TRANSCRIPTIONAL REGULATION OF THE GM-CSF GENE IN T LYMPHOCYTES

CAMERON STUART OSBORNE B. Sc.

Thesis submitted for the degree of

Doctor of Philosophy

in

University of Adelaide

(Faculty of Science)

Division of Human Immunology,

Hanson Centre for Cancer Research.

Department of Microbiology and Immunology,

University of Adelaide.

Adelaide, South Australia

September, 1996

TABLE OF CONTENTS

Addendum

The following revisions to the thesis entitled 'Transcriptional Regulation of the GM-CSF Gene in T Lymphocytes' by Cameron Stuart Osborne, should be noted:

The sentence on page 3, line 8 should reference Bagby and Segal, 1995.

The sentence on page 4, line 9-10 should reference Shaw and Kamen, 1986.

Page 5, Line 20 should read 'IL-3 induces the differentiation of B cell progenitors into mature cells'.

Page 7, Line 24 should read 'an intracellular phosphatase'.

The sentence 'Calcineurin functions by dephosphorylating the cytosolic component of NFAT, allowing its translocation into the nucleus (Luo et al., 1996)' should be added after page7, line24.

Page 10, Line 7 should read 'transcription initiation site'.

Page 13, Line 12 should read 'Secondly, actively transcribed genes such as the osteocalcin gene are often associated with the nuclear matrix'.

Page 16, Line 9 should read 'Sp1 is a constitutive transcription factor'.

Page 18, Line 2 should read 'between -49 and -63 from the transcriptional start site'

Page 18, Line 23 should read 'T cell specificity of IL-3 gene expression'.

Page 18, Line 3 should read 'The GM-CSF gene in endothelial cells'.

Page 19, Line 10 should read 'Like the CK-1 and CK-2 elements in the IL-2 and GM-CSF gene promoters'.

Page 19, Line 19 should read 'DB1 is able to synergise with Tax to promote constitutive IL-3 expression.'

Page 19, Line 20 should read 'upstream of the IL-3 gene'.

Page 19, Line 25 should read 'that binds to an unidentified repressor.'

Page 20, Line 17 should read 'blocks the formation of the NFAT complex. (Flanagan et al, 1991)'.

Page 22, Line16 should read 'apparent molecular weight'.

Page 22, Line 22 should read ' calf intestinal phosphatase'.

Page 23, Line 9 should read 'NFATp is indeed a component of this complex.'

Page 23, Line 10 should read 'cytosolic component of NFAT'.

Page 34, Line 4 should read 'Another subset of AML's, however, constitutively produce cytokines that appear to promote their growth in an autocrine manner.'

Page 47, Line 17 should read '2 M acetic acid'.

Page 57, Line 2-3 should read 'Equivalent amounts of protein were added'.

Page 109, Line 15 should read 'may be co-ordinately expressed.'

SUMMARY

Cytokines play an important role in regulating haemopoiesis and inflammation. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are two cytokines whose genes are very closely linked, just 10 kb apart on human chromosome 5, and 14 kb apart on mouse chromosome 11. Previous work in this laboratory had identified an enhancer, located 3 kb upstream of the human GM-CSF gene, that is thought to be involved in the transcriptional regulation of GM-CSF and possibly IL-3.

This thesis describes the investigation as to whether the mouse GM-CSF and IL-3 genes are regulated in a similar manner as those of the human, focussing on regulation through an enhancer.

Initial work found a mouse homologue to the human GM-CSF gene enhancer located 2.0 kb upstream of the mouse GM-CSF gene. This element contained 76% sequence homology with the human GM-CSF gene enhancer within a core region of 417 bp, with many conserved transcription factor consensus binding sites. Transient transfection assays demonstrated that the mouse homologue to the human enhancer had enhancer activity, as it was able to increase the inducible activity of heterologous promoters such as the herpes simplex thymidine kinase promoter or the human GM-CSF promoter. The mouse enhancer was also able to increase the induction through the mouse IL-3 gene promoter. The mouse GM-CSF enhancer had little effect, however, when tested in its natural context, upstream of the mouse GM-CSF gene promoter. The mouse GM-CSF gene promoter

alone was sufficient for high induction, to a level comparable to constructs containing the human GM-CSF gene promoter and enhancer.

Considering the human and mouse GM-CSF gene proximal promoters share a very high degree of sequence homology, yet have different induction capacities, the nature of these differences was investigated. Transcription factor binding studies demonstrated that the mouse GM-CSF promoter contained higher affinity binding sites for Sp1 and TBP, however attempts to correlate these differences with functional differences in transient transfection assays were largely unsuccessful.

Putative binding sites for the transcription factor core-binding factor were conserved in the promoter and enhancer of the GM-CSF gene. Functional assays were carried out to determine whether these sites contribute significantly to regulation of the GM-CSF gene. Specific mutations to the CBF site within the promoter significantly reduced the inducible activity of the promoter in transient transfection assays. Coupling the enhancer CBF site to a truncated GM-CSF promoter increased the inducible activity. These results suggested an important role for CBF in regulating GM-CSF transcription.

This study has highlighted both similarities and differences of the GM-CSF/IL-3 locus regulation in the mouse and human. The nature of the differences remains unknown. This may represent an evolution of the mouse GM-CSF gene with less dependence upon an enhancer for its activation. Alternatively, it may suggest a greater role of the enhancer beyond that of a classical enhancer, perhaps in regulating transcription by altering chromatin structure, as has been found for locus control regions.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

_____ 30/9/96

Cameron Stuart Osborne Date

ACKNOWLEDGEMENTS

I would like to thank Professor Mathew Vadas for helping me find a niche in the Division of Human Immunology at the Hanson Centre, and for his interest and support throughout my studies.

Much thanks go to my supervisor, Dr. Peter Cockerill for accepting me as his student, and for his supervision and wealth of knowledge throughout this project.

I am particularly grateful for my co-workers Andrew Bert, Kym Duncliffe and Greg Ryan, and others within the Division for all their help, advice and wonderful friendship over the years.

Thanks also go to those outside the Hanson Centre, such as the Adelaide University Skindiving Club, and the fine makers of Cooper's Scotch Ale, for making my stay in Adelaide most pleasurable.

Finally, I would like to thank my family; John, Eleanor, Catherine and Alison for their unfaltering love and support across great distances.

Thanks to all!

CHAPTER 1.

INTRODUCTION

1.1. HAEMOPOIESIS AND IMMUNITY

A constant turnover of blood cells requires the production of 10^{10} erythrocytes and 10^8-10^9 leukocytes every hour in an adult human. This rate of renewal is increased in times of stress, such as blood loss or infection. All blood cells are derived from a common, self-renewing precursor cell, called the stem cell (reviewed in Williams, 1995). These stem cells, which are located in adult bone marrow, proliferate to keep a constant pool of stem cells, yet they have the potential to differentiate into any blood cell type, if given the appropriate signals to do so. Once a stem cell begins to differentiate into a given cell lineage, it becomes committed to that differentiation pathway. In the initial steps of differentiation, the stem cells commit to either a lymphoid of myeloid cell lineage. These partially differentiated progenitor cells can also proliferate, or may undergo subsequent differentiating steps to change into more defined cell types. The final differentiating steps yield mature blood cell types that can carry out the requirements of the circulatory system.

A major function of blood is to protect the individual from infection. This requires pathogens to be recognised from host cells, and be killed. Immunity is the domain of the leukocytes, and is divided in to two classifications: cell-mediated immunity and humoral immunity (reviewed in André-Schwartz and Schwartz, 1995; Fitch, 1992; Berek, 1992). The cell-mediated response involves a direct recognition of pathogen infected host cells by cytotoxic T cells. The infected cell displays a fragment of the pathogen on a class I major

histocompatability complex (MHC) molecule. This is recognised by a cytotoxic T cell which directly kills the infected cell. B cells mediate the humoral immunity. Macrophages presenting antigen on a class II MHC molecule activate helper T cells through the T cell receptor. The activated T cells, in turn, act on B cells that produce immunoglobulins specific to the antigen, causing them to clonally proliferate and secrete immunoglobulin.

1.1.1. CYTOKINES IN HAEMOPOIESIS AND IMMUNITY

The cell-to-cell interactions of both haemopoiesis and immunity rely on an intricate communication network. This network is mediated in part by intercellular signalling polypeptides called cytokines. Cytokines that signal the haemopoietic cells to proliferate and to differentiate are collectively called haemopoietic growth factors (HGFs) (Bagby and Segal, 1995). These HGFs, when supplemented *in vitro*, can induce progenitor cells to proliferate or differentiate into various cell lineages, depending on the HGFs that are provided. Many of these HGFs also participate in the functioning of the immune system. This highlights a major characteristic of HGFs; they have multiple biological activities. For example, granulocyte colony-stimulating factor (G-CSF) supports the proliferation of stem cells, but also activates the phagocytic function of neutrophils (Ikebuchi et al., 1988; Nathan, 1989). Another important characteristic of HGFs is that they often function synergistically with other HGFs to elicit various functions. Interleukin-6 (IL-6), in conjunction with IL-4,

synergistically supports T cell proliferation (Hodgkin et al., 1988). IL-6 and granulocyte-macrophage CSF (GM-CSF), however, synergistically supports the differentiation of granulocytic progenitor cells (Caracciolo et al., 1989), demonstrating that different combinations of HGFs have varying effects. Clearly, the regulation of when and where HGFs are expressed plays a key role in controlling proper haemopoiesis and immune responses. Dysregulated cytokine production is associated with many diseases. Activities and expression patterns of the individual HGFs are described in Bagby and Segal, (1995). The properties of GM-CSF and IL-3, two HGFs that are central to this thesis, will be discussed.

1.1.2. REGULATION OF CYTOKINE GENE EXPRESSION

Tight regulation of cytokines production is necessary to ensure a rapid response to host infection by the appropriate cell types, and that they are not produced when and where they are not needed. Cytokines can be regulated at several intracellular levels. A condensed chromatin environment can render a gene locus inaccessable to binding of transcription factors (Davie, 1995). It is interesting to note that genes for multiple human cytokines are situated in relative close proximity on chromosome 5 (Thangavelu et al., 1992), hinting there may be a general encompassing regulatory mechanism for these genes in certain cell types. Gene transcription of cytokines is usually found to be tightly regulated. Genes such as IL-2 and G-CSF are responsive to rapid induction by a number of pre-existing transcription factors such

as NFATp and NF-kB, and transcription factors that are rapidly transcribed such as fos and jun (Riegel et al., 1992; Shannon et al., 1992). These cytokine genes also often contain binding sites for transcription suppressors in order to maintain tight control in inactivated cells, and to rapidly halt transcription when needed. Some cytokines exhibit some degree of post-transcriptional control by altering the rate at which their mRNA is degraded (Shaw and Kamen, 1986). This allows mRNA that is transcribed at basal levels to be quickly degraded. During cell activation, the mRNA is stabilised to increase the amounts of cytokine translated. Newly translated cytokine can also be the point of regulation. Cytokines can be stored in a precursor form that can be quickly processed upon cell activation. For example, IL-1 precursor is enzymatically cleaved by IL-1-converting enzyme and subsequently secreted from monocytes, possibly in response to apoptotic signals (Hogquist et al., 1991). As they are central to the work presented in this thesis, the regulation of two cytokines, GM-CSF and IL-3, will be discussed in detail.

1.1.3. GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

GM-CSF is a pluripotent cytokine with roles in both haemopoiesis and immunity. It is a heavily glycosylated single peptide, approximately 23 kDa (Nicola et al., 1979b). GM-CSF is expressed by many cell types. In fact, most tissues isolated from mice that have been injected with endotoxin will produce GM-CSF when cultured *in vitro* (Nicola et al.,

1979a). Major sources of GM-CSF include T cells, macrophages, endothelial cells and fibroblasts (Gough and Nicola, 1990). The general roles of GM-CSF are three-fold: proliferation, differentiation and activation. GM-CSF supports the proliferation of both stem cells and myeloid progenitor colonies (Metcalf et al., 1986). GM-CSF also promotes the maturation of neutrophils, macrophages and eosinophils (Metcalf et al., 1986). Activating functions of GM-CSF include the stimulation of eosinophils, neutrophils, monocytes and macrophages (Gasson et al., 1984; Lopez et al., 1986; Morrisey et al., 1989).

1.1.4. INTERLEUKIN-3

Interleukin-3 is a single peptide glycoprotein of approximately 28 kDa (Morris et al., 1990). Unlike GM-CSF, IL-3 is expressed by very few cell types. Its predominant source is activated T cells, although it can also be expressed in mast cells (Niemeyer et al., 1989; Wodnar-Filipowicz et al., 1989). The biological activities of IL-3 are mainly proliferative and, to a lesser degree, differentiating. The proliferative activities of IL-3 are more general than GM-CSF, encompassing the stem cells as well as both myeloid and lymphoid progenitor cells (Lopez et al., 1988; Clark and Kamen, 1987). IL-3 stimulates the differentiation of B cell progenitor into mature cells (Tadmori et al., 1989).

1.1.5. THE GM-CSF/IL-3 GENE LOCUS

The genes for GM-CSF and IL-3 are located on chromosome 5q23-q31 in humans and chromosome 11 subband B1 in mice (Yang et al., 1988; Lee and Young, 1989). In both species, these genes are very closely linked; they are only 9 kb apart in humans, and 14 kb apart in mice. The human genes reside in a cluster of cytokine and cytokine receptor genes that includes IL-4, IL-5, M-CSF, M-CSFR, and PDGFR (Thangavelu et al., 1992). The human and mouse GM-CSF genes are similarly arranged, both with four exons, and they share approximately 54% amino acid sequence homology (Miyatake et al., 1985). In addition, their promoters share 80% DNA sequence homology within 350 bp, and 90% homology within 120 bp of the transcription start site. The human and mouse IL-3 genes are also similarly arranged, both consisting of five small exons (Yang and Clark et al., 1988). However, the amino acid homology of the mouse and human IL-3 proteins is only 29%. The 5' flanking regions are more conserved with 59% homology within 350 bp from the coding regions (Yang and Clark et al., 1988).

In T cells, both GM-CSF and IL-3 are produced in response to T cell receptor (TCR) signalling (reviewed in Masuda et al., 1993a). This occurs when the TCR comes into contact with antigen coupled to an antigen presenting cell. TCR signalling activates at least two separate intracellular signalling pathways, increasing cytosolic calcium levels, and activating protein kinase C. These pathways can be individually activated with calcium ionophore, which causes a calcium influx, and

phorbol esters which are analogues of diacylglycerol and activate protein kinase C. TCR induced expression of GM-CSF and IL-3 can be inhibited by the immunosuppressive drug cyclosporin A (CsA) (Kelso and Gough, 1989; Tocci et al., 1989). For maximal GM-CSF and IL-3 production, a secondary signalling pathway transduced through the CD28 receptor must be activated as well (Thompson et al., 1989). T cells can also produce GM-CSF in response to IL-1 stimulation, and in response to IL-2 stimulation. These stimuli, however, result in lower levels of GM-CSF production than through TCR signalling (Kelso et al., 1986). Macrophages produce GM-CSF in response to lipopolysaccharides, whereas endothelial cells and fibroblasts produce GM-CSF in response to TNFα and IL-1 signalling (Gough and Nicola, 1990). IL-3 is produced in mast cells after immunoglubulin E signalling (Wodnar-Filipowicz et al., 1989).

CsA is a lipid soluble immunosuppressive drug isolated from a fungus, and is able to block T cell, B cell and mast cell activation and proliferation by inhibiting calcium dependent signalling pathways (reviewed in Schreiber and Crabtree, 1992; Kunz and Hall, 1993). It is commonly administered to organ transplant patients for its ability to prevent organ rejection. In T cells, CsA inhibition affects the expression of cytokine genes such as IL-2, IL-3, IL-4, IL-5, GM-CSF, TNFα and IFNγ (Tocci et al., 1989). CsA has been demonstrated to bind cyclophilin, and thereby inhibit the cyclophilin dependent activation of calcineurin, and intracellular phosphatase (Liu et al., 1991). The activity of calcineurin has been directly linked to the expression of

cytokine genes (Kubo et al., 1994; O'Keefe et al., 1992; Tsuboi et al., 1994).

There is evidence for post-transcriptional regulation of both GM-CSF and IL-3. Shaw and Kamen (1986) described a sequence located in the 3' untranslated region of human GM-CSF that confers selective mRNA stability. This element has been further defined as $AUUUA^{A}/_{U}{}^{A}/_{U}$ (Lagnado et al., 1994). The rate at which IL-3 mRNA degrades has also been shown to be slower in stimulated T cell than in resting T cells (Ryan et al., 1991). Of note, there is 59% nucleotide sequence homology in the 3' untranslated regions of the mouse and human IL-3 genes suggesting the presence of conserved elements (Yang and Clark, 1988).

1.2. REGULATION OF GENE TRANSCRIPTION

The transcription of eukaryotic protein-encoding genes into mRNA requires the recruitment of RNA polymerase II (RNAPII) (reviewed in Zawel and Reinberg, 1995). This recruitment is mediated by a core DNA sequence element, the TATA box, which is usually 30 bp upstream of the transcription start site, and a multiprotein complex of general transcription factors (GTFs) that bridges between the TATA box and the RNAPII. Some genes possess an initiator site either in conjunction with or instead of a TATA box, that spans the transcription start site and binds to the RNAPII recruiting GTFs. The initial step in TATA box mediated recruitment of RNAPII involves the

binding to the TATA box by a multi-subunit GTF, called TFIID. The DNA binding ability of TFIID is contained within a single protein subunit, TATA binding protein (TBP). The other components of TFIID, the TBP-associated factors (TAFs), associate strongly with TFIID, and are able to associate with other GTFs and with transcriptional activators binding to *cis*-acting DNA sequences. The second step occurs once TFIID has bound to TBP. TFIID then binds to TFIIB, a GTF that, with the aid of TFIIF, recruits RNAPII to the transcription initiation site. RNAPII can then engage the DNA at the transcription start site. The final step involves the release of RNAPII from the initiation complex. This requires the recruitment of two additional GTFs, TFIIE and TFIIH. TFIIH possesses kinase activity and is able to phosphorylate the C-terminal domain of RNAPII. Once RNAPII is phosphorylated, it is released from the initiation complex, and begins to transcribe RNA.

The GTFs are both necessary and sufficient to initiate transcription (Tyree et al., 1993). However both *cis*- and *trans*-acting elements can either promote or suppress transcription initiation. The transcription factor binding sites that are located immediately upstream of the transcription initiation site are collectively called the promoter. Transcription factors that promote or suppress transcription initiation are referred to as activators and repressors respectively. Some activators promote transcription initiation by directly or indirectly associating with the TAFs to stabilise the binding of TFIID at the TATA box. For example, the activator Sp1 can directly interact with TAF110 to stabilise TFIID binding (Hoey et al., 1993). Conversely, repressors

suppress transcription initiation by either directly or indirectly interfering with the binding of TFIID, or the recruitment of other GTFs. For example, the thyroid hormone receptor, when unbound to thyroid hormone, can directly inhibit the formation of the initiation complex (Fondell et al., 1996).

Genes are often partially regulated by elements that are distal to the transciption initiation site. These include enhancers, which are elements that promote transcription, and silencers, which are elements that suppress transcription. These elements are special in that they can operate at great distances from the gene, at least up to 50 kb, as seen in the ß-globin locus (Tuan et al., 1989), either upstream or downstream. Whereas promoters are required to be in a set orientation with respect to the gene to function properly, enhancers and silencers can function in either orientation (reviewed in Atchison, 1988). Enhancers and silencers cannot replace the requirement of promoters, but appear to work in conjunction with the promoters. A type of regulatory element that has come into prominence over the past few years is the locus control region (LCR). LCRs are composed of arrays of regulatory elements distal to one or more genes within a gene locus (Dillon and Grosveld, 1993). Like enhancers, they are able to convey increased rates of transcription. In contrast, LCRs can exert their effect, regardless of the local chromatin environment. LCRs have been identified in a number of gene loci. For example, in the human ß-globin locus, at least four far upstream regulatory elements act in synergy to sequentially enhance the

expression of five developmental stage and cell type specific globin genes (Grosveld et al., 1987, reviewed in Wood, 1996).

It is unclear how distal elements can exert their effects from such great distances. One model suggests that these regulatory elements can loop over to the promoter to allow protein:protein interactions between their respectively bound transcription factors (Ptashne, 1986). Another model referred to at the tracking model, hypothesises that the binding of transcription factors to distal regulatory elements sends transcriptional activating signals, perhaps by modifying chromatin structure of the locus (Travers, 1992). An additional model suggests that distal regulatory elements may direct its locus into compartments in the nucleus that are transcriptionally active (Stein et al., 1995). It is important to note that none of these models of action for distal regulatory elements are mutually exclusive.

The chromatin structure of DNA is likely to play a role in the regulation of gene transcription. All chromosomal DNA is packaged by histone proteins into structures called nucleosomes, each encompassing 146 bp of DNA (Richmond et al., 1984). Lengths of nucleosome-packaged DNA are further condensed into varying higher order structures (reviewed in Woodcock and Horowitz, 1995). When DNA is packaged in a condensed chromatin structure, transcription factors are generally excluded from binding. Regions of chromosomes that contain frequently transcribed genes tend to be packaged in a less condensed chromatin structure than those that are rarely or never transcribed. The ß-globin locus has a more relaxed chromatin

configuration in cell types expressing the globin genes (Forrester et al., 1990). This would suggest that a condensed chromatin packaging of DNA may act to suppress transcription of genes. DNaseI is an enzyme that can cleave unprotected DNA. Inactive loci are tightly packaged in chromatin, and are generally insensitive to DNaseI cleavage. Active loci, however, have a loosened chromatin structure, and hence have an increased sensitivity to DNaseI (Elgin, 1988). Within these active loci, some small regions of approximately 200 bp have a heightened sensitivity to DNaseI, and are called DNaseI hypersensitive (DH) sites (reviewed in Gross and Garrard, 1988). DH sites are thought to represent sites of altered chromatin structure due to the presence of DNA-binding proteins. It is thought that some transcription factors can displace nucleosomes and thereby open up the binding sites of factors that are normally excluded (Becker, 1994; Felsenfeld, 1992).

The nucleus is a highly structured organelle with discrete domains that separate nuclear processes such as the transcription of genes encoding proteins, the transcription of genes encoding ribosomal RNA, DNA replication, DNA repair and RNA processing (reviewed in Nickerson et al., 1995). Within the nucleus is an extensive network of protein scaffolding called the nuclear matrix (Berezney, 1974). The nuclear matrix may play a role in compartmentalising the various processes. The chromatin is anchored by proteins to the nuclear matrix at sequences called nuclear matrix association regions (MARs) (Cockerill and Garrard, 1986). The sequence between MARs form loops which range in size between 5 and 200 kb (Jackson et al, 1990). Studies of the human apolipoprotein B gene locus and the chicken

lysozyme gene locus show that the relaxed chromatin structure that is associated with these loci, ends at their flanking MAR sites (Phi-Van and Stratling, 1988; Levy-Wilson and Fortier, 1989). Some MARs are found within active loci, particularly near enhancers; the immunoglobulin kappa enhancer, for example, is closely associated with a MAR (Cockerill and Garrard, 1986) There is growing evidence that gene transcription is also associated with the nuclear matrix. Firstly, there are cell type and developmental stage specific nuclear matrix proteins, suggesting that they may play a role in recruiting specific loci to the nuclear matrix in a regulated manner (Fey and Penman, 1988; Bidwell et al., 1993; Dworetzky et al., 1992). Secondly, actively transcribed genes are often associated with the nuclear matrix, such as the osteocalcin gene (Jackson and Cook, 1985; Dworetzky et al., 1992). Finally, many transcription factors such as c-Myc, Sp1, Oct1, and AP-1 can associate with the nuclear matrix (Waitz and Loidl, 1991; van Wijnen et al., 1993). The nuclear matrix has been suggested to coordinate gene transcription. One model put forth by Cook and colleagues suggests that transcription occurs in highly localised foci on the nuclear matrix in so-called transcriptional 'factories' (reviewed in Jackson and Cook, 1995). Evidence that supports this hypothesis includes the observation that RNA polyermases associate with the nuclear matrix (Jackson and Cook, 1985). Furthermore, when cells were pulsed with bromodeoxyuridine (Br-dUTP), and then immunolabeled with a fluorescently labeled antibody against Br-dUTP, 300-500 foci appear in the nucleus (Jackson et al., 1993). If cells are pretreated with a RNAPII specific inhibitor, the foci fail to form. Based on immunofluorescence staining

intensity, there are an estimated 50 active polymerases in each transcription focus (Jackson et al., 1993). Stein et al. (1994) expand the transcription factory model in suggesting that transcription factors are bound to the matrix in these foci, thereby concentrating transcription factors in sites of transcription.

1.2.1. GM-CSF GENE TRANSCRIPTIONAL REGULATION

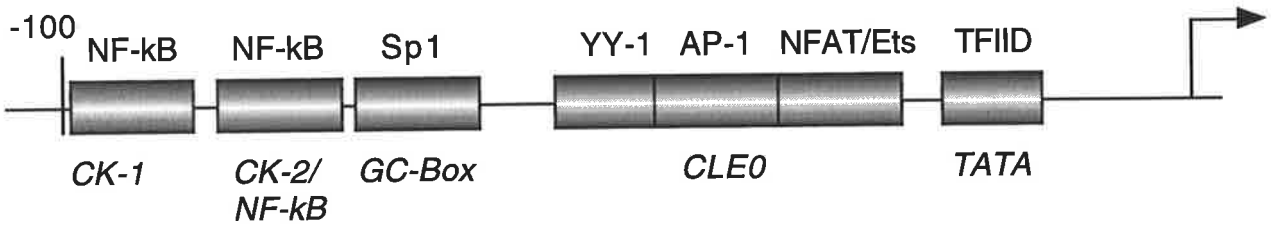
Considerable effort has been spent in elucidating the transcriptional regulatory mechanisms of the GM-CSF gene in T cells. In initial studies by Chan et al. (1986), the 5' flanking sequences of the human GM-CSF gene were linked to a chloramphenicol acetyl transferase (CAT) reporter gene (Chan et al., 1986). They found that a fragment containing sequence up to 660 bp 5' of transcriptional start was able to increase CAT expression in T cells when the cells were activated. Nimer et al. (1988) further characterised the 5' regulatory regions of human GM-CSF by testing serially deleted 5' sequence constructs in T cells, and found that sequences contained within 53 bp of transcriptional start were sufficient for full inducible activity. Furthermore, Nimer et al. found that these sequences contained a high constitutive activity suggesting that upstream elements were responsible for inhibiting basal activity. However, this finding is in contrast to that which is observed by others in both the mouse and human GM-CSF promoters (Miyatake et al., 1988a; Himes et al., 1993; Jenkins et al., 1995). These workers found that sequences up to 96 bp upstream of the mouse GM-CSF transcriptional start site are required

for a full response to phorbol ester and calcium ionophore signalling (Miyatake et al., 1988a). Indeed, others have found that the human GM-CSF promoter truncated at -53 bp is considerably less active than a promoter construct that contains sequence up to -620 bp (Himes et al., 1993; Jenkins et al., 1995). It is unclear as to the reasons for this discrepancy, however it is of note that the plasmid vectors used by Nimer et al. differ from those used by both Himes et al. and Jenkins et al.

It is convenient to separately consider the sequences upstream and downstream of -53 bp in the human GM-CSF promoter (equivalent to -60 bp in the mouse). The sequence contained between -53 bp and -30 bp (the location of the TATA box) has been shown to be important for GM-CSF promoter function (Nimer et al., 1990; Miyatake et al., 1991), and in fact, an element termed the conserved lymphokine element 0 (CLE0), located between -54 and -40 bp, is loosely conserved in the IL-4 and IL-5 gene promoters (Miyatake et al., 1991). In the GM-CSF promoter, a binding site for the transcription factor AP-1 has been identified within the CLE0, at 42 bp upstream of transcriptional start (Tokumitsu et al., 1993; Masuda et al., 1993b; Wang et al., 1994). Immediately 3' to the AP-1 binding site is a site that can bind NFATp, Elf-1 and Ets-1 (Tokumitsu et al., 1993; Masuda et al., 1993b; Tsuboi et al., 1994; Jenkins et al., 1995; Wang et al., 1994; Thomas et al., 1995; Nimer et al., 1996). The binding sites for both AP-1 and NFATp/Elf-1/Ets-1 have been demonstrated to function as activators of GM-CSF expression. AP-1 and NFATp appear to be able to bind to their sites co-operatively (Jenkins et al., 1995).

Fig. 1.1. Regulatory elements and binding proteins of the GM-CSF gene promoter.

Schematic representation of the major transcriptional regulatory elements of the GM-CSF gene promoter within 100 bp of the transcription start site are shown as boxes and labelled in italics. Putative transcription factors which may bind these sites are indicated above the map. The arrow indicates the transcription start site.


-100
NF-kB
NF-kB
Sp1
YY-1
AP-1
NFAT/Ets
TFIID
CK-1
CK-2/
NF-kB
GC-Box
CLE0
TATA

Immediately upstream of the AP-1 binding site is a site that has been suggested to bind YY-1, a transcription factor that can function as an activator in some gene promoters, and as a repressor in others (Hahn, 1992). In the GM-CSF gene promoter, it appears that YY-1 functions as a transcriptional repressor (Ye et al., 1994).

The sequences upstream of -53 bp of GM-CSF also have transcription factor binding sites. The transcriptional activator Sp1 has been demonstrated to bind to a GC-rich region in the mouse GM-CSF promoter (Masuda et al., 1994). Sp1 is a constitutively present transcription factor that is involved in the transcriptional regulation of a wide variety of cellular and viral genes (McKnight and Tjian, 1986; Kadonaga et al., 1987). Upstream of the GC-rich region are two elements, CK-1 and CK-2 (also called CLE1 and CLE2 respectively), that are conserved in the regulatory elements of other cytokine genes. The CK-2 element, which is also found in the IL-3 promoter, while the CK-1 element is conserved in the regulatory elements of G-CSF, IL-2, IL-3, IL-4, IL-5 and IL-6. Deletion of the CK-1 and CK-2 elements from mouse GM-CSF promoter reporter plasmids led to a decrease of inducible activity (Miyatake et al., 1988b). NF-kB like transcription factors bind to a site that overlaps the CK-2 and GC-rich region (Tsuboi et al., 1991; Koyano-Nakagawa et al., 1993). The CK-1 element is responsive to CD28 signalling (Fraser and Weiss, 1992), which in both the GM-CSF and IL-2 gene promoters, has been shown to interact with NF-kB related transcription factors (Dunn et al., 1994; Ghosh et al., 1993). Human T cell leukaemia virus I (HLTV I)-infected T cells encode a viral protein, tax, that activates transcription of GM-CSF and

other genes such as IL-2, IL-3 and IL-5, through the CK-1 and CK-2 elements (Miyatake et al., 1988b; Himes et al., 1993; Himes et al., 1996)

Regulatory elements upstream of the GM-CSF promoter have been suggested to regulate GM-CSF expression. Of note, an inducible enhancer is present in the human GM-CSF locus, approximately 3.0 kb upstream of the GM-CSF gene, and may regulate its expression (Cockerill et al., 1993). The chromatin structure within this enhancer undergoes significant modification upon T cell activation as evident by the formation of a DNaseI hypersensitive (DH) site. Just as the TCR induced expression of GM-CSF is blocked by CsA, so too is the formation of the GM-CSF DH site, suggesting that CsA may directly affect the enhancer's functioning. The enhancer contains four binding sites for NFAT, three of which can function as enhancers of transcription in their own right (Cockerill et al., 1993; Cockerill et al., 1995b). These NFAT sites are composed of adjacent AP-1 and NFATp binding sites that co-operatively bind their factors (Cockerill et al., 1995b).

Another regulatory element has been identified between -192 and -161 bp upstream of the human GM-CSF gene that consists of a repeat element (Staynov et al., 1995). While initial studies show that it binds several activating factors, they remain unidentified.

There have been some studies of the transcriptional regulation of GM-CSF expression in cell types other than T cells. Endothelial cells

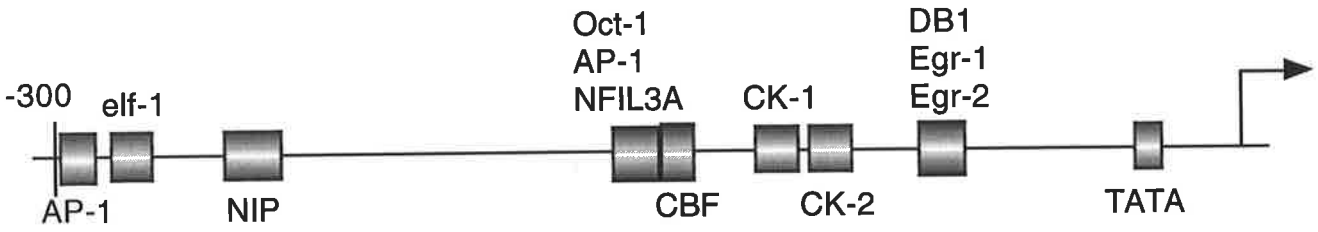
produce GM-CSF in response to IL-1 signalling, which is dependent upon sequences between -49 bp and -63 bp of transcriptional start (Kaushansky, 1989). GM-CSF in endothelial cells is also regulated by the enhancer 3.0 kb upstream of the GM-CSF gene (Cockerill et al., 1995a). In fibroblast cells, a transcription factor that is related to cold shock domain proteins binds to a single strand of the CK-1 and CK-2 elements, thereby repressing GM-CSF expression (Coles et al., 1994; Coles et al., 1996).

1.2.2. IL-3 GENE TRANSCRIPTIONAL REGULATION

Like the GM-CSF gene, many laboratories are attempting to dissect the transcriptional regulation of the IL-3 gene (Fig. 1.2). Serially deleted IL-3 promoter fragments linked to reporter genes have led to the demonstration that there are both activating and repressing factors that bind to the promoter, and that sequence up to -300 bp can convey maximal promoter activity (Mathey-Prevot et al., 1990; Park et al., 1993; Gottschalk et al., 1993; Ryan et al., 1994; Cameron et al., 1994) . Prominent activating elements include a site called ACT-1 (or NF-IL3-A). This is the binding site for the constitutive transcription factor Oct-1 and AP-1 (Shoemaker et al., 1990; Davies et al., 1993; Park et al., 1993). Recently, a transcription factor called NF-IL3A has been cloned, and demonstrated to bind to the NF-IL3-A binding site in an inducible, T cell-specific manner (Zhang et al., 1995). Therefore, NF-IL3A may contribute to the T cell specificity of the IL-3 gene expression. Directly downstream of the ACT-1 site is a binding site for

Fig. 1.2. Transcription factor binding elements of the interleukin-3 gene promoter.

Schematic representation of the transciption factor binding sites within 300 bp of the transcription start site. The DNA elements are shown as boxes, and the putative transcription factors are indicated. The arrow indicates the transcription start site.


-300
elf-1
AP-1
NIP
Oct-1
AP-1
NFIL3A
CBF
CK-1
CK-2
DB1
Egr-1
Egr-2
TATA

the transcription factor core binding factor (CBF) (Cameron et al., 1994). CBF binding appears to functionally synergise with the binding factors of ACT-1. CBF will be discussed further in section 1.3.2.

Between -128 and -109 bp upstream of transcriptional start of the human IL-3 gene are consensus sequences for CK-1 and CK-2/GATA elements. Limited work has been performed on these elements, however some evidence suggests that this region is responsible for repression of basal transcription (Ryan et al., 1994). This repression appears heightened in T cells that have been infected with HTLV I (Wolin et al., 1993). Like the CK-1 and CK-2 elements in IL-2 and GM-CSF, mutation of these elements in the IL-3 gene promoter abrogated its response to CD28 signalling (Fraser and Weiss, 1992). A CT/GC-rich region is located at position -76 to -47 and appears to bind to transcriptional activators, and be a target of HTLV I tax transactivation (Nishida et al., 1991). Proteins binding this region have been identified to be Egr-1, Egr-2 and DB1 (Koyano-Nakagawa et al., 1994). Egr-1 and Egr-2 appear to have roles in the inducible activation of IL-3, while DB1 maintains basal transcription levels. DB1 is able to synergise with tax to constitutively express IL-3.

A strong activator is located approximately 300 bp upstream of IL-3 (Mathey-Prevot et al., 1990; Shoemaker et al., 1990). This element consists of a binding site for AP-1 adjacent to an Elf-1 binding site, both which are required for maximal IL-3 expression (Gottschalk et al., 1993). Downstream of the AP-1/Elf-1 site is a repressor element, nuclear inhibitory protein (NIP), that binds to a yet undetermined

repressor (Mathey-Prevot et al., 1990; Shoemaker et al., 1990; Engeland et al., 1995).

1.3. TRANSCRIPTION FACTORS FOR CYTOKINE GENES

A comparison of the regulatory elements of cytokines demonstrate that while there are many different transcription factors involved in their regulation, there are some transcription factors that are common. Two transcription factors, nuclear factor of activated T cells (NFAT), and core-binding factor (CBF), are involved in the regulation of several cytokine and receptor genes; they are discussed below.

1.3.1. NUCLEAR FACTOR OF ACTIVATED T CELLS

Nuclear factor of activated T cells (NFAT) is a heterodimeric transcription factor that has been implicated in the transcriptional regulation of some cytokine genes. It was initially identified as a factor that footprinted to the IL-2 enhancer in activated T cells, appearing within 25 minutes of activation (Shaw et al., 1988). Subsequently, it was shown that CsA and FK506, which block IL-2 production and that of other cytokines, blocks the binding of NFAT (Flanagan et al., 1991). Combining either unstimulated cytosolic extracts or cytosolic extracts stimulated in the presence of CsA or FK506, with stimulated nuclear extracts with or without CsA or FK506, led to the formation of the complex. This indicated that NFAT forms

through the association of a pre-existing component that is translocated to the nucleus in a CsA sensitive manner, and a CsA insensitive component that is synthesised *de novo* (Flanagan et al., 1991).

Activator protein-1 (AP-1) is an inducible heterodimeric transcription factor that appears in the nucleus very soon after cell activation through protein kinase C signalling pathways (Jain et al., 1992b). It was first identified as a protein that bound to the human metallothionein IIA gene promoter and the SV40 enhancer (Lee et al., 1987). It has subsequently been shown to regulate a wide variety of both cellular and viral genes. AP-1 is composed of two subunits, from the *fos* and *jun* gene families (Bohmann et al., 1987, Franza et al., 1988). These families encode basic region/leucine zipper proteins where the basic region binds to DNA and the leucine zipper is required for heterodimeric interaction. The Jun family members, c-Jun, JunB and JunD, can form homodimers or heterodimers with Fos family members (Halazonetis et al., 1988; Nakabeppu et al., 1988). The Fos family members, c-Fos, Fra-1, Fra-2 and fosB appear to only form heterodimers with Jun family members (Chiu et al., 1988). Both c-Fos and FosB are able to interact with TATA binding protein suggesting a role in recruiting and stabilising the basal transcriptional machinery (Metz et al., 1994a, Metz et al., 1994b). A *c-jun* gene disruption is lethal (Johnson et al., 1992), while mice with disruptions to the *c-fos* gene have many developmental and neurological defects (Johnson et al., 1992; Wang et al., 1992), showing the importance of these families of transcription factors.

AP-1 was demonstrated to be the CsA-insensitive component of NFAT (Jain et al., 1992a). Binding studies of the NFAT complex showed the formation of two DNA-protein complexes with the IL-2 NFAT site in stimulated T cells. The formation of the lower mobility complex could be blocked with the addition of AP-1 binding sites as cold competitors. The formation of the complex could also be blocked with antibodies specific to c-fos, fra-1, c-jun and junB. In experiments linking an NFAT binding site to reporter genes, cotransfection with constitutively expressing c-jun, junD, c-fos and fra-1 plasmids could overcome the need for signalling through the protein kinase C pathway, leaving the NFAT driven expression under the control of calcium signalling alone. (Northrop et al., 1993). This shows that the AP-1 component of NFAT can contain various members of the fos and jun families.

The CsA sensitive component of NFAT was identified soon after by two groups. Rao and coworkers purified NFATp (p=pre-existing), and found it to have an apparent moeclular weight of 110-140 kDa in resting T cells, and 90-125 kDa in activated T cells (McCaffrey et al., 1993a). Purified NFATp was shown to form a DNA-protein complex with recombinant jun homodimers and fos-jun heterodimers (Jain et al., 1993). The difference in size in resting and activated cells was believed to represent a dephosphorylated state in activated cells, as treatment of NFATp from resting cells with calf intestinal phosphotase yielded a protein of equivalent size to the NFATp isolated from activated cells (Jain et al., 1993). Furthermore, it was shown that NFATp was a target for calcineurin; NFATp underwent a decrease in

its apparent molecular weight in the presence of calcineurin in vitro, which was blocked by calcium chelators such as EGTA, or by specific peptide inhibitors of calcineurin. NFATp was subsequently cloned (McCaffrey et al., 1993b; Hoey et al., 1995; Masuda et al., 1995). NFATp expressed from a truncated cDNA clonewas demonstrated to bind to the IL-2 enhancer NFAT site and complex with fos and jun. Antibodies raised against NFATp reacted with the NFAT complex from nuclear extracts bound to the IL-2 enhancer NFAT site thereby confirming that NFATp is indeed a component. Crabtree and colleagues also purified a cytosolic component to NFAT, called NFATc (c=cytosolic). It was found to have a molecular weight between 94 and 116 kDa (Northorp et al., 1993). Upon cloning of NFATc, it was revealed it has similarities with NFATp, yet is distinct (Northorp et al., 1994). When co-transfected, NFATc was able to increase the transcription through a NFAT binding site driven reporter plasmid, demonstrating its ability to transactivate through this site. Significantly, NFATc mRNA expression appears to be inducible in T cells in response to PMA and calcium ionophore signalling, but NFATp mRNA is not inducible in these conditions (Northorp et al., 1994).

To date, four family members of NFAT have been described: NFATp, NFATc, NFAT3 and NFAT4 (or NFATx) (McCaffrey et al., 1993b; Northorp et al., 1994; Hoey et al., 1995; Masuda et al., 1995). All four members contain a highly conserved domain that is similar to the DNA binding and dimerisation domain of the Rel family of transcription factors. Other similarities within the NFAT family have been noted. There are conserved serine and proline rich regions in the N-terminal

regions (Hoey et al., 1995). The N-terminal regions have recently been shown to be responsible for the association with calcineurin (Luo et al., 1996).

In contrast to its name, the NFAT family of transcription factors are expressed in a cell types other than T cells. NFATp RNA is detected in almost all human tissues, with especially high levels present in the placenta, spleen and in leukocytes (Northorp et al., 1994; Hoey et al., 1995; Masuda et al., 1995). NFATc RNA is detected at low levels in many cell types, with high levels present in skeletal muscle and thymus. NFAT3 RNA is highly expressed in placenta, lung, kidney, ovary and testis (Hoey et al., 1995). However its expression is very weak in tissues associated with the immune system. NFAT4 is strongly expressed in skeletal muscle, and to a lesser extent in thymus and kidney (Hoey et al., 1995; Masuda et al., 1995).

The ability of NFATp/c/3/4 to tranlocate into the nucleus is tightly associated with its phosphorylation state. T cell stimulation leads to a rapid dephosphorylation of NFATp which occurs before its translocation (Shaw et al., 1994). This dephosphorylation is very closely linked to the activity of calcineurin, as the addition of CsA or EGTA to calcium ionophore stimulated T cells, or the removal of calcium ionophore, leads to a rapid rephosphorylation of NFATp, and its return to the cytosol. A recent report has demonstrated that calcineurin is translocated into the nucleus upon calcium-dependent signalling (Shibasaki et al., 1996). When signals stimulating calcineurin are removed from the cell, both calcineurin and NFAT4 are

shuttled back into the cytosol. Another report has shown that protein kinase C signalling contributes to gradual NFATp rephosphorylation, demonstrating an active return to resting state after TCR stimulation (Loh et al, 1996a).

Mice with disrupted NFATp genes have been generated (Hodge et al., 1996; Xanthoudakis et al., 1996). Interestingly, mice lacking functional NFATp exhibit heightened immune response with increases in leukocyte numbers. These mice did, however, show a major early defect in IL-4 expression, and to a lesser extent, GM-CSF, IL-13 and TNFα (Hodge et al., 1996). IL-4 levels were raised at later time points to levels greater than that which is seen in normal mice, suggesting a dynamic role for NFATp.

Numerous genes appear to be regulated by NFAT in T cells. Within the IL-2 gene regulatory elements are five NFAT sites (Rooney et al., 1995). NFAT also regulates cytokine genes such as GM-CSF, IL-3, IL-4, IL-5, TNFα, and gp39 (Cockerill et al., 1995b; Szabo et al., 1993; Tsuruta et al., 1995; Goldfeld et al., 1993; Schubert et al., 1995; Tsytsykova et al., 1996; McCaffrey et al., 1993b). A comparison of NFATp/c/3/4 binding sites has highlighted a consensus binding sequence of $^{A}/_{T}GGAR A^{R}/_{C}{}^{A}/_{T}$ (Cockerill et al., 1995b; R=purine, Y=pyrimidine). The binding of the NFAT complex is often co-operative, as is seen in NFAT sites of the human GM-CSF enhancer and IL-2 promoter, with strong binding of the complex, yet only weak binding of the individual components (Cockerill et al., 1995b; Rooney et al., 1995). NFATp/c/3/4 is able to cooperatively bind with partners

other than AP-1. The TNFα promoter binds NFATp/c/3/4 in conjunction with ATF-2/jun binding to a cAMP responsive element (Tsai et al., 1996). In T cells, activation of the IL-4 gene involves AP-1 in the NFAT complex, whereas in mast cells, AP-1 does not appear to bind (Weiss et al., 1996). Hence the partner with which NFATp/c/3/4 associates may influence cell type specific regulation of certain genes.

1.3.2. CORE BINDING FACTOR

Core Binding Factor (CBF) is a heterodimeric transcription factor that binds to regulatory elements of genes involved in cell differentiation. CBF was independently isolated by numerous groups. Ito and colleagues isolated CBF as a factor (PEBP2) that bound to the polyomavirus enhancer A in mice (Satake et al., 1988; Kamachi et al., 1990). PEBP2 was shown to be undetectable in F9 embryonal carcinoma cells, yet was upregulated when the cells began to differentiate (Kryszke et al., 1987; Satake et al., 1988; Furukawa et al., 1990). Speck and co-workers identified CBF as a DNA-binding protein that associated with the Molony murine leukaemia virus enhancer core site (Wang et al., 1992), a site that is highly conserved in many enhancers of Mammalian Type C retroviruses (Golemis et al., 1990). Purification of CBF/PEBP2 showed that it was composed of two families of polypeptides, α and ß (Kamachi et al., 1990), and that it could also bind to the enhancer core motif of a simian immunodeficiancy virus, and to cellular enhancers of the immunoglobulin μ chain and T-cell receptor γ-chain genes (Wang et al.,

1992). Isolation of cDNA clones for CBFα/PEBP2α revealed that this protein is encoded by the *AML1* gene (Bae et al., 1993; Ogawa et al., 1993b; Wang et al., 1993), that was identified by Miyoshi et al. (1991). Furthermore, CBFα has a strikingly high degree of homology with a region in the *Drosophila* segmentation protein, Runt, and appear to belong to a family of transcription factors (Bae et al., 1993; Ogawa et al., 1993b; Wang et al., 1993). The ß subunit of CBF was cloned by several groups (Liu et al., 1993; Ogawa et al., 1993a; Wang et al., 1993). While CBFß was found to not directly bind to DNA, it could increase the binding affinity of the CBFα subunit (Ogawa et al, 1993a; Wang et al., 1993).

The Runt family of transcription factors all contain a highly conserved Runt domain that is required for binding to DNA and CBFß (Ogawa et al. 1993b; Lenny et al., 1995). Outside the runt domain, the sequence diverges considerably (Ogawa et al., 1993b). A nuclear localisation signal sequence is present in both the Runt domain and in the C-terminal sequences to the Runt domain (Lu et al., 1995). The transactivation potential of these proteins appears to also reside in the C-terminal domain (Bae et al., 1994).

Three related genes, *AML1*, *AML2* and *AML3* have been found to express CBFα subunits in humans. These genes are homologous to mouse genes *PEBP2αB*, *PEBP2αC* and *PEBP2αA* respectively. AML1 is expressed primarily in haemopoietic cells (Satake et al., 1995). Hence, it is expressed in the thymus, spleen, foetal liver, and bone marrow (Satake et al., 1995). It is also transcribed in low amounts in the

heart, lungs, skeletal muscle and testes (Satake et al., 1995; Zhu et al., 1994). The *AML1* gene can generate at least three alternatively spliced mRNA isoforms, AML1a, b and c (Miyoshi et al., 1995; Bae et al., 1993). Whereas AML1b and c are both able to transactivate and positively regulate transcription, AML1a, a smaller isoform missing the C-terminus, is able to bind to DNA with a greater affinity than AML1b and c, but is unable to transactivate, and hence appears to act as a negative regulator (Bae et al., 1994; Tanaka et al., 1995; Miyoshi et al., 1995). The *AML2* gene generates at least two alternatively spliced isoforms and is ubiquitously expressed (Speck et al., 1995; Levanon et al., 1994). *AML3* appears to be expressed in the thymus, primarily in T cells (Ogawa et al.,1993b; Satake et al., 1995).

Thus far, only one gene for CBFß, which is located on human chromosome 16, has been isolated although at least four isoforms of this gene exist (Zaiman et al., 1995). In *Drosophila*, two genes have been isolated that are able to interact with Runt, both which show considerable homology with CBFß within the domain required for dimerisation with AML1 (Golling et al., 1996; Hajra et al., 1995). Interestingly, whilst AML1 is restricted in the types of cells in which it is expressed, CBFß appears to be ubiquitously expressed, suggesting that it may have other roles than associating with AML1 in cells that don't express AML1 (Ogawa et al., 1993a; Satake et al., 1995; Wang et al., 1993).

The AML1/Runt family of binding proteins binds to the consensus sequence YGYGGTY (Y=pyrimidine) (Melnikova et al., 1993).

Interestingly, the most commonly found AML1 binding site in mammalian genes is TGTGGTTT, whereas mammalian retroviruses usually contain TGTGGTCA (Melnikova et al., 1993). The Runt protein has been implicated in Drosophila developmental genes involved in segmentation, sex determination and neurogenesis (Gergen et al., 1985; Duffy et al., 1991a; Duffy et al., 1991b). Recently, a Runt homologue, lozenge, was found to be required for proper Drosophila eye development (Daga et al., 1996). AML1 has been shown to be required for the expression of a number of haemopoietic related genes such as the T-cell receptor-γ,-δ, and -β genes, M-CSF receptor gene, interleukin-3 gene, myeloperoxidase gene, neutrophil elastase gene, granzyme B gene, and the CD36 gene (Giese et al., 1995; Redondo et al., 1992; Hsiang et al., 1993; Prosser et al., 1992; Zhang et al., 1994; Cameron et al., 1994; Nuchprayoon et al., 1994; Wargnier et al., 1995; Armesilla et al., 1996). It has also been found to regulate a bone specific gene, osteocalcin, involved in osteoblast differentiation (Merriman et al., 1995).

In all cases reported, CBF acts as a positive regulator of transcription. Mutagenesis and transactivation experiments show that CBF is necessary for full activation of TCR–α, –δ, –γ, CSF-1R and CD36 genes, and in some cases, CBF is able to synergise with adjacent transcription factors such as c-Myb, Ets-1 and C/EBP, to activate transcription (Hernandez-Munain et al., 1994; Hernandez-Munain et al., 1995; Hsiang et al., 1995; Wotton et al., 1994; Zhang et al., 1996). One report has found that whilst c-Myb and CBF functionally synergise, they bind independently, suggesting that they act on different aspects of

transcription (Hernandez-Munain et al., 1995). In contrast, CBF and Ets-1 have been demonstrated to cooperatively bind to their sites in the T-cell receptor α and ß enhancer sites (Giese et al., 1995; Wotton et al., 1994), suggesting that CBF interacts differently with certain transcription factors.

When AML1b was overexpressed in NIH3T3 cells the cells became transformed, suggesting a role in myeloid proliferation and differentiation (Kurokawa et al., 1996). Deletions of the *AML1* gene are lethal, with the mice dying around embryonic day 12.5 with a lack of foetal liver haemopoiesis, and haemorrhaging in the central nervous system (Okuda et al., 1996; Wang et al., 1996). Chimaeric mice that contained both wild type and homozygous AML1 gene knockout embryonic stem cells had only wild type embryonic stem cells contributing to haemopoiesis suggesting that AML1 is required for the production of all haemopoietic cell types (Okuda et al., 1996).
A study of CBF function in regulating the osteocalcin gene has been informative. NMP-2, a osteoblast specific AML-1 homologue, is able to transcriptionally activate the gene through its promoter (Merriman et al., 1995; Banerjee et al., 1996). NMP-2 has been shown to be exclusively associated with the nuclear matrix. suggesting a role of nuclear compartmentalisation (Merriman et al., 1995; Banerjee et al., 1996). Hiebert et al. (1996) have also shown that AML-1b is partitioned in nuclear fractions isolated under high salt conditions. This suggests that AML1 is also associated with the nuclear matrix. Indeed, preliminary studies from our laboratory have demonstrated that T cells immunofluorescently stained with antibodies to AML-1

show that AML1 is concentrated in highly localised foci in the nuclei, remeniscent of transcriptional 'factories' where transcription is postulated to take place (G. Cockerill, unpublished data; Jackson et al., 1993; Cook, 1995).

Chromosomal translocations within both the α and ß subunits of CBF have been associated with acute myelogenous leukaemias (AML) (Miyoshi et al., 1991; Mitani et al., 1994; Golub et al., 1995; Liu et al., 1993). AML is one of the most common haemopoietic disorders, affecting the proliferation of haemopoietic precursor cells, resulting in the overproduction of immature myeloid cells (Rowley, 1990). The genes for AML1 and CBFß are often found to be affected by chromosomal translocations in AML's resulting in the production of a fusion protein. Several AML1 fusion proteins have been described (Erickson et al., 1992; Miyoshi et al., 1993; Mitani et al., 1994; Nucifora et al., 1993; Golub et al., 1995; Liu et al., 1993).

The (8;21)(q22;q22) translocation is found in approximately 40% of all AML-M2 cases (Nucifora and Rowley, 1995). This translocation yields a fusion of AML1 of chromosome 21 with ETO of chromosome 8, a gene with an unknown function. The resulting fusion encodes a 83kD protein that contains the first 177 of 250 amino acids of AML1 and the last 575 of 604 amino acids of ETO (Miyoshi et al., 1991). This protein retains the runt homology domain of AML1, and can bind to DNA and to CBFß (Miyoshi et al., 1991; Meyers et al., 1995). However, AML1/ETO is unable to transactivate genes with AML1 regulatory elements suggesting that either a section of the AML1 transactivation

domain is missing, or the ETO fusion is able to interfere with AML1 action (Meyers et al., 1995). Furthermore, when the AML1/ETO protein is mixed with wildtype AML1b (the active isoform of AML1), the AML1/ETO fusion protein is able to block transactivation by AML1b, showing that it is able to function as a dominant negative regulator (Meyers et al., 1995; Lenny et al., 1995). When antisense oligonucleotides complementary to the RNA of the chimaeric protein was introduced into a leukaemic t(8;21) cell line, the uninhibited proliferation of these cells was halted and they were induced to differentiate, suggesting that the AML1/ETO fusion gene is central to the leukaemic phenotype (Sakakura et al., 1994).

The AML1/Evi1 fusion protein is a result of the (3;21)(q26;q22) translocation and is found in a small percentage of leukaemia patients (Nucifora and Rowley, 1995). Evi1 is a DNA binding protein whose expression is spatially and temporally controlled, and is required for proper development of the kidneys, the brain and the peripheral nervous system (Nucifora and Rowley, 1995). The *AML1/Evi1* fusion truncates the *AML1* gene at the end of the runt domain and links it to the entire *Evi1* gene, including a region of its 5' untranslated region (Mitani et al., 1994). Like the AML1/ETO chimaera, the AML1/Evi1 fusion protein is able to bind DNA and is likely to bind CBFß, but is unable to transactivate at AML1 binding sites (Tanaka et al., 1995). In addition, it is also able to dominantly suppress the binding of AML1b (Tanaka et al., 1995). However, it appears that the Evi1 region of the protein may also contribute to the leukaemic phenotype. Both Evi1 and AML1/Evi1 are able to transactivate the c-fos and c-jun

promoters, thereby increasing cellular AP-1 activity (Tanaka et al., 1995; Kurokawa et al., 1995). This activation is dependent on a zinc finger domain in the Evi1 protein (Kurokawa et al., 1995). AML1/Evi1 is able to transform Rat1 fibroblasts, but deletion of this zinc finger in AML1/Evi1 inhibited its ability to transform fibroblasts (Kurokawa et al., 1995). Cellular AP-1 levels correlated with the transformation of these cells. This is consistent with the proto-oncogenic potentials of c-Fos and c-Jun .

CBFß gene chromosomal translocations has also been implicated in subtype M4Eo AML's in which the *CBFß* gene is fused to a smooth muscle myosin heavy chain gene (*SMMHC*) via a chromosome 16 inversion (Liu et al., 1993). This inversion links the first 165 of 187 amino acids of CBFß to various portions of the SMMHC protein (Liu et al., 1994). This fusion protein is able to complex with AML1, but the transactivation potential is disturbed (Hajra et al., 1995). CBFß/SMMHC is able to transform NIH3T3 fibroblasts and form rod-like structures in the nucleus, possibly due to the aggregation of the SMMHC component (Wijmenga et al., 1996).

Proper cytokine regulation is required for normal differentiation of haemopoietic cells. AML's are often characterised by an inability of haemopoietic precursor cells to differentiate into the various myeloid cell lineages One would expect a nonfunctional CBF transcription factor to downregulate the production of cytokines and their receptors, contributing to this block in the differentiation potential of haemopoietic cells. Indeed, some AML cell lines can be forced into

differentiation by the administration of cytokines such as GM-CSF and IL-3 (Lotem et al., 1988; Shabo et al., 1990; Bassan et al., 1994), implying a deficit in the production of differentiation inducing factors. However, another subset of AML's constitutively produce cytokines and induce themselves to proliferate in an autocrine loop. Cytokines such as GM-CSF, G-CSF, M-CSF, IL-1 and IL-6 have been found to be expressed in some AML cells cultured *in vitro* (Young et al., 1988; Russell, 1992; Rogers et al., 1994). In some cases, AML blast cell growth can be inhibited with the treatment of antisense GM-CSF or anti-GM-CSF antibody showing that, for these AML lines, GM-CSF plays a major role in their tumourigenicity (Rogers et al., 1994; Freedman et al., 1993). The mechanisms by which some AML's proliferate and others differentiate in response to cytokines, and their relationship to CBF and its fusion proteins remains undetermined.

1.4 THESIS AIMS

The aims of this project were as follows:

1). To determine whether a homologue to the human GM-CSF gene enhancer exists in the mouse GM-CSF gene locus.

2). To compare the mechanisms of gene regulation of the mouse and human GM-CSF gene loci.

3). To investigate whether the transcription factor CBF participates in the regulation of the GM-CSF locus.

CHAPTER 2.

MATERIALS AND METHODS

2.1. ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CK-1	cytokine-1
CLE	conserved lymphokine element
CsA	cyclosporin A
dbcAMP	dibutyryl cyclic adenosine monophosphate
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol-bis-(ß-amino-ethyl ether)N,N'-tetra-acteic acid
FCS	foetal calf serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
IL	interleukin
kb	kilobase
kDa	kilodalton
LUC	luciferase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate
PMSF	phenyl-methyl sulphonyl fluoride

RT	room temperature
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
SV	Simian Virus
TAE	Tris/Acetic Acid/EDTA buffer
TBE	Tris/Boric Acid/EDTA buffer
TE	Tris/EDTA buffer
TK	thymidine kinase
Tris	tris(hydroxymethyl) aminomethane

2.2. CHEMICALS AND REAGENTS

Major sources of important reagents and chemicals were as follows:

ampicillin, EGTA, HEPES, Tris base, PMA, PMSF, aprotinin, leupeptin, dibutyryl cyclic AMP: Sigma Chemical Company, St. Louis, MO.;

agarose, DTT: 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: BDH Chemicals, Kilsyth Vic.;

phenol: Wako Pure Chemical, Osaka, Japan;

deoxyribonucleotide triphosphates (dNTPs): Pharmacia, Uppsala, Sweden;

calcium ionophore A23187, D-luciferin, deoxyribonucleotide triphosphates (dNTPs): Boehringer Mannheim Australia, Castle Hill, NSW;

PHA: Wellcome Pharmaceuticals, Kent, UK;

forskolin: Calbiochem, San Diego, CA.;

Proteinase K: Merck, Rahway, NJ.;

Super-base sequencing kit: Bresatec, Adelaide, SA.;

CsA: gift of Sandoz, Basel, Switzerland

2.3. RADIOCHEMICALS

Radiochemicals were obtained from the following sources:

[α-^{32}P]dATP, [γ-^{32}P]dATP: Bresatec, Adelaide, Australia.

[^{35}S]dATP, [γ-^{33}P]dATP: NEN-Dupont.

2.4. ENZYMES

All restriction enzymes were purchaced from Amersham, New England Biolabs, Beverly, MA, Pharmacia, or United States Biochemical Corporation (USB), Ohio.

Other enzymes were obtained from the sources listed:

acetyl coenzyme A, calf instestinal phosphatase: Boehringer Mannheim;

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

ribonuclease A: Sigma;

deoxyribonucleaseI: Worthington, Freehold, NJ.

2.5. BUFFERS

luciferase harvest buffer: 100 mM KH_2PO_4 pH 7.4, 2 mM DTT, 10 mM EDTA

luciferase assay buffer: 100mM KH_2PO_4 pH 7.4, 20mM DTT, 80 mM $MgSO_4$, 7.5 mM ATP, 1.75 mM Coenzyme A

PBS: 136 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.3

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

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

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

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

2.6. CLONING VECTORS

pXP1luc and pTK81luc were gifts from Dr. S. Nordeen, University of Colorado, Denver, USA (Nordeen et al,. 1988)

pGL3basic and pGL2promoter were purchased from Promega, Madison, WI.

2.7. CLONED DNA SEQUENCES

pAOGM (Stanley et al., 1988) and pMUP were gifts from Drs. E. Stanley and A. Dunn, Ludwig Institute for Cancer Research, Melbourne, Australia.

pGMluc was a gift from Dr. R. Himes, this department (Himes et al., 1993).

pGMEluc was a gift from Dr. F. Jenkins, this department (Jenkins et al., 1995).

2.8. SYNTHETIC OLIGONUCLEOTIDES

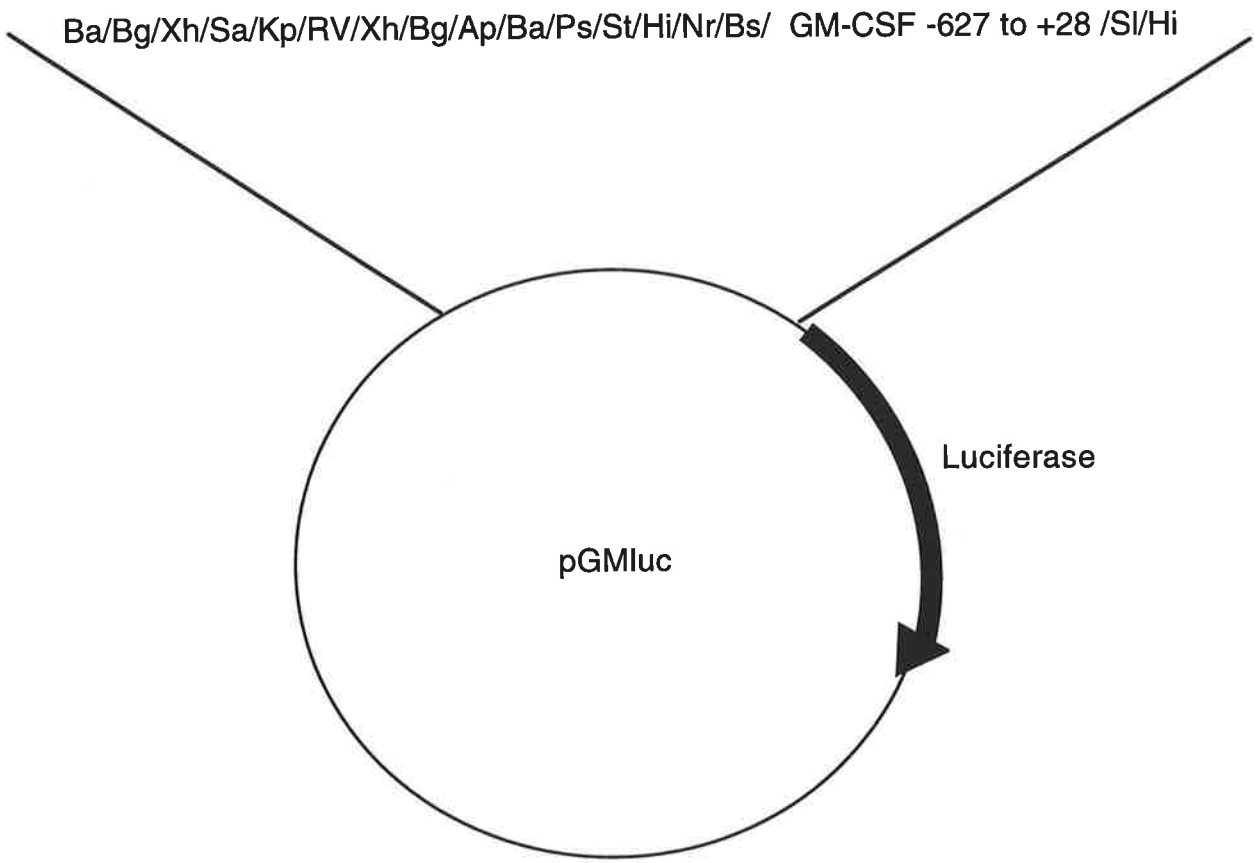
Early in the project, oligonucleotides were synthesised on an Applied Biosystems model 381A DNA synthesiser. Subsequent oligonucleotides were prepared by Bresatec, Adelaide. All oligonucleotides were electrophoresed on a 0.5x TBE, 10% polyacrylamide gel, and excised using UV shadowing. The purified oligonucleotide was eluted from the gel fragment in 5 ml 50 mM NaAcetate at 56°C. The supernatant was transferred to a new tube, its volume reduced by butanol extraction, and then ethanol precipitated and dissolved in TE. Oligonucleotide concentration was determined by measuring its absorbance at 260 nm where OD_{260} 30 = 1 mg/ml. Complimentary oligonucleotides were annealed into duplexes by heating 5 ug of the two strands to 90°C for 2 minutes in 25 ul 50 mM NaCl, and allowing the mixture to cool slowly to room temperature. Sequences for oligonucleotides are indicated in section 2.13.1.

2.9. CONSTRUCTION OF LUCIFERASE PLASMIDS

The plasmid pGM was made in this department by R. Himes (Himes et al., 1993) (Fig. 2.1). pGME was made by F. Jenkins, this department, by cloning the 716 bp *BglII* human GM-CSF enhancer fragment into a *BglII* site in the polylinker of pGM. Similarly, pGM+M703 was made by cloning the 703 bp *MscI* mouse GM-CSF enhancer fragment of pAOGM into a *StuI* site within the polylinker of pGM.

Fig. 2.1 Schematic diagram of the luciferase reporter gene construct, pGMluc.

The expanded section of the plasmid map represents the multiple cloning sites, into which the human GM-CSF gene promoter (-627 bp to +28 bp relative to the transcription start site) was cloned. The following abbreviations have been used for restriction enzyme sites: Ap, ApaI; Ba, BamHI; Bg, BglII; Bs, BstEII; Hi, HindIII; Kp, KpnI; Nr, NruI; Ps, PstI; RV, EcoRV; Sa, SacI; Sl, SalI; St, StuI; Xh, XhoI. The luciferase reporter gene is represented as a thick black arrow.


Ba/Bg/Xh/Sa/Kp/RV/Xh/Bg/Ap/Ba/Ps/St/Hi/Nr/Bs/ GM-CSF -627 to +28 /Sl/Hi
Luciferase
pGMluc

pMGM3.1 was constructed by digesting pGM with *SacI* to remove the sequence between -19 bp of the human GM-CSF gene promoter, and a *SacI* site within the polylinker, upstream of the promoter. Into this vector, a 3.1 kb *SacI* mouse GM-CSF gene promoter fragment, isolated from pAOGM (Stanley et al., 1985), was cloned. All subsequent mouse GM-CSF gene promoter constructs were derived from pMGM3.1. pMGM3.1ΔE was made by removing a 703 bp *MscI* fragment from pMGM3.1, and religating. pMGM2.3 was constructed by digesting pMGM3.1 with *BglII*, before religation, to remove all insert sequence upstream of -2.3 kb. pMGM1.6 was made by cutting pMGM3.1 with *BglII* and *MscI*, to remove all insert sequence upstream of -1.6 kb, before end-filling and religating. pMGM1.6+B716 was made by inserting a 716 bp *BglII* human enhancer fragment into a *BamHI* site within the upstream polylinker of pMGM1.6. pMGM0.2 was constructed by digesting pMGM3.1 with *BglII* and *StuI*, to remove all insert sequence between -0.2 kb and the upstream polylinker, before end-filling and religating. pMGM0.06 was constructed by cutting pMGM3.1 with *BglII* and *BstEII*, to remove all insert sequence between -60 bp and the upstream polylinker, before end-filling and religating. The *B716* human enhancer fragment and the *M703* mouse enhancer fragment were subsequently cloned into the polylinker of pMGM0.06 to make pMGM0.06+B716 and pMGM0.06+M703, respectively.

pTK+M703 and pTK+M703R were made by inserting the 703 bp *MscI* mouse GM-CSF enhancer fragment, in both orientations, into a *SmaI* site within the polylinker of pTK81 (Nordeen, 1988). Likewise,

pTK+B176 was constructed by inserting the 716 bp *BglII* human GM-CSF enhancer fragment into a *BamHI* site within the polylinker of pTK81.

pSV40+M703 and pSV40+B716 were made by inserting the mouse and human enhancer fragments into the polylinker of the SV40 promoter driven luciferase plasmid (pGL2-promoter), respectively.

The pMIL3 construct was made by inserting a 560 bp *HindIII* PCR IL-3 promoter fragment from the pMUP plasmid into a *HindIII* site within the polylinker of pXP1 (Nordeen, 1988). pMIL3+M703 and pMIL3+B716 were subsequently constructed by inserting the 703 bp *MscI* mouse enhancer fragment and the 716 bp *BglII* human enhancer fragment, respectively, upstream of the mouse IL-3 promoter in the pMIL3 plasmid.

The constructs pGM116, pHSp1 and pHCAT were constructed by inserting an oligonucleotide with *XhoI* and *BstEII* ends, into *XhoI/BstEII* cut pGM. The constructs pMGM119, pHM116, pMSp1 and pMCAT were all constructed by inserting an oligonucleotide with *XhoI* and *BstEII* ends, into *XhoI/BstEII* cut pMGM3.1. The constructs pM4bp and pMTATA were constructed by inserting oligonucleotides with *BstEII* and *SacI* ends, into the *BstEII/SacI* cut pMGM119. Likewise, pH4bp and pHTATA were made by inserting oligonucleotides with *BstEII* and *SacI* ends, into *BstEII/SacI* cut pGM116. These plasmids are described in chapter 4.

pGM627, pGM69, pGM69ΔCBF, pGM627ΔCBF and pGM55 were designed by P. Cockerill, A. Bert and J. Burrows (this laboratory) (Cockerill et al., 1996). pGM47 was created by inserting a 31 bp oligonucleotide sequence, containing bases -47 bp to -20 bp of the human GM-CSF gene promoter, into a pGM627luc construct that had been cut with *SacI* to excise the promoter from -19 bp to -627 bp. This created a plasmid that contained the human GM-CSF promoter up to -47 bp. The pGM47CBFe construct was created by inserting a 52 bp oligonucleotide sequence, containing -47 bp to -20 bp of the human GM-CSF promoter, linked to a 19 bp fragment encompassing the GM-CSF enhancer CBF site, into a *SacI* cut pGM627 vector. pGM627CBF3e and pGM55CBF3e were constructed by inserting *XhoI/NruI* cut multimers of three enhancer CBF sites (designed by A. Bert), into *XhoI/SmaI* cut pGM627 and pGM55 respectively.

2.10. BACTERIAL CULTURE

The *E. coli* strains XL1blue and JM109 were used as a host for recombinant plasmids. 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).

Where appropriate, media and plates were supplemented with 100 ug/ml ampicillin after autoclaving.

2.11. RECOMBINANT DNA METHODS

2.11.1 ISOLATION OF DNA FRAGMENTS

Typically, DNA was cleaved with restriction enzymes as per the recommended digestion conditions supplied by New England Biolabs. DNA digests were then electrophoresed through a 0.7 cm thick 0.8% agarose gel in 1x TA buffer with 0.5 ug/ml ethidium bromide. The band of interest was then cut out of the gel over a UV light box with a scapel blade. The DNA band was removed from the gel fragment using a Gene Clean kit (Bresatec). The DNA fragment was quantitated by electrophoresing a sample volume on an agarose gel along with a standard marker of known concentration.

2.11.2 LIGATION OF DNA FRAGMENTS

In a 10 ul reaction, 100 ng of vector (linearised and phosphatased) was ligated to a three molar excess of the insert fragment. The ligation buffer contained 1x one-phor-all buffer (Pharmacia), 2.0 mM DTT and 0.5 mM ATP. 0.5 ul of T4 ligase (1 unit/ul) was added and the reaction was incubated at 14°C for one hour.

2.11.3 PREPARATION OF COMPETENT BACTERIA

A colony from a plate of a bacterial strain such as XL1blue or JM109 was picked and used to inoculate a small volume of LB broth and grown overnight at 37°C. This starter culture was used to inoculate 500 ml of LB broth which was grown at 37°C until the culture reached an OD_{650} of 0.5. The culture was then transferred to 50 ml plastic Falcon tubes and incubated on ice for 30 minutes. The cells were then centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatant was removed and the cells were pooled and resuspended in 250 ml of ice cold 0.1 M $CaCl_2$. The resuspended cells were incubated on ice for one hour after which time, 2.6 ml of glycerol was added. Cells were then aliquoted into 520 ul volumes in Eppendorf tubes and stored at -70°C for future use.

2.11.4 BACTERIAL TRANSFORMATION

One hundred microlitres of freshly thawed competent bacteria were added to 5 ul of the ligation mixture, and incubated on ice for 10 minutes. The cells were then heat shocked at 42°C for 1.5 minutes, and then cooled on ice. Fifty microlitres of the cells were then added to 150 ul of LB broth, and incubated at 37°C for 45 minutes. These cells were then plated on plates containing ampicillin, and incubated overnight at 37°C. Colonies on plates of transformed bacteria were screened for the correct plasmid construct by picking the colony with a sterile toothpick, growing it in LB broth with 100 ug/ml ampicillin,

preparing a miniprep purification of the plasmid, and then either performing diagnostic restriction enzyme digests and/or sequencing the insert region of the plasmid.

2.11.5 SMALL SCALE PREPARATION OF PLASMID DNA -ALKALINE LYSIS METHOD

A sterile 5 ml polysterene tube was used to grow a 2 ml culture of the plucked colony. The miniculture was grown overnight in LB broth plus 100ug/ml ampicillin, in a 37°C shaking incubator. One and a half millilitres of culture was transferred to a 1.5 ml Eppendorf tube, and centrifuged for one minute in a microcentrifuge at 6000 rpm. The LB broth was completely aspirated, and the cell pellet was resuspended by vortexing in 100 ul of cold 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 0.02% sodium azide. The cells were incubated on ice for 2 minutes, and then 200 ul 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. To this mixture, 150 ul of cold 3 M potassium acetate, 2 acetic acid (pH 5.8) was added. The mixture was again mixed gently by inverting the tube several times, and then incubated on ice for 10 minutes. The mixture was then spun at 13000rpm for 10 minutes at 4°C. The supernatant was transferred to a new Eppendorf tube, and an equal volume of 1:1 phenol:chloroform was added. The tube was vortexed, centrifuged, and the supernatant was transferred to a new Eppendorf tube. To precipitate the plasmid DNA, 900 ul of 100% ethanol was added, the

tube was incubated at -20°C for 20 minutes, and then centrifuged at 13000 rpm for 10 minutes. The DNA pellet was washed with 200 ul 75% ethanol, 50 mM NaCl, and then with 200 ul 100% ethanol. The pellets were allowed to dry with open lids in a 37°C heating block for 5 minutes, then dissolved in 40 ul TE, 20 ug/ml RNaseA, and incubated for one hour at 37°C with periodic mixing. DNA preps were then stored at -20°C for future use.

2.11.6 SMALL SCALE PREPARATION OF PLASMID DNA-COCKY PREP METHOD

This is a rapid small scale plasmid preparation devised by Dr. P. Cockerill that yields plasmid DNA that is suitable for enzymatic digestion but not for DNA sequencing. Essentially, 1.5 ml of overnight bacterial culture was centrifuged for one minute at 6000 rpm in an microcentrifuge. After removing the supernatant, 50 ul TE was added, and the pellet was resuspended by vortexing. An Eppendorf tube containing approximately 500 mg of silicone grease was spun down briefly to bring the grease to the bottom of the tube. To the resuspended pellet, 100 ul phenol/chloroform was added, and the tube was mixed gently by inverting the tube several times. The contents of the tube were then transferred to the silicone grease containing tube, and spun at high speed for one minute. Two hundred microlitres of chloroform was added, and the tube was mixed gently before spinning for one minute. The liquid left at the top of the silicone grease that contains the plasmid in approximately 50 ul TE.

This was transferred to a new Eppendorf tube and mixed with 5 ul of 3 M NaAcetate and 100 ul ethanol. The tube was incubated on ice for 5 minutes before spinning in a microfuge for 5 minutes at 13000 rpm. The ethanol was removed, the pellet was dissolved in 50 ul TE, 20 ug/ml RNaseA, and incubated at 37°C for 30 minutes. The DNA was stored at -20°C for future use.

2.11.7 LARGE SCALE PREPARATION OF CsCl PURIFIED PLASMID

The following method typically yielded 0.5-4 mg of DNA per 250 ml bacterial culture, depending on the plasmid grown up. A small starter culture from a freshly picked colony or a mid-log phase glycerol stock was grown up during the day in 2 ml LB broth, 100 ug/ml ampicillin. One hundred microlitres of this culture was used to inoculate 250 mL LB broth, 100 ug/ml ampicillin in a 2 litre flask, which was incubated overnight in a 37°C shaking incubator. The confluent culture was then poured into a 500 ml Beckman centifuge pots and centrifuged for 10 minutes at 4000 rpm (Beckman JA10 rotor) at 4°C. The supernatant was poured off, and the pellet was suspended in 8 ml of cold 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 0.02% sodium azide, 100 ug/ml RNase A. This was transferred to a 40 ml Oakridge tube and chilled on ice. Sixteen millilitres of 1% SDS, 0.2 M NaOH was added, and the tube was mixed very gently by inverting the tube several times. Then 12 ml of chilled 3 M Potassium Acetate, 2M Acetic acid was added, the tube was again mixed very gently, and stood on ice for 10 minutes with occasional gentle mixing. The tube

was spun at 18000 rpm (Beckman JA20 rotor) for 25 minutes at 4°C. The supernatant was carefully removed with a pipette and transferred to a 50 ml Falcon tube. To this supernatant, 0.6 volumes of isopropanol was added, and the tube was incubated at RT for 10 minutes before spinning at 3700 rpm in a Beckman benchtop centrifuge. The pellet was washed in 5 ml 70% ethanol and dissolved in 3 ml TE. When the pellet completely dissolved, 3.0 g CsCl was added and dissolved, followed by 60 ul ethidium bromide. This was then transferred to a Beckman TLN100 centrifuge tube, and the volume was brought up with TE to fill the tube. Tubes were then balanced within 0.005 g of their partners, 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 extracting the band in a volume of approximately 400-500 ul using a 1 ml syringe and 19 gauge needle, and transferred to an Eppendorf tube. The ethidium bromide was removed by 7 butanol extractions, and the volume was made up to 500 ul with H_2O. The plasmid was then ethanol precipitated with the addition of 1.5 ml 50 mM NaAcetate and 4.0 ml ethanol. This was mixed gently and incubated at -20°C for 30 minutes. The tube was spun at 3700 rpm for 15 minutes in a Beckman benchtop centrifuge at 4°C. The supernatant was removed, and the pellet was dissolved in 400 ul TE for 20 minutes at 37°C. The DNA was transferred to an Eppendorf tube, and mixed with 20 ul 3 M NaAcetate and 900 ul ethanol. This mixture was incubated on ice for 10 minutes before spinning at 13000 rpm in a microcentrifuge. The supernatant was removed, the pellet

was washed in 500 ul 70% ethanol, dried briefly, and redissolved in 500 ul sterile TE at 37°C for an hour. The plasmid concentration was quantitated by taking an absorbance reading at 260 nm, where an OD_{260} of 1 contains 50 ug/ml. The quality and quantity of plasmid was confirmed by electrophoresing a sample on an agarose gel along with standards of known concentration.

2.11.8 DNA SEQUENCING

The Sequenase, version 2.0 (United States Biochemical) method was generally followed. Essentially, 8 ul of miniprep DNA was mixed with 2 ul 2 M NaOH for 5 minutes to denature the plasmid. The DNA was then ethanol precipitated with the addition of 30 ul 400 mM NaAcetate and 100 ul 100% ethanol. The DNA was precipitated at -20°C for 20 minutes before spinning in a microfuge at 13000 rpm for 10 minutes at 4°C. The DNA pellet was washed with 100 ul 70% ethanol and dried briefly, then dissolved in 7 ul H_2O. Two microlitres of reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) was added with 5 ng of DNA primer. The tube was heated at 65°C for 2 minutes, and then allowed to cool slowly to room temperature over an hour. To the annealed template-primer, a cocktail containing 1.0 ul 0.1 M DTT, 2 ul 1x labelling mix (1.5 uM dGTP, dCTP, dTTP), 0.5 ul 10 uM [α-^{33}P]-dATP, and 2 ul T7 polymerase (5.25 units, diluted in 10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA) was add. This mixture was incubated at RT for 2 minutes to extend the primer. To terminate the reaction, 3.5 ul of the

mixture was added to each of four 2.5 ul aliquots of termination mix (ddGTP, ddATP, ddTTP, ddCTP), prewarmed to 37°C. These termination reactions were incubated at 37°C for 5 minutes before the addition of 4 ul of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). These reactions were then loaded on to a seqencing gel immediately or stored at -20°C for future use. To prepare a sequencing gel, sequencing plates were cleaned and rinsed, and then washed with ethanol. The plates were assembled using 0.4 mm spacers and taped together. A 6% denaturing acrylamide was prepared by dissolving 50 g urea in 20 ml 5x TBE, 20 ml 30% acrylamide solution (29.2:0.8 w/w acrylamide:bisacrylamide), 600 ul 10% ammonium persulphate, H_2O up to 100 ml. Just prior to pouring the gel, 48 ul TEMED was added. The gel solution was mixed well and poured into the plates. Two 24 well sharks tooth combs were inserted with the straight surface down to create a flat interface. The top of the gel was then wrapped in Gladwrap and allowed to set for at least two hours. To electrophorese the sequencing reactions, the gel was assembled in the electrophoresis apparatus. The upper and lower buffer chambers were filled with 1x TBE. The comb was removed, and excess urea was flushed from the top of the gel. The gel was pre-electrophoresed for one hour at 55 W. The samples were heated to 80°C for 2 minutes prior to loading. The sharks tooth combs were replaced with the teeth facing down, excess urea was flushed once again, and 4 ul of the samples were loaded. The gel was run at 80 W until the bromophenol blue dye front reached 30 cm from the top of the gel. NaAcetate was added to the lower reservoir to an approximate concentration of 300 mM, and the gel was

electrophoresed until the bromophenol blue dye front reached the bottom of the gel. The gel apparatus was then disassembled, and the gel was fixed by soaking in 10% acetic acid, 20% methanol for 20 minutes. The gel was then blotted on to Whatman 3MM paper and dried at 80°C before setting up autoradiography. Sequencing gel autoradiographs were read manually to determine the sequence.

2.11.9 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) was carried out to isolate a 560 bp mouse IL-3 promoter fragment from the mouse IL-3 gene containing plasmid, MuP (-502 to +64, Stanley et al., 1985). PCR primers were designed with internal *HindIII* restriction enzyme sites to facilitate cloning. The PCR reaction was performed using 10 ng MuP plasmid in 50 ul containing 10 mM NaCl, 5 mM Tris-HCl pH 7.9, 1 mM $MgCl_2$, 0.1 mM DTT, 200 ng of each primer, 0.2 mM each of dATP, dCTP, dGTP and dTTP. One and a quarter units of *Pfu* polymerase was added to the reaction, which was then overlayed with wax. The PCR protocol consisted of 30 cycles: 94°C for one minute to denature the DNA template; 52°C for 2 minutes to anneal the primers, and 72°C for one minute for *Pfu* polymerase extension. The PCR product was then phenol extracted, ethanol precipitated, dissolved in water, and digested with *HindIII*. The digest was then electrophoresed on a 0.8% agarose gel, and the PCR product band was cut out and extracted from the gel using a Gene Clean kit (Bresatec).

2.12. EUKARYOTIC CELL CULTURE AND PROCEDURES

2.12.1 CELL CULTURE

Cell lines used were Jurkat cells, a human T lymphoblastoid cell line provided by Dr. W Greene, Gladstone Institute of Virology and Immunology, San Francisco, USA; and the murine thymoma cell line, EL4, obtained from Dr. J Gamble, this department. All cells were maintained at 37°C, 5% CO_2 and 95% humidity in a NAPCO 4100 CO_2 incubator. Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS (CSL, Parkville, VIC.) , 20mM HEPES pH 7.3, 1 mM L-glutamine, 28 mM $NaHCO_3$, 100U/ml penecillin, and 40 ug/ml gentamycin. Cells were maintained between a density of $5x10^4$ and $1x10^6$ cells/ml.

2.12.2 TRANSIENT TRANSFECTION OF PLASMID CONSTRUCTS IN TISSUE CULTURE CELLS

Jurkat or EL4 T cells were grown to a density of approximately $5x10^5$ cells/ml. Cells were pelleted by centrifugation for 5 minutes at 1000xg and resuspended in RPMI 1640 medium containing 20% FCS at a concentration of $1.33x10^7$ cells/ml. Three hundred microlitre (4x106 cells) were mixed with either 5 or 10 ug of reporter plasmid in a 0.4 cm electroporation cuvette (Biorad). After standing at room temperature for 10 minutes, cuvettes were gently flicked to mix the contents and supend the cells, then exposed to a single voltage pulse

of 270 V from the 960 uF capacitor of a Bio-Rad Gene Pulser electroporation unit. Cells were allowed to recover for 10 minutes before being plated in a 25 cm^3 culture flask containing 10 ml of fresh medium. Cells were left for 20-24 hours (approximately one cell division cycle) to allow the plasmid to be packaged in the nucleus. Cells were then left unstimulated, or stimulated with combinations of PMA (20 ng/ml), calcium ionophore A23187 (2 uM), cyclosporin A (0.1 uM), PHA (1 ug/ml), forskolin (10 uM) and dibutyryl cAMP (1 mM). Cells were incubated with stimuli for nine hours before harvesting To harvest, cells were pelleted, washed once in 10 ml PBS, and resuspended in 100 ul harvest buffer (0.1 M K_2HPO_4 pH 7.8, 2mM DTT, 10 uM EDTA), before freezing at -20°C. The extracts were then assayed using the luciferase assay.

2.12.3 STABLE TRANSFECTION OF PLASMID CONSTRUCTS IN TISSUE CULTURE CELLS

The luciferase reporter plasmids were digested with *ScaI* to linearise the plasmid, and the marker plasmid, pE°TKneo, was digested with *PvuI* to linearise it. Ten micrograms of linearised reporter plasmid and 1 ug of linearised pE°TKneo 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 ug/ml G418 for 15 days, after which time cells in an unelectroporated control flask were all dead.

To determine the copy number and number of integration events, nuclei were isolated from the stable cell lines, and digested with *EcoRI* and *SpeI*, respectively. The copy number and integration was analysed by Southern blot using a 850 bp *EcoRI/EcoRV* luciferase gene probe.

To test the activity of the constructs, cells were removed from G418 selection for 24 hours before stimulating $1x10^6$ cells in 10 ml with PMA (20 ng/ml) and calcium ionophore A23187 (2uM). Cells were stimulated for nine hours, and then harvested by pelleting the cells, washing in 10 ml PBS, and resuspending in harvest buffer (0.1 M K_2HPO_4 pH 7.8, 2 mM DTT, 10 uM EDTA), and freezing at -20°C. The extracts were then assayed using the luciferase assay.

2.12.4 LUCIFERASE ASSAY

Extracts were prepared from transfected cells, and harvested as described above. After removing cells from the -20°C freezer, cells were lysed by two additional freeze-thaw rounds in liquid N_2 and room temperature water. Cell debris was removed by centrifugation at 3700 rpm in a Beckman benchtop centrifuge for 10 minutes. The supernatant was transferred to Eppendorf tubes and placed on ice. The protein concentration of the cytoplasmic extracts were determined using a BioRad protein assay kit. Equal amounts of protein were assayed, generally between 0.5 and 20 ug, depending on the activity of the constructs being tested. If required, samples were

diluted in harvest buffer containing 0.5 mg/ml BSA to ensure that volumes pipetted ranged between 5 and 30 ul. Equivalent amounts of protein was added to 400 ul assay buffer (0.1 M K_2PO_4 pH 7.8, 8 mM $MgSO_4$, 2 mM DTT, 750 uM ATP, 150 uM co-enzyme A) and mixed well. Eighty microlitres of 1 mM D-luciferin was added to commence the reaction, and the sample was immediately quantitated by using a scintillation counter with the coincidence curcuit switched off.

2.12.5 PREPARATION OF GENOMIC DNA

To investigate the integration of the plasmids into stable cell lines, genomic DNA was prepared. $2x10^7$ cells of each stable cell line were pelleted was washed twice in 10 ml PBS. The cell pellet was then resuspended in 5 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K). The cell suspension was mixed on a rotating wheel for one hour and then incubated at 56°C overnight. The DNA was phenol extracted, ethanol precipitated and dissolved in TE.

2.12.6 DNaseI HYPERSENSITIVITY ANALYSIS

EL4 cells were cultured to $6.5x10^5$ cells/ml in 120 ml of RPMI medium. Cells were either left unstimulated or were stimulated with PMA (20 ng/ml) and calcium ionophore (2 uM), with or without CsA (0.1 uM), and incubated for 7 hours. The following steps to isolate the

nuclei were performed in an ice bucket, in the cold room. Cells were centrifuged at 4°C in three 40 ml volumes, resuspended in ice cold PBS, and pooled together. The volume was brought up to 40 ml with ice cold PBS to wash the cells, and the cells were centrifuged. The PBS was then decanted. Five millilitres of cold lysis buffer (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, 300 mM sucrose, 0.1 mM EGTA, 0.5 mM PMSF, 5 ug/ml leupeptin, 10 ug/ml aprotinin, 0.1% NP40) was added, and the cells were suspended by forcing them five times out of a 10 ml pipette held hard on the bottom of a 50 ml Falcon tube. The volume was brought up to 20 ml with cold lysis buffer, and then spun at 1500 rpm in a Beckman benchtop centrifuge for 5 minutes, stopping with no brake when the speed reached 300 rpm upon deceleration. The supernatant was drawn off with a pipette, and the tube was briefly respun to draw off the final traces of supernatant. The pellet was then resuspended in 1 ml cold resuspension buffer (60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, 300 mM sucrose, 0.1 mM EGTA). The nuclei were quantitated by mixing 10 ul nuclei with 1 ul 15 mM $CaCl_2$ and 0.5 ul 2 mg/ml DNaseI. This mixture was incubated for one minute at room temperature, after which time, 500 ul 1 M NaOH was added and the mixture was vortexed. The light absorbance was taken at 260 nm where OD_{260} 0.54 = 1 mg/ml. The samples were diluted to 0.4 mg/ml. The nuclei then were digested with a series of DNaseI concentrations. Four hundred microlitres of the diluted nuclei were added to a 10 ml polypropylene tube containing 4 ul of 100 mM $CaCl_2$. The samples were incubated in a 22°C water bath for 3 minutes to equilibrate to room temperature. To the nuclei, 40 ul of diluted DNaseI (diluted in

resuspension buffer) was added, and incubated at room temperature for 3 minutes. The digestion was stopped with the addition of 3.5 ml 0.3 M NaAcetate, 0.5% SDS, 5 mM EDTA, 0.1 mg/ml proteinase K. The samples were then incubated at 55°C for one hour. An aliquot of each sample was electrophoresed on a 0.7% agarose gel in TA buffer. Samples that had a slight smear from the main band to approximately 20 kb were deemed to be suitable for the assay. To these samples, an additional 40 ul of 0.1 mg/ml proteinase K was added, and these samples were digested for another hour at 55°C. The samples were then extracted with phenol, then phenol-chloroform, then chloroform. The DNA was then ethanol precipitated. The pellets were dissolved in 100 ul TE (1/4 of the original digest volume). Ten micrograms of DNA from each sample was digested with 30 units *KpnI* for 3 hours. The samples were then analysed by Southern blot using a 900 bp *KpnI/SacI* fragment of the mouse GM-CSF gene.

2.12.7 SOUTHERN BLOT ANALYSIS

After restriction enzyme digestion, the samples were electrophoresed on a 0.7 cm thick, 15 cm long, 0.8% agarose gel in TA buffer and 0.5 ug/ml ethidium bromide at 30 volts for 15 hours. The gel was washed 2 x 15 minutes in 0.5 M NaOH, 1.5 M NaCl, then 2 x 15 minutes in 1M Tris pH 7, 1.5 mM NaCl. The DNA was transferred to a Hybond N nylon membrane (Amersham) in 20x SSC buffer overnight. The membrane was washed briefly in 2x SSC, dried, then UV crosslinked for 30 seconds. The membrane was prehybridised

overnight in a sealed bag with 20 ml Rapid Hyb (Amersham) and 0.25 mg/ml sonicated herring DNA, at 68°C. ^{32}P-labelled DNA probes for Southern blot analysis were prepared using a GIGAPrime kit (Bresatec). Two nanograms of radioactively labelled probe was added to the bag in 1 ml Rapid Hyb, and incubated at 68°C for 3 hours. The filter was then washed twice in 500 ml of 2x SSC, 25 mM $NaPO_4$ pH 7, 0.1% SDS at room temperature for 10 minutes, then twice in 250 ml of 0.1x SSC, 1 mM $NaPO_4$ pH 7, 0.1% SDS at 68°C for 15 minutes. Then membrane was then blotted dry, wrapped in plastic wrap, and autoradiographed.

2.13. PROTEIN:DNA BINDING ANALYSES

2.13.1 OLIGONUCLEOTIDES

To study Sp1 and TBP binding to the mouse and human GM-CSF gene promoters, the following oligonucleotides were synthesised and annealed as described earlier. The binding sites are underlined:

Sp1-M 22-mer mouse GM-CSF -85 to -64

5'AGTTCCCCCGCCCCCCTGGAGT3'

Sp1-H 22-mer human GM-CSF -82 to -61

5'AGTTCCCCCGCCTCCCTGGCAT3'

TATA-M 46-mer mouse GM-CSF -55 to -10

5'CACCATTAATCATTTCCTCTAACTGTGTATATAAGAGCTCTTTTGC3'

TATA-H 42-mer human GM-CSF -52 to -11

5'CACCATTAATCATTTCCTCTGTGTATTTAAGAGCTCTTTTGC3'

To prepare 5' end-labelled probes, 20 ng of double stranded oligonucleotide was diluted in 10 ul of a buffer containing 50 mM Tris-HCl pH7.4, 10 mM MgCl2 and 50uCi [γ-^{32}P]ATP. One microlitre of T4 kinase was added and the reaction was incubated at room temperature for 30 minutes. The kinase reaction was then purified on a 1x TAE, 7.5% polyacrylamide gel, and the labelled oligonucleotide band was cut from the gel and eluted in 300 ul 50mM NaCl in TE. 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.

2.15.2 Sp1 BINDING ASSAYS

One nanogram of recombinant human Sp1 (Promega) was incubated with 0.2 ng of Sp1M or Sp1H probe oligonucleotide and 0, 0.5, 1.0, 2.0, 4.0, 6.0, 10.0 ng of Sp1M or Sp1H cold competitor oligonucleotide in a buffer including 100 uM ZnSO4, 5 ug/ml leupeptin, 5 ug/ml aprotinin, 12.5 mM HEPES pH 7.4, 2 mM DTT, 0.1 mM PMSF, 10% glycerol. The mixture was incubated at room temperature for 25 minutes before loading on a pre-electrophoresed 4% acrylamide, 0.5x TBE gel. The gel was electrophoresed at 250 V until the BPB dye front reached 9.5 cm

from the bottom of the wells. The gel was fixed in 0.1% CTAB and dried. DNA binding was measured using a PhosphorImager (Molecular Dynamics, CA.) coupled with ImageQuant Software version 3.21.

2.13.3 TBP BINDING ASSAYS

0.2 ng of TATA-M or TATA-H probe was mixed with 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 ng of recombinant human TBP (Promega) in a buffer containing 10% glycerol, 20 mM Tris, 80 mM KCl, 10 mM $MgCl_2$, 2 mM DTT. The mixture was incubated at room temperature before electrophoresis on a 9% acrylamide gel with 0.5x TBE, 4 mM MgCl2 and 0.2% NP40 . The gel was run at 250 V for 40 minutes, fixed in 0.1% CTAB, dried. DNA binding was measured using a PhosphorImager (Molecular Dynamics, CA.) coupled with ImageQuant Software version 3.21.

CHAPTER 3.

ISOLATION AND CHARACTERISATION OF AN ENHANCER UPSTREAM OF THE MOUSE GM-CSF GENE

3.1. INTRODUCTION

At the initiation of the work described in this thesis, an enhancer had been identified in the human GM-CSF/IL-3 locus, approximately 3 kb upstream of the GM-CSF gene (Cockerill et al, 1993). This enhancer was first identified as a DNaseI hypersensitive (DH) site that, like the expression of GM-CSF and IL-3, was inducible upon signalling through T cell receptor pathways, and inhibitable by the immunosuppressive drug cyclosporin A (CsA). In this study, segments of DNA from upstream of the human GM-CSF gene were subcloned into plasmid constructs in front of the chloramphenicol acetyl transferase (CAT) reporter gene, and transient transfection assays showed that the inclusion of the DH site significantly increased the level of inducible CAT expression, indicating that the DH site possesses inducible enhancer activity.

The human GM-CSF enhancer was found to be contained within a 716 bp *BglII* fragment has been further defined as a 425 bp *BamHI/MscI* fragment (Cockerill et al. 1995b). The enhancer contains the bindings sites for several transcription factors that could contribute to its function. Three of four binding sites for the transcription factor NFAT that are present in the enhancer have been shown to have the capacity to support enhancer activity, and are thought to be largely responsible for the inducible, CsA sensitivity of the enhancer.

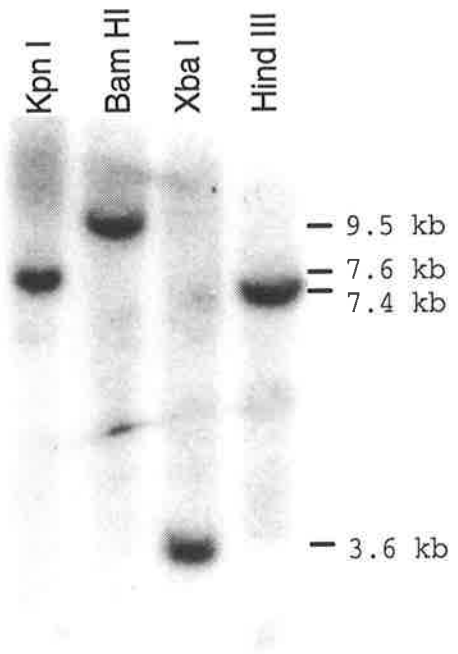
At the start of the work described here, Dr. Peter Cockerill had shown by Southern blot analysis that a element homologous to the human

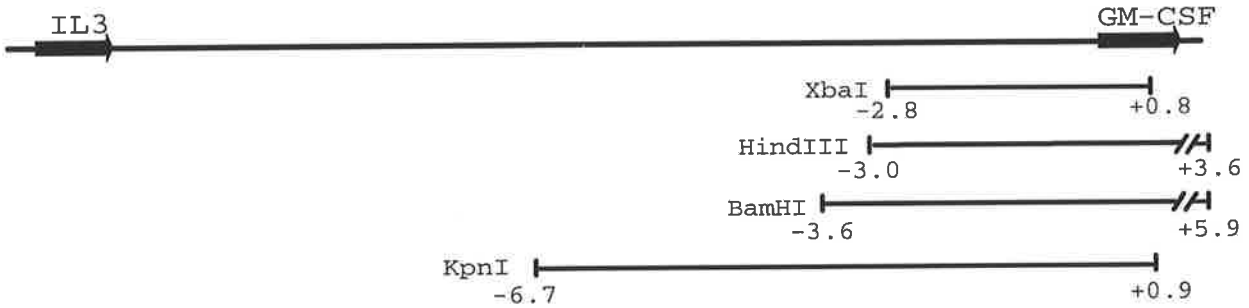
GM-CSF enhancer existed in the mouse genome (Fig. 3.1; Osborne et al., 1995). The experiments detailed in this chapter were aimed at isolating and characterising the mouse homologue of the human GM-CSF enhancer. This work was carried out in order to more closely define the essential enhancer core through sequence homology, and to investigate the practicality of initiating transgenic studies to analyse the role of the enhancer *in vivo*.

Fig. 3.1. Southern blot hybridisation analysis of an element in the mouse genome homologous to the human GM-CSF enhancer.

Mouse genomic DNA isolated from EL4 T cells was cut with *KpnI*, *BamHI*, *XbaI* or *HindIII*, and probed by Southern blot analysis with the 716 bp *BglII* human GM-CSF enhancer fragment.

A restriction enzyme map indicating the DNA fragments containing regions homologous to the human GM-CSF enhancer is shown.


Kpn I
Bam HI
Xba I
Hind III
9.5 kb
7.6 kb
7.4 kb
3.6 kb


IL3
GM-CSF
XbaI
-2.8
+0.8
HindIII
-3.0
+3.6
BamHI
-3.6
+5.9
KpnI
-6.7
+0.9

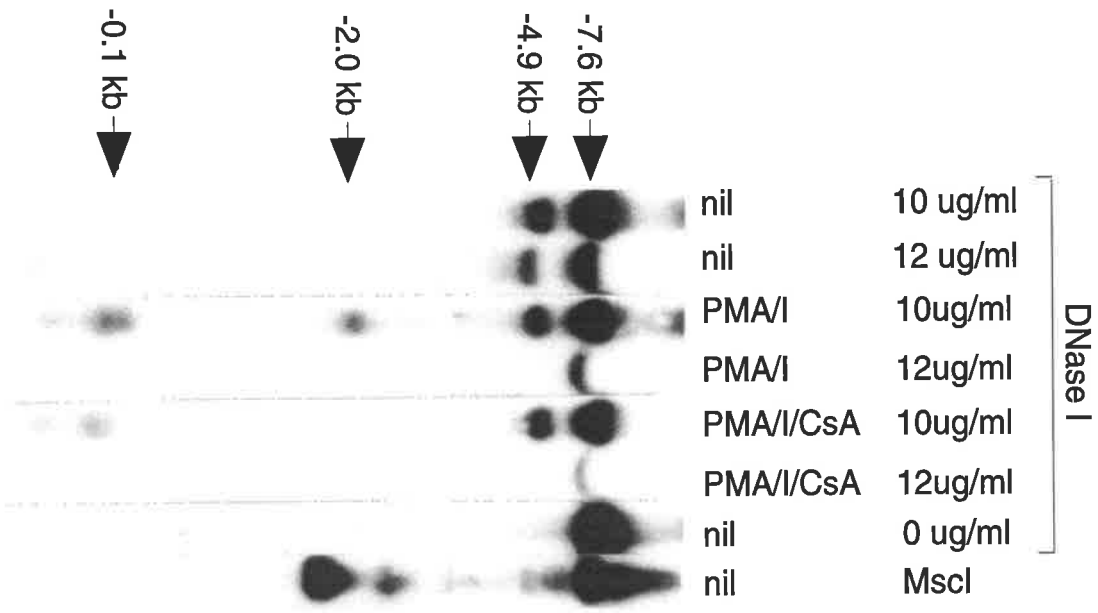
3.2. RESULTS

3.2.1. FORMATION OF A DNaseI I HYPERSENSITIVE SITE IN THE MOUSE GM-CSF LOCUS.

To determine whether an inducible, CsA-sensitive DH site could form in the mouse GM-CSF/IL-3 locus, similar to the one that forms over the human GM-CSF enhancer, nuclei isolated from a mouse EL4 T cell line were partially digested with DNaseI, purified, digested with *KpnI*, electrophoresed on a 0.8% agarose gel and blotted on to a nitrocellulose membrane that was probed with a ^{32}P-labelled 0.9 kb *SacI/KpnI* fragment of mouse GM-CSF gene DNA. This method of indirect end-labelling identifies the location of upstream DH sites with respect to a fixed, radioactively labelled *KpnI* site within the mouse GM-CSF gene. Three DH sites were identified upstream of the GM-CSF gene, within a DNA fragment extending from a *KpnI* site at 0.9 kb within the gene, to a *KpnI* site at 6.7 kb upstream (Fig. 3.2). One site, located 4.9 kb upstream of the gene, was strongly present in both unstimulated and stimulated cells, and was unaffected in the presence of CsA. The two remaining sites were inducible upon stimulation with calcium ionophore and phorbol ester (PMA). One of these inducible DH sites, at -0.1 kb, was partially suppressed by CsA, and encompassed the GM-CSF proximal promoter. The other inducible DH site was located 2.0 kb upstream of the GM-CSF gene and was completely suppressed by CsA. This site resembled the DH site that forms over the human GM-CSF enhancer.

Fig. 3.2. DNaseI hypersensitivity of the mouse GM-CSF locus in mouse EL4 T cells.

Nuclei of EL4 cells that were unstimulated (nil, lanes 1 and 2), stimulated for 6 hours with PMA (20 ng/ml) and calcium ionophore (2 uM) (PMA/I, lanes 3 and 4), or PMA and calcium ionophore with cyclosporin A (0.1 uM)(PMA/I/CsA, lanes 5 and 6), were partially digested with either 10 or 12 ug/ml DNaseI, then to completion with *KpnI*, and electrophoresed on an agarose gel. The DNA was blotted on to a membrane and probed with a fragment of the mouse GM-CSF gene. The upper band at -7.6 kb is the intact *KpnI* fragment, whereas the lower bands at -4.9, -2.0 and-0.1 kb upstream of the GM-CSF gene are fragments generated by DNaseI digestion. The DNA in lane 7 was not digested with DNaseI prior to *KpnI* digestion. Lane 8 contains EL4 cell DNA partially digested with *MscI*, indicating that the DH site at -2.0 kb is midway between two *MscI* sites.


-0.1 kb
-2.0 kb
-4.9 kb
-7.6 kb
nil
10 ug/ml
nil
12 ug/ml
PMA/I
10ug/ml
PMA/I
12ug/ml
PMA/I/CsA
10ug/ml
PMA/I/CsA
12ug/ml
nil
0 ug/ml
nil
MscI
DNase I

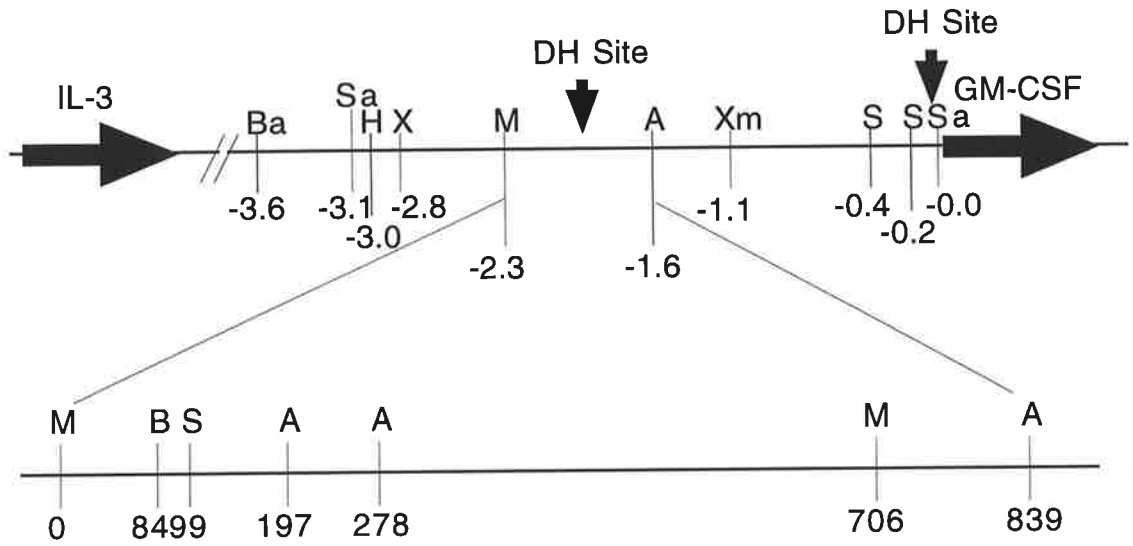
The DH site located 2.0 kb upstream of the mouse GM-CSF gene was encompassed by a 839 bp *MscI/ApaI* fragment (Fig. 3.3). This fragment was subcloned into pUC19 and sequenced in both directions using sets of oligonucleotide primers designed to produce overlapping sequences. The mouse DNA sequence exhibits a high degree of homology with the human GM-CSF enhancer (Fig. 3.4). A segment of 417 bp of DNA within the *MscI* fragment possesses 76% sequence homology with the human GM-CSF enhancer, corresponding largely to the functional core of the human enhancer . Sequence homology breaks down significantly outside this core region. Within the highly conserved region of the mouse *MscI* fragment are several putative transcription factor binding sites, many of which are conserved in the human enhancer. Conserved sites include those for general transcription factors such as Sp1 and AP-1. There are, however, conserved putative binding sites for transcription factors implicated in the regulation of genes involved in haemopoiesis, including conserved potential binding sites for Ets, GATA, E2A, CBF, and NFATp/c families of transcription factors.

Of interest is any conservation of NFAT sites, as these may account for the CsA-sensitivity of the element. However, only two of the four NFAT sites present in the human GM-CSF enhancer are conserved to any extent in the mouse. The GM170 NFATp/AP-1 site is absent in the mouse enhancer homologue. The GM330 NFAT site has a conserved AP1 site but the NFATp/c site is not present in the mouse. However, a NFATp/c site that is not present in the human enhancer is present in the mouse, 25 bp downstream of the GM330 AP1 site. This

Fig. 3.3. Sequence spanning the DH site at -2.0 kb upstream of the mouse GM-CSF gene.

A. A restriction enzyme map of the GM-CSF locus. Letters represent restriction enzyme cleavage sites: A, *ApaI*; B, *BglII*; Ba, *BamHI*; H, *HindIII*; M, *MscI*; S, *StuI*; Sa, *SacI*; X, *XbaI*; and Xm, *XmnI*. Numbers on top map represent -kb from transcriptional start of GM-CSF. Numbers in expanded map represent bp from *MscI* site at -2.3 kb.

B. Nucleotide sequence of the *MscI/ApaI* 839 bp fragment between -2.3 and -1.6 kb upstream of the mouse GM-CSF gene. The fragment was subcloned from pAOGM (Stanley et al., 1985) into pUC19 and sequenced in both directions using sets of oligonucleotide primers designed to produce overlapping sequence. Restriction enzyme cleavage sites are underlined.



1 TGGCCAGAAT TTGCTGTGTG GCCCATTCCC CTAGAGCAGG AGAAAGCATC CATGTATGGA
MscI

61 CTGAGCTGCA AGTGGTCTCT TAGATCTCAG ACTCCATTAA GGACGAAGGC CTCCCTGAAG
BglII StuI

121 AATGTTGATC ATCAGGATGG GTCAGACCAT CTTAAATGCT CATGTGTCTG TCCCGTGGGA

181 TGAGAGGGAC TTTGGGGCCC ATTTCCTTCC CTCCTGGTGT TGACTTCCTC TTGTGATAAA
ApaI

241 GATCCTGGGG CAGCCACACG CTTCTGGTTC CCCCAGGGCC CATGTCAAGG GACACTCCCC
ApaI

301 GGAAACTCCT TCCAGAGGGT TCTCTCCTGG CCTTGTGGGC TGGGAGGCCA AAGAGGGGTC

361 ACTCCACCCA TCTGGTCCCC ACCTCCGCCC TGCCACAGCC CCACTGAGGC CCTTGAGTCA

421 GCAAGGCTGT CTGATGCTAT TGCCGCTTCT TTCCATGCGT GCAATCTAGG AATGCCTGCC

481 CGTGTCACCG CCTCCACCCT GATGTCATCT TTCTAGGAGA AAGATACATA TCTACCCACA

541 AACTGCATCC CCAAGCCCTT AGAATCCTGC CCCATAAGTG ACAACTTTGG CACCTGTAGA

601 CCATATCCTT GACGTGAGTC TTGACTCTGG GGCCTCTGCT TTCCTCTTAG CCACCACCCT

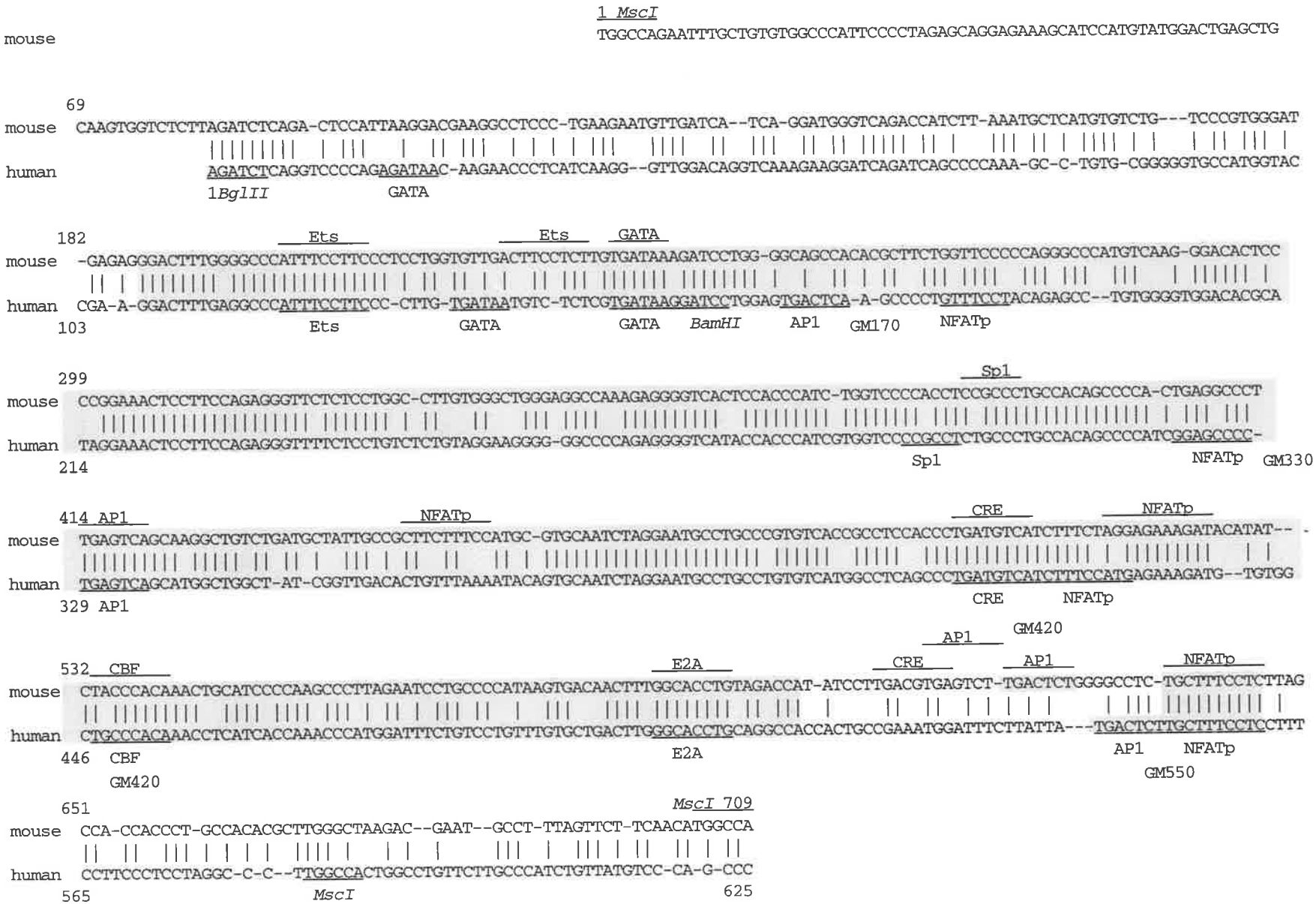
661 GCCACACGCT TGGGCTAAGA CGAATGCCTT TAGTTCTTCA ACATGGCCAT TCTGGCCCCC
MscI

721 CAGTCTCTCT TTCTACATGA TTCCTGTGGT CTTGTCCTGA GTTCCACCTG TTATCTGACC

781 CTCGAAAGCC CTCACTTCTG TCTGGTTTCA TCAGTCCACT GAATCAGCTT CTTGGGCCC
ApaI

Fig. 3.4. Alignment of the 703 bp MscI mouse fragment with a 625 bp human GM-CSF enhancer fragment.

Sequence alignment was performed using the DNASIS computer program. Maximum sequence homology of 76% was obtained within a 417 bp span (large shaded region). An additional region of high homology corresponding to the GM550 human enhancer site is also indicated (small shaded regions). Sequences were analysed for putative transcription factor binding sites using the Signal Scan computer program. Sites that are likely to be relevent in mammalian haemopoietic cells are cited and marked by bars. Restriction enzyme sites are shown in italics.


1 MscI
mouse
TGGCCAGAATTTGCTGTGTGGCCCATTCCCCTAGAGCAGGAGAAAGCATCCATGTATGGACTGAGCTG
69
mouse CAAGTGGTCTCTTAGATCTCAGA-CTCCATTAAGGACGAAGGCCTCCC-TGAAGAATGTTGATCA--TCA-GGATGGGTCAGACCATCTT-AAATGCTCATGTGTCTG---TCCCGTGGGAT
human AGATCTCAGGTCCCCAGAGATAAC-AAGAACCCTCATCAAGG--GTTGGACAGGTCAAAGAAGGATCAGATCAGCCCCAAA-GC-C-TGTG-CGGGGGTGCCATGGTAC
1BglII
GATA
182
Ets
Ets
GATA
mouse -GAGAGGGACTTTGGGGCCCATTTCCTTCCCTCCTGGTGTTGACTTCCTCTTGTGATAAAGATCCTGG-GGCAGCCACACGCTTCTGGTTCCCCCAGGGCCCATGTCAAG-GGACACTCC
human CGA-A-GGACTTTGAGGCCCATTTCCTTCCC-CTTG-TGATAATGTC-TCTCGTGATAAGGATCCTGGAGTGACTCA-A-GCCCCTGTTTCCTACAGAGCC--TGTGGGGTGGACACGCA
103
Ets
GATA
GATA
BamHI
AP1
GM170
NFATp
299
Sp1
mouse CCGGAAACTCCTTCCAGAGGGTTCTCTCCTGGC-CTTGTGGGCTGGGAGGCCAAAGAGGGGTCACTCCACCCATC-TGGTCCCCACCTCCGCCCTGCCACAGCCCCA-CTGAGGCCCT
human TAGGAAACTCCTTCCAGAGGGTTTTCTCCTGTCTCTGTAGGAAGGGG-GGCCCCAGAGGGGTCATACCACCCATCGTGGTCCCCGCCTCTGCCCTGCCACAGCCCCATCGGAGCCCC-
214
Sp1
NFATp
GM330
414 AP1
NFATp
CRE
NFATp
mouse TGAGTCAGCAAGGCTGTCTGATGCTATTGCCGCTTCTTTCCATGC-GTGCAATCTAGGAATGCCTGCCCGTGTCACCGCCTCCACCCTGATGTCATCTTTCTAGGAGAAAGATACATAT--
human TGAGTCAGCATGGCTGGCT-AT-CGGTTGACACTGTTTAAAATACAGTGCAATCTAGGAATGCCTGCCTGTGTCATGGCCTCAGCCCTGATGTCATCTTTCCATGAGAAAGATG--TGTGG
329 AP1
CRE
NFATp
GM420
AP1
532 CBF
E2A
CRE
AP1
NFATp
mouse CTACCCACAAACTGCATCCCCAAGCCCTTAGAATCCTGCCCCATAAGTGACAACTTTGGCACCTGTAGACCAT-ATCCTTGACGTGAGTCT-TGACTCTGGGGCCTC-TGCTTTCCTCTTAG
human CTGCCCACAAACCTCATCACCAAACCCATGGATTTCTGTCCTGTTTGTGCTGACTTGGGCACCTGCAGGCCACCACTGCCGAAATGGATTTCTTATTA---TGACTCTTGCTTTCCTCCTTT
446 CBF
GM420
E2A
AP1
NFATp
GM550
651
MscI 709
mouse CCA-CCACCCT-GCCACACGCTTGGGCTAAGAC--GAAT--GCCT-TTAGTTCT-TCAACATGGCCA
human CCTTCCCTCCTAGGC-C-C--TTGGCCACTGGCCTGTTCTTGCCCATCTGTTATGTCC-CA-G-CCC
565
MscI
625

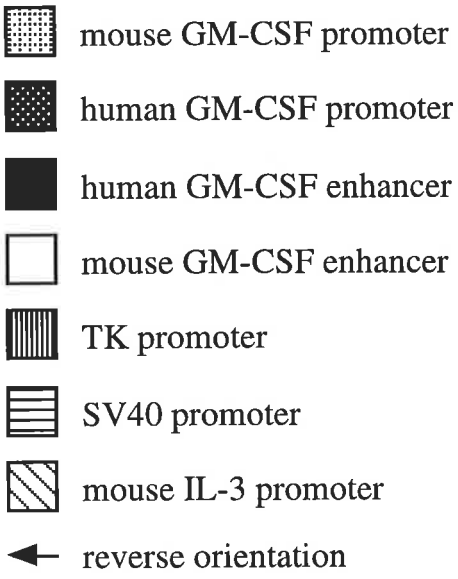
putative NFATp/c site has a sequence that is identical to the human NFATp/c site within the GM420 element. The two NFAT sites at GM420 and GM550 are not really conserved. Within the GM420 element, the CRE is strictly conserved, but a two base pair difference has likely disrupted the NFATp/c site yet formed another potential site immediately downstream; this site, however, is in an opposite orientation, and may not be functional in this context. The NFATp/c site at GM550 is conserved, however, the spacing between the AP-1 and NFATp/c binding sites differ in the two species. Another putative AP1 and a CRE binding site is present directly upstream of the mouse GM550 site. A CBF site is conserved, and is 25 bp downstream of the CRE site in both the mouse and human. It is unclear whether NFATp/c can bind cooperatively with AP-1 and CREB at their respective sites in the mouse GM330 and GM420 sites as their binding sites are not directly coupled as is seen in the functional sites of both the human GM-CSF enhancer and the IL-2 promoter. (Cockerill et al., 1995b; Jain et al., 1992a).

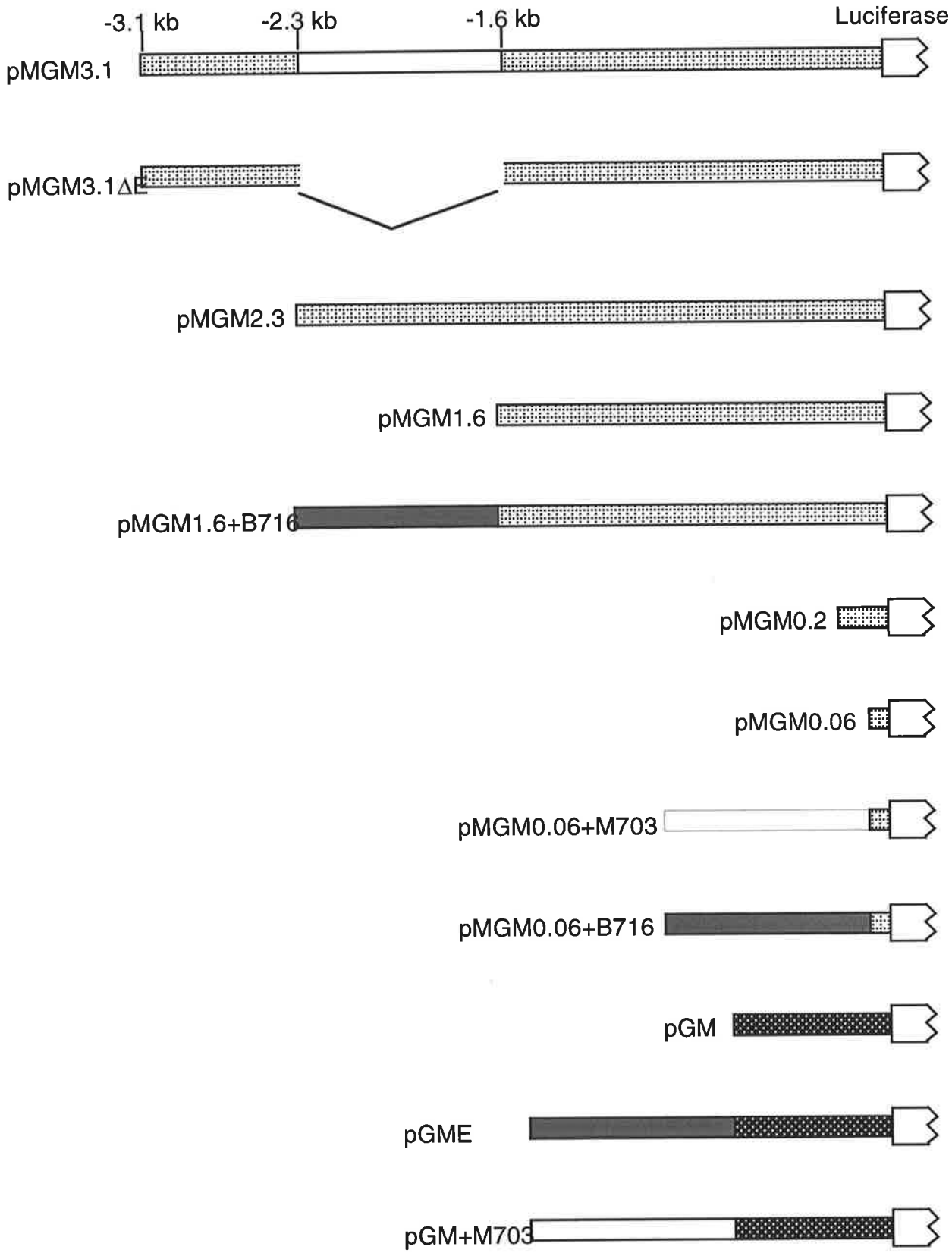
3.2.2. FUNCTIONAL STUDIES OF THE MOUSE GM-CSF ENHANCER.

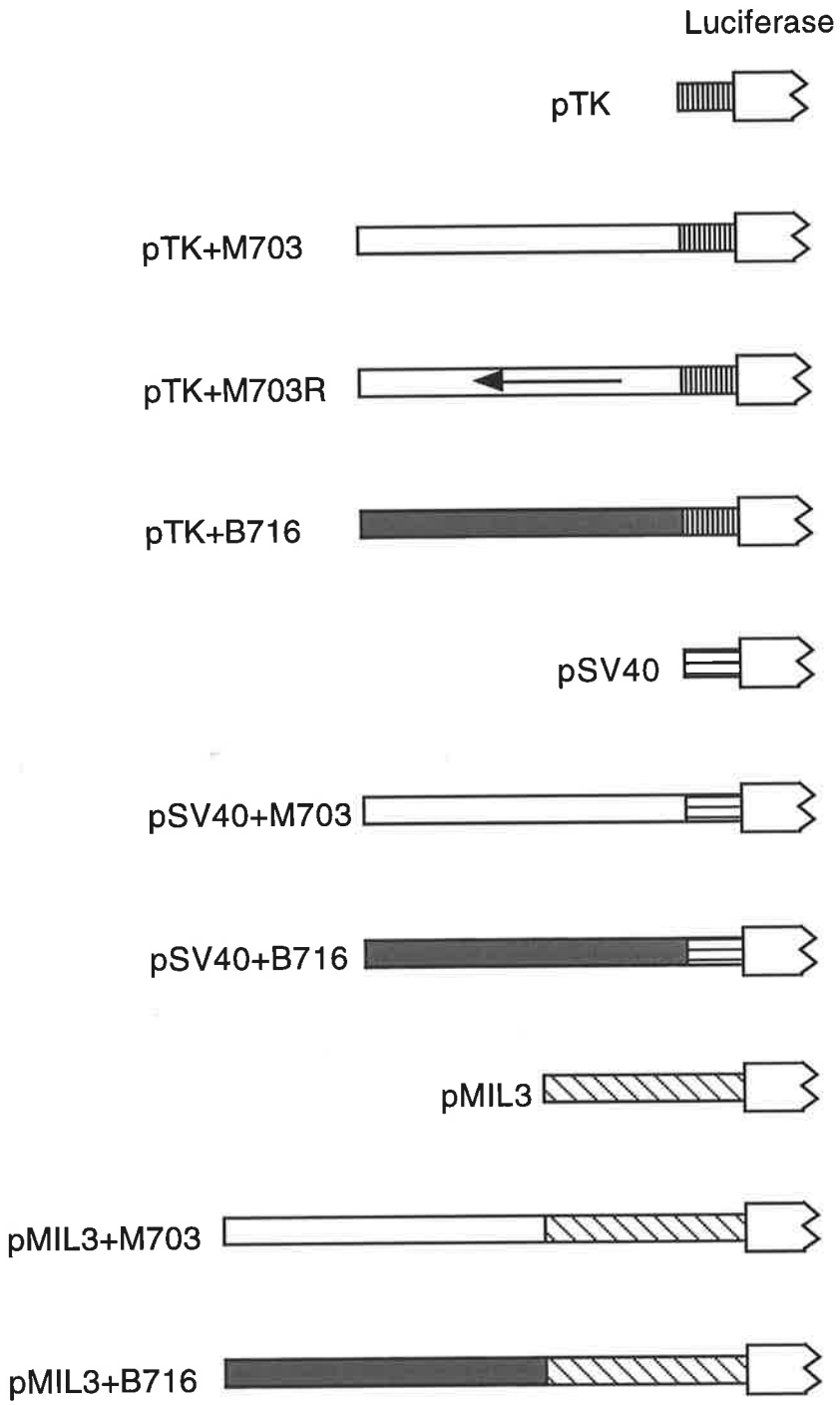
To determine the functional role of the mouse homologue to the GM-CSF enhancer, plasmids were constructs linking various regions from upstream of the mouse GM-CSF gene to a luciferase reporter gene (Fig 3.5. Two plasmid constructs containing DNA sequences from upstream of the mouse GM-CSF promoter were transiently transfected into Jurkat T cells, and the samples were harvested at various times. One

Fig. 3.5. Schematic representation of luciferase reporter constructs used for detection of enhancer activity.

The mouse GM-CSF promoter constructs contain 5' serial deletions of the mouse GM-CSF 5' UTR sequence from -3.1 kb to -23 bp, and human GM-CSF 5' UTR from -22 to +28 bp. The human GM-CSF promoter constructs contain human GM-CSF sequence from -627 bp to +28 bp. The mouse IL-3 promoter constructs contain mouse IL-3 promoter sequence from -502 to +64 bp. All constructs contain a transcription start site.
For details of cloning, see chapter 2, section 2.9.




-3.1 kb
-2.3 kb
-1.6 kb
Luciferase
pMGM3.1
pMGM3.1ΔE
pMGM2.3
pMGM1.6
pMGM1.6+B716
pMGM0.2
pMGM0.06
pMGM0.06+M703
pMGM0.06+B716
pGM
pGME
pGM+M703


Luciferase
pTK
pTK+M703
pTK+M703R
pTK+B716
pSV40
pSV40+M703
pSV40+B716
pMIL3
pMIL3+M703
pMIL3+B716

construct, pMGM3.1, contained mouse GM-CSF promoter sequence up to -3.1 kb from the gene. The second construct, pMGM3.1ΔE contained a 700 bp internal deletion that removed enhancer homologue from the pMGM3.1 construct. After stimulation, luciferase levels for both pMGM3.1 and MGM3.1ΔE steadily increased reaching a peak between 8 and 12 hours. Luciferase levels then declined, reaching near background levels by 20 hours (Fig. 3.6). Based on this time course, all subsequent assays were harvested at nine hours in order to harvest the maximum amount of luciferase enzyme. Unexpectedly, the construct that did not contain the enhancer homologue was two to three fold more active than the construct containing the enhancer homologue.

A deletion series containing various lengths of sequence 5' to the mouse GM-CSF gene linked to a luciferase gene was made (Fig. 3.5). These constructs were transiently transfected into Jurkat T cells. Again, the inclusion of DNA containing the mouse enhancer homologue did not significantly increase the levels of induction of luciferase over those constructs that contained the mouse GM-CSF promoter alone, suggesting that the mouse homologue does not exert an effect on the mouse GM-CSF promoter in these transient transfection assays (Fig. 3.7). Furthermore, a construct that included only 200 bp of the promoter of the mouse GM-CSF gene (pMGM0.2) gave level of induction equivalent to that obtained with a the construct that contained the enhancer homologue (pMGM2.3). This suggested that there were no regulatory elements up to and including the enhancer homologue that can exert an effect on the induction of the mouse GM-

Fig. 3.6. Time course for luciferase activity of mouse GM-CSF promoter constructs in transient transfection assays in Jurkat T cells.

Jurkat T cells were transfected with pMGM3.1 and pMGM3.1ΔE, and stimulated 20 hours later with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM). Cells were then harvested at the indicated time points, and assayed for luciferase activity. Luciferase activity is expressed as raw counts per minute.

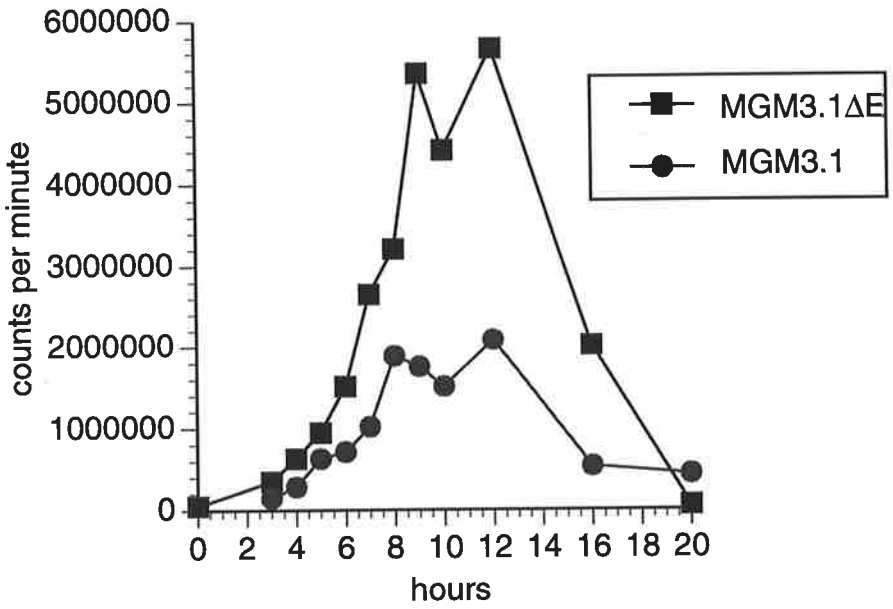
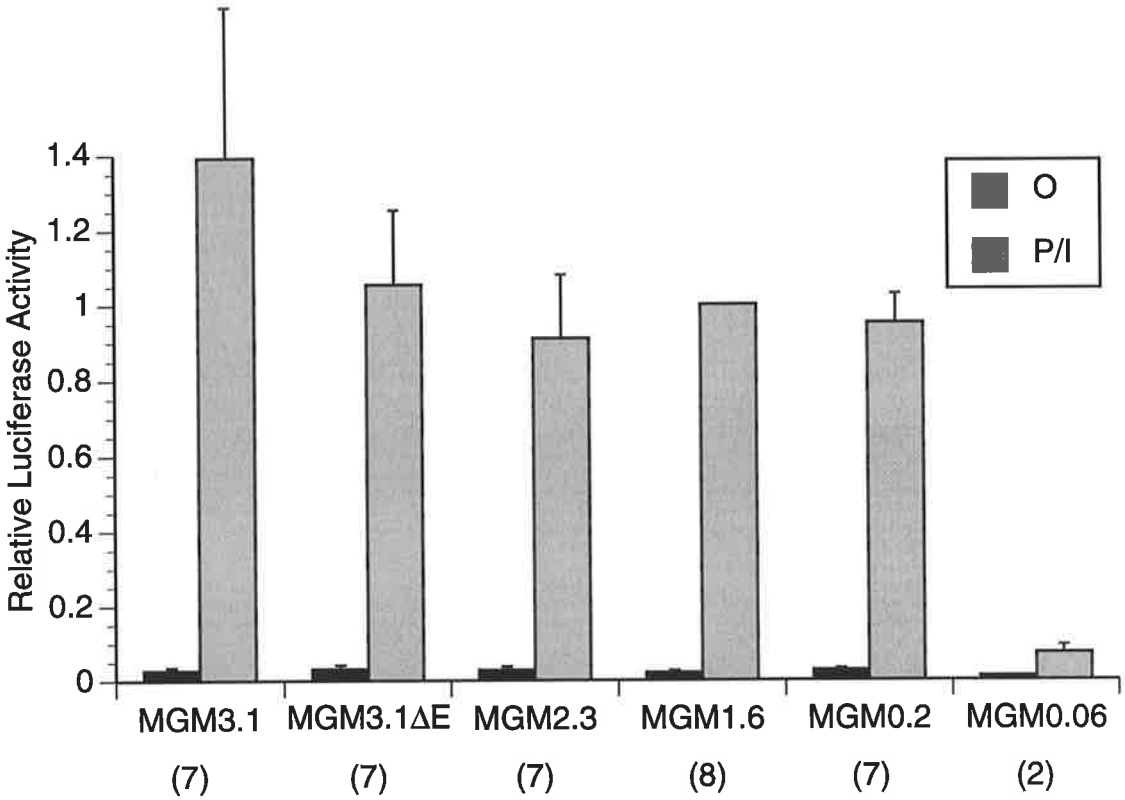

6000000
5000000
4000000
3000000
2000000
1000000
0
counts per minute
0 2 4 6 8 10 12 14 16 18 20
hours
MGM3.1ΔE
MGM3.1

Fig. 3.7. Relative transcriptional activity of serially deleted mouse GM-CSF upstream constructs in Jurkat T cells in transient transfection assays.

Jurkat T-cells were transfected with 10ug of pMGM3.1, pMGM3.1ΔE, pMGM2.3, pMGM1.6, pMGM0.2 and pMGM0.06. After 20 hours, cells were either left unstimulated (O) or stimulated with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM) (P/I), then harvested nine hours later and assayed for luciferase activity. Luciferase activity is expressed relative to the level of activity in stimulated pMGM1.6 samples, assigned a value of 1. Error bars represent the standard error and numbers in parentheses are the number of assays performed.


Relative Luciferase Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
O
P/I
MGM3.1
MGM3.1ΔE
MGM2.3
MGM1.6
MGM0.2
MGM0.06
(7)
(7)
(7)
(8)
(7)
(2)

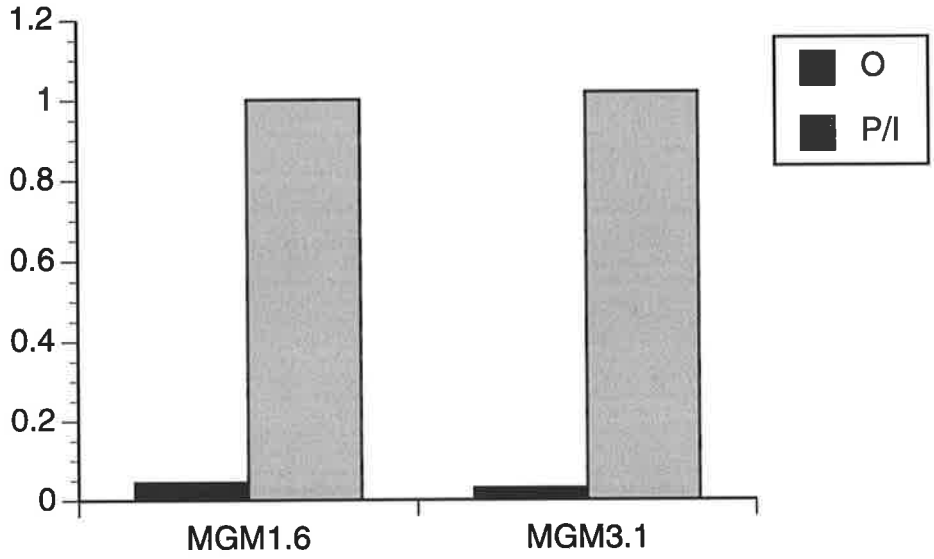
CSF gene in transient transfection assays. A construct that contained sequence upstream of the enhancer homologue up to -3.1 kb (pMGM3.1) was able to increase induction levels, however this increase was not statistically significant. A construct that contained only sequence up to -60 bp was greatly less induced than constructs contain more upstream sequences, indicating that there are important regulatory elements between -60 and -200 bp.

It is possible that the mouse enhancer needs to function in the context of a mouse cell to be able to exert an effect on mouse GM-CSF transcription. There may be factors that are lacking in the human Jurkat T cells that are needed for mouse GM-CSF gene transcription. To determine if there are species-specific differences in the signalling of these regulatory elements between humans and mice, the mouse GM-CSF gene promoter constructs were also transfected into a mouse EL4 T cell line. As was observed with the Jurkat T cell transfections, no significant increase of induction was observed in the construct containing the mouse enhancer homologue compared that which lacked it (Fig. 3.8).

It was of interest to examine how the regulation of the mouse GM-CSF promoter and promoter/enhancer constructs compared to the regulation of the human promoter and promoter/enhancer constructs. Both sets of the human and mouse constructs were transiently transfected into Jurkat T cells It was observed that the level of induction that was obtained through the proximal promoter of the mouse GM-CSF gene was comparable to that which was obtained with

Fig. 3.8. Relative transcriptional activity of mouse GM-CSF upstream constructs in transient transfection assays in EL4 cells.

EL4 cells were transfected with pMGM3.1 and pMGM1.6, and stimulated 20 hours later with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM) (P/I), or left unstimulated (O). Cells were harvested nine hours later and assayed for luciferase activity. Luciferase activity is expressed relative to the level of activity in stimulated pMGM1.6, which has been assigned a value of 1. Each sample was assayed once.


1.2
1
0.8
0.6
0.4
0.2
0
MGM1.6
MGM3.1
O
P/I

the human GM-CSF promoter linked to the human enhancer (Fig. 3.5, Fig. 3.9). Nevertheless, an increase in induction over that of the mouse GM-CSF promoter was observed when the human enhancer was linked to the mouse GM-CSF promoter (MGM1.6+B716). This shows that the mouse GM- CSF promoter activity is still able to be augmented by a strong enhancer.

It is possible that the strength of the mouse GM-CSF gene promoter greatly masks the effects of the mouse GM-CSF enhancer homologue. Alternatively, the mouse GM-CSF enhancer homologue may not be functional. To determine whether the mouse GM-CSF enhancer homologue had any inducible enhancer activity in other contexts, it was linked to the promoter of the herpes simplex virus thymidine kinase (TK) gene, coupled to a luciferase reporter gene, and tested in transient transfection assays (Fig. 3.5). The TK promoter was chosen for this purpose because it was poorly induced by T cell activation, mediating just a 1.7-fold induction of luciferase in response to PMA and calcium ionophore in the human Jurkat T cell line, and hence would be less likely to mask any weak enhancer activity. When the mouse 703 bp MscI fragment (M703) was linked to the TK promoter, a 4.3-fold induction of the luciferase gene activity was obtained in response to PMA and calcium ionophore, indicating that this element does indeed function as an enhancer (Fig. 3.10). When the M703 fragment was linked in reverse orientation to the TK promoter, a 4.2-fold induction of luciferase was obtained. In each case, the activation of the enhancer/TK constructs were suppressed in the presence of CsA. For comparison, the 716 bp fragment containing the human

Fig. 3.9. Comparison of the relative transcriptional activity of the mouse and human GM-CSF loci in transient transfection assays in Jurkat T cells.

pMGM1.6, pMGM2.3, pGM and pGME were transfected into Jurkat T cells and left unstimulated (O), or stimulated with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM) (P/I) 20 hours later. After nine hours, cells were harvested and luciferase activity was assayed. Luciferase activity is expressed relative to the stimulated level of pMGM1.6, assigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.

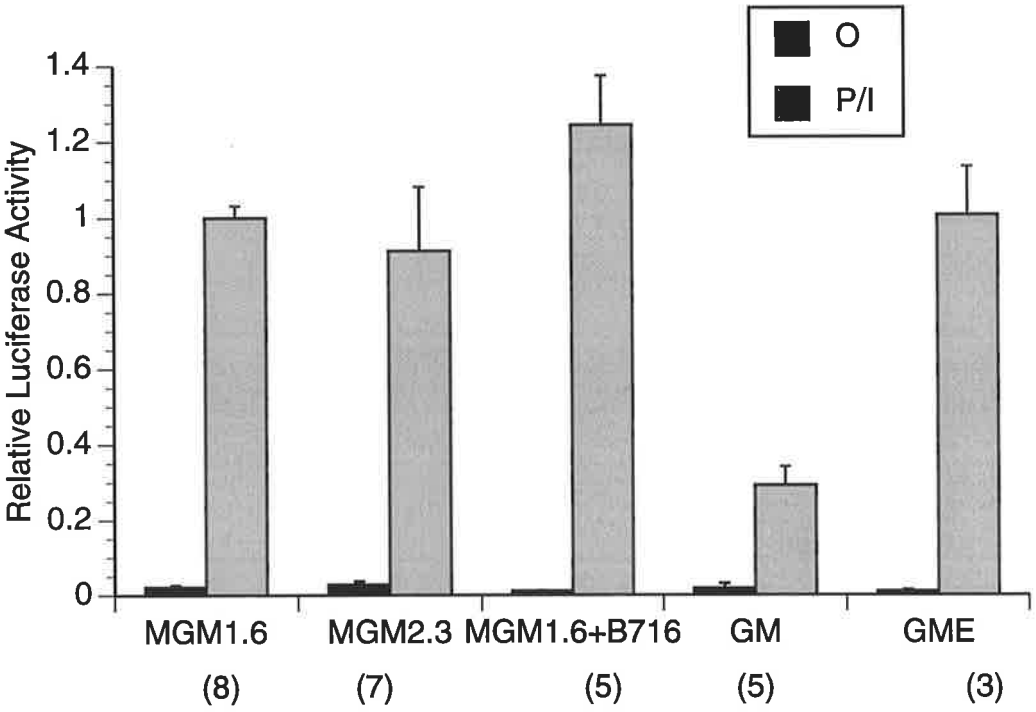
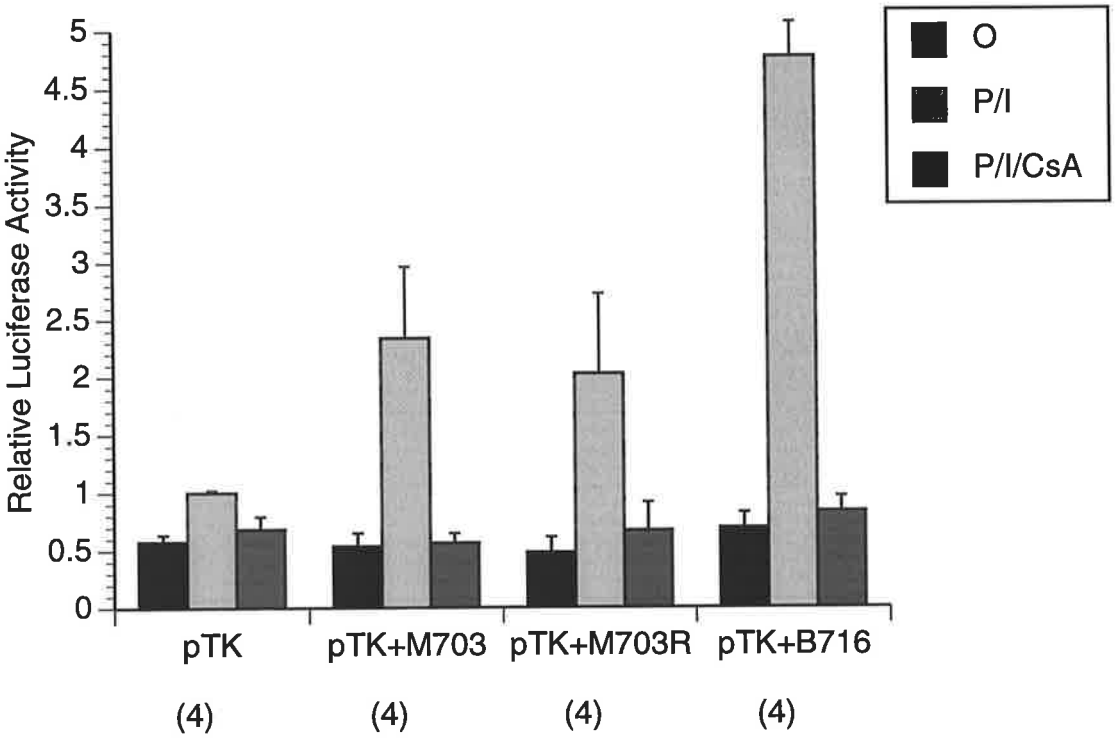

O
P/I
Relative Luciferase Activity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MGM1.6
MGM2.3
MGM1.6+B716
GM
GME
(8)
(7)
(5)
(5)
(3)

Fig. 3.10. Relative transcriptional activities cells of the mouse and human GM-CSF enhancers when coupled to the TK promoter in transient transfection assays in Jurkat T.

Jurkat T cells were transfected with pTK, pTK+M703, pTK+M703R and pTK+B716. After 24 hours, cells were stimulated with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM)(P/I), or with PMA, calcium ionophore A23187 and CsA (0.1 uM)(P/I/CsA), or left unstimulated (O). Cells were harvested after nine hours, and assayed for luciferase activity. Luciferase activity is expressed relative to the stimulated level of pTK, assigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.

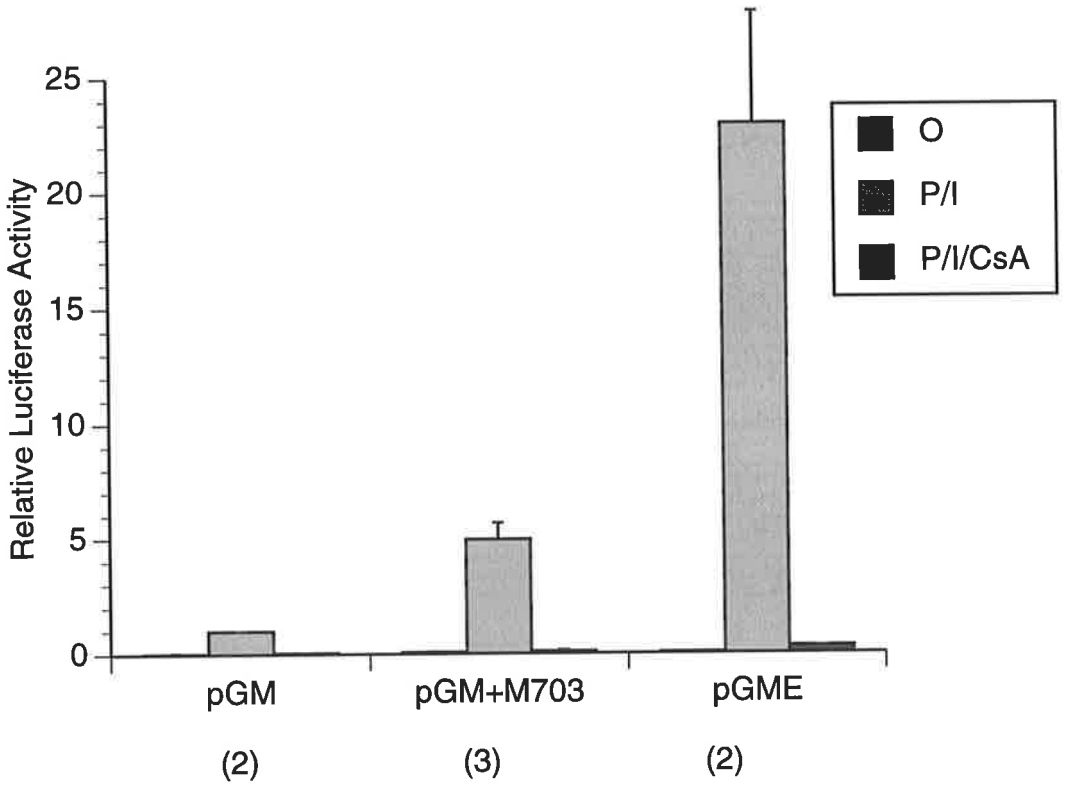

Relative Luciferase Activity
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
O
P/I
P/I/CsA
pTK
pTK+M703
pTK+M703R
pTK+B716
(4)
(4)
(4)
(4)

enhancer (B716) was linked to the TK promoter coupled to the luciferase gene, and tested in transient transfection assays (Fig. 3.5). Here, a 6.9-fold induction was obtained from the luciferase gene. It therefore appeared that while the mouse enhancer is not as efficient as its human counterpart, it can function as an enhancer (Fig. 3.10).

Considering that the human GM-CSF promoter has weaker inducible activity than its mouse counterpart, it was felt that the mouse GM-CSF enhancer may be able increase its induction Therefore, the M703 mouse enhancer fragment was next coupled to the human GM-CSF promoter in front of a luciferase gene (Fig. 3.5). Unlike the mouse GM-CSF promoter, the human GM-CSF promoter is dependent on its enhancer for high level of induction of the human GM-CSF gene. When tested in transient transfection assays in Jurkat T cells, the human GM-CSF promoter alone gave 27-fold induction of luciferase in response to PMA and calcium ionophore (Fig. 3.11). When linked to the M703 mouse enhancer fragment, induction through the human GM-CSF promoter was increased to 72-fold over unstimulated levels. Again, the induction was suppressed to basal levels in the presence of CsA. When the B716 human enhancer fragment was linked to the human GM-CSF promoter a 374-fold induction over unstimulated levels was obtained. In comparing the absolute levels of the induced constructs, the construct containing the mouse enhancer was induced five-fold higher than the human GM-CSF promoter alone. The construct containing the human enhancer was induced 23-fold higher than the human GM-CSF promoter alone This data supports evidence

Fig. 3.11. Relative transcriptional activities of the mouse and human GM-CSF enhancers when coupled to the human GM-CSF promoter in transient transfection assays in Jurkat T cells.

Jurkat T cells transfected with pGM, pGM+M703 and pGME. After 24 hours, cells were stimulated with PMA (20 ng/ml) plus calcium ionophore A23187 (2 uM) (P/I), or PMA plus calcium ionophore plus cyclosporin A (0.1 uM) (P/I/CsA), or left unstimulated (O), then harvested nine hours later and assayed for luciferase activity. Luciferase activity is expressed relative to the stimulated level of pGM, assigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.


Relative Luciferase Activity
25
20
15
10
5
0
O
P/I
P/I/CsA
pGM
pGM+M703
pGME
(2)
(3)
(2)

suggesting that the human enhancer is stronger however the mouse enhancer is indeed functional.

To determine whether the mouse enhancer needs to function in the context of a mouse cell to induce transcription at a level comparable to the human GM-CSF enhancer, the human GM-CSF promoter constructs coupled to the human or mouse enhancers were transiently transfected into mouse EL4 T cells. Similar to what was seen in the Jurkat T cells, the mouse enhancer yielded a 2.7-fold increase in inducible activity over the human GM-CSF promoter alone, compared to a 5.8-fold increase by the human enhancer (Fig. 3.12).

The mouse and human GM-CSF enhancers were also tested in front of an SV40 promoter (Fig. 3.5). In contrast to what was seen with the TK and human GM-CSF promoter constructs, the presence of the mouse enhancer actually decreased the level of induced transcription, whereas the human enhancer contributed to a 4.7-fold increase in induced transcription (Fig. 3.13). In each sample, the addition of CsA inhibited any increase in inducible activity. It is noteworthy, however, that the uninduced levels of expression by the mouse enhancer containing construct was half that of the SV40 promoter alone and a quarter of that seen with the human enhancer containing plasmid. When induction relative to the unstimulated sample is analysed, the mouse enhancer containing plasmid is induced to a slightly higher level (3.2-fold) than the promoter alone (2.3-fold), yet less than the human enhancer containing construct.

Fig. 3.12. Relative transcriptional activity of the mouse and human GM-CSF enhancers when coupled to the human GM-CSF promoter in transient transfection assays in EL4 T cells.

EL4 T cells were transfected with pGM, pGM+M703 and pGME, and were stimulated 24 hours later with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM) (P/I), or left unstimulated (O). After nine hours, cells were harvested, and luciferase activity was assayed. Luciferase activity is expressed relative to the stimulated level of pGM, assigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.

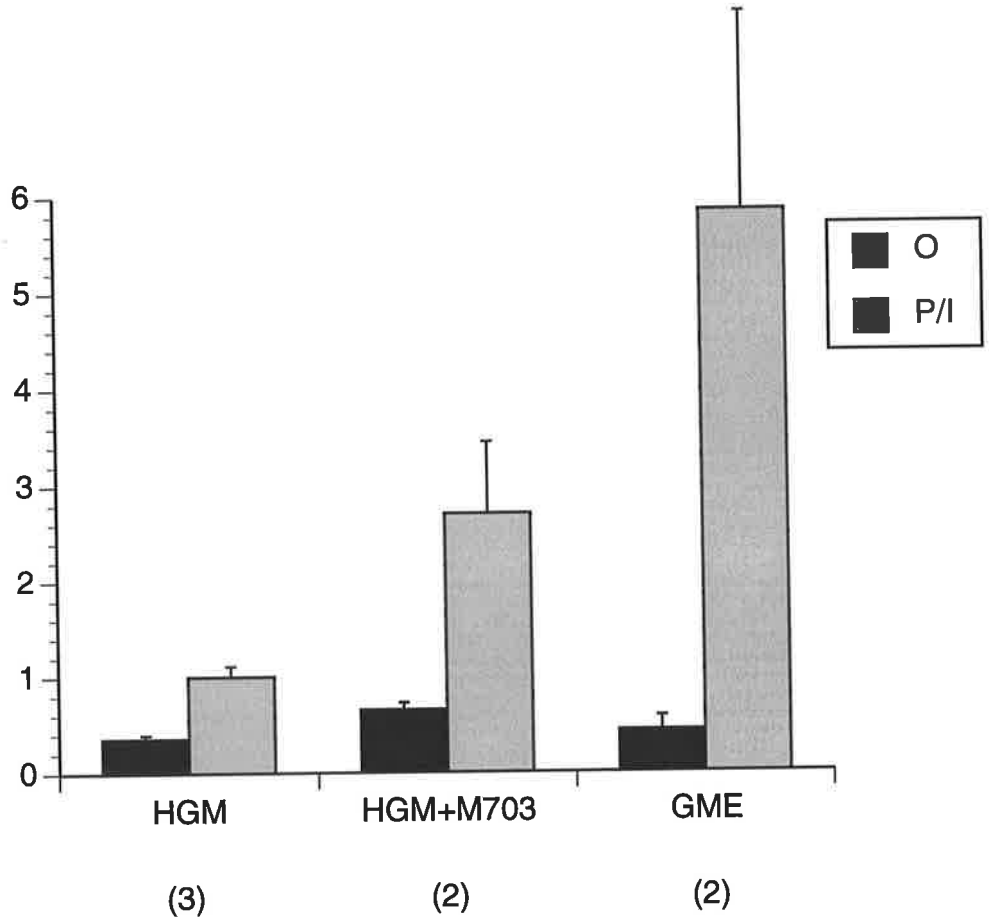
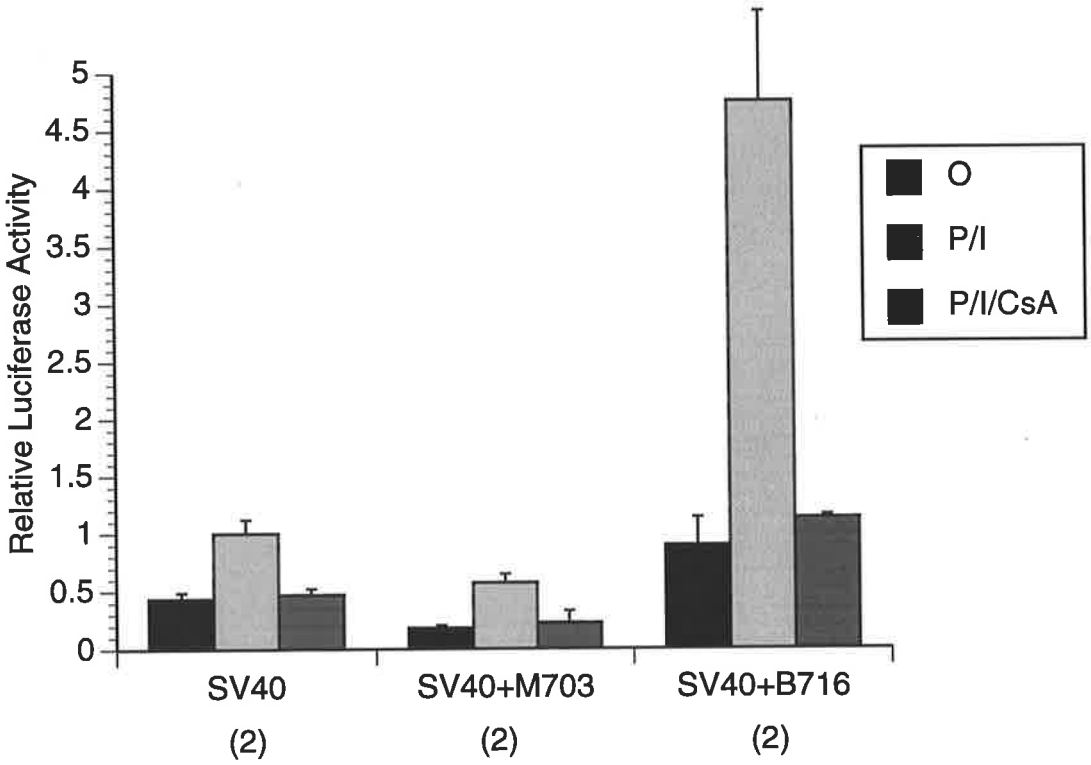

6
5
4
3
2
1
0
O
P/I
HGM
HGM+M703
GME
(3)
(2)
(2)

Fig. 3.13. Relative transcriptional activity of the mouse and human GM-CSF enhancers when coupled to the SV40 promoter in transient transfection assays in Jurkat T cells.

Jurkat T cells were transfected with pSV40, pSV40+M703 and pSV40+B716. After 24 hours, cells were stimulated with PMA (20 ng/ml) plus calcium ionophore A23187 (2 uM) (P/I), or PMA plus calcium ionophore plus cyclosporin A (0.1 uM) (P/I/CsA), or left unstimulated (O), then harvested nine hours later and assayed for luciferase activity. Luciferase activity is expressed relative to the stimulated level of pSV40, assigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.


Relative Luciferase Activity
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
O
P/I
P/I/CsA
SV40
(2)
SV40+M703
(2)
SV40+B716
(2)

It was of interest to determine whether the mouse enhancer could function through a truncated mouse promoter, as upstream promoter elements may mask its action. The mouse enhancer was linked to a 60 bp minimal mouse GM-CSF promoter upstream of luciferase and transiently transfected into Jurkat T cells (Fig. 3.5). No increase in inducible activity was observed when compared to the 60 bp promoter alone (Fig. 3.14). However, the human enhancer, when linked to the 60 bp mouse promoter, yielded a large increase in inducible activity, suggesting that the lack of function of the mouse enhancer on the full length mouse promoter is not merely due to masking by the strongly induced promoter.

One possible role of the mouse GM-CSF enhancer is to regulate the mouse IL-3 gene which is located 12 kb upstream of the enhancer. To ascertain whether the mouse enhancer could exert an effect on mouse IL-3 induction, the M703 mouse enhancer fragment was linked directly upstream of the mouse IL-3 promoter, and transiently transfected into Jurkat T cells (Fig. 3.5). Upon stimulation with PMA and calcium ionophore, a 4.4-fold increase in induction was observed over the mouse IL-3 promoter alone (Fig. 3.15). As was seen with the assays using TK and human GM-CSF promoters, the human GM-CSF enhancer was able to exert a greater effect on the mouse IL-3 promoter.

To investigate whether the mouse and human GM-CSF enhancers respond to different stimuli, promoter only, and promoter with enhancer constructs of both the mouse and human GM-CSF genes were

Fig. 3.14. Relative transcriptional activity of the mouse and human GM-CSF enhancers when coupled to a 60 bp minimal mouse GM-CSF promoter in transient transfection assays in Jurkat T cells.

pMGM0.06, pMGM0.06+M703 and pMGM0.06+B716 were transfected into Jurkat T cells and stimulated 24 hours later with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM) (P/I), or left unstimulated (O). Cells were harvested nine hours later and assayed for luciferase activity. Luciferase activity is expressed relative to the level of stimulated pMGM0.06, assigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.

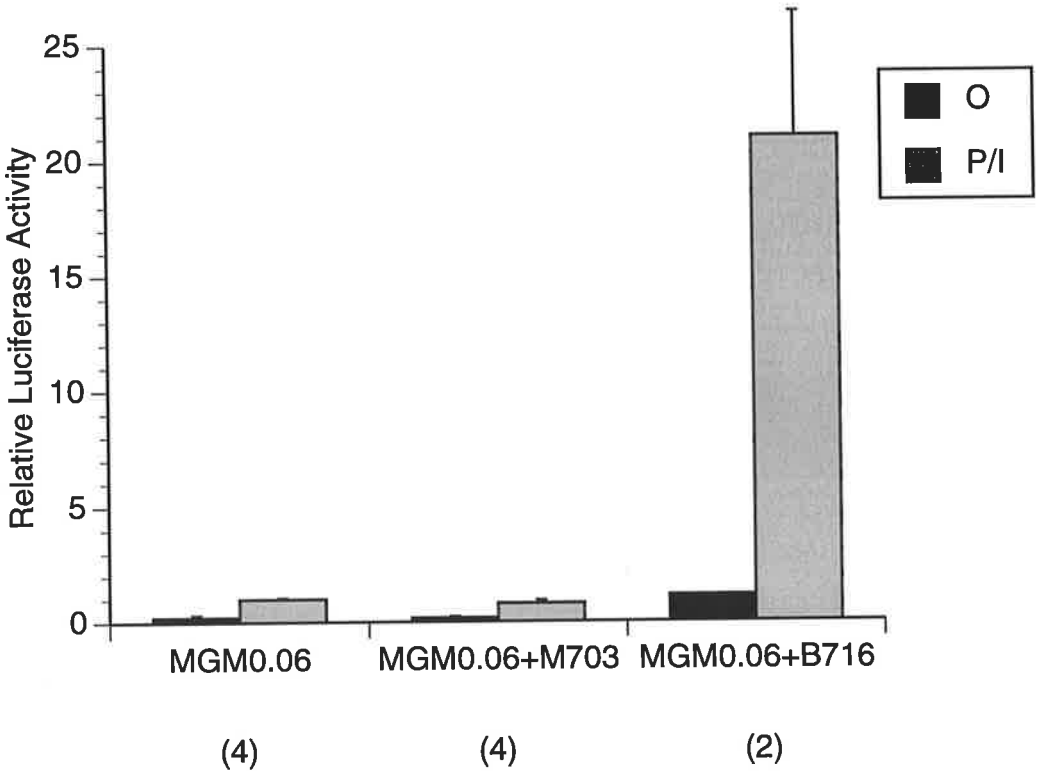
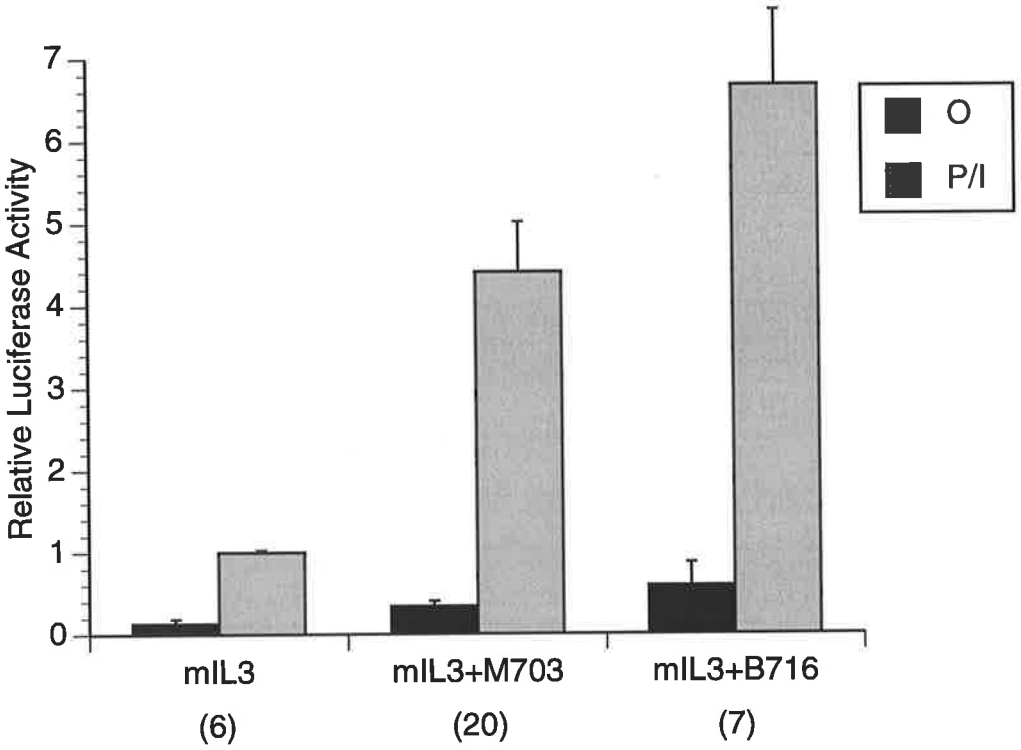

Relative Luciferase Activity
25
20
15
10
5
0
O
P/I
MGM0.06
MGM0.06+M703
MGM0.06+B716
(4)
(4)
(2)

Fig. 3.15. Relative transcriptional activity of the mouse and human GM-CSF enhancers when coupled to the mouse IL-3 promoter in transient transfection assays in Jurkat T cells.

Jurkat T cells were transfected with pMIL3, pMIL3+M703 and pMIL3+B716. After 24 hours, cells were stimulated with PMA (20 ng/ml) and calcium ionophore A23187 (2 uM) (P/I), or left unstimulated (O). Cells were harvested nine hours later and assayed for luciferase activity. Luciferase activity is expressed relative to the level of stimulated pMIL3, assigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.

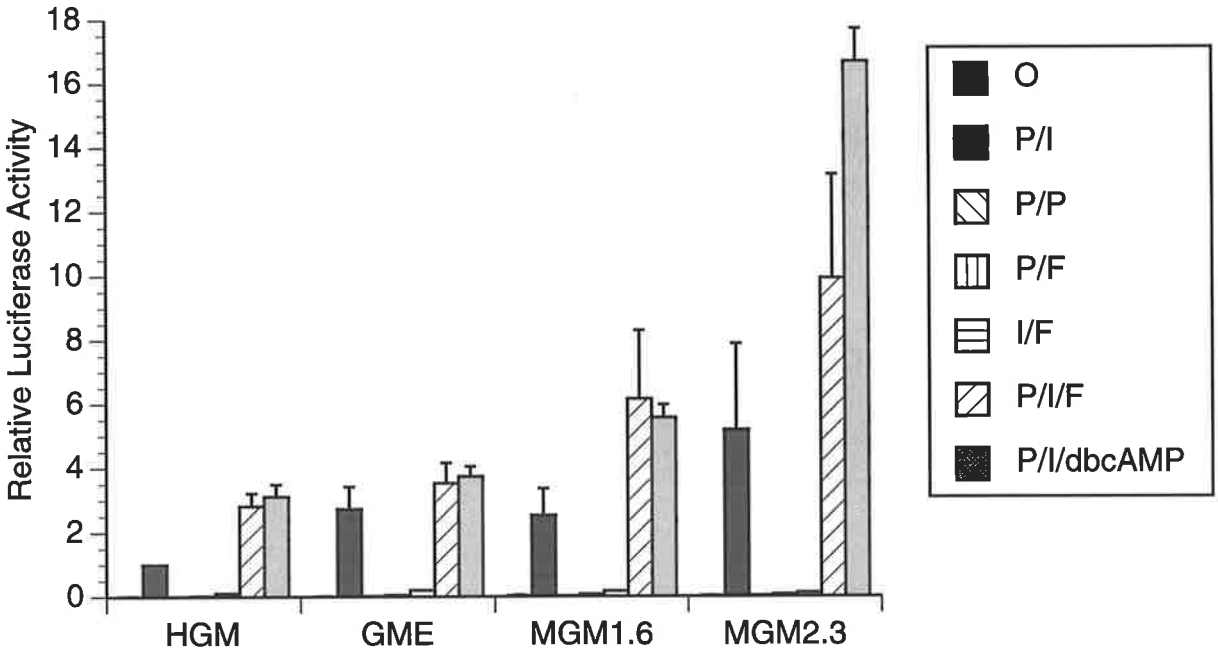

Relative Luciferase Activity
0
1
2
3
4
5
6
7
O
P/I
mIL3
(6)
mIL3+M703
(20)
mIL3+B716
(7)

transiently transfected into Jurkat T cells and stimulated with a panel of stimuli. Cells were stimulated with combinations of PMA and Ca^{2+} ionophore with, phytohaemagglutinin (PHA), forskolin or dibutyryl cAMP. PHA is a lectin that can activate the TCR. Forskolin and dibutyryl cAMP are both activators of the cAMP signalling pathways. Combinations of PMA and PHA, PMA and forskolin, or Ca^{2+} ionophore and forskolin had little effect on the induction of either the human or mouse constructs containing or lacking the enhancer (Fig. 3.16). Forskolin and dibutyryl cAMP were both able to synergise with PMA/Ca^{2+} ionophore to increase the human GM-CSF promoter activation (pGM). pGME did not appear to be significantly more responsive to PMA/calcium ionophore/forskolin or PMA/calcium ionophore/dbcAMP than was pGM. The mouse promoter (pMGM1.6) also responded to the addition of forskolin or dbcAMP to PMA/Ca^{2+} ionophore with greater than a two-fold increase in induction of luciferase. This induction increased even further when the construct contained the mouse enhancer (pMGM2.3), but in this series of experiments, the pMGM2.3 construct was almost twice as inducible as the pMGM1.6 construct, in contrast to what had been observed in previous assays. Nevertheless, it appears that the mouse GM-CSF constructs are more responsive to cAMP signalling pathways in combination with PMA/Ca^{2+} ionophore than the human GM-CSF constructs.

The mouse GM-CSF enhancer may function in the context of chromatin structure; studying its role in transient transfection assays may not be an appropriate measure of its activity. To investigate the role of the

Fig. 3.16. Response of the mouse and human GM-CSF loci to various stimuli in transient transfection assays in Jurkat T cells.

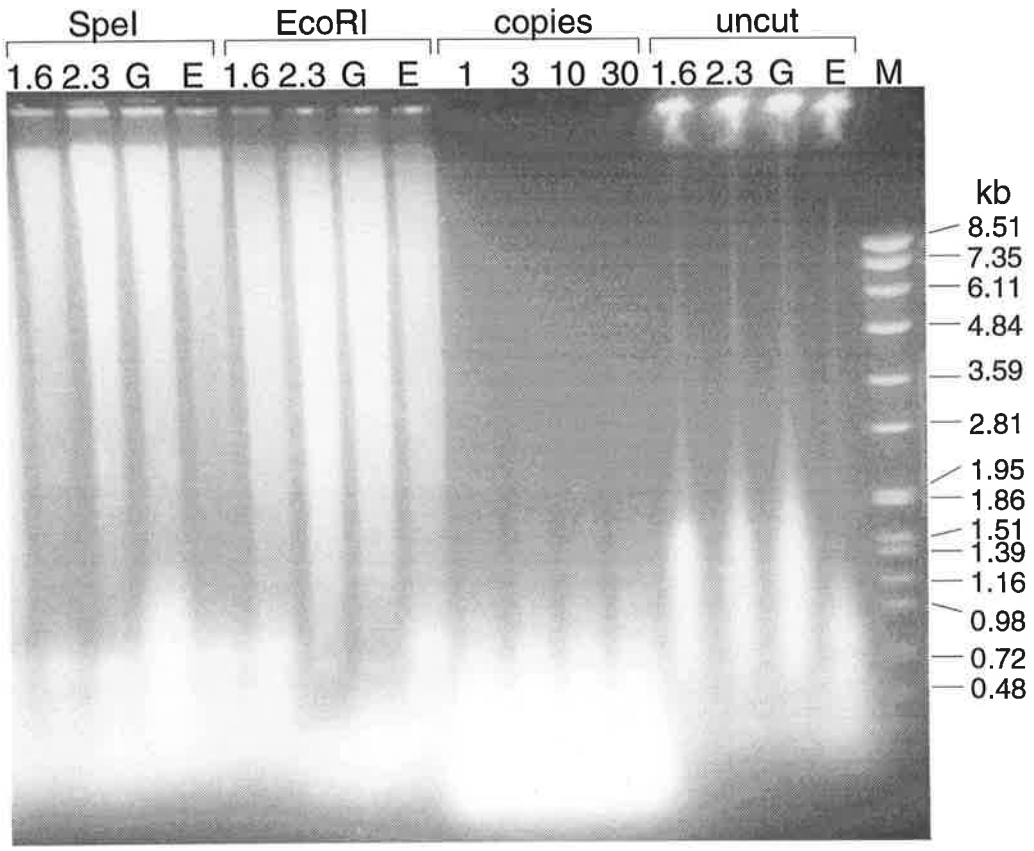
Jurkat T cells were transfected with pGM, pGME, pMGM1.6 and pMGM2.3. After 24 hours, cells were either left unstimulated (O) or were stimulated with PMA (20 ng/ml) and calcium ionophore (2 uM) (P/I), PMA and PHA (1 ug/ml) (P/P), PMA and forskolin (10 uM) (P/F), calcium ionophore and forskolin (I/F), PMA and calcium ionophore and forskolin (P/I/F), or PMA and calcium ionophore and dibutyryl cAMP (1 mM) (P/I/dbcAMP). Cells were harvested after nine hours and assayed for luciferase activity. Luciferase activity is expressed relative to the level of PMA/calcium ionophore stimulated pHGM, assigned a value of 1. Error bars represent standard deviation of two experiments.

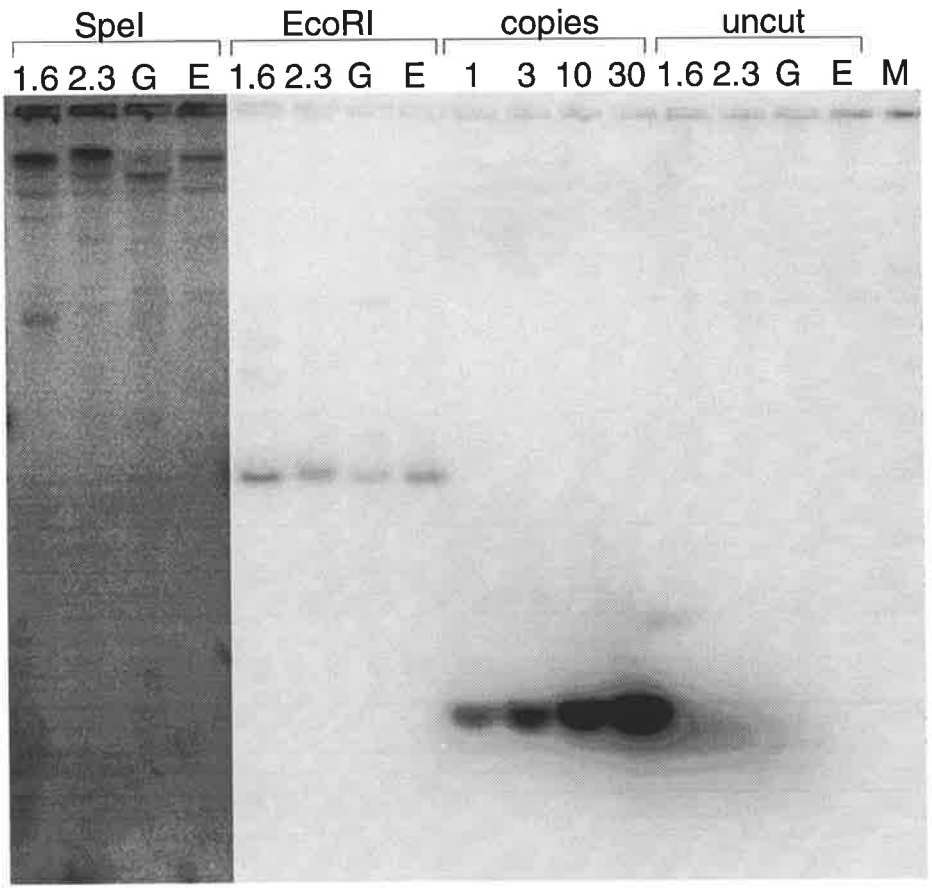

Relative Luciferase Activity
18
16
14
12
10
8
6
4
2
0
HGM
GME
MGM1.6
MGM2.3
O
P/I
P/P
P/F
I/F
P/I/F
P/I/dbcAMP

mouse enhancer in a more natural chromatin environment, mouse GM-CSF promoter (pMGM1.6) and promoter/enhancer (pMGM2.3) constructs linked to luciferase were stably transfected into Jurkat T cells. In parallel, human GM-CSF promoter (pGM) and promoter/enhancer (pGME) luciferase constructs were also stably transfected. In an effort to control for plasmid integration position effects, each transfection was grown without isolating individual clones in order to prepare a polyclonal population. A Southern blot was performed on the resulting cell populations to determine the number of integration events and the average copy number of the construct per cell (Fig. 3.17). Digestion of the stable cell line genomic DNA with *SpeI*, a restriction enzyme that does not cut within the reporter construct, revealed that each stable line is polyclonal, as evident by the presence of multiple bands (Fig. 3.17, lanes 1 through 4). Digestion of the genomic DNA with *EcoRI* produced a 2.7 kb fragment that was recognised by the probe. This demonsrated that all four stable cell lines had an average of less than one copy of the construct per cell: pMGM1.6 had an average of 0.24 copies/cell; pMGM2.3 had an average of 0.17 copies per cell; pGM had an average of 0.07 copies per cell, and pGME had an average of 0.15 copies per cell (stable lines, lanes 5 through 8; standards, lanes 9 through 12). The fact that there appear to be less than one copy per cell could mean that there are clones within the population that contain only the neomycin selectable marker construct, but not the reporter construct. It is also possible that, because DNA fragments of different sizes can transfer to membranes with different efficiencies, the 850 bp copy number standard fragments transferred more efficiently than the 2.7

Fig. 3.17. Southern blot showing integration site number and copy number of mouse and human GM-CSF loci constructs stably transfected into Jurkat T cells.

Genomic DNA isolated from stably transfected Jurkat T cell lines, and 10 ug samples were digested with restriction enzymes to determine the integration site number and copy number of the constructs, pMGM1.6 (1.6), pMGM2.3 (2.3), pGM (G), and pGME (E). *SpeI* did not cut within the constructs, and was used to detect the number of integration events (lanes 1 through 4). *EcoRI* excised a 2.7 kb fragment from the construct, and was used to determine the average number of copies of the construct per cell (lanes 5 through 8). A 850 bp *EcoRI/EcoRV* fragment of the luciferase gene corresponding to 1 copy, 3 copies, 10 copies and 30 copies per genome, combined with 10 ug salmon sperm carrier DNA, was used as a standard (lanes 9 through 12). Uncut genomic DNA samples were run in lanes 13 through 16. A *EcoRI* cut SPP1 marker (M), was included (lane 17), and fragment sizes are indicated. The top figure shows the ethidium bromide stained agarose gel. The bottom figure shows the Southern blot, probed with a radiolabelled 850 bp *EcoRI/EcoRV* luciferase gene fragment.

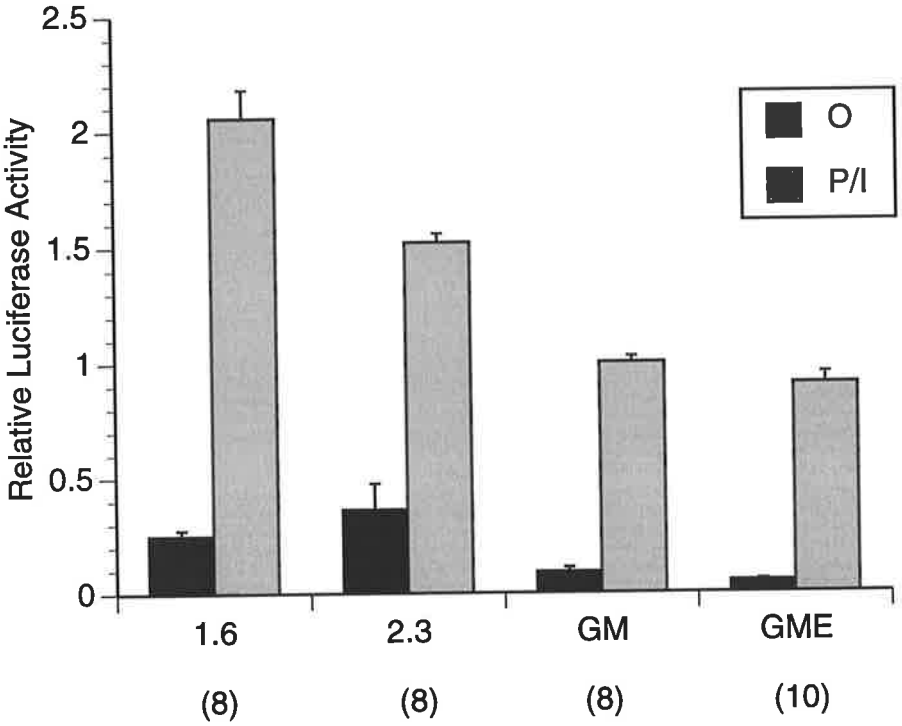

SpeI
EcoRI
copies
uncut
1.6 2.3 G E 1.6 2.3 G E 1 3 10 30 1.6 2.3 G E M
kb
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
0.48


SpeI
EcoRI
copies
uncut
1.6 2.3 G E 1.6 2.3 G E 1 3 10 30 1.6 2.3 G E M

kb reporter construct fragments. Uncut genomic DNA from the stable lines was included to determine whether any of the constructs remained unintegrated (bands 13 through 16). No band was visible suggesting that all constructs had integrated into the genomic DNA, however, no band was seen at the top of the gel, suggesting that the DNA may not have entered the gel. The stably transfected cell lines were stimulated with PMA and Ca^{2+} ionophore and tested for luciferase activity after nine hours. Surprisingly, neither the inclusion of the human nor mouse enhancer affected the induction of the human or mouse GM-CSF enhancer respectively (Fig. 3.18).

Fig. 3.18. Comparison of the relative transcriptional activity of the mouse and human GM-CSF loci in stable transfection assays in Jurkat T cells.

Jurkat T cells were transfected with linearised pMGM1.6, pMGM2.3, pGM, pGME along with linearised pE°TKneo. Cells with stably integrated plasmid were selected in neomycin containing medium for 3 weeks. Cells were then stimulated with PMA (20 ng/ml) and calcium ionophore (2 uM) (P/I) or left unstimulated (O). After nine hours, cells were harvested and assayed for luciferase activity. Luciferase activity is expressed relative to stimulated pGM levels, asigned a value of 1. Error bars represent standard error, and numbers in parentheses represent the number of assays performed.


Relative Luciferase Activity
2.5
2
1.5
1
0.5
0
O
P/I
1.6
2.3
GM
GME
(8)
(8)
(8)
(10)

3.3. DISCUSSION

The work described in this chapter was undertaken to isolate and characterise the element homologous to the human GM-CSF enhancer. The identification of this mouse enhancer has added to the considerable structural conservation of the mouse and human GM-CSF/IL-3 loci. With the high degree of conservation between the two species with respect to gene distance, orientation, intron/exon organisation and promoter conservation, it is not surprising to find a homologous enhancer that also forms an inducible CsA sensitive DH site.

As a guide to which elements are functionally important within the enhancers, it is useful to look at the regions that are conserved. It is of note that the area of highest homology almost directly corresponds to the 425 bp *BamHI/MscI* fragment defined as the full length human enhancer (Cockerill et al., 1995b). It's likely that this region also is central to the functioning of the mouse enhancer. Indeed, a 230 bp human enhancer fragment that spans this region, including the only two conserved NFAT sites (GM420 and GM550), is able to convey the full human enhancer activity (Cockerill et al., 1995b). In contrast, the human GM170 site, which is not conserved at all in the mouse enhancer is the weakest of all four NFAT sites (Cockerill et al., 1995b). The sequence conservation has also highlighted other elements which may play important roles in the enhancer function. A core-binding factor site is well conserved, very close to the GM420 NFAT site. This binding site will be discussed in Chapter Five. Sequence homology studies have also highlighted putative binding sites for other

haemopoietic specific transcription factors such as E2A, Ets and GATA family members. These factors may contribute to the tissue/stage-specific regulation of GM-CSF expression. The presence of long stretches of homology with no obvious transcription factor binding sites may suggest that these sequences contain transcription factor binding sites that are yet to be defined. DNA footprinting and methylation interference of these regions need to be performed to help identify such factors.

Despite there being a high degree of sequence homology, there are also some significant differences between the mouse and human enhancers. Only two of the four NFAT sites present in the human enhancer are conserved to any extent in the mouse enhancer (GM420 and GM550), and neither of these are strictly conserved. In both cases the AP-1 and NFATp/c components are spaced further apart in the mouse, and are therefore less likely to cooperatively bind. Gel shift assays need to be performed to address the question binding affinity of NFAT to the NFAT-like elements in the mouse enhancer.

With such a structural similarity in the organisation of the locus and a high degree of homology between the mouse and human enhancers, it is surprising that they appear to function differently. Whilst both enhancers are able to augment the inducible activity of heterologous promoters such as the TK promoter, the mouse enhancer had no significant effect on the activation through the mouse GM-CSF promoter in transient transfection assays. This lack of function appears to be compensated by a higher inducible activity of the

mouse GM-CSF promoter, compared to a weaker inducible activity of the human GM-CSF promoter. The differences between the mouse and human GM-CSF promoters will be discussed in Chapter Four. The apparent lack of function of the mouse enhancer on the mouse promoter cannot be simply explained as a masking of its effects by the already strongly inducible promoter. Linking the enhancer to a truncated mouse promoter (pMGM0.06) that was weakly inducible did not support an increase in activation, whereas the human enhancer was still able to augment this minimal promoter's activity. Additionally, the mouse enhancer was unable to function on the SV40 promoter, suggesting that the human enhancer may be more indiscriminate than the mouse enhancer.

It is unclear why the TK and SV40 promoters were sensitive to CsA. It casts doubt as to whether the CsA-mediated suppression of the constructs containing the enhancer is due to a CsA sensitivity of the enhancer, or due to more general cellular inhibitory mechanisms. Some of these effects may be due to CsA sensitive sites within the vector, or perhaps a CsA sensitivity of other transcription factors

Although the mouse enhancer is able to increase induction through the mouse IL-3 promoter, this does not necessarily suggest that this is indeed a function of the mouse enhancer, merely that these two elements are compatible. In these assays, both the human and mouse enhancers were linked directly upstream of the mouse IL-3 promoter. In the natural context, there may be structural barriers such as

boundary elements that block interactions between the IL-3 promoter and GM-CSF enhancer.

It is important to recognise the limitations of transient transfection assays. Some regulatory elements may need to be packaged in a chromatin context to be able to function, and may operate by altering the chromatin to promote transcription. These characteristics are usually associated with LCR activity. Within the ß globin locus, only one of the four elements composing the LCR has classical enhancer activity when tested in transient transfection assays (Tuan et al., 1989), but all four elements are required *in vivo* for proper globin gene regulation (Grosveld et al, 1987). Supercoiled circular plasmids may not generate some higher orders of chromatin structure on DNA, and hence these chromatin modifying abilities may be unnecessary in this context. If the role of the mouse GM-CSF enhancer is largely to alter chromatin structure, this function would be less likely to be detected in transient transfection assays. Studying the role of this element when stably integrated into DNA is more likely to address this issue. In the one stable transfection assay performed, neither the human nor the mouse enhancers had any significant effect on their respective promoters. However, this study is not conclusive. As the constructs can be integrated anywhere in the genome, and in numerous array configurations, variability can be expected depending on the degree of packaging of the chromatin structure of the integration site, and interaction between multiple copies at each integration site. To normalise for these effects, these stable transfections should be repeated several times. Also, these constructs

may not be appropriate if the enhancers need to interact with other regulatory elements within the locus. The mouse enhancer may need to interact with upstream elements that are not included in these constructs, such as sequences immediately upstream; the construct containing sequence up to -3.1 kb was more inducible than the construct up to -2.3 kb. Alternatively, the mouse enhancer may need to function with more distal elements, such as the constitutive DH site that is located 4.9 kb upstream of the GM-CSF gene. Larger fragments of the mouse GM-CSF locus need to be stably integrated to address this. The lack of function of the human enhancer was surprising. This might be due, however, to the fact that in this construct the enhancer is directly coupled to the promoter without any of the intervening sequence. This length of sequence may be too short to allow the enhancer to loop over and interact with the promoter. A construct containing the human enhancer in its natural position with respect to the promoter may be more appropriate. An additional explanation for lack of apparent enhancer function may be that the luciferase expression kinetics are different when the constructs are stably integrated into DNA. A new time course mapping luciferase expression would be required to determine this.

The mouse GM-CSF locus appears to be more responsive than the human GM-CSF locus to cAMP signals in combination with PMA and Ca^{2+} ionophore, but it is difficult to say how much of this effect is elicited by the promoter and how much by the enhancer. In these assays, the mouse enhancer construct pMGM2.3 was more highly induced with respect to the mouse promoter construct pMGM1.6, that

had been previously observed, hence its corresponding activities seen in the presence of cAMP signals may be artificially high. Another manner by which one could address the cAMP responsiveness of the enhancer would be to link it to a non-cAMP responsive promoter and compare its activity in response to PMA/Ca^{2+} ionophore versus PMA/Ca^{2+} ionophore/dbcAMP. The generally higher responsiveness to cAMP signals in the mouse locus is not conclusive. The mouse promoter construct contains 1600 bp of upstream sequence whereas the human promoter construct contains 627 bp. It is possible that a cAMP responsive element is present in the mouse construct but its counterpart is not included in the human construct.

The are other possible reasons for the inability of the mouse enhancer to activate the mouse GM-CSF promoter. The mouse GM-CSF enhancer may not be required for the rapid induction of GM-CSF expression in response to T cell activation. Its role may be involved in the developmentally programmed regulation of the GM-CSF locus during haemopoiesis. A mouse GM-CSF enhancer transgene model would be useful for investigating this possibility as this would provide information on its temporal and tissue specific requirements for function. Another possibility is that the mouse GM-CSF enhancer responds to the activation of pathways other than those invoked through T cell receptor signalling. Whilst a small panel of stimuli was tested, a more extensive search would be beneficial. The human enhancer has been shown to function in other cell types such as endothelial cells (Cockerill et al., 1995a), and preliminary evidence from this laboratory shows that an inducible DH site forms over the

human GM-CSF enhancer in fibroblasts and monocytes (P. Cockerill, unpublished data). However, the physiological stimuli involved in these cells have not been determined. The mouse enhancer may activate the mouse GM-CSF promoter in response to the activation of pathways other than those invoked through T cell receptor signalling, and in cell types other than T cells.

CHAPTER 4.

COMPARISON OF THE MOUSE AND HUMAN GM-CSF GENE PROMOTERS

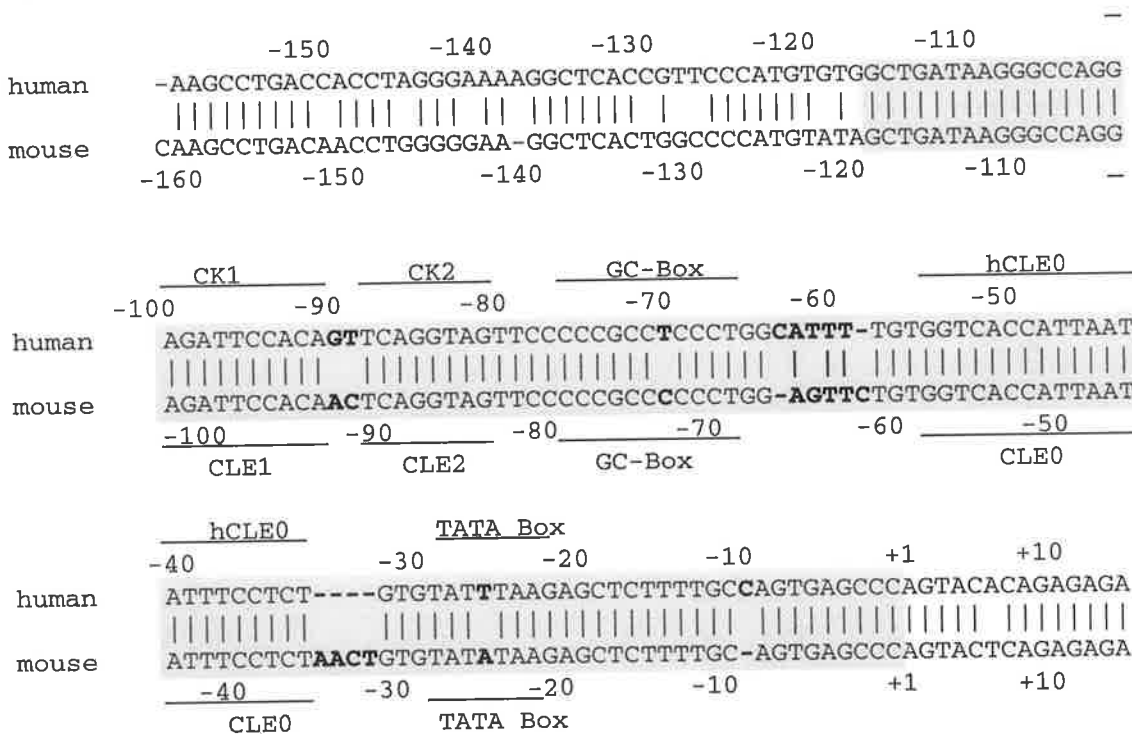
4.1. INTRODUCTION

An interesting observation to emerge from the study of the mouse GM-CSF enhancer was the apparent difference in inducible potentials of the mouse and human GM-CSF gene promoters. These studies showed that the induced activity of the mouse GM-CSF gene promoter was approximately three-fold higher than that of its human counterpart. Indeed, Miyatake et al. (1988b) also observed a significant difference in the induced activities of these promoters. Such a difference is surprising considering the high degree of DNA sequence homology between the human and mouse GM-CSF promoters; there is approximately 80% homology within 350 bp of the transcriptional start site, and approximately 90% homology within 120 bp (Fig. 4.1).

Deletion analyses of the mouse and human GM-CSF gene promoters suggest that full inducible activity is contained within 120 bp from the transcriptional start site (Miyatake et al., 1988a; Nimer et al., 1988). Within this sequence are conserved elements; the CK-1, CK-2 and CLE0 elements are all strictly conserved, as is a potential GATA binding site. There are differences, however, which could affect transcription factor binding. In the sequence between -120 bp and the *BstEII* site, at -54 bp and -57 bp in the human and mouse promoters respectively, there are three sequence differences that may account for the different inducible activities observed: a two bp difference is located between the CK-1 and CK-2 elements at -89 bp in the human; a one bp difference exists within the GC-rich region at -70

Fig. 4.1. Alignment of the mouse and human GM-CSF gene promoters within 120 bp of the transcriptional start site.

Sequence alignment between the mouse and human GM-CSF gene promoters was performed using the DNASIS computer program. Sequence is numbered from the transcriptional start site at +1. Within 119 bp of the mouse transcription start site, and 116 bp of the human transcription start site, there is 90% DNA sequence homology, and is shown shaded. Sequence differences between the mouse and human promoters within the -119 to +1 bp region are printed in bold.


-150 -140 -130 -120 -110
human -AAGCCTGACCACCTAGGGAAAAGGCTCACCGTTCCCATGTGTGGCTGATAAGGGCCAGG
mouse CAAGCCTGACAACCTGGGGGAA-GGCTCACTGGCCCCATGTATAGCTGATAAGGGCCAGG
-160 -150 -140 -130 -120 -110
CK1
CK2
GC-Box
hCLE0
-100 -90 -80 -70 -60 -50
human AGATTCCACAGTTCAGGTAGTTCCCCCGCCTCCCTGGCATTT-TGTGGTCACCATTAATC
mouse AGATTCCACAACTCAGGTAGTTCCCCCGCCCCCCTGG-AGTTCTGTGGTCACCATTAATC
-100 -90 -80 -70 -60 -50
CLE1
CLE2
GC-Box
CLE0
hCLE0
TATA Box
-40 -30 -20 -10 +1 +10
human ATTTCCTCT----GTGTATTTAAGAGCTCTTTTGCCAGTGAGCCCAGTACACAGAGAGA
mouse ATTTCCTCTAACTGTGTATATAAGAGCTCTTTTGC-AGTGAGCCCAGTACTCAGAGAGA
-40 -30 -20 -10 +1 +10
CLE0
TATA Box

bp in the human; and difference in the most distal of three CATT repeats at -59 bp in the human. Downstream of the *BstEII* site, there are three sequence differences that could account for the differing inducible activities. There are four additional bases between the CLE0 element and the TATA box in the mouse, and there is a one bp difference within the TATA box. There is also an additional one base pair in the human promoter, 10 bp from the transcription start site.

The aim of this study was to determine which DNA sequence differences could account for the induction differences of the mouse and human GM-CSF gene promoters. A series of luciferase reporter gene plasmid constructs containing mutations within the mouse and human GM-CSF gene promoters were made, and tested in transiently transfected Jurkat T cells. DNA-protein binding studies were also conducted to test the affinities of Sp1 binding to the human and mouse GC-boxes, and TATA-binding protein (TBP) binding to the human and mouse TATA boxes.

4.2. RESULTS

4.2.1. TRANSCRIPTION FACTOR BINDING STUDIES OF THE MOUSE AND HUMAN GM-CSF GENE PROMOTERS

The GC-boxes of the mouse and human GM-CSF gene promoters were tested for their ability to bind to the transcription factor Sp1. Differential binding affinities of the sites could contribute to the differences in inducible activity. Recombinant human Sp1 was able to bind to both radioactively labelled mouse and human probes containing the GM-CSF gene promoter GC-box (Fig. 4.2). Binding of Sp1 to the mouse GM-CSF GC-box has also been observed by Masuda et al. (1994). Unlabelled oligonucleotides of the mouse and human GM-CSF GC-boxes were used to compete for Sp1 binding with the probes. The mouse GC-box functioned as a stronger competitor for Sp1 binding than the human GC-box. Against the human GC-box probe, the mouse GC-box competitor competed 1.5 to 2 times more strongly than the human GC-box competitor. Against the mouse GC-box probe, the mouse GC-box competitor competed 2 to 3 times more strongly than the human GC-box competitor. Such a difference in binding affinities for Sp1 could partially account for differences seen in inducible activity levels.

The mouse and human GM-CSF gene TATA boxes were also tested for their binding to TBP in a DNA-protein binding assay. Radioactively labelled oligonucleotides probes spanning the mouse and human TATA boxes were incubated with varying amounts of recombinant

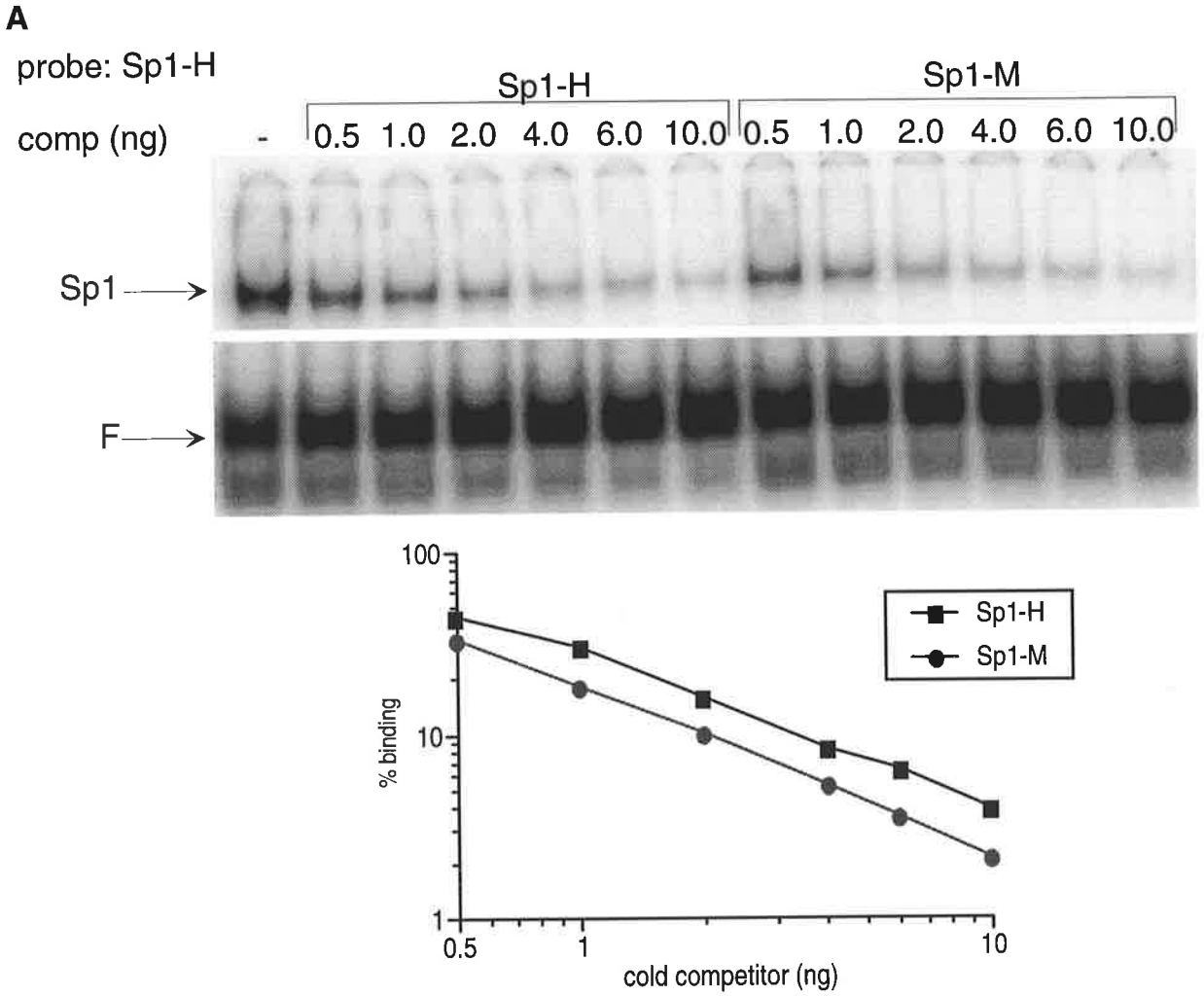
Fig. 4.2. Competition for recombinant human Sp1 binding to the GC-boxes of the human and mouse GM-CSF promoters.

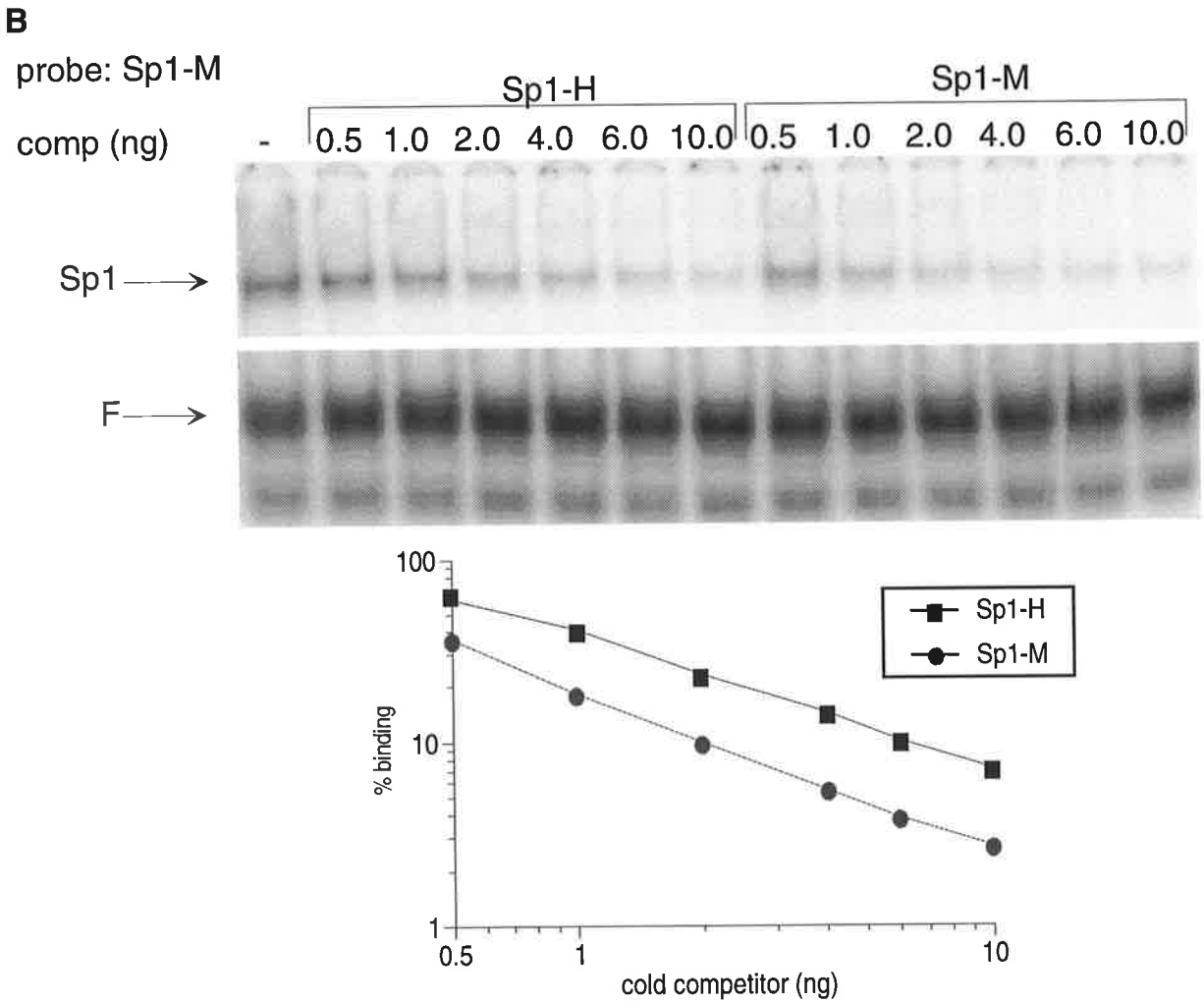
A. Recombinant human Sp1 (1.0 ng) was combined with radiolabelled human GC-box oligonucleotide (0.2 ng)(Sp1-H). Unlabelled competitor oligonucleotides were added to the binding reactions in the nanogram amounts indicated. Lanes 2 through 7 contained Sp1-H competitor, and lanes 8 through 13 contained Sp1-M competitor. Lane 1 had no competitor added. The Sp1 complex (Sp1) and unbound probe (F) are indicated.

The amount of shifted Sp1-H probe was quantitated as a percentage of total probe. Values are presented as percent binding where no competitor represents 100% binding.

B. Recombinant human Sp1 (1.0 ng) was combined with radiolabelled mouse GC-box oligonucleotide (0.2 ng)(Sp1-M). Unlabelled competitor oligonucleotides were added to the binding reactions in the nanogram amounts indicated. Lanes 15 through 20 contained Sp1-H competitor, and lanes 21 through 26 contained Sp1-M competitor. Lane 14 had no competitor added. The Sp1 complex (Sp1) and unbound probe (F) are indicated.

The amount of shifted Sp1-M probe was quantitated as a percentage of total probe. Values are presented as percent binding where no competitor represents 100% binding.


A
probe: Sp1-H
Sp1-H
Sp1-M
comp (ng)
-
0.5 1.0 2.0 4.0 6.0 10.0
0.5 1.0 2.0 4.0 6.0 10.0
Sp1
F
100
10
1
% binding
0.5
1
10
cold competitor (ng)
Sp1-H
Sp1-M


B
probe: Sp1-M
Sp1-H
Sp1-M
comp (ng)
-
0.5 1.0 2.0 4.0 6.0 10.0
0.5 1.0 2.0 4.0 6.0 10.0
Sp1
F
100
10
1
% binding
0.5
1
10
cold competitor (ng)
Sp1-H
Sp1-M

human TBP. TBP was able to bind to both the mouse and human probes (Fig 4.3). However, TBP bound more 2 to 3 times strongly to the mouse TATA box probe than the human probe. This difference may also partially account for differences observed in the inducible activities of the two promoters.

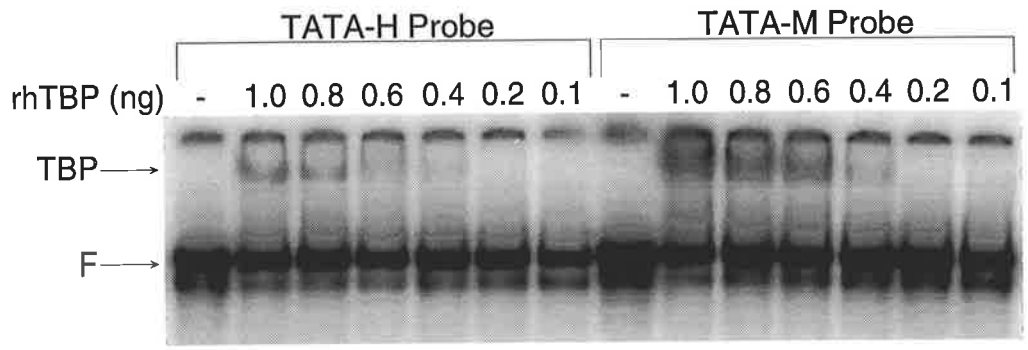
4.2.1. FUNCTIONAL STUDIES OF THE MOUSE AND HUMAN GM-CSF GENE PROMOTERS

To directly compare the inducible activities of the human and mouse GM-CSF gene promoters, it was necessary to test promoters of equivalent lengths. DNA sequences contained within 116 bp and 119 bp of the human and mouse transcription start sites, respectively, were chosen because these regions have been previously demonstrated to contain full inducible activity (Miyatake et al., 1988; Nimer et al., 1988), and DNA sequence homology is reduced upstream of this region. The inducible activities of the 116 bp human GM-CSF gene promoter construct (pGM116) and the 119 bp mouse GM-CSF gene promoter construct (pMGM119) were compared to the activities of the 627 bp human and 200 bp mouse promoters (pGM and pMGM0.2, respectively) (Fig. 4.4; Fig. 4.5). The induced activity of pGM116 was over 4-fold higher than pGM, and the induced activity of pMGM119 was over 3-fold higher than pMGM0.2. This suggests that there may be negative regulatory elements that are contained within pGM and pMGM0.2 that are not present in their truncated promoters. The fold difference of induced pMGM119 over pGM116 appeared to

Fig. 4.3. Binding of the mouse and human GM-CSF TATA boxes to recombinant human TBP.

Nanogram amounts of recombinant human TBP were combined with radiolabelled 0.2 ng human TATA box oligonucleotide (TATA-H, lanes 2 through 7), or 0.2 ng mouse TATA box oligonucleotide (TATA-M, lanes 9 through 14). Lanes 1 and 8 had no TBP added. The TBP complex (TBP) and unbound probe (F) are indicated.

The extent of binding of TBP to the mouse and human TATA box probes was quantitated by ImageQuant version 3.2.1 computer software. The amount of probe shifted by TBP binding is presented as a percentage of total probe.


TATA-H Probe
TATA-M Probe
rhTBP (ng) - 1.0 0.8 0.6 0.4 0.2 0.1 - 1.0 0.8 0.6 0.4 0.2 0.1
TBP
F

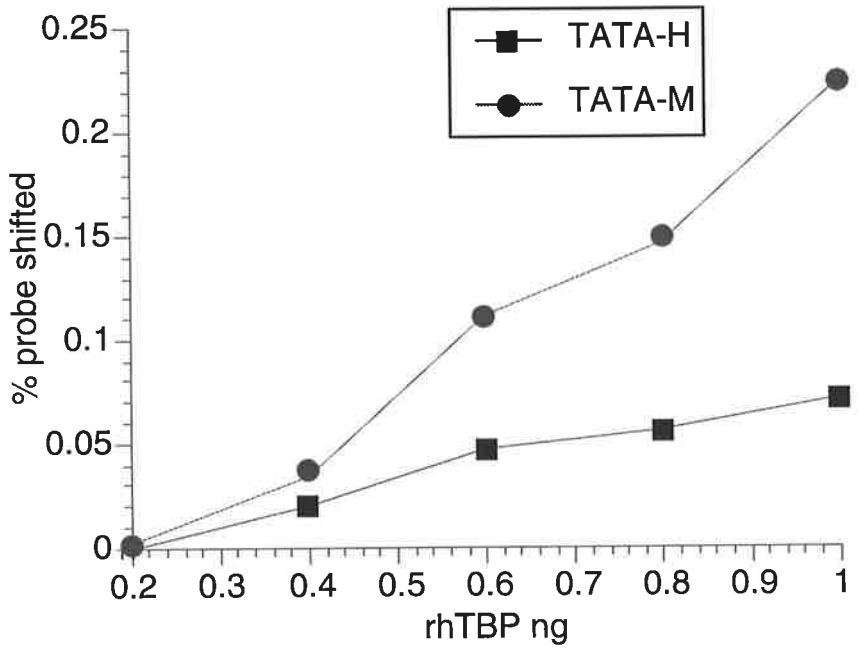

TATA-H
TATA-M
% probe shifted
0.25
0.2
0.15
0.1
0.05
0
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
rhTBP ng

Fig. 4.4. Schematic representation of luciferase constructs used to detect differences between the mouse and human GM-CSF promoters.

Oligonucleotides were cloned into mouse and human GM-CSF gene promoter constructs to create promoters with specific mutations. The white shapes represent sequence motifs of the mouse GM-CSF promoter. The black shapes represent sequence motifs of the human GM-CSF promoter. Both the mouse and human constructs contain human GM-CSF promoter sequence from -19 bp to +28 bp. The arrows indicate a deletion between the NFAT-1 site and the TATA box of pM4bp or an insertion between the NFAT-1 site and the TATA box of pH4bp. For details of cloning, refer to chapter 2, section 2.9.

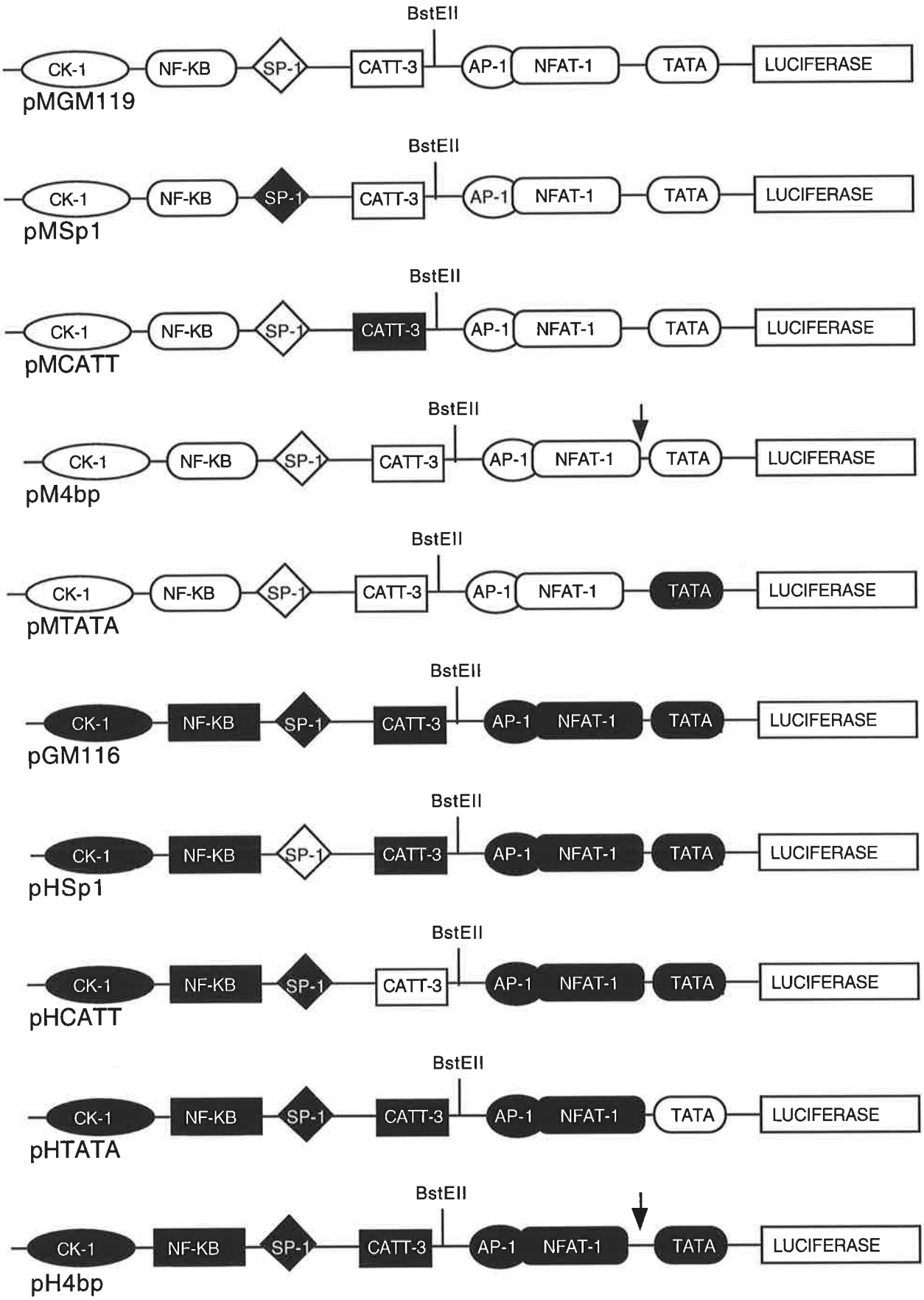
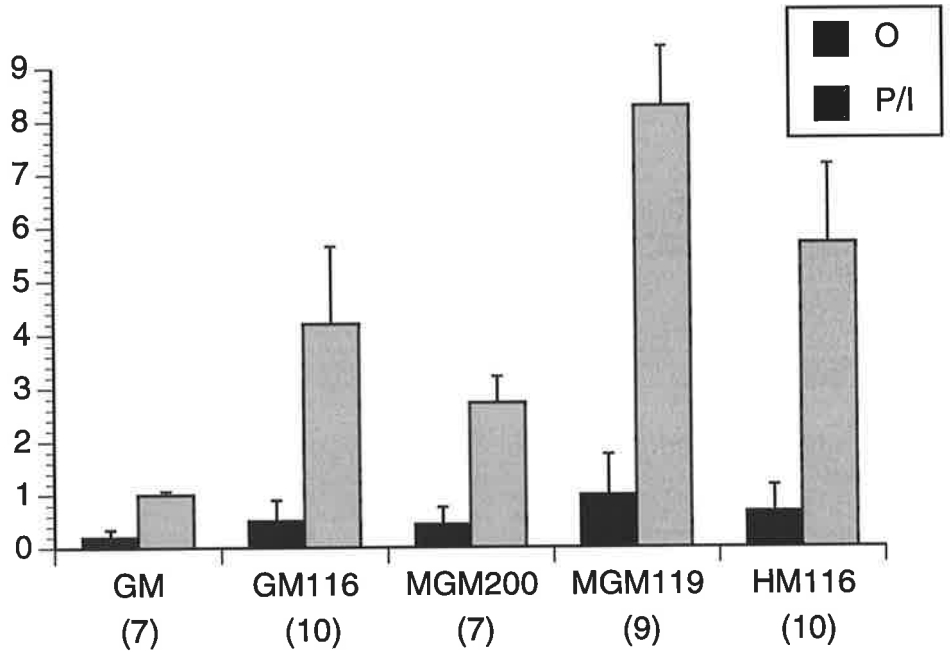

BstEII
CK-1
NF-KB
SP-1
CATT-3
AP-1
NFAT-1
TATA
LUCIFERASE
pMGM119
pMSp1
pMCATT
pM4bp
pMTATA
pGM116
pHSp1
pHCATT
pHTATA
pH4bp

Fig. 4.5. Relative transcriptional activity of human and mouse GM-CSF gene promoter constructs in transient transfection assays in Jurkat T cells.

Jurkat T cells were transfected with 5ug of pGM, pGM116, pMGM0.2, pMGM119 and pHM116. After 24 hours, cells were either left unstimulated (O) or stimulated with PMA (20ng/ml) and calcium ionophore (2uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Luciferase activity is expressed relative to the level of activity in stimulated pGM samples, assigned a value of 1. Error bars represent the standard error and numbers in parentheses are the number of assays performed.


O
P/I
9
8
7
6
5
4
3
2
1
0
GM
(7)
GM116
(10)
MGM200
(7)
MGM119
(9)
HM116
(10)

be less than that of pMGM0.2 over pGM. There may be elements upstream of -119 bp that contribute to the different inducible activities. There is still, however, a two-fold difference in induced activity of the pMGM119 and pGM116 constructs, implying that there are differences within this sequence that contributes to variance of induction levels.

A chimaeric 116 bp GM-CSF gene promoter plasmid was constructed which coupled mouse promoter sequence downstream of the *BstEII* restriction enzyme site at -57 bp, with human promoter sequence from the *BstEII* site to -116 bp (pHM116). This plasmid was compared to pMGM119 and pGM116 to determine whether sequence differences contributing to the difference in inducible activities resided upstream or downstream of the *BstEII* site. The induced activity of pHM116 was midway between the activities of pGM116 and pMGM119. Its activity, however, was not significantly different from either pGM116 or pMGM119, as determined by a student T test (Fig. 4.5).

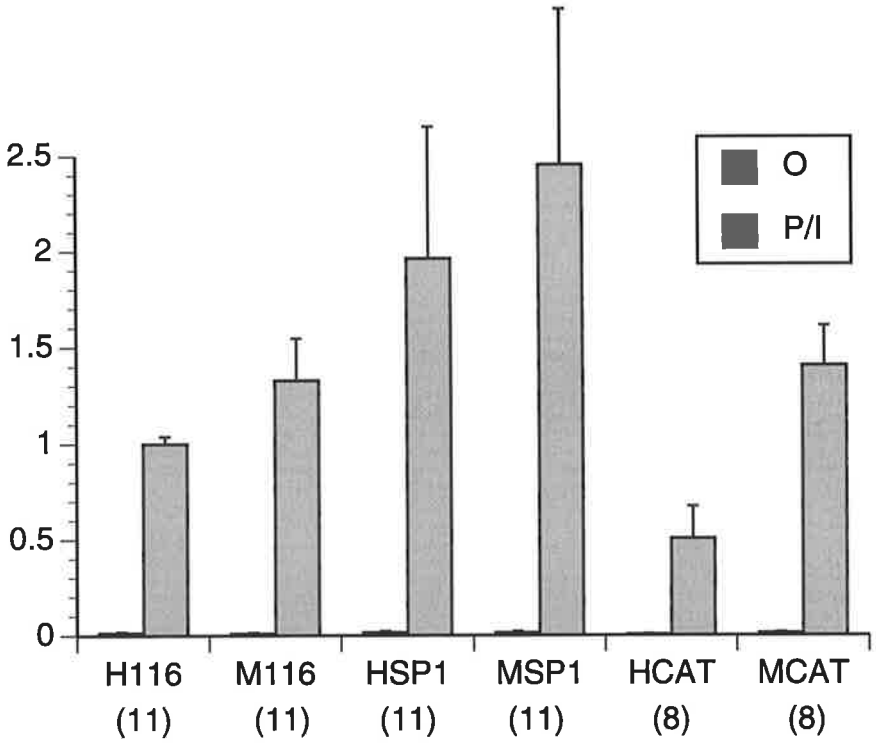
To determine the significance of individual sequence differences between the mouse and human GM-CSF gene promoters, mutations were introduced into pGM116 and pMGM119. Upstream of the *BstEII* site, two sequence differences between the mouse and human promoters were investigated. The GC-box forms a good Sp1 consensus binding site (Kadonaga et al., 1986) in the mouse promoter; a 1 bp difference in the human GC-box is likely to make it a less favourable Sp1 binding site. A mutation was introduced to pGM116 at the GC-box

to form a mouse GC-box (pHSp1). Likewise, a mutation was introduced into pMGM119 to form a human GC-box (pMSp1). The furthest upstream of three CATTA/T repeats (termed CATT-3) present in the human GM-CSF gene promoter is absent in the mouse GM-CSF promoter. This site has been suggested to bind to a transcriptional repressor (Nimer et al., 1990; Fraser et al., 1994), and hence could account for the induction differences between the mouse and human promoters. Mutations were introduced into pMGM119 to form a CATT-3 element (pMCAT). Again, a complementary mutation was introduced into pGM116 to replace the CATT-3 element with the mouse equivalent (pHCAT). The GC-box and CATT-3 mutant constructs were compared to the wild type pMGM119 and pGM116 constructs in transient transfection assays (Fig. 4.6). In these assays, there was no significant difference between the inducible activities of pGM116 and pMGM119, in contrast to what had been observed previously. The activities of pHSp1 and pMSp1 were both higher than those of pGM116 and pMGM119, but were variable. pHCAT was significantly less active than both pGM116 and pMGM119. There was no significant difference between the activities of pMCAT and the wild type promoters.

Downstream of the *BstEII* site within the GM-CSF gene promoter, two differences between the mouse and human were considered. An additional 4 bp are present in the mouse GM-CSF promoter between the binding sites for Ets/NFATp/c and TATA box binding protein (TBP). There are approximately 10 bp within one helical twist of double stranded DNA, hence, this 4 bp difference would constitute

Fig. 4.6. Relative transcriptional activity of human and mouse GM-CSF gene promoter constructs containing mutations in the GC-box or the CATT-3 box, in transient transfection assays in Jurkat T cells.

Jurkat T cells were transfected with 5ug of pGM116, pMGM119, pHSp1, pMSp1, pHCAT and pMCAT. After 24 hours, cells were either left unstimulated (O) or stimulated with PMA (20ng/ml) and calcium ionophore (2uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Luciferase activity is expressed relative to the level of activity in stimulated pGM116 samples, assigned a value of 1. Error bars represent the standard error and numbers in parentheses are the number of assays performed.

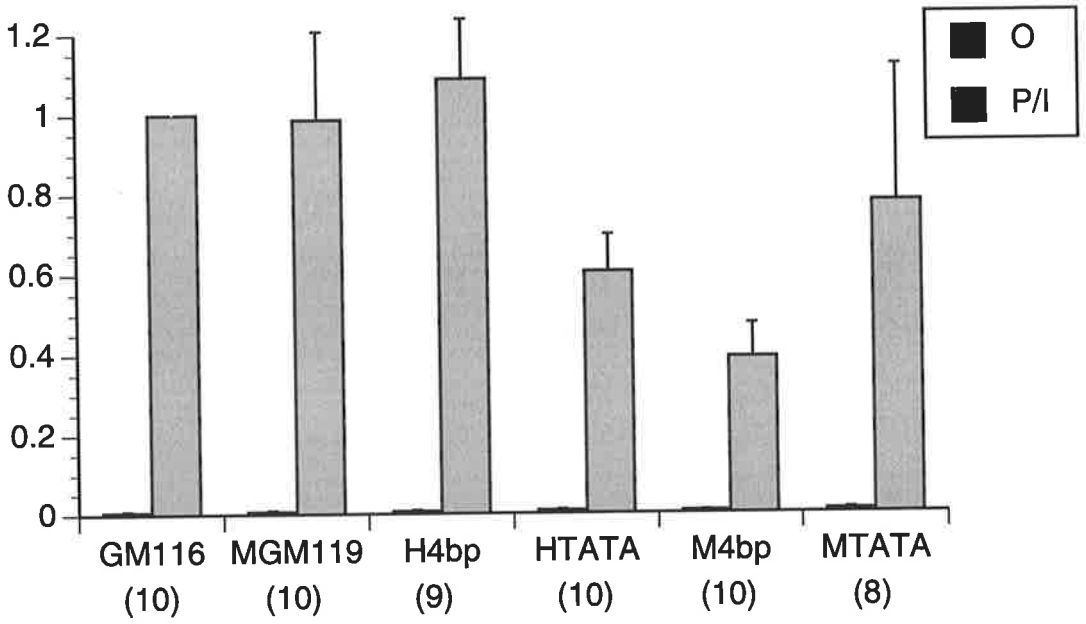

2.5
2
1.5
1
0.5
0
O
P/I
H116
(11)
M116
(11)
HSP1
(11)
MSP1
(11)
HCAT
(8)
MCAT
(8)

approximately half a twist. This may affect protein-protein interactions between the transcription initiation complex and the activator and repressors of the promoter. A mouse GM-CSF gene promoter construct was made that contained a mutation which deleted the additional 4 bp (pM4bp). Conversely, a human GM-CSF gene promoter construct that contained the additional 4 bp was made (pH4bp). There is a one bp difference within the TATA boxes of the mouse and human GM-CSF gene promoters. As the mouse TATA box more closely matched the selected TBP consensus binding site (Wong and Bateman, 1994), it was felt that this may also contribute to the differences in inducible activities. Mouse and human GM-CSF gene promoter constructs were made that each contained the TATA box of the other species (pMTATA and pHTATA). When these constructs were assayed in transiently transfected cells, unexpected results were seen (Fig. 4.7). Again, no significant difference in inducible activity of the wild type promoters, pMGM119 and pGM116, was observed. pH4bp had a similar activity to pMGM119 and pGM116, whereas pHTATA was significantly less active. The pM4bp construct was less active than both wild type promoter construct. pMTATA was not significantly different to the wild type promoter constructs.

The reasons for the discrepancies between the induction differences of earlier and later experiments are unclear. DNA sequencing confirmed that the promoters within all constructs were all as expected. Multiple large scale DNA preparations were performed from separate isolates of each construct, but the activities of pGM116 and pMGM119 remained equivalent. A series of plasmid preps of pMGM119 were

Fig. 4.7. Relative transcriptional activity of human and mouse GM-CSF gene promoter constructs containing mutations in the TATA box, and region immediately upstream of the TATA box, in transient transfection assays in Jurkat T cells.

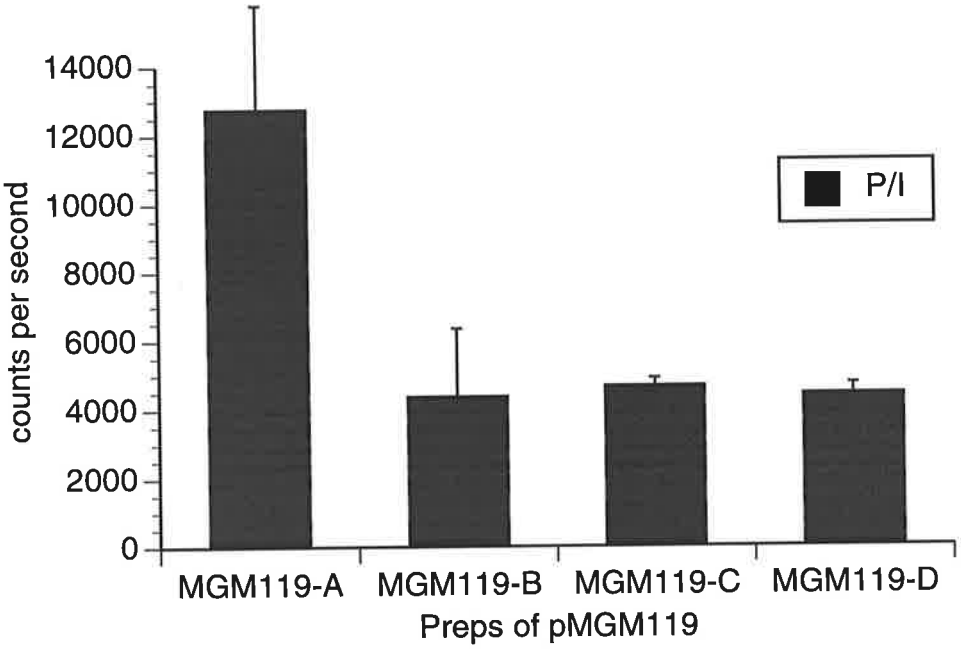
Jurkat T cells were transfected with 5ug of pGM116, pMGM119, pH4bp, pM4bp, pHTATA and pMTATA. After 24 hours, cells were either left unstimulated (O) or stimulated with PMA (20ng/ml) and calcium ionophore (2uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Luciferase activity is expressed relative to the level of activity in stimulated pGM116 samples, assigned a value of 1. Error bars represent the standard error and numbers in parentheses are the number of assays performed.


1.2
1
0.8
0.6
0.4
0.2
0
O
P/I
GM116
(10)
MGM119
(10)
H4bp
(9)
HTATA
(10)
M4bp
(10)
MTATA
(8)

compared, including the prep which was used to generate the data in figure 4.5. The plasmid prepared in the first large scale preparation was significantly more inducible than subsequent preps (fig. 4.8). Because of these difficulties with obtaining consistent data, transient transfection assays were not continued.

Fig. 4.8. Transcriptional activity of a panel of mouse 119 bp GM-CSF promoter constructs in transient transfection assays in Jurkat T cells.

Jurkat T cells were transfected with 5 ug of pMGM119 from different large scale preparations, A, B, C and D. After 24 hours, cells were stimulated with PMA (20 ng/ml) and calcium ionophore (2 uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Luciferase activity is expressed as raw counts per second. Error bars represent the standard deviation of two assays.


14000
12000
10000
8000
6000
4000
2000
0
counts per second
P/I
MGM119-A
MGM119-B
MGM119-C
MGM119-D
Preps of pMGM119

4.3. DISCUSSION

The work described in this chapter was initiated based on the observation that the activity obtained through the mouse GM-CSF promoter in activated cells was much greater than that of the human promoter. In fact, its level of induction was comparable to that which is seen when the human GM-CSF promoter is coupled to the human GM-CSF enhancer. This suggested that the mouse GM-CSF promoter evolved as a more active promoter than its human counterpart to compensate for the presence of a weaker enhancer in the mouse locus.

Studies carried out to observe the functional significance of differences between the mouse and human GM-CSF promoter were troublesome. The differences in inducible activities that were seen in early experiments became insignificant in subsequent experiments. Part of the problem lies with the degree of variability obtained with the luciferase assays. Experiments must be repeated many times in order to get statistically relevant data. This is generally not a problem when one is comparing constructs with large differences in activities. When one is comparing constructs with similar activities, however, it is difficult to obtain meaningful data. For example, in this study, the construct pHM116 was made to determine whether sequence differences upstream and/or downstream of the *BstEII* site were responsible for the different inducibilities. While the activity of pHM116 was in between those of pGM116 and pMGM119, suggesting that there may be elements contributing to the differences on both sides of the *BstEII* site, this finding was not statistically significant,

despite a reasonable number of samples being tested. If there were actually multiple sequence differences contributing to the different inducible activities, each may be expected to contribute only a minor portion of the difference. These individual differences may be too subtle to detect with this assay system.

The difficulties encountered with the drop in activities of constructs are puzzling. The initial preparations of plasmids gave significant differences in the activities of pGM116 and pMGM119. All subsequent preparations, however, showed no difference in their induced activities. Multiple preparations from different isolates of the constructs did not account for the changes seen. Sequencing of the inserts of these constructs confirmed that there were not errors in this region. There may, however, be mutations outside this region that have arisen which could affect the activities of the pMGM119 construct and those derived from it. It could also be argued that the activity of the first pMGM119 preparation was artificial, and that the subsequent preparations represent the true activity of this construct. Regardless, it was decided that the differences being sought were too subtle for this assay system.

Binding affinities for Sp1 and TBP were found to be greater in the mouse GM-CSF gene promoter than in the human GM-CSF gene promoter. The differential binding affinity of both factors could possibly contribute to the differential inducible activities. However, without the functional data to support it, it is difficult to speculate as

to the degree of effect these binding differences would cause in inducible activity.

CHAPTER 5.

REGULATION OF THE HUMAN GM-CSF GENE LOCUS BY CORE-BINDING FACTOR

5.1. INTRODUCTION

Sequence analyses led to the identification of conserved putative binding sites for the transcription factor Core Binding Factor (CBF) in the proximal promoters and enhancers of both the human and mouse GM-CSF genes. CBF has been shown to play an important role in the regulation of many haemopoietic specific genes such as T cell receptor β, γ, δ chains , IL-3, and M-CSF receptor (Prosser, et al., 1992; Hsiang et al., 1993; Redondo et al., 1992; Cameron et al., 1994; Zhang et al., 1994). A comparison of the the putative human and mouse CBF binding sites within the GM-CSF promoter and enhancer shows a high degree of homology with the known CBF binding sites of other genes (Fig. 5.1). These putative CBF binding sites are located in the minimal promoter and the enhancer core, in close proximity to other transcription factor binding sites. CBF has been shown to functionally co-operate with other transcription factors such as c-Myb and Ets-1 in similar configurations (Herndandez-Munain et al., 1994; Wotton et al., 1994)(Fig. 5.2). We therefore postulated that it plays a role in the regulation of the GM-CSF gene. CBF is expressed in many cell types in which GM-CSF is expressed (Nucifora and Rowley, 1995). Additionally, Acute Myeloid Leukaemia patients are sometimes found to have abnormal GM-CSF expression (Young et al., 1988; Rogers et al., 1994; Freedman et al., 1993).

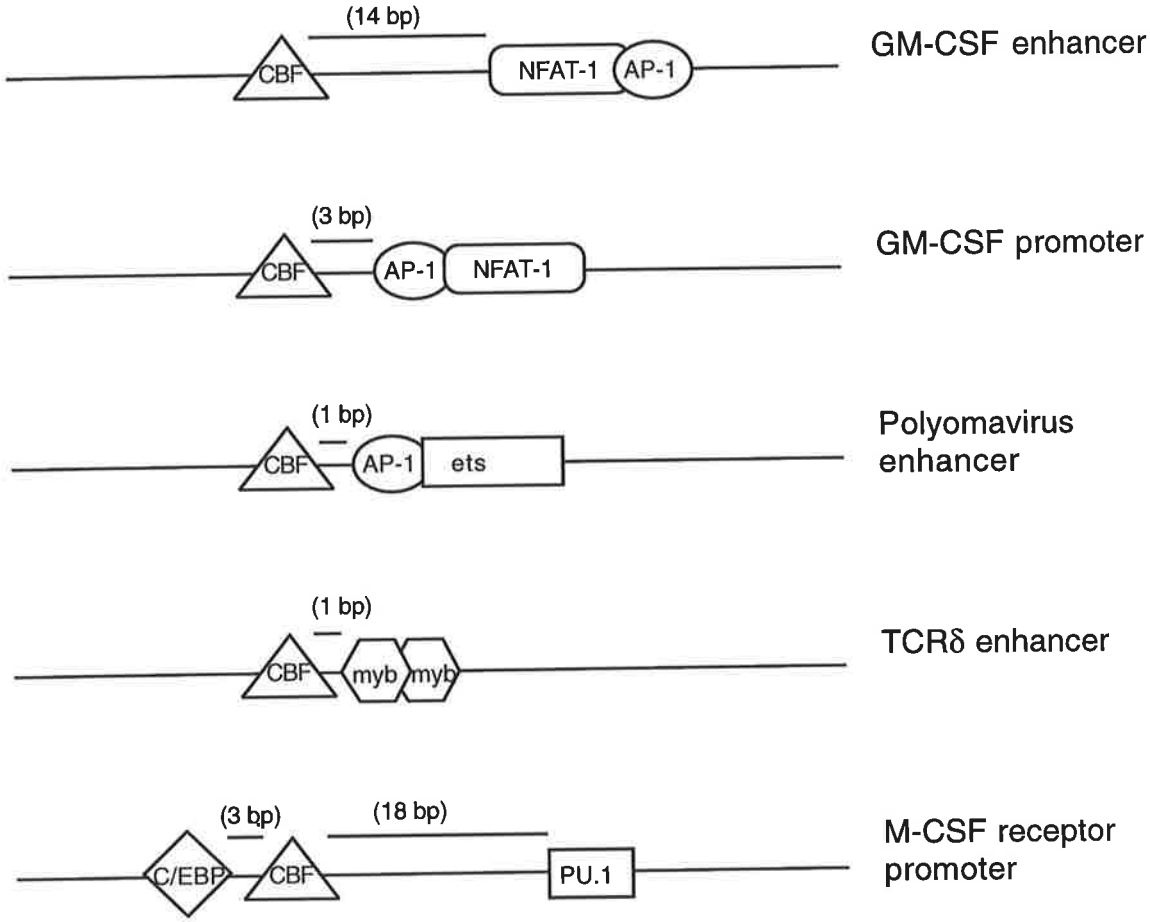
The aim of the work described in this chapter was to determine whether the CBF site within the human GM-CSF promoter played a significant role in the expression of the GM-CSF gene, and whether the

Fig. 5.1. Comparison of Core Binding Factor binding sites.

Regulatory Element	Sequence	Reference
Polyomavirus enhancer	TGTGGTTT	Kamachi et al., 1990
Soule murine leukaemia virus enhancer	TGTGGTCA	Golemis et al., 1990
Molony murine leukemia virus enhancer	TGTGGTAA	Wang and Speck, 1992
M-CSF receptor promoter	TGTGGTTG	Zhang et al., 1994
T-cell receptor γ enhancer	TGTGGTTT	Hsiang et al., 1993
T-cell receptor δ enhancer	TGTGGTTT	Redondo et al., 1991
Interleukin-3 promoter	TGTGGTTT	Cameron et al., 1994
human GM-CSF promoter	TGTGGTCA	
mouse GM-CSF promoter	TGTGGTCA	
human GM-CSF enhancer	TGTGGGCA	
mouse GM-CSF enhancer	TGTGGGTA	
consensus binding site as defined by oligo site selection	PyTPyGGTPy	Melnikova et al., 1993

Fig. 5.2. Comparison of transcription factor binding sites associated with CBF binding sites.

Numbers in parentheses represent base pairs separating the CBF binding site and adjacent transcription factor binding sites.


(14 bp)
CBF
NFAT-1
AP-1
GM-CSF enhancer
(3 bp)
CBF
AP-1
NFAT-1
GM-CSF promoter
(1 bp)
CBF
AP-1
ets
Polyomavirus enhancer
(1 bp)
CBF
myb
myb
TCRδ enhancer
(3 bp)
(18 bp)
C/EBP
CBF
PU.1
M-CSF receptor promoter

enhancer CBF site could influence transcriptional induction when directly linked to the human GM-CSF proximal promoter. Work performed concurrently in the laboratory by Mr. Andrew Bert and Ms. Rina Grotto showed that a DNA binding protein from a nuclear extract prepared from Jurkat T cells could bind to the GM-CSF promoter and enhancer CBF sites, and in both cases, be competed by a known CBF binding sequence, and supershifted by both CBFα and CBFß specific antibodies (Cockerill et al., 1996). Furthermore, CBF binding to the mouse and human GM-CSF promoter has recently been confirmed by other groups (Takahashi et al., 1995; Frank et al., 1995.).

In order to test the functional importance of these potential CBF sites, I investigated their contribution to the function of the human GM-CSF promoter and enhancer in transient transfection assays.

5.2. RESULTS

5.2.1. DELETION ANALYSIS OF THE HUMAN GM-CSF PROMOTER

To determine the effect of serially deleting the promoter past the CBF site, a series of truncated human GM-CSF promoters in a luciferase vector were designed (Fig. 5.3).

When transiently transfected into Jurkat T cells, a plasmid construct containing 627 bp of the proximal promoter of the human GM-CSF gene (the full length promoter) gave a 20-fold induction of luciferase when cells were stimulated with PMA and Ca^{2+} ionophore (Fig. 5.4). When a construct containing the CBF site, but truncated just before the Sp1 site, was assayed (pGM69) it was found to have approximately one third the inducible activity of the full length promoter. Note that this construct has a higher constitutive expression of luciferase suggesting that elements negatively regulating uninduced expression have been deleted. A construct that was truncated within the CBF site (pGM55) dropped its inducible activity to approximately 15% of that of the full length promoter, or half that of pGM69, suggesting the loss of a positive regulatory element. A construct that was further truncated to delete the YY-1 binding site, yet retain the AP-1 binding site (pGM47), showed no significant differences to the pGM55 construct.

Fig. 5.3. Schematic representation of luciferase reporter constructs used for detection of CBF binding site function

Human GM-CSF promoter fragments were cloned into the multiple cloning sites upstream of the luciferase gene. The full length promoter construct, pGM627, contains sequence from -627 bp to +28 bp. Other constructs are 5' deletions of pGM627. Details of cloning are described in chapter 2, section 2.9. The white shapes represent transcription factor binding sites within the human GM-CSF promoter. The cross through the CBF binding site indicates a specific mutation within the CBF site. The thickened line and black CBF box represent human GM-CSF enhancer sequence, and the parentheses indicate an array of three copies.

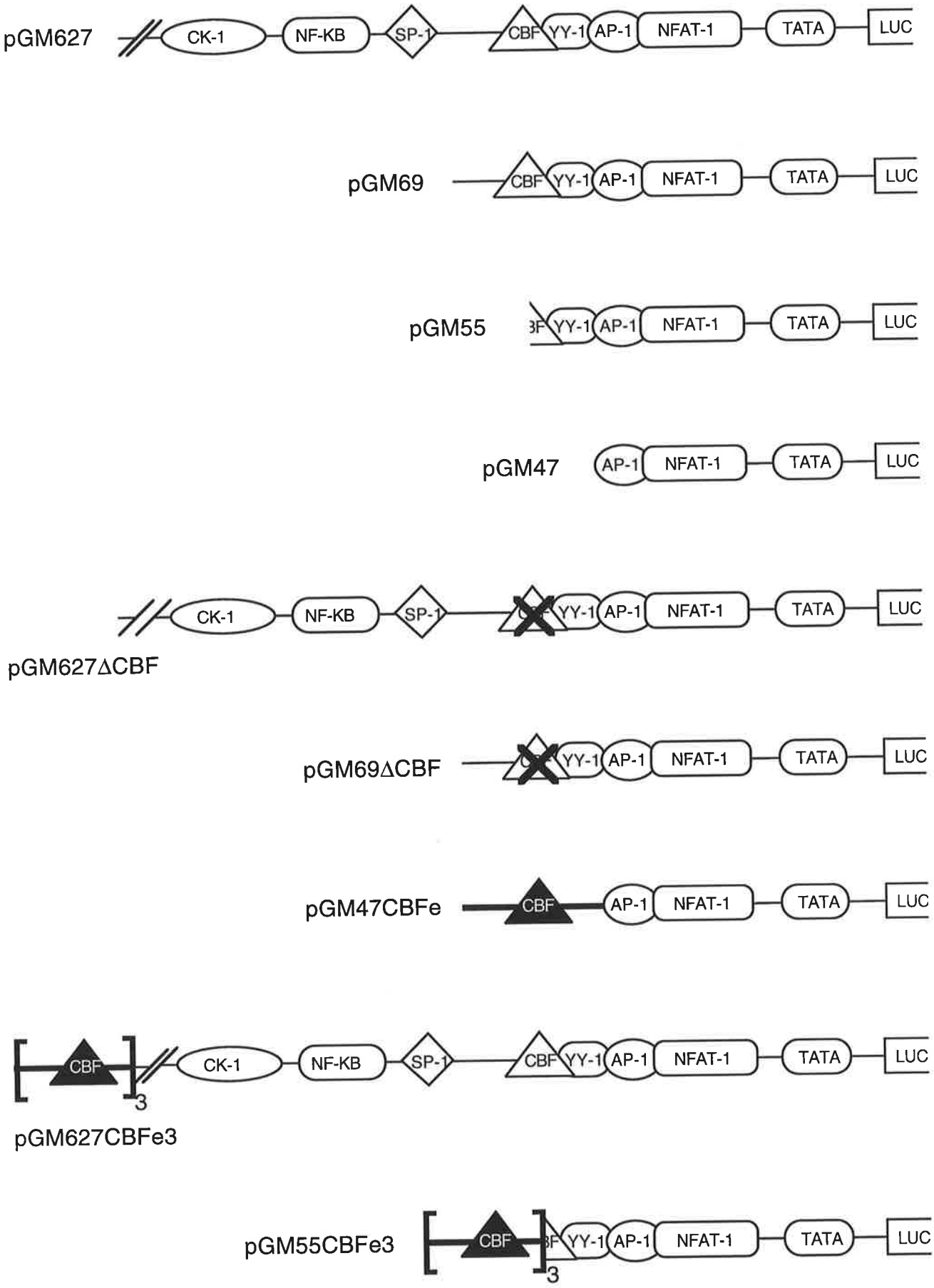
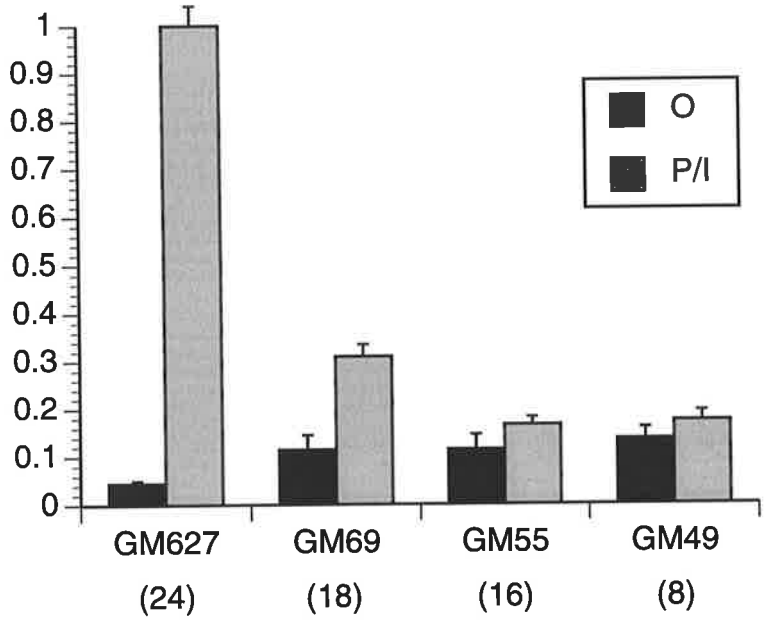

pGM627
CK-1
NF-KB
SP-1
CBF
YY-1
AP-1
NFAT-1
TATA
LUC
pGM69
pGM55
pGM47
pGM627ΔCBF
pGM69ΔCBF
pGM47CBFe
pGM627CBFe3
3
pGM55CBFe3

Fig. 5.4. Relative transcriptional activity of serially deleted human GM-CSF promoter constructs in Jurkat T-cells.

Jurkat T cells were transfected with 5ug of pGM627, pGM69, pGM55 and pGM47. After 24 hours, cells were either left unstimulated (O) or stimulated with PMA (20ng/ml) and calcium ionophore (2uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Error bars represent the standard error and numbers in parentheses are the number of assays performed.


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O
P/I
GM627
(24)
GM69
(18)
GM55
(16)
GM49
(8)

5.2.2. FUNCTIONAL ANALYSIS OF THE PROMOTER CBF SITE

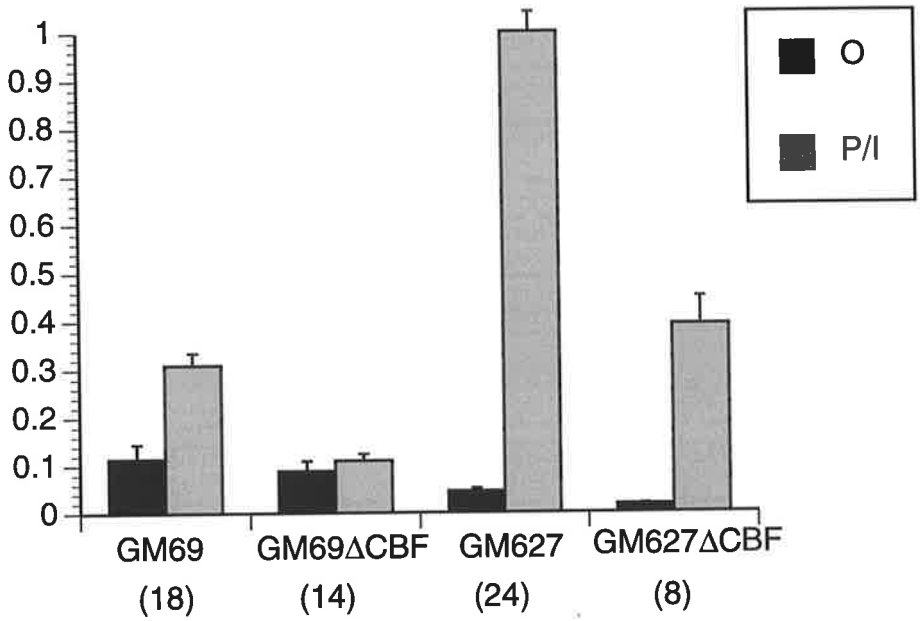
To further determine the functional role of the GM-CSF promoter CBF site, wild type promoter constructs were compared to those which contain a 2 bp mutation that inhibits CBF binding (Fig. 5.3), in transiently transfected Jurkat T cells. When compared to the wild type pGM69 construct, the pGM69ΔCBF construct, which contains the specific mutation within the CBF binding site (Fig. 5.3), gave only a third of the inducible activity (Fig. 5.5), suggesting that this binding site is crucial for the induction of this truncated promoter. Likewise, the construct pGM627ΔCBF, containing the same specific mutation as pGM69ΔCBF in the full length GM-CSF promoter (Fig. 5.3), was able to be induced only from a third to half of the level that was seen for the wild type promoter, pGM627 (Fig. 5.5). This also shows that the CBF site plays an important role in the regulation of the full length promoter. However, factors binding upstream of the CBF site were able to partially compensate for its loss.

5.2.3. FUNCTIONAL ANALYSIS OF THE ENHANCER CBF SITE

To determine whether the enhancer CBF site can function to increase transcription, the enhancer CBF site was linked to the pGM47 proximal promoter construct so as to locate it in a similar position as the promoter CBF site, in a construct resembling pGM69 (pGM47CBFe, Fig. 5.3). When assayed in transiently transfected Jurkat T cells, this construct gave approximately a two-fold increase in inducible activity

Fig. 5.5. Relative transcriptional activity of human GM-CSF promoter constructs containing mutations in the CBF binding site.

Jurkat T-cells were transfected with 5ug of pGM69, pGM69ΔCBF, pGM627 and pGM627ΔCBF. After 24 hours, cells were either left unstimulated (O) or stimulated with PMA (20ng/ml) and calcium ionophore (2uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Luciferase activity is expressed relative to the level of activity in stimulated pGM627 samples assigned a value of 1. Error bars represent the standard error and numbers in parentheses are the number of assays performed.


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
O
P/I
GM69
(18)
GM69ΔCBF
(14)
GM627
(24)
GM627ΔCBF
(8)

over that of pGM47 (Fig. 5.6). This level of inducible activity was comparable to that which is seen for pGM69. This result suggested that the GM-CSF enhancer CBF binding site does indeed function as a transcriptional activator.

5.2.4. THE EFFECTS OF A CBF MULTIMER LINKED TO THE GM-CSF PROMOTER

To test the enhancer CBF site for its abilty to function as a distal enhancer of transcription of the human GM-CSF promoter, an array of three enhancer CBF sites was inserted upstream of either the full length 627 bp (pGM627CBF3e) promoter or the truncated 55 bp (pGM55CBF3e) promoter in a luciferase reporter gene plasmid (Fig. 5.3). When tested in transiently transfected Jurkat T cells, both CBF array constructs gave a lower activity in both stimulated and unstimulated cells than those of their wild type counterparts (Fig. 5.7). This result suggests that the enhancer CBF array may not function as a transcriptional activator, and indeed may act as a negative regulator of transcription.

Fig. 5.6. Relative transcriptional activity of human GM-CSF promoter constructs linked to a human enhancer CBF binding site.

Jurkat T-cells were transfected with 5ug of pGM47, pGM47CBFe and pGM69. After 24 hours, cells were either left unstimulated (O) or stimulated with PMA (20ng/ml) and calcium ionophore (2uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Luciferase activity is expressed relative to the level of activity in stimulated pGM627 samples assigned a value of 1 (data not shown). Error bars represent the standard error and numbers in parentheses are the number of assays performed.

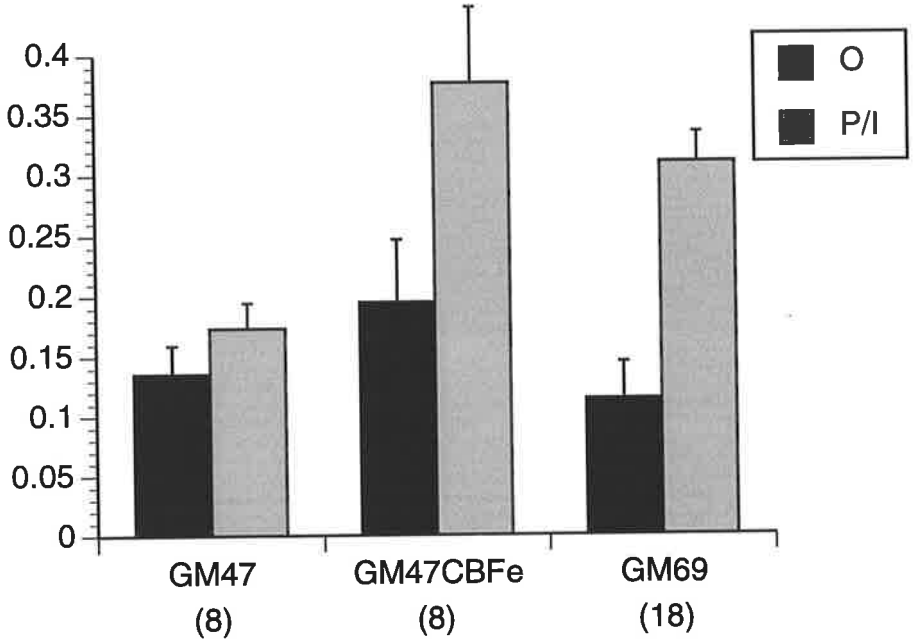
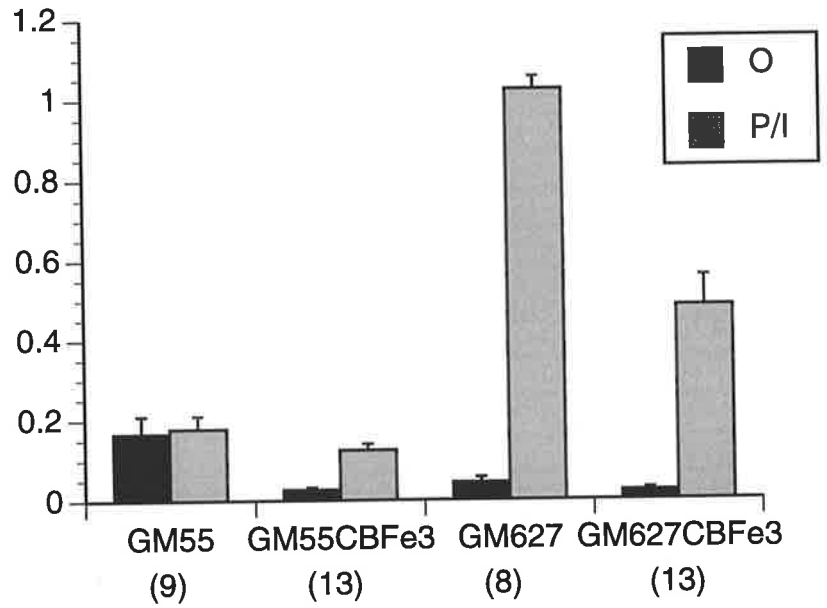

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
O
P/I
GM47
(8)
GM47CBFe
(8)
GM69
(18)

Fig. 5.7. Relative transcriptional activity of human GM-CSF promoter constructs linked to an array of three enhancer CBF binding sites.

Jurkat T-cells were transfected with 5ug of pGM55, pGM55CBFe3, pGM627 and pGM627CBFe3 After 24 hours, cells were either left unstimulated (O) or stimulated with PMA (20ng/ml) and calcium ionophore (2uM) (P/I), then harvested nine hours later and assayed for luciferase activity using 20ug cytoplasmic extract. Luciferase activity is expressed relative to the level of activity in stimulated pGM627 samples assigned a value of 1. Error bars represent the standard error and numbers in parentheses are the number of assays performed.


1.2
1
0.8
0.6
0.4
0.2
0
O
P/I
GM55
(9)
GM55CBFe3
(13)
GM627
(8)
GM627CBFe3
(13)

5.3. DISCUSSION

The work described in this chapter details the functional importance of CBF in the regulation of GM-CSF. It adds GM-CSF to a growing list of genes involved in haemopoiesis and differentiation that are regulated by CBF.

Serially deleted human GM-CSF promoter constructs were made to more finely study the CBF site in the promoter. A 3-fold decrease in inducible activity was observed when the promoter was truncated to -69 bp from the full length promoter (pGM627luc), presumably due to the loss of the Sp1, NF-κB and CD28RE sites that sit immediately upstream of -69 bp. The next deletion, pGM55luc, truncated the CBF site and decreased activity by apprximately 7.5-fold from that of the full length promoter. This construct has been reported to contain a large constitutive activity (Nimer et al. 1990), yet in our hands, we observe a very low activity compared to the 627 bp promoter. The next truncation deleted the promoter to 47 bp, which removed a reported YY1 binding site (Ye et al. 1994). Although this site has been reported to have negative regulatory properties on GM-CSF transcription, no change in activity was seen by us upon its deletion.

Binding studies performed in this laboratory (Cockerill et al., 1996) revealed that the 2 bp mutation incorporated in pGM69ΔCBF was sufficient to eliminate CBF binding to the GM-CSF promoter sequence. This 2 bp mutation dropped inducible activity by 3-fold in the 69 bp construct in transient transfection assays. A comparable result was

obtained by Frank et al. (1995) where they completely obliterated the CBF site with a 5 bp mutation. It is interesting that the pGM69ΔCBF mutation drops the inducible activity below that of pGM55 and pGM47. A possible reason for this could be due to a reported negative regulatory transcription factor binding to the upstream CATT box (termed CATT-3) (Nimer et al. 1990); in the absence of CBF binding to the pGM69ΔCBF construct, the CATT-3 element may exert a more noticable inhibitory effect. To determine this, one could add a mutation to this CATT-3 region in the pGM69ΔCBF construct to see if an alleviation of low activity is observed. Alternatively, one could make a murine pGM69ΔCBF which lacks this CATT-3 box. An additional reason for a very low inducible activity of pGM69ΔCBF could be that the YY-1 site, with the absence of CBF binding, may be able to bind to its factor. The CBF and YY-1 binding sites overlap by about 2 bp. The inhibition of CBF binding may allow YY-1 binding which may normally be outcompeted by a stronger binding affinity of CBF. It is unclear however why the pGM55 construct, which lacks a complete CBF binding site yet retains a YY-1 bindings, does not show a reduced inducible activity. This may be due to YY-1 requiring some specific flanking sequence for its binding, or perhaps YY-1 is not involved in GM-CSF regulation in our Jurkat cells. The comparison of the full length promoter, pGM627 with the pGM627ΔCBF plasmid also showed that there was a three-fold decrease in inducible activity. A decrease of such magnitude suggests that CBF plays a crucial role in the inducible activity of the GM-CSF gene.

To investigate the role of the CBF binding site within the enhancer, the CBF binding site was linked to a pGM47 promoter construct. This was designed to place the enhancer in a position comparable to that of the promoter CBF site, thereby giving the enhancer CBF site the opportunity to interact within the promoter environment in the same manner as the promoter CBF site. When linked to the pGM47 construct, the enhancer CBF site led to approximately a two-fold increase in inducible activity, compared to a minimal increase of induction seen in the pGM47 construct. The stimulated level of induction was comparable to that which is seen for the pGM69 construct in stimulated cells. Of note, the overall activity of the pGM47CBFe construct is higher than those of pGM47 and pGM69. This cannot be explained by CBF binding affinities, as the promoter CBF binding site appears to be stronger than that of the enhancer (Cockerill et al., 1996). The higher activity of pGM47CBFe may be due to flanking sequences present in the enhancer CBF insert (ie. upstream of the enhancer CBF site), which may bind positive regulatory proteins. Alternatively, the absence of a YY-1 binding site may alleviate some general basic repression. It is also possible that the absence of the CATT-3 box may also prevent some basic inhibition. A more definitive test of the enhancer CBF site function would be to observe it in its natural context. To this aim, a mutation of the enhancer CBF site within a construct containing the enhancer linked to the promoter would need to be compared to a natural enhancer/promoter construct.

Unexpectedly, an array of three CBF sites linked to the full length GM-CSF construct actually decreased activity. It is possible that CBF needs to function in a specific context with other transcription factors. This might explain a lack of activity, but perhaps not a decrease in activity. A plausible explanation correlates with the suggestion that CBF is a transcription factor that can interact with the nuclear matrix; CBF binding sites within a promoter may localise the promoter into regions of active tarnscription (Merriman et al. 1995; Meyers et al. 1995; G. Cockerill and P. Cockerill, unpublished data). Multiple copies of CBF sites that are distal to the promoter may bind very strongly to the nuclear matrix, and due to their distance from the other elements in the promoter, they may localise the promoter elements aways from sites of active transcription. Another explanation is that the array of CBF sites may bind so strongly to the nuclear matrix that it hinders the transcription of the gene.

The environmental context in which CBF is located is likely to be important in GM-CSF regulation. Indeed, a recent report has demonstrated that the CBF site within the human IL-3 promoter is dependent on its position relative to other transcription factor binding sites to affect IL-3 transcription (Taylor et al,. 1996). As has been found for many regulatory elements containing CBF sites, the GM-CSF CBF sites are closely linked with other key transcription factor binding sites. Because both the enhancer and promoter CBF sites are situated in the functional cores of their respective elements adjacent to AP-1/NFATp/c binding sites, it is feasible that this array of transcription factors can act as a functional unit. Upon T cell activation, NFATp/c is

quickly translocated into the nucleus (Flanagan et al., 1991). Data from our laboratory has demonstrated that an array of three NFATp/c binding sites is sufficient to form an inducible DH site when stably integrated in T cells (P. Cockerill, unpublished data). This suggests that NFATp/c has the capacity to disrupt chromatin structure. AP-1 is rapidly transcribed in activated T cells. It is able to directly interact with TFIID to stabilise its formation (Metz et al. 1994a). One could envision a scenario where upon T cell activation, NFATp/c disrupts the chromatin structure, thereby presenting binding sites to CBF and AP-1, which may localise the gene into a region of active transcription, and stabilise the RNAPII initiation complex, respectively.

It is unclear what role CBF plays in the regulation of GM-CSF gene expression in AML patients. One would predict that the dominant negative effects possessed by most AML-1 and CBFß fusion proteins would downregulate the expression of GM-CSF. This would be consistent with a subset AML's that avoid programmed differentiation by suppressing the expression of differentiation causing genes, such as GM-CSF or other cytokines and their receptors (Bassan et al., 1994; Shabo et al., 1990).

A proportion of AML's possess a constitutive expression of GM-CSF (Young et al., 1988; Rogers et al., 1994; Freedman et al., 1993). This possibly represents a form of AML's that don't involve AML-1, but do affect another factor involved in myeloid differentiation. However, it is possible that whilst the fusion of AML-1 to another protein inhibits the action of CBF , the fusion counterpart, now under the

transcriptional control of the *AML-1* promoter, may act on other genes, which may affect GM-CSF expression indirectly. An example of an AML-1 fusion protein that retains the activity of the fused partner is the result of the t(3;21) translocation fusing *AML-1* to *Evi-1* (Kurokawa et al., 1995). The aberrant expression of Evi-1 in the form of this fusion protein has been shown to increase levels of c-Fos and c-Jun, thereby increasing cellular AP-1 levels (Tanaka et al. 1995, Kurokawa et al. 1995). Artificially high AP-1 levels have been associated with cellular transformation, and GM-CSF expression has been associated with elevated AP-1 levels in some myeloid leukaemic cell lines (Shabo et al., 1990). Other factors may be induced by the AML1/Evi-1 fusion protein and other AML1 fusion proteins that also indirectly activate GM-CSF expression.

CHAPTER 6.

CONCLUDING DISCUSSION

The studies described in this thesis were commenced to isolate and characterise regulatory elements within the mouse GM-CSF gene locus. Primary studies focussed on the isolation of a homologue to the human GM-CSF gene enhancer. Identification of such a homologue would help further define the function of the human GM-CSF gene enhancer, and could lead to the establishment of a model system to determine if the GM-CSF gene expression is dependent on the enhancer; enhancer knockouts could be made to determine the importance of the enhancer *in vivo*, and GM-CSF regulatory region-driven transgenes could elucidate the temporal and spatial dependencies of the GM-CSF gene on its enhancer.

While a transcriptional enhancer homologous to the human GM-CSF gene enhancer was found in the mouse GM-CSF gene locus, it was shown to function differently than the human GM-CSF enhancer. In fact, in transient transfection assays, the mouse and human GM-CSF genes appear to have reciprocal promoter and enhancer functions; the mouse GM-CSF gene has a strongly inducible promoter, with little or no contribution from its enhancer, while the human GM-CSF gene has a weaker promoter that is several times more active in the presence of the upstream enhancer. The different regulatory mechanisms of the mouse and human GM-CSF gene appear to be characteristics of the mouse and human GM-CSF loci, and not the source of T cells; the differences are seen in both human Jurkat and mouse EL4 T cell lines. The outcome, however, is that the combination of the mouse promoter and enhancer is induced to a comparable level to that of the human, suggesting that while the contribution of individual elements may

vary, the overall induction is equivalent. This illustrates that the mouse GM-CSF gene locus may not be a suitable model for defining the function of regulatory elements in the human GM-CSF gene locus. Although there is much conservation of the GM-CSF/IL-3 locus with regards to overall gene structure and regulatory mechanisms, there are some significant differences, and one must be cautious with assumptions that are made.

Although the mouse GM-CSF enhancer is not required for strong inducible activity through the mouse GM-CSF promoter in transient transfection assays, there exist a remarkable degree of conservation between the human and mouse GM-CSF enhancers. As there is no apparent need for the mouse GM-CSF enhancer in transient transfection assays, there may be other, perhaps more important roles for both the human and mouse GM-CSF enhancers. As the mouse and human enhancer can both increase induction through the IL-3 promoter, it might participate in its activation in T cells. Alternatively, another role of the GM-CSF gene enhancer may be to increase GM-CSF expression in another cell type, another developmental stage, or in response to signalling pathways other than those invoked in T cell stimulation; GM-CSF is expressed by a wide variety of cell types in response to varying signals. This putative role of the GM-CSF enhancer, however, may be separable from that of a classical transcriptional enhancer. It may, for instance, participate in governing gene regulation by organising changes in the chromatin structure of the locus, akin to a locus control region. Such a role may not be detectable in a transient transfection assay system, as LCRs

often need to be stably integrated into chromatin to exert an effect. The GM-CSF enhancer may require interactions with other regulatory elements to exert an effect, thus larger fragments of the GM-CSF/IL-3 locus may need to be studied. One approach that has been used to investigate the regulation of the ß-globin gene locus has been to insert large fragments of the locus, up to 230 kb, into a yeast artificial chromosome (YAC) (Gaensler et al., 1990). These YACs can be transferred into cell lines, or microinjected into mouse germlines to produce transgenic animals. Efficient homologous recombination in yeast allows for simple introduction of mutations to the regulatory elements. This would provide a system in which to study the effects of regulatory elements in the context of an intact locus.

The identification of functional core-binding factor binding sites within the GM-CSF gene promoter and enhancer has added to a greater understanding of the mechanisms involved in GM-CSF transcriptional control. Evidence that suggests CBF associates with the nuclear matrix would imply that GM-CSF transcriptional regulation is also associated with the nuclear matrix, and perhaps localises the GM-CSF gene into transcriptionally active regions of the nucleus. Other genes that contain CBF binding sites within their regulatory regions include genes which are involved in cell differentiation and in haemopoiesis. These genes are often transiently expressed. Rapid, transient expression of these genes may require nuclear matrix associating transcription factors such as CBF to quickly localise the gene into sites of active transcription.

A clearer description of the events that occur after T cell receptor signalling, resulting in the transcription of GM-CSF and other cytokine genes, is emerging. The T cell receptor signalling cascade is believed to culminate in the binding of NFAT to sites within the GM-CSF gene enhancer and promoter. Additionally, combinations of T cell receptor activation and costimulation of the CD28 cell surface molecule likely results in the binding of NF-κB related transcription factors to sites within the GM-CSF gene promoter. These transcription factors, in conjunction with other transcription factors such as CBF, Sp1, Ets family members, and GATA family members may interact to allow properly regulated transcription of the GM-CSF gene. Some of these transcription factor binding motifs are conserved in many cytokine regulatory elements, suggesting that there are common themes to the activating signals of these cytokines, and that in some instances, they may co-ordinately expressed.

BIBLIOGRAPHY

André-Schwartz, J. and Schwartz, R.S. (1995). Structure and function of the immune system. In Hematology: basic principles and practise. R. Hoffman, E.J. Benz, S.J. Shattil, B. Furie, H.J. Cohen, and L.E. Silberstein, eds. (New York: Church Livingstone), pp. 86-108.

Armesilla, A.L., Calvo, D., and Vega, M.A. (1996). Structural and functional characterization of the human CD36 gene promoter: identification of a proximal PEBP2/CBF site. J. Biol. Chem. *271*, 7781-7787.

Atchison, M.L. (1988). Enhancers: mechanisms of action and cell specificity. Annu. Rev. Cell Biol. *4*, 127-153.

Babgy, G.C. and Segal, G.M. (1995). Growth factors and the control of hematopoiesis. In Hematology: basic principles and practise. R. Hoffman, E.J. Benz, S.J. Shattil, B. Furie, H.J. Cohen, and L.E. Silberstein, eds. (New York: Church Livingstone), pp. 207-241.

Bae, S.C., Yamaguchi-Iwai, Y., Ogawa, E., Maruyama, M., Inuzuka, M., Kagoshima, H., Shigesada, K., Satake, M., and Ito, Y. (1993). Isolation of PEBP2 alpha B cDNA representing the mouse homolog of human acute myeloid leukemia gene, AML1. Oncogene. *8*, 809-814.

Bae, S.C., Ogawa, E., Maruyama, M., Oka, H., Satake, M., Shigesada, K., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., and Ito, Y. (1994). PEBP2 alpha B/mouse AML1 consists of multiple isoforms that possess differential transactivation potentials. Mol. Cell Biol. *14*, 3242-3252.

Banerjee, C., Hiebert, S.W., Stein, J.L., Lian, J.B., and Stein, G.S. (1996). An AML-1 consensus sequence binds an osteoblast-specific complex and transcriptionally activates the osteocalcin gene. Proc. Natl. Acad. Sci. USA *93*, 4968-4973.

Bassan, R., Rambaldi, A., Amaru, R., Motta, T., and Barbui, T. (1994). Unexpected remission of acute myeloid leukemia after GM-CSF. Br. J. Haematol. *87*, 835-838.

Becker, P.B. (1994). The establishment of active promoters in chromatin. Bioessays *16*, 541-547.

Berek, C. (1992). Humoral immunity. In Encyclopedia of Immunology. I.M. Roitt, ed. (San Diego: Academic Press), pp. 700-702.

Berezney, R. and Coffey, D.S. (1974). Identification of a nuclear protein matrix. Biochem. Biophys. Res. Commun. *60*, 1410-1417.

Bidwell, J.P., van Wijnen, A.J., Fey, E.G., Dworetzky, S., Penman, S., Stein, J.L., Lian, J.B., and Stein, G.S. (1993). Osteocalcin gene promoter-binding factors are tissue-specific nuclear matrix components. Proc. Natl. Acad. Sci. U. S. A. *90*, 3162-3166.

Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., and Tjian, R. (1987). Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science *238*, 1386-1392.

Cameron, S., Taylor, D.S., TePas, E.C., Speck, N.A., and Mathey-Prevot, B. (1994). Identification of a critical regulatory site in the human interleukin-3 promoter by *in vivo* footprinting. Blood *83*, 2851-2859.

Caracciolo, D., Clark, S.C., and Rovera, G. (1989). Human interleukin-6 supports granulocytic differentiation of hematopoietic progenitor cells and acts synergistically with GM-CSF. Blood. *73*, 666-670.

Chan, J.Y., Slamon, D.J., Nimer, S.D., Golde, D.W., and Gasson, J.C. (1986). Regulation of expression of human granulocyte/macrophage colony- stimulating factor. Proc. Natl. Acad. Sci. U. S. A. *83*, 8669-8673.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell *54*, 541-552.

Clark, S.C. and Kamen, R. (1987). The human hematopoietic colony-stimulating factors. Science *236*, 1229-1237.

Cockerill, G.W., Bert, A.G., Ryan, G.R., Gamble, J.R., Vadas, M.A., and Cockerill, P.N. (1995a). Regulation of granulocyte-macrophage colony-stimulating factor and E- selectin expression in endothelial cells by cyclosporin A and the T- cell transcription factor NFAT. Blood. *86*, 2689-2698.

Cockerill, P.N. and Garrard, W.T. (1986). Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell *44*, 273-282.

Cockerill, P.N., Shannon, M.F., Bert, A.G., Ryan, G.R., and Vadas, M.A. (1993). The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible, cyclosporin A-sensitive enhancer. Proc. Natl. Acad. Sci. USA *90*, 2466-2470.

Cockerill, P.N., Bert, A.G., Jenkins, F., Ryan, G.R., Shannon, M.F., and Vadas, M.A. (1995b). Human granulocyte-macrophage colony-stimulating factor enhancer function is associated with cooperative interactions between AP-1 and NFATp/c. Mol. Cell. Biol. *15*, 2071-2079.

Cockerill, P.N., Osborne, C.S., Bert, A.G., and Grotto, R.J.M. (1996). Regulation of GM-CSF gene transcription by core-binding factor. Cell Growth Diff. *7*, 917-922.

Coles, L.S., Occhiodoro, F., Vadas, M.A., and Shannon, M.F. (1994). A sequence-specific single-strand DNA binding protein that contacts repressor sequences in the human GM-CSF promoter. Nucleic Acids Res. *22*, 4276-4283.

Coles, L.S., Diamond, P., Occhiodoro, F., Vadas, M.A., and Shannon, M.F. (1996). Cold shock domain proteins repress transcription from the GM-CSF promoter. Nucleic Acid Res. *24*, 2311-2317.

Cook, P.R. (1995). A chromomeric model for nuclear and chromosome structure. J. Cell Sci. *108*, 2927-2935.

Daga, A., Karlovich, C.A., Dumstrei, K., and Banerjee, U. (1996). Patterning of cells in the *Drosophila* eye by Lozenge, which shares homologous domains with AML1. Genes Dev. *10*, 1194-1205.

Davie, J.R. (1995). The nuclear matrix and the regulation of chromatin organization and function. Int. Rev. Cytol. *162A*, 191-250.

Davies, K., TePas, E.C., Nathan, D.G., and Mathey-Prevot, B. (1993). Interleukin-3 gene expression by activated T cells involves an

inducible T-cell-specific factor and an octamer binding protein. Blood *81*, 928-934.

Dillon, N. and Grosveld, F. (1993). Transcriptional regulation of multigene loci: multilevel control. Trends. Genet. *9*, 134-137.

Duffy, J.B. and Gergen, J.P. (1991a). The Drosophila segmentation gene runt acts as a position-specific numerator element necessary for the uniform expression of the sex- determining gene Sex-lethal. Genes. Dev. *5*, 2176-2187.

Duffy, J.B., Kania, M.A., and Gergen, J.P. (1991b). Expression and function of the Drosophila gene runt in early stages of neural development. Development. *113*, 1223-1230.

Dunn, S.M., Coles, L.S., Lang, R.K., Gerondakis, S., Vadas, M.A., and Shannon, M.F. (1994). Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. Blood. *83*, 2469-2479.

Dworetzky, S.I., Wright, K.L., Fey, E.G., Penman, S., Lian, J.B., Stein, J.L., and Stein, G.S. (1992). Sequence-specific DNA-binding proteins are components of a nuclear matrix-attachment site. Proc. Natl. Acad. Sci. U. S. A. *89*, 4178-4182.

Elgin, S.C. (1988). The formation and function of DNase I hypersensitive sites in the process of gene activation. J. Biol. Chem. *263*, 19259-19262.

Engeland, K., Andrews, N.C., and Mathey-Prevot, B. (1995). Multiple proteins interact with the nuclear inhibitory protein repressor element in the human interleukin-3 promoter. J. Biol. Chem. *270*, 24572-24579.

Erickson, P., Gao, J., Chang, K.S., Look, T., Whisenant, E., Raimondi, S., Lasher, R., Trujillo, J., Rowley, J., and Drabkin, H. (1992). Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood. *80*, 1825-1831.

Felsenfeld, G. (1992). Chromatin as an essential part of the transcriptional mechanism. Nature *355*, 219-224.

Fey, E.G. and Penman, S. (1988). Nuclear matrix proteins reflect cell type of origin in cultured human cells. Proc. Natl. Acad. Sci. U. S. A. *85*, 121-125.

Fitch, F.W. (1992). Cell-mediated immunity. In Encyclopedia of Immunology. I.M. Roitt, ed. (San Diego: Academic Press), pp. 315-318.

Flanagan, W.M., Corthesy, B., Bram, R.J., and Crabtree, G.R. (1991). Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A [see comments]. Nature *352*, 803-807.

Fondell, J.D., Brunel, F., Hisatake, K., and Roeder, R.G. (1996). Unliganded thyroid hormone receptor alpha can target TATA-binding protein for transcriptional repression. Mol. Cell Biol. *16*, 281-287.

Forrester, W.C., Epner, E., Driscoll, M.C., Enver, T., Brice, M., Papayannopoulou, T., and Groudine, M. (1990). A deletion of the human beta-globin locus activation region causes a major alteration in chromatin structure and replication across the entire beta-globin locus. Genes. Dev. *4*, 1637-1649.

Frank, R., Zhang, J., Uchida, H., Meyers, S., Hiebert, S.W., and Nimer, S.D. (1995). The AML1/ETO fusion protein blocks transactivation of the GM-CSF promoter by AML1B. Oncogene. *11*, 2667-2674.

Franza, B.R.,Jr., Rauscher, F.J., Josephs, S.F., and Curran, T. (1988). The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 binding sites. Science *239*, 1150-1153.

Fraser, J.D. and Weiss, A. (1992). Regulation of T-cell lymphokine gene transcription by the accessory molecule CD28. Mol. Cell. Biol. *12*, 4357-4363.

Fraser, J.K., Guerra, J.J., Nguyen, C.Y., Indes, J.E., Gasson, J.C., and Nimer, S.D. (1994). Characterization of a cell-type-restricted negative regulatory activity of the human granulocyte-macrophage colony-stimulating factor gene. Mol. Cell Biol. *14*, 2213-2221.

Freedman, M.H., Grunberger, T., Correa, P., Axelrad, A.A., Dube, I.D., and Cohen, A. (1993). Autocrine and paracrine growth control by granulocyte-monocyte colony-stimulating factor of acute lymphoblastic leukemia cells. Blood *81*, 3068-3075.

Furukawa, K., Yamaguchi, Y., Ogawa, E., Shigesada, K., Satake, M., and Ito, Y. (1990). A ubiquitous repressor interacting with an F9 cell-specific silencer and its functional suppression by differentiated cell-specific positive factors. Cell Growth Diff. *1*, 135-147.

Gaensler, K.M., Burmeister, M., Brownstein, B.H., Taillon-Miller, P., and Myers, R.M. (1991). Physical mapping of yeast artificial chromosomes containing sequences from the human beta-globin gene region. Genomics. *10*, 976-984.

Gasson, J.C., Weisbart, R.H., Kaufman, S.E., Clark, S.C., Hewick, R.M., Wong, G.G., and Golde, D.W. (1984). Purified human granulocyte-macrophage colony-stimulating factor: direct action on neutrophils. Science *226*, 1339-1342.

Gergen, J.P. and Wieschaus, E.F. (1985). The localized requirements for a gene affecting segmentation in Drosophila: analysis of larvae mosaic for runt. Dev. Biol. *109*, 321-335.

Ghosh, P., Tan, T.-H., Rice, N.R., Sica, A., and Young, H.A. (1993). THe interleukin-2 CD28-responsive complex contains at least three members of the NF-κB family: c-Rel, p50, and p65. Proc. Natl. Acad. Sci. U. S. A. *90*, 1696-1700.

Giese, K., Kingsley, C., Kirshner, J.R., and Grosschedl, R. (1995). Assembly and function of a TCR alpha enhancer complex is dependent on LEF-1-induced DNA bending and multiple protein-protein interactions. Genes. Dev. *9*, 995-1008.

Goldfeld, A.E., McCaffrey, P.G., Strominger, J.L., and Rao, A. (1993). Identification of a novel cyclosporin-sensitive element in the human tumor necrosis α gene promoter. J. Exp. Med. *178*, 1365-1379.

Golemis, E.A., Speck, N.A., and Hopkins, N. (1990). Alignment of U3 region sequences of mammalian type C viruses: identification of

highly conserved motifs and implications for enhancer design. J Virol. *64*, 534-542.

Golling, G., Li, L., Pepling, M., Stebbins, M., and Gergen, J.P. (1996). Drosophila homologs of the proto-oncogene product PEBP2/CBF beta regulate the DNA-binding properties of Runt. Mol. Cell. Biol. *16*, 932-942.

Golub, T.R., Barker, G.F., Bohlander, S.K., Hiebert, S.W., Ward, D.C., Bray-Ward, P., Morgan, E., Raimondi, S.C., Rowley, J.D., and Gilliland, D.G. (1995). Fusion of the TEL gene on 12p13 to the AML1 gene on 21q22 in acute lymphoblastic leukemia. Proc. Natl. Acad. Sci. U. S. A. *9295*, 4917-4921.

Gottschalk, L.R., Giannola, D.M., and Emerson, S.G. (1993). Molecular regulation of the human IL-3 gene: inducible T cell restricted expression requires intact AP-1 and Elf-1 nuclear protein binding sites. J. Exp. Med. *178*, 1681-1692.

Gough, N.M. and Nicola, N.A. (1990). Granulocyte-macrophage colony-stimulating factor. Immunology Series *9*, 111-153.

Gross, D.L. and Garrard, W.T. (1988). Nuclease hypersensitive sites in chromatin. Ann. Rev. Biochem. *57*, 159-197.

Grosveld, F., Blom van Assendelft, G., Greaves, D., and Kollias, G. (1987). Position-independent, high-level expression of the human β-globin gene in transgenic mice. Cell *51*, 975-985.

Hahn, S. (1992). The ying and the yang of mammalian transcription. Current Biology *2*, 152-154.

Hajra, A., Liu, P.P., Wang, Q., Kelley, C.A., Stacy, T., Adelstein, R.S., Speck, N.A., and Collins, F.S. (1995). The leukemic core binding factor beta-smooth muscle myosin heavy chain (CBF beta-SMMHC) chimeric protein requires both CBF beta and myosin heavy chain domains for transformation of NIH 3T3 cells. Proc. Natl. Acad. Sci. U. S. A. *92*, 1926-1930.

Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E., and Leder, P. (1988). c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. Cell *55*, 917-924.

Hernandez-Munain, C. and Krangel, M.S. (1994). Regulation of the T-cell receptor delta enhancer by functional cooperation between c-Myb and core-binding factors. Mol. Cell Biol. *14*, 473-483.

Hernandez-Munain, C. and Krangel, M.S. (1995). c-Myb and core-binding factor/PEBP2 display functional synergy but bind independently to adjacent sites in the T-cell receptor delta enhancer. Mol. Cell Biol. *1555*, 3090-3099.

Hiebert, S.W., Sun, W., Davis, J.D., Golub, T.R., Shurtleff, S., Buijs, A., Downing, J.R., Grosveld, G., Roussel, M.F., Gilliland, D.G., Lenny, N., and Meyers, S. (1996). The t(12;21) translocation converts AML-1B from an activator to a repressor of transcription. Mol. Cell. Biol. *16*, 1349-1355.

Himes, S.R., Coles, L.S., Katsikeros, R., Lang, R.K., and Shannon, M.F. (1993). HTLV-1 tax activation of the GM-CSF and G-CSF promoters requires the interaction of NF-κB with other transcription facotr families. Oncogene *8*, 3189-3197.

Himes, S.R., Katsikeros, R., and Shannon, M.F. (1996). Costimulation of cytokine gene expression in T cells by the human T leukemia/lymphotropic virus type 1 trans activator Tax. J. Virol. *70*, 4001-4008.

Hodge, M.R., Ranger, A.M., Charles de la Brousse, F., Hoey, T., Grusby, M.J., and Glimcher, L.H. (1996). Hyperproliferation and dysregulation of IL-4 expression in NF-ATp- deficient mice. Immunity. *4*, 397-405.

Hodgkin, P.D., Bond, M.W., O'Garra, A., Frank, G., Lee, F., Coffman, R.L., Zlotnik, A., and Howard, M. (1988). Identification of IL-6 as a T cell-derived factor that enhances the proliferative response of thymocytes to IL-4 and phorbol myristate acetate. J. Immunol. *141*, 151-157.

Hoey, T., Weinzierl, R.O., Gill, G., Chen, J.L., Dynlacht, B.D., and Tjian, R. (1993). Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell *72*, 247-260.

Hoey, T., Sun, Y.L., Williamson, K., and Xu, X. (1995). Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. Immunity. *2*, 461-472.

Hogquist, K.A., Nett, M.A., Unanue, E.R., and Chaplin, D.D. (1991). Interleukin 1 is processed and released during apoptosis. Proc. Natl. Acad. Sci. U. S. A. *88*, 8485-8489.

Hsiang, Y.H., Spencer, D., Wang, S., Speck, N.A., and Raulet, D.H. (1993). The role of viral enhancer "core" motif-related sequences in regulating T cell receptor-gamma and -delta gene expression. J Immunol *150*, 3905-3916.

Hsiang, Y.H., Goldman, J.P., and Raulet, D.H. (1995). The role of c-myb or a related factor in regulating the T cell receptor γ gene enhancer. J. Immunol. *154*, 5195-5204.

Ikebuchi, K., Ihle, J.N., Hirai, Y., Wong, G.G., Clark, S.C., and Ogawa, M. (1988). Synergistic factors for stem cell proliferation: further studies of the target stem cells and the mechanism of stimulation by interleukin-1, interleukin-6, and granulocyte colony-stimulating factor. Blood. *72*, 2007-2014.

Jackson, D.A. and Cook, P.R. (1985). Transcription occurs at a nucleoskeleton. EMBO J. *4*, 919-925.

Jackson, D.A., Dickinson, P., and Cook, P.R. (1990). The size of chromatin loops in HeLa cells. EMBO J. *9*, 567-571.

Jackson, D.A., Hassan, A.B., Errington, R.J., and Cook, P.R. (1993). Visualization of focal sites of transcription within human nuclei. EMBO J. *12*, 1059-1065.

Jackson, D.A. and Cook, P.R. (1995). The structural basis of nuclear function. International Review of Cytology *162A*, 125-149.

Jain, J., McCaffrey, P.G., Valge-Archer, V.E., and Rao, A. (1992a). Nuclear factor of activated T cells contains Fos and Jun. Nature *356*, 801804.

Jain, J., Valge-Archer, V.E., Sinskey, A.J., and Rao, A. (1992b). The AP-1 site at -150 bp, but not the NF-kappa B site, is likely to

represent the major target of protein kinase C in the interleukin 2 promoter. J. Exp. Med. *175*, 853-862.

Jain, J., McCaffrey, P.G., Miner, Z., Kerppola, T.K., Lambert, J.N., Verdine, G.L., Curran, T., and Rao, A. (1993). The T-cell trancription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature *365*, 352-355.

Jenkins, F., Cockerill, P.N., Bohmann, D., and Shannon, M.F. (1995). Multiple signals are required for function of the human granulocyte-macrophage colony-stimulating factor gene promoter in T cells. J. Immunol. *155*, 1240-1251.

Johnson, R.S., Speigelman, B.M., and Papaioannou, V. (1992). Pleiotropic effects of a null mutation in the c-fos proto-oncogene. Cell *71*, 577-586.

Kadonaga, J.T., Jones, K.A., and Tjian, R. (1986). Promoter-specific activation of RNA polymerase II transcription by Sp1. Trends Biochem. *11*, 20-23.

Kamachi, Y., Ogawa, E., Asano, M., Ishida, S., Murakami, Y., Satake, M., Ito, Y., and Shigesada, K. (1990). Purification of a mouse nuclear factor that binds to both the A and B cores of the polyomavirus enhancer. J Virol. *64*, 4808-4819.

Kaushansky, K. (1989). Control of granulocyte-macrophage colony-stimulating factor production in normal endothelial cells by positive and negative regulatory elements. J. Immunol. *143*, 2525-2529.

Kelso, A., Metcalf, D., and Gough, N.M. (1986). Independent regulation of granulocyte-macrophage colony-stimulating factor and multi-lineage colony-stimulating factor production in T lymphocyte clones. J. Immunol. *136*, 1718-1725.

Kelso, A. and Gough, N.M. (1989). Differential inhibition by cyclosporin A reveals two pathways for lymphokine synthesis in T cells. Growth Factors *1*, 165-177.

Koyano-Nakagawa, N., Nishida, J., Arai, N., Arai, K., and Yokota, T. (1993). Reconstitution of the functional granulocyte macrophage colony stimulating factor promoter: evidence for distinct activation

mechanisms that mediate the response to phorbol ester/calcium and human T cell leukemia virus type I Tax signals. Int. Immunol. *5*, 345-352.

Koyano-Nakagawa, N., Nishida, J., Baldwin, D., Arai, K., and Yokota, T. (1994). Molecular cloning of a novel human cDNA encoding a zinc finger protein that binds to the interleukin-3 promoter. Mol. Cell Biol. *14*, 5099-5107.

Kryszke, M.H., Piette, J., and Yaniv, M. (1987). Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. Nature *328*, 254-256.

Kubo, M., Kincaid, R.L., Webb, D.R., and Ransom, J.T. (1994). The Ca2+/calmodulin-activated, phosphoprotein phosphatase calcineurin is sufficient for positive transcriptional regulation of the mouse IL-4 gene. Int. Immunol. *6*, 179-188.

Kunz, J. and Hall, M.N. (1993). Cyclosporin A, FK506 and rapamycin: more than just immunosuppression. Trends. Biochem. Sci. *18*, 334-338.

Kurokawa, M., Ogawa, S., Tanaka, T., Mitani, K., Yazaki, Y., Witte, O.N., and Hirai, H. (1995). The AML1/Evi-1 fusion protein in the t(3;21) translocation exhibits transforming activity on Rat1 fibroblasts with dependence on the *Evi-1* sequence. . Oncogene *11*, 833-840.

Kurokawa, M., Tanaka, T., Tanaka, K., Ogawa, S., Mitani, K., Yazaki, Y., and Hirai, H. (1996). Overexpression of the AML1 proto-oncoprotein in NIH3T3 cells leads to neoplastic transformation depending on the DNA-binding and transactivational potencies. Oncogene. *12*, 883-892.

Lagnado, C.A., Brown, C.Y., and Goodall, G.J. (1994). AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). Mol. Cell Biol. *14*, 7984-7995.

Lee, J.S. and Young, I.G. (1989). Fine-structure mapping of the murine IL-3 and GM-CSF genes by pulse-field gel electrophoresis and molecular cloning. Genomics *5*, 359-362.

Lee, W., Mitchell, P., and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell *49*, 741-752.

Lenny, N., Meyers, S., and Hiebert, S.W. (1995). Functional domains of the t(8;21) fusion protein, AML-1/ETO. Oncogene. *11*, 1761-1769.

Levanon, D., Negreanu, V., Bernstein, Y., Bar-Am, I., Avivi, L., and Groner, Y. (1994). AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. Genomics. *23*, 425-432.

Levy-Wilson, B. and Fortier, C. (1989). The limits of the DNase I-sensitive domain of the human apolipoprotein B gene coincide with the locations of chromosomal anchorage loops and define the 5' and 3' boundaries of the gene. J. Biol. Chem. *264*, 21196-21204.

Liu, J., Farmer, J.D.,Jr., Lane, W.S., Friedman, J., Weissman, I., and Schreiber, S.L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP- FK506 complexes. Cell *66*, 807-815.

Liu, P., Tarle, S.A., Hajra, A., Claxton, D.F., Marlton, P., Freedman, M., Siciliano, M.J., and Collins, F.S. (1993). Fusion between transcription factor CBFβ/PEBP2β and a myosin heavy chain in acute myeloid leukemia. Science *261*, 1041-1044.

Liu, P., Seidel, N., Bodine, D., Speck, N., Tarle, S., and Collins, F.S. (1994). Acute myeloid leukemia with Inv (16) produces a chimeric transcription factor with a myosin heavy chain tail. Cold. Spring. Harb. Symp. Quant. Biol. *59*, 547-553.

Loh, C., Carew, J.A., Kim, J., Hogan, P.G., and Rao, A. (1996). T-cell receptor stimulation elicits an early phase of activation and a later phase of deactivation of the transcription factor NFAT1. Mol. Cell. Biol. *16*, 3945-3954.

Lopez, A.F., Williamson, D.J., Gamble, J.R., Begley, C.G., Harlan, J.M., Klebanoff, S.J., Waltersdorph, A., Wong, G., Clark, S.C., and Vadas, M.A. (1986). Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and

eosinophil function, surface receptor expression, and survival. J. Clin. Invest. *78*, 1220-1228.

Lopez, A.F., Dyson, P.G., To, L.B., Elliott, M.J., Milton, S.E., Russell, J.A., Juttner, C.A., Yang, Y.C., Clark, S.C., and Vadas, M.A. (1988). Recombinant human interleukin-3 stimulation of hematopoiesis in humans: loss of responsiveness with differentiation in the neutrophilic myeloid series. Blood. *72*, 1797-1804.

Lotem, J. and Sachs, L. (1988). In vivo control of differentiation of myeloid leukemic cells by recombinant granulocyte-macrophage colony-stimulating factor and interleukin 3. Blood. *71*, 375-382.

Lu, J., Maruyama, M., Satake, M., Bae, S.C., Ogawa, E., Kagoshima, H., Shigesada, K., and Ito, Y. (1995). Subcellular localization of the alpha and beta subunits of the acute myeloid leukemia-linked transcription factor PEBP2/CBF. Mol. Cell Biol. *15*, 1651-1661.

Luo, C., Burgeon, E., Carew, J.A., McCaffrey, P.G., Badalian, T.M., Lane, W.S., Hogan, P.G., and Rao, A. (1996). Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. Mol. Cell. Biol. *16*, 3955-3966.

Masuda, E.S., Naito, Y.T., Arai, K., and Arai, N. (1993a). Expression of lymphokine genes in T cells. Immunologist *1*, 198-203.

Masuda, E.S., Tokumitsu, H., Tsuboi, A., Shlomai, J., Arai, K., and Arai, N. (1993b). The granulocyte-macrophage colony-stimulating factor promoter *cis*-acting element CLE0 mediates induction signals in T cells and is recognized by factors related to AP1 and NFAT. Mol. Cell. Biol. *13*, 7399-7407.

Masuda, E.S., Yamaguchi-Iwai, Y., Tsuboi, A., Hung, P., Arai, K., and Arai, N. (1994). The transcription factor Sp1 is required for induction of the murine GM-CSF promoter in T cells. Biochem. Biophys. Res. Commun. *205*, 1518-1525.

Masuda, E.S., Naito, Y., Tokumitsu, H., Campbell, D., Saito, F., Hannum, C., Arai, K., and Arai, N. (1995). NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. Mol. Cell Biol. *15*, 2697-2706.

Mathey-Prevot, B., Andrews, N.C., Murphy, H.S., Kreissman, S.G., and Nathan, D.G. (1990). Positive and negative elements regulate human interleukin-3 expression. Proc. Natl. Acad. Sci. USA *87*, 5046-5050.

McCaffrey, P.G., Luo, C., Kerppola, T.K., Jain, J., Badalian, T.M., Ho, A.M., Burgeon, E., Lane, W.S., Lambert, J.N., Curran, T., Verdine, G.L., Rao, A., and Hogan, P.G. (1993a). Isolation of the cyclosporin-sensitive T cell transcription factor NFATp. Science *262*, 750-754.

McCaffrey, P.G., Perrino, B.A., Soderling, T.R., and Rao, A. (1993b). NFATp, a T lymphocyte DNA binding protein that is a target for calcineurin and immunosuppressive drugs. J. Biol. Chem. *268*, 3747-3752.

McCaffrey, P.G., Goldfeld, A.E., and Rao, A. (1994). The role of NFATp in cyclosporin A-sensitive tumor necrosis factor-α gene transcription. J. Biol. Chem. *269*, 30445-30450.

Melnikova, I.N., Crute, B.E., Wang, S., and Speck, N.A. (1993). Sequence specificity of the core-binding factor. J Virol. *67*, 2408-2411.

Merriman, H.L., van Wijnen, A.J., Hiebert, S., Bidwell, J.P., Fey, E., Lian, J., Stein, J., and Stein, G.S. (1995). The tissue-specific nuclear matrix protein, NMP-2, is a member of the AML/CBF/PEBP2/runt domain transcription factor family: interactions with the osteocalcin gene promoter. Biochemistry *34*, 13125-13132.

Metcalf, D., Begley, C.G., Johnson, G.R., Nicola, N.A., Vadas, M.A., Lopez, A.F., Williamson, D.J., Wong, G.G., Clark, S.C., and Wang, E.A. (1986). Biologic properties in vitro of a recombinant human granulocyte- macrophage colony-stimulating factor. Blood. *67*, 37-45.

Metz, R., Bannister, A.J., Sutherland, J.A., Hagemeier, C., O'Rourke, E.C., Cook, A., Bravo, R., and Kouzarides, T. (1994a). c-Fos-induced activation of a TATA-box-containing promoter involves direct contact with TATA-box-binding protein. Mol. Cell Biol. *14*, 6021-6029.

Metz, R., Kouzarides, T., and Bravo, R. (1994b). A C-terminal domain in FosB, absent in FosB/SF and Fra-1, which is able to interact with

the TATA binding protein, is required for altered cell growth. EMBO J. *13*, 3832-3842.

Meyers, S., Lenny, N., and Hiebert, S.W. (1995). The t(8:21) fusion protein interferes with AML-1B-dependent transcriptional activation. Mol. Cell. Biol. *15*, 1974-1982.

Mitani, K., Ogawa, S., Tanaka, T., Miyoshi, H., Kurokawa, M., Mano, H., Yazaki, Y., Ohki, M., and Hirai, H. (1994). Generation of the AML1-EVI-1 fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. EMBO J *13*, 504-510.

Miyatake, S., Otsuka, T., Yokota, T., Lee, F., and Arai, K. (1985). Structure of the chromosomal gene for granulocyte-macrophage colony-stimulating factor: comparison of the mouse and human genes. EMBO J. *10*, 2561-2568.

Miyatake, S., Seiki, M., Malefijt, R.D., Heike, T., Fujisawa, J., Takebe, Y., Nishida, J., Shlomai, J., Yokota, T., Yoshida, M., Arai, K., and Arai, N. (1988a). Activation of T cell-derived lymphokine genes in T cells and fibroblasts: effects of human T cell leukemia virus type I $p40^{x}$ protein and bovine papilloma virus encoded E2 protein. Nucleic Acid Res. *16*, 6547-6566.

Miyatake, S., Seiki, M., Yoshida, M., and Arai, K. (1988b). T-cell activation signals and human T-cell leukemia virus type I-encoded $p40^{x}$ protein activate the mouse granulocyte-macrophage colony-stimulating factor gene through a common DNA element. Mol. Cell. Biol. *8*, 5581-5587.

Miyatake, S., Shlomai, J., Arai, K., and Arai, N. (1991). Characterization of the mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) promoter: nuclear factors that interact with an element shared by three lymphokine genes-those for GM-CSF, interleukin-3 and interleukin-5. Mol. Cell. Biol. *11*, 5894-5901.

Miyoshi, H., Shimizu, K., Kozu, T., Maseki, N., Kaneko, Y., and Ohki, M. (1991). t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc. Natl. Acad. Sci. U. S. A. *88*, 10431-10434.

Miyoshi, H., Kozu, T., Shimizu, K., Enomoto, K., Maseki, N., Kaneko, Y., Kamada, N., and Ohki, M. (1993). The t(8;21) translocation in acute myeloid leukemia results in production of an AML1-MTG8 fusion transcript. EMBO J *12*, 2715-2721.

Miyoshi, H., Ohira, M., Shimizu, K., Mitani, K., Hirai, H., Imai, T., Yokoyama, K., Soeda, E., and Ohki, M. (1995). Alternative splicing and genomic structure of the AML1 gene involved in acute myeloid leukemia. Nucleic Acid Res. *23*, 2762-2769.

Morris, C.F., Young, I.G., and Hapel, A.J. (1990). Molecular and cellular biology of interleukin-3. In Colony Stimulating Factors: Molecular and Cellular Biology. T.M. Dexter, J.M. Garland, and N.G. Testa, eds. (New York: Marcel Dekker), pp. 177-214.

Morrissey, P.J., Grabstein, K.H., Reed, S.G., and Conlon, P.J. (1989). Granulocyte/macrophage colony stimulating factor. A potent activation signal for mature macrophages and monocytes. Int. Arch. Allergy Appl. Immunol. *88*, 40-46.

Nakabeppu, Y., Ryder, K., and Nathans, D. (1988). DNA binding activities of three murine Jun proteins: stimulation by Fos. Cell *55*, 907-915.

Nathan, C.F. (1989). Respiratory burst in adherent human neutrophils: triggering by colony- stimulating factors CSF-GM and CSF-G. Blood. *73*, 301-306.

Nickerson, J.A., Blencowe, B.J., and Penman, S. (1995). The architectural organisation of nuclear metabolism. International Review of Cytology *162A*, 67-123.

Nicola, N.A., Burgess, A.W., and Metcalf, D. (1979a). Similar molecular properties of granulocyte-macrophage colony-stimulating factors produced by different mouse organs in vitro and in vivo. J. Biol. Chem. *254*, 5290-5299.

Nicola, N.A., Metcalf, D., Johnson, G.R., and Burgess, A.W. (1979b). Separation of functionally distinct human granulocyte-macrophage colony- stimulating factors. Blood. *54*, 614-627.

Nicola, N.A. (1989). Hemopoietic cell growth factors and their receptors. Annu. Rev. Biochem. *58*, 45.

Niemeyer, C.M., Sieff, C.A., Mathey-Prevot, B., Wimperis, J.Z., Bierer, B.E., Clark, S.C., and Nathan, D.G. (1989). Expression of human interleukin-3 (multi-CSF) is restricted to human lymphocytes and T-cell tumor lines. Blood. *73*, 945-951.

Nimer, S.D., Morita, E.A., Martis, M.J., Wachsman, W., and Gasson, J.C. (1988). Characterisation of the human granulocyte-macrophage colony-stimulating factor promoter region by genetic analysis: correlation with DNaseI footprinting. Mol. Cell. Biol. *8*, 1979-1984.

Nimer, S.D., Fraser, J., Richards, J., Lynch, M., and Gasson, J. (1990). The repeated sequence CATT(A/T) is required for granulocyte-macrophage colony-stimulating factor promoter activity. Mol. Cell. Biol. *10*, 6084-6088.

Nimer, S.D., Zhang, W., Kwan, K., Wang, Y., and Zhang, J. (1996). Adjacent, cooperative elements form a strong, constitutive enhancer in the human granulocyte-macrophage colony-stimulating factor gene. Blood. *87*, 3694-3703.

Nishida, J., Yoshida, M., Arai, K., and Yokota, T. (1991). Definition of a GC-rich motif as regulatory sequence of the human IL-3 gene: coordinate regulation of the IL-3 gene by CLE2/GC box of the GM-CSF gene in T cell activation. Int. Immunol. *3*, 245-254.

Nordeen, S.K. (1988). Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques *6*, 454.

Northrop, J.P., Ullman, K.S., and Crabtree, G.R. (1993). Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NFAT) complex. J. Biol. Chem. *268*, 2917-2923.

Northrop, J.P., Ho, S.N., Chen, L., Thomas, D.J., Timmerman, L.A., Nolan, G.P., Admon, A., and Crabtree, G.R. (1994). NF-AT components define a family of transcription factors targeted in T-cell activation [see comments]. Nature *369*, 497-502.

Nuchprayoon, I., Meyers, S., Scott, L.M., Suzow, J., Hiebert, S., and Friedman, A.D. (1994). PEBP2/CBF, the murine homolog of the human myeloid AML1 and PEBP2 beta/CBF beta proto-oncoproteins, regulates the murine myeloperoxidase and neutrophil

elastase genes in immature myeloid cells. Mol. Cell Biol. *14*, 5558-5568.

Nucifora, G., Begy, C.R., Erickson, P., Drabkin, H.A., and Rowley, J.D. (1993). The 3;21 translocation in myelodysplasia results in a fusion transcript between the AML1 gene and the gene for EAP, a highly conserved protein associated with the Epstein-Barr virus small RNA EBER 1. Proc. Natl. Acad. Sci. U. S. A. *90*, 7784-7788.

Nucifora, G. and Rowley, J.D. (1995). AML1 and the 8;21 and 3;21 translocations in acute and chronic myeloid leukemia. Blood *86*, 1-14.

O'Keefe, S.J., Tamura, J., Kincaid, R.L., Tocci, M.J., and O'Neill, E.A. (1992). FK506- and CsA sensitive activation of the interleukin-2 promoter by calcineurin. Nature *357*, 692-694.

Ogawa, E., Inuzuka, M., Maruyama, M., Satake, M., Naito-Fujimoto, M., Ito, Y., and Shigesada, K. (1993a). Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. Virology. *194*, 314-331.

Ogawa, E., Maruyama, M., Kagoshima, H., Inuzuka, M., Lu, J., Satake, M., Shigesada, K., and Ito, Y. (1993b). PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. Proc. Natl. Acad. Sci. U. S. A. *90*, 6859-6863.

Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., and Downing, J.R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell *84*, 321-330.

Osborne, C.S., Vadas, M.A., and Cockerill, P.N. (1995). Transcriptional regulation of mouse granulocyte-macrophage colony-stimulating factor/IL-3 locus. J. Immunol. *155*, 226-235.

Park, J.H., Kaushansky, K., and Levitt, L. (1993). Trancriptional regulation of interleukin 3 in primary human T lymphocytes. J. Biol. Chem. *268*, 6299-6308.

Phi-Van, L. and Stratling, W.H. (1988). The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. EMBO J. *7*, 655-664.

Prosser, H.M., Wotton, D., Gegonne, A., Ghysdael, J., Wang, S., Speck, N.A., and Owen, M.J. (1992). A phorbol ester response element within the human T-cell receptor beta- chain enhancer. Proc. Natl. Acad. Sci. U. S. A. *89*, 9934-9938.

Ptashne, M. (1986). Gene regulation by proteins acting nearby and at a distance. Nature *322*, 697-701.

Redondo, J.M., Pfohl, J.L., Hernandez-Munain, C., Wang, S., Speck, N.A., and Krangel, M.S. (1992). Indistinguishable nuclear factor binding to functional core sites of the T-cell receptor delta and murine leukemia virus enhancers. Mol. Cell Biol. *12*, 4817-4823.

Richmond, T.J., Finch, J.T., Rushton, B., Rhodes, D., and Klug, A. (1984). Structure of the nucleosome core particle at 7 A resolution. Nature *311*, 532-537.

Riegel, J.S., Corthesy, B., Flanagan, W.M., and Crabtree, G.R. (1992). Regulation of the interleukin-2 gene. Chem. Immunol. *51*, 266-298.

Rogers, S.Y., Bradbury, D., Kozlowski, R., and Russell, N.H. (1994). Evidence for internal autocrine regulation of growth in acute myeloblastic leukemia cells. Exp. Hematol. *22*, 593-598.

Rooney, J.W., Sun, Y.L., Glimcher, L.H., and Hoey, T. (1995). Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. Mol. Cell Biol. *15*, 6299-6310.

Rowley, J.D. (1990). Recurring chromosome abnormalities in leukemia and lymphoma. Seminars in Hematology *27*, 122.

Russell, N.H. (1992). Autocrine growth factors and leukaemic haemopoiesis. Blood. Rev. *6*, 149-156.

Ryan, G.R., Milton, S.E., Lopez, A.F., Bardy, P.G., Vadas, M.A., and Shannon, M.F. (1991). Human interleukin-3 mRNA accumulation is controlled at both the transcriptional and posttranscriptional level. Blood. *77*, 1195-1202.

Ryan, G.R., Vadas, M.A., and Shannon, M.F. (1994). T-cell functional regions of the human IL-3 proximal promoter. Mol. Reprod. Dev. *39*, 200-207.

Sakakura, C., Yamaguchi-Iwai, Y., Satake, M., Bae, S.C., Takahashi, A., Ogawa, E., Hagiwara, A., Takahashi, T., Murakami, A., and Makino, K. (1994). Growth inhibition and induction of differentiation of t(8;21) acute myeloid leukemia cells by the DNA-binding domain of PEBP2 and the AML1/MTG8(ETO)-specific antisense oligonucleotide. Proc. Natl. Acad. Sci. U. S. A. *91*, 11723-11727.

Satake, M., Furukawa, K., and Ito, Y. (1988). Biological activities of oligonucleotides spanning the F9 point mutation within the enhancer region of polyomavirus DNA. J. Virol. *62*, 970-977.

Satake, M., Nomura, S., Yamaguchi-Iwai, Y., Takahama, Y., Hashimoto, Y., Niki, M., Kitamura, Y., and Ito, Y. (1995). Expression of the Runt domain-encoding PEBP2 alpha genes in T cells during thymic development. Mol. Cell Biol. *15*, 1662-1670.

Schreiber, S.L. and Crabtree, G.R. (1992). The mechanisms of action of cyclosporin A and FK506. Immunol. Today *13*, 136.

Schubert, L.A., King, G., Cron, R.Q., Lewis, D.B., Aruffo, A., and Hollenbaugh, D. (1995). The human gp39 promoter. Two distinct nuclear factors of activated T cell protein-binding elements contribute independently to transcriptional activation. J. Biol. Chem. *270*, 29624-29627.

Shabo, Y., Lotem, J., and Sachs, L. (1990). Induction of genes for transcription factors by normal hematopoietic regulatory proteins in the differentiation of myeloid leukemic cells. Leukemia *4*, 797-801.

Shannon, M.F., Coles, L.S., Fielke, R.K., Goodall, G.J., Lagnado, C.A., and Vadas, M.A. (1992). Three essential promoter elements mediate tumour necrosis factor and interleukin-1 activation of the granulocyte-colony stimulating factor gene. Growth. Factors. *7*, 181-193.

Shaw, G. and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell *46*, 659-667.

Shaw, J.P., Utz, P.J., Durand, D.B., Toole, J.J., Emmel, E.A., and Crabtree, G.R. (1988). Identification of a putative regulator of early T cell activation genes. Science *241*, 202-205.

Shaw, K.T.-Y., Ho, A.M., Raghavan, A., Kim, J., Jain, J., Park, J., Sharma, S., Rao, A., and Hogan, P.G. (1995). Immunosuppressive drugs prevent a rapid dephosphorylation of transcription factor NFAT1 in stimulated immune cells. Proc. Natl. Acad. Sci. U. S. A. *92*, 11205-11209.

Shibasaki, F., Price, E.R., Milan, D., and McKeon, F. (1996). Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature *382*, 370-373.

Shoemaker, S.G., Hromas, G.R., and Kaushansky, K. (1990). Transcriptional regulation of interleukin-3 gene expression in T lymphocytes. Proc. Natl. Acad. Sci. USA *87*, 9650-9654.

Speck, N.A. and Stacy, T. (1995). A new transcription factor family associated with human leukemias. Critical Reviews in Eukaryotic Gene Expression *5*, 337-364.

Stanley, E., Metcalf, D., Sobieszuk, P., Gough, N.M., and Dunn, A.R. (1985). The structure and expression of the murine gene encoding granulocyte-macrophage colony-stimulating factor: evidence for utilization of alternative promoters. EMBO J. *4*, 2569.

Staynov, D.Z., Cousins, D.J., and Lee, T.H. (1995). A regulatory element in the promoter of the human granulocyte- macrophage colony-stimulating factor gene that has related sequences in other T-cell-expressed cytokine genes. Proc. Natl. Acad. Sci. U. S. A. *92*, 3606-3610.

Stein, G.S., van Wijnen, A.J., Stein, J.L., Lian, J.B., Bidwell, J.P., and Montecino, M. (1994). Nuclear architecture supports integration of physiological regulatory signals for transcription of cell growth and tissue-specific genes during osteoblast differentiation. J. Cell Biochem. *55*, 4-15.

Stein, G.S., van Wijnen, A.J., Stein, J., Lian, J.B., and Montecino, M. (1995). Contributions of nuclear architecture to transcriptional control. Int. Rev. Cytol. *162A*, 251-278.

Szabo, S.J., Gold, J.S., Murphy, T.L., and Murphy, K.M (1993). Identification of cis acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NFATc. Mol. Cell. Biol. *13*, 4793-4805.

Tadmori, W., Feingersh, D., Clark, S.C., and Choi, Y.S. (1989). Human recombinant IL-3 stimulates B cell differentiation. J. Immunol. *142*, 1950-1955.

Takahashi, A., Satke, M., Yamaguchi-Iwai, Y., Bae, S.C., Lu, J., Maruyama, M., Zhang, Y.W., Oka, H., Arai, N., and Arai, K. (1995). Positive and negative regulation of granulocyte-macrophage colony-stimulating factor promoter activity by AML1-related transcription factor, PEBP2. Blood *86*, 607-616.

Tanaka, T., Mitani, K., Kurokawa, M., Ogawa, S., Tanaka, K., Nishida, J., Yazaki, Y., Shibata, Y., and Hirai, H. (1995). Dual functions of the AML1/Evi-1 chimeric protein in the mechanism of leukemogenesis in t(3;21) leukemias. Mol. Cell. Biol. *15*, 2383-2392.

Taylor, D.S., Laubach, J.P., Nathan, D.G., and Mathey-Prevot, B. (1996). Cooperation between core binding factor and adjacent promoter elements contributes to tissue-specific expression of interleukin-3. J. Biol. Chem. *271*, 14020-14027.

Thangavelu, M., Neuman, W.L., Espinosa, R., Nakamura, Y., Westbrook, C.A., and Le Beau, M.M. (1992). A physical and genetic linkage map of the distal long arm of human chromosome 5. Cytogenet. Cell Genet. *59*, 27-30.

Thomas, R.S., Tymms, M.J., Seth, A., Shannon, M.F., and Kola, I. (1995). ETS1 transactivates the human GM-CSF promoter in Jurkat T cells stimulated with PMA and ionomycin. Oncogene *11*, 2135-2143.

Thompson, C.B., Lindsten, T., Ledbetter, J.A., Kunkel, S.L., Young, H.A., Emerson, S.G., Leiden, J.M., and June, C.H. (1989). CD28 activation pathway regulates the production of multiple T-cell-

derived lymphokines/cytokines. Proc. Natl. Acad. Sci. U. S. A. *86*, 1333-1337.

Tocci, M.J., Matkovich, D.A., Collier, K.A., Kwok, P., Dumont, F., Lin, S., Degudicibus, S., Siekierka, J.J., Chin, J., and Hutchinson, N.I. (1989). The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. J. Immunol. *143*, 718-726.

Tokumitsu, H., Masuda, E.S., Tsuboi, A., Arai, K., and Arai, N. (1993). Purification of the 120 kDa component of the human nuclear factor of activated T cells (NF-AT): reconstitution of binding activity to the cis-acting element of the GM-CSF and IL-2 promoter with AP-1. Biochem. Biophys. Res. Commun. *196*, 737-744.

Travers, A.A. (1992). The reprogramming of transcriptional competence. Cell *69*, 573-575.

Tsai, E.Y., Jain, J., Pesavento, P.A., Rao, A., and Goldfeld, A.E. (1996). Tumor necrosis factor alpha gene regulation in activated T cells involves ATF-2/Jun and NFATp. Mol. Cell. Biol. *16*, 459-467.

Tsuboi, A., Sugimoto, K., Yodoi, J., Miyatake, S., Arai, K., and Arai, N. (1991). A nuclear factor NF-GM2 that interacts with a regulatory region of the GM-CSF gene essential for its induction in responses to T-cell activation: purification from human T-cell leukemia line Jurkat cells and similarity to NF-kappa B. Int. Immunol. *3*, 807-817.

Tsuboi, A., Masuda, E., Naito, Y., Tokumitsu, H., Arai, K., and Arai, N. (1994). Calcineurin potentiates activation of the granulocyte-macrophage colony-stimulating factor gene in T cells: involvement of the conserved lymphokine element 0. Mol. Biol. Cell. *5*, 119-128.

Tsuruta, L., Lee, H.J., Masuda, E.S., Yokota, T., Arai, N., and Arai, K. (1995). Regulation of expression of the IL-2 and IL-5 genes and the role of proteins related to nuclear factor of activated T cells. J. Allergy Clin. Immunol. *96*, 1126-1135.

Tsytsykova, A.V., Tsitsikov, E.N., and Geha, R.S. (1996). The CD40L promoter contains nuclear factor of activated T cells-binding motifs which require AP-1 binding for activation of transcription. J. Biol. Chem. *271*, 3763-3770.

Tuan, D.Y.H., Solomon, W.B., London, I.M., and Lee, D.P. (1989). An erythroid-specific, developmental-stage-dependent enhancer far upstream of the human "β-like globin genes. Proc. Natl. Acad. Sci. USA *86*, 2554-2558.

Tyree, C.M., George, C.P., Lira-DeVito, L.M., Wampler, S.L., Dahmus, M.E., Zawel, L., and Kadonaga, J.T. (1993). Identification of a minimal set of proteins that is sufficient for accurate initiation of transcription by RNA polymerase II. Genes. Dev. *7*, 1254-1265.

van Wijnen, A.J., Bidwell, J.P., Fey, E.G., Penman, S., Lian, J.B., Stein, J.L., and Stein, G.S. (1993). Nuclear matrix association of multiple sequence-specific DNA binding activities related to SP-1, ATF, CCAAT, C/EBP, OCT-1, and AP-1. Biochemistry *32*, 8397-8402.

Waitz, W. and Loidl, P. (1991). Cell cycle dependent association of c-myc protein with the nuclear matrix. Oncogene. *6*, 29-35.

Wang, C.Y., Bassuk, A.G., Boise, L.H., Thompson, C.B., Bravo, R., and Leiden, J.M. (1994). Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires co-operative binding of Elf-1 and AP-1 transcription factors. Mol. Cell. Biol. *14*, 1153-1159.

Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A.H., and Speck, N.A. (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. Proc. Natl. Acad. Sci. U. S. A. *93*, 3444-3449.

Wang, S., Wang, Q., Crute, B.E., Melnikova, I.N., Keller, S.R., and Speck, N.A. (1993). Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol. Cell Biol. *13*, 3324-3339.

Wang, S.W. and Speck, N.A. (1992). Purification of core-binding factor, a protein that binds the conserved core site in murine leukemia virus enhancers. Mol. Cell Biol. *12*, 89-102.

Wang, Z.-Q., Grigoriadis, A.E., Möhle-Steinlein, U., Rüther, U., and Wagner, E.F. (1992). Bone and haematopoietic defects in mice lacking c-fos. Nature *360*, 741-745.

Wargnier, A., Legros-Maida, S., Bosselut, R., Bourge, J.F., Lafaurie, C., Ghysdael, C.J., Sasportes, M., and Paul, P. (1995). Identification of human granzyme B promoter regulatory elements interacting with activated T-cell-specific proteins: implication of Ikaros and CBF binding sites in promoter activation. Proc. Natl. Acad. Sci. U. S. A. *92*, 6930-6934.

Weiss, D.L., Hural, J., Tara, D., Timmerman, L.A., Henkel, G., and Brown, M.A. (1996). Nuclear factor of activated T cells is associated with a mast cell interleukin-4 transcription complex. Mol. Cell. Biol. *16*, 228-235.

Wijmenga, C., Gregory, P.E., Hajra, A., Schrock, E., Ried, T., Eils, R., Liu, P.P., and Collins, F.S. (1996). Core binding factor beta-smooth muscle myosin heavy chain chimeric protein involved in acute myeloid leukemia forms unusual nuclear rod- like structures in transformed NIH 3T3 cells. Proc. Natl. Acad. Sci. U. S. A. *93*, 1630-1635.

Williams, D.A. (1995). Stem cell model of hematopoiesis. In Hematology: basic principles and practise. R. Hoffman, E.J. Benz, S.J. Shattil, B. Furie, H.J. Cohen, and L.E. Silberstein, eds. (New York: Church Livingstone), pp. 180-192.

Wodnar-Filipowicz, A., Heusser, C.H., and Moroni, C. (1989). Production of the haemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation. Nature *339*, 150-152.

Wolin, M., Kornuc, M., Hong, C., Shin, S.K., Lee, F., Lau, R., and Nimer, S. (1993). Differential effect of HTLV infection and HTLV tax on interleukin 3 expression. Oncogene *8*, 1905-1911.

Wong, J.M. and Bateman, E. (1994). TBP-DNA interactions in the minor groove discriminate between A:T and T:A base pairs. Nucleic Acids Res. *22*, 1890-1896.

Wood, W.G. (1996). The complexities of β-globin gene regulation. Trends Genet. *12*, 204-206.

Woodcock, C.L. and Horowitz, R.A. (1995). Chromatin oragnization re-viewed. Trends in Cell Biology *5*, 272-277.

Wotton, D., Ghysdael, J., Wang, S., Speck, N.A., and Owen, M.J. (1994). Cooperative binding of Ets-1 and core binding factor to DNA. Mol. Cell Biol. *14*, 840-850.

Xanthoudakis, S., Viola, J.P., Shaw, K.T., Luo, C., Wallace, J.D., Bozza, P.T., Curran, T., and Rao, A. (1996). An enhanced immune response in mice lacking the transcription factor NFAT1. Science *272*, 892-895.

Yang, Y-C. and Clark, S.C. (1988a). Molecular cloning of a primate cDNA and the human gene for interleukin 3. In *Lymphokines.* Vol 15. *Interleukin 3: the Pan Specific Hemopoietin.*. J.W. Schrader, ed. (New York: Academic Press), pp. 375-391.

Yang, Y-C., Koacic, R., Kriz, S., Wolf, S., Clark, S.C., Wellems, A., Nienhuis, A., and Epstein, N. (1988b). The human genes for GM-CSF and IL-3 are closely linked in tandem on chromosome 5. Blood *71*, 958-961.

Ye, J., Young, H.A., Ortaldo, J.R., and Ghosh, P. (1994). Identification of a DNA binding site for the nuclear factor YY1 in the human GM-CSF core promoter. Nucleic Acids Res. *22*, 5672-5678.

Young, D.C., Demetri, G.D., Ernst, T.J., Cannistra, S.A., and Griffin, J.D. (1988). In vitro expression of colony-stimulating factor genes by human acute myeloblastic leukemia cells. Exp. Hematol. *16*, 378-382.

Zaiman, A.L., Lewis, A.F., Crute, B.E., Speck, N.A., and Lenz, J. (1995). Transcriptional activity of core binding factor-alpha (AML1) and beta subunits on murine leukemia virus enhancer cores. J Virol. *69*, 2898-2906.

Zawel, L. and Reinberg, D. (1995). Common themes in assembly and function of eukaryotic transcription complexes. Annu. Rev. Biochem. *64*, 533-561.

Zhang, D., Fujioka, K., Hetherington, C.J., Shapiro, L.H., Chen, H., Look, A.T., and Tenen, D.G. (1994). Identification of a region which directs the monocytic activity of the colony-stimulating factor (macrophage colony-stimulating factor) receptor promoter and binds PEBP2/CBF (AML1). Mol. Cell. Biol. *14*, 8085-8095.

Zhang, D.E., Hohaus, S., Voso, M.T., Chen, H.M., Smith, L.T., Hetherington, C.J., and Tenen, D.G. (1996). Function of PU.1 (Spi-1), C/EBP, and AML1 in early myelopoiesis: regulation of multiple myeloid CSF receptor promoters. Curr. Top. Microbiol. Immunol. *211*, 137-147.

Zhang, W., Zhang, J., Kornuc, M., Kwan, K., Frank, R., and Nimer, S.D. (1995). Molecular cloning and characterization of NF-IL3A, a transcriptional activator of the human interleukin-3 promoter. Mol. Cell Biol. *15*, 6055-6063.

Zhu, X., Yeadon, J.E., and Burden, S.J. (1994). AML1 is expressed in skeletal muscle and is regulated by innervation. Mol. Cell Biol. *14*, 8051-8057.