# DEFINING THE MOLECULAR EVENTS REQUIRED FOR GM-CSF GENE ACTIVATION IN T CELLS.

**A thesis submitted in fulfillment of the requirements of the degree of doctor of philosophy**

**by**

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**Discipline of Biochemistry**

**University of Tasmania**

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# DECLARATION

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgement is made in the text.

Kate Helen Brettingham-Moore

I assess my contribution to the work described in each chapter to be the following;

Chapter 3: 100%

Chapter 4: 100%

Chapter 5: 95%; microarray hybridizations were undertaken in collaboration with Dr. Kaiman Peng at the Biomolecular Resource Facility, JCSMR, ANU.

(i) This thesis is less than 100,000 words in length not including tables, figure legends and bibliographies

(ii) Papers arising from the period of my PhD candidature

1. **Brettingham-Moore K. H.**, Rao S., Juelich T., Shannon M.F. and A. F. Holloway (2005) GM-CSF promoter chromatin remodelling and gene transcription display distinct signal and transcription factor requirements. ***Nuc. Acids Res.*** **33 (1)**: 225-234

2. Shannon M.F., Chen X., **Brettingham-Moore KH**. and AF. Holloway (2006) Chromatin remodelling; distinct molecular events during differentiation and activation of T cells. ***Curr. Imm. Rev.*** **2**: 273-289

3. **Brettingham-Moore KH.,** Chen X., Oakford, P., Shannon MF. and AF Holloway (2006). Enrichment of histone acetylation and the Brg1 protein generates a transcriptionally competent environment at the GM-CSF promoter. (Submitted).

## ABSTRACTS

Holloway, A. F., Rao, S., **Brettingham-Moore, K.,** and M. F. Shannon. Selective Remodelling of a Single Nucleosome Precedes GM-CSF Transcription in T cells. *Proceedings of the Australian Society for Biochemistry and Molecular Biology,* ComBio, Sydney, NSW. Sym 58-04 (2002).

**Brettingham-Moore, K.**, Rao, S., Shannon, M. F., and A. Holloway. Dissecting the Molecular Events Involved in Activation of GM-CSF Gene Expression in T cells. *24th Annual Conference on the Organisation and Expression of the Genome.* Lorne, Victoria. Poster 1-25 (2003).

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# ABREVIATIONS

| | |
|---|---|
| 3' | three prime |
| 5' | five prime |
| APC | antigen presenting cell |
| APS | ammonium persulfate |
| ATP | adenosine triphosphate |
| BAF | brg1 associated factor |
| bp | base pairs |
| BPB | bromophenol blue |
| Brg1 | brahma related gene 1 |
| Brm | brahma |
| BSA | bovine serum albumin |
| $CaCl_2$ | calcium chloride |
| CBP | CREB binding protein |
| CD28RR | CD28 response region |
| CD28RE | CD28 response element |
| cDNA | complimentary deoxyribonucleic acid |
| CHART PCR | chromatin accessibility by real-time PCR |
| ChIP | chromatin immunoprecipitation |
| CHX | cycloheximide |
| $CO_2$ | carbon dioxide |
| cRNA | complimentary ribonucleic acid |
| CsA | cyclosporin A |
| DAG | diacylglycerol |
| DMSO | dimethylsulfoxide |
| DMEM | Dulbecco's modified Eagle medium |
| DNA | deoxyribonucleic acid |
| dNTPs | deoxyribonucleotides |
| DTT | dithiothreotol |
| E. Coli | Escherichia coli |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid |

| | |
|---|---|
| EMSA | electrophoretic mobility shift assay |
| FACS | fluorescently activated cell sorting |
| FCS | fetal calf serum |
| g | relative centrifugal field |
| g | grams |
| GAPDH | glyceraldehyde phosphate dehydrogenase |
| GFP | green fluorescent protein |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| H | histone |
| HAT | histone acetyltransferase |
| HCl | hydrochloric acid |
| HDAC | histone deactylase |
| I | ionophore |
| IgG | immunoglobin |
| IL | interleukin |
| IFN | interferon |
| IP | immunoprecipitate |
| $IP_3$ | inositol 1, 4, 5 triphosphate |
| IVT | *in vitro* transcription |
| K | lysine |
| kb | kilobase |
| KCl | potassium chloride |
| kDa | kilodaltons |
| LiCl | lithium chloride |
| LPS | lipopolysaccharide |
| M | molar |
| M-CSF | macrophage colony stimulating factor |
| MDa | megadaltons |
| mg | milligrams |
| $MgCl_2$ | magnesium chloride |
| mGM-I | murine GM primer set I |
| mGM-I | murine GM primer set -I |
| mGM+I | murine GM primer set +I |
| MHC | major histocompatibility complex |

| | |
|---|---|
| mIFNγ | murine IFNγ primer set |
| mIFNγP | murine IFNγ promoter primer set |
| mIL4 | murine IL4 primer set |
| mIL5 | murine IL5 primer set |
| mIL4p | murine IL4 promoter primer set |
| mIL5p | murine IL5 promoter primer set |
| mM-CSF | murine M-CSF primer set |
| mM | millimolar |
| MNase | micrococcal nuclease |
| MOPS | 3-(N-morpholio)propanesulfonic acid |
| mRNA | messenger ribonucleic acid |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| NFAT | nuclear factor of activated T cells |
| NF-κB | nuclear factor kappa B |
| nm | nanometre |
| nM | nanomolar |
| NS | non stimulated |
| NTC | no template control |
| O.D. | optical density |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| P/I | PMA and ionophore |
| PI3K | phosphatidyl inositol-3 kinase |
| $PIP_2$ | phosphatidyl inositol (4, 5) biphosphate |
| PKC | protein kinase C |
| pM | picomolar |
| PMA/P | phorbol myristate acetate |
| PMSF | phenylmethylsulphonylfluoride |
| PTX | pentoxifylline |
| R | arginine |
| RNA | ribonucleic acid |
| RNase | ribonuclease |

| | |
|---|---|
| RPMI | Roswell park memorial institute |
| SAPE | streptavidin-phycoerythrin |
| SDS | sodium dodecylsulphide |
| SDS PAGE | sodium dodecylsulphide polyacrylamide gel electrophoresis |
| TCR | T cell receptor |
| TE | tris/EDTA |
| TEMED | N,N,N',N'-Tetramethylethylenediamine |
| Th | T helper cell |
| TNT | tris/sodium/tween solution |
| TSA | trichostatin A |
| U | units |
| µg | micrograms |
| µM | micromolar |

# ABSTRACT

Granulocyte macrophage colony stimulating factor (GM-CSF) is a potent regulator of haemopoiesis and is vital for immune function. GM-CSF is rapidly, but transiently activated in response to T cell activating signals. It is now well established that activation of the GM-CSF gene in T cells is accompanied by distinct changes in chromatin structure across the promoter region. The aim of this thesis was to investigate these chromatin remodelling events and their association with gene transcription.

Analysis of GM-CSF promoter accessibility and transcription in response to various pharmacological stimulations demonstrated that the processes of transcription and promoter chromatin remodelling were distinct, with each requiring different factors and signals. While chromatin remodelling was found to be dependent on factors activated downstream of PKC signalling, transcription required both PKC and calcium signalling pathways. Nuclear activation of the NF-κB transcription factor, c-Rel was strongly correlated with chromatin remodelling events. In contrast NFAT transcription factors were demonstrated to be required for GM-CSF transcription but not chromatin remodelling. In addition, remodelling of the GM-CSF promoter was found to be relatively stable in contrast to the more transient profile observed for transcription.

The ATPase component of the SWI/SNF chromatin remodelling complex has previously been shown to associate with the GM-CSF promoter *in vitro*. To determine whether Brg1 was involved in activation of the GM-CSF gene *in vivo*, T cells were transfected with an ATPase defective Brg1 mutant construct. Analysis of these cells demonstrated that efficient activation of the GM-CSF gene is dependent on Brg1. Surprisingly, chromatin immunoprecipitation experiments revealed that Brg1 is bound to the GM-CSF promoter in resting T cells and is depleted concomitant with chromatin remodelling. These data lead to the hypothesis that Brg1 is involved in forming a basal chromatin state that is transcriptionally competent. In support of this, Brg1 is not bound to the GM-CSF promoter in B cells, which do not express GM-CSF. However a competent chromatin environment can be created in these cells by increasing histone acetylation levels.

Data presented here are consistent with a model in which the basal state of the GM-CSF promoter is maintained in a transcriptionally competent state in resting T cells via histone acetylation and Brg1 recruitment. Such a chromatin environment may ensure that GM-CSF can be activated rapidly in response to T cell activation signals. Microarray analysis was subsequently used to identify genes which may be similarly poised to respond to T cell

activation signals by the constitutive recruitment of Brg1. A number of cytokine genes were identified as Brg1 targets. One of these, Interferon gamma (IFNγ) was found to share a similar activation profile to GM-CSF and data presented here suggests it may be regulated by a common mechanism. As observed for GM-CSF, Brg1 is constitutively poised at the IFNγ promoter in resting EL-4 T cells and lost from the promoter concomitant with gene activation.

# CHAPTER 1.
# INTRODUCTION

## 1.1 IMMUNE GENE REGULATION

The immune system is a complex network of cells, tissues and organs which provide host defense against invading pathogens. Capable of providing remarkable surveillance, the immune system helps to fight bacterial and viral infections and eradicate cancerous body cells. The innate immune system includes cells which provide the initial line of defense against infection and helps to detect and eliminate invading pathogens before infection can take hold. Adaptive immunity occurs once immune cells gain the ability to recognize a pathogen and allows the immune system to mount an even stronger attack. The primary response to infection is inflammation which results in chemokine mediated recruitment of innate immune cells to the site of injury or infection (reviewed in Schluger and Rom, 1997). These cells move from capillaries into infected tissue to scavenge bacteria and cellular debris and help to prevent the spread of infection. A range of cell types derived from myeloid and lymphoid lineages help to mediate the immune response including T cells, B cells, macrophages, neutrophils, mast cells, granulocytic and dendritic cells. Helper T cells, also known as CD4 positive (CD4+) T cells, aid in both cellular and humoral immunity. They help to trigger antibody production by B cells and activate other T cells and macrophages. CD8 positive (CD8+) T cells, also known as cytotoxic T cells, can differentiate into killer T cells which attack and destroy infected cells. Infection stimulates the differentiation and proliferation of immune cells by triggering changes in gene expression programs and cytokine profiles. The swift responses characteristic of the immune system, rely on the rapid and co-ordinate activation and silencing of various genes (reviewed in Holloway *et al*, 2002). The activation of these immune genes helps to protect the organism from disease, however when the regulation of these genes is compromised, the ability to defend against infection and disease is weakened. Therefore, understanding the precise mechanisms underlying immune gene regulation is vital.

## 1.1.1 T CELL ACTIVATION

T cell activation plays a central role in cell mediated immunity. During infection, antigens are taken up by antigen presenting cells (APCs) which process and present them to T cells. APCs (including macrophages, B cells and dendritic cells) present the processed antigen bound to major histocompatibility complex (MHC) antigens. These self-antigens aid T cells in the recognition of foreign antigens (reviewed in Stefanova *et al*, 2003). T cell activation is a complex process requiring two signals, which are occupancy of the T cell receptor (TCR) and interaction with costimulatory ligands on the APC. When a T cell comes in contact with an APC, the TCR is stimulated along with the co-stimulatory molecule. The TCR recognises and binds to antigen fragments presented together with MHC molecules (reviewed in van der Merwe and Davis, 2003). The second signal is provided by stimulation of the costimulatory molecule which augments TCR activation. The main costimulatory molecules are CD2, LFA-1 and CD28 which bind the LFA-3, ICAM-1 and B7 ligands respectively (reviewed in Wingren *et al*, 1995). A specialized junction formed between the APC and T cell is stabilized by costimulators and helps to sustain and amplify signalling from the TCR (reviewed in Acuto and Cantrell, 2000).

T cell stimulation triggers an intracellular cascade of signalling which leads to the nuclear translocation of numerous transcriptional activators required for activating immune genes. TCR activation and stimulation of the costimulatory molecule initiate signalling cascades by activating protein tyrosine kinases (reviewed in Acuto and Cantrell, 2000). The tyrosine kinases regulate the metabolism of inositol phospholipids via the Ras and Rho family of GTPases. Changes in inositol phospholipid metabolism directly influence calcium levels and the activity of numerous serine/threonine kinases including PKC and phosphatidyl inositol-3 kinase (PI3K). The TCR controls the production of inositol 1, 4, 5 triphosphate ($IP_3$) and diacylglycerol (DAG) through the hydrolysis of phosphatidylinositol (4, 5) biphosphate ($PIP_2$) (reviewed in Gupta, 1989). Therefore these tyrosine kinases help to regulate the intracellular levels of calcium and the activity of protein kinase C (PKC; Figure 1.1) which plays a significant role in immune responses (reviewed in Tan and Parker, 2003). These signalling cascades ultimately result in the activation of an array of cytokine genes which help trigger the proliferation and differentiation of cells required for an immune response.

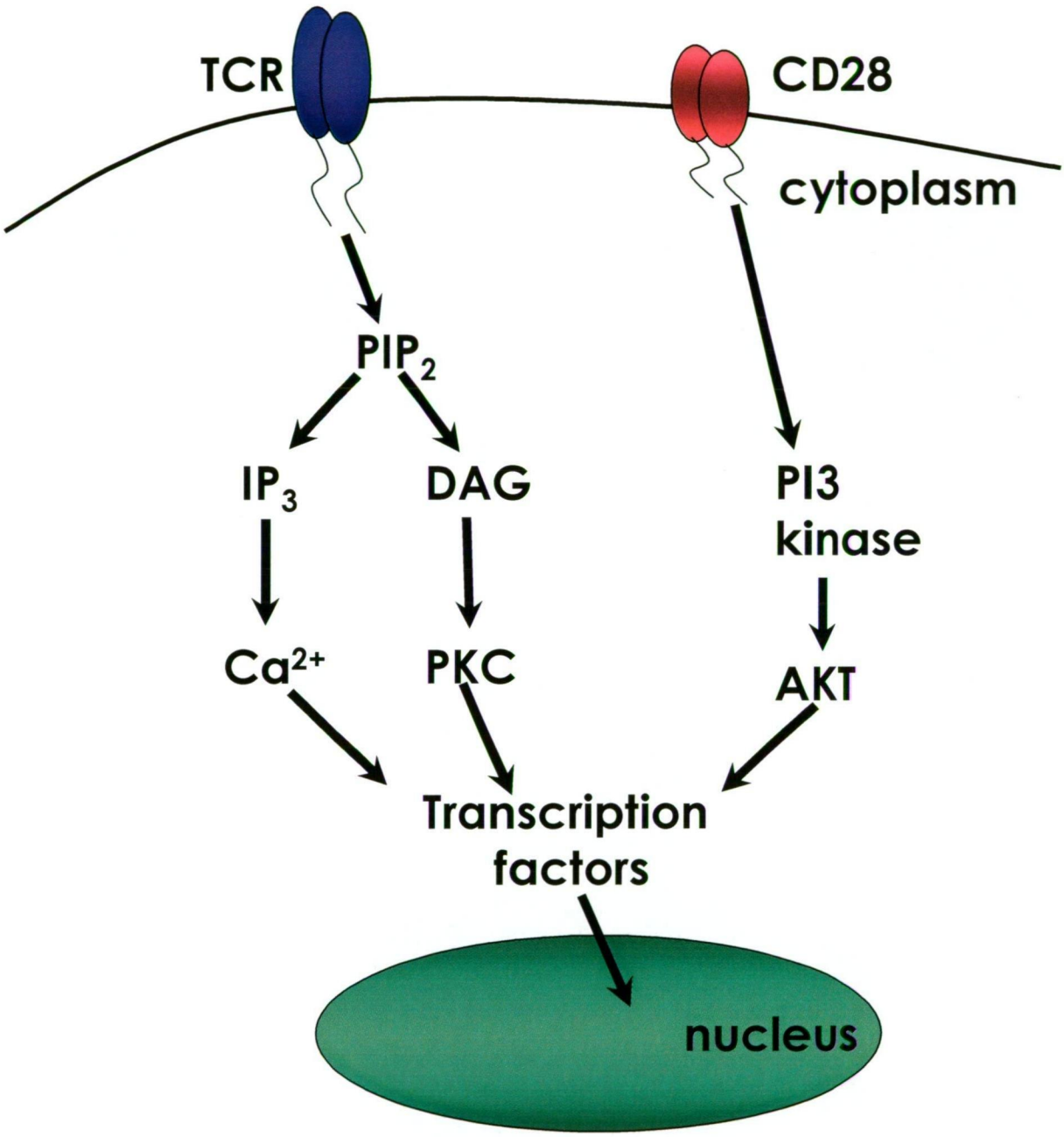



**Figure 1.1. Overview of the signalling pathways leading to cytokine gene expression following TCR ligation and CD28 stimulation.**

## 1.1.2 CYTOKINES

Activated T cells produce an array of cytokines which relay signals between cells within the immune system. These small protein hormones play an important role in the development of the immune system and the immune response. The types of cytokines produced depend on the cell type, stimulus and the environment and can enhance or inhibit cellular functions such as cell growth and differentiation (Lotem *et al*, 1991; Gasson, 1991). Most cytokines act locally in a paracrine manner and stimulate the proliferation of progenitor blood cells in the bone marrow. Cytokine expression is controlled primarily at the level of transcription. The genes encoding cytokines are often highly inducible and are rapidly switched on and off during an immune response. Cytokine genes are often silent or expressed at very low levels in resting cells, however following immune stimulation, expression is induced to high levels for a short period of time following which levels return to basal (reviewed in Holloway *et al*, 2002).

Cytokine gene expression is restricted to particular cell types and often only expressed following exposure to a specific set of signals. While some cytokines are widely expressed across a range of cell types, certain cytokines are restricted to a single cell type. Restricted cytokine expression profiles are observed in differentiated Th1 and Th2 cells. Naïve T cells are capable of differentiating into two distinct subtypes, Th1 and Th2 cells which are characterized by distinct patterns of cytokine gene expression. Th1 cells produce IFN-γ and are involved in protecting against intracellular bacterial and viral infections. Th2 cells express IL-4, IL-5 and IL-13 to fight extracellular parasites. The types of cytokines produced therefore depend on the stimulus.

# 1.2 GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)

Granulocyte-macrophage colony-stimulating factor (GM-CSF or CSF2) is a 22 kDa glycoprotein consisting of 127 amino acids derived from a 144 amino acid precursor. It was first purified from mouse lung conditioned medium almost 30 years ago (Burgess *et al*, 1977) and identified as a factor which could stimulate cell proliferation from immature bone-marrow derived progenitors (reviewed in Gasson, 1991). GM-CSF is a cytokine

involved in regulating the differentiation, maturation and survival of inflammatory cells. It plays a role in host defense and homeostatic maintenance of myelopoiesis. Compared to other cytokines, GM-CSF is relatively widely expressed. Following immune stimulation a wide range of cell types including Th1 and Th2 cells, macrophages, fibroblasts and endothelial cells secrete GM-CSF (reviewed in Gasson *et al*, 1990). Specific high affinity receptors are found on the surface of target cells which include multipotential haemopoietic stem cells, mature neutrophils, monocytes and macrophages. The major progeny of haemopoietic progenitor cells stimulated by GM-CSF are granulocytes and macrophages. *In vitro* research has shown that GM-CSF stimulates the growth and differentiation of GM lineage cells from bone marrow progenitor cells (Burgess *et al*, 1977). *In vivo* studies indicate that GM-CSF is predominantly involved in the recruitment and activation of myeloid lineage cells at sites of inflammation (Mock and English, 1990).

The role of GM-CSF in immune regulation has been highlighted by studies involving GM-CSF knockout mice. While the GM-CSF$^{-/-}$ mouse is viable with no abnormalities in haemopoiesis for up to 12 weeks, lung development is abnormal as is the accumulation of surfactant and protein in the alveolar spaces, suggesting that GM-CSF has a role in pulmonary homeostasis (Stanley *et al*, 1994). In addition the response to infection is significantly reduced in GM-CSF deficient mice demonstrating that this cytokine is critical for proper immune function (Lieschke *et al*, 1994; Zhan *et al*, 1998). Transgenic mice overexpressing GM-CSF have enlarged livers and spleens and increased numbers of macrophages and granulocytes (Burke *et al*, 2004) providing further evidence of a role for GM-CSF in regulating haemopoesis.

Aberrant expression of GM-CSF has been linked to a number of disease states including asthma (Stanley *et al*, 1994), atopy (Cousins *et al*, 1996), rheumatoid arthritis (Campbell *et al*, 1997) and myeloid leukemias (Young *et al*, 1987). Myeloid leukemias are clonal neoplasms of the granulocyte-macrophage precursor cells. This type of leukemia is thought to arise from the over-expression of GM-CSF via the action of viral or cellular oncogenes (Metcalf, 1985). GM-CSF has also been suggested as a tumor marker due to its critical role in regulating haemopoetic growth and differentiation (reviewed in Mroczko and Szmitkowski, 2004). In asthma it is thought GM-CSF plays a role in establishing the level of surfactant in the lungs (Stanley *et al*, 1994) and inflammation of the synovial fluid

in the arthritic joint is exacerbated by GM-CSF (reviewed in Feldmann *et al*, 1996).

## 1.2.1 REGULATION OF GM-CSF

GM-CSF expression is regulated primarily at the level of transcription (Chan *et al*, 1986). The rapid induction of GM-CSF gene expression following immune stimulation involves the activation and nuclear translocation of a range of transcription factors which bind to regulatory regions of the GM-CSF gene. The human GM-CSF gene is located on chromosome 5q31.1 (Miyatake *et al*, 1985). The murine equivalent is located on sub-band B1 on chromosome 11 (Barlow *et al*, 1987). Both the murine and human genes are approximately 2.5kb in length and comprise 4 exons and 3 introns (Miyatake *et al*, 1985). Expression of the GM-CSF gene is controlled by the proximal promoter comprising of the first 120 bp upstream of the transcription start site and an enhancer located a further 3 kb upstream of the human promoter (Cockerill *et al*, 1993) and 2 kb upstream of the mouse promoter (Osborne *et al*, 1995). Both the promoter and enhancer elements are required for the induction of GM-CSF gene expression. The GM-CSF gene in mice and humans is highly conserved with their promoters sharing approximately 90% sequence homology (Miyatake *et al*, 1985). However sequence analysis of cloned human and murine GM-CSF cDNA revealed only a 54% homology in the protein coding region (Wong *et al*, 1985) suggesting that while the gene products may differ, they are regulated in a similar fashion.

The GM-CSF promoter has been relatively well defined. It contains an array of transcription factor binding sites including the CD28 response region (CD28RR) and conserved lymphokine element 0 (CLE0). It contains binding sites for NFAT, NF-κB, SP1, Ets, CBF and AP1 transcription factors (Dunn *et al*, 1994; Wang *et al*, 1994; Himes *et al*, 1996; Cockerill *et al*, 1996; Schreck and Baeuerle, 1990; Shang *et al*, 1999) as outlined in Figure 1.2. The CD28RR consists of the CK-1 region (also termed the CD28RE), a 10bp element which responds specifically to signals delivered to T cells via the CD28 surface receptor, and an adjacent NF-κB and a Sp1 binding site. The CK-1 region is a variant NF-κB site which binds Rel A homodimers and c-Rel containing complexes (Himes *et al*, 1996). The CLE0 is shared between a number of cytokine genes including GM-CSF, IL-4 and IL-5. This element binds NFAT, Ets and AP-1 transcription factors (Masuda *et al*,

1993).

Studies in transgenic mice and cell lines determined that the GM-CSF enhancer is essential for the highly inducible activation of the human GM-CSF gene (Cockerill *et al*, 1999). The GM-CSF enhancer element is approximately 400bp in mice (Osborne *et al*, 1995) and 716bp in humans (Cockerill *et al*, 1993). It contains binding sites for Sp1, AML1, CBF, AP1 transcription factors and four NFAT binding sites (Cockerill *et al*, 1993; Cockerill *et al*, 1995; Cockerill *et al*, 1996).

## 1.2.2 Factors involved in transcriptional activation of the GM-CSF promoter

### 1.2.2.1 NFAT

The nuclear factor of activated T cells (NFAT) family of transcription factors regulates expression of a number of cytokine genes. Members include NFATc1 (NFATc), NFATc2 (NFATp), NFATn, NFATc3 (NFAT4 or NFATx) and NFATc4 (NFAT3), all of which share a conserved DNA binding domain. These factors are activated via the calcium dependent protein phosphatase, calcineurin following TCR ligation (Jain *et al*, 1993; Luo *et al*, 1996). In resting T cells, NFAT proteins can be found in the cytoplasm in a phosphorylated form with low affinity for DNA. T cell activation results in increased intracellular calcium levels. This leads to the dephosphorylation of NFAT by the calcium-dependent phosphatase, calcineurin and their subsequent nuclear translocation (Beals *et al*, 1997). While NFATp is constitutively expressed, NFATc is newly synthesized in response to T cell activating signals (Chuvpilo *et al*, 1999).

The involvement of NFAT in GM-CSF expression has been studied in considerable detail and subsequently its role in activating GM-CSF expression is well established. Using the electrophoretic mobility shift assay (EMSA) recombinant NFATp proteins were found to bind to the CD28RR of a GM-CSF promoter probe (Shang *et al*, 1999). However, reporter assays involving the cotransfection of Jurkat T cells with an NFATp construct and a construct containing two copies of the CD28RR attached to a luciferase reporter demonstrated that NFATp alone is not capable of activating this region. NFATp can however cooperate with signals which mimic T cell activation to enhance transcription

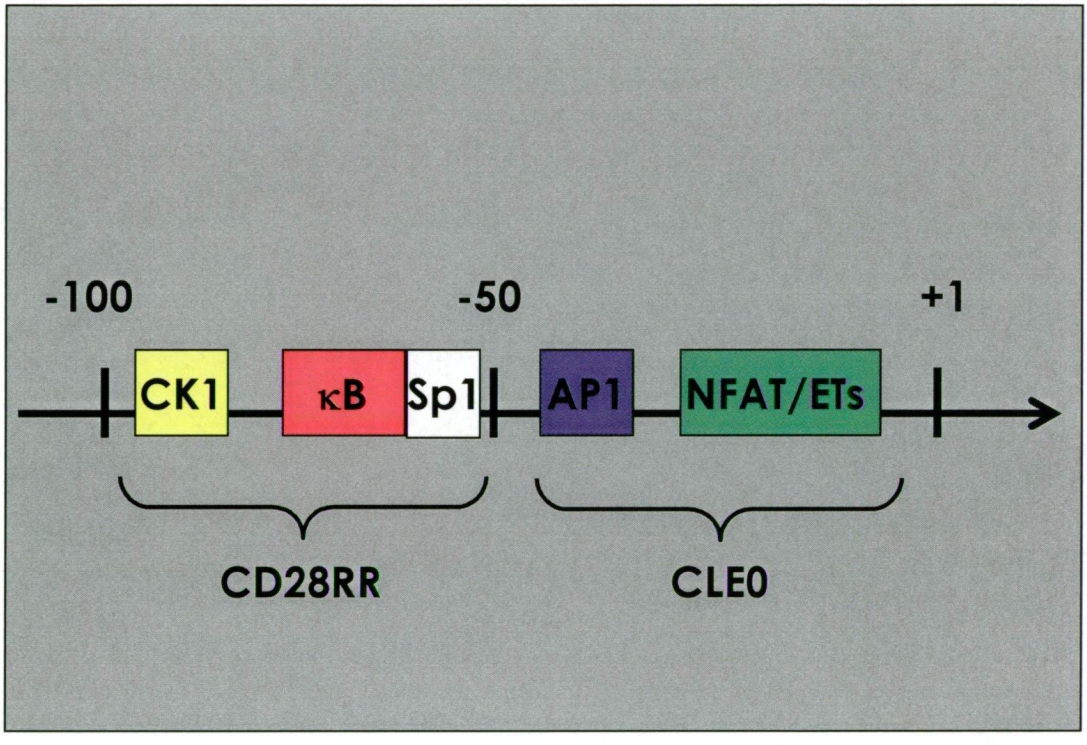



**Figure 1.2. Schematic representation of transcription factor binding sites across the GM-CSF promoter.**

from this element (Shang *et al*, 1999). NFAT has also been shown to bind to the CLE0 region of the GM-CSF promoter (Tsuboi *et al*, 1994). Furthermore, it is clear that NFAT members play an important role in GM-CSF induction as inhibition of NFAT activation using the immunosuppressant Cyclosporin A prevents GM-CSF transcriptional activation (Cockerill *et al*, 1993).

### 1.2.2.2 NF-κB

The NF-κB family of transcription factors is responsible for mediating immune and inflammatory responses by coordinating the expression of an array of immune genes. There are five mammalian NF-κB/Rel family members; p65 (Rel A), c-Rel, RelB, p50 (NF-κB1) and p52 (NF-κB2) (reviewed in Baldwin, 1996). The NF-κB family members share the Rel homology domain consisting of approximately 300 amino acids. NF-κB binds as homo and heterodimers to decameric sequence motifs within promoter and enhancer elements. While all members bind to DNA only the Rel A, c-Rel and Rel B factors have carboxy-terminal transactivation functions (Verma *et al*, 1995). Many target genes produce proteins which are quickly expressed in immune and inflammatory responses. NF-κB transcription factors are rapidly activated in response to a number of stimuli and thus are involved in rapid gene induction. NF-κB exists in the cytoplasm of resting cells bound to its inhibitory protein partner IκB which masks its nuclear localisation signal to sequester NF-κB in the cytoplasm (Antonsson *et al*, 2003). Numerous extracellular signals, including T cell activation, activate the IκB kinase complex which phosphorylates serine residues on the inhibitory IκB targeting it for ubiquitination and degradation. NF-κB is then free to translocate into the nucleus (reviewed in Baldwin, 1996). NF-κB proteins are activated in two waves following T cell stimulation with levels of Rel A and p50 peaking within 30 minutes. Levels of c-Rel and Rel B accumulate during the second wave of activation, peaking at approximately 6 hours following T cell activation (Kalli *et al*, 1998).

NF-κB binds at two sites in the CD28RR of the GM-CSF promoter (at positions -82 to -91 and -98 to -108) as determined by EMSA (Schreck and Baeuerle, 1990). These sites are referred to as the CK-1 element and the classical NF-κB/Sp1 site. Following on from this, the NF-κB family members c-Rel and Rel A were found to bind to the CK-1 element

and were reported as being vital for the activity of the CD28RR *in vitro* (Himes *et al*, 1996). Previous work has also identified the NF-κB/Sp1 region of the CD28RR in the GM-CSF promoter as critical for GM-CSF induction in primary T cells (Cakouros *et al*, 2001). GM-CSF transcription was inhibited in transgenic mice carrying the human GM-CSF transgene with an NF-κB/Sp1 mutation. This mutation exhibited an effect only when integrated into chromosomal DNA, either in transgenic mice or stably transfected cell lines while reporter expression was unaffected in cells transiently transfected with the mutant NF-κB/Sp1 construct. These data suggest that the NF-κB/Sp1 region of the GM-CSF promoter plays an important role in the activation of the GM-CSF gene in a chromatin context.

## 1.3 CHROMATIN

### 1.3.1 CHROMATIN STRUCTURE

Within the eukaryotic nucleus DNA is assembled into a DNA-protein complex called chromatin. The concept that DNA is organized into small repeating units was proposed more than thirty years ago by Hewish and Burgoyne (1973) when they found that a DNA ladder was produced when chromatin was digested. These repeating units called nucleosomes were subsequently found to consist of DNA associated with an octamer of histone proteins, consisting of two copies each of histones H2A, H2B, H3 and H4 (Thomas and Kornberg, 1975). The structure of the nucleosome core particle was resolved many years later by X-ray crystallography (Luger *et al*, 1997) and showed 147 base pairs of DNA helix super-coiled around the histone octamer, contacting the histone proteins with approximately two turns aligned with grooves between the histone proteins. Neighboring nucleosomes are then linked by short segments of DNA of variable length referred to as linker DNA (Morris, 1976). The four core histones (H2A, H2B, H3 and H4) are small, proteins which consist of a highly conserved histone fold domain (Zhong *et al*, 1983) and a less conserved N-terminal tail. The histone fold domains mediate histone-histone and histone-DNA interactions which are involved in formation of the core nucleosome structure (reviewed in Luger and Richmond, 1998). The amino-terminal tails have random coil segments extending out of the nucleosome core and while not involved in the formation of

the nucleosome structure facilitate the assembly of nucleosomes into higher order chromatin structures (reviewed in Peterson and Laniel, 2004). These higher order structures are then stabilized by linker histone H1, which is structurally unrelated to the core histones (reviewed in Lu and Hansen, 2003). Nucleosomal arrays are coiled together to form a 30 nm chromatin filament, which is then further condensed to form the interphase chromosomes or the more highly compacted metaphase chromosomes.

## 1.3.2 REGULATION OF GENE EXPRESSION

While the role of chromatin in packaging DNA into the confines of the nucleus has long been established (Eikbush and Moudrianakis, 1978), a second equally important role it plays in gene regulation has emerged only more recently (reviewed in Struhl, 1999). The assembly of DNA into nucleosomes is generally considered inhibitory to the binding of transcription factors. For example, in the case of the yeast *PHO5* promoter the Pho4 transcription factor can bind to the active promoter yet is unable to bind to the repressed promoter (Venter *et al*, 1994). It has long been known that more tightly compacted chromatin structures form a repressive transcriptional environment (Wasylyk and Chambon, 1980). For example, on a global scale chromatin within the cell nucleus can be classified as the more condensed heterochromatin, which is generally gene poor and inhibitory to gene transcription and euchromatin which corresponds to more relaxed chromatin domains associated with actively transcribed regions of the genome (reviewed in Yasuhara and Wakimoto, 2006). However even within these classifications it is now clear that chromatin is highly dynamic and can change in both structure and composition in response to cellular signals. Furthermore, it is clear that such changes are often required to facilitate gene activation. There are two general mechanisms by which changes in chromatin can occur broadly classified as histone modification and chromatin remodelling.

## 1.3.3 HISTONE MODIFYING COMPLEXES

Chromatin structure can be altered directly or indirectly through covalent modifications to the N-terminal tails of the core histone proteins (reviewed in Turner, 2000). Intense study over the last decade has uncovered a vast number of sites in the N-

terminal tails of histones which can be subjected to a variety of post translational modifications. These include acetylation, methylation, phosphorylation, or ubiquitylation (Alfrey *et al*, 1964; Stevely and Stocken, 1966; Henry *et al*, 2003).

While the addition of acetyl and phosphate groups to histone proteins can change the charge of the protein and alter chromatin by electrostatic means, such changes are unlikely to alter nucleosome structure although they may influence higher order chromatin packaging (Shogren-Knaak *et al*, 2006). However the major mechanism by which the tail modifications are thought to act is by facilitating interactions with non-histone proteins (Jenuwein and Allis, 2001). While most study has focused on modifications to histone tails, with the use of mass spectroscopy in studying histones it has become clear that residues outside of the N-terminal tail can also be modified (reviewed in Mersfelder and Parthun, 2006). Initial work demonstrated that lysine 79 on histone H3 within the core domain could be methylated in yeast (Ng *et al*, 2002) and since then a number of other core modifications have been identified. Unlike modifications to the histone tails, these core modifications are thought to influence the structure of the nucleosome.

The modification of histone proteins is carried out by enzymes which catalyze the addition and removal of covalent groups. Interestingly, many of these histone modifying proteins such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases and kinases were initially identified as transcriptional activators and repressors (reviewed in Nar *et al*, 2001). These enzymes have been shown to play a central role in modifying histones to alter chromatin structure. For example, the coactivator CREB binding protein (CBP) was one of the first HATs identified and reported to have intrinsic acetyltransferase activity (Bannister and Kouzarides, 1996) while in contrast class I, II and III histone deacetylases are found in corepressor complexes (reviewed in Verdin *et al*, 2003).

Initially histone modifications were considered to disrupt chromatin stability however it is emerging that the major mechanism by which the tail modifications function is by acting as docking sites for non-histone proteins which are able to modify chromatin structure and function (reviewed in de la Cruz *et al*, 2005). There is now evidence that specific histone marks can be recognized and bound by particular proteins and this has given rise to the histone code hypothesis. This hypothesis suggests that just as DNA

provides the code for every mRNA and protein required by an organism, the code present in histone modifications acts as a means of adding to the information held within the genetic code by stipulating when genes should be turned on or off (Jenuwein and Allis, 2001). Thus, site specific and domain wide histone modifications help to dictate the transcriptional potential of a gene by tagging genes with activating or repressive marks. Acetylation of histone proteins is generally associated with gene activation in euchromatic domains while heterochromatin corresponds with transcriptionally repressed, deacetylated chromatin environment (reviewed in Kornberg and Lorch, 1999). The acetylation of lysine residues on H3 and H4 are associated with active genes while methylation of H3-K9 and H3-K27 is linked to repression (Zhang and Reinberg, 2001). Therefore certain methylating marks are associated with activation while others are linked to repression.

The code established by various histone marks is recognized by particular proteins. Chromodomains are common domains found in numerous regulators of chromatin structure. These domains have been found to interact with histones, DNA and RNA. Heterochromatin protein 1 (HP1) directs the binding of other proteins to regulate chromatin structure and transcription. The chromodomain of HP1 recognizes methylation of lysine 9 in histone H3 (Nielsen *et al*, 2002). Bromodomains, present in a number of HAT co-activators, have been shown to act as acetyl-lysine-binding domains (Dhalluin *et al*, 1999) and are likely to be important in the assembly and activity of multiprotein complexes during transcriptional activation.

In addition to the covalent modification of histone proteins, the core histone proteins can also be altered by the inclusion of histone variants (reviewed in Hake and Allis, 2006). Recently several H3 variants were identified as having either repressive marks (H3.2) or activating marks (H3.3) while the histone variant H3.1 contained both activating and repressive marks (Hake *et al*, 2006). The histone variant H2A.Z is often associated with regions of chromatin which have potential to be actively transcribed (Allis *et al*, 1986) and is considered to stop the spreading of silenced chromatin (Meneghini *et al*, 2003).

## 1.3.4 CHROMATIN REMODELLING

Chromatin remodelling refers to any detectable change in either a single nucleosome or chromatin domain. Chromatin remodelling may therefore involve global or

localized changes and can be detected using a number of different assays which rely on changes to DNA accessibility using endonucleases. The complexes involved in remodelling chromatin have been identified in a wide range of organisms from yeast to humans (Peterson and Herskowitz, 1992; Kwon *et al*, 1994) indicating the inherent importance of chromatin remodelling complexes in transcription of the eukaryotic genome. These chromatin remodelling complexes are large multi-subunit protein complexes which use the energy from ATP hydrolysis to move histones by disrupting the DNA-histone bonds (reviewed in Becker and Horz, 2002). A number of ATP dependent chromatin remodeling complexes have been identified including the ISWI, Mi-2 and SWI/SNF complexes (reviewed in Vignali *et al*, 2000). The SWI/SNF complex is the most widely studied ATP dependent chromatin remodelling complex and is well conserved from yeast to humans (Kwon *et al*, 1994). SWI/SNF was first discovered in yeast as having DNA dependent ATPase activity (Laurent *et al*, 1993). Its name hails from its function in activating particular genes involved in mating type **swi**tching and sucrose **n**on-**f**ermenting metabolism. It comprises 10-15 subunits, with a combined molecular mass of approximately 2 MDa. The ATPase component of the mammalian SWI/SNF complex, Brg1 or Brm, is central to its function. Other Brg1/Brm associated factors (BAFs) are found within SWI/SNF and are important in regulating the interaction between SWI/SNF and other factors. For example BAF57 mediates the interaction of SWI/SNF with estrogen and androgen receptors (Belandia *et al*, 2002).

Histone loss and nucleosome sliding have both been proposed as mechanisms by which SWI/SNF drives the displacement of nucleosomes from gene regulatory regions. Previous *in vitro* work demonstrated that SWI/SNF can displace histone octamers from one piece of DNA to another (Lorch *et al*, 1999). Nucleosome sliding was first demonstrated *in vitro* with the detection of short range nucleosome mobility in response to the action of the ATP dependent GAGA factor remodelling complex (Varga-Weisz *et al*, 1995). SWI/SNF has also been shown to slide nucleosomes to an acceptor site on the same molecule of DNA *in vitro* (Whitehouse *et al*, 1999). There is also evidence to support both mechanisms *in vivo*. Histone loss was demonstrated *in vivo* at the yeast *PHO5* and *PHO8* promoters (Reinke and Horz, 2003; Adkins *et al*, 2004). By embedding the *PHO5* promoter within a small plasmid which limits the ability for nucleosome sliding, Korber *et al* (2004)

demonstrated that histone loss at the *PHO5* promoter is most likely due to histone eviction in *trans* rather than nucleosome sliding. The nucleosome transfer observed at the *PHO5* and *PHO8* promoters requires the histone chaperone, Asf1 (Korber *et al*, 2006). Histone chaperones aid the displacement of nucleosomes through the disassembly of the histone octamer (Walter *et al*, 1995). Nucleosome sliding has been demonstrated *in vivo* by Lomvardas and Thanos (2001) who showed that the nucleosome encompassing the IFN-β promoter slides in response to virus infection. Thus, it appears that different nucleosome remodelling mechanisms may operate at different genes.

The SWI/SNF complex lacks DNA sequence specific binding and is therefore thought to be recruited to specific promoter and enhancer regions via interactions with specific transcription factors and histone modifications. This is supported by evidence in which components of the SWI/SNF complex interact with a variety of activators and factors which bind to specific DNA sites (Nie et al, 2000; Zhao *et al*, 2005). BAF250 has been shown to directly bind to the beta-globin locus and in addition, it is capable of interacting with the glucocorticoid receptor thereby conferring binding specificity to the SWI/SNF complex (Nie *et al*, 2000). Thus, BAF250 has been suggested to recruit the complex to gene regulatory elements by directly binding DNA or interacting with other sequence specific transcription factors. *In vitro* work has demonstrated that SWI/SNF interacts with the transcription factor EKLF and the recruitment of the SWI/SNF complex to the beta-globin promoter is via an interaction with this transcription factor (Lee *et al*, 1999; Brown *et al*, 2002). The SWI/SNF complex has also been found to associate with the RNA polymerase II holoenzyme (Wilson *et al*, 1996). Recent reports have shown that RNA polymerase II is associated with the mediator complex, CBP and other proteins suggesting that the holoenzyme can be recruited as a unit to promoter regions therefore supporting the role of Brg1 in helping to initiate gene transcription.

It is becoming clear that ATP dependent remodelling complexes act cooperatively along side histone modifying proteins. Histone marks and the recruitment of chromatin remodelling activities help define whether or not a particular gene will be activated or not. Acetylated histones have been found to stabilize SWI/SNF association (Hassan *et al*, 2002) and this is due to the interaction of the bromodomain present in the ATPase subunit of the SWI/SNF complex with acetylated histones. Conversely, the HDAC Rpd3, causes

transcriptional repression by inhibiting the recruitment of SWI/SNF (Deckert and Struhl, 2002). SWI/SNF is recruited to the IFN-β promoter by prior acetylation of lysine 8 on histone H4 (Agalioti *et al*, 2002) and to the myogenin promoter following hyperacetylation of H4 (de la Serna *et al*, 2005). This facilitated by bromodomains within the complex acting as acetyl-lysine-binding domains (Dhalluin *et al*, 1999).

Although BAF complexes comprise multiple subunits it is now evident that only four of these are essential *in vitro* for the complete known range of activities (Narlikar *et al*, 2002). Of particular interest, Brg1 alone was found to be capable of increasing accessibility to a nucleosome which is unable to slide due to the position of neighboring nucleosomes (Fan *et al*, 2003). Other components of the SWI/SNF complex may exert their role by interacting with targets and co-activators or controlling the extent of the remodelling. It is also possible that they possess other important roles yet to be identified.

### 1.3.5 BRG1

The SWI/SNF complex in mammalian cells has two different catalytic subunits; Brahma (Brm, also called SNF2α or Smarca*2*) and Brahma related gene 1 (Brg1, also known as Snf2β or Smarca*4*). Brm and Brg1 are the ATPase components of the SWI/SNF complex and therefore central to its function. Disruption of Brm increases cellular proliferation (Reyes *et al*, 1998) while the targeted deletion of Brg1 in mice is embryonic lethal (Bultman *et al*, 2000) with the embryo dying during the peri-implantation stage. The heterozygote survives but develops exancephaly and tumors (Bultman *et al* 2000).

While the genes encoding Brg1 and Brm share 70% sequence homology, the two proteins differ in some of their activities and properties. While showing some functional redundancy in some cases, both Brg1 and Brm have been found to interact with the Rb protein to suppress the cancerous phenotype displayed in tumor lines (Strobeck *et al*, 2002). However these proteins display distinct tissue and developmentally specific patterns of expression. Work carried out by Reisman *et al* (2005) demonstrated the differential expression of the Brg1 and Brm components of the SWI/SNF complex. While Brg1 was found predominantly in tissues undergoing self renewal, Brm was consistently present in tissues such as brain, liver and endothelial which undergo limited proliferation. In addition, the non-conserved regions of Brg1 and Brm allow binding to specific transcription factors

or co-activators permitting recruitment to different gene promoters and enhancers. In support of this Brg1 has been found to bind zinc finger proteins via its unique N-terminal domain while Brm lacks this capability (Kadam and Emerson, 2003).

Loss of Brg1 has also been associated with increased DNA methylation. Banine *et al* (2005) demonstrated that the CD44 and E-cadherin promoters are hypermethylated in non-expressing SW13 and C33A cell lines which are deficient in Brg1 and Brm. The restoration of Brg1 function in these cells resulted in the demethylation of the CD44 and E-cadherin promoter. This study suggests that the loss of Brg1 may result in increased DNA methylation in some cases. Therefore the loss of SWI/SNF mediated transcription is a possible mechanism to increase DNA methylation in cancer cells. In addition they found that the dominant negative Brg1 and Brm proteins still interact with the promoters of these genes however transcription is inhibited. Thus while the ATPase domain is essential for transcriptional activity, recruitment to promoter regions is regulated by a different domain.

### 1.3.6 CHROMATIN REMODELLING IN THE IMMUNE SYSTEM

The rapid and precisely controlled expression of a range of genes, including cytokines, in response to immune and inflammatory signals is critical for the orchestration of an effective and appropriate immune response. Data accumulated over the last decade has begun to reveal the critical role that chromatin plays in facilitating activation of these genes (reviewed in Smale and Fisher, 2002). It is now becoming clear that the activation of genes in a chromatin context involves a complex interplay between transcription factors, chromatin modifiers and chromatin remodellers.

Initial work demonstrated the appearance of inducible DNase I hypersensitive sites in gene regulatory regions. The appearance of these sites was thought to represent changes in accessibility brought about by changes in chromatin structure. Early studies examined the position and appearance of DNase I hypersensitive sites across the IFN-β gene upon induction. Inducible DNase I hypersensitive sites were detected 100, 430 and 580bp upstream of the transcription start site indicating changes in chromatin structure during IFN-β activation (Higashi, 1985). Since these earlier studies, the accessibility of hundreds

of genes has been mapped to varying levels. Inducible DNase I hypersensitive sites have been detected in a number of immune gene promoters and enhancer elements demonstrating that transcriptional induction of genes such as GM-CSF, IL-2, IL-13 and IL-4 is associated with changes in chromatin structure (Cockerill *et al*, 1993; Ward *et al*, 1998; Takemoto *et al*, 1998).

Basal chromatin environment is now considered to play a vital role in regulating immune gene expression. For example, the appearance of DNase I hypersensitive sites is associated with cytokine gene expression programs in Th1 versus Th2 cells (Agarwal and Rao, 1998). Following TCR ligation naïve T cells differentiate into two distinct T helper (Th) subsets, Th1 and Th2 cells. Th1 cells are capable of producing IFN-γ while Th2 cells are capable of producing IL-4. Differentiated Th1 and Th2 cells show differences in chromatin structure and DNA methylation status at these particular gene loci. Avni and colleagues (2002) showed that culturing naïve T cells in polarizing conditions created a selective pattern of histone acetylation on the IL-4 and IFN-γ genes. Chromatin immunoprecipitation revealed that the IL-4 locus is hyperacetylated in Th2 cells (Avni *et al*, 2002; Yamashita *et al*, 2004; Grogan *et al*, 2003) while the IFN-γ promoter is hyperacetylated in Th1 cells (Avni *et al*, 2002) with acetylation status strongly correlated with gene expression. In addtition DNA methylation of the IFN-γ and IL-4 promoters in Th1 versus Th2 cells has been correlated with gene expression. While the IFN-γ promoter is hypomethylated in Th0 and Th1 cells, it undergoes hypermethylation during Th2 differentiation (Winders *et al*, 2004). Conversely, the 5' region of the IL-4 locus is hypermethylated in naïve T cells and undergoes extensive demethylation during Th2 differentiation (Lee *et al*, 2002).

While these studies demonstrate that histone modifications play an important role in immune gene regulation, numerous studies have also demonstrated that changes in nucleosome structure accompany the induction of cytokine gene transcription (Cockerill *et al*, 1993; Rao *et al*, 2001; Weinmann *et al*, 1999). Activation of the IL-2 gene in EL-4 and primary T cells is accompanied by changes in chromatin structure restricted to the first 300bp covering the proximal promoter (Rao *et al*, 2001). Increased accessibility is detected within the first 2 hours of T cell activation and maintained for at least 16 hours post-stimulation. In addition the kinetics of remodelling preceded transcription of the IL-2 gene.

Similarly rapid and selective remodelling is observed at the IL-12 p40 promoter (Weinmann *et al*, 1999) with nucleosome remodelling detected within one hour of macrophage activation with LPS.

The precise manner in which signals, transcription factors, chromatin remodellers and the transcriptional machinery interact to drive remodelling and transcription has only been elucidated for a handful of eukaryotic genes (Agalioti *et al*, 2000; de la Serna *et al*, 2005). One of the best defined activation profiles is that of the IFN-β gene which is activated in response to viral infection. A nucleosome positioned over the TATA box and start site of transcription is remodelled following a series of ordered recruitment events (Yie *et al*, 1999; Agalioti *et al*, 2000; Lomvardas and Thanos, 2001; Agalioti *et al*, 2002). Agalioti *et al* (2000) found that following infection, an enhanceosome is assembled at the nucleosome free enhancer region of the IFN-β gene. The enhanceosome directs the ordered recruitment of factors required for chromatin remodelling and transcription with the HAT, GCN5 initially recruited to the enhancer region followed by the CBP/PolII holoenzyme complex. Histone acetylation by GCN5 then acts as a signal for CBP mediated SWI/SNF recruitment. SWI/SNF then remodels the nucleosome covering the transcriptional start site and transcription of the IFN-β gene is then activated by the recruitment of TFIID.

Activation of the genes encoding the cytokines GM-CSF, IL-2 and IL-12 is also likely to be dependent on the recruitment of chromatin modifying activities as the transcription factor binding sites in the promoter regions of these genes are covered by nucleosomes (Weinmann *et al*, 1999; Rao *et al*, 2001; Holloway *et al*, 2003). However it is possible that these genes activate in a similar manner to the yeast *PHO5* promoter which is activated in the presence of low phosphate. The binding of Pho4, a key *PHO5* activator, is inhibited by nucleosomes encompassing the *PHO5* promoter region. While Pho4 can bind to the active promoter it is unable to bind to the repressed promoter (Venter *et al*, 1994). Svaren *et al* (1994) showed that deletion of the Pho4 activation domain prevented remodelling of the nucleosome encompassing the *PHO5* promoter suggesting it recruited remodelling activities. This was confirmed in later work by Barbaric *et al* (2003), who demonstrated that Pho4 was required for the recruitment of the histone acetyltransferase complex, SAGA. Histones are then lost from the promoter at a rate determined by SWI/SNF recruitment (Gaudreau *et al*, 1997).

## 1.3.7 GM-CSF ACTIVATION IN A CHROMATIN CONTEXT

There is substantial evidence that chromatin remodelling plays a vital role in regulation of the GM-CSF gene. The discovery of an inducible DNase I hypersensitive site in the GM-CSF enhancer following T cell stimulation provided the first evidence that the chromatin structure of the GM-CSF gene is altered following immune stimulation (Cockerill *et al*, 1993). Further investigations revealed that the appearance of this site was restricted to cells capable of GM-CSF expression, therefore highlighting the importance of chromatin remodelling in GM-CSF activation (Cockerill *et al*, 1999). The NFAT family of transcription factors were implicated in GM-CSF enhancer remodelling as treatment of Jurkat T cells with cyclosporin A prevented the appearance of the DNase I hypersensitive site (Cockerill *et al*, 1993). Using DNase footprinting Johnson *et al* (2004) recently demonstrated that GM-CSF enhancer core elements are spanned by two adjacent nucleosomes. Following T cell activation remodelling is not restricted to the enhancer region, but rather spans a 3 kb domain. NFAT dependent chromatin remodelling at the GM-CSF enhancer was also confirmed by site directed mutagenesis, with mutations in NFAT binding sites preventing GM-CSF enhancer remodelling (Johnson *et al*, 2004).

The detection of an inducible DNase I hypersensitive site at the GM-CSF promoter following T cell stimulation suggested the chromatin structure of the promoter is also altered during T cell activation (Cockerill *et al*, 1999). Carrying on from this, Holloway *et al* (2003) mapped accessibility across the GM-CSF promoter region and demonstrated that a single nucleosome is positioned across the resting GM-CSF promoter between -174 and +24. Following T cell stimulation this nucleosome is rapidly and selectively remodelled. Mutation of the NF-κB/Sp1 site within the GM-CSF promoter has been shown to limit remodelling in primary mouse lymphocytes (Cakouros et al, 2001). Further evidence of a role for NF-κB in GM-CSF promoter remodelling was provided by Holloway *et al*, (2003) who demonstrated that the stable expression of an IκBα mutant construct, which prevents NF-κB nuclear translocation, inhibited GM-CSF promoter chromatin remodelling events. Most recently, Chen *et al* (2005) demonstrated that the increase in GM-CSF promoter accessibility following T cell activation is due to histone loss. However, the mechanism behind the loss of histones from the GM-CSF promoter following T cell stimulation and

the signals and factors involved in this process remain to be elucidated, and this is the focus of the studies outlined in this thesis.

## 1.4 RESEARCH PROJECT AIMS

The aim of this study was to identify the role of signals and factors activated following T cell stimulation in regulating chromatin remodelling and transcription of the murine GM-CSF promoter. The specific aims were to:

1) Identify the signals, factors and kinetics involved in increasing accessibility to the GM-CSF promoter

2) Determine the role of Brg1 in activating the GM-CSF gene

3) Determine whether other genes are activated in a similar manner.

# CHAPTER 2.
# MATERIALS AND METHODS

## 2.1.1 Cell culture

Murine EL-4 T cells (ATCC) were cultured in RPMI 1640 medium (Gibco®) supplemented with 10% FCS (JRH Biosciences), 2mM L-glutamine (JRH Biosciences), 100U/ml penicillin and 100μg/ml streptomycin (JRH Biosciences). The murine A-20 B cell line (provided by Prof. Lyn Corcoran, WEHI) was grown in DMEM (Gibco®) supplemented with 10% FCS (JRH Biosciences), 2mM L-glutamine (JRH Biosciences), 100U/ml penicillin, 100μg/ml streptomycin (JRH Biosciences) and 0.05mM β-mercaptoethanol (Sigma-Aldrich). Cultures were incubated at 37°C and 5% $CO_2$ in humidified air. EL-4 T cells and A-20 B cells were maintained at a density between $2x10^5$ and $5x10^5$ cells/ml by addition of fresh medium every 24 to 48 hours.

## 2.1.2 Freezing cells

Cells (50ml at $5x10^5$ cells/ml) were centrifuged at 500g for 5 minutes. The supernatant was removed and the cells were resuspended in 2.5ml of culture medium. DMSO (500μl) was added to 2ml of medium then added slowly to the cells. The cell suspension was aliquoted between 5 cryovials, wrapped in cotton wool to allow gradual freezing and stored at -80°C or the vapour phase of liquid nitrogen.

## 2.1.3 Thawing cells

Frozen stocks of EL-4 T cells or A-20 B cells were thawed in a water bath at 37°C. The cell suspension was then transferred to a 15ml Falcon tube along with 10ml of culture medium and centrifuged at 500g for 5 minutes. The supernatant, containing DMSO was then discarded. The pelleted cells were resuspended in 1ml of media which was transferred to a $25cm^2$ culture flask with another 9ml of medium. The cells were then cultured at 37°C and 5% $CO_2$ in humidified air (section 2.1.1).

## 2.1.4 Stimulation of cultured cells

**Table 2.1. Reagents used to treat EL-4 T cells and A-20 B cells.**

| Stimuli | Stock concentration | Treatment |
|---|---|---|
| Phorbol myristate acetate (PMA/P; Boehringer Mannheim) | 1mg/ml in DMSO | 20ng/ml for various times as indicated |
| Calcium ionophore A23187 (I, Sigma-Aldrich) | 10mM in DMSO | 1μM for various times as indicated |
| Cyclohexamide (CHX; Calbiochem) | 100mg/ml in ethanol | 10μg/ml for 30 minutes |
| Cyclosporin A (CsA; Calbiochem) | 100 mg/ml in ethanol | 50ng/ml for 30 minutes |
| Ro-32-0432 (Roche Pharmaceuticals) | 10mM in DMSO | 10μM for 1 hour |
| Trichostatin A (TSA; Sigma-Aldrich) | 1mg/ml in DMSO | 200ng/ml for 4/16 hours |

In some experiments stimuli were withdrawn from EL-4 T following treatment. In this case cells were treated with P/I for 2 hours, before pelleting cells at 500g, washing cells twice in fresh RPMI medium before resuspending in RPMI medium free of stimuli.

## 2.2 Analysis of RNA by real-time PCR

Cytokine mRNA levels in EL-4 T cells and A-20 B cells were determined by real-time PCR according to the following protocols.

## 2.2.1 Isolation of RNA

Total RNA was isolated from $5x10^5$-$1x10^6$ cells. Following centrifugation at 700g at room temperature, the pelleted cells were lysed by the addition of 500μl of Tri-reagent (Sigma) with repeated pipetting. The cell lysates were then transferred to eppendorf tubes and incubated at room temperature for 5 minutes. Chloroform (100μl) was added to each sample tube which was then shaken vigorously for approximately 15 seconds. The samples were incubated at room temperature for 10 minutes. Samples were then centrifuged at 12000g for 15 minutes at 4°C. The upper aqueous phase was transferred to new eppendorf tubes, to which 300μl of isopropanol was added. Each tube was gently inverted several

times and then placed at –80°C for 30 minutes to overnight to precipitate the RNA. The samples were centrifuged at 13000g for 10 to 30 minutes at 4°C and the supernatant removed. The pellets were washed with 75% ethanol and air dried for several minutes. MilliQ water (20µl to 50µl depending on pellet size) was added to each pellet which was then vortexed and heated at 60°C for several minutes on a heating block. The RNA was snap frozen in liquid nitrogen and stored at –80°C.

## 2.2.2 Quantitation of RNA

Isolated RNA was diluted 1 in 20 with MilliQ water and absorbance readings were determined using the BioRad SmartSpec 3000™ spectrophotometer. Absorbance at 260, 280 and 320 nm was measured for each sample. RNA was quantified according to the formula 1 O.D. at 260nm = 40.0µg/ml. RNA quality was assessed by agarose gel electrophoresis. RNA (500ng) and 2.5µg of Lambda molecular weight marker (New England Biolabs) were separated on a 1% agarose gel in 1xTAE (refer to section 2.9.1) at 100V for 1 hour before staining with ethidium bromide.

## 2.2.3 cDNA synthesis

RNA (0.5µg to 1µg) was added to an eppendorf tube along with 2µl of 5x first strand buffer (Invitrogen) and 1µl of 1U/µl DNase (Sigma). MilliQ water was added to a total volume of 10µl. This reaction was then incubated at 37°C for 30 minutes and the DNase then inactivated at 75°C for 5 minutes. Another 2µl of first strand buffer was added to each tube along with 1µl of 1µg/µl oligodT (Sigma) and 3.5µl of MilliQ water. This reaction was incubated at 70°C for 10 minutes and then cooled on ice. A 2µl aliquot of 0.1M DTT (Invitrogen), 1µl of 10mM dNTPs (Promega) and 0.5µl of Superscript reverse transcriptase (Invitrogen) was added to each reaction. This mixture was incubated at 42°C for 50 minutes and then the enzyme inactivated at 70°C for 15 minutes. The cDNA was stored at –20°C.

## 2.2.4 Real-time PCR

## 2.2.4.1 Primers

The primer sequences used in the amplification of target cDNA are outlined in the table below.

**Table 2.2 Primers used in the quantification of cytokine mRNA by real-time PCR.**

| Name | Sequence (5' to 3') | Amplicon (bp) | Reference |
|---|---|---|---|
| mGM+II Forward primer | AAGGTCCTGAGGAGGATGTG | 140 | Holloway *et al*, 2003 |
| mGM+II Reverse primer | GAGGTTCAGGGCTTCTTTGA | | |
| mM-CSF Forward primer | CGAGTCAACAGAGCAACCAA | 236 | |
| mM-CSF Reverse primer | TGCTTCCTGGGTCAAAAATC | | |
| mIL-4 Forward primer | TCAACCCCCAGCTAGTTGTC | 177 | |
| mIL-4 Reverse primer | TGTTCTTCGTTGCTGTGAGG | | |
| mIL-5 Forward primer | ATGGAGATTCCCATGAGCAC | 177 | |
| mIL-5 Reverse primer | CCCACGGACAGTTTGATTCT | | |
| mIFNγ Forward primer | GCTTTGCAGCTCTTCCTCAT | 162 | |
| mIFNγ Reverse primer | GTCACCATCCTTTTGCCAGT | | |
| mGAPDH Forward primer | AAGTATGATGACATCAAGAAGGTGGT | 67 | |
| mGAPDH Reverse primer | AGCCCAGGATGCCCTTTAGT | | |

All primers were supplied by Sigma.

## 2.2.4.2 SYBR green real-time PCR

cDNA was diluted 10ng/μl with MilliQ water and 5μl (50ng) was used in each reaction. A PCR was prepared containing 12.5μl Qiagen QuantiTect SYBR green master mix, 1.5μl forward primer (5μM), 1.5μl reverse primer (5μM) and 4.5μl MilliQ water to give a total reaction volume of 25μl. A no template control (NTC) was prepared for each primer set to detect any contaminating DNA. This reaction replaced 5μl of cDNA with 5μl of MilliQ water. The Rotor-gene 2000 real-time PCR machine (Corbett Research, Australia) was used for the amplification of cDNA. The optimized PCR cycle conditions were; 95°C for 15 minutes (hold); 95°C for 15 seconds, 60°C for 60 seconds (30-45 cycles, acquiring to channel 1); 60°C to 95°C increasing 1°C every 5 seconds (melt). Reactions were prepared in an area designated "PCR only" to reduce the occurrence of

contamination. Melt analysis was used to assess the presence of a single PCR species and this was verified by separating PCR products on a 2% agarose gel in 1x TAE (section 2.9.1) at 100V for 40 minutes. Molecular weight marker (2.5μg of 100 bp ladder from New England BioLabs) was also electrophoresed on each gel. PCR products were visualized using ethidium bromide.

Standard curves for each primer set were used to quantify cytokine mRNA levels and glyceraldehyde phosphate dehydrogenase (GAPDH) expression was used for normalization of data. A mouse GAPDH PCR plasmid containing a 67bp fragment of the mouse GAPDH gene was prepared by cloning the PCR product formed using the GAPDH primers into the PCR® 2.1 vector (Section 2.5). This plasmid was used in the construction of a GAPDH standard curve. The mouse GM-CSF construct, AOGM provided by Dr. P Cockerill (Osborne *et al*, 1995) was used for construction of the GM-CSF standard curve. The mM-CSF, mIL-4 and mIL-5 standard curves were generated using serial dilutions of quantified gel extracted PCR product.

### 2.2.4.3 Gel extraction of PCR product

PCR products were extracted from 2% agarose gels using the Qiagen Gel Extraction kit according to the manufacturers' protocol. Briefly, the DNA fragment was excised from the gel and weighed before adding 3 volumes of buffer QG. The sample was then incubated at 50°C for 10 minutes with occasional vortexing. Isopropanol (1 gel volume) was then added to the sample and mixed by inversion. The sample was then applied to the MinElute column and centrifuged for 1 minute. The flow-through was discarded and the column washed with 750μl of buffer PE by centrifuging again for 1 minute. The flow-through was discarded and the sample centrifuged for 1 minute at 10,000g. The column was then transferred to a fresh eppendorf tube and 10μl of MilliQ water applied to the membrane. The sample was incubated for 1 minute at room temperature before centrifuging once again for 1 minute.

## 2.3 Chromatin Accessibility by real-time PCR (CHART PCR)

Accessibility of regions of cytokine genes to MNase and restriction enzymes was determined by CHART-PCR (Rao *et al*, 2001) according to the following protocols.

### 2.3.1 Isolation of nuclei

Nuclei were isolated from EL-4 T cells and A-20 B cells as described previously (Schreiber *et al*, 1989). The entire protocol was carried out in the cold room at 4°C. Briefly, cells ($2x10^6$) were pelleted at 500g for 5 minutes. The media was aspirated and cells washed in ice cold PBS (ICN Biomedicals). The supernatant was removed and the pellet was resuspended in 0.5ml of nuclei buffer (10mM Tris, pH 7.5, 10mM NaCl, 3mM $MgCl_2$, 0.1mM EDTA, 0.5% Igepal, 16.5nM Spermine (Sigma-Aldrich) and 0.32μM Spermidine (Sigma-Aldrich)) and transferred to eppendorf tubes, using cut pipette tips to prevent cell lysis. The cells were incubated for 5 minutes on ice and then centrifuged at 700g for 4 minutes. The supernatant was discarded and the nuclei were resuspended in 400μl of nuclei wash buffer (10mM Tris pH 7.5, 50mM NaCl, 10mM $MgCl_2$, 0.2mM EDTA, 0.2mM EGTA, 18nM Spermine and 0.32μM Spermidine). The nuclei were then centrifuged at 700g for 4 minutes. The wash buffer was removed and the nuclei were gently resuspended in 250μl of restriction enzyme buffer diluted 1:10 with MilliQ water (New England Biolabs buffer 2) or 200μl of MNase buffer (10mM Tris pH 7.5, 15mM NaCl, 60mM KCl, 18nM spermine and 0.32μM spermidine) supplemented with the following protease inhibitors; Aprotinin (2μg/ml; Calbiochem), Leupeptin (1μg/ml; Calbiochem) and PMSF (100μM; Sigma-Aldrich).

### 2.3.2 Restriction digest

For each treatment, 97μl aliquots of EL-4 nuclei resuspended in restriction enzyme buffer were transferred to two eppendorf tubes. *Hin*fI (150 units; New England Biolabs) was added to one sample (as determined empirically) and the other sample was left untreated. Both samples were incubated at 37°C for 30 minutes with occasional flicking to

resuspend nuclei. Samples were then removed from the heating block and 100µl of PBS added to each sample and DNA isolated immediately (Section 2.3.4).

## 2.3.3 MNase digest

EL-4 or A-20 nuclei resuspended in MNase buffer (94µl) were transferred to eppendorf tubes containing 1µl of ice cold $CaCl_2$ (0.1M). MNase (5U/ µl: Boehringer) was then added to one tube and the other was left untreated. Both samples were then incubated at room temperature for 5 minutes before adding 20µl stop buffer (0.1M EDTA, pH8 and 50mM EGTA, pH 8) and placing on ice. A further 80µl of MNase buffer was added and DNA isolated immediately (Section 2.3.4).

## 2.3.4 DNA isolation

DNA was isolated from cell nuclei using the Qiagen QIAamp DNA blood mini kit according to the manufacturers' instructions. Briefly, 20µl of Qiagen protease was added to each cell nuclei sample along with 4µl of RNase (Roche; 10mg/ml in 0.01M sodium acetate, pH 5.2) and 200µl of buffer AL. The samples were then vortexed for 15 seconds, incubated at 56°C for 10 minutes and then centrifuged briefly. Ethanol (200µl) was added to each sample and pulse vortexed for 15 seconds. The samples were then centrifuged briefly. The contents in each eppendorf tube were then applied to QIAamp spin columns. The tubes were centrifuged at 8000g for 1 minute. The filtrate was discarded and the column was placed in a new collection tube. Buffer AW1 (500µl) was carefully applied to each column. The spin columns were then centrifuged at 8000g for 1 minute. The filtrate was again discarded and the column was placed in a fresh collection tube. Buffer AW2 (500µl) was then carefully applied to each column. The samples were centrifuged at 13000g for 3 minutes. The collection tube was discarded and the spin column was placed in a 1.5ml microcentrifuge tube. Buffer AE (200µl) was then applied to each column and incubated at room temperature for 5 minutes. The tubes were then centrifuged at 8000g for 1 minute. The spin column was discarded and the flow through containing DNA was stored at -20°C.

## 2.3.5 Quantification of DNA

Prior to PCR amplification the concentration of DNA was determined using a spectrophotometer. Isolated DNA was diluted 1:20 with MilliQ water and absorbance readings determined using the BioRad SmartSpec 3000$^{TM}$ spectrophotometer. Absorbance at 260, 280 and 320nm was measured for each sample. DNA was quantified according to the formula 1 O.D. at 260nm=50µg/ml. DNA quality was assessed by agarose gel electrophoresis. DNA (500ng) and 2.5µg of the Lambda molecular weight marker (New England Biolabs) were separated on a 1% agarose gel in 1xTAE (section 2.9.1) at 100V for 1 hour before staining with ethidium bromide.

## 2.3.6 Real-time PCR of CHART samples

## 2.3.6.1 Primers

The primers used in the amplification of genomic DNA in the CHART assay are outlined below.

**Table 2.3. Primers used in the quantification of genomic DNA by real-time PCR.**

| Name | Sequence (5' to 3') | Amplicon (bp) | Reference |
|---|---|---|---|
| mGM-I Forward primer | GCCTGACAACCTGGGGGAAG | 116 | Holloway *et al*, 2003 |
| mGM-I Reverse primer | TGATTAATGGTGACCACAGAACTC | | |
| mGM+I Forward primer | GAGTTCTGTGGTCACCATTAATCA | 147 | Holloway *et al*, 2003 |
| mGM+I Reverse primer | CACATCCTCCTCAGGACCTT | | |
| mGM-V Forward primer | TGGAATGAGCCACCAGAGTA | 75 | Holloway *et al*, 2003 |
| mGM-V Reverse primer | GGCTCTTGCTTCCATAGCAC | | |
| mIL-4E Forward primer | GCACCAGGGCACTTAAACAT | 158 | |
| mIL-4E Reverse primer | CTGTGCAGTGCCACAATGAT | | |
| mIL-5p Forward primer | ACCCTGAGTTTCAGGACTCG | 94 | Wang *et al*, 2006 |
| mIL-5p Reverse primer | TCCCCAAGCAATTTATTCTCTC | | |
| mIFN-γp Forward primer | AACATGCCACAAAACCATAGC | 156 | |
| mIFN-γp Reverse primer | CACCTCTCTGGCTTCCAGTT | | |

All primers were supplied by Sigma.

### 2.3.6.2 Real-time PCR

DNA was diluted to 10ng/μl for real-time PCR amplification. A PCR was prepared containing 12.5μl SYBR, 1.5μl forward primer (5μM), 1.5μl reverse primer (5μM), and 4.5μl MilliQ water to which 5μl (50ng) of DNA was added to give a total reaction volume of 25μl. A NTC reaction was set up for each primer set which included 5μl of MilliQ water in place of DNA. Amplification of DNA was performed by and detected with the Rotor-gene 2000 real-time PCR machine (Corbett Research, Australia). The PCR conditions were; 95°C for 15 minutes seconds (hold); 95°C for 15 seconds followed by 60°C for 60 seconds (35-45 cycles, acquiring to channel 1); 60°C to 95°C increasing by 1°C every 5 seconds (melt). Reactions were set up in an area designated "PCR only" to reduce contamination. Melt analysis was used to assess the presence of a single PCR species and this was verified by separating PCR products on a 2% agarose gel in 1x TAE (section 2.9.1) at 100V for 40 minutes. Molecular weight marker (2.5μg of 100 bp ladder from New England BioLabs) was also electrophoresed on each gel. Electrophoresed PCR products were visualized with ethidium bromide. PCR products were quantified using standard curves for each primer set. Accessibility was determined by correlating the Ct values from the amplification plots to a standard curve for each primer set and was expressed as a percentage of undigested genomic DNA for each primer set.

The mouse GM-CSF construct, AOGM was used for construction of the mGM-I, mGM+I and mGM-V standard curves. The IFNγp, IL-4p and IL-5p standard curves were generated separately using quantified and serially diluted gel extracted PCR product (section 2.2.4.3).

## 2.4 Analysis of nuclear proteins

Nuclear levels of various transcription factors in EL-4 T cells were determined according to the following protocols.

## 2.4.1 Preparation of nuclear extracts

Nuclear extracts were prepared as described previously (Schreiber *et al*, 1989). Cells ($5x10^5$ cells/ml) were centrifuged at 500g for 5 minutes. The supernatant was removed and the pellet washed in PBS. Cell pellets were resuspended in 1.25 ml buffer A (10mM Tris pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 0.1mM EDTA pH 8 and 0.5% Igepal) and transferred to eppendorf tubes. The samples were then incubated on ice for 5 minutes and centrifuged at 700g for 5 minutes. The supernatant was removed and the pellets were resuspended in 1.25ml of buffer A minus Igepal. The tubes were then centrifuged at 700g for 5 minutes. The supernatant containing the cytoplasmic extracts was removed and transferred to a new eppendorf tube. The pelleted nuclei were resuspended in 25µl of buffer C (400mM NaCl, 7.5mM $MgCl_2$, 0.2mM EDTA pH 8 and 1mM DTT with protease inhibitors added just prior to use; aprotinin (10µg/ml), leupeptin (5µg/ml) and PMSF (500µM). The nuclei were then incubated in buffer C for 15 minutes on ice then centrifuged at 13000g for 5 minutes. The supernatant containing the nuclear proteins was then transferred to a new eppendorf tube. The nuclear extracts were stored at -80°C.

## 2.4.2 Quantification of protein

Protein concentrations of nuclear extracts were determined using the Bradford protein assay (Bradford, 1976) using bovine serum albumin (BSA) as the protein standard. Serial dilutions of BSA were prepared from 1.0 to 0.1mg/ml for construction of a standard curve. Nuclear extracts were diluted 1:10. Bradford dye reagent (BioRad) was prepared by filtering 1 part dye to 4 parts water through filter paper (Whatman, Number 1). The dye reagent (1ml) was added to 10µl of the standards and samples. The standards and samples were vortexed and incubated at room temperature for 5 minutes. Absorbance was measured at 595nm using the BioRad SmartSpec 3000™ spectrophotometer. The concentration of the nuclear extracts was determined from the BSA standard curve.

## 2.4.3 Separation and transfer of nuclear proteins

### 2.4.3.1 SDS PAGE and Western Blotting

Nuclear extracts (3-15μg) were added to an equal volume of 2x sample buffer (section 2.9.2) and heated at 95°C for 5 minutes. A 12.5% acrylamide gel was poured and assembled in a gel apparatus (BioRad). The tank was filled with 1x SDS PAGE running buffer (section 2.9.2). The samples were then loaded onto the gel along with 5μl of Multimark® multi-coloured standard (Invitrogen). The samples were electrophoresed at 125V for 90 minutes. The gel was removed from its cassette and placed into the transfer apparatus (BioRad). The chambers were filled with Western transfer buffer (section 2.9.2) and proteins were transferred to a nitrocellulose membrane (Micro Filtration Systems) at 20V for 18 hours at 4°C.

The membrane was placed in blocking solution (section 2.9.2) and incubated on a platform shaker at room temperature for 1 hour. Following this the membrane was sealed in plastic with the appropriate primary antibody (Table 2.4) diluted with 1x TNT (section 2.9.2) and incubated for another hour at room temperature on a shaker. The membrane was washed three times in 1x TNT (10 minutes per wash) and sealed in plastic along with the appropriate secondary antibody dilution in 1x TNT. The membrane was incubated in this secondary antibody solution for 1 hour at room temperature on a shaker then washed another three times (10 minutes per wash) in 1x TNT. The membrane was then sealed in plastic and incubated for 5 minutes in 2.5ml of Pierce SuperSignal ® West Pico Luminol enhancer solution and 2.5ml of stable peroxide solution (Pierce). The solution was removed and the membrane was exposed to high performance autoradiography film (Amersham Pharmacia Biotech) for 1 minute. The film was then agitated in developer (Kodak) for 1 minute, rinsed in water and placed in fixing solution (Ilford) for 1 minute. Shorter or longer exposures were carried out as required.

**Table 2.4. Antibodies used in the analysis of proteins in EL-4 T cells.**

| Primary Antibody | Concentration | Secondary Antibody | Concentration |
|---|---|---|---|
| Anti–p65 (Rel A) | 1:1000 | Goat anti-rabbit | 1:1000 |
| Anti-c-Rel | 1:1000 | Goat anti-rabbit | 1:1000 |
| Anti-Sp1 | 1:1000 | Goat anti-rabbit | 1:1000 |
| Anti NFATp | 1:1000 | Rabbit anti-mouse | 1:1000 |
| Anti-NFATc | 1:1000 | Rabbit anti-mouse | 1:1000 |
| Anti-HA | 1:1000 | Goat anti-rabbit | 1:1000 |
| Anti-Brg1 | 1:1000 | Goat anti-rabbit | 1:1000 |

All primary antibodies were provided by Santa Cruz Biotechnology. Peroxidase-conjugated secondary antibodies were provided by Dako.

### 2.4.3.2 Stripping and Reprobing Blots

Antibodies were removed from blots to enable reprobing for analysis of additional proteins. Blots were incubated at 65°C in stripping buffer (section 2.9.2) for 30 minutes with shaking, followed by three washes (10 minutes per wash) in 1x TNT. The membrane was then placed in blocking solution for 1 hour. The remainder of the protocol was carried out as described in section 2.4.3.1.

## 2.5 Cloning

### 2.5.1 Ligation

PCR product was ligated into the appropriate vector by combining fresh PCR product, 1µl of 10x ligation buffer (Invitrogen), 2µl PCR® 2.1 vector (50ng total; Invitrogen), 1µl T4 DNA ligase (New England Biolabs) and MilliQ water to a total volume of 10µl. The ligation reaction was then incubated for a minimum of 4 hours at 14°C.

### 2.5.2 Preparation of competent cells

L-broth (100ml; section 2.9.1) was inoculated with 1ml of a saturated MC1061 E.Coli culture and incubated with shaking at 37°C. The optical density of the cells was then measured at 650nm using the BioRad SmartSpec 3000™ spectrophotometer. Once the

bacterial culture had reached an $O.D_{650}$ of 0.4 to 0.5 the culture was transferred to centrifuge tubes and centrifuged at 7000g for 5 minutes. The bacterial pellet was then resuspended in 20ml of ice cold 50mM $CaCl_2$ and incubated on ice for 30 minutes. The cells were then pelleted at 7000g for 5 minutes and resuspended in 3ml ice cold 50mM $CaCl_2$.

## 2.5.3 Transformation of competent cells

A 200µl aliquot of the freshly prepared competent cells was used for each transformation. A 2µl aliquot of the ligation reaction was pipetted into the competent cells and mixed well prior to incubating on ice for 30 minutes. The cells were then heat shocked at 42°C for 30 seconds and placed on ice. SOC medium (250µl; section 2.9.1) was added to each sample and the vials placed on a shaking platform at 37°C for 1 hour at 225rpm. Transformed cells (10-200µl) were then spread on LB agar plates (section 2.9.1) containing antibiotic. The plates were inverted and incubated at 37°C for 18 hours before incubating at 4°C for 3 hours to allow colour development. A single colony was then inoculated into 2ml of L-broth containing the appropriate antibiotics for further propagation and preparation of stocks. Glycerol stocks of transformed bacterial cultures were prepared by adding 850µl bacterial culture to 150µl of sterile glycerol in an eppendorf tube. Stocks were stored at -80°C.

## 2.6 Transfection of EL-4 T cells

EL-4 T cells were transfected with plasmids targeting NFAT or Brg1 action according to the following protocols.

## 2.6.1 Plasmids

The mutant Brg1 construct, pBJ5-brg1K/R and the pBJ5 plasmid were provided by Dr G. Crabtree (Khavari *et al*, 1993). The pEGFP-VIVIT and pEGFP plasmids were provided by Dr. A. Rao (Aramburu *et al*, 1999). The $K^K$II plasmid used for magnetically

activated cell sorting was provided with the MACSelect kit (Miltenyi Biotech). Plasmids were propagated in MC1061 E.Coli cells.

## 2.6.2 Plasmid purification

Plasmids were isolated either by alkaline lysis/$CsCl_2$ purification or using the Qiagen Midi prep kit. In each case, glycerol stocks of MC1061 cells, transformed with the relevant plasmids, were inoculated in 2ml of L-broth containing either ampicillin (100μg/ml) or kanamycin (100μg/ml) depending on the resistance provided by the plasmid. The starter cultures were incubated at 37°C in a shaking incubator for 6 to 8 hours before transferring to larger (100-400ml) cultures (containing antibiotics) for overnight incubation.

### 2.6.2.1 Plasmid preparation by alkaline lysis

The pEGFP and pEGFP-VIVIT plasmids were isolated by alkaline lysis and subsequent cesium chloride ($CsCl_2$) purification, essentially as described previously (Sambrook and Russel, 2001). Bacterial cultures grown overnight were transferred to 500ml centrifuge bottles and bacteria pelleted at 3200g for 15 minutes at 4°C. The supernatant was removed and the pellet resuspended in 8ml alkaline lysis buffer 1 (50mM glucose, 25mM Tris HCl pH 7.4, 10mM EDTA pH 8) with repeated pipetting and transferred to sorvall tubes. Lysozyme (80mg) was then added to each sample and incubated at room temperature for 5 minutes. Freshly prepared lysis buffer 2 (16ml; 0.2M NaOH, 1% SDS) was then added and the samples inverted several times before incubating on ice for 10 minutes. Ice cold buffer 3 (12ml; 5M KAc, pH 4.8) was then added to each sample and mixed by inverting several times. The lysate was incubated on ice for another 10 minutes before centrifuging at 17000g for 20 minutes at 4°C. The supernatant was then transferred to a new tube and 0.6 volumes of isopropanol was added and mixed well before incubating at room temperature for 30 minutes. The samples were then centrifuged at 12000g for 15 minutes at room temperature and the pellets resuspended in 1 ml TE (100mM Tris pH 7.4, 10mM EDTA pH 8). RNase (Roche; 100μg/ml) was then added to each sample and incubated at 37°C for 30 minutes. TE (9ml) and $CsCl_2$ (10.9g) was added

to each sample. Ethidium bromide (5mg) was added and the centrifuge tubes balanced and sealed. The tubes were then centrifuged at 120,000g for 40 hours in the Beckman L8-70M Ultracentrifuge. The plasmid bands were extracted using a 5ml syringe and transferred to 50ml falcon tubes. Ethidium bromide was removed by extracting several times with an equal volume of MilliQ saturated butanol. Once the lower DNA phase was colourless, 2 volumes of water was added along with 0.1 volume of 3M sodium acetate (pH 6.5) and 2.5 volumes ethanol (100%). The DNA was then precipitated at -20°C for 6 hours before transferring to sorval tubes and pelleting at 12000g for 15 minutes. The pellet was then washed in 70% ethanol and resuspended in 400µl TE. DNA was then reprecipitated by adding 40µl 3M sodium acetate (pH 6.5) and 1ml ethanol before incubation at -20°C for 48 hours. The plasmid DNA was then pelleted at 13000g for 15 minutes. The pellet was washed in 70% ethanol and allowed to air dry before resuspending in 200µl TE. The DNA was then quantitated by measuring absorbance at $A_{260}$ (section 2.3.5), the plasmid stock diluted to 1µg/µl and stored at -20°C.

### 2.6.2.2 Plasmid purification using the Qiagen Midi Prep kit

Plasmid DNA was extracted from MC1061 E.Coli bacterial cultures transformed with the pBJ5, pBJ5-brg1K/R and $K^K$II plasmids using the Qiagen midiprep kit according to the manufacturers' instructions, with some modification as outlined below.

Overnight bacterial cultures (400ml) were pelleted at 6000g for 15 minutes at 4°C. Pellets were resuspended in 20ml of buffer P1 to which 20ml of buffer P2 was added before inverting several times. Lysates were incubated at room temperature for 5 minutes, 20ml of chilled buffer P3 added and samples mixed by inverting several times before incubating on ice for 30 minutes. Samples were then centrifuged at 20000g for 30 minutes at 4°C following which the supernatant was removed and centrifuged again at 20000g for 15 minutes. The supernatant was transferred to a fresh tube and DNA was precipitated by adding 0.7 volumes of isopropanol. After inverting to mix, samples were centrifuged at 15000g for 30 minutes at 4°C. The supernatant was then carefully removed and the pellet resuspended in 500µl TE and RNase treated (100µg/ml) for 30 minutes at 37°C. Samples were then phenol/chloroform extracted and ethanol/NaAc precipitated (section 2.9.3). DNA

was resuspended in 500µl TE and 4.5ml of buffer QBT was added. The columns (Qiagen-tip 100) were equilibrated by washing with 4ml of buffer QBT and allowed to empty by gravity flow. The DNA solution was then applied to the column and emptied by gravity flow. The column was washed twice with 10ml of buffer QC and plasmid DNA was eluted with 5ml of buffer QF into a glass sorvall tube. Isopropanol (3.5ml) was added to precipitate the DNA. Samples were mixed before centrifuging at 15000g for 30 minutes at 4°C. The pellet was washed in 70% ethanol, air dried for 10 minutes then resuspended in 400µl TE. Plasmid DNA was then reprecipitated and resuspended in a smaller volume before quantitation by spectrophotometry reading at $A_{260}$ (Section 2.3.5) and diluting to 1µg/µl. Plasmid stocks were stored at -20 °C.

## 2.6.3 Electroporation of EL-4 T cells

EL-4 T cells were co-transfected by electroporation with 8µg of either pBJ5, pBJ5-brg1K/R, pEGFP or pEGFP-VIVIT plasmids along with 3µg of the $K^K$II selection plasmid in 4mm cuvettes using the BioRad Gene Pulser X cell. Briefly, EL-4 T cells at a density of $5x10^5$ cells/ml were pelleted at 1000g for 5 minutes. The supernatant was removed and the cells resuspended in RPMI containing 20% FCS at $1.5x10^7$ cells/ml. Plasmid DNA was then added to the side of each cuvette (BioRad) followed by the addition of 300µl of the cell suspension. Cells were then electroporated at 270V with a capacitance of 975µF. RPMI medium (1ml) was then added to each cuvette and the cells left to recover for 5 minutes. The transfected cells were then transferred to $75cm^2$ culture flasks containing the appropriate amount of medium (5ml per electroporation) and returned to the incubator for 24 hours.

## 2.6.4 Magnetic labelling and sorting of transfected cells

Transfected cells were enriched 24 hours post transfection using the MACSelect magnetically activated cell sorting kit (Miltenyi Biotech) according to the manufacturers' instructions. Transfected cells ($2x10^7$) were pelleted at 200g for 5 minutes and resuspended in 1ml PBS supplemented with 5mM EDTA pH 8. The cell suspension was then layered over 2ml of FCS in a 15ml Falcon tube before pelleting once again at 200g for 5 minutes to

remove dead cells. The cell pellet was resuspended in 320µl degassed PBE (PBS containing 5mM EDTA and 0.5% BSA) per $10^7$ cells and 80µl of MACSelect $K^K$II microbeads were added. The cells and beads were mixed well and incubated at 4°C for 15 minutes, with occasional flicking. The cell suspensions were made up to 2ml with PBE. Two $MS^+/RS^+$ columns (Miltenyi Biotech) were set up in the magnetic separation apparatus and washed with 500µl PBE. The magnetically labelled cells were then applied to the columns in 4x 500µl aliquots (half over each column). The columns were then washed twice with 500µl PBE before removing from the magnetic field and placing into a 15ml Falcon tube. RPMI medium (500µl) was then added to the column and the plunger used to force cells through. Cells were then transferred to 6 well culture plates at a density of $5x10^5$ cells/ml.

## 2.7 Chromatin Immunoprecipitation (ChIP)

ChIP analysis of Brg1 binding was performed according to the Upstate protocol (http://www.upstate.com/misc/protocol) with some modifications. EL-4 T cells or A-20 B cells (15ml at $5x10^5$ cells/ml) were incubated in RPMI or DMEM respectively containing 1% formaldehyde for 15 minutes with shaking at room temperature to crosslink chromatin. Crosslinking was stopped by adding glycine to a final concentration of 0.125M and incubating for 10 minutes with shaking. Cells were collected after two PBS washes (supplemented with 2µg/ml Aprotinin, 1µg/ml Leupeptin and 100µM PMSF) at 500g for 4 minutes. Cells ($2x10^6$) were resuspended in 250µl lysis buffer (1% SDS, 10mM EDTA, 50mM Tris HCl pH 8.1 supplemented with protease inhibitors). Following incubation on ice for 10 minutes samples were sonicated five times with 30 second pulses at output 2 (Microson XL 2000) to generate DNA fragments ranging from 200 to 1000bp. Samples were diluted to 1.25ml with IP dilution buffer (1.2mM EDTA pH8, 0.1% SDS, 1% Triton X, 16.7mM Tris HCl pH 8.1, 167mM NaCl) and pre-cleared with 60µl salmon sperm DNA/Protein A agarose (Upstate) for 30 minutes at 4°C. The protein A agarose was pelleted by centrifuging at 500g for 1 minute and 100µl of the supernatant removed for the total input. The remaining supernatant was aliquoted as either a no antibody control or immunoprecipitated with 1µg of Brg-1 antibody (Santa Cruz Biotechnology) overnight at

4°C with rotation. Immune complexes were recovered by incubation at 4°C for one hour with 60µl salmon sperm DNA/Protein A agarose. Samples were washed for 3 minutes on the rotary wheel at 4°C followed by pelleting at 500g for 1 minute. Immune complexes were washed once in low salt buffer (2mM EDTA, 0.1% SDS, 1% Triton X, 20mM Tris HCl pH 8.1, 150mM NaCl), high salt buffer (2mM EDTA, 0.1% SDS, 1% Triton X, 20mM Tris HCl pH 8.1, 500mM NaCl) and LiCl buffer (1mM EDTA, 10mM Tris HCl pH 8.1, 250mM LiCl, 1% Igepal, 1% deoxycholate) and washed twice with TE (1mM EDTA pH 8, 10mM Tris HCl pH 8.1). All wash buffers were supplemented with protease inhibitors. Chromatin was eluted by adding 200µl of elution buffer (1% SDS, 0.1M $NaHCO_3$) and incubating at room temperature for 1 hour on the rotary wheel. Protein A agarose was pelleted by centrifuging at 500g for 1 minute and the supernatant containing chromatin removed. The elution step was repeated and supernatants combined to give 400µl total volume. Crosslinks were reversed with 0.2M NaCl overnight at 65°C. DNA was then purified by phenol/chloroform extraction and ethanol/NaAc precipitation (section 2.9.3). DNA was resuspended in 50µl of MilliQ water and amplified by real-time PCR using primer sets outlined in Table 2.5.

**Table 2.5. Primers used in real-time PCR analysis of ChIP samples.**

| Name | Sequence (5' to 3') | Amplicon (bp) | Reference |
|---|---|---|---|
| mGM-I Forward primer | GCCTGACAACCTGGGGGAAG | 116 | Holloway *et al*, 2003 |
| mGM-I Reverse primer | TGATTAATGGTGACCACAGAACTC | | |
| mGM+I Forward primer | GAGTTCTGTGGTCACCATTAATCA | 147 | Holloway *et al*, 2003 |
| mGM+I Reverse primer | CACATCCTCCTCAGGACCTT | | |
| mGM-V Forward primer | TGGAATGAGCCACCAGAGTA | 75 | Holloway *et al*, 2003 |
| mGM-V Reverse primer | GGCTCTTGCTTCCATAGCAC | | |
| mIL-4E Forward primer | GCACCAGGGCACTTAAACAT | 158 | |
| mIL-4E Reverse primer | CTGTGCAGTGCCACAATGAT | | |
| mIL-5p Forward primer | ACCCTGAGTTTCAGGACTCG | 94 | Wang *et al*, 2006 |
| mIL-5p Reverse primer | TCCCCAAGCAATTTATTCTCTC | | |
| mIFN-γp Forward primer | AACATGCCACAAAACCATAGC | 156 | |
| mIFN-γp Reverse primer | CACCTCTCTGGCTTCCAGTT | | |
| mGM-3' Forward primer | ATTTGGGCATAGGTGGAGTG | 89 | |
| mGM-3' Reverse primer | CCTCGATTTCACCTCCCTTT | | |
| mGM-5' Forward primer | GAGCTTCTGGAGAGGGAGGT | 60 | |
| mGM-5' Reverse primer | TCCCAGGCTTAGTCTGTTGC | | |
| mGAPDH Forward primer | AAGTATGATGACATCAAGAAGGTGGT | 67 | Brettingham-Moore *et al*, 2005 |
| mGAPDH Reverse primer | AGCCCAGGATGCCCTTTAGT | | |

All primers were supplied by Sigma. Real-time PCR was carried out as described in section 2.3.6.2. Brg1 recruitment was determined as the percentage of immunoprecipitated sample, minus the "No Antibody" control, compared to the total input.

## 2.8 Microarrays

The expression of T cell genes in response to the introduction of an ATPase defective Brg1 mutant construct (pBJ5-brg1K/R) was determined by microarray analysis according to the following protocols.

### 2.8.1 Sample preparation from Brg1 mutant cells

EL-4 T cells co-transfected with either the pBJ5 or pBJ5brg1K/R and $K^{K}$II plasmids were sorted for transfected cells (section 2.6.3 and 2.6.4) then either left untreated

or stimulated for 6 hours in the presence of P/I as described in sections 2.1.4. Total RNA was isolated as described in section 2.2.1.

## 2.8.2 RNA quality assessment

The quality of the RNA to be used in the microarray experiments was assessed using the Agilent 2100 BioAnalyzer which determines sample integrity and the NanoDrop® which determines sample concentration and purity. cRNA was prepared and microarrays hybridized with Dr. Kaiman Peng at the Biomolecular Resource Facility, John Curtin School of Medical Research at the Australian National University, according to the recommended Affymetrix protocols as outlined below.

## 2.8.3 First strand cDNA synthesis

RNA (3μg) was made up to a total volume of 8μl with MilliQ water. RNA along with 2μl PolyA RNA control (Invitrogen Life Technologies) and 2μl T7-(dT)24 oligomer (Affymetrix) were combined into 100μl PCR tubes. The reaction components were mixed and incubated at 70°C for 10 minutes before cooling at 4°C for 2 minutes. To each sample 4μl of 5x first strand reaction mix (Invitrogen Life Technologies), 2μl 0.1 M DTT (Invitrogen Life Technologies) and 1μl 10mM dNTPs was added along with 1μl Superscript II (Invitrogen Life Technologies). The reactions were incubated at 42°C for 1 hour before cooling for 2 minutes at 4°C.

## 2.8.4 Second strand cDNA synthesis

Following first strand cDNA synthesis, 91μl DEPC treated water, 30μl 5x second strand reaction (Invitrogen Life Technologies), 3μl 10mM dNTPs (Invitrogen Life Technologies), 1μl 10U/μl E. coli DNA ligase (Invitrogen Life Technologies), 4μl 10U/μl E. coli DNA polymerase I (Invitrogen Life Technologies) and 1μl 2U/μl E. coli RNase H (Invitrogen Life Technologies) were added to each sample. Each sample was then incubated at 16°C for 2 hours. T4 DNA polymerase (2μl; Invitrogen Life Technologies) was then added and the samples incubated at 16°C for a further 5 minutes. Following this

10µl of 0.5M EDTA was added. Double stranded cDNA was purified using phase lock gel GeneChip sample clean up modules (Affymetrix).

## 2.8.5 Synthesis of Biotin labelled cRNA

In Vitro Transcription (IVT) and biotin labelling of cRNA was carried out using the BioArray HighYield RNA transcript labelling kit (Affymetrix). Briefly, template cDNA (12µl) was transferred to an RNase free microfuge tube along with 8µl RNase free water, followed by 12µl labelling NTP mix (Affymetrix), 4µl 10x IVT labelling buffer (Affymetrix) and 4µl IVT labelling enzyme mix (Affymetrix). The reactions were incubated at 37°C for 16 hours.

## 2.8.6 Purification and quantification of cRNA

Unincorporated NTPs were removed from the cRNA using the Genechip sample clean up module (Affymetrix). IVT reactions were made up to 100µl with RNase free water and mixed by vortexing for 3 seconds. IVT cRNA binding buffer (350µl; Qiagen) was added and vortexed, followed by 250µl of 100% ethanol which was mixed by pipetting. The sample was then applied to the IVT cRNA clean up spin column which was then centrifuged for 15 seconds at 8000g. The flow through was discarded and IVT cRNA wash buffer (500µl; Qiagen) was added to each column followed by centrifugation at 8000g for 15 seconds before discarding the flow through. This step was repeated using 500µl of 80% ethanol. The spin column was then centrifuged at maximum speed for 5 minutes before discarding the collection tube and its contents. RNase free water (35µl) was pipetted directly onto the membrane before centrifuging at 13000g for 1 minute and the eluate collected in a new collection tube. Another 35µl of RNase free water was added to the column and the eluate collected by centrifugation at 13000g for 1 minute. The concentration and purity of the cRNA was determined using the NanoDrop®.

## 2.8.7 Fragmentation of cRNA

Fragmentation reactions were set up using 20µg cRNA along with 6.4µl 5x

Fragmentation buffer (Affymetrix) and RNase free water to a total volume of 32µl. cRNA was then incubated at 94°C for 35 minutes. A small aliquot of non fragmented and fragmented cRNA was analyzed using the BioAnalyzer (Agilent) to determine concentration and purity. Samples were stored at -20°C before hybridization.

## 2.8.8 Target hybridization

A hybridization mixture was prepared containing 15µg fragmented cRNA, 50pM control oligonucleotide B2 (Affymetrix), 1x eukaryotic hybridization buffer (Affymetrix), 100pM DMSO, 1.5/5/25/100pM controls (bioB, bioC, bioD, cre; Affymetrix), 0.1mg/ml herring sperm DNA (Affymetrix) in a total volume of 300µl.

cRNA hybridization cocktails were initially hybridized to Affymetrix Test3 arrays to ensure sample quality before proceeding onto the Mouse Genome 430 2.0 GeneChips®. The arrays were equilibrated to room temperature and the hybridization cocktails were heated at 99°C for 5 minutes then 45°C for 5 minutes. The hybridization cocktails were then centrifuged at 13000g for 5 minutes to remove insoluble material. The array was wet with 1x hybridization buffer (Affymetrix) by pipetting through one of the septa, and incubated in the hybridization oven for 10 minutes with rotation at 45°C. Buffer solution was then removed from the array chamber and the chamber filled with 80µl of the hybridization cocktail. The arrays were then placed in the hybridization oven at 45°C and rotated at 60rpm for 16 hours.

## 2.8.9 Washing and staining the arrays

Arrays were washed and scanned using the GeneChip® Operating System (GCOS, Affymetrix). Arrays were removed from the hybridization oven and the hybridization cocktail removed. The array was then filled with 100µl of non stringent wash buffer after equilibrating to room temperature. The following dye and antibody solutions were prepared and 600µl of each placed into eppendorfs and fitted to the fluidics station.

**SAPE Stain solution**

| | |
|---|---|
| 2x MES stain buffer (Affymetrix) | 600µl |
| 50mg/ml acetylated BSA (Affymetrix) | 48µl |
| 1mg/ml SAPE (Affymetrix) | 12µl |
| $ddH_2O$ | 540µl |
| Total volume | 1200µl |

**Antibody solution**

| | |
|---|---|
| 2x MES stain buffer (Affymetrix) | 300µl |
| 50mg/ml acetylated BSA (Affymetrix) | 24µl |
| 10mg/ml goat IgG (Affymetrix) | 6µl |
| 0.5mg/ml biotinylated antibody (Affymetrix) | 3.6µl |
| $ddH_2O$ | 266.4µl |
| Total volume | 600µl |

After washing the lines in the fluidics station and placing the wash and stain solutions in the station, the arrays were fitted to the station and the wash and dye steps carried out according to the program set. The arrays were then scanned using the Affymetrix GeneChip® Scanner 3000.

## 2.9 General Techniques

### 2.9.1 General reagents

**TAE buffer (50x)**

24.2g Tris base

10µl 0.5M EDTA

5.7 ml Glacial acetic acid

Distilled water to 100mls

pH to 7.0 using acetic acid

**Agarose gel loading buffer**

0.25g sucrose

12.5µl 0.5M EDTA

125µl 10% SDS

0.25% BPB

MilliQ water to 1ml

**L-Broth**

5g tryptone

2.5g yeast extract

2.5g NaCl

200 µl 5M NaOH

MilliQ water to 500 ml

1.5g agar

**LB agar plates**

1g tryptone

0.5g yeast extract

0.5g NaCl

MilliQ water to 100ml

40µl 5M NaOH

**SOC medium**

20g tryptone

5g yeast extract

0.5g NaCl

2.5ml 1M KCl

MilliQ water to 1000ml

pH to 7 with NaOH

20ml sterile glycerol

## 2.9.2 Reagents for SDS PAGE and western blotting

**10x SDS-PAGE running buffer**

14.5g Tris base

72g Glycine

5g SDS

To 500ml with distilled water

Distilled water to10ml

β-mercaptoethanol (10%) added before use.

**2x Sample buffer**

| | |
|---|---|
| 0.5M Tris HCl, pH 6.8 | 2.5 ml |
| Glycerol | 2.0 ml |
| 10% SDS | 4.0 ml |
| 0.1% BPB | 0.5 ml |

**SDS-PAGE Upper buffer**

30g Tris Base

2g SDS

pH to 6.8 with HCl

Distilled water to 500ml

**SDS-PAGE Lower buffer**

91g Tris Base

2g SDS

pH to 8.8 with HCl

Distilled water to 500ml

**SDS-PAGE Running gel**

2ml lower buffer

3.4ml 30% acrylamide

20µl 10% APS

10µl TEMED

2.7ml distilled water

**SDS-PAGE Stacking gel**

0.7ml Upper buffer

0.4ml 30% acrylamide

15µl 10% APS

5µl TEMED

1.5ml distilled water

**Western transfer buffer**

2.4g Tris

11.2g Glycine

200ml Methanol

1.0g SDS

Distilled water to 1000ml

**10x TNT, pH 8**

12.11g Tris

87.6g NaCl

5ml Tween

Distilled water to 1000ml

**Blocking solution**

Western blocking reagent (Roche) diluted 1:10 with 1x TNT

**Stripping buffer**

6.25ml 1M Tris

0.7ml β-mercaptoethanol

20ml 10% SDS

Distilled water to 100ml

### 2.9.3 Phenol/chloroform extraction and ethanol precipitation of DNA

DNA was extracted and concentrated by adding an equal volume of phenol:chloroform to samples. Samples were vortexed and centrifuged at 10000g for 10 minutes. The upper phase was then removed and 0.1 volumes of NaAc pH 6.5 and 2 volumes of 100% ethanol added. Samples were then precipitated at -20°C overnight before centrifuging at 13000g for 20 minutes. The DNA pellet was then washed with 70% ethanol and air-dried for 5 minutes before resuspending in an appropriate amount of MilliQ water.

# CHAPTER 3.
# GM-CSF PROMOTER REMODELLING AND TRANSCRIPTION ARE DISTINCT PROCESSES.

## 3.1 Introduction

The induction of cytokine gene expression is primarily controlled at the level of transcription, through the assembly of specific transcription factor complexes across gene regulatory regions. It is now evident that the transcriptional machinery must first access the relevant DNA binding sites within a repressive chromatin environment which provides an additional level of regulatory control (reviewed in Smale and Fisher, 2002; Holloway *et al*, 2002). While it is well recognized that GM-CSF transcription is induced when stimulated by signals arising from T cell activation, (reviewed in Shannon *et al*, 1997; Jenkins *et al*, 1995) the role of the pathways and factors activated downstream of TCR ligation in chromatin remodelling events at the GM-CSF gene remain largely unknown.

The rapid increase in GM-CSF gene expression following T cell activation is regulated by a proximal promoter limited to 100 bp upstream of the transcription start site as well as an upstream enhancer (reviewed in Shannon *et al*, 1997). Earlier work by Cockerill *et al* (1999) has identified an inducible DNase I hypersensitive site in the GM-CSF promoter in response to stimulation with PMA and ionophore. The presence of this inducible DNase I hypersensitive site suggests that the chromatin structure of the GM-CSF promoter is altered following T cell stimulation. Indeed, a more detailed analysis by Holloway *et al* (2003) has revealed that GM-CSF gene expression is preceded by changes in chromatin structure across the proximal promoter region of the gene. While the GM-CSF promoter is slightly accessible to agents such as MNase and restriction enzymes in the resting T cell, immune stimulation promotes a dramatic rise in accessibility. Upon T cell stimulation changes in chromatin structure occur across the GM-CSF promoter in the region between -174 and +24, suggesting the targeted remodelling of a single nucleosome encompassing the proximal promoter (Holloway *et al*, 2003).

Limited data is available regarding the activation of the endogenous GM-CSF

promoter, although a number of studies have considered the role of various transcription factors and signals in GM-CSF reporter driven expression (Jenkins *et al*, 1995; Shang *et al*, 1999). An important role for NF-κB in GM-CSF gene activation has been identified as mutation of the NF-κB site in the human GM-CSF promoter reduces luciferase reporter expression by half (Jenkins *et al*, 1995). In addition, Shang *et al* (1999) demonstrated using gel shift assays, that NFAT binds the GM-CSF promoter to drive expression of a GM-CSF reporter construct in response to P/I signalling.

While it is clear that inducible transcription factors play a major role in activating transcription from the GM-CSF promoter (Shang *et al*, 1999; Cakouros *et al*, 2001), activation of the endogenous GM-CSF gene is accompanied by remodelling of the proximal promoter region (Holloway *et al*, 2003) and the signals and factors responsible for increasing GM-CSF promoter accessibility have yet to be elucidated. However, previous work has implicated NF-κB proteins in GM-CSF promoter remodelling (Cakouros *et al*, 2001; Holloway *et al*, 2003). Mutation of the NF-κB/Sp1 region in the GM-CSF promoter has been shown to drastically reduce GM-CSF expression, and this effect was only observed in a chromatin context and not in transiently transfected plasmids (Cakouros *et al*, 2001). Adding further support to a role for NF-κB in GM-CSF promoter remodelling, Holloway *et al* (2003) stably transfected EL-4 T cells with a mutant IκB construct which prevented the nuclear translocation of NF-κB following T cell stimulation. T cells expressing this mutant construct failed to display the typical increase in promoter accessibility following T cell stimulation. This suggests that chromatin remodelling across the GM-CSF promoter region may be regulated by NF-κB proteins. In contrast, NFAT family members have been implicated in remodelling of the GM-CSF enhancer region (Cockerill *et al*, 1993; Johnson *et al*, 2004), with inhibition of NFAT by the immunosuppressant, Cyclosporin A, preventing the appearance of an inducible DNase I hypersensitive site.

While strong evidence for the NF-κB and NFAT transcription factors in regulating the GM-CSF promoter has been provided in the past, the precise role of individual factors in activating the endogenous GM-CSF gene have yet to be identified. This chapter aims to address how the GM-CSF gene is activated in a chromatin context. More precisely, roles of the PKC and calcium signalling pathways, along with the NF-κB and NFAT family

members were investigated.

## 3.2 Results

### 3.2.1 Distinct signals are required for chromatin remodelling and gene transcription at the GM-CSF promoter.

In order to determine the transcription factor and signal requirements for GM-CSF promoter remodelling and gene transcription, these two events were examined following the activation of different intracellular signalling pathways in T cells. T cell receptor ligation activates two major signalling pathways involving the mobilization of PKC and the increase in free intracellular calcium levels (Altman *et al*, 1992). Phorbol esters and calcium ionophores are often used to activate T cells *in vitro* to mimic the activation of the PKC and calcium signalling pathways respectively (Takahama and Nakauchi, 1996). These agents are therefore able to trigger T cell activation and have previously been shown to induce the transcription of a number of cytokine genes including GM-CSF (Tsuboi *et al*, 1994).

Previous work by the author has demonstrated that both the PKC and calcium sigalling pathways are required for full transcriptional induction of GM-CSF gene expression (Brettingham-Moore, 2002, Honours Thesis). Little GM-CSF mRNA was detected in resting EL-4 T cells, however following the combined P/I treatment, GM-CSF mRNA levels increased approximately 200 fold (Figure 3.1a). In contrast I alone had minimal impact on GM-CSF transcription (approximately 4 fold increase) while treatment with P resulted in only a small (20 fold) increase. In addition these studies found using a real-time PCR based accessibility assay (CHART PCR, Rao *et al*, 2001) that a *Hin*fI site in the CK1 element in the GM-CSF promoter has an inherent basal accessibility of approximately 40% in non stimulated cells (Figure 3.1b). Following stimulation with P/I, *Hin*fI accessibility increased to approximately 70%. Similar accessibility levels were observed at the GM-CSF promoter in P stimulated cells, while accessibility in I treated cells remained at basal levels (Brettingham-Moore, 2002, Honours thesis).

Next it was necessary to determine whether accessibility changes observed at the *Hin*fI site were seen across the entire promoter and had the same signal requirements.

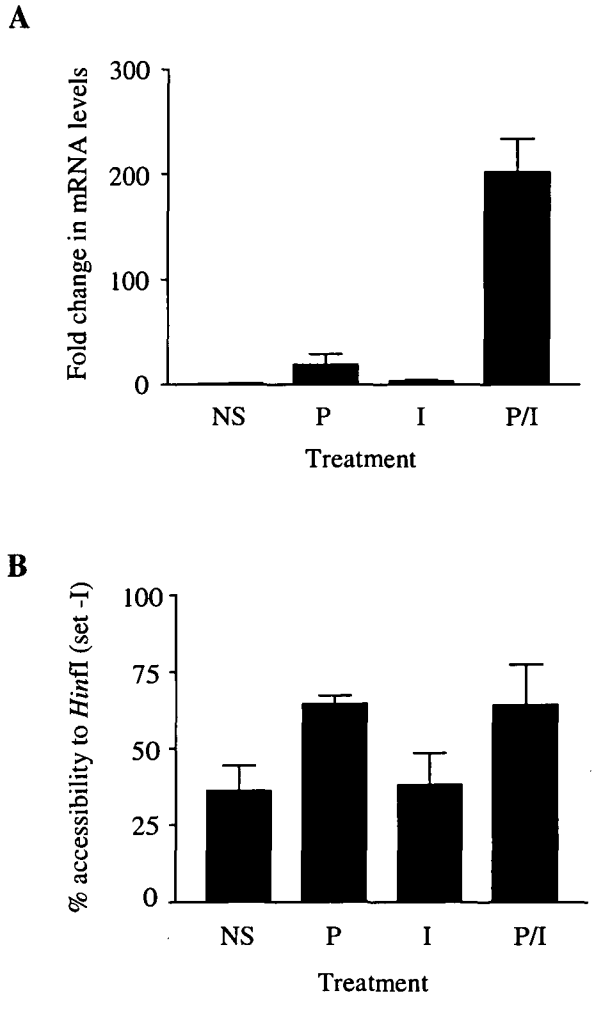



**Figure 3.1. Distinct signals are required for GM-CSF gene transcription and promoter remodelling.** (Brettingham-Moore, 2002, Honours thesis). (A) GM-CSF mRNA levels were determined by real-time PCR analysis of cDNA prepared from EL-4 T cells either left untreated (NS) or stimulated for 4 hours with P and I, alone or in combination. Data is graphed as fold change in GM-CSF mRNA levels compared to non-stimulated. The mean and standard error of three replicate assays are shown. (B) Nuclei from NS EL-4 T cells or cells stimulated for 4 hours with P and I as indicated were incubated with *Hin*fI. Genomic DNA was analyzed by real-time PCR with primer set –I. The mean and standard error of three replicate assays are shown.

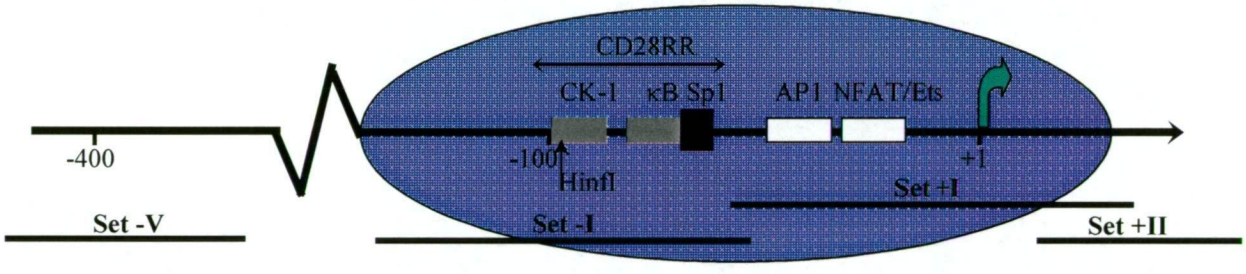



**Figure 3.2. Schematic representation of the GM-CSF promoter with transcription factor binding sites, the CD28RR, *Hin*fI cutting site and regions amplified by primer sets –V, -I, +I and +II.**

Therefore the effect of the different stimuli on the accessibility of the promoter region to MNase digestion was assessed using primer sets mGM-I, mGM+I and mGM-V (Figure 3.2). Primer set -I covers the CD28RR and the *Hin*fI recognition sequence, while primer set +I (-63 to +44) spans the transcription start site and the AP1, NFAT/Ets transcription factor binding sites. In addition, accessibility at a site spanned by primer set –V, which binds further upstream of the transcription start site was analyzed (Figure 3.2). While *Hin*fI cuts at a specific site, MNase will cleave all internucleosomal sites. EL-4 T cells were left untreated or stimulated with P, I or P/I for 4 hours. Nuclei were then isolated and chromatin digested using MNase. Accessibility across the promoter region mirrored the results previously obtained using *Hin*fI. In unstimulated cells both the CD28RR (primer set -I, Figure 3.3a) and NFAT/AP-1 region (primer set +I, Figure 3.3b) showed an inherent accessibility to MNase of approximately 50%. Stimulation with P alone or P/I resulted in an increase in accessibility to approximately 75% across the entire promoter. I alone however was not able to increase accessibility above basal levels. Previous data (Holloway *et al*, 2003) has shown that changes in accessibility are confined to the GM-CSF promoter region. In agreement with this a lower basal accessibility was observed in the region amplified by primer set -V, located -422 to -347 base pairs upstream of the transcription start site. In addition stimulation with P, I and P/I failed to cause any change in accessibility in this region (Figure 3.3c). Put together, these data provide evidence for a role for factors activated downstream of PKC signalling in chromatin remodelling events at the GM-CSF promoter. However, full transcriptional induction of the GM-CSF gene requires factors activated downstream of the PKC and calcium signalling pathways.

The GM-CSF promoter contains an array of transcription factor binding sites including sites for NF-κB and NFAT proteins and these transcription factor families have been implicated in GM-CSF gene expression (Shang *et al*, 1999; Cakouros *et al*, 2001). In order to determine which transcription factors are likely to be involved in directing promoter remodelling compared to transcription, the presence of different transcription factors in the nucleus was correlated with different signalling events. Nuclear extracts were prepared from EL-4 T cells either left untreated (NS) or stimulated with P, I or P/I for 4 hours. The presence of NF-κB family members, c-Rel, Rel A along with NFAT family members, NFATc and NFATp in the nucleus was determined by western blotting. It is

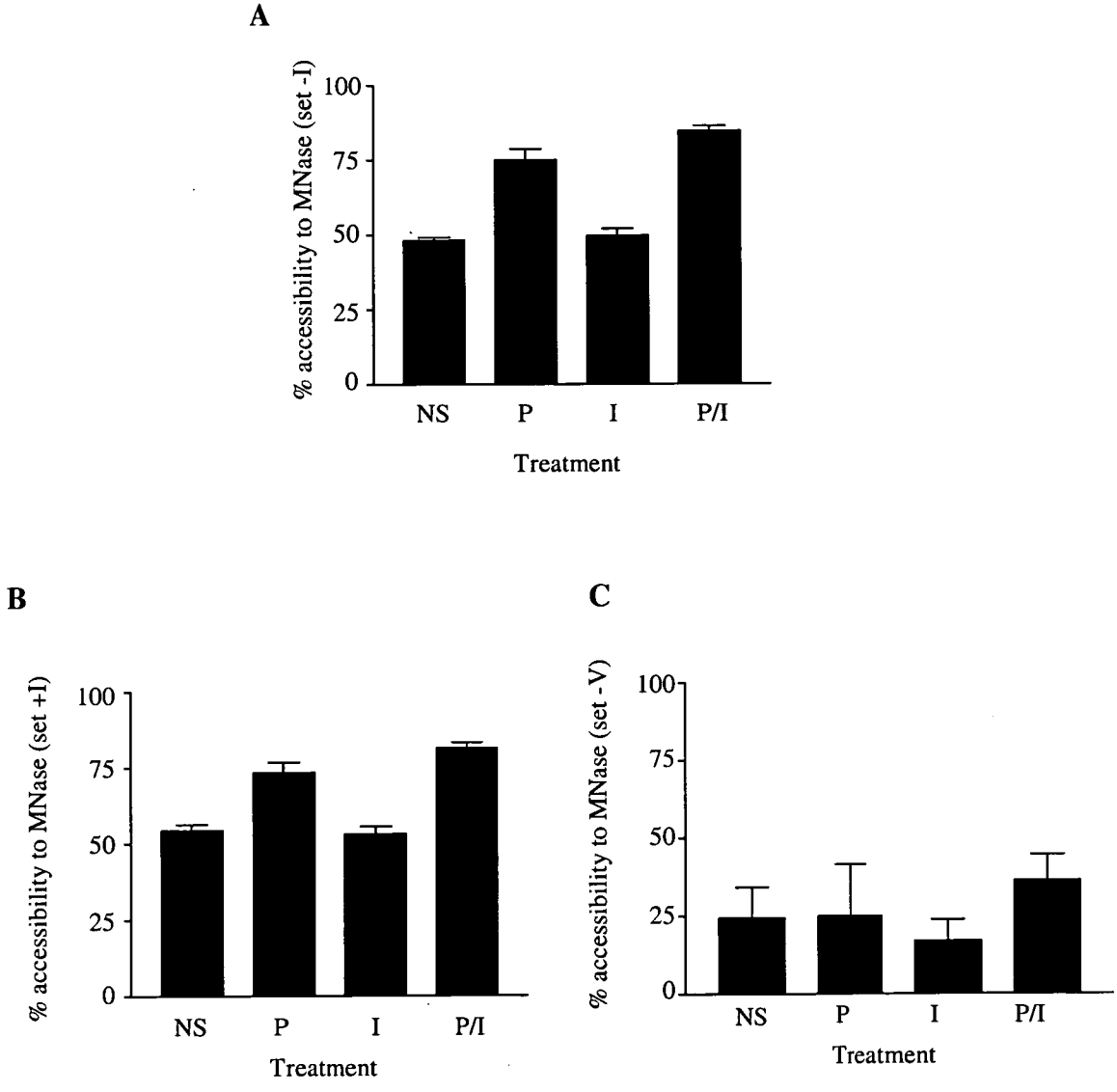



**Figure 3.3. PKC signalling is sufficient to induce GM-CSF promoter remodelling.** Nuclei were isolated from non-stimulated (NS) EL-4 T cells or cells stimulated for 4 hours with P and I as indicated and incubated with MNase. Genomic DNA was analyzed by real-time PCR with primer set -I (A), +I (B) and –V (C). The mean and standard error of three replicate assays are shown.

clear that while P can induce the nuclear translocation of c-Rel and Rel A, stimulation with P/I results in an increased accumulation of these proteins (Figure 3.4). Stimulation with P alone can induce the nuclear translocation of NFATc and once again stimulation with P/I results in an increase in nuclear accumulation. Combined P/I stimulation is required for the nuclear translocation of NFATp. I is capable of allowing a small amount of NFATc to enter the nucleus. Combined PKC (P) and calcium (I) signals ensures the nuclear translocation of all 4 factors. The blot was reprobed using an Sp1 antibody to ensure even loading of all samples. From these results it is clear that the presence of c-Rel, Rel A and NFATc in the nucleus correlate with chromatin remodelling (Figure 3.3a-b) and suggest that these factors could play a role in increasing accessibility to the GM-CSF promoter in P treated cells.

The PKC inhibitor, Ro-32-0432 (Wilkinson *et al*, 1993) was used to confirm the role of PKC signalling in GM-CSF promoter remodelling and gene transcription. To ascertain whether the PKC inhibitor had an effect on transcription factor activation EL-4 T cells were either left untreated or pre-treated with Ro-32-0432 for 1 hour before being stimulated with P/I for 4 hours. Nuclear extracts were then prepared and analyzed by western blotting (Figure 3.5). The inhibition of PKC signalling prevented the nuclear translocation of both NFATc and NFATp along with c-Rel and to a lesser extent Rel A. The blot was reprobed using an Sp1 antibody to ensure even loading of all samples.

The effect of PKC inhibition on GM-CSF transcription was then determined by real-time PCR analysis of cDNA prepared from Ro-32-0432 treated EL-4 T cells. cDNA was analyzed by real-time PCR using the mGM+II primer set and primers designed to the "housekeeping" gene GAPDH (Table 2.2 chapter 2). PCR amplification was monitored by SYBR green incorporation and standard curves used to correlate Ct values with copy number. GM-CSF mRNA levels were then normalized to GAPDH. Cells activated in the absence of Ro-32-0432 showed an approximately 150 fold increase in GM-CSF expression (Figure 3.6a). In contrast pre-treatment with the PKC inhibitor markedly inhibited GM-CSF transcription in response to T cell activation. To determine whether the reduction in GM-CSF activation was due to the inhibition of GM-CSF promoter remodelling, EL-4 T cells were either left untreated or pre-treated with Ro-32-0432 prior to incubating with or without P/I. Accessibility of the GM-CSF promoter to *Hin*fI was then determined using CHART-PCR. While control treated cells displayed a typical increase in accessibility

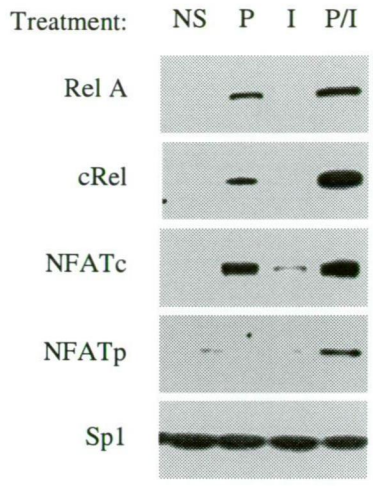



**Figure 3.4. The nuclear translocation of NF-κB and NFAT proteins require distinct signals.** Nuclear extracts from EL-4 T cells either left untreated (NS) or stimulated for 4 hours with P and I or P/I were subjected to SDS-PAGE and analyzed by western blotting with the indicated antibodies.

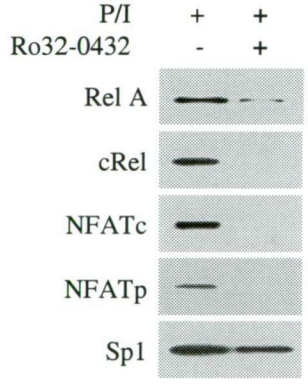
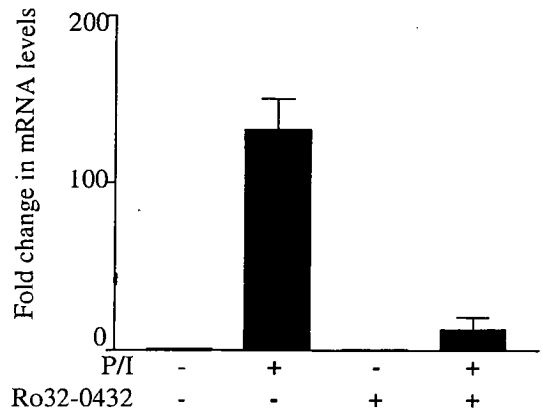



**Figure 3.5. The inhibition of PKC signalling prevents the nuclear accumulation of NF-κB and NFAT proteins.** Nuclear extracts from EL-4 T cells pre-treated with or without the PKC inhibitor Ro-32-0432 followed by stimulation with P/I for 4 hours were subjected to SDS-PAGE and analyzed by western blotting with the indicated antibodies.

A




B

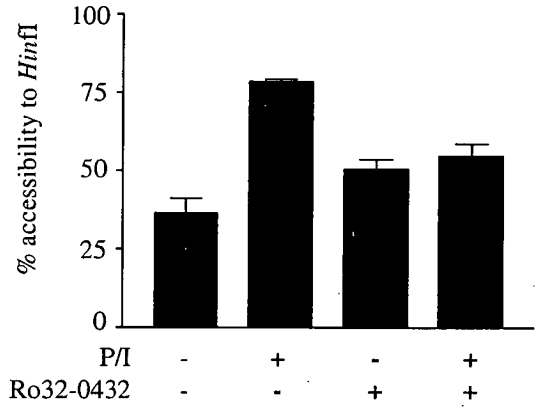



**Figure 3.6. The PKC signalling pathway is required for transcription and GM-CSF promoter remodelling.** (A) EL-4 T cells were either untreated or pre-treated with Ro-32-0432, then incubated with or without P/I for 4 hours before isolating RNA. cDNA was then prepared and GM-CSF mRNA levels were determined by real-time PCR analysis. The mean and standard error of three replicate assays are shown. (B) Nuclei from EL-4 T cells as treated in A were incubated with *Hin*fI and genomic DNA analyzed by real-time PCR using primer set -I. The mean and standard error of three replicate assays are shown.

across the GM-CSF promoter following T cell activation, chromatin remodelling was prevented in cells treated with the PKC inhibitor (Figure 3.6b). Therefore, the inhibition of the PKC signalling pathway prevented remodelling from occurring at the GM-CSF promoter in stimulated cells and confirms that PKC signalling is essential for remodelling.

In order to verify the role of calcium signalling in GM-CSF gene transcription and remodelling the immunosuppressant cyclosporin A (CsA) was used to block this pathway. CsA binds cyclophillin and inhibits the action of the phosphatase calcineurin which is required to dephosphorylate NFAT for nuclear translocation (Liu *et al*, 1991). To determine the transcription factors affected by inhibition of calcium signalling by CsA nuclear extracts were prepared from control and CsA pre-treated EL-4 T cells either left unstimulated or P/I stimulated. Western analysis revealed that pre-treatment with CsA blocked the nuclear accumulation of NFATc and NFATp (Figure 3.7). In addition, CsA was also found to slightly inhibit the translocation of Rel A and c-Rel to the nucleus.

To assess the role of NFAT proteins in GM-CSF promoter remodelling and gene transcription genomic DNA was analyzed by CHART-PCR. Nuclei isolated from EL-4 T cells either left untreated or treated with CsA before incubating with or without P/I for 4 hours, were digested with *Hin*fI and the accessibility of the GM-CSF promoter determined by real-time PCR analysis. Compared to the control treated cells, treatment with CsA did not alter the basal or stimulated levels of accessibility at the GM-CSF promoter (Figure 3.8a). This result demonstrates that NFATc and NFATp are not required for chromatin remodelling events at the GM-CSF promoter in response to P/I. However, the reduction of NFATc and NFATp brought about by CsA pretreatment resulted in a dramatic decrease in GM-CSF mRNA levels (Figure 3.8b). While P/I induced a 300 fold increase in GM-CSF mRNA levels in control cells, only a 100 fold increase was detected in CsA treated cells.

These data suggest that NFAT is required for transcription of the endogenous GM-CSF gene. To confirm this, NFAT nuclear accumulation was specifically inhibited using the VIVIT peptide (Aramburu *et al*, 1999). In order to assess the role of NFAT further and in a more specific manner EL-4 T cells were transfected with a pEGFP-VIVIT expression construct. This peptide prevents the nuclear translocation of NFAT proteins by blocking the calcineurin binding site thereby preventing dephosphorylation (Aramburu *et al*, 1999). EL-

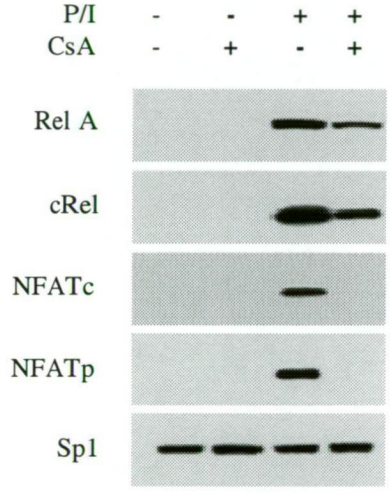



**Figure 3.7. Cyclosporin A prevents the nuclear accumulation of NFAT proteins.** Nuclear extracts from EL-4 T cells either left untreated or pre-treated with CsA before incubating with or without P/I for 4 hours were subjected to SDS-PAGE and analyzed by western blotting with the indicated antibodies.

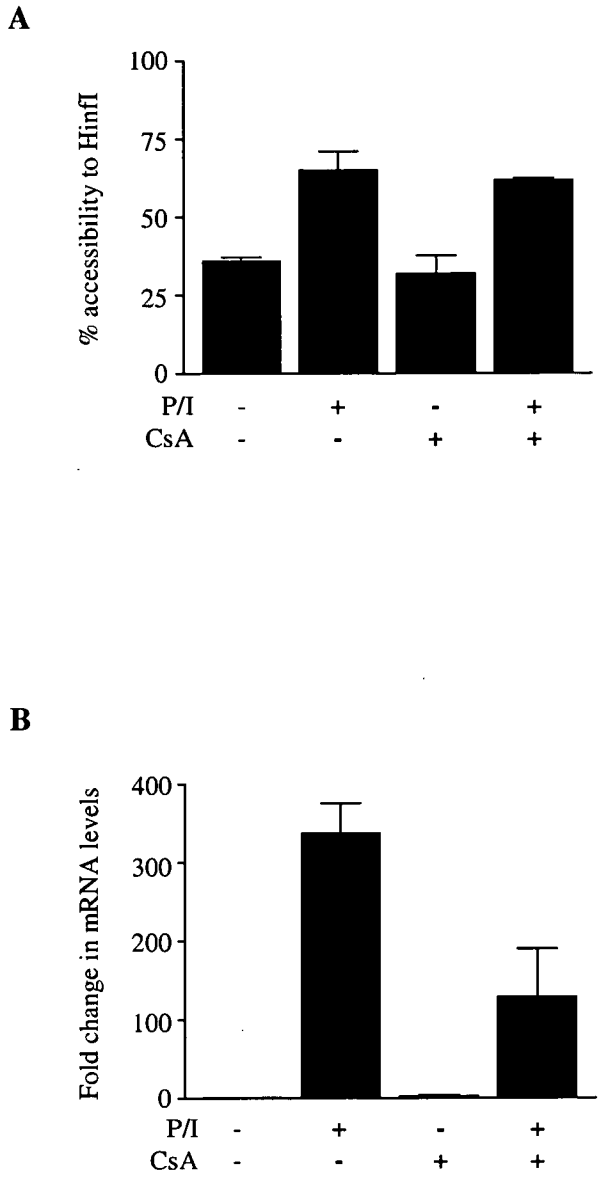



**Figure 3.8. CsA inhibits GM-CSF gene transcription but not promoter remodelling in response to P/I.** (A) Nuclei from EL-4 T cells either left untreated or treated with CsA before incubating with or without P/I for 4 hours were incubated with *Hin*fI and genomic DNA analyzed by real-time PCR using primer set -I. The mean and standard error of three replicate assays are shown. (B) cDNA prepared from EL-4 T cells as treated in A was analyzed by real-time PCR. The mean and standard error of three replicate assays are shown.

4 T cells were co-transfected with the $K^K$II plasmid and the control pEGFP plasmid or the pEGFP-VIVIT plasmid. The $K^K$II plasmid drives the expression of the mouse H-$2K^k$ receptor protein with a truncated cytoplasmic domain. This enables attachment of transfected cells to magnetic beads coated with a monoclonal antibody directed against the surface marker encoded by the $K^K$II plasmid. The transfected cells were sorted 24 hours post transfection by virtue of expression of the H-$2K^k$ receptor. Cells were then either left untreated or stimulated for 4 hours before isolating RNA and analyzing by real-time PCR. While the cells transfected with the control plasmid showed a 160 fold increase in GM-CSF expression following stimulation, transcription was reduced 5 fold in pEGFP-VIVIT transfected cells (Figure 3.9). Therefore, while not required for promoter remodelling, NFAT is essential for the optimal induction of GM-CSF transcription.

To determine whether new protein synthesis is required for chromatin remodelling at the GM-CSF promoter and subsequent transcriptional activation, EL-4 T cells were treated with the protein synthesis inhibitor cyclohexamide (CHX). Nuclear translocation of the NF-κB and NFAT transcription factors in response to P/I was determined by western analysis. As seen previously Rel A, c-Rel, NFATp and NFATc were all absent from the nuclei of non-stimulated cells and all four factors translocated to the nucleus following stimulation with P/I (Figure 3.10a). CHX pre-treatment blocked the nuclear translocation of c-Rel and NFATc, while Rel A and NFATp were unaffected. This result is in keeping with Rel A and NFATp being retained in the cytoplasm of resting T cells and translocated to the nucleus in response to P/I (reviewed in Karin and Ben-Neriah, 2000; Northrop *et al*, 1994) but c-Rel and NFATc being newly synthesized in response to T cell activation. Interestingly, Rel A appeared in the nuclei of non-stimulated cells treated with CHX in keeping with the findings of Han and Brasier (1997) who demonstrated that this is due to the role of the CHX sensitive factor, IκB in the cytoplasmic retention of Rel A. Reprobing with the Sp1 antibody demonstrated equivalent loading in each lane.

The effect of CHX pre-treatment on transcription and chromatin remodelling was then examined. EL-4 T cells were pre-treated with CHX for 30 minutes before being stimulated with P/I for 4 hours or left untreated. GM-CSF mRNA levels were then determined by real-time PCR. While cells untreated with CHX displayed a 350 fold increase in GM-CSF mRNA levels, T cells pretreated with the protein synthesis inhibitor

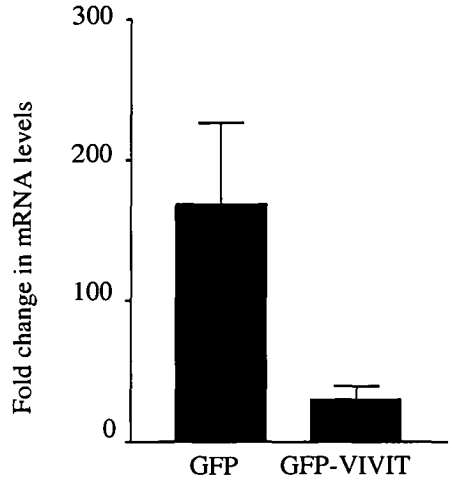



**Figure 3.9. Inhibition of NFAT proteins reduces GM-CSF transcription.** EL-4 T cells were co-transfected with the $K^{K}II$ and GFP or GFP-VIVIT plasmids prior to magnetically activated cell sorting before incubating with or without P/I for 4 hours. cDNA was prepared and levels of GM-CSF mRNA analyzed by real-time PCR. The mean and standard error of three replicate assays are shown.

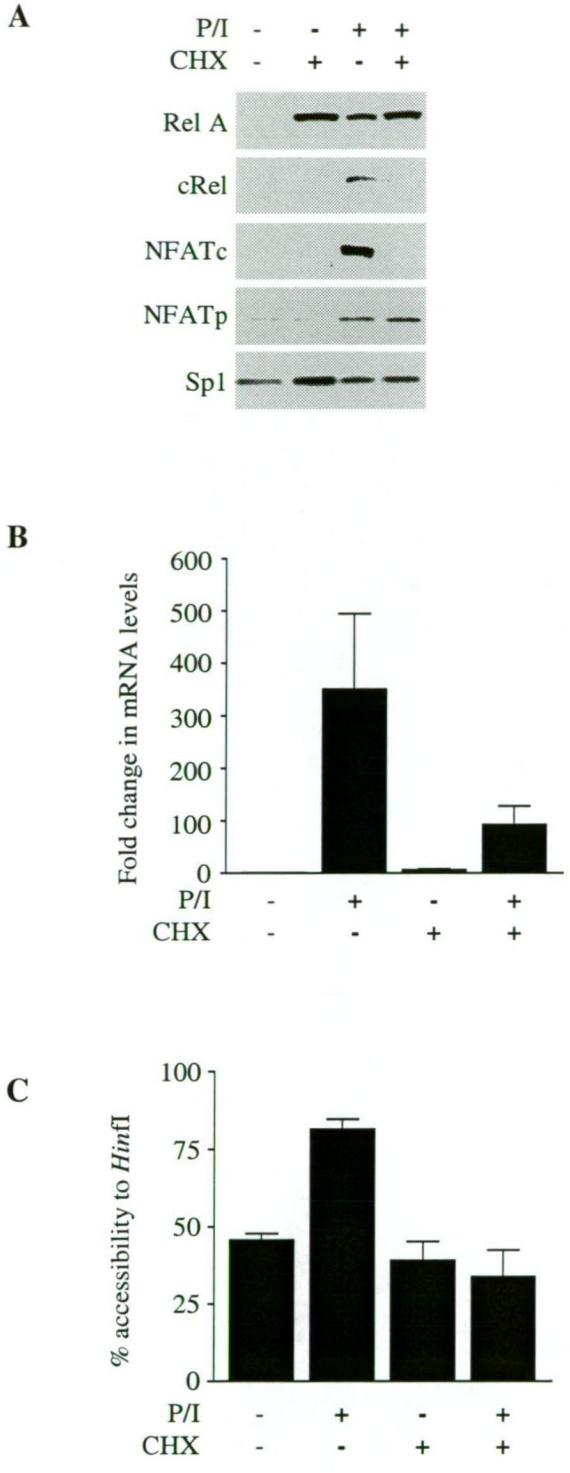



**Figure 3.10. New protein synthesis is required for GM-CSF promoter remodelling but not transciption.** (A) Nuclear extracts from EL-4 T cells either untreated or pre-treated with cyclohexamide (CHX) before incubating with or without P/I for 4 hours were subjected to SDS-PAGE and analyzed by western blotting with the indicated antibodies. (B) cDNA prepared from EL-4 T cells as treated in A was analyzed by real-time PCR. The mean and standard error of three replicate assays are shown. (C) Nuclei prepared from EL-4 T cells as treated in A were digested with *Hin*fI and genomic DNA analyzed by real-time PCR with primer set -I. The mean and standard error of three replicate assays are shown.

displayed only a 100 fold increase in GM-CSF mRNA levels (Figure 3.10b). Next, the possibility that this reduction in transcription was due to inhibition of promoter remodelling was investigated. Nuclei were isolated from cells treated as above and promoter accessibility to *Hin*fI analyzed by CHART-PCR. As observed previously, non-stimulated cells displayed an inherent basal accessibility (~50%) which increased to 80% following T cell stimulation (Figure 3.10c). EL-4 T cells pre-treated with CHX, showed similar basal accessibility (50%) however remodelling of the promoter region following T cell stimulation was inhibited. These data suggest that chromatin remodelling requires new protein synthesis and implicates c-Rel or NFATc in this process, however previous results (Figure 3.8a) have ruled NFAT proteins out as contributing to remodelling. The results also demonstrate that some gene transcription can occur in the absence of promoter remodelling.

### 3.2.2 GM-CSF gene transcription and promoter remodelling display distinct kinetics.

The data outlined above demonstrate that the processes of chromatin remodelling across the GM-CSF promoter and gene transcription are distinct events. To determine whether the kinetics of these events were also distinct, each process was studied over time following T cell activation. EL-4 T cells were stimulated with P/I at various time points, up to 48 hours before extracting RNA and analyzing GM-CSF mRNA levels by real-time PCR. GM-CSF mRNA levels increased within 2 hours post stimulation and continued to increase to 600 fold at 6 hours (Figure 3.11a). Levels had decreased considerably by 24 and 48 hours, however GM-CSF expression remained elevated for at least 48 hours following stimulation. In order to determine whether the kinetics of GM-CSF promoter remodelling were similar to transcription, accessibility to the promoter over time was analyzed. Nuclei from EL-4 T cells stimulated for 0, 2, 6, 24 and 48 hours with P/I were incubated with *Hin*fI before analyzing genomic DNA by real-time PCR. Accessibility of the *Hin*fI site in the GM-CSF promoter increased from 40% to 60% within 2 hours of the initial stimulation (Figure 3.11b) and continued to increase for at least 24 hours when accessibility reached maximal levels of approximately 80%. By 48 hours accessibility had begun to decline. To

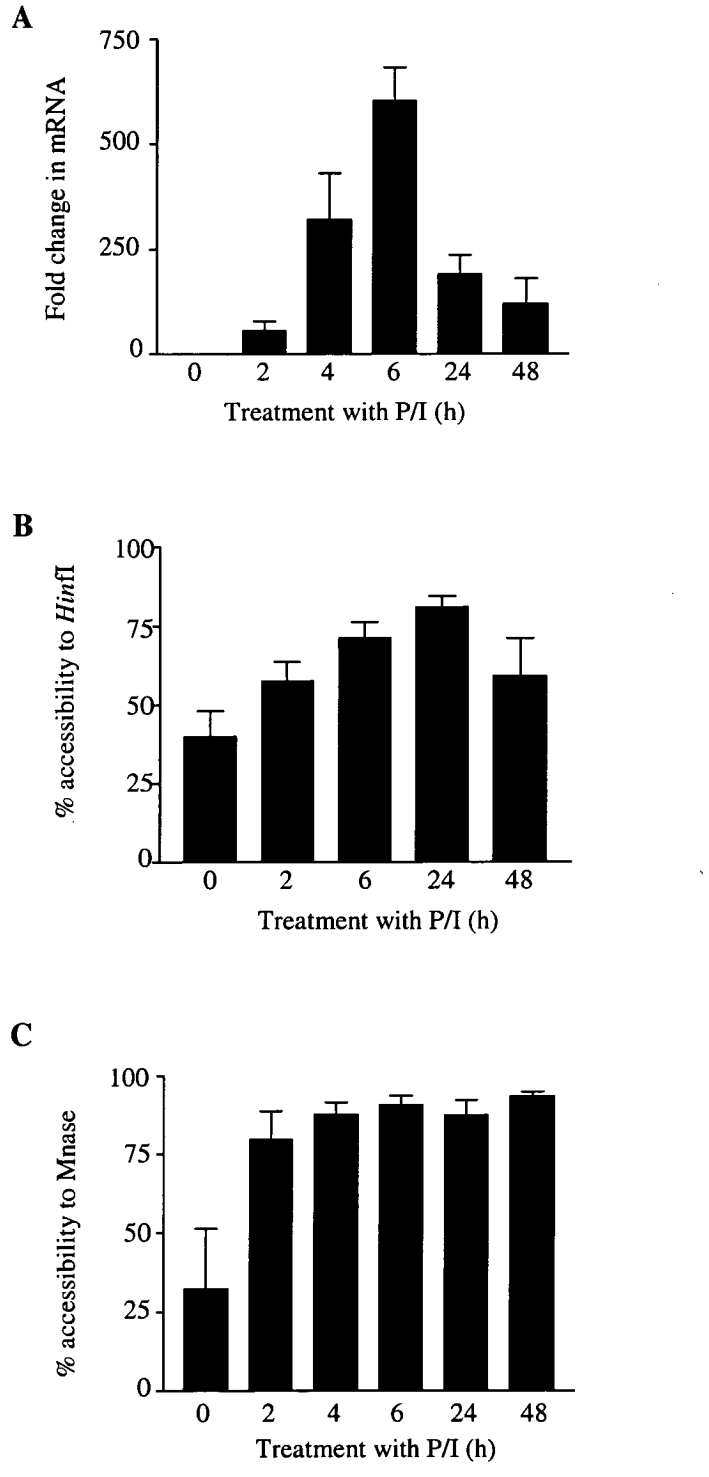



**Figure 3.11. GM-CSF promoter remodelling and gene transciption display distinct kinetics.** (A) GM-CSF mRNA levels were determined by real-time PCR analysis of cDNA prepared from EL-4 T cells stimulated with P/I for the indicated time periods. The mean and standard error of 3 replicate assays are shown. Nuclei from cells stimulated with P/I for the indicated time periods were incubated with *Hin*fI (B) or MNase (C). Genomic DNA was analyzed by real-time PCR using primer set -I. The mean and standard error of three replicate assays are shown.

determine whether similar kinetics were observed across the entire promoter nuclei were again isolated from EL-4 T cells stimulated with P/I for up to 48 hours before digesting with MNase and analyzing genomic DNA by real-time PCR. Analysis of accessibility across the GM-CSF promoter to MNase showed slightly different kinetics to accessibility at the single *Hin*fI site. Within 2 hours following stimulation there was a dramatic increase in accessibility across the promoter region, reaching maximal levels of 80%, by 4 hours and this was maintained for at least 48 hours (Figure 3.11c). Thus while GM-CSF mRNA levels gradually increased following stimulation before peaking at 6 hours and declining by 24 hours, chromatin changes at the GM-CSF promoter were relatively stable, being maintained in a highly accessible state for at least 48 hours.

Next, the timing of transcription factor nuclear translocation was determined to correlate transcription factor presence with GM-CSF gene transcription and chromatin remodelling events. Nuclear extracts were prepared from EL-4 T cells stimulated at various time points over a 24 hour time period and analyzed by western blotting with Rel A, c-Rel, NFATp and NFATc antibodies. Rel A and NFATp showed similar nuclear translocation profiles (Figure 3.12). Both of these factors translocated to the nucleus within 30 minutes of T cell stimulation, and remained in the nucleus for at least 8 hours. By 16 and 24 hours post stimulation the levels of Rel A declined significantly, and NFATp was barely detectable in the nucleus. Meanwhile c-Rel entered the nucleus within 2 hours of stimulation and persisted for at least 24 hours. Similarly, NFATc translocated to the nucleus by 4 hours following stimulation and persisted for at least 24 hours. Thus NFATp and Rel A presence appears to correlate well with the transcriptional profile of GM-CSF, while the persistence of c-Rel and NFATc in the nucleus for a prolonged time period correlated with the more stable, longer term chromatin remodelling events at the promoter.

While GM-CSF promoter remodelling appears to be a relatively stable event following T cell stimulation, transcription is a more transient process. To determine whether the remodelled state needed to be stably maintained by continued stimulation, stimulus withdrawal experiments were conducted. In order to determine whether stimulus withdrawal had an effect on GM-CSF transcription, EL-4 T cells were stimulated for 2 hours before stimulus withdrawal. Cells were left for a further 4 or 22 hours before isolating RNA. EL-4 T cells were also stimulated with P/I in parallel for 0, 2, 6, and 24

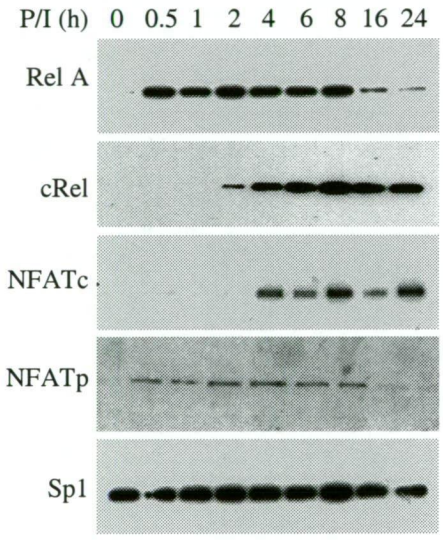



**Figure 3.12. The kinetics of NF-κB and NFAT nuclear translocation.** Nuclear extracts prepared from EL-4 T cells stimulated with P/I for the indicated time periods were subjected to SDS-PAGE and analyzed by western blotting with the indicated antibodies.

hours before isolating RNA. GM-CSF mRNA levels were then determined by real-time PCR analysis of cDNA. Cells treated with P/I displayed a transient increase in GM-CSF mRNA levels which peaked at 6 hours with a 500 fold induction before levels decreased to 300 fold by 24 hours (Figure 3.13). In the stimulus withdrawal treatment GM-CSF mRNA levels at 6 hours only reached 40 fold. Following stimulus removal for 24 hours GM-CSF mRNA levels remained at basal levels. Thus in the absence of persistent stimulation GM-CSF mRNA levels fail to reach the peak in expression seen in the control samples.

The stability of GM-CSF promoter remodelling in the absence of persistent stimulation was then analyzed. EL-4 T cells were either left untreated or stimulated for 2 hours with P/I at which time chromatin remodelling had occurred at the promoter (see Figure 3.11b and c). The stimulus was then withdrawn and cells were left for a further 4 or 22 hours. Nuclei were then harvested from these samples along with cells stimulated for up to 24 hours and accessibility of the promoter *Hin*fI site examined by CHART-PCR. As seen before, stimulation resulted in an increase in GM-CSF promoter accessibility to 60% within 2 hours which increased further to 70% by 6 hours and was maintained for 24 hours (Figure 3.14a). Cells stimulated for 2 hours, followed by a 4 hour stimulus withdrawal only showed a slight decrease in accessibility (to approximately 65%). In comparison, promoter accessibility in EL-4 T cells stimulated for 2 hours followed by a 22 hour stimulus withdrawal decreased 20-25% compared to cells undergoing persistent stimulation for the same period (24 hours). However, even after stimulus removal for 22 hours accessibility had not returned to basal levels.

In order to determine when accessibility returns to basal levels following stimulus withdrawal the effect of stimulus withdrawal over a longer time period was analyzed. EL-4 T cells were either left untreated or stimulated with P/I for 24, 48 and 72 hours before isolating nuclei, digesting with MNase and analyzing by CHART-PCR. Nuclei from EL-4 T cells treated for 2 hours to facilitate chromatin remodelling before stimulus withdrawal for 22, 46 and 70 hours were also isolated and analyzed by CHART-PCR in parallel. MNase accessibility across the GM-CSF promoter increased from a basal level of approximately 25% to 85% by 24 hours, remained at this level at 48 hours post stimulation (Figure 3.14b) and at 72 hours had decreased to 70%. In contrast, when the stimulus was withdrawn at 2 hours, promoter accessibility to MNase had decreased at 24 hours

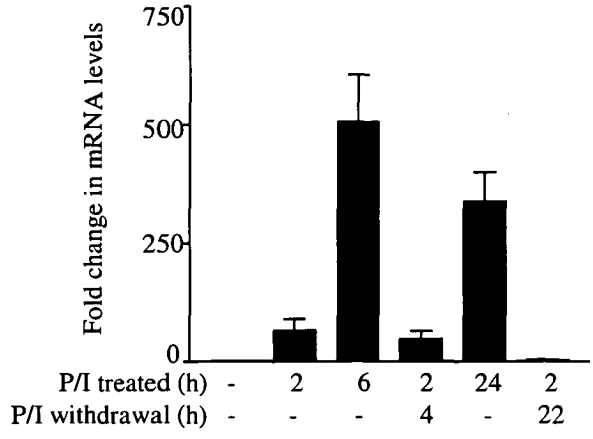



**Figure 3.13. The effect of brief versus continuous T cell stimulation on GM-CSF gene transcription.** EL-4 T cells were either left unstimulated or stimulated with P/I for 2 hours, before the stimulus was withdrawn and the cells incubated for the indicated time periods. GM-CSF mRNA levels were determined by real-time PCR analysis of cDNA. The mean and standard error of three replicate assays are shown.

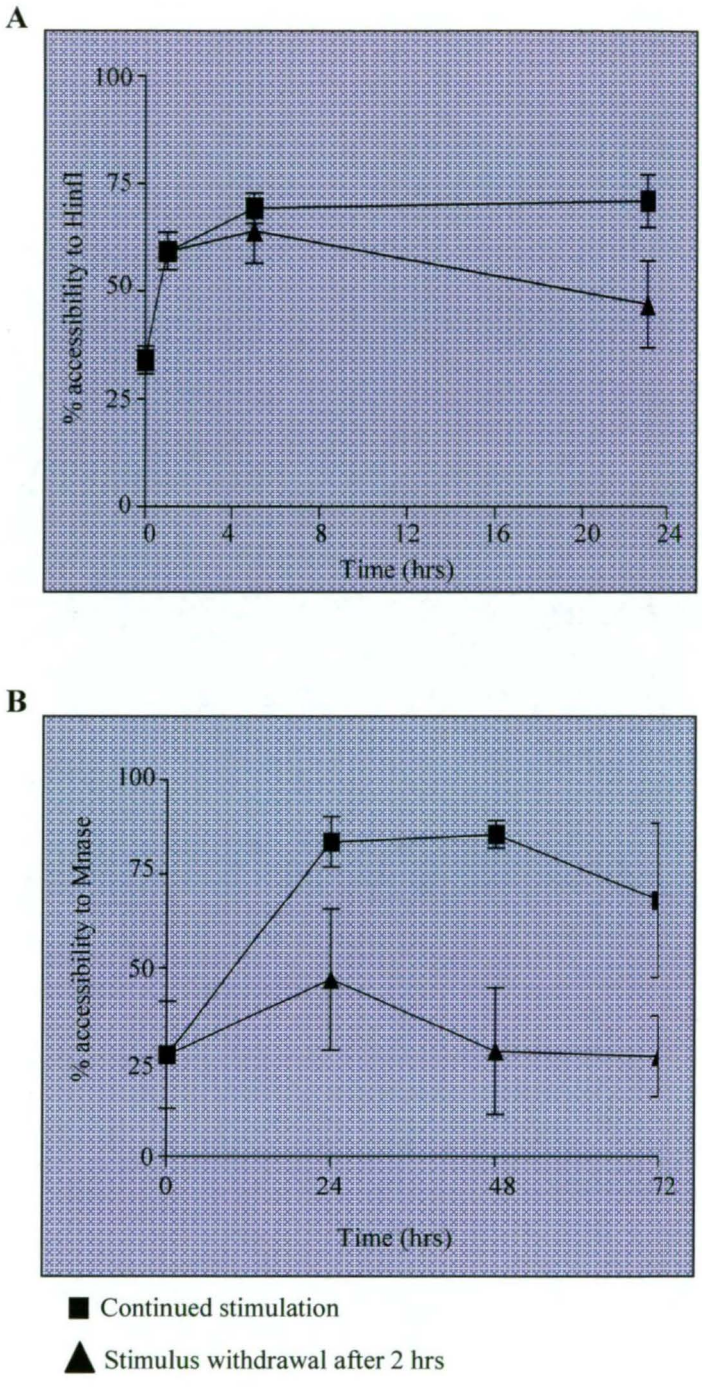



**Figure 3.14. The effect of stimulus withdrawal on GM-CSF promoter remodelling.** EL-4 T cells were incubated with P/I for 2 hours before the stimulus was withdrawn. In parallel, EL-4 T cells were stimulated with P/I for the indicated time periods. Nuclei were prepared and digested with *Hin*fI (A) or MNase (B). Genomic DNA was analyzed by real-time PCR using primer set -I. The mean and standard error of three replicate assays are shown.

compared to cells undergoing persistent stimulation and had returned to basal levels by 48 hours. It is therefore clear that the remodelled GM-CSF promoter returns to its basal state following stimulus withdrawal although with significantly delayed kinetics compared to mRNA levels.

Put together these data show GM-CSF transcriptional activation needs persistent stimulation while chromatin remodelling is relatively stably maintained. In order to determine how the nuclear translocation of transcription factors correlates with this, the effect of stimulus withdrawal on the nuclear accumulation of NF-κB and NFAT was determined. Nuclear extracts were prepared from EL-4 T cells either left untreated, stimulated for 2, 6 and 24 hours or stimulated for 2 hours followed by a 4 or 22 hour stimulus withdrawal. Rel A was detected in the nucleus at 2 hours (Figure 3.15). At 6 hours post stimulation Rel A protein levels peaked but had dramatically decreased by 24 hours. However if stimulation was withdrawn after 2 hours, levels of Rel A decreased considerably by 4 hours and were undetectable by 22 hours. c-Rel nuclear translocation occurred within 2 hours post-stimulation and increased further at 6 and 24 hours. Stimulus withdrawal for 4 hours caused a slight decrease in c-Rel and by 22 hours the levels of c-Rel were barely detectable. In contrast NFATc and NFATp could not be detected in the nucleus following stimulus withdrawal. Therefore the more stable presence of NF-κB correlates with remodelling while the pattern of NFAT nuclear translocation correlates more closely with transcription.

GM-CSF promoter remodelling is a stable event with chromatin accessibility remaining high for up to 72 hours post stimulation. In order to determine whether remodelling at the promoter is the rate limiting step in GM-CSF gene activation, subsequent reactivation of the gene was analyzed. EL-4 T cells were either left untreated or stimulated with P/I for 2 hours. The stimulus was then withdrawn and cells left for a further 22 hours at which time mRNA levels return to basal but the promoter remains accessible (Figure 3.11). Cells were then restimulated with P, I or P/I. Stimulation with P alone had a minimal impact on GM-CSF transcription only causing a 20 fold increase in mRNA levels (Figure 3.16a). Restimulation with I caused no increase in transcription while restimulation with P/I resulted in a 300 fold increase in GM-CSF mRNA levels. These data suggest that while remodelling has already occurred in these previously stimulated cells, both PKC and

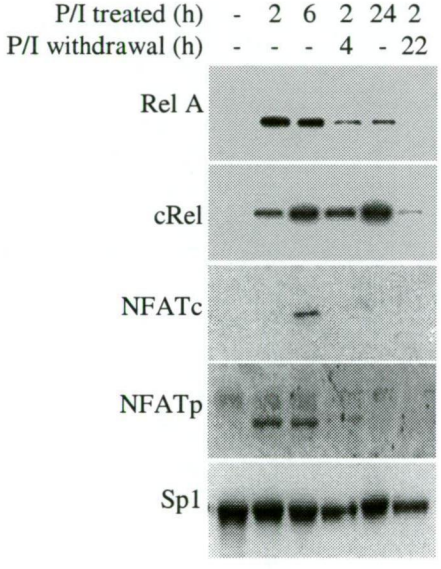



**Figure 3.15. The stability of NF-κB and NFAT proteins in the nucleus.** Nuclear extracts from EL-4 T cells which were either left unstimulated or stimulated with P/I for 2 hours, before the stimulus was withdrawn and the cells incubated for the indicated time periods, were subjected to SDS-PAGE and analyzed by western blotting with the indicated antibodies.

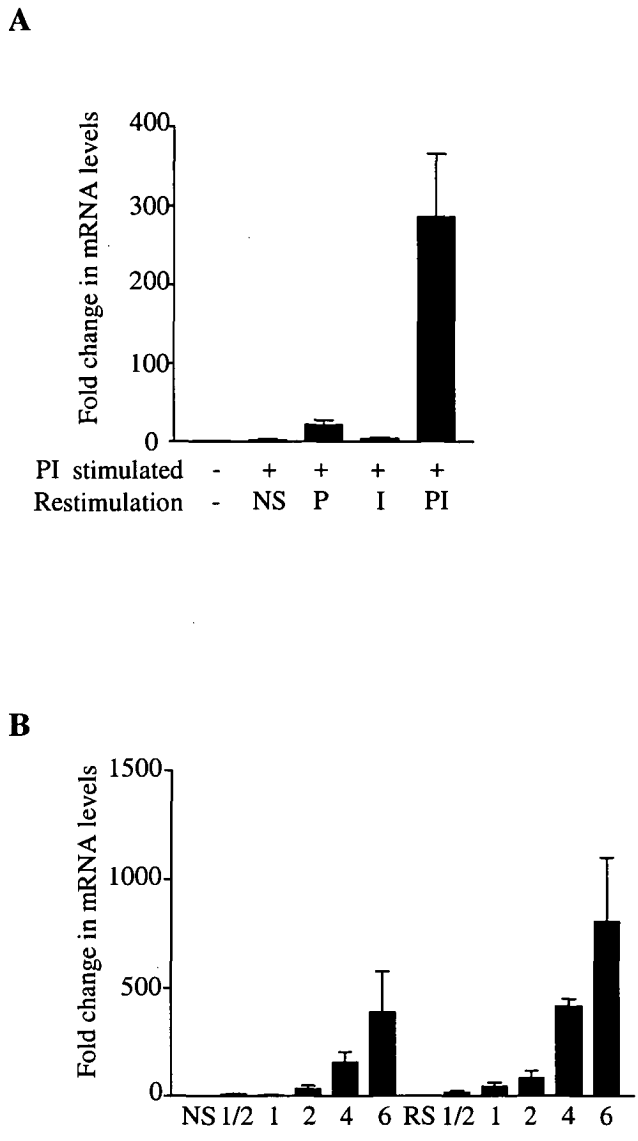



**Figure 3.16. Restimulation of EL-4 T cells causes a more rapid and robust GM-CSF transcriptional response.** (A) GM-CSF mRNA levels were determined by real-time PCR analysis of cDNA prepared from EL-4 T cells stimulated for 2 hours followed by 22 hours of stimulus withdrawal. Cells were then left untreated (NS) or restimulated with P or I alone or in combination. The mean and standard error of three replicate assays are shown. (B) cDNA was prepared from EL-4 T cells either left untreated or stimulated with P/I over the indicated time periods. In parallel, cDNA was prepared from EL-4 T cells stimulated with P/I for 2 hours prior to 22 hours of stimulus withdrawal. Cells were then restimulated over the indicated time periods. GM-CSF mRNA levels were determined by real-time PCR analysis. The mean and standard error of three replicate assays are shown.

calcium signalling pathways are still required for full transcriptional induction of GM-CSF.

Next the effect of restimulation with P/I over time was analyzed. Following stimulation with P/I for 2 hours, stimulus was withdrawn for 22 hours before restimulating with P/I for 0 to 6 hours. cDNA was also prepared from EL-4 T cells stimulated for 0 to 6 hours. Cells stimulated with P/I showed a typical profile of GM-CSF mRNA accumulation (Figure 3.16b) and levels reached approximately 400 fold by 6 hours. In contrast, in EL-4 T cells which were restimulated an approximately 800 fold increase in mRNA levels was detected by 6 hours. Restimulated cells showed a much more rapid induction of transcription. Therefore, cells in which remodelling has already occurred, due to prior stimulation, are capable of more rapid reactivation.

## 3.3 DISCUSSION

The data outlined in this chapter demonstrate that GM-CSF promoter remodelling and subsequent gene transcription are independent events. These two distinct events have different signal and transcription factor requirements. While remodelling of the GM-CSF promoter requires PKC activated factors, full transcriptional induction requires both the PKC and calcium signalling pathways. The PKC pathway may influence chromatin remodelling events at the GM-CSF promoter by activating transcription factors which bind to the GM-CSF promoter and recruit chromatin remodelling activities. In support of this NF-κB, which can be activated by PKC signalling alone, has been implicated in GM-CSF promoter remodelling (Cakouros *et al*, 2001; Holloway *et al*, 2003; Brettingham-Moore *et al*, 2005). Alternatively PKC signalling may lead to the activation of co-activators and remodelling complexes required for these remodelling events or may directly affect histone proteins. PKC has been found to directly phosphorylate a serine residue on histone H3 *in vitro* (Huang *et al*, 2004) which could directly influence chromatin accessibility at the promoter. Interestingly, remodelling of the GM-CSF promoter by PKC signalling alone caused a slight increase in transcription. This suggests that the increase in accessibility is sufficient to drive a low level of transcription, however other factors are clearly required for optimal gene expression. It is also interesting to note that the inhibition of PKC signalling caused a very slight increase in the basal accessibility of the promoter indicating

that perhaps a PKC activated factor is not only responsible for increasing promoter accessibility, but also necessary for maintaining the basal chromatin structure of the GM-CSF promoter.

While remodelling of the GM-CSF promoter can occur following PKC mobilization alone, other signals generated from the calcium signalling pathway are required to induce high levels of GM-CSF gene transcription. The requirement for both signalling pathways in full GM-CSF expression is supported by Jenkins *et al* (1995) whose work revealed that the activity of the human GM-CSF promoter attached to a luciferase reporter relies on both signals. The data presented in this chapter demonstrates that NFAT proteins are not essential for GM-CSF promoter remodelling, however, the nuclear translocation of NFAT appears to be critical for the optimal activation of transcription. CsA, which inhibits the nuclear translocation of NFAT, has previously been shown to inhibit endogenous GM-CSF gene transcription in Jurkat T cells (Tsuboi *et al*, 1994) and this finding was confirmed by specific inhibition of NFAT proteins using the VIVIT peptide. NFAT has previously been shown to play a role in GM-CSF enhancer remodelling (Cockerill *et al*, 1993), therefore the decrease in GM-CSF transcription due to the absence of NFAT may also be due to limited enhancer remodelling and activity. Another possibility is highlighted by the findings of Johnson *et al* (2004) who revealed that NFAT has the potential to cooperate with other co-activators at the GM-CSF enhancer.

c-Rel has previously been implicated in GM-CSF induction in T cells (Himes *et al*, 1996; Gerondakis *et al*, 1996). The results here clearly demonstrate that nuclear translocation of the NF-κB family member c-Rel mirrors the increase in accessibility to the GM-CSF promoter across a range of treatments (Table 3.1). Remodelling of the GM-CSF promoter has been detected as early as 1 hour post stimulation (Holloway *et al*, 2003) and c-Rel has previously been detected in the nuclei of EL-4 T cells within 1 hour of stimulation (Rao *et al*, 2001). The delayed nuclear activation of c-Rel is concomitant with promoter remodelling and a large increase in protein binding to the CD28RR (Himes *et al*, 1996; Shannon *et al*, 1995). Therefore it is possible that c-Rel is involved in the initial chromatin remodelling events at the GM-CSF promoter and/or maintaining increased accessibility at the remodelled promoter. The observation that CsA caused a slight reduction in c-Rel levels yet remodelling was not prevented would suggest that a certain

**Table 3.1. Correlation between transcription factor presence in the nucleus and chromatin remodelling at the GM-CSF promoter.**

| Treatment | Rel A | cRel | NFATc | NFATp | Chromatin Remodelling |
|---|---|---|---|---|---|
| PMA | ✔ | ✔ | ✔ | X | ✔ |
| Ro-32-0432 | ✔ | X | X | X | X |
| CsA | ✔ | ✔ | X | X | ✔ |
| CHX | ✔ | X | X | ✔ | X |
| 24 hour stim | ✔ | ✔ | ✔ | X | ✔ |
| 24 hour stim removal | X | ✔ | X | X | ✔ |

threshold of this NF-κB member is sufficient for chromatin remodelling. The role of c-Rel in chromatin remodelling events at the GM-CSF promoter is supported by findings that inhibition of c-Rel by Pentoxifylline inhibits the increase in GM-CSF promoter accessibility following T cell activation (Brettingham-Moore, 2002, Honours Thesis). In addition, Shannon and colleagues found that stimulation of c-Rel$^{-/-}$ T cells failed to induce GM-CSF promoter remodeling (Brettingham-Moore *et al*, 2005). It is interesting to note that NF-κB proteins have been found to have DNA bending properties *in vitro* (Schreck *et al*, 1990). This feature may help c-Rel to access DNA binding sites within the repressive chromatin environment and aid other factors in gaining access.

Similar to the findings presented here for GM-CSF, c-Rel has also been identified as critical for IL-2 gene transcription in a reporter based system (Wang *et al*, 1997) and the accumulation of c-Rel has also been correlated with IL-2 promoter accessibility changes. Accessibility to the CD28RR in the IL-2 promoter upon stimulation was reduced in T cells from c-Rel$^{-/-}$ mice (Rao *et al*, 2003). In contrast, c-Rel does not play a role in IL-12 promoter remodelling as increased accessibility in response to IFN-γ and LPS stimulation was still observed in c-Rel$^{-/-}$ macrophages (Weinmann *et al*, 2001). However, c-Rel was found to be important for the transcription of IL-12. These differences highlight the specific way in which each gene is likely to be activated, with c-Rel playing different roles in different circumstances.

This work also highlights the different roles played by different transcription factor family members. The CHX data demonstrated that while Rel A and NFATp are pre-existing, both NFATc and c-Rel are newly synthesized, or require continual replacement. The appearance of Rel A in the nuclei of non stimulated CHX treated cells indicates that Rel A alone is incapable of remodelling the GM-CSF promoter. It is also evident from the CHX data that transcription of the GM-CSF gene can occur without any detectable changes in promoter accessibility. It is therefore possible that the transcriptional machinery can be assembled at the promoter and drive transcription at a low level in the absence of any changes to chromatin structure. This may be attributed to the inherent basal accessibility of the GM-CSF promoter or alternatively the influence of the enhancer element, either of which may allow low levels of transcription without full promoter remodelling. However, CHX has also previously been shown to block the appearance of an inducible DNase

hypersensitive site in the GM-CSF enhancer (Cockerill *et al*, 1993).

Chromatin remodelling at the GM-CSF promoter is a relatively stable event while transcription is a transient event. The stability of GM-CSF promoter remodelling is biologically relevant in regard to the immune system as previously activated T cells may be able to respond more swiftly to subsequent attacks on the immune system. Restimulation of T cells results in a more rapid and robust GM-CSF transcriptional response suggesting that remodelling of the GM-CSF promoter is the rate limiting step for transcription. It also suggests that the altered structure of the promoter and stability of this event helps confer a type of "memory" to the cells. EL-4 T cells divide every 16 to 24 hours, thus the remodelled state would appear to be retained following cell division. It is possible that the stability of this increased accessibility is due to active maintenance of the remodelled state through cell division. The nuclear accumulation of c-Rel correlates with the stability of the remodelled promoter. Therefore it is possible that c-Rel may be involved in maintaining the increased accessibility. Alternatively, continuous signalling may allow the altered chromatin structure to be reset following cell division. In agreement with this, BAF remodelling complexes have been shown to be phosphorylated and inactivated during mitosis (Sif *et al*, 1998) indicating that remodelling is prevented during cell division and chromatin must be reset following division. Yet another possibility is the generation of transient, short-term epigenetic marks. Histone modifications in activated cells may help mark certain regions of the genome and impart memory to daughter cells (Smith *et al*, 2002).

It is also important to note, that as with any technique which studies cell populations rather than individual cells, an average value is obtained and thus it is impossible to determine whether the results from the CHART assay reflect the accessibility of all cells within the population or a small number within a heterogeneous population. For example an accessibility value of 50% may reflect a population in which 50% of the cells have 100% accessibility or alternatively a case in which 100% of cells have 50% accessibility. Therefore it is possible that accessibility remains at an increased level in some cells which have failed to divide over the time period examined.

The data presented here are consistent with a two step model for GM-CSF activation in which remodelling precedes transcription. The first step in GM-CSF

activation being initiated by the activation of the PKC signalling pathway downstream of TCR ligation and resulting in chromatin remodelling and increased accessibility at the GM-CSF promoter. This induces the nuclear translocation of NF-κB proteins. These transcription factors may then be able to bind to the promoter region due to the inherent basal accessibility of the GM-CSF promoter. c-Rel may then interact with chromatin remodelling factors to increase accessibility to the promoter region. This step ensures the second stage of activation can occur, by permitting other transcription factors and transcriptional machinery to access their relevant DNA binding sites. Only then, can GM-CSF gene expression occur.

# CHAPTER 4.

# BRG1 IS POISED AT THE GM-CSF PROMOTER IN T CELLS AND IS REQUIRED FOR GENE ACTIVATION.

## 4.1 INTRODUCTION

The data presented in the previous chapter and published reports have now clearly demonstrated that activation of the GM-CSF gene in T cells involves changes to the chromatin structure across its proximal promoter region (Cockerill *et al,* 1999; Holloway *et al*, 2003, Brettingham-Moore *et al*, 2005). However, very little is known about the mechanisms which underpin these chromatin remodelling events. Recent work by Chen *et al* (2005) has demonstrated using ChIP that total histone levels and histone acetylation at the GM-CSF promoter *decrease* following T cell activation, implying that remodelling involves the loss of histones from the promoter region. However, the complexes responsible for this chromatin remodelling and the mechanisms involved remain unidentified.

The exact mechanism underlying the activation of inducible genes in a chromatin context has only been elucidated for a small number of mammalian genes. One of the best examples is the step by step assembly of an enhanceosome at the enhancer region of the IFN-β gene following viral infection (Agalioti *et al*, 2000). IFN-β transcription is activated 6 hours post infection, prior to which a highly ordered recruitment profile has occurred. This involves the binding of transcription factors to the nucleosome-free enhancer, followed by local increases in histone acetylation, subsequent SWI/SNF recruitment and finally remodelling of adjacent nucleosomes (Agalioti *et al*, 2000). During skeletal muscle differentiation a slightly different Brg1 recruitment profile is observed at the myogenin promoter. A two-step mechanism was observed in which the homeodomain factor Pbx1 is constitutively bound to the myogenin promoter and indirectly helps to recruit the transcription factor, MyoD. Following induction of myogenic differentiation increased H4

acetylation was observed followed by MyoD dependent binding of Brg1 (de la Serna *et al*, 2005). Brg1 binding facilitates and strengthens MyoD binding even further and results in transcriptional induction of myogenin.

Similar to the histone loss detected at the GM-CSF promoter (Chen *et al*, 2005) histone depletion has also been observed at the yeast *PHO5* (Reinke and Horz, 2003; Korber *et al*, 2004) and *PHO8* promoters (Adkins *et al*, 2004). In addition this histone loss has been shown to be dependent on SWI/SNF recruitment. While *PHO8* activation requires SWI/SNF and GCN5 histone acetyltransferase activity (Gregory *et al*, 1999), the *PHO5* promoter is still remodelled in the absence of SWI/SNF and GCN5 but with delayed kinetics (Gadreau *et al*, 1997). The different requirements for *PHO5* and *PHO8* activation have recently been shown to be due to the inherent differences in chromatin structure, with the nucleosomes encompassing the *PHO8* promoter being more stable than those at the *PHO5* promoter (Hertel *et al*, 2005). Histone acetylation has also been demonstrated to precede promoter remodelling at the *PHO5* promoter (Reinke and Horz, 2003) and this appears to be a common feature for gene activation (Agalioti *et al*, 2001; de la Serna *et al*, 2005).

Previous *in vitro* work by Holloway *et al* (2003) has suggested a role for the ATPase component of the SWI/SNF complex, Brg1, in GM-CSF gene activation. An immobilized GM-CSF promoter template was incubated with nuclear extracts from unstimulated and stimulated Jurkat T cells to demonstrate that Brg1 could interact with the GM-CSF promoter in an NF-κB dependent manner (Holloway *et al* 2003). Brg1 has previously been implicated in immune gene activation and, following T cell activation, the SWI/SNF complex has been found to associate with chromatin in a Brg1 dependent manner (Zhao *et al*, 1998). However whether or not this ATPase interacts directly with the endogenous GM-CSF promoter is yet to be determined.

Therefore, the role of Brg1 in GM-CSF gene activation was analyzed and Brg1 binding at the GM-CSF promoter was determined using the ChIP assay. In addition, the role of histone acetylation in GM-CSF activation was investigated. The basal state of the GM-CSF promoter in expressing and non-expressing cells was also analyzed to investigate whether the inherent chromatin environment determines GM-CSF expression capabilities.

## 4.2 RESULTS

### 4.2.1 Brg1 is required for GM-CSF gene activation

In order to determine whether the Brg1-containing SWI/SNF complex is required for GM-CSF gene activation, GM-CSF expression was monitored in EL-4 T cells transfected with an ATPase mutant Brg1 construct (pBJ5-brg1K/R) in which lysine 774 has been mutated to an arginine (Khavari *et al*, 1993). This Brg1 mutant protein has been found to assemble into the SWI/SNF complex, but is unable to remodel nucleosomes (de la Serna *et al*, 2000). The Brg1 cDNA is cloned in frame with a C-terminal haemoglutanin (HA) tag which can be used to monitor expression of the mutant protein. To ensure the Brg1 mutant protein was expressed in the transfected cells, EL-4 T cells were transfected with the control pBJ5 plasmid or pBJ5-brg1K/R plasmid. Nuclear extracts were prepared, subjected to SDS-PAGE and analyzed by western blotting with the HA antibody. A band of over 180 kDa was detected in the cells transfected with the pBJ5-brg1K/R plasmid but not in the control transfected cells (Figure 4.1a) confirming expression of the mutant Brg1 protein in transfected cells.

While the mutant protein can be expressed in EL-4 T cells, the transfection efficiency of these cells is relatively low (~15%) and therefore it was necessary to enrich for the transfected cells in order to monitor the functional effect of the Brg1 mutant on GM-CSF gene activation. EL-4 T cells were therefore transfected with the pBJ5-brg1K/R plasmid or the empty pBJ5 control vector along with the $K^KII$ plasmid. This plasmid enables the expression of a truncated H-$2K^K$ receptor protein. Transfected cells expressing H-$2K^K$ can bind to magnetic beads coated with antibody directed against this cell surface marker to allow the transfected cell population to be enriched. Sorted cells were either left unstimulated or stimulated with P/I for 6 hours and GM-CSF expression monitored by real-time PCR. GM-CSF mRNA levels increased approximately 500 fold following P/I stimulation in cells transfected with the control plasmid. In contrast, accumulation of GM-CSF mRNA was reduced by 50% in cells expressing the Brg1 ATPase mutant (Figure 4.1b). This demonstrates that Brg1 is required for optimal activation of the GM-CSF gene.

It was hypothesised that the reduction in GM-CSF expression in cells transfected with the Brg1 mutant could be due to the inability of the ATPase defective protein to

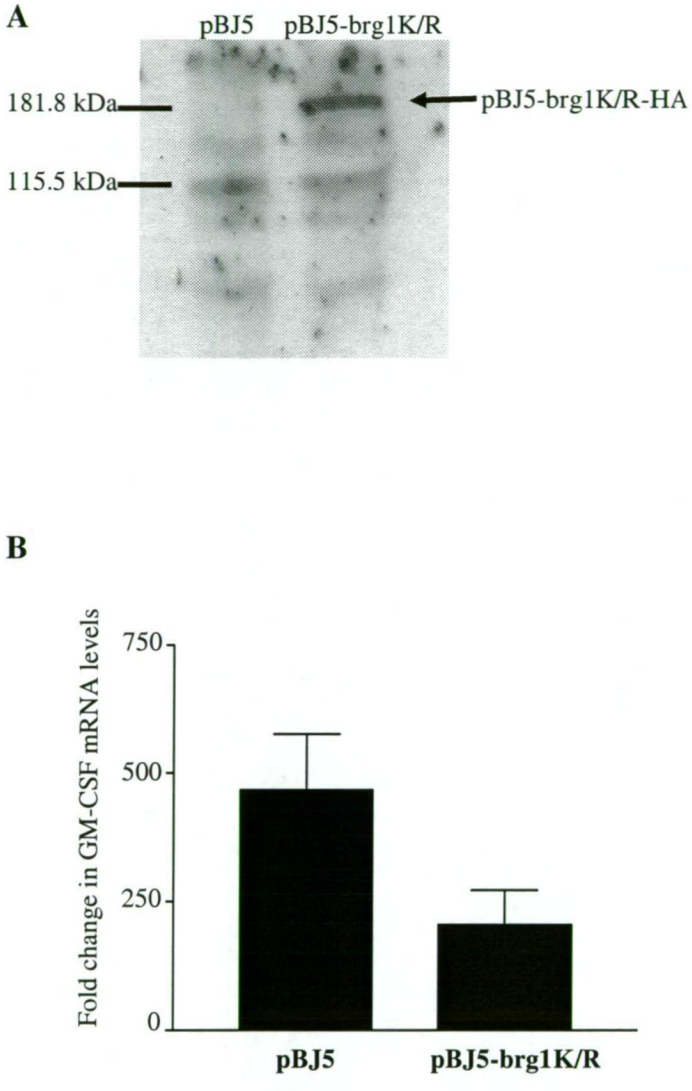



**Figure 4.1. Brg1 is required for efficient transcription of the GM-CSF gene.** (A) Nuclear extracts prepared from EL-4 T cells transfected with either the pBJ5 or pBJ5-brg1K/R-HA plasmid were subjected to SDS-PAGE and analyzed by western blotting using an anti-HA antibody. (B) EL-4 T cells were co-transfected with the $K^K$II and pBJ5 or pBJ5-brg1K/R plasmids prior to magnetically activated cell sorting before incubating with or without P/I for 6 hours. cDNA was prepared and levels of GM-CSF mRNA analyzed by real-time PCR. The mean and standard error of three replicate assays are shown.

remodel the GM-CSF promoter. To determine whether Brg1 is involved in chromatin remodelling events at the GM-CSF promoter EL-4 T cells were transfected with either the pBJ5 or pBJ5-brg1K/R constructs along with the $K^{K}$II plasmid. Following cell sorting 24 hours post-transfection, cells were either left unstimulated or stimulated with P/I for 6 hours. Nuclei were then isolated and incubated with or without MNase. Accessibility to the GM-CSF promoter was then determined by CHART-PCR analysis of genomic DNA using primer set –I which covers the proximal promoter. An inherent level of accessibility to MNase of approximately 50% was observed at the GM-CSF promoter in unstimulated cells transfected with the control plasmids (Figure 4.2) as seen previously at the GM-CSF promoter (Chapter 3). Accessibility to the promoter region increased to approximately 80% following stimulation. While a similar increased level of accessibility was detected in Brg1 mutant expressing cells following stimulation, these cells displayed a slightly higher basal level of accessibility prior to stimulation in addition to increased variability. Surprisingly, these data suggest that Brg1 is not required to generate the remodelled state across the promoter upon stimulation but may be involved in generating the basal chromatin structure.

### 4.2.2 Brg1 is enriched at the GM-CSF promoter in resting T cells and is lost from the promoter upon stimulation.

Previous *in vitro* analysis of proteins recruited to the GM-CSF promoter has implicated Brg1 in GM-CSF gene activation (Holloway *et al*, 2003). Therefore in order to determine whether Brg1 is associated with the GM-CSF promoter in T cells, ChIP analysis was performed on EL-4 T cells with Brg1 antibodies, before and after stimulation with P/I for 4 hours. Immunoprecipitated DNA was measured by real-time PCR using PCR primers designed to regions of the GM-CSF gene and the constitutively expressed GAPDH gene (Figure 4.3a). Brg1 recruitment was calculated as a percentage of the DNA levels prior to immunoprecipitation (i.e. % total input). Using primer sets –I and +I which cover the GM-CSF promoter, Brg1 was found to be associated with the promoter in resting T cells however, Brg1 levels at the promoter decreased upon T cell stimulation (Figure 4.3b). In contrast, there was little change in Brg1 levels associated with distal 5' and 3' regions of the gene or the constitutively expressed GAPDH gene following stimulation (Figure 4.3b).

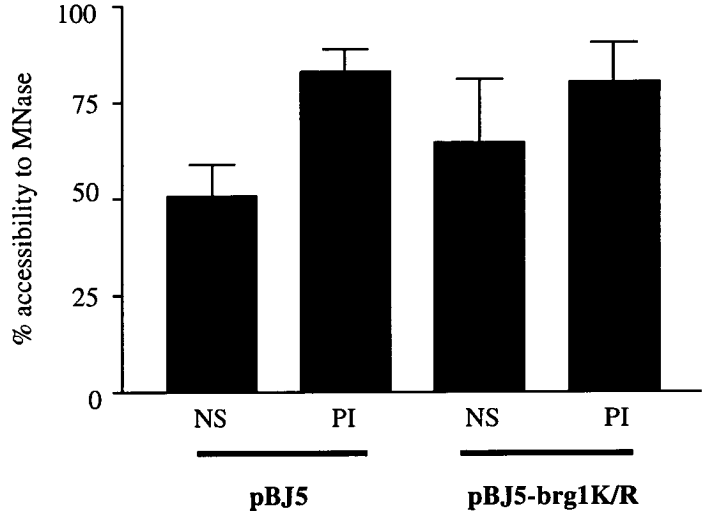



**Figure 4.2. Brg1 is not required for chromatin remodelling of the GM-CSF promoter.** EL-4 T cells were co-transfected with the $K^K$II and pBJ5 or pBJ5-brg1K/R plasmids prior to magnetically activated cell sorting before incubating with or without P/I for 6 hours. Nuclei were isolated and digested with MNase and genomic DNA analyzed by real-time PCR using primer set -I. The mean and standard error of three replicate assays are shown.

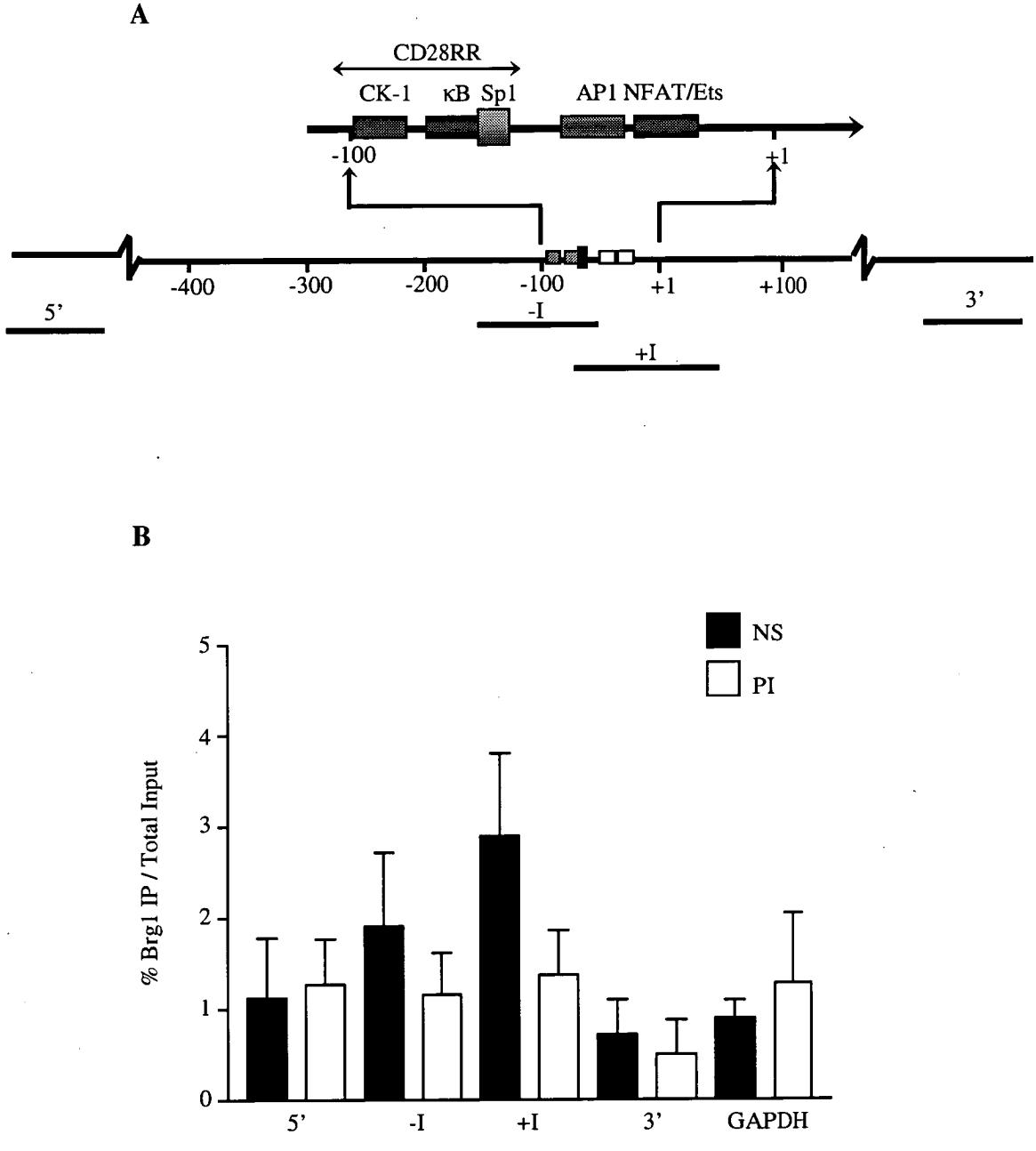



**Figure 4.3. Brg1 is bound to the GM-CSF promoter in resting T cells.** (A) Schematic representation of the GM-CSF promoter with positions of primer sets 5', -I, +I and 3'. (B) EL-4 T cells were stimulated with P/I for 4 hours before performing ChIP with Brg1 antibodies. Enrichment of Brg1 at the GM-CSF promoter, a 5' region, a 3' region and at the GAPDH gene was determined by real-time PCR. The mean and standard error of 3 replicate assays are shown.

In addition, in resting cells the relative levels of Brg1 were considerably higher at the GM-CSF promoter (primer sets –I and +I) than the 3' and 5' regions of the gene or the GAPDH gene suggesting enrichment of Brg1 at the promoter region. These data therefore suggest that Brg1 is enriched at the GM-CSF promoter in resting T cells and lost from the promoter region following T cell activation, concomitant with chromatin remodelling. Put together, these data suggest a role for Brg1 in establishing the basal chromatin environment at the GM-CSF promoter that is conducive to gene activation.

### 4.2.3 Increasing promoter acetylation facilitates Brg1 recruitment and GM-CSF gene activation

The data presented above demonstrate that Brg1 is enriched at the GM-CSF promoter in resting T cells. This raises the question of how Brg1 might be constitutively recruited to the GM-CSF promoter. The recruitment of Brg1 has previously been linked to increased histone acetylation (Hassan *et al*, 2002). Therefore, to determine whether histone acetylation levels influence GM-CSF gene activation and chromatin remodelling, the effect of the histone deacetylase inhibitor, TSA on these two processes was examined. EL-4 T cells were pre-treated with TSA for 4 hours and left unstimulated or stimulated with P/I for 4 hours. GM-CSF mRNA levels were then determined by real-time PCR analysis of cDNA. GM-CSF was induced 400 fold following stimulation with P/I (Figure 4.4). TSA pre-treatment augmented GM-CSF expression in stimulated cells to approximately 1200 fold (Figure 4.4). Treatment of EL-4 T cells with TSA has been shown to increase global acetylated H3 levels and specifically increase acetylated histone H3 levels across the GM-CSF gene (Chen and Shannon, personal communication). Therefore, increasing histone acetylation at the GM-CSF promoter results in increased gene transcription.

In order to determine how increasing histone acetylation levels influenced chromatin remodelling events at the GM-CSF promoter, chromatin accessibility was monitored using CHART-PCR. EL-4 T cells were either left untreated or pre-treated with TSA for 4 hours prior to incubating with or without P/I for 4 hours. The GM-CSF promoter displayed an inherent accessibility to MNase of approximately 50% in the control treatment as expected, and following stimulation, this accessibility increased further to approximately

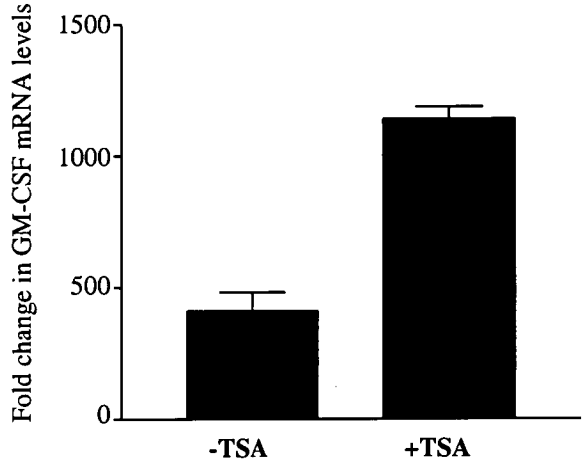



**Figure 4.4. Increased acetylation increases GM-CSF gene transcription.** EL-4 T cells were either left untreated or pre-treated with TSA prior to incubating with or without P/I for 4 hours. GM-CSF mRNA levels were determined by real-time PCR analysis of cDNA. The mean and standard error of 3 replicate assays are shown.

80% (Figure 4.5a). Cells which were pre-treated with TSA similarly displayed approximately 50% accessibility in untreated cells, increasing to 80% upon stimulation. Thus, despite enhancing GM-CSF gene transcription, TSA pre-treatment did not alter either basal or induced levels of accessibility at the GM-CSF promoter. This result failed to answer the question of how increasing histone acetylation augmented GM-CSF mRNA accumulation. Therefore to determine whether pre-treatment with TSA affected the kinetics of chromatin remodelling across the GM-CSF promoter. EL-4 T cells were either left untreated or pre-treated with TSA for 4 hours before incubating with P/I for 0, 0.5, 1 or 4 hours. In the absence of TSA little change in chromatin accessibility across the GM-CSF promoter was observed 30 minutes following P/I stimulation and only a small increase in accessibility was observed 1 hour after P/I stimulation. As seen previously, a dramatic increase in accessibility was then observed 4 hours post stimulation with P/I (Fig 4.5b). In contrast, in cells pre-treated with TSA, increased chromatin accessibility was observed within 30 minutes post-stimulation and had almost reached maximal induced accessibility by 1 hour (Figure 4.5b) increasing only slightly further at 4 hours. These results suggest that increasing histone acetylation levels across the GM-CSF gene, while not affecting basal accessibility, allowed chromatin remodelling events to occur more rapidly following stimulation.

To investigate the possibility that Brg1 may be enriched at the GM-CSF promoter through association with acetylated histones, Brg1 recruitment at the promoter was measured using the ChIP assay. EL-4 T cells were either left untreated or pre-treated with TSA for 16 hours before incubating with or without P/I for 4 hours. Chromatin was then crosslinked and immunoprecipitated with the Brg1 antibody. Immunoprecipitated DNA was amplified by real-time PCR using primer set +I. As seen previously (Figure 4.3b), Brg1 was enriched at the GM-CSF promoter in unstimulated T cells with levels decreasing upon stimulation (Figure 4.6). Treatment with TSA increased Brg1 levels at the promoter by approximately 3 fold although the typical decrease in Brg1 levels was not detected following P/I treatment in TSA treated cells. These data suggest that increasing acetylation at the GM-CSF promoter increases Brg1 levels at the GM-CSF promoter, and are consistent with Brg1 recruitment via acetylated histones.

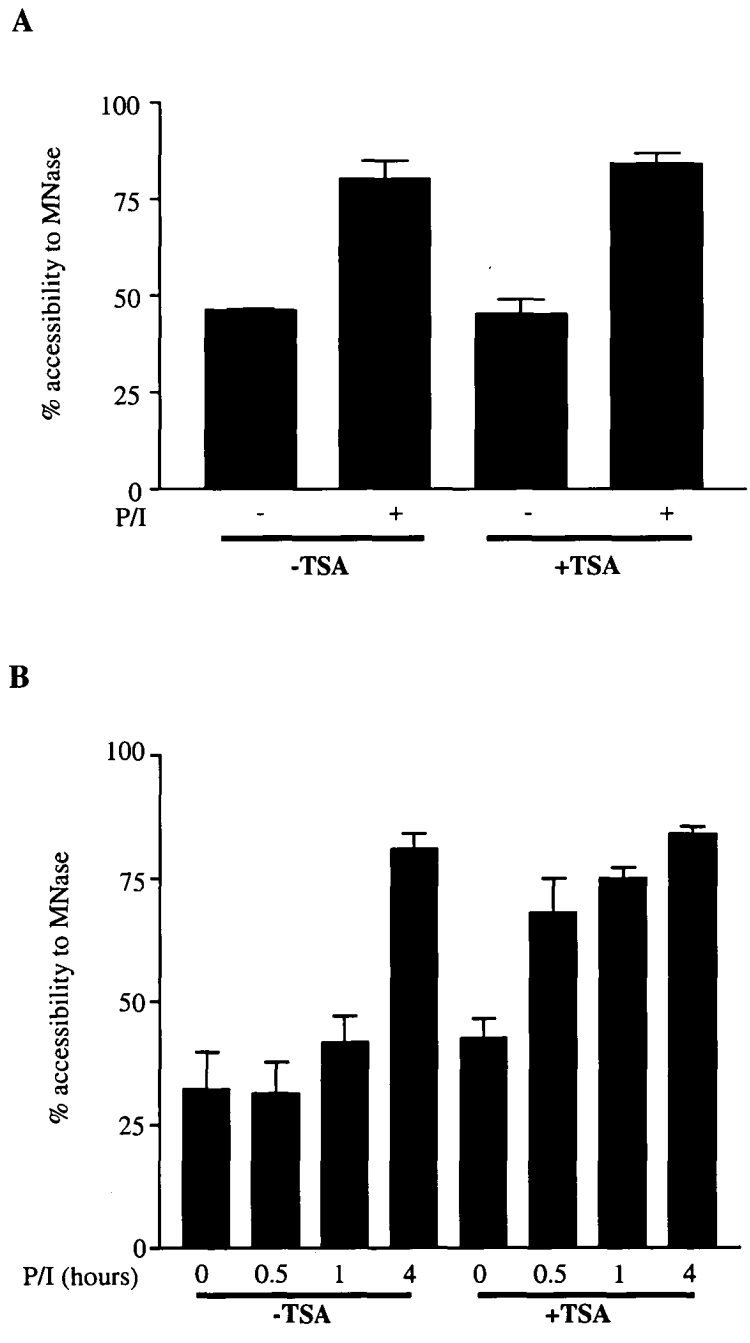



**Figure 4.5. Increased acetylation increases the rate of remodelling at the GM-CSF promoter.** (A) EL-4 T cells were either left untreated or pre-treated with TSA prior to incubation with or without P/I for 4 hours. Nuclei were then isolated, incubated with MNase and accessibility at the GM-CSF promoter determined by real-time PCR analysis of genomic DNA using primer set –I. The mean and standard error of 3 replicate assays are shown. (B) EL-4 T cells were treated as in A prior to stimulating with P/I for the indicated times. Promoter accessibility was determined as in A. The mean and standard error of 3 replicate assays are shown.

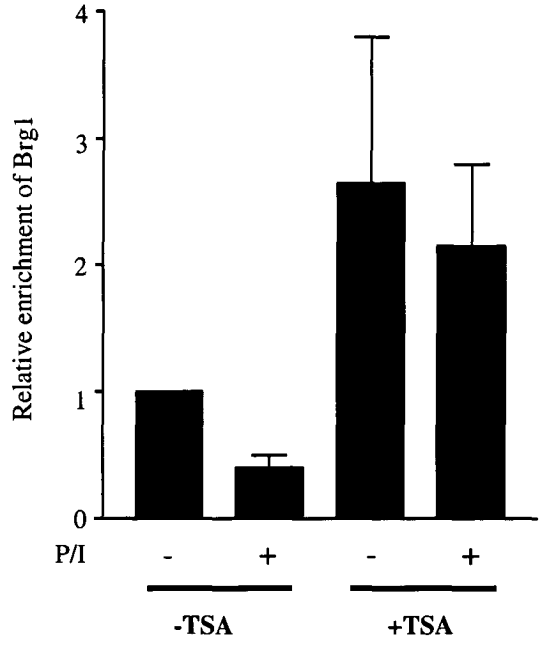



**Figure 4.6. TSA increases Brg1 recruitment to the GM-CSF promoter.** EL-4 T cells were either left untreated or treated with TSA for 16 hours prior to incubating with or without P/I for 4 hours. Chromatin was then crosslinked and immunoprecipitated using Brg1 antibodies. The relative enrichment of Brg1 compared to the unstimulated control was determined by real-time PCR analysis of DNA using primer set +I. The mean and standard error of 3 replicate assays are shown.

### 4.2.4 Chromatin remodelling at the GM-CSF promoter is inhibited in non-expressing cell types

The data presented so far suggests that enrichment of Brg1 at the GM-CSF promoter maybe a requirement of GM-CSF gene activation. To investigate this further, chromatin remodelling events were analyzed in B cells which do not normally express GM-CSF. EL-4 T cells and A-20 B cells were either left untreated or stimulated with P/I for 4 hours and GM-CSF mRNA levels determined by real-time PCR. As expected, GM-CSF was highly inducible in EL-4 T cells by P/I stimulation with an approximately 600 fold induction, while its expression was essentially uninducible in the B cell line (Table 4.1). In order to determine whether the difference in GM-CSF expression was due to disparity in chromatin accessibility across the GM-CSF promoter, non-stimulated and stimulated EL-4 T cells and A-20 B cells were analyzed by CHART-PCR using primer set -I. As seen previously, EL-4 T cells displayed inherent basal accessibility to MNase which increased dramatically following stimulation with P/I (Figure 4.7). While A-20 B cells displayed a similar level of basal accessibility, no increase in accessibility was observed upon stimulation with P/I (Figure 4.7). Therefore, chromatin analysis of the A-20 cells suggests that their inability to express GM-CSF maybe due to a blockage in chromatin remodelling events at the promoter.

Brg1 is associated with the GM-CSF promoter in T cells, and is required for GM-CSF gene activation. Therefore ChIP assays were used to investigate whether there is a difference between the association of Brg1 with the GM-CSF promoter in B cells compared to T cells. EL-4 T cells and A-20 B cells were either left untreated or stimulated with P/I for 4 hours before crosslinking chromatin followed by immunoprecipitation with Brg1 antibodies. Immunoprecipitated DNA was then quantitated by real-time PCR analysis using primer set +I. As seen previously, Brg1 was detected at the GM-CSF promoter in unstimulated T cells and levels decreased 2 fold upon stimulation (Fig 4.8). In contrast, very little Brg1 was detected at the GM-CSF promoter in B cells in both resting and stimulated cells. Importantly, the Brg1 levels detected in either unstimulated or stimulated B cells were lower than observed in T cells even following stimulation. Therefore it appears that Brg1 is absent from the GM-CSF promoter in B cells.

**Table 4.1. GM-CSF mRNA copy number in EL-4 T cells and A-20 B cells.** EL-4 T cells and A-20 B cells were either left untreated or stimulated for 4 hours with P/I. cDNA was prepared and GM-CSF mRNA levels determined by real-time PCR.

| Sample | GM-CSF copies (normalized to GAPDH) | Fold Change (relative to EL-4 NS) |
|---|---|---|
| EL-4 NS | 6.6 | 1 |
| EL-4 P/I | 4315 | 652 |
| A-20 NS | 0 | 0 |
| A-20 P/I | 3.8 | 0.58 |

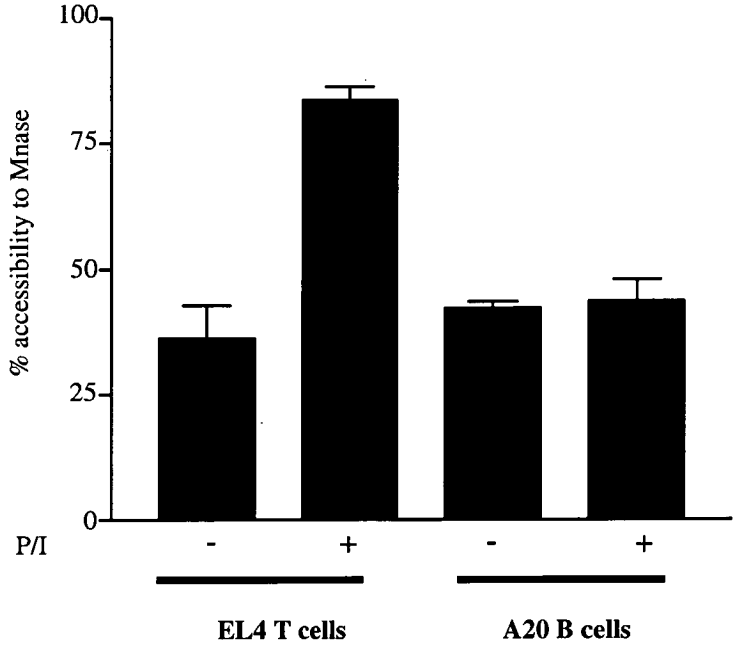



**Figure 4.7. The GM-CSF promoter is not remodelled in non expressing B cells.** EL-4 T cells and A-20 B cells were either left untreated or stimulated with P/I for 4 hours. Nuclei were isolated and incubated with MNase prior to real-time PCR analysis of genomic DNA using primer set –I. The mean and standard error of 3 replicate assays are shown.

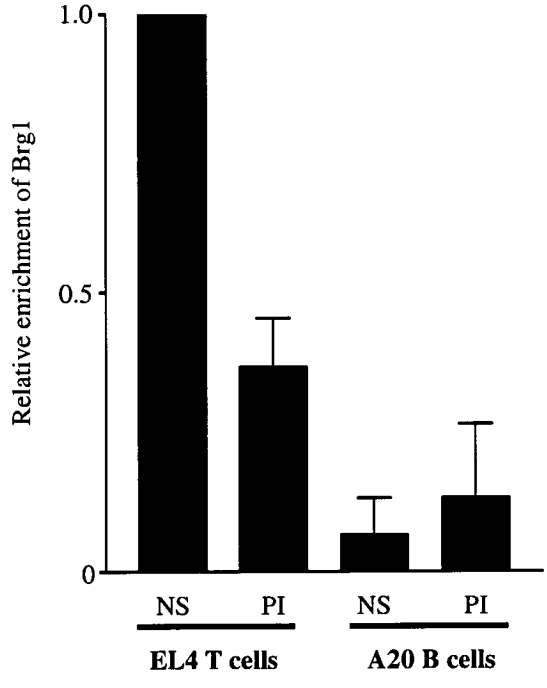



**Figure 4.8. Brg1 is not bound to the GM-CSF promoter in B cells.** EL-4 T cells and A-20 B cells were incubated with or without P/I for 4 hours before crosslinking chromatin and immunoprecipitating against Brg1. Recruitment of Brg1 was then determined by real-time PCR analysis of DNA using primer set +I. The mean and standard error of three replicate assays are shown.

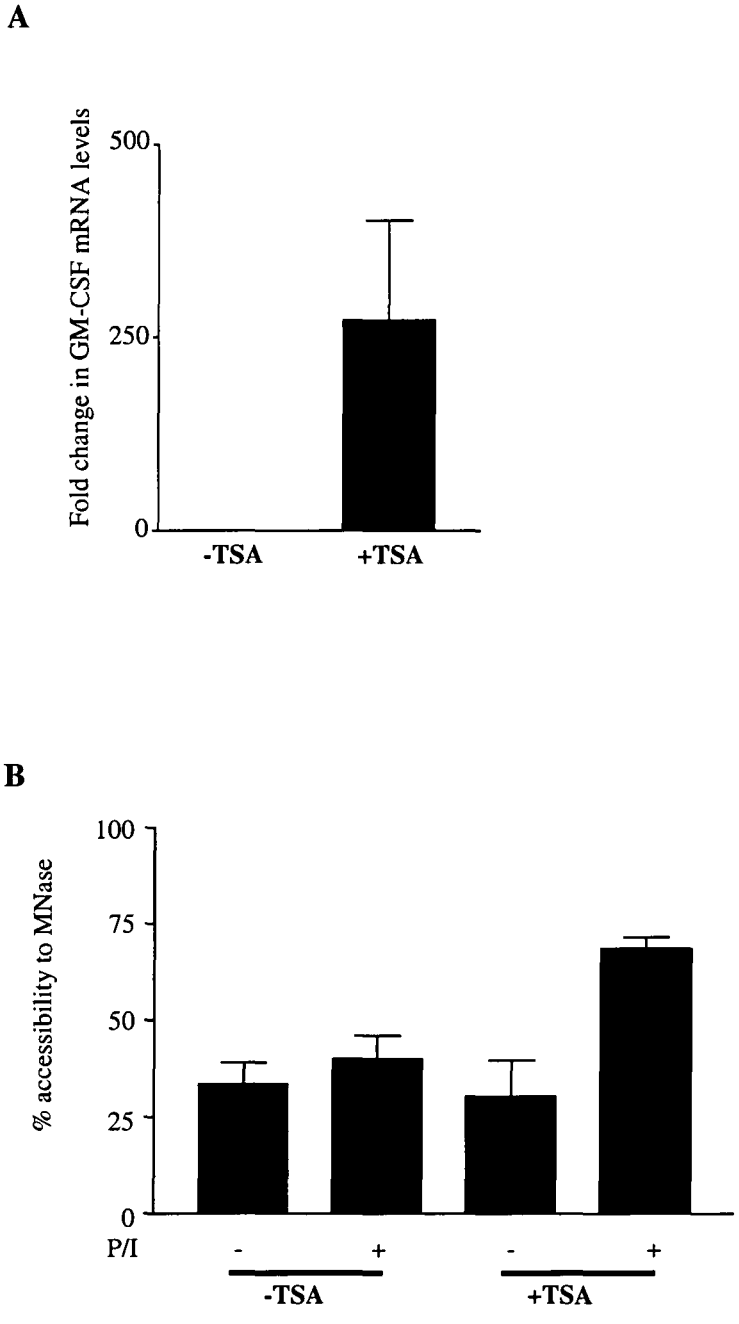



**Figure 4.9. Increasing acetylation in B cells overcomes the block in GM-CSF expression by facilitating remodelling of the GM-CSF promoter.** (A) A-20 B cells were either left untreated or treated with TSA prior to incubating with P/I for 4 hours. GM-CSF mRNA levels were then determined by real-time PCR analysis of cDNA. The mean and standard error of 3 replicate assays are shown. (B) A-20 B cells were either left untreated or pre-treated with TSA before incubating with or without P/I for 4 hours. Nuclei were then isolated and digested with MNase before analyzing genomic DNA by real-time PCR analysis using primer set –I. The mean and standard error of 3 replicate assays are shown.

Increasing histone acetylation levels was found to augment GM-CSF gene activation and increase the kinetics of chromatin remodelling events in T cells (Figure 4.5). Therefore to determine whether increasing histone acetylation could also alter the inducibility of the GM-CSF gene in B cells, A-20 B cells were pre-treated with TSA for 4 hours. Cells were then either left untreated or stimulated with P/I for 4 hours before measuring GM-CSF mRNA levels using real-time PCR. As before, little GM-CSF mRNA was detected in B cells following stimulation with P/I. However, there was a dramatic 200 fold increase in GM-CSF mRNA levels following stimulation with P/I in B cells pre-treated with TSA (Figure 4.9a). Therefore, increasing acetylation of the GM-CSF promoter in B cells allows transcription to occur in response to P/I. However, it should be noted that while GM-CSF expression could be induced in B cells by pre-treatment with TSA, these levels are still much lower than those typically observed in T cells (Figure 4.4).

In order to determine whether increasing histone acetylation levels by TSA treatment also facilitated chromatin remodelling events at the GM-CSF promoter, A-20 B cells were either left untreated or TSA pre-treated for 4 hours before incubating with or without P/I for 4 hours. Accessibility of the GM-CSF promoter to MNase was determined by CHART-PCR using primer set -I. As before, in non-stimulated B cells, the GM-CSF promoter displayed an inherent basal accessibility which remained unchanged following stimulation with P/I. Pre-treatment of B cells with TSA did not alter basal chromatin accessibility across the promoter, however it facilitated an increase in chromatin accessibility in response to P/I stimulation with levels increasing to 70% (Figure 4.9b), which is similar to levels observed in T cells upon stimulation.

Collectively, these data indicate that Brg1 is not associated with the GM-CSF promoter in B cells, which do not express GM-CSF in response to P/I stimulation. However chromatin remodelling events and GM-CSF gene expression can be induced by modification of the histone acetylation status of the gene.

## 4.3 DISCUSSION

Activation of the GM-CSF gene in T cells involves targeted chromatin remodelling events that result in increased accessibility across the promoter region (Holloway *et al*,

2003; Brettingham-Moore *et al*, 2005). Recently, this increased accessibility was shown to be due to the loss of histones from the GM-CSF promoter (Chen *et al*, 2005). The data presented in this chapter demonstrate that the ATPase component of the SWI/SNF remodelling complex, Brg1, is required for GM-CSF gene activation. Using a Brg1 dominant negative approach it was shown that Brg1 is essential for the optimal activation of GM-CSF expression. ChIP analysis confirmed a direct role for Brg1 in GM-CSF activation. Brg1 is bound to the GM-CSF promoter in resting T cells and lost from the promoter region upon T cell activation. Combining this result with the findings of Chen *et al* (2005), it is evident that Brg1 is poised at the GM-CSF promoter in resting T cells and lost concomitant with histone loss following T cell stimulation.

The association of Brg1 with the GM-CSF promoter in the resting state was somewhat unexpected, however Brg1 has previously been found constitutively associated with a number of other promoters including the M-CSF (Lui *et al*, 2001), CIITA pIV promoter (Ni *et al*, 2005) and IFITM1 promoters (Cui *et al*, 2004). These genes are also rapidly activated, suggesting that the chromatin structure of these promoters is poised for swift transcriptional induction. The activation of the IFN responsive IFITM1 gene is remarkably similar to GM-CSF. It is expressed constitutively at low levels and induced rapidly following stimulation with IFNα or IFNγ. In addition, the promoter has increased basal levels of H4 acetylation and the promoter is partially accessible to restriction digestion indicating that the nucleosome is partially disrupted in the basal state (Cui *et al*, 2004). Brg1 is constitutively bound to the nucleosome covering the IFITM1 promoter region. Therefore, the chromatin environment of the IFITM1 gene suggests that it maybe prepared in anticipation of IFNα or IFNγ signalling.

Recently, Liang *et al* (2006) showed that the interleukin-1β (IL-1β) gene is poised for rapid activation. Liang *et al* (2006) demonstrated that in resting monocytic cells capable of rapid transcriptional activation, the IL-1β promoter is assembled into a poised chromatin architecture, with the promoter nucleosome free in the basal state as determined by CHART-PCR and ChIP analysis of histone acetylation. In addition, it was demonstrated that promoter accessibility alone is not sufficient for transcription. Some promoters, such as those belonging to heat-shock genes exist in an open chromatin structure, sometimes with pre-initiated paused RNA polymerase II molecules. This indicates that not all genes require

nucleosome remodelling for transcriptional activation (Boehm *et al*, 2003).

The constitutive recruitment of Brg1 to the GM-CSF promoter may help establish a basal chromatin environment permissive to the rapid induction of transcription. Adding support to this hypothesis, the delayed recruitment of Brg1 to the IFN-β promoter correlates with the delayed up-regulation of transcription 6 hours post-infection (Agalioti *et al*, 2000). This slower transcriptional activation can be attributed to the requirement for histone acetylation and Brg1 recruitment before the nucleosome covering the promoter can be remodelled. In contrast, histone loss is detectable across the GM-CSF promoter as early as 30 minutes after stimulation (Chen *et al*, 2005), with GM-CSF transcription increasing rapidly after this time and peaking at 6-8 hours (Chapter 3). It is possible that such rapid induction of GM-CSF gene expression can occur because the promoter is already primed for activation with an inherent basal level of acetylation and the presence of Brg1. In the case of IFN-β activation, Brg1 is associated with the promoter transiently, for only a few hours, while transcription continues for close to 24 hours (Agalioti *et al*, 2000). This suggests that Brg1 functions within a short time frame to remodel the promoter region which remains in an accessible configuration for an extended period. The stability of the accessible state at the IFN-β promoter is comparable to the stability of GM-CSF promoter remodelling (Chapter 3).

Brg1 may not only prime the GM-CSF gene for rapid activation by establishing a competent chromatin environment, it may also help to recruit other transcription factors to the GM-CSF promoter. Chromatin remodellers have been suggested to accelerate transcription factor interactions with non-specific DNA sites by speeding up genome wide scanning (Karpova *et al*, 2004). In fact, Brg1 has been found to be responsible for the initial recruitment of the STAT1 co-activator to the CIITA promoter (Ni *et al*, 2005). It is therefore possible that Brg1 enhances recruitment of the transcription factors required to drive GM-CSF expression.

Adding further support to the hypothesis that Brg1 recruitment is associated with priming genes for activation, Brg1 was not detected at the promoter of non expressing B cells. While Brg1 maintains a transcriptionally competent state in T cells it is possible that this signal can be overridden by increased histone acetylation in B cells. However, even though GM-CSF can be activated in TSA treated B cells the levels of activation are much

lower than those observed in T cells suggesting that Brg1 binding is required for the optimal activation of transcription. This is supported by the reduction in GM-CSF transcription observed in T cells expressing the Brg1 ATPase mutant.

There are a number of possibilities to explain the mechanism behind the constitutive recruitment of Brg1 at the GM-CSF promoter in T cells. In the case of the *PHO5* promoter, chromatin remodelling and Brg1 recruitment is dependent on prior histone hyperacetylation (Reinke and Horz, 2003). Similarly, a transient increase in histone acetylation occurs prior to Brg1 recruitment to the IFN-β promoter (Agalioti *et al*, 2000). Brg1 is known to associate with acetylated histones via its bromodomain (Hassan *et al*, 2002). This raises the possibility that Brg1 is associated with the GM-CSF promoter via interactions with acetylated histones. In support of this, increasing histone acetylation by TSA treatment resulted in an associated increase in Brg1 levels at the promoter and an increase in the rate of remodelling. It is important to note that the typical decrease in Brg1 levels was not detected following P/I treatment in TSA treated cells. This may possibly be due to saturation of the nucleosome with Brg1 and equilibrium between nucleosome assembly and disassembly. Alternatively, increasing histone acetylation may increase Brg1 recruitment across the entire gene and loss of Brg1 did not occur on the neighbouring nucleosomes as detected in the ChIP assay. Acetylated histones are enriched at the GM-CSF promoter (Chen and Shannon, unpublished data) and this may potentially enable the constitutive recruitment of Brg1. This would also explain why a transient increase in histone acetylation, as observed at the *PHO5* and IFN-β promoters (Reinke and Horz, 2003; Agalioti *et al*, 2000), is not required at the GM-CSF promoter prior to chromatin remodelling (Chen *et al*, 2005).

Alternatively, Brg1 may be recruited to the GM-CSF promoter via an interaction with a constitutively expressed transcription factor. Liu *et al* (2002) have revealed that Sp1 binds Brg1 and recruits SWI/SNF to the IFN-α responsive IFITM3 locus and perhaps a similar mechanism operates at the GM-CSF promoter. Indeed, Brg1 has previously been shown to be recruited in a NF-κB/Sp1 dependent manner to the GM-CSF promoter *in vitro* (Holloway *et al*, 2003). This region has previously been shown as vital for GM-CSF promoter remodelling and transcription and thus makes it a potential candidate for the basal recruitment of Brg1. Alternatively, an unidentified, constantly bound transcription factor

may recruit Brg1. Whether Brg1 is constitutively bound to the GM-CSF promoter in T cells throughout the cell cycle or if, following cell division, the basal state must be re-established remains to be elucidated. Interestingly, Brg1 has previously been found to be excluded from condensed chromosomes during mitosis (Muchardt *et al*, 1996) suggesting that the basal chromatin state must be re-set following cell division.

Unexpectedly, chromatin remodelling events across the GM-CSF promoter were still able to occur in the presence of the Brg1 ATPase mutant. However, the basal accessibility of the GM-CSF was altered in Brg1 mutant cells. This suggests that Brg1 may be involved in maintaining a basal chromatin structure which is permissive to gene activation. In addition, it is possible that remodelling may occur less efficiently in the ATPase mutant cells, and this may have not been detected at the 6 hour time point examined. In support of this, chromatin remodelling can still occur at the *PHO5* promoter in the absence of SWI/SNF but with delayed kinetics (Gaudreau *et al*, 1997; Barbaric *et al*, 2001). In addition, wild type Brg1 would still be operating in the transfected cells. It is also interesting to note that the ATPase domain of Brg1 is not only required for its remodelling function but also for interactions with other factors (Bultman *et al*, 2005). Therefore the reduction in GM-CSF transcription in Brg1 mutant cells may be attributed to interactions with other transcription factors being inhibited. Bultman *et al* (2005) generated a mutant Brg1 in which the ATPase domain was mutated but ATPase activity was restored. They found that while the mutant Brg1 is still recruited to the β-globin locus, remodelling and transcription were inhibited. By uncoupling ATPase activity and chromatin remodelling in this case it became evident that the ATPase domain is also essential for interactions with other co-activators

GM-CSF is constitutively expressed at very low levels in resting T cells and rapidly induced in response to T cell activation signals. The promoter is relatively accessible in the resting state suggesting that the nucleosome is remodelled incompletely and constitutively. It is therefore conceivable that GM-CSF is a few steps further along in its activation profile. These data are in keeping with a model in which in resting T cells, Brg1 is bound to the GM-CSF promoter due to increased basal acetylation, priming the gene for activation by establishing an inherent level of basal accessibility. Following stimulation an entire nucleosome is then lost from the promoter in a c-Rel dependent manner. c-Rel may drive or

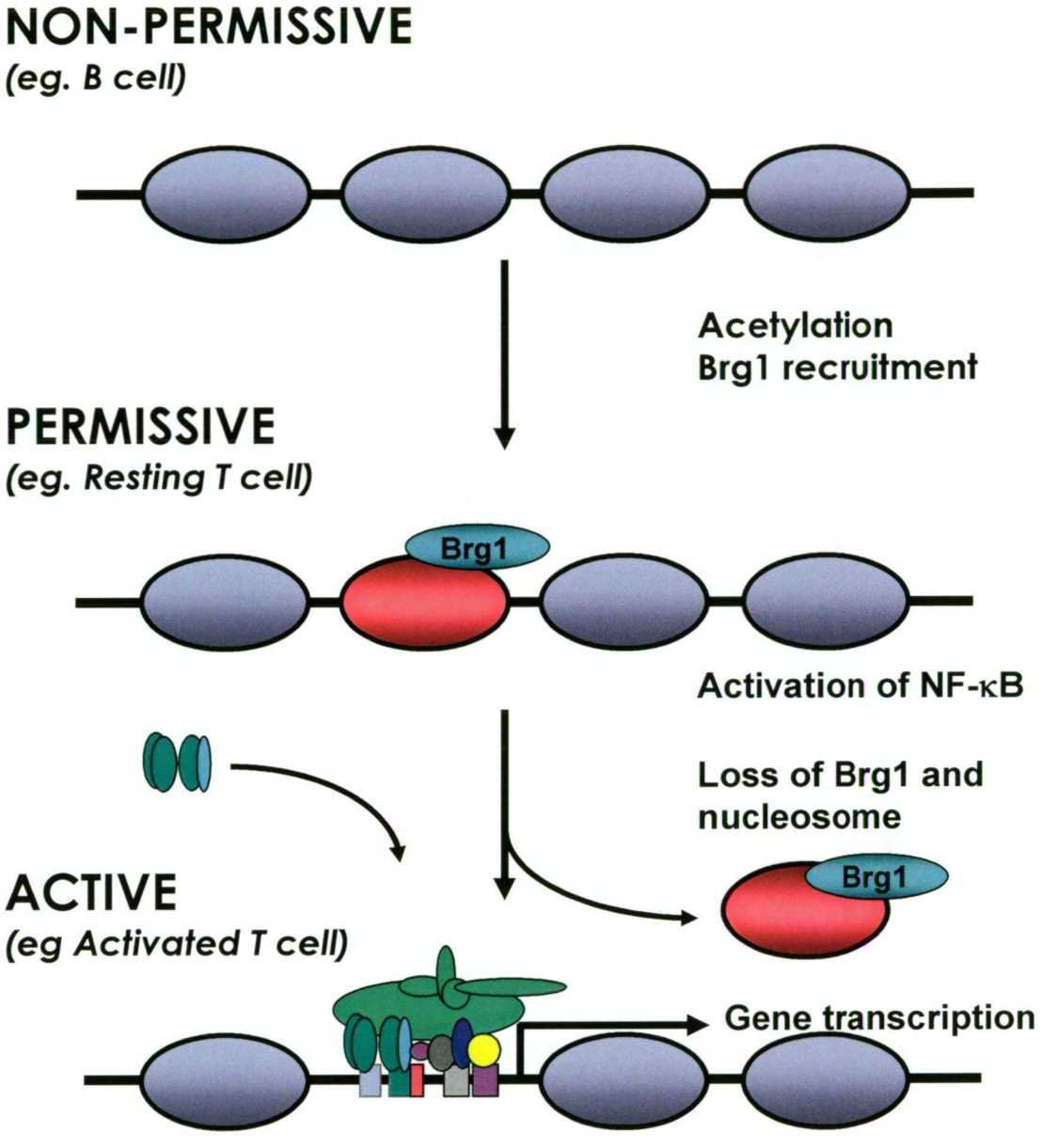



**Figure 4.10. Model for activation of the GM-CSF gene.** Schematic representation of the GM-CSF promoter in the resting and activated state in B and T cells showing increased histone acetylation and Brg1 recruitment in resting T cells.

maintain Brg1 loss from the promoter thereby exposing DNA to the transcriptional machinery required for switching on gene expression (Figure 4.10). Many examples in the literature have demonstrated a delayed recruitment of Brg1 to activated promoters. However, for immune genes such as GM-CSF which are rapidly activated, the basal recruitment of the SWI/SNF complex at sites which need to be remodelled may ensure the activating signal can operate much more efficiently.

# CHAPTER 5.

# IFN-γ, IL-4 AND IL-5 GENE ACTIVATION IN T CELLS IS BRG1 DEPENDENT.

## 5.1 INTRODUCTION

While a number of studies have investigated the role of Brg1 in activating various genes, little work has been done to determine the role of Brg1 in T cells (Yeh *et al*, 2002; Holloway *et al*, 2003; Gebuhr *et al*, 2003). To date, the involvement of Brg1 during T cell activation is yet to be determined. The previous chapter highlighted a role for Brg1 in GM-CSF gene activation, raising the question of whether other genes are activated in a similar Brg1 dependent manner in T cells.

The role of Brg1 in gene activation has been studied using a variety of different approaches. While knockout of the Brg1 gene is embryonic lethal in mice (Bultman *et al*, 2000) conditional Brg1 knockouts have been used successfully in the past to elucidate Brg1 function. Previous research has demonstrated that Brg1 is vital for immune development and the immune response. The specific deletion of Brg1 in thymocytes using the Cre-*lox*P conditional mutagenesis system has revealed a role for this protein in T cell development (Gebuhr *et al*, 2003). Profound thymic abnormalities were observed in T cell specific Brg1 deficient mice. Staining of these Brg1 deficient cells with 5'-bromo-2'-deoxyuridine demonstrated that Brg1 was required for thymocyte proliferation and survival. In agreement with Gebuhr *et al* (2003), Vradii *et al* (2006) recently demonstrated that Brg1 is critical for haematopoietic cell maturation. A dominant negative Brg1 mutant was stably expressed in myeloid cells. The forced expression of the mutant Brg1 was found to inhibit G-CSF dependent differentiation of myeloid cells towards the granulocytic stage.

Indeed, a vital role for Brg1 in immune gene regulation has begun to emerge (reviewed in Chi, 2004). Cui *et al* (2004) revealed that the inhibition of Brg1 reduces a cells ability to control viral growth by reducing the expression of IFN-β and other antiviral proteins. Cells were transfected with siRNA directed against the SWI/SNF component BAF47. The inhibition of BAF47 using this siRNA approach was shown to down-regulate

Brg1 and its remodelling activity which in turn reduced the activity of IFN-β. In addition, Brg1 appears to be essential for cytokine induced gene regulation and is responsible for the activation of IFN-α responsive genes (Huang *et al*, 2002). Brg1 has also previously been shown to play an important role in T cell activation. Zhao *et al* (1998) found that T cell receptor signalling results in the rapid association of the Brg1 complex with chromatin. This suggests that SWI/SNF might be involved in regulating the expression of genes typically activated following immune stimulation. This evidence strongly implicates Brg1 in immune gene regulation and therefore highlights the importance of determining the targets of Brg1 in T cells.

A number of microarray studies investigating the role of Brg1 have been conducted in the past. The ALAB, SW-13 and C33A cell lines all lack functional Brg1 protein. These cell lines are derived from breast carcinoma, adenocarcinoma and cervical carcinomas respectively. The reintroduction of wildtype Brg1 into these deficient cell lines has proved to be a useful means for elucidating the function of Brg1. In a study by Liu *et al* (2001) SW-13 cells were transfected with a wildtype Brg1 construct. Brg1 expressing cells were then enriched by FACS and differentially activated transcripts determined by microarray analysis. This group demonstrated that Brg1 was involved in the activation of approximately 80 genes including the cytokine, M-CSF.

Brg1 has been shown to play a role in the regulation of cellular proliferation (Dunaief *et al*, 1994) and is frequently deleted or mutated in tumour cell lines (Wong *et al*, 2000). It is therefore extremely important to determine the targets of this ATPase. Previous work by Hendricks *et al* (2004) used microarrays to identify novel Brg1 targets in ALAB cells, a breast tumor cell line. This cell line lacks Brg1 due to a premature stop codon mutation in exon 10. The adenovirus mediated reintroduction of Brg1 into these cells resulted in the activation of numerous genes involved in cell adhesion, proliferation and motility. Targets included the protooncogenes, cJun and cMyc. Of greatest clinical relevance however, the reintroduction of Brg1 into this tumor cell line was shown to induce growth arrest. In another microarray study, de la Serna *et al* (2005) used stably transfected myocytes capable of the inducible expression of a dominant negative Brg1 protein for microarray analysis. This group found that the activation of one third of myoD induced genes were dependent on Brg1. Therefore, there are a variety of different ways which have

been used successfully in the past to elucidate targets of Brg1.

Studies in yeast have shown that SWI/SNF is only required for the normal transcription of a small subset of genes. Expression studies have revealed that SWI/SNF regulates the transcription of 5-6% of the yeast genome (Sudarsanam *et al*, 2000). Furthermore, a genome wide study in yeast found that SWI/SNF dependent genes were scattered through out the genome suggesting that this complex controls transcription at the level of individual genes and not at the level of chromosomal domains (Sudarsanam *et al*, 2000). Further complexity in Brg1 function became evident as SWI/SNF has been found to have activating (DiRenzo *et al*, 2000; Liu *et al*, 2001) and repressive functions (Murphy *et al*, 1999; Pal *et al*, 2003). Thus, gene regulation by Brg1 is extremely complex and is likely to be slightly different at each target gene.

Very few direct targets of Brg1 have been confirmed in mammalian cells. However, ChIP analysis has been successfully used to demonstrate that Brg1 directly interacts with a number of promoters. Brg1 has been shown to bind to the IFN-β promoter in HeLa cells following viral stimulation (Agalioti *et al*, 2000). ChIP analysis also revealed that Brg1 is recruited to the myogenin promoter during myocyte differentiation (de la Serna *et al*, 2005) and Brg1 has been found to be constitutively associated with the M-CSF promoter in the WI-38 fibroblast cell line (Liu *et al*, 2001). The P21 and metallothionein-1 promoters have also been identified as direct targets of Brg1 (Hendricks *et al*, 2004; Datta *et al*, 2005). In each instance its recruitment appears to be cell type specific and requires different factors and signals.

While the role of Brg1 in regulating gene expression has been analyzed in a number of cell types, there is a lack of array studies investigating the role of Brg1 in T cell function. The aim of this chapter is to identify T cell gene targets which rely on Brg1 for activation. Gene targeting experiments in mice have demonstrated that while Brm is dispensable (Reyes *et al*, 1998) Brg1 is essential for development (Bultman *et al*, 2000) and therefore the use of the Brg1 knockout is not possible for studying the function of Brg1 in T cells. Hence, the transient introduction of a dominant negative ATPase mutant (K→R) Brg1 followed by cell sorting was used to assess the effect of Brg1 on gene activation in EL-4 T cells. Microarrays were used here for the first time to identify a subset of Brg1 dependent inducible T cell genes.

## 5.2 RESULTS

### 5.2.1 Experimental design

To determine the set of inducible genes in T cells which are dependent on Brg1 for their activation, EL-4 T cells in which Brg1 activity is disrupted were generated and subsequently analyzed using microarrays. SWI/SNF function was disrupted by over-expression of the ATPase defective Brg1 K774R mutant (Khavari *et al*, 1993). Cells expressing this mutant have previously been used to determine Brg1 target genes in mouse NIH 3T3 fibroblasts (de la Serna *et al*, 2000).

Attempts were made at generating stably transfected pBJ5-brg1K/R EL-4 T cells. However, while the stable transfection of the pBJ5 control was successful, the cells transfected with the Brg1 mutant failed to grow (Ray and Holloway, personal communication). Therefore cells were transiently transfected with the Brg1 mutant construct and the transfected cell population purified and analyzed using microarrays since this strategy had been used successfully to investigate the role of Brg1 in GM-CSF gene activation (Figure 4.1b, Chapter 4). In addition Liu *et al* (2001) used a similar technique to demonstrate a role for Brg1 in M-CSF activation by transient transfection followed by FACS. Liu and co-workers transfected Brg1 deficient SWI 13 cells with a GFP tagged Brg1 expression vector. Transfected cells were then enriched by FACS 24 hours post transfection and gene expression changes determined by microarray analysis.

EL-4 T cells were transfected with either the pBJ5 parent vector or pBJ5-brg1K/R plasmids along with the $K^K$II plasmid which expresses a truncated form of the $K^K$II receptor (as described in Material and Methods and Chapter 4). After 24 hours transfected cells were purified by virtue of expressing the $K^K$II receptor and incubated with or without P/I for 6 hours. RNA was then extracted from each of these four cell samples for microarray analysis (Table 5.1). The experiment was then repeated to generate a second replicate set of RNA.

**Table 5.1 RNA samples generated for microarray analysis.**

| Sample I.D. | Transfection | P/I (hrs) |
|---|---|---|
| NS-pBJ5-1 | pBJ5 | 0 |
| PI-pBJ5-1 | pBJ5 | 6 |
| NS-Brgm-1 | pBJ5-brg1K/R | 0 |
| PI-Brgm-1 | pBJ5-brg1K/R | 6 |
| NS-pBJ5-2 | pBJ5 | 0 |
| PI-pBJ5-2 | pBJ5 | 6 |
| NS-Brgm-2 | pBJ5-brg1K/R | 0 |
| PI-Brgm-2 | pBJ5-brg1K/R | 6 |

## 5.2.2 Quality Assurance

### 5.2.2.1 RNA quality

The quality of data generated from microarray experiments is to a large extent a reflection of sample quality. Therefore the RNA was subjected to a number of quality assurance assessments before the microarray experiments were undertaken. RNA quality was verified using the Agilent 2100 BioAnalyzer which can accurately determine sample integrity. This chip based capillary electrophoresis system was used to determine the ratio of the 28S ribosomal peak to the 18S ribosomal peak. All samples were within an acceptable range of 1.6 to 1.9 (Table 5.2) which confirmed that the starting RNA was of high quality. Sample concentration and A260/280 ratios were determined using the NanoDrop®. Each ratio was within the acceptable range of 1.8-2.1 suggesting the RNA was relatively pure (Table 5.2).

### 5.2.2.2 Confirmation of functional depletion of Brg1

Since disruption of Brg1 function is known to affect GM-CSF gene activation (Chapter 4) GM-CSF mRNA levels were analyzed in the RNA samples in order to confirm a functional effect from the introduction of the Brg1 mutant. An aliquot of each RNA sample was converted to cDNA and analyzed by real-time PCR to determine GM-CSF mRNA levels. In cells transfected with the control pBJ5 plasmid a greater than 500 fold increase in GM-CSF mRNA was detected following stimulation with P/I for 6 hours

**Table 5.2. The A260/280 and 28S/18S rRNA ratios of the microarray samples.** The integrity of RNA isolated from $K^K$II and pBJ5 or pBJ5-brg1K/R co-transfected cells was determined using the Agilent 2100 BioAnalyzer and RNA purity determined using the NanoDrop®.

| Array | A260/280 | 28S/18S rRNA ratio |
|---|---|---|
| NS pBJ5-1 | 2.11 | 1.8 |
| PI pBJ5-1 | 1.96 | 1.7 |
| NS Brgm-1 | 2.10 | 1.6 |
| PI Brgm-1 | 2.13 | 1.6 |
| NS pBJ5-2 | 2.01 | 1.7 |
| PI pBJ5-2 | 2.07 | 1.7 |
| NS Brgm-2 | 2.03 | 1.9 |
| PI Brgm-2 | 2.09 | 1.6 |

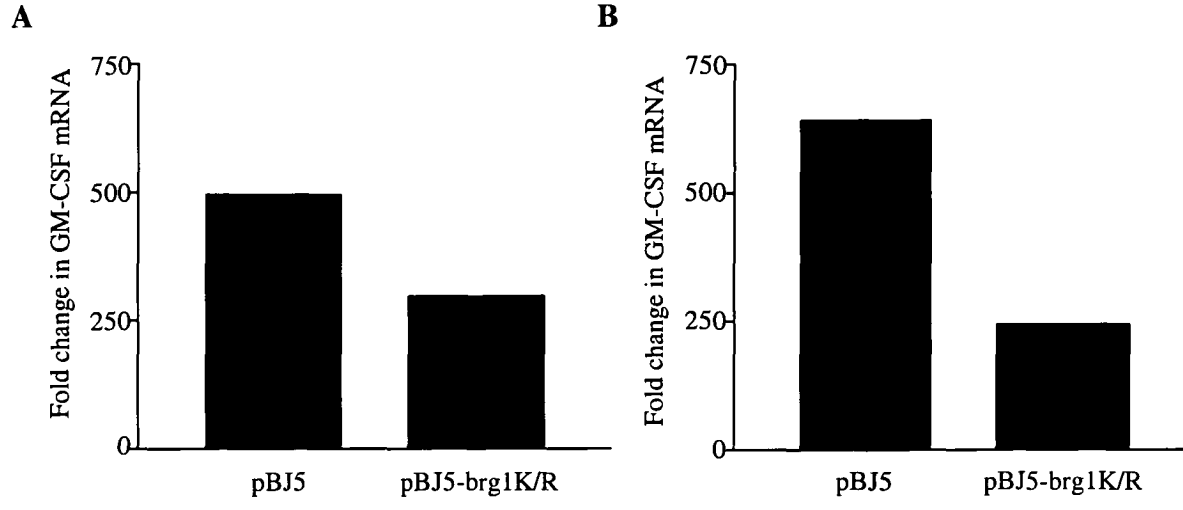



**Figure 5.1. Brg1 is required for transcription of the GM-CSF gene.** EL-4 T cells were co-transfected with the $K^K$II and pBJ5 or pBJ5-brg1K/R plasmids prior to magnetically activated cell sorting before incubating with or without P/I for 6 hours. cDNA was prepared and levels of GM-CSF mRNA analyzed by real-time PCR. The data for replicate 1 (A) and replicate 2 (B) are shown.

(Figure 5.1; 500 fold in replicate 1, 600 fold in replicate 2). As seen previously (Chapter 4) GM-CSF activation in response to P/I was reduced almost 50% in cells transfected with the pBJ5-brg1K/R plasmid (Figure 5.1) with cells expressing the mutant construct only capable of producing a 300 (replicate 1) or 250 fold (replicate 2) induction in GM-CSF mRNA upon T cell activation. This demonstrated that Brg1 function had been disrupted in the cells containing the Brg1 mutant protein, and that this translated into reduced expression of a known Brg1 candidate gene, and suggested that these samples would be useful to screen for novel Brg1 targets.

### 5.2.2.3 Sample processing and array quality

#### 5.2.2.3.1 Test Arrays

Affymetrix GeneChip® Test3 arrays were used as a verification of sample quality and sample processing from total RNA through to the hybridization. These test arrays provide an accurate means of determining the quality of the labelled target prior to analysis on the GeneChip® Mouse Genome 430 2.0 arrays. The test arrays comprise probes representing a subset of characterised genes from various organisms. For each gene represented, probes derived from the 5', middle and 3' portions of the gene are represented thereby enabling the identification of samples containing degraded RNA or inefficiencies in cDNA synthesis which would lead to poor experimental results. The control hybridization probes are identical to those used on the GeneChip® Mouse Genome 430 2.0 arrays and include a subset of mouse housekeeping genes. Briefly, RNA was converted to fragmented cRNA and hybridized to the Test3 arrays overnight before scanning. The 5' to 3' ratios of two housekeeping genes were analyzed. This ratio is an indication of sample integrity, input, the number of full length transcripts and cDNA reaction efficiency. For each experiment 3'/5' ratios for the housekeeping genes β-actin and GAPDH were close to 1, ranging from 0.74 to 1.37 (Table 5.3), indicating efficient cDNA synthesis.

Background values reflect the autofluorescence of the array and non-specific binding of target or stain molecules. Typical background values on arrays analyzed through the JCSMR Biomolecular Resource Facility are approximately 45 (S. Rao, personal communication) and should be similar within the one experiment to ensure accurate

comparisons can be made. High background levels result in an overall loss of sensitivity in the experiment. The test arrays had an average background of approximately 45, ranging from 34.33 to 53.93 (Table 5.3). Together, these data suggested that the 8 samples were of high quality and therefore were hybridized to the GeneChip® Mouse Genome 430 2.0 arrays to screen for Brg1 target genes.

## 5.2.2.3.2 Affymetrix Mouse Genome 430 2.0 Array Quality

The Affymetrix Mouse Genome 430 2.0 arrays allow the analysis of over 39,000 transcripts and variants, including 34,000 well characterized mouse genes. Oligonucleotide probes (11 pairs of 25-mers) including perfect match and mismatch probes are used to measure the level of transcription for each gene represented on the array. Before analyzing the data generated from the Mouse Genome 430 2.0 Arrays, the quality of sample processing and hybridization was confirmed. As seen with the Test3 Arrays, the 5' to 3' ratio for the housekeeping genes β-actin and GAPDH were close to 1 (Table 5.4) and the arrays had an average background of 45 ranging from 41.67 to 54.34 for the 8 arrays indicating consistency across the arrays (Table 5.5).

Each Affymetrix array represents a separate experiment therefore data must be normalized to account for variability in starting amounts of RNA, sample processing, staining and scanning. Arrays must be scaled or normalized to one target intensity to allow comparison between experiments. The signal intensities were imported into GeneChip® Operating Software version 1.2 (GCOS; Affymetrix) and all arrays were normalized using the MAS 5.0 algorithm for comparison purposes to a target intensity (TGT) of 150. Optimally, scale factors should be very close to one another (within three-fold) indicating that minimal scaling of the data is required. The scale factor for the 8 arrays in this experiment ranged from 1.1 to 2.1 which indicated that the scaling due to background across the arrays was minimal (Table 5.5).

The number of probe sets called "present" relative to the entire number of probe sets on the array is dependent on the type of cell, stimuli and overall RNA quality. The call of "presence" or "absence" was determined by signal intensity above or below background respectively. Extremely low percentage of genes present values are often an indication of poor sample quality or hybridization. The percentage of genes present or absent was similar

**Table 5.3. Efficiency of cDNA synthesis and levels of non specific binding on the Affymetrix Test3 arrays.** RNA from EL-4 T cells co-transfected with the pBJ5 or pBJ5-brg1K/R and $K^K$II plasmids was processed and hybridized to the Affymetrix Test3 arrays. Signal intensities were measured and 5' to 3' ratio for 2 housekeeping genes generated. Background signal levels were also measured on the test arrays.

| Array | Beta actin signal 3'/5' | GAPDH signal 3'/5' | Background |
|---|---|---|---|
| NS pBJ5-1 | 1.13 | 0.78 | 34.33 |
| PI pBJ5-1 | 1.26 | 0.86 | 53.52 |
| NS Brgm-1 | 1.16 | 0.81 | 36.01 |
| PI Brgm-1 | 1.37 | 0.77 | 41.08 |
| NS pBJ5-2 | 1.17 | 0.74 | 51.85 |
| PI pBJ5-2 | 1.16 | 0.90 | 46.15 |
| NS Brgm-2 | 1.19 | 0.78 | 53.93 |
| PI Brgm-2 | 1.24 | 0.90 | 45.89 |

**Table 5.4. Efficiency of cDNA synthesis for the Affymetrix Mouse Genome 430 2.0 arrays.** RNA from EL-4 T cells co-transfected with the pBJ5 or pBJ5-brg1K/R and $K^K$II plasmids was processed and hybridized to the Affymetrix GeneChip® mouse genome 430 2.0 arrays. The arrays were scanned and signal intensities were measured to calculate the 5' to 3' ratio for 2 housekeeping genes.

| Array | Beta-actin signal (3'/5') | GAPDH signal (3'/5') |
|---|---|---|
| NS pBJ5-1 | 1.14 | 0.78 |
| PI pBJ5-1 | 1.26 | 0.82 |
| NS Brgm-1 | 1.13 | 0.83 |
| PI Brgm-1 | 1.20 | 0.79 |
| NS pBJ5-2 | 1.15 | 0.81 |
| PI pBJ5-2 | 1.14 | 0.83 |
| NS Brgm-2 | 1.15 | 0.78 |
| PI Brgm-2 | 1.19 | 0.79 |

**Table 5.5. Relative hybridization efficiency, non specific binding and % genes present on the arrays.** Following sample hybridization and scanning, the signal intensities of each array were used to generate the scale factor, background and percentage of genes present using GCOS.

| Array | Scale Factor | Average background | % genes present |
|---|---|---|---|
| NS pBJ5-1 | 1.474623561 | 42.44 | 45.4 |
| PI pBJ5-1 | 1.773011446 | 54.34 | 40.0 |
| NS Brgm-1 | 2.098287106 | 41.67 | 42.6 |
| PI Brgm-1 | 1.551655054 | 41.79 | 44.1 |
| NS pBJ5-2 | 1.158938408 | 50.61 | 46.1 |
| PI pBJ5-2 | 1.895966768 | 44.09 | 42.4 |
| NS Brgm-2 | 1.10244453 | 44.39 | 46.3 |
| PI Brgm-2 | 1.63254261 | 41.95 | 43.3 |

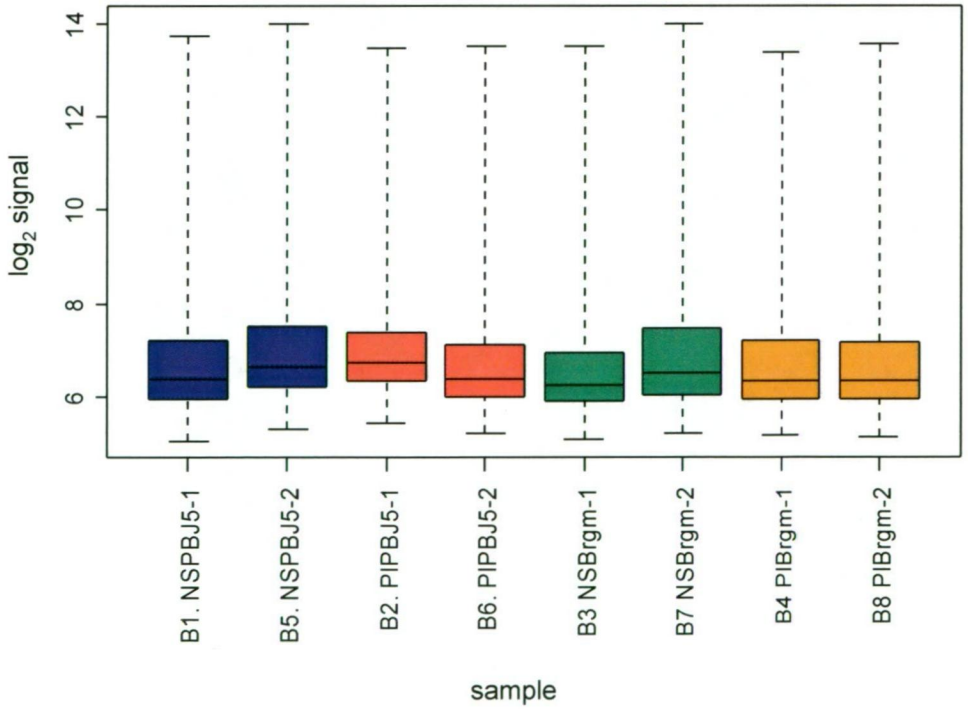



**Figure 5.2. Box plots of the $\log_2$ Affymetrix signal values for all the samples as output by Bioconductor.**

across the replicate arrays with the % present ranging between 40.0 to 46.3% (Table 5.5). This result is typical of array experiments which have been conducted in the JCSMR Biomolecular Resource Facility (S. Rao, personal communication). Together these analyses confirmed that the 8 RNA samples and hybridizations were of high quality.

Statistical analysis was initially carried out by Dr. Stephen Ohms of the Biomolecular Resource Facility. Plots of the $\log_2$ CEL file intensities were generated using the box plot function in the base package R (Bioconductor version 1.8.0) with all input parameters set to default values. The 8 arrays were found to be highly reproducible with background equivalent among all samples as demonstrated in Figure 5.2. Using unbiased statistical analysis it was shown that all arrays had performed with equal efficiency. Therefore it was concluded that all 8 arrays were of good quality and suitable for comparison across samples.

## 5.2.3 Microarray data analysis

### 5.2.3.1 Absolute analysis

Once the quality of the samples and arrays had been confirmed absolute data analysis was performed using the computational analysis program GCOS. Since GM-CSF is known to be regulated by Brg1 and this had been confirmed in the samples prior to array analysis GM-CSF data was extracted to confirm that changes in expression could be detected on the arrays and the target intensity values (TGTs) are shown in Table 5.6. For both replicates an increase in GM-CSF expression was detected following stimulation to a TGT of 1600 and 1100 for replicates 1 and 2 respectively. In the mutant transfected cells TGTs of approximately 800 were detected (Table 5.6). This demonstrated that expression of the Brg1 mutant resulted in reduced GM-CSF expression in both replicates (Figure 5.3) confirming the real-time PCR analysis of these samples (Figure 5.1).

### 5.2.3.2 Comparative analysis

Normalized data was transferred to GeneSpring 7.2 (Silicon Genetics) for comparative analysis. The extremely defined aim of this analysis was to identify inducible T cell genes which, like GM-CSF, were Brg1 dependent. The loss of Brg1 was found to

**Table 5.6. GM-CSF TGTs for the two replicate experiments.** EL-4 T cells were co-transfected with either the pBJ5 or pBJ5-brg1K/R and $K^K$II plasmids prior to magnetically activated cell sorting and incubation with or without P/I for 6 hours. Fragmented cRNA was prepared and hybridized to the Affymetrix GeneChip® mouse genome 430 2.0 arrays. The arrays were scanned and signal intensities (TGTs) used to determine the relative levels of GM-CSF mRNA.

| Sample | Replicate 1 GM-CSF TGT | Replicate 2 GM-CSF TGT |
|---|---|---|
| NS pBJ5 | 15.2 | 20.0 |
| PI pBJ5 | 1593.5 | 1091.3 |
| NS Brgm | 24.2 | 11.2 |
| PI Brgm | 884.8 | 889.3 |

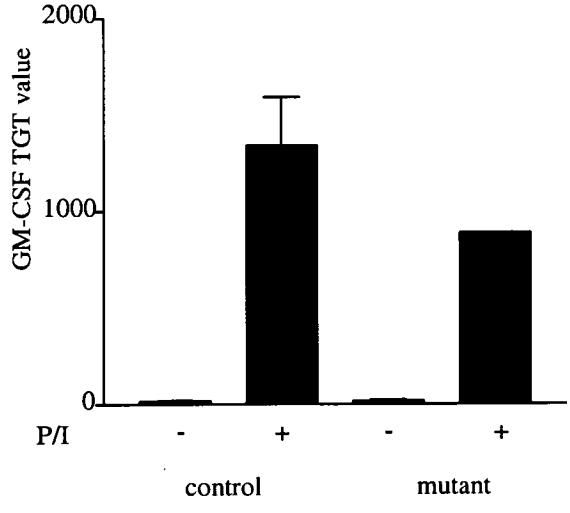



**Figure 5.3. GM-CSF TGTs for the two replicate experiments.** EL-4 T cells were co-transfected with either the pBJ5 or pBJ5-brg1K/R and $K^K$II plasmids prior to magnetically activated cell sorting and incubation with or without P/I for 6 hours. Fragmented cRNA was prepared and hybridized to the Affymetrix GeneChip® mouse genome 430 2.0 arrays. The relative levels of GM-CSF mRNA were determined by scanning signal intensities (TGTs). The mean and standard error of the two replicate assays are shown.

disrupt the activation of 0.3% of genes in EL-4 T cells. Altered basal expression in the mutant transfected cells was also determined. Upregulated basal levels of 2 fold or greater were observed in 32 genes (data not shown). In addition, 47 genes were down-regulated 2 fold or greater in the Brg1 mutant cells (both replicates).

Data was filtered to determine the genes differentially expressed between the control and Brg1 mutant samples. The Advanced Filter tool in GeneSpring was used to generate a list of inducible genes which had a reduction in activation in the Brg1 mutant expressing cells compared to the control cells (i.e. genes with flags present in P/I control AND genes induced greater than 2 fold in P/I control compared to the NS control NOT genes induced greater than 1.4 fold in the P/I control compared to the P/I mutant). The stringency of the filters set was adapted to generate a list of genes with similar profiles to GM-CSF. In replicate 1, 1071 genes were affected according to the filters set and 2570 genes were affected in replicate 2 (Figure 5.4). The difference betweent the genes identified in each replicate can be explained by slight variations in signal intensity across replicate arrays. A number of genes may be excluded from replicate 1 as they fall just outside the 2 or 1.4 fold cutoffs. The gene lists generated for each experimental replicate were combined and genes common to both lists and therefore reproducibly differentially expressed identified. Of the genes induced greater than 2 fold in response to P/I stimulation, 101 displayed reduced expression in the pBJ5-brg1K/R cells compared to the control stimulated cells and are listed in Appendix A. A subset of "known" genes is displayed in Table 5.7.

These genes were then grouped according to functional classification by using the "Build Ontology" function in GeneSpring. It was evident that genes involved in a wide range of processes were affected by the Brg1 mutant including a large number of genes involved in cell communication (Figure 5.5). As GM-CSF was not included in the combined data (Table 5.7) a less stringent means of identifying Brg1 targets was used. The cytokine and immune genes which fell into the cell communication category for replicate 1 were identified and are listed along with signal intensity values in Table 5.8. This group included M-CSF, IL-4, IL-5, IL-25, GM-CSF, and IFN-γ. Genes differentially expressed in both replicates included IL-4 and IFN-γ.

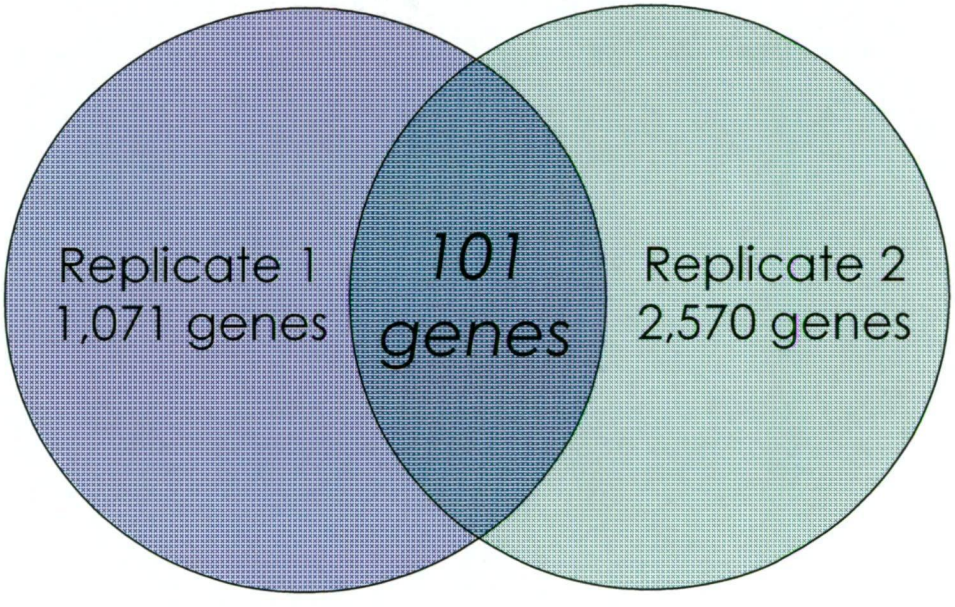



**Figure 5.4. Venn diagram of genes identified as inducible Brg1 targets in EL-4 T cells for replicate 1 and 2.**

**Table 5.7. Genes with reduced activation in the Brg1 mutant cells.** Data from the microarray experiments was imported into GeneSpring and inducible genes which were downregulated in the presence of the Brg1 mutant in both replicates identified using the advanced filter. Only a subset of genes are shown.

| Gene Name | Description |
|---|---|
| 1445521_at | ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R) |
| 1449235_at | tumor necrosis factor (ligand) superfamily, member 6 |
| 1449298_a_at | phosphodiesterase 1A, calmodulin-dependent |
| 1426176_a_at | prokineticin 2 |
| 1447647_at | wingless-related MMTV integration site 7A |
| 1441298_at | spectrin beta 3 |
| 1420888_at | Bcl2-like |
| 1441662_at | cytochrome P450, family 4, subfamily x, polypeptide 1 |
| 1440558_at | ATPase, class II, type 9B |
| 1425947_at | interferon gamma |
| 1460271_at | triggering receptor expressed on myeloid cells 3 |
| 1451837_at | adaptor-related protein complex 3, beta 2 subunit |
| 1452028_a_at | cadherin 23 (otocadherin) |
| 1449864_at | interleukin 4 |
| 1449988_at | immunity-associated protein |
| 1437937_at | chemokine binding protein 2 |
| 1450716_at | a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 |
| 1450869_at | fibroblast growth factor 1 |
| 1450829_at | tumor necrosis factor, alpha-induced protein 3 |
| 1450375_at | Persephin |
| 1425071_s_at | neurotrophic tyrosine kinase, receptor, type 3 |
| 1424376_at | CDC42 effector protein (Rho GTPase binding) 1 |
| 1424647_at | gamma-aminobutyric acid (GABA-A) receptor, pi |
| 1432155_at | Wiskott-Aldrich syndrome-like (human) |
| 1425225_at | Fc receptor-like 3 |
| 1426071_at | progressive ankylosis |
| 1419431_at | Epiregulin |
| 1422397_a_at | interleukin 15 receptor, alpha chain |
| 1426180_a_at | submaxillary gland androgen regulated protein 2 |
| 1420593_a_at | TEA domain family member 3 |
| 1420532_at | activin receptor interacting protein 1 |
| 1438927_x_at | Similar to 60S ribosomal protein L23a (LOC270584), mRNA |
| 1433573_x_at | protease, serine, 2 |
| 1425155_x_at | colony stimulating factor 1 (macrophage) |

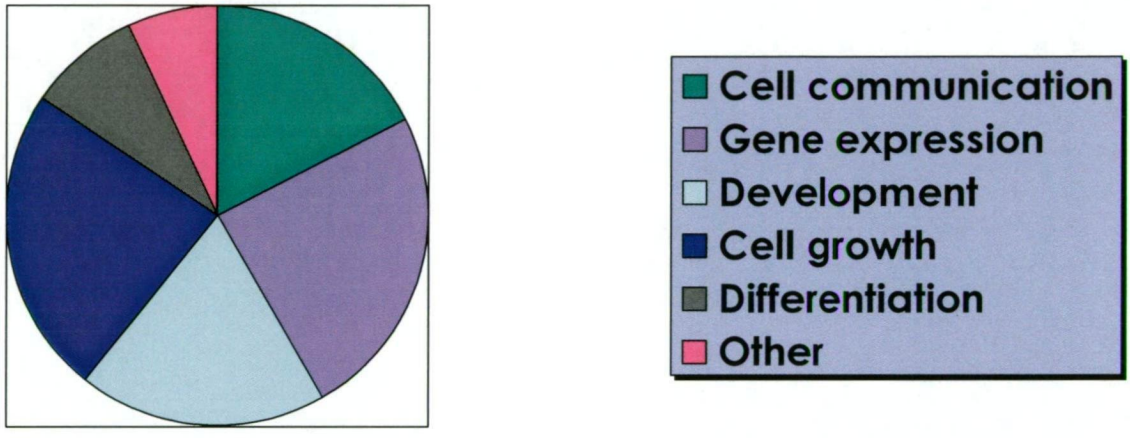



**Figure 5.5. Functional classification of genes differentially expressed in the pBJ5-brg1K/R transfected cells.** Data from the microarray experiments was imported into GeneSpring and inducible genes which were downregulated by the expression of the Brg1 mutant were grouped according to function using the build ontology tool.

**Table 5.8. Immune genes with reduced activation in the Brg1 mutant.** Data from the microarray experiments was imported into GeneSpring and inducible genes which were downregulated in the presence of the Brg1 mutant (m) in replicate 1 identified using the advanced filter. Genes were then sorted according to ontology and immune genes which fell into the "cell communication" category are listed.

| Gene Name | Description | NS TGT | PI TGT | NSm TGT | PIm TGT |
|---|---|---|---|---|---|
| 1424254_at | interferon induced transmembrane protein 1 | 13.2 | 62 | 53.5 | 25.6 |
| 1425947_at | interferon gamma | 23.7 | 1037.3 | 20 | 588.6 |
| 1449988_at | immunity-associated protein | 32.7 | 101.7 | 37.3 | 49.7 |
| 1427429_at | colony stimulating factor 2 (granulocytemacrophage) | 15.2 | 1593.5 | 24.4 | 884.8 |
| 1449864_at | interleukin 4 | 22.7 | 139.5 | 20 | 79.8 |
| 1418126_at | chemokine (C-C motif) ligand 5 | 9.4 | 150.9 | 32 | 86.5 |
| 1428856_at | histocompatibility 13 | 10.4 | 53.3 | 30.8 | 31.8 |
| 1421174_at | interferon regulatory factor 4 | 52.2 | 641.4 | 31.8 | 395.4 |
| 1422938_at | B-cell leukemia/lymphoma 2 | 46.4 | 223.4 | 69.2 | 138.7 |
| 1450550_at | interleukin 5 | 10.4 | 237.9 | 20.5 | 149.5 |
| 1433632_at | interferon regulatory factor 2 binding protein 2 | 159.7 | 414.7 | 143.9 | 261 |
| 1425155_x_at | colony stimulating factor 1 (macrophage) | 4.3 | 91.2 | 28.6 | 60.9 |
| 1416696_at | interleukin 25 | 347.3 | 734.3 | 418.2 | 494.4 |
| 1419529_at | interleukin 23, alpha subunit p19 | 2.2 | 596.4 | 18.6 | 416.1 |
| 1449903_at | cytotoxic and regulatory T cell molecule | 5.5 | 552.7 | 2.9 | 377 |

## 5.2.4 Expression of the cytokines M-CSF, IFN-γ, IL-4 and IL-5 is dependent on Brg1

Of the genes found to be dependent on Brg1 for activation in T cells, a number were cytokines, including M-CSF, IFN-γ, IL-4 and IL-5. To validate the Brg1 dependence of these genes, EL-4 T cells were again co-transfected with $K^{K}$II and pBJ5 or the pBJ5-brg1K/R mutant plasmids and transfected cells purified 24 hours later. Cells were then left unstimulated or stimulated with P/I for 6 hours before isolating RNA. M-CSF, IFN-γ, IL-4 and IL-5 mRNA levels were then determined by real-time PCR analysis of cDNA. The disruption of Brg1 function in EL-4 T cells reduced M-CSF mRNA accumulation (Figure 5.6a). An 11 fold increase in M-CSF mRNA levels was observed in control transfected cells following stimulation with P/I. In contrast, M-CSF mRNA only increased 7 fold in P/I stimulated cells expressing the Brg1 mutant. Stimulation of control transfected cells produced a 1500 fold increase in IFN-γ mRNA levels, however the response to stimulation in the mutant transfected cells was reduced to 500 fold (Figure 5.6b). The Brg1 mutant also inhibited IL-4 expression (Figure 5.6c). Control transfected cells displayed an approximately 60 fold increase in IL-4 mRNA levels following stimulation, while the Brg1 mutant transfected cells exhibited only a 10 fold increase in IL-4 expression. IL-5 expression was almost completely abolished by Brg1 mutant expression. While control transfected cells displayed a 60 fold induction in IL-5 mRNA levels, IL-5 transcription was inhibited in cells transfected with the Brg1 mutant (Figure 5.6d). These data validated the microarray results and confirmed that Brg1 is involved in the expression of M-CSF, IFN-γ, IL-4 and IL-5. M-CSF has previously been identified as a target of Brg1 by Liu *et al* (2000) and the identification of a known Brg1 target therefore confirms the validity of data generated from the microarrays.

## 5.2.5 IFN-γ, IL-4 and IL-5 display distinct activation profiles

As M-CSF has previously been identified as a direct target of Brg1 (Lui *et al*, 2001), the remaining candidates IFN-γ, IL-4 and IL-5 were chosen for further analysis. In order to determine the activation profiles of the IFN-γ, IL-4 and IL-5 genes in response to P/I stimulation in EL-4 T cells, RNA was isolated from EL-4 T cells stimulated with P/I for

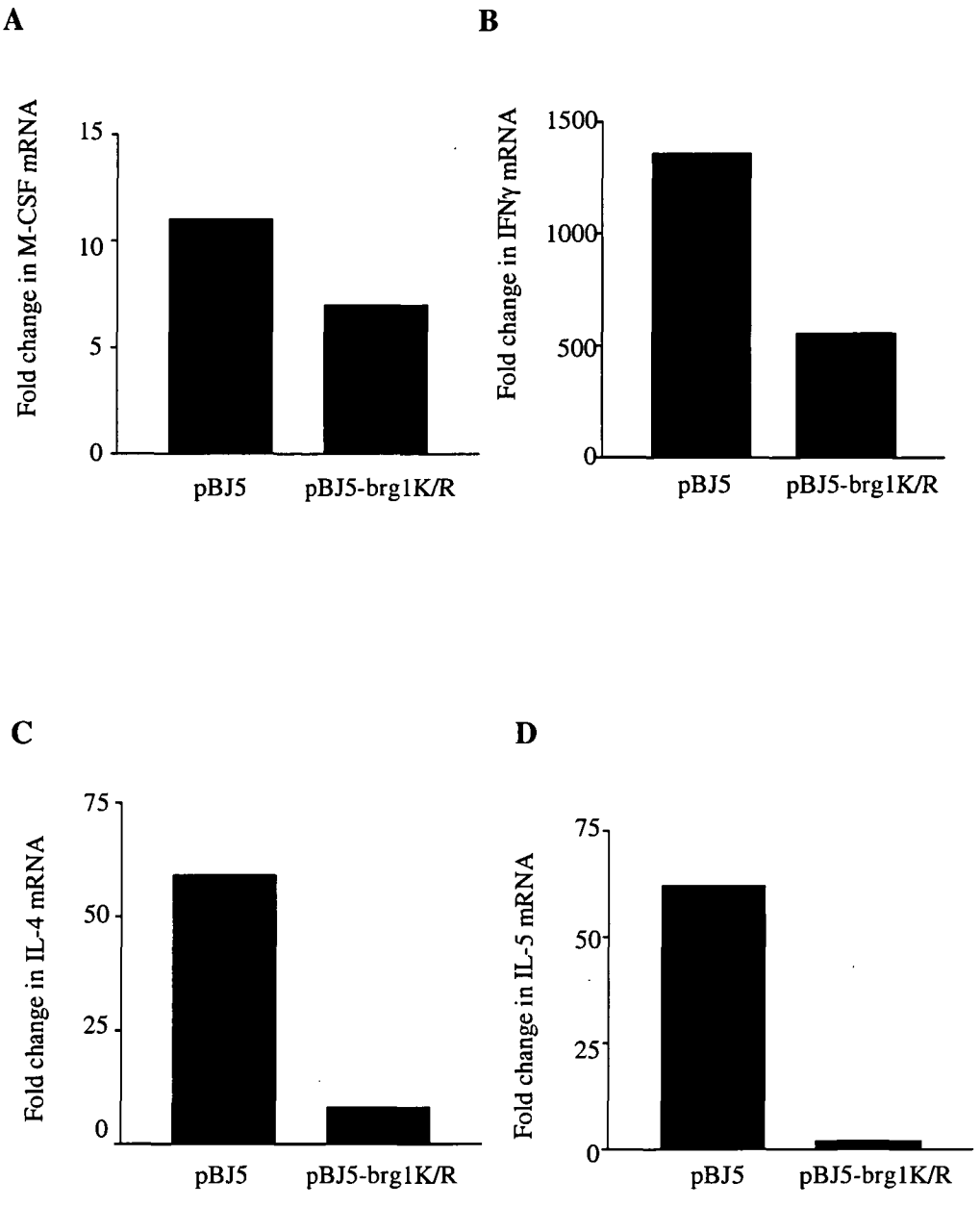



**Figure 5.6. Induction of M-CSF, IFN-γ, IL-4 and IL-5 genes is Brg1 dependent.** EL-4 T cells were transfected with the pBJ5 or pBJ5-brg1K/R plasmids. Transfected cells were purified before incubation with or without P/I for 6 hours. cDNA was prepared and levels of M-CSF (A), IFN-γ (B), IL-4 (C) and IL-5 (D) mRNA determined by real-time PCR. The results for a single assay are shown.

0, 0.5, 2, 4, 6 and 24 hours. cDNA was prepared and IFN-γ, IL-4 and IL-5 mRNA levels determined by real-time PCR. An increase in IFN-γ mRNA levels was detected within 30 minutes of stimulation and peaked at 6 hours with a 300 fold induction before returning to near basal levels by 24 hours (Figure 5.7a). The IL-4 gene was induced more rapidly with mRNA levels peaking at 4 hours post-stimulation with levels reaching approximately 60 fold basal levels, declining after this time and returning to basal levels by 24 hours post-stimulation (Figure 5.7b). The IL-5 mRNA time course displayed distinctly different kinetics with little increase in mRNA levels detected before 6 hours of stimulation but had significantly increased by 24 hours to 30 fold basal levels (Figure 5.7c). Thus while the IFN-γ and IL-4 genes, like GM-CSF are rapidly activated in response to P/I, the IL-5 gene showed a more delayed activation profile.

### 5.2.6 The IFN-γ, IL-4 and IL-5 promoters display distinct remodelling profiles

To determine whether the different activation profiles of the IFN-γ, IL-4 and IL-5 genes reflected differences in basal chromatin structure and remodelling kinetics chromatin accessibility across their promoters was examined prior to and following stimulation. EL-4 T cells were either left untreated or stimulated with P/I for 4 and 24 hours. Nuclei were then isolated and digested with MNase. Accessibility was determined by real-time PCR analysis of genomic DNA using primer sets designed to the IFN-γ, IL-4 or IL-5 promoters. A low level of basal accessibility was detected at the IFN-γ promoter (20%) which increased to 30% at 4 hours post-stimulation and by 24 hours had decreased to approximately 25% (Figure 5.8a). The IL-4 promoter had a higher inherent level of basal accessibility, with accessibility in the non-stimulated cells at 45% (Figure 5.8b). Stimulation for 4 hours resulted in increased promoter accessibility with levels increasing to 60% and remained at this level for 24 hours. The IL-5 promoter was relatively inaccessible in resting T cells with basal accessibility at approximately 20%. Accessibility remained at this level at 4 hours post stimulation, however at 24 hours post stimulation the promoter had opened up to approximately 50% (Figure 5.8c). Chromatin accessibility changes at the IFN- γ, IL-4 and IL-5 promoters reflected the transcriptional activation

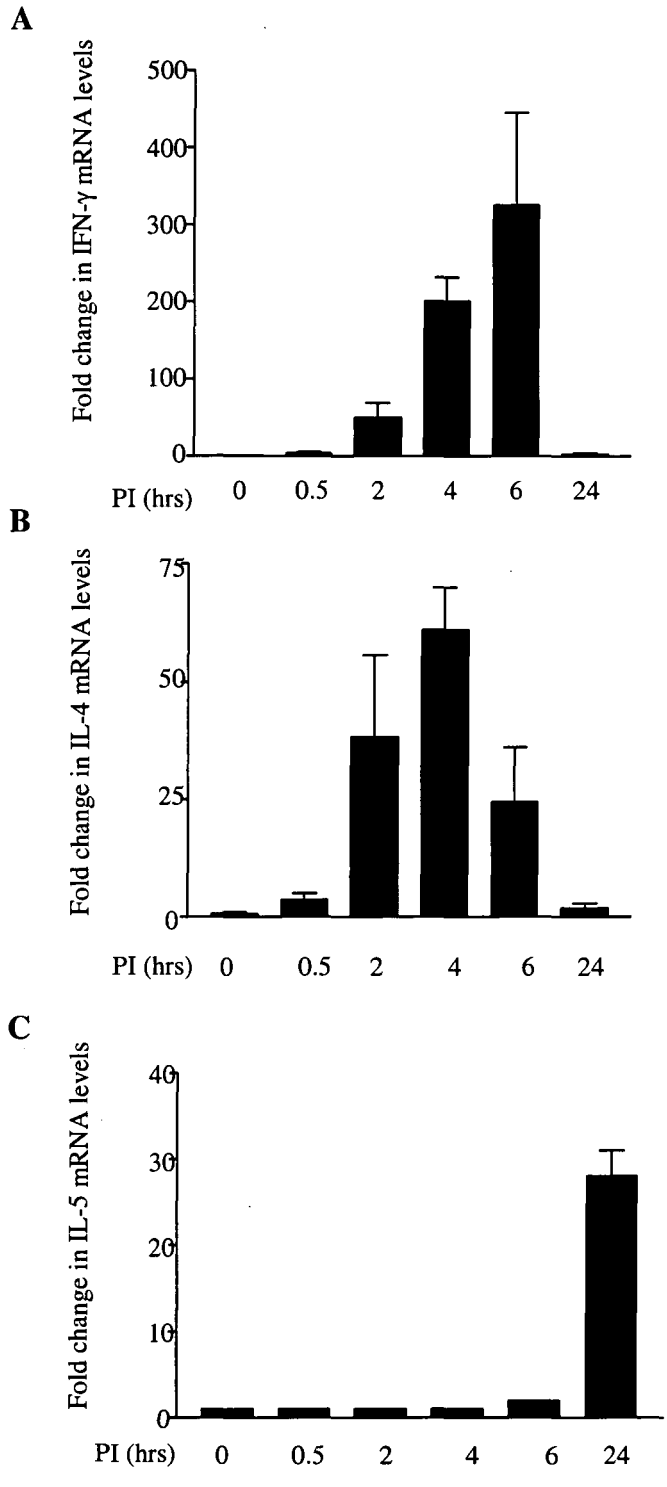



**Figure 5.7. The IFN-γ, IL-4 and IL-5 genes show distinct activation profiles.** cDNA was prepared from EL-4 T cells stimulated with P/I for the indicated time periods. IFN-γ (A) IL-4 (B) and IL-5 (C) mRNA levels were then analyzed by real-time PCR. The mean and standard error of three replicate assays are shown.

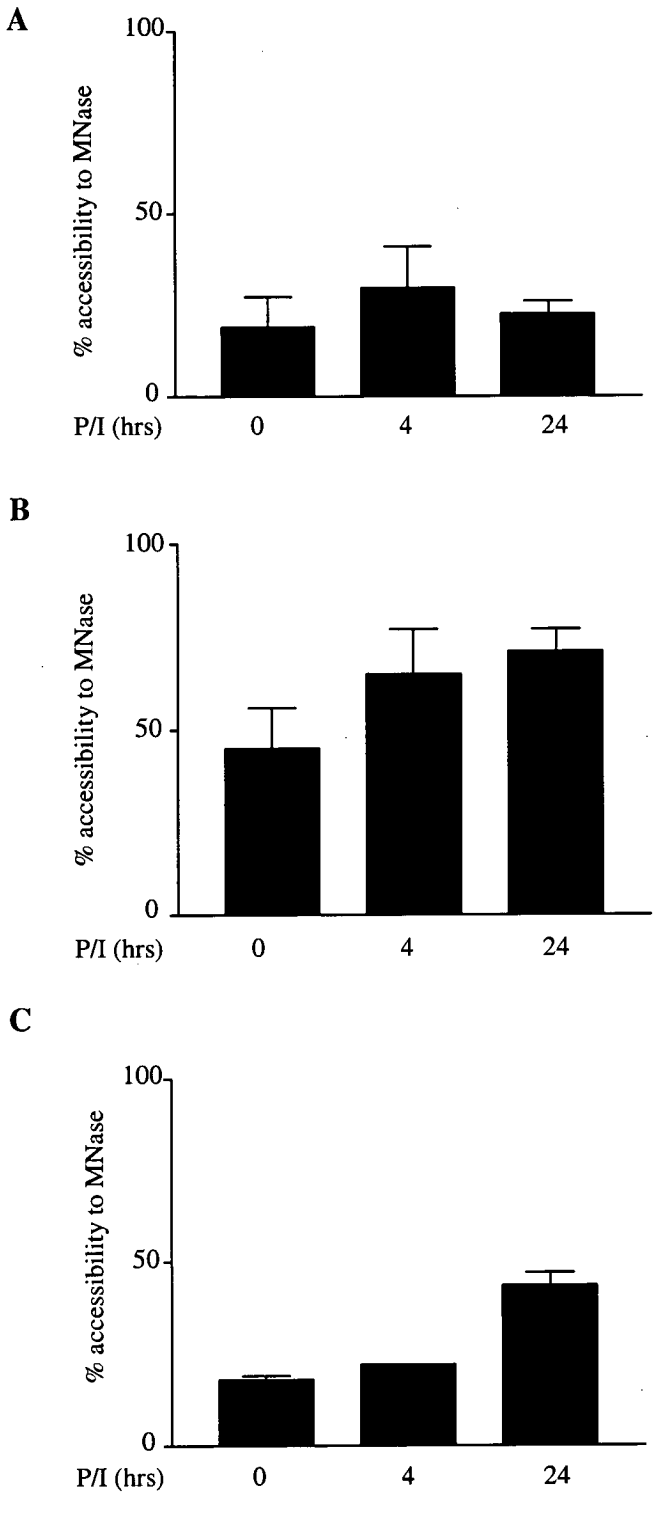



**Figure 5.8. The IFN-γ, IL-4 and IL-5 promoters display different remodelling profiles.** Nuclei isolated from EL-4 T cells stimulated for the indicated time periods were digested with MNase before analyzing genomic DNA by real-time PCR with primers designed to the IFN-γ (A) IL-4 promoter (B) and IL-5 promoter (C). The mean and standard error for two replicate assays are shown

kinetics of these genes.

### 5.2.7 Brg1 is bound to the IFN-γ promoter in resting cells and is recruited to the IL-4 promoter following stimulation

IFN-γ, IL-4 and IL-5 expression in activated T cells, like GM-CSF, is dependent on Brg1. To determine whether Brg1 is directly involved in the activation of these genes, ChIP assays were used to investigate whether Brg1 is associated with the gene promoters. EL-4 T cells were left unstimulated or stimulated for 4 hours with P/I before cross linking and immunoprecipitating chromatin using a Brg1 antibody. ChIP revealed that Brg1 is enriched at the IFN-γ promoter in resting T cells, and Brg1 levels decrease following stimulation (Figure 5.9a). In contrast only relatively low levels of Brg1 were detected at the IL-4 promoter in resting cells, however following stimulation Brg1 levels increased 3 fold (Figure 5.9b). In contrast, little Brg1 was detected at the IL-5 promoter in resting and stimulated cells (Figure 5.9c). Therefore, IFN-γ shows a similar Brg1 recruitment profile to GM-CSF with Brg1 bound to the resting promoter and lost following stimulation. In contrast, while IL-4 has a similar activation profile to GM-CSF and IFN- γ, Brg1 is not present at the IL-4 promoter in the resting state but is recruited following stimulation. Brg1 could not be detected at the IL-5 promoter at the time points analyzed.

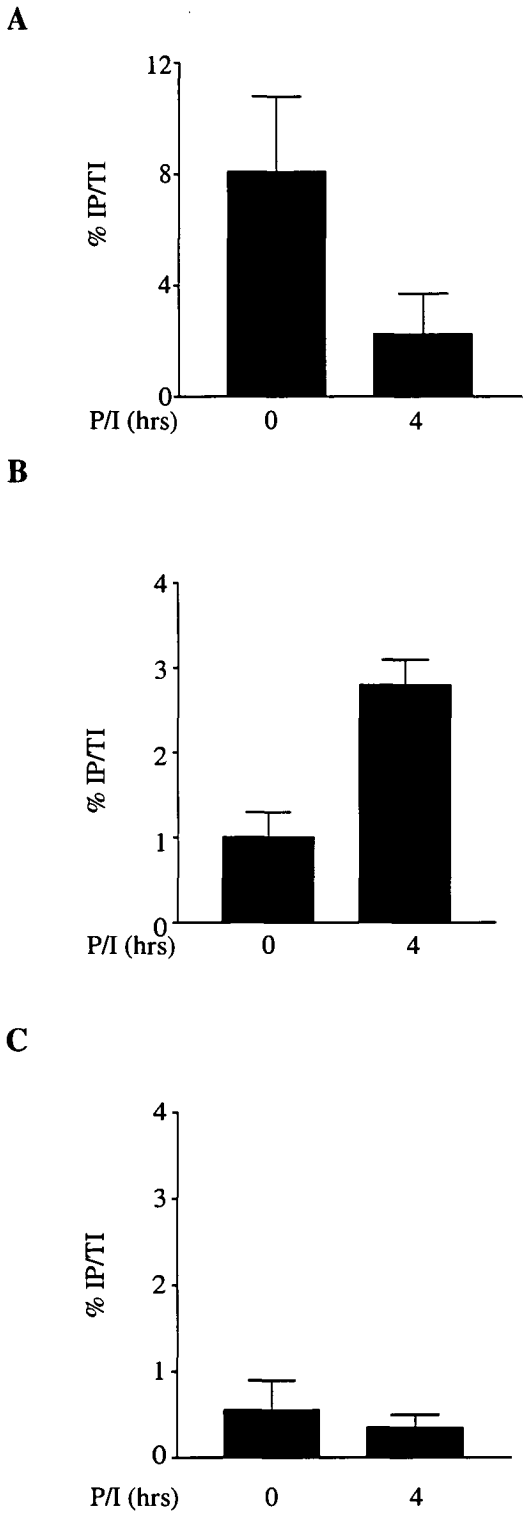



**Figure 5.9. Brg1 binding at the IFN-γ, IL-4 and IL-5 promoters.** EL-4 T cells were either left untreated or stimulated for 4 hours with P/I before immunoprecipitating chromatin using a Brg1 antibody. Enrichment of Brg1 was determined by real-time PCR analysis using primers designed to the IFN-γ (A) IL-4 (B) and IL-5 (C) promoter regions. The mean and standard error of three replicate assays are shown

## 5.3 DISCUSSION

Microarray experiments have proved to be incredibly powerful tools for determining the function of Brg1 (Liu *et al*, 2001; Hendricks *et al,* 2004; de la Serna *et al,* 2005). Here, microarray analysis was used to address the extremely focussed question of whether other immune genes are regulated by Brg1 in a similar manner to GM-CSF in response to T cell activation. This study identified 101 targets of Brg1 from 34,000 known mouse transcripts which were reproducibly differentially expressed. Other studies have found similarly small sets of genes regulated by Brg1. Hendricks *et al* (2004) found 70 genes induced 2 fold or more following the restoration of Brg1 in SW-13 and ALAB cells and 65 genes repressed 2 fold or greater, out of 40,000 genes screened. While Liu *et al* (2001) found 80 genes induced 3 fold or greater and 2 repressed 3 fold or more out of 22,000 genes in SW-13 cells following reintroduction of Brg1. The data mining techniques used differ between research groups and depend on the aim of the experiment. The number of genes identified is therefore restricted by the cut-offs used in data analysis and of course the cell type, experimental system and treatments used. In this case GM-CSF, a previously identified target of Brg1, was actually omitted from the gene list generated for replicate 2. It is therefore clear that less stringent filtering could be used to identify more Brg1 dependent genes. Thus, Brg1 targets which are activated via different signalling cascades would be omitted from this study along with genes which failed to meet the restrictions set during data analysis.

The microarray data presented in this chapter demonstrates that Brg1 is essential for the activation of a number of cytokine genes in T cells. M-CSF, IFN-γ, IL-4 and IL-5 were all identified as targets of Brg1. The fact that M-CSF has previously been identified as a Brg1 target in a past microarray study validates the data presented in this chapter (Liu *et al,* 2001). IFN-γ expression is associated with Th1 cells, while IL-4 and IL-5 are Th2 cytokines. EL-4 T cells have Th1 and Th2 expression capabilities and are thus considered Th0 like. It is therefore interesting to note that both Th1 and Th2 cytokines were identified as targets of Brg1.

This is not the first time a role for Brg1 in cytokine signalling has been identified. Pattenden *et al* (2002) have previously shown that Brg1 is linked to immune surveillance as Brg1 interacts with the CIITA promoter in an IFN-γ inducible manner. Recent evidence has

pointed to a role for Brg1 in immune regulation (Zhao *et al*, 1998; Huang *et al*, 2002; Cui *et al*, 2004) and Brg1 has previously been identified as playing a major role in the immune response due to its requirement in inducing IFN-γ and IFN-α responsive genes (Pattenden *et al*, 2002; Ni *et al*, 2005). The findings presented in this chapter add further support to the role of Brg1 in immune regulation.

While IFN-γ, IL-4 and IL-5 were all identified as Brg1 targets, each has a distinct activation profile. IFN-γ is rapidly activated, with an increase in promoter accessibility detected within a short time frame. In addition, Brg1 was found to be constitutively associated with the IFN-γ promoter. These features suggest that IFN-γ gene is poised for activation in a similar manner to GM-CSF. The data presented in this chapter supports the very recent findings of Zhang and Boothby (2006) in which Brg1 was shown to be constitutively associated with the IFN-γ promoter in mouse primary Th1 cells. Their work demonstrated that, like GM-CSF, the rapid activation of IFN-γ is associated with basal recruitment of Brg1. Three days following differentiation under Th1 or Th2 conditions Brg1 was found to be associated with the IFN-γ promoter in Th1 but not Th2 cells with recruitment therefore being associated with locus activation. The association of Brg1 with the promoter region was also linked to increased accessibility and transcriptional competence. Similar to the findings for GM-CSF and IFN-γ, Brg1 has also been found constitutively associated with the M-CSF promoter in SW-13 and WI38 cells (Liu *et al*, 2001), suggesting a similar mechanism for gene activation. It is also interesting to note however, that unlike GM-CSF, the IFN-γ promoter is relatively inaccessible in its basal and activated state. It would therefore be important to study remodelling of the IFN-γ gene over a series of time points and at a number of different sites.

Precisely which factor is responsible for anchoring Brg1 to the promoter region remains unclear. Constitutively expressed factors or basal histone acetylation may be involved in the basal recruitment of Brg1 to the IFN-γ and GM-CSF promoters. Indeed, the data presented in Chapter 4 indicates that the basal recruitment of Brg1 to the GM-CSF promoter may be attributed to increased basal histone acetylation. In support of this hypothesis de la Serna *et al* (2005) have shown that histone H4 hyperacetylation precedes Brg1 recruitment to the myogenin promoter. Recently, it has been suggested that Brg1

association with the IFN-γ promoter in Th1 cells is dependent on NFAT members as Brg1 recruitment is CsA sensitive and Brg1 can co-immunoprecipitate with NFAT (Zhang and Boothby, 2006). This hypothesis may be flawed however, as NFAT only translocates to the nucleus following T cell activation. In addition, this study failed to address whether basal histone acetylation of the IFN-γ promoter influences Brg1 recruitment. Increased histone acetylation has previously been observed at the IFN-γ locus in Th1 cells (Avni *et al*, 2002), which correlates with the basal Brg1 recruitment identified by Zhang and Boothby (2006). However, the role of histone acetylation in Brg1 recruitment needs to be studied in more detail.

Unlike GM-CSF and IFN-γ, which share a common mechanism of activation with promoter priming by basal Brg1 recruitment, the IL-4 gene is activated using a different Brg1 dependent mechanism. Intriguingly, while the IL-4 gene displayed a slightly more rapid activation profile than IFN-γ and GM-CSF, Brg1 recruitment was delayed. However, the peak in IL-4 expression and promoter remodelling did occur concomitant with Brg1 recruitment highlighting the importance of Brg1 in IL-4 activation. While Brg1 was not detected at the IL-4 promoter in resting cells, it may be recruited to the IL-4 promoter relatively quickly, at a time point omitted from the ChIP experiment. Alternatively, the delay in Brg1 binding may be due to a more central role for histone acetylation in IL-4 activation. The IL-4 promoter in naïve and Th2 cells is modified in the basal state, with the promoter region immunoprecipitating with acetylated H3 (Grogan *et al* 2003). This basal chromatin modification may help to establish a permissive chromatin environment and prime the gene for rapid induction as IL-4 transcription is dependent on hyperacetylation of the promoter region (Valapour *et al*, 2002; Grogan *et al,* 2003). Thus, it appears that the IL-4 promoter is primed for activation in a manner distinct to GM-CSF, with increased basal accessibility and histone acetylation playing a primary role in rapid activation with SWI/SNF binding occurring with more delayed kinetics. Perhaps this permissive chromatin environment helps mark the IL-4 promoter for rapid recruitment of Brg1.

In comparison to GM-CSF, IFN- γ and IL-4, the IL-5 gene is significantly delayed in its activation with levels elevated only at 24 hours post stimulation. It is therefore surprising that IL-5 was identified as a target of Brg1 in the microarray experiments as these cells only received 6 hours of P/I stimulation. This appears to be due to IL-5 being

more responsive to P/I in transfected cells as this result was validated by replicate real-time PCR experiments. The IL-5 promoter is highly compacted with basal accessibility extremely low with only a slight increase observed 24 hours post stimulation concomitant with transcriptional induction. This is supported by the findings of Siegel *et al*, (1995) who found that the IL-5 promoter region is protected from DNase I digestion. In addition increased sensitivity to DNase I was observed in regions bordering this site suggesting that like GM-CSF, the IL-5 proximal promoter is covered by a nucleosome (Cousins *et al*, 2000). The delayed activation of IL-5 may therefore be attributed to the highly compacted promoter region. It is also possible that other co-activators are delayed in their nuclear translocation. In support of this, the activation of an IL-5 promoter-driven luciferase reporter in EL-4 T cells has previously been observed to take approximately 9 to 12 hours to become fully activated post stimulation (Wang *et al*, 2006). It is clear that activation in a chromatin context would be considerably slower due to the chromatin remodelling requirement. The delay in IL-5 activation may also be attributed to the delay in Brg1 recruitment. At the two time points analyzed, Brg1 was not detected at the IL-5 promoter, however it would be interesting to analyze Brg1 recruitment over 24 hours to correlate gene activity and the presence of Brg1. Alternatively, IL-5 may be a secondary target of Brg1.

It is evident that Brg1 is involved in the activation of genes switched on rapidly and others which are activated with more delayed kinetics. Previous work in macrophages in which the ATPase was disrupted through the retroviral delivery of siRNA hairpins directed against Brg1 found that Brg1 is involved in the activation of late primary and secondary response genes (Ramirez-Carrozzi *et al*, 2006). Brg1 was also found to be constitutively associated with the promoter regions of early primary response genes which parallels the findings presented here for the rapidly activated GM-CSF and IFN-γ genes, although Brg1 did not appear to be required for their subsequent activation.

The data presented in this chapter highlight a role for Brg1 in immune regulation. Microarray analysis was successfully used to identify T cell genes dependent on Brg1 for their activation. A number of cytokine genes were identified as Brg1 targets including IFN-γ, IL-4 and IL-5. While each of these cytokines requires Brg1 for optimal expression, there is some distinction between the transcription and chromatin remodelling profiles for each

gene. The divergent regulation of IFN-γ, IL-4, IL-5 and GM-CSF suggests that although each gene requires Brg1 for activation, IL-4 and IL-5 adopt a slightly different mechanism to GM-CSF and IFN-γ for transcriptional induction. The inaccessible nature of the IL-5 promoter and absence of Brg1 recruitment may contribute to delayed transcription of the IL-5 gene. While the rapidly activated IL-4 gene exhibits increased basal promoter accessibility, IL-4 is not primed for activation by basal recruitment of Brg1. IFN-γ was found to share a similar activation profile to GM-CSF and data presented here suggests it may be regulated by a common mechanism. As observed for GM-CSF, Brg1 is constitutively poised at the IFN-γ promoter in resting EL-4 T cells and lost from the promoter concomitant with gene activation.

# CHAPTER 6
# CONCLUSIONS AND FUTURE DIRECTIONS

Activation of the GM-CSF gene following immune stimulation has been studied extensively in the past however the mechanism underlying activation of the GM-CSF gene in a chromatin context has remained largely unstudied. The data presented in this thesis clearly demonstrate that GM-CSF promoter remodelling and gene transcription are distinct events. Each process can occur independently of the other and has different signalling and transcription factor requirements and different kinetics. While GM-CSF gene transcription relies on PKC and calcium signalling pathways, GM-CSF promoter remodelling requires the action of PKC alone. In addition the transcription factors activated downstream of T cell stimulation are involved in different processes. NFAT members are required for the optimal induction of GM-CSF gene transcription but are dispensable for promoter remodelling and this may be attributed to the requirement of NFAT in enhancer remodelling (Cockerill *et al*, 1993; Johnson *et al*, 2004). In contrast, NF-κB members, in particular c-Rel, are essential for chromatin remodelling events at the GM-CSF promoter. It is also clear that GM-CSF gene transcription and promoter remodelling show quite different kinetics. While transcription displays a transient profile, peaking within several hours of T cell activation, remodelling events at the GM-CSF promoter were relatively stable, being maintained over several days. The stability of promoter remodelling was closely correlated with the nuclear translocation of c-Rel, suggesting this factor may be involved in maintaining the remodelled state. The stability of remodelling also raises the question of whether increased accessibility to the GM-CSF promoter is maintained through cell division or reset at the beginning of the cell cycle and this would be interesting for further investigation.

The precise mechanism involved in remodelling the GM-CSF promoter remains largely unknown. However, recently Chen *et al* (2005) demonstrated that the increase in GM-CSF promoter accessibility, observed during T cell activation, is due to loss of histones from the promoter. Here, an important role for the ATPase component of the SWI/SNF complex, Brg1 was identified in GM-CSF transcriptional activation. Brg1 is

essential for the optimal transcriptional activation of the GM-CSF gene, as a reduction in GM-CSF mRNA production was detected in T cells expressing a Brg1 mutant protein. While Brg1 defective T cells were still capable of GM-CSF promoter remodelling upon T cell stimulation, basal levels of accessibility were slightly increased and displayed more variability suggesting Brg1 may be involved in maintaining the basal state of the promoter. Interestingly, the *PHO5* promoter can still be remodelled in the absence of SWI/SNF, however with delayed kinetics (Gadreau *et al*, 1997) thus it is possible that the kinetics of GM-CSF promoter remodelling was affected in a similar manner which was not detectable at the time point analyzed.

A direct role for Brg1 in GM-CSF gene activation was confirmed using the ChIP assay, which unexpectedly demonstrated that Brg1 was constitutively recruited to the GM-CSF promoter in resting T cells and lost from the promoter concomitant with histone loss, following T cell activation. Analysis of Brg1 enrichment at the GM-CSF promoter in non-expressing B cells demonstrated that Brg1 is absent, providing further support for a role for Brg1 in GM-CSF activation in T cells. This raised the question as to how Brg1 is constitutively associated with the GM-CSF promoter in T cells. Evidently, the classic model in which SWI/SNF is recruited to gene regulatory elements by inducible transcription factors (eg. Agalioti *et al*, 2000, de la Serna *et al*, 2005) does not hold true in this case. It is possible that a constitutively expressed factor helps tether Brg1 to the GM-CSF promoter and this has been demonstrated in the case of the IFITM3 gene with Sp1 recruiting Brg1 (Liu *et al*, 2002). Alternatively, Brg1 may bind to the resting GM-CSF promoter via an interaction with acetylated histones as Brg1 contains a bromodomain capable of such an interaction (Hassan *et al*, 2002). The data presented here suggest that the basal recruitment of Brg1 may in fact be due to increased basal levels of histone acetylation as increasing the levels of acetylation lead to increased Brg1 recruitment at the promoter and increased the rate of remodelling. In support of this increased histone acetylation has been detected across the GM-CSF promoter in T cells (Chen and Shannon, personal communication).

The signal that drives histone loss and remodelling of the GM-CSF promoter remains unclear. Combining the Brg1 data with the findings from Chapter 3 it is possible that the NF-κB family member, c-Rel, which is associated with GM-CSF promoter

remodelling, interacts with Brg1 following T cell activation to drive histone loss. Confirmation of a role for c-Rel in GM-CSF promoter remodelling could be provided by determining Brg1 recruitment and histone loss in c-Rel$^{-/-}$ or PTX treated T cells. A role for PKC signalling in chromatin remodelling at the GM-CSF promoter was also highlighted in Chapter 3. Previous work by Rando and colleagues (2002) found that phosphatidylinositol, activated downstream of PKC signalling, interacts with Brg1 *in vitro* and enhances its affinity for chromatin. In addition, Zhao *et al*, (1998) also found that SWI/SNF binding after T cell activation was dependent on phosphoinositol. Therefore, it is possible that PKC signalling helps to increase the activity of Brg1 and its affinity with chromatin to drive the loss of the nucleosome encompassing the GM-CSF promoter. PKC has also been found to directly phosphorylate a serine residue on histone H3 *in vitro* (Huang *et al*, 2004) and this ability to modify histone marks may influence the docking of other factors which direct nucleosome loss. Determining the role of histone chaperones in the loss of histones from the GM-CSF promoter would help to elucidate how this process occurs.

The constitutive basal recruitment of Brg1 to the GM-CSF promoter in T cells may be involved in generating a poised state with increased basal acetylation and increased Brg1 recruitment priming the gene for rapid activation. Work by Ramirez-Carrozzi *et al* (2006) supports this hypothesis as Brg1 was found to be constitutively associated with a number of early response genes including *Cxcl2*, *Tnf* and *Ptgs2* in macrophages which are activated within minutes of LPS stimulation. However, in this case Brg1 was not found to be essential for the transcriptional activation of these genes.

The data presented in this thesis is consistent with a model in which GM-CSF is primed for rapid activation in T cells (Figure 6.1). An enrichment of acetylated histones is evident at the resting GM-CSF promoter (Chen and Shannon, unpublished data). This histone mark may potentially act as a docking site for the constitutive basal recruitment of Brg1. The enrichment of Brg1 at the promoter may then help to establish a competent chromatin environment which can be remodelled rapidly. In response to T cell activating signals the nuclear translocation of c-Rel may direct the loss of histones from the GM-CSF promoter creating a highly accessible promoter region capable of binding the transcriptional machinery. Nucleosome loss is stably maintained potentially via prevention of nucleosome reassembly by c-Rel (Figure 6.1). Future work investigating the role of Brg1

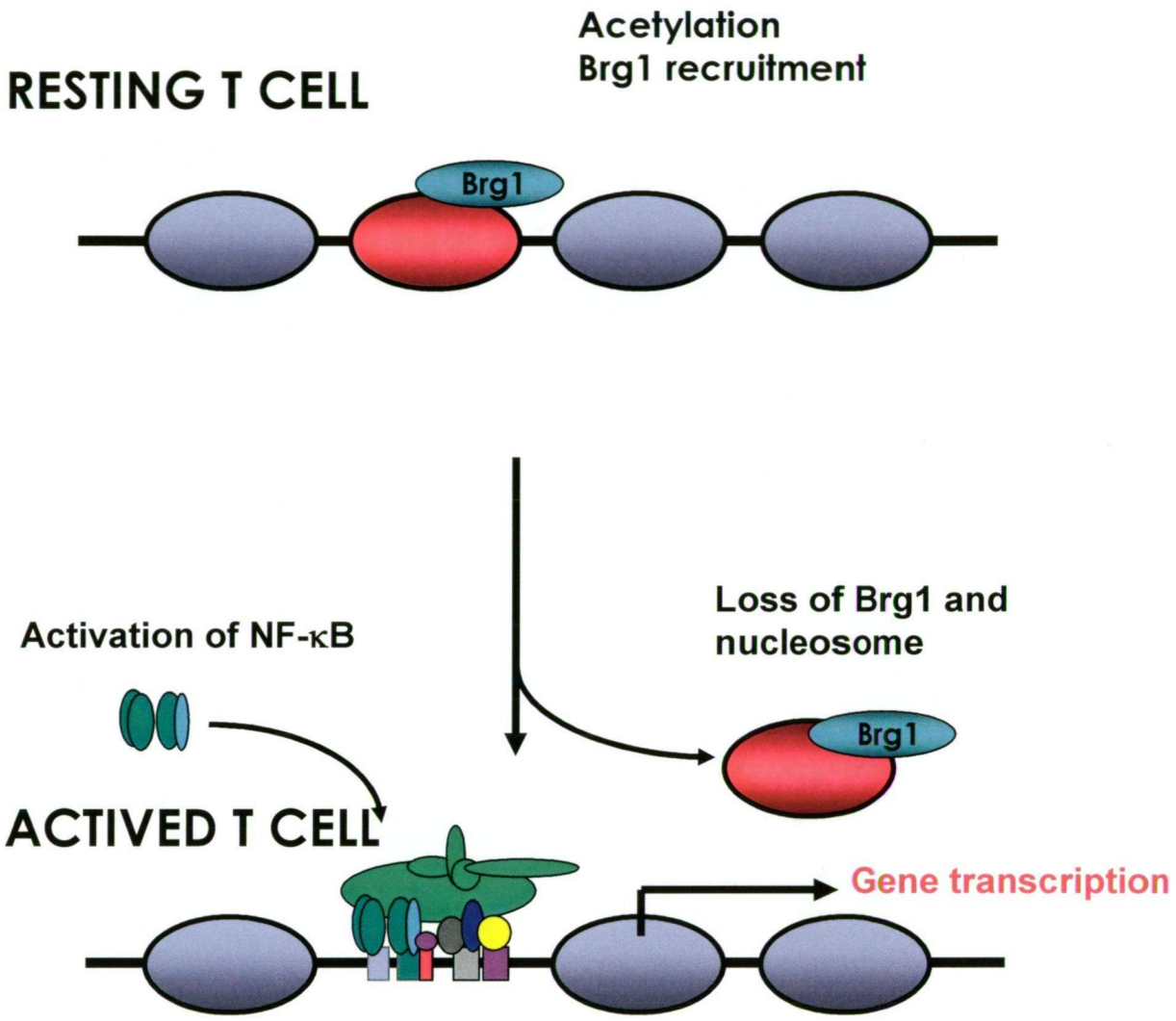



**Figure 6.1. Model for activation of the GM-CSF gene.** Schematic representation of the GM-CSF promoter in the resting and activated state.

in remodelling the GM-CSF enhancer and the precise order of transcription factor recruitment events at the promoter would help to further clarify this model.

In order to determine whether this mechanism is common to immune genes microarray analysis of Brg1 defective T cells was used to identify Brg1 targets. Transcripts from resting and activated T cells expressing a Brg1 mutant construct were analyzed following hybridization to Affymetrix mouse genome arrays. An extremely defined question was asked in order to identify immune genes which were activated in a Brg1 dependent manner similar to GM-CSF. The cytokines IFN-γ, IL-4 and IL-5 were identified as potential Brg1 targets and chosen for futher analysis as these genes share a similar role in immune regulation as GM-CSF. It is important to note that only a small portion of the microarray data was used in this study and therefore these data could be further analyzed to consider the role of Brg1 in other aspects of T cell activation. Analysis of the activation and remodelling profiles for each of the candidate genes demonstrated that while IL-4 and IFN-γ shared similar activation profiles to GM-CSF, the IL-5 gene was relatively delayed in its activation and promoter remodelling. Brg1 recruitment was found to be different at each of these promoters. Brg1 was bound to the IFN-γ promoter in resting T cells and lost following T cell activation. In contrast, recruitment of Brg1 to the IL-4 promoter required T cell activating signals. Brg1 was not detected at the IL-5 promoter in resting T cells or those which had been activated for 4 hours and therefore could not be confirmed as a direct Brg1 target. Future work should define Brg1 recruitment over an extended time course in order to determine whether Brg1 is recruited to the IL-4 promoter rapidly and the IL-5 promoter in a more delayed manner.

IL-4 and IFN-γ have similar activation and remodelling profiles and ChIP demonstrated they are direct targets of Brg1. The constitutive recruitment of Brg1 to the IFN-γ and GM-CSF promoters suggests they may share a common model of activation. Perhaps IFN-γ is poised in a similar manner to GM-CSF with increased levels of basal acetylation acting as a docking site for Brg1 recruitment. Following T cell activation, the nucleosome covering the IFN-γ promoter is rapidly remodelled, allowing the transcriptional machinery to assemble at a much faster rate. The delayed recruitment of Brg1 to the IL-4 promoter is consistent with a number of published reports detailing a more traditional model of transcription factor mediated recruitment of remodelling activities

(Agalioti *et al*, 2000; de la Serna *et al*, 2005). In the case of IL-4, the promoter is relatively accessible in the basal state perhaps with slightly increased levels of histone acetylation. T cell activation signals are then required to trigger relatively rapid Brg1 binding. Brg1 can then further increase accessibility to the IL-4 promoter and enhance binding of the transcriptional machinery. The IL-5 gene is activated with much more delayed kinetics and this may be attributed to the requirement for histone acetylation and Brg1 recruitment before chromatin remodelling can occur.

It is possible that both the IFN-γ and IL-4 promoters have slightly increased levels of histone acetylation to enhance Brg1 recruitment. During Th1 differentiation the IFN-γ locus undergoes extensive H3 and H4 acetylation (Morinobu *et al*, 2004) and it is therefore possible that the constitutive recruitment of Brg1 is via histone acetylation in Th1 cells. Meanwhile, the IL-4 promoter is associated with acetylated H3 in Th2 cells (Grogan *et al*, 2003) which could serve to mark the gene for rapid Brg1 recruitment following T cell activation.

The mechanisms involved in remodelling cytokine promoters to facilitate gene activation are known for only a small number of genes, however a common theme appears to be emerging. At the IFN-β promoter, NF-κB is bound first, within 2 hours of viral infection, followed by recruitment of the HAT, GCN5 at 5 hours. Brg1 is then recruited at 6 hours, at which point mRNA levels increase (Agalioti *et al*, 2000). The data presented in this thesis suggests that there may be a unifying theme in cytokine gene regulation. While each gene is activated in a very specific, tightly co-ordinated manner, there appear to be some common features. The delay in IL-5 activation may be attributed to delays in histone acetylation and Brg1 recruitment. Thus, transcription following T cell activation is slower, taking up to 24 hours for mRNA to accumulate. IFN-β is potentially a few steps further along the activation profile, with acetylation and Brg1 recruitment occurring more rapidly to ensure mRNA accumulates within 6 hours of viral infection (Agalioti *et al*, 2000). GM-CSF and IFN-γ activate even more rapidly, with increased transcription detected within less than an hour. This faster activation profile could be attributed to promoter priming, with increased levels of basal histone acetylation and Brg1 recruitment. Even faster activation is observed at the IL-1β gene in macrophages, with transcription increasing within a matter of minutes of activation with LPS and peaking within three to four hours (Liang *et al*, 2006).

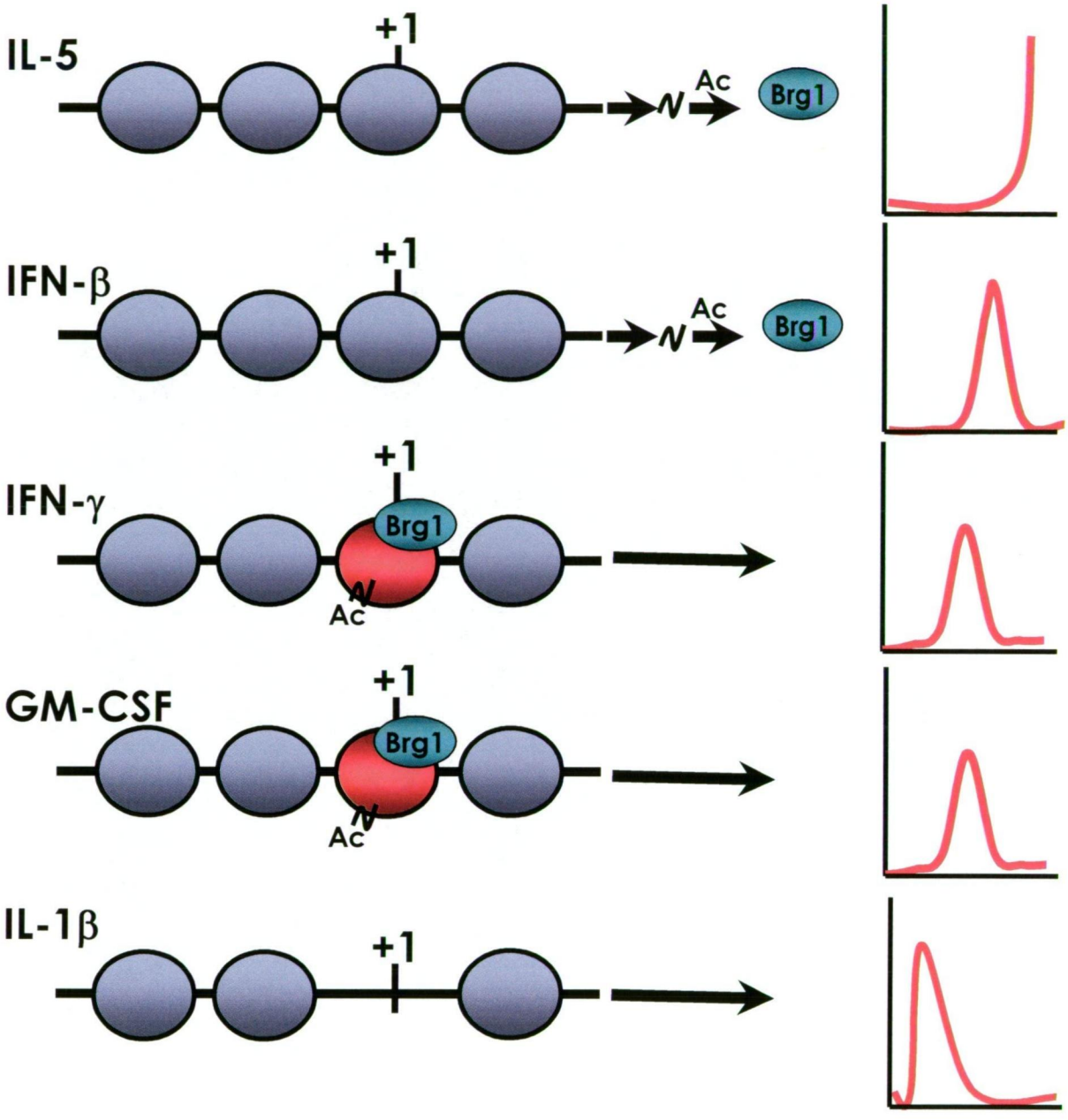



**Figure 6.2. An emerging theme for the activation of cytokine genes.** Schematic representation of the IL-5, IFN-β, IFN-γ, GM-CSF and IL-1β promoters, their remodelling requirements and transcriptional profiles showing the role of histone acetylation and Brg1 in activation.

The IL-1β promoter is nucleosome free in the basal state with high levels of accessibility. This means that the only rate limiting step in transcription in this case is the assembly of the transcriptional machinery (Figure 6.2).

The role of Brg1 in the activation of early versus delayed response immune genes is interesting to consider. A clear example of a role for Brg1 in activating immune genes is provided by studies on the IL-12b promoter. Brg1 is required for IL-12b promoter and enhancer remodelling as depletion of Brg1 by siRNA in macrophages inhibits LPS induced increases in accessibility (Ramirez-Carrozzi *et al*, 2006). Similar to GM-CSF, IL-12b mRNA accumulates approximately 2 hours post stimulation, however unlike GM-CSF, Brg1 is recruited to the IL-12b promoter following LPS stimulation of macrophages. The loss of Brg1 did not impact on expression of early primary response genes (activated within minutes of stimulation) however while Brg1 was found to be recruited to late primary response and secondary response genes in an LPS induced manner, Brg1 was actually found to be constitutively associated with the early primary response promoters. This suggests that Brg1 may establish or maintain a poised chromatin environment for rapidly activated genes in macrophages (Ramirez-Carrozzi *et al*, 2006).

The link between Brg1 and DNA methylation in priming genes for activation is also interesting to consider. Banine *et al* (2005) found that the restoration of Brg1 to deficient cells lead to demethylation of target promoters. Aberrant DNA methylation has long been established as a feature of cancerous cells (reviewed in Baylin, 2005) and therefore the loss of Brg1 and its role in cancer requires further study. Indeed, a number of published reports have revealed that Brg1 is mutated in cancerous cell lines (Wong *et al*, 2000; Reisman *et al*, 2002) and primary tumors (Medina *et al*, 2004). Therefore, in terms of the clinical implications of this work, it will be important to screen myeloid leukemias in which GM-CSF is aberrantly expressed for mutations in Brg1 and investigate how Brg1 impacts the methylation status of the GM-CSF gene. Investigating changes in the methylation status of the GM-CSF gene in cells transfected with the Brg1 mutant could provide further insight into the mechanisms involved in GM-CSF gene activation. Indeed, the GM-CSF promoter has found to be methylated in Brg1 deficient C33A cells and is demethylated in expressing EL-4 T cells (Sprod and Holloway, personal communication).

By defining the molecular events in GM-CSF gene activation, some insight has

been gained into the mechanisms operating to activate immune genes in a chromatin context. Highly ordered and specific signals and recruitment events are required to drive remodelling and transcription of the GM-CSF gene. Most importantly, all these features contribute to the most important aspect of GM-CSF induction, its rapid and transient nature. In addition, from these data, together with studies emerging in the literature, common themes underlying activation of immune genes are beginning to emerge.

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# APPENDIX A

***Combined gene list for replicate 1 and 2 for genes with reduced activation in Brg1 mutant expressing cells.***

| Gene Name | Description |
|---|---|
| 1445937_at | Transcribed sequences |
| 1446082_at | 15 days embryo head cDNA, RIKEN full-length enriched library, clone:D930048E06 product:unknown EST, full insert sequence |
| 1445521_at | ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R) |
| 1446430_at | RIKEN cDNA A630025O09 gene |
| 1445097_at | L0210E07-3 NIA Mouse Newborn Ovary cDNA Library Mus musculus cDNA clone L0210E07 3', mRNA sequence. |
| 1448039_at | uj04e02.x1 Sugano mouse liver mlia Mus musculus cDNA clone IMAGE:1890938 3' similar to gb:M29281 Mouse complement receptor (MOUSE);, mRNA sequence. |
| 1449235_at | tumor necrosis factor (ligand) superfamily, member 6 |
| 1449298_a_at | phosphodiesterase 1A, calmodulin-dependent |
| 1447087_at | Transcribed sequences |
| 1447135_at | Transcribed sequences |
| 1447041_at | BB124417 RIKEN full-length enriched, adult male urinary bladder Mus musculus cDNA clone 9530097L15 3', mRNA sequence. |
| 1447647_at | wingless-related MMTV integration site 7A |
| 1447374_at | 0 day neonate lung cDNA, RIKEN full-length enriched library, clone:E030033D05 product:weakly similar to DNA-BINDING PROTEIN [Rattus norvegicus], full insert sequence |
| 1441298_at | spectrin beta 3 |
| 1441044_at | 16 days embryo head cDNA, RIKEN full-length enriched library, clone:C130002M15 product:unknown EST, full insert sequence |
| 1442178_at | Transcribed sequences |
| 1441662_at | cytochrome P450, family 4, subfamily x, polypeptide 1 |
| 1440716_at | Adult male spinal cord cDNA, RIKEN full-length enriched library, clone:A330051C14 product:unknown EST, full insert sequence |
| 1440893_at | RIO kinase 1 (yeast) |
| 1441738_at | C0502C08-3 NIA Mouse E13.5 VMB Dopamine cell cDNA Library Mus musculus cDNA clone C0502C08 3', mRNA sequence. |
| 1440742_at | Transcribed sequences |
| 1440558_at | ATPase, class II, type 9B |
| 1443474_at | Transcribed sequences |
| 1443919_at | RIKEN cDNA B230206N24 gene |

| Gene Name | Description |
|---|---|
| 1442550_at | Transcribed sequences |
| 1442600_at | 0 day neonate cerebellum cDNA, RIKEN full-length enriched library, clone:C230008G15 product:unknown EST, full insert sequence |
| 1457423_at | Transcribed sequence with moderate similarity to protein pdb:1LBG (E. coli) B Chain B, Lactose Operon Repressor Bound To 21-Base Pair Symmetric Operator Dna, Alpha Carbons Only |
| 1457608_at | BB236014 RIKEN full-length enriched, 3 days neonate thymus Mus musculus cDNA clone A630056O03 3', mRNA sequence. |
| 1456847_at | Transcribed sequence with strong similarity to protein sp:P00722 (E. coli) BGAL_ECOLI Beta-galactosidase |
| 1456564_at | RIKEN cDNA C030046I01 gene |
| 1459447_at | Transcribed sequences |
| 1460271_at | triggering receptor expressed on myeloid cells 3 |
| 1460088_at | AV209518 RIKEN full-length enriched, adult male testis Mus musculus cDNA clone 1700120A01 3', mRNA sequence. |
| 1460104_at | vacuolar protein sorting 4b (yeast) |
| 1458025_at | Transcribed sequences |
| 1452309_at | RIKEN cDNA 4933421H10 gene |
| 1451837_at | adaptor-related protein complex 3, beta 2 subunit |
| 1452028_a_at | cadherin 23 (otocadherin) |
| 1449864_at | interleukin 4 |
| 1449903_at | cytotoxic and regulatory T cell molecule |
| 1449900_at | 601776377F1 NCI_CGAP_Lu29 Mus musculus cDNA clone IMAGE:4017906 5', mRNA sequence. |
| 1449988_at | immunity-associated protein |
| 1449652_at | AV318995 RIKEN full-length enriched, 13 days embryo male testis Mus musculus cDNA clone 6030403F05 3', mRNA sequence. |
| 1450716_at | a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 |
| 1450869_at | fibroblast growth factor 1 |
| 1450829_at | tumor necrosis factor, alpha-induced protein 3 |
| 1450375_at | persephin |
| 1455186_a_at | RIKEN cDNA 1190003J15 gene |
| 1453075_at | RIKEN cDNA 1600012P17 gene |
| 1453210_at | Mus musculus 8 days embryo whole body cDNA, RIKEN full-length enriched library, clone:5730507C01 product:similar to ZINC FINGER PROTEIN 125 (FRAGMENT) [Mus musculus], full insert sequence. |
| 1453874_at | RIKEN cDNA 4933401B06 gene |
| 1453437_at | RIKEN cDNA A230020K05 gene |
| 1425071_s_at | neurotrophic tyrosine kinase, receptor, type 3 |
| 1424376_at | CDC42 effector protein (Rho GTPase binding) 1 |

| Gene Name | Description |
|---|---|
| 1424647_at | gamma-aminobutyric acid (GABA-A) receptor, pi |
| 1425569_a_at | signaling lymphocytic activation molecule family member 1 |
| 1425471_x_at | Mus musculus cDNA clone MGC:6439 IMAGE:3601769, complete cds. |
| 1425720_at | CDNA clone MGC:12025 IMAGE:3603243, complete cds |
| 1425225_at | Fc receptor-like 3 |
| 1423061_at | armadillo repeat gene deleted in velo-cardio-facial syndrome |
| 1422397_a_at | interleukin 15 receptor, alpha chain |
| 1427828_at | Mus musculus murine retrovirus readthrough RNA sequence. |
| 1426632_at | Similar to hypothetical protein MGC2376 (LOC233529), mRNA |
| 1425947_at | interferon gamma |
| 1426180_a_at | submaxillary gland androgen regulated protein 2 |
| 1426176_a_at | prokineticin 2 |
| 1426071_at | progressive ankylosis |
| 1419431_at | epiregulin |
| 1418900_at | RIKEN cDNA 1810045K17 gene |
| 1418018_at | carboxypeptidase D |
| 1417496_at | BB332449 RIKEN full-length enriched, 6 days neonate medulla oblongata Mus musculus cDNA clone B730048G11 3', mRNA sequence. |
| 1419651_at | RIKEN cDNA 2610200G18 gene |
| 1419628_at | C. elegans ceh-10 homeo domain containing homolog |
| 1420593_a_at | TEA domain family member 3 |
| 1420888_at | Bcl2-like |
| 1420887_a_at | Bcl2-like |
| 1420700_s_at | folate receptor 4 (delta) |
| 1420532_at | activin receptor interacting protein 1 |
| 1435523_s_at | 11 days embryo head cDNA, RIKEN full-length enriched library, clone:6230415F21 product:unknown EST, full insert sequence |
| 1435508_x_at | RIKEN cDNA 0610009D07 gene |
| 1439043_at | RIKEN cDNA 1500010G04 gene |
| 1438927_x_at | Similar to 60S ribosomal protein L23a (LOC270584), mRNA |
| 1439110_at | RIKEN cDNA A930012O16 gene |
| 1439816_at | Transcribed sequences |
| 1439485_at | RIKEN cDNA 4932417D18 gene |
| 1437937_at | chemokine binding protein 2 |
| 1438570_at | Transcribed sequences |
| 1437360_at | RIKEN cDNA B530002L05 gene |
| 1430070_at | RIKEN cDNA 1500035N22 gene |
| 1430843_at | RIKEN cDNA 1110027M19 gene |
| 1428943_at | RIKEN cDNA 4933433B15 gene |
| 1429657_at | UI-M-BH1-anr-b-02-0-UI.s1 NIH_BMAP_M_S2 Mus musculus cDNA clone UI-M-BH1-anr-b-02-0-UI 3', mRNA sequence. |
| 1433573_x_at | protease, serine, 2 |

| Gene Name | Description |
|---|---|
| 1432971_at | Adult male testis cDNA, RIKEN full-length enriched library, clone:4921518B13 product:unclassifiable, full insert sequence |
| 1432891_at | dehydrogenase/reductase (SDR family) member 7 |
| 1432888_at | Adult male testis cDNA, RIKEN full-length enriched library, clone:4930455M05 product:unclassifiable, full insert sequence |
| 1432864_at | 10 days lactation, adult female mammary gland cDNA, RIKEN full-length enriched library, clone:D730050C22 product:unclassifiable, full insert sequence |
| 1432959_at | Adult male testis cDNA, RIKEN full-length enriched library, clone:4930447I22 product:unknown EST, full insert sequence |
| 1431626_at | Adult male testis cDNA, RIKEN full-length enriched library, clone:4933429K18 product:unclassifiable, full insert sequence |
| 1432795_at | Adult male tongue cDNA, RIKEN full-length enriched library, clone:2310058F05 product:unclassifiable, full insert sequence |
| 1432155_at | Wiskott-Aldrich syndrome-like (human) |