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**Analysis of the Promoter Region of the Interleukin-2 Receptor Alpha Chain
(CD25) Gene in Human B Lymphocytes**

by

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Philosophy, November 1999.

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Abstract

In T and B cells, attenuation of an undefined negative regulatory element binding protein (NRE-BP) binding to a negative regulatory element (NRE) removes the suppression of transcriptional activity upon the promoter of a unique 55kDa IL-2 receptor subunit, CD25. This event leads to the expression of CD25 at the cell surface. In human B cells IL-4, a potent activator of CD25 transcription abolishes NRE binding activity via a cyclic adenosine monophosphate (cAMP)/ cAMP-dependent kinase (PKA)-sensitive pathway. Studies of the IL-4-regulated cAMP/PKA pathway showed that the catalytic subunit of PKA (PKAc) did not have a direct effect on NRE-BP *in vitro*, suggesting that PKAc has an indirect involvement in the loss of NRE binding activity. Studies with the intracellular signalling modulators Wortmannin and Rapamycin revealed that IL-4 does not attenuate NRE binding activity via PI-3 kinase signalling. Electrophoretic mobility shift assay (EMSA) analysis showed that IL-4 does not directly affect NF- κ B activity.

The attenuating effect of IL-4 on NRE binding activity was also observed for protein binding to the entire NRR_I region. Signalling through the IL-13 receptor, which shares the IL-4R α subunit with IL-4R, failed to attenuate NRR_I binding activity suggesting that IL-4 causes a loss of protein binding to NRR_I through the signalling generated by the γ c subunit of the IL-4R complex. EMSA studies using consensus and mutant NRR_I oligonucleotides showed that NRE is the dominant site for protein binding within NRR_I, although the consensus AP-1 site was required for maximum binding. Supershift experiments showed that the transcription factors YY1 and Ets were unlikely to be NRE-BP and that CBP/p300 is unlikely to form a bridging complex over proteins binding to NRR_I. Western blot and EMSA experiments characterised the putative AP-1 binding site as a *bona fide* AP-1 binding site; an antibody against the AP-1-family protein c-fos supershifted one protein species binding to radiolabelled CD25-NRR_I.

Transient-transfection of P3HR1 B cells with NRR_I deletion mutants confirmed that NRE is the dominant suppressive region of NRR_I. Lymphoid cells transfected with CD25 STAT6 deletion mutant constructs showed an increase in reporter activity following treatment with phorbol myristate acetate (PMA). This finding is the first demonstration that PRR_{II} is both functional and mitogen-sensitive in B cells. IL-4-sensitivity was assessed in lymphoid cells transfected with STAT6 CD25-CAT reporter constructs. These studies demonstrated that a putative STAT6 site overlapping NRR_{II} was sensitive to IL-4. The remaining sites were insensitive to IL-4, suggesting they are not functional STAT6 sites. This finding suggests that a second IL-4 signalling mechanism, activation of the JAK/STAT pathway, may be involved in the upregulation of CD25 expression.

In summary, IL-4 up-regulates the expression of CD25 in human B cells through a cAMP/PKA-sensitive pathway, which attenuates protein binding to NRR_I and may also activate the JAK/STAT pathway. NRE binding activity is unaffected by direct action of PKAc, although this kinase is likely to exert an indirect effect. NRE is the dominant protein binding and functional region of NRR_I, and supershift experiments characterised the retinoid sensitive region as an AP-1 binding site. The identity of NRE-BP candidates remains unknown and supershift experiments exclude YY1 and Ets as NRE-BP and show that CBP/p300 does not form a bridging complex linking NRE-BP and AP-1. Studies with deletion mutants of putative CD25 STAT6 sites showed for the first time in human B cells, that PRR_{II} is responsive to mitogenic stimulation and identified a STAT6 site close to NRR_{II} as IL-4 sensitive.

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This thesis is dedicated to Leigh Wallace who has believed in me and put up with me for longer than she would like to remember!

How do ye walk. Well ye put one foot in front of the other and fall very slowly, very slowly, just that one foot and then the next yin, just very slowly, ye catch up with yerself, that's the boy. Ye get going.'

James Kelman. *How late it was, how late.*

'Seven times down, eight times up.'

Anon

"What are the chances of that happening!"

Harry Hill

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ABBREVIATIONS

| | |
|-------|------------------------------------|
| Ab | Monoclonal Antibody |
| Ag | Antigen |
| AC | Adenylate Cyclase |
| AET | 2-Aminoethylisothiuronium Bromide |
| AFC | Antigen Forming Cell |
| AKAP | A-Kinase Anchoring Protein |
| AP-1 | Activator Protein-1 |
| ATF-1 | Activating Transcription Factor-1 |
| BCA | Bicinchonic Acid |
| BCGF | B Cell Growth Factor |
| BCL-6 | B Cell Lymphoma/Leukaemia-6 |
| BCR | B Cell Antigen Receptor Complex |
| bHLH | Binding Helix-Loop-Helix |
| BiP | Binding Protein |
| BLNK | B Cell Linker Protein |
| bp | Base Pair |
| BSA | Bovine Serum Albumin |
| BSAP | B Cell Specific Activator Protein |
| Btk | Brutons Tyrosine Kinase |
| bZIP | Basic Region Leucine Zipper |
| c | Catalytic |
| cAMP | Cyclic Adenosine Monophosphate |
| CAT | Chloramphenicol Acetyl Transferase |
| CBP | CREB Binding Protein |
| CD | Cluster of Differentiation |
| CD23 | Low Affinity Receptor for IgE |
| CD25 | IL-2 Receptor α Chain |

| | |
|--------|--|
| CHO | Chinese Hamster Ovary |
| CIS | Cytokine-Inducible SH2 Domain Containing Protein |
| CKb-11 | Chemokine b-11 |
| ConA | Concanavalin A |
| CRE | cAMP Response Element |
| CREB | cAMP Response Element Binding Protein |
| CREM | cAMP Response Element Modulator |
| CSF | Colony Stimulating Factor |
| CVID | Common Variable Immunodeficiency |
| D | Diversity Region |
| DAG | Diacylglycerol |
| DNA | Deoxyribonucleic Acid |
| DTT | Dithiothreitol |
| EBF | Early B Cell Factor |
| ECC | Enhanced Chemiluminescence |
| EDTA | Ethylenediaminetetra-acetic Acid |
| EMSA | Electrophoretic Mobility Shift Assay |
| ER | Endoplasmic Reticulum |
| ERK | Extracellular Signal-Related Kinase |
| FACS | Fluorescence Activated Cell Sorter |
| FcR | Fc Receptor |
| FCS | Foetal Calf Serum |
| FDC | Follicular Dendritic Cell |
| FITC | Fluorescein Isothiocyanate |
| 4PS | IL-4 Phosphorylation Substrate |
| g | Force of Gravity |
| G | GTP-Binding |
| GAP | GTPase Activating Protein |
| GAS | Gamma Interferon Activation Site |

| | |
|-----------------|--|
| γ c | Common γ Subunit |
| GDP | Guanosine Diphosphate |
| GFP | Green Fluorescence Protein |
| Gi | GTP-Binding Inhibitory Subunit |
| Gs | GTP-Binding Stimulatory Subunit |
| GSK-3 | Glycogen Synthase Kinase-3 |
| GTP | Guanosine Triphosphate |
| H | Heavy Chain |
| HCl | Hydrochloric Acid |
| HIV | Human Immunodeficiency Virus |
| HMG-1(Y) | High Mobility Group Protein-1(Y) |
| HRP | Horse Radish Peroxidase |
| HTLV | Human T-Cell Leukemia Virus |
| IDC | Interdigitating Cells |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IGF | Insulin-Like Growth Factor |
| I κ B | Inhibitor- κ B |
| I κ K | Inhibitor- κ B-Kinase |
| IL | Interleukin |
| IL-2R | IL-2 Receptor |
| IL-4R | IL-4 Receptor |
| IL-13R | IL-13 Receptor |
| IP ₃ | Inositol 1,4,5-Trisphosphate |
| IRS | Insulin Receptor Substrate |
| ITAM | Immunoreceptor Tyrosine-Based Activation Motif |
| ITIM | Immunoregulatory Tyrosine Inhibitory Motif |
| J | Joining Region |
| JAB | JAK Binding Protein |

| | |
|----------------|--|
| JAK | Janus Kinase |
| JNK | c-jun N-Terminal Kinase |
| KID | Kinase-Inducible Domain |
| Kd | Dissociation Constant |
| kDa | Kilodalton |
| L | Light Chain (of antibody) |
| LARC | Liver and Activation Regulated Chemokine |
| LGL | Large Granular Lymphocyte |
| LTR | Long Terminal Repeat |
| M | Mutant |
| MAPK | Mitogen Activated Protein Kinase |
| MAPKK | MAPK Kinase |
| MCIF | Macrophage Colony Inhibitory Factor |
| MCSF | Macrophage-Colony Stimulatory Factor |
| MDC | Macrophage Derived Cytokine |
| MHC | Major Histocompatibility Complex |
| mIg | Membrane Ig |
| MIRR | Multichain Immune Recognition Receptor |
| mRNA | Messenger RNA |
| N | Nucleotide Region |
| NF- κ B | Nuclear Factor- κ B |
| NF-AT | Nuclear Factor |
| NK | Natural Killer |
| NIK | NF- κ B-Inducing Kinase |
| NRE | Negative Regulatory Element |
| NRE-BP | NRE-Binding Protein |
| NRR | Negative Regulatory Region |
| OBF-1 | Oct Binding Factor-1 |
| Oct | Octamer |

| | |
|------------------|--|
| OD | Optical Density |
| PAGE | Polyacrylamide Gel Electrophoresis |
| PALS | Periarteriolar Lymphoid Sheath |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PDE | Phosphodiesterase |
| p85 | 85kDa Subunit of PI-3 Kinase |
| PH | Pleckstrin Homology |
| PIAS | Protein Inhibitor of Activated STAT |
| PIP | Phosphatidylinositol 3-Phosphatase |
| PIP ₂ | PI 3, 4/4, 5 P |
| PIP ₃ | PI 3, 4, 5 P |
| PIR-B | Paired Ig-Type Receptor B |
| PI-3 Kinase | Phosphatidylinositol-3 Kinase |
| PKA | Protein Kinase A/cAMP-Dependent Kinase |
| PKB | Protein Kinase B |
| PKC | Protein Kinase C |
| PKI | PKA Inhibitor |
| PLC γ | PI Lipid-Specific Phospholipase γ |
| PMA | Phorbol Myristate Acetate |
| p110 | 110kDa Subunit of PI-3 Kinase |
| PTP | Phosphotyrosine Phosphatase |
| PTK | Protein Tyrosine Kinase |
| PRR | Positive Regulatory Region |
| R | Regulatory |
| RA | Retinoic Acid |
| RACKS | Receptors for Activated C-Kinases |
| RAG | Recombinase Activating Gene |
| RARE | Retinoic Acid Response Element |

| | |
|--------|--|
| rE | Response Element |
| RPE | R-Phycoerythrin |
| RSK | Ribosomal S6 Kinase |
| SAPK | Stress Activated Protein Kinase |
| SDS | Sodium Dodecyl Sulphate |
| sCD23 | Soluble CD23 |
| SDF-1 | Stromal Cell Derived Factor-1 |
| SEAP | Secreted Alkaline Phosphatase |
| SHIP | SH2-Containing Inositol-5-Phosphatase |
| SHP | SH2-Containing Phosphatase |
| SH2 | Src-Homology 2 Domain |
| SLC | Surrogate Light Chain |
| SLP-65 | SH2 Domain Containing Leukocyte Protein of 65kDa |
| SOCS | Suppressor of Cytokine Signalling |
| Sos | Son of Sevenless |
| SRBC | Sheep Red Blood Cells |
| src | Sarcoma |
| SRF | Serum Response Factor |
| SSI | STAT-Induced STAT Inhibitor |
| STAT | Signal Transducer and Activator of Transcription |
| SV-40 | Simian Virus-40 |
| TCR | T Cell Antigen Receptor Complex |
| TECK | Thymus-Expressed Cytokine |
| TdT | Terminal Deoxynucleotide Transferase |
| TH1 | T-Helper Cell |
| TK | Thymidine Kinase |
| TLC | Thin Layer Chromatography |
| TNF | Tumour Necrosis Factor |
| TRAF | TNF Receptor Associated Factor |

| | |
|--------|---|
| Tris | Tris(hydroxymethyl)methylamine |
| UV | Ultra Violet |
| V | Variable Region |
| VCAM | Vascular Cell Adhesion Molecule |
| Xid | X-Linked Immunodeficiency |
| XLA | X-Linked Agammaglobulinemia |
| X-SCID | X-Linked Severe Combined Immunodeficiency |

CHAPTER ONE
INTRODUCTION

1.1 B-LYMPHOCYTE DEVELOPMENT

B lymphocytes, in common with all haematopoietic cells, are derived from pluripotent stem cells which are produced in the bone marrow (Kee and Paige, 1995). Pluripotent stem cells give rise to all cells of the immune system and such cells are classed as leukocytes. Leukocytes can be categorised into B lymphocytes and T lymphocytes, as well as the broadly classed polymorphonuclear phagocytes and auxiliary cells (including platelets and mast cells). B lymphopoiesis is a fundamental event in the functioning of the immune system, and the ultimate products, namely high affinity antibody secreting plasma cells, form the main part of an immediate immune response.

In both human and murine bone marrow, B lymphocyte differentiation has two main stages of development, the antigen-independent stage and the antigen-dependent stage. B cell development can be further divided into a succession of immunoglobulin (Ig) gene rearrangements (Hardy et al., 1991; Melchers et al., 1995) or the ordered expression of B cell-related markers (Osmond et al., 1994), culminating in the formation and expression of IgM. B lymphopoiesis takes a very similar course in both mouse and human bone marrow and efforts have been made to clarify development in both species, and to integrate the different models which describe B cell development (Ghia et al., 1998; LeBien, 1998; Osmond et al., 1998).

1.1.1 Antigen-Independent B Cell Development

The antigen-independent stage is a developmental stage which occurs in foetal liver and in human and murine bone marrow. Differentiation through pro- and pre-B lymphocytes provides the B cell with a functional cell surface receptor for antigen and early B cell development is dependent on growth factors derived from stromal cells, such as IL-7 (van Freeden-Jeffry, 1995). B cell development was initially characterised using A-MuLV transformed cell lines which demonstrated an ordered Ig heavy (H) chain

rearrangement of three elements, variable (V), diversity (D) and joining (J) gene regions (Alt et al., 1984). The heavy chain μ is expressed following successful rearrangement of Ig heavy chain D and J regions at the pro-B cell stage, followed by the pre-B cell step of allelic exclusion, which is achieved by successful $V_H(D)J_H$ rearrangement. Allelic exclusion was first identified by work in Balb/c mice embryos which demonstrated that both homologous chromosomes expressing $V_H(D)J_H$ form complete gene regions, although only one of two Ig H chains is expressed, the other failing to complete successful gene rearrangements (Hozumi and Tonegawa, 1976). This is possibly a result of the random nature of H chain rearrangement, making it highly unlikely that both alleles would simultaneously make a productive $V_H(D)J_H$ rearrangement. In addition, the successful rearrangement of one allele may inhibit further $V_H(D)J_H$ rearrangements at the other allele. Recent evidence has suggested that developing B cells which make a productive $V_H(D)J_H$ rearrangement in the first instance will quickly move into the next stage of development, preventing any further $V_H(D)J_H$ rearrangement at the other allele (Chang et al, 1999).

B cell differentiation was first described in adult mouse bone marrow tissue (Hardy et al., 1991). Different cell surface markers are expressed on developing B cells in mouse and human although there are also some similarities (Gbia et al., 1998). The phosphotyrosine phosphatase B220, an isoform of CD45, is found on the pro-B cell surface, as are CD43 and CD19, at an early stage of development. Terminal deoxynucleotide transferase (TdT), is found in the nucleus at this stage. TdT is an intranuclear enzyme which is expressed during H chain V region gene rearrangement and works in tandem with recombinase activating gene (RAG) 1 and RAG 2 to regulate an ordered sequence of gene rearrangements at the Ig loci. Developing B cells gradually decrease TdT and CD43 expression while increasing B220. The differentiating pro-B cell becomes a pre-B-I cell, which initiates H-chain rearrangement by binding a D gene, the TdT-formed extra nucleotide (N) region and a J region. In forming a DNJ_H gene; the developing B cell enters the pre-B-II stage and increases in size. A V region gene is then

joined to form a $V_{H}N-DNJ_{H}$ gene which is capable of producing a μ -H chain. In the endoplasmic reticulum (ER) in some pre-BI and in most large pre-BII cells, $V_{pre-B(1/2)}$ and $\lambda 5$ (murine) or $\lambda 5/14.1$ (human) are expressed. These Ig like proteins are able to bind together via an extra β strand located in the V_{pre-B} chain to form a surrogate light chain (SLC) (Minegishi et al, 1999). In mice, the $\lambda 5$ chain is encoded by one gene, whereas the V_{pre-B} chain is encoded by two genes. Mutational studies of V_{pre-B1} homozygous knockout mice show that either isoform of the V_{pre-B} chain can interact with $\lambda 5$ to form the SLC in B cell development (Martensson et al., 1999). Once formed, the SLC binds to the newly-formed μ -H chain, displaces the ER chaperone binding protein BiP from the heavy chain and forms a complex in association with a signal transducing heterodimer consisting of two $Ig\alpha$ and $Ig\beta$ subunits, CD79a and CD79b. The complex leaves the ER with an $\alpha\beta$ heterodimer flanking each side of the SLC-heavy chain arrangement and translocates to the cell surface where it is expressed as a pre-B cell receptor (BCR). In some pro-B or pre-BI cells, surrogate light chains translocate to the cell surface without μ -H chains and interact with different molecular weight glycoproteins to form complexes which may function as receptors. An example of this is their expression on the surface of pro-B cells in association with the integral membrane protein, calnexin (Nagata et al., 1997). The functions of this and other such receptor complexes have yet to be determined (Karasuyama et al, 1993; Sanz and de la Hera, 1996).

The role of the pre-BCR is not clearly defined, however expression at the cell surface enables the B cell to enter the cell cycle, expand and divide. Failure of pre-BCR expression can block further development of B cells. This can be observed partially in $\lambda 5^{-/-}$ mice and completely in common variable immunodeficiency (CVID) in humans (LeBien, 1998). The next step is driven by the transition of large mitotic pre-BII cells to small resting pre-B-II cells (Figure 1.1). This involves the rearrangement of κ L-chain genes which in most cells will lead to the production of a κ chain. Those which have non-productive rearrangements, may go on to rearrange and produce λ L-chains. When

a cell has formed a complete in-phase κ or λ gene it can express an L-chain which can associate with an H chain and the $Ig\alpha/Ig\beta$ heterodimer complex and be processed to the cell surface as a complete BCR called surface immunoglobulin (sIg)-M (Figure 1.1) (Melchers et al., 1995).

1.1.2 Receptor Editing Events

Emerging B cells are subject to challenge by autoantigens which can activate the BCR. The fate of B cells at this stage is determined by positive or negative selection. Positive selection occurs when the BCR is altered by receptor editing, is no longer receptive to autoantigen challenge and the B cell is able to continue its development. Successful receptor editing is achieved by establishing tolerance to autoantigens through the upregulation of RAG1 and RAG2 expression, leading to the induction of secondary L-chain rearrangements and the replacement of the autoreactive BCR. By altering the antigen binding regions of IgM, this process enables immature B cells to avoid reacting with autoantigens. Due to the short life span of the immature B cell, if the IgM fails to lose specificity to autoantigen binding over a certain time, the B cell will undergo negative selection and die through apoptosis. A recent study on transgenic mice which expressed green fluorescent protein (GFP) under the regulation of RAG measured the expression of RAG throughout B cell development and reported GFP expression in all immature B cells in the bone marrow and spleen. It was proposed from this study that immature B cells which do not undergo autoantigen challenge decrease RAG levels and accelerate immediate B cell development to the stage of high IgM expression. B cells which are engaged by autoantigen arrest cell development at a stage where IgM levels are low and RAG levels are high, to increase editing efficiency. In addition, low levels of autoantigen challenge may stimulate editing without increasing RAG expression by slowing development from the RAG-expressing immature B cell in the bone marrow to the mature B cell in the spleen which does not express RAG (Yu et al., 1999). Immature murine B cells which exist in the bone marrow benefit from a bias in BCR signalling

which inhibits autoreactive deletion and promotes the induction of RAG1 and RAG2. Immature B cells which have left the bone marrow and entered into the periphery lose the BCR signalling bias and undergo apoptosis (Sandel and Monroe, 1999). This bias may be a result of phosphatidylinositol bis phosphate (PIP₂) hydrolysis which generates the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) (Bijsterbosch et al., 1985). IP₃ is known to play an important role in mobilising calcium from internal stores and DAG can activate several isotypes of protein kinase C (PKC) such as the conventional PKC- α , - β and - γ as well as the novel PKC- δ and - ϵ (Newton, 1995). PKC activation has been reported to rescue immature B cells from apoptosis. Furthermore, PKC depleted or inhibited mature B cells are driven into apoptosis suggesting an important role for this kinase in the negative selection of immature B cells (King et al, 1999). Owing to the diversity of H and L chain rearrangement, at the point of sIgM expression, B cells are committed to an antigen binding specificity. Therefore, an individual B cell can only produce one type of antibody (Burnet, 1959). Furthermore, antibody produced by immunoglobulin can only induce production of antibodies of the same specificity, work on healthy mice showed that injections of IgM antibodies induced direct plaque-forming cells of the same specificity as the injected antibody (Forni et al, 1979). At this stage, B cells are termed sIgM⁺ or virgin cells and are ready to enter the antigen dependent phase of development.

1.1.3 Antigen-Dependent B Cell Development

Immature cells which have not been subject to autoantigen challenge or have undergone successful receptor editing increase surface levels of IgM and enter the antigen dependent stage of B cell development (Figure 1.1). This is followed by the appearance of IgD in low amounts. At this stage, B cells enter a transitional state and migrate from the bone marrow to the periphery. If there is no further autoantigen challenge then the B cells develop further, and the levels of IgD are increased and exceed the levels of IgM. The B cells progress to the mature stage of development and migrate to secondary

lymphoid organs, such as the spleen or lymph nodes. B cells enter the secondary organs through a multi-step process which involves L-, P- and E-selectin-mediated attachment and rolling (Montoya et al., 1999), activation of chemokines, adhesion by activated integrins and finally, transendothelial migration (Butcher and Picker, 1996). The recruitment of B cells is achieved by the chemoattractant properties of small secretory or membrane-bound proteins (6-14kDa) called chemokines. The interaction of B cells with chemokines enables their entry into secondary lymphoid organs through high endothelial venules in lymph nodes or the endothelial cell layer lining sinuses in the spleen. Chemokines found on lymph nodes and spleen include; liver and activation-regulated chemokines (LARC), macrophage derived chemokine (MDC), stromal cell-derived factor (SDF-1), secondary lymphoid tissue chemokine (SLC), Chemokine (CK) β -11, macrophage colony inhibitory factor (MCIF) and thymus-expressed chemokine TECK (Kim and Broxmeyer, 1999).

When presented with antigen, the mature B cell can respond in two ways. The first response involves a rapid clonal expansion and plasma-cell differentiation to give rise to a high antigen-affinity antibody producing cell. Mature B cells which do this are found in concentrated T lymphocyte areas such as the periarteriolar lymphoid sheath (PALS) of the spleen and are activated by interaction with CD4⁺ T-helper (II) cells (Tarlinton, 1998). The second response is a complex process which involves rapid clonal expansion of the B cell within the microenvironment of the germinal centre reaction to form affinity matured, long-lived B cells called memory B cells (Hess et al., 1998).

1.1.4 The Germinal Centre Reaction

Germinal centres are areas found within B cell rich areas of secondary lymphoid organs. Antigen activated B cells are recruited to germinal centre reactions by TH cells which have been primed and activated by interdigitating cells (IDC) found in the PALS. The activated cells then migrate to primary follicles where they undergo rapid clonal

expansion, forming a secondary follicle called the germinal centre. The germinal centre polarizes into 'light' or 'dark' zone regions, the former is rich in follicular dendritic cells (FDC) and macrophages, while the latter is adjacent to T-cell rich regions and contains rapidly dividing B cell blasts called centroblasts. In the dark zone, centroblasts clonally expand and diversify their BCRs by somatic hypermutation of V regions and receptor editing. Re-expression of RAG1 and RAG2 in germinal centre B cells has been demonstrated, suggesting that receptor editing is achieved by the same mechanisms by which B cell tolerance is achieved during the emergence of sIgM⁺ cells (Han et al., 1996) After this phase of development the cells enter a dormant phase and are defined as centrocytes. The centrocytes express their altered BCRs and enter the light zone where they encounter challenge by FDC-bound antigen. If BCR binding to the antigen is insufficient, the centroblast undergoes apoptosis and is quickly removed by macrophage clearance. Centrocytes which demonstrate high affinity binding to the antigen undergo positive selection. This event leads to one of two fates; the centrocyte may re-enter into the dark zone for further modification and selection, or secondly, the cell leaves the germinal centre reaction and remains in the secondary lymphoid tissue often for many years as a memory B cell (Lane, 1996).

Re-exposure to the antigen leads to another phase of development in T and B cell rich regions of secondary lymphoid organs. In the T cell rich regions, memory B cells undergo rapid clonal expansion and develop into antibody-forming plasma cells (AFCs) to give an increased response to the antigen which is greater than in the primary response. Re-activated B cells which enter the B-cell rich regions again enter the germinal centre and are processed by the same mechanisms as the primary response. Memory B cells can be divided by Ig isotype expression ie. IgE, IgG, IgA, or by antibody secretion. Non-secretory memory B cells are thought to act as precursors for the rapid antibody secreting response and in antigen processing and presentation for the secondary response. However, any mechanism which maintains non-secretory memory B cells has yet to be identified (McHeyzer-Williams and Ahmed, 1999).

1.1.5 Transcriptional Regulation of B Cell Development

The signalling pathways and patterns of gene expression which control the maturation of plasma cells and memory B cells are many and well documented (Henderson and Calame, 1998; Mess et al., 1998; Tarlinton, 1998). In particular, the B cell specific activator protein, Octamer (Oct) binding factor (OBF)-1 which is also known as OCA-B or Bob-1 has a prominent role in the formation of memory B cells. OBF-1 interacts with the Oct binding family of transcription factors Oct-1 and Oct-2 to bind to the highly conserved octamer site of Ig promoters where it is involved in upregulating the transcription of Ig genes. Studies with OBF-1 knockout mice showed that it was not required for early transcription of Ig genes or for B cell development, but was essential for the B cell response following antigen challenge, and was necessary for the formation of germinal centres (Schubart et al., 1996). This is supported by recent evidence which has shown that in the absence of OBF-1, germinal centre formation is blocked completely but primary B cell follicles, the marginal zone and plasma cells are all intact. The failure of germinal centre development was shown to be through the inhibition of the capacity of OBF-1 to integrate BCR, CD40 ligand (L) and IL-4 mediated signals to the germinal centre reaction (Qin et al., 1998).

Another important transcription factor in the formation of the germinal centre is the Ets transcription factor family member Spi-B. Spi-B is closely related to PU.1, an Ets transcription factor which is essential for the production of monocytes and lymphocytes. Spi-B knockout mice show defective secondary responses to T-cell dependent antigen challenge and demonstrate a defect in germinal centre formation and maintenance (Su et al., 1997). Spi-B activity is understood to be regulated by BCR signal transduction, as is PU.1. Both Ets transcription factors have recently been shown in knockout mice to be essential for normal BCR signalling events. Deletion of both genes leads to the formation of defective germinal centres and the reduced capacity of B cells to proliferate in

response to IgM cross linking *in vitro* (Garret-Sinha et al., 1999). A further transcription factor, Spi-C has been identified, and although not involved in the formation of germinal centre reactions, may be responsive to BCR activity in peripheral B cells (Bemark et al., 1999).

The transcription factor B cell lymphoma/leukemia (BCL)-6 has been shown to act as a suppressor of signal transducer and activator of transcription (STAT) genes such as the IL-4-regulated protein, STAT6. The BCL-6 gene is expressed in the germinal centre of human tonsils, although not in plasma cells. The function of BCL-6 is not fully understood, however it appears to have an important role in B cell development (Dent et al., 1997). Mice deficient in RAG1 were reconstituted with bone marrow cells from BCL-6 deficient mice and were found to have normal levels of B cells in primary lymphoid tissue. In addition, these mice were able to produce IgG antibodies which were specific for T-cell dependent antigens, but did not have any germinal centres. Lack of BCL-6 did not affect levels of plasma cells indicating that BCL-6 expression is specifically required for germinal centre formation (Fukuda et al., 1997). In direct contrast to BCL-6 is the tumour necrosis factor (TNF) family member OX40-ligand (OX40L) which is expressed by activated B cells and CD40-activated human dendritic cells (Oshima et al., 1997). The receptor for OX40L is OX40 (CD134) and is expressed on activated T cells. Transgenic mice which constitutively expressed OX40L on dendritic cells showed increased levels of CD4⁺ T cells within splenic B cell follicles in comparison with wild type mice (Brocker et al., 1999). A block of the OX40/OX40L interaction in mice lead to a diminution of CD4⁺ T cells in B cell follicles and, as a result, impaired formation of AFCs. Both germinal centre and memory cell formation were unaffected (Stuber and Strober, 1996). Conversely, in transgenic studies, BCL-6 knockout mice failed to develop germinal centres but formed normal AFCs. These findings demonstrate that the developmental pathways which lead to the formation of germinal centres and AFCs become independent of each other after a stage of development, although the critical stage is as yet, undefined.

Other important transcription factors in B cell development include; Early B cell Factor (EBF), Ikaros, E2A proteins and Pax5. EBF is expressed during primary B cell development and is essential for the progression of pro-B cell to pre-B cell development. EBF knockout mice show pro-B cells which lack DJ_H rearrangements, $Ig\alpha$ and β , surrogate light chain and RAG-1 and RAG-2 expression, therefore EBF is an essential early-stage B cell transcription factor (Hagman et al., 1993; Lin and Grosschedl, 1995). The Ikaros gene encodes a protein which can form homo- or heterodimers which bind DNA and activate transcription (Sun et al, 1996). Ikaros is an important protein throughout B cell development and its expression appears to increase as B cells mature. Ikaros knockout mice showed a complete block in early B cell development, prior to the pro-B cell stage. Ikaros is a ubiquitous transcription factor in B cells and potential sites for Ikaros regulation include the genes encoding RAG1, the IL-2 receptor, $Ig\alpha$, $\lambda 5$ and TdT (Georgopoulos et al, 1997; Sun et al, 1996). Ikaros has been shown to interact with a similar lymphocytic transcription factor, Aiolos, in the regulation of lymphoid differentiation (Morgan et al., 1997).

E2A is a widely expressed transcription factor which is part of a larger family of transcription factors that share homology in their DNA binding domains and in their helix-loop-helix binding motif, and as such are called bHLH proteins (Kadesch, 1992). The expression of E2A appears to be specific and essential for B cell development. B lymphopoiesis is completely blocked in E2A knockout mice, whereas production of other haematopoietic lineages, including T cells, are unaffected (Zhuang, Soriano and Weintraub, 1994). The Pax5 gene encodes B cell specific activator protein (BSAP) which is important for the progression of B cell development from the pro-B to pre-B stage of development (Busslinger and Urbanek, 1995). Pax5 knockout mice show pro-B cells which have completed DJ_H rearrangements, express c-kit, B220, CD43 and receptors for IL-7 although CD19 is absent and V_HDJ_H recombination is markedly reduced. As a result, B cell development in Pax5 knockout mice is arrested at the pro-B

cell stage (Urbanek et al., 1994). A recent report showed that Pax5 was essential not only for the progression of pro-B cell development to the pre-B cell stage but vital for B cell lineage commitment. Pro-B cells derived from Pax5^{-/-} mice were shown to differentiate into functional lymphoid or myeloid lineages, following treatment with the appropriate cytokine. An example of this was the culture of Pax5^{-/-} pro B cells with macrophage colony-stimulating factor (M-CSF)-producing ST2 cells which led to the production of mature macrophages. The restoration of Pax5 activity using retroviral transduction prevented the pro-B cells from differentiating into other hematopoietic lineages, therefore showing that Pax5 is essential for B cell commitment (Nutt et al., 1999).

1.2 BCR SIGNALLING

When challenged by antigen or anti-receptor antibody, the BCR aggregates and stimulates signal transduction through the Ig α and Ig β subunits (see Figure 1.2). Critical to the understanding of BCR signalling has been the identification of immunoreceptor tyrosine-based activation motifs (ITAMs) within these subunits (Reth, 1989). The ITAM motif, D/E-X₇-D/E-X₂-Y-X₂-L-X₇-Y-X₂-L/I is found in the cytoplasmic domains of the multi-chain immune recognition receptor (MIRR) family, which as well as the BCR, includes the T cell receptor (TCR), and receptors for the Fc portions of IgG (Fc γ RI, Fc γ RIIA and C, Fc γ RIIIA) and IgE (Fc ϵ RI) as members (Keegan and Paul, 1992). BCR aggregation leads to the recruitment of three distinctive groups of non-receptor protein tyrosine kinases (PTKs) to the ITAM motifs. Src-family kinases consisting of Lyn, Fyn, Blk and Fgr, Syk/ZAP-70 kinases such as Syk, and Tec kinases, including Bruton's tyrosine kinase (Btk), Itk and Tec activate ITAMs by phosphorylating tyrosine residues. PTKs then interact with the activated ITAMs via Src Homology 2 (SH2) domains leading to PTK phosphorylation and resulting downstream tyrosine phosphorylation events. This mutual phosphorylation serves two purposes; firstly, signal amplification through a positive feedback effect created by the cumulative recruitment of PTKs and

secondly, signal diversification through the recruitment of multiple PTKs and the subsequent activation of their specific downstream signalling pathways. Signalling diversity in the BCR enables the B cell to undergo the developmental progressions of receptor editing, proliferation, maturation, survival and apoptosis (Benschop and Cambier, 1999; Tamir and Cambier, 1998).

Activation of the BCR leads to aggregation of the receptor and increased phosphorylation of tyrosine residues within the ITAMs of Ig α and Ig β . This is achieved through the recruitment of Src-family PTKs which also become phosphorylated at the BCR receptor. Important to Src-family PTK phosphorylation is the role of the transmembrane tyrosine phosphatase CD45, which regulates BCR signalling by dephosphorylating Ig α , Ig β and the negative regulatory regions of Src-kinases such as Lyn (Satterthwaite and Witte, 1996). Another cell surface molecule which is involved in Src-mediated BCR signalling is CD19. Interactions with downstream molecules such as phosphatidylinositol (PI) 3-kinase and the adaptor protein Vav define CD19 as an important effector molecule in BCR signalling (Sato et al., 1997; Weng et al., 1994). A recent study on CD19 knockout mice showed a reduction in Lyn kinase activity, accompanied by a decrease in BCR phosphorylation when stimulated with anti-IgM antibody (Fujimoto et al., 1999). This demonstrates that CD19 is able to further influence BCR signal transduction by regulating Src-family kinase activity.

1.2.1 Src -Family Activity

Src-family PTK activation results in the initiation of the p21^{ras} (Ras) pathway which leads to a series of downstream tyrosine phosphorylation events through further PTK activation (Figure 1.2). This is initiated by the recruitment of the adaptor protein Shc. Shc binds the phosphorylated ITAM motif via its' SH2 domain and recruits another adaptor protein, again via SH2 domain interaction, called Grb2. Grb2 contains a Src

Homology 3 (SH3) domain, which upon activation, recruits the GDP nucleotide exchange protein, Son of sevenless (Sos) (Li et al., 1993). Sos promotes GDP-GTP exchange in the serine/threonine kinase Ras, via the activation of GTPase activating protein (GAP) and of the small GTP binding protein Rho (Boriak-Sjodin et al., 1998). Ras is found at the inner surface of the plasma membrane and upon activation forms an association with the cytoplasmic serine/threonine kinase Raf-1 and translocates the protein to the plasma membrane where it becomes phosphorylated. Activated Raf-1 then phosphorylates two mitogen activated protein kinase kinases (MAPKK) also referred to as MEK1 and MEK2. The activated MEKs phosphorylate serine and threonine residues in two MAP kinases (MAPK), termed extracellular signal-related kinase (ERK) 1 and 2 or p42 and p44, respectively (Hashimoto et al., 1998). Activated MAP-kinases can then phosphorylate and regulate cytosolic proteins such as cytoskeletal proteins or, after prolonged activation, dimerise and translocate to the nucleus where phosphorylation of transcriptional regulatory proteins such as cyclic-AMP response element binding protein (CREB), c-Fos, c-Jun and members of the Ets family can occur (Cohen, 1997). Additionally, MAP kinase can activate other kinases such as the p90S6^{rsk} serine/threonine kinase which is involved in the regulation of protein synthesis (Campbell et al., 1998).

1.2.2 The Role of Syk

Kinetic studies have shown that, upon BCR aggregation, activation of the Src-family PTKs Lyn and Blk occurs before the increased activity of Btk and Syk, indicating that Src-family activation may be responsible for the activation of additional substrates (Saouaf et al., 1994). Therefore, Syk activation may be achieved by recruitment to ITAMs which have already been phosphorylated through interactions with Src-family kinases. In T-cells, there is evidence to suggest an activating function of Syk which is independent of Src-PTK through studies involving a chimeric transmembrane protein model of the TCR in human Jurkat T cells. This model consisted of a CD16 extracellular

domain and either a Src-family or Syk kinase intracellular domain which were activated by anti-CD16 antibody-mediated clustering of the chimeras. Chimeric proteins which contained a Src-family intracellular domain failed to respond; however Syk containing chimeras initiated calcium mobilization indicating a positive response to antibody-induced stimulation (Kolanus et al, 1993). In addition, a recent study demonstrates that BCR aggregation induces the phosphorylation and activation of the serine/threonine kinase p70^{s6k} in wild type and in Lyn-deficient DT40 B cells, but not Syk-deficient cells, where phosphorylation is blocked (Li et al, 1999). By way of contrast, loss of Lyn in DT40 B cells demonstrates a profound decrease in BCR-induced Syk activation (El-Hillal et al., 1997). This information suggests that some Syk phosphorylation events on ITAM motifs in T cells can occur independently of Src-family and other, downstream PTKs in B cells, however Src-family PTKs clearly have a significant role in Syk activation at ITAM motifs in B cells. The activation of Syk leads to the tyrosine phosphorylation of phosphatidylinositol-lipid-specific phospholipase C γ (PLC γ), which occurs by recruitment to phosphorylated Syk and binding via SH2 domains, as well as interaction with activated Btk (Tamir and Cambier, 1998). The most common PLC γ enzyme in B cells is PLC γ 2 and, following its recruitment to the membrane and subsequent activation, mediates the hydrolysis of PIP₂, and the resultant production of DAG and IP₃ (Figure 1.2 and described earlier).

1.2.3 The Role of Btk

Btk activation is, like Syk, stimulated through Src-family kinase-mediated phosphorylation. Studies in human COS B cells over-expressing Btk and Src-family kinases show that BCR stimulation leads to Src-PTK-mediated phosphorylation and increased activity of Btk, up to five times greater than when Btk is expressed alone (Rawlings et al., 1996). Studies in Lyn-deficient DT40 B cells found that initial BCR-induced Btk phosphorylation was significantly reduced before levels of phosphorylation gradually returned to wild-type amounts. Conversely, BCR activation in Syk-deficient

DT40 cells showed initial Btk phosphorylation in common with wild-type cells, but this decreased over a prolonged period of activation. The failure of Lyn⁻/Syk⁻ DT40 cells to phosphorylate Btk over any length of time following BCR activation suggests that while either Lyn or Syk alone are capable of phosphorylating Btk, both are required to exert complete phosphorylation of Btk in BCR signalling (Kurosaki and Kurosaki, 1997). Therefore, the activation of Btk in BCR signalling may be sustained by two stages of tyrosine phosphorylation, the first stage is the initial stage generated through the rapid phosphorylation and activation of Src-family members (namely Lyn) and a second stage involving a long term activation mainly through interactions with Syk (Kurosaki, 1999). Further insight into the mechanisms involved in the activation of Btk can be drawn from a recent study of CD19 knockout mice. This demonstrated that BCR-stimulated CD19 phosphorylation initiates Btk activation through the recruitment and activation of PI-3 kinase. A product of PI3-kinase activation, PI 3,4,5-trisphosphate, may then recruit Btk to the phosphorylated ITAM motif environment by binding its pleckstrin homology (PH) domain (Buhl and Cambier, 1999). The role of Btk in BCR-mediated signalling is also important in B cell development. The B cell immunodeficiencies X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice are a result of Btk abnormalities. XLA is characterised by the arrest of B cell development at the pre-B cell stage, while Xid mice show, amongst other aberrations, decreased production of mature B cells (Satterthwaite et al, 1998).

1.2.4 The Activation of PLC γ 2

The activation of PLC γ 2 is initiated by Btk and Syk which phosphorylate tyrosine residues on PLC γ 2 SH2 domains (Figure 1.2). This was identified in DT40 B cells which were Btk deficient. Tyrosine phosphorylation of PLC γ 2 upon BCR stimulation was reduced leading to the loss of PI hydrolysis and calcium mobilisation (Takata and Kurosaki, 1996). As Syk and Btk are recruited to the ITAM motifs by different mechanisms, a shared protein or complex is required to co-localise both PTKs and

activate PLC γ 2. In B cells a candidate molecule is B cell linker protein (BLNK), also termed Src homology (SH2) domain containing leukocyte protein of 65kDa (SLP-65), which shows homology to a signalling element of the T cell receptor, SLP-76 (Wienands et al., 1998). Studies in DT40 B cells in which BLNK was over-expressed showed an increased tyrosine phosphorylation of PLC γ 1 and 2, and transcriptional activation of nuclear factor of activated T cells (NF-AT). BLNK-deficient cells showed the opposite effects. Further co-expression experiments between BLNK and Syk revealed an induction of tyrosine phosphorylation and activation of PLC γ suggesting that BLNK links BCR-activated PTKs with PLC γ -driven signalling pathways (Fu et al., 1998). Figure 1.2 describes a possible mechanism of PLC γ 2 activation. Activated Syk phosphorylates BLNK and initiates its recruitment to the membrane, where it lies proximal to the ITAM motif region. Phosphorylated BLNK recruits PLC γ 2 to the membrane and to activated Syk, where it becomes tyrosine phosphorylated. Btk, present at the membrane as a result of the earlier interaction between PI 3,4,5-P $_3$ and its PH domain, phosphorylates other tyrosine residues, thereby fully activating PLC γ 2 (Ishiai et al., 1999).

The products of PLC γ 2 catalytic activity, the second messengers IP $_3$ and DAG, generate an increase in intracellular calcium and activation of PKC. Increased calcium leads to the activation of calmodulin-dependent protein kinase II and the serine/threonine phosphatase calcineurin. The activation of calcineurin leads to the activation of the transcription factor; NF-AT. Despite their name, NF-AT proteins are found not only in T cells, but in other immune system cells, such as B cells, mast cells, basophils and macrophages. NF-AT proteins consist of a cytoplasmic component and a nuclear component. When calcineurin activates the cytoplasmic NF-AT it translocates to the nucleus where it combines with the nuclear NF-AT, which was identified as the activator protein (AP)-1 complex of Fos and Jun family members (Jain et al., 1992). The activated NF-AT complex regulates transcriptional events, such as the expression of IL-2 (Rao et al., 1997). Activated PKC isoenzymes are serine/threonine kinases which are recruited to

the inner membrane on activation and anchored by receptors for activated C-kinase (RACKs), perinuclear binding protein PICK1 and cyclic adenosine monophosphate (cAMP) dependent A-kinase anchoring proteins (AKAPS). In BCR signalling, DAG and calcium-activated PKC isoforms are most likely to be bound to RACKs (Liu and Heckman, 1998). The regulatory mechanisms of the effects of PKC isoforms on cell growth, differentiation, and gene expression as a result of BCR signalling or any other system is largely unknown. PKC α , however, has been shown to directly phosphorylate and activate Raf-1 in a Ras-GTP-Raf-1 complex, indicating that PKC α may serve as a secondary activation kinase to the Ras signalling pathway (Kolch et al., 1993; Marais et al., 1998). Studies in DT40 B cells which were deficient in PLC γ 2 showed inhibition of MAP kinase following BCR aggregation, further supporting a role for PKC in the Ras pathway (Hashimoto et al., 1998). In addition, PKC θ has been shown to activate the stress activated protein kinase (SAPK), c-Jun N-terminal kinase (JNK), through interaction with calcineurin (Werlen et al., 1998).

1.3 CYTOKINES AND THEIR RECEPTORS

The role of cytokines and cytokine receptor signalling is of paramount importance to the development and function of B cells. Cytokines are small pleiotropic proteins between 8-80kDa which stimulate auto- or paracrine effects on lymphoid cells. Cytokines include; Interleukins (IL), interferons (IFN), colony stimulating factors (CSF), tumour necrosis factors (TNF) and can act in synergy to tightly regulate the behaviour of B cells and other cells of the immune system. Cytokines are involved in the differentiation, expansion and survival of B cells and act by binding specific cell-surface receptors which leads to receptor tyrosine phosphorylation and initiation of signal transduction pathways (Baird et al, 1999). The observed effects of B cells at the site of an immune response are generally a result of the combination of cytokines acting in a synergistic and/or inhibitory way. Examples of inhibitory combinations include IL-4 and IFN γ (Rabin et al., 1986) and IL-4 and IL-2 (Jelinek and Lipsky, 1988).

1.3.1 The JAK/STAT Pathway

Cytokine receptors are linked to a family of PTKs called the Janus kinases (JAKs) (Wilks et al., 1991). Cytokine receptor ligand binding causes a dimerisation of the receptor subunits, leading to the activation of JAKs and receptor phosphorylation. JAK family members include JAK 1-3 and Tyk2, all of which are broadly expressed, with the exception of JAK3, which appears to be restricted to lymphoid and myeloid cells (Rane and Reddy, 1994). JAKs are the linking factor between receptor ligation and tyrosine phosphorylation of signalling proteins, in particular, a cytoplasmic family of transcriptional activators called the signal transducers and activators of transcription (STATs). STAT family members number seven in total; STAT 1-4, STAT5A, STAT5B and STAT6, all of which contain a carboxy-terminal SH2 domain, an SH3 domain and a conserved DNA binding region. Phosphotyrosines on cytokine receptors are bound by STAT proteins via their SH2 domains. STATs are tyrosine-phosphorylated and form homo or heterodimers with other STAT proteins through SH2-tyrosine phosphorylation interactions. Dimerised STATs then translocate to the nucleus where they bind specific DNA sequences. These sequences are similar to the nucleotide consensus motif TTNCNNNAA found in the regulatory element of the promoter of IFN- γ inducible genes called the gamma interferon activated site (GAS) (Karras et al., 1996). STATs binding to GAS sequences regulate gene expression (Chen et al., 1998). In addition, some STATs also require serine phosphorylation for optimal transcriptional activation following stimulation with IFN- γ (Darnell Jr., 1997). JAKs are capable of activating more than one distinct signal transduction pathway. The IL-2 driven activation of JAK1 and 3 leads to the initiation of the Ras/MAPK pathway and the activation of STAT5a and 5b, respectively (Evans et al., 1995; O'Shea, 1997).

1.3.2 Negative Regulation of Cytokine-Induced Signalling

Recently, two different classes of negative regulators of cytokine-induced signalling have been reported. The first type have various names including; JAK-binding protein (JAB), suppressor of cytokine signalling (SOCS), STAT-induced STAT inhibitor (SSI), and cytokine-inducible SH2 domain containing protein (CIS) (Aman and Leonard, 1997). These inhibitor proteins act by binding to and blocking the kinase activity of JAKs and are activated by cytokines themselves, indicating they may constitute a negative feedback mechanism on cytokine signalling. A study of IL-2 signalling in human T cells and peripheral blood lymphocytes demonstrates a role for the SOCS-3 inhibitor protein in suppressing STAT5 phosphorylation and lymphocyte proliferation, mainly through its involvement with tyrosine phosphorylated JAK1 and the IL-2 receptor β subunit (Cohney et al., 1999). Work done on primary human monocytes demonstrated that the inhibitory function of IFN on IL-4-stimulated STAT6 induction and gene expression was shown to be at least partly regulated by IFN-induced expression of SOCS-1 (Dickensheets et al., 1999). A recent investigation of JAB activity in M1 myelogenous leukemia cells identified a JAB binding site as a specific tyrosine residue (Y1007) in the activation loop of JAK2. The study demonstrated that JAB bound this region via SH-2 domain interaction, blocking JAK2 phosphorylation and its resultant activity (Yasukawa et al., 1999). The second class of inhibitory proteins are called protein inhibitor of activated STAT (PIAS) which bind to a specific STAT protein and block DNA binding and transcriptional activation by the formation of homo or heterodimers (Chen et al., 1998). A study of IL-6 induced STAT1 and STAT3 activation in murine myeloblast M1 cells showed that PIAS3 bound to STAT3 and not STAT1 (Chung et al., 1997). Additionally, work on human Daudi B cells identified a STAT1 specific inhibitor PIAS1 which blocked IFN stimulation through STAT1 but not STAT2 or STAT3 (Liu et al., 1998). Both these findings suggest that each STAT protein has a specific PIAS inhibitor.

1.4 IL-4 AND THE IL-4 RECEPTOR

Interleukin-4 is a type 1 cytokine which is synthesised by TH2 cells, CD4⁺ T cells, basophils and mast cells (Nelms et al., 1999). IL-4 was first reported as a B cell growth factor (BCGF), distinct from IL-2, in the supernatant of phorbol myristate acetate (PMA)-treated murine EL4 thymoma cells, where it caused an enhanced proliferation of normal mouse B cells in response to anti-Ig antibodies (Howard et al., 1982). In B cells, IL-4 stimulates a pleiotropic response which includes; the induction of cellular proliferation and differentiation (Keegan et al., 1994a), expression of class II major histocompatibility complex (MHC) (Noelle et al., 1984), stimulation of Ig class switching to IgE and IgG4 in human B cells or IgE and IgG1 in mouse B cells (Gascan et al., 1991; Vitetta et al., 1985), and the upregulation of the expression of the low affinity Fc receptor for IgE, CD23 (Bonney et al., 1997a). Studies on concanavalin A (Con A)-stimulated spleen cells from IL-4 knockout mice showed diminished serum levels of IgG1 and IgE, when compared with wild type cells, although T and B cell development was normal (Kuhn et al., 1991). This observation was repeated in IL-4 receptor (R)- α knockout mice which showed a similar decrease in IgG1 and IgE production (Noben-Trauth et al., 1997). Mutations in human IL-4R α have been linked to allergy and this has been investigated in studies measuring the signalling effects of IL-4 on mutated human IL-4 receptors which have been transfected into murine cells. Identification of all allergy-linked IL-4R α mutations may be required before these studies are able to generate further information (Wang et al., 1999).

The IL-4 receptor is broadly expressed on many haematopoietic and non-haematopoietic cell surfaces. It consists of a main 140kDa IL-4R α subunit which interacts with a 64kDa γ chain (γ c) that, in addition to IL-4, is common to the receptors of IL-2, IL-7, IL-9 and IL-15 (Raskin et al., 1998). In humans and mice, mutations in γ c lead to a defective IL-2 receptor which profoundly diminishes levels of peripheral blood T cells and although B cells are produced normally, they are non-functional. This is a condition

which occurs in males, and which without a bone marrow transplant, is fatal. The disease is termed X-linked severe combined immunodeficiency (XSCID) (Leonard, 1996). When IL-4 binds to its receptor it forms a complex with the IL-4R α chain with which the γ c chain then interacts and initiates intracellular signalling pathways (Figure 1.3) (Letzelter et al, 1998). The IL-4R α subunit is not unique to IL-4, as IL-13 additionally binds IL-4R α although it does not interact with γ c, indicating an unique mechanism of action (Figure 1.3) (Lin et al., 1995). The IL-13R α chain was identified following work on monocytic U937 cells which showed that IL-4 could induce STAT6 tyrosine phosphorylation and DNA binding activity in the absence of γ c, and in doing so, defined a 70kDa receptor component which could facilitate IL-4 signalling via STAT6 activation (Dawson et al, 1997). The cytoplasmic domain of IL-13R α has recently been characterised, revealing that receptor signalling is mainly driven via Tyk2 activation (Orchansky et al., 1999). Two types of human IL-13 receptor chains have been cloned. The first receptor was cloned from the Caki-1 human renal carcinoma cell line and is called IL-13R α . In addition, IL-13R α was characterised and shown to have a 50% homology with the human IL-5R α chain (Caput et al., 1996). The second IL-13 receptor is identified as IL-13R α' and was cloned from the human T-cell leukemia virus (HTLV)-1-infected MT-2 cell line, using murine IL-13R cDNA. The human IL-13R α' receptor in common with the cloned mouse IL-13R, has no homology with any other cytokine receptors. This is in contrast to the IL-13R α chain (Aman et al., 1996). Work done on Chinese hamster ovary (CHO) cells demonstrated that in cell types where γ c is not expressed, the IL-4 receptor complex is made up of the IL-4R α and the IL-13R α' chain (Murata et al, 1998). In B cells, the main action of IL-4 on its receptor is to induce heterodimerisation of the IL-4R α chain with the γ c chain, leading to the activation of IL-4R signal transduction pathways (Kammer et al., 1996).

1.4.1 IL-4R Signalling Pathways

Activation of the IL-4 receptor results in the activation of tyrosine kinases which phosphorylate protein substrates on tyrosine residues and initiate intracellular signalling cascades such as the PI-3 kinase or the previously described PLC γ pathways (Figure 1.3) (Miyajima et al., 1992). The IL-4 receptor does not have any endogenous kinase activity therefore requires the involvement of receptor-associated tyrosine kinases to initiate signalling, namely JAK kinases (Taniguchi, 1995). Three members of the JAK kinase family; JAK1, JAK2 and JAK3 are activated in response to IL-4R ligation. JAK1 in most cell lines and JAK2 in certain cell lines associate with the IL-4R α subunit (Miyazaki et al., 1994; Murata, Noguchi and Puri, 1996). A study of a female human with autosomal recessive SCID which showed an absence of JAK3, and the findings that JAK3 knockout mice display immunodeficiencies similar to XSCID, demonstrate that JAK3 is associated with the γ_c chain (Nosaka et al., 1995; Park et al., 1995; Russell et al., 1995). JAK1 and 3 activation leads to the recruitment and activation of STAT6 which is essential for the induction of several IL-4 responsive genes, such as CD23 and MHCII (Kaplan et al., 1996; Shimoda et al., 1996). STAT6 homodimers translocate to the nucleus where they bind GAS sequences, in particular the nucleotide sequence TTCCANGGAA, and activate transcription (Ihle, 1996). How STAT6 or STATs in general are able to activate transcription is unclear although it is thought that activated STATs may form complexes with other transcriptional factors in the nucleus such as with nuclear factor- κ B (NF- κ B) (Shen and Stavnezer, 1998). In nematode pre-infected STAT6 knockout mice, IL-4-induced up-regulation of MHC Class II expression, CD23, IgE and IgG1 were all profoundly reduced. In addition, IL-4-supported T and B cell proliferation were diminished (Takeda et al., 1996). A recent study of IL-4R stimulation in the murine pro-B cell line Ba/F3 and peripheral human T cells showed that in addition to STAT6 activation, IL-4R heterodimers containing γ_c phosphorylate tyrosine residues of both STAT5a and 5b. This demonstrates that IL-4R signalling can follow several different transduction pathways, depending on the components of the receptor complex

(Lishke et al., 1998). Further to this study, mutated human IL-4R subunits stably expressed in Ba/F3 cells were used to establish that disruption of a proline rich 'box1' motif in either the IL-4R α or γ c subunit abolished IL-4-induced tyrosine phosphorylation of JAK1 and JAK3. These mutations also blocked IL-4-induced STAT5 activation and cell proliferation indicating that either subunit may activate STAT5 activity via JAK1 and JAK3 following IL-4 stimulation through a specific cytoplasmic domain (Friedrich et al., 1999). IL-4R-induced STAT5 activity has also been reported in primary human B cells (Rolling et al., 1996).

1.4.2 IL-4R Interactions with IRS

Another signalling component affected by IL-4R activation was identified when a 170-kDa phosphoprotein was found to be specifically phosphorylated by IL-4 induced signalling. This protein was called the IL-4 phosphorylation substrate (4PS) and shown to be related to insulin receptor substrate (IRS)-1, a substrate phosphorylated following treatment of non-haematopoietic cells with insulin or insulin-like growth factor (IGF)-1. The homology between 4PS and IRS-1 was so high that 4PS was renamed as IRS-2. Stable transfectants of the factor-dependent myeloid progenitor cell line 32D, which expressed IRS-1 or IRS-2, phosphorylated their particular substrate in response to IL-4 treatment. In addition, IL-4 stimulation appeared to initiate cell growth suggesting that IRS-1 and 2 link IL-4R signalling pathways to cellular proliferation (Sun et al., 1995; Wang et al., 1993). Figure 1.3 shows that IRS-1 and 2 become associated with the IL-4R α and γ c chain following IL-4R activation. The IRS-1 and 2 activating region of the IL-4R α is contained within a 120 base-pair cytoplasmic region between amino acids 437 and 557. This region shows a considerable homology to sequences in insulin and IGF-1 receptors which activate IRS-1 and 2. Mutation of the tyrosine residue (Y497) in this region results in decreased proliferation in response to IL-4 and inhibition of IRS-1 and 2 phosphorylation. This activating region is termed the insulin IL-4 receptor, or the I4R motif (Keegan et al., 1994b). Although it appears that IL-4R α is the predominant

chain, studies have also shown that γc is required for tyrosine phosphorylation of IRS1 and 2 suggesting that other cytokines which share this subunit may also use IRS activation to promote cellular proliferation (Johnston et al., 1995; Russell et al., 1993). The link between activation of the IL-4 receptor and tyrosine phosphorylation of IRS1 and 2 is thought to involve the JAK kinases. JAKs 1, 2 and 3 have been shown to directly phosphorylate IRS-1 and experiments on JAK-deficient human fibrosarcoma cells indicate that JAK1 is essential for IL-4-driven stimulation of IRS-1 (Wang et al., 1997; Yin et al., 1995). IRS1 and 2 contain several tyrosine phosphorylation sites which are bound by SH2 domains enabling interactions with other SH2 containing signalling molecules (Sun et al., 1991). Grb-2 interacts with phosphorylated IRS-1 and 2 leading to the activation of the previously described Ras/MAP kinase signalling pathway, and interacts with the regulatory subunit of PI-3 kinase activating an unique signalling cascade. However IL-4 is incapable of activating Ras in B cells and other hematopoietic tissues and therefore the single most important signalling pathway linked to IRS1 and 2 phosphorylation in B cells is the PI-3 kinase pathway (Welham et al, 1994).

1.4.3 The PI-3 Kinase Signalling Pathway

PI-3 kinase is a dual specificity lipid kinase which has several isoforms. PI-3 kinase catalyzes the addition of a phosphate to the 3-position of the inositol ring of phosphoinositides and can also phosphorylate serine and threonine residues. There are four different lipid products generated by the lipid-phosphorylating activity of all PI-3 kinase isoforms; phosphatidylinositol 3-phosphate (PIP), PI-3,4-P₂, PI-3,5-P₂ and PI-3,4,5-P₃ (PIP₃) (Rameh and Cantley, 1999). Nine isoforms have been identified in mammalian cells and they are grouped into three classes with respect to their activity and structural characteristics (Domin and Waterfield, 1997). Of the PI-3 kinase isoforms, only one is activated in response to IL-4, and is described in Figure 1.3. This particular complex consists of an 85kDa regulatory subunit (p85) and a 110kDa catalytic subunit (p110). The p85 subunit contains two SH2 domains in the C-terminus and an N-terminal

SH3 domain and is an adaptor molecule which links the p110 catalytic subunit to tyrosine phosphorylated proteins. IL-4 receptor activation therefore recruits p85 to tyrosine phosphorylated IRS-1 and 2 via SH2 domain interaction. This interaction results in a conformational change in the PI-3 kinase complex and leads to the activation of p110. The activated p110 subunit has the capacity to phosphorylate membrane lipids as well as serine and threonine residues (Dhand et al., 1994). A recent study investigated the role of PI-3 kinase in IL-4 receptor signalling using the pro B cell line Ba/F3. Over expression of the proto-oncogene c-Cbl enhanced the mitogenic signalling and survival effects of IL-4, which were blocked when cells were treated with the PI-3 kinase inhibitor Wortmannin (Ueno et al., 1998). In addition, c-Cbl has been reported to function as a ligand for the SH3 domains of signalling molecules as well as interacting with Grb2 and PI-3 kinase in Jurkat T cells (Meisner et al., 1995). These studies suggest that c-Cbl may link phosphorylated IRS1 and 2 with PI-3 kinase signalling pathways. When PI-3 kinase is activated it immediately generates PIP₂ and PIP₃. These products activate the intracellular kinases PKC ζ and Akt kinase, otherwise known as protein kinase B (PKB), both of which have important roles in cell survival (Franke et al, 1997). The activation of PKB is dependent on 3-phosphoinositide-dependent protein kinase 1 (PDK1) and leads to the serine phosphorylation and resultant inactivation of glycogen synthase kinase -3 (GSK-3) (Alessi et al., 1997). A study in hematopoietic cells demonstrated that Wortmannin blocked the capacity of IL-4 to prevent apoptosis, further underlining the importance of PI-3 kinase in IL-4 mediated signalling (Zamorano et al., 1996).

1.4.4 Regulation of IL-4R Signalling by SHP and SHIP

Tyrosine phosphorylation events driven by IL-4 are modulated, like other cytokine signalling, by the dephosphorylating actions of phosphotyrosine phosphatases (PTP). Important to IL-4 signalling are the SH2-containing phosphatases SHP-1, SHP-2 and the SH2-containing inositol-5-phosphatase (SHIP) (Scharenberg and Kinet, 1996). SHP-

1 and 2 have a high degree of homology to each other and share two SH2 domains found in the N-terminal region whereas SHIP has one SH2 domain but two SH3 binding motifs. These domains are important in linking SHP-1/2 and SHIP to phosphorylated molecules in order that they may dephosphorylate them. In B cells this involves a low affinity receptor for IgG called FcγRIIb1, which can be activated along with sIg and inhibits B cell proliferation, and a integral membrane protein called paired immunoglobulin-type receptor B (PIR-B) which is activated when co-ligated to the BCR (Ashman et al, 1996;Blery et al., 1998). The SH2 domains of SHP-1/2 and SHIP are particularly sensitive to a domain found in the cytoplasmic regions of FcγRIIb1 and PIR-B called the immunoregulatory tyrosine inhibitory motif (ITIM). Upon phosphorylation, ITIM motifs attract SHP-1/2 and SHIP by acting as a docking site for their SH2 domains. Studies of pervanadate-induced phosphatase inhibition in Daudi B cells show that JAK1 and STAT6 become activated. Given the involvement of JAKs and STATs in IL-4 signalling, these findings indicate a regulatory role of SHP-1/2 and SHIP in IL-4R signalling (Haque et al., 1997). SHP-1 has been shown to interact with the IL-4Rα chain after IL-4 treatment and to dephosphorylate p85 (Imani et al., 1997). A PIR-B-stimulated inhibitory role of SHP-1 over Syk and Btk has also been demonstrated in DT40 B cells (Maeda et al., 1999). SHP-2 has recently been shown to interact with the p85 subunit of PI-3 kinase, along with SHIP, in ligand-stimulated BaF/Flt3 haematopoietic cells (Zhang et al, 1999) and with FcγRIIb, following BCR activation in the human Burkitt lymphoma cell line BL41 (Sarmay et al., 1999). A potential role for SHIP is to dephosphorylate PIP₃ and initiate the PIP₂ driven activation of PKB (Franke et al., 1997). The roles of SHP-1/2 and SHIP are therefore important, not only in IL-4R signalling, but in B cell regulation as a whole.

1.4.5 Activation of cAMP Signalling

In tonsillar B cells, IL-4 has been reported to stimulate an increase in IP₃ and calcium levels, followed by a delayed accumulation of cAMP. Both events were shown to be

essential for the induction of CD23 expression (Finney et al., 1990). An increase in IP_3 and intracellular calcium leads to the activation of DAG and PKC (previously described in text and shown in Figure 1.2). The activation of the cAMP pathway is another important feature of IL-4-induced B cell signalling (Daniel et al, 1998). The cAMP-dependent signaling pathway is usually activated when a ligand binds to a membrane receptor which becomes activated and, in turn, activates a linked guanosine triphosphate (GTP)-binding (G) protein. The G protein complex consists of an α subunit of which there are three types; G_s which is stimulatory and G_i and G_o which are inhibitory and a β/γ subunit heterodimer. When a G-protein is activated, the G_α subunit dissociates from the heterotrimeric complex and binds to GTP where it modulates effector proteins until a GTPase enzyme ends the association and G_α returns to the complex (Hamm and Gilchrist, 1996). Activation of G_{α_s} protein and to a lesser extent the dissociated β/γ , stimulates the activation of the enzyme adenylate cyclase (AC) which has nine different isoforms distributed over many tissue types (Simmonds, 1999). Activation of AC catalyzes the conversion of ATP to cAMP. The effect of cAMP activity is dependent on the intracellular concentration of a diverse group of proteins called phosphodiesterases (PDEs) which degrade intracellular cAMP and appear to counteract the role of AC, particularly where AC is expressed in high levels. There are over forty isoforms of PDE which have been grouped into seven families named PDE1-7, with respect to their specificity for cyclic nucleotides. PDEs which show the greatest affinity for cAMP are PDE4 and 7, although 1,2 and 3 show a weak involvement (Houslay and Milligan, 1997).

1.5 THE cAMP SIGNALLING PATHWAY

Cyclic AMP activates cAMP dependent protein kinase, or protein kinase A (PKA). PKA is tetrameric complex which consists of two catalytic (C) subunits bound to a homodimer of two regulatory (R) subunits. There are three isoforms of C subunits; C_α , C_β and C_γ , which have similar properties, and two R subunits; RI and RII, which have

specific cAMP binding properties and are localised differentially within a cell. There are two isoforms of PKA; type I PKA consists of a RI α and RI β complex which is found mainly in the cytoplasm, and type II PKA which consists of a RII α and RII β complex which is mainly associated with cellular structures and organelles (Taylor et al, 1990). The subcellular localisation of type II PKA is due to its association with AKAPs. AKAPs are a large family of proteins which can promote activation of PKA by locating it near a site of stimulation, or it can limit PKA activation by localising it with enzymes which are inhibitory to cAMP (Colledge and Scott, 1999). Binding of cAMP exerts a conformational change on the PKA complex and the catalytic subunits become activated and released. PKA specifically phosphorylates serine and threonine residues found within RRXS/T motifs leading to phosphorylation of ion channels, cytoskeletal proteins and intracellular enzymes (Kennelly and Krebs, 1991). Another function of PKA is the phosphorylation and activation of PDEs, which results in an increase in cAMP degradation. A study in bovine vascular smooth muscle cells showed that PKA could activate the cAMP specific PDE4 indicating that PKA regulates a negative feedback effect on cAMP by the phosphorylation of PDEs (Eckholm et al., 1997). The cAMP pathway terminates in the regulation of several transcription factors which include; CREB, cAMP response element modulator (CREM), activating transcription factor (ATF)-1 and NF- κ B.

1.5.1 cAMP-Regulated Transcriptional Regulation

The cAMP responsive element (CRE) is a DNA sequence which has the consensus nucleotide sequence TGACGTCA, found within gene promoter regions and relays cAMP signalling activity to the gene (Montminy et al., 1986). CREB belongs to a group of transcription factors which contain basic region leucine zippers (bZIP) (Landshulz et al, 1988). The cAMP-responsive bZIP transcription factors form a distinct group which also includes AP-1, CREM and ATF-1 (Meyer and Habener, 1993). CREB, CREM and ATF-1 share an amino-terminal proximal kinase-inducible domain (KID) which is

flanked by a glutamine-rich transactivation domain. Transcriptional activity of CREB is regulated within KID, in particular, by the phosphorylation of serine residue 133 (Gonzalez and Montminy, 1989). Although this residue is the target for PKA activity, other kinases can also phosphorylate serine 133. The ribosomal S6 kinase RSK2 has been reported to function as a CREB kinase as has PKC indicating a potential amplification of cAMP signalling by other transduction pathways (Xing et al, 1996; Yamamoto et al., 1988). The maximal effect of phosphorylation of serine 133 within the KID domain of CREB is achieved through the association of a 256kDa nuclear factor which acts as a transcriptional coactivator called CREB binding protein (CBP) (Kwok et al., 1994). CBP recognises phosphorylation of serine 133 through a domain called KIX which enables CBP binding to CREB on an arginine residue found close to serine 133 (Parker et al., 1996). A nuclear factor called p300 is homologous to CBP and can additionally interact with CREB. CBP and p300 can both be bound by the adenovirus E1A protein which has an inhibitory effect on CREB activity. CBP and p300 function to regulate other transcriptional events, enabling signals, most of which regulate differentiation, to integrate with gene expression (Shikama et al, 1997). Other transcription factors which act on CBP/p300 include; the transcriptional coactivator p/CIP, AP-1, and STAT complexes (Torchia et al., 1997).

1.5.2 cAMP Activation of NF- κ B

The transcription factor NF- κ B is widely expressed and plays an important part in immune and stress responses by regulating gene expression. It is comprised of protein homodimers or heterodimers that share a 300 amino acid homology domain region and belong to the Rel family of proteins. Members of the Rel family found in mammalian cells include c-Rel, p105, p100, RelA (p65) and RelB (Baldwin, 1996). In *Drosophila melanogaster* cells there are Rel proteins which have similar characteristics to mammalian homologues, examples are; Dorsal, important in dorso-ventral patterning, and Dif, an inducible transcription factor which is important in immune responses (May

and Ghosh, 1998). NF- κ B is found in the cytoplasm of most cells, bound to inhibitory proteins called inhibitor (I) κ B of which there are three isoforms; I κ B- α , β and ϵ . When NF- κ B is induced by such factors as TNF α and IL-1, serine residues at positions 32 and 36 of I κ B become phosphorylated. This phosphorylation is induced by I κ B kinases (IKK) which are activated by signalling elements which have been induced by TNF α and IL-1, called TNF receptor associated factor (TRAF)-2 and TRAF6 respectively. TRAFs interact with a MEK kinase called NF- κ B-inducing kinase (NIK) which leads to the activation of IKK and the resultant phosphorylation of I κ B serine residues. This leads to polyubiquitination and degradation of I κ B by the 26S proteasome enabling NF- κ B to translocate to the nucleus where it is able to regulate gene expression (DiDonato et al., 1997; Maniatis, 1997). cAMP and PKA exert cell-specific effects on NF- κ B-induced reporter gene expression. A study showed that elevation of intracellular cAMP resulted in the inhibition of NF- κ B induced activation of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and TNF α in human monocytic and endothelial cells (Ollivier et al., 1996). The effect of PKA on NF- κ B may be independent of cAMP, since the catalytic subunit of PKA has been shown to directly phosphorylate and activate the p65 Rel protein of NF- κ B following the degradation of I κ B. The same study revealed that cAMP-induced PKA did not significantly affect NF- κ B activity (Zhong et al., 1997). The p65 NF- κ B subunit has also been reported to interact with CBP and p300 (Gerritsen et al., 1997).

1.6 IL-2 AND THE IL-2 RECEPTOR COMPLEX

IL-2 was originally identified as a growth factor for T cells (Morgan, Ruscetti and Gallo, 1976). It is a multifunctional cytokine that modulates the growth, differentiation and apoptosis of lymphocytes including; T, B and natural killer (NK) cells, as well as monocytes and some haematopoietic cells (Smith, 1988). IL-2 is produced mainly by CD4⁺ T_H cells and to a lesser extent by CD8⁺ cells and large granular lymphocytes. In common with the IL-4R complex, the IL-2 receptor has no intracellular kinase activity

and relies upon the activity of non-receptor tyrosine kinases to activate IL-2-induced signalling pathways. The IL-2 receptor consists of three subunits; the constitutively expressed 75kDa β and 64kDa γ chains and an unique, inducible 55kDa α chain (see Figure 1.4). These subunits are also termed CD122, CD132 and CD25, respectively. The IL-2R β subunit is additionally found in the IL-15 receptor complex along with γ ; however, both IL-15R and IL-2R have different α subunits (Waldmann, Tagaya and Bamford, 1998). The constitutive expression of IL-2R β and γ creates a heterodimeric IL-2 receptor complex which has a dissociation constant (K_d) of 10^{-9} M for IL-2 and is referred to as the intermediate affinity complex. The induction of IL-2R α (CD25) expression leads to the formation of a heterotrimeric complex with IL-2R β and γ and an increased K_d of 10^{-11} M for IL-2. This complex is recognised as the high affinity complex. CD25 expressed alone generates a low affinity binding complex for IL-2, with a K_d value of 10^{-8} M (Minami et al., 1993). Although CD25 is unable to activate intracellular signalling pathways, experiments with chimeric IL-2R molecules on leukemic large granular lymphocytic (LGL) cells and T cells indicated that CD25 is able to present IL-2 to an IL-2R β / γ complex on adjacent cells, thereby promoting IL-2 signalling (Eicher and Waldmann, 1998). The role of CD25 in the receptor complex can therefore be defined as one which enhances ligand affinity and effect, while the role of IL-2R β and γ is to initiate intracellular signalling events. IL-2 is an important biological activator of the expression of CD25 in T cells (Malek and Ashwell, 1985). A recent study on a mouse T cell line grown in IL-2-containing medium showed that, *in vitro*, IL-2 has a wide and essential role on the expression of genes which encode cytoskeletal proteins, oncogene regulators and transcription factors (Herblot et al., 1999). IL-2 binding to the intermediate or high affinity receptor complex in lymphoid cells leads to the activation of several previously described signalling pathways (Figure 1.4). These are; the Ras/MAP kinase pathway which regulates mitogenesis, the PI-3 kinase pathway which is involved in cytoskeletal organisation and the JAK/STAT pathway, in particular JAK1 and JAK3 which activate STAT3 and STAT5, which exert multiple effects via transcriptional regulation (Nelson and Willerford, 1998).

1.6.1 The Expression of CD25

CD25 is expressed at an early stage of development and marks the initiation of T cell receptor (TCR) rearrangement and commitment to the T cell lineage. Following successful TCR β rearrangement, constitutive CD25 expression is lost and is not observed at any other stage of T cell development (Godfrey and Zlotnick, 1993). CD25 expression is induced in mature resting T cells upon TCR stimulation or by non-TCR activation in response to IL-1 or TNF α (Plaetinck et al., 1990; Rothenberg, 1992). In murine B cells, there is a similar observation of expression as CD25 is found on pre-B cells (Rolink et al., 1994). In murine and human mature B cells, CD25 is upregulated in response to BCR activation (Benschop and Cambier, 1999). In addition to BCR induced expression, IL-4 has also been reported to upregulate CD25 expression in resting human tonsillar B cells by inducing a loss of activity on proteins binding to a negative regulatory region which suppresses CD25 transcription (Hewitt et al., 1997). Other compounds which have been reported to upregulate CD25 expression in B cells are; CD40 specific antibody (Ab) and anti-Ig Ab (Burlinson et al., 1995). The reported effect of IL-4 on B cells is in direct contrast to the effect on T cells, where IL-4 is reported to have an inhibitory effect on CD25 expression (Jankovich et al., 1989).

1.6.2 Human CD25 Promoter Regulation

Expression of human CD25 is regulated by three positive regulatory regions (PRR_{I-III}) and two negative regulatory regions (NRR_{I-II}) in the proximal promoter region of the CD25 gene (Figure 1.5a). PRR_I is located between nucleotides -276 and -244, relative to the major transcription start site. It contains binding sites for NF- κ B and serum response factor (SRF) which have been shown through transient transfection studies to control PRR_I activity (Pierce et al., 1995). PRR_I is responsive to mitogenic and antigenic signals resulting from TCR activation or stimulation by phorbol ester, IL-1 and

TNF α . PRR_{II} is a T-cell specific promoter and is found between nucleotides -137 and -64. PRR_{II} contains binding sites for the Ets family protein, Elf-1 (Graves and Petersen, 1998) and the non-histone chromatin-associated protein HMG-1(Y) (Landsman and Bustin, 1993). This region is principally involved in basal promoter activity and in T-cell specific CD25 expression (John et al., 1995). Antigen is the primary stimulus which induces CD25 transcription, although IL-2 is required to elicit a maximum and prolonged expression (Sperisen et al., 1995). PRR_{III} is an IL-2 responsive element (rE) located between nucleotides -3780 to -3703 (or according to another group, approximately 80 nucleotides around position -4150) and is similar to PRR_{II} as it also contains binding sites for Elf-1 and HMG-1(Y). In addition, PRR_{III} contains a GATA site and a binding site for STAT5 which is activated in response to IL-2 ligation of the IL-2R, leading to an upregulation of CD25 expression. While the binding site for STAT5 has an enhancing effect on PRR_{III} induction, Elf-1 appears to negatively regulate the constitutive expression of CD25 in unstimulated T cells (John et al, 1996; Lecine et al., 1996).

1.6.3 The Human CD25 Negative Regulatory Regions

Transient transfection studies using deletion mutants of the CD25 promoter identified two negative regulatory regions; NRR_{I-II} between nucleotides -400 and -368, and -300 to -313 respectively. Figure 1.5b shows that within the NRR_I 31 base pair (bp) region is an 11bp core element (TTCATCCCAGG) which regulates both basal and mitogen induced transcription in Jurkat T cells and is referred to as the negative regulatory element (NRE). UV cross-linking studies indicated that NRE is bound by a 50kDa NRE-binding protein (NRE-BP) which leads to the repression of NRE activity. NRE-BP was also found to repress an element related to NRE in the promoter region of the 5' long terminal repeat (LTR) of type 1 human immunodeficiency virus (HIV-1) (Smith and Greene, 1989). In human tonsillar B cells, transient transfection studies using deletion mutants lacking both the NRE and the entire NRR_I region demonstrated that NRE is a

functional repressor of CD25 transcription in B cells. DNA-binding protein activity which binds specifically to the NRE region was lost and CD25 expression induced when the cells were treated with human recombinant IL-4 (Hewitt et al., 1997). The identity of NRE-BP is undefined, however an IFN-regulated gene called stimulated trans-acting factor of 50kDa (Staf-50) was shown to down-regulate transcription regulated by the promoter region of HIV-1 LTR, suggesting a possible candidate for this protein (Tissot and Mechti, 1995; Tissot et al., 1996). Immediately 3' to NRE is a retinoid sensitive region (GCTGAC). Retinoic acid has been reported to induce transcriptional regulation of CD25 in human B cells (Bhatti and Sidell, 1994). In addition, treatment with IL-4 stimulates a loss of binding activity for retinoic acid response element (RARE)-specific oligonucleotides. While it is clear the retinoid-sensitive sequence has an active role in the CD25 promoter gene, protein binding activity to this region has yet to be characterised (Hewitt et al., 1997).

Overlapping the putative RARE sequence, is a further element (TGACTCC) which is highly homologous to the consensus AP-1 binding site (Figure 1.5b). In lymphocytes, the role of AP-1 is important in the regulation of gene expression which induces lymphocyte differentiation and activation (Foletta et al, 1998). AP-1 is a homo- or heterodimeric complex which binds the consensus nucleotide sequence TGACTCA (Vogt and Bos, 1990). The complex is made up of two protein families called fos and jun that dimerise via a leucine zipper interaction. The fos family members include c-fos, fos-related antigen-1 (fra-1), fra-2, fosB and its truncated form, fosB2. There are three jun family members; c-jun, junB and junD. Jun proteins can homodimerise or heterodimerise with fos, whereas fos is unable to homodimerise and cannot bind DNA alone (Halazonetis et al., 1988). AP-1 complexes belong to the same family of bZIP proteins as the previously described CREB, CREM and ATF-1, but can also interact and form protein complexes with NF- κ B enhancing DNA binding and function. A study of p65 and NF- κ B interaction demonstrated *in vitro* a synergistic activation of the 5' LTR of the the HIV-1 virus in human cell lines (Stein et al., 1993). An important mediator of

the transcriptional regulation of AP-1 is the MAP kinase signal transduction pathway (Angel and Karin, 1991; Karin et al., 1997). The MAP kinase pathway regulates the level of gene transcription which transcribes fos and jun proteins, shortly after cell stimulation. AP-1 protein function is activated mainly by phosphorylation (Whitmarsh and Davis, 1996). In B cells, another previously described pathway which is important in AP-1 induction is the PKC signalling pathway. A study co-cross-linking sIg with the CD40 receptor in B cells demonstrated that activation of the PKC pathway was essential for cellular proliferation, as PKC depletion blocked cell cycle progression (Kawakami and Parker, 1993).

1.6.4 AP-1 Expression in B Cells

In primary B cells and B cell lines, surface Ig cross-linking was shown to induce c-fos expression (Chan et al., 1993). A study of murine B cells examined the effect of B cell activation through the individual stimulation of the sIg and CD40 receptors on AP-1 regulation, using anti-Ig Ab and CD40 ligand. Both forms of stimulation lead to the expression of junB and junD but not c-jun. JunB induction by anti-Ig Ab was dependent on PKC whereas CD40 mediated induction was independent of PKC. In addition, both forms of receptor stimulation lead to the expression of c-fos, fosB and fra-1 but not fra-2, however fosB was only induced by anti-Ig Ab activation. Both c-fos and fosB were induced independently of PKC signalling whereas fra-1 expression was PKC-dependent (Huo and Rothstein, 1995; Huo and Rothstein, 1996). AP-1 is involved not only in positive regulatory transcriptional activity, but also negative regulatory activity. A truncated form of fosB has been reported to inhibit fos and jun transcriptional activity and junB has been found to be less active in AP-1 complexes than c-jun (Chiu et al., 1989; Nakabeppu and Nathans, 1991). In B cells, AP-1 has been reported to be associated with the activation of the κ -light chain promoter and the IgH chain gene, indicating a role for AP-1 in Ig synthesis (Grant et al., 1995; Schanke et al., 1994). Given the reported role of AP-1 in B cell promoter regions, it is highly likely that the

TGACTCC region of NRR_I , if bound by AP-1, has an important role in the regulation of expression of CD25.

1.6.5 The Murine CD25 Promoter

In common with the human CD25 promoter region, IL-2-driven stimulation of the mouse CD25 promoter is controlled by an IL-2rE. The murine IL-2rE is found 1.3kb upstream of the major transcription start site, spanning positions -1377 to -1312. The IL-2rE contains three elements which are involved in enhancer activity, site I (CTTCTGAGAAG) site II, (TTCTGATAA) and site III (AACTTCCTGA) (Sperisen et al., 1995). Sites I and II contain regions which are bound by STAT proteins. A study using a hybrid between a mouse cytolytic T cell line and a rat thymic lymphoma (PC60 cell line) demonstrated STAT5 bound sites I and II and mediated IL-2 stimulation of CD25 transcription (Meyer et al., 1997). In addition, studies in STAT5A knockout mice showed a severe reduction in IL-2-induced CD25 expression (Nakajima et al., 1997). Site III contains two consensensus sequences for Ets proteins and studies on PC60 cell nuclear extract demonstrated that Elf-1 binds to this site and regulates IL-2 responsiveness (Serdobova et al., 1997). The binding regions of sites I,II and III in the murine CD25 IL-2rE are fully conserved in the IL-2rE of the human CD25 promoter gene (Bucher et al., 1997).

A recent report demonstrated that IL-2, in addition to activating STAT5, may prime the CD25 gene for transcription by making CD25 chromatin available to transcription factors on the IL-2rE (Rusterholz et al, 1999). Transcriptional control of the murine CD25 gene is regulated by an additional promoter proximal region (PPR) and is induced by the activating effects of IL-1. It is located between nucleotides -584 to -54 and has been shown to be necessary for the rapid appearance of CD25 mRNA following IL-1 stimulation, in PC60 cells (Freimuth et al., 1989). In common with the human counterparts PRR_I and PRR_{II} , mouse PPR was shown to be sensitive to the

transcriptional activities of NF- κ B (Nabholz et al., 1995). A further region has recently been identified on the CD25 promoter. The NF-AT family members NF-ATp and NF-ATc have been reported to bind two sites located between positions -585 and -650, upstream of the proximal CD25 promoter in murine T cells (Schuh et al., 1998).

1.7 AIMS OF PROJECT

The aims of this project are three-fold. The first aim is to study and characterise the signalling mechanisms generated by IL-4 that lead to the loss of NRE binding activity and the up-regulation of CD25 expression. It has been shown, in this laboratory, that IL-4-driven CD25 expression is mediated via a cAMP/PKA-sensitive pathway. The effects of addition of the catalytic subunit of PKA, PKAc, to cell extracts on NRE-BP activity will be investigated. Use of cell signalling inhibitors and transient-transfection of model cell lines with CD25-CAT reporter constructs will address other potential signalling pathways through which IL-4 may up-regulate CD25 expression.

Secondly, the nature of protein binding to the NRR₁ region will be studied. It has already been shown that IL-4 attenuates NRE-BP binding to NRE, but the effect of this cytokine on the entire NRR₁ region is unknown. NRE-BP has yet to be defined and several proteins will be assessed as candidates for this important molecule. NRR₁ contains a retinoid sensitive region which is characterised by a consensus AP-1 binding site. Protein binding to NRE and the consensus AP-1 binding region within NRR₁ will be assessed by EMSA analysis. In addition, protein binding to the consensus AP-1 binding site will be investigated in an attempt to characterise the region.

The transcriptional activity of NRE and the consensus AP-1 binding region will be assessed by transiently-transfecting lymphoid cells with NRR₁ deletion mutant constructs. Finally, four putative STAT6 binding sites within the proximal promoter region of CD25 will be investigated by transfecting lymphoid cells with CD25 deletion mutant reporter constructs and then examining the effects of IL-4 stimulation. This project will investigate the signalling events that up-regulate CD25, the identity and interactions of proteins binding to NRR₁ and the transcriptional activity of the region, as well as other specific areas within the CD25 promoter.

Figure 1.1. Human B Cell Development

Cellular stages of B cell development in human bone marrow (antigen-independent) and periphery (antigen-dependent). Antigen-independent model based on figure presented by (Ghia et al., 1998).

The differentiating pro-B cell develops into a pre-B-I cell, which initiates H-chain rearrangement by binding a D gene, the TdT-formed extra nucleotide (N) region and a J region. In forming a DNJ_H gene; the developing B cell enters the pre-B-II stage and increases in size. A V region gene is then joined to form a V_HN-DNJ_H gene which is capable of producing a μ -H chain. In the endoplasmic reticulum (ER) in some pre-BI and in most large pre-BII cells, V_{pre-B}(1/2) and λ_5 (murine) or $\lambda_5/14.1$ (human) are expressed. These Ig like proteins are able to bind together via an extra β strand located in the V_{pre-B} chain to form a surrogate light chain (SLC). Once formed, the SLC binds to the newly-formed μ -H chain and forms a complex in association with a signal transducing heterodimer consisting of two Ig α and Ig β subunits, CD79a and CD79b. The complex leaves the ER with an $\alpha\beta$ heterodimer flanking each side of the SLC-heavy chain arrangement and translocates to the cell surface where it is expressed as a pre-B cell receptor (BCR). Immature cells which have not been subject to autoantigen challenge or have undergone successful receptor editing increase surface levels of IgM and enter the antigen dependent stage of B cell development

When presented with antigen, the mature B cell can undergo rapid clonal expansion and plasma-cell differentiation to give rise to a high antigen-affinity antibody producing cell. Alternatively, the cell enters the germinal centre reaction and remains in the secondary lymphoid tissue often for many years as a memory B cell.

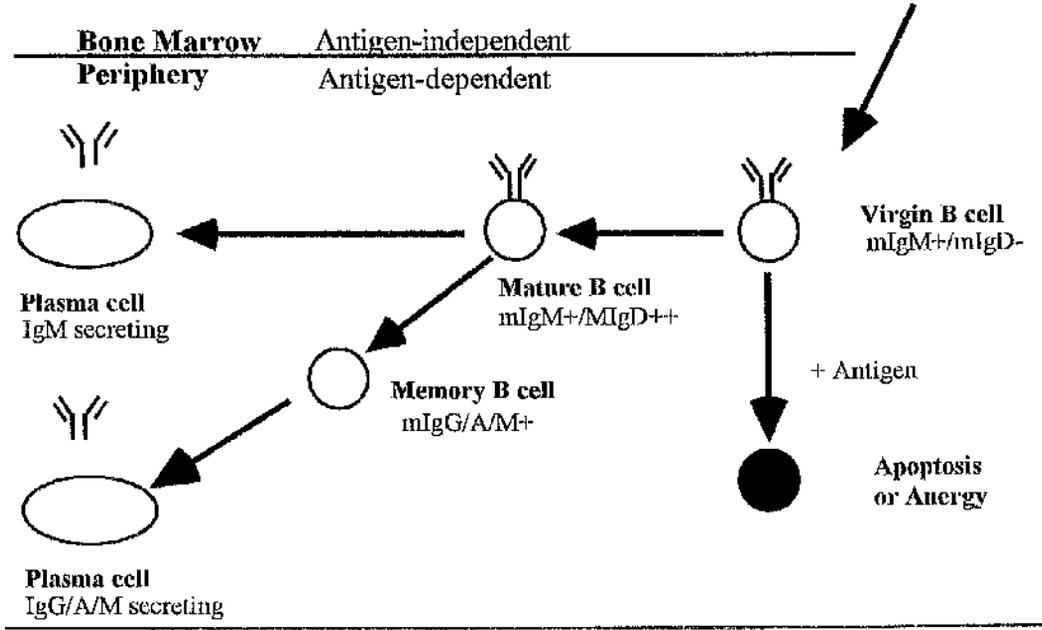
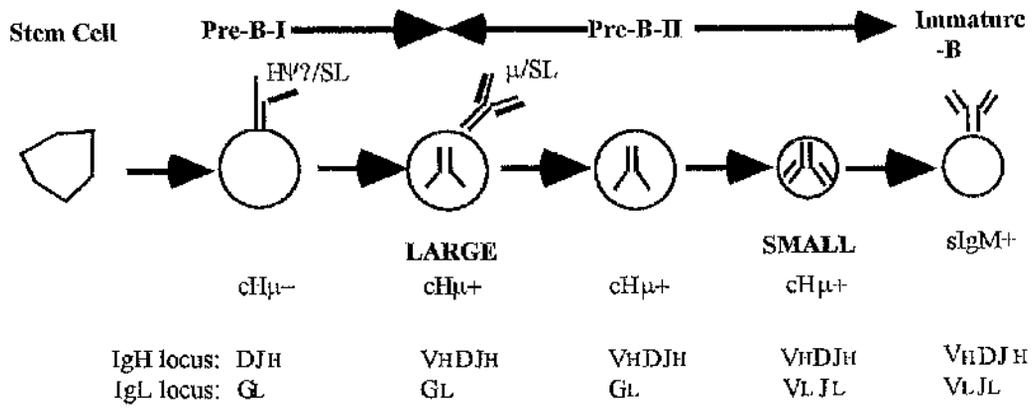


Figure 1.2. B Cell Receptor and Associated Signal Transduction Cascades.

When bound by antigen or anti-receptor antibody, the BCR aggregates and initiates signal transduction pathways through the Ig α and Ig β subunit-ITAM motifs. The main signalling pathways shown are driven by a cascade of phosphorylation events involving cytoplasmic and membrane proteins.

PLC γ 2 activation is achieved following BCR stimulation. Src-family member Lyn becomes activated leading to the tyrosine phosphorylation of ITAM motifs in Ig α and Ig β subunits. Syk is recruited to the phosphorylated ITAMs via SH2 interaction. Lyn-activated PI-3 kinase produces PI-3,4,5-P $_3$ which recruits Btk to the membrane by binding its PH domain. Lyn then phosphorylates tyrosine residues in Syk and Btk leading to their activation. Syk phosphorylates BLNK and recruits it to the membrane where a Syk-BLNK-Btk complex is formed. Phosphorylated BLNK then recruits PLC γ 2 to the complex where it becomes tyrosine phosphorylated and activated, initiating a signal transduction pathway. Adapted from (Kurosaki, 1999).

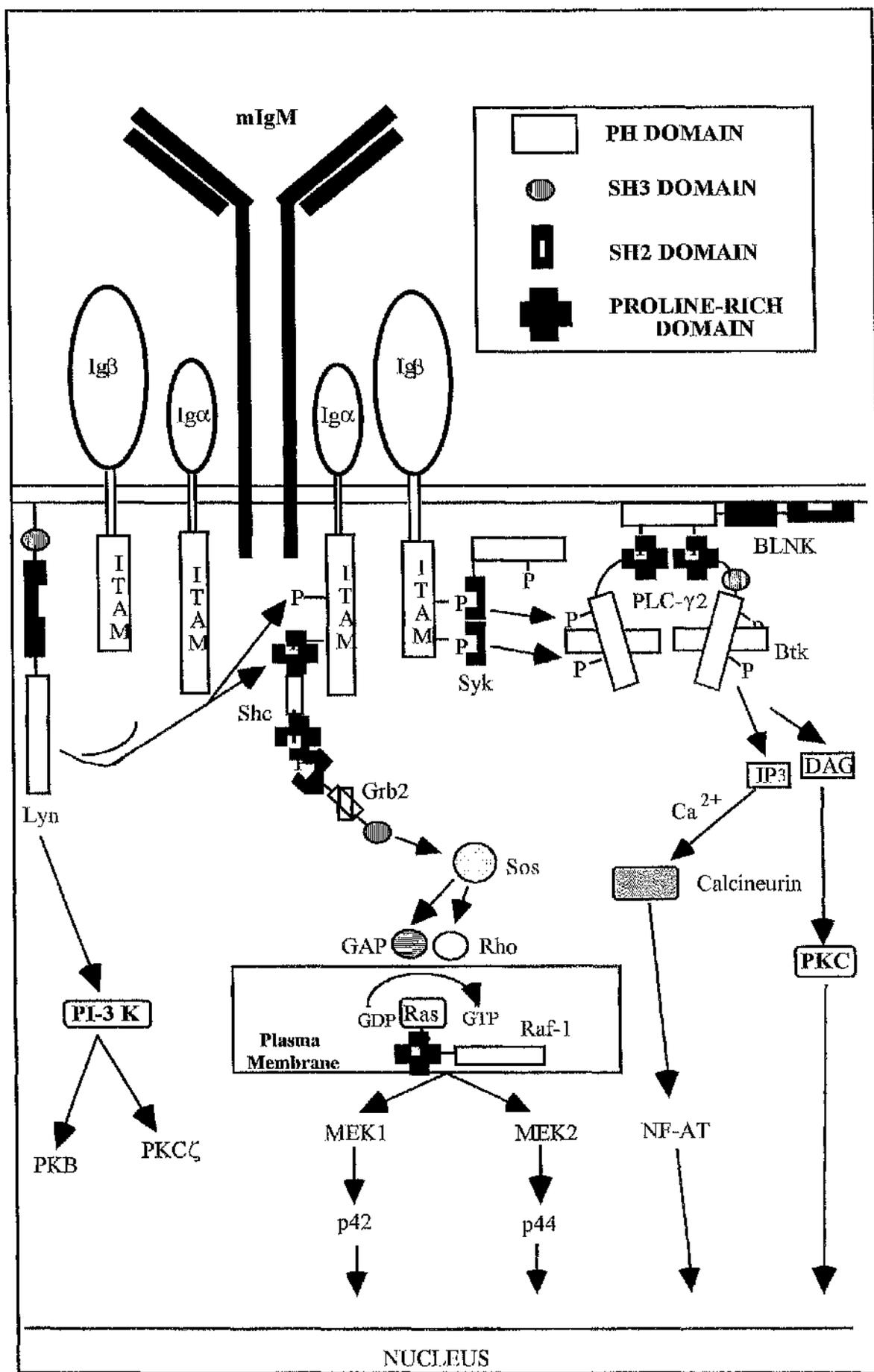


Figure 1.3. IL-4 Receptor Complexes with γ c and IL-13R Subunits and Signalling Pathways.

In B cells, the most common IL-4 receptor complex is formed by IL-4R α and γ c. Signalling pathways initiated by IL-4 binding are the PI-3 kinase pathway and several JAK/STAT interactions.

The PI-3 kinase pathway activates downstream proteins such as PKB and PKC ζ stimulating cell growth and survival. JAK1 and JAK2 are activated via phosphorylation at the IL-4R α receptor. Both forms initiate the dimerisation of STAT5a and STAT5b which migrate into the nucleus and regulate transcriptional events culminating in cellular proliferation. JAK3, phosphorylated by the γ c receptor subunit, activates STAT6 dimerisation events resulting in the expression of CD23 and MHC Class II expression at the cell surface.

This model is partially based on a figure from (Nelms et al., 1999).

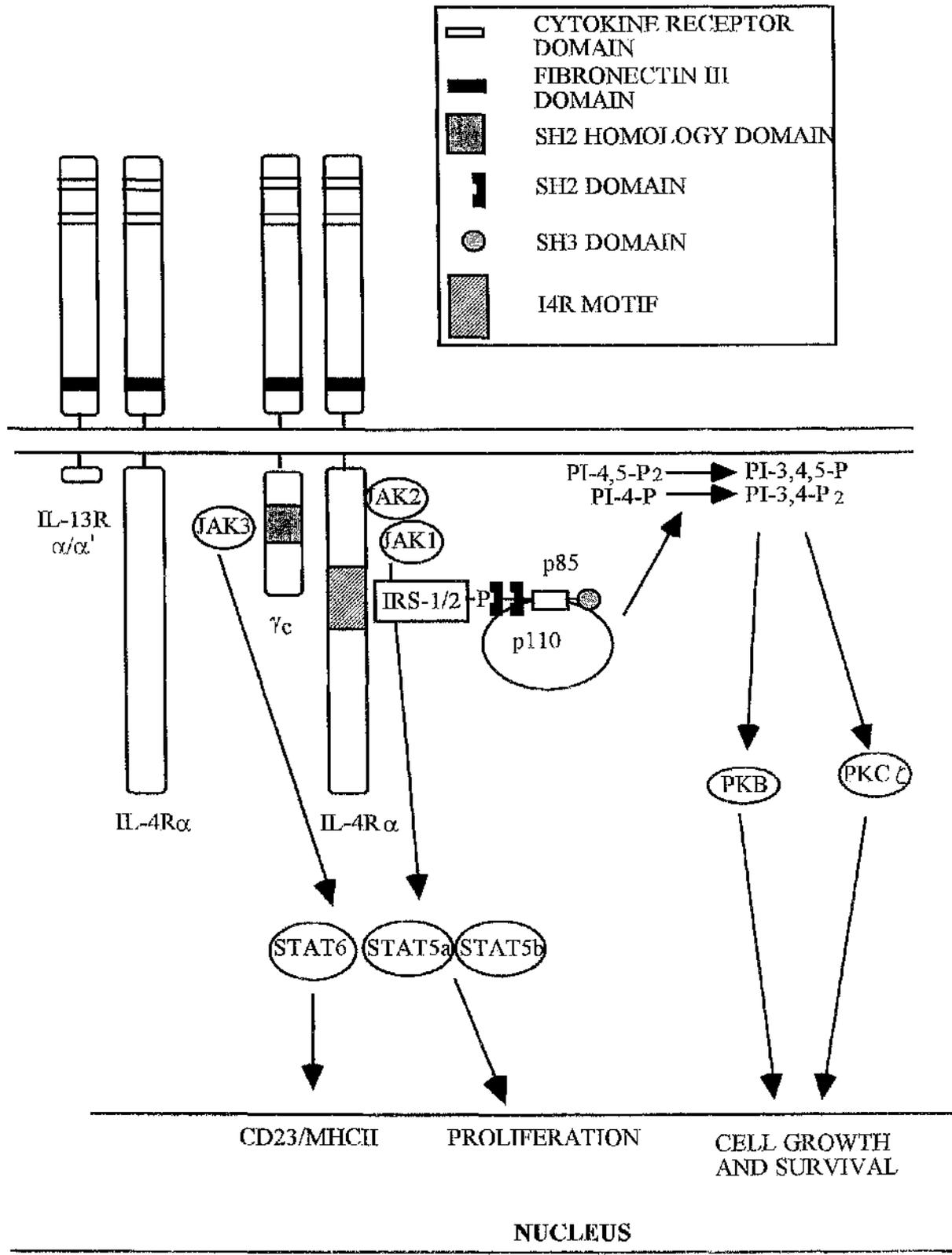


Figure 1.4. High Affinity IL-2 Receptor Complex and Associated Signalling Pathways.

Expression of the unique CD25 subunit at the cell surface creates a complex with the β and γ subunits which binds IL-2 with high affinity. IL-2R activation leads to the initiation of signalling events via the β and γ subunits, including JAK/STAT, Ras/MAP kinase and PI-3 kinase pathways. The pathways terminate at the nucleus where they regulate transcriptional activity.

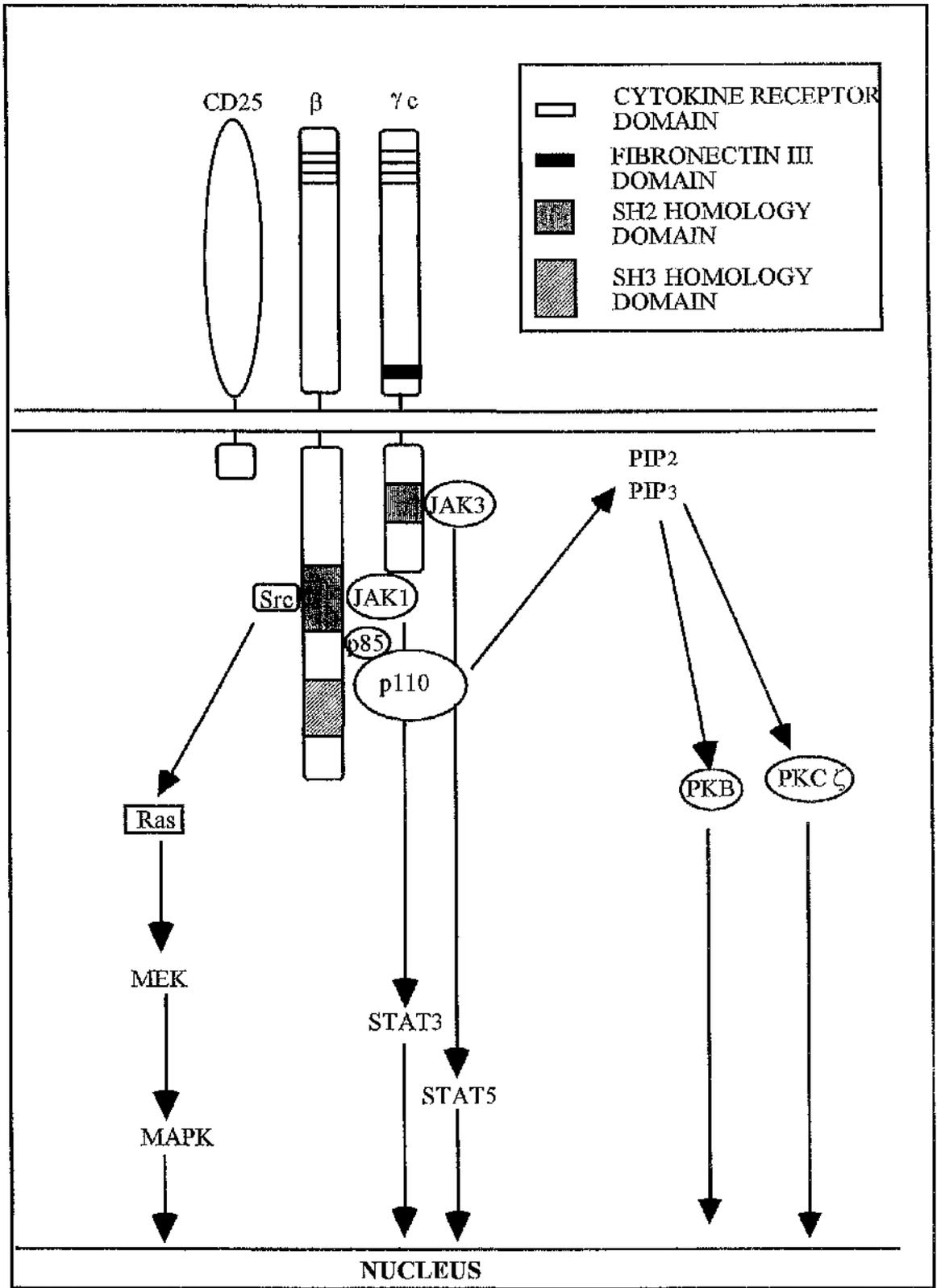
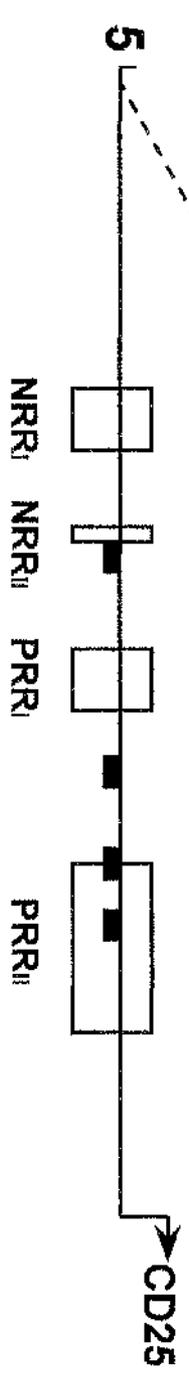
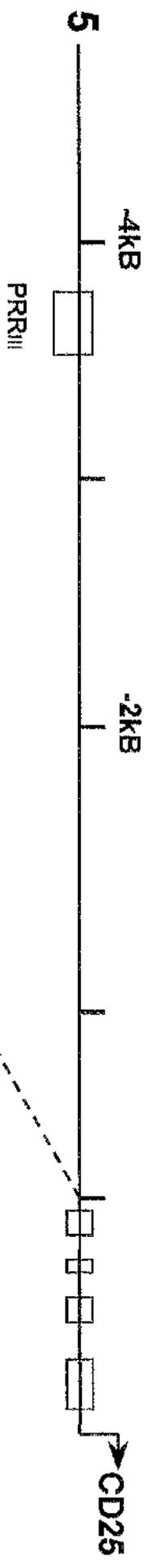


Figure 1.5 a). The Human CD25 Promoter Region.

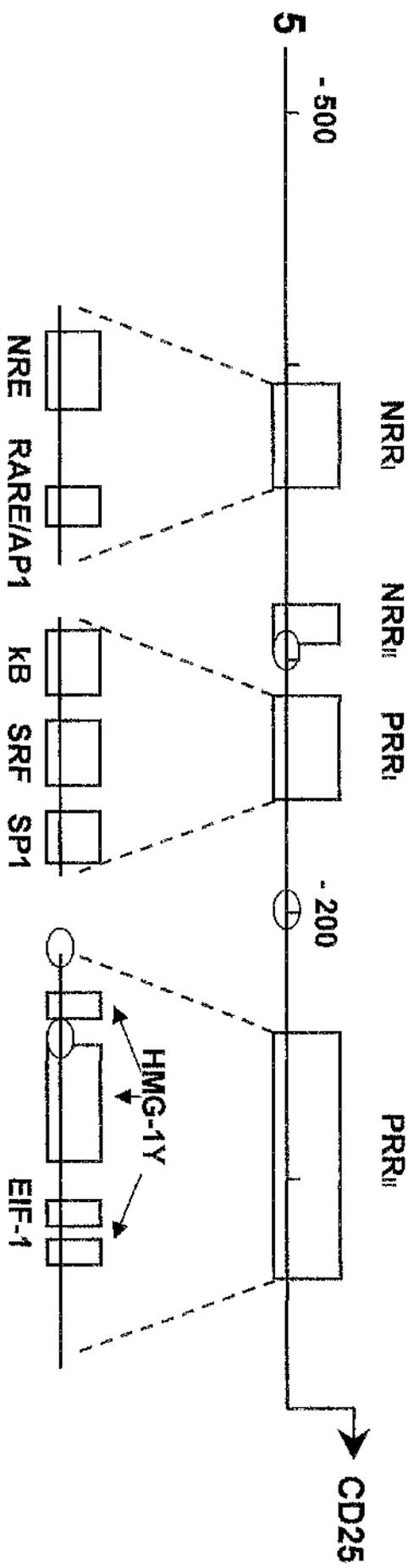
Schematic diagram of the human CD25 promoter region including the distal PRR_{III} and the proximal regions; PRR_{I-II} and NRR_{I-II}. In addition, four putative STAT6 sites consensus and non-consensus to the TTCNNNGAA GAS sequence, are outlined as open boxes.

Figure 1.5 b). The Human CD25 Proximal Promoter Region

Diagram of the proximal region of the CD25 promoter gene. Shown are the NRE and RARE/AP-1 binding regions of NRR_I which form the main negative regulatory domain of the CD25 promoter. This region forms the main area of investigation. Adjacent to NRR_I are the smaller negative regulatory region NRR_{II} and the promoter regions; PRR_I and PRR_{II}.



■ Putative STAT6 Binding Domain



○ Putative STAT6 Binding Domain

CHAPTER 2
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

All chemicals used were purchased from Sigma-Aldrich Ltd., Dorset, UK., unless otherwise stated.

2.1.2 Oligonucleotides

Consensus NRE and AP-1 sequences shown in **bold**, and mutated sites are shown in *italics*.

CD25-NRR_I 5'-GCTCCT**TCAT**CCCAGGTGGTCCCGGC**TGACT**CCTGAGGA-3'
CD25-NRR_I-M1 5'-GCTCCT**TAGATCT**AGGTGGTCCCGGCT**GACT**CCTGAGGA-3'
CD25-NRR_I-M2 5'-GCTCCT**TCAT**CCCAGGTGGTCCCGGCC**GACT**CCTGAGGA-3'
CD25-NRR_I-M3 5'-GCTCCT**TAGATCT**AGGTGGTCCCGGCC**GACT**CCTGAGGA-3'
CD25-AP1 5'-GCT**GACT**CCTG-3'
CD25-AP1m 5'-GCC**GACT**CCTG-3'
CD25-NRE 5'-CCT**TCAT**CCCAGGTG-3'
CD25-NREm 5'-CCT**TAGATCT**AGGTG-3'

The above oligonucleotides were synthesised and supplied by CRC Beatson Laboratories, Glasgow, UK.

CD25-NRE 5'-C**TT**CA**TCCC**AGGT-3'
CD25-NREm 5'-ACT**TGGG**ATGAAG-3'

The above oligonucleotides were synthesised and supplied by Cruachem Ltd., Glasgow, UK.

CD25-NRE 5'-**TTCATCCCAGG**-3'
 CD25-NREm 5'-*AAGTAGGGTCC*--3'

The above oligonucleotides were synthesised and supplied by Genosys Biotechnologies, Cambridgeshire, UK.

YY1 5'-CGCTCCGCGGCCATCTTGGCGGCTGGT-3'
 YY1m 5'-CGCTCCGCGATTATCTTGGCGGCTGGT-3'

The above oligonucleotides were synthesised and supplied by Santa Cruz Biotechnology, Inc., Middlesex, UK.

NF- κ B 5'-AGTTGAGGGGACTTTCCCAGGC-3'

The above oligonucleotide was synthesised and supplied by Promega, Southampton, UK

2.1.3 PCR Primers

Full length CD25 5' primer 5'-GACAAGCTTGAATTCTCAGGATCCTTCAGTTC-3'
 NRE deletion 5' primer 5'-GCAAAGCTTTGGTCCCGGCTGACTCCTGAG-3'
 NRR_I deletion 5' primer 5'-GCAAAGCTTTGAGGACGTTACAGCCCTGA-3'
 PRR_I-NF- κ B 5' primer 5'-CTTCAAGCTTGGGAACTCCCTCT-3'
 PRR_I STAT6A deletion 5'-TTCAAAGCTTCTCATCAACCCAGC-3'
 5' primer
 PRR_{II} STAT6B deletion 5'-TTGAAAGCTTACCGCAAATA-3'
 5' primer
 PRR_{II} STAT6C deletion 5'-CAAACAAGCTTGTCATCAAAA-3'
 5' primer

3' universal primer

5'-GCAGCAGTCGACCTGCAGAAGGCCAGTTGC-3'

The above PCR primers were synthesised and supplied by CRC Beatson Laboratories, Glasgow, UK. Restriction enzyme sites are contained within the primers for cloning purposes. Shown in **bold** is the *Hind* III site and in *italics*, the *Sal* I site.

2.1.4 Antibodies

| Antibody | Supplier |
|---|---|
| Rabbit polyclonal IgG anti-CBP | Santa Cruz Biotechnology, Inc. |
| Rabbit polyclonal IgG anti-Ets-1/Ets-2 | Santa Cruz Biotechnology, Inc. |
| Rabbit polyclonal IgG anti-IL-4 Stat | Santa Cruz Biotechnology, Inc. |
| Mouse monoclonal IgG _{2a} anti-phospho-Tyr | Santa Cruz Biotechnology, Inc. |
| Rabbit polyclonal IgG anti-YY1 | Santa Cruz Biotechnology, Inc. |
| Anti-mouse Ig, horseradish peroxidase (HRP)-linked whole sheep antibody | Amersham Life Science Ltd., Amersham, UK. |
| Anti-rabbit Ig, HRP-linked whole donkey antibody | Amersham Life Science Ltd., Amersham, UK. |
| Monoclonal rabbit anti-human IgM (μ -chain specific) | Sigma-Aldrich, Ltd. |

2.1.5 Intracellular Signalling Modulators

| Compound | Name and Mode of Action | Supplier |
|------------------|---|---|
| Cytokine | Recombinant Human IL-4. Stimulates IL-4 Receptor. | Genzyme Diagnostics, Cambridge, UK. |
| Enzyme Inhibitor | Wortmannin (KY 12420). Blocks activation of PI-3 kinase. | Calbiochem-Novabiochem Ltd., Nottingham, UK. |

| | | |
|------------------|---|-----------------------------|
| Immuno- | Rapamycin. | Calbiochem-Novabiochem |
| modulator | Inhibits activation of p70 ^{S6} kinase. | Ltd. |
| Protein | PD98059 (2'-Amino-3'-methoxyflavone). | Calbiochem-Novabiochem Ltd. |
| Kinase Inhibitor | Inhibits MAP kinase activation by blocking ERK phosphorylation. | |
| | PKI. Inhibitor of PKA activation. | Sigma-Aldrich, Ltd. |
| | H-89. Inhibitor of PKA. | Sigma-Aldrich, Ltd. |

2.1.6 Tissue Culture Materials

| Material | Supplier |
|-----------------------------------|---------------------------------------|
| RPMI-1640 medium | Gibco-BRL Life Sciences, Paisley, UK. |
| Glutamine/Penicillin/Streptomycin | Gibco-BRL Life Sciences, Paisley, UK |
| Foetal Calf Serum | Gibco-BRL Life Sciences, Paisley, UK. |

| Complete RPMI 1640 medium | Incomplete RPMI-1640 medium |
|-----------------------------------|------------------------------------|
| 100U/ml penicillin | 100U/ml penicillin |
| 100ng/ml streptomycin | 100ng/ml streptomycin |
| 5mM glutamine | 5mM glutamine |
| 10% (v/v) Foetal Calf Serum (FCS) | |

2.1.7 Isolation of High Density Tonsillar B cells

Human tissue was obtained from juvenile tonsillectomy with the kind consent of parents and courtesy of Mr. Sadiq at the Royal Hospital for Sick Children, Glasgow, UK.

Percoll, Histopaque and 2-Aminoethylisothiuronium bromide (AET) were purchased from Sigma Aldrich Ltd.

Sheep Red Blood Cells in Alsever's Solution were supplied by SAPU, Carlisle, UK.

2.1.8 Electrophoresis and Western Blotting

Acrylamide/bis-acrylamide was obtained from Biorad Laboratories, U.K. N,N,N',-N'-tetramethylethylenediamine, Tween 20 and the Kodak X-ray exposure cassettes were from Sigma, Poole, Dorset, U.K. The 220kDa-14.3kDa molecular weight markers, nitro-cellulose and Enhanced Chemiluminescence (ECL) kits were from Amersham Life Science Ltd., Amersham, UK. Fuji Medical X-ray film was from Genetic Research Instrumentation, Essex, U.K. The Atto dual chamber mini gel electrophoresis kit was supplied by GRI, Sussex, U.K.

2.1.9 Electrophoretic Mobility Shift Assay (EMSA)

[γ -³²P]ATP was obtained from Amersham Life Sciences Ltd., Buckinghamshire, UK. Oligonucleotides and antibodies were acquired as described in the above Tables. Gels were mounted and electrophoresed on a Vertical Gel Electrophoresis System, Bethesda Research Laboratories, Gaithersburg, USA using a Shandon Vokam 500-150 power pack.

2.1.10 Transient-Transfection of Lymphoid Cells

All cells were electroporated in 4mm electroporation cuvettes obtained from Flowgen, Kent, UK, using a Biorad Gene Pulser and Capacitance Extender. pCAT-E' basic vector was obtained from Promega Life Sciences, Southampton, UK. Great EscAPE pSEAP control vector and Chemiluminescent SEAP Assay were purchased from Clontech,

Hampshire, UK. CD25 proximal promoter region plasmids were generated in this laboratory, as described (Hewitt et al., 1997). *D-threo*-[*dichloroacetyl*-1- C^{14}] Chloramphenicol was purchased from Amersham Life Sciences Ltd., Buckinghamshire, UK and Acetyl Coenzyme A was from Sigma-Aldrich, Dorset, UK. Thin layer chromatography was performed using PE SIL G silica gel plates, purchased from Whatman Ltd., Kent, UK. BAS-III phosphorimage plates and X-ray film were from Fuji Photo Film, Japan. Phosphorimage analysis was performed using a Fujix BAS-1000 Phosphorimager. Chemiluminescent SEAP assay analysis was carried out on an SLT Spectra Luminometer. Solvent evaporation was achieved using a speedivac unit consisting of a Jouan RC 10.22. centrifuge connected to a Jouan RCT 90 refrigeration unit and JAVAC DD75 High Vacuum Pump.

2.1.11 Plasmid Purification

Plasmid purification and QIAquick gel extraction kits were purchased from Qiagen, West Sussex, UK. Restriction enzymes and buffers were purchased from Promega, Southampton, UK and Boehringer Mannheim, East Sussex, UK. Centrifugation steps were carried out on a Beckman J2-HS centrifuge using type JA-14 and type JA-20 rotors.

2.1.12 Flow Cytometry

All flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson, UK) and all information processed using either a Becton Dickinson Consort 30 system containing; a Hewlett Packard 3000 series computer, 91533B hard disk drive, 9144 cassette drive and a 46020A keyboard, or the Becton Dickinson application for Macintosh, CELLQuest. RPE- and FITC-conjugated monoclonal mouse antibodies were obtained from Dako, Glostrup, Denmark. Streptavidin quantum red (SA-QR) and propidium iodide were purchased from Sigma-Aldrich, Dorset, UK.

2.2 METHODS

All solutions are described in the Appendix; section 8 pp. 189-193.

2.2.1 Preparation of 2-Aminoethylisothiuronium Bromide (AET)-Erythrocytes

Five millilitres of sheep red blood cell (SRBC) suspension was pelleted then washed twice in 25ml incomplete RPMI-1640 medium by centrifugation at 350 x g. The washed erythrocytes were resuspended in 4ml of filter-sterilised AET solution and incubated at 37°C for 20 min. The erythrocytes were then washed a further five times in 10ml incomplete RPMI-1640. Washed AET-SRBC were retained in 10ml incomplete RPMI-1640 at 4°C for a maximum of one week.

2.2.2 Isolation of High-Density Resting Tonsillar B Cells

Freshly excised tonsils were spilled into incomplete RPMI-1640 medium. Following passage through a fine wire mesh, the cell suspension was washed three times in incomplete RPMI 1640 then aliquoted onto 10ml of Ficoll-Hypaque and centrifuged for 15 min at 350 x g. The mononuclear cells were harvested from the interface, pooled and subjected to three further washes before being pelleted with 1ml AET-SRBC for five minutes at 90 x g. T cell populations were removed by rosette formation following 30 min incubation on ice with 1ml foetal calf serum (FCS) and centrifugation through 10ml of Ficoll for 20 min at 350 x g. The non-rosetted cell population was harvested from the interface, washed and then centrifuged for 30 min through a five-step, discontinuous percoll gradient made from dilutions in a incomplete RPMI-1640 medium/10x sterile PBS buffer to give densities of 1.055g/ml, 1.065g/ml, 1.075g/ml, 1.08g/ml and 1.09g/ml at 1000 x g. Quiescent tonsillar B cells were isolated in this gradient forming a high density solution within the 1.09g/ml-1.08g/ml interface. Cells were removed and washed

twice in incomplete RPMI-1640 medium then once in complete RPMI 1640 medium. Cells were rested for 1h in a 37°C incubator before treatment and then returned for the required incubation period.

2.2.3 Preparation of Whole Cell Extracts

High density tonsillar B cells were incubated for 16h in complete RPMI-1640 medium prior to extraction. Whole cell extracts were prepared from human B and T lineage cells which had been subjected to 24h incubation in fresh complete RPMI-1640 medium before stimulation. Following stimulation, the cells were washed in ice-cold PBS, resuspended in 1ml RIPA buffer and incubated on ice for 20 min. The lysates were then centrifuged at 11,592 x g in a bench-top microfuge to remove cellular debris. The supernatant was retained and stored at -20°C. Ten micrograms of lysate were boiled with 5µl of 2x sodium dodecyl sulphate (SDS) loading buffer before addition to the SDS-PAGE gel.

2.2.4 Preparation of Cytosolic and Nuclear Cell Extracts

Primary tonsillar B cells or B cell lines were cultured overnight in complete RPMI-1640 medium prior to extraction. A total of 5×10^6 cells were harvested for each extraction and washed in 10ml ice-cold PBS. Pellets were then resuspended in 300µl cytosolic extraction buffer and incubated on ice for 30 min. A volume of 250µl of 1% (w/v) NP-40 was added to lyse the cellular membrane and the nuclei were immediately separated by centrifugation at 322 x g in a benchtop centrifuge. The supernatant was retained as cytosolic extract, with the addition of 60µl 50% (w/v) glycerol. Extracts were stored at -70°C. The nuclear pellet was resuspended in 100µl of nuclear extraction buffer and placed on a revolving vertical platform for 30 min at 4°C. Nuclear fragments were pelleted at 11,598 x g on a Hareus biofuge for 15 min and supernatant retained as extract and stored at -70°C.

2.2.5 SDS-PAGE Electrophoresis and Western Blotting

A 10% (w/v) acrylamide separating gel was prepared using a 'Biorad' mini rig. The gel was poured and a layer of $(\text{CH}_3)_2\text{CHOH}$ added at the top which was drained when the gel had polymerised. A 5% (w/v) acrylamide stacking gel was poured on top and a teflon comb inserted. Once set, the gels were transferred to an Atto dual chamber mini gel electrophoresis kit and fully submerged in Tris-glycine electrophoresis buffer. The comb was removed and up to 15 μl of each of the samples was loaded in a pre-determined order. An equal volume of SDS loading buffer was loaded into any unused wells. The electrophoresis kit was attached to a Shandon power pack and an initial current of 15 milli-Amperes (mA) was applied until the sample had moved in to the 10% separating gel whereupon the current was increased to 25mA until the samples had migrated to the bottom of the separating gel.

Whatman 3MM paper and nitrocellulose membrane, cut to the same size of the gel were soaked in protein transfer buffer and carefully assembled with the gel and two porous pads in a Biorad protein transfer apparatus. The nitrocellulose membrane was layered over the gel facing the anode side and the 3MM paper and porous pads were layered over both the anode- and cathode-facing sides. The apparatus was filled with transfer buffer and packed in ice, then subjected to 80V over 1.5h. After this time the apparatus was disconnected and the nitrocellulose membrane retained. The membrane was treated with a 10% (w/v) non-fat dried milk or a 5% (v/v) Tween-20 (Polyoxyethylene-sorbitan monolaurate) solution, both made up in PBS, overnight at 4°C and then washed three times in PBS. The membrane was then treated with a rabbit polyclonal IgG primary antibody in 1% (w/v) non-fat dried milk or 0.1% (v/v) Tween-20, both made up in PBS, for 4 hours at 4°C on a Luckham R1000 rotator. Excess antibody was removed by three 10 min washes of the membrane with 0.1% (w/v) Tween-20 in PBS before a secondary horse-radish-peroxidase (HRP) antibody in 1% (w/v) non-fat dried milk or 0.1% (w/v)

Tween-20 in PBS, as above, was added for 1.5h at 4⁰C on the rotator. Excess antibody was removed by washing, in 0.1% (v/v) Tween-20 in PBS, as described.

The membrane was transferred to a Kodak X-ray exposure holder and treated with 10ml of combined ECL detection reagents for 1 min. The membrane was then exposed to X-ray medical film, for a time ranging from 30s to 5 min. The X-ray film was developed using a Kodak X-omat processor.

2.2.6 Restriction Enzyme Double Digestion

Ten microlitres of plasmid was mixed with 10 units of restriction enzyme and 2 μ l of restriction enzyme buffer, both specific for the particular cleavage site, to a final volume of 20 μ l and incubated at 37⁰C for 1h. For double digests, the least isotonic restriction enzyme buffer was used in the first instance. After the first incubation, the DNA was precipitated by addition of 1 μ l 3M CH₃COONa and 50 μ l 100% (v/v) CH₃CHOH then incubated for 1h at -70⁰C. Centrifugation at 11,592 x g for 5 min in a Biofuge pelleted the DNA, the supernatant was removed and the pellet washed in 70% ice-cold CH₃CHOH. A repeat centrifugation repelleted the DNA and the CH₃CHOH was discarded. Plasmid DNA was resuspended in 10 μ l of TE buffer and further digested by mixing with appropriate restriction enzyme and buffer as previously described.

When final restriction digests were complete, 2 μ l of 2x loading buffer was added to the mixture. Twelve microlitre aliquots were loaded into wells in a 1% agarose gel containing 4ng/ml ethidium bromide. Samples were run alongside 1 kilo base and 100 base-pair DNA ladders at 150V or 100V (if low melting point agarose was used in the gel). The gel was then transferred to a transilluminator, exposed to UV light and the digested DNA analysed and photographed using a high intensity video camera and processor.

2.2.7 Radiolabelling of Oligonucleotides

Oligonucleotides (3.5pmol) were radiolabelled with 1 μ l (10mCi/ml of 3000Ci/mmol) [γ -³²P]ATP, and mixed with 1 μ l 10x T4 polynucleotide kinase buffer and 1 μ l T4 polynucleotide kinase with 7 μ l dH₂O. Following a 10 min incubation at 37⁰C, the reaction was stopped by the addition of 1 μ l 0.5mM EDTA pH 8.3 and 89 μ l TE buffer.

2.2.8 Electrophoretic Mobility Shift Assay (EMSA)

Samples containing 20% (w/v) 5x binding buffer and 5 μ g protein extract, were incubated with and without the presence of excess unlabelled oligonucleotide for 10 min at room temperature. For supershift EMSA, 0.1 μ g of antibody was added to the sample, then incubated for 30 min at room temperature. All samples were then incubated at room temperature for 20 min with 3.5pmol radiolabelled oligonucleotide in a final volume of 10 μ l.

Samples were loaded on to either a 5% (w/v) or 7% (w/v) non-SDS denaturing gel and electrophoresed on a vertical gel electrophoresis system at 150V for 1.5 h. Migration of samples was marked by 10 μ l of 2x loading buffer in an adjacent well. Gels were then placed on Whatman 3MM filter paper and dried on a gel drier at 80⁰C under vacuum for 1h. The gels were transferred to a high-speed developing cassette then exposed to X-ray film for a variable time and stored at -70⁰C. X-ray film was developed using a Kodak x-omat procesor.

2.2.9 Transient-Transfection of Lymphoid Cells

Tonsillar B lymphocytes, B lineage cells and T lineage cells were cultured overnight in fresh complete RPMI-1640 medium. Cells were washed in incomplete RPMI-1640, resuspended at 5x10⁶cells/ml then transferred to 4mm electroporation cuvettes

containing 40µg of appropriate plasmid and 40µg of p-SEAP control plasmid and incubated on ice for 10 min. Cells were electroporated at 0.35kV/960µF for 10s then returned to ice for a further 10 min incubation. Cells were added to 10mls complete RPMI-1640 and incubated at 37°C for a minimum of 2h. For phorbol ester and IL-4 treatments, all cell lineages were treated with and without 10nM Phorbol-13 Myristate Acetate (PMA) and recombinant human IL-4 at doses indicated in individual figure legends in the Results section. Cells were incubated for 24-48h prior to harvest and assay.

2.2.10 Chloramphenicol Acetyl Transferase (CAT) Assay

Transfected cells were harvested from culture and washed in 10mls PBS. Cells were resuspended in 1ml PBS, transferred to an Eppendorf tube, and pelleted at 322 x g for 10 min on a Heraeus 'Biofuge'. The supernatant was discarded and the pellet resuspended in 100µl of 250mM Tris-HCl, pH 7.8. Extracts were made by three continuous cycles of freeze-thaw in a dry ice/ethanol mixture followed by a 37°C hot plate. After centrifugation at 11,578 x g for 10 min, a 10µl aliquot was taken from each sample and retained for protein analysis using a protein assay, as described in section 2.2.12 or 2.2.13. Cellular acetylases were deactivated by heating the extract to 65°C for 10 min. Extracts were then incubated on a 37°C hot plate and 10µl of 50mM acetyl coenzyme A and 1µl of 2.5µCi *D-threo*-[*dichloroacetyl*-1-¹⁴C] chloramphenicol was added to each sample. Tonsillar B cell extracts were incubated for 12-16hrs and B and T cell lineage extracts were incubated for 90 min, all at 37°C. Addition of 600µl ethyl acetate and brief vortexing terminated the reaction. Organic extraction of samples was performed by centrifugation at 11,578 x g for 2 min and the upper layer was retained. Samples were dried down in a speedivac for 2h and the lyophilisate resuspended in 20µl of ethyl acetate. Samples were spotted on to a thin layer chromatography plate which was developed in a tank containing 200mls 5% CH₃OH/95% CHCl₃. Plates were

developed for 1h then air dried before exposure to a phosphorimage plate for 2h, then X-ray film for 48h.

2.2.11 Chemiluminescent Secreted Alkaline Phosphatase (SEAP) Assay

Aliquots of 1ml supernatant were retained from every transiently-transfected sample, following cell harvest after 48h incubation. Fifteen microlitres of each sample was added to a 96-well microtiter plate in triplicate and assayed using the Great EscAPE Chemiluminescent secreted alkaline phosphatase (SEAP) Assay. Samples were mixed with 45µl of a dilution buffer (as supplied by the manufacturers) and heated to 65°C for 30 min to eliminate endogenous alkaline phosphatase activity. Samples were ice cooled to room temperature then incubated with 60µl of an assay buffer which contains L-homoarginine for 5 min at room temperature. Chemiluminescence was achieved by addition of 60µl of a chemiluminescent substrate (CSPD) diluted 20-fold by a chemiluminescent enhancer and a further 10 min incubation at room temperature. The level of SEAP reporter gene activity, expressed as units of alkaline phosphatase (AP) activity, for each sample was determined by reading plates in a S.I.T Spectra luminometer and taking a mean of each triplicate assay. The following calculation was used to normalise CAT reporter activity values derived from quantitative analysis of TLC resolved samples, to individual AP values. Baseline values were defined by CAT and AP activity measured from untreated cells, transiently-transfected with the full length CD25 plasmid, pCD25 CATE'.

X = Baseline AP Value

Y = Co-Transfection AP Value

Z = CAT Value

A = Corrected CAT Value

B = Baseline CAT Value

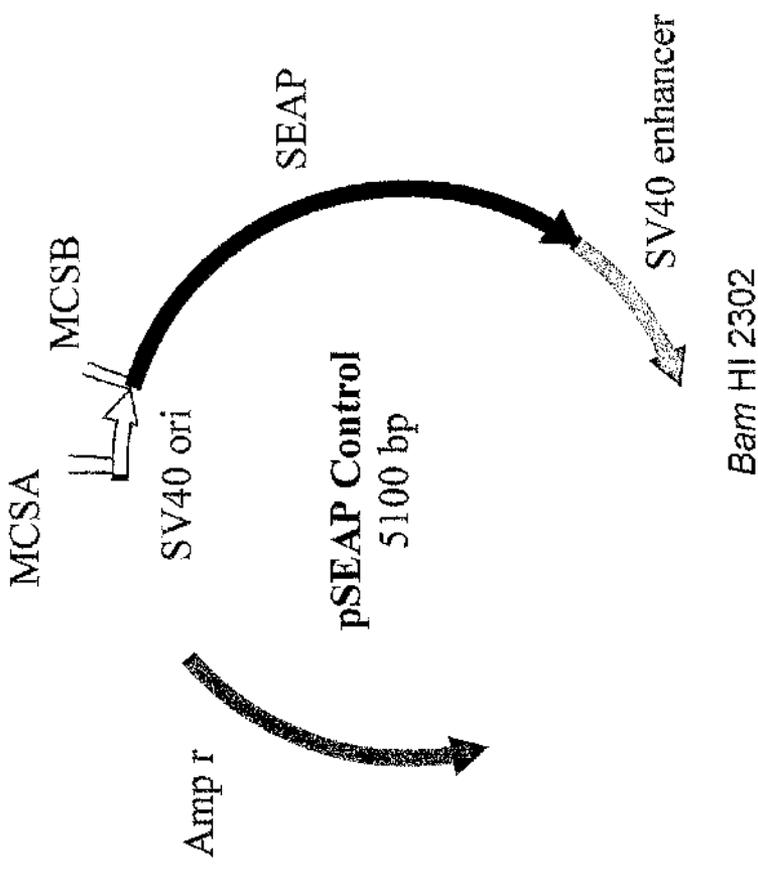
C = AP Normalised CAT Reporter Activity

$$\frac{Z}{Y/X} = A$$

$$\text{Therefore; } \frac{A}{B} = C$$

Figure 2.1 The Restriction Enzyme Map of pSEAP.

The pSEAP2-control plasmid restriction enzyme map. The region coding for SEAP is located between positions 245 and 1794, relative to the origin (ori), next to the SV40 enhancer region. The plasmid contains an ampicillin resistance gene and is punctuated by two multiple cloning sites (MCS) at positions 1-41 and 245-264.



2.2.12 Bicinchonic Acid (BCA) Protein Assay

BCA assay solution was made by mixing a solution containing 1% (w/v) 4,4 dicarboxy-2,2 biquinoline, disodium salt, 2% (w/v) Na carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% NaOH, 0.95% (w/v) NaH_2CO_3 pH 11.25, with 4% (w/v) CuSO_4 in a 1:49 ratio. Standards consisted of bovine serum albumin (BSA) diluted in either RIPA, nuclear or cytosolic buffer and made up in 0.2mg/ml increments from 0 to 2mg/ml in a final volume of 10 μ l. The samples were aliquoted into 10 μ l volumes and all were added to a 96 well plate and then mixed with 0.2mls of BCA assay solution. The plate was incubated at room temperature for 30 min then analysed at an absorbance of 492nm on an SLT Spectra plate reader. Unknown sample protein concentrations were calculated by plotting a best-fit straight line of the absorbance at 492nm versus each standard protein concentration then using the absorbance data reading for unknown values from that line.

2.2.13 Protein Assay

"BioRad" protein assay buffer was diluted 1 in 5 in distilled H_2O and filtered through Whatman 3MM paper. BSA protein standards were made up in Eppendorf tubes as previously described, in either RIPA buffer or nuclear extract buffer to a volume of 20 μ l. A 1:10 dilution of sample was prepared by extracting 2 μ l and mixing with the appropriate buffer in a final volume of 20 μ l. The assay buffer was added to each tube in a final volume of 1ml. After brief vortexing, the standards and samples were transferred to disposable 1ml spectrophotometer cuvettes and analysed on a spectrophotometer at an absorbance of 595nm. Sample protein concentrations were derived from a standard curve, as previously described, then multiplied by the appropriate dilution factor to give a final protein concentration.

2.2.14 Preparation of Competent Cells

A sample of approximately 2µl of JM109 or DH5-α *Escherichia (E.) coli* cells was collected from aliquots stored at -70°C and transferred to 10ml Luria Bertani (LB) media. The inoculated media was incubated at 37°C overnight in a shaker at 300rpm. A 2ml aliquot was transferred to 200ml and incubated in a 37°C shaker at 300rpm. Regular 1ml samples were withdrawn from the culture and optical density (OD) was determined by spectrophotometry at an absorbance of 550nm. The culture was allowed to grow until an OD value of 0.45 was reached for both cell types. The culture was then transferred to 200ml centrifuge tubes, incubated on ice for 15 min then pelleted by centrifugation at 3000 x g at 4°C for 5 min in a JA14 rotor. The supernatant was discarded and pellet resuspended by addition of 80ml TfbI buffer, then incubated on ice for 15 min. Cells were pelleted as previously described, the supernatant discarded and the cells resuspended in 8ml of TfbII bufer. Cells were aliquoted and either used immediately or frozen by exposure to an ethanol-dry ice mixture and stored at -70°C.

2.2.15 Transformation of Competent Cells

Competent JM109 or DH5α *E. coli* cells were added in 100µl amounts to pre-chilled sterile Eppendorfs at 4°C. Two microlitres of each plasmid was added to the cells and gently mixed before incubating on ice for 30 min. The mixture was heat-shocked by direct transfer to a hot plate at 42°C for 40s, then returned to ice. Each sample was treated with 1ml SOC medium then incubated at 37°C on an orbital shaker at 300rpm for 1h. LB agar plates containing 50µg/ml ampicillin were inoculated with the transformed cells by spreading, and allowed to dry before inversion and 37°C incubation overnight.

2.2.16 Plasmid Purification

A single colony of transformed *E. coli* JM109 or DH1 α cells was picked from a freshly streaked LB agar plate with a sterile tip then cultured in 10ml LB media containing 50 μ g/ml ampicillin. The inoculated media was incubated at 37 $^{\circ}$ C overnight in a shaker at 300rpm. A 1ml aliquot of culture was retained and assessed by mini-prep purification and restriction enzyme digest for the presence of the ligated DNA.

2.2.17 Mini-Prep

A 1ml aliquot was retained from the 10ml overnight culture and pelleted by centrifugation at 3944 x g for 5 min. The pellet was resuspended in 100 μ l of a 90% (v/v) sucrose-tris-EDTA- Triton-X (STET)/10% (v/v) 10mg/ml lysozyme solution and denatured by boiling in water for 2 min. Centrifugation at 11,598 x g for 10 min pelleted cell debris and the supernatant was retained in a fresh Eppendorf. DNA was precipitated by the sequential addition of 400 μ l 0.3M CH₃COONa and 500 μ l (CH₃)₂CHOH then incubated at -20 $^{\circ}$ C for 1h. DNA was pelleted by centrifugation at 11,598 x g for 10 min. The DNA pellet was washed with 100 μ l 70% (v/v) CH₃CHOH then retained following centrifugation at 11,598 x g for 5 min. The supernatant was discarded and excess ethanol evaporated by air drying for 5 min. Purified DNA was resuspended in 30 μ l of TE buffer and analysed by double restriction enzyme digest as previously described but with the inclusion of 1 μ l 25mg/ml RNAse A.

2.2.18 Maxi-Prep

Inoculated media were further diluted to 200mls in LB medium containing 50 μ g/ml ampicillin. The culture was grown at 37 $^{\circ}$ C for 12-16h on a shaker at 300rpm. Bacterial cells were then harvested by centrifugation at 6000 x g for 15 min at 4 $^{\circ}$ C in a Beckman JA-20 rotor. All plasmid DNA was isolated and purified using a Qiagen plasmid

purification kit. Cells were resuspended in 10ml buffer P1 then treated with 10ml lysis buffer P2. Samples were gently mixed then incubated at room temperature for 5 min. Ten millilitres of ice-cold neutralization buffer P3 was added then samples gently mixed before incubation on ice for 20 min. The lysis mixture was pelleted by centrifugation at $20,000 \times g$ for 30 min at 4°C using a JA-20 rotor. The supernatant was transferred in a fresh tube then centrifuged at $20,000 \times g$ for 15 min at 4°C . Plasmid DNA was collected by column gravity filtration of the supernatant, then washed in buffer QC. DNA was eluted by washing the column with 15ml buffer QF and was collected in a fresh tube. To precipitate DNA, 10.5ml room temperature $(\text{CH}_3)_2\text{CHOH}$ was added to the samples which were then pelleted by centrifugation at $15,000 \times g$ for 30 min at 4°C using a JA-20 rotor. The pellet was washed in 70% (v/v) room temperature CH_3CHOH , repelleted by centrifugation (as above) for 10 min then air dried for 5 min. The plasmid DNA pellet was resuspended in 700 μl TE buffer and DNA concentration determined by spectrophotometric analysis of a 1:100 dilution in a quartz cuvette at an absorbance of 260nm.

2.2.19 Polymerase Chain Reaction (PCR)

Human CD25 STAT6 deletion mutant constructs were synthesised by PCR in a volume of 100 μl , consisting of a 100ng pCD25 CAT-E' basic template, 20% (v/v) 10 x Taq buffer, 0.5% (v/v) Taq polymerase, 1.5mM MgCl_2 , 250 μM deoxynucleotide triphosphate (pNTP) mix, 0.5pmol/ μl universal primer and 50pmol/ μl STAT6 deletion mutant primer. The mixture was placed in a thermal cycler and subjected to a 3 min 94°C denaturing phase, 35 x 30s cycles of amplification at 94°C , two sequential 1 min phases at 55°C and 72°C , and a final 10 min phase at 72°C . PCR products were purified by electrophoresis on a 1% low melting point agarose gel containing 4ng/ml ethidium bromide at 100V, UV exposure and the amplified nucleic acid bands cut out.

Purification was achieved using a QIAquick gel extraction kit which involved weighing the gel slice, then adding three volumes of extraction buffer to one volume of gel. The mixture was heated at 50°C, for 10 min, with occasional vortexing. Addition of approximately 10µl of 3M CH₃COONa, (pH 5.0) lowered the pH of the mixture to 7.5 which prevented DNA degradation. One gel volume equivalent of (CH₃)₂CHOH was added to increase the yield of DNA fragments which were then bound to a column by drawing the mixture through a vacuum manifold. The DNA was washed with 70% ethanol which was removed by applying a vacuum. Excess buffer was removed by transferring the column to an eppendorf and centrifugation at 8,050 x g for 1 min on a biofuge. DNA was eluted in 50µl TE buffer and then collected in a sterile Eppendorf tube by centrifugation at 8,050 x g.

Restriction fragments with cohesive ends were generated by restriction enzyme double digest with *Hind* III and *Sal* I. The digestion products were separated by electrophoresis on a 1% (w/v) low melting point agarose gel and identified by running alongside 1 kilobase and 100 base-pair ladders and cut out using a sterile scalpel. The desired fragment was purified as described above. The same restriction enzyme double digest was employed with a pCAT-E' basic vector which contained *Hind* III and *Sal* I cutting regions in a multiple cloning site. The digested vector was separated by electrophoresis on a 1% (w/v) low melting point gel, the cut band was discarded and the vector was retained and purified as before. Vector concentration was determined at an absorbance of 260nm in a quartz cuvette and adjusted to 100ng/µl in TE buffer.

Ten nanograms of purified cut pCAT-E' basic vector was mixed with approx 700ng of restriction fragments with cohesive ends, 1µl DNA ligase and 1µl DNA ligase buffer then incubated for 12h at 17°C. The pCAT CD25 STAT6 deletion mutant construct was then inserted into competent *E. coli* cells by transformation. Transformed *E. coli* cells were then cultured in LB broth and transferred to a larger volume until sufficient growth

was achieved. The plasmid DNA was isolated from the culture and purified as described in section 2.2.18, then stored at -20°C .

2.2.20 Cyclic-AMP Dependent Kinase (PKA) Assay

Tonsillar B cell or B cell lineage extracts at a concentration of 3mg/ml were added in a volume of $10\mu\text{l}$ to a PKA reaction mixture consisting of $4\mu\text{l}$ $5\times$ PKA buffer, $2\mu\text{l}$ 1.25mM ATP and $2\mu\text{l}$ $100\mu\text{M}$ okadaic acid. Extracts were pre-incubated at 30°C for 10 min then treated with or without $50\mu\text{g/ml}$ PKA catalytic subunit in the presence of $2\mu\text{l}$ dH_2O , $2\mu\text{l}$ of the PKA inhibitor PKI, at a concentration of 1mg/ml or $2\mu\text{l}$ of the PKA inhibitor, H-89 at a concentration of $0.5\mu\text{M}$. Aliquots of $2\mu\text{l}$ were taken from every reaction mixture after 0, 5, 10, 20, 30, 60 and 120 min incubation at 30°C and were added to Eppendorfs containing 20% (v/v) $5\times$ binding buffer then incubated with and without the presence of excess unlabelled oligonucleotide for ten minutes at room temperature. Each sample was then mixed with radiolabelled probe and subjected to EMSA as described above.

2.2.21 FACScan Analysis of B Lymphoid Cells

After incubation of either treated or untreated tonsillar B cells or B cell lines, 5×10^6 cells/ml were harvested and washed twice in PBS by centrifugation at $250 \times g$. The pellet was then resuspended in $100\mu\text{l}$ PBS and transferred to a $12 \times 75\text{mm}$ plastic tube. Cells were treated with $3\mu\text{l}$ of RPE-conjugated anti-human B cell CD19, $3\mu\text{l}$ of FITC-conjugated anti-human B cell CD25 or $3\mu\text{l}$ of FITC-conjugated anti-human T cell CD3, then after brief vortexing, incubated in dark conditions on ice for 30 min. Cells were pelleted by centrifugation at $250 \times g$ then resuspended in $500\mu\text{l}$ PBS. Samples were treated with $5\mu\text{l}$ 1mg/ml propidium iodide to enable gating of dead cells, then analysed by flow cytometry. CD25 expression was calculated by dividing the number of $\text{CD19}^+/\text{CD25}^+$ B cells with the combined total of $\text{CD19}^+/\text{CD25}^-$ and $\text{CD19}^+/\text{CD25}^+$ B

cells. The value obtained was multiplied by 100 to give the percentage of B cells expressing CD25. The value of percentage CD3 or CD19 expression was calculated using the same method.

2.2.22 Statistical Analysis

All statistics were calculated as a Standard Error Mean (SEM) using Student's Paired t Test.

CHAPTER 3
CELLULAR SIGNALLING MECHANISMS IN IL-4 STIMULATED B CELLS.

3.1 Introduction

IL-4 stimulates several cell signalling pathways in B cells through the IL-4R complex, leading to a number of cellular events which regulate B cell function. The events that follow the binding of IL-4 to its receptor involve receptor-associated kinases which phosphorylate specific signalling molecules. This leads to the induction of B cell proliferation, differentiation and gene activation, leading to the expression of immunologically important molecules, such as MHC class II, CD23 and CD25. Signal transduction pathways important in IL-4R signalling in B cells include cAMP (McKay and Cushley, 1996), PLC γ , PI-3 kinase and the induction of the JAK/STAT activation mechanism (Finney et al., 1990; Nelms et al., 1999).

Adjacent to the NRR_{I-II} regions of the human CD25 promoter region lies PRR_I. The PRR_I region is sensitive to antigen and mitogen-induced signalling and contains binding sites for SRF and the ubiquitous transcription factor NF- κ B. Both cAMP and PKA have been shown to regulate NF- κ B either directly or indirectly (Baeurle and Henkel, 1994; Ollivier et al., 1996). The fact that NF- κ B has a multifactorial role in transcriptional regulation, is sensitive to cAMP and PKA, and binds to PRR_I suggests that it may be involved in IL-4-induced CD25 expression.

3.2 Isolation of Quiescent Tonsillar B Cells and their Activation by IL-4.

Quiescent tonsillar B cells were isolated as described in section 2.2.2. Samples were retained before and after treatment with SRBC-AET, which induced T cell rosetting. Aliquots were stained with FITC-labelled anti-CD3 and RPE-labelled anti-CD19 to quantitate the populations of T and B cells, respectively. Samples were analysed by FACScan using two-colour flow cytometry and all CD3⁻/CD19⁺ and CD3⁺/CD19⁻ cells were assessed. Figure 3.1a shows that samples analysed before SRBC-AET treatment had a large population of CD3⁻/CD19⁺ (upper left quadrant) cells and a similar population of CD3⁺/CD19⁻ (lower right quadrant) cells. Figure 3.1b shows samples analysed after SRBC-AET treatment, which had enriched the population of CD3⁻/CD19⁺ (upper left quadrant) cells and had significantly reduced the population of CD3⁺/CD19⁻ cells (lower right quadrant). The decrease in CD3⁺/CD19⁻ cells after SRBC-AET treatment suggests that a proportion of T cells have been rosetted and subsequently removed, leaving a population which is enriched for B cells. The isolated tonsillar B cells were treated with recombinant human (rh) IL-4 and incubated over a period of 24-48h. Cells were harvested and stained with RPE-anti-CD19 and FITC-anti-CD25 then analysed by flow cytometry for CD19⁺/CD25⁺ B cells. Figure 3.2 panel a) shows a population of untreated tonsillar B cells after 24h incubation. A high percentage of cells were CD19⁺ and are identified in the upper left quadrant. In the upper right quadrant, there is a population of CD19⁺/CD25⁺ B cells. Figure 3.2b shows a population of tonsillar B cells which had been treated with rh-IL-4. In the upper left quadrant, there remained a subset of CD19 single positive B cells. In the upper right quadrant there was a modest increase in the population of B cells which were CD19⁺/CD25⁺, suggesting that IL-4 treatment leads to the up-regulation of CD25 expression in quiescent tonsillar B cells.

3.3 EMSA Studies of NRE Binding Activity in IL-4-Treated Tonsillar B Cells.

The IL-4-driven up-regulation of CD25 expression is mediated, at least in part, by a cAMP/PKA signalling pathway. This signalling pathway was investigated by treating quiescent tonsillar B cells with pharmacological compounds which modified cAMP/PKA activity. Quiescent tonsillar B cells were treated with and without IL-4 and, over a four hour incubation, samples were taken and cell extracts prepared. The extracts were incubated with a radiolabelled CD25-NRE oligonucleotide, then assessed for protein binding activity by EMSA analysis. Experiments were performed to assess the effect of IL-4 treatment and to examine the effect of an adenylate cyclase stimulating compound, Forskolin, on NRE binding activity in tonsillar B cells. Figure 3.3 panel a shows the single specifically shifted species indicative of a protein binding to the radiolabelled CD25-NRE oligonucleotide which was competed by the addition of an unlabelled five-fold excess of CD25-NRE. Treatment of B cells with either IL-4 or Forskolin caused a loss of binding activity over a four hour period (Figures 3.3b and 3.3c, respectively). A similar result was observed following treatment with the $G_{s\alpha}$ -protein activator, Cholera Toxin (data not shown).

3.4 EMSA Studies of the Effects of PKA Catalytic Subunit on Quiescent Tonsillar B cells.

The role of the cAMP/PKA signalling pathway in IL-4-induced CD25 expression was further investigated using an assay which assessed a possible direct effect of PKA on NRE-BP. IL-4 stimulated loss of NRE-BP binding activity in the CD25 promoter in tonsillar B cells is mimicked by the cAMP-inducing agent Forskolin. Previous work in this laboratory showed that the PKA-specific inhibitor, H-89, could block the effects of IL-4, whereas the PKC-specific inhibitor, bis-indolylmaleimide, had no effect (McKay et al., 1999). From this information, an experiment was designed to assess whether the direct action of the catalytic subunit of PKA (PKAc) could abrogate the DNA binding

activity of NRE-BP over a short period of time. EMSA experiments on samples taken during the incubation reaction would demonstrate, if PKAc acted directly on NRE-BP, that protein binding to radiolabelled CD25 oligonucleotide would be lost. If the action of PKAc was indirect then protein binding would be unaffected in samples taken throughout the reaction (Figure 3.4). An incubation reaction containing EDR B cell whole cell extract was treated with and without PKAc and with or without the presence of one of two specific PKA inhibitors, H-89 and protein kinase A inhibitor (PKI); each reaction mixture also contained the phosphatase inhibitor, Okadaic acid. Samples were taken over a two hour period then extracts prepared and incubated with a radiolabelled oligonucleotide sequence which either contained the short CD25-NRE sequence, or the NRE-intact/AP-1-mutant oligonucleotide, CD25-NRR_T-M2, then analysed by EMSA.

Figure 3.5i (left panel) shows oligonucleotide binding activity in EDR B cell whole cell extract to radiolabelled CD25-NRE oligonucleotide which is competed by the addition of an unlabelled two-fold excess of CD25-NRE. In control reactions lacking PKAc, protein binding was unaffected over 20 minutes of incubation (figure 3.5ii) and up to two hours of incubation (data not shown). Figure 3.5iii shows protein binding activity in samples taken from incubation reactions with PKAc. A single protein species bound to radiolabelled CD25-NRE oligonucleotide which was unaffected over the first 20 minutes of incubation and up to two hours of incubation (data not shown). The PKA inhibitors PKI and H-89 had no effect on protein species binding to radiolabelled CD25-NRE oligonucleotide or CD25-NRR_T (data not shown). These findings were also shown in repeated EMSA studies on PKA assay samples using radiolabelled CD25-NRR_T-M2 oligonucleotide (Figures 3.5i-iii, right panel).

3.5 Effect of Signalling Inhibitors on IL-4-Driven CD25 Expression.

The signalling mechanisms by which IL-4 initiates the up-regulation of CD25 expression were investigated. Freshly-isolated quiescent tonsillar B cells were pre-treated with cell

signalling inhibitors, before stimulation with IL-4. In particular, a potential role of PI-3 kinase on IL-4-driven CD25 upregulation was tested by treating cells with the PI-3 kinase inhibitor, Wortmannin. A further inhibitor, Rapamycin, was used to study the effect of blocking the activation of a downstream PI-3 kinase-sensitive serine/threonine kinase, p70^{S6} kinase. As IL-4R signalling does not utilise the Ras/MAP kinase signalling pathway in B cells (Welham et al, 1994), the MEK kinase inhibitor PD98059 was used as an internal control. After inhibitor pre-treatment, tonsillar B cells were treated with and without IL-4 and incubated for 24-48 hours. Samples were removed at 24 and 48 hours and the B cells were analysed for CD25 expression by flow cytometry.

Figure 3.6 shows a graph outlining the effect of inhibitors on the capacity of IL-4 to upregulate CD25 expression. IL-4 treatment of tonsillar B cells over 24 and 48 hours of incubation lead to a three- and four-fold increase in the expression of CD25, respectively. Pre-treatment of tonsillar B cells with 2 μ M or 20 μ M PD98059 over 24 and 48 hours had no effect on the capacity of IL-4 to upregulate CD25 expression, as anticipated. Pre-treatment of tonsillar B cells with 5nM and 50nM Wortmannin over 24 and 48 hours did not effect IL-4-driven CD25 expression. Finally, pre-treatment with 500pM and 50pM Rapamycin failed to affect IL-4-mediated upregulation of CD25 expression in tonsillar B cells over 24 and 48 hours of incubation. Inhibition of PI-3 kinase and p70^{S6} kinase activation appears to have no effect on the capacity of IL-4 to increase CD25 expression in tonsillar B cells.

3.6 EMSA Studies of NF- κ B Expression in IL-4 Treated EDR B cells.

The effect of IL-4R signalling on NF- κ B was investigated using EMSA analysis of EDR B cell whole cell extract, cytosolic extract and nuclear extract. Firstly, EDR B cells were assessed for the expression of NF- κ B both in untreated and IL-4-treated whole cell extracts which were prepared over a four hour incubation period. Secondly, the activation of NF- κ B was assessed. In most cells, inactive NF- κ B is located in the cell

cytoplasm, bound by I κ B. Activation of NF- κ B is achieved by the IKK-induced phosphorylation of I κ B, which leads to degradation of the complex and the subsequent uncoupling of I κ B from NF- κ B. The transcription factor then migrates to the nucleus where it can regulate gene expression (May and Ghosh, 1998). Therefore, in order to investigate NF- κ B activation, both cytosolic and nuclear extracts were prepared from EDR B cells which were treated with and without IL-4. Extracts were prepared over a four hour incubation period then incubated with radiolabelled NF- κ B oligonucleotide and analysed by EMSA.

In whole cell, cytosolic and nuclear extracts, two slow mobility species specifically bound to radiolabelled NF- κ B oligonucleotide. Figure 3.7 panel a shows a study of EDR B cell whole cell extracts. Protein binding to radiolabelled NF- κ B was competed by an unlabelled five-fold excess of NF- κ B oligonucleotide. Figure 3.7b shows that protein binding to radiolabelled NF- κ B was unaffected in untreated samples taken over four hours. Extracts which were generated from cells treated with IL-4 showed that protein binding to radiolabelled NF- κ B was also unaffected over four hours. Figure 3.7c shows a study of EDR B cell cytosolic extracts. Protein binding to radiolabelled NF- κ B was unaffected in both treated and untreated samples generated over four hours of incubation. Figure 3.7d shows a study of EDR B cell nuclear extract. Protein binding to radiolabelled NF- κ B was unaffected in samples generated over four hours without IL-4 treatment. Extracts prepared from IL-4-treated cells showed that protein binding to radiolabelled NF- κ B in samples generated over three hours of incubation was unaffected; however protein binding was slightly diminished in samples generated after the fourth hour of incubation.

3.7 Discussion.

IL-4 has been shown to up-regulate CD25 expression in human B cells (Burlinson et al., 1995; Butcher et al., 1990; Zola et al., 1991). This important immunological function was investigated by studying the signalling pathways that are activated by IL-4R stimulation. These include cAMP/PKA, PKC, PI-3 kinase and JAK/STAT pathways. JAK/STAT signalling is discussed in Chapter 5 and therefore will not be considered in this section. IL-4 up-regulates CD25 expression in human quiescent tonsillar B cells. EMSA studies of IL-4-treated B cells shown in Figure 3.3b demonstrate that IL-4 abrogates NRE binding activity over four hours of incubation. This effect was mimicked by the pharmacological activator, Forskolin (Figure 3.3c), which increases the intracellular concentration of cAMP via the stimulation of AC. These findings support previous studies, and indicate that IL-4 causes the expression of CD25 in human B cells by abolishing NRE-BP binding to NRE via a cAMP/PKA sensitive mechanism.

A potential role of IL-4-activated PKAc is direct phosphorylation of NRE-BP, thus attenuating NRE binding activity and enabling CD25 expression. Failure of PKAc to abolish protein binding to three different NRE sequence-containing oligonucleotides, demonstrates that PKAc does not have a direct phosphorylating effect on NRE-BP in human B cells (Figure 3.5b). This was unexpected as the finding that H-89 blocked the effects of IL-4 on NRE binding activity suggested that PKAc may directly uncouple or degrade NRE binding proteins. A possible explanation is that PKA acts to stimulate further downstream effector molecules, and has no direct contact with NRE-BP. A candidate for PKA-induced activation which may induce NRE-BP degradation is the cAMP/PKA-regulated transcription protein CREB. The shared cAMP response element and ATF-1 binding region (TGACGTCA) is homologous to the putative AP-1 site (TGACTCC) within NRR_I indicating a potential site of interaction for CREB (Lee and Masson, 1993). Therefore, activated CREB, which interacts with CBP, may bind to the

putative AP-1 site and enable CD25 transcription by activating the region, thus inducing the loss of NRE binding activity.

The role of the multi-functional PI-3 kinase on IL-4-driven CD25 expression was investigated using two inhibitors; Wortmannin, the PI-3 kinase-specific inhibitor and Rapamycin, a specific inhibitor of the PI-3 kinase-inducible p70^{S6} kinase. Both Wortmannin and Rapamycin did not affect the capacity of IL-4 to up-regulate CD25 expression on tonsillar B cells over 24 and 48 hours (Figure 3.6). IL-4R stimulation leads to the activation of PI-3 kinase signal transduction; however, the failure of either Wortmannin or Rapamycin to block CD25 up-regulation strongly suggests that PI-3 kinase is not involved in IL-4-driven CD25 expression in human B cells. The finding that PI-3 kinase inhibition failed to affect IL-4-driven CD25 regulation additionally suggests the involvement of downstream proteins such as PKB and PKC ζ , as well as p70^{S6} kinase is unlikely. The Ras/MAP kinase signalling pathway is unstimulated by IL-4 in B lymphocytes. Previous work in this laboratory indicates that PKC activation has no effect in IL-4-driven CD25 expression, therefore any involvement of PLC- γ 2, IP₃ or NF-AT is unlikely (McKay et al., 1999).

Finally, the role of the ubiquitous transcription factor NF- κ B in IL-4-regulated CD25 expression was investigated. Figure 3.7b shows that whole cell extract protein binding to the NF- κ B probe was unaffected by IL-4 treatment. This study serves as a control for the expression of NF- κ B in B cells. The fact that NF- κ B is constitutively expressed and that its activation state is dependent on I κ B-binding suggests that analysis of whole cell extract can only determine the expression of NF- κ B, rather than its activation (DiDonato et al., 1997). Cytosolic extracts were probed with radiolabelled NF- κ B oligonucleotide (Figure 3.7c) and in common with whole cell extract, EMSA analysis showed that protein binding was unaffected in cells treated with IL-4. This suggests that IL-4 does not directly activate NF- κ B. IL-4-induced phosphorylation of I κ B would lead to the uncoupling of the I κ B-NF- κ B complex and the migration of NF- κ B to the

nucleus. This may be indicated by an attenuation of protein binding to the NF- κ B probe in cytosolic extract. Figure 3.7d showed that nuclear extracts bound protein to the NF- κ B probe which in untreated cells was unaffected over four hours. In IL-4-treated cells, binding was unaffected after three hours although after four hours, binding was somewhat diminished. Given the capacity of IL-4 to abolish NRE binding activity after approximately one hour of incubation (Figure 3.3b), it is unlikely that the diminution of NF- κ B-binding is directly linked to IL-4 signalling. In addition, the failure of IL-4 to abrogate protein binding to radiolabelled NF- κ B in cytosolic extracts over four hours (Figure 3.7c) suggests that any effect of IL-4 on NF- κ B within the nucleus is likely to be a result of secondary effects. This finding was unexpected as PRR₁, which lies adjacent to NRE, contains a binding domain for NF- κ B which has been reported to be sensitive to mitogen stimulation in T cells (Pierce et al., 1995).

A possible mechanism of secondary action may be through cAMP/PKAc activation of CREB. Studies have shown that CBP, the transcriptional protein which binds CREB, can interact with the p65 subunit of NF- κ B and potentiate transcriptional activity. The interaction of CBP with NF- κ B is dependent on CBP involvement with CREB (Gerritsen et al., 1997). Another possible role of IL-4R signalling in NF- κ B activation is the activation of cAMP-independent PKAc. Studies have shown that PKAc is contained within the NF- κ B-I κ B complex in an inactive state. When NF- κ B is activated by the degradation of I κ B, the PKAc subunit also becomes activated and phosphorylates the NF- κ B p65 subunit. This leads to a marked increase in the transcriptional activity of NF- κ B (Zhong et al., 1997). If PKAc is able to stimulate the NF- κ B p65 subunit from within the complex then it is possible that PKAc generated outwith the complex may either directly activate NF- κ B or potentiate the activation of NF- κ B by combining with complex PKAc upon the phosphorylation of I- κ B. Therefore, a cAMP-independent or dependent activation of PKAc can enhance NF- κ B transcriptional activity. An increase in NF- κ B transcriptional activity leads to an increase in the expression of I κ B, therefore it is possible that inactivation and relocation of NF- κ B in the cytosol by complexing with

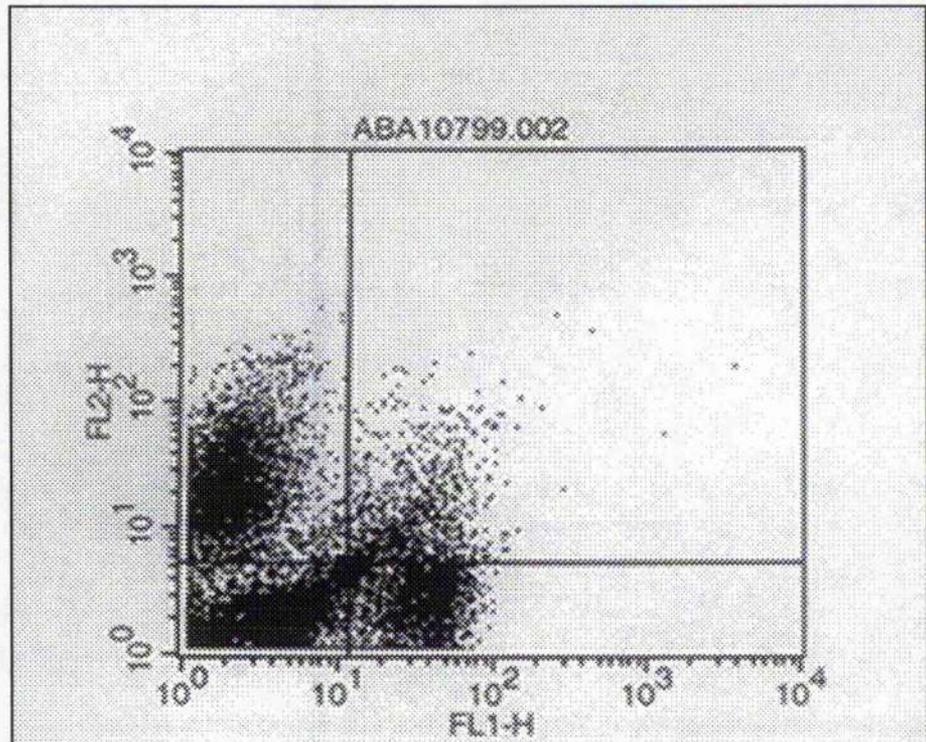
I κ B may explain the loss of binding after four hours. The possible IL-4-driven loss of NF- κ B binding activity in nuclear extracts is not consistent with the unaffected protein binding of the cytosolic extract.

In summary, IL-4 treatment of human B cells up-regulates the expression of the unique IL-2 receptor component, CD25. EMSA studies on EDR B cells demonstrated that IL-4 achieves CD25 up-regulation by removing NRE binding activity, via a cAMP/PKA-sensitive pathway. The exact mechanisms coupling the IL-4R to cAMP accumulation are unclear. Experiments on EDR B cell extracts showed that PKAc does not have a direct effect on NRE-BP, suggesting that PKAc activates further unidentified downstream molecules which abolish NRE-BP binding to NRE. Experiments investigating signalling pathways failed to identify a further mechanism involved in the IL-4-driven up-regulation of CD25 expression. Inhibitor experiments which blocked the activity of PI-3 kinase and p70^{S6} kinase failed to prevent IL-4-induced up-regulation of CD25. EMSA studies demonstrated that NF- κ B activity was diminished after four hours of IL-4 treatment, however this is unlikely to be a direct effect of the IL-4-stimulated cAMP/PKA pathway.

Figure 3.1 Isolation of Tonsillar B Cells from a Mononuclear Cell Population.

Aliquots of tonsillar mononuclear cells (Panel a) and isolated tonsillar B cells (Panel b) (1×10^6 cells) were stained with RPE-conjugated monoclonal anti-CD19 antibody and FITC-conjugated monoclonal anti-CD3 antibody and analysed by flow cytometry. Populations of CD3⁻/CD19⁺ cells are located in the upper left quadrant of both panels and CD3⁺/CD19⁻ cells in the lower right quadrants.

a)



b)

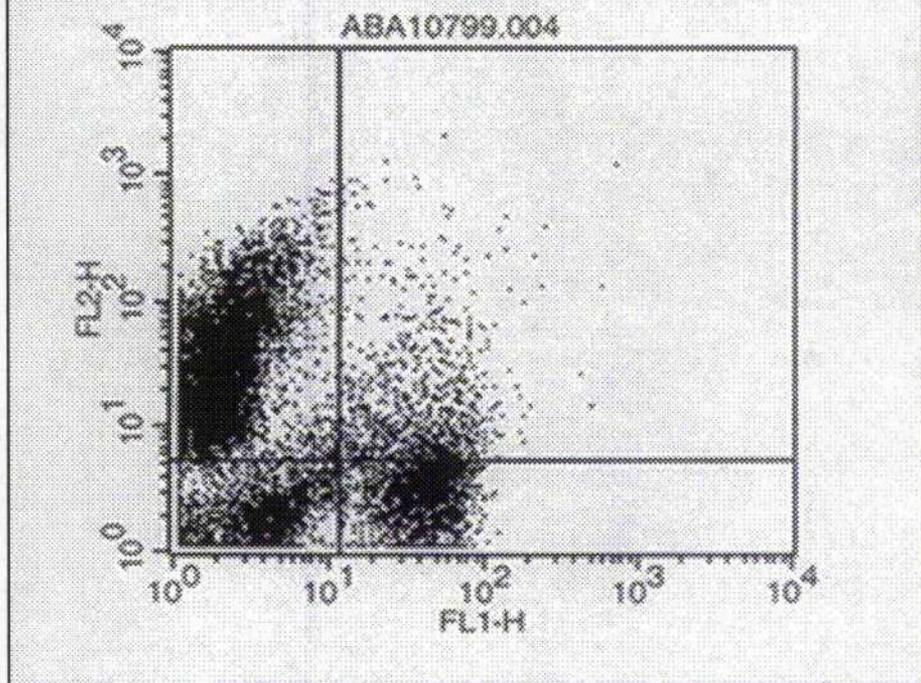
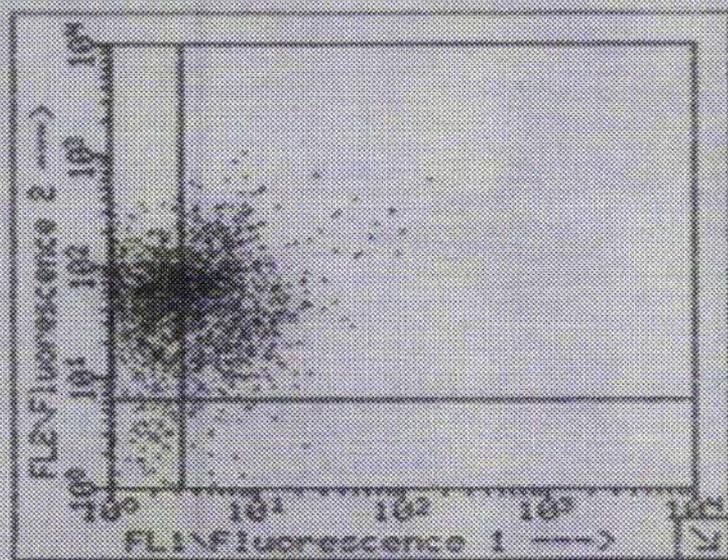


Figure 3.2 IL-4 Induces CD25 Expression in Tonsillar B Cells.

Aliquots of unstimulated tonsillar B cells (1×10^6 cells) from healthy donors were harvested after 24h treatment in the absence (Panel a) or presence (Panel b) of 500U/ml IL-4, and stained with RPE-conjugated monoclonal CD19-specific antibody and FITC-conjugated monoclonal CD25-specific antibody, then analysed by flow cytometry. In both panels, CD19⁺/CD25⁻ cells are located in the upper left quadrant and CD19⁺/CD25⁺ cells are in the upper right quadrant. Data was generated from the analysis of 10,000 events (1 event = 1 cell).

a)



b)

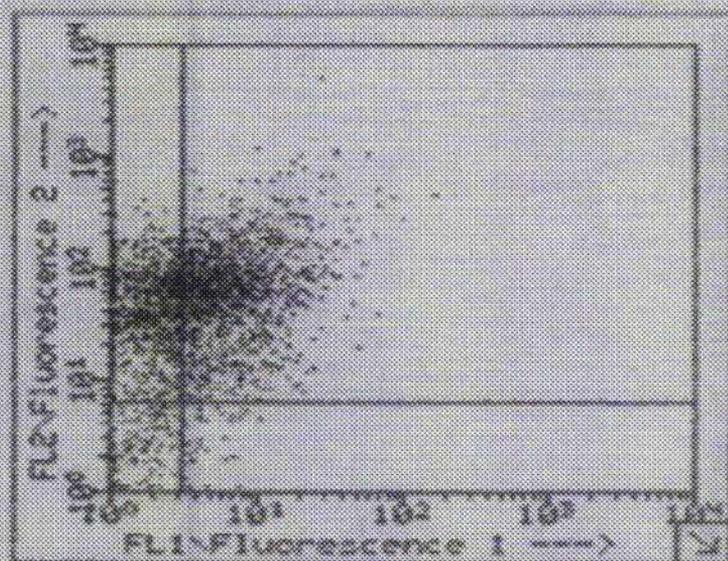
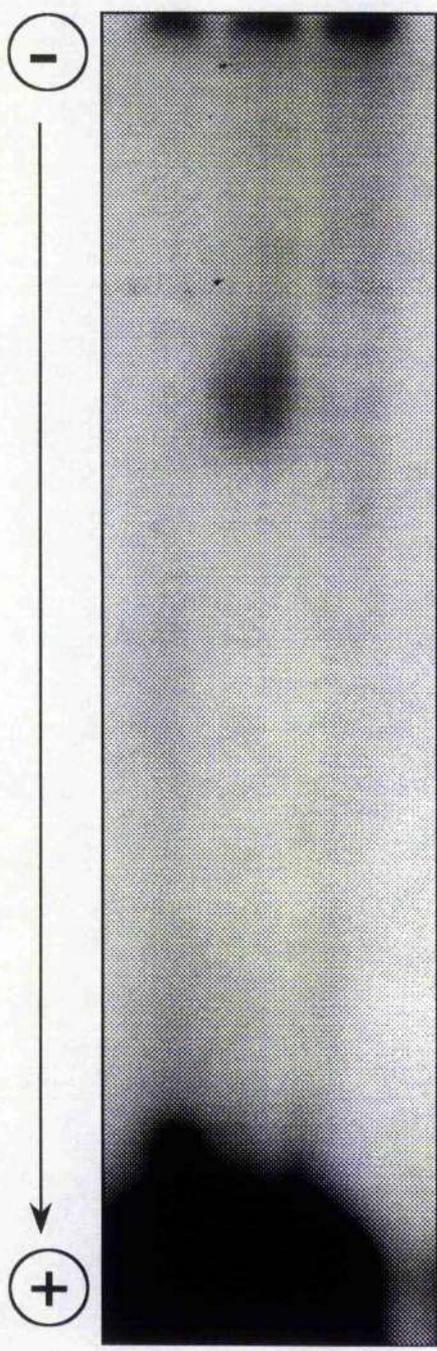


Figure 3.3 EMSA Analysis on the Effect of cAMP/PKA Activation on NRE-BP.

Figure 3.3a shows analysis of 5µg of untreated EDR whole cell extract by EMSA for binding to ³²P-labelled CD25-NRE oligonucleotide in the absence or presence of 2-fold excess unlabelled CD25-NRE oligonucleotide as competitor. Whole cell extracts were prepared at hourly intervals from quiescent tonsillar B cells after incubation with 500U/ml IL-4 (Fig. 3.3b) or 100µM Forskolin (Fig 3.3c), and 5µg of protein extract was analysed by EMSA for binding to ³²P-labelled CD25-NRE oligonucleotide. Specific binding activity for NRE-BP is indicated by horizontal arrows

a) Specificity of Binding

- + Comp



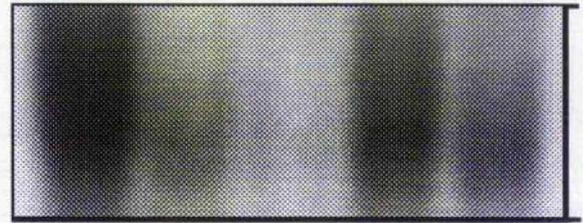
← NRE-BP

Free Probe

b) IL-4 Treatment

Time (h)

0 1 2 3 4



c) Forskolin Treatment

Time (h)

0 1 2 3 4

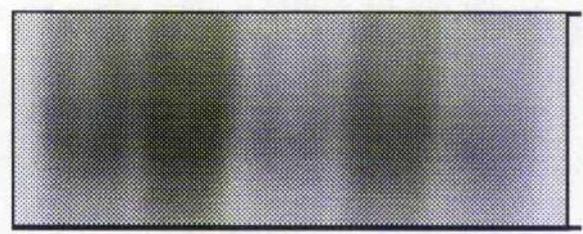
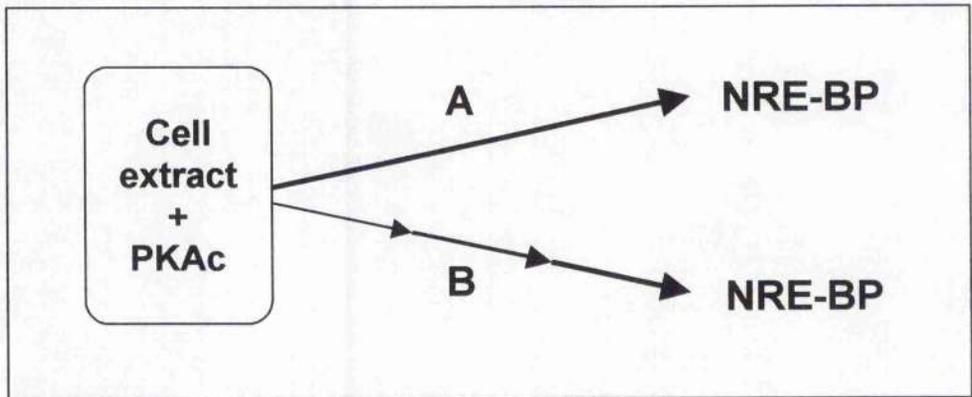
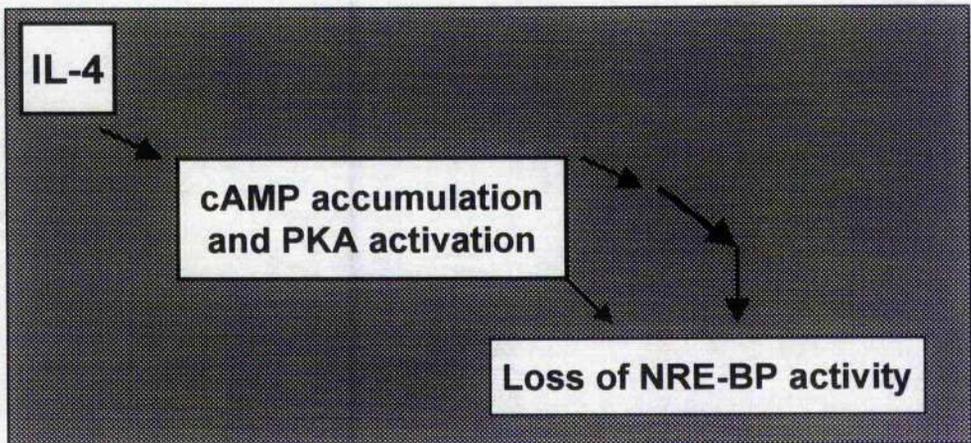


Figure 3.4 Hypothesis on the Effect of PKAc on NRE Binding Activity.

IL-4R signalling stimulates a cAMP/PKA sensitive pathway which leads to the loss of NRE binding activity. The loss of NRE-BP to NRE is sensitive to PKAc and this may be through a direct or indirect mechanism of action (upper panel). A proposed model to explain the activity of PKAc on NRE binding activity is shown in the middle panel. Treatment of B cell extracts with activated PKAc may lead to either a direct (arrow A) or indirect effect (arrow B) on NRE binding activity, indicating whether PKAc acts to directly attenuate NRE-BP binding or stimulates further downstream elements which affect NRE binding activity. Predicted EMSA results are indicated in the bottom panel.



Predicted EMSA

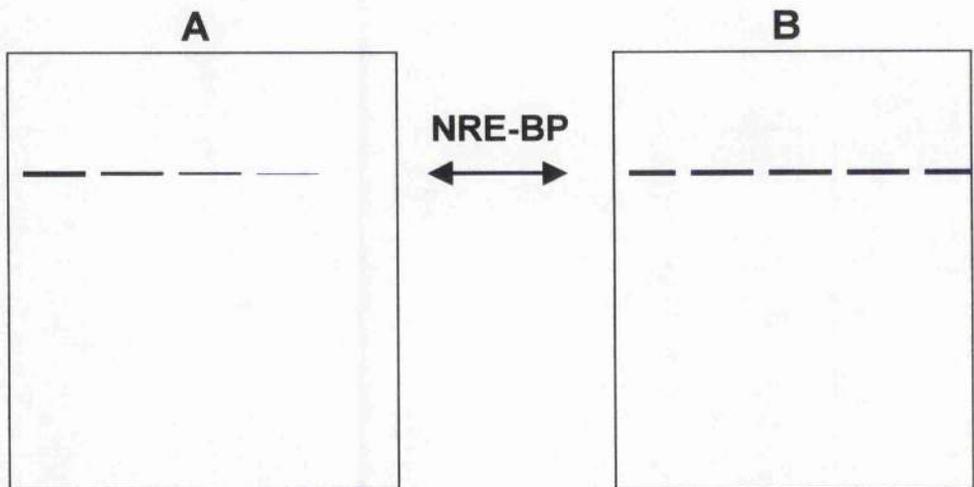


Figure 3.5 EMSA Analysis on the Effect of PKAc on NRE Binding Activity.

Whole cell extracts were prepared from EDR B cells (5×10^6 cells). Aliquots of 30 μ g were mixed with 4 μ l 5x PKA buffer, 2 μ l 1.25mM ATP and 2 μ l 100 μ M okadaic acid and incubated at 30 $^{\circ}$ C with or without 2 μ l of 50 μ g/ml PKA catalytic subunit, 2 μ l of 1mg/ml PKI and 2 μ l of 0.5pM H-89. Aliquots were taken after 0, 5,10,20,30,60 and 120 min. The left hand panel shows analysis of 5 μ g of untreated EDR whole cell extract by EMSA (Fig 3.5i) for binding to 32 P-labelled CD25-NRE oligonucleotide in the absence or presence of 2-fold excess unlabelled CD25-NRE oligonucleotide as competitor. Figure 3.5ii shows the binding activity of a control, untreated extract taken over 20 min. Figure 3.5aiii shows binding activity of an identical extract exposed to 5 μ g PKAc over the same period. The right hand panel shows analysis of 5 μ g of untreated whole cell extract by EMSA (Fig 3.5bi) for binding to 32 P-labelled CD25-NRR $_{\Gamma}$ -M2 oligonucleotide in the absence or presence of 2-fold excess unlabelled CD25-NRR $_{\Gamma}$ -M2 oligonucleotide as competitor. Figure 3.5bii shows the binding activity of a control, untreated extract taken over 20 min. Figure 3.5biii shows binding activity of an identical extract exposed to 5 μ g PKAc over the same period. Specific binding activities for untreated extracts are indicated by horizontal arrows.

Probe: NRE

Probe: M2 (NRE+/AP1-)

i) Specificity

i) Specificity

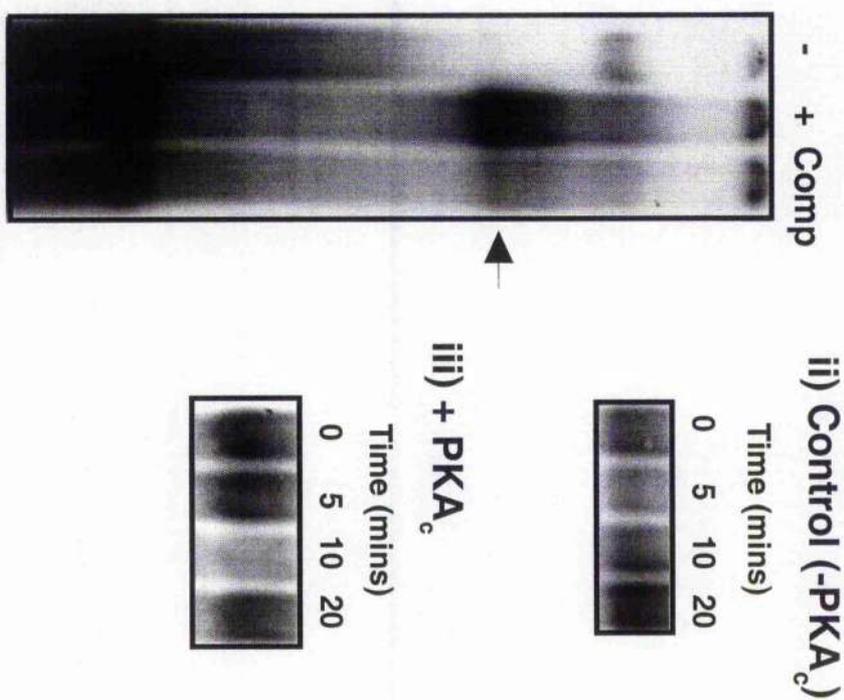
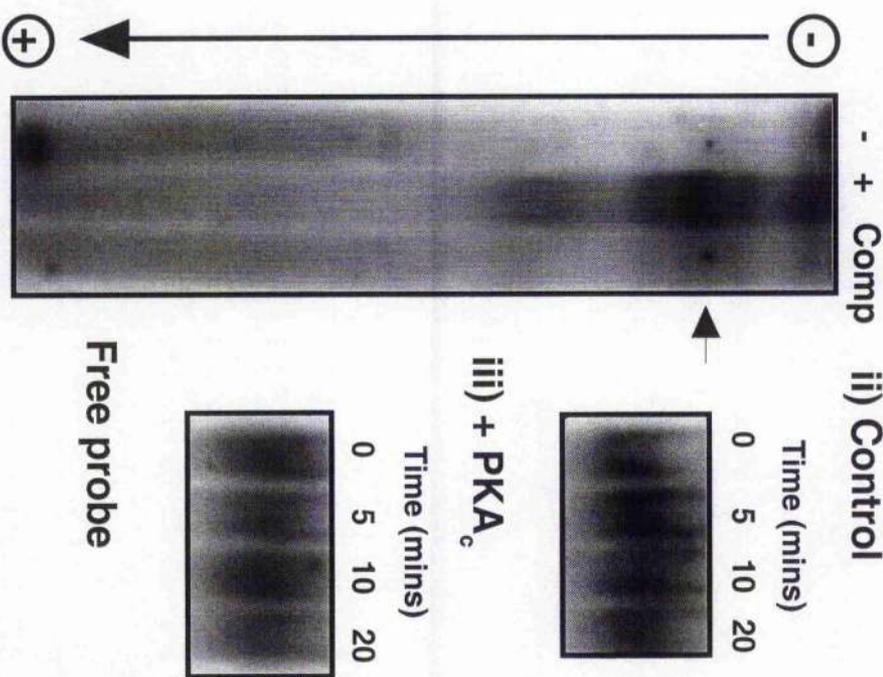


Figure 3.6 Effect of Signalling Pathway Inhibitors on IL-4-Driven CD25 Expression in Tonsillar B Cells.

Aliquots of 1×10^6 tonsillar B cells were harvested after 24h and 48h treatment in the absence or presence of 50pM and 500pM Rapamycin, 5nM and 50nM Wortmannin, 2 μ M and 20 μ M PD98059 and 500U/ml IL-4, then stained with RPE-conjugated monoclonal anti-CD19 antibody and FITC-conjugated monoclonal anti-CD25 antibody and analysed by flow cytometry. Both graphs are representative of CD19⁺/CD25⁺ cells as a percentage of the total population of CD19⁺/CD25⁻ and CD19⁺/CD25⁺ cells. Values represent means of relative reporter activity (\pm SEM) from ten independent assays

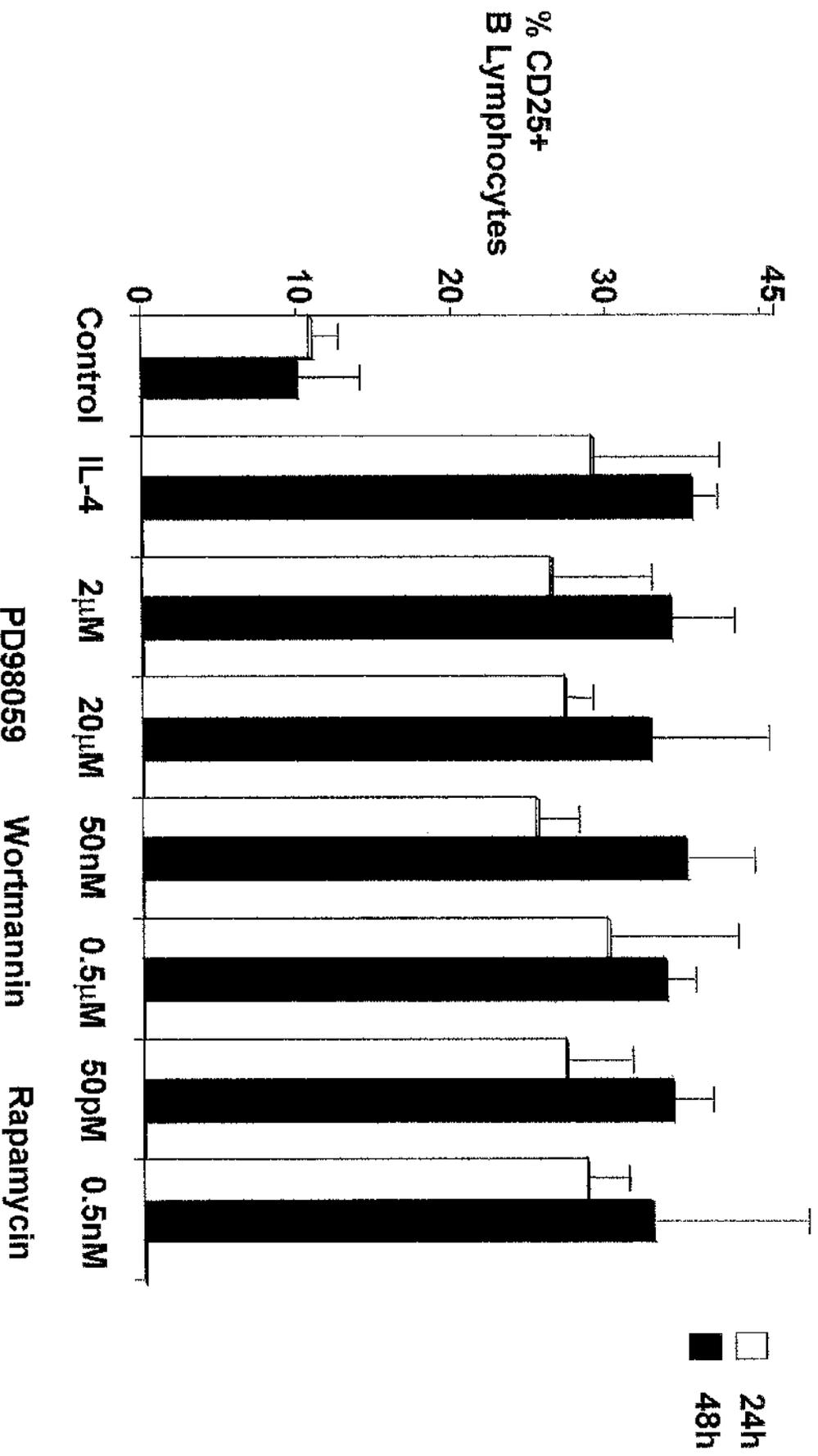
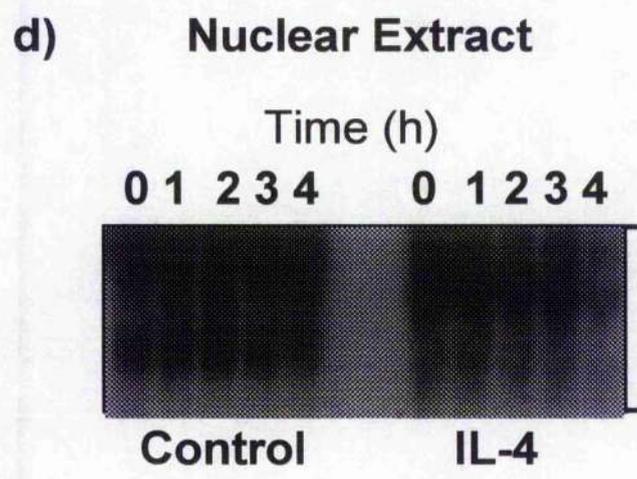
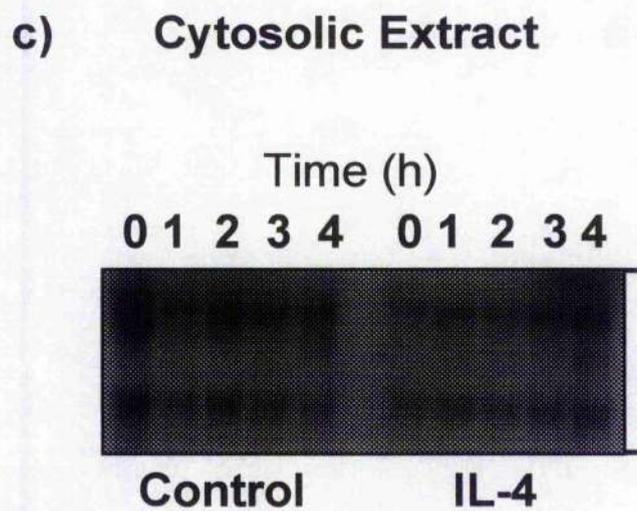
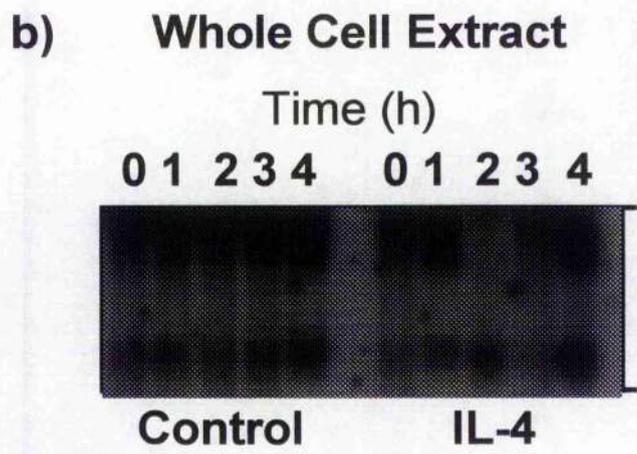
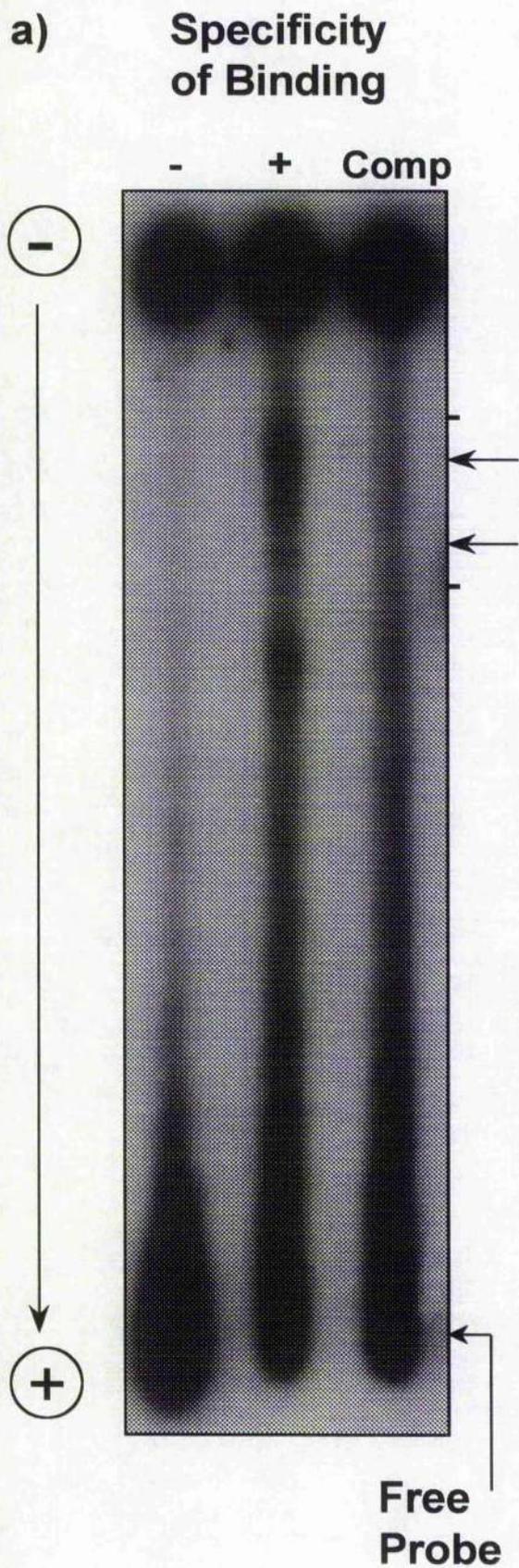


Figure 3.7 Effect of IL-4 on NF- κ B Expression in EDR B Cells.

The binding activity of 5 μ g aliquots of protein extract prepared from unstimulated EDR B cells for 32 P-labelled NF- κ B oligonucleotide was assessed by EMSA (Fig. 3.7a) in the absence or presence of unlabelled 5-fold excess NF- κ B oligonucleotide. Whole cell (Fig 3.7b), cytosolic (Fig 3.7c) and nuclear extracts (Fig3.7d) were prepared at hourly intervals from EDR B cells after incubation in the absence or presence of 500U/ml IL-4, and 5 μ g of protein extract assessed by EMSA for binding to 32 P-labelled NF- κ B oligonucleotide. Specific binding activities are indicated by horizontal arrows.



CHAPTER 4
STUDIES OF PROTEIN INTERACTIONS WITH NEGATIVE REGULATORY
REGION 1 (NRR₁) OF THE HUMAN CD25 PROMOTER.

4.1 Introduction.

The NRR_I region of the human CD25 promoter is characterised by an 11-bp NRE region and a retinoid-sensitive region characterised by the presence of a consensus AP-1 binding site. In T cells, the NRE region (TTCATCCCAGG) is suppressed by a 50kDa silencer protein which was identified in UV cross-linking studies (Smith and Greene, 1989). Binding of this protein to NRE leads to the prevention of CD25 gene expression in T cells. A possible candidate for NRE-BP is the ubiquitous transcription factor YY1. Although greater than 50kDa in mass, NRE has the minimal 5'-CAT-3' binding region for this 65kDa transcription factor and YY1 has been reported to have a negative regulatory effect on gene transcription (Shi et al., 1991). Other molecules of interest include the Ets family of transcription factors because NRE contains a 3'-GGA-5' core Ets binding site on the antisense strand, and the nuclear factor CBP/p300, which interacts with the cAMP/PKA stimulated CREB. The putative AP-1 binding site, adjacent to NRE, is homologous to the cAMP response element which is bound by CREB (Montminy et al., 1986). Given the size of CBP/p300, some 300kDa in mass, it is unlikely to fulfil the role of NRE-BP. However, CBP/p300 may form a complex with the proteins binding to NRR_I, possibly acting to bridge them together.

Retinoic acid (RA) also has an inhibitory effect on B cell function; studies in human B cells show that RA-treatment inhibits IL-4-induced IgE production and also prevents apoptosis (Lomo et al., 1998; Worm et al., 1998). In addition, RARE-specific oligonucleotide binding is blocked by IL-4, and RA has been shown to influence CD25 expression in human B cells (Bhatti and Sidell, 1994). This indicates that proteins binding to NRE and the retinoid sensitive region may have a synergistic effect on silencing CD25 expression. The role of AP-1 proteins binding to the putative TGACTCC binding site may have a positive or negative regulatory effect on gene transcription. In lymphocytes, AP-1 proteins have been shown to induce differentiation

and activation (Foletta et al, 1998). Conversely, a truncated form of fosB can inhibit fos and jun transcriptional activity (Nakabeppu and Nathans, 1991).

Work in Chapter 3 has described NRE binding activity in control and IL-4-stimulated cells. What is unknown is the nature of protein binding to NRR_I as a whole and whether the interaction between NRE and NRE-BP is affected by other proteins. In addition, the identity of NRE-BP is still unknown, although there are several candidate proteins which require investigation. The putative AP-1 binding site has yet to be characterised, and if this site does bind AP-1 family members, the role of this region in NRE binding activity requires clarification.

4.2 Origins and Effect of IL-4R Signalling on Protein-Binding to NRR_I.

The two binding regions, putative or otherwise, found within NRR_I indicate that this sequence may be bound by several protein species. The fact that IL-4 can abolish NRE binding activity has been established; however, the effect of IL-4R signalling on protein binding to NRR_I has not. In addition, IL-4R signalling can be driven by a complex of the IL-4R α subunit with either the γ c chain or the IL-13R subunit (Dawson et al, 1997). Although the IL-4R complex in lymphocytes is comprised of the IL-4R α subunit and γ c, the subunit-origin of IL-4R signalling which leads to the attenuation of NRE binding activity has not been determined.

EDR B cell whole cell extracts and quiescent tonsillar B cell nuclear extracts were prepared from cells treated with and without IL-4 over four hours. Extracts were probed with radiolabelled CD25-NRR_I oligonucleotide and analysed by EMSA. Figure 4.1 shows a study of EDR whole cell extracts. Figure 4.1a shows that three protein species bound to radiolabelled CD25-NRR_I, the uppermost of which was competed by an unlabelled two-fold excess of CD25-NRR_I oligonucleotide. In extracts prepared from untreated cells (Figure 4.1b), protein binding was unaffected over four hours. In

contrast, cell extracts prepared from IL-4-treated cells failed to bind protein to radiolabelled CD25-NRR_I after two hours (Figure 4.1c). Figure 4.2 shows a study on tonsillar B cell nuclear extracts. Figure 4.2 shows that three protein species bound radiolabelled CD25-NRR_I, all of which were competed by an unlabelled two hundred-fold excess of CD25-NRR_I oligonucleotide. Protein binding in untreated cells was unaffected (data not shown); however, in common with EDR B cells, protein binding to the NRR_I region was abrogated after two hours of IL-4 treatment (Figure 4.2).

In order to assess the subunit origins of IL-4R signalling, the effect of IL-13 on protein binding to NRR_I was investigated. Quiescent tonsillar B cells were treated with a range of concentrations of IL-13 for four hours. Samples were taken hourly and nuclear extracts generated then probed with radiolabelled CD25-NRR_I oligonucleotide then analysed by EMSA. Three protein species bound to radiolabelled CD25-NRR_I, all of which were competed by the addition of an unlabelled excess of CD25-NRR_I oligonucleotide. Figure 4.3a shows that two protein species bound radiolabelled CD25-NRR_I, both of which were competed by an unlabelled two hundred-fold excess of CD25-NRR_I oligonucleotide. Figure 4.3b shows that protein binding was unaffected throughout the four hour incubation in untreated extracts and Figure 4.3c shows binding was unaffected in extracts generated from cells treated with 3ng/ml of IL-13. Figures 4.3d and 4.3e show protein binding was unaffected in extracts generated from cells treated with 6ng/ml and 12ng/ml of IL-13, respectively. This experiment was repeated, except extracts were probed with the NRE-intact/AP-1-mutant CD25-NRR_I-M2 oligonucleotide. Protein binding to radiolabelled CD25-NRR_I-M2 was unaffected with and without treatment with a range of IL-13 concentrations (data not shown). These data are consistent with the interpretation that IL-13 does not affect protein binding to NRE or NRR_I.

4.3 EMSA Studies of Oligonucleotide Binding in Human Lymphoid Cells.

The NRE region and the putative AP-1 binding region in the NRR_T sequence were analysed by EMSA experiments on cell extracts using four consensus and mutant oligonucleotides (described in section 2.1.2). Binding to NRE and the putative AP-1 binding site was investigated in an attempt to characterise protein binding to the NRR_T region. Cell extracts of untreated tonsillar B cells and a panel of B and T-lineage cells were probed with radiolabelled NRR_T oligonucleotides and then analysed by EMSA. Extracts were probed with radiolabelled CD25-NRR_T, then protein binding was challenged by the individual addition of an unlabelled two hundred-fold excess of CD25-NRR_T, CD25-NRR_T-M1, CD25-NRR_T-M2 and CD25-NRR_T-M3 oligonucleotides. Each oligonucleotide was radiolabelled and used to probe cell extracts. Binding was challenged by the individual addition of all four unlabelled oligonucleotides in a two hundred-fold excess, starting with the same oligonucleotide that was used as the probe. This procedure was repeated until each oligonucleotide had been used to probe the cell extracts and had been challenged by all four excess unlabelled oligonucleotides.

Figure 4.4 shows a study of tonsillar B cell nuclear extracts. The first EMSA panel in figure 4.4a shows that protein binding to radiolabelled CD25-NRR_T was competed by an unlabelled two hundred-fold excess of CD25-NRR_T and CD25-NRR_T-M2 but not CD25-NRR_T-M1 or CD25-NRR_T-M3. The second EMSA panel shows that two main protein species bound to radiolabelled CD25-NRR_T-M1. These bands were not competed by an unlabelled two hundred-fold excess of CD25-NRR_T-M1, CD25-NRR_T-M2 or CD25-NRR_T-M3 but partially competed by CD25-NRR_T oligonucleotide. The first EMSA panel in figure 4.4b shows that three faster-migrating protein species bound to radiolabelled CD25-NRR_T-M2. The middle band was competed by an unlabelled two hundred-fold excess of CD25-NRR_T-M2 and CD25-NRR_T but not by CD25-NRR_T-M1 or CD25-NRR_T-M3 oligonucleotides. The second EMSA panel shows that four protein species bound to radiolabelled CD25-NRR_T-M3. This binding was not competed by an

unlabelled two hundred-fold excess of CD25-NRR_T-M3, CD25-NRR_T-M1 or CD25-NRR_T-M2 but partially competed by CD25-NRR_T oligonucleotide.

Table 4.1 shows a summary of the same experiments on a range of B and T cell extracts. Experiments on P3HR1 B cell extract strongly suggested that, in common with tonsillar B cell nuclear extract, protein binding to radiolabelled CD25-NRR_T oligonucleotide was competed by an unlabelled two hundred-fold excess of CD25-NRR_T and CD25-NRR_T-M2, but not by an excess of CD25-NRR_T-M1 or CD25-NRR_T-M3 oligonucleotides. Similarly, no specific binding was evident for radiolabelled CD25-NRR_T-M1 or CD25-NRR_T-M3 oligonucleotides and binding to radiolabelled CD25-NRR_T-M2 was competed by an unlabelled two-hundred fold molar excess of CD25-NRR_T and CD25-NRR_T-M2. EDR B cell nuclear extracts bound protein to radiolabelled CD25-NRR_T oligonucleotide. This binding was competed by an unlabelled two hundred-fold excess of CD25-NRR_T and CD25-NRR_T-M2 but not CD25-NRR_T-M3 oligonucleotides. Protein binding was partially competed by an unlabelled two hundred-fold excess of CD25-NRR_T-M1 oligonucleotide. These findings were also observed from studies in SMS-SB pre-B cell whole cell extracts and Jurkat T cell nuclear extracts. Data from studies of the IB4 B cell line whole cell extracts are in agreement with the other cell extracts, however protein binding to radiolabelled CD25-NRR_T was not competed by an unlabelled two hundred-fold excess of CD25-NRR_T-M1 oligonucleotide. EDR B cell nuclear extracts bound protein to radiolabelled CD25-NRR_T-M2 oligonucleotide. This binding was competed by an unlabelled two hundred-fold excess of CD25-NRR_T-M2 but not by CD25-NRR_T or CD25-NRR_T-M3 oligonucleotides. Protein binding was partially competed by an unlabelled two hundred-fold excess of CD25-NRR_T-M1 oligonucleotide. The failure of unlabelled two hundred-fold excess CD25-NRR_T to compete protein binding to radiolabelled CD25-NRR_T-M2 was also observed in IB4 B cell extracts and in Jurkat T cell extracts, although unlabelled excess CD25-NRR_T-M1 had no competing effects. SMS-SB pre-B cell extracts failed to bind radiolabelled CD25-NRR_T-M2 oligonucleotide. Radiolabelled CD25-NRR_T-M1 or CD25-NRR_T-M3 oligonucleotides

failed to bind specific protein species to all extracts in Table 1. These data show that an intact NRE region is required for protein to bind to CD25-NRR_I oligonucleotides, however in contrast to tonsillar B cell and P3HR1 B cell extracts, protein binding to radiolabelled CD25-NRR_I was partially competed by an unlabelled excess of CD25-NRR_I-M1 oligonucleotides and protein binding to radiolabelled CD25-NRR_I-M2 was not competed by an unlabelled two hundred-fold excess of CD25-NRR_I oligonucleotide.

4.4 EMSA Studies of a CD25-NRR_I-M1 Titration on Radiolabelled CD25-NRR_I Protein Binding.

Experiments on NRR_I-protein binding showed that in most cell lines, protein binding to radiolabelled CD25-NRR_I was partially competed by the addition of excess CD25-NRR_I-M1 oligonucleotide (Table 4.1). To further investigate this, a titration of unlabelled CD25-NRR_I-M1 was performed with samples containing EDR B cell nuclear extract and radiolabelled CD25-NRR_I oligonucleotide. Figure 4.5a shows that three main protein species bound to radiolabelled CD25-NRR_I. The uppermost band was competed by an unlabelled two hundred-fold excess of CD25-NRR_I oligonucleotide. Figure 4.5b indicates that increasing concentrations of unlabelled CD25-NRR_I-M1 diminished protein binding to radiolabelled CD25-NRR_I. Protein binding was completely blocked by concentrations greater than or equal to an unlabelled two hundred-fold excess of CD25-NRR_I-M1 oligonucleotide.

4.5 Characterisation of YY1 and AP-1 Protein in Human B Cells.

The 65kDa ubiquitous transcription factor YY1 was proposed as a possible NRE-BP candidate. The pattern of expression of YY1 in resting and stimulated B cells was determined. Tonsillar B cells were treated with and without IL-4 and anti- μ -antibody over a period of four hours, samples were taken every hour and whole cell extracts prepared. Figure 4.6a shows a Western blot of nuclear extract derived from the cells,

and anti-YY1-antibody bound a protein species of approximately 65kDa. The level of expression of this protein was unaffected in samples which had been treated with IL-4 or anti- μ -antibody, relative to control, unstimulated B cells.

EDR B cell extracts were treated with and without IL-4 over a period of four hours, samples taken every hour and whole cell extracts generated. Cell extracts were assessed for AP-1 family member expression by Western blotting and incubation with antibodies against c-fos and c-jun. Figure 4.6b shows that anti-c-fos antibody bound a protein species of approximately 60kDa which was unaffected in samples treated with IL-4, when compared to unstimulated cells. Anti-c-jun antibody failed to bind protein in both treated and untreated samples (data not shown).

4.6 Characterisation of YY1 Binding to NRE.

In order to determine if YY1 was in fact identical to NRE-BP, two EMSA experiments were designed. The first experiment involved probing untreated tonsillar B cell whole cell extracts with radiolabelled YY1 oligonucleotide. Protein binding to YY1 was then challenged with excess unlabelled YY1 oligonucleotide or CD25-NRE oligonucleotide. Untreated extracts were probed with radiolabelled CD25-NRE and protein binding was challenged by excess unlabelled CD25-NRE and YY1 oligonucleotide. Figure 4.7 shows that protein binding to radiolabelled YY1 was specifically competed by an unlabelled one- or two-fold excess of YY1 oligonucleotide, but not by an unlabelled one- or five-fold excess of CD25-NRE. One slower-migrating species bound to radiolabelled CD25-NRE. This band was competed by an unlabelled one- or five-fold excess of CD25-NRE but not by an unlabelled one- or two-fold excess of YY1 oligonucleotide.

The second EMSA experiment was a supershift of protein binding to radiolabelled YY1 oligonucleotide with anti-YY1-antibody. Supershifted bands were challenged with excess YY1 oligonucleotide then excess CD25-NRE. Figure 4.8 shows that one species

of protein bound to radiolabelled YY1 oligonucleotide. Additional treatment with anti-YY1-antibody caused a slower migratory species shift which was competed by an unlabelled one- or two-fold excess of YY1 oligonucleotide. The shift was not competed by an unlabelled one- or five-fold excess of CD25-NRE oligonucleotide.

4.7 EMSA Supershift Analysis of NRE-BP Candidates and AP-1 Binding.

The investigation of candidate proteins for NRE-BP continued with studies of protein binding to NRR_I using EDR B cell whole cell extract in EMSA supershift experiments. The candidate proteins were YY1, Ets and CBP/p300. The latter protein was considered for a possible "bridging" role with proteins binding to NRR_I. In addition, these experiments were performed to characterise the putative AP-1 binding site (TGACTCC) found within the NRR_I region. Figure 4.9 shows that three protein species bound to radiolabelled CD25-NRR_I. The two uppermost bands were competed by an unlabelled two-fold excess of CD25-NRR_I oligonucleotide. The addition of anti- YY1, Ets and CBP/p300 antibodies failed to shift either of the two upper bands. Addition of anti-c-fos antibody shifted the lower of the two uppermost bands. Addition of anti-c-jun antibody failed to shift either band. These data suggest that c-fos binds to NRR_I, whereas c-jun, YY1, Ets and CBP/p300 fail to show binding to nucleotide sequences in this region.

4.8 Discussion.

The NRR_I sequence of the human CD25 promoter gene is 31bp long and contains several protein binding regions, the most important of which is an 11bp NRE sequence that is bound, in T cells, by a silencer protein of approximately 50kDa in mass. Previous studies of the NRE region showed that IL-4 diminished NRE-BP binding over a period of four hours. The effect of IL-4 on protein binding to the entire NRR_I region was unknown. Figures 4.1 and 4.2 show that IL-4 treatment of both tonsillar B cells and EDR B cells abrogated protein binding to radiolabelled CD25-NRR_I after two hours of

stimulation. This suggests that IL-4-induced up-regulation of CD25 expression in human B cells is achieved by the attenuation of protein binding to the entire NRR_I region and is not simply confined to the NRE region.

In B cells, the IL-4 receptor subunit mainly consists of the IL-4R α subunit in complex with the γ c chain. Both components are involved in signalling and it has not been determined whether signalling from the IL-4R α subunit, the γ c chain, or both is involved in the loss of protein binding activity for NRR_I. To address this question, tonsillar B cells were treated with rh-IL-13 as the IL-13 receptor complex consists of the IL-13R subunit and the IL-4R α subunit, therefore removing the signalling input of the γ c chain. Figure 4.3 shows that protein binding to radiolabelled NRR_I is unaffected by IL-13. This indicates that the effect of IL-4R signalling on protein binding to the NRR_I region is likely to be mediated through the γ c chain and not through IL-4R α .

Adjacent to the NRE region is a retinoid-sensitive region which is characterised by the presence of a consensus AP-1 binding domain. Little is known of the role of the consensus AP-1 binding site in protein binding to the NRR_I region. Figure 4.4 and Table 4.1 show that, in tonsillar and P3HR1 B cells, protein binding to a series of NRR_I oligonucleotides is dependent upon sequences containing an intact NRE region. Both EDR and tonsillar B cell extract proteins fail to bind oligonucleotides containing mutated NRE sequences. Compared with protein binding to the consensus CD25-NRR_I oligonucleotide, the migration of protein species binding to radiolabelled CD25-NRR_I-M2 was faster. This indicates that the mutation of the AP-1-homology region leads to an alteration in protein binding to NRR_I and suggests that protein binds specifically to the AP-1-homology region as part of the NRR_I-binding complex. Protein binding to oligonucleotides containing consensus AP-1 sequences was noted only when an intact NRE region was present indicating that NRE is required to facilitate protein binding to the putative AP-1 binding site. This suggests that the NRE region is the dominant region for protein binding in the NRR_I sequence. Table 4.1 shows a summary of the

oligonucleotide study carried out in a range of B cells and the Jurkat T cell line. With the exception of SMS SB pre-B cell extracts, which showed no specific binding activity for CD25-NRR_T-M2, all other extracts showed that protein binding to radiolabelled CD25-NRR_T-M2 was competed by the addition of an unlabelled two-fold excess of CD25-NRR_T-M2 but was not competed by CD25-NRR_T. Only tonsillar B cell and P3HR1 B cell extracts showed competition of protein binding to radiolabelled CD25-NRR_T-M2 with excess unlabelled CD25-NRR_T. One explanation which may account for these inconsistent binding patterns is that the consensus and mutant oligonucleotides contained relatively minor differences and, as opposed to comparing YY1 oligonucleotide binding with CD25-NRE, the comparison between the CD25-NRR_T oligonucleotides was of a more subtle nature.

Figure 4.5 shows the effect of a titration of unlabelled CD25-NRR_T-M1 on protein-binding to radiolabelled CD25-NRR_T. Protein binding was blocked by concentrations greater than or equal to an unlabelled two-fold excess of CD25-NRR_T-M1. Therefore, CD25-NRR_T-M1 can compete protein binding to the consensus CD25-NRR_T oligonucleotide. This finding was unexpected because EMSA experiments showed that NRR_T specific protein species failed to bind radiolabelled oligonucleotides which contained a mutated NRE region (Figure 4.4 and Table 4.1). This suggests that the competition of protein binding to the consensus AP-1 binding region leads to the eventual loss of NRE binding activity. Therefore, proteins binding to the consensus AP-1 binding site may have a role in NRE binding activity and as a result, negative regulation of the CD25 promoter.

The transcription factor YY1 was a possible candidate for NRE-BP. NRE has a minimal 5'- CAT -3' binding site for YY1 and the negative regulatory capabilities of this transcription factor are documented (Shi et al., 1991). Figure 4.6a shows a constitutively expressed protein of approximately 65kDa in treated and untreated tonsillar B cell extract. This is the same mass as YY1 and suggests that this transcription factor is

expressed in both quiescent and stimulated tonsillar B cells. Figure 4.7 shows that protein binding to radiolabelled YY1 oligonucleotide was not competed by excess unlabelled CD25-NRE and vice versa. Figure 4.8 demonstrates that protein binding to radiolabelled YY1 was supershifted by the addition of anti-YY1 antibody. This shift was competed by the addition of excess unlabelled YY1 oligonucleotide but not by excess unlabelled CD25-NRE. The supershift EMSA increases certainty of the identity of the protein binding to the oligonucleotide. This data supports the Western blot findings that YY1 is present in tonsillar B cells and the initial EMSA experiments which showed no competition in binding between YY1 and CD25-NRE oligonucleotides. The failure of CD25-NRE to compete both normal and supershifted binding to radiolabelled YY1 oligonucleotide indicates that YY1 is not NRE-BP.

The supershift EMSA experiment was extended to the full-length NRR_I sequence. Figure 4.9 shows that anti-YY1 antibody failed to shift protein binding to radiolabelled CD25-NRR_I indicating again that YY1 does not bind NRE, or any other region in NRR_I. In addition, anti-Ets antibodies failed to shift protein binding to radiolabelled CD25-NRR_I indicating that the Ets is not NRE-BP. The failure of anti-CBP/p300 antibody to supershift protein binding to radiolabelled CD25-NRR_I indicates that this larger transcriptional regulator is not involved in a bridging complex with proteins binding to NRR_I. In an attempt to characterise the putative AP-1 binding site, supershift experiments assessed the common AP-1 family members, c-fos and c-jun. Figure 4.9 shows that anti-c-fos supershifted a protein species binding to radiolabelled CD25-NRR_I. Treatment with anti-c-jun failed to supershift these species. This is consistent with Figure 4.6b which shows that c-fos is constitutively expressed in IL-4-stimulated or untreated EDR B cells, whereas c-jun is not (data not shown). These findings are in agreement with reported AP-1 expression in B cells (Foletta et al, 1998). The capacity of c-fos to supershift protein binding to CD25-NRR_I indicates that the TGACTCC region of NRR_I is a *bona fide* site for the binding of AP-1 proteins. The binding site for AP-1 is within the retinoid-sensitive sequence of NRR_I. RA upregulates CD25

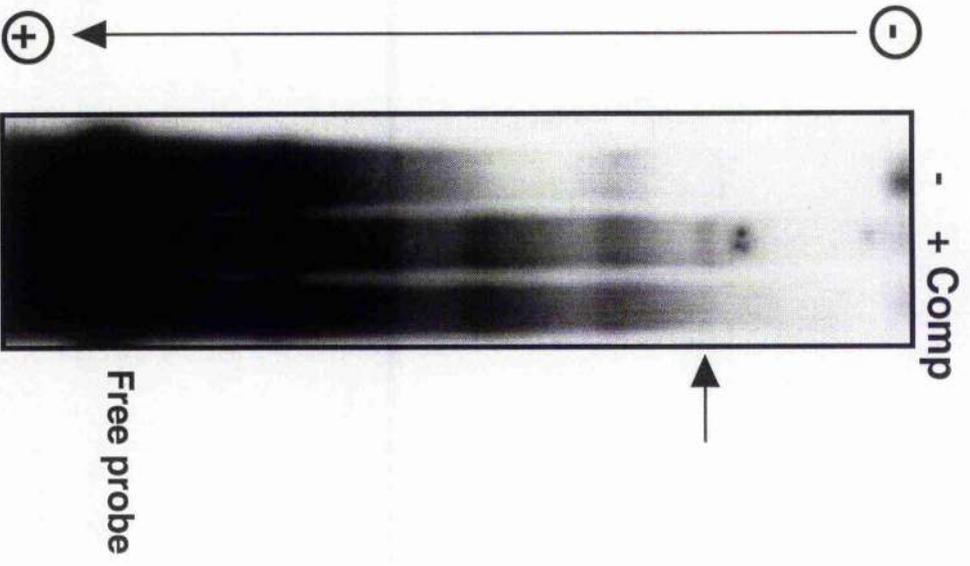
expression in human B cells (Bhatti and Sidell, 1994), therefore it is possible that both RA and AP-1 may interact with one another, leading to the loss of NRE binding activity. A possible mechanism of action is that binding of RA to the AP-1 binding site causes the alteration of AP-1 protein binding. This may result in the inactivation of the AP-1 protein, possibly disrupting NRE-BP binding and initiating the transcription of the CD25 promoter. Therefore, RA may in fact up-regulate CD25 in B cells via AP-1 inactivation.

In summary, there are two main binding regions in NRR_I : NRE and an AP-1 binding site. In addition to inducing loss of NRE-binding activity, IL-4 treatment abrogates protein binding to the entire NRR_I sequence. IL-4R signalling which leads to this is likely to be mediated through the γ c chain of the IL-4R complex. EMSA studies on lymphoid cells indicate that NRE is the dominant binding region of NRR_I and is required for AP-1 protein binding. In addition, competition of AP-1 binding leads to the loss of NRR_I binding activity suggesting that AP-1 may have a role in the suppression of CD25 transcription. NRE-BP has yet to be identified, however supershift experiments revealed that it is not YY1 or Ets. CBP/p300 does not form a bridging complex with NRR_I binding proteins. Supershift experiments confirmed that the TGACTCC region of NRR_I is an AP-1 binding region. The AP-1 family member c-fos binds the AP-1 region of NRR_I .

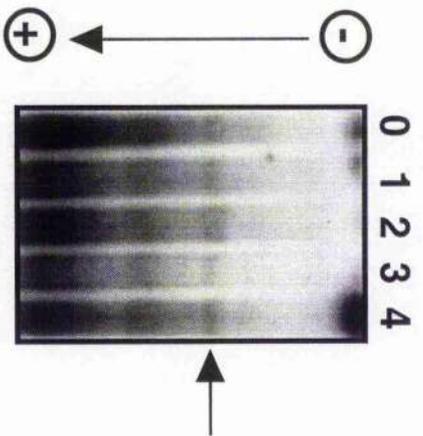
Figure 4.1 EMSA Analysis on the Effect of IL-4R Signalling on NRR_I Binding Activity in EDR B Cells.

The binding activity of 5µg aliquots of EDR B cell whole cell extract to ³²P-labelled CD25-NRR_I was analysed by EMSA (Fig 4.1a) in the absence or the presence of unlabelled two hundre-fold excess CD25-NRR_I oligonucleotide as competitor. Whole cell extracts were prepared at hourly intervals from EDR B cells after incubation in the absence (Fig. 4.1b) or presence (Fig 4.1c) of 500U/ml IL-4, and 5µg of protein extract assessed by EMSA for binding to ³²P-labelled CD25-NRR_I. Specific binding activities are indicated with horizontal arrows.

a) Specificity



b) Control



c) IL-4

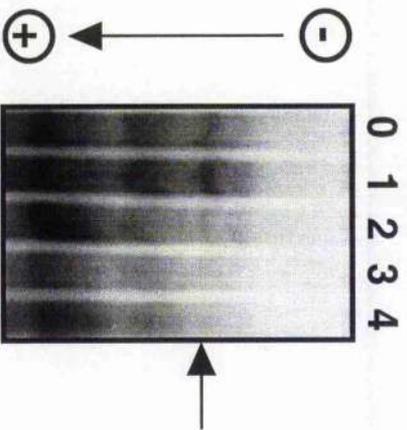


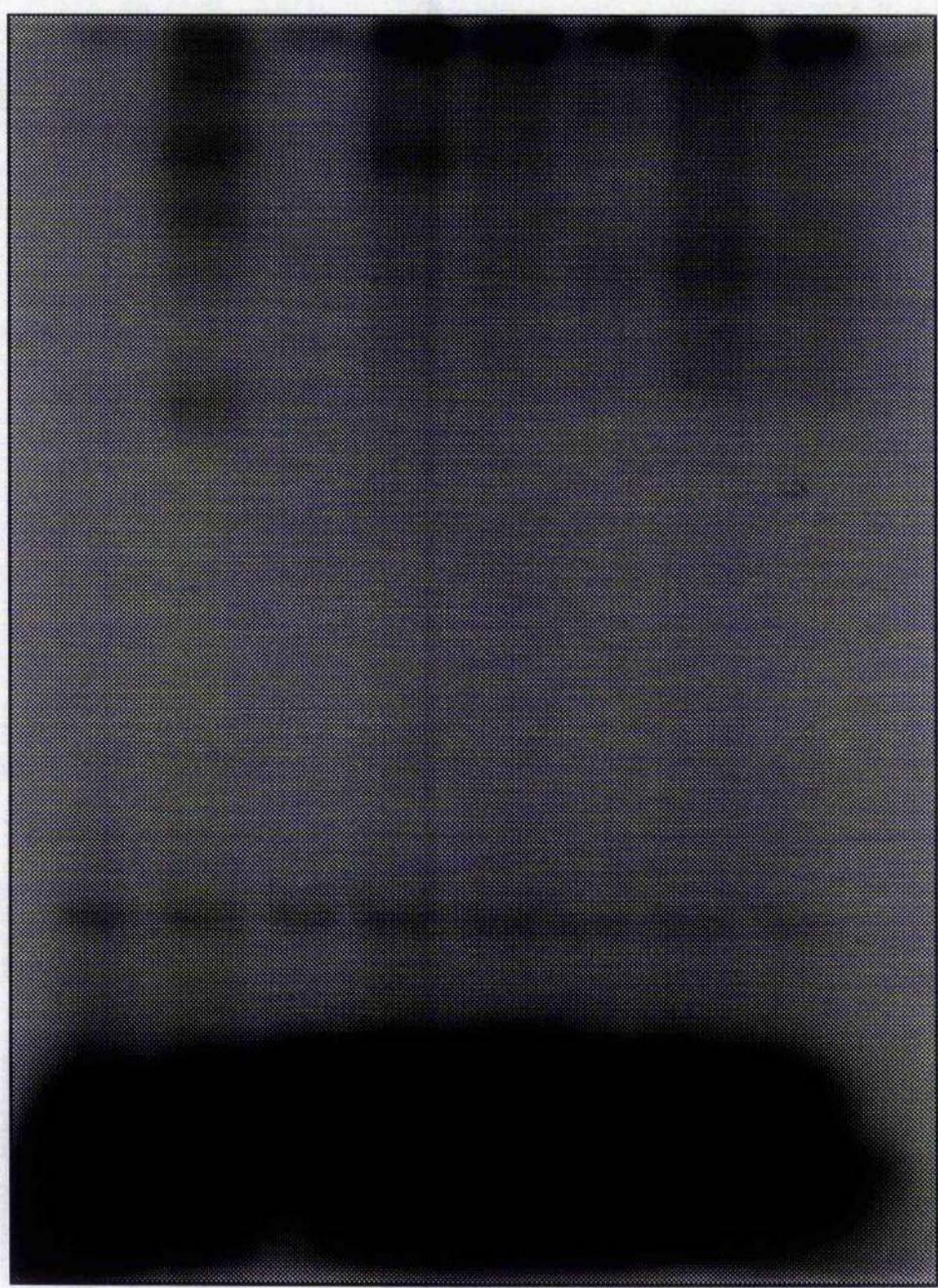
Figure 4.2 EMSA Study on the Effect of IL-4R Signalling on NRR₁ Binding Activity in Tonsillar B Cells.

The binding activity of 5µg aliquots of tonsillar B cell nuclear extract for ³²P-labelled CD25-NRR₁ was analysed by EMSA in the absence or the presence of unlabelled two hundred-fold excess CD25-NRR₁ oligonucleotide as competitor. Nuclear extracts were prepared at hourly intervals from tonsillar B cells after incubation in the presence of 500U/ml IL-4, and 5µg of protein extract assessed by EMSA for binding to ³²P-labelled CD25-NRR₁. Specific binding activity is indicated by a horizontal arrow.

Time (h)

- + C 0 1 2 3 4

(-)



←

← Free Probe

Figure 4.3 Effect of IL-13R Signalling on NRR_I Binding Activity in Tonsillar B Cells.

The binding activity of 5µg aliquots of tonsillar B cell nuclear extract for ³²P-labelled CD25-NRR_I was analysed by EMSA (Fig. 4.3a) in the absence or the presence of unlabelled two hundred-fold excess CD25-NRR_I oligonucleotide as competitor. Nuclear extracts were prepared at hourly intervals from tonsillar B cells after incubation in the absence (Fig. 4.3b) or presence (Figs. 4.3c, 4.3d and 4.3e) of 3ng/ml, 6ng/ml and 12ng/ml IL-13, and 5µg of protein extract assessed by EMSA for binding to ³²P-labelled CD25-NRR_I. Specific binding activities are indicated by the horizontal arrow.

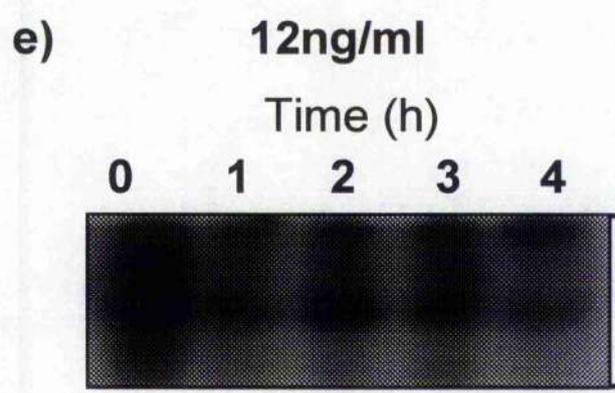
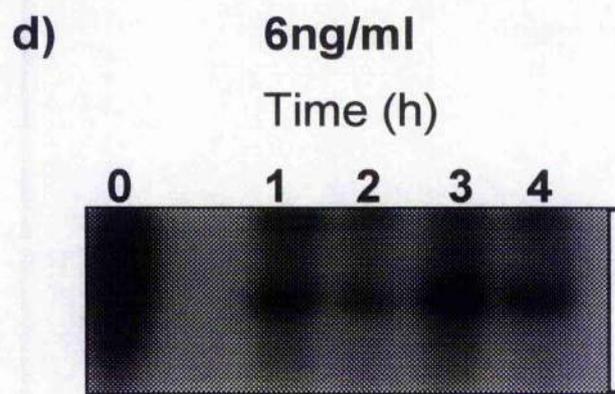
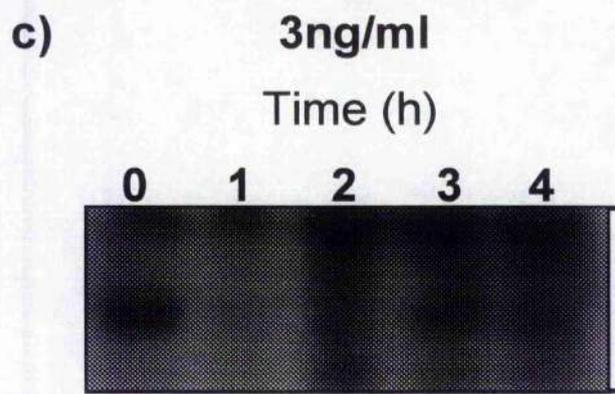
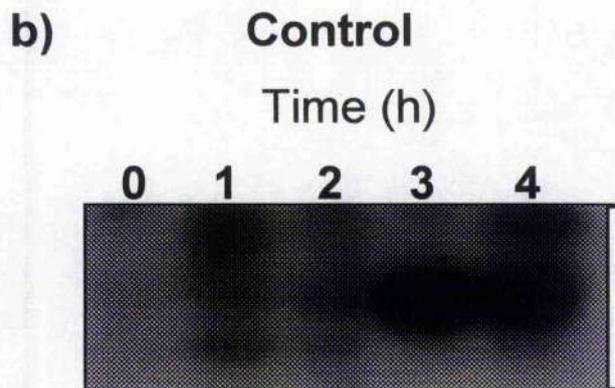
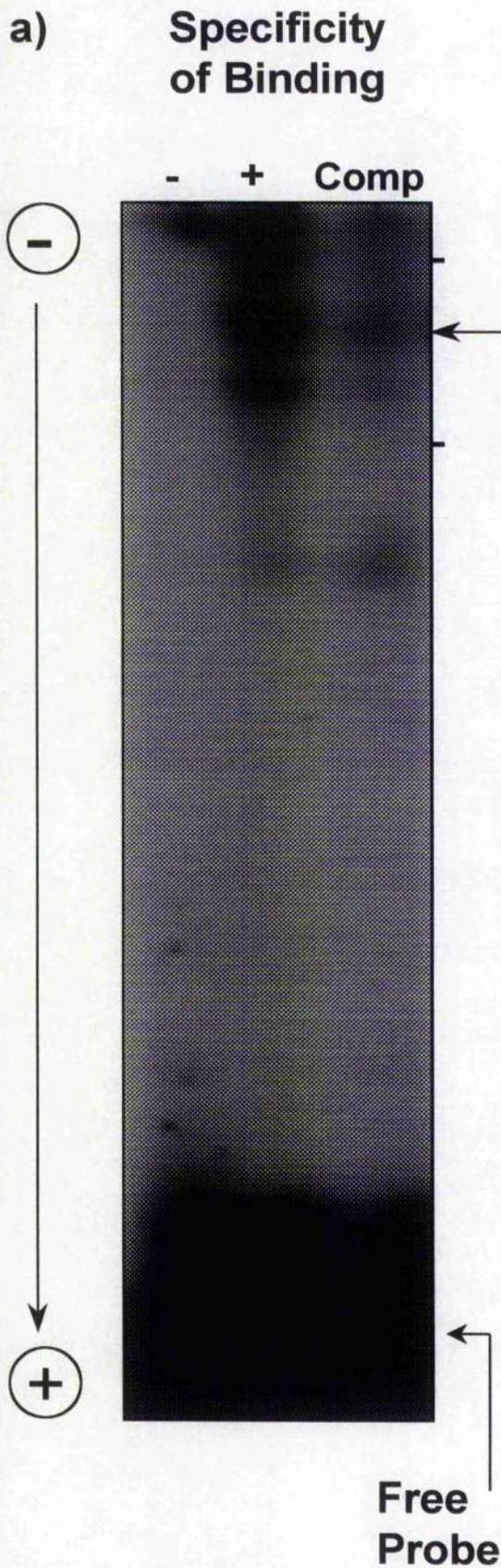


Figure 4.4 EMSA Analysis of Protein Binding to NRR_I in Tonsillar B Cells.

Nuclear extracts were prepared from resting tonsillar B cells (1×10^7 cells) which had been cultured overnight in fresh complete medium. The binding activity of 5 μ g aliquots of protein to ³²P-labelled CD25-NRR_I (Fig. 4.4a), CD25-NRR_I-M1 (Fig. 4.4a), CD25-NRR_I-M2 (Fig 4.4b), and CD25-NRR_I-M3 (Fig 4.4bi) oligonucleotide was assessed by EMSA in the absence or presence of unlabelled two hundred-fold excess CD25-NRR_I (0), CD25-NRR_I-M1 (1), CD25-NRR_I-M2 (2), and CD25-NRR_I-M3 (3) oligonucleotide as competitor, respectively. Specific binding activities are indicated with horizontal arrows.

a)

CD25-NRR₁

CD25-NRR₁-M1

- + 0 1 2 3 - + 1 0 2 3

⊖



⊕



Free Probe

b)

CD25-NRR₁-M2

CD25-NRR₁-M3

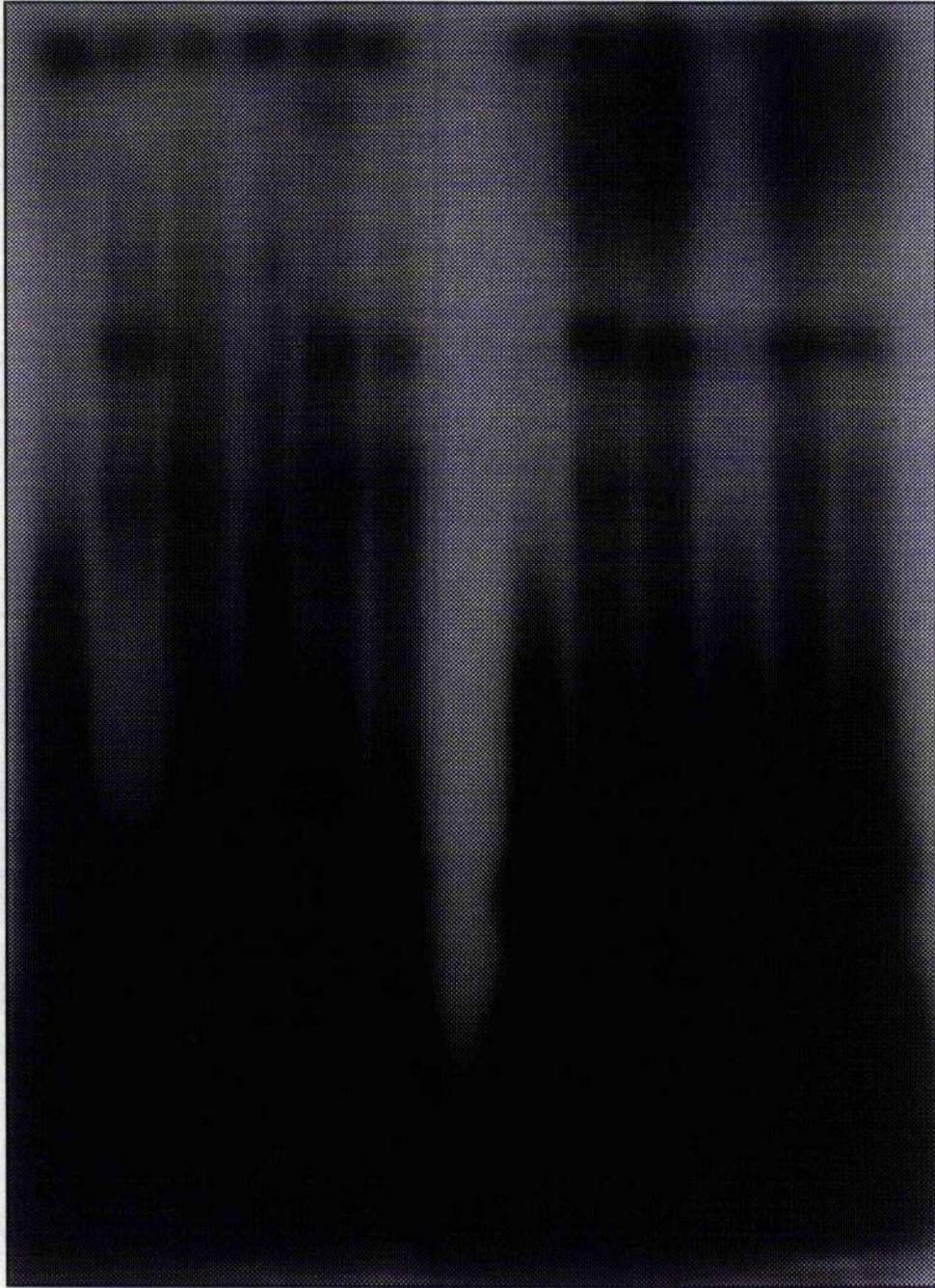
- + 2 0 1 3

- + 3 0 1 2

⊖



⊕



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←

Free
Probe

Table 4.1 Summary of NRR_T Binding Activity in Lymphoid Cells.

Cell extracts were prepared from P3HR1, EDR, IB4, SMS B cells or Jurkat T cells. The binding activity of 5µg aliquots of protein for ³²P-labelled CD25-NRR_T, CD25-NRR_T-M1, CD25-NRR_T-M2, and CD25-NRR_T-M3 oligonucleotides were assessed by EMSA in the absence or presence of unlabelled two hundred-fold excess CD25-NRR_T, CD25-NRR_T-M1, CD25-NRR_T-M2, and CD25-NRR_T-M3 oligonucleotides. Table 4.1 shows the effect of each unlabelled two hundred-fold excess oligonucleotide on protein binding for each ³²P-labelled oligonucleotide in P3HR1 B cells (panel 1), EDR B cells (panel 2), IB4 B cells (panel 3), SMS-SB B cells (panel 4), and Jurkat T cells (panel 5). Complete competition of protein binding for ³²P-labelled oligonucleotide by unlabelled 2-fold excess oligonucleotide is indicated by YES, incomplete competition of binding is indicated by PARTIAL, and no competition of binding is indicated by NO.

$\gamma^{32}\text{P}$ 

| P3HR1 B Cell | CD25-NRR ₁ | CD25-NRR ₁ -M1 | CD25-NRR ₁ -M2 | CD25-NRR ₁ -M3 |
|---------------------------|-----------------------|---------------------------|---------------------------|---------------------------|
| CD25-NRR ₁ | YES | NO | YES | NO |
| CD25-NRR ₁ -M1 | NO | NO | NO | NO |
| CD25-NRR ₁ -M2 | YES | NO | YES | NO |
| CD25-NRR ₁ -M3 | NO | NO | NO | NO |

| EDR B Cell | CD25-NRR ₁ | CD25-NRR ₁ -M1 | CD25-NRR ₁ -M2 | CD25-NRR ₁ -M3 |
|---------------------------|-----------------------|---------------------------|---------------------------|---------------------------|
| CD25-NRR ₁ | YES | NO | NO | NO |
| CD25-NRR ₁ -M1 | PARTIAL | NO | PARTIAL | NO |
| CD25-NRR ₁ -M2 | YES | NO | YES | NO |
| CD25-NRR ₁ -M3 | NO | NO | NO | NO |

| IB4 B Cell | CD25-NRR ₁ | CD25-NRR ₁ -M1 | CD25-NRR ₁ -M2 | CD25-NRR ₁ -M3 |
|---------------------------|-----------------------|---------------------------|---------------------------|---------------------------|
| CD25-NRR ₁ | YES | NO | NO | NO |
| CD25-NRR ₁ -M1 | NO | NO | NO | NO |
| CD25-NRR ₁ -M2 | YES | NO | YES | NO |
| CD25-NRR ₁ -M3 | NO | NO | NO | NO |

| SMS SB B Cell | CD25-NRR ₁ | CD25-NRR ₁ -M1 | CD25-NRR ₁ -M2 | CD25-NRR ₁ -M3 |
|---------------------------|-----------------------|---------------------------|---------------------------|---------------------------|
| CD25-NRR ₁ | YES | NO | NO | NO |
| CD25-NRR ₁ -M1 | PARTIAL | NO | NO | NO |
| CD25-NRR ₁ -M2 | YES | NO | NO | NO |
| CD25-NRR ₁ -M3 | NO | NO | NO | NO |

Unlabelled Two Hundred-Fold Excess Oligonucleotide

Unlabelled Two Hundred-Fold Excess Oligonucleotide

$\gamma^{32}\text{P}$



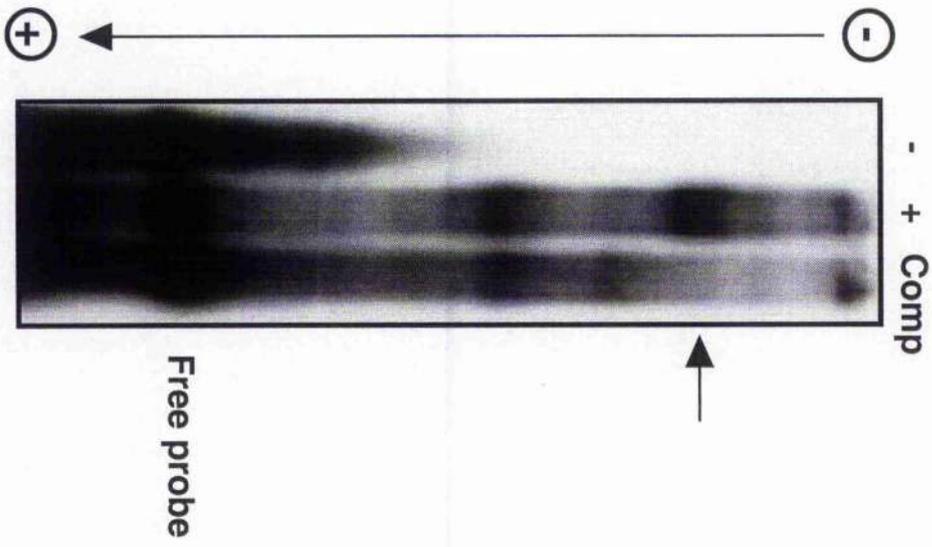
| Jurkat T Cell | CD25-NRR _i | CD25-NRR _i -M1 | CD25-NRR _i -M2 | CD25-NRR _i -M3 |
|---------------------------|-----------------------|---------------------------|---------------------------|---------------------------|
| CD25-NRR _i | YES | NO | NO | NO |
| CD25-NRR _i -M1 | PARTIAL | NO | NO | NO |
| CD25-NRR _i -M2 | YES | NO | YES | NO |
| CD25-NRR _i -M3 | NO | NO | NO | NO |



Figure 4.5 Analysis on the Effect of CD25-NRR_I-M1 Competition of CD25-NRR_I Binding Activity.

The binding activity of 5µg aliquots of EDR B cell nuclear extracts for ³²P-labelled CD25-NRR_I oligonucleotide was analysed by EMSA (Fig. 4.6a) in the absence or presence of unlabelled two hundred-fold excess CD25-NRR_I oligonucleotide as competitor. Figure 4.6b shows the binding activity of 5µg aliquots of EDR B cell nuclear extracts for ³²P-labelled CD25-NRR_I oligonucleotide in the absence or presence of unlabelled two hundred-fold excess CD25-NRR_I oligonucleotide as competitor, in the presence of unlabelled 25, 50, 75, 1, 125 and one hundred and fifty--fold CD25-NRR_I-M1 oligonucleotide. Specific binding activities for protein binding to CD25-NRR_I are indicated by the bold horizontal arrow.

a) Specificity



b) Titration

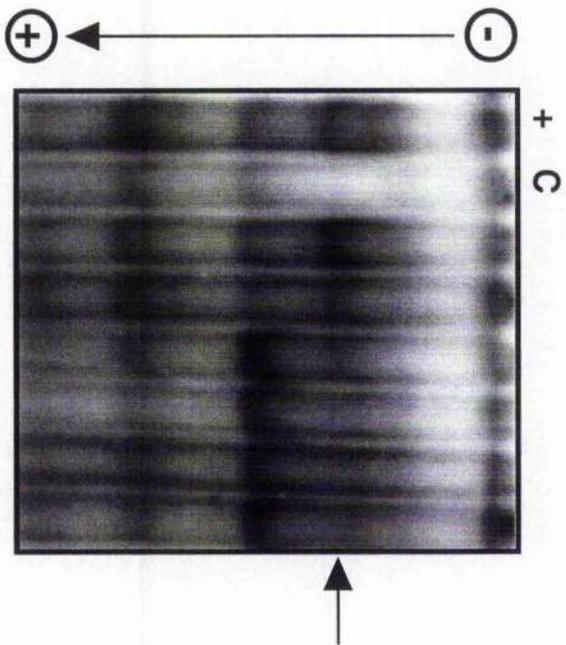


Figure 4.6 Western Blot Analysis of YY1 and AP-1 Protein in Human B Cells.

Whole cell extracts were prepared at hourly intervals from quiescent tonsillar B cells which were either resting, treated with 500U/ml IL-4 or treated with 1 μ g/ml rabbit anti- μ chain antibody, over a four hour period. 30 μ g of protein extract analysed by Western blotting with rabbit anti-YY1 antibody (Fig. 4.7a). Whole cell extracts were also generated at hourly intervals from EDR B cells which were treated with 500U/ml IL-4 over a four hour period, 30 μ g of extract was analysed with rabbit anti-c-fos antibody (Fig 4.7b).

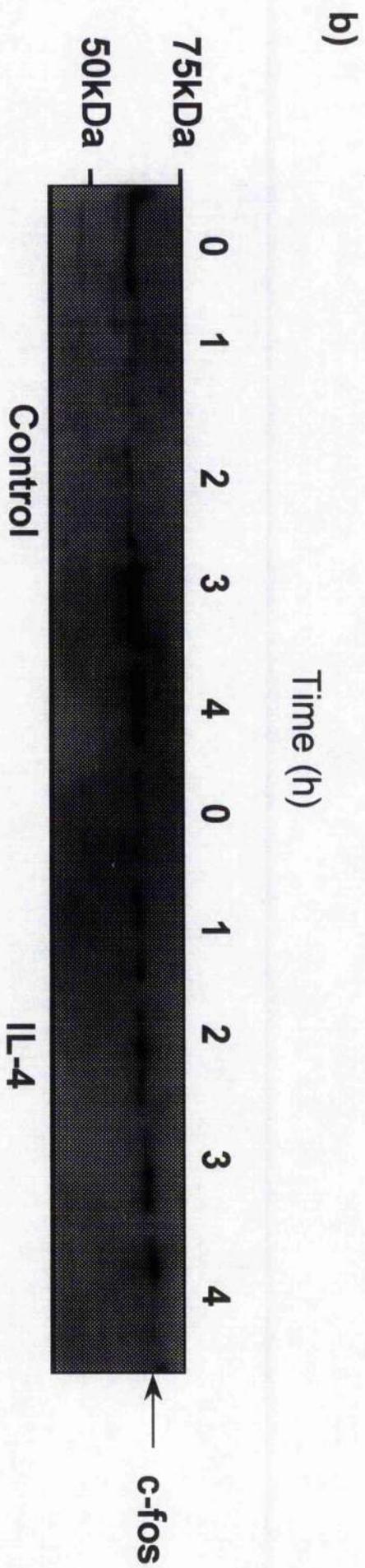
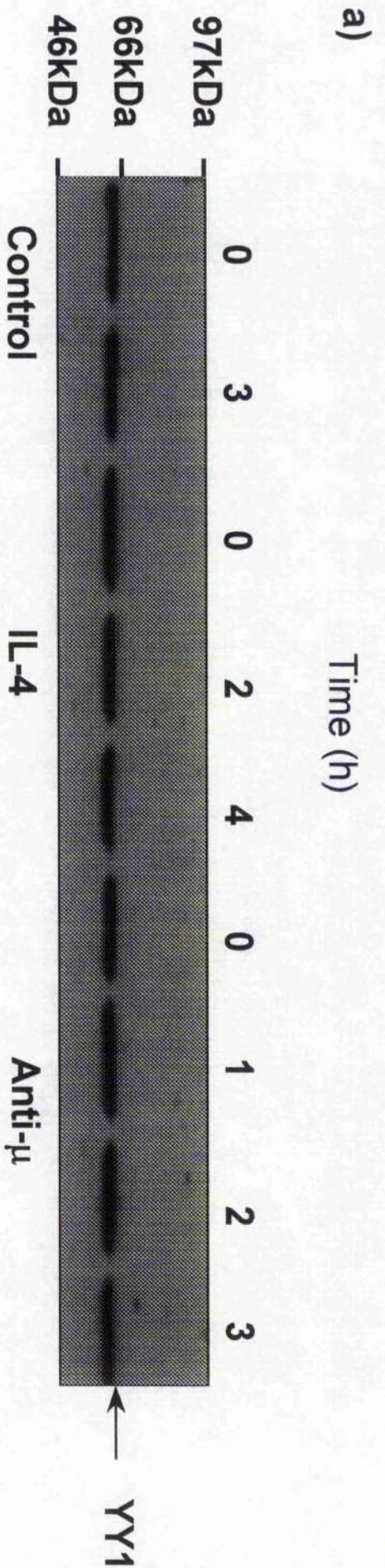


Figure 4.7 EMSA Study of Protein Binding to YY1 and NRE in Tonsillar B Cells.

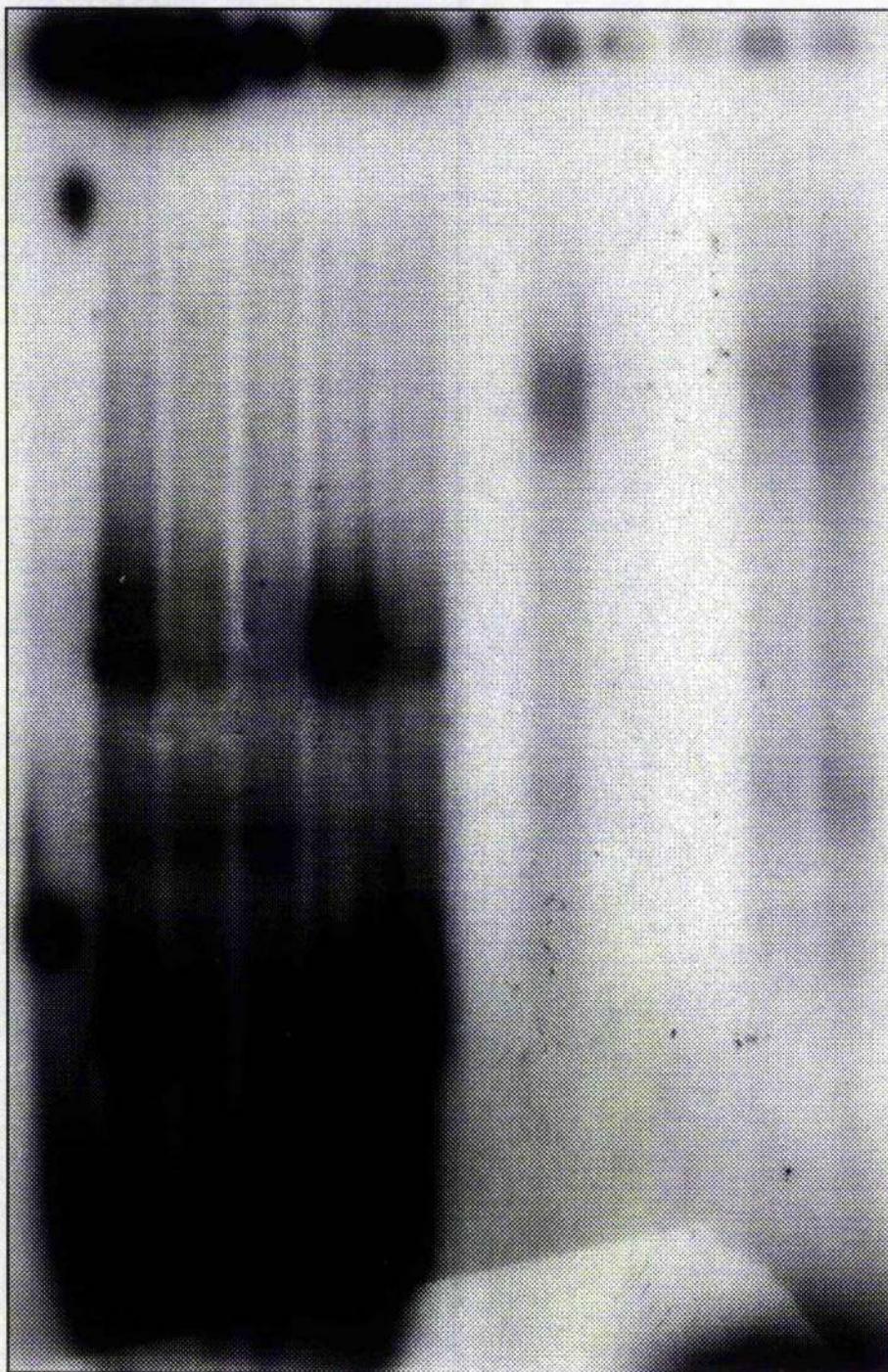
Whole cell extracts were prepared from resting tonsillar B cells (5×10^6 cells), which had been cultured overnight in fresh complete medium. The binding activity of $5\mu\text{g}$ aliquots of protein for ^{32}P -labelled YY1 and CD25-NRE oligonucleotide was assessed by EMSA, in the absence or presence of unlabelled 1- and 2-fold excess YY1 oligonucleotide and unlabelled 1- and 5-fold excess CD25-NRE oligonucleotide. Specific binding activities are indicated with horizontal arrows.

$\gamma^{32}\text{-P}$ YY1

$\gamma^{32}\text{-P}$ CD25-NRE

- + YY1 NRE - + NRE YY1

-



← NRE-BP

← YY1

← Free
Probe

Figure 4.8 EMSA Supershift Analysis of Protein Binding to YY1.

Whole cell extracts were prepared from tonsillar B cells (5×10^6 cells) which had been cultured overnight in fresh medium. The binding activity of $5 \mu\text{g}$ protein samples for ^{32}P -labelled YY1 oligonucleotide was assessed by EMSA supershift, in the presence of $1 \mu\text{g}$ rabbit anti-YY1 antibody, in the absence or presence of 1- and 2-fold excess unlabelled YY1 oligonucleotide as competitor or in the presence of 1- and 5-fold CD25-NRE oligonucleotide. Specific binding activities are indicated with horizontal arrows.

| | | | YY1 | | NRE | |
|------------|---|---|-----|---|-----|---|
| | | | ▲ | | ▲ | |
| Anti YY1 | - | - | + | + | + | + |
| Competitor | - | - | - | - | - | - |
| Probe | - | + | + | + | + | + |

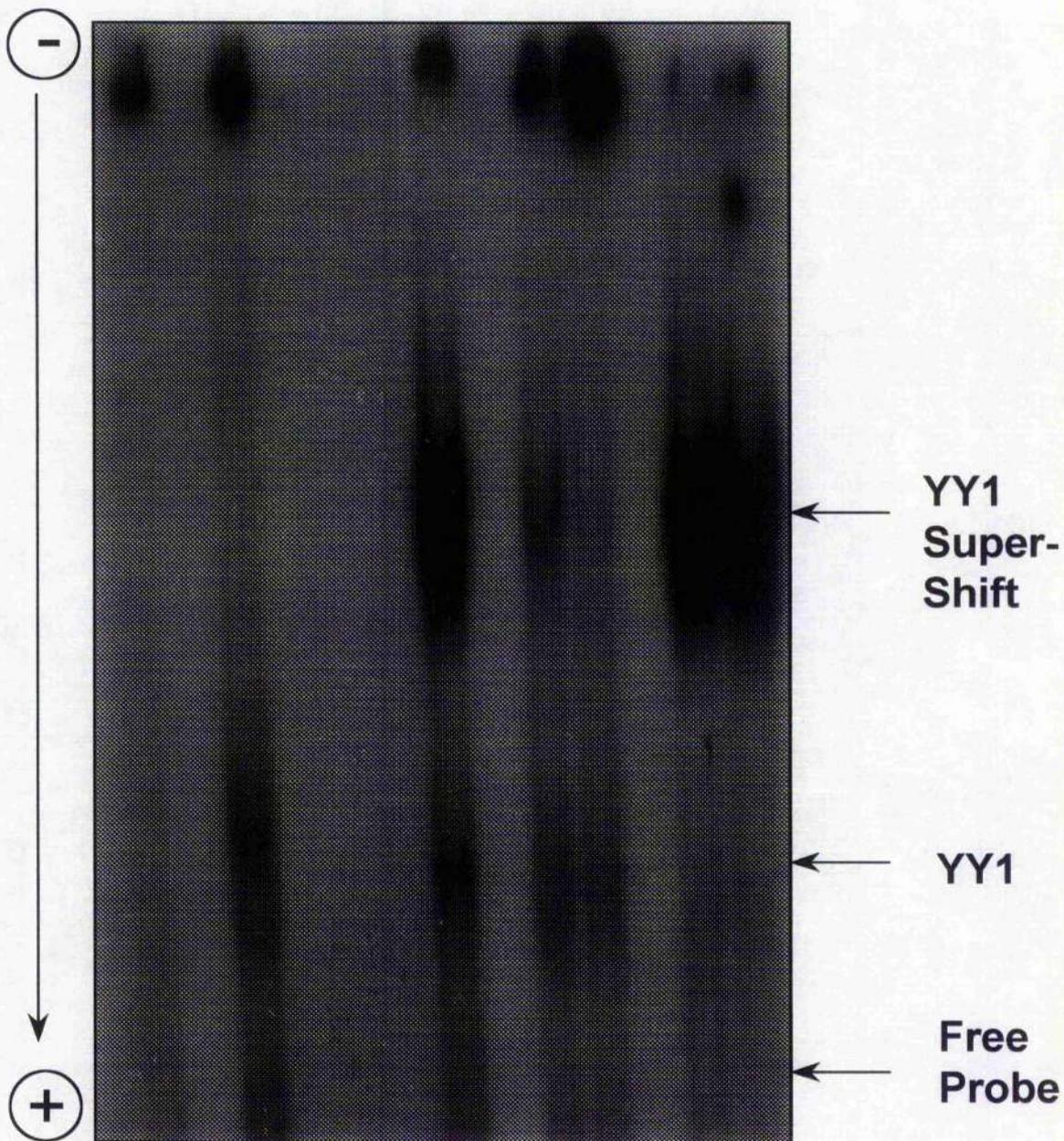


Figure 4.9 EMSA Supershift Analysis of Protein Binding to NRR_I.

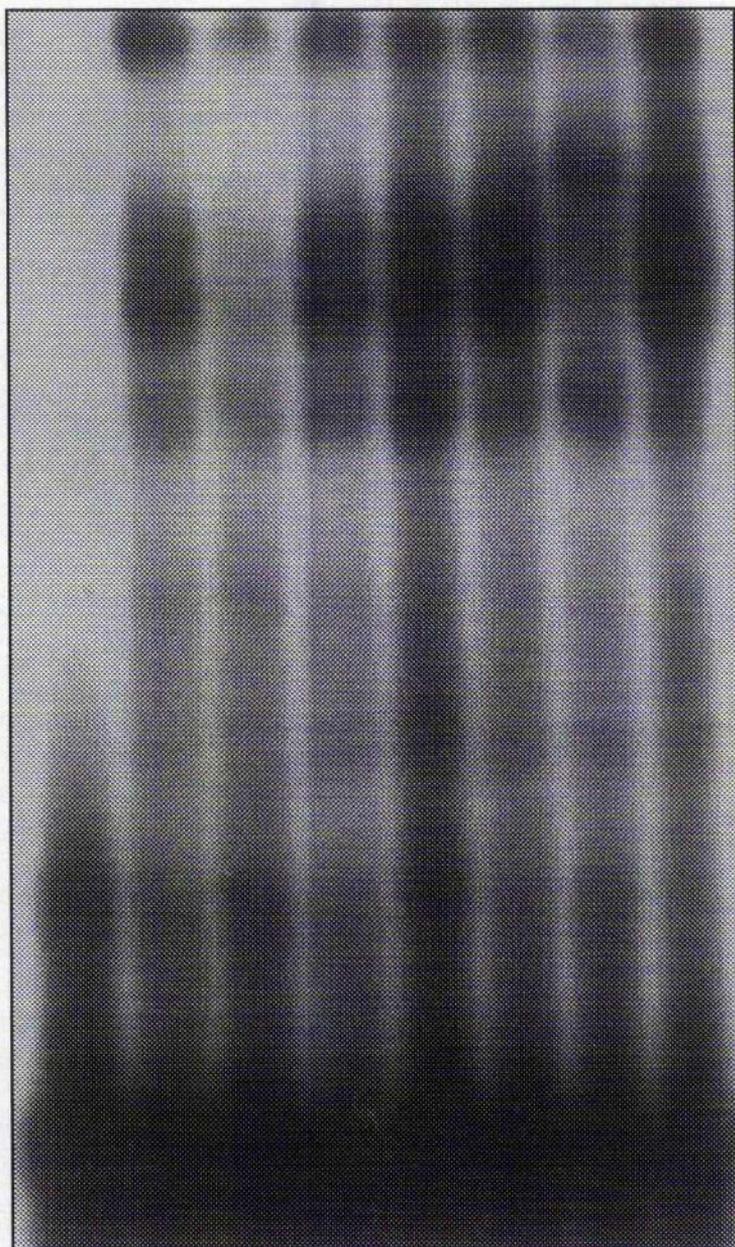
Whole cell extracts were prepared from EDR B cells (5×10^6 cells). The binding activity of $5 \mu\text{g}$ protein samples for ^{32}P -labelled CD25-NRR_I oligonucleotide was assessed by EMSA supershift, in the absence or presence of 2-fold excess unlabelled CD25-NRR_I oligonucleotide as competitor or in the presence of $1 \mu\text{g}$ of the following rabbit antibodies; anti-YY1 antibody, anti-CBP antibody, anti-Ets antibody anti-c-fos-antibody and anti-c-jun-antibody. Specific binding activities are indicated with horizontal arrows.

| | <u>YY1</u> | | <u>CBP</u> | | <u>Ets</u> | | <u>Fos</u> | | <u>Jun</u> | |
|------------|------------|---|------------|---|------------|---|------------|---|------------|---|
| Ab | - | - | - | + | + | + | + | + | + | + |
| Competitor | - | - | + | - | - | - | - | - | - | - |
| Probe | - | + | + | + | + | + | + | + | + | + |

(-)



(+)



Free Probe

CHAPTER 5
FUNCTIONAL STUDIES OF THE HUMAN CD25 PROXIMAL PROMOTER
REGION.

5.1 Introduction.

The expression of the human CD25 gene is controlled by the regulatory regions PRR_{I-III} and NRR_{I-II} (see figure 1.5). With the exception of PRR_{III}, these regions are found adjacent to one another between nucleotides -400 and -64, relative to the major transcription start site. Within the NRR_I region are a core 11bp NRE region and a retinoid-sensitive region characterised by the presence of an AP-1 binding site. When NRE binding activity is diminished, the suppression of promoter activity is lost, allowing the transcription of the CD25 gene. Work detailed in Chapter 4 identified that NRE was the dominant protein binding region of NRR_I and that the AP-1 region was bound by c-fos. Having demonstrated protein binding to NRR_I, the next step involved an investigation of the transcriptional activity of the region. Previous work in this laboratory has established the transcriptional activity of NRE in EDR B cells. Deletion of the NRE sequence lead to an elevation of CAT expression, relative to control levels. However, the increase in reporter activity was not further enhanced upon the deletion of the entire NRR_I region (Hewitt, Ozanne and Cushley, 1997).

Chapter 3 assessed the potential involvement of a number of IL-4R signalling pathways in the up-regulation of CD25. Data from these studies indicate that IL-4 up-regulates CD25 expression via a cAMP/PKA-sensitive pathway. A final pathway which may be involved in IL-4-driven CD25 expression is the JAK/STAT pathway, in particular, the pathway that leads to the induction of STAT6 activity. STAT6 is essential for the cellular responses of IL-4R signalling in lymphoid cells (Takeda et al., 1996). There are no STAT6 binding regions within the NRR_I sequence itself although distal to this region are four putative STAT6 binding regions. The first putative binding site is located partially in NRR_{II} between nucleotides -306 and -297, relative to the major transcriptional start site. The second site is found between PRR_I and PRR_{II} and is located between nucleotides -204 and -195. The final two sites are found within the PRR_{II} region and are located between positions -139 to -131 and -124 to -115,

respectively (John et al., 1996). These sites are additionally described in figure 1.5 and figure 5.1. By creating deletion constructs of these particular regions, their sensitivity to IL-4-induced reporter activity could be determined.

5.2 Transient-Transfection Studies of NRR₁ Deletion Mutants.

Previous work has investigated the transcriptional activity of the NRR₁ region in EDR B cells. In section 4.3, EMSA studies of P3HRI B cells revealed strong protein binding activity within NRR₁, but the transcriptional activity of NRR₁ has not been characterised in this cell line. P3HRI B cells were transiently-transfected with NRR₁ deletion mutant constructs containing consensus CD25, NRE or NRR₁-deleted sequences. These plasmids were named; pCD25 CAT-E', pANRE and pΔNRR₁, respectively. NRR₁ deletion mutant constructs were generated in-house, as described in section 2.1.3 and 2.2.19. P3HRI B cells were co-transfected with pCAT-E' CD25 plasmids and the control plasmid pSEAP. Cells transfected with pSEAP expressed and secreted human placental alkaline phosphatase (AP) which was measured by assay of culture supernatants (see section 2.2.11). The amount of CAT reporter activity was quantitated relative to AP production in each transfection. Transiently-transfected cells were then stimulated with and without the mitogen phorbol myristate acetate (PMA). To control for CAT reporter activity, a sample of cells were transfected with the TK-promoter plasmid pLW2.

Figure 5.2a shows the analysis of pCD25 CAT-E', pANRE- and pΔNRR₁-transiently-transfected P3HRI B cells which were measured for reporter gene activity, by thin layer chromatography (TLC). Figure 5.2b shows a graph of quantitative data obtained from phosphorimage analysis of the TLC plate and adjusted for relative alkaline phosphatase (AP) expression. Cells transfected with pCD25 CAT-E' showed a low level of CAT reporter activity that was enhanced by PMA stimulation. Transfection of cells with pANRE lead to an approximately two-fold increase in basal CAT activity and a similar

increase in PMA-treated cells, relative to the control pCD25 CAT-E' reporter activity. Cells transfected with p Δ NRR_I showed a two-fold increase in basal reporter activity and a three-fold increase in PMA-stimulated cells, relative to control.

5.3 Western Blot Analysis of STAT6 Expression in Tonsillar B cells.

The levels of STAT6 protein in quiescent tonsillar B cells stimulated with IL-4 or IL-13 was assessed. Whole cell extract was prepared at hourly intervals over four hours, then again after 24 hours of stimulation. The extracts were analysed for the presence of STAT6 by Western blotting, followed by incubation with anti-STAT6 antibody. Figure 5.3 shows a band of approximately 100kDa which is bound by anti-STAT6 antibody in each lane. Figure 5.3a shows samples prepared from untreated tonsillar B cells, this band was consistently expressed over 24 hours. In samples taken from IL-4-treated cells (Figure 5.3b), the band increased in intensity up to and including four hours of treatment, although the band returned to a basal-level intensity after 24h (data not shown). In IL-13-treated cells (Figure 5.3c) the band was consistently expressed at basal levels over 24h of incubation.

5.4 Creation of pCAT-E' CD25 STAT6 Deletion Mutant Constructs.

The role of the putative STAT6 binding regions in the regulation of CD25 promoter activity was investigated by engineering STAT6 deletion constructs for each proposed region. In common with the NRR_I deletion constructs made by other workers, a pCAT-E' plasmid was used for this purpose. Figure 5.4a shows a restriction map of pCAT-E' which contains an ampicillin resistance gene, a simian virus (SV)-40 enhancer region, a CAT reporter structural gene and a multiple cloning site. The STAT6 deletion primer sequences were amplified by PCR, as described in section 2.2.19, then purified and inserted into pCAT-E' between the *Hind* III and *Sal* I restriction sites within the multiple cloning region. The pCAT-E' CD25 STAT6 deletion mutant construct was inserted into

competent *E. coli* cells then amplified by culturing transformed cells in LB broth. The plasmids were purified by maxi-prep, as described in section 2.2.18. Plasmids containing the mutant constructs were called pPRR_I, pΔPRR_I, pΔS6-1 and pΔS6-2 with respect to the sequential order of putative STAT6 sites, as outlined in section 5.1. Figure 5.4b shows a restriction enzyme double-digest of pCD25 CAT-E', pPRR_I, pΔPRR_I, pΔS6-1 and pΔS6-2, cut by the restriction enzymes *Hind* III and *Sal* I. All digested plasmids were loaded on to a 1% agarose gel, along with 1Kbp and 100bp DNA-molecular weight markers and the fragments separated by electrophoresis. The restriction enzyme digest of pCD25 CAT-E' cut a fragment which was approximately 425bp. The restriction enzyme digests of pPRR_I, pΔPRR_I, pΔS6-1 and pΔS6-2 cut fragments of 390bp, 380bp, 360bp and 355bp, respectively. All plasmids were sequenced in-house by co-workers.

5.5 Transient-Transfection Studies of STAT6 Activity in the Human CD25 Proximal Promoter.

In order to assess a possible role for IL-4-stimulated STAT6 in CD25 expression in a range of lymphoid cells, equal amounts of pCD25 CAT-E', pPRR_I, pΔPRR_I, pΔS6-1 and pΔS6-2 were co-transfected with pSEAP into tonsillar B cells, EDR B cells and Jurkat-T cells. Initial experiments were performed to assess the effect of PMA stimulation on both PRR_{I-II} regions. A second set of experiments involved the stimulation of transiently-transfected cells with and without IL-4 to assess the sensitivity of putative STAT6 binding sites. After 48h incubation, whole cell extracts were prepared from each sample, then assayed for CAT reporter gene activity.

Figure 5.5 shows the effect of PMA on transiently transfected Jurkat T and EDR B cells. In both Jurkat T and EDR B cells, transfection of pCD25 CAT-E' showed low basal levels of reporter activity, which were enhanced when stimulated with PMA. EDR B cells which were transfected with with pPRR_I showed higher basal levels of reporter

activity than control pCD25 CAT-E' transfectants. Jurkat T cell reporter activity was comparable to control levels. PMA treatment of Jurkat T and EDR B cells increased reporter activity three-fold in both cell lines. Transfection of cells with p□PRR_I increased basal levels of reporter activity, relative to control levels, which were enhanced by PMA stimulation. Lymphoid cells transfected with pΔS6-1 showed low levels of reporter activity which were enhanced six-fold by stimulation with PMA. Cells transfected with pΔS6-2 expressed lower basal levels which were enhanced by PMA stimulation.

The TLC analysis of the reporter activity of transiently-transfected tonsillar B cells is shown in Figure 5.6a. Figure 5.6b shows the AP-normalised graph of CAT reporter activity. Cells which were transfected with pCD25 CAT-E' showed a low basal reporter activity which was increased three-fold by treatment with IL-4. Cells transfected with pPRR_I and pΔPRR_I showed an approximately three-fold increase in basal reporter activity, relative to pCD25 CAT-E' basal levels although IL-4 treatment failed to enhance reporter activity. Cells transfected with pΔS6-1 showed a two-fold increase in basal reporter activity, relative to control. Stimulation of cells with IL-4 minimally enhanced CAT expression. Cells transfected with pΔS6-2 showed basal reporter activity that was similar to pCD25 CAT-E' basal levels. IL-4 treatment minimally enhanced reporter activity.

Figure 5.7a shows shows TLC analysis of the reporter activity of transiently-transfected Jurkat T cells. Figure 5.7b shows the AP-normalised graph of reporter activity. The basal reporter activity in cells which were transfected with pCD25 CAT-E' was increased four-fold by treatment with IL-4. Cells transfected with pPRR_I showed a higher basal level of reporter activity, which was not enhanced by IL-4 stimulation. The basal reporter activity of cells transfected with pΔPRR_I was low and was not enhanced by IL-4 treatment. Jurkat T cells which were transfected with pΔS6-1 and pΔS6-2 showed low basal levels of reporter activity, similar to pΔPRR_I levels. Treatment with

IL-4 did not enhance either p Δ S6-1 or p Δ S6-2 reporter activity. This data leads to the conclusion that in lymphoid cells, the putative STAT6 binding site which overlaps NRR_{II} is sensitive to IL-4 stimulation.

5.6 Discussion.

Chapter 4 investigated protein binding to the NRR_I region and established that NRE had a dominant role with a secondary involvement of AP-1 proteins. Transcriptional activity of NRR_I was assessed to determine whether NRE had the same dominant role in the regulation of transcription as it does in protein binding activity. Figure 5.2 demonstrates that basal and PMA-stimulated reporter activity in P3HR1 B cells transfected with either p Δ NRE or p Δ NRR_I is markedly increased, relative to the basal reporter activity of pCD25 CAT'-E' transfected cells. These findings are in agreement with previous work on EDR B cells and indicate that NRE has a dominant role in NRR_I transcriptional activity.

STAT6 is an important mediator of IL-4 signalling. Investigation of the CD25 promoter sequence lead to the identification of four putative STAT6 sites (Figure 5.1), located distal to NRR_I. Figure 5.3 shows that STAT6 is constitutively expressed in tonsillar B cells. STAT6 expression was slightly increased in tonsillar B cells which had been treated with IL-4 but not with IL-13. The finding that STAT6 is expressed in B cells and is stimulated by IL-4 suggests that putative STAT6 binding sites which lie distal to NRR_I may have an important role in mediating IL-4R signals which lead to the loss of NRE-BP and the initiation of CD25 transcription.

Initial experiments investigated mitogen-sensitivity in cells transfected with deletion mutant constructs, designed to investigate each putative STAT6 binding region, within CD25. Figure 5.5 shows that PMA increased reporter activity in all transiently-transfected Jurkat T cells and EDR B cells. This was unexpected as p Δ S6-1 and p Δ S6-2 delete the influence of the mitogen-sensitive PRR_I, leaving only PRR_{II}. This region has

been shown to be involved in T cell-specific CD25 expression but there have been no reports of a similar role in B cells (John et al., 1995). Therefore, PRR_{II} is sensitive to PMA stimulation and appears to be involved in CD25 expression in B cells.

Transient-transfection studies of lymphoid cells with the STAT6 deletion mutants pPRR_I, pΔPRR_I, pΔS6-1 and pΔS6-2 assessed the capacity of each putative STAT6 site to respond to IL-4 stimulation. Figure 5.6 demonstrates that tonsillar B cells transfected with the STAT6 deletion mutant pPRR_I showed an approximately three-fold increase in reporter activity, relative to the basal levels expressed by pCD25 CAT-E'. This was expected as the transfection of cells with pPRR_I removes the influence of both negative regulatory elements on the CD25 promoter, thereby removing all suppression of CD25 transcriptional activity. Therefore, the functional deletion of NRR_{I-II} in tonsillar B cells leads to a slight enhancement of the reporter activity of the CD25 gene. Individual transfection of cells with pΔPRR_I showed a further increase in reporter activity which was unexpected, given that the PRR_I region is removed by this plasmid. Cells transfected with pΔS6-1 and pΔS6-2 showed a sequential decrease of the influence of promoter regions PRR_I and PRR_{II} on levels of reporter activity. This was expected, as the deletion of the influence of PRR_I and the partial deletion of PRR_{II} removes NF-κB, SRF and HMG-1(Y) binding regions which would be predicted to reduce the transcriptional capacity of the CD25 promoter. IL-4 stimulation of tonsillar B cells transfected with pCD25 CAT-E' increased levels of reporter activity, which was expected, given the capacity of IL-4 to up-regulate CD25 expression (as outlined in Chapters 3 and 4). Figure 5.6 additionally shows that IL-4 had no influence on the reporter activity of cells transfected with pPRR_I and pΔPRR_I, although pΔS6-1 and pΔS6-2-transfectant reporter activity was slightly enhanced. This is in agreement with other experiments which indicated that IL-4 only enhances reporter activity from cells transfected with pCD25 CAT-E' (data not shown). The finding that IL-4 consistently up-regulates reporter activity from cells transfected with pCD25 CAT-E' but not pPRR_I suggests that the first putative STAT6 binding site, which overlaps NRR_{II}, is influential in IL-4-mediated up-

regulation of CD25 expression. The failure of IL-4 to influence the reporter activity from cells transfected with pAPRR_I, pΔS6-1 and pΔS6-2 suggests that the only putative STAT6 site which may be sensitive to IL-4 in tonsillar B cells is the site that overlaps NRR_{II}. Transient-transfection of tonsillar B cells with pCD25 CAT-E' and the STAT6 deletion mutants showed low levels of reporter activity in all experiments thus making interpretation of results problematic. This was likely to be due to high cell death which occurred as a result of electroporation of highly sensitive, freshly isolated quiescent tonsillar B cells which do not possess the resilience of transformed B cell lines.

Figure 5.7 shows that Jurkat T cells transiently-transfected with pCD25 CAT-E' expressed a level of basal CAT activity that was increased when cells were stimulated with IL-4. This is in contrast to the finding that IL-4 blocks CD25 expression in T cells (Jankovich et al., 1989). Transfection with STAT6 deletion mutants showed a decrease in reporter activity which, reflected the scale of the CD25 deletion. IL-4 treatment failed to enhance the reporter activity of cells transiently-transfected with each STAT6 deletion mutant.

Therefore, in lymphoid cells, IL-4 treatment of cells transiently-transfected with pCD25 CAT-E' increases basal levels of reporter activity. The failure of IL-4 to raise basal levels of reporter activity from cells transfected with pPRR_I, pΔPRR_I, pΔS6-1 and pΔS6-2 indicates that the putative STAT6 site which overlaps NRR_{II} may be sensitive to IL-4 treatment. Further work which could be done on this study would be to transfect lymphoid cells with pΔNRR_I, in addition to the four STAT6 deletion mutants. The deletion of NRR_I would leave NRR_{II} intact and if IL-4 stimulation of transfected cells enhanced reporter activity, then a more definitive role for this STAT6 site might be demonstrated.

In summary, transient-transfection studies of NRR_I deletion mutants on P3HRI B cells confirms that NRE is the dominant region in the regulation of NRR_I transcriptional activity. Western blot studies show that STAT6 is expressed in tonsillar B cells. Studies of PMA-sensitivity in lymphoid cells transfected with STAT6 deletion mutants show for the first time, that the PRR_{II} region of CD25 is involved in the regulation of transcriptional activity in human B cells. Transient-transfection experiments of STAT6 deletion mutants in lymphoid cells show that IL-4 sensitivity may be lost to the CD25 promoter upon deletion of the NRR_{I-II} regions. Therefore, the putative STAT6 binding site which lies in the NRR_{II} region appears to be sensitive to IL-4 and may be involved in the transcription of the CD25 gene promoter. In addition to cAMP/PKA signalling, IL-4 may also upregulate CD25 via a JAK/STAT pathway which leads to the activation of STAT6.

Figure 5.1 The Proximal Region of the Human CD25 Promoter.

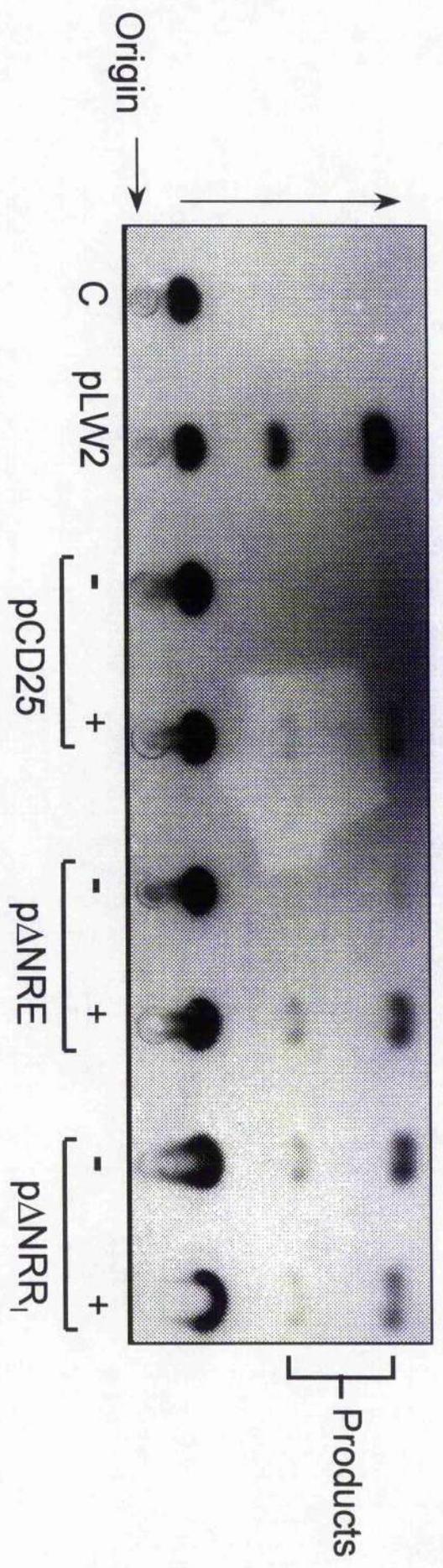
The nucleotide sequence (-482 to +176) of the human CD25 promoter region. In **bold italics** are the NRE and AP-1 regions, contained within NRR_I (-400 to -368, encompassing a putative RARE site). In **bold** is the NF- κ B site, found within the PRR_I region (-276 to -244, also containing an SRF binding region). Underlined are four putative STAT6 binding sites which conform to a consensus (T>C)TNNNNNNNAA sequence. The first sequence overlaps NRR_{II} and lies between positions -306 to -297. The second site lies between PRR_I and PRR_{II}, at nucleotides -204 and -195. The remaining sites are positioned between nucleotides -139 to -131 and -124 to -115, overlapping PRR_{II} (-137 to -64, which additionally contains Elf-1 and HMG-1(Y) binding sites). The human CD25 promoter nucleotide sequence was obtained from (John et al, 1996)

GAATTCCTCAGGATTCCTTCAGTTPCCGCCGCAITCCTTCICCATTAATTTGAATATTGGAGGCTGGCCTGACCAGAATCTTGTGAG
GACTTTGCTCC**TTCAITCCCA**GGTGTGTCGCCGGCT**GA**CTTCTGAGGACGTTACAGCCCTGAGGGAGGACTCAGCTTATGAAG
TGCTGGGTGAGACCACCTGCCAAGAAGTCTTGTCTCACTACCTTGAACGGCAGGGGAAT**CT**CCCTCTCTTTAATGGCG
TAGCTGAAGAAAGGATTCATAAATGAAAGTTCAATCC**TTCTC**ATCAAAACCCACAGCCCAACCTCCAGCAATTGAACCTTGAAA
AAAAAAAAA**CO**CTGTTTGA**AAAAATT**ACCGCAAA**ACT**ATATTTGT**CAT**CAAAAAAAAAAAAAAAAAAAAAAAAA**ACA**CTTCCCTATATTTG
AGATGAGAGAGAGAGTGTAGGCAGT**TTCC**TGGCTGAAACACCGCCAGCCCAAT**ACT**TAAAGAGAGCAACTCCCTGACTCCG
ATAGAGACTGGATGGACCCCA**CA**AGGGT**GAC**AGCCAGGGACCGATCTTCCCATCCCA**CA**TCCCTCCGGCGGATGCCAA
AAAGAGGCTGACCGCA**ACT**GGCCCTTCTG**CAG**AGAAAGACCTCCGCTT**CA**CTGCCCCGGCTGTCCCAAGGGT**CA**AGGAAG
ATGGATTCATACCTGCTG

Figure 5.2 Activity of CD25-CAT Reporter Gene Constructs in P3H1 B Cells.

Aliquots of P3H1 B cells (5×10^6 cells) were electroporated with the indicated plasmids at 0.35kV, at a capacitance of 960 μ F, then after a 1h rest period, were incubated in the absence or presence of 1nM PMA for 48h. Whole cell extracts were prepared and assayed for CAT reporter gene activity. Acetylated [14 C]-chloramphenicol products were resolved by TLC (Fig. 5.2a) and quantitated by phosphorimage analysis (Fig. 5.2b). Values represent means of relative reporter activity (\pm SEM) from five independent assays.

a)



b)

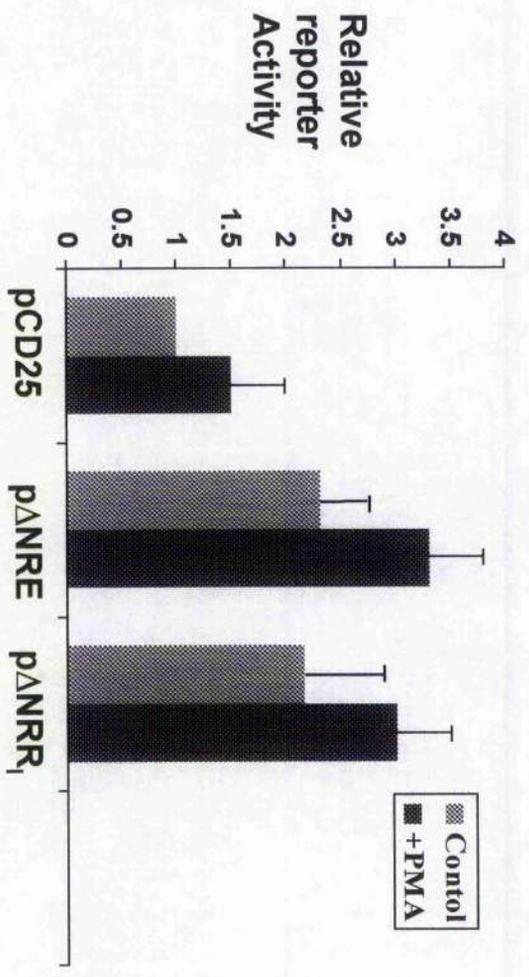
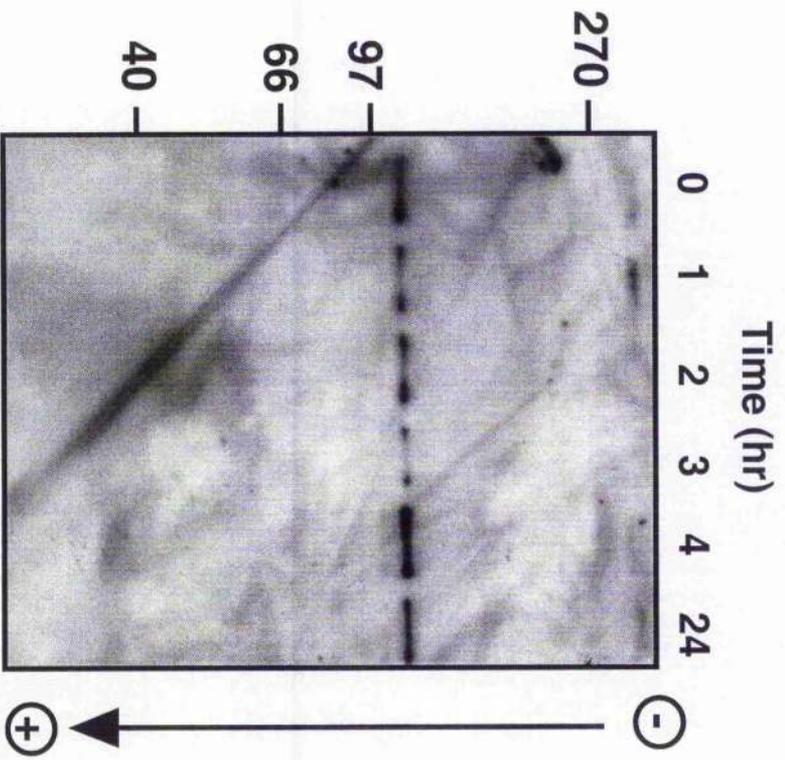


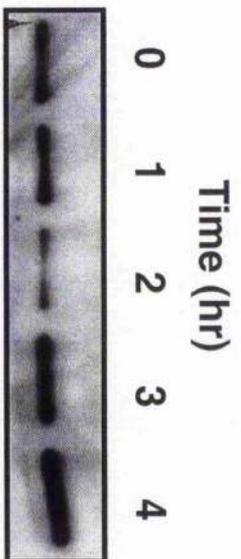
Figure 5.3 Western Bot Analysis of STAT6 in Tonsillar B Cells.

Whole cell extracts were prepared at hourly intervals from quiescent tonsillar B cells which were either resting (Fig 5.3a), treated with 500U/ml IL-4 (Fig 5.3b) or treated with 12ng/ml IL-13 (Fig 5.3c), and 30 μ g of protein extract analysed by Western blotting for binding to rabbit anti-STAT6 antibody.

a) Control



b) IL-4



c) IL-13

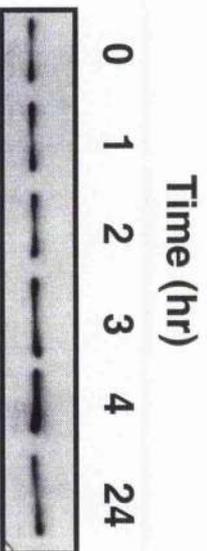
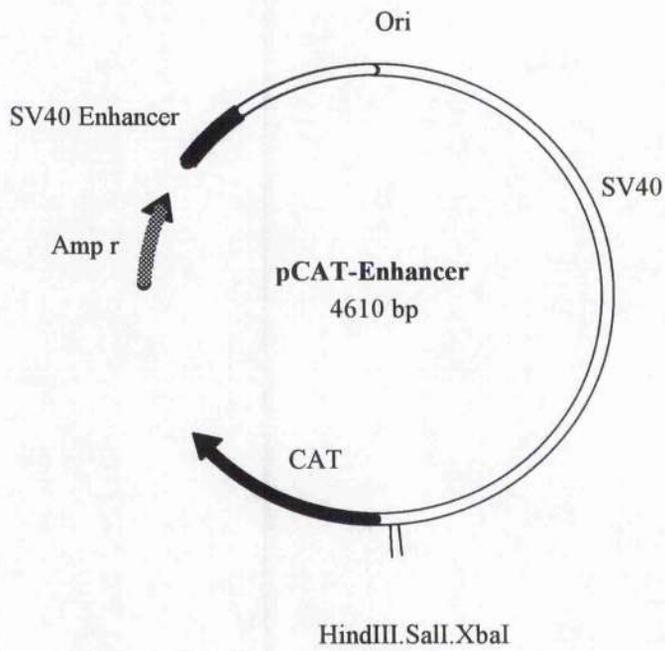


Figure 5.4 Restriction Enzyme Map of the pCAT Enhancer Plasmid and Restriction Enzyme Digest of CD25-CAT Reporter Gene Constructs.

The pCAT Enhancer (E') plasmid restriction enzyme map (Figure 5.4a). The region coding for CAT is located between positions 2315 (start site) and 2974 (stop site) and lies next to a multiple cloning site which contains regions for *Hind* III (2242) and *Sal* I (2260). The plasmid also contains an ampicillin resistance gene and SV40 enhancer region.

Aliquots of pCD25 CAT-E', pPRR_L, pΔPRR_L, pΔS6-1 and pΔS6-2 plasmids (1μg) were double-digested (Figure 5.4b) by incubation with *Hind* III and *Sal* I then samples were assessed by gel electrophoresis and UV analysis along with 1kb and 100bp molecular weight markers.

a)



b)



Figure 5.5 Activity of CD25-CAT Reporter Gene Constructs in Lymphoid Cells.

Aliquots of EDR B cells (Fig. 5.5a) or Jurkat T cells (Fig. 5.5b) (5×10^6 cells) were electroporated with the indicated plasmids at 0.35kV, at a capacitance of 960 μ F. After a 1h rest period, samples were incubated in the absence or presence of 1nM PMA (5.5a) or 10nM PMA (5.5b), respectively, for 48h. Whole cell extracts were prepared and assayed for CAT reporter gene activity. Acetylated [14 C]-chloramphenicol products were resolved by TLC and quantitated by phosphorimage analysis.

This figure is included with the kind permission of Tom Carr.

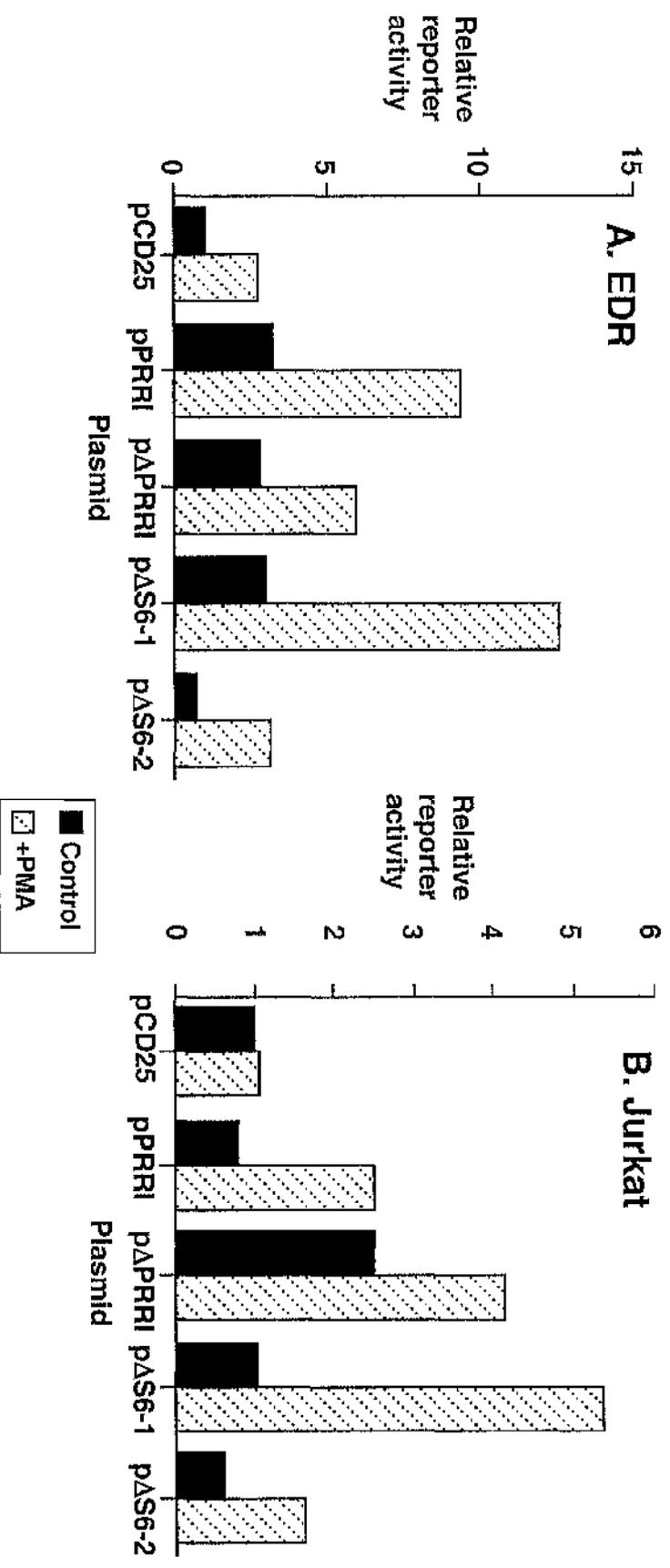
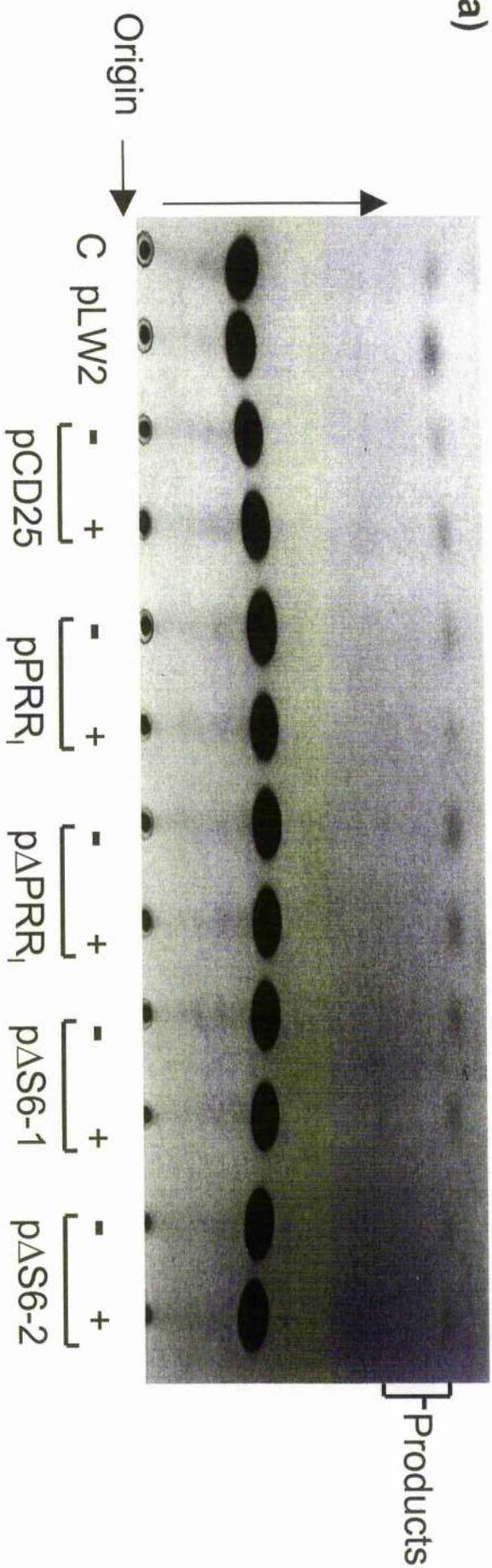


Figure 5.6 Effect of IL-4 on Activity of CD25-CAT Reporter Gene Constructs in Tonsillar B Cells.

Aliquots of quiescent tonsillar B cells (5×10^6 cells) were electroporated with the indicated plasmids at 0.35kV, at a capacitance of 960 μ F. After a 1h rest period, samples were incubated in the absence or presence of 500U/ml IL-4 for 48h. Whole cell extracts were prepared and assayed for CAT reporter gene activity. Acetylated [14 C]-chloramphenicol products were resolved by TLC (Figure 5.6a) and quantitated by phosphorimage analysis (Figure 5.6b). This figure shows one experiment, representative of five.

a)



b)

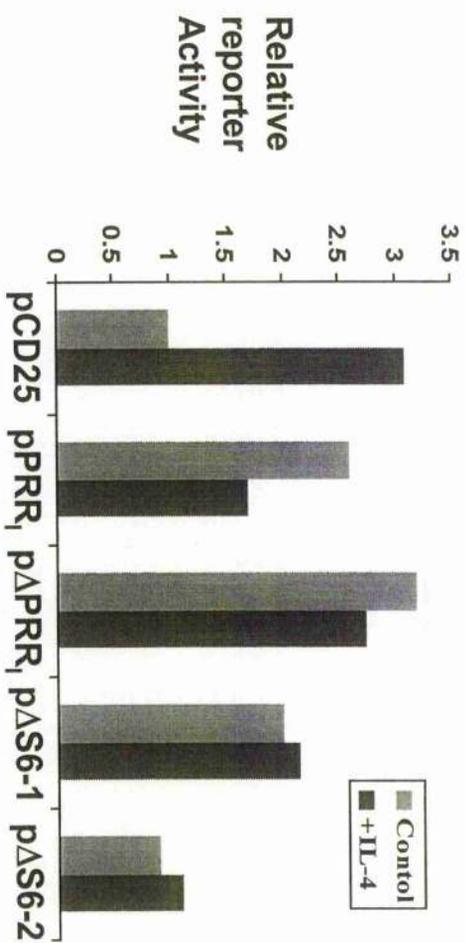
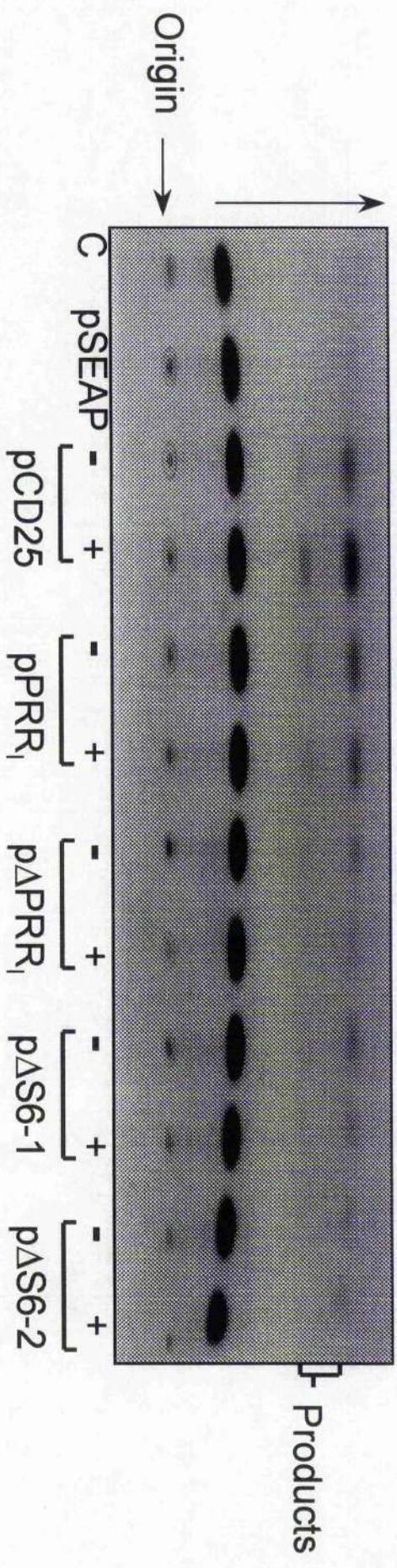


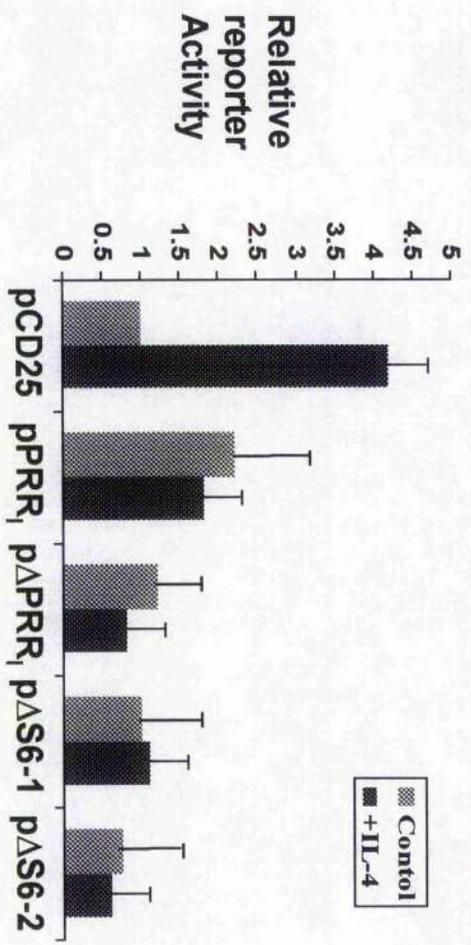
Figure 5.7 Effect of IL-4 on Activity of CD25-CAT Reporter Gene Constructs in Jurkat T Cells.

Aliquots of Jurkat T cells (5×10^6 cells) were electroporated with the indicated plasmids at 0.35kV, at a capacitance of 960 μ F. After a 1h rest period, samples were incubated in the absence or presence of 500U/ml IL-4 for 48h. Whole cell extracts were prepared and assayed for CAT reporter gene activity. Acetylated [14 C]-chloramphenicol products were resolved by TLC (Figure 5.7a) and quantitated by phosphorimage analysis (Figure 5.7b). Values represent means of relative reporter activity (\pm SEM) from ten independent assays.

a)



b)



CHAPTER 6
GENERAL DISCUSSION

The high affinity IL-2 receptor consists of constitutively expressed β (75kDa) and γ_c (64kDa) subunits, and a unique inducible 55kDa α subunit, called CD25. The IL-2R β and γ_c subunits are expressed at the cell surface and have an intermediate affinity for IL-2. Expression of CD25 leads to the formation of a heterotrimeric IL-2 receptor complex which has a high affinity for IL-2. In generating a high affinity receptor for IL-2, CD25 expression potentiates the cellular signalling events generated by the IL-2R β and γ_c subunits. IL-2 has an important role in the differentiation and apoptosis of lymphocytes, NK cells, and monocytes.

The human CD25 promoter has been well studied in human T cells, but little is known about the regulation of the gene in B cells. The expression of CD25 in T cells is stimulated by molecules such as PMA, TNF and IL-1 (Plaetinck et al., 1990; Toledano et al., 1990). Previous work in this laboratory demonstrated that while PMA stimulated a response in B cells, TNF and IL-1 failed to up-regulate CD25 expression. Molecules which were shown to upregulate CD25 expression in B cells were μ chain specific antibody, CD40 specific antibody and IL-4 (Burlinson et al., 1995). In particular, further work on IL-4 identified that CD25 expression was regulated by the removal of NRE binding activity and that the IL-4-driven loss of NRE-BP binding was achieved via a cAMP/PKA-sensitive pathway (McKay et al., 1999).

Figure 3.2 shows that IL-4 up-regulates CD25 expression in tonsillar B cells over 24 hours. IL-4 abrogates NRE-BP binding to NRE, thereby removing the suppressive influence on the CD25 promoter and enabling transcription (Figure 3.3b). B cells treated with Forskolin, which up-regulates the production of cAMP by activating adenylate cyclase, provoked a similar loss of NRE binding activity over the same period of treatment (Figure 3.3c). These findings indicate that IL-4 influences CD25 expression by abolishing NRE binding activity via a cAMP/PKA-sensitive pathway. This is in agreement with the findings of McKay (McKay, 1996). The precise mechanism and targets of cAMP/PKA signalling in this process are unclear. The ligation of the IL-4

receptor is unlikely to activate a G-protein complex as a reported intracellular rise of cAMP occurs ten to fifteen minutes after stimulation (McKay et al., 1999). If IL-4 stimulated a G-protein complex, then the rise in cAMP would be evident within seconds (Daniel et al, 1998). The fact that it is delayed suggests that IL-4 stimulates an indirect increase in cAMP levels.

A possible mechanism by which IL-4 may increase intracellular cAMP is by stimulating the expression of CD23 at the cell surface (Figure 6.1). CD23 is the low affinity FcεRII receptor for IgE which can also be released in a soluble form into the extracellular environment. IL-4 treatment induces a marked up-regulation of CD23 in B cells. Soluble (s)CD23 has an auto/paracrine effect on surrounding B cells and can induce cell signalling by binding receptors such as CD11a, CD11b, CD21 and possibly CD23 itself (Bonnefoy et al., 1997a). The interaction between CD23 and other binding receptors in B cells has a wide range of effects including; the regulation of IgE production, the anti-apoptotic role in germinal centres and mediation of T-B cell interactions (Bonnefoy et al., 1997b). Given the multifunctional roles of CD23 and CD21 it is possible that a target receptor for soluble sCD23 may be CD21, which may lead to its activation and the generation of signalling events. One such signalling pathway is a cAMP/PKA-sensitive pathway (Mossalayi et al, 1997; Paul-Eugene et al., 1992). Thus, a potential mechanism of action is that IL-4 treatment of B cells increases the expression of surface CD23 which, in turn, leads to a rise in the extracellular concentration of sCD23. The auto/paracrine effect of CD23 causes an increase in the production of cAMP, activating PKA which attenuates NRE-BP binding via the activation of downstream signalling molecules. A possible experiment which could test this hypothesis is the treatment of quiescent tonsillar B cells with sCD23. After a period of incubation, cells could be analysed for increased CD25 expression by flow cytometry. A further experiment which may clarify the involvement of cAMP/PKA signalling would be the pre-treatment of quiescent tonsillar B cells with the PKA-specific inhibitor H-89 before stimulation with sCD23. The inhibition of CD25 expression would indicate that sCD23 may up-regulate

CD25 via a cAMP/PKA-sensitive pathway. Such a finding would also provide a possible explanation for the slow kinetics of the IL-4-stimulated rise in intracellular cAMP concentrations.

Another possible explanation of the delayed elevation in cAMP levels is a decrease in PDE activity. In most cells, a ratio of basal PDE activity to AC activity regulates cAMP signalling. If AC becomes activated then the balance swings away from PDE until the G-protein stimulation has diminished or the levels of PDE have raised sufficiently to counteract the AC activity (Houslay and Milligan, 1997). Thus, a possible stimulatory action of IL-4 may be to down-regulate the intracellular activity of PDE. The balance would be in the favour of AC and would therefore increase cAMP levels within the cell, resulting in the activation of a cAMP/PKA-sensitive pathway. A possible experiment which could test this theory is the treatment of resting tonsillar B cells with a methylxanthine-family compound i.e caffeine, theobromine and theophylline. Methylxanthines are a class of pharmacological compounds which have a broad therapeutic range, being useful in the treatment of asthma, the central nervous and cardiovascular systems, and in kidney diuretics. One of the mechanisms of action of methylxanthines is the inhibition of PDE activity. Thus, if quiescent tonsillar B cells expressed CD25 in response to treatment with, for example, theophylline then this would be induced by stimulation of the cAMP/PKA-sensitive pathway via a decrease in PDE activity. (Rang et al., 1995). The delay in accumulation of cAMP which follows IL-4 stimulation may be due to the period of time required to sufficiently lower PDE activity levels.

A possible direct phosphorylation of NRE-BP by the active catalytic subunit of PKA, (PKAc) was proposed and an assay (as outlined in Figure 3.4) was set up to investigate this hypothesis. The assay demonstrated that PKAc had no direct effect on NRE-BP (Figure 3.5), but could not rule out an indirect effect. Although PKAc did not induce loss of NRE binding activity, it is difficult to rule out any indirect effect on NRE-BP

using this assay. Potential downstream effectors of PKAc which act on NRE-BP have not been identified and, owing to the dilution factor for the incubation reaction, it is unknown whether enough of these molecules would be stimulated to affect NRE binding activity. IL-4-driven CD25 expression clearly involves the stimulation of a cAMP/PKA-sensitive pathway, therefore it is probable that PKAc does have an indirect effect on the loss of NRE binding activity (Figure 6.1). The pleiotropic nature of IL-4 suggested that other IL-4-stimulated signalling pathways may have a role in the up-regulation of CD25 in B cells. The activation of IL-4R initiates a number of signalling pathways including the stimulation of the PI-3 kinase and JAK/STAT pathways, mainly through IRS signalling. Experiments, shown in Figure 3.6, demonstrated that Wortmannin and Rapamycin had no effect on the capacity of IL-4 to induce CD25 expression in tonsillar B cells. This suggests that PI-3 kinase and p70^{S6} kinase are not involved in the IL-4-driven upregulation of CD25. Further work could focus on the effect of PI-3 kinase signalling on cAMP levels within the cell. Pre-treatment of quiescent tonsillar B cells with Wortmannin and Rapamycin before IL-4 stimulation will inhibit PI-3 kinase signalling and after a period of incubation cAMP accumulation assays could be performed to determine whether cAMP signalling was affected.

The investigation of JAK/STAT signalling involved transfection experiments which studied the effect of IL-4 treatment on cells that had been transfected with one of four CD25 promoter deletion mutants (Figure 6.3), where STAT6 sites had been eliminated (Figures 5.6 and 5.7). This study indicated that IL-4-driven CD25 expression may involve the activation of the JAK/STAT pathway. The putative STAT6 site which overlaps NRR_{II} may be sensitive to IL-4 although further transfections with an NRR_I deletion mutant plasmid (p Δ NRR_I) are required to verify this hypothesis. If IL-4 sensitivity was demonstrated in cells transfected with p Δ NRR_I and lost in cells transfected with pPRR_I, then the STAT6 site located on NRR_{II} would be clearly identified as being STAT6-sensitive.

EMSA studies on the role of NF- κ B in IL-4 signalling in B cells indicated that in whole cell or cytosolic extracts, IL-4 failed to directly effect the expression of NF- κ B (Figure 3.7). This was unexpected given the proximity of an NF- κ B binding site to NRR_I (John et al., 1995). These data suggest that STAT6 activation may be the dominant factor in upregulating CD25 transcription, following the IL-4-driven loss of NRE-BP activity. Previous work in this laboratory indicated that PKC activation has no effect on the expression of CD25 (McKay and Cushley, 1996) and the fact that IL-4 does not stimulate Ras/MAP kinase signalling in B cells (Welham et al., 1994) suggests that it is unlikely any other IL-4 stimulated pathways are involved in the up-regulation of CD25 expression. Therefore, IL-4 appears to up-regulate CD25 expression via two specific signalling pathways; a cAMP/PKA-sensitive pathway stimulating effector molecules further downstream of PKAc to abolish NRE binding activity and remove the suppression of the CD25 gene, and the possible stimulation of the JAK/STAT pathway, that leads to the activation of STAT6 (Figure 6.1). Thus, IL-4 may upregulate CD25 primarily by stimulating a cAMP/PKA-sensitive pathway which leads to the loss of NRE binding activity. The loss of NRE-BP removes the suppressive effect on the CD25 promoter, allowing transcription. IL-4 may also activate JAK/STAT signalling which stimulates a STAT6 binding region overlapping NRR_{II}. This would further potentiate the transcriptional activity of CD25 and enhance gene expression.

Further studies revealed that the capacity of IL-4 to abolish NRE binding activity is also observed on protein binding to the entire NRR_I (Figures 4.1 and 4.2). The nature of IL-4 signalling was investigated to determine whether the diminution of NRE and NRR_I binding activity was a result of signalling mediated via the IL-4R α or γ c subunit. Figure 4.3 shows that stimulation of quiescent tonsillar B cells by IL-13 fails to abolish NRR_I binding activity. The IL-13 receptor complex consists of an IL-13R subunit and the IL-4R α chain. The failure of IL-13 to abolish NRR_I binding activity indicates that the loss of protein binding to NRR_I is likely to be delivered via IL-4 signalling through the γ c subunit. A second experiment which would verify this observation would be to analyse

peripheral blood B cells from an XSCID patient and compare the effect of IL-4 stimulation on CD25 expression, with normal B cells. If the XSCID B cells failed to up-regulate CD25 expression then this would provide further evidence that IL-4-driven loss of NRE binding activity is dependent on γc signalling. XSCID patients express mutated forms of γc leading to a loss of intracellular signalling capabilities and serious immunodeficiency (Uribe and Weinberg, 1998). Previous work in this laboratory showed that treatment of quiescent tonsillar B cells with IL-4 stimulated an intracellular rise in the concentration of cAMP; IL-13 did not induce cAMP accumulation (McKay, 1996). These findings further support the hypothesis that IL-4-driven CD25 expression is partially dependent on signalling through the IL-4R γc subunit. In addition, the analysis of potential signalling pathways for IL-4 in Chapter 3 showed a number of signalling mechanisms which were not involved in CD25 expression. These pathways are stimulated by IRS signalling which is stimulated by the IL-4R α subunit. The finding that IL-4 does not up-regulate CD25 expression via IL-4R α signalling is further support for the hypothesis that signalling events generated via the IL-4R γc subunit are required for full CD25 expression (Figure 6.1).

The identity of proteins binding to NRR_I remains to be fully defined. Of two potential binding sites, NRE is bound by a 50kDa silencer protein in T cells and RA may upregulate CD25 expression by attenuating binding of AP-1 proteins to their binding site, which overlaps the retinoid sensitive region. The identity of NRE-BP is unknown and work is being undertaken in this laboratory by other workers to characterise this protein. Some proteins, were proposed as candidates for NRE-BP. The 11bp TTCATCCCAGG NRE region contains a minimal 5'- CAT -3' binding region for the transcription factor YY1 and although 65kDa in mass, has been reported to have negative regulatory capabilities (Shi et al., 1991). EMSA studies of B cell extract showed that the protein species which bound radiolabelled YY1 oligonucleotide was different to the species which bound radiolabelled CD25-NRE oligonucleotide (Figure 4.7). The failure of unlabelled excess CD25-NRE oligonucleotide to compete the supershift of

proteins binding to anti-YY1 antibody and radiolabelled YY1 oligonucleotide was further evidence that YY1 is unlikely to be NRE-BP (Figure 4.8). A second candidate for NRE-BP was Ets on account of the minimal 3'- GGA -5' core binding site found on the antisense strand, within NRE. EMSA studies showed that protein binding to radiolabelled CD25-NRR₁ was not supershifted by anti-Ets antibody, therefore it is unlikely that an Ets family member is NRE-BP. The IFN-regulated protein Staf-50 has been reported to negatively regulate the transcriptional activity of the promoter region of the HIV-1 3' LTR region by binding to a sequence which is highly homologous (9/11 bases) to NRE (Tissot and Mechti, 1995). The hypothesis that Staf-50 is identical to NRE-BP is currently under evaluation.

The putative AP-1 binding site was investigated by Western blot, screening B cell extracts for the expression of AP-1 family members (Figure 4.6b) then assessing the capacity of some identified family members to supershift protein binding to radiolabelled CD25-NRR₁ oligonucleotide. The AP-1 family member c-fos (Figure 4.9), caused a shift of one protein species binding to CD25-NRR₁ whereas c-jun, which is not expressed in B cells, failed to do so. This is in agreement with Figure 4.6b as c-fos was expressed in EDR B cells although c-jun was not present. This is also consistent with the fact that c-jun is not expressed in B lymphocytes (Foletta et al., 1998). Therefore, AP-1 family members are expressed in B cells and the capacity for c-fos antibody to induce a supershift of protein binding to radiolabelled CD25-NRR₁ suggests that NRR₁ contains an active AP-1 binding site. In addition, the fact that this binding site overlaps a putative retinoid sensitive region suggests that RA may up-regulate CD25 expression in B cells via an influence upon AP-1 transcription. Further work is needed to fully characterise the AP-1 family members which bind to this region. This would be established by further supershift experiments, using antibodies against AP-1 family members which are expressed in B cells and, as a control, those which are not.

The proteins which bind to NRR_I appear to be NRE-BP and AP-1. EMSA experiments have determined that NRE is the dominant region of protein binding although the AP-1 site is also involved. EMSA experiments in tonsillar B cell and P3HR1 B cell extracts (Figure 4.4 and Table 4.1) showed that different patterns of binding to NRE and the entire NRR_I region. In addition, the partial competition of protein binding to radiolabelled CD25- NRR_I in some cell lines by CD25- NRR_I -M1 (Table 4.1 and Figure 4.5) suggests that NRE-BP and AP-1 proteins may bind NRR_I as a complex. A possible explanation of the complex is a larger protein "bridging" NRE-BP and AP-1. A candidate molecule for such a bridging effect was CBP/p300 owing to the fact that it is activated by cAMP/PKA signalling events (Kwok et al., 1994) and the AP-1 binding site within the NRR_I sequence is also homologous to a CREB binding site. EMSA supershift studies (Fig 4.9) showed that anti-CBP/p300 antibody failed to supershift protein species which bound to radiolabelled CD25- NRR_I . Therefore if such a bridging protein exists, then it is unlikely to be CBP/p300.

The precise mechanism by which NRE-BP is lost from NRE has yet to be determined, but is likely to involve the entire NRR_I region. Several hypotheses for the role of NRE-BP on the suppression of CD25 transcription can be considered (Figure 6.2). The first proposes the simple loss of NRE binding activity in response to IL-4 (or other) treatment. This involves the loss of NRE-BP from NRE. A loss of NRE-BP removes the suppression of CD25 transcription and as a result enables positive factors such as NF- κ B or SRF to initiate gene expression. The AP-1 proteins binding to NRR_I may possibly be removed or exchanged for different family members. It is unclear whether the AP-1 region has a positive or negative regulatory role in the loss of NRE-BP or on the transcription of the CD25 gene. This model is referred to as the "handbrake" model, as it envisages a straight-forward removal of NRE-BP from NRE resulting in transcription, in much the same way as a handbrake is released before driving off in a car. By extension of this analogy, positively-acting factors (i.e NF- κ B or STAT6) would function as

accelerators, and therefore this is referred to as the "handbrake" model of CD25 transcription.

The second model focuses on the AP-1 binding site and proposes that stimulation may initiate an exchange of AP-1 family members leading to transcriptional activation. This model is referred to as the "exchange" model. This activation could affect the rest of NRR_I and lead to the loss of NRE-BP binding, enabling CD25 transcription. Such a model requires the full characterisation of the family members binding to the AP-1 binding region before it can be fully tested. The third and final model considers a potential role of a bridging protein, which may link NRE-BP and AP-1 together. Thus, stimulation via IL-4 signalling may remove the bridging protein from NRE-BP and AP-1 and result in the uncoupling of NRE-BP from NRE. AP-1 may or may not be affected. Although studies show that it is unlikely that CBP/p300 (Figure 4.10) is involved in this bridging complex, the finding that unlabelled excess CD25-NRR_I-M1 can compete protein binding to radiolabelled CD25-NRR_I although protein cannot bind to radiolabelled CD25-NRR_I-M1 itself, indicates that a protein complex may link NRE-BP and AP-1 together. This model is referred to as the bridging model. The findings from EMSA studies of NRR_I (Figure 4.4 and Table 4.1) show that NRE is the dominant binding region and the fact that IL-4 treatment of B cells stimulates a loss of protein binding to both CD25-NRE and CD25-NRR_I oligonucleotides indicates that the loss of NRE-BP may result in concomitant loss of AP-1 binding. This is reflected in the loss of "total" NRR_I binding activity. These data suggest that the favoured model for the loss of the suppression of CD25 transcription is the "handbrake" model.

NRE is the dominant region of protein binding in NRR_I, and the AP-1 binding site appears to have a minor role. This relationship was also studied at a transcriptional level by the transient transfection of P3HR1 B cells with NRR_I deletion mutants that either deleted the NRE region or the NRR_I region in its entirety. Both deletion mutants showed a marked increase in both basal and PMA-stimulated levels of reporter activity,

in comparison to full-length CD25 reporter activity (Figure 5.2). These findings indicate that the deletion of NRE leads to the loss of suppression of the CD25 promoter thus enhancing levels of reporter activity. Complete deletion of the NRR_I region showed an increase in reporter activity, which was comparable to the NRE deletion. The finding, over a number of experiments, that the complete removal of NRR_I did not further increase reporter activity is consistent with the interpretation that NRE is the dominant transcriptional region within NRR_I.

Four putative STAT6 sites were identified at positions along the proximal CD25 promoter (Figures 1.5 and 5.1), each one with the potential to respond to IL-4 stimulation and up-regulate transcriptional activity. Reporter constructs were designed to assess these regions by deleting the appropriate sequence; one overlapping NRR_{II}, one between PRR_I and PRR_{II} and the final two, within PRR_{II} itself (Figure 6.3). The STAT6 deletion mutants assessed the effect of PMA on the reporter activity levels of transfected lymphoid cells (Figure 5.5). Both PRR_{I-II} regions appeared to contribute to the rise in basal and PMA stimulated reporter activity, upon the deletion of NRR_{I-II}. Cells transfected with each STAT6 deletion mutant showed that stimulation with PMA enhanced basal reporter activity levels. The further increase in reporter activity expressed by cells transfected with p Δ PRR_I suggests that PRR_{II} may be more sensitive to mitogen in the absence of PRR_I. The finding that PRR_{II} is sensitive to PMA is the first time that this region has been shown to be mitogen-responsive in B cells.

Lymphoid cells transfected with a full-length pCD25 CAT-E' plasmid showed basal reporter activity levels which were enhanced by IL-4 stimulation (Figures 5.6 and 5.7). Each cell line transfected with the STAT6 deletion mutants showed basal reporter activity levels which were unaffected by IL-4 stimulation. This indicates that only one STAT6 site is sensitive to IL-4 stimulation. Cells transfected with pCD25 CAT-E' showed increased levels of reporter activity after stimulation with IL-4 although cells transfected with pPRR_I did not. As transfection with pPRR_I deletes both NRR_{I-II}

regions, this suggests that the putative site which overlaps NRR_{II} is IL-4 sensitive and therefore may be a *bona fide* STAT6 binding site. These findings suggest that IL-4 stimulates a STAT6 binding site which lies close to NRE and enhances CD25 promoter activity following the loss of NRE-BP.

In summary, the human CD25 promoter region in B cells contains a negative regulatory region, NRR_I, comprised of an 11bp core negative regulatory element and a retinoid sensitive region bound by AP-1 proteins. NRE is regulated, in T cells, by an unidentified 50kDa NRE-binding protein. IL-4 has been shown to up-regulate the expression of CD25 in human B cells through a cAMP/PKA-sensitive pathway which attenuates the binding of NRE-BP to NRE. NRE binding activity is not lost by the direct actions of PKAc, although it is likely that PKAc has an indirect effect via the activation of unidentified downstream molecules. Studies with cell signalling inhibitors and the transient-transfection of lymphoid cells showed that IL-4 up-regulates CD25 expression via a cAMP/PKA-sensitive pathway and possibly via the activation of a JAK/STAT pathway which stimulates a STAT6 binding site overlapping NRR_{II}. IL-4 signalling is mediated via the γ c chain of the IL-4R complex. EMSA studies showed that NRE is the dominant protein binding region of NRR_I, and supershift experiments characterised the retinoid sensitive region as an AP-1 binding site for c-fos. Models for the loss of the suppressive effect of NRE-BP on CD25 transcription were proposed covering possible roles of NRE-BP, AP-1 and a complex formed by a bridging protein, in response to stimulation. Data indicate the most likely model is the so-called "handbrake" model which proposed that stimulus-induced CD25 expression is caused by the loss of NRE-BP binding, which may or may not affect AP-1 binding, that leads to the loss of a suppressive effect on CD25 transcription. The identity of NRE-BP remains unknown; supershift experiments revealed that YY1 and Ets are not identical to NRE-BP. The same experiments showed that CBP/p300 does not form a bridging complex over NRE-BP and AP-1. Transfection studies involving NRR_I deletion mutants revealed that NRE is the dominant transcriptional region within NRR_I. Studies with deletion mutants

suggested that a STAT6 site adjacent to NRR_{II} is IL-4 sensitive and showed for the first time that PRR_{II}, in common with PRR_I, is responsive to mitogenic stimulation in B cells.

The CD25 promoter responds to signalling from a range of surface receptors. It remains to be resolved which intracellular signalling pathways influence the activities of transcriptional regulatory proteins associated with individual regions of the CD25 promoter and, in particular, those signals which positively and negatively regulate the activities of the NRE-BP and AP-1 proteins interacting with NRR_I. Further work is required to identify NRE-BP and to define the role of the AP-1 binding site in regulation of CD25 transcription.

Figure 6.1 Model of IL-4R Signalling Involved in the Removal of Suppression of CD25 Transcription.

A model of the signalling events generated by IL-4 which lead to the loss of NRE binding activity and the suppression of CD25 expression. Included is the cAMP/PKA-sensitive pathway and an assessment of other IRS-linked signalling pathways as outlined in Chapter 3, the NRR₁ region and signalling generated by the IL-4R γ c subunit, as described in Chapter 4, and the putative STAT6 sites within the CD25 proximal promoter region, described in Chapter 5.

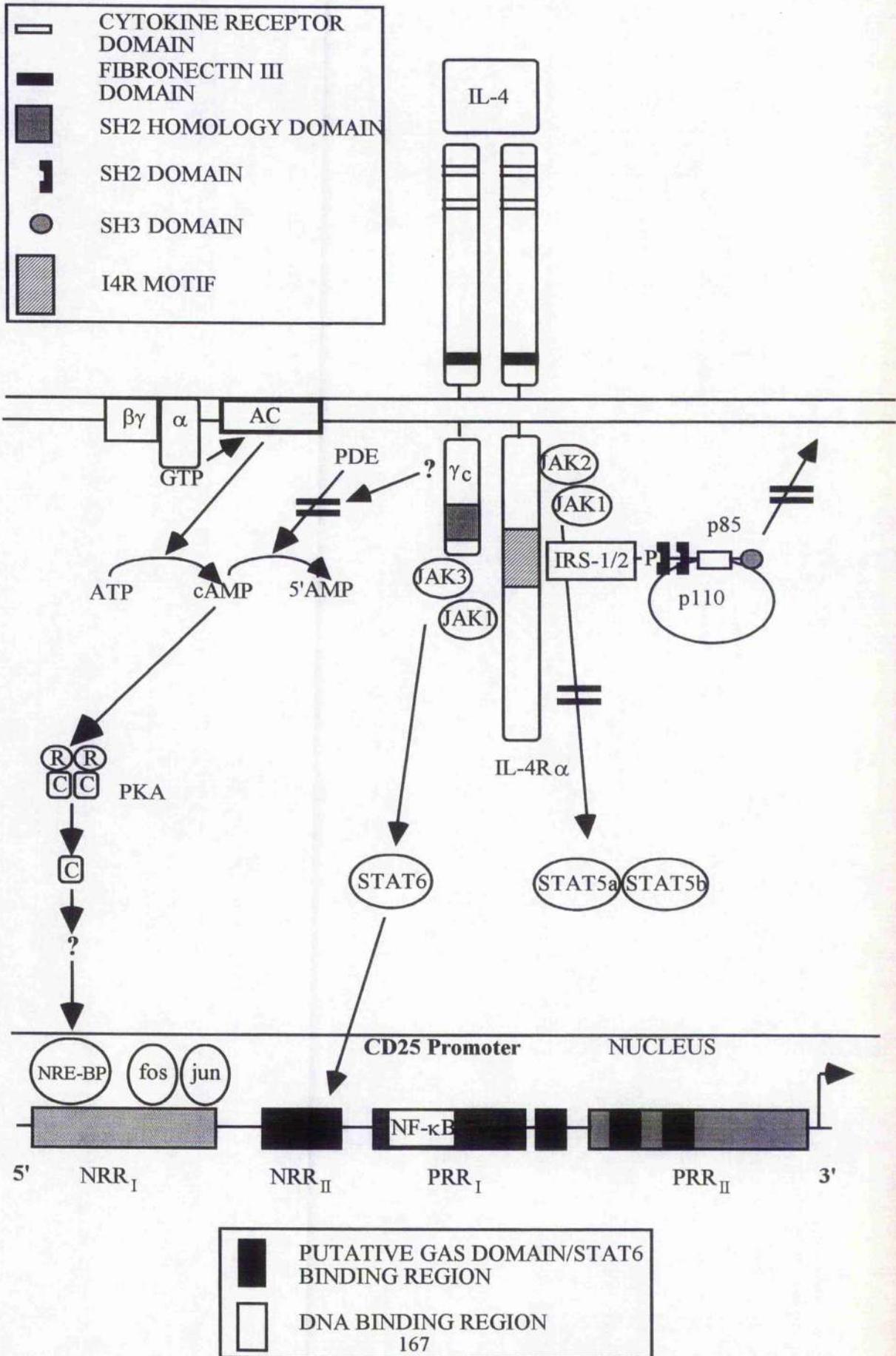


Figure 6.2 Hypothesis for the Regulation of NRE by NRR_I Binding Proteins Which Suppress CD25 Transcription.

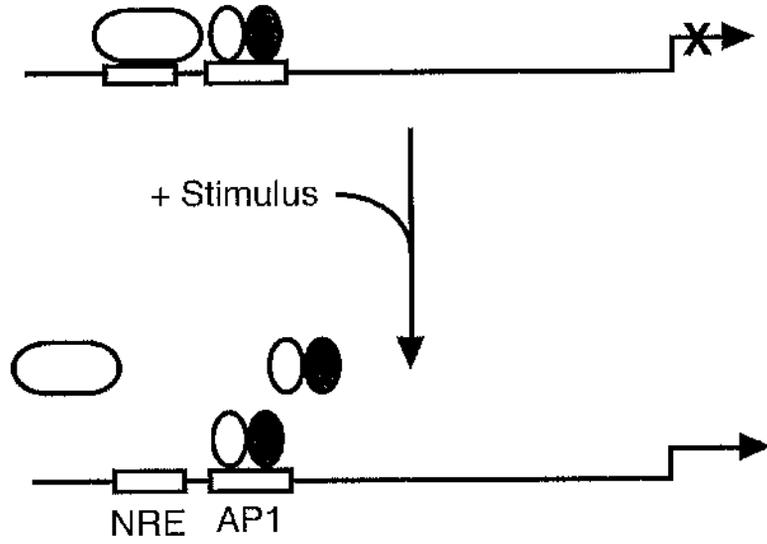
Three models are proposed for the suppressive effect of NRE-BP on CD25 transcriptional activity.

Model A (Fig 6.1A) shows that NRE-BP and AP-1 proteins bind to NRR_I in the resting state. Upon stimulation ie. by IL-4, the stimulus acts on NRE-BP and possibly AP-1 proteins to attenuate binding to NRR_I. This leads to a loss of negative regulation of the CD25 promoter gene and the initiation of transcriptional activity. This will result in the expression of CD25. The removal of NRE-BP, coupled with the possible activation of the AP-1 region is analogous to releasing the handbrake (NRE-BP) when driving a car. This model is hence called the "handbrake" model.

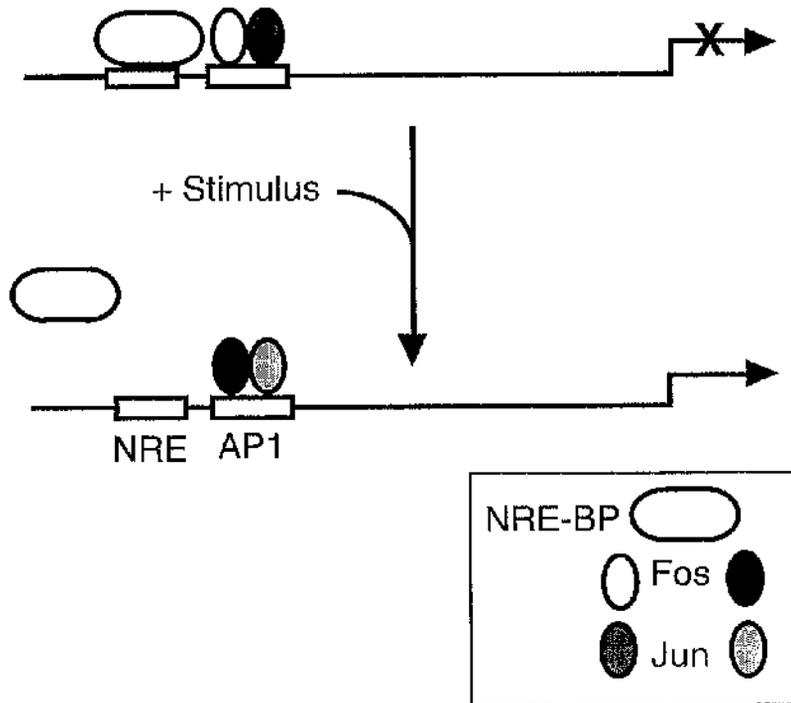
Model B (Fig 6.1B) centres on the role of AP-1 and shows that a stimulus causes an exchange of proteins binding to the AP-1 region of NRR_I. The alteration of AP-1 family members binding to NRR_I activates the region leading to the removal of NRE-BP and the initiation of CD25 transcriptional activity. This model is termed the "exchange" model.

Model C (Fig 6.1C) shows that in a resting state, NRR_I is bound by NRE-BP, AP-1 family proteins and a larger protein complex which "bridges" the proteins. The stimulus directly attenuates the binding of the large protein complex to NRE-BP and AP-1. Loss of binding also removes the binding of NRE-BP and possibly AP-1 to NRR_I. NRE-BP binding to NRE is lost leading to CD25 promoter transcription. The linking of NRE-BP and AP-1 by the large protein complex is termed the "bridging" model.

A. "Handbrake" Model



B. "Exchange" Model



C. "Bridged" Model

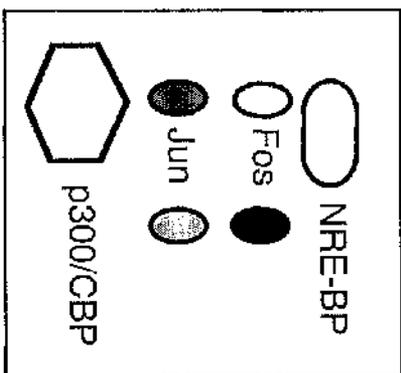
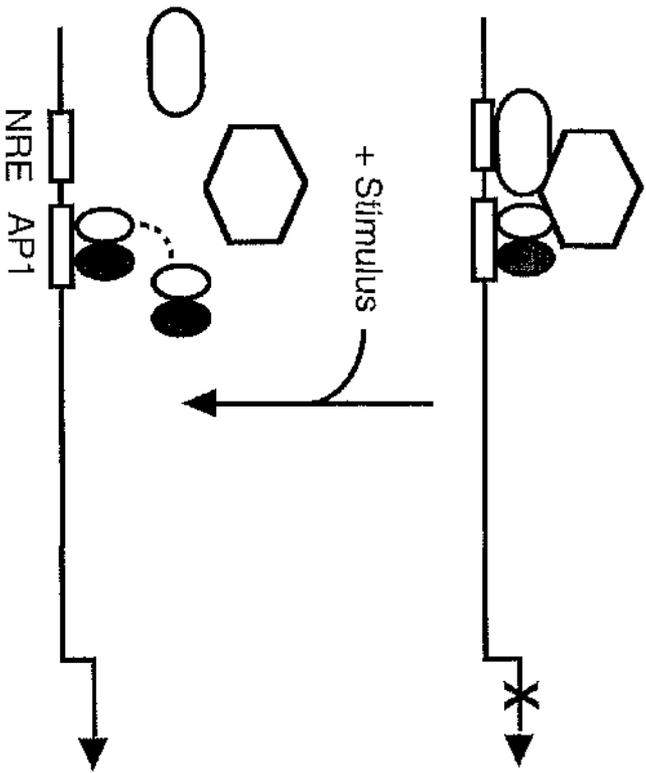
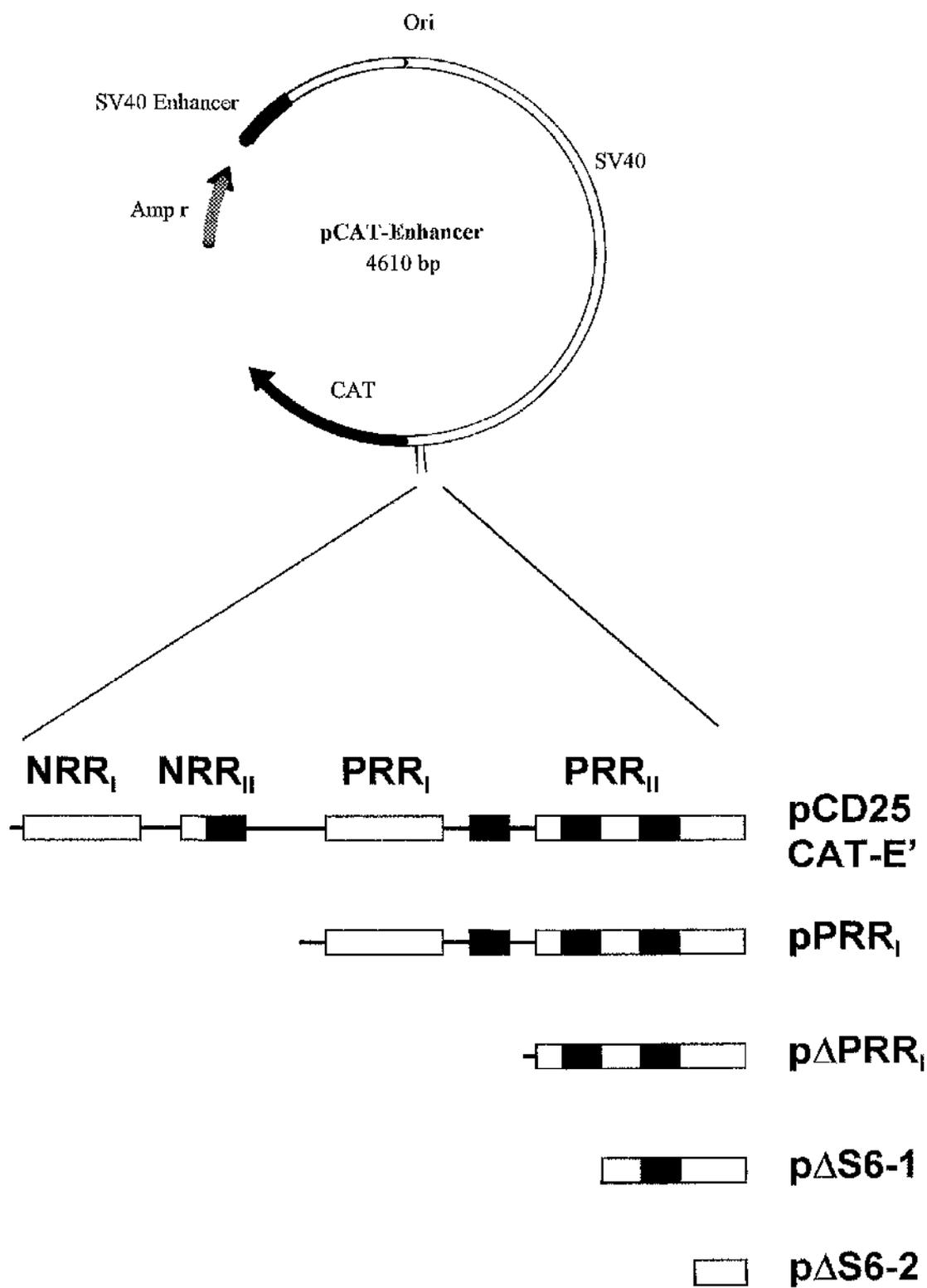


Figure 6.3 Summary of Deletion Mutant Plasmids for Putative STAT6 Binding Regions Within the CD25 Promoter.

Deletion mutant constructs for putative STAT6 sites within the CD25 promoter were created by inserting mutated sequences for CD25 within the *Hind* III and *Sal* I restriction sites of a pCAT-Enhancer plasmid. A control plasmid, pCD25 CAT-E' contained the full length proximal CD25 promoter sequence. pPRR_I contained a deletion mutation for a putative STAT6 site found within the NRR_{I-II} regions. pΔPRR_I further deleted the second STAT6 site between PRR_{I-II}. The third and fourth putative STAT6 sites within PRR_{II} were deleted by the plasmids pΔS6-1 and pΔS6-2, respectively. DNA binding regions are indicated by open boxes, putative STAT6 sites are shown as black boxes.



REFERENCES

- Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., and Cohen, P. (1997). *Curr. Biol.* 7, 261-269.
- Alt, F. W., Yancopoulos, G. D., Blackwell, T. K., Wood, C., Thomas, E., Boss, M., Coffman, R., Rosenberg, N., Tonegawa, S., and Baltimore, D. (1984). *EMBO J.* 3, 1209-1219.
- Aman, M. J., and Leonard, W. J. (1997). *Curr. Biol.* 7, R784-R788.
- Aman, M. J., Tayebi, N., Obiri, N. I., Puri, R. K., Modi, W. S., and Leonard, W. J. (1996). *J. Biol. Chem.* 271, 29265-29270.
- Angel, P., and Karin, M. (1991). *Biochim. Biophys. Acta* 1072, 129-157.
- Ashman, R. F., Peckham, D., and Stunz, L. L. (1996). *J. Immunol.* 157, 5-11.
- Baurle, P. A., and Henkel, T. (1994). *Annu. Rev. Immunol.* 12, 141-179.
- Baird, A. M., Gerstein, R. M., and Berg, L. J. (1999). *Curr. Opin. Immunol.* 11, 157-166.
- Baldwin, A. S. (1996). *Annu. Rev. Immunol.* 14, 649-681.
- Bemark, M., Martensson, A., Liberg, D., and Leanderson, T. (1999). *J. Biol. Chem.* 272, 10259-10267.
- Benschop, R. J., and Cambier, J. C. (1999). *Curr. Opin. Immunol.* 11, 143-151.
- Bhatti, L., and Sidell, N. (1994). *Immunology* 81, 273-279.
- Bijsterbosch, M. K., Meade, C. J., Turner, G. A., and Klaus, G. G. B. (1985). *Cell* 41, 999-1006.

- Blery, M., Kubagawa, H., Chen, C. C., Vely, F., Cooper, M. D., and Vivier, E. (1998). *Proc. Natl. Acad. Sci. USA* 95, 2446-2451.
- Bonnefoy, J., Lecoanet-Henchoz, S., Gauchat, J., Graber, P., Aubry, J., Jeannin, P., and Plater-Zyberk, C. (1997a). *Intern. Rev. Immunol.* 16, 113-128.
- Bonnefoy, J.-F., Aubry, J.-P., Gauchat, J. F., Graber, P., and Lecoanet-Henchoz, S. (1997b). (M. M. Harnett, K. P. Rigley, ed.), p. 65-71. John Wiley & Sons Ltd.
- Boriak-Sjodin, P., Margarit, S. M., Bar-Sagi, D., and Kuriyan, J. (1998). *Nature* 394, 337-343.
- Brocker, T., Gulbranson-Judge, A., Flynn, S., Riedinger, M., Raykundafia, C., and Lane, P. (1999). *Eur. J. Immunol.* 29, 1610-1616.
- Bucher, P., Corthesy, P., Imbert, J., and Nabholz, M. (1997). *Immunobiol.* 198, 136-143.
- Buhl, A. M., and Cambier, J. C. (1999). *J. Immunol.* 162, 4438-4446.
- Burlinson, E. L., Pringle, H. M., Ozanne, B. W., and Cushley, W. (1995). *Immunol. Lett.* 45, 93-98.
- Burnet, M. F. (1959). "The clonal selection theory of aquired immunity", Cambridge University Press. London.
- Busslinger, M., and Urbanek, P. (1995). *Curr. Opin. Genet. Dev.* 5, 595-601.
- Butcher, E. C., and Picker, L. J. (1996). *Science* 272, 60-66.
- Butcher, R. D. J., McGarvie, G. M., and Cushley, W. (1990). *Immunology* 69, 57-64.
- Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., and Der, C. J. (1998). *Oncogene* 17, 1395-1413.

- Caput, D., Laurent, P., Kaghad, M., Lelias, J. M., Lefort, S., Vita, N., and Ferrara, P. (1996). *J. Biol. Chem.* 271, 16921-16926.
- Chan, M. A., McGrath, T. H., and Gelfand, E. W. (1993). *Cell. Immunol.* 146, 300-312.
- Chang, Y., Bosma, M. J., and Bosma, G. C. (1999). *J. Exp. Med.* 189, 1295-1305.
- Chen, E. H., Gadina, M., Galon, J., Chen, M., and O'Shea, J. J. (1998). *Immunol. Today* 19, 338-341.
- Chiu, R., Angel, P., and Karin, M. (1989). *Cell* 59, 979-986.
- Chung, C. D., Liao, J., Liu, B., Xiaoping, R., Jay, P., Berta, P., and Shuai, K. (1997). *Science* 278, 1803-1805.
- Cohen, P. (1997). *Trends Cell Biol.* 7, 353-361.
- Cohneney, S. J., Sanden, D., Cacalano, N. A., Yoshimura, A., Mui, A., Migone, T. S., and Johnston, J. A. (1999). *Mol. Cell. Biol.* 19, 4980-4988.
- Colledge, M., and Scott, J. D. (1999). *Trends Cell Biol.* 9, 216-221.
- Daniel, P. B., Walker, W. H., and Habener, J. F. (1998). *Annu. Rev. Nutr.* 18, 353-383.
- Darnell Jr., J. E. (1997). *Science* 277, 1630-1635.
- Dawson, C. II., Brown, B. L., and Dobson, P. R. M. (1997). *Biochem. Biophys. Res. Comms.* 233, 279-282.
- Dent, A. L., Schaffner, A. L., Yu, X., Allman, D., and Staudt, L. M. (1997). *Science* 276, 589-592.
- Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., Yonezawa, K., Kasuga, M., Courtneidge, S. A., and Waterfield, M. D. (1994). *EMBO J.* 13, 522-533.

- Dickensheets, H. L., Venkataraman, C., Schindler, U., and Donnelly, R. P. (1999). *Proc. Natl. Acad. Sci. USA* 96, 10800-10805.
- DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandhi, E., and Karin, M. (1997). *Nature* 388, 548-554.
- Domin, J., and Waterfield, M. D. (1997). *FEBS Lett.* 410, 91-95.
- Eckholm, D., Belfrage, P., Manganiello, V., and Degerman, E. (1997). *Biochim. Biophys. Acta* 1356, 64-70.
- Eicher, D. M., and Waldmann, T. A. (1998). *J. Immunol.* 161, 5430-5437.
- El-Hillal, O., Kurosaki, T., Yamamura, H., Kinet, J., and Scharenberg, A. M. (1997). *Proc. Natl. Acad. Sci. USA* 94, 1919-1924.
- Evans, G. A., Goldsmith, M. A., Johnstone, J. A., Xu, W. D., Weiler, S. R., Erwin, R., Howard, O. M. Z., Abraham, R. T., O'Shea, J. J., Greene, W. C., and Farrar, W. L. (1995). *J. Biol. Chem.* 270, 28858-28863.
- Finney, M., Guy, G. R., Michell, R. H., Gordon, J., Dugas, B., Rigley, K. P., and Callard, R. E. (1990). *Eur. J. Immunol.* 20, 151-156.
- Foletta, V. C., Segal, D. H., and Cohen, D. R. (1998). *J. Leuk. Biol.* 63, 139-152.
- Forni, L., Coutinho, A., Kohler, G., and Jerne, N.K. (1979). *Proc. Natl. Acad. Sci. USA* 77, 1125-1128
- Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997). *Cell* 88, 435-437.
- Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997). *Science* 275, 665-668.
- Freimuth, W. W., Depper, J. M., and Nabel, G. J. (1989). *J. Immunol.* 143, 3064-3068.

- Friedrich, K., Kammer, W., Erhardt, I., Brandlein, S., Sebald, W., and Moriggl, R. (1999). *Int. Immunol.* 11, 1283-1293.
- Fu, C., Turck, C., Kurosaki, T., and Chan, A. C. (1998). *Immunity* 9, 93-103.
- Fujimoto, M., Poe, J. C., Jansen, P. J., Sato, S., and Tedder, T. F. (1999). *J. Immunol.* 162, 7088-7094.
- Fukuda, T., Yoshida, T., Okada, S., Hatano, M., Miki, T., Ishibashi, K., Okabe, S., Koseki, H., Hirose, S., Taniguchi, M., Miyasaka, N., and Tokuhisa, T. (1997). *J. Exp. Med.* 186, 439-448.
- Garret-Sinha, L. A., Su, G. H., Rao, S., Kabak, S., Hao, Z., Clark, M. R., and Simon, M. C. (1999). *Immunity* 10, 399-408.
- Gascan, H., Gauchat, J. F., Roncarolo, M. G., Yssel, H., Spits, H., and de Vries, J. E. (1991). *J. Exp. Med.* 173, 747-750.
- Georgopoulos, K., Winandy, S., and Avitahl, N. (1997). *Annu. Rev. Immunol.* 15, 155-176.
- Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997). *Proc. Natl. Acad. Sci. USA* 94, 2927-2932.
- Ghia, P., Boekel, E., Rolink, A. G., and Melchers, F. (1998). *Immunol. Today* 19, 480-485.
- Godfrey, D. L., and Zlotnick, A. (1993). *Immunol. Today* 14, 547-553.
- Gonzalez, G. A., and Montminy, M. R. (1989). *Cell* 59, 675-680.
- Grant, P. A., Thompson, C. B., and Pettersson, S. (1995). *EMBO J.* 14, 4501-4513.
- Graves, B. J., and Petersen, J. M. (1998). *Adv. Cancer Res.* 75, 1-55.

- Hagman, J., Belanger, C., Travis, A., Turck, C. W., and Grosschedl, R. (1993). *Genes Dev.* 7, 760-773.
- Halazonetis, T., Georgopoulos, K., Greenberg, M. E., and Leder, P. (1988). *Cell* 55, 917-924.
- Hamm, H. E., and Gilchrist, A. (1996). *Curr. Opin. Cell. Biol.* 8, 189-196.
- Han, S., Zheng, B., Schatz, D. G., Spanopoulo, E., and Kelsoe, G. (1996). *Science* 274, 2094-2097.
- Haque, S. J., Wu, Q., Kammer, W., Friedrich, K., Smith, J. M., Kerr, I. M., Stark, G. R., and Williams, B. R. G. (1997). *Proc. Natl. Acad. Sci. USA* 94, 8563-8568.
- Hardy, R. R., Carmack, C. E., Shinton, S. A., Kemp, J. D., and Hayakawa (1991). *J. Exp. Med.* 173, 1213-1225.
- Hashimoto, A., Okada, H., Jiang, A., Kurosaki, M., Greenberg, S., Clark, E. A., and Kurosaki, T. (1998). *J. Exp. Med.* 188, 1287-1295.
- Henderson, A., and Calame, K. (1998). *Annu. Rev. Immunol.* 16, 163-200.
- Herblot, S., Chastagner, P., Samady, L., Moreau, J. L., Demaison, C., Froussard, P., Liu, X., Bonnet, J., and Theze, J. (1999). *J. Immunol.* 162, 3280-3288.
- Hess, J., Laumen, H., Muller, K. B., and Wirth, T. (1998). *J. Cell. Physiol.* 177, 525-534.
- Hewitt, E. I., Ozanne, B. W., and Cushley, W. (1997). *Cytokine* 9, 982-991.
- Houslay, M. D., and Milligan, G. (1997). *TIBS* 22, 217-224.
- Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T., and Paul, W. E. (1982). *J. Exp. Med.* 155, 914-923.

- Hozumi, N., and Tonegawa, S. (1976). *Proc. Natl. Acad. Sci. USA.* 73, 3628-3632.
- Huo, L., and Rothstein, T. L. (1995). *J. Immunol.* 154, 3300-3309.
- Huo, L., and Rothstein, T. L. (1996). *J. Immunol.* 157, 3812-3818.
- Ihle, J. N. (1996). *Cell* 84, 331-334.
- Imani, F., Rager, K. J., Catipovic, B., and Marsh, D. G. (1997). *J. Biol. Chem.* 272, 7927-7931.
- Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A. C., and Kurosaki, T. (1999). *Immunity* 10, 117-125.
- Jain, J., McCafferty, P. G., Valge, A. V., and Rao, A. (1992). *Nature* 356, 801-804.
- Jankovich, D. L., Gibert, M., Baran, D., Ohara, J., Paul, W. E., and Theze, J. (1989). *J. Immunol.* 142, 3113-31320.
- Jelinek, D. F., and Lipsky, P. E. (1988). *J. Immunol.* 141, 164-173.
- John, S., Reeves, R. B., Lin, J.-X., Child, R., Leiden, J. M., Thompson, C. B., and Leonard, W. J. (1995). *Mol. Cell. Biol.* 15, 1786-1796.
- John, S., Robbins, C. M., and Leonard, W. J. (1996). *EMBO J.* 15, 5627-5635.
- Johnston, J. A., Wang, L. M., Hanson, E. P., Sun, X. J., White, M. F., Oakes, S. A., Pierce, J. H., and O'Shea, J. J. (1995). *J. Biol. Chem.* 270, 28527-28530.
- Kadesch, T. (1992). *Immunol. Today* 13, 31-36.
- Kammer, W., Lischke, A., Morrigl, R., Groner, B., Ziemiecki, A., Gurniak, C. B., Berg, L. J., and Friedrich, K. (1996). *J. Biol. Chem.* 271, 23634-23637.
- Kaplan, M. H., Schindler, U., Smiley, S. T., and Grusby, M. J. (1996). *Immunity* 4, 313-319.

- Karasuyama, H., Rolink, A., and Melchers, F. (1993). *J. Exp. Med.* 178, 469-478.
- Karin, M., Liu, Z. G., and Zandi, E. (1997). *Curr. Opin. Cell Biol.* 9, 240-246.
- Karras, J. G., Wang, Z., Coniglio, S. J., Frank, D. A., and Rothstein, T. L. (1996). *J. Immunol.* 157, 39-47.
- Kawakami, K., and Parker, D.C. (1993). *Eur. J. Immunol.* 23, 77-84
- Kee, B. L., and Paige, C. J. (1995). *Int. Rev. Cytol.* 157, 129-179.
- Keegan, A. D., Nelms, K., Wang, L., Pierce, J. H., and Paul, W. E. (1994a). *Immunol. Today* 15, 423-431.
- Keegan, A. D., Nelms, K., White, M., Wang, L. M., Pierce, J. H., and Paul, W. E. (1994b). *Cell* 76, 811-820.
- Keegan, A. D., and Paul, W. E. (1992). *Immunol. Today* 13, 63-68.
- Kennelly, P. J., and Krebs, E. G. (1991). *J. Biol. Chem.* 266, 15555-15558.
- Kim, C. H., and Broxmeyer, H. E. (1999). *J. Leukoc. Biol.* 65, 6-15.
- King, I. B., Norvel, A., and Monroe, J. G. (1999). *J. Immunol.* 162, 2655-2662.
- Kofanus, W., Romeo, C., and Seed, B. (1993). *Cell* 74, 171-183.
- Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, D., Marme, D., and Rapp, U. R. (1993). *Nature* 364, 249-252.
- Kuhn, R., Rajewsky, K., and Muller, W. (1991). *Science* 254, 707-710.
- Kurosaki, T. (1999). *Annu. Rev. Immunol.* 17, 555-592.
- Kurosaki, T., and Kurosaki, M. (1997). *J. Biol. Chem.* 272, 15595-15598.

- Kwok, R. P., Lundblad, J. R., Chrivia, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G. E., Green, M. R., and Goodman, R. H. (1994). *Nature* 370, 223-226.
- Landshulz, W. H., Johnson, P. F., and McKnight, S. L. (1988). *Science* 240, 1759-1764.
- Landsman, D., and Bustin, M. (1993). *BioEssays* 15, 539-546.
- Lane, P. (1996). *Curr. Opin. Immunol.* 8, 331-336.
- LeBien, T. W. (1998). *Curr. Opin. Immunol.* 10, 188-195.
- Lecine, P., Algarte, M., Rameil, P., Beadling, C., Bucher, P., Nabholz, M., and Imbert, J. (1996). *Mol. Cell. Biol.* 16, 6829-6840.
- Lec, K. A. W., and Masson, N. (1993). *Biochim. Biophys. Acta* 1174, 221-223.
- Leonard, W. J. (1996). *Annu. Rev. Med.* 47, 229-239.
- Letzelter, F., Wang, Y., and Sebald, W. (1998). *Eur. J. Biochem.* 257, 11-20.
- Li, H., Davis, W., and Pure, E. (1999). *J. Biol. Chem.* 274, 9812-9820.
- Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993). *Nature* 363, 85-88.
- Lin, H., and Grosschedl, R. (1995). *Nature* 376, 263-267.
- Lin, J., Migone, T., Tsang, M., Friedmann, M., Weatherbee, J. A., Zou, L., Yamauchi, A., Bloom, E. T., Mietz, J., John, S., and Leonard, W. J. (1995). *Immunity* 2, 331-339.
- Lishke, A., Morigg, R., Brandlein, S., Berchtold, S., Kammer, W., Sebald, W., Groner, B., Liu, X., Hennighausen, L., and Freidrich, K. (1998). *J. Biol. Chem.* 273, 31222-31229.

- Liu, B., Liao, J., Rao, X., Kushner, S. A., Chung, C. D., Chang, D. D., and Shuai, K. (1998). *Proc. Natl. Acad. Sci. USA* 95, 10626-10631.
- Liu, W. S., and Heckman, C. A. (1998). *Cell. Signal.* 10, 529-542.
- Lomo, J., Smeland, E. B., Ulven, S., Natarajan, V., Blomhoff, R., Gandhi, U., Dawson, M. I., and Blomhoff, H. K. (1998). *J. Cell. Physiol.* 175, 68-77.
- Maeda, A., Scharenberg, A. M., Tsukada, S., Bolen, J. B., Kinet, J., and Kurosaki, T. (1999). *Oncogene* 18, 2291-2297.
- Malek, T. R., and Ashwell, J. D. (1985). *J. Exp. Med.* 161, 1575.
- Maniatis, T. (1997). *Science* 278, 818-819.
- Marais, R., Light, Y., Mason, C., Paterson, H., Olson, M. F., and Marshall, C. J. (1998). *Science* 280, 109-112.
- Martensson, A., Argon, Y., Melchers, F., Dul, J. L., and Martensson, I. (1999). *Int. Immunol.* 11, 453-460.
- May, M. J., and Ghosh, S. (1998). *Immunol. Today* 19, 80-88.
- McHeyzer-Williams, M. G., and Ahmed, R. (1999). *Curr. Opin. Immunol.* 11, 172-179.
- McKay, C. E. (1996). Ph.D. Thesis. "Interleukin-4-Mediated Regulation of CD25 Gene Expression in Human B Lymphocytes". University of Glasgow.
- McKay, C. E., Hewitt, E. L., Ozanne, B. W., and Cushley, W. (1999). *In Press*.
- McKay, C. E., and Cushley, W. (1996). *Cytokine* 8, 305-312.
- Meisner, H., Conway, B. R., Hartley, D., and Czech, M. P. (1995). *Mol. Cell. Biol.* 15, 3571-3578.

- Melchers, F., Rolink, A., Grawunder, U., Winkler, T. H., Karasuyama, H., Gbia, P., and Andersson, J. (1995). *Curr. Opin. Immunol.* 7, 214-227.
- Meyer, T. E., and Habener, J. F. (1993). *Endoc. Rev.* 14, 269-290.
- Meyer, W. K. H., Reichenbach, P., Schindler, U., Soldain, E., and Nabholz, M. (1997). *J. Biol Chem.* 272, 31821-31828.
- Minami, Y., Kono, T., Miyazaki, T., and Taniguchi, T. (1993). *Ann. Rev. Immunol.* 11, 245-67.
- Minegishi, Y., Hendershot, L. M., and Conley, M. E. (1999). *Proc. Natl. Acad. Sci. USA* 96, 3041-3046.
- Miyajima, A., Kitamura, T., Harada, N., Yokota, T., and Arai, K. (1992). *Ann. Rev. Immunol.* 10, 295-331.
- Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z. J., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Ihle, J. N., and Taniguchi, T. (1994). *Science* 266, 1045-1047.
- Montminy, M. R., Sevarino, K. A., Wagner, K. A., Mandel, G., and Goodman, R. H. (1986). *Proc. Natl. Acad. Sci. USA* 83, 6682-6686.
- Montoya, M. C., Holtmann, K., Snapp, K. R., Borges, E., Sanchez-Madrid, F., Luscinskas, F. W., Kansas, G., Vestweber, D., and de Landazuri, M. O. (1999). *J. Clin. Invest.* 103, 1317-1327.
- Morgan, B., Sun, L., Avithal, N., Andrikopoulos, K., Ikeda, T., Gonzalez, E., Wu, P., Neben, S., and Georgopoulos, K. (1997). *EMBO J.* 16, 2004-20013.
- Morgan, D. A., Ruscetti, F. W., and Gallo, R. (1976). *Science* 193, 1007-1008.
- Mossalayi, M. D., Arock, M., and Debre, P. (1997). *Int. Rev. Immunol.* 16, 129-146.

- Murata, T., Noguchi, P. D., and Puri, R. K. (1996). *J. Immunol.* 156, 2972-2978.
- Murata, T., Taguchi, J., and Puri, R. K. (1998). *Blood* 91, 3884-3891.
- Nabholz, M., Soldaini, E., Sperisen, P., Pla, M., Wang, S. M., MacDonald, R., Reichenbach, P., Beerman, F., and Bucher, P. (1995). *Immunobiol.* 193, 259-262.
- Nagata, K., Nakamura, T., Kitamura, F., Kuramochi, S., Taki, S., Campbell, K. S., and Karasuyama, H. (1997). *Immunity* 7, 559-570.
- Nakabeppu, Y., and Nathans, D. (1991). *Cell* 64, 751-759.
- Nakajima, H., Liu, X.-W., Wynshaw-Boris, A., Rosenthal, L. A., Imada, K., Finbloom, D. S., Henighausen, L., and Leonard, W. J. (1997). *Immunity* 7, 1-20.
- Nelms, K., Kegan, A. D., Zamorano, J., Ryan, J. J., and Paul, W. E. (1999). *Annu. Rev. Immunol.* 17, 701-738.
- Nelson, B. H., and Willerford, D. M. (1998). *Adv. Immunol.* 70, 1-79.
- Newton, A. C. (1995). *J. Biol. Chem.* 270, 28495-28498.
- Noben-Trauth, N., Shultz, L. D., Brombacher, F., Urban Jr., J. F., Gu, H., and Paul, W. E. (1997). *Proc. Natl. Acad. Sci. USA* 94, 10838-10843.
- Noelle, R., Krammer, P. H., Ohara, J., Uhr, J. W., and Vitetta, E. S. (1984). *Proc. Natl. Acad. Sci. USA* 81, 6149-6153.
- Nosaka, T., van Deursen, J. M. A., Tripp, R. A., Thierfelder, W. E., Witthuhn, B. A., McMickle, A. P., Doherty, P., Grosveld, G. C., and Ihle, J. N. (1995). *Science* 270, 800-802.
- Nutt, S. L., Heavey, B., Rolink, A. G., and Busslinger, M. (1999). *Nature* 401, 556-562.
- O'Shea, J. J. (1997). *Immunity* 7, 1-11.

- Ollivier, V., Parry, G. C. N., Cobb, R. R., de Prost, D., and Mackman, N. (1996). *J. Biol. Chem.* 271, 20828-20835.
- Orchansky, P. L., Kwan, R., Lee, F., and Schrader, J. W. (1999). *J. Biol. Chem.* 274, 20818-20825.
- Oshima, Y., Tanaka, Y., Tozawa, H., Takahashi, Y., Maliszewski, C., and Delespesse, G. (1997). *J. Immunol.* 159, 3838-3848.
- Osmond, D. G., Rico-Vargas, S., Valenzona, H., Fauteux, L., Liu, L., Janani, R., Lu, L., and Jacobsen, K. (1994). *Immunol. Rev.*, 209-230.
- Osmond, D. G., Rolink, A., and Melchers, F. (1998). *Immunol. Today* 19, 65-68.
- Park, S. Y., Saijo, K., Takahashi, T., Osawa, M., Arase, H., Hirayama, N., Miyake, K., Nakauchi, H., Shirasawa, T., and Saito, T. (1995). *Immunity* 3, 771-782.
- Parker, D., Ferreri, K., Nakajima, T., LaMorte, V. J., Evans, R., Koerber, S. C., Hoeger, C., and Montminy, M. R. (1996). *Mol. Cell. Biol.* 16, 694-703.
- Paul-Eugene, N., Kolb, J.-P., Abadie, A., Gordon, J., Delespesse, G., Sarfati, M., Mencia-Huerta, J.-M., Braquet, P., and Dugas, B. (1992). *J. Immunol.* 149, 3066-3071.
- Pierce, J. W., Jamieson, C. A., Ross, J. L., and Sen, R. (1995). *J. Immunol.* 155, 1972-1980.
- Plaetinck, G., Combe, M. C., Corthesy, P., Sperisen, P., Kanamori, H., Honjo, T., and Nabholz, M. (1990). *J. Immunol.* 145, 3340-3347.
- Qin, X., Reichlin, A., Luo, Y., Roeder, R. G., and Nussenzweig, M. C. (1998). *EMBO J.* 17, 5066-5075.
- Rabin, E. M., Mond, J. J., Ohara, J., and Paul, W. E. (1986). *J. Immunol.* 137, 1573-1576.

- Ramch, L. E., and Cantley, L. C. (1999). *J. Biol. Chem.* 274, 8347-8350.
- Rane, S. G., and Reddy, E. P. (1994). *Oncogene* 9, 2415-2423.
- Rang, H. P., Dale, M. M., and Ritter, J. M. (1995). "Pharmacology." 3rd ed., Churchill Livingstone.
- Rao, A., Luo, C., and Hogan, P. G. (1997). *Annu. Rev. Immunol.* 15, 707-747.
- Raskin, N., Jakubowski, A., Sizing, I., Olson, D. L., Kalled, S. L., Hession, C. A., Benjamin, C. D., Baker, D. P., and Burkly, L. C. (1998). *J. Immunol.* 161, 3474-3483.
- Rawlings, D. J., Scharenberg, A. M., Park, H., Wahl, M. I., Lin, S., Kato, R. M., Fluckiger, A. C., Witte, O. N., and Kinet, J. P. (1996). *Science* 271, 822-825.
- Reth, M. (1989). *Nature* 338, 383-384.
- Rolink, A., Grawunder, U., Winkler, T. H., Karasuyama, H., and Melchers, F. (1994). *Int. Immunol.* 6, 1257-1264.
- Rolling, C., Treton, D., Pellegrini, S., Glanaud, P., and Richard, Y. (1996). *FEBS Lett.* 393, 53-57.
- Rothenberg, E. V. (1992). *Adv. Immunol.* 51, 85-214.
- Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E., and Leonard, W. J. (1993). *Science* 262, 1880-1883.
- Russell, S. M., Tayebi, N., Nakajima, H., Riedy, M. C., Roberts, J. L., Aman, M. J., Migonc, T. S., Noguchi, M., Markert, M. L., Buckley, R. II., O'Shea, J. J., and Leonard, W. J. (1995). *Science* 270, 797-800.
- Rusterholz, C., Henrioud, P. C., and Nabholz, M. (1999). *Mol. Cell. Biol.* 19, 2681-2689.

- Sandel, P. C., and Monroe, J. G. (1999). *Immunity* 10, 289-299.
- Sanz, E., and de la Hera, A. (1996). *J. Exp. Med.* 183, 2693-2698.
- Saouaf, S. J., Mahajan, S., Rowley, R. B., Kut, S. A., Fargnoli, J., Burkhardt, A. L., Tsukada, S., Witte, O. N., and Bolen, J. B. (1994). *Proc. Natl. Acad. Sci. USA* 91, 9524-9528.
- Sarmay, G., Koncz, G., Pecht, I., and Gergely, J. (1999). *Immunol. Lett.* 68, 25-34.
- Sato, S., Jansen, P. J., and Tedder, T. F. (1997). *Proc. Natl. Acad. Sci. USA* 94, 13158-13162.
- Satterthwaite, A., and Witte, O. (1996). *Annu. Rev. Immunol.* 14, 131-154.
- Satterthwaite, A. B., Li, Z., and Witte, O. N. (1998). *Semin. Immunol.* 10, 309-316.
- Schanke, J. T., Marcuzzi, A., Podzorski, R. P., and Van Ness, B. (1994). *Nucleic Acids Res.* 22, 5425-5432.
- Scharenberg, A. M., and Kinet, J. P. (1996). *Cell* 87, 961-964.
- Schubart, D. B., Rolink, A., Kosco-Vilbois, M. H., Botteri, F., and Matthais, P. (1996). *Nature* 383, 538-542.
- Schuh, K., Twardzik, T., Kneitz, B., Hoyer, J., Schimpl, A., and Serfling, E. (1998). *J. Exp. Med.* 188, 1369-1373.
- Serdobova, I., Pla, M., Reichenbach, P., Sperisen, P., Ghysdael, J., Wilson, A., Freeman, J., and Nabholz, M. (1997). *J. Exp. Med.* 185, 1211-1221.
- Shen, C. H., and Stavnezer, J. (1998). *Mol. Cell. Biol.* 18, 3395-3404.
- Shi, Y., Seto, E., Chang, L.-S., and Shenk, T. (1991). *Cell* 67, 377-388.
- Shikama, N., Lyon, J., and La Thangue, N. B. (1997). *Trends Cell Biol.* 7, 230-236.

- Shimoda, K., van deursen, J., Sangster, M. Y., Sarawar, S. R., Carson, R. T., Tripp, R. A., Chu, C., Quelle, F. W., Nosaka, T., Vignali, D. A. A., Doherty, P. C., Grosveld, G., Paul, W. E., and Ihle, J. N. (1996). *Nature* 380, 630-633.
- Simmonds, W. F. (1999). *Trends Pharm. Sci.* 20, 66-73.
- Smith, K. A. (1988). *Science* 240, 1169-1176.
- Smith, M. R., and Greene, W. C. (1989). *Proc. Natl. Acad. Sci. USA* 86, 8526-8530.
- Sperisen, P., Wang, S. M., Soldaini, E., Pla, M., Rusterholz, C., Bucher, P., Cothesy, P., Reichenbach, P., and Nabholz, M. (1995). *J. Biol. Chem.* 270, 10743-10753.
- Stein, B., Baldwin Jr., A. S. J., Ballard, D. W., Greene, W. C., Angel, P., and Herrlich, P. (1993). *EMBO J.* 12, 3879-3891.
- Stuber, E., and Strober, W. (1996). *J. Exp. Med.* 183, 979-989.
- Su, G. H., Chen, H., Muthusamy, N., Garrett-Sinha, L. A., Baunoch, D., Tenen, D. G., and Simon, M. C. (1997). *EMBO J.* 16, 7118-7129.
- Sun, L., Liu, A., and Georgopolous, K. (1996). *EMBO J.* 15, 5358-5369.
- Sun, X. J., Rothenburg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991). *Nature* 352, 73-77.
- Sun, X. J., Wang, L. M., Zhang, Y., Yenush, L., Myers Jr., M. G., Glasheen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995). *Nature* 377, 173-177.
- Takata, M., and Kurosaki, T. (1996). *J. Exp. Med.* 184, 31-40.
- Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. (1996). *Nature* 380, 627-630.
- Tamir, I., and Cambier, J. C. (1998). *Oncogene* 17, 1353-1364.

- Taniguchi, T. (1995). *Science* 268, 251-255.
- Tarlinton, D. (1998). *Curr. Opin. Immunol.* 10, 245-251.
- Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990). *Annu. Rev. Biochem.* 59, 971-1005.
- Tissot, C., and Mechti, N. (1995). *J. Biol. Chem.* 270, 14891-14898.
- Tissot, C., Taviaux, S. A., Diriong, S., and Mechti, N. (1996). *Genomics* 34, 151-153.
- Toledano, M. B., Roman, D. G., Halden, N. F., Lin, B. B., and Leonard, W. J. (1990). *Proc. Natl. Acad. Sci. USA* 87, 1830-1834.
- Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997). *Nature* 387, 677-84.
- Ueno, H., Sasaki, K., Honda, H., Nakamoto, T., Yamagata, T., Miyagawa, K., Mitani, K., Yazaki, Y., and Hirai, H. (1998). *Blood* 91, 46-53.
- Urbanek, P., Wang, Z. Q., Fetka, I., Wagner, E. F., and Busslinger, M. (1994). *Cell* 79, 901-912.
- Uribe, L., and Weinberg, K. I. (1998). *Semin. Hematol.* 35, 299-309.
- van Freeden-Jeffry, U. (1995). *J. Exp. Med.* 181, 1519-1526.
- Vitetta, E. S., Ohara, J., Myers, C. D., Layton, J. E., Krammer, P. H., and Paul, W. E. (1985). *J. Exp. Med.* 162, 1726-1731.
- Vogt, P. K., and Bos, T. J. (1990). *Adv. Cancer Res.* 55, 1-35.
- Waldmann, T., Tagaya, Y., and Bamford, R. (1998). *Intern. Rev. Immunol.* 16, 205-226.

- Wang, H. Y., Shelburne, C. P., Zamorano, J., Kelly, A. E., Ryan, J. J., and Keegan, A. D. (1999). *J. Immunol.* 162, 4385-4389.
- Wang, H. Y., Zamorano, J., Yoerkie, J. J., Paul, W. E., and Keegan, A. D. (1997). *J. Immunol.* 158, 1037-1040.
- Wang, L. M., Myers Jr., M. G., Sun, X. J., Aaronson, S. A., White, M., and Pierce, J. H. (1993). *Science* 261, 1591-1594.
- Welham, M. J., Duronio, V., and Schrader, J. W. (1994). *J. Biol. Chem.* 269, 5865-5873.
- Weng, W. K., Jarvis, J., and LeBien, T. W. (1994). *J. Biol. Chem.* 269, 32514-32521.
- Werlen, G., Jacinto, E., Xia, Y., and Karin, M. (1998). *EMBO J.* 17, 3101-3111.
- Whitmarsh, A. J., and Davis, R. J. (1996). *J. Mol. Med.* 74, 589-607.
- Wienands, J., Schweikert, J., Wollscheid, B., Jumaa, H., Nielsen, P. J., and Reth, M. (1998). *J. Exp. Med.* 188, 791-795.
- Wilks, A. F., Harpur, A. G., Kurban, R. R., Ralph, S. J., Zurcher, G., and Ziemiecki, A. (1991). *Mol. Cell. Biol.* 11, 2057-2065.
- Worm, M., Krah, J. M., Manz, R. A., and Henz, B. M. (1998). *Blood* 92, 1713-1720.
- Xie, H., Wang, Z., and Rothstein, T. L. (1996). *Cell. Immunol.* 169, 264-270.
- Xing, J., Ginty, D. D., and Greenberg, M. E. (1996). *Science* 273, 959-963.
- Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H. D., and Montminy, M. R. (1988). *Nature* 334, 494-498.

- Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imiaizumi, T., Matsuda, T., Ihle, J. N., and Yoshimura, A. (1999). *EMBO J.* 18, 1309-1320.
- Yin, T. G., Keller, S. R., Quelle, F. W., Witthuhn, B. A., Tsang, M. L. S., Lienhard, G. E., Ihle, J. N., and Yang, Y. C. (1995). *J. Biol. Chem.* 270, 20497-20502.
- Yu, W., Nagaoka, H., Jankovich, M., Misulovin, Z., Suh, H., Rolink, A., Melchers, F., Meffre, E., and Nussenzweig, M. C. (1999). *Nature* 400, 682-687.
- Zamorano, J., Wang, H. Y., Wang, L. M., Pierce, J. H., and Keegan, A. D. (1996). *J. Immunol.* 157, 4926-4934.
- Zhang, S., Mantel, C., and Broxmeyer, H. E. (1999). *J. Leukoc. Biol.* 65, 372-380.
- Zhong, H., Suyang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997). *Cell* 89, 413-424.
- Zhuang, Y., Soriano, P., and Weintraub, H. (1994). *Cell* 79, 875-884.
- Zola, II., Weedon, H., Thompson, G. R., Fung, M. C., Ingley, E., and Hapel, A. J. (1991). *Immunol.* 72, 167-173.

Appendix

AET Solution

50mM Tris-HCl, pH 8.0

10mM EDTA

Buffer P1

100 μ g/ml RNase A

102mg/ml AET, pH 9.0

1 x PBS

Buffer P2

200mM NaOH

1% (w/v) SDS

Buffer P3

3M CH₃COOK, pH 5.5

Buffer QBT

0.75M NaCl

50mM 3-[N-Morpholino]propane
sulfonic acid (MOPS), pH 7.0

15% (v/v) (CH₃)₂CHOH

0.15% (v/v) Triton-X-100

Buffer QC

1M NaCl

50mM MOPS, pH 7.0

15% (v/v) (CH₃)₂CHOH

Buffer QF

1.25M NaCl, pH 8.5

50mM Tris-HCl

15% (v/v) (CH₃)₂CHOH

Cytosolic Extraction Buffer

1mM HEPES-KOH, pH 8.0

2mM MgCl₂

15mM KCl

0.1mM EDTA

0.1mM EGTA

1mM DTT

0.25mM PMSF

2 μ g/ml leupeptin

2 μ g/ml aprotinin

Discontinuous Percoll Gradient

Percoll

10 x filter-sterile PBS

5x Binding Buffer

20% glycerol

5mM MgCl₂

2.5mM DTT

250mM NaCl

50mM Tris-HCl, pH 7.5

0.25mg/ml poly (dI-dC).poly (dI-dC)

5x PKA Buffer

0.2M MOPS, pH 7.0

0.16M (CH₃COO)₂Mg.4H₂O**LB Agar**

1.5% (w/v) bacto agar

1% (w/v) bacto tryptone

0.5% (w/v) bacto yeast

1% (w/v) NaCl

pH 7.5

5% (w/v) Acrylamide Stacking Gel

13% (v/v) acrylamide

40% (w/v) bis acrylamide

26% (v/v) 0.5M Tris-HCl, pH 6.8

1% (v/v) (NH₄)₂S₂O₈

0.1% (w/v) SDS

0.07% (v/v) TEMED

5% Non-SDS Denaturing Gel

8% (v/v) acrylamide

40% (w/v) bis acrylamide

5% (v/v) 5 x TBE, pH 8.3

0.5% (v/v) (NH₄)₂S₂O₈

05% (v/v) TEMED

5 x TBE

0.9M Tris base, pH 8.3

0.9M H₃BO₃0.02M disodium EDTA.2H₂O**1.B Media**

10g/l bacto tryptone

5g/l bacto yeast

0.17M NaCl

pH 7.5