

REGULATION OF THE HUMAN INTERLEUKIN-3 GENE



By

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SUMMARY

To understand the regulation of the human IL-3 gene, this study has investigated the functions of DNase I hypersensitive (DH) sites located upstream and downstream of the gene and focused on a prominent pair of sites located 4.5 kb and 4.1 kb upstream of the IL-3 gene. A 245 bp SacI/StuI DNA fragment (SS245) encompassing the -4.5 kb site increased the activity of a minimal promoter 20 fold in luciferase reporter gene plasmid upon transfection into Jurkat T cells and activation with a combination of phorbol ester (PMA) and calcium ionophore (CaI). In contrast the -4.1 kb DH site did not increase the activity of the promoter in transient transfection assays, indicating an absence of enhancer activity.

The SS245 DNA element functioned as an inducible cyclosporin A-sensitive enhancer. Following stimulation with PMA and CaI, this enhancer increased the activity of a constitutively active thymidine kinase promoter three fold. The enhancer was not active in cells other than T cells, indicating that it was a T cell-specific enhancer. Binding assays showed that AML1, AP-1, Sp1, and NFAT bind to specific sites within the enhancer. Deletion and mutational analyses showed that the enhancer was activated primarily by NFAT family proteins that bound to three specific sites in the enhancer, whereas the AML1, AP-1, and Sp1 binding sites were not required for *in vitro* enhancer function in Jurkat T cells.

The 135 bp StuI/BamHI fragment (SB135) located between the -4.5 kb and -4.1 kb DH sites was shown to eliminate the activity of the SS245 enhancer when it was placed between the enhancer and a minimal promoter in transient transfection assays. However, an unrelated DNA fragment of similar size had the same effect on SS245 enhancer when

tested in a parallel assay. Hence, the evidence for the presence of a potential repressor element in that region was not conclusive.

The region downstream of the IL-3 gene contains three ubiquitous DH sites. The 3' most of these sites encompasses a TATA box upstream of an open reading frame and it functioned as a promoter in a luciferase reporter gene plasmid. The activity of this promoter like element was further increased upon addition of the central DH site suggesting that it may represent an enhancer. However, no transcripts could be detected from this region of the endogenous IL-3 locus in cell lines by reverse transcriptase-coupled PCR.

To test the region immediately upstream of the IL-3 gene for the presence of a locus control region (LCR), a 5.2 kb fragment containing five DH sites was linked to a luciferase gene in a reporter plasmid and stably transfected into Jurkat T cells and analysed in single cell clones. This construct had twice as much promoter activity as a shorter construct with the -4.1 kb and -4.5 kb sites deleted. The activity of both of these fragments increased a further three fold upon linking an additional inducible enhancer located 14 kb upstream of IL-3 gene. This indicates that the two enhancers upstream of the IL-3 gene cooperate to activate the IL-3 promoter. Although the -4.1 kb site had no activity *in vitro* we predict that it may function as an enhancer *in vivo* as it encompasses a potential GATA and Eklf sites and thus resembles DH site 3' of the β -globin LCR. Hence, we hypothesise that the sites at -4.1 kb and -4.5 kb cooperate *in vivo* to provide an LCR-like activity required for the correct developmental regulation of the IL-3 locus *in vivo*, while the -14 kb enhancer and the -4.5 kb respond to T cell receptor signalling.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if it is accepted for the award of the Ph.D. degree.

Abbas M. Hawwari

DEDICATION

This thesis is dedicated for my wife, Areyb Al-barghouthy, and our two daughters, Yara and Arwa. Their sacrifices have been immense and without which this work would have been impossible.

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CHAPTER 1: INTRODUCTION

1.1. HAEMOPOIESIS

In adults, blood cells originate from primitive cells called stem cells that reside primarily in the bone marrow (reviewed in Metcalf, 1984). Some stem cells can be found in the circulation or in the spleen. Stem cells give rise to the two branches of the haematopoietic system: the myeloid and the lymphoid compartments. The myeloid branch includes erythroid cells, granulocytes, monocytes, eosinophils, megakaryocytes and mast cells. The lymphoid cells include T and B lymphocytes. Stem cells have the capacity for self-renewal and the ability to differentiate into the many cell types that exist in the blood (Ogawa, 1994). It is estimated that for a 70 kg man, one trillion cells are produced each day.

Early in embryonic development, stem cells develop in the blood islands of the yolk sac (reviewed Metcalf, 1984; Cambiaggi and Vivier, 1999). Once the liver develops, the stem cells then migrate from the yolk sac to the foetal liver. As the spleen, bone marrow, thymus, and lymph nodes develop, they then become the home of stem and progenitor cells.

1.2. CYTOKINES ROLE IN THE REGULATION OF THE IMMUNE SYSTEM AND HAEMOPOIESIS:

In order for the pluripotential stem cells to differentiate into the many cells of the blood, they have to be induced by cytokines (Nicola, 1989). Stem cells develop into erythroid

cells, granulocytes, monocytes, megakaryocytes, and lymphoid cells (Hoffbrand and Pettit, 1995). Cytokines or interleukins (IL) are secreted growth factors that are produced by the haematopoietic cells to regulate the proliferation, differentiation, and activity of the haematopoietic cells (Nicola, 1989). Non-haematopoietic cells such as stromal cells, endothelial cells, epithelial cells and fibroblast also produce many cytokines. These factors are small extracellular glycosylated proteins of 100 to 200 amino acids in length (Baird et al., 1995).

The cytokines function in a network to promote differentiation, proliferation, survival, and activation of haematopoietic cells. Cytokines are produced constitutively or induced in response to inflammatory signals or others (Hoffbrand and Pettit, 1995). For example, antigens stimulate macrophages to release IL-1 and TNF. IL-1 and TNF stimulate other cells like T cells, endothelial cells and fibroblast to make IL-3, GM-CSF, G-CSF, M-CSF, IL-6 and others. These factors in turn act on other cells to stimulate their proliferation and differentiation. Some cytokines induce the production of other cytokines or stimulate the production of cytokine receptors. Some of these cytokines like IL-8, TGF- β , and interferons inhibit cell proliferation (Alexander, 1998). Some cytokines control the proliferation of many cell types and others induce differentiation of progenitor cells to develop into a one specific mature cell type (Ploemacher, 1999). Cytokines also inhibit apoptosis. Cytokines exert their effects through receptors on target cells. Some cytokines such as M-CSF can also be expressed as trans-membrane molecules, which may contribute to haemopoiesis through cell-to-cell interactions (Alexander, 1998).

When an antigen conjugates the TCRs on the surface of a T cell, a signal is transmitted to the nucleus as described in more detail later. This results in the production of many factors like IL-2, IL-3, IL-4, IL-5, IL-6, γ -interferon, and others (Kishimoto et al., 1994). Leukemia inhibitory factor (LIF) and IL-6 induce precursor cells to differentiate to macrophages (Kishimoto et al., 1994). IL-2 enhances the T cell and NK responses to antigenic challenge (Gillis, 1989). IL-3 is active on the earliest marrow progenitors and promotes proliferation of basophils, mast cells, granulocytes, macrophages, megakaryocytes, and eosinophils (Gillis, 1989). GM-CSF activities overlap with IL-3 and are observed to increase the number of granulocytes, neutrophils, eosinophils, and macrophages in circulation (Gillis, 1989).

IL-1, IL-3, IL-6, IL-11, Steel Factor (SF), LIF, and G-CSF act early on progenitors cells (Ogawa 1994). These factors induce the proliferation of multi-potential progenitors. In contrast, some factors are lineage specific such as M-CSF (specific for macrophages/monocytes) and IL-5 (specific for eosinophil) (Ogawa 1994). Cytokines synergise to stimulate the proliferation and differentiation of progenitor cells. For example, IL-1 and IL-6 enhance the effect of IL-3 and GM-CSF on survival and differentiation of progenitor cells to maintain a pool of progenitor cells (Hoffbrand and Pettit, 1995). These cells are then acted on by other cytokines in response to a specific need to increase the production of specific lineages.

1.3. B AND T CELL DEVELOPMENT

Lymphoid cells are made up of B, T, and NK cells and they originate from a common precursor, which is derived from the stem cell population in the bone marrow. B cells

start their developmental pathway in the bone marrow (Fougereau and Schiff, 1999). Pro-B cells develop as a result of precursors interacting with the stromal cells in the bone marrow. Stromal cells produce factors such as macrophage-colony stimulating factor (M-CSF), IL-7, and IL-11 (Arai, 1992). Late pro-B cells express both CD34, which is a marker of stem cells, and CD19, which is a B cell marker (Fougereau and Schiff, 1999). When pro-B cells are stimulated with IL-7, they develop to the pre-B cell stage. During this stage of differentiation, immunoglobulin gene (Ig) rearrangements take place. B cells develop to the immature B cell stage where they express surface IgM. At this stage, self-reacting B cells are clonally deleted by apoptosis. Those cells that are not self-reacting leave the bone marrow into the circulation. They then populate the lymphoid organs where they become mature B cells and express both IgM and IgD. Later stages of development depend on the presence of foreign antigens. After antigenic stimulation with the help of T cells, B cells develop into the last stage of development, plasma cells. At this stage, IgG, IgA and IgE are produced. Some mature B cells develop into memory cells.

Precursors of T cells and NK cells appear in the human liver as early as 6 weeks of gestation (Cambiaggi and Vivier, 1999). T cell precursors migrate to the thymus where they complete their differentiation. At this stage, these cells are CD34⁺, CD33⁺, and CD45RA⁺ and they are known as the triple negative (i.e. they do not have CD4, CD8 or CD3). These cells are not committed to either T cells or NK cells. Later, cells that express CD1, CD2, CD5 and an MHC I-like protein on the surface are committed to the T cell lineage, at which point TCR rearrangement starts (Cambiaggi and Vivier, 1999). Pre-TCR α along with rearranged TCR β chain (pre-TCR complex) appears on the surface along with CD3 in the double negative stage. The formation of the pre-TCR complex

appears to play a role in the suppression of any further rearrangement of the TCR β locus, initiates TCR α rearrangement, and plays a role in the expression of CD4 and CD8. Once T cells express both CD4 and CD8 markers they then are in the double positive stage. When the mature TCR appears on the surface, T cells undergo selection. Only cells that are not reactive to self go into further differentiation and develop into single positive cells; either helper (CD4⁺) or cytotoxic (CD8⁺) T cells, which are restricted to MHC II and MHC I respectively. Self-reacting T cells are eliminated. It is hypothesised that the elimination of self-reacting T cells or survival of non-self specific T cells depends on the strength of the interaction between the T cells and the MHC (Cambiaggi and Vivier, 1999). Only T cells that have weak interaction with self-MHC are allowed to survive. No interacting or very weak interacting cells are neglected and die. Strong interacting cells go through apoptosis. After this process of selection, T cells are mature but naive and leave the thymus into the periphery. When naive T cells encounter an antigen presented in conjunction with MHC I and II by antigen presenting cells (APC) and receive a second signal like CD28, they become activated and develop into effector cells or memory cells (Cambiaggi and Vivier, 1999). T helper cells differentiate into either T Helper 1 (Th1) or T helper 2 (Th2) which are facilitated by IL-12 and IL-4 (reviewed in Kiani et al., 1997; Viola and Rao, 1999).

Th1 and Th2 cells have different profiles of cytokine synthesis. Although Th1 and Th2 cells both produce IL-3 and GM-CSF, Th1 cells produce IL-2 and interferon (IFN)- γ , while Th2 cells also produce IL-4, IL-5, IL-10, and IL-13 (Takahiko and Miyajima, 1999). Th1 cells are involved in cell-mediated immune response, while Th2 cells are involved in humeral responses (reviewed in Kuo and Leiden, 1999).

In response to an infection, T cells produce cytokines that lead to the recruitment and activation of white blood cells, which combat the infection. However, cytokine synthesis can also cause damage to the host. Hence, T cells have developed mechanisms to tightly control the expression of cytokines, and cytokine expression is rapidly turned on and off in response to an infection. Uncontrolled cytokine production leads to septicaemic shock, fever, hypotension, acute tubular necrosis, metabolic acidosis, hepatic dysfunction, respiratory failure, and death (Slifka and Whitton, 2000). For this reason, it is essential that T cells maintain tight control over cytokine production. Cytokine expression can be controlled at the level of receptor signalling, transcription, RNA stability, and protein synthesis.

1.4. MYELOID DEVELOPMENT

The myeloid compartment includes cells like neutrophils, eosinophils, basophils, monocytes, and macrophages. These types of cells develop in the adult bone marrow. Only the mature and fully differentiated cells leave the bone marrow to the circulation and then to the tissues (Fibbe and Ploemacher, 1999; Metcalf, 1989; Metcalf, 1999). In response to antigenic stimulation or to the presence of pro-inflammatory cytokines like IL-1, and TNF- α , endothelial, fibroblast, marrow stromal and T cells produce many cytokines such as G-CSF, GM-CSF, M-CSF, IL-3 and IL-6 in addition to IL-1 and TNF- α . These cytokines induce the differentiation of myeloid precursor cells in the bone marrow. Once these cells are fully differentiated, they leave the bone marrow. This results in a rapid increase in these cells concentration in the circulation. GM-CSF promotes the differentiation of the granulocyte, macrophage, eosinophil, monocyte, megakaryocytic and basophil lineages. GM-CSF synergises with other cytokines like M-

CSF or G-CSF, for example, to stimulate the development of macrophages or granulocytes respectively. Moreover, GM-CSF along with IL-3 in the presence of erythropoietin increases the number of multipotent progenitors. G-CSF stimulates committed lineage-restricted progenitor cells to develop into neutrophils and increases the phagocytic activity of neutrophils. M-CSF is also a lineage-specific factor and is believed to be a survival factor for monocytes and macrophages. IL-3, on the other hand, is an early acting factor. It induces the growth and differentiation of granulocytes, macrophages, erythrocytes, megakaryocytes, eosinophils, and mast cells. It also acts in synergy with GM-CSF, G-CSF, erythropoietin, IL-6 and IL-11 to promote the development of single lineages. For example, eosinophil development requires IL-5 along with IL-3 and GM-CSF. Stem cell factor (SCF) synergises with IL-3 and GM-CSF to induce the production of megakaryocytes.

1.5. CHROMATIN STRUCTURE AND TRANSCRIPTION

To understand some of the mechanisms by which cells maintain tight control over cytokine production, one needs to look at the organization and mechanisms of gene expression at the level of chromatin structure and transcription. In eukaryotes, DNA is associated with histone proteins to form repeat structures known as nucleosomes. Each nucleosome is composed of 146 bp of DNA wrapped twice around a histone core particle and the average nucleosomal repeat length is about 200 bp (reviewed in Redner et al., 1999; Fletcher and Hansen, 1996; Wolffe and Guschin, 2000). The core particle consists of two of each of H2A, H2B, H3, and H4 histone molecules to form the octamer core. H3 and H4 form a hetero-dimer, which associates to form a tetramer (H3₂-H4₂), which in turn associates with two H2A-H2B hetero-dimers to either side to form the octamer core.

Each of the core histones has a globular body and an N-terminal tail. The inter-nucleosomal linker DNA is typically associated with one molecule of H1 Histone and other non-histone proteins.

Chromatin exists in vivo as highly compacted fibre. Several layers of folding result in at least 1000 fold compaction of the DNA length within chromosomes (reviewed in Redner et al., 1999; Mizzen et al., 1998; Fletcher and Hansen, 1996; Wolffe and Guschin, 2000). A decondensed array of nucleosomes appears under the microscope as a string of beads 10 nm in diameter. The 10 nm fibre is normally coiled in helical array, solenoid, or zigzag arrangement to form 30 nm diameter fibres. The exact structure of the 30 nm fibre is not very well defined. However, according to the solenoid model, each turn of the helix in the 30 nm fibre contains 6 nucleosomes. The linker histone, H1 histone, is not required for the folding of the 10 nm to the 30 nm fibre but it stabilises the higher order structure and may be important for further folding of the chromatin fibre (figure 1.1). The N- and C-terminal tails of H1 bind to DNA between two nucleosome cores. These tail domains contain many basic residues, which neutralise the charges on the DNA, hence facilitating the folding of the 10 nm fibres into a higher order structure. The assembly of the nucleosome onto genes occurs during DNA replication and represses initiation of transcription by RNA polymerase II. Chromatin assembly can also prevent transcription factors from binding to DNA, and leaves regions of DNA inaccessible to the transcription machinery.

The 30 nm fibre forms long loops thousands of nucleosomes long (Elliott and Elliot, 1997-pp. 246-248). These loops are attached to central scaffolding proteins. These loops form other coils and/or folds. The DNA in the metaphase chromosomes is in its

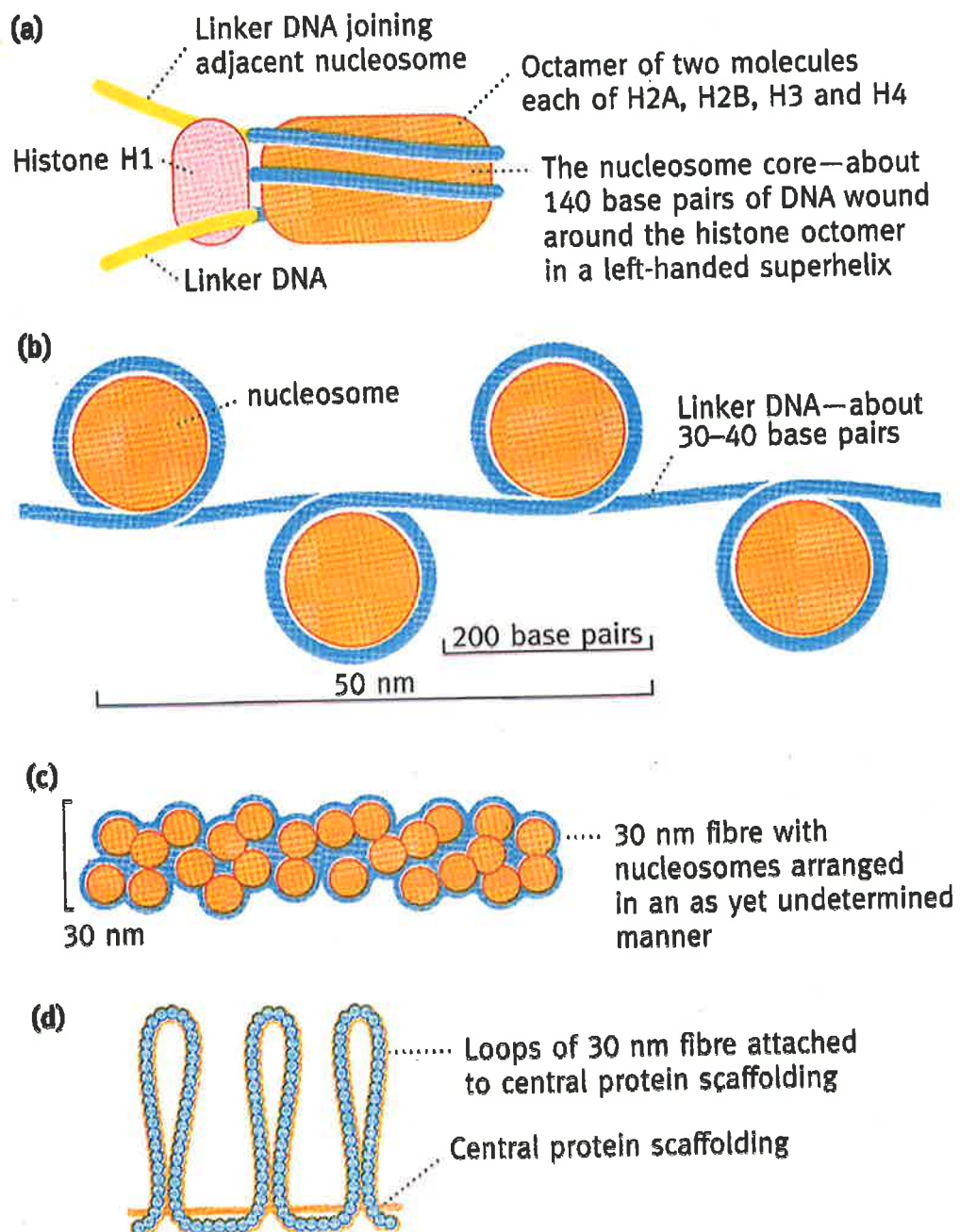


Fig. 1.1 The structure of chromatin organization.

Diagram showing the folding of chromatin fibres. (a) The DNA is wrapped around the outside of nucleosome, showing the histone core and the H1 binding to linker DNA. (b) The panel shows the arrangement of the 10 nm fibres. (c) The 10 nm fibre coils in a solenoid fashion to form the 30 nm fibre. (d) The 30 nm fibre loops which attach to the scaffold at the base of the loops. Taken from Elliott, 1997.

highest compactness state up to 10,000 times to allow for the translocation of replicated chromosomes into daughter cells. Once the sister chromatids separate and the cell divides, chromosomes in interface nuclei become less compact. Chromosomes carry functional parts that have coded information organised in genes. Active parts of the chromosomes are partially unwound to facilitate transcription and are known as euchromatin. Other non-coding regions often exist as large blocks of condensed chromatin. These areas include the centromere and telomere and are called heterochromatin. The chromatin folding is an efficient way to pack 1-2 meters of DNA into the small volume of the nucleus.

In *Drosophila*, heterochromatin protein 1 (HP1) was found to associate with heterochromatin along with other proteins to form a heterochromatin multi-protein complex (Lamond and Earnshaw, 1998). Polycomb proteins were found to bind to sites in euchromatin and may be involved in silencing euchromatin genes as illustrated in figure 1.2.

During the course of gene activation, a significant degree of chromatin remodelling and decondensation occurs. Transcription factors play a major role in this process of chromatin remodelling. For example, resting T cells have small-compacted nuclei and their cytokine genes are silent. Soon after stimulation with an antigen, the nuclei size increases about 5-10 fold and euchromatin structures appear (Zhao et al., 1998). Most of the work in this field currently is focused on the downstream changes at the 10 nm fibre that directly affect gene activation and repression. These changes involve histone acetylation, DNA methylation and nucleosome remodelling. The net effect of these

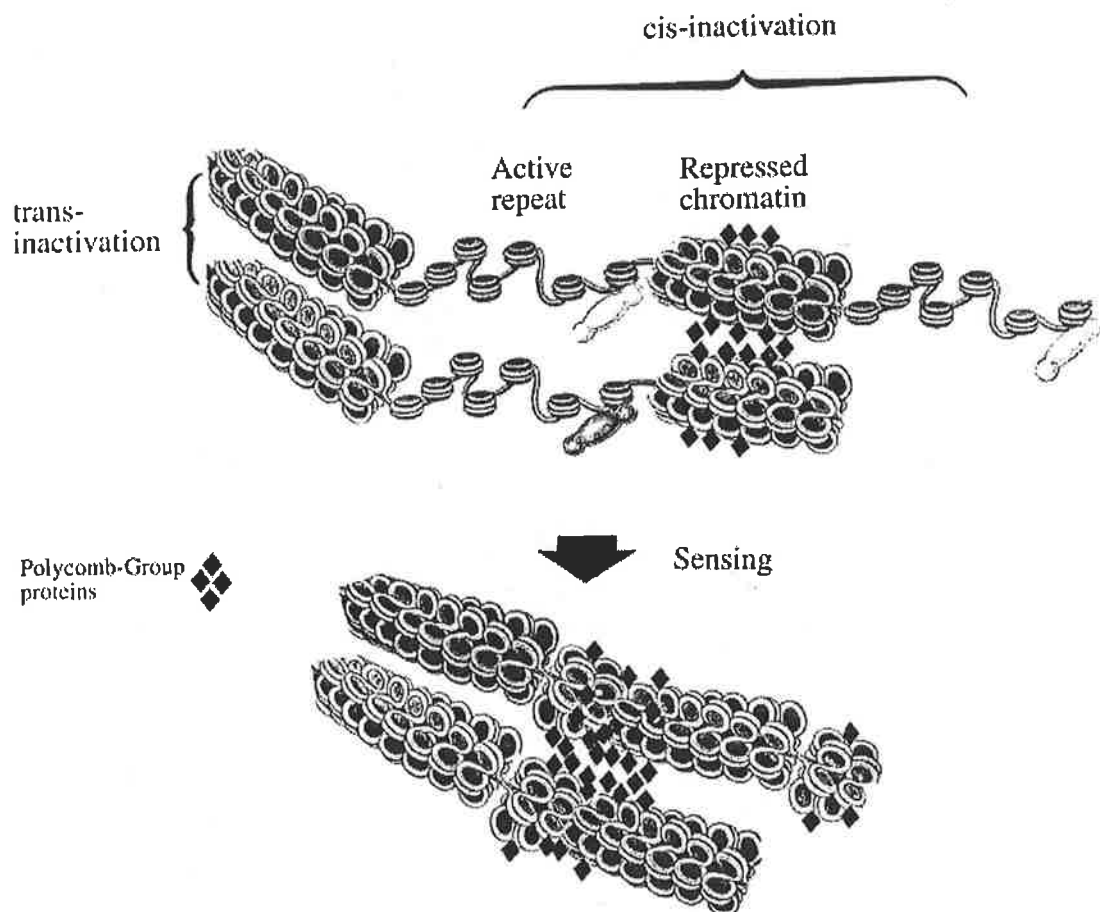


Fig. 1.2 Active and inactive chromatin structure

Areas of inactive chromatin are shown as a condensed structure. The active areas are shown as uncondensed structure. The diagram shows spreading of inactivation either by *trans* or *cis* through sensing mechanism, which leads to recruitment of Polycomb proteins. Taken from (Vermaak and Wolffe, 1998).

changes is to form relatively hypersensitive regions to DNase I digestion known as DNase hypersensitive (DH) sites.

DNA in chromatin is relatively protected from DNase I digestion compared to naked DNA. Active chromatin domains are generally more sensitive to DNase I digestion than inactive domains. This general sensitivity can extend up to 200 kb of DNA. However, regions where nucleosomes have been remodelled, are free of nucleosomes, are sensitive to DNase I digestion (hypersensitive). These sites are known as DH sites (reviewed in Gross and Garrard, 1988). It is believed that the presence of these sites is an indication of an open chromatin structure that allows transcription factors access to the DNA. Hence, DH sites are often associated with the presence of *cis*-regulatory elements and DH site mapping is often used to localise positions of regulatory elements. DH sites are found in regulatory elements such as, promoters, enhancers, silencers, and are also found at origins of replication, centromeres and recombination sequences. DH sites can be either inducible or constitutive. DH sites also usually correlate well with the tissue specificity of gene expression (Agarwal and Rao, 1999). For example, Agarwal et al. have identified a DH site 3' of the IL-4 gene that functions as an enhancer element in Th2 cells but not in Th1 (Agarwal et al., 2000). This DH site appears only in stimulated Th2 cells but not in stimulated Th1 cells. Moreover, the IFN- γ locus is completely silenced in Th2 cells but active in Th1 cells.

1.5.1. Replication timing, nuclear compartmentalisation, and nuclear scaffolding

As a general rule, actively transcribed genes replicate early while silenced genes replicate late during S-phase. An origin of replication (OR) is found in the vicinity of the

β -globin locus, which is active only in erythroid cells (Dhar et al., 1988). The β -globin OR is active in both erythroid and non-erythroid cells. However, in erythroid cells, the locus is in an open conformation, transcriptionally active, and replicates early, while the opposite is the case in non-erythroid cells.

The interphase nucleus is organised into compartments (reviewed in Lamond, 1998). Each chromosome occupies a “chromosomal territory”. The areas between chromosomal territories are called “inter-chromosomal domains”. Some evidence has been obtained that transcription occurs at the internal surfaces of the territories. RNA is then released into the inter-chromosomal domain for further processing. Hence active genes tend to be localised in the interior of the nucleus and to the periphery of the chromosomal territories, while inactive genes in heterochromatin tend to be located near the nuclear membrane. Areas around the centromeres and telomeres exist primarily as heterochromatin.

It has been observed that when active genes are translocated into heterochromatin, gene expression can be affected by the silencing effect of heterochromatin and in some cases results in variegated expression whereby only a small proportion of the cells express the gene. Many proteins have been identified in yeast, drosophila and mammals that are responsible for the heterochromatin silencing effect. Ikaros is one of these proteins in mammals, which is involved in lymphoid development (Brown et al., 1997) and is described in more detail later. It was proposed that during B cell development active genes at one stage of development are recruited by Ikaros to the heterochromatin areas near the centromere at another stage of development for inactivation.

Within the chromosome, the chromatin fibre is organised as looped domains approximately 30-100 kb in length on average. In interphase nuclei the DNA is anchored to a skeletal structure termed the nuclear matrix via matrix association regions (MARs) (Cockerill and Gerard, 1986). The scaffold is a proteinaceous structure that runs the length of the chromosome (reviewed in Paul and Ferl, 1999). The proteins that form the scaffold have protein motifs that help in chromosomal condensation and expansion. MARs and Scaffold attachment regions (SAR) are A/T-rich and are found associated with topoisomerase II (topo II) cleavage sites (Cockerill and Gerard, 1986, Jarman and Higgs, 1988).

1.5.2. Histone acetylation

The N and C terminal tails of each of the histones H2A, H2B, H3, and H4 are sites of acetylation, methylation and phosphorylation. Histone tail modifications occur in parallel with DNA replication, gene expression, and chromatin assembly (Luger and Richmond 1998; Wu, 1997). Many transcription factors recruit histone acetyltransferases (HAT). HATs acetylate all of the four core histones at specific lysine residues on the N-terminal tails. Hence, the specificity of histone acetylation comes from the recruitment of a specific transcription factor to the promoter or enhancer of the specific gene to be transcribed. Hyperacetylation of histones is correlated with gene activation. De-acetylation of histone tails is linked with gene silencing. Histone acetylation neutralises the positive charge on histone tails, which lessens the interaction of these tails with DNA, thus, allowing transcription factors access to DNA.

Histones can also undergo methylation, but this modification is less well understood than acetylation or phosphorylation (reviewed in Strahl and Allis, 2000). Histone H3 phosphorylation has been implicated in both chromosome condensation and decondensation. Kinases such as MSK1 activated by growth factor have been shown to be involved with H3 phosphorylation, which help the chromatin decondense (reviewed in Strahl and Allis, 2000).

1.5.3. Chromatin remodelling

Histone acetylation paves the way for chromatin remodelling factors to further disrupt the nucleosome structure and thereby facilitate the assembly and function of the transcription machinery. Remodelling complexes have been identified in yeast, *Drosophila*, humans and others. The SWI/SNF and ISWI complexes were identified in yeast (reviewed in Kornberg 1999). The process of remodelling of nucleosomes is ATP dependent. The SWI/SNF complex disrupts the core-particle structure and shift nucleosomes, while the ISWI complexes shift the locations of nucleosomes. It has been proposed that the remodelling events can result in the formation of DNase hypersensitive (DH) sites.

SWI/SNF-like complexes have also been identified in higher eukaryotes (Zhao et al., 1998). The Brahma (Brm) complex and a Brahma related gene (BRG) protein containing complex, BRG1 associated factors (BAF), was identified in *Drosophila* and in mammals respectively. Three non-mutually exclusive models have been suggested to understand the mechanism of remodelling complex function. These models suggest that the remodelling complex is recruited by (1) sequence-specific transcription factors, (2)

the RNA polymerase II holoenzyme, and/or (3) the complex itself binding DNA directly in a similar fashion to high mobility group (HMG) proteins, a characteristic not found in the yeast SWI/SNF complex. Wang, et al. have cloned BAF57 genes, a core component of the BAF complex, from mouse and human and showed that the BAF57 sequence contains an HMG domain as well as a kinesin domain (Wang, et al., 1998). They also showed that this domain by itself binds to DNA with the same efficiency as the entire BAF complex. The importance of the HMG domain was shown (Zhao et al., 1998) in T cells from transgenic mice producing physiological levels of dominant negative mutants lacking the HMG domain but not the kinesin domain of the BAF57. These T cells when stimulated do not get activated except that they proliferate normally indicating that the binding activity of the HMG domain within the BAF complex is essential for chromatin remodelling activity of activated genes.

1.5.4. Silencing effects of chromatin

As discussed earlier, nucleosomes assemble onto DNA to form a 10 nm diameter “beads-on-a-string” fibre. The H1:H1 interactions on different nucleosomes cause the 10 nm fibre to coil with 6 nucleosome per turn in a solenoidal fashion to form the 30 nm diameter fibre. Other proteins are involved in maintaining this structure such as the HMG family of proteins. The 30 nm filament is even further condensed in the nucleus. The most condensed structure is called heterochromatin, which is not permissive for transcription. Heterochromatin spreads from the edge of the silenced loci or from the telomeres and centromeres. In yeast, proteins like Rap1 bind to telomeric C₁₋₃A repeats, and Sir 3 and Sir 4 proteins interact with Rap 1, Histone H3 and H4 (reviewed in

Edmonson, and Roth, 1996). The Sir 3 and 4 complex then polymerises to form a heterochromatin-like structure while Sir 2 de-acetylates Histone H4.

In order for a gene be activated and overcome the heterochromatin silencing, two mechanisms have been proposed for chromatin de-repression (reviewed in Felsenfeld, 1992). The first one speculates that transactivators may bind to sites in linker DNA and displace histone H1, to sites near the entrance or exit points of the core histone, to sites located at one superhelical turn away from the entrance point, and/or to sites within the nucleosome as a result of the destabilisation effect of factor binding nearby. Thus, this results in disruption of the nucleosome particle. In contrast to the first mechanism, the second mechanism of activation requires DNA replication (reviewed in Felsenfeld, 1992). This model assumes that DNA binding sites are inaccessible to activator because chromatin folds into higher order structures. During replication, trans-activators bind to target DNA before chromatin assembly at the replication fork. This results in nucleosome-free areas that are maintained until the next round of DNA replication mainly because of the unavailability of free histones in resting cells.

1.5.5. DNA methylation

DNA methylation is associated with transcriptional silencing of genes by methylation of CpG sequences (reviewed in Wolffe and Guschin, 2000). Active genes are hypomethylated. Methylated DNA bind proteins such as MeCP1 and 2 that recruit corepressors and de-acetylases. To de-methylate and activate a gene, de-methylases are recruited during cell division by specific transcription complexes.

During cellular differentiation, some genes are turned off while others are turned on. The activation process involves chromatin-remodelling events such as DNA demethylation, and histone acetylation. Activated T cells produce a substantial amount of cytokines as a result of increased accessibility of these genes to the transcription machinery. Some of these cytokine genes are only expressed at high levels after differentiation down specific pathways. For example, the IFN- γ locus remains methylated in Th2 cells, which, do not make IFN- γ but becomes hypomethylated in Th1 cells, which make IFN- γ (Young, et al., 1994). DH site analysis also showed that the IL-4, IL-5, and IL-13 loci are remodelled only in Th2 cells, which express these genes, but not in Th1 cells, which do not. It has been proposed that the first step in chromatin remodelling of cytokines genes in Th2 cells involves activation of NFAT and STAT proteins which in turn activate GATA3 and Maf which may bind to DH sites and recruit chromatin-remodelling factors (Viola and Rao, 1999; Agarwal and Rao, 1998; Agrawal and Rao, 2000). The last stage of gene activation involves other transcription factors such as NFAT, AP-1 and NF- κ B that have gained access to the DNA.

1.6. MECHANISMS OF GENE EXPRESSION

Genes are expressed (transcribed) through the action of RNA polymerases (pol). Messenger RNA (mRNA) is synthesised by pol II, processed in both the nucleus and cytoplasm, and then translated into proteins. Proteins can then undergo further post-translational modifications. After synthesis, proteins can be either sequestered until a signal is received to release them or released directly to sites where they function.

Many types of gene regulatory elements have been identified, and these include promoters, enhancers, locus control regions (LCRs), boundary elements and repressors or silencers. Enhancers and LCRs can exist far upstream or downstream of genes, and also exist in introns in some genes. In order for regulatory elements to activate gene transcription, they need to be accessible to transcription factors (reviewed in Edmondson and Roth, 1996). Three mechanisms by which this can be achieved are: (1) activator factors may bind during DNA replication and hence compete with nucleosome assembly, (2) activator proteins may bind to the DNA within the nucleosomes, hence weakening the DNA: nucleosome interaction and (3) chromatin remodelling complexes such as Brg1/BAF may disrupt nucleosomes as discussed in more detail earlier.

1.6.1. Promoters and the basic transcription machinery

Promoters normally are located immediately upstream of the transcription start site which usually contains a pyrimidine-rich initiator element. By definition, the transcription start site is given a +1 number. All the 3' nucleotides (downstream) are given sequentially positive numbers and all of the 5' nucleotides (upstream) are given negative numbers. Core promoter elements usually contain a TATA box which is located at about -25 to -30 bp, and a "pyrimidine rich initiator element" which overlaps with the transcription start site (reviewed in Nikolov and Burley, 1997; Edmondson and Roth, 1996; Macfarlane, 2000; Blackwood and Kadonaga, 1998). The transcriptional activation of all genes is dependent upon recruitment of TFIID complex to the proximal region of the promoter. The TATA element usually associates with the TBP component of the TFIID complex. TFIID complex also contains TBP-associated factors (TAFs) that can mediate recruitment of TFIID complex by other specific transcription factors binding

upstream of the TATA-box. Transcription factors binding to upstream elements in the promoter also function to recruit the HATs and SWI/SNF-like complexes that mediate the chromatin remodelling events required for efficient assembly of the TFIID complex on the promoter and the subsequent recruitment of other components of the basic transcription apparatus. The association of TBP with the promoter results in the separation of the two strands of DNA, thus providing an entry point for pol II.

Another effect of TBP binding is to facilitate TFIIB binding to the TFIIB recognition element (BRE) (reviewed in Nikolov and Burley, 1997; Edmondson and Roth, 1996; Macfarlane, 2000; Blackwood and Kadonaga, 1998). This complex is then recognised by a pre-formed complex of Pol II and TFIIF. Pol II assembles at the initiator element (Inr). The start site seems to be determined by the TFIIB factor. TFIIE and TFIIH follow. TFIIE binds to both the TFIIF and pol II. TFIIE then recruits TFIIH to bind to both TFIIE and pol II. TFIIH is a helicase that is responsible for unwinding of the DNA helix for a short 10 bp. This complex that has assembled over the TATA box is called the pre-initiation complex (PIC). At the completion of PIC assembly, the N-terminal of Pol II is phosphorylated and transcription is initiated. Once Pol II is released from the promoter, and begins transcription only TFIIF remains attached to pol II. All of the other components of the PIC dissociate. RNA elongation continues and new PIC is assembled over the TATA box to initiate new rounds of transcription. Elongation is terminated when pol II reaches a termination signal, which causes the pol II to pause. As a result of pausing the pol II loses its phosphate and falls off.

Some promoters do not have a TATA box and are called TATA-less promoters (Kutach and Kadonaga, 2000). Instead, they often contain a downstream promoter element

(DPE). In this case the DPE is located about 30 bp downstream of the transcription start site. The DPE includes a G(A/T)CA sequence. The DPE is the site of TFIID binding in this type of promoter. The DPE interacts with the Inr to facilitate the PIC formation. Kutach and Kadonaga found that the spacing between the Inr and the DPE is important for promoter activity (Kutach and Kadonaga, 2000). Any change in the spacing eliminates promoter activity. In a screen of 205 promoters that the transcription start site has been defined for in *Drosophila*, they found that 29%, 26%, 14%, and 31% of these promoters contain TATA box only, DPE only, both TATA-box and DPE, and neither respectively. This showed that the frequency of TATA-box occurrence is similar to that of DPE. They suggested that the Inr and other transcription factors like Sp1 might drive Promoters that do not have either a TATA-box or a PDE.

1.6.2. Enhancers

Enhancers are DNA elements that are present in eukaryotes that activate gene transcription from a distance (reviewed in Atchison, 1988, Macfarlane, 2000). Based on the early work on the SV40 early region, Atchison has defined enhancers as elements that “(a) increase transcription of cis-linked promoters, (b) operate in an orientation independent manner, (c) exert an effect over large distances independent of position, and (d) enhance the expression of heterologous promoters” (Atchison, 1988). Some genes may contain multiple enhancers.

Enhancers are found upstream or downstream of genes as well as within introns of some genes. Enhancers bind activator proteins that interact with the transcription machinery to increase the transcription rate. Some enhancers can also bind proteins that function in

bending the DNA to facilitate Protein-protein interactions (reviewed in Hertel et al., 1997). For example, the TCR α and IFN- β enhancers bind HMG-family proteins that function as architectural proteins bending the DNA to form an enhanceosome (Falvo et al., 1995; Thanos and Maniatis, 1995; Carey, 1998). The bending of the DNA facilitates the interaction of activators like NF- κ B, AML1 and others with the basal transcription machinery (reviewed in Carey, M., 1998).

The mechanism of enhancer function described thus far implies that the function of enhancers is to increase the rate of transcription of a promoter. However, Walters et al., 1995, proposed an alternative “probability” model for enhancer mechanism. According to this model, enhancers increase the number of cells expressing the reporter gene rather than increasing the rate at which each cell transcribes the gene (Walters et al., 1995). The increase of transcription observed when an enhancer is linked to a promoter in gene reporter transfection assays is not due to an increase of the rate of transcription from a particular promoter but rather the rate of transcription remain the same in cells expressing the gene. The addition of the enhancer increases the probability of the gene transcription.

1.6.3. Locus control regions

Locus control regions (LCRs) are *cis* acting elements that induce accessibility to genetic loci. LCRs were defined based on studies of the β -globin locus in transgenic mice (Grosveld et al., 1987). Expression of these transgenes was low and subject to the influence of the site of integration unless the transgene contained a fragment from the 5' distal end of the β -globin locus. As a result of these studies, the LCR was found to

influence the chromatin structure across a 100 kb region. In addition, it conferred position independence, copy number dependence, and high level of expression comparable to the level of endogenous gene (reviewed in Ernst and Smale, 1995).

LCRs can often exist as clusters of tissue-specific DH sites. The human β -globin LCR consists of 4 erythroid-specific (numbers 1-4) and a ubiquitous (number 5) DH site and is located 5' of the β -globin gene cluster (figure 1.3) (Tuan et al., 1985). DH site 1 is located 6.4 kb upstream of the ϵ -globin gene. DH sites 2, 3, 4, and 5 are located 11, 15.2, 17.5, and 21.5 kb from the ϵ -globin gene respectively. The gene cluster contains ϵ , γ , δ , and β -globin genes, linked in a 5' to 3' orientation. Each gene is expressed at a specific stage of development. The ϵ -gene is expressed at the embryonic stage of development, the γ -gene expression is initiated in the embryo and is predominant at the foetal stage in the liver and the β -gene is expressed soon after birth (reviewed in Li et al., 1999; reviewed in Baron, 1997). Without the LCR, the expression of the human β -globin gene is extremely low compared to the expression of the endogenous β -globin gene in transgenic mice (reviewed in Li et al., 1999; reviewed in Baron, 1997). The LCR increases the activity of the transgene to the level of the endogenous gene. This enhancer activity has been mapped to DH sites 2 to 4. DH sites 3 and 4 have enhancer activity only in the context of the chromatin and do not show any enhancer function when tested in transient transfection assays. However, DH site 2 functions as an enhancer in transient transfection assays. It was shown that the DH site 3 directs the β -globin expression in one copy transgene while DH site 2 does not (Ellis et al., 1996). DH site 5 functions as an insulator and is not required for the LCR function.

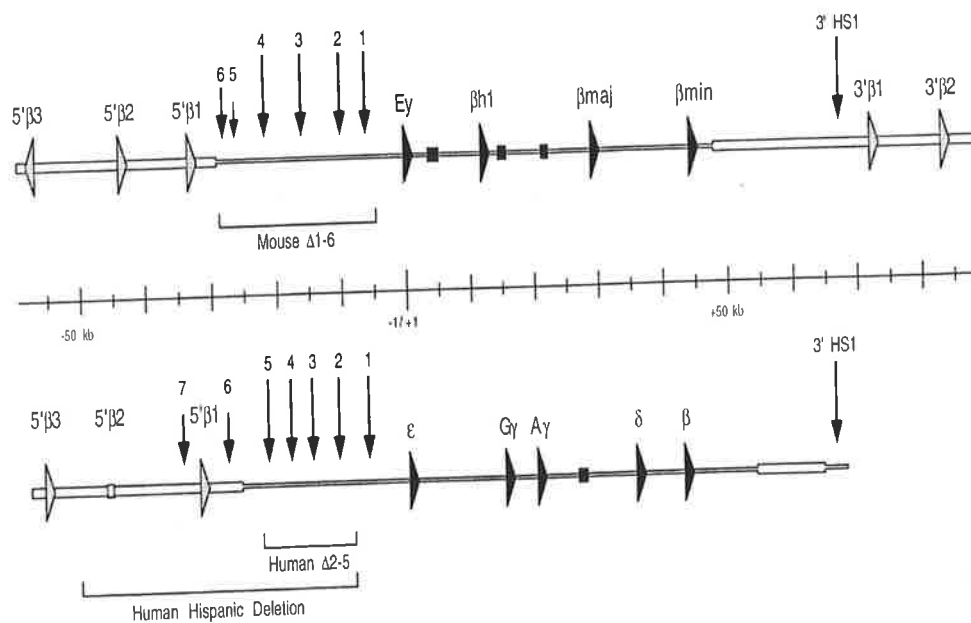


Fig. 1.3 The structure of the mouse and the human β -globin locus.

The upper diagram is the mouse β -globin locus and the lower one is of the human. The globin genes, represented as filled arrows, are designated $E\gamma$, β_{h1} , β_{maj} , β_{min} for mouse and ϵ , $G\gamma$, $A\gamma$, δ , and β for human. Olfactory genes are shown scattered around both the human and the mouse globin locus and represented by shaded arrows. The filled or shaded boxes are pseudogenes. Vertical arrows represent DH sites; long arrows represent more intense DH sites than smaller ones. The scale between the mouse and human diagram is set so that the cap of the $E\gamma$ gene and the ϵ gene are at +1. (Taken from Bulger et al., 1999).

In naturally occurring deletions in human such as the Hispanic β -thalassaemias, which is the smallest deletion of all β -thalassaemias, where the LCR (DH site 2 to 5) is deleted in addition to a about 25 kb of DNA further upstream, the β -globin locus is transcriptionally silenced, condensed, and replicates late (Bender et al., 1998; Bender et al., 2000).

It has been shown with the β -globin locus that each DH site in the LCR is involved in the expression of globin genes in a tissue-specific or developmental stage- specific manner. Each of DH sites 1, 2, 3, and 4 were individually linked to a 36 kb *Sma*I-*Cla*I fragment containing all of the β -globin like genes except the ϵ -globin gene and tested in transgenic mice. DH site 3 regulates the expression of the γ -globin gene and it is the most active site in the embryo while DH site 4 is most active in the adult stage when the β -globin gene is expressed (Fraser et al., 1993). This implies that specific sequences within the DH sites of the LCR interact specifically with the promoters of specific genes at specific stages of development. This idea is further supported by recent studies that deleted specific sites within the whole LCR. When a 234 bp fragment spanning the core of DH site 3 was deleted from a 248 kb β -locus yeast artificial chromosome (YAC), the ϵ -globin gene expression was abolished at all stages of development. Moreover, γ -globin expression was abolished in the definitive stage in the foetal liver but was normal in the embryonic stage. However, β -globin gene expression was low and variable indicating that β -globin gene expression was affected by position of integration (Navas et al., 1988). Hence, DH site 3 is critical for the protection against position of integration effects. This supports the hypothesis that the DH site 3 is important for the ϵ -globin at all stages and for γ -globin gene expression in the definitive stage of erythropoiesis in the

liver but not in the yolk sac. It is believed that proteins binding to a promoter of a β -like globin gene interact through DNA looping with proteins binding to a specific site within the LCR (Baron, 1997). Hence, the availability of stage-specific transcription factors determines which gene is to be expressed.

In another study, transgenic mice with either of two YACs containing larger deletions of 2.3 and 1.9 kb of DH site 3 and 2 respectively were produced (Peterson et al., 1996). DH site-3 deletion resulted in a significant decrease of the ϵ -globin and an increase in the γ -globin transcripts. This again suggests that specific DH sites may interact with specific globin gene promoters. However, DH site 2 deletion resulted in only a small decrease of all β -globin like transcripts at all stages of development. Moreover, neither deletion affected gene switching. Hence, it was suggested that elements within the LCR are redundant and hence not all of these elements are required.

Twenty kb regions further upstream of the LCR in both human and mouse were sequenced and analysed as described below (Bulger et al., 1999). Similar to the LCR (Moon and Ley, 1990), this region is highly conserved between mouse and human indicating a functionally important region. Additional DH sites were found (Bulger et al., 1999) further upstream of DH site 5 as shown in figure 1.3. These were numbered 6 and 7. The naturally occurring deletion in the Hispanic β -thalassaemias includes this region. The importance of this region is indicated by loss of DNase I sensitivity and silencing of the β -globin locus. This is in contrast to studies which will be described next where targeted deletion of the LCR DH site 1-5 in human transgene and native locus in the mice reduced expression of β -globin like gene but did not affect the general

DNase hypersensitivity of this locus. As a result of all of these studies, it was suggested to expand the LCR region to include DH site 6 and 7.

Bulger et al. (1999) also showed in the same study mentioned above, that DH site 7 is located 5' of the first olfactory receptor gene 5' of the human β -globin locus. Olfactory receptor genes have been identified upstream and down stream of β -globin locus in both human and mouse. It seems that the β -globin locus is situated in the middle of a larger olfactory receptor gene locus. Hence it was suggested that the β -globin LCR function is more complicated than originally thought. It may be involved in the regulation of the olfactory receptor locus as well as the β -globin locus or simply to protect the β -globin locus from the influence of the olfactory receptor genes or visa versa since the olfactory genes are expressed in a different tissue type than the β -globin genes.

The main problem with transgenic experiments is that genes are normally regulated in their native locations. Randomly integrated constructs may behave differently in these new locations than in their native locations. Experiments recently have been carried out to study the LCR functions in its native location (Reik et al., 1998; Cimborra et al., 2000). These studies have shown a quite different picture compared to what transgenic experiments have shown. Targeted deletions of the human DH site 2 to 4 in cell lines resulted in greatly reduced transcription from all β -globin like genes as has been seen in transgenic experiments. However, the general DNase I-sensitivity of the locus is not affected nor is the timing of replication altered. These experiments suggested three things: firstly that the LCR as defined by transgenic experiments (DH site 2 to 4) is not required for maintaining an open chromatin conformation, secondly that an open conformation can be maintained in the absence of transcription and third, that the LCR

does not affect replication timing. In contrast to this, the Hispanic deletion abolishes open chromatin structure, replication of the locus is late, and transcription is abolished.

To understand the role of the β -globin LCR in nuclear localisation and chromatin remodelling, human chromosome 11 from lymphocytes where the β -globin LCR was normally silenced was transferred to a mouse erythroid leukemia (MEL) cell line (an erythroid background) (Aladiem et al., 1995; Cimbora et al., 2000). At least three different MEL hybrids were generated. The chromosomes 11 transferred were (1) a wild type, (2) containing a Hispanic deletion from β -thalassemia patient, (3) a targeted deletion by homologous recombination of DH site 2 to 5. The β -globin gene in the MEL cell line, which contains the Hispanic deleted chromosome 11 was transcriptionally silent, co-localised with centromeres, hypoacetylated and in a heterochromatin configuration. In the other two MELs, it was hyperacetylated, and was not associated with centromeric heterochromatin. However, only the WT chromosome 11 had a transcriptionally active globin locus. This suggested that the LCR is required for transcriptional activity but not required for directing the locus away from heterochromatin compartments in the nucleus. Although both the deleted DH site 2 to 5 and wt human β -globin genes were hyperacetylated, the hyperacetylation was greater in the wt than the deleted one, suggesting a role for LCR in hyperacetylation of the locus.

The above studies in cell lines, although informative, do not take into account the developmental program of the whole organism. For that reason, studies of the β -globin LCR were carried out by creating mice with LCR deletions in its native locus. Bender, et al. (2000) used homologous recombination to generate mice with deletions in the native LCR. Deletion of mouse 5' DH sites 1 to 6 did not affect the general DNase I

hypersensitivity of the locus, and all of the globin genes were expressed at the correct stage of development. However, the expression level was much lower than normal. This confirmed the results from the tissue culture experiments and reinforced the idea that the LCR is needed for the correct level of expression but not for the correct developmental expression or for establishing an open chromatin structure (Bender et al., 2000).

LCRs have also been shown to be responsible for the control of tissue specificity and developmental regulation of gene expression in many other loci such as the human CD2 (Lang et al., 1988, Greaves et al., 1989, Festenstein et al., 1996), Chicken lysozyme (Bonifer et al., 1990), T cell receptor (TCR) α/δ and MHC (Diaz et al., 1994). A series of T cell-specific DH sites 3' of the TCR α and δ gene locus has been shown to confer site of integration independence on the linked gene. Since the TCR α and δ gene are expressed in different T cell types and at different stages of development, the TCR LCR has the capacity to regulate mutually exclusive programs of gene expression in the TCR locus.

1.6.4. Boundary elements (or insulators)

Boundary elements are defined by two functional assays (reviewed in Gerasimova and Corces, 1999). The first one is an enhancer-blocking assay. If a boundary element (BE) is inserted between a promoter and an enhancer, then the enhancer is blocked and unable to increase transcription above the level attributed to the promoter alone. However, if the BE is inserted on either side of the promoter-enhancer or enhancer-promoter, the BE can't influence the transcriptional level. Although BEs repress the activation of a promoter by an enhancer if it is placed between them, the enhancer remains free to

activate another linked promoter on the other side of it. This characteristic distinguishes BEs from classical repressors. The second property defining the BE is their ability to insulate a transgene from heterochromatin effects (variegation) and from the influence of the neighbouring gene regulatory elements.

The chicken β -globin BE located at the 5' end of the locus (DH site 4) is one of the well-studied insulators (Chung et al., 1993). DH site 4 is constitutive and its core, a 250 bp G+C rich element, containing all of the activity of the insulator (Chung et al., 1997). Upstream of the DH site 4, Prioleau et al. (1999) found an independently regulated folate receptor gene that is expressed in pro-erythroid cells only before the expression of the β -globin genes. The folate receptor gene is separated from the β -globin locus by a 16 kb of heterochromatin region that is methylated and condensed. Hence it was proposed that the insulator, DH site 4, is important in separating the β -globin from the upstream region and to insure its independent activation and regulation (Prioleau et al., 1999). The 3'-end of the chicken β -globin locus is marked also by a constitutive DH site. This site has been characterised and was shown to function as a BE in an enhancer blocking assay. An odorant receptor gene is found just 2 kb downstream of the 3' BE (Saitoh et al., 2000). Both BEs of the chicken β -globin locus (Saitoh et al., 2000; Bell et al., 1999) bind to CTCF protein as was shown for BEs of other loci such as H19/igf2 locus (Bell and Felsenfeld, 2000), T cell receptor α/δ locus (Bell et al., 1999), chicken lysozyme (Burcin et al., 1997), and chicken and human myc-genes (Filippova et al., 1996).

Because CTCF contains 11-zinc finger DNA binding domains, the binding motifs for CTCF protein in all of the BE of the genes mentioned above are different. Different combinations of these fingers with different binding specificity bind to different BEs.

The mechanism of action of CTCF was suggested by Lutz et al. (2000). They found that CTCF binds to the co-repressor Sin3A and that recombinant CTCF binds to histone deacetylases from Hela cell nuclear extracts. This suggested that CTCF recruits histone deacetylases (Lutz et al., 2000).

Other well-studied insulator elements are the *scs* and the *scs'* elements flanking the heat shock protein 70 (*hsp70*) genes in *Drosophila* (Kellum and Schedl, 1992; Dunaway et al., 1997; Krebs and Dunaway, 1998). Strong DH sites are present on the location of the two insulators. They have been shown to block enhancer activation of a reporter gene as well as to protect a transgene from position of integration effects. Another insulator in *Drosophila* is the *gypsy* insulator present in the *gypsy* retrotransposon (reviewed in Gdula et al., 1996; Cai and Levine, 1997). The function of this element when inserted at specific sites during development is to silence transcription of adjacent genes. Proteins that bind to BEs in *Drosophila* have also been identified (reviewed in Gerasimova and Corces, 1999). The 32-kDa-boundary element associated factors A and B (BEAF-32A and BEAF-32B) bind to sites containing 3 motifs of CGATA in the *scs'* sequence. Another factor that binds the *gypsy* BE is called suppressor of hairy-wing [*su(Hw)*] protein, which contains 12 zinc fingers.

1.6.5. Repressors

Genes may be negatively regulated by binding of negative regulatory factors (repressors). Repressors could function by a combination of the following mechanisms (reviewed in Macfarlane, 2000). Repressors may bind on a site that overlaps the TATA box and prevent the PIC from forming. Second, repressors may bind to enhancers,

which cause the DNA to loop and bind in a similar fashion to activator factors and block or prevent pol II from being activated. Third, repressors may compete with transcription factors for the same binding sites. Fourth, repressors may interact with transcription factors without binding to DNA. In this case repressors may inactivate the transcription factors, prevent them from binding to DNA or/and allow transcription factors to bind DNA but prevent transcription factors from interacting with each other or with co-activators.

1.7. T CELL ACTIVATION

T lymphocytes respond to antigens, in the cases of extracellular antigens, only when presented on the surface by antigen presenting cells (APC) in conjunction with the major histocompatibility complex (MHC) class II (reviewed in Pieters, 2000). APCs internalise antigens either through phagocytosis or through receptor mediated events. Antibodies bind to antigens, which the APC recognises through its Fc receptor, and the internalised antigen is degraded into peptides. The peptides are then bound to the MHC II and transported to the surface. MHC II consists of two glycoproteins, α and β . There are different classes of MHC II and many different proteins are generated from each class with different antigenic specificity. In addition, for each locus there are multiple alleles. This allows the MHC II greater range of antigen binding specificity. Intracellular antigens such as viruses and mycobacterium are processed and presented in conjunction with the MHC I. MHC I molecules are present in all cell types.

TCRs are dimers of two proteins, either $\alpha\beta$ or $\gamma\lambda$. These proteins arise as a result of gene rearrangement to produce a large repertoire of TCRs specific for different antigens

(reviewed in Malissen and Malissen, 1996). The TCR $\alpha\beta$ cells make up more than 85% of the peripheral blood lymphocytes. The TCR is linked non-covalently to the CD3 receptor. The TCR is responsible for antigen recognition and binding while the CD3 is responsible for signal transduction. CD3 is composed of ξ homodimer, and $\gamma\epsilon$ and $\delta\epsilon$ heterodimer protein chains. The TCR-CD3 complex assembles in the endoplasmic reticulum (ER). Initially, the TCR α and TCR β chains associate with CD3 $\delta\epsilon$ and CD3 $\gamma\epsilon$ dimers respectively. Then, the TCR α associate with TCR β by disulfide bonds. This, then, is followed by the association of the CD3 ξ homodimer that has separately associated through sulphide bonding. Once this assembly is complete, the complex exits the ER and is transported to the cell surface.

Both CD4+ (helper) and CD8+ (cytotoxic) T cells have the TCR-CD3 complex (Roitt, 1997). CD4+ T cells recognise antigens on the surface of APC in conjunction with MHC II while CD8+ T cells recognise antigens in conjunction with MHC I. As a result of this recognition CD4+ cells release a variety of interleukins and cytokines, which in turn activate other cells of the immune system (hence the term helper T cells). On the other hand, the main function of CD8+ cells is the direct killing of other cells. Recognition of antigen in conjunction with MHC I result in CD8+ cells killing the cells presenting the antigen.

The TCR-CD3-CD4/CD8-MHC complex conjugated to antigen on the cell surface is not enough to efficiently activate T cells (reviewed in Lensch et al., 1996). T cells need a second signal for full activation. The major costimulatory signal is the activation of the CD28 receptor on T cells through binding to B7 ligands. CD28 is expressed on the surface of all CD4+ T cells and 50% of CD8+ T cells. Following T cell stimulation, and

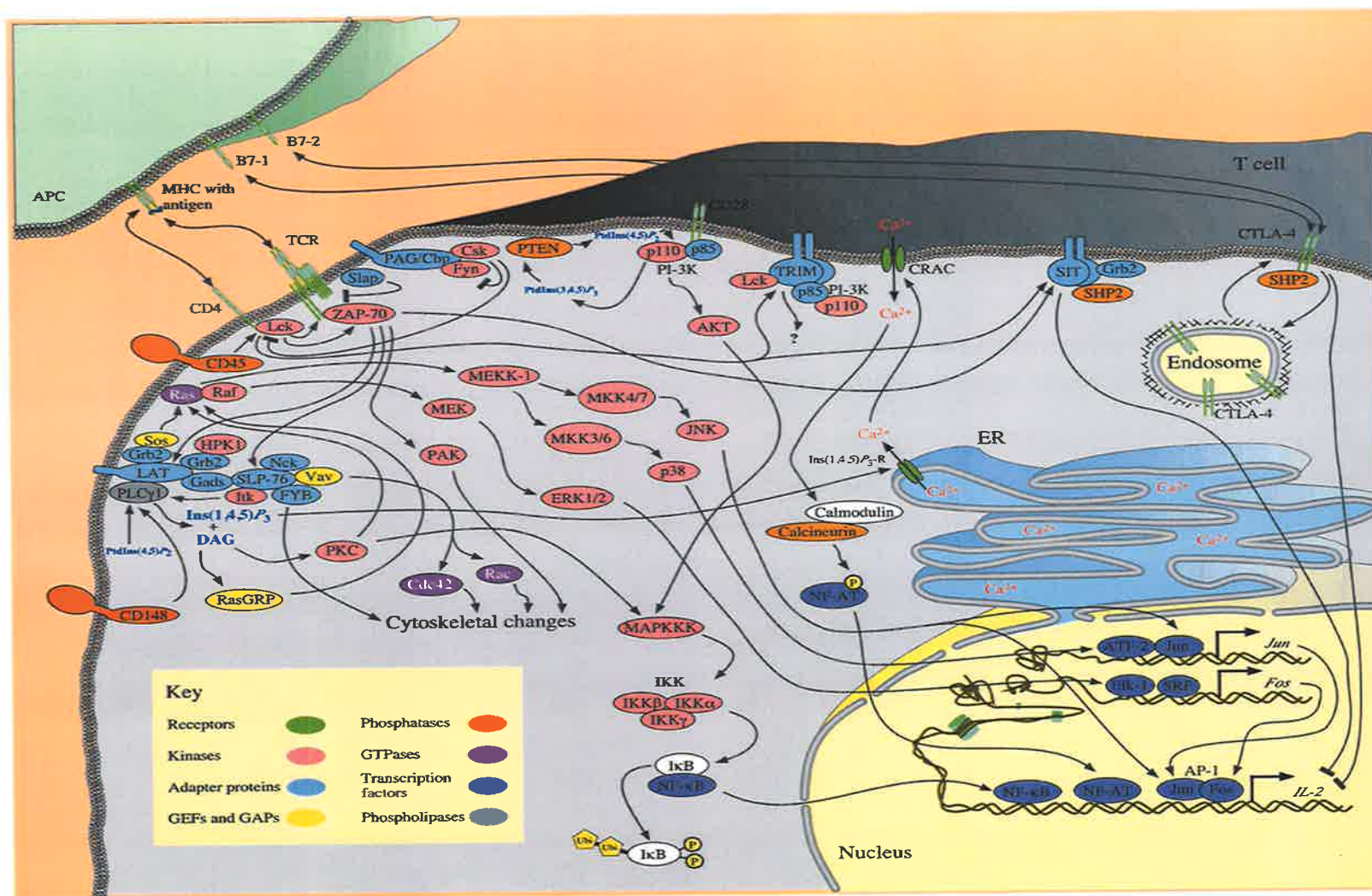
induction of cytokine gene expression, CD28 is down regulated and CTLA-4 expression is induced which antagonises CD28 signalling by competing for B7 binding (Chambers and Allison, 1999). Both CD28 and CTLA-4 are glycoprotein homodimers crosslinked by disulfide bond. Treatment of T cells with anti-CTLA-4 Ab resulted in the down regulation of T cell activation suggesting a role for CTLA-4 in negative regulation of T cells. B7 on the surfaces of many APCs is the ligand for CD28 and CTLA-4 (reviewed in Lensch et al., 1996).

Another consequence of TCR cross-linking is the activation of tyrosine kinases such as Lck and ZAP-70, which are required for calcium mobilisation and phospholipase C activation in T cells (reviewed in Rao et al., 1997) (see figure 1.4). TCR cross-linking also activates protein kinase C, Ras, and Raf. These pathways can also be activated more directly by using the phorbol ester (PMA), which activates the protein kinase C pathway downstream of phospholipase C. Raf activates MEK1, which in turn activates ERK, which leads to the activation of *c-Fos*. In addition to Raf, Ras activates Rac, which lead to the activation of c-Jun. Activation of both c-Fos and c-Jun gene expression results in a functional AP-1 complex.

When the TCR is stimulated, phospholipase C is activated (figure 1.4) (reviewed in Rao et al., 1997). As a result, the inositol triphosphate concentration in the cytoplasm is increased which induces release of Ca^{2+} stores in the endoplasmic reticulum. The pharmacological agent, ionomycin releases the calcium stores directly. The rise in free intracellular calcium activates calcineurin, which is a calmodulin-dependent phosphatase. Calcineurin binds to a region near the N terminus of the NFAT regulatory domain, which also contains a nuclear localisation signal. NFAT in the cytoplasm then

Fig. 1.4. Signal transduction pathways of the TCR.

A diagrammatic representation of the major TCR signalling pathways is shown. The diagram also shows the costimulatory signalling pathways of CD28 and CTLA-4 as well as CD4 signalling. (Taken from Lin and Weiss, 2001).



becomes dephosphorylated by calcineurin, which exposes its nuclear localisation signal and the NFAT is translocated into the nucleus. The calmodulin/calcineurin pathway is inhibited by cyclosporin A and FK506 which are potent inhibitors of cytokine gene expression. In the absence of co-stimulatory signals stimulation through the TCR alone leads to anergy and apoptosis (Rincón and Flavell 1994). Over-expression of CTLA-4 at the expense of CD28 also results in anergy. Little IL-2 is produced when only the TCR is stimulated. Efficient IL-2 transcription requires CD28 and TCR activation.

1.8. TRANSCRIPTIONAL REGULATION OF T LYMPHOCYTE DEVELOPMENT AND FUNCTION:

Transcription factors maintain control over T cell development and cytokine gene expression. Some of the factors important in T cells are reviewed in this section.

1.8.1. Ikaros

Ikaros contains four zinc-finger domains, three of which are required to bind to DNA (reviewed in Kuo and Leiden, 1999). The evidence for Ikaros involvement in lymphoid cell development came from the observation that its expression is detected in haematopoietic stem cells in the yolk sac, then in the liver, and later in the bone marrow in the same time frame as stem cell migration from the yolk sac to the liver then to the bone marrow. In Ikaros knockout mice, B cell development was arrested before the immature pro-B cell stage, and the foetal thymus of these mice had no lymphocytes. However, soon after birth T cells began to populate the thymus. These T cells have a number of deficiencies including a CD4⁺/CD8⁺ ratio that is higher than normal, antigen

presenting and NK cells numbers that are much lower than normal, thymic and splenic T cells that hyperproliferate in response to an antigen, and defective TCR $\gamma\delta$ T cells. Knockout of Ikaros and other related factors by a dominant negative form of Ikaros abolish T, B and NK cells development while the other haematopoietic lineages are unaffected. As a result, a model was proposed that Ikaros and other related proteins are required for the differentiation of a T and B common progenitor. It was observed that Ikaros associates with inactive genes and localises to heterochromatin compartments (Brown et al., 1997).

1.8.2. GATA3

GATA proteins are zinc finger proteins that bind (A/T)GATA(A/G) consensus sequences (reviewed in Kuo and Leiden, 1999). GATA1 protein is required for erythrocyte development while GATA3 is required for T and NK cells development. GATA3 is required for the development of CD4/CD8 double positive thymocytes. It is also required for the development of Th2 cells.

Reducing GATA3 protein level by stably transfecting GATA3 anti-sense RNA in a Th2 cell line abolished IL-4, IL-5, IL-6, IL-10 and IL-13 expression, while IL-3 expression was not affected (Zheng and Flavell, 1997). Further support for the role of GATA3 in Th2 development was obtained from a line of transgenic mice that was created with the GATA3 gene under the control of the CD4 promoter, which resulted in all CD4⁺ cells expressing GATA3. Both Th1 and Th2 cells from these animals expressed IL-4, IL-5, IL-6, and IL-10 (Zheng and Flavell, 1997).

1.8.3. LKLF

LKLF is a 40-kD transcription factor and member of the zinc-finger Kruppel-like factor (KLF) protein family that also includes EKLF, GKLF, and BKLF that bind to CACCC boxes (reviewed in Kuo and Leiden, 1999). EKLF was shown to bind to the β -globin gene promoter and is required for the switch from γ - to β -globin. LKLF is expressed in areas where T cells reside in the thymic medulla and the white pulp of the spleen. It is expressed at high levels in CD4 and CD8 single positive resting T cells but not in double positive or double negative T cells. After activation of T cells, no LKLF mRNA or protein can be detected. Hence, LKLF may function to prevent spontaneous activation of T cells.

1.8.4. NFAT

NFAT is a family of Ca^{2+} -inducible transcription factors required for the activation of many cytokine genes in T cells. NFAT proteins reside in the cytoplasm of resting T cells, and rapidly migrate to the nucleus in response to Ca^{2+} mobilisation (reviewed in Rao et al., 1997). NFAT typically functions in a complex with Fos and Jun on composite NFAT/AP-1 elements, but it can also synergise with other factors such as Oct family proteins. When T cells become activated, NFAT becomes dephosphorylated which results in the appearance of NFAT in the nucleus as described earlier and increased affinity for DNA. In addition to the N- and C-terminal trans activation domains (TAD), NFAT members have two major domains: the DNA-binding domain (DBD) and the NFAT homology region (NHR). The DBD is moderately similar to the DBD of rel-family proteins. The NHR is the region that contains the nuclear transport signal and it is

the site that is phosphorylated in resting T cells. Calcineurin binds to the NHR and dephosphorylates it. The TADs are not conserved.

There are four closely related NFAT proteins that have recently been designated as NFATC1-4 by the HUGO nomenclature committee. NFATC1 (NFAT2 or NFATc) and NFATC2 (NFAT1 or NFATp) are found in T cells and thymocytes. In addition, NFATC2 is expressed in monocytes, pancreas, and placenta. NFATC1 is expressed in muscles and testis and it is implicated in cardiac valve development. NFATC4 (NFAT3) is expressed in many tissues except T cells. NFATC3 (NFATx or NFAT4) expression, however, is tightly restricted to T cells and skeletal muscle (Kuo and Leiden, 1999).

T cells from NFATC2^{-/-} mice were defective in the induction of IL-3, IL-13, TNF α , GM-CSF, FasL, and CD40L genes after CD3 stimulation (Kiani et al., 1997). These T cells also developed more Th2 T cells than normal and low numbers of Th1 T cells. Early IL-4 production was abolished but late production was prolonged and more elevated than normal. Hence NFATC2 seems to play a negative role in Th2 development and seems to induce early IL-4 expression and play a role in termination of IL-4 gene expression. IL-2 expression was normal. These cells appear to be hyperproliferative in response to antigen stimulation. These observations indicate that NFATC2 plays an important negative role in the maintenance of IL-4 as well as the proliferation response of T cells. In addition, NFATC2 is important in the expression of IL-3, IL-13, TNF α , GM-CSF, FasL, and CD40L (reviewed in Kuo and Leiden, 1999).

NFATC1^{-/-} knockout experiments show that T cell development is blocked at the CD4/CD8 double negative stage (reviewed in Kuo and Leiden, 1999). These T cells

show impaired proliferative responses when activated. IL-4, and IL-6 production was significantly reduced, while IL-2 expression was not affected.

NFATC1 and NFATC2 appear to be important at different stages of T cell development. While NFATC1 appears to play a role at an earlier stage of development as well as a positive role in IL-4 expression and Th2 development, NFATC2 plays a role at a later stage and a negative role in IL-4 expression and Th2 development (reviewed in Kuo and Leiden, 1999). Because loss of either NFATC1 or NFATC2 did not seem to affect IL-2 expression, it was suggested that NFATC3 might be more important for IL-2 expression than NFATC1 or NFATC2.

NFAT5 has been recently cloned. It is a 1,455-aa nuclear phosphoprotein that shares homology with the four cytoplasmic NFATC proteins only in its Rel-like DNA-binding domain (López-Rodríguez et al., 1999). It differs from the others in that it does not bind cooperatively with AP-1 due to its lack of Fos/Jun contact residues, and it is constitutively present in the nucleus regardless of cell activation.

1.8.5. Maf

The proto-oncogene c-maf belongs to the b-ZIP superfamily of transcription factors (reviewed in Kuo and Leiden, 1999; Viola and Rao 1999). The b-ZIP family includes AP-1 and CREB/ATF transcription factor families. C-Maf is a 42-kDa protein that forms homo- and heterodimers. It is preferentially expressed in Th2 cells and binds to the sequence: TGCTGAC(-/G)TCAGCA, which also contains an AP-1 binding site. Cotransfection of c-Maf but not AP-1 with NFATC2 expression plasmids into the M12 B

cell line resulted in trans-activation of the IL-4 promoter in a reporter gene plasmid (Ho et al., 1996). These experiments suggested a role for c-Maf in Th2 development.

1.8.6. Activator Protein-1 (AP-1)

AP-1 is a dimer of Jun and Fos proteins, which are members of the leucine zipper family (reviewed in Foletta, 1996). Both *fos* and *jun* family proteins have a DNA binding domain located N-terminal to the leucine zipper domain, which is required for the heterodimerisation. Growth factors, serum, calcium, phorbol esters, UV light, and cAMP have been shown to induce the *c-fos* and *c-jun* genes. Serum response factor (SRF) binds to the serum response element (SRE) on the *c-fos* promoter in both stimulated and unstimulated states. Activation of *c-fos* and *c-jun* occurs when specific receptors on the surface of a cell are conjugated or when chemicals like PMA are used to activate the cells. PMA leads to the activation of protein kinase C, which leads via kinase signalling cascade to the phosphorylation of proteins like TCF/Elk-1 (see figure 1.4). Phosphorylated TCF/Elk-1 proteins have increased binding activity to the SRE and associate with the SRF on *c-fos* promoter, which leads to the transcription of *c-fos*. The newly synthesised c-Fos protein then binds to the *c-jun* promoter along with pre-existing c-Jun protein to activate the *c-jun* gene.

When protein kinase C is activated, it activates Jun phosphatase, which in turn dephosphorylates the c-Jun DNA binding domain (reviewed in Foletta, 1996). The dephosphorylated c-Jun along with c-Fos binds to the *c-jun* promoter. In addition, the c-Jun N terminal kinase (JNK) phosphorylates the N-terminal domain of c-Jun to stimulate the transcriptional activity of *c-jun*.

1.8.7. Core Binding Factor (CBF)

CBF is a heterodimer of a CBF α and a CBF β protein (reviewed in Speck and Stacy, 1995). Only CBF α can independently bind to the DNA, and when CBF β dimerises with CBF α , it increases the affinity of CBF α for DNA. The CBF α proteins are encoded by three genes, which code for CBF α 1, CBF α 2, CBF α 3 proteins. The CBF β protein is encoded by only one gene. The CBF α 2 gene was originally called the AML1 gene (Miyoshi et al., 1991). AML1 was identified as a gene that is often rearranged in patients with acute myeloid leukemia (AML). In about 20% of AML patients the AML1 gene on chromosome 21 is translocated to chromosome 8, in juxtaposition with the ETO gene. Analysis of the resulting fusion protein led to the cloning of the AML1 gene. This chimerical protein contains the first 177 aa of the AML1 protein and 575 aa of the ETO protein. The AML1 portion of the fusion protein is homologous to the *Drosophila* segmentation gene, *runt* (Erickson et al, 1992). The *runt* homology domain (RHD) contains the DNA binding domain as well as the CBF β binding domain (Lenny et al., 1995). The AML1/ETO protein binds to DNA but can't participate in the transactivation of genes because it has lost its transactivating domain (Zhang et al., 1995). As a result it prevents other transcription factors like the other AML1 protein from the second allele as well as other isoform of the AML proteins from binding, and thereby acts as a repressor. It was shown that the AML1/ETO fusion protein blocks the GM-CSF promoter transactivation (Frank et al, 1995).

Only the CBF α protein contains nuclear localisation signals and is found in the nucleus (reviewed in Speck and Stacy, 1995; Meyers and Hiebert, 1995). The CBF β protein is expressed ubiquitously and found in the cytoplasm. It is translocated to the nucleus only

when the CBF α protein is expressed. This suggests that CBF β is translocated to the nucleus only when it is associated with CBF α . The involvement of AML1 in leukemia suggests that it plays an important role in haematopoietic differentiation and proliferation. AML1 protein binds to the consensus sequence: TG(T/C)GGTT that is found in many T, B, and myeloid cell specific genes. It was shown to activate the IL-3 promoter (Cameron et al., 1994), GM-CSF promoter and enhancer (Cockerill et al., 1996; Takahashi et al., 1995; Frank et al., 1995), and TCR (Redondo et al., 1992).

1.8.8. Sp1

The transcription factor Sp1 is a member of a family of zinc finger proteins and it is ubiquitously expressed (reviewed in Philipsen, and Suske, 1999). Sp1 was named after the method that purified it which used sephacryl and phosphocellulose. This family has at least 16 members that have a conserved C-terminus three-zinc finger DNA binding domain. Sp1, Sp3, and Sp4 bind to a 9 bp GC box that spans the sequence GGGCGG. Sp2, however, does not bind to the classical GC box but it binds to a GT box. This is because Sp2 differs from the other Sp proteins in the first finger where a histidine residue is replaced with a leucine. In addition to the DNA-binding domain, the Sp family proteins contain glutamine-rich activation domains. Sp2 is significantly different than the other Sp proteins, which are closely related. Other groups related to this family are the Kruppel-like factors (KLFs), the basic transcription element binding protein (BTEB) and the early growth response (EGR) gene.

It has been shown that Sp1 is involved in the regulation of many genes as well as cell cycle genes. For example, the mouse Thymidine Kinase (TK), which is involved in

DNA replication, is regulated by Sp1 and E2F proteins. The promoter of the TK gene was shown to be regulated by the interaction of Sp1 with E2F transcription factor (Karlseder et al., 1996). In addition to TK, many of the genes that are expressed during the cell cycle such as *c-fos*, *c-myc*, adenosine deaminase, dihydrofolate reductase, retinoblastoma, cyclin A and E and DNA polymerase α are regulated by E2F interaction with Sp1 on their promoters. Promoters of some of these genes do not have a TATA box (Lin et al., 1996). Hence, Sp1 and E2F have been implicated in these promoters for the initiation of transcription in cell cycle regulated genes. Sp1 was also implicated in preventing CpG island methylation (Macleod et al., 1994; Brandeis et al., 1994). Sp1 binding to HS3 of the human β -globin locus was shown to be essential for an LCR function (Philipsen et al., 1993).

The importance of Sp1 has been demonstrated in Sp1 knockout mice (Marin et al., 1997). The phenotype of Sp1^{-/-} knockout is embryonic lethal at day 11. However, during these early days of embryonic development, the ES cells continued to develop and differentiate normally until day 9.5 and then died at day 11. Methylation free islands were maintained, cell cycle genes expression was normal, and the β -globin locus was active. Hence, Sp1 does not seem to be important in early development. However, the MeCP2 gene expression was severely reduced in the Sp1^{-/-} mice. Sp1 regulates the gene expression of MeCP2 protein, which binds to methylated DNA to maintain the differentiated state by maintaining the methylation status. This suggested that Sp1 is important in later stages of development.

1.8.9. NF- κ B

NF- κ B is a dimer of members of the Rel family of proteins (Hatada et al., 2000; Baldwin, 1996). This family consists of RelA (P65), *c-Rel*, RelB, p50, and p52. P50 and P52 are derived from the precursors p105 and p100 respectively. All of the NF- κ B proteins contain a Rel homology domain (RHD). The RHD interacts with the inhibitors of NF- κ B (I κ B) proteins through the c-terminal ankyrin repeats motifs, and functions in dimerisation and DNA binding. Ankyrin repeats prevent DNA binding as well as nuclear transport. These proteins form either homo- or hetero-dimers. In resting cells, I κ B- α and I κ B- β proteins bind NF- κ B and inactivate it (Blackwell and Christman 1997). The precursors p100 and p105 can dimerise with RelA. In these cases, the c-terminal of p100 and p105 contain ankyrin repeats termed I κ B- δ and I κ B- γ that interact with the RHD and mask the nuclear localisation signal and prevent NF- κ B from going to the nucleus.

Sustained NF- κ B stimulation requires two signals (reviewed in Gerondakis et al., 1998). In T cells it requires TCR and CD28 signalling. Single signals induced by agents such as PMA leads to phosphorylation and degradation of only I κ B- α . The I κ B- α gene is activated by NF- κ B, which leads to the inactivation of NF- κ B very rapidly. However, CD28 signalling leads to the phosphorylation and degradation of both of I κ B- α and I κ B- β . NF κ B does not regulate I κ B- β gene. Hence, NF κ B activation is sustained. An early step in the phosphorylation of I κ B- α and I κ B- β is the activation of I κ B- α kinases (IKK α) and I κ B- β kinases (IKK β) (Hatada et al., 2000).

Phosphorylation of I κ B leads to its ubiquitination, which results in the degradation of I κ B by the proteosome (reviewed in Baeuerle and Baichwal, 1997). This exposes the nuclear localisation signal on NF- κ B, which facilitates its nuclear translocation. Once NF- κ B is in the nucleus it binds to its target DNA and participates in gene activation.

NF- κ B is involved in the regulation of many genes involved in inflammatory and immune responses. NF- κ B binding sites are found in CD28 response elements, which are present in the IL-2, IL-3, and GM-CSF promoters (Himes et al., 1996). NF- κ B is also important for IL-1, IL-6 and IL-8 gene expression (Smith et al., 1997; Makarov, et al., 1997; Medzhitov, 1997).

1.8.10. Oct

Octamer (Oct) transcription factors are members of the POU (Pit-1, Oct-1, Oct-2, and Unc-86) family of transcription factors that bind to the octameric consensus ATGCAAAT (reviewed in Latchman, 1999). In addition to the original four members of the POU family, other members have been identified such as Brn-2, Brn-3 and Brn-4. All members of the POU family have two POU homology domains that interact with separate halves of the octamer sequence. One of the POU homology domains is unique to POU family member, while the other POU homology domain is related to the homeodomain of homeobox proteins. Many of the POU family members have been shown by gene knockout mice experiments to be critical for the development of the nervous system (reviewed in Latchman, 1999).

Oct-1 protein is ubiquitously expressed and binds to ubiquitously active promoters such as the histone H2B gene promoters (Fletcher et al., 1987). Oct-2 was initially thought to be a B cell-specific transcription factor involved in the expression of B cell-specific genes such as the immunoglobulin genes, and in B cell development. It was found at low level in pro- or pre B cells but at high concentration in mature B cells. However, Oct-2 was subsequently found in T cells and other haematopoietic lineages (Cockerill and Klinken, 1990), and in cells from the nervous system (reviewed in Matthias, 1998). Oct-2 knockout mice die soon after birth (Corcoran et al., 1993). B cells from these mice are found at normal numbers, but are unresponsive to LPS, and the number of IgM⁺ cells is reduced. Moreover, transcription of B cell-specific genes containing Oct binding sites was unaffected. This study indicated that transcription of B cell-specific gene could proceed without Oct-2, and that Oct-2 is important for final stages of development rather than early stages. RAG1^{-/-} mice reconstituted with Oct-2^{-/-} B cells confirmed the previous observations and showed that late Ig transcription is impaired even though the early Ig transcription is unaffected (Humbert and Corcoran, 1997). It was suggested that in these mice Oct-1 might have maintained B cell development at early stages not at later stages. T cells in these mice were normal.

An Oct co-factor was isolated by different laboratories and was called OCA-B, OBF-1, or Bob-1 (Luo and Roeder, 1995; Strubin et al, 1995; Gstaiger et al, 1995). This co-factor interacts with both Oct-1 and Oct-2, but not with other Oct family proteins (Sauter and Matthias, 1997). OCA-B is found in both B and T cells. OCA-B^{-/-} knockout mice were born normal, and early B cell development was normal in the bone marrow (Schubart et al., 1996; Kim et al., 1996; Schubart et al., 2000). However, their spleens have reduced number of B cells, and mature B cell numbers were significantly reduced.

Although IgM levels were normal, IgG and IgA levels were significantly reduced. The capacity of these mice to mount an immune response was impaired, which correlated with the lack of germinal centre formation. T cell functions in these mice are normal. These studies showed that OCA-B is critical for B cell development as B cells exit from the bone marrow. However, the low number of mature B cells observed in these knockout mice came from the foetal liver and not from the bone marrow. Although, these studies unexpectedly showed that Oct-2 and OCA-B are not essential for Ig gene expression a more recent study showed that promoters that contain octamer motifs like the κ Ig promoter are totally inactive in OCA-B^{-/-} B cells (Laumen et al., 2000). The authors of this study suggested that in transfection experiments, truncated promoters do not include all of the regulatory elements present in endogenous genes. Therefore, there may be other factors that are not present in short promoter/enhancer constructs that compensate for the lack of OCA-B or Oct proteins.

In a transient transfection study of a T cell-specific IL-3 enhancer, Bert et al. (2000a) found that a composite element (IL-190) of NFAT and Oct is T cell-specific. The T cell-specific activation of this element required the presence of OCA-B and the NFAT co-factor NIP45 in addition to NFAT and Oct family proteins. Furthermore, this element could be activated in other transfected cell types by restoring the full complement of four factors (Bert et al, 2000a).

1.9. IL-3 AND GM-CSF LOCUS

The IL-3 and GM-CSF genes are located in the 5q31 region of chromosome 5 within a cluster of cytokines in a 1-Mb region (Le Beau et al., 1987; Yang et al., 1988; Frazer et al., 1997). Other cytokines in the cluster are IL-4, IL-5, and IL-13.

In vitro studies have shown that IL-3 is a cytokine that regulates the differentiation and proliferation of haematopoietic precursor cells of granulocytes (basophils, neutrophils), erythrocytes, monocytes (macrophages, dendritic cells), megakaryocytes, eosinophils, and mast cells (reviewed in Nimer and Uchida, 1995). GM-CSF is a cytokine that regulates the growth, differentiation and activation of a more restricted panel of cells of the granulocyte and macrophage lineages. While GM-CSF is produced by many cell types such as T cells, monocytes, macrophages, natural killer (NK) cells, mast cells, basophils, eosinophils, megakaryocytes, endothelial cells and fibroblasts, IL-3 is produced primarily by activated T cells, NK cells, mast cells, basophils and eosinophils.

The role of IL-3 is to stimulate the growth and differentiation of progenitor cells. GM-CSF acts at a later stage of differentiation to stimulate the differentiation into macrophages and granulocytes, while G-CSF promotes the differentiation of neutrophils, and IL-5 promotes the final stages of differentiation of eosinophils (Niemeyer et al., 1989; Plaut et al., 1989). IL-3, IL-4, IL-5, and GM-CSF are closely linked and produced by activated T cells and mast cells in specific situations (i.e. inflammation and immune response) to expand specific subset of hematopoietic cells and to promote immune and inflammatory responses.

The IL-3 and GM-CSF receptors are found at low numbers on the surface of myeloid cells (Reviewed in Miyajima, 1992; Woodcock et al., 1999). The IL-5, IL-3 and GM-CSF receptors have a common β -chain that is responsible for signalling. Each one of these receptors has a specific α chain that directs the specific binding to cytokine and the α -chains alone function as low affinity receptors. When IL-3, IL-5, or GM-CSF binds to its specific α chain, the common β (β_c) chain is recruited, which results in the formation of a high affinity complex (reviewed in Miyajima, 1996). The β_c then transduces a signal to intracellular pathways to activate a number of genes, which initiates the differentiation or activation process. In mice there is another β chain, which is specific to IL-3 and it is called β_{IL-3} . Mouse IL-3 can transduce through both of β -chain types (Nishinakamura et al., 1995).

Mice have been generated recently that lack β_c chain, and the IL-3 protein (Nishinakamura et al., 1995; Nishinakamura et al., 1996). Hence, these mice lack the entire IL-3, IL-5, and GM-CSF function. The surprising results of these studies were that these mice have normal haematopoiesis. The defects in these mice are pulmonary alveolar proteinosis, reduced numbers of eosinophils and the lack of eosinophilic response to parasites, which is similar to mice deficient only in β_c , which leaves the IL-3 function intact. Moreover, both of these mouse gene knockouts have lymphocytic infiltration in the lung. These results contradict the model that IL-3, IL-5, and GM-CSF are produced by activated T cells to promote haemopoiesis. In addition, since both of these knockout mice have similar pathology, one could conclude that the pathology observed is due to the lack of IL-5 and GM-CSF function only. It was suggested that the function of these cytokines is redundant and that there may be other mechanisms sufficient for myeloid haemopoiesis. However, β_c chain activates the JAK2 and STAT5

signalling pathway, which is required for cell-cycle progression and the Ras pathway, which is required for the survival of the activated cells (reviewed in Hara and Miyajima, 1996; Scott, and Begley 1999). Both of these signalling pathways are required for the proliferation of cells. This suggests a proliferative, anti-apoptotic function for the IL-3, IL-5, and GM-CSF cytokine.

A mouse IL-3 gene knockout was shown to have normal mast cells and basophils. However, these mice failed to increase the number of mast cells and basophils in tissues in response to parasitic infection. This clearly indicated that IL-3 is important for increasing the number of immune cells needed for immunity to parasites (Lantz et al., 1998). Moreover, it was found that delayed hypersensitivity was impaired in these mice (Mach et al., 1998).

GM-CSF, IL-3, and IL-5 have been shown to be involved in allergy and diseases such as leukemia and asthma (Kay et al., 1991). IL-3, IL-5, and GM-CSF gene expression is increased in infiltrating cells in allergen-induced reactions in skin biopsies. In asthmatic patients, activated eosinophils accumulate in the bronchioles as a result of IL-5 and GM-CSF released from infiltrating CD4⁺ but not CD8⁺ T cells (Till et al., 1995). Peripheral blood CD4⁺ Th2 T cells were responsible for the increased production of IL-5 and GM-CSF. IL-3 expression was not increased in either CD4⁺ or CD8⁺ populations. Anti-GM-CSF antibodies, and to a lesser degree the anti-IL-5 antibodies, inhibited the prolonging of survival and activation of eosinophils (Corrigan et al., 1995).

Acute myelogenous leukemias (AMLs) have been reported to express low levels of retinoblastoma (RB), an inactive form of p53, and constitutively active Ras protein

(Preisler et al., 1997). Because RB and p53 suppress IL-6 expression, RB and p53 abnormalities may be responsible for the increased production of IL-6 in AML. Moreover, some AMLs produce IL-1b autonomously, which induces GM-CSF expression. It has been suggested that GM-CSF can act as an intracellular autocrine growth factor (Young, 1987), which gives the AML cell an advantage over the normal cells. IL-1b also stimulates leukemic cells to produce IL-6, TNF α , and stem cell factor (SCF) (Preisler et al., 1997). It also stimulates normal cells to produce IL-3, GM-CSF, and G-CSF. TNF α increases the expression of cytokine receptors on the surface cells. IL-6 and SCF enhance the effects or proliferative responses to GM-CSF and IL-3 and induces the production of more leukemic cells.

Juvenile chronic myelogenous leukemia (JCML) is characterised by excessive proliferation of immature myeloid cells in children and GM-CSF has been shown to promote the survival of JCML cells (Iversen et al., 1996; Iversen et al., 1997). It has been shown also that JCML cells produce GM-CSF and monoclonal antibodies have been shown to inhibit colony formation of JCML. Moreover, the GM-CSF antagonist named E21R that binds to the α chain of the GM-CSF receptor without recruiting the β chain resulted in the induction of apoptosis in JCML cells.

The defect in chronic myelogenous leukemia (CML) is the translocation of the abl oncogene from chromosome 9 to the breakpoint cluster region (bcr) of chromosome 22 (known as Philadelphia chromosome) (Groffen et al., 1984). This translocation results in an 8 kb mRNA encoding the fused abl and bcr genes (Shtivelman et al., 1985), which is translated into a 210-kDa (P210) phosphoprotein (Ben-Neriah et al., 1986). The resulting fusion protein is expressed constitutively and has an enhanced kinase activity

(reviewed in Tefferi, 1999). It has been shown that this kinase activity activates the phosphatidylinositol 3-kinase (PI3K) signal transduction pathway, which is essential for leukemogenesis by activating Akt. The effect of Akt activation is the autocrine production of IL-3, which leads to the expression of the anti-apoptotic protein, Bcl-2 (Skorski et al., 1997). The involvement of constitutive cytokine expression in the maintenance of leukemic cells highlighted the importance of understanding the mechanism of cellular regulation of cytokines.

The GM-CSF and IL-3 genes are 10 kb apart on chromosome 5 (Frolova et al., 1991) and are expressed in T cells in response to activation by the TCR (reviewed in Nicola, 1989; Chan et al., 1986). IL-3 and GM-CSF production is regulated at different levels. At the cellular level, the cells making these cytokines need to be stimulated by conjugation of specific receptors on the surface of these cells (eg TCR, IL-1b, etc.) (Reviewed in Malter, 1998). An intracellular signal is then transduced that activates transcription factors that activate transcription of IL-3 and GM-CSF genes as will be described in detail later. Another level of regulation is at the level of m-RNA stability. IL-3 and GM-CSF mRNA have 3' AU-rich sequences (AUUUA), which make these RNA unstable. In resting T cells, mRNA of IL-3 and GM-CSF are not detected by northern, RNase protection or PCR, but detected in nuclear run-on experiments, which suggests that these RNAs may be synthesised even in the resting state but are unstable. However, IL-3 and GM-CSF mRNA become stable and accumulate to a high level after cellular stimulation.

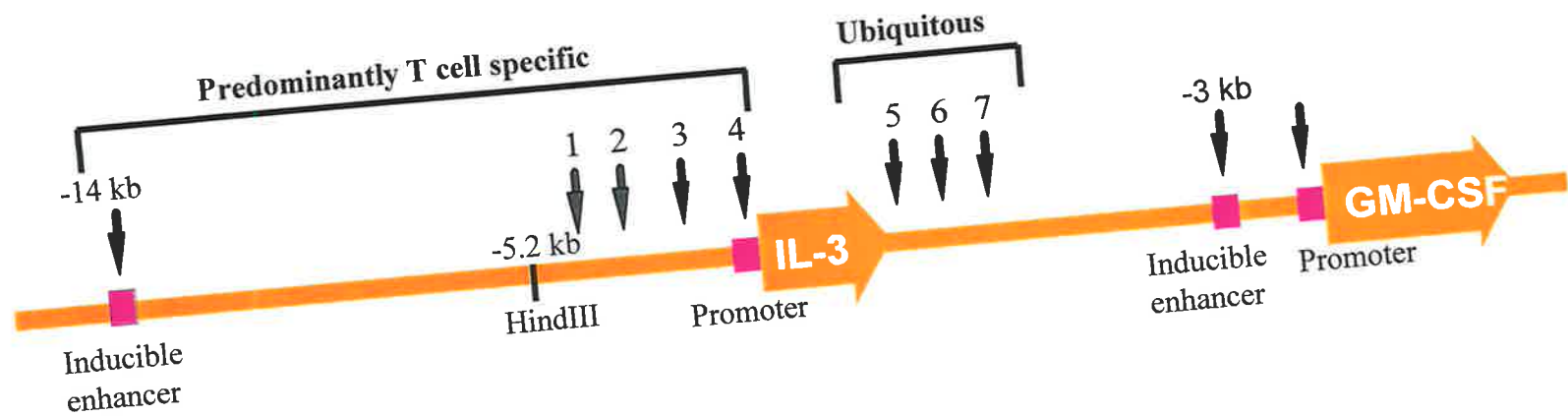


Fig. 1.5 Representation of the IL-3 and GM-CSF locus

Diagram showing the IL-3 and GM-CSF gene locus. The solid vertical arrows represent the location of the DH sites. The solid squares represent well-defined regulatory elements as indicated in the text.

hand, a second construct where the GM-CSF enhancer was deleted had a lower and more variable level of activity. This suggested that the enhancer is sufficient to correctly regulate the expression of GM-CSF and to protect the transgene from the effects of the surrounding genes (Cockerill et al., 1999).

1.9.2. IL-3 Promoter regulation

The IL-3 promoter is activated in T cells by TCR signalling pathways that can be directly activated by the combination of the PMA and the CaI. The IL-3 promoter is located within 330 base pairs immediately upstream of the transcription initiation site (figure 1.6).

The region upstream of the TATA box between -47 and -76 contains binding sites for two inducible zinc finger proteins, Egr-1 and Egr-2 and a constitutive factor, DB1 which maintain a low level basal transcription in unstimulated T cells (Koyano-Nakagawa et al., 1994). The region from -109 to -128, which contains the CK-1, CK-2/GATA have been identified on the basis of sequence homology with other regulatory regions. Mutations in the CK1 and CK2 elements abrogated the activation of the IL-3 promoter in response to CD28 signalling (Fraser and Weiss, 1992). It has been suggested that this region is involved in down regulating the basal transcription machinery and that CD28 signalling relieves this repression (Ryan et al., 1994).

In addition to the TATA box and the CK-1 and CK-2/GATA elements, several activating elements have been identified in the promoter region as well as an inhibitory element. These elements were defined by deletion analysis. The first activating element is located

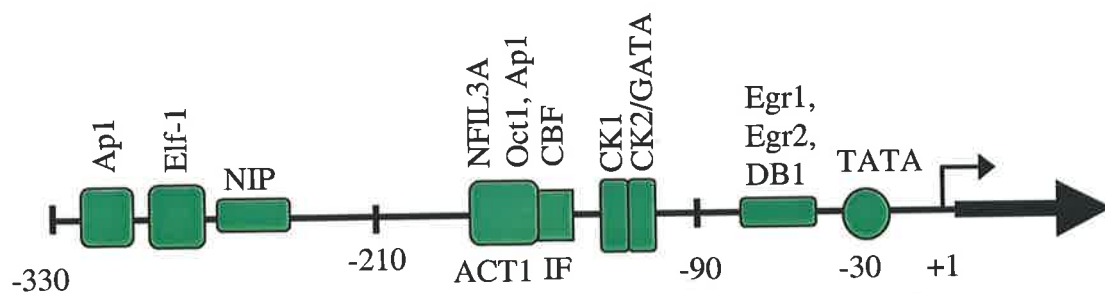


Fig. 1.6 Structure of the IL-3 promoter.

The IL-3 gene is represented as a filled arrow. A 90°-bent arrow represents the start of transcription. The diagram shows binding of transcription factors to the promoter upstream of the transcription start site as discussed in the text. The numbers under the line are the distance in base pair from the transcription start site.

between -143 and -173 and is called the ACT-1 (NFIL-3A). Oct1, and AP-1 were found to bind to this region (Mathey-Prevot et al., 1990; Gottschalk et al., 1993; Davies et al., 1993; Shoemaker et al., 1990; Park et al., 1993). A T cell-specific factor was also found to bind to this region and was called NFIL-3A (Zhang et al., 1995). Cameron et al. identified the IF-1 element immediately downstream of the ACT-1 element that binds CBF (Cameron et al., 1994). IF-1 is required, but is not sufficient for IL-3 expression. A functional IF-1 site is required by AP-1 and ACT-1 for activation of IL-3 expression (Cameron et al., 1994). The CBF site has been shown to cooperate with the ACT-1 site and contribute to the tissue specificity of the promoter (Taylor et al., 1996). The second activating element is the AP-1/Elf-1 motif located between -271 and -315 (Mathey-Prevot et al., 1990; Gottschalk et al., 1993; Davies et al., 1993; Shoemaker et al., 1990). The AP-1/ Elf-1 region also contains an NFAT-like element, and this region is required for maximum promoter activity (Gottschalk et al., 1993).

The IL-3 promoter contains a NIP site between -241 and -270 bp, which functions as a repressor and it is present in many different tissues and species (Engeland et al., 1995). Engeland et al. (1995) suggested that NIP might function to repress IL-3 in all cell types. Recently, Ye et al. (1999) found that both YY1 (Yin-Yang 1) and an AP-2-like protein bind to the NIP region. The AP-2-like protein, which binds to DNA sequences similar to AP-2, was named an AP-2 sequence recognising factor (ASRF). The ASRF binding is required for the silencing activity of NIP. YY1 also binds to the NIP region as well. YY1 acts as an inhibitor of ASRF. Hence it was proposed that YY1 acts as a positive stimulator of the promoter transcription by inhibiting ASRF (Ye et al., 1999).

1.9.3. IL-3 Enhancer regulation

Initially, it was thought that the GM-CSF enhancer might regulate both the IL-3 and the GM-CSF genes. However, the GM-CSF gene as well as the enhancer is active in a variety of cell types that do not make IL-3 (Cockerill et al., 1995b; Cockerill et al., 1999). Duncliffe et al. (1997) identified a second enhancer in the IL-3/GM-CSF locus, located 14 kb upstream of the IL-3 gene, that functions as an inducible T cell-specific enhancer in transient transfection assays. This enhancer was located within an inducible DH site that forms in response to PMA and CaI stimulation and is inhibited by CsA. The enhancer is composed of four NFAT binding sites: IL70, IL140, IL190, and IL280. The IL70 element was not able to activate the luciferase gene above the promoter level in Jurkat cells. The other three functioned as enhancers similar to the full-length enhancer. Both the IL140 and the IL190 elements are located at the centre of the DH site. It was suggested that these two elements might be involved in the DH site formation. The IL190 element binds Oct factors and NFAT at the same time to overlapping sites (Duncliffe et al., 1997).

To examine the basis for the T cell specificity of the IL-3 enhancer, Bert et al. (2000a) studied the IL190 composite NFAT/Oct binding site. This element was shown to be T cell-specific, although both Oct and NFAT are present in cells other than T cells. It was shown that the NIP45 and OCA-B cofactors, which are lymphoid-specific but not necessarily T cell specific, are needed along with the NFAT and Oct factors for the T cell specificity of the element. In cell lines where the IL190 element was not active, it was shown that reconstituting the missing factors in co-transfection experiments restored the activity of the IL190 element. This work demonstrated that cell specificity is achieved

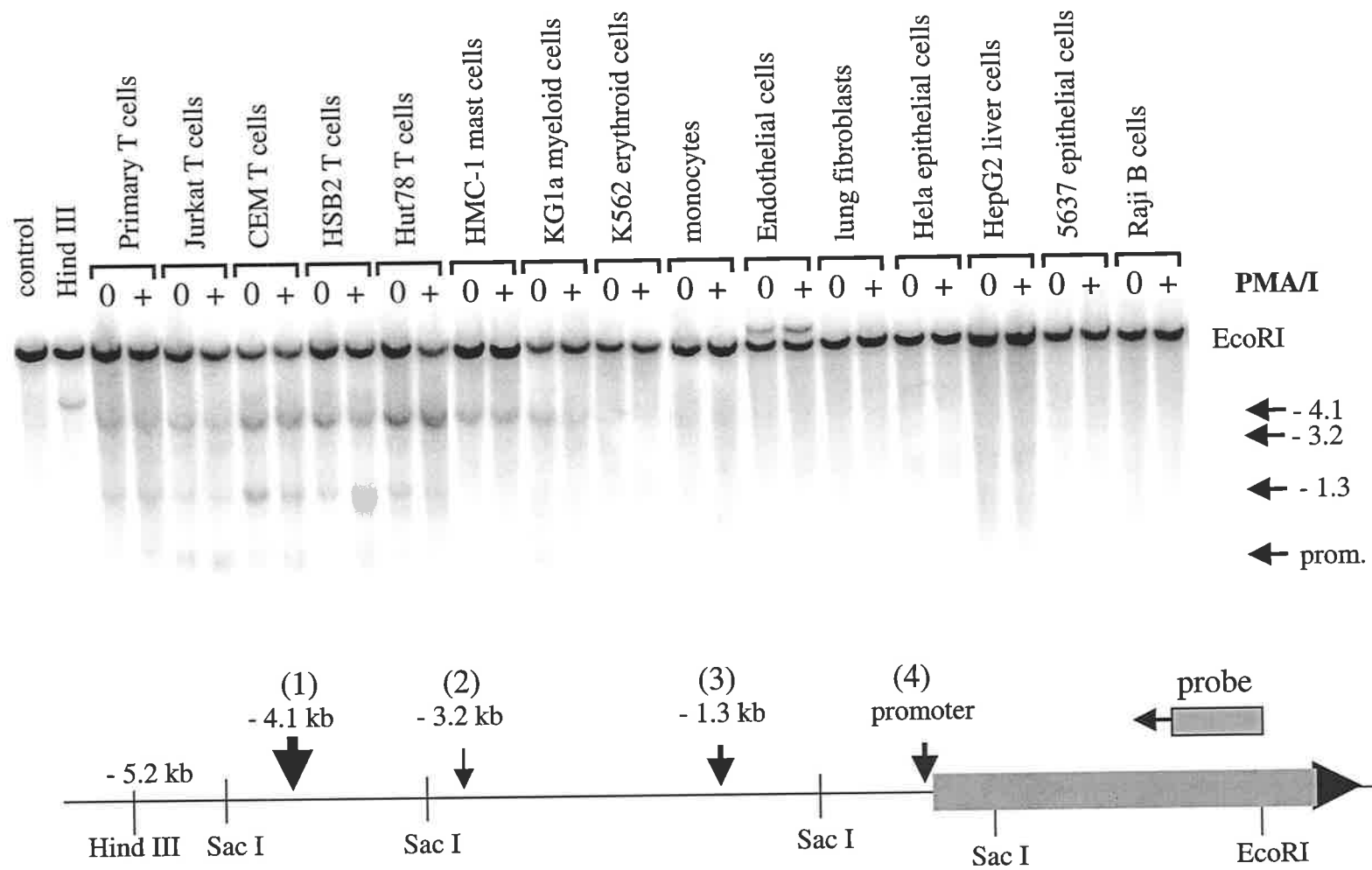
by the combinatorial arrangement of factors and co-factors that are not necessarily cell specific on their own. In this case, the presence of all four factors in T cells provided the T cell specificity of the IL190 element (Bert et al., 2000a).

1.9.4. Other DH sites flanking the IL-3 gene

Two distinct clusters of constitutive DH sites have also been identified just upstream and downstream of the IL-3 gene (figure 1.5) (Cockerill, 1993). The upstream cluster includes up to four DH sites that are primarily restricted to T cells (sites 1 to 4) and extends approximately 4.1 kb upstream of the IL-3 gene. The upstream cluster of DH sites was mapped by Peter Cockerill by Southern blot hybridisation using a probe from the 3' EcoRI site on DNA from haematopoietic and non-haematopoietic cells (figure 1.7). Blood T cells, and all of the T cell lines, have DH sites at -1.3 kb and -4.1 kb, and just Jurkat cells had the fourth DH site at -3.2 kb. The DH sites in the promoter and at -4.1 kb were also present in primitive undifferentiated CD34⁺ KG1a myeloid cells and just the -4.1 kb DH site was present in the HMC-1 mast cell line. Additional studies performed by Peter Cockerill have also found that the -4.1 kb DH site is the only member of the cluster present in HPB-ALL T cells and in one primary CD34⁺ AML sample (Cockerill, unpublished data) as was seen in HMC-1 cells. None of these sites are present in other cell types that were tested such as K562 cells, monocytes, endothelial cells, fibroblasts, epithelial cells, or B cells. This upstream cluster of sites, or at least the -4.1 kb site, seem to appear early in haematopoietic development and may activate the IL-3 locus in primitive haematopoietic cells committed to develop into IL-3-producing cells and it may therefore function as a tissue-specific Locus Control Region (LCR).

Fig. 1.7. DH site Assay

Southern blot hybridisation mapping of DH sites upstream of the IL-3 EcoRI site is presented. The primary cell lines tested are indicated above. Each cell type was tested without (0) or with PMA/CaI (+) stimulation. The lower diagram represents the location of the DH sites and their distance upstream of the transcription start site. The numbers within parenthesis are the DH site numbers. The major restriction enzyme sites are shown. The probe used in this assay is also shown. Data taken from Cockerill unpublished data.



The downstream cluster of three DH sites (DH sites 5 to 7) is ubiquitous, and occurs uniformly in all cell types (Cockerill et al, 1993 and 1999). One possibility is that this region may function as a boundary element to prevent the IL-3 gene from being activated by the GM-CSF enhancer in cells other than T cells that express GM-CSF but not IL-3.

1.10. AIMS AND HYPOTHESIS

The IL-3 gene is expressed in very few types of cells. It is predominantly produced by mature T cells activated by TCR stimulation. It has also been reported to be produced by mast cells (Ishizuka et al., 1999; Lorentz et al., 2000; Moller et al., 1998; Nilsson et al., 1995; Wodnar-Filipowicz et al, 1989), basophils (Lippert et al., 2000) and eosinophils (Kita et al., 1991; Fujisawa et al. 1994). One of the aims of this study is to understand the mechanisms by which this cell specificity and the developmental restriction of IL-3 gene are achieved. The DH sites in the cluster immediately upstream of IL-3 gene are present constitutively but are tissue-specific. The T cell lines studied in this laboratory resemble different stages of T cell development, and DH site 1 is invariably the most prominent site in the T cell lines, and in primitive myeloid cells such as KG1a. The presence of this site in primitive cells is particularly interesting as it suggests that this site is developmentally important.

We hypothesised that the -4.1 kb region may function as a locus control region that regulates the tissue-specific activation of the IL-3 locus. Because DH site 1 is the most prominent site and since it seems that it is involved in the development of the locus, we hypothesised that this site may contain a positive element that could play a major role in remodelling the locus and prime the cells to develop into IL-3-producing cells.

The region downstream of the IL-3 gene contains 3 ubiquitous DH sites. This region has been sequenced and analysed. The most 3' DH site has a structure that looks like a promoter element that includes a TATA box-like element upstream of an open reading frame that potentially codes for a proline rich protein. It is hypothesised that there may be another gene that is located in the region between the GM-CSF and the IL-3 genes. However, it is also possible that this cluster of ubiquitous sites represents a boundary element that segregates the IL-3 and GM-CSF genes into independently regulated domains.

The aims of this study were:

- (1) to assess the function of the DH site 1 in cell lines as a possible enhancer element,
- (2) to study the mechanism of function of any positive or negative element(s) that is identified in (1),
- (3) to study the function of the intact cluster of DH sites 1 to 4 in the 5.2 kb region upstream of the IL-3 gene in luciferase reporter gene plasmids in stable transfection assays with or without the enhancer located at -14 kb upstream as well as in a transgenic mice, and
- (4) to study the function(s) of the elements immediately downstream of the IL-3 gene as a possible promoter/ enhancer elements.

CHAPTER 2: MATERIALS AND METHODS

2.1 ABBREVIATION

ATP	adenosine triphosphate
Ac	Acetate
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
C	Centigrade
CsA	cyclosporin A
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol-bis-(β -amino-ethyl ether)N,N'-tetra-acetic acid
FCS	foetal calf serum
g	gram
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
IL	interleukin
kb	kilobase
LB	L broth
μ Ci	microcurie

μF	microfarad
μl	microlitre
μg	microgram
μM	micromolar
M	Molar
ml	millilitre
mM	millimolar
MOPS	3-[N-Morpholino]propanesulfonic acid
MQ H ₂ O	Millipore MilliQ-purified water
nl	nanolitre
nM	nanomole
ng	nanogram
pM	picomole
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PMSF	phenyl-methyl sulphonyl fluoride
RNA	ribo-nucleic acid
rpm	round per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SV40	Simian Virus
TK	thymidine kinase
Tris	tris(hydroxymethyl) aminomethane

2.2 CHEMICALS

The chemicals used in this study and suppliers are listed bellow:

Ampicillin, EGTA, HEPES, Tris base, PMA, PMSF, aprotinin, leupeptin, piperidine, Spermine, Spermidine, Xylene thiocyanate, cetrimide: Sigma Chemical Company, St. Louis, MO.;

Agarose, DTT, RNase inhibitor (RNA Guard): Promega Corporation, Madison, WI.;

SDS, acrylamide, bisacrylamide, N, N, N', N'-tetramethyl-ethene-diamine (TEMED): Bio-RAD, Richmond, CA;

BSA; CSL, Melbourne, Vic.;

EDTA, urea, formamide, chloroform, Formic acid, Bromo-Phenol Blue: BDH chemicals, Kilsyth, Vic.;

Gentamicin (Gentamycin): GibcoBRL, Rockville, MD.

Phenol: Wako Pure Chemical, Osaka, Japan, or BDH chemicals;

Deoxyribonucleotide triphosphate (dNTP), poly dI:dC, Concanavalin A: Pharmacia, Uppsala, Sweden;

Calcium ionophore A23187, D-luciferin, tRNA, recombinant mouse IL-2 (rmIL-2): Boehringer Mannheim, Australia, Castle Hill, NSW;

CsA: Sandoz, Basel, Switzerland.

Herring sperm DNA: Merck KGaA, Darmstadt, Germany.

Diethyl Pyrocarbonate (DEPC): ICN Biomedical Inc., Aurora, Ohio.

2.3 ENZYMES

All enzymes used in this study are listed along with their suppliers:

Restriction enzymes: New England Biolabs, Beverly, MA, Pharmacia, Promega, or Fermentas, Amherst, NY.

Calf intestinal alkaline phosphatase, Acetyl coenzyme A: Boehringer Mannheim;

Ribonuclease A: Sigma;

T4 ligase, T4 polymerase, T7 polymerase, T4 polynucleotide kinase: New England Biolabs, Beverly, MA;

Deoxyribonuclease I: Worthington, Freehold, NJ;

Proteinase K: Merck,

Pfx polymerase, SuperscriptII RNase H⁻ Reverse Transcriptase, Taq polymerase⁺ Gibco BRL;

2.4 RADIONUCLEOTIDES

Radionucleotides were obtained from the following sources:

[α -³²P]dATP, [α -³²P]dCTP, and [γ -³²P]dATP: Bresatec, Adelaide, Australia.

2.5 BUFFERS

Luciferase lysis buffer: 100 mM K₂HPO₄, 2 mM DTT, 10 mM EDTA, pH 7.4

Luciferase assay buffer: 100 mM K₂KPO₄, 20 mM DTT, 80 mM MgSO₄, 7.5 mM ATP, 1.75 mM Coenzyme A, pH 7.4

PBS: 136 mM NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.3

SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.1

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

TBE: 50 mM Tris-HCl, 50 mM boric acid, 1 mM EDTA, pH 8.3

TE: 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4

2.6 KITS

The kits used and their suppliers were are listed bellow:

- BresaClean: Bresa, Adelaide, SA, Australia.
- GigaPrime: Amersham, Little Chalfont, England.
- ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit: Applied BioSystems, Foster City, CA.
- Quiagen midi, Quiagen Qiaquick Nucleotide removal column: Quiagen, Valencia, CA.
- Bio-RAD P30 Bio-Spin 30 Chromatography column: Bio-Rad, Hercules, CA.

2.7 CLONING VECTORS

- pXP1 was a gift from Dr. S. Nordeen, University of Colorado, Denver, USA (Nordeen, 1988). This vector contains a luciferase reporter gene and it contains poly(A) addition signals upstream and down stream of the luciferase gene designed to block read-through transcription.

- pXPG is a modification of pXP1 (Bert et al., 2000b). It contains a novel mutation in the origin of replication that makes it a high copy number plasmid. The luciferase gene was replaced by a luciferase gene from pGL3 (Sherf and Wood, 1994), which lacks a peroxisomal targeting sequence.
- pXPG-GM55 (Bert et al., 2000b), was created by cloning a 101 bp HindIII fragment containing the minimal GM-CSF promoter was cloned into the HindIII site of pXPG (See figure 2.1 for a map of this plasmid).
- Bluescript: Promega
- pIC19H (Marsh et al., 1984).
- pE^otk.neo (Blasquez et al., 1989).
- pIC19H-MENK is a modification of pIC19H to expand the polylinker and include the following restriction enzyme sites: MluI, EagI, NcoI, and KpnI. pIC19H plasmid was digested with EcoRI and BglII and the following double stranded oligo was added by ligation:

5' -aattcgcccgacgcgtatcgatggtaccatggatatcta.....-3'
 3' -.....gccggctgcgcatagctaccatggtacctatagatctag-5'

- pXPG-TK750 was previously cloned in this laboratory by Joanna Burrows by cloning a 0.75 kb HindIII/BglII fragment of Herpes Simplex Virus (obtained from the Promega pRL-TK vector) into HindIII/BglII cut pXPG.
- pXPG-TK750-B383 was generated by cloning a 383 bp BamHI fragment from pIL3H-S1.3 encompassing the SS245 enhancer into the BamHI site of pXPG-TK750.

BamHI/BglII/XhoI/SacI/KpnI/SmaI/Sall/HindIII

GM55 Luciferase

pGM55

5'-AAGCTTCGCGAGGGTCACCATTAATCATTTCCTCTG
HindIII

TATA box
 TGTTATTTAAGAGCTCTTTTGCCAGTGAGCCCAGTAC

ACAGAGAGAAAGGCTAAAGTTCTGTCGACAAAGCTT-3'
HindIII

Organisation of the pXPG-GM55 plasmid (A), the polylinker is shown above the plasmid and the GM55 minimum promoter is cloned in the HindIII site of pXPG upstream of the luciferase gene. Panel (B) shows the sequence of the GM55 promoter. It shows the TATA box the YY1 and the AP-1/NFAT binding sites.

- PXPG-TK229-SS245 was generated by cutting pXPG-TK750-B383 with StuI/PvuII and religation. This removed all of the sequences 5' of the PvuII in the TK750 promoter and all of the sequences 3' of the StuI site in the B383 element.
- pXPG-TK229 was generated by cloning a 229 bp HindII-PvuII fragment spanning the TK promoter (obtained from pXPG-TK750) into HindII/Ecl136II cut pXPG.
- pXPG-TK229-SV40E was generated by cloning a 175 bp BamHI fragment containing sequences from 113 to 270 (from the SV40 enhancer region) (Duncliffe, et al. 1997) into the BamHI site of pXPG-TK229.
- pXPG-GM55-SV40E was generated by cloning a 175 bp BamHI fragment from the SV40 enhancer region into the BamHI site of pXPG-GM55.
- pIL3H (Duncliffe et al., 1997) contains the IL3 promoter which was generated by PCR using primers that contain HindIII restriction sites. The PCR product carries the HindIII sites at -559 and +50. The product was cleaved with HindIII and cloned into the HindIII sites of pXP1 plasmid.
- pXPG-IL3H was generated by subcloning a 611 bp HindIII IL-3 promoter fragment from pIL3H into HindIII cut pXPG.
- pIL3H-S1.3 and pXPG-IL3H-S1.3 were generated by cloning a 1.3 kb SacI fragment from 5' of the IL-3 locus into the SacI site of pIL3H and pXPG-IL3H respectively.
- pIL3H-B1.2 (Duncliffe et al., 1997) and pXPG-IL3H-B1.2 were generated by cloning a 1.2kb BglII fragment spanning the IL-3 enhancer into the BglII sites of pIL3H and pXPG-IL3H.
- pXPG-IL3H-NA330 was generated by cutting pXPG-GM55-NA330 (Duncliffe et al., 1997) with HindIII, (which removes the GM55 promoter) and then cloning in a 611 bp HindIII IL-3 promoter fragment.

- PXPG-GM55-GME contains a 425 bp BamHI/MscI fragment from the GM-CSF enhancer cloned into the BamHI/MscI sites of pXPG-GM55 (Bert et al., 2000b).
- pIL3H5.2 was generated by cloning a 5.0 kb HindIII/ ScaI fragment from the region immediately upstream of the IL-3 gene into a HindIII/ScaI partially cut pIL3H (excising most of the IL-3 promoter). The resulting plasmid contains the entire 5.2 kb region upstream of the transcription start site of the IL-3 gene.
- pXPG-H5.2 was generated by cloning a 5.2 kb HindIII fragment excised from pIL3H5.2 into a HindIII linearised pXPG plasmid.
- pXPG-GM55-SS245 was generated by cloning a 245 bp Ecl136II/StuI fragment in the SmaI site of pXPG-GM55.
- pXPG-GM55-SE546 was generated by cloning a 550 bp StuI/EcoRV (SE546) fragment encompassing -4.1 DH site into the SmaI site of pXPG-GM55.
- pXPG-GM55-SE1.3 A plasmid containing a 1.3 kb SmaI/EcoRV fragment (encompassing the HindIII to EcoRV DNA of H5.2 region from the pIL3H5.2 plasmid) cloned into the SmaI site of pXPG-GM55.
- pBlue-B1.2-H5.2 plasmid was generated by cloning a 1.2 kb BglII fragment (spanning the IL-3 enhancer), -14 kb upstream of the IL-3 gene and a 5.2 kb HindIII fragment from pXPG-H5.2 plasmid into the BamHI and HindIII sites.
- PblueSK-FRT-CD4-(Sfi/BsI), A plasmid containing the human CD4 gene, was a gift from Dr. Jerry Adams of the Walter and ELIZA Hall Institute of Medical Research, Melbourne, Vic. (Ogilvy, 1999).
- pBlue-B1.2-H5.2-CS1.7. A 1.7 kb MluI/NruI fragment was excised from pBlueSK-FRT-CD4- (Sfi/BsI) plasmid. The 1.7 kb fragment contains the CD4 coding sequence, an SV40 Intron and an SV40 poly A signal. This fragment was cloned into an MluI-SmaI cut pIC19H-MENK to give pIC19H-MENK-MN1.7cD4 (pIC-cD4). The cD4 gene

was then excised from pIC-cD4 with ClaI and SalI restriction enzymes and cloned into a ClaI/SalI cut pBlue-B1.2-H5.2 to give pBlue-B1.2-H5.2-CS1.7.

2.8 BACTERIAL CULTURE

A competent JM 109 strain of *E. coli* was used for preparation of most of the recombinant DNA plasmids. Large plasmids prepared for transgenic mice work, were introduced into super competent *Epicurian Coli* strain (Strain: Sure) supplied by Stratagene, LaJolla, CA. Cultures were grown in LB-broth or on LB-agar plates.

- LB-broth: 1% (w/v) Tryptone (Gibco), 0.5% (w/v) yeast extract (Gibco), 1% (w/v) NaCl, pH 7.0.
- LB-agar plates: LB-broth containing 1.5% (w/v) agar (Gibco).

Both media were sterilised by autoclaving. All recombinant plasmids contained the Ampicillin gene and this was utilised as a selectable marker. Ampicillin was added to the LB or the LB-agar to a final concentration of 100 µg/ml at less than 50°C.

2.9 RESTRICTION DIGESTS

Restriction enzymes were used to cut DNA, according to the manufacturer recommended reaction conditions. DNA fragments were then separated by electrophoresis through a 7 to 10 mm thick 0.8-1.2% agarose gel (depending on the sizes of the fragments to be separated) in 1X TA or 1X TAE buffer with 0.5 µg/ml Ethidium Bromide in both the gel and the running buffer. In the cases where DNA fragments were purified for cloning;

Ethidium Bromide was not used during electrophoresis, the gel was stained for 15 minutes with 0.5 µg/ml Ethidium Bromide after electrophoresis instead.

2.10 DNA CLONING

2.10.1 Purification of DNA fragments from gel (Bresa clean kit)

After staining the gel with Ethidium Bromide, the DNA was visualised with the aid of a UV light box and the required DNA fragment was cut out of the gel using a scalpel blade. The DNA was purified from the gel fragment using a BresaClean kit (Bresatec). The DNA was quantitated in a 0.8% gel agarose gel by comparing its intensity with that of a comparable size fragment of known concentration.

2.10.2 Ligation

Vector DNA was linearised with the appropriate restriction enzyme and terminal phosphates were removed by Calf Intestinal Alkaline Phosphatase (CIP). The ligation reaction was carried out in a 10 µl volume. A three molar excess of the inserted fragment was ligated to 100 ng of vector with 1 µl of T4 ligase (10 units/µl) at 4°C overnight or at 15°C for 3-5 hours. The T4 ligation buffer supplied by the manufacturer was used.

2.10.3 Competent cells

One colony from a freshly grown culture of *Escherichia Coli*, (JM109 strain from glycerol stock) was inoculated into 2 ml of LB in 10 ml polystyrene tubes (Sarstedt,

Germany) and grown overnight. This starter culture was used to inoculate 500 ml of LB, which was grown at 37°C, until the culture reached an Optical Density at 650 nm (OD₆₅₀) of 0.5. The cells were incubated on ice for 10 minutes and centrifuged at 2500 rpm for 10 minutes at 4°C. The cells were then washed with 250 ml of ice cold 0.1 M MgCl₂, and spun at 2500 rpm for 10 minutes at 4°C. The resulting pellet was then resuspended in 25 ml of ice cold 0.1 M CaCl₂ and incubated on ice for one hour, and Glycerol was added to a concentration of 10 %. Cells were stored in 550 µl aliquots at -70°C for future use.

2.10.4 Super-competent cells

A starter culture of 2 ml of ψ broth (2% (w/v) Tryptone (Gibco, BRL), 0.5% (w/v) yeast extract (Gibco BRL), and 0.5% (w/v) MgSO₄, pH adjusted to 7.6 with 0.1 M KOH) was inoculated with a colony from an LB plate of Epicurian *E. Coli*, Sure strain, freshly streaked from a glycerol stock and grown overnight at 37°C in a shaking incubator. Three hundred and thirty µl (1/30 volume) of the starter culture was used to inoculate 10 ml of ψ broth, which was grown at 37°C until the culture reached an OD₆₀₀ of 0.6. Five ml of the 10 ml culture was transferred into 100 ml of pre-warmed ψ broth and grown at 37°C until the culture reached an OD₆₀₀. The cells were incubated on ice for 5 minutes and centrifuged in a GPR Beckman centrifuge at 2500 rpm for 5 minutes at 4°C. The cell pellets were resuspended with 40 ml of ice-cold filter sterilised transformation buffer 1 (30 mM KAc, 100 mM RbCl, 10 mM CaCl₂(2H₂O), 50 mM MnCl₂(4H₂O), 15% Glycerol, adjusted pH to 5.8 with 0.2 M acetic acid) and incubated on ice for 5 minutes. The cells were then spun at 2500 rpm for 5 minutes at 4°C, and the pellet was resuspended in 4 ml of ice cold filter sterilised transformation buffer 2 (10 mM MOPS,

10 mM RbCl, 75 mM CaCl₂(2H₂O), 15% Glycerol, adjusted PH to 6.5 with 0.1 M KOH) and incubated on ice for 15 minutes. Cells were then stored in 550 µl aliquots at -70°C for future use.

2.10.5 Transformation

Competent bacteria were thawed just before use and 100 µl was added to a maximum of 10 µl of the ligation reaction or to 10 ng of plasmid DNA and incubated on ice for 15 to 20 minutes. The cells were then heat shocked at 42°C for 2 minutes and cooled on ice for 10 minutes. Four hundred µl of LB was added to the DNA and incubated at 37°C for 45 to 60 minutes. LB plates containing 100 µg/ml Ampicillin were inoculated with 200 µl of the transformed cells and incubated overnight at 37°C. Six to 10 colonies from each transformation were picked and each was inoculated into 2 ml LB for a mini DNA preparation.

2.10.6 Mini plasmid DNA preparation by the alkaline/SDS method

Colonies were inoculated into 2 ml of LB containing 100 µg/ml Ampicillin in a sterile 10 ml polystyrene tubes (Sarstedt) and grown overnight in a 37°C shaking incubator. One and a half ml of the culture was centrifuged for 2 minutes in a microcentrifuge at 13000 rpm. The cell pellet was resuspended in 100 µl of cold 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 0.02% sodium azide by pipetting up and down. The cells were incubated on ice for at least 5 minutes. Two hundred µl of freshly prepared 0.1 M NaOH, 1% SDS solution was added to the cells. This mixture was mixed gently by inverting the tube several times and placed on ice for a maximum of 5 minutes. To this,

150 µl of cold 5 M potassium acetate (pH 5.8) was added. This mixture was again mixed gently by inverting the tube several times and then incubated on ice for at least 10 minutes. The mixture was then spun at 13000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new 1.5 ml Eppendorf tube containing 500 µl of 1:1 phenol:chloroform. The tube was vortexed, centrifuged, and the supernatant was transferred to a 1.5 ml new Eppendorf tube containing 1.0 ml of 100% Ethanol to precipitate the plasmid DNA. The tube was incubated at -20°C for at least one hour and centrifuged at 13000 rpm for 10 minutes. The DNA pellet was washed with 200 µl of 75% ethanol, 50 mM NaCl. The pellets were dried in a 37°C heating block for 5 minutes with the tube lids open to air. The DNA was then dissolved in 40 µl TE containing 20 ng/µl RNaseA and incubated for one hour at 37°C. The DNA was then stored at -20°C for future use. The identity of the plasmid DNA was confirmed by either restriction enzyme digestion and/ or sequencing.

2.10.7 Sequencing DNA

The IMVS automated sequencing facility was utilised. ABI PRISM™ Dye, Big Dye, or Big Dye v.2 Terminator kits were used as recommended by the manufacturer. Briefly, 2 µl of mini plasmid DNA prep or 1 µg of DNA were mixed with 60 ng of oligonucleotide primer and 6 µl of the Dye, Big Dye, or Big Dye v2 mix in a 20 µl volume. Sequencing fragments were generated by Polymerase Chain Reaction (PCR) using the following conditions. The DNA was denatured at 96°C for 10 seconds, then the primer annealed to the DNA at 50°C for 5 seconds, which was followed by DNA synthesis by the polymerase at 65°C for 4 minutes. This was repeated 25 times. At the end of the last cycle, the DNA was kept at 4°C until ready. When ready, the DNA was precipitated

with 2 μ l of 3 M NaAc and 60 μ l of 100% ethanol at -20°C for at least one hour or with 80 μ l of 75% isopropanol for 15 minutes at RT. The precipitate was spun at 13000 rpm for 10 to 20 minutes. Pellets were washed with 250 μ l of either 75% ethanol or 75% isopropanol, spun down again for 5 minutes, and dried in a heating block for 10 minutes at 37°C with the lids of the tubes open to air. Samples were then sent to the sequencing facility.

2.10.8 Glycerol stocks

Fifty μ l of miniprep culture were inoculated into 2 ml LB containing 100 $\mu\text{g/ml}$ Ampicillin in sterile 10 ml polystyrene tubes (Sarstedt, Germany) and grown in a 37°C shaking incubator for 5-8 hours. Seven hundred μ l was mixed with 300 μ l of 50% Glycerol by pipetting up and down. The culture was then stored at -70°C

2.10.9 Large Scale Plasmid DNA Preparation

A colony from freshly streaked LB, 100 $\mu\text{g/ml}$ Ampicillin plates (inoculated from a glycerol stock) was inoculated into a 2 ml LB, 100 $\mu\text{g/ml}$ Ampicillin starter culture and grown to mid-log phase in about 5-7 hours in a 37°C shaking incubator at 200 rpm. Two hundred and fifty ml of LB, 100 $\mu\text{g/ml}$ Ampicillin in a 2 litre flask was inoculated with 100 μ l of the starter culture and incubated overnight in a 37°C shaking incubator at 200 rpm. The overnight culture was centrifuged in 500 ml Beckman centrifuge bottles for 7 minutes at 5000 rpm (Beckman JA10 rotor) at 4°C . The pellets were suspended in 8 ml of ice cold 50 mM Tris-HCl, 10 mM EDTA (pH 8.0) and transferred to 40 ml Oakridge tubes and kept on ice. Fifteen ml of 1% SDS, 0.1 M NaOH was added and the tubes

were mixed very gently by inverting the tube several times, and left on ice for no more than 5 minutes. Then, 10 ml of chilled 3 M Potassium Acetate, 2M Acetic acid was added, and the tube was again mixed very gently and stood on ice for at least 15 minutes with occasional gentle mixing. The tube was spun at 18000 rpm (Beckman JA20 rotor) for 15 minutes at 4°C. Thirty-ml of the supernatant were transferred to a 50 ml Falcon tube containing 20 ml of isopropanol and incubated at RT for 15 minutes. The tube was then spun at 3700 rpm in a Beckman benchtop centrifuge for 20 minutes. The pellet was washed in 20 ml of 70% ethanol. The DNA was then dissolved in 2.9 ml TE. When the pellets completely dissolved, 3.2 g of CsCl was added and dissolved, followed by 100 µl of 10 mg/ml Ethidium Bromide. A Beckman TLN100 centrifuge tube was filled to the line on the neck of the tube with the DNA/CsCl mix. Tubes were then heat sealed, and spun overnight at 85000 rpm in a Beckman benchtop ultracentrifuge at 20°C. The centrifuge was then stopped with no deceleration program, and the plasmid bands were harvested by piercing the top of the tube and harvesting the band in a volume of approximately 1 ml using a 3 ml syringe and a 19-gauge needle, and transferred to a new Beckman TLN100 centrifuge tube. The tube was topped up with CsCl solution of the same density as in the previous run. No additional Ethidium Bromide was added. Tubes were then heat sealed, and spun overnight at 85000 rpm under the same condition as the previous run. Occasionally, one of the two ultra-centrifugations was done at 95,000 rpm for 5 hours. After the second spin, 500–700 µl of the plasmid band was harvested as before and placed into an Eppendorf tube. The sample was extracted with butan-1-ol 6 times, and the volume was made up to 2 ml with 50 mM NaAc. DNA was precipitated with 4.0 ml ethanol. This was mixed gently and incubated on ice for 10-15 minutes. The tube was spun at 3700 rpm for 15 minutes in a Beckman benchtop centrifuge at RT. The supernatant was removed and the pellet was dissolved in 400 µl TE for 20 minutes

at 37°C. The DNA was transferred to a 1.5 ml Eppendorf tube, and mixed with 20 µl of 3 M NaAc and 900 µl ethanol gently until fibres appeared, and left at RT for 5 minutes before spinning at 13000 rpm in a microcentrifuge for 2 minutes. The supernatant was removed, and the pellet was washed in 500 µl of 70% ethanol, dried briefly, and redissolved in 300-500 µl sterile TE at 37°C for an hour. The plasmid concentration was quantitated by taking an absorbance reading at 260 nm, where an OD₂₆₀ of 1 contains 50 µg/ml. The quality and quantity of plasmid was confirmed by electrophoresing a 200 ng sample on an agarose gel along with standards of known concentration. To confirm the identity of the DNA, diagnostic restriction digests and/or sequencing were performed.

2.10.10 Plasmid DNA preparation using the Qiagen kit

A glycerol stock was used to prepare fresh colonies on plates containing LB with 100 µg/ml ampicillin. Colonies were inoculated into 2 ml LB, 100 µg/ml Ampicillin in 10 ml polystyrene tubes (Sarstedt) and grown to a mid-log phase during the day in about 5-7 hours in a 37°C shaking incubator at 200 rpm. One hundred ml of LB, 100 µg/ml Ampicillin in a 1 litre flask was inoculated with 50 µl of the starter culture and incubated overnight in a 37°C shaking incubator at 200 rpm. Cells from the overnight culture were centrifuged in 500 ml Beckman centrifuge bottles for 7 minutes at 5000 rpm (Beckman JA10 rotor) at 4°C. The pellets were resuspended and cells lysed with the reagents provided and according to the manufacturer instruction in a 40 ml Oakridge tube and on ice. The tube was mixed very gently and stood on ice for at least 15 minutes. The tube was spun at 18000 rpm (Beckman JA20 rotor) for 15 minutes at 4°C. The supernatant was passed through a filter supplied with the kit then passed through the supplied column

that had been equilibrated with the supplied reagents. The column was then washed and eluted according to the manufacturer instructions. The eluant was aliquoted into 1.5 ml Eppendorf tubes and precipitated with an equal volume of isopropanol for 15 minutes at RT. The DNA was spun down in a micro-centrifuge at 13000 rpm and the pellets washed with 500 μ l of 75% ethanol. The DNA was then dissolved with an appropriate volume of TE at 37°C for one hour. DNA was quantitated as described in the section titled large-scale preparation plasmid DNA.

2.11 POLYMERASE CHAIN REACTION

In order to facilitate cloning of required DNA regions where no restriction site was available, restriction sites were introduced by PCR. A SmaI site was introduced by designing an oligonucleotide that carried the restriction enzyme site and which could be used to amplify the region of interest. This process is summarised below.

2.11.1 Oligonucleotides

The following are Oligonucleotide sequences that were used as PCR primers to amplify regions within the Ecl136/EcoRV region in pXPG-GM55-EE0.8 (see sequence for the region amplified in figure 3.18). These fragments were used to map the SS245 enhancer boundaries.

5' → 3' Primers

1. AGGAGGCCCGGGCTCTCCACC (from -40)
2. TGTCACCCGGGCCACCGCGG (from +100)

3. TCTGACCCGGGTGATGCCATGG (from +150)

3' → 5' primers

A. TGGACCCGGGTCTAATGGATCCC (from +377)

B. TGGAAGCCCGGGCCCTCAGGGTC (from +312)

C. AGAACCCGGGCCTCAGTCAC (from +203)

2.11.2 Amplification

The reaction was carried out in 40 µl containing 100 ng of template DNA and 100 ng of each primer (one 5' to 3' and one 3' to 5'). The template/primer mixture was boiled for 5 minutes then chilled on ice for 5 minutes, spun down briefly to collect condensation, and 1.5 µl of a dNTP mix of 10 mM of each nucleotide and 4 µl of the 10x Pfu buffer were added on ice. The tubes were then placed in the thermocycler and the cycles started. Once the temperature reached 92°C, the machine paused and 2 units of Pfu enzyme was added and the thermocycler was allowed to continue at 95°C for two minutes. The cycle conditions used were 95°C for 20 sec, followed by 30 sec at 60°C and 1 minute at 72°C for 25 cycles. After the last cycle the samples stayed at 72°C for an extra 3 minutes. The samples were then stored at 4°C. The DNA was checked on either a 1.5 % agarose or 7.5 % PAGE gel. If the products of the expected size appeared then the PCR products were extracted with an equal volume of 1:1 phenol/chloroform, and equal volume of chloroform. Glycogen and NaAc were added to a final concentration of 67 ng/µl and 0.3 M respectively. DNA was then precipitated with 2.5 volumes of 100% ethanol at -20°C for 2 hours and the tubes were spun at 13000 rpm for 30 minutes. Pellets were washed with 100 µl of 75% ethanol and dried at 37°C for 5 minutes with the lids open to air.

DNA was dissolved in 10 µl of MQ H₂O at 37°C for one hour. PCR products were then digested with the appropriate restriction enzymes.

Digested PCR products were ligated to plasmid vectors, mini preparation of plasmid DNA were then screened with restriction enzymes, and sequenced to identify clones.

2.12 TISSUE CULTURE

Cell lines used were

- 1) Jurkat cells, a human T-lymphoblastoid cell line provided by Dr. Warner Greene, Gladstone Institute of Virology and Immunology, San Francisco, USA;
- 2) CEM (Bert et al., 2000a)
- 3) HMC-1 mast cells were provided by Dr. Joseph Butterfield at the Mayo Clinic, Minneapolis, USA (Butterfield et al., 1989).
- 4) KG1a (Fukuda et al., 1981).
- 5) K562 (Klein et al., 1976).
- 6) Raji B cells (Epstein et al., 1966).

All cells were maintained at 37°C, 5% CO₂ and 95% humidity in a NUAIRE US Autoflow water-Jacket incubator. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (FCS), 25 mM HEPES pH 7.3, 2 mM L-glutamine, 28 mM (2 g/L) NaHCO₃, 50U/ml penicillin, and 50 mg/ml Streptomycin. Cells were maintained between a density of 1.2×10^5 and 1×10^6 cells/ml.

2.12.1 Transient Transfection

Cells were cultured to a density of approximately 5×10^5 to 7×10^6 cells/ml. For each transfection, 4.5×10^6 cells were pelleted by centrifugation for 5 minutes at 1250 to 1500 rpm in a bench top Beckman GPR Centrifuge and the supernatants removed by aspiration. Cells were then resuspended in 300 or 500 μ l of RPMI 1640 medium containing 20% FCS. Three or five hundred μ l (4×10^6 cells) was mixed with either 5 or 10 μ g of reporter plasmid in a 0.4 cm electroporation cuvette (BioRad). After standing at room temperature for at least 10 minutes, cuvettes were gently flicked to resuspend the contents and exposed to a single voltage pulse of 270 V from the 950 to 975 μ F capacitor of a Bio-Rad Gene Pulsar electroporation unit. Cells were allowed to recover for at least 10 minutes before being plated in a 25 cm² culture flask containing 10 ml of fresh medium. Cells were left for 20 to 24 hours (approximately one cell division cycle) to allow the plasmids to be equilibrated in the nucleus. Cells were then left unstimulated, or stimulated with a combination of PMA (25 ng/ml) and calcium ionophore A23187 (1.2 μ M), in the presence or absence of cyclosporin A (0.1 μ M) and incubated for nine hours before harvesting. To harvest, cells were pelleted, washed once in 10 ml PBS, and frozen at -20°C with or without a 100 μ l lysis buffer (0.1 M K₂HPO₄ pH 7.8, 2 mM DTT, 10 μ M EDTA).

2.12.2 Stable transfection

The luciferase reporter gene plasmids were digested with *FspI* to linearise the plasmid, and the marker plasmid, pE^oTKneo, was digested with *PvuI* to linearise it. Ten μ g of linearised reporter plasmid and 1 μ g of linearised pE^oTKneo were transfected into Jurkat

cells using the method described in the transient transfection section. Cells were allowed to recover for 2 days before commencing G418 selection. The cells were incubated with 600 µg/ml G418 for 20 or 21 days, after which time cells in the unelectroporated control flask were all dead. Cells were removed from G418 selection for at least 24 hours before stimulating 1×10^6 cells in 10 ml with PMA (25 ng/ml) and calcium ionophore A23187 (1.2 µM). Cells were stimulated for nine hours, and then harvested by spinning the cells at 1250 rpm for 5 minutes, and washing them in 10 ml PBS. The pellets were then either stored at -20°C without buffer or with lysis buffer (0.1 M K_2HPO_4 pH 7.8, 2 mM DTT, 10 µM EDTA).

2.12.3 Luciferase Assay

After removing cells from the -20°C freezer, they were thawed at RT, and the pellets were resuspended in lysis buffer. Cells were lysed by two or three additional freeze-thaw rounds as follow: cells were frozen in liquid nitrogen, placed in water bath to thaw then placed on ice, and then vortexed. Lysed cells were spun at 3700 rpm at 4°C to remove cell debris in a Beckman benchtop GPR centrifuge for 10 minutes. Samples were kept on ice. The supernatants were transferred to Eppendorf tubes and placed on ice. The protein concentrations of the cytoplasmic extracts were determined using BioRad Bradford reagents. Equal amounts of protein were assayed, generally between 20 and 75 µg depending on the concentration of the samples, a minimum of 5 µl volume was used. Equal amounts of protein were added to 200 µl assay buffer (0.1 M K_2PO_4 pH 7.8, 8 mM $MgSO_4$, 2 mM DTT, 750 µM ATP, 150 µM co-enzyme A) in 96 well black plates (Packard) and mixed well. Forty µl of 1 mM D-luciferin was added to

commence the reaction, and the plate was read using Top Count reader (Packard Topcount Microplate Scintillation Counter, model B9904V1).

2.12.4 Preparation of genomic DNA

To investigate the integration of plasmids into stable cell lines, genomic DNA was prepared. Ten million cells of each stable cell line were pelleted by centrifugation at 1250 rpm for 5 minutes and resuspended in 2 ml of digestion buffer I (300 mM NaAc, 5 mM EDTA). After all the cells had been resuspended, 2 ml of digestion buffer II was added (300 mM Na Ac., 1% SDS, and 0.2 mg/ml proteinase K). Lysate were incubated at 55°C for one hour, and then moved to 37°C overnight. 40 µl of 10mg/ml proteinase K was added and the cell suspension was mixed on a rotating wheel for one hour and then incubated at 55°C for another hour. The DNA was extracted with equal volumes (4 ml) of phenol, 1:1 phenol/chloroform, and chloroform by mixing on a rotating wheel for one hour and spinning at 1500 rpm for 5 minutes. The DNA was then precipitated with 6.6 ml of 100% ethanol at -20°C for at least one hour and spun down at RT for 10 min at 3700 rpm. Pellets were washed with 1 ml of 75% ethanol and dried at 37°C for 5 minutes with the lids open to air. DNA pellets were dissolved in 0.5 ml of TE at 37°C for one hour or at RT overnight. The DNA was quantitated as described in section 2.10.9.

2.12.5 Southern Blot Hybridisation Analysis

Ten µg of genomic DNA were digested with the appropriate restriction enzymes in a 15 µl volume. The digests were mixed with 2 or 3 µl of 20% or 15% Ficoll loading buffer

containing 0.01% Bromo-phenol-blue and were electrophoresed on a 0.8% agarose gel in TAE buffer supplemented with 0.5 µg/ml Ethidium bromide at 25 volts for 15 hours or at 75 volts for 5 hours. The gel was washed twice for at least 15 minutes each time with 0.5 M NaOH, 1.5 M NaCl. The gel was then neutralised twice for at least 20 minutes each time in 1M Tris pH 7, 1.5 mM NaCl. The DNA was transferred to a Hybond N Nylon membrane in 10x SSC buffer overnight. The membrane was washed briefly in 2X SSC, dried, then UV crosslinked for 30 seconds on a trans-illuminator. The membrane was prehybridised for at least 3 hours at 65°C, in bottles in a rotating Hyb-Aid oven with 20 ml Rapid-Hyb (Amersham) buffer and 0.25 mg/ml boiled herring DNA. DNA probes labelled with ³²P for Southern blot hybridisation analysis were prepared using a GIGA Prime kit (Bresatec). Boiled radiolabeled probe was added to the bag in 1 ml Rapid-Hyb buffer, and incubated at 65°C for at least 3 hours. The filter was then washed twice in 250 ml of 2X SSC, 25 mM NaPO₄ pH 7, 0.1% SDS at room temperature for at least 10 minutes, then twice in 250 ml of 0.1X SSC, 1 mM NaPO₄ pH 7, 0.1% SDS at 65°C for at least 15 minutes. The wet membrane was then wrapped in plastic film (Cling Wrap), and auto-radiographed and/or exposed to a phosphor-imager screen.

2.13 PREPARATION OF NUCLEAR EXTRACTS – SCHIBLER METHOD

The Schibler method (Lopez-Molina, L., et al. 1997) was used as described here. Jurkat cells were grown to a density of 4-6 X10⁵ cells /ml and stimulated with 20 to 25 µM PMA and 50 to 1.2 µM CaI for 2 hours. An additional 200 ml culture of cells was left unstimulated. Each culture was spun down at 1250 rpm for 5 minutes at 4°C in 4 50 ml falcon tubes. Pellets were resuspended and combined in 50 ml ice cold PBS, spun again at 1250 rpm, resuspended in another 50 ml of ice cold PBS, and spun at 1250 rpm.

Pellets were resuspended in 5 times the pellet volume of a hypotonic buffer (solution 1: 10 mM HEPES pH 8.0, 10 mM KCl, 0.75 mM Spermidine, 0.15 mM Spermine, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml Aprotinin, and 10 µg/ml Leupeptin) and incubated on ice for 10 minutes to lyse the cells. Nuclei were spun down and resuspended with 5 times the pellet size of solution 1. The cell suspensions were homogenised with a Dounce glass homogeniser (Pestle A) 5 to 10 times until all of the cells were lysed based on their appearance under the microscope. Nuclei suspensions were transferred to new 10 ml tubes and 0.1 the volume of solution 2 (50 mM HEPES, pH 8.0, 10 mM KCl, 0.75 mM Spermidine, 0.15 mM Spermine, 0.2 mM EDTA, 70% Sucrose, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin) was added to stop the lysis. The nuclei were pelleted and resuspended in nuclei suspension buffer (NSB) (20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% Glycerol) so that the nuclei concentration was at 4×10^6 nuclei/µl. Nine times the volume of NaCl-urea-NP-40 buffer (NUN) buffer (1.1 M Urea, 0.33 M NaCl, 1.1% NP-40, 27.5 mM HEPES, pH 7.6, 1.1 mM DTT) was then added drop wise. After all the NUN buffer was added, samples were vortexed at the lowest speed and left on ice for 15 minutes, which is enough for all of the nuclei to lyse. Nuclei lysates were then spun at 13,000 rpm in a microcentrifuge for 15 minutes, the supernatants were transferred to new tubes and glycerol concentrations were increased to 10%. Nuclear extracts were then aliquoted and stored at -70°C.

2.14 PREPARATION OF RADIOLABELED OLIGONUCLEOTIDE PROBES BY END-LABELLING

Oligonucleotides were 5' end-labelled with either [α - ^{32} P]dATP or [α - ^{32} P]dCTP as follows. Twenty ng of double stranded oligonucleotide was labelled in a 10 μ l volume reaction which contained 1 μ l of 10 times concentrated T4 DNA polymerase buffer, 2 μ l of a mix of 0.5 mM of each of dGTP, dTTP and either dATP or dCTP, 5 μ l of 10 μ Ci/ μ l of either [α - ^{32} P]dATP or [α - ^{32} P]dCTP, and 1 μ l of T4 DNA polymerase and was incubated at RT for 15 minutes. The labelling reaction was then purified on a TAE, 7.5% polyacrylamide gel, and the labelled oligonucleotide band was cut from the gel and eluted in 300 μ l 50mM NaCl in TE at RT overnight. Probe concentration was estimated by measuring the counts per second of both the gel slice and supernatant, and the supernatant alone to estimate the percent recovery. The volume of the supernatant was measured to calculate the probe concentration.

2.15 PREPARATION OF RADIOLABELED PROBES BY KINASING

Restriction enzyme digested fragments were treated with Calf Intestinal phosphatase (CIP) by adding 1-2 units at the end of the restriction digest for about an hour to remove the phosphate group from the end of the DNA fragments. The CIP treated DNA fragments were then extracted once with an equal volume of 1:1 phenol/ chloroform, and NaAc was then added to a final concentration of 0.6 M. The DNA was then precipitated with 2.5 volume of 100% ethanol at -20°C of at least 1 hour, spun down in a microcentrifuge for 10 minutes at 13,000 rpm, washed with 75% ethanol, dried and dissolved in 20 μ l TE.

Three pM ends of CIP treated DNA was labelled in 70 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 5 mM DTT, 0.1 mM Spermidine, 50 µCi, and 10 units of polynucleotide kinase at 37°C. After 10 minutes, the reaction was stopped by adding EDTA to a final concentration of 50 mM. Ammonium acetate was added to 2.5 M and DNA precipitated with 2 volumes of ice-cold 100% ethanol at -20°C for 30 minutes. DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes. Pellets was redissolved in 2 M Ammonium acetate and re-precipitated as before. Pellets were washed with ice-cold 500 µl of 70% ethanol, dried, and dissolved in MQ-H₂O. If only one end was required to be labelled then the DNA was cut with a restriction enzyme that had a site close to the end that does not need to be labelled. In these cases, the DNA was gel purified using a Bresa-Clean kit.

2.16 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Binding was performed in 20 µl reaction volumes that contain gel shift buffer (10 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1 M NaCl), 2 µg poly dI:dC, 10% Glycerol, 0.03 % NP-40, 1 mM DTT, 1 mM EDTA, 2 mM EGTA, 0.2 ng end-filled ³²P-labelled probes and 5 µg of nuclear extract. Where oligonucleotide duplex competitors were needed, 20 ng of competitor was added in 1 µl volumes. Binding was performed at room temperature for 20 minutes. When antibodies were used, the extracts were pre-incubated with antibodies on ice for 20 minutes and then all the other components were added. A 15 cm long, 1.5 mm thick 4% polyacrylamide gel was pre-run at 200 volts for 30 to 60 minutes. Samples were loaded and gel run at 250 volts until the Bromophenol Blue dye reaches about 9.5 cm from the bottom of the wells. The Gels were then fixed with 0.1%

cetrimide for 20 minutes and dried for one hour at 80°C under vacuum. The dried Gels were then exposed to either a Phosphor-imager screen or to X-ray films.

2.16.1 Oligonucleotides used in EMSA

The following double-stranded oligonucleotides were used for binding assays: (mutated nucleotides are represented with small letters.)

PIL62,	GCTGCCTTCTGTGGTGACAAAACCAGGCG
PIL103,	CCACCAGCGGAAATACAACCGAGGTCATGGG
PIL150A,	TGGAAAGGGTGGCGGAGGAGCTTGTCACAGTG
PIL150B,	CTTGTGATGCCATGGAAAGGGYGGCGGAGGAGCT
PIL150C,	TGCCATGGAAAGGGTGGCGGAGGAGCTTGT
PIL220,	CGGGGTACCCCAGAAAATTCCACTAGGCCTGTG
PIL62ΔAML1	GCTGCCTTCTGT _{cc} TGACAAAACCAGGC
PIL62ΔAP-1	GCTGCCTTCTGTGGTGAC _{cg} AACCAGGC
PIL150CΔNFAT	TGCCA _{Tcc} AAAGGGTGGCGGAGGAGCTTGT
PIL150CΔSp1	TGCCATGGAAAGGGT _{tt} CaaAGGAGCTTGT
PIL220ΔNFAT	CGGGTACCCCAGAAAATT _{gg} ACTAGGCCTGTG

Competitor oligonucleotides used were:

GM170	GATCCTGGAGTGACTCAAGCCCCTGTTTCCTACAG
GM430,	GATCTCACACATCTTTCTCATGGAAAGATGA
Oct,	GATCCCTAATTTGCATG
NF-κB,	GTTTTTGGATGCCATTGGGGATTTCCTCTTTACTGGATG
TCR-AML1,	GATCCGGCAATGCATGTGGTTTCCAACCGTTAATG

Stromelysin AP-1, GATCTGGATCACCCGCAGCTTGACTCATCCTTGCA
Sp1, GATTCGATCGGGGCGGGGCGAGC

The GM170 and GM 430 elements are derived from the GM-CSF enhancer and contain AP-1/NFAT and NFAT binding sites respectively (Cockerill et al., 1995b). The AML1 site is derived from the TCR δ enhancer (Cockerill et al., 1996)

The AP-1 site is derived from the stromelysin gene promoter (Cockerill et al., 1993). The NF- κ B site is derived from the E-selectin gene (Cockerill et al., 1995a)

Recombinant proteins: The rAP-1 expression vectors were obtained from Donna Cohen of the John Curtin School of Medical Research, ACT, Australia (see Abate, 1990); rNFAT expression vector was a gift from Anjana Rao of the Dana-Farber Cancer Institute, Boston, MA, USA (see Cockerill et al., 1995b). The proteins were prepared from these vectors by Peter Cockerill (Cockerill et al., 1995b).

Antibodies: α CBF α directed against the DNA binding domain was a gift from Dr. Nancy Speck of the Dartmouth Medical School, Hanover, NH, USA.; α NFATp was a gift from Dr. Anjana Rao.

2.17 IN VITRO FOOTPRINTING ASSAY

2.17.1 Maxam and Gilbert (G and A) sequencing

DNA was cleaved at A and G residues by the Maxam and Gilbert sequencing method (Maxam and Gilbert, 1977). Thirty thousand counts per minute of each kinase labelled probe was mixed with 1 µg of poly dI:dC and treated with 1 µl of 1 M formic acid for 20–30 minutes at 37°C in a 10 µl volume. An additional 30,000 cpm of the probe was added and incubated at 37°C for 5 minutes. The DNA was diluted by adding 80 µl of MQ H₂O, cleaved with 10 µl piperidine at 90°C for 30 minutes, and chilled on ice. The DNA was precipitated by adding 1 µl of 5 mg/ml t-RNAs and 1 ml of Butan-1-ol, vortexed, and incubated at RT for 5 minutes. Pellets were washed with 200 µl of 100% ethanol and dissolved in 100 µl of 1% SDS at RT for 15 minutes. DNA was mixed vigorously with 1 ml of Butan-1-ol. DNA was then re-precipitated at RT for 15 minutes. The pellets were washed with 200 µl of 100 % ethanol, dried and dissolved in 10 µl of loading buffer (80% Formamide, 1 X TBE, 0.05 % Bromo-Phenol-Blue, 0.05 % Xylene thiocyanate) and 5 µl was loaded on the gel.

2.17.2 DNase I Footprinting Assay.

The binding reaction was carried out on ice in a 20 µl volume containing 15 µl of 30,000 cpm of kinase-labelled probe, 1 µg poly dI:dC, 25 mM HEPES, pH 7.6, and 5 mM MgCl₂ and 5 µl of either NUN buffer or nuclear extract at the required concentration for 15 minutes. The Nuclear extracts were diluted to the required concentration with NUN

buffer. Two μl of either 5.25 $\mu\text{g}/\mu\text{l}$ or 10.5 $\mu\text{g}/\mu\text{l}$ DNase I in 10 mM HEPES, pH 7.6, 25 mM CaCl_2 were added to each binding reaction without or with nuclear extract respectively on ice for 3 minutes.

At the end of the three minutes, 82 μl of stop buffer was added (20 mM Tris-HCl, pH 8.0, 20 mM EDTA, 250 mM NaCl, 0.5% SDS, 4 μg of Herring DNA and 10 μg of proteinase K) and incubated at 37°C for 2 hours. Samples were then extracted with 100 μl of 1:1 phenol / chloroform, and 100 μl chloroform and then precipitated with 200 μl of 100% ethanol at -20°C for at least one hour. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. Pellets were washed with 1.0 ml of 75% ethanol, dried and dissolved in 10 μl of loading buffer.

2.17.3 Restriction Enzyme Digests

Kinase-labelled probes were digested with restriction enzymes separately in 20 μl reactions. Reactions were stopped with 82 μl stop buffer and DNA purified as described above for the footprint experiment. Two hundred and fifty cpm from each digest were mixed together to a total volume of 10 μl .

2.17.4 Running the gel

Five μl of each of the samples from the Maxam and Gilbert sequencing, restriction enzyme digestions, and the footprinting assays were loaded on a gel. The gel was run in TBE running buffer at 80 watts until the xylene dye was 13 cm from the bottom of the wells. At this point 200 ml of 3 M NaAc was added to the bottom chamber of the

electrophoresis unit. This retards the migration of the smaller DNA fragments and prevented their loss from the gel while allowing better resolution of the larger fragments. When the xylene dye reached 20 cm from the bottom of the well, the run was stopped. The gel was fixed in 300 ml fix solution (30% methanol, 10% Acetic acid, 0.1% CTAB) for 30 minutes. The Gel was then dried under vacuum at 80°C for one hour and exposed to a Phosphorimager screen and to an x-ray film.

2.18 MUTAGENESIS

2.18.1 Primers

Mutated nucleotides are represented with small letters.

M1a-PIL58ΔAML1 CTGAGCTGCCTTCTGT_{cc}TGACAAAACCAGGCGATG

M1b-PIL58ΔAML1 CATCGCCTGGTTTTGTCA_{gg}ACAGAAGGCAGCTCAG

M2a-PIL58ΔAp-1 CCTTCTGTGGTGAC_{cg}AACCAGGCGATGTCATC

M2b-PIL58ΔAp-1 GATGACATCGCCTGGTT_{cg}GTCACCACAGAAGG

M3a-PIL160ΔNFAT CACTTGTGATGCCAT_{cc}AAAGGGTGGCGGAGG

M3b-PIL160ΔNFAT CCTCCGCCACCCTTT_{gg}ATGGCATCACAAGTG

M4a-PIL160ΔSP1

GCCATGGAAAGGGT_{tt}CaaAGGAGCTTGTCACAGTGACTGAGGC

M4b-PIL160ΔSP1

GCCTCAGTCACTGTGACAAGCTCCTttGaaACCCTTTCCATGGC

M5a-PIL220ΔNFAT GGTACCCCAGAAAATTggACTAGGGGGGTCGACAAGC

M5b-PIL220ΔNFAT GCTTGTCGACCCCCTAGTccAATTTTCTGGGGTACC

2.18.2 Mutagenesis by PCR

Two hundred ng of template DNA was used per mutagenesis reaction. The template was pXPG-GM55-EE0.8 for all mutagenesis reactions except for those performed with the M5a-PIL220ΔNFAT/ M5b-PIL220ΔNFAT set of primers. For this set of primers, the pXPG-GM55-SS245 vector was used. The reaction was assembled in 50 µl reaction volumes on ice in the supplied Pfx buffer supplemented with 1 mM MgSO₄, 0.3 mM of each of the dNTPs, and 10 µM of each primer of a pair of primers; one for each strand. The conditions of amplification were: 95°C for 2 minutes, followed by 20 cycles of 95°C for 30 sec, 55°C for 1 minute and 68°C for 10 minutes. The 20th cycle was followed by 68°C for 10 minutes. The samples then were stored at 4°C until ready. Initially, the reaction tubes were placed in the thermal cycler to warm up, when the temperature reached 94°C, 2.5 units of Pfx polymerase was added. This PCR amplification resulted in the amplification of the entire plasmid. After the run, the PCR products were digested with 30 units of DpnI restriction enzyme, which cuts only the methylated plasmid DNA and not the PCR products. The digests were then transformed into JM109 strain of *E. coli* as described earlier. Colonies were screened by the mini-plasmid preparation procedure and sequencing of the mutated region as described earlier. Because the Pfx polymerase can generate errors, the fragments containing the mutations were isolated

from the plasmid and subcloned into pXPG-GM55. The identities of these constructs were confirmed again by sequencing and restriction enzyme mapping.

2.19 PREPARATION OF TOTAL RNA

All solutions and glassware were DEPC-treated to inactivate any RNase contamination. Ten million cells were spun down at 1250 rpm for 5 minutes in a bench-top Beckman GPR centrifuge. Pellets were washed with 10 ml cold PBS solution, spun down at 1250 rpm for 5 minutes, resuspended in 4 ml of RNA lysis solution (4 M Guanidinium Thiocyanate, 25 mM Sodium Citrate, 0.5% Sarcosyl, and 0.1 M β -mercaptoethanol) and vortexed. Two hundred and sixty six μ l of 3 M NaAc, 4 ml of H₂O saturated phenol, and 800 μ l of 49:1 chloroform/Iso-Amyl alcohol was added sequentially to the lysates. Samples were then extracted by mixing them vigorously and placing them on ice for 15 minutes. Samples were spun down at 3700 rpm for 15 minutes at 4°C. RNA was precipitated from the aqueous layer by sequentially adding an equal volume (4 ml) of isopropanol, mixing vigorously by shaking, incubating at -20°C for a maximum of one hour and spinning at 4200 rpm for 30 minutes at 4°C in a Beckman J6 ME rotor. RNA pellets were dissolved in 0.6 ml of RNA lysis solution and transferred to 1.5 ml Eppendorf tubes. RNA was precipitated by sequentially adding an equal volume (0.6 ml) of isopropanol, mixing the tubes vigorously, and incubating them at -20°C for a maximum of 15 minutes and spinning at 13000 rpm in microcentrifuge. Pellets were washed with 1.5 ml of 75% ethanol, dried and dissolved in 0.1 mM EDTA at 37°C for one hour. RNA was then quantitated using a spectrophotometer by measuring the OD at a wavelength of 260 nm.

2.20 NORTHERN BLOT HYBRIDISATION ANALYSIS

The Electrophoresis unit, gel casting tray, and combs were soaked in 0.1 M NaOH for 30 minutes to inactivate any RNase contamination, rinsed several times with DEPC treated water, and filled with running buffer (20 mM MOPS, 1 mM EDTA, and 5 mM Na Ac.). Ten μg of total RNA was dried along with 3 μg of 0.24–9.5 kb RNA ladder (GibcoBRL) in a Speedivac (Savant Speedivac, model SVC100) and dissolved in 10 μl of sample buffer (50% formamide, 6.25% formaldehyde, and 0.25 $\mu\text{g}/\mu\text{l}$ Ethidium Bromide; in running buffer) at 65°C for 5 minutes. Samples were then mixed with 2 μl of loading dye (0.25% Bromophenol blue, 0.25% xylene cyanol, and 20% Ficoll), loaded into an agarose gel (1% agarose, 6.3% Formaldehyde; in running buffer) and electrophoresed until the BPB dye was 10 cm from the bottom of the wells.

Following electrophoresis, the gel was soaked in 0.05 M NaOH for 20 minutes, rinsed twice with MQ H₂O and soaked in 10X SSC for 15 to 30 minutes. RNA was then transferred to a Hybond N⁺ nylon filter (Amersham) as described in the Southern blot section. After transfer, filters were washed with 2x SSC and dried in air. When the filter was completely dried, RNA was crosslinked by exposing the filter to UV light for 30 seconds. The filters were prehybridised and hybridised with DNA probes, washed and exposed to x-ray film and/or a phosphor-imager screen as described in the Southern blot section.

2.21 PREPARATION OF POLY (A)⁺ RNA

Approximately 10^8 cells were spun down at 1250 rpm for 5 minutes and resuspended in 15 ml of lysis buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K) at 37°C for one hour. Lysates were homogenised in a Polytron for 10 seconds at 2500 rpm. The lysates were then mixed with 0.2 g of oligo-(dT)-cellulose (Boehringer Mannheim) and the concentration of NaCl was raised to 0.5 M. The suspension was mixed on a shaker for an hour and spun at 2000 rpm for 5 minutes. The pellets were resuspended in 1 ml of wash buffer 1 (0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 % SDS), packed in an Econocolumn and washed again with 1 ml of the same wash buffer 6 times. The column was washed with wash buffer 2 (0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 % SDS) twice. Poly (A)⁺ RNA was eluted with 0.3 ml of elution buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.05% SDS) four times. RNA was precipitated by adding NaAc to a final concentration of 0.3 M and 2.5 volume of 100 % ethanol and precipitated at -20°C for one hour. RNA pellets were washed with 1.0 ml of 75% ethanol, dried, and dissolved in TE at 37°C for 1 hour. RNA concentrations were determined by measuring OD at a wavelength of 260 nm. RNA was stored at -20°C.

2.22 REVERSE TRANSCRIPTASE LINKED PCR

To make cDNA, 5 µg of total RNA was mixed with either random primer or oligo-(dT) primer. The mix was heated to 65°C for 5 minutes and then chilled on ice for 5 minutes. The reaction was carried out in the supplied RT buffer supplemented with 10 mM DTT,

0.5 mM of each of the four dNTP, and 20,000 unit of RNase inhibitor (RNA Guard), 200 units of RT buffer was added and incubated at 37°C for one hour.

Two µl of each cDNA reaction was then amplified by PCR in Taq buffer supplemented with 50 µM of each of the four dNTPs, 1.5 mM MgCl₂, 10 µM of each of the two primers, and 3 units of Taq polymerase. The samples were heated to 94°C for 2 minutes, then followed by 35 cycles of 94°C for 1 minute, 50°C for 30 seconds, and 72°C for 30 seconds. Samples were then stored at 4°C. The oligonucleotides used were (1) 5' to 3': GGGAAAGCGTATAT, and (2) 3' to 5': TCCTCAGAGCAGTTGC. Half of each PCR product was mixed with 2 µl of loading buffer (0.1% BPB, TE, and 20% Ficoll), loaded onto a 2% agarose gel and a Southern blot performed.

2.23. GENERATION OF TRANSGENIC MICE

pBlue-B1.2-H5.2-CS1.7 plasmid was digested with SacII and XhoI enzymes. The SacII/XhoI digest was mixed with loading dye and run on a 0.7% preparative agarose gel (to separate the 8.1 kb fragment from the Bluescript vector fragment) until the dye reached the bottom of the gel. TA buffer was used without any Ethidium bromide. The gel was cut vertically on one side so that only a few millimetre of the digest was included. The gel slice was stained with 0.5 µg/ml Ethidium Bromide. The DNA was visualised with UV light and the required DNA band was marked with a needle full of Indian ink. The gel was reassembled and the DNA band of interest was cut out of the main body of the gel using the Indian ink as a guide. The gel slice was inserted into 15 mm dialysis tubing that was pre-washed with water. Five hundred µl of TA buffer was added to the tubing. The DNA was eluted from the gel slice by placing the dialysis

tubing in the electrophoresis tank and electrophoresing for two hours at 60 volts. The gel slice was removed from the dialysis tubing leaving all the liquid behind, and dialysed over night in TE. The DNA was purified on Qiagen Qiaquick Nucleotide removal columns according to the manufacturers instructions. The DNA was purified further by desalting on a Bio-RAD P30 Bio-Spin 30 Chromatography column. The column was prewashed with 500 µl of transgenic injection buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4). The column was spun at 1000 rpm for 2 minutes and the wash through was discarded. The DNA was added to the column and topped with 20 µl of transgenic injection buffer. The column was spun at 1000 rpm for 2 minutes. The eluted DNA was quantitated as described in section 2.10.1.

The DNA was injected into eggs by Donna Roberts at 1 ng/µl. When the mice are at a suitable age, the tip of their tail was cut and used to prepare genomic DNA. To the tail tip, 700 µl of transgenic tail buffer (50 mM Tris, pH 8, 10 mM EDTA, 100 mM NaCl, 1% SDS) was added. Ten µl of 10 mg/ml proteinase K were added and incubated overnight at 55°C. DNA was then extracted with an equal volume of pH 8 phenol, 1:1 phenol/chloroform and chloroform as described before. DNA was precipitated, washed with 75% ethanol, dried, dissolved in TE, and quantitated.

DNA from transgenic mice and from normal non-transgenic mice and from Jurkat cells was cut with either SacI or NsiI/NotI restriction enzymes. Southern blot hybridisation was carried out using a 3 kb SacI fragment from the 5' region of the mouse GM-CSF gene and/or a 599 bp NcoI/HindIII from human IL-3 promoter. To calculate copy number, a mixture of the two probes was used in the SacI Southern blot. To identify the size of the human transgene construct as complete and not rearranged during integration,

the human IL-3 probe was used alone on the NsiI/NotI Southern. Mice containing intact transgenes were bred and second generation mice were screened in the same way and tested for transgene expression.

Single cell suspensions were made from spleens of transgenic mice by squashing the spleen between two slides in 3 ml of supplemented Iscoves Modified Dulbecco's Medium (IMDM) with 10% FCS, 50 μ M β -mercapto-ethanol, Penicillin-G at 50 units/ml, and Streptomycin-sulphate at 50 μ g/ml. The cells were transferred to a 10 ml tube and separated from extracellular debris by underlaying the cells with 2 ml of IMDM media, which was then left to settle for 5 minutes. Four μ l were taken from the top and transferred to a fresh tube. Cells were plated at 1.5×10^6 cells/ml in 10 ml volumes of IMDM. To the 10 ml of culture, 10 μ l of 2 mg/ml of Concanavalin A was added and left for 2 days at 37°C in a tissue culture incubator with 5% CO₂ and humidity. After 2 days, T cell blasts had developed. Cells were counted and 2×10^6 cells were spun down and resuspended in 10 ml of IMDM. Ten μ l of recombinant mouse IL-2 at 5 μ g/ml was added to give a 10 units/ml activity. Cells were incubated for 2 days in a 37°C incubator as before. The cell density was monitored during the two days so as not to exceed 6×10^5 cells/ml.

For FACS analysis, 1×10^6 cells for each staining reaction were spun down and the pellets resuspended in 50 μ l media. Cells were stained with FITC-conjugated α -mouse Thy-1 Ab, PE-conjugated α -human CD4, and a negative control Ab for both PE and FITC. Antibodies were added according to the manufacturer's recommended concentration. Staining reactions were carried out on ice for 30 minutes. Cells were washed with 2 ml PBS, spun down and the pellet resuspended in 0.5 ml FACS fix buffer (10 g of D-

Glucose, 0.1 g of Na Azide, and 5 ml of Formaldehyde dissolved in 500 ml of PBS) and read on the Epics flow cytometer machine.

CHAPTER 3: DEFINITION OF AN ENHANCER UPSTREAM OF THE HUMAN IL-3 GENE

3.1. INTRODUCTION

A cluster of constitutive DH sites upstream of the human IL-3 gene was identified as described in figures 1.5 and 1.7. The DH site number 1 in the cluster of DH sites was found to be the most prominent site. However, in some cases where peripheral blood lymphocytes or Jurkat T cells were used the DH site 1 appeared as a doublet (Cockerill, unpublished data). To map this site with a higher resolution, Dr. Peter Cockerill repeated the DH site assays as presented in figures 3.1 and 3.2.

To map these sites with higher resolution, a probe from the 5' SpeI site was used as shown in figure 3.1 (Cockerill, unpublished data). This Southern blot showed an additional DH site upstream of the -4.1 kb site in both Jurkat and primary T cells at -4.5 kb. This site (-4.5) was clearly inducible by PMA/CaI in T lymphoblasts, Jurkat T cells, HSB2 T cells, and HuT 78 T cells and it was clearly suppressed by the addition of CsA in Jurkat T cells, and HSB2 T cells. It was weak or absent in the KG1a and HMC-1 myeloid cell lines. The -4.1 and -4.5 kb DH sites are separated by a StuI and a BamHI sites that are about 130 bp apart (figures 3.1 and 3.2). The -4.5 and -4.1 sites are both located between two SacI sites, 1.3 kb apart, and the BamHI site separating them are shown in figure 3.2 where the DH sites were mapped by probing from a XbaI site (Cockerill, unpublished data). These figures also show the other constitutive DH sites at -3.3 kb, -1.3 kb and the IL-3 promoter, as well as the ones downstream of the IL-3 gene.

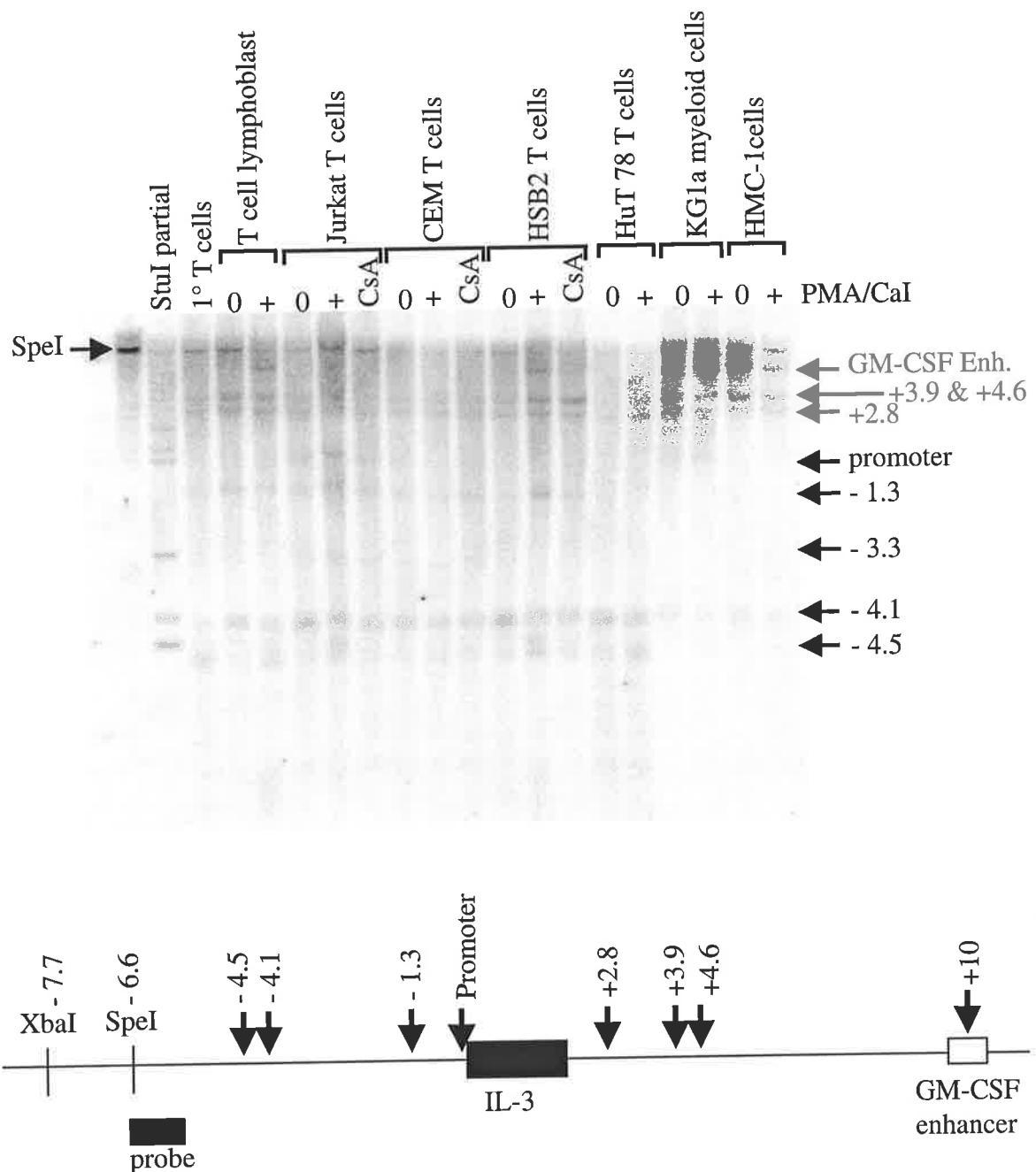


Fig. 3.1 DNase I Hypersensitive site assay.

Nuclei from the indicated cell lines were digested with DNase I. DNA was purified and digested with SpeI. Southern blot hybridisation was performed using a probe from the 5' end of the SpeI digest. Lane 1 shows a non-DNase I treated DNA digested with SpeI. A StuI partial digest is shown on lane 2. Lanes 3 to 19 show DNase I treated DNA from cell lines that have been (0) unstimulated, (+) PMA and CaI, and PMA, CaI, and CsA treated. The locations of the DH sites were marked on the side with arrows. The probe was a 470 bp SpeI/BamHI fragment of DNA. Figure taken from Cockerill, unpublished data.

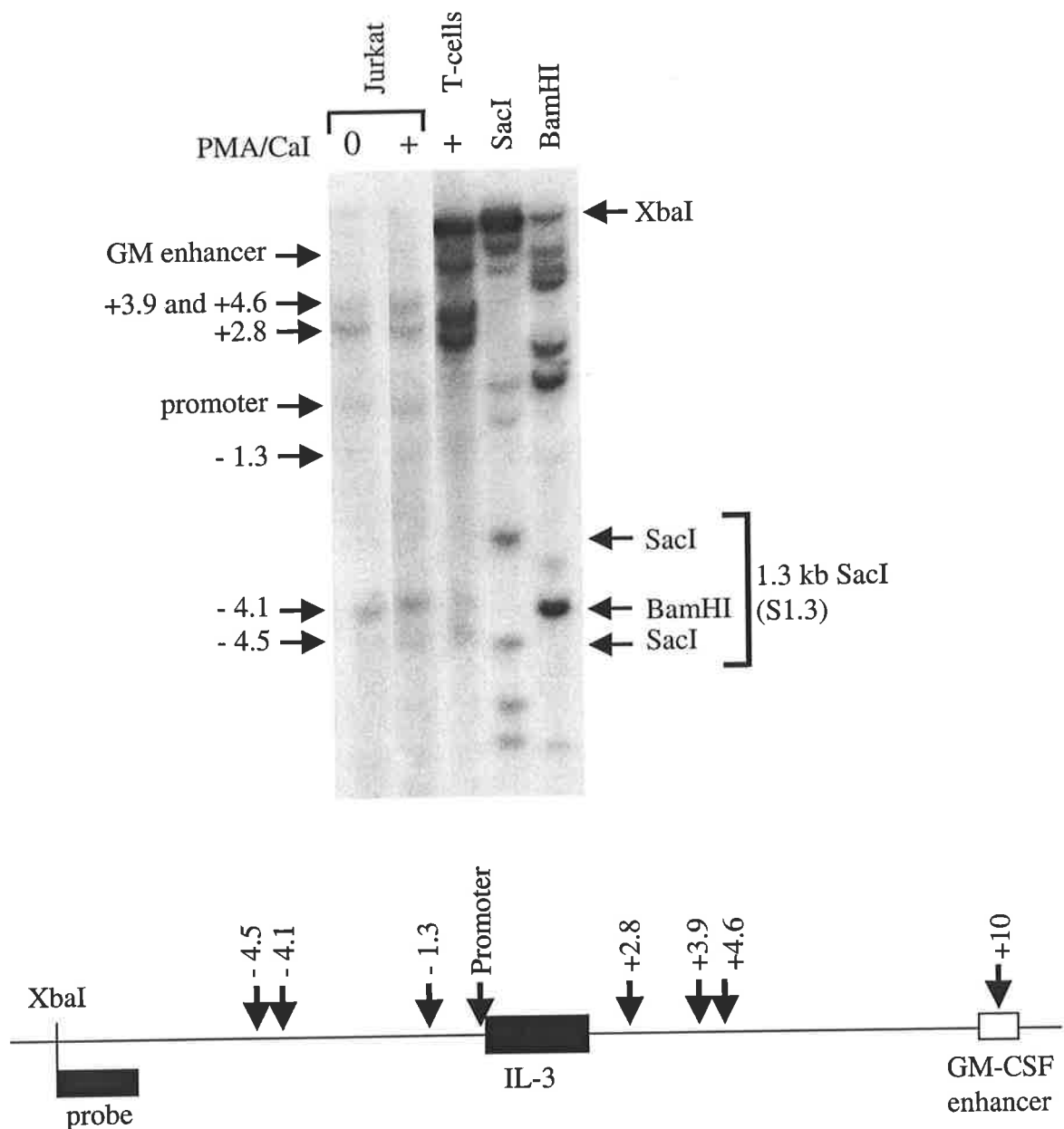


Fig. 3.2. DNase I-hypersensitive Site Assay of Jurkat T cell and primary T cells.

Cells were either unstimulated (0) or stimulated (+) with PMA and CaI. The -4.5 DH site was clearly inducible. SacI and a BamHI partial digests were included to help orientate the location of the DH sites. The -4.5 and -4.1 kb sites are separated by a BamHI site and are both located within a 1.3 kb SacI fragment (S1.3). The probe was a 1.6 kb XbaI/BamHI fragment. Figure taken from Cockerill, unpublished data.

The work described in this chapter focuses in defining and characterising the -4.5 kb DH site as an enhancer element.

3.2 IL-3 AND GM-CSF MRNA PRODUCTION IN CELL LINES

Northern blot hybridisation was used to assess IL-3 and GM-CSF production in cell lines that were used to test the elements described in this study. Figure 3.3 shows that Jurkat and CEM, (T cell lines) and HMC-1 (mast cell line) made IL-3 and GM-CSF, KG1a (myeloid cell line) and K562 (erythroid cell line) made only GM-CSF, and Raji (B cell line) made neither. The GAPDH gene was used as a standard control. Although HMC-1 makes IL-3 and GM-CSF, the amount made as a result of PMA/CaI stimulation is very small compared to the T cell lines. The expression of IL-3 and/or GM-CSF was inducible in each case where expression was seen.

3.3 THE -4.5 kb DH SITE FUNCTIONS AS AN ENHANCER

Both of the -4.5 and -4.1 DH sites are located between two SacI sites as shown in figure 3.4A. To test the function of the -4.5 kb and -4.1 kb DH sites, a 245 bp Ecl136II (isoschizomer of SacI)/StuI (SS245) fragment and a 550 bp StuI/EcoRV (SE546) fragment encompassing the -4.5 and the -4.1 DH sites respectively were cloned in the SmaI site of a pXPG-GM55 luciferase plasmid driven by a minimal GM-CSF promoter GM55 spanning bp -55 to +28 to give pXPG-GM55-SS245 and pXPG-GM55-SE546. As a positive control we also assayed PXPG-GM55-GME, which contains the 717 bp BglII fragment of the human GM-CSF enhancer. These constructs were transiently transfected into Jurkat T cells along with the pXPG-GM55. The unstimulated

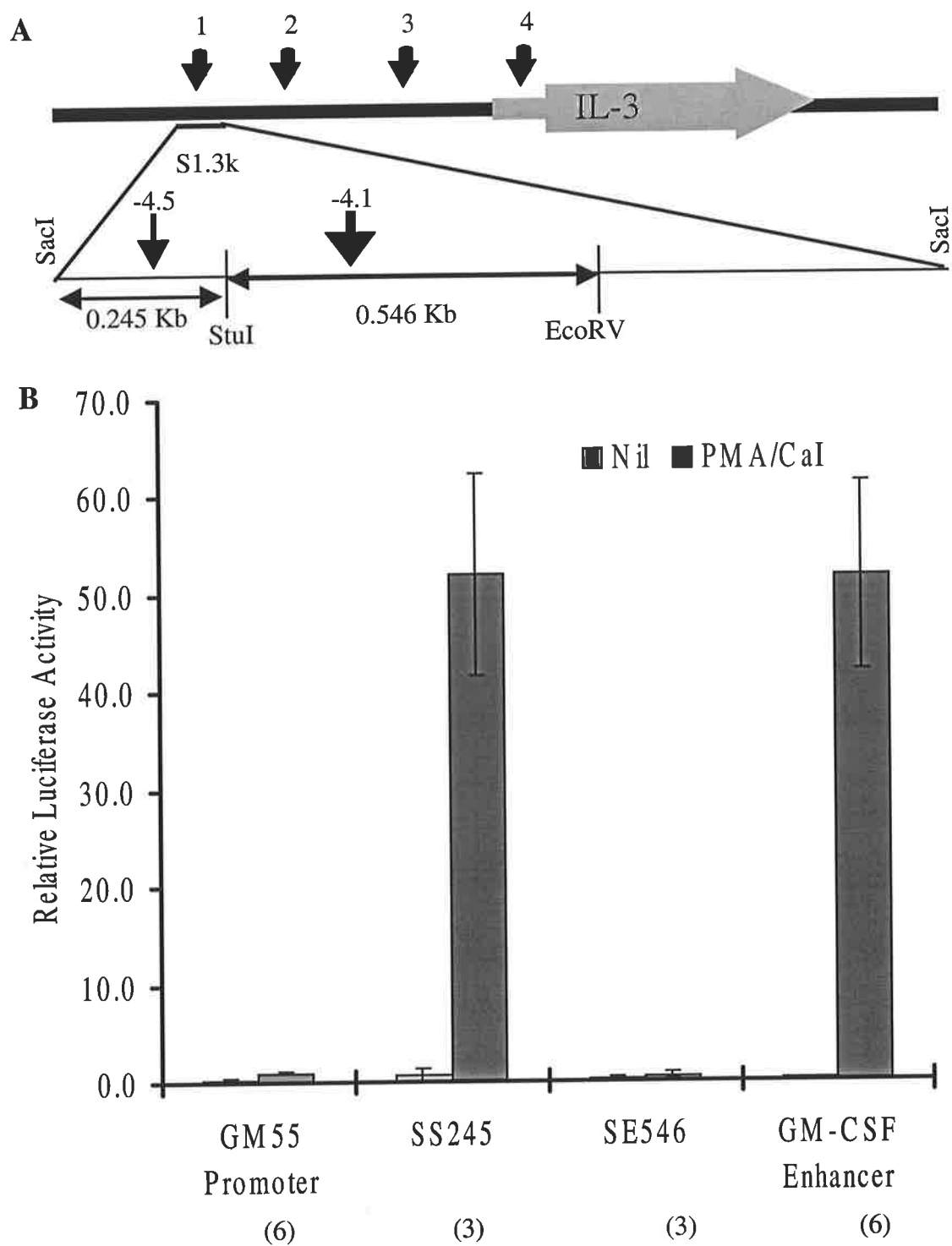


Fig. 3.4 Transient transfection assay of constructs containing the -4.5 and the -4.1DH sites.

(A) A schematic diagram of the 5' region of the IL-3 locus with DH sites marked by arrows. The 1.3 kb SacI region is expanded and the location of the -4.5 and -4.1 DH sites are shown. (B) Transient transfection assay showing the activity of luciferase reporter gene constructs in Jurkat T cells in the presence or absence of stimulation by PMA and CaI. Activities are expressed on a scale where the promoter alone has an activity in stimulated cells of 1. Error bars indicate standard error of the mean, and the numbers of independent stimulated transfections are shown in parentheses.

transfected cells had very low luciferase activity. Upon stimulation with PMA and CaI, the SS245 fragment increased the activity of the promoter by 50 fold, similar to the GM-CSF enhancer control, while the SE546 fragment did not increase the activity of the promoter (figure 3.4B). This showed that the SS245 fragment contained a strong enhancer while the SE546 fragment had no enhancer activity in Jurkat cells in transient transfection assays.

3.4 THE SS245 ENHANCER IS T CELL SPECIFIC

To test the specificity of the SS245 enhancer, the pXPG-GM55 constructs with the SS245 and GM-CSF enhancers were transiently transfected into Jurkat T cells, CEM T cells, KG1a cells, and K562 cells and stimulated with PMA and CaI as described previously. The results are presented in figure 3.5. The activities were expressed relative to the GM-CSF enhancer construct having an activity of 100%. The SS245 enhancer had an inducible activity in Jurkat and CEM T cell lines of approximately 60–80% of that supported by the GM-CSF enhancer. In contrast, the activity of the SS245 enhancer was only about 10% of the GM-CSF enhancer in KG1a cells and was completely inactive in the K562 pro-erythroid cell line. The SS245 and GM-CSF enhancers were essentially inactive in the absence of stimulation.

To test the CsA-sensitivity of the SS245 enhancer, the SS245 construct was transiently transfected into Jurkat T cells and stimulated with PMA and CaI with or without CsA. As is shown in figure 3.6, the treatment with CsA completely abolished enhancer activity.

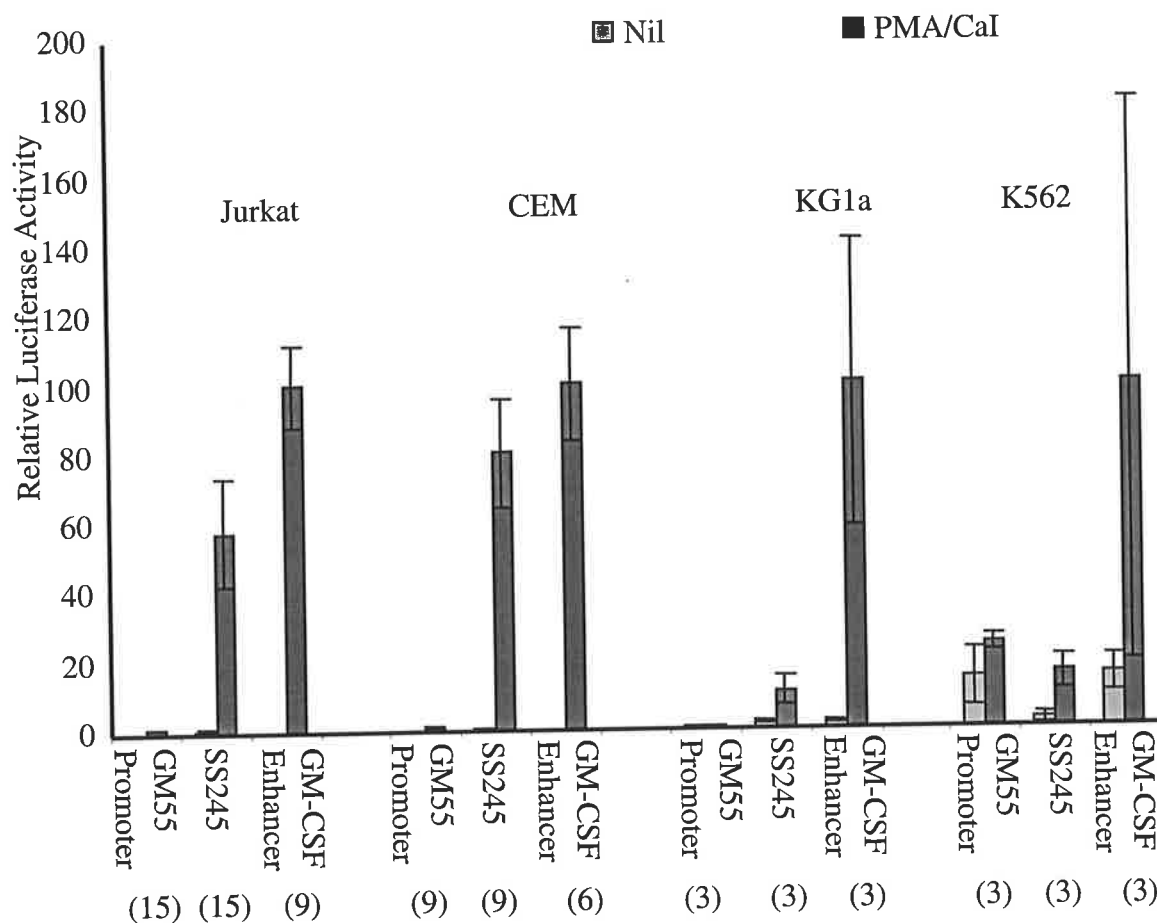


Fig 3.5. Activity of the SS245 enhancer in different cell lines.

The SS245 and GM-CSF enhancers were inserted in the luciferase reporter gene plasmid pXPG-GM55 and assayed in independent transient transfection assays in Jurkat T cell, CEM, KG1a, and K562 cells. Transfected cells were either left untreated or stimulated with PMA and CaI. In each case the relative luciferase activities were expressed on a scale where the GM-CSF enhancer activity was assigned a value of a 100. Activities were expressed as a mean of the number of independent transfections. Error bars are the standard error of the mean. Numbers within parenthesis are the number of stimulated independent transfections.

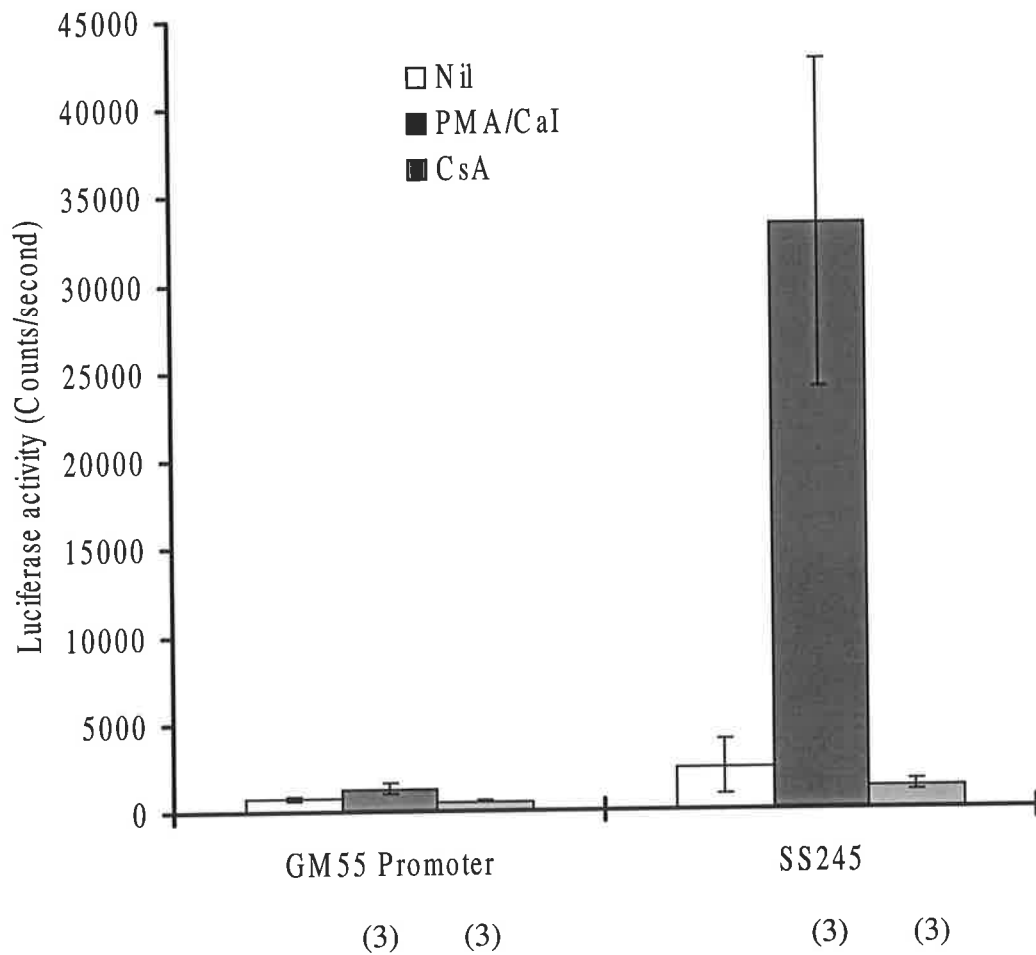


Fig 3.6. Effect of CsA on the SS245 enhancer activity.

Transient transfection assay of pXPG luciferase plasmids containing the GM55 promoter alone or the promoter and the SS245 enhancer in Jurkat T cell. Transfected cells were either left untreated, stimulated with PMA and CaI alone, or stimulated in the presence of 0.1 uM CsA. Activities were expressed as a mean of 3 independent transfections and the standard errors of the mean are represented by error bars.

Since the minimal GM55 promoter is an inducible promoter driven by the AP-1, Ets, and NFAT transcription factors, at least some of the inducible CsA-sensitive properties of the pXPG-GM55-SS245 may be due to the promoter. To assay the enhancer in a different context the SS245 fragment was cloned upstream of the constitutively active thymidine kinase promoter (TK229) in a luciferase reporter plasmid (pXPG-TK229). For a control, a 175 bp fragment of the SV40 enhancer, which is widely active, was cloned upstream of the TK229 promoter to give pXPG-TK229-SV40E plasmid. These plasmids were used to test the specificity and the inducibility of the enhancer as presented in figure 3.7. These plasmids were transiently transfected into Jurkat T cells, HMC-1 mast cells, Raji B cells, KG1a myeloblastic cells, and K562 pro-erythroid cells and stimulated with PMA and CaI. The enhancer was only active in Jurkat T cells and HMC-1 mast cells where it increased inducible, but not constitutive promoter activity, by 2 to 3-fold. The SS245 enhancer was not active in KG1a, K562 or Raji cells, in contrast to the SV40 enhancer that was active in all of the cell lines and functioned to increase both the inducible and the constitutive activity of the TK promoter.

3.5 THE SS245 ENHANCER IS CAPABLE OF ACTIVATING THE FULL LENGTH IL-3 PROMOTER

To test whether the SS245 enhancer is capable of activating its natural promoter element, it was cloned in the SmaI site upstream of a 611 bp full length IL-3 promoter element, encompassing IL-3 gene sequences from -559 to +50, in a pXPG-IL3H luciferase reporter gene plasmid. The SS245 enhancer increased the inducible activity of the IL-3 promoter in Jurkat and CEM T cells by 2 and 1.75 fold respectively as shown in figure

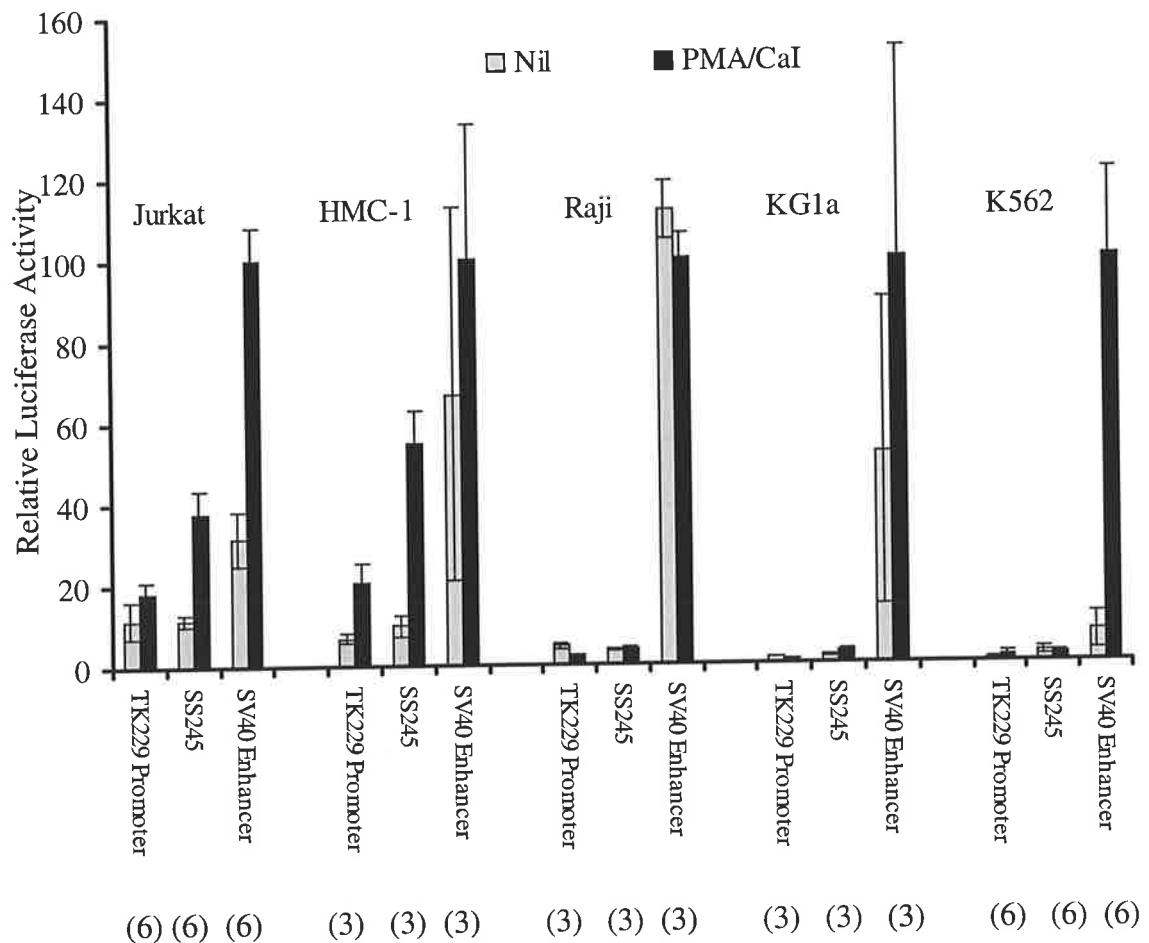


Fig. 3.7. Activation of the TK promoter by the SS245 enhancer in transient transfection assays.

The SS245 fragment and the SV40 enhancer were cloned upstream of the constitutively active TK promoter and tested in Jurkat T cell, HMC-1 mast cells, Raji B cells, KG1a cells and K562 cells in the presence and absence of PMA and CaI. Activities are shown as relative to the inducible activity of the SV40 enhancer set at 100. Activities are expressed as an average of 3 to 6 independent transfections and the standard errors are expressed as error bars. The numbers of stimulated transfections are indicated within parenthesis.

3.8. In this context the SS245 enhancer was approximately half as active as the -14 kb IL-3 enhancer (NA330), which increased inducible promoter activity by 2.5 to 3 fold.

3.6 PROTEIN BINDING TO THE SS245 ENHANCER

To characterise the mechanism of SS245 enhancer function, a 366 bp SacI / BamHI fragment which encompasses the SS245 enhancer was footprinted for protein bound in a DNase I assay of DNA bound to Jurkat cell nuclear extracts. This fragment was radiolabeled with ^{32}P at the SacI site. Figure 3.9 shows the result of the footprinting experiment. In this experiment both unstimulated and PMA and CaI stimulated nuclear extracts were used at 5, 10, 20, and 30 μg and were reacted with the ^{32}P labelled DNA on ice for 15 minutes and then digested with 21 ng of DNase I for 3 minutes on ice. Naked DNA was also digested with 10.5 ng of DNase I on ice for 3 minutes. After proteinase K treatment and phenol/chloroform extraction and ethanol precipitation, the DNA was dissolved in loading buffer and loaded on a 6% acrylamide/urea gel. In addition, restriction enzyme digests and G and A Maxam and Gilbert (Maxam and Gilbert, 1977) sequencing reactions were also loaded.

The protected areas are marked in figure 3.9 with vertical lines. On the side of the gel is a diagram that indicates the position of predicted protein-binding sites. Figure 3.10 shows the sequence of this region and a number of potential transcription factor binding sites. Some of these sites were subsequently shown to bind by the gel electrophoretic mobility shift assays (EMSA) (see below). Four areas were tested in EMSA. These were (1) AML1/AP-1, (2) NFAT/AP-1, (3) NFAT/Sp1, and (4) NFAT areas. Probes were made spanning these areas and are marked between 90° bent arrows as shown in

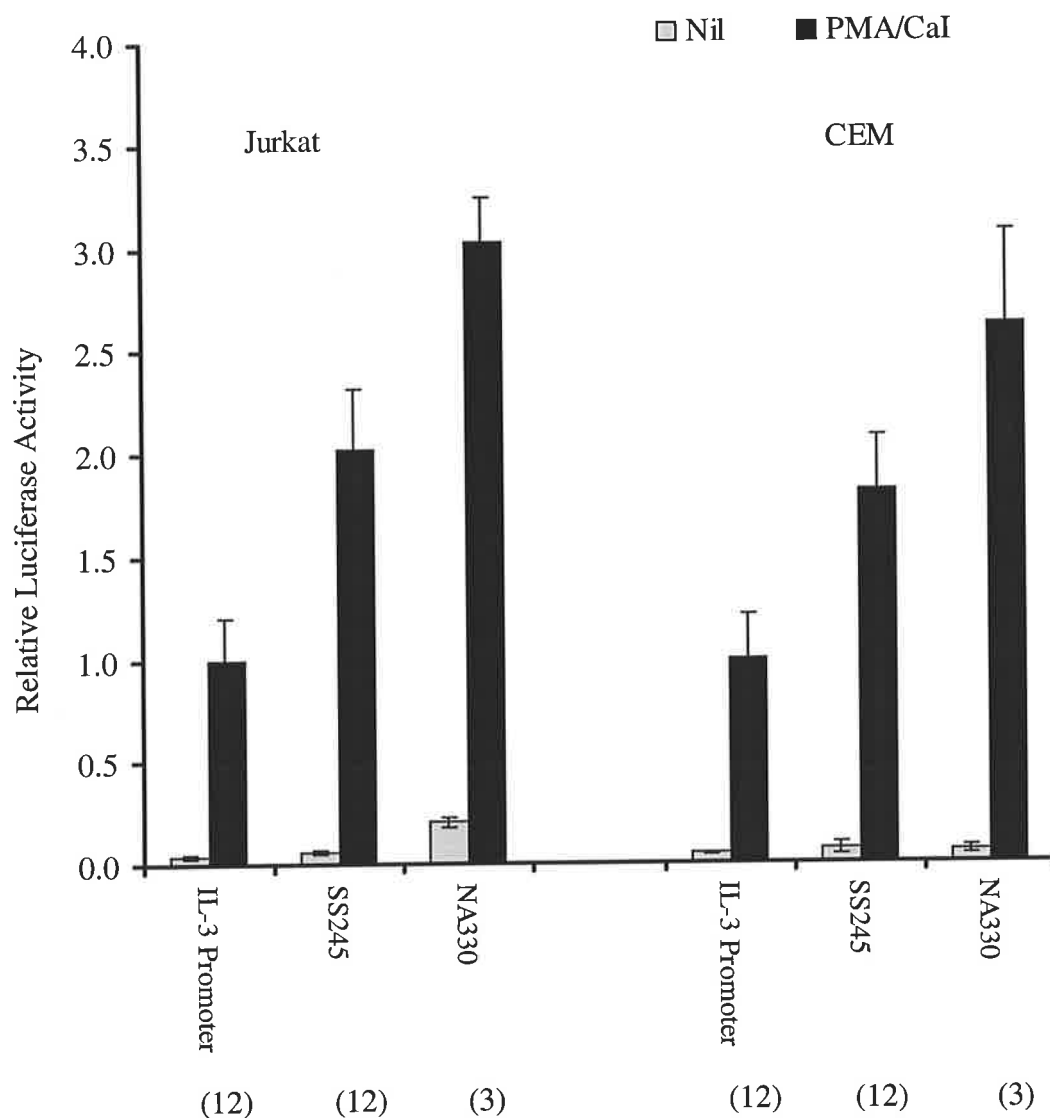
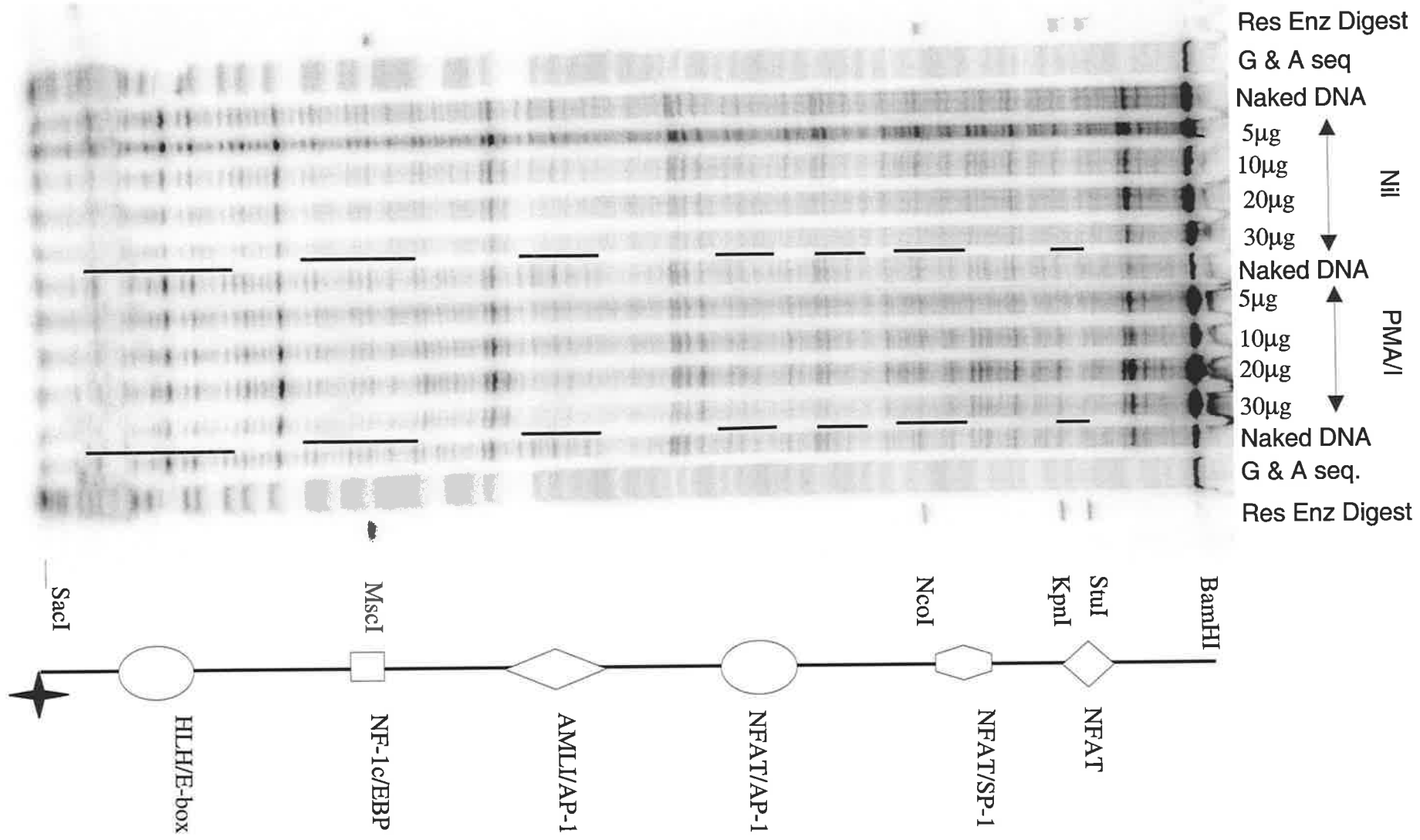


Fig. 3.8. The activity of the SS245 enhancer upstream of the full length IL-3 promoter.

The SS245 fragment was cloned upstream a 611 bp full length IL-3 promoter in pXPG and transfected in Jurkat T cell and CEM T cells. A 330 bp NsiI/AccI fragment of the -14 kb IL-3 enhancer (NA330) was also inserted upstream of the IL-3 promoter to provide a control. Transfected cells were either left untreated, or stimulated with PMA and CaI. The luciferase activities are shown relative to the inducible activity of the IL-3 promoter alone, which is assigned a value of 1. Activities of the stimulated transfections were presented as an average of 3 to 12 of independent transfections (indicated within parenthesis) and the standard error of the mean as error bars.

Fig. 3.9. DNaseI footprint analysis of the Sac I–BamH I region spanning the SS245 enhancer.

The region SacI to BamHI containing the SS245 enhancer was foot-printed using Jurkat T cell nuclear extracts at 5, 10, 20 and 30 μ g of nuclear extracts from either unstimulated or PMA and CaI-stimulated Jurkat T cells. Restriction enzyme digests, G and A sequencing DNA ladder, and naked DNA partially digested with DNase-I were also shown. Protected areas were marked with vertical lines. On the side a schematic diagram of the potential binding factors to the protected areas are indicated.



-51 ACAGGAGGCCAGGGCTCTCCACCTGGGCCCCTCGGTGGCTTTTTCCT

Ecl136II
(Sac I)
↓

-3 GAGCTCTCTGCTCAGGTGAGCAGGGGACATCTGGTTGCACTGCTTGGCTG
1

48 GCCAAGAACCCTGAGCTGCCTTCTGTGGTGACAAAACCAGGCGATGTCAT
MscI PIL62 AML-1 AP-1 ←

98 CAGGGCCACCAGCGGAAATACAACCGAGGTCATGGGCAGATTAAATCTGA
PIL103 NFAT AP-1 ←

148 CACTTGTGATGCCATGGAAAGGGTGGCGGAGGAGCTTGTCTACAGTGACTG
PIL150 Nco I NFAT Sp1 ←

198 AGGCCCAGGTTCTTGCCTGTGGCGGGTACCCAGAAAATTCCACTAGGCC
PIL220 KpnI NFAT Stu I ↑

248 TGTGGGGGTGAGTGATGGGAAGAAGGAGGCATGTACCTCTAGTTGGACTG
←

298 GACCCTGAGGGCCAGGACTTCCAACAATGTGCCCACCTCATAGGGATGGGG

348 TCAGGCCACAGGGATCCATTAGACAGGGGTCCAGAGAC
BamHI

Fig. 3.10. Sequence of the SS245 enhancer.

The sequence of DNA spanning the SS245 enhancer from 51 bp upstream of the SacI site to 141 bp downstream of the StuI site is presented. The four oligonucleotide probes used in EMSA are indicated between 90° bent arrows. The potential binding sites for the transcription factors tested are indicated. Note that there are several overlapping segments that potentially bind Sp1 in the PIL150 region.

figure 3.10 as PIL62, PIL103, PIL150, and PIL220 respectively. The HLH/E-box and the NF-1/c-EBP-like areas were not tested in EMSA and are not highlighted.

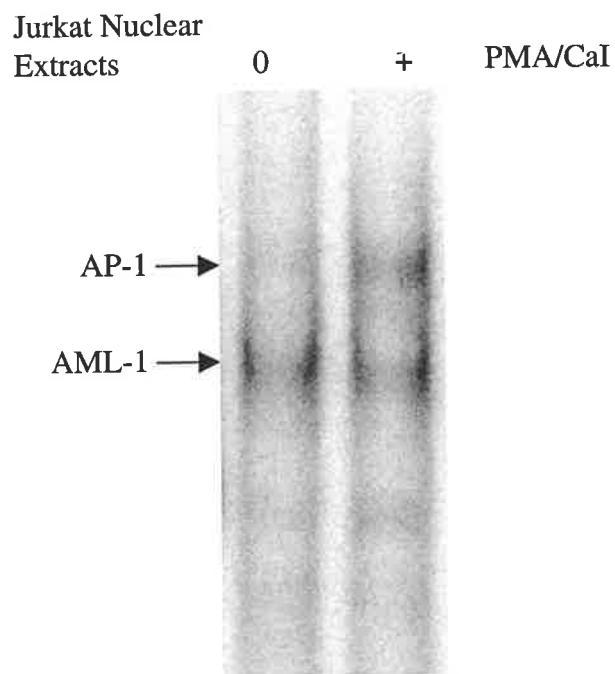
3.6.1 AML1 and AP-1 bind to PIL62 region

The PIL62 probe was used in EMSA using nuclear extracts from unstimulated and PMA/CaI stimulated Jurkat T cells. Figure 3.11A clearly shows an inducible and a constitutive band binding to this probe, which contains AML1 and AP-1-like elements. In panel B, the proteins that bind were identified using an antibody to CBF α and oligonucleotide competitors. The anti-CBF α Ab caused most of the AML1-like band to super-shift as a more slowly migrating complex. Moreover, the control TCR AML1 oligonucleotides competed away the AML1 binding. The AP-1-like band was competed away by the GM170 oligonucleotide duplex, which contains an AP-1 site from the GM-CSF enhancer (Cockerill et al, 1993). Recombinant AP-1 (rAP-1) protein also bound efficiently to the PIL62 probe. Figure 3.12 shows another EMSA where probes carrying AML1 or AP-1 mutations and the wild type probe were assayed. Again the wild type probe showed both the AP-1 and the AML1 binding and they were competed with the AP-1 and TCR-AML1 consensus oligonucleotide competitors. An Sp1 consensus probe was used as an unrelated control competitor that did not inhibit formation of either the AP-1 or the AML1 complex. The PIL62 Δ AML1 probe lost all AML1-like binding and showed a modest decrease in AP-1 binding. Because the AML1 and AP-1 binding sites overlap, and this mutation flanks the AP-1 site, it may also have some impact on AP-1 binding. Conversely, the PIL62 Δ AP-1 mutation eliminated AP-1 binding but not AML1 binding. A third band that is present with lower mobility than the AML1 band was not

Fig. 3.11. Binding of AML1 and AP-1 proteins to the PIL62 element.

(A) EMSA showing binding of the AML1 and AP-1 transcription factors in 4 μ g of each of unstimulated (0) and PMA and CaI-stimulated (+) Jurkat T cell nuclear extracts with 2 μ g of poly dI:dC. (B) EMSA showing inhibition of AML1 and AP-1-like complexes formed with 4 μ g of nuclear extracts of activated Jurkat cells with 2 μ g of poly dI:dC. Lane 2 included the α -CBF α antibodies. Lane 3 included the GM170 oligonucleotide duplex as an AP-1 competitor. Lane 4 included an oligonucleotide duplex spanning an AML1 site from the TCR locus. Recombinant AP-1 (2 ng) was used in place of nuclear extract in lane 5 with 0.1 μ g poly dI:dC. The sequence of the PIL62 probe is displayed below. Probe, and competitors were used at 0.2 ng, and 20 ng respectively.

A



B

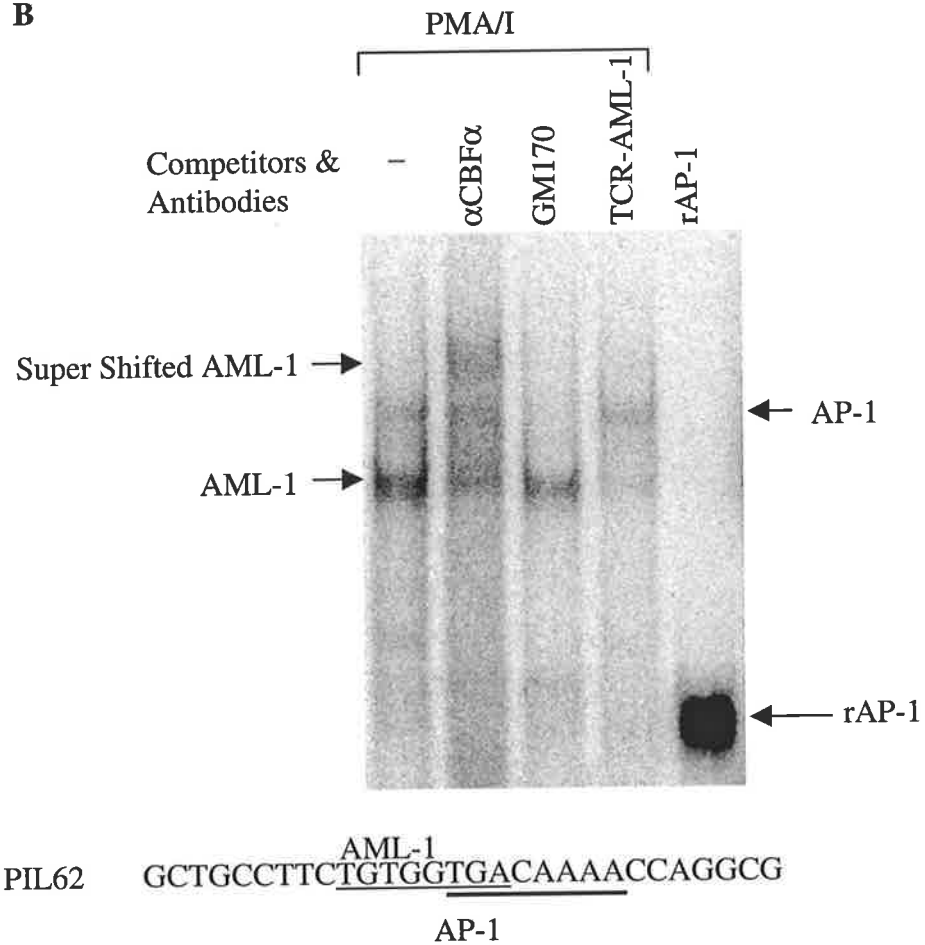
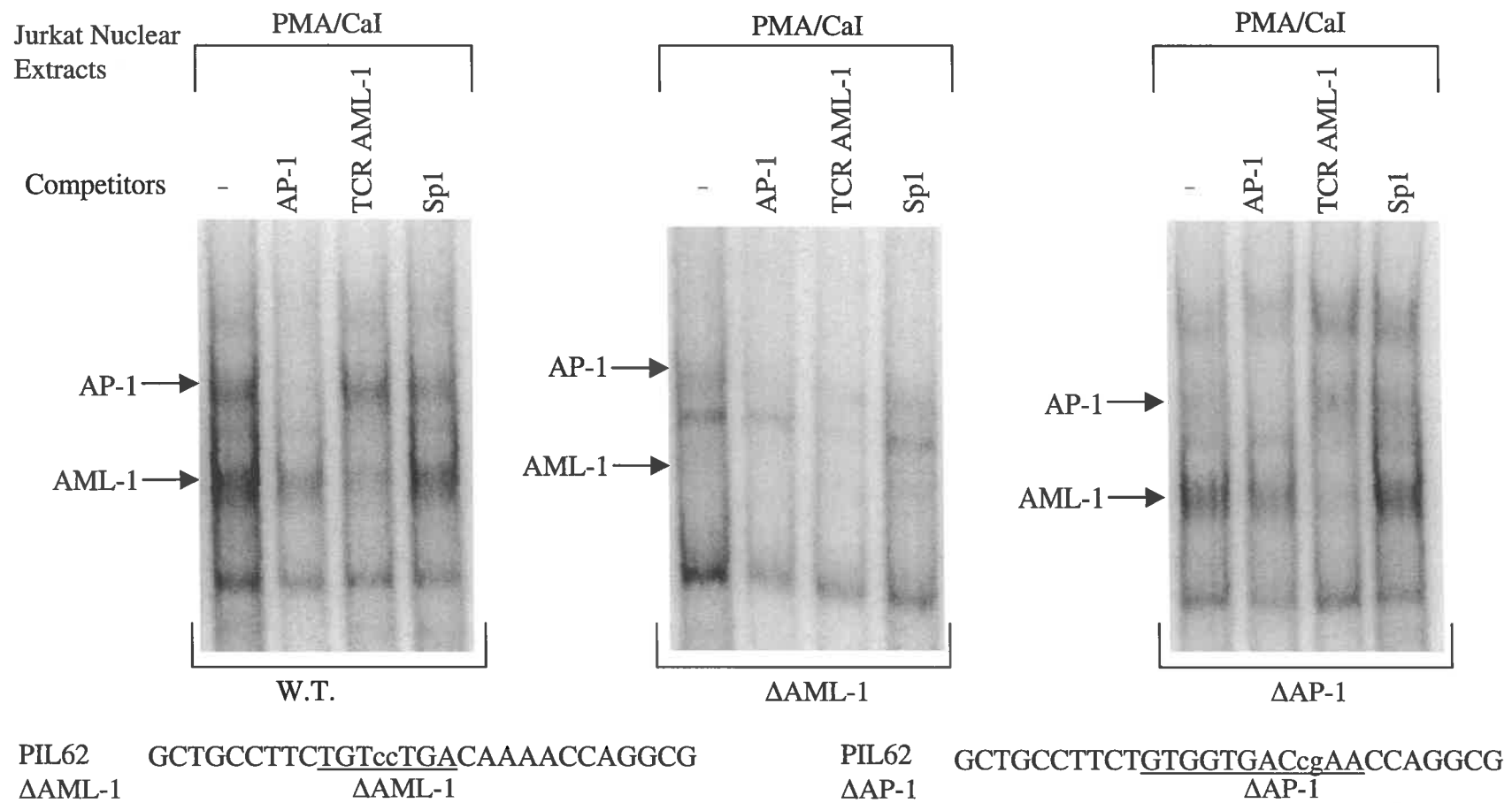


Fig. 3.12. EMSA of AML1 or AP-1 mutant PIL62 oligonucleotides.

Oligonucleotide probes (0.2 ng) that have the AML1 or AP-1 element mutated were tested in EMSA with 5 µg of nuclear extracts of stimulated (PMA/CaI) Jurkat cells. Oligonucleotides encompassing a TCR locus AML1 site and a stromelysin gene AP-1 site were used as specific competitors (20 ng) to confirm the identity of the complexes. An unrelated Sp1 element was used as a control competitor. The sequences of the mutated oligonucleotides are depicted below with the altered bases highlighted in lower case. Poly dI:dC was used in each of the binding at 2 µg.



competed away with any of the competitors used. This band may represent non-specific binding or it could be specific binding of an unidentified protein.

3.6.2 NFAT and AP-1 bind to PIL103 region

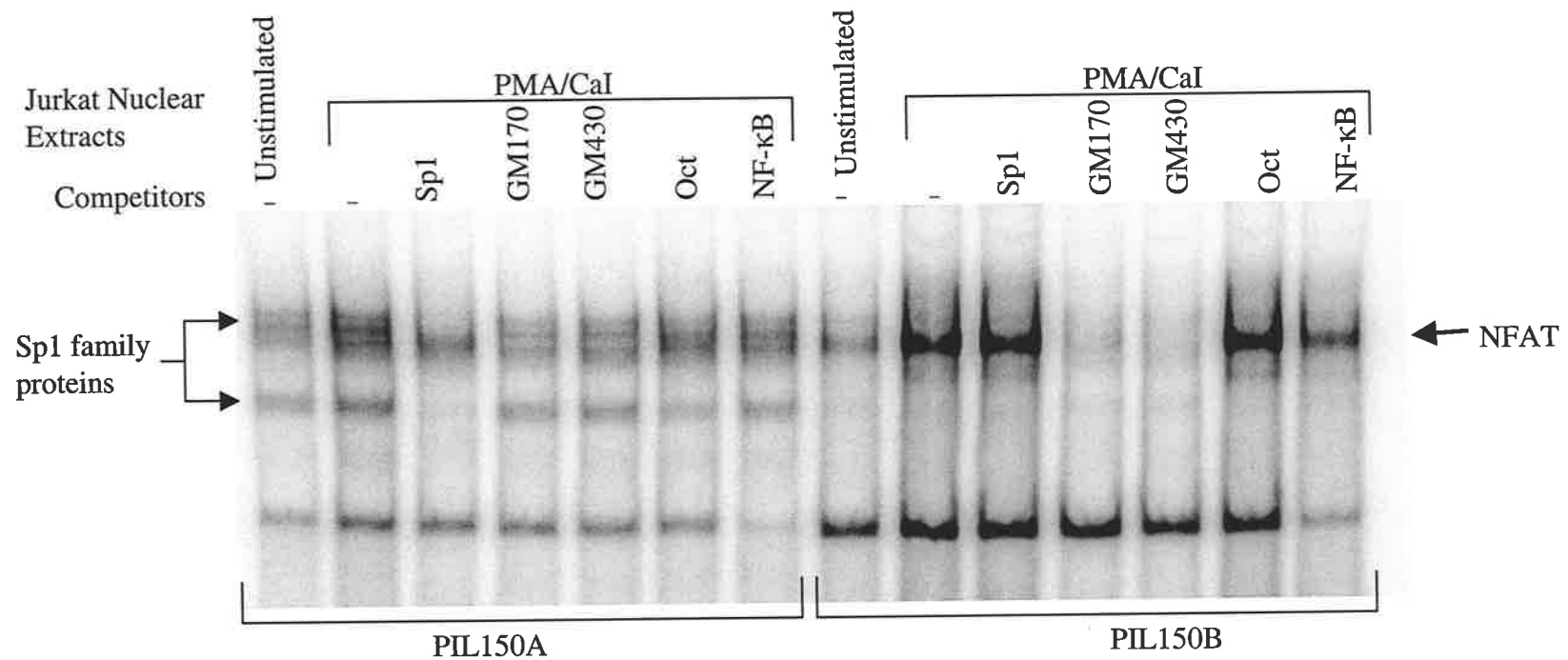
NFAT and AP-1 binding to the PIL103 probe was tested with recombinant proteins only. As is shown in figure 3.13, both NFAT and AP-1 recombinant proteins bound to this probe. Unlike many other composite NFAT/AP-1 sites (Cockerill et al., 1995b), no cooperative binding is expected here since the binding sites for the proteins are separated by 4 bases, and this aspect was not investigated.

3.6.3 NFAT and Sp1 bind to PIL150 region

The sequence between positions 150 and 193 (figure 3.10) was investigated for potential NFAT and Sp1 binding sites. Three probes were synthesised to investigate NFAT and Sp1 binding in this region. The first probe (PIL150A) runs from positions 162 to 193. This probe had the binding site for Sp1 family proteins and for NFAT as is shown in figure 3.14. It was expected that Sp1 family proteins would bind to the sequence GTGGCGGAG from 170 to 178 and at least 3 Sp1-like bands were seen which might represent Sp1, Sp2 and Sp3. The Sp1 protein binding was competed away by the Sp1 consensus oligonucleotides but not with GM170, GM430, Oct, or NF- κ B consensus oligonucleotides. Because this probe starts at the edge of the NFAT binding site, NFAT protein binds to this probe weakly as is evident from the competition with GM170 and GM430. However, when the PIL150B probe was used, that runs from 150 to 183, the NFAT binding affinity increased dramatically, and Sp1 binding was greatly diminished

Fig. 3.14. NFAT and Sp1 binding to the PIL150 element.

EMSA showing binding of the Sp1 and NFAT proteins from 5 μ g Jurkat T cells nuclear extracts of cells that have been either unstimulated and PMA and CaI stimulated. The bands representing the NFAT and Sp1 proteins are indicated by arrows. Twenty ng of GM170 and GM430 NFAT specific and OCT and NF- κ B non-specific competitors were used to identify the binding factors. Two μ g of poly dI:dC and 0.2 ng of each probe were used.



PIL150A NFAT
TGGAAAGGGTGGCGGAGGAGCTTGTTCACAGTG (From 162 to 193)

PIL150B CTTGTGATGCCAT**TGGAAAGGGTGGCGGAGGAGCT** (From 150 to 183)

Potential Sp1 binding sites

GTGGCGGAG

GCGGAGGAG

GAGGAGCTT

GGAGCTTGT

compared to PIL150A. This suggested that the Sp1 site overlapped or was close to position 183, and was probably not the predicted sequence GTGGCGGAG but rather was further downstream. Hence, the binding site for Sp1 could be either GCGGAGGAG from 173 to 181, GAGGAGCTT from 176 to 184 or GGAGCTTGT from 178 to 186, based on the published consensus (Kadonaga et al., 1987).

To further examine which of the potential binding sites the Sp1 protein bound to, a third probe was used which runs from 157 to 186 (see figure 3.15). This probe bound specifically to both NFAT and Sp1 protein as evident by the competition with the consensus oligonucleotides. Two more oligonucleotides were tested with specific mutations to NFAT and Sp1 binding sites. These mutations knocked out specifically the NFAT and Sp1 binding. These data taken together eliminate the GGAGCTTGT as a binding site for Sp1 since this sequence is not touched by the mutation. That leaves the sequences GAGGAGCTT and GCGGAGGAG as the most likely sites for Sp1 binding. However, the result of the GG to AA mutation that eliminates binding makes it more likely that GCGGAGGAG is the binding site because this alters two highly conserved bases in the GCGGAGGAG sequence, but just one poorly conserved base in the less ideal GAGGAGCTT sequence.

3.6.4 NFAT binds to the PIL220 region

EMSA of Jurkat nuclear extracts indicated that the PIL220 region contained a binding site for NFAT (figure 3.16A). The NFAT band was competed away specifically with the GM430 and GM170 oligonucleotides which both contain an NFAT binding site but not with Sp1, Oct., AP-1, or NF- κ B consensus sequences. The NFAT band was super-

Fig. 3.15. The Sp1 and NFAT binding to mutant PIL150 oligonucleotides.

EMSA showing binding of 4 μ g of unstimulated or PMA/CaI stimulated Jurkat nuclear extracts to 20 ng of wild type (W.T.) probes or probes containing mutation in the binding sites of NFAT or Sp1. Sp1 and NFAT (Gm430) consensus probes were also used. The same consensus probes were used as cold competitors at 20 ng. The sequences of the probes used are shown except for the consensus probes (see material and methods). The bands corresponding to the Sp1 and NFAT proteins are indicated by arrows. The Sp1 consensus is derived from Kadonaga et al., 1987. poly dI:dC was used at 4 μ g for each binding reaction.

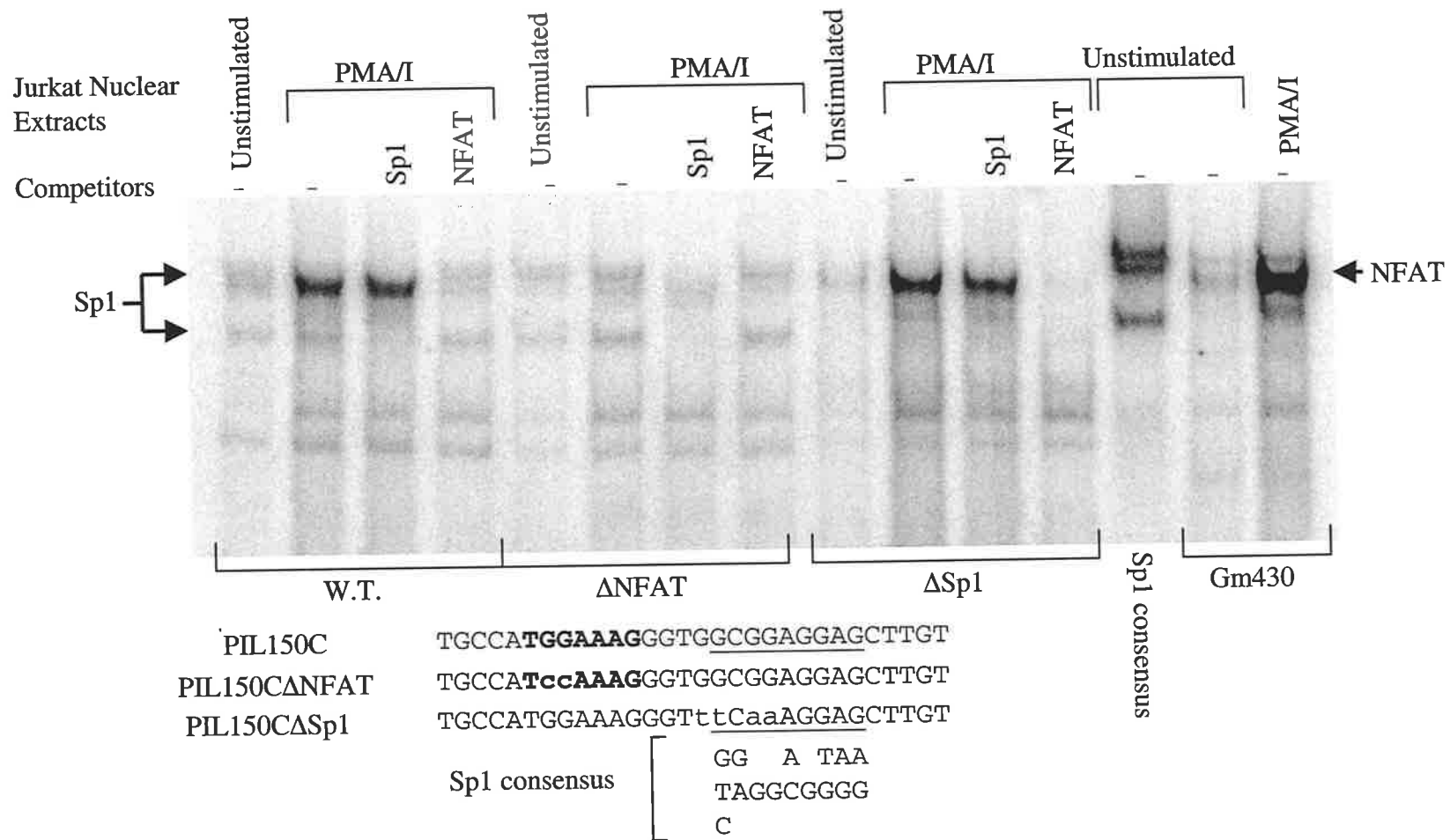
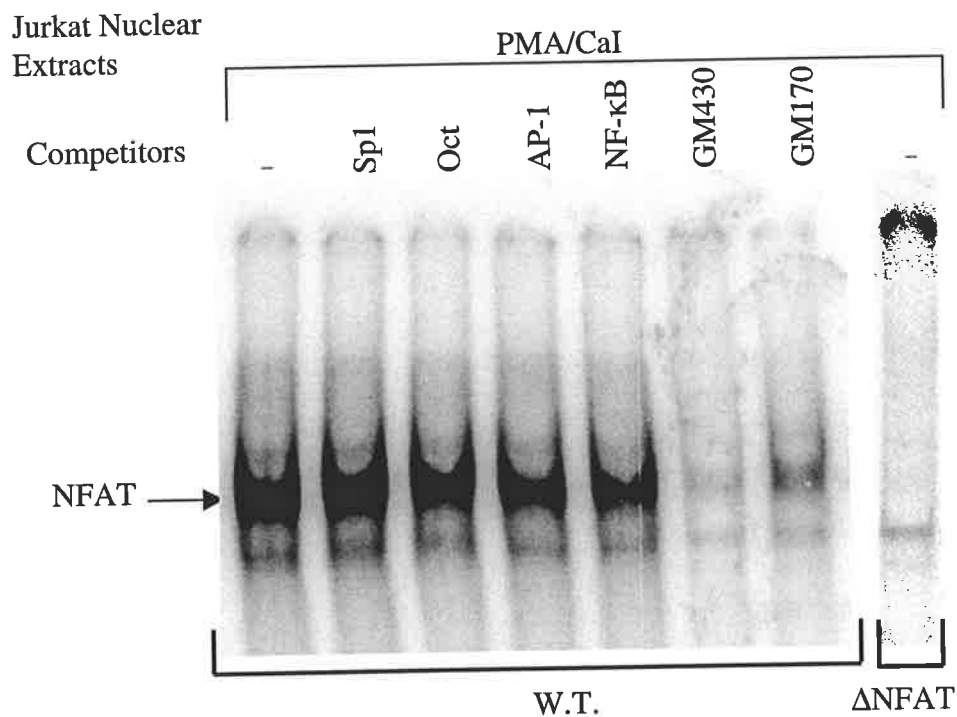


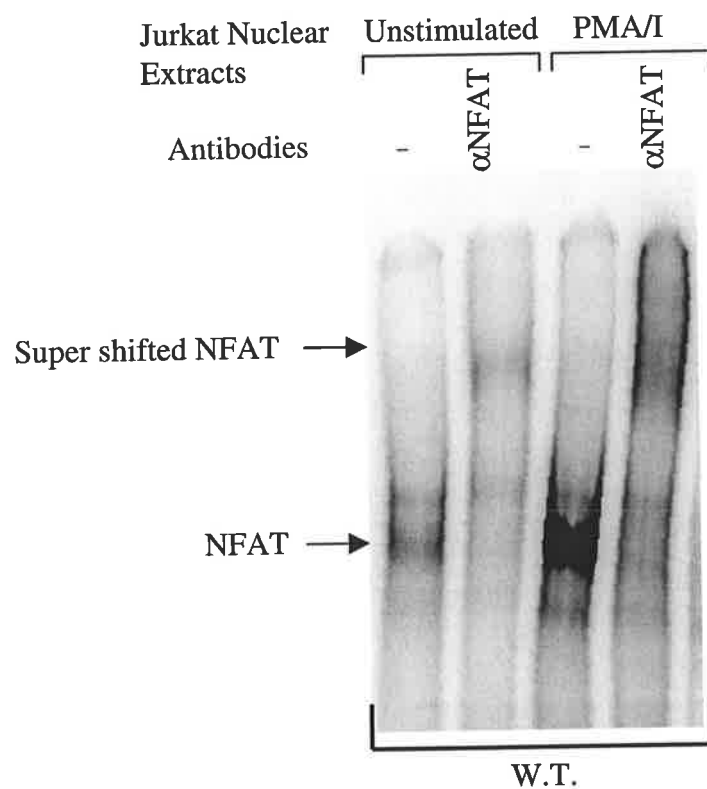
Fig. 3.16. NFAT binding to the PIL220 probe.

Binding of the NFAT protein in 4 µg of nuclear extracts from PMA and CaI (PMA/CaI)-stimulated Jurkat T cells to 0.2 ng of the PIL220 probe or a similar probe containing a deletion in the NFAT binding site (PIL220ΔNFAT). In A, Sp1, Oct, Stromelysin AP-1, and NF-κB oligos were used at 20 ng as non-specific competitors and GM430 and GM170 as NFAT specific competitors. In B, rabbit antibody to NFAT was added to lane 2 and lane 4. Four µg of poly dI:dC was used.

A



B



PIL220

PIL220ΔNFAT

NFAT

CGGGTACCCAGAAAATTCCACTAGGCCTGTG

Kpn I

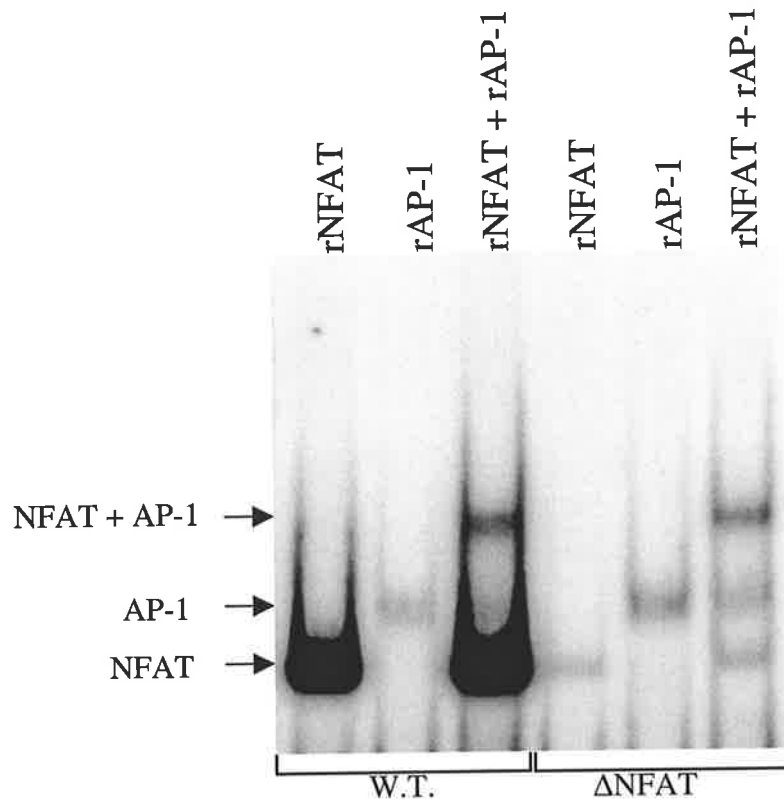
CGGGTACCCAGAAAATTggACTAGGCCTGTG

shifted when antibodies to NFATp were included. Another probe was made that changed the NFAT binding site from AAATTCCA to AAATTggA, and this mutation abolished all NFAT binding as shown in figure 3.16A, and in figure 3.17 with recombinant protein. Figure 3.17 shows binding of NFAT and AP-1 recombinant proteins to the wild type and the mutant oligonucleotides. Only NFAT bound significantly to the probe. The AP-1 band was very faint and could represent non-specific binding. When both NFAT and AP-1 recombinant proteins were added to the wild type oligonucleotides, a second AP-1/NFAT band in addition to the NFAT band was present. This weak higher order complex may result from recruitment of AP-1 by the overlapping NFAT-like sequence AGAAAAT on the opposite strand. Furthermore, this complex was not affected by the CC to GG mutation in PIL220ΔNFAT that lies outside AGAAAAT.

3.7 THREE NFAT SITES DRIVE SS245 ENHANCER FUNCTION

To define the boundaries of the SS245 enhancer within the -4.5 kb region, and to study the function of specific elements, serial 5' and 3' deletions and specific mutations were created by a PCR cloning strategy (figure 3.18). Oligonucleotides carrying introduced SmaI sites were used to amplify specific regions of the enhancer. The PCR products were cleaved by digesting with restriction enzymes and cloned upstream of the GM55 promoter in pXPG-GM55 plasmid as described in materials and methods. These were assayed alongside the SS245 enhancer fragment.

Figure 3.19A shows the DNA fragments with 3' serial deletions of the enhancer generated by PCR. SacI and SmaI were used to cut PCR products generated by PCR A,



PIL220 NFAT
 CGGGTACCCCAGAAAATTCCACTAGGCCTGTG
Kpn I

PIL220ΔNFAT CGGGTACCCCAGAAAATTggACTAGGCCTGTG

Fig 3.17. Recombinant protein binding to the PIL220

EMSA showing binding of recombinant NFAT and AP-1 proteins to PIL220 and PIL220ΔNFAT probes (0.2 ng). Recombinant NFAT protein was added to lane 1, 3, 4, and 6 at 0.2 ng and rAP-1 was added to lanes 2, 3, 5, and 6 at 2 ng of each of Fos and Jun proteins. 0.1 ng of poly dI:dC was added to each lane.

(1) AGGAGGCCcGGGCTCTCCACC-->
-51 ACAGGAGGCCAGGGCTCTCCACCTGGGCCCCTCGGTGGCTTTTTCCT

Ecl136II
(Sac I)
↓
-3 GAGCTCTCTGCTCAGGTGAGCAGGGGACATCTGGTTGCACTGCTTGGCTG
1

MscI
PIL62 (2) TGTCAc
48 GCCAAGAACCCTGAGCTGCCTTCTGTGGTGACAAAACCAGGCGATGTCAT
AML-1 AP-1

CcGGGCCACCAGCGG --> PIL103 (3) TCTGA
98 CAGGGCCACCAGCGGAAATACAACCGAGGTCATGGGCAGATTAAATCTGA
NFAT AP-1

CACccGgGATGCCATGG--> PIL150
148 CACTTGTGATGCCATGGAAAGGGTGGCGGAGGAGCTTGTACAGTGACTG
NFAT Sp1 <--CACTGAC

KpnI PIL220 Stu I
198 AGGCCCAGGTTCTTGCCTGTGGCGGGTACCCCAGAAAATTCCACTAGGCC
TCCGGGCCGAAGA (C) NFAT ↑

248 TGTGGGGGTGAGTGATGGGAAGAAGGAGGCATGTACCTCTAGTTGGACTG

298 GACCCTGAGGGCCAGGACTTCCAACAATGTGCCCCACTCATAGGGATGGGG
<--CTGGGACTCCCGGgCCcGAAGGT (B)

BamHI
348 TCAGGCCACAGGGATCCATTAGACAGGGGTCCAGAGAC
<--CCCTAGGTAATCTGgGCCAGGT (A)

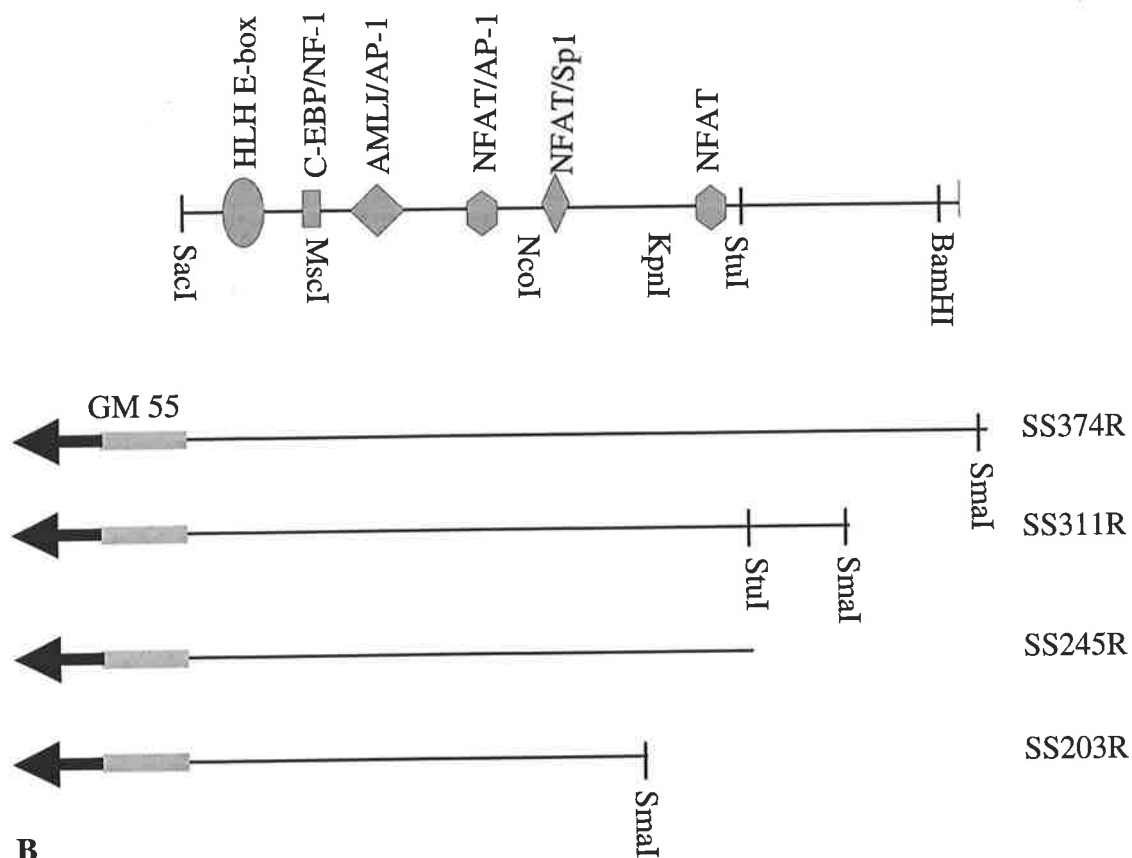
3.18 Location of the PCR oligonucleotides within the SacI to BamHI sequence.

The sequence shows the location of the PCR oligonucleotides and the direction of amplification within the SacI–BamHI region. The transcription binding sites are indicated to highlight their presence within the amplified products. The restriction enzymes used to cleave the PCR products are also indicated.

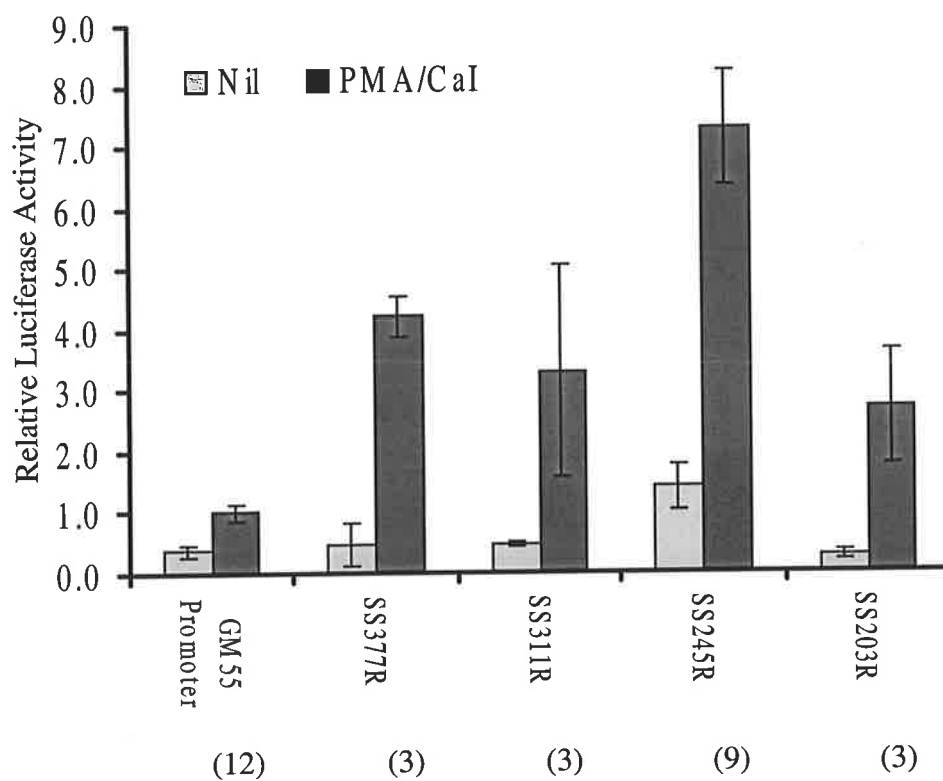
Fig. 3.19. Activity of the 3' deletion of the SS245 enhancer.

Schematic diagram (A) showing constructs with 3' deletions of the SS245 enhancer and cloned in reverse orientation upstream of the GM55 promoter in pXPG-GM55 plasmid. The SS374R, SS311R, and the SS203R were generated by PCR as described in materials and method. These constructs were transfected into Jurkat T cells. In (B), the unstimulated (Nil) and the stimulated (PMA/CaI) activity of these constructs were compared. Activities were expressed as means relative to the stimulated activity of the GM55 promoter alone, which was assigned a value of one. The numbers of transfections are indicated within parenthesis and the standard errors of the mean are represented as error bars.

A



B

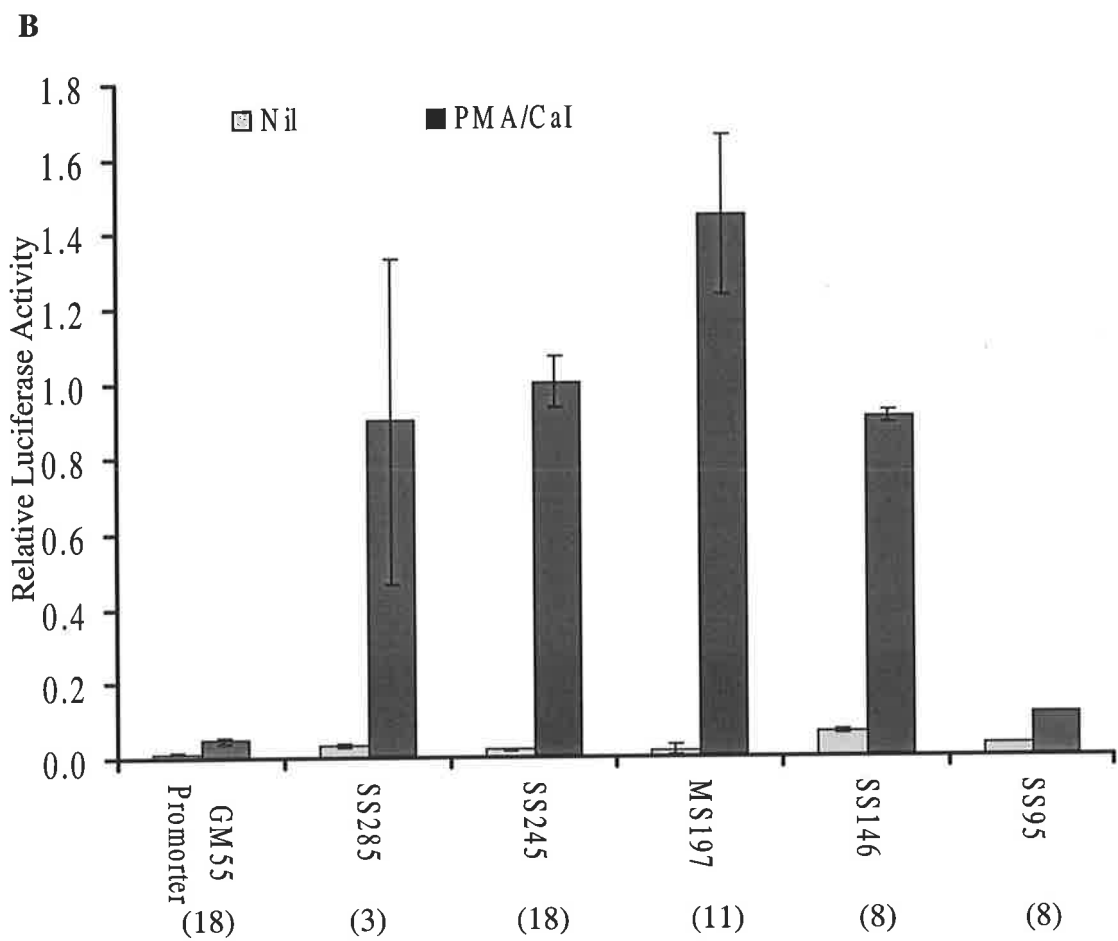
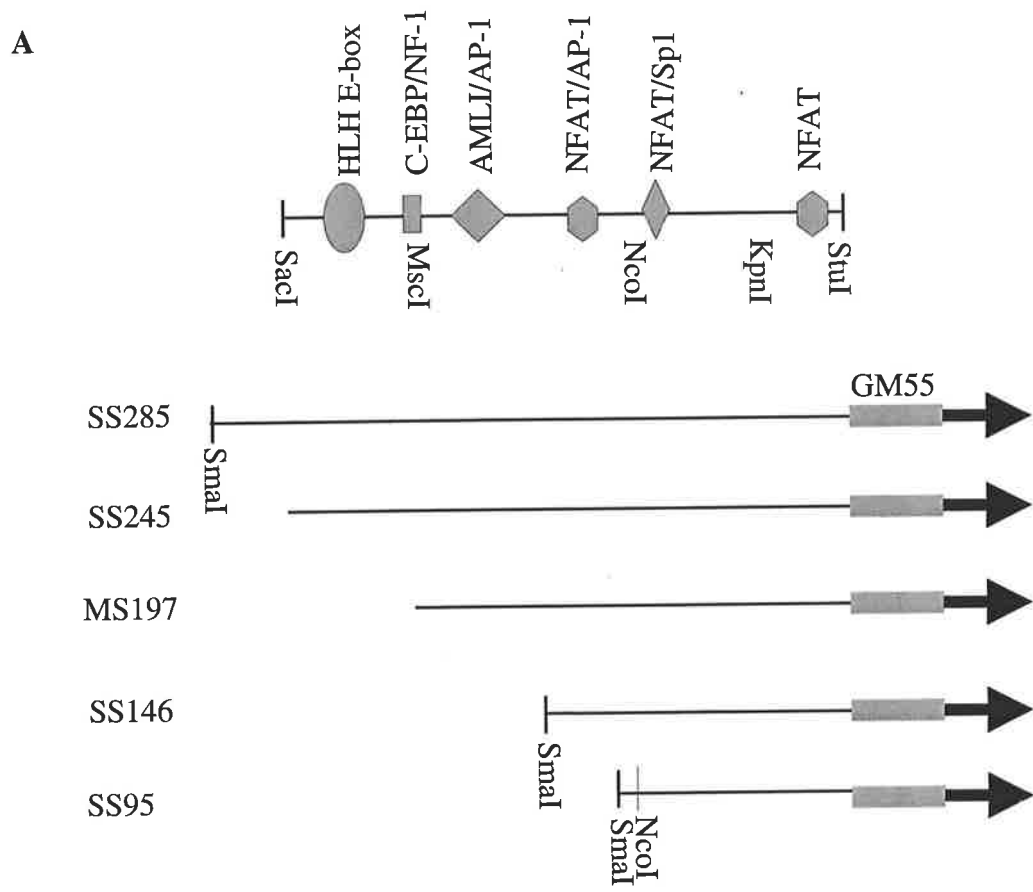


B, and C with PCR 1 primers (see materials and method), to generate the SS374, SS245 and SS203 fragments respectively. These fragments were cloned in the opposite orientation to normal so that the 5' end of the enhancer is the same in all the constructs and is immediately upstream of the GM55 promoter in pXPG-GM55. This is important so that the distance between the promoter and the factor binding sites remain the same relative to the promoter and that the only thing changing is the 3' end. The SS245R construct had the highest activity of all the constructs when tested in transiently transfected Jurkat cells (figure 3.19B). The longer SS311R and SS374R fragments and the shorter SS203R fragment each had enhancer activities that ranged from approximately 37% and 58% of that seen with SS245R. The reduction of enhancer activity that occurred as a result of adding sequences from downstream of the StuI site may indicate the presence of a repressor, and this will be discussed in the next chapter. The two thirds decrease in activity that was observed when a region encompassing the PIL220 NFAT element was deleted in the SS203R construct suggests that this element plays a significant role in enhancer function.

Figure 3.20A illustrates the enhancer fragments with 5' serial deletions that were cloned upstream of the GM55 promoter in their normal orientation. StuI and SmaI were used to cut PCR products generated by using PCR B with PCR 1, 2, and 3 to get SS285, SS146, and SS95. The SS285 fragment extending 5' of the SacI site, the previously defined SS245 enhancer fragment, the MS197 fragment which missing the potential HLH E-box and C-EBP/NF-1 binding sites, and the SS146 fragment missing the AML1/AP-1 binding sites all had similar activities, as shown in figure 3.20B. The only significant reduction in activity observed in this deletion series was with the SS95 construct, suggesting that the PIL103 NFAT/AP-1 element is also required for enhancer function.

Fig. 3.20. Activity of the 5' deletions of the SS245 enhancer constructs.

Schematic diagram (A) showing constructs with 5' deletions of the SS245 enhancer and cloned in normal orientation upstream of the GM55 promoter in pXPG-GM55 plasmid and transiently transfected into Jurkat T cells. The SS285, SS146, and SS95 were generated by PCR as described in materials and methods. SS245 and MS197 were generated as *Ecl*1136II/*Stu*I and *Msc*I/*Stu*I fragments. In (B), the activity of these constructs in unstimulated (Nil) and stimulated with PMA and CAI are compared. The numbers of transfections stimulated are indicated within parenthesis. Data is expressed as the mean with error bars indicating the standard error of the mean.



In this construct the enhancer activity went down to 12% of the SS245 enhancer activity. From these studies we can conclude that the core of the enhancer is contained within 146 bp upstream of the Stu I site.

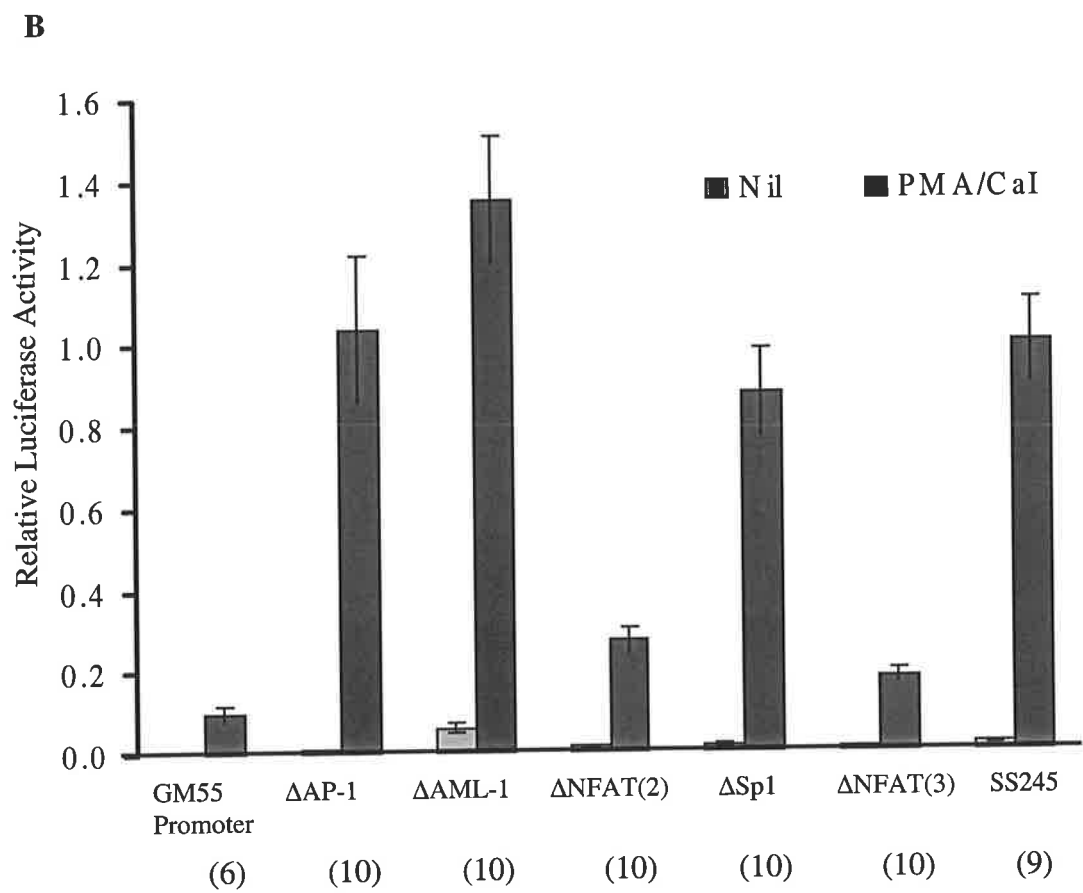
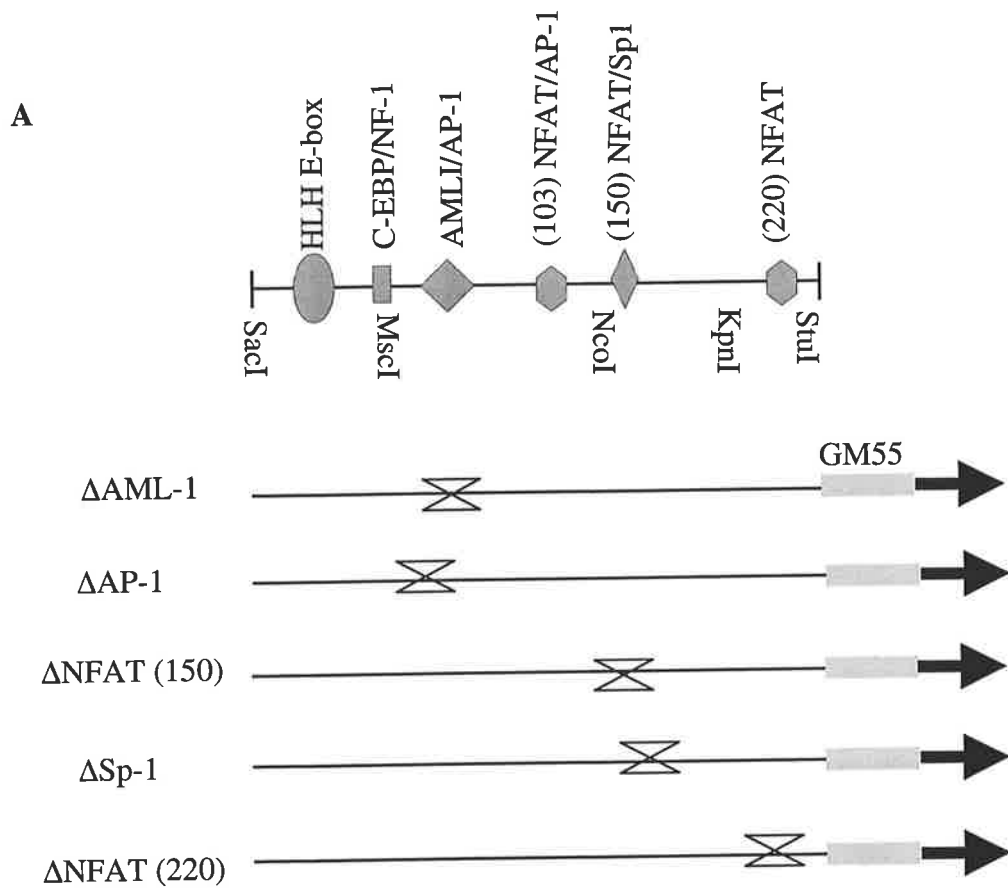
The above studies deleted binding sites sequentially by making the enhancer progressively smaller. In some instances multiple sites were deleted at the same time. For example when the AML1/AP-1 binding sites were deleted, the HLH E-box and the C-EBP/NF-1 sites were deleted as well. To study the effect of knocking out specifically one site at a time without affecting the other sites and without changing the distances between sites, mutagenesis was carried out by PCR as described in materials and methods. These mutated enhancer fragments were cloned into pXPG-GM55, and figure 3.21A shows the sites that were knocked out. These constructs were transiently transfected into Jurkat T cells and stimulated with PMA and CaI. The results are shown in figure 3.21B. The only knockouts that affected enhancer function in this analysis were knocking out either the NFAT site at 170 (site 2, PIL150) or the NFAT site at 240 (site 3, PIL220). These mutations reduced enhancer activity to 27% and 18% respectively. Although the PIL103 NFAT/AP-1 region was also implicated in enhancer function (figure 3.20), these NFAT and AP-1 sites were not investigated in this mutagenesis study.

3.8 DISCUSSION

The aim of this chapter was to characterise and identify functions for a closely linked pair of DH sites located 4.1 and 4.5 kb upstream of the IL-3 gene. Although no function has been identified for the prominent constitutive DH site at -4.1 kb, the inducible DH

Fig. 3.21. The SS245 enhancer activity with specific transcription site mutations.

As shown in (A) the mutations that were introduced into the binding sites of the indicated transcription factors and tested in EMSA and was shown to abolish binding of the specific transcription factor were cloned upstream of the GM55 promoter in pXPG-GM55 plasmid and tested in transient transfection assays (B). The luciferase activity is presented as a relative mean of the number of transfections indicated within parenthesis. The mean activity of the wild type enhancer (SS245) was assigned a value of one. Error bars are a measure of standard error of the mean.



site located at -4.5 kb was demonstrated to function as an inducible CsA-sensitive enhancer in a similar manner to other regulatory elements of cytokine genes expressed by activated T cells. CsA has been found to similarly inhibit inducible expression of the endogenous IL-2 (Graneli-Piperno, 1988; Kronke et al., 1984), IL-3 (Herold et al., 1986), IL-4 (Tocci et al., 1989), GM-CSF (Kelso and Gough, 1989), IFN- γ (Herold et al., 1986), and other cytokine genes (Tocci et al., 1989). CsA was also found to suppress the activity of the IL-2 promoter (Emmel et al., 1989), -14 kb IL-3 enhancer (Duncliffe et al., 1997), and GM-CSF enhancer (Cockerill et al., 1993) in transfection studies through its inhibition of NFAT activity (reviewed in Matsuda and Koyasu, 2000).

The function of the -4.5 kb region was initially tested by assaying a 245 bp SacI/StuI fragment encompassing this region (SS245) in the context of a luciferase reported gene plasmid containing the minimal core of the GM-CSF promoter (pXPG-GM55). However, because the GM55 promoter contains overlapping AP-1, Ets, and NFAT sites, the inducible activities of the GM55 promoter/enhancer constructs described here will arise from the combined properties of the promoter and enhancer. For this reason the SS245 enhancer was also studied in the context of the TK promoter, which is constitutively active in essentially all cell types. The TK promoter studies allowed the confirmation that the SS245 enhancer was both highly inducible and tissue specific, whereby it functioned in both T cells and mast cells.

One advantage of the TK promoter is that, unlike the GM55 promoter, it is a full-length promoter and more likely to identify enhancer functions. However, an advantage of the GM55 short promoter, composed of a composite AP-1/NFAT/Ets binding site, was that it responded with a 50 fold increase in activity in the presence of the enhancer. In

contrast, the TK229 system showed that the SS245 enhancer is capable of increasing the activity of a heterologous full-length promoter by about two fold. This 2 fold increase was, however, similar to the increase supported by the GM-CSF enhancer. This finding was confirmed when a 610 bp full length IL-3 promoter was used to test the enhancer function. Hence, the SS245 enhancer was also capable of increasing the activity of the IL-3 promoter by about two fold in Jurkat T cells and may therefore serve this function in its natural context.

In this study, PMA and CaI were used to activate cells *in vitro*, and the effect of these two agents is similar to the effect of TCR conjugation. Activation through the TCR leads to the activation of both the protein kinase and the calcineurin pathways as described earlier. This activation system provided the advantage that it allowed the activation of the same pathways in cells from non-T cell lineages which do not have the TCR on their surface as in T cells. As a result, the activity of gene regulatory elements in T cells could be compared with non-T cells, and this enabled us to determine that the same pathways activated the SS245 enhancer in mast cells, but not in other myeloid cells or B cells.

It was significant that the enhancer was shown to have activity in HMC-1 mast cells as well as T cells because HMC-1 cells were shown to make IL-3 and GM-CSF (figure 3.3). As such, the activity of this enhancer in HMC-1 cells was not unexpected. The enhancer activity was not tested in the context of the full length IL-3 promoter in HMC-1 cells and it would be interesting to show that both the IL-3 promoter and the SS245 enhancer are active in HMC-1 cells. In KG1a cells, which do not express IL-3, the SS245 enhancer also increased the activity of the GM55 promoter, but only at 11% of

the GM-CSF enhancer level of activation, but it did not increase significantly the activity of a full length TK229 promoter. This can be explained in two ways. On the one hand, the increase in activity seen in the GM55 system may be an artefact of using a minimum promoter system. However, in a more natural context when a full length promoter was used no significant increase was observed in KG1a cells when the SS245 enhancer was included. It was not possible to use the IL-3 promoter in KG1a cells since the IL-3 promoter is inactive in KG1a cells. Moreover, because KG1a cells do not make IL-3, there is no reason why the SS245 enhancer should be active in this context. Even if the small increase in activity observed in KG1a cells with heterologous promoters in transient transfection assays is real, this does not reflect what happens *in vivo* in the context of the IL-3 promoter. On the other hand, one can argue that since the locus is beginning to open in CD34+ cells as it potentially develops into IL-3 producing cells, the observed increase of activity could reflect the priming of the locus and the commitment process of becoming IL-3 producing cells.

Along these lines, the presence of the -4.1 DH site in KG1a and HMC-1 cells was also particularly interesting. KG1a is a CD34+ cell line that may represent an early stage of myeloid development (Koeffler et al., 1980). Cells from this stage typically develop to myeloid lineages that include basophils, eosinophils and mast cells, and it has been shown that basophils, eosinophils and mast cells make IL-3. Although, KG1a does not make IL-3, as shown in the Northern blot analysis (figure 3.3), these observations may suggest that the presence of the -4.1 site is a developmental marker for primitive myeloid cells that develop into IL-3 producing cells. Hence, the early stages of basophil, eosinophil, and mast cells development may be initiated by the appearance of the -4.1 DH site which primes the locus for its subsequent activation. In this study, however, the

–4.5 DH site was shown to be an enhancer and no enhancer function has been detected for the –4.1 site by the *in vitro* approaches used here.

In this study, transcription factor binding sites within the SS245 enhancer were also characterised. Four footprinted areas, as defined by the footprint experiments, were tested in EMSA and factors that bind to these areas were identified. In two other areas of footprints HLH/E-box proteins and NF-1/c/EBP were predicted to bind, but the deletion analysis indicated that they do not have an effect on the activity of the enhancer *in vitro* in transient transfection assays. They may have a significant role in the function of the enhancer within this locus *in vivo*. The role of these elements should be tested *in vivo* in conjunction with other elements in the locus.

The binding assays showed that the transcription factors AML1, and AP-1 associated with the AML1 site, and that Sp1 family factors bind to the enhancer. However, these proteins bound weakly to the enhancer DNA as is shown by the weak intensity of their respective bands, and they were not required for enhancer function. These proteins, although bound *in vitro*, may not bind *in vivo*. The binding of these factors to the enhancer needs to be demonstrated *in vivo* by using *in vivo* techniques such as *in vivo* footprinting or chromatin immuno-precipitation (CHIPS) assays. On the other hand, these proteins may prove to be important for the enhancer function *in vivo*.

The contribution of these transcription factors to the activity of the SS245 enhancer was not assessed in mast cells where the enhancer can also function. AML1 has been shown to be critical for hematopoiesis (Castilla et al., 1996; Okuda et al., 1996), and AML1 protein (CBF) is expressed in both T cells and myeloid cells. AML1 was shown to be

involved in the regulation of the GM-CSF promoter and enhancer (Takahashi et al. 1995; Cockerill et al., 1996), IL-3 promoter (Soemaker et al., 1990), CSF-1 receptor promoter (Zhang et al. 1994), TCR α (Hernandez-Munain et al., 1998), TCR δ (Hernandez-Munain and Krangel, 1995), TCR γ (Hsiang et al. 1993) and TCR β (Prosser et al., 1992) enhancers. In the GM-CSF promoter a two thirds reduction in activity was observed when the AML1 site was deleted (Cockerill et al., 1996). However, the AML1 site within the GM-CSF enhancer binds AML1 very weakly and it does not seem to affect enhancer function (Cockerill et al. 1996). The AML1 site in both cases of the GM-CSF promoter and enhancer was located in the proximity of NFAT and AP-1 sites. This provides the potential for AML1 to interact with the NFAT/AP-1 complex. Furthermore, AML1 was previously shown to interact with c/EBP α in the M-CSF receptor promoter (Petrovick et al., 1998). Hence, AML1 may interact with the potential c/EBP site and or NFAT to regulate the expression of the SS245 enhancer in IL-3 producing myeloid cells such as mast cells, and in T cells.

NFAT proteins have been shown to regulate cytokine gene expression (De Boer et al., 1999). NFAT was found to bind to five sites of the IL-2 promoter and four of these sites are composite NFAT and AP-1 (Rooney et al., 1995b). NFAT was also shown to cooperatively bind with AP-1 to the IL-4 promoter in the CLE0 region, which is conserved in the IL-3, IL-4, IL-5, and GM-CSF promoters (Miyatake et al., 1991) and that both are required for the full activity of the promoter (Rooney et al., 1995a). Moreover, c-maf and NFAT were found to synergistically activate the IL-4 promoter in a tissue specific manner (Ho et al. 1996). In addition, NFAT and AP-1 bind cooperatively to three sites in the GM-CSF enhancer (Cockerill et al., 1995b). In the IL-3 enhancer, NFAT binds to four sites, one of which (the IL190 element) functions synergistically

with an overlapping Oct element to induce T cell-specific activation of the IL-3 enhancer (Duncliffe et al., 1997). The IL280 element within the IL-3 enhancer also contains a composite NFAT/AP-1 site. Although the conserved CLE0 region binds NFAT in both GM-CSF and IL-4 promoters cooperatively with AP-1, it doesn't bind NFAT in the IL-5 promoter. However, AP-1 binds to the IL-5 CLE0 region.

Of all the transcription factor sites identified in this study, only the NFAT binding to the SS245 enhancer at the three locations was strong as judged by the strong intensity of the bands. The deletion and mutagenesis studies implied that the three NFAT sites in the SS245 were primarily responsible for enhancer function. Binding of NFAT to two of the three NFAT sites were demonstrated using nuclear extracts. The third NFAT binding site at position 115 of the SS245 enhancer was only demonstrated by recombinant NFAT protein. In this case the portion of the probe shifted was about a quarter of the total probe loaded, which is likely to represent significant binding as it was comparable to the other NFAT sites tested in this study (data not shown). AP-1 recombinant protein bound to the AP-1 binding site at position 130 of the SS245 enhancer and two thirds of the probe was shifted in this assay. The NFAT and AP-1 sites in the PIL103 element were both deleted during the creation of the SS95 enhancer fragment that had lost most of its activity. Hence, the contribution of these sites individually was not assessed. A better approach would have been to mutate these sites individually and to test these mutants in the context of the full length enhancer.

The GM170 element in the GM-CSF enhancer, which binds both NFAT and AP-1 independently, has no enhancer activity because the sites are too far apart to support cooperative binding (Cockerill et al., 1995b). Hence, the 115 NFAT and the 130 AP-1

sites of the SS245 enhancer might not have synergistic enhancer activity since they are separated by a large distance that is unlikely to support cooperative binding. However, both of the NFAT and AP-1 proteins may still functionally interact as in the case of the CLE0 element in the GM-CSF promoter (Miyatake et al., 1991). NFAT and AP-1 bind independently to the CLE0 element but knocking out binding of either inhibits promoter activity. Hence, the determination of the role of the 115 NFAT and the 130 AP-1 sites of the SS245 enhancer may prove to be critical for the regulation of the SS245 enhancer function.

The significance of the Sp1 binding was not determined in the SS245 enhancer. Sp1 was reported to be critical for the activity of the IL-2 response element in the Fas ligand promoter (Xiao et al., 1999). The IL-2 response element contains an overlapping binding site for both NFAT and Sp1 proteins. The PIL160 region of the SS245 enhancer contains an overlapping site for both the NFAT and Sp1 sites. However, knocking out Sp1 site did not affect enhancer function in Jurkat T cells stimulated by PMA and CaI. Whether the Sp1 binding in this region is important for other signalling mechanism remain to be seen. Alternatively, the Sp1 could simply function in the SS245 enhancer to maintain a methylation free region as described for other elements (Macleod et al., 1994; Brandeis et al., 1994).

In conclusion, the mechanism of action of this enhancer was shown to be driven predominantly by the three regions encompassing NFAT sites. Deleting or knocking out any one of them reduced the enhancer activity to between 10% and 30% of the full enhancer activity. NFAT has been shown previously to activate genes in both T cells and in non-T cells. The GM-CSF enhancer (Cockerill, 1995b) has been shown to

function in T cells, KG1a cells, K562 cells and endothelial cells, and that this enhancer is primarily driven by NFAT/AP-1 composite elements. However, in contrast to the NFAT/AP-1 elements, an NFAT/Oct composite element was shown to drive the T cell specificity of the IL-3 enhancer located 14 kb upstream of the IL-3 gene. However, in this case it was shown that the combinatorial effect of NFAT, Oct, and the cofactors OCA-B and NIP45 were responsible for the T cell specificity of this enhancer (Duncliffe et al., 1997). So, what is responsible for the tissue-specificity of the SS245 enhancer? To answer this question, more work needs to be done to identify all of the factors that bind to this enhancer and to assess their relative contribution to its function in T cells and mast cells. Future studies will also need to address the role of the enhancer in other cell types such as eosinophils and basophils that express IL-3 and NFAT. Moreover, the nature of the interactions between the different factors that bind to this enhancer needs to be assessed.

CHAPTER 4: INVESTIGATION OF A POTENTIAL SILENCER DOWNSTREAM OF THE SS245 ENHANCER

4.1 INTRODUCTION

Genes are not only turned on in response to a positive stimulus, but genes are also turned off once the positive stimulus is removed or a negative stimulus is introduced. Negative regulatory elements (NREs) have been identified in many systems. These elements are called repressors or silencers. Their mechanism of action is different from the silencing that occurs through heterochromatin, and the general DNase I sensitivity of the locus is not affected by the presence of these NREs. In general, these elements can be either position- and orientation-independent or position-dependent elements (reviewed in Ogbourne and Antalis, 1998).

Silencers have been identified in many systems by deletion analysis in transient transfection assays. As an example, the plasminogen activator inhibitor type-2 (PAI-2) gene, which is involved in the mononuclear phagocytes response to inflammation, contains a silencer element at between -1977 to -1675 (Antalis et al., 1996). It was found to repress transcription in a non cell-specific, and position and orientation independent manner. The inhibition mediated by the silencer is overcome by the interaction of the promoter with another positive regulatory element located further upstream between -5100 and -3300 in a cell-specific manner. The minimum silencer motif was localised to 28 bp containing the sequence CTCTCTAGAGAG. The protein that binds to this sequence was called PAI-2-upstream silencer element-1 (PAUSE-1) binding factor.

Another example of a silencer is the one found in the chicken ovalbumin locus between –308 and –88 (Haecker et al., 1995). The ovalbumin gene is activated by estrogens or glucocorticoids. The silencer element represses transcription in the absence of steroid but cooperates with a regulatory element upstream between –892 and –780 in the presence of steroid to activate transcription. Oviduct-specific proteins were found to bind to this silencer. Other silencers were found in the chicken lysozyme gene (Arnold et al., 1996), and platelet-derived growth factor A-chain (Liu et al., 1996).

Position-dependent repressors have been identified in many loci. Some repressors function by prematurely terminating RNA elongation as in the *c-myc* (Bentley and Groudine, 1988), *c-fos* (Plet et al., 1995), and *c-myb* (Pinaud and Mirkovitch, 1998) promoters. In the ϵ -globin gene, a silencer (–278 to –258) overlaps with YY1 and GATA-1 consensus binding sites (Peters et al., 1993). It was suggested, based on mutational studies, that a repressor protein functions by competing with YY1 and GATA-1 to repress transcription. Other repressor proteins can bind to silencer elements and cause DNA bending, resulting in steric hindrance of positive regulatory elements (Natesan and Gilman, 1993). This chapter describes studies attempting to define the region immediately downstream of the SS245 enhancer as a silencer element.

4.2. THE 116 bp IMMEDIATELY DOWNSTREAM OF THE SS245 ENHANCER SILENCE THE ENHANCER ACTIVITY

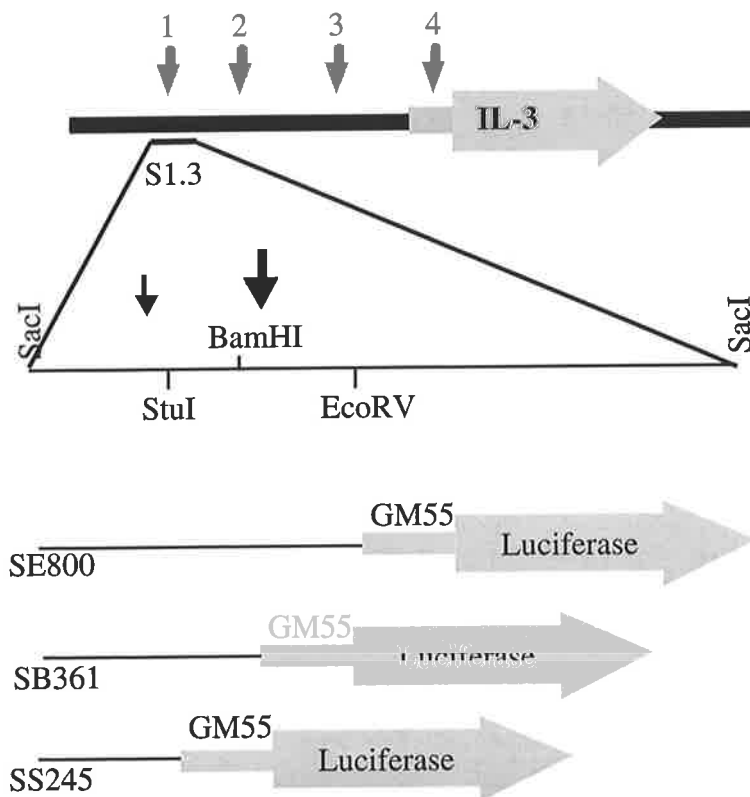
During the course of defining the SS245 enhancer, it was observed that when more sequences downstream of the enhancer were included and cloned upstream of the GM55

promoter in pXPG-GM55 in the normal orientation, the activity of the enhancer was eliminated (figure 4.1). An 800 bp *Ecl*136II/*Eco*RV (SE800) fragment, which extends the SS245 enhancer by 550 bp and encompasses the -4.1 kb DH site, was cloned in the *Sma*I site of pXPG-GM55. The 361 bp *Sac*I/*Bam*HI (SB361) fragment, which extends the SS245 enhancer by an additional 116 bp was sub-cloned from pIL3H-S1.3 as a *Bam*HI fragment and cloned into the *Bam*HI site of pXPG-GM55. The pXPG-GM55, SS245, SB361, and SE800 constructs were transiently transfected into Jurkat cells and were either left unstimulated or stimulated with PMA and *CaI*. All of the enhancer activity of the SS245 construct above the GM55 promoter level was abolished in the SE800 and the SB361 constructs by the introduction of sequences downstream of the SS245 enhancer. This loss of activity resulting from the inclusion of additional sequences may be due to one of three possibilities. The first possibility is that a silencer element exist downstream of the SS245 enhancer. This potential silencer element, based on this experiment, is contained within the 116 bp immediately downstream of the SS245 enhancer. The second possibility is that this apparent repression is due to the use of a minimum promoter system that is composed of only a TATA box and a composite NFAT/AP-1/Ets binding site. Consequently, the minimum promoter may be sensitive to distant effects when enhancers are placed further upstream. When a positive element is placed immediately upstream of a minimum promoter, all transcription factors synergise with the basal transcription machinery to increase the transcription rate of the linked reporter gene. However, when the same positive element is placed further upstream, the transcription factors that bind to this element may not be in the right context to synergise with the basal transcription machinery in the same fashion. This would occur if, for example, the minimum promoter system lacks binding sites for critical factors that allow the bridging of the promoter and the enhancer from a distance and thereby allow the

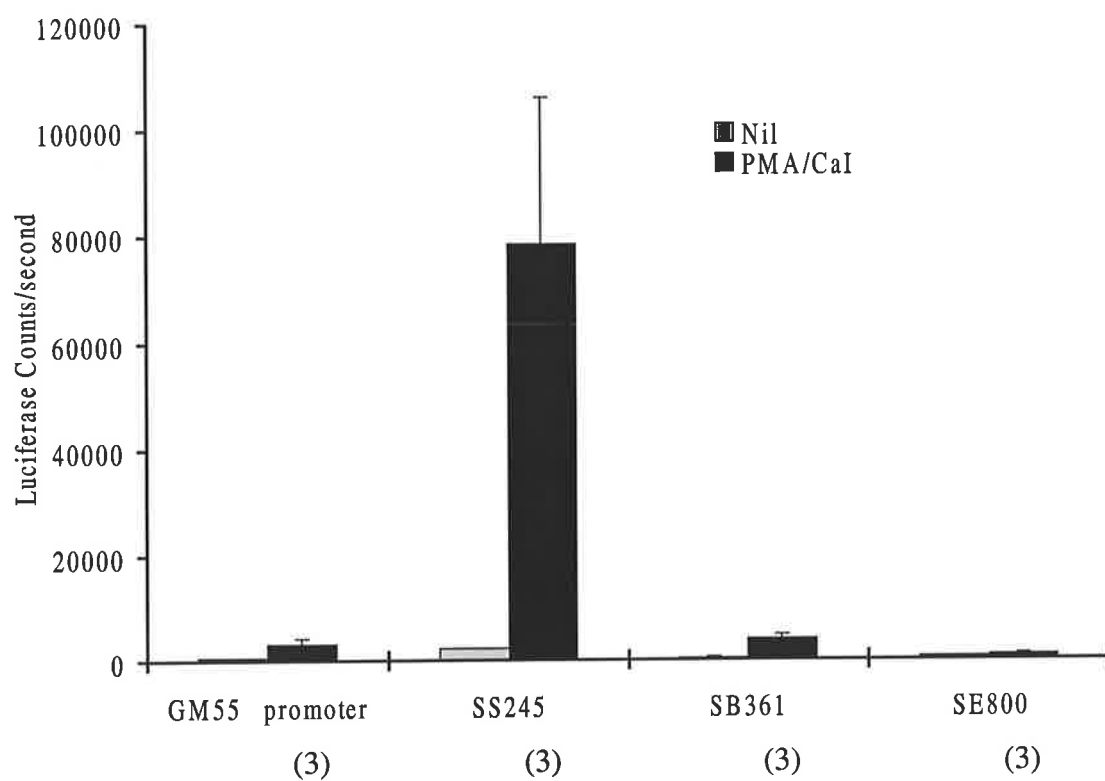
Fig. 4.1. The SS245 enhancer activity is abolished by downstream sequences.

(A) Schematic diagrams of constructs of the SS245 enhancer without or with additional downstream sequences. The 800 bp SacI/ EcoRV (SE800), 361 SacI/BamHI (SB361), and the SS245 enhancer regions were cloned upstream of GM55 promoter in a luciferase reporter pXPG-GM55 plasmid were transfected into Jurkat T cells. (B) Mean of the luciferase activity of the above constructs without (Nil) or with PMA/CaI stimulation. The numbers of transfections are indicated within parenthesis and the standard errors of the mean are represented by error bars.

A



B



effective increase in transcription rate. Hence, the apparent abolishment of enhancer activity could be due to distance rather than actual repression. The third possibility is that the SS245 element may be functioning as a promoter rather than an enhancer, and the additional sequences present in the SB361 fragment may interfere with translation rather than transcription of the luciferase gene. This would occur if, for example, the ATG site at position 278 was employed as an efficient initiation codon because the in frame stop codon at position 287 would then suppress translation of the luciferase gene. This possibility should not be discounted because the sequence AGGCATG that encompasses the ATG at 278 does indeed form part of the translation initiation consensus sequence (Kozak, 1984).

To resolve this issues concerning the differences between the SS245 and SB361 constructs, this chapter describes additional experiments that address whether there is a repressor or not.

4.3 DNASE I FOOTPRINT OF THE 116 bp STUI/BAMHI FRAGMENT

To assess the mechanism by which the 116 bp StuI/BamHI sequence repressed enhancer function, it was DNase I footprinted for protein binding activity (figure 4.2). The footprinting was performed using nuclear extracts from unstimulated and PMA/Cal stimulated Jurkat T cells as described earlier. There was one clear footprint immediately downstream of the StuI site marked by a bracket and three not so obvious sites marked with vertical lines. The footprint that is marked with a bracket appeared to bind a protein that is present in both unstimulated and stimulated extracts. However, the unstimulated nuclear extract is able to protect the DNA at 5 µg while the stimulated nuclear extract

Fig. 4.2. DNase I footprint analysis of the putative silencer region.

DNase I footprinting of the 116 bp StuI/BamHI region upstream of the IL-3 gene with nuclear extracts from unstimulated (Nil) and PMA/CaI-stimulated Jurkat T cells. 5, 10, 20, or 30 µg of protein were incubated with the P³²-labeled DNA and digested with DNase I. DNA digested with DNase I in the absence of protein (Naked DNA), and Maxam and Gilbert G and A sequencing reactions were also included. The locations of the BamHI and StuI sites and position 311 bp were indicated. The star is to indicate the end that was radiolabelled. The bracket is to indicate the definitive footprint area. Vertical lines are to indicate weaker footprint areas.

★
BamHI

311 bp →

StuI ↓

Res Enz Digest
G & A seq

Naked DNA
5μg

10μg

20μg

30μg

Naked DNA

5μg

10μg

20μg

30μg

Naked DNA

G & A seq.

Res Enz Digest

↑
NiI
↓

↑
PMA/CaI
↓

┌

was able to protect only when the protein amounts used were at 20 µg or more. This difference could, however, be due to variations in protein extraction or degradation between samples, and may not be significant. Alternatively, it could indicate that less protein is able to bind following stimulation. The other three areas of possible footprints were also more strongly protected by unstimulated rather than by stimulated Jurkat T cell nuclear extracts.

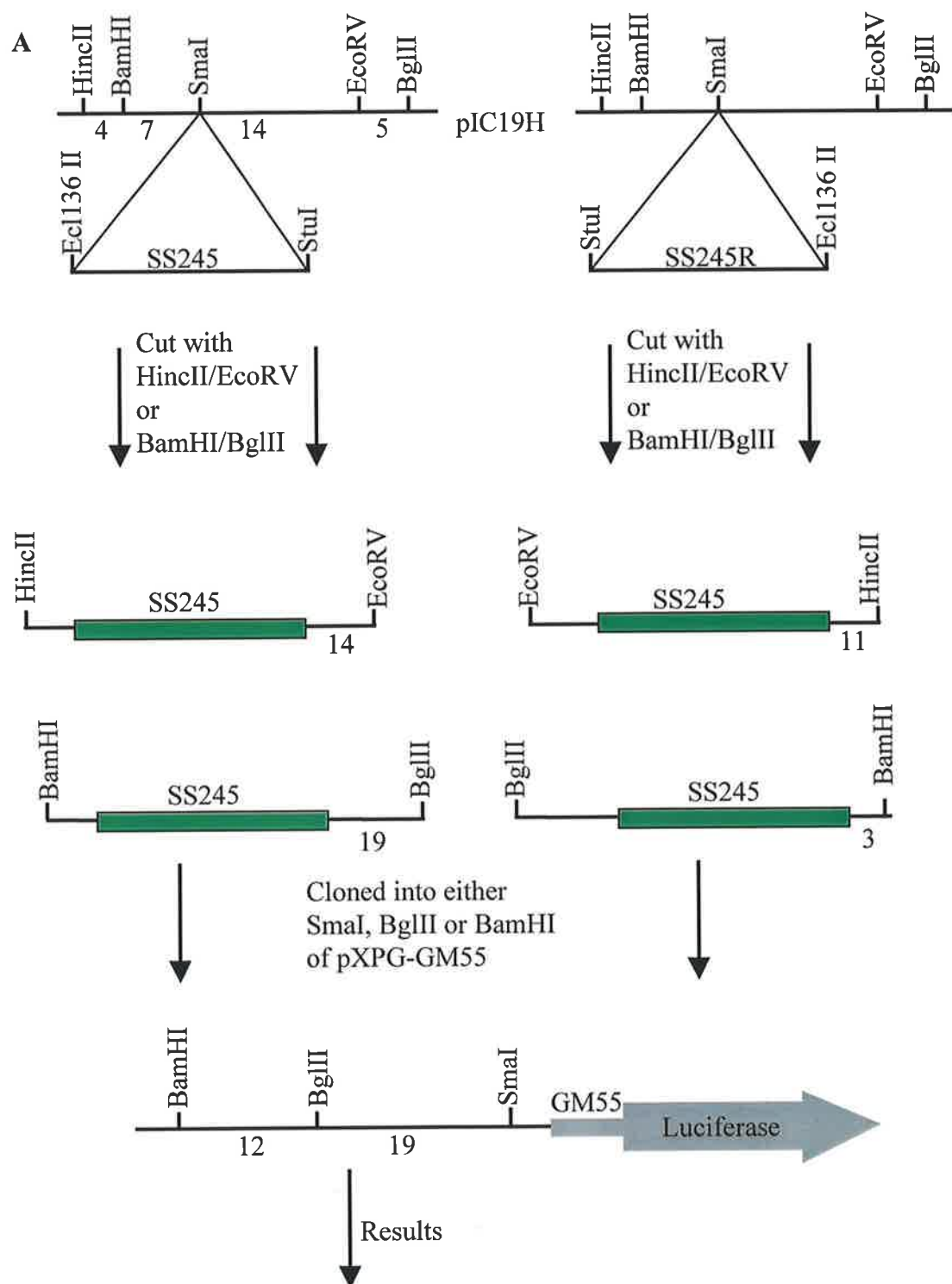
4.4. A 66 bp FRAGMENT FROM THE 5' OF THE 138 StuI/BamHI FRAGMENT ELIMINATED THE ACTIVITY OF THE SS245 ENHANCER

The SB361 clone used in section 4.2 was cloned in the BamHI site while the SS245 enhancer was cloned in the SmaI site of pXPG-GM55. Since there are extra polylinker sequences (31 bp) between the BamHI and the SmaI sites, these extra polylinker sequences may contribute to the negative effect observed. In addition, these polylinker sequences are not from the native sequence. Hence, to make the SB361 and the SS245 comparable, a PCR cloning strategy was adopted to introduce SmaI sites at 374 bp (see map) and at site 311 bp. PCR oligonucleotides 1 with either A or B (see figure 3.18) were used to amplify the region as described in the materials and methods. The two PCR products were digested with Eco136II and SmaI, which resulted in the SS374 and SS311 fragments respectively. The SS311 fragment extends the SS245 enhancer by 66 bp and contains 2 of the 4 footprinted regions described in figure 4.2. The SS374 fragment extends the SS245 enhancer by 129 bp and includes all four footprinted areas. Both of these fragments were cloned in the SmaI site of pXPG-GM55 in the normal orientation and transiently transfected into Jurkat T cells and either stimulated with PMA/CaI or left unstimulated. The SS245 construct was used as a positive control. In addition, a clone

that has 50 bp of additional poly-linker sequences added between the SS245 and the GM55 promoter was employed to control for distance effects (SS245+50). This control was constructed by re-cloning the SS245 fragment into the BamHI site of pXPG-GM55 as a BamHI/BglII fragment from pIC-SS245, which was created by cloning the SS245 fragment into the SmaI site of pIC19H (Marsh et al., 1984) (see figure 4.3). Figure 4.4 shows a transient transfection assay of these plasmids. As was the case for the SB361 fragment, the SS374 fragment had no enhancer activity. Whereas the SS245+50 construct had an activity not significantly different to the SS245 construct ($P = 0.13$), the 66 bp sequences downstream of the SS245 enhancer in the SS311 construct was able to abolish the enhancer activity. This experiment showed that the enhancer-promoter interaction was not significantly affected by distance up to 50 bp and suggested that the 66 bp downstream of the StuI site was a silencer. Nevertheless, it is a formal possibility that the 66 bp sequence in the SS311 construct could generate a much greater distance effect than the 50 bp polylinker. It also remains a possibility that transcription is initiating within the SS245 fragment, and that translation is initiating and terminating within the 66 bp sequence, as discussed above.

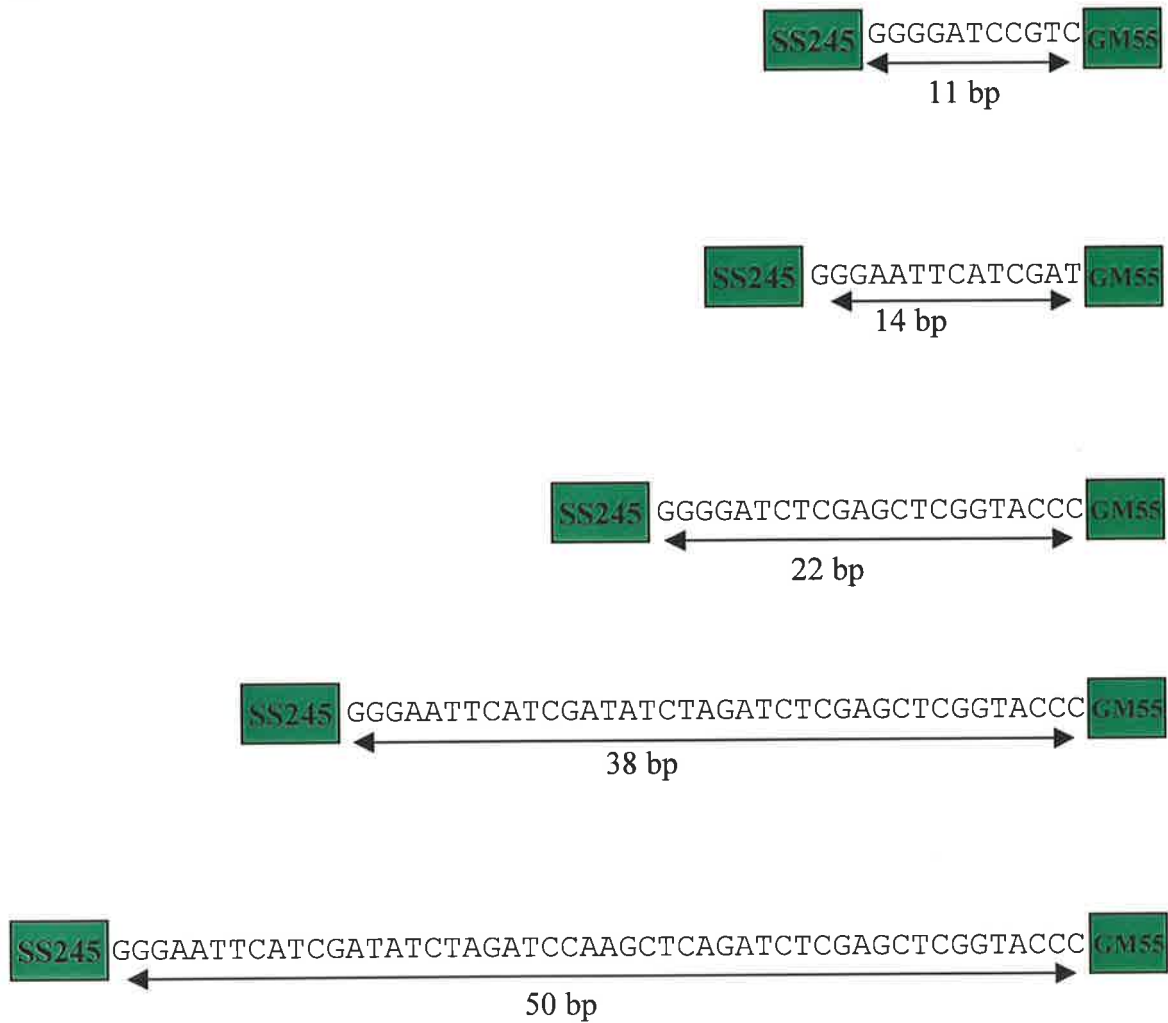
4.5 THE EFFECT OF SPACING ON ENHANCERS IN THE CONTEXT OF GM55 PROMOTER

To further study the effect of spacing between the GM55 promoter and enhancers, a well-characterised enhancer was chosen, the GM-CSF enhancer. The GM-CSF enhancer has a similar structure to the SS245 enhancer. Both enhancers encompass NFAT, Sp1, AP-1 and AML1 binding sites. Four fragments, which encompass the GM-CSF enhancer (see figure 4.5A), were cloned upstream of the GM55 promoter in pXPG-



- 1) SS245+11
- 2) SS245+14
- 3) SS245+3 +19 = SS245 + 22
- 4) SS245+3+12+19 = SS245 + 34 (not used)
- 5) SS245+19+19 = SS245 + 38
- 6) SS245+19+12+19 = SS245 + 50

B



4.3. Cloning strategy of the SS245 enhancer at different distances from the GM55 promoter.

(A) A flow chart depicting the steps taken to introduce different spacing between the SS245 enhancer and the GM55 promoter in the pXPG-GM55 plasmid. Initially the SS245 enhancer was cloned in both orientations in the *Sma*I site of pIC19H plasmid (pIC-SS245 or pIC-SS245R). Four fragments were generated by digesting these plasmids with either *Hinc*II/*Eco*RV or *Bam*HI/*Bgl*II. The two *Hinc*II/*Eco*RV fragments were cloned into the *Sma*I site, and the two *Bam*HI/*Bgl*II fragments were cloned into either *Bgl*II, or *Bam*HI sites of pXPG-GM55 plasmid to generate 6 plasmids. (B) The resulting constructs with the additional poly-linker sequences are shown.

Fig. 4.4. A 66 bp of the potential silencer is sufficient to abolish enhancer function.

(A) A schematic diagram of constructs of the SS245 enhancer with 66 or 129 additional base pairs of sequences 3' of the StuI site. In addition, the SS245 and the SS245+50 enhancer constructs are shown. (B) The activity of these plasmids in unstimulated or PMA/CaI-stimulated transiently transfected Jurkat T cells. The luciferase activity and the standard error of the mean are presented as means of the number of transfections indicated within parenthesis and error bars respectively.

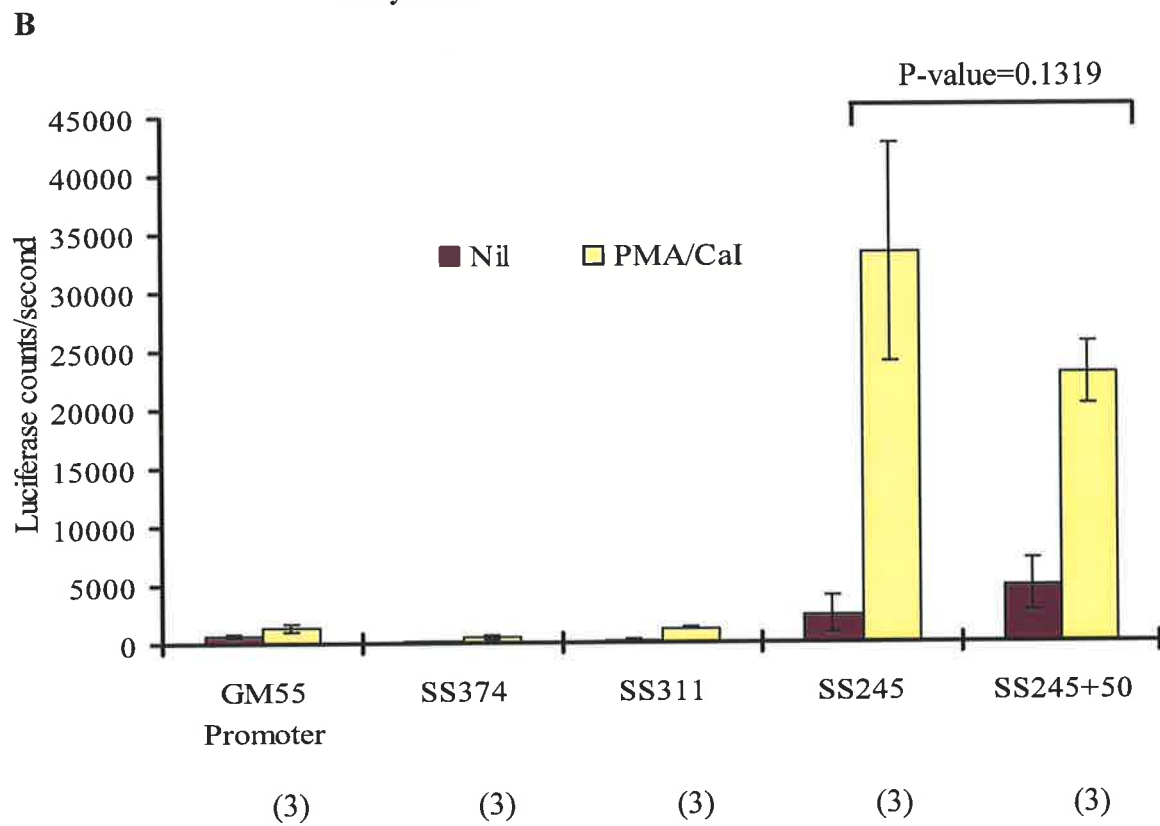
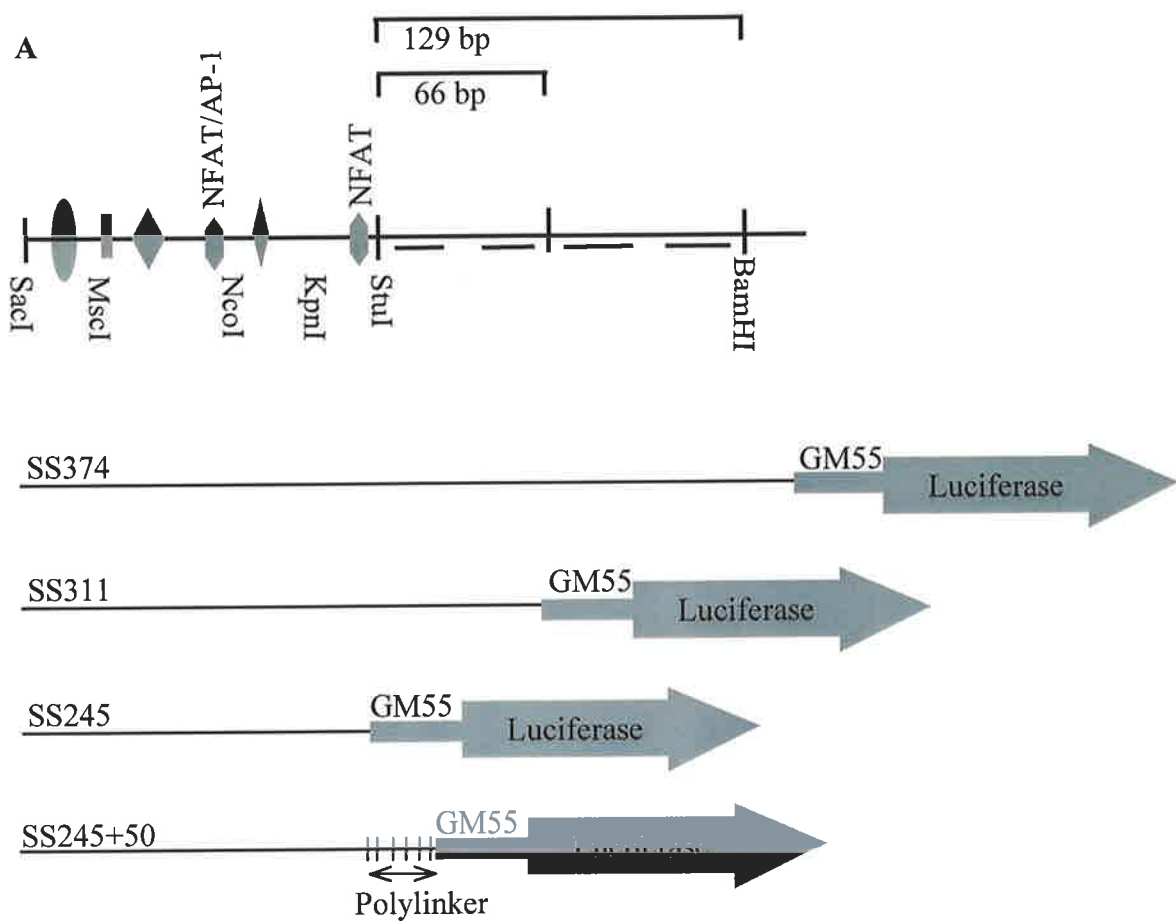
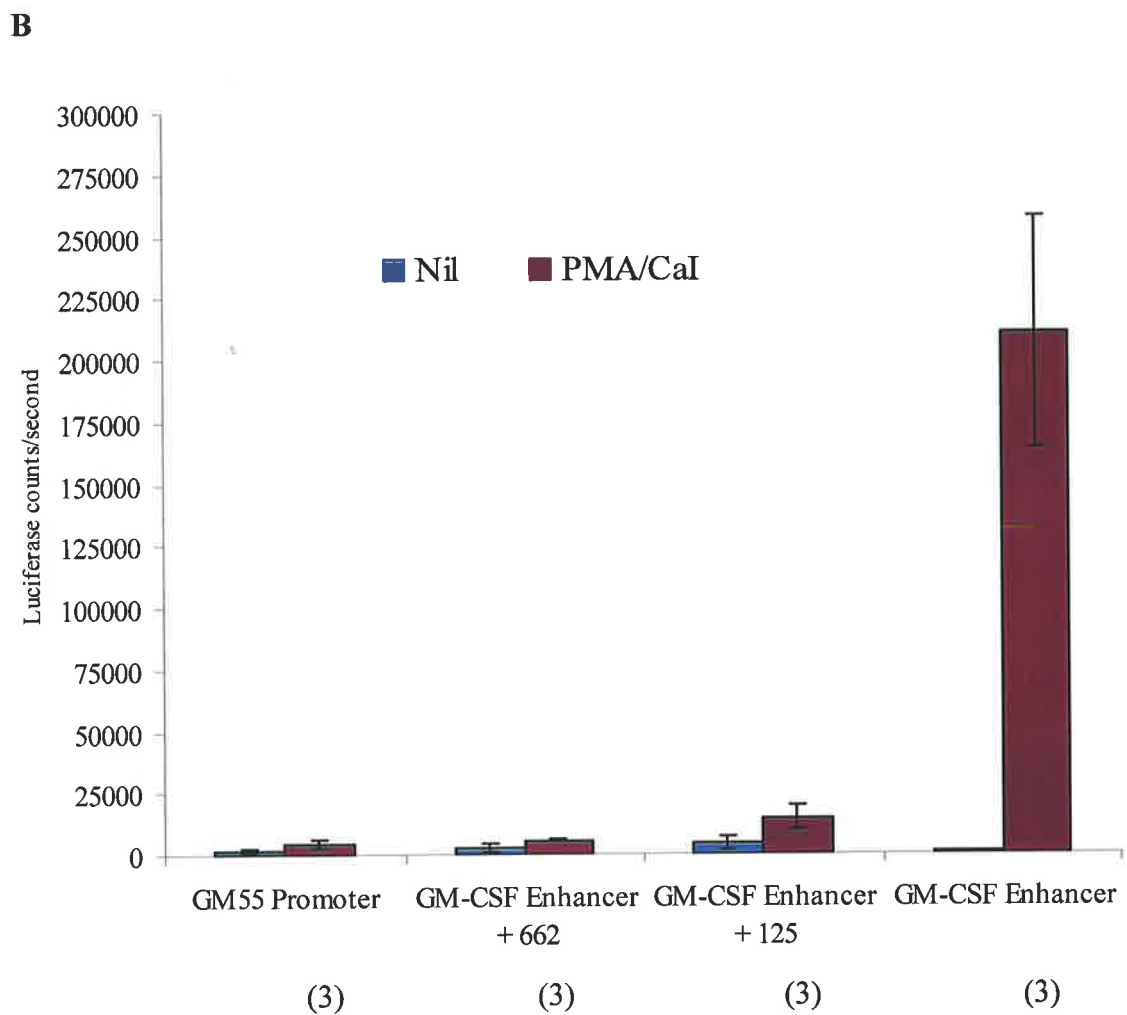
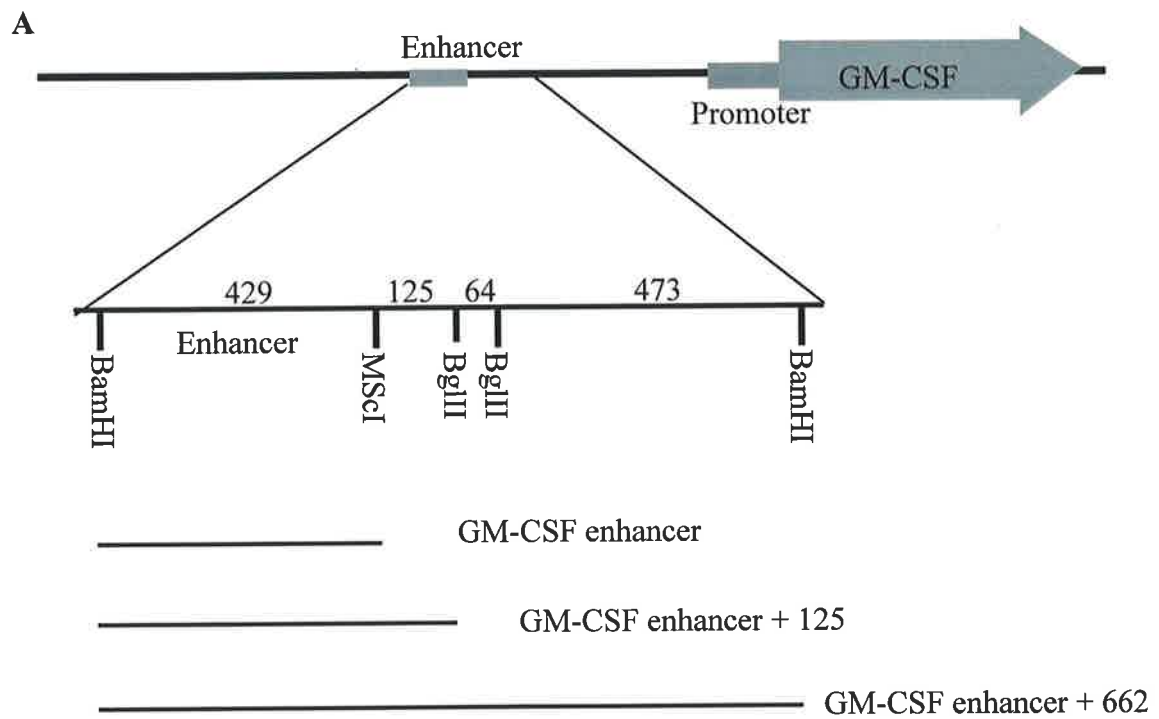


Fig. 4.5. Effect of distance on the GM-CSF enhancer.

(A) A restriction map of the GM-CSF enhancer showing the 429 bp BamHI/MscI enhancer core. Downstream restriction enzyme sites used in the cloning of constructs described in the text are indicated. (B) Luciferase activity of the different GM-CSF enhancer constructs in transiently transfected Jurkat T cells without or with PMA/CaI stimulation are shown. Activities are expressed as mean luciferase counts and the error bars are the standard error of the mean. The numbers of independent transfections stimulated by PMA/CaI are indicated within parenthesis.



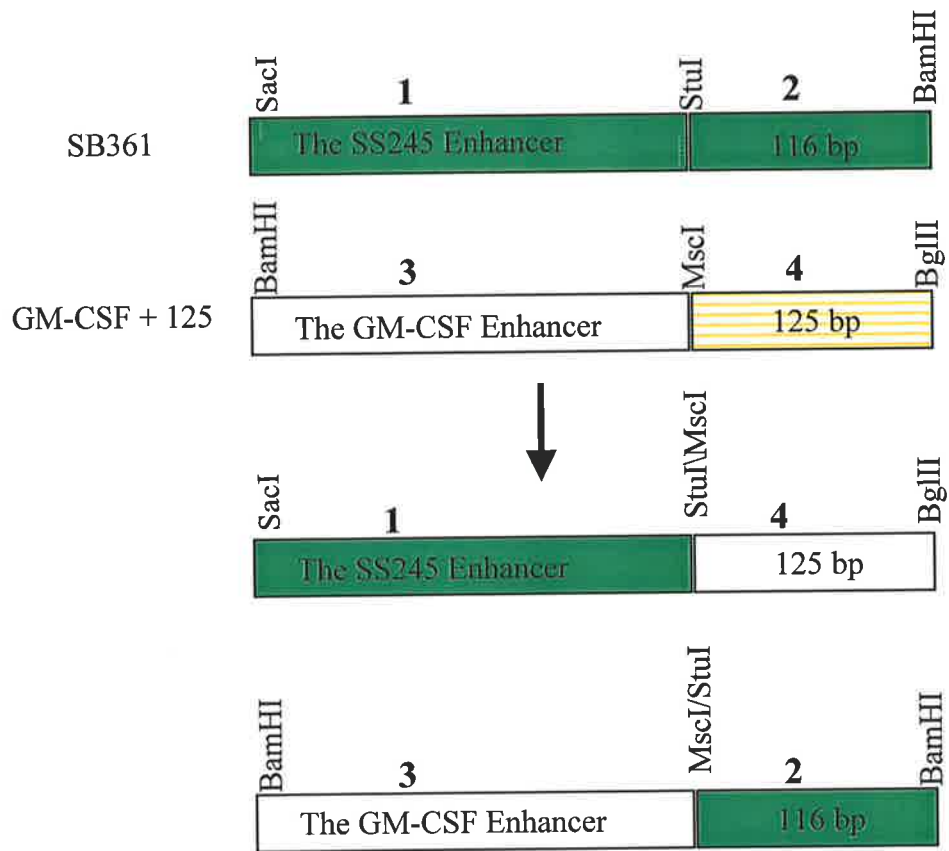
GM55. These fragments were the core of the GM-CSF (429 bp BamHI-MscI), the core+125 bp (554 bp BamHI-BglII), and the core+662 bp (1091 bp BamHI). They were cloned in the BamHI/SmaI, BamHI/BglII and BamHI sites of pXPG-GM55 respectively. These constructs, along with pXPG-GM55, were transfected into Jurkat T cells and either stimulated with PMA/CaI or left unstimulated (figure 4.5B). The addition of the 125 bp greatly reduced the enhancer activity. Whereas the GM-CSF enhancer construct was 51.9 times as active as the GM55 construct, the GM-CSF enhancer+125 bp construct was only 3.6 times as active. Furthermore, the addition of 662 bp to the core GM-CSF enhancer abolished the activity of the enhancer almost completely.

Since the GM-CSF+125 enhancer fragment was able to increase the activity of the GM55 promoter by three fold and it has a structural similarity to the SB361, which contains the core SS245 enhancer+116 bp, the 125 bp MscI-BglII fragment was removed from the GM-CSF+125 and the 116 bp StuI-BamHI IL-3 gene fragment was cloned in its place. Similarly, the 116 bp StuI- BamHI was removed from the SB361 and the 125 bp MscI-BglII was cloned in its place (figure 4.6A). The idea being tested here is that if the 116 bp StuI-BamHI is a silencer, it should abolish the GM-CSF enhancer activity in a similar fashion as it did with the SS245 enhancer. In contrast, the 125 bp MscI-BglII GM-CSF enhancer fragment should not repress the SS245 enhancer completely but is likely to exert a distance effect. These constructs were transiently transfected into Jurkat T cells and either stimulated with PMA/CaI or left unstimulated (figure 4.6B). The 116 bp StuI-BamHI fragment did not abolish the activity of the GM-CSF enhancer, but behaved like the 125 bp GM-CSF enhancer-flanking fragment, because the GM-CSF+116 construct had an activity similar to the GM-CSF+125 construct. However, the 125 bp MscI-BglII fragment abolished the SS245 enhancer activity. This experiment

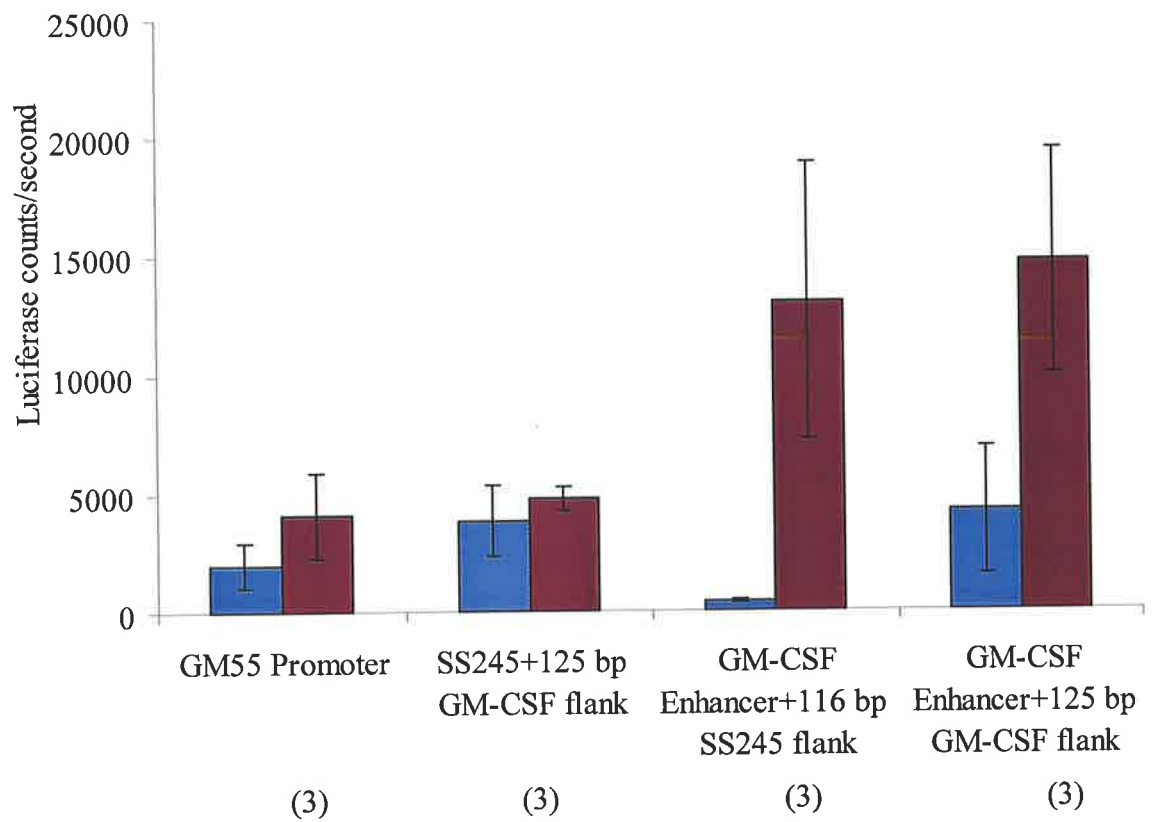
Fig. 4.6. Effect of the putative silencer element on the GM-CSF enhancer.

(A) A schematic diagram showing the putative silencer (116 bp *StuI/BamHI*) downstream of the SS245 enhancer, and the GM-CSF enhancer with its 3'-flanking 125 bp *MscI/BglII* fragment. Shown below the native fragments are two rearranged DNA segments constructed by swapping the sequences that flank the two enhancers. (B) The unstimulated and PMA/CaI-stimulated luciferase activity of pXPG-GM55 constructs containing the above DNA segments in transiently transfected Jurkat T cells. The activities are expressed as the mean of a number of independent transfections. The standard errors of the means are represented by error bars. The numbers of stimulated transfections are indicated within parenthesis.

A



B



indicated that the 116 bp *Stu*I-*Bam*HI fragment was not able to silence the GM-CSF enhancer, but most likely did exert a distance effect.

To further study the effects of inserting short poly linker sequences, 11, 14, 22, 38, and 50 bp of additional polylinker sequences were placed between the SS245 enhancer and the GM55 promoter. As shown in figure 4.3 and 4.7, there was no effect of spacing up to 50 bp on the enhancer function. There was no significant difference between the activities of the different enhancer constructs.

4.6 THE EFFECT OF THE 66 bp ELEMENT ON THE SV40 ENHANCER

Since the 66 bp *Stu*I/*Sma*I fragment (in SS311) was able to suppress the SS245 enhancer, as described earlier (figure 4.4), it was tested in the presence of an unrelated enhancer. The 66 bp *Stu*I/*Sma*I fragment was cloned between the SV40 enhancer and the GM55 promoter in both orientations in the *Sma*I site of pXPG-GM55-SV40E. In addition, to control for distance effect, a 72 bp *Hae*III fragment from ϕ X174 viral DNA was cloned in the *Sma*I site between the SV40 enhancer and the GM55 promoter in pXPG-GM55-SV40E. The ϕ X174 spacer DNA reduced the activity of the SV40 enhancer by about 72.6% (figure 4.8). However, even though the 66 bp *Stu*I/*Sma*I is smaller than the spacer control, it reduced the activity of the SV40 enhancer in both orientations by 87.4% to 91.1%, and this further reduction in activity was statistically significant ($P=0.0229$ for normal orientation and $P=0.0205$ for the reverse orientation by student one tail and equal variance t-test).

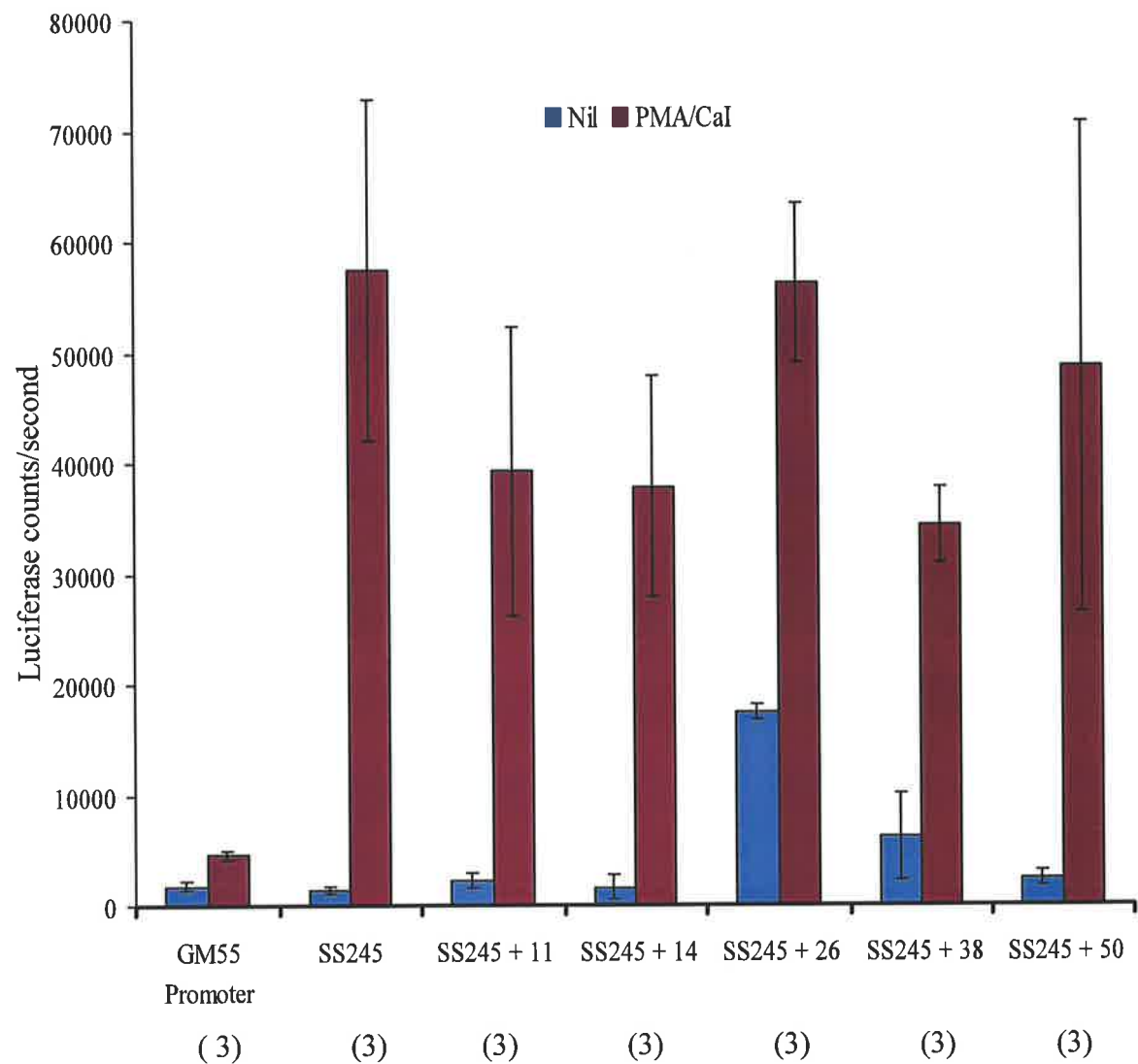


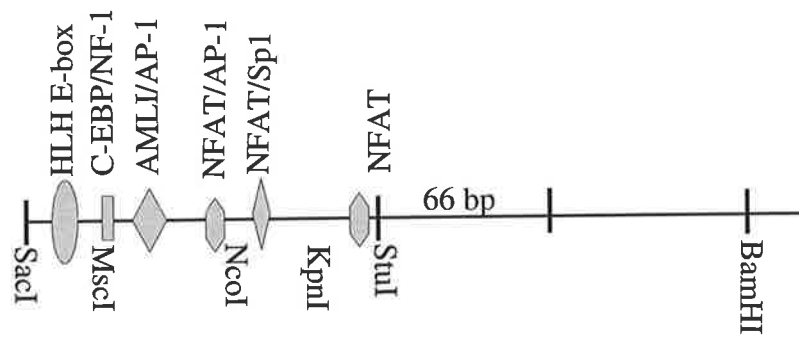
Fig 4.7. Effect of small distances on the SS245 enhancer activity.

Plasmids generated as described in figure 4.3, were transiently transfected into Jurkat T cells and either left unstimulated or stimulated with PMA/CaI. Activities are expressed as means and the standard errors as error bars. The numbers of independent PMA/CaI-stimulated transfections are indicated within parenthesis.

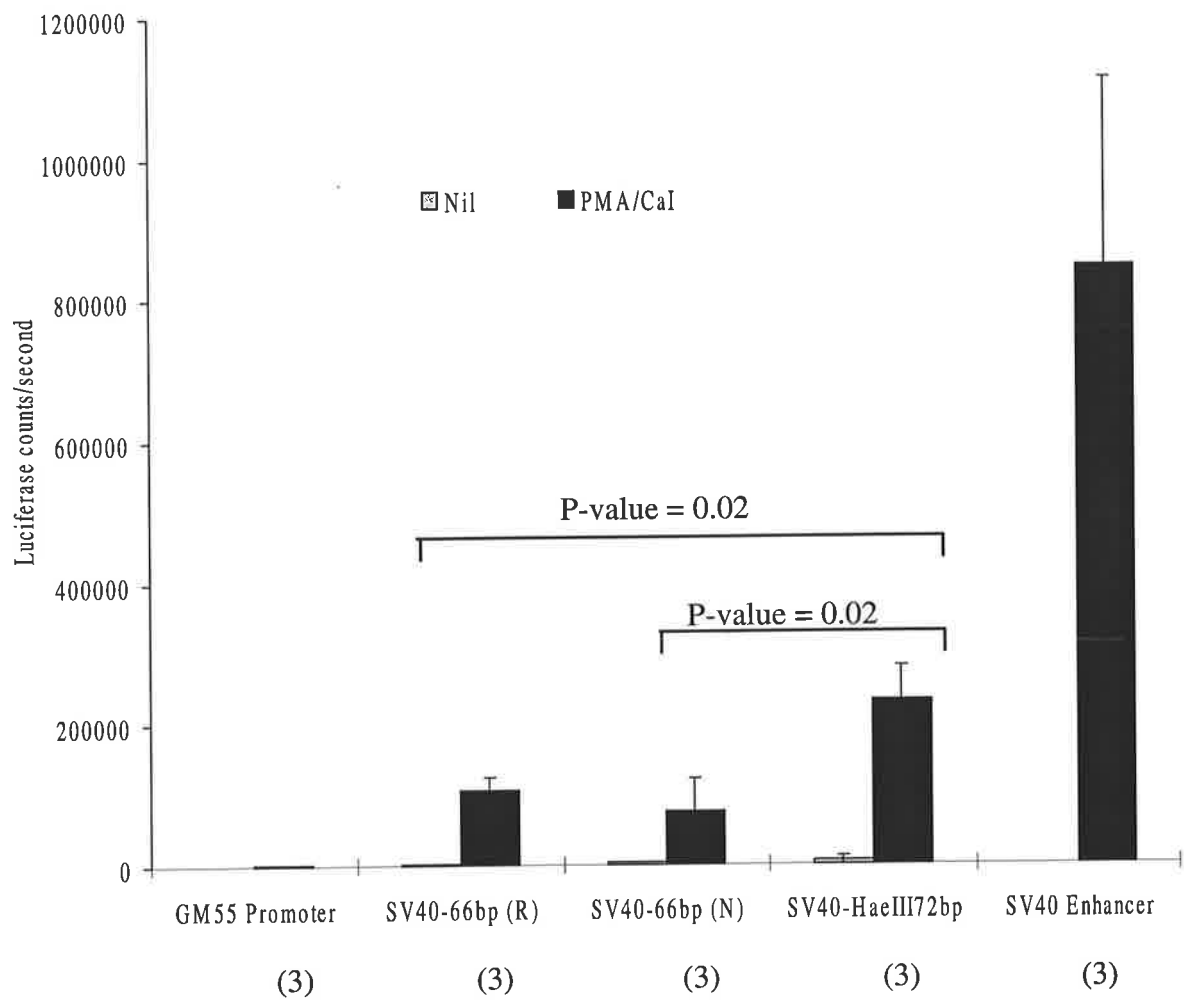
Fig. 4.8. Effect of the putative 66 bp silencer fragment on the SV40 enhancer in both orientations.

The SS66 fragment was cloned in both orientations between the GM55 promoter and the SV40 enhancer as depicted in A. As a spacing control, a 72 bp HaeIII ϕ X174 fragment was also cloned between the GM55 and SV40 enhancer. These constructs, along with pXPG-GM55-SV40E and pXPG-GM55, were transiently transfected into Jurkat T cells that then were either left unstimulated or PMA/CaI-stimulated. The activities are expressed as means and standard errors as error bars. The numbers of stimulated independent transfections are indicated within parenthesis.

A



B



4.7 THE 116 bp StuI/BamHI FRAGMENT DOES NOT ELIMINATE THE SS245 ENHANCER ACTIVITY IN THE CONTEXT OF THE FULL LENGTH IL-3 PROMOTER.

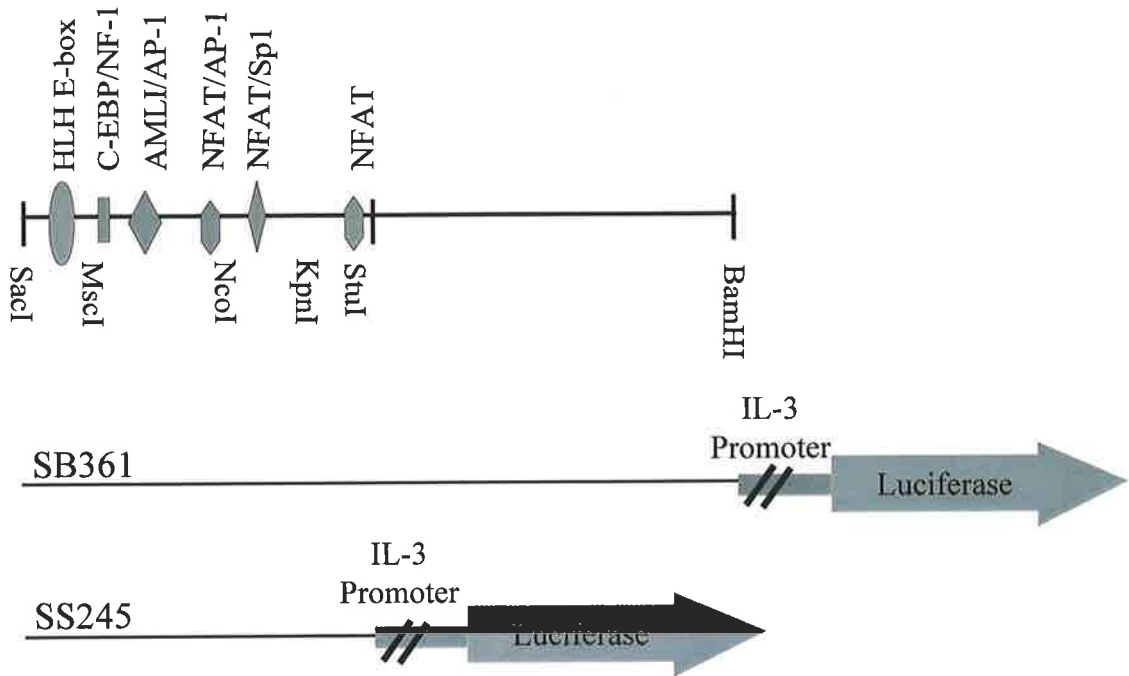
A full-length promoter several hundred bp in length is likely to be less sensitive to the effect of distances that separate it from an enhancer. Furthermore, enhancers in their natural context activate promoters from large distances. Therefore, to test the potential silencer in a more natural context, the 116 bp StuI/BamHI DNA was tested for silencer activity upstream of a full length (-559 to +50) IL-3 promoter. For that purpose, two clones were generated. The SS245 enhancer and the SB361 fragment containing the SS245 and the 116 bp element were cloned in the SmaI and BamHI cells respectively upstream of the IL-3 promoter in pXPG-IL3H and transiently transfected into Jurkat T cells and stimulated with PMA/CaI. The SV40 enhancer and the -14 kb IL-3 enhancer (B1.2) were cloned into the BamHI and BglII sites of pXPG-IL3H as controls. Figure 4.9 shows that the activity of the SS245 enhancer was not affected by the addition of the 116 bp StuI/BamHI fragment. Both plasmids activated a full-length promoter in similar manner by about two fold similar to the B1.2 IL-3 enhancer, which activated the IL-3 promoter by about 2.5 fold. The SV40 enhancer activated the IL-3 promoter by about 4 fold. This experiment suggested that the 116 bp is not a repressor in the normal context of the IL-3 locus.

Similarly, to test the effect of the StuI/BamHI 116 bp fragment on a heterologous full-length promoter, the SS245 and the SB361 fragments were cloned into the Ecl136II (SacI) and BamHI sites respectively of the pXPG-TK229 plasmid. Along with these constructs the pXPG-TK229 and pXPG-TK229-SV40E constructs were transfected in

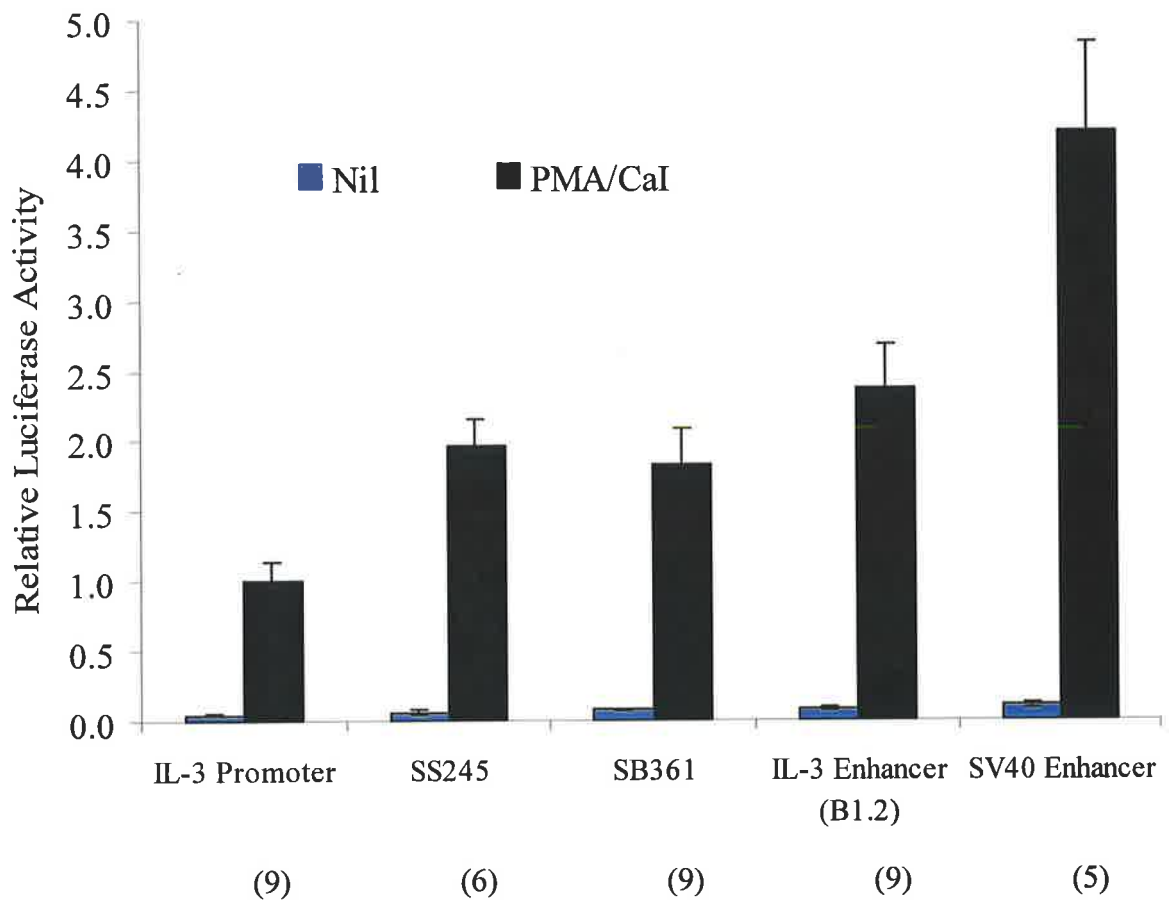
Fig. 4.9. Testing the putative silencer in the context of full length IL-3 promoter.

The SB361 fragment containing the SS245 enhancer and the putative SB116 silencer were cloned upstream of the 611 bp IL-3 full-length promoter as shown in A. Panel B shows the activity of these constructs along with the SV40 enhancer, the -14 kb IL-3 enhancer, and the SS245 enhancer constructs compared to the IL-3 promoter alone. These constructs were transiently transfected into Jurkat T cells and were either left unstimulated or PMA/CaI-stimulated. The activities are expressed as means relative to the mean of the stimulated IL-3 promoter alone construct, which was assigned a value of one. Error bars represented the standard error of the means. The numbers within parenthesis are the numbers of stimulated independent transfections.

A



B



Jurkat T cells and stimulated with PMA/CaI (figure 4.10). In contrast to the IL-3 promoter results, the SB116 fragment was able to abolish the SS245 enhancer activity in Jurkat T cells to the promoter alone level in the TK229 construct. Furthermore, the constitutive activity of the TK promoter was also reduced in the SB361 construct.

4.8 DISCUSSION

The work described in this chapter attempted to resolve whether the abolishment of enhancer function observed when the SS245 enhancer included more sequences downstream in the context of a minimum GM-CSF promoter was due to the presence of a silencer or was due to a distance effect. It was established that the GM55 promoter is sensitive to the distance that separates it from any positive regulatory element when placed upstream of it. The distance effect was observed when the space between the SS245 enhancer and the GM55 promoter was at least 72 bp, since distances of 50 bp or less did not seem to affect the SS245 enhancer significantly. This was shown with the 72 bp HaeIII fragment from ϕ X174 viral DNA when placed between the GM55 promoter and the SV40 enhancer. This distance effect was also observed when sequences, downstream of the GM-CSF enhancer, were included in the GM-CSF enhancer constructs upstream of GM55 promoter. One could argue that these additional sequences from downstream of GM-CSF enhancer also contain a silencer since the 125 bp immediately downstream of the GM-CSF enhancer reduced the activity of the GM-CSF enhancer by 95% but retained a 3-fold induction over the GM55 promoter alone. However, when this 125 bp was placed between the SS245 enhancer and the GM55 promoter, the enhancer activity was reduced by 100%, to the promoter alone level. The SB116 fragment had a similar effect on the GM-CSF enhancer as the 125 bp fragment. In other words, both of

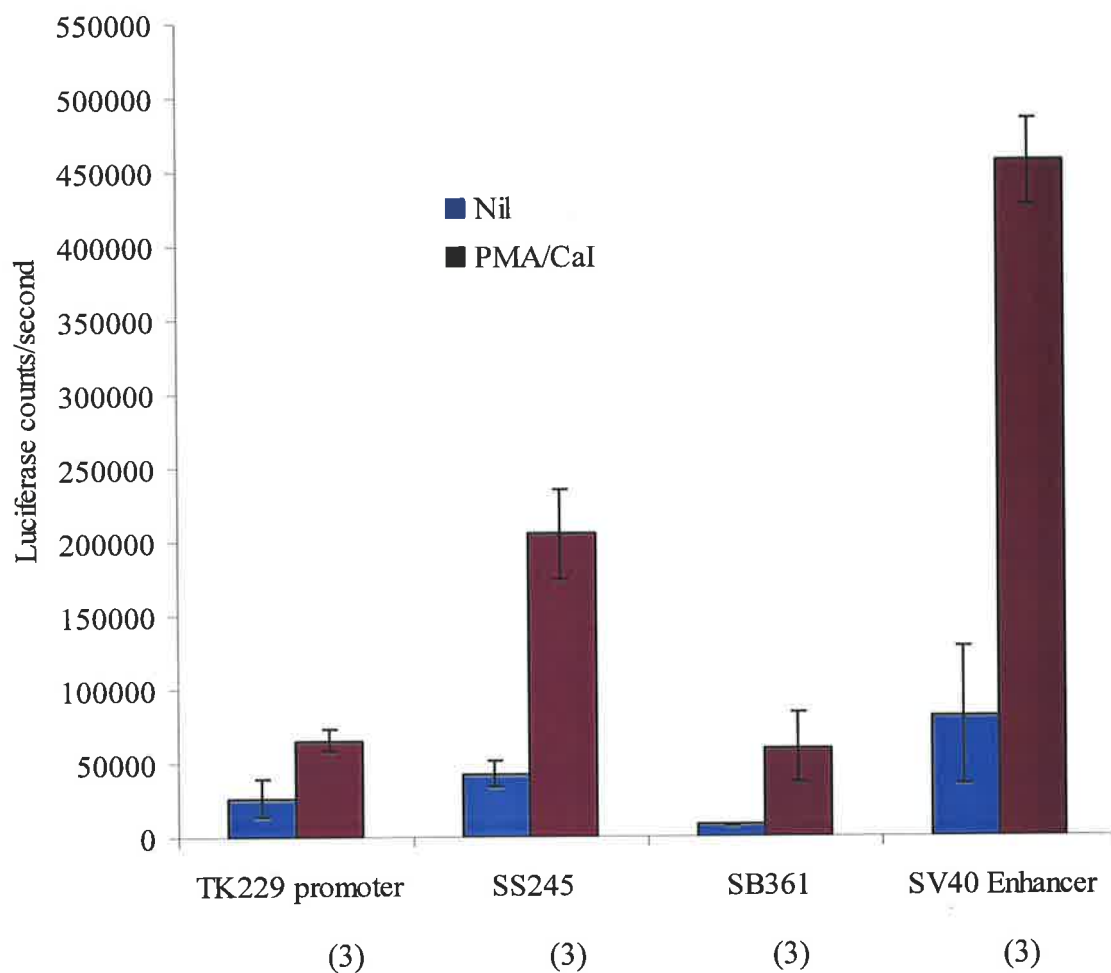


Fig. 4.10. Effect of the putative silencer on the full length TK229 promoter.

The SB361 fragment was cloned upstream of the TK229 promoter in pXPG-TK229 and assayed in parallel with constructs containing the SS245 or SV40 enhancers cloned upstream of the TK229 promoter. Plasmids were transiently transfected into Jurkat T cells that were then either left either unstimulated or PMA/CaI-stimulated. The numbers of stimulated independent transfections are indicated within parenthesis. The luciferase activities are expressed as mean of the activities of the independent transfections. The error bars represent the standard error of the means.

the 125 bp GM-CSF enhancer-flanking fragment and the SB116 fragments affected each of the GM-CSF and the SS245 enhancer in similar ways. Accordingly, it was not possible to tell from this experiment whether the effect observed was due to a distance or silencer effect.

The evidence that supported the presence of a silencer element in the SB116 fragment was obtained from using only 66 bp from the 5' end of the SB116. When this 66 bp fragment was placed in either orientation between the GM55 promoter and the SV40 enhancer, it was able to reduce the enhancer activity by more than the reduction observed with the 72 bp spacer control. This experiment supported the hypothesis that there is a silencer that seems to repress the enhancer activity when cloned in both orientations.

Further evidence was obtained from the DNase I footprinting experiment where the 66 bp contained an area that seemed to be protected from DNase I digestion indicating the presence of protein(s) that binds within this area. Although binding activity was shown within this region in the footprinting study, this was not followed up by EMSA to identify the proteins that may bind there. Furthermore, the sequence did not give any clue of the identity of the proteins that may bind. Mutagenesis studies should be carried out to first abolish the binding to this region and second to test these mutants for their ability at least partially to restore enhancer function to a level expected with an unrelated spacer such as the 72 bp ϕ X174 fragment. Moreover, it was not clear from the footprint whether the binding was constitutive or whether it was regulated, because some reduction in binding was seen in the nuclear extract prepared from stimulated cells. This should become clearer if EMSAs are employed in future studies of this element. Since

different proteins have different mobility, EMSAs give a better indication of whether the same proteins bind from unstimulated or stimulated nuclear extracts.

Since the GM55 promoter is so sensitive to distance effects, it was thought that using a full-length promoter where the distance is less of an issue would be more appropriate. Hence, two promoters were used: a 611 bp IL-3 promoter and a 229 bp TK promoter. However, the SS245 enhancer behaved differently in these two promoters in Jurkat T cells. The IL-3 promoter was not affected by the presence of the SB116 fragment present between the promoter and the SS245 enhancer. However, the TK229 promoter was affected and the activity was reduced to the promoter alone level. This can potentially be explained as follows. The SS245 enhancer is a natural enhancer for the IL-3 promoter. Moreover, Jurkat T cells express IL-3 when stimulated and both the IL-3 promoter and the SS245 enhancer are active in these cells. Hence both of the promoter and the SS245 enhancer cooperate to overcome the repression. In the case of the TK229 promoter, cooperation with the SS245 enhancer to overcome the repression may not have been possible. Hence, this could be evidence for specificity in enhancer-promoter communications.

Future studies will also need to determine whether the repressor effect is acting at the level of translation rather than transcription. The SS245 enhancer encompasses several transcription factor binding sites, has an A/T-rich region at position 137 (ATTAAAT) that might function as a TATA element, and might support promoter as well as enhancer activity. In the SS245 construct transcription might initiate within the SS245 fragment without influencing the use of the luciferase gene ATG as the predominant translation initiation site. In the case of the SS311 and SB361 constructs, transcription from within

the SS245 region might be followed by translation initiation and termination within the SS66 represor-like region, and aborted luciferase protein synthesis.

All the experiments described in this chapter were carried out in Jurkat T cells. However, because activated Jurkat cells make IL-3, any repressors in the IL-3 locus might be expected to be switched off in the natural setting in this context. It was not possible to study the SB361 fragment (containing the SS245 enhancer and the SB116 region) in other cell lines that do not make IL-3 because the enhancer was not active in these cells. However, because the GM55 promoter is active in a wider range of cells than the IL-3 gene (Bert et al., 2000a), the SB116 effect on the SV40 enhancer in the context of the GM55 promoter could have been studied in a variety of cell lines. Nevertheless, because of the sensitivity of the GM55 promoter to distance effect these kinds of studies were not undertaken.

Future studies of the potential repressor region in other cell lines should be carried out, but in the context of the TK229 promoter, or even larger TK promoter fragments. The SB116 or the SS66 fragment should be placed between the SV40 enhancer and the TK229 promoter and tested in cell lines such as KG1a, K562, and Raji where both the SV40 enhancer and the TK229 promoter are active. The IL-3 locus is inactive in cell lines such as K562 and Raji, as is shown by the lack of upstream DNase I hypersensitive sites. The lack of DH sites may suggest that the potential silencer is not important in these cell lines. However, there would still be an advantage to studying the silencer in the context of the TK229 promoter and the SV40 enhancer so as to study whether the silencing effect is a generalised one or T cell-specific. Hence, one may initially want to study the binding activity of the silencer to proteins present in nuclear extracts from

these cell lines and to the same region as in Jurkat cells. Methylation interference assays could be used to more precisely map the bases of the binding of the potential repressor proteins

The *in vitro* approaches employed above to study potential silencers have not been very informative. Furthermore, positive and negative regulatory elements normally interact with each other *in vivo* in the context of the chromatin and over large distances. In addition, transient transfection assays typically study these elements when they are in close proximity to each other. Hence, the observed effects may not reflect what happens *in vivo*. Future studies of the potential silencer should therefore be carried out *in vivo*. Initially, *in vivo* binding may be carried out to establish whether there is any *in vivo* binding of proteins to this region and whether the observed *in vitro* binding is an artefact of the *in vitro* binding assays. In addition, *in vivo* functional assays could be carried out by knock out of the silencer element from its natural location by homologous recombination in both cell lines and mice. Transgenic animals could also be generated with constructs that carry deletion or mutations in the silencer regions.

The region of the IL-3 locus containing the SS245 enhancer and the -4.1 DH site may be important for opening of the chromatin. The -4.1 DH site is the most prominent site of the locus. It is present in all of the T cell lines that were examined and it also present in the primitive myeloid cell line KG1a. However, this site is not always linked to IL-3 gene expression because not all of the T cell lines make IL-3, and KG1a does not make IL-3 either. IL-3 is normally produced in T cells, mast cells, basophils, and eosinophils but not in other differentiated haemopoietic cells such as B cells or monocytes. It is likely that the -4.1 DH site appears early in development because KG1a cells are

primitive CD34+ cells. The presence of a silencer in this region could provide a mechanism to suppress IL-3 expression during early stages of development when the -4.1 DH site appears. The putative silencer separates both the SS245 enhancer and the -14 kb IL-3 enhancer from the promoter. One can hypothesise that this silencer plays an important role in regulating or preventing IL-3 activation in undifferentiated cells that have their chromatin remodelled and poised for transcription.

Negative regulatory elements (NRE) are as important as positive elements in gene regulation. It was mentioned in the introduction section of this chapter that NREs have been found in many loci. NREs may play a role in the tissue-specific regulation of genes such as the Hypoxanthine phosphoribosyltransferase (HPRT) gene (Rincon-Limas et al., 1995). HPRT is expressed constitutively at high levels in the brain. Whereas its expression in other tissues is low, an element was identified that suppressed expression of the HPRT gene in non-neuronal tissues. Moreover, proteins that bind to this element, although unidentified, are different in neuronal and non-neuronal tissues. The human ϵ -globin gene silencer is another example of silencers. It suppresses the ϵ -globin gene expression by 10-fold in non-erythroid cells in transient transfection assays (Gumucio et al., 1993). This silencer was found to contain (a) CCACC, (b) an overlapping GATA and YY1 and (c) a GATA motif (Raich et al., 1995). Mutating any of these motifs results in an increased level of expression of the ϵ -globin gene in the adult erythroid cells indicating a role for the silencer in the developmental regulation of gene expression. YY1 is also shown to be involved in the silencer element from the avian embryonic ρ -globin gene (Wandersee et al., 1996). The human CD4 gene silencer located within the first intron of the CD4 gene has been shown to silence the CD4 gene expression in single positive CD8 cells (reviewed in Ellmeier, 1999). Inclusion of the CD4 silencer

downstream of the CD4 enhancer and promoter using the human CD2 gene as a reporter in transgenic mice ensured the expression of the CD2 in CD4+ cells only. However, when this silencer was deleted, the CD2 gene was expressed in double negative and CD8+ mature cells (Sawada et al., 1994). All of these studies indicate that silencers play an important role in the tissue specificity and timing of gene expression. Hence, the SB116 may play a similar role in IL-3 expression.

As discussed earlier in the introduction of this chapter, the PAI-2 (Antalis et al., 1996), and ovalbumin (Haecker et al., 1995) genes contain silencers that contain the sequences CTCTCTAGAGAG and TCTCTCCNA respectively. The proteins that bind to these sequences have not been identified. Another silencer was found in the platelet-derived growth factor A-chain gene, which contains the sequence GGGGAGGGGG that is shown to be necessary for silencer function (Liu et al., 1996). CTCF protein is shown to block enhancer activity in many loci such as insulin-like growth factor 2 (Igf2) and H19 (Bell and Felsenfeld, 2000), β -globin (Chung et al., 1997), T cell receptor (Zhong and Krangel, 1997). The consensus sequence for CTCF was determined as CCGCGNGGNGGCAG (Chung et al., 1997). The sequence present in the SS66 element was compared to the sequences of well-characterised silencers as described above and summarised in figure 4.11. The alignment of these sequences showed poor matches with the footprinted areas in the SS66 region. However, the sequences CTCTCTAGAGAG and TCTCTCCNA from the PAI-2 and the ovalbumin seem to be the closest to the lower strand of the footprinted sequence, CCTTCCCATCACTC, which is purine rich. In this study no EMSA was done to determine whether the same proteins bind to these sequences or whether they compete binding to the SS66 region. No YY-1 or GATA-like sequences was identified in the SS66 region. Although, the consensus-binding site for

A

SS66 region

AGGCCTGTGGGGGT**GAGTGATGGGAAGAAGGAGGCA**
 StuI

TGTACCTCTAGTT**GGACTGGACCCT**GAGGGCCcGGg
 SmaI

B

SS66 proximal Foot-printed area

GAGTGATGGGAAGG
 CTCACTACCCTTCC

ε-globin

GGTGG
 CCACC

PAI-2

CTCTCTAGAGAG
 GAGAGATCTCTC

Ovalbumin

TCTCTCCNA
 AGAGAGGNT

Platelet-derived growth factor A-chain gene

GGGGAGGGGG
 CCCCTCCCCC

H19 and β-globin CTCF

CCGCGNGGNGGCAG
 GGCGCNCCNCCGTC

Fig. 4.11. Sequence of the SS66 fragment.

The putative silencer region (SS66) sequence is presented and the DNase I foot-printed areas are underlined and bold (A). Alignment of some of the sequences reported to have silencer activity and to bind to repressor proteins.

the CTCF protein is different to the sequence of the footprinted region, CTCF contains 11 zinc fingers and may bind to a wide range of sequences. Hence, the footprinted region may bind to CTCF (Bell et al., 1999), but internal with a different subset of zinc fingers to those used in the above consensus. In this case, we would not be able to predict the existence of a CTCF site on the basis of sequence alone, but would have to employ EMSAs and other binding studies.

The spacing problem encountered in this study can be solved by making mutations within the potential silencer to relieve the repression without changing the spacing between the enhancer and the promoter. The studies mentioned above have used this technique to mutate within protein binding areas to increase the expression of the linked gene. For example, mutation within the CCACC site of the ϵ -globin silencer resulted in the expression of the ϵ -globin gene in adult erythroid cells in transgenic mice (Raich, 1995). Mutational analysis could also establish whether the A/T-rich element in the SS245 fragment functions as a promoter element, and whether the GCCATG and TAG elements in the SS66 fragment function as translation initiation and termination elements.

CHAPTER 5: THE 5.2 KB REGION UPSTREAM OF THE IL-3 GENE IS NOT SUFFICIENT FOR AN LCR FUNCTION

5.1 INTRODUCTION

IL-3 is expressed primarily by T cells in response to antigenic stimulation of the T cell receptor, and also by several cell types in the myeloid lineage that include mast cells, basophils, and eosinophils. This mode of activation has been discussed in detail in chapter one. As has been discussed in earlier chapters of this thesis, the 5.2 kb region immediately upstream of IL-3 transcription start site contains up to five DH sites. The -4.1 kb site is invariably present in T cell lines regardless of their stage of development or the transcriptional status of the IL-3 gene. The -4.1 kb site is also often found in myeloid cell lines and myeloid leukaemias, including primitive CD34 positive cells such as the cell line KG1a and M1 class AMLs. Based on these observations we formulated a hypothesis that this region may function as an LCR because the -4.1 kb site is apparently forming early in development, it is a good candidate for a regulatory element having LCR-like properties and activating the locus at a different level to subsequent TCR-signalling pathways which ultimately trigger IL-3 gene transcription.

LCRs have been defined as a result of characterisation of the β -globin locus. Accordingly, LCRs are supposed to confer position-independent regulation of gene expression. In transgenic animal or stable line experiments, LCRs are expected to protect the integrated DNA from the effects of surrounding genes or from heterochromatin silencing effects. LCRs function as powerful enhancers that are capable of positively regulating the expression of linked genes. LCRs can also determine tissue-

specificity and the developmental stage at which the gene is supposed to be expressed. For the IL-3 gene, we hypothesised an LCR to exist upstream of IL-3 gene that is controlling the stages of T cell and myeloid cell development at which IL-3 can be produced.

This chapter describes work that was carried out to test the function of the 5.2 kb region upstream of the human IL-3 gene as an LCR in both stably transfected Jurkat cell lines and transgenic mice. However, the results obtained indicate that this region failed to function as an LCR.

5.2 SEQUENCING OF THE H5.2 REGION

During the course of this study a complete sequence of the IL-3 upstream region extending up to the -5.2 kb HindIII site was compiled. Previously, just the 1.3 kb SacI fragment (Cockerill et al., unpublished data) and the IL-3 promoter region up to -855 bp had been sequenced (Gottchalk et al., 1993). The rest of the region was completely sequenced as part of this study. The compiled sequence is presented in appendix A.

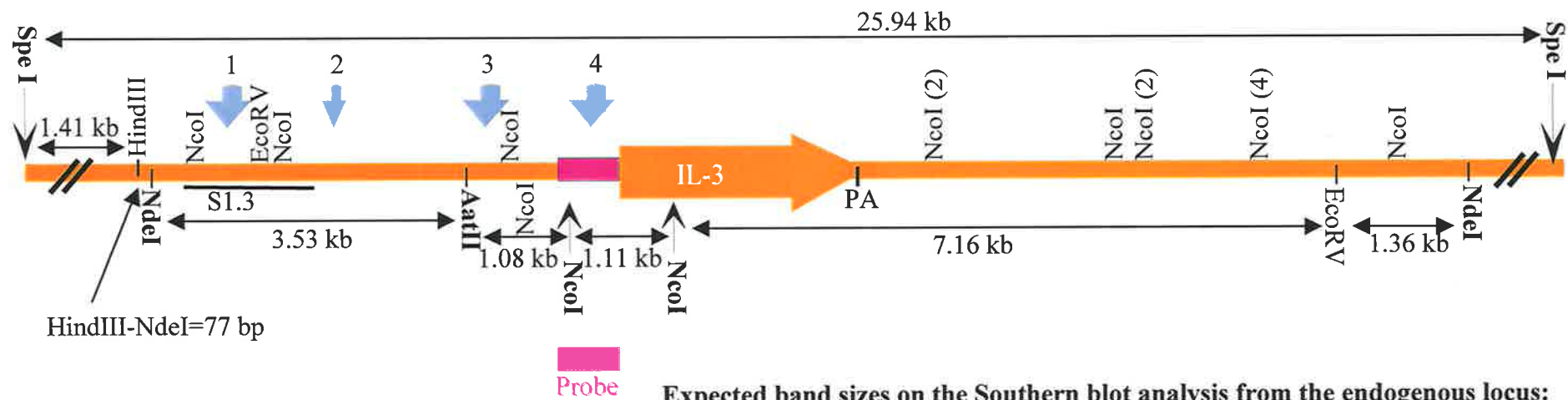
5.3 VARIABILITY OF POLY-CLONAL STABLY TRANSFECTED CELL LINES

The IL-3 promoter (-559 to +50) and the H5.2 (-5146 to +50) fragments were cloned in the pXP1 luciferase plasmid (described in materials and method) to give pIL3H and pIL3H5.2. The 1.2 kb BglII (B1.2) fragment encompassing the -14 kb IL-3 enhancer (Duncliffe et al., 1997) and the 1.3 kb SacI (S1.3) fragment encompassing both the -4.5 kb and -4.1 kb DH sites were cloned in the BglII and SacI sites upstream of the IL-3

promoter in the pIL3H plasmid to generate pIL3H-B1.2 (Duncliffe et al., 1997) and pIL3H-S1.3. A clone containing the S1.3 fragment in reverse orientation relative to the IL-3 promoter (pIL3H-S1.3R) was also used. These plasmids were linearised with FspI as shown in figure 5.1. A neomycin gene containing plasmid (pE^otk.neo) was also co-transfected with each of the four constructs mentioned in a 1:10 ratio as described in materials and method chapter. Two days after transfection, the transfected cells along with un-transfected cells were grown in media containing Geneticin (G418) as described in materials and methods. Following selection of G418 resistant cells, the surviving transfected cells were tested for luciferase activity and for the presence of DNA by Southern at day 21, 48, and 109 following transfection. DNA from each transfection was digested with (1) NcoI/EcoRV, (2) NdeI, (3) NdeI/AatII, and (4) SpeI. Restriction maps of the endogenous locus and the DNA constructs are presented in figure 5.2 and 5.3. The expected banding patterns from the four digests are as follow.

NcoI/EcoRV digests were used to estimate relative copy numbers. The relative copy numbers were calculated from the ratio of band intensities from the inserted plasmid and the endogenous IL-3 gene on a Southern blot hybridisation analysis. A 1.1 kb NcoI and a 2 kb NcoI/EcoRV band from the endogenous IL-3 locus and the transfected DNA respectively were visualised with a ³²P-labelled 611 bp HindIII IL-3 promoter probe excised from pIL3H.

The NdeI enzyme is expected to generate a 14.24 kb fragment from the endogenous IL-3 locus. Since the NdeI enzyme cuts the transfected DNA only once, discrete bands will be generated as a result of multimerised insert DNA integrated into the genomic DNA. In addition, other bands will be generated which depend on the number of independent

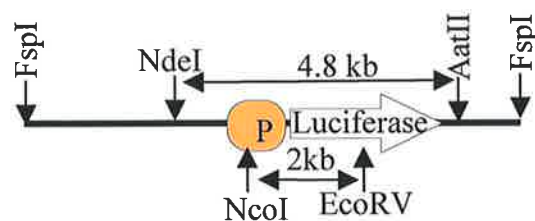


- 1) NcoI/EcoRV: - a 1.11 kb band
- 2) NdeI:- a 14.24 kb band
- 3) NdeI/AatII:- a 10.71 kb band
- 4) SpeI:- a 25.94 kb band

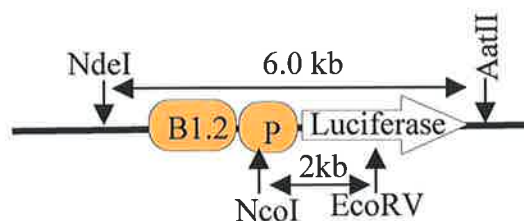
Fig. 5.2. Restriction enzyme map of the human IL-3 locus.

The sites of the restriction enzymes used in the Southern blot hybridisation are indicated. The distance between these sites are indicated and marked with arrows. The locations of the DH sites upstream of the IL-3 locus are shown. The numbers within parenthesis indicate the number of restriction site at the specified location. The probe used is shown. The expected bands from the Southern blot hybridisations are listed.

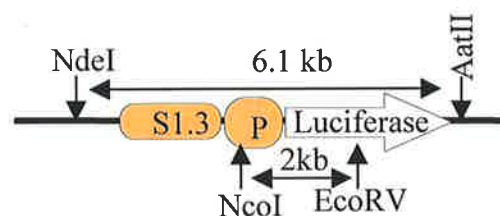
pIL3H



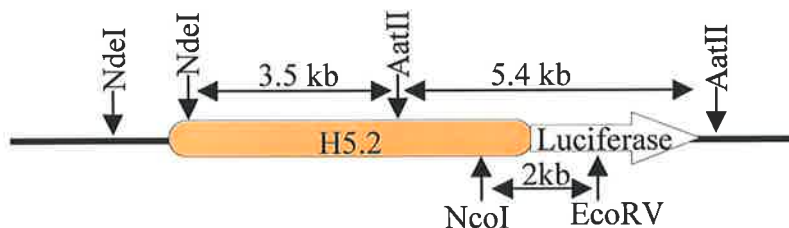
pIL3H-B1.2



pIL3H-S1.3



pIL3H5.2



 probe

Fig. 5.3. Schematic diagrams of the constructs used in the stable transfection

Linearised plasmid restriction maps of the constructs used in the stable line transfections are presented. P stands for the IL-3 promoter. The distances between the sites are marked with arrows.

integration events into the genomic DNA. Hence, in a pool of stably transfected cells that has a lot of independent integration events, a smear may be expected on the Southern blot hybridisation assay in addition to a few discrete bands generated by multimerised constructs. However, if the transfection resulted in few independent integration events, then only a few bands will be generated in addition to those ones resulting from multimerised constructs.

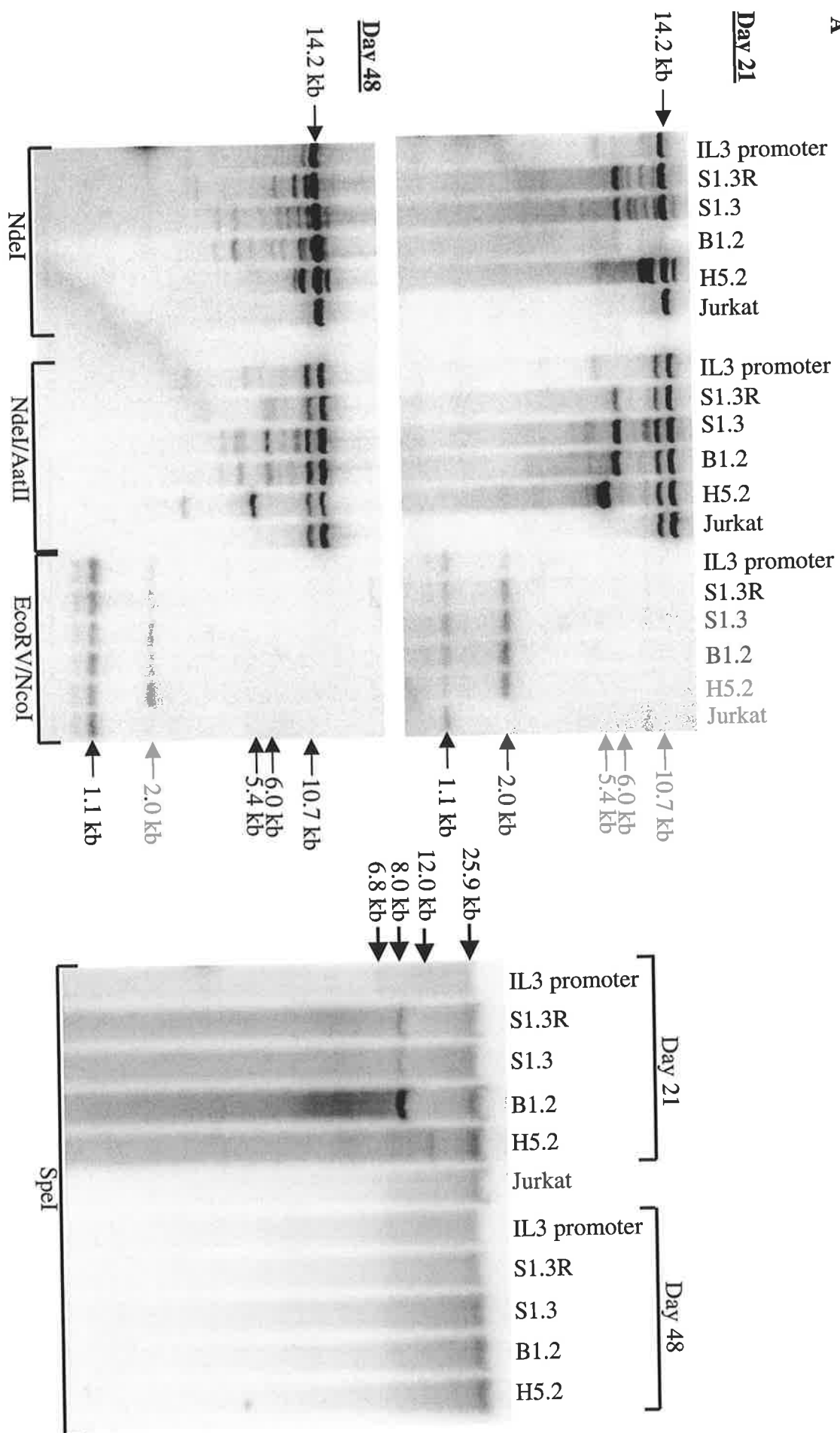
The NdeI/AatII enzyme will generate different bands from the different constructs as well as a 10.71 kb band from the endogenous IL-3 locus. The purpose of this digest was to test for rearrangement, deletion or recombination of integrants. The SpeI enzyme does not cut within the constructs but generates a 25.94 kb band from the genomic DNA. Any additional bands appearing will be from the sites of integrations.

The first analysis of the DNA taken at day 21 after transfection is presented below (figure 5.4A) and compared with repeated analyses performed at day 48 and 109 (figure 5.4B). The number of independent integration events in this transfection was low as suggested by the NdeI Southern. Moreover, the band intensities changed with time reflecting differences in growth rates between the different clones. Hence, with passaging the cells, the faster growing cells were selected while the slower clones were eliminated. As a result, the copy number calculated from the NcoI/EcoRV Southern changed with time. Unexpected results were obtained from the SpeI digests where discrete bands 8 to 12 kb in length, corresponding to the linear plasmid sizes, were observed at day 21 indicating the presence of unintegrated plasmid DNA.

Fig. 5.4. Southern blot hybridisation analysis of the first stable transfection assay.

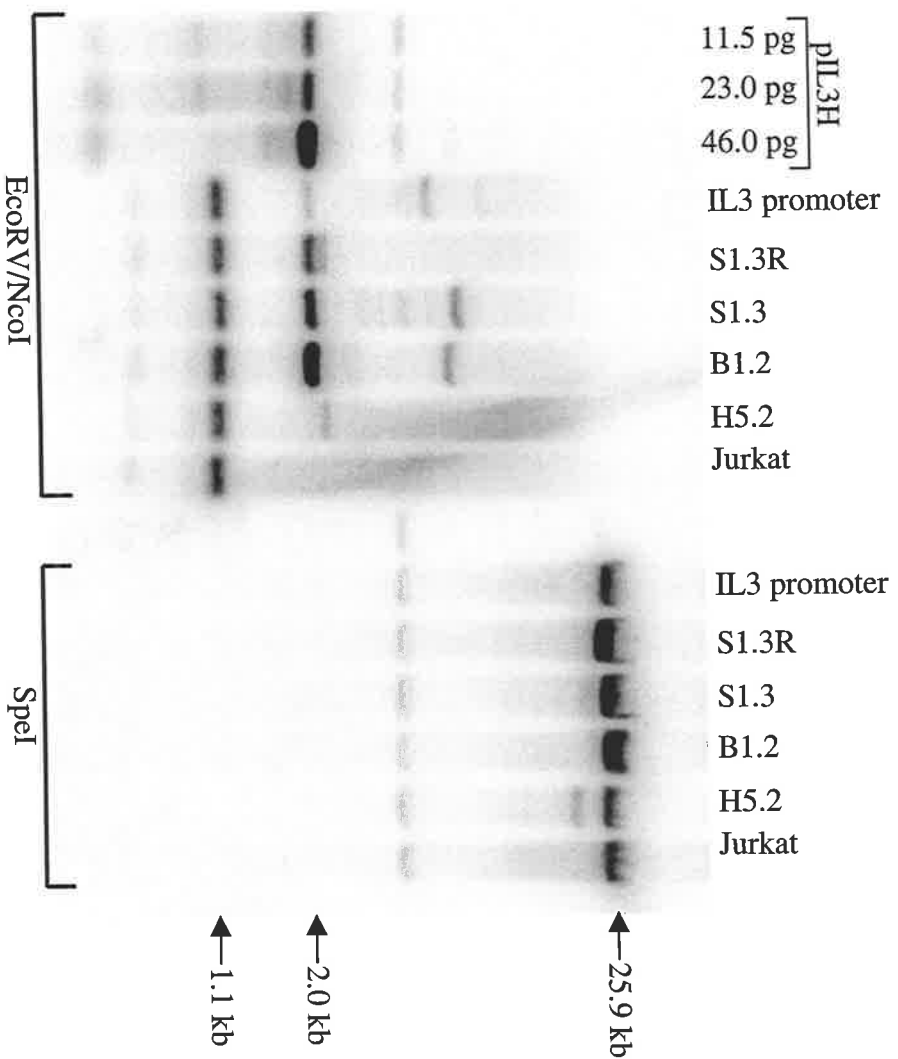
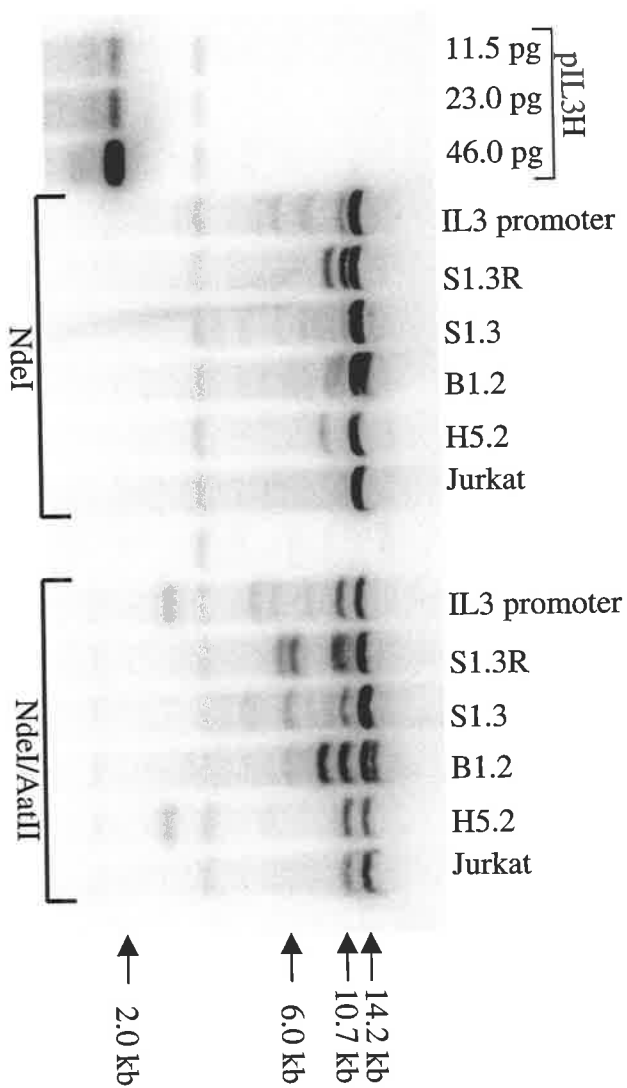
(A) DNA prepared from each of the stable cell transfections at day 21 and day 48 after transfection was digested with the indicated restriction enzymes. The names of the constructs used in these transfections are indicated on the top of the gels. The sizes of the bands generated from these digests were indicated. Note that in lane 4 of the NdeI digest at day 21 the DNA appears to under-represented. (B) Repeat Southern blot hybridisation analysis of DNA purified from transfected cells at day 109. The NdeI digest shows the endogenous 14.2 kb band. The other bands are due to multimerisation of the insert or due to bands generated from the position of integration. The NdeI/AatII digests show the 14.2 and 10.7 kb bands from the endogenous locus. The other bands are insert-specific. The EcoRV/NcoI digest shows a 2.0 kb band from the inserts and a 1.1 kb from the endogenous locus. The ratio of the 2.0 kb to the 1.1 kb intensities was used to calculate copy number. The SpeI digests show the 25.9 kb endogenous band. Any other band appearing comes from either episomal plasmid DNA or from DNA fragments from the site of integration spanning the inserted plasmid.

A



B

Day 109



However, these bands disappeared by day 48. The *SpeI* digest also generated a high molecular weight band consistent with the expected 25.9 kb band from the endogenous IL-3 locus. The lack of smears and/or discrete bands at day 48 probably indicated the presence of a large population of cells that do not carry the IL-3 constructs. This also was indicated by the reduction of copy number in these transfections. To address this problem, Geneticin (G418) selection was reapplied on day 80 to day 94. On day 109, Southern blot hybridisation and luciferase assays were performed. The proportion of cells carrying the IL-3 constructs was increased as shown by the *NcoI/EcoRV* Southern. At this stage it was clear that only few independent clones were present in each transfection as indicated by the *NdeI* Southern blot hybridisation assays. For *SpeI* the integrated plasmid existed predominantly as high molecular weight *SpeI* bands co-migrating with the genomic band. The *AatII* enzyme was affected by CpG methylation and did not cut the genomic IL-3 DNA to completion. The Southern blot hybridisation assay with the *NdeI/AatII* enzymes resulted in a partial digest of *AatII* sites, which gave rise to a 14.24 kb fragment from *NdeI* and a 10.71 kb fragment from *NdeI/AatII*. In contrast, the transfected plasmid DNA did not seem to be affected by the CpG methylation and resulted in complete digestion resulting in discrete bands consistent with what is expected from the constructs.

The luciferase activity of these cells also varied at the different time points in parallel with the change in the clonal composition of these cells. Figure 5.5 shows the results of the luciferase assays at the different time points as both corrected or uncorrected for copy number. The copy number correction may not be meaningful, however, since the percent composition of each of the different clones with independent integration events in each transfection were not known and changed with time. Before correction for copy

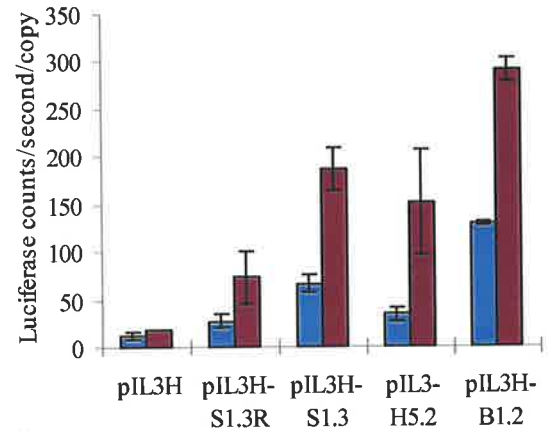
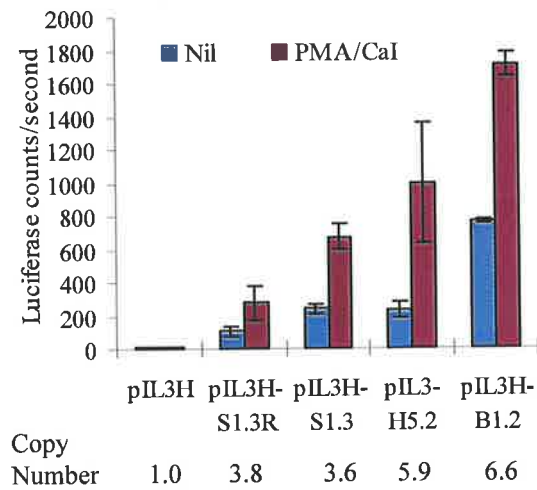
Fig. 5.5. Luciferase activity of Jurkat T cells stably transfected with IL-3 promoter constructs: first transfection.

Graphs of mean luciferase activities measured from two to three luciferase measurements at day 26, 48, and 109 post transfection in the first round of stable transfection studies. The error bars represent standard error of the mean. The graphs on the left are luciferase activities not corrected for copy number. The graphs on the right are the luciferase activities after correction for copy number.

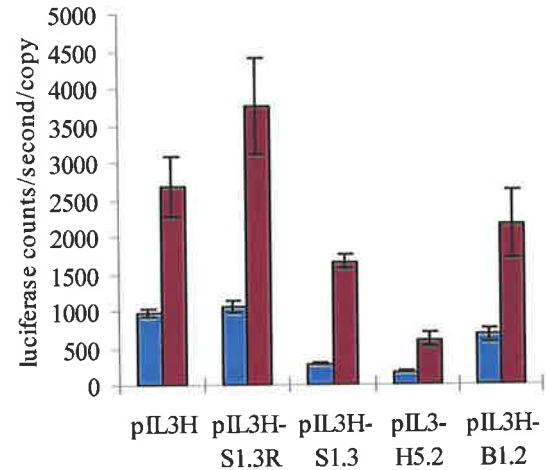
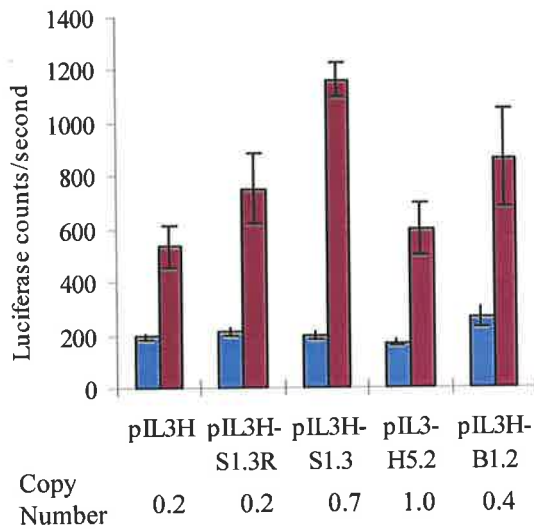
Not corrected for copy number

Corrected for copy number

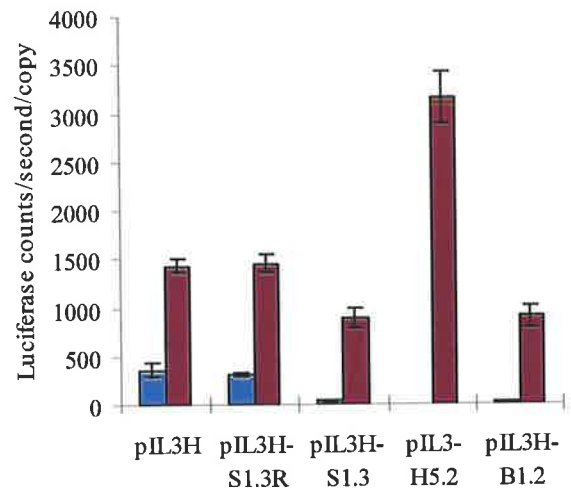
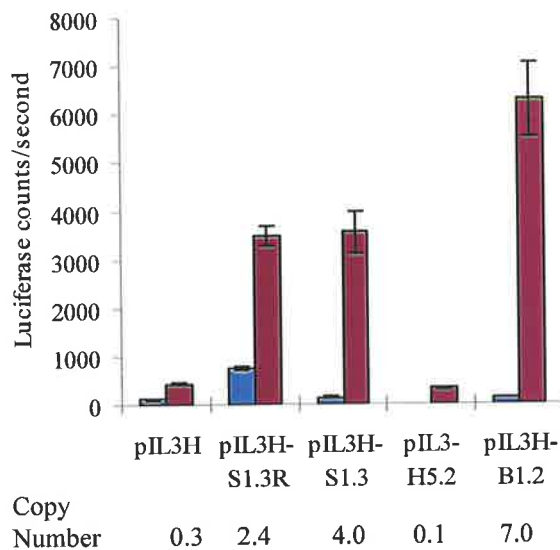
Day 26



Day 48



Day 109



number S1.3, S1.3R and the B1.2 constructs were all more active than pIL3H at each of the three time points but to different degrees. The level of increase changed with time reflecting the change in composition of the cells. The H5.2 construct activity was high at day 20 but declined at later time points as result of eliminating populations carrying this constructs as indicated by the drop in copy number to 0.1 copy. To have such a low copy number indicates that most of the cells carry the pE^otk.neo plasmid only and very few cells carry both the pIL3H5.2 and the pE^otk.neo. After correcting for copy number, the S1.3, S1.3R, H5.2, and B1.2 transfected cells each had higher luciferase activities than pIL3H at day 20 but not in most cases at day 48 and 109.

The same constructs used in the first transfection were used again in a second transfection experiment. To increase the likely hood of obtaining many independent integration events, and more significant data, two independent transfections were performed for pIL3H and pIL3H-S1.3. This time only one time point was taken, at day 30. This time point was sufficient because no episomal DNA was detected and the copy number was high (figure 5.6). The discrete bands in the NdeI digest patterns suggested that there were many independent integration events, and that most of the DNA existed in multicopy arrays. The SpeI patterns again indicated integration within large SpeI fragments migrating above the endogenous band.

The luciferase activities in this second transfection study are shown in figure 5.7. Only the B1.2 construct increased the activity of the IL-3 promoter in this transfection both with or without copy number adjustment. The S1.3 R showed increased transcription level only after the activity was adjusted for copy number. Overall, it seems that the S1.3, the H5.2 and the B1.2 fragments are all capable of enhancing the activity of the IL-

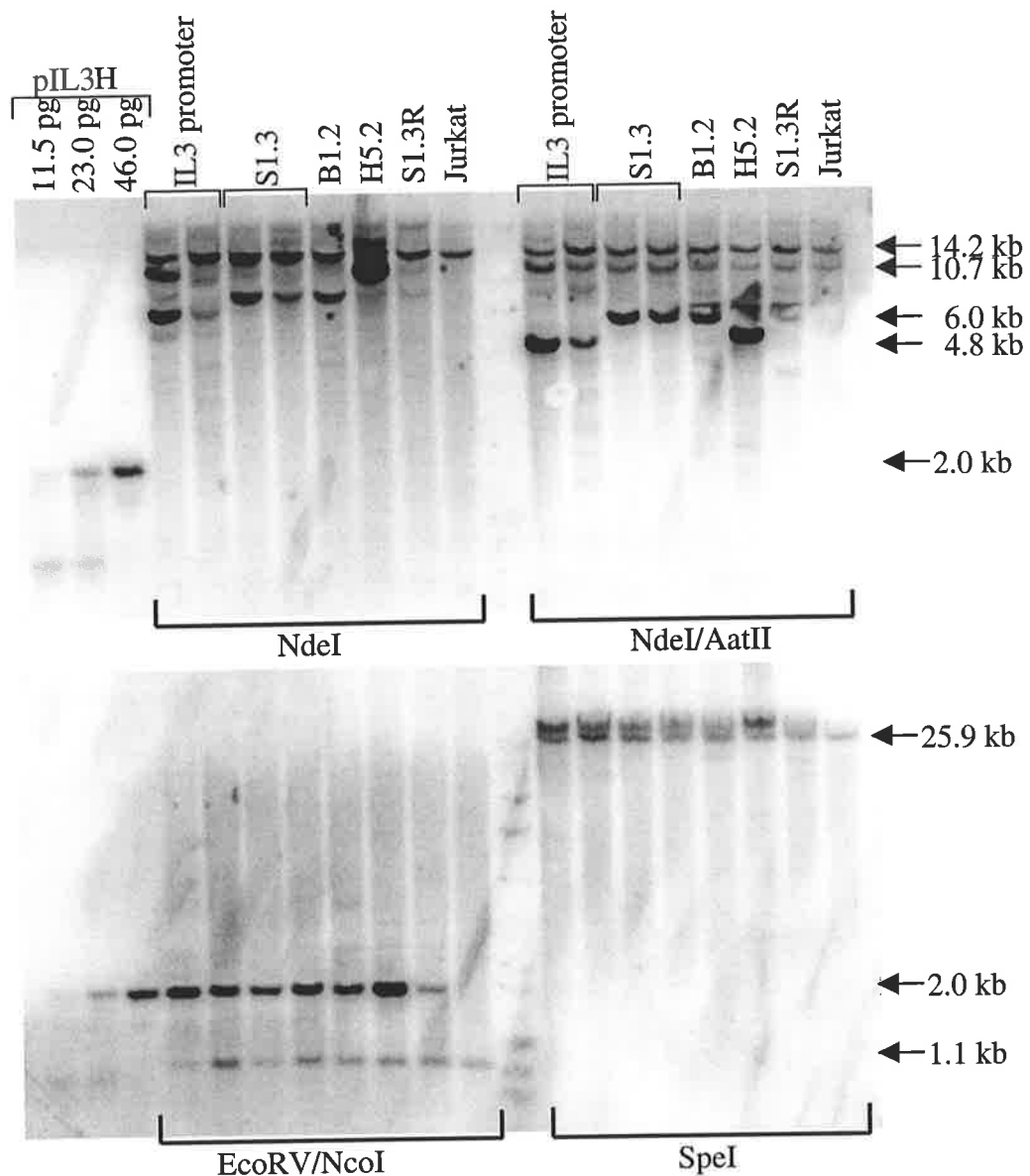


Fig. 5.6. Southern blot hybridisation analysis of the second stable transfection study.

DNA prepared from each of the pools of stably transfected cells was digested with the indicated restriction enzymes. The names of the constructs used in these transfections are indicated on the top of the gels. The sizes of the bands generated from these digests are indicated. The NdeI digest shows the endogenous 14.2 kb band. The other bands are due to multimerisation of the insert or due to bands generated from the position of integration. The NdeI/AatII digests show the 14.2 and 10.7 kb bands from the endogenous locus. The other bands are insert-specific and identify the plasmid constructs. The EcoRV/NcoI digest shows a 2.0 kb band from the inserts and a 1.1 kb from the endogenous locus. The ratio of the 2.0 kb to the 1.1 kb intensities was used to calculate copy number. The SpeI digests show the 25.9 kb endogenous band. Any other band appearing comes from either episomal plasmid DNA or from DNA fragments from the site of integration spanning the inserted plasmid.

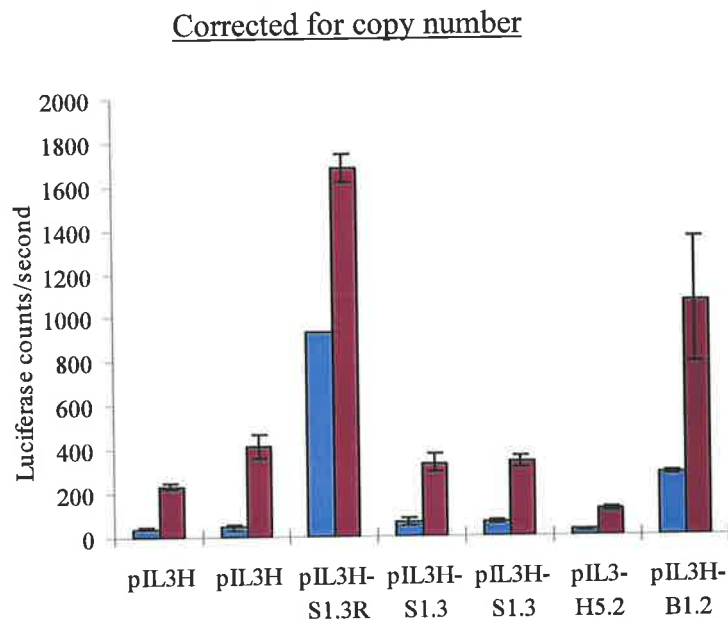
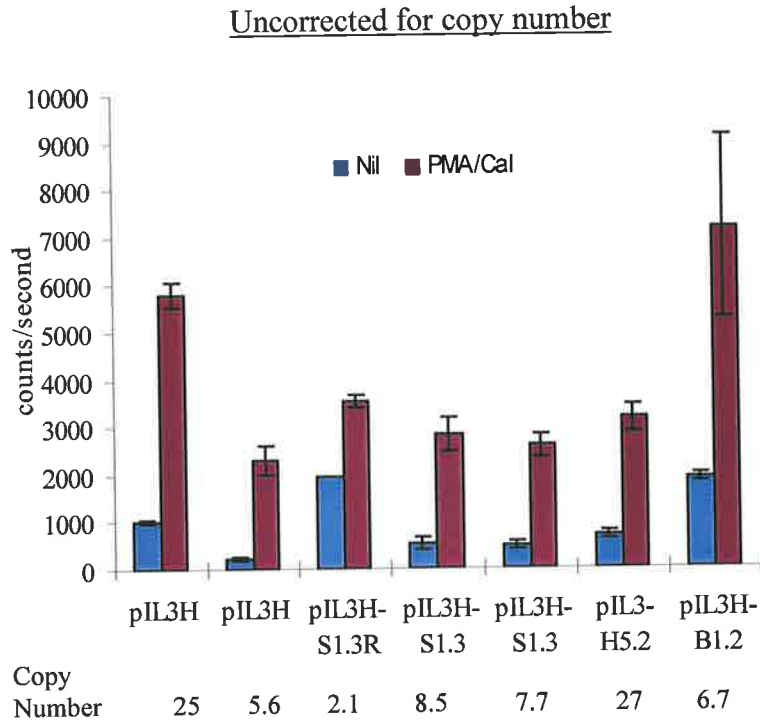


Fig. 5.7. Luciferase activity of the stably transfected Jurkat T cells: second transfection.

Panel A is a graph of the mean activities of 2 to 3 luciferase measurements of the second Jurkat T cell stable transfection assay. Error bars represent standard error of the mean. Copy number from each stable line is indicated. The second panel show the luciferase activity after correction for copy number.

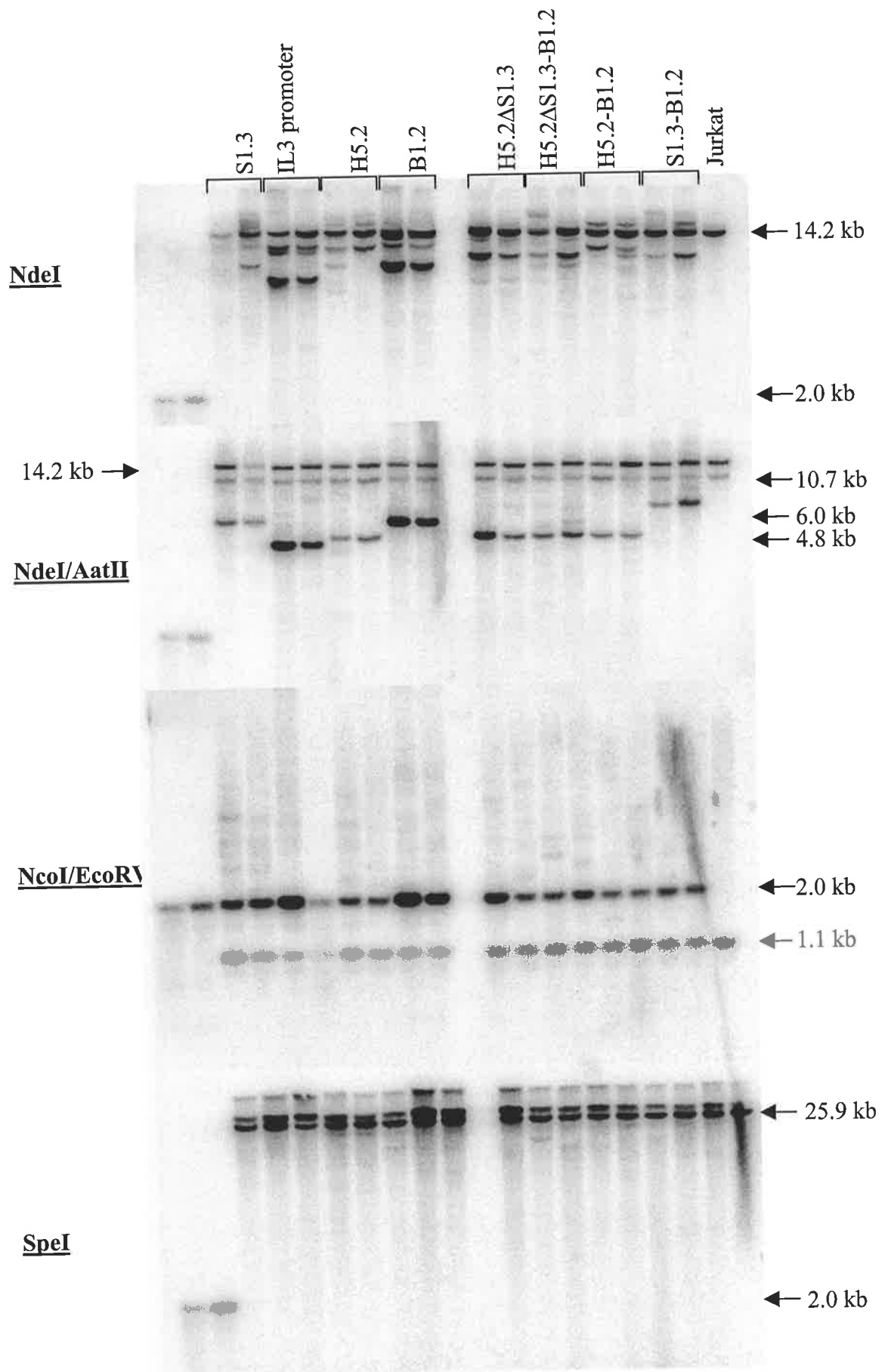
3 promoter but the variability in this assay system means that this effect cannot be demonstrated as reproducible phenomena.

5.4 COOPERATIVITY BETWEEN TWO ENHANCER ELEMENTS

In the previous two stable transfection experiments, there was some indication that both the B1.2 and the S1.3 fragments in either orientation were able to increase the activity of the IL-3 promoter to varying degrees. Since the two transfection experiments produced conflicting results, a third stable transfection experiment with additional constructs and two independent transfections of each construct was performed. The effect of both enhancers from the IL-3 locus on the IL-3 promoter together or separately was studied. The B1.2 -14 kb IL-3 enhancer fragment was cloned in the BglII site upstream of the 0.6 or 5.2 kb promoter fragments in pIL3H-S1.3 and pIL3H5.2 to generate the pIL3H-S1.3-B1.2 and pIL3H5.2-B1.2. In addition, the S1.3 region was deleted from the H5.2 construct to generate the pIL3H5.2 Δ S1.3. The B1.2 fragment was cloned in the BglII site of pIL3H5.2 Δ S1.3 to generate pIL3H5.2 Δ S1.3-B1.2. Restriction maps of these additional constructs are presented in figure 5.8. These plasmids were transfected into Jurkat T cells in duplicate along with pTKneo plasmid at 10:1 molar ratio. On day 23 after transfection, DNA was obtained from these cells and luciferase assays were performed. Southern blot hybridisation analysis was performed on the DNA as described previously. Results of the Southern blot hybridisation assay are presented in figure 5.9. Similar to the previous stable transfection studies, the number of independent integration events as judged by the NdeI and the SpeI Southern was low. However, the copy numbers of the constructs were relatively high.

Fig. 5.9. Southern blot hybridisation analysis of the third stable transfection assay.

DNA prepared from each of the pools of stably transfected cells was digested with the indicated restriction enzymes. The names of the constructs used in these transfections are indicated on the top of the gels. The sizes of the bands generated from these digests are indicated. The NdeI digest shows the endogenous 14.2 kb band. The other bands are due to multimerisation of the insert or due to bands generated from the position of integration. The NdeI/AatII digests show the 14.2 and 10.7 kb bands from the endogenous locus. The other bands are insert-specific and identify the plasmid constructs. The EcoRV/NcoI digest shows a 2.0 kb band from the inserts and a 1.1 kb from the endogenous locus. The ratio of the 2.0 kb to the 1.1 kb intensities was used to calculate copy number. The SpeI digests show the 25.9 kb endogenous band. Any other band appearing comes from either episomal plasmid DNA or from DNA fragments from the site of integration spanning the inserted plasmid.



The luciferase activities of these cells are shown in figure 5.10. In the plot where the luciferase activities are not corrected for copy number, the pIL3H-S1.3, the pIL3H-B1.2, pIL3H-S1.3-B1.2, and pIL3H5.2ΔS1.3 showed increased activities at least 50% over the IL-3 promoter alone construct. None of the other constructs showed any increase significantly over the promoter alone construct. Moreover, this plot shows that the addition of the B1.2 to the pIL3H-S1.3 enhanced the activity of the promoter by a further two fold.

When the luciferase activities were corrected for copy number, the activity within each pair of transfections was different. The two promoter alone transfections, pIL3H, gave totally different activities, which made determination of the base line activity difficult. However, it is possible for errors to creep into copy number determinations and it is unclear how reliable these values are. In this instance it is probable that the NcoI/EcoRV digests used to estimate copy number of the second pIL3H transfection was under-loaded, thus skewing the results based on copy number. The corrected activity of the pIL3H-S1.3 and pIL3H-S1.3-B1.2 showed similar increase as in the not corrected. The activities increased stepwise with the addition of each of the S1.3 and B1.2 elements to the IL-3 promoter. The level of activation for pIL3H5.2, pIL3H5.2ΔS1.3, and pIL3H-B1.2 was similar to the more active of the two pIL3H-transfections. The pIL3H5.2-B1.2 and pIL3H5.2ΔS1.3-B1.2 activities were higher than the promoter alone.

Because of the discrepancy between the activities of the duplicate transfections, an average was taken of the copy number corrected luciferase activities within each pair and presented in figure 5.11. This plot shows that the S1.3 element enhances the activity of the IL-3 promoter by about 3.4 fold over the promoter alone. The B1.2 enhancer

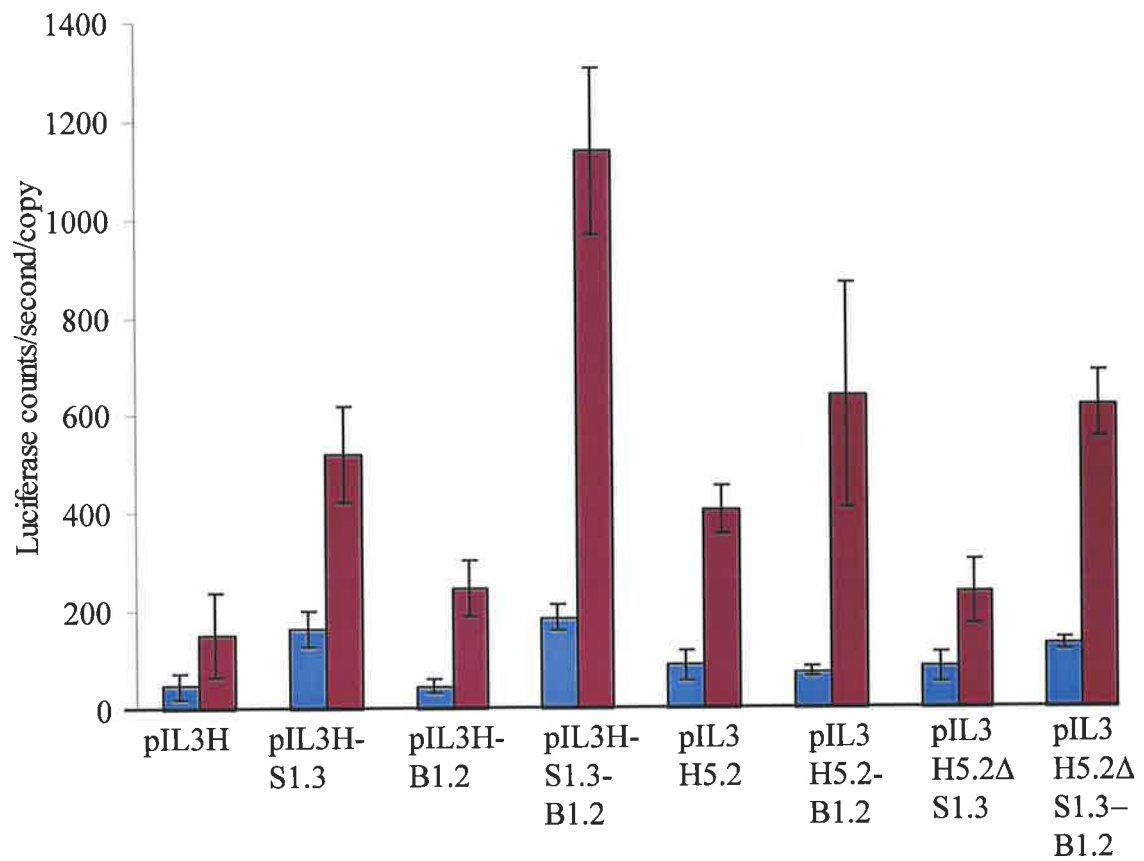


Fig. 5.11. Average of the Luciferase activity from two independent transfections: third transfection.

The stable luciferase activities of stably transfected cells were calculated after correction for copy number and the mean determined for the two independent transfections. Error bars represent standard error of the mean.

unexpectedly did not significantly increase the IL-3 promoter transcription level since the error bars overlap. The H5.2 mean luciferase activity was 2.7 fold more than the promoter alone. Both the S1.3 and the H5.2 fragment contained the SS245 enhancer characterised in chapter 3. The enhancement activity seen in this experiment may be attributed to the SS245 enhancer. When the S1.3 fragment was deleted from the H5.2 segment (pIL3H5.2 Δ S1.3), the activity was reduced to 1.6 fold over the promoter alone. The addition of the B1.2 enhancer to the constructs containing the SS245 enhancer in pIL3H-S1.3-B1.2, and pIL3H5.2-B1.2 increased the activity by a further 2.2 and 1.6 fold respectively. The pIL3H5.2 Δ S1.3-B1.2 had an activity that is 2.6 and 4.1 fold over the pIL3H5.2 Δ S1.3 and the promoter alone. This experiment suggested that the two IL-3 enhancers at -4.5 and -14 kb upstream of the IL-3 gene cooperate to activate the IL-3 gene when stimulated with PMA and CaI.

5.5 THE CLUSTER OF DH SITES WITHIN THE 5.2 REGION UPSTREAM IS NOT AN LCR

The stable transfection experiments described so far are variable. In these experiments, the neomycin gene was on a different plasmid (pE^otk.neo) and co-transfected along with the IL-3 constructs at a 1:10 ratio. It is possible that some of the cells may have got only either the pE^otk.neo or the IL-3 plasmid constructs. All cells that did not get the pE^otk.neo plasmid should die off. However, cells that got only the pE^otk.neo will survive along with the cells that got both the pE^otk.neo and the IL-3 constructs.

Another problem with the above experiment was that a determination of an LCR function for the H5.2 fragment was not possible. To establish an LCR function one

needs to relate transcriptional activity to the number of copies integrated. To address these problems and test for an LCR function, another transfection was carried out using one of each of the following constructs per transfection: pIL3H5.2ΔS1.3, pIL3H5.2ΔS1.3-B1.2, pIL3H5.2-B1.2 and pIL3H5.2. These were co-transfected with pE^otk.neo at 10:1 molar ratio. Two days after transfection, the cells were counted and plated on the first row of two 96 well plates per transfection. The cells were serially diluted down the plates in media containing G418. All wells with cells growing in them were screened for the presence of IL-3 DNA constructs by Southern blot hybridisation analysis.

The DNA from these cells was digested with HpaI. HpaI does not cut the luciferase gene or any of the IL-3 fragments, but it cuts elsewhere in the vector DNA (figure 5.12). A 1.65 kb XbaI fragment from pIL3H encompassing the luciferase gene was used as a probe. The expected sizes from the HpaI digest are 6.54, 7.77, 9.07, and 7.84 kb from pIL3H5.2ΔS1.3, pIL3H5.2ΔS1.3-B1.2, pIL3H5.2-B1.2 and pIL3H5.2 respectively. No band is expected from the genomic DNA since luciferase DNA is not present. As a result only one band is expected from each stable line carrying the luciferase constructs. The presence of multiple bands or bands with different sizes than expected would imply rearrangement. All clones with no DNA or with DNA that gave unexpected sizes or multiple DNA bands were discarded. Figure 5.13 shows an example of a Southern blot from this screen. Cells were growing in 76 wells. Only 49 of the 76 were positive for luciferase DNA and only 22 clones with DNA that was not rearranged. The distribution of these 49 is presented in table 5.1.

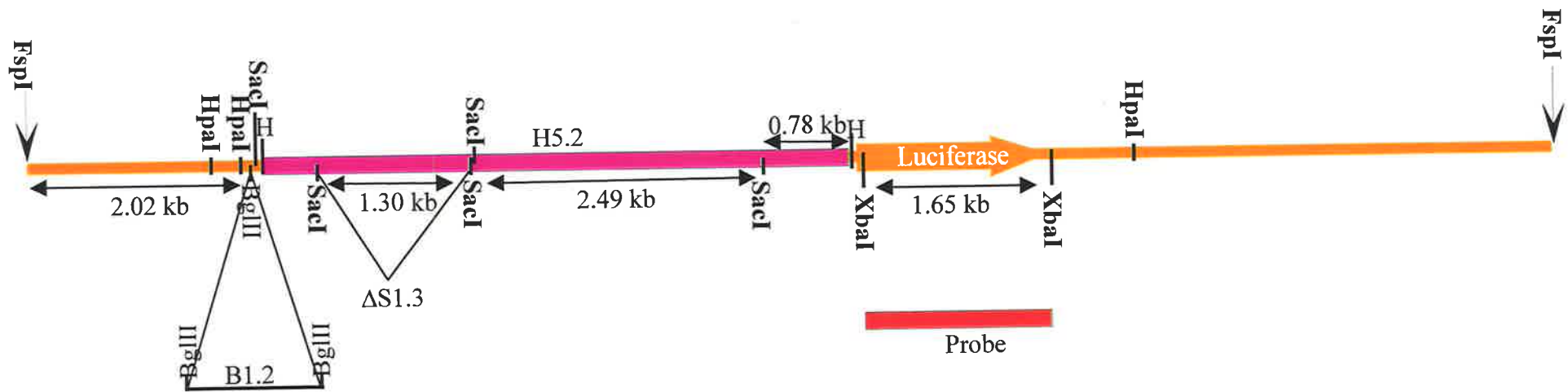


Fig 5.12. A schematic diagram of the H5.2 construct used in the fourth-stable transfection.

The H5.2 construct is shown. The site of the B1.2 ligation and the S1.3 deletion are shown. Restriction enzyme digests used in the Southern blot hybridisation assay were marked. H stands for HindIII. The distances between the restriction enzymes are indicated and marked with arrows.

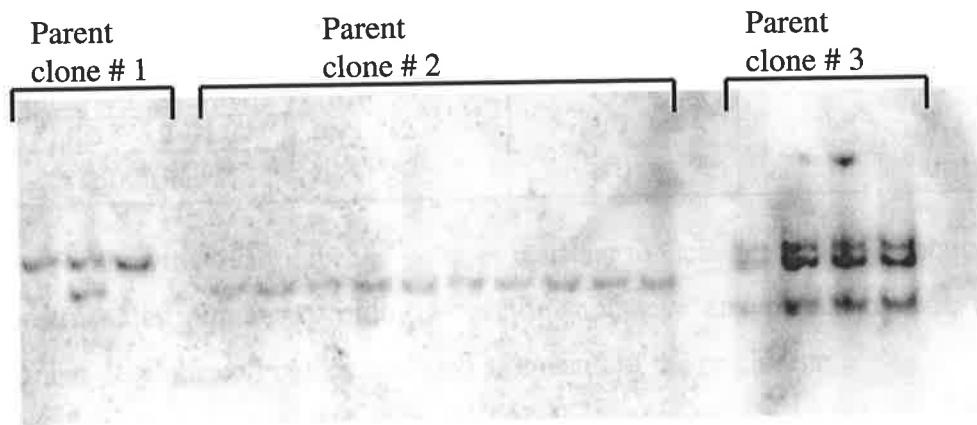
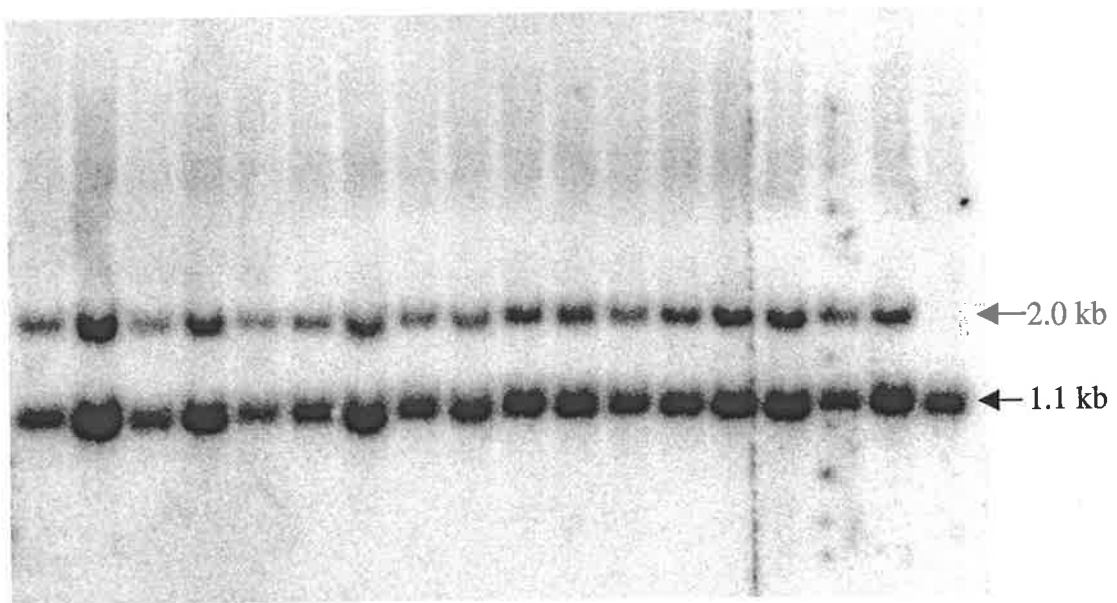
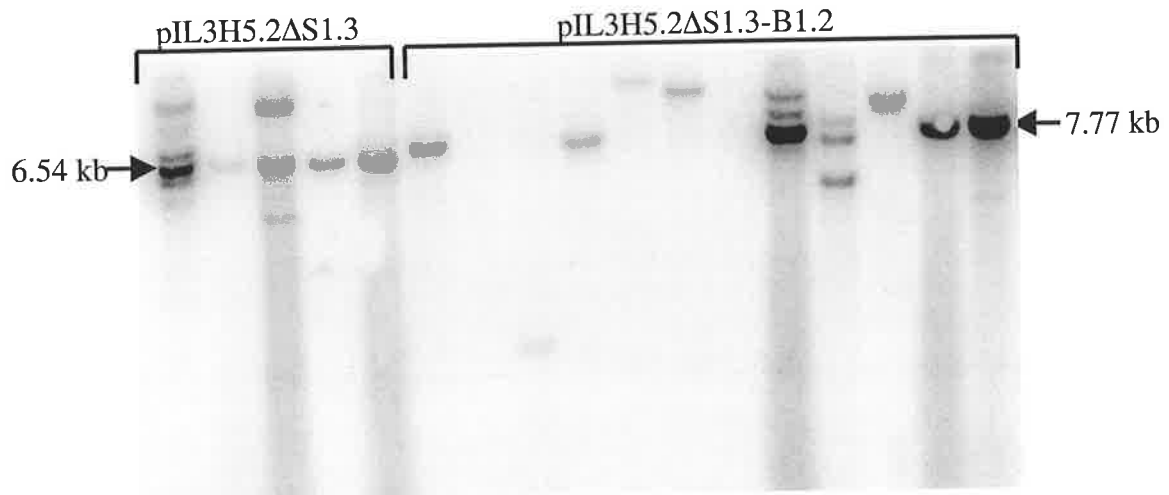
A**B**

Fig 5.13. Screening single cell clones for the presence of luciferase constructs

Genomic DNA prepared from single cell clones that were resistant to Gentacin (G418) were screened for the presence of luciferase DNA constructs. DNA was digested with HpaI. Southern blot hybridisation analysis used an XbaI luciferase DNA fragment as a probe. The presence of multiple bands was an indication of rearrangement of the inserted DNA. The absence of bands indicated that the luciferase DNA was not present. Only clones with one band were selected. The size of this band identified the constructs used and their sizes are indicated. Panel A and B show few examples of the single cell cloned analysed.

A *Sac*I digest



B *Nco*I/*Eco*RV digest

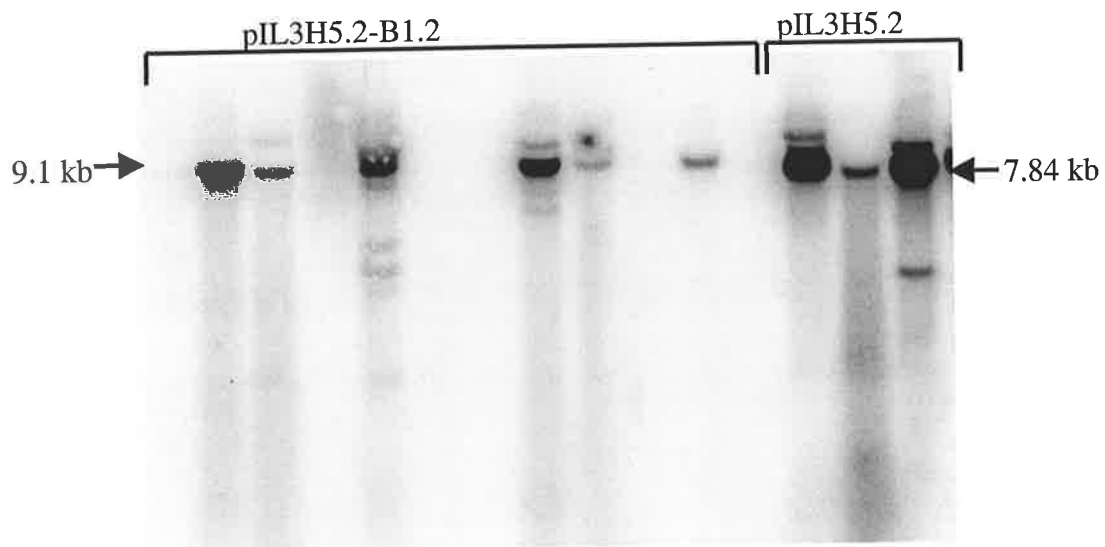


Fig 5.14. Southern blot hybridisation analysis of DNA from the single cell clones.

DNA from each of the single cell clones was digested with either *Sac*I or *Nco*I and *Eco*RV restriction enzymes. Panel A shows an example of the *Sac*I southern blot hybridisation assay. Single cell clones from three parent lines are shown. The first three lanes are from the same parent line while lanes 5-14 are for clones from the second parent line and lanes 16 to 19 are from clones from the third parent line. The second lane has a banding pattern different from lane one and three. This indicated that parent line-1 is a mixture of two lines. Multiple bands in some of the clones are a result of multiple inserts integrated. Panel B show the 2.0 kb band from the inserted DNA and the 1.2 kb from the endogenous locus as shown for previous Southern blot hybridisation assay. This blot was used to calculate the relative copy number.

The recloning resulted in 27 clones with independent integration sites. The luciferase activities of each of the 27 daughter cells were determined in two separate batches and compared in figures 5.15 and 5.16. In these analyses, pIL3H5.2ΔS1.3-B1.2 was more active than the pIL3H5.2ΔS1.3 in by at least two fold. Moreover, pIL3H5.2-B1.2, which contains two enhancers (the B.12 and the SS245) increased the activity further in some of the clones when compared to clones having the same copy number of the pIL3H5.2ΔS1.3-B1.2. Overall there seem to be an additive effect of the two enhancers on the transcription level of the luciferase gene. This may indicate that the two enhancers cooperate to increase the activity of the IL-3 promoter.

From the results obtained with single clones, there did not seem to be any correlation between activity and copy number in any of the constructs used. Some of the higher copy number clones gave similar activity as low copy number clones. This result indicates that the H5.2 fragment is not sufficient to protect the linked gene from the effect of the position of integration.

5.6 TRANSGENIC MICE

Since stable transfection experiments do not take into consideration the developmental program of the whole animal, we also assessed the functions of IL-3 gene regulatory elements in a transgenic mouse model using the human CD4 gene as a reporter gene. Transgenic mice were produced by Donna Roberts in our laboratory carrying the construct depicted in figure 5.17 and described in materials and methods. In this construct, the human CD4 gene is controlled by the H5.2 region from immediately upstream of the human IL-3 gene. The IL-3 enhancer (B1.2) was cloned upstream of the

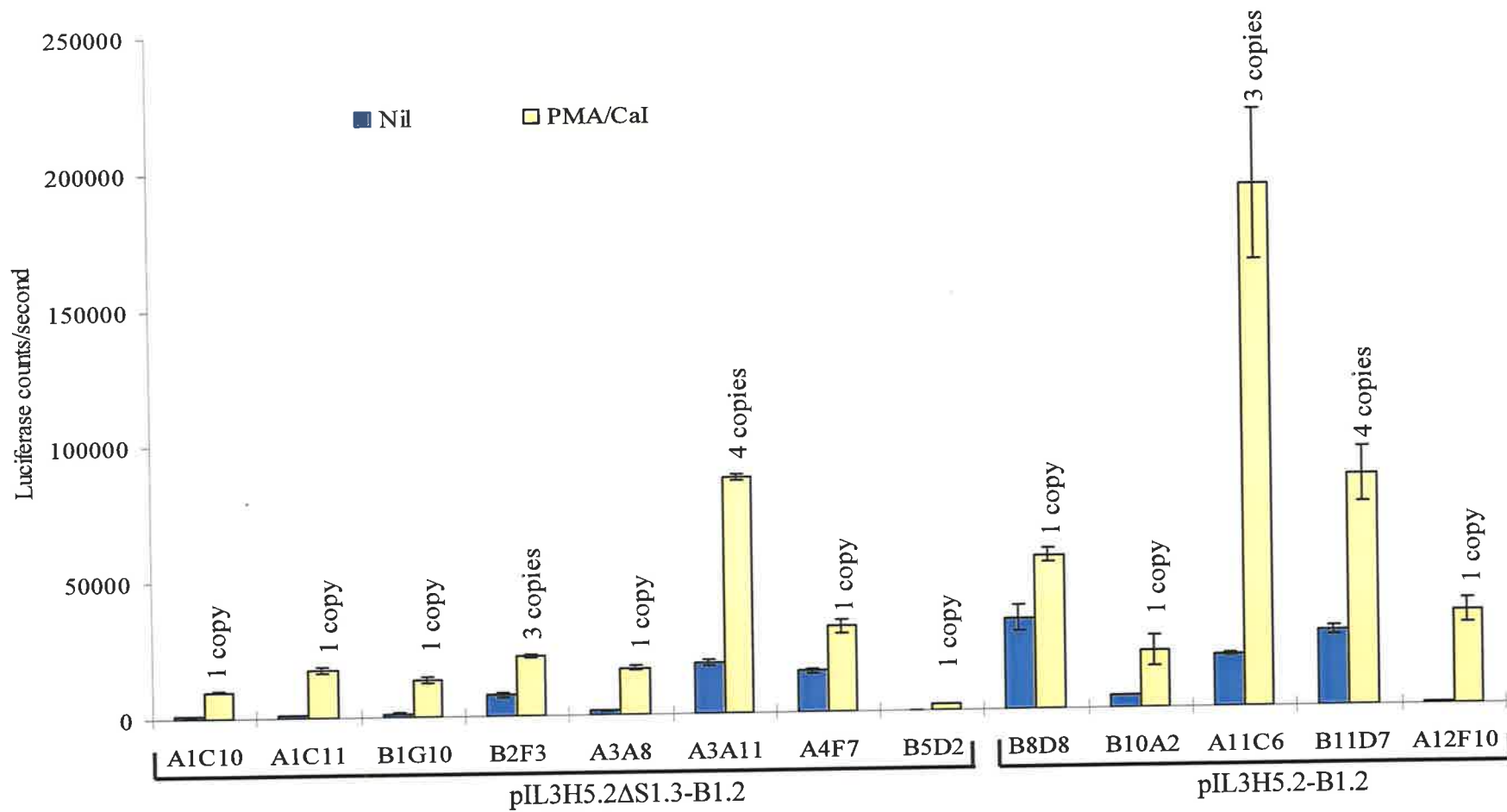


Fig. 5.15. Luciferase activity of the single cell clones I

Average luciferase activity of 2-3 measurements of the unstimulated (Nil) and the PMA and CaI (PMA/CaI) stimulated single cell clones. Error bars represent standard error of the mean. Clones from two constructs (pIL3H5.2ΔS1.3-B1.2 and pIL3H5.2-B1.2) are presented.

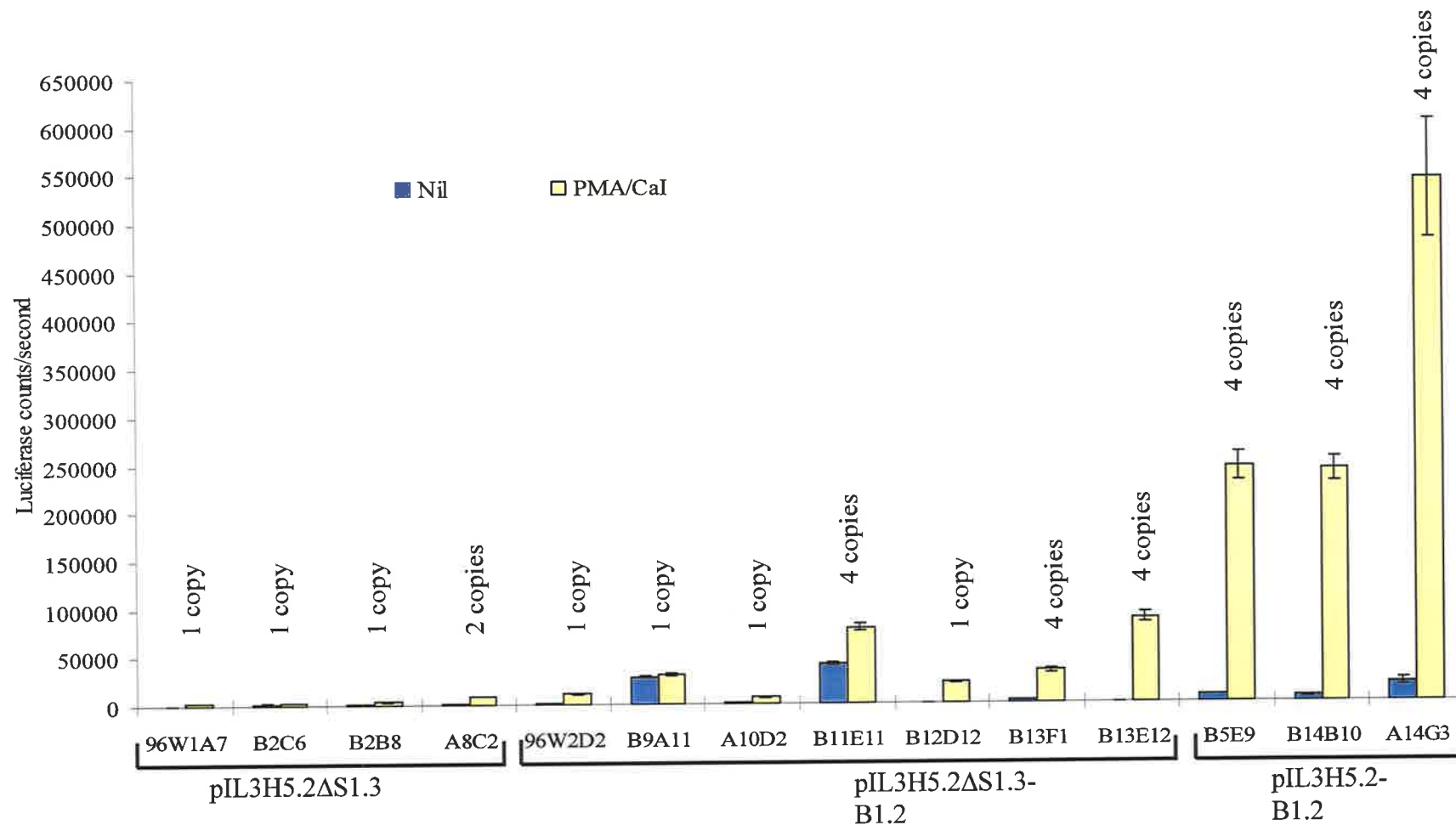


Fig. 5.16. Luciferase activity of the single cell clones II

Average luciferase activity of 2-3 measurements of the unstimulated (Nil) and the PMA and CaI (PMA/CaI) stimulated single cell clones. Error bars represent standard errors of the mean. Clones from three constructs (pIL3H5.2ΔS1.3, pIL3H5.2ΔS1.3-B1.2 and pIL3H5.2-B1.2) are presented.

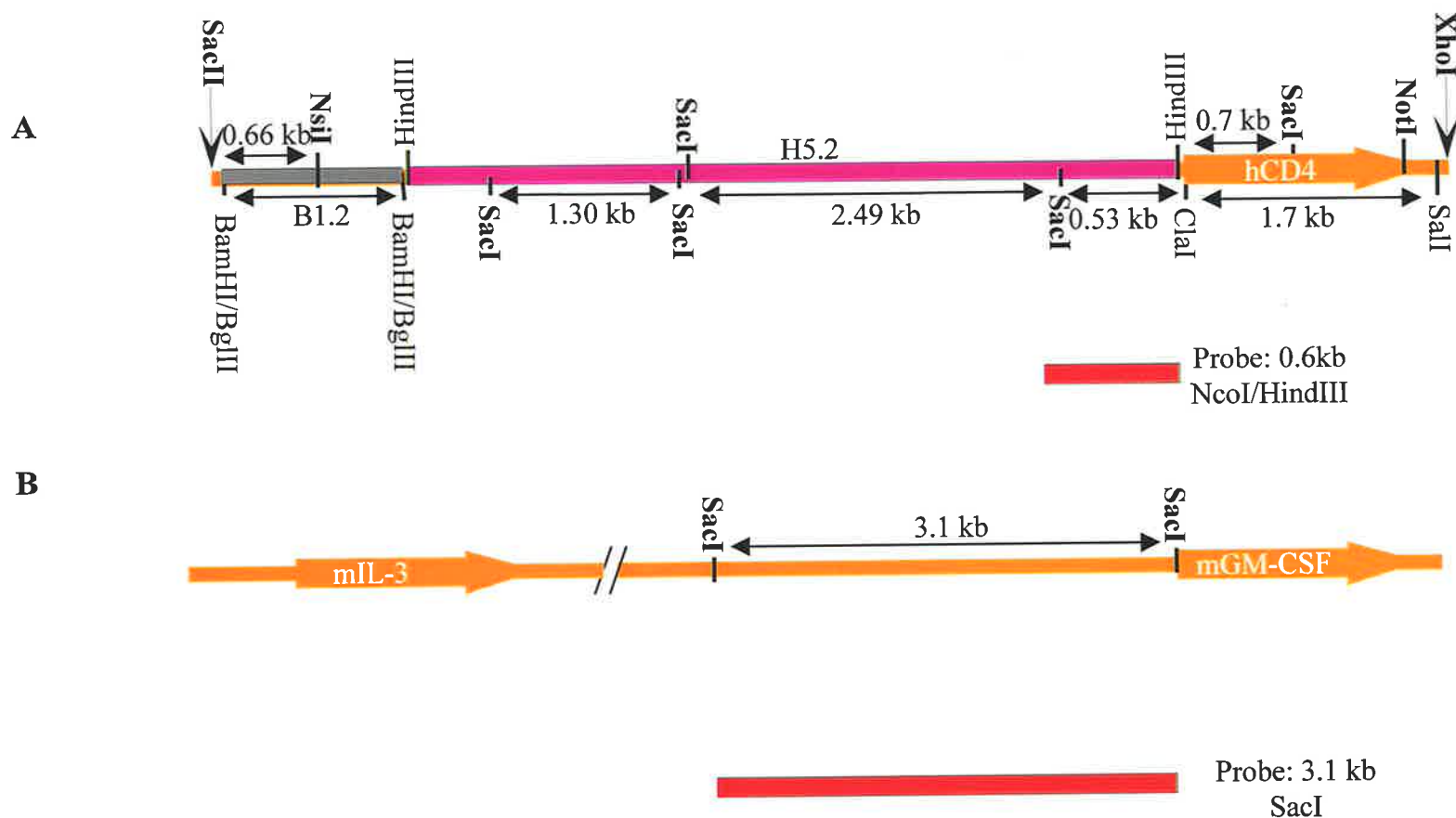


Fig 5.17. Schematic representation of the human CD4 construct and the mouse GM-CSF locus.

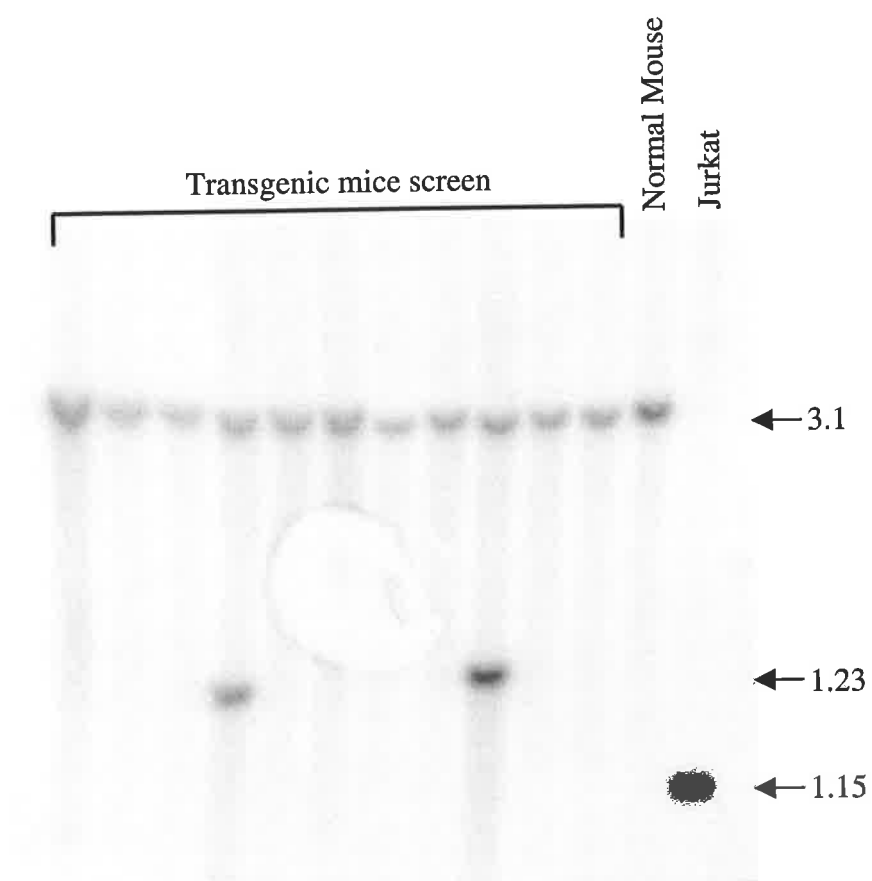
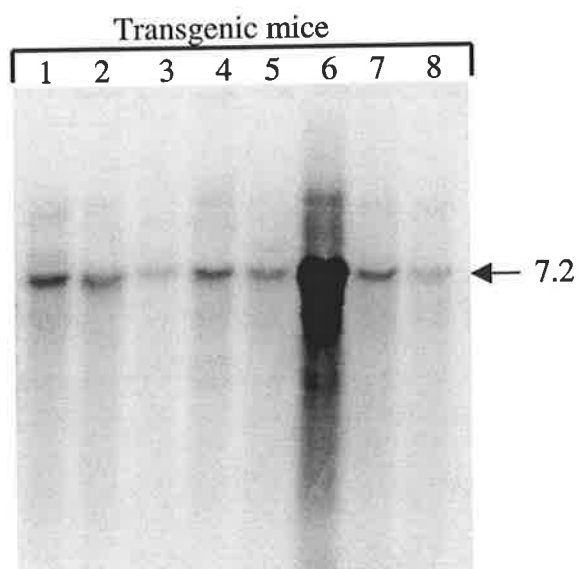
(A) A schematic representation of the XhoI/SacII fragment used to generate transgenic mice from the human CD4 construct as described in the text. The locations of the restriction enzymes used in the Southern blot hybridisation analysis are indicated. The distances between some of these sites are indicated and marked with arrows. Also shown is the 0.6 kb NcoI/HindIII fragment from the human IL-3 promoter used as a probe. (B) Location of the SacI sites and the 3.0 kb SacI probe in the endogenous mouse GM-CSF used in figure 5.18 to estimate copy number.

H5.2 region. Pups born from eggs injected with these constructs were screened for the presence of the transgene by Southern blot hybridisation analysis. DNA extracted from tail cells was digested with SacI. Restriction maps of both the transgene and the endogenous mouse IL-3/GM-CSF locus are presented in figure 5.18. Southern blot filters were hybridised with a ^{32}P -labelled mixture of a 0.6 kb NcoI/HindIII fragment of the human IL-3 promoter excised from pIL3H and a 3.0 kb fragment from the upstream region of the mouse GM-CSF. Two bands should be generated if the mouse DNA contained the transgene (1.23 and 3.1 kb) but only one band from normal mouse DNA (3.1 kb). The ratio of the intensity of the transgene band over the mouse genomic band gave the relative number of copies of the transgene are present as shown in figure 5.18A. Eight transgenic mice were obtained. The DNA of the eight transgenic mice was further tested for rearrangement of the transgene by digesting with NsiI/NotI and hybridising the filter with the 0.6 kb NcoI/HindIII IL-3 promoter probe. Both NsiI and NotI cut only once in the transgene. NsiI enzyme cut in the B1.2 just outside the 5' end of the core IL-3 enhancer, while the NotI enzyme cuts just 3' of the translation termination signal at the end of the hCD4 gene. Since the probe is a human probe only one band is expected if the integrated DNA is intact. On the other hand if one or more copies of the transgene is rearranged, multiple bands will be shown. The result of this Southern blot is shown in figure 5.18B. Most of the transgenes appear to be intact except for number 6, which has a very large copy number and may have a small degree of rearrangement.

Analysis of human CD4 expression in these mice was performed by Andrew Bert in this laboratory. Splenocytes from progeny of all of the eight transgenic mice were prepared. T cells were generated by culturing with concanavalin A and Murine IL-2 as described in materials and methods. Cells were either unstimulated or stimulated with PMA/Cal as

Fig. 5.18. Example of Southern blot hybridisation assay of transgenic mouse tail DNA

Transgenic mouse DNA was digested with *SacI*. Southern blots from these DNA digests were hybridised with a mixture of the human 0.6 kb *NcoI*/*HindIII* IL-3 probe and a 3.1 kb *SacI* fragment from the mouse GM-CSF locus. The ratio of the 1.23 kb human band to the 3.1 kb mouse band was used to determine a relative copy number. Panel A shows the analysis of one of the Southern blot hybridisation assays. Only two transgenic mice are shown from this screen. Normal mouse DNA and Jurkat T cell DNA were used as controls. Jurkat T cell DNA generated a 1.15 kb band from the human IL-3 locus. A 1.23 kb band was generated from the human CD4 construct. Panel B shows the Southern blot hybridisation analysis of transgenic mouse DNA digested with *NsiI* and *NotI* and hybridised with DNA from the human CD4 gene. Only one band (7.2 kb) is expected resulting from the transgene. Appearance of multiple bands indicates that the transgene is rearranged. Panel C gives the relative copy number of the transgenic mouse.

A**B****C**

Mouse	Copy number
1	13
2	6
3	2
4	20
5	20
6	59
7	5
8	3

described for 9 hours. Cells were stained with either PE-conjugated non-specific antibody or PE-conjugated mouse anti human CD4 antibody and analysed on the FACS machine. Only three mice (mouse # 1, 3 and 8) expressed the transgene, and in a maximum of 28% of the T cell blasts. The expression did not correlate with the transgene copy number. This experiment provided further evidence that the H5.2 region is not sufficient for an LCR function. FACS analysis results are presented in figure 5.19 from one positive and one negative transgenic mouse. In addition as a positive control, the human CEM CD4+ T cell line was used to control for the CD4 staining (figure 5.19).

5.7. EFFECT OF DELETING THE SS245 ENHANCER AND THE -4.1 DH SITE FROM THE H5.2 REGION IN TRANSIENT TRANSFECTION ASSAYS

To further examine the role of the SS245 enhancer it was studied in its natural promoter context in the H5.2 fragment in transient transfection assays. The SS245 enhancer was deleted by partially digesting pBlue-B1.2-H5.2 with *Ecl136II* and *StuI* and re-ligating the plasmid. Similarly, the -4.1 DH site was deleted by partially digesting the pBlue-B1.2-H5.2 with *StuI* and *EcoRV* to remove a 550 bp fragment. Then the resulting plasmids were cut with *HindIII* and 4.95 and 4.65 kb fragments were isolated and cloned into *HindIII*-linearised pXPG-B1.2 plasmid. Three plasmids (pXPG-H5.2-B1.2, pXPG-H5.2 Δ SS0.245-B1.2 and pXPG-H5.2 Δ SE0.55-B1.2) were transiently transfected into Jurkat T cells and stimulated with PMA and *CaI*.

Figure 5.20 shows the result of the transfection experiment. Although there was reduction in the level of luciferase activity of both plasmids carrying a deletion, that reduction was statistically insignificant between each of pXPG-H5.2 Δ ES0.245-B1.2 and

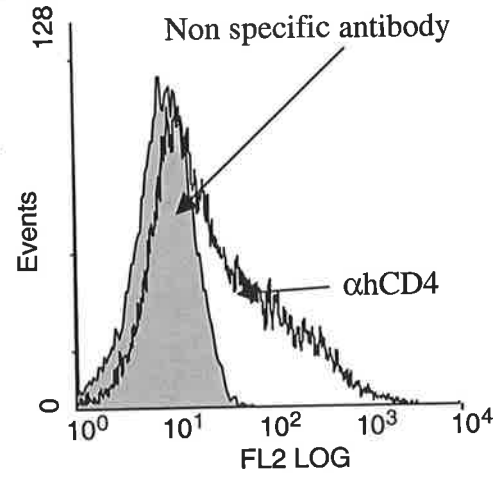
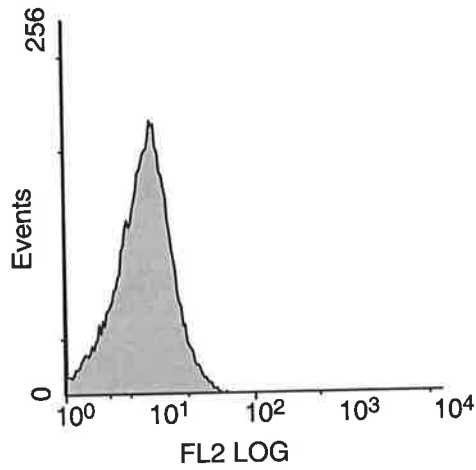
Fig. 5.19. FACS analysis of T cell blasts from CD4 transgenic mice.

One expressing (Phoenix) and one non-expressing (Maxwell) human CD4 transgenic mouse T cell blast analysis are shown. T cell blasts from these mice and human CEM T cells were stained with either anti-human CD4 or a non-specific PE conjugated antibodies. Fluorescent activity from unstimulated and PMA and CaI stimulated T cell blasts are shown. The CEM cells were used as a positive control.

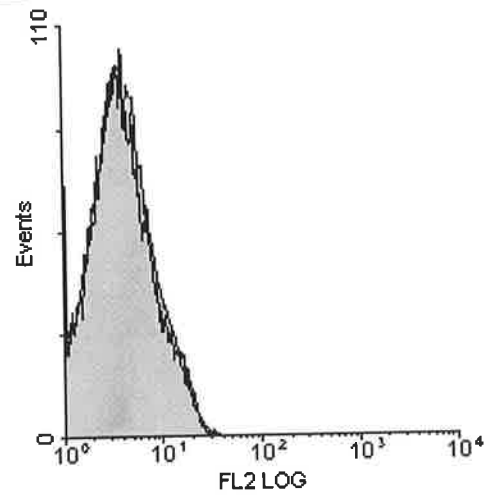
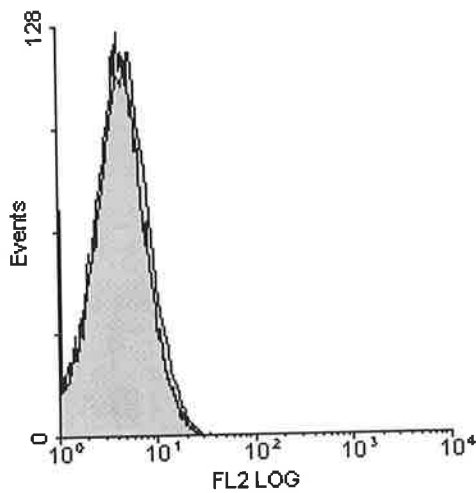
Not stimulated

PMA/CaI stimulated

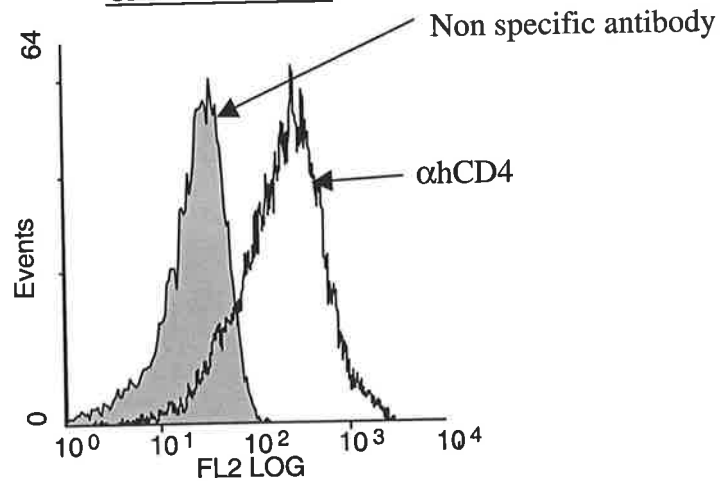
Mouse #1 (13 copies)



Mouse # 4 (20 copies)



CEM T Cell Line



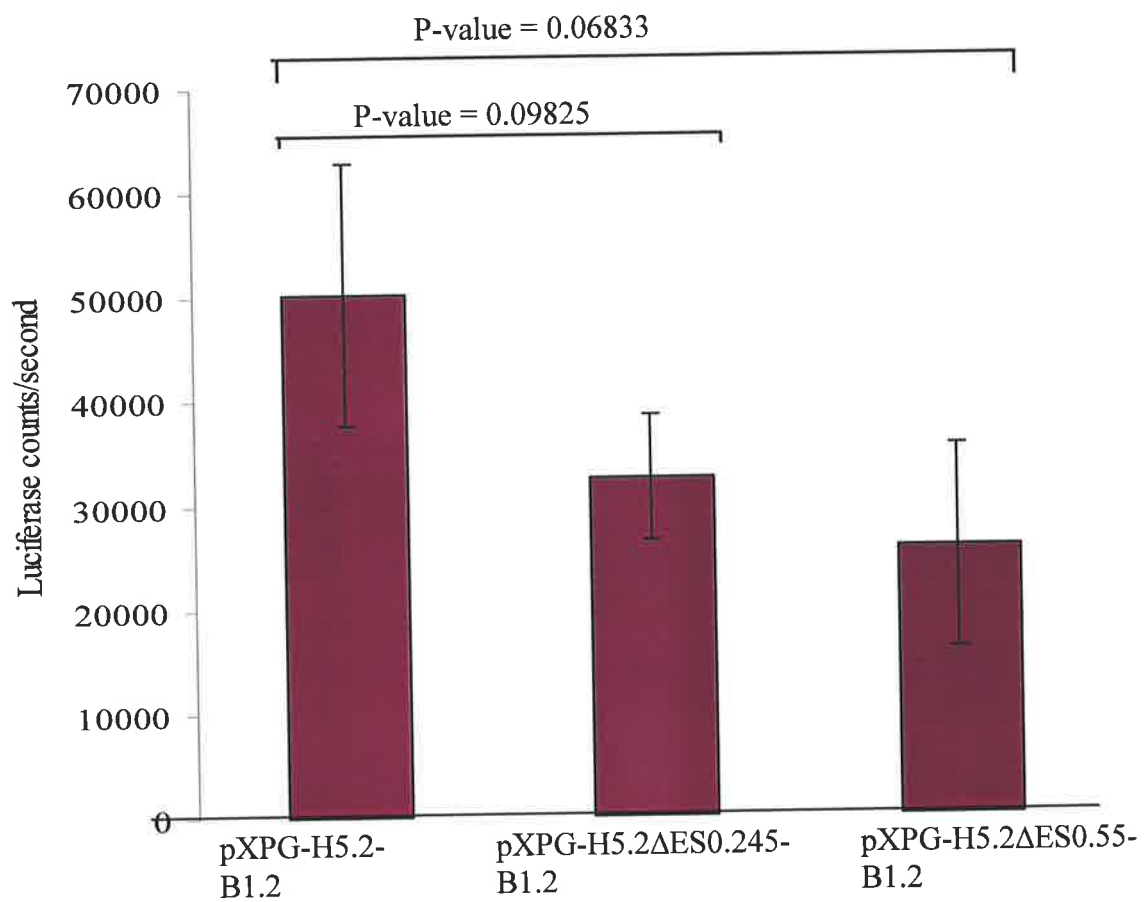


Fig. 5.20. Luciferase activity of the H5.2 constructs in transiently transfected Jurkat T cells.

The pXPG-H5.2-B1.2, pXPG-H5.2ΔES0.245-B1.2, and pXPG-H5.2ΔSE0.55-B1.2 constructs were transiently transfected into Jurkat T cells. Cells were stimulated with PMA and CaI. The luciferase activities are presented as means of 5 to 6 independent transfections. The error bars represent standard error of the means.

pXPG-H5.2 Δ ES0.55-B1.2 plasmids and pXPG-H5.2-B1.2 ($p=0.09825$ and 0.06833 respectively).

5.8. DISCUSSION

The experiments described in this chapter sought to identify functions for the DH sites present in the H5.2 region upstream of the IL-3 gene in the context of the chromatin. In chapter 3, an enhancer was found (SS245) next to a prominent DH site within the S1.3 region. All the previous work described for the enhancer and the S1.3 fragment was performed in transient transfection assays. In transient transfection assays, the constructs introduced into cells stay un-integrated. Hence, expressions of these constructs are not affected by the chromatin environment of the whole genome. Expression of endogenous genes is affected by the interplay between transcription factors and chromatin. For this reason, the studies described in this chapter sought to understand how the DH sites upstream of the IL-3 gene function in the context of the chromatin.

Initially, these constructs were tested in cell lines generated by stably integrating plasmid constructs in the chromosomal DNA of Jurkat cells. Polyclonal stable line experiments were performed. At least some of these experiments showed that each of the enhancers, which have been defined in transient transfection assays (the B1.2 and the SS245) increased the activity of the IL-3 promoter. In addition, when both of these enhancers were linked together, the IL-3 promoter activation was further increased in an additive effect. This implied that both of these enhancers cooperate together to insure a high level of expression of the IL-3 gene when stimulated. None of the other 2 DH sites

present between the IL-3 promoter and the S1.3 region had any effect on the IL-3 promoter activation level as judged by the activity of the pIL3H5.2ΔS1.3 construct.

The results from the three stable transfection experiments were variable. The luciferase activities of the different constructs changed with passaging the cells over a long period of time. It is possible that when the introduced DNA integrated into the chromosomal DNA, some may have integrated within genes that influence cell growth. Hence some of the cells may divide at a faster or slower rate than others. Hence, with the continuous passaging of cells the slow dividing cells may be eliminated while the fast growing cells would take over. In the first experiment, it was evident that the cells which had the IL-3 constructs and the pE^otk.neo were growing at a slower rate than the cells which had only the pE^otk.neo plasmid as indicated by the drop of copy numbers.

Another problem with stable transfection is that inserted DNA tends to integrate at random which make their expression position-dependent. If these DNA fragments integrated next to a gene that is controlled by a strong enhancer, then the expression of the inserted reporter gene is expressed at a higher level. However, if the same construct is integrated in a silenced area (in heterochromatin) the expression becomes variegated or repressed. That is, progeny of a cell may express the reporter gene at the same level; some may be silenced, some may express at a high level, and others may express in between.

It is reported that LCRs protect integrated genes from the position of integration effect in many systems as discussed in chapter one. In this study, we tested the hypothesis that the 5.2 kb region upstream of the IL-3 gene contains an LCR. For that purpose, single

cell clones from stably transfected Jurkat T cell lines were generated. Analysis of these single cell clones showed that the H5.2 region is not sufficient to protect the luciferase reporter gene from position effects. Some of the single cell clones that had the same copy numbers had high activity and some had low activity. Moreover, the expression did not correlate with relative copy number. The luciferase expression level of some clones that had 3 copies was the same as some clones that had one copy.

Transformed cell lines often have genetic abnormalities as a result of the transformation process. Because these stable lines were generated from transformed cell lines (Jurkat T cells), the result could be due to the abnormalities of these cells. It may be that a critical factor for an LCR function is missing in these cells. In addition, stably transfected cell line experiments do not take into consideration the developmental program that leads to the activation of specific locus. Hence, we wanted to confirm the result obtained in stable lines in transgenic mice. The H5.2 and the B1.2 regions were linked upstream of the human CD4 gene. Since CD4 is a surface marker, the use of the CD4 gene as a reporter gene provided an advantage over the luciferase gene.

The transgenic mice experiment also indicated that the H5.2 is not sufficient to confer high level expression, position independence and copy number dependent expression on the human CD4 gene. Out of eight transgenic mice generated, only three mice expressed the transgene. All three expressed CD4 when their T cells were stimulated with PMA and CaI. Hence the expression was inducible. However, the expression was low and only detected in between 20 and 30 % of all the T cells and did not correlate with copy number.

These experiments indicated that more sequences upstream or downstream of the IL-3 gene might be needed to confer high level, copy number-dependent, and position-independent expression. The DH sites downstream of the IL-3 gene may be important for such an LCR function. Future experiments should include these DH sites. Another possibility is that the IL-3 gene in its native position may not need an LCR. The IL-3 gene seems to be regulated by an enhancer that is located 14 kb upstream and is separated from the next gene locus upstream by a large distance (Frazer et al., 1997). So, this large distance that separates the IL-3 locus from the next 5' locus may be enough to minimise the influence of that locus on IL-3. Another possibility is that there is normally a boundary element upstream of the IL-3 gene that in conjunction of the DH sites downstream of the IL-3 gene are needed to protect the IL-3 gene from the effect of the surrounding genes.

Future experiments with stably transfected cells or transgenic mice to study the functions of the DH sites upstream of the IL-3 gene could adopt two approaches. The first approach is to determine the minimum region of the IL-3 locus that confers independence of expression on the transgene. Once that is determined, then specific DH sites could be deleted and the effect on the transgene expression or the loss of LCR function may be assessed. The other approach is to link specific DH site or multiple sites to a transgene as was done in this study but to add insulator elements like the scs or scs' elements (Kellum and Schedl, 1992) around the transgene to protect them from position effects. The function of the linked elements can then be assessed. Another approach that can be taken is to delete DH site elements from the endogenous mouse IL-3 locus in a similar fashion to the studies recently been done in the β -globin locus ((Reik et al., 1998; Cimborra et al., 2000). This would, however, require a comprehensive analysis of the

chromatin structure and regulation of the mouse IL-3 locus, which is poorly characterised when compared to the human IL-3 gene.

LCRs have been shown to function in a tissue-specific manner. For example, the β -globin LCR functions only in erythroid cells (Grosveld et al., 1987), while the TCR (Diaz et al., 1994), CD2 (Lang et al., 1988), and CD4 (ref) LCRs function only in T cells. Moreover, linking the CD2 (Graves et al., 1989) or the TCR (Ortiz et al., 1997) LCRs to the β -globin gene confers expression of the β -globin gene in T cells only. Although these constructs contain the β -globin promoter and enhancer, which are erythroid-specific, expression was only seen in T cells. These experiments suggest that the tissue specificity of the promoters can be over-ridden by dominant-acting LCRs. Hence, a T cell-specific LCR will only open the chromatin in T cells and maintains a silenced chromatin conformation in non-T cells. Ortiz et al. (1997) also showed that when the DH sites 2 to 6 of the TCR α LCR are linked to the β -globin reporter gene, the chromatin opened ubiquitously in many tissues such as liver, heart, spleen, kidney, lung and the thymus (Ortiz et al., 1997). However, when DH sites 1, 7 and 8 are added, general sensitivity of the locus and expression pattern of the reporter β -globin gene becomes restricted to T cells and inhibited in other cell types (Ortiz et al., 1997). Moreover, DH sites 1, 7, and 8 are not sufficient to confer an LCR function on their own (Diaz et al., 1994).

The IL-3 locus LCR may be activated in a similar manner to the TCR α LCR. In the IL-3 locus the 3' DH sites are constitutively present and may open the chromatin ubiquitously but the tissue-specific expression of IL-3 may be controlled by the 5' elements. Hence, the 5' elements may not be responsible for chromatin-opening activity of the IL-3 locus. That would explain the result that was obtained when experiments in stable lines and

transgenic mice using the 5' locus gave variable results. Moreover, the transgenic experiment showed that the CD4 reporter gene expression was not active in five out of eight transgenic mice and the other three had a low expression level but T cells. This indicated that the 5' DH sites might not be able to open chromatin sufficiently to obtain high level and T cell specific expression. The DNase sensitivity of the transgene in these transgenic mice was not studied. In future studies the chromatin structure of the 5' DH sites in these transgenic mice should be studied to ascertain whether they can open the chromatin. The low expression level seen in the three transgenic mice could be due to the integration of the transgene next to a locus that is poised for expression.

The -4.5 kb and the -4.1 kb DH sites resemble the DH sites 2 and 3 of the β -globin LCR. DH site 2 has a classic enhancer activity in transient transfection experiment (Ryan et al., 1989; Philipsen et al., 1990) similar to the -4.5 kb DH site spanning the SS245 enhancer. The -4.5 kb DH site may be involved in the developmental activation of the locus and may contain an open chromatin activity similar to that of the DH site 3 of the β -globin locus (Bungert et al., 1995). Although, DH site 3 of the β -globin locus has an enhancer activity only in the context of the chromatin, the -4.1 kb DH site was not tested for enhancer activity in the context of the chromatin. It would be interesting to test a fragment spanning the -4.5 kb DH site for enhancer activity in stable lines or transgenic mice. The other 2 DH sites located between the -4.1 kb DH site and the promoter did not have any detectable function in stable lines. A fragment containing these sites has an activity similar to the promoter alone. Not all T cell lines that were tested contain these sites at -1.3 and -3.3 in the IL-3 locus. They may be markers for intermediate stages of T cell development. If the mouse IL-3 locus contains similar DH sites then mice with these sites deleted may help to elucidate their function.

CHAPTER 6: FUNCTION OF THE 3' IL-3 DH SITES

6.1 INTRODUCTION

The human IL-3 gene is separated from the GM-CSF gene by about 10.5 kb. Both genes are transcribed in T cells, mast cells, basophils and eosinophils. In addition to these cells, GM-CSF but not IL-3 is produced by endothelial cells, monocytes and many other cell types such as fibroblasts and epithelial cells. One of the questions of interest here is how this differential regulation is achieved. The GM-CSF enhancer has been shown to activate the IL-3 promoter if placed upstream (Cockerill et al., 1993). The question becomes then what stops the GM-CSF enhancer from activating the IL-3 gene from its normal downstream location in endothelial cells for example. It was proposed that the ubiquitous constitutive sites downstream of the IL-3 gene might function as a boundary element to insulate the IL-3 gene from the effect of the GM-CSF enhancer. However, the studies in this chapter identified what may be a pseudo-gene in this region.

Analysis of the three DH sites immediately downstream of the human IL-3 gene showed that these sites were found in all of the cell types that have been tested, that included endothelial, epithelial, mast, erythroid, myeloid, T and B cells (Cockerill et al., 1999; Cockerill unpublished data). The sequence between the human IL-3 and GM-CSF genes was analysed and found to contain an open reading frame just downstream of the distal 3' IL-3 DH site (Cockerill, P. personal communication). The sequence of this region and the open reading frame is presented in figure 6.1 and 6.2. The sequence of the DNA spanning DH site 7 resembled a promoter. It has a TATA box like sequence and computer analysis predicted binding for E-box, Oct, and NF- κ B proteins. Further

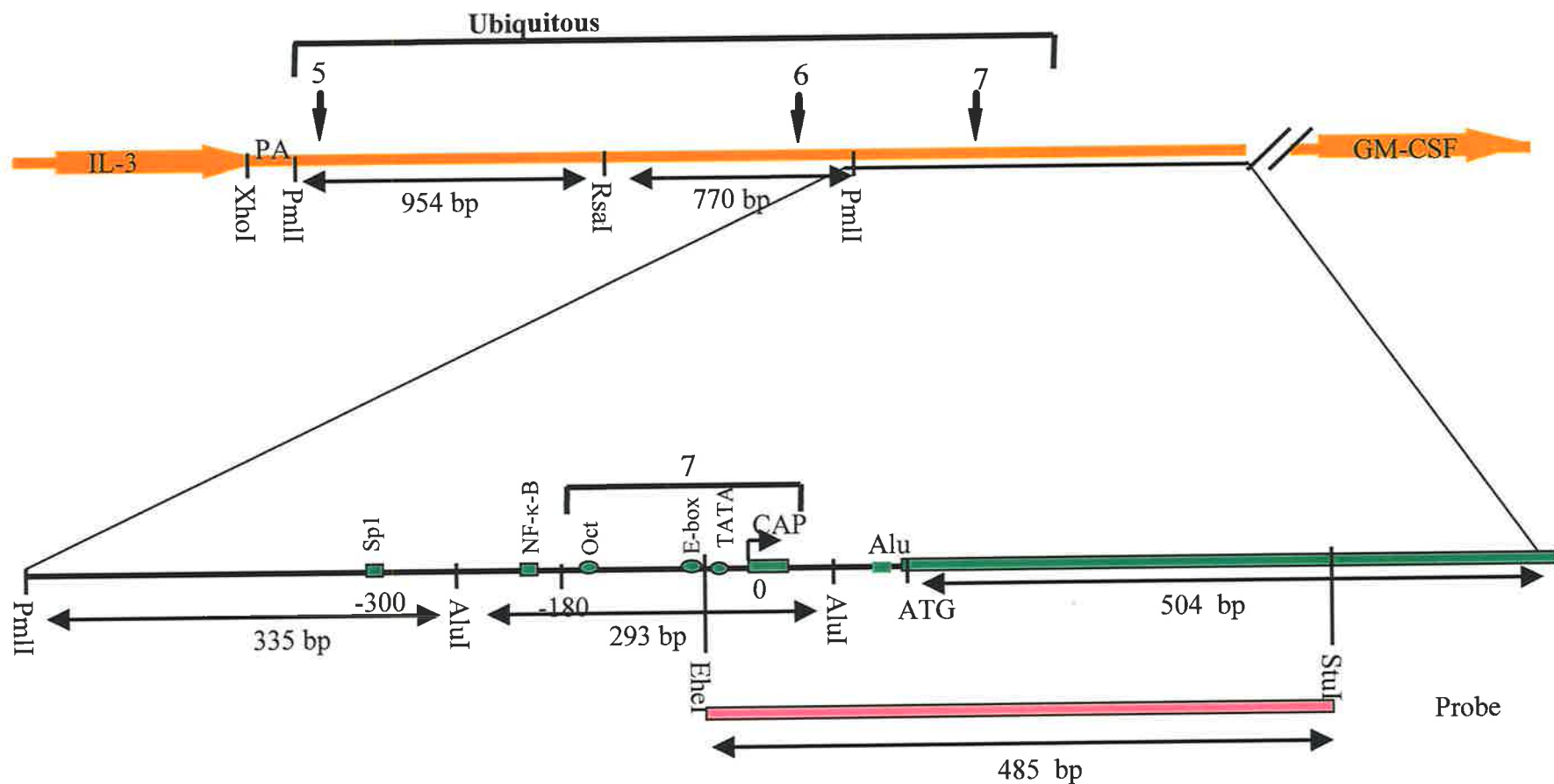


Fig 6.1. Map of the downstream region of the human IL-3 gene

The location of the DH sites downstream of the IL-3 gene and the restriction enzyme sites used for the cloning of the DNA spanning the DH sites are shown. The region spanning the DH site 7 and the downstream DNA containing the open reading frame is enlarged. Location of the TATA box, E-box, CAP site, Oct, NF-κB, Sp1, and the Alu repeat element are shown. The open reading frame is represented by a long box and the ATG potential initiation of translation for the open reading frame is shown. Distances between restriction enzyme sites were indicated and represented by arrows.

Fig. 6.2. Sequence of the open reading frame and the putative promoter.

The sequence of DNA spanning DH site 7 and the open reading frame. Locations of some of the potential transcription binding sites are shown. The TATA-box, E-box, and RNA cap signal are shown. The Alu repeat element is underlined and in bold. The potential translation start site is marked by a 90°-bent arrow. The horizontal arrows mark the CMV homology region. The vertical arrows indicate the location of a potential intron. The predicted amino acid sequence is shown.

Sp1

CTGGCGGAGGGTGGCACGCGGAGGTGACATGCTGGAGAGGGCAGTGGGCGGACCTTTTCAGATGCGCTG

AluI

GGGGCTCCTCGCTGACCTTCTGGAATAGTATAGACTGCAGCTTAAAGCCAGACTGGGGCCAGAACCAAG

NF-kB

TGGCAGTCAGTTCCTGGACAGGCTGGGGGAATTTCTACATGCTTTGACAGAAGGTTTATGTTCCCTCCA

Oct

AATTTGTATGTTAAATGAATCCCCACTGTGATGGCATTAGGATGTGGAGCCTGTCTTTGGGCCAGG

E-box

TATA

TCTGTGTGTCCAGATGGCGCCAGGGGGCAGCATCGCCCCATTATAAATCCCAGAACCACCAGAGAAGGC

RNA Cap site

CCAAGTTCCTGCCTTCCACATCCCAGGTGTCTGCTCAGGGGAAAGGTGGATTACAGCTCTCAGCCAGG

Alu repeat element

AATTTTATTTTCACTTAAAACTATTGTTGGAGCGAGACTCCATCTCAAAAAAAAAAACAAAACTA

ATG GGA AAG CGT ATA TAC TCT TTA CTG AAA AAC
 Met Gly Lys Arg Ile Tyr Ser Leu Leu Lys Asn

→ CMV homology

TTG GAA ATG CAG AAA AAT AGA ACA AAG GGA AAA TTG ACA GAA AGG CAA GTG
 Leu Glu Met Gln Lys Asn Arg Thr Lys Gly Lys Leu Thr Glu Arg Gln Val

CCT AAG GAG GGC AGA CCT CAG AAG GCA CAA CAA GCC TCA CCC CTT GAC AGG
 Pro Lys Glu Gly Arg Pro Gln Lys Ala Gln Gln Ala Ser Pro Leu Asp Arg

AAT CCT CCC ACT CCA GGA AGG GGC TCT GTT CCT CCT GCC CTG GAC TCA AGC
 Asn Pro Pro Thr Pro Gly Arg Gly Ser Val Pro Pro Ala Leu Asp Ser Ser

CCC CAT TTG ACT CAG TGG AGC AAC TGC TCT GAG GAC CCT CCA CGG CTC CCC
 Pro His Leu Thr Gln Trp Ser Asn Cys Ser Glu Asp Pro Pro Arg Leu Pro

↓ Intron ?

CAG GCT GGG GCG GGC ATA GGT GAG ACA CTG GGC CAG GCC TTT CAG GCC TCC
 Gln Ala Gly Ala Gly Ile Gly Glu Thr Leu Gly Gln Ala Phe Gln Ala Ser

← end of CMV homology

TGG CCT TGT GCA GTT GGC CCA GCA CAG TGC GGT GGG GGT CAT TAC AGA CAG
 Trp Pro Cys Ala Val Gly Pro Ala Gln Cys Gly Gly Gly His Tyr Arg Gln

↓
 CAC AGG ACC GAT GGA TGG GCG TGG ACC TCA TCG GAG GCA TCC ATG GTG GCA
 His Arg Thr Asp Gly Trp Ala Trp Thr Ser Ser Glu Ala Ser Met Val Ala

CAG AAG GGG CCT GAG AGA GAA GTT CAT CCA GCA GAG AAG GGT GGG AAA AGC
 Gln Lys Gly Pro Glu Arg Glu Val His Pro Ala Glu Lys Gly Gly Lys Ser

ACA GGG AAG GAA GGA GGC AGA TGC CTG GGG GAG GAG GGC ATG GAG GTC GAG
 The Gly Lys Glu Gly Gly Arg Cys Leu Gly Glu Glu Gly Met Glu Ala Glu

AAG ACA AGG TAA GCGGAGGCACAGGAGAGACTAAGCAGTGGAGAGGCAGAGTGTGGCTGGGTG
 Lys Thr Arg Stop

CAAGTGTCAAGGTGAAGGCTGTGCCTGGGTACGTCTTTTCCT

upstream a potential Sp1 site is predicted. Immediately downstream of the TATA box are a potential RNA cap site and Alu repeat element. The open reading frame spans about 0.5 kb. The open reading frame contains a CMV homology region as marked in figure 6.2. The predicted amino acid sequence indicates a proline rich protein. The open reading frame may also contain an intron.

Although one of our original hypotheses was that these sites may function as a boundary element, this study focused in determining whether the open reading frame codes for an actual gene. Moreover, this study focused in determining whether the three DH sites represent regulatory elements of this new gene.

6.2 THE 3' MOST DH SITE FUNCTIONED AS A PROMOTER

Since the DH site 7 DNA sequence indicated that this site might function as a promoter element, a 293 bp AluI fragment spanning DH site 7 was cloned in the SmaI site of pXPG plasmid to make pXPG-A0.3. A 175 bp BamHI fragment from the SV40 enhancer was cloned in the BamHI site of pXPG-A0.3 to make pXPG-A0.3-B175 (pXPG-A0.3-SV40E). Both of these plasmids were transfected into Jurkat T cells and stimulated with PMA and CaI. Figure 6.3 shows the result of this transfection experiment. The A0.3 fragment was able to activate a low level of transcription of the luciferase gene. Moreover, the SV40 enhancer was able to substantially further increase transcription rate. The A0.3 kb element is also able to support a significant level of constitutive transcription as shown by the non-stimulated activity.

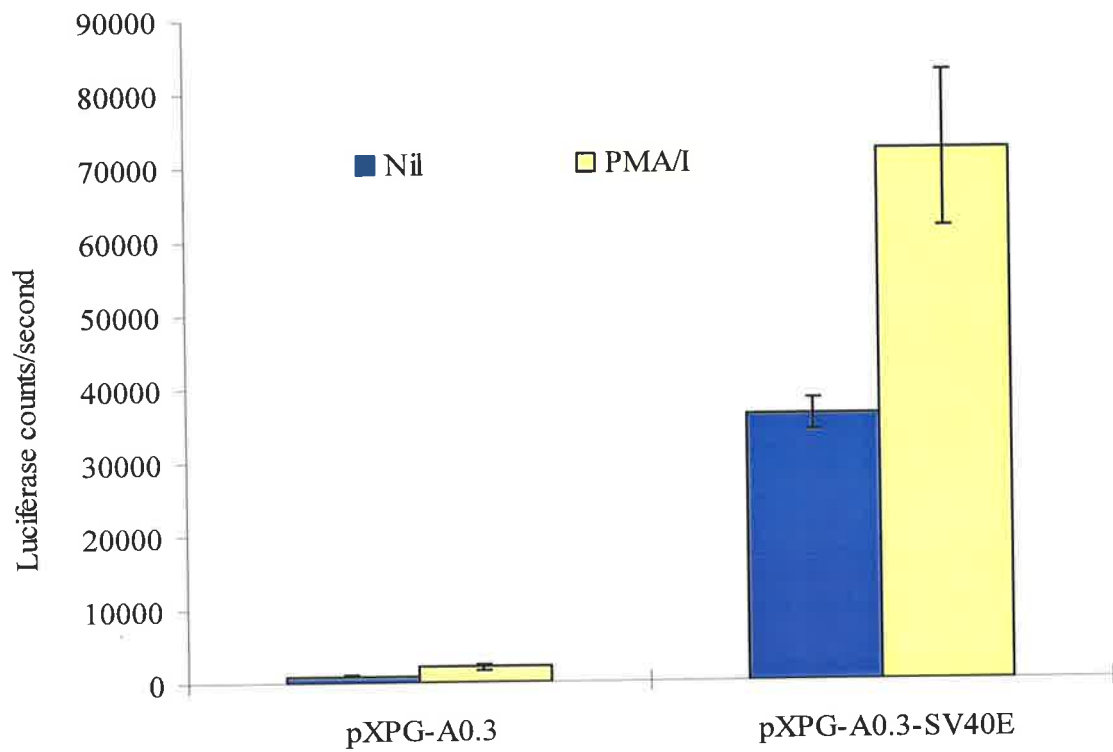


Fig. 6.3. Luciferase activity of the putative 0.3 kb promoter.

The 0.3 kb AluI DNA fragment spanning DH-7 was linked upstream of the luciferase gene in pXPG to make pXPG-A0.3. The 0.175 kb BamHI SV40 enhancer was linked upstream of the 0.3 kb AluI promoter element in pXPG-A0.3 to make pXPG-A0.3-SV40E. Constructs were transfected into Jurkat T cells. Activities are expressed as the mean of two unstimulated (Nil) and three PMA and CaI stimulated (PMA/CaI) independent transfections. The error bars represent the standard error of the mean.

6.3 THE MIDDLE SITE INCREASED THE ACTIVITY OF GM55 AND THE 3' ELEMENT

Since the A0.293 fragment was shown to activate transcription, the question then was whether DH sites 5 and 6 function as enhancers. For that purpose, two fragments were generated. A 0.77 kb PmlI/RsaI and a 1.7 kb PmlI fragment (figure 6.1) were cloned in the Ecl136II site of pXPG-A0.3 upstream of the A0.3 promoter element and in the SmaI site of pXPG-GM55 upstream of the GM55 promoter. Figure 6.4 shows the results of a transient transfection assay in Jurkat T cells with the above four constructs in addition to pXPG-GM55 and pXPG-A0.3. Cells were either left unstimulated or stimulated with PMA and CaI. The DNA fragments spanning either the DH sites 6 or 5 and 6 together did not have any enhancer activity upstream of the A0.3 promoter element. Although the DNA element spanning DH site 6 increased the activity of the luciferase gene driven by GM55 by about three fold (P-value = 0.04995), a fragment spanning both DH sites 5 and 6 increased the activity of the GM55 promoter only slightly (P-value = 0.16). Hence, the existence of enhancers upstream of potential promoter was not convincingly demonstrated.

6.4 NO TRANSCRIPTS OF THE OPEN READING FRAME WERE DETECTED BY NORTHERN BLOT ANALYSIS OR BY REVERSE TRANSCRIPTASE LINKED PCR

To determine whether the open reading frame structure results in production of actual RNA *in vivo*, total RNA was prepared from unstimulated and PMA/CaI stimulated Jurkat T cells, KG1a cells and K562 cells as described in materials and methods. Twenty µg

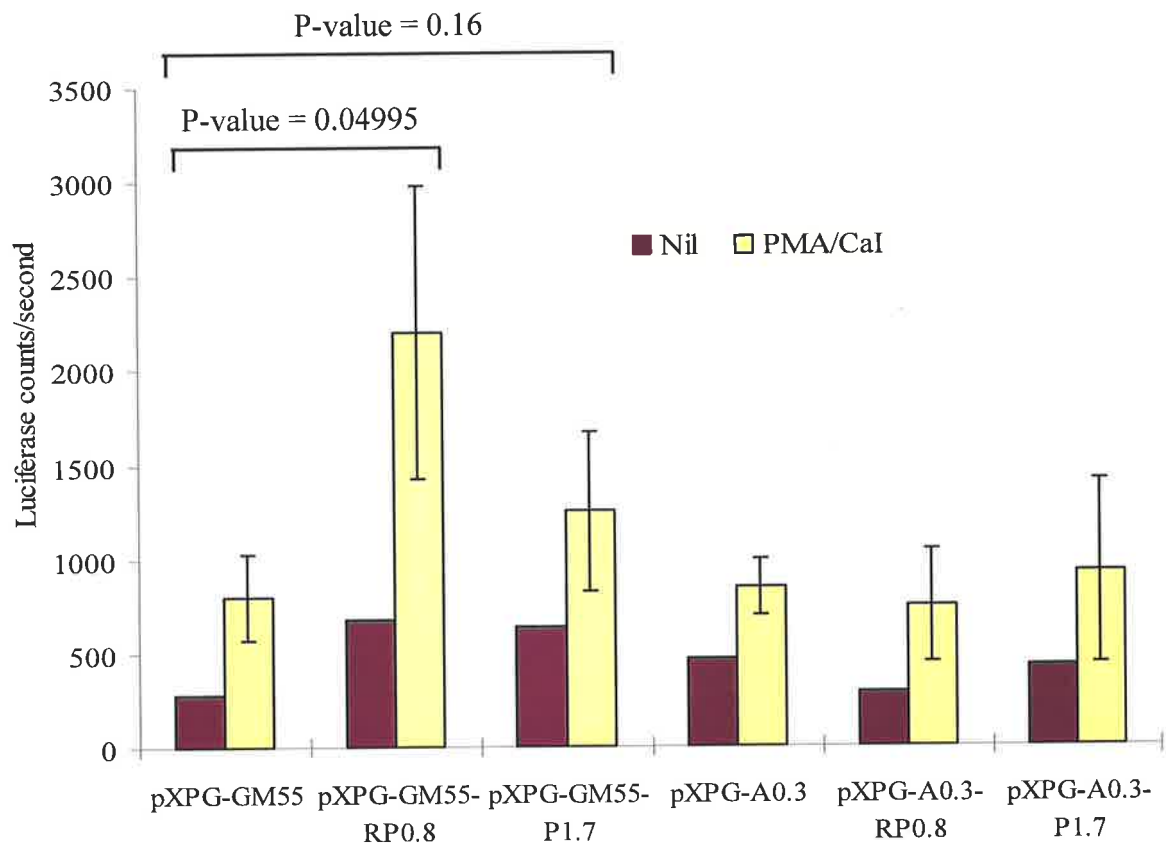


Fig. 6.4. Regulatory activity of DNA spanning DH sites 6 and 7.

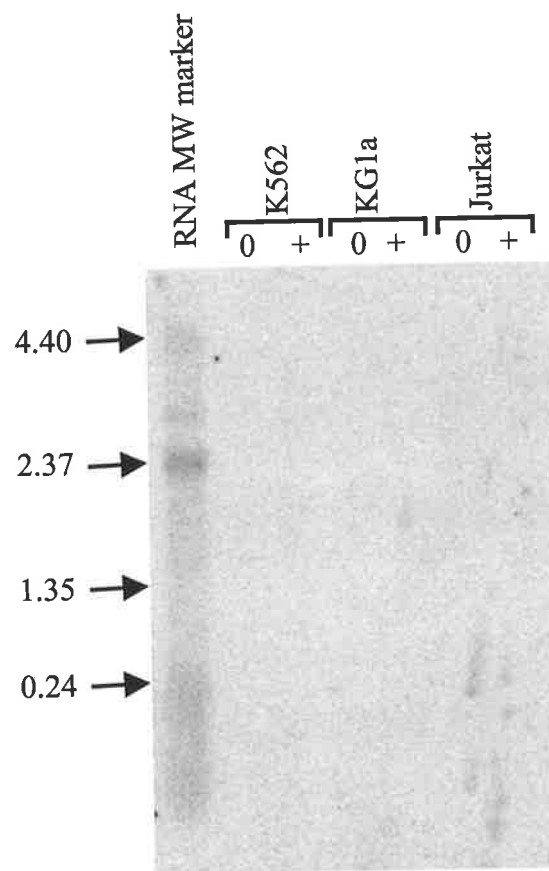
A 0.8 kb *RsaI*/*PmlI* and a 1.7 kb *PmlI* fragments spanning DH site 6 or DH sites 6 and 5 respectively were linked upstream of the promoters in either pXPG-GM55 or pXPG-A0.3. Constructs transfected into Jurkat T cells and 2 and 3 transfections were unstimulated (Nil) or stimulated with PMA and CaI (PMA/CaI) respectively. Activities are represented as means and error bars as standard error of the means.

were separated by electrophoresis and transferred to a Hybond N nylon filter. The filter was hybridised with 0.5 kb EheI/StuI fragment from the open reading frame region shown in figure 6.1, but no specific transcripts were detected (figure 6.5). To make sure that these results are true and not a result of RNA quality, the filter was hybridised with a 0.7 kb EcoRI/BamHI fragment from the human GM-CSF gene. As expected, only RNA from stimulated cells was positive, and a very strong GM-CSF band was detected indicating that the RNA was intact. cDNA was prepared from these RNA samples as described in materials and methods. PCR was performed using a pair of primers described in materials and methods section to amplify the open reading frame area. A 241 bp band was expected. Also included in this experiment was genomic DNA from Jurkat and KG1a cells as a control for the amplification. A fraction of these samples were loaded on an agarose gels and separated by electrophoresis. As shown in figure 6.6, products were generated in all samples, although PCR products from cDNA samples had different sizes to that of the genomic controls. A Southern blot hybridisation analysis was performed using PCR products from the experiment described above. In addition, other PCR products from cDNA of Stromal, U937, Nalm6, Molt4 and Hut 78 cells present in the laboratory from previous work were loaded on the gel. DNA was transferred to a Hybond N Nylon filter and hybridised with the 0.5 kb EheI/StuI probe. Bands appeared from PCR products of genomic DNA but not from cDNA samples except K562 cDNA PCR in lane 6. This band probably was generated from genomic DNA contaminant in the PCR reaction mix since the other K562 cDNA PCR reaction in lane 11 did not generate any band.

Fig. 6.5. Screening for mRNA production from the open reading frame by Northern blot analysis.

RNA from unstimulated (0) and stimulated with PMA and CaI (+) K562, KG1a, and Jurkat T cells were purified and tested in Northern blot hybridisation analysis with a probe from the open reading frame (A). RNA molecular size markers and their sizes are shown. No bands were detected. In B, hybridisation of the same filter with GM-CSF probe is shown. GM-CSF RNA was detected as expected from stimulated cells.

A



B

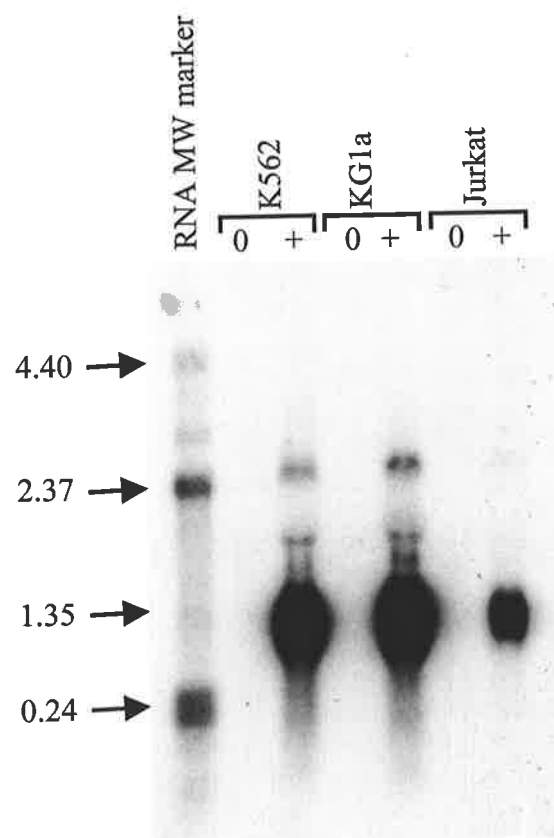
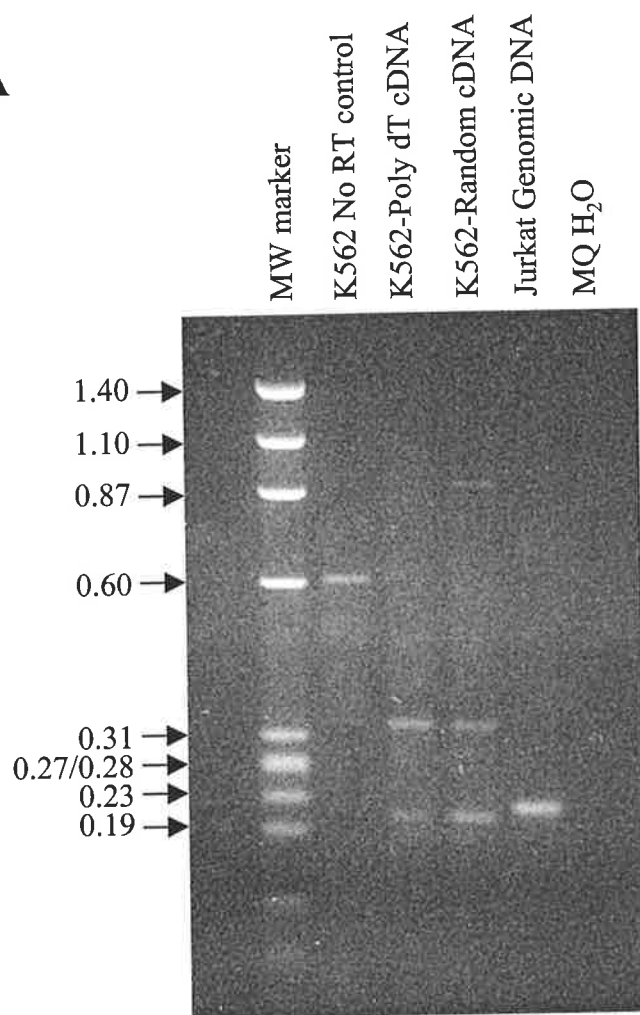


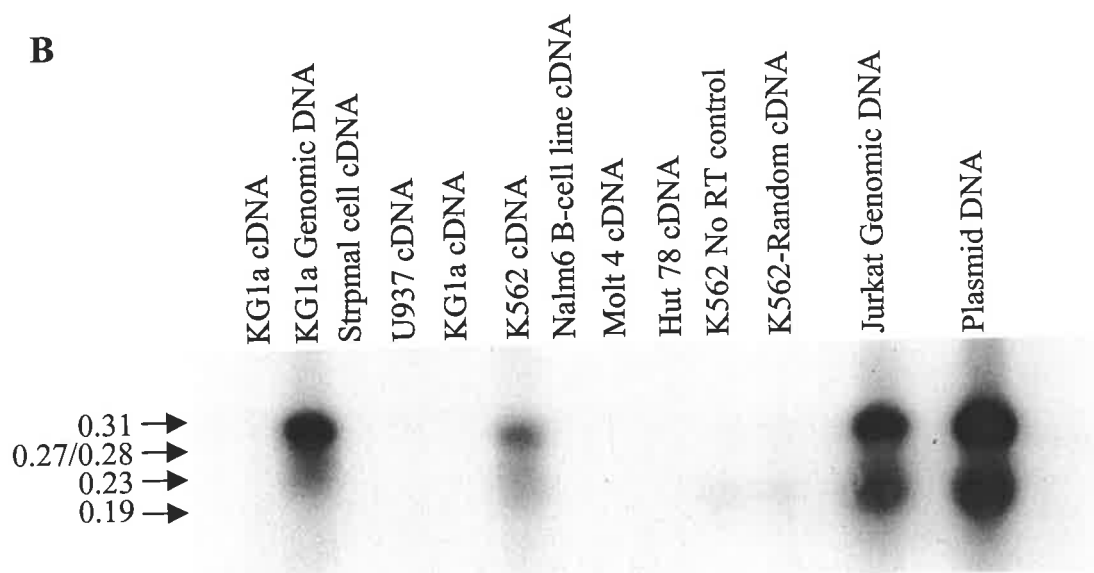
Fig. 6.6. Reverse Transcriptase based PCR.

Reverse transcriptase-based PCR was performed using RNA prepared from Jurkat T cell, K562, and KG1a. Panel A shows the PCR products from some of the samples. The MW marker, no RT control and two cDNA from K562 prepared either by poly dT or random primer, and a water control. Jurkat T cells DNA was used as a PCR positive control. The two RT K562 samples resulted in two PCR products with sizes close to 0.2 and 0.3 kb bands. The Jurkat T cells positive control produce one band a little bit larger than 0.2 kb. A 0.6 kb band resulted unexpectedly, from no RT cDNA negative control. Panel B shows a Southern blot hybridisation of the RT-PCR products (lanes 10 to 12) and some additional RT-PCR products already present in the laboratory from previous experiments (lanes 1-9 and 13). Two specific bands resulted from the plasmid and Jurkat T cell DNA at 0.2 and 0.3 kb. KG1a genomic DNA resulted in only one band. K562 cDNA PCR in lane 6 showed a band, which is most likely generated from a genomic DNA contaminant since the other K562 cDNA PCR in lane 11 did not generate any band. None of the other cDNA samples gave any bands.

A



B



6.5 DISCUSSION

This chapter investigated the possibility that a gene existed downstream of the IL-3 gene. Previously, it was observed that the sequence downstream has a gene-like structure as was discussed in the introduction to this chapter. Analysis of the DH site immediately upstream of the open reading frame indicated that this DH site could function as a promoter element to derive luciferase gene expression. A fragment spanning the middle DH site (DH site 6) did not increase the activity of this putative promoter element. However, it increased the activity of the GM55 promoter. This element may function as an enhancer although it is not clear why it did not activate the putative promoter element. The increased activity of the GM55 promoter could be simply an artefact of the transient transfection experiment and may not be due to a real enhancer.

No transcripts were detected by Northern blot analysis or by reverse transcriptase based PCR on Jurkat, KG1a, Molt4, U937, Nalm6, Hut 78, K562 or stromal cells except for one K562 reaction, which was suspected as a contaminant. These experiments indicated that this putative gene might be a pseudogene that is not functional *in vivo*. The DNA sequence of this open reading frame indicated a potential proline rich protein. Proline rich proteins are not common in humans. It is still possible that this gene may put out transcripts but at very low rate that can't be detected by the assays used here. None of the nine cell lines mentioned contained any transcripts from the open reading frame. It remains possible, however, that this gene-like element is expressed in other cell types outside of the range of cells tested here.

Pseudogenes arise either by gene duplication or as a result of insertion of genetic elements such as retrotransposons (reviewed in Mighell et al., 2000). Duplicated genes either evolve to become a new gene or are neutralised to become pseudogenes. Pseudogenes resulting from duplication may retain regulatory elements such as promoters and may make non-functional RNA or proteins due to mutations. Some gene duplications are not complete. Hence these sequences would not be functional from the start. Pseudogenes resulting from duplication are generally near the gene of origin. For example, two of the V-genes of the immunoglobulin λ light chain are believed to have evolved by a single duplication and translocation before gorilla and human diverged (Fripiat, et al., 1997).

Pseudogenes resulting from retrotransposon insertion are characterised by the presence of Alu or LINE repeats (Maestre et al., 1995), and 3'-polyadenylation signal (Loguercio and Wilkins, 1998; Ramos-Onsins and Aguda, 1998). Some pseudogenes may have a promoter like structure but lack any intron (Loguercio and Wilkins, 1998). Alu sequences are not found in the coding regions of transcribed genes (Mighell et al, 1997). The presence of Alu sequences in the coding regions of retrotransposons indicates the presence of a pseudogene. Most pseudogenes are unlikely to be transcribed. However, some make RNA such as the phosphoglycerate kinase (PGK) pseudogene (McCarrey, et al., 1996).

The open reading frame identified downstream of IL-3 is consistent with the presence of a retrotransposon pseudogene since it does not have any homology to any known human gene. Hence, this pseudogene can't arise by gene duplication. A region within the open reading frame is weakly homologous to CMV DNA. This is consistent with a viral origin

for this region, although it differs from the previously reported retrotransposon pseudogenes. Although it contains an Alu repeat, this Alu repeat is not in the coding region but it is just upstream of the ATG translation start site. There was some weak indication that an intron may exist within the open reading frame. Alternatively, it is possible that this region could be equivalent to any random piece of genomic DNA. This region was not compared to the corresponding region in the mouse genome. Hence, the sequence homology between human and mouse in this region was not established.

A possible function of this region is as an insulator element that insulates the IL-3 gene from the effect of the GM-CSF gene. The presence of a pseudogene may be unrelated to the main function of this region. This region should be tested for function either as an enhancer activity blocker and/or as a heterochromatin insulator that protects the transgene from the silencing effect of the chromatin.

CHAPTER 7: OVERALL DISCUSSION

In this study, we attempted to identify mechanisms controlling the transcriptional regulation of the IL-3 gene. Previously, a T cell specific enhancer has been identified at -14 kb upstream of the IL-3 gene that can increase the activity of the IL-3 promoter by at least two fold. In this thesis, work is presented that attempted to identify functions for the DH elements within the 5.2 kb region upstream of the IL-3 gene and for the DH sites within the 3 kb region downstream of the IL-3 gene. As indicated in the previous chapters, the DH sites in the region upstream of the IL-3 gene are restricted to T cells and some myeloid cell lineages such as mast cells and eosinophils. These 5' DH sites, particularly the DH site at -4.1 kb, are also sometimes found in some of the primitive AMLs and in primitive myeloid leukemic cell lines such as KG1a. Although this study did not find a function for the -4.1 kb DH site, it has identified another enhancer located at -4.5 kb. As discussed in chapter three, this enhancer was found to be active only in Jurkat T cells, CEM T cells, and mast T cells, but not active in Raji B cells, KG1a cells, and K562 cells. This pattern of activity mirrors the IL-3 gene expression profile.

The -4.5 kb enhancer (SS245) contained binding sites for NFAT, Sp1, AP-1, and AML1 and was active in Jurkat T cells which have these factors. However, NFAT seems to be primarily responsible for driving the activity of this enhancer in Jurkat T cells. None of the other transcription factors seem to influence the level of activation in this enhancer as their sites could be deleted with little effect on enhancer activity. Although this enhancer was active in HMC-1 cells, detailed analysis of what drives the enhancer in HMC-1 cells has not been undertaken. However, it has been determined by Cockerill et al. (unpublished data) that HMC-1 cells makes NFAT, but it is probable that it also makes

AML1, AP-1, and Sp1. The contributions of NFAT, AML1, Sp1, and AP-1 to the enhancer function in HMC-1 cells are not known. It is possible that the AML1 in conjunction with the NFAT and/or c/EBP drives the expression of the enhancer in myeloid cells such as HMC-1.

Since the DH site at -4.1 was found in primitive myeloid cells and leukemic cells, this site may prime the locus early in development and initiate chromatin remodelling, thereby allowing these cells to become IL-3 producers. The DNA sequence spanning the -4.1 kb DH site indicates that there are potential binding sites for GATA and EKLF like proteins (figure 7.1). DH site 3 in the β -globin LCR also contains binding sites for the GATA and EKLF proteins and it may perform a similar role. Moreover, the arrangement in the IL-3 locus of an enhancer located at -4.5 kb next to the -4.1 DH site that potentially has a chromatin opening activity is similar to the arrangement of DH sites 2 and 3 in the β -globin locus.

As discussed earlier, β -globin LCR DH site 2 has been shown to function as a classical enhancer in both transient and transgenic transfection experiments (Langdon and Kaufman, 1998; Tuan et al., 1989; Philipsen et al., 1990; Curtin et al., 1989; Ryan et al., 1989). Experiments *in vivo* showed that a fragment spanning DH site 2 alone directs tissue-specific high-level expression in transgenic animals, up to 50% of the endogenous mouse level (Ryan et al., 1989; Philipsen et al., 1990). This enhancer was found to contain binding sites for erythroid-specific and ubiquitous transcription factors. The enhancer activity of β -globin LCR DH site 2 was shown to be driven by multiple AP-1 binding sites that bind to the erythroid specific NF-E2 factor (Neyn et al., 1990a,b). The NF-E2 was shown to account for most of the enhancer activity of DH site 2 (Ney 1990a).

GGATCCATTAGACAGGGGTCCAGAGACCCCTCCTGAAACCTCCAG
BamHI

GGCTTTGGCAGACCCTGGGCTGCAGTCTTGGGCTGGCTCTCCTGT
PstI

CCGGGCCCTGGCACACCCTCCCTCATGCCTGCCCCAGGGGTGGGC
Eklf Eklf

ACGTGTGCTCATTTCCTTCTTGATAGCAATCAGCCCAGCCCACA
GATA

AAGGAGGTTT

Fig. 7.1. Sequence of the DNA spanning the –4.1 kb DH site.

The location of the putative EKLF and GATA sites in the DNA sequence of the region that span the –4.1 kb DH site downstream of the BamHI site are shown and underlined. The BamHI and PstI sites are also shown.

In addition, it was found that Sp1, GATA-1, and USF proteins bind to this enhancer (Philipsen et al., 1990; Caterina et al., 1994). Although these sites do not affect the enhancer activity of DH site 2, they seem to be involved in the opening activity of the chromatin. However, DH site 2 was found to function as an LCR only in multicopy but not in single copy transgenes (Ellis et al., 1993). Moreover, when a reporter gene construct containing the DH site 2 integrated in heterochromatin such as the centromere in single copy, the transgene was inactivated.

In contrast to DH site 2, β -globin LCR DH site 3 seems to dominantly open the chromatin (Bungert et al., 1995), and it directs expression of linked gene in single copy clones (Ellis, et al., 1996). DH site 3 does not have enhancer activity in transient transfection assays but it enhances the activity of reporter gene in the context of the chromatin (Fraser et al., 1990). DH site 3 contains binding site for EKLF, and EKLF knockout mouse resulted in the loss of DH site 3 formation (Wijgerde, 1996). Binding of Sp1 and EKLF has been shown to protect against position effect variegation of expression of transgenes (McMorrow et al., 2000). The core of DH site 3 contains 6 footprints that bind to three Sp1/EKLF sites (Philipsen et al., 1993). These sites bind to footprints 2, 4 and 6. GATA binds to footprint 1, 5 and to the 3' end of footprint 4. Although footprint 2 has been shown to bind Sp1, EKLF, and Sp3 *in vitro*, it binds EKLF *in vivo* (Gillemans et al., 1998). Footprint 1-3 is the smallest fragment that can activate the β -globin gene in transgenic mice (Philipsen, et al., 1993).

The region between the -4.1 and -4.5 kb IL-3 gene DH sites may function as a negative regulatory element. As was discussed in chapter 4, this region may play an important role in controlling IL-3 gene expression. This region may function to modulate the

interaction between the -4.5 with the -4.1 DH site. Moreover, it may insulate the two enhancers at -14 and -4.5 kb from interacting inappropriately with the IL-3 promoter as discussed in chapter 5.

It was hypothesised that the region within the 5.2 kb upstream of IL-3 may function as an LCR. Experiments in stably transfected Jurkat T cells and transgenic animals showed that this region was not sufficient to function as an LCR and that sequences upstream or downstream are needed for an LCR function. The DH sites downstream of the IL-3 gene may also be required for efficient and correctly regulated IL-3 gene expression. Since the DH sites downstream of the IL-3 gene are ubiquitously present, as described earlier, these sites may activate the locus first. The DH sites upstream of the IL-3 gene may get activated second. Early in development the remodelling of the IL-3 locus may absolutely need the locus partially remodelled, which could be achieved by the 3' elements. Hence the 3' elements may have two potential functions. On one hand, it may create an environment suitable for activation of the IL-3 locus. Alternatively, it may act as a boundary element to insulate the IL-3 locus from the influences of the GM-CSF enhancer. This may explain the result obtained from the transgenic mouse study as 3 out of 8 transgenic mice expressed the transgene at a low level. These 3 transgenic mice that were able to express the transgene may be integrated next already remodelled chromatin. With the IL-3 locus partially open at the 3' region, the DH site at -4.1 kb may be able to act early in development in a tissue-specific manner to remodel the 5' region of the IL-3 locus and prime the locus for further tissue specific activation and expression.

Alternatively, the IL-3 gene may be protected from the influence of the surrounding chromatin environment or the GM-CSF gene by the presence of boundary elements.

Boundary elements may exist further upstream yet to be identified that in conjunction with the DH sites downstream of the IL-3 locus may protect the IL-3 gene. In this case the 5' element function may be to drive the tissue-specific and developmental expression of the IL-3 gene.

In conclusion, this study has highlighted the complexity involved with IL-3 gene regulation. The functions of the DH sites 5' and 3' of the IL-3 gene are still not very well understood. Their function cannot be determined by *in vitro* experiments such as transient transfection assays. These sites need to be studied *in vivo* in transgenic mice and/or by knock out of specific site from the mouse endogenous locus.

APPENDIX A: SEQUENCE OF THE IL-3 UPSTREAM REGION TO THE HindIII SITE AT -5.2 KB.

-5156 AAGCTTTGTA TTTCAGGTGG GTAACAAGAG AGTGACAGAC ACTGAAAGCC CATAACTTAT
 -5096 TAATAACAAT AGGAACCATA TGGCCACCCC AGGTATCCAG ATATTGGAGC CCTGGATTGA
 -5036 GGCCAACAGA TGGAGCACCT GCAGCCCCAA ACCCAGCACT CCCTAGGGAA CACTTCTTCT
 -4976 GTGGCTTGCC TGGCTCCAGA TCATCCTTTA GGTTCATAGCT GTCCTTTCCT CCTGTCTTCT
 -4916 CTGATGGTGC AGGCTGGATG AGGCTGCCTC ATTTCTGGGC TTCTGTCTCC CACAGGCATA
 -4856 TCATTCTAAA TGGGGATTCT CCAACTGTCT TCTCTATTAG ACTGGGCCCTG GGATGGAGGG
 -4796 TCTGCTCTGC ACCGACTGGG CCCCAGGAA GCCATGACCC TGCAGCAGGG AATAGAAAGG
 -4736 CGAGGGTGCA CAGGTGGAAG CTGTGTAGGT CTTGGGGATG GAGTGCAGAG CCAGGTGGGG
 -4676 ACAGGAGGCC AGGGCTCTCC ACCTGGGCCC GCTCGGTGGC TTTTTCCTGA GCTCTCTGCT
 -4616 CAGGTGAGCA GGGGACATCT GGTGCACTG CTTGGCTGGC CAAGAACCCT GAGCTGCCTT
 -4556 CTGTGGTGAC AAAACCAGGC GATGTCATCA GGGCCACCAG CGGAAATACA ACCGAGGTCA
 -4496 TGGGCAGATT AAATCTGACA CTTGTGATGC CATGGAAAGG GTGGCGGAGG AGCTTGTCAC
 -4436 AGTGACTGAG GCCCAGGTTC TTGCCTGTGG CGGGTACCCC AGAAAATTCC ACTAGGCCTG
 -4376 TGGGGGTGAG TGATGGGAAA GAAGGAGGCA TGTACCTCTA GTTGGACTGG ACCCTGAGGG
 -4316 CCAGGACTTC CAACAATGTG GCCCACTCCA TAGGGATGGG GTCAGGCCCA CAGGGATCCA
 -4256 TTAGACAGGG GTCCAGAGAC CCCTCCTGAA ACCTCCAGGG CTTTGGCAGA CCCTGGGCTG
 -4196 CAGTCTTGGG CTGGCTCTCC TGTCCGGGCC CTGGCACACC CTCCCTCATG CCTGCCCCAG
 -4136 GGGTGGGCAC GTGTGCTCAT TTCCCTTCTT GATAGCAATC AGCCCAGCCC ACAAAGGAGG
 -4076 TTTCCCTTAA GCCGGGCTGG GTTGACACAA AGGCCTGGGG TGAATCACC ATCTTACAGG
 -4016 GGACCAGGTC TCTCCTCAAG GGCTGGCAGG ACTGTCGCCT CTGGAGGAGG GAAGTGGTGC
 -3956 CCATGATTTT GCATGAAGCT CCTATAGAGT CAAGGCAAAC CTTGAAGGAG ACCCCCAGGA
 -3896 TGACCCAGGC TATGCCTCGG TCCAAGGGCT CACGGAAGCG CTGTGGTGGC CAACTACCCA
 -3836 GATATCCTTG GCAGCCCCTT TGAATGAGG TTTGCTTTTA GGAACATGTC CCTCCATTCC
 -3776 TCCCTGGTTC ATCGAACGGC AGCAGGTATT GGGCTGCAGT CACTGGGCCT CTCCGGGGTC
 -3716 CGGGGCTGCA CCACCTCCTG CCTCCTTTGC TCTGCCCAGC TGGCCTCCCT GCCCATCCCA
 -3656 GCCTCCTCCC ATGGCCCCAC TCCCTGCCTC TGGGGCCTTC CATTCATAT TCTTGCTGCTG
 -3596 CAAGGCCCTG GGTCCCTCCA ACCTCCTCGC CCTCTGACCC CAGGCTGCCA GCCCCTCAAG
 -3536 GTCACCTCCA GGTAGTCACA GCTCAAGCCA CCAAACCTCA CCACCTTAT TGCTTCTAGC
 -3476 CCCACCTCAG GGGCTGACTT GACCTCTATT TCACAGAGAA CACAGAAGCC ATTGTCCCTT
 -3416 CCCACCACAA ACCTGTGGAC CAACCAGGCC CACCCCACTG CACCTACCCC ACCCAGATGG
 -3356 ACTGGGCCAC CCAGGCAGCC CTTCCTGAG CTCTCTGCGA GGAGCCCGTG ACAGCTGATG
 -3296 GTGGGGCTCT GCCTGTGCTG GCCGGCCAGC CATCTCACCC ATTCCCGGCT CACCTCCTTA
 -3236 AGAGAGGAAG GAGCTCAAAC TTCTGTGTGG AAGGTGCGTC TCTGACCCTG TGACTCCTCT
 -3176 GGCTGCCCCA TCCCCCTCTT TCCTTCAAAA TAAACTTCA AAGAATGTTC TACCTCCGT
 -3116 CTCACCGTCA CTCGCTTCTC AGCCCACTGC CAGAGGCTTT GCCCACCAT ACACTGAAAC
 -3056 TACTCCGCCA GAGGCCTCAG GGATCTCCCC GTCAGTAATC AGAGTGGTTA CATCTCATCC

-2996 TCACCCTGCT GTCCCCCTCCT GCCACATGGC ATCATCCTTT AAGGCCGTCA TTGTCCGATG
 -2936 CTGCCCAGGG GGTGCTCAGC CTCTGGGTTG GACTGTTCTG CTTATCTCAG TCCCAGTGAT
 -2876 GTCACCTCCT AGCTACATCA GCTCAGCTTC TCTGAAACTC AGTTTCTTCA TCTGAAAAAT
 -2816 GGGGCTAATA ACAGCACCTG CCTCACAGTT CATCATGAGG ATTAACAAGT TCAGCCATGA
 -2756 AAAGAACTTA GTTCTCCACA CTTCAACAAGT GCCACTCAAT AGAAGTCAGC TGTGAGAGCG
 -2696 CTTCCCTTCA CCCAGTCCTC TCCACCCCGT AAGAACCTAC CTAGTCCAGC TTCCCACCAC
 -2636 CTGTGTTCCA GAGGACTCCC AATATCTCCT CACTGGCCAC CCTGCTGCCT TGAACCTTCT
 -2576 CATCCTTTCC CCCACAGCAG GTGCACCTGA GCTTCACCTC GCCTCCCCTG GTTGAAAACC
 -2516 CCGCAAGTCC TCCAATGCTC CTAGCATGAA GCCTCAACCT CTTTTGCCTT TAATAAACTA
 -2456 TGTGTATATA TATAATTTTT TTGGAGAGAT GGTCTCACTC CAACACCCAG GCTGGAGTGC
 -2396 AGTGGCACAA TCACAACTCA CTGCAGCCTT GACCTCCCAG GCTCAGGTGG TCCTCCCACA
 -2336 TCATCCTCCT AAGTAGCTGG GACCACAGGT GTGCACCACC ACGCCTGGCT AATTTTTTGT
 -2276 ATTTTTTGTA GAGATGAGGT TTCGCCAAAT TGCTCAGGCT GGTATATATT CATTTTTTAA
 -2216 ATGAGAAAAA CACTACAAAT GTCAAGATTT ACAATACTAC TATTCAGGAT TTTGTGTGCA
 -2156 TGTGTGGTTA CAACACAAAG TCTTGACTTT TAAAGAAAACA AAAAAAATA CAGGAATGTT
 -2096 TTGTCTCCAC AGCTTTAAAT TCATTGAACT TTAAAAATCC CTAGAAAAGC CCACCTACAC
 -2036 TAAAAAAAT ACAGCTTTTC TCTGTAAGGA ATCACATTAT TTGTGCACAA GTCCCCAAAA
 -1976 CGTTTACATA GACAGTAACA CCAAGGTACA GTTGAGTATG CAAGGAACAA ACTCTGGGTC
 -1916 CGTGGGGCAA TAGGGGACCC TACCGGAGAT AAGATTGCTG AGGTAGTTAG ATCAGAGCCC
 -1856 CTCAAATGCA CTGAATCACT GAGGATCTTA TCAAAATGCG ATTTTTGATT CAGAAAGTCT
 -1796 GGGCTGGGGC CTGAGGGTTG ACACCGCTAA CAAGCTCCCG GGCAGGGCTG ACCTGCAGGT
 -1736 CTGGGACCAC AGGAAGGGTA GCAAGACTGG AAATCTGTCT CTCACAAGAC TGAGCTGCTA
 -1676 GTGGAGGTTA CCATGTCTGT TTTATTCACA GCAAGTGCTT TGTAGACGTC TGTTGAATGA
 -1616 ATTGTGCCTA CGGAGTACGC AAGCTGGTGC TAAGGCTCCC CTCTGTGTGT TGAGTTGTGC
 -1556 CTGTCAGACT CCACTCCGGG TCCAGAACCT AGGGCTCTTC CTGGTCCAGG GCCCAGCCTG
 -1496 GGGCTGAGCC AGGAGGGCAT ATCAGCTACA GAAATCATCC TCACAGCATG GCCTTGAGTC
 -1436 ACAGCCTCTT CACCTGCCTC CCCACAGCCA CATCCTGGTC CTTGTCTTCA CCTGATCTGC
 -1376 TTATGCTCAG AAGCCCAAAG CACCCAAATT CCGCTGCCTC AGCATAACCT CCTATCCCGC
 -1316 ATCCCCCACA CTGATCACAA CACACCGCAC CATGACCCAG ACCCTGGCCT TTCCTTACTG
 -1256 CTCTCCTCTC CCTCCCACCC GGGGCCAGTC ATCTTGAAGA AGTCTTCCAC ATGCCCCTGT
 -1196 CACACTCATC CCTTTACCAA AAGCCCCTAC CCCATGGGGT GGGTCAGGCA GGCCCCAAGA
 -1136 CAGGCCCCGTA TCAGGAGGAC CCCTCTTCTC TCAGGGGCTG CCCTCTGGGA TAACCACCCC
 -1076 CGCCCTTCTG GGTTCCTGC TTCCTATCTG GCTGCAGTTT CTCAGGTCCC TTGTGGATTT
 -1016 CCCCATGGTC TGTCCCCACT CACATCCCCT CTCTGCAAAC CTTGCCTACT GGGCCTGCAC
 -956 CTGGCAAATC CATGCTCAGC ACAGACGGGG ATCAAGACCT CTCAATACAA CTGTCTCCTG
 -896 CCAATCCCTG CCCCAGCAGC CTGAGGCCCA GTCTGAAACC AGGGAGTTGC TCTCCTTTCT
 -836 CCTCCCTTGA CCTCACCCCT CAGACCATGC CAATTCTGCC TCCTAAACCT CCCAGGCCAG
 -776 CCCCTCCCCC AGCTCCCAGT GACAGTGTCC TCAGGTACCT GAGCTCAGCT CTCGGTGCTA
 -716 CCAGAGGGAC TGCCAGGGCT GCAGCCGGGC CTCCTGCAGA GGCTGAGTCC CACACGCAGG
 -656 GAACAGCCAT GCCACTGCTA GCAGACCAGT AAGAGAATGG CCACCTGGGG CCTGAGCGCC
 -596 CTCGGCCATC CACCAGAAAC AAAGTGTCAG GGAGAAGCTG CCCGAAGCCC ATGGGACAAA

-536 CCACTGGGGA CTGGAACACC AGTAATTCTG TATTGGGAAG CGGCACCAAG AGATGTGCTT
 -476 CTCAGAGCCT GAGGCTGAAC GTGGATGTTT AGCAGCGTGA CCGGCTACCA GACAAACTCT
 -416 CATCTGTTCC AGTGGCCTCC TGGCCACCCA CCAGGACCAA GCAGGGCGGG CAGCAGAGGG
 -356 CCAGGGTAGT CCAGGTGATG GCAGATGAGA TCCCACTGGG CAGGAGGCCT CAGTGAGCTG
 -296 AGTCAGGCTT CCCCTTCCTG CCACAGGGGT CCTCTCACCT GCTGCCATGC TTCCCATCTC
 -236 TCATCCTCCT TGACAAGATG AAGTGATACC GTTTAAGTAA TCTTTTTTCT TGTTTCACTG
 -176 ATCTTGAGTA CTAGAAAGTC ATGGATGAAT AATTACGTCT GTGGTTTTCT ATGGAGGTTT
 -116 CATGTCAGAT AAAGATCCTT CCGACGCCTG CCCCACACCA CCACCTCCCC CGCCTTGCCC

-56 GGGGTTGTGG GCACCTTGCT GCTGCACATA TAAGGCGGGA GCTGTTGCCA ACTCTTCAGA
 +1

+5 GCCCCACGAA GGACCAGAAC AAGACAGAGT GCCTCCTGCC GATCCAAACA TGAGCCGCCT
 +64

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