Regulation of granulocyte macrophage-colony stimulating factor by Cold Shock Domain proteins

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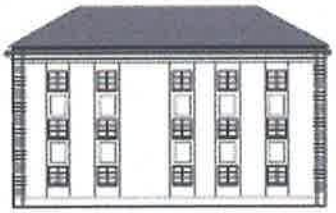

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ABSTRACT

Cold shock domain (CSD) family members, as a whole, have been shown to play roles in either transcriptional activation or repression of many genes in various cell types. Previous experiments undertaken in our laboratory indicate that CSD proteins, dbpAv and dbpB (also known as YB-1) act to repress granulocyte macrophage-colony stimulating factor (GM-CSF) transcription in human embryonic lung (HEL) fibroblasts. This repression is mediated via binding of CSD factors to single stranded DNA regions across the non-coding (-) strand of domain 1 in the GM-CSF proximal promoter. Subsequent work, presented here, identifies two additional CSD binding sites on the opposite coding (+) strand in domain 2 of the GM-CSF proximal promoter. These additional CSD binding sites bind nuclear and recombinant CSD factors and also function as repressors sites in the GM-CSF promoter in HEL fibroblasts. Deletion of specific domains in dbpB show that the highly conserved, central CSD domain, implicated in sequence specific DNA binding, is essential for the observed transcriptional repression in HEL fibroblasts. These results lend further evidence to previous work suggesting that CSD factors function to repress GM-CSF transcription via DNA binding to single stranded regions across the proximal promoter.

Since Jurkat T cells express high levels of GM-CSF in response to T cell receptor and co-receptor signalling, experiments were designed to examine if CSD factor regulation of GM-CSF transcription in Jurkat T cells involved the same mechanisms as identified in HEL fibroblasts. Transient transfection assays with the GM-CSF proximal promoter and CSD factors show that the CSD factors (dbpAv and dbpB) that repress GM-CSF transcription in HEL fibroblasts are involved in GM-CSF transcriptional activation in Jurkat T cells but only when the cells are stimulated to mimic T cell receptor activation. Unlike the mechanisms of CSD repression in HEL fibroblasts, CSD mediated activation in Jurkat T cells is not mediated through DNA binding but presumably through

protein:protein interactions via the C-terminus of the CSD protein with transcription factors such as NF-κB p50 / RelA. Analysis of HEL fibroblasts and Jurkat T cell nuclear extracts using CSD specific antibodies, DNA competitions and South-Western assays demonstrate that Jurkat T cells lack truncated CSD factor subtypes present in HEL fibroblasts. This observation raises the possibility that the cellular content of CSD proteins may determine their final role as activators or repressors.

Statement of Originality

This thesis contains no material that has been accepter for the award of any degree or diploma by any other university. To the best of my knowledge it contains no material that has previously been published by any other person, except where due reference has been made in the text. I consent to this thesis, when deposited in the university library, being available for photocopying and loan.

30/3/2001

Peter Diamond

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Publications arrising from work presented in this thesis

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Coles, L. S., Diamond, P., Occhiodoro, F., Vadas, M. A., and Shannon, M. F. (2000) An ordered array of cold shock domain repressor elements across tumor necrosis factor-responsive elements of the granulocyte-macrophage colony-stimulating factor promoter. *J Biol Chem* **275**, 14482-14493.

Diamond, P., Shannon, M. F., Vadas, M. A., and Coles, L. S. (2001) Cold shock domain factors activate the granulocyte-macrophage colony- stimulating factor promoter in stimulated Jurkat T cells. *J Biol Chem* **276**, *7943-7951*

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ABBREVIATIONS

All abbreviations used throughout this thesis are in accordance with those described in the *Journal of Biological Chemistry*, additional and alternate abbreviations are shown below.

APS	ammonium persulphate
ATP	adenosine triphosphate
Amp	ampicillin
bp	base pairs
BSA	bovine serum albumin
CIP	calf intestinal phosphatase
DMEM	Dulbecco's modified Eagle's Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
FCS	foetal calf serum
Gel Shift	(EMSA) Electrophoretic Mobility Shift Assay
GST	Glutathione-S-Transferase
GM-CSF	granuloctye macrophage colony stimulating factor
HEL	Human Embryonic Lung
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kDa	kilodalton
mA	milliamps
mins	minutes
mRNA	messenger RNA
nt	nucleotides
NP-40	Nonident ® P 40
OD	optical density

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluroide
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
secs	seconds
SDS	sodium dodecyl sulphate
TAE	tris-acetic acid EDTA
TBE	tris-boric acid EDTA
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethyl-ethene-diamine
TNFα	Tumour Necrosis Factor α
Tween 20	polyoxyethylene-sorbitan monolaurate
UV	ultra-violet

Chapter 1

Introduction

From the moment I picked up your book up until I laid it down I was convulsed with laughter. Some day I intend reading it.

Groucho Marx

1.1 The Immune system

The body's immune system is made up of a vast number of cells, functioning by an array of mechanisms designed to identify and eliminate a wide variety of invading pathogens. This array of mechanisms include physical barriers, phagocytic cells (present both in the blood and tissues), lymphocytes and various blood-borne molecules (like complement), all of which play integral parts in defending the body from invading pathogens. The immune system can be divided into two broad categories, governing innate and specific or adaptive immunity.

Mechanisms that make up innate immunity do not discriminate among most foreign substances, are present prior to exposure to foreign macromolecules or infectious pathogens and are not enhanced by exposures to the pathogen. Specific or adaptive immune system mechanisms are induced or stimulated by exposure to foreign substances, are very specific for distinct macromolecules and increase in magnitude and defensive capabilities with each successive exposure to a particular invading pathogen. The specific immune responses can be further classified into two types based on the components of the immune system that mediate the response. The first type is humoral immunity, which is defined as an immune response that can be transferred to unimmunised individuals by cell free portions of the blood (plasma or serum). Humoral immunity is mediated by the B lymphocyte population, which produce antibodies whose physiological function is to neutralise and facilitate the elimination of the antigen that induce their formation. The second type of specific immunity is cell-mediated immunity. Cell-mediated immunity can be transferred to naive individuals with T lymphocytes from an immunised individual but not with plasma or serum. Cell mediated immunity is a response of T lymphocytes recognising foreign molecules presented in the context of major histocompatibility complex (MHC) class I or II on the surface of cells. The class of MHC molecule in which the foreign epitope is presented dictates the

subset of T lymphocyte activated. Virally infected cells express viral antigens on their surface in the context of MHC class I, resulting in activation of cytotoxic T lymphocytes, which in turn destroy the virally infected cell. Professional antigen presenting cells process endogenous foreign antigens and display them on their surface in the context of MHC class II molecules. Foreign molecules presented in the context of MHC class II activate helper T lymphocytes which in turn promote proliferation and differentiation of both T and B lymphocytes as well as macrophages.

The components of the immune system, from the cells that first detect inflammatory signals to the lymphocytes and various effector cells that clear antigen and repair tissue damage, travel the body in the circulation, travel through organs and tissues and sometimes form more organised structures in the lymphoid tissues. The transitory state of the cells of the immune system, presents a challenge to a system that must raise a rapid, integrated response to invading pathogens anywhere in the body. Cells of the immune system require communication networks that can act locally or at a distance, specifically or globally, and transiently or in a sustained manner. The networks that allow such sophistication in the mammalian immune response exploit a variety of cell membrane-bound and soluble messengers (Kelso 1998). One group of these messengers are cytokines.

1.2 Cytokines in haemopoisis and immunity

Cytokines are a large group of non-enzymatic soluble or membrane bound protein hormones whose actions are diverse and affect wide and overlapping target cell populations (Kelso 1998); (Metcalf 1989). Cytokines are produced in conjunction with both the innate and specific immune systems and their secretion is usually brief and well controlled. Most cytokines are pleiotropic, exhibiting a wide range of biological effects on different cells. Cytokines can be redundant, with several cytokines acting on the same cell type to mediate

similar effects. For example interleukins (IL) IL-6, IL-2, IL-4, IL-5 and gamma interferon (γ-INF) can all induce antibody production in B cells (Kishimoto *et al.*, 1994). Cytokines often influence the synthesis of other cytokines, for example IL-1 induces fibroblasts, endothelial and bone marrow cells to synthesise G-CSF, GM-CSF, M-CSF, IL-6, TNFα as well as IL-1 itself (Bagby *et al.*, 1986); (Fibbe *et al.*, 1988); (Zuculi *et al.*, 1986). Cytokines often influence the action of other cytokines, they can have opposite effects, for example IL-3 and GM-CSF act to stimulate proliferation of haemopoietic progenitor cells while IL-8 and TGF-β act to inhibit progenitor cell proliferation (Alexander 1998). Cytokines can also act synergistically, for example, IL-1 and IL-6 enhance the effect of IL-3 and GM-CSF on survival and differentiation of haemopoietic progenitor cells (Hoffbrand *et al.*, 1995). Cytokines initiate their action by binding to specific receptors on the surface of target cells. The relevant target cells may be the same cell that secreted the cytokine (autocrine stimulation), a nearby cell (paracrine stimulation) or a distant cell by secreting the cytokine into the circulation (endocrine stimulation). Cytokines can also act as growth factors, stimulating differentiation and cell division.

For the body to maintain an effective immune system a vast quantity of cells are required, it is estimated that there is a constant turnover of 10^{10} erythrocytes and 10^{9} leukocytes every hour in a human adult. This rate of renewal is increased in times of stress, such as blood loss or infection. Blood cells originate from a self-renewing population of multipotential haemopoietic stem cells located mainly in the bone marrow, which generate progenitor cells committed irreversibly to one or other of the various haemopoietic lineages (Metcalf 1992). One of the major factors that control and direct haematopoiesis are cytokines. Cytokines that signal haemopoietic cell proliferation and differentiation are collectively called haemopoetic growth factors (HGF) (Metcalf 1989). HGF are produced by

haemopoietic cells but are also produced by many non-haemopoietic cells such as stromal, endothelial, epithelial and fibroblast cells (Baird *et al.*, 1995).

1.3 Granulocyte macrophage-colony stimulating factor

The work presented in this thesis will focus on the HGF, granulocyte macrophage-colony stimulating factor (GM-CSF). GM-CSF is a pluripotent cytokine with roles in survival, activation, proliferation and differentiation of cells from the haematopoietic lineage of granulocytes and macrophages (Cleavinger *et al.*, 1996); (Shannon *et al.*, 1997); (Swamynathan *et al.*, 1997). GM-CSF is a heavily glycosylated, single peptide of approximately 23 kDa (Nicola 1989), which is expressed by a wide variety of cells including myeloid, mesenchymal and lymphoid cells in response to stress signals such as those derived from infection, inflammation and blood loss (Reviewed in Shannon *et al.*, 1997). GM-CSF receptors have been identified on most types of myeloid progenitors and on mature monocytes, neutrophils, eosinophils, basophils and dendritic cells (Nicola 1994). The GM-CSF receptor consists of a ligand-specific α subunit (GMRα) (Gearing *et al.*, 1989) and a β subunit (βc) which is common between a range of cytokine receptors like IL-3 and IL-5 (Hayashida *et al.*, 1990). The α subunit is the major ligand-binding subunit but on its own does not seem to transduce any of the biological activities ascribed to GM-CSF in haemopoietic cells. The βc subunit, on the other hand, enables high affinity GM-CSF binding by the receptor and is responsible for most, if not all, of the signal transduction (Reviewed by Guthridge *et al.*, 1998).

1.4 GM-CSF mediated proliferation, activation and survival of mature haematopoietic cells

GM-CSF supports the proliferation of both stem cells and myeloid progenitor cells (Metcalf 1986). GM-CSF has the unusual property of stimulating the proliferation of a progressively broader spectrum of progenitor cells as concentrations are increased: first macrophage progenitors, then granulocyte-macrophage, followed by granulocyte, eosinophil, megakaryocytic and finally multipotential progenitors (Metcalf 1989). Cytokines are essential for the progression of haematopoietic cells through the cell cycle. Induction of cells from the resting G_0 state to G_1 in the cell cycle is mediated by competence factors (G-CSF, SCF, IL-6 and IL-11), with progression factors (GM-CSF, IL-3 and M-CSF) needed to enter the S-G_2 phase. High levels of these factors results in a decrease in the cell cycle times of progenitor cells, thereby increasing the number of progeny cells generated (Metcalf 1980).

In addition to it's proliferative effect, GM-CSF has also been shown to stimulate the function of mature end cells. GM-CSF acts in a paracrine or autocrine fashion being released at local sites of infection, which attracts effector cells to the site of infection and in turn directly activates these cells (Fibbe *et al.*, 1999). For example GM-CSF is directly chemotactic for neutrophils (Wang *et al.*, 1987), it also primes neutrophils for oxidative bursts (Weisbart *et al.*, 1987), enhances phagocytosis and increases the expression of certain adhesion molecules on their surface (Arnaout *et al.*, 1986). GM-CSF also activates eosinophils, monocytes and macrophages (Metcalf 1989).

When haematopoietic cells are grown *in vitro* in the absence of exogenously added growth factors the cells undergo apoptosis (Williams *et al.*, 1990). It has been shown that apoptosis can be prevented by the addition of cytokines like IL-1, GM-CSF or M-CSF, with cytokine mediated cell survival occurring at all stages of differentiation (Mangal *et al.*,

1991). Since the secretion of cytokines is only transitory during times of inflammation and infection, it is thought that one of the functions of apoptosis, in the absence of cytokines, may be a physiological mechanism to rapidly reduce the number of cells in a population that has been expanded by the action of cytokines after successful removal of the invading pathogen (Fibbe *et al.*, 1999).

1.5 Mechanisms of GM-CSF regulation

GM-CSF is expressed by a wide variety of cells but of particular interest to the work presented in this thesis both T lymphocytes and fibroblasts produce GM-CSF in response to a variety of immune and inflammatory stimuli. Fibroblasts express GM-CSF in response to activation by IL-1 and tumor necrosis factor alpha (TNFα). GM-CSF expression in activated T cells is triggered by activation of the T cell receptor by antigen complexed with either MHC class I or II molecules on the surface of antigen-presenting cells (Fibbe *et al.*, 1999).

GM-CSF expression is regulated at both the transcriptional and post transcriptional levels (Gasson 1991). Very briefly the post transcriptional control is mediated at the level of mRNA stability, with AUUUAA/UA/U sequences located in the 3' untranslated region conferring selective stability (Lagnado *et al.*, 1994). However, GM-CSF expression is primarily controlled at the level of transcription and the data presented in this thesis will focus on this area. The human GM-CSF gene is located on chromosome 5q23-q31 and resides in a cluster of cytokine and cytokine receptor genes that include IL-3, IL-4, IL-5, IL-13, M-CSF, M-CSF receptor and PDGF receptor (Thangavelu *et al.*, 1992). A detailed examination of the GM-CSF gene has identified a promoter and an upstream enhancer (3 kb upstream of the transcription initiation site), both of which are essential for efficient GM-CSF expression (Cockerill *et al.*, 1993); (Cockerill *et al.*, 1999).

Transcriptional regulation is mediated by two mechanisms, global regulation of a region of DNA in the context of chromatin and gene specific transcription factors. Both of these mechanisms are intimately linked and appear to play important roles in GM-CSF regulation. As with all cytokines, GM-CSF expression is tightly regulated. Most experiments to try and understand the mechanism of gene regulation have primarily focussed on gene activation but the correct regulation, of not only GM-CSF but most other genes, requires not only gene activation but also gene silencing. A recent increase in interest in this area of research has identified that silencing gene expression and maintaining genes in this silenced state is a highly organised, multi-protein, ATP-dependent process. Mechanisms of gene activation and repression, both of which are critical for correct GM-CSF expression, will be discussed in detail.

1.6 Mechanisms of gene regulation

1.6.1 DNA architecture mediated regulation of GM-CSF expression

1.6.1.1 Chromatin

Recent work has begun to identify the importance of the structure or architecture of DNA as a mechanism for transcriptional regulation. In eukaryotes, DNA is packaged with histone proteins to form structures known as nucleosomes. The nucleosome consists of dimers of the four histone proteins H2A, H2B, H3 and H4 binding to each other to form an octamer histone core, with approximately 143 bp of DNA wrapped twice around the core (Fletcher *et al.*, 1996). A fifth class of histone proteins, the HI or "linker" histones, bind at the exit and entry points of DNA from the nucleosome core. The presence of H1 is necessary for the condensation of nucleosome core. The presence of H1 is necessary for the condensation of nucleosome chains into higher order chromatin structures (Thomas 1984).

The formation of chromatin is a physiological mechanism that allows up to one thousand-fold compaction of the DNA length in chromosomes. The histone proteins have a globular body and a highly basic tail, which helps neutralise the overall negative charge on DNA, hence facilitating DNA folding into this compact chromatin structure (Wolffe *et al.*, 2000b). It was once thought that nucleosomes were static, non-participating structures merely utilised as a scaffold for DNA storage in a compact state, now it is clear that histone/DNA interactions are an integral and dynamic part of the machinery responsible for regulating gene transcription.

The degree of DNA compaction of chromatin appears to be one of the major controlling factors in regulation of gene expression. DNA that is tightly packed in chromatin (heterochromatin) is associated with regions of no or poor gene activity whereas DNA that is not tightly packed in chromatin (euchromatin) is associated with regions of high gene expression. During the course of gene activation, a significant degree of chromatin remodelling occurs. For example, circulating, resting T cells contain nuclei which are small and compacted, whereas soon after T cell receptor and co-receptor stimulation the nucleus increases up to ten fold with regions of euchromatin appearing (Zhao *et al.*, 1998a).

1.6.1.2 Histone modification

One possible mechanism responsible for the degree of DNA compaction in the nucleosomes is dependent on post translational modifications of the core histones. The histone tails are sites for acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation, all of which affect the chromatin architecture by altering histone/DNA and histone/histone interactions (Van Holde 1998).

Of all the histone modifications listed acetylation has been the most extensively studied. The acetylation state of histones has been correlated with whether associated DNA

regions are active or silenced. Hyper acetylated histones are associated with transcriptionally active regions of chromatin, whereas deacetylated histones are associated with transcriptionally silent regions of chromatin (Strahl *et al.*, 2000). However, a direct link between chromatin function and acetylation was only established by the discovery that coactivation complexes required for transcriptional activation contained histone acetyltransferases, whereas co-repressor complexes contained histone deacetylases (Wolffe *et al.*, 2000a).

Acetylation occurs on specific lysine residues present on the N-terminal histone tails. Acetylation of histone tails neutralises the positive charges on histones, decreasing the affinity of histones with other components of chromatin, including nucleosomal DNA, linker DNA and the histones of adjacent nucleosomes, thereby setting up an open and potentially transcriptionally active chromatin structure (Strahl *et al.*, 2000). Deacetylation of histones leads to stabilisation of the nucleosome and higher order chromatin structure, setting up a transcriptionally inactive chromatin structure (Covault *et al.*, 1980).

1.6.1.3 DNase I Hypersensitive sites

The changes in nucleosome arrangement have been identified and examined via experiments using DNase I enzyme digestion. DNase I cleaves DNA that is not associated with protein, (these regions are known as DNase I hypersensitive sites (DH sites)). Therefore, DNA in the context of tightly packaged nucleosomes is relatively protected from DNase I digestion compared to naked DNA. It has been shown that regions of chromatin that are transcriptionally active or have been remodelled so they contain no nucleosomes, are generally more sensitive to DNase I digestion than regions of inactive chromatin (Gross *et al.*, 1988). DH sites appear to co-localise with euchromatic regions of chromatin and hence

suggest that the DNA is in an open structure facilitating transcription factor binding and initiation of transcription.

Examination of the GM-CSF regulatory regions has identified two DH sites, one within the proximal promoter and the other in a region 3 kb upstream, which was later identified as an enhancer region (Cockerill *et al.*, 1993). These DH sites were found to be inducible by stimulation of T cells, myeloid cells and endothelial cells with factors know to stimulate GM-CSF production in these cells (Cockerill *et al.*, 1999). In T cells the DH site formation was blocked by cyclosporin A treatment, which has been shown to block signals transmitted by the T cell receptor, indicating that T cell receptor signals were responsible for this DH site formation (Cockerill *et al.*, 1993). Work done in the Cockerill laboratory has recently shown that the GM-CSF DH sites are not present in resting T cells but formation is induced upon T cell receptor stimulation (P.N. Cockerill personal communication). In activated T cells the GM-CSF proximal promoter and enhancer DH sites are either maintained, even in the absence of T cell receptor stimulation, or are able to form much more rapidly on subsequent T cell receptor activation. This observation suggests a mechanism for the rapid response and synthesis of GM-CSF in activated T cells in response to T cell receptor and co-stimulatory stimulation without a great deal of chromatin rearrangement (Cockerill laboratory, unpublished).

1.6.1.4 Nucleosome modification complexes

Destabilisation of the nucleosome complex by histone modification is involved in both activation and repression, but on its own appears not to be sufficient to confer either active or silent states of chromatin. Disrupting the nucleosome core is thought to create a platform for other chromatin remodelling factors to interact with the histones to either bring about activation or repression. In yeast, two of these nucleosome-remodelling complexes,

the SWI/SNF and ISWI, have been studied in detail. The SWI/SNF complexes have been shown to be involved in further disruption of the histone core and display some ability to shift nucleosomes while the ISWI complex is involved in shuttling the nucleosome along DNA (reviewed by Kornberg 1999). SWI/SNF like complexes have been identified in higher order eukaryotes, for example Brahma related gene (BRG) protein complex, BRG1 associated factors (BAF) (Wang *et al.*, 1998) and Mi-2 (Wade *et al.*, 1999).

There are many examples that now show nucleosome modification proteins are targeted to sites of transcriptional activation or repression. Both activation and repression of transcription involves the coordinated binding of many factors to form a functional unit. When these units form, leading to activation of a gene, the unit has been termed an enhanceosome. Factors within this enhanceosome actively recruit nucleosome rearranging factors like SWI/SNF family members BAF, and histone acetyltransferases p300, CREB-binding protein (CBP) and p300/CBP-associated factor directly to sites of transcriptional activation (Li *et al.*, 1999). It has been shown that T cell receptor signalling induces the rapid association of the BAF complex with chromatin. BAF is recruited to sites of chromatin remodelling by a complex containing PIP2, whose function is regulated by T cell receptor activation signalling. This recruitment is specific, with PIP2 having no effect on other related chromatin remodelling complexes containing SWI/SNF or ISWI like factors. Purification and peptide sequencing of the subunits of the complex revealed many proteins, which act as docking platforms for enhanceosome formation and interaction with chromatin (Zhao *et al.*, 1998b). These results, in conjunction with the results observed by Cockerill, indicate that T cell receptor signals can directly mediate chromatin regulation.

Large protein units that form on DNA to repress transcription have been shown to recruit histone deacetylases and nucleosome rearranging co-repressor complexes like SIN 3 and MI-2 (Vermaak *et al.*, 1999) (Wade *et al.*, 1999). Unlike activation, where a gene is

targeted specifically for activation by processes like T cell receptor mediated signalling, it is not well understood how a region of DNA is actively repressed. It has been shown that repression is not merely due to the absence of activation factors but by specific mechanisms.

One mechanism by which site specific repression is achieved is CpG island methylation of DNA. Methylated DNA is often associated with regions of silenced chromatin (El Osta *et al.*, 2000) (Wolffe *et al.*, 2000b). Methyl-CpG-binding proteins, MeCp1, MeCp2, MBO2 and MB03 have been shown to recruit co-repressor complexes like SIN 3 and Mi-2 (El Osta *et al.*, 2000). The Mi-2 complex consists of 6 proteins but is often found associated with major nucleosome reorganising complexes consisting of up to forty proteins (Xue *et al.*, 1998). The Mi-2 protein itself is a nucleosomal ATPase from the SWI/SNF family and is involved in nucleosome destabilisation (Guschin *et al.*, 2000). Two major factors often found associated with these co-repressor complexes are the histone binding protein RbAp48 and associated histone deacetylases (Wolffe *et al.*, 2000b). RbAp48 has been shown to bind to the histone core but is unable to gain access to the histone core when assembled into nucleosomes (Vermaak *et al.*, 1999). Therefore the Mi-2 complex is recruited, which disrupts the nucleosome structure, facilitating RbAp48 binding to the histone core. RbAp48 can also directly interact with histone deacteylases, enhancing their activity, presumably by positioning the deacetylase next to it target site on the core histone, thereby setting up a transcriptionally silent chromatin structure (Wolffe *et al.*, 2000b).

1.6.1.5 High mobility group 1 proteins

Another family of proteins that have been shown to affect DNA architecture and are important in GM-CSF promoter regulation are the high mobility group I (HMGI) family of proteins. The HMGI family of proteins consists of three family members HMGI, HMG(Y) and HMGI-C. These proteins are approximately 10 kDa in size and have in common three

conserved DNA binding motifs. They bind to the minor groove of A/T rich DNA and appear to recognise structure rather than sequence of DNA. Because of the presence of three DNA binding domains in these proteins it is postulated that they can contact DNA at several regions along a stretch of DNA, thereby significantly altering DNA structure (Shannon *et al.*, 2001). HMGI proteins have been shown to: alter DNA supercoiling, both enhance and inhibit transcription factor binding to sites that are overlapping or adjacent to their A/T rich binding sites, enhance transcription factor binding through protein:protein interactions, alter the DNA packaging into nucleosomes and may play a role in nucleosome positioning and shuffling (Reviewed by Shannon *et al.*, 2001). HMGI proteins can be modified by both phosphorylation and acetylation, both of which affect their protein:DNA interactions. Of particular interest to GM-CSF gene regulation HMGI proteins have been shown to interact with the transcription factors NF-κB/c-Rel, NFAT and AP-1. HMGI(Y) has been shown to be important in enhanceosome formation on the interferon β promoter by recruiting transcription factors, in particular NF-κB (Thanos *et al.*, 1995) (Yie *et al.*, 1997). HMGI(Y) is able to be acetylated by a co-activator protein CBP, which results in a loss in HMGI(Y)'s ability to enhance NF-κB binding and general enhanceosome formation. It is thought that this may be a mechanism by which to disassemble the enhanceosome complex thereby switching off gene transcription (Shannon *et al.*, 2001).

1.6.2 Transcription factor mediated regulation of GM-CSF expression

1.6.2.1 T cell receptor and co-receptor mediated signalling of GM-CSF expression

GM-CSF is expressed in a variety of cell types but of interest to the work presented in this thesis, I will focus in particular on GM-CSF expression in T cells. GM-CSF is

expressed in activated T cells, of both the Th1 and Th2 phenotype. GM-CSF expression is triggered by intracellular signalling cascades resulting from activation of the T cell receptor by interactions with antigen presented in the context of major histocompatibility class I or II molecules on the surface of antigen presenting cells. Activation of T cells through the T cell receptor alone is not sufficient for high levels of cytokine production. Full T cell activation requires both the activation of the T cell receptor and costimulatory signals provided by antigen presenting cells, resulting in the induction of a sustained and proliferative T cell response (Weiss *et al.*, 1987). These costimulatory signals arise from paracrine stimulation by cytokines or from the interaction of ligands on antigen-presenting cells with receptors on the T cell surface. For example the antigen presenting cell ligand B7 interacts with the CD28 receptor on the T cell surface initiating costimulatory signalling (Bluestone 1995). Both resting and activated T cell recognition of antigen in the absence of costimulatory signals induces either T cell apoptosis or functional unresponsiveness, characterised by the failure to proliferate or synthesise cytokines on re-exposure to antigen presented with the correct costimulatory signals (Ragazzo *et al.*, 2001); (Shang *et al.*, 1999). Costimulatory signals are a crucial for adequate responses to infectious pathogens but are also important in preventing inappropriate responses to self-antigens.

T cell receptor activation initiates two major intracellular signalling cascades which result in the activation of a series of transcription factors needed for GM-CSF transcription. One of these T cell receptor mediated signals results in an increase of intracellular calcium leading to activation of the calmodulin-dependent phosphatase, calcineurin. The second signal activates a number of tyrosine kinases that subsequently leads to activation of protein kinase C. T cell receptor activation can be mimicked by the treatment of T cells with phorbol myristate acetate (PMA) and calcium ionophore (Ca^{2+} ionophore).

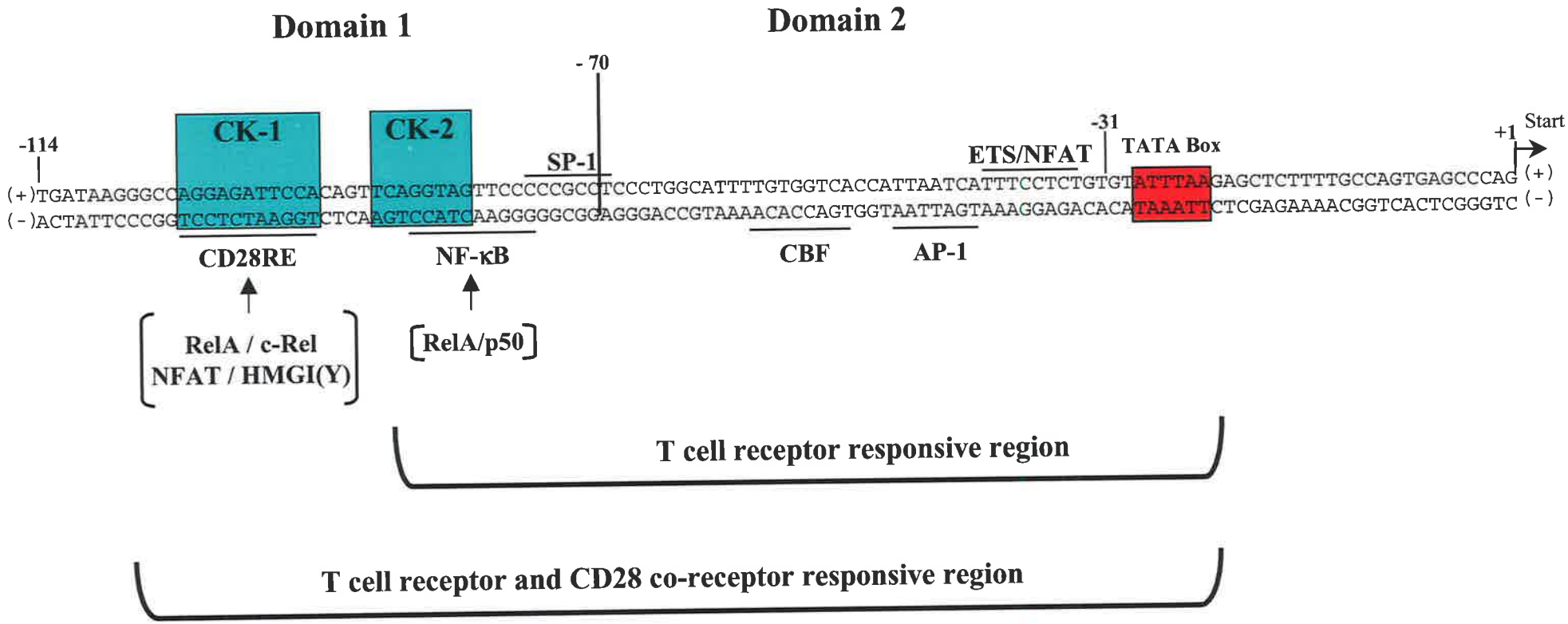
GM-CSF expression is regulated at the level of transcription by a proximal promoter and enhancer region, found 3 kb upstream. The GM-CSF proximal promoter can be divided into functional domains, domain 1 and domain 2 (Figure 1.1). Domain 1 (-114 to -71) contains the CK-1 (also known as the CD28-responsive element CD28RE) and CK-2 elements conserved in a number of cytokine genes and is responsive to T cell receptor activators and costimulators, like CD28 (Himes *et al.*, 1996a); (Himes *et al.*, 1996b); (Jenkins *et al.*, 1995). This regions binds a number of transcription factors including NF-κB/Rel, SP-1, NFAT and HMGI(Y) (Gasson 1991); (Shannon *et al.*, 1995); (Shannon *et al.*, 1997). Domain 2 (-70 to -31) binds CBF, AP1, ETS and NFAT transcription factors, and also responds T cell receptor activation (Cockerill *et al.*, 1996); (Jenkins *et al.*, 1995); (Thomas *et al.*, 1997). The CBF, AP1, ETS and NFAT transcription factor binding sites have been shown to be essential for T cell receptor signalling in conjunction with the domain 1 NF-κB site (Cockerill *et al.*, 1996); (Jenkins *et al.*, 1995); (Thomas *et al.*, 1997).

Signalling mediated via the CD28 co-receptor functions through the CD28RE, located upstream of the NF-κB site in domain 1 (Figure 1.1). The CD28RE is a variant NF-κB binding site which has been shown to bind not only NF-κB/Rel family members but NFAT and HMGI(Y) as well (Shang *et al.*, 1999) (Himes *et al.*, 1996a). The CD28RE, however, can not function on its own, requiring downstream sequences including the NF-κB and SP-1 binding sites to function (Figure 1.1) (Himes *et al.*, 1996a); (Himes *et al.*, 1996b); (Shannon *et al.*, 1997). This observation correlates with the observation that CD28RE activity is only seen when both of the T cell receptor and co-receptor signals are provided, indicating the need for transcription factors upregulated by these signals to function (Shang *et al.*, 1999). This region including the CD28RE, NF-κB and SP-1 binding sites has been collectively called the CD28 responsive region (CD28RR).

Figure 1.1

The sequence of the GM-CSF proximal promoter

Domain 1 (-114 to –71) and Domain 2 (-70 to –31) regions are indicated. The binding sites for double stranded transcription factors which mediate GM-CSF expression including: NF-κB/Rel family members (RelA, p50), CBF, SP-1, AP-1, ETS and NFAT are underlined {8} {260}. The CD28 response element (CD28RE) and NF-κB sites are indicated. Transcription factors known to bind to each of these sites are shown underneath. GM-CSF proximal promoter regions responsive to T cell receptor stimulation and CD28 co-receptor stimulation are indicated.


Domain 1
Domain 2
- 70
-114
CK-1
CK-2
SP-1
-31
ETS/NFAT
TATA Box
+1
Start
(+)TGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTCCCCCGCCTCCCTGGCATTTTGTGGTCACCATTAATCATTTCCTCTGTGTATTTAAGAGCTCTTTTGCCAGTGAGCCCAG (+)
(-)ACTATTCCCGGTCCTCTAAGGTCTCAAGTCCATCAAGGGGGCGGAGGGACCGTAAAACACCAGTGGTAATTAGTAAAGGAGACACATAAATTCTCGAGAAAACGGTCACTCGGGTC (-)
CD28RE
NF-κB
CBF
AP-1
RelA / c-Rel
NFAT / HMGI(Y)
RelA/p50
T cell receptor responsive region
T cell receptor and CD28 co-receptor responsive region

In addition to the GM-CSF proximal promoter, an enhancer has been identified 3 kb upstream of the transcriptional start site (Cockerill *et al.*, 1993). The GM-CSF enhancer has cell specific activity that mirrors GM-CSF expression, being active only in cells that are known to express GM-CSF and hence is postulated to play an important role in regulating high levels of cell specific GM-CSF expression (Cockerill *et al.*, 1993) (Bert *et al.*, 2000a) (Cockerill *et al.*, 1999). GM-CSF enhancer function is readily blocked by cyclosporin A, which has been shown to disrupt T cell receptor mediated calcium signalling, suggesting that factors upregulated by T cell receptor stimulation were involved in its regulation. Examination of this region identified three composite NFAT/AP-1 binding sites. Co-operative binding of NFAT/AP-1 complexes to these enhancer binding sites are critical for GM-CSF enhancer function (Cockerill *et al.*, 1999) (Cockerill *et al.*, 1993) (Bert *et al.*, 2000a) (Cockerill *et al.*, 1995). The relative roles in transcriptional regulation of GM-CSF expression by NFAT and NF-κB/Rel family members will be now discussed in detail.

1.6.2.2 Nuclear factor of activated T cell (NFAT)

One of the important factors for GM-CSF activation is NFAT. NFAT is a family of at least four related proteins that regulate inducible cytokine gene transcription not only in T cells but also in myeloid and endothelial cells (Rao *et al.*, 1997). NFAT does not usually function alone, but in strict co-operation with other factors where it has been implicated in different patterns of expression of certain cytokines. This specific regulation of cytokine expression is mediated by NFAT protein:protein interactions with specific transcription factors. For example, NFAT has been shown to be important in T cell specific expression of IL-2, IL-3 and IL-4 by interacting with AP-1, Oct, NIP45 and Oca-B proteins (Bert *et al.*, 2000a). NFAT is activated by T cell receptor mediated activation of the phosphatase, calcineurin (Rao *et al.*, 1997). Calcineurin has been shown to dephosphorylate NFATp, a

preformed cytoplasmic NFAT family member which enables its translocation into the nucleus where it has a high affinity for DNA (Shaw *et al.*, 1988). T cell receptor signalling also results in activation of activator protein –1 (AP-1) (Weiss *et al.*, 1994). NFATp and AP-1 proteins cooperatively bind in the nucleus to form a functional higher-order complex, binding with significantly higher affinity than either of the individual proteins (Crabtree 1999). The main role of NFAT/AP-1 in mediating GM-CSF transcription is via its binding to the enhancer region, where three composite NFAT/AP-1 sites have been identified (Bert *et al.*, 2000a); (Cockerill *et al.*, 1995). The ability of NFAT and AP-1 to cooperatively bind to these sites is primarily determined by the relative spacing of the binding sites to one another (Cockerill *et al.*, 1995).

Domain 2 of the GM-CSF proximal promoter also contains both NFAT and AP-1 sites, but unlike the enhancer, no cooperative NFAT/AP-1 binding is observed. The change in order and the slight change in the spacing of the AP-1 and NFAT binding sites in the proximal promoter, compared to the functional NFAT/AP-1 sites in the enhancer, is thought to be the reason for the lack of cooperative binding (Bert *et al.*, 2000a); (Jenkins *et al.*, 1995). NFAT has been shown to also bind the CD28RE and is thought to play a role in the activity of the GM-CSF CD28RR (Shang *et al.*, 1999). Gel shift experiments have shown that AP-1 is not able to bind to the CD28RE. NFAT is only able to transactivate CD28RR when both T cell receptor signals are provided suggesting that NFAT interacts with factors, other that AP-1, which are induced by T cell receptor activation (Shang *et al.*, 1999).

In addition to directly recruiting factors like AP-1, NFAT is also thought to play a major role in decondensing chromatin and mediating the formation of DH sites by directly recruiting the histone acetyl transferase CBP/p300 (Avots *et al.*, 1999); (Garcia-Rodriguez *et al.*, 1998). The NFAT/AP-1 complex has even greater potential to remodel chromatin because AP-1 can also directly disrupt nucleosome organisation (Ng *et al.*, 1997). NFAT's

function is therefore postulated to be more than just a docking site for factors. NFAT is thought to indirectly increase transcription factor binding by recruiting chromatin remodelling machinery and thereby increasing access to their binding sites (Bert *et al.*, 2000a). NFAT may also rely on recruiting partners that can interact directly with the polymerase complex to facilitate transcriptional activation. In the case of the NFAT/AP-1 complex, AP-1 is a strong activator of the TFIID complex, suggesting a potential direct link between the enhancer and proximal promoter (Ng *et al.*, 1997).

1.6.2.3 Nuclear factor - kappa B (NF-κB)

NF-κB/Rel factors are activated by T cell receptor and co-receptor mediated signals, and are essential in the regulation of GM-CSF transcription via the CD28RE and NF-κB sites present in domain 1 of the proximal promoter. NF-kappa B/Rel family members are found in essentially all cell types and are important regulators of a large number of genes important in response to infection, inflammation, and other cellular stress signals requiring rapid reprogramming of gene expression. The NF-κB/Rel family members consists of NF-κB1 (also know as p50, which is derived from the NF-κB1p105 precursor), NF-κB2 (also known as p52, which is derived from the NF-κBp100 precursor), and the Rel proteins RelA (also known as p65), RelB and c-Rel. The Rel proteins share a highly conserved N-terminal 300 amino acid region called the Rel homology domain (RHD). The RHD is responsible for dimerisation, DNA binding, protein:protein interactions and also contains a nuclear localisation sequence (Reviewed by Karin *et al.*, 2000).

The NF-κB/Rel proteins bind DNA as either homo or heterodimers, each of which have different DNA specificity and function. Homodimeric complexes of p50 and p52 are proposed to have inhibitory function, whereas protein complexes containing RelA, RelB and c-Rel proteins activate transcription (reviewed by (Ghosh *et al.*, 1998)). In unstimulated

cells, NF-κB/Rel proteins are sequestered in the cytoplasm complexed with specific inhibitor proteins, called IκBs. All IκBs contain ankyrin repeats which mediate binding to the RHD of Rel proteins, masking the nuclear localisation signal thereby leading to cytoplasmic retention (Ghosh *et al.*, 1998).

Of all the IκB's (IκBα, IκBβ, IκBγ and IκBε, NF-κB1p105 and NF-κBp100) IκBα and IκBβ have been the best characterised. Activation stimuli, including signals derived from T cell receptor and co-receptor stimulation, result in rapid phosphorylation of IκBα. Once phosphorylated, IκBα is ubiquitinated and becomes a target for degradation by the ubiquitin-26S proteosome pathway. Phosphorylation, ubiquitination and proteosome degradation all take place while the IκBα is still bound to NF-κB/Rel proteins in the cytoplasm (Chen *et al.*, 1995b). Once IκBα is degraded, the nuclear localisation signal of NF-κB/Rel proteins is exposed resulting in binding to karyopherins and translocation into the nucleus. NF-κB/Rel and IκBα regulate each other via an autoregulatory feedback loop. Nuclear NF-κB/Rel induces the synthesis of IκBα, which in turn translocates to the nucleus where it binds to NF-κB/Rel proteins shuttling them back to the cytoplasm. This shuttling is mediated via a nuclear export sequence located in both the C- and N-terminal regions of IκBα (Johnson *et al.*, 1999); (Schmid *et al.*, 2000). The IκBβ protein is also regulated by similar mechanisms upon T cell receptor and co-receptor stimuli. Unlike IκBα, IκBβ is thought to play a role in both repressing and prolonging the action of NF-κB/Rel proteins. Phosphorylated IκBβ acts in a similar manner to IκBα, in that it inhibits the DNA-binding ability of NF-κB/Rel proteins, via shutting them back into the nucleus. However, newly synthesised, unphosphorylated IκBβ acts as a chaperone, binding to DNA associated NF-κB/Rel proteins, thereby blocking the inhibitory effect of IκBα in the nucleus. By this process IκBβ is able to maintain NF-κB/Rel activity even after IκBα resynthesis and is

thought to be initiated by costimulatory signals like CD28 costimulation (Suyang *et al.*, 1996).

The specific response of regions of the GM-CSF promoter to either T cell receptor or CD28 signals is related to the formation of distinct NF-κB/Rel complexes. Regulation is determined by which NF-κB/Rel subunits are activated and the selective binding abilities of the different NF-κB/Rel dimers. The GM-CSF domain 1 NF-κB site, which is responsive to T cell receptor or PMA/Ca^{2+} Ionophore mediated signals only binds RelA/p50 dimers (Figure 1.1). Upon CD28 stimulation, the CD28RE is able to bind RelA and c-rel NF-κB/Rel family members resulting in high levels of GM-CSF expression levels (Figure 1.1). High level c-rel binding to the CD28RE is dependent on the presence of HMGI(Y). This HMGI(Y)/c-rel binding appears to be specific, with the binding of RelA to either the CD28RE or the NF-κB site not affected by HMGI(Y) (Himes *et al.*, 1996a).

Many of the proteins described above, have been shown to have multiple associations with other transcription factors and chromatin remodelling proteins. For example, RelA has been shown to associate with the histone actyl transferase CBP/p300 (Gualerzi *et al.*, 1990) and HMGI(Y) has been associated with not only c-rel but also NFAT (Shang *et al.*, 1999), suggesting that the recruitment of these DNA remodelling factors is important in creating a open chromatin structure for efficient GM-CSF transcription. The NFAT/AP-1/NF-κB/Rel factors also have been shown to synergistically interact with other transcription factors including Elf-1, ETS1, CBF and SP-1 potentially forming an enhaceosome structure for efficient GM-CSF transcription (Himes *et al.*, 1996a); (Meier *et al.*, 1992); (Ozer *et al.*, 1990a); (Thomas *et al.*, 1997).

1.7 Cold shock domain (CSD) factors

CSD factors, also known as Y-box proteins, were originally identified in bacteria where they are involved in the cold-shock response (Jones *et al.*, 1987). The cold shock response is a specific pattern of gene expression that occurs in response to abrupt shifts to low temperatures. This pattern includes the induction of CSD proteins, synthesis of proteins involved in transcription and translation and the specific repression of heat shock proteins (Jones *et al.*, 1987); (Jones *et al.*, 1992). The major bacterial CSD protein identified is CspA, and has been shown to play an essential role in the bacterial cold shock response.

Sequence comparison of CSD family members has identified a central 100 amino acid domain called the Cold Shock Domain, which is highly conserved throughout evolution in CSD proteins isolated from bacteria to plants and humans, with the notable exception of *Saccharomyces cerevisiae* (Graumann *et al.*, 1998); (Wolffe *et al.*, 1992); (Wolffe 1993). In bacteria, CSD factors act as RNA chaperones, preventing RNA secondary structure, thereby keeping RNA in a linear state which is an essential prerequisite for efficient initiation of transcription in prokaryotes (Gualerzi *et al.*, 1990); (Jiang *et al.*, 1997). Bacterial CSD factors have also been shown to bind DNA and contribute to transcriptional control by sustaining the expression of genes necessary for cell growth at low temperatures (Jones *et al.*, 1994).

CSD factors of higher organisms have been reported to bind double and single stranded DNA and RNA and can interact with a wide and diverse array of proteins. By virtue of their diverse nucleic acid and protein interaction abilities, CSD proteins have been shown to be involved in multiple aspects of gene regulation. CSD proteins are involved in transcriptional repression and activation and in mRNA packaging, transport, localisation, masking, stability and translation (Wolffe *et al.*, 1992) (Wolffe 1993) (Graumann *et al.*,

1998) (Ladomery *et al.*, 1995) (Sommerville *et al.*, 1996) (Matsumoto *et al.*, 1998) (Sommerville 1999) (Shannon *et al.*, 1997).

1.7.1 CSD structure

CSD proteins have three functional domains, an N-terminal domain, the central highly conserved cold shock domain and the C-terminal domain. The crystal structure for the bacterial CSD proteins CspA and CspB have been resolved (Figure 1.2), lending structural evidence to functional studies that have determined the role of each of the domains (Newkirk *et al.*, 1994); (Schindelin *et al.*, 1993); (Schindelin *et al.*, 1994); (Schnuchel *et al.*, 1993).

The N-terminal region of the CSD protein has not been well characterised. The N-terminal region on its own has no intrinsic function, but when in the context of the whole protein has been shown to contribute to single stranded DNA binding and protein:protein interactions (Ansari *et al.*, 1999); (Kolluri *et al.*, 1992); (Nambiar *et al.*, 1998). The central, highly conserved cold shock domain is composed of five antiparallel β-strands forming a closed, five stranded β-barrel (Newkirk *et al.*, 1994); (Schindelin *et al.*, 1993); (Schindelin *et al.*, 1994); (Schnuchel *et al.*, 1993) (Figure 1.2). The cold shock domain also contains two highly conserved motifs termed RNP1 and RNP2 which are conserved in a number of single strand nucleic acid binding proteins (Graumann *et al.*, 1996); (Landsman 1992) and are required for sequence-specific, single stranded DNA and RNA binding (Bouvet *et al.*, 1995); (Kolluri *et al.*, 1992); (Schroder *et al.*, 1995). Three consecutive β-strands, β1, β2 (which contains the RNP1 motif) and β3 (which contains the RNP2 motif) create a surface rich in aromatic and basic residues that are involved in nucleic acid binding (Figure 1.2) (Schindelin *et al.*, 1993).

Figure 1.2

A) Sequence alignment of CSD region from different species of Cold shock domain proteins.

Sequence alignment of the CSD domain from different cold shock domain proteins. In the alignment, identical residues are idicated by dashes and insertions by colons. The percent identity with the bacterial CSD protein CspA is shown on the right and is calculated by one-by-one comparisons of the aligned sequences.

E. coli, Escherichia coli

B. Sub., Bacillus subtilis

S. Clav., Streptomyces clavuligerus

Figure reproduced from Schindelin *et al.* 1994

B) Three-dimensional structure of the bacterial CSD protein CspA.

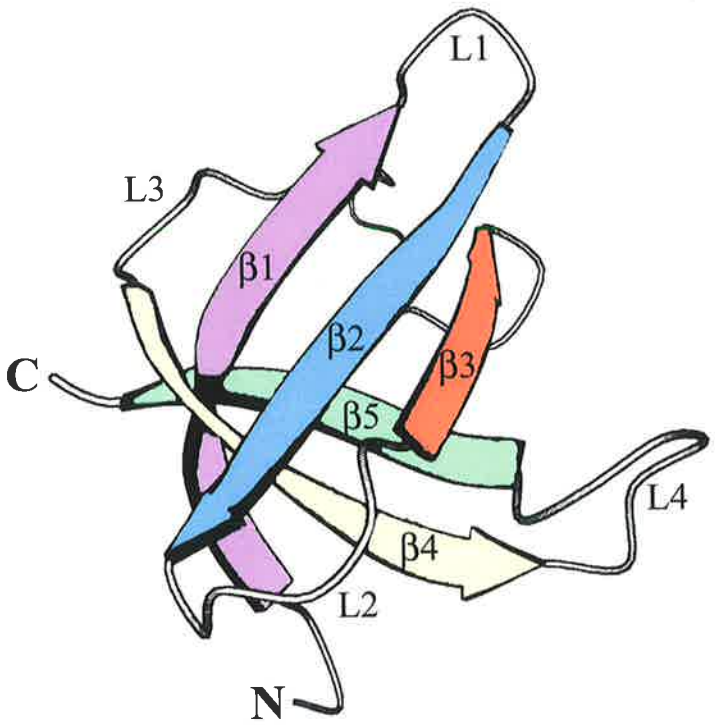
β-Strands are given as coloured curved arrows and numbered β1-β5 and loops between β-strands are numbered L1-L4.

Figure reproduced from Schindelin *et al.* 1994

A

```
CspA   E. coli    MSGKMTGIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDG::::YKSLDEGQKVSFTIESGAKGPAAGNVTSL
CspB   E. coli    --N----L----------S-V----------N::::-RT-F-----T-S----------A--IITD          80%
CspC   E. coli     MA-IK-Q-----ES--------A----------GN-::::F-T-A---N-E-E-QD-Q-----V---AI     68%
CspD   E. coli       MEK-T-----NA------C-EG-GE-I-A-Y-T--M--::::-RT-KA--S-Q-DVHQ-P--NH-SVIVPVEVEAAVA  48%
CspB   B. sub.       MLE-K-----SE------EVE:-QD----------GE-::::F-T-E---A---E-VE-NR--Q-A---KEA  61%
Csp7   S. clav.      MA--T------E------AQ-G-GP-----Y---NAT-::::FR--E-N-V-N-DVTH-E:--Q-E--SPA   56%
CbfR   Rat        IAT-VL-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKYLR-VGD-ET-E-DVVE-E--AE-A---GPG  43%
CbfM   Mouse      IAT-VL-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKYLR-VGD-ET-E-DVVE-E--AE-A---GPG  43%
CbfH   Human      IAT-VL-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKYLR-VGD-ET-E-DVVE-E--AE-A---GPG  43%
DbpA   Human      LAT-VL-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKYLR-VGD-ET-E-DVVE-E--AE-A---GPD  43%
Yb1H   Human      IAT-VL-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKYLR-VGD-ET-E-DVVE-E--EE-A---GPG  43%
Yb1X   Xenopus    IAT-VL-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKYLR-VGD-ET-E-DVVE-E--AE-A---GPE  43%
Yb2X   Xenopus    LATQVQ-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKFLR-VGD-ET-E-DVVE-E--AE-A---GPG  41%
Yb3X   Xenopus    IAT-VL-T-----VRN-Y---NRN-TKE-----QT--KKNNPRKYLR-VGD-ET-E-DVVE-E--AE-A---GPG  43%
```

B



The C-terminal domain of CSD proteins are generally composed of alternating basic and acidic regions, each about 30 amino acids in length. The alternating blocks of basic and acidic amino acids are proposed to function as a charge zipper domain, mediating protein:protein interactions (Ozer *et al.*, 1990a). The C-terminal domain has been implicated in both non-sequence specific RNA binding and protein:protein interactions with transcription factors like RelA, Zo-1, TBP, NF-Y, YY-1 and Ap-2 and structural proteins such as actin. These proteins have been shown to interact with different regions of the C-terminus, providing the option for CSD factors to interact with a number of partners, or factors on different parts of the promoter (Shnyreva *et al.*, 2000) (Li *et al.*, 1997) (Ansari *et al.*, 1999) (Ise *et al.*, 1999) (Moorthamer *et al.*, 1999) (Ruzanov *et al.*, 1999). The alternating basic and acidic regions found in the C-terminal domain of CSD proteins are similar to sequences found on proteins that bind to ribonucleoprotein complexes and shuttle between the nucleus and cytoplasm (Meier *et al.*, 1992). CSD factors were originally identified in humans as ribosome associated proteins, and while CSD factors lack an obvious nuclear localisation sequence, they are found in both the nuclear and cytoplasmic compartments (Shnyreva *et al.*, 2000). Sequence similarities with these shuttling proteins suggests that the C-terminus may play a role in the shuttling of CSD proteins (Ranjan *et al.*, 1993). CSD proteins have been shown *in vitro* to homodimerise and form large protein aggregates. This homodimerisation is dependent on C-terminal mediated protein:protein interactions (Bouvet *et al.*, 1995); (Nambiar *et al.*, 1998); (Wolffe *et al.*, 1992). Unlike the Cold shock domain, the C-terminal domains of different CSD factors display large variations, which are attributed to the wide and varied functions of CSD factors. The main role, however, for the C-terminus is in transcriptional activation (Ansari *et al.*, 1999); (Kerr *et al.*, 1994).

1.7.2 CSD family subtypes

There have been a large number of CSD proteins reported from many species. Of all the ubiquitously expressed mammalian and avian CSD protein reported, sequence analysis had deduced they could be grouped into two subtypes. The first subtype consists of homologues of dbpB, also commonly known as YB-1 (Human), they include EFIA (chicken), p50 (rabbit), MSY1 (mouse) and FRGY1 (*Xenopus*) (Evdokimova *et al.*, 1995); (Ozer *et al.*, 1990b); (Sapru *et al.*, 1996); (Tafuri *et al.*, 1993); (Tekur *et al.*, 1999). The second subtype is dbpA (human) with homologues reported EFII (chicken), M1Y/M1Ya (mouse), YB-2 (human) and the recently described CSD protein ZONAB (dog) (Balda *et al.*, 2000); (Cleavinger *et al.*, 1996); (Sapru *et al.*, 1996); (Swamynathan *et al.*, 1997). The remaining CSD proteins can be subdivided into two groups. The first of these groups represents germ cell specific CSD factors, Contrin (human), MSY2 (mouse) and FRGY2 (*Xenopus*) (Bouvet *et al.*, 1995); (Gu *et al.*, 1998); (Tekur *et al.*, 1999). The main role of this subset of CSD factors appears to be in RNA binding. For example, during oogenesis, the *Xenopus laevis* CSD factor FRGY2, binds specific nascent mRNA during transcription, sequestering them in the cytoplasm, preventing translation and degradation (Bouvet *et al.*, 1995). The C-terminus of FRGY2 binds mRNA in a non-sequence specific manner mediating the translational repression, while sequence specificity is conferred by the RNP1 and RNP2 motifs in the cold shock domain (Matsumoto *et al.*, 1996). Post-fertilisation, phosphorylation of residues in the C-terminus of FRGY2 results in the specific release of mRNA from CSD proteins, enabling translation (Sommerville *et al.*, 1996). The final subset of CSD factors includes CSD-related proteins. An example of a protein that falls into this subset is UNR. UNR has been identified in humans, guinea pigs and *D. melanogaster* and contains several repeats of cold shock domain motifs but lacks a C-terminal like domain region (Doniger *et al.*, 1992); (Thieringer *et al.*, 1997).

Work done in our laboratory and by others have identified variants of CSD factors. These variants are proposed to arise from either splice variants or post translational proteolytic cleavage. The major splice variant, reported in chicken, arises from the dbpA gene and has been termed YB-2 and has been shown to bind to the Rous sarcoma long terminal repeat promoter. This splicing event results in a 69 amino acid deletion in the C-terminal region which does not affect single strand DNA binding but could have an effect on the ability of the truncated protein to interact with other regulatory proteins (Cleavinger *et al.*, 1996); (Swamynathan *et al.*, 1997). Human homologues of YB-2 have also been identified (Balda *et al.*, 2000); (Kudo *et al.*, 1995). Post-translational proteolytic cleavage of CSD proteins has been demonstrated in dbpB. In this example the C-terminal region of dbpB is cleaved resulting in its translocation into the nucleus where it is involved in the thrombin inducibility of PDGF B-chain expression (Stenina *et al.*, 2000). Since full length dbpB is found in the nuclear compartment in other systems, it is known that C-terminal cleavage is not required for import of CSD factors into the nucleus.

1.8 CSD factors and transcriptional regulation

1.8.1 CSD factors and transcriptional activation

CSD factor mediated transcriptional activation has been reported in a few cellular genes (α1 (I) procollagen and MM-2/gelatinase A (Dhalla *et al.*, 1998); (Mertens *et al.*, 1997)) but the most extensively studied cases have been on viral promoters. CSD factors have been implicated in transcriptional activation of human immunodeficiency virus, Rous sarcoma virus, human T lymphotrophic virus and human neutropic JV polymavirus (JCV) promoters (Greuel *et al.*, 1990); (Kashanchi *et al.*, 1994); (Kerr *et al.*, 1994); (Sawaya *et al.*,

1998). The mechanisms by which CSD factors mediate transcriptional activation are not well understood but appear to involve both DNA binding and protein:protein interactions.

1.8.1.1 CSD activation of human neutropic JV polymavirus (JCV)

Of all the viral promoters studied, the role of CSD factors in activation of the JCV promoter is the most understood and represents a good example of both DNA binding and protein:protein interactions for CSD factors. The primary role of CSD factors in JCV activation involves a complex interplay between host cell factors Purα and NF-κB/Rel and the JCV T-antigen protein. The JCV regulatory domain contains the origin of replication, promoters for early and late gene transcription, a NF-κB site, two 98 bp tandem repeats and a region called the D domain (Kerr *et al.*, 1994) (Chen *et al.*, 1995a) (Safak *et al.*, 1999). A pentameric repeat sequence called the lytic control element (LCE) found in the 98 bp tandem repeats, is responsible for modulating viral early and late promoters and contributes to tissue specificity and viral DNA replication (Tada *et al.*, 1992). The JCV genome is hypervariable and variants have been identified that contain a 23bp insertion in the LCE. These variants show changes in virulence and tissue specificity, indicating the importance of the LCE (Safak *et al.*, 1999). Binding studies on the LCE demonstrated that two single stranded DNA binding proteins dbpB and Purα bound to opposite strands of the JCV LCE. Purα bound to the LCE early strand, inducing viral early gene transcription and dbpB bound to the complementary LCE late strand, inducing viral late gene transcription (Chen *et al.*, 1995a) (Safak *et al.*, 1999). Purα and dbpB are able to modulate each others binding to their respective LCE targets. Purα increases binding of dbpB to its DNA target but in contrast Purα binding to its strand of the LCE is decreased by dbpB (Chen *et al.*, 1995a) (Gallia *et*

al., 1998). Purα early gene transcription leads to synthesis of the viral T-antigen protein. T-antigen has been shown to induce late gene transcription and is also able to increase dbpB binding to the late promoter, thereby further increasing the level of viral late gene transcription. Purα reduces the extent of T-antigen late gene transactivation, however T-antigen is able to in turn, decrease the induced level of early gene transcription by Purα. The functional antagonism observed between Purα and T-antigen is determined by protein:protein interactions with one another (Gallia *et al.*, 1998).

The model for regulation of the JCV viral promoter by this interplay between Purα, dbpB and T-antigen is as follows. Early stages of infection result in strong binding of Purα to the LCE leading to early viral gene transcription (including the synthesis of T-antigen). As the infection progresses the level of T-antigen increases, which in turn stabilises dbpB binding to the LCE late strand. Stabilised binding of dbpB to the LCE results in the dissociation of Purα from the early strand of the LCE which leaves dbpB and T-antigen free to enhance late viral gene transcription (Gallia *et al.*, 1998) (Chen *et al.*, 1995a) (Safak *et al.*, 1999).

The binding of Purα and dbpB to the JCV promoter in variant strains containing the 23 bp insertion within the LCE is however, dramatically different. Where previously Purα and dbpB affected early and late gene transcription respectively, in variant JCV strains both Purα and dbpB act synergistically to stimulate both early and late gene transcription (Safak *et al.*, 1999). The different activity of dbpB and Purα on the variant JCV promoter suggests that structural organisation of the viral promoter can dictate the activities of these proteins.

Another layer of control and complexity of JCV transcriptional regulation is added by the introduction of the NF-κB/Rel family members. The JCV promoter contains two NF-κB/Rel-responsive regions, a consensus NF-κB site and a region called the D domain, both

of which affect JCV early/late gene expression (Raj *et al.*, 1996). The NF-κB site binds NF-κB/Rel family members p50, p52 and p65 whereas the D domain binds all these NF-κB/Rel family members and the CSD factor dbpB. NF-κB binding to these sites is independent of Purα and T-antigen but is regulated by interactions with dbpB. JCV late gene transcription is decreased when p50 and p52 bind to the NF-κB site but transcription is increased when p65 binds to the NF-κB site. The converse pattern is seen on the D domain, where p50 and p52 NF-κB/Rel factors act to increase transcription and p65 acts to decrease transcription. These results can be explained by p65 and dbpB being able to modulate each others binding to DNA. It is known that p50/p52 NF-κB/Rel factors often have inhibitory effects whereas p65 activates viral late gene expression (Ghosh *et al.*, 1998). dbpB augments the affinity of p65 binding to the JCV NF-κB site thereby synergistically activating viral late gene expression. On the D domain p65 binds to dbpB, reducing dbpB binding to its target thereby repressing late viral gene expression. This p65 mediated repression is relieved by p50/p52 NF-κB/Rel family members titrating p65 away from interactions with dbpB, enabling dbpB to bind to the D domain enhancing JCV late gene transcription (Raj *et al.*, 1996).

1.8.2 CSD factors and transcriptional repression

Whereas CSD factors have primarily been shown to act as activators of viral genes, they act as repressors of many cellular genes. The majority of genes regulated by CSD factors fall into three categories, growth factor genes (GM-CSF, G-CSF, IL-3 & Erb B2) (Coles *et al.*, 2000) (Balda *et al.*, 2000), stress response genes (MHC Class I & II, thyrotropin receptor, grp78, nicotine acetylcholine receptor δ and multidrug resistance gene I) (Hu *et al.*, 2000); (Li *et al.*, 1997); (Ohmori *et al.*, 1996); (Sapru *et al.*, 1996); (Ting *et al.*, 1994), and the cell cycle gene thymidine kinase (Kim *et al.*, 1997).

The mechanisms by which CSD factors are proposed to act to repress transcription are two fold. The most common of the two mechanisms is brought about by the ability of CSD proteins to bind to single stranded DNA. CSD factors have been shown *in vitro* to induce or stabilise single strand regions within double strand DNA containing CSD sites (Horwitz *et al.*, 1994); (Kolluri *et al.*, 1992); (MacDonald *et al.*, 1995); (Mertens *et al.*, 1998); (Schwartzbauer *et al.*, 1998). The binding of CSD factors to single stranded regions of DNA would thereby prevent the binding of activator factors which are dependent on double stranded DNA for binding. For example, dbpB has been shown to bind to two single stranded regions of DNA in the thyrotropin receptor gene promoter, repressing insulin and cAMP mediated induction of transcription (Ohmori *et al.*, 1996). Another example is regulation of the cell stress induced protein chaperone, grp78. The transcription factor YY1 binds to the grp78 promoter, activating gene transcription under periods of cellular stress. CSD factors, dbpA and dbpB, repress this stress mediated activation of grp78 by binding to single stranded DNA regions across the grp78 promoter, thereby preventing YY1 from binding to its double stranded DNA target (Li *et al.*, 1997). There is some evidence that CSD factors themselves can induce single stranded regions of DNA (MacDonald *et al.*, 1995) but the latest evidence suggests that their role is more one of stabilisation of the single stranded DNA structure. CSD proteins have recently been found associated with protein complexes containing nucleosome remodelling factors suggesting a potential mechanism by which regions of single stranded DNA are induced (Shnyreva *et al.*, 2000).

The second mechanism of CSD mediated transcriptional repression is via protein:protein interactions. Protein:protein interactions involving CSD factors generally are associated with transcriptional activation but in some reported cases CSD factors have been shown to bind to activator proteins, thereby preventing them binding to their DNA targets. For example, CSD factors dbpA and dbpB have been shown to regulate the expression of the

MHC class I-Aβ. Efficient transcription of I-Aβ requires the formation of the NF-YA/NF-YB complex. CSD proteins repress activation of the I-Aβ gene by blocking formation of the NF-YA/NF-YB complex via two mechanisms. The first mechanism is by dbpA directly binding to NF-YA, replacing NF-YB and forming a non-functional NF-YA/dbpA complex. The second mechanism is not fully understood, but involves dbpB preventing the formation of a functional NF-YA/NF-YB complex (lloberas *et al.*, 1995).

Due to the type of genes that CSD factors have been shown to regulate (growth factor, stress response, cell cycle) repression of these genes in the absence of appropriate stimuli is important. The role of CSD factors in the regulation of these genes may be critical in preventing inappropriate cell growth and disease.

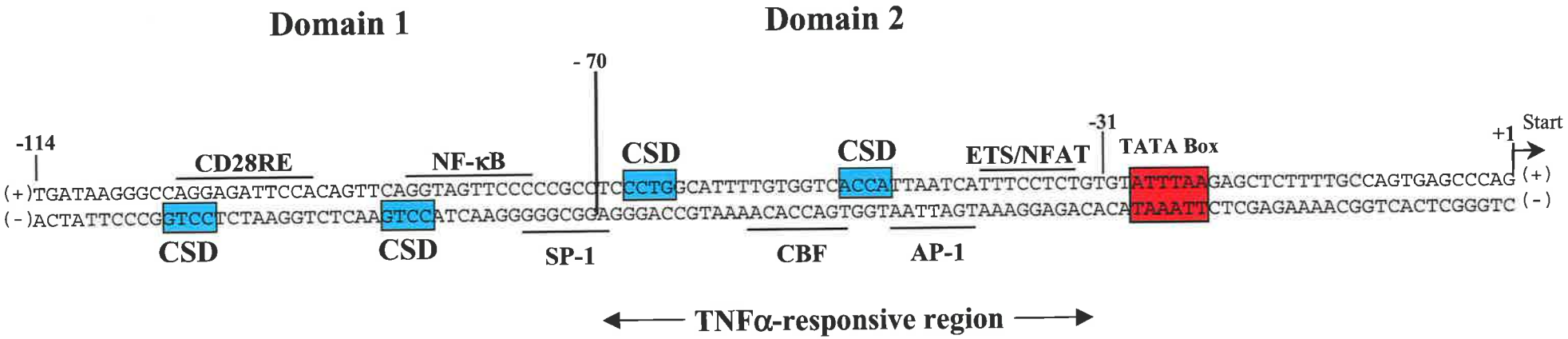
1.9 Regulation of GM-CSF expression by CSD factors in human embryonic lung (HEL) fibroblasts

Experiments performed in our laboratory to examine the regulation of GM-CSF transcription identified that the GM-CSF promoter could be divided into two functional domains: Domain 1 (-114 to –71) and Domain 2 (-70 to –31). Domain 1 contains the CD28RE and NF-κB sites and Domain 2 contains the binding sites for several transcription factors (Figure 1.3). Using HEL fibroblasts and transfection of chloramphenicol acetyl-transfease (CAT) GM-CSF proximal promoter report constructs, it was observed that domain 2 of the GM-CSF proximal promoter was highly TNFα inducible (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). This TNFα induction of domain 2 is mediated via TNFα inducible and constitutive factors CBF, AP1 and ETS/NFAT binding to sites in domain 2 (Figure 1.3)(Coles *et al.*, 2000). Interestingly, when the reporter construct was extended to encompass domain 1, there was a dramatic reduction in the level of TNFα inducible expression. Mutational analysis of sequences in Domain 1 relieved this repression,

Figure 1.3

Cold Shock Domain binding sites in Domain 1 and Domain 2 of the GM-CSF proximal promoter

Domain 1 (-114 to –71) and Domain 2 (-70 to –31) regions are indicated. The CD28 responsive element (CD28RE) and the binding sites for double stranded transcription factors which mediate GM-CSF expression including: NF-κB/Rel family members (RelA, p50), CBF, SP-1, AP-1, ETS and NFAT are underlined {8} {260}. Binding sites identified for nuclear NF-GMb/c complexes are indicated on the non-coding (-) strand of domain 1 and the coding strand (+) of domain 2. The Domain 2, TNFα responsive region is also highlighted.


Domain 1
Domain 2
- 70
-114
-31
+1
Start
CD28RE
NF-κB
CSD
CSD
ETS/NFAT
TATA Box
(+)TGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTCCCCCGCCTCCCTGGCATTTTGTGGTCACCATTAATCATTTCCTCTGTGTATTTAAGAGCTCTTTTGCCAGTGAGCCCAG (+)
(-)ACTATTCCCGGTCCTCTAAGGTCTCAAGTCCATCAAGGGGGCGGAGGGACCGTAAAACACCAGTGGTAATTAGTAAAGGAGACACATAAATTCTCGAGAAAACGGTCACTCGGGTC (-)
CSD
CSD
SP-1
CBF
AP-1
TNFα-responsive region

suggesting that Domain 1 contained repressor binding sites. Gel shift experiments using ^{32}P-labelled oligonucleotides spanning domain 1 and nuclear extracts from HEL fibroblasts and HUT78 T cells, identified two complexes, NF-GMb and a faster migrating complex NF-GMc that bound only to single stranded oligonucleotides across this region. Fine mutational analysis across domain 1, identified that these two complexes were contacting two repeated 5'-CCTG-3' sequences (one overlapping the CD28RE and the other overlapping the NF-κB element) on the non-coding strand of the GM-CSF domain 1 (Figure 1.3). Binding of the NF-GMb complex required the presence of both sequences whereas the NF-GMc complex could form when only one site was present. Binding of NF-GMb and NF-GMc complexes to the sites identified in the GM-CSF domain 1 region was implicated in repression of the domain 2 TNFα response (Coles *et al.*, 1994). UV cross-linking experiments carried out on these complexes identified that the NF-GMb complex was composed of 42 and 22 kDa proteins, while the NF-GMc complex was composed of only a 22 kDa protein (Coles *et al.*, 1994); (Coles *et al.*, 2000).

In an attempt to isolate the protein components of the NF-GMb/c complexes, a single stranded oligonucleotide spanning the domain 1 region, was used to screen a HUT78 T cell λgt11 cDNA expression library. This screen isolated two cDNA clones, which encoded proteins with NF-GMb-like binding activity. Sequencing of these clones identified two proteins from the group of proteins known as cold shock domain (CSD) proteins. One of the sequences was identified as the CSD factor dbpB and the other was a variant of the CSD factor dbpA, which was called dbpAv (Coles *et al.*, 1996). Subsequent gel shift and UV cross-linking analysis using single stranded DNA oligonucleotides spanning domain 2, identified that NF-GMb/c complexes could also form on the non-coding strand of domain 2. Mutational analysis of domain 2 identified a further two NF-GMb/c CSD binding sites. One of the CSD binding sites identified was the same as the two identified in domain 1 5'-CCTG-

3' while the other site was identified as 5'-ACCA-3' (Figure 1.3). Mutational analysis of the domain 2 NF-GMb/c CSD binding sites resulted in an increase of domain 2 TNFα-inducible expression, implying that these sites were also acting as repressor elements (Coles *et al.*, 2000). The spacing between the four CSD sites in domains 1 and 2 was conserved, bringing about an ordered regularly spaced arrangement of CSD repressor sites across the GM-CSF promoter. It is proposed that CSD factors themselves or interactions with chromatin remodelling machinery induce regions of single stranded DNA across the GM-CSF promoter. Binding of CSD factors to these single stranded binding sites is postulated to prevent the double stranded DNA activating factors from binding and initiating transcription.

In addition to GM-CSF, the promoter sequences of two other myeloid growth factor genes, the human G-CSF and IL-3 genes, were analysed. G-CSF and IL-3 have overlapping patterns of expression with GM-CSF. It was observed that the unique arrangement of CSD binding sites seen in GM-CSF was present across the G-CSF and IL-3 proximal promoters. In each of the three genes the domain 1 NF-GMb/c CSD binding sites were on the non-coding strand, whereas the domain 2 sites were on the coding strand. Similar to what was observed in the GM-CSF promoter, CSD sites in the G-CSF and IL-3 genes overlap or are adjacent to activator sites like SP-1, CBF, NF-κB and the CD28-responsive element (Coles *et al.*, 2000). The sequence, spacing and strand conservation of CSD sites in the three genes suggests an important role not only for binding of CSD proteins to DNA but also for CSD interactions with other regulatory proteins and ultimately, the architectural DNA structure resulting from binding of these factors.

1. 20 Project Rationale

An objective of our laboratory over the years has been to investigate the transcriptional regulation of the human GM-CSF proximal promoter. Most of the emphasis has been on positive regulatory factors like NF-κB/Rel and HMGI-Y which are important in T cell receptor and co-receptor mediated signalling of GM-CSF expression. Another focus of our laboratory has been on negative regulatory elements of the GM-CSF promoter which have led us to investigate cold shock domain (CSD) proteins. CSD proteins are expressed in all cell types and appear to play a role in the strict regulation of expression of genes involved in growth regulation and stress response. Therefore we wanted to determine if CSD factors could regulate GM-CSF transcription in Jurkat T cells, since they express high levels of GM-CSF in response to T cell receptor and co-receptor signalling.

At the time this project commenced nuclear CSD complexes (NF-GMb and NF-GMc) had been detected binding to single stranded DNA regions across the GM-CSF proximal promoter in HEL fibroblasts. Mutational analysis across the GM-CSF proximal promoter identified four CSD binding sites, two across the non-coding strand of domain 1 and two across the coding region of domain 2 (Coles *et al.*, 1994) (Coles *et al.*, 1996). Transient transfection experiments identified that these CSD binding sites were acting as repressor sites in the GM-CSF promoter in HEL fibroblasts. The work presented in this thesis continues work previously undertaken in our laboratory examining the role of CSD proteins in HEL fibroblasts and extends this work to examine the role of CSD proteins in the regulation of the GM-CSF promoter in Jurkat T cells.

1. 21 The specific aims of this project are:

1) To compare GM-CSF promoter transcriptional regulation by CSD factors dbpAv and dbpB in HEL fibroblasts and Jurkat T cells.

2) To determine which regions of the CSD proteins were involved in transcriptional repression and activation.
3) To express recombinant CSD GST-fusion proteins dbpAv-GST, dbpB-GST and to examine binding to CSD sites across the GM-CSF proximal promoter.
4) To generate CSD specific antibodies.
5) To characterise the sub-components of nuclear CSD NF-GMb and NF-GMc complexes in HEL fibroblasts and Jurkat T cells.
6) To identify potential mechanisms for CSD mediated activation in Jurkat T cells.

Chapter 2

Materials and Methods

Once you eliminate the impossible, no matter how improbable, must be the truth

Sherlock Holmes

2.1 Chemicals, reagents and consumables

The following chemicals were obtained from Sigma Chemicals: agarose (type 1), ampicillin, ATP (disodium, grade I), β-mercaptoethanol, BSA, bromophenol blue, xylene cynol, DTT, ethidium bromide, PMSF, Tween 20, lysozyme and Triton X-100. TEMED, acrylamide and bisacrylamide were purchased from BioRad Laboratories. Agar, foetal calf serum, ammonium persulphate were purchased from Gibco BRL. Trypsin, DMEM, RPMI, L-glutamine, Penicillin, Gentamycin were purchased from CSL laboratories. Phenol was purchased from WAKO Pure Chemical Industries Ltd. Poly(dI:dC), Protein A Sepharose and Glutathione Sepharose 4B were purchased from Amersham Pharmacia Biotec. D(-)-Luciferin, acetyl Co-A were purchased from Boehringer Mannheim. All other fine chemicals were purchased from Merck.

2.2 Enzymes and protein molecular weight markers

All restriction enzymes were purchased from New England Biolabs or Promega. T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs. Calf intestinal phosphatase and RNAseA were purchased from Boehringer Mannheim. Benchmark™ protein ladder and Benchmark™ prestained protein ladder were purchased from GIBCO BRL.

2.3 Radiochemicals

[γ-^{32}P]ATP (4,000 Ci/mmol) and L – ^{35}S Methionine (1175 Ci/mmol) were purchased GeneWorks. ^{14}C Chloramphenicol (0.2 μCi/4 nmol) was purchased from NEN.

2.4 Buffers and solutions

1 x TBE	50 mM Tris, 1 mM EDTA, 42 mM Boric Acid, pH 8.3
1 x TAE	40 mM Tris, 20 mM Acetic acid, 0.9 mM EDTA
1 x SDS-PAGE Buffer	250 mM Tris, 34 mM SDS, 1.9 M Glycine
3 x Urea Load Buffer	4 M Urea, 50% Sucrose (w/v), 50 mM EDTA, 0.1% Bromophenol blue (w/v)
5 x Acrylamide load	50% Sucrose (w/v), 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.1% Bromophenol blue (w/v), 0.1% Xylene cynol (w/v)
Formamide load	80% deionized formamide (w/v), 0.01% bromophenol blue (w/v), 0.01% Xylene cynol (w/v), 0.1 mM EDTA in 0.5 x TBE
2 x SDS-PAGE load	125 mM Tris, 20% glycerol (v/v), 2% SDS (w/v), 2% 2-β-mercaptoethanol (v/v), 0.001% Bromophenol blue (w/v), adjusted to pH6.8 with HCl
TE Buffer	10 mM TrisHCl pH 7.5, 0.1 mM EDTA.
LB broth:	1% Bacto-tryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v), adjusted to pH 7.0 with NaOH.
LB agar plates:	Made by adding 1.5% Bacto-agar (w/v) to the LB broth

2.5 *E. coli* Bacterial Strains

MC1061 F^-, hsdR2, araD139, Δ(araABC-leu)7696, ΔlacX74 galE15, galU, galK16, rpsL, (Str^r), (r_K^- m_K^+), mcrA, mcrB1, thi

JM109 F'[traD36, proAB, $lacI^qZ$ Δ M15], recA1, endA1, gryA96, (Nal^r), thi, hsdR17, (r_K^- m_K^+), supE44, relA1, Δ(lac-proAB), mcrA (Stratagene)

BL21 (DE3) F^-, ompT, Ion, $hsdS_B$, (r_B^- m_B^+), λ DE3 (Stratagene)

2.6 Plasmids

pSG5 with modified polylinker (Stratagene) (Figure 2.1)

pXP1 luciferase vector (Promega) modified by Peter Cockerill HCCR (Bert *et al.*, 2000b) (Figure 2.2)

pGEX-4T-1 (Amersham Pharmacia Biotech) (Figure 2.3)

pRcCMV (Invitrogen)

pBLCAT2 (Luckow *et al.*, 1987)

pSV2CAT (Gorman *et al.*, 1982)

2.7 Eukaryotic cell lines

HUT78 a T-Lymphoblastoid cell line derived from a patient with Sezary syndrome (Gazdar *et al.*, 1980)

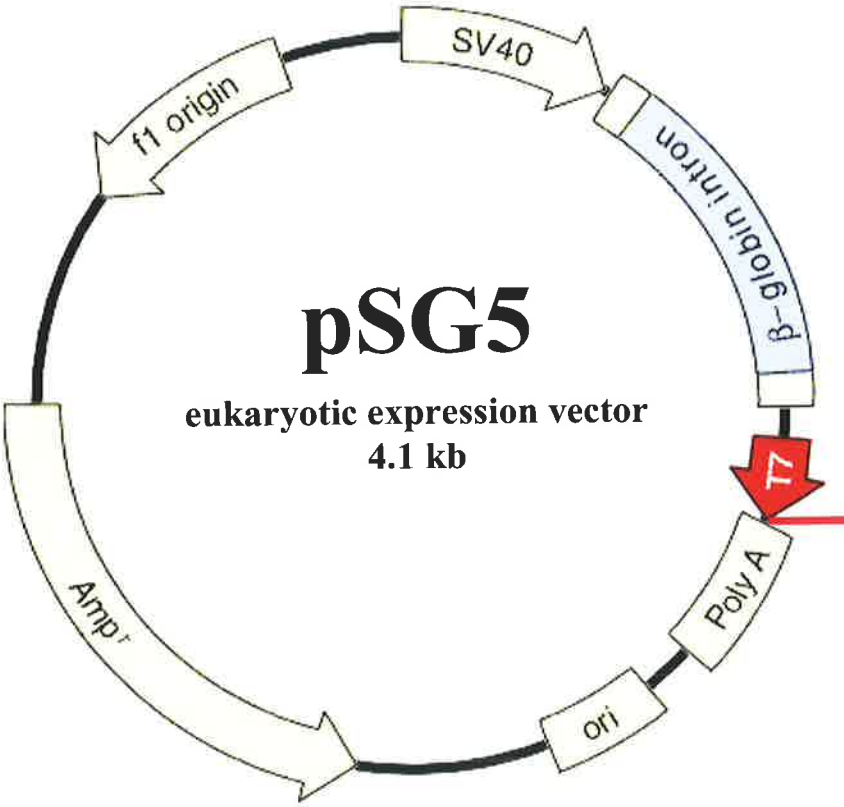
HEL 299 Human Embryonic Lung fibroblast (ATCC # CCL-137)

Jurkat T lymphocyte line derived from a patient with acute T cell leukaemia

Figure 2.1

Eukaryotic Expression vector pSG5

The eukaryotic expression vector (pSG5) used in all transient transfection experiments is shown. The restriction enzyme sites present in the modified multiple cloning site are also shown.



pSG5

eukaryotic expression vector
4.1 kb

MCS

BclI
EcoRV
EcoRI
Asp718
BanI
KpnI
XhoII
BstYI
BglII
CcrI
PacR71
XhoI
AvaI
AguI
BanI
EheI
AayI
Aha2
NarI
BbeI
HaeII
PstI
NheI
XhoII
BstYI
BglII
CfrI
EaeI
EagI
Eco521
NotI
XmaIII
BglI
SfiI
CfnI
EdeI

Figure 2.2

Plasmid map of the luciferase reporter pXP1

The plasmid map of the luciferase reporter pXP1 is shown (Bert, 2000). The restriction enzyme sites in the modified multiple cloning site are also indicated.

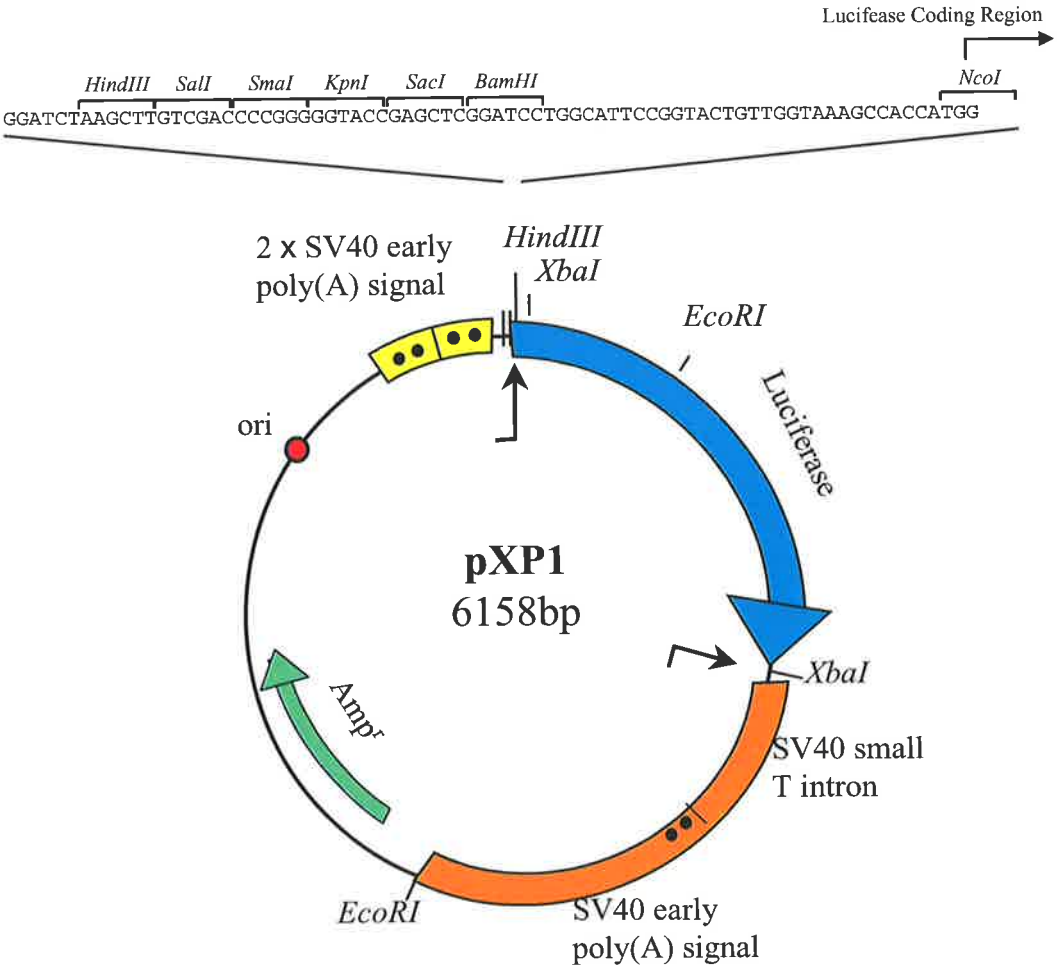
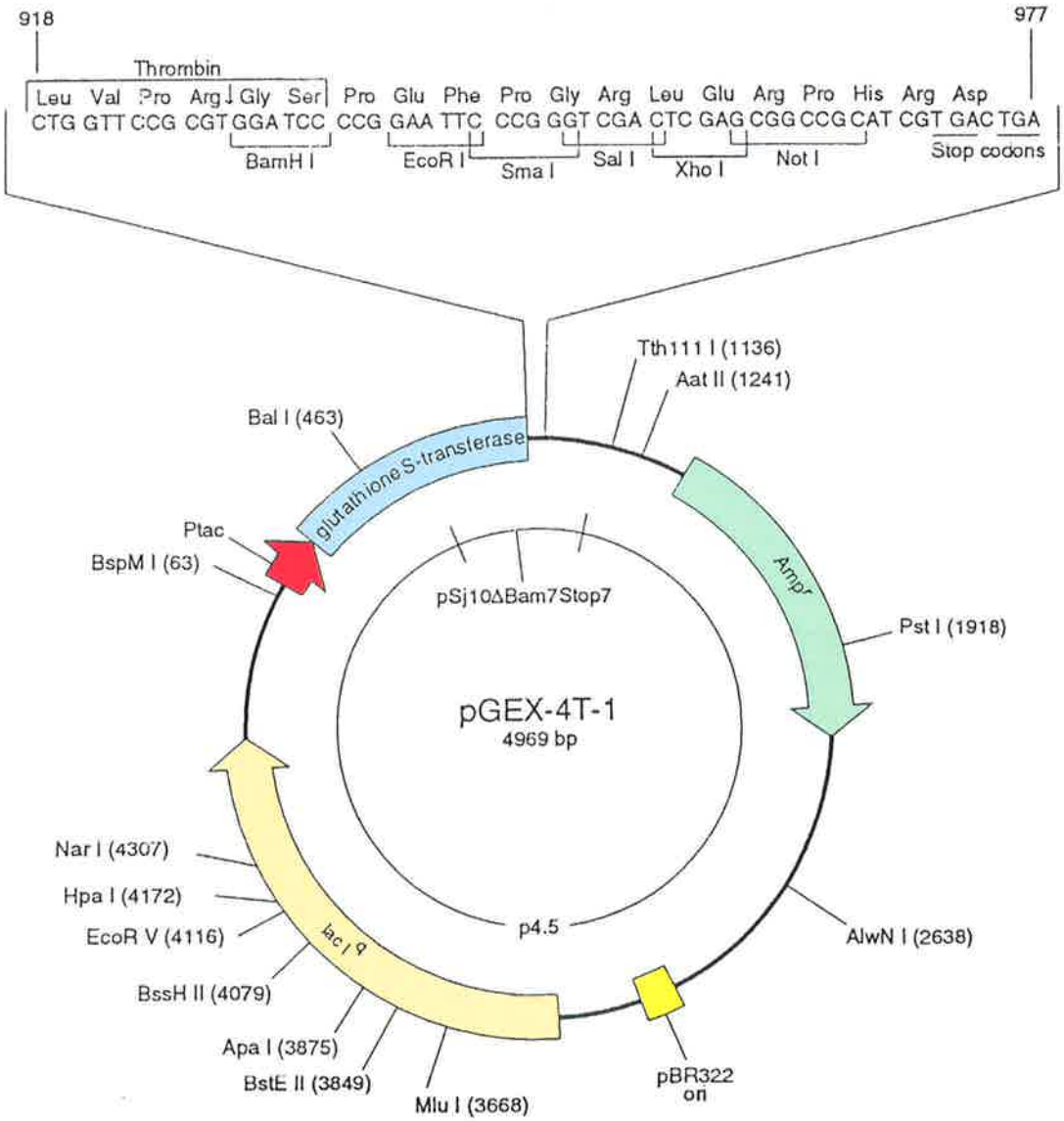

Lucifease Coding Region
HindIII SalI SmaI KpnI SacI BamHI
NcoI
GGATCTAAGCTTGTCGACCCCGGGGGTACCGAGCTCGGATCCTGGCATTCCGGTACTGTTGGTAAAGCCACCATGG
2 x SV40 early
poly(A) signal
HindIII
XbaI
EcoRI
Luciferase
ori
pXP1
6158bp
XbaI
Amp
SV40 small
T intron
EcoRI
SV40 early
poly(A) signal

Figure 2.3

Plasmid map of the bacterial expression vector pGEX-4T-1

The plasmid map of pGEX-4T-1 is presented, highlighting the restriction enzyme sites present in the multiple cloning site, the origin of replication (ori), Ptac promoter and Ampicillin, Lac I^q and glutathione S-transferase coding regions.


918
977
Thrombin
Leu Val Pro Arg↓Gly Ser Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT CGT GAC TGA
BamH I
EcoR I
Sma I
Sal I
Xho I
Not I
Stop codons
Tth111 I (1136)
Aat II (1241)
Bal I (463)
glutathione S-transferase
Ptac
BspM I (63)
pSj10ΔBam7Stop7
Ampr
Pst I (1918)
pGEX-4T-1
4969 bp
Nar I (4307)
Hpa I (4172)
EcoR V (4116)
lacIq
p4.5
AlwN I (2638)
BssH II (4079)
Apa I (3875)
BstE II (3849)
Mlu I (3668)
pBR322
ori

2.8 Mini-Prep purification of plasmid DNA (Alkaline Lysis Method)

A single colony was used to inoculate 3 mls of L-Broth + 100 μg/ml Ampicillin and grown overnight in a 37°C shaking incubator. One and a half millilitres of the culture was transferred to a 1.5 ml Microcentrifuge tube and centrifuged at 13000 xg for 20 seconds. (The rest of this method was done at room temperature unless otherwise indicated.) The L-Broth supernatant was completely removed by aspiration and the cell pellet was resuspended in 100 μl of Solution 1 (25 mM Tris-HCL pH 8.0, 10 mM EDTA pH 7.6, 15% Sucrose) plus a small amount of Lysozyme powder. The cells were incubated for 1 minute then 200 μl of Solution 2 was added (0.2 M NaOH, 1% SDS). The tubes were inverted to achieve mixing and then incubated for 5 minutes. To this mixture 125 μl of 3 M Sodium Acetate pH 4.6 was added and again the tubes were inverted to achieve mixing and incubated for 15 minutes. The mixture was then spun at 13000 xg for 15 minutes to pellet chromosomal and cellular debris. The supernatant was removed to a new 1.5 ml Microcentrifuge tube, 2 μl of a 10 mg/ml solution of RNaseA added and incubated at 37°C for 15 minutes to digest RNA. After incubation 100 μl of TE buffered phenol and 100 μl of chloroform were added, the tube vortexed briefly, spun at 13000 xg for 5 minutes and the supernatant was removed to a new Microcentrifuge tube. To precipitate the plasmid DNA 1 ml of 100% ethanol was added and spun at 13000 xg for 5 minutes. The DNA pellet was washed with 30 μl of 70% ethanol and spun again for 1 minute. The DNA pellet was then resuspended in 20 μl of TE Buffer.

2.9 Large Scale purification of plasmid DNA (Alkaline Lysis)

Glycerol stocks of bacterial strain MC1061, transformed with the appropriate plasmid were used to inoculate 400 mls of L-Broth + 100 μg/ml Ampicillin and the culture was grown overnight in a 37°C, shaking incubator. The following day the culture was spun at

2830 xg for 10 minutes at 4°C to pellet the bacteria. The supernatant was removed and the pellet was resuspended in 3 mls of Solution 1(25 mM Tris-HCL pH 8.0, 10 mM EDTA pH 7.6, 15% sucrose). The cell suspension was transferred to an Oakridge tube where a small amount of lysozyme was added, the mixture mixed and incubated on ice for 10 minutes. Six millilitres of Solution 2 (0.2 M NaOH, 1% SDS) was added, gently mixed and incubated on ice for 5 minutes. Next 3.75 mls of 3 M Sodium Acetate pH 4.6 was added mixed gently and incubated on ice for 10 minutes. The mixture was spun at 39200 xg for 20 minutes to pellet chromosomal DNA and cellular debris. The supernatant was transferred to a new Oakridge tube, 60 µl of a RNaseA solution (10 µg/µl) was added and then incubated at 37°C for 30 minutes. The solution was extracted twice using Phenol/Chloroform, the DNA precipitated by adding 20 mls of ice cold 100% ethanol to the Phenol/Chloroform supernatant and then pelleted by spinning at 18600 xg for 10 minutes. The resulting DNA pellet was washed with 30 mls of 70% ethanol and resuspended in 0.8 mls of dH_2O. When fully resuspended 0.2 mls of 4 M NaCl and 1 ml of Polyethylene Glycol was added, mixed thoroughly and incubated on ice for 1 hour. The mixture was then separated into 2 Microcentrifuge tubes and spun at 13000 xg for 10 minutes. The supernatant was removed and the DNA pellet was resuspended in 200 µl of dH_2O. The tubes were vortexed until the pellet was fully dissolved then 20 µl of 3 M Sodium Acetate pH 5.5 and 500 µl of 100% ethanol was added. The tubes were inverted to mix the solutions then spun at 13000 xg to pellet the DNA. The supernatant was removed and the pellet dissolved in 100 µl of TE buffer.

2. 10 Purification of DNA fragments using the GeneClean kit (Gene Works)

A slice of agarose containing a DNA fragment was excised under UV-transillumination and put in a 1.5 ml microtube. DNA was eluted from the agarose gel fragment according to procedure of manufacturer.

2. 11 DNA Ligation

Plasmid DNA and the DNA insert were digested with restriction enzymes that generated compatible ends. The ligation was routinely carried out in a total volume of 10 µl containing DNA insert:vector of 3:1 (molar ratio), 1 x ligation buffer (50 mM Tris-HCL pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and 2 units of T4 DNA ligase at room temperature for one hour.

2. 12 Preparation of Competent bacteria for Calcium Chloride mediated transformation

A single colony was inoculated into 10 mls of L-Broth grown overnight in a 37°C shaking incubator. One millilitre of this starter culture was used to inoculate 50 mls of L-broth and was grown at 37°C, shaking until the culture reached OD_{600} of 0.6. The bacteria were pelleted in pre-cooled tubes at 3000 xg, 4°C for 5 minutes and the cells resuspended in ice cold 0.1 M $MgCl_2$. The cells were then pelleted again at 3000 xg, 4°C for 5 minutes. The pellet was resuspended in 2 mls of ice cold 0.1 M $CaCl_2$ and incubated on ice for one hour. (200 µl of cells were used per transformation).

2. 13 Transformation of bacterial Competent cells

For transformation of ligations, 2 µl of the ligation reaction was added to 200 µl of competent cells, gently mixed and incubated on ice for 30 minutes. The cells were heat shocked at 42°C for 2 minutes then placed back on ice for 5 minutes. One millilitre of L-Broth was added and incubated at 37°C for 30 minutes. The cells were briefly pelleted, resuspended in a small volume of L-Broth and plated onto L-Agar plates containing 100

μg/ml Ampicillin and incubated at 37°C overnight. Transformed colonies were picked and used to inoculate 3 mls of L-Broth with 100 μg/ml Ampicillin and grown overnight in a 37°C shaking incubator. Miniprep purification of the plasmid was then undertaken followed by diagnostic restriction enzyme digests and sequencing analysis.

2. 14 Sequencing

Plasmid DNA was isolated and purified as described previously. Sequencing was undertaken using the ABI PRISM™ dye terminator cycle sequencing ready reaction kit according to the procedure of the manufacturer, Perkin Elmer. Sequencing reactions were analysed by the sequencing service at the Institute of Medical and Veterinary Science, Adelaide.

2. 15 Glutathione S-Transferase (GST)-fusion protein induction and purification

Glycerol stocks of bacterial strain MC1061, transformed with the appropriate plasmid were used to inoculate 40 mls of L-Broth + 100 μg/ml Ampicillin and supplemented with glucose to a final concentration of 2%. The culture was grown overnight in a 37°C, shaking incubator. The following day the overnight culture was diluted 1/10 in L-Broth + 100 μg/ml Ampicillin (supplemented with glucose, 2% final), and grown in a 37°C, shaking incubator for 2 hours (OD_{260} 0.5). Once the cells had reached the appropriate optical density IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added to a final concentration of 0.1 mM (for dbpB-GST expression) or 0.5 mM (for dbpAv-GST expression) and the cells were grown for either 3 hours for dbpB-GST or 4 hours for dbpAv-GST expression. The cells were pelleted at 4420 xg for 5 minutes, the supernatant removed and the pellet resuspended in PBS plus Triton X-100 (1% final), Lysozyme (5 mg), incubated for 30 minutes on ice. The pellet was

then sonicated three times for 10 seconds, on ice for complete lysis of the bacteria. The cellular debris was removed by centrifugation 13000 xg for 15 minutes at 4 degrees. The supernatant was removed and 150 µl of 50% glutathione sepharose beads was added. The mixture was mixed on a rotating wheel at room temperature for 30 minutes. The beads were pelleted at 500 xg for 5 minutes. The beads were washed 3 times with 10 mls of PBS.

a) GST-fusion proteins were eluted from the sepharose via three rounds of 150 µl reduced glutathione (15 mM reduced glutathione in Tris-HCL pH 7.6). The elutions were assayed for protein concentration via Bradford assay. The recombinant proteins were stored at 4°C.

b) CSD protein without a GST tag were produced by cleaving the GST tag via thrombin. Once the GST-CSD fusion proteins had been bound to the glutathione sepharose column and washed the beads were resuspended in 300 µl of 1 x PBS and 3 units of thrombin and left on a rotating wheel at room temperature for 12 hours. The beads were pelleted at 500 xg for 5 minutes and the supernatant containing thrombin cleaved CSD proteins collected. The elutions were assayed for protein concentration via Bradford assay. The recombinant proteins were stored at 4°C.

2. 16 Fractionation of Nuclear and Cytoplasmic Proteins

Nuclear and Cytoplasmic extracts were prepared by two alternate methods, labelled technique 1 and technique 2. Technique 1 is the method described by Dignam *et al.* (Dignam *et al.*, 1983). Technique 2 is a modification of the method described by Schriber *et al.* (Schreiber *et al.*, 1989) 50 mls of cells ($6x10^5$ cells/ml) were spun at 1300 xg for 5 minutes at 4°C and washed twice with ice cold PBS. The pellet was resuspended in 800 µl of Buffer A (10 mM HEPES pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM

EGTA, 1 mM PMSF, 10 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1 mM DTT) and left on ice for 15 minutes for the cells to swell. The cells were lysed upon addition of 50 μl of 10% Nonident P-40 and vortexed for 30 seconds. Lysates were then microcentrifuged for 30 seconds at 13000 xg to collect the nuclei. The supernatant containing the cytoplasmic fraction was removed and stored at -70°C. The nuclear pellets were then resuspended in 75 μl of Buffer C (400 mM NaCl, 7.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 25% glycerol (v/v), 10 μg/ml Aprotinin, 5 μg/ml Leupeptin) and left shaking for 15 minutes on ice, to disrupt the nuclear membrane. Spinning at 13000 xg for 1 minute pelleted the nuclear debris and the supernatant containing the nuclear proteins was removed and stored at -70°C.

2. 17 Oligonucleotides and ^{32}P-labelled Probe Preparation

All oligonucleotides were purchased from GeneWorks and full-length product was purified from non-denaturing polyacrylamide gels (Maniatis *et al.*, 1982). Single stranded DNA probes for gel retardation assays were prepared by end-labelling 100 ng of oligonucleotide with T4 polynucleotide kinase and [γ-^{32}P] ATP in a 10 μl reaction. The reaction was incubated for 1 hour at 37°C. Following incubation 3μl of Formamide load was added and the sample heat denatured at 100°C for 3 minutes. Full length end labelled oligonucleotide was separated on a 10%-12% non-denaturing polyacrylamide gel, exposed to X-ray film, bands excised and oligonucleotide eluted from gel in 200 μl of TE overnight at 4°C.

2. 18 Gel shift Analysis

Gel retardations were done using 0.25 ng of either single or double stranded ^{32}P-labelled oligonucleotide probes in a 10 µl reaction mix of 0.5 x TM buffer (1 x TM 50 mM Tris-HCL pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% Glycerol, 1 mM DTT) containing 200 mM KCL, 0.4 µg of poly (dI-dC) and either 1 µg of nuclear extract or 25 ng of recombinant CSD fusion proteins or recombinant RelA. Retardation assays using recombinant protein also contained 1 µg of bovine serum albumin. Reactions were incubated at room temperature for 20 minutes then acrylamide load was added and the reactions were run on 12% (for nuclear extracts) or 6% (for recombinant proteins) non-denaturing polyacrylamide gels run in 0.5 x TBE (Shannon *et al.*, 1989). The gels were dried and exposed to X-ray film overnight.

2. 19 UV Cross-Linking

Nuclear extracts from HUT78 T cells, HEL Fibroblasts and Jurkat T cells were bound to ^{32}P-labelled single stranded DNA probes in a gel retardation reaction. The UV cross linking experiment was performed in a 25 µl reaction mix volume of 0.5 X TM buffer ((Coles *et al.*, 1994); (Coles *et al.*, 1996); (Shannon *et al.*, 1988)) containing 200 mM KCL, 0.4 µg of poly (dI-dC), 4 µg of nuclear extract and 1 ng of ^{32}P-labelled single stranded DNA probe. The reaction was left for 20 minutes at room temperature then the complexes were separated on a 12% non-denaturing polyacrylamide gel run in 0.5 x TBE buffer. The gel was exposed to UV light (340 nm) for 15 minutes to cross-link bound protein to the DNA. The gel was exposed to X-ray film for 12 hours at 4°C and the retarded complexes were excised. The resulting gel slices were incubated in 100 µl of SDS-PAGE load buffer for 30 minutes at 37°C. The gel slices were then placed into the wells of a 12% Laemmli SDS-polyacrylamide

gel (Laemmli 1970) and electrophoresed at 30 mA for 2-3 hours to fractionate the individual proteins in the complex on the basis of their size (Coles *et al.*, 1994); (Maniatis *et al.*, 1982). The gels were dried down and exposed to X-ray film overnight at -70°C using an intensifying screen.

2. 20 Competitions

Competitions with unlabelled single strand oligonucleotides were performed by adding protein and unlabelled probe into a reaction mix as described above, followed by immediate addition of the ^{32}P-labelled probe (Coles *et al.*, 1996). The reaction was left to incubate for 20 minutes as room temperature before being analysed on 12% (for nuclear extracts) and 6% (for recombinant proteins) non-denaturing polyacrylamide gels run in 0.5 x TBE. The gels were dried and exposed to X-ray film overnight.

2. 21 South Westerns

Proteins were separated on a Laemmli SDS-PAGE protein gel (Laemmli 1970) and then transferred to nitrocellulose using a BIO-RAD protein transfer apparatus in South/Western transfer buffer (125 mM Tris, 950 mM Glycine) at 100 mA over night. After transfer, the filter was washed briefly in transfer buffer then the proteins were renatured on the filter in renaturation buffer (10 mM Tris-HCL pH 7.5, 150 mM NaCl, 2.5% NP-40, 10% Glycerol, 5% Skim milk powder, 10 mM DTT) at room temperature for 1 hour, then the buffer was replaced twice with new buffer each time with a further 1 hour incubation. The filter was rinsed briefly in Renaturation Buffer minus poly(dI:dC) (25 mM Tris-HCL pH 7.9, 6.3 mM $MgCl_2$, 0.5 mM EDTA, 10% Glycerol, 200 mM KCl, 0.125% Skim milk powder, 10 µg/ml Poly(dI:dC)). ^{32}P-labelled oligonucleotide probe was added at a concentration of 25 ng/ml to complete Binding Buffer, and incubated with the filter overnight gently rocking at

room temperature. The filter was washed 2 times for 5 minutes in ice cold Wash buffer 1 (100 mM Tris-HCL pH 7.5, 100 mM KCl) then once for 5 minutes in ice cold Wash buffer 2 (10 mM Tris-HCL pH 7.5, 200 mM KCl). The filter was sealed in plastic and exposed to x-ray film overnight.

2. 22 Antibody production and purification

Antibodies were made in rabbits to both full-length recombinant dbpA-GST and dbpB-GST and 3 short peptides to regions of dbpA and dbpB. Peptides were conjugated to the hapten keyhole limpet hemocyanin via the Pierce Imjet® Immunogen EDC conjugation kit. The Veterinary Services Division of the Institute of Medical and Veterinary Science injected conjugated peptides or GST-fusion proteins into rabbits. Blood from immunised rabbits was collected and allowed to clot, serum was collected and stored at 4°C. Immunoglobulins were partially purified via ammonium sulphate precipitations (Harlow *et al.*, 1988). Proteins were removed from the sera via precipitation with 25% (final) ammonium sulphate (mixing on a wheel at 4°C overnight) and then centrifugation at 1600 xg, 30 minutes. The supernatant was removed and immunoglobulins precipitated with 50% (final) ammonium sulphate (mixing on wheel at 4°C for 24 hours) before centrifugation at 1600 xg, 30 minutes. The immunoglobulin pellet was washed 3 times with saturated ammonium sulphate with centrifugation step (1600 xg, 30 minutes) in between each wash. The immunoglobulin pellet was resuspended in a tenth of the original volume of PBS and dialysed against PBS for 24 hours. The IgG immunoglobulin fraction was isolated via Protein A Sepharose (Amersham Pharmacia Biotec). A 50% protein A slurry was made in PBS and added to a chromatography column. Ammonium sulphate purified sera was added to the column and allowed to drip through. The column was washed 8 times with 2 column volumes of wash buffer (25 mM Tris-HCL pH 8, 100 mM NaCl, 0.02% Tween 20). Bound

IgG was eluted from the column with 4 column volumes of 0.1 M Glycine-HCL pH 2.7. The eluted antibody was brought to a neutral pH with un-pH'ed 1 M Tris-HCL.

2. 23 Western Blot

Proteins were separated on a Laemmli SDS-PAGE protein gel (Laemmli 1970) and then transferred to nitrocellulose using a BIO-RAD protein transfer apparatus in SDS-PAGE transfer buffer at 100 mA over night. (20 mM Tris-HCL, 150 mM Glycine, 20% Methanol (v/v) 0.1% SDS). The filter was then blocked in a 1% Boehringer blocking reagent made up in a PBS/Tween 20 solution, (PBS plus 0.2% Tween 20 (v/v)) for 1 hour. The blocking reagent was then removed, the filter placed in an appropriate volume of PBS/Tween 20 solution plus 1% BSA (w/v) and primary antibody and left gently shaking for 1 hour. To remove any excess binding the filter was washed 2 times in PBS/Tween 20 + 1% BSA followed by 4 washes in the PBS/Tween 20 solution. Each wash was for a duration of 10 minutes. The secondary antibody (Horseradish peroxidase conjugated goat-anti-rabbit DAKO) was diluted 1:2000 in PBS/Tween 20 solution and incubated with the filter for 1 hour, gently shaking. The secondary antibody solution was removed and the filters were washed as described above. Antibody binding was detected using ECL reagents as described by the manufacturer Amersham Pharmacia Biotec and exposed to X-ray film.

2. 24 Antibody blocking experiments

Antibody blocking experiments were performed by adding protein and antibody to a gel retardation reaction mixture as described above with the exception that the poly (dI:dC) concentration was increased to 2 μg. The antibody and protein were incubated for 5 minutes at room temperature before adding 0.25 ng of the appropriate ^{32}P-labelled probe. The reaction was then incubated for a further 20 minutes at room temperature before being

analysed on 10% or 12% polyacrylamide gels run in 0.5 x TBE buffer. The gels were dried and exposed to X-ray film overnight.

2. 25 Human Embryonic Lung (HEL) Transient Transfections and CAT assays

HEL (passage 7) cells were thawed from liquid nitrogen storage and grown in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml Penicillin and 100 U/ml Gentamycin antibiotics and 0.25% sodium bicarbonate until passage 9. Cells were then trypsinised and seeded into 10 cm petri dishes containing DMEM and left to grow for 2 days to semi-confluence. Two days later all of the media was aspirated off and 4 mls of fresh DMEM was added. The DNA (15 μg for each transfection) was diluted into 80μl of TBS (1.3 M NaCl, 51 mM KCl, 5.6 mM Na_2HPO_4, 0.25 mM Tris, 0.1 mM $CaCl_2$, 50 mM $MgCl_2$, pH'd to 7.5) (Maniatis *et al.*, 1982)and mixed. This mixture was then added drop-wise to 160 μl of DEAE-Dextran (10 mg/ml stock of 2 x 10^6 molecular weight Dextran made up in TBS) and mixed well. The mixture was then added drop-wise around the plate and the plate gently rocked to disperse the DEAE-Dextran/DNA mix. The plates were then incubated in a 37°C tissue culture incubator for 2 hours. Two hours later the media was removed from the cells by aspiration and the cells were 'shocked' with 5 mls of 10% DMSO (DMSO diluted in PBS). The DMSO solution was left for 1 minute then removed by aspiration, followed by a 5 ml PBS wash. The PBS was aspirated, 10 mls of fresh DMEM added and the cells left to recover overnight in the incubator. The next day the cells were stimulated with TNFα (100 units per ml of media final) and left for 24 hours. For each transfection there was a stimulated and un-stimulated sample. The next day the media was aspirated and the cells washed once with 5 mls of PBS. The cells were then scraped off the plate into 1 ml of ice cold PBS and pelleted for 1 minute 13000 xg. The supernatant was removed and the cells

were resuspended in 50-200 µl of ice cold 0.25 M Tris- HCL pH 7.6 (depending on pellet size). The cells were lysed via three freeze thaw cycles in liquid nitrogen and a 37°C water bath respectively. The cell debris was removed by centrifugation at 13000 xg for 5 minutes at 4°C and the resulting supernatant extract removed to a new Microcentrifuge tube and kept on ice. The protein concentration in the extracts was determined using Bradford reagent comparing to known concentrations of BSA standards. Forty micrograms of each protein extract was used in the Chloramphenicol Acetyl Transferase (CAT) assay. (40 µg of protein extract, 0.2 M Tris-HCL pH 8, 0.7 mM Acetyl Co-enzyme A, 0.2 µCi (4 nM) ^{14}C Chloramphenicol) The mixture was incubated for 60 minutes at 37°C. Five hundred microliters of ethylacetate was added to each tube, vortexed and the phases separated by centrifugation, 5 minutes at 13000 xg. The top phase was removed to a new Microcentrifuge and the ethylacetate dried down using the spedi-vac vacuum dryer. The pellet was resuspended in 5 µls of ethylacetate and spotted onto a TLC chromatography plate. The acetylated and un-acetylated ^{14}C Chloramphenicol was separated using a 95% chloroform, 5% methanol (v/v). When the chloroform/methanol mixture had run three quarters up the TLC plate, the plate was removed from the mixture and allowed to dry. It was then wrapped in plastic wrap and exposed to a phosphoimager plate for 2 days before analysis. The phosphoimager plate was scanned using Molecular Dynamics Phosphoimager and Image Quant software. The acetylated and un-acetylated fractions for each transfection (both stimulated and unstimulated) were measured and compared to the backbone vectors control (SV2CAT + SG5) to give a final relative measurement.

2. 26 Jurkat T cell Transient Transfections and Luciferase Assays

The Jurkat T cell line was cultured in RPMI medium containing 10% heat inactivated foetal calf serum (FCS), supplemented with 2 mM L-glutamine, 100 U/ml Penicillin and 100

U /ml Gentamycin antibiotics and 0.25% sodium bicarbonate. Jurkat T cells were pelleted at 400 xg for 5 minutes in a bench top centrifuge, the supernatant removed by aspiration and the cell pellet resuspended to 4.5 x 10^6 cells per 500 µl in RPMI medium with 20% FCS. DNA was added to electroporation cuvettes (5 µg of reporter plasmid and 10 µg of expression plasmid) then the 500 µl of Jurkat T cells was added and the cell/DNA mix allowed to sit for 10 minutes prior to electroporation. Electroporation with a Bio-Rad Gene Pulser was used for transfection at 270 V and a capacitance of 960 µF. The cells were left to rest for 10 minutes post electroporation then 1 ml of RPMI medium was added and the entire contents of the cuvette transferred to a tissue culture flask with 9 mls of RPMI medium. The cells were left for 24 hours to recover then stimulated with 20 ng/ml (final) Phorbol-12-myristate-13-acetate (PMA Sigma) and 1 µM (final) Calcium ionophore (A23187 Boehringer Mannheim) and 8 hours post-stimulation, were assayed for luciferase activity. Cells were pelleted at 400 xg for 5 minutes and washed 2 times in PBS. The resulting pellet was then resuspended in 200 µl of Lysis Buffer (100 mM potassium phosphate buffer, 2 mM DTT, 0.01 mM EDTA) and freeze thawed 3 times in liquid nitrogen and a 37°C water bath. The cellular debris was pelleted by centrifugation for 5 minutes at 1500 rpm and the protein concentration was assayed using Bradford reagent. Using a 96 well black Packard Lite-Plate™ 400 µl of Luciferase Assay Buffer (100 mM potassium phosphate buffer, 2 mM DTT, 10 mM $MgSO_4$, 320 mM Coenzyme A and 500 mM ATP) was placed into the appropriate amount of wells then 20 µg of protein extract was added. Just before reading the plate in a Packard Topcount Luminometer 40 µl of 1 mM D-Luciferin was added to each well. Luminocity was measured in counts per second and all values were compared back to the empty vector unstimulated transfection to give a relative measurement.

Chapter 3

Differential regulation of the human GM-CSF proximal promoter by CSD proteins in fibroblasts and T cells

Smithers, release the hounds!

Mr Burns

3.1 Introduction

At the commencement of this project, repressor sites that functioned in Human Embryonic Lung (HEL) fibroblasts had been identified in both Domain 1 (-114 to –71) and Domain 2 (-70 to –31) regions of the proximal human granulocyte macrophage-colony stimulating factor (GM-CSF) promoter. Two sites were identified on the non-coding (-) strand of domain 1 (5'-CCTG-3') and two sites on the coding (+) strand of domain 2 (5'-CCTG-3' and 5-ACCA-3') (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). Analysis of these sites identified the binding of single stranded DNA specific nuclear factors called NF-GMb and NF-GMc (Coles *et al.*, 1994). These factors were subsequently cloned and identified as members of the Cold Shock Domain (CSD) family of proteins (Coles *et al.*, 1996). Cold shock domain family members generally have three domains, 1) a N-terminal domain, which has no defined function but is postulated to aid in the overall functions of the protein, 2) the highly conserved central cold shock domain, which is responsible for sequence specific DNA and RNA interactions and, 3) a C-terminal region which is implicated in protein:protein interactions. The two CSD family members isolated were dbpB (also known as YB-1, p50, EF1A) and a variant of dbpA called dbpAv (Coles *et al.*, 1996). The variant dbpAv has four amino acid changes and an additional 30 C-terminal amino acids compared to the original dbpA in the Genbank database (Coles *et al.*, 1996). The two CSD factors identified have a high degree of homology, especially within the cold shock domain region, but display variation within the C-terminal protein:protein interaction domain.

Here I further investigate the mechanisms of GM-CSF promoter repression by CSD proteins dbpAv and dbpB in HEL fibroblasts. One of the major focuses in the laboratory is also the study of GM-CSF regulation in T cells. Since T cells are major sources of GM-CSF *in vivo* and differences have been observed in the regulation of the GM-CSF promoter

in T cells and fibroblasts (Shannon *et al.*, 1997), the role of CSD proteins in GM-CSF regulation in Jurkat T cells was also investigated.

3.2 Construction of GM-CSF reporter constructs for analysis in fibroblasts and T cells.

To analyse GM-CSF promoter function, a series of promoter constructs were made in either chloramphenicol acetyltransferase (CAT) or luciferase reporter vectors. Reporter vectors pBLCAT2 (Luckow *et al.*, 1987) and pXP1 (Bert *et al.*, 2000b) are shown in Figures 1 and 2 in the material and methods section and all reporter constructs used in this chapter are shown in Figure 3.1. The sequence of the human GM-CSF proximal promoter (-114 to +28) from which constructs are derived, is shown in Figure 3.1A. Sequences contained within CAT (Figure 3.1B) and luciferase (Figure 3.1C) reporter constructs used in transient transfection experiments are indicated. The regions of the GM-CSF promoter (-114 to -31 for pGM43-CAT and -65 to -31 for pGM41-CAT) that were cloned upstream of the basal thymidine kinase (TK) promoter present in the CAT reporter vector pBLCAT2 as shown in Figure 3.1B. The pGM43-CAT and pGM41-CAT constructs were obtained from Dr. Leeanne S. Coles (Coles *et al.*, 1994).

Sequences contained within luciferase reporter constructs pGMCK-1(2)-TK/Luc, pGM1-Luc, pGM1(mut1)-Luc, pGM2-Luc, pGM4-Luc and pGM-Luc are indicated in Figure 1C. The number following the pGM in each construct name indicates how many CSD binding sites are present. The pGMCK-1(2)-TK/Luc construct was created by cloning 2 copies of the GM-CSF domain 1 region (-114 to +28) upstream of the minimal TK promoter present in the pTK81 luciferase reporter vector. The full GM-CSF promoter (-627 to +28) was cloned into the pXP1 luciferase vector to create pGM-Luc. The pGMCK-1(2)-TK/Luc and pGM-Luc constructs were obtained from Dr S. Roy Himes (Himes *et al.*, 1993); (Himes *et al.*, 1996a). All other luciferase reporter plasmids represented in Figure 3.1C were constructed by inserting oligonucleotides encoding human GM-CSF promoter fragments into the pXP1 luciferase vector. The plasmids pGM1-Luc, pGM2-Luc and pGM4-Luc were constructed by cloning oligonucleotides (with *Hind*III 5'

Figure 3.1

GM-CSF promoter and transfection constructs used in transient transfections of HEL fibroblast and Jurkat T cells.

A) The sequence of the human GM-CSF proximal promoter.

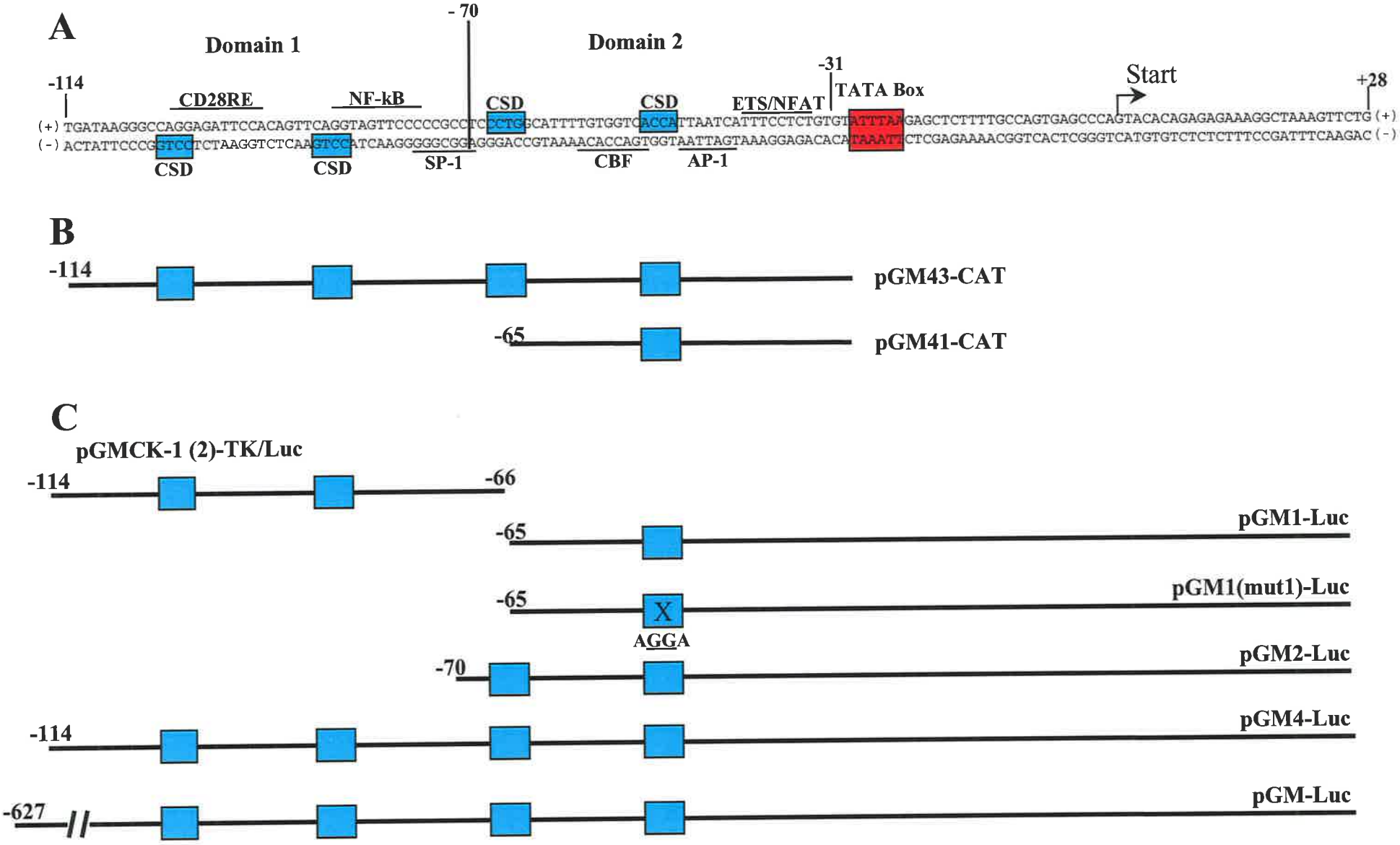
Domain 1 (-114 to –71) and Domain 2 (-70 to –31) regions are indicated. The binding sites for many double stranded transcription factors which mediate GM-CSF expression including: NF-κB, CBF, SP-1, AP1, ETS/NFAT and the CD28RE are underlined (Shannon *et al.*, 1995); (Shannon *et al.*, 1997). Nuclear NF-GMb/c and recombinant CSD factor binding sites are indicated on the non-coding (-) strand in Domain 1 and the coding (+) strand in Domain 2.

B) GM-CSF promoter CAT reporter constructs used in HEL fibroblast transient transfections.

GM-CSF promoter, CAT reporter constructs used, pGM43-CAT and pGM41-CAT are shown diagrammatically. Numbers indicate distance from the transcriptional start site and boxes represent wild type CSD binding sites.

C) GM-CSF promoter luciferase reporter constructs used in Jurkat T cell transient transfections.

GM-CSF promoter, luciferase reporter constructs used, pGMCK-1(2)-TK/Luc, pGM1-Luc, pGM1(mut1)-Luc, pGM2-Luc, pGM4-Luc and pGM-Luc are shown diagrammatically. Numbers indicate distance from the transcriptional start site and boxes represent wild type CSD binding sites. Mutation of the CSD binding site in pGM1(mut1)-Luc is represented by a box containing a 'X' and the altered sequence is given below it.


A
Domain 1
Domain 2
- 70
-114
-31
+28
Start
CD28RE
NF-kB
CSD
CSD
ETS/NFAT
TATA Box
(+) TGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTCCCCCGCCTCCCTGCATTTTGTGGTCACCATTAATCATTTCCTCTGTGTTATTTAAGAGCTCTTTTGCCAGTGAGCCCAGTACACAGAGAGAAAGGCTAAAGTTCTG (+)
(-) ACTATTCCCGGTCGTCTAAGGTCTCAAGTCCATCAAGGGGGCGGAGGGACCGTAAAACACCAGTGGTAATTAGTAAAGGAGACACATAAATTCTCGAGAAAACGGTCACTCGGGTCATGTGTCTCTCTTTCCGATTTCAAGAC (-)
CSD
CSD
SP-1
CBF
AP-1
B
-114
pGM43-CAT
-65
pGM41-CAT
C
pGMCK-1 (2)-TK/Luc
-114
-66
pGM1-Luc
-65
pGM1(mut1)-Luc
-65
X
AGGA
pGM2-Luc
-70
pGM4-Luc
-114
pGM-Luc
-627

and *Bam*HI 3' ends) spanning regions of the GM-CSF promoter (-65 to +28), (-70 to +28) and (-114 to +28) respectively into the *Hind*III/*Bam*HI sites of the multiple cloning site of pXP1 (Figure 3.1C). The Stratagene QuickChange™ Site-Directed Mutagenesis kit was used to mutate the CSD binding site in pGM1-Luc from 5'-ACCA-3' to 5'-AGGA-3' to create the pGM1-(mut1)-Luc reporter plasmid. Mutation of the CSD binding site in the construct pGM1(mut1)-Luc is represented by a box with an "X" in it with the altered sequence given below it (Figure 3.1C).

3.3 CSD factors dbpAv and dbpB repress the GM-CSF promoter function in HEL fibroblasts.

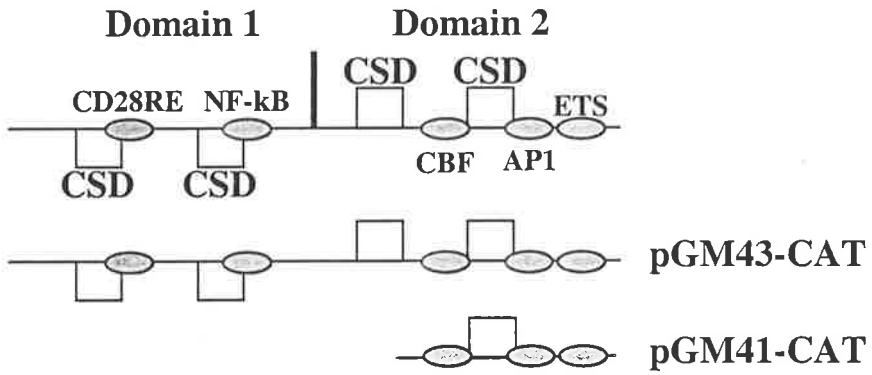
It has been previously reported in HEL fibroblasts that the domain 2 region of the GM-CSF promoter is TNFα inducible (via ETS, AP1, CBF binding sites, Figure 1A) and that the upstream domain 1 region contained sequences which acted to repress this TNFα-mediated induction (Coles *et al.*, 1996); (Coles *et al.*, 2000). Domain 1 sequences were also found, in part, to contribute to TNFα activation through the NF-κB sites (Coles *et al.*, 1996). From binding analysis of HEL fibroblast nuclear CSD complexes, four CSD sites have been identified across the proximal (-114 to +28) region of the human GM-CSF promoter, 2 sites 5'-CCTG-3' on the non-coding (-) strand along domain 1 (-114 to -71) and 2 sites along the coding strand (+) of domain 2 (-70 to -31) a 5'-CCTG-3' and 5'-ACCA-3' (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000) (Figure 1A). At the commencement of this work, it had been confirmed by mutation analysis that the domain 1 CSD sites acted as repressor sequences (Coles *et al.*, 1994). To determine the function of the cloned CSD factors dbpAv and dbpB on the GM-CSF proximal promoter in HEL fibroblasts, transient transfection overexpression experiments were performed (Section 2.25 of the materials and methods section).

The eukaryotic expression constructs for dbpAv (pSGdbpAv) and dbpB (pSGdbpB), used in all the transient transfection experiments, were created by cloning the respective dbpAv and dbpB coding regions from λgt cDNA expression library clones into the eukaryotic overexpression vector pSG5 (Figure 2.1 in materials and methods section). The pSGdbpAv and pSGdbpB constructs were obtained from Dr. Leeanne S. Coles (Coles *et al.*, 1996). CAT reporter constructs containing both the domain 1 and 2 regions of the human GM-CSF proximal promoter pGM43-CAT (-114 to -31, all 4 CSD sites) or only the domain 2 region pGM41-CAT (-70 to -31, only 2 CSD sites) were used in the transient transfections. These GM-CSF promoter CAT reporter constructs were co-transfected into HEL fibroblasts with either the empty expression vector (pSG5), or expression constructs for either full-length dbpAv (pSGdbpAv) or dbpB (pSGdbpB) and cells were treated with or without TNFα. As reported previously, TNFα stimulation of HEL fibroblasts resulted in activation of the GM-CSF promoter. As expected when the GM-CSF promoter construct pGM43-CAT, which contains the domain 1 CSD repressor sites and domain 2, was co-transfected into HEL cells with the CSD factors dbpAv and dbpB, they both acted to repress the TNFα mediated activation (Figure 3.2A). When the domain 2 GM-CSF promoter construct pGM41-CAT, which is highly inducible with TNFα, was co-transfected with CSD factors dbpB or dbpAv, TNFα induced expression was also repressed (Figure 3.2B). Subsequent experiments in our laboratory, where CSD sites were mutated, revealed that the domain 2 CSD sites, as for domain 1, acted as repressor binding sites (Coles *et al.*, 2000). Hence four CSD binding repressor sites, located across both GM-CSF promoter domains involved in response to TNFα, have been identified (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). To test if the CSD factors were having an effect on the backbone vector, dbpAv and dbpB were co-transfected with pBLCAT2 into HEL fibroblasts. No significant change in CAT activity was observed with either

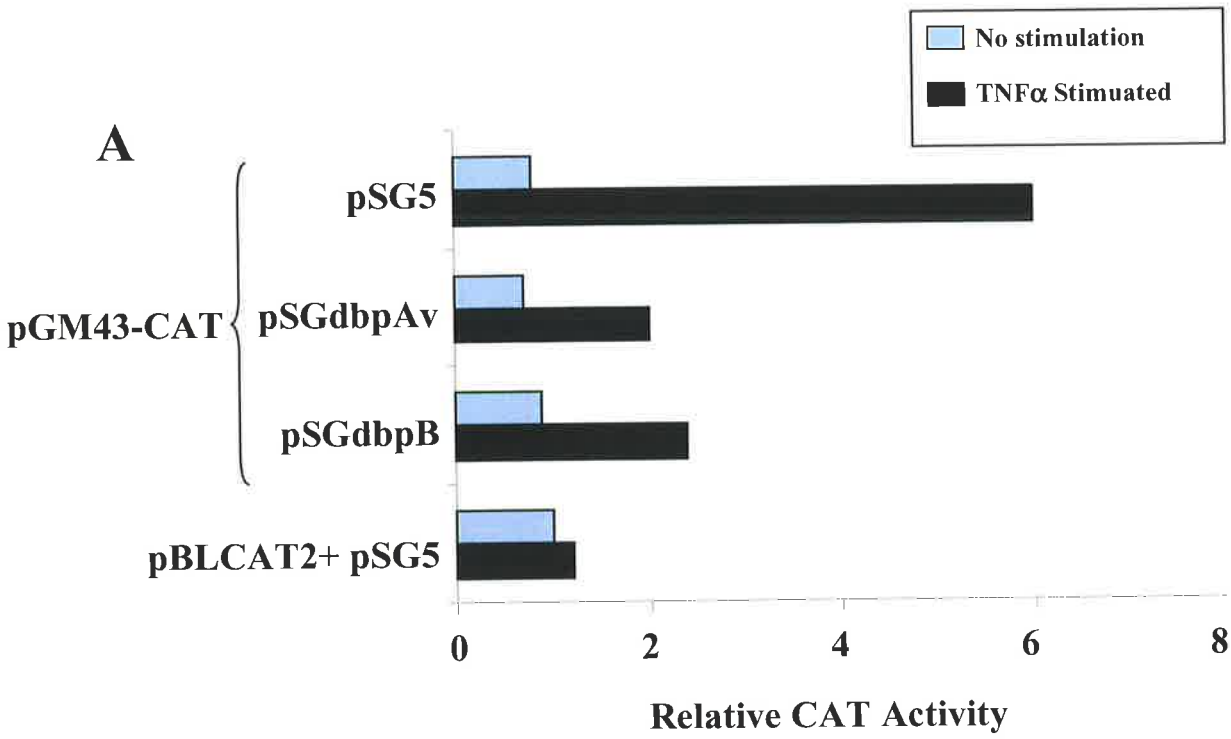
Figure 3.2

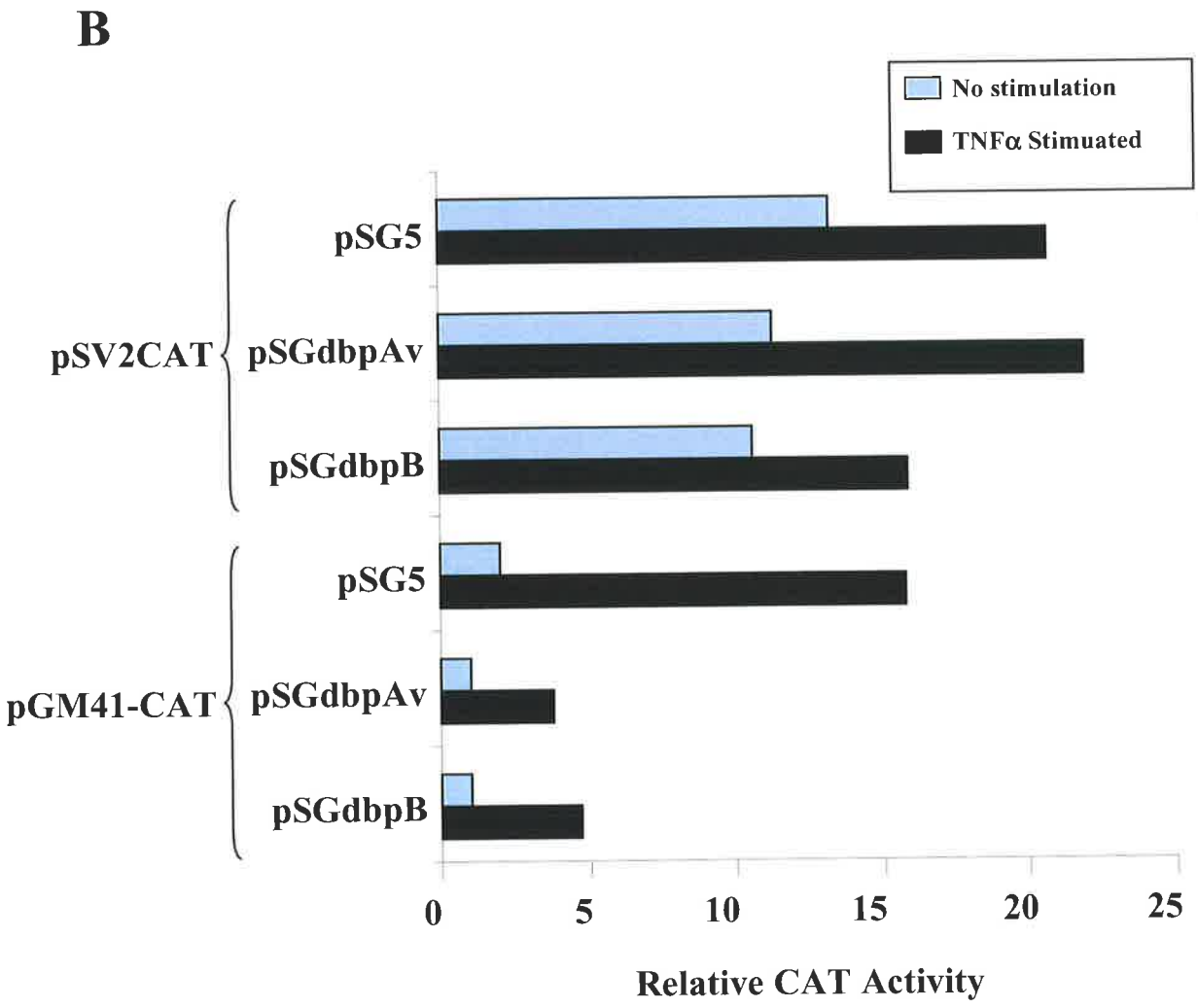
Overexpression of dbpAv and dbpB results in repression of the GM-CSF promoter in HEL fibroblasts.

The reporter plasmids pBLCAT2 and pGM43-CAT (**Figure A**) and pGM41-CAT and pSV2CAT (**Figure B**) were co-transfected into HEL Fibroblasts with empty pSG5 expression vector and either the expression plamids encoding for full length dbpAv (pSGdbpAv) or full length dbpB (pSGdbpB). Cells were treated with or without TNFα for 24 hours, harvested and assayed for CAT activity. The transfection results represent the average of three replicates. All CAT activities are given relative to untreated pBLCAT2 + SG5, which is given a value of 1. The graphed values have standard errors of less than 20%.



The human GM-CSF proximal promoter and CAT reporter plasmids used in these experiments are represented diagrammatically above (sequences are shown in Figure 3.1). Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factor as defined in the diagram above it.


A
No stimulation
TNFα Stimuated
pGM43-CAT
pSG5
pSGdbpAv
pSGdbpB
pBLCAT2+ pSG5
0
2
4
6
8
Relative CAT Activity


B
No stimulation
TNFα Stimuated
pSV2CAT
pSG5
pSGdbpAv
pSGdbpB
pGM41-CAT
pSG5
pSGdbpAv
pSGdbpB
0
5
10
15
20
25
Relative CAT Activity

dbpAv or dbpB irrespective of TNFα stimulation (data not shown). As a control, a construct containing the viral SV40 early promoter was used (Gorman *et al.*, 1982). The pSV2CAT construct was co-transfected into HEL fibroblasts with CSD factors dbpAv and dbpB. As can be seen in Figure 3.2B, dbpAv had no effect on the pSV2CAT promoter construct while dbpB repressed it slightly. It has been reported that *Xenopus* CSD proteins can effect translation (Wolffe *et al.*, 1992); (Wolffe 1993) and since there were only minor effects of dbpAv and dbpB overexpression on pSV2CAT this ruled out major, general effects on CAT reporter protein levels due to changes in translation.

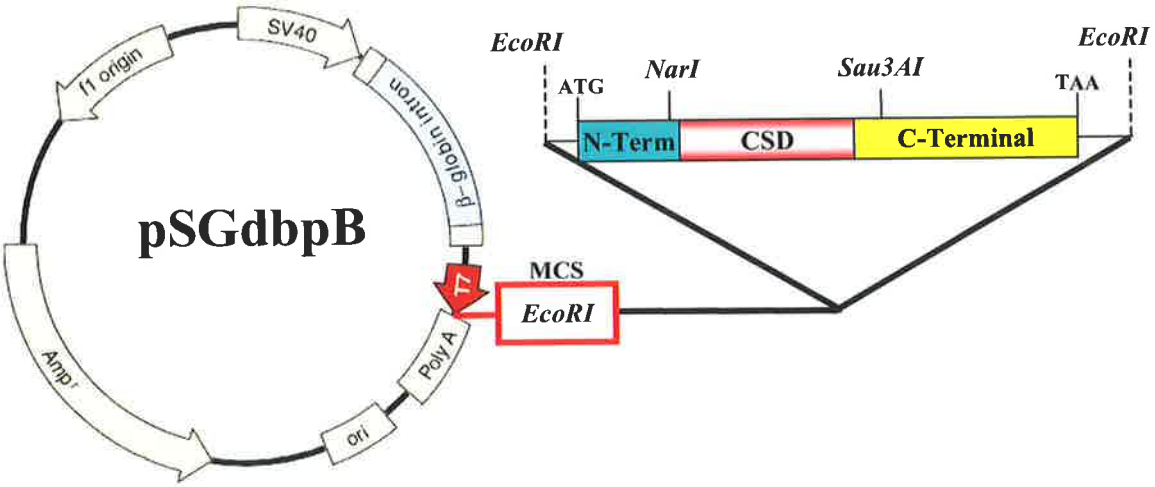
3.4 The Central CSD region of CSD proteins is required for repression of the GM-CSF promoter in HEL fibroblasts.

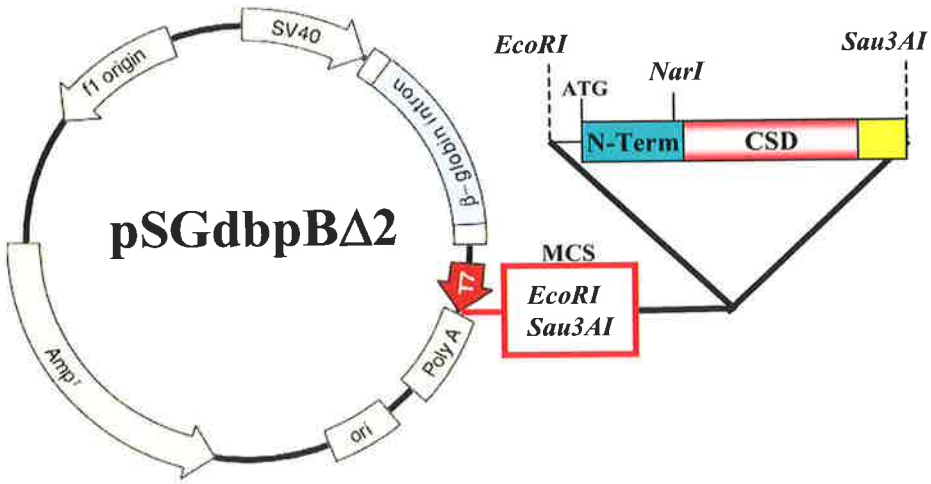
CSD proteins have three functional domains: the N-terminal regions, the function of which is not well defined though it has been attributed with some DNA binding function; the highly conserved CSD domain, essential for sequence specific DNA and RNA binding and the C-terminal region implicated in protein:protein interactions (Nambiar *et al.*, 1998) (Kolluri *et al.*, 1992) (Schroder *et al.*, 1995) (Landsman 1992) (lloberas *et al.*, 1995) (Li *et al.*, 1997) (Raj *et al.*, 1996). To determine which region of the CSD proteins was involved in repression of the GM-CSF promoter in HEL fibroblasts truncations of dbpB were made, removing either the C-terminal (pSGdbpBΔ1) or C-terminal plus CSD domains (pSGdbpBΔ2) of the protein (Figure 3.3). pSGdbpBΔ1, which encodes the first 47 amino acids of dbpB, (Figure 3.3), was created by digesting the eukaryotic dbpB expression construct pSGdbpB with *Nar*I, followed by religation. pSGdbpBΔ2, which encodes the first 173 amino acids of dbpB, (Figure 3.3), was created by digesting pSGdbpB with *Eco*RI and *Sau*3AI, and ligating this fragment into *Eco*RI/*Bgl*II digested pSG5.

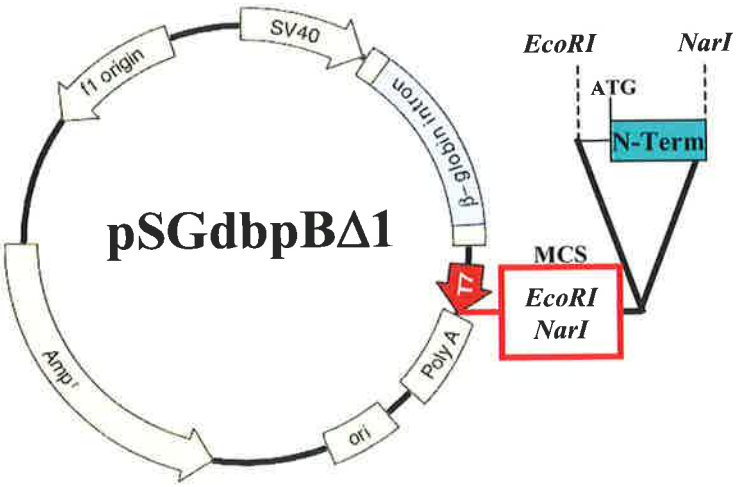
Figure 3.3

Eukaryotic expression constructs encoding for dbpB and truncations

The coding regions of dbpB contained in the expression constructs pSGdbpB, pSGdbpBΔ1 and pSGdbpBΔ2 are shown. Translational start and stop codons and restriction enzyme sites used to ligate dbpB fragments into pSG5 are indicated both at the end of the DNA inserts and in the pSG5 vector multiple cloning site (MCS).


pSGdbpB
f1 origin
SV40
β-globin intron
Amp r
ori
Poly A
MCS
EcoRI
EcoRI
ATG
NarI
Sau3AI
TAA
EcoRI
N-Term
CSD
C-Terminal


pSGdbpBΔ2
f1 origin
SV40
β-globin intron
Amp r
ori
Poly A
MCS
EcoRI
Sau3AI
EcoRI
ATG
NarI
Sau3AI
N-Term
CSD


pSGdbpBΔ1
f1 origin
SV40
β-globin intron
Amp r
ori
Poly A
MCS
EcoRI
NarI
EcoRI
NarI
ATG
N-Term

To determine which region of the CSD protein was involved in repression of the GM-CSF promoter observed in HEL fibroblasts, constructs expressing full length dbpB (pSGdbpB) and truncations of dbpB (pSGdbpBΔ1 and pSGdbpBΔ2) were co-transfected with the CAT reporter constructs pGM43-CAT and pGM41-CAT. As was previously observed, dbpB (pSGdbpB) was able to repress the TNFα mediated activation on both GM-CSF reporter constructs (Figure 3.4A & B). Removal of the potential protein:protein interacting C-terminal domain of dbpB, in dbpBΔ2 (pSGdbpBΔ2), resulted in a some loss in the ability of this protein to repress the TNFα mediated activation on both pGM41-CAT and pGM43-CAT constructs (Figure 3.4A & B). This result suggested that the C-terminal region and hence protein:protein interactions probably plays some role in the repression observed. Truncating the CSD dbpB protein further, in dbpBΔ1 (pSGdbpBΔ1) removing both the C-terminal region and the single stranded DNA interaction CSD domain, resulted in a reduced ability of this truncated protein to repress the TNFα mediated activation on both promoter constructs (Figure 3.4A & B). This result suggested, that the ability of the CSD protein dbpB to repress the GM-CSF promoter, was determined mainly by the CSD domain and hence most likely its ability to bind to DNA.

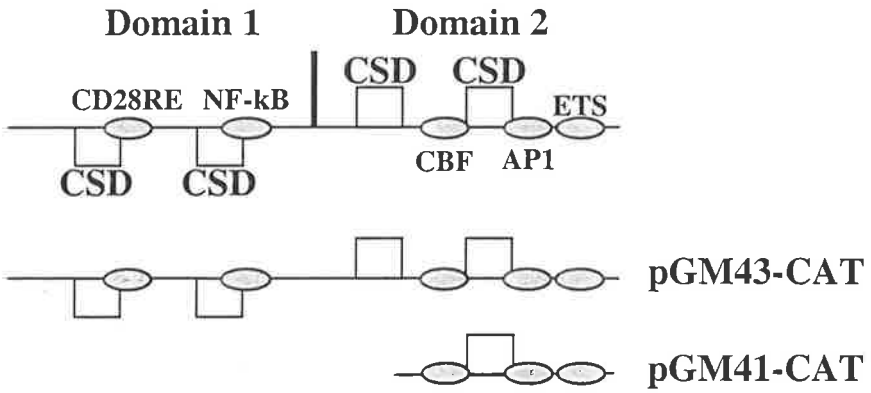
3.5 Effect of overexpression of CSD factors dbpAv and dbpB on GM-CSF expression in Jurkat T cells.

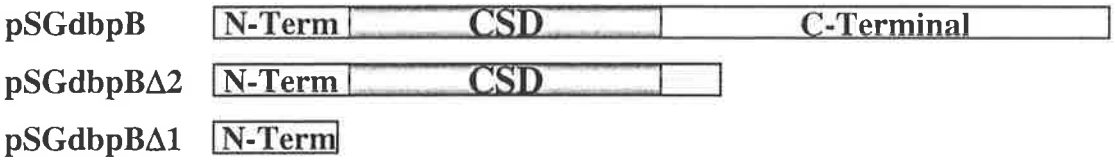
Having shown that the CSD factors, dbpAv and dbpB repress TNFα mediated activation of the human GM-CSF proximal promoter in HEL fibroblasts, we wished to determine if CSD proteins also acted as repressors of the GM-CSF promoter in Jurkat T cells. To determine this, the whole human GM-CSF promoter (-627 to +28) luciferase construct pGM-Luc, was initially co-transfected into Jurkat T cells with CSD overexpression constructs for dbpAv (pSGdbpAv) and dbpB (pSGdbpB). Surprisingly,

Figure 3.4

The CSD domain is important for the dbpAv and dbpB mediated repression of the GM-CSF promoter in HEL fibroblasts.

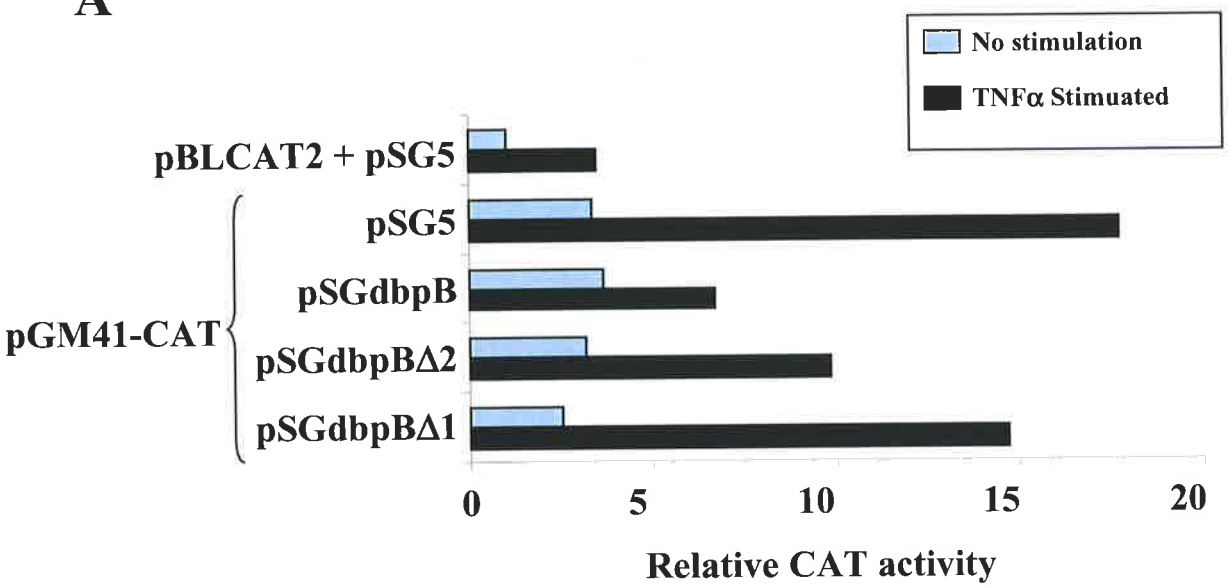
GM-CSF promoter CAT reporter plasmids pGM41-CAT (**Figure A**) and pGM43-CAT (**Figure B**) were co-transfected into HEL fibroblasts with: empty vector pSG5, expression plamsids encoding full length dbpAv (pSGdbpAv), full length dbpB (pSGdbpB) and expression vectors encoding dbpB deletions dbpbΔ1 (pSGdbpBΔ2) and dbpBΔ1 (pSGdbpBΔ1). Cells were treated with or without TNFα for 24 hours, harvested and assayed for CAT activity. The transfection results represent the average of three replicates. All CAT activities are given relative to untreated pBLCAT2 + pSG5 which is given a value of 1. The graphed values have standard errors of less than 20%.



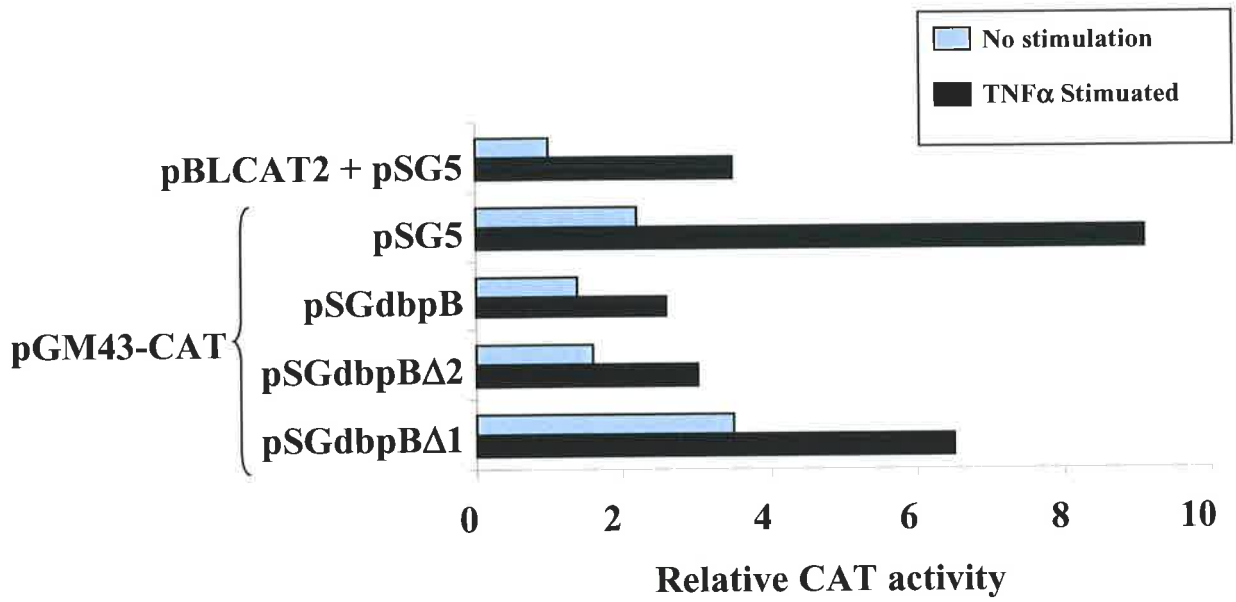


The human GM-CSF proximal promoter and CAT reporter plasmids used in these experiements are represented diagrammatically above (sequences are shown in Figure 3.1). Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factor as defined in the diagram above. The regions of dbpB encoded by the deletion constructs are also shown.

A


No stimulation
TNFα Stimuated
pBLCAT2 + pSG5
pGM41-CAT
pSG5
pSGdbpB
pSGdbpBΔ2
pSGdbpBΔ1
0
5
10
15
20
Relative CAT activity

B


No stimulation
TNFα Stimuated
pBLCAT2 + pSG5
pGM43-CAT
pSG5
pSGdbpB
pSGdbpBΔ2
pSGdbpBΔ1
0
2
4
6
8
10
Relative CAT activity

dbpAv and dbpB were found to activate pGM-Luc (Figure 3.5A). This activation was only observed when the cells were stimulated with PMA/Ca^{2+} ionophore to mimic T cell receptor activation (Figure 3.5A). Since the CSD factors dbpAv and dbpB were only involved in activation of the GM-CSF promoter construct when the cells were stimulated with PMA/Ca^{2+} ionophore, this indicates that the CSD proteins need to be activated to function or that they co-operate with other inducible proteins to function.

To determine the minimal region needed for the observed CSD mediated activation of the GM-CSF promoter, a shorter construct was made (pGM4-Luc) containing only the GM-CSF proximal promoter (-114 to +28) which included the four defined CSD sites (Figure 3.1C). The luciferase reporter construct pGM4-Luc was similar to the CAT reporter construct pGM43-CAT, used in the previous HEL fibroblast transient transfection experiments, except pGM4-Luc contained the GM-CSF promoter TATA box and transcriptional start site (-31 to +28). When pGM4-Luc was co-transfected into Jurkat T cells with expression constructs for dbpAv (pSGdbpAv) and dbpB (pSGdbpB) they again acted as activators only when the cells were stimulated with PMA/Ca^{2+} ionophore (Figure 3.5B). The level of co-activation observed over PMA/Ca^{2+} ionophore activation levels on the shorter pGM4-Luc construct was similar when compared to the longer pGM-Luc construct approximately three fold activation by pSGdbpAv over pSG5 and two fold activation by pSGdbpB over pSG5 (Figure 3.5A & B). This indicated that the minimal region needed for the observed CSD mediated activation of the GM-CSF promoter was contained within the first 114 bases of the proximal promoter.

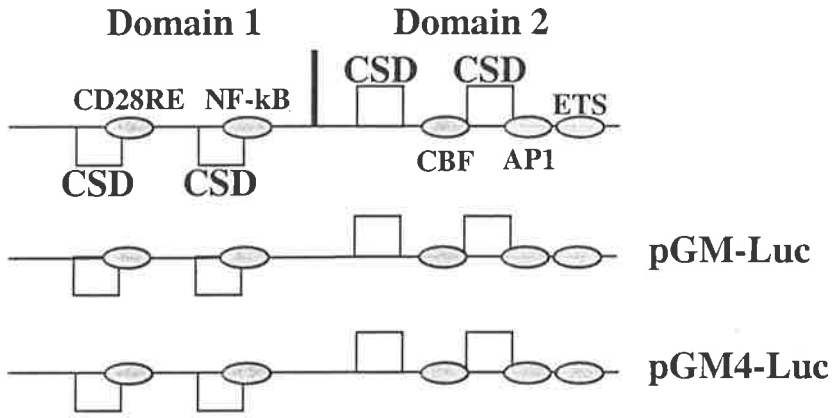
3.6 Determination of regions of the GM-CSD promoter required for CSD mediated activation

I wished to determine which region of the GM-CSF proximal promoter (-114 to +28) was required for CSD mediated activation and the role, if any, that the four CSD

Figure 3.5

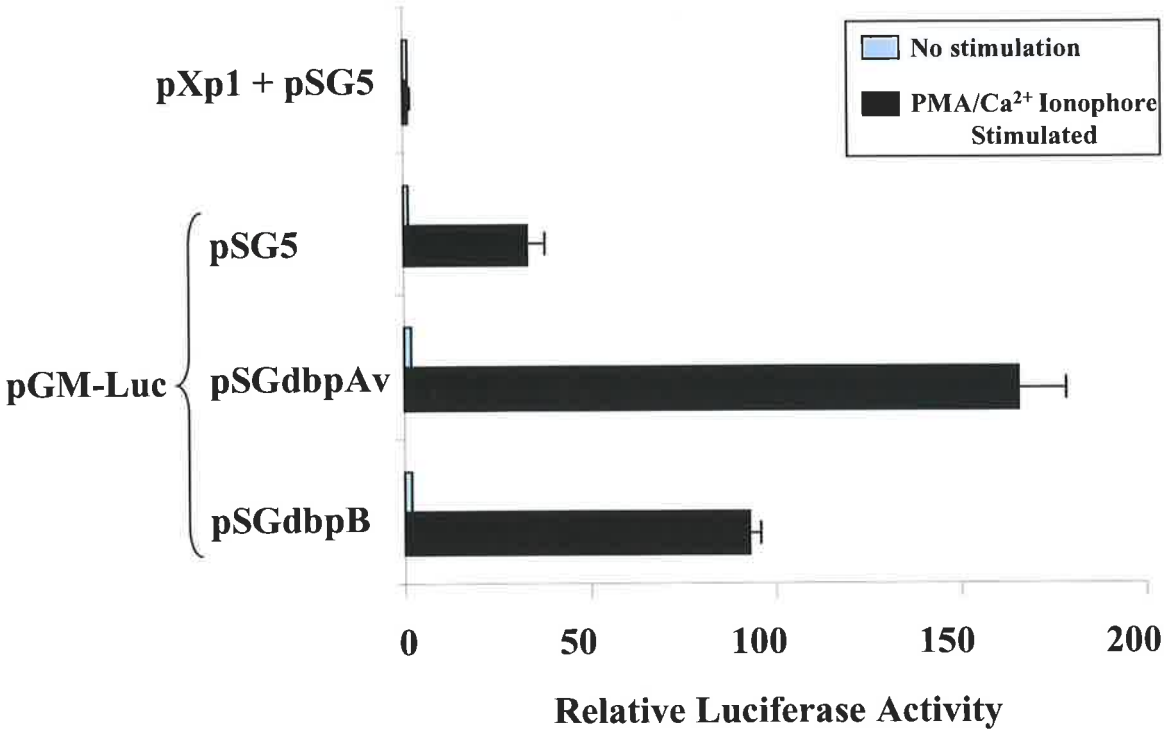
Overexpression of dbpAv and dbpB results in co-activation of the GM-CSF promoter in Jurkat T cells.

The reporter plasmids pGM-Luc, (**Figure A**) and pGM4-Luc (**Figure B**) were co-transfected into Jurkat T cells with the empty pSG5 expression vector and with expression plasmids containing full length dbpAv (pSGdbpAv) and full length dbpB (pSGdbpB). Cells were treated with or without PMA/Ca^{2+} Ionophore for 8 hours, harvested and assayed for luciferase activity. The transfection results represent the average of three replicates. All luciferase activities are given relative to untreated pXP1 co-transfected with pSG5, which is given a value of 1.

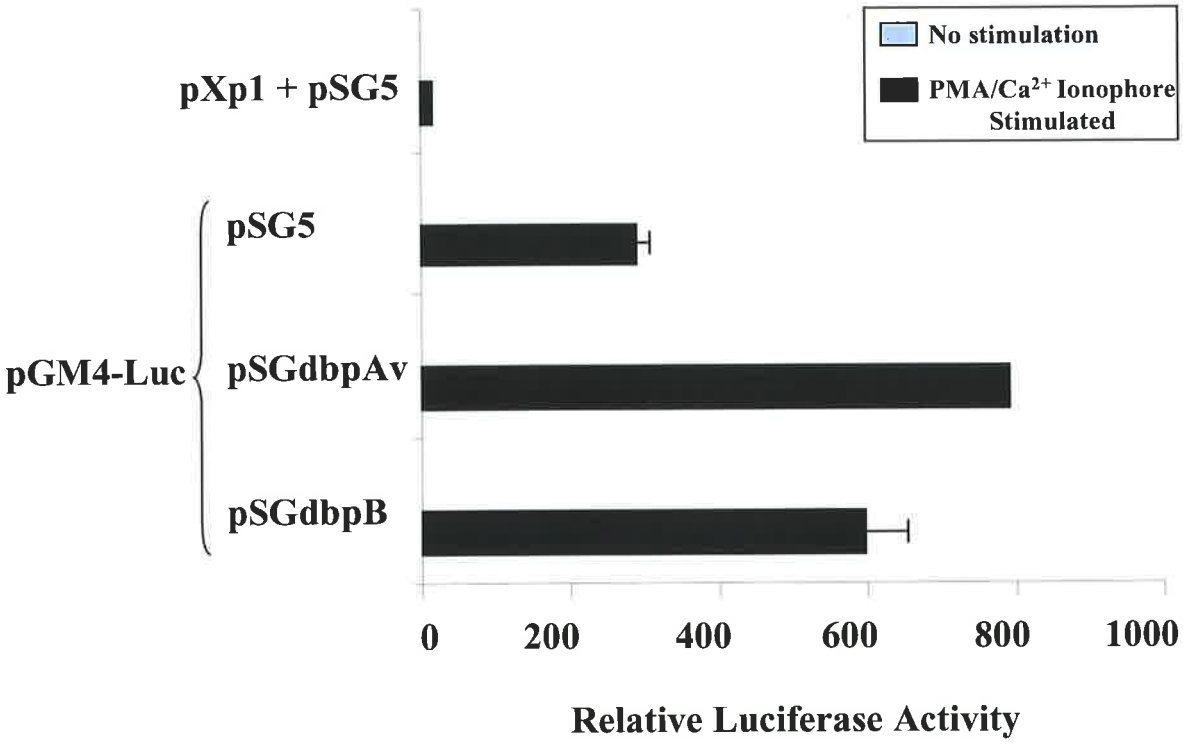


The human GM-CSF proximal promoter and Luciferase plasmids used in these experiments are represented diagrammatically above (sequences are shown in Figure 3.1). Boxes represent CSD binding sites, and ovals the binding sites of the corresponding transcription factor in the top diagram.

A


No stimulation
PMA/Ca2+ Ionophore Stimulated
pXp1 + pSG5
pGM-Luc
pSG5
pSGdbpAv
pSGdbpB
0
50
100
150
200
Relative Luciferase Activity

B


No stimulation
PMA/Ca2+ Ionophore Stimulated
pXp1 + pSG5
pGM4-Luc
pSG5
pSGdbpAv
pSGdbpB
0
200
400
600
800
1000
Relative Luciferase Activity

binding sites played in this activation. To do this, CSD overexpression constructs were co-transfected into Jurkat T cells with GM-CSF promoter luciferase constructs containing variable numbers of CSD sites (Figure 3.6). Initially the pGMCK-1(2)-TK/Luc construct (-114 to –70) containing only the two domain 1 CSD sites of the GM-CSF promoter was used in experiments. Co-transfections showed that both CSD factors (pSGdbpAv and pSGdbpB) could co-activate this construct in the presence of PMA/Ca^{2+} ionophore (Figure 3.6). Since the domain 1 region was cloned upstream of the basal thymidine kinase (TK) promoter in pGMCK-1(2)-TK/Luc, CSD factors were tested for activator function on the TK promoter. As shown in Figure 3.6, CSD factors could not activate the TK promoter in the presence of PMA/Ca^{2+} ionophore. A construct containing only the domain 2 sites was also tested to determine if they too were targets for CSD mediated activation. When pGM2-Luc (-71 to +28, containing only the domain 2 CSD sites) was used in co-transfection experiments, CSD mediated activation was still observed but both basal (pSG5) and CSD (pSGdbpAv, pSGdbpB) co-activated expression was dramatically reduced relative to pGM4-Luc (Figure 3.6). When, however the pGM2-Luc construct was truncated by five bases to create pGM1-Luc, higher levels of activity and CSD factor mediated activation were restored (Figure 3.6). The five base truncation removed the 5' CSD site in domain 2, leaving only the single 3' domain 2 CSD site. These results therefore indicated that the 5' domain 2 CSD binding sites had repressor activity.

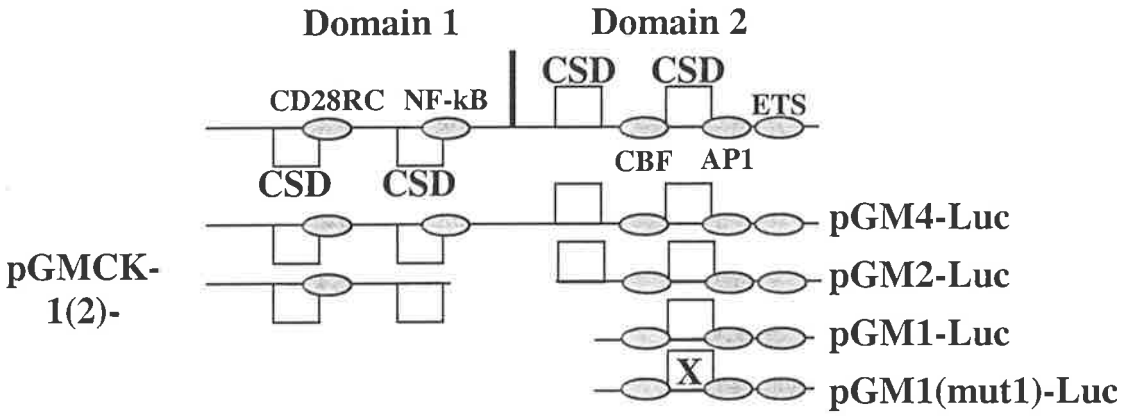
To determine if CSD mediated co-activation of pGM1-Luc was acting through the single CSD site in this construct, co-activation levels were compared between pGM1-Luc (which contains one CSD site) and pGM1(mut1)-Luc (where the one CSD site has been mutated) (Figure 3.6). Both pGM1-Luc and pGM1(mut1)-Luc were co-transfected into Jurkat T cells with constructs encoding for CSD factors dbpAv and dbpB. The PMA/Ca^{2+} ionophore stimulated levels of both basal and CSD mediated co-activation in pGM1(mut1)-Luc were greater than those seen on pGM1-Luc (up to 2 fold greater) (Figure

Figure 3.6

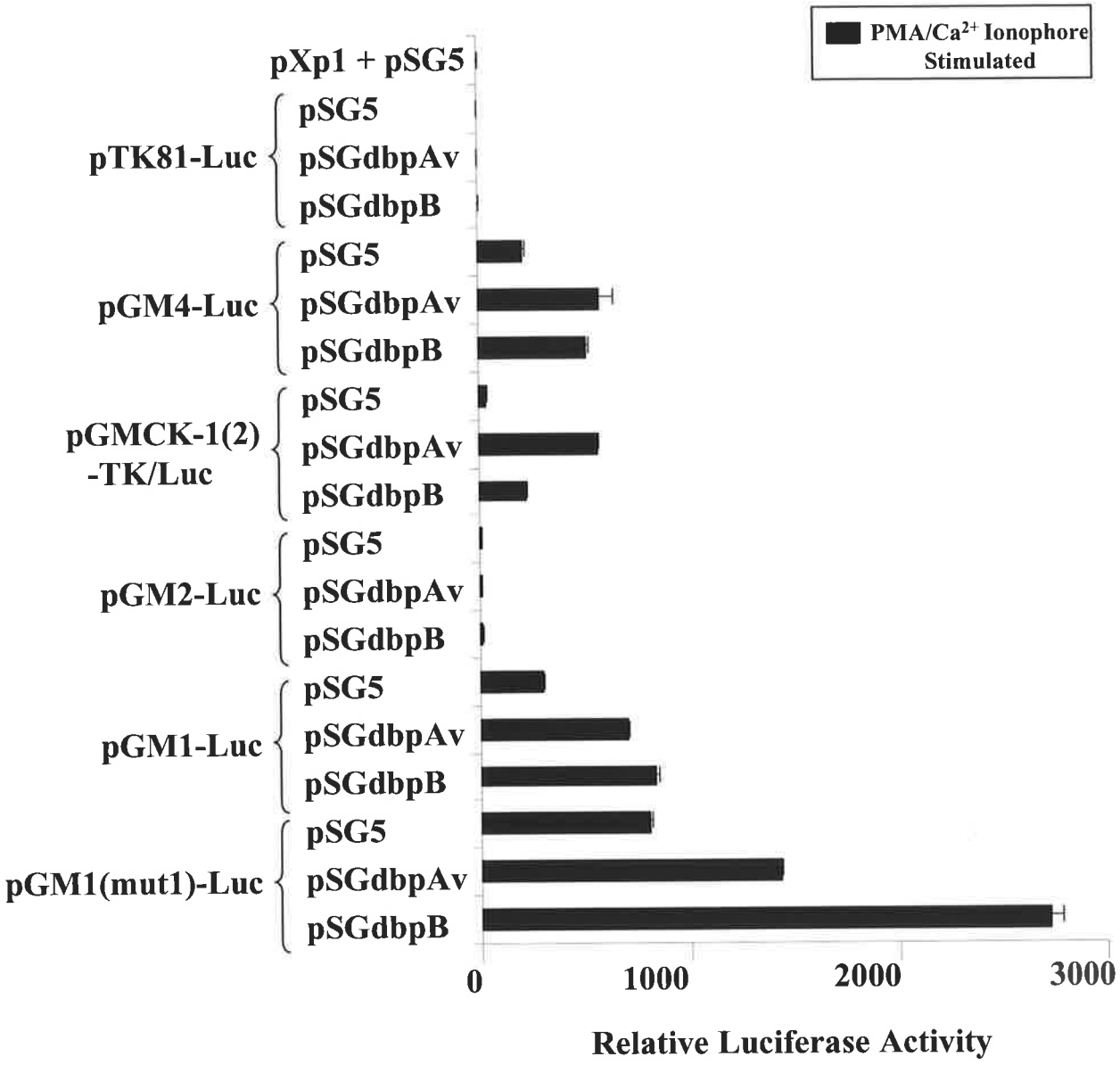
Overexpression of dbpAv and dbpB on GM-CSF promoter truncations.

The reporter plasmids pTK81-Luc, pGM4-Luc, pGMCK-1(2)-TK/Luc, pGM2-Luc, pGM1-Luc and pGM1(mut1)-Luc were co-transfected into Jurkat T cells with the empty pSG5 expression vector and with expression plasmids containing full length dbpAv (pSGdbpAv) and full length dbpB (pSGdbpB).

Cells were treated with or without PMA/Ca^{2+} ionophore for 8 hours, harvested and assayed for luciferase activity. The transfection results represent the average of three replicates. All luciferase activities are given relative to untreated pXP1 co-transfected with pSG5, which is given a value of 1. Only PMA/Ca^{2+}Ionophore treated values are shown, all untreated values were below a relative luciferase activity of 10.



The human GM-CSF proximal promoter and Luciferase plasmids used in these experiments are represented diagrammatically above (sequences are shown in Fig 3.1). Boxes represent CSD binding sites, crossed boxes where the CSD binding sites have been mutated and ovals the binding sites of the corresponding transcription factor in the top diagram. The regions of dbpB encoded by the deletion constructs are also shown.


PMA/Ca2+ Ionophore Stimulated
pXp1 + pSG5
pTK81-Luc
pSG5
pSGdbpAv
pSGdbpB
pGM4-Luc
pSG5
pSGdbpAv
pSGdbpB
pGMCK-1(2) -TK/Luc
pSG5
pSGdbpAv
pSGdbpB
pGM2-Luc
pSG5
pSGdbpAv
pSGdbpB
pGM1-Luc
pSG5
pSGdbpAv
pSGdbpB
pGM1(mut1)-Luc
pSG5
pSGdbpAv
pSGdbpB
0
1000
2000
3000
Relative Luciferase Activity

3.6). This data taken altogether, therefore demonstrates that both the Domain 2 CSD sites have repressor activity. These results also show that the co-activation effects of CSD proteins on the GM-CSF promoter did not require contact with these CSD sites. I was unable to repeat similar experiments on domain 1 CSD sites due to the overlap of the NF-κB binding sites with the CSD sites. Mutation of the domain 1 NF-κB sites results in a loss of GM-CSF promoter activity (Jenkins *et al.*, 1995); (Shannon *et al.*, 1997).

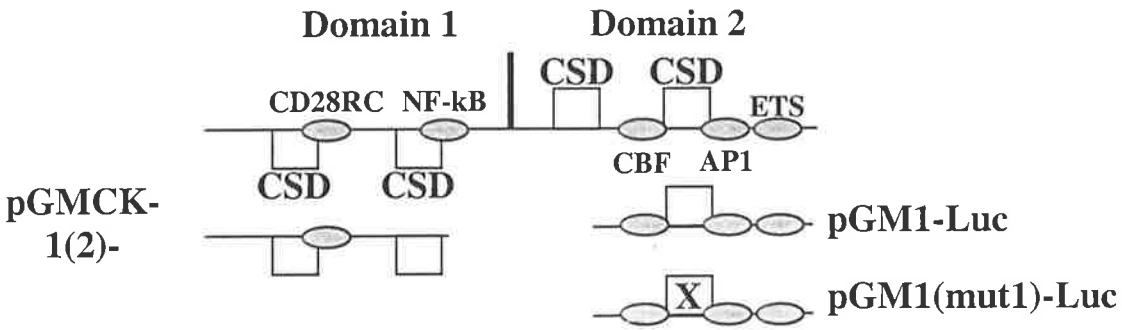
3.7 The C-terminal domain of dbpB is required for CSD mediated activation of the GM-CSF proximal promoter.

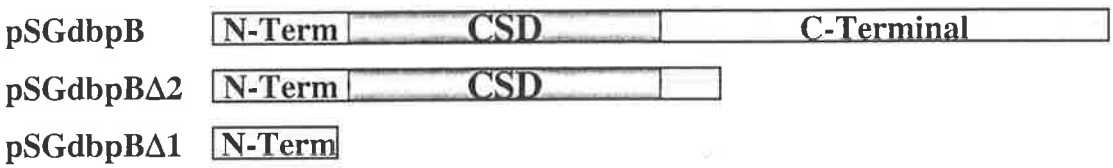
As interactions between CSD factors and other transcriptional regulators have been implicated in mechanisms of activation of a number of viral and cellular genes, a potential mechanism for the co-activation effect seen here was via interaction of CSD factors with other proteins. The C-terminal domain of dbpB has been demonstrated to be required for protein:protein interactions (Balda *et al.*, 2000) (Shnyreva *et al.*, 2000) (lloberas *et al.*, 1995) (Li *et al.*, 1997) (Mertens *et al.*, 1998) (Raj *et al.*, 1996) (Safak *et al.*, 1999) (Ise *et al.*, 1999). To explore the possibility that the co-activation effect was mediated via CSD protein:protein interactions, expression constructs were made encoding truncations of the dbpB CSD protein pSGdbpBΔ1 and pSGdbpBΔ2, as described above. The dbpB full length and truncation constructs were co-transfected into Jurkat T cells with either pGM1-Luc, pGM1(mut1)-Luc domain 2 constructs or the pGMCK-1(2)-TK/Luc domain 1 construct and the cells stimulated with PMA/ Ca^{2+} ionophore. Removal of most of the C-terminal region of dbpB in pSGdbpBΔ2 resulted in a significant decrease in the level of PMA/ Ca^{2+} ionophore co-activation on all constructs, implying that this region was important for co-activation (Figure 3.7). Removal of both the C-terminal and CSD domain of dbpB in pSGdbpbΔ1 resulted in reduced co-activation on both domain 1 and 2 constructs (Figure 3.7).

Figure 3.7

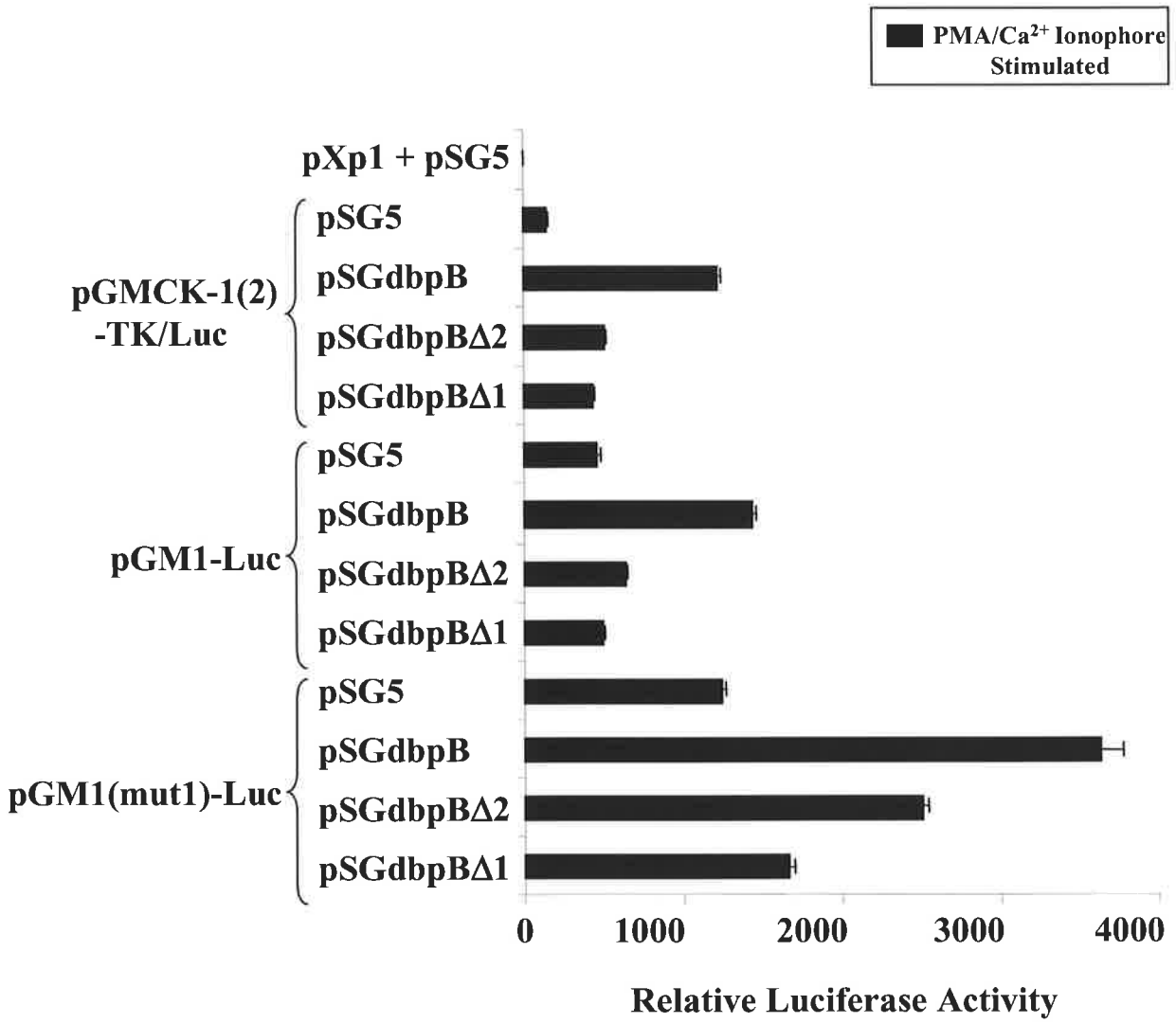
The C-terminal region of dbpAv and dbpB is required for co-activation

The reporter plasmids pGM1-Luc, pGMCK-1(2)-TK/Luc and pGM1(mut1)-Luc were co-transfected into Jurkat T cells with the empty pSG5 expression vector and with expression plasmids containing full length dbpAv (pSGdbpAv), dbpB (pSGdbpB) and expression vectors encoding dbpB deletions dbpBΔ2 (pSGdbpBΔ2) and dbpBΔ1 (pSGdbpBΔ1). Cells were treated with or without PMA/Ca^{2+} ionophore for 8 hours, harvested and assayed for luciferase activity. The transfection results represent the average of three replicates. All luciferase activities are given relative to untreated pXP1 co-transfected with pSG5, which is given a value of 1. Only PMA/Ca^{2+}ionophore treated values are shown, all untreated values were below a relative luciferase activity of 10.





The human GM-CSF proximal promoter and Luciferase plasmids used in these experiments are represented diagrammatically above (sequences are shown in Fig 3.1). Boxes represent CSD binding sites, crossed boxes where the CSD binding sites have been mutated and ovals the binding sites of the corresponding transcription factor in the top diagram. The regions of dbpB encoded by the deletion constructs are also shown.


PMA/Ca2+ Ionophore Stimulated
pXp1 + pSG5
pGMCK-1(2) -TK/Luc
pSG5
pSGdbpB
pSGdbpBΔ2
pSGdbpBΔ1
pGM1-Luc
pSG5
pSGdbpB
pSGdbpBΔ2
pSGdbpBΔ1
pGM1(mut1)-Luc
pSG5
pSGdbpB
pSGdbpBΔ2
pSGdbpBΔ1
0
1000
2000
3000
4000
Relative Luciferase Activity

3.8 Summary and Discussion

Experiments described here and other work done in our laboratory show that the function of the CSD binding sites in HEL fibroblasts is clearly one of repression (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). CSD factor overexpression experiments and CSD binding site mutational analysis suggests that the CSD factors act via these sites to repress TNFα mediated induction of the GM-CSF proximal promoter in HEL fibroblasts. CSD factor truncations indicate that the central CSD domain mediates the repressor function seen in HEL fibroblasts. The CSD domain has been shown to be essential for sequence specific single stranded DNA binding (Kolluri *et al.*, 1992) (Bouvet *et al.*, 1995) (Schroder *et al.*, 1995) and therefore suggests a DNA binding mechanism for the observed CSD mediated repression of the GM-CSF promoter in HEL fibroblasts. Since CSD factors dbpAv and dbpB have been shown to only bind single stranded regions of the GM-CSF promoter this may prevent double stranded transcription factors from binding and hence result in repression. Work done in our laboratory has found that the IL-3 and granulocyte-colony stimulating factor (G-CSF) genes have the same arrangement of CSD sites across their proximal promoters as observed in the GM-CSF promoter (Coles *et al.*, 2000). As for the GM-CSF gene, these sites are adjacent to or overlap activator elements, some of which are in common with the GM-CSF gene (SP-1, CBF, NF-κB and the CD28-responsive element sites). Transient transfection experiments show that CSD factors also act to repress TNFα mediated activation of the G-CSF promoter in HEL fibroblasts (unpublished results), suggesting a potential common means of repression of these cytokine genes. A similar mechanism of transcriptional repression, where CSD factors bind to single stranded regions of DNA thereby preventing the binding of double stranded transcription factors, has also been reported in the thyrotropin receptor and grp78 promoters (Ohmori *et al.*, 1996) (Pelletier *et al.*, 2000).

In contrast to what was observed in HEL fibroblasts, when CSD factors were co-trasfected into Jurkat T cells with the GM-CSF promoter, they acted as co-activators of PMA/Ca^{2+} ionophore mediated activation. This co-activation acted primarily via the c-terminal domain of dbpB. The C-terminal domain of CSD proteins has been implicated in protein:protein interactions where these interactions has been shown to regulate gene transcription (Ozer *et al.*, 1990a) (Shnyreva *et al.*, 2000) (Li *et al.*, 1997) (Ansari *et al.*, 1999). These results suggest that protein:protein interactions is the mechanism for the observed CSD mediated activation of the GM-CSF promoter in Jurkat T cells. A potential duel role for CSD factors in GM-CSF transcriptional regulation in Jurkat T cells was discovered by mutational analysis of the CSD binding sites across the GM-CSF proximal promoter. CSD experiments with GM-CSF domain 2 promoter constructs containing CSD binding site mutations, resulted in increased expression. This suggested that at least the domain 2 CSD binding sites acted as repressor elements. A potential model for CSD function in Jurkat T cells could be that the pool of CSD factors in the nucleus can act either as repressors (via DNA binding) or co-activators (via protein interactions) in the same system, but that any co-activation effect achieved by CSD factors is enough to overcome the repression. A similar mechanism whereby CSD factors act as both activators and repressors in the same system has also been reported for JCV early/late viral gene transcriptional regulation (Gallia *et al.*, 1998) (Chen *et al.*, 1995a) (Safak *et al.*, 1999).

This is the first example where CSD factors have been shown to regulate the expression of a cellular gene differently in two different cell types that normally express the gene. These results also suggest that CSD factors may play multiple roles in gene transcription in the one system.

Chapter 4

Characterisation of nuclear cold shock domain complexes in fibroblasts and T cells

Is it an "Evil" petting zoo?

Dr. Evil

4.1 Introduction

In the previous chapter I demonstrated that overexpression of the CSD factors dbpAv and dbpB had a different affect in Jurkat T cells compared with HEL fibroblasts. CSD co-trasfection experiments demonstrated that CSD factors could repress TNFα induction of the GM-CSF promoter in HEL fibroblasts, whereas the same CSD factors functioned to co-activate the GM-CSF promoter in Jurkat T cells, but only in the presence of PMA/ Ca^{2+} ionophore. To investigate this difference initial experiments focused on examining the CSD factors present in these cell types. Using gel shift analysis, we have previously demonstrated in this laboratory, that nuclear CSD proteins in HEL fibroblasts and HUT78 T cells form as two complexes NF-GMb and NF-GMc and bind to single stranded CSD binding sites across the GM-CSF proximal promoter (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). Four CSD binding sites have been identified across the GM-CSF proximal promoter, two sites on the non-coding strand of domain 1 and two sites on the coding strand of domain 2 (Figure 4.1). UV cross-linking of the HEL fibroblasts/HUT78 T cell NF-GMb/c complexes that were shown to form on these sites identified that the NF-GMb complex consisted of 42, 25 and 22 kDa CSD proteins, while the NF-GMc complex consisted of only the 25 and 22 kDa CSD proteins (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). Here I utilise gel shift, UV cross-linking and DNA competition techniques to determine if the same CSD factors and corresponding CSD factor subtypes identified in HEL fibroblasts are present in Jurkat T cells.

4.2 Nuclear CSD complex formation on the human GM-CSF promoter domain 1 and 2 regions in Jurkat T cells

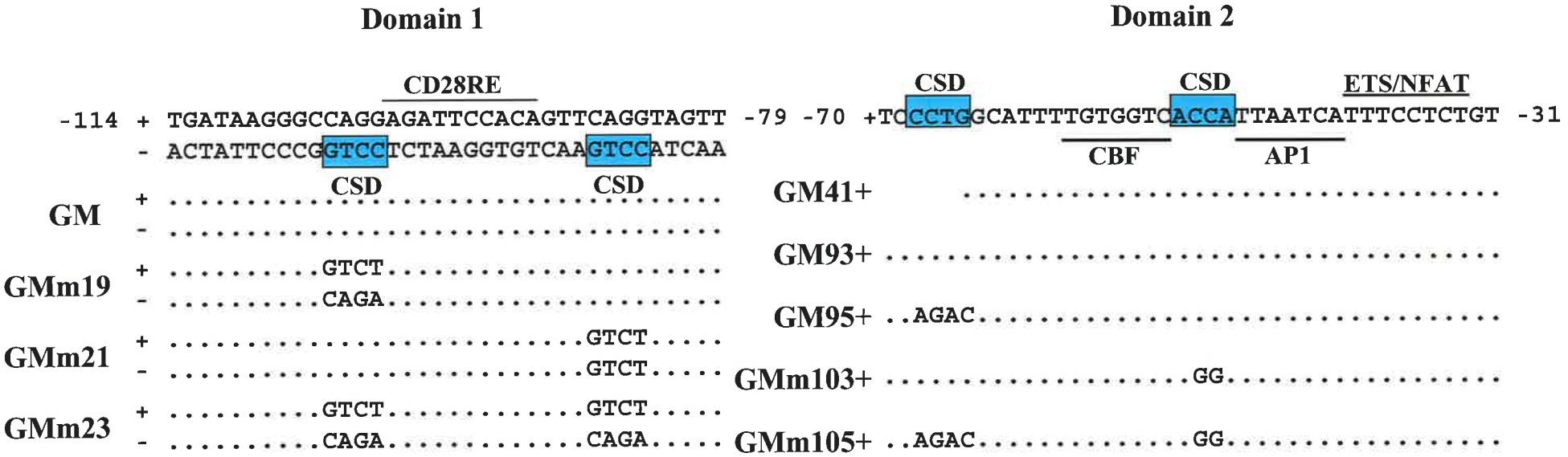
Previous work undertaken in our laboratory has shown that both HEL fibroblasts and HUT78 T cells contain nuclear CSD proteins which bind to the human GM-CSF proximal promoter as two complexes NF-GMb and NF-GMc (Coles *et al.*, 1994); (Coles *et*

Figure 4.1

GM-CSF promoter and domain 1 & 2 oligonucleotides used in gel shift assays.

The sequence of the coding (+) and non-coding (-) wildtype domain 1 (-114 to –79) oligonucleotides GM and GM- are shown. Sequences needed for nuclear NF-GMb/c and recombinant CSD factor binding to the non-coding strand are indicated by a box. Base changes in CSD binding sites of the non-coding (-) strand for each mutant oligonucleotide GMm19-, GMm22-, GMm23- are shown (Coles *et al.* 1996).

The sequence of the wild type coding strand (+) domain 2 GM93+ oligonucleotide (-70 to –31) is given with CSD, CBF, AP1 and ETS/NFAT sites. Nuclear and recombinant CSD binding is exclusively to the coding (+) strand along domain 2. Base changes in the CSD binding sites of the coding (+) strand for each mutant oligonucleotide GMm95+, GMm103+, GMm105+ are shown.


Domain 1
Domain 2
CD28RE
CSD
CSD
CSD
CSD
ETS/NFAT
CBF
AP1
-114 + TGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTT -79 -70 +TCCCTGGCATTTTGTGGTCACCATTAATCATTTCCTCTGT -31
- ACTATTCCCGGTCCTCTAAGGTGTCAAGTCCATCAA
GM + ...
- .....
GMm19 + ...GTCT.....
- .....CAGA.....
GMm21 + ...GTCT.....
- .....GTCT.....
GMm23 + ...GTCT.....GTCT.....
- .....CAGA.....CAGA.....
GM41+ .....
GM93+ .....
GM95+ ..AGAC.....
GMm103+ .....GG.....
GMm105+ ..AGAC.....GG.....

al., 1996); (Coles *et al.*, 2000). These nuclear CSD complexes were detected binding only to the non-coding (-) strand of domain 1 (GM-) and the coding (+) strand of domain 2 (GM93+) of the human GM-CSD proximal promoter (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000) (Figure 4.1). No NF-GMb/c complex formation was detected on the opposite strands ie. either the coding strand of domain 1 (GM+) or the non-coding strand of domain 2 (GM93-) (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). This CSD factor complex formation was consistent with the arrangement of CSD binding sites along the GM-CSF promoter; two sites along the non-coding strand of domain 1 and two sites along the coding strand of domain 2 (Figure 4.1). Originally nuclear extracts were made as described by (Dignam *et al.*, 1983) (labelled as 1 in Figures 4.2 and 4.5 [eg. HUT1 = HUT78 T cell technique 1]) but subsequently nuclear extracts in our laboratory were made by a simpler and more efficient procedure as described by (Schreiber *et al.*, 1989) (labelled as 2 in Figures 4.2 and 4.5 [eg. HUT2 = HUT78 T cell technique 2]).

To directly compare the two isolation techniques, nuclear extracts prepared by either technique were used in gels shift assays and run side by side. Gel shift and UV cross-linking experiments have shown that the nuclear CSD complexes that form on both GM-CSF proximal promoter domain 1 and 2 in HEL fibroblasts/HUT78 T cells prepared via technique 1, are the same (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). Therefore, HUT78 T cell nuclear extract prepared via technique 1 was compared to HUT78 T cell and HEL fibroblasts nuclear extracts prepared via technique 2. Technique 1 HUT78 T cell nuclear extract material formed the two previously described complexes NF-GMb and NF-GMc on both GM-CSF domain 1 and 2 oligonucleotides (Figure 4.2, lane 2 & 6).

When nuclear extracts were prepared from HEL fibroblasts and HUT78 T cells via technique 2 and bound to both domain 1 and 2 GM-CSF oligonucleotides in a gel shift assay, the binding seen was again only to the non-coding (-) strand GM- of domain 1 and

Figure 4.2

Jurkat T cell NF-GMb complexes form an NF-GMb-like complex

Jurkat T cell, HUT78 T cell (Technique 1 and 2) and HEL fibroblast Technique 2 nuclear extracts were bound to ^{32}P-labelled wild type domain 1, non-coding (-) strand GM- (Lanes 1-4) and domain 2, coding (+) strand GM41+ (Lanes 5-8) single stranded oligonucleotides in a gel shift assay.

(x represents non-specific binding)

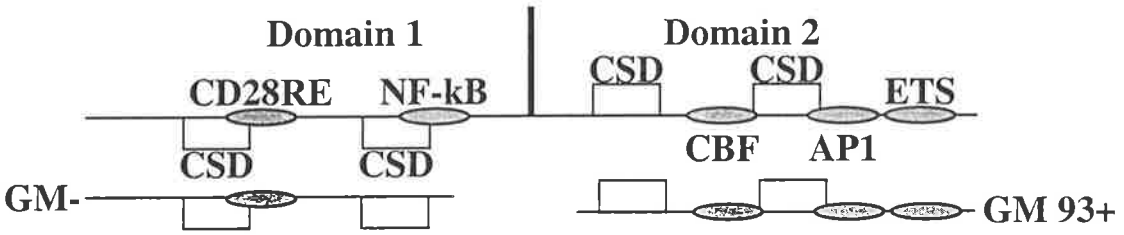
(ss indicates free ^{32}P-labelled single stranded oligonucleotides)

Jkt: Jurkat T cell

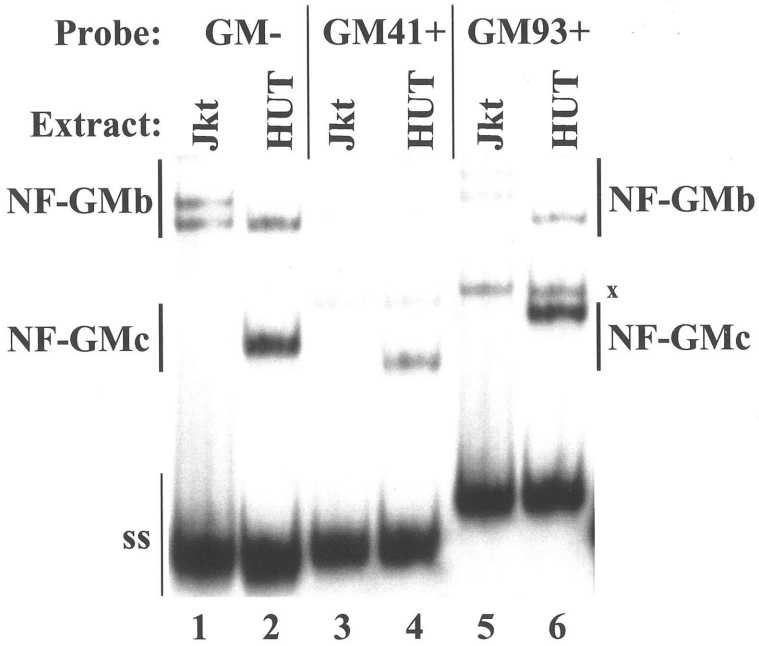
HUT 1: HUT78 T cell (Technique 1)

HUT 2: HUT78 T cell (Technique 2)

Fibro 2: HEL fibroblast (Technique 2)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


Probe:
GM-
GM41+
GM93+
Extract:
Jkt
HUT
Jkt
HUT
Jkt
HUT
NF-GMb
NF-GMb
x
NF-GMc
NF-GMc
ss
1
2
3
4
5
6

the coding (+) strand GM93+ of domain 2. When nuclear extracts prepared via technique 2 from HUT78 T cells and HEL fibroblasts were bound to the non-coding (-) strand of domain 1 GM-, the NF-GMb complex migrated in multiple apparent conformational forms NF-GMb(1) and NF-GMb(2) (Figure 4.2, lane 3 & Dr. L. Coles unpublished) whereas the NF-GMc complex again was only represented by a single band (Figure 4.2, lane 4). Using HUT78 T cell and HEL fibroblast nuclear extracts in a gel shift assay with the coding (+) strand of Domain 2 (GM93+), three bands were observed making up the NF-GMb complex, while only a single NF-GMc band was observed (Figure 4.2, lanes 7 & 8). Extensive analysis performed in our laboratory, some of which is presented later in this chapter, demonstrated that the NF-GMb/c complexes isolated from HUT78 T cells or HEL fibroblasts by either technique contain the same CSD protein subtypes despite the apparent multiple conformational forms of NF-GMb observed in the gel shift experiments.

To determine if Jurkat T cells contained CSD proteins, Jurkat T cell nuclear extract was prepared via technique 2 and bound in a gel shift assay to the same single stranded oligonucleotides spanning domain 1 and domain 2 of the human proximal GM-CSF promoter (Figure 4.2). When Jurkat T cell nuclear extract was used with the domain 1 GM- oligonucleotide, complexes were observed that co-migrated with the HUT78 T cell and HEL fibroblast technique 2 NF-GMb doublet, but a NF-GMc like complex could not be detected (Figure 4.2, lane 1). Jurkat T cell nuclear extract bound to the coding (+) strand of domain 2 (GM93+) gave two bands which co-migrated with the two slowest migrating HUT78 T cell and HEL fibroblast NF-GMb bands and again no NF-GMc like complex was seen (Figure 4.2, lane 5).

To further examine if the complex formation seen in Jurkat T cells represented CSD factor binding, nuclear extracts from both Jurkat and HUT78 T cells were bound to three GM-CSF oligonucleotides: the full domain 1 oligonucleotide GM-, the full domain 2

oligonucleotide GM93+ (both GM- and GM93+ contain 2 CSD binding sites) and to GM41+ which is a shorter domain 2 oligonucleotide containing only one CSD binding site (Figure 4.1). Experiments using HEL fibroblasts and HUT78 T cell nuclear extracts have demonstrated that to get full NF-GMb complex formation in these cell types two CSD binding sites are required, whereas NF-GMc complex formation required only one CSD site (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). Binding Jurkat or HUT78 T cell nuclear extracts to either full length domain 1 or 2 oligonucleotides gave the previously reported binding pattern, that is, NF-GMb/c complex formation in HUT78 T cells and only a NF-GMb-like complex in Jurkat T cells (Figure 4.3, lanes 1,2,5 & 6). When Jurkat T cell nuclear extract was bound to the shorter domain 2 GM41+ oligonucleotide, no NF-GMb like complex formed, as expected, given that this oligonucleotide contained only a single CSD binding site (Figure 4.3, lane 3). Consistent with binding to GM- and GM93+ oligonucleotides no NF-GMc complex formation was observed on GM41+ from Jurkat T cell nuclear extracts (Figure 4.3, lane 3). In contrast, when HUT78 T cell nuclear extract was bound to the shorter domain 2 GM41+ oligonucleotide, which contains one CSD binding site; only the NF-GMc complex was detected (Figure 4.3, lane 4). Therefore the NF-GMb like complexes in Jurkat and HUT78 T cells appear to bind to the GM-CSF oligonucleotides in a similar manner but in contrast to HUT78 T cells and HEL fibroblasts, the Jurkat T cells lacked a NF-GMc complex.

4.3 The Jurkat T cell NF-GMb-like complex is competed with CSD binding site oligonucleotides.

To verify that the Jurkat T cell NF-GMb-like complexes seen on Domain 1 and 2 were authentic CSD-containing complexes, competition assays, using oligonucleotides containing CSD binding sites, were performed. Competitions on Domain 1, non-coding (-)

Figure 4.3

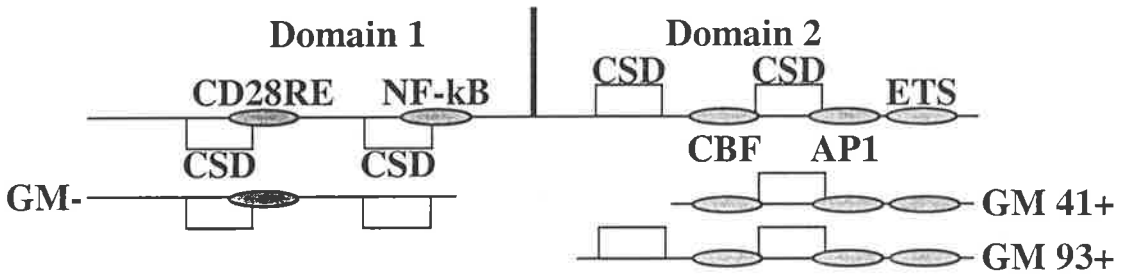
Jurkat T cells lack the NF-GMc complex.

Jurkat T cell and HUT78 T cell nuclear extracts were bound to GM-CSF promoter ^{32}P-labelled single stranded oligonucleotides: non-coding (-) strand of domain 1 GM- (Jurkat [Lane 1], HUT [Lane 2]), the coding (+) strand of domain 2 GM41+ (Jurkat [Lane 3], HUT [Lane 4]) and the extended coding (+) strand of domain 2 GM93+ (Jurkat [Lane 5], HUT [Lane 6])

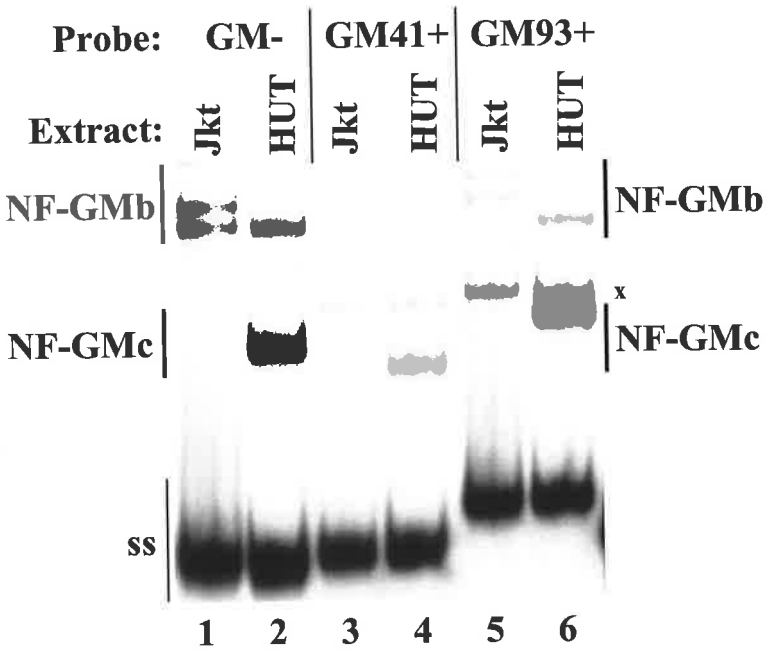
ss indicates free ^{32}P-labelled single stranded oligonucleotides

x represents non-specific binding

Jkt: Jurkat T cell **HUT**: HUT78 T cell



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


Probe:
GM-
GM41+
GM93+
Extract:
Jkt
HUT
Jkt
HUT
Jkt
HUT
NF-GMb
NF-GMb
x
NF-GMc
NF-GMc
ss
1
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6

strand GM- and Domain 2 coding (+) strand GM93+ oligonucleotides showed the same results. As shown in Figure 4.4A & B, the unlabelled, wildtype, non-coding (-) oligonucleotide GM- (contains 2 CSD sites), when used as a competitor inhibited formation of the Jurkat T cell NF-GMb-like complex on both Domain 1 (GM-) (Figure 4.4A, Lane 2), and Domain 2 (GM93+) probes (Figure 4.4B, Lane 2). Competing with the GMm23-, Domain 1, non-coding strand oligonucleotide, which has both CSD sites mutated, had no effect on NF-GMb-like complex formation (Figures 4.4A & B, Lane 3). As a positive control an oligonucleotide from the coding (+) strand of the human papillomavirus 18 enhancer (HPV+) which has been shown to bind recombinant CSD proteins, was used (Coles *et al.*, 1996); (Spitkovsky *et al.*, 1992). Adding the same amount of HPV+ oligonucleotide as GM- oligonucleotide to the competition reaction indicated that HPV+ was able to compete the Jurkat T cell NF-GMb-like complex binding almost as well as the wildtype GM- oligonucleotide (Figures 4.4A & B, Lane 4). The NF-GMb-like complex was not competed by the oligonucleotide (N.S.) that we and others have shown is unable to bind nuclear or recombinant CSD proteins (Figures 4.4A & B, Lane 5) (Coles *et al.*, 1996); (Horwitz *et al.*, 1994); (Kolluri *et al.*, 1992). These competition experiments suggest that the Jurkat T cell NF-GMb-like complex is an authentic CSD containing complex and that both bands represent CSD factor binding.

4.4 UV cross-linking analysis of the NF-GMb-like nuclear complex in Jurkat T cells.

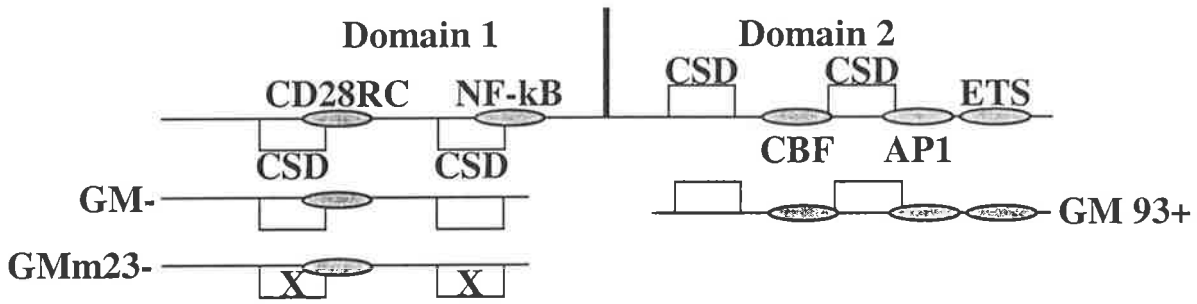
To further verify that the Jurkat T cell NF-GMb-like complexes contained CSD proteins the complex was analysed by UV cross-linking, which allows determination of the size of proteins within a protein:DNA complex. Nuclear extracts derived either via technique 1 or 2 from HEL fibroblasts, Jurkat and HUT78 T cells were again bound to the full length GM-CSF domain 1 oligonucleotide (GM-) and the domain 2 oligonucleotide (GM93+) (Figure 4.5A). Nuclear extracts from each of the cell types gave rise to the

Figure 4.4

Jurkat T cell NF-GMb complex contains CSD proteins.

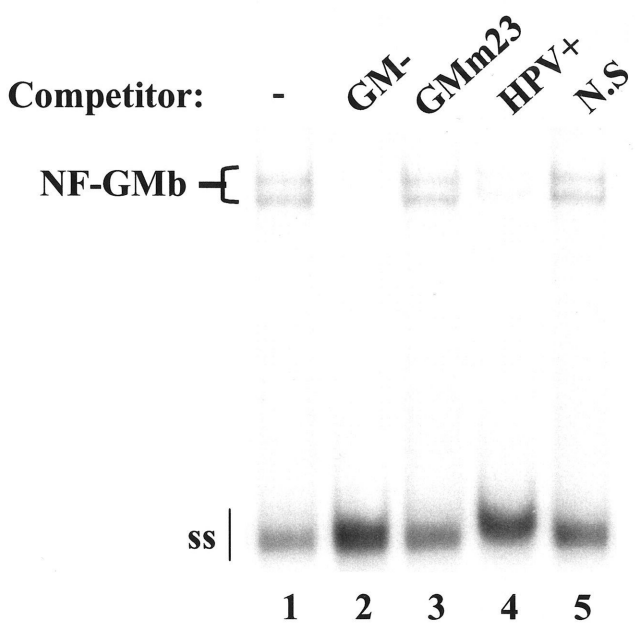
Jurkat T cell nuclear extract was bound in a gel shift assay to labelled GM-CSF Domain 1 non-coding (-) strand oligonucleotide GM- (**Figure A**, Lanes 1 to 5) and to Domain 2 coding (+) strand oligonucleotide GM93+ (**Figure B**, Lanes 6-10). A minus sign indicates no competitor. The NF-GMb-like complex was competed with (GM-) (Lane 2, Figures A & B), the CSD site mutant (GMm23-) (Lane 3, Figures A & B), the control CSD-binding site (HPV+) (Lanes 4, Figures A & B) and a non-specific single stranded oligonucleotide (N.S) (Lanes 5, Figures A & B).

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)

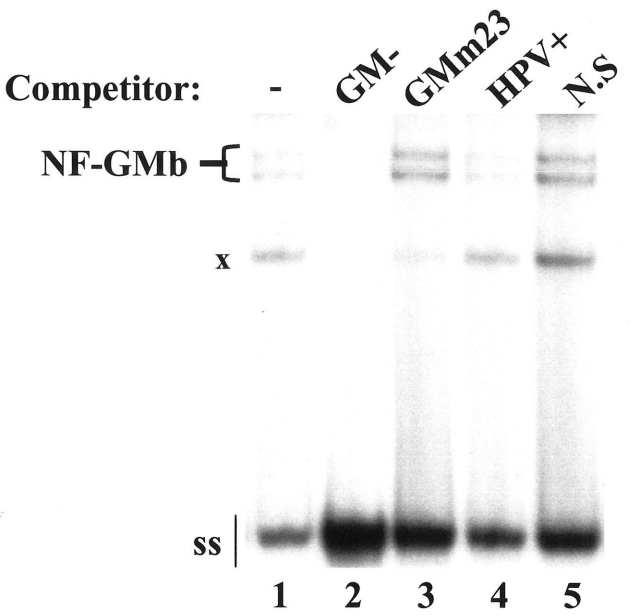


The human GM-CSF proximal promoter and oligonucleotides used in these experiements are represented diagrammatically above. Boxes represent CSD binding sites, boxes with an 'X' in them indicate mutated CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.

A


Probe: GM-
Competitor: - GM- GMm23 HPV+ N.S
NF-GMb
ss
1 2 3 4 5

B


Probe: GM93+
Competitor: - GM- GMm23 HPV+ N.S
NF-GMb
x
ss
1 2 3 4 5

previously described complexes (HEL fibroblasts/HUT78 T cell giving rise to NF-GMb/c complexes, while Jurkat T cells only formed a NF-GMb complex, Figure 4.5A). Using UV cross-linking, it has been previously shown that the NF-GMb/c complexes seen with both HUT78 T cells and HEL fibroblasts (prepared using technique 1) contain proteins of 42, 25 and 22 kDa (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). As can be seen in Figure 4.5B and reported previously (Coles *et al.*, 1994); (Coles *et al.*, 2000), the HUT78 T cell and HEL fibroblast (technique 1) NF-GMb complex formed on the non-coding (-) strand of domain 1 (GM- oligonucleotide) contained both 42 and 22 kDa proteins (HUT78 data Figure 4.5B, lane 3), whereas the NF-GMc complex contained only a 22 kDa protein (HUT78 data Figure 4.5B, lane 4). The 42 kDa protein apparently represents full length CSD, while published reports suggest that proteins in the 22-25 kDa size range are potential splice variants of the full length product. When HUT78 T cell nuclear extract prepared using technique 2 was bound to GM-, the NF-GMb complex was seen to form as a doublet (Figure 4.5A, lane 3). UV cross-linking both of the complexes (top labelled NF-GMb(1) and the bottom NF-GMb(2)) identified that these complexes were comprised of the same size, 42 and 22 kDa proteins, as seen in the NF-GMb HUT78 T cell, technique 1, complex. (Figure 4.5B, lanes 5 & 6 compared to lane 3). The NF-GMc complex from HUT78 T cell Technique 2 also was comprised of the same sized 22 kDa protein seen in HUT78 T cell Technique 1 NF-GMc complex (Figure 4.5B, lane 7 compared to, lane 4). UV cross-linking either band of the Jurkat T cell NF-GMb-like complex doublet formed on domain 1, GM- oligonucleotide (Figure 4.5A, Lane 1), identified only a 42 kDa protein (Figure 4.5B, lane 1 & 2).

On the coding (+) strand oligonucleotide of domain 2 (GM93+) in HUT T cells prepared by technique 1, the NF-GMb complex contained the 42, 22 and an extra 25 kDa protein (Figure 4.5B, lane 10) whereas the NF-GMc complex contained only the 22 and 25 kDa sized proteins (Figure 4.5B, lane 11). UV cross-linking any of the HUT78 T cell

Figure 4.5

Jurkat T cell NF-GMb complexes contain CSD-like proteins.

A) Jurkat T cell, HUT78 T cell (Technique 1 and 2) and HEL fibroblast Technique 2 nuclear extracts were bound to ^{32}P-labelled wild type domain 1, non-coding (-) strand GM- (Lanes 1,2,3,4) and domain 2, coding (+) strand GM41+ (Lanes 5,6,7,8) single stranded oligonucleotides in a gel shift assay.

(x represents non-specific binding)

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)

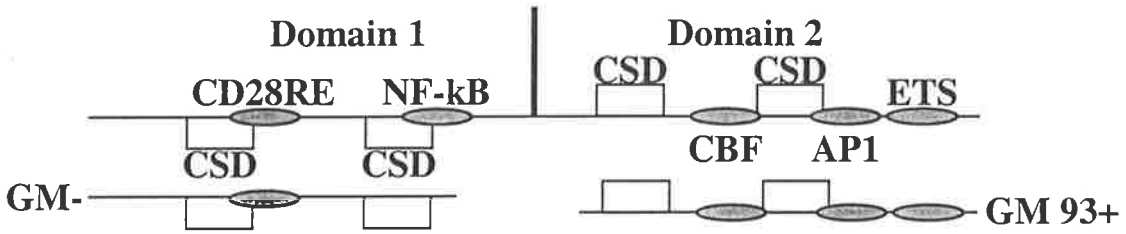
B) Jurkat T Cell and HUT78 T cell (Technique 1 and 2) NF-GMb and NF-GMc gel shift complexes UV crosslinked and Laemmli SDS-polyacrylamide protein gel fractionated. Fractionated NF-GMb/c gel shift complexes from Jurkat T cell and HUT78 T cell nuclear extracts bound to Domain 1, non-coding (-), GM- wild type ^{32}P-labelled oligonucleotide [Jurkat Lanes 1 & 2], [HUT (Technique 1) Lanes 3 to 5], [HUT (Technique 2) Lanes 5 to 7] and bound to Domain 2, coding (+), GM93+ wild type ^{32}P-labelled oligonucleotide [Jurkat Lanes 8 & 9], [HUT (Technique 1) Lanes 10 & 11], [HUT (Technique 2) Lanes 12 to 15].

Jkt: Jurkat T cell

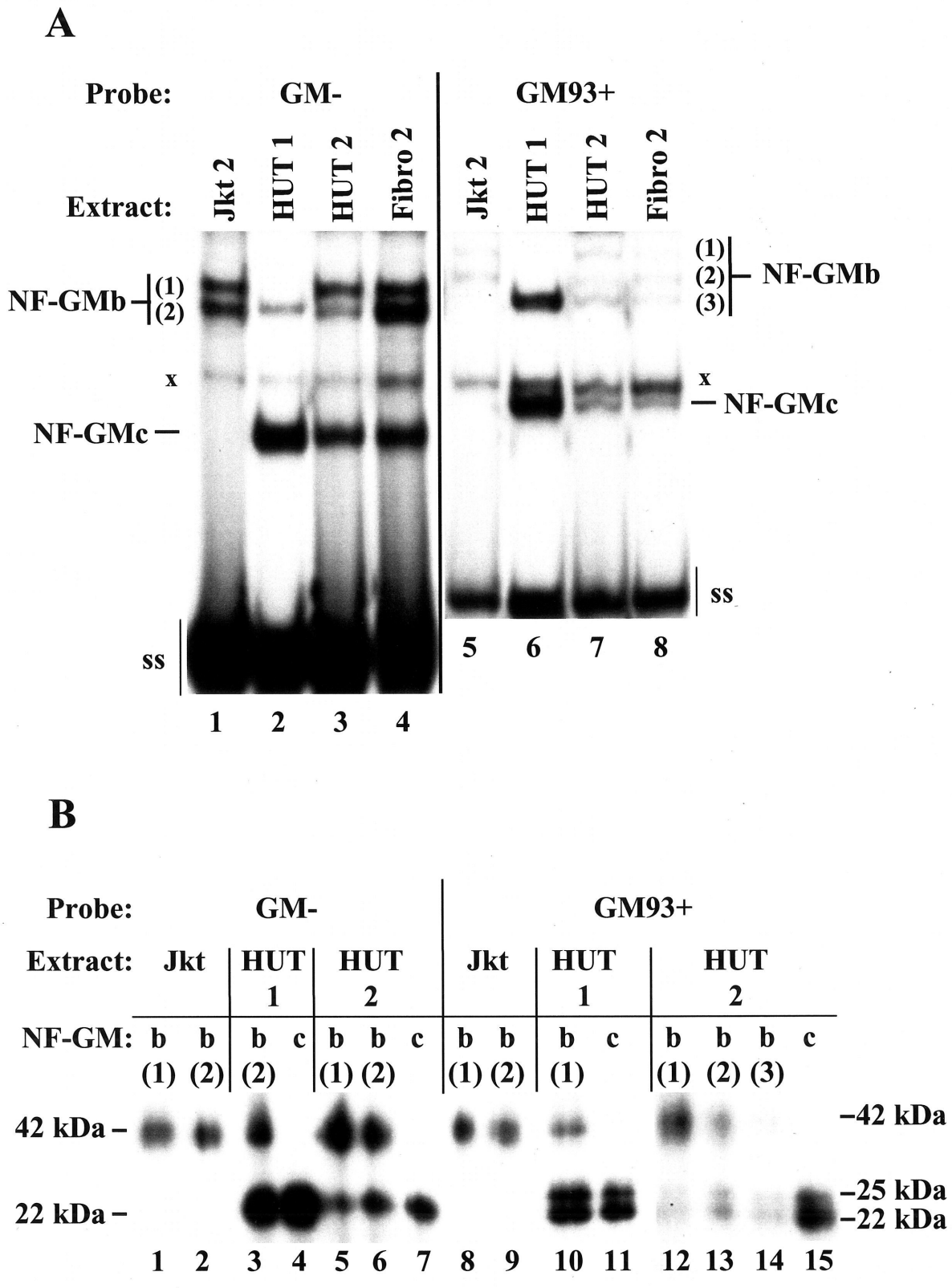
HUT 1: HUT78 T cell (Technique 1)

HUT 2: HUT78 T cell (Technique 2)

Fibro 2: HEL fibroblast (Technique 2)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


A
Probe:
GM-
GM93+
Extract:
Jkt 2
HUT 1
HUT 2
Fibro 2
Jkt 2
HUT 1
HUT 2
Fibro 2
NF-GMb
(1)
(2)
x
NF-GMc
ss
(1)
(2)
(3)
NF-GMb
x
NF-GMc
ss
1 2 3 4
5 6 7 8
B
Probe:
GM-
GM93+
Extract:
Jkt
HUT 1
HUT 2
Jkt
HUT 1
HUT 2
NF-GM:
b b b c b b c
(1) (2) (2) (1) (2)
b b b c b b b c
(1) (2) (1) (1) (2) (3)
42 kDa
22 kDa
42 kDa
25 kDa
22 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

(technique 2) complexes from the NF-GMb triplet (Figure 4.5A, lane 7) identified proteins of 42, 22 and 25 kDa in size (Figure 4.5B, lanes 12 – 14). UV cross-linking the HUT78 T cell technique 2 NF-GMc complex seen on domain 2, GM93+ oligonucleotide, resulted in proteins of the size 22 and 25 kDa (Figure 4.5B, lane 15). In Jurkat T cells again, only the 42 kDa protein could be detected in either of the NF-GMb-like complex doublet bands formed on the domain 2 GM93+ oligonucleotide (Figure 4.5B, lanes 8 & 9).

The multiple NF-GMb complexes observed with Technique 2 Jurkat and HUT78 T cell nuclear extracts may be explained by the 42 kDa protein binding to DNA in multiple conformational forms. Another possibility could be post-translation modification of the protein, although there has been no evidence in the literature to support this, and finally there may be other proteins in this complex which do not cross-link to the DNA and hence are not detected by this method. This method of UV cross-linking, as described in the materials and methods section, does not result in cross-linking between proteins.

4.5 Binding of the Jurkat T cell nuclear NF-GMb-like complex to the GM-CSF promoter

To determine if the 42 kDa protein in the Jurkat T cell NF-GMb complex bound to the GM-CSF promoter CSD binding sites, nuclear extract was used in a gel shift analysis with wildtype Domain 1 (GM-) and Domain 2 (GM93+) oligonucleotides (each contain two CSD binding sites) and their respective CSD binding site mutants. These CSD binding site mutations (see Figure 4.1) have been previously described and characterised for HUT78 T cell and HEL fibroblast CSD binding (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000). Gel shift analysis using the CSD mutant oligonucleotides on the non-coding (-) strand of Domain 1 were compared to the binding on the wild type domain 1 GM- oligonucleotide (Figure 4.6, Lane 2). Mutating one CSD site, in GMm19-, decreased binding significantly (Figure 4.6, Lane 3) whereas mutating the other CSD

Figure 4.6

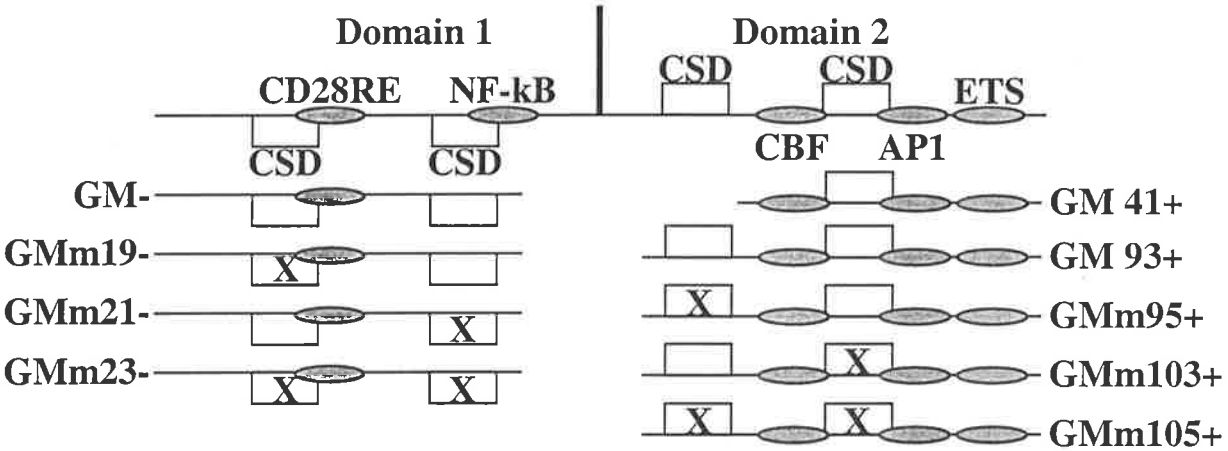
Jurkat T cell nuclear extract binding to GM-CSF promoter mutant oligonucleotide series.

Jurkat T cell nuclear extract was bound to ^{32}P-labelled wild type domain 1 coding (+) and non-coding (-) oligonucleotides GM+, GM- and corresponding mutant oligonucleotides GMm19-, GMm21-, GMm23- (lanes 1-5). Nuclear extract was also bound to ^{32}P-labelled wild type domain 2 coding (+) and non-coding (-) oligonucleotides GM93+, GM93- and corresponding mutant oligonucleotides GMm95+, GMm103+, GMm105+ (lanes 6-10).

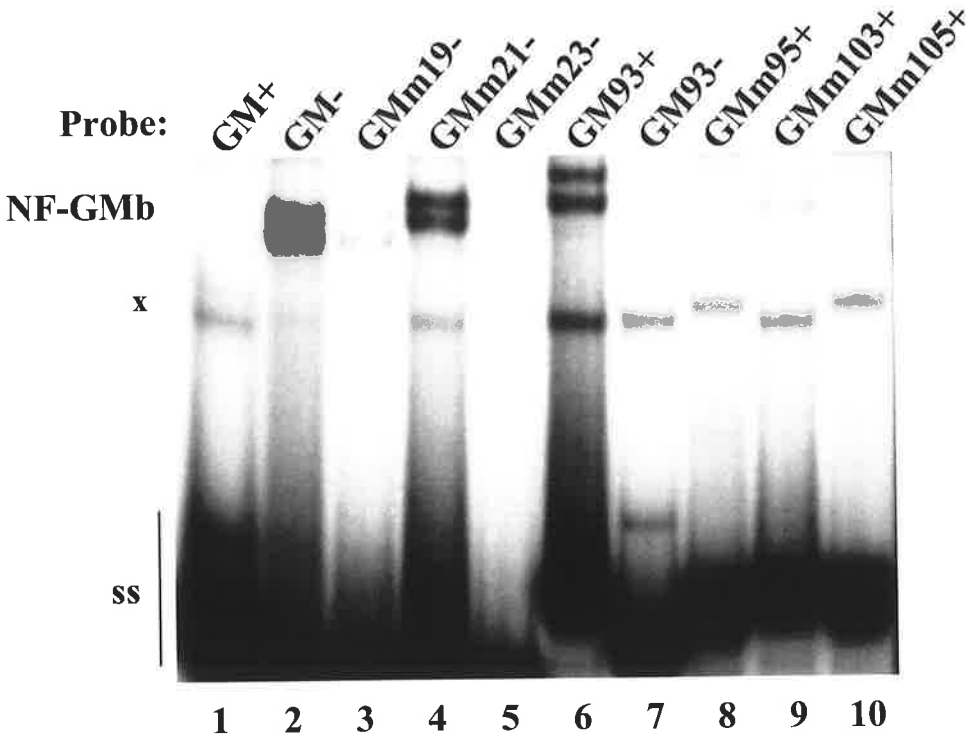
ss indicates free ^{32}P-labelled single stranded oligonucleotides

x represents non-specific binding

Jkt: Jurkat T cell **HUT**: HUT78 T cell



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites, boxes with an 'X' in them indicate mutated CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


Probe:
GM+
GM-
GMm19-
GMm21-
GMm23-
GM93+
GM93-
GMm95+
GMm103+
GMm105+
NF-GMb
x
ss
1
2
3
4
5
6
7
8
9
10

site, in GMm21-, resulted in an altered mobility of the complex but no decrease in NF-GMb binding was observed (Figure 4.6, Lane 4). Only when both CSD sites were mutated, in the GMm23- oligonucleotide, was all binding abolished (Figure 4.6, Lane 5). Similar results were seen as observed for domain 1 when binding of Jurkat T cell nuclear extract on the wild type coding (+) strand of Domain 2 (GM93+) (Figure 4.6, Lane 6) was compared to the CSD binding site mutant oligonucleotides. Mutating one CSD binding site in GMm95+ almost abolished binding (Figure 4.6, Lane 8) and mutating the other CSD site in GMm103+ also significantly reduced binding (Figure 4.6, Lane 9). Again, it was not until both CSD binding sites were mutated, in the oligonucleotide GMm105+, that all binding was lost (Figure 4.6, Lane 10), indicating that both CSD binding sites were needed for full binding. These results agree with the previous observations for NF-GMb/CSD complex binding in HUT78 T cells and HEL fibroblasts.

4.6 Summary and Discussion

Previous work performed in our laboratory identified that HEL fibroblast/HUT78 T cell nuclear extracts could form two complexes (NF-GMb, NF-GMc) on CSD binding sites contained within single stranded DNA oligonucleotides that spanned the GM-CSF proximal promoter (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). UV cross-linking experiments identified that these NF-GMb/c complexes contained factors of the size 42, 25 and 22 kDa, which has been re-examined and confirmed in this chapter. The 42 kDa protein apparently represents full length CSD, while published reports where CSD proteins have been isolated in the range of 22-25 kDa, indicate that these proteins are potential splice variants or proteolytic cleavage products of the full length protein (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000) (Balda *et al.*, 2000) (Cleavinger *et al.*, 1996) (Kudo *et al.*, 1995) (Swamynathan *et al.*, 1997) (Stenina *et al.*, 2000).

Work presented in this chapter identified that Jurkat T cells contain CSD-like proteins that can form NF-GMb-like complexes on domains 1 and 2 of the GM-CSF proximal promoter. Competition analysis with oligonucleotides containing CSD binding sites from the GM-CSF proximal promoter (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000) and the human papillomavirus 18 enhancer (Spitkovsky *et al.*, 1992) showed that these oligonucleotides could readily compete Jurkat T cell NF-GMb complex binding. These experiments suggested that the Jurkat T cell NF-GMb-like complex did contain CSD factors. This was verified by mutational analysis across the GM-CSF proximal promoter, identifying that Jurkat T cell NF-GMb complex formation was dependent on the presence of the previously defined CSD binding site. However, unlike other cells previously examined, Jurkat T cell nuclear extracts did not form a NF-GMc complex on the GM-CSF promoter. UV cross-linking experiments on the Jurkat T cell NF-GMb-like complex identified only a 42 kDa protein while the 25 and 22 kDa CSD factor subtypes identified in HEL fibroblasts/HUT78 T cells are lacking in Jurkat T cells.

These experiments identified a primary difference between HEL fibroblasts/HUT78 T cells and Jurkat T cells in respect to CSD factor subtypes. This difference is examined in further chapters and is discussed as a possible mechanism for the functional differences observed in CSD transient transfection assays between HEL fibroblasts and Jurkat T cells.

Chapter 5

Recombinant CSD protein expression and binding to CSD sites spanning the GM-CSF proximal promoter

One of the symptoms of an approaching nervous breakdown is the belief that one's work is terribly important.

Bertrand Russel

5.1 Introduction

Work done previously by Dr. L. S. Coles identified two CSD proteins (dbpAv and dbpB) from a HUT78 T cell cDNA expression library that bound to sequences containing CSD binding sites, including those in the GM-CSF promoter, human papilloma virus 18 enhancer, c-myc promoter and epidermal growth factor receptor promoter (Coles *et al.*, 1996). The coding sequences of these two CSD proteins were inserted into an eukaryotic expression vector and used in transient transfection assays with the GM-CSF proximal promoter. CSD factors functioned to repress the TNFα mediated activation of the GM-CSF promoter in HEL fibroblasts while acting as co-activators of PMA/Ca^{2+} ionophore mediated GM-CSF activation in Jurkat T cells (chapter 3). Results presented in chapter 4 suggested that the NF-GMb/c complexes observed binding to the CSD binding sites across the GM-CSF proximal promoter consisted of both potential full length and truncated CSD factors.

To further analyse CSD factor binding to the GM-CSF proximal promoter CSD binding sites, full length recombinant GST fusion proteins (dbpAv-GST and dbpB-GST) were produced. The bacterial - GST fusion system was chosen because bacterial expression systems produce high levels of proteins and the GST tag facilitated purification of full length recombinant CSD proteins. Conditions were determined for optimal expression of the recombinant CSD-GST fusion proteins and purified protein was used in binding assays to the GM-CSF domain 1 and domain 2 regions. This work was performed to further verify that the nuclear NF-GMb/c binding sites defined on the GM-CSF promoter (chapters 3 & 4) were authentic CSD binding sites.

The recombinant CSD-GST fusion proteins were also used to raise polyclonal antibodies in rabbits (discussed in chapter 6). The GST tag was useful here since it is highly immunogenic, circumventing the need to conjugate a chemical hapten to the

immunising protein. A highly immunogenic hapten is generally required to elicit an effective specific immune response to the injected protein.

Finally recombinant CSD proteins were used for structure function experiments designed to dissect regions of the CSD protein involved in DNA binding (discussed in this chapter) and potential protein:protein interactions (discussed in chapter 7).

5.2 Construction of CSD-GST fusion protein expression plasmids and recombinant CSD protein purification

The coding regions of CSD factors dbpAv and dbpB were sub-cloned into the bacterial expression vector pGEX-4T-1 (Figure 2.3 material and methods section). The bacterial expression construct for dbpAv (pdbpAv-GST) was created by digesting the eukaryotic dbpAv expression construct pSGdbpAv with *EcoRI*, isolating the 1.4 kb fragment and ligation into the *EcoRI* site of the bacterial GST fusion expression plasmid pGEX-4T-1 (Figure 5.1A). The bacterial expression construct for dbpB (pdbpB-GST) was created by digesting the eukaryotic dbpB expression construct pSGdbpB with *EcoRI*, isolating the 1.04 kb fragment and ligation into the *EcoRI* site of the bacterial GST fusion expression plasmid pGEX-4T-1 (Figure 5.1B).

In a preliminary experiment to determine if the bacterial expression and glutathione sepharose purification systems were working efficiently, recombinant GST was bacterially expressed and purified. To achieve this, the bacterial expression vector pGEX-4T-1, which encodes for GST alone, was transformed into the bacterial strain MC1061. Unpurified MC1061 bacterial lysate containing recombinant GST (Figure 5.2A, lane 6) and recombinant GST purified from this lysate by glutathione sepharose (Figure 5.2A, lane 5) were analysed on a Laemmli SDS-PAGE gel and stained with coomassie blue to visualise the protein bands. Many bands were seen in the unpurified material, including an intense band at approximately 27 kDa, which is the predicted size for recombinant GST (Figure 5.2A, lane 6). The lane containing purified GST material contained only the single 27 kDa GST band with no discernible protein degradation (Figure 5.2A, lane 5), indicating that the bacterial expression and purification systems were both working efficiently.

The bacterial expression constructs for dbpAv (pdbpAv-GST) and dbpB (pdbpB-GST) were transformed into two different bacterial strains, JM109 and MC1061 which were chosen for their different growth and protein expression profiles (Section 2.5 material

Figure 5.1

Construction of GST-fusion bacterial expression constructs for dbpAv (pdbpAv-GST) and dbpB (pdbpB-GST).

A) & B) The coding regions of either dbpAv (Figure A) or dbpB (Figure B) were excised from the SG5 based eukaryotic expression vectors and cloned into the pGEX-4T-1 bacterial expression vector.

Abbreviations: ori, bacterial origin of replication; Amp^r, ampicillin resistance gene; $lacI^q$, lactose operon repressor protein; T7, T7 transcriptional start site; MCS multiple cloning site

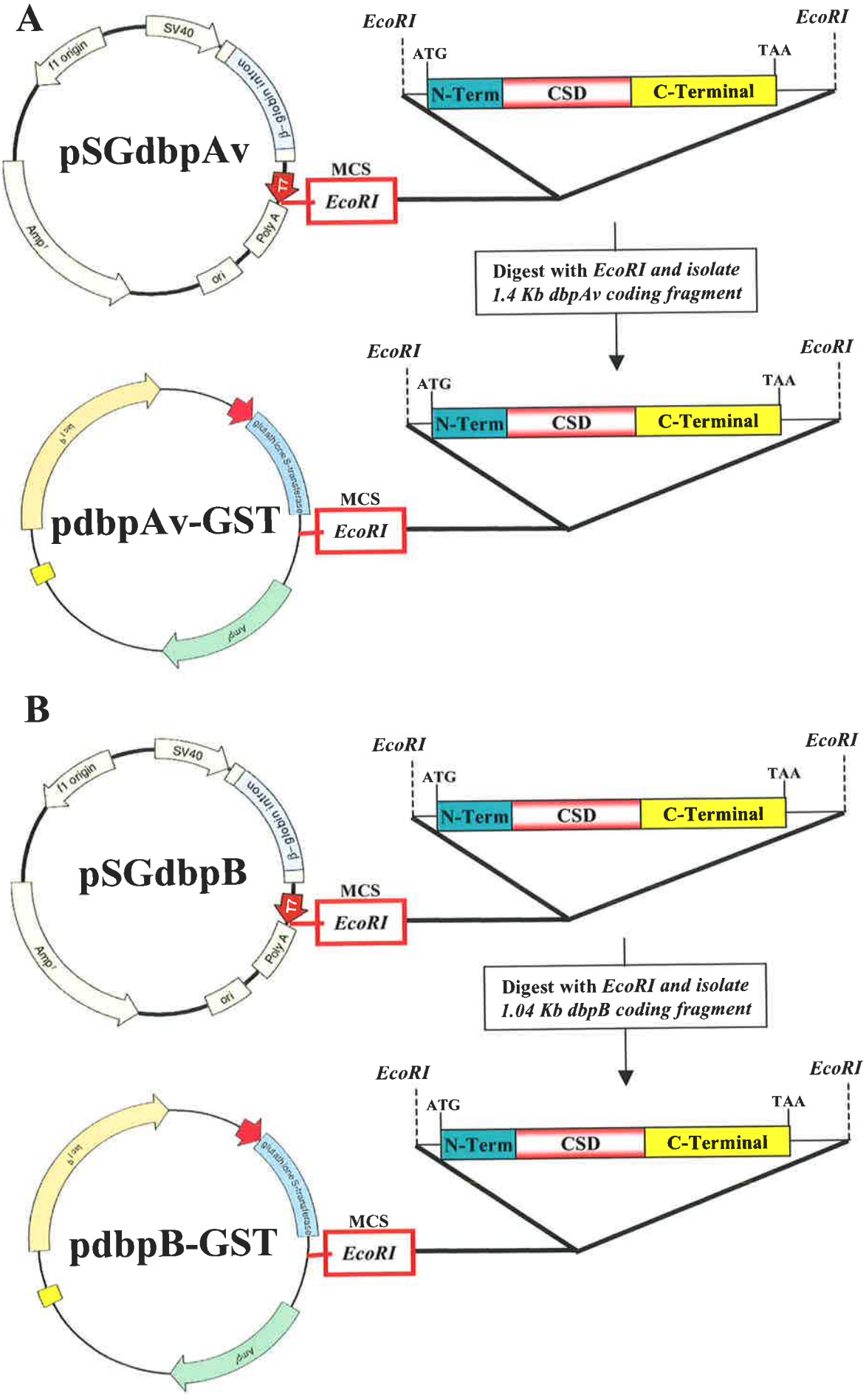

A
pSGdbpAv
SV40
f1 origin
β-globin intron
T7
MCS
EcoRI
Poly A
ori
Amp'
EcoRI
EcoRI
ATG
TAA
N-Term
CSD
C-Terminal
Digest with EcoRI and isolate
1.4 Kb dbpAv coding fragment
pdbpAv-GST
MCS
EcoRI
B
pSGdbpB
Digest with EcoRI and isolate
1.04 Kb dbpB coding fragment
pdbpB-GST

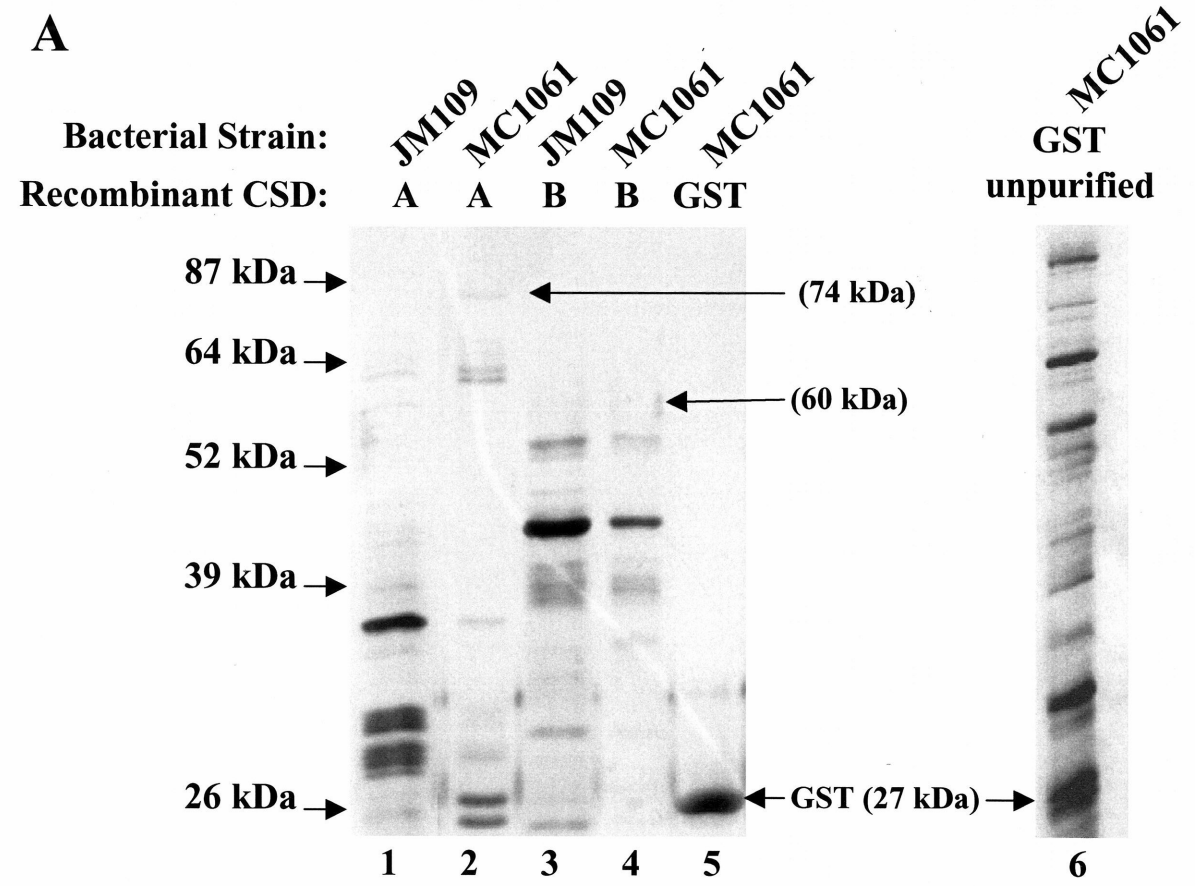
Figure 5.2

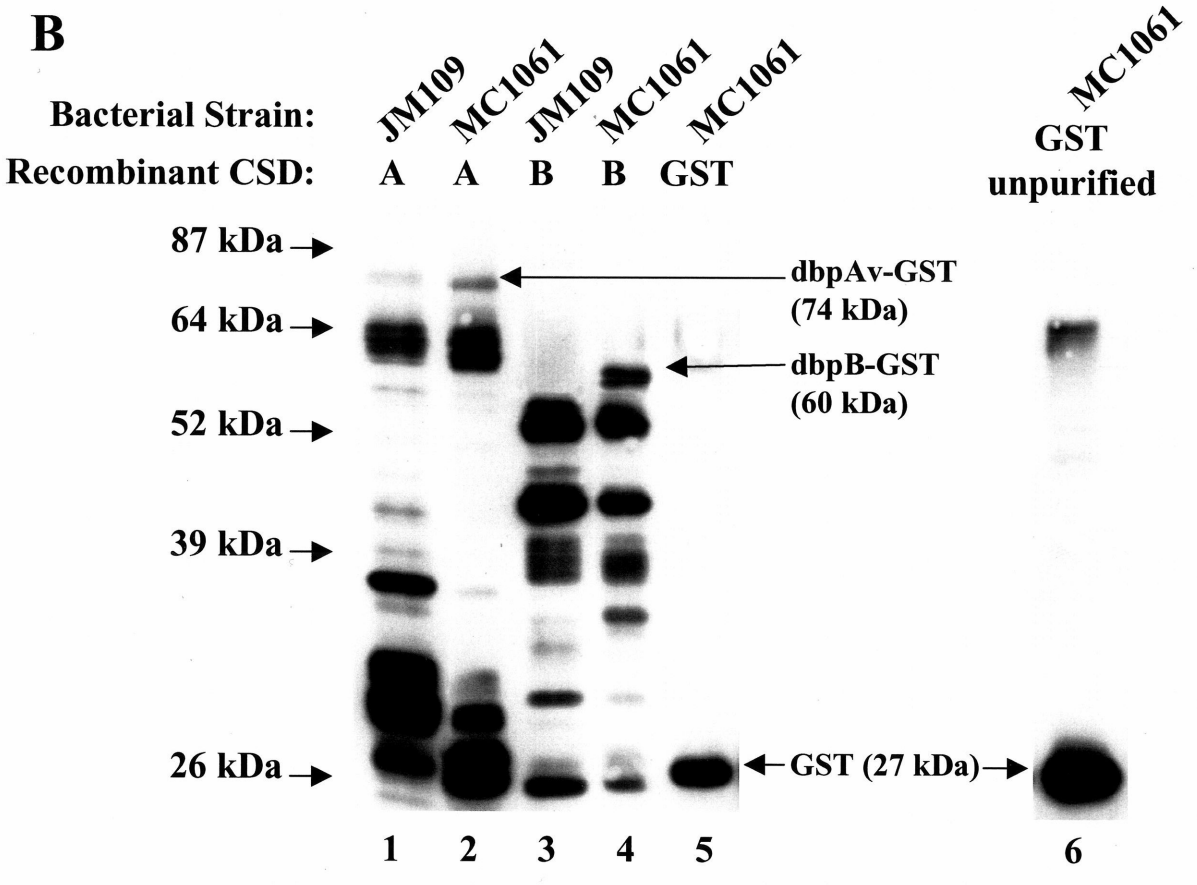
Recombinant CSD protein expression.

Recombinant GST fusion proteins dbpAv-GST and dbpB-GST were expressed in either JM109 or MC1061 bacterial strains then purified by binding to Glutathione Sepharose beads and eluting GST fused proteins using reduced glutathione (as described in the materials and methods section).

A) The eluted GST-fusion proteins were run on a 12% Laemmli SDS-PAGE protein gel [dbpAv-GST (expressed in: (JM109, Lane 1) (MC1061, Lane 2)], [dbpB-GST (expressed in: (JM109, Lane 3) (MC1061, Lane 4)], GST (expressed in MC1061, Lane 5), unpurified GST bacterial lysate (MC1061, Lane 6) and stained using coomassie stain to visualise the protein bands.

B) A replica of the 12% Laemmli SDS-PAGE protein gel in Figure A was transferred to a nitrocellulose filter using a BIO-RAD protein transfer apparatus and probed in a Western blot with an antibody raised to recombinant GST (as described in the materials and methods section).


A
Bacterial Strain:
JM109
MC1061
JM109
MC1061
MC1061
MC1061
GST unpurified
Recombinant CSD:
A
A
B
B
GST
87 kDa
64 kDa
52 kDa
39 kDa
26 kDa
(74 kDa)
(60 kDa)
GST (27 kDa)
1
2
3
4
5
6


B
Bacterial Strain:
JM109
MC1061
JM109
MC1061
MC1061
MC1061
GST unpurified
Recombinant CSD:
A
A
B
B
GST
87 kDa
64 kDa
52 kDa
39 kDa
26 kDa
dbpAv-GST
(74 kDa)
dbpB-GST
(60 kDa)
GST (27 kDa)
1
2
3
4
5
6

and methods section). The transformed bacteria were initially induced with IPTG for 3 hours, lysed and CSD-GST fusion proteins, dbpAv-GST and dbpB-GST, purified by binding to glutathione sepharose beads and eluted with reduced glutathione (Section 2.15 material and methods section). To determine protein yield and integrity, glutathione sepharose purified dbpAv-GST and dbpB-GST were analysed on a Laemmli SDS-PAGE gel and stained with coomassie blue to visualise the bands (Figure 5.2A). Many bands were seen in both the dbpAv-GST and dbpB-GST fusion protein materials, regardless of expression in MC1061 or JM109 bacterial strains but slightly more bands were present in the JM109 expressed material (Figure 5.2A, lanes 1-4). Since the purification of recombinant GST was so effective and the same method of purification was used to purify dbpAv-GST and dbpB-GST, this suggested that these extra bands represented degradation products. These degradation products were present despite the addition of protease inhibitors to all solutions used to make the bacterial lysates. The predicted size for the GST fusion protein dbpA-GST is 74 kDa and dbpB-GST is 70 kDa. A faint band possibly representing full length dbpAv-GST (74 kDa) can be seen in MC1061 expressed protein (Figure 5.2A, lane 2). Although a band of the expected size for full length dbpB-GST (70 kDa) was not observed, a very faint band of approximately 60 kDa was the largest product identified (Figure 5.2A, Lanes 3 & 4). Work performed in our laboratory and reported by others (Stenina *et al.*, 2000) have observed that dbpB does not migrate true to its predicted molecular weight on SDS-PAGE, therefore I have predicted that this 60 kDa band represents full length dbpB-GST fusion protein.

To confirm that the observed proteins identified on SDS-PAGE were GST-fusion proteins, duplicates of the gels in Figure 5.2A were transferred to nitrocellulose filters and probed in a Western blot with an antibody generated against GST (kindly supplied by Joanne Woodcock) (Figure 5.2B). To determine the specificity of the GST antibody, protein extracts containing purified and unpurified recombinant GST were again examined.

The GST antibody recognised two major bands in the unpurified material, a band migrating at approximately 27 kDa representing GST and another band at approximately 64 kDa representing an unknown protein (Figure 5.2B, lane 6). GST antibody recognised only the 27 kDa GST protein in the GST purified material (Figure 5.2B, lane 5) demonstrating that the GST antibody was highly specific. The GST antibody recognised all bands observed on the coomassie blue stained gel (Figure 5.2A), confirming that these bands represented CSD-GST fusion protein degradation products (Figure 5.2B, lanes 1-4).

As indicated in Figure 5.2B, the degradation seen in both CSD recombinant proteins was slightly more pronounced when the proteins were synthesised in the JM109 bacterial cell type, with a higher proportion of full length protein, albeit small, in the MC1061 synthesised proteins (Figure 5.2B compare lanes 1 & 2 for dbpAv-GST and lanes 3 & 4 for dbpB-GST). In MC1061 the full length dbpAv-GST protein (74 kDa) is better visualised (Figure 5.2B, lanes 1 & 2) and again no band larger that 60 kDa was detected for dbpB-GST (Figure 5.2B, lanes 3 & 4). The 60 kDa protein therefore probably represents full length dbpB-GST. Again as previously mentioned, others have observed similar differences when comparing the predicted molecular weight and apparent molecular weight of dbpB by mobility on SDS-PAGE (Stenina *et al.*, 2000).

To further test the recombinant CSD proteins, glutathione sepharose purified dbpAv-GST and dbpB-GST isolated from both JM109 and MC1061 bacterial cell types were bound to wildtype, domain 1 GM- single stranded oligonucleotide in a gel shift assay. As can be clearly seen in Figure 5.3A, for both dbpAv-GST and dbpB-GST, proteins expressed in MC1061 gave a single complex whereas proteins expressed in JM109 gave multiple bands. These extra bands observed in the JM109 expressed material presumably arise from the higher percentage of degradation products observed in JM109 expressed material compared to MC1061 where more full length protein was observed (Figure 5.2B).

Figure 5.3

Recombinant CSD protein purification.

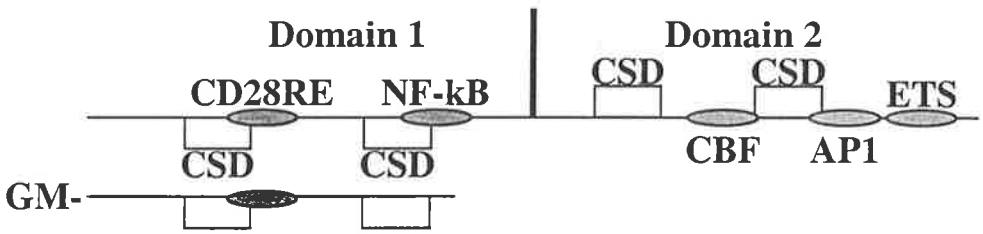
A) Purified recombinant CSD fusion proteins dbpAv-GST and dbpB-GST expressed in either JM109 or MC1061 bacterial strains were bound in a gel shift assay to ^{32}P-labelled GM-CSF domain 1 non-coding (-) strand GM- oligonucleotides.

Recombinant dbpAv-GST expressed in JM109 [Lane 1 (0.15ng), Lane 2 (0.25ng)], and expressed in MC1061 [Lane 3 (0.15ng), Lane 4 (0.25ng)].

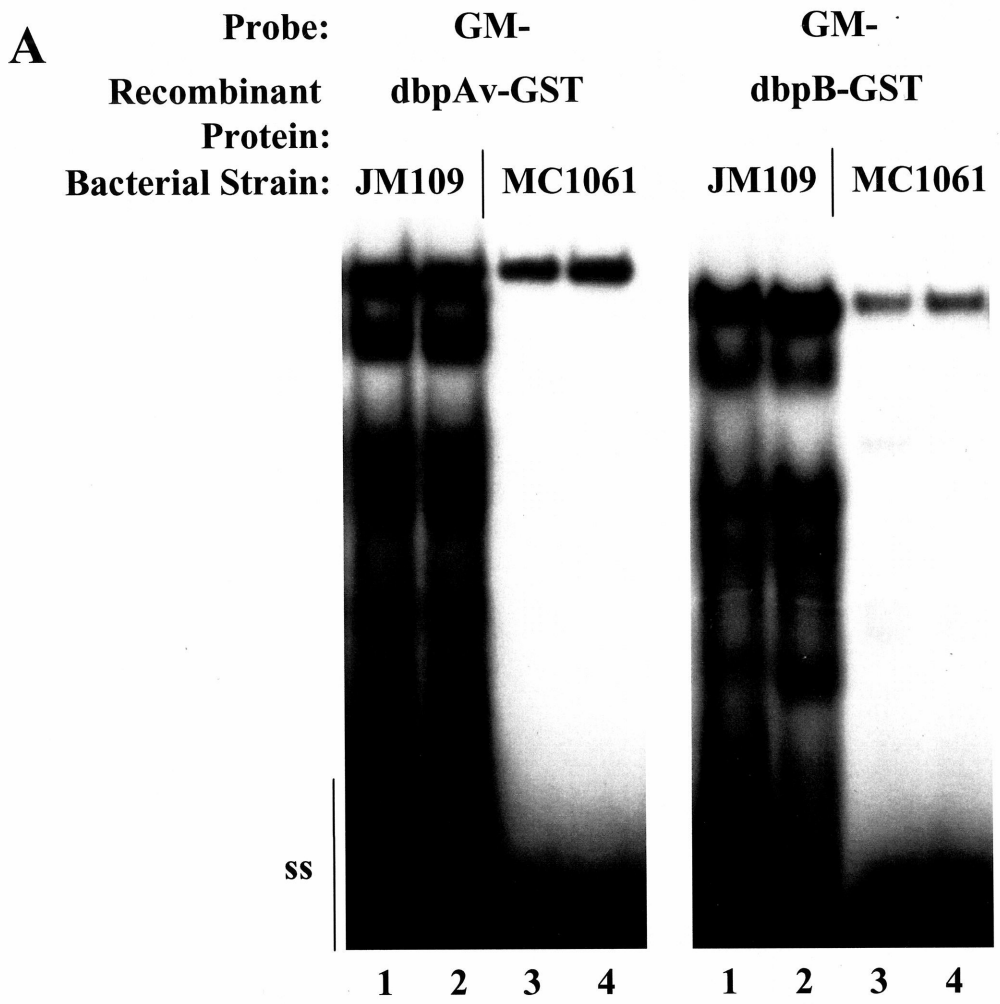
Recombinant dbpB-GST expressed in JM109 [Lane 5 (0.15ng), Lane 6 (0.25ng)], and expressed in MC1061 [Lane 7 (0.15ng), Lane 8 (0.25ng)].

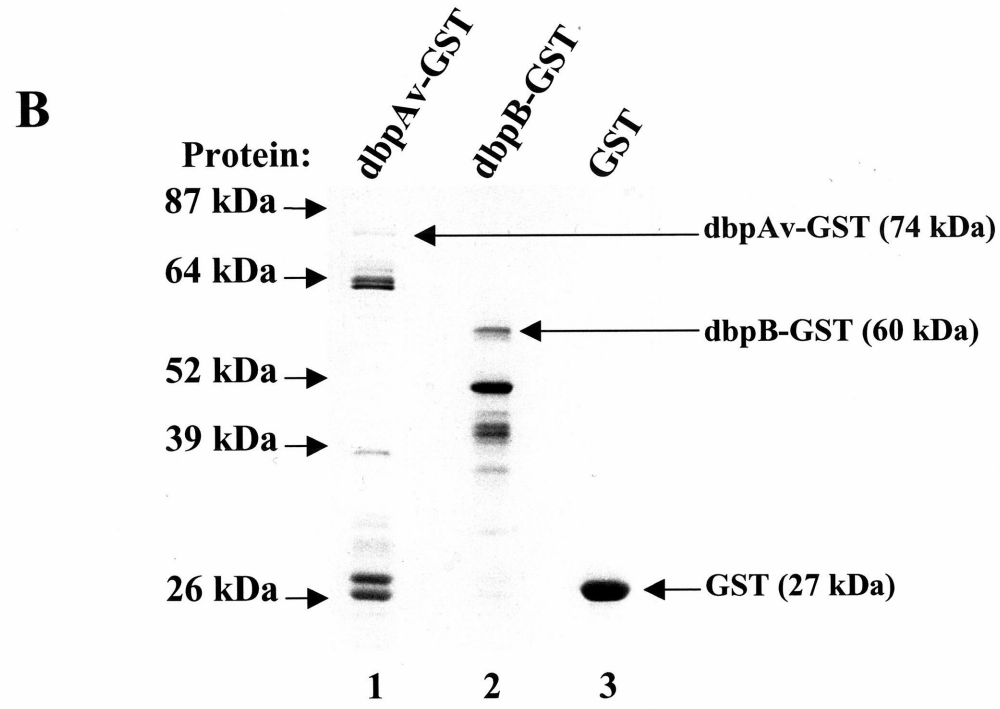
(ss indicates free ^{32}P-labelled single stranded oligonucleotides)

B) Final purified recombinant CSD fusion proteins dbpB-GST (Lane 1), dbpAv-GST (Lane 2) and GST (Lane 3) fractionated on a 12% Laemmli SDS-PAGE protein gel and stained with coomassie to visualise the protein bands.



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites and ovals represent the transcription factor binding site as indicated above. The oligonucleotide sequences are presented in Figure 4.1.


A
Probe:
GM-
GM-
Recombinant Protein:
dbpAv-GST
dbpB-GST
Bacterial Strain:
JM109
MC1061
JM109
MC1061
ss
1
2
3
4
1
2
3
4


B
Protein:
dbpAv-GST
dbpB-GST
GST
87 kDa
64 kDa
52 kDa
39 kDa
26 kDa
dbpAv-GST (74 kDa)
dbpB-GST (60 kDa)
GST (27 kDa)
1
2
3

In an effort to increase both yield and protein integrity many different strategies were undertaken, including experimenting with bacterial concentration before induction, final concentration of IPTG used to induce protein synthesis, induction time and bacterial cell type (data not shown). The major difficulty encountered was obtaining reasonable yields of proteins without protein degradation. In an attempt to overcome this problem the protease deficient bacterial strain BL21(DE3) was used but this again did not improve either yield or protein integrity (data not shown). The poor yield and integrity of these proteins may be due to toxicity of the proteins to bacteria or that the proteins are intrinsically unstable and easily degraded. Problems were also encountered with storage, with the recombinant CSD proteins unable to be frozen for storage, once thawed these proteins lost all DNA binding ability (Data not shown). Therefore recombinant proteins were stored at 4°C and in some instances had a shelf life of only a couple of weeks. Individual conditions were deduced for optimal yield and integrity for both dbpAv-GST and dbpB-GST. Optimal expression of dbpAv-GST was achieved with a 4 hour 0.5 mM (final concentration) IPTG induction, whereas optimal expression of dbpB-GST was achieved with a 3 hour 1 mM (final concentration) IPTG induction. Recombinant CSD proteins expressed in a MC1061 bacterial strain using these optimised conditions were used in all subsequent experiments and the material visualised on a coomassie blue stained SDS-PAGE gel is presented in Figure 5.3B.

5.3 Recombinant dbpAv-GST and dbpB-GST binding to CSD binding site mutant oligonucleotides spanning the GM-CSF promoter.

To determine if recombinant, glutathione sepharose purified CSD proteins bound to the GM-CSF promoter CSD binding sites, recombinant CSD proteins (dbpAv-GST and dbpB-GST) were bound in gel shift assays to wildtype domain 1 (GM-) and domain 2 (GM93+) single stranded oligonucleotides (each containing two CSD binding sites) and to

oligonucleotides containing CSD binding site mutants. Gel shift analysis with recombinant CSD protein dbpAv-GST, indicated that binding was only to the non-coding (-) strand of the wildtype domain 1 oligonucleotide (GM- Figure 5.4A, Lanes 4 and 5), as seen with nuclear CSD NF-GMb/c factors in Jurkat and HUT78 T cells and HEL fibroblasts (Chapter 4). Mutating any of the CSD binding sites in domain 1 (GMm 19-, 21- & 23-) resulted in loss of dbpAv-GST binding (Figure 5.4A, Lanes 1 to 3). When the full domain 2 oligonucleotide GM93+ was used in a gel shift binding was the same as nuclear cold shock domain proteins, i.e. strong binding only to the coding (+) strand (Figure 5.4A, lanes 6 & 7). Using the domain 2 CSD mutant oligonucleotide GMm103+, which has one CSD binding site mutated, resulted in a significant decrease in dbpAv-GST binding (Figure 5.4A, Lane 8). Mutating both domain 2 CSD binding sites in GMm105+ resulted in complete loss of binding (Figure 5.4A, Lane 9). Therefore these results suggest that dbpAv-GST binding to the GM93+ oligonucleotide requires both CSD binding sites for complete binding.

To determine if both CSD proteins had the same binding affinity and specificity, recombinant CSD, dbpB-GST was also bound to the same series of GM-CSF domain 1 and 2 wildtype single stranded oligonucleotides and mutants. Recombinant CSD protein dbpB-GST bound only to the non-coding (-) strand of domain 1 and the coding (+) strand of domain 2 of the GM-CSF proximal promoter (Figure 5.4B, GM lanes 1 & 2). This binding pattern was the same as that observed for either the Jurkat and HUT78 T cells and HEL fibroblast NF-GM complexes or recombinant dbpAv-GST CSD protein. When dbpB-GST was bound to domain 1, single CSD site mutant oligonucleotides (Figure 5.4B, GMm19-, Lane 3 and GMm21-, Lane 4, dbpB-GST) binding was reduced but not abolished. It was only when both CSD binding sites were mutated in GMm23-, that all dbpB-GST binding was lost (Figure 5.4B, Lane 5). As expected when the full domain 2 GM93+ oligonucleotide was used dbpB-GST binding was only seen to the coding (+) strand

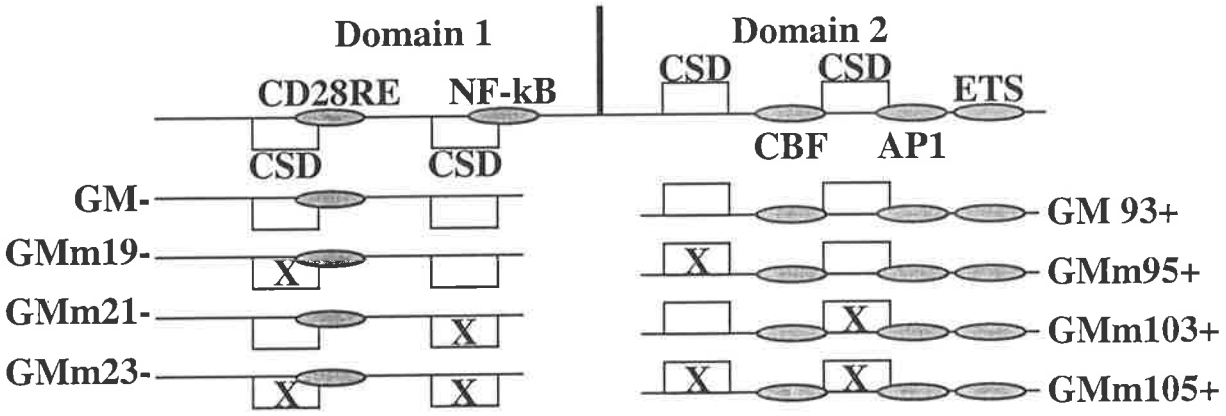
Figure 5.4

Recombinant dbpAv-GST and dbpB-GST binding to GM-CSF promoter mutant oligonucleotides.

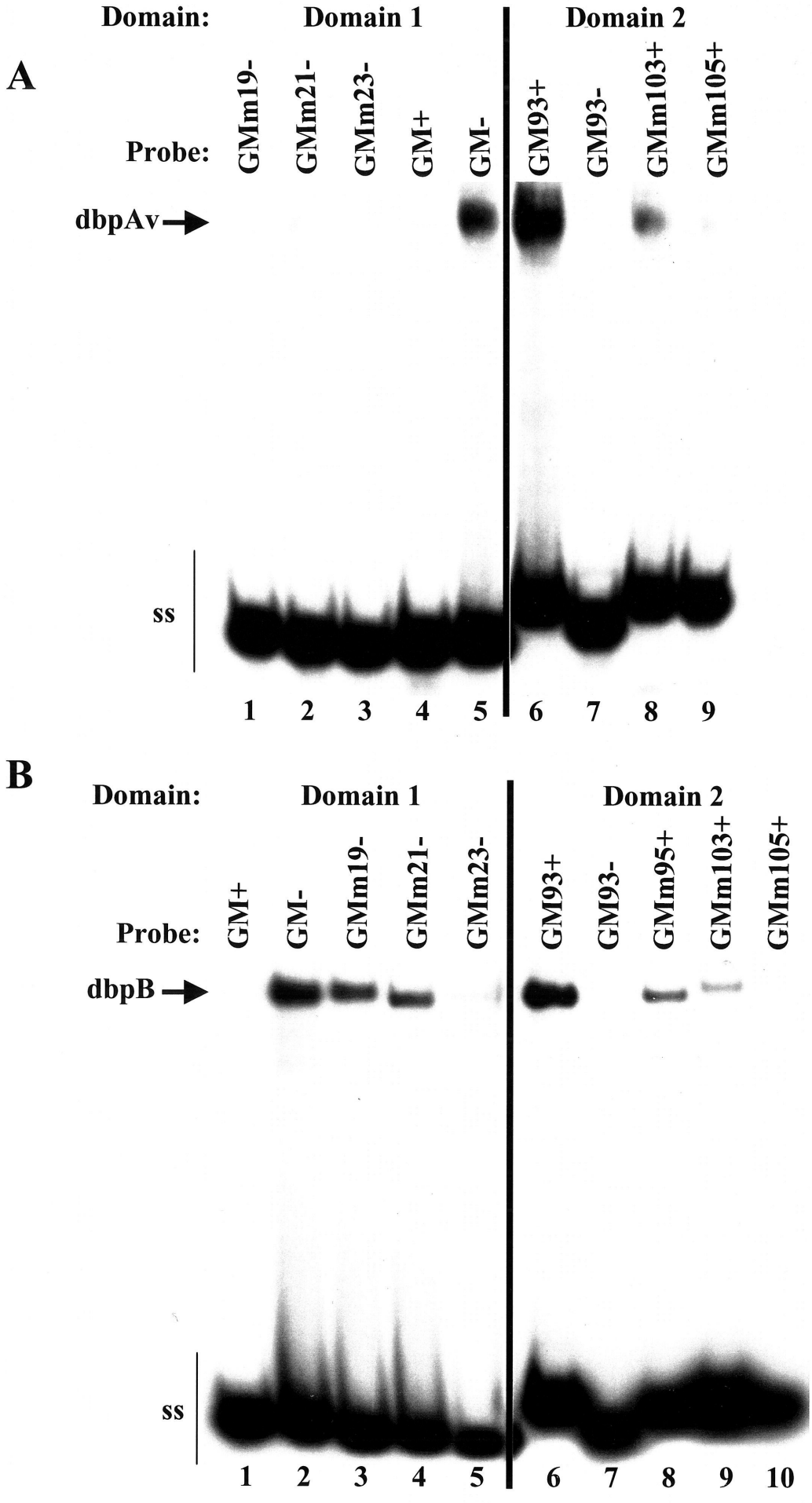
A) Recombinant dbpAv-GST was bound to ^{32}P-labelled wild type domain 1 coding (+) and non-coding (-) oligonucleotides GM+, GM- and corresponding mutant oligonucleotides GMm19-, GMm21-, GMm23- (lanes 1-5). Recombinant dbpAv-GST was also bound to ^{32}P-labelled wild type domain 2 coding (+) and non-coding (-) oligonucleotides GM93+, GM93- and corresponding mutant oligonucleotides GMm95+, GMm103+, GMm105+ (lanes 6-9).

B) Recombinant dbpB-GST was bound to ^{32}P-labelled wild type domain 1 coding (+) and non-coding (-) oligonucleotides GM+, GM- and corresponding mutant oligonucleotides GMm19-, GMm21-, GMm23- (lanes 1-5). Recombinant dbpB-GST was also bound to ^{32}P-labelled wild type domain 2 coding (+) and non-coding (-) oligonucleotides GM93+, GM93- and corresponding mutant oligonucleotides GMm95+, GMm103+, GMm105+ (lanes 6-10).

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)



The human GM-CSF proximal promoter oligonucleotides used in these experiemtns are represented diagrammatically above. Boxes represent binding sites and boxes with an 'X" in them indicate mutated CSD binding sites, ovals represent the transcription factor binding site as indicated above. The oligonucleotide sequences are presented in Figure 4.1.


A
Domain:
Domain 1
Domain 2
Probe:
GMm19-
GMm21-
GMm23-
GM+
GM-
GM93+
GM93-
GMm103+
GMm105+
dbpAv
ss
1 2 3 4 5 6 7 8 9
B
Domain:
Domain 1
Domain 2
Probe:
GM+
GM-
GMm19-
GMm21-
GMm23-
GM93+
GM93-
GMm95+
GMm103+
GMm105+
dbpB
ss
1 2 3 4 5 6 7 8 9 10

(Figure 5.4B, lanes 6 & 7). Mutating either of the domain 2 CSD binding sites resulted in a decrease in dbpB-GST binding sites (Figure 5.4B GMm95+, lane 8 and GMm103+, lane 9) but not until both sites were mutated in GMm105+ was all dbpB-GST binding lost (Figure 5.4B, lane 10). These experiments indicate that the two recombinant CSD proteins recognise the same NF-GMb/c CSD binding sequences on the GM-CSF proximal promoter. While there appears to be some differences in binding affinity between the two, with CSD binding site mutations on domain 1 having a greater affect on dbpAv-GST binding than dbpB-GST, the same principle applies to either protein, that both CSD binding sites are required in each domain for full CSD binding. Consistently both CSD binding sites in either domain 1 or domain 2 are also required for full binding of the nuclear NF-GMb/c complexes in Jurkat T cells (Chapter 2) and HUT78 T cells and HEL fibroblasts (Coles *et al.*, 1994); (Coles *et al.*, 1996); (Coles *et al.*, 2000).

5.4 Thrombin cleavage of dbpB-GST and potential multimerisation of CSD proteins

The recombinant CSD proteins expressed from the pGEX-4T-1 bacterial expression plasmid were expressed as GST fusions to enable purification. To more accurately determine the apparent molecular weight and to determine if the GST tag affected binding or protein structure, the GST tag was cleaved from the CSD proteins with thrombin. Recombinant dbpB-GST was digested with thrombin and the resulting dbpB protein bound to wildtype domain 1 GM- oligonucleotide alongside dbpB-GST to compare binding affinity and complex formation (Figure 5.5A). Recombinant dbpB-GST was bound to GM- resulting in a single band of strong binding intensity as seen previously (Figure 5.5A, lane 1). When dbpB thrombin-cleaved material was bound to the GM- oligonucleotide two complexes were seen, (complexes b and c, Figure 5.5A, lane 2). Neither of the two complexes observed in the dbpB thrombin-cleaved material co-migrated with the full length dbpB-GST complex and hence could not be attributed to incomplete

Figure 5.5

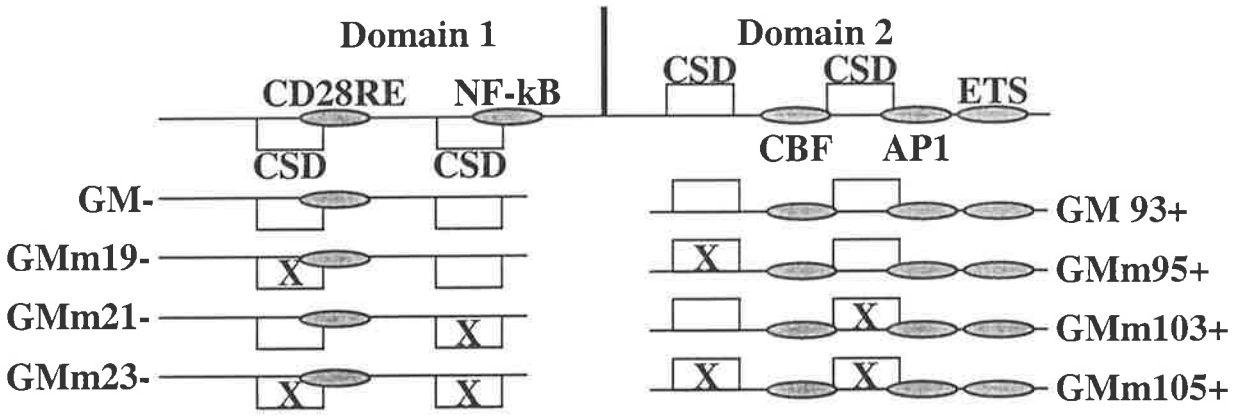
Binding of recombinant dbpB-GST compared to dbpB thrombin cleaved.

A) Recombinant GST fusion protein dbpB-GST (Lane 1) and dbpB-GST with the GST tag cleaved via thrombin (Lane 2) were bound in a gel shift assay to ^{32}P-labelled GM-CSF domain 1 non-coding (-) strand GM- oligonucleotide.

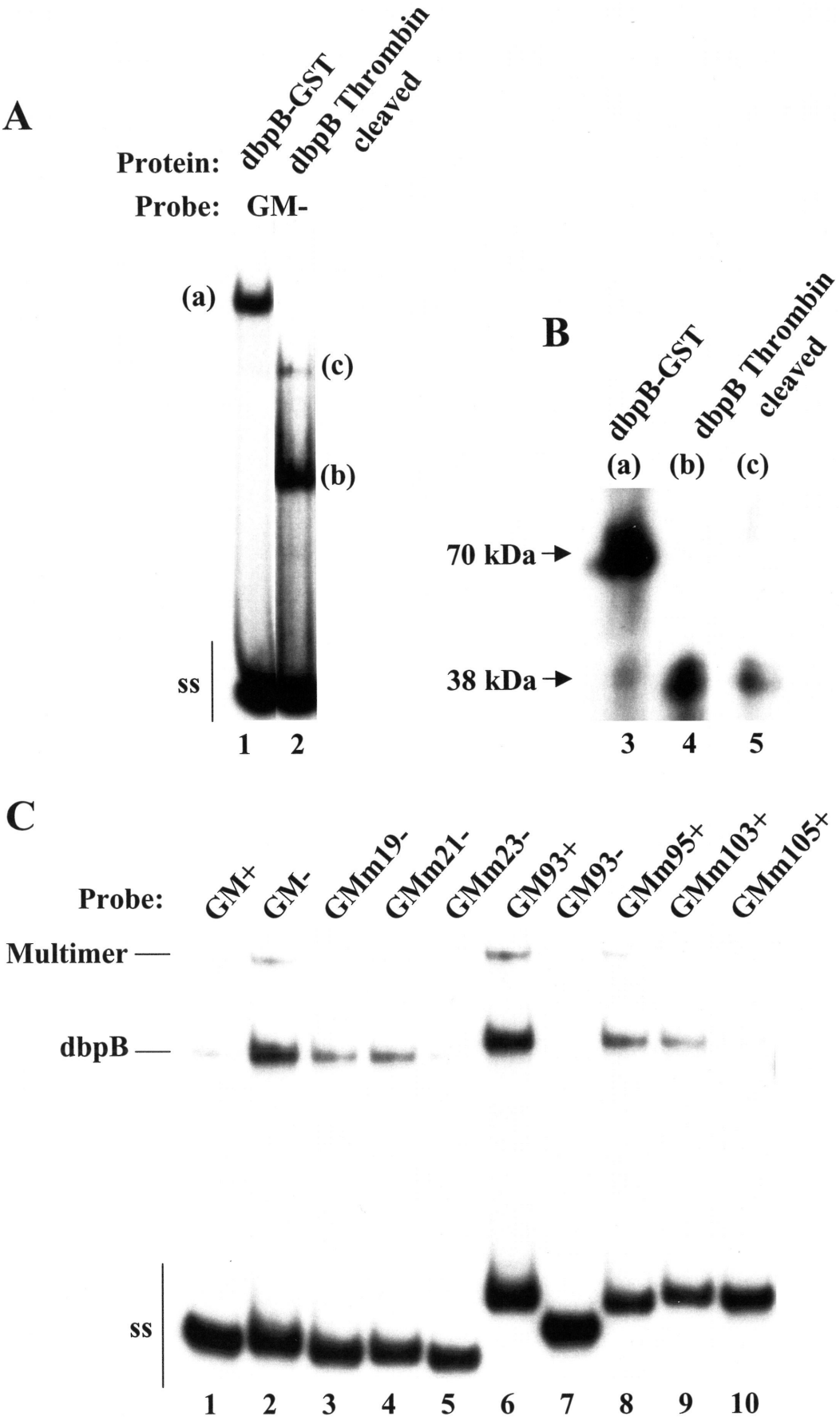
B) Gel shift complexes observed in Figure A were UV cross-linked and fractionated on a Laemmli SDS-PAGE protein gel. Figure B shows the resulting UV cross-linked complexes, recombinant dbpB-GST (Lane 3), the single unit complex (b) from the GST cleaved dbpB (Lane 4) and the GST cleaved dbpB multimer complex (c) (Lane 5). Protein sizes are indicated in kilodaltons and determined by subtracting the size of the free probe (13.5 kDa).

C) Recombinant GST cleaved dbpB was bound to ^{32}P-labelled wild type domain 1 coding (+) and non-coding (-) oligonucleotides GM+, GM- and corresponding mutant oligonucleotides GMm19-, GMm21-, GMm23- (lanes 1-5). Recombinant GST cleaved dbpB was also bound to ^{32}P-labelled wild type domain 2 coding (+) and non-coding (-) oligonucleotides GM93+, GM93- and corresponding mutant oligonucleotides GMm95+, GMm103+, GMm105+ (lanes 6-10).

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent binding sites and boxes with an 'X' in them indicate mutated CSD binding sites, ovals represent the transcription factor binding site as indicated above. The oligonucleotide sequences are presented in Figure 4.1.


A
Protein:
dbpB-GST
dbpB Thrombin cleaved
Probe: GM-
(a)
(c)
(b)
ss
1 2
B
dbpB-GST
dbpB Thrombin cleaved
(a) (b) (c)
70 kDa
38 kDa
3 4 5
C
Probe:
GM+
GM-
GMm19-
GMm21-
GMm23-
GM93+
GM93-
GMm95+
GMm103+
GMm105+
Multimer
dbpB
ss
1 2 3 4 5 6 7 8 9 10

thrombin cleavage of the GST tag. A scan of the dbpB sequence did not identify any known internal thrombin cleavage sites and experiments done by Stenia *et al.* (Stenina *et al.*, 2000) confirm that thrombin does not cleave dbpB.

To determine the size of these two dbpB thrombin-cleaved complexes in relation to the full length dbpB-GST protein, all three complexes were subjected to UV cross-linking analysis. The molecular weight of the proteins in UV cross-linking analysis is determined by subtracting the molecular weight of the oligonucleotide probe (13.5 kDa for GM-) from the observed molecular weight on the gel. The kDa sizes shown in Figure 5.5 have had the molecular weight of the probe removed. UV cross-linking analysis of full length recombinant dbpB-GST identified a protein with the correct predicted molecular weight of 70 kDa (45 kDa dbpB plus 27 kDa GST) (Figure 5.5B, complex (a), lane 1). This was in contrast to the observed molecular weight of recombinant dbpB-GST on SDS-PAGE, which gave an approximate molecular weight of 60 kDa. As stated previously I and others (Stenina *et al.*, 2000) have shown that CSD proteins on their own do not migrate true to their predicted molecular weight on SDS-PAGE. Experiments in our laboratory show that size estimation for CSD proteins via UV cross-linking can be more accurate that SDS-PAGE analysis ((Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000) & unpublished results). UV cross-linking of the (b) and (c) complexes seen in the dbpB thrombin-cleaved material identified that neither of the bands represented thrombin-cleaved dbpB migrating to its predicted size of 45 kDa. Surprisingly both (b) and (c) complexes UV cross-linked a protein of approximately 38 kDa (Figure 5.5B, lanes 4 & 5). These results suggest that the bottom (b) complex seen in the gel shift assay represents thrombin-cleaved protein binding (even though it doesn't migrate to its predicted molecular weight) and the higher (c) complex represents multimer binding of this dbpB thrombin cleaved protein. Multimerisation of CSD proteins has also been observed for *Xenopus* CSD factors (Tafuri *et al.*, 1992).

To determine the GM-CSF CSD binding site specificity of dbpB thrombin cleaved material and if the number of CSD binding sites could effect the ability of the protein to form multimers, dbpB thrombin-cleaved material was bound to GM-CSF domain 1 and 2 oligonucleotides and respective CSD binding site mutants (Figure 5.5C). As expected the binding specificity of dbpB was the same as that seen for dbpB-GST with binding only to the non-coding (-) strand of domain 1 and to the coding (+) strand of domain 2 (Figure 5.5C, lanes 1,2 domain 1 & 6,7 domain 2). Mutation of either CSD binding site reduced binding and mutation of both CSD sites resulted in loss of binding (Figure 5.5C, lanes 3 to 5 domain 1 and lanes 8 to 10 domain 2). Potential multimerisation was observed on oligonucleotides containing one or two CSD sites, hence two CSD sites are not required for multimerisation to occur (Figure 5.5C). The thrombin cleaved dbpB material was very unstable, rapidly losing DNA binding ability and therefore was not used in further gel shift experiments (data not shown).

5.5 The CSD domain of dbpB is essential for binding to the GM-CSF promoter

Experimental analysis of CSD proteins has lead to the division of CSD proteins into three domains, each of which has been attributed function: the C-terminal region for protein:protein interaction and non-sequence specific RNA interactions, the CSD domain for sequence specific single stranded DNA/RNA interactions and the N-terminus which contributes to single stranded DNA binding (Shannon *et al.*, 2001). In HEL fibroblasts and HUT78 T cells a 22 kDa protein was identified in both the NF-GMb and NF-GMc complexes (Chapter 4 & (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000)). This protein is proposed to be a splice or proteolytic cleavage variant of the full length CSD protein (42 kDa) which lacks a significant proportion of the C-terminal protein:protein interaction domain. To determine if a CSD protein lacking either the C-terminus or both CSD and C-terminal domains could bind to DNA, dbpB bacterial expression constructs

encoding dbpB truncations were made. The truncations of dbpB used were the same as the dbpB truncations encoded by the eukaryotic expression constructs pSGdbpBΔ1 and pSGdbpBΔ2 used in previous transient transfections.

The bacterial expression construct for the dbpB deletion dbpBΔ1 (pdbpBΔ1-GST) was created by digesting the dbpBΔ1 eukaryotic expression vector (pSGdbpBΔ1) with *EcoRI / NotI* to release a fragment which encodes for the first 47 amino acids of dbpB followed by ligation into the *EcoRI / NarI* sites of pGEX-4T-1 (Figure 5.6). The bacterial expression construct for the dbpB deletion dbpBΔ2 (pdbpBΔ2-GST) was created by digesting the dbpBΔ2 eukaryotic expression vector (pSGdbpBΔ2) with *EcoRI / NotI* releasing a fragment which encodes for the first 173 amino acids of dbpB followed by ligation into the *EcoRI / BglII* sites of pGEX-4T-1 (Figure 5.6).

Full length recombinant dbpB-GST and the dbpB-GST truncation proteins, encoded by bacterial expression constructs pdbpB-GST, pdbpBΔ1-GST and pdbpBΔ2-GST, were synthesised using the Promega *E. coli* T7 S30 extract *in vitro* transcription translation system and the [^{35}S]methionine labeled proteins were run on a Laemmli SDS-PAGE gel to check for appropriate expression (Figure 5.7). Full length dbpB was not efficiently transcribed/translated in the Promega *in vitro* kit (Figure 5.7A, lane 1). The majority of the bands seen in this track were also observed in the no DNA control reaction, indicating that they were non-specific (Figure 5.7A, compare lanes 1 & 4). Previous experiments where recombinant dbpB-GST was analysed by SDS-PAGE, indicated the mobility of dbpB-GST was approximately 60 kDa (Figures 5.2B & 5.3B). In this experiment a very faint band can be seen migrating at this molecular weight (Figure 5.7A, lane 1). This result again indicates the apparent instability of the full length dbpB protein. Intense bands were observed for protein migrating at the predicted molecular weight for dbpBΔ1-GST (32 kDa, Figure 5.7A, lane 2) and dbpBΔ2-GST (46 kDa, Figure 5.7A, lane

Figure 5.6

Construction of the bacterial expression dbpB truncation constructs pdbpBΔ1-GST and pdbpBΔ2-GST.

A) & B) The coding region from the eukaryotic dbpB truncation expression constructs for either dbpBΔ1 (**Figure A**) or dbpBΔ2 (**Figure B**) were excised and cloned into the bacterial expression vector pGEX-4T-1.

Abbreviations: ori, bacterial origin of replication; Amp^r, ampicillin resistance gene; $lacI^q$, lactose operon repressor protein; T7, T7 transcriptional start site; MCS multiple cloning site

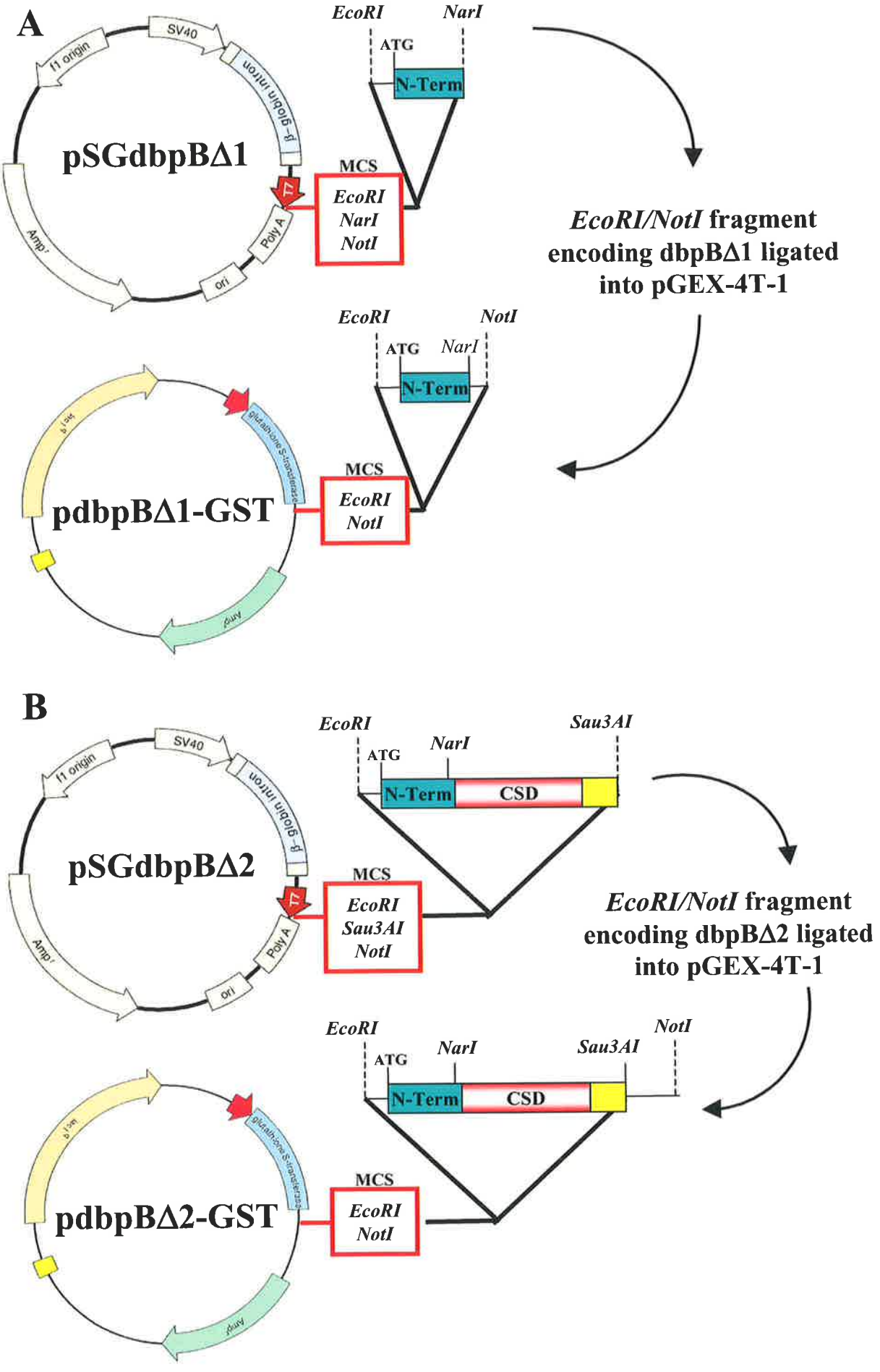
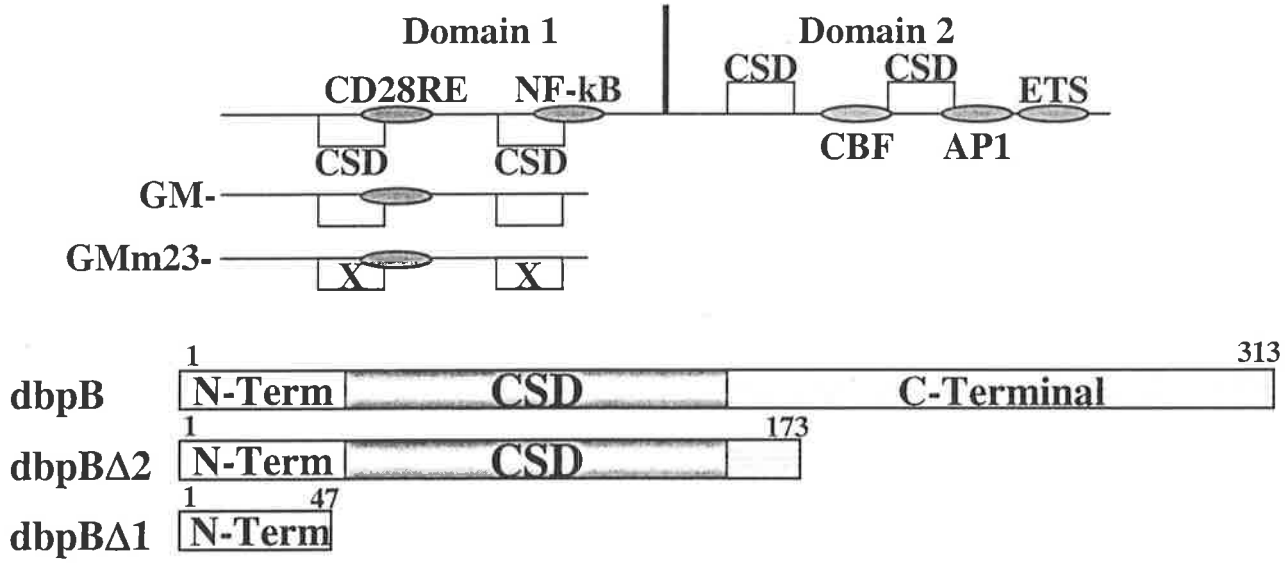

A
EcoRI
NarI
ATG
N-Term
MCS
EcoRI
NarI
NotI
pSGdbpBΔ1
SV40
f1 origin
β-globin intron
T7
Poly A
ori
Amp r
EcoRI/NotI fragment encoding dbpBΔ1 ligated into pGEX-4T-1
EcoRI
NotI
ATG
NarI
N-Term
MCS
EcoRI
NotI
pdbpBΔ1-GST
lacI
glutathione S-transferase
Amp r
B
EcoRI
NarI
Sau3AI
ATG
N-Term
CSD
MCS
EcoRI
Sau3AI
NotI
pSGdbpBΔ2
SV40
f1 origin
β-globin intron
T7
Poly A
ori
Amp r
EcoRI/NotI fragment encoding dbpBΔ2 ligated into pGEX-4T-1
EcoRI
NarI
Sau3AI
NotI
ATG
N-Term
CSD
MCS
EcoRI
NotI
pdbpBΔ2-GST
lacI
glutathione S-transferase
Amp r

Figure 5.7

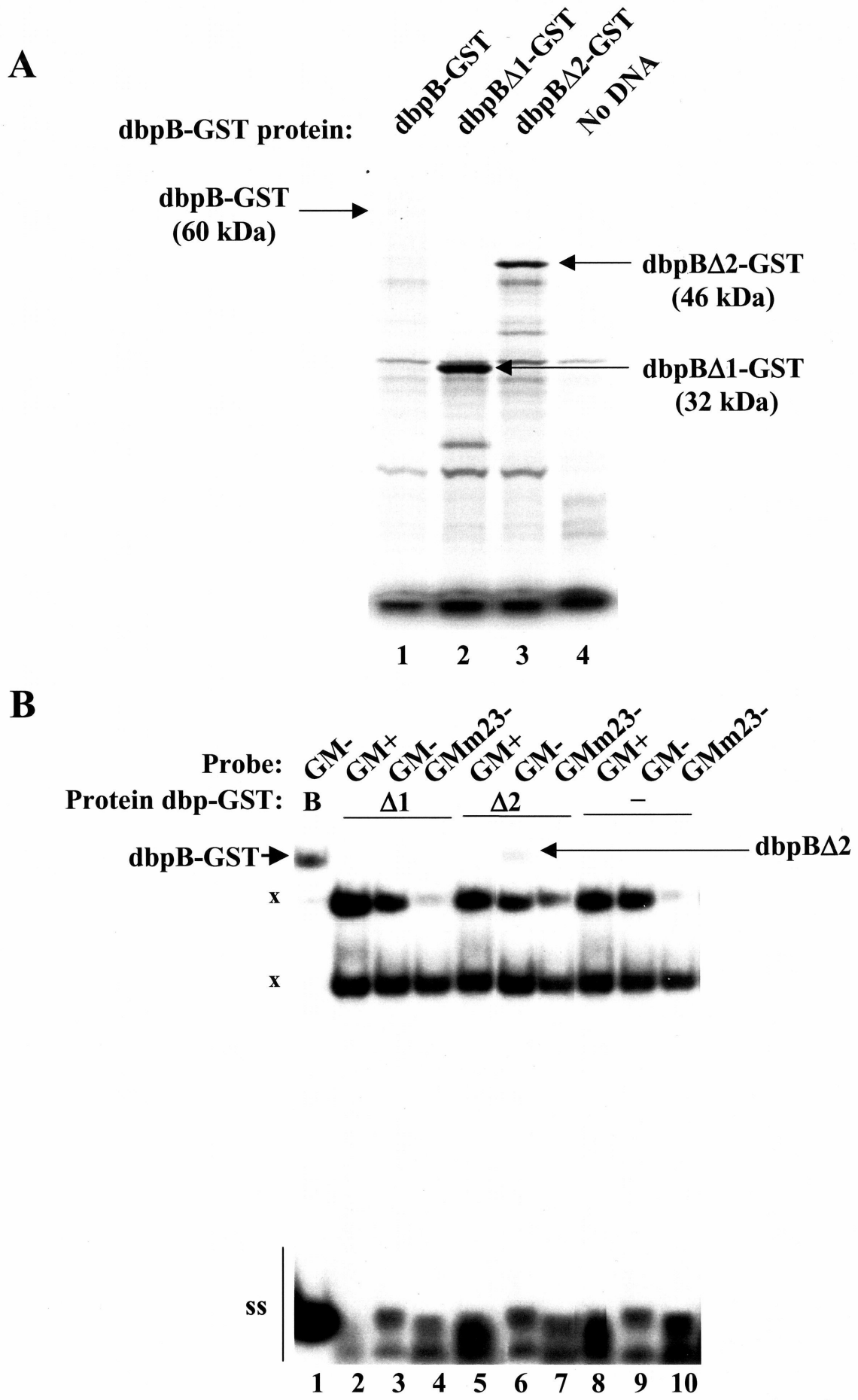
***In vitro* transcribed/translated dbpB-GST truncations.**

A) The coding region for full length recombinant dbpB-GST (encoding 313 amino acids) was truncated to give 2 constructs encoding for the first 173 amino acids (pdbpBΔ2-GST) and the first 47 amino acids (pdbpBΔ1-GST). Constructs for full length and dbpB-GST truncations were used to express [^{35}S]Methionine labelled recombinant truncated dbpB-GST proteins in a bacterial *in vitro* transcription/translation system. The expressed proteins: dbpB-GST (lane 1), dbpBΔ1-GST (lane 2), dbpBΔ2-GST (lane 3) were run out on a 12% Laemmli SDS-PAGE protein gel, along with a no DNA control reaction (lane 4).

B) The *in vitro* transcribed/translated dbpB-GST truncation proteins dbpBΔ1-GST and dbpBΔ2-GST along with bacterial expressed full length dbpB-GST and a negative control no DNA *in vitro* reaction mix were bound to ^{32}P-labelled wild type domain 1, non-coding (-) strand GM- (lanes 1,3,6 & 9), coding (+) strand GM+ (lanes 2, 5 & 8) and the non-coding (-) strand CSD mutant GMm23- (Lanes 4, 7 & 10) single stranded oligonucleotides. (ss indicates free ^{32}P-labelled single stranded oligonucleotides)
(x represents non-specific binding)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent binding sites, boxes with an 'X' in them indicate mutated CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above.. The oligonucleotide sequences are presented in Figure 4.1. The regions of dbpB encoded by the deletion constructs are also shown with numbers indicating amino acid number.


A
dbpB-GST protein:
dbpB-GST
dbpBΔ1-GST
dbpBΔ2-GST
No DNA
dbpB-GST
(60 kDa)
dbpBΔ2-GST
(46 kDa)
dbpBΔ1-GST
(32 kDa)
1 2 3 4
B
Probe:
GM- GM+ GM- GMm23- GM+ GM- GMm23- GM+ GM- GMm23-
Protein dbp-GST:
B
Δ1
Δ2
–
dbpB-GST
dbpBΔ2
x
x
ss
1 2 3 4 5 6 7 8 9 10

3). Many smaller bands were visible in these tracks but the full length bands were of a higher intensity.

To determine the function of the CSD and C-terminal domains of CSD proteins in relation to binding to the GM-CSF proximal promoter, these *in vitro* expressed proteins were bound in a gel shift to wildtype non-coding (-) and coding (+) domain 1 GM-CSF promoter oligonucleotides and the double CSD domain 1 mutant oligonucleotide GMm23- (Figure 5.7B). Since the full length dbpB-GST protein was not efficiently transcribed/translated in the *in vitro* system, bacterially expressed full length dbpB-GST was used in the gel shift assay as a positive control instead. The bacterially expressed dbpB-GST material bound strongly as a single complex to the GM-CSF domain 1 GM- oligonucleotide (Figure 5.7B, lane 1). When either of the *in vitro* transcribed/translated proteins dbpBΔ1-GST, dbpBΔ2-GST were bound to oligonucleotides in the gel shift assay, two major bands were seen (Figure 5.7B, lanes 2-7). To determine if these bands represented specific CSD protein binding, a no DNA control reaction mix from the *in vitro* kit, was also used in the gel shift assay as a negative control. These two major bands were also seen when the no DNA control reaction mix was used in the gel shift (Figure 5.7B, lanes 8-10), indicating that they did not represent *in vitro* transcribed/translated dbpBΔ1-GST or dbpBΔ2-GST. Therefore, these bands may represent non-specific bacterial single stranded DNA binding proteins present in the *E. coli* extract included in the *in vitro* transcription/translation kit.

When the dbpB truncation dbpBΔ1-GST (lacks both the C-terminus and CSD domain) was used in the gel shift assay, apart from the two previously discussed non-specific bands, no binding could be detected to any of the oligonucleotides (Figure 5.7B, lanes 5-7). This result confirms those observed by others indicating that while the N-terminal region aids in single stranded DNA binding, it is not sufficient to confer binding on its own. When the dbpBΔ2-GST truncated protein (lacks the C-terminal domain) was

used in the gel shift assay, in addition to the two non-specific bands an extra faint band could be seen binding to the non-coding (-) strand of GM (Figure 5.7B, lane 6). Surprisingly this band representing truncated dbpB-GST, co-migrated with the full length dbpB-GST. The reason for this observation is unclear and would require further experiments like UV cross-linking and shorter dbpB deletion constructs to be undertaken to further investigate this. This complex was not detected on either the coding (+) strand of GM or the double CSD mutant oligonucleotide suggesting that it represented dbpBΔ2-GST binding (Figure 5.7B, lanes 5 & 7). Since dbpBΔ2-GST was *in vitro* derived material and the recombinant full length dbpB-GST was bacterially derived, relative binding intensities between the two proteins could not be compared. Consistent with what has been observed in other gene systems these results suggest that the C-terminal region of dbpB is not essential for DNA binding to the GM-CSF proximal promoter, whereas the CSD domain appears to be required (Kolluri *et al.*, 1992); (Schroder *et al.*, 1995); (Shannon *et al.*, 2001); (Wang *et al.*, 2000).

5.6 Summary and Discussion

In summary, conditions were determined for optimal expression of recombinant CSD-GST fusion proteins dbpAv-GST and dbpB-GST. Although examination of these recombinant proteins by SDS-PAGE revealed that these proteins were highly unstable, giving rise to several truncated products. Recombinant CSD proteins expressed in the bacterial strain MC1061 gave rise to the formation of a single complex when used in gel shift experiments. Presumably the smaller sized protein products observed on SDS-PAGE had lost some essential part of the CSD protein required to bind DNA. Full length recombinant CSD factors dbpAv and dbpB bound the CSD binding sites across the GM-CSF proximal promoter in the same fashion as did the nuclear NF-GMb/c complexes (Chapter 4 (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000)). Recombinant

CSD factor binding to the GM-CSF proximal promoter was observed on the non-coding (-) strand of domain 2 and the coding (+) strand of domain 1. While it appears that there are some differences in binding affinity to individual CSD sites between dbpAv and dbpB, both factors bound in a similar fashion to CSD nuclear proteins, requiring both CSD binding sites in domain 1 or both sites in domain 2 for full binding to each of the domains (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). It is not known what affect, if any, that these differences between dbpAv and dbpB would have to the function of CSD proteins *in vivo*. Analysis of dbpB truncations demonstrated that the C-terminus of dbpB is not essential for DNA binding to the GM-CSF proximal promoter CSD sites whereas the CSD domain is (Kolluri *et al.*, 1992) (Bouvet *et al.*, 1995) (Schroder *et al.*, 1995). While the N-terminus of the protein may aid in DNA binding as previously suggested (Nambiar *et al.*, 1998), in our system it is not sufficient alone to allow DNA binding. The differences in binding affinity to individual CSD sites between dbpAv and dbpB may be related to the very different C and N-terminal regions of the proteins. (An amino acid comparison of the two CSD proteins is presented in the following chapter Figure 6.1.) As mentioned previously regions outside the CSD domain, which do not bind DNA directly, have been shown to influence DNA binding via the CSD domain. These differences in binding affinity may also correlate to the observed differences in activation and repression ability of the individual CSD proteins on GM-CSF promoter domain 1 and 2 regions in Jurkat T cells and HEL fibroblasts respectively (Chapter 3). Fine and detailed mutational analysis of the C and N-terminal regions of dbpAv and dbpB would have to be undertaken to determine if this is the case.

Results of binding studies of CSD factors to the GM-CSF proximal promoter suggest that this is the first example where CSD binding sites are proposed to act as pairs. Full nuclear and recombinant CSD complexes are only observed when two CSD binding sites in domain 1 or domain 2 are present. The interleukin 3 (IL-3) and granulocyte-colony

stimulating factor (G-CSF) proximal promoters also contain this array of CSD binding sites, two on the coding strand and two on the non-coding stand, with the spacing between the CSD sites similar to what is observed in the GM-CSF promoter (Coles *et al.*, 2000, and L.S. Coles unpublished results). Binding experiments to determine CSD binding to one or two of the CSD binding site pairs in these promoters has not been undertaken. The observed conservation in arrangement of CSD sites between GM-CSF, IL-3 and G-CSF promoters suggests that CSD binding to these sites may be similar and CSD factors may regulate transcription of these cytokine genes in a similar way.

Chapter 6

Generation of antibodies specific for cold shock domain proteins

TIMMY Shhhhh! timmy

Timmy and Stan

6.1 Introduction

In this chapter I discuss the generation and purification of antibodies specific for CSD proteins. I have described previously (Chapter 4) that gel shift and UV cross-linking analysis identified only a 42 kDa protein in Jurkat T cell NF-GMb complexes but in HEL fibroblast and HUT78 T cells NF-GMb/c complexes contained the 42 kDa protein with extra 25 and 22 kDa proteins as well. All these proteins are capable of binding DNA and are proposed to be CSD family members. Antibodies were generated primarily to directly examine the components of the NF-GMb and NF-GMc complexes that formed on GM-CSF proximal promoter oligonucleotides (Chapter 4) and to compare CSD protein sub-populations between HUT78 and Jurkat T cells. CSD proteins are highly conserved between rabbits and humans (Matsumoto *et al.*, 1998) (Grant *et al.*, 1993) and the generation of antibodies to highly conserved proteins in rabbits is often difficult. Immunization of rabbits with highly conserved proteins often results in poor or no antibody response due to immune self tolerance (Ye *et al.*, 1996). In an attempt to circumvent these difficulties two strategies were undertaken to generate specific CSD antibodies. The two strategies used to generate CSD antibodies involved using either the whole CSD-GST fusion protein (which may induce self tolerance) and peptides encoding for specific regions of CSD proteins (which are less likely to generate a self tolerance response but may not result in high levels of specific antibody production) (Harlow *et al.*, 1988). A majority of this chapter is devoted to strategies undertaken to purify polyclonal antibodies raised against either the recombinant CSD-GST fusion protein or CSD peptides. Once this was achieved the partially purified serum was then used in Western blot assays to determine if the 22 and 25 kDa nuclear CSD proteins identified in HUT78 T cells and HEL fibroblasts by UV cross-linking (Chapter 4) were completely lacking from Jurkat T cells or simply unable to bind DNA. Finally, antibody/gel shift experiments were used to

observe the effect of polyclonal CSD antibody on NF-GMb/c complex formation in both Jurkat and HUT78 T cells.

6.2 Generation of Antibodies

6.2.1 CSD immunogens used to raise polyclonal antibodies

Antibodies were generated to three CSD peptides Peptide A, Peptide B and Peptide CSD (Figure 6.1). The peptides, pepA (AGATTTTTTTLPQA) amino acids 4-18 from dbpAv and pepB (AETQQPPAAPPA) amino acids 5-16 from dbpB were generated from sequences at the N-terminus end of the respective CSD proteins (Figure 6.1). These peptides, pepA and pepB were chosen because they represent distinct sequences from the CSD proteins and hence subsequent antibodies raised to these peptides would have a greater possibility of being specific for either dbpAv (pepA antibody) or dbpB (pepB antibody). A general CSD antibody was also raised to a peptide pepCSD (IKKNNPRKYLRSVGD amino acids 89-103 in dbpB and 121-136 in dbpAv), which represents a region within the highly conserved CSD domain with 100% homology between dbpAv and dbpB. X-ray crystallography studies on bacterial CSD proteins suggest that this region loops out of the core protein structure therefore presenting a potentially good epitope for antibody binding (Figure 6.1) (Matsumoto *et al.*, 1998); (Newkirk *et al.*, 1994); (Schindelin *et al.*, 1993); (Schindelin *et al.*, 1994); (Schnuchel *et al.*, 1993). To stimulate an immune response against the peptides, (since peptides themselves are rarely immunogenic), they were chemically coupled to the highly immunogenic KLH. With the recombinant CSD-GST fusion proteins the GST tag is likely to be sufficiently immunogenic to elicit an immune response. Rabbits were inoculated four times with either the full length CSD-GST proteins or the KLH-coupled peptides and polyclonal sera collected after approximately 16 weeks for analysis. Pre-immune serum, before inoculation was also collected. All the sera collected were initially fractionated by ammonium sulphate precipitation to obtain an enriched fraction of total immunoglobulin.

Figure 6.1

Amino acid alignment of dbpB and dbpAv.

The CSD proteins dbpB and dbpAv have been aligned and separated into the three previously defined regions N-Terminal, CSD Domain and C-Terminal. A key is presented at the bottom of the figure. The RNP-1 & 2 sequences are highlighted as well as the predicted loop region in the CSD Domain. The basic regions are highlighted in both the CSD and C-Terminal domains. The amino acid sequence for the peptides used to make antibodies: **pepB** (**AETQQPPAAPPA**), **pepA** (**AGATTTTTTTLPQA**) and **pepCSD** (**IKKNNPRKYLRSVGD**) are indicated.

N-TERMINAL

```
dbpB   MSSEAET---------QQPPAAPPAAPALSAADTKPGTTGSGAGSGGPGGLTSAAPAG
dbpAv  **EAG*ATTTTTTTLP*A*TE*AA***QDP*PKSPV*SGAPQ*AAPA*AAHVAGN*G*

dbpB   G----------------------D 51
dbpAv  DAAPAATGTAAAASLATAAGSEDAE 84
```

CSD DOMAIN

```
                                  RNP-1        RNP-2        LOOP
dbpB   KKVIATKVLGTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGE
dbpAv  ***L**************************************************

dbpB   TVEFDVVEGEKGAEAANVTGPGGVPVQGSKYAADRNHYRR--YPRRRGPPR 159
dbpAv  **********************D****E**R*****RR***GY*G******* 284
```

C-TERMINAL

```
dbpB   NYQQNYQNSESGEKNEGSESAPEGQ----------AQQRRPYRRRRFPPYYMRRPYGRR
dbpAv  **AGE-EEE*GSGSS**FDPPATDRQFSGARNQLRRP*Y*PQ**Q******HVGQTFD**

dbpB   PQYSNPPVQGEVMEGADNQGAGEQGRPVRQNMYRG--YRPRFR-RGPPRQRQPREDGNEE
dbpAv  SRVLPH*NRIQAG*IGEMKDGVPEGAQLQGPVH*NPT****Y*S*****P*PAPAV*EA*

dbpB   DKENQGDETQGQQPPQRR-YRRNFNYRRRRPENPKP-QDGKETKAADPPAENSSAPEAEQ
dbpAv  *****QATSGPN**SV**G***PY*****PRPPNA*S*****A**GEA*T**P-**PTQ*

dbpB   GGAE 324
dbpAv  SS** 372
```

PepB Peptide specific for dbpB

PepA Peptide specific for dbpAv

PepCSD Peptide general for CSD

Basic Regions

\- Indicates a gap

* Indicates same amino acid as dbpB

6.2.2 Analysis of polyclonal antibodies to recombinant full length CSD-GST fusion proteins.

To test the specificity of the purified rabbit polyclonal antibodies raised to both full length recombinant CSD-GST fusion proteins and to CSD peptides, equal amounts of a series of proteins and peptides (dbpB, dbpAv (with the GST tags cleaved off), dbpB-GST, dbpAv-GST, pepA, pepB, pepCSD, a non-specific peptide and GST) were dotted onto nitrocellulose filters for use in Western blot assays (Figure 6.2A). To determine the optimal titre of polyclonal serum required in Western blot assays to be able to discriminate between specific and non-specific binding, ammonium sulphate fractionated rabbit pre-immune serum was used to probe the panel of recombinant CSD proteins and peptides. No signal to any of the proteins or peptides was detected at dilutions 1:1000 or below using rabbit pre-immune serum (Figure 6.2B). Ammonium sulphate fractionated antibodies, raised to each of the recombinant CSD-GST fusion proteins were therefore diluted at 1:1000, 1:3000 and 1:10000 and used to probe the protein/peptide panel in Western blot assays (Figures 6.2). Since CSD-GST fusion proteins were used to immunize the rabbits, it was observed that the majority of the immune response was directed against the highly immunogenic GST tag (Figure 6.2C & D). As can be seen in Figures 6.2C & D, both antibodies raised against the CSD-GST fusion proteins (α-dbpAv-GST and α-dbpB-GST) recognized both the CSD-GST fusion proteins dotted onto the membrane. Therefore, to analyze for antibody/CSD specificity, recombinant CSD proteins with the GST tag cleaved off, were also included in the panel. It was observed that both dbpAv-GST and dbpB-GST antibodies cross-reacted to both GST-cleaved dbpAv and dbpB recombinant CSD proteins (Figure 6.2C & D). This result was not surprising given the high sequence similarity in the CSD domain between the two proteins (Figure 6.1). Another possible explanation for this result could be antibodies raised to the GST tag are cross-reacting to residual GST present in the GST cleaved dbpA and dbpB recombinant material. Neither of these antibodies

Figure 6.2

Antibodies generated to full length dbpAv-GST and dbpB-GST.

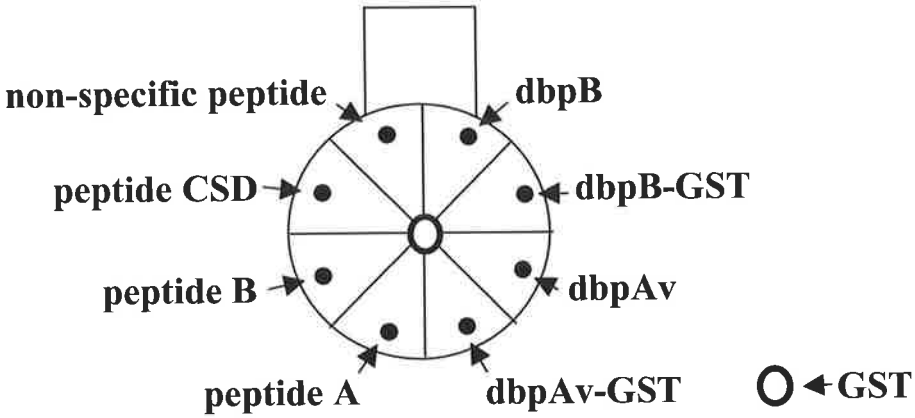
A) Key for following figures indicating where each of the proteins/peptides (dbpB and dbpAv (with GST tag cleaved) dbpB-GST, dbpAv-GST, peptides A, B & CSD, a non-specific peptide and GST) has been dotted onto the nitrocellulose filter. Immunoglobulins were purified from all the sera using ammonium sulphate precipitations, and were standardised to the same protein concentration as assayed via Bradford reagent. Dilutions of sera were made and used to probe the filters in a Western blot.

B) Rabbit pre-immune sera

C) Rabbit anti-dbpAv-GST sera

D) Rabbit anti-dbpB-GST sera

A

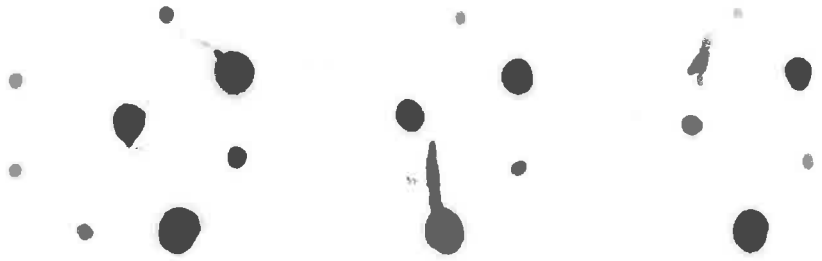


B Pre-Immune Sera

1:300 1:1000 1:3000 1:10000

C

α-dbpAv-GST



1:1000 1:3000 1:10000

D

α-dbpB-GST

1:1000 1:3000 1:10000

displayed a strong specific cross reactivity with any of the CSD peptides or the negative control peptide (Figure 6.2C & D).

6.2.3 Analysis of antibodies generated to CSD peptides

Ammonium sulphate fractionated antibodies raised to the three CSD peptides were also tested for specificity and cross-reactivity at dilutions between 1:1000 and 1:10000 in a Western blot against the same array of CSD proteins and peptides as shown in Figure 6.2. Western blot data using either the pepA or pepB antibodies demonstrated that they both weakly cross-reacted with both peptide A and peptide B at the 1:1000 dilution but could not be seen cross-reacting to any of the peptides in the lower dilutions (Figures 6.3B & C). Even though the peptide A and B antibodies were designed to be specific for dbpAv and dbpB respectively, they both appeared to cross-react with both full-length dbpAv and dbpB equally, even at the lowest 1:10000 dilution (Figure 6.3B & C). Peptide CSD was designed to a highly conserved, potential looped out region of the CSD domain (Figure 6.1). This region is identical in both dbpAv and dbpB and therefore should react equally well with both proteins. When used in a Western blot the peptide CSD antibody recognized the CSD peptide and cross-reacted well to both dbpAv and dbpB CSD proteins (Figure 6.3D). All three of the CSD peptide antibodies appear to recognise GST in the 1:1000 dilution but the level of cross-reactivity to GST drops well below that of the CSD proteins in the 1:3000 and 1:10000 dilutions of sera (Figure 6.3). This suggests that this cross-reactivity is background. Taking these results into account, further purification strategies were undertaken to optimise experiments using these sera.

6.2.4 Further purification of CSD antibodies

Initial experiments where the ammonium sulphate fractionated antibodies were used in Western blot assays to screen nuclear extracts for CSD proteins resulted in very

Figure 6.3

Antibodies raised to peptides.

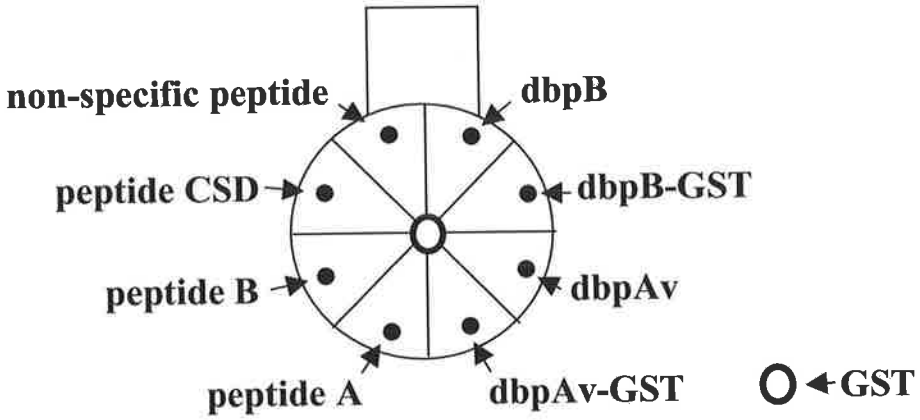
A) Key for following figures indicating where each of the proteins/peptides (dbpB and dbpAv (with GST tag cleaved) dbpB-GST, dbpAv-GST, peptides A, B & CSD, a non-specific peptide and GST) has been dotted onto the nitrocellulose filter. Immunoglobulins were purified from all the sera using ammonium sulphate precipitations, and were standardised to the same protein concentration as assayed via Bradford reagent. Dilutions of sera were made and used to probe the filters in a Western blot.

B) Rabbit anti-peptide A sera

C) Rabbit anti-peptide B sera

D) Rabbit anti-peptide CSD sera

A

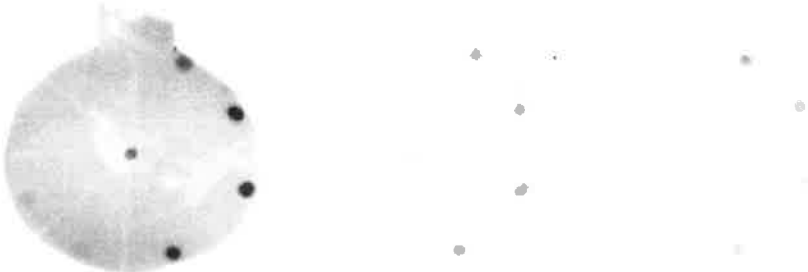


B

α - peptide A

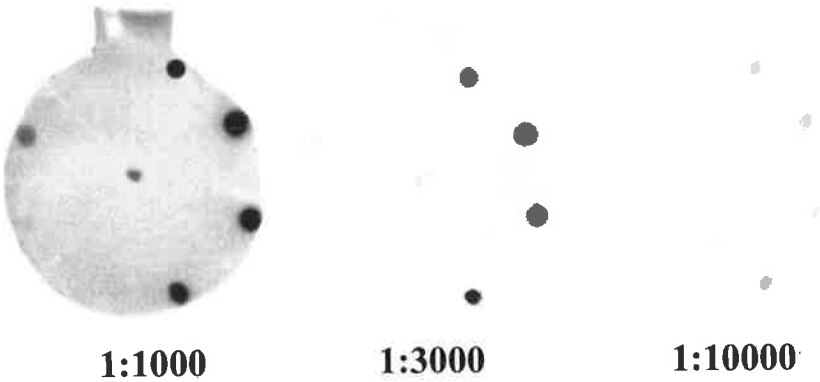
C

α - peptide B



D

α - peptide CSD



high background levels which were too high to discern specific CSD binding (data not shown). Therefore a strategy of further antibody purification was undertaken.

6.2.4.1 Protein sepharose A purification

Due to the time period of inoculations of rabbits, (over a period of 16 weeks), the primary group of antibodies generated against the injected immunogen would be of the IgG class (Ye *et al.*, 1996). To further purify the antibody, the IgG antibodies were fractionated via protein A sepharose (as described in the material and methods section). Antibody raised against peptide B was initially fractionated via ammonium sulphate precipitations to isolate all classes of immunoglobulin followed by IgG purification with protein A sepharose. Antibody at each stage of purification was collected and then used in a Western blot against a panel of peptides and proteins to determine yield and specificity. The panel of peptides consisted of peptide B, peptide B coupled to KLH, a non-specific peptide, dbpB with the GST tag cleaved off and BSA (Figure 6.4A). As can be seen in Figure 6.4C, ammonium sulphate fractionation of the peptide B antibody resulted in a reduction of background binding compared to crude serum (Figure 6.4B) while still retaining good cross reactivity to GST cleaved dbpB but displayed poor cross-reactivity to peptide B. Protein A purification of peptide B antibody resulted in a very poor yield of antibody, which while still weakly cross-reacting with GST cleaved dbpB, did not cross-react with peptide B (Figure 6.4D). These results indicated that purification of CSD antibodies by Protein A was not effective for future experiments.

6.2.4.2 Affinity purification of the CSD peptide antibody.

It appeared, from the previous Western blots, that the majority of the antibodies being purified were directed towards the hapten, GST in the case of the full-length proteins or KLH in the case of the peptides (Figures 6.2 & 6.3). Pre-clearing the serum with either

Figure 6.4

Purification of α-peptide B antibodies

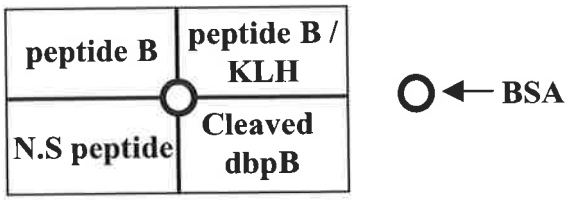
A) Key for following figures indicating where each of the proteins/peptides (peptide B, peptide B coupled to KLH, a non-specific (N.S) peptide, bovine serum albumin (BSA) and dbpB with the GST tag cleaved) have been dotted onto the nitrocellulose filter.

B) Western blot using rabbit anti-peptide B crude sera

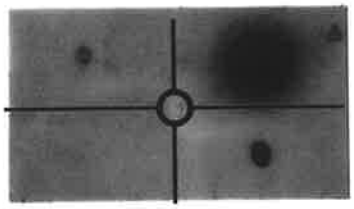
C) Western blot using rabbit anti-peptide B ammonium sulphate purified sera

D) Western blot using rabbit anti-peptide CSD Protein A purified sera

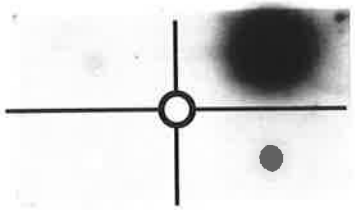
A



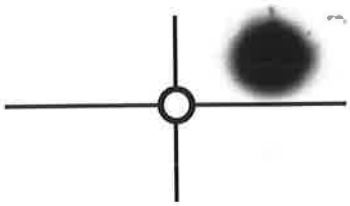
B α - peptide B Crude Sera



C α - peptide B Ammonium Sulphate purified



D α - peptide B Protein A purified



of the haptens bound to a column had little or no effect despite several rounds of pre-clearing (data not shown). Therefore binding the immunogen, without hapten (either full-length CSD protein or CSD peptide) to a column and affinity purifying the specific antibodies was proposed. However, since CSD proteins with the GST tag cleaved off appeared to be very unstable, purification of the antibodies to full length recombinant CSD-GST fusion proteins by this means was not possible. Hence, an antibody generated to the CSD peptide, was purified by these means. Peptide CSD was bound to a CNBr-activated sepharose column as described in the material and methods section. A sample of CSD peptide taken before coupling to the column and effluent taken from the column after several rounds of coupling were analyzed on HPLC (Kindly performed by Mark Van Der Hoek, IMVS). HPLC results indicated that all the CSD peptide was bound to the column after three rounds of coupling (Figure 6.5A). This CSD peptide coupled CNBr-sepharose column was then used to affinity purify antibodies specific for the CSD peptide. A panel of peptide and proteins (dbpB-GST, peptide CSD, peptide CSD coupled to KLH, GST and BSA) were dotted onto nitrocellulose membranes and CSD peptide antibodies purified by either ammonium sulphate or CSD peptide affinity column were used to probe the panel in a Western blot (Figure 6.5B). The ammonium sulphate fractionated antibodies cross-reacted well to recombinant dbpB-GST, CSD peptide coupled to KLH and to a lesser extent the CSD peptide (Figure 6.5C). When this material was further purified using the CSD coupled CNBr-sepharose column the yields were very low. Even though the affinity purification of the CSD specific antibodies appeared to work, with no KLH antibodies coming through the purification procedure (observed by no cross reaction to the peptide CSD coupled to KLH), the antibody did not cross react with peptide CSD alone (Figure 6.5D). Considerably more CSD peptide CNBr-column purified antibody, compared to ammonium sulphate purified material, was needed to detect dbpB-GST in this Western Blot (Figure 6.5D). Since only an estimation of the amount of immunoglobulin present in

Figure 6.5

Purification of α-CSD peptide antibody.

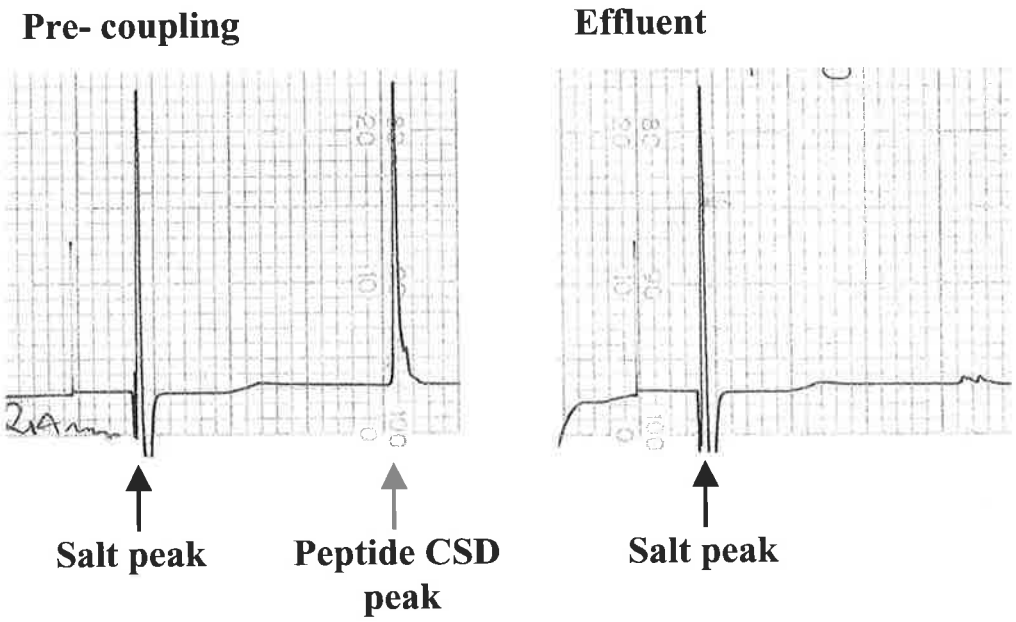
A) CSD peptide was coupled to CNBr-activated sepharose and used to purify anti-CSD specific antibodies. High Pressure Liquid Chromatography graphed results showing CSD peptide before coupling and the effluent from the column after several rounds of coupling.

B) Key for following figures indicating where each of the proteins/peptides (dbpB-GST, bovine serun albumin (BSA), gutathione-S-transferase (GST), peptide CSD and peptide CSD coupled to KLH) have been dotted onto the nitrocellulose filter.

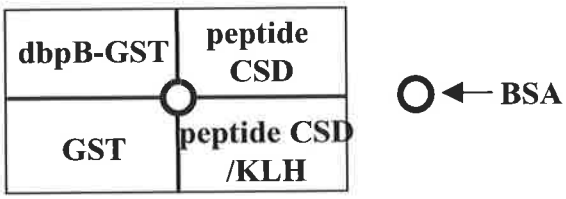
C) Western blot using rabbit anti-CSD ammonium sulphate purified sera

D) Western blot using rabbit anti-CSD purified using CSD coupled to CNBr-activated sepharose

A

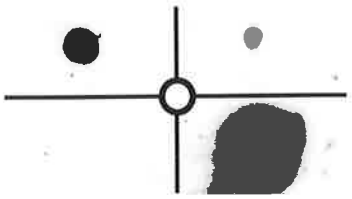


B



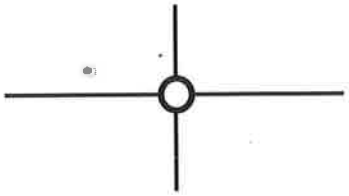
C

Ammonium Sulphate purified α - CSD



D

CSD peptide column purified α - CSD



the ammonium sulphate fractionated material could be undertaken it was not possible to directly ascertain the efficiency of CSD specific antibody purification by CNBr-column purification. What can be deduced from this experiment however, is that CNBr-column purification did not result in sufficient immunoglobulin material for use in experiments and hence made purification of the CSD specific antibodies by this means not optimal for future experiments.

6.2.4.3 Multiple Ammonium sulphate precipitations

Therefore, to decrease the background levels seen, when the ammonium sulphate fractionated antibodies were used in Western blots against nuclear extracts, a third protocol was attempted. This protocol involved a further round of ammonium sulphate precipitation with a slight variation in final ammonium sulphate concentration (final concentration of ammonium sulphate 30%) (Ye *et al.*, 1996). This extra purification of the CSD peptide antibody resulted in Western blots with less background, while the antibody retained its ability to recognize both the CSD peptide and recombinant dbpB-GST (data shown in subsequent experiment, Figure 6.6). This double ammonium sulphate fractionated material was then used in all future antibody experiments.

6.3 Use of CSD antibodies to analyse nuclear NF-GMb/c complexes

Gel shift and UV cross-linking experiments have shown differences in CSD factors between Jurkat T cells and HEL fibroblasts/HUT78 T cells (Chapter 4). The Jurkat T cell nuclear NF-GMb complex has been detected binding to both domain 1 and 2 of the GM-CSF proximal promoter and UV cross-linking experiments show that both domain 1 and 2 NF-GMb complexes contain a 42 kDa protein. Nuclear extracts from HEL fibroblasts and HUT78 T cells form NF-GMb and NF-GMc complexes on both GM-CSF domain 1 and 2 oligonucleotides. UV cross-linking of these NF-GMb/c complexes identified that as well

as the 42 kDa protein identified in Jurkat T cells NF-GMb/c complexes that formed on domain 1 contained a 22 kDa protein and NF-GMb/c complexes that formed on domain 2 contained the 42, 22 and a further 25 kDa protein (Chapter 4).

6.3.1 Jurkat T cell nuclear extracts lack the 22 and 25 kDa nuclear CSD proteins identified in HUT78 T cells and HEL fibroblasts

To determine if the 22 and 25 kDa proteins identified in HEL fibroblasts and HUT78 T cells by UV cross-linking (Chapter 2) were completely lacking from Jurkat T cells or simply unable to bind DNA, Western and South-Western experiments were performed. Jurkat and HUT78 T cell nuclear extract and purified recombinant dbpB-GST fusion proteins were fractionated on a Laemmli SDS-PAGE gel and transferred to nitrocellulose membranes resulting in five replicate filter panels. One panel was probed with the double ammonium sulphate fractionated CSD peptide antibody in a Western blot. The CSD antibody recognized a single protein of approximately 60 kDa for recombinant dbpB-GST which is the approximate size for full length dbpB-GST previously seen on Laemmli SDS-PAGE (Chapter 5) (Figure 6.6A, lane 3). In the Jurkat and HUT78 T cell nuclear extract lanes the CSD antibody cross-reacted with many proteins (Figure 6.6A, lanes 1 & 2). Proteins of the approximate size for the CSD proteins identified in the UV cross-linking experiments (42, 25 & 22 kDa) were detected. A strongly reacting band of approximately 42 kDa was seen in both Jurkat and HUT78 T cell extracts, whereas the smaller 25 & 22 kDa proteins were seen only in the HUT78 T cell extract (Figure 6.6A, lanes 1 & 2).

To confirm that these were the CSD factors identified by previous UV cross-linking experiments, South-Western experiments were performed. In the South-Western experiments one panel was probed with the wildtype GM-CSF, domain 1 GM-oligonucleotide (contains two CSD binding sites Figure 6.6B, lanes 1-3), and the other

Figure 6.6

Detection of CSD proteins in Jurkat T cells and HUT78 T cells.

Jurkat T cell and HUT78 T cell nuclear extracts and recombinant dbpB-GST were fractionated by Laemmli SDS-PAGE and transferred to nitrocellulose membranes reaulting in five identical panels.

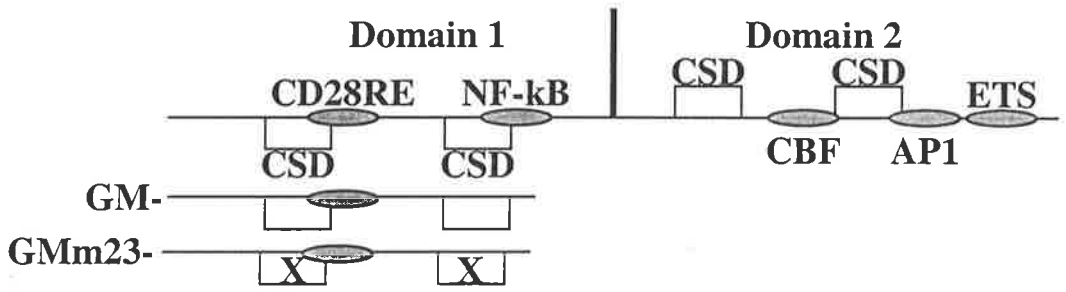
A) The first filter was used for a Western blot using the ammonium sulphate purified antibody raised to the CSD peptide to identify all CSD proteins present. The 42, 25 and 22 kDa CSD proteins are indicated.

B) The second and third filters were probed with either the wild type domain 1, non-coding (-) strand GM- oligonucleotide or the corresponding CSD mutant GMm23- oligonucleotide in a South-Western. The 42, 25 and 22 kDa CSD proteins are indicated.

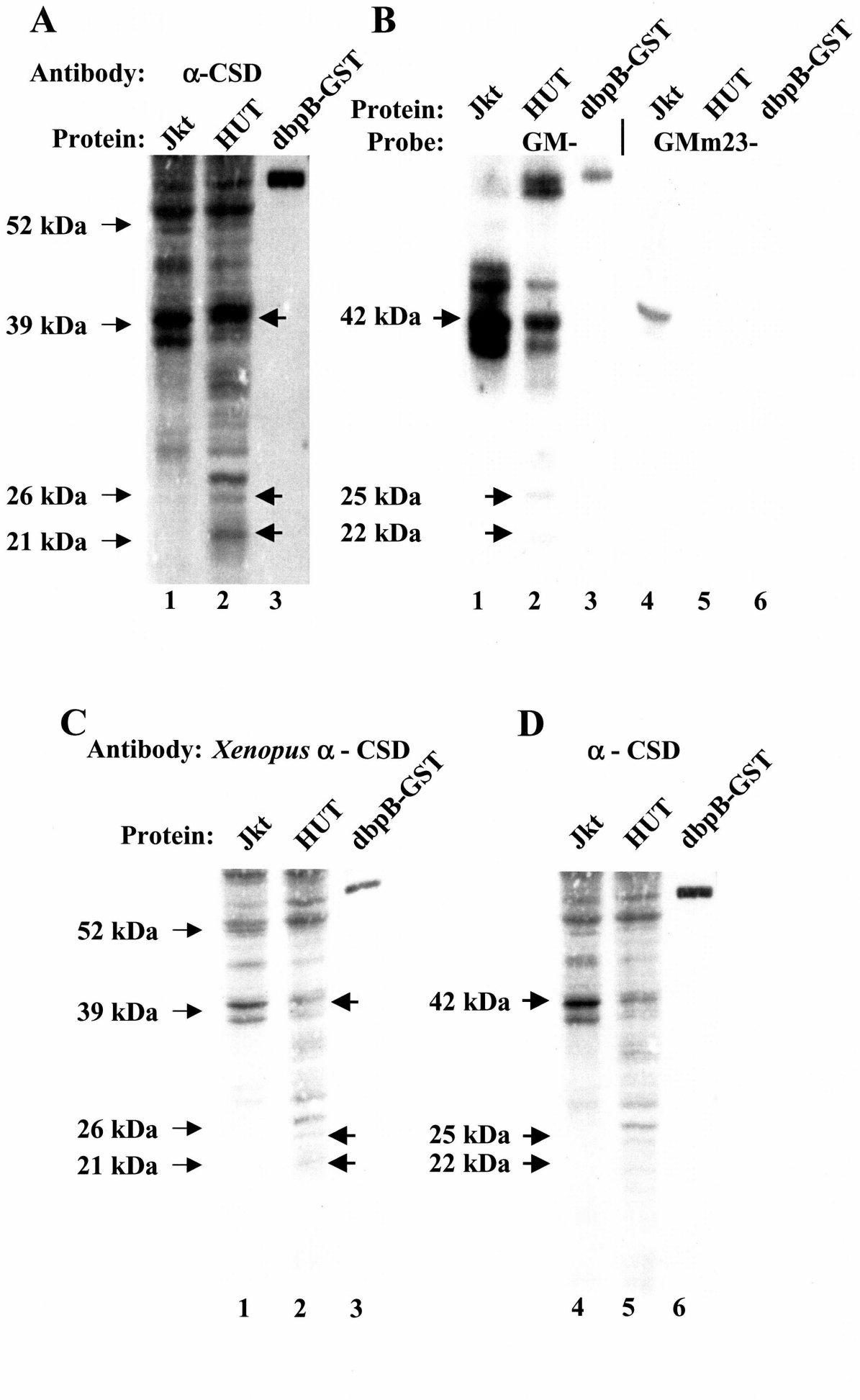
C) The fourth filter was used for a Western blot using an antibody raised to the *Xenopus* CSD protein FRGY2 (obtained from Alan Wolffe). The 42, 25 and 22 kDa CSD proteins are indicated.

D) The fifth filter was used for a Western blot using the ammonium sulphate purified antibody raised to the CSD peptide. The 42, 25 and 22 kDa CSD proteins are indicated.

Jkt: Jurkat T cells **HUT**: HUT78 T cells



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent binding sites, boxes with a 'X' in them indicate mutated CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


A
Antibody: α-CSD
Protein: Jkt HUT dbpB-GST
52 kDa
39 kDa
26 kDa
21 kDa
1 2 3
B
Protein: Jkt HUT dbpB-GST Jkt HUT dbpB-GST
Probe: GM- | GMm23-
42 kDa
25 kDa
22 kDa
1 2 3 4 5 6
C
Antibody: Xenopus α - CSD
Protein: Jkt HUT dbpB-GST
52 kDa
39 kDa
26 kDa
21 kDa
1 2 3
D
α - CSD
Jkt HUT dbpB-GST
42 kDa
25 kDa
22 kDa
4 5 6

panel was probed with the double CSD binding site mutant of the GM- oligonucleotide, GMm23- (Figure 6.6B, lanes 4-6). Recombinant dbpB-GST can be seen to bind to the wildtype GM- probe (Figure 6.6B, lane 3) but not to the CSD mutant GMm23- probe as expected (Figure 6.6B, lane 6). Proteins of the same size as detected by UV cross-linking the HUT78 T cell NF-GMb/c complexes (42, 25 & 22 kDa), were detected binding to the GM- probe in this assay (Figure 6.6B, lane 2), but were significantly reduced or not detected binding to the GM23- mutant oligonucleotide (Figure 6.6B, lanes 4 & 5). Several other bands were also observed that might represent CSD family members. In Jurkat T cell extracts, a band of 42 kDa (and a smaller protein of 39 kDa) were the strongest bands observed when the GM- oligonucleotide was used as a probe. Significantly the 22 and 25 kDa bands were not observed in Jurkat T cells but were present in HUT78 T cell nuclear extract (Figure 6.6B, lane 1). Other proteins are seen binding to the GM- probe and not to the CSD mutant GMm23-. CSD proteins of greater than 42 kDa in size have been detected in nuclear extracts by other groups and these proteins, seen binding here to the GM- probe, may represent other CSD family members which do not form part of the NF-GMb/c complexes.

To further confirm these results a CSD antibody raised to the *Xenopus* CSD protein FRGY2 (kindly provided by Alan Wolffe (Tafuri *et al.*, 1992)) was also used to probe one panel of the same recombinant CSD protein and nuclear extracts. The same banding pattern was detected when either the *Xenopus* CSD antibody (Figure 6.6C) or the CSD peptide antibody (Figure 6.6D) was used in the Western blots. Importantly, the NF-GMb/c CSD proteins identified by UV cross-linking experiments (chapter 4) (42, 25, 22 kDa) were also detected by the *Xenopus* CSD antibody (Figure 6.6C).

These experiments confirm UV cross-linking results of Jurkat T cell NF-GMb complexes and HEL fibroblast/HUT78 T cell NF-GMb/c complexes indicating that Jurkat T cells lack the 25 and 22 kDa CSD proteins.

6.3.2 CSD peptide antibody blocks dbpB-GST binding to DNA

To determine if the addition of CSD specific antibodies could affect NF-GMb/c complex formation gel shift antibody assays were performed. Antibodies could either block NF-GMb/c complex formation, if the antibody binds to the CSD domain, or result in a further retardation of the complex (or supershift) if the antibody binds to a region of the protein which is not involved in binding. To determine if the CSD peptide antibody had blocking or supershifting ability it was initially used in a gel shift assay with the recombinant CSD-GST fusion protein dbpB-GST. Since the antibody was raised against the CSD peptide and not full length CSD-GST fusion protein any blocking or supershifting seen could be attributed to a specific CSD/antibody interaction. As can be seen in Figure 6.7, increasing the amount of antibody in the gel shift assay with recombinant dbpB-GST demonstrated that the CSD peptide antibody could block recombinant CSD protein binding to the GM- domain 1 oligonucleotide with no corresponding antibody/CSD protein supershift complex observed (Figure 6.7, Lanes 2 to 6). The addition of pre-immune (P.I) serum, to the binding reaction mixture, at the maximal concentration of CSD antibody used, showed no effect on the mobility or complex formation of the recombinant dbpB-GST protein (Figure 6.7, lane 7).

6.3.3 CSD peptide antibody blocks binding of NF-GMb/c complexes in Jurkat and HUT78 T cells on GM-CSF domain 1.

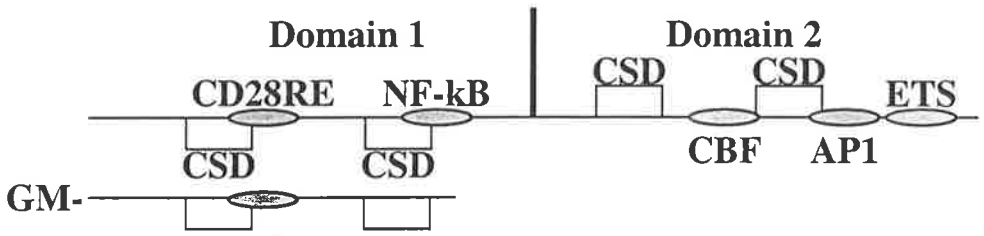
Gel shift analysis of Jurkat and HUT78 T cell nuclear extracts identified that NF-GMb/c complexes formed in HUT78 T cells whereas only NF-GMb complexes formed in Jurkat T cells. UV cross-linking experiments identified a 42 kDa protein present in the Jurkat T cells and 42 and 22 kDa proteins in HUT78 T cells when nuclear extracts were bound to GM-CSF domain 1 oligonucleotides (Chapter 4).

Figure 6.7

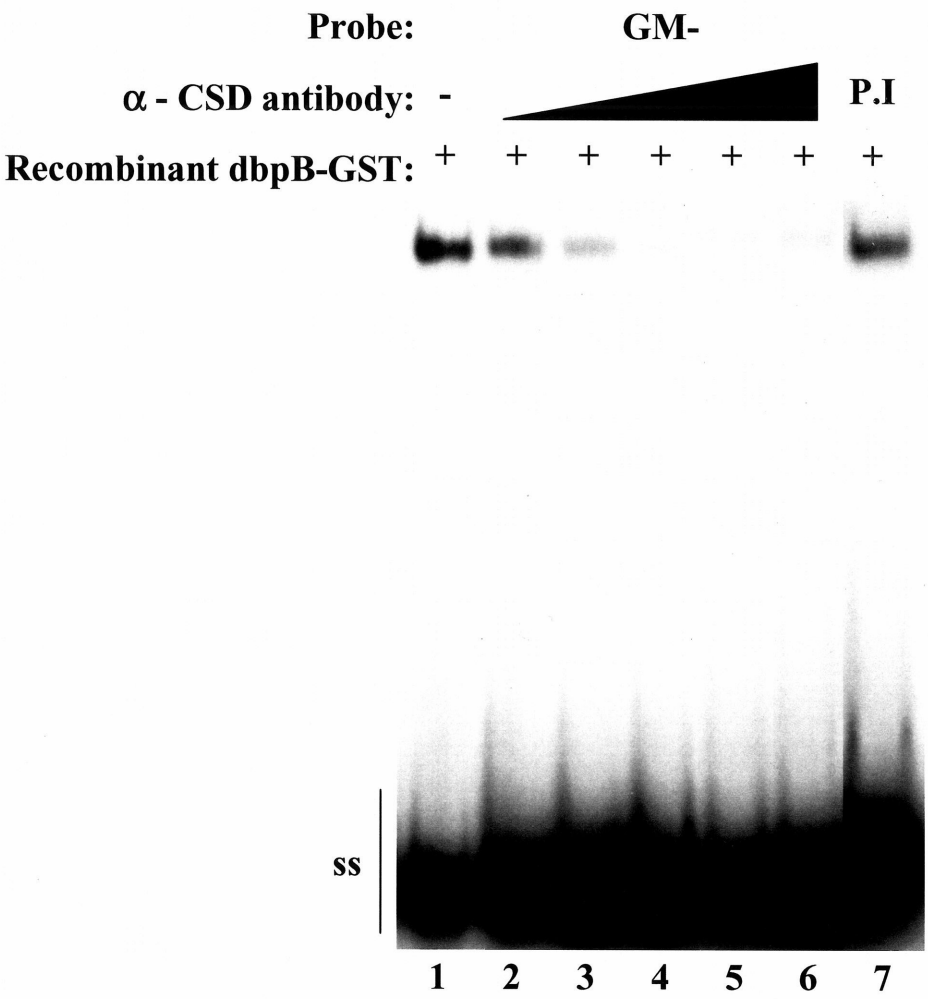
α-CSD peptide antibody blocks dbpB-GST binding.

Recombinant dbpB-GST was bound to ^{32}P-labelled wild type domain 1, non-coding (-) strand GM- oligonucleotide in a gel shift assay. Increasing amounts of anti-CSD peptide antibody was added to the reaction (Lanes 2 to 6). No antibody was added to the reaction (denoted by a minus (-) sign) in Lane 1 and rabbit pre-immune was added to the reaction as a negative control (denoted by P.I) in Lane 7.

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)



The human GM-CSF proximal promoter and oligonucleotide used in this experiment is represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


Probe:
GM-
α - CSD antibody:
-
P.I
Recombinant dbpB-GST:
+ + + + + + +
ss
1 2 3 4 5 6 7

To test if the NF-GMb/c complexes seen forming on domain 1 of the GM-CSF promoter in Jurkat and HUT78 T cell nuclear extracts contained CSD factors, the CSD peptide antibody was added to gel shift reactions to determine if it had any affect on NF-GMb/c complex formation. Increasing amounts of CSD antibody in the gel shift reaction resulted in a decrease, and at higher concentrations, a total block of NF-GMb complex formation (Figure 6.8A, lanes 3 to 6), indicating that CSD proteins are present in the Jurkat T cells and are essential for NF-GMb doublet formation. Pre-immune serum had no effect on Jurkat T cell NF-GMb binding or mobility and no complex was seen when antibody alone was added to the gel shift reaction (Figure 6.8A, Lanes 7 & 1). To test if the HUT78 T cell nuclear NF-GMb/c complexes also contained CSD factors, HUT78 T cell nuclear extract was bound to GM-CSF domain 1, GM- oligonucleotide in a gel shift assay in the presence of CSD antibody (Figure 6.8B). Adding increasing amounts of CSD antibody to the gel shift reaction resulted in a decrease and then total blocking of both NF-GMb (42 kDa) and NF-GMc (42 and 22 kDa) complex formation (Figure 6.8B, Lanes 2 to 5). Pre-immune serum used at the same maximum antibody concentration had no effect on HUT78 T cell NF-GMb/c complex formation or mobility (Figure 6.8B, lane 6). These results confirm that the 42 and 22 kDa proteins identified in HEL fibroblast, Jurkat and HUT78 T cells by UV cross-linking experiments were CSD factors.

6.3.4 The CSD peptide antibody specifically blocks the binding of the 22 and 25 kDa HUT78 T cell NF-GMc proteins to GM-CSF domain 2

As described previously (Chapter 4) UV cross-linking experiments identified that the Jurkat T cell NF-GMb complex detected binding to the GM-CSF domain 2 GM93+ oligonucleotide consisted of the same 42 kDa protein identified binding to the GM-CSF domain 1 GM- oligonucleotide. UV cross-linking experiments on HUT78 T cell showed that both NF-GMb and NF-GMc complexes formed on the GM-CSF domain 2 GM93+

Figure 6.8

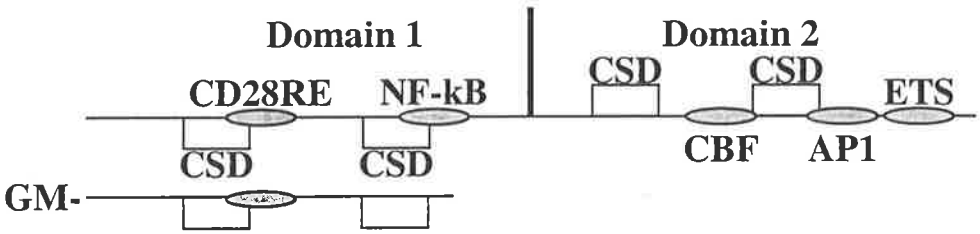
α-CSD peptide antibody blocks binding of NF-GMb/c complexes in Jurkat and HUT78 T cells.

A) Jurkat T cell nuclear extract was bound to ^{32}P-labelled wild type domain 1, non-coding (-) strand GM- oligonucleotide in a gel shift assay. Increasing amounts of anti-CSD peptide antibody was added to the reaction (Lanes 3 to 6)(2, 3 & 5 fold more antibody in each lane). No antibody was added to the reaction (denoted by a minus (-) sign) in Lane 2, as negative controls anti-CSD sera was added to the reaction without Jurkat T cell nuclear extract (Lane 1) and rabbit pre-immune was added to the reaction (denoted by P.I) in Lane 7.

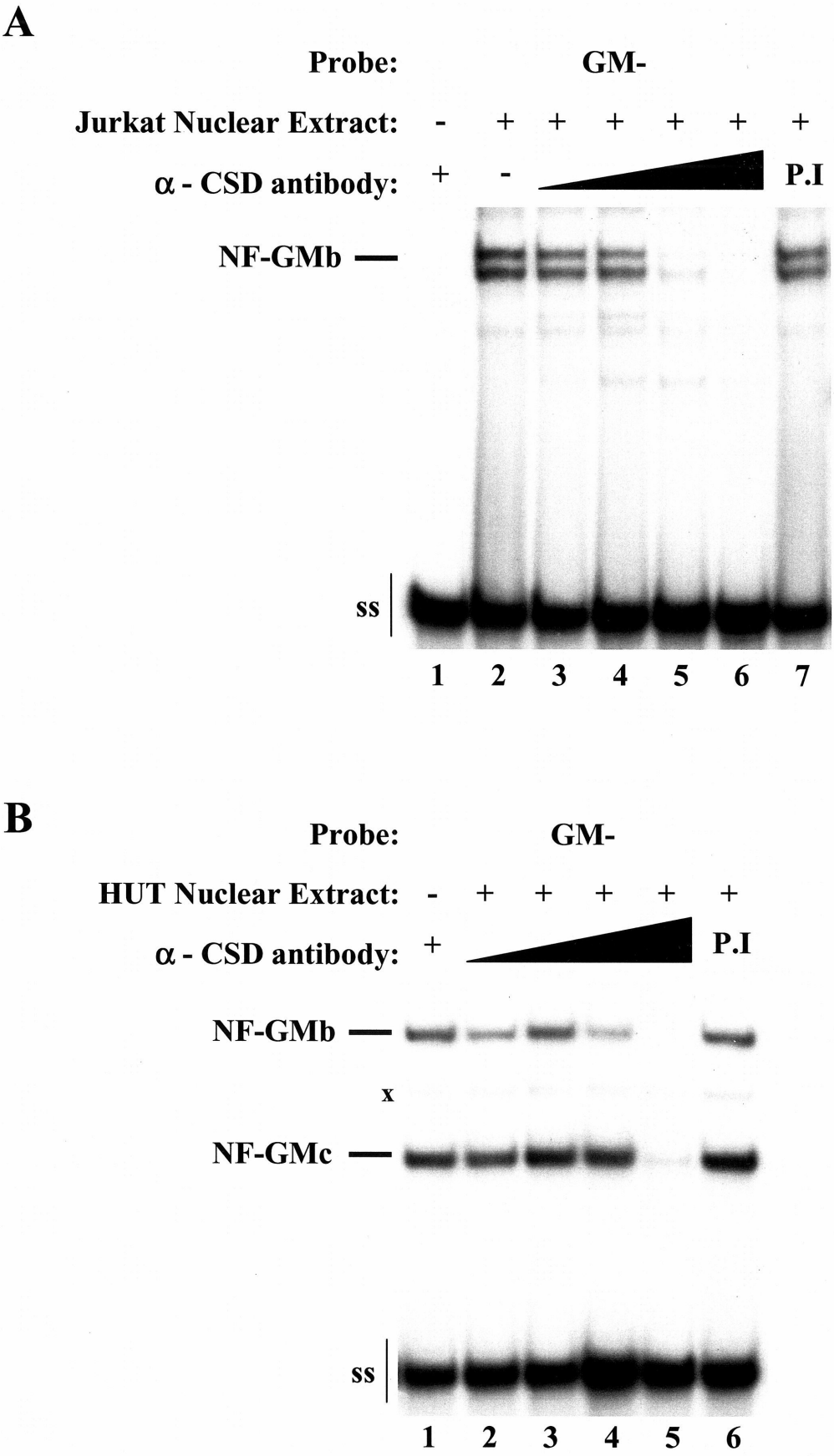
B) HUT78 T cell nuclear extract was bound to ^{32}P-labelled wild type domain 1, non-coding (-) strand GM- oligonucleotide in a gel shift assay. Increasing amounts of anti-CSD peptide antibody was added to the reaction (Lanes 2 to 5) (2, 3 & 5 fold more antibody in each lane). No antibody was added to the reaction (denoted by a minus (-) sign) in Lane 1, and rabbit pre-immune was added to the reaction (denoted by P.I) in Lane 7 as a negative control.

(x denotes non-specific binding)

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)



The human GM-CSF proximal promoter and oligonucleotide used in this experiment is represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


A
Probe:
GM-
Jurkat Nuclear Extract:
- + + + + + +
α - CSD antibody:
+ - P.I
NF-GMb
ss
1 2 3 4 5 6 7
B
Probe:
GM-
HUT Nuclear Extract:
- + + + + +
α - CSD antibody:
+ P.I
NF-GMb
x
NF-GMc
ss
1 2 3 4 5 6

oligonucleotide. The HUT78 T cell domain 1 NF-GMc complex consisted of only a 22 kDa protein whereas the HUT78 T cell domain 2 NF-GMc complex consisted of 22 and 25 kDa proteins. By using CSD binding site mutant oligonucleotides the 22 kDa protein has been shown to bind exclusively to the domain 2, 5' CSD binding site and the 25 kDa protein to the 3' CSD binding site (Coles *et al.*, 2000). To directly observe the 22 and 25 kDa proteins present in the HUT78 T cell NF-GMc complex, domain 2 oligonucleotides with either the 5' or 3' CSD binding sites mutated were used in gel shift experiments. As seen previously full NF-GMb complex formation requires both CSD binding sites (Figure 4.3A) whereas NF-GMc complex formation requires only one CSD binding site, therefore on these mutant oligonucleotides only NF-GMc complexes form. Using these CSD binding site mutant oligonucleotides it was possible to directly observe if the CSD antibody had an effect on either the 22 or 25 kDa proteins. Firstly HUT78 T cell nuclear extract was bound to wildtype GM-CSF domain 2 GM93+ oligonucleotide with increasing amounts of CSD peptide antibody. As seen on domain 1 both the NF-GMb and NF-GMc complexes were blocked by the addition of the highest concentration of the CSD antibody (Figure 6.9, Lanes 1 to 4). Using the domain 2 CSD mutant oligonucleotide GMm95+, which mutates the 5' domain 2 CSD site, results in a loss of the HUT78 T cell NF-GMb complex with the remaining NF-GMc complex containing only the 22 kDa protein (Figure 6.9, lane 5) (Coles *et al.*, 2000). Addition of increasing amounts of CSD antibody resulted in blocked NF-GMc complex formation (Figure 6.9, lanes 6 to 8). Using the domain 2 CSD mutant oligonucleotide GMm103+, which mutates the 3' CSD site, also results in a loss of the HUT78 NF-GMb complex while retaining the NF-GMc complex containing only the 25 kDa protein (Figure 6.9, lane 9) (Coles *et al.*, 2000). Again addition of the CSD antibody readily blocked NF-GMc complex formation (Figure 6.9, lanes 10 to 12). NF-GMb/c complex formation or mobility was not affected by the addition of pre-immune

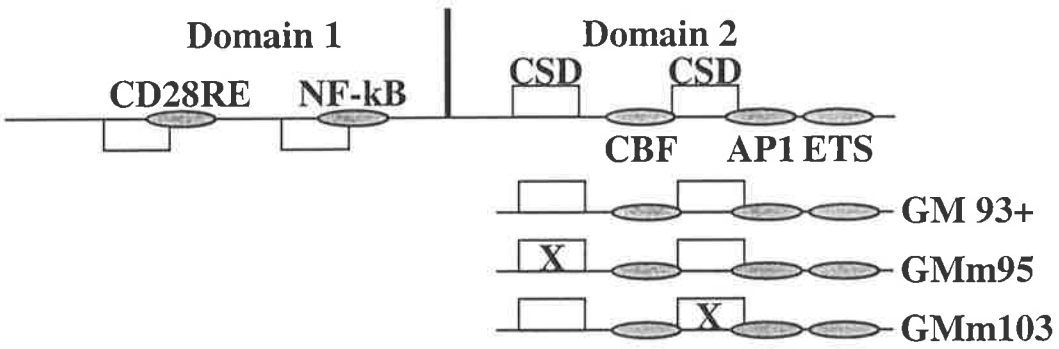
Figure 6.9

α-CSD peptide antibody specifically blocks the 22 and 25 kDa HUT78 T cell NF-GMc proteins.

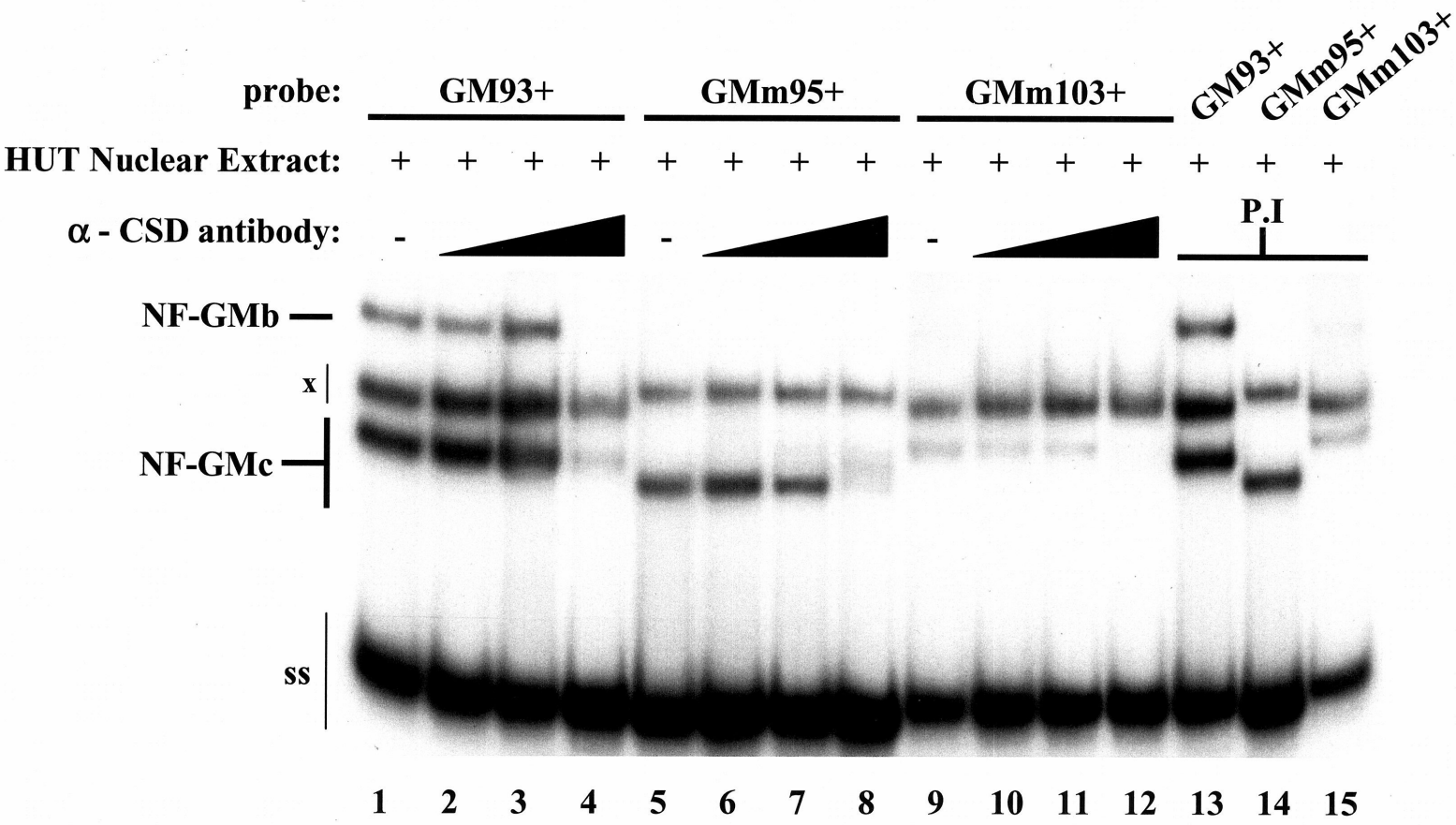
HUT78 T cell nuclear extract was bound in a gel shift assay to ^{32}P-labelled GM-CSF wild type domain 2 coding (+) strand oligonucleotide GM93+ and to the mutant GMm95+ and GMm103+ oligonucleotides. Increasing amounts of anti-CSD antibody (2 fold and 5 fold more antibody) was added to the reaction with each oligonucleotide (Lanes 2 to 4 with GM93+), (Lanes 6 to 8 with GMm95+) and (lanes 10 to 12 with GMm103+). No antibody was added in the reaction with each oligonucleotide (indicated by a minus (-) sign Lanes 1,5 and 9) as was pre-immunne as a negative control (indicated by P.I, Lanes 13 to 15).

(x represents non-specific binding)

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)



The human GM-CSF proximal promoter and oligonucleotides used in these experiements are represented diagrammatically above. Boxes represent CSD binding sites, boxes with a 'X' in them indicate mutated CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 4.1.


probe:
GM93+
GMm95+
GMm103+
GM93+
GMm95+
GMm103+
HUT Nuclear Extract:
+ + + + + + + + + + + + + + +
α - CSD antibody:
- - -
P.I
NF-GMb
x
NF-GMc
ss
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

on any of the oligonucleotides (Figure 6.9, lanes 13 to 15). These results strongly suggest that both the 22 and 25 kDa proteins are CSD proteins.

6.4 Summary and Discussion

Sequence alignment of CSD factors from different species have shown that they are highly conserved throughout evolution, especially the CSD region which has a 43% homology between bacteria and humans (Wolffe 1993). Experiments described in the literature indicate that generation of antibodies in rabbits to such highly conserved proteins is often problematic, resulting in low specific antibody titres due to immune self tolerance (Harlow *et al.*, 1988). This characteristic poor antibody response was observed when the highly conserved full length CSD factors dbpAv and dbpB were used to immunize rabbits. New techniques in antibody production indicate that generation of antibodies to highly conserved proteins are best undertaken in chickens, where large titres of specific antibodies have been isolated from eggs. If these experiments were to be repeated, chickens would be the ideal choice of immunizing animals.

Generation of antibodies to peptides usually results in generation of lower serum titres of specific antibody compared to when proteins are used as immunogens but usually are not constrained by immune self tolerance. Antibodies were raised in rabbits to three peptides, peptide A, peptide B and peptide CSD (Figure 6.1). Difficulties were encountered when trying to purify the peptide antibodies, which was attributed to the low titre of antibody present in the rabbit serum. Therefore, partial purification of peptide CSD antibodies by two rounds of ammonium sulphate fractionation was found to be the most effective means of obtaining antibody material useful for subsequent experiments.

UV cross-linking experiments, described in chapter 4, identified that Jurkat T cell NF-GMb complexes contained only a 42 kDa protein whereas HUT78 T cell NF-GMb/c complexes contained 42, 25 and 22 kDa proteins. To determine if the 25 and 22 kDa

proteins were not present at all in Jurkat T cells or present and did not bind to the GM-CSF proximal promoter, western blot analysis was undertaken. Western blot analysis showed that the peptide CSD antibody cross reacted with a large number of proteins in both Jurkat and HUT78 T cell nuclear extracts. South western analysis of the same nuclear extracts highlighted fewer bands but of particular interest confirmed that the 42 kDa protein was present in both Jurkat and HUT78 T cells whereas the 25 and 22 kDa proteins were only present in HUT78 T cells. Several other bands were identified in both the western and south western experiments. This suggested that there could be other CSD factor family members present in HUT78 and Jurkat T cells that are not part of the NF-GMb/c complexes that form on the GM-CSF proximal promoter. Several CSD factors of various sizes have been reported which fall into the size range identified in these experiments (Matsumoto *et al.*, 2000) (Hipfel *et al.*, 2000) (Thieringer *et al.*, 1997) (Balda *et al.*, 2000) (Swamynathan *et al.*, 1997). This was confirmed when an antibody raised to a Xenopus CSD protein was used to probe the same set of nuclear extracts from Jurkat and HUT78 T cells. The banding pattern observed with the Xenopus CSD antibody was identical to the peptide CSD antibody suggesting that these other bands did represent CSD family members.

To directly examine the components of the HUT78 and Jurkat T cell NF-GMb/c complexes antibody gel shift experiments were performed. Peptide CSD antibody could block complex formation of NF-GMb/c complexes in both Jurkat and HUT78 T cells as well as recombinant CSD factor binding, indicating that the 42, 25 and 22 kDa proteins identified in the UV cross-linking experiments were CSD factors. These results confirm all previous results indicating that Jurkat T cells lack the 25 and 22 kDa CSD protein identified in HEL fibroblasts and HUT78 T cells.

Chapter 7

Potential CSD protein:protein interactions

There was metho in his madness!

J. D. Bontwood

7.1 Introduction

In Chapter 3, data was presented demonstrating that CSD factors dbpAv and dbpB, acted to co-activate the GM-CSF proximal promoter in Jurkat T cell co-transfection assays in the presence of PMA/Ca^{2+} ionophore. CSD binding site mutational analysis also showed that the co-activation affect of CSD proteins seen on the GM-CSF promoter did not require contact with the CSD sites. When the C-terminal deletion of dbpB was used in co-transfection experiments, the PMA/ Ca^{2+} ionophore mediated co-activation was significantly reduced, indicating that the co-activation was mediated primarily through the C-terminal domain of the CSD protein. The C-terminal domain of many CSD factors are composed of alternating basic and acid regions which have been implicated in protein:protein interactions (Wolffe *et al.*, 1992); (Wolffe 1993). Several examples of transcriptional regulation by CSD factors involving protein:protein interactions have been reported. For example CSD factors have been shown to interact with the transcription factors Purα and viral T antigen which regulate early and late viral gene expression in human neutropic JV polyomavirus (JCV) (Safak *et al.*, 1999) (Chen *et al.*, 1995a). Another interesting example is CSD factor interaction with the tight junction associated protein, ZO-1. This CSD:ZO-1 interaction has been shown to regulate ErbB-2 expression, indicating that tight junctions directly participate in the control of gene expression (Balda *et al.*, 2000) (Sakura *et al.*, 1988). Other proteins shown to interact with CSD factors include hnRNPk, NF-Y, SP-1 and the viral Tat protein (Shnyreva *et al.*, 2000) (Iloberas *et al.*, 1995) (Sawaya *et al.*, 1998) (Ansari *et al.*, 1999). Many transcription factors involved in GM-CSF gene regulation are upregulated upon PMA/ Ca^{2+} Ionophore stimulation, but of particular interest to our laboratory are the p50 and RelA NF-κB proteins. We have shown that NF-κB proteins are key regulators of the GM-CSF gene in T cells in response to T cell receptor and co-receptor signalling (Jenkins *et al.*, 1995); (Shannon *et al.*, 1997). Of particular interest to the work presented here, Raj *et. al.* have demonstrated an interaction

between CSD factors and NF-κB/Rel family members involved in the regulation of a viral promoter (Raj *et al.*, 1996). Since the GM-CSF NF-κB/Rel and CSD binding sites in domain 1 overlap (Figure 7.1) and experiments show that CSD mediated activation acts through the C-terminus of the CSD proteins only when cells are PMA/ Ca^{2+} ionophore stimulated, NF-κB/Rel factors presented a potential CSD:protein interaction candidate in T cells. Therefore, a series of experiments were designed to look for a potential interaction between CSD factors dbpAv and dbpB with the NF-κB/Rel factors p50 and RelA, resulting in effects on factor binding to the GM-CSF proximal promoter. In this chapter I discuss these potential CSD:NF-κB/Rel interactions and show that CSD factors and RelA can synergistically activate the GM-CSF promoter.

Figure 7.1

GM-CSF promoter and oligonucleotides used in gel shift assays.

The sequence of the coding (+) and non-coding (-) wildtype human GM-CSF proximal promoter is shown. Domain 1 (-114 to –71) and Domain 2 (-70 to –31) regions are indicated. The NF-kB p50/RelA site and the CD28-responsive complex are shown. Nuclear NF-GMb/c and recombinant CSD factor binding sites are indicated on the non-coding (-) strand in domain 1 and the coding (+) strand in domain 2. Below are the sequences of the coding (+) and non-coding (-) wildtype oligonucleotides GM+/- and GM35+/-. The coding (+) strand of the mutant oligonucleotides GMm49+, GMm51+, GMm53+ is shown with the altered bases indicated.


Domain 1
-70
Domain 2
CD28RE
NF-κB
CSD
CSD
-114 + TGATAAGGGCCAGGAGATTCCACAGTTCAGGTAGTTCCCCCGCCTCCCTGGCATTTTGTGGTCACCATTAATCATTTCCTCTGT+ -31
- ACTATTCCCGGTCCTCTAAGGTGTCAAGTCCATCAAGGGGGCGGAGGGACCGTAAAACACCAGTGGTAATTAGTAAAGGAGACA-
CSD
CSD
GM35 + ... - .....
GMm49 + ...GTCT.....
GMm51 + ...CGG.....
GMm53 + ...AAA.....
GM- + ... - .....

7.2 Recombinant CSD factor dbpB binds to a new CSD site in the GM35 region of the GM-CSF proximal promoter.

It is known from all the previous work presented here and performed in our laboratory, that nuclear NF-GMb/c complexes and recombinant CSD factors dbpAv and dbpB can only bind to single stranded DNA, whereas NF-κB proteins p50 and RelA will only bind to double stranded DNA (Shannon *et al.*, 1997). The GM-CSF proximal promoter contains two NF-κB binding sites, the CD28RE and the NF-κB site, both of which are present in domain 1 of the promoter (Figure 7.1). The binding of NF-κB/Rel factors p50 and RelA to these sites has been extensively studied in our laboratory. Experiments show that both p50 and RelA can bind to the NF-κB site, whereas only RelA can bind to the CD28RE site (Shannon *et al.*, 1997) (Ranjan *et al.*, 1993) (Jenkins *et al.*, 1995). In the GM-CSF proximal promoter the CD28RE, NF-κB and CSD binding sites overlap (See Figure 7.1), therefore binding of one protein may preclude the binding of the other protein due to the requirement of double or single stranded targets respectively. Using this difference, I was able to directly examine the effect of either CSD factors on NF-κB binding to double stranded DNA or the effect of NF-κB factors on CSD factor binding to single stranded DNA. NF-κB/Rel:CSD factor interaction was examined by gel shift experiments using two GM-CSF proximal promoter oligonucleotides. The first of these was the GM oligonucleotide (-114 to -75) (Figure 7.1), which has been used for gel shift analysis in previous chapters. The GM oligonucleotide contains both domain 1 CSD binding sites but only the CD28RE and was used to examine RelA/CSD factor interactions. The second oligonucleotide used was GM35 (-90 to -66), which spans the 3' end of domain 1 and the 5' end of domain 2 of the GM-CSF proximal promoter. The GM35

oligonucleotide contains the NF-κB site and only one CSD binding site (Figure 7.1). The GM35 oligonucleotide was used to examine NF-κB p50/CSD factor interactions.

Before this could be done it was necessary to determine if the CSD factors could bind to the GM35 oligonucleotide. All previous binding experiments were done using either GM-CSF domain 1 or 2 oligonucleotides, whereas the GM35 oligonucleotide spans a small proportion of both of these regions and contains only one CSD binding site on the non-coding (-) strand (Figure 7.1). Previous mutational analysis (Chapter 5) indicated that recombinant CSD factors could bind to only one CSD site but with reduced efficiency. Since it was not known if the sequence surrounding the domain 1 and 2 CSD binding sites were critical for CSD binding it was not known if the CSD factors would bind to the GM35 oligonucleotide. Recombinant CSD-GST fusion proteins, dbpAv-GST and dbpB-GST, were bound to the GM-CSF domain 1 (GM-) oligonucleotide (shown previously to bind CSD factors, Chapters 4 & 5) and to both the coding (+) and non-coding (-) strands of GM35 in a gel shift assay. Both dbpAv-GST and dbpB-GST bound well to GM- (Figure 7.2, lanes 1 & 2), but surprisingly the CSD factors bound to the coding (+) strand of GM35 and not to the CSD site present on the non-coding (-) strand of GM35 (Figure 7.2, lanes 3-6). This binding was surprising, firstly because of the lack of binding to the GM35-oligonucleotide. This indicated that in this context this CSD site was not able to bind CSD factors, even though in previous gel shift experiments, recombinant CSD factors were able to bind to an oligonucleotide (GMm19-) containing this single CSD binding site (Chapter 5 Figure 5.4B). Even more surprising was the binding to the GM35+ oligonucleotide since there were no apparent CSD binding sites on the GM35+ coding strand. The partial domain 2, CSD binding site present in this GM35+ oligonucleotide is not sufficient for binding (Figure 7.1). These results indicated the presence of another CSD binding site in the GM-CSF proximal promoter.

Figure 7.2

Jurkat T cell nuclear NF-GMb complex, dbpAv-GST and dbpB-GST bind to the 35+ oligonucleotide.

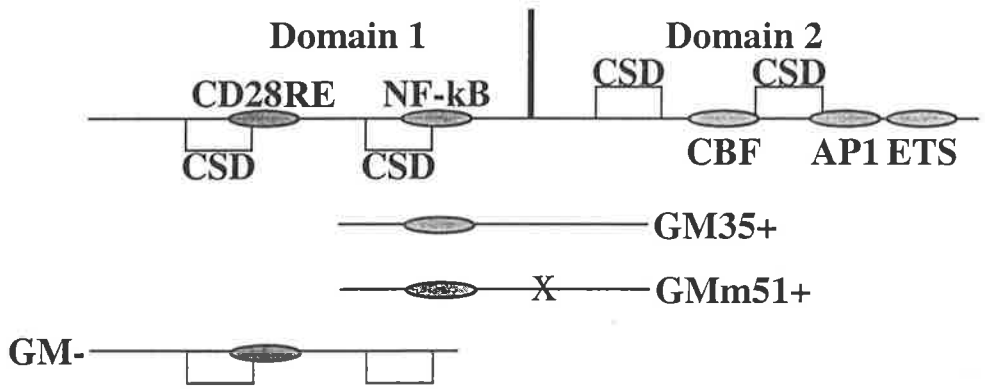
A) Recombinant dbpB-GST and dbpAv-GST were bound in a gel shift assay to ^{32}P-labelled wild type GM-CSF domain 1 non-coding (-) strand GM- oligonucleotide (Lanes 1 & 2) and to the wild type coding (+) strand GM35+ oligonucleotide (Lanes 3 & 4).

B) Recombinant dbpB-GST was bound in a gel shift assay to ^{32}P-labelled coding (+) and non-coding (-) strand oligonucleotides GM35+ and GM35- and to the coding (+) strand mutants GMm49+, GMm51+ & GMm53+ (Lanes 1-6). Jurkat T cell nuclear extract was bound in a gel shift assay to ^{32}P-labelled coding (+) and non-coding (-) strand GM35 oligonucleotides (lanes 6 & 7) and to the coding (+) strand of the mutant GMm51 oligonucleotide (lane 8)

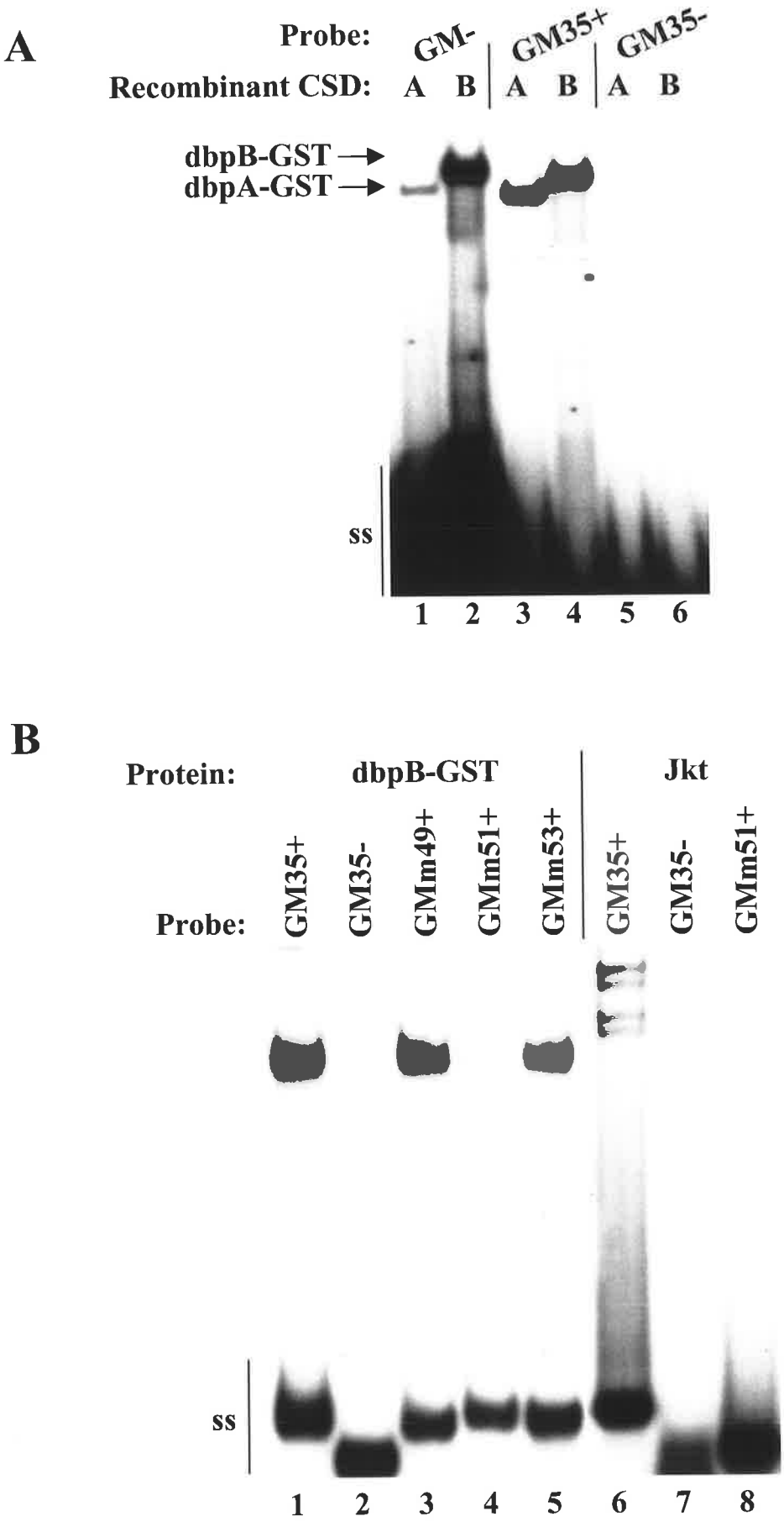
A: dbpAv-GST

B: dbpB-GST

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites, a 'X' represents mutated GM-CSF promoter sequence and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 7.1.


A
Probe:
GM-
GM35+
GM35-
Recombinant CSD:
A B A B A B
dbpB-GST
dbpA-GST
ss
1 2 3 4 5 6
B
Protein:
dbpB-GST
Jkt
Probe:
GM35+
GM35-
GMm49+
GMm51+
GMm53+
GM35+
GM35-
GMm51+
ss
1 2 3 4 5 6 7 8

To determine where the CSD binding site was on the GM35+ coding strand, recombinant dbpB-GST was bound to the coding (+) strand of the mutant oligonucleotides (GMm49+, GMm51+ and GMm 53+) which were originally designed to examine NF-κB binding (Figure 7.2). Of the three GM35+ oligonucleotide mutations, GMm49+, GMm51+ and GMm53+, only the mutation in GMm51+ had an effect on dbpB-GST binding (Figure 7.2B, lanes 1 to 5). This mutation alters bases from 5'-GGTAGTTCCC-3' to 5'-GGTAGCGGCC-3'. While there is no CSD factor consensus binding site, CSD factors have a preference for CT rich regions of DNA. Binding studies have also shown that while the core binding site is important, flanking sequence also play an important role in CSD factor binding (Wolffe *et al.*, 1992) (Wolffe 1993) (Shannon *et al.*, 1997) (Ohmori *et al.*, 1996). The mutation present in the GM51+ oligonucleotide suggest that the core of the potential new CSD binding site is TTCC.

7.3 Jurkat T cell NF-GMb and HUT78 T cell NF-GMc nuclear CSD complexes can form on the GM35+ oligonucleotide

To determine if the nuclear NF-GMb complex could also form on this new potential CSD binding site, Jurkat T cell nuclear extract was bound in a gel shift assay to both coding (+) and non-coding (-) strands of GM35 and the GM51+ mutant oligonucleotide. When Jurkat T cell nuclear extract was used in the gel shift, two doublet complexes formed on the GM35+ oligonucleotide (Figure 7.2B, lane 6). As for recombinant dbpB-GST neither of these complexes formed on either the GM35- or GMm51+ oligonucleotides (Figure 7.2B, lanes 7 & 8) suggesting that these complexes could represent CSD factor binding.

To further examine CSD factor binding to the GM35+ oligonucleotide Jurkat and HUT78 T cell nuclear extracts were bound to both GM- and GM35+ oligonucleotides in a gel shift assay. The expected pattern of complex formation on GM- domain 1

oligonucleotide was observed for both Jurkat T cell (NF-GMb complex doublet only Figure 7.3A, lane 1) and HUT78 T cell (single NF-GMb and NF-GMc complexes Figure 7.3A, lane 2). Since previous experiments have indicated that full NF-GMb complex formation requires 2 CSD binding sites whereas NF-GMc complex formation requires only 1 CSD binding site, it was not surprising to see only HUT78 T cell NF-GMc complex formation on the GM35+, which has only one proposed CSD site (Figure 7.3A, lane 3). The two doublet NF-GMb complexes were however, seen when Jurkat T cell nuclear extract was bound to the GM35+ oligonucleotide (Figure 7.3A, lane 4). To determine the size of the components of the complexes seen forming on the GM35+ oligonucleotide, complexes were subjected to UV cross-linking analysis. As a comparison the previously characterised NF-GMb/c complexes formed on domain 1 GM- oligonucleotide (Chapter 5) were also UV cross-linked. As seen previously the GM- Jurkat T cell NF-GMb complex contained only the 42 kDa protein, the HUT78 T cell NF-GMb complex contained both the 42 and 22 kDa proteins and the HUT78 T cell NF-GMc complex contained only the 22 kDa protein (Figure 7.3B, lanes 1 to 3). UV cross-linking analysis of the GM35+ HUT78 T cell NF-GMc complex also only contained a 22 kDa protein (Figure 7.3B, lane 4). The differences in mobility of the HUT78 T cell NF-GMc complex on GM35+ compared to GM- is due to differences in probe length (Figure 7.1). On the GM35+ oligonucleotide Jurkat T cell nuclear extract bound as 2 doublet NF-GMb complexes. The higher complex (denoted NF-GMb(1)), when UV cross-linked contained a 52 kDa protein and the lower complex (denoted NF-GMb(2)), contained the 42 kDa protein seen previously (Figure 7.3B, lanes 5 & 6). CSD proteins of approximately 55 kDa in size have been reported (Evdokimova *et al.*, 1995), suggesting that the 52 kDa protein identified here may represent another CSD factor. The reason for formation of the 42 kDa NF-GMb complex on GM35+ in Jurkats but only the truncated 22 kDa NF-GMc complex in HUT78 T cells is not clear (Figure 7.3B, lane 4). The Jurkat T cell results however are consistent with

Figure 7.3

Jurkat T cell nuclear NF-GMb and HUT78 T cell NF-GMc complexes bind to the 35+ oligonucleotide.

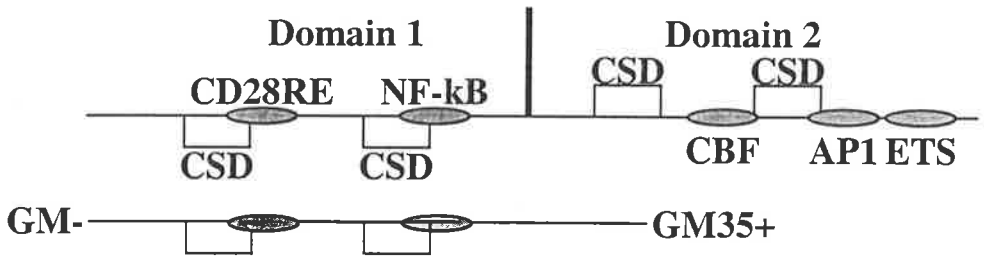
A) Jurkat T and HUT78 T cell nuclear extracts were bound in a gel shift assay to ^{32}P-labelled GM-CSF domain 1, non-coding (-) strand GM- oligonucleotide (lanes 1 & 2) and to coding (+) strand GM35+ oligonucleotide (lanes 3 & 4).

B) Jurkat and HUT78 T cell NF-GMb and NF-GMc gel shift complexes were UV cross-linked and Laemmli SDS-polyacrylamide protein gel fractionated. Fractionated NF-GMb/c gel shift complexes from Jurkat and HUT78 T cells formed on domain 1 non-coding (-) strand GM- (Jurkat, lane 1 & HUT78, lanes 2 & 3) and complexes formed on coding (+) strand GM35+ (HUT78, lane 4 & Jurkat, lanes 5 & 6) ^{32}P-labelled oligonucleotides.

Jkt: indicates Jurkat T cell nuclear extract

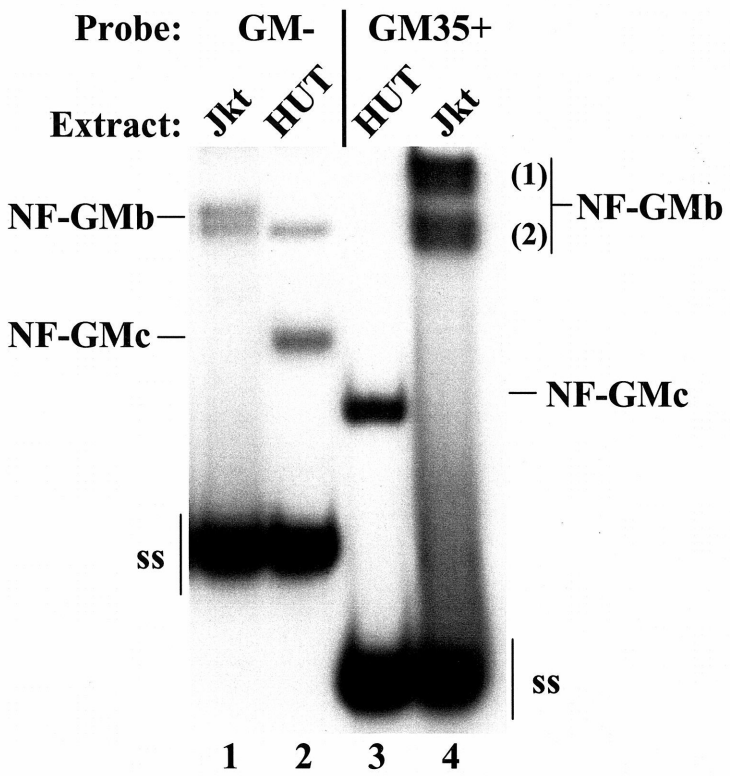
HUT: indicates HUT78 T cell nuclear extract

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)

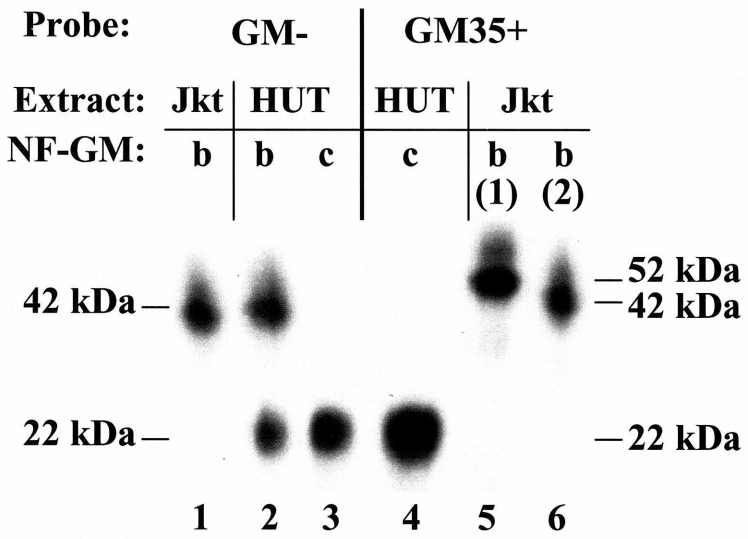


The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 7.1.

A


Probe:
GM-
GM35+
Extract:
Jkt
HUT
HUT
Jkt
NF-GMb
NF-GMc
ss
(1)
(2)
NF-GMb
NF-GMc
ss
1
2
3
4

B


Probe:
GM-
GM35+
Extract:
Jkt
HUT
HUT
Jkt
NF-GM:
b
b
c
c
b (1)
b (2)
42 kDa
22 kDa
52 kDa
42 kDa
22 kDa
1
2
3
4
5
6

results described previously (Chapter 4) where some NF-GMb complex formation is detected on oligonucleotides containing only one CSD site, but maximal levels of NF-GMb complex binding require two CSD binding sites.

7.4 CSD factor dbpAv-GST is able to disrupt RelA binding to its double stranded DNA target

Having shown that CSD factors can bind to the GM35 oligonucleotide I was able to proceed with experiments outlined previously, which were designed to examine the possible interaction of CSD and NF-κB factors. Recombinant RelA was obtained from Dr. S. Gerondakis and recombinant p50 was obtained from Promega Corp. Double stranded domain 1 GM+/- oligonucleotide was used in the first gel shift experiment, to detect RelA binding but only the GM- strand of the double stranded probe (which does not bind CSD factors) was ^{32}P labelled. This was done to prevent any detection of CSD binding to single stranded DNA that may be present due to probe denaturation. This recombinant RelA material has been previously characterised by gel shift analysis using both RelA specific antibodies and GM-CSF NF-κB mutant oligonucleotides (Shannon *et al.*, 1995) and Dr. L. S. Coles unpublished) and can be seen binding to the double stranded GM+/- probe in Figure 7.4A, lane 3. As mentioned, the CSD factors dbpAv and dbpB do not bind to double stranded DNA GM+/-, this can be seen in Figure 7.4A, lanes 1 & 2. To determine if the presence of CSD proteins could affect RelA binding to its double stranded DNA target, increasing concentrations of either dbpAv-GST or dbpB-GST were mixed with a constant amount of RelA in a gel shift reaction. Mixing CSD factor dbpAv-GST with recombinant RelA acted to inhibit, and at higher concentrations of dbpAv-GST to block RelA binding to the GM+/- double stranded oligonucleotide (Figure 7.4A, lanes 4 to 6). Mixing CSD factor dbpB-GST with RelA appeared to have no effect on RelA binding to

Figure 7.4

dbpAv-GST is able to disrupt RelA binding to its double stranded DNA target.

A) Recombinant dbpAv-GST, dbpB-GST and RelA were bound in a gel shift assay separately or in combination to a wildtype, domain 1, double stranded oligonucleotide GM+*/GM- where only the coding (+) strand (that does not bind CSD factors) was ^{32}P-labelled (denoted by *). Recombinant dbpAv-GST, dbpB-GST and RelA were bound to the GM+*/GM- double stranded oligonucleotide on their own (Lanes 1,2 & 3 respectively). Progressively increasing amounts of dbpAv-GST was added to a constant amount of RelA in lanes 4 to 6. Progressively increasing amounts of dbpB-GST was added to a constant amount of RelA in lanes 7 to 9.

(ds indicated free double stranded ^{32}P-labelled oligonucleotide)

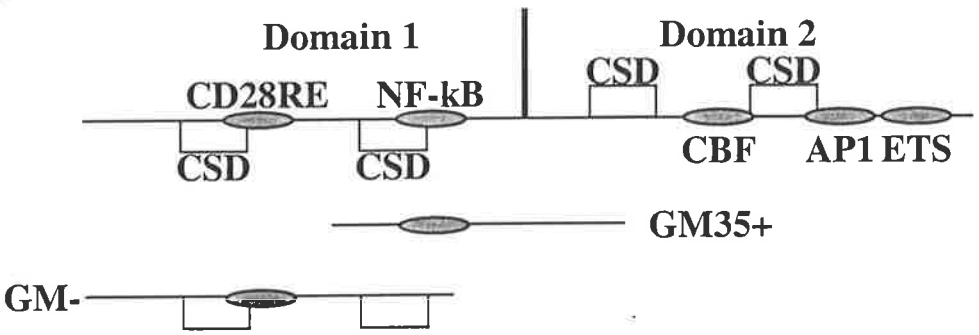
RelA is able to disrupt dbpB-GST binding to its single strand DNA target.

B) Recombinant dbpAv-GST, dbpB-GST and RelA were bound in a gel shift assay separately or in combination to a ^{32}P-labelled, wildtype, non-coding (-) strand, domain 1 GM-oligonucleotide. Recombinant RelA, dbpAv-GST and dbpB-GST were bound to the GM- oligonucleotide on their own (Lanes 1,2 & 3 respectively). Progressively increasing amounts of RelA was added to a constant amount of dbpAv-GST in lanes 4 to 7 and to a constant amount of dbpB-GST in lanes 8 to 11.

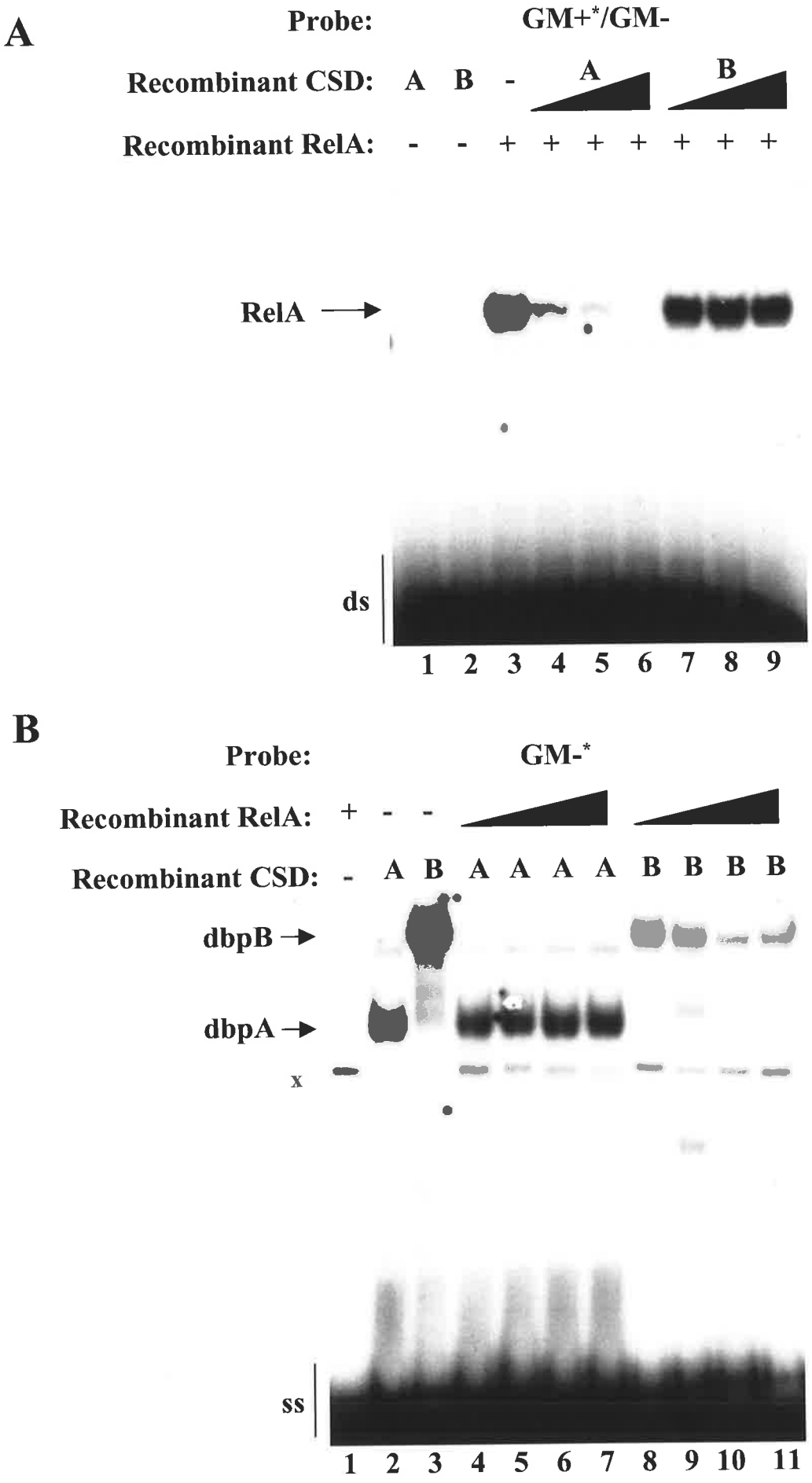
(ss indicated free single stranded ^{32}P-labelled oligonucleotide)

(x represents non-specific binding)

A: indicates dbpAv-GST **B**: indicates dbpB-GST)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 7.1.


A
Probe:
GM+*/GM-
Recombinant CSD:
A B -
A
B
Recombinant RelA:
- - + + + + + + +
RelA
ds
1 2 3 4 5 6 7 8 9
B
Probe:
GM-*
Recombinant RelA:
+ - -
Recombinant CSD:
- A B A A A A B B B B
dbpB
dbpA
x
ss
1 2 3 4 5 6 7 8 9 10 11

its double stranded DNA target at any concentration used (Figure 7.4A, lanes 7 to 9). To determine if the presence of RelA could effect CSD factor binding to its single stranded DNA target a constant amount of CSD factors dbpAv-GST and dbpB-GST were each mixed with increasing amounts of RelA. RelA requires a double stranded binding site to bind and therefore did not bind to the single stranded GM- oligonucleotide (Figure 7.4B, lane 1). In contrast to what was seen on double stranded DNA, RelA had no effect on dbpAv-GST binding, at any concentration (Figure 7.4A, lanes 4 to 7), whereas increasing amounts of RelA acted to inhibit dbpB-GST binding to its single stranded target (Figure 7.4B, lanes 8 to 11). A band (marked x in Figure 7.4B) was detected binding to the single stranded GM- oligonucleotide in the recombinant RelA extract. This band was present whenever recombinant RelA was added to the reaction but did not increase in intensity when the amount of RelA was increased and therefore was deemed non-specific. Previous experiments done in our laboratory by Dr. L. S. Coles has also shown that binding of this non-specific band is not affected by NF-κB mutations or NF-κB specific antibodies (Dr. L. S. Coles unpublished).

Since NF-κB p50 can bind the NF-κB site on the GM-CSF promoter (Figure 7.1) (Shannon *et al.*, 1995), similar experiments were undertaken to determine if there was a similar NF-κB p50/CSD interaction. To assay for NF-κB p50 binding, the GM35+/- double stranded oligonucleotide was used in gel shift assays. This recombinant NF-κB p50 material has been previously characterised by gel shift analysis using both NF-κB p50 specific antibodies and GM-CSF NF-κB mutant oligonucleotides (Shannon *et al.*, 1995 and Dr. L. S. Coles unpublished) and can be seen binding to the double stranded GM35+/- oligonucleotide in Figure 7.5A, lane 3. Again, as for the RelA experiments only one strand, GM35- (which does not bind CSD factors) was ^{32}P labelled. Neither CSD factor dbpAv-GST or dbpB-GST could be detected binding to this double stranded probe,

Figure 7.5

dbpAv-GST is able to disrupt NF-κB p50 binding to its double stranded target.

A) Recombinant dbpAv-GST, dbpB-GST and NF-κB p50 were bound in a gel shift assay separately or in combination to a wildtype, domain 1, double stranded oligonucleotide GM35+/GM35* where only the non-coding (-) strand (that does not bind CSD factors) was ^{32}P-labelled (denoted by *).

Recombinant dbpAv-GST, dbpB-GST and NF-κB p50 were bound to the GM35+/GM35* double stranded oligonucleotide on their own (Lanes 1,2 & 3 respectively). Progressively increasing amounts of dbpAv-GST was added to a constant amount of NF-κB p50 in lanes 4 to 6. Progressively increasing amounts of dbpB-GST was added to a constant amount of NF-κB p50 in lanes 7 to 9.

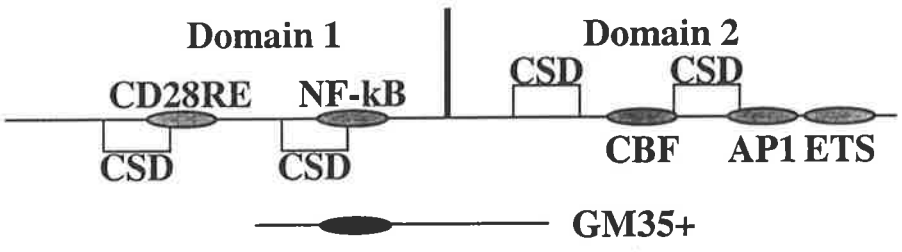
(ds indicated free double stranded ^{32}P-labelled oligonucleotide)

NF-κB p50 had no effect on dbpAv-GST or dbpB-GST binding to their single strand target.

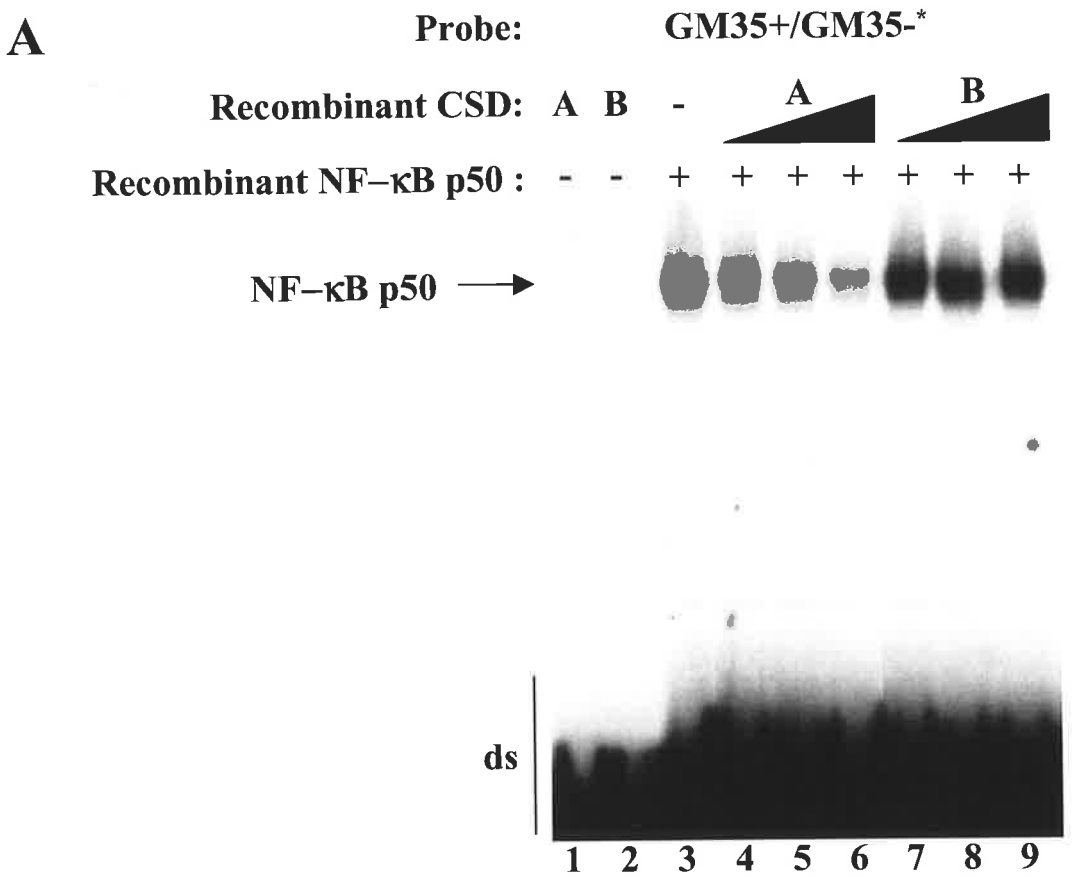
B) Recombinant dbpAv-GST, dbpB-GST and NF-κB p50 were bound in a gel shift assay separately or in combination to a ^{32}P-labelled, wildtype, coding (+) strand, domain 1 GM35+ oligonucleotide.

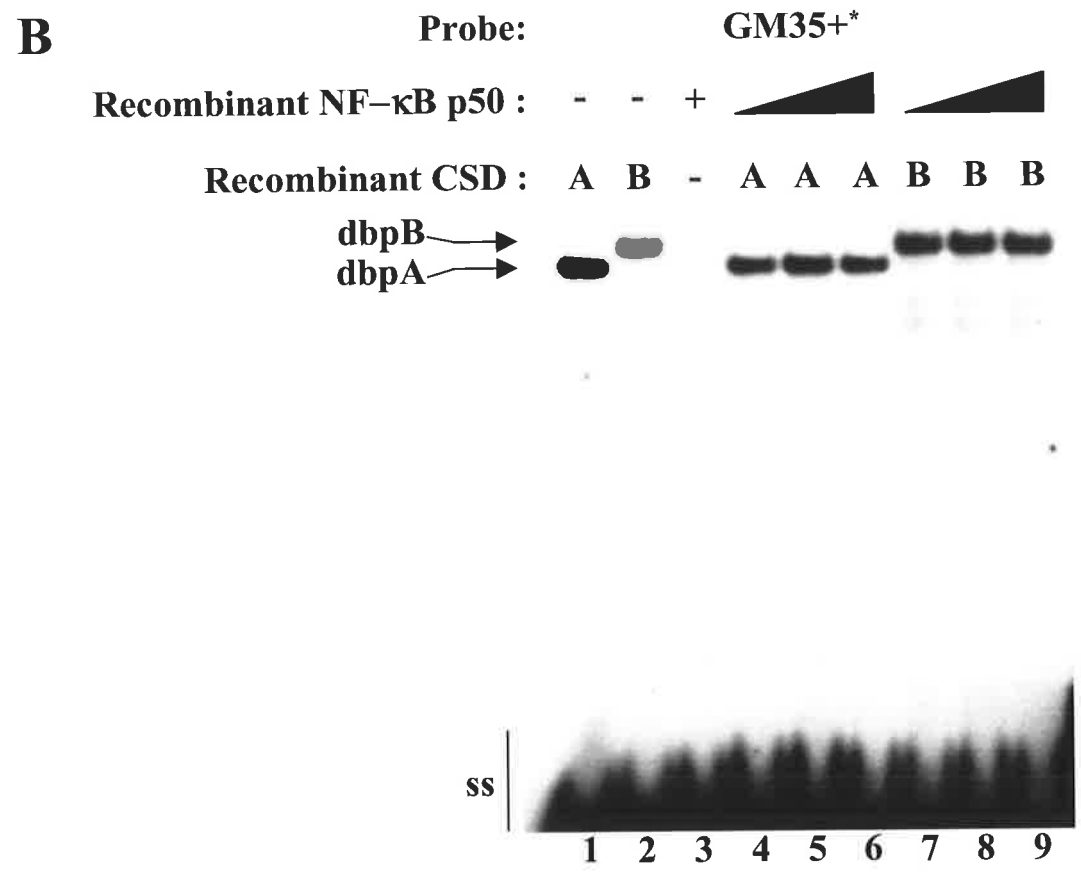
Recombinant dbpAv-GST, dbpB-GST and NF-κB p50 were bound to the GM35+ oligonucleotide on their own (Lanes 1,2 & 3 respectively). Progressively increasing amounts of NF-κB p50 was added to a constant amount of dbpAv-GST in lanes 4 to 6 and to a constant amount of dbpB-GST in lanes 7 to 9.

(ss indicated free single stranded ^{32}P-labelled oligonucleotide)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Ovals represent the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 7.1.


A
Probe:
GM35+/GM35-*
Recombinant CSD:
A B -
A
B
Recombinant NF–κB p50 :
- - + + + + + + +
NF–κB p50
ds
1 2 3 4 5 6 7 8 9


B
Probe:
GM35+*
Recombinant NF–κB p50 :
- - +
Recombinant CSD :
A B - A A A B B B
dbpB
dbpA
ss
1 2 3 4 5 6 7 8 9

whereas NF-κB p50 bound well (Figure 7.5A, lanes 1 to 3). When CSD factor dbpAv-GST was mixed with NF-κB p50, dbpAv-GST could inhibit NF-κB p50 binding to its double stranded target, as was seen with RelA (Figure 7.5A, lanes 4-6). CSD factor dbpB-GST had no effect on NF-κB p50 binding to its double stranded DNA target at any of the concentrations used (Figure 7.5A, lanes 7-9).

To determine the effect of NF-κB p50 on CSD binding, mixing experiments were performed using the GM35+ single stranded oligonucleotide. As seen previously both CSD factors dbpAv-GST and dbpB-GST can bind to the single strand oligonucleotide whereas NF-κB p50 can not (Figure 7.5B, lanes 1 to 3). It was observed that NF-κB p50 had no effect, at any concentration, on either CSD factor dbpAv or dbpB binding to the GM35+ oligonucleotide (Figure 7.5B, lanes 4 to 6 for dbpAv-GST, lanes 7 to 9 for dbpB-GST).

To determine if RelA could act to dissociate dbpB-GST once it was bound to its single stranded target, dbpB-GST was first bound to single stranded DNA (GM- Domain 1) for 10 minutes prior to the addition of RelA for a further ten minutes. As can be seen in Figure 7.6, dbpB-GST binding after 10 minutes was the same as that of protein bound for 20 minutes (Figure 7.6, lanes 1 & 2). The addition of RelA after 10 minutes of dbpB-GST incubation resulted in a decrease in the level of dbpB-GST binding seen (Figure 7.6, lane 3). To make sure that this effect was apparent on all CSD binding sites the same experiment was repeated on the GM35+ oligonucleotide. The same results were seen on the GM35+ as the GM- oligonucleotide, RelA could reduce dbpB-GST binding after it had bound to its single strand DNA target (Figure 7.6, lanes 5 to 7). Again the non-specific band seen when recombinant RelA was bound to GM- was observed whenever RelA was added to the gel shift mix (marked x in Figure 7.6, lanes 3 & 4) and can be seen binding faintly to the GM35+ oligonucleotide (Figure 7.6, lanes 7 & 8).

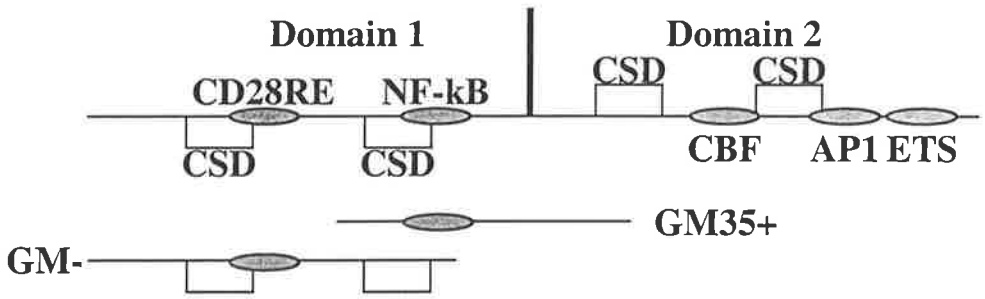
Figure 7.6

RelA is able to dissociate dbpB-GST from its single strand DNA target.

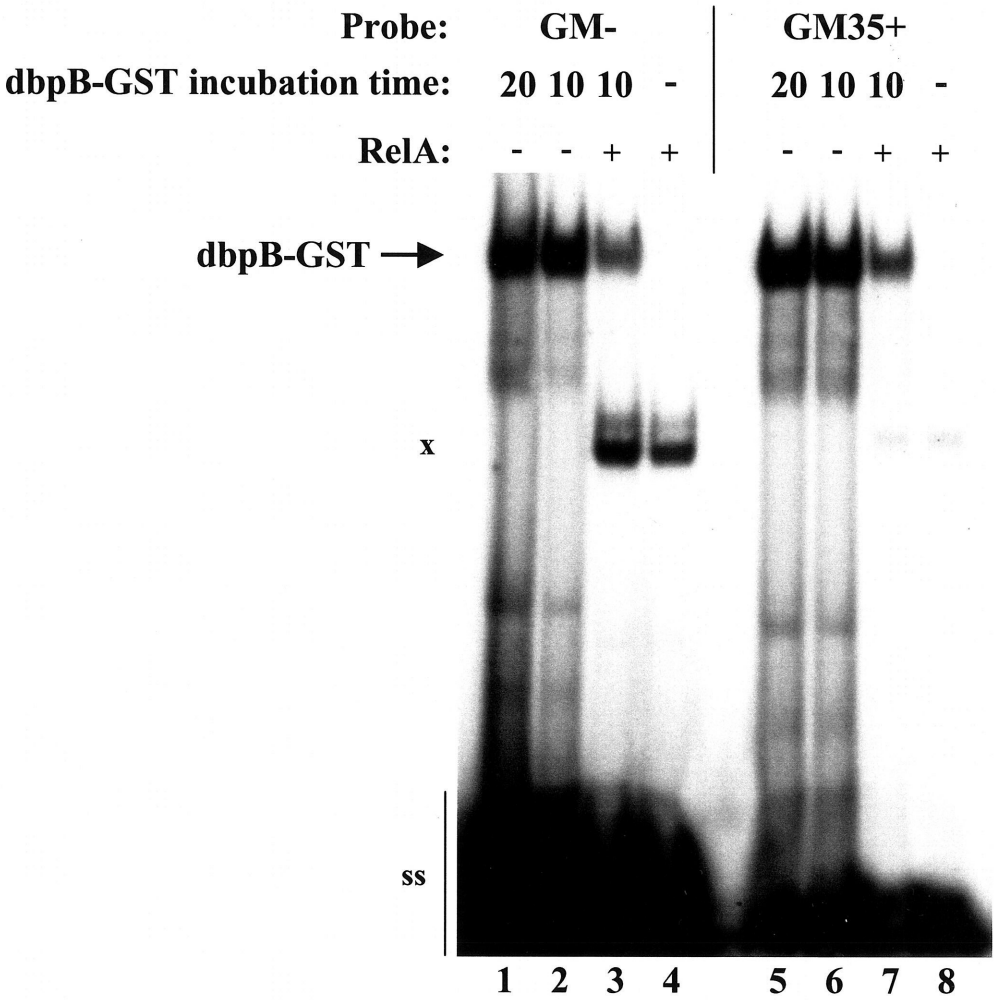
Recombinant dbpB-GST and RelA were bound separately or together in a gel shift assay to ^{32}P-labelled wildtype, domain 1, non-coding (-) strand GM- or to wildtype, coding (+) strand GM35+ oligonucleotides. Recombinant dbpB-GST was incubated with labelled GM- or GM35+ oligonucleotides for a period of 20 minutes (Lanes 1 & 5 respectively). Recombinant dbpB-GST was also incubated with labelled GM- or GM35+ oligonucleotides for a period of 10 minutes followed by addition of RelA and incubated for a further 10 minutes (Lanes 3 & 7 respectively). Recombinant RelA alone was incubated with either GM- or GM35+ oligonucleotides for a period of 20 minutes as a negative control (Lanes 4 & 8 respectively).

(x indicates non-specific binding)

(ss indicates free ^{32}P labelled oligonucleotide)



The human GM-CSF proximal promoter and oligonucleotides used in these experiments are represented diagrammatically above. Ovals represent the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 7.1.


Probe:
GM-
GM35+
dbpB-GST incubation time:
20 10 10 -
20 10 10 -
RelA:
- - + +
- - + +
dbpB-GST
x
ss
1 2 3 4 5 6 7 8

Experiments undertaken to determine if CSD factors and RelA could affect each other binding indicated differences between dbpAv and dbpB. The CSD factor dbpAv was able to inhibit RelA binding to its double stranded DNA target while dbpB had no effect on RelA binding. However, RelA had no effect on dbpAv binding to its single stranded binding site while inhibiting the binding of dbpB. This difference in effect suggests differences in the mechanism of interaction of RelA with the two different CSD factors. The effect of CSD factors on NF-κB p50 binding was limited to dbpAv inhibiting binding to its double stranded target.

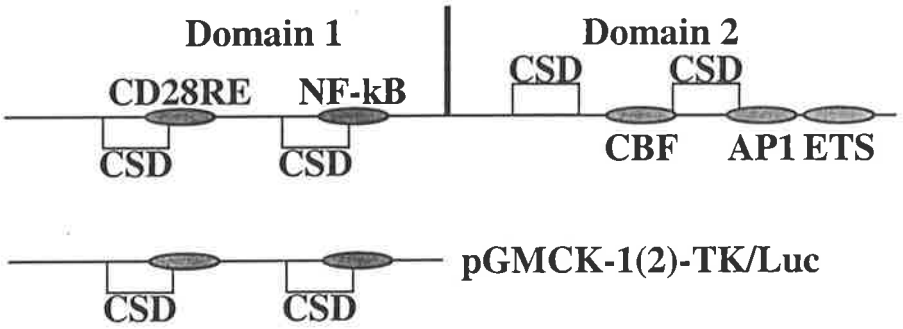
7.5 CSD factors and RelA co-operatively activate the GM-CSF proximal promoter

To investigate the potential role of a CSD:RelA interaction, in the CSD mediated activation of the GM-CSF promoter domain 1 sequences, RelA and CSD expression constructs were co-transfected with the GM-CSF domain 1 reporter construct pGMCK-1(2)TK-Luc into Jurkat T cells. In Figure 7.7 it can be seen, as previously reported, that RelA was able to activate the pGMCK-1(2)TK-Luc construct in unstimulated cells whereas the CSD factors could not (Chapter 3 and Shang *et al.*, 1999). When the pGMCK-1(2)-TK/Luc reporter construct was co-transfected with expression constructs for both RelA (pRcCMVRelA) and dbpAv (pSGdbpAv) or dbpB (pSGdbpB) co-operative activation was observed (Figure 7.7). Repeating the triple co-transfection with the dbpB C-terminal truncation (pSGdbpBΔ2) significantly reduced the co-operative activation (Figure 7.7), suggesting that the C-terminus of the CSD protein dbpB protein may be involved in interactions with RelA to bring about activation. Co-transfecting with the dbpB construct pSGdbpBΔ1 (missing both the C-terminal and CSD domains) further reduced the activation to near basal pSG5 levels (Figure 7.7). These experiments show that RelA and

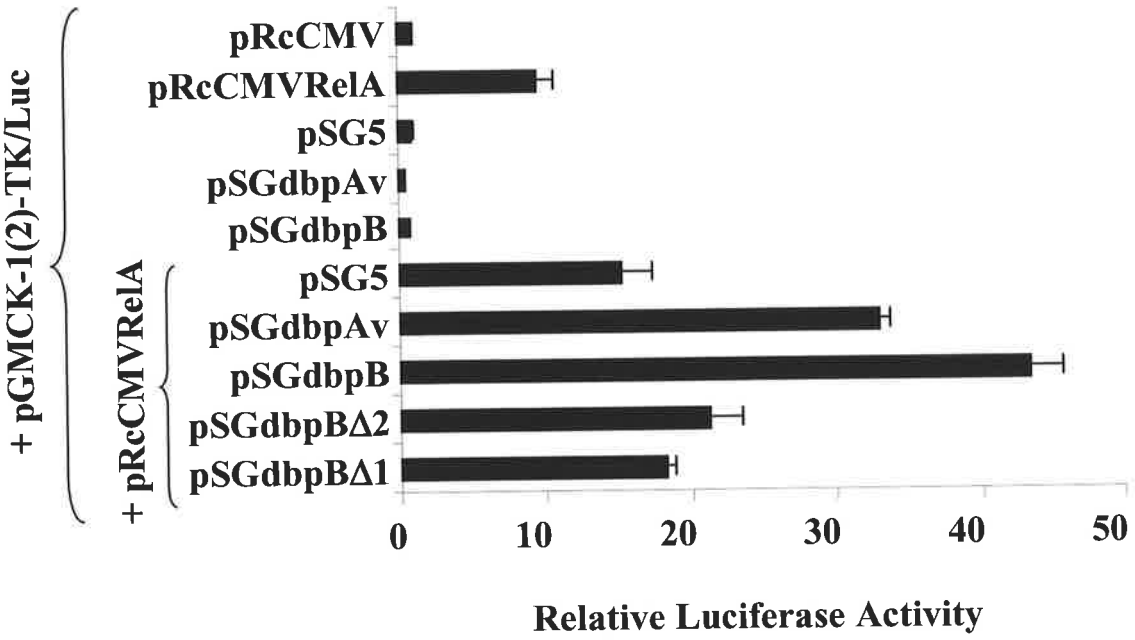
Figure 7.7

RelA and dbpAv or dbpB are able to co-operatively activate the GM-CSF promoter.

A) The GM-CSF promoter luciferase reporter plasmid pGMCK-1(2)-TK/Luc was co-transfected into Jurkat T cells with: pRcCMV, pRcCMVRelA, pSG5, pSGdbpAv and pSGdbpB. pGMCK-1(2)-TK/Luc and pRcCMVRelA were co-transfected together into Jurkat T cells with: pSG5, pSGdbpAv, pSGdbpB, pSGdbpBΔ2 and pSGdbpBΔ1. Cells were harvested 24 hours post transfection and assayed for luciferase activity. All luciferase activities are given relative to pGMCK-1(2)-TK/Luc co-transfected with pRcCMV which is given a value of 1.



Above is a diagrammatic representation of the GM-CSF proximal promoter and luciferase reporter construct used in this experiment. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The sequence of the GM-CSF promoter contained within pGMCK-1(2)-TK/Luc construct is shown in Figure 3.1.


+ pGMCK-1(2)-TK/Luc
+ pRcCMVRelA
pRcCMV
pRcCMVRelA
pSG5
pSGdbpAv
pSGdbpB
pSG5
pSGdbpAv
pSGdbpB
pSGdbpBΔ2
pSGdbpBΔ1
0
10
20
30
40
50
Relative Luciferase Activity

the CSD proteins can co-operate to activate GM-CSF promoter function and that the C-terminus of dbpB is essential for this co-operation.

7.6 PMA/Ca^{2+} Ionophore stimulation does not alter CSD factor levels

As well as protein:protein interactions being involved in the PMA/Ca^{2+} ionophore co-activation of the GM-CSF promoter by CSD factors, we also considered the possibility that levels of CSD proteins themselves may be affected by PMA/Ca^{2+} ionophore stimulation. In preliminary experiments designed to determine the sub-cellular localisation of CSD proteins, it was observed that CSD proteins were present in abundance in both the nuclear and cytoplasmic fractions (data not shown). A possible explanation for the observed CSD mediated activation only when the cells were PMA/Ca^{2+} ionophore stimulated was that CSD factor levels in the nucleus were increased due to stimulation. To determine if mimicking T cell receptor stimulation with PMA/ Ca^{2+} ionophore resulted in increases of cellular CSD proteins, Jurkat T cells were stimulated with PMA/ Ca^{2+} ionophore and cells harvested at time points 0, 0.5, 1, 1.5, 3 and 5 hours post stimulation. Cytoplasmic and nuclear proteins were prepared and used in gel shift assays with the wildtype domain 1 GM- oligonucleotide. As can be seen in Figure 7.8, stimulating the cells with PMA/ Ca^{2+} ionophore did not result in an increase of either cytoplasmic or nuclear CSD factors. These results suggest that changes in CSD levels are probably not involved in the CSD mediated co-activation of the GM-CSF promoter seen in previous experiments (Chapter 3).

7.7 Summary and Discussion

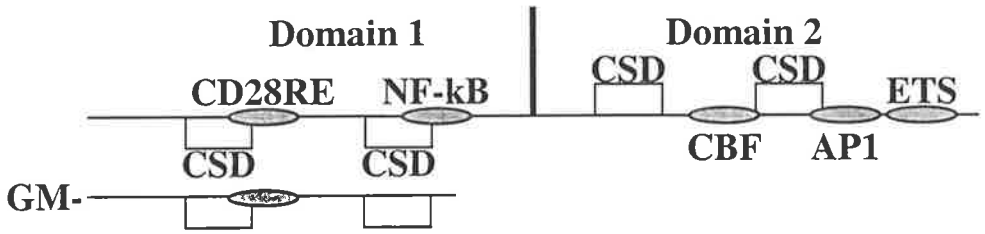
Previous characterisation of CSD factor binding across the GM-CSF proximal promoter identified four CSD binding sites, two on the non-coding strand of domain 1 and

Figure 7.8

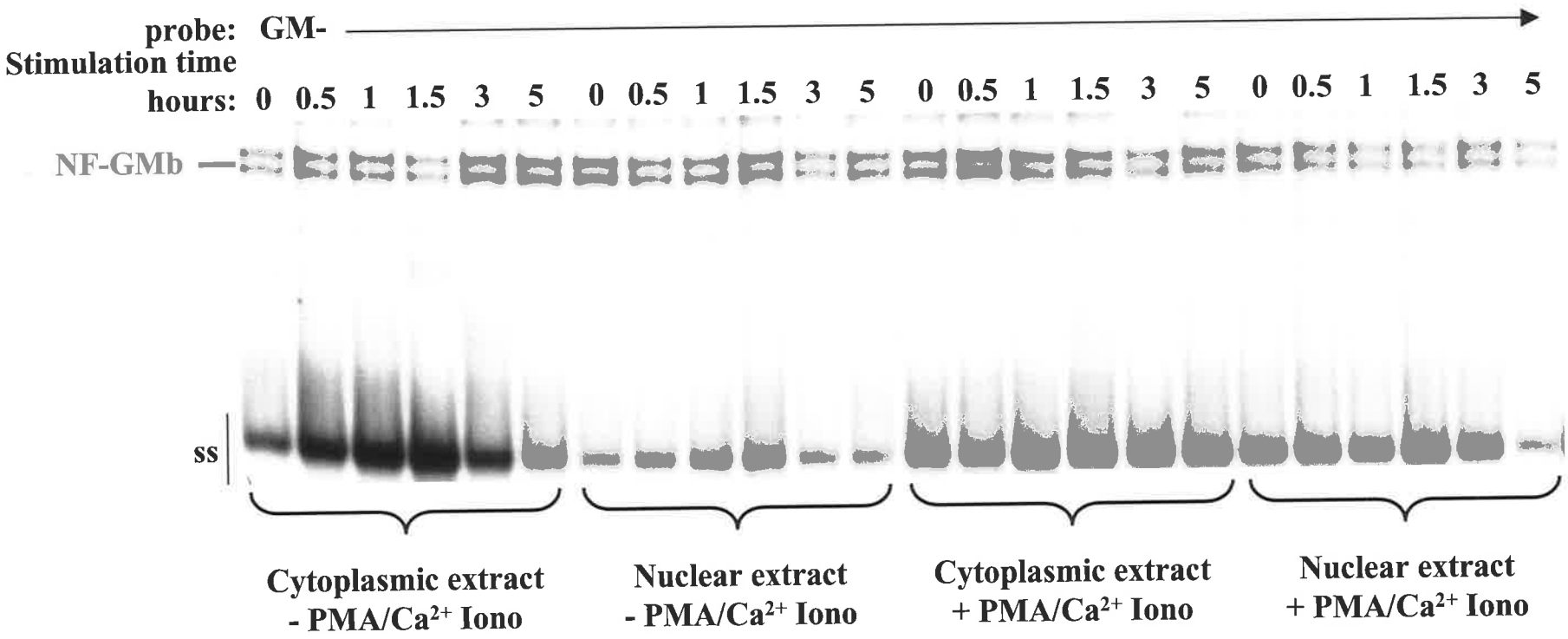
The level of Jurkat T cell NF-GMb in the nucleus and cytoplasm does not change with PMA and Ca^{2+} Ionophore stimulation.

Jurkat T cells were either unstimulated or stimulated with PMA and Ca^{2+} Ionophore over a time course of 5 hours with samples of cells being taken at 0, 0.5, 1, 1.5, 3 and 5 hour post stimulation time points. The cells were then lysed and the nuclear and cytoplasmic proteins fractionated. The cytoplasmic extracts and nuclear extracts from both unstimulated and stimulated cells were bound in a gel shift assay to ^{32}P-labelled GM-CSF domain 1, non-coding (-) strand oligonucleotide GM-.

(ss indicates free ^{32}P-labelled single stranded oligonucleotides)



The human GM-CSF proximal promoter and oligonucleotide used in this experiment are represented diagrammatically above. Boxes represent CSD binding sites and ovals the binding sites of the corresponding transcription factors indicated above. The oligonucleotide sequences are presented in Figure 7.1.


probe: GM-
Stimulation time
hours: 0 0.5 1 1.5 3 5 0 0.5 1 1.5 3 5 0 0.5 1 1.5 3 5 0 0.5 1 1.5 3 5
NF-GMb
ss
Cytoplasmic extract
- PMA/Ca2+ Iono
Nuclear extract
- PMA/Ca2+ Iono
Cytoplasmic extract
+ PMA/Ca2+ Iono
Nuclear extract
+ PMA/Ca2+ Iono

two on the coding strand of domain 2 (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). Gels shift analysis using oligonucleotides spanning either domain 1 (GM-) or domain 2 (GM93+) indicated that CSD factors could bind to one CSD site but both sites were needed for full CSD factor binding (Chapter 4). However, gel shift analysis using the GM35- oligonucleotide (which spans a region of both domain 1 and 2 and contains only the downstream domain 1 CSD binding site), indicated that in the context of this GM35- oligonucleotide, CSD factors could not bind to this one CSD binding site. This result was in contrast to what had been observed previously where this same single downstream CSD binding site was present in an oligonucleotide spanning only domain 1 and could bind CSD factors (Chapter 5). These results lend further evidence to experiments done in our laboratory which suggest that not only was the core CSD binding site important for binding but flanking sequences could also dictate CSD factor binding (Coles *et al.*, 1994). Surprisingly, CSD factor binding was observed on the opposite, coding strand of GM35. Mutational analysis across this oligonucleotide identified a new CSD binding site at the end of the domain 1 region but on the opposite strand to the two previously identified domain 1 CSD binding sites. This binding site consists of a CT rich sequence, and is in keeping with previous reports indicating that CSD factors show a preference for CT rich sequences (Wolffe *et al.*, 1992) (Wolffe 1993) (Shannon *et al.*, 1997). Gel shift analysis using recombinant and nuclear extracts from both Jurkat and HUT78 T cells demonstrated that CSD factor complexes could form on this sequence, identifying it as a real CSD binding site.

Co-transfection data presented in chapter 3, indicated that CSD mediated co-activation of the GM-CSF proximal promoter only occurred when the cells were stimulated with PMA/Ca^{2+} ionophore. One possible explanation for this observation was that PMA/Ca^{2+} ionophore stimulation resulted in an increase in the level of CSD proteins. Increases in levels of CSD proteins have been previously reported when cells undergo

periods of stress and DNA damage, for example during treatment with chemotherapeutic agents or exposure to UV-light (Li *et al.*, 1997) (Ohga *et al.*, 1997) (Metcalf 1991) (Bargou *et al.*, 1997). Increases in nuclear CSD factors have also been reported in breast cancer and ovarian serous adenocarcinoma and are implicated in the pathogenesis of the disease (Bargou *et al.*, 1997) (Kamura *et al.*, 1999). Gel shift analysis using extracts from unstimulated and PMA/Ca^{2+} ionophore stimulated Jurkat T cells indicated that both the total level of CSD factors and the cytoplasmic to nuclear ratio of CSD factors did not change with stimulation. This suggests that the PMA/Ca^{2+} ionophore CSD mediated activation of the GM-CSF promoter was not due to increases in the level of CSD factors.

Co-transfection data also presented in chapter 3, showed that removal of the C-terminal protein:protein interaction domain of the CSD factor dbpB, resulted in a reduction of CSD mediated co-activation. This suggested that protein:protein interactions were a potential mechanism for CSD mediated activation of the GM-CSF promoter. The interaction of CSD factors with transcription factors has been implicated in the mechanism of activation of several viral and cellular promoters (Reviewed by (Shannon *et al.*, 2001)). Experiments designed to directly examine the effect of CSD/NF-κB interactions indicated that each of the CSD and NF-κB/Rel factors had different effects on each others binding. The CSD factor dbpAv acted to inhibit RelA binding to its double stranded DNA target while RelA had no effect on dbpAv binding to its single stranded target. In contrast, dbpB had no effect on RelA binding to its double stranded target, while RelA could readily inhibit dbpB binding to its single stranded target even after dbpB had already bound. Experiments where NF-κB p50 was mixed with CSD factors indicated that only dbpAv had an effect on NF-κB p50 binding to its double stranded target, whereas dbpB had no effect. NF-κB p50 had no effect on either CSD factors binding to their single stranded DNA target. Since RelA and CSD factors required double and single stranded binding sites respectively, the effect on each others binding was not one of simple binding site

competition, therefore suggesting a direct protein:protein interaction. Such a CSD/NF-κB interaction has also been described by Raj *et al.* (Raj *et al.*, 1996). One possible explanation for the differences seen in effect of RelA on dbpAv and dbpB binding and vice versa, could be attributed to the CSD C-terminal region. It has been shown that different regions of the CSD protein C-terminus can interact with various heterologous proteins, providing the option for CSD proteins to interact with a number of partners (Shnyreva *et al.*, 2000) (Li *et al.*, 1997) (Ansari *et al.*, 1999) (Safak *et al.*, 1999) (Ise *et al.*, 1999) (Moorthamer *et al.*, 1999). In this instance RelA may interact with different regions of the dbpAv and dbpB C-terminal regions. It is also possible that the C-terminus between the two CSD factors is sufficiently different for there to be a different affect on binding. Interactions between RelA and dbpAv effect RelA's binding but not dbpAv's suggesting that dbpAv may block or cover the DNA binding region of RelA, whereas RelA doesn't block dbpAv's DNA binding region. The opposite situation may apply to the dbpB and RelA interactions. While no evidence was provided here for a direct NF-κB/CSD protein:protien interaction Raj *et al.* have shown by co-immunoprecipitation experiments that recombinant RelA and CSD factors could interact (Raj *et al.*, 1996).

RelA and CSD factors were also co-transfected into Jurkat T cells to determine if the effect on each others binding had a downstream effect on transcriptional regulation of the GM-CSF promoter. Co-transfection of CSD factors and RelA indicated that they could co-operatively activate domain 1 of the GM-CSF proximal promoter, in the absence of PMA/Ca^{2+} ionophore stimulation. This co-operative activation was also dependent on the C-terminus of the CSD protein, again suggesting a protein:protein interaction. The ability of CSD and NF-κB/Rel factors to co-operate and activate transcription has also been demonstrated to be important for regulation of the JCV early and late viral genes (Raj *et al.*, 1996). The co-operative activation seen in the co-transfection experiments however, was inconsistent with the recombinant gel shift data, where neither of the CSD factors

acted to increase RelA binding to its double stranded DNA target. One possible explanation for this inconsistency may be the lack of post-translational modifications of bacterially expressed recombinant proteins. The post-translational modifications of proteins that interact with NF-κB factors have been shown to be important in regulating NF-κB function. For example, the IκB protein, IκB-β when un-phosphorylated maintains NF-κB binding to its target, whereas when IκB-β is phosphorylated it acts to dissociate NF-κB from its binding site, shuttling it out of the nucleus (Suyang *et al.*, 1996). A similar mechanism may apply for CSD/NF-κB interactions.

The ability of NF-κB/Rel and CSD factors to decrease each others binding may play an important role in the transition from activation to repression. Removing transcription activators like NF-κB/Rel factors from their double stranded DNA binding targets could be an important step in the transition from activated to repressed transcription states. Conversely removing CSD factors from their single stranded DNA binding sites, which has been implicated in transcriptional repression, could be an important step in transitions from repressed to activated states of transcription. The co-trasfection results suggest that once CSD factors have been removed from their single stranded DNA targets they then are able to function with RelA via protein:protien interactions to activate the GM-CSF promoter, indicating that CSD factors are able to play multiple roles in transcriptional regulation.

Chapter 8

Final Discussion

We all agree that your theory is crazy, but is it crazy enough?

Niels Bohr

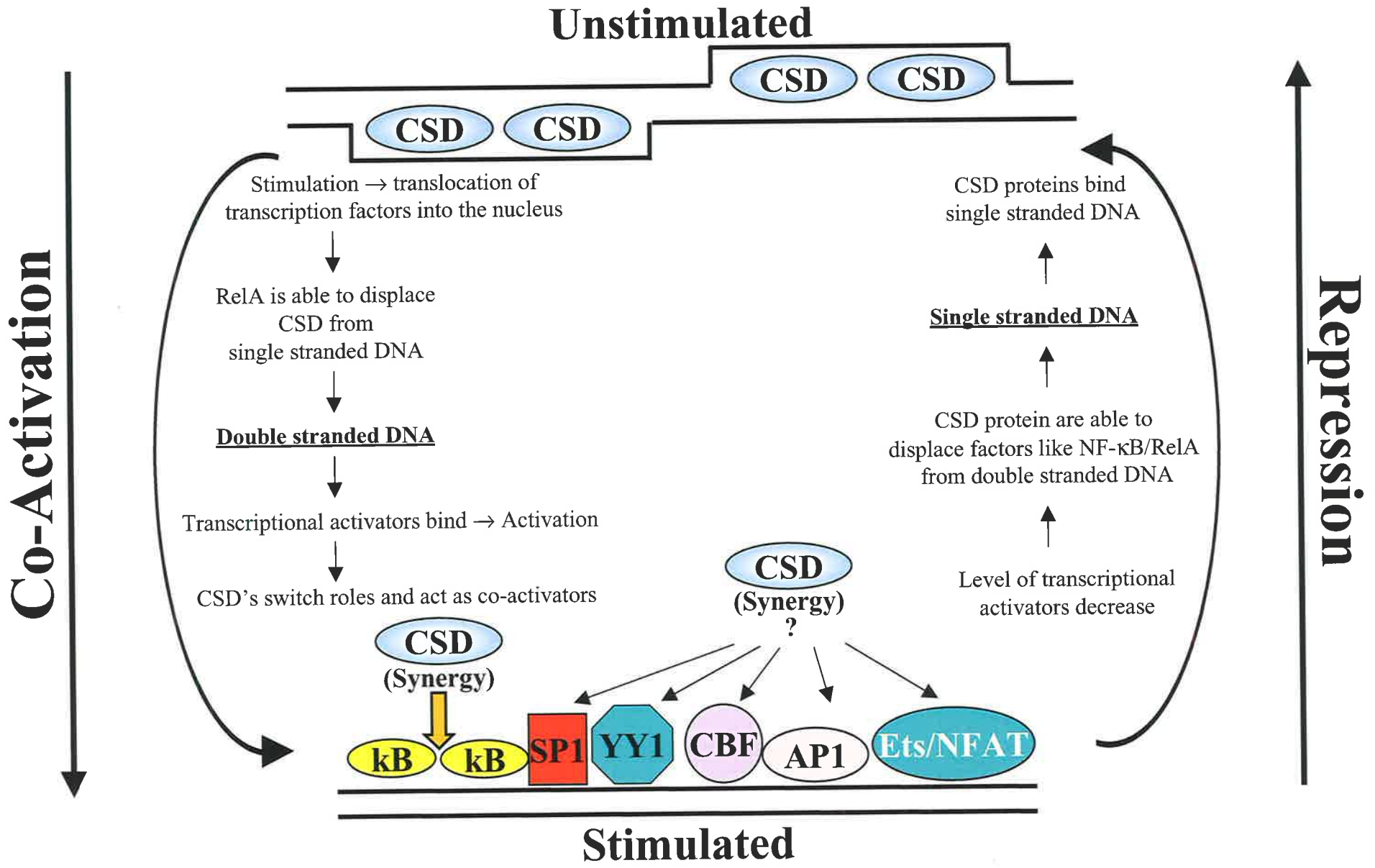
8.1 Final Discussion

Cold shock domain family members have been associated with diverse functions including the ability to both activate and repress transcription and translation. The main aim of the work presented in this thesis was to investigate the role of the CSD proteins dbpAv and dbpB in regulation of the GM-CSF promoter in HEL fibroblasts and Jurkat T cells. Work presented here and done previously in our laboratory has shown that HUT78 T cell and HEL fibroblast nuclear CSD proteins bind to defined single stranded regions along domain 1 and domain 2 of the GM-CSF promoter as NF-GMb and NF-GMc complexes (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). UV cross-linking experiments indicated that the NF-GMb complex contained 42, 25 and 22 kDa factors, whereas the NF-GMc complex contained only 25 and 22 kDa factors. Transient transfections in HEL fibroblasts showed that the CSD proteins, dbpAv and dbpB repressed the TNFα induction of the GM-CSF proximal promoter. Mutational analysis of the CSD binding sites in both domains of the GM-CSF promoter indicated that the CSD repression was mediated via DNA binding. This was confirmed by transient transfections where a dbpB deletion construct, removing the central CSD domain, was co-transfected with the GM-CSF promoter into HEL fibroblasts. This CSD/dbpB deletion lost the ability to repress GM-CSF transcription. The highly conserved central CSD domain has been shown to be involved in sequence specific DNA binding (Kolluri *et al.*, 1992) (Bouvet *et al.*, 1995) (Schroder *et al.*, 1995), and hence indicated that DNA binding was required for GM-CSF transcriptional repression in HEL fibroblasts.

It has been proposed that the binding of nuclear NF-GMb/c or recombinant CSD proteins along the GM-CSF promoter in HEL fibroblasts resulted in or stabilised a single stranded DNA structure, thereby preventing the binding of transcriptional activators that are dependent on double stranded DNA for binding and activity (Figure 8.1). This proposed structure would be an efficient means of silencing the GM-CSF promoter and

Figure 8.1

A potential model for transition between repression and activation states of GM-CSF transcription.


Unstimulated
CSD
CSD
CSD
CSD
Co-Activation
Repression
Stimulation → translocation of transcription factors into the nucleus
RelA is able to displace CSD from single stranded DNA
Double stranded DNA
Transcriptional activators bind → Activation
CSD's switch roles and act as co-activators
CSD (Synergy)
CSD (Synergy) ?
kB
kB
SP1
YY1
CBF
AP1
Ets/NFAT
CSD proteins bind single stranded DNA
Single stranded DNA
CSD protein are able to displace factors like NF-κB/RelA from double stranded DNA
Level of transcriptional activators decrease
Stimulated

many other promoters where NF-GMb/c CSD proteins have been shown to act as repressors of transcription (Coles *et al.*, 2000) (Ting *et al.*, 1994) (MacDonald *et al.*, 1995) (Saji *et al.*, 1997) (Ohmori *et al.*, 1996) (Sapru *et al.*, 1996) (Li *et al.*, 1997). The specific arrangement of NF-GMb/c CSD binding sites observed on the GM-CSF promoter, a pair of distal sites binding on the non-coding strand and a pair of proximal sites binding the coding strand, can also be found in the promoters of the granulocyte-colony stimulating factor and interlukin-3 cytokine genes, which have overlapping patterns of expression with GM-CSF (Nagata *et al.*, 1986) (Yang *et al.*, 1988).

Analysis of Jurkat T cell extracts identified nuclear CSD proteins binding to the CSD binding sites across the GM-CSF promoter as a NF-GMb-like complex, with no NF-GMc-like complex detected. Competitions with single stranded DNA, CSD site sequences and CSD antibody experiments in addition to UV cross-linking, indicated that the Jurkat T cell NF-GMb complex was made up of only a 42 kDa CSD protein. With the lack of the NF-GMc complex in Jurkat T cells a corresponding lack of the 22 and 25 kDa CSD proteins previously identified in HEL fibroblasts and HUT78 T cells was also observed (Coles *et al.*, 1994) (Coles *et al.*, 2000). These 22 and 25 kDa CSD proteins probably represent CSD splice variants or proteolytic cleavage products, as reported by others, which lack the C-terminal protein:protein interaction domain (Coles *et al.*, 1996) (Saji *et al.*, 1997) (Dhalla *et al.*, 1998) (Swamynathan *et al.*, 1997) (Kerr *et al.*, 1994) (Stenina *et al.*, 2000).

Transient transfections in Jurkat T cells showed that in contrast to the repression observed in HEL fibroblasts, the CSD proteins, dbpAv and dbpB, functioned as co-activators on this 140 bp region of the human GM-CSF proximal promoter. This activation was only observed when T cells were activated with PMA/Ca^{2+} ionophore. Experiments showed that both the GM-CSF domain 1 region (containing NF-κB/RelA sites) and the domain 2 region (containing the CBF, AP1 and ETS/NFAT sites) are able to

independently respond to CSD mediated activation. Experiments also showed that this response to CSD mediated activation did not require the CSD binding sites, at least in the –60 to +28 region of domain 2. The CSD binding sites, however, still retain repressor function as defined by deletion and mutation analysis.

CSD factors have been reported to activate a few cellular genes including c-myc, α1(I) procollagen and metaloproteinase (MMP-2) and also regulate many viral promoters including HIV LTR, HTLV LTR, RSV LTR and the JCV viral promoter (Kolluri *et al.*, 1992) (Dhalla *et al.*, 1998) (Mertens *et al.*, 1997) (Kashanchi *et al.*, 1994) (Swamynathan *et al.*, 1997) (Kerr *et al.*, 1994). To determine the mechanism of CSD mediated activation in Jurkat T cells experiments were performed to examine the possible role of CSD protein interactions with other transcription factors. The data indicates that CSD factors dbpB and dbpAv are able to act with RelA in transient transfections to co-operatively activate the GM-CSF promoter in the absence of PMA/Ca^{2+} ionophore stimulation. These results suggests that the co-activation of GM-CSF by CSD and RelA proteins is via protein:protein interactions. This was demonstrated by truncating dbpB, removing the C-terminus of the protein, which has been shown to be essential for CSD protein:protein interactions (Shnyreva *et al.*, 2000) (Li *et al.*, 1997) (Ansari *et al.*, 1999) (Safak *et al.*, 1999) (Ise *et al.*, 1999) (Moorthamer *et al.*, 1999). When the dbpB deletion construct removing the C-terminus was used, PMA/Ca^{2+} ionophore mediated co-activation was significantly reduced as was the ability of dbpB and RelA to co-operativetly activate, indicating that the co-activation was mediated primarily through the C-terminal region of the CSD protein and therefore most likely via protein:protein interactions. Raj *et al.* have found that CSD and RelA can interact in solution and that this interaction leads to increased RelA binding to the JCV viral promoter (Raj *et al.*, 1996) (Kerr *et al.*, 1994). In these experiments RelA conversely decreased CSD/dbpB binding to its single stranded DNA binding site. This CSD:RelA interaction was implicated in activation of the JCV

promoter (Kerr *et al.*, 1994). Similarly, experiments described here using recombinant proteins indicate that RelA can decrease CSD/dbpB binding to its GM-CSF single stranded DNA binding site. Conversely it was demonstrated here that CSD/dbpAv can decrease RelA binding to its GM-CSF double stranded DNA binding site. Unlike the experiments described by Raj (Raj *et al.*, 1996) no CSD mediated increase in RelA binding was observed. This was in contrast to the results shown in chapter 7, where CSD factors and RelA functioned to co-operatively activate the GM-CSF promoter in the absence of PMA/Ca^{2+} ionophore. The reason for this difference is unknown but may be due to the lack of post-translational modifications in bacterially expressed recombinant proteins. The mechanisms for the co-activation by RelA and CSD factors of the GM-CSF promoter is unclear but may require post-translational modifications of either CSD or RelA proteins to bring about effects on each others binding. Such effects may not be revealed by use of bacterial recombinant proteins, which are not modified.

Taken together our data suggests a model for CSD mediated regulation of GM-CSF transcription. Firstly in unstimulated Jurkat T cells, CSD proteins bind to single stranded DNA in a structure as described for the repression of the GM-CSF gene by CSD proteins in HEL fibroblasts (Figure 8.1) (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). This model has been suggested for a number of other genes repressed by CSD proteins (MacDonald *et al.*, 1995) (Ohmori *et al.*, 1996) (Horwitz *et al.*, 1994). Upon T cell stimulation (mimicked by PMA/Ca^{2+} ionophore) it has been well documented that many transcriptional activators (like NF-κB p50 and RelA) are upregulated. Binding experiments with Jurkat T cell cytoplasmic and nuclear fractions indicated, however, that there was no changes in the overall level of CSD proteins and subcellular localisation in response to PMA/Ca^{2+} ionophore stimulation (chapter 7 and reviewed in (Shannon *et al.*, 1995) & (Shannon *et al.*, 1997)). As experiments described here and those done by others show, RelA is able to dissociate dbpB from it's single stranded target (Raj *et al.*, 1996). It

is proposed that this interaction results in destabilisation of the single stranded DNA structure facilitating a return of the DNA to its double stranded conformation (Shannon *et al.*, 1997) (Coles *et al.*, 1996) (Coles *et al.*, 2000) (Shannon *et al.*, 2001). This would allow the transcriptional activators (like NF-κB, AP-1, CBF, ETS/NFAT) to bind initiating transcriptional activation and may aid in enhanceosome formation (Figure 8.1).

I have presented data here that indicates a functional CSD/RelA interaction, which is involved in activation of the GM-CSF promoter Domain 1 region. This requires CSD factors to have a dual function, swapping from repressors to co-activators. I have also shown CSD mediated activation via the domain 2 region that contains no RelA sites. Hence CSD proteins may also be able to interact with other transcription factors binding to this region. There is a growing list of proteins where interactions with CSD protein have been implicated to affect promoter function (NF-κB, mtSSB, ZO-1, hnRNPk, TBP, NF-Y, YY-1, AP-2, SP-1, Tat, PuRα) (Raj *et al.*, 1996) (Coles *et al.*, 2000) (Balda *et al.*, 2000) (Kolluri *et al.*, 1992) (Shnyreva *et al.*, 2000) (lloberas *et al.*, 1995) (Li *et al.*, 1997) (Mertens *et al.*, 1999) (Sawaya *et al.*, 1998) (Ansari *et al.*, 1999) (Chen *et al.*, 1995a). Potential targets, for CSD interactions on the GM-CSF promoter domain 2, could be SP1 and YY1, both of which have been shown to regulate GM-CSF function in T cells (Figure 8.1) (Shannon *et al.*, 1997) (Ye *et al.*, 1996) (Ye *et al.*, 1994).

While activation of gene transcription is obviously important in correct gene expression in response to stimulus, equally important is 'shutting off' and maintaining a transcriptionally silent state upon the withdrawal of stimulus. Withdrawal of the stimulus results in a decrease in the level of many transcriptional activators in the nucleus, but the level of CSD factors remains the same (reviewed in (Shannon *et al.*, 1995) & (Shannon *et al.*, 1997) and unpublished results). Binding experiments, discussed in chapter 7, indicate CSD factors are able to effect RelA binding to its double stranded DNA target, thereby displacing RelA and freeing overlapping CSD binding sites. CSD factors may be directly

involved in the formation of a single stranded structure across the promoter or may bind to a pre-existing structure to stabilise it. This may involve recruitment of chromatin remodelling machinery. This would therefore return the promoter to a transcriptionally repressed state (Figure 8.1).

The complement of CSD protein subtypes in the cell may also affect CSD function. Experiments show that in HEL fibroblasts (with 42, 22 and 25 kDa CSD proteins) that CSD proteins acted to repress activation, whereas in T cells (with only the 42 kDa protein) CSD factors function to activate, when the cells were PMA/Ca^{2+}ionophore stimulated. The reason for this difference between the cell types is as yet unclear, but may be related to the proposed lack of C-terminal protein interaction sequences in the 22 and 25 kDa CSD subtypes observed in HEL fibroblasts (Coles *et al.*, 1996) (Saji *et al.*, 1997) (Dhalla *et al.*, 1998) (Swamynathan *et al.*, 1997) (Kerr *et al.*, 1994) (Stenina *et al.*, 2000). Taking into account previously reported results and the reported function associated with each of the CSD protein domains, the 42 kDa CSD factor is implicated in repression (via the CSD domain) and activation (via the C-terminal domain) while the 22 kDa CSD factors may be involved only in repression (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). In fibroblasts, where CSD factors act to repress GM-CSF promoter function, UV cross-linking experiments indicated that the 22 kDa CSD protein sub-population makes up a greater proportion than the 42 kDa sub-population suggesting that the CSD intracellular environment is predisposed towards truncated forms of the CSD proteins and hence repression (Coles *et al.*, 1994) (Coles *et al.*, 1996) (Coles *et al.*, 2000). In Jurkat T cells, where CSD factors act to mediate activation of the GM-CSF promoter, only the full length 42 kDa CSD protein is present. The 42 kDa CSD protein while still being able to bind DNA and potentially repress in the unstimulated state, is also able to interact with proteins in a stimulated cell via the C-terminal protein:protein interaction domain to bring about activation. Therefore the differences in effect when full length CSD factors were

overexpressed in Jurkat T cells and HEL fibroblasts may be due to the intrinsic difference in the ratio of CSD factor subtypes and resulting intracellular environment already present in the two cell types.

In this thesis I have shown that CSD proteins can act to repress or activate the GM-CSF gene in different cell types. Repression is associated with the CSD binding sites located across the promoter whereas activation may relate to CSD co-operation with other transcription factors. I also raise the possibility that the cellular content of CSD proteins pre-determines the functional outcome in the transcription response.

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Corrections and discussion of examiners comments for the PhD thesis of Peter Diamond "Regulation of granulocyte macrophage-colony stimulating factor by cold shock domain proteins"

Typographical errors

Abstract, line 10: repressors should read repressor

P 10, line 5: focuses should read focused

p 65, line 9: duel should read dual

p67, line 15: gel shifts should read gel shift

p79, line 13: that should read than

Examiners Comments

Figure 4.5 A is the same gel as presented in Figure 4.2. This diagram is presented in Figure 4.5 A only to enable quick reference to the different NF-GMb/c complexes UV cross-linked in Figure 4.5 B.

GM-CSF gene expression has not been examined in HUT78 T cells and therefore no direct comparison can be made between NF-GMb/c complex formation and GM-CSF expression in HUT78 T cells, Jurkat T cells and HEL fibroblasts. HUT78 T cells are a tumour derived cell line which have not been extensively analysed for T cell receptor mediated gene expression, whereas Jurkat T cells have been extensively studied and are the model system for determining T cell receptor activation of gene expression.

Nuclear proteins prepared from HUT78 T cell by technique 1 and 2 gave different migrational patterns when used in gel shift assays (Chapter 4, p68). Despite the presence of these different conformational forms, UV cross-linking experiments indicated that the same CSD subtypes are present. There was not an obvious explanation for these observed results but potential reasons could include differences in salt and pH conditions between the two extraction techniques.

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An Ordered Array of Cold Shock Domain Repressor Elements across Tumor Necrosis Factor-responsive Elements of the Granulocyte-Macrophage Colony-stimulating Factor Promoter*

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The tumor necrosis factor-α-responsive region of the human granulocyte-macrophage colony-stimulating factor (GM-CSF) promoter (−114 to −31) encompasses binding sites for NF-κB, CBF, AP-1, ETS, and NFAT families of transcription factors. We show both here and previously that mutation of any one of these binding sites greatly reduces tumor necrosis factor-α induction of the GM-CSF promoter. Interspersed between these elements are sequences that when mutated lead to an increase in GM-CSF promoter activity. We have previously shown that two of these repressor elements bind proteins known as cold shock domain (CSD) factors and that overexpression of CSD proteins leads to repression of GM-CSF promoter activity in fibroblasts. CSD proteins are single strand DNA- and RNA-binding proteins that contact 5′-CCTG-3′ sequences in the GM-CSF repressor elements. We show here that two newly identified repressor sequences in the proximal promoter can also bind CSD proteins. We have characterized the CSD-containing protein complexes that bind to the GM-CSF promoter and identified a novel protein related to mitochondrial single strand binding protein that forms part of one of these complexes. The four CSD-binding sites on the promoter occur in pairs on opposite strands of the DNA and appear to form an ordered array of binding elements. A similar ordered array of CSD sites are present in the promoters of the granulocyte colony-stimulating factor and interleukin-3 genes, implying a common mechanism for negative regulation of these myeloid growth factors.

Granulocyte-macrophage colony-stimulating factor (GM-CSF)[1] is one of a family of hematopoietic growth factors that control the survival, proliferation, and differentiation of hemopoietic progenitor cells as well as the functional activation of mature cells. GM-CSF functions in particular to regulate hematopoietic cells of the myeloid lineage. GM-CSF expression is normally tightly regulated, and it is produced by a number of cell types following appropriate stimulation. These include myeloid, mesenchymal (fibroblast and endothelial cells), and lymphoid cells (1–4). Inappropriate or constitutive expression of GM-CSF is implicated in a number of disease states, including myeloid leukemia, prostate and colorectal cancers, arthritis, and asthma (1, 2, 5–9). Because the GM-CSF gene is primarily regulated at the level of transcription, it is important to investigate the mechanisms of repression as well as activation of this gene. Such studies are necessary to define the means by which the GM-CSF gene is maintained in a strictly silent state in the absence of stimulation and also to determine the mechanisms of rapid derepression of the gene and subsequent activation upon stimulation. This will then allow identification of defective regulatory pathways in diseases where GM-CSF disregulation is important.

Numerous transcriptional activators bind to and regulate the proximal GM-CSF promoter, which can be divided into two functional domains (see Fig. 1). Domain 1 (−114 to −71) contains the CK-1 and CK-2 elements conserved in a number of cytokine genes and binds a number of transcription factors including NF-kB, Sp1, and the CD28-responsive complex (1–4). This region is responsive to T cell receptor activators and costimulators (10–12), is involved in response to TNF-α in fibroblasts (13, 14), and is required for constitutive expression in juvenile myelomonocytic leukemia cells (15). The NF-kB site is critical for expression in these cell types. Domain 2 (−70 to −31) binds CBF, AP1, ETS, and NFAT transcription factors (10, 16–18). This region responds to TNF-α and interleukin (IL)-1 stimulation of fibroblast (13, 14) and endothelial (19) cells and T cell receptor activation (10, 16–18) and is required for constitutive expression in certain acute myeloid leukemia cell lines (20). The CBF, AP1, and ETS/NFAT transcription factor-binding sites have been shown to be essential for T cell receptor signaling in conjunction with the domain 1 NF-kB site (10, 16, 18), but it is not known which of these sites in domain 2 are required for activation in fibroblasts and endothelial cells.

In addition to the activators described above, we have identified nuclear complexes called NF-GMa, NF-GMb, and NF-GMc that bind to domain 1 of the GM-CSF promoter (13, 14, 21, 22). The NF-GMa complex was found to also bind to the CK-1/CK-2 equivalent regions of the genes for two other myeloid growth factors, granulocyte colony-stimulating factor (G-CSF) and IL-3 (23, 24). The GM-CSF gene is coordinately regulated with the G-CSF gene in fibroblasts (25) and with the IL-3 gene in T cells (2), respectively, in response to certain stimuli. This complex was found to be TNF-α-inducible in fibroblasts and was implicated in G-CSF promoter activation (23, 24, 26). The

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[1] The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF, tumor necrosis factor; CSD, cold shock domain; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; CAT, chloramphenicol acetyl transferase; HPV, human papillomavirus; mtSSB, mitochondrial single strand binding protein; HS, heparin-Sepharose.

protein composition of this complex was not determined. In contrast, the NF-GMb/c complexes are implicated in repression. These complexes bound to two repressor sites in domain 1 that were functional in fibroblasts (3, 4, 13, 14). NF-GMb contains two separate complexes, one containing a 42-kDa protein and the other containing a dimer of a 22-kDa protein, whereas NF-GMc represents the binding of a single 22-kDa protein. We identified these proteins as cold shock domain (CSD) proteins (4, 27–32) by cloning of factors contacting the repressor elements and by subsequent analysis of the NF-GMb/c complexes (4, 14). An interesting property of these proteins is that they bind to single-stranded DNA and in the case of the GM-CSF sites, to two repeated 5′-CCTG-3′ elements on the noncoding (−) strand of domain 1 (13, 14). CSD factors are expressed in all cell types, and consistently we have observed NF-GMb/c complexes in all cell types examined, including fibroblasts, endothelial cells, T cells, and myeloid cells (13, 14).[2] CSD factors in addition to binding to single strand DNA can bind to double strand DNA and RNA. By virtue of their varied binding activities, these proteins are observed to be involved in transcriptional repression and activation and also in translational regulation (27–32). In particular CSD factors appear to play a role in the strict regulation of expression of genes involved in growth regulation and stress responses (13, 14, 29, 33–36). Analysis of CSD protein function on hematopoietic growth factor genes is at present restricted to the GM-CSF gene, where we found that overexpression of recombinant CSD proteins led to repression of domain 1 activity (14). Surprisingly, overexpression of CSD proteins was also shown to directly repress domain 2 in the absence of a similar arrangement of CSD-binding sites (14). The reason for this repression was unknown.

We now report the identification of two new CSD sites across domain 2 of the GM-CSF promoter that function as repressor elements in fibroblasts. We also define the TNF-α-responsive sequences in domain 2 and find that they flank the CSD sites. The CSD-binding sites across domains 1 and 2 form an ordered regularly spaced array of repressor elements across the entire TNF-α-responsive proximal GM-CSF promoter. We also find a similar array of CSD sites across the promoter regions of the G-CSF and IL-3 genes. We performed a detailed analysis of the protein composition of NF-GMb/c and NF-GMa complexes and find that distinct nuclear complexes bind to the different CSD sites across the GM-CSF promoter and determine that NF-GMa is also composed of CSD proteins. We propose mechanisms by which the different CSD complexes regulate growth factor promoter expression.

EXPERIMENTAL PROCEDURES

Plasmid Constructs —The human GM-CSF promoter constructs pGM41 and pGM43 have previously been described and were constructed by cloning the oligonucleotides GM41 (−65 to −31) and GM43 (−114 to −31), respectively, into the pBLCAT2 reporter vector (13). The construct pGM93 (−70 to −31) and the mutant constructs pGMm89, pGMm87, pGMm81, pGMm85, and pGMm95 were constructed by cloning respective oligonucleotides (with *Hin*dIII 5′ and *Bam*HI 3′ ends) into pBLCAT2. Oligonucleotide sequences are shown in Fig. 1*c*. The bacterial expression vector pGEXBT contains the large *Eco*RI fragment from the B5 λgt11 DbpB CSD cDNA expression clone (14) inserted into pGEX-4T-1 (Promega). This construct expresses a protein lacking the last 10 amino acids of DbpB CSD protein. It has previously been demonstrated that these last 10 amino acids do not affect recombinant DbpB binding to single strand DNA (37, 38).

Oligonucleotides and Probe Preparation—All oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. Full-length oligonucleotides for retardations or cloning into reporter vectors (see Fig. 1, *b* and *c*) were purified from nondenaturing polyacrylamide gels (39). Single strand DNA probes for gel retardation assays were prepared by end-labeling coding (+) or noncoding (−) strand oligonucleotides with [γ-^{32}P]ATP and T4 polynucleotide kinase followed by gel purification.

Preparation of Recombinant Protein—The *Escherichia coli* strain JM109 transformed with pGEXBT was induced with isopropyl-1-thio-β-D-galactopyranoside to produce recombinant GST-DbpB fusion protein, which was purified on glutathione-Sepharose beads as described by the manufacturer (Promega).

Preparation of Nuclear Protein, Affinity Purification, and Protein Sequencing—Crude nuclear extracts were prepared from HUT78 T cells as previously reported by us for extraction of NF-GMb/CSD complexes (13, 21, 22). Extracts contain NF-GMb/c and NF-GMa binding activity (see Fig. 5*a*). Crude HUT 78 nuclear extracts were heparin-Sepharose (HS) enriched for either NF-GMb/c or NF-GMa complexes as described previously (22). HS fractions enriched for NF-GMb/c (HSGMb; see Figs. 3 and 5) contained no detectable NF-GMa, and conversely fractions enriched for NF-GMa (HSGMa; see Fig. 5) were free of NF-GMb/c (13, 21, 22). For affinity purification concentrated HSGMa protein in 0.5× TM buffer (22) containing a final concentration of 200 mM KCl and 10 μg/ml poly(dI-dC) was applied to a 1-ml DNA affinity column. DNA affinity chromatography was carried out as described (40) except that the ligated oligonucleotides were coupled to Affi-Gel 15 matrix (Bio-Rad) in 0.1 M Hepes, pH 7.5. The oligonucleotide contains the IL-3 CK-1/CK-2 region, which is homologous to the GM-CSF CK-1/CK-2 domain 1 region (24). Of the three myeloid growth factor genes, GM-CSF, G-CSF, and IL-3, we previously found that the IL-3 region has the best affinity for NF-GMa (24). Specifically bound protein was eluted from the column with TM buffer containing 1 M KCl and rerun on a second affinity column. Protein eluates were monitored by both gel retardation assays and SDS-polyacrylamide gel electrophoresis. The 16-kDa affinity purified protein was transferred to polyvinylidene fluoride membrane, eluted, and analyzed by microsequencing (R. Simpson, Walter and Eliza Hall Institute, Melbourne, Australia).

Gel Retardation Analysis and UV Cross-linking—Gel retardation assays were performed using 0.25 ng of single strand ^{32}P-labeled oligonucleotide probe in a 10-μl reaction mix of 0.5× TM buffer (13, 14, 22) containing 200 mM KCl, 0.4 μg of poly(dI-dC) and either 0.2 μg of HS-enriched extract (HSGMa or HSGMb), 1.0 μg of crude nuclear extract, 25 ng of recombinant CSD fusion protein (GST-DbpB), or 1 ng of affinity purified material. Retardation assays using recombinant protein also contained 2 μg of bovine serum albumin. Reactions were incubated at room temperature for 20 min and analyzed on 12% nondenaturing polyacrylamide gels in 0.5× TBE (21). Competition with unlabeled single strand oligonucleotides was performed by addition of protein and unlabeled probe, followed by immediate addition of the ^{32}P-labeled probe (14).

For UV cross-linking, crude nuclear extracts were bound to ^{32}P-labeled single strand DNA probes in a 25-μl retardation reaction and fractionated on a polyacrylamide gel as described above. The gel was exposed to UV light (340 nm) for 15 min, and retarded complexes were excised after exposure to x-ray film. Protein in excised bands was analyzed on 12% SDS-polyacrylamide gels (13, 39).

Cell Culture and Transfections—Human embryo lung fibroblasts (Commonwealth Serum Laboratories) were grown in Dulbecco's modified Eagle's medium and 10% fetal calf serum. These cells were used for passages 14–20 in all experiments. Human embryo lung fibroblasts were cotransfected with 15 μg of reporter constructs using DEAE-dextran as described (13, 14). 24 h following transfection, cells were stimulated with TNF-α (100 units/ml) or left untreated for an additional 24 h. Cells were then harvested and CAT assays were performed (13, 14). The percentage of [^{14}C]chloramphenicol conversion to acetylated forms via CAT activity in extracts was determined using PhosphorImager analysis (Molecular Dynamics).

RESULTS

Identification of Overlapping TNF-responsive Elements and Repressor Elements in the GM-CSF Domain 2 Region—We previously reported that a domain 2 reporter construct (pGM41, −65 to −31; Ref. 14) was responsive to TNF-α in fibroblasts and that this activity was repressed by overexpression of the CSD proteins, DbpB and DbpA, that were cloned as GM-CSF domain 1 repressor site-binding proteins (14). To define the sequences responsible for activation and repression, mutations were made in the pGM41 construct (Fig. 1*c*) and transfected into human embryo lung fibroblasts, and cells were

[2] L. S. Coles, P. Diamond and M. F. Shannon, unpublished data.

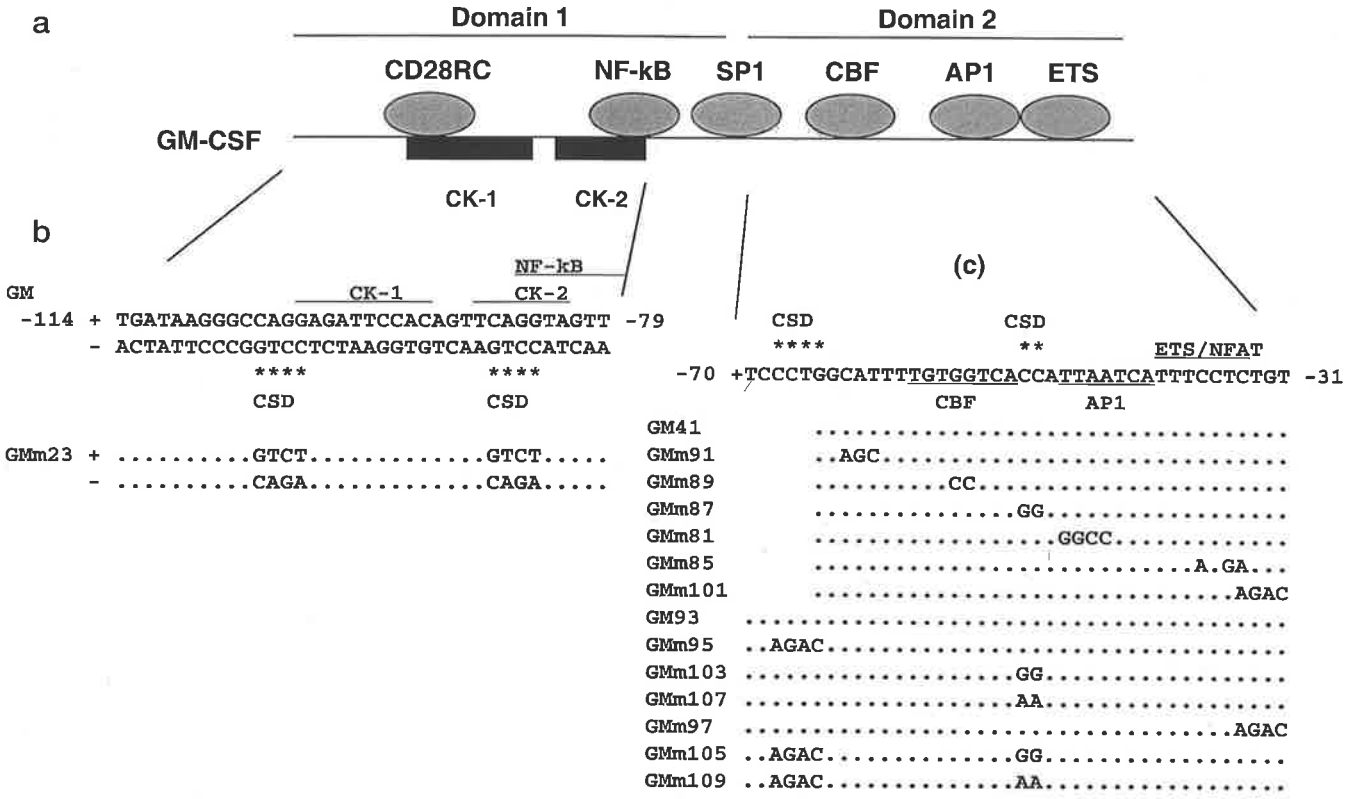

FIG. 1. **Human GM-CSF proximal promoter oligonucleotide sequences.** *a*, the GM-CSF proximal promoter is shown diagrammatically. The relative locations of conserved CK-1 and CK-2 elements and transcription factor-binding sites are indicated. The domain 1 (−114 to −71) and domain 2 (−70 to −31) regions are marked. The NF-kB site and the domain 2 region are responsive to TNF-α in fibroblasts (13, 14). The NF-kB, CBF, AP1, and ETS/NFAT sites are required for T cell receptor signaling, and the CD28-responsive complex site is required for T cell costimulatory signaling (3, 4). *b*, the sequence of coding (+) and noncoding (−) strand wild type domain 1 CK-1/CK-2 region (−114 to −79) oligonucleotides (GM+ and GM−, respectively) are shown (13, 14). Sequences required for nuclear (NF-GMb/c) and recombinant CSD factor binding to the noncoding (−) strand are marked with *asterisks*. These sequences are repressor elements in fibroblasts (13, 14). Base changes in the mutant GMm23 oligonucleotides are shown (13, 14). *c*, the sequence of the wild type coding (+) strand of domain 2 (−70 to −31) is given with CSD (data presented here), CBF, AP1, and ETS/NFAT sites (3, 4) marked. Nuclear and recombinant CSD binding is exclusive to the coding (+) strand. Coding (+) strand oligonucleotide sequences are listed under the domain 2 sequence. Only those bases that vary from the wild type sequence are indicated.

treated with TNF-α or left untreated (Fig. 2). Mutations included specific base changes previously shown to disrupt CBF, AP1 and ETS/NFAT binding. Mutation of the CBF, AP-1, or ETS/NFAT elements reduced both basal and TNF-α-induced activity (Fig. 2). A repressor element was also identified. Mutation of a 5′-CC-3′ (pGMm87) within a 5′-ACCA-3′ sequence located between the CBF and AP1 sites resulted in a 50% increase in basal and TNF-α-induced expression. An extended construct (pGM93) containing an extra five bases with a 5′-CCTG-3′ sequence identical to the domain 1 CSD-binding sites was also analyzed (Fig. 2). The extended sequences caused a decrease in TNF-α-inducible and basal expression. Mutation of the 5′-CCTG-3′ element in this extended construct (pGM95) restored promoter expression, identifying this site as a second repressor element. Hence domain 2 contains at least three sites required for TNF-α response, and these are overlapped/flanked by two repressor elements.

Nuclear and Recombinant CSD Proteins Bind to Repressor Elements across Domain 2 on the Opposing Strand to CSD-binding Sites Identified on Domain 1—To determine whether the repressor elements identified were CSD-binding sites, domain 2 single strand wild type and mutant oligonucleotides (Fig. 1*c* and Refs. 13 and 14) were analyzed in gel retardation assays for binding with HUT78 T cell extracts enriched for NF-GMb and NF-GMc CSD-containing nuclear complexes (HS-GMb) (Fig. 3*a*). Binding was compared with the GM- oligonucleotide (Fig. 3*a*, *lane 1*), which contains the noncoding (−) strand of the GM-CSF domain 1 CK-1/CK-2 region (−114 to −79) (Fig. 1*b*) and supports NF-GMb and NF-GMc complex formation. NF-GMb complex formation represents protein binding to both the 5′-CCTG-3′ CSD repressor sites, whereas NF-GMc represents binding to only one site on the GM- oligonucleotide (Fig. 3*a*, *lane 1*) (13, 14). The domain 2 GM93 coding (+) strand oligonucleotide containing the two newly identified repressor elements supports both NF-GMb and NF-GMc-like complex formation while the GM41(-65 to -31) and GMm95 (-70 to -31; 5′ repressor site mutated) coding (+) strand oligonucleotides, containing only one repressor element, form only the NF-GMc-like complex (Fig. 3*a*, *lanes 11*, *7*, and *15*, respectively). No complex formation was observed on noncoding (−) strand domain 2 sequences (data not shown). Competition assays verified that the complexes forming on domain 2 were authentic NF-GMb/c complexes. As shown in Fig. 3*a*, the NF-GMb/c complexes formed on domain 2 coding (+) strand oligonucleotides (GM41+, GM93+, and GMm95+) were competed to a much greater extent by the wild type GM-CSF domain 1 noncoding (−) strand oligonucleotide (GM−) (*lanes 8*, *12*, and *16*) than by the GMm23− oligonucleotide (*lanes 9*, *13*, and *17*) containing mutations in both the CK-1/CK-2 region NF-GMb/CSD sites (13, 14). Consistent with these results the NF-GMb/c complexes on GM− were readily competed with the domain 2 oligonucleotides (*lanes 4–6*). These data mapped one NF-GMb/c site to the 5′ repressor site in domain 2 and the other to the −65 to −31 region containing the 3′ repressor site.

Subsequent analysis of mutants in the GM41+ sequence (−65 to −31) mapped the second NF-GMb/c site to the 3′

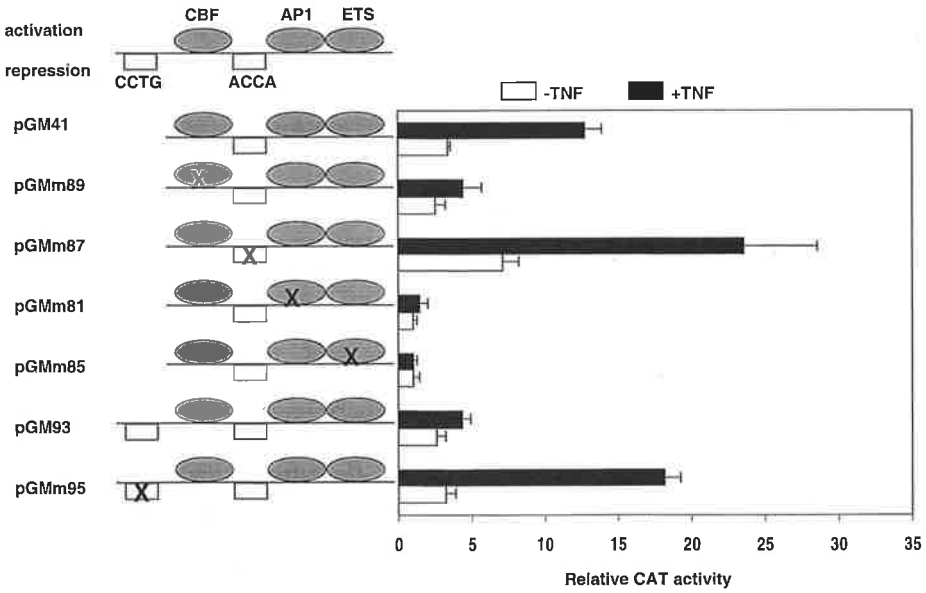

FIG. 2. **Identification of TNF-responsive elements and repressor elements in the GM-CSF domain 1.** Wild type (pGM41 and pGM93) and mutant (pGMm89, pGMm87, pGMm81, pGMm85, and pGMm95) GM-CSF promoter reporter constructs were transfected into human embryo lung fibroblasts, followed by treatment with (+) or without (−) TNF-α and CAT activity determined. CAT activity levels (average of at least three experiments) relative to unstimulated pBLCAT2, given as 1.0, are shown. Promoter constructs are shown diagrammatically. Repressor and activator elements are marked with *boxes* and *circles*, respectively. Sequences contained within promoter constructs are given in Fig. 1*c*.

repressor site, identified above, as mutation of the 5′-CC-3′ within the 5′-ACCA-3′ repressor element (GMm87+) abolished complex formation (Fig. 3*b*, *lane 4*). This type of binding site for CSD factors has only been reported for a viral gene (41, 42) and has not been reported in a genomal gene. No other mutations affected NF-GMc complex formation, but there were shifts in mobility on the different mutant sequences. Competition with the wild type GM41+ oligonucleotide indicated that the complexes forming on these mutant sequences were authentic NF-GMc complexes (data not shown). This suggests therefore that NF-GMc may have a different conformation on the different mutant sequences and that the way NF-GMc complex formation occurs depends not only on the complexes binding site but also on the nature of surrounding sequences. To further confirm the requirement for both repressor elements in the formation of NF-GMb/c, mutations were made in one or the other or both sites across the extended GM93+ sequence (−70 to −31) (Fig. 3*c*). Mutation of any of the sites (GMm95+, GMm103+, or GMm107+) resulted in loss of the NF-GMb complex but did not result in loss of the NF-GMc complex on GMm95+ (5′-CCTG-3′ site mutated), and caused some reduction in NF-GMc on GMm103+ and GMm107+ (5′-ACCA-3′ mutated) (*lanes 2–4*). Mutation of both sites (GMm105+ and GMm109+) resulted in loss of all complex formation (*lanes 6* and *7*). These data are consistent with the way we observed NF-GMb/c complex formation on the domain 1 region where NF-GMb complex formation requires both sites for binding, whereas NF-GMc complexes can form on either CSD site (13, 14).

We have previously demonstrated the binding of recombinant CSD protein to domain 1 NF-GMb/c sites (14). Domain 2 oligonucleotides were also tested for binding of recombinant CSD (Fig. 3*d*). As for nuclear NF-GMb/c CSD-containing complexes, recombinant GST-DbpB CSD protein binds exclusively to the coding (+) strand of domain 2 (GM41+ and GM93+; *lanes 3* and *6*) and requires the NF-GMb/c sites for binding. As shown, mutation of the single 5′-ACCA-3′ binding element in the GM41+ oligonucleotide essentially abolished CSD binding (GMm87+; Fig. 3*d*, *lane 5*). When the longer fragment containing two NF-GMb/c sites (GM93+) was used in binding, mutation of the individual repressor elements (GMm95+ and GMm103+) reduced binding, whereas mutation of both sites (GMm105+) completely abolished binding (Fig. 3*d*, *lanes 8–10*). Consistent with our observations for nuclear NF-GMb/c complexes, the binding of recombinant CSD protein to the mutant sequences results in altered mobility. Binding data for nuclear and recombinant CSD protein are summarized (Fig. 3*d*). Therefore, as observed for domain 1, we have shown that domain 2 contains a pair of repressor elements that bind both nuclear and recombinant NF-GMb/CSD factors, and we have identified a novel 5′-ACCA-3′ NF-GMb/CSD-binding site.

Analysis of Nuclear NF-GMb/c CSD-containing Nuclear Complexes Reveals a Novel 25-kDa Protein Binding to the 3′ Domain 1 5′-ACCA-3′ Repressor Element—The nature of the proteins in CSD-containing nuclear complexes binding to domain 2 were examined by UV cross-linking (Fig. 4*a*). As previously reported (13), the NF-GMb complex forming on domain 1 (GM- oligonucleotide) contains both a 42- and a 22-kDa protein (*lane 1*), whereas the NF-GMc complex contains only a 22-kDa protein (*lane 2*). The NF-GMb and NF-GMc complexes formed on domain 2 (GM93+) also contained 42- and 22-kDa proteins, but in addition these complexes contain a unique 25-kDa protein (*lane 3* and *4*) (Fig. 4*a*). To determine which of the NF-GMb/CSD sites binds this new protein, UV cross-linking was performed using mutants in domain 2. This revealed that the 22-kDa protein bound to the 5′ NF-GMb/CSD site in domain 2 (GMm103+), whereas the 25-kDa protein bound to the 3′ site (GMm95+) (Fig. 4*b*, *lane 3* and *4*, respectively). Hence the distal three NF-GMb/CSD sites across the GM-CSF promoter with a 5′-CCTG-3′ consensus bind the 22-kDa protein, whereas the newly identified 5′-ACCA-3′ site binds the novel 25-kDa protein. In contrast, the 42-kDa protein, as we have previously shown on domain 1 (13), requires both domain 2 sites for binding (Fig. 4*b*, compare *lanes 1*, *3*, and *4*). Data are summarized diagrammatically in Fig. 4*b*. The nature of the 25-kDa protein is not known, but we have confirmed that this protein is a CSD factor by competition of the GMm95+ complex with a CSD polyclonal antibody.[2] Given the differences in CSD protein composition of complexes binding to 5′-CCTG-3′ and 5′-ACCA-3′ CSD sites, it is of interest that the GM- oligonucleotide (binding 42- and 22-kDa proteins) can compete for the GMm95+ complex (25 kDa). This is most probably due to the ability of all nuclear CSD-binding oligonucleotides to bind all CSD subtypes at the amounts of competitor required to observe a competition effect. That this is possible is suggested by experiments competing the GMm95+ complex (25 kDa) with an oligonucleotide that only binds the 22-kDa protein (GMm103+). We found that over a wide range of competitor amounts GMm103+ could compete for GMm95+ complex for-

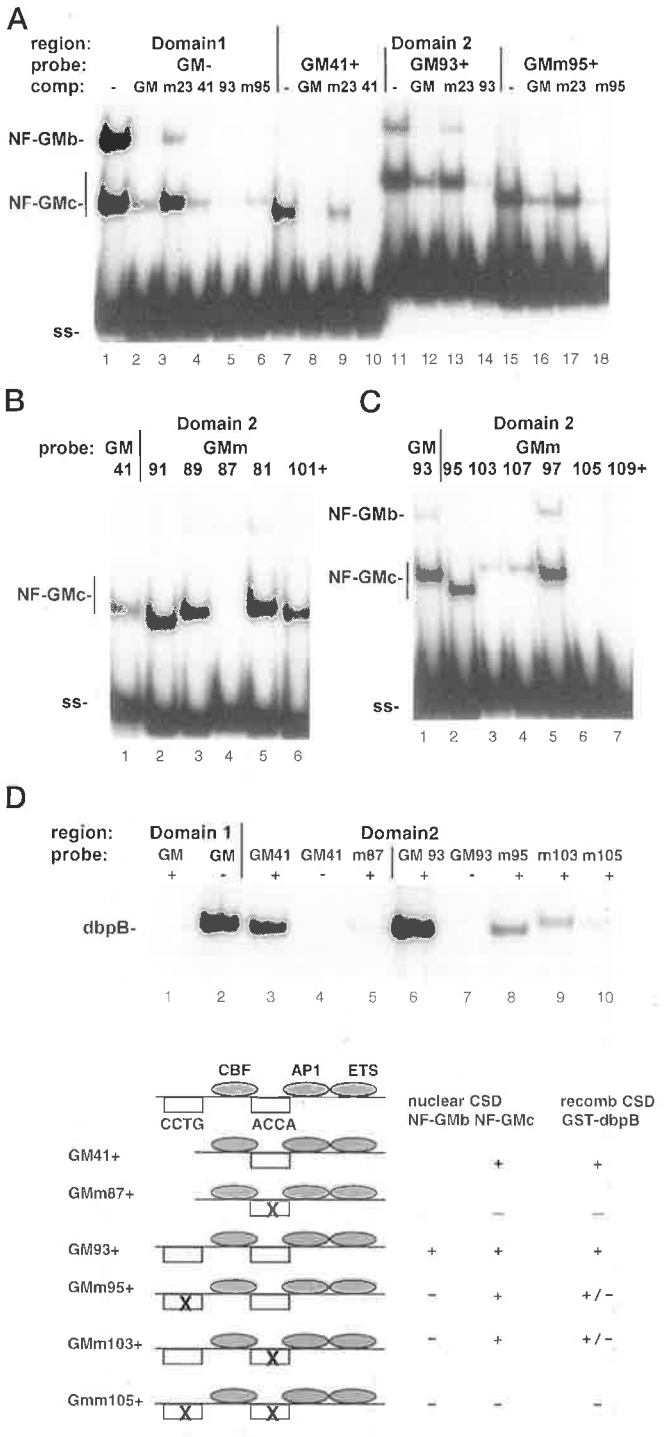

FIG. 3. **Nuclear and recombinant CSD proteins bind to repressor elements on the coding (+) strand of domain 2.** *a*, HUT78 T cell nuclear extract enriched for NF-GMb/c complexes by heparin-Sepharose chromatography (HSGMb) was bound to a ^{32}P-labeled wild type domain 1 (GM−) single strand oligonucleotide probe or to domain 2 wild type (GM41+ and GM93+) and mutant (GMm95+) probes. Complexes were competed (*comp*) with 5 ng of unlabeled wild type (GM−) and mutant (GMm23−) domain 1 oligonucleotides or with domain 2 oligonucleotides (GM41+, GM93+, and GMm95+). Tracks with no competitor are marked with a *minus sign*. *b*, HUT 78 T cell nuclear extracts enriched for NF-GMb/c (HSGMb) were bound to wild type (GM41+) and mutant (GMm series; Fig. 1*c*) domain 2 coding (+) strand oligonucleotides. *c*, HUT 78 T cell nuclear extracts enriched for NF-GMb/c (HSGMb) were bound to wild type (GM93+) and mutant (GMm series; Fig. 1*c*) domain 2 coding (+) strand oligonucleotides. NF-GMb and NF-GMc complexes and free oligonucleotide (*ss*) are marked. *d*, the bacterially expressed CSD fusion protein (GST-DbpB) was bound to wild type coding (+) and noncoding (−) domain 1 (GM) and domain 2 (GM41 and GM93) oligonucleotides and also to coding (+) strand oligonucleotides containing mutant domain 2 NF-GMb/CSD (GMm95, 103, 105)-binding sites. Binding of nuclear and recombinant CSD proteins to domain 2 sequences is summarized.

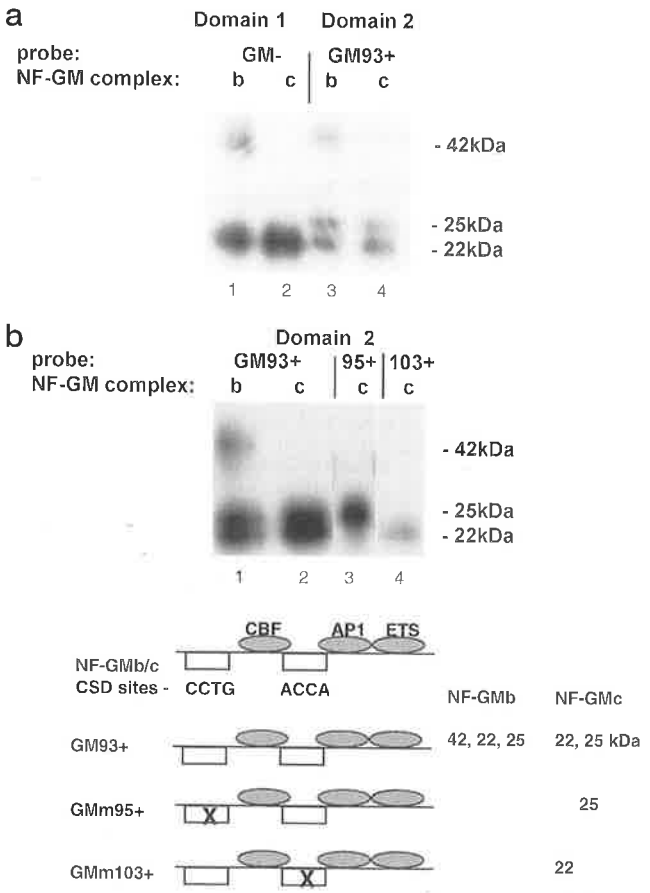

FIG. 4. **Domain 2 nuclear CSD complexes contain a novel 25-kDa protein that contacts the 3′ CSD repressor site.** *a*, NF-GMb and NF-GMc complexes formed after binding of HUT78 T cell nuclear extracts enriched for NF-GMb/c complexes (HSGMb) to domain 1 (GM−) and domain 2 (GM93+) oligonucleotides were irradiated with UV light and analyzed by SDS-polyacrylamide gel electrophoresis. *b*, UV cross-linked complexes formed on domain 2 wild type (GM93+) and NF-GMb/c-binding site mutant (GMm95+ and GMm103+) oligonucleotides are shown. The sizes of individual proteins cross-linked to DNA are indicated. Protein binding to domain 2 sequences is summarized.

mation but that this competition was less efficient than competing with the self GMm95+ sequence. The other explanation for cross-competition between sequences binding the different 25- and 22-kDa CSD proteins is that the 25-kDa protein represents the binding of the 22-kDa protein to the 5′-ACCA-3′ sequence in altered conformation relative to the way it binds to the 5′-CCTG-3′ sequence. This is feasible given the mobility shifts seen for nuclear NF-GMb/c and recombinant CSD complexes observed on different domain 2 mutant sequences as discussed above (Fig. 3).

NF-GMa Represents a Higher Order Complex of CSD Proteins Binding to Single Strand DNA—Studies performed above used extracts that were heparin-Sepharose enriched for NF-GMb/c binding activity. We have found that binding of crude extract to the domain 1 noncoding (−) strand oligonucleotide (GM−) reveals, in addition to NF-GMb/c, the binding of a more slowly migrating complex that we previously called NF-GMa (Fig. 5*a*, *lane 1*) (21, 22). This complex also binds to the CK-1 regions of two other myeloid growth factor genes, G-CSF and IL-3, and it was found to be TNF-α-inducible in fibroblasts (23, 24, 26). As for NF-GMb/c, NF-GMa binding activity could be

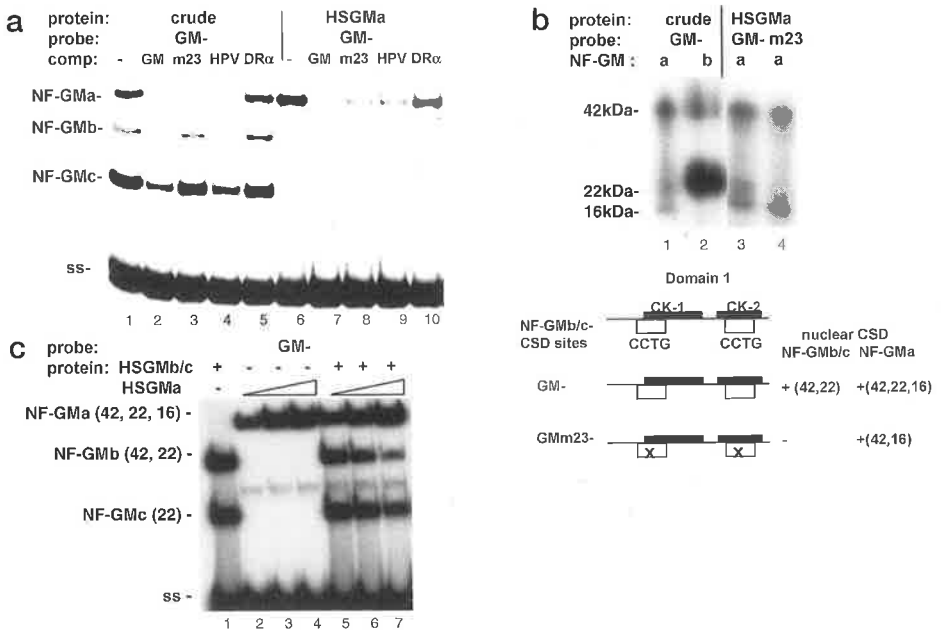

FIG. 5. **NF-GMa contains 42- and 22-kDa nuclear CSD proteins and a novel 16-kDa protein.** *a*, HUT78 T cell crude nuclear extract (*crude*) and extract enriched for NF-GMa (HSGMa) were bound to ^{32}P-labeled GM− oligonucleotide probe and competed (*comp*) with unlabeled single strand self (GM−) oligonucleotide, NF-GMb/CSD-binding site mutant (GMm23−), the CSD-binding site sequence from the HPV18 enhancer (*HPV*; Ref. 43), and a control sequence from the major histocompatibility complex DRα gene (*Drα*; Ref. 79). HPV and DRα represent the coding (+) strands of their respective promoter regions (14). Nuclear complexes are indicated. *b*, HUT78 T cell crude nuclear extracts (*crude*) and HUT78 extract enriched for NF-GMa by heparin-Sepharose chromatography (*HSGMa*) were bound to GM− and GMm23− domain 1 oligonucleotides, and the resulting nuclear complexes were analyzed by UV cross-linking. The sizes of cross-linked proteins are indicated. Data are summarized below the cross-linking gel. *c*, nuclear extract enriched for NF-GMb/c (*HSGMb*) and NF-GMa (*HSGMa*) were bound to labeled domain 1 (GM−) oligonucleotide either alone (*tracks 1–4*) or together (*tracks 5–7*). Increasing amounts of HSGMa were used (*tracks 2–4* and *5–7*). Complexes are indicated.

enriched from crude extracts and separated from NF-GMb/c activity by heparin-Sepharose chromatography (21, 22). Given the overlapping binding sites of NF-GMb/c and NF-GMa complexes, it was possible that NF-GMa was a higher order complex of CSD proteins or that it could act as a competitor for NF-GMb/c binding. To determine the relationship of NF-GMa and NF-GMb/c, NF-GMa was further investigated.

Competition experiments were carried out to determine the requirements for NF-GMa binding to the domain 1 CK-1/CK-2 region GM− oligonucleotide. As shown in Fig. 5*a*, the NF-GMa complexes from crude nuclear extracts (*lanes 1–5*) or extracts enriched for NF-GMa (HSGMa; *lanes 6–10*) were competed by a control CSD-binding site oligonucleotide from the coding (+) strand of the HPV18 enhancer (HPV+; *lanes 4* and *9*). This oligonucleotide competes for NF-GMb/c complexes and has been shown to be a good binding site for recombinant CSD proteins (14, 43). The NF-GMa complex is not competed by a sequence (DRα+; *lanes 5* and *10*) that we and others have found is unable to bind nuclear or recombinant CSD factors (14, 37, 38). As for NF-GMa, the NF-GMb/c complexes are not competed by this sequence (Fig. 5*a*, *lane 5*) (14). Even though NF-GMa and NF-GMb/c complexes show the same binding characteristics to the control CSD-binding sequence (HPV+), the NF-GMa complex does not appear to require the NF-GMb/CSD-binding sites defined in the GM− sequence. This is demonstrated by the ability of the GMm23− mutant (both 5′-CCTG-3′ sites mutated) to compete for NF-GMa complex formation (Fig. 5*a*, *lanes 3* and *8*) but not for NF-GMb/c complexes as described previously (Fig. 5*a*, *lane 3*, and Refs. 13 and 14); hence NF-GMa readily binds this sequence.

UV cross-linking revealed that the NF-GMa complex formed from both crude nuclear extract (crude) and extract heparin-Sepharose enriched for NF-GMa (HSGMa) on the domain 1 GM- oligonucleotide, contained 42- and 22-kDa proteins, similar in size to that observed in the NF-GMb complex, as well as a new 16-kDa protein (Fig. 5*b*, *lanes 1–3*). The NF-GMa complex formed on GMm23− was also analyzed by UV cross-linking (Fig. 5*b*, *lane 4*). NF-GMa on the mutant sequence contains the 42- and 16-kDa proteins but not the 22-kDa protein. This implies that the 42-kDa protein forms part of the NF-GMa complex without the need for binding to the CSD repressor sites but that the 22-kDa protein is dependent on these sites. These results imply that at least three CSD-containing complexes, NF-GMa, NF-GMb, and NF-GMc, can form on the GM-CSF domain 1 each with distinct sequence requirements for binding. A similarly migrating complex to NF-GMa was observed on domain 2, but cross-linking revealed the presence of a single 36-kDa protein (data not shown). The NF-GMa complex is therefore specific to the domain 1 CK-1/CK-2 region and does not form on domain 2.

As well as changing nuclear CSD factor DNA binding characteristics, it was possible that NF-GMa could compete with NF-GMb/c repressor complexes for binding to domain 1. This was examined in a retardation assay where addition of increasing amounts of heparin-Sepharose enriched NF-GMa (HSGMa) to enriched NF-GMb/c (HSGMb) resulted in inhibition of NF-GMb/c complex formation (Fig. 5*c*). This effect was prevented by inclusion of a competing GMm23− oligonucleotide that will titrate out NF-GMa but not NF-GMb/c complexes (data not shown). Hence the effect on the NF-GMb/c complexes was due specifically to the addition of NF-GMa. It appears therefore that NF-GMa may play a dual role in modulating CSD repressor function by competing with repressive NF-GMb/c complexes for binding to repressor elements and by altering the DNA binding characteristics of the 42-kDa protein so that it no longer contacts the domain 1 repressor elements.

Identification of the 16-kDa NF-GMa Component—To further characterize the 16-kDa component of the NF-GMa com-

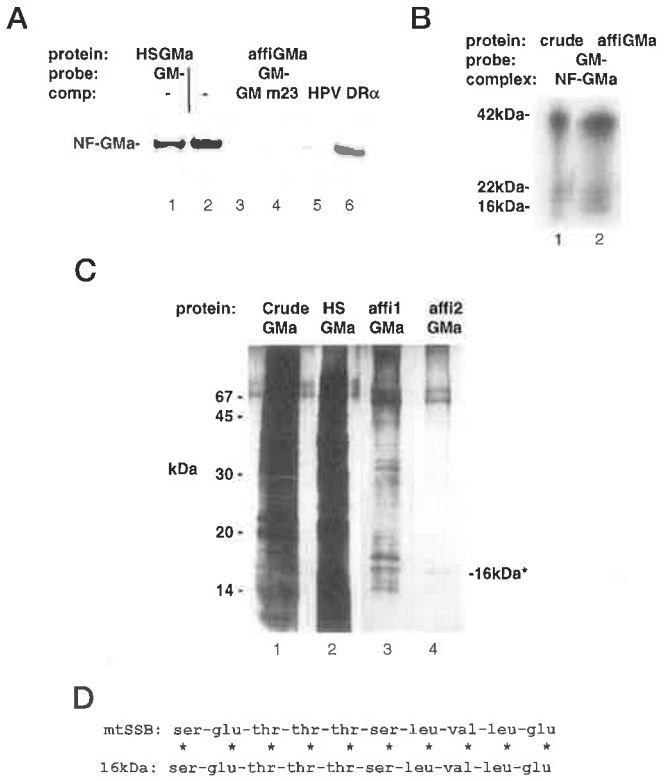

FIG. 6. **Characterization of the 16-kDa component of NF-GMa.** *a*, HUT78 T cell nuclear extracts enriched for NF-GMa (*HSGMa*) and affinity purified NF-GMa (*affiGMa*) were bound to labeled GM-CSF domain 1 noncoding (−) strand oligonucleotide (GM−) and assayed by gel retardation. The apparent NF-GMa complex formed using affinity purified NF-GMa material was competed with itself (GM−), the CSD site mutant (GMm23−), the control CSD-binding site (*HPV*+; Refs. 14 and 43), and the nonspecific DRa coding (+) strand oligonucleotides (Refs. 14 and 79 and Fig. 5). A *minus sign* indicates no competitor. *b*, the NF-GMa complexes from crude and affinity purified (*affiGMa*) HUT78 extracts were analyzed by UV cross-linking. The size of cross-linked proteins is indicated. *c*, SDS-polyacrylamide gel electrophoresis of protein fractions from different steps of the NF-GMa purification. Tracks were loaded with crude, heparin-Sepharose (*HSGMa*), first round affinity (*affi1 GMa*), or second round affinity (*affi2 GMa*) material. Protein was visualized by silver staining. Positions of molecular mass markers are shown. The 16-kDa protein is indicated. *d*, a comparison of the N-terminal amino acid sequences of the mature human mtSSB and purified 16-kDa protein are shown. Because the nature of the first amino acid from the N terminus of the 16-kDa protein could not be determined, the presented sequence commences at the second amino acid of both the 16-kDa and mtSSB proteins.

plex, DNA affinity chromatography was performed. To do this HUT78 T cell nuclear extract enriched for NF-GMa complex formation (HSGMa) was subjected to two rounds of affinity purification using the IL-3 CK-1/CK-2 region as a target for NF-GMa complex formation. The IL-3 CK-1/CK-2 region shares homology with the GM-CSF domain 1 CK-1/CK-2 region (see Fig. 7, *a* and *b*) and was shown to have a higher affinity for NF-GMa (24). The specifically bound protein was eluted in 1.0 M KCl. NF-GMa activity was assayed by gel retardation using the GM-CSF domain 1 noncoding (−) strand oligonucleotide. As shown in Fig. 6*a* the affinity purified material forms a complex (*lane 2*) that migrates at the same position as the NF-GMa complex formed from binding of the heparin-Sepharose purified material (HSGMa) (*lane 1*). Consistent with this complex being authentic NF-GMa, the affinity purified complex was competed by the GM-, GMm23−, and HPV+ sequences but not by the DRα sequence (compare Fig. 6*a*, *lanes 3–6*, with Fig. 5*a*). The complex also contained the appropriate 42-, 22-, and 16-kDa proteins expected for NF-GMa as determined by UV cross-linking (Fig. 6*b*). Analysis of second round affinity purified NF-GMa on an SDS-polyacrylamide gel revealed the presence of a 16kDa protein band after silver staining (Fig. 6*c*, *lane 4*). The 42- and 22-kDa proteins were, however, not visible. This protein was isolated from the gel and prepared for microsequencing. A sequence of 10 amino acids was obtained from the N terminus of the protein (Fig. 6*d*). Data base searches revealed that the sequence matched the N-terminal sequence of mature human mitochondrial single strand binding protein (mtSSB) (44). The mature human mtSSB binds to single strand DNA and is similar in size (15.2 kDa) to the 16-kDa component of the purified NF-GMa complex (44, 45). SSB proteins have also been shown to be able to both homodimerize and heterodimerize (44, 46–48). Gel filtration chromatography of heparin-Sepharose-enriched NF-GMa (HSGMa) under native conditions showed that the protein in the NF-GMa complex had an apparent molecular mass of 62 kDa (data not shown). We have previously determined that the NF-GMb complex represents a mixture of two different types of complex, one containing a single 42-kDa protein and the other containing a pair of 22-kDa proteins (13, 14). A molecular mass of 62 kDa is therefore consistent with the NF-GMa complex being composed of a single 42-kDa protein with a single 16-kDa protein (58 kDa) or a pair of 22-kDa proteins with a single 16-kDa protein (60 kDa). These results now implicate a second single-stranded binding protein, the mtSSB or a related protein, together with the CSD proteins, in the complexes that can bind to the domain 1 CK-1/CK-2 region of the GM-CSF gene.

Recombinant and Nuclear CSD Proteins Bind to the G-CSF and IL-3 Myeloid Growth Factor Genes—Two other myeloid growth factor genes, G-CSF and IL-3, share conserved regulatory elements with GM-CSF, such as the CK-1 region, and have overlapping patterns of regulation (2, 23–25). These genes also bind the NF-GMa complex (23, 24). Given this we examined the proximal promoter sequences of these genes for CSD-binding sites (Fig. 7*a*). Alignment of the domain 1 CK-1-containing regions did not show the repeated 5′-CCTG-3′ elements, found on the noncoding (−) strand of the GM-CSF sequence, although there was a pair of CSD-like sites (Fig. 7, *a* and *b*). However, closer to the transcription start site a 5′-CCTG-3′/5′-ACCA-3′ pair of potential CSD-binding sites identical to those on the domain 2 coding (+) strand of the GM-CSF promoter were observed in both G-CSF and IL-3 promoters (Fig. 7, *a* and *b*). Single-stranded oligonucleotides spanning these sequences were tested for their ability to form NF-GMb/c-like complexes in gel retardation assays. As expected coding (+) strand oligonucleotides spanning the conserved 5′-CCTG-3′/5′-ACCA-3′ domain 2-like sequences from both G-CSF (D2) and IL-3 (D2) formed complexes that comigrated with NF-GMb and NF-GMc when incubated with HUT78 nuclear extracts enriched for NF-GMb/c (HSGMb) (Fig. 7*b*, *lanes 7* and *13*). The complexes formed with similar intensity to those formed on the GM− oligonucleotide (*lane 1*). Consistently these complexes were competed to a much greater extent by the GM− wild type oligonucleotide (*lanes 8* and *14*) than by the NF-GMb/CSD-binding site mutant oligonucleotide, GMm23− (*lanes 9* and *15*), suggesting that these complexes are authentic NF-GMb/c. The G-CSF domain 1 noncoding (−) strand oligonucleotide (D1) formed an apparent NF-GMc-like complex and a weak NF-GMb-like complex, whereas the IL-3 domain 1 oligonucleotide (D1) formed both complexes weakly (Fig. 7*b*, *lanes 4* and 10). These complexes were also shown to be authentic by competition assays (*lanes 5*, *6*, *11*, and *12*). The decreased complex formation on the domain 1 sequences is consistent with the reduced conservation of potential NF-GMb/CSD-binding sites in the G-CSF and IL-3 genes. The presence of both NF-GMb

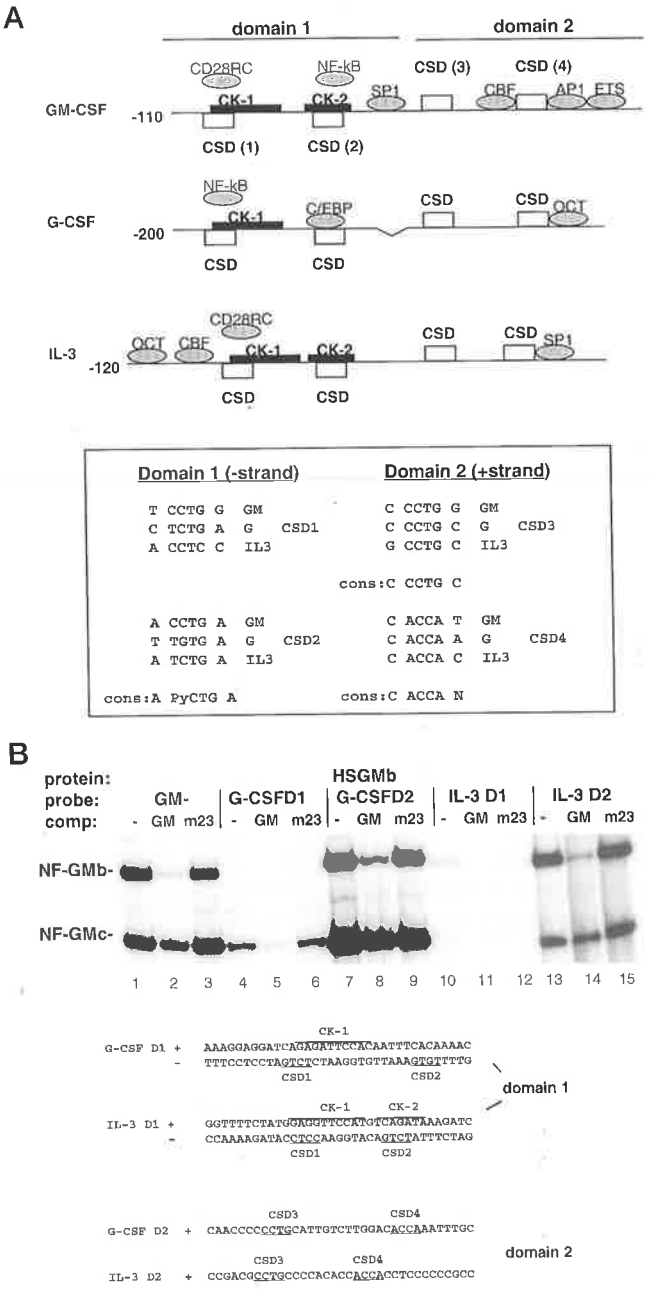

FIG. 7. **A conserved arrangement of CSD sites across the GM-CSF, G-CSF, and IL-3 genes.** *a*, the proximal promoter regions of the human GM-CSF, G-CSF and IL-3 genes are shown diagrammatically. Transcription factor-binding sites and CSD sites are indicated by *circles* and *boxes*, respectively. CSD sites are labeled *1–4*. The sequences of potential CSD sites are shown below the diagram and are aligned with the GM-CSF sequences. Consensus (*cons*) sequences for domain 1 and 2 CSD sites are given. *Py* represents C/T residues. In general CSD-binding sites in nonviral genes have a preference for C/T residues (37, 48, 43). *b*, HUT78 T cell nuclear extracts enriched for NF-GMb/c complexes (*HSGMb*) were bound to noncoding (−) strand domain 1 ^{32}P-labeled sequences from GM-CSF (*GM−*), G-CSF (*G-CSFD1*), and IL-3 (*IL-3D1*) or to coding (+) strand domain 2 sequences from G-CSF (*G-CSFD2*) and IL-3 (*IL-3D2*). Complexes were competed (*comp*) with 10 ng of unlabeled wild type GM− (*GM*) or mutant GMm23− (*m23*) GM-CSF domain 1 oligonucleotides. Tracks with no competitor are marked with *minus signs*. NF-GMb and NF-GMc complexes are indicated. The sequences of oligonucleotides containing regions of the human G-CSF and IL-3 gene promoters (56, 57) with homology to GM-CSF domain 1 (*D1*) and domain 2 (*D2*) are shown below. The predicted CSD sites are shown. Domain 1 and domain 2 oligonucleotides used in retardation assays are, respectively, the noncoding (−) and coding (+) strand sequences. *c*, recombinant CSD fusion protein (*GST-DbpB*) was bound to domain 1 and domain 2 oligonucleotides from GM-CSF, G-CSF, and IL-3. The recombinant protein-DNA complex is indicated.

and NF-GMc complexes forming on the G-CSF and IL-3 oligonucleotides, however, suggests the presence of a pair of CSD sites in each domain as predicted. Consistent with these results, recombinant CSD fusion protein (GST-DbpB) also binds to the G-CSF and IL-3 domain 1 and domain 2 oligonucleotides with binding being strongest to the domain 2 regions (Fig. 7*c*). Given the conservation of a specific arrangement of CSD sites across the promoters of three myeloid growth factor genes, it is apparent that these genes may be subject to a common mechanism of repression and that the spatial arrangement of CSD sites is important to bring about this repression.

DISCUSSION

Common GM-CSF Proximal Promoter Elements Respond to Appropriate Signals in T Cells and Fibroblasts—In analysis of the human GM-CSF promoter in fibroblasts, we previously demonstrated the involvement of the domain 1 NF-kB site and domain 2 sequences in response to TNF-α (13, 14). We now show that the CBF, AP1, and ETS/NFAT sites (Fig. 1) are absolutely required for TNF-α response of domain 2. The NF-kB, CBF, AP1, and ETS/NFAT sites across domains 1 and 2 have been shown to be required for maximal activation in T cells in response to T cell receptor signals (3, 4, 10–12, 16–18). Extensive studies have not been performed regarding functional GM-CSF promoter elements in other cell types. Deletion studies in endothelial cells suggest a role for all three binding sites in domain 2 for IL-1 response (19), whereas mutation studies suggest that sequences across the AP1 and ETS/NFAT-binding sites may be required for constitutive expression in some acute myeloid leukemia cell lines (20). A basic promoter unit may therefore be required for GM-CSF promoter function in a number of different cell types. The fact that mutation of any one transcription factor-binding site abolishes promoter activity suggests that all the sites act as a functional unit. A similar cooperative complex of factors has been described for the interferon-β promoter and termed an enhanceosome (49). The IL-2 promoter appears to operate in a similar manner (50). CBF and AP1 could clearly be involved in expression in fibroblasts because AP1 is widely expressed and TNF-α-inducible, and CBF is constitutively expressed (4, 16, 18). The relevance of ETS/NFAT in fibroblasts is less clear because they have primarily been investigated in lymphoid gene expression (4, 51). We have not been able to detect NFAT protein in fibroblasts[2]; hence, the most proximal site may bind an ETS family member (51). The way in which the promoter responds in different cell types and to different stimuli will depend on variations in levels of constitutive factors between cell types and the degree to which inducible factors respond to stimuli.

An Ordered Arrangement of Repressor Elements Binding CSD Proteins across the Proximal Promoters of the GM-CSF, G-CSF, and IL-3 Genes—We previously observed the binding of nuclear NF-GMb and NF-GMc complexes to two repeated 5′-CCTG-3′ sequences on the noncoding (−) strand of domain 1 of the GM-CSF promoter (Figs. 1 and 7*a*). We subsequently found

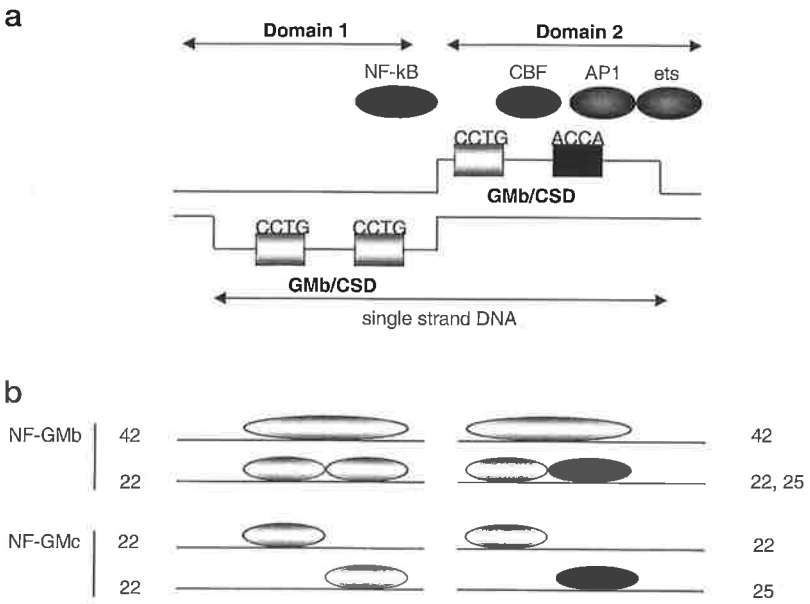

FIG. 8. **A repressive single strand structure across the GM-CSF promoter and the composition of CSD nuclear complexes.** *a*, the proposed single strand structure of the GM-CSF promoter is shown diagrammatically. CSD sites are indicated. The binding of CSD factors to single strand DNA of the noncoding strand of domain 1 and the coding strand of domain 2 may form an extensive single strand structure, preventing the binding of positive transcription factors requiring double strand DNA for binding and hence maintaining the promoter in a completely silenced state in the absence of appropriate stimuli. Proteins that complex with CSD factors such as mtSSB may be involved in reversing the effects of CSD proteins allowing for regulated gene expression. *b*, the predicted composition of CSD-containing nuclear complexes as determined from retardation analysis and UV cross-linking analysis is indicated. NF-GMb complexes contain a single 42-kDa nuclear CSD protein bound to DNA or a pair of truncated CSD proteins (22/25 kDa) bound to DNA, whereas NF-GMc represents the binding of truncated CSD proteins to one or other CSD site within domain 1 and 2.

that mutation of these sites resulted in an increase in TNF-α-inducible expression directed by domain 1 and 2 TNF-α-responsive regions (13). This identified the 5′-CCTG-3′ sites as repressor elements. By screening a cDNA library with a single strand domain 1 probe, we determined that the 5′-CCTG-3′ elements bound CSD proteins. Given we observed that recombinant and nuclear NF-GMb/c complexes bound common single strand DNA sequences and that NF-GMb/c complexes were competed by CSD consensus sequences and by CSD antibodies, we determined that NF-GMb/c represented nuclear CSD complexes (14). We now show that nuclear NF-GMb/c-like CSD complexes and recombinant CSD protein (DbpB) bind across the TNF-α-responsive elements in domain 2. As for domain 1, two NF-GMb/CSD sites were identified, but in contrast to domain 1 the 5′ and 3′ sites were, respectively, a 5′-CCTG-3′ and a novel 5′-ACCA-3′ sequence and were on the coding (+) strand of domain 2 (Figs. 1 and 7*a*). Mutation of either one of these sites, as for domain 1, resulted in an increase in TNF-α-inducible expression, identifying these elements as repressors. The spacing between the four CSD sites in domains 1 and 2 was conserved bringing about an ordered regularly spaced arrangement of CSD repressor sites across the GM-CSF promoter. Overexpression of DbpB and DbpA CSD proteins confirmed that CSD proteins were the mediators of repression via the NF-GMb/CSD sites (14).

We previously proposed that the binding of NF-GMb/CSD proteins to single strand domain 1 DNA resulted in a local single strand structure blocking the binding of transcriptional activators that are dependent on double strand DNA for binding and activity (4, 13, 14). This proposed single strand structure can now be extended to contain the entire TNF-α-responsive GM-CSF promoter, covering all activator binding sites and hence providing an efficient means of completely silencing the promoter. This model is shown in Fig. 8*a*. Even though individual NF-GMb/CSD elements can act as repressor elements, our previous and present transfection data reveal that all four CSD sites are required for maximal GM-CSF promoter repression. For example, the pGM93 construct containing two sites is repressed to a greater extent than pGM41 containing one site, whereas a construct containing all four sites is maximally repressed (13, 14). This is consistent with the idea of an extensive single structure across the GM-CSF promoter requiring binding to all four sites. Consistent with this model, single strand regions within double strand DNA, coinciding with CSD-binding sites have been detected *in vitro* (37, 38, 52). *In vivo* studies will confirm such a model. The binding of CSD proteins to opposite strands of the promoter may allow stabilization of such a single strand structure by interaction of CSD proteins (27, 28) bound to either strand.

CSD proteins have also recently been shown to repress a number of genes including those for thyrotropin receptor (36), nicotinic acetylcholine receptor δ (53), major histocompatibility complex class I and II genes (34, 54, 55), and the *grp78* gene (35). CSD proteins also bind to repressor sequences in the γ globin genes (38). Extensive characterization of CSD-binding sites across promoter elements has only been performed for the major histocompatibility complex DRα (52) and thyrotropin receptor (36) genes, but neither study reveals the ordered arrangement of CSD sites reported here. In the thyrotropin receptor gene, three CSD sites have been detected, one on the noncoding and two on the coding strand (36). These sites have a common sequence but are separated by large distances. The major histocompatibility complex DRα gene has two CSD-binding sites on opposing strands, but the exact location of the sites has not been determined (52). The study of the GM−CSF promoter therefore reveals a unique arrangement of CSD sites that can efficiently function to repress an entire proximal promoter.

The location of the CSD sites in the GM-CSF promoter suggests that CSD proteins may be involved in repression of the GM-CSF promoter not only in fibroblasts but also in T cells and potentially in endothelial and myeloid cells. Consistently, sequences containing the 5′ repressor site in domain 2 have been shown to have repressor activity in myeloid leukemic cell lines

and in Jurkat, MLA144, and primary human T cells (4, 20). In addition, sequences containing the 3′ domain 2 repressor element have repressor activity in an acute myeloid leukemia cell line (20), and domain 1 has been reported to have repressor activity in endothelial cells (19). The sequences identified in the acute myeloid leukemia cell line bind a 45-kDa protein (20), consistent with the size of a CSD protein, and we have also identified CSD binding to the GM-CSF promoter in extracts from a number of GM-CSF expressing cells (data not shown). CSD proteins may be involved in maintaining tight regulation of the GM-CSF promoter in all expressing cell types. The signaling pathways and transcription factors activated in different cell types will dictate the ability of different signals to overcome the repressive effects of CSD binding.

In addition to GM-CSF, we analyzed the promoter sequences of two other myeloid growth factor genes, the human G-CSF and IL-3 genes (56, 57). These genes have overlapping patterns of expression with GM-CSF (2, 4, 25). We find here that the unique arrangement of CSD sites in the GM-CSF gene is also apparent across the G-CSF and IL-3 proximal promoters (56, 57). In each of the three genes the domain 1 NF-GMb/CSD sites are on the noncoding (−) strand, whereas the domain 2 sites are on the coding (+) strand. CSD sites in the G-CSF and IL-3 genes overlap or are adjacent to activator sites in these genes (2, 58–64). Some of these sites are in common with GM-CSF gene activator sites including SP1, CBF, NF-kB, CK-1, and CD28-responsive complex sites (3, 4). The finding of a conserved arrangement of CSD sites suggests a common means of repression of the growth factor genes. Consistent with this, both domain 1 and domain 2 regions in the IL-3 gene have been shown to have repressor activity in T cells (62), and domain 2 in the G-CSF gene has repressor activity in CHU-2 cells (65). In addition we have confirmed by mutation analysis that the most 5′ G-CSF domain 1 CSD site is required for nuclear CSD binding and that overexpression of CSD protein represses the TNF-α-inducible expression of the G-CSF promoter in fibroblasts.[2] The sequence, spacing, and strand conservation of CSD sites in the three genes suggests an important role not only for binding of CSD proteins to DNA but also for CSD interactions with other regulatory proteins (35, 51), and ultimately, the structure of the complex formed on the DNA resulting from binding these factors. The idea of a common single strand structure across all three genes is supported by the presence of CT-rich regions flanking the CSD sites. Such sites are susceptible to single strand DNA formation and could act as entry sites for CSD proteins (37, 38) to enable them to bind and form a repressive single strand structure common to the three myeloid growth factor genes.

Distinct CSD-containing Nuclear Complexes Can Bind to the Domain 1 and Domain 2 Sites in the GM-CSF Promoter—From our previous analysis of the binding of nuclear NF-GMb/c complexes to mutant domain 1 oligonucleotides and from analysis of the protein composition of complexes by UV cross-linking, we interpreted our data (13, 14) as follows: The NF-GMb complex represents two separate complexes, one containing a single 42-kDa protein requiring both CSD sites for maximal binding and the other containing two 22-kDa proteins, with one 22-kDa protein bound to each of the CSD sites. The NF-GMc complex represents the binding of the 22-kDa protein to one or other CSD site. This is summarized in Fig. 8*b*. The 42-kDa protein is the correct size for a full-length CSD protein as determined by Western analysis of nuclear extracts from a number of cell lines (43, 52). Consistently full-length recombinant CSD proteins (DbpA and DbpB) also require both CSD sites for full binding to domain 1 (14). The 22-kDa protein probably represents a truncated CSD protein (discussed below). We now analyze the protein composition of nuclear NF-GMb/c across domain 2 and interpret our data as summarized in Fig. 8*b*. As for domain 1, NF-GMb contains two complexes, one containing one single 42-kDa protein requiring both the 5′-CCTG-3′ and 5′-ACCA-3′ sites for full complex formation (Figs. 3*c* and 4*b*). Consistently recombinant DbpB CSD protein requires both sites for full binding (Fig. 3*d*). In contrast to domain 1, the second complex in domain 2 NF-GMb represents binding of both a 22-kDa protein to the 5′-CCTG-3′ site and a novel 25-kDa protein binding to the 5′-ACCA-3′ site (Fig. 4*b*). We have confirmed that the 25-kDa protein, like the 42- and 22-kDa proteins, is a cold shock protein by use of a CSD antibody.[2] Our data suggest that the 25-kDa protein is a separate subtype or an altered conformation of the 22-kDa protein contacting 5′-ACCA-3′. The NF-GMc complex on domain 2 most likely represents the binding of either a single 22- or 25-kDa protein. Taken all together, for the whole GM-CSF promoter (Fig. 8), a single 42-kDa CSD protein can contact the pair of sites in domain 1 or domain 2, a single 22-kDa protein can contact each of the first three 5′-CCTG-3′ sites, and the 25-kDa protein contacts the 5′-ACCA-3′ site. Hence the four repressor sites across the GM-CSF promoter that are required for full promoter repression bind a series of different CSD subtypes.

We are at present screening for cDNAs encoding the 22/25-kDa CSD subtypes. Proteins in the 22/25-kDa size range could be produced from reported alternatively spliced DbpA CSD cDNA sequences (42, 52, 66, 67) or from potentially functional DbpB pseudogenes (67–69). Interestingly a chicken CSD factor binding to an 5′-ACCA-3′ sequence in the Rous sarcoma virus long terminal repeat promoter represents a truncated form of DbpA, called YB-2 (41, 42). Human homologues of YB-2 have also been identified (66, 67); hence the 25-kDa protein that binds to a 5′-ACCA-3′ sequence may represent a YB-2-type protein. The function of human YB-2 is unknown, but it is known that it lacks sequences in the C-terminal region relative to full-length CSD proteins. CSD proteins have three functional domains, an N-terminal, a central highly conserved cold shock, and a C-terminal domain (28, 31, 32). The central CSD domain is involved in single strand DNA binding and repression mechanisms (4, 14, 34–36, 52–55), and the C-terminal region is involved in interaction with other transcription factors (28, 35, 51, 70–72). Such protein interaction appears to be involved in both the mechanisms of repression and subsequent derepression upon stimulation (35, 51, 71). It can be seen therefore that full-length and truncated proteins may have different abilities to repress and also vary in the degree to which their repressive action can be reversed.

We cannot yet determine the precise role that each CSD subtype is playing at each repressor site until the subtypes are cloned, but our data do demonstrate that the full-length 42-kDa protein binds to sequences in common with those binding either the truncated 22- or 25-kDa protein. As we have observed that the relative levels of the 42-kDa *versus* 22/25-kDa factors vary between cell types,[2] it is probable that full-length and truncated proteins compete for binding sites *in vivo*. The relative amounts of the different CSD types may determine the ability of a gene to be repressed and derepressed in different cell types. It will also be of interest to determine the differences between 22- and 25-kDa proteins that dictate their binding to different sequences. At present the functional consequences of binding to different sequences is not apparent, but from mutation studies, however, the 22-kDa site (5′-CCTG-3′) in domain 2 does appear to be a stronger repressor element than the 25-kDa (5′-ACCA-3′) site (Fig. 2). The presence of alternative CSD subtypes or alternative conformational forms binding to both different and common elements enables a set up that can

be manipulated *in vivo* to bring about appropriate gene regulation in different cell types.

CSD proteins have been shown to interact with transcription factors in solution (35, 71, 72) or to complex with a transcription factor on its double strand DNA-binding site (51). A complex of CSD proteins with heterologous proteins on single strand DNA has not, however, previously been reported. We show here that CSD proteins can interact with the GM-CSF promoter in association with a heterologous single strand DNA-binding protein to form the NF-GMa complex. The NF-GMa complex was originally identified as binding to the CK-1 regions of the GM-CSF, G-CSF, and IL-3 genes (23, 24). We now show that NF-GMa is a complex of 42- and 22-kDa nuclear CSD proteins with a novel 16-kDa protein that forms on the noncoding (−) strand of the GM-CSF CK-1/CK-2 region in domain 1. We have determined the N-terminal sequence of the 16-kDa protein and found it to be identical to the N-terminal sequence of mature human mtSSB (44). Consistent with the properties of the 16-kDa protein, mtSSB has a molecular mass of 15.2 kDa and binds to single strand mitochondrial DNA (44, 45). Human mtSSB belongs to a family of SSB proteins conserved from *E. coli* to mammals (44–46, 73, 74). In *E. coli* and in the mitochondria of higher organisms these proteins have been implicated in the processes of DNA replication, recombination, and repair (74–46) and in transcriptional derepression (75). In higher organisms mtSSB is primarily detected in mitochondria. Trace amounts have, however, been detected in the nucleus (77, 78), and it has been demonstrated that overexpression of mtSSB can result in the activation of a nuclear gene, Aα fibrinogen (78). Interestingly, a nuclear protein with N-terminal sequence conserved with mature mtSSB was isolated as binding to an IL-6 response element in the Aα fibrinogen gene (78). This element, 5′-GAATTTCTGGGA-3′, has a similar sequence to that observed across the CK-1 region CSD site 1 in the growth factor genes we have investigated. The homology is particularly striking with the GM-CSF and G-CSF genes (Figs. 1*b* and 7, *a* and *b*). mtSSB or related proteins may therefore have a broader role in the regulation of genes involved in growth and stress responses as is also the case for CSD proteins. Our identification of the 16-kDa component of NF-GMa as an mtSSB-like protein strengthens the idea that mtSSB type proteins can have a function in both mitochondria and the nucleus.

We have also shown that the association of the 16-kDa mtSSB related protein with the 42- and 22-kDa nuclear CSD proteins changes the specificity of the CSD factors for binding to domain 1. The 42-kDa protein no longer requires the NF-GMb/CSD sites to bind to domain 1 DNA when it is part of the NF-GMa complex. The 22-kDa protein may still need to interact with these elements because it is not present in the NF-GMa complex formed on the GMm23 mutant sequence that lacks functional CSD-binding sites. The NF-GMa complex can probably also form in solution in the absence of DNA because it can be separated by heparin-Sepharose chromatography from NF-GMb/c nuclear complexes containing only the 42- and 22-kDa CSD proteins. These findings are consistent with the reported abilities of both CSD and SSB proteins to complex with other proteins (44, 46–48). We have also shown that the NF-GMa complexes can compete with NF-GMb/c complexes for binding to domain 1. Given the effects of the 16-kDa protein on CSD factor binding to repressor elements, formation of the NF-GMa complex may prevent the repressive function of the CSD proteins. This is supported by our observation of an increase in NF-GMa complex formation upon TNF stimulation of fibroblasts (23, 24). Such a function for NF-GMa is consistent with the observed involvement of *E. coli* SSB in transcriptional derepression (75) and for mammalian mtSSB in the activation of the Aα fibrinogen gene (78). Hence NF-GMa complex formation may be part of a mechanism to ensure rapid derepression of very tightly regulated genes such as GM-CSF and the other growth factor genes, G-CSF and IL-3, that also bind both the repressive NF-GMb/c complexes and the potentially antagonistic NF-GMa complex.

We have now characterized the entire TNF-α-responsive proximal promoter of the human GM-CSF gene. We have identified the sequences required for both activation and repression of this promoter and have characterized a family of nuclear complexes containing single strand DNA-binding proteins that bind across these sequences. In doing so we determined a role for these single strand proteins in both the mechanisms of repression of the GM-CSF gene and potentially other growth factor genes and possibly in their subsequent activation.

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Cold Shock Domain Factors Activate the Granulocyte-Macrophage Colony-stimulating Factor Promoter in Stimulated Jurkat T Cells*

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Cold shock domain (CSD) family members have been shown to play roles in either transcriptional activation or repression of many genes in various cell types. We have previously shown that CSD proteins dbpAv and dbpB (also known as YB-1) act to repress granulocyte-macrophage colony-stimulating factor transcription in human embryonic lung (HEL) fibroblasts via binding to single-stranded DNA regions across the promoter. Here we show that the same CSD factors are involved in granulocyte-macrophage colony-stimulating factor transcriptional activation in Jurkat T cells. Unlike the mechanisms of CSD repression in HEL fibroblasts, CSD-mediated activation in Jurkat T cells is not mediated through DNA binding but presumably through protein-protein interactions via the C terminus of the CSD protein with transcription factors such as RelA/NF-κB p65. We demonstrate that Jurkat T cells lack truncated CSD factor subtypes present in HEL fibroblasts, which raises the possibility that the cellular content of CSD proteins may determine their final role as activators or repressors of transcription.

Cold shock domain (CSD)[1] proteins were originally identified in bacteria and have been shown to be highly conserved throughout evolution from bacteria to humans (1–3). CSD proteins have three functional domains: an N terminus, the central CSD, and a C-terminal domain. The N terminus region of the protein has not been well characterized but has been shown to contribute to single-stranded DNA binding (4, 5). The highly conserved central cold shock domain (from which this family of proteins derives its name) contains an RNP1 motif that is essential for sequence-specific DNA and RNA binding (5–9) (see Fig. 1*C*). The C terminus of the protein has alternating basic and acidic domains and has been implicated in both nonsequence specific RNA binding and protein-protein interactions with transcriptional regulators like RelA, ZO-1, TATA binding protein, NF-Y, YY-1, and AP-2 (10–15) (Fig. 1*C*). As a whole, the family of CSD proteins has been reported to bind to double- and single-stranded DNA and RNA and is involved in transcriptional repression and activation and in mRNA packaging, transport, localization, masking, stability, and translation (1–3, 16–20).

An important role of CSD proteins is in transcriptional regulation of genes involved in growth and stress responses (13, 21–28). The CSD proteins dbpB (also known as YB-1) and dbpA have been shown to act as activators of viral genes (15, 29–32) and both as activators and repressors of many cellular genes (13, 14, 25–28, 33–37), including the gene for the hemopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) (21–23).

GM-CSF belongs to a family of growth factors that control survival, proliferation, and differentiation of cells from the hematopoietic lineage of granulocytes and macrophages (20, 38–41). GM-CSF is expressed by a wide variety of cells including myeloid, mesenchymal, and lymphoid cells in response to stress signals such as those derived from infection, inflammation, and blood loss (20, 28, 38, 39). GM-CSF expression is triggered in T cells primarily via T cell receptor and coreceptor activation and is tightly regulated at the level of transcription (20, 38). The human GM-CSF proximal promoter can be conveniently divided into two domains (domain 1, −114 to −71 and domain 2, −70 to −31), which are both important for GM-CSF regulation (21–23) (Fig. 1*A*). The two domains have many binding sites for transcription factors including: NF-κB/Rel, CBF, AP-1, ETS/NFAT, and CD28-responsive complex (CD28RC), which all act to mediate GM-CSF expression (reviewed in Refs. 20 and 38). In addition to the double-stranded DNA binding transcription factors mentioned above, we have shown previously that there are four CSD binding sites along the GM-CSF proximal promoter. There are two single-stranded DNA CSD binding sites along the noncoding (−) strand of domain 1 and two along the coding (+) strand of domain 2 (Fig. 1, *A* and *B*) (21–23). We demonstrated that these CSD sites acted as repressor sites in human embryonic lung (HEL) fibroblasts, that CSD proteins could bind to these sites, and that overexpression lead to repression of TNFα-mediated activation of GM-CSF (21–23).

T cells are a major source of GM-CSF *in vivo*, and we wished to determine whether CSD proteins played a role in GM-CSF transcription in these cells. We now report that CSD proteins are present in Jurkat T cells and bind to the defined CSD binding sites along the GM-CSF promoter. The Jurkat T cell nuclear CSD complexes that form on the GM-CSF promoter are distinct from those found in HEL fibroblasts. Using transient transfection assays, we previously showed that the CSD binding sites acted as repressor elements in HEL fibroblasts. However, when Jurkat T cells are stimulated with PMA/Ca^{2+} ionophore to mimic T cell receptor activation, the CSD factors behave as coactivators. The coactivation seen does not function through the CSD binding sites but through potential protein-

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[1] The abbreviations used are: CSD, cold shock domain; TK, thymidine kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; HEL, human embryonic lung; PMA, phorbol 12-myristate 13-acetate; GST, glutathione *S*-transferase; CBF, core binding factor; NFAT, nuclear factor of activated T cell.

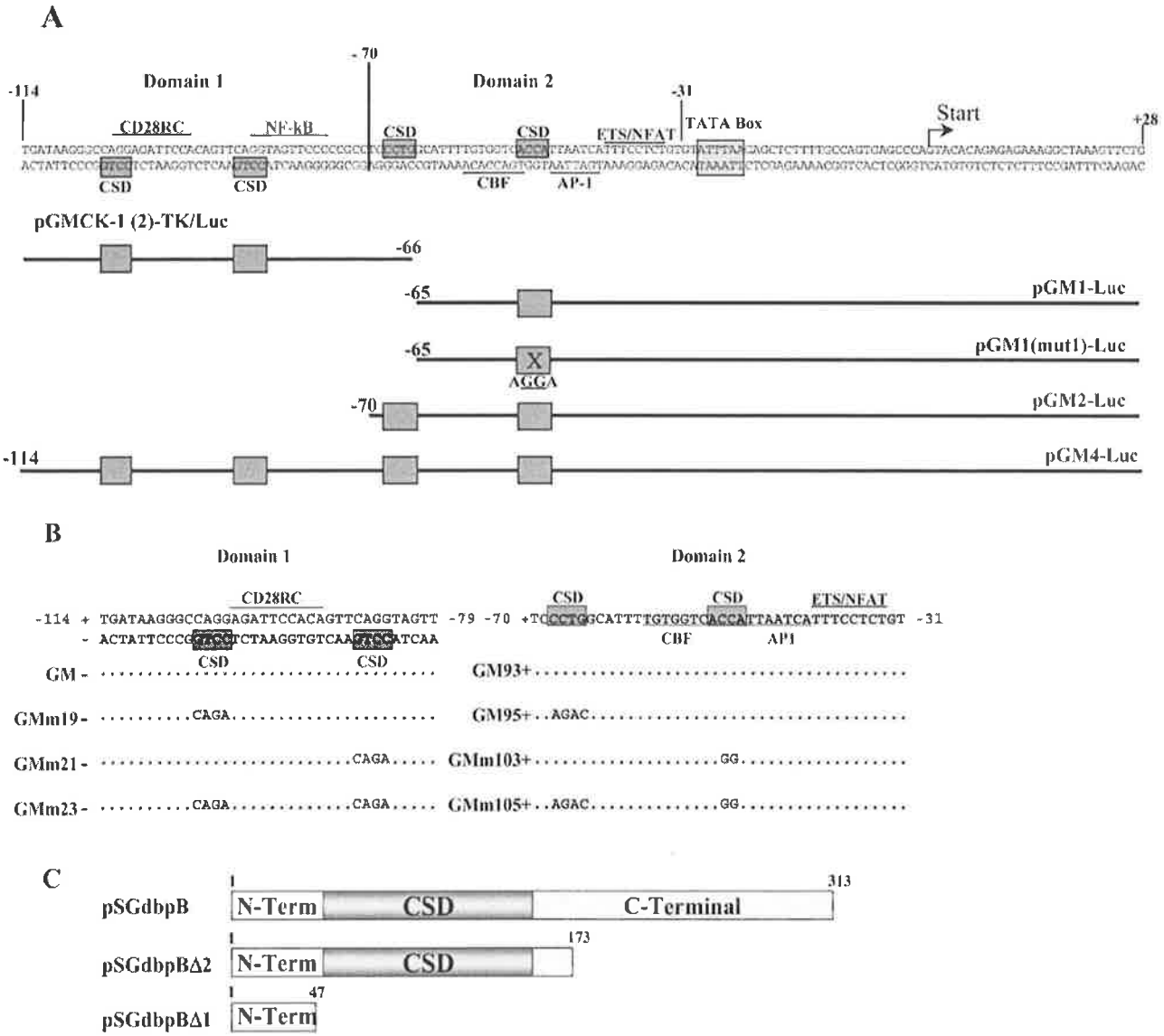

FIG. 1. **The GM-CSF proximal promoter and CSD factor dbpB truncation constructs.** *A*, the sequence of the human GM-CSF proximal promoter is shown. Domain 1 (−114 to −71) and domain 2 (−70 to −31) regions are indicated. The binding sites for many double-stranded DNA binding transcription factors that mediate GM-CSF expression, including NF-kB, CBF, AP1, ETS/NFAT, and CD28RC, are *underlined* (20, 38). Nuclear NF-GMb/c and recombinant CSD factor binding sites are indicated on the noncoding (−) strand in domain 1 and the coding (+) strand in domain 2 with *shaded boxes*. GM-CSF promoter, luciferase reporter constructs used, pGMCK-1(2)-TK/Luc, pGM1-Luc, pGM1(mut1)-Luc, pGM2-Luc, and pGM4-Luc are shown diagrammatically underneath. *Numbers* indicate distance from the transcriptional start site, and *boxes* represent CSD binding sites. Mutation of the CSD binding site in pGM1(mut1)-Luc is represented by a *box* with an X in it, and the altered sequence is given below it. *B*, the sequence of the coding (+) and noncoding (−) wild-type domain 1 (−114 to −79) oligonucleotides, GM and GM−, are shown (21, 22). Sequences needed for nuclear NF-GMb/c and recombinant CSD factor binding to the noncoding strand are indicated by a *box*. Base changes in CSD binding sites of the noncoding (−) strand for each mutant oligonucleotide GMm19, GMm22, and GMm23 are shown (22). The sequence of the wild-type-coding strand (+) domain 2 GM93+ oligonucleotide (−70 to −31) is given with CSD, CBF, AP1, and ETS/NFAT sites shown (21, 22). Base changes in the CSD binding sites of the coding (+) strand for each mutant oligonucleotide GMm95+, GMm103+, and GMm105+ are shown (23). *C*, the coding regions of dbpB contained in the expression plasmids pSGdbpB, pSGdbpBΔ1, and pSGdbpbBΔ2 are shown schematically. The N-terminal, CSD, and C-terminal regions of the protein are represented, and the numbers represent the amino acid number. The construct pSGdbpB encodes for full-length dbpB, pSGdbpBΔ2 (the first 173 amino acids), and pSGdbpbBΔ1 (the first 47 amino acids).

protein interactions mediated via the C-terminal domain of CSD proteins.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Luciferase reporter plasmids were constructed by inserting oligonucleotides encoding human GM-CSF promoter fragments into the pXP1 luciferase vector (Promega). The number after the pGM in each luciferase construct name indicates how many CSD binding sites are present. The plasmids pGM1-Luc, pGM2-Luc, and pGM4-Luc were constructed by cloning oligonucleotides (with *Hin*dIII 5′ and *Bam*HI 3′ ends) spanning regions of the GM-CSF promoter (−65 to +28), (−70 to +28), and (−114 to +28) into the *Hin*dIII/*Bam*HI sites of pXP1 (Fig. 1*A*). The QuikChange™ site-directed mutagenesis kit (Stratagene) was used to mutate the CSD binding site in pGM1-Luc from 5′-ACCA-3′ to 5′-AGGA-3′ to create the pGM1-mut1-Luc reporter plasmid (Fig. 1*A*). The backbone expression vector pRcCMV was obtained from Invitrogen. Construction of the RelA expression plasmid has been described previously (42). Construction of the expression plasmids pSGdbpAv and pSGdbpB has been described previously (22). The dbpB deletion plasmid pSGdbpBΔ1 was created by digesting pSGdbpB with *Nar*I, which removes the CSD and C-terminal domain of dbpB, followed by religation (pSGdbpBΔ1 contains the first 47 amino acids of

dbpB) (Fig. 1*C*). The dbpB deletion plasmid pSGdbpBΔ2 was created by digesting pSGdbpB with *Eco*RI and *Sau*3AI, which removes most of the C-terminal domain of dbpB, and ligating this fragment into an *Eco*RI/*Bgl*II-digested pSG5, pSGdbpBΔ2, containing the first 173 amino acids of dbpB (Fig. 1*C*).

Oligonucleotides and Probe Preparation—All oligonucleotides were purchased from GeneWorks (Adelaide, Australia), and full-length product was purified from nondenaturing polyacrylamide gels (43). Single-stranded DNA probes for gel retardation assays were prepared by end-labeling oligonucleotides with T4 polynucleotide kinase and [γ-^{32}P]ATP followed by gel purification.

Preparation of Recombinant Proteins—Construction of the bacterial expression construct for CSD protein dbpB (pGEXBT) has been previously described (23). The *Escherichia coli* strain MC1061 transformed with pGEXBT was induced with isopropyl-1-thio-β-D-galactopyranoside to produce recombinant GST-dbpB fusion protein. Recombinant GST-dbpB was purified on glutathione-Sepharose beads as described by the manufacturer (Amersham Pharmacia Biotech). The bacterial expression construct for recombinant RelA was a gift of Dr. Steven Gerondakis, and protein was prepared according to the procedure described by Dunn *et al.* (42).

Gel Retardation Analysis, Competitions, and Antibody Blocking Experiments—Nuclear extracts were prepared from HUT78 T cells and Jurkat T cells as previously reported (44 45). Gel retardations were carried out using 0.25 ng of single-stranded ^{32}P-labeled oligonucleotide probes (Fig. 1*A*) in a 10-μl reaction mix of 0.5× TM buffer (21, 22, 45) containing 200 mM KCl, 0.4 μg of poly(dI-dC), and either 1 μg of nuclear extract or 25 ng of recombinant CSD fusion protein (GST-dbpB) or recombinant RelA. Retardation assays using recombinant protein also contained 2 μg of bovine serum albumin. Reactions were incubated at room temperature for 20 min and analyzed on 12% (for nuclear extracts) and 6% (for recombinant proteins) nondenaturing polyacrylamide gels run in 0.5× TBE (46). Competitions with unlabeled single strand oligonucleotides were performed by mixing protein and unlabeled probe followed by the immediate addition of the ^{32}P-labeled probe (22). Antibody blocking experiments were performed by adding protein and antibody and incubating for 5 min at room temperature before adding the ^{32}P-labeled probe. The reaction was then incubated for an additional 20 min at room temperature before being analyzed on polyacrylamide gels.

UV Cross-linking—For UV cross-linking, nuclear extracts were bound to ^{32}P-labeled single-stranded DNA probes (Fig. 1*B*), and the complexes were separated on a 12% polyacrylamide gel as described above. The gel was exposed to UV light (340 nm) for 15 min to cross-link bound protein to the DNA. The gel was exposed to x-ray film for 12 h at 4 °C, and the retarded complexes were excised. Protein in the excised bands was analyzed on a 12% SDS-polyacrylamide gel (21, 43).

Antibodies—The anti-CSD peptide antibody was raised by immunizing rabbits with the peptide (IKKNNPRKYLRSVGD) (dbpB amino acids 89–103) conjugated to keyhole limpet hemocyanin (Imject conjugation kit, Pierce). Immunoglobulins were partially purified via ammonium sulfate precipitation. The specificity of the anti-CSD peptide antibody was verified by dot blot immunoblots against the CSD peptide, peptides to different regions of the dbpB protein, and recombinant glutathione *S*-transferase (data not shown).

Cell Culture, Transfections, and Luciferase Assays—The Jurkat T cell line was cultured in RPMI medium containing 10% fetal calf serum supplemented with L-glutamine, penicillin, and gentamycin antibiotics. Electroporation with a Bio-Rad Gene Pulser was used for transfection of Jurkat T cells at 270 V and a capacitance of 960 microfarad. 5×10^6 cells were electroporated in 500 μl of RPMI with 20% fetal calf serum per transfection with 5 μg of reporter plasmid and 10 μg of expression plasmid. Cells were stimulated 24 h post-transfection at a final concentration of 20 ng/ml PMA and 1 μM calcium ionophore (A23187) and, 8 h post-stimulation, were assayed for luciferase activity as described by Osborne *et al.* (47).

Western Blot and Southwestern Analysis—Nuclear extracts from Jurkat and HUT78 T cells were isolated as described previously (44 45), and 10 μg of protein was separated by 12% SDS-polyacrylamide gel electrophoresis before transfer to a nitrocellulose membrane via a Bio-Rad protein transfer apparatus. The filter was probed with CSD peptide antibody (1:1000 dilution) and developed with an ECL detection kit according to the recommendations of the manufacturer (Amersham Pharmacia Biotech). For Southwestern analysis, nuclear extracts were prepared as described above, and the filter was probed with ^{32}P-labeled oligonucleotides as described by Silva *et al.* (48).

RESULTS

Detection of CSD Complexes in Jurkat T Cells—We have previously shown that both HEL fibroblasts and HUT78 T cells contain nuclear CSD proteins and that they bind to the human GM-CSF proximal promoter (21–23). We have also shown previously that the nuclear CSD complexes in HEL fibroblasts and HUT78 T cells are the same (21–23). To determine whether Jurkat T cells contained CSD proteins, Jurkat T cell nuclear extract was bound in a gel shift assay to single-stranded oligonucleotides spanning domain 1 and domain 2 of the human proximal GM-CSF promoter (Fig. 1*B*) and compared with HUT78 T cell CSD factor binding on the same oligonucleotides. When HUT78 T cell nuclear extract was used in a gel shift assay with the noncoding (−) strand of domain 1 (GM−), which contains two CSD binding sites, we observed the previously reported NF-GMb and NF-GMc nuclear complexes (Fig. 2*A*, *lane 2*). Specific competitions, cross-linking, and antibody analysis have previously demonstrated that these HUT78 T cell complexes contain CSD proteins (21–23). In HEL fibroblast and HUT78 T cells, the nuclear NF-GMb complex migrates in multiple apparent conformational forms NF-GMb (1) and NF-GMb (2), and we have shown the different conformational forms have identical sequence specificity and protein content (21–23).[2] When Jurkat T cell nuclear extract was used in a gel shift assay we saw complexes that comigrated with the HUT78 T cell NF-GMb complexes but could not detect a NF-GMc-like complex (Fig. 2*A*, *lane 1*). Using HUT78 T cell nuclear extract in a gel shift with the coding (+) strand of domain 2 (GM93+), which also contains two CSD binding sites, we observed three bands making up the NF-GMb complex and a single NF-GMc band (Fig. 2*A*, *lane 4*) (21–23).[2] Jurkat T cell nuclear extract gave two bands that comigrated with the two slowest-migrating HUT78 T cell NF-GMb bands, and again, no NF-GMc-like complex was seen (Fig. 2*A*, *lane 3*).

To verify that the Jurkat T cell NF-GMb complexes seen on domains 1 and 2 were authentic CSD-containing NF-GMb complexes, competition assays were performed. As shown in Fig. 2*B*, the unlabeled, wild-type, noncoding (−) oligonucleotide GM− (contains two CSD sites), when used as a competitor, inhibited formation of the Jurkat T cell NF-GMb complex on both domain 1 (GM−) (Fig. 2*B*, *lane 7*) and domain 2 (GM93+) probes (Fig. 3*A*, *lane 7*). Competing with GMm23, domain 1 noncoding strand oligonucleotide, which has both CSD sites mutated, had no effect on NF-GMb complex formation (Fig. 2*B*, *lanes 3* and *8*). As a positive control, we used an oligonucleotide from the coding (+) strand of the human papillomavirus 18 enhancer (HPV+), which has been shown to bind recombinant and nuclear CSD proteins (22, 49). HPV+ was able to compete the Jurkat T cell NF-GMb complex binding almost as well as the wild-type GM− oligonucleotide (Fig. 2*B*, *lanes 4* and *9*). The NF-GMb complex was not competed by an oligonucleotide (nonspecific) that we and others have shown is unable to bind nuclear or recombinant CSD proteins (Fig. 2*B*, *lanes 5* and *10*) (5, 22, 50).

As further evidence that the Jurkat T cell NF-GMb complex contained CSD factors, we made polyclonal antibodies to the peptide (IKKNNPRKYLRSVGD, dbpB amino acids 89–103) that represents a small region of the highly conserved CSD domain which is required for single-stranded DNA binding (7). This region of the CSD domain has identical sequence in both CSD factors, dbpB and dbpAv. The antibody raised to the CSD peptide was used in a gel shift with recombinant CSD protein dbpB (23). Increasing amounts of antibody in a gel shift assay

[2] P. Diamond, M. F. Shannon, M. A. Vadas, and L. S. Coles, unpublished results.

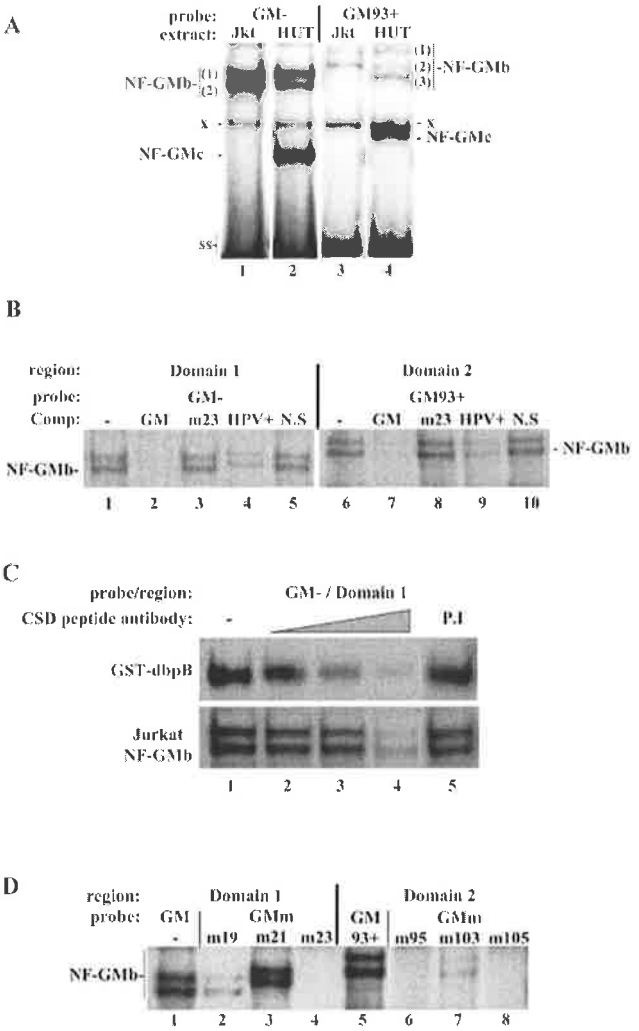

FIG. 2. **CSD proteins are present in Jurkat T cells and bind to the GM-CSF proximal promoter.** *A*, Jurkat T cell and HUT78 T cell nuclear extracts were bound to ^{32}P-labeled wild type domain 1 noncoding (−) strand GM− (*lanes 1* and *2*), and domain 2 coding (+) strand GM93+ (*lanes 3* and *4*) single-stranded oligonucleotides in a gel shift assay. Different conformational forms of NF-GMb are represented by *(1)*, *(2)*, and *(3)*. × represents nonspecific binding, and *ss* represents free single stranded labeled oligonucleotide. *B*, Jurkat T cell nuclear extract was bound in a gel shift assay to labeled GM-CSF domain 1 noncoding (−) strand oligonucleotide GM- (*lanes 1–5*) and to domain 2 coding (+) strand oligonucleotide GM93+ (*lanes 6–10*). The NF-GMb complex was competed with (GM−) (*lanes 2* and *7*), the CSD site mutant (GMm23−) (*lanes 3* and *8*), the control CSD binding site (HPV+) (*lanes 4* and *9*), and a nonspecific oligonucleotide (*N.S.*) (*lanes 5* and *10*). *C*, Jurkat T cell nuclear extract and recombinant dbpB were bound to labeled domain 1 noncoding (−) strand GM− oligonucleotide in a gel shift assay. Increasing amounts of anti-CSD peptide antibody were added to the reaction (*lanes 2–4*). 2-Fold more antibody than in *lane 2* was added in *lane 3*, and 5-fold more was added in *lane 4*. As controls, no antibody was added to the reaction (denoted by a *minus* (−) sign, *lane 1*), and rabbit pre-immune (*P.I*) was added in the reaction as a negative control (*lane 5*). *D*, Jurkat T cell nuclear extract was bound to wild-type domain 1 noncoding (GM−) oligonucleotide (*lane 1*), domain 1 CSD mutants (GMm19−, GMm21−, GMm23− (*lanes 2–4*)), wild-type domain 2 noncoding (GM93+) oligonucleotide (*lane 5*), and domain 2 CSD mutants (GMm95+, GMm103+, GMm105+ ((*lanes 6–8*)).

with recombinant dbpB demonstrated that the CSD peptide antibody could block recombinant CSD protein dbpB binding to the GM− domain1 oligonucleotide (Fig. 2*C*, *top panel* (*GST-dbpB*), compare *lane 1* to *lanes 2–4*). In the bottom *panel* of Fig. 2*C* we show that the CSD peptide antibody was also able to block binding of the Jurkat NF-GMb complex, demonstrating

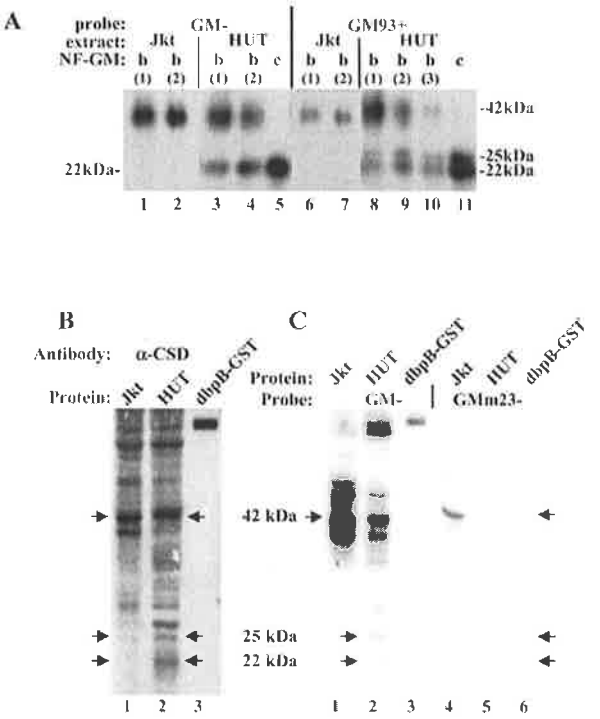

FIG. 3. **Jurkat T cells lack the 22- and 25-kDa nuclear CSD proteins.** *A*, Jurkat and HUT78 T cell NF-GMb and NF-GMc gel shift complexes were UV-cross-linked and separated by SDS-polyacrylamide gel electrophoresis. NF-GMb/c gel shift complexes from Jurkat T cell and HUT78 T cell nuclear extracts bound to either domain 1 noncoding GM− (*lanes 1* and *2*, Jurkat; *lanes 3–5*, HUT78) or domain 2 coding GM93+ (*lanes 6* and *7*, Jurkat; *lanes 8–11*, HUT78) wild-type ^{32}P-labeled oligonucleotides are shown. Proteins of the sizes 42, 25, and 22 kDa are indicated. See Fig. 2 for explanation of *(1)* and *(2)*. *B*, Jurkat and HUT78 T cell nuclear extracts and recombinant GST-dbpB were fractionated by Laemmli SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with the CSD peptide antibody in a Western blot to identify all CSD proteins present (*lanes 1–3*). Potential CSD proteins of the size identified in UV-cross-linking assays in HUT78 T cells (42, 25, and 22 kDa) and Jurkat T cells (42 kDa) are indicated. *C*, Nitrocellulose membranes with the same fractionated proteins, as in *B*, were probed with either the wild-type GM-CSF, domain 1, GM-^{32}P-labeled ologonucleotide (*lanes 1–3*), or the double CSD binding site mutant of the GM-^{32}P-labeled oligonucleotide, GMm23- (*lanes 4–6*). Potential CSD proteins identified of the size 42 kDa (in both HUT78 and Jurkat T cells), 25, and 22 kDa (in HUT78 T cells) are indicated.

that the Jurkat NF-GMb complex contained CSD factors (Fig. 2*C*, *bottom panel* (Jurkat nuclear extract), compare *lane 1* to *lanes 2–4*). The addition of pre-immune antibody (*P.I*) to the binding reaction mixture at the maximal concentration of CSD peptide antibody used showed no effect on mobility or complex formation in either the recombinant dbpB protein or the Jurkat nuclear extract gel shift (Fig. 2*C*, compare *lanes 1* and *5* in both *panels*). The CSD peptide antibody was also able to block binding of the recombinant dbpB and Jurkat T cell nuclear extract to the coding (+) strand of domain 2 (GM93+) (data not shown).

To determine whether the Jurkat T cell NF-GMb CSD-containing complex bound to the GM-CSF promoter CSD binding sites, nuclear extract was used in a gel shift analysis with wild-type domain 1 (GM−) and domain 2 (GM93+) oligonucleotides (each contain two CSD binding sites) and their respective CSD binding site mutants. These CSD binding site mutations (see Fig. 1*B*) have been previously described and characterized for HUT78 T cell and HEL fibroblast CSD binding (21, 23).[2] Gel shift analysis using the CSD mutant oligonucleotides on the noncoding (−) strand of domain 1 were compared with the

binding on the wild type oligonucleotide GM− (Fig. 2*D*, *lane 1*). Mutating one CSD site in GMm19 decreased binding significantly (Fig. 2*D*, *lane 2*), whereas mutating the other CSD site in GMm21 actually increased CSD binding but also resulted in an altered mobility of the complex (Fig. 2*D*, *lane 3*). Only when both CSD sites were mutated, in the GMm23 oligonucleotide, was all binding abolished (Fig. 2*D*, *lane 4*). Similar results were seen when we compared binding of Jurkat T cell nuclear extract on the wild-type coding (+) strand of domain 2 (*GM93+*) (Fig. 2*D*, *lane 5*) to the CSD mutant oligonucleotides. Mutating one CSD binding site in GMm95 almost abolished binding (Fig. 2*D*, *lane 6*) and mutating the other CSD site in GMm103 also significantly reduced binding (Fig. 2*D*, *lane 7*). Again, it was not until we mutated both CSD binding sites, in the oligonucleotide GMm105, that we lost all binding (Fig. 2*D*, *lane 8*), indicating that both CSD binding sites were needed for full binding. These results agree with previous observations for NF-GMb CSD complex formation in HUT78 T cells and HEL fibroblasts (21–23).

In summary, Jurkat T cells contain CSD-like proteins that can form a NF-GMb complex on domains 1 and 2 of the GM-CSF proximal promoter. This binding is dependant on the presence of the previously defined CSD binding sites. However, unlike other cells previously examined, Jurkat T cell nuclear extracts do not form a NF-GMc complex on the GM-CSF promoter.

Jurkat T Cells Lack the 22- and 25-kDa Nuclear CSD Proteins—To determine the complement of CSD proteins present in the Jurkat T cell NF-GMb-containing complexes, UV cross-linking experiments were performed. As reported previously (21, 23), the HUT78 T cell NF-GMb complexes, which form on the noncoding (−) strand of domain 1 (GM− oligonucleotide), contain both 42- and 22-kDa proteins (Fig. 3*A*, *lanes 3* and *4*). The NF-GMc complex, shown to be lacking from Jurkat T cells (Fig. 2*A*), contained only a 22-kDa protein (Fig. 3*A*, *lane 5*). The 42-kDa protein apparently represents full-length CSD, whereas published reports where CSD proteins in the 22-kDa protein size range have been identified indicate that the 22-kDa protein is a potential splice variant or proteolytic cleavage product of the full-length protein (10, 23, 31, 51–53). On the noncoding (−) strand of domain 1 (GM− oligonucleotide), the Jurkat T cell NF-GMb complex contained only the 42-kDa protein (Fig. 3*A*, *lanes 1* and *2*). On the coding (+) strand oligonucleotide of domain 2 (GM93+) in HUT78 T cells, the NF-GMb complex contained the 42-, 22-, and an extra 25-kDa protein (Fig. 3*A*, *lanes 8–10*), whereas the NF-GMc complex contained only the 22- and 25-kDa proteins (Fig. 3*A*, *lane 11*). The 22-kDa protein has been shown to bind the most 5′ CSD binding site on domain 2 and the 25-kDa protein, the 3′ CSD binding site (21–23). The 25-kDa protein may represent an additional CSD splice variant or a different conformational binding of the 22-kDa protein (10, 23, 31, 51–53). In Jurkat T cells, we could only detect the 42-kDa protein in the NF-GMb complexes (Fig. 3*A*, *lanes 6* and *7*), and the 22- and 25-kDa proteins were lacking.

To determine whether the 22- and 25-kDa proteins were completely lacking from Jurkat T cells or simply unable to bind DNA, Western and Southwestern experiments were performed. Jurkat and HUT78 T cell nuclear extracts and recombinant dbpB-GST fusion protein were fractionated on a Laemmli SDS-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose, resulting in three replicate filter panels. One panel was probed with the CSD peptide antibody in a Western blot. The CSD peptide antibody recognized a single protein of ~75 kDa for recombinant dbpB-GST, which is the predicted size for the dbpB-GST fusion protein (Fig. 3*B*, *lane 3*). In the Jurkat and HUT78 T cell nuclear extract lanes the CSD antibody cross-reacted with many proteins (Fig. 3*B*, *lanes 1* and *2*). Proteins of the approximate size for the CSD factors identified in UV-cross-linking experiments (42, 25, and 22 kDa) were detected. A strongly reacting band of ~42 kDa was seen in both Jurkat and HUT78 T cell extracts, whereas the smaller CSD factors 25 and 22 kDa were seen only in the HUT78 T cell extract (Fig. 3*B*, *lanes 1* and *2*).

To confirm that these were the CSD factors identified by UV-cross-linking analysis above, Southwestern experiments were performed. In the Southwestern experiments, one panel was probed with the wild-type GM-CSF domain 1 GM- oligonucleotide (contains two CSD binding sites; Fig. 3*C*, *lanes 1–3*), and the other panel was probed with the double CSD binding site mutant of the GM− oligonucleotide, GMm23- (Fig. 3*C*, *lanes 4–6*). Recombinant dbpB-GST can be seen to bind to the wild-type GM− probe (Fig. 3*C*, *lane 3*) but not to the CSD mutant GMm23− probe (Fig. 3*C*, *lane 6*). Proteins of the same size, as detected by UV cross-linking the HUT78 T cell NF-GMb/c complexes (42, 25, and 22 kDa), were detected binding to the GM− probe in this assay (Fig. 3*C*, *lane 2*). Several other bands were also observed that may represent CSD family members. In Jurkat T cell extracts, a band of 42 kDa (and a smaller protein of 39 kDa) was the strongest band observed when the GM− oligonucleotide was used as a probe; of most significance, the 22- and 25-kDa bands were not observed (Fig. 3*B*, *lane 1*).

In summary, UV cross-linking the Jurkat T cell NF-GMb complex identified only a 42-kDa protein, which is also detected here by the CSD peptide antibody, and as expected, the 22- and 25-kDa proteins seen in the HUT78 T cell nuclear extract were not detected in Jurkat T cells (Fig. 3*B*, *lane 1*). As expected, the 42-, 25-, and 22-kDa proteins either did not bind or the binding was significantly less to the CSD mutant probe GM23− (Fig. 3*C*, *lanes 4–6*). Other proteins are seen binding to the GM− probe and not to the CSD mutant GMm23−. CSD proteins of greater than 42 kDa in size have been detected in nuclear extracts by other groups, and these proteins, seen binding here to the GM− probe, may represent other CSD family members that do not form part of the NF-GMb/c complexes.

CSD Factors Activate the GM-CSF Proximal Promoter in Jurkat T Cells—We have previously reported that overexpression of CSD proteins repress tumor necrosis factor α-mediated activation of the human GM-CSF proximal promoter in HEL fibroblasts (22). We wished to determine whether CSD proteins also acted as repressors of GM-CSF in Jurkat T cells. Surprisingly, we saw that when the GM-CSF reporter plasmid (pGM4-Luc), which contained all four CSD binding sites, was cotransfected into Jurkat T cells with CSD overexpression constructs for dbpAv (pSGdbpAv) and dbpB (pSGdbpB), that the CSD proteins behaved as activators in the presence of PMA/Ca^{2+} ionophore (Fig. 4*A*). That they were only involved in activation of the GM-CSF promoter construct when the cells were stimulated with PMA/Ca^{2+} ionophore indicated the CSD proteins either needed to be activated to function or that they cooperate with other proteins to function.

We wished to determine which region of the GM-CSF proximal promoter was required for CSD-mediated activation and the role, if any, that the four CSD sites across the promoter played in this activation. To do this, CSD overexpression constructs were cotransfected with reporter constructs containing variable numbers of CSD sites (Fig. 4*B*). Initially the pGMCK-1(2)-TK/Luc construct (−114 to −70) containing only the two domain 1 CSD sites of the GM-CSF promoter was used in experiments. Cotransfections showed that both CSD factors (pSGdbpAv and pSGdbpB) could coactivate this construct in the presence of PMA/Ca^{2+} ionophore (Fig. 4*B*). Since the do-

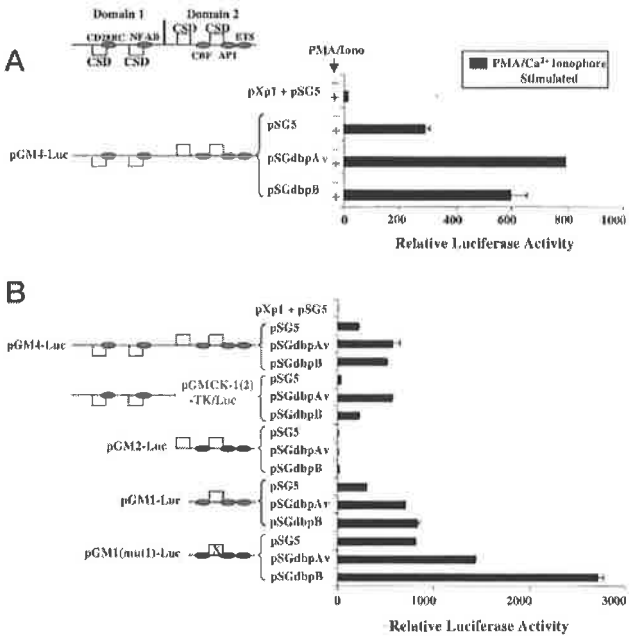

FIG. 4. **CSD factors activate the GM-CSF proximal promoter in Jurkat T cells.** The human GM-CSF proximal promoter and luciferase plasmids used are represented diagrammatically (sequences are shown in Fig. 1*A*). *Boxes* represent CSD binding sites, the *box* with an X in it represents where the CSD binding site has been mutated, and *ovals* represent the binding sites of the corresponding transcriptional factor in the top diagram. In all transfection experiments cells were treated with or without PMA/Ca^{2+} ionophore for 8 h, and luciferase activities are given relative to untreated pXP1 background plasmid cotransfected with pSG5, which is given a value of 1. All untreated values were below a relative luciferase activity of 10. *A*, the reporter plasmid pGM4-Luc was cotransfected into Jurkat T cells with the empty pSG5 expression vector and with expression plasmids containing full-length dbpAv (pSGdbpAv) and full-length dbpB (pSGdbpB). *B*, each GM-CSF promoter luciferase reporter plasmid, pGM4-Luc, pGMCK-1(2)-TK/Luc, pGM2-Luc, pGM1-Luc, and pGM1(mut1)-Luc, was cotransfected into Jurkat T cells with pSG5, pSGdbpAv, and pSGdbpB. Only PMA/Ca^{2+} ionophore-treated values are shown; all untreated values were below a relative luciferase activity of 10.

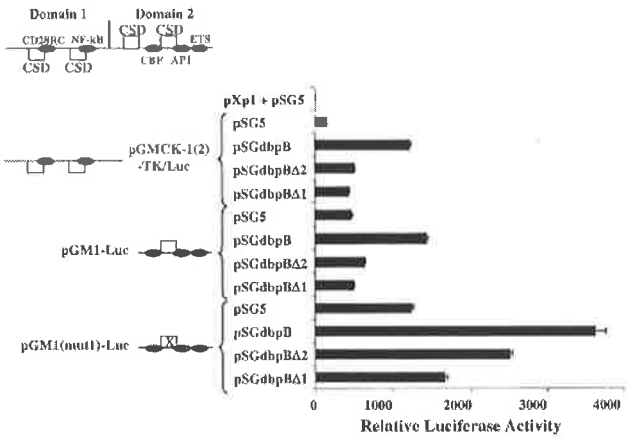

FIG. 5. **The C-terminal domain of dbpB is required for CSD mediated activation of the GM-CSF proximal promoter.** GM-CSF promoter luciferase reporter plasmids pGMCK-1(2)-TK/Luc, pGM1-Luc, and pGM1(mut1)-Luc were cotransfected into Jurkat T cells with pSG5, pSGdbpAv, pSGdbpB, and expression vectors encoding dbpB deletions pSGdbpBΔ2 and pSGdbpBΔ1. Only PMA/Ca^{2+} ionophore values are shown, with all unstimulated values below a relative luciferase activity of 10.

main 1 region was cloned upstream of the basal thymidine kinase (TK) promoter in pGMCK-1(2)-TK/Luc, CSD factors were tested for activator function on the TK promoter. We observed that CSD factors could not activate the TK promoter in the presence of PMA/Ca^{2+} ionophore (data not shown). We next tested a construct containing only the domain 2 CSD sites to determine whether they too were targets for CSD-mediated activation. When pGM2-Luc (−71 to +28, containing only the domain 2 CSD sites) was used in cotransfection experiments, CSD-mediated activation was still observed, but both basal (pSG5)- and CSD (pSGdbpAv, pSGdbpB)-coactivated expression was dramatically reduced relative to pGM4-Luc (Fig. 4*B*). When however, the pGM2-Luc construct was truncated by five base pairs to create pGM1-Luc, higher levels of activity and CSD factor-mediated activation were restored (Fig. 4*B*). The five-base pair truncation removed the 5′ CSD site in domain 2, leaving only the single 3′ domain 2 CSD site. These results therefore indicated that the 5′ CSD binding sites had repressor activity.

To determine whether CSD-mediated coactivation of pGM1-Luc was acting through the single CSD site in this construct, coactivation levels were compared between pGM1-Luc (which contains one CSD site) and pGM1(mut1)-Luc (where the one CSD site has been mutated) (Fig. 4*B*). Both pGM1-Luc and pGM1(mut1)-Luc were cotransfected into Jurkat T cells with constructs encoding for CSD factors dbpAv and dbpB. The PMA/Ca^{2+} ionophore stimulated levels of both basal- and CSD-mediated coactivation in pGM1(mut1)-Luc were greater than those seen on pGM1-Luc (up to 2-fold greater) (Fig. 4*B*). These data taken altogether therefore demonstrate that both the domain 2 CSD sites have repressor activity. Our results also show that the coactivation effect of CSD proteins on the GM-CSF promoter did not require contact with these CSD sites. We were unable to repeat similar experiments on domain 1 CSD sites due to the overlap of the NF-κB sites with the CSD sites. Mutation of the domain 1 NF-κB sites results in loss of GM-CSF promoter activity (54).

The C-terminal Domain of dbpB Is Required for CSD-mediated Activation of the GM-CSF Proximal Promoter—Because interactions between CSD factors and other transcriptional regulators have been implicated in mechanisms of activation of a number of viral and cellular genes, a potential mechanism for the coactivation effect seen here was via interaction of CSD factors with other proteins. The C-terminal domain of dbpB has been demonstrated to be required for protein-protein interactions (10–15). To explore the possibility that the coactivation effect was mediated via CSD protein-protein interactions, expression constructs were made encoding truncations of the dbpB CSD protein. In the dbpB deletion construct pSGdbpBΔ2, most of the C-terminal region of dbpB was deleted, and in pSGdbpBΔ1, both the C-terminal and CSD domain were deleted (Fig. 1*B*). The dbpB and dbpB deletion constructs were cotransfected into Jurkat T cells with either the pGM1-Luc or pGM1(mut1)-Luc domain 2 constructs or the pGMCK-1(2)-TK/Luc domain 1 construct, and the cells were stimulated with PMA/Ca^{2+} ionophore. Removal of most of the C-terminal region of the dbpB in pSGdbpBΔ2 resulted in a significant decrease in the level of PMA/Ca^{2+} ionophore coactivation on all constructs (Fig. 5). Removal of both the C-terminal and CSD domain of dbpB in pSGdbpbΔ1 resulted in a return to near basal levels of expression on both domain 1 and 2 constructs (Fig. 5).

Taken altogether the results presented in Figs. 4 and 5 indicate, first, that at least the domain 2 CSD binding sites in the GM-CSF proximal promoter act as repressor elements, second, that the PMA/Ca^{2+} ionophore coactivation by CSD proteins is not associated with the CSD binding sites (at least in domain 2), and third, that the coactivation seen is mediated primarily via the C-terminal protein interaction domain of dbpB.

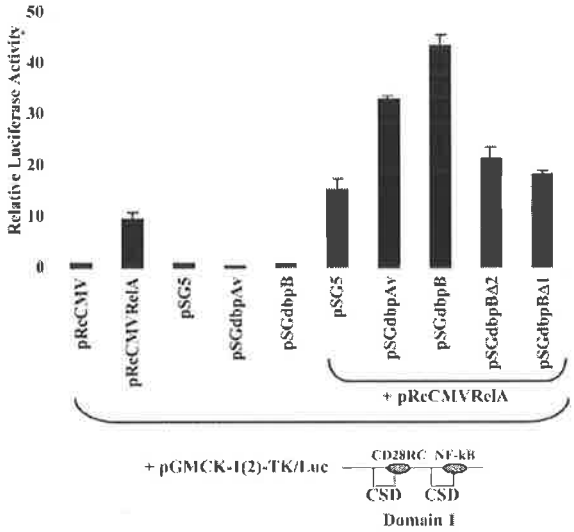

FIG. 6. **CSD factors and RelA act to synergistically activate the GM-CSF proximal promoter.** The GM-CSF promoter domain 1 luciferase reporter plasmid pGMCK-1(2)-TK/Luc was cotransfected into Jurkat T cells with pRcCMV, pRcCMVRelA, pSG5, pSGdbpAv, or pSGdbpB. pGMCK-1(2)-TK/Luc and pRcCMVRelA were cotransfected together into Jurkat T cells with pSG5, pSGdbpAv, pSGdbpB, pSGdbpBΔ2, and pSGdbpBΔ1. Values are shown relative to pGMCK-1(2)-TK/Luc cotransfected with the pRcCMV backbone plasmid, which is given a value of 1.

CSD Factors and RelA Act to Synergistically Activate the GM-CSF Proximal Promoter—The C-terminal domain of CSD proteins has been shown to be involved in interactions with other transcription factors and, to date, of the transcription factors acting across the GM-CSF proximal promoter; only RelA/NF-κB p65 has been reported to interact with CSD proteins (10–15, 55). To investigate the potential role of a CSD-RelA interaction in the CSD-mediated activation of the GM-CSF promoter domain 1 sequences (which contains RelA/NF-κB p65 sites; Refs. 20 and 54), RelA and CSD expression constructs were cotransfected with the GM-CSF domain 1 reporter construct pGMCK-1(2)-TK-Luc (Fig. 6). In Fig. 6, we show as previously reported that RelA/NF-κB p65 stimulated the pGMCK-1(2)-TK-Luc construct in unstimulated cells, whereas the CSD factors could not (56). When the pGMCK-1(2)-TK/Luc reporter construct was cotransfected with expression constructs for both RelA (pRcCMVRelA) and dbpAv (pSGdbpAv) or dbpB (pSGdbpB), synergistic activation was observed (Fig. 6). Repeating the triple cotransfection with the dbpB C-terminal truncation (pSGdbpBΔ2) significantly reduced the synergistic activation (Fig. 6), suggesting that the C terminus of the CSD protein dbpB protein may be involved in interactions with RelA to bring about activation. Cotransfecting with the dbpB construct pSGdbpBΔ1 (missing both the C-terminal and CSD domains) further reduced the activation to basal pSG5 levels (Fig. 6). These experiments show that RelA/NF-κB p65 and the CSD proteins can cooperate to activate GM-CSF promoter function and that the C terminus of dbpB is essential for this cooperation.

DISCUSSION

Cold shock domain family members have been associated with diverse functions including the ability to both activate and repress transcription and translation. Here we investigated the role of the CSD proteins dbpAv and dbpB in regulation of GM-CSF promoter in Jurkat T cells. We have previously reported that HUT78 T cell and HEL fibroblast nuclear CSD proteins bind to defined single-stranded regions along domain 1 and domain 2 of the GM-CSF promoter as NF-GMb and NF-GMc complexes (21–23). We proposed that the binding of NF-GMb/c CSD proteins along the GM-CSF promoter resulted in or stabilized a single-stranded DNA structure, thereby preventing the binding of transcriptional activators that are dependent on double-stranded DNA for binding and activity. This proposed structure would be an efficient means of silencing the GM-CSF promoter and many other promoters where NF-GMb/c CSD proteins have been shown to act as repressors of transcription (13, 23, 25–27, 33, 57). The specific arrangement of NF-GMb/c CSD binding sites observed on the GM-CSF promoter, a pair of distal sites binding on the noncoding strand and a pair of proximal sites binding the coding strand, can also be found in the promoters of the granulocyte-colony stimulating factor and interleukin-3 cytokine genes with overlapping GM-CSF expression patterns (58, 59).

Analysis of Jurkat T cell extracts identified nuclear CSD proteins binding to the CSD binding sites across the GM-CSF promoter as a NF-GMb-like complex. Competitions with single-stranded DNA CSD site sequences and CSD antibody experiments in addition to UV cross-linking indicated that the Jurkat T cell NF-GMb complex was made up of only a 42-kDa CSD protein. With the lack of the NF-GMc complex in Jurkat T cells, we also observed a lack of the 22- and 25-kDa CSD proteins previously identified in HEL fibroblasts and HUT78 T cells (21, 23). These 22- and 25-kDa CSD proteins probably represent CSD splice variants or proteolytic cleavage products, as reported by others, that lack the C-terminal protein-protein interaction domain (22, 31–33, 35, 53).

Transient transfection in Jurkat T cells showed that in contrast to the repression observed in HEL fibroblasts, the CSD proteins, dbpAv and dbpB, functioned as coactivators on a 140-base pair region of the human GM-CSF proximal promoter. This activation was only observed when T cells were activated with PMA/Ca^{2+} ionophore. We showed that both the GM-CSF domain 1 region (containing NF-kB/RelA sites) and the domain 2 region (containing the CBF, AP1, and ETS/NFAT sites) are able to independently respond to CSD-mediated activation. We also showed that this response to CSD-mediated activation did not require the CSD binding sites, at least in the −60 to +28 region of domain 2. The CSD binding sites, however, still retain repressor function as defined by deletion and mutation.

CSD factors have been reported to activate a few cellular genes including c-Myc, α1(I) procollagen, and metalloproteinase (MMP-2) and also regulate many viral promoters such as human immunodeficiency virus, human T-cell lymphotrophic virus, and Rous sarcoma virus long terminal repeats and human polyomavirus JC late viral promoter (5, 31, 32, 35, 36, 60), but the precise mechanisms of CSD-mediated activation has not been well defined. To determine the mechanism of CSD-mediated activation in Jurkat T cells, we examined the possible role of CSD protein interactions with other transcription factors. Our data indicate that CSD factors dbpB and dbpAv are able to act with RelA in transient transfections to synergistically activate the GM-CSF promoter in the absence of PMA/Ca^{2+} ionophore stimulation. Our data suggest that this coactivation of GM-CSF by CSD and RelA proteins is via protein-protein interactions. We demonstrated this by truncating dbpB, removing the C terminus of the protein, which has been shown to be essential for CSD protein-protein interactions (11, 13, 30, 61–63). When the dbpB deletion construct removing the C terminus was used, PMA/Ca^{2+} ionophore-mediated coactivation was significantly reduced as was the ability of dbpB and RelA to synergize, indicating that the coactivation was mediated primarily through the C-terminal region of the CSD pro-

tein and, therefore, most likely via protein-protein interactions. Raj *et al.* (15, 32) find that CSD and RelA can interact in solution and that this interaction leads to increased RelA binding to the human polyomavirus JC viral promoter. In these experiments RelA conversely decreased CSD binding to its single-stranded DNA binding site. This CSD-RelA interaction was implicated in activation of the human polyomavirus JC early promoter (32). Similarly, we have found that RelA can decrease CSD binding to its GM-CSF single-stranded DNA binding site (data not shown).

Taken together our data suggest a model whereby, first, in unstimulated Jurkat T cells, CSD proteins bind to single-stranded DNA in a structure as described for the repression of the GM-CSF gene by CSD proteins in HEL fibroblasts (21–23). This model has been suggested for a number of other genes repressed by CSD proteins (26, 27, 50). Upon T cell stimulation (mimicked by PMA/Ca^{2+} ionophore) it has been well documented that many transcriptional activators (like NF-κB p50, and RelA) are up-regulated, whereas the level of CSD proteins in the nucleus remains constant (reviewed in Refs. 38 and 20).[2] We and others (15)[2] have shown that RelA is able to dissociate dbpB from its single-stranded target, and we propose that this results in destabilization of the single-stranded DNA structure, facilitating a return of the DNA to its double-stranded conformation . This would allow the transcriptional activators (like NF-κB, AP-1, CBF, ETS/NFAT) to bind initiating transcriptional activation.

We have presented data here that indicate a functional CSD-RelA interaction that is involved in activation of the GM-CSF promoter, domain 1 region. We have also shown CSD mediated activation via the domain 2 region, which contains no RelA sites, and hence, CSD proteins may also be able to interact with other transcription factors binding to this region. There is a growing list of proteins where CSD protein interactions have been implicated to affect promoter function (NF-κB, mitrochondrial SSB, ZO-1, heterogeneous nuclear ribonucleoprotein K, TBP, NF-Y, YY-1, AP-2, SP-1, Tat, PuRα) (5, 10–13, 15, 23, 29, 30, 55, 64). Potential targets on the GM-CSF promoter domain 2 for CSD interactions could be SP1 and YY1, both of which have been shown to regulate GM-CSF function in T cells (20, 65, 66).

The complement of CSD protein subtypes in the cell may also affect CSD function. We have previously observed in HEL fibroblasts (with 42-, 22-, and 25-kDa CSD proteins) that CSD proteins acted to repress activation, whereas in T cells (with only the 42-kDa protein) CSD factors function to activate when the cells were PMA/Ca^{2+} ionophore-stimulated. The reason for this difference between the cell types is as yet unclear but may be related to the proposed lack of C-terminal-protein interaction sequences in the 22- and 25-kDa CSD subtypes observed in HEL fibroblasts (22, 31–33, 35, 53). Taking into account our previous results and the reported function associated with each of the CSD protein domains, the 42-kDa CSD factor is implicated in repression (via the CSD domain) and activation (via the C-terminal domain), whereas the 22-kDa CSD factors may be involved only in repression (21–23). In fibroblasts, where CSD factors act to repress GM-CSF promoter function, UV-cross-linking experiments indicated that the 22-kDa CSD protein sub-population makes up a greater proportion than the 42-kDa sub-population, suggesting that the CSD intracellular environment is predisposed toward truncated forms of the CSD proteins and, hence, repression (21–23). In Jurkat T cells, where CSD factors act to mediate activation of the GM-CSF promoter, only the full-length 42-kDa CSD protein is present. The 42-kDa CSD protein, although still able to bind DNA and potentially repress in the unstimulated state, is also able to interact with proteins in a stimulated cell via the C-terminal protein-protein interaction domain to bring about activation. Therefore the differences in effect when full-length CSD factors were overexpressed in Jurkat T cells and HEL fibroblasts may be due to the intrinsic difference in the ratio of CSD factor subtypes and resulting intracellular environment already present in the two cell types.

We have shown, therefore, that CSD proteins can act to repress or activate the GM-CSF gene in different cell types. Repression is associated with the CSD binding sites located across the promoter, whereas activation may relate to CSD cooperation with other transcription factors. We also raise the possibility that the cellular content of CSD proteins predetermines the functional outcome in the transcription response.

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