcription. The possibility is intriguing that there may be as yet unidentified transcription regulatory elements within the 5' -untranslated regions of the IL-3 mRNA which collaborate with the upstream activation elements to increase IL-3 transcription in stimulated cells.

Upstream of the consensus TATA box, the most 3' regulatory domain of the IL-3 promoter, termed here ACT0 (Figure 6.1), contains a CT/CG rich region (-47bp to -76bp) that is conserved in the murine IL-3 gene but has not been identified in other lymphokine gene promoters. This region of the IL-3 promoter was bisected in the construction of our -60bp reporter construct which did not respond to T-cell stimulation. Koyano-Nakagawa *et al.* (1994) however, have recently shown that a longer fragment of the IL-3 promoter (-86bp to +47bp) containing the CT/GC motif induced reporter gene transcription 7 fold in transfected Jurkat cells. These authors cloned three transcriptional regulatory proteins that bind to this fragment, EGR1, EGR2 and DB1. The DNA binding activity of one of these, EGR1, is inducible by stimuli that cause T-cell activation. However, co-transfection of either EGR1 or EGR2 expression vectors did not augment the response from -86bp IL-3 reporter constructs or from heterologous promoters containing multiple copies (3) of the CT/CG element. These results indicate that the minimal CT/CG element by itself does not have the

Figure 6.1 Regulatory elements in the IL-3 promoter

The sequence of the IL-3 promoter and 5' flanking region is shown from the first few codons of the leader peptide to base -857 relative to the start of transcription which is marked with an arrow and labeled +1. Functionally active domains of the promoter are shaded alternately light and dark gray and are labeled to the right of the diagram. The position of each restriction site used in this study to generate the truncated promoter reporter constructs is indicated, above the sequence, in bp relative to the start of transcription. The domain boundaries correspond to the ends of the promoter/reporter constructs that were used in this study and by others. The position of the TATA box is indicated by a dark shaded rectangle. The binding sites of protein complexes or of DNase I protection assay footprints are indicated by darkly shaded shapes positioned above or below the recognition element. Each protein complex is marked with the names of the binding proteins. The regulatory elements or consensus sequence elements are labeled with their name and synonym where possible. The extent of each of the elements is indicated by a horizontal bar. The inverted imperfect palindrome within the CK-1 element is indicated by converging arrows. The position of the CACC repeats is indicated above the sequence at -75bp and -80bp.

aggcccagtt gaaaccaggg agttgctctc ctttctcctc ccttgacctc acccctcaga
ccatgccaat tctgctctct aaacctccca ggcagcccc tccccagct ccagtgaca
gtgtctcag gtacctgagc tcagctctcg gtgctaccag agggactgca gggctgcaga

-674 NIP like
ggctgagtcc cacacgcagg gaacagccat gccactgcta gcagaccagt aagagaatgg
ccacctgggg cctgagcgcc ctgggccatc caccagaaac aaagtgtcaa ggagaagctg

-550
cccgaagccc atgggacaaa ccactgggga ctggaacacc agtaattctg tattgggaag
cggcaccaag agatgtgctt ctcagagcct gaggetgaac gtggatgttt agcagcgtga
ccggctacca gacaaactct catctgttcc agtggcctcc tggccacca ccaggacca

ACT3
Domain

SP1 5'- CK1
gcagggcggg cagcagaggg ccagggtagt ccaggtgatg gcagatgaga tcccactggg
-315 ICK-1
caggaggcct cagtgagctg agtcaggctt ccccttctctg ccacaggggt cctctcacct

NIP2
Domain

ACT2
Domain

NIP/ICK-1 EBS
gctgccatgc ttcccatctc tcctctctct tgacaagatg aagtgatacc gtttaagtaa

NIP

-173
tcttttttct tgtttcactg atcttgagta ctagaaagtc atggatgaat aattacgtct
IF-1 CK1/CD28 CK2/GATA3 (ACT-1B) (ACT-1A)
CBE NF-GMa NF-IL-3B AP-1 OCT-1
gtggtttct atggaggctc catgtcagat aaagatcctt ccgagccttg ccccacacca
NFIL-3A CRE

ACT1
Domain

CACC SP1 AP-1 FOOTPRINT
ccacctccc cgccttgccc ggggttgtag gcaccttgcg gctgcacata taaggcggga
EGR1 or 2 DBP CK3

ACT0
Domain

gctgttgcca actcttcaga gccccacgaa ggaccagaac aagacagagt gcctcctgcc

+1 Start of Transcription

gatccaaada tgagccgcc

Coding Sequences

ability to respond to T-cell activation signals transmitted through EGR1 or EGR2 (Koyano-Nagawa *et al.*, 1994).

In contrast to EGR1, the gene for DB1 is expressed constitutively and ubiquitously in human tissues. DB1 also binds specifically to the 5' end of the IL-3 CT/CG rich element and mutations of its recognition sequence decreased basal transcription activity but co-transfection with expression vectors of DB1 did not alter the transcriptional activity of IL-3 reporter plasmids, in either stimulated or unstimulated cells (Koyano-Nakagawa *et al.*, 1994). These results suggest the role of DB1 may be to regulate constitutive transcription. Interestingly, Cameron *et al.* (1994) detected an inducible DNase I footprint (-83bp to -73bp) overlapping the EGR1/2 and DB1 binding elements. The footprint was centered over two CACC repeats. Furthermore, similar motifs found in the adult β -globin are required for maximal expression and an erythroid-specific transcription factor that recognises this repeat has been cloned by Miller *et al.* (1993). However, the implied role of DB1 and the CACC repeat elements in regulation of IL-3 transcription has yet to be thoroughly examined.

The ACT0 region of the IL-3 promoter was also originally defined as a responsive region for the HTLV-1 transactivator, Tax (Nishida *et al.*, 1991). Although co-transfection of either EGR1 or EGR2 with the IL-3 reporter constructs had no effect on the IL-3 promoter transcriptional response in Tax expressing cells, co-transfection of DB1 expression plasmids, however, caused a significant increase in transcription from the -86bp IL-3 promoter (Koyano-Nakagawa *et al.*, 1994). At present the mechanism by which Tax transactivates transcription is unclear. While several sequences including the 21bp enhancer of the HTLV I long terminal repeat (Fujisawa *et al.*, 1989), NF- κ B binding sites (Ballard *et al.*, 1988) and the serum

response element (Alexandre and Verrier 1991) have been reported as Tax-responsive elements, none share homology with the CT/CG rich element of IL-3. Moreover, there is in general, no sequence homology among the Tax-responsive elements and no direct binding of Tax to those sequences has been demonstrated. However an association of DNA binding proteins with Tax has been reported (Fujii *et al.*, 1992, Hirai *et al.*, 1992, Suzuki *et al.*, 1993 and Wagner and Green 1993).

The ACT0 region also contains an element resembling the consensus sequence for the cAMP responsive transcription factor AP-2 and at its 5' end an overlapping GC rich element, predicted by computer homology comparison to bind the transcription factor SP1. Our *in vitro* DNase I protection analysis detected a broad footprint and indicated that purified AP-2 protein can bind to IL-3 promoter sequences in the region between -67 bp to -40bp. Furthermore, our comparison of the IL-3 and GM-CSF proximal promoters revealed an additional conserved 10bp element (-50bp to -59bp in the GM-CSF promoter and -43bp to -53bp in the IL-3 promoter) (Figure 6.1). This element, termed here CK3, exhibits only a one base mismatch between the two human genes, although there are 2bp differences in the murine GM-CSF gene sequence and it is not at all conserved in the murine IL-3 gene. Of interest, this element in the GM-CSF promoter is found between the 2nd and 3rd CATT/A element and covers half of the inverted repeat constituting the GM-CSF ICK-1 transcription inhibitor binding element that was identified by Nomiya *et al.* (1993). More recently, Fraser *et al.* (1994) identified a 45kDa negative regulatory factor, GM-NRF-I, which binds to the GM-CSF CK3 element and possibly a juxtaposed 3' region to the exclusion of positive regulatory factors. It is not known whether this complex can bind to the CK3 element in the context of the IL-3 promoter. Given the abundance of potential binding

motifs in the ACT0 domain, their presence in other cytokine gene promoters and the ACT0 region's proximity to the TATA box, it seems likely that this region will be of great importance in regulation of expression of the IL-3 promoter and may contribute to its T-cell specificity. Rather than a simple deletional analysis which may alter the critical spacing pattern of promoter elements in this TATA proximal region, transfection experiments with *in vitro* mutagenised reporter constructs which exclude the binding of one or more proteins would be required to unravel the transcription factor interactions within this region and their possible interactions with the upstream and distant regulatory elements.

The second activation domain, ACT1, lies further 5' to that of ACT0 (Figure 6.1). This region was defined by the -173bp reporter construct and is the smallest promoter fragment used in this study that maintained the ability to respond to T-cell activation with PHA/PMA. The ACT1 domain contains the conserved cytokine elements CK-1 and CK-2 (Shannon, Gamble and Vadas 1988). Initial deletion analysis of the IL-3 cytokine elements (Shoemaker, Hromas and Kaushansky, 1990 and Mathey-Prevot *et al.*, 1990) in PHA/PMA stimulated or unstimulated cells, failed to establish a transcriptional regulatory role for these elements. Although, given the compact nature of these elements, it is not surprising that removal of an individual element and the consequent disruption of the spacing arrangement of the remaining elements or the changing of the surrounding context of neighbouring elements could alter their function. Transfection of cells with our reporter constructs containing multiple copies of the CK-1/CK-2 elements within the context of a heterologous constitutive promoter, suggested that together these elements confer a constitutive negative regulatory activity in T-cells but that they can also respond to PHA/PMA stimulation.

To further investigate the regulatory activities of these elements and to avoid the limitations of a deletional approach, future functional studies should employ promoter constructs in which the CK-1/CK-2 elements are altered by *in vitro* mutagenesis rather than deletion. Initial studies to define the binding sites of NF-GMa, NF-IL3b and NF-IL3c, identified mutations which may interfere with the binding of one or more of the CK-1/CK-2 complexes (Figure 5.2) and provide a good starting point for the design of CK-1/CK-2 mutated promoter constructs. Furthermore, while the SDS-renaturation-EMSA experiments revealed the presence of a number of additional complexes capable of binding to the CK-1/CK-2 elements their role in the function of these elements remains unsupported by EMSA utilising crude nuclear extracts. Alternative techniques such as UV crosslinking of BrdUdr labeled EMSA probes may enable, under more physiologic conditions, the *in vitro* determination of the number and characteristics of proteins capable of binding to these elements. The development of protein enrichment procedures for extracts prepared from primary T-cells or PHA blasts will also assist in defining the precise recognition sequences for the specific transcription factor complexes by facilitating methylation interference footprinting and DNase I protection assays. Moreover the development of more efficient techniques for *in vivo* footprinting (Hornstra and Yang, 1993 and Garrity and Wold, 1992) may assist in determination of the physiological relevance of factor binding activity *in vivo*.

Other compelling evidence that the conserved cytokine elements play an important role in IL-3 regulation is provided by cells costimulated with α -CD28 antibodies. Although in this study the activation of transcription from the proximal promoter fragments in response to CD28 stimulation was marginal, Fraser and Weiss (1993)

have shown that both the IL-3 and GM-CSF as well as the IL-2 and IFN γ promoters respond to stimulation of the CD28 receptor. Such a discrepancy may be due to a lesser extent of crosslinking of the CD28 receptor in this study as a result of the use of very high levels of α -CD28 antibody (Ledbetter *et al.*, 1990). Fraser and Weiss (1993) detected a CD28 inducible complex, CD28RC, that binds to both the IL-3 and GM-CSF CK-1 elements and showed that mutation of the IL-3 or GM-CSF CK-1 elements eliminated the transcriptional response to stimulation through the CD28 receptor. These results suggest that the interaction of CD28 with its counter receptor on antigen presenting cells may play a crucial role in coordinating the transcription of several cytokines secreted by activated T-lymphocytes.

While the transcription factors involved in the CD28 response have not been fully characterised, evidence suggests that activation of IL-2 *via* stimulation of CD28 is mediated through a CK-1 like element, CD28RE (Fraser *et al.*, 1991). More recent observations suggest that CD28 stimulation in concert with stimulation of the TCR-CD3 complex (Romano *et al.*, 1994) or alternatively with stimulation of the CD2 pathway (Costello *et al.*, 1993) can accelerate the nuclear translocation of c-Rel, NF- κ B p50 (NF κ B1) and p65 (RelA) (Bryan *et al.*, 1994). In addition CD28 costimulation can induce the formation of NF κ B1/RelA heterodimers as well as c-Rel homodimer or a c-Rel/RelA heterodimer. Moreover, Gosh *et al.* (1993) has recently shown that c-Rel, RelA and NF κ B1 are all associated with the IL-2 CD28RE in CD28 costimulated cells. Moreover, Dunn *et al.* (1994) have shown that RelA (p65) can bind to the related GM-CSF CK-1 element. The implied role of these factors and the IL-3 cytokine elements in the IL-3 CD28 response however, requires further confirmation.

In addition to their role in the CD28 mediated response described above, evidence of the involvement of the HTLV transactivator Tax in the transcriptional response from the conserved cytokine elements lends further weight to the proposal that the conserved elements may play an important role in the regulation of IL-3 and other T-cell cytokine genes. Involvement of the Tax transactivator in regulation of IL-3 transcription in transiently transfected T-cells was initially demonstrated by Miyatake *et al.* (1988). More recently, transient co-transfection studies with an IL-3 CAT reporter construct and an HTLV Tax expression construct demonstrate that Tax can transactivate the IL-3 promoter in HTLV-uninfected cells and that sequences within the ACT1 domain are required for the transactivation activity (Wolin *et al.*, 1993).

These observations in Tax expressing cells suggest that members of the NF- κ B or CREB families of transcription factors may be involved in IL-3 transcriptional regulation. Of interest, Lanoix *et al.* (1994) have shown that NF- κ B2 (p100) and c-Rel are over expressed in HTLV infected or Tax expressing cells. The same authors suggested that there is a Tax dependent correlation between expression of NF- κ B2 and processing to the DNA binding NF- κ B2 (p52) form. Furthermore, they propose that altered NF- κ B2 expression and processing allows continuous nuclear expression of this otherwise cytoplasmic transcription factor and in conjunction with overexpression of c-Rel, NF- κ B2 alters the NF- κ B signalling pathway. Tax dependent transactivation of transcription may also be transmitted through pathways involving the CREB/ATF family of transcription factors (Zhao and Giam, 1992). Interestingly, the transactivating activities of Tax with the NF- κ B or CREB/ATF families of transcription factors are mediated through distinct functional domains of the Tax protein and can be differentiated by missense mutations in the Tax gene (Smith and

Green 1990). Recent results from this laboratory suggest that Tax transactivates the human GM-CSF promoter through a 44bp region containing the CK-1 element, a potential CREB/ATF binding site which overlaps the CK-2 element and the NF- κ B binding site (Himes *et al.*, 1993). Moreover, as discussed above, Dunn *et al.* (1994) have shown that the NF- κ B family member RelA (p65) can bind to the GM-CSF CK-1 element and recent results suggest that Tax can form a direct association with RelA (p65) (Lacoste *et al.*, 1994). Utilising mutated Tax proteins, Himes *et al.* (1993) have shown that activation of both NF- κ B and CREB/ATF family proteins is essential for Tax transactivation of GM-CSF transcription. They also speculate that the response of the IL-3 promoter to Tax may be at least partially explained by RelA (p65) binding to the CK-1 element. It is possible that arrangement of the CRE like site, ACT-1A, (Figure 6.1 and discussion below) immediately upstream of the CK-1 element may be functionally equivalent to the arrangement of Tax responsive factor binding sites found in the GM-CSF promoter.

Paradoxically, while the IL-3 promoter reporter constructs are transactivated by Tax IL-3 mRNA cannot be detected in HTLV infected cell lines, and in contrast GM-CSF is constitutively expressed in HTLV infected cells (Wolin *et al.*, 1993). Differences in the IL-3 promoter binding proteins in infected *versus* uninfected cells may account for this discrepancy. DNase I protection analysis of the proximal IL-3 promoter detected a strong footprint over the CK-1 and CK-2 elements in HTLV infected cells but not in uninfected cells although protection of the NF-IL3-A and the CREB like elements (ACT-1A/B) was observed in either case (Wolin *et al.*, 1993). Importantly, deletion of the CK-1/CK-2 containing region resulted in a significant increase in the basal and induced IL-3 promoter activity in HTLV-infected but not uninfected cells. These data

suggest that the region containing the CK-1/CK-2 elements plays a negative role in HTLV-infected cells that is not observed in uninfected cells. The paradox of the lack of IL-3 gene expression in infected cells and the Tax responsiveness of the IL-3 promoter has not been observed for other genes. Wolin *et al.* (1993) have speculated that alterations in the nuclear transcription factor complement of T-cells by HTLV infection (e.g. the CK-1/CK-2 binding complex) is probably not mimicked by the transient expression of Tax in virally uninfected cells and that such changes may require more prolonged exposure to Tax or the expression of other HTLV-inducible genes. It is possible that HTLV viral infection may be revealing mechanisms whereby IL-3 expression can be suppressed without the suppression of related cytokine genes such as GM-CSF. Determination of the components of the HTLV infection induced nuclear complex that occupies the IL-3 CK-1/CK-2 region should therefore be of high priority. However, inasmuch as these observations are suggestive of either CREB or NF- κ B involvement in the Tax transactivation, it still remains to be determined whether these complexes are of crucial importance in the transactivation of the IL-3 promoter constructs. Cotransfection experiments utilising the Tax missense mutations of Smith and Green (1990) should be of use in determining their role or may reveal other physiological activators that contribute to regulation of IL-3 expression.

As well as the conserved cytokine elements the ACT1 domain contains a sequence similar to the Oct-1 motif (Emmel *et al.*, 1989) known as the NF-IL3-A or ACT-1 element (Shoemaker, Hromas and Kaushansky 1990, Park, Kaushansky and Levitt, 1993 and Davies *et al.*, 1993). An additional motif immediately 3' to the NF-IL3-A site is similar to a cAMP-responsive element (CRE, Montmini *et al.*, 1986). These two elements have also been referred to as ACT-1B and ACT-1A respectively

(Gottschalk *et al.*, 1993). Other groups have shown by mutational analysis that both the ACT-1A and ACT-1B sites are critical for the expression of IL-3 in stimulated T-cells (Park, Kaushansky and Levitt, 1993 and Davies *et al.*, 1993). Evidence suggests that the ACT-1B element is targeted by at least two transcription factors, Oct-1 and an inducible factor, possibly AP-1 or the octamer associated complex OAP⁴⁰, which is comprised of junD and c-jun (Ullman *et al.*, 1993, Park Kaushansky and Levitt 1993 and Davies *et al.*, 1993). Recently, however, Kaushansky *et al.* (1994) characterised a nuclear protein complex capable of binding to this site which consists of two peptides of 43kDa and 45kDa and by using antibodies to junD and c-jun, showed that they are not antigenically related to the components of AP-1 or OAP⁴⁰. Interestingly, sequences from the octamer like regions of both GM-CSF and IL-5 competed for the binding of both Oct-1 and the p43/p45 complexes to the IL-3 ACT-1B. Moreover, both Oct-1 and the p43/p45 complex can bind to a sequence within the critical GM-CSF promoter CATTATA/T repeats (CLE0 -38bp to -50bp) (Kaushansky *et al.*, 1994). These observations suggest that these complexes or some of their components may play an important role in coordinating transcription of several cytokine genes. Notwithstanding the detection of complexes binding to the ACT-1B element by *in vitro* EMSA studies, it is surprising that under seemingly more physiologic conditions in either stimulated or unstimulated cells, *in vivo* footprint studies did not detect nuclear complexes bound to this element (Cameron *et al.*, 1994). Therefore further characterisation of the nature of the complexes that bind *in vivo* to the IL-3 ACT-1B promoter element is a prerequisite to a better understanding of its function.

In vivo footprint analysis by Cameron *et al.* (1994) did, however, detect enhanced protection over other promoter elements in stimulated MLA144 cells. A weak footprint was detected over the ACT-1A (CRE) element and more prominently over a previously unrecognised element, IF-1^{IL-3}, which is immediately 3' to ACT-1A (Figure 6.1) (Cameron *et al.*, 1994). The IF-1^{IL-3} element is related or identical to the viral core enhancers of SV40, polyoma and Moloney murine leukaemia viruses (Johnson *et al.*, 1987). It is notable also that a similar element has recently been found in the critical core sequences of the IL-3 and GM-CSF locus intergenic enhancer (Cockerill *et al.*, 1995a). In addition, an analogous site (δ E3) was described in the T-cell-receptor δ promoter (Redondo *et al.*, 1992). Linear deletion analysis of the IL-3 promoter had failed to detect the activity of the IF-1^{IL-3} element in part because of its proximity to the ACT-1 elements and because of its inability to mediate expression on its own. Its action appears to be mediated *via* interactions with either the neighbouring ACT-1 sites or an upstream AP-1 element (-300bp to -292bp, Figure 6.1)(Cameron *et al.*, 1994). The same authors, using antibodies against the transcription factor CBF, a complex known to bind the δ E3 element, were able to show that CBF interacts with IF-1^{IL-3}. Although CBF is not a T-cell specific complex, Cameron *et al.* (1994) have suggested that its role may be to further the T-cell tropism of IL-3 expression by interacting with the nearby tissue-specific activators at the ACT-1 elements.

In addition to the ACT-1 and IF-1^{IL-3} elements, prominent inducible *in vivo* footprints were also detected by Cameron *et al.* (1994) over the conserved cytokine element, CK-2, and over the 5' end of a motif similar to that of the Ets transcription factor family binding elements (EBS, Figure 6.1) (Gottschalk *et al.*, 1993 and Cameron *et al.*, 1994). The role of the putative EBS remains to be determined, although it is

intriguing that this site lies close to the CK-1/2 elements which were shown here by our DNase I protection analysis to be capable of binding purified AP-1. Such an arrangement of AP-1 and EBS binding sites (-301bp to -294bp and -288bp to -278bp respectively, Figure 6.1) is also found further upstream in the IL-3 promoter, within a third activation domain, ACT2.

The promoter constructs containing the ACT2 region's AP-1 and EBS motifs (-315bp) (Figure 6.1) gave the highest reporter gene activity. Deletion analysis by Gottschalk *et al.* (1993) demonstrated that this promoter fragment is, unlike the shorter fragments, active only in T-cells but not HeLa cells or a B cell line. Direct binding of AP-1 and Elf-1 to their respective recognition elements in this region of the IL-3 promoter (Figure 6.1) has been demonstrated (Shoemaker, Hromas and Kaushansky, 1990, Park, Kaushansky and Levitt, 1993 and Gottschalk *et al.*, 1993). Importantly, Gottschalk *et al.* (1993) also observe that the AP-1 site may bind distinct factors in the induced or uninduced state. Other evidence supports the suggestion that multiple regulatory complexes may bind to these elements. Nuclear factor complexes containing c-fos and c-jun proteins were detected by Park *et al.* (1993), although α -c-jun and α -c-fos antibodies only partly blocked complex formation and a different pattern of antibody interference was detected with extracts from primary T-cells and Jurkat cells. Because it is likely that protein interactions between various Fos and Jun related transcription factors mediate the differential effects observed with the AP-1 transcription factor, examination of the individual constituents of the complex binding to the ACT2 -AP-1 site will be of importance. Interestingly, a number of studies have shown that the regulatory regions of several other inducible T-cell cytokine genes contain an AP-1 binding site in close proximity to an Ets family member binding site,

and that such sites are important for inducible expression (Wasylyk *et al.*, 1990 and Boise *et al.*, 1993). Recently, Wang *et al.* (1994) have shown that Elf-1, c-fos and c-jun bind to adjacent sites in the CLE0 element of GM-CSF. This site has been shown to be an important regulator of GM-CSF transcription in T-cells. Therefore the presence of this arrangement of AP-1 and Elf-1 sites may be of importance in the co-ordinated regulation of the cytokine genes expressed early after T-cell activation. Neither AP-1 or Elf-1 transcription factors are found only in T-cells, therefore the manner in which they might confer T-cell specificity is of interest. It is possible that they interact with other T-cell specific transcription factors which do not bind DNA or with as yet undefined regulatory elements, or alternatively that they are modified *in vivo* leading to the T-cell restricted expression. Moreover, Rincon and Flavell (1994) showed recently that the DNA binding activity of AP-1 could be dissociated from its transcriptional activation capacity in murine primary T-lymphocytes. Binding of the complex was induced by activation of PKC but transcriptional activity required an increase in intracellular calcium and a second signal that could be delivered by stimulation of the CD28 receptor pathway. The dissociation of these activities is likely due, at least partially, to post-translational phosphorylation of the AP-1 complex provided by CD28 stimulation. Such a modification activates the transcriptional activity of AP-1 and likely other T-cell specific protein complexes such as NFAT or OAP⁴⁰ that contain Jun and Fos proteins.

In addition to the distinct binding sites discussed above, several other promoter elements have been implicated in the regulation of IL-3 gene transcription. Factors binding to these other sites are as yet poorly characterised. Evidence for the negative regulation of IL-3 transcription has implicated the NIP binding element between the

ACT-1 and ACT2 domains (Mathey-Prevot et al., 1990). A less well defined negative regulatory domain (NIP2) which lies 5' to the ACT2 domain was identified here. In either case the negative regulatory activity can be overcome by elements positioned directly upstream, the ACT2 and ACT3 domains respectively. Overall many of these observations suggest that *cis*-acting elements in the IL-3 promoter may be functionally interdependent on one another to optimally regulate IL-3 gene expression. Such an arrangement of multiple and overlapping or interdependent negative and positive regulatory sites may be important for maintaining the tight control over IL-3 gene expression. Similar observations have been made for the elements that regulate other early activated T-cell genes whose promoters bind partially overlapping sets of ubiquitous and lymphoid-specific transcription factors. Comparison of the IL-2 and IL-3 promoters shows that although they do not share extensive sequence homology they display a similar arrangement of regulatory elements (Davies *et al.*, 1993). For example both promoters contain motifs that bind AP-1 and Oct-1 proteins and intervening or flanking negative regulatory elements. This implication of common sets of transcription regulatory proteins in the coordinate regulation of a range of T-cell expressed cytokines has important consequences for the control of the immune response. For example, evidence suggests that both IL-5 and GM-CSF share the same OCT-1 and associated protein complex as that of IL-3 (Kaushansky *et al.*, 1994). Such an arrangement may be of importance in situations where eosinophil activation by the T-cell is required. On the other hand, differential expression of these cytokines must rely on subtle differences in the content and arrangement of the cooperating promoter elements and their binding proteins. For example, Oct-1 binds to the T-cell specific promoters of both IL-3 and IL-2, but in the IL-2 promoter Oct-1 associates with

OAP⁴⁰ while proteins distinct from OAP⁴⁰ are associated with Oct-1 in the IL-3 promoter. Such an overlapping yet distinct profile of transcription factor binding may therefore allow the T-cell to alter the pattern of cytokine transcription depending on the specific stimulus applied.

Collectively, the observations above suggest that the proximal 5' flanking sequences of the IL-3 gene contain nuclear binding protein sites which play a major role in conferring both inducible and T-cell specific expression to the IL-3 gene. However, transcription in stimulated T-cells directed by the IL-3 -5500bp promoter was considerably reduced compared to that of the endogenous IL-3 gene. Furthermore, reporter gene transcription, unlike that of the endogenous gene, was insensitive to cyclosporin A (CSA) treatment (Cockerill *et al.*, 1993). Surprisingly, in contrast to its effects on endogenous IL-3 gene expression, CSA treatment produced a consistent increase in reporter gene expression in Jurkat cells. Although the mechanism for the increase is unknown, it was evident with both the shorter proximal promoter constructs and the -5500bp construct (Cockerill *et al.*, 1993 and G. Ryan, unpublished results). These observations may be partially explained by the capacity of CSA to modulate the level of stability of IL-3 mRNA. CSA treatment has been shown to inhibit IL-3 mRNA accumulation through mechanisms dependent on the AREs (Nair *et al.*, 1994) and these elements are absent from the IL-3 reporter constructs. In addition, DNase I hypersensitivity analysis (DH) and functional studies of the IL-3/GM-CSF locus revealed an intergenic enhancer downstream of the IL-3 gene (Cockerill *et al.*, 1993). This enhancer confers highly inducible expression on the proximal IL-3 promoter and its activity action is blocked by CSA treatment (Cockerill *et al.*, 1993). Interestingly, the appearance of the DH site within the enhancer preceded

the onset of IL-3 and GM-CSF mRNA accumulation in T-cells suggesting that activation of the IL-3 and GM-CSF promoters requires prior activation of the enhancer. The activity of enhancer containing IL-3 reporter constructs was induced by an order of magnitude when the cells were stimulated with TCR-like signals, suggesting that there is considerable cooperation between elements in the intergenic enhancer and the IL-3 promoter. Highly synergistic activation of the GM-CSF promoter by the intergenic enhancer was also detected (Cockerill *et al.*, 1993). Evidence was subsequently obtained in this laboratory and by others, showing that the T-cell specific transcription factor NFAT associates with four NFAT sites in the enhancer and at least one site, CLE0, in the GM-CSF promoter (Masuda *et al.*, 1993, Cockerill *et al.* 1993 Cockerill *et al.* 1995a and Cockerill *et al.*, 1995b). The NFAT complex, which was initially thought to be T-cell specific, consists of a complex of AP-1 family proteins and NFATp or NFATc. Recently, the genes for NFATp and NFATc have been cloned and sequence analysis reveals that they are both similar over a limited domain to the NF- κ B/Rel family of transcription factors (Northrop *et al.*, 1994). Of interest, it has also been suggested that the interactions between NFATp or NFATc and AP-1 may be quite similar to those that occur between the Rel family proteins and AP-1 (Nolan, 1994). Moreover, cooperation between NFAT, AP-1 and NF- κ B binding elements in the GM-CSF promoter (the CLE0, CK-1 and NF- κ B motifs) appears to be partly responsible for the synergy between TCR and CD28 costimulation signals. The additional interaction between the promoter and the NFAT elements in the enhancer appears to augment this response. However, the actual importance of NFAT binding in the enhancer to the molecular mechanisms controlling the activity of the enhancer and IL-3 promoter is presently unknown. It will therefore

be of importance to investigate in transient transfection which of the IL-3 proximal promoter elements are necessary or sufficient to allow interaction of promoter and enhancer bound transcription factors which activate (or repress) transcription.

Notwithstanding the ability of the intergenic enhancer to activate the IL-3 promoter in circular plasmid constructs, its capacity to activate transcription from its natural position 10kb downstream of the IL-3 promoter still remains in question. The recent identification in this laboratory, of a homologous intergenic enhancer in the murine IL-3/GM-CSF locus may resolve this question by providing the opportunity to obtain mice with defined deletions or mutations of the enhancer through *in vivo* homologous recombination in murine stem cells (C. Osborne and P. Cockerill unpublished results). Alternatively, regulatory changes in the chromatin structure of the IL-3/GM-CSF locus and long distance regulation of both genes could be examined *in vivo* by transfer of large (~100kb) P1 or PAC/BAC plasmids (Sternberg, 1992) containing the whole locus and a large part of its flanking region into transgenic animals or *in vitro* by transfer to cultured cells. Defined deletions and subtle mutations can be made in these plasmids by *in vivo* homologous recombination within bacteria (G. Ryan, unpublished results). The use of such very large DNA constructs may allow the *in vivo* examination of the activity of many different mutant constructs without the delays and expense inherent in developing homologous recombinant embryonic stem cell lines and chimeric animals. Such transgenic approaches have been used to identify, in addition to enhancers, cis-acting elements that regulate alterations in the chromatin structure and organisation and which can dramatically impact on the ability of transcriptional machinery to access underlying elements and permit or suppress gene expression (Aronow *et al.*, 1995).

In conclusion, this study focused on the importance of transcriptional and post-transcriptional mechanisms that are involved in the regulation IL-3 expression, the transcriptional regulatory role of the conserved cytokine elements CK-1 and CK-2 and the nuclear proteins that bind to this region of the IL-3 proximal promoter. Initial clues to the functional activity of the conserved CK-1/CK-2 elements were revealed in the context of an heterologous promoter. A more complete characterisation of the putative suppression and activation roles of the cytokine elements in the normal promoter context remains for future studies. Finally, while these studies afford a glimpse of the regulatory factor network that is responsible for the integrated responses of the T lymphocyte, the molecular mechanisms by which single or multiple combinations of *cis*-acting elements enhance transcription in response to one or more signals remains to be clarified and it is likely that such mechanisms will not be revealed simply by a reductionist approach, studying relatively short regions of DNA, and that some form of overall systems analysis will be required. These observations do however, provide the foundation for continued studies aimed at a better understanding of the role of transcription in controlling gene expression and the role of cytokine genes in the regulation of the immune system.

6.2 Summary

The work presented in this thesis has shown that both induction of transcription and stabilisation of mRNA have important roles in the regulation of IL-3 gene expression in T-lymphocytes. IL-3 expression was absent in resting peripheral blood lymphocytes (PBLs) and in unstimulated Jurkat T-lymphocytic leukaemia cells which were used as a model for normal human T-cells in these studies. Jurkat cells express IL-3 mRNA with similar transient kinetics and require stringent stimulation conditions to elicit

IL-3 expression, as do normal PBLs. Rapid induction of IL-3 mRNA transcription, mRNA accumulation and protein expression followed the stimulation of the T-cell surface receptor glycoproteins by PHA in conjunction with a second signal. The second signal could be effectively delivered by maximally activating the PKC signalling pathway by phorbol ester treatment or by raising intracellular Ca^{2+} levels with an ionophore. An increase in intracellular Ca^{2+} , usually a consequence of TCR signalling, is an obligatory requirement for transcriptional induction of the IL-3 gene. Furthermore, the maximum activation of IL-3 mRNA expression was obtained when Jurkat cells were stimulated through the CD28 costimulatory pathway in addition to PHA and PMA treatment. In Jurkat cells these stimuli caused, within one hour, the transient induction of IL-3 transcription, which peaked at approximately 4 hours and rapidly returned to basal levels by 6 hours. Changes in the half-life of the accumulated IL-3 mRNA contributed to the accelerated decay of the mRNA at times later in the timecourse of expression and allowed the reduction of accumulated IL-3 mRNA to background levels 24 hours after stimulation.

The transcriptional component of IL-3 induction in Jurkat cells was dependent on nascent protein translation. IL-3 gene transcription may therefore require the *de novo* synthesis of transcription regulatory factors which in turn activate the IL-3 gene. The rapid induction of IL-3 gene transcription and its dependence on nascent protein synthesis, resembles that of other cytokines which are collectively expressed following T-cell activation. These genes comprise a set of factors that are expressed early in the procession of events following T-cell activation. Included in this group, among others are, GM-CSF, IL-2 through 6 and IFN- γ . This coordinate induction, and repression, of groups of cytokine genes including IL-3 in activated T-lymphocytes

suggests that T-cell activation regulates expression of the cytokine genes by common pathways. Although it is likely that each step in the pathway from gene expression to final polypeptide is exploited as a means of cytokine regulation, the signals generated by surface receptors target not least an array of promoter regulatory elements and their cognate nuclear binding proteins that control gene transcription.

Functional assays that employed IL-3 promoter-CAT reporter gene constructs were used here to define critical promoter regions and elements that are involved in IL-3 transcriptional regulation. In these studies, serially truncated versions of the IL-3 promoter were co-transfected with an internal control reporter plasmid into Jurkat cells. This approach identified both activation and inhibitory regions of the IL-3 promoter including a novel upstream activation region between -398 bp and -550 bp and a negative regulatory region between -398 bp and -315 bp. Functional assays have also shown that the conserved IL-3 cytokine elements, CK-1/CK-2 can function in T-cells as a repressor of constitutive expression, at least from the heterologous thymidine kinase promoter.

The properties of the cytokine elements CK-1 and CK-2 are of particular interest because of their appearance in the promoters of other cytokine genes. This work has identified at least three constitutive nuclear factor complexes, NFGMa, NFIL-3b and NFIL-3c, that bind to the IL-3 CK1/CK2 elements. One of these, NFGMa, has been previously characterised in this laboratory by Shannon *et al.* (1988) as a factor that binds to the GM-CSF CK1 element. Cross-competition EMSA with GM-CSF and IL-3 probes show that NFIL-3c, also recognises the related cytokine elements of the GM-CSF promoter. Interestingly, mobility shift assays employing competing binding sites suggest that NFIL-3b binds exclusively to the IL-3 sequences and probably to the

CK2 element and that both NFGMa and NFIL-3c may bind to the sequences within the CK1 element. Heparin sepharose and hydroxylapatite chromatography procedures were developed for the characterisation of these factors and these matrices were evaluated for their usefulness in the enrichment and purification of the three nuclear factors. These preliminary data will be of use in subsequent endeavours to purify and clone these putative transcription factors.

Finally, although the expression of IL-3 in activated T-cells is easily detected, IL-3 has not been detected in the bone marrow and is rarely detected in the circulation. These observations have led to the concept that the role of IL-3 may lie in regulation of local effector cells in situations of infection and in allergic inflammatory responses where the rapid supply of functionally active effector cells is required. Under conditions where high doses of IL-3 may be needed, induction of IL-3 expression is achieved by both increasing gene transcription, presumably by induction of one or several transcription factors, and by stabilising the newly synthesised IL-3 mRNA. The lack of a central role in constitutive haemopoiesis would also be consistent with the divergence in the structure of the gene that is seen among species. Indeed inhibiting the expression of IL-3 together with several other T cell cytokines by CSA or FK506 administration *in vivo*, does not seem to be harmful to either steady-state or recovering haemopoiesis. In conclusion, these studies therefore provide preliminary insights into the regulatory mechanisms underlying the pleiotropic response of the immune system and may contribute to a better understanding of the etiology of conditions in which lymphokine gene expression is inappropriate.

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